STUDIES ON K12, A MAMMALIAN TEMPERATURE-SENSITIVE CELL CYCLE MUTANT

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ABSTRACT

K12 is a temperature-sensitive Chinese hamster fibroblast cell cycle mutant which arrests at a point in mid-G1 when incubated at the nonpermissive temperature (npt) of 40°C. The effect of growth medium composition on the cell's viability at the npt was studied, using temperature shift experiments on both random and mitotically-synchronized cultures. Reducing the sodium bicarbonate level of the medium was found to significantly increase the survival time at 40°C, by allowing the execution point of the mutation to be passed for one or more extra cycles before eventual arrest at the block point. It would appear likely that bicarbonate, acting *via* its effects on the nutritional quality or pH of the medium, can, directly or indirectly, alter the activity of the K12 temperature-sensitive molecule. This observation implies that the activity of specific cell cycle events within this cell line, and possibly others, can be greatly influenced by the composition of the growth medium. In addition, the use of 7 mM $NaHCO_3$ MCDB-302 medium allowed for the isolation of revertant K12 cells at a relatively high frequency which were capable of indefinite growth at 40°C, and which may have undergone amplification of the mutant gene.

Attempts were also made to clone the K12 defective gene by transfection with various human cDNA and genomic libraries, in order to elucidate the nature of the gene product of interest. However, it seemed likely that the human homologue of the required sequence was unable to function in a Chinese hamster background, or was expressed in an inappropriate manner. An alternative route for the isolation of the gene by *in vivo* linkage of genomic DNA from the parental cell line, Wg-1A, and the selective marker pSV2neo was subsequently taken, but proved unlikely to result in its successful recovery.

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ABBREVIATIONS USED

AIT	average intermitotic time
AM	acetoxymethyl ester
AMP	adenosine monophosphate
ATP	adenosine triphosphate
BCECF	2',7'-bis(carboxyethyl)-5,6-carboxyfluorescein
BES	N,N-bis(2-hydroxyethyl)-2-aminoethanesulphonic acid
BHK	baby hamster kidney
cAMP	cyclic AMP
cdc	cell division cycle
CDK	cyclin-dependent protein kinase
cGMP	cyclic GMP
CHO	Chinese hamster ovary
cpm	counts per minute
CRE	cAMP-response element
CREB	cAMP-response element binding protein
CS	calf serum
CSF-1	colony-stimulating factor-1
DAG	diacylglycerol
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
dGTP	deoxyguanosine triphosphate
DM	double minute
DMSO	dimethyl sulphoxide
dNTP	deoxynucleotide triphosphate
dTTP	deoxythymidine triphosphate
dUTP	deoxyuridine triphosphate
EBNA	Epstein-Barr virus nuclear antigen
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EOP	efficiency of plating
FCS	foetal calf serum
G protein	guanine nucleotide-binding protein
GAP	GTPase-activating protein
GMP	guanosine monophosphate
gpt	xanthine-guanine phosphoribosyl transferase

GRP	glucose-regulated protein
HEPES	N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulphonic acid]
hGH	human growth hormone
HGPRT	hypoxanthine-guanine phosphoribosyl transferase
HPV	human papilloma virus
HSR	homogeneously staining region
HSV	Herpes Simplex virus
IE	immediate early
IP_3	inositol (1,4,5) trisphosphate
kb	kilobases
kD	kilodaltons
MAP	microtubule-associated protein
MEM	Minimal Essential Medium Eagle (Modified) with Earle's salts
MPF	M phase-promoting factor
NBT	nitroblue tetrazolium
npt	nonpermissive temperature
OD	optical density
PDGF	platelet-derived growth factor
PEG	polyethylene glycol
PFGE	pulsed-field gel electrophoresis
pfu	plaque forming units
pH_{ex}	extracellular pH
pH_i	intracellular pH
PI	phosphoinositol
PIP_2	phosphoinositol (4,5) bisphosphate
PKC	protein kinase C
PLC	phospholipase C
pt	permissive temperature
SDS	sodium dodecyl sulphate
SPF	S phase-promoting factor
SRE	serum-response element
SRF	serum response factor
SV40	Simian virus 40
tk	thymidine kinase
ts	temperature-sensitive
UV	ultraviolet
wt	wild-type

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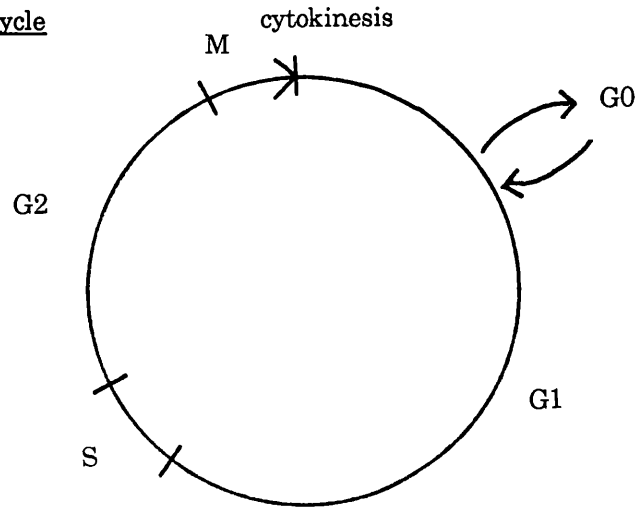
SECTION 1

INTRODUCTION

1.1 The basic eukaryotic cell division cycle

In order to reproduce, eukaryotic cells pass through characteristic cycles of growth and division. The cell division cycle (cdc) consists of a complex and multiply-coordinated network of events during which cells assimilate nutrients from their environment, increase in mass and then divide, the overall process being achieved in four distinct phases. All eukaryotic cells conform to the same basic plan for cell growth and division, although different species and cell types exhibit widely varying cdc lengths (Baserga, 1976).

Figure 1.1 The eukaryotic cell cycle



The synthesis of cellular DNA is confined exclusively to the S, or synthetic phase (Howard and Pelc, 1951), during which each chromosome is duplicated to form a pair of sister chromatids. Distribution of the newly-replicated chromosomes between the two daughter cells which are formed as a result of cell division (cytokinesis) is accomplished by mitosis, otherwise known as the M phase. In addition to the synthetic and mitotic stages, there are two interphase or gap periods, G1 and G2. The term gap, however, is somewhat misleading since both phases are periods of intense metabolic activity. G2 represents the post-DNA synthesis, pre-mitotic interval which prepares for, and leads up to mitosis and cytokinesis. Upon completion of cell division the two daughter cells enter G1, during which the cell increases in mass and prepares chemically for the replication of its DNA.

A considerable number of the biochemical processes which proceed in successive

stages of the cell cycle have been revealed, but we remain a long way from arriving at a complete and precise temporal map of the many integrated events that occur in each phase, and of their functional interrelationships. The G1 phase is concerned with the growth of the cell and the synthesis or activation of those components necessary for the initiation, progression and termination of DNA replication. Early G1 is marked by a general increase in the membrane transport of low-molecular-weight compounds, such as amino acids and nucleosides (Sander and Pardee, 1972; Costlow and Baserga, 1973). This is followed by a period of sequential gene activation, where alternating rounds of RNA and protein synthesis produce the molecules required for the onset of DNA synthesis (Baserga, 1976). Late in G1, close to the G1/S boundary, the expression of those enzymes directly involved in DNA replication e.g. dihydrofolate reductase (Hendrickson *et al.*, 1980; Farnham and Schimke, 1985), thymidine kinase (Johnson *et al.*, 1982; Thompson *et al.*, 1985), thymidylate synthetase (Nagarajan and Johnson, 1989) and proliferating cell nuclear antigen (Almendral *et al.*, 1988), increases significantly, as does the pool of the precursors for DNA synthesis, the deoxyribonucleotides (Tobey *et al.*, 1974).

During the S phase of the cell cycle the entire genome, along with its associated chromosomal proteins, must be efficiently and accurately duplicated. This involves a complex series of individual reactions which together constitute the separate but coordinated and highly regulated processes of replication of the DNA itself, and of the histones and the large number of other chromosomal proteins which contribute to the structure and function of the chromatin. At the G1/S boundary, the enzymes responsible for the process of DNA synthesis migrate to the nucleus and coalesce into a multienzyme complex, referred to as the replitase (Reddy and Pardee, 1980), containing DNA polymerase α and δ, which serve as the lagging and leading strand replicases respectively (So and Downey, 1988), DNA primase, DNA ligase, unwinding enzymes and various cofactors and accessory molecules (Laskey *et al.*, 1989). DNA synthesis subsequently initiates simultaneously at many points in the genome, clusters of replicons being activated in a specific temporal order (Hand, 1978; Lau and Arrighi, 1981) during S. The biosynthesis of histones begins at or near the G1/S interface (Robbins and Borun, 1967), coupled to DNA replication by a variety of transcriptional and post-transcriptional regulatory mechanisms (Schumperli, 1988) which ensure that the newly synthesized DNA associates with these major structural constituents of chromatin. Strict controls must be exercised to ensure that the genome is replicated only once per cell cycle, but the mechanism for this is unclear at present, as is the signal for the termination of S phase.

G2 activities in the cell have been only poorly documented in mammalian cells, but it seems likely that this phase represents the time required by the cell to

synthesize the various components, such as microtubular proteins and microtubule-associated proteins, required during the subsequent mitosis. At mitosis the chromosomes condense, the nuclear envelope breaks down and a mitotic spindle forms, upon which the chromosomes align. The separation of each chromosome into two identical parts precedes the movement of each half to opposite cell poles along the mitotic spindle, following which the spindle disassembles, the nuclear envelope reforms, the chromosomes decondense and cytokinesis occurs. This overall process (see McIntosh and Koonce, 1989 for review) ensures the equal distribution of nuclear material and cytoplasm into the daughter cells, which have now returned to the start of the G1 phase.

1.2 Control of the cell cycle

Although many of the biochemical events which occur during cell proliferation have been elucidated, the mechanisms which underlie their activation, and thus control the progression of cells through the cycle, are still unresolved. Identification at the molecular level of such key regulatory control points, which ensure the orderly reproduction of normal cells, is clearly of major importance, as hopefully this will provide insights into the nature of the defects in growth regulation which result in the unrestrained and inappropriate proliferation of transformed cells. There are a number of lines of evidence to suggest that the growth of mammalian cells is regulated in the G1 phase:

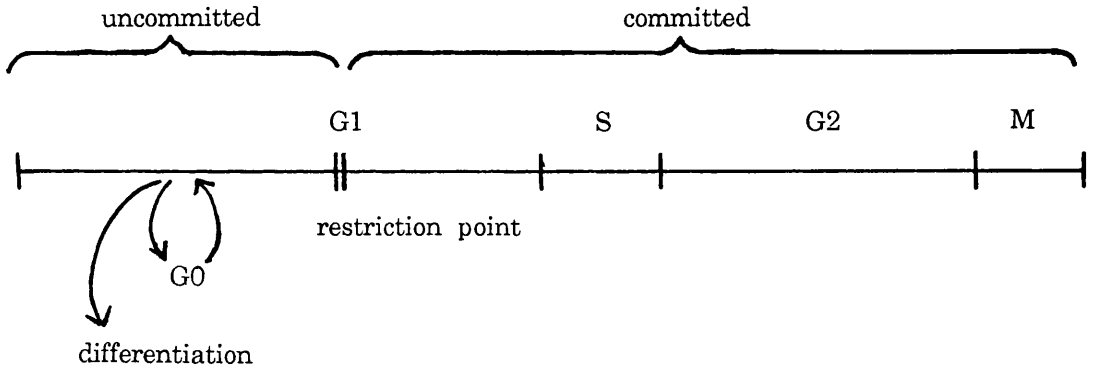
1. Most of the variation between different species and cell types in cycle length can be accounted for by variability in the duration of G1 (Prescott, 1968; Shields, 1977), with the time taken to traverse S, G2 and M being similar in all cells. This implies that passage through G1 regulates the rate of cell proliferation as a whole.
2. Under a variety of suboptimal conditions, such as overcrowding or deprivation of an essential nutrient or growth factor, nontransformed cells leave the cdc at a point in G1 (see Fig. 1.1) and enter a stable resting state, known as quiescence or G0, in which there is no DNA synthesis, cell division or growth (Nilausen and Green, 1965; Ley and Tobey, 1970; Pardee, 1974). It has been proposed that G0 simply represents a slow traversal of G1 under inhibiting conditions (Prescott, 1968; Smith and Martin, 1973; Shields and Smith, 1977). However, although the molecular machinery necessary for accumulating cells into G0 is as yet unclear, cell kinetic studies which demonstrate that the lag time between growth stimulation of quiescent cells and entry into S exceeds the length of time required for traversal of G1 during proliferative growth, thereby implying the necessity for carrying out steps not present in G1 (Baserga, 1985), biochemical data showing both quantitative and qualitative

differences between G0 and G1 cells in their patterns of gene expression (Pehrson and Cole, 1980; Wang, 1985a; 1985b; Schneider *et al.*, 1988; Bedard *et al.*, 1989), and the existence of G0-specific cdc mutants (Crane and Thomas, 1976; Ide *et al.*, 1984) all suggest that G0 actually resembles an out-of-cycle state fundamentally distinct from the G1 phase. G0 cells have a low metabolic activity, with significantly decreased rates of RNA and protein synthesis and an increase in protein degradation (Hershko *et al.*, 1971), which may allow for survival in an environment unsupportive of cell proliferation. Most cells *in vivo* exist in this non-proliferating state, but remain viable and capable of growth for an extended period of time (Dell'Orco *et al.*, 1973; Augenlicht and Baserga, 1974), and re-enter the cell cycle in G1 after the appropriate stimulation (Pardee, 1974). However, transformed cells, which display unrestricted growth, demonstrate a reduced ability to enter G0 in response to suboptimal conditions in which normal cells are blocked (Stoker, 1972), which, although permitting them to continuously proliferate, also increases their susceptibility to adverse conditions. Instead, if severely restricted nutritionally, the proliferation rate of the transformed cell gradually slows until it eventually arrests and dies at a random point in the cell cycle (Paul, 1973; Medrano and Pardee, 1980). This strongly suggests that malignancy may be the result of an inability to take the appropriate decisions about transition between proliferation and quiescence, the control of which is exerted in G1 phase.

3. Pardee (1974) demonstrated the existence of a single switching point, the restriction or R point in mid-G1, which regulates entry into a new round of the cell cycle in response to environmental cues, an idea also supported by the studies of Zetterberg and Larsson (1985). Prior to the R point, the uncommitted post-mitotic mammalian cell may follow one of three alternative developmental pathways. If the environment is favourable for growth, the cell will start a mitotic programme leading inexorably to DNA replication and cell division. Alternatively, the cell may withdraw from the cdc before this point and either enter G0 in suboptimal conditions, or it may differentiate (Scott *et al.*, 1982). Once the cell has traversed the restriction point it becomes committed to completing the remainder of the cycle (except, that is, in rare cases of G2 arrest e.g. see Gelfant, 1981; Melchers and Lernhardt, 1985; Gomez-Lechon and Castell, 1987), which is consequently thought to be insensitive to the influence of extracellular factors and thus under the control of purely intracellular signals. The regulation of cell cycle initiation at the R point is thought to depend upon the accumulation of a particular labile initiator protein, the restriction or R protein, to a certain critical threshold level in G1, which then triggers the initiation of DNA synthesis (Rossow *et al.*, 1979). The synthesis of this protein is proposed to be both growth factor-dependent and exquisitely sensitive to conditions which inhibit

protein synthesis, such as nutrient deprivation, thus coupling the cell to external environmental changes and allowing it to make the appropriate proliferative response (see Fig. 1.2). Growth factors therefore appear to exert their effects on the control of initiation of DNA synthesis in G1, presumably at the restriction point, acting via a complex system involving specific receptors, membrane signalling systems, activation of protein kinases and changes in gene expression (see Sections 1.10 and 1.11). It is unclear what relationship this restriction protein bears to the dose-dependent cytoplasmic inducer of DNA replication demonstrated to trigger entry into S phase in cell fusion experiments carried out by Rao and Johnson (1970), although it seems likely that they may represent the same molecule. The variations in the length of G1 observed in different cells could be accounted for by differences in the rate of R protein induction, which would determine the amount of time the cell spends in the pre-restriction point G1 interval. Furthermore, the inability of transformed cells to enter G0 under suboptimal conditions may be explained by an increased stability and/or rate of synthesis of the R protein (Campisi *et al.*, 1982; Croy and Pardee, 1983), thus rendering them insensitive to environmental alterations.

Figure 1.2 Restriction point control of the cell cycle in G1



In summary, it thus appears likely that the primary site of control of the cell cycle by external factors is in G1, and that processes carried out here may determine the programming of the cdc as a whole. However, the nature of the proteins involved, and the interactions between them, are only just beginning to be revealed.

1.3 The use of mammalian temperature-sensitive mutants in the study of the cell cycle

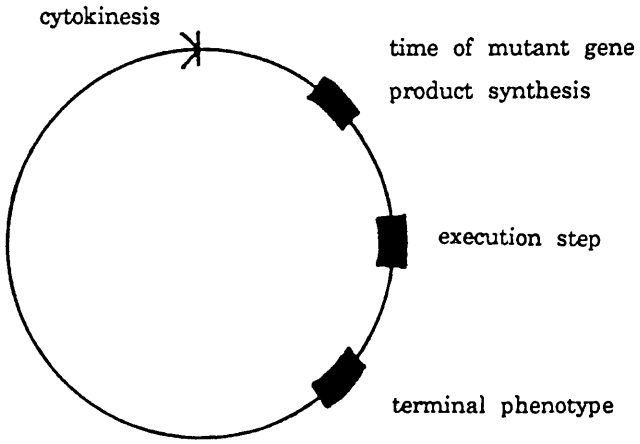
In order to identify the temporal order of events involved in cell cycle progression,

and to elucidate the causal relationships between them, it would clearly be of use to examine the consequences of mutations in the individual gene products concerned. A mutation in a gene encoding a protein essential for passage through the cdc will of necessity be lethal, since the cells will be unable to propagate fully, thus those mutants isolated are generally of the conditional lethal type i.e. expression of the defect is observed only under certain conditions. Nearly all of the studies so far have involved mutants of the temperature-sensitive (ts) type. A ts mutation in a protein will render it inactive (or less active) at the nonpermissive temperature (npt), whereas below this threshold, at the permissive temperature (pt), the protein exhibits normal, or near normal activity. Ts mutations are usually missense mutations, involving substitutions of a single amino acid in the gene product. All ts mutants analysed to date carry a recessive lesion, and thus are only expressed if a double mutation occurs in both sets of the gene, which would be an extremely rare event, or if they occur on the X chromosome, or at a locus which is rendered functionally hemizygous by chromosomal rearrangements during evolution of the cell line (Siminovitch, 1976).

In practice, ts mutants are detected as growth mutants that become arrested at a specific phase of the cell cycle when placed at the npt, due to temperature inactivation of a gene product essential for cell cycle progression. Following a shift to the npt, cell division ceases only when the cell reaches the particular stage-specific event which requires the mutated function, thus all cells in an initially asynchronous population with a given mutation will ultimately stop cycling at a fixed cell cycle stage, known as the terminal phenotype (Fig. 1.3), that is characteristic for each mutant. General ts mutants, by contrast, show random growth arrest after the temperature shift as a consequence of a defect in some process essential for viability at all cdc stages. The execution point of the mutation (Fig. 1.3) represents the stage at which the function of the normal gene product is required for progression through the ongoing cycle and completion of cell division (Pringle, 1978), and may extend over several hours or, alternatively, may define a discrete point in the cell cycle. Consequently, when an asynchronous population of ts cdc mutant cells is placed at the npt, those cells which have already performed the deficient function at the pt complete the current cycle and divide once, before arresting at the execution point in the subsequent cycle, whereas the cells that have not yet done so halt without completing the present cycle. The last stage at which the function in question is required can therefore be determined experimentally as the point in the cdc beyond which a temperature upshift no longer prevents the mutant cell from successfully completing the ongoing cycle, and may, in theory, precede the arrest point, whereas the beginning of the time period covered by the execution point may be defined by

temperature shift-down experiments where synchronized cells are stimulated to grow at the npt, and the length of incubation at this temperature required in order to delay the passage of the mutant through the cell cycle is assessed. It is also of importance to make a distinction between the execution point of the gene product and the time at which it is synthesized, since a cell cycle protein may be produced continuously throughout the cdc, yet act only within a discrete period of time.

Figure 1.3 Diagram of the cell cycle showing times relevant to the consideration of a hypothetical ts cell cycle mutant (adapted from Pringle, 1978)



Many ts cdc mammalian cell mutants, defective in each stage of the cell cycle, have been isolated, mainly from rodent cell lines such as mouse fibroblasts (Liskay, 1974; Sheinin, 1976; Mita *et al.*, 1980), rat fibroblasts (Ohno *et al.*, 1984; Zaitsu and Kimura, 1985) and hamster cells (Burstin *et al.*, 1974; Crane and Thomas, 1976; Wang, 1976). The study of such mutants has undoubtedly provided a clearer picture of the overall organization of the cell cycle, which is seen to be driven by a sequence of stage-specific interdependent biosynthetic reactions catalyzed by cdc proteins. As described in Section 1.2, an important objective is to identify control points for cellular proliferation, which appear to reside mainly in G1 in mammalian cells. Ts G1 mutants are thus of particular interest in this context. The majority of ts mammalian cdc mutants isolated so far have in fact been in G1 (Simchen, 1978). This is perhaps due to the fact that these cells are better able to survive the commonly employed selection regimen for the isolation of ts cdc mutants, consisting of high temperature incubation for several generation period equivalents in the presence of agents lethal for cycling cells, than cells arresting in other stages of the cycle (Basilico, 1978). However, despite the plethora of G1 mutants, the characterization of the primary lesion at a molecular and biochemical level is lacking in the majority. In those few cases where the defect has been subjected to rigorous analysis, the

results have not proved to be particularly informative; either the gene responsible for the G1 arrest has been cloned and the protein product shown to have no homologies to known proteins, and thus its function is unclear (Greco *et al.*, 1987; Ittman *et al.*, 1987; Sekiguchi *et al.*, 1988), or the lesion has been demonstrated to reside in some aspect of general protein synthesis, and therefore to affect the production of many proteins, only one of which is likely to be responsible for the cdc block (Rossini and Baserga, 1978; Tenner and Scheffler, 1979; Ortwerth *et al.*, 1984; Watanabe *et al.*, 1991). Progress in genetically defining the precise steps regulating mammalian cell cycle progression has thus been limited.

1.4 Employment of yeast temperature-sensitive mutants in the study of the cell cycle

In contrast to the situation with mammalian ts mutants, where the diploid nature of the genome means that recessive mutants cannot be readily isolated, and the analysis of the underlying genetic defect is time-consuming and complex, the predominantly haploid lifestyle of the simple eukaryotic organism yeast renders it amenable to both mutant isolation and genetic manipulation. Both the fission yeast *Schizosaccharomyces pombe*, and the budding yeast *Saccharomyces cerevisiae* have proved invaluable models in which to study the basic mechanism of the control of cell cycle regulation, the fundamental mechanisms of which appear to be conserved throughout the eukaryotic world. A large number of ts cell cycle mutants have been isolated in these two yeasts (Hartwell *et al.*, 1973; Nurse *et al.*, 1976; Reed, 1980; Nasmyth and Nurse, 1981), facilitating the identification of genes involved at key control points in the cell cycle.

Two major cdc control points have been identified in fission yeast. The first is located in G1 at a point known as Start (Nurse and Bissett, 1981). This represents the stage at which the cell becomes committed to the mitotic cell cycle, if the extracellular conditions are appropriate, rather than to the alternative developmental pathways of conjugation, sporulation or entry into stationary phase (Hartwell, 1974), and thus can be considered analogous to the mammalian G1 restriction point (Section 1.2). Traverse through Start is a major rate-limiting step in the fission yeast cell cycle, and requires growth to a critical cell size (Fantes, 1977; Nurse and Thuriaux, 1977; Nasmyth *et al.*, 1979), thus ensuring the coordination between the growth and division cycles which is necessary for balanced proliferation. Mutations in two genes, *cdc2* and *cdc10*, block the cell cycle at Start (Nurse and Bissett, 1981). In addition, *cdc2* is also required for passage through the second control point in the cell cycle of fission yeast (Nurse and Bissett, 1981), which acts during G2 and determines the timing of the initiation of mitosis (Nurse, 1975). This has also been delineated as a

rate-limiting step, since *wee* mutants, which show a premature advancement into M phase, have been isolated (Nurse and Thuriaux, 1980), and growth to a particular cell size is once again required before the mitotic control point can be passed (Fantes and Nurse, 1977).

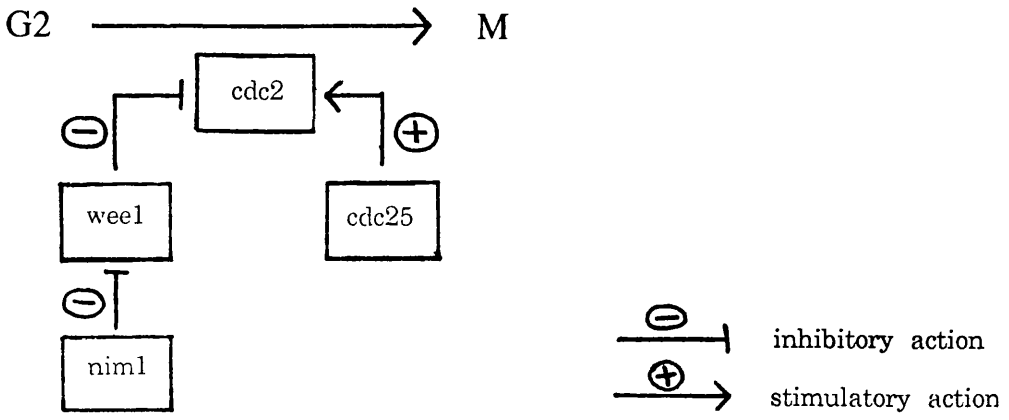
In contrast to fission yeast, which shows a typical eukaryotic cdc pattern consisting of G1, S, G2 and M phases, *S. cerevisiae* has no real G2 period, with mitosis following directly on from S phase (Nurse, 1985), and this difference in organization is reflected in its cell cycle control mechanism. The major rate-limiting control point in budding yeast is located in G1 at Start (Hartwell, 1974), passage through which requires the attainment of a certain cell size (Hartwell and Unger 1977; Carter and Jagadish, 1978; Lord and Wheals, 1980) and the function encoded by the gene *CDC28* (Reed, 1980). Although an equivalent of the fission yeast G2 control point has not been clearly identified in *S. cerevisiae*, a requirement for a mitotic function of *CDC28* in addition to its role at Start has been demonstrated, acting soon after passage through Start (Piggott *et al.*, 1982; Reed and Wittenberg, 1990), consistent with the early initiation of mitosis in this organism.

The *cdc2* and *CDC28* genes thus appear to play pivotal roles in the cell cycles of their respective organisms, controlling both the transition from G1 to S phase (and therefore commitment to the mitotic cycle) and the initiation of mitosis. *CDC28* has been shown to be functionally interchangeable with the *cdc2* gene, as it can complement mutants of *S. pombe* deficient in *cdc2* function, allowing for growth under restrictive conditions (Beach *et al.*, 1982). Furthermore, each gene has been sequenced (Hindley and Phear, 1984; Lorincz and Reed, 1984) and the protein products shown to be identical at 62% of their amino acid positions. The functional and structural conservation of these two proteins in such phylogenetically distant organisms as fission and budding yeast clearly points to their fundamental role in the cell cycle.

A number of additional genes have been shown to be involved in the timing of the initiation of mitosis in *S. pombe*, interacting closely with the *cdc2* function. Mutations in the gene *wee1* result in a premature advancement into mitosis, indicating that this gene may encode an inhibitor of mitotic control (Russell and Nurse, 1987a). This activity is counterbalanced by the *cdc25* gene, which has been demonstrated to encode a mitotic inducer, since its overexpression causes mitotic advancement (Russell and Nurse, 1986) whereas a ts mutation in the gene blocks the fission yeast cell in G2 (Fantes, 1979). However, in the absence of functional *wee1*, the deletion of *cdc25* does not cause cell cycle arrest (Russell and Nurse, 1986), suggesting that the timing of entry into mitosis is coordinately regulated through a balance between the activity of the gene products of the inducer *cdc25* and the inhibitor *wee1*. Certain dominant mutations in *cdc2* can also advance cells into mitosis

early, and analysis of such *cdc2* wee alleles has shown that they can be divided into two classes (Russell and Nurse, 1987a), one producing cells largely unresponsive to *wee1* inhibition but requiring some *cdc25* function for premature advancement, and the second resulting in cells independent of *cdc25* but still responsive to *wee1*. This indicates that the signals generated by the *wee1* and *cdc25* gene products act *via* two independent pathways to directly modulate the induction of mitosis by the cdc2 protein (Fig. 1.4). A further gene product, *nim1*, is implicated in this network as a negative regulator of *wee1* activity (Russell and Nurse, 1987b). A functional homologue of *cdc25*, the *MIH1* gene, has been demonstrated in *S. cerevisiae* (Russell *et al.*, 1989), suggesting that a similar mechanism for controlling the timing of entry into mitosis is also operational in this organism.

Figure 1.4 Scheme for mitotic control in *S. pombe* (after Lee and Nurse, 1988)



In order to elucidate the mechanism by which *cdc2/CDC28* controls entry into both S phase and mitosis, it was necessary to characterize their protein products. Sequencing of both genes (Hindley and Phear, 1984; Lorincz and Reed, 1984) suggested that each had the potential to encode a 34 kD protein with significant homology to protein kinases. Antibodies raised against the gene products of *cdc2* (Simanis and Nurse, 1986) and *CDC28* (Reed *et al.*, 1985) each recognized a 34 - 36 kD phosphoprotein with protein kinase activity, hereafter referred to as p34. Mutations in the kinase-encoding domain of *cdc2* were shown to abolish its function (Booher and Beach, 1986), suggesting that *cdc2* exerts its control over the cell cycle through the phosphorylation of key proteins. The level of p34 does not change appreciably during the cell cycle of either *S. pombe* (Simanis and Nurse, 1986) or *S. cerevisiae* (Mendenhall *et al.*, 1987), implying that the entry into S phase or mitosis is not triggered by an accumulation of p34 to a threshold level. However, the protein kinase activity of p34 oscillates with the cdc, reaching its highest levels in G1 in

budding yeast (Mendenhall *et al.*, 1987), and at the G2/M transition in fission yeast (Booher *et al.*, 1989; Moreno *et al.*, 1989). It thus appears that cyclical changes in the kinase activity of p34 are correlated with traverse through the cell cycle, and that commitment to S phase in *S. cerevisiae* and to initiation of mitosis in *S. pombe* are brought about by an increase in this protein kinase function. Clues as to how this kinase activity is itself regulated came not from genetic analysis of yeast mutants, however, but rather from biochemical studies carried out in higher eukaryotes.

1.5 Identification of further components involved in cell cycle regulation in higher eukaryotes

The presence of a protein activity, termed maturation-promoting factor (MPF), in mature *Xenopus laevis* oocytes which enables immature oocytes, naturally arrested just before meiotic M phase, to enter meiosis in the absence of appropriate hormonal stimulation, and which thus presumably plays a role at the G2/M transition, has long been known (Masui and Markert, 1971). The activity of MPF was demonstrated to oscillate during the cell cycle, being high during mitosis and low at other stages (Gerhart *et al.*, 1984; Newport and Kirschner, 1984). MPF has been purified from *Xenopus*, and found to contain two major protein components, one of 32 kD and a second of 45 kD (Lohka *et al.*, 1988). Both immunoprecipitation and immunoblotting experiments demonstrated that the smaller component is the *Xenopus* homologue of $p34^{cdc2}$ and has protein kinase activity, as assayed by histone H1 phosphorylation (Gautier *et al.*, 1988). M phase-promoting factors from a variety of other organisms e.g. starfish (Arion *et al.*, 1988; Labbe *et al.*, 1988; 1989a), *Tetrahymena* (Roth *et al.*, 1991) and mammalian cells (Brizuela *et al.*, 1989; Langan *et al.*, 1989) have since been purified and shown to also contain $p34^{cdc2}$ homologues (Table 1.1), which display a high degree of structural and functional conservation with the yeast molecule. It thus appears that proteins that function in the control of mitosis in yeast have counterparts in many eukaryotes, implying that there is a fundamental similarity in the mechanism of regulation of their respective cell cycles, and that $p34^{cdc2}$ plays a pivotal role in this process. Indeed, homologues of $p34^{cdc2}$ have also been isolated in algal and higher plant cells (John *et al.*, 1989; Hirayama *et al.*, 1991), further underlining the widespread and fundamental importance of this protein.

The additional subunit of MPF from both clam and starfish was shown to be a cyclin (Draetta *et al.*, 1989; Labbe *et al.*, 1989b), a class of proteins, initially described in sea urchin eggs, which characteristically accumulate during interphase, reach a peak at metaphase and are specifically and rapidly destroyed by proteolysis as the cells enter anaphase (Evans *et al.*, 1983). The mitotic cyclin family can be classified

into two types, cyclin A and cyclin B, on the basis of their amino acid sequences and patterns of expression (Hunt, 1989). Purified MPF (now considered to stand for M phase-promoting factor in view of its general role in mitotic initiation) from all the cell types analysed to date has been shown to contain a cyclin subunit in addition to the catalytic p34 component, the nature of the cyclin molecule involved varying according to the particular organism in question (Table 1.1). Cyclins from plant cells have been shown to be capable of inducing maturation of *Xenopus* oocytes (Hata *et al.*, 1991), indicating that like $p34^{cdc2}$, these molecules display a remarkable degree of functional conservation.

Table 1.1 Components of MPF in various systems (adapted from Lewin, 1990)

Cell type	**Catalytic subunit**	**Regulatory subunit**
S. pombe	cdc2 = p34	cdc13 = p56 = cyclin B
S. cerevisiae	CDC28 = p34	G1 cyclins
X. laevis	p34	cyclins A, B1 and B2
Human	p34	p60 = cyclin A p62 = cyclin B
Starfish	p34	p60 = cyclin B

Evidence that the formation of a complex between $p34^{cdc2}$ and the cyclin subunit is essential for the mitosis-inducing kinase activity of MPF came initially from genetic studies on the *cdc13* gene of *S. pombe*, which has been demonstrated to encode a 56 kD protein (p56) with a strong homology to the mitotic cyclins (Goebl and Byers, 1988; Hagan *et al.*, 1988; Solomon *et al.* 1988). Deletion of *cdc13* results in G2 arrest (Booher and Beach, 1988; Hagan *et al.*, 1988), and low $p34^{cdc2}$ kinase activity (Moreno *et al.*, 1989), thus $p56^{cdc13}$ is required for MPF kinase activation at mitosis in this organism. A partial function mutant, *cdc13-117*, allows $p34^{cdc2}$ kinase activation (Moreno *et al.*, 1989), but only some mitotic events take place (Hagan *et al.*, 1988), thus the *cdc13* gene product is required both to initiate mitosis and also plays a continuing role during M phase. Mitosis in *Xenopus* oocyte extracts was shown to be blocked by the specific destruction of cyclin B mRNA (Minshull *et al.*, 1989; Murray and Kirschner, 1989), but on addition of exogenous cyclin B mRNA the cell cycle was resumed (Murray and Kirschner, 1989), further emphasizing the requirement for a cyclin molecule at mitotic initiation. The importance of cyclins in cell cycle regulation is also underlined by the finding that they have been implicated in the oncogenesis of parathyroid tumours (Motokura *et al.*, 1991) and hepatocellular carcinoma (Wang *et al.*, 1990). However, although interaction between a cyclin molecule

and p34^{cdc2} is clearly required to turn on the kinase activity of the latter protein, this association does not in itself appear to constitute the activating event, since the timing of entry into mitosis has been shown to be independent of the rate of cyclin accumulation (Lehner and O'Farrell, 1989). The responsibility for triggering mitosis thus lies with an further set of regulatory molecules, described in Section 1.6.

The existence of two classes of cyclin, cyclin A and cyclin B, and the observation that p34^{cdc2} does not appear to bind the two cyclin types simultaneously (Draetta *et al.*, 1989), suggest that one function of the cyclin subunit may be to modify the substrate specificity of p34^{cdc2}, an association with alternative cyclin types resulting in the phosphorylation of different target molecules (Draetta *et al.*, 1989; Hunt, 1989). Differences noted in the timing of the accumulation of cyclins A and B (Minshull *et al.*, 1990; Pines and Hunter, 1990; Whitfield *et al.*, 1990) may also provide a degree of temporal regulation in the specific kinase activities of the molecule and, in addition, each p34^{cdc2}-cyclin complex may be recognized differently by regulatory molecules, and hence be differentially controlled. It has also been proposed that interaction with the cyclin molecule may mediate a relocation of MPF (Booher *et al.*, 1989; Pines and Hunter, 1991a), different cyclins thus targetting the active protein to substrates in particular subcellular compartments.

In addition to its role in the initiation of mitosis, destruction of the cyclin molecule by proteolysis at anaphase has been causally implicated in the inactivation of p34^{cdc2} kinase activity required to exit from mitosis, since proteolysis-resistant cyclin A and B mutations prevent both MPF inactivation and the return to interphase (Murray *et al.*, 1989; Luca *et al.*, 1991). Little is known, however, about the signal for cyclin degradation, although the observed phosphorylation of the cyclin subunit by p34^{cdc2} itself (Pines and Hunter, 1989) has been proposed to be involved (Felix *et al.*, 1990). Proteolysis of the cyclin molecule appears to be mediated by the ubiquitin pathway of protein degradation, which recognizes a conserved N-terminal destruction sequence common to all cyclins, thus it is possible that p34^{cdc2} kinase brings about cyclin destruction at anaphase by modulating the activity of a cyclin-specific ubiquitin-conjugating enzyme (Glotzer *et al.*, 1991). The activation of MPF would therefore provide a negative feedback loop, making mitosis a self-limiting process.

1.6 Regulatory molecules determining the timing of mitosis

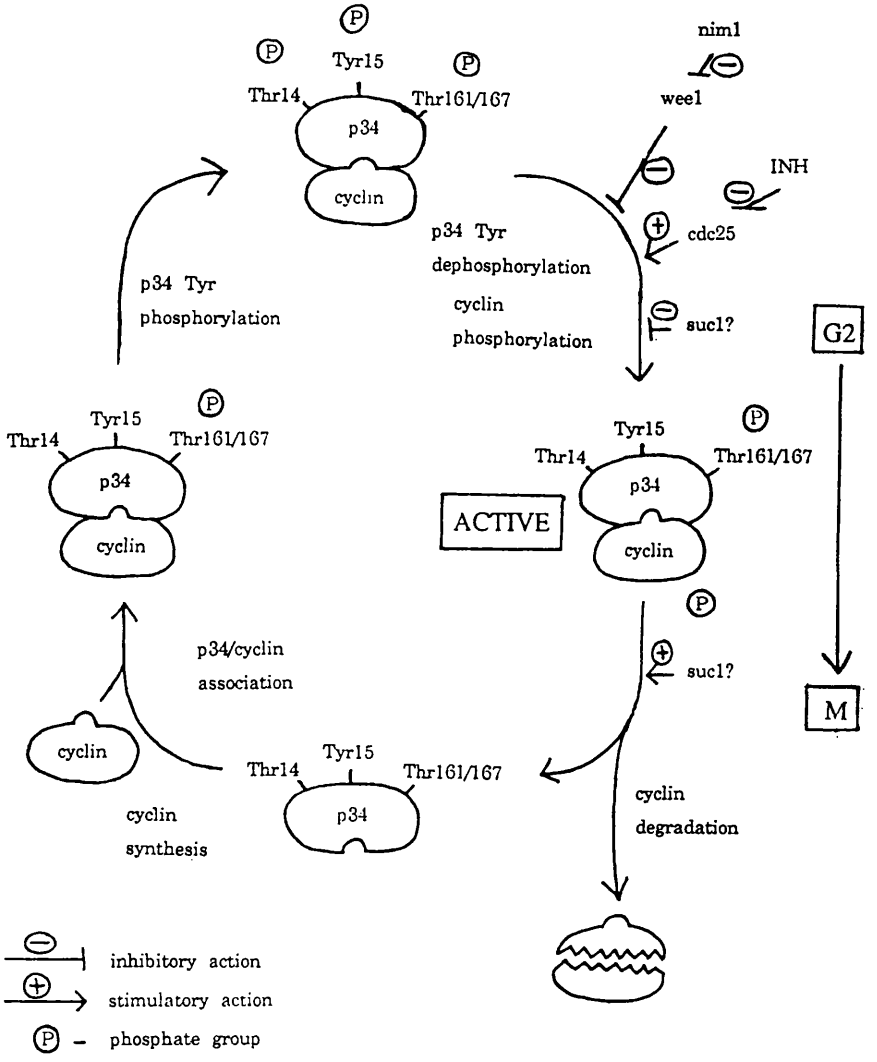
There is good evidence to suggest that the activation of p34^{cdc2} kinase at mitosis is correlated with the dephosphorylation of the protein molecule in starfish (Labbe *et al.*, 1989a), *Xenopus* eggs (Gautier *et al.*, 1989), mammalian cells (Morla *et al.*, 1989)

and fission yeast (Gould and Nurse, 1989). In *S. pombe*, the specific target of this regulatory phosphorylation/dephosphorylation event was mapped to Tyr15, a residue within the presumptive ATP-binding site (Gould and Nurse, 1989). The substitution of Tyr15 with a non-phosphorylatable residue, phenylalanine, caused fission yeast cells to enter mitosis prematurely (Gould and Nurse, 1989), suggesting that the $p34^{cdc2}$ kinase is most active in its dephosphorylated form. Furthermore, removal of the phosphate group from Tyr15 in wild-type $p34^{cdc2}$ isolated from G2 cells was shown to be sufficient for the activation of its kinase activity (Gould *et al.*, 1990). During interphase, when this tyrosine is phosphorylated, $p34^{cdc2}$ kinase activity is inhibited, presumably because the presence of the phosphate group hinders the binding or use of ATP. Removal of the Tyr15 phosphate group at the G2/M boundary thus appears to be the event triggering entry into mitosis by allowing the kinase activity of $p34^{cdc2}$ to appear. The regulation of $p34^{cdc2}$ protein kinase activity by phosphorylation/dephosphorylation of Tyr15 is conserved in vertebrate cells (Krek and Nigg, 1991a; Norbury *et al.*, 1991). However, evidence suggests that there is an additional inhibitory phosphorylation site on the adjacent amino acid, threonine 14, in these cells (Krek and Nigg, 1991a; Norbury *et al.*, 1991), and that activation of the kinase requires removal of the phosphates from both residues (Krek and Nigg, 1991b; Norbury *et al.*, 1991). In addition to the specific dephosphorylation of Thr14 and Tyr15, activation of $p34^{cdc2}$ at mitosis also involves the phosphorylation of a threonine residue, Thr167 in fission yeast (Gould *et al.*, 1991) and Thr161 in vertebrates (Krek and Nigg, 1991a), perhaps due to the observed requirement for Thr161/167 phosphorylation for tight association between $p34^{cdc2}$ and the cyclin molecule (Ducommun *et al.*, 1991; Gould *et al.*, 1991). This phosphorylation event is believed to occur during S phase (Gould *et al.*, 1991).

The overall scheme for the activation of $p34^{cdc2}$ kinase at the G2/M transition is thus now beginning to be pieced together (Fig. 1.5). During interphase, $p34^{cdc2}$ is in an inactive state, despite the phosphorylation of Thr161/167 and the dephosphorylation of its Thr14 and Tyr15 residues, due to its lack of association with a cyclin partner. However, cyclin accumulates and interacts with the $p34^{cdc2}$ molecule during G2. The binding of cyclin induces inhibitory phosphorylation of Thr14 and Tyr15 (Solomon *et al.*, 1990; Meijer *et al.*, 1991), perhaps in order to produce an abrupt switch, rather than a gradual transition, between G2 and M phase; the accumulation of large quantities of inactive complex may allow for a sudden change when the balance between the phosphorylation and dephosphorylation of Thr14 and Tyr15 alters slightly to permit partial activation of the kinase, which may then in turn act in an autocatalytic manner to produce large quantities of active complex (Cyert and Kirschner, 1988). Inactivation of the kinase at the end of mitosis could,

in principle, be caused by rephosphorylation of Thr14/Tyr15. However, in mammalian cells, rephosphorylation of Tyr15 has been shown to occur long after the inactivation of $p34^{cdc2}$ (Morla *et al.*, 1989), thus it appears more likely that either cyclin degradation (Murray *et al.*, 1989; Luca *et al.*, 1991) or Thr161/167 dephosphorylation (Gould *et al.*, 1991) is responsible.

Figure 1.5 Scheme for the roles of $p34^{cdc2}$ and cyclins in the induction of mitosis (adapted from Lewin, 1990)



The gene product of *S. pombe cdc25*, an 80 kD phosphoprotein (Moreno *et al.*, 1990), has been clearly implicated in the dephosphorylation of $p34^{cdc2}$ Tyr15. As described in Section 1.4, its involvement in the timing of mitosis was first indicated by genetic studies which showed that overproduction of wild-type *cdc25* transcripts led to mitotic advancement, whereas its deletion blocked the yeast cell in mitosis (Russell and Nurse, 1986). This, together with the observation that the level of $p80^{cdc25}$ increases

as cells proceed through G2, reaching a peak just before the onset of M phase (Moreno *et al.*, 1990), suggested that the accumulation of $p80^{cdc25}$ to a critical level in the cell may have a key role in determining the timing of mitotic onset in *S. pombe*. Homologues of *cdc25* have been demonstrated in budding yeast (Russell *et al.*, 1989), *Drosophila* (Edgar and O'Farrell, 1989) and mammalian cells (Sadhu *et al*, 1990; Galaktionov and Beach, 1991; Kakizuka *et al.*, 1992), further emphasizing its importance in cell cycle control. Ample genetic evidence exists in *S. pombe* to suggest that regulation of the G2/M transition by $p80^{cdc25}$ is achieved through its activation of $p34^{cdc2}$ by Tyr15 dephosphorylation (Gould and Nurse, 1989; Moreno *et al.*, 1989), and this has been substantiated by the demonstration that purified $p80^{cdc25}$ can both tyrosine dephosphorylate and activate $p34^{cdc2}$ in cell-free extracts (Gautier *et al.*, 1991; Kumagi and Dunphy, 1991; Millar *et al.*, 1991; Strausfeld *et al.*, 1991). In addition, the amino acid sequence of $p80^{cdc25}$ exhibits a degree of homology with the vaccinia virus VH1 protein tyrosine phosphatase (Moreno and Nurse, 1991), implying that direct dephosphorylation of Tyr15 of $p34^{cdc2}$ by the *cdc25* gene product is responsible for regulation of entry into mitosis. It appears likely that $p80^{cdc25}$ can also dephosphorylate the inhibitory Thr14 residue in vertebrate $p34^{cdc2}$ (Gautier *et al.*, 1991; Strausfeld *et al.*, 1991). However, the fact that fission yeast can divide in the absence of both *cdc25* and *wee1* functions (Russell and Nurse, 1986) indicates that there are additional controls on the activation of MPF which have yet to be identified. The action of $p80^{cdc25}$ on $p34^{cdc2}$ also seems to play a role in the mechanism which couples S phase to mitosis in *S. pombe* (Enoch and Nurse, 1990), ensuring that M phase cannot occur before DNA replication is complete. The activity of $p80^{cdc25}$ itself has been proposed to be regulated by dephosphorylation, in this case by protein phosphatase 2A, a component of a factor termed INH (Lee *et al.*, 1991) which has been suggested to inhibit the activation of $p34^{cdc2}$ through its effect on $p80^{cdc25}$ (Clarke and Karsenti, 1991), and it is believed that cyclin B may be responsible for the activation of the $p80^{cdc25}$ phosphatase function (Galaktionov and Beach, 1991), which may account for the requirement for interaction with a cyclin molecule for $p34^{cdc2}$ kinase activation at mitosis.

As described in Section 1.4, the *wee1* gene of *S.pombe* encodes an inhibitor of mitosis (Russell and Nurse, 1987a). *Wee1* has been demonstrated to encode a serine/threonine protein kinase (Russell and Nurse, 1987b) which can also phosphorylate tyrosine residues *in vitro* (Featherstone and Russell, 1991). Coexpression of wild-type (wt) *cdc2* and *wee1* in insect cells has been shown to result in phosphorylation of $p34^{cdc2}$ on Tyr15, and this phosphorylation is greatly stimulated by coexpressed cyclin (Parker *et al.*, 1991), strongly suggesting that *wee1* inhibits entry into mitosis by directly or indirectly inactivating $p34^{cdc2}$ *via* the Tyr15

phosphorylation which occurs upon cyclin binding. Wee1 protein activity is controlled in turn by the action of the *nim1* gene product, which exhibits sequence homology with serine/threonine kinases (Hanks *et al.*, 1988). The kinase responsible for the phosphorylation of Thr14 and Thr161/167 has yet to be determined, but it is clear that the activity of $p34^{cdc2}$, and therefore the timing of cell division, is controlled by a complex regulatory network of protein kinases and phosphatases, and that entry into mitosis must be achieved by a shift in the balance of these stimulatory/inhibitory reactions in favour of $p34^{cdc2}$ activation.

An additional component of this regulatory system is the 13 kD protein product of the *suc1* gene, first identified in *S. pombe* as a suppressor of certain ts alleles of *cdc2* (Hayles *et al.*, 1986a), and subsequently demonstrated to interact directly with $p34^{cdc2}$ (Brizuela *et al.*, 1987). Homologues of *suc1* have been identified in *S. cerevisiae* (Hadwiger *et al.*, 1989a), and in higher eukaryotes (Draetta *et al.*, 1987). The overexpression of fission yeast wild-type *suc1* delays entry into mitosis (Hayles *et al.*, 1986b; Hindley *et al.*, 1987), and the addition of $p13^{suc1}$ to *Xenopus* cell-free extracts prevents both initiation of M phase (Dunphy *et al.*, 1988) and tyrosine dephosphorylation of $p34^{cdc2}$ (Dunphy and Newport, 1989), suggesting a negative role for *suc1* in kinase activation at the G2/M transition. It seems likely that this may be accounted for by the observed inhibition of the phosphatase activity of $p80^{cdc25}$ by $p13^{suc1}$ (Galaktionov and Beach, 1991; Gautier *et al.*, 1991). $p13^{suc1}$ may also be involved in the inactivation of $p34^{cdc2}$ kinase on exit from mitosis; in *S. pombe* cells deleted for *suc1*, $p34^{cdc2}$ kinase is activated normally, but this activity remains at a high level and cells become arrested in M phase (Moreno *et al.*, 1989). However, the mechanism by which $p13^{suc1}$ promotes the inactivation of $p34^{cdc2}$ kinase at the M/G1 transition remains unclear.

1.7 How does $p34^{cdc2}$ bring about the events of mitosis?

A dramatic reorganization of both nuclear and cytoplasmic structures must occur for cell division to take place. Chromatin condensation is required to permit the segregation of the chromosomes prior to cytokinesis, and the nuclear envelope must be dissolved and a mitotic spindle formed. In addition, transcription and translation are inhibited during mitosis (Taylor, 1960; Baserga, 1962; Prescott and Bender, 1962; Konrad, 1963). The role of active MPF in these processes is beginning to be elucidated, but much work remains to be done. $p34^{cdc2}$ is a serine/threonine protein kinase and its consensus recognition sequence, Ser/Thr-Pro followed by a basic residue (Langan, 1978), is found in a large number of proteins. Many potential substrates have been demonstrated to be phosphorylated *in vitro* by $p34^{cdc2}$ (Table 1.2), but in

the majority of cases it remains to be seen whether these represent true substrates *in vivo*, and the functional consequences of their phosphorylation have generally not yet been determined.

Table 1.2 Potential $p34^{cdc2}$ substrates

Target for $p34^{cdc2}$	**Possible role in mitosis**	**Reference**
H1 histone	chromatin condensation	Bradbury *et al.*, 1974; Langan, 1978; Arion *et al.*, 1988; Labbe *et al.*, 1988; Langan *et al.*, 1989
HMG-1 nonhistone protein	chromatin condensation	Reeves *et al.*, 1991
Lamins	nuclear envelope breakdown	Peter *et al.*, 1990a; Ward and Kirschner, 1990
Nucleolin	nucleolar disassembly	Belenguer *et al.*, 1990; Peter *et al.*, 1990b
MAP kinase	microtubule reorganization	Gotoh *et al.*, 1991
$pp60^{c\text{-}src}$	cytoskeletal rearrangements	Morgan *et al.*, 1989; Shenoy *et al.*, 1989
RNA polymerase II	transcription inhibition	Cisek and Corden, 1989
EF-1β, EF-1γ	translation inhibition	Belle *et al.*, 1989
Vimentin	intermediate filament reorganization	Chou *et al.*, 1990

Perhaps the clearest evidence for a direct role for MPF in mitotic events is provided by the observation that not only does $p34^{cdc2}$ phosphorylate nuclear envelope lamin molecules on serine residues shown to be required in a phosphorylated state for lamina disassembly (Heald and McKeon, 1990), but that active MPF can cause solubilization of the nuclear lamina in cell-free extracts (Peter *et al.*, 1990a; Ward and Kirschner, 1990; Dessey *et al.*, 1991). The phosphorylation of $pp60^{c\text{-}src}$ by $p34^{cdc2}$ is also of interest since this has been suggested to be correlated with an increase in the $pp60^{c\text{-}src}$ tyrosine kinase activity (Shenoy *et al.*, 1989), thus activation of MPF at mitosis may set in motion a cascade of regulatory kinases, with the potential to affect the phosphorylation state of a large number of divergent substrates, the combined action of which bring about the complex interplay of events

known as mitosis. In addition, it has been suggested (Moreno and Nurse, 1990) that the $p34^{cdc2}$ consensus phosphorylation site may constitute a DNA-binding motif, and that phosphorylation by $p34^{cdc2}$ may disrupt this structure and thus remove the protein from the DNA (Churchill and Suzuki, 1989; Suzuki, 1989). An additional role for active MPF at mitosis may therefore be in the displacement of proteins from chromatin in order to facilitate the required chromosome condensation.

Reentry into interphase requires the dephosphorylation of the mitotic substrates of $p34^{cdc2}$. In contrast to the situation with cell cycle protein kinases, much less is known about the protein phosphatases which presumably act at the M/G1 transition. However, genes encoding type 1 phosphatases have been identified in both *S. pombe* (*bws1/dis2* - Booher and Beach, 1989; Ohkura *et al.*, 1989) and *Aspergillus nidulans* (*bimG* - Doonan and Morris, 1989) which are involved in the mitotic control pathway, and microinjection of mammalian cells with neutralizing anti-phosphatase type 1 antibodies before division caused metaphase arrest (Fernandez *et al.*, 1992), clearly implicating phosphatase activity in regulation of the cell cycle, although how this phosphatase activity is controlled is not known at present.

1.8 Involvement of $p34^{cdc2}$ at the G1/S transition

In addition to disrupting entry into mitosis, mutations in *S. cerevisiae CDC28* and *S. pombe cdc2* have been demonstrated to block the G1/S transition (Reed, 1980; Nurse and Bissett, 1981; Piggott *et al.*, 1982). The function of $p34^{cdc2}$ in G1 is only starting to be characterized, however, mainly due to a lack of good assays for its G1 kinase activity. Genetic evidence in *S. cerevisiae* suggests the existence of a distinct class of G1-specific cyclins, encoded by the *CLN* genes, which are associated with the function of $p34^{cdc2}$ in G1. Budding yeast cells with mutations in the gene *CLN3* (previously known as *WHI-1* or *DAF-1*) were shown to progress through Start prematurely (Sudbery *et al.*, 1980; Cross, 1988; Nash *et al.*, 1988), suggesting that the gene product is a rate-limiting activator of Start. Two similar genes, *CLN1* and *CLN2*, were found in a screen for high copy-number suppressors of *CDC28* mutations (Hadwiger *et al.*, 1989a). Sequencing of all three genes revealed weak but significant homology to mitotic cyclins (Nash *et al.*, 1988; Hadwiger *et al.*, 1989b), and *CLN2* has been shown to directly associate with $p34^{CDC28}$ to form an active protein kinase complex (Wittenberg *et al.*, 1990). A fourth G1 cyclin, *HCS26/CLN4*, has also recently been identified in *S. cerevisiae* (Ogas *et al.*, 1991). Like the mitotic cyclins, the G1 cyclin level varies through the cell cycle, but in this case peak levels are attained in G1, and they are subsequently degraded during S phase (Wittenberg *et al.*, 1990). Cells harbouring single deletions of *CLN1*, *CLN2* or *CLN3* remain viable, but elimination of

all three results in G1 arrest, thus the G1 cyclins appear to be members of a functionally redundant family, with passage through Start requiring the gene product of at least one CLN gene (Richardson *et al.*, 1989). The accumulation of a critical threshold level of the G1 cyclins appears to be required for activation of $p34^{CDC28}$, as the presence of multiple copies of the wt *CLN* alleles, as well as normal expression of certain dominant mutations in these genes, advances the G1/S transition, with the cells passing directly from mitosis to S phase (Sudbery *et al.*, 1980; Cross, 1988; Nash *et al.*, 1988; Hadwiger *et al.*, 1989b). Analysis of such dominant mutations (Cross, 1988; Nash *et al.*, 1988; Hadwiger *et al.*, 1989b) demonstrated that premature stop codons within the genes resulted in truncated gene products lacking the carboxy-terminal PEST region common to all G1 cyclins, the presence of which causes intrinsic instability of proteins (Rogers *et al.*, 1986), thereby confirming that untimely accumulation of cyclins due to a lack of degradation is capable of advancing the cell cycle. The G1 cyclins are therefore thought to act as rate-limiting inducers of S phase. *CLN1* and *CLN2* are transcribed in late G1 (Wittenberg *et al.*, 1990), thus the periodicity of accumulation of their protein products may be accounted for by transcriptional regulation, which appears to be under the positive control of *CLN3*, active $p34^{cdc2}$ (Dirick and Nasmyth, 1991) and the transcriptional regulators *SWI4* and *SWI6* (Nasmyth and Dirick, 1991; Ogas *et al.*, 1991), implying the existence of a positive feedback loop. However, CLN3 mRNA is expressed constitutively (Nash *et al.*, 1988; Wittenberg *et al.*, 1990), thus regulation of CLN3 protein abundance presumably occurs at the post-transcriptional level.

It thus appears that, at least in *S. cerevisiae*, passage through Start i.e. commitment to the mitotic cell cycle, occurs when G1-specific cyclins accumulate to a threshold level and activate $p34^{CDC28}$. At this point in the cdc, external conditions determine whether the cell proceeds through the rest of the cycle, enters stationary phase or undergoes conjugation (Hartwell, 1974). The underlying basis for this decision-taking is now beginning to be explained by the observed effect of the environment on G1 cyclin accumulation, accounting for the mechanism by which external signals may influence the activity of $p34^{CDC28}$ kinase. Nutritional depletion has been demonstrated to prevent the accumulation of G1 cyclins, and therefore the activation of $p34^{CDC28}$, presumably due to an decrease in the rate of protein synthesis, and thus causes entry into stationary phase at Start (C. Wittenberg and S.I. Reed, unpublished work quoted in Reed, 1991). Mating factors also block the *S. cerevisiae* cell cycle at Start, and allow the cells to be diverted to the conjugation pathway (Bucking-Throm *et al.*, 1973). α-factor appears to function by preventing the accumulation of *CLN1* and *CLN2* mRNA (Wittenberg *et al.*, 1990), with *CLN2* transcription possibly being blocked by the pheromone-induced *FAR-1* gene (Chang and

Herskowitz, 1990), and expression of the conjugation-specific *FUS3* gene inhibits both the transcription of the *CLN1* and *CLN2* genes and the accumulation of CLN3 protein (Elion *et al.*, 1991). It thus appears that the appropriate proliferative response to extracellular signals may be mediated by the G1 cyclins.

The substrates for active $p34^{CDC28}$ kinase which, on phosphorylation, bring about the process of DNA replication in S phase are not yet known. Prior to S phase, a transcriptional programme is set in train which results in the formation of the gene products required for DNA synthesis (see Section 1.1), thus it is possible that $p34^{CDC28}$ may phosphorylate and activate transcription factors involved in this process. However, there are no data to support this idea as yet, or to confirm the hypothesis that $p34^{CDC28}$ may be involved in the chromatin and cytoskeletal reorganizations that occur at S phase. Of interest, though, is the observation that $p34^{cdc2}$ phosphorylates the SV40 large T antigen *in vitro*, and that this phosphorylation is required for T antigen to be active in SV40 DNA replication (McVey *et al.*, 1989), removal of the *cdc2* gene product from *Xenopus* egg extracts eliminates their ability to initiate DNA replication *in vitro* (Blow and Nurse, 1990), and $p34^{cdc2}$ has also been demonstrated to be a component of RF-S, a factor shown to activate DNA synthesis in cell-free extracts (D'Urso *et al.*, 1990), the first pieces of evidence linking its activity directly to S phase processes.

Although the primary control point for regulation of the mammalian cdc appears to be in G1 (Section 1.2), at present there is no mutational evidence for a G1 function for $p34^{cdc2}$ homologues or for the presence of G1 cyclins in mammalian cells, presumably due mainly to the inherent difficulties in genetic manipulation of higher eukaryotes. However, candidate G1 cyclins have been identified in human (Koff *et al.*, 1991; Lew *et al.*, 1991; Motokura *et al.*, 1991) and murine (Matsushime *et al.*, 1991; Kiyokawa *et al.*, 1992) cells, expression of which may be induced by growth factor treatment (Matsushime *et al.*, 1991), mammalian cyclin A has been suggested to play a role in the G1/S phase in addition to its mitotic function (Girard *et al.*, 1991; Pagano *et al.*, 1992), and the elimination of *cdc2* expression in human cells by antisense RNA prevents entry into S phase (Furukawa *et al.*, 1990). In addition, Pardee's restriction point (Pardee, 1974), the traverse of which requires the accumulation of a labile regulatory molecule, the restriction or R protein (Rossow *et al.*, 1979), shows obvious similarities to Start control in budding yeast, and it is possible that the R protein may represent a human homologue of the *S. cerevisiae* G1 cyclins. It also seems likely that the cytoplasmic S phase-initiating factor, indicated by mammalian cell fusion studies as accumulating in late G1 (Rao and Johnson, 1970), will be identified as an activated $p34^{cdc2}$ homologue complexed with G1 cyclins. Indeed, the human *cdc2/CDC28* homologue has been shown to complement

mutations of *CDC28* in *S. cerevisiae* (Wittenberg and Reed, 1989), and thus can presumably form productive complexes with budding yeast G1 cyclins and perform the function of an active $p34^{cdc2}$ kinase in G1, implying the existence of similar G1-specific cyclins in mammalian cells. However, such evidence is indirect, and confirmation of a role for $p34^{cdc2}$ in G1 in higher eukaryotes must await further study.

1.9 A universal control mechanism for the eukaryotic cell cycle?

Taking these studies as a whole, a unifying hypothesis for the control of the cell cycle can be postulated, and it appears that this mechanism is highly conserved in all eukaryotic cells thus far examined. The central molecule in this scheme is $p34^{cdc2}$, whose kinase activity is required to bring about the processes of both DNA replication and mitosis. This protein may be considered to exist in two different states, as an S phase-promoting factor (SPF) and an M phase-promoting factor (MPF), distinguished by its association with various cyclin molecules. The latter proteins appear to act as regulatory subunits, altering the substrate specificity and compartmentalization of $p34^{cdc2}$, and thus allowing a functional, spatial and temporal separation of its cell cycle roles. The G1-specific cyclins accumulate during G1 at a rate influenced by the external environment, and when a threshold level is reached the active SPF, consisting of $p34^{cdc2}$ complexed with a G1 cyclin, triggers entry into S phase. In G2, the association of $p34^{cdc2}$ with mitotic cyclins to form MPF, and its subsequent activation by the rate-limiting dephosphorylation of Tyr15 on $p34^{cdc2}$ by $p80^{cdc25}$ causes initiation of mitosis.

This model predicts that in all eukaryotic cells, both a G1/S and a G2/M control point exists. The observed role of $p34^{cdc2/CDC28}$ in both the G1 and G2 phases of *S. cerevisiae* and *S. pombe* (Nurse and Bissett, 1981; Piggott *et al.*, 1982; Reed and Wittenberg, 1990) suggests that this may indeed be the case in yeasts. In addition, G1-like cyclins have recently been identified in *S. pombe* (Bueno *et al.*, 1991; Forsburg and Nurse, 1991), and mitotic cyclins have been found in *S. cerevisiae* (Ghiara *et al.*, 1991; Surana *et al.*, 1991), tending to support this hypothesis. The existence of a G2 control mechanism in mammalian cells has not been widely studied, perhaps due to the relative paucity of G2 mutants. However, several lines of evidence point to the presence of mitotic control in mammalian cells, namely the demonstration that cells can arrest stably in G2 (Gelfant, 1981; Melchers and Lernhardt, 1985; Gomez-Lechon and Castell, 1987), the isolation of a number of ts cdc mutants that halt in both G1 and G2 at the npt (Chen and Wang, 1982; Ohno *et al.*, 1984; Zaitsu and Kimura, 1985) and of a *cdc2* mutation that blocks murine cells in G2 (Th'ng *et al.*, 1990), and

the widespread distribution of MPF among higher eukaryotic cells (Arion *et al.*, 1988; Labbe *et al.*, 1988; 1989a; Brizuela *et al.*, 1989; Langan *et al.*, 1989), thus direct study of this question is clearly required.

Despite the great advances made in understanding the regulation of the eukaryotic cell cycle in recent years, a number of unanswered questions nonetheless remain:

a. What is the molecular mechanism by which the cell division cycle is coupled to the growth cycle? It seems likely that this may be achieved through those components of the system which are rate-limiting for cell cycle traverse i.e. the G1 cyclins and $p80^{cdc25}$, but further work is necessary to determine whether this is the case.

b. It is becoming clear that $p34^{cdc2}$ belongs to a cyclin-dependent protein kinase (CDK) family with an increasing membership (see Pines and Hunter, 1991b for review), raising the possibility that varying combinations of p34 homologues and cyclin molecules could have separate functional roles in the cell cycle, and indeed there is some evidence to suggest that this may be the case (Fang and Newport, 1991).

c. Analysis of cdc mutants in *Aspergillus nidulans* has identified other genes, including *nimA* (Osmani *et al.*, 1987; 1988a) and *bimE* (Osmani *et al.*, 1988b), which, although unrelated to known yeast cell cycle genes, may act as regulators of mitosis. It is not yet known whether homologues of these genes exist in other eukaryotes, or how they fit into the universal mechanism of cell cycle control.

d. It is unclear at present how the different signals originating from growth factors and other mitogens, which presumably all have an increase in the kinase activity of $p34^{cdc2}$ as their common endpoint, bring about this activation. A detailed knowledge of the precise details of the signalling pathways utilized by growth factors would clearly be of great value in answering this question.

1.10 Growth factor signal transduction pathways

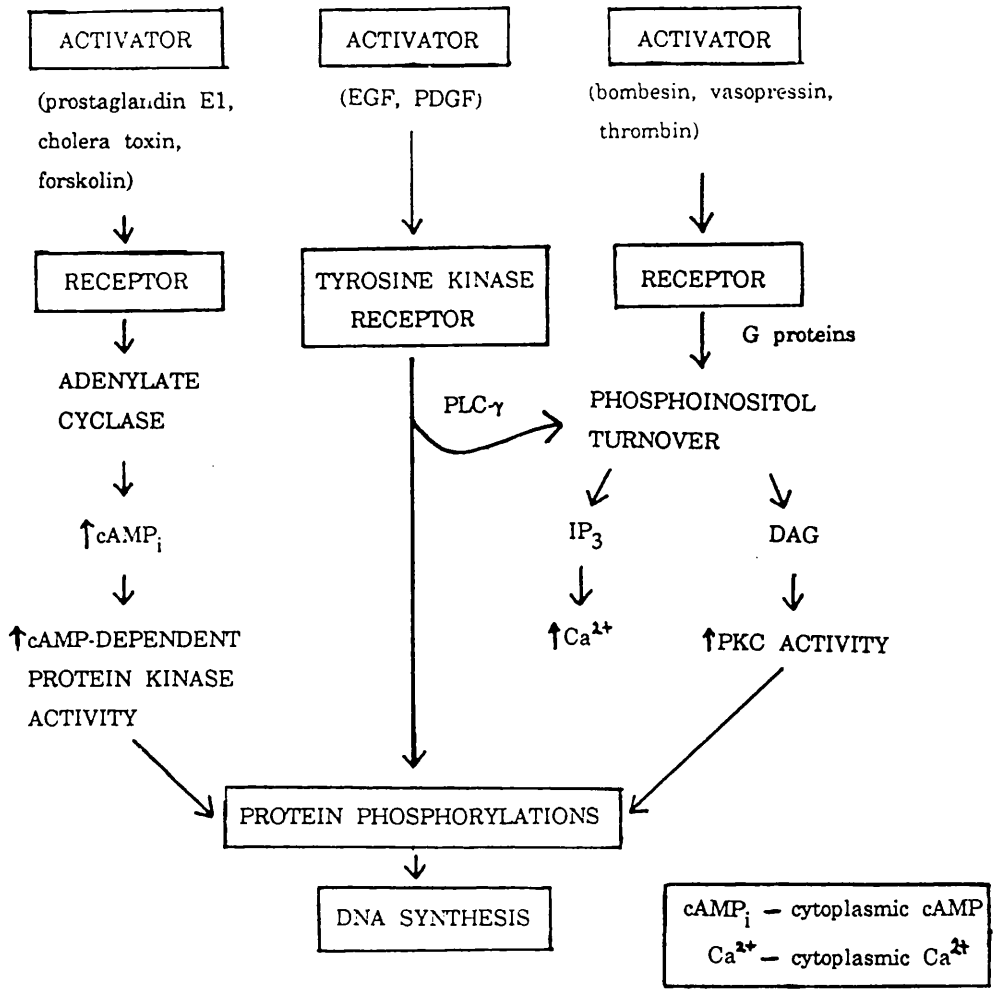
Stimulation by polypeptide growth factors, regulatory peptides or hormones, acting singly or in synergistic combinations, is required to induce mitogenesis in cultured mammalian cells, and such mitogens appear to exert their effects on the control of initiation of DNA synthesis in G1 (see Section 1.2). The proliferative response of cells is thus determined by the extracellular environment, the precise combination of mitogens required and the cellular response to a given factor varying among different cell types (see Sporn and Roberts, 1988 for review). The mechanism by which the external mitogenic signal is transduced across the plasma membrane and is subsequently propagated into the nucleus, resulting in the necessary alterations in

gene expression, is starting to be unravelled, but many of the molecular aspects of the flow of information leading to a mitogenic response remain unclear. Three main classes of signal transduction pathways utilized by mitogens, each producing a variety of second messengers, have been delineated, and these ultimately converge into a common response leading to DNA synthesis and mitosis (Fig. 1.6). Synergistic and inhibitory interactions between various components of each pathway occur, and a given growth factor may induce only one or any combination of the various mechanisms available in each cell type.

1.10.i Phosphoinositol turnover and protein kinase C activation

The interaction of a wide variety of mitogens, such as bombesin, vasopressin and thrombin, with their cell surface receptors, has been demonstrated to provoke the hydrolysis of inositol phospholipids in the plasma membrane of many different cell types (Michell, 1975; Michell *et al.*, 1981; Creba *et al.*, 1983; Berridge, 1984). The initiating reaction in phosphoinositol turnover is the activation of the receptor-coupled enzyme phospholipase C (PLC) on ligand binding, which catalyzes the breakdown of phosphoinositol (4,5) bisphosphate (PIP_2) to the second messengers diacylglycerol (DAG) and inositol (1,4,5) trisphosphate (IP_3) (Michell *et al.*, 1981; Berridge, 1983; Abdel-Latif *et al.*, 1986; Majerus *et al.*, 1986). Numerous studies have implied that the coupling of receptor function to PLC is mediated by an unspecified guanine nucleotide-binding (G) protein (Cockcroft and Gomperts, 1985; Litosch *et al.*, 1985; Taylor *et al.*, 1986; Cockcroft, 1987; Fain *et al.*, 1988). Although the precise nature of the G protein involved has, in most cases, yet to be elucidated, there is evidence to suggest that differing but closely related G proteins may perhaps mediate this process in different cells and/or at different receptors (Martin *et al.*, 1985; Cockcroft and Stutchfield, 1988; Ashkenazi *et al.*, 1989). DAG can also be generated from agonist-induced phosphatidyl choline hydrolysis, catalyzed by either PLC or phospholipase D (Besterman *et al.*, 1986; Muir and Murray, 1987; Pai *et al.*, 1991), without producing IP_3, thus adding a further level of complexity to the system. Many isozymes of PLC exist (Rhee *et al.*, 1989), with tissue-specific patterns of expression (Gerfen *et al.*, 1988; Suh *et al.*, 1988), suggesting that each G protein may be responsible for selectively coupling a distinct receptor population to a specific PLC isoform (Ashkenazi *et al.*, 1989), thus producing a different response according to the mitogen in question. Alternatively, it may be that different G proteins and PLC isozymes are operative in various cell types, allowing for cell-specific reactions to a given growth factor.

Figure 1.6 Generalized working hypothesis of mitogenic signal transduction pathways (adapted from Dumont *et al.*, 1989 - see text for details)



The function of DAG in signal transduction is to mediate the activation of protein kinase C (PKC), a serine/threonine kinase (Nishizuka *et al.*, 1984; Nishizuka, 1986) which requires calcium and phospholipids for its activity (Takai *et al.*, 1979a). PKC is also a member of a gene family encoding several closely related yet distinct isozymes (Nishizuka *et al.*, 1988; Nishizuka, 1989) with different tissue-specific patterns of expression (Ohno *et al.*, 1987; 1988; Brandt *et al.*, 1987), cofactor requirements and substrate specificities (K.-P. Huang *et al.*, 1988; Marais and Parker, 1989). It thus appears that cells can call upon a wide repertoire of signal transduction components, including a number of G proteins and isoforms of PLC and PKC, which may provide the cell with the ability to generate subtly different effects from one basic pathway, and thus produce a diversity of responses in different tissues and cell types to varying external stimuli. The primary effect of DAG is to greatly increase the

affinity of PKC for calcium, fully activating the enzyme without any change in cytosolic calcium levels (Takai *et al.*, 1979b; Kishimoto *et al.*, 1980; Nishizuka, 1983). PKC has a broad substrate specificity, phosphorylating many proteins *in vitro*, including vinculin (Werth *et al.*, 1983), histone H1 (Martelli *et al.*, 1989) and lamin B (Hornbeck *et al.*, 1988), but the nature of its physiological substrates in most tissues, and the biological significance of their phosphorylation has remained largely unexplored. However, phosphorylation by PKC has been postulated to regulate the activity of a number of transcription factors, such as the Jun/Fos heterodimer (Nishizuka, 1989), the cyclic AMP-response element binding protein (CREB) (Yamamoto *et al.*, 1988) and NF-κβ (Shirakawa and Mizel, 1989), and PKC may regulate the cell's capacity for protein synthesis through the phosphorylation and activation of ribosomal S6 protein (Le Peuch *et al.*, 1983) and eukaryotic initiation factor 2 (Schatzman *et al.*, 1983), thus activation of PKC could potentially produce the rapid changes in gene expression that are required prior to the initiation of DNA synthesis. A further level of regulation is suggested by the observation that an endogenous protein becomes phosphorylated on tyrosine residues when cells are treated with synthetic DAG (Gilmore and Martin, 1983), implying a possible cascade reaction from PKC to tyrosine kinases. It therefore appears that the activation of PKC has a widespread effect upon the expression and activity of a large number of cellular proteins, but the precise mechanism by which this brings about mitogenesis has still to be resolved. The issue is further complicated by the fact that the PKC pathway is known to regulate a large array of cellular processes, including metabolism, secretion and differentiation (Miao *et al.*, 1978; Vandenbark *et al.*, 1984; Hokin, 1985; Nishizuka, 1986; 1989; Berridge and Michell, 1988), such that those specifically relevant to the proliferative response must be determined. The importance of PKC in the proper regulation of growth control is, however, emphasized by the demonstration that phorbol esters, compounds which interact directly with PKC to activate it in the absence of the appropriate external stimulation (Castagna *et al.*, 1982; Ashendel, 1985), are potent tumour promoters, and mutations in the PKC molecule itself have also been associated with tumourigenesis (Megadish and Mazurek, 1989).

In addition to the production of DAG, the hydrolysis of PIP_2 produces IP_3, which acts as an intracellular mediator of calcium mobilization (Streb *et al.*, 1983; Berridge and Irvine, 1984); IP_3 causes the release of Ca^{2+} ions from internal stores, thought to be principally located in the endoplasmic reticulum (Streb *et al.*, 1984; Mignery *et al.*, 1989), *via* its action on a specific receptor molecule, which has been purified (Supattapone *et al.*, 1988a) and shown to act as a ligand-gated Ca^{2+} channel (Ferris *et al.*, 1989; Gill, 1989). PKC activation and calcium mobilization have been

demonstrated to act synergistically to elicit the full physiological response to growth factors utilizing the PKC pathway, and neither is sufficient alone for complete signal transduction (Kaibuchi *et al.*, 1982; 1983; Kajikawa *et al.*, 1983; Nishizuka, 1983; Yamanishi *et al.*, 1983). The role of increased cytosolic calcium in this process remains unclear, however.

The production of both DAG and IP_3 is transient, and each compound is rapidly degraded (Nishizuka, 1984; Majerus *et al.*, 1986; Joseph and Williamson, 1989; Petersen, 1989), ensuring that the proliferative signal, once received and transduced, is terminated. In addition, a number of negative feedback loops are employed by the system; for example, DAG may ultimately be metabolized to prostaglandin endoperoxide and arachidonate peroxide, each of which activates guanylate cyclase (Graff *et al.*, 1978) to produce cyclic GMP (cGMP), which inhibits receptor-linked phosphoinositol turnover (Takai *et al.*, 1981; Nishizuka, 1983). The activation of PKC by phorbol esters in the absence of mitogens does not produce DAG and therefore would not involve such a cGMP feedback loop, thus a persistent proliferative signal may result, accounting perhaps for the ability of these compounds to promote tumour formation.

1.10.ii Receptor tyrosine kinases

Many growth factors e.g. platelet-derived growth factor (PDGF), epidermal growth factor (EGF), insulin, insulin-like growth factors and colony-stimulating factor-1 (CSF-1), mediate their pleiotropic actions by binding to cell surface receptors with an intrinsic protein tyrosine kinase activity (Yarden and Ullrich, 1988). Such growth factor receptors all display a similar molecular topology; each possesses a glycosylated extracellular ligand-binding domain, a hydrophobic transmembrane region and a cytoplasmic portion with tyrosine kinase catalytic activity (Schlessinger, 1988; Yarden and Ullrich, 1988; Williams, 1989). The best-studied receptors of this class are the PDGF and EGF receptors. In each case, binding of the ligand stimulates the tyrosine kinase activity of the receptor (Carpenter *et al.*, 1979; Ushiro and Cohen, 1980; Heldin *et al.*, 1989), which has been shown, by analysis of mutant receptors, to be essential for signal transduction and mitogenesis (Chen *et al.*, 1987; Honegger *et al.*, 1987; Escobedo *et al.*, 1988; Moolenaar *et al.*, 1988). Ligand binding induces receptor dimerization (Yarden and Schlessinger, 1987; Cochet *et al.*, 1988; Bishayee *et al.*, 1989; Heldin *et al.*, 1989; Seifert *et al.*, 1989), and it is believed that this plays a role in the subsequent kinase activation (Boni-Schetzler and Pilch, 1987; Yarden and Schlessinger, 1987; Heldin *et al.*, 1989). Binding of PDGF to its receptor causes autophosphorylation of the receptor molecule at two major sites (Kazlauskas and

Cooper, 1989); a consensus tyrosine residue, Tyr 857, identified in all tyrosine kinases analysed to date (Hanks *et al.*, 1988), and Tyr 751 in the kinase insert region of the receptor, a stretch of hydrophobic amino acids which interrupts the kinase region and is thought to modulate the interaction of the receptor with its substrates (Escobedo and Williams, 1988; Kazlauskas and Cooper, 1989; Severinsson *et al.*, 1990). The interaction of EGF with its receptor also induces autophosphorylation of tyrosine residues in the carboxy-terminal tail of the glycoprotein (Margolis *et al.*, 1989a). It is thought that receptor dimerization may facilitate the autophosphorylation of each type of receptor by allowing intermolecular cross-phosphorylation of tyrosine residues (Honegger *et al.*, 1989; 1990; Heldin and Westermark, 1990). Mutational analyses suggest that autophosphorylation of Tyr 751 in the PDGF receptor, and of the carboxy-terminal tyrosine residues of the EGF receptor, allows the receptor molecule to adopt a conformation in which the binding site for its substrates becomes available (Bertics *et al.*, 1988; Honegger *et al.*, 1988; Kazlauskas and Cooper, 1989; Margolis *et al.*, 1990). Following transduction, the signal is terminated by receptor internalization, followed by either degradation or recycling of the molecule to the cell surface (Carpenter and Cohen, 1976; Cohen *et al.*, 1979; Fox *et al.*, 1979; Nilsson *et al.*, 1983; Rosenfeld *et al.*, 1984; Sorkin *et al.*, 1989).

A number of substrates have been demonstrated to become phosphorylated on tyrosine residues in response to the stimulation of cells with either PDGF or EGF, but the functional consequences of the phosphorylation events, and the particular substrates important for the induction of DNA synthesis, have in most cases still to be elucidated. PLC-γ forms a complex with the appropriate activated receptor on ligand binding (Kumjian *et al.*, 1989; Margolis *et al.*, 1989b; Morrison *et al.*, 1990). It is thought that this results in direct tyrosine phosphorylation and consequent activation of PLC-γ by each receptor type (Margolis *et al.*, 1989b; Meisenhelder *et al.*, 1989; Nishibe *et al.*, 1989; 1990a; 1990b; Wahl *et al.*, 1989a; 1989b; Kim *et al.*, 1991), and that this activation is responsible for the phosphoinositol turnover, PKC activation, IP_3 formation and increased cytosolic calcium observed on stimulation of certain cell types with PDGF or EGF (Sawyer and Cohen, 1981; Hepler *et al.*, 1987; Ives and Daniel, 1987; Johnson and Garrison, 1987; Pike and Eakes, 1987; Wahl *et al.*, 1987; Kazlauskas and Cooper, 1988). However, activation of PLC-γ may not be obligatory for the induction of DNA synthesis by PDGF (Hill *et al.*, 1990), nor is it sufficient on its own to evoke a growth response, since mutant PDGF receptors exist which retain the ability to complex PLC-γ and to induce phosphoinositol turnover but are unable to evoke mitogenesis (Morrison *et al.*, 1990), indicating that additional pathways are also required.

The activated PDGF and EGF receptors have also been shown to associate with a

number of other potential substrates, such as phosphoinositol (PI) 3-kinase, which complexes with the kinase insert domain of the receptor and is believed to be activated by tyrosine phosphorylation (Coughlin *et al.*, 1989; Kazlauskas and Cooper, 1989; Bjorge *et al.*, 1990). This enzyme catalyzes the phosphorylation of inositol phosphate and phosphoinositol phosphates at the 3' position on the inositol ring (Whitman *et al.*, 1988), thus generating novel phosphoinositol polyphosphates, which may also be involved in transduction of the PDGF- and EGF-induced mitogenic response (Auger *et al.*, 1989). PI 3-kinase has been demonstrated to associate with both the activated *v-abl* oncoprotein (Varticovski *et al.*, 1991) and the polyoma middle T antigen:pp60$^{c\text{-}src}$ complex in transformed cells (Courtneidge and Heber, 1987), and this interaction is thought to be important for the tumourigenic activity of the oncogenes (Talmage *et al.*, 1989; Varticovski *et al.*, 1991). In addition, a mutant PDGF receptor that was unable to associate with PI 3-kinase but still caused phosphoinositol turnover had lost mitogenicity (Coughlin *et al.*, 1989), further suggesting that this enzyme has an important role to play in the control of cell division.

pp60$^{c\text{-}src}$ itself has also been shown to bind to the activated EGF and PDGF receptors (Anderson *et al.*, 1990), and it is possible that this association may play a causal role in the increase in tyrosine kinase activity of pp60$^{c\text{-}src}$ observed on PDGF stimulation of fibroblasts (Kypta *et al.*, 1990). An additional protein thought to associate with and be tyrosine phosphorylated by both the PDGF and EGF receptors is the GTPase-activating protein, GAP (Molloy *et al.*, 1989; Ellis *et al.*, 1990; Kaplan *et al.*, 1990; Kazlauskas *et al.*, 1990), which is believed to be involved in the regulation of G protein-mediated coupling of receptors to mitogenic signalling systems (Hall, 1990; Parsons, 1990; Bourne *et al.*, 1991), and may thus link receptor tyrosine kinases to a further cascade of signal transduction molecules. It is thought that PLC-γ, GAP, PI 3-kinase and pp60$^{c\text{-}src}$ all bind to the activated receptor *via* a common homologous domain, the SH2 domain, which is present in each protein and could account for the observed association of many structurally diverse molecules with each receptor (Anderson *et al.*, 1990; Koch *et al.*, 1991). This also raises the possibility that they may bind as a single complex, allowing for regulatory interactions between them (Kaplan *et al.*, 1990; Morrison *et al.*, 1990).

In addition to causing tyrosine phosphorylation of intracellular proteins, EGF and PDGF have also been shown to activate multiple serine/threonine kinases as part of a cascade of tyrosine kinase action; these include casein kinase II (Ackerman *et al.*, 1990), ribosomal S6 kinase (Ballou *et al.*, 1988), microtubule-associated protein (MAP)-2 kinase (Hoshi *et al.*, 1988) and Raf-1 kinase, which is directly activated *in vitro* by association with, and tyrosine phosphorylation by, the receptor kinase (Morrison *et al.*,

1989).

It thus appears that mitogens utilizing tyrosine receptor kinase receptors produce a multiplicity of molecular changes in their target cells, phosphorylating and activating a wide range of substrates, but the precise causal role of each of these processes in the subsequent stimulation of cell division is unclear. In common with the EGF and PDGF receptors, the insulin tyrosine kinase receptor is activated by intermolecular autophosphorylation on ligand binding (Boni-Schetzler *et al.*, 1988; Ballotti *et al.*, 1989), and this tyrosine kinase activity is essential for mitogenesis (Chou *et al.*, 1987). The activities of ribosomal S6 kinase (Tabarini *et al.*, 1985), PI 3-kinase (Ruderman *et al.*, 1990), Raf-1 kinase (Blackshear *et al.*, 1990) and MAP-2 kinase (Hoshi *et al.*, 1988) are stimulated by this activation, but there is no evidence for phosphorylation of PLC-γ (Nishibe *et al.*, 1990b) or increased phosphoinositol turnover (Pouyssegur *et al.*, 1988). It seems therefore that although receptor tyrosine kinase cellular signalling pathways may overlap, differences do exist between them, presumably allowing the cell to respond in a specific manner to each growth factor. However, until an understanding of the role of each component of the various transduction systems is attained, the significance of these differences will remain unclear.

1.10.iii Adenylate cyclase-linked receptors

A third class of receptors, which bind such mitogens as cholera toxin, prostaglandin E1 and forskolin, is coupled to adenylate cyclase *via* a G protein (Gilman, 1984) to produce a single second messenger, namely cyclic AMP (cAMP) (Dumont *et al.*, 1989). This stimulates the activity of cAMP-dependent protein kinase (Hoppe and Wagner, 1979; Hemmings, 1985), a serine/threonine kinase (Cohen, 1985; Edelman *et al.*, 1987) which phosphorylates and activates CREB (Gonzalez and Montminy, 1989), the transcription factor (Montminy and Bilezikjian, 1987; Hoeffler *et al.*, 1988; Yamamoto *et al.*, 1988) which recognizes the cAMP-response element (CRE) found in the promoter of genes whose expression is induced by an increase in cytoplasmic cAMP e.g. the somatostatin (Montminy *et al.*, 1986; Montminy and Bilezikjian, 1987; Yamamoto *et al.*, 1988), human glycoprotein α-subunit (Delegeane *et al.*, 1987; Silver *et al.*, 1987), *c-fos* (Sassone-Corsi *et al.*, 1988a, Berkowitz *et al.*, 1989), proenkephalin (Comb *et al.*, 1986) and insulin (Philippe and Missotten, 1990) genes.

There is considerable regulatory cross-talk between the components of the cAMP signalling system and the PKC pathway, with cAMP inhibiting phosphoinositol turnover (Nishizuka, 1983; Kato *et al.*, 1986; Neylon and Summers, 1988) and IP_3-induced calcium release (Supattapone *et al.*, 1988b), but inducing expression of the PDGF receptor (Weinmaster and Lemke, 1990). E-type prostaglandins, produced from

DAG (Nishizuka, 1984), have also been shown to stimulate cAMP release (Chlapowski *et al.*, 1975).

In summary, the cell possesses multiple pathways for the transduction of mitogenic signals, allowing for cell type- and growth factor-specific responses depending upon the complement of receptors present and the signalling system utilized. The issue is further complicated by the fact that some growth factors, such as transforming growth factor-β (see Moses *et al.*, 1990 for review), can be inhibitory for cell growth in certain cell types. In theory, each element involved in signal transduction would be expected to have an oncogenic potential, since a mutation resulting in its constitutive activity would release the cell from dependence on an extracellular initiating event, and thus result in inappropriate proliferation, and indeed many components at different levels have been demonstrated to have oncogenic counterparts: *v-sis*, the transforming gene of simian sarcoma virus, has been shown to code for a PDGF-like growth factor (Doolittle *et al.*, 1983; Waterfield *et al.*, 1983); the *neu/c-erb B2* oncogene encodes a molecule which exhibits homology with the EGF receptor (Schechter *et al.*, 1984) but which has constitutive tyrosine kinase activity due to a single point mutation in the transmembrane region (Bargmann *et al.*, 1986; Bargmann and Weinberg, 1988); mutations in *v-fms*, which codes for a CSF-1 receptor (Sherr *et al.*, 1985), also result in ligand-independent activation (Roussel *et al.*, 1988; Woolford *et al.*, 1988); the *ras* oncogenes encode proteins with homology to signal-transducing G proteins (Barbacid, 1987) which have been shown to activate PKC (Morris *et al.*, 1989), and the serine/threonine kinase Raf-1 was originally identified as the transforming gene of murine sarcoma virus (Rapp *et al.*, 1983).

1.11 Transduction of the mitogenic signal to the nucleus

The treatment of quiescent cells with growth factors has been demonstrated to induce the rapid but transient expression of a set of specific genes, known as immediate early (IE) genes, whose products appear to mediate the growth response (for review see Denhardt *et al.*, 1986). The induction of IE gene expression is achieved by transcriptional activation in the absence of *de novo* protein synthesis, suggesting that their induction is under the control of transactivating factors that are constitutively present within the cell, but are only activated following post-translational modification by some component of the growth factor signal transduction pathways (Cochran *et al.*, 1983; 1984; Kelly *et al.*, 1983; Kruijer *et al.*, 1984; Muller *et al.*, 1984). The pattern of IE genes induced depends upon both the cell type and the nature of its stimulation, and each clone shows different kinetics of induction,

suggesting that varying control mechanisms, as yet largely unidentified, may be responsible for their activation. A number of these IE genes have been isolated by differential screening of cDNA libraries prepared from the mRNA of quiescent cells stimulated to proliferate by mitogens (Cochran *et al.*, 1983; Lau and Nathans, 1985; 1987; Lim *et al.*, 1987; Milbrandt, 1987; Sukhatme *et al.*, 1987; Almendral *et al.*, 1988). However, an increase in the expression of a gene on exit from G0, the criterion by which such clones were isolated, should not be taken as proof that the gene product has a causative role to play in the induction of DNA synthesis. On entry into quiescence, the expression of many proteins decreases as a consequence of a general lowering of RNA and protein synthetic rates (Hershko *et al.*, 1971). Transcription of such growth-associated genes on mitogenic stimulation may thus simply reflect a return to a constant pre-quiescent level on the resumption of growth, rather than representing a triggering factor for cell proliferation, thus casting doubt on the relevance of such studies to processes exhibited by actively proliferating cells as they progress from G1 into S phase. Investigation of patterns of IE gene expression in non-growth-arrested cycling cells have indeed demonstrated that the level of the protein product of a number of IE genes is invariant throughout the cell cycle, suggesting that they may not play a significant role in G1 progression (Riddle and Pardee, 1980; Hann *et al.*, 1985), while others continue to show a peak in early G1 (Cosenza *et al.*, 1991). It thus appears that further study is required before each IE gene can be assigned a direct role in the determination of proliferative processes.

The relationship of many of the IE gene products, such as β-actin, α-tropomyosin, the β-subunit of the fibronectin receptor, fibronectin (Ryseck *et al.*, 1989), tissue factor (Hartzell *et al.*, 1989), and the potential cytokines JE (Rollins *et al.*, 1988) and KC (Oquendo *et al.*, 1989), to cell proliferation is unclear. However, a subset of the IE genes has been shown to encode known or putative transcription factors, including *c-fos* (Greenberg and Ziff, 1984; Kruijer *et al.*, 1984; Muller *et al.*, 1984), *c-jun* (Lamph *et al.*, 1988; Ryder and Nathans, 1988), *jun-B* (Ryder *et al.*, 1988), *fosB* (Zerial *et al.*, 1989), *fra-1* (Cohen and Curran, 1988), *c-myc* (Kelly *et al.*, 1983; Greenberg and Ziff, 1984), NGF1-A/*egr1/zif268* (Milbrandt, 1987; Sukhatme *et al.*, 1987; 1988; Christy *et al.*, 1988), NGF1-B/*nur77* (Hazel *et al.*, 1988; Milbrandt, 1988), *Krox-20* (Chavrier *et al.*, 1988) and *Krox-24* (Lemaire *et al.*, 1988). It is believed that these transcriptional regulators represent the nuclear intermediaries in the signal transduction cascade, coupling the cytoplasmic arm of the growth factor-induced signalling pathways to the changes in gene expression required for commitment to DNA synthesis and cell proliferation. Each transcription factor may recognize an independent binding site and thus regulate a distinct set of secondary response genes, allowing for cell type- and stimulus-specific responses depending on the particular combination of transcription

factors induced in a given cell type by an individual mitogen.

The most extensively studied of the IE gene transcription factors is the Jun/Fos heterodimer. The potential importance of both *c-jun* and *c-fos* in the control of cellular proliferation is clearly indicated by the observation that viral homologues of these genes are responsible for the oncogenic transformation induced by avian sarcoma virus 17 (Maki *et al.*, 1987) and FBJ murine osteosarcoma virus (Curran and Teich, 1982) respectively, and that overexpression of *c-jun* (Schutte *et al.*, 1989) or *c-fos* (Miller *et al.*, 1984) from a retroviral promoter results in cell transformation. In addition, the elimination of *c-fos* expression by antisense RNA or injection of anti-Fos antibodies prevents the mitogen-induced G0 to S transition in cultured cells (Nishikura and Murray, 1987; Riabowol *et al.*, 1988). A number of lines of evidence suggested that both the *c-jun* and *c-fos* protein products were components of the mammalian transcription factor AP-1 (Bohmann *et al.*, 1987; Angel *et al.*, 1988a; Franza *et al.*, 1988; Rauscher *et al.*, 1988a; Chui *et al.*, 1989), described initially as a complex of polypeptides required for optimum expression of the human metallothionein IIA promoter (W. Lee *et al.*, 1987a; 1987b), and subsequently demonstrated to bind to a specific palindromic DNA sequence, TGACTCA, which functions as a *cis* element mediating the transcriptional response of a number of genes to phorbol esters, and thus presumably also to those growth factors which transduce signals *via* an increase in PKC activity (Angel *et al.*, 1987; W. Lee *et al.*, 1987b).

In vitro studies have established that Fos and Jun form a heterodimeric complex that interacts with the AP-1 binding site to increase transcription of the linked gene (Rauscher *et al.*, 1988b; Sassone-Corsi *et al.*, 1988b; 1988c). Fos is unable to interact with AP-1 sites in the absence of Jun, and Jun homodimers bind the DNA with only low affinity, thus the function of Fos appears to be to enhance the binding affinity of Jun for its consensus recognition sequence, and consequently to stimulate its transcriptional activation function (Halazonetis *et al.*, 1988; Kouzarides and Ziff, 1988; Nakabeppu *et al.*, 1988; Rauscher *et al.*, 1988b; Sassone-Corsi *et al.*, 1988d). The association of Fos and Jun is mediated by interaction between the leucine zipper region of each molecule (Kouzarides and Ziff, 1988; Sassone-Corsi *et al.*, 1988d; Gentz *et al.*, 1989), a conserved structural motif found in many DNA-binding proteins and believed to facilitate the formation of dimers through hydrophobic interactions (Landschulz *et al.*, 1988). Regions of basic residues adjacent to the leucine zipper are thought to constitute the DNA-binding domain (Kouzarides and Ziff, 1988; Landschulz *et al.*, 1988; Gentz *et al.*, 1989; Turner and Tjian, 1989), suggesting that dimerization is required to juxtapose DNA contact regions from each subunit to form a complete binding site, with each palindromic half of the consensus recognition sequence interacting with the DNA-binding site of one molecule of the dimer. The region

responsible for transcriptional activation appears to be located close to the DNA-binding domain of each molecule (Bohmann and Tjian, 1989; Abate *et al.*, 1990). AP-1-binding sites have been found in the regulatory regions of numerous genes, including the collagenase (Angel *et al.*, 1987), osteocalcin (Schule *et al.*, 1990), stromelysin (Angel *et al.*, 1987), histone H3.2 (Sharma *et al.*, 1989), adipocyte P2 (Distel *et al.*, 1987) and metallothionein IIA (Angel *et al.*, 1987; W. Lee *et al.*, 1987b) genes, and it appears likely that the Jun/Fos heterodimer may mediate the induction of many of the genes required for entry into S phase. However, further investigation is required in order to determine those target genes relevant to the initiation of cell proliferation.

The activation of *c-fos* itself by growth factors appears to be mediated, at least in part, by an upstream *cis* sequence known as the serum response element or SRE (Treisman, 1985; 1986; Gilman *et al.*, 1986; Fisch *et al.*, 1987; Greenberg *et al.*, 1987; Gilman, 1988). This represents the binding site for the serum response factor or SRF (Treisman, 1986; 1987; Prywes and Roeder, 1987), the binding of which is thought to be responsible, at least in part, for the transcriptional induction of *c-fos*, *zif268* and *Krox-20* expression (Prywes and Roeder, 1986; Chavrier *et al.*, 1989; Christy and Nathans, 1989) as well as that of additional genes, such as the α-actin gene (Walsh, 1989), on serum stimulation. SRF binding activity requires its phosphorylation on serine and threonine residues (Prywes *et al.*, 1988), identifying it as a potential target for one of the many serine/threonine kinase activities implicated in signal transduction pathways, and indeed casein kinase II has been shown to increase the DNA-binding activity of SRF (Manak *et al.*, 1990) and Raf-1 kinase has been demonstrated to activate *c-fos* transcription (Jamal and Ziff, 1990). SRF phosphorylation and subsequent activation of *c-fos* transcription may thus provide one of the mechanisms by which mitogen-stimulated signalling systems bring about a cascade of changes in gene expression. The *c-fos* promoter also contains a CRE (Sassone-Corsi *et al.*, 1988a; Berkowitz *et al.*, 1989) and is inducible by cAMP (Greenberg *et al.*, 1985; Kruijer *et al.*, 1985), thus multiple pathways may activate *c-fos* expression. The *c-jun* promoter has been shown to harbour an AP-1 site (Hattori *et al.*, 1988; Nishimura and Vogt, 1988), and binding of Jun to this site is known to activate its own transcription (Angel *et al.*, 1988b), thus control of *c-jun* expression appears to involve a positive autoregulatory loop. Phosphorylation of sites in the transactivating domain of pre-existing Jun by MAP kinase, resulting in an increase in its transcriptional activity (Pulverer *et al.*, 1991), in addition to dephosphorylation of residues which inhibit DNA binding by an unidentified phosphatase (Boyle *et al.*, 1991), are thought to be the initial activating steps on mitogen stimulation. The mechanism by which both Jun and Fos expression is down-regulated once the proliferative signal has been received and transduced is unclear, although the

observation that Fos represses its own transcription (Sassone-Corsi *et al.*, 1988c; Schonthal *et al.*, 1988) suggests that negative feedback may be involved. In addition, Jun B has been shown to inhibit the induction of *c-jun* (Chui *et al.*, 1989), thus *c-jun* and *c-fos* expression appears to be controlled by a complex regulatory network.

The stimulation of cells by mitogens also leads to the induction of a number of proteins homologous to Fos and Jun e.g. Jun B (Ryder *et al.*, 1988), Jun D (Ryder *et al.*, 1989), Fos B (Zerial *et al.*, 1989) and the Fos-related antigens (Cohen and Curran, 1988; Nishina *et al.*, 1990), suggesting that both proto-oncogenes are members of multigene families. All members of the Fos family can form heterodimers with each member of the Jun family (Nakabeppu *et al.*, 1988; Cohen *et al.*, 1989; Zerial *et al.*, 1989; Kovary and Bravo, 1991), thus the combinatorial repertoire of heterodimers is large, and it seems likely that the transcriptional activity of each differs (Chui *et al.*, 1989; Yang-Yen *et al.*, 1990). In addition, it is believed that alternative combinations of Fos and Jun proteins may display different affinities for AP-1 sites with varying flanking regions (Ryseck and Bravo, 1991), and thus may be responsible for the transcriptional activation of specific subsets of AP-1-responsive genes, allowing for a cell type- and mitogen-specific response according to the complement of Jun and Fos proteins expressed on stimulation. A further temporal level of regulation is suggested by the observation that Jun and Fos family members show different kinetics of induction and turnover on the treatment of cells with growth factors (Kovary and Bravo, 1991), thus allowing specific target genes to be induced at varying times post-stimulation depending on the concentrations of the various heterodimers present. Both Fos and Jun have also been shown to undergo extensive post-translational modification (Curran *et al.*, 1984; Barber and Verma, 1987; D. Carroll *et al.*, 1988; Binetruy *et al.*, 1991; Boyle *et al.*, 1991; Kovary and Bravo, 1991; Pulverer *et al.*, 1991), which may also play a role in determining the transcriptional activity of the Fos/Jun heterodimer. The number of potential target genes is increased still further by the ability of the Fos/Jun complex to bind to the CRE and to activate transcription of the linked gene (Nakabeppu *et al.*, 1988; Rauscher *et al.*, 1988b; Cohen *et al.*, 1989), and a degree of cross-talk between the Fos/Jun and CREB signalling pathways is also implied by the observation that members of the CREB family can form heterodimers with those of the Jun family (Hai and Curran, 1991).

It thus appears that mitogen stimulation can bring about widespread changes in gene expression through the induction of a series of transcription factors which mediate the activation (and in some cases repression e.g. see Distel *et al.*, 1987; Sassone-Corsi *et al.*, 1988c; Schonthal *et al.*, 1988; Schule *et al.*, 1990) of a diverse set of genes, whose only common feature is the possession of an appropriate consensus response element, but which together somehow elicit the process of DNA synthesis.

However, the link between the cytoplasmic events of signal transduction and transcription factor induction has yet to be established (and will most likely differ in the case of each transcription factor), as have the precise molecular events which trigger the transition from G1 to S phase i.e. signal a commitment to cell proliferation, as opposed to simply being required for DNA synthesis and cell growth. Given the central role of the $p34^{cdc2}$/cyclin complex in the control of cell proliferation, it appears probable that the various signalling pathways will ultimately converge in its activation, perhaps *via* transcription factor-mediated stimulation of the expression of G1 cyclins. Although the induction of a candidate human G1 cyclin by the growth factor CSF-1 has been demonstrated (Matsushime *et al.*, 1991), suggesting that mitogenic stimulation may indeed result in commitment to DNA synthesis and cell division through G1 cyclin-mediated activation of $p34^{cdc2}$, the means by which this occurs remains obscure at present, and it is unclear whether this represents a general phenomenon.

It is becoming increasingly evident that in addition to stimulatory proteins, the induction of which is required for the initiation of cell proliferation, the cell cycle is also under the control of a number of negative regulatory elements, encoded by tumour suppressor genes. The first of such genes to be cloned was the retinoblastoma gene (Friend *et al.*, 1986; Fung *et al.*, 1987; W.-H. Lee *et al.*, 1987a). The loss or alteration of both functional alleles of the retinoblastoma gene has been implicated as the underlying cause of tumour formation in all retinoblastomas (Horowitz *et al.*, 1990), and many sarcomas (Friend *et al.*, 1987; Weichselbaum *et al.*, 1988), small cell lung carcinomas (Harbour *et al.*, 1988; Yokota *et al.*, 1988; Horowitz *et al*, 1990), breast carcinomas (T'Ang *et al.*, 1988) and bladder carcinomas (Horowitz *et al.*, 1990), suggesting that it may play a role in constraining cell proliferation, and indeed transfection of the wild-type gene into transformed cells lacking functional retinoblastoma protein resulted in a loss of tumourigenicity (H.-J.S. Huang *et al.*, 1988; Bookstein *et al.*, 1990). Further evidence, albeit indirect, for the involvement of the retinoblastoma protein, a 110 - 114 kD nuclear phosphoprotein (W.-H. Lee *et al.*, 1987b) termed pRB and expressed in all human tissues so far examined (Friend *et al.*, 1986), in negative regulation of cell proliferation is the observation that it is complexed by the structurally diverse transforming proteins of three different tumour viruses: the E1A protein of adenovirus (Whyte *et al.*, 1988), the human papillomavirus (HPV) 16 and 18 E7 proteins (Dyson *et al.*, 1989; Munger *et al.*, 1989), and the SV40 T antigen (DeCaprio *et al.*, 1988), each of which binds to the same region of pRB (Hu *et al.*, 1990; Kaelin *et al.*, 1990). This suggests that each virus may, at least in part, transform the cell through the inactivation of pRB by complex formation (although in the absence of an assay for the biochemical function of pRB this

remains speculative), and this interaction does indeed appear to be important for transformation of cells by these viruses since mutations in E1A and T antigen which prevent them from binding to pRB, also simultaneously abolish their transforming powers (DeCaprio *et al.*, 1988; Whyte *et al.*, 1989). The adenovirus E1A protein has also been shown to bind to cyclin A (Pines and Hunter, 1990), which may represent an additional mechanism by which the virus perturbs the normal regulatory processes of the cell.

The function of pRB in the cell cycle is at present unclear. The phosphorylation state of the protein is cell cycle-regulated; pRB is unphosphorylated in G0, differentiated and early G1 cells, becomes phosphorylated on serine and threonine residues (Ludlow *et al.*, 1989; Shew *et al.*, 1989) on passage into S and G2, and is dephosphorylated in late mitosis (Buchkovich *et al.*, 1989; Chen *et al.*, 1989; DeCaprio *et al.*, 1989; Ludlow *et al.*, 1990). This implies that phosphorylation of pRB may be required for exit from G1, and that the unphosphorylated molecule is active in constraining proliferation, a hypothesis supported by the observation that SV40 T antigen binds only the unphosphorylated form (Ludlow *et al.*, 1989). pRB is known to bind to a number of nuclear proteins (Huang *et al.*, 1991; Kaelin *et al.*, 1991), and of particular interest is the observation that the domain of pRB which complexes these molecules is the same as that which interacts with the viral transforming proteins above (Hu *et al.*, 1990; Huang *et al.*, 1990; 1991; Kaelin *et al.*, 1990; 1991), suggesting that the oncoproteins might transform by sequestering pRB away from its natural targets. In addition, mutations which inactivate pRB activity are often found in this same domain (Hu *et al.*, 1990), and may thus have equivalent functional consequences i.e. causing the disruption of associations with cellular proteins. The cellular proteins with which pRB interacts include the putative transcription factors c-Myc and N-myc (Rustgi *et al.*, 1991), DRTF-1 (Bandara and LaThangue, 1991) and E2F (Chellappan *et al.*, 1991; Chittenden *et al.*, 1991). The interaction between pRB and E2F has been demonstrated to repress the latter molecule's ability to stimulate transcription (Hiebert *et al.*, 1992), and E2F is known to regulate the expression of several proliferation-related genes (Blake and Azizkhan, 1989; Hiebert *et al.*, 1989; Thalmeier *et al.*, 1989; Mudryj *et al.*, 1990). Unphosphorylated pRB may thus prevent the induction of DNA synthesis by the inactivation of transcriptional regulators responsible for turning on the expression of the required genes. It has also been suggested that the pRB molecule itself may possess a transcriptional regulatory function, since it has affinity for DNA (W.-H. Lee *et al.*, 1987b) and its sequence predicts the presence of a putative leucine zipper region (Hong *et al.*, 1989), a characteristic of many transcription factors (Landschulz *et al.*, 1988). However, further work is clearly necessary to fully define the role of pRB in the mammalian cell cycle. $p34^{cdc2}$ has

been demonstrated to associate with pRB *in vivo* (Hu *et al.*, 1992) and to phosphorylate this molecule *in vitro* on the same sites as are phosphorylated *in vivo* (Taya *et al.*, 1989; Bryan *et al.*, 1991; Lees *et al.*, 1991; Lin*et al.*, 1991) thus, although the functional consequences of this modification are unknown, it may be that one of the requirements for $p34^{cdc2}$ kinase activation at the G1/S transition is to relieve a block to proliferation imposed by the presence of unphosphorylated pRB.

Many more tumour suppressors have subsequently been isolated, including the Wilms' tumour gene (Gessler *et al.*, 1990; Rose *et al.*, 1990), the neurofibromatosis type 1 gene (Cawthon *et al.*, 1990; Viskochil *et al.*, 1990; Wallace *et al.*, 1990), the protein tyrosine phosphatase γ gene (LaForgia *et al.*, 1991) and the gene encoding p53. Mutations in the p53 gene represent the most common genetic alteration underlying a wide variety of cancer types (see Hollstein *et al.*, 1991 for review). The normal allele encodes a 53 kD nuclear phosphoprotein (Lane and Crawford, 1979; Linzer and Levine, 1979) which suppresses the transformation of cultured cells by various oncogenes (Eliyahu *et al.*, 1989; Finlay *et al.*, 1989) and the growth and tumourigenicity of transformed cells (Baker *et al.*, 1990; Chen *et al.*, 1990; Diller *et al.*, 1990; Mercer *et al.*, 1990), causing cell cycle arrest in G1, and thus appears to negatively regulate cell growth.

As is the case with pRB, p53 binds to tumour virus oncoproteins, namely adenovirus E1B (Sarnow *et al.*, 1982), HPV 16 and 18 E6 (Werness *et al.*, 1990) and SV40 T antigen (Lane and Crawford, 1979; Linzer and Levine, 1979). Interaction with HPV 16 or 18 E6 protein has been shown to promote degradation of p53 through the ubiquitin protease system (Scheffner *et al.*, 1990), further implying that one of the pathways by which these viruses mediate transformation involves removal of proliferation suppressor molecules. In addition, the observation that each virus has evolved two separate proteins whose combined action inactivates both pRB and p53 suggest that these tumour suppressor molecules act in different pathways. However, the molecular role of p53 in the cell cycle has yet to be elucidated. p53 has been demonstrated to block the DNA replication function of SV40 T antigen (Braithwaite *et al.*, 1987; Gannon and Lane, 1987; Wang *et al.*, 1989), thus it is possible that p53 may interact with a cellular homologue of T antigen which is involved in DNA synthesis, and in this way inhibit entry into S phase. It has also been suggested that p53, which has been shown to bind to DNA (Lane and Gannon, 1983; Kern *et al.*, 1991), may act as a transcription factor (Fields and Jang, 1990; Raycroft *et al.*, 1990), and could therefore either positively regulate the expression of other growth-inhibitory genes, or repress the transcription of genes whose protein products are required for the G1/S transition. Indeed, wt p53 has been shown to down-modulate the expression of a number of serum-inducible genes (Ginsberg *et al.*, 1991), although

the mechanism by which this is achieved is unclear at present. In addition, p53 associates with (Milner *et al.*, 1990) and is believed to be phosphorylated in a cell cycle-dependent manner by $p34^{cdc2}$ (Bischoff *et al.*, 1990), suggesting that like pRB, its activity may be regulated by this central cell cycle control molecule, although the functional significance of the phosphorylation event is unclear.

In conclusion, it appears that the decision taken by the mammalian cell in G1 of whether or not to replicate its DNA and divide depends on a balance between the activity of both negative and positive regulatory molecules, and that mitogenic signal transduction pathways alter this equilibrium and thus allow the cell to respond appropriately to environmental cues. Cell transformation may therefore occur in one of two ways: by the overexpression of a stimulatory component (which would thus represent a proto-oncogene in its normal form) to provide a constitutive growth signal, or by the inactivation of a molecule which acts to restrain proliferation i.e. a tumour suppressor protein. A complex network of events separates the initial stimulation of the cell from the ultimate goal, DNA synthesis and mitosis, although the early processes all appear to converge at a point known as the commitment or restriction point (which may represent activation of $p34^{cdc2}$ kinase), following which a common genetic program ensues. However, despite the considerable advances made in recent years regarding the precise molecular mechanisms involved in regulating the transition through G1 into S phase, a complete picture of G1 events has yet to be attained, especially in the case of mammalian cells. The further identification of cdc-controlling processes by the analysis of mammalian ts G1 mutants would thus clearly be of great benefit, particularly in identifying the events that link the activation of diverse kinases by mitogenic signal transduction pathways with the induction of specific gene expression and an increase in $p34^{cdc2}$ activity.

1.12 Properties of the K12 cell line

One such ts G1 mutation is found in K12 (a.k.a cell line ts K/34c or H3.5 - Lee *et al.*, 1986), derived from the Chinese hamster cell line Wg-1A following mutagenization with ethyl methane sulphonate (Roscoe *et al.*, 1973a). At the permissive temperature of 33°C, K12 shows a normal spread configuration and good growth, but at 40°C (the npt) the cells cease to cycle and are unable to form colonies, whereas the parental line Wg-1A grows well at both temperatures (Roscoe *et al.*, 1973a). On incubation at the npt, K12 cells retain their ability to form colonies, if returned to the pt, for 15 - 20 hours after the temperature shift, and time-lapse cinematographic studies of this cell line at 33° and 40°C revealed a normal rate of mitosis at the npt for up to 15 hours (Roscoe *et al.*, 1973b). However, viability is lost gradually

during the following 20 hours at 40°C as the cells fail to divide, become spindle-shaped, begin to lose contact with the underlying substrate, and eventually round up completely before their subsequent death (Roscoe *et al.*, 1973b). The rounding phenotype enabled the original isolation of this mutant by selective detachment (Roscoe *et al.*, 1973a), a process analogous to the mitotic shake-off synchronization procedure of Terasima and Tolmach (1963), since a sharp tap of the culture flask is sufficient to dislodge the loosely attached rounded ts cells preferentially and efficiently from the plastic surface on which they are grown. Although it has been suggested that cell shape may be a crucial element in growth control (Folkman and Greenspan, 1975), and that the reorganization of actin-containing filaments might play a role in the induction of DNA synthesis and cell division (Edelman, 1976; Maness and Walsh, 1982; Miyashita, 1987), it seems unlikely, given the late expression of the rounding-up phenotype in K12 at the npt, that this in itself could be responsible for the cell cycle arrest. Furthermore, K12 cultures continue to cycle normally at the pt when grown in soft agar as rounded cells (Roscoe *et al.*, 1973a). Net protein synthesis in K12 at 40°C occurs normally for approximately 24 hours but ceases as the cells round up, suggesting that the ts defect is not manifested as an early generalized cessation of protein synthesis (Smith and Wigglesworth, 1973).

Temperature shift experiments on both synchronous and random cultures of K12 have been used to map the execution point of the mutation. Roscoe *et al.* (1973b) observed that when random cultures of K12 were shifted to 40°C, the rate of DNA synthesis remained normal for 3 - 4 hours but then decreased markedly, reaching 95% inhibition twenty four hours after the temperature shift. Conversely, cells incubated at the npt for 16 hours and subsequently shifted to 33°C showed an increase in DNA synthesis approximately 5 hours after shift-down, and mitosed 13 hours later. These results were interpreted as indicating the existence of a critical step, designated K, in the cycle in mid-G1, 3 - 4 hours before the start of S phase, the unsuccessful completion of which at the npt prevents K12 from initiating DNA synthesis and thus arrests the cell in G1 (although the data presented could also be construed as measuring the final boundary of the execution point, since the method utilized is unable to distinguish between an execution point which is operational at a discrete point in the cycle, and one which extends over several hours). When the cells are returned to the pt following a 16 hour incubation at 40°C, during which time the population has become synchronized by arrest at K, it was postulated that the cells require 1 - 2 hours to recover, and 3 - 4 hours to initiate S phase following traverse of K. Temperature shift-up and shift-down experiments carried out on synchronous K12 cultures by Ashihara *et al.* (1978) and Melero and Fincham (1978) confirm the placing of the execution point of the mutation as in mid- to late

G1. It seems likely that a threshold level of activity of a protein defective in K12 at the npt is required to pass the execution point, and that this activity is brought below the threshold at 40°C, either directly or indirectly, by expression of the K12 mutation. The most likely cause for this loss of activity is a reduction in the heat stability of the protein, which would lead to its gradual collapse at the npt. Alternatively, it may be that the protein's activity is directly reduced by a mutation in the active site or elsewhere in the molecule, whose effects are manifested only at 40°C. It is also conceivable that the molecule causing the G1 block is itself not defective, but that a protein which controls its expression or activity shows aberrant functioning at the npt due to mutation, and thus indirectly causes the observed cell cycle arrest.

A number of biochemical defects occur on incubation of K12 at the npt, but their significance is as yet unclear. Kit and Jorgensen (1976) demonstrated that the tenfold increase in activity of two enzymes of deoxyribonucleotide metabolism, thymidine kinase and deoxycytidilate deaminase, normally seen at the initiation of DNA synthesis in K12 at the pt, was grossly inhibited at 40°C. The mutation appears to act at the transcriptional or post-transcriptional level to block the formation of these proteins, rather than affecting their activity. Although a reduction in the levels of these vital components of the DNA replication machinery would be expected to block the exit of cells from G1, it appears likely that the failure to induce these enzymes, rather than actually causing the cell cycle to arrest, simply reflects the inability of the cell to pass the mid-G1 block, thus preventing the triggering of certain downstream events which are dependent on traverse of mid-G1, a hypothesis which may also account for the lack of induction of histone H3 transcription in late G1 in K12 at the npt (Artishevsky *et al.*, 1984). Indeed, in contrast to Wg-1A, K12 cells fused with mature inactive chick erythrocytes fail to reactivate DNA synthesis in the chick nuclei at 40°C (Dubbs and Kit, 1976), suggesting that the cytoplasmic S phase-initiating factor, identified by Rao and Johnson (1970) in cell fusion experiments (and possibly representing the active $p34^{cdc2}$ kinase/G1 cyclin complex - see Section 1.8), is not present in K12 at the npt. It thus appears likely that K12 is unable to pass the restriction point at 40°C, and that this lack of commitment to S phase is reflected in a failure to derepress the proteins required for DNA replication. The execution point for the mutation thus seems to reside upstream of the restriction point.

The incubation of K12 at the npt also leads to the induction, at the transcriptional level, of two proteins known as the glucose-regulated proteins (GRPs), GRP78 and GRP94 (Melero and Fincham, 1978; Melero and Smith, 1978; Lee *et al.*, 1981; 1983; Melero, 1981), which are major constituents of the mammalian

endoplasmic reticulum (Zala *et al.*, 1980; Munro and Pelham, 1986). Both proteins are synthesized constitutively in detectable amounts under normal growth conditions in many cell types, but are induced by a variety of stress conditions, including glucose starvation, treatment with the calcium ionophore A23187, or with drugs which inhibit cellular glycosylation (Olden *et al.*, 1979; Welch *et al.*, 1983; Lee, 1987), and they appear to be both structurally and functionally related to the cytoplasmic heat shock proteins (Munro and Pelham, 1986; Pelham, 1986; Subjeck and Shyy, 1986). Their function seems to be involved with the folding, assembly and transport of exocytotic and membrane proteins (Pelham, 1986; Hendershot *et al.*, 1987; Vogel *et al.*, 1990), to which they bind transiently, and are presumably released on proper folding of these molecules. In addition, transcription of the GRPs is increased by the presence of malfolded proteins in the endoplasmic reticulum (Kozutsumi *et al.*, 1988), to which they bind stably (Bole *et al.*, 1986; Gething *et al.*, 1986; Pelham, 1986; Dorner *et al.*, 1987; 1988; Kassenbrock *et al.*, 1988), and they may thus also play a protective role by preventing the progress of abnormal proteins, such as those formed under conditions of stress, to the Golgi body for secretion. GRP78 has been identified as the immunoglobulin heavy chain binding protein, BiP (Munro and Pelham, 1986; Kozutsumi *et al.*, 1989), which plays a role in the assembly of immunoglobulin molecules (Bole *et al.*, 1986).

No increase in the level of these proteins has been noted during G1 in the normal cell cycle (Lee *et al.*, 1983) or in other G1 ts mutants, indicating that their overproduction, rather than being indirectly caused by the G1 arrest at the npt, is a direct consequence of the expression of the K12 mutation. Hybrid cells produced by fusion of K12 with other ts mutants showed suppression of the change in GRP level at the npt, favouring the idea of a defect in the appropriate regulation of transcription of these proteins in K12 at 40°C (Melero and Fincham, 1978). The induction of the GRPs in K12 at the npt is thought to be an indirect consequence of a defect in the protein *N*-linked glycosylation pathway, manifested at the stage of transfer of the oligosaccharide core from the dolichol-linked intermediate to the nascent peptide chain, which is expressed rapidly on a shift to 40°C in this cell line (Tenner *et al.*, 1976; Tenner and Scheffler, 1979; Melero; 1981). Such abnormal glycosylation may prevent or delay the folding and assembly of glycoproteins, leading to the accumulation of malfolded proteins in the endoplasmic reticulum, and thereby triggering the increased transcription of the GRP genes. Indeed, a 3T3 mutant cell line, AD6, which is defective in the acetylation step of glycosylation, also shows overexpression of the GRPs (Pouyssegur *et al.*, 1977), although this cell line does not exhibit cell cycle arrest (Pouyssegur and Pastan, 1976). The nature of the induction signal is unclear, although a *cis*-acting enhancer-like element in the promoters of

GRP78 and GRP94 has been identified which increases the transcription of these genes in response to A23187 treatment of cells, the presence of conformationally abnormal proteins in the endoplasmic reticulum, and expression of the K12 ts mutation at 40°C, and presumably represents the target for a *trans*-acting transcription factor (the CCAAT-binding factor CTF/NF-1 has been suggested as a putative mediator of this response [Wooden *et al.*, 1991]) which is responsible for the coordinate induction of both GRP genes under the appropriate conditions (Lin *et al.*, 1986; Chang *et al.*, 1987; 1989; Resendez *et al.*, 1988; Wooden *et al.*, 1991). The treatment of K12 with tunicamycin at the pt induces GRP production (Olden *et al.*, 1979) but fails to inhibit entry into S phase (Feige and Scheffler, 1987), thus it seems unlikely that overexpression of the GRPs in this cell line at the npt is the primary cause of the cell cycle arrest.

It has been reported that treatment of a human Burkitt lymphoma cell line (Nishikawa *et al.*, 1980) and mouse 3T6 cells (Engstrom and Larsson, 1988) with the *N*-linked glycosylation inhibitor tunicamycin, arrests the cells in G1, suggesting that the failure in glycosylation in K12 at the npt may be the primary defect underlying the cell cycle blockage. Since all known growth factor receptors are glycoproteins, and glycosylation appears to be essential for their function (e.g. Pratt and Pastan, 1978; Heldin *et al.*, 1983; Jacobs *et al.*, 1983; Soderquist and Carpenter, 1984; 1986), one could postulate that G1 arrest in K12 at 40°C is due to a reduction in functional growth factor receptors, and a consequent insensitivity to mitogenic stimulation. Alternatively, a particular cell cycle protein involved in the commitment process in G1 may require glycosylation for its folding and stability, as is the case with many other proteins (Bole *et al.*, 1986; Machamer and Rose, 1988), and may thus be inactive at the npt in K12, causing the observed block in G1. However, as mentioned above, treatment of K12 with tunicamycin to mimic the defect at the pt had no effect on DNA synthesis or cell cycle progression (Feige and Scheffler, 1987), thus aberrant glycosylation also appears to be an indirect effect of expression of the K12 mutation, and the primary defect has still to be identified.

K12 displays an unusually low spontaneous reversion frequency (less than 1 in 60 million) when incubated under non-selective conditions for prolonged periods (Roscoe *et al.*, 1973a), and in seven years of continuous maintenance in the laboratory of Dr. D.H. Roscoe, no spontaneous revertants have been detected. However, Scharff *et al.* (1982) isolated a single spontaneous revertant following incubation at the npt, and revertants can be obtained at a frequency of approximately 1 in 10^5 - 10^6 following mutagenization with ethyl methane sulphonate (Roscoe *et al.*, 1973a; Tenner *et al.*, 1976). All the effects of the mutation are reversed in K12 revertants (Tenner *et al.*, 1976; Scharff *et al.*, 1982), indicating that a single mutation may be responsible for

each of the diverse aspects of the K12 phenotype at the npt, which may thus reside in a molecule of a regulatory nature. The mutation was shown to be recessive and complemented in interspecific hybrids (Smith and Wigglesworth, 1973; Marin and Labella, 1976; Ming *et al.*, 1979), implying that the cell line is probably functionally hemizygous for the allele in question.

The picture of K12 that emerges from the studies carried out so far is of a cell line which harbours a single mutation having pleiotropic effects at the npt, inhibiting the derepression of certain proteins required for DNA replication, abolishing ordered regulation of GRP production *via* a defect in protein glycosylation, and preventing commitment to the initiation of DNA synthesis. The pleiotropic consequences of the mutation suggest that the defect is of a regulatory nature, and is thus of particular interest in the study of cell cycle events. Furthermore, given the observed placing of the defect in mid-G1 (Roscoe *et al.*, 1973b; Ashihara *et al.*, 1978), the widespread nature of its effects, and the inability of K12 to reactivate DNA replication in chick erythrocyte nuclei at the npt (Dubbs and Kit, 1976), it seems likely that the protein defective in K12 acts upstream of the restriction point, somewhere in the scheme of events linking mitogenic stimulation to the commitment to initiation of DNA synthesis, and is therefore likely to play a part in the regulation of entry of the cells into the cell cycle. Elucidation of the role which the wild-type protein defined by this defect plays in this scheme, and therefore of its relationship to the various signal transduction pathways and to the central cell cycle regulatory protein, $p34^{cdc2}$ kinase, may thus add a further element to the growing knowledge of the overall pattern of events controlling passage through the cell cycle, an understanding of which is required in order to investigate how these functions may be subverted to produce the inappropriate proliferation manifested as malignancy.

1.13 Aims of the current study

Two methods of approach were available for the further investigation of the nature of the K12 defect, namely molecular and cellular biological studies, both of which have proved invaluable in clarifying the details of the current unifying hypothesis of cell cycle control. The most direct pathway for understanding the role of the protein defined by the K12 mutation in the regulation of cell proliferation is clearly through the cloning of the wild-type gene, and the investigation of the nature of the protein product and its functional relationship to known cell cycle proteins. The isolation of a K12-complementing clone from various human cDNA and genomic libraries was thus attempted with this aim in view. However, complementary studies on the phenotype caused by the mutation of this protein in K12 were also carried out, with the

potential of shedding further light on the nature of the defect.

SECTION 2

MATERIALS AND METHODS

2.1 CELL LINES AND CULTURE CONDITIONS

Stocks of Wg-1A and K12 Chinese hamster lung fibroblasts were maintained at 33°C in Dulbecco's modified Eagle medium containing a low sodium bicarbonate concentration of 26 mM (E4 low bic) and supplemented with 5% foetal calf serum (FCS). Cells were grown on 6 cm plastic tissue culture dishes in a humidified incubator with an atmosphere of 5% CO_2, and stock transfers were carried out at 4 - 5 day intervals.

2.2 CHEMICALS AND SOLUTIONS USED

Analytical grade chemicals (see Appendix I for list of suppliers) were used for all experiments, and deionized water was used to make up all solutions. Solutions for tissue culture were sterilized either by autoclaving for 15 minutes at 15 lbs/sq.in., or by passage through a 0.2 μm pore size filter. All solutions used for DNA work were rendered nuclease-free by autoclaving at 15 lbs/sq.in. for 15 minutes.

2.3 FROZEN STOCKS OF CELLS

In addition to actively growing cell stocks, reserves of each cell line used were also stored frozen in liquid nitrogen.

2.3.i Freezing down cells

Cells were prepared for freezing in liquid nitrogen as described in Freshney (1987). The medium was removed from a subconfluent 10 cm plate of cells, and the monolayer was washed twice in 2.5 ml of trypsin. Once detached, the cells were resuspended in 4 ml of freeze mix (70% E4 low bic, 20% FCS, 10% glycerol) containing glycerol as a cryoprotectant. The resulting cell suspension was dispensed into 1.8 ml cryotubes at 1 ml/tube, and allowed to stand at room temperature for 15 - 30 minutes. The vials were wrapped in a 5 cm layer of cotton wool and stored at -70°C overnight, before transfer to a liquid nitrogen container (L'Air Liquide, BT55)

the following day.

2.3.ii Thawing cells from liquid nitrogen

The required vial was removed from the liquid nitrogen container, and the contents were thawed in a 37°C water bath. The surface of the vial was swabbed with 70% ethanol, and the cells were resuspended vigorously in 1 ml of E4 low bic supplemented with 15% FCS, and plated in the same medium. After an overnight incubation at 33°C, the medium was removed, replaced with 5% FCS E4 low bic, and the plate was returned to the incubator, ready for further manipulation.

2.4 PREPARATION OF CELL SUSPENSIONS

The cells were detached from their underlying substrate using the proteolytic enzyme trypsin, suspended in the appropriate medium, and counted before replating. Subconfluent stock plates were used in all experiments, in order to provide exponentially growing cells. The medium was removed from the fibroblasts, and the plate was washed twice with 1.5 ml of trypsin (0.25% trypsin in Tris-saline). Once the cells had detached (2 - 3 minutes), they were resuspended in 5 ml of the appropriate medium and placed on ice. The cell number was counted in a haemocytometer, and the suspension was diluted in medium to the required concentration. Cells were replated at 5 ml per 6 cm dish or 25 cm^2 tissue culture flask, or 10 ml per 10 cm plate, and incubated overnight at 33°C before any further manipulation, in order to allow time for attachment of the cells to the substrate.

2.5 STAINING AND COUNTING COLONIES

Colonies were stained using Leishman's stain, which contains eosin methylene blue. The medium was removed from the plate of cells, and 1.5 ml of Leishman's stain was added per 6 cm plate. After 10 minutes at room temperature, the plate was filled to the rim with distilled water and left for a further 30 minutes. The stain was then poured off, and the plate was rinsed in tap water and air-dried. Colonies (defined as a group of greater than 20 cells) were counted under a Wild Heerbrugg M3 dissecting microscope, using a x16 objective. The efficiency of plating (E.O.P.), which provides a reliable measure of survival and proliferative capacity by showing the percentage of cells seeded that grew and divided to form a colony under the

particular experimental treatment, was then calculated from the number of colonies per plate using the following formula:

$$\text{E.O.P. (\%)} = \frac{\text{number of colonies formed}}{\text{number of cells seeded}} \times 100$$

2.6 VIABILITY CURVES

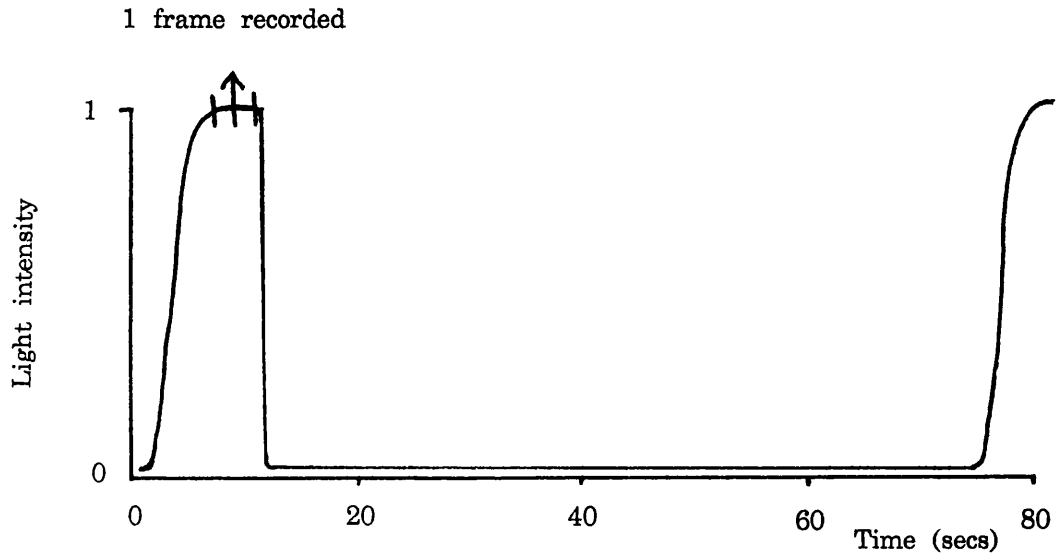
In order to measure the time course of the loss of proliferative capacity of K12 at 40°C, a cell suspension was made from a single subconfluent plate of the cell line, and diluted down to 80 cells/ml in the appropriate medium. If more than one type of medium was used in the study, a common cell suspension was diluted to 8,000 cells/ml in 5% FCS E4 low bic, and the final 1 in 100 dilution was carried out with the particular medium required as the diluent. The suspension was replated and incubated overnight at 33°C. Plates were shifted up to 40°C, and pairs of dishes were returned at intervals to 33°C. Controls for each temperature were provided by incubating duplicate plates for each medium type continuously at either 33° and 40°C. One week after plating, all dishes were stained and the number of colonies was counted.

2.7 TIME-LAPSE STUDIES

For continuous observation of the cells at 33° or 40°C, time-lapse video recordings were made. A cell suspension was prepared, replated in a 25 cm^2 tissue culture flask at a concentration of $4x10^4$ cells/ml, and placed in a 33°C incubator for 4 hours, with the lid of the flask loose to allow its interior atmosphere to equilibrate to 5% CO_2. The lid was then tightened, the flask was placed under a Wild Heerbrugg M-40 inverted microscope with an attached Hitachi CCTV video camera linked to a Panasonic NV-8051 time-lapse video recorder, and the recording was begun. The microscope was housed within a dark, insulated cabinet, whose internal temperature was regulated by an Accuron temperature controller. The flask was maintained at 33°C for 16 - 20 hours, before the temperature was raised to 40°C. In order to protect the medium components from light-induced degradation, the microscope bulb was controlled by a Swift supply unit, which had been modified to

switch the bulb on for a 10 second period once in every 74 seconds. When the bulb had warmed up to full brightness, a pulse activated the video recorder and the equivalent of a single frame was recorded on Scotch videotape (see Fig. 2.1). The final recording interval was thus one frame per 74 seconds. The condition of the cells was followed on a Hitachi black and white TV monitor linked to the video recorder, with an overall magnification on screen of x300. The recording was terminated when all the cells appeared to be dead. For analysis, recordings were viewed on the TV monitor, and the time of division of each cell, of its progeny, of its progeny's progeny *etc.*, was noted and processed further, as described in Section 3.2.ii.

Figure 2.1 Pulse recording of time-lapse data



2.8. PREPARATION OF SYNCHRONIZED CELL SUSPENSIONS BY MITOTIC HARVEST

The method for mitotic synchronization was adapted from Terasima and Tolmach (1963). A cell suspension was plated in the appropriate medium at a concentration of $4x10^5$ cells/ml in a 25 cm^2 tissue culture flask, and incubated overnight at 33°C. Twenty hours after plating, the medium was removed, replaced with fresh medium, and the flask was returned to the incubator for a further 2 hours. The medium was then changed again, to remove any dead cells, and the flask was tapped sharply on the side several times, in order to dislodge mitotic cells. The medium containing the mitotic cells was transferred to an empty 25 cm^2 flask, which had been equilibrated

in a 33°C 5% CO_2 incubator overnight, and the empty flask was kept upright during the transfer to prevent escape of the CO_2. The lid was then tightened, the flask was placed in the time-lapse cabinet directly at 40°C, and the progress of the cells was followed as described in Section 2.7.

2.9 MEASUREMENT OF THE pH OF GROWTH MEDIA UNDER INCUBATION CONDITIONS

In order to determine the pH of a particular growth medium under incubation conditions i.e. at 40°C in a 5% CO_2 atmosphere, a lidless Universal glass bottle containing 15 ml of the medium in question was placed in a 40°C incubator under an atmosphere of 5% CO_2. The contents were agitated with a magnetic stirrer for 1 - 2 hours, before the final stable pH was measured within the incubator using a Radiometer pH electrode.

2.10 FLOW CYTOFLUOROMETRY OF K12 AND Wg-1A CELLS

The protocol found to be most suitable for the preparation of K12 and Wg-1A cells for flow cytofluorometry was that of Fried *et al.* (1978), where cells are rapidly stained *in situ* with the fluorescent dye propidium iodide. Cells were plated on 10 cm dishes in 10 ml of the appropriate medium, at a concentration of 10^4 cells/ml. Plates were incubated overnight at 33°C, and those requiring incubation at 40°C were shifted to this temperature the following day. After the appropriate time interval, the medium was removed from the plates and the cells were washed in 3 ml of PBSA (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4; pH 7.3). The monolayer was then rinsed with 3 ml of cold (4°C) freshly prepared stain solution (0.1% [v/v] Triton X-100, 0.1% [w/v] sodium citrate), and 1.25 ml of cold stain solution containing 100 µg/ml of propidium iodide was added. The plates were placed in a refrigerator at 4°C for 45 minutes, and the cells were subsequently resuspended by repeated pipetting, filtered through cotton wool to remove large debris, and kept in an ice-bath protected from light until analysis (within 3 hours). Flow cytofluorometry was carried out on a Becton-Dickinson Facscan. 10^4 cells were counted for each sample, and the machine was rinsed through with PBSA between each measurement, in order to prevent cross-contamination of samples. Data from the Facscan was processed with the Consort 30 programme, and used to estimate the percentage of cells in the G1 phase of the cell cycle, on the assumption that the

amount of fluorescence emitted is proportional to the DNA content of the nucleus, and that the latter is determined by cell cycle stage (see Section 3.15).

2.11 MEASUREMENT OF THE INTRACELLULAR pH OF K12 AND Wg-1A

Solutions used

BCECF-AM The acetoxymethyl ester of BCECF (2',7'-bis(carboxyethyl)-5,6-carboxyfluorescein) was dissolved in dimethyl sulphoxide (DMSO) at an appropriate concentration (such that the volume of DMSO added to the tissue culture plate did not exceed 0.1%) and 200 mg/ml of pluronic F-127 was added.

BCECF-free acid The free acid form of BCECF was made up in deionized water at a concentration of 1 μM.

Nigericin Nigericin was made up as a 1 mM solution in 100% ethanol.

All solutions were stored at -20°C in the dark.

2.11.i Single cell measurements

The staining method was adapted from Tonnessen *et al.* (1987). A sterile glass coverslip (22 x 22 mm) was placed in the bottom of a 6 cm tissue culture dish, and overlaid with 5 ml of a cell suspension containing $2x10^4$ - 10^5 cells/ml in 5% FCS E4 low bic. The plate was incubated overnight at 33°C and, if required, shifted to 40°C the following day. Twenty four hours later, the coverslip was transferred to a fresh plate containing 2 ml of 5% FCS E4 low bic supplemented with 1 μm BCECF-AM, and incubated for a further hour at either 33° or 40°C, as appropriate. The coverslip was washed twice in 5 ml of 5% FCS E4 low bic, in order to remove any unincorporated extracellular dye, and transferred to a perfusion chamber on the stage of an inverted microscope for the measurement of emitted fluorescence. The cells were perfused with growth medium, bubbled with 5% CO_2 and warmed to the appropriate temperature, at a rate of 1.2 ml/min. Using a black and white TV monitor to observe the cells, each single cell to be measured was centred in the field of view, and a window was applied to eliminate light emitted from the surrounding area. Excitation light from a xenon lamp was applied in alternating one second pulses of two different wavelengths - 430 and 500 nm - and the fluorescence emitted from the cells at 535 nm was passed through a photomultiplier tube before its display on a

chart recorder. Background fluorescence was determined for each cell measured by recording the fluorescence intensity of a near-by cell-free area with the same size window. Once the relationship between fluorescence and pH was revealed by calibration, the ratio of fluorescences measured at excitation wavelengths of 500 and 430 nm, when corrected for background levels, provided an estimate of intracellular pH (pH_i) essentially independent of intracellular dye concentration.

Both *in vitro* and *in vivo* calibration measurements were made in a calibration solution consisting of 140 mM KCl, 1.2 mM $MgCl_2$ and 10 mM HEPES (N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulphonic acid]), adjusted to pH values of 5.5, 7.0, and 8.5 with potassium hydroxide. *In vivo* calibration involved recording of the fluorescence intensity ratios when the last stained cell to be measured was perfused with each calibration solution supplemented with 10 μM nigericin. *In vitro* calibration was performed by perfusing the empty chamber with 1 μM BCECF-free acid dissolved in each of the calibration solutions, and measuring the fluorescence intensity ratios at each pH value.

In order to convert the experimental readings to absolute pH values, the following calculations were used:

For *in vitro* calibration,

$$\text{let } R = \frac{\text{fluorescence intensity at 500 nm}}{\text{fluorescence intensity at 430 nm}}$$

where each measurement is corrected for background levels.

$$\begin{aligned} \text{Let } R_{min} &= R \text{ at pH 5.5} \\ R_7 &= R \text{ at pH 7.0} \\ R_{max} &= R \text{ at pH 8.5} \end{aligned}$$

From a modification of the Henderson-Hasselbach equation, at pH 7,

$$7 = pK + \log \frac{R_7 - R_{min}}{R_{max} - R_7}$$

thus the pK was calculated as follows:

$$pK = 7 - \log \frac{R_7 - R_{min}}{R_{max} - R_7}$$

The process was repeated using the *in vivo* calibration R values. The pK values thus obtained from each calibration experiment were used to convert the fluorescence intensity ratio (R value) for each cell to absolute pH_i values, by applying the formula:

$$pH_i = pK + \log \frac{R - R_{min}}{R_{max} - R}$$

The pH_i was therefore calculated for each cell using the pK, R_{min} and R_{max} values from either the *in vitro* or *in vivo* calibrations.

2.11.ii Cell population measurements

Cells were seeded in 10 cm tissue culture dishes at a concentration of $5x10^5$ cells/plate in 5% FCS E4 low bic, and incubated overnight at 33°C. The following day, plates were moved, if appropriate, to 40°C. After 16.5 hours at this temperature, plates requiring a change of medium were rinsed with 5 ml, and refed with 10 ml of the appropriate medium. Two hours later, the medium was removed from all dishes, replaced with 5 ml of medium supplemented with 5 μM BCECF-AM, and the plates were returned to the incubators at either 33° or 40°C for 30 minutes. The medium was removed from each dish, and the cells were rinsed with 5 ml, and refed with 10 ml of medium, and incubated at the required temperature for a further hour. Plates were then trypsinized, and the cells were resuspended in 2 ml of calibration solution or the appropriate 5% CO_2-equilibrated medium, and transferred to 15 ml screw-cap polystyrene conical tubes, which had also been equilibrated with 5% CO_2. The cell suspensions were counted, and all cells were diluted down to a common concentration, defined by the suspension with the lowest cell number (generally approximately 10^6 cells/ml). The tubes were kept in a 33° or 40°C water bath until analysis (within 2 hours). Fluorescence was measured on a Perkin-Elmer 650-105 fluorescence spectrophotometer, with an excitation scan range of 420 - 525 nm. Emission at 535 nm was displayed on a chart recorder, with a scan speed of 60 nm/minute. A variety of excitation and emission slit widths were used, depending on the intensity of the fluorescence emitted. 1 ml of each sample was loaded into a glass cuvette (1 cm light path), and a rubber bung was used to seal the cuvette and thus slow down the escape of CO_2 from the medium. During the scan, the temperature of the sample was maintained at either 33° or 40°C, as appropriate, by a thermostatic control unit within the spectrophotometer reading chamber, and the cells were kept in suspension with a magnetic stirrer. *In vivo*

calibration was performed by applying 10 μM nigericin immediately before measurement to cells which had been stained with BCECF-AM and resuspended in calibration solutions (140 mM KCl, 1.2 mM $MgCl_2$, 10 mM HEPES) with pH values of 6.0, 7.2, 7.8 or 8.0. 1 ml samples of each calibration solution containing 10 nm BCECF-free acid were used to construct an *in vitro* calibration curve. The background level of cell and medium autofluorescence was assessed using a sample of unstained cells in growth medium, and calibration solution with no added BCECF-free acid was also scanned, as a control for the *in vitro* calibrations.

2.12 ETHANOL PRECIPITATION OF DNA

The monovalent cation concentration of the DNA solution was adjusted by the addition of either 0.1 volumes of 1 M sodium chloride, or 0.083 volumes of 3 M sodium acetate (pH 5.5). Two volumes of cold (-20°C) ethanol were added, mixed well, and the solution was placed at -20°C for 1 - 24 hours to allow the DNA to precipitate. The DNA was recovered by centrifugation at 13,000 rpm in an MSE microfuge for 10 minutes at 4°C, the supernatant was discarded, and the pellet was washed in cold (4°C) 70% ethanol and recentrifuged. The pellet was then drained, traces of ethanol were removed by vacuum dessication for 10 minutes, and the DNA was resuspended in the required volume of TE, pH 8.0 (10 mM Tris, 1 mM EDTA [ethylenediaminetetraacetic acid]).

2.13 SPECTROPHOTOMETRIC DETERMINATION OF DNA CONCENTRATIONS

The DNA solution was diluted to an appropriate level in TE (pH 8.0), and transferred to a glass cuvette with a 0.5 cm light path. A control cuvette contained TE (pH 8.0) alone. A Beckman DU-7 visible/UV spectrophotometer was used to scan the optical density (OD) of the solution over a range of 220 - 300 nm, and the DNA concentration was calculated from the OD reading at 260 nm, assuming that a DNA solution of 50 μg/ml gives an OD_{260} reading of 1.0 when a 1 cm light path is used (Maniatis *et al.*, 1982). The purity of the DNA i.e. the degree of protein or phenol contamination, was estimated by the ratio between the readings at 260 and 280 nm (OD_{260}/OD_{280}); a good DNA preparation should have a ratio of greater than 1.8 (Maniatis *et al.*, 1982).

2.14 PHENOL/CHLOROFORM/ISOAMYL ALCOHOL EXTRACTION OF DNA SOLUTIONS

2.14.i Preparation of phenol/chloroform/isoamyl alcohol

300 g of phenol crystals was dissolved in 300 ml of chloroform, and 0.3 g of 8-hydroxyquinoline and 6 ml of isoamyl alcohol were added. The mixture was shaken with 300 ml of 1 M Tris (pH 8.0), allowed to settle, and the aqueous layer was removed and discarded. The process was repeated with 300 ml of 0.1 M Tris (pH 8.0), and the pH of the aqueous layer was measured. If the pH was lower than 7.6, the phenol was re-extracted with deionized water until the pH rose (DNA will partition into the organic phase at acid pH). For short-term storage, the solution was kept at 4°C in a foil-wrapped bottle or, for longer storage periods, it was frozen at -20°C.

2.14.ii Extraction procedure

The DNA solution was transferred to a polypropylene tube, an equal volume of phenol/chloroform/isoamyl alcohol was added, and the solution was mixed well until an emulsion formed (mixing was achieved by brief vortexing for DNA molecules of < 10 kb, or the tube was mixed gently on a top-over-top rotator for 15 minutes for larger DNA molecules). The layers were then separated by centrifugation; volumes of under 1 ml were centrifuged at 13,000 rpm in an MSE microfuge for 2 minutes, whilst larger volumes were centrifuged in a Gallenkamp Labspin at 3,000 rpm for 10 minutes. The aqueous layer (containing the DNA) was transferred to a fresh tube, leaving the interface and organic layer behind, and was re-extracted until it was clear in appearance.

2.15 GROWTH AND PURIFICATION OF THE OKAYAMA-BERG λcDNA LIBRARY

Media and Solutions

LB (Luria-Bertani) medium

Per litre:

Bacto-tryptone	10g
Bacto-yeast extract	5g
NaCl	10g

The pH was adjusted to pH 7.5 with sodium hydroxide, and the medium was autoclaved at 15 lbs/sq.in. for 15 minutes.

TB medium

Per litre:

Bacto-tryptone	10g
NaCl	5g

The medium was autoclaved at 15 lbs/sq.in. for 15 minutes, and 10 ml of sterile 1 M $MgSO_4$ was added.

2.15.i Plaque assays

A loopful of the indicator bacterium *E. coli* JC8679, grown on a 1.5% (w/v) nutrient agar plate, was inoculated into 5 ml of LB broth supplemented with 0.2% (w/v) maltose, and incubated overnight at 37°C with shaking. The overnight culture was diluted tenfold into fresh LB broth containing 0.2% (w/v) maltose, and shaken for a further two hours. An appropriate dilution series of the phage suspension to be assayed was constructed with SM buffer (100 mM NaCl, 8 mM $MgSO_4$, 50 mM Tris [pH 7.5], 0.01% [w/v] gelatin) as the diluent, and 50 µl of each viral dilution was mixed with 0.2 ml of the indicator bacteria. After 15 minutes of incubation at room temperature, to allow for adsorption of the phage to the bacteria, 2.5 ml of melted 0.75% (w/v) nutrient agar in LB broth was added, and the resulting mixture was poured into a 10 cm petri dish containing a base layer of 1.5% (w/v) nutrient agar in LB broth. Duplicate plates were assayed for each phage dilution. Once set, the plates were inverted and incubated overnight at 37°C. The number of plaques on each plate was counted and used to estimate the concentration of phage particles in the original suspension, expressed as plaque forming units (pfu)/ml, on the assumption that each plaque represents initial infection of a single bacterium by a single virus.

2.15.ii Bulk growth of λcDNA

Stocks of the λ-based cDNA library of Okayama and Berg (1985), kindly donated by Prof. G. Stark (Imperial Cancer Research Fund), were prepared by the plate lysate method (Maniatis *et al.*, 1982). A culture of *E. coli* JC8679 was grown up overnight at 37°C, with shaking, in 5 ml of TB broth supplemented with 0.2% (w/v) maltose. $5x10^6$ pfu of λcDNA in SM buffer were added to 0.25 ml of the overnight culture, and incubated at room temperature for 15 minutes. 3 ml of melted 0.7% (w/v) nutrient agar in TB broth was added, and the mixture was poured onto a 10 cm petri dish containing a base layer of 1.5% (w/v) nutrient agar in TB broth supplemented with 0.2% (w/v) glucose. The plates were incubated at 37°C, without inversion, until confluent lysis was achieved (5 - 7 hours). To harvest the virus, the soft agar top layer was scraped into a 50 ml polypropylene conical tube using a sterile glass rod. The base layer surface was washed twice with 3 ml of SM, which was pooled with the soft agar. The tubes were centrifuged in a Gallenkamp Labspin at 4,000 rpm for 5 minutes and the supernatant, containing the phage, was poured off and retained. The pellet was re-extracted with 5 ml of SM buffer, the centrifugation was repeated, and the supernatants were pooled. 100 µl of chloroform was added to the crude lysate to prevent bacterial contamination, and it was stored at 4°C. The concentration of phage particles was estimated by a plaque assay.

2.15.iii Purification of λcDNA by caesium chloride centrifugation

The bacterial debris was removed from the crude lysate by centrifugation at 4°C in an MSE-18 centrifuge (6 x 50 ml rotor) for 5 minutes at 10,000 rpm. The phage was pelleted from the resulting supernatant by further centrifugation for 2.5 hours at 20,000 rpm and 4°C. The supernatant was poured off immediately, and the viral pellet was covered with 1 ml of SM buffer and left overnight at 4°C. The pellet was then resuspended by gentle pipetting, and any remaining debris was removed by centrifugation in an MSE microfuge at 13,000 rpm for 2 minutes. The supernatant was transferred to a 14 ml Sorvall centrifuge tube, and 1.28 g of caesium chloride per gram weight of viral suspension was added, to give a caesium chloride density of 1.7 g/ml. This was overlaid by 3 ml of 1.6 density caesium chloride, followed by an equal volume of 1.4 density caesium chloride, and SM was added to fill the tube. The position of each interface was marked on the outside of the tube prior to its centrifugation at room temperature in a Sorvall Ultracentrifuge OTD65B for 1.5 hours at 33,000 rpm. The phage collected at the interface between the 1.6 and 1.4 density caesium chloride layers, and was removed by puncturing the side of the tube with a

hypodermic syringe at the appropriate position and drawing out the viral band. In order to remove any contaminating caesium chloride, the phage was dialyzed at 4°C against SM buffer for 5 days, followed by 1x HBS, pH 6.85 (25 mM HEPES, 140 mM NaCl, 0.38 mM Na_2HPO_4, 0.38 mM NaH_2PO_4) for 2 hours. The resulting viral suspension was stored at 4°C, and the concentration of phage was estimated by plaque assay.

2.15.iv Purification of λcDNA by polyethylene glycol precipitation

An alternative method for the purification of λ particles, which avoids the use of caesium chloride centrifugation, was adapted from Silhavy *et al.* (1984). In order to remove the nucleic acids released from the lysed bacteria, the crude lysate was incubated for 30 minutes at room temperature with 1 μg/ml each of pancreatic DNase and RNase. Solid sodium chloride was then added, to a final concentration of 1 M (29.2 g NaCl/500 ml of lysate), and dissolved by gentle swirling. Following incubation for 1 hour on ice, the debris was removed by centrifugation at 4°C in an MSE-18 centrifuge (6 x 50 ml rotor) at 11,500 rpm for 10 minutes. The supernatants were pooled in a conical flask, and solid polyethylene glycol (PEG) 6000 was added to a final concentration of 10% (w/v). The PEG was dissolved by slow stirring on a magnetic stirrer at room temperature, and the flask was then cooled in ice water for 2 hours in order to precipitate the viral particles. The phage was recovered by centrifugation at 4°C in an MSE-18 centrifuge (6 x 50 ml rotor) for 10 minutes at 11,500 rpm, and the supernatant was removed and discarded. The pellet was gently resuspended in SM buffer (160 μl SM/10 ml pellet) using a wide-bore pipette, and the walls of the centrifuge bottle were washed thoroughly to remove any adhering viral precipitate. An equal volume of chloroform was added, and mixed thoroughly by vortexing. The organic and aqueous phases were separated by centrifugation at 4°C in an MSE-18 centrifuge (6 x 50 ml rotor) at 4,000 rpm for 15 minutes, and the aqueous phase (containing the virus) was recovered, leaving the PEG behind at the interface. The chloroform layer was re-extracted with 0.2 ml of SM, and the aqueous layers were pooled. The pure viral suspension was stored at 4°C, with the addition of 50 μl of chloroform to maintain sterility. The concentration of the virus was assessed by plaque assay.

2.16. GROWTH AND PURIFICATION OF THE OKAYAMA-BERG PLASMID cDNA LIBRARY AND pSV2neo

Media

X broth

Per litre:

Bacto-tryptone	10g
Bacto-yeast extract	5g
NaCl	5g

The medium was autoclaved for 15 minutes at 15 lbs/sq. in., and the following compounds were then added:

$MgCl_2$	1g
Glucose	5g
Diaminopimelic acid	0.1g
Thymidine	0.04g

2.16.i Bulk growth of the Okayama-Berg plasmid cDNA library

The Okayama-Berg plasmid cDNA library (Okayama and Berg, 1983) was obtained, via Prof. P. Nurse (Imperial Cancer Research Fund), as a frozen ampoule containing 1 ml of *E. coli* χ1776 transformed with the plasmid library. The contents of the ampoule were inoculated into 10 ml of X broth supplemented with 15 μg/ml ampicillin, and incubated overnight at 37°C with shaking. In order to maintain stocks of the library as similar as possible in composition to the original library, aliquots of the overnight culture were frozen down before any further growth, using the glycerol freezing method (Maniatis *et al.*, 1982); 0.85 ml of overnight culture was transferred to a sterile vial containing 0.15 ml of glycerol, and the contents were mixed thoroughly by vortexing and stored at -20°C. Bulk growth of the plasmid cDNA library was achieved by the addition of 4 ml of the overnight culture to 1 litre of X broth containing 15 μg/ml of ampicillin. The flask was incubated at 37°C with vigorous shaking, until the optical density of the culture at 600 nm (OD_{600}) reached a value of 0.4 - 0.5. 5 ml of chloramphenicol solution (34 mg/ml in ethanol) was then added, in order to increase the plasmid copy number, and incubation with

shaking at 37°C was continued for a further 16 hours.

2.16.ii Bulk growth of pSV2neo

E. coli HB101 harbouring the pSV2neo plasmid was stored at -20°C as a frozen glycerol stock. The surface of a frozen suspension was scratched with a sterile wire loop, and the bacteria were streaked onto a 1.5% (w/v) nutrient agar plate supplemented with 15 μg/ml ampicillin. The plate was inverted and incubated overnight at 37°C. A colony from this plate was inoculated into 10 ml of nutrient broth containing 15 μg/ml ampicillin, and incubated overnight at 37°C with shaking. Bulk growth of the bacteria was carried out as described in Section 2.16.i, with the exception that nutrient broth was used in place of X broth.

2.16.iii Purification of the Okayama-Berg plasmid cDNA library and pSV2neo by polyethylene glycol precipitation

Both plasmid types were purified by a method adapted from Godson and Vapnek (1973). The bacteria were pelleted by centrifugation of the overnight culture at 4°C in an MSE-18 centrifuge (6 x 250 ml rotor) at 7,000 rpm for 10 minutes. The resulting pellets were resuspended in 2 ml of lysis buffer (25% [w/v] sucrose, 50 mM Tris [pH 8.0], 1 mg/ml lysozyme), pooled, and placed on ice for 15 minutes. χ1176 proved refractory to lysis in preliminary attempts, thus the lysozyme concentration was increased to 2 mg/ml in subsequent preparations involving this organism. EDTA was added to a final concentration of 10 mM, and a further 15 minute incubation on ice was carried out. 5 ml of 3x Triton buffer (1.5% [v/v] Triton X-100, 75 mM Tris [pH 8.0], 94 mM EDTA) was then added for every 10 ml of bacterial suspension, mixed gently, and left on ice for 30 minutes to complete the lysis of the bacteria. The bacterial debris was removed by centrifugation at 4°C in an MSE-18 centrifuge (6 x 50 ml rotor) for 60 minutes at 12,500 rpm, and the supernatant was transferred to a 50 ml polypropylene centrifuge tube. Solid sodium chloride was added to a final concentration of 0.5 M, the protein was removed by extracting twice with phenol/chloroform/isoamyl alcohol (Section 2.14.ii) and the latter process was subsequently repeated twice with an equal volume of chloroform alone, in order to remove any contaminating phenol. 10% (w/v) PEG 6000 was added to the aqueous layer, dissolved by shaking at 37°C, and the solution was left at 4°C overnight to allow for DNA precipitation. The plasmid was collected by centrifugation at 10,000 rpm in an MSE-18 centrifuge (6 x 50 ml rotor) for 20 minutes at 4°C, and the pellet was redissolved in 0.5 ml of 0.1 M Tris (pH 8.0) and transferred to a 1.5 ml

Eppendorf tube. Contaminating RNA was degraded by the addition of pancreatic RNase to a final concentration of 0.2 mg/ml, and incubation at 37°C for 30 minutes. An equal volume of a solution containing 10 mM Tris (pH 8.0), 1 mM EDTA, 1 M NaCl and 20% (w/v) PEG 6000 was added, and the mixture was left on ice for 1 hour. The DNA was recovered by centrifugation at 13,000 rpm in an MSE microfuge for 10 minutes at room temperature, and the pellet was redissolved in 400 µl of 10 mM Tris (pH 8.0), 0.5 M NaCl. Two phenol/chloroform/isoamyl alcohol extractions and two chloroform extractions were subsequently carried out, and the plasmid was precipitated from the final aqueous layer by ethanol precipitation (Section 2.12), and resuspended in 1 ml of TE (pH 8.0). The concentration and purity of the DNA was estimated by spectrophotometric determination (Section 2.13).

2.17 ISOLATION OF HIGH-MOLECULAR-WEIGHT DNA FROM TISSUE CULTURE CELLS

The method used for the preparation of high-molecular-weight chromosomal DNA was a modification of the protocol published by Dillela and Woo (1985). Thirty 10 cm plates of the cell line in question were grown to confluence in 5% FCS E4 low bic, the medium was removed, and each dish was washed with 5 ml of PBSA. 1 ml of PBSA was added per plate, and the monolayer was scraped from the base of the dish with a sterile rubber policeman. The PBSA (now containing the cells) was transferred to a 50 ml polypropylene conical tube and the plate was washed once more with 2.5 ml PBSA, which was pooled into the same tube. The cells were recovered by centrifugation at 2,000 rpm in a Gallenkamp Labspin for 5 minutes, the supernatant was gently removed by aspiration, and the pellet was resuspended in 0.5 ml of PBSA. 20 ml of lysis buffer (50 mM NaCl, 10 mM EDTA, 10 mM Tris; pH 8.1) was added to each tube, and proteinase K and sodium dodecyl sulphate (SDS) were added to give final concentrations, respectively, of 100 µg/ml and 1% (w/v). The tubes were incubated overnight at 37°C, and each tube was subsequently extracted with phenol/chloroform/isoamyl alcohol (Section 2.14.ii). The DNA was then precipitated with isopropanol; the final aqueous layer was adjusted to contain 0.1 M sodium acetate (pH 5.5), and one volume of isopropanol was added. Samples were mixed for 15 minutes on a top-over-top rotator, and the precipitated DNA was spooled out from the interface with a sterile glass rod and washed in 2 ml of 70% ethanol. The pellet was touched to the side of a sterile tube to drain out the ethanol, and allowed to dissolve overnight at 4°C without shaking in 3 ml of TE (pH 8.0). Contaminating RNA was degraded by incubation with 100 µg/ml of pancreatic RNase at 37°C for 1

hour, and any protein remaining in the sample was digested by treatment with 100 μg/ml proteinase K and 1% (w/v) SDS at 37°C for 3 hours. The solution was then extracted with phenol/chloroform/isoamyl alcohol, and the DNA was precipitated with isopropanol, as described above, and dissolved in 5 ml of TE (pH 8.0). The concentration and purity of the DNA was assessed by spectrophotometry (Section 2.13).

2.18 TRANSFECTION OF K12 CELLS

2.18.i Standard calcium phosphate precipitation method

The calcium phosphate-mediated transfection protocol used was adapted from that of Graham and van der Eb (1973). A cell suspension was prepared from a subconfluent plate of K12 cells, replated in 5% FCS E4 low bic at a concentration of $4x10^5$ cells/6 cm plate, and incubated overnight at 33°C. The appropriate amount of DNA was diluted with water to give a volume of 0.225 ml, and 0.225 ml of 2x HBS (pH 6.85) was added. 0.05 ml of 1.25 M $CaCl_2$ was then added slowly, mixed well, and the solution was allowed to stand at room temperature for 15 minutes, by which time a visible precipitate had formed. For transfections where 10 cm plates were used, cell numbers were increased to 10^6cells/plate and precipitate volumes were doubled. The precipitate was transferred to the plate of cells dropwise, mixed by swirling, and the dishes were returned to a 33°C incubator for 4 hours. Following this incubation period, the medium was removed and the plates were washed in 2 ml of 1x HBS (pH 7.1). In order to increase the transfection efficiency, the cells were glycerol-shocked by incubation in 1 ml of 15% glycerol in 1x HBS (pH 7.1) at room temperature for 4 minutes. Plates were washed twice with 2 ml of 1x HBS (pH 7.1), fresh medium was added, and the cells were returned to a 33°C incubator. After a 24 hour recovery period, the selective agent was applied. The medium, supplemented with the selective agent at the appropriate concentration, was then changed at 4 - 5 day intervals, and plates were shifted to 40°C 1 - 3 weeks after transfection.

2.18.ii High-frequency transfection method

In order to increase the efficiency of calcium phosphate precipitation, the protocol of Chen and Okayama (1987) was followed. K12 cells were plated in 5% FCS E4 low bic at a concentration of $5x10^5$ - $1.2x10^6$ cells/10 cm dish, and incubated overnight at 33°C. 20 μg of DNA was mixed with 0.5 ml of 0.25 M $CaCl_2$, 0.5 ml

of 2x BBS (50 mM BES [N,N-bis(2-hydroxyethyl)-2-aminoethanesulphonic acid], 280 mM NaCl, 1.5 mM Na_2HPO_4; pH 6.95) was added, and the mixture was incubated for 15 minutes at room temperature. The precipitate was added dropwise to the plate of cells, mixed gently, and the dishes were incubated for 20 hours at 33°C under a 3% CO_2 atmosphere. The medium was then removed, plates were washed twice with 5 ml of growth medium, and the cells were fed with 10 ml of medium and returned to a 33°C incubator with a 5% CO_2 atmosphere. In order to prevent monolayers growing to superconfluence and dying at the high transfection efficiencies attained, cells were split at an appropriate ratio by trypsinization and resuspension after a 24 hour incubation period, and the selective agent was applied the following day. The growth medium, supplemented with the selective agent, was subsequently changed every 4 - 5 days, and the plates were shifted to 40°C 1 - 3 weeks after transfection.

2.19 PRODUCTION AND SCREENING OF K12-HUMAN HYBRID CELLS

Solutions

PEG solution 5 g of solid PEG 1500 was autoclaved at 15lbs/sq.in. for 15 minutes and, while still molten, 5 ml of serum-free E4 low bic was added. The pH was adjusted with sterile 10% (w/v) sodium bicarbonate until the medium became pink (pH 7.4 - 7.6).

2.19.i Fusion of cells and selection of hybrids

Human fibroblasts (a generous donation from Dr. S. Povey, University College London) were grown in 10% FCS minimal Eagle's medium at 33°C in a sealed $75cm^2$ flask until approximately 50% confluent. A subconfluent plate of K12 cells was trypsinized, and $2.4x10^6$ cells suspended in 1 ml of 10% FCS E4 low bic were added to the flask containing the human fibroblasts, and incubated at 33°C. Five hours later, when the K12 cells had settled and attached, the medium was removed to the last drop, and the cells were washed with 10 ml of serum-free E4 low bic. With the flask upright, 5 ml of PEG solution was added, the flask was laid flat, and the cells were incubated with the PEG solution for exactly 1 minute at room temperature. Before the minute had elapsed, the flask was tilted slightly to let the PEG run off the cells, and at precisely 1 minute, 10 ml of serum-free E4 low bic was added to dilute out the PEG. The medium was removed, the cells were washed

three times with 10 ml of serum-free E4 low bic, 15 ml of 10% FCS E4 low bic was added, and the flask was incubated at 33°C with the lid loose. Twenty four hours later, the cells were trypsinized, seeded into twenty 6 cm dishes, and placed in a 40°C incubator in order to select against unfused K12 fibroblasts. The growth medium was subsequently changed twice weekly. Many unfused human fibroblasts were still present one month after fusion, thus the medium was supplemented with 0.728 μg/ml of ouabain, a Na^+/K^+-ATPase inhibitor (Skou, 1965) to which human cells are more sensitive than rodent-human hybrids (Baker *et al.*, 1974), and medium changes were reduced to once per week.

2.19.ii Producing hybrid clones for chromosome screening

For chromosome screening purposes, it was necessary to produce a number of independent clonal populations of cells. In order to achieve this, the medium was removed from the plate of interest and well-separated, independent hybrid colonies were each surrounded by a metal cloning cylinder. Trypsinization was carried out within the cloning cylinders, and the resuspended cells from each single colony were replated on separate 6 cm dishes in 5 ml of 10% FCS E4 low bic supplemented with 0.728 μg/ml ouabain. Incubation was continued at 40°C, in order to allow a large healthy stock of independent hybrid clones to grow. Each clone was frozen down into liquid nitrogen (Section 2.3.i) once a healthy line had been established.

2.19.iii Subcloning of hybrids

In order to select for rapidly growing subclones, the required hybrid line was seeded at a low inoculum on a 6 cm plate in 5% FCS E4 low bic supplemented with 0.728 μg/ml ouabain, and incubated at 40°C. The medium was removed from the dish, and the largest colony i.e. the clone with the most rapid growth rate, was scraped off with a fine glass tip, resuspended in 5 ml of 5% FCS E4 low bic containing 0.728 μg/ml ouabain, and replated. The process was repeated every 6 - 7 days.

2.20 RESTRICTION ENZYME DIGESTS

Solutions used

i. Digestion buffers

10x high salts buffer	100 mM NaCl, 50 mM Tris (pH 7.5), 10 mM $MgCl_2$, 1 mM dithiothreitol.
10x medium salts buffer	50 mM NaCl, 10 mM Tris (pH 7.5), 10 mM $MgCl_2$, 1 mM dithiothreitol.

Digestion buffers were stored at -20°C.

ii. 5x gel loading buffer	0.1% (w/v) bromophenol blue, 50% (w/v) sucrose, 0.05 M EDTA (pH 8.0), 4 M urea.

Gel loading buffer was stored at 4°C.

Restriction digests

Restriction digests were carried out as outlined in Maniatis *et al.*. (1982). The DNA sample was diluted in deionized water, such that the total volume of restriction enzyme to be added (10 units/µg of DNA) did not exceed 10% of the total reaction volume (restriction enzymes were supplied in 50% glycerol, which inhibits the digestion reaction if present in significant quantities). 0.1 volumes of the appropriate 10x digestion buffer was added, followed by 5 units of restriction enzyme/µg DNA. The solution was mixed well, and incubated in a 37°C water bath for 90 minutes. A further 5 units of restriction enzyme/µg DNA were added, and the sample was mixed and incubated for 60 minutes at 37°C. If the digest was to be electrophoretically separated without further manipulation, 0.25 volumes of 5x gel loading buffer were added, and the sample was vortexed and loaded onto an agarose gel (Section 2.21). If the use of gel loading buffer to stop the reaction was to be avoided e.g. for probe preparation, the reaction was stopped by the addition of 10 mM EDTA. Alternatively, if the DNA was to be concentrated down to a smaller volume after digestion, the restriction enzyme was removed by phenol/chloroform/isoamyl alcohol extraction (Section 2.14.ii), and the digested DNA was recovered by ethanol precipitation (Section 2.12) and resuspended in the appropriate volume of deionized water.

2.21 AGAROSE GEL ELECTROPHORESIS

2.21.i Standard electrophoresis

For a 20 x 17 x 0.5 cm gel size, 170 ml of a 1% (w/v) agarose solution made up

in 1x TAE buffer (40 mM Tris, 1 mM EDTA; adjusted to pH 8.0 with glacial acetic acid) was boiled in a microwave until the agarose dissolved, cooled to 50°C, and poured into a gel-former, using a 20-well comb. The gel was placed in an electrophoresis tank, and the tank was filled with sufficient 1x TAE to cover the surface of the gel by approximately 2 mm. The DNA samples were loaded into the wells, and 0.4 - 0.6 µg of either a λ*Hin*d III or a λ*Eco* RI digest was loaded, to allow for the sizing of bands after separation. In order to prevent diffusion of the samples into the running buffer, the DNA was forced rapidly into the gel by the application of a voltage of 3 V/cm for 10 minutes. The voltage was subsequently maintained at 1 V/cm until the gel loading buffer had migrated approximately 75% of the gel length (16 - 20 hours). The gel was then removed from the tank, and stained by immersion for 1 hour in 0.5 µg/ml of ethidium bromide in 1x TAE with continuous agitation. Non-specific background stain was reduced by rinsing in deionized water for 1 - 2 minutes, and the gel was viewed on a UV transilluminator (Ultraviolet Products Inc, Chromato-Vue C-63) at a wavelength of 302 nm. A Polaroid 600SE land camera was used to record the electrophoretic separation of the DNA on Polaroid film (Type 665).

2.22.ii Minigels

A minigel tank was used for rapid analysis of DNA samples, with a gel size of 10 x 7.5 x 0.5 cm and an 8-well comb. The gel was poured using 45 ml of a 0.8% (w/v) agarose solution in 1x TBE (89 mM Tris, 89 mM boric acid, 2 mM EDTA [pH 8.0]), placed in the gel tank, and immersed in 40 ml of 1x TBE containing 0.5 µg/ml ethidium bromide. Samples, including a λ*Eco* RI or λ*Hin*d III marker (0.4 - 0.6 µg), were loaded, and a voltage of 2 - 3 V/cm was applied for 3 - 4 hours until the gel loading buffer was close to the end of the gel. After removal from the tank, the gel was rinsed for 1 - 2 minutes in deionized water, and viewed and photographed on a UV transilluminator.

2.22.iii Pulsed-field gel electrophoresis

When good separation of large DNA fragments was required, pulsed-field gel electrophoresis (PFGE) was carried out, using an LKB 2015 Pulsaphor system with a hexagonal electrode. In order to prevent overheating of the apparatus at the high voltages required, the conductivity of the running buffer (TBE) was reduced by lowering the EDTA concentration from its usual value of 2 mM. 110 ml of 1.2% agarose-NA in 1x TBE low EDTA (100 mM Tris, 100 mM boric acid, 0.2 mM EDTA;

pH 8.5) was used for a 15 x 15 x 0.5 cm gel with a 16-well comb, and the gel was placed in the electrophoresis tank when set. The unit was filled with 2.5 litres of 1x TBE low EDTA, and the DNA samples were loaded. Marker tracks contained 1 μg of whole λ DNA, 0.75 μg of a λ*Hin*d III digest, and 1 μg of a λ concatamer sample (kindly supplied by Miss J. Woodall, University College London). A Multitemp II controller was used to cool the running buffer to 14°C during the run, and a voltage of 8 V/cm (with a 10 second pulse time) was applied to the gel for 16 - 17 hours. Once the run was completed, the gel was removed from the tank and stained for 1 hour on an orbital shaker in 0.5 μg/ml ethidium bromide in 1x TBE low EDTA. Destaining was achieved by rinsing the gel for 1 - 2 minutes in deionized water, and the gel was transferred to a transilluminator for photographic documentation.

2.22 TRANSFER OF DNA TO NITROCELLULOSE BY BLOTTING

2.22.i Capillary blotting (adapted from Maniatis *et al.*, 1982)

If capillary blotting was required, the gel was transferred to a plastic box and immersed in 400 ml of depurination solution (0.25M HCl) following the documentation of the electrophoretic separation of the DNA samples on a transilluminator. The gel was agitated on an orbital shaker for 15 minutes, the depurination solution was discarded, and the gel was rinsed three times in deionized water. The gel was subsequently shaken in 400 ml of denaturation solution (1.5 M NaCl, 0.5 M NaOH) for 30 minutes. The denaturation solution was removed, and the gel was washed three times in deionized water, before the addition of 400 ml of neutralization solution (1.5 M NaCl, 0.5 M Tris; pH 7.5) and agitation for a further 30 minutes. This solution was discarded, replaced with 400 ml of fresh neutralization solution, and the gel was shaken for a further 15 minutes. The DNA was then transferred to nitrocellulose (Schleicher and Schuell, 0.45 μm pore size) using the method described by Southern (1975). A plastic dish was part-filled with the transfer buffer, 20x SSC (0.3 M tri-sodium citrate, 3 M NaCl; pH 7.0), and a supporting platform was made with a glass plate and covered with a wick, consisting of three sheets of Whatman 3MM filter paper saturated with 20x SSC. The gel was placed on the 3MM paper, with the well-openings facing downwards, and any air bubbles trapped beneath it were smoothed out. To prevent the transfer buffer being absorbed directly into the paper towels above, the edges of the gel were masked with Saran wrap. A sheet of nitrocellulose, cut to the exact size of the gel and with the top left-hand corner cut

off for orientation purposes, was pre-wet briefly in deionized water, followed by a 15 minute immersion in 20x SSC, and placed on top of the gel. Air bubbles were squeezed out, and three sheets of 3MM paper, cut to size and wetted in 20x SSC, were positioned on top of the nitrocellulose. A 5 - 7 cm stack of absorbent paper tissues was placed on the 3MM paper, and held down with a weight of approximately 750 g. Transfer was allowed to proceed for 16 - 20 hours. The blotting stack was then dismantled, and the position of each well on the nitrocellulose was marked with a chinagraph pencil. To remove any adhering agarose, the nitrocellulose was washed in 6x SSC with gentle agitation for 5 minutes, and allowed to air-dry. The DNA was fixed to the membrane by sandwiching the blot between two sheets of 3MM paper, and baking in an 80°C oven for 2 hours. In order to confirm the transfer of the DNA to the nitrocellulose, the gel was restained in 0.5 μg/ml ethidium bromide in gel running buffer for 1 hour, and viewed on a transilluminator.

2.22.ii Vacuum blotting

A Hybaid Vacu-aid blotting system, which uses a vacuum to effect the transfer of the DNA fragments to nitrocellulose more rapidly than capillary blotting, was also employed. A piece of 3MM paper, slightly larger than the gel, was pre-wet in 20x SSC and placed on the blotting apparatus. This was overlaid with a piece of nitrocellulose, also pre-wet in 20x SSC, and the gel was positioned on top, with the well-openings facing upwards. Any air bubbles trapped between each of the layers were smoothed out, and a vacuum of 80 cm of water was applied. 25 ml of depurination solution was placed on the surface of the gel, and left for 15 minutes to draw through. Excess solution was removed with an aspirator, and 25 ml of denaturation solution was pipetted onto the gel surface and allowed to pass through the gel for 20 minutes. Any remaining solution was removed, and neutralization was achieved by two 15 minute washes with 25 ml of neutralization solution per wash. The surface of the gel was then covered in 20x SSC, and transfer was allowed to proceed for 1 hour with continual replenishment of the transfer buffer as it was drawn through the gel. The vacuum pump was turned off, and the blotting stack dismantled. The position of the wells on the nitrocellulose was marked, and it was subsequently washed and baked as for capillary blotting (Section 2.22.i), and the gel was restained to confirm that complete transfer had been achieved.

2.22.iii Dot blotting

The most effective protocol for dot blotting was found to be that outlined by

Amersham for use with Hybond membranes. The DNA samples to be spotted were denatured by heating to 95°C in a water bath for 10 minutes, and chilled on ice for 3 minutes. An equal volume of 20x SSC was added, and the samples were diluted down to appropriate levels with 10x SSC as the diluent. A 3 cm stack of absorbent paper towels was placed on a glass plate, and overlaid with two sheets of dry Whatman 3MM filter paper. A third sheet of 3MM paper, soaked in 10x SSC, was added to the stack, and the nitrocellulose, also pre-wet in 10x SSC, was laid on top. The DNA samples were spotted onto the membrane, in aliquots of no more than 5 µl, and the spots were allowed to dry between the application of each aliquot. Once the samples were dry, the nitrocellulose was placed on a wad of 3MM paper, presoaked in denaturing solution (1.5 M NaCl, 0.5 M NaOH), for 5 minutes, and then immersed in neutralizing solution (1.5 M NaCl, 0.5 M Tris [pH 7.2], 0.001 M EDTA [pH 8.0]) for 1 minute. The nitrocellulose was air-dried, and the DNA was fixed to the membrane by baking in an 80°C oven for 2 hours.

2.23 PROBING OF BLOTS WITH THE AMERSHAM ECL SYSTEM

Labelling, hybridization and detection of the probe was carried out according to the manufacturer's instructions;

2.23.i Preparation of labelled probe

The linearized DNA probe, at a concentration of 10 ng/µl, was denatured by incubation for 5 minutes in a boiling water bath, and placed on ice for 3 minutes. An equal volume of the supplied DNA labelling reagent, a derivative of horseradish peroxidase (Renz and Kurz, 1984), was added and mixed thoroughly. The same volume of supplied glutaraldehyde solution was also added, and the tube was vortexed and incubated at 37°C for 10 minutes. Following incubation, the probe was stored on ice until ready for use (within 20 minutes).

2.23.ii Hybridization

All volumes given apply to a 100 cm^2 filter, and were scaled up or down as necessary. The dry blot was placed inside a heat-sealable plastic bag, 12.5 ml of the supplied prehybridization buffer was added, and the bag was sealed, and incubated in a 42°C shaking water bath for 15 minutes. 1 ml of the prehybridization solution was removed from the bag, mixed with 250 ng of labelled probe DNA, replaced, and

the bag was resealed. The blot was incubated for a further 16 - 20 hours at 42°C, with agitation. Following hybridization, any unbound probe was removed by placing the nitrocellulose in a plastic box, and washing the filter twice with 200 ml of primary wash buffer (6 M urea, 0.014 M SDS, 0.5x SSC) in a 42°C shaking water bath for 20 minutes. The blot was then transferred to a fresh container, immersed in 200 ml of secondary wash buffer (2x SSC), and placed on an orbital shaker at room temperature for 5 minutes. The secondary wash buffer was discarded, and the wash was repeated. The washed blot was drained, transferred to a fresh container, and taken to a darkroom for detection.

2.23.iii Detection of the signal

In order to visualize the bound labelled probe, 12.5 ml of the supplied detection reagent was pipetted onto the surface of the blot, and incubated for precisely 1 minute at room temperature. Excess detection buffer was drained off, and the blot was wrapped in Saran wrap and placed (DNA-side up) in an X-ray film holder. The darkroom lights were switched off, the appropriate type of X-ray film was placed on top of the blot, and the cassette was closed. After an exposure time of 1 hour, the film was removed and developed.

2.24 PROBING OF BLOTS USING A ^{35}S-LABELLED PROBE

2.24.i Preparation of labelled probe

The linear probe DNA was labelled using a BRL Nick Translation Reagent Kit, according to the manufacturer's protocol. 0.5 µg of linear probe DNA in deionized water was placed in an Eppendorf tube on ice, 5 µl of the supplied dNTP mixture (0.2 mM dCTP, 0.2 mM dGTP, 0.2 mM dTTP, 500 mM Tris [pH 7.8], 50 mM $MgCl_2$, 100 mM 2-mercaptoethanol, 100 µg/ml nuclease-free bovine serum albumin) and 156 pmoles of ^{35}S-dATP (800 C/mmole) were added, and the volume was made up to 50 µl with deionized water. The contents of the tube were mixed briefly, and 5 µl of a DNA polymerase I/DNase I solution (0.4 units/µl DNA polymerase I, 40 pg/µl DNase I, 50 mM Tris [pH 7.5], 5 mM magnesium acetate, 1 mM mercaptoethanol, 0.1 mM phenylmethylsulphonyl fluoride, 100 µg/ml nuclease-free bovine serum albumin, 50% [w/v] glycerol) was added. The solution was mixed gently, centrifuged in an MSE microfuge at 13,000 rpm for 5 seconds, and incubated for 2 hours at 15°C. The reaction was stopped by the addition of 5 µl of 0.3 M EDTA (pH 8.0). Unincorporated ^{35}S-dATP was removed from the labelled probe by passage

through a Sephadex bead column, as described in Maniatis *et al.* (1982). The bottom of the barrel of a 1 ml disposable plastic syringe was plugged with autoclaved siliconized glass wool, and the syringe was filled with a homogeneous Sephadex G-50 bead suspension (30 g Sephadex G-50/250 ml TE, pH 8.0). The syringe was supported on a 1.5 ml lidless Eppendorf tube, and placed inside a 15 ml polystyrene conical tube. The tube was centrifuged for 5 minutes in a Gallenkamp Labspin at 3,000 rpm and the Eppendorf, now containing TE, was discarded and replaced with a fresh tube. The DNA sample was loaded onto the top of the column, the incubation tube was washed with 50 µl of TE (pH 8.0) which was also added to the column, and the tube was centrifuged under exactly the same conditions as in the previous step. Unincorporated ^{35}S-dATP was retained on the column, which was discarded, and the labelled probe was recovered from the Eppendorf. The degree of incorporation of ^{35}S into the probe was assessed by liquid scintillation counting. Total counts were determined by spotting 1 µl of the DNA sample onto a 2 x 2 cm square of Whatman 3MM filter paper. Acid-insoluble counts i.e. counts from the nucleic acid, were measured by spotting 1 µl of DNA on a 2 x 2 cm square of 3MM paper, which was subsequently immersed in two batches of ice-cold 5% trichloroacetic acid, and two batches of ice-cold 70% ethanol. Both squares of 3MM paper were air-dried and immersed in Optiphase scintillation fluid. Counting was performed on an LKB 1211 Minibeta Liquid Scintillation Counter, and values were expressed as counts per minute (cpm)/µg DNA.

2.24.ii Hybridization

The hybridization method followed that outlined in Maniatis *et al.* (1982). The blot was soaked in 6x SSC for 2 minutes, placed inside a heat-sealable plastic bag, and 0.2 ml of warm (68°C) prehybridization solution (6x SSC, 0.5% [w/v] SDS, 5x Denhardt's solution, 100 µg/ml denatured salmon sperm DNA) was added per cm^2 of membrane. Air bubbles were squeezed out, and the bag was sealed, and incubated for 2 - 4 hours in a 68°C shaking water bath. The prehybridization fluid was removed from the bag, and replaced with 50 µl of hybridization solution (6x SSC, 0.01 M EDTA [pH 8.0], 5x Denhardt's solution, 0.5% [w/v] SDS, 100 µg/ml denatured salmon sperm DNA, 2x10^6 cpm ^{35}S-labelled freshly denatured probe DNA) per cm^2 of nitrocellulose. Air bubbles were eliminated, the bag was resealed, and incubation was continued for a further 16 - 20 hours at 68°C with agitation. The blot was removed from the bag and submerged in 2x SSC, 0.5% (w/v) SDS at room temperature for 5 minutes. The nitrocellulose was then transferred to a fresh container, and gently shaken for 15 minutes at room temperature in 2x SSC, 0.1% (w/v) SDS. The blot

was subsequently agitated at 68°C for 2 hours in 0.1x SSC, 0.5% (w/v) SDS, before the wash buffer was changed, and incubation was continued for a further 30 minutes. The nitrocellulose was then air-dried, and taken to a darkroom for detection.

2.24.iii Detection of the signal

The blot was placed in an X-ray film holder (DNA-side up) and the darkroom lights were switched off. A sheet of DuPont Cronex MRF-31 medical recording film was placed on top, and the cassette was closed and wrapped in aluminium foil. After a 3-day exposure period at -70°C, the film was removed and developed.

2.25 PROBING OF BLOTS WITH THE BOEHRINGER MANNHEIM DIGOXIGENIN-BASED LABELLING AND DETECTION KIT

Labelling, hybridization and detection of the probe was carried out according to the manufacturer's instructions;

2.25.i Preparation of labelled probe

The linear template DNA (1 µg) was denatured by incubation in a boiling water bath for 10 minutes, and placed on ice for 3 minutes. With the Eppendorf tube still on ice, 2 µl of the supplied 10x hexanucleotide reaction mixture, and 2 µl of 10x dNTP labelling mixture (1 mM dATP, 1 mM dCTP, 1 mM dGTP, 0.65 mM dTTP, 0.35 mM Dig-dUTP; pH 6.5) were added, and the volume was made up to 19 µl with deionized water. Two units of Klenow enzyme, in a volume of 1 µl, were added, mixed well, and the tube was centrifuged at 13,000 rpm in an MSE microfuge for 5 seconds before incubation at 37°C for 20 hours. The reaction was stopped by the addition of 2 µl of 0.2 M EDTA (pH 8.0), and the DNA was recovered, free of unincorporated dNTPs, by adding 1.83 µl of 3 M sodium acetate (pH 5.5) and 75 µl of cold (-20°C) ethanol. The tube was placed at -70°C for 30 minutes, and the precipitated DNA was collected by centrifugation at 13,000 rpm in an MSE microfuge for 10 minutes at 4°C. The pellet was washed with 40 µl of cold (-20°C) 70% ethanol, respun, and the supernatant was discarded. Traces of ethanol were removed by vacuum dessication for 10 minutes, and the DNA was dissolved at 37°C for 30 minutes in 50 µl of 10 mM Tris, 1 mM EDTA (pH 8.0), 0.1% (w/v) SDS.

2.25.ii Hybridization

The blot was sealed into a plastic bag containing 0.2 ml of hybridization solution (5x SSC, 0.1% [w/v] sodium N-lauroylsarcosine, 0.02% [w/v] SDS, 1% [w/v] supplied blocking agent) per cm^2 of membrane, and incubated in a shaking water bath at 68°C for 1 - 4 hours. The prehybridization fluid was discarded, replaced with 25 µl of hybridization solution (containing 30 ng/ml of freshly denatured labelled probe DNA) per cm^2 of nitrocellulose, and incubation was continued for a further 16 - 20 hours. Filters were washed twice at room temperature with 0.5 ml of 2x SSC, 0.1% (w/v) SDS per cm^2 of membrane, and were twice shaken for 15 minutes at 68°C with the same volume of 0.1x SSC, 0.1% (w/v) SDS. The nitrocellulose was then air-dried, and used for detection.

2.25.iii Detection of the signal

All incubations were carried out on an orbital shaker at room temperature, and all volumes given apply to a 100 cm^2 filter, and were scaled up or down as necessary. The blot was washed for 1 minute in 150 ml of buffer 1 (100 mM Tris, 150 mM NaCl; pH 7.5), followed by a 30 minute incubation with 100 ml of buffer 2 (0.5% [w/v] supplied blocking agent in buffer 1). The filter was then washed again, briefly, in 150 ml of buffer 1, and 20 ml of anti-digoxigenin antibody-conjugate (150 mU/ml polyclonal sheep anti-digoxigenin Fab fragments conjugated to alkaline phosphatase, dissolved in buffer 1) was applied. After a 30 minute incubation period, unbound antibody was removed by two 15 minute washes in 100 ml of buffer 1. The membrane was then equilibrated for 2 minutes with 20 ml of buffer 3 (100 mM Tris, 100 mM NaCl, 50 mM $MgCl_2$; pH 9.5), and sealed inside a plastic bag containing 10 ml of buffer 3, to which 45 µl of NBT solution (75 mg/ml nitroblue tetrazolium salt in 70% [v/v] dimethylformamide) and 35 µl of X-phosphate solution (50 mg/ml 5-bromo-4-chloro-3-indolyl phosphate, toluidinium salt, in dimethylformamide) had been added. The filter was incubated at room temperature in the dark (with no agitation) for 3 days, and the reaction was stopped by washing the membrane in 50 ml of TE (pH 8.0) for 5 minutes. The pattern of bands or spots was recorded by photocopying the membrane.

SECTION 3

RESULTS

3.1 CONFIRMATION OF THE TEMPERATURE-SENSITIVE NATURE OF K12

The aim of this experiment was to compare the survival of K12 and the parental strain, Wg-1A, at 33° and 40°C, and thus to confirm the temperature-sensitive nature of the growth of K12. In addition, the cell lines were grown at each temperature in two different types of serum - foetal calf serum (FCS) and calf serum (CS) - in order to investigate whether the composition of the serum used to supplement the medium has any effect on the growth of the fibroblasts at either temperature. For each cell line, a suspension containing 80 cells/ml was prepared (Section 2.4) in E4 low bic supplemented with either 5% FCS or 10% CS, and four 6 cm dishes were plated for each medium type. Following an overnight incubation at 33°C, two plates from each set of four were moved to 40°C, and all dishes were stained and counted (Section 2.5) one week after plating.

The results, expressed as E.O.P. values (Tables 3.1 and 3.2) show that although K12 was capable of forming healthy colonies at 33°C, no growth was seen at 40°C in either serum, confirming the temperature-sensitive nature of this cell line. In contrast, growth of Wg-1A, in terms of E.O.P., was equivalent at 33° and 40°C for a given serum type, and colonies were significantly larger at the higher temperature. This implies that the frequency of establishment of Wg-1A colonies is the same at each temperature, but that the growth and division rate of the cells is faster at 40°C, perhaps as a consequence of the general acceleration of enzymatic reactions at higher temperatures. For both cell lines at a given temperature, higher E.O.P. values were achieved when the cells were grown in medium supplemented with 5% FCS rather than 10% CS. This suggests that the composition of the former serum allows for the better establishment and growth of each cell line than that of the latter, thus medium containing 5% FCS was subsequently used routinely for the culture of both Wg-1A and K12. Of particular interest was the observation that K12 displayed a higher E.O.P. than Wg-1A at 33°C for a given serum type. Since these results appeared to be reproducible for cells grown in E4 low bic (see Tables 3.9, 3.10, 3.26 and 3.27), it seems unlikely that this may be explained by a general effect related to differences in the preparation of the two cell suspensions e.g. to variations in the length of trypsinization or in the growth stage of the stock plate, or to faults in aliquotting the cell suspension due to cell clumping. Consequently, the data appear to indicate that K12 and Wg-1A differ in their behaviour even at 33°C, implying that

the K12 ts protein may not show full wild-type activity at the pt, and that this may affect the E.O.P. of this cell line at this temperature. Alternatively, it is possible that the two cell lines differ at more than one locus, only one of which is concerned with cell cycle progression; another may influence colony establishment.

Table 3.1 EFFICIENCY OF PLATING OF K12 AT 33° AND 40°C IN E4 LOW BIC

SERUM	TEMPERATURE	COLONY NUMBER	E.O.P.	AVERAGE E.O.P.
FCS	33°C	254	63.5	
		270	67.5	65.5
FCS	40°C	0	0	
		0	0	0
CS	33°C	189	47.3	
		214	53.5	50.4
CS	40°C	0	0	
		0	0	0

Table 3.2 EFFICIENCY OF PLATING OF Wg-1A AT 33° AND 40°C IN E4 LOW BIC

SERUM	TEMPERATURE	COLONY NUMBER	E.O.P.	AVERAGE E.O.P.
FCS	33°C	182	45.5	
		206	51.5	48.5
FCS	40°C	187	46.8	
		215	53.8	50.3
CS	33°C	150	37.5	
		168	42.0	39.8
CS	40°C	165	41.3	
		154	38.5	39.9

3.2 FOLLOWING THE TIME COURSE OF THE LOSS OF VIABILITY OF K12 AT 40°C IN MEDIUM SUPPLEMENTED WITH TWO DIFFERENT SERA

In order to determine how rapidly K12 cells lose viability when plated at 40°C in medium supplemented with either 5% FCS or 10% CS, two alternative methods were used - viability curve experiments and time-lapse studies - and the results were compared with those obtained by Roscoe *et al.* (1973b) shortly after the isolation of the cell line. Loss of viability was defined as the loss of ability to divide, a consequence of the K12 cell cycle block. In the case of viability curve studies, this was reflected by the inability of cells to form colonies on return to 33°C, whereas time-lapse experiments directly followed the rate of mitosis in the population.

3.2.i Viability curve studies

A viability curve experiment, comparing the loss of the proliferative capacity of K12 at the npt in E4 low bic supplemented with either 5% FCS or 10% CS, was carried out as described in Section 2.6. The E.O.P. value for each time point was converted to % relative E.O.P. i.e. to a percentage of the value for plates incubated continuously at 33°C (Tables 3.3 and 3.4), and the results were expressed graphically (Fig. 3.2). Figure 3.2 demonstrates that, in both types of medium, K12 cells began to lose viability after 20 - 30 hours at 40°C, and the loss of colony-forming ability was almost complete by the 64.5 hour time point. The composition of the serum used to supplement the medium therefore appears to have little effect on the rate of loss of viability of K12 at the npt. When a similar experiment was carried out by Roscoe *et al.* in 1973, with K12 cells grown in 10% CS, the loss of viability occurred more rapidly; it began 15 - 20 hours after the temperature shift and was complete 20 hours later.

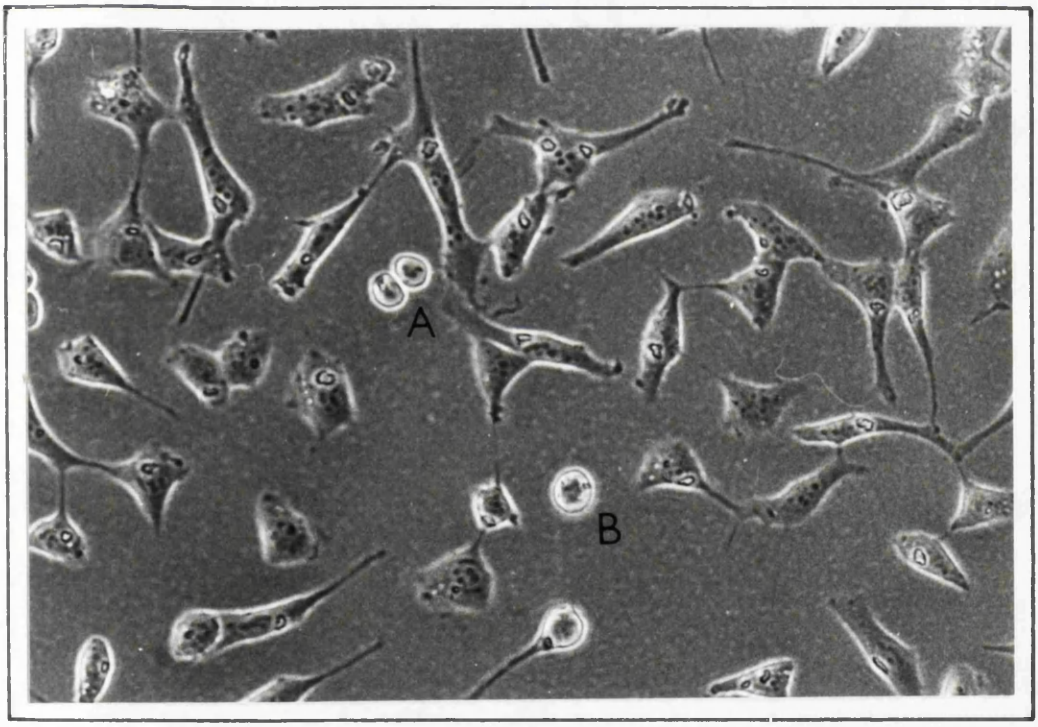
3.2.ii Time-lapse studies

An alternative approach to studying the loss of viability of K12 at 40°C was also used, in order to confirm and extend the results provided by the viability curve experiments. The chosen approach was that of time-lapse cinematography, which involves the study of single cells rather than cell populations. Experiments were carried out according to the protocol outlined in Section 2.7, using K12 cells suspended in E4 low bic supplemented with either 5% FCS or 10% CS. By constantly monitoring a field of 20 - 30 cells, it was possible to follow accurately the loss of viability for each cell under the various experimental conditions over a

continuous time spectrum, rather than by sampling the situation at a small number of time points, as was the case with the viability curve studies.

Preliminary experiments used cells that had been incubated overnight at 33°C before being placed on the time-lapse equipment. However, it was found that cells within the field of view were synchronized to a significant degree, perhaps since adjacent cells were likely to be sister cells formed during the overnight incubation. These tended to divide with approximately the same timings, leading to long periods where no cells were dividing, thus giving an incomplete representation of whether K12 cells at a particular time point had the ability to divide again. This problem was circumvented by placing cells on the time-lapse equipment only 4 hours after plating, thus allowing insufficient time for a significant number of divisions before the video was started, and consequently providing a field of mainly unrelated cells.

Figure 3.1 Phase-contrast micrograph of K12 fibroblasts growing at 33°C in 5% FCS E4 low bic



x280 magnification

A - cell that has recently completed division

B - mitotic cell

Analysis of the video consisted of following individual cells on the TV screen, and recording their times of division, the times of division of their daughters, their

daughter's daughters *etc.*, until a 'family tree' of division times was constructed for each of the cells originally in the field of view at the start of the recording (see Appendix IIa for an example). The time of division was defined as the first point at which the existence of two cells became apparent (Fig. 3.1 - cell A), rather than the point at which the cell rounded up for mitosis (Fig. 3.1 - cell B). This data was then used to construct two different kinds of graph, each giving information about the nature of the K12 defect.

a. Cumulative mitoses plots

For this plot, the cells were arranged in a table (see Appendix IIb) in order of time of mitosis, from earliest to latest, each mitosis was scored as 1, and the cumulative number of mitoses in the field was plotted against the time of each cell division. The resulting graph showed the rate of mitosis as the experiment progressed, and provided an indication of the loss of viability of K12 at the npt analogous to the situation in the viability curve experiments, since cells that can no longer divide would not be expected to form colonies in viability curve studies.

When a cumulative mitosis graph was plotted for K12 grown in 5% FCS E4 low bic (Fig. 3.3a), the rate of mitosis appeared to remain normal for approximately 30 hours after the shift to 40°C, but then decreased markedly, and there were no divisions later than 45 hours after the temperature increase. The cumulative mitosis graph for K12 cultured in 10% CS (Fig. 3.3b) showed a similar pattern - the mitotic rate began to decrease 35 hours after the shift, and the last cell division was seen 13 hours later - lending further support to the hypothesis that the type of serum used in the medium has no significant effect on the behaviour of K12 at the npt. These figures correspond fairly closely with those of the viability curve experiments (Fig. 3.2), where proliferative capacity was inhibited by 90% by the 50 hour time point. That cells in the viability curve studies appeared to survive for slightly longer than those in the time-lapse experiments may be a reflection of the fact that the time-lapse technique records only the time of the last mitosis, which is a few hours before the execution point, thus cells will remain viable for a short interval after their last division before reaching the block, and would be expected to form colonies if returned to 33°C. In addition, the blockage in K12 is to some extent reversible i.e. cells that have halted at the execution point at 40°C retain the capacity to proliferate for up to 15 - 20 hours if subsequently returned to the pt (Roscoe *et al.*, 1973b). The viability curve plots will therefore include a 15 - 20 hour period during which the cell, although blocked from cycling at the npt, is able to proliferate and form colonies if returned to 33°C, and will thus indicate a significantly slower loss

of viability than is seen with time-lapse cumulative mitosis graphs. Time-lapse plots therefore provide an accurate indication of the rate at which the cell cycle becomes blocked in K12 at 40°C, whereas viability curves will demonstrate the time taken for the cell line to lose its colony-forming ability, an event which happens some time after the initial arrest.

When these results were compared with those obtained using similar techniques by Dr. D.H. Roscoe in 1973 (unpublished data), once again K12 appeared to be losing viability more slowly than when first isolated. In the original studies, carried out in 10% CS, a cumulative mitosis plot (Fig. 3.4) indicated that the cells maintained a normal rate of mitosis for only 11 - 13 hours at 40°C, and the time of the last cell division was 20 hours after the temperature shift. It thus seemed reasonable to conclude that K12 cells were undergoing one or more extra cycles of division at the npt which were not seen in 1973. This was confirmed by studying the raw 'family trees', which show that many cells (but not all) divided twice, and some even three times after the temperature shift, whereas when initially isolated, K12 cycled until it reached the execution point of the mutation for the first time at high temperature and halted there, thus dividing a maximum of only once at 40°C.

b. Plots of intermitotic time

The time of each cell division was also plotted against the time taken for the progeny of that division to divide again (Appendix IIc), in order to determine whether the intermitotic time changed with the length of incubation at the npt. Such plots for K12 grown in either of the two sera (Figs. 3.5a and 3.5b) seem to indicate that the intermitotic time altered little when cells were shifted to 40°C, thus K12 cells appear to cycle normally until they arrest at the execution point. The average intermitotic time (A.I.T.) for cells grown in 10% CS, calculated from a total of 65 cells, was 21.3 hours (standard deviation = 5.55), whereas K12 cells cultured in 5% FCS showed an A.I.T. (from 89 cells) of only 17.3 hours (standard deviation = 5.00), further supporting the hypothesis, first proposed in Section 3.1, that medium supplemented with FCS allows for better growth of K12 cells than that containing CS.

In conclusion, whereas K12 behaved as a classic cell cycle mutant when first isolated, in that it was unable to pass the execution point of the mutation even once at the npt, at present the cells appear to have gained the ability to pass this point once, or in some cases even twice at 40°C, before finally halting. There are two possible explanations for this change; either the cell line itself, or the conditions

under which the cells are grown have altered. It is possible that the properties of K12 have changed at some point in the years since its isolation. If, as proposed in Section 1.12, the cell cycle block occurs when the activity of the K12 ts protein falls below a threshold level required to pass the execution point, due to either a direct or an indirect reduction in its activity or stability, then a compensating mutation may have occurred, either in the gene encoding the K12 ts protein or elsewhere in the genome, which prolongs the high activity level of the defective protein for just enough time to allow for one or two extra divisions at the npt, but not for long enough to allow for prolonged growth. This may be achieved by an increase in either the stability or activity of the ts protein. Alternatively, the composition of the medium in which the cells are grown may have altered significantly since 1973, and it is conceivable that this may have a considerable effect on the K12 phenotype i.e. an unknown medium component may, directly or indirectly, affect the activity or stability of the K12 ts protein. The current medium formulation may thus allow for an increase in the stability or activity of the protein defective in K12, and therefore allow for the present-day prolonged survival.

Unfortunately, original isolates of K12 from 1973 are no longer available, thus it was not possible to compare the behaviour of these cells with the current K12 cell line. Studies were thus confined to the examination of the effects of growth medium composition on the time course of loss of viability of K12 at 40°C, in order to determine whether a difference in medium formulation could indeed account for the alteration in the behaviour of K12 since its isolation. Since serum composition appeared to have no significant effect on the viability of K12 at the npt, as determined by both viability curve and time-lapse experiments, studies were concentrated on determining the effects of variations in the formulation of the basic growth medium on the survival of K12 at 40°C. However, any slight alterations in the behaviour of K12 which could be caused by batch-dependent differences in serum composition were reduced as far as possible by using the same batch of serum for each set of experiments.

Table 3.3 LOSS OF VIABILITY OF K12 AT 40°C IN 5% FCS E4 LOW BIC

HOURS OF INCUBATION AT 40°C	COLONY NUMBER	E.O.P.	AVERAGE E.O.P.	% RELATIVE E.O.P.
continuous	0	0		
	0	0	0	0
0	274	68.5		
	250	62.5	65.5	100
16.5	261	65.3		
	242	60.5	62.9	96.0
24.0	238	59.5		
	254	63.5	61.5	93.9
40.5	82	20.5		
	84	21.0	20.8	31.7
48.0	32	8.0		
	64	16.0	12.0	18.3
64.5	0	0		
	8	2.0	1.0	1.5

Table 3.4 LOSS OF VIABILITY OF K12 AT 40°C IN 10% CS E4 LOW BIC

HOURS OF INCUBATION AT 40°C	COLONY NUMBER	E.O.P.	AVERAGE E.O.P.	% RELATIVE E.O.P.
continuous	0	0		
	0	0	0	0
0	180	45.0		
	228	57.0	51.0	100
16.5	285	71.3		
	244	61.0	66.1	129.7
24.0	222	55.5		
	194	48.5	52.0	102.0
40.5	48	12.0		
	33	8.3	10.1	19.9
48.0	39	9.8		
	68	17.0	13.4	26.2
64.5	5	1.3		
	8	2.0	1.6	3.2

Figure 3.2 LOSS OF VIABILITY OF K12 AT 40°C IN E4 LOW BIC SUPPLEMENTED WITH DIFFERENT SERA

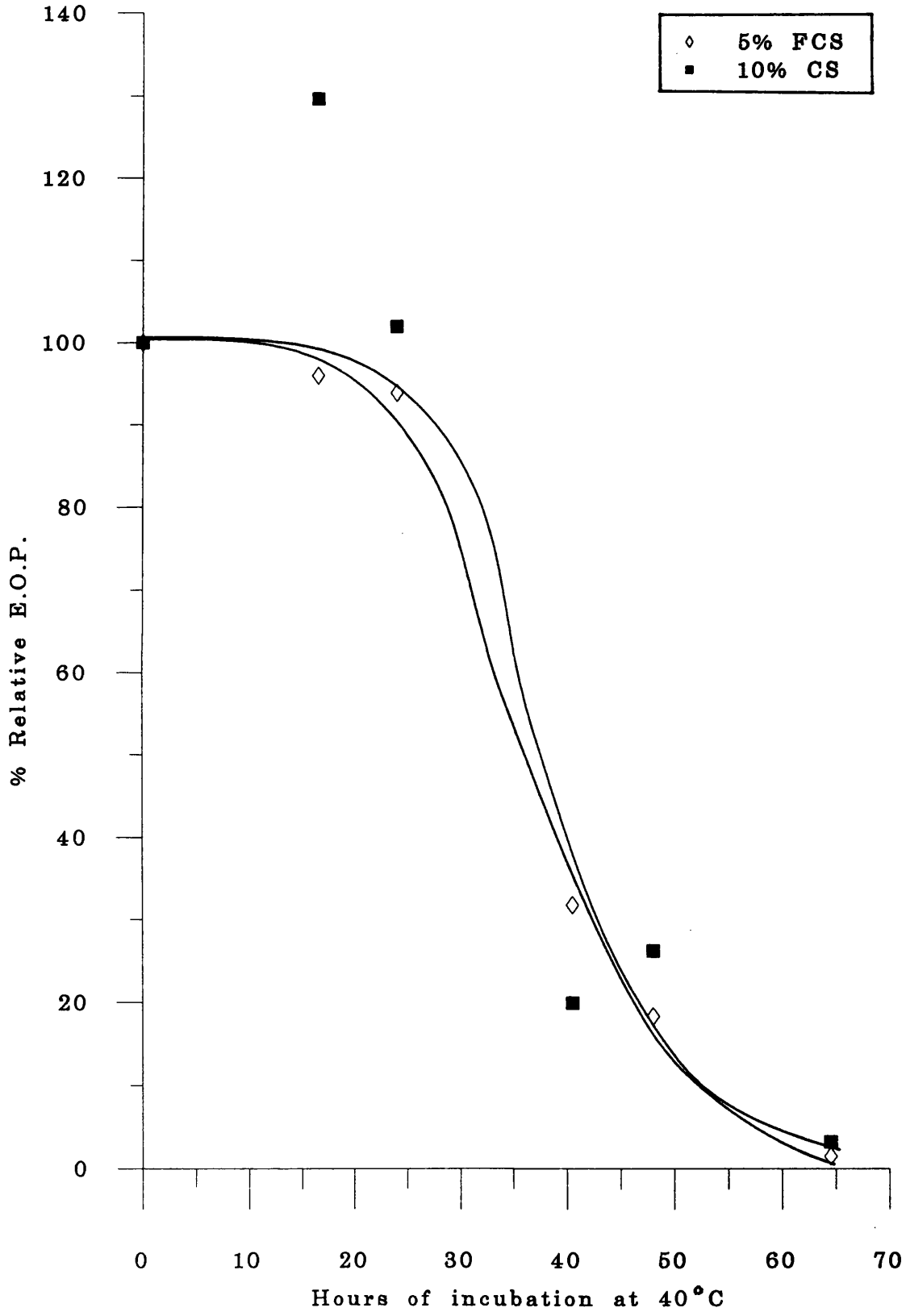
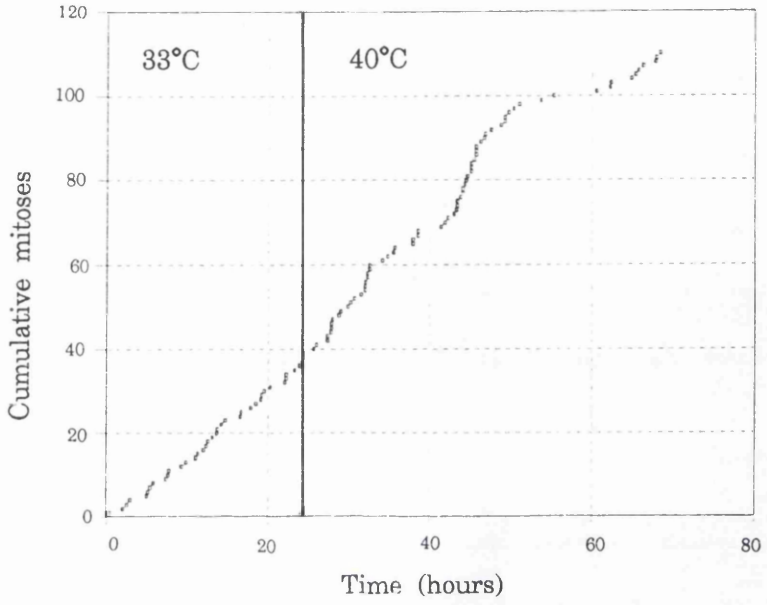


Figure 3.3 CUMULATIVE MITOSES PLOTS USING TIME-LAPSE DATA FOR K12 GROWN IN E4 LOW BIC SUPPLEMENTED WITH DIFFERENT SERA

a. 5% FCS



b. 10% CS

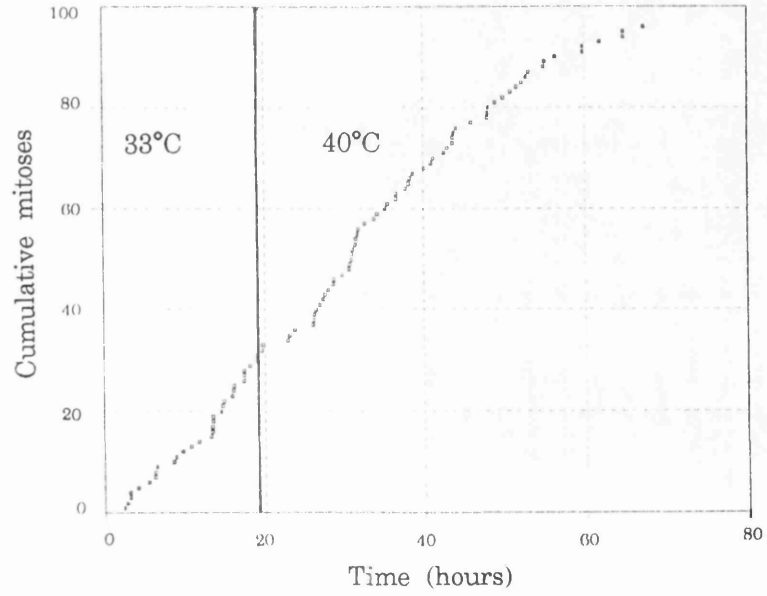


Figure 3.4 CUMULATIVE MITOSES PLOT USING TIME-LAPSE DATA FOR K12 GROWN IN 10% CS E4 LOW BIC (Dr D.H. Roscoe, unpublished data)

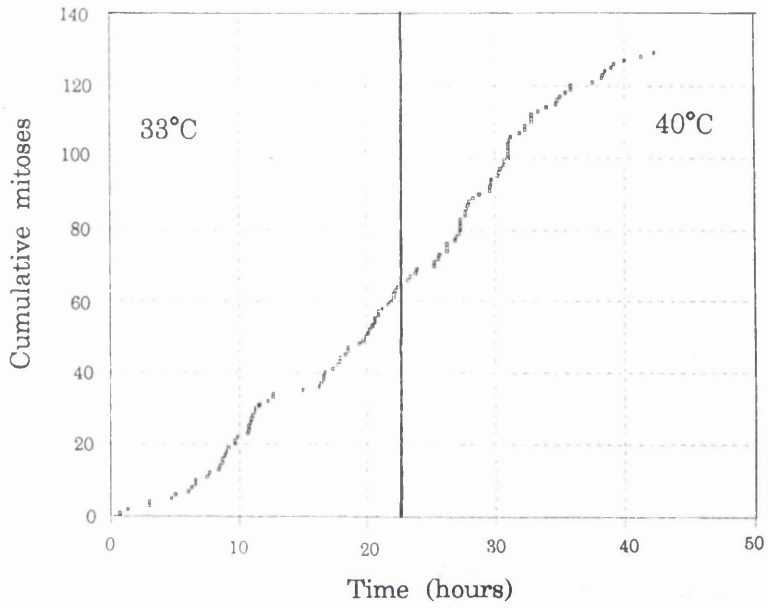
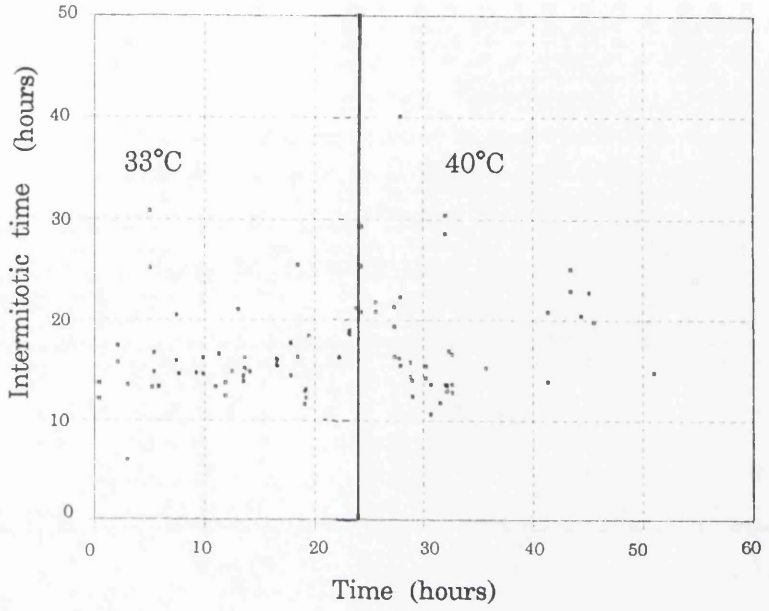
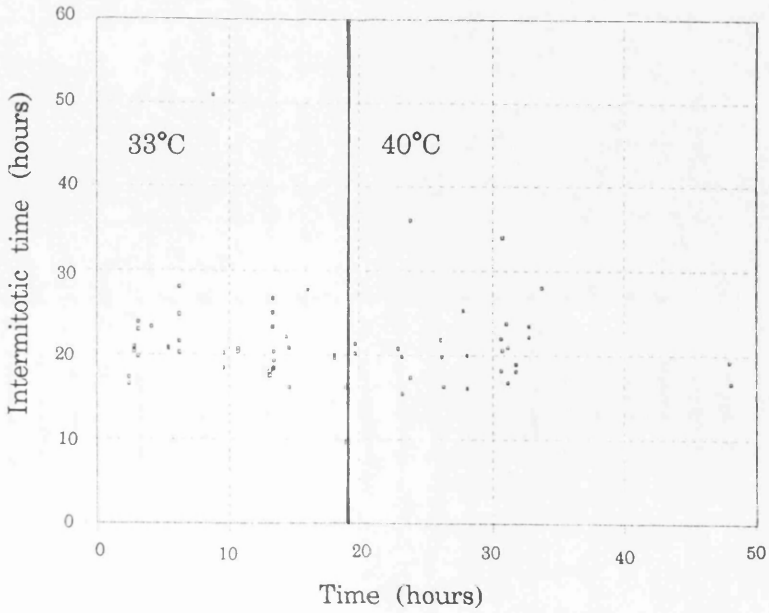


Figure 3.5 INTERMITOTIC TIME PLOTS USING TIME-LAPSE DATA FOR K12 GROWN IN E4 LOW BIC SUPPLEMENTED WITH DIFFERENT SERA

a. 5% FCS



b. 10% CS



3.3 EFFECTS OF TEMPERATURE ON THE LOSS OF VIABILITY OF K12

In viability curve studies where the effects of alterations in the medium composition on the survival of K12 at the npt was examined (see Sections 3.4, 3.6, 3.7, 3.9 and 3.12), even if the medium composition was identical on experiments carried out on different occasions, the viability curves produced differed significantly, casting doubt on the validity of using this technique for studying the phenomenon. One could postulate that such differences could be caused by slight variations in the method of preparation of the cell suspensions, such as a failure to harvest the stock plate at the same growth stage, or to standardize the length of trypsinization, but it seems unlikely that such minor changes would be reflected as significant alterations in the behaviour of K12 at the npt. Alternatively, it was considered possible that observed day-to-day fluctuations in the temperature of the '40°C' incubator, such that the actual temperature varied between 39° and 40.2°C, may have had an effect on the rate of loss of viability of K12 at the npt. In order to test this hypothesis, viability curves were thus carried out (Section 2.6) with K12 cells grown in 5% FCS E4 low bic, using nonpermissive incubator settings of 39.2°C (range 39.0° - 39.7°C) or 40°C (range 39.5° - 40.3°C). The results, expressed as % relative E.O.P. (Tables 3.5 and 3.6), were represented graphically (Fig. 3.6).

The results clearly show that even minor variations in the npt used have a significant effect on the rate of loss of viability of K12, with cells grown at 39.5° - 40.3°C losing viability 15 - 20 hours before those incubated at 39° - 39.7°C. This suggests that the loss of activity or structural collapse of the K12 ts protein, rather than being an 'all-or-none' effect, is a gradual process, and that the rate of loss or collapse can be accelerated by raising the npt from 39° to 40.3°C. The magnitude of the variation in the timing of the viability curves demonstrated here is similar to that observed when identical viability curve experiments were carried out on separate occasions, suggesting that the latter phenomenon could indeed be explained by fluctuations in the incubator temperature. Since it proved impossible to control the temperature of the 40°C incubator used with a greater degree of accuracy, a mid-range setting of 39.8°C was chosen (range 39.3° - 40.2°C). As a degree of variation would always be present, each study on the influence of alterations in the medium composition on the behaviour of K12 at the npt was therefore carried out as a complete set of viability curves on a single occasion, in order to minimize the possible effects of slight fluctuations in incubation temperature (any temperature variation would then be identical for all test plates), and thus produce reliable curves. Curves produced in separate sets of experiments were not considered to be comparable, as small differences in the incubation temperature may have had

additional significant effects on the rate of the loss of viability of this cell line.

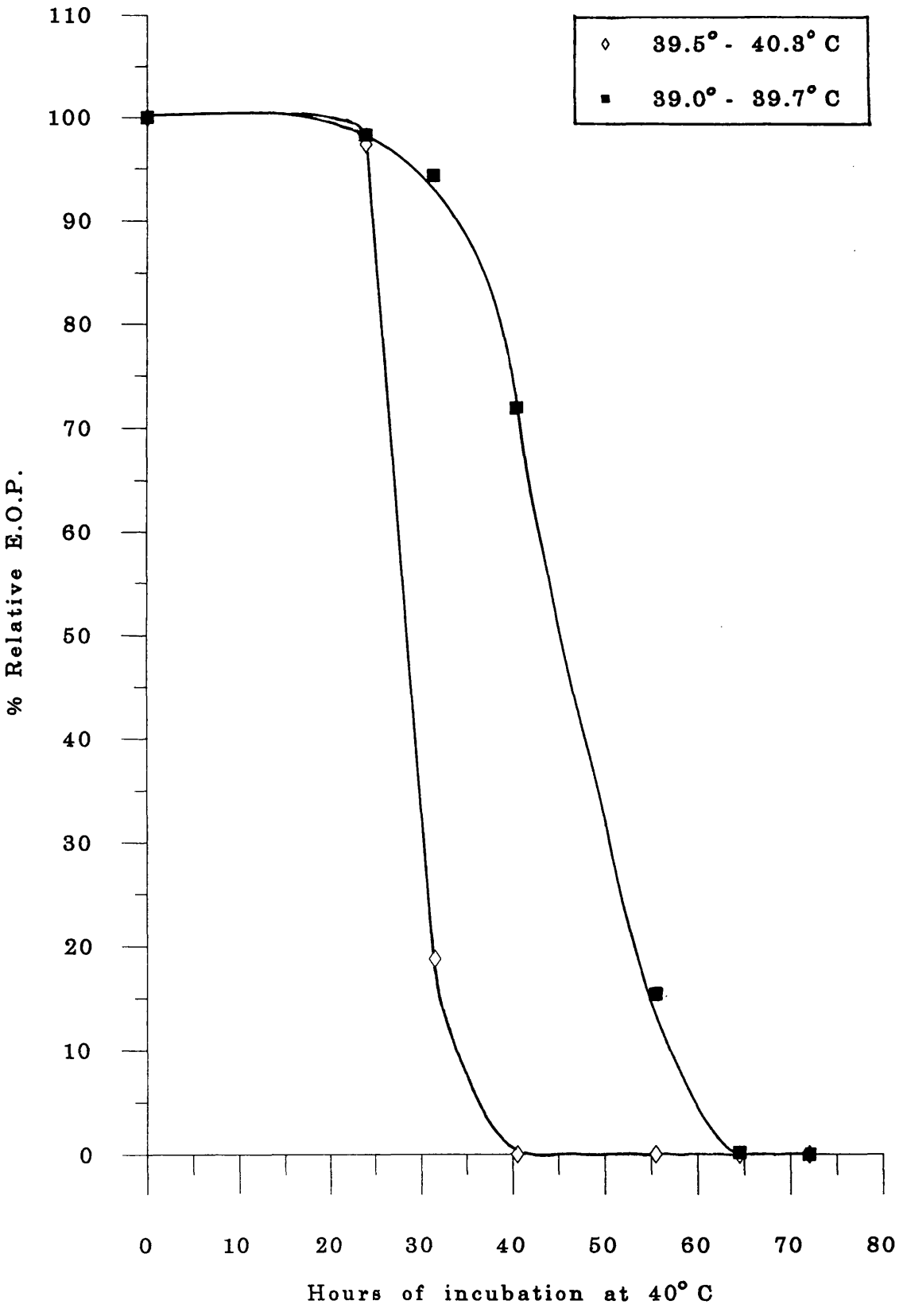
Table 3.5 LOSS OF VIABILITY OF K12 IN 5% FCS E4 LOW BIC USING A NONPERMISSIVE TEMPERATURE OF 39° - 39.7°C

HOURS OF INCUBATION AT THE NPT	COLONY NUMBER	E.O.P.	AVERAGE E.O.P.	% RELATIVE E.O.P.
continuous	0	0		
	0	0	0	0
0	203	50.8		
	221	55.3	53.0	100
24.0	221	55.3		
	196	49.0	52.1	98.3
31.5	206	51.5		
	194	48.5	50.0	94.3
40.5	157	39.3		
	148	37.0	38.1	71.9
55.5	37	9.3		
	28	7.0	8.1	15.3
64.5	1	0.3		
	0	0	0.1	0.2
72.0	0	0		
	0	0	0	0

Table 3.6 LOSS OF VIABILITY OF K12 IN 5% FCS E4 LOW BIC USING A NONPERMISSIVE TEMPERATURE OF 39.5° - 40.3°C

HOURS OF INCUBATION AT THE NPT	COLONY NUMBER	E.O.P.	AVERAGE E.O.P.	% RELATIVE E.O.P.
continuous	0	0		
	0	0	0	0
0	230	57.5		
	191	47.8	52.6	100
24.0	191	47.8		
	219	54.8	51.3	97.4
31.5	42	10.5		
	37	9.3	9.9	18.8
40.5	0	0		
	0	0	0	0
55.5	0	0		
	0	0	0	0
64.5	0	0		
	0	0	0	0
72.0	0	0		
	0	0	0	0

Figure 3.6 LOSS OF VIABILITY OF K12 IN 5% FCS E4 LOW BIC AT DIFFERENT INCUBATION TEMPERATURES



3.4 COMPARISON OF THE LOSS OF VIABILITY OF K12 GROWN IN E4 LOW BIC OR MCDB-302 AT 40°C

In order to investigate whether the composition of the growth medium in which K12 is cultured can influence the behaviour of this cell line at the npt, viability curves (Section 2.6) using 200 cells/plate were carried out with K12 grown in each of two different media; 5% FCS E4 low bic or 5% FCS MCDB-302, a medium originally formulated for the serum-free growth of Chinese hamster ovary (CHO) cells (Hamilton and Ham, 1977). The results (Tables 3.7 and 3.8) are displayed in Figure 3.7.

The viability curves clearly show that K12 cells survived for approximately 8 hours longer in MCDB-302 than in E4, thus the timing of the expression of the cell cycle block appears to be influenced in this cell line by the extracellular conditions. This phenomenon could occur either *via* a general effect on cell growth, or in a more specific manner. It could be postulated that a particular constituent of the medium has a specific influence, either directly or indirectly, on the rate of loss of activity or heat stability of the K12 ts protein. Alternatively, it was considered possible that the growth of K12 in MCDB-302 is slower than in E4 low bic, thus the cells would take longer to reach the block point in the former medium at 40°C, and loss of viability would therefore occur later than in E4. This seemed unlikely, however, since at equivalent time points in the viability curves, colonies of cells grown in MCDB-302 were in general significantly larger than those cultured in E4 low bic, suggesting that they actually display a faster growth rate, which would in fact be expected to accelerate the loss of proliferative capacity by reducing the time taken to reach the execution point. Nonetheless, this hypothesis was investigated further in Section 3.5 by studying the influence of the medium composition on the growth of K12 at 33°C.

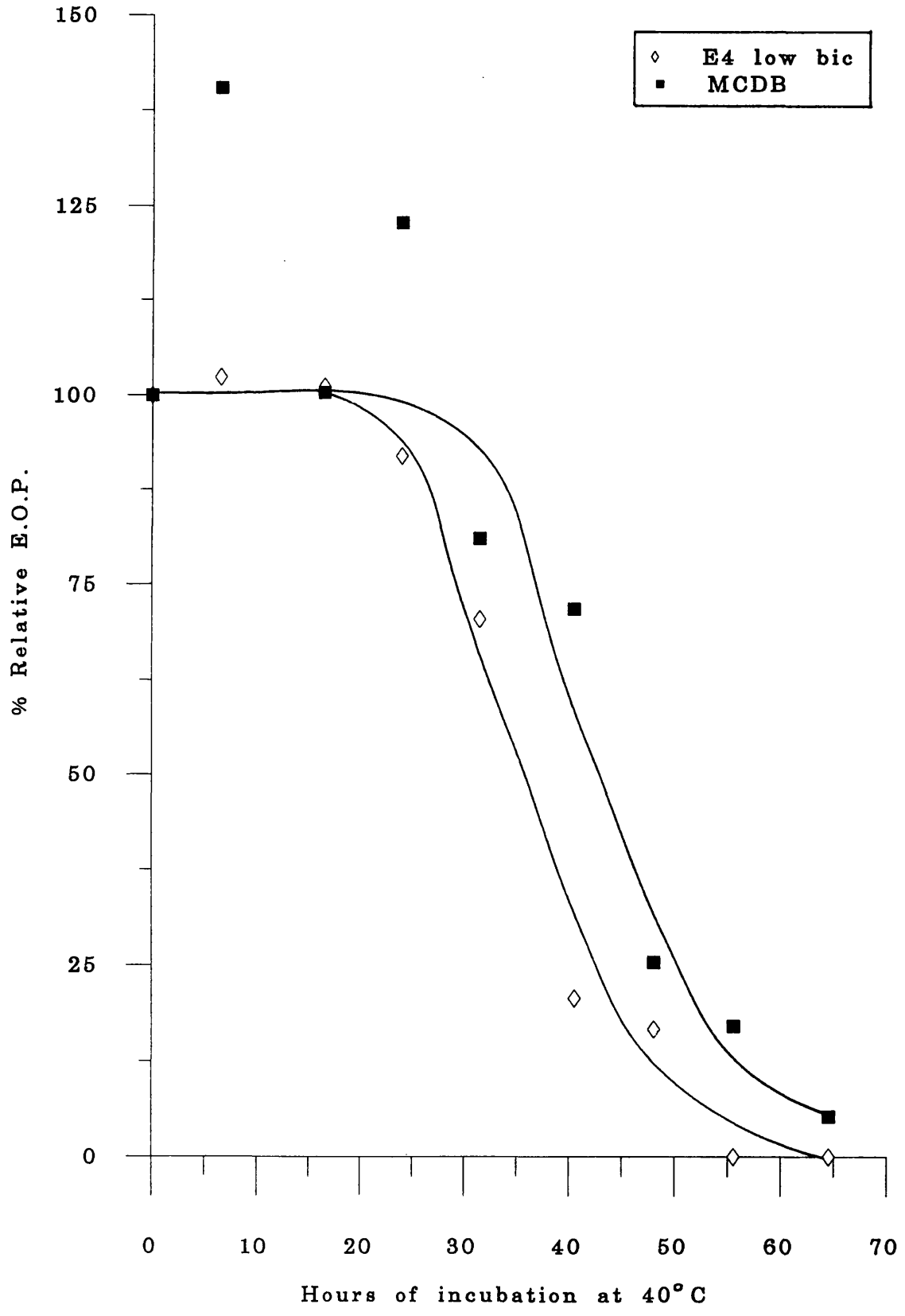
Table 3.7 LOSS OF VIABILITY OF K12 AT 40°C IN 5% FCS E4 LOW BIC

HOURS OF INCUBATION AT 40°C	COLONY NUMBER	E.O.P.	AVERAGE E.O.P.	% RELATIVE E.O.P.
continuous	0	0		
	0	0	0	0
0	133	66.5		
	118	59.0	62.8	100
6.5	132	66.0		
	125	62.5	64.3	102.4
16.5	128	64.0		
	126	63.0	63.5	101.2
24.0	114	57.0		
	117	58.5	57.8	92.0
31.5	81	40.5		
	96	48.0	44.3	70.5
40.5	26	13.0		
	26	13.0	13.0	20.7
48.0	10	5.0		
	32	16.0	10.5	16.7
55.5	0	0		
	0	0	0	0
64.5	0	0		
	0	0	0	0

Table 3.8 LOSS OF VIABILITY OF K12 AT 40°C IN 5% FCS MCDB-302

HOURS OF INCUBATION AT 40°C	COLONY NUMBER	E.O.P.	AVERAGE E.O.P.	% RELATIVE E.O.P.
continuous	0	0		
	0	0	0	0
0	contaminated			
	114	57.0	57.0	100
6.5	168	84.0		
	152	76.0	80.0	140.4
16.5	110	55.0		
	119	59.5	57.3	100.4
24.0	134	67.0		
	146	73.0	70.0	122.8
31.5	77	38.5		
	108	54.0	46.3	81.1
40.5	82	41.0	41.0	71.9
	contaminated			
48.0	35	17.5		
	23	11.5	14.5	25.4
55.5	21	10.5		
	18	9.0	9.8	17.1
64.5	2	1.0		
	10	5.0	3.0	5.3

Figure 3.7 LOSS OF VIABILITY OF K12 AT 40°C IN 5% FCS E4 LOW BIC AND 5% FCS MCDB-302



3.5 COMPARING THE GROWTH OF K12 AND Wg-1A AT 33°C IN MCDB-302 AND E4 LOW BIC

If the prolonged viability of K12 at the npt exhibited by cells grown in MCDB-302 rather than E4 low bic was caused by a general effect on basic cell growth e.g. a slowing down of the cell cycle so that the execution point takes longer to reach, then such a phenomenon should also be manifested at 33°C. In order to investigate whether this was the case, the growth of both K12 and its wild-type parent, Wg-1A, was studied at 33°C in each of the two different media. A cell suspension was prepared for each cell line (Section 2.4), diluted down to 100 cells/ml in the appropriate medium supplemented with 5% FCS, and plated at 5 ml/6 cm dish. Duplicate dishes were plated for each medium and cell type, and placed in a 33°C incubator. Colonies were stained and counted (Section 2.5) one week after plating.

The results (Tables 3.9 and 3.10) show that when Wg-1A was cultured in MCDB-302 rather than E4 low bic, both the E.O.P. and colony size increased, suggesting that MCDB-302 allows for both a higher frequency of establishment of the cells and a faster growth rate. With K12, the E.O.P. was lower in MCDB-302 than in E4 low bic, implying a lower frequency of establishment, but once cells were established, growth appeared to be faster, producing larger colonies. The observed patterns of growth were reproducible (see Tables 3.26 and 3.27). Two conclusions may be drawn from these results. The first is that K12 does not appear to be fully wild-type at 33°C (as first suggested in Section 3.1), since its behaviour does not mimic that of the parental cell line - growth seems to be slower than Wg-1A for a given medium type, as colonies are smaller, and although MCDB-302 increases the growth rate over that displayed in E4 low bic for both cell lines, it has opposite effects on the frequency of establishment in each cell type. This implies that the K12 ts protein does not show completely normal levels of activity at 33°C; a sufficient amount of activity is present to allow the cell to cycle, but at a slower rate than Wg-1A. Alternatively, K12 may have accumulated other mutations since 1973, independent of the original ts mutation, which result in an increase in the intermitotic time.

The second conclusion suggested by the results is that the use of MCDB-302 rather than E4 low bic does indeed have a general effect on the growth properties of K12 and Wg-1A, influencing both the frequency of establishment (E.O.P.) and the growth rate (colony size) of the cells. Both cell lines grew more rapidly in this medium than in E4 low bic, implying that MCDB-302 contains either a higher concentration of growth promoting substances, or a decreased level of inhibitory components. However, this would be expected to accelerate the loss of viability of K12 at the npt, since the time taken to reach the execution point would be reduced,

thus this does not appear to provide an explanation for the prolonged survival of K12 at 40°C which is observed in MCDB-302. Experiments were thus concentrated on discovering whether a specific component of MCDB-302 is responsible for this phenomenon.

Table 3.9 GROWTH OF Wg-1A AT 33°C IN E4 LOW BIC OR MCDB-302 SUPPLEMENTED WITH 5% FCS

MEDIUM	COLONY NUMBER	E.O.P.	AVERAGE E.O.P.	COLONY SIZE
E4	172	34.4		
	205	41.0	37.7	large
MCDB	250	50.0		
	272	54.4	52.2	very large

Table 3.10 GROWTH OF K12 AT 33°C IN E4 LOW BIC OR MCDB-302 SUPPLEMENTED WITH 5% FCS

MEDIUM	COLONY NUMBER	E.O.P.	AVERAGE E.O.P.	COLONY SIZE
E4	313	62.6		
	278	55.6	59.1	medium
MCDB	178	35.6		
	178	35.6	35.6	large

N.B. Throughout this study as a whole, colony sizes were defined as follows:

Colony size	Approximate diameter of colony
small	< 1 mm
medium	1 mm
large	2 mm
very large	> 2 mm

3.6 INVESTIGATION OF THE EFFECTS ON K12 AT 40°C OF ALTERING THE SODIUM BICARBONATE CONCENTRATION OF MCDB-302

In order to identify a component of the MCDB-302 medium which could potentially alter the viability of K12 at the npt, the compositions of MCDB-302 and E4 low bic were compared (see Appendix III), and found to vary in the levels of a large number of constituents, any one, or combination of which may be responsible for the observed effects. When the exact protocol of the viability curves carried out in 1973 (Roscoe *et al.*, 1973b) was studied carefully, it was found that the original experiments, where cells lost viability significantly faster than at the present time, were conducted in 10% CS E4 containing 44 mM $NaHCO_3$, whereas the present studies used 5% FCS E4 low bic (26 mM $NaHCO_3$). The results from Section 3.2 imply that the difference in serum composition was unlikely to affect the behaviour of K12 at 40°C, raising the possibility that bicarbonate levels may play a critical role in determining the rate of loss of proliferative capacity of K12 at the npt, with a reduction in the $NaHCO_3$ concentration of the medium prolonging viability. Further support was lent to this hypothesis by the observation that K12 cells survive for approximately 8 hours longer at the npt in MCDB-302, which contains only 13.3 mM $NaHCO_3$, than in E4 low bic (Section 3.4).

To test the proposition that $NaHCO_3$ levels affect the survival of K12 at the npt, viability curves were therefore carried out (Section 2.6) with K12 cells cultured in either 5% FCS E4 low bic (26 mM $NaHCO_3$), 5% FCS MCDB-302 (13.3 mM $NaHCO_3$), or 5% FCS MCDB-302 supplemented with 12.7 µl/ml of 1 M $NaHCO_3$, to give a final $NaHCO_3$ concentration of 26 mM. The results were expressed as % relative E.O.P. (Tables 3.11, 3.12 and 3.13) and plotted (Fig. 3.8). Initial experiments were hampered by high levels of contamination since, unlike E4, MCDB-302 contains no antibiotics, thus the latter medium was subsequently supplemented with streptomycin (100 µg/ml) and ampicillin (50 µg/ml).

The results show that the variation in the time course of loss of the proliferative capacity of K12 at the npt in MCDB-302 and E4 low bic can be accounted for almost entirely by the difference in their sodium bicarbonate levels, since an increase in the $NaHCO_3$ concentration of MCDB-302 to the E4 low bic level of 26 mM produced a viability curve virtually indistinguishable from that demonstrated in E4 low bic (Fig. 3.8), thus supporting the hypothesis that the bicarbonate concentration of the medium has a significant effect on the behaviour of K12 at the npt. It appears likely that the differences in the bicarbonate level of E4 also explain the prolonged survival of K12 at the present time when compared with the 1973 results.

Table 3.11 LOSS OF VIABILITY OF K12 AT 40°C IN 5% FCS E4 LOW BIC

HOURS OF INCUBATION AT 40°C	COLONY NUMBER	E.O.P.	AVERAGE E.O.P.	% RELATIVE E.O.P.
continuous	0	0		
	0	0	0	0
0	211	52.8		
	172	43.0	47.9	100
23.5	145	36.3		
	107	26.8	31.5	65.8
31.0	102	25.5		
	98	24.5	25.0	52.2
40.5	46	11.5		
	28	7.0	9.3	19.3
55.5	1	0.3		
	0	0	0.1	0.3
64.5	1	0.3		
	1	0.3	0.3	0.5
71.5	2	0.5		
	2	0.5	0.5	1.0

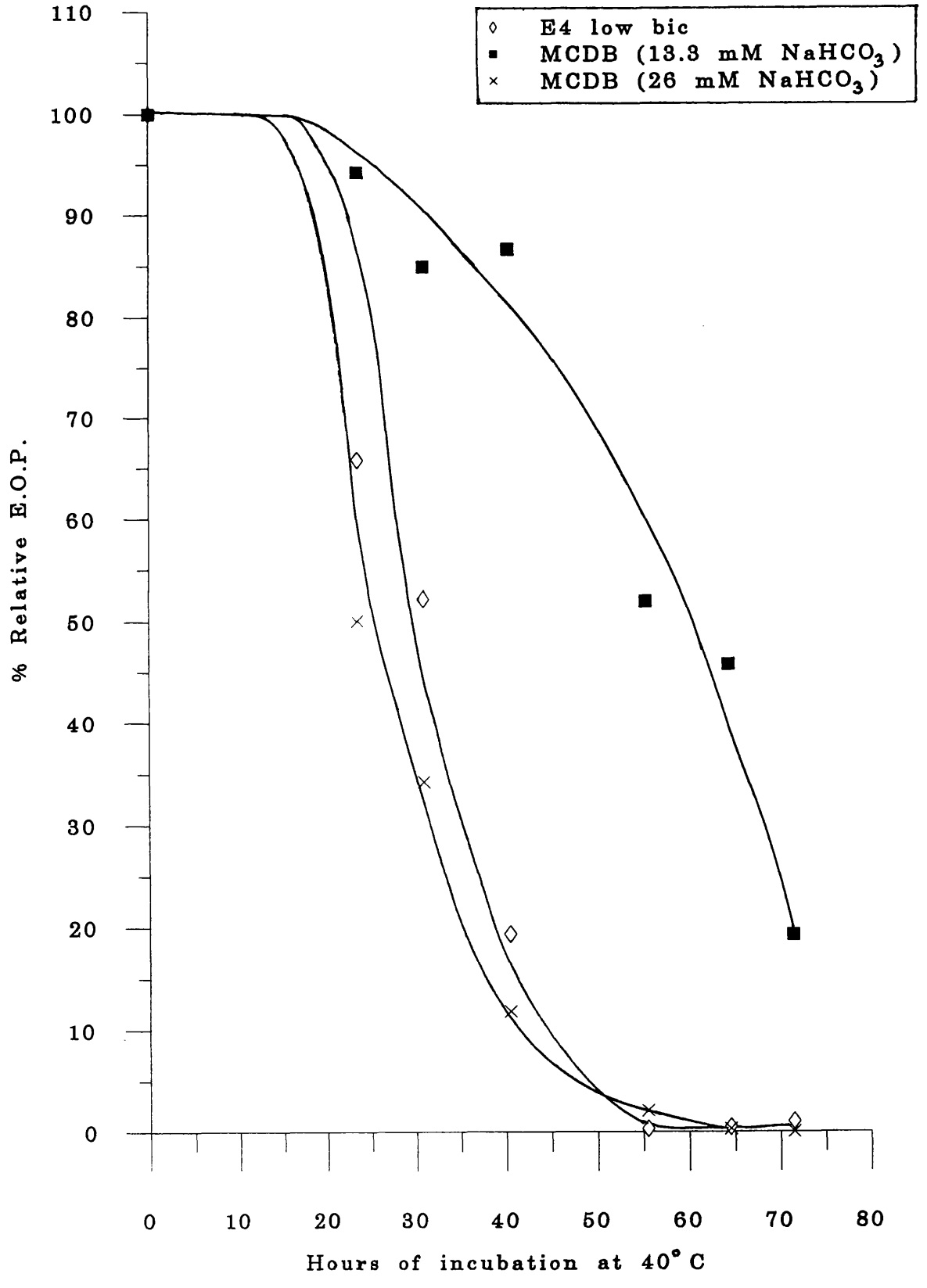
Table 3.12 LOSS OF VIABILITY OF K12 AT 40°C IN 5% FCS MCDB-302 (13.3 mM $NaHCO_3$)

HOURS OF INCUBATION AT 40°C	COLONY NUMBER	E.O.P.	AVERAGE E.O.P.	% RELATIVE E.O.P.
continuous	0	0		
	0	0	0	0
0	221	55.3		
	228	57.0	56.1	100
23.5	194	48.5		
	229	57.3	52.9	94.2
31.0	184	46.0		
	197	49.3	47.6	84.9
40.5	202	50.5		
	187	46.8	48.6	86.6
55.5	111	27.8		
	122	30.5	29.1	51.9
64.5	97	24.3		
	108	27.0	25.6	45.7
71.5	32	8.0		
	54	13.5	10.8	19.2

Table 3.13 LOSS OF VIABILITY OF K12 AT 40°C IN 5% FCS MCDB-302 (26 mM $NaHCO_3$)

HOURS OF INCUBATION AT 40°C	COLONY NUMBER	E.O.P.	AVERAGE E.O.P.	% RELATIVE E.O.P.
continuous	0	0		
	0	0	0	0
0	163	40.8		
	176	44.0	42.4	100
23.5	84	21.0		
	86	21.5	21.3	50.1
31.0	56	14.0		
	60	15.0	14.5	34.2
40.5	16	4.0		
	24	6.0	5.0	11.8
55.5	4	1.0		
	3	0.8	0.9	2.1
64.5	0	0		
	1	0.3	0.1	0.3
71.5	0	0		
	0	0	0	0

Figure 3.8 LOSS OF VIABILITY OF K12 AT 40°C IN 5% FCS-SUPPLEMENTED MEDIA WITH VARYING $NaHCO_3$ LEVELS



3.7 FURTHER MANIPULATION OF THE SODIUM BICARBONATE LEVELS OF E4 AND MCDB-302

Since the previous experiment suggested that the bicarbonate level of the medium plays a critical role in determining the rate of loss of proliferative capacity of K12 at 40°C, the $NaHCO_3$ concentrations of E4 and MCDB-302 were manipulated further, to both higher and lower levels, to study the effect on the behaviour of K12 at the npt. In order to bring the $NaHCO_3$ concentrations below their usual values in these media, MCDB-302 and E4 containing no bicarbonate were used as the starting point for all curves, and were supplemented with 1 M $NaHCO_3$ to the appropriate level (any differences in the dilution of the media caused by the addition of different volumes of 1 M $NaHCO_3$ were eliminated by adding water so that the total volume of liquid added, determined by the amount of 1 M $NaHCO_3$ required by the medium with the highest bicarbonate level, was equal in all cases).

Two sets of viability curves were carried out on two separate occasions. In the first set, K12 cells were plated in each of the following media - 5% FCS E4 containing 13.3, 26 or 44 mM $NaHCO_3$, or 5% FCS MCDB-302 supplemented with 13.3, 26 or 44 mM $NaHCO_3$ - and viability curves were carried out, as described in Section 2.6. The results (Tables 3.14 - 3.19) were represented graphically (Fig. 3.9), and clearly demonstrate that an increase in the bicarbonate level of the medium accelerated the rate of loss of viability of K12 at the npt, with equivalent $NaHCO_3$ levels in each of the two media producing similar viability curves. Loss of viability was, indeed, slowed down so greatly in 13.3 mM $NaHCO_3$ MCDB-302 that two medium-sized, healthy colonies were detected even 6 days after the temperature shift to 40°C. An increase in the E4 bicarbonate level from 26 to 44 mM brought the viability curve for K12 at the npt much closer to that seen in the experiments carried out when the cell line was first isolated (Roscoe *et al.*, 1973b), although cells in the present-day curve still took slightly longer to die, suggesting perhaps that an alteration in bicarbonate level, although it can account for much of the difference, is not entirely responsible for the current prolonged viability, and that other factors may therefore also be exerting an influence.

In the second set of viability curves, attempts were made to raise or lower the bicarbonate levels still further in each of the two media. Viability curves were carried out on K12 cells plated in either 5% FCS E4 supplemented with 7, 26 or 65 mM $NaHCO_3$, or in 5% FCS MCDB-302 containing 7, 26 or 65 mM $NaHCO_3$ (see Tables 3.20 - 3.25 and Fig. 3.10). When the $NaHCO_3$ concentration was increased in MCDB-302 to 65 mM, the loss of viability of K12 at the npt was even more rapid than that demonstrated in the original 1973 studies, with a % relative E.O.P. of

only 29% at the 24 hour time point. With 7 mM $NaHCO_3$ MCDB-302, however, there was no reduction in cell number even 72 hours after the temperature shift, although following one week of incubation at 40°C, when the plates were stained and counted, the % relative E.O.P. had fallen to 4%. Those colonies which survived for a week at the npt were healthy, and of a size comparable to colonies maintained at 33°C for the same length of time, thus it appeared that K12 cells were capable of cycling normally for prolonged periods at 40°C in this medium. K12 cells grown in E4 seemed unable to tolerate such a wide range of $NaHCO_3$ levels; growth was extremely poor in E4 containing either 7 mM or 65 mM $NaHCO_3$, even at 33°C. The viability of K12 at 40°C in such bicarbonate concentrations could not therefore be studied in E4. Sodium bicarbonate acts as a buffer, thus it is possible that the $NaHCO_3$ concentrations used alter the pH of E4 to a level unacceptable for cell growth (see Section 3.10). Alternatively, it is conceivable that such extremes of bicarbonate levels place the osmolality of E4 outside the range compatible with good growth of K12.

In conclusion, these experiments confirm that the concentration of bicarbonate in the growth medium is critical with regard to the behaviour of K12 at 40°C, with an increase in the $NaHCO_3$ level leading to an acceleration of the rate of loss of viability of the cells at the npt. The purpose of sodium bicarbonate in tissue culture media is twofold; it acts as both a buffer, to keep the pH at a level optimal for cell growth, and as a nutrient. It is possible that changes in either the pH or the nutritional quality of the medium may influence the behaviour of K12 at the npt *via* some general growth effect, with low bicarbonate levels perhaps providing suboptimal growth conditions, either in terms of nutrient limitation or inappropriate pH values, and thereby slowing down the cell cycle and delaying the eventual blockage at 40°C. This may be tested by determining the effect on the growth properties of K12 at 33°C of manipulating the $NaHCO_3$ concentrations of each medium (see Section 3.8). Alternatively, sodium bicarbonate may influence the viability of K12 at the npt by a more specific effect on the activity or stability of the K12 ts protein, once again acting either in its capacity as a nutrient or by altering the pH of the medium, thus further experiments were also carried out in order to investigate whether this may be the case.

Table 3.14 LOSS OF VIABILITY OF K12 AT 40°C IN 5% FCS E4 (13.3 mM $NaHCO_3$)

HOURS OF INCUBATION AT 40°C	COLONY NUMBER	E.O.P.	AVERAGE E.O.P.	% RELATIVE E.O.P.
continuous	0	0		
	0	0	0	0
0	189	47.3		
	197	49.3	48.3	100
24.0	171	42.8		
	198	49.5	46.1	95.6
31.5	195	48.8		
	201	50.3	49.5	102.6
40.5	153	38.3		
	168	42.0	40.1	83.2
55.5	184	46.0		
	167	41.8	43.9	90.9
64.5	104	26.0		
	118	29.5	27.8	57.5
72.0	39	9.8		
	69	17.3	13.5	28.0

Table 3.15 LOSS OF VIABILITY OF K12 AT 40°C IN 5% FCS E4 (26 mM $NaHCO_3$)

HOURS OF INCUBATION AT 40°C	COLONY NUMBER	E.O.P.	AVERAGE E.O.P.	% RELATIVE E.O.P.
continuous	0	0		
	0	0	0	0
0	216	54.0		
	180	45.0	49.5	100
24.0	179	44.8		
	187	46.8	45.8	92.4
31.5	188	47.0		
	200	50.0	48.5	98.0
40.5	131	32.8		
	129	32.3	32.5	65.7
55.5	116	29.0		
	39	9.8	19.4	39.1
64.5	12	3.0		
	15	3.8	3.4	6.8
72.0	1	0.3		
	1	0.3	0.3	0.5

Table 3.16 LOSS OF VIABILITY OF K12 AT 40°C IN 5% FCS E4 (44 mM $NaHCO_3$)

HOURS OF INCUBATION AT 40°C	COLONY NUMBER	E.O.P.	AVERAGE E.O.P.	% RELATIVE E.O.P.
continuous	0	0		
	0	0	0	0
0	213	53.3		
	224	56.0	54.6	100
24.0	170	42.5		
	198	49.5	46.0	84.2
31.5	152	38.0		
	190	47.5	42.8	78.3
40.5	61	15.3		
	127	31.8	23.5	43.0
55.5	58	14.5		
	46	11.5	13.0	23.8
64.5	0	0		
	0	0	0	0
72.0	0	0		
	0	0	0	0

Table 3.17 LOSS OF VIABILITY OF K12 AT 40°C IN 5% FCS MCDB-302 (13.3 mM $NaHCO_3$)

HOURS OF INCUBATION AT 40°C	COLONY NUMBER	E.O.P.	AVERAGE E.O.P.	% RELATIVE E.O.P.
continuous	2	0.5		
	0	0	0.3	0.5
0	216	54.0		
	195	48.8	51.4	100
24.0	198	49.5		
	195	48.8	49.1	95.6
31.5	219	54.8		
	197	49.3	52.0	101.2
40.5	206	51.5		
	196	49.0	50.3	97.8
55.5	176	44.0		
	157	39.3	41.6	81.0
64.5	162	40.5		
	145	36.3	38.4	74.7
72.0	101	25.3		
	92	23.0	24.1	47.0

Table 3.18 LOSS OF VIABILITY OF K12 AT 40°C IN 5% FCS MCDB-302 (26 mM $NaHCO_3$)

HOURS OF INCUBATION AT 40°C	COLONY NUMBER	E.O.P.	AVERAGE E.O.P.	% RELATIVE E.O.P.
continuous	0	0		
	0	0	0	0
0	213	53.3		
	242	60.5	56.9	100
24.0	218	54.5		
	220	55.0	54.8	96.3
31.5	189	47.3		
	214	53.5	50.4	88.6
40.5	173	43.3		
	182	45.5	44.4	78.0
55.5	152	38.0		
	174	43.5	40.8	71.6
64.5	80	20.0		
	61	15.3	17.6	31.0
72.0	9	2.3		
	21	5.3	3.8	6.6

Table 3.19 LOSS OF VIABILITY OF K12 AT 40°C IN 5% FCS MCDB-302 (44 mM $NaHCO_3$)

HOURS OF INCUBATION AT 40°C	COLONY NUMBER	E.O.P.	AVERAGE E.O.P.	% RELATIVE E.O.P.
continuous	0	0		
	0	0	0	0
0	230	57.5		
	224	56.0	56.8	100
24.0	189	47.3		
	167	41.8	44.5	78.4
31.5	178	44.5		
	167	41.8	43.1	76.0
40.5	143	35.8		
	146	36.5	36.1	63.7
55.5	31	7.8		
	56	14.0	10.9	19.2
64.5	13	3.3	3.3	5.7
	contaminated			
72.0	1	0.3		
	3	0.8	0.5	0.9

Figure 3.9 LOSS OF VIABILITY OF K12 AT 40°C IN 5% FCS-SUPPLEMENTED E4 AND MCDB-302 WITH VARYING $NaHCO_3$ LEVELS

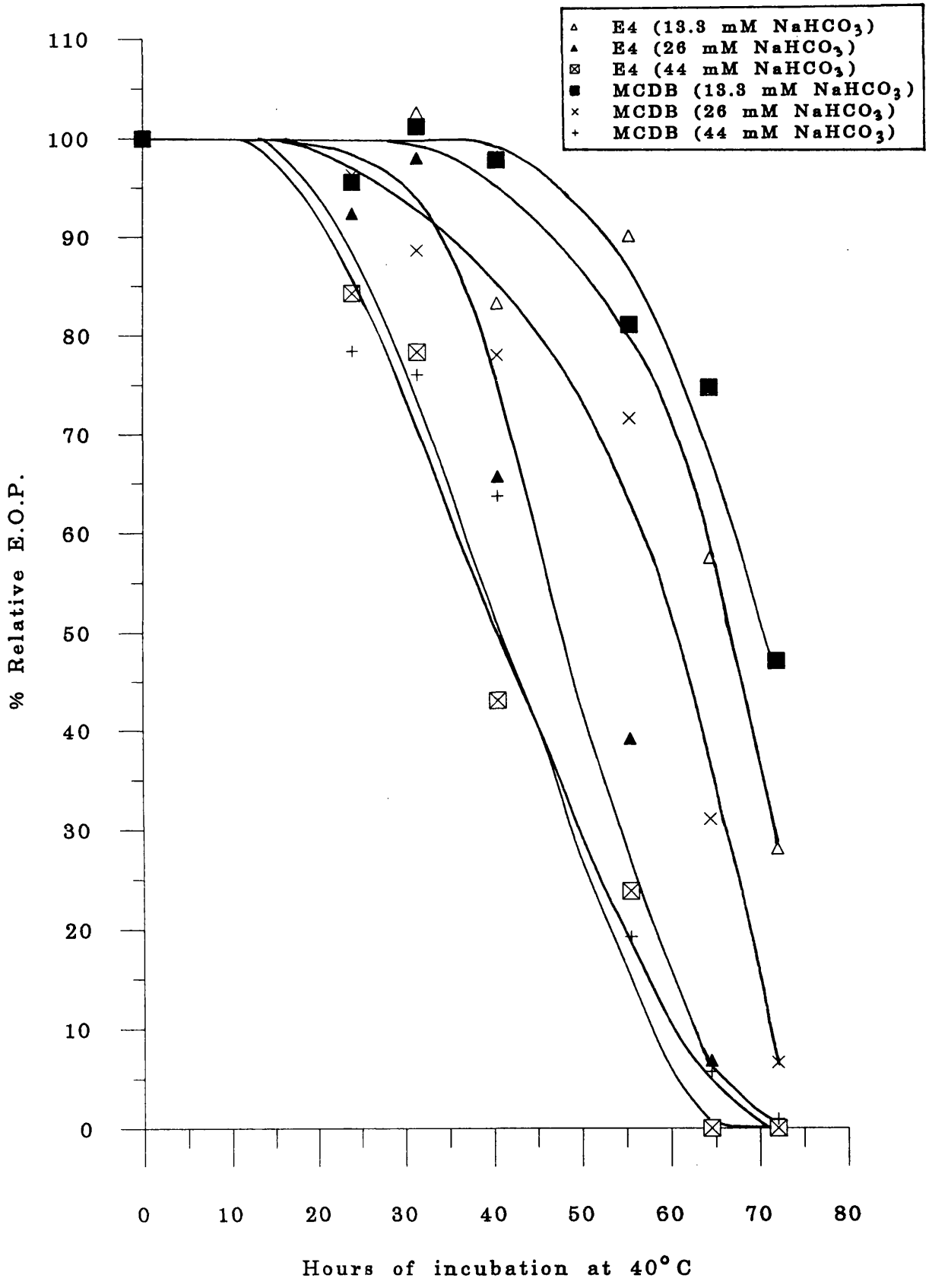


Table 3.20 LOSS OF VIABILITY OF K12 AT 40°C IN 5% FCS E4 (7 mM $NaHCO_3$)

HOURS OF INCUBATION AT 40°C	COLONY NUMBER	E.O.P.	AVERAGE E.O.P.	% RELATIVE E.O.P.
continuous	0	0		
	0	0	0	/
0	0	0		
	0	0	0	/
24.0	0	0		
	0	0	0	/
31.5	0	0		
	0	0	0	/
41.0	0	0		
	0	0	0	/
55.5	1	0.3		
	0	0	0.1	/
64.5	0	0		
	1	0.3	0.1	/
72.0	1	0.3		
	1	0.3	0.3	/

Table 3.21 LOSS OF VIABILITY OF K12 AT 40°C IN 5% FCS E4 (26 mM $NaHCO_3$)

HOURS OF INCUBATION AT 40°C	COLONY NUMBER	E.O.P.	AVERAGE E.O.P.	% RELATIVE E.O.P.
continuous	0	0		
	0	0	0	0
0	233	58.3		
	187	46.8	52.5	100
24.0	223	55.8		
	238	59.5	57.6	109.8
31.5	167	41.8		
	187	46.8	44.3	84.3
41.0	162	40.5		
	152	38.0	39.3	74.8
55.5	24	6.0		
	35	8.8	7.4	14.0
64.5	0	0		
	2	0.5	0.3	0.5
72.0	0	0		
	2	0.5	0.3	0.5

Table 3.22 LOSS OF VIABILITY OF K12 AT 40°C IN 5% FCS E4 (65 mM $NaHCO_3$)

HOURS OF INCUBATION AT 40°C	COLONY NUMBER	E.O.P.	AVERAGE E.O.P.	% RELATIVE E.O.P.
continuous	0	0		
	0	0	0	0
0	69	17.3		
	49	12.3	14.8	100
24.0	4	1.0		
	10	2.5	1.8	11.9
31.5	0	0		
	0	0	0	0
41.0	0	0		
	0	0	0	0
55.5	0	0		
	0	0	0	0
64.5	0	0		
	0	0	0	0
72.0	0	0		
	0	0	0	0

Table 3.23 LOSS OF VIABILITY OF K12 AT 40°C IN 5% FCS MCDB-302 (7 mM $NaHCO_3$)

HOURS OF INCUBATION AT 40°C	COLONY NUMBER	E.O.P.	AVERAGE E.O.P.	% RELATIVE E.O.P.
continuous	10	2.5		
	6	1.5	2.0	4.3
0	192	48.0		
	182	45.5	46.8	100
24.0	235	58.8		
	218	54.5	56.6	121.1
31.5	189	47.3		
	202	50.5	48.9	104.5
41.0	224	56.0		
	198	49.5	52.8	112.8
55.5	188	47.0		
	200	50.0	48.5	103.7
64.5	192	48.0		
	170	42.5	45.3	96.8
72.0	204	51.0		
	209	52.3	51.6	110.4

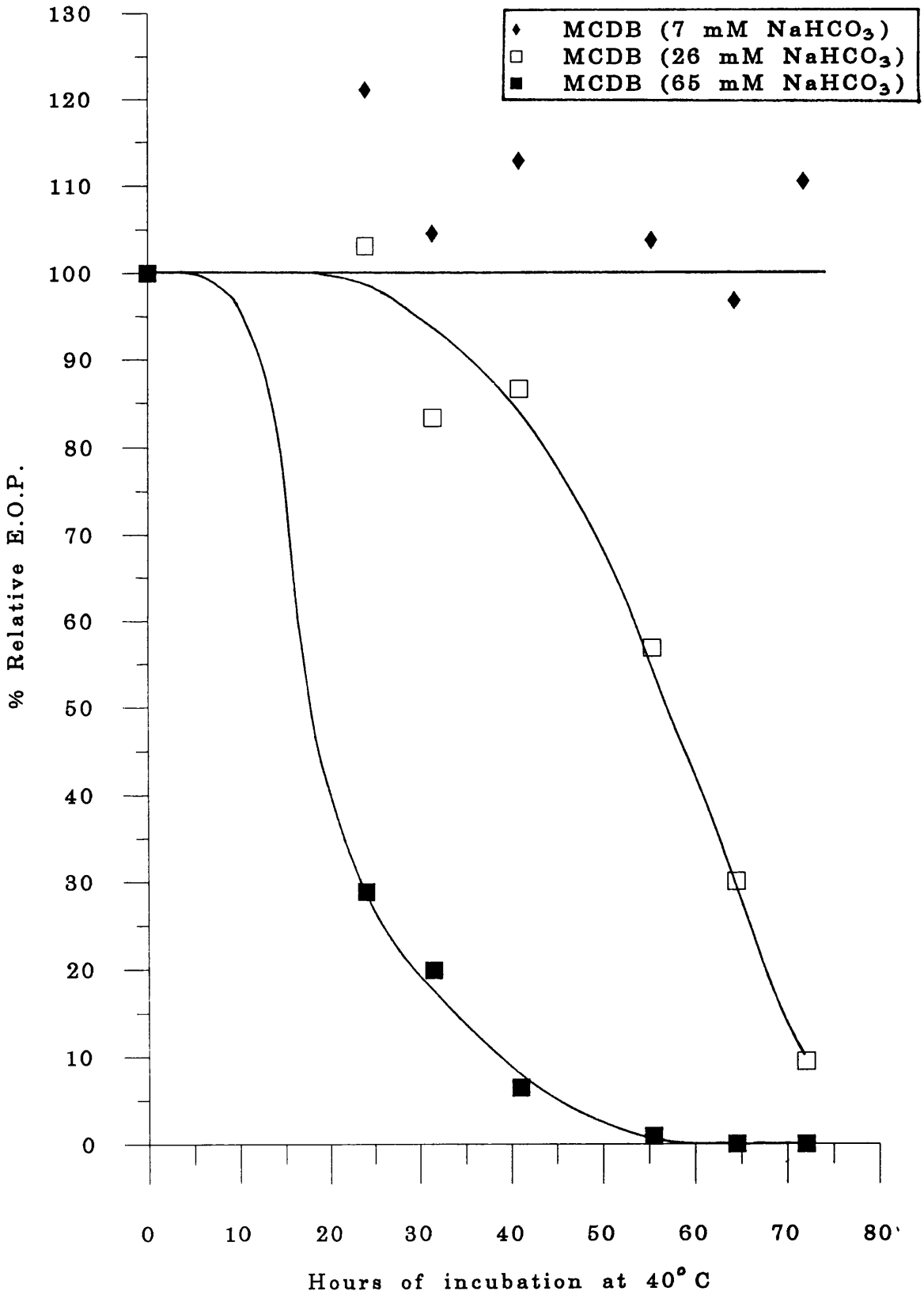
Table 3.24 LOSS OF VIABILITY OF K12 AT 40°C IN 5% FCS MCDB-302 (26 mM $NaHCO_3$)

HOURS OF INCUBATION AT 40°C	COLONY NUMBER	E.O.P.	AVERAGE E.O.P.	% RELATIVE E.O.P.
continuous	0	0		
	0	0	0	0
0	221	55.3		
	205	51.3	53.3	100
24.0	205	51.3		
	234	58.5	54.9	103.1
31.5	183	45.8		
	172	43.0	44.4	83.3
41.0	192	48.0		
	177	44.3	46.1	86.6
55.5	106	26.5		
	136	34.0	30.3	56.8
64.5	49	12.3		
	79	19.8	16.0	30.0
72.0	16	4.0		
	24	6.0	5.0	9.4

Table 3.25 LOSS OF VIABILITY OF K12 AT 40°C IN 5% FCS MCDB-302 (65 mM $NaHCO_3$)

HOURS OF INCUBATION AT 40°C	COLONY NUMBER	E.O.P.	AVERAGE E.O.P.	% RELATIVE E.O.P.
continuous	0	0		
	0	0	0	0
0	177	44.3		
	159	39.8	42.0	100
24.0	40	10.0		
	57	14.3	12.1	28.9
31.5	16	4.0		
	51	12.8	8.4	19.9
41.0	7	1.8		
	15	3.8	2.8	6.5
55.5	2	0.5		
	1	0.3	0.4	0.9
64.5	0	0		
	0	0	0	0
72.0	0	0		
	0	0	0	0

Figure 3.10 LOSS OF VIABILITY OF K12 AT 40°C IN 5% FCS MCDB-302 WITH VARYING $NaHCO_3$ LEVELS



3.8 COMPARING THE GROWTH OF K12 AND Wg-1A AT 33°C IN BICARBONATE-SUPPLEMENTED MEDIA

If changes in bicarbonate level act to alter the survival time of K12 at the npt by a general effect on cell growth properties, then manipulation of the $NaHCO_3$ concentration of the medium should also alter the growth characteristics of this cell line at 33°C. K12 cells grown in 5% FCS MCDB-302 (13.3 mM $NaHCO_3$) at the npt show a larger colony size (i.e. a faster growth rate) and a higher E.O.P. (frequency of establishment) than those cultured in 5% FCS E4 (26 mM $NaHCO_3$) (Section 3.5), suggesting that a difference in bicarbonate levels may indeed influence the general pattern of cell growth at 33°C. In order to determine whether this is the case, the effect of raising the MCDB-302 $NaHCO_3$ concentration from 13.3 mM to 26 mM (the level found in E4 low bic) on the growth of both K12 and the parental cell line Wg-1A at 33°C was studied.

A cell suspension was prepared for each cell line (Section 2.4), diluted down to 80 cells/ml in one of the following media supplemented with 5% FCS - E4 (26 mM $NaHCO_3$), MCDB-302 (13.3 mM $NaHCO_3$) or MCDB-302 (26 mM $NaHCO_3$) - and plated on 6 cm dishes. Duplicate dishes were plated for each medium and cell type, and placed in a 33°C incubator. All dishes were stained and the colonies counted (Section 2.5) one week after plating.

The results (Tables 3.26 and 3.27) demonstrate that raising the MCDB-302 bicarbonate content from 13.3 mM to the E4 low bic level did not alter the growth pattern of either Wg-1A or K12. Each cell line retained the characteristic growth properties exhibited in MCDB-302 irrespective of the bicarbonate concentration; the higher E.O.P. and larger colony size of Wg-1A when grown in MCDB-302 (13.3 mM $NaHCO_3$) rather than in E4 low bic did not change when the $NaHCO_3$ level was raised, and neither did the lower E.O.P. but larger colony size of K12 in MCDB-302. This implies that the difference in the growth properties manifested by each cell line in the two media, MCDB-302 (13.3 mM $NaHCO_3$) and E4 low bic, is not caused by their differing bicarbonate contents, and further suggests that variations in $NaHCO_3$ level, rather than altering the viability of K12 at 40°C by a general growth promoting or inhibiting effect, most likely act in a more specific manner to influence the activity or stability of the K12 ts protein itself, an effect which may therefore only be apparent at the npt.

Table 3.26 GROWTH OF K12 AT 33°C IN MEDIA WITH VARYING $NaHCO_3$ LEVELS

MEDIUM	COLONY NUMBER	E.O.P.	AVERAGE E.O.P.	COLONY SIZE
E4	203	50.8		
(26 mM $NaHCO_3$)	220	55.0	52.9	medium
MCDB-302	190	47.5		
(13.3 mM $NaHCO_3$)	183	45.8	46.6	large
MCDB-302	180	45.0		
(26 mM $NaHCO_3$)	189	47.3	46.1	large

Table 3.27 GROWTH OF Wg-1A AT 33°C IN MEDIA WITH VARYING $NaHCO_3$ LEVELS

MEDIUM	COLONY NUMBER	E.O.P.	AVERAGE E.O.P.	COLONY SIZE
E4	162	40.5		
(26 mM $NaHCO_3$)	160	40.0	40.3	large
MCDB-302	288	72.0		
(13.3 mM $NaHCO_3$)	268	67.0	69.5	very large
MCDB-302	292	73.0		
(26 mM $NaHCO_3$)	275	68.8	70.9	very large

3.9 LOSS OF VIABILITY OF K12 IN MEDIA WITH VARYING SODIUM BICARBONATE LEVELS BUT CONSTANT OSMOLALITY

Altering the $NaHCO_3$ level of a medium will also change its osmolality, which will increase as the bicarbonate concentration is raised. The optimum range of medium osmolality values for growth of most cell types is considered to be 260 - 320 mOsm/kg (Waymouth, 1970). Elevating the $NaHCO_3$ level of MCDB-302 from 0 to 13.3 mM increases the osmolality of the medium from 260 to 290 mOsm/kg, and raising the E4 $NaHCO_3$ concentration from 0 to 44 mM changes its osmolality from 255 to 331 mOsm/kg, thus when it is considered that the viability curve experiments (Section 3.7) manipulated the $NaHCO_3$ contents of the media used over a range of 7 to 65 mM, significant changes in the osmolality of the media (and therefore in the osmotic pressure of the cells) must have occurred. In order to confirm that it was the alteration in $NaHCO_3$ levels, rather than the associated change in osmolality of the medium, which affects the rate of loss of viability of K12 at the npt, the bicarbonate concentration of each medium was varied without altering its osmolality. This was achieved by adding choline chloride to the medium, so that the sum of the concentrations of choline chloride and sodium bicarbonate, and thus the osmolality, was constant for all bicarbonate levels used. Any differences in the dilution of the media caused by the addition of varying volumes of $NaHCO_3$ and choline chloride solutions were avoided by also adding water, such that the total volume of liquid added was equivalent in each case.

Initially, attempts were made to balance the osmolality to the highest concentration of $NaHCO_3$ used in the medium (65 mM for MCDB-302, 44 mM for E4), but cells were unable to grow in the high concentrations of choline chloride necessary, thus the medium was balanced only to the 26 mM level. A viability curve experiment (Section 2.6) was carried out using K12 cells suspended in one of the following media - MCDB-302 (7 mM $NaHCO_3$), MCDB-302 (7 mM $NaHCO_3$ + 19 mM choline chloride), MCDB-302 (26 mM $NaHCO_3$), E4 (13.3 mM $NaHCO_3$), E4 (13.3 mM $NaHCO_3$ + 12.7 mM choline chloride) or E4 (26 mM $NaHCO_3$), each supplemented with 5% FCS - and the results (Tables 3.28 - 3.33) were plotted (Figs. 3.11 and 3.12).

The results demonstrate that the addition of 19 mM choline chloride to 7 mM $NaHCO_3$ MCDB-302, in order to balance the osmolality to that found in 26 mM $NaHCO_3$ MCDB-302, did not produce a viability curve comparable to that for K12 grown in 26 mM $NaHCO_3$ MCDB-302 and similarly, cells grown in 13.3 mM $NaHCO_3$ E4 produced roughly equivalent viability curves irrespective of the presence or absence of choline chloride. This implies that changes in osmolality cannot account

for the alterations in the rate of loss of viability of K12 cells at 40°C observed when the bicarbonate level of either MCDB-302 or E4 was varied (Sections 3.6 and 3.7), and that this phenomenon is therefore truly due to an effect of the bicarbonate ion itself.

Table 3.28 LOSS OF VIABILITY OF K12 AT 40°C IN 5% FCS MCDB-302 (7 mM $NaHCO_3$)

HOURS OF INCUBATION AT 40°C	COLONY NUMBER	E.O.P.	AVERAGE E.O.P.	% RELATIVE E.O.P.
continuous	0	0		
	0	0	0	0
0	235	58.8		
	229	57.3	58.0	100
24.0	182	45.5		
	212	53.0	49.3	84.9
31.5	197	49.3		
	219	54.8	52.0	89.7
40.5	211	52.8		
	195	48.8	50.8	87.5
55.5	95	23.8		
	95	23.8	23.8	40.9
64.5	67	16.8		
	45	11.3	14.0	24.1
72.0	21	5.3		
	27	6.8	6.0	10.3

Table 3.29 LOSS OF VIABILITY OF K12 AT 40°C IN 5% FCS MCDB-302 (7 mM $NaHCO_3$ + 19 mM choline chloride)

HOURS OF INCUBATION AT 40°C	COLONY NUMBER	E.O.P.	AVERAGE E.O.P.	% RELATIVE E.O.P.
continuous	0	0		
	0	0	0	0
0	199	49.8		
	210	52.5	51.1	100
24.0	157	39.3		
	137	34.3	36.8	71.9
31.5	178	44.5		
	162	40.5	42.5	83.1
40.5	170	42.5		
	175	43.8	43.1	84.4
55.5	112	28.0		
	114	28.5	28.3	55.3
64.5	119	29.8		
	114	28.5	29.1	57.0
72.0	82	20.5		
	85	21.3	20.9	40.8

Table 3.30 LOSS OF VIABILITY OF K12 AT 40°C IN 5% FCS MCDB-302 (26 mM $NaHCO_3$)

HOURS OF INCUBATION AT 40°C	COLONY NUMBER	E.O.P.	AVERAGE E.O.P.	% RELATIVE E.O.P.
continuous	0	0		
	0	0	0	0
0	223	55.8		
	234	58.5	57.1	100
24.0	214	53.5		
	223	55.8	54.6	95.6
31.5	186	46.5		
	201	50.3	48.4	84.7
40.5	190	47.5		
	183	45.8	46.6	81.6
55.5	2	0.5		
	5	1.3	0.9	1.5
64.5	8	2.0		
	6	1.5	1.8	3.1
72.0	0	0		
	0	0	0	0

Table 3.31 LOSS OF VIABILITY OF K12 AT 40°C IN 5% FCS E4 (13.3 mM $NaHCO_3$)

HOURS OF INCUBATION AT 40°C	COLONY NUMBER	E.O.P.	AVERAGE E.O.P.	% RELATIVE E.O.P.
continuous	0	0		
	0	0	0	0
0	323	80.8		
	304	76.0	78.4	100
24.0	283	70.8		
	292	73.0	71.9	91.7
31.5	283	70.8		
	309	77.3	74.0	94.4
40.5	152	38.0		
	187	46.8	42.4	54.1
55.5	13	3.3		
	16	4.0	3.6	4.6
64.5	23	5.8		
	11	2.8	4.3	5.4
72.0	9	2.3		
	2	0.5	1.4	1.8

Table 3.32 LOSS OF VIABILITY OF K12 AT 40°C IN 5% FCS E4 (13.3 mM $NaHCO_3$ + 12.7 mM choline chloride)

HOURS OF INCUBATION AT 40°C	COLONY NUMBER	E.O.P.	AVERAGE E.O.P.	% RELATIVE E.O.P.
continuous	0	0		
	0	0	0	0
0	248	62.0		
	233	58.3	60.1	100
24.0	231	57.8		
	239	59.8	58.8	97.7
31.5	268	67.0		
	268	67.0	67.0	111.4
40.5	183	45.8		
	171	42.8	44.3	73.6
55.5	31	7.8		
	42	10.5	9.1	15.2
64.5	11	2.8		
	20	5.0	3.9	6.4
72.0	22	5.5		
	3	0.8	3.1	5.2

Table 3.33 LOSS OF VIABILITY OF K12 AT 40°C IN 5% FCS E4 (26 mM $NaHCO_3$)

HOURS OF INCUBATION AT 40°C	COLONY NUMBER	E.O.P.	AVERAGE E.O.P.	% RELATIVE E.O.P.
continuous	0	0		
	0	0	0	0
0	338	84.5		
	371	92.8	88.6	100
24.0	265	66.3		
	279	69.8	68.0	76.7
31.5	230	57.5		
	261	65.3	61.4	69.3
40.5	105	26.3		
	138	34.5	30.4	34.3
55.5	0	0		
	5	1.3	0.6	0.7
64.5	3	0.8		
	3	0.8	0.8	0.8
72.0	0	0		
	3	0.8	0.4	0.4

Figure 3.11 LOSS OF VIABILITY OF K12 AT 40°C IN 5% FCS MCDB-302 WITH VARYING OSMOLALITIES

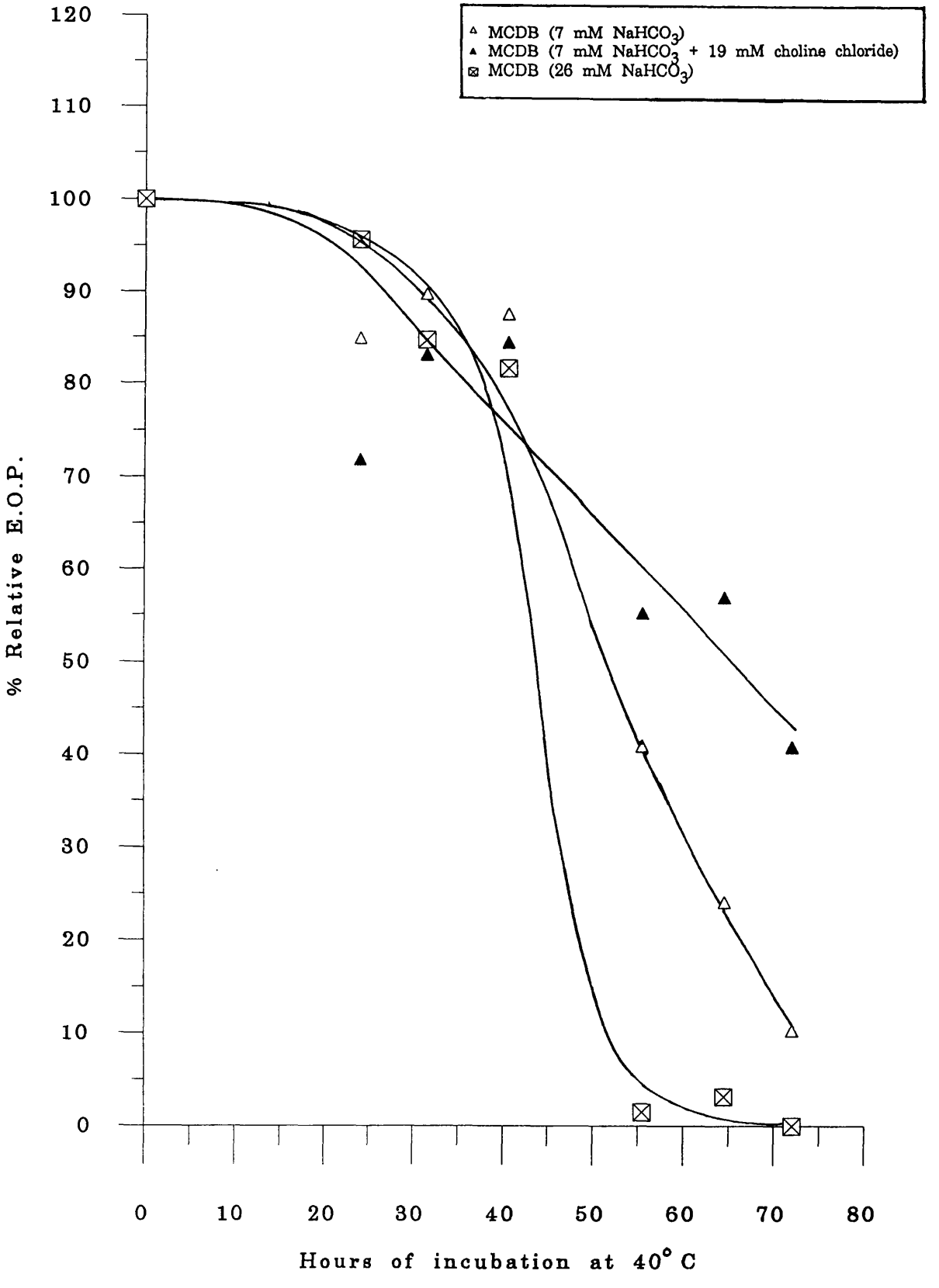
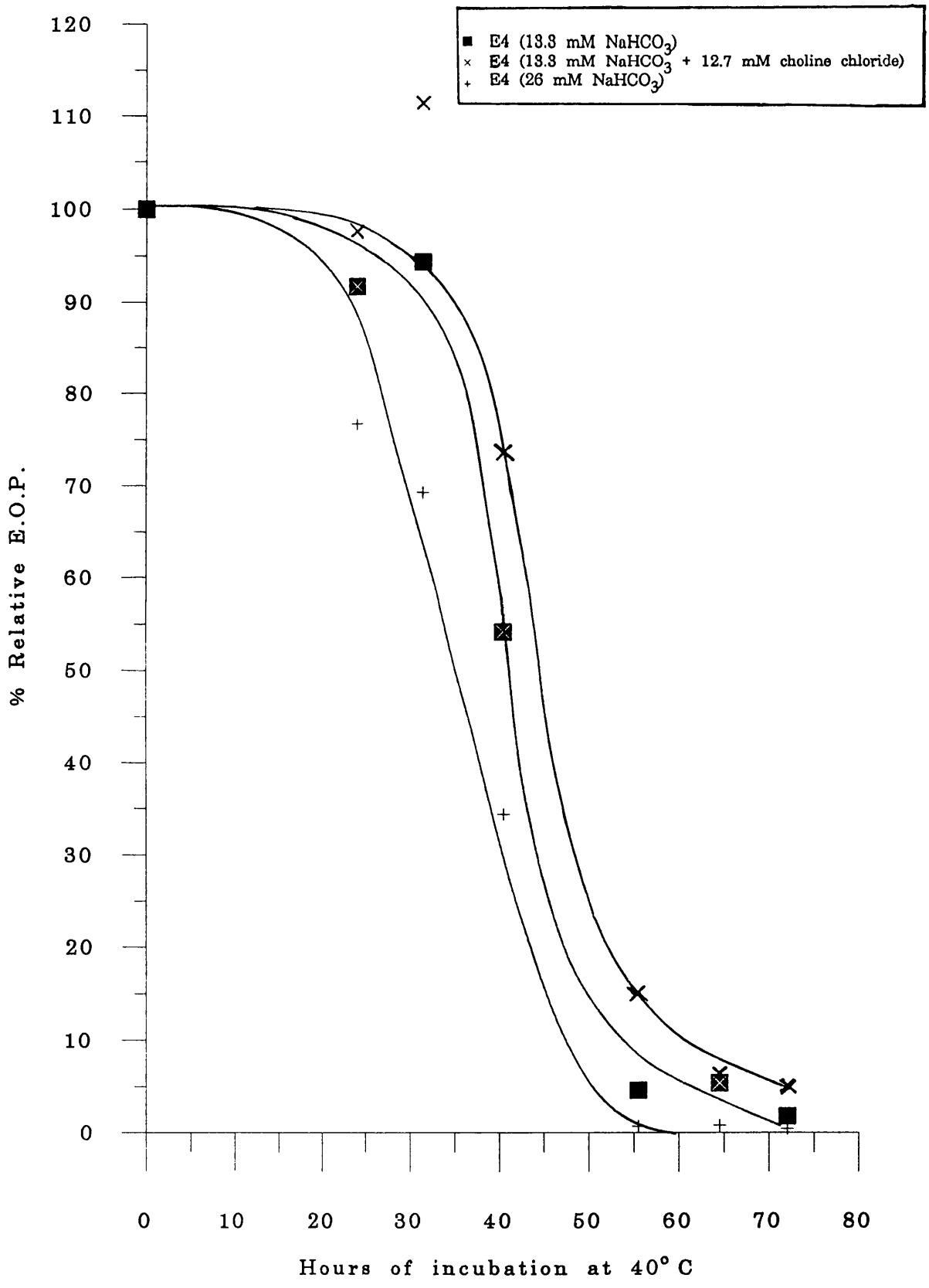


Figure 3.12 LOSS OF VIABILITY OF K12 AT 40°C IN 5% FCS E4 WITH VARYING OSMOLALITIES



3.10 MEASUREMENT OF THE pH OF E4 AND MCDB-302 CONTAINING VARYING SODIUM BICARBONATE LEVELS

As noted in Section 3.7, it is possible that the manipulation of the $NaHCO_3$ level acts to influence the behaviour of K12 at the npt by changing the pH of the medium. If this is the case, the range of bicarbonate concentrations used in the viability curve experiments (Section 3.7) must alter the pH of the media significantly in order to exert any effect on the cells. To determine the extent of the changes in pH that occur when the $NaHCO_3$ level is altered over such a range, the pH values of 5% FCS MCDB-302 and 5% FCS E4 at $NaHCO_3$ concentrations of 7, 13.3, 26, 44 and 65 mM were measured under incubation conditions i.e. under a 5% CO_2 atmosphere at 40°C (Section 2.9).

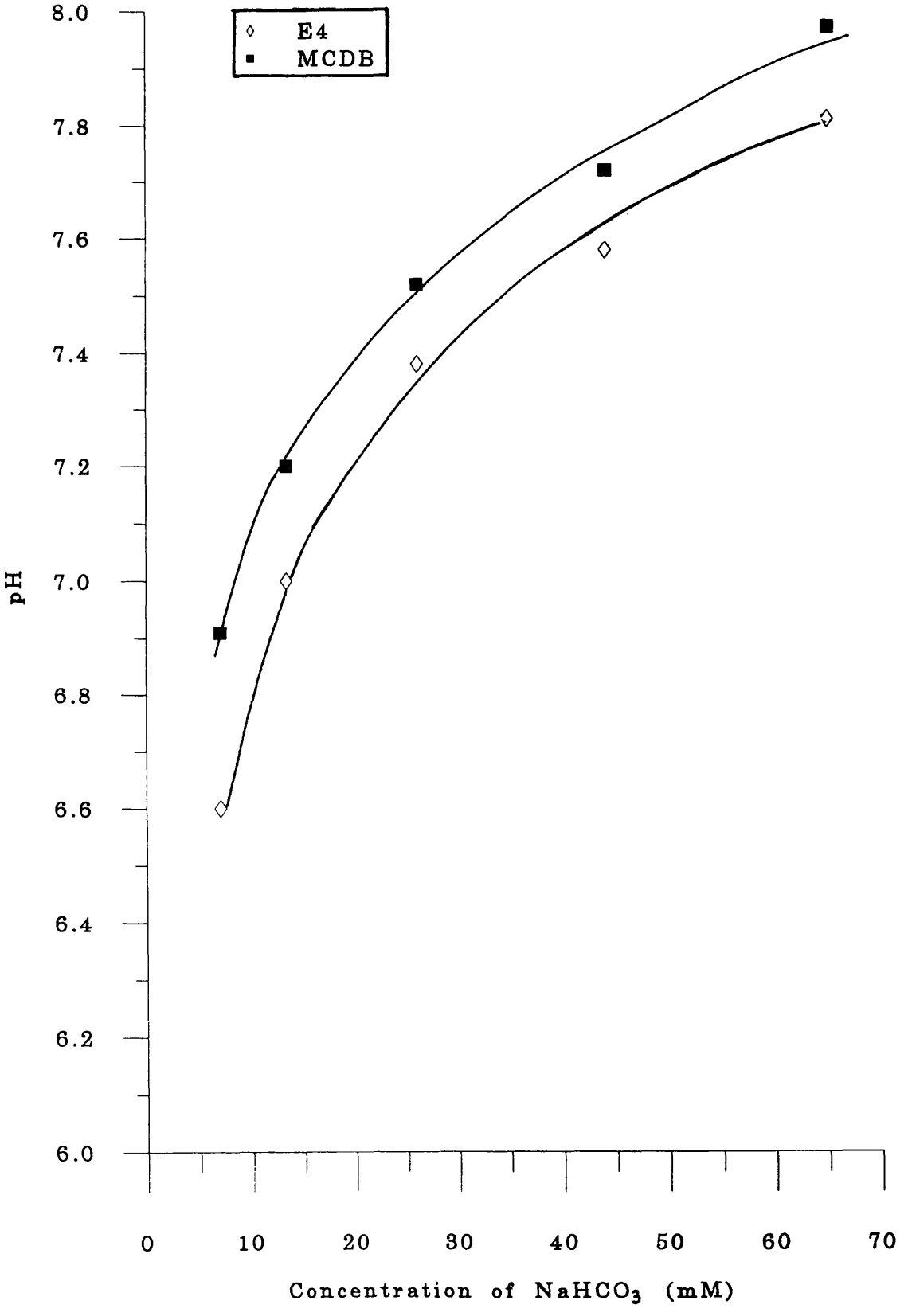
Table 3.34 pH of 5% FCS MCDB-302 and 5% FCS E4 at 40°C with varying $NaHCO_3$ levels

	pH	
[$NaHCO_3$] mM	E4	MCDB-302
7	6.60	6.91
13.3	7.00	7.20
26	7.38	7.52
44	7.58	7.72
65	7.81	7.97

The results (Table 3.34 and Fig. 3.13) demonstrate that the pH of the media increased as the bicarbonate level was raised, but for a given concentration of $NaHCO_3$, E4 consistently had a pH value 0.1 - 0.3 pH units below that of MCDB-302. This may be due to differing levels of additional buffering components, such as amino acids, in the two media. The results also suggest that the poor growth of K12 cells in 7 mM $NaHCO_3$ E4 observed in the viability curve studies (Section 3.7) may be explained by the extremely low pH (pH 6.6) of this medium (most fibroblasts perform best within the pH range of 7.0 - 7.7 [Ceccarini and Eagle, 1971; Rubin, 1971; 1973; Eagle, 1973]).

If the changes in the pH of the medium brought about by the manipulation of $NaHCO_3$ levels were solely responsible for the alteration in the rate of loss of viability of K12 at 40°C, with a decrease in the pH leading to an increase in the survival time, then cells grown in E4 would be expected to lose viability more slowly than cells cultured in MCDB-302 with an equivalent bicarbonate concentration, since E4 shows a pH value lower than that of MCDB-302 for a given $NaHCO_3$ content. However, this was not the case (see Fig. 3.9); cells in E4 consistently died more rapidly than those grown in MCDB-302 with an equivalent $NaHCO_3$ level. This implies either that additional medium components may alter the behaviour of K12 at the npt, and these differ in concentration between E4 and MCDB-302 or, alternatively, that the effect of variations in $NaHCO_3$ level on the rate of loss of viability of the cells may not be solely due to its influence on the pH of the medium; its function as a nutrient may also play a role.

Figure 3.13 STANDARD CURVE RELATING $NaHCO_3$ CONCENTRATION OF 5% FCS-SUPPLEMENTED MEDIA TO pH



3.11 ATTEMPTS TO GROW K12 IN NON-BICARBONATE-BUFFERED MEDIA

In order to investigate whether alterations in $NaHCO_3$ level exert their observed effects on the viability of K12 at the npt through either an alteration in the nutritional status of the cell or, alternatively, through an influence on the pH of the medium, the dual functions of bicarbonate must be dissociated. One way to achieve this would be to manipulate the pH of the medium whilst keeping its $NaHCO_3$ level constant, to determine whether a change in pH alone can mimic the effects that alterations in $NaHCO_3$ concentration have on the behaviour of K12 at the npt. The reaction scheme for the HCO_3^-/CO_2 buffer system is as follows:

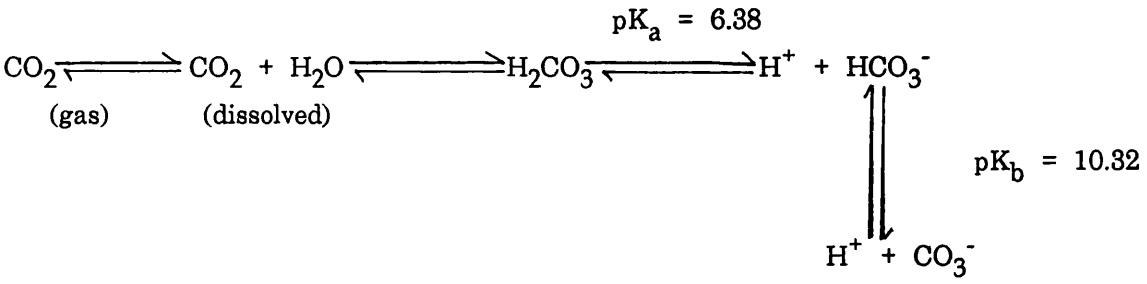
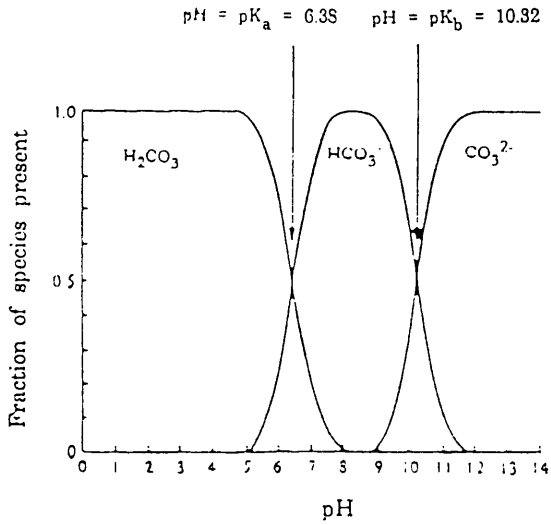


Figure 3.14 pK curve for the HCO_3^-/CO_2 buffer system



This system acts to resist changes in pH, since raising the pH, i.e. increasing the proton concentration, will shift the equilibrium of the reaction to the left, converting the hydrogen ions to water and thus reducing the pH, and conversely, lowering the pH will move the reaction scheme to the right, producing more protons and thereby increasing the pH. The point of equilibrium for the system is dependent on the pH and bicarbonate concentration of the medium and the partial pressure of CO_2 in the incubator atmosphere. As a consequence, if any bicarbonate is present in the medium,

altering the pH by alternative mechanisms (e.g. by addition of acid or alkali) will also change the proportion of bicarbonate present in its ionic form, by altering the equilibrium for the dissociation of both the bicarbonate ion and carbonic acid. Since the pK value for the latter reaction (pK = 6.38) is fairly close to the pH range covered in the viability curve studies of Section 3.7 (pH 6.91 - 7.97), even a small change in the pH within this range will lead to a large change in the ratio of the species present (Fig. 3.14). As a consequence, even if $NaHCO_3$ is added to the medium in only small quantities in order to satisfy its requirement as a nutrient, the extent to which it is present in its dissociated form will vary significantly in media of different pH values e.g. if $NaHCO_3$ is present at a concentration of 7 mM, raising the pH of the medium from 6.91 to 7.97, the range covered in Section 3.7, will increase the proportion of HCO_3^- ions present by approximately 50% i.e. to a level of 10 - 11 mM, which may produce significant changes in the rate of loss of viability of K12 at the npt. In addition, since CO_2 dissolves in the medium and dissociates to form HCO_3^- ions, and the extent to which it does so is, once again, partly determined by the pH of the medium (with a pK for the overall reaction $CO_2 + H_2O \rightleftharpoons H^+ + HCO_3^-$ of 6.1, still fairly close to the range of pH values used in Section 3.7), if CO_2 is present in the incubator atmosphere, the concentration of bicarbonate ions present will continue to vary when the pH of the medium is manipulated by means other than by altering the $NaHCO_3$ level, even if no exogenous bicarbonate is added to the medium. It thus appears that unless both $NaHCO_3$ and CO_2 can be totally eliminated from the incubation conditions, the pH cannot be altered independently of the bicarbonate level, and variations in the HCO_3^- ion concentration will continue to play a role in determining both the pH and the nutritional value of the medium.

As a consequence, attempts were made to buffer bicarbonate-free media with two alternatives to $NaHCO_3$; HEPES, with a pK value of 7.31 and an effective buffering range of pH 6.6 - 8.0 at 37°C, and BES, which has a pK value of 6.90 and an effective buffering range of pH 6.2 - 7.6 at 37°C (Good *et al.*, 1966). Initial experiments with HEPES-buffered media used two different levels of HEPES, 10 or 20 mM, in order to determine the optimum level for cell growth under the conditions applied. Varying levels of $NaHCO_3$ were also added, to ascertain the minimum requirement of K12 for this nutrient. The media used were Minimal Essential Medium Eagle (Modified) with Earle's salts (MEM), containing 20 mM HEPES and no $NaHCO_3$, and E4 containing no $NaHCO_3$ (E4 no bic) was supplemented with 10 mM HEPES and the pH was adjusted to 7.4 with 1 M NaOH. A suspension of K12 cells, at a concentration of 80 cells/ml, was prepared (Section 2.4) in each of the two media, which were supplemented with 5% FCS and either 0, 4 or 7 mM $NaHCO_3$.

For each bicarbonate level used, one 25 cm^2 flask and one 6 cm dish was plated, in order to investigate whether a closed (flask) or open (dish) system was best for cell growth. The flask lids were tightened, and the cells were placed in a 33°C incubator under atmospheric CO_2 for one week, after which time the colonies were stained and counted (Section 2.5). A control dish containing 80 K12 cells/ml in 5% FCS E4 low bic was also plated and maintained under 5% CO_2 for one week before staining and counting.

The results (Table 3.35) suggest that K12 cells were unable to grow in E4 medium containing only 10 mM HEPES under these conditions, perhaps because this level of HEPES is insufficient for adequate buffering of the medium in the presence of low concentrations of bicarbonate. However, this cell line did appear to be capable of some growth in 20 mM HEPES-buffered modified MEM in atmospheric CO_2, when the $NaHCO_3$ concentration was 4 mM or higher and when cultured in flasks, but growth was poor; colonies were small and unhealthy in appearance, and the E.O.P. was low. No growth was seen in this medium when bicarbonate was omitted, or when the cells were grown on plates. This implies that K12 cells have an absolute requirement for a certain threshold level of $NaHCO_3$. When growth medium containing a low level of bicarbonate is placed under atmospheric conditions in dishes, the equilibrium of the buffer system is shifted towards the left, the bicarbonate leaves the medium in the form of CO_2, diffuses out from the plate and is lost permanently from the medium, thus cells are unable to grow. When this diffusion is limited by incubation in a sealed flask, the bicarbonate is lost to a lesser extent since the CO_2 concentration in the flask will gradually rise and move the equilibrium of the reaction back towards the right to a certain degree. However, some bicarbonate will still be lost from the medium, leaving enough for only limited growth.

The experiments carried out with BES-buffered media support the hypothesis that K12 requires a critical $NaHCO_3$ level for growth. 5% FCS E4 no bic was supplemented with 10, 20 or 30 mM BES, the pH was adjusted to 7.4 with sodium hydroxide, a suspension of K12 cells was prepared at a concentration of 80 cells/ml in each of the three media (Section 2.4), and each medium type was supplemented with either 0 or 4 mM $NaHCO_3$. Duplicate 25 cm^2 flasks were plated for each medium, the lids were tightened, and all cells were placed in a 33°C incubator. Duplicate control dishes of K12 in 5% FCS E4 low bic were also plated at 80 cells/ml, and maintained under a 5% CO_2 atmosphere at 33°C. One week after plating, colonies were stained and counted (Section 2.5). The results (Table 3.36) suggest that a BES concentration of 20 mM is best for cell growth, and a $NaHCO_3$ level of 4 mM is required. However, growth even in the presence of 4 mM $NaHCO_3$

was extremely poor, with a low E.O.P. and small colony size, thus the bicarbonate level provided was again sufficient for only suboptimal growth.

Clearly, K12 cells show only limited growth at low $NaHCO_3$ or CO_2 levels, even when the pH is maintained within the optimum range by the use of alternative buffers (the medium remained red throughout the majority of the experiments, indicating a pH of greater than approximately 7.3), confirming the obligatory role of bicarbonate as a nutrient. In order to determine whether growth of this cell line could be achieved in the absence of exogenous $NaHCO_3$ under any conditions, the CO_2 concentration in the incubator was raised to 2%. A K12 suspension of 80 cells/ml (Section 2.4) was plated either in 5% FCS MEM containing 20 mM HEPES and no $NaHCO_3$ or in 5% FCS E4 low bic, with duplicate 6 cm plates for each medium type. The cells were placed in a 33°C incubator; a 2% CO_2 atmosphere was used for cells plated in MEM, and 5% CO_2 for those in E4. One week after plating, the colonies were stained and counted (Section 2.5). The results (Table 3.37) indicate that the use of a 2% CO_2 atmosphere compensated for the lack of any exogenously supplied $NaHCO_3$, presumably because the CO_2 dissolved to form bicarbonate ions and thus allowed for good growth with large, healthy colonies. It appears, therefore, that K12 cells have an obligatory requirement for $NaHCO_3$, a property shared with many other cell lines (Geyer and Chang, 1958; Swim and Parker, 1958). As a consequence, the pH of the medium cannot be manipulated independently of the $NaHCO_3$ concentration, and the differential effects of bicarbonate's nutrient and buffering roles cannot be dissociated unless a medium can be found which will allow for growth of K12 cells with a nutrient that can substitute for $NaHCO_3$. The majority of growth media contain at least 4 mM $NaHCO_3$, in order to satisfy the nutrient requirement of cells, and those compounds which have been used as substitutes for $NaHCO_3$ e.g. sodium pyruvate in Leibowitz L-15 medium (Leibowitz, 1963) or sodium glycerophosphate (Waymouth, 1979), do so by enabling cells to increase their endogenous production of CO_2, thus making them independent of CO_2 and exogenously added bicarbonate. Since the CO_2 produced in such a situation will dissolve in the medium to form bicarbonate ions to a degree determined by the pH of the medium, the problems of varying bicarbonate levels will continue to exist. It thus seems that although the level of $NaHCO_3$ in the medium appears to play a critical role in determining the survival time of K12 at 40°C, whether this is due to changes in the pH of the medium or to alterations in the nutritional conditions is unclear, and cannot be fully resolved unless K12 can be cultured in the absence of bicarbonate ions.

Table 3.35 GROWTH OF K12 AT 33°C UNDER ATMOSPHERIC CO_2 IN HEPES-BUFFERED MEDIA

MEDIUM	[$NaHCO_3$] mM	TYPE OF GROWTH VESSEL	COLONY NUMBER	E.O.P.
20 mM HEPES MEM	0	plate	0	0
		flask	0	0
	4	plate	0	0
		flask	68	17.0
	7	plate	0	0
		flask	68	17.0
10 mM HEPES E4	0	plate	0	0
		flask	0	0
	4	plate	0	0
		flask	0	0
	7	plate	0	0
		flask	0	0
E4 low bic	26	plate	192	48.0

Table 3.36 GROWTH OF K12 AT 33°C UNDER ATMOSPHERIC CO_2 IN BES-BUFFERED E4

[BES] mM	[$NaHCO_3$] mM	COLONY NUMBER	E.O.P.	AVERAGE E.O.P.
10	0	0	0	
		0	0	0
	4	0	0	
		0	0	0
20	0	0	0	
		0	0	0
	4	29	7.3	
		1	0.3	3.8
30	0	0	0	
		0	0	0
	4	contaminated		
		5	1.3	1.3
E4 low bic	26	191	47.8	
		185	46.3	47.0

Table 3.37 GROWTH OF K12 AT 33°C IN E4 LOW BIC OR 20 mM HEPES-BUFFERED MEM

MEDIUM	COLONY NUMBER	E.O.P.	AVERAGE E.O.P.	COLONY SIZE
E4 low bic	183	45.8		
	181	45.3	45.5	large
20 mM HEPES MEM	147	36.8		
	148	37.0	36.9	large

3.12 VIABILITY CURVE STUDIES USING MCDB-302 AND E4 MEDIA OF EQUIVALENT pH VALUES

If the alterations in the pH of the media caused by varying the $NaHCO_3$ level were solely responsible for the observed effects on the viability of K12 at the npt, then MCDB-302 and E4 media with equivalent pH values but varying bicarbonate concentrations would be expected to produce similar viability curves. In order to investigate whether this was indeed the case, a viability curve study was undertaken in MCDB-302 and E4 brought to three different equivalent pH values by manipulation of the $NaHCO_3$ content. The concentration of bicarbonate needed to produce the required pH was calculated using the standard curve (Fig. 3.13) from Section 3.10, which relates $NaHCO_3$ concentration to pH for each of the two media. The pH values chosen for the study were pH 7.0, 7.4 and 7.7, since pH values outside this range required $NaHCO_3$ levels in E4 that were unacceptable for good cell growth (see Section 3.7).

A viability curve experiment was carried out (Section 2.6) using K12 cells suspended in one of the following media, each supplemented with 5% FCS - 13.3 mM $NaHCO_3$ E4 (pH 7.0), 8.5 mM $NaHCO_3$ MCDB-302 (pH 7.0), 27.5 mM $NaHCO_3$ E4 (pH 7.4), 20 mM $NaHCO_3$ MCDB-302 (pH 7.4), 52 mM $NaHCO_3$ E4 (pH 7.7) or 38 mM $NaHCO_3$ MCDB-302 (pH 7.7) - and the results (Tables 3.38 - 3.43) were plotted (Fig. 3.15).

The viability curves produced demonstrate that at a pH value of 7.0, K12 cells plated in either MCDB-302 or E4 showed a similar rate of loss of viability at 40°C, with cells starting to lose their proliferative capacity approximately 30 hours after the temperature shift. However, at a pH of 7.4, although the cells lost viability more rapidly than at pH 7.0 in each medium, cells plated in E4 began to lose their colony-forming ability 5 - 10 hours before those cultured in MCDB-302, and when the pH was further manipulated to give a pH value of 7.7, K12 now lost viability 10 - 15 hours faster in E4 than in MCDB-302. It thus appears that variations in the bicarbonate level do not alter the survival time of K12 at the npt simply by an effect on the pH of the medium, since media with equivalent pH values did not give identical viability curves. In fact, there appears to be a better correlation between the concentration of $NaHCO_3$ and the rate of loss of viability at the npt, with an increased $NaHCO_3$ level accelerating death, than between the pH and the survival time, and the increased differential between curves at the higher pH values could be explained by the greater difference between the $NaHCO_3$ concentrations needed to set the required pH for each medium type as the pH value is raised. However, the degree of correlation between $NaHCO_3$ level and survival time is also imperfect, thus

although no firm conclusions regarding the precise mechanism by which $NaHCO_3$ influences the behaviour of K12 at 40°C can be drawn from this experiment, it is clear that these effects are not simply attributable either to the changes in the pH of the medium, or to the variations in the nutritional status of the cell which are brought about by manipulation of the medium bicarbonate level, but that a more complex situation exists. It seems probable that the two functions of bicarbonate, as both a buffer and a nutrient, may each play a role, in combination. Alternatively, other components of the medium which differ markedly in concentration between E4 and MCDB-302 e.g. ions, may also be involved in determining the rate of loss of viability of K12 at the npt, and this proposal was further studied in Section 3.13.

Table 3.38 LOSS OF VIABILITY OF K12 AT 40°C IN 5% FCS 13.3 mM $NaHCO_3$ E4 (pH 7.0)

HOURS OF INCUBATION AT 40°C	COLONY NUMBER	E.O.P.	AVERAGE E.O.P.	% RELATIVE E.O.P.
continuous	0	0		
	0	0	0	0
0	235	58.8		
	215	53.8	56.3	100
24.0	214	53.5		
	212	53.0	53.3	94.7
31.5	222	55.5		
	209	52.3	53.9	95.8
40.5	203	50.8		
	181	45.3	48.0	85.3
55.8	8	2.0		
	7	1.8	1.9	3.3
64.5	12	3.0		
	12	3.0	3.0	5.3
72.0	0	0		
	1	0.3	0.1	0.2

Table 3.39 LOSS OF VIABILITY OF K12 AT 40°C IN 5% FCS 27.5 mM $NaHCO_3$ E4 (pH 7.4)

HOURS OF INCUBATION AT 40°C	COLONY NUMBER	E.O.P.	AVERAGE E.O.P.	% RELATIVE E.O.P.
continuous	0	0		
	0	0	0	0
0	222	55.5		
	209	52.3	53.9	100
24.0	170	42.5		
	205	51.3	46.9	87.0
31.5	136	34.0		
	178	44.5	39.3	72.9
40.5	104	26.0		
	141	35.3	30.6	56.8
55.8	contaminated			
	0	0	0	0
64.5	0	0		
	1	0.3	0.1	0.2
72.0	0	0		
	0	0	0	0

Table 3.40 LOSS OF VIABILITY OF K12 AT 40°C IN 5% FCS 52 mM $NaHCO_3$ E4 (pH 7.7)

HOURS OF INCUBATION AT 40°C	COLONY NUMBER	E.O.P.	AVERAGE E.O.P.	% RELATIVE E.O.P.
continuous	0	0		
	0	0	0	0
0	203	50.8		
	209	52.3	51.5	100
24.0	114	28.5		
	108	27.0	27.8	53.9
31.5	79	19.8		
	77	19.3	19.5	37.9
40.5	22	5.5		
	28	7.0	6.3	12.1
55.8	0	0		
	0	0	0	0
64.5	0	0		
	0	0	0	0
72.0	0	0		
	0	0	0	0

Table 3.41 LOSS OF VIABILITY OF K12 AT 40°C IN 5% FCS 8.5 mM $NaHCO_3$ MCDB-302 (pH 7.0)

HOURS OF INCUBATION AT 40°C	COLONY NUMBER	E.O.P.	AVERAGE E.O.P.	% RELATIVE E.O.P.
continuous	0	0		
	0	0	0	0
0	186	46.5		
	167	41.8	44.1	100
24.0	157	39.3		
	178	44.5	41.9	94.9
31.5	154	38.5		
	165	41.3	39.9	90.4
40.5	142	35.5		
	146	36.5	36.0	81.6
55.8	18	4.5		
	44	11.0	7.8	17.6
64.5	25	6.3		
	19	4.8	5.5	12.5
72.0	3	0.8		
	1	0.3	0.5	1.1

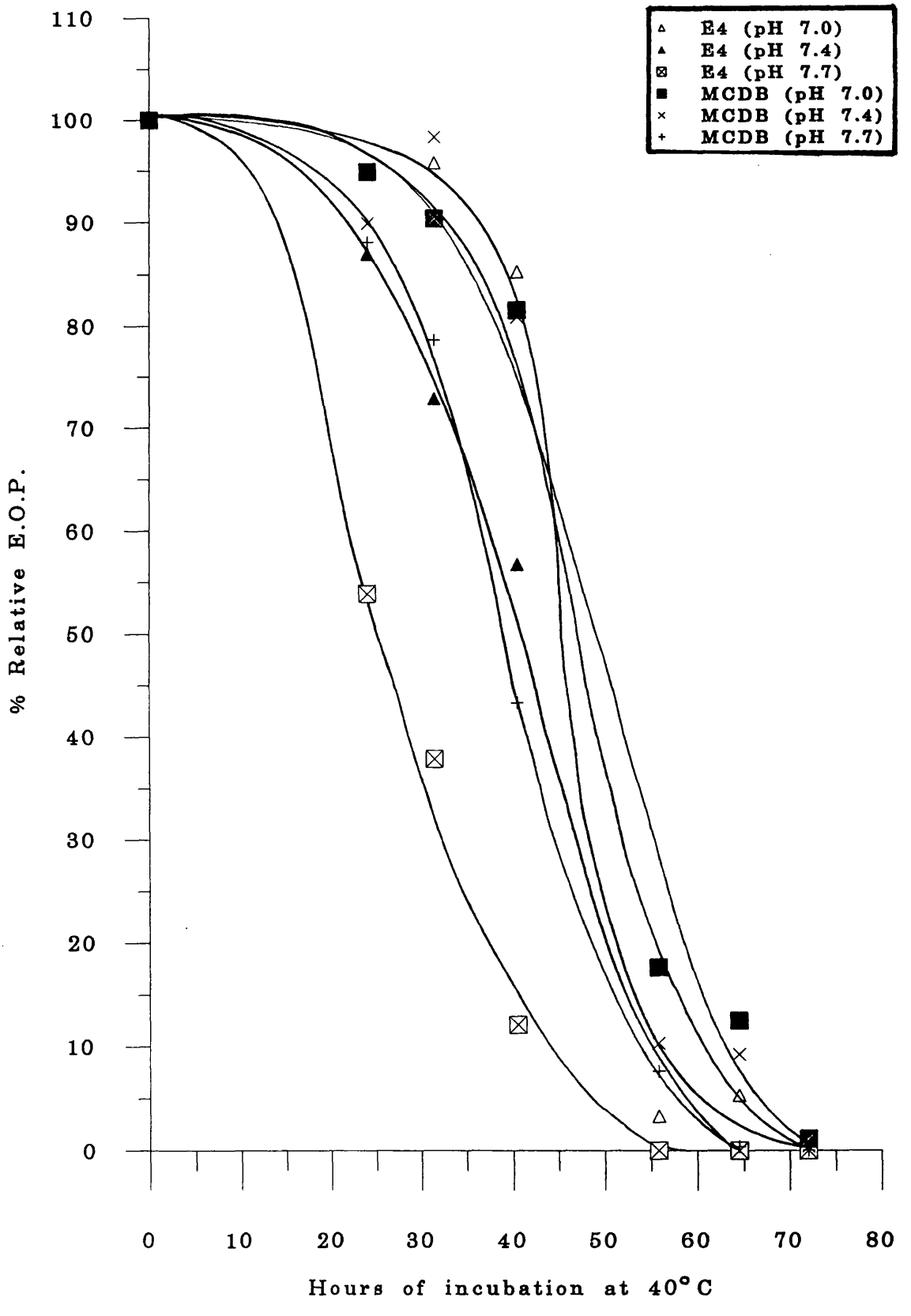
Table 3.42 LOSS OF VIABILITY OF K12 AT 40°C IN 5% FCS 20 mM $NaHCO_3$ MCDB-302 (pH 7.4)

HOURS OF INCUBATION AT 40°C	COLONY NUMBER	E.O.P.	AVERAGE E.O.P.	% RELATIVE E.O.P.
continuous	0	0		
	0	0	0	0
0	245	61.3		
	221	55.3	58.3	100
24.0	219	54.8		
	200	50.0	52.4	89.9
31.5	220	55.0		
	238	59.5	57.3	98.3
40.5	187	46.8		
	190	47.5	47.1	80.9
55.8	24	6.0		
	contaminated		6.0	10.3
64.5	21	5.3		
	22	5.5	5.4	9.2
72.0	0	0		
	2	0.5	0.3	0.4

Table 3.43 LOSS OF VIABILITY OF K12 AT 40°C IN 5% FCS 38 mM MCDB-302 (pH 7.7)

HOURS OF INCUBATION AT 40°C	COLONY NUMBER	E.O.P.	AVERAGE E.O.P.	% RELATIVE E.O.P.
continuous	0	0		
	0	0	0	0
0	258	64.5		
	254	63.5	64.0	100
24.0	223	55.8		
	228	57.0	56.4	88.1
31.5	201	50.3		
	202	50.5	50.4	78.7
40.5	112	28.0		
	110	27.5	27.8	43.4
55.8	22	5.5		
	17	4.3	4.9	7.6
64.5	1	0.3		
	1	0.3	0.3	0.4
72.0	0	0		
	0	0	0	0

Figure 3.15 LOSS OF VIABILITY OF K12 AT 40°C IN 5% FCS-SUPPLEMENTED MEDIA OF EQUIVALENT pH VALUES



3.13 INVESTIGATION OF THE EFFECTS OF VARIOUS IONS ON THE GROWTH OF K12 AT 40°C

In order to identify medium components which, in addition to sodium bicarbonate, may influence the survival time of K12 at the npt, and thus account for the difference in behaviour of K12 at 40°C in MCDB-302 and E4 with either equivalent pH values or $NaHCO_3$ concentrations, the compositions of the two media were compared (Appendix III), and it was found that MCDB-302 contains six ions (Zn^{2+}, VO_3^-, SeO_3^{2-}, Mn^{2+}, Mo^{6+} and Cu^{2+}) that are absent in E4, and one ion (Fe^{2+}) is present at an approximately eightfold higher concentration in MCDB-302 than in E4. To determine whether the difference in levels of any of these ions could influence the viability of K12 at the npt, 5% FCS E4 low bic was supplemented with each ion in turn such that the concentration of the ion in question was brought to equivalence in the two media. Viability curves (Section 2.6) were carried out on K12 grown in each of the supplemented media, and compared with those obtained with cells cultured in 5% FCS MCDB-302 or unsupplemented 5% FCS E4 low bic.

The results (Tables 3.44 - 3.52 and Figs. 3.16 and 3.17) indicate that the addition of five of the seven ions, namely VO_3^-, SeO_3^{2-}, Zn^{2+}, Cu^{2+} and Mn^{2+}, to E4 low bic significantly prolonged the survival time of K12 at 40°C, suggesting that these medium components can indeed influence the behaviour of this cell line at the npt. The E.O.P. of K12 cells grown at 33°C in E4 low bic supplemented with iron (Table 3.52) was low, suggesting either that the $FeSO_4$ solution used may have contained a toxic contaminant or, alternatively, the iron may have oxidised and precipitated on to the cells in a deleterious manner. No single ion prolonged the survival of K12 to the degree found when cells were grown in MCDB-302, presumably due to the additional effects of differing $NaHCO_3$ concentrations in the two media. The large number of ions involved in the observed increased viability suggests that it may have been caused by general growth promoting or inhibiting effects brought about by the ions in question, rather than by a specific action of each ion on the activity or stability of the K12 ts protein. Indeed, the E.O.P. of K12 at 33°C varied widely in media supplemented with the different ions, despite the fact that all cells originated from a common cell suspension, implying that each ion may affect the general growth characteristics of this cell line. However, there appears to be no clear relationship between the E.O.P. value and the survival time at 40°C.

When E4 low bic was supplemented simultaneously with the five ions that increased the survival time of K12 at 40°C (VO_3^-, SeO_3^{2-}, Zn^{2+}, Cu^{2+} and Mn^{2+}), the rate of loss of viability at the npt was similar to that found in unsupplemented E4 low bic (Tables 3.53 - 3.55 and Fig. 3.18). This suggests that although an

increased level of each of these ions in isolation can alter the viability and general growth characteristics of this cell line, their ability to promote the survival of K12 at the npt is lost when they interact. Since the higher concentration of each ion in question is present simultaneously in MCDB-302, it thus seems unlikely that the difference in behaviour of K12 at the npt in MCDB-302 and E4 media with either equivalent pH values or $NaHCO_3$ concentrations can be explained by the differing levels of these particular ions. In addition, variations in the concentration of these ions did not appear to be responsible for the increased colony size and lower E.O.P. observed when K12 is grown at 33°C in MCDB-302 rather than in E4 low bic (see Section 3.5 and Tables 3.53 - 3.55), implying that an additional medium component may influence the behaviour of K12 at the npt.

A comparison of the compositions of MCDB-302 and E4 low bic (Appendix III) also indicates that E4 low bic contains a higher level of K^+, Ca^{2+} and Mg^{2+} ions than MCDB-302. Viability curves were thus carried out (Section 2.6) where 5% FCS MCDB-302 was supplemented with each ion in turn to bring the concentration of the ion of interest to equivalence in the two media, and the survival time of K12 at the npt in each of the supplemented media was compared to that found in 5% FCS E4 low bic or in unsupplemented 5% FCS MCDB-302. The results (Tables 3.56 - 3.60 and Fig. 3.19) suggest that the differences in the levels of these ions in MCDB-302 and E4 low bic cannot explain the varying rates of loss of viability of K12 at 40°C observed in these two media. Raising the Mg^{2+} level of MCDB-302 to that found in E4 low bic appeared to prolong the survival of the cells for significantly longer than when unsupplemented MCDB-302 was used, but given the consistently high (> 100%) % relative E.O.P. values of the 23.5 - 55.5 hour time points, it seems probable that the 33°C control plates received fewer cells than the rest of the set, perhaps due to pipetting inaccuracies, thus distorting the succeeding % relative E.O.P. values. If these were adjusted so that the first four time point values fell around the 100% relative E.O.P. level, the viability curve would then appear to be similar to that found with unsupplemented MCDB-302.

The overall conclusion to be drawn from these experiments is that variations in the ionic levels of MCDB-302 and E4 are unlikely to account for the differences in the rate of loss of viability of K12 at the npt observed in E4 or MCDB-302 media of equivalent $NaHCO_3$ concentrations or pH values. However, since all ions were not tested simultaneously in this experiment, and interactions between them appear to alter their effects on K12 at 40°C, the possibility that the particular combination of ions present in each medium may be responsible for the observed differences in behaviour remains. This could be tested by taking E4 and MCDB-302 media with either an equivalent $NaHCO_3$ level or pH value, concurrently bringing all the ion

concentrations of one medium to equivalence with those of the other, and comparing the viability of K12 at the npt in the two media. Unfortunately, since each medium contains some ions at a higher concentration and a number at a lower level than the other, this would involve making each medium up from its basic constituents to the required ionic concentrations, and there was insufficient time available to carry this procedure out.

Table 3.44 LOSS OF VIABILITY OF K12 AT 40°C IN 5% FCS E4 LOW BIC

HOURS OF INCUBATION AT 40°C	COLONY NUMBER	E.O.P.	AVERAGE E.O.P.	% RELATIVE E.O.P.
continuous	0	0		
	0	0	0	0
0	132	33.0		
	188	47.0	40.0	100
24.0	118	29.5		
	107	26.8	28.1	70.3
48.0	1	0.3		
	0	0	0.1	0.3
72.5	0	0		
	0	0	0	0

Table 3.45 LOSS OF VIABILITY OF K12 AT 40°C IN 5% FCS MCDB-302

HOURS OF INCUBATION AT 40°C	COLONY NUMBER	E.O.P.	AVERAGE E.O.P.	% RELATIVE E.O.P.
continuous	0	0		
	0	0	0	0
0	152	38.0		
	159	39.8	38.9	100
24.0	170	42.5		
	151	37.8	40.1	103.0
48.0	76	19.0		
	50	12.5	15.8	40.5
72.5	0	0		
	1	0.3	0.1	0.3

Table 3.46 LOSS OF VIABILITY OF K12 AT 40°C IN 5% FCS E4 LOW BIC SUPPLEMENTED WITH $3x10^{-3}$ mM $ZnSO_4.7H_2O$

HOURS OF INCUBATION AT 40°C	COLONY NUMBER	E.O.P.	AVERAGE E.O.P.	% RELATIVE E.O.P.
continuous	0	0		
	0	0	0	0
0	215	53.8		
	183	45.8	49.8	100
24.0	148	37.0		
	149	37.3	37.1	74.6
48.0	23	5.8		
	21	5.3	5.5	11.1
72.5	0	0		
	0	0	0	0

Table 3.47 LOSS OF VIABILITY OF K12 AT 40°C IN 5% FCS E4 LOW BIC SUPPLEMENTED WITH 10^{-5} mM $CuSO_4.5H_2O$

HOURS OF INCUBATION AT 40°C	COLONY NUMBER	E.O.P.	AVERAGE E.O.P.	% RELATIVE E.O.P.
continuous	0	0		
	0	0	0	0
0	133	33.3		
	141	35.3	34.3	100
24.0	113	28.3		
	124	31.0	29.6	86.5
48.0	6	1.5		
	15	3.8	2.6	7.7
72.5	0	0		
	0	0	0	0

Table 3.48 LOSS OF VIABILITY OF K12 AT 40°C IN 5% FCS E4 LOW BIC SUPPLEMENTED WITH 10^{-6} mM $MnSO_4.14H_2O$

HOURS OF INCUBATION AT 40°C	COLONY NUMBER	E.O.P.	AVERAGE E.O.P.	% RELATIVE E.O.P.
continuous	0	0		
	0	0	0	0
0	151	37.8		
	195	48.8	43.3	100
24.0	155	38.8		
	147	36.8	37.8	87.3
48.0	28	7.0		
	54	13.5	10.3	23.7
72.5	0	0		
	0	0	0	0

Table 3.49 LOSS OF VIABILITY OF K12 AT 40°C IN 5% FCS E4 LOW BIC SUPPLEMENTED WITH 10^{-5} mM $[NH_4]_6Mo_7O_{24}.4H_2O$

HOURS OF INCUBATION AT 40°C	COLONY NUMBER	E.O.P.	AVERAGE E.O.P.	% RELATIVE E.O.P.
continuous	0	0		
	0	0	0	0
0	117	29.3		
	99	24.8	27.0	100
24.0	68	17.0		
	65	16.3	16.6	61.6
48.0	5	1.3		
	7	1.8	1.5	5.6
72.5	0	0		
	0	0	0	0

Table 3.50 LOSS OF VIABILITY OF K12 AT 40°C IN 5% FCS E4 LOW BIC SUPPLEMENTED WITH 4.57×10^{-6} mM Na_2SeO_3

HOURS OF INCUBATION AT 40°C	COLONY NUMBER	E.O.P.	AVERAGE E.O.P.	% RELATIVE E.O.P.
continuous	0	0		
	0	0	0	0
0	145	36.3		
	159	39.8	38.0	100
24.0	148	37.0		
	143	35.8	36.4	95.7
48.0	30	7.5		
	20	5.0	6.3	16.4
72.5	0	0		
	0	0	0	0

Table 3.51 LOSS OF VIABILITY OF K12 AT 40°C IN 5% FCS E4 LOW BIC SUPPLEMENTED WITH 10^{-5} mM NH_4VO_3

HOURS OF INCUBATION AT 40°C	COLONY NUMBER	E.O.P.	AVERAGE E.O.P.	% RELATIVE E.O.P.
continuous	0	0		
	0	0	0	0
0	170	42.5		
	156	39.0	40.8	100
24.0	163	40.8		
	144	36.0	38.4	94.2
48.0	55	13.8		
	38	9.5	11.6	28.5
72.5	0	0		
	0	0	0	0

Table 3.52 LOSS OF VIABILITY OF K12 AT 40°C IN 5% FCS E4 LOW BIC SUPPLEMENTED WITH $3x10^{-3}$ mM $FeSO_4.7H_2O$

HOURS OF INCUBATION AT 40°C	COLONY NUMBER	E.O.P.	AVERAGE E.O.P.	% RELATIVE E.O.P.
continuous	0	0		
	0	0	0	0
0	39	9.8		
	44	11.0	10.4	100
24.0	1	0.3		
	1	0.3	0.3	2.4
48.0	0	0		
	0	0	0	0
72.5	0	0		
	0	0	0	0

Figure 3.16 LOSS OF VIABILITY OF K12 AT 40°C IN 5% FCS E4 LOW BIC SUPPLEMENTED WITH VARIOUS IONS - I

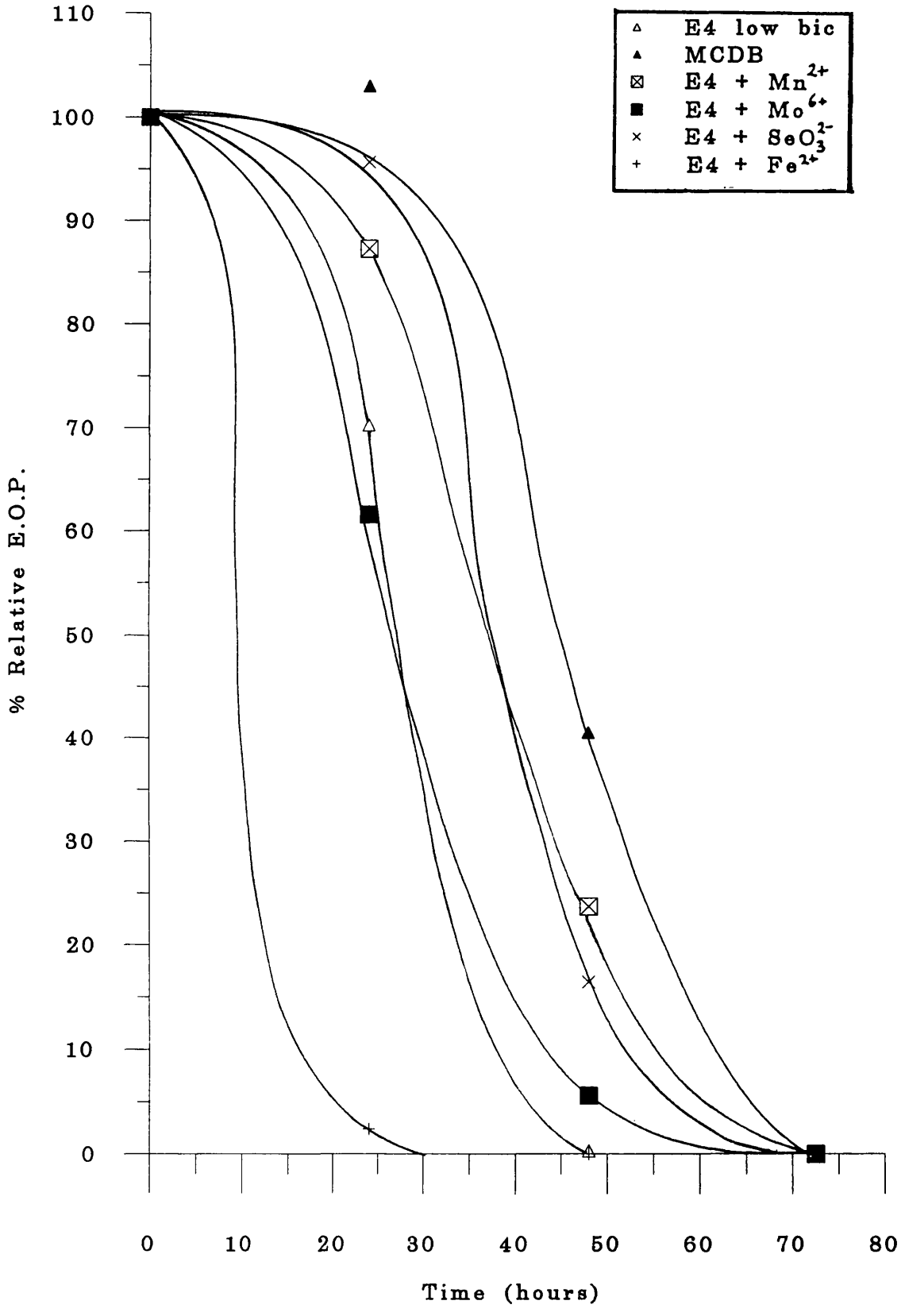


Figure 3.17 LOSS OF VIABILITY OF K12 AT 40°C IN 5% FCS E4 LOW BIC SUPPLEMENTED WITH VARIOUS IONS - II

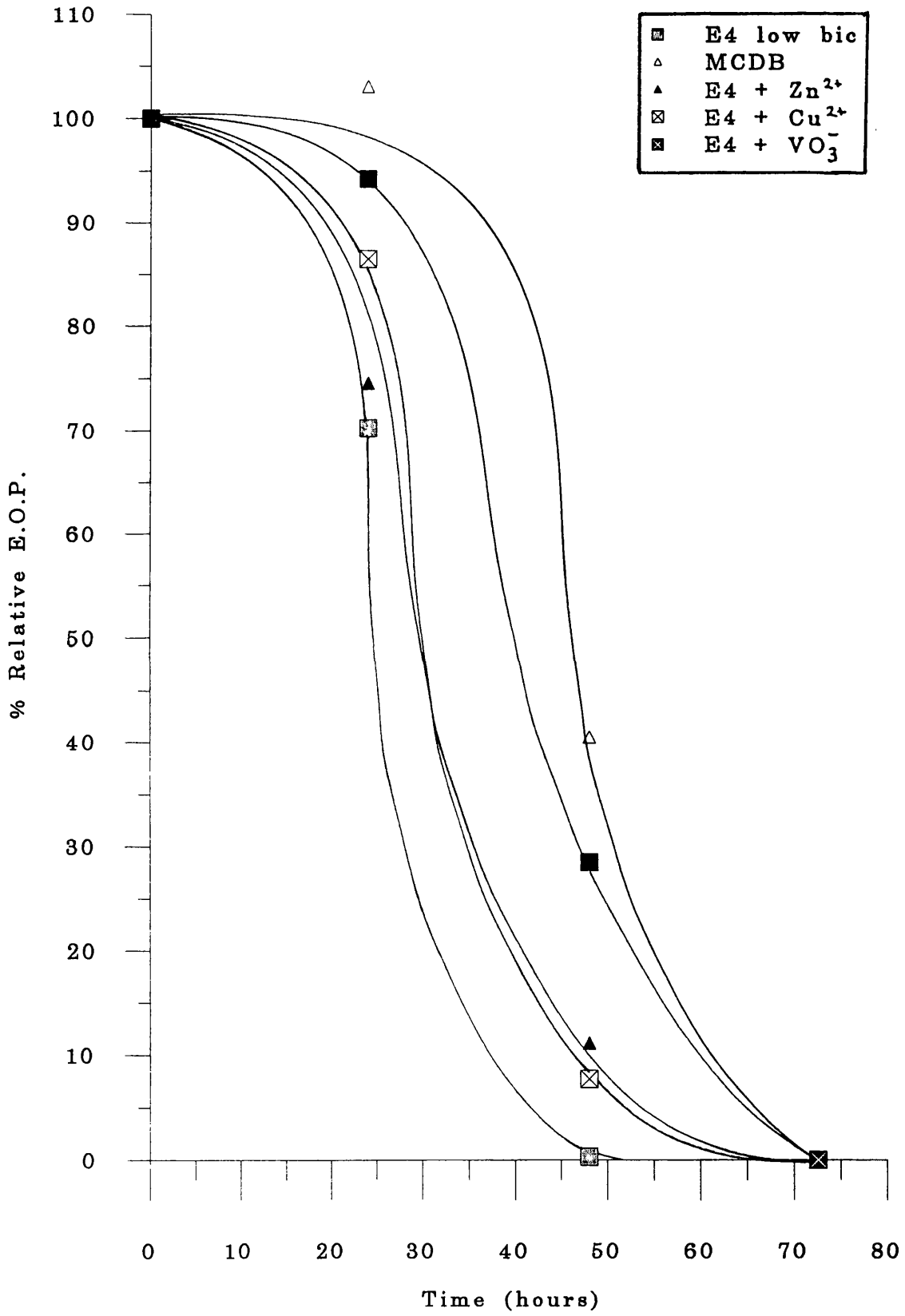


Table 3.53 LOSS OF VIABILITY OF K12 AT 40°C IN 5% FCS E4 LOW BIC

HOURS OF INCUBATION AT 40°C	COLONY NUMBER	E.O.P.	AVERAGE E.O.P.	% RELATIVE E.O.P.
continuous	0	0		
	0	0	0	0
0	191	47.8		
	198	49.5	48.6	100
24.0	213	53.3		
	222	55.5	54.4	111.8
31.5	251	62.8		
	222	55.5	59.1	121.6
48.0	48	12.0		
	60	15.0	13.5	27.8
72.0	0	0		
	0	0	0	0

Table 3.54 LOSS OF VIABILITY OF K12 AT 40°C IN 5% FCS MCDB-302

HOURS OF INCUBATION AT 40°C	COLONY NUMBER	E.O.P.	AVERAGE E.O.P.	% RELATIVE E.O.P.
continuous	0	0		
	0	0	0	0
0	100	25.0		
	96	24.0	24.5	100
24.0	101	25.3		
	115	28.8	27.0	110.2
31.5	108	27.0		
	122	30.5	29.8	117.3
48.0	125	31.3		
	144	36.0	33.6	137.1
72.0	26	6.5		
	17	4.3	5.4	22.0

Table 3.55 LOSS OF VIABILITY OF K12 AT 40°C IN 5% FCS E4 LOW BIC SUPPLEMENTED WITH 10^{-5} mM NH_4VO_3, 4.57×10^{-6} mM Na_2SeO_3, 3×10^{-3} mM $ZnSO_4.7H_2O$, 10^{-5} mM $CuSO_4.5H_2O$ AND 10^{-6} mM $MnSO_4.4H_2O$

HOURS OF INCUBATION AT 40°C	COLONY NUMBER	E.O.P.	AVERAGE E.O.P.	% RELATIVE E.O.P.
continuous	0	0		
	0	0	0	0
0	217	54.3		
	224	56.0	55.1	100
24.0	271	67.7		
	222	55.5	61.6	111.8
31.5	176	44.0		
	195	48.8	46.4	84.1
48.0	45	11.3		
	45	11.3	11.3	20.4
72.0	1	0.3		
	0	0	0.1	0.2

Figure 3.18 LOSS OF VIABILITY OF K12 AT 40°C IN 5% FCS E4 LOW BIC SUPPLEMENTED SIMULTANEOUSLY WITH FIVE DIFFERENT IONS

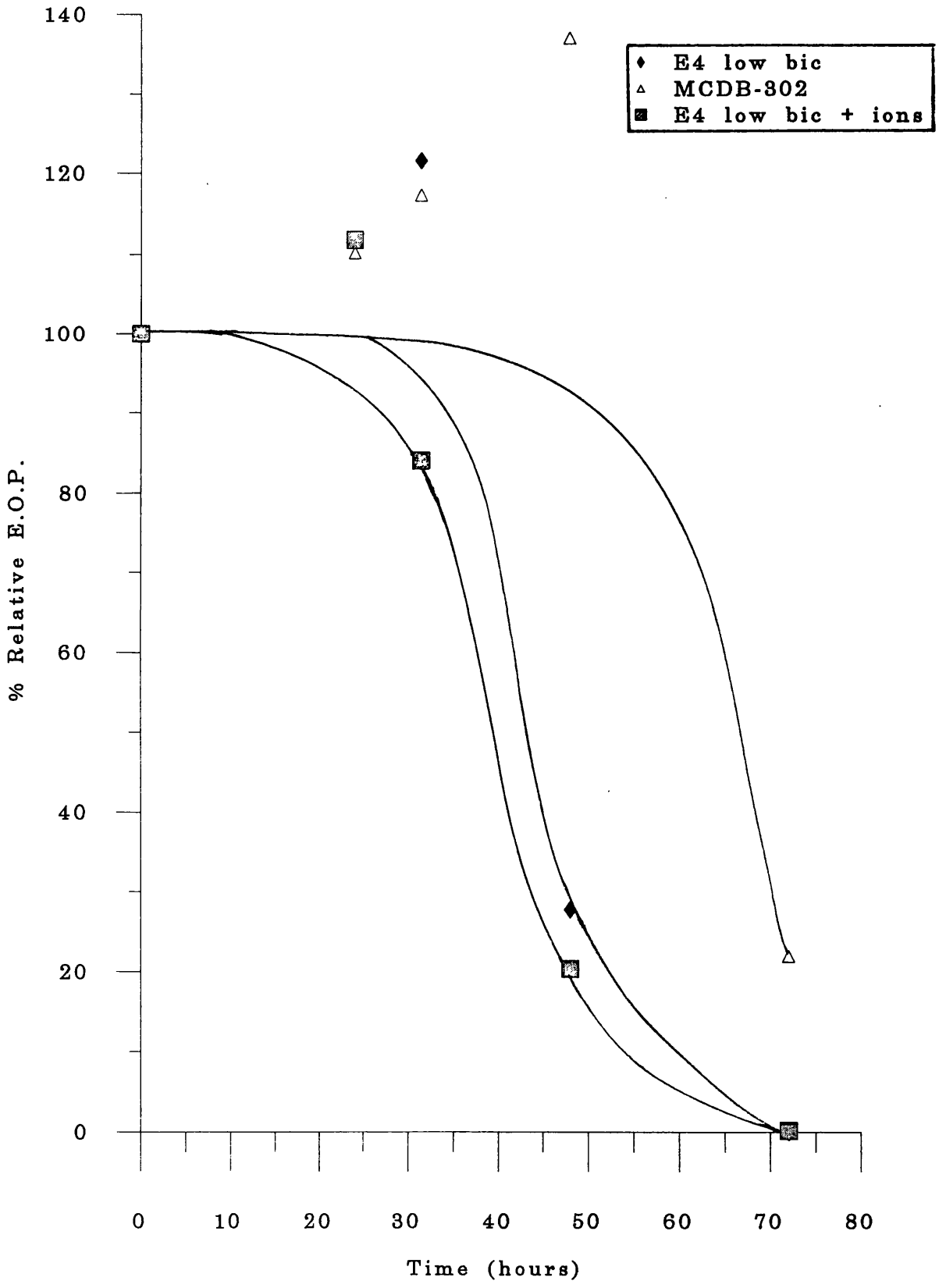


Table 3.56 LOSS OF VIABILITY OF K12 AT 40°C IN 5% FCS E4 LOW BIC

HOURS OF INCUBATION AT 40°C	COLONY NUMBER	E.O.P.	AVERAGE E.O.P.	% RELATIVE E.O.P.
continuous	0	0		
	0	0	0	0
0	137	34.3		
	125	31.3	32.8	100
23.5	110	27.5		
	100	25.0	26.3	80.2
31.0	109	27.3		
	132	33.0	30.1	92.0
40.5	59	14.8		
	58	14.5	14.6	44.7
55.5	26	6.5		
	44	11.0	8.8	26.7
64.5	10	2.5		
	30	7.5	5.0	15.3
71.5	0	0		
	8	2.0	1.0	3.1

Table 3.57 LOSS OF VIABILITY OF K12 AT 40°C IN 5% FCS MCDB-302

HOURS OF INCUBATION AT 40°C	COLONY NUMBER	E.O.P.	AVERAGE E.O.P.	% RELATIVE E.O.P.
continuous	1	0.3		
	4	1.0	0.6	1.4
0	197	49.3		
	152	38.0	43.6	100
23.5	168	42.0		
	129	32.3	37.1	85.1
31.0	132	33.0		
	133	33.3	33.1	75.9
40.5	145	36.3		
	147	36.8	36.5	83.7
55.5	117	29.3		
	109	27.3	28.3	64.8
64.5	122	30.5		
	107	26.8	28.6	65.6
71.5	56	14.0		
	89	22.3	18.1	41.5

Table 3.58 LOSS OF VIABILITY OF K12 AT 40°C IN 5% FCS MCDB-302 SUPPLEMENTED WITH 2.36 mM KCl

HOURS OF INCUBATION AT 40°C	COLONY NUMBER	E.O.P.	AVERAGE E.O.P.	% RELATIVE E.O.P.
continuous	1	0.3		
	0	0	0.1	0.4
0	137	34.3		
	125	31.3	32.8	100
23.5	109	27.3		
	130	32.5	29.9	91.2
31.0	120	30.0		
	130	32.5	31.3	95.4
40.5	93	23.3		
	123	30.8	27.0	82.4
55.5	82	20.5		
	82	20.5	20.5	62.6
64.5	82	20.5		
	66	16.5	18.5	56.5
71.5	29	7.3		
	33	8.3	7.8	23.7

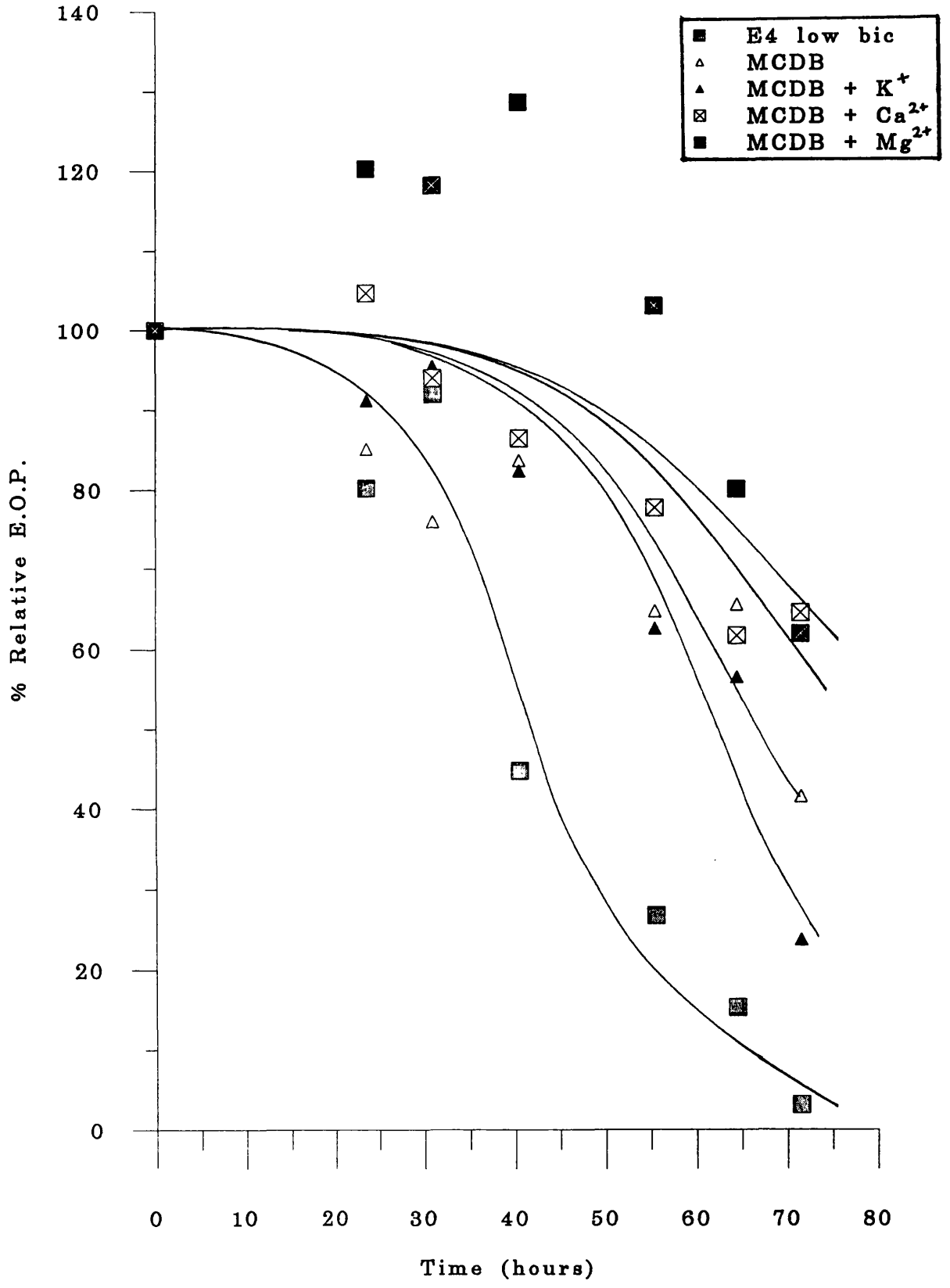
Table 3.59 LOSS OF VIABILITY OF K12 AT 40°C IN 5% FCS MCDB-302 SUPPLEMENTED WITH 1.2 mM $CaCl_2.2H_2O$

HOURS OF INCUBATION AT 40°C	COLONY NUMBER	E.O.P.	AVERAGE E.O.P.	% RELATIVE E.O.P.
continuous	0	0		
	0	0	0	0
0	145	36.3		
	137	34.3	35.3	100
23.5	154	38.5		
	141	35.3	36.9	104.6
31.0	123	30.8		
	142	35.5	33.1	94.0
40.5	142	35.5		
	102	25.5	30.5	86.5
55.5	102	25.5		
	117	29.3	27.4	77.7
64.5	80	20.0		
	94	23.5	21.8	61.7
71.5	86	21.5		
	96	24.0	22.8	64.5

Table 3.60 LOSS OF VIABILITY OF K12 AT 40°C IN 5% FCS MCDB-302 SUPPLEMENTED WITH 0.211 mM $MgCl_2.6H_2O$

HOURS OF INCUBATION AT 40°C	COLONY NUMBER	E.O.P.	AVERAGE E.O.P.	% RELATIVE E.O.P.
continuous	2	0.5		
	2	0.5	0.5	1.7
0	118	29.5		
	113	28.3	28.9	100
23.5	135	33.8		
	143	35.8	34.8	120.3
31.0	145	36.3		
	128	32.0	34.1	118.2
40.5	156	39.0		
	141	35.3	37.1	128.6
55.5	111	27.8		
	127	31.8	29.8	103.0
64.5	97	24.3		
	88	22.0	23.1	80.1
71.5	83	20.8		
	60	15.0	17.9	61.9

Figure 3.19 LOSS OF VIABILITY OF K12 AT 40°C IN 5% FCS MCDB-302 SUPPLEMENTED WITH VARIOUS IONS



3.14 TIME-LAPSE STUDIES ON K12 CELLS GROWN AT 40°C IN MCDB-302 AND E4 WITH VARYING SODIUM BICARBONATE CONCENTRATIONS

In viability curve experiments where the effect of varying the $NaHCO_3$ concentration on the behaviour of K12 at the npt was studied (Sections 3.6, 3.7 and 3.9), curves carried out in the same media on different occasions showed significant variations in shape, most likely due to fluctuations in the incubation temperature (Section 3.3). Time-lapse studies were thus carried out on K12 cultured in each of the media used in Section 3.7, since the temperature control of the time-lapse cabinet was superior to that of the incubators used (the temperature fluctuation range was only 0.2°C), and would therefore allow the further investigation of this phenomenon under more controlled conditions. Time-lapse work, however, offered a major additional advantage; because it permitted the measurement of the number and timing of mitoses, it could be used to determine whether the prolonged viability of K12 at 40°C in low bicarbonate media was caused by the cell's ability to pass the execution point of the mutation and divide normally an increased number of times at the npt, or whether the cells are protected from death simply by a slowing down of the cell cycle such that it takes longer for the cell to reach the block point and halt. Viability curve studies, which only measure the time period over which K12 retains the ability to form colonies when returned to 33°C, are unable to distinguish between these two situations.

Time-lapse studies were thus carried out (Section 2.7) on K12 cells grown in each of the following media: 5% FCS E4 supplemented with either 13.3, 26 or 44 mM $NaHCO_3$, and 5% FCS MCDB-302 containing 7, 26 or 65 mM $NaHCO_3$. The data from the raw 'family trees' (data not shown) were plotted as cumulative mitoses graphs (Figs. 3.20a - 3.25a), from which the time taken for K12 to halt at 40°C could be determined (see Tables 3.61 and 3.62), and also as plots of intermitotic time (Figs. 3.20b - 3.25b). In addition, the raw data were used to calculate the average intermitotic time (A.I.T.) for K12 cells growing in each of the media employed (Tables 3.61 and 3.62).

The cumulative mitoses plots demonstrate that the rate of mitosis remained normal at 40°C for an increasing length of time as the bicarbonate level of each medium was lowered, suggesting that the cells continued dividing normally at the npt for longer as the $NaHCO_3$ concentration was dropped, and were therefore passing the execution point of the mutation several times. This was confirmed by studying the raw 'family tree' data where, in the case of K12 grown in 7 mM $NaHCO_3$ MCDB-302, a large number of cells divided 4, and some even 5 times after the temperature shift, whereas the maximum number of divisions at 40°C in cells cultured in 65 mM

$NaHCO_3$ MCDB-302 was one (Table 3.62). As was the case with the viability curve studies (Section 3.7), the survival time did not seem to be correlated directly with the concentration of bicarbonate in the medium, since cells grown in 26 mM $NaHCO_3$ E4 halted considerably more rapidly at the npt than those cultured in 26 mM $NaHCO_3$ MCDB-302. In addition, although in general it appeared that the time taken to arrest increased as the pH of the medium was lowered, cells grown in 26 mM $NaHCO_3$ MCDB-302 (pH 7.52) took significantly longer to halt at 40°C than those cultured in 26 mM $NaHCO_3$ E4 (pH 7.38), implying that pH was not the principal determinant of the rate of loss of viability either. This further supports the hypothesis that the effect of $NaHCO_3$ on the survival time of K12 at the npt may be due to a combination of its roles as both a nutrient and as a buffer.

The intermitotic time plots show no consistent correlation between the $NaHCO_3$ concentration or the pH and changes in intermitotic time with increasing incubation at 40°C; the intermitotic time remained roughly constant throughout the experiment for cells grown in 65 mM $NaHCO_3$ MCDB-302, 26 mM $NaHCO_3$ MCDB-302 or 13.3 mM $NaHCO_3$ E4, and increased for cells cultured in 26 mM $NaHCO_3$ E4, 44 mM $NaHCO_3$ E4 or 7 mM $NaHCO_3$ MCDB-302. However, in most cases the degree of scatter around the A.I.T. increased as the study progressed, implying perhaps some degree of loss of appropriate cell cycle timing. It thus appears that low bicarbonate levels do not protect K12 against early death at the npt by allowing the cells to cycle more slowly under these potentially suboptimal growth conditions, and thus increasing the time taken for K12 to reach the execution point and die. In fact, it seems that lowering the $NaHCO_3$ level in each medium actually decreased the A.I.T. (Tables 3.61 and 3.62), although to a greater degree in E4 than in MCDB-302. Cells in low bicarbonate media thus appeared to be cycling normally at the npt, passing the execution point of the mutation several times prior to their eventual arrest. Some of the later divisions in 7 mM $NaHCO_3$ MCDB-302 and 13.3 mM $NaHCO_3$ E4 seemed to be somewhat abnormal, however, since a small number of cells divided into three unusually small daughters, only one or two of which subsequently divided again, suggesting that cell cycle control had begun to break down after such a prolonged period at 40°C.

The pattern of loss of viability observed in this experiment closely followed that found in the viability curve studies carried out in identical media in Section 3.7, confirming the validity of each set of measurements. However, when the 44 mM $NaHCO_3$ E4 cumulative mitoses plot (Fig. 3.22) was compared with that constructed from time-lapse work carried out on K12 in this same medium in 1973 shortly after isolation of the cell line (Fig. 3.4), the current cell population appeared to retain viability for 24 hours longer at the npt than the original isolates, implying either

that additional medium components, which have changed in level in the intervening period, affect the behaviour of K12 at the npt, or that the cell line itself has altered by the accumulation of additional mutations, either in the same gene as the original mutation or elsewhere in the genome.

The figures for the maximum number of divisions at 40°C presented in Tables 3.61 and 3.62 were produced using random populations of cells and do not, however, precisely reflect the number of times K12 is capable of passing the execution point at the npt, since a cell which is incapable of passing this point even once at 40°C may or may not divide again, depending on its position within the cell cycle at the time of the temperature shift; if it is before the execution point, it will halt with no further divisions, whereas if it has passed it at the time of the shift, it will divide once before it eventually arrests. In order to assess this value more accurately, the cells must be synchronized at a known point in the cell cycle, and placed directly on the time-lapse equipment at 40°C. The chosen method of synchronization was the mitotic shake-off procedure (Terasima and Tolmach, 1963), which takes advantage of the observation that cells round up and become more loosely attached to their underlying substrate during mitosis (see Fig. 3.1), and can therefore be efficiently and preferentially harvested by a sharp tap on the side of the culture vessel. Since this method involves no artificial blockage of the cycle, the cells undergo a minimal degree of perturbation, and no recovery time is required. An additional advantage of this technique is that cells are harvested at the beginning of the cycle, prior to the K12 execution point, thus the number of divisions each cell carries out at the npt will directly reflect the number of times the execution point has been passed. The time-lapse studies were therefore repeated using mitotically-synchronized K12 populations (Figs. 3.26 - 3.30 and Tables 3.63 and 3.64), as described in Section 2.8, and the degree of synchronization was found to be high. Wg-1A cells cultured in 5% FCS E4 low bic were also monitored by time-lapse, using both random and mitotically-synchronized populations, and were found to continue growing normally at 40°C until they reached superconfluence, died, and detached from the base of the flask, confirming that the method of study itself, and the protocol used for synchronization have no adverse effects on the cells.

K12 cells synchronized at mitosis consistently arrested more rapidly at 40°C than random populations for a given medium type (Tables 3.63 and 3.64), presumably due to the fact that all cells in a mitotically-synchronized culture are located just before the execution point when placed at the npt, whereas in a random population cells may be nearly a whole cycle length before this point at the time of the temperature shift, yet will still arrest after passing the same number of block points, and will thus take longer to cease division. The results also demonstrate that within each cell

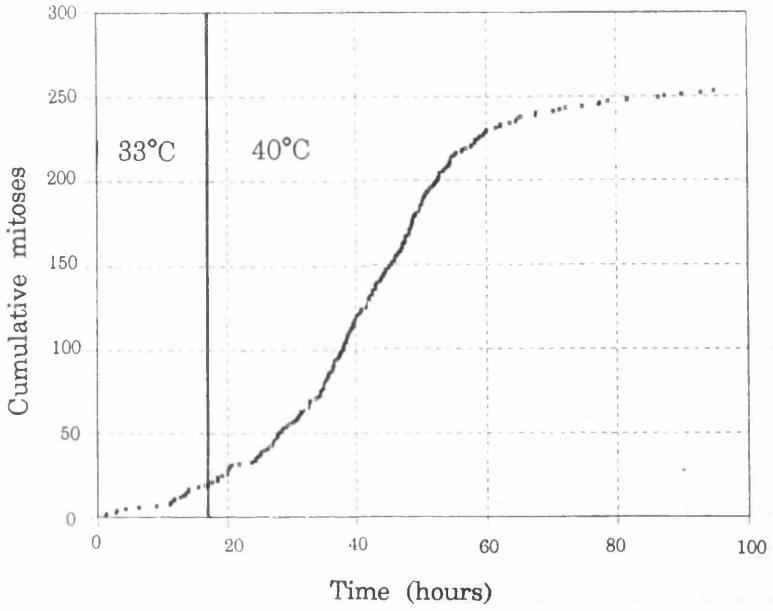
population there was a wide range in the number of times that individual cells were capable of dividing at 40°C e.g. in 13.3 mM $NaHCO_3$ E4, some cells underwent mitosis only twice at the npt, whereas a number were seen to divide five times. This can be explained in part by the wide spread of intermitotic times (which seems to be an inherent property of the cell line, since sister cells often showed large divergences in cycle length) displayed by individual cells within each population (see Figs. 3.26 - 3.30); if the ts protein loses its activity or heat stability at 40°C at a constant rate in all cells, those cells with a longer cycle time will be able to complete fewer divisions at the npt than those with shorter intermitotic times.

The pattern of loss of viability at the npt with mitotically-synchronized populations was similar to that observed with random cultures, with a decrease in the $NaHCO_3$ level generally increasing the time taken for K12 to arrest at the npt, and once again there was no precise correlation between the rate of loss of proliferative capacity at 40°C and either the pH or the bicarbonate concentration of the medium. In most cases, the intermitotic time plots (Figs. 3.26b - 3.30b) suggest that the cycle time lengthened in each medium as the time of incubation at 40°C increased, implying perhaps, as suggested in Section 1.12, that a threshold level of the K12 ts protein is required to pass the execution point, and that the progressive reduction in its activity at the npt may increase the time required to reach this critical concentration, and thus slow the cycle down. The K12 ts protein may thus act as a rate-limiting protein in its defective form. Eventually its activity may become so low that the threshold level is never attained, and the cell consequently halts at the execution point. The average intermitotic times calculated for cells in mitotically-synchronized cultures appeared to be generally shorter than those for random populations for a given medium type (Tables 3.61 - 3.64). This may be a reflection of the fact that values calculated from random cultures included cycles completed at both the pt and the npt, whereas cells in synchronized populations were studied only at 40°C, and small differences in the intermitotic time of K12 at the two temperatures may have affected the results.

In conclusion, the time-lapse studies presented here provide direct evidence to support the idea that lowering the $NaHCO_3$ concentration of the growth medium prolongs the survival of K12 at the npt by allowing the cell to pass the execution point an increasing number of times.

Figure 3.20 TIME-LAPSE PLOTS FOR UNSYNCHRONIZED K12 CELLS GROWN IN 5% FCS E4 CONTAINING 13.3 mM $NaHCO_3$

a. Cumulative mitoses plot



b. Intermitotic time plot

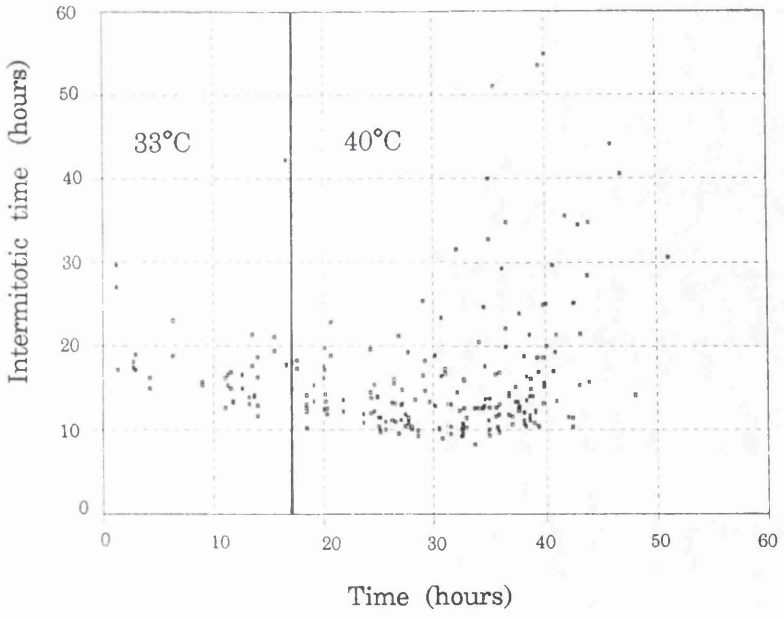
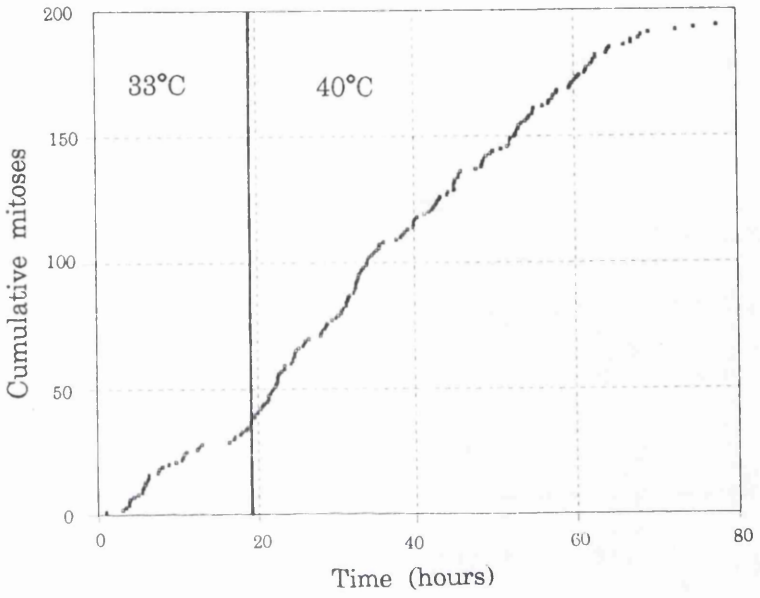


Figure 3.21 TIME-LAPSE PLOTS FOR UNSYNCHRONIZED K12 CELLS GROWN IN 5% FCS E4 CONTAINING 26 mM $NaHCO_3$

a. Cumulative mitoses plot



b. Intermitotic time plot

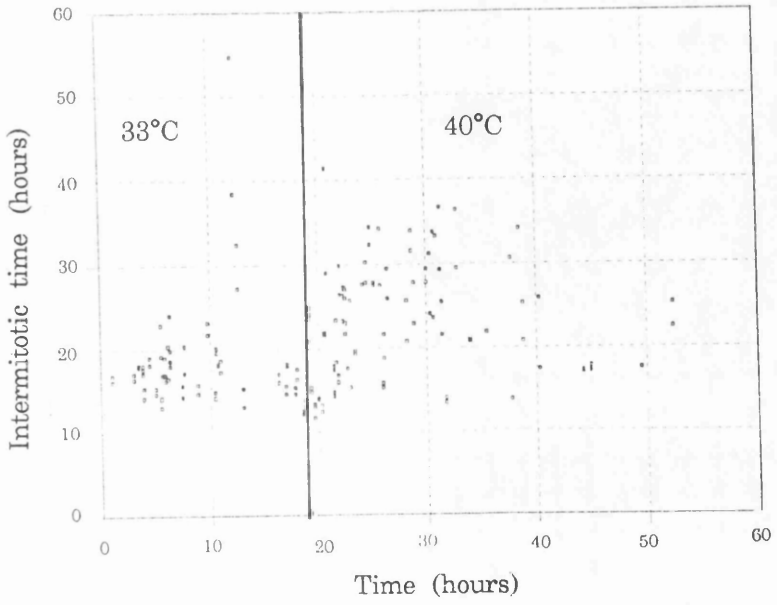
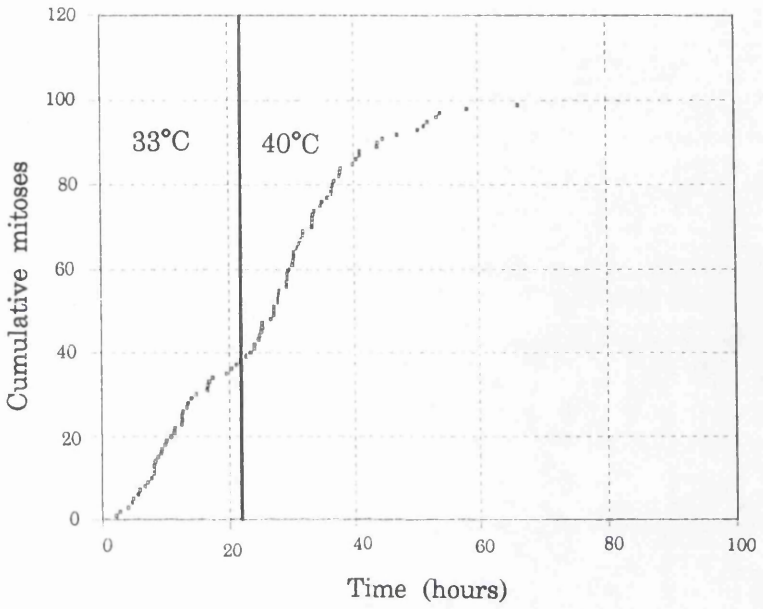


Figure 3.22 TIME-LAPSE PLOTS FOR UNSYNCHRONIZED K12 CELLS GROWN IN 5% FCS E4 CONTAINING 44 mM $NaHCO_3$

a. Cumulative mitoses plot



b. Intermitotic time plot

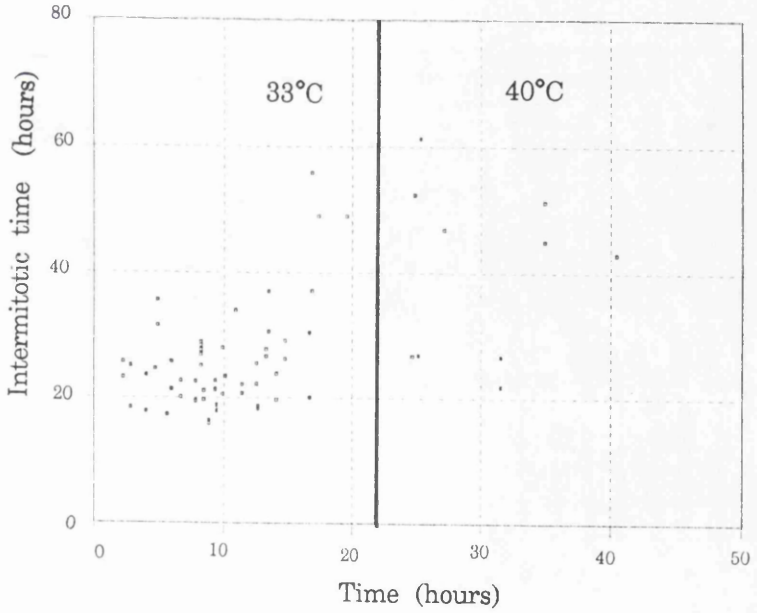
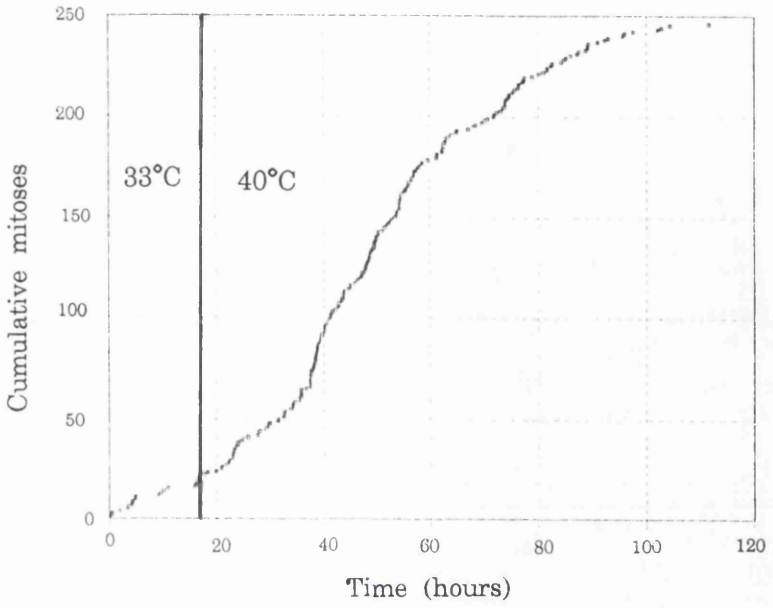


Figure 3.23 TIME-LAPSE PLOTS FOR UNSYNCHRONIZED K12 CELLS GROWN IN 5% FCS MCDB-302 CONTAINING 7 mM $NaHCO_3$

a. Cumulative mitoses plot



b. Intermitotic time plot

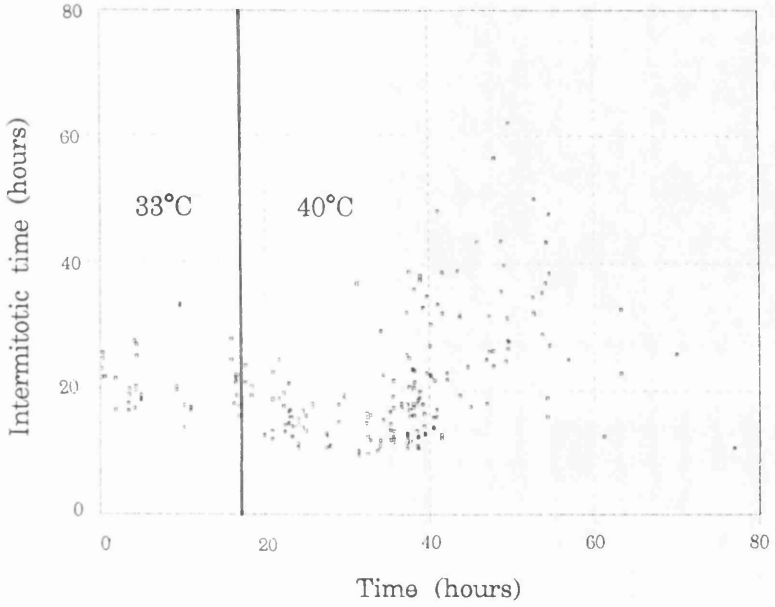
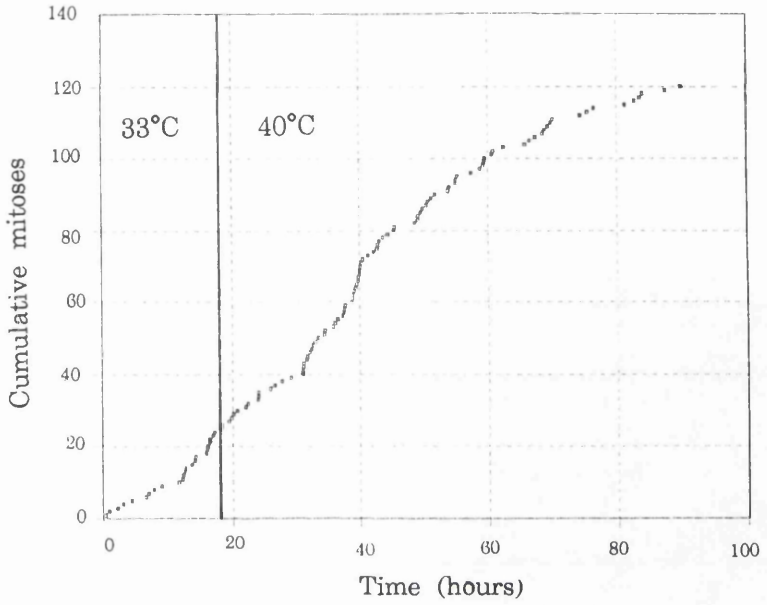


Figure 3.24 TIME-LAPSE PLOTS FOR UNSYNCHRONIZED K12 CELLS GROWN IN 5% FCS MCDB-302 CONTAINING 26 mM $NaHCO_3$

a. Cumulative mitoses plot



b. Intermitotic time plot

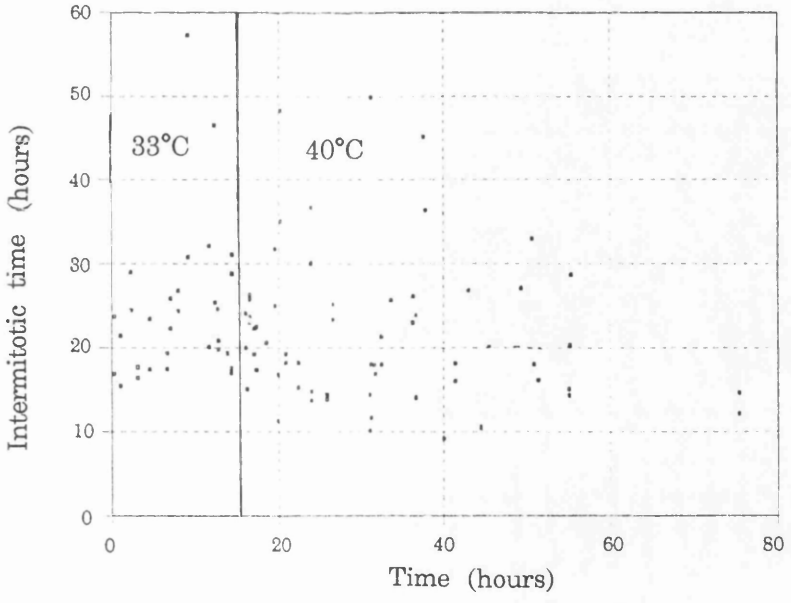
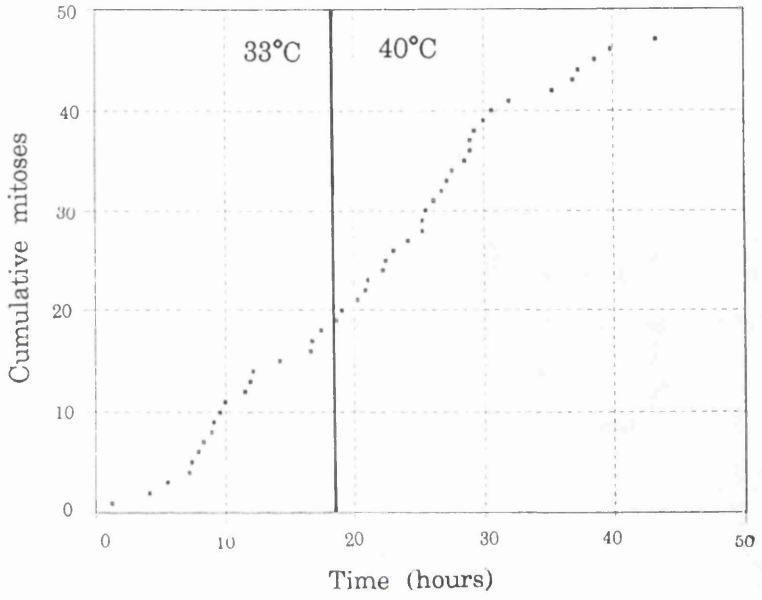


Figure 3.25 TIME-LAPSE PLOTS FOR UNSYNCHRONIZED K12 CELLS GROWN IN 5% FCS MCDB-302 CONTAINING 65 mM $NaHCO_3$

a. Cumulative mitoses plot



b. Intermitotic time plot

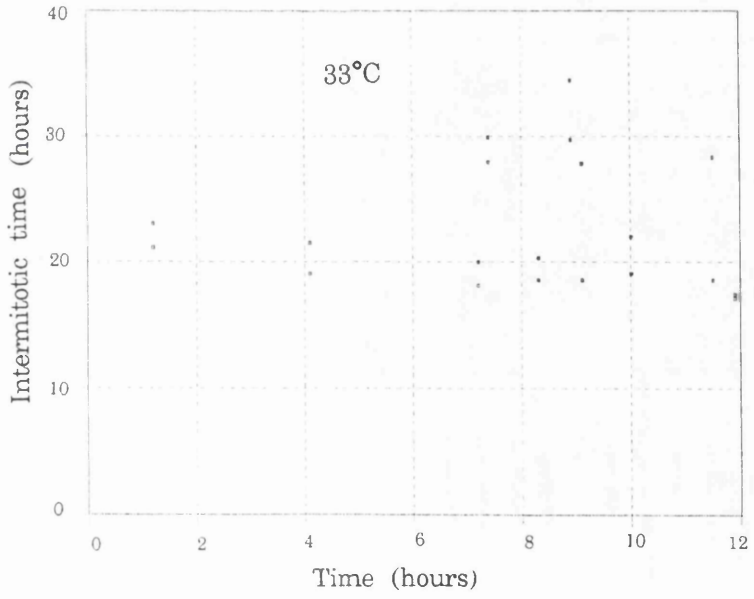


Table 3.61 TIME-LAPSE STUDIES ON UNSYNCHRONIZED K12 CELLS AT 40°C IN 5% FCS E4 WITH VARYING $NaHCO_3$ CONCENTRATIONS

[$NaHCO_3$] mM	TIME TAKEN TO HALT AT 40°C (hours)	AVERAGE INTERMITOTIC TIME [a] (hours)	STANDARD DEVIATION OF A.I.T.	MAXIMUM NUMBER OF DIVISIONS AT 40°C (hours)
13.3	78	16.3 (227)	7.63	4
26	58	21.0 (162)	7.05	3
44	44	27.8 (65)	10.48	2

[a] Number in brackets represents the number of cells used to calculate the average.

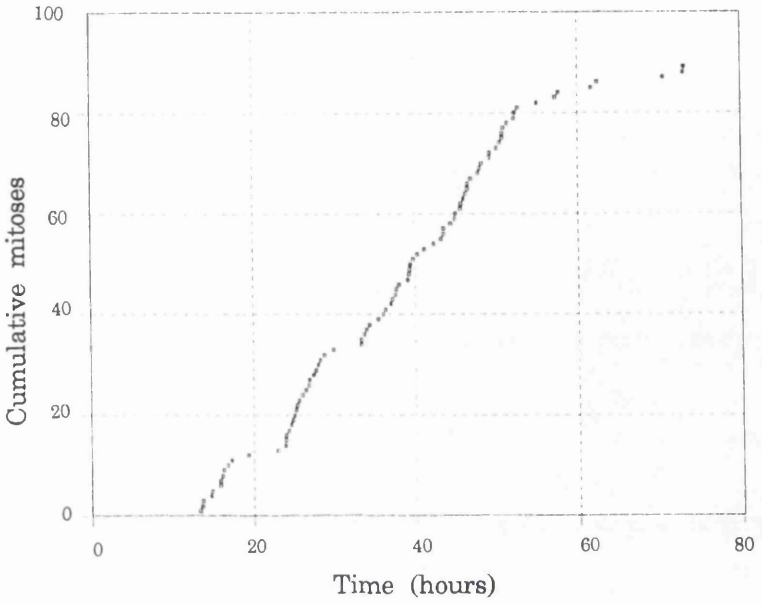
Table 3.62 TIME-LAPSE STUDIES ON UNSYNCHRONIZED K12 CELLS AT 40°C IN 5% FCS MCDB-302 WITH VARYING $NaHCO_3$ CONCENTRATIONS

[$NaHCO_3$] mM	TIME TAKEN TO HALT AT 40°C (hours)	AVERAGE INTERMITOTIC TIME [a] (hours)	STANDARD DEVIATION OF A.I.T.	MAXIMUM NUMBER OF DIVISIONS AT 40°C (hours)
7	94	20.8 (223)	9.18	5
26	71	22.5 (93)	8.89	4
65	25	22.6 (20)	5.13	1

[a] Number in brackets represents the number of cells used to calculate the average.

Figure 3.26 TIME-LAPSE PLOTS FOR MITOTICALLY-SYNCHRONIZED K12 GROWN IN 5% FCS E4 CONTAINING 13.3 mM $NaHCO_3$

a. Cumulative mitoses plot



b. Intermitotic time plot

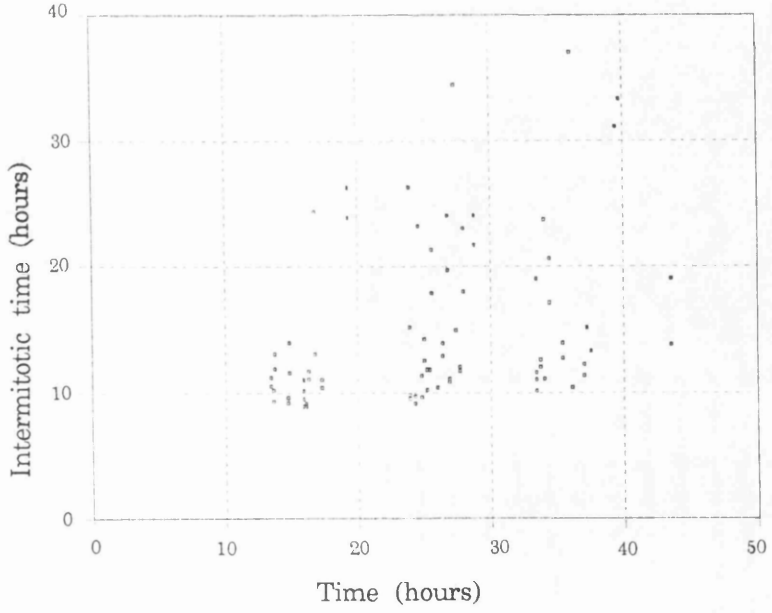
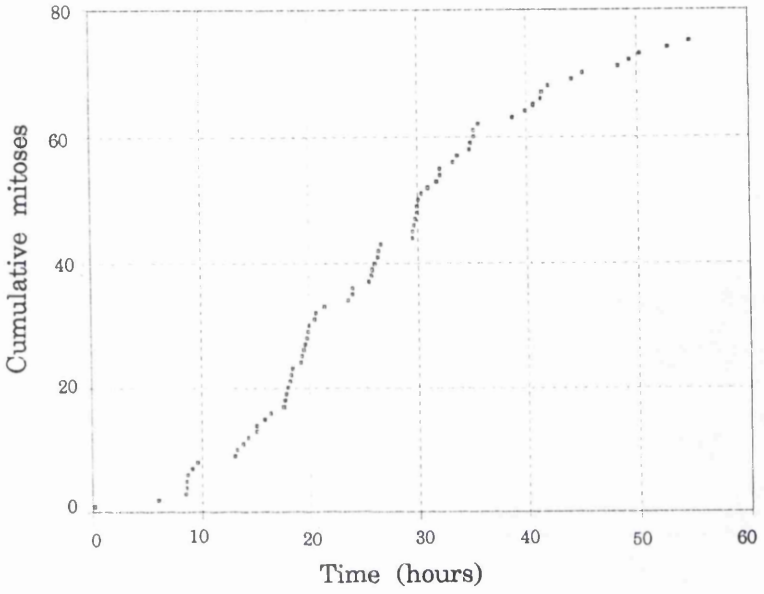


Figure 3.27 TIME-LAPSE PLOTS FOR MITOTICALLY-SYNCHRONIZED K12 CELLS GROWN IN 5% FCS E4 CONTAINING 26 mM $NaHCO_3$

a. Cumulative mitoses plot



b. Intermitotic time plot

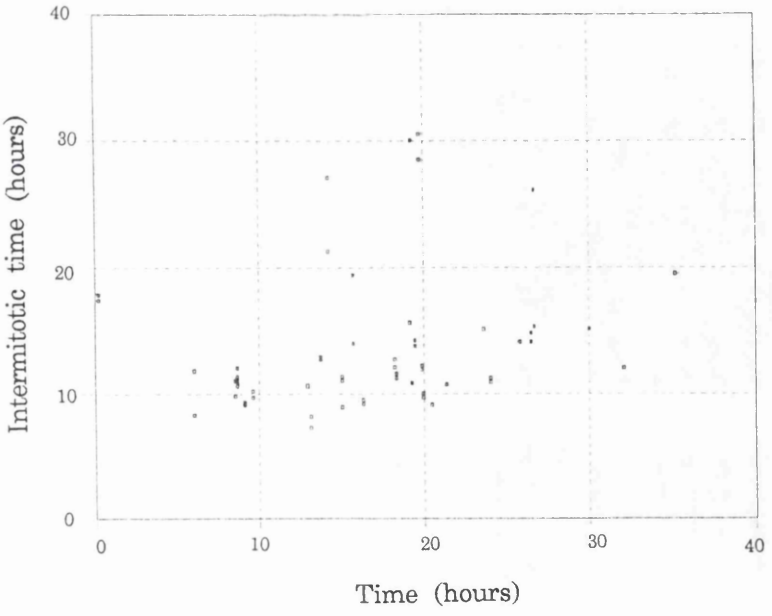
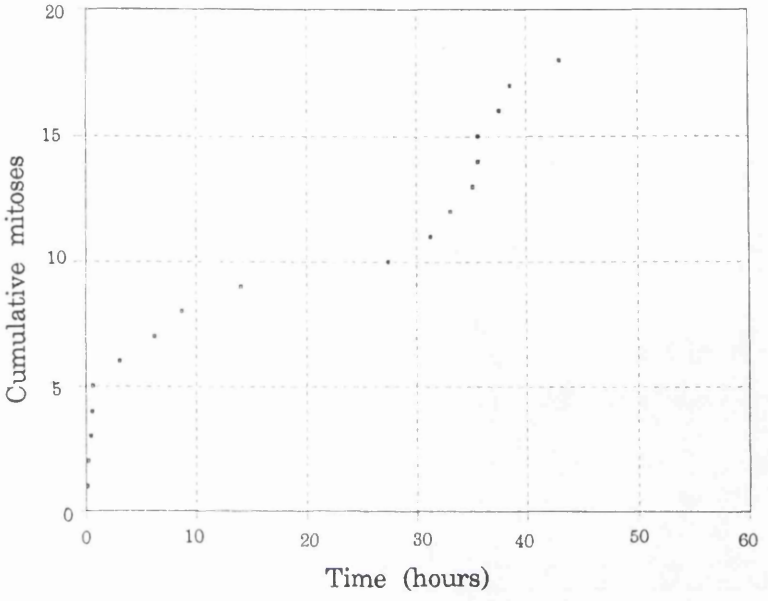


Figure 3.28 TIME-LAPSE PLOTS FOR MITOTICALLY-SYNCHRONIZED K12 CELLS GROWN IN 5% FCS E4 CONTAINING 44 mM $NaHCO_3$

a. Cumulative mitoses plot



b. Intermitotic time plot

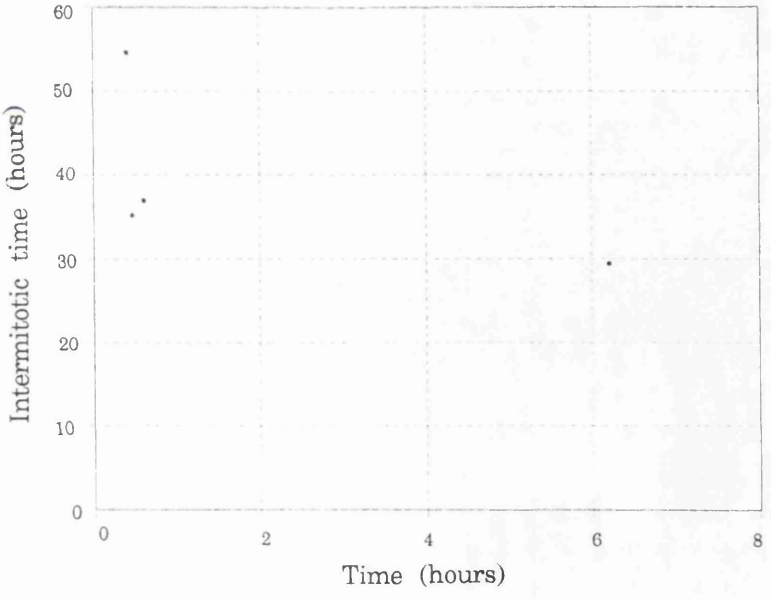
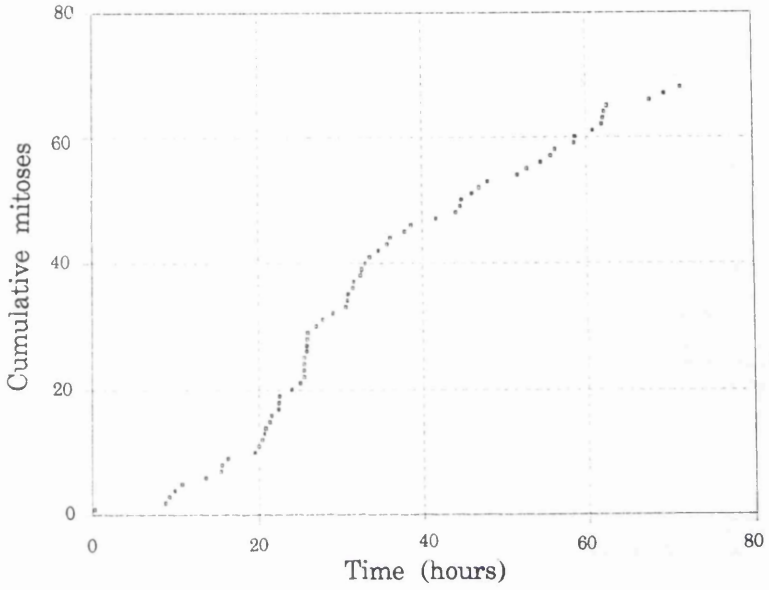


Figure 3.29 TIME-LAPSE PLOTS FOR MITOTICALLY-SYNCHRONIZED K12 CELLS GROWN IN 5% FCS MCDB-302 CONTAINING 7 mM $NaHCO_3$

a. Cumulative mitoses plot



b. Intermitotic time plot

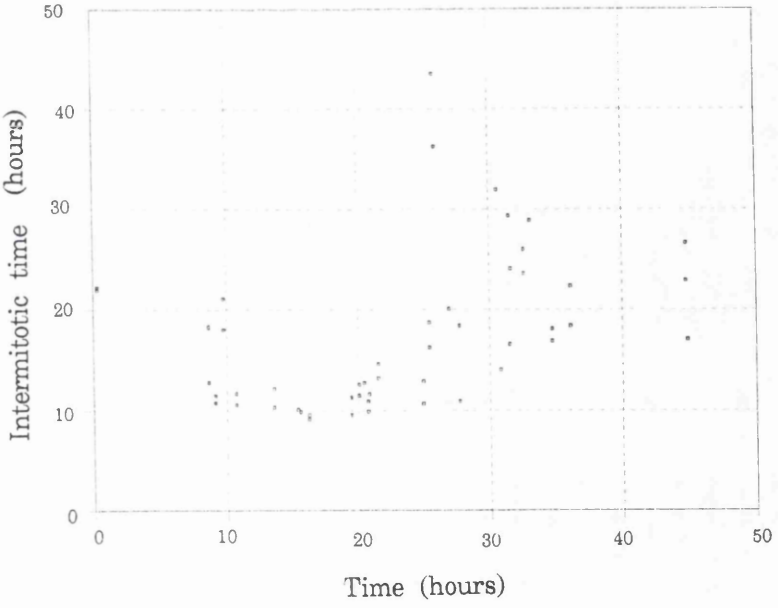
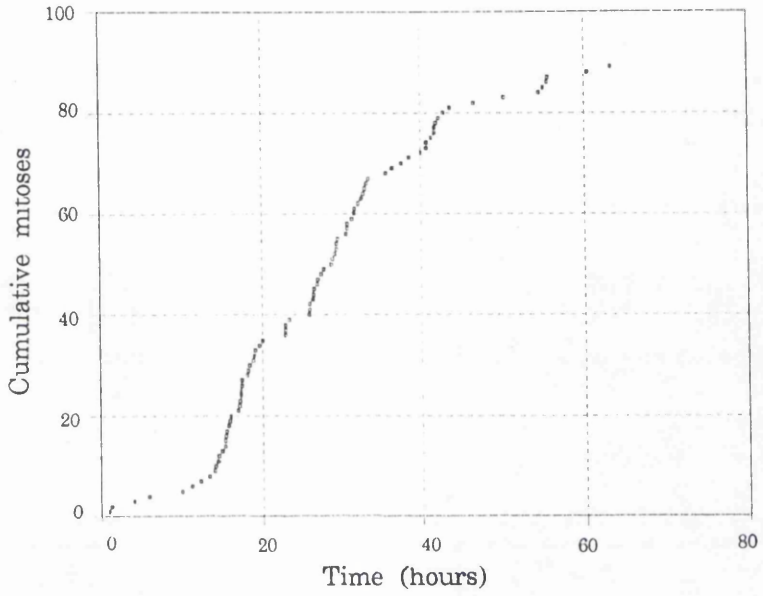


Figure 3.30 TIME-LAPSE PLOTS FOR MITOTICALLY-SYNCHRONIZED K12 CELLS GROWN IN 5% FCS MCDB-302 CONTAINING 26mM $NaHCO_3$

a. Cumulative mitoses plot



b. Intermitotic time plot

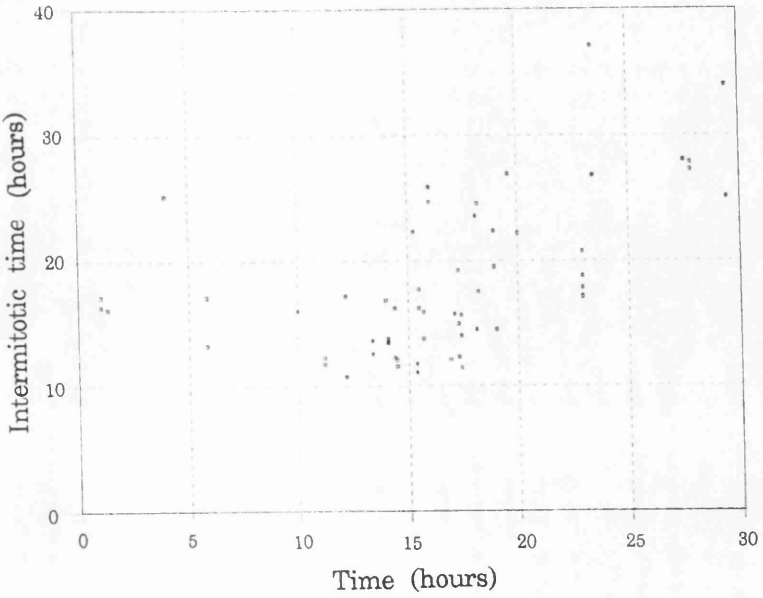


Table 3.63 TIME-LAPSE STUDIES ON MITOTICALLY-SYNCHRONIZED POPULATIONS OF K12 AT 40°C IN 5% FCS E4 WITH VARYING $NaHCO_3$ CONCENTRATIONS

$[NaHCO_3]$ mM	TIME TAKEN TO HALT AT 40°C (hours)	AVERAGE INTERMITOTIC TIME [a] (hours)	STANDARD DEVIATION OF A.I.T.	MAXIMUM NUMBER OF DIVISIONS AT 40°C [b] (hours)
13.3	73	15.0 (77)	6.54	5 (2-5)
26	55	13.7 (56)	5.52	4 (1-4)
44	43	39.0 (4)	10.57	2 (0-2)

[a] Number in brackets represents the number of cells used to calculate the average.
[b] Number in brackets represents the range of number of divisions.

Table 3.64 TIME-LAPSE STUDIES ON MITOTICALLY-SYNCHRONIZED POPULATIONS OF K12 AT 40°C IN 5% FCS MCDB-302 WITH VARYING $NaHCO_3$ CONCENTRATIONS

$[NaHCO_3]$ mM	TIME TAKEN TO HALT AT 40°C (hours)	AVERAGE INTERMITOTIC TIME [a] (hours)	STANDARD DEVIATION OF A.I.T.	MAXIMUM NUMBER OF DIVISIONS AT 40°C [b] (hours)
7	72	17.1 (51)	7.52	4 (1-4)
26	63	18.1 (58)	5.97	3 (1-3)
65	no divisions			0

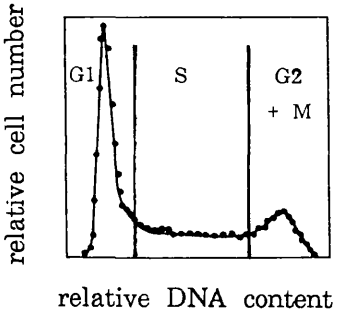
[a] Number in brackets represents the number of cells used to calculate the average.
[b] Number in brackets represents the range of number of divisions.

3.15 STUDYING THE POSITION OF THE CELL CYCLE BLOCK IN K12 AT 40°C IN MEDIA WITH VARYING SODIUM BICARBONATE CONCENTRATIONS

The time-lapse studies carried out in Section 3.14 demonstrate that the prolonged viability of K12 at 40°C in low bicarbonate media is due to the ability of the cell line to pass its execution point a number of times in these growth conditions, before finally halting. In order to confirm that the cells continued to block at the execution point of the mutation in medium containing reduced $NaHCO_3$ concentrations, the site of arrest was determined by flow cytometry studies.

Since replication of the genome is confined to S phase (Howard and Pelc, 1951), the DNA content of a cell is dependent on its position within the cell cycle; cells in G2 and M will contain twice as much DNA as those in G1, and S phase cells will have an amount intermediate between these two values. It thus follows that the position of a cell in the cycle can be determined by measurement of its DNA content. In flow cytofluorometry, cells are stained with a fluorescent DNA-specific dye, such as propidium iodide (Hudson *et al.*, 1969), which binds to cellular DNA and fluoresces in a quantitative manner i.e. the amount of emitted fluorescence is directly proportional to the amount of bound dye, and is thus an indirect measure of the quantity of DNA within the cell. The intensity of the emitted fluorescence is determined using a fluorescence-activated cell sorting machine, in which individual cells within a stained population are passed in single file across a laser beam of exciting light. As the cells pass through the beam, the emitted fluorescence is collected by a photomultiplier and passed for analysis to a computer, which plots the degree of fluorescence of each cell within the population i.e. the relative DNA content, against the relative cell number, thus providing a cell cycle profile of the population indicating the proportion of cells within each stage of the cycle (Fig. 3.31).

Figure 3.31 Generalized DNA content distribution of a hypothetical asynchronous exponentially growing cell population (adapted from Dean, 1990)



Initial flow cytofluorometry experiments on K12 cells grown at 40°C in media of varying $NaHCO_3$ levels used a staining method adapted from Crissman *et al.* (1975), where the cells were trypsinized, collected by centrifugation, fixed in cold 95% ethanol, incubated with RNase, and stained in 50 μg/ml propidium iodide in 1.12% sodium citrate. Cells were washed in PBSA and collected by centrifugation between each treatment. However, when K12 was stained according to this protocol, only 1 - 5% of the initial cell number was recovered at the end of the procedure. Numerous modifications of the method and testing of the cell number at each stage identified the centrifugation steps in PBSA as the point at which cells were lost, with only 10 - 20% of cells being recovered after a single spin of 2,000 rpm for 10 minutes in a Gallenkamp Labspin, compared with a 90 - 95% recovery value when cells were centrifuged in the growth medium. Increasing the speed or the length of centrifugation did not improve the efficiency of recovery, and similar results were obtained with three different batches of PBSA, including one made up from tablets (Sigma). The reason for this loss is unclear; although some cells were found in the supernatant after each spin, there was an insufficient number to account for the high loss levels. In addition, K12 suspensions allowed to stand for one hour at room temperature in PBSA showed no reduction in cell number, thus lysis of the cells in PBSA did not appear to offer a tenable explanation.

An alternative staining method (Section 2.10) was therefore investigated, in which centrifugation steps are avoided by staining *in situ*. The propidium iodide solution was added directly to the attached monolayer under hypotonic conditions, in order to lyse the cells and thus allow for the efficient staining of the isolated nuclei, which were then simply resuspended for immediate analysis. Flow cytofluorometry was carried out on K12 cells grown in the following media, each supplemented with 5% FCS: E4 containing 13.3, 26 or 44 mM $NaHCO_3$, and MCDB-302 supplemented with 7, 26 or 65 mM $NaHCO_3$. For each medium type, two different lengths of incubation at 40°C were chosen, by reference to the time-lapse cumulative mitoses plots (Figs. 3.20a - 3.25a), such that at the time of analysis the majority of cells would be expected to have arrested at the execution point, but loss of fibroblasts from the plate as a consequence of cell death would not yet have begun to occur. Cells from corresponding control plates were stained and analysed after identical incubation periods at 33°C, and duplicate plates were studied for each time point. Twin plates for each medium type were also serum-starved for 48 hours at 33°C, according to the protocol outlined by Ashihara *et al.* (1978), in order to provide controls showing the DNA distribution pattern expected in a G1-arrested population. Cells for serum starvation were plated in the growth medium supplemented with 5% FCS, and incubated overnight at 33°C. The following day, they were washed with 5 ml of the

appropriate medium containing 0.5% FCS, refed with 10 ml of 0.5% FCS-supplemented growth medium, and returned to a 33°C incubator for a further 48 hours before staining. An additional control was provided by the analysis of Wg-1A cells grown in 5% FCS E4 low bic, and unstained cells were also included for each medium type, in order to determine the degree of cellular autofluorescence.

Data processed by the Becton-Dickinson Facscan was displayed as a cell cycle profile, where the relative degree of fluorescence (i.e. DNA content) was plotted against the relative cell number (Figs. 3.32 - 3.38). Gates were set at fluorescence levels corresponding to the beginning of the G1 peak, at the end of this peak, and after the peak consisting of S, G2 and M phase cells (see Figs. 3.32 - 3.38). These were used by the Consort 30 computer program to calculate the proportion of cells in the total population which were in G1 at the time of analysis, and also the percentage which fell within the rest of the cell cycle, and the results from duplicate plates were averaged and tabulated (Tables 3.65 - 3.71). In each cell population measured, a peak of fluorescence appeared at the far left-hand end of the profile, indicating the presence of extremely small stained components; it is possible that this may represent the binding of propidium iodide to mitochondrial DNA or, alternatively, fragments of cellular debris may have become non-specifically stained. The height of this peak varied widely between samples, but appeared to be similar in populations prepared from duplicate plates. However, the peak height did not appear to be correlated with either the incubation temperature, the $NaHCO_3$ concentration of the medium or the degree of G1 arrest, thus the cause of the variation in its size is unclear.

For each medium type, an unstained sample of cells was processed by the Facscan, and neither K12 or Wg-1A was found to exhibit any background autofluorescence (e.g. see Fig. 3.33l). The stability of the dye signal was also tested by remeasuring the first cell population to be studied at the end of each analysis session (generally within 3 hours of staining). The two cell cycle profiles consistently showed a high degree of similarity (e.g. see Fig. 3.33k), indicating that the fluorescence signal was not quenched during the time period of measurement.

When K12 cells were grown at 33°C in 5% FCS E4 or 5% FCS MCDB-302 supplemented with 26 mM $NaHCO_3$, the percentage of the population in the G1 phase of the cell cycle ranged from 46 - 51% (Tables 3.66 and 3.69). This value increased when the $NaHCO_3$ concentration of each medium was raised or lowered, implying perhaps that high or low bicarbonate media provide suboptimal growth conditions in which the time taken for K12 cells to traverse G1 is lengthened or, alternatively, a proportion of the cells may arrest in G0. However, evidence from the viability curve (Section 3.7) and time-lapse (Section 3.14) studies implies that the

growth of K12, in terms of E.O.P., colony size and average intermitotic time, does not appear to be significantly inhibited in any of these media at either 33° or 40°C, suggesting that although alterations in $NaHCO_3$ level may have some slight effects on the general growth characteristics of this cell line at 33°C, this is unlikely to account for the observed influence of the bicarbonate concentration on the rate of loss of viability of K12 at the npt. Populations of K12 cultured in 13.3 mM $NaHCO_3$ E4 showed a particularly high percentage of cells in G1 (75 - 80%) at 33°C (Table 3.65), and this value was not further increased by incubation at 40°C, implying that a large proportion of cells were arrested in G1 even at the pt. How this can be reconciled with the viability curve data showing good growth of K12 cells in this medium at 33°C (Table 3.14), and the short average intermitotic time (15 - 16 hours) and large number of cell divisions (2 - 5) at the npt demonstrated in time-lapse studies (Tables 3.61 and 3.63) is unclear. It is possible, perhaps, that a miscalculation in the preparation of the cell suspension led to an excess of cells being plated, which subsequently became confluent and had entered G0 by the time of analysis.

When K12 cells were grown in either 7 mM $NaHCO_3$ MCDB-302 or 13.3 mM $NaHCO_3$ E4, a noticeable trailing of the signal on the left-hand side of the G1 peak under all incubation conditions tested appeared to indicate the presence of a number of undersized nuclei in the population. This phenomenon did not occur when K12 cells were cultured in media with higher bicarbonate levels, and may be related to the observation noted during the time-lapse studies (Section 3.14), that when low bicarbonate media were used for growth of this cell line, a number of mitotic cells divided into three abnormally small daughters. These cells would presumably show a low relative DNA content, since it seems likely that under these circumstances the proper partitioning of the genetic material would have broken down, thus nuclei from such cells would be expected to give a signal to the left of the G1 peak on a cell cycle profile. This effect was particularly pronounced in the case of 7 mM $NaHCO_3$ MCDB-302, and indeed the largest number of such abnormal divisions was observed during time-lapse studies carried out with this medium.

As expected, serum starvation of K12 for 48 hours at 33°C in each medium type caused the proportion of cells in G1 to increase significantly, presumably because a large fraction of the population entered G0 under such suboptimal conditions (Pardee, 1974). Once again, the percentage of the population within G1 was highest when K12 cells were cultured in high or low bicarbonate media rather than in 26 mM $NaHCO_3$ MCDB-302 or E4, perhaps due to the underlying differences in cell cycle distribution observed at 33°C in 5% FCS-supplemented media with varying bicarbonate levels. Arrest of the population was not complete in any medium. This

was not entirely unexpected, since both K12 and Wg-1A are capable of anchorage-independent growth (Roscoe *et al.*, 1973a), a characteristic of transformed cells (MacPherson and Montagnier, 1964), and such cells show a reduced ability to halt in G1 in response to serum starvation (Stoker, 1972), presumably due to a lower serum requirement for growth. Serum starvation appeared to be even less effective at halting Wg-1A in G1; the proportion of Wg-1A cells in G1 increased by only 5 - 10% when the serum concentration of the medium was reduced, even after 72 hours of serum starvation. Wg-1A thus seems to be able to cope better with low serum concentrations than K12, providing further evidence that K12 cells do not show fully wild-type patterns of behaviour at the pt.

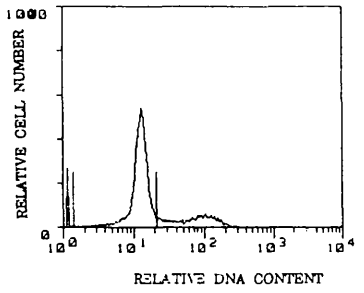
The incubation of K12 cells at 40°C caused the accumulation of the population within G1 in each medium type tested, suggesting that the cells continue to arrest in G1, presumably at the execution point of the mutation, under such conditions. In contrast, the percentage of Wg-1A cells within G1 showed no significant change when the temperature was raised, thus confirming that the observed G1 arrest in K12 was caused by the specific defect in the K12 ts protein. The percentage of K12 cells in G1 at the npt did not exceed 87% in any medium, presumably since the time points for analysis were carefully chosen such that the population was harvested before death had begun, at which stage a significant fraction of the cells may still have been cycling normally.

When K12 cells were grown in 44 mM $NaHCO_3$ E4 for 24 hours at 40°C, the proportion of the population within G1 was 84.2% (Table 3.67). This figure agrees closely with that obtained by Melero (1979), who carried out flow cytofluorometry on K12 cultured in the same medium for 24 hours at the npt, with mithramycin as the fluorescent dye, and obtained a G1 percentage of 82.0%. This suggests that the properties of K12 at 40°C with regard to the rate of G1 arrest may not have changed appreciably since this time.

In conclusion, the flow cytofluorometry data from these experiments clearly demonstrate that although the manipulation of the medium $NaHCO_3$ level appears to alter the rate of loss of viability of K12 at the npt, the cells ultimately arrested in G1 in each of the medium types tested, thus supporting the idea that low bicarbonate concentrations act to prolong the survival of K12 at 40°C by simply slowing down the loss of activity of the ts protein for sufficient time to allow for a few extra divisions, but that they cannot prevent the eventual halt of the cell at the execution point.

Figure 3.32 CELL CYCLE DISTRIBUTION OF K12 GROWN IN 5% FCS E4 (13.3 mM $NaHCO_3$)

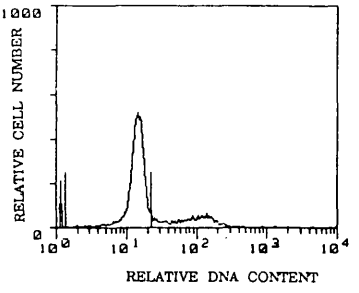
a. 33°C for 40 hours



% cells in G1 = 75.8

% cells in S, G2 and M = 24.2

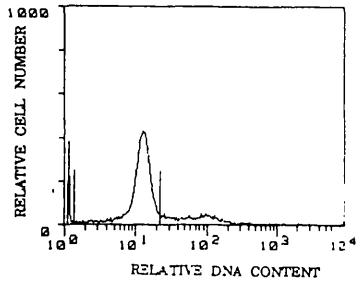
b. 33°C for 40 hours



% cells in G1 = 74.5

% cells in S, G2 and M = 25.5

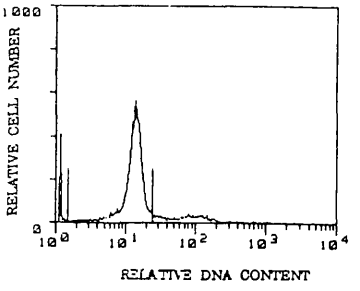
c. 33°C for 64 hours



% cells in G1 = 76.3

% cells in S, G2 and M = 23.7

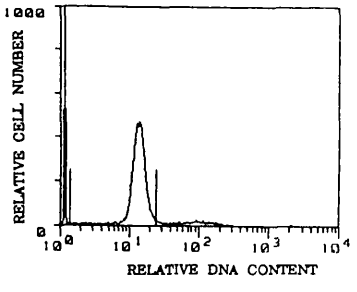
d. 33°C for 64 hours



% cells in G1 = 83.1

% cells in S, G2 and M = 16.9

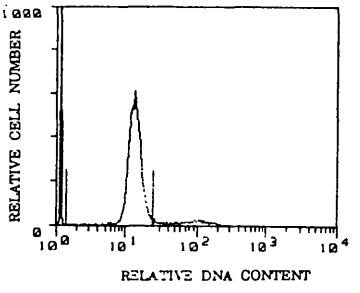
e. 33°C serum starved for 48 hours



% cells in G1 = 87.4

% cells in S, G2 and M = 12.6

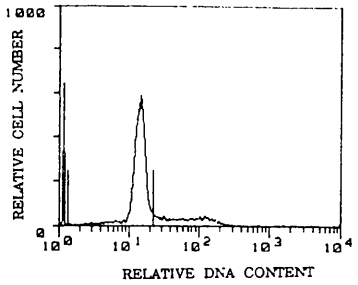
f. 33°C serum starved for 48 hours



% cells in G1 = 88.2

% cells in S, G2 and M = 11.8

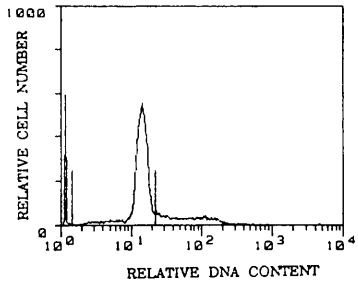
g. 40°C for 40 hours



% cells in G1 = 76.0

% cells in S, G2 and M = 24.0

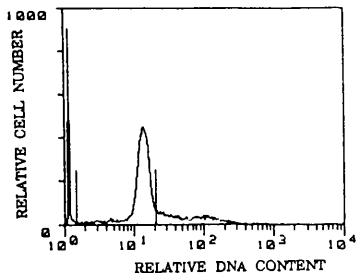
h. 40°C for 40 hours



% cells in G1 = 76.7

% cells in S, G2 and M = 23.3

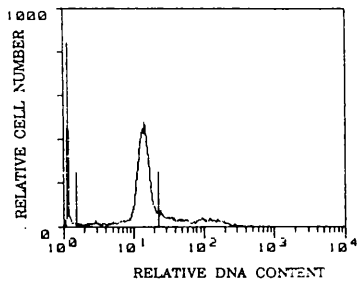
i. 40°C for 64 hours



% cells in G1 = 73.6

% cells in S, G2 and M = 26.4

j. 40°C for 64 hours

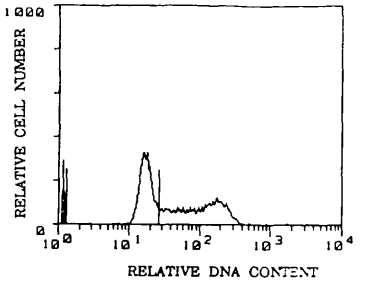


% cells in G1 = 76.4

% cells in S, G2 and M = 23.6

Figure 3.33 CELL CYCLE DISTRIBUTION OF K12 GROWN IN 5% FCS E4 (26 mM $NaHCO_3$)

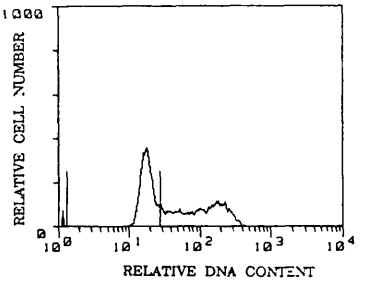
a. 33°C for 24 hours



% cells in G1 = 45.7

% cells in S, G2 and M = 54.3

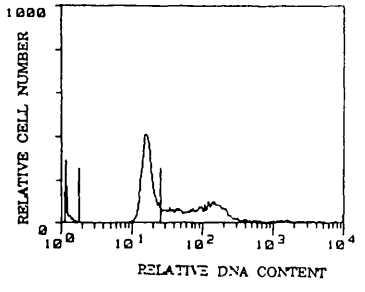
b. 33°C for 24 hours



% cells in G1 = 46.1

% cells in S, G2 and M = 53.9

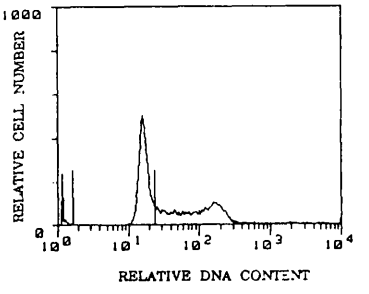
c. 33°C for 40 hours



% cells in G1 = 51.4

% cells in S, G2 and M = 48.6

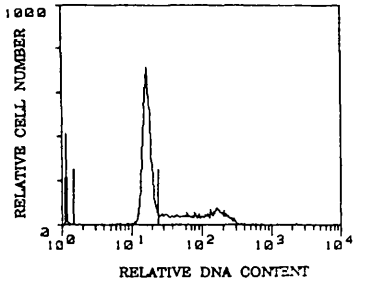
d. 33°C for 40 hours



% cells in G1 = 51.0

% cells in S, G2 and M = 49.0

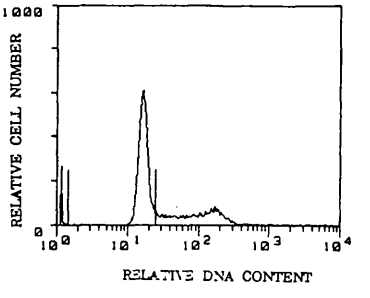
e. 33°C serum starved for 48 hours



% cells in G1 = 68.3

% cells in S, G2 and M = 31.7

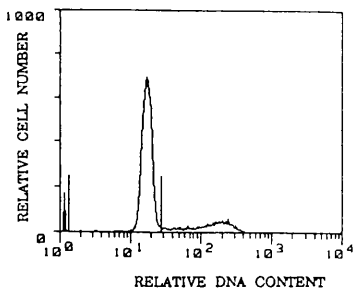
f. 33°C serum starved for 48 hours



% cells in G1 = 63.9

% cells in S, G2 and M = 36.1

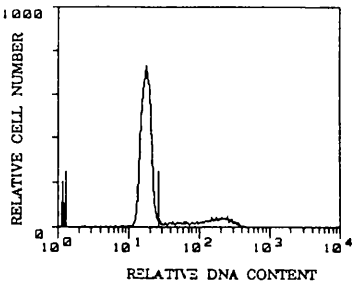
g. 40°C for 24 hours



% cells in G1 = 80.1

% cells in S, G2 and M = 19.9

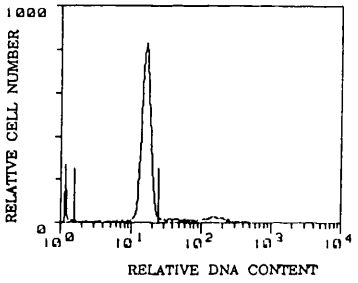
h. 40°C for 24 hours



% cells in G1 = 81.9

% cells in S, G2 and M = 18.1

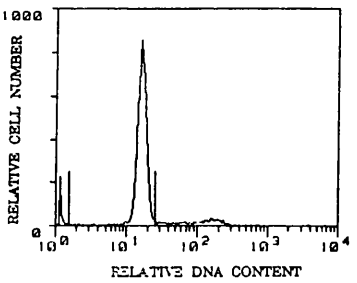
i. 40°C for 40 hours



% cells in G1 = 87.0

% cells in S, G2 and M = 13.0

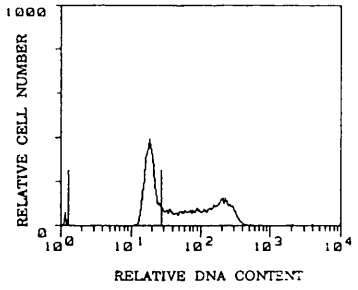
j. 40°C for 40 hours



% cells in G1 = 86.2

% cells in S, G2 and M = 13.8

k. Repeat of 33°C for 24 hours (a) 2 hours after first analysis



% cells in G1 = 45.0

% cells in S, G2 and M = 55.0

l. 33°C for 24 hours unstained

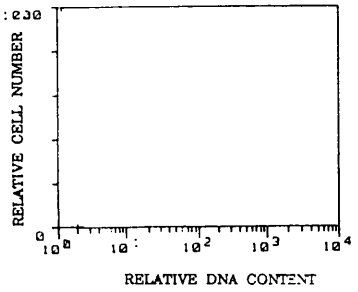
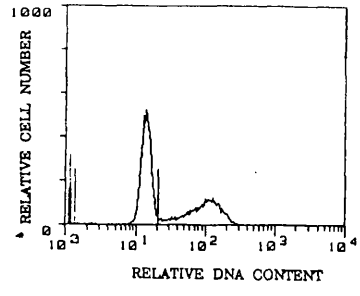


Figure 3.34 CELL CYCLE DISTRIBUTION OF K12 GROWN IN 5% FCS E4 (44 mM $NaHCO_3$)

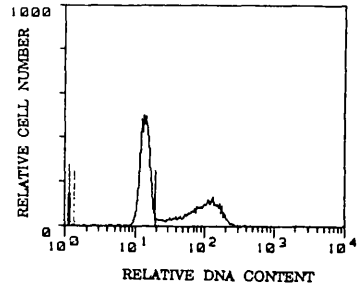
a. 33°C for 16 hours



% cells in G1 = 57.9

% cells in S, G2 and M = 42.1

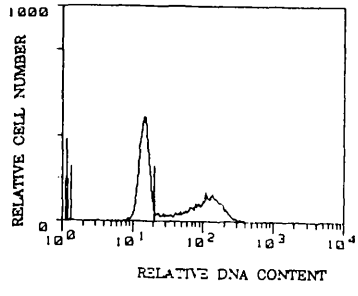
b. 33°C for 16 hours



% cells in G1 = 55.4

% cells in S, G2 and M = 44.6

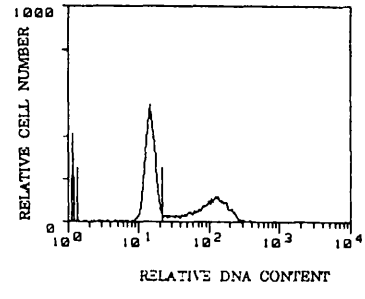
c. 33°C for 24 hours



% cells in G1 = 57.0

% cells in S, G2 and M = 43.0

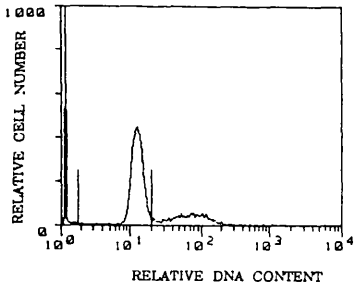
d. 33°C for 24 hours



% cells in G1 = 58.8

% cells in S, G2 and M = 41.2

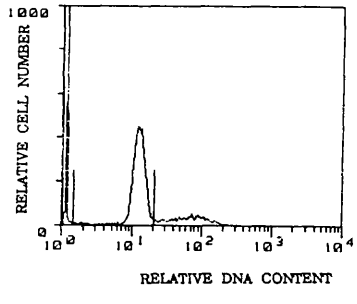
e. 33°C serum starved for 48 hours



% cells in G1 = 76.8

% cells in S, G2 and M = 23.2

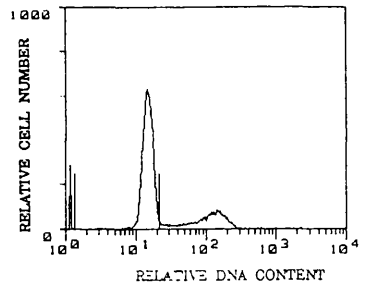
f. 33°C serum starved for 48 hours



% cells in G1 = 71.3

% cells in S, G2 and M = 28.7

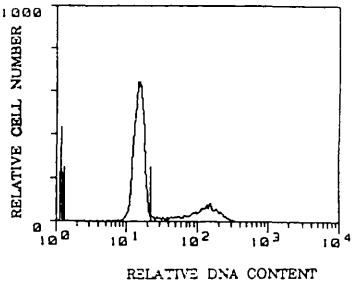
g. 40°C for 16 hours



% cells in G1 = 72.9

% cells in S, G2 and M = 27.1

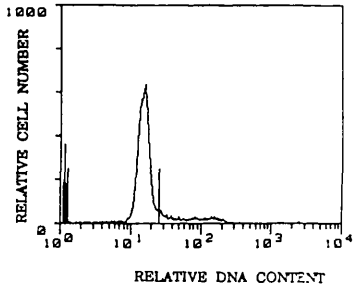
h. 40°C for 16 hours



% cells in G1 = 75.2

% cells in S, G2 and M = 24.8

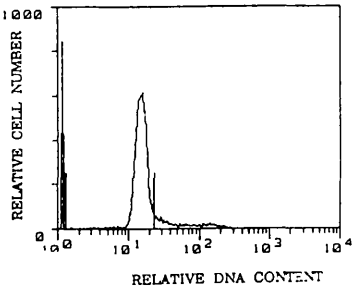
i. 40°C for 24 hours



% cells in G1 = 84.0

% cells in S, G2 and M = 16.0

j. 40°C for 24 hours

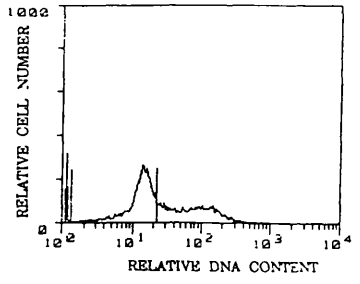


% cells in G1 = 84.4

% cells in S, G2 and M = 15.6

Figure 3.35 CELL CYCLE DISTRIBUTION OF K12 GROWN IN 5% FCS MCDB-302 (7 mM $NaHCO_3$)

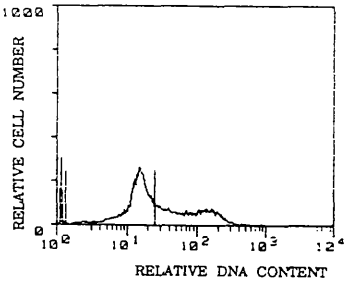
a. 33°C for 40 hours



% cells in G1 = 54.9

% cells in S, G2 and M = 45.1

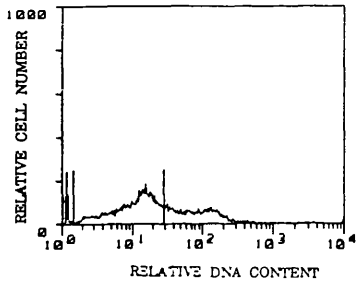
b. 33°C for 40 hours



% cells in G1 = 55.1

% cells in S, G2 and M = 44.9

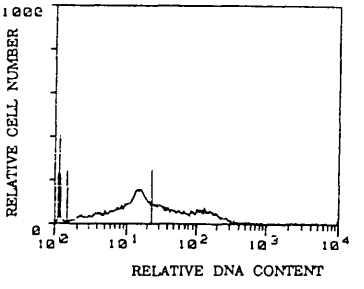
c. 33°C for 64 hours



% cells in G1 = 61.1

% cells in S, G2 and M = 38.9

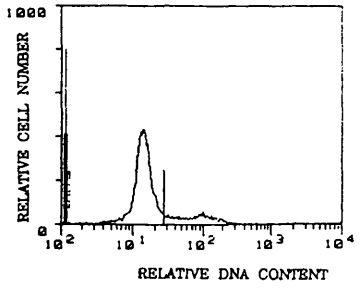
d. 33°C for 64 hours



% cells in G1 = 54.0

% cells in S, G2 and M = 46.0

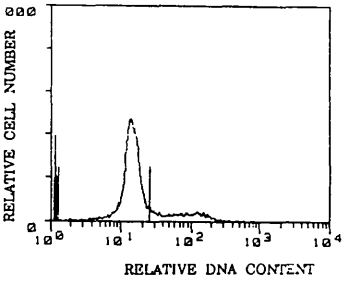
e. 33°C serum starved for 48 hours



% cells in G1 = 76.9

% cells in S, G2 and M = 23.1

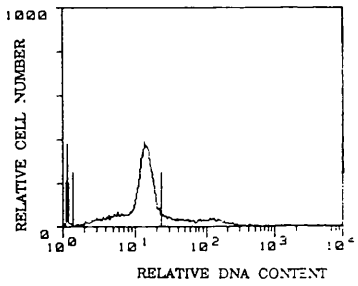
f. 33°C serum starved for 48 hours



% cells in G1 = 76.9

% cells in S, G2 and M = 23.1

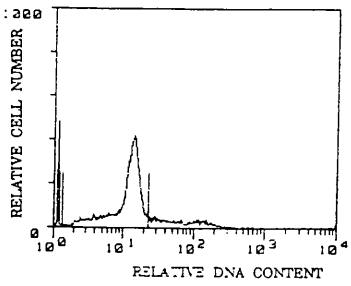
g. 40°C for 40 hours



% cells in G1 = 76.4

% cells in S, G2 and M = 23.6

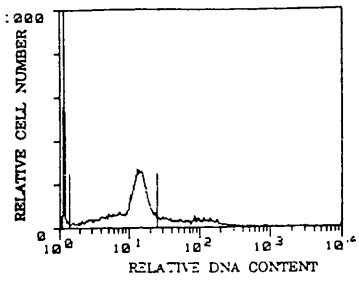
h. 40°C for 40 hours



% cells in G1 = 77.7

% cells in S, G2 and M = 22.3

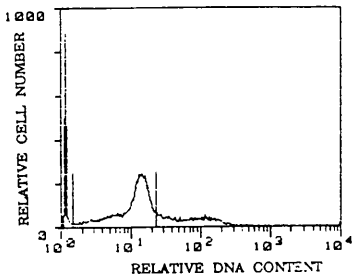
i. 40°C for 64 hours



% cells in G1 = 73.9

% cells in S, G2 and M = 26.1

j. 40°C for 64 hours

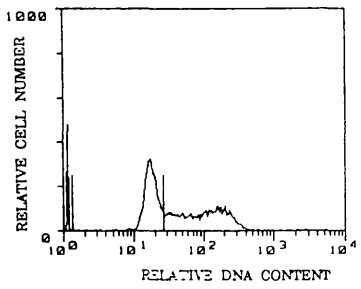


% cells in G1 = 69.3

% cells in S, G2 and M = 30.7

Figure 3.36 CELL CYCLE DISTRIBUTION OF K12 GROWN IN 5% FCS MCDB-302 (26 mM $NaHCO_3$)

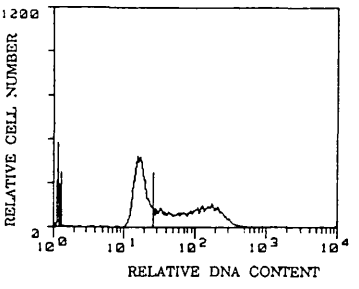
a. 33°C for 24 hours



% cells in G1 = 45.5

% cells in S, G2 and M = 54.5

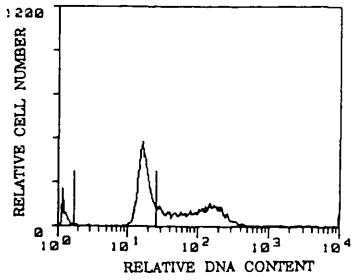
b. 33°C for 24 hours



% cells in G1 = 46.6

% cells in S, G2 and M = 53.4

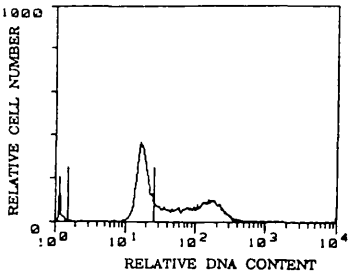
c. 33°C for 40 hours



% cells in G1 = 46.9

% cells in S, G2 and M = 53.1

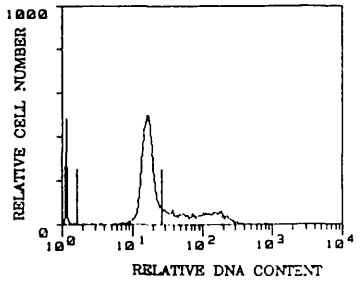
d. 38°C for 40 hours



% cells in G1 = 48.3

% cells in S, G2 and M = 51.7

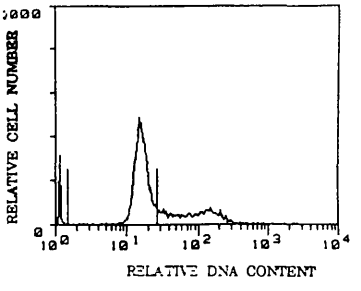
e. 33°C serum starved for 48 hours



% cells in G1 = 68.9

% cells in S, G2 and M = 31.1

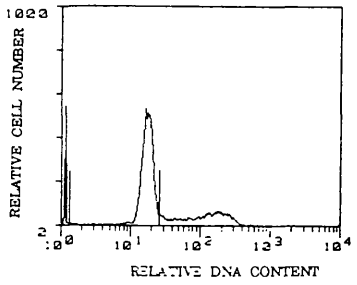
f. 33°C serum starved for 48 hours



% cells in G1 = 65.7

% cells in S, G2 and M = 34.3

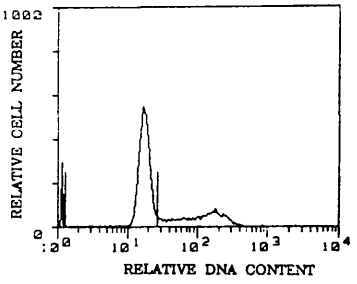
g. 40°C for 24 hours



% cells in G1 = 67.8

% cells in S, G2 and M = 32.2

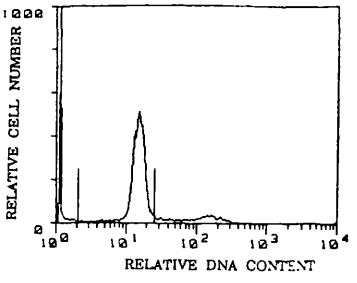
h. 40°C for 24 hours



% cells in G1 = 68.0

% cells in S, G2 and M = 32.0

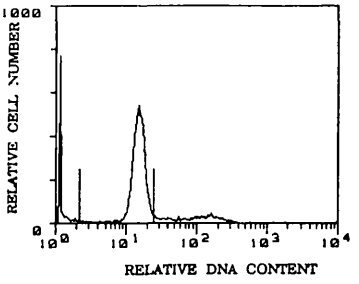
i. 40°C for 40 hours



% cells in G1 = 82.0

% cells in S, G2 and M = 18.0

j. 40°C for 40 hours

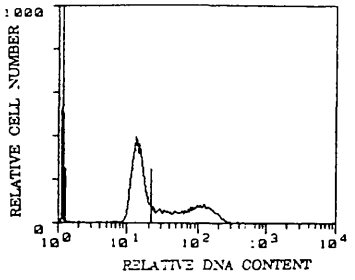


% cells in G1 = 81.7

% cells in S, G2 and M = 18.3

Figure 3.37 CELL CYCLE DISTRIBUTION OF K12 GROWN IN 5% FCS MCDB-302 (65 mM $NaHCO_3$)

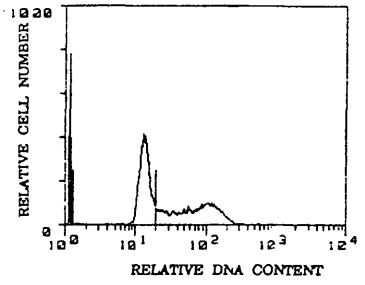
a. 33°C for 16 hours



% cells in G1 = 55.0

% cells in S, G2 and M = 45.0

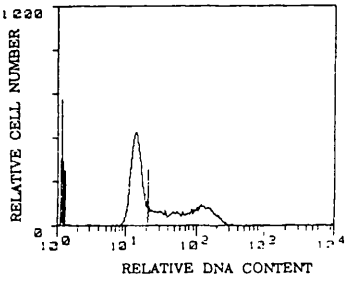
b. 33°C for 16 hours



% cells in G1 = 51.3

% cells in S, G2 and M = 48.7

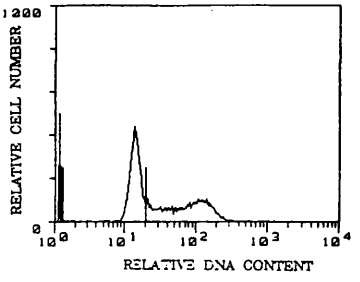
c. 33°C for 24 hours



% cells in G1 = 53.5

% cells in S, G2 and M = 46.5

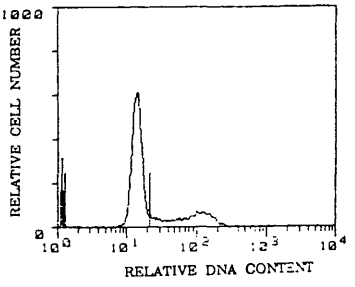
d. 33°C for 24 hours



% cells in G1 = 49.4

% cells in S, G2 and M = 50.6

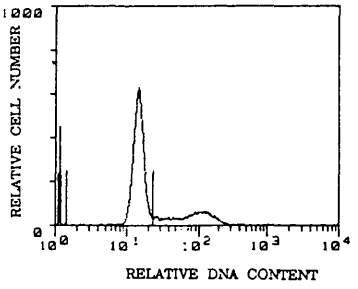
e. 33°C serum starved for 48 hours



% cells in G1 = 70.9

% cells in S, G2 and M = 29.1

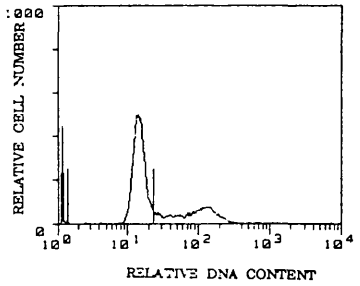
f. 33°C serum starved for 48 hours



% cells in G1 = 70.9

% cells in S, G2 and M = 29.1

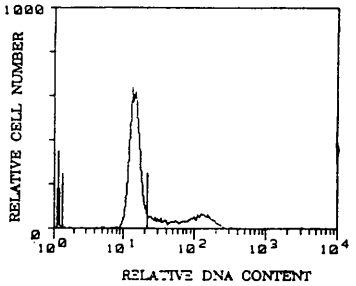
g. 40°C for 16 hours



% cells in G1 = 66.6

% cells in S, G2 and M = 33.4

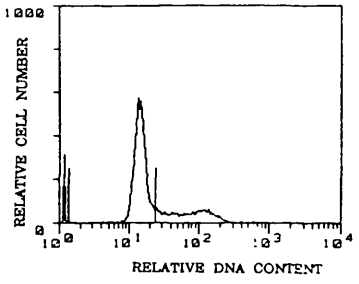
h. 40°C for 16 hours



% cells in G1 = 72.3

% cells in S, G2 and M = 27.7

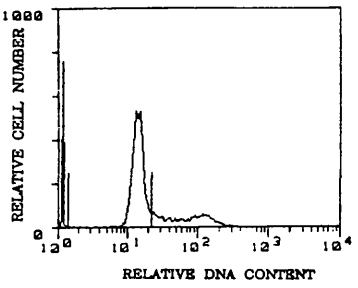
i. 40°C for 24 hours



% cells in G1 = 70.6

% cells in S, G2 and M = 29.4

j. 40°C for 24 hours

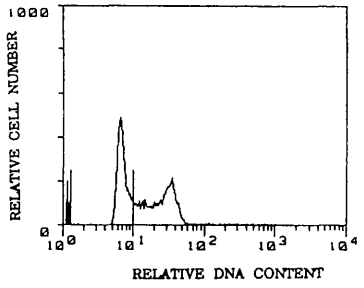


% cells in G1 = 71.0

% cells in S, G2 and M = 29.0

Figure 3.38 CELL CYCLE DISTRIBUTION OF Wg-1A GROWN IN 5% FCS E4 LOW BIC

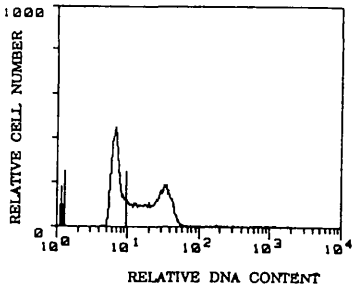
a. 33°C for 24 hours



% cells in G1 = 46.3

% cells in S, G2 and M = 53.7

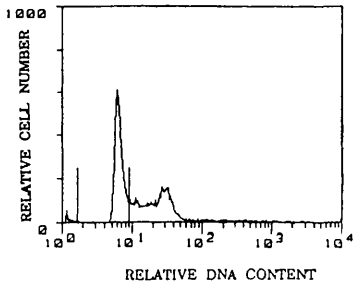
b. 33°C for 24 hours



% cells in G1 = 43.1

% cells in S, G2 and M = 56.9

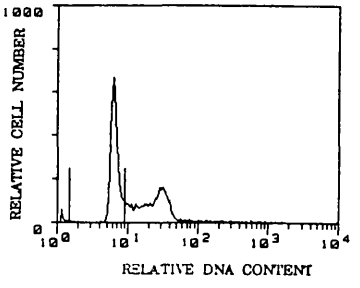
c. 33°C for 40 hours



% cells in G1 = 50.4

% cells in S, G2 and M = 49.6

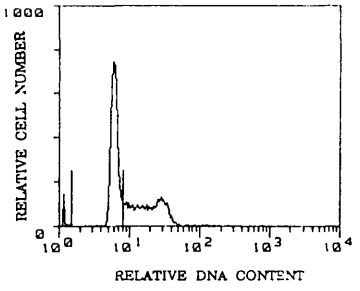
d. 33°C for 40 hours



% cells in G1 = 51.6

% cells in S, G2 and M = 48.4

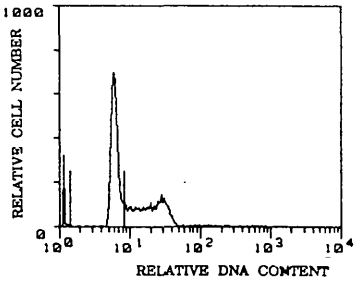
e. 33°C serum starved for 48 hours



% cells in G1 = 56.3

% cells in S, G2 and M = 43.7

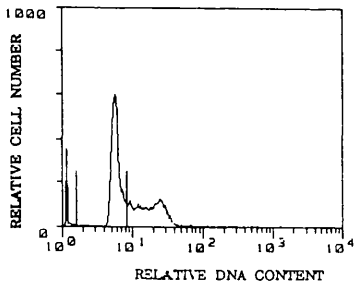
f. 33°C serum starved for 48 hours



% cells in G1 = 55.2

% cells in S, G2 and M = 44.8

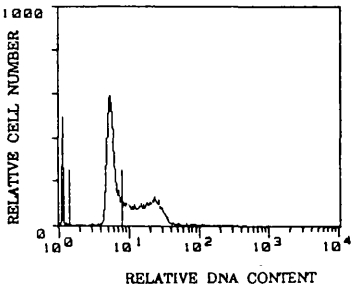
g. 33°C serum starved for 72 hours



% cells in G1 = 56.5

% cells in S, G2 and M = 43.5

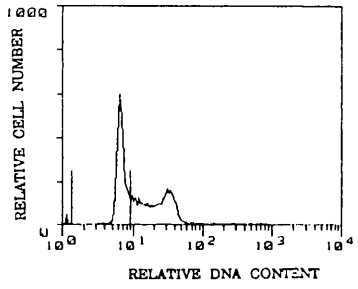
h. 33°C serum starved for 72 hours



% cells in G1 = 53.9

% cells in S, G2 and M = 46.1

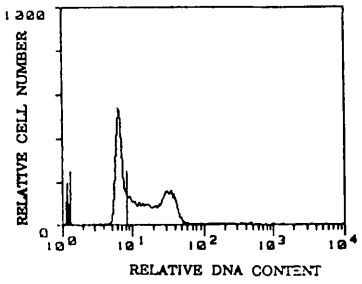
i. 40°C for 24 hours



% cells in G1 = 47.1

% cells in S, G2 and M = 52.9

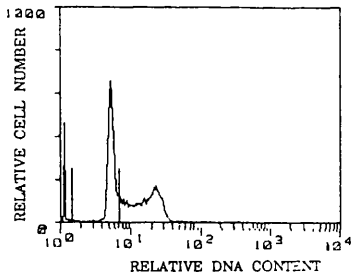
j. 40°C for 24 hours



% cells in G1 = 42.0

% cells in S, G2 and M = 58.0

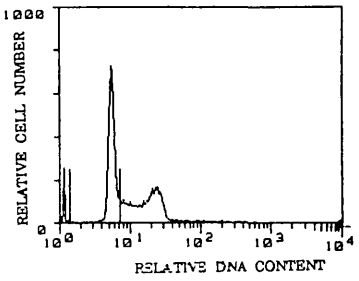
k. 40°C for 40 hours



% cells in G1 = 49.4

% cells in S, G2 and M = 50.6

l. 40°C for 40 hours



% cells in G1 = 50.6

% cells in S, G2 and M = 49.4

Table 3.65 CELL CYCLE DISTRIBUTION OF K12 GROWN IN 5% FCS E4 (13.3 mM $NaHCO_3$)

TEMPERATURE	HOURS OF INCUBATION AT REQUIRED TEMPERATURE	AVERAGE % OF CELLS IN G1	AVERAGE % OF CELLS IN S, G2 AND M
33°C	40	75.2	24.9
	64	79.7	20.3
40°C	40	76.4	23.7
	64	75.0	25.0
33°C serum-starved	48	87.8	12.2

Table 3.66 CELL CYCLE DISTRIBUTION OF K12 GROWN IN 5% FCS E4 (26 mM $NaHCO_3$)

TEMPERATURE	HOURS OF INCUBATION AT REQUIRED TEMPERATURE	AVERAGE % OF CELLS IN G1	AVERAGE % OF CELLS IN S, G2 AND M
33°C	24	45.9	54.1
	40	51.2	48.8
40°C	24	81.0	19.0
	40	86.6	13.4
33°C serum-starved	48	66.1	33.9

Table 3.67 CELL CYCLE DISTRIBUTION OF K12 GROWN IN 5% FCS E4 (44 mM $NaHCO_3$)

TEMPERATURE	HOURS OF INCUBATION AT REQUIRED TEMPERATURE	AVERAGE % OF CELLS IN G1	AVERAGE % OF CELLS IN S, G2 AND M
33°C	16	56.7	43.4
	24	57.9	42.1
40°C	16	74.1	26.0
	24	84.2	15.8
33°C serum-starved	48	74.1	26.0

Table 3.68 CELL CYCLE DISTRIBUTION OF K12 GROWN IN 5% FCS MCDB-302 (7 mM $NaHCO_3$)

TEMPERATURE	HOURS OF INCUBATION AT REQUIRED TEMPERATURE	AVERAGE % OF CELLS IN G1	AVERAGE % OF CELLS IN S, G2 AND M
33°C	40	55.0	45.0
	64	57.6	42.5
40°C	40	77.1	23.0
	64	71.6	28.4
33°C serum-starved	48	76.9	23.1

Table 3.69 CELL CYCLE DISTRIBUTION OF K12 GROWN IN 5% FCS MCDB-302 (26 mM $NaHCO_3$)

TEMPERATURE	HOURS OF INCUBATION AT REQUIRED TEMPERATURE	AVERAGE % OF CELLS IN G1	AVERAGE % OF CELLS IN S, G2 AND M
33°C	24	46.1	54.0
	40	47.6	52.4
40°C	24	67.9	32.1
	40	81.9	18.2
33°C serum-starved	48	67.3	32.7

Table 3.70 CELL CYCLE DISTRIBUTION OF K12 GROWN IN 5% FCS MCDB-302 (65 mM $NaHCO_3$)

TEMPERATURE	HOURS OF INCUBATION AT REQUIRED TEMPERATURE	AVERAGE % OF CELLS IN G1	AVERAGE % OF CELLS IN S, G2 AND M
33°C	16	53.2	46.9
	24	51.5	48.6
40°C	16	69.5	30.6
	24	70.8	29.2
33°C serum-starved	48	70.9	29.1

Table 3.71 CELL CYCLE DISTRIBUTION OF Wg-1A GROWN IN 5% FCS E4 (26 mM $NaHCO_3$)

TEMPERATURE	HOURS OF INCUBATION AT REQUIRED TEMPERATURE	AVERAGE % OF CELLS IN G1	AVERAGE % OF CELLS IN S, G2 AND M
33°C	24	44.7	55.3
	40	51.0	49.0
40°C	24	44.6	55.5
	40	50.0	50.0
33°C serum-starved	48	55.8	44.3
	72	55.2	44.8

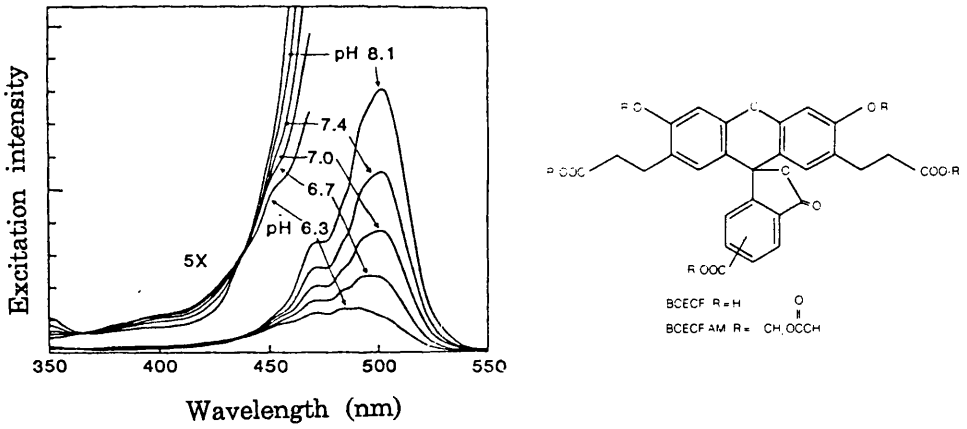
3.16 MEASUREMENT OF THE INTRACELLULAR pH OF K12 AND Wg-1A UNDER DIFFERENT GROWTH CONDITIONS

If, as first suggested in Section 3.7, manipulation of the $NaHCO_3$ level influences the rate of loss of viability of K12 at the npt through alteration of the pH of the medium, then in order to exert such an effect it would be expected that the consequent changes in the extracellular pH (pH_{ex}) would be reflected in variations in the intracellular pH (pH_i) of the cell. The internal pH of eukaryotic cells is generally maintained at a precise level under physiological conditions, usually within the pH range of 7.0 - 7.4 (Madshus, 1988), the exact value depending on the cell type in question. This is achieved by a number of different mechanisms, including the use of transmembrane ion transport systems (see Section 4.1.iia for detailed discussion). Raising or lowering the external pH by a certain degree is therefore unlikely to be reflected by an increase or decrease of a similar magnitude in the pH_i of the cell. In fact, it has been demonstrated in a variety of cell types that the external pH must be reduced to a value of 6.5 - 6.8 before the pH_i falls from its steady-state level (Deutsch *et al.*, 1982; Mikkelsen *et al.*, 1985; Musgrove *et al.*, 1987), and an pH_{ex} of 7.6 or higher is required before any significant alkalinization of the cytoplasm occurs (Pouyssegur *et al.*, 1985; Szwergold *et al.*, 1989). In the viability curve (Section 3.7) and time-lapse (Section 3.14) studies, the pH of the media was varied over the pH range 6.91 - 7.97 as a consequence of the alterations in $NaHCO_3$ concentration (Section 3.10). In order to investigate whether such changes alter the internal pH of the cell to any significant degree, and if so, to determine the size of this effect, the pH_i of K12 and Wg-1A cells was measured in each of the growth media used in the earlier studies.

It is also conceivable that the G1 block in K12 is caused not by a mutation in a cell cycle protein, but rather is a consequence of a defect in one of the cell's pH_i-regulatory ion transport systems, such that expression of the mutation leads to a gradual raising of the pH_i at the npt. If a particular cell cycle protein, required to pass the K12 execution point, has a pH optimum at acidic values, such an increase in the pH_i could cause the activity of this molecule to fall below a threshold level, and therefore arrest the cell at this point. A reduction in the bicarbonate content, and therefore the pH_{ex} of the medium, would then compensate transiently for the increase in pH_i caused by the faulty ion pump by acidifying the cytosol to some extent, thus allowing for the prolonged survival of the cell line at 40°C. In order to determine whether K12 does indeed exhibit defective pH_i regulation at the npt, the internal pH values of K12 and Wg-1A at both 33° and 40°C were also measured and compared.

The measurement of pH_i was carried out on both single cells and cell populations by application of the fluorescent pH indicator BCECF (Section 2.11). The acetoxymethyl ester of BCECF, BCECF-AM, is a non-fluorescent membrane-permeant molecule, but once in the cytoplasm it is hydrolyzed by cellular esterases to its fluorescent free acid form, which shows a decreased ability to cross the membrane due to its high negative charge, and thus becomes trapped within the cell (Rink *et al.*, 1982). The intensity of fluorescence of the dye, emitted at a wavelength of 535 nm, is pH-dependent, with an excitation peak at 500 - 505 nm (Fig. 3.39). The pH_i of cells loaded with BCECF-AM was thus calculated by measurement of the intensity of fluorescence emitted at an excitation wavelength of 500 - 505 nm. However, since this value is also dependent on the concentration of the dye, which may vary from cell-to-cell due to differing levels of uptake, esterase activity and leakage, the measurements were rendered independent of intracellular dye concentration by calculating the ratio of fluorescence intensities emitted at excitation wavelengths of 430 nm, where emission is pH-independent (Fig. 3.39), and at 500 nm.

Figure 3.39 Excitation spectrum (emission at 535 nm) and molecular structure of BCECF (adapted from Molecular Probes catalogue)



Such ratios were subsequently converted to absolute pH values by calibration of the dye's fluorescence intensity, using both *in vivo* and *in vitro* calibration measurements. For *in vitro* calibration, BCECF-free acid was suspended in solutions of varying pH and the fluorescence intensity ratios were measured. However, the excitation peak and pK of BCECF alter slightly when in an intracellular environment (Rink *et al.*, 1982), thus *in vivo* methods should produce more reliable calibration values. The application of nigericin, an electroneutral K^+/H^+ antiporter, brings the pH_i and pH_{ex} values to equivalence (Thomas *et al.*, 1979) if the calibration solution is adjusted such that there is no K^+ ion gradient across the membrane. *In vivo*

calibration of BCECF fluorescence was thus achieved by recording the fluorescence intensity ratios of nigericin-treated stained cells whose pH_i value was defined by manipulations of the pH_{ex}.

Measurements of the fluorescence intensity ratios of single cells of K12 and Wg-1A grown at either 33° or 40°C in 5% FCS E4 low bic were performed according to the protocol described in Section 2.11.i. The degree of fluorescence emitted by each cell at 535 nm, with excitation wavelengths of 430 and 500 nm, was displayed on a chart recorder (see Figs. 3.40 - 3.43) and used to calculate an R value (Tables 3.72 - 3.75) for each cell. The R values were then converted to absolute pH values (see Section 2.11.i for method of calculation) by reference to results from both *in vitro* and *in vivo* calibrations carried out on the same occasion as the measurement of the cells (Figs. 3.40 - 3.43).

Two points regarding the calculated results need to be considered:

a. *In vitro* calibration measurements carried out on separate occasions, using identical conditions, should give very similar R and pK values for the dye. In fact, as shown in Tables 3.72 - 3.75, the R values varied quite considerably. Since 500/430 nm ratios were used to calculate R values, it seems unlikely that this could be a result of slight variations in the concentration of BCECF, and a single batch of each calibration solution was used for all the experiments in order to ensure a constant pH. It thus appears likely that the amount of emitted fluorescence detected and recorded by the machine at each excitation wavelength was subject to day-to-day fluctuation. This variation in R values was reflected in a significant degree of inconstancy in the calculated pK values, which on one occasion (Table 3.74) deviated quite markedly (by 0.26 pH units) from the published value of pH 6.97 calculated by Rink *et al.* (1982) under comparable conditions. R and pK values also varied when calculated from *in vivo* calibration measurements, and in the case of the measurements carried out on K12 at 40°C (Table 3.75) the R_{max} was lower than most of the recorded experimental values, suggesting an pH_i in excess of 8.5 for the majority of cells, compared with an average pH_i of 6.98 when calculated from *in vitro* results. There were thus, quite clearly, problems with the calibration of fluorescence and therefore with the calculation of absolute pH values.

b. Individual cells within a single measured population showed a wide degree of variation in their calculated pH_i values, a finding also noted by Tanasugarn *et al.* (1984) when determining the pH_i of 3T3 cells with a fluorescent dye. Although the pH_i of a cell is believed to be cell cycle-dependent (Musgrove *et al.*, 1987), it seems unlikely that such large deviations could be accounted for by the measurement of cells at different stages of the cycle. Bright *et al.* (1989) observed during the staining of 3T3 fibroblasts that in a number of cells BCECF-AM compartmentalizes into the

mitochondria, which have a more alkaline pH than the cytoplasm (Thomas, 1986), and can thus indicate misleading intracellular pH values. It is possible that this phenomenon may account for some of the variations in the apparent pH_i observed in the present study.

Due to the problems associated with the variability in both calibration and individual cell measurements, the absolute pH values calculated for each cell were considered fairly inaccurate, and it seems that broad generalizations only can be drawn from the results. Internal pH values for the majority of cells in each population studied fell within the range of 6.8 - 7.5. However, the extremely low BCECF pK value of 6.71 calculated from the *in vitro* calibration for K12 cells at 33°C (Table 3.74) suggested pH_i values for the cells of 5.95 - 6.27. Such low pH_i values seem unlikely to be compatible with continued growth, given the observed strict maintenance of a pH_i value of 7.0 - 7.4 within a range of eukaryotic cell types (Madshus, 1988), yet the measured cells appeared to be healthy, thus this set of figures was deemed unreliable, as were the pH_i values calculated using *in vivo* calibration results for K12 at 40°C, which implied pH_i values in excess of 8.5 (Table 3.75). For Wg-1A at either temperature, pH_i values calculated from *in vivo* calibration figures were consistently higher than those using *in vitro* measurements, an observation also noted by Rink *et al.* (1982). Since *in vivo* measurements should provide a more reliable indication of the true situation within the cell, pH_i values calculated on the basis of *in vivo* calibration figures were considered to be a more accurate reflection of the actual pH_i. The results (Tables 3.72 and 3.73) suggest that Wg-1A has a pH_i of approximately 7.34 at 33°C, and that this value increases slightly, to pH 7.39, when the incubation temperature is raised to 40°C. K12 appears to have a significantly lower pH_i value (pH 7.14) than Wg-1A at 33°C (Table 3.74), providing additional evidence that this cell line may not be fully wild-type at the pt. Unfortunately, since the *in vivo* calibration provided unreliable results for K12 measured at 40°C, any alteration in this pH_i value following incubation of the cells at the npt could not be determined with any degree of accuracy. Calculations based on the *in vitro* calibration suggest an average pH_i of 6.98 for K12 at 40°C (Table 3.75); if it is assumed that the same differential between *in vitro* and *in vivo*-based pH calculations (approximately 0.3 - 0.4 pH units) applies to K12 as to Wg-1A at 40°C, the actual pH_i value of K12 at the npt may be estimated at around 7.3 - 7.4. This suggests that the internal pH of K12 does indeed increase somewhat at the npt, but to a value closer to the wild-type figure, which would make it unlikely that a defect in a pH_i-regulatory ion transport system could account for the cell cycle block. However, more rigorous investigation of the pH_i of these cell lines at each temperature was clearly necessary.

Once the fluorescence intensity ratio measurements had been carried out on nine K12 cells at 40°C, the population was perfused with each of the following media in turn - 5% FCS E4 supplemented with 13.3, 26 or 44 mM $NaHCO_3$, and 5% FCS MCDB-302 containing 7, 26 or 65 mM $NaHCO_3$ - and the fluorescence emitted in each of the different media was recorded (Fig. 3.44 and Table 3.76) using cell number 9. Unfortunately, a number of complications also beset this experiment:

i. The chosen cell was found, when calculation was performed after completion of the experiment, to have an unusually low pH_i (6.50) in the normal growth medium (5% FCS 26 mM $NaHCO_3$ E4), thus the absolute values calculated for each medium type seem unlikely to be representative of an average cell within the population.

ii. The pH_i of cell 9 was calculated as 7.02 when first measured in 5% FCS 26 mM $NaHCO_3$ E4 (Table 3.75). This value had dropped to pH 6.50 by the time the measurements in varying media had begun (Table 3.76), and when the cell was returned to 5% FCS 26 mM $NaHCO_3$ E4 at the end of the set of readings, the pH_i was now 6.29, despite the fact that a total of only 25 - 30 minutes had elapsed between the three recordings, suggesting perhaps that the pH_i-regulatory properties of this cell were distinctly abnormal. This did not appear to be caused by the onset of death at the npt, since the cell in question had a healthy, flattened morphology, but whether this gradual lowering of the pH_i was representative of the situation in the population as a whole, as a consequence of the expression of the K12 mutation, is unclear. However, since there was clearly an underlying alteration in the pH_i of the cell, superimposed on any changes induced by variations in the external pH of the growth medium, the relative differential between pH_i values calculated in each of the different media can be taken as only a rough estimate.

iii. This same cell was also used for *in vivo* calibration, and produced R and pK figures suggesting that K12 cells cultured at 40°C in 5% FCS E4 low bic had an average pH_i of greater than 8.5 (Table 3.75), which was clearly inaccurate. It therefore seemed that the cell chosen for this set of analyses was not representative of the population as a whole.

The results from this experiment thus proved unreliable, and although it appeared that the pH_i of K12 altered significantly when the external pH was changed (Table 3.76), a more accurate analysis was required to confirm this. The major problems encountered in these studies appeared to be a wide variation in the calculated pH_i values for individual cells within a population, casting doubt on the degree to which averages taken from a small number of cells reflected the situation as a whole, and inherent day-to-day variabilities in the degree of detection of the fluorescence signal by the recording equipment, resulting in unreliable calibration figures. In an attempt to overcome these difficulties, measurements were thus also carried out on whole cell

populations, in order to provide a more accurate representation of the average pH_i of each cell line under the different growth conditions. This required the use of a fluorescence spectrophotometer for detection and measurement of the fluorescence signal, and it was hoped that the change of equipment might, in addition, eliminate the variabilities in the calibration measurements.

The pH_i measurements on populations of K12 and Wg-1A were carried out as described in Section 2.11.ii. Each cell line was measured at both 33° and 40°C in 5% FCS E4 low bic, and at 40°C in each of the following media: 5% FCS E4 containing 13.3 or 44 mM $NaHCO_3$, and 5% FCS MCDB-302 supplemented with 7, 26 or 65 mM $NaHCO_3$. The recordings were made on trypsinized cells and may not, therefore, accurately reflect the pH_i of flattened cells, but should provide an indication of the extent to which the pH_i changes when the composition of the medium or the temperature of incubation is altered. Initial experiments used a BCECF-AM concentration of 0.5 μM, but it was found that a large degree of cellular autofluorescence or scattering of the incident light at excitation wavelengths greater than 490 nm obscured the BCECF fluorescence peak at 505 nm. Staining of the cells with carboxy-SNARF-1/AM acetate, a fluorescent dye with an excitation peak (548 nm; Molecular Probes catalogue) away from the area of cell scattering, was attempted, but the dye was not taken up by either of the cell lines. The concentration of BCECF-AM was thus raised to 5 μM, in order to increase the degree of fluorescence and therefore obscure the cell scattering or autofluorescence peak.

For this set of experiments, the excitation wavelength was plotted against the intensity of fluorescence emitted at 535 nm (Figs. 3.45 - 3.48). The traces for unstained samples of Wg-1A and K12 demonstrate that cellular autofluorescence or scattering of the light continued to affect readings taken in the 420 - 490 nm excitation range, with a peak of emission at an excitation wavelength of 470 nm. The fluorescence intensity in this area would therefore be partly determined by the concentration of cells in the suspension, and although attempts were made to standardize this value before measurement, there still appeared to be significant differences in cell number in the samples, since the traces started at varying levels of fluorescence at 420 nm. This irregularity in cell concentration may have been caused by cell clumping, since suspensions were allowed to stand for up to 2 hours in conical tubes prior to analysis, and resuspension of the clumps which formed over this time may not have been complete. As cellular autofluorescence or light scattering predominated at an excitation wavelength of 430 nm, the BCECF fluorescence intensity at this value could no longer be used in the calculation of R values for *in vivo* calibration or for experimental cell measurements, since it was determined

primarily by the variable cell concentration. Although fluorescence intensities at 505 nm could still be compared between samples, this value was partly dependent on the intracellular concentration of the dye, which may have varied between populations e.g. cells stained at different temperatures or in varying medium compositions may show unequal degrees of uptake, hydrolysis or leakage of BCECF. Moreover, a variety of slit widths (which determine the amount of light used for excitation and the proportion of the emitted light which is recorded) was employed during the measurement period, since the intensity of the emitted fluorescence did indeed differ greatly from sample to sample, and this would also be expected to considerably alter the height of the 505 nm fluorescence peak. Since these variables would no longer be eliminated from calculations by taking a ratio of fluorescence intensities at two wavelengths, any comparisons would thus show a significant degree of inaccuracy. Furthermore, *in vitro* calibrations could no longer be used to calculate cellular pH_i values, since the concentration of BCECF in the calibration solutions and in the cytoplasm was unlikely to be the same, thus *in vitro* calibration was not carried out for the analysis of Wg-1A cell populations.

Once again, *in vivo* calibration appeared to give unreliable results, as was found with the single cell measurements. When *in vivo* calibration was performed at 40°C with Wg-1A cells, external solutions of pH 7.2 and 6.0 gave equivalent 505 nm fluorescence peaks (Fig. 3.48), and K12 cells suspended in a calibration solution of pH 6.0 appeared to have a markedly higher pH_i than those in solutions of pH 7.2 and 7.8 (Fig. 3.46b). It would thus seem that nigericin does not have the expected effect of bringing the pH_{ex} and pH_i values to equivalence in each cell line under these conditions, although the reasons for this are unclear; nigericin did not appear to kill the cells, since the consequent lysis would have released the dye into the calibration solution and the pattern of fluorescence would then have mimicked that seen in the *in vitro* calibration.

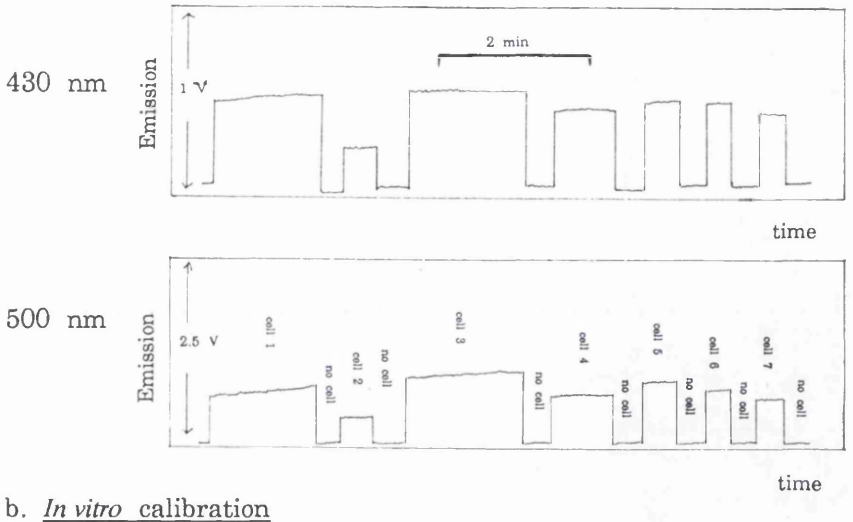
Since no reliable calibration figures were obtained for this set of results, only rough conclusions could be drawn. In the case of K12 grown and suspended in MCDB-302 at 40°C, the internal pH of the cells appeared to increase significantly when the $NaHCO_3$ concentration of the medium was raised (Fig. 3.45b). However, no such clear correlation between cytoplasmic and medium pH values was observed when E4 was used as the suspending medium (Fig. 3.45a). K12 cells suspended in 5% FCS E4 low bic produced similar fluorescence intensities at 33° and 40°C, suggesting that the pH_i of K12 changes little when the temperature is altered. For Wg-1A cells, there appeared to be no relationship between the pH_{ex} and pH_i for either medium at 40°C (Fig. 3.47), and cells grown at 33° or 40°C in 5% FCS E4 low bic showed significantly different fluorescence intensities (Fig. 3.47a). The most likely explanation

for the lack of any consistent correlation between external and internal pH values (given that in the single cell studies, where ratios could be taken in order to eliminate concentration-dependent effects, the pH_i increased as the pH_{ex} was raised) seemed to be differences in the intracellular dye concentration in each population, for the reasons indicated earlier, which would consequently alter the height of the fluorescence intensity peaks. It is also possible that maintaining the cells in suspension at the appropriate temperature for up to 2 hours before analysis, although not causing lysis, may have led to defects in pH_i regulation, thus resulting in abnormal internal pH values. In addition, it is noticeable that in each cell line, the fluorescence peaks at 505 nm were markedly smaller in E4 than in MCDB-302, suggesting that a component of E4 interferes with the uptake, hydrolysis or leakage of BCECF or, alternatively, quenches its fluorescence.

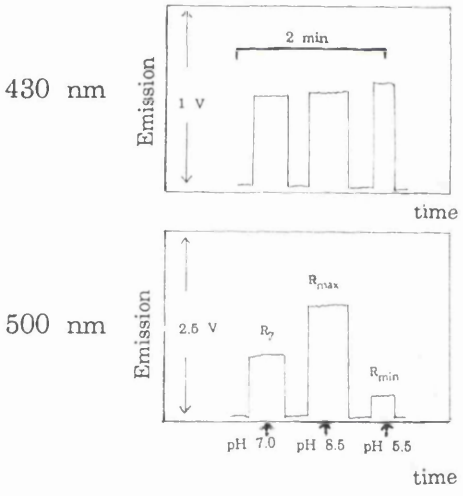
Given the complications inherent in both the single cell and cell population pH_i measurements, no clear conclusions could be drawn from these experiments. Nonetheless, it does appear probable that altering the $NaHCO_3$ level of the medium may significantly influence the pH_i of the cell (Table 3.76), thus supporting the theory that bicarbonate could exert its effects on the viability of K12 at 40°C by altering the intracellular pH. The results also suggest that it is unlikely that the K12 cell cycle block is caused by a major defect in pH_i regulation at the npt, since the internal pH values of K12 and Wg-1A did not appear to be markedly different at 40°C (Tables 3.73 and 3.75). It is clear, however, that a more reliable method of pH_i measurement was required in order to confirm and extend the results of these studies.

Figure 3.40 INTRACELLULAR pH MEASUREMENTS ON SINGLE Wg-1A CELLS AT 33°C IN 5% FCS E4 LOW BIC

a. Cell measurements



b. *In vitro* calibration



c. *In vivo* calibration

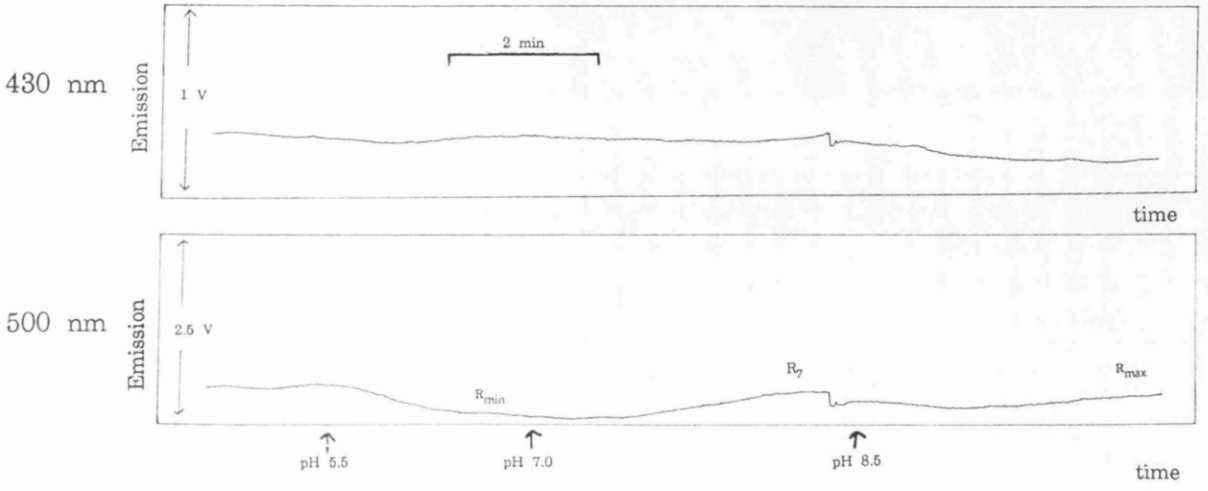
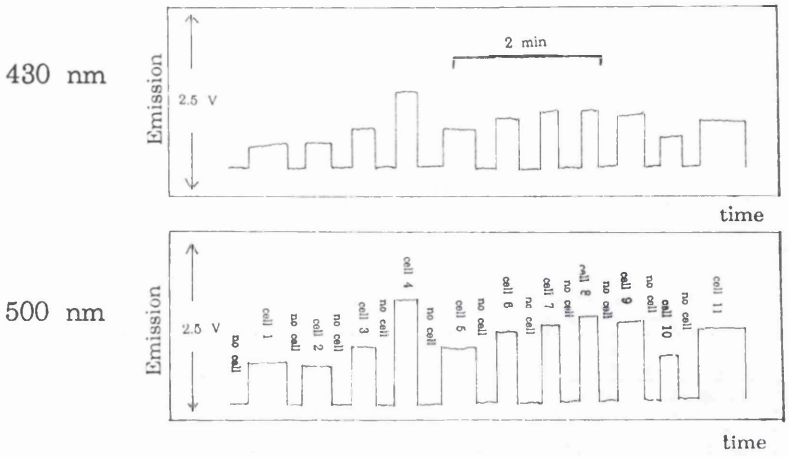
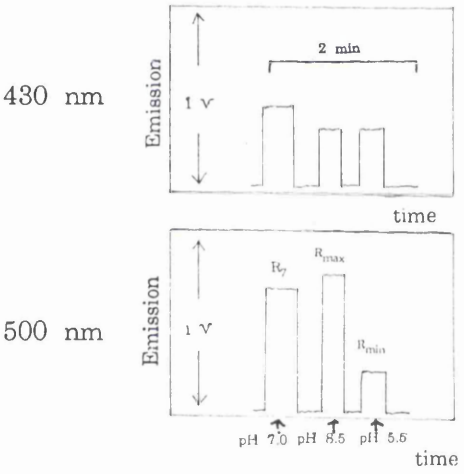


Figure 3.41 INTRACELLULAR pH MEASUREMENTS ON SINGLE Wg-1A CELLS AT 40°C IN 5% FCS E4 LOW BIC

a. Cell measurements



b. *In vitro* calibration



c. *In vivo* calibration

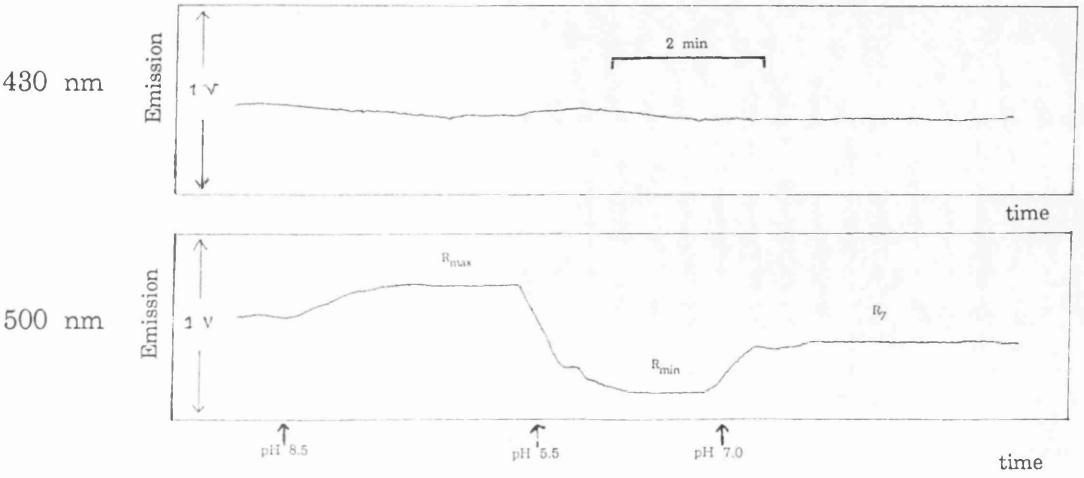
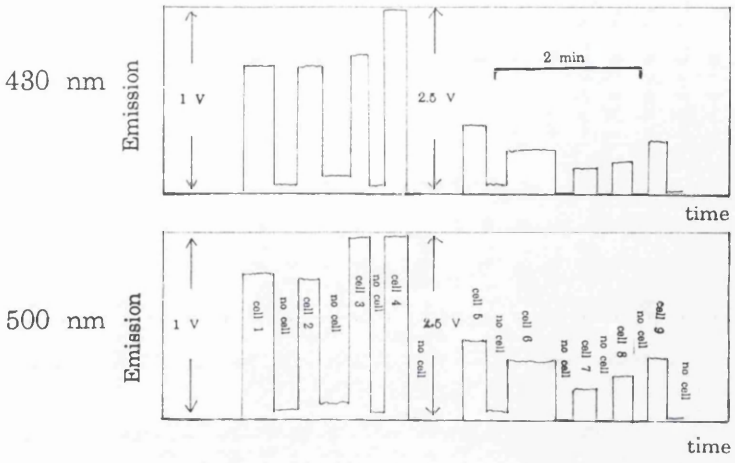
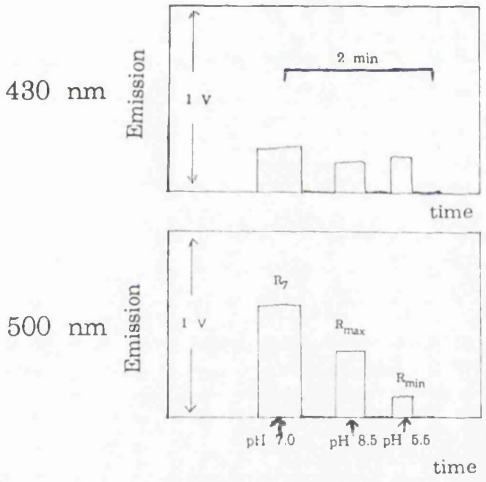


Figure 3.42 INTRACELLULAR pH MEASUREMENTS ON SINGLE K12 CELLS AT 33°C IN 5% FCS E4 LOW BIC

a. Cell measurements



b. *In vitro* calibration



c. *In vivo* calibration

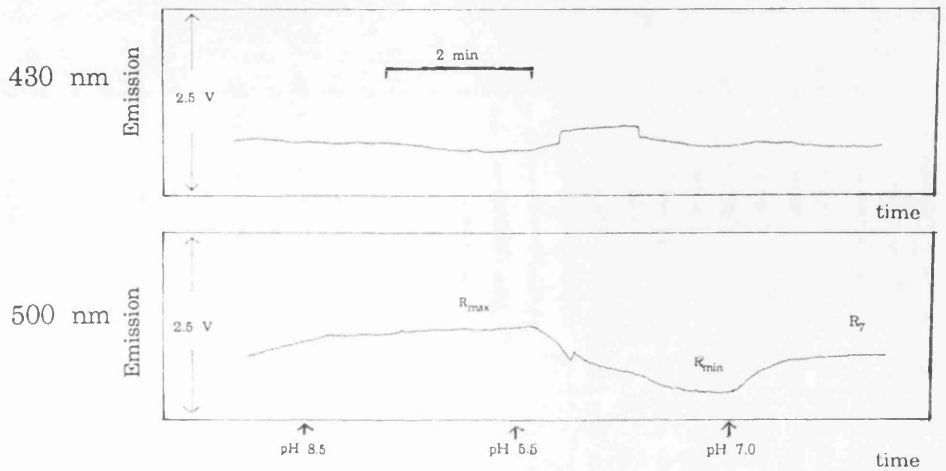
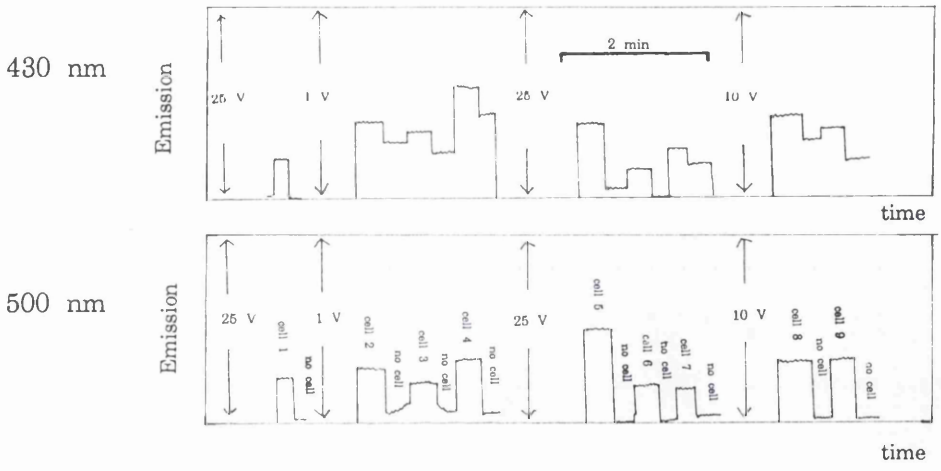
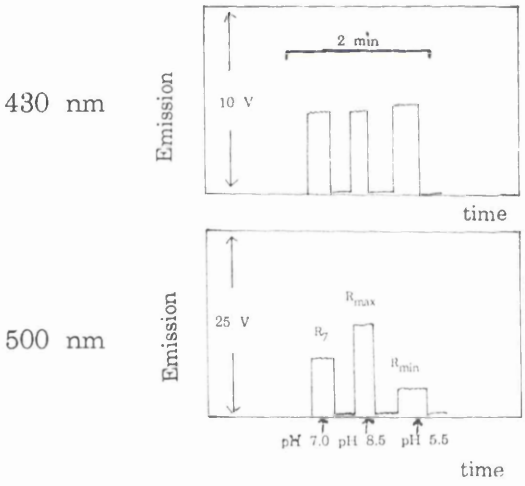


Figure 3.43 INTRACELLULAR pH MEASUREMENTS ON SINGLE K12 CELLS AT 40°C IN 5% FCS E4 LOW BIC

a. Cell measurements



b. *In vitro* calibration



c. *In vivo* calibration

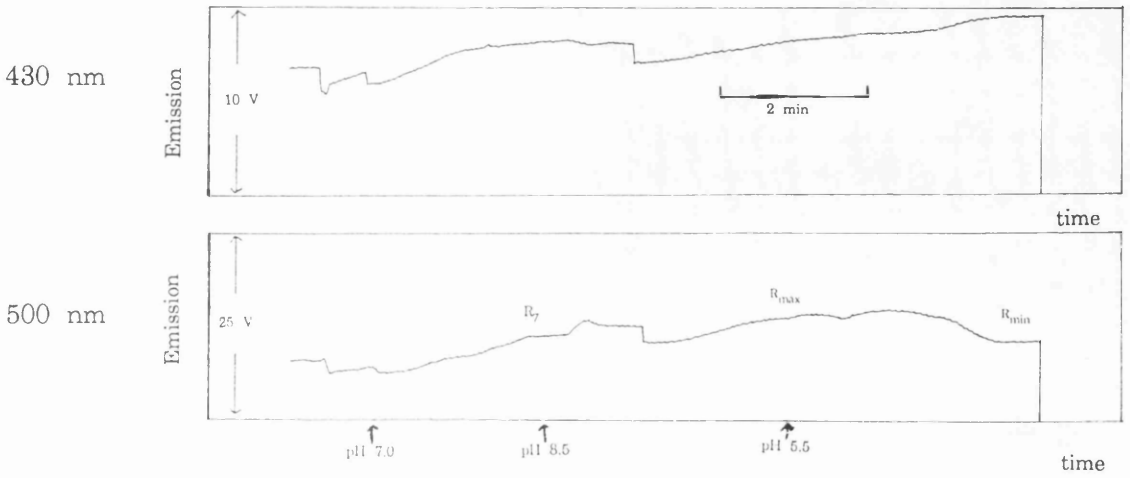


TABLE 3.72 INTRACELLULAR pH MEASUREMENTS ON Wg-1A AT 33°C IN 5% FCS E4 LOW BIC

CALIBRATION

	R_{min}	R_7	R_{max}	pK
In vitro calibration	0.58	1.77	3.13	7.06
In vivo calibration	0.36	1.25	2.50	7.15

CELL	R VALUE	pH_i (*in vitro* calibration)	pH_i (*in vivo* calibration)
1	1.63	6.90	7.31
2	1.54	6.84	7.24
3	1.88	7.07	7.53
4	1.55	6.84	7.25
5	1.83	7.04	7.49
6	1.67	6.93	7.35
7	1.51	6.82	7.22
	AVERAGE pH_i	6.92	7.34
	STANDARD DEVIATION	0.10	0.12

TABLE 3.73 INTRACELLULAR pH MEASUREMENTS ON Wg-1A AT 40°C IN 5% FCS E4 LOW BIC

CALIBRATION

	R_{min}	R_7	R_{max}	pK
In vitro calibration	0.76	1.48	2.26	7.04
In vivo calibration	0.34	1.24	1.92	6.87

CELL	R VALUE	pH_i (*in vitro* calibration)	pH_i (*in vivo* calibration)
1	1.64	7.19	7.55
2	1.64	7.19	7.55
3	1.45	6.97	7.26
4	1.39	6.90	7.17
5	1.54	7.08	7.39
6	1.46	6.98	7.27
7	1.48	7.01	7.30
8	1.52	7.04	7.34
9	1.59	7.13	7.45
10	1.69	7.25	7.64
11	1.50	7.03	7.32
	AVERAGE pH_i	7.07	7.39
	STANDARD DEVIATION	0.11	0.15

TABLE 3.74 INTRACELLULAR pH MEASUREMENTS ON K12 AT 33°C IN 5% FCS E4 LOW BIC

CALIBRATION

	R_{min}	R_7	R_{max}	pK
In vitro calibration	0.78	2.36	3.17	6.71
In vivo calibration	0.39	1.12	2.30	7.21

CELL	R VALUE	pH_i (*in vitro* calibration)	pH_i (*in vivo* calibration)
1	1.19	6.03	7.07
2	1.14	5.95	7.01
3	1.34	6.20	7.21
4	1.16	5.98	7.03
5	1.29	6.15	7.16
6	1.33	6.19	7.20
7	1.41	6.27	7.27
8	1.27	6.12	7.14
	AVERAGE pH_i	6.11	7.14
	STANDARD DEVIATION	0.11	0.09

TABLE 3.75 INTRACELLULAR pH MEASUREMENTS ON K12 AT 40°C IN 5% FCS E4 LOW BIC

CALIBRATION

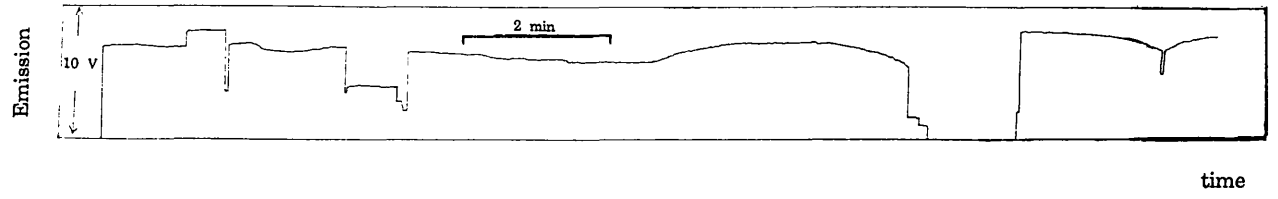
	R_{min}	R_7	R_{max}	pK
In vitro calibration	0.82	1.86	2.84	6.97
In vivo calibration	1.11	1.34	1.63	7.10

CELL	R VALUE	pH_i (*in vitro* calibration)	pH_i (*in vivo* calibration)
1	1.83	6.98	>8.5
2	1.83	6.98	>8.5
3	1.73	6.89	>8.5
4	1.93	7.06	>8.5
5	1.37	6.55	7.10
6	1.40	6.58	7.20
7	2.33	7.45	>8.5
8	2.21	7.32	>8.5
9	1.88	7.02	>8.5
	AVERAGE pH_i	6.98	
	STANDARD DEVIATION	0.30	

Figure 3.44 INTRACELLULAR pH MEASUREMENTS ON SINGLE K12 CELLS AT 40°C IN MEDIA WITH VARYING $NaHCO_3$ CONCENTRATIONS

Cell measurements

430 nm



500 nm

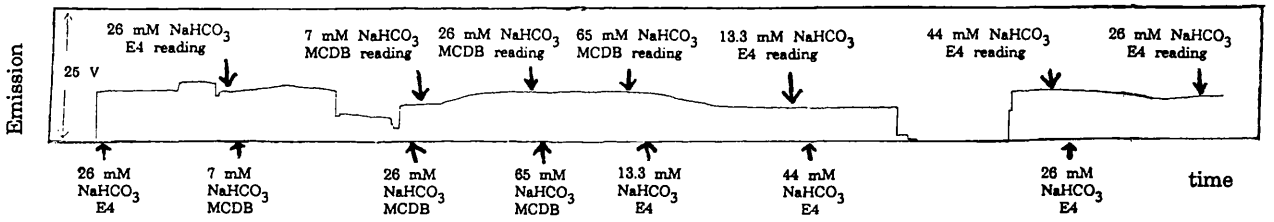
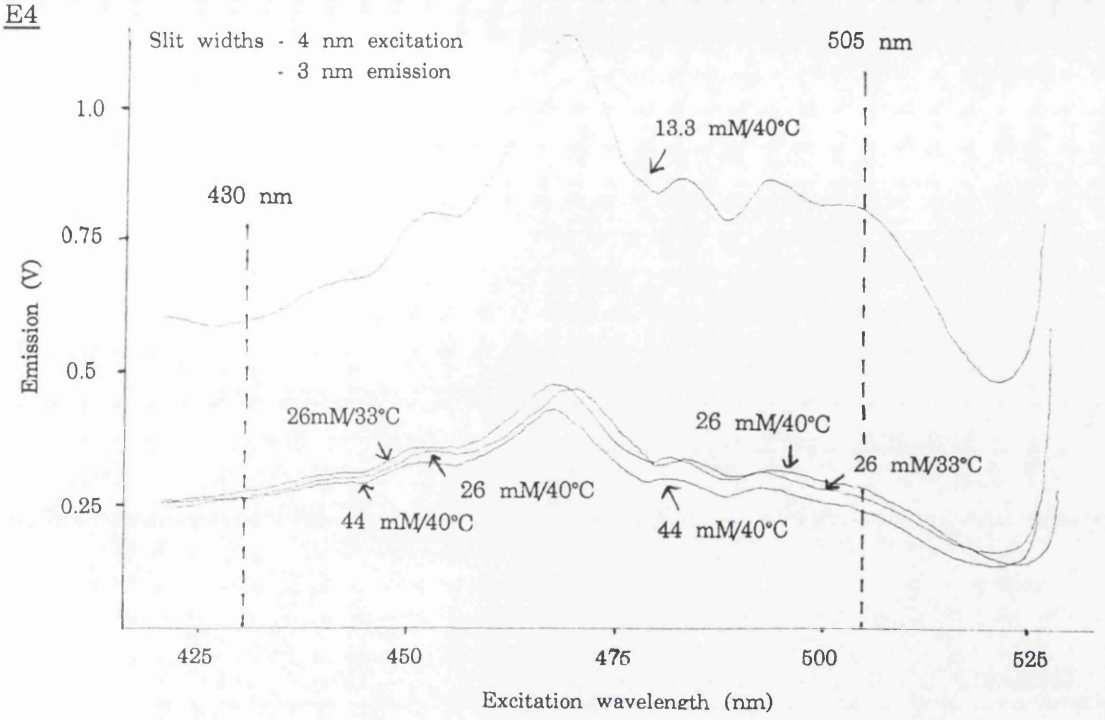


TABLE 3.76 INTRACELLULAR pH MEASUREMENTS ON K12 AT 40°C IN MEDIA WITH VARYING $NaHCO_3$ CONCENTRATIONS

MEDIUM	$[NaHCO_3]$ mM	pH_{ex}	R VALUE	pH_i (*in vitro* calibration)
E4	26	7.38	1.32	6.50
MCDB	7	6.91	1.09	6.17
	26	7.52	1.55	6.73
	65	7.97	1.70	6.86
E4	13.3	7.00	0.96	5.84
	44	7.58	1.22	6.37
	26	7.38	1.16	6.29

Figure 3.45 INTRACELLULAR pH MEASUREMENTS ON K12 POPULATIONS AT 33° AND 40°C IN MEDIA WITH VARYING $NaHCO_3$ CONCENTRATIONS

a. E4



b. MCDB

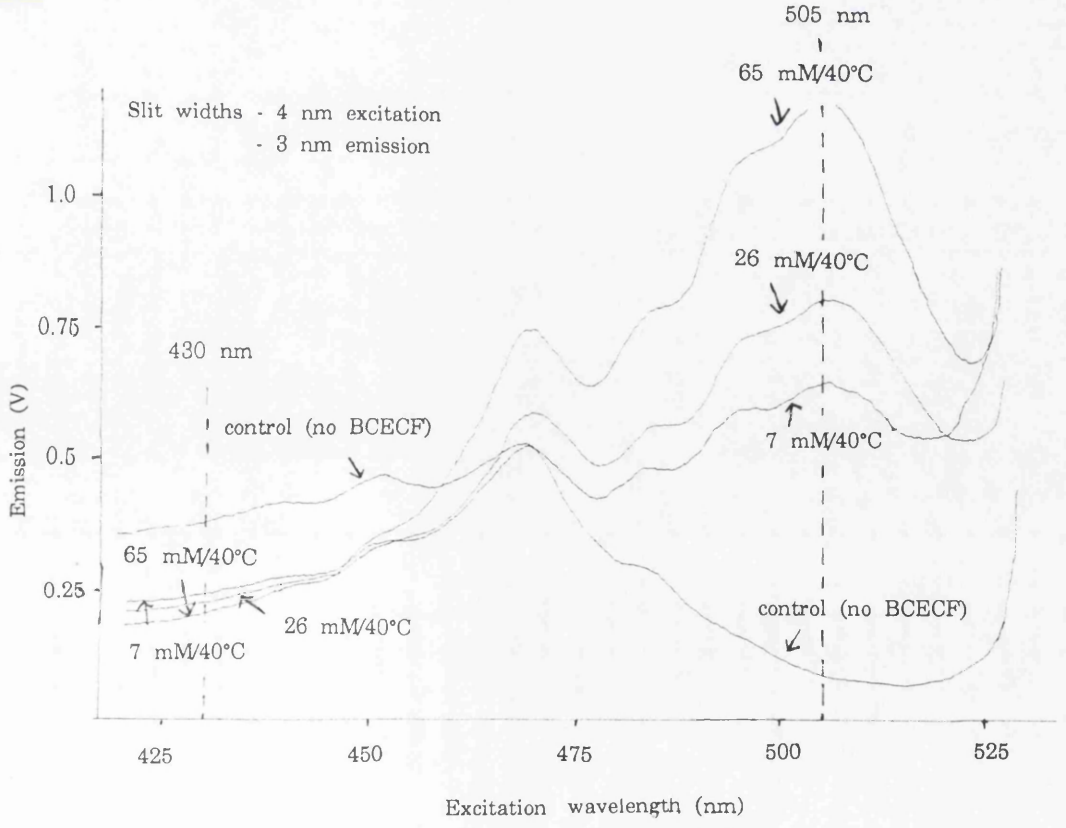
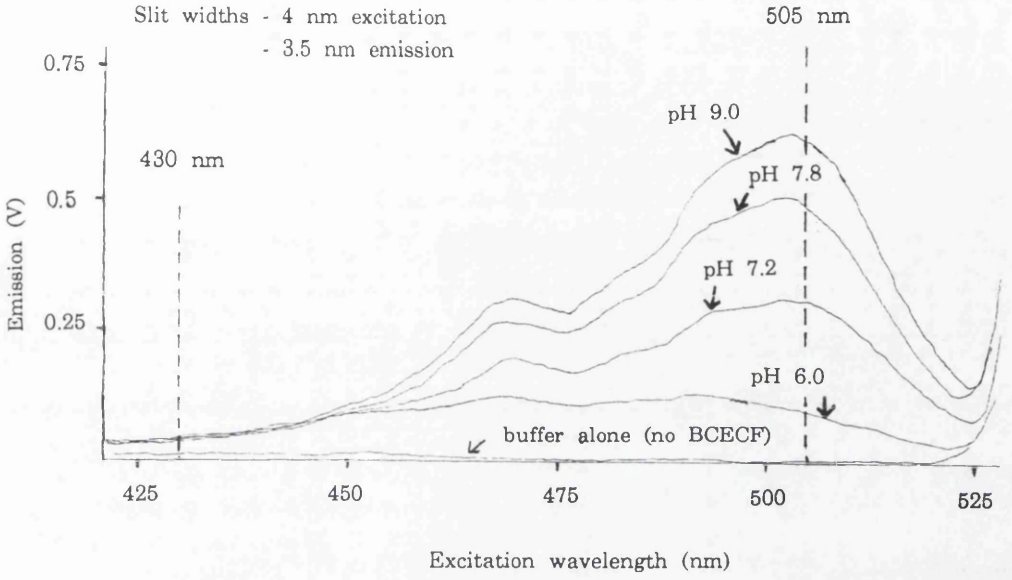


Figure 3.46 CALIBRATION AT 40°C FOR INTRACELLULAR pH MEASUREMENTS ON K12 POPULATIONS

a. *In vitro* calibration



b. *In vivo* calibration

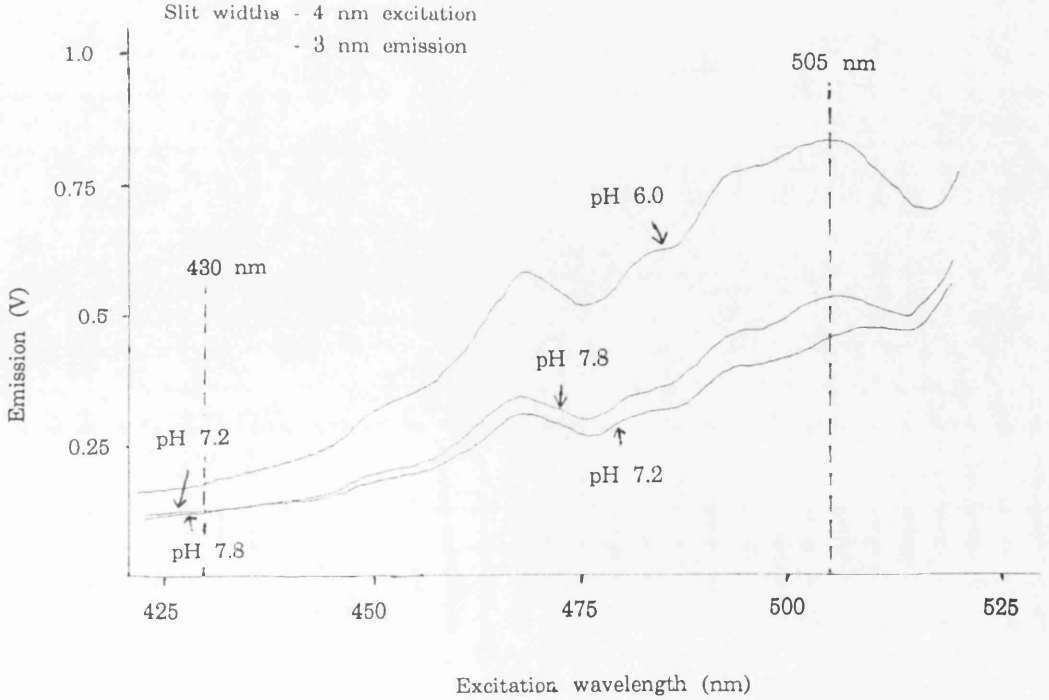
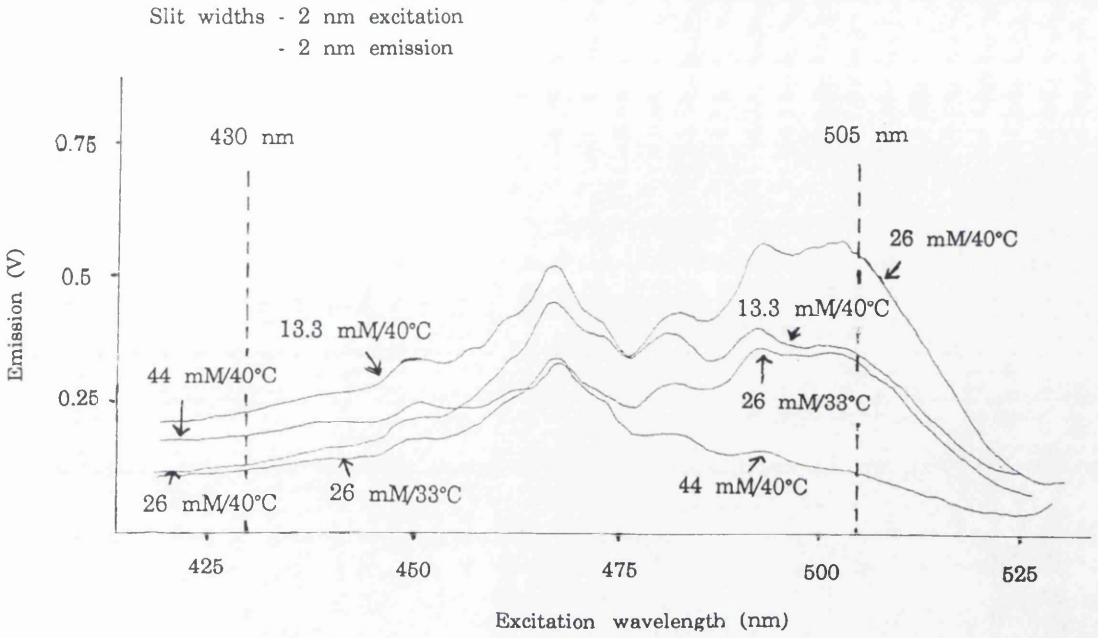


Figure 3.47 INTRACELLULAR pH MEASUREMENTS ON Wg-1A POPULATIONS AT 33° AND 40°C IN MEDIA WITH VARYING $NaHCO_3$ CONCENTRATIONS

a. E4



b. MCDB

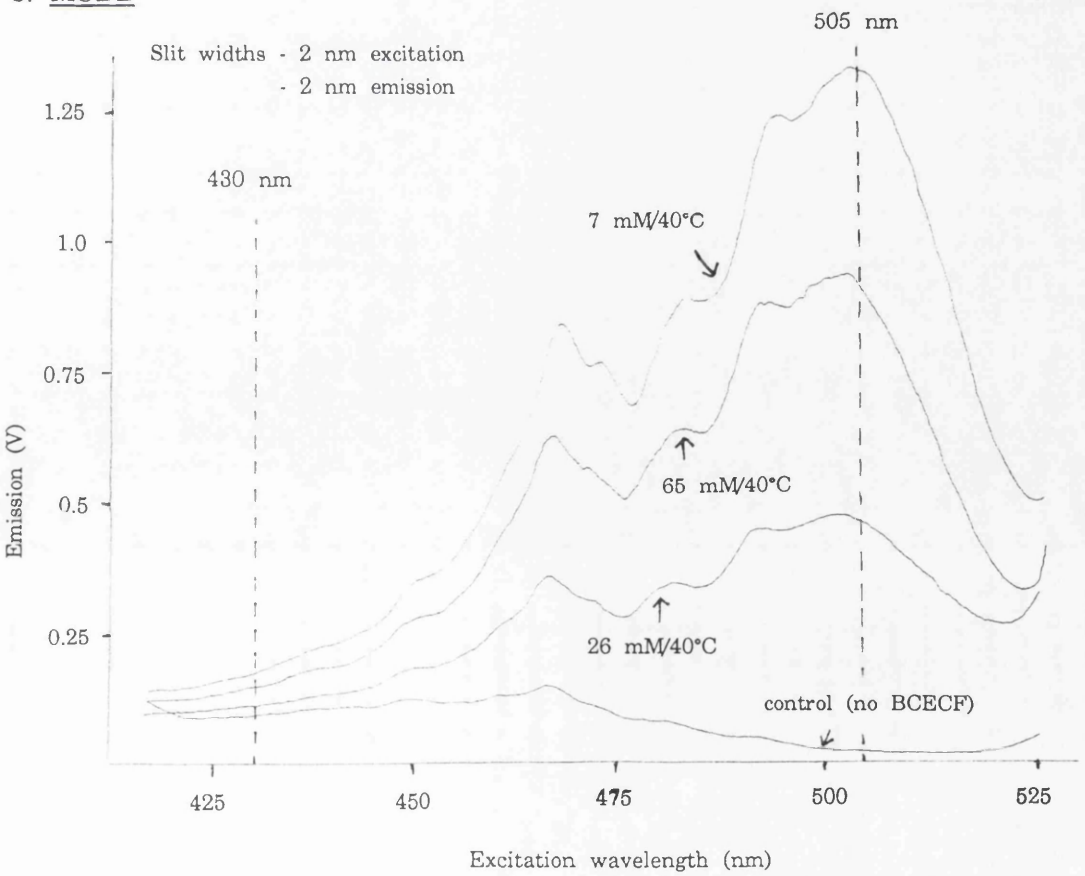
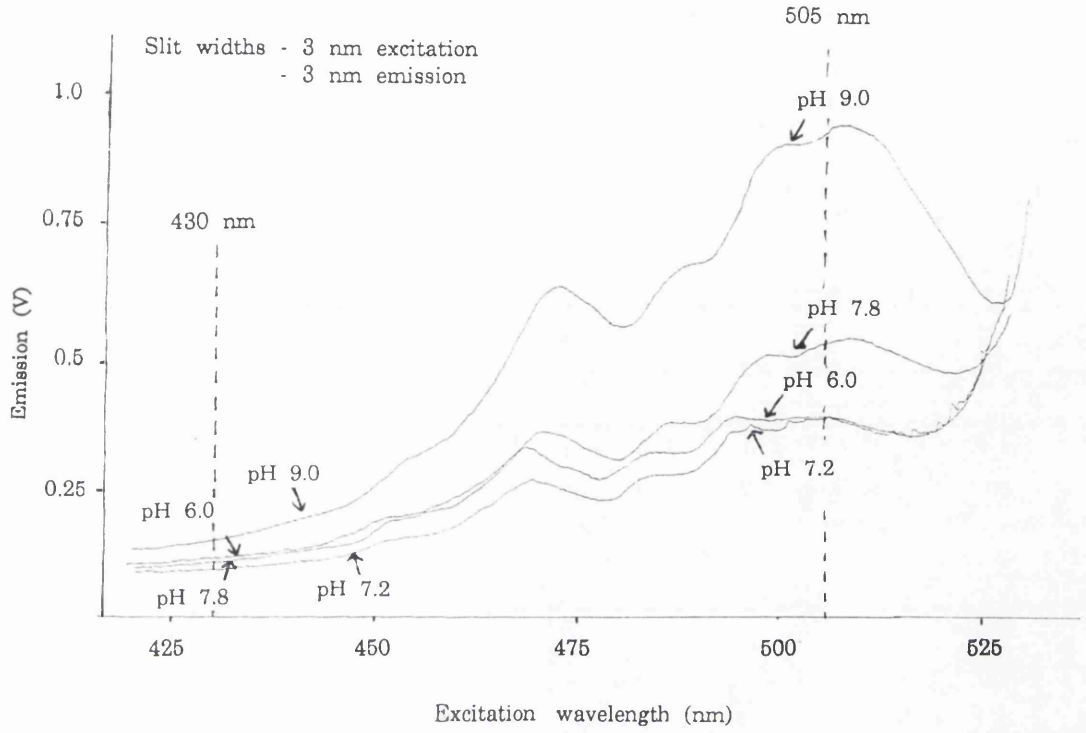


Figure 3.48 CALIBRATION AT 40°C FOR INTRACELLULAR pH MEASUREMENTS ON Wg-1A POPULATIONS

In vivo calibration



3.17 ISOLATION OF REVERTANTS OF K12 AT 40°C IN 7 mM $NaHCO_3$ MCDB-302

In the course of the viability studies (Section 3.7), it was noted that large healthy colonies of K12 were found at a low frequency on control plates which had been incubated in 7 mM $NaHCO_3$ MCDB-302 at 40°C for one week, despite the fact that time-lapse studies suggested that no divisions occurred after 94 hours at the npt under these conditions (Table 3.62). Given that these colonies had a healthy morphology, and were of a size equivalent to those found on control plates incubated at 33°C for the same time period in this medium, it appeared possible that these may be formed by revertant cells, capable of indefinite good growth at 40°C, whose presence was induced or selected for in some way by the use of 7 mM $NaHCO_3$ MCDB-302 as growth medium. Such colonies were not observed on 40°C control plates containing K12 cells grown in any of the other media used, and only a single spontaneous revertant of K12 has ever been previously detected (Scharff *et al.*, 1982), thus their appearance at such a relatively high frequency seems to be specific to the use of 7 mM $NaHCO_3$ MCDB-302.

In order to investigate the hypothesis that the growth of K12 cells at 40°C in this medium leads to the formation of revertant colonies, suspensions of K12 cells were prepared (Section 2.4) at a concentration of $2x10^4$ cells/ml in 5% FCS MCDB-302 containing either 7 or 26 mM $NaHCO_3$, and duplicate 10 cm dishes were plated for each medium type. All plates were placed directly at 40°C, and the medium was changed twice weekly. Eleven days after plating, although no cells remained on the dishes with 26 mM $NaHCO_3$ MCDB-302 as the growth medium, a number of large, healthy colonies were observed to be growing on the plates containing 7 mM $NaHCO_3$ MCDB-302 (Table 3.77). Such cells appeared to be capable of indefinite good growth at 40°C (albeit with a slower division rate than the wt parent Wg-1A), since they remained healthy during several months of continuous passaging, before being retained as frozen stocks only.

It thus seems that the use of 7 mM $NaHCO_3$ MCDB-302 as growth medium at the npt did indeed allow the appearance of revertant K12 cells which had overcome the ts defect. The high frequency with which this reversion occurred (approximately 1 in 6,000 cells, although this may be an overestimate due to the opportunity for formation of secondary colonies during the repeated medium changes - see Section 3.19) suggests that it was unlikely to have been caused by a back-mutation of the defective gene to a wild-type form, since the spontaneous mutation frequency for any given gene is generally considered to be in the order of 10^{-6}/cell/generation (DeMars and Held, 1972). Growth of the revertants at 40°C was also significantly slower than

that of Wg-1A, implying that the cells had not become fully wild-type. It is therefore possible that the appearance of such revertants may not be a consequence of a genetic change, but rather they may be 'phenotypic' revertants i.e. they may represent a small subpopulation of K12 cells, perhaps with an above average expression level of the defective protein which, in combination with the low bicarbonate content of the growth medium, allows for a required threshold level of activity of the protein to be attained.

The question of whether the observed reversion was genotypic or phenotypic was thus investigated. If it represents an alteration in the genetic composition of the cells, growth at 40°C should still occur even when the revertants are plated in 26 mM $NaHCO_3$ MCDB-302, whereas phenotypic revertants will only be capable of continued division in the low bicarbonate medium in which they were first isolated. Suspensions of revertant, K12 and Wg-1A cells were prepared (Section 2.4) at a concentration of 80 cells/ml in one of the following media - 5% FCS MCDB-302 containing either 7 or 26 mM $NaHCO_3$, or 5% FCS E4 supplemented with 13.3 or 26 mM $NaHCO_3$ - and four 6 cm dishes were plated for each medium type. All plates were placed in a 33°C incubator, and duplicate dishes for each medium type were shifted to 40°C the following day. One week after plating, all dishes were stained and the number of colonies was counted (Section 2.5).

A number of conclusions can be drawn from the results displayed in Tables 3.78 - 3.80:

a. A comparison of the growth of K12 and Wg-1A at 33°C in the different media provides further evidence that K12 is not fully wild-type at the pt, since K12 cells grew equally well in all of the media used, whereas Wg-1A exhibited a significantly reduced E.O.P. when the bicarbonate concentration of the medium was lowered. It thus seems likely that the K12 ts protein does not show full activity at 33°C, and as a consequence, this cell line is better able to cope with low medium bicarbonate levels than the wild-type parent.

b. The pattern of E.O.P. values for the revertant cell line at the two temperatures in varying media did not mimic the situation found with Wg-1A cells; the E.O.P. for the revertants was generally significantly lower for a corresponding medium type, and overall growth in E4 was very poor. This strongly suggests that the reversion was unlikely to be caused by a direct back-mutation of the defective gene in K12 to the wt form since, although the revertants were capable of growth at 40°C, full wild-type behaviour was not observed.

c. In 7 mM $NaHCO_3$ MCDB-302, the medium in which they were first isolated, revertant cells formed large healthy colonies at 40°C, but the E.O.P. was fairly low. This implies that although the reversion has to some extent eliminated the effects of

the ts mutation at the npt, the defect has not been fully overcome. The E.O.P. of the cells was further reduced when 33°C was used as the incubation temperature, suggesting that the effects of the reversion may actually be deleterious to the cell at the lower temperature. However, it appears that the harmful consequences may be overcome to some extent by raising the bicarbonate level of MCDB-302 to 26 mM.

d. Evidence that the reversion process has a genetic component is provided by the observation that healthy colonies of revertant cells grew at the npt in 26 mM $NaHCO_3$ MCDB-302. Since K12 cells are unable to grow, and revertants do not arise in this medium at 40°C, the observed reversion must, by implication, involve a genetic, heritable change which allows for some growth of the cell at the npt irrespective of the bicarbonate level of MCDB-302. However, the E.O.P. of the revertant cells at 40°C was lower when grown in 26 mM rather than 7 mM $NaHCO_3$ MCDB-302, thus the bicarbonate concentration of the medium continues to play a role in determining the behaviour of this cell line. Good growth of the revertants at 40°C is thus clearly achieved by a combination of the underlying genetic change brought about during reversion, which compensates to a large degree for the ts defect, and the use of a low bicarbonate growth medium.

e. Although the overall pattern of behaviour of revertant cells grown in E4 was similar to that seen in MCDB-302 i.e. a lowering of the $NaHCO_3$ level increased the E.O.P. value at 40°C, and allowed for better growth at the npt than at the pt, overall E.O.P. values were extremely low. Of particular interest was the observation that revertants grown in E4 medium of either bicarbonate concentration grew very poorly, or not at all, at 33°C, despite the fact that K12 and Wg-1A showed high E.O.P. values in the same media. This further implies that whereas the reversion process promotes growth at 40°C, an inhibitory effect is observed at the pt, and that the balance between these positive and negative aspects is affected by the level of $NaHCO_3$ in the medium, as was also observed with MCDB-302. However, it seems that due to the additional differences in composition between E4 and MCDB-302, when revertant cells are cultured in E4 the deleterious effects of the reversion predominate over those that are growth promoting.

The overall conclusion that may be drawn from these results, therefore, is that the use of 7 mM $NaHCO_3$ MCDB-302 as a growth medium for K12 cells at 40°C causes the emergence of a population of revertant cells which have undergone a distinct genetic change, allowing for their continuous growth at the npt. However, the precise nature of the genetic alteration is unclear, and requires further investigation.

Table 3.77 GROWTH OF K12 CELLS AT 40°C IN 5% FCS MCDB-302 CONTAINING 26 OR 7 mM $NaHCO_3$

MEDIUM	COLONY NUMBER	AVERAGE COLONY NUMBER
26 mM $NaHCO_3$ MCDB	0	
	0	0
7 mM $NaHCO_3$ MCDB	33	
	37	35

Table 3.78 GROWTH OF K12 IN MEDIA WITH VARYING $NaHCO_3$ CONCENTRATIONS

MEDIUM	TEMPERATURE	COLONY NUMBER	E.O.P.	AVERAGE E.O.P.
7 mM $NaHCO_3$ MCDB	33°C	234	58.5	
		225	56.3	57.4
	40°C	0	0	
		0	0	0
26 mM $NaHCO_3$ MCDB	33°C	236	59.0	
		231	57.8	58.4
	40°C	0	0	
		0	0	0
13.3 mM $NaHCO_3$ E4	33°C	220	55.0	
		262	65.5	60.3
	40°C	0	0	
		0	0	0
26 mM $NaHCO_3$ E4	33°C	218	54.5	
		216	54.0	54.3
	40°C	0	0	
		0	0	0

Table 3.79 GROWTH OF Wg-1A IN MEDIA WITH VARYING $NaHCO_3$ CONCENTRATIONS

MEDIUM	TEMPERATURE	COLONY NUMBER	E.O.P.	AVERAGE E.O.P.
7 mM $NaHCO_3$ MCDB	33°C	184	46.0	
		181	45.3	45.6
	40°C	270	67.5	
		266	66.5	67.0
26 mM $NaHCO_3$ MCDB	33°C	233	58.3	
		244	61.0	59.6
	40°C	275	68.8	
		276	69.0	68.9
13.3 mM $NaHCO_3$ E4	33°C	53	13.3	
		50	12.5	12.9
	40°C	196	49.0	
		188	47.0	48.0
26 mM $NaHCO_3$ E4	33°C	263	65.8	
		271	67.8	66.8
	40°C	260	65.0	
		244	61.0	63.0

Table 3.80 GROWTH OF REVERTANT CELLS IN MEDIA WITH OF VARYING $NaHCO_3$ CONCENTRATIONS

MEDIUM	TEMPERATURE	COLONY NUMBER	E.O.P.	AVERAGE E.O.P.
7 mM $NaHCO_3$ MCDB	33°C	67	16.8	
		51	12.8	14.8
	40°C	114	28.5	
		117	29.3	28.9
26 mM $NaHCO_3$ MCDB	33°C	84	21.0	
		95	23.8	22.4
	40°C	68	17.0	
		52	13.0	15.0
13.3 mM $NaHCO_3$ E4	33°C	2	0.5	
		3	0.8	0.6
	40°C	29	7.3	
		27	6.8	7.0
26 mM $NaHCO_3$ E4	33°C	0	0	
		0	0	0
	40°C	0	0	
		0	0	0

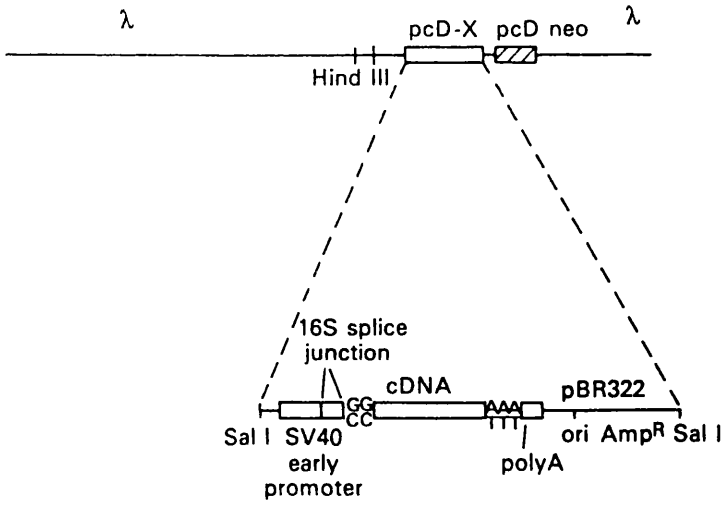
3.18 TRANSFECTION OF K12 WITH THE OKAYAMA-BERG λcDNA LIBRARY

In order to understand the mechanism by which the single gene product that is defective in K12 leads to cell cycle arrest and therefore, by implication, to elucidate the role of this protein in the normal cell cycle, attempts were made to clone the gene encoding it. K12 was transfected with various sources of DNA, and transfectants were selected for by subsequent incubation at 40°C; since untransfected K12 cells are unable to grow at the npt and display an extremely low spontaneous reversion frequency (Roscoe *et al.*, 1973a), any clone that grows at this temperature would be expected to contain DNA encoding a function that can complement the K12 defect.

The first type of DNA used in the cloning experiments was the Okayama-Berg λcDNA expression library (Okayama and Berg, 1985), where human cDNA clones of up to 9 kb have been inserted into a λNMT vector under the control of the SV40 early region promoter (Fig. 3.49) and packaged, allowing for a high efficiency of transfection into mammalian cells. The vector also incorporates the Tn5 *neo* gene from pSV2neo, which expresses neomycin phosphotransferase and consequently confers resistance to the antibiotic G418, thus providing a dominant selectable marker for transfected mammalian cells (Colbere-Garapin *et al.*, 1981). Evidence suggests that direct selection for some phenotypes may not yield transfectants even if the complementing gene is present in the library (Okayama and Berg, 1985); it seems likely that it is necessary for a certain level of the required gene product to be accumulated before selection is implemented for full resistance to the selecting conditions to be achieved. A sequential selection procedure was thus applied to transfected K12 cells; the transfectants were held at 33°C for 4 weeks in the presence of 500 μg/ml G418, in order to eliminate untransfected cells and to allow time for accumulation of the K12-complementing gene product, and were subsequently incubated at 40°C.

The λNMT vector has the additional advantage of containing both SV40 and pBR322 origins of replication. As a consequence, recombinant molecules may be recovered from transfectants by their fusion with COS cells. These cells, a derivative of the CV-1 monkey cell line, constitutively express the large T antigen of SV40 (Gluzman, 1981) which, in concert with additional factors provided by the COS cells, allows for the replication of the recombinant molecule from the integrated SV40 origin. The replicated DNA is subsequently excised to form episomal circular molecules, and may be isolated from the hybrid cells, free of genomic DNA, by a simple plasmid preparation. The recombinant DNA may then be cloned by transformation into *E. coli*, in which it can replicate using the pBR322 origin of replication, and may be selected for by the presence of an ampicillin resistance gene.

Figure 3.49 Structure of the Okayama-Berg λcDNA library (adapted from Okayama and Berg, 1985)



λ - λ-derived DNA

Amp^R - ampicillin resistance gene from pBR322

pcD neo - neomycin phosphotransferase gene from pSV2neo

The λcDNA library was grown up (Section 2.15.ii) and purified for transfection. Initial purification methods involved caesium chloride centrifugation (Section 2.15.iii), but the efficiency of recovery of intact phage was found to be extremely low (97% of the phage was lost during the caesium chloride centrifugation step), suggesting either that the virus was inactivated during centrifugation, or that the physical process of recovery itself was inefficient. Subsequent preparations of λcDNA molecules thus used the polyethylene glycol protocol (Section 2.15.iv), which gave significantly improved efficiencies of recovery; approximately 40% of the virus present in the crude lysate was retrieved following purification.

In order to determine the optimum level of virus for efficient transfection, $4x10^5$ K12 cells/6 cm dish were plated, and transfected with λcDNA according to the protocol described in Section 2.18.i. A range of viral concentrations ($4.2x10^9$ - $1.7x10^{10}$ pfu/plate) was applied to duplicate dishes, and transfectant cells were selected for by the application of 500 μg/ml G418 to the growth medium 24 hours after transfection. To confirm that this level of G418 selected with 100% efficiency against untransfected cells, two plates received the HBS/$CaCl_2$ transfection solution but no phage. The dishes were maintained at 33°C for 4 weeks, before the transfection efficiency was assessed (assuming an initial cell density at the time of transfection of $8x10^5$ cells/plate) by counting visible colonies on plates held up to the light (Table 3.81). All dishes were subsequently incubated at 40°C, but no colony survived for more than a few days, thus indicating the absence of any cells

containing a K12-complementing gene.

The efficiency of transfection to G418-resistance was extremely low (at best, approximately 0.01%). Using this library (purified by caesium chloride centrifugation) and a similar transfection method, Okayama and Berg (1985) achieved transfection efficiencies of 0.5 - 1% with mouse L cells. Although tenfold higher concentrations of virus were used by Okayama and Berg, given the hundredfold difference in transfection efficiencies observed, it seems likely that polyethylene glycol precipitation of the virus may significantly reduce the transfection efficiencies which may be attained. Okayama and Berg (1985) suggest that for a rare gene (i.e. present at a frequency of around 10^{-5} in the library), approximately 10^{6} transfected cells are required in order to detect the clone in question. As the present experiment produced a maximum of only 110 G418-resistant colonies/plate, this implied that a total of 10^{4} plates would be required for isolation of the K12-complementing gene from this library. Since this was clearly impractical, and adequate quantities of caesium chloride-purified virus could not be produced for large-scale transfections of K12, an alternative plasmid form of the library was used, in the hope that transfection efficiencies could be improved.

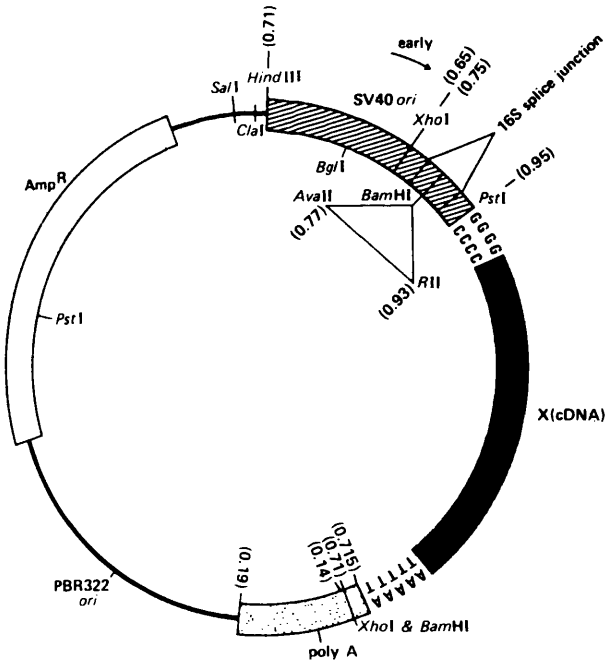
Table 3.81 TRANSFECTION EFFICIENCIES FOR K12 TRANSFECTED WITH THE OKAYAMA-BERG λcDNA LIBRARY

λcDNA (pfu)	COLONY NUMBER	AVERAGE COLONY NUMBER	TRANSFECTION EFFICIENCY (%)
0	0		
	0	0	0
$4.2x10^9$	1		
	16	8.5	$1x10^{-3}$
$8.4x10^9$	21		
	41	31.0	$4x10^{-3}$
$1.3x10^{10}$	4		
	6	5.0	$6x10^{-4}$
$1.7x10^{10}$	110		
	13	61.5	$8x10^{-3}$

3.19 TRANSFECTION OF K12 WITH THE OKAYAMA-BERG PLASMID cDNA LIBRARY

The Okayama-Berg cDNA expression library was originally constructed within the plasmid vector pCD (Okayama and Berg, 1983; Fig. 3.50). The λcDNA library was subsequently formed by partial digestion of the pCD vector at its unique *Sal* I site, and insertion of the entire molecule into the *Sal* I site of λNMT (Okayama and Berg, 1985). The use of the plasmid form of the library, where no restriction endonuclease digestions were carried out on the double-stranded cDNA molecules during its formation, thus avoids the potential problems inherent in the nature of the construction of the λcDNA library, where *Sal* I cleavage of the gene of interest may have occurred (although this seems unlikely, since *Sal* I cuts only rarely within the human genome). As a consequence, the probability that the gene of interest is present in a full-length form is increased. Recovery and isolation of the molecule from transfectants follows the procedure used for the λ form i.e. fusion to COS cells, plasmid purification, and subsequent transformation into *E. coli*.

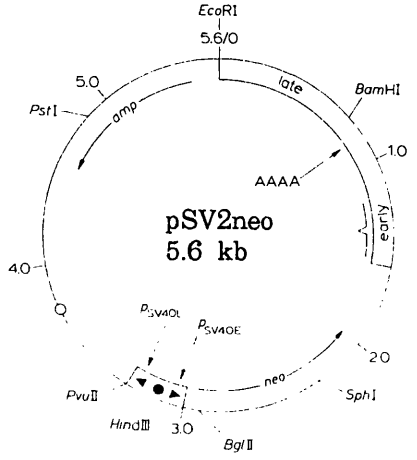
Figure 3.50 Structure of the Okayama-Berg plasmid cDNA library (adapted from Okayama and Berg 1985)



Amp^R - ampicillin resistance gene from pBR322

The main advantage of the pCD-cDNA library for the present purposes, however, was that the Chen and Okayama (1987) high-efficiency transfection procedure could be used for the transfection of K12 cells. This method (Section 2.18.ii), which can only be applied with circular DNA molecules, is based on the standard calcium phosphate precipitation technique, but rather than the usual incubation of the cells at 5% CO_2 for 4 hours in the presence of the precipitate, the cells are incubated under 3% CO_2 for 24 hours, and the buffer 2x BBS (pH 6.95) is used in preference to 1x HBS (pH 6.85) for mixture of the calcium chloride and DNA prior to transfection. The gradual formation of the calcium phosphate-DNA complex which occurs under such conditions appears to be responsible for the increase in the transfection efficiency, perhaps by allowing a larger proportion of the cells to take up the precipitate. However, the underlying basis for this phenomenon is unclear. Using this method, Chen and Okayama obtained transfection efficiencies for the pCD vector of 10 - 50% with a range of mammalian cell lines, compared with efficiencies of approximately 0.5 - 1% for λcDNA when standard calcium phosphate precipitation was used with mouse cells (Okayama and Berg, 1985). As described in Section 3.18, a total of 10^6 transfected cells would be required to detect the K12-complementing gene if it is present at a low frequency in the library. If such high transfection efficiencies could be attained by using this protocol with K12, an initial population of at most 10^7 untransfected cells should therefore be necessary for isolation of the gene of interest. Since the pCD vector lacks a dominant selectable marker, cotransfection with the plasmid pSV2neo (Fig. 3.51), which carries the Tn5 *neo* gene (Pouwels *et al.*, 1985) and thus encodes resistance to G418, was necessary, in order to allow for the sequential selection of transfectants by G418 and temperature.

Figure 3.51 Structure of plasmid pSV2neo (adapted from Pouwels *et al.*, 1985)



amp - ampicillin resistance gene from pBR322

neo - neomycin phosphotransferase gene from Tn5

Both plasmids were grown up (Sections 2.16.i and 2.16.ii) and purified by polyethylene glycol precipitation (Section 2.16.iii). In order to assess the transfection efficiency attainable with the Chen and Okayama high-frequency method, K12 cells were initially transfected with pSV2neo alone, and the proportion of surviving cells which had acquired G418-resistance was calculated as described by Chen and Okayama (1987). Six 10 cm plates of K12 were seeded at a concentration of $5x10^5$ cells/plate, incubated overnight at 33°C, and four of the dishes - A, B, C and D - were transfected with 20 μg of pSV2neo using the high-frequency protocol (Section 2.18.ii). Plates E and F received the calcium chloride-2x BBS transfection solution but no DNA, in order to confirm the potency of 500 μg/ml G418 against untransfected cells. Twenty four hours later, the cells were trypsinized and replated at a concentration of 10^3 cells/dish. Following a further overnight incubation, cells on plates A, B, E and F were selected by the addition of 500 μg/ml G418 to the growth medium. All plates were incubated at 33°C, and the medium was subsequently changed twice weekly. Two weeks after transfection, the colonies on plates C and D were large and near to detaching, and consequently the dishes were stained and the number of colonies was counted (Section 2.5). However, the application of G418 appeared to slow down the growth of transfectant cells in the early stages of selection, perhaps due to an initially insufficient accumulation of the *neo* gene product for full resistance to the antibiotic, thus the remaining plates were stained eleven days later, by which time colonies had become clearly visible.

The results (Table 3.82) show that no colonies were obtained when untransfected cells (plates E and F) were selected with G418, confirming the effectiveness of this antibiotic against mammalian cells. The E.O.P. value for transfected K12 cells unselected by G418 (dishes C and D), which includes both transfectant and nontransfectant colonies and therefore represents the total population of cells surviving transfection, was 23.9%, whereas transfected plates receiving 500 μg/ml G418 (dishes A and B), and thus containing only cells which had been successfully transfected, showed an E.O.P. of 49.4%. This suggests a transfection efficiency of 206%, clearly an overestimate. It seems probable that this inaccuracy was a consequence of the different times of staining of the two sets of plates. Each time the medium is changed on a dish of cells, there is the possibility for secondary colony formation i.e. loosely attached cells, such as those undergoing mitosis, may be washed away from one colony, settle down elsewhere on the dish and start up a new colony. Since plates A and B received three extra medium changes after the staining of plates C and D, the opportunity for secondary colony formation was greater, potentially leading to an overestimation of the true number of transfectant cells.

In order to investigate whether repeated medium changes do indeed lead to a significant alteration in E.O.P. values through secondary colony formation, four 6 cm dishes of K12 cells were seeded at a concentration of 50 cells/plate in 5% FCS E4 low bic (Section 2.4), and incubated at 33°C. The medium was changed three days after plating, and duplicate plates were stained (Section 2.5) four days later. The remaining two plates were stained after a further three medium changes at three-day intervals. The results (Table 3.83) confirm that repeated medium changes substantially increase the E.O.P. (by a factor of seven- to eightfold). As a consequence, the true figure for the efficiency of transfection of K12 to G418-resistance with pSV2neo must be reduced to approximately 30% to correct for this factor. Based on this figure, an initial population of only $3x10^6$ cells should be sufficient to isolate the K12-complementing gene if it is present, even at a low frequency, in the library. However, since Chen and Okayama (1987) demonstrate that the transfection efficiency is reduced somewhat by the use of cotransfection, a starting population of approximately 10^7 cells was transfected.

Twelve 10 cm dishes of K12 were plated at a concentration of $5x10^5$ cells/plate in 5% FCS E4 low bic, and incubated at 33°C. Twenty four hours later, high-frequency transfection (Section 2.18.ii) was carried out according to the following pattern: plates 1 - 10 received 18 μg of the pCD-cDNA library and 2 μg of pSV2neo, 2 μg of pSV2neo alone was added to plate 11, and plate 12 received no DNA. Cells were split at a ratio of 1 in 5 the following day and selection, in the form of 500 μg/ml G418, was applied after a further overnight incubation at 33°C. All plates were shifted to 40°C two weeks after transfection, by which time there were no cells on dish 12, confirming the lethal nature of 500 μg/ml G418 for untransfected cells. Nine days later, each plate was examined for colonies under a dissecting microscope. Plate 11 contained no viable cells, indicating that the presence of the *neo* gene alone is unable to complement the defect in K12 or to cause its reversion. Of the cotransfected plates (plates 1 - 10), five dishes each contained a single colony. However, the colonies were small, and the constituent cells were rounded and unhealthy in appearance, thus the plates were moved to a 33°C incubator in an attempt to prevent the cells from dying. The following day, each colony was trypsinized within a cloning cylinder. Since the cells were loosely attached, the second trypsin wash was combined with the resuspended fibroblasts, to ensure the transfer of all cells, and the resulting suspension, containing 500 μg/ml G418, was split between two 6 cm dishes, one of which was placed at 33°C and the other at 40°C. Two weeks after this transfer, no viable cells were found on any plate. It appears likely that these cells contained the K12-complementing gene, since they survived for 9 days at 40°C, whereas untransfected cells generally lose viability under these

conditions within 3 days (Table 3.61). If it is assumed, using the Chen and Okayama (1987) figure of a fivefold reduction in transfection efficiencies when cotransfection is used, that the cotransfection efficiency was roughly 6%, the frequency of transfection to temperature-resistance (approximately 1 in 10^5 transfected cells) suggested that the gene in question is found at a low frequency in the library. However, the presence of this gene within the cell appeared to be unable to allow for prolonged survival at the high temperature. This could be for a number of reasons:

a. It is possible that one of the cotransfected plasmids was unstable and was lost from the cell line during incubation at 40°C, leading to cell death. Indeed, Alder *et al.* (1989) also noted this phenomenon when attempting to complement the defect in several hamster ts cell cycle mutants by transfection with a clone from the pCD-cDNA library; transfectants grew for several weeks at the npt, but subsequently degenerated rapidly, suggesting loss of the pCD plasmid (Alder *et al.*, 1989; Prof. R. Baserga, personal communication).

b. The SV40 early region promoter, used for expression of the cDNA insert, acts as a constitutive promoter in the transfected cell. It is conceivable that uncontrolled expression of the K12-complementing gene, although allowing for transient complementation of the defect, becomes lethal if the protein product accumulates in high quantities or is consistently present at an inappropriate time in the cell cycle. However, if this is the case, it seems unlikely that colonies would survive for as long as the observed period of 3 - 4 weeks following transfection.

c. The human homologue of the gene of interest may be unable to fully compensate for the defect in the Chinese hamster background. Although the human homologue has been shown to be capable of complementing the defect in K12-human hybrid cells (Ming *et al.*, 1979), it is possible that in order to do so the gene product in question must interact with a further human protein for full activity. In the K12 transfectant cells, where no additional human genes may be present, the hamster homologue of the required protein may be unable to fulfil the same role to an adequate extent, thus survival is prolonged at 40°C, but only transiently.

d. It may be that cell cycle progression in K12 requires a high level of the protein defined by the ts mutation at a particular point in the cycle, and that expression of the K12 gene encoding it is normally increased at this time by interactions between its promoter and specific regulatory molecules. If the hamster molecules are unable to interact with the SV40 promoter to enhance expression of the human homologue at the required time, it is possible that despite the fact that the gene product is constitutively produced throughout the cycle, insufficient quantities are present when needed, thus the cell is incapable of prolonged survival.

In conclusion, this experiment suggests that although there is a human homologue

to the gene defective in K12, it may not be isolated by using the Okayama-Berg cDNA expression library.

Table 3.82 EFFICIENCY OF TRANSFECTION OF K12 BY pSV2neo

PLATE	CONCENTRATION OF pSV2neo RECEIVED (μg)	G418 SELECTION	COLONY NUMBER	E.O.P.	AVERAGE E.O.P.
A	20	yes	657	65.7	
B	20	yes	330	33.0	49.4
C	20	no	241	24.1	
D	20	no	237	23.7	23.9
E	0	yes	0	0	
F	0	yes	0	0	0

Table 3.83 EFFECTS OF REPEATED MEDIUM CHANGES ON THE E.O.P. OF K12 AT 33°C

NUMBER OF MEDIUM CHANGES	COLONY NUMBER	E.O.P.	AVERAGE E.O.P.
1	35	70	
	38	76	73
4	326	652	
	199	398	525

3.20 PRODUCTION OF K12-HUMAN HYBRID CELLS IN ORDER TO ENRICH FOR THE HUMAN HOMOLOGUE OF THE GENE DEFECTIVE IN K12

The transfection of K12 with human genomic DNA, where each gene retains its own promoter, should eliminate one of the potential problems inherent in the Okayama-Berg cDNA libraries, namely the use of an SV40 promoter for expression of the human insert. In order to isolate the human homologue of the K12 defective gene for further analysis, the DNA from any temperature-resistant transfectants could be used to construct a genomic library, and screened for the presence of human DNA with the BLUR-8 probe (Rubin *et al.*, 1980), which hybridizes to the short highly repetitive human-specific *Alu* sequences which are found every few kilobases along the human genome (Houck *et al.*, 1979). However, primary K12 transfectants obtained using this method may contain a number of human genes, since evidence suggests that each primary transfectant cell incorporates approximately 1000 kb of exogenous DNA into its genome (Perucho *et al.*, 1980), and only one of these genes, presumably, would be responsible for complementing the K12 defect. In order to eliminate the irrelevant human sequences, and therefore to facilitate the identification and isolation of the gene of interest, a second round of transfection, using DNA prepared from the primary transfectants, must be carried out, since the majority of the additional DNA acquired by the transfected cell would then be expected to originate from the K12 genome.

Preliminary experiments carried out by Dr. D.H. Roscoe (unpublished work), where K12 was cotransfected with pSV2neo and genomic DNA from the human tumour line A431, resulted in the isolation of temperature-resistant primary transfectants at a frequency of 1 in 10^4 - 10^5 transfectant cells. However, growth of these cells at the npt was unhealthy, and attempts to transfer the human gene of interest to secondary transfectants were unsuccessful; colonies were obtained at a low frequency (approximately 1 in $3x10^4$ G418-resistant colonies), but they grew at the npt only poorly and died after several weeks at 40°C. This secondary transfection step would be rendered superfluous, however, if the amount of irrelevant human DNA present in the primary transfectants could be significantly reduced. In order to achieve this, it was decided to use K12-human hybrid cells as the source of genomic DNA for transfection.

When hamster and human cells are fused, the hybrid cell retains all the hamster DNA, but human chromosomes inessential for survival are gradually eliminated (Kao and Puck, 1970). Thus, if K12 and human cells were fused and the hybrids were maintained at an incubation temperature of 40°C, the chromosome carrying the human homologue of the gene defective in K12 would be retained, since its presence

would be necessary for growth, but much of the additional human DNA would be lost. As a consequence, genomic DNA prepared from the hybrids should contain a small number of human sequences, including the gene of interest, present in a Chinese hamster background. This would increase the likelihood that the human K12-complementing gene would be the sole human sequence in temperature-resistant K12 cells transfected with such DNA, thus obviating the requirement for a second round of transfection. In the unlikely event that a small number of additional human sequences were present in the transfectant cell, the relevant molecule could be determined following screening simply by its ability to complement the K12 defect on transfection.

K12 and human fibroblasts were fused by the polyethylene glycol fusion method, according to the protocol outlined in Section 2.19.i. Initially, selection was imposed upon unfused fibroblasts simply by maintaining the cells at 40°C, since neither parental cell line should grow at this temperature. However, many unfused human cells, which are elongated and needle-like in appearance in contrast to the polygonal hybrid cells, were still present one month after fusion. Such parental human fibroblasts were eliminated by the addition of ouabain to the medium at a level which is lethal to human cells but to which hybrid cells are resistant. After a further month of growth, 17 large hybrid colonies were detected on a total of 6 plates. In order to produce independent clones, each colony was transferred to a separate dish, using cloning cylinders for isolated trypsinization (Section 2.19.ii), and the cells were grown up for further analysis.

It has previously been shown that human chromosome 14 is the only human chromosome invariably retained in K12-human hybrids maintained at 40°C, and thus presumably carries a gene complementing the K12 mutation (Ming *et al.*, 1979). The hybrids produced in the current study were therefore tested for the retention of human chromosome 14 by Dr. S. Povey (University College London). In addition, the presence of human chromosomes 11, 12 and 19 was also assessed for each clone, in order to provide an indication of the number of human chromosomes remaining two months after fusion. The protocol followed for chromosome complement analysis was that described by Siciliano and White (1987). Since many of the enzymes shared by human and Chinese hamster cells exhibit species-specific variations in their amino acid sequences, the human and hamster forms of the proteins in question can often be distinguished by differences in their migration patterns on starch gel electrophoresis of cell homogenates. If the chromosomal location of the gene encoding such an enzyme is known, the presence of the human form of the protein can thus be used as a marker to indicate the retention of a particular human chromosome within a hybrid cell. Enzymes displaying such species-specific differences in

electrophoretic migration have been described for each of the human chromosomes. The hybrid cell may therefore be screened for the retention of a specific human chromosome by starch gel electrophoresis of a cell homogenate, followed by the use of a colourimetric assay system designed to visualize the presence of an enzyme associated with the chromosome in question. Comparison of the resultant pattern of bands with control lanes containing only human or hamster cell preparations will indicate whether the human form of the protein, and therefore by implication its associated chromosome, is present within the cell. The presence of the trimeric protein nucleoside phosphorylase was used to test for the retention of chromosome 14 in the K12-human hybrid clones, lactate dehydrogenase A was used for chromosome 11, lactate dehydrogenase B for chromosome 12, and glucose phosphate isomerase for chromosome 19.

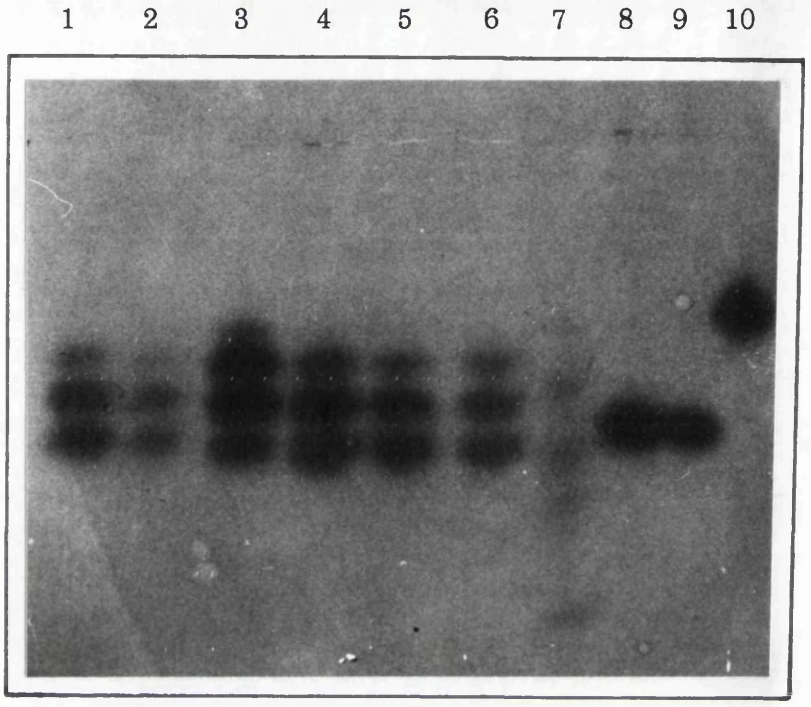
The results (Table 3.84, and see Fig. 3.52 for an example of starch gel analysis) indicate that all 17 hybrids contained human chromosome 14, and each of the other human chromosomes screened was present in only a limited number of hybrid clones, thus supporting the hypothesis that chromosome 14 carries the human K12-complementing gene (Ming *et al.*, 1979). However, unless each clone is screened for the presence or absence of all of the human chromosomes and chromosome 14 is shown to be the only one invariably retained, this cannot be stated for certain.

For the purposes of providing a source of genomic DNA for transfection, it was necessary to reduce the number of irrelevant human chromosomes present in the hybrids as far as possible. Since hybrids 4, 12 and 13 all contained only one of the additional three human chromosomes tested for, these were further screened for the presence of human chromosomes 6 and 20. However, each hybrid clone contained both of these chromosomes, thus it appeared that much superfluous DNA had yet to be eliminated. Hybrid 4 was therefore grown up, and rapidly dividing cells were selected by continuous subcloning (Section 2.19.iii), in order to maximize the rate of loss of irrelevant human DNA; it seems likely that inessential chromosomes are lost at the time of cell division (Ruddle, 1972; Handmaker, 1973), thus a faster rate of growth will provide more opportunities for their elimination. Following three months of such subcloning, hybrid 4 was screened for the retention of human chromosomes 1, 3, 5, 6, 9, 10, 11, 12, 14, 15, 17, 18, 19 and 21, and chromosomes 1, 3, 6, 10, 12, 14, 18 and 21 were still present. Since so much irrelevant human DNA remained seven months after the initial fusion of the parental fibroblasts, this method of providing a source of genomic DNA for transfection of K12 that would obviate the need for a secondary transfection step was deemed impractical, given the time scale required to achieve its final aim, and was thus abandoned.

Table 3.84 HUMAN CHROMOSOME ANALYSIS OF K12-HUMAN HYBRIDS TWO MONTHS AFTER FUSION

HYBRID	HUMAN CHROMOSOME 14	HUMAN CHROMOSOME 11	HUMAN CHROMOSOME 12	HUMAN CHROMOSOME 19
1	present	present	present	present
2	present	present	present	present
3	present	absent	present	present
4	present	absent	present	absent
5	present	present	present	present
6	present	present	present	present
7	present	present	present	absent
8	present	absent	present	present
9	present	absent	present	present
10	present	present	present	present
11	present	present	present	present
12	present	absent	present	absent
13	present	absent	present	absent
14	present	present	present	present
15	present	present	present	present
16	present	present	absent	present
17	present	present	present	present

Figure 3.52 STARCH GEL OF K12-HUMAN HYBRID CELL HOMOGENATES STAINED FOR NUCLEOSIDE PHOSPHORYLASE

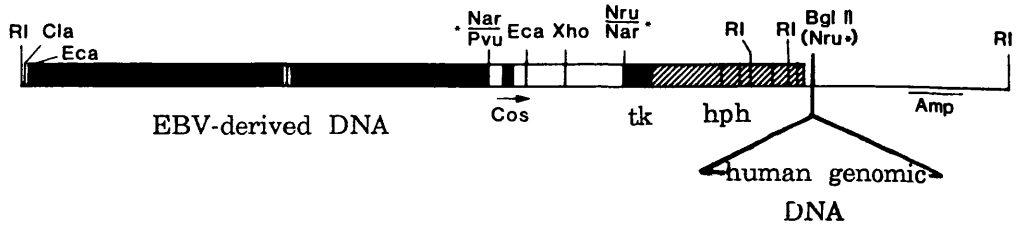


Track 1 - hybrid 15 Track 2 - hybrid 10 Track 3 - hybrid 8
Track 4 - hybrid 13 Track 5 - hybrid 2 Track 6 - hybrid 17
Track 7 - hybrid 5 Track 8 - Wg-1A Track 9 - K12
Track 10 - Namalwa (human) cell line

3.21 USE OF AN EPSTEIN-BARR VIRUS-BASED HUMAN GENOMIC LIBRARY FOR TRANSFECTION OF K12

In an alternative approach for circumventing the potential problems caused by the employment of a heterologous SV40 promoter for expression of the human insert in the Okayama-Berg cDNA libraries, an Epstein-Barr virus-based cosmid human genomic DNA library (Kioussis *et al.*, 1987) was used for transfection of K12, in an attempt to isolate a K12-complementing gene. This library incorporates a number of useful features: (i.) The cosmid vector (cos202) carries the Epstein-Barr virus origin of replication, oriP, and the *EBNA-1* gene (Fig. 3.53). The product of this gene, the EBNA-1 antigen, interacts with oriP to allow the molecule to replicate autonomously within the transfected cell (Yates *et al.*, 1985), thus stable expression of the human insert does not require integration of the vector into the host's chromosomes, and the level of transcription of the gene is no longer dependent on the integration site. (ii.) Hygromycin B, an inhibitor of protein synthesis in both prokaryotes and eukaryotes (Gonzalez *et al.*, 1978), can be used for the selection of transfectant cells, since the cosmid contains the hygromycin B-resistance gene, *hph*, which encodes hygromycin B phosphotransferase (Gritz and Davies, 1983). (iii.) The vector incorporates the *cos* site from phage λ and the bacterial origin of replication and ampicillin-resistance gene from plasmid pBR322, therefore once purified as an episomal molecule from the transfectants, may be packaged by a λ packaging extract and cloned directly into *E. coli*. (iv.) The human genomic DNA insert is large (35 - 40 kb) thus, even if the required gene is rare, the probability that it is present in a full-length form, complete with all necessary regulatory sequences, is high.

Figure 3.53 Structure of cos202 (adapted from Kioussis *et al.*, 1987)



Cos - phage λ cos site

Amp - ampicillin-resistance gene from pBR322

tk - thymidine kinase promoter from herpes simplex virus (HSV)

hph - hygromycin B-resistance gene from *E. coli*

However, Kioussis *et al.* (1987) found that the transfection frequency of HeLa cells with the cos202 library was low (10 colonies/5 μg DNA/5x10^5 cells). The efficiency increased approximately one-hundredfold when this cell line was first transfected with the *EBNA-1*-bearing plasmid pSVoB-H2.9 (Yates *et al.*, 1985), and thus stably expressed the EBNA-1 antigen. This clearly implies that the cos202 *EBNA-1* gene functions inadequately in the cosmid background, thus the vector is unable to replicate in cells not already expressing the EBNA-1 antigen. It appears likely that the low frequency of transfection to hygromycin B-resistance in EBNA$^-$ HeLa cells was a consequence of cosmid integration into the genome and subsequent EBNA-1-independent replication.

K12 was transfected with the cos202 library, using both the standard calcium phosphate precipitation method and the Chen-Okayama high-frequency protocol, in order to investigate whether cos202 was capable of autonomous replication in this cell line. To determine the level of hygromycin B required to select against untransfected cells with 100% efficiency, six 6 cm dishes were each seeded with 3x10^5 K12 cells in 5% FCS E4 low bic (Section 2.4), and placed in a 33°C incubator. Two days later, hygromycin B was added to the dishes at a concentration of 0, 100, 175, 250, 300 or 350 μg/ml. The plates were returned to the 33°C incubator, and the dishes were stained and the colonies counted (Section 2.5) one week later. The results (Table 3.85) suggested that a hygromycin B level of 100 μg/ml was sufficient to select against untransfected K12 cells.

The cosmid library (obtained from Dr. P. Brickell, University College London) was supplied in four aliquots, each representing one quarter of the total library. Standard calcium phosphate transfection was carried out as described in Section 2.18.i, using five 6 cm dishes of K12 seeded at a concentration of 4x10^5 cells/plate. One plate received no DNA, in order to confirm the efficacy of hygromycin B selection against untransfected cells, and duplicate plates were each transfected with either 10 or 15 μg of Aliquot 1 of the cos202 library. Twenty four hours after transfection, each dish was split at a ratio of 1 in 5 (Kioussis *et al.* [1987] suggest that this procedure results in a five- to tenfold increase in the number of hygromycin B-resistant colonies), and after a further day of incubation at 33°C, all plates were selected with 100μg/ml hygromycin B. The medium was subsequently changed twice weekly. One week after the initial selection, no healthy cells could be seen on any plate. Incubation was continued for a further two weeks, but no colonies appeared to have survived selection.

For high-frequency transfection (Section 2.18.ii), three 10 cm dishes of K12 were seeded at a concentration of 5x10^5 cells/plate. Duplicate plates each received 20 μg of Aliquot 1 of the cosmid library, and the transfection buffer alone was added to the

remaining control dish. Each plate was split at a ratio of 1 in 5 twenty four hours after transfection, 100 μg/ml hygromycin B was added to all plates the following day, and incubation was continued at 33°C with twice weekly medium changes. All cells died within one week of selection.

The inability to obtain hygromycin B-resistant cells in these experiments clearly suggests that cos202 is unable to replicate within K12, presumably due to the inadequate functioning of the vector *EBNA-1* gene, and is consequently lost from the transfected population. As described earlier, Kioussis *et al.* (1987) overcame this problem in HeLa cells by first transfecting the culture with the *EBNA-1*-bearing plasmid pSVoB-H2.9. However, Yates *et al.* (1985) provide evidence to suggest that mouse BALB/c3T3 cells carrying the integrated pSVoB-H2.9 plasmid and expressing detectable levels of EBNA-1, still did not allow for the replication of an oriP-bearing plasmid within the cell and, in addition, a number of rodent cell lines transfected with plasmids incorporating oriP, a functional *EBNA-1* gene, and *hph* were incapable of supporting autonomous replication of the molecules, despite the fact that these same plasmids were maintained episomally in a variety of human, monkey and dog cell lines. A low frequency (1 - $9x10^{-4}$) of transfection to hygromycin B-resistance was achieved, however, by integration of the plasmid molecule into the rodent cell genome. It thus appears that the EBNA-1 antigen is incapable of functioning within a rodent cell background.

In the preliminary transfections of K12 with cos202, no hygromycin B-resistant cells were isolated even with an initial population of 2 - $3x10^6$ cells. This suggests either that the cos202 vector integrates with an extremely low frequency within the K12 genome, or that expression of the hygromycin B-resistance gene from the integrated molecule is inadequate in the hamster background. The *hph* gene is under the control of the HSV thymidine kinase promoter, which is a fairly weak promoter (Gorman *et al.*, 1983). It is therefore possible that the accumulation of an adequate level of hygromycin B phosphotransferase requires the replication of the cos202 vector and a resultant increase in the *hph* gene copy number (Kioussis *et al.* [1987] suggest a cos202 copy number of 1 - 10 in transfected human fibrosarcoma 143/EBNA$^+$ cells). In EBNA$^-$ cell lines, such as K12, survival under hygromycin B selection may therefore require the integration of a number of cos202 molecules within a single transfectant cell, and in addition, transcription of the *hph* gene may be suppressed in particular integration sites, thus significantly reducing the transfection efficiency. In order to ensure the survival of any transfectant cells in which the cos202 molecule had become integrated, K12 was therefore cotransfected with both the cos202 library and pSV2neo (the latter molecule had been shown in previous transfections [Sections 3.18 and 3.19] to act as an efficient selective marker in K12), and the cells were

selected with G418 rather than with hygromycin B.

Small-scale cotransfection experiments, using both the standard calcium phosphate precipitation and the high-frequency transfection methods, were initially carried out, with the aim of assessing and comparing transfection efficiencies. For the standard transfection method (Section 2.18.i), eight 6 cm plates of K12 were seeded at a concentration of $4x10^5$ cells/dish. After overnight incubation at 33°C, plates 1 and 2 received no DNA, in order to confirm the efficacy of G418 selection, plates 3 and 4 were each transfected with 1 μg of pSV2neo, and the remaining four dishes each received 1 μg of pSV2neo and 1 μg of Aliquot 2 of the cos202 library. The following day, the cells on plates 3, 4, 7 and 8 were trypsinized, counted, and split at a ratio of 1 in 5. The cells from plates 5 and 6 were also trypsinized and counted, but these were replated at a concentration of 10^3 cells/dish, in order to calculate the proportion of cells surviving the process of transfection. All plates, with the exception of 5 and 6, were selected with 500 μg/ml G418 twenty four hours later, and the medium was subsequently changed twice weekly. One week after the initial selection, plates 1, 2, 5 and 6 were stained and counted (Section 2.5). The number of colonies on plates 3, 4, 7 and 8 was estimated by eye, and the incubation temperature was increased to 40°C. Ten days after the temperature shift, all colonies were dead.

The results (Table 3.86) demonstrate that the E.O.P. for transfected K12 cells unselected by G418 (plates 5 and 6), which includes both transfectant and nontransfectant colonies, was approximately 44%. This value decreased to 1.2% when the cells were selected with 500 μg/ml G418, suggesting that roughly 2.7% of those fibroblasts surviving transfection had undergone successful integration of the pSV2neo molecule. The transfection efficiency was similar in cells receiving pSV2neo alone, implying that the presence of the cosmid does not inhibit pSV2neo uptake. Given such a high transfection efficiency, and if it is assumed that the majority of G418-resistant K12 cells also take up a cosmid molecule (a study by Pellicer *et al.* [1980] on the cotransfection process in rat cells suggested that approximately 80% of selected cotransfectants may also contain the unselected gene), then it seemed reasonable to conclude that it should be possible to isolate the human homologue of the defective gene in K12 from the library; even if the gene is rare i.e. present at a frequency of around 1 in 10^5, and therefore requiring up to 10^6 transfected cells for detection, an initial population of approximately $3x10^7$ K12 cells should be sufficient for its recovery. A trial pSV2neo/cos202 cotransfection was also carried out using the high-frequency protocol (Section 2.18.ii), but the efficiency of transfection to G418-resistance was only 0.03%, suggesting that this method does not work well with molecules as large as the cosmid library.

A full-scale cotransfection of K12 with pSV2neo and the cos202 library was

therefore carried out. For each of the four aliquots of the library, five 10 cm plates of K12 were seeded at a concentration of $1.2x10^6$ cells/dish. Following overnight incubation at 33°C, each plate was transfected with 3 μg of pSV2neo and 3 μg of the cosmid library using the standard calcium phosphate precipitation method (Section 2.18.i). In order to confirm the selective nature of 500 μg/ml G418, a single 10 cm dish was also seeded with $1.2x10^6$ K12 cells, but received no DNA. Twenty four hours after transfection, each cotransfected plate was split at a ratio of 1 in 5, and all dishes were selected with 500 μg/ml G418 the following day. The medium was subsequently changed twice weekly. One week after the initial selection, each cotransfectant plate was approximately 50% confluent, and all dishes were moved to a 40°C incubator. No colonies were visible on the control plate. Three weeks after the temperature shift, no healthy colonies were observed on any plate. However, each cotransfectant dish which had received DNA from Aliquot 2 of the cosmid library contained several rounded-up colonies of between 10 and 100 cells, which died and detached soon afterwards. Given the similarity between this pattern of results and those found with the Okayama-Berg plasmid cDNA library (Section 3.19) i.e. in each case, several colonies survived for a few weeks at the npt, but subsequently died, it appears likely that the human K12-complementing gene is present in each of these libraries, but even if its expression is regulated by its natural promoter, although capable of prolonging the viability of K12 cells at 40°C, it is unable to support their continued survival. This may be due to an inappropriate pattern of expression of the gene product, or to a requirement for interaction of the protein in question with an accessory human molecule, for which the Chinese hamster homologue is unable to substitute, as discussed in detail in Section 3.19.

Table 3.85 EFFECTS OF VARYING LEVELS OF HYGROMYCIN B ON THE GROWTH OF K12 CELLS AT 33°C

HYGROMYCIN B CONCENTRATION (μg/ml)	COLONY NUMBER	E.O.P.
0	confluent	high
100	0	0
175	0	0
250	0	0
300	0	0
350	0	0

Table 3.86 EFFICIENCY OF COTRANSFECTION OF K12 WITH cos202/pSV2neo

PLATE	CELLS PLATED AFTER SPLIT	COLONY NUMBER	E.O.P.	AVERAGE E.O.P.
1	/	0	0	
2	/	0	0	0
3	1.9×10^5	~2000	1.1	
4	2.5×10^5	~2000	0.8	0.9
5	10^3	459	45.9	
6	10^3	418	41.8	43.9
7	1.8×10^5	~2000	1.1	
8	1.6×10^5	~2000	1.3	1.2

3.22 TRANSFECTION OF K12 WITH Wg-1A GENOMIC DNA

In order to confirm that the defective K12 gene can indeed be complemented by the process of transfection, and that the problems in the previous cloning experiments were therefore caused by the nature of the libraries used, K12 was cotransfected with genomic DNA from the wild-type parent, Wg-1A, and the selective marker pSV2neo; this method should avoid the potential difficulties of incompatibility of genes or gene products.

High-molecular-weight Wg-1A and K12 genomic DNA was prepared according to the protocol outlined in Section 2.17 (Fig. 3.55), and the standard calcium phosphate precipitation method of transfection was used (Section 2.18.i). Seventeen 10 cm plates were each seeded with 10^6 K12 cells, and incubated overnight at 33°C. The following day, plates 1 and 2 received no DNA, to confirm the selective nature of 500 μg/ml G418. Plates 3 and 4 were each transfected with 1 μg of pSV2neo, and plates 5 - 7 received 1 μg of pSV2neo and 20 μg of K12 genomic DNA, in order to demonstrate that neither pSV2neo nor K12 DNA is capable of complementing the K12 defect. The remaining ten plates were each cotransfected with 1 μg of pSV2neo and 20 μg of Wg-1A genomic DNA. Twenty four hours after transfection, 500 μg/ml G418 was applied, and the medium was subsequently changed twice weekly.

One week after the initial selection, although no healthy cells were observed on control plates 1 and 2, dishes 3 - 17 each contained approximately 2,000 colonies. The transfectant plates attained 25 - 50% confluence three days later, at which point the temperature was increased to 40°C. Each dish was examined after a further week of incubation. No colonies were detected on plates 3 - 7, and four of the dishes transfected with Wg-1A DNA were fungally contaminated. However, three of the remaining six Wg-1A/pSV2neo cotransfectant plates each contained several large, healthy colonies; a total of 12 temperature-resistant transfectant colonies (four on plate 8, three on plate 11 and five on plate 12) was isolated. Given that 2000 cells/plate were successfully transfected to G418-resistance, and evidence suggests that each transfectant cell integrates an average of 1000 kb of exogenous DNA (Perucho *et al.*, 1980), it would appear that a total of approximately 1.2×10^7 kb of DNA was taken up on the six cotransfected dishes. The mammalian cell genome contains in the order of 3×10^6 kb of DNA (Winnacker, 1987), thus the figure of 12 temperature-resistant transfectant clones per four genome's worth of transfected DNA suggested that there are roughly three copies of the K12-complementing gene/Wg-1A genome. However, this figure should be regarded as an upper estimate, since the 3 - 5 ts^+ transfectant colonies obtained on each successfully cotransfected plate may not represent independent clones, due to the opportunity for secondary colony formation

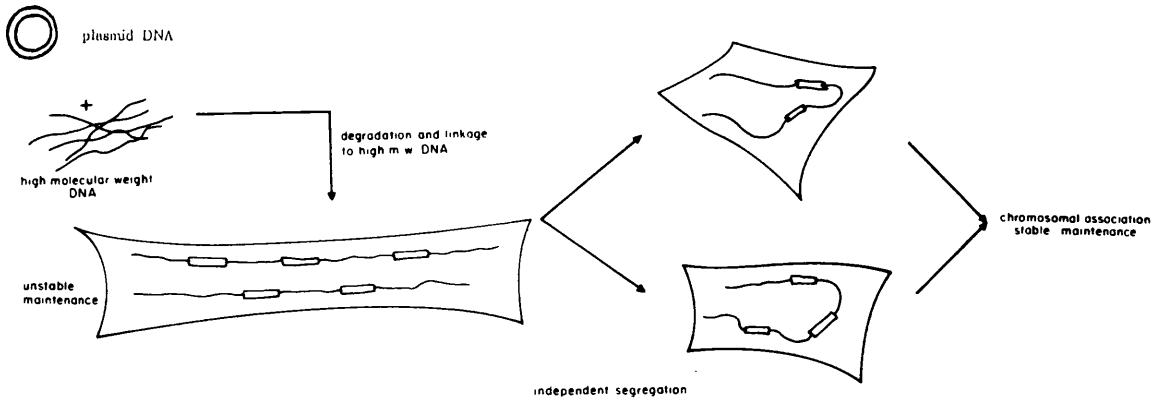
during the repeated medium changes, and it seems more likely that the K12 ts gene is a single copy sequence.

Although this study clearly demonstrates that Wg-1A genomic DNA can successfully complement the defect in K12 cells, the gene of interest cannot be detected in and isolated from the temperature-resistant transfectant cells, since the DNA from the two cell lines should be indistinguishable in all aspects except for the single mutation in the gene in question; in order to achieve this, the Wg-1A DNA must be tagged with a physical marker to allow for its identification in the K12 background. The *neo* gene from pSV2neo would seem to be an attractive candidate for such a purpose; the ligation of *neo* to Wg-1A genomic DNA would provide both a physical tag for the associated Wg-1A genes (recombinant clones containing Wg-1A DNA may then be isolated from a transfectant cell cosmid library by probing for the presence of the linked *neo* gene with pSV2neo), and would act as a selective marker, thus obviating the need for cotransfection.

However, the procedure of *in vitro* linkage is time-consuming, thus a potentially shorter option was chosen. A number of lines of evidence suggest that when high-molecular-weight DNA is cotransfected with a plasmid-borne selectable marker, the large DNA fragments are degraded, the plasmid molecules are linearized, and the unrelated DNA sequences are linked together by random blunt-end ligations to generate a large concatamer or transgenome (Perucho *et al.*, 1980; Scangos *et al.*, 1981; see Fig. 3.54). This structure has an average size of approximately 1000 kb, and consists of plasmid sequences interspersed with the non-selectable DNA. The transgenome is initially present in an episomal form within the cell, and is thus unstable, but if selective pressure is continuously applied, subsequently integrates at a unique site in the host's chromosomes, and is stably maintained (Scangos *et al.*, 1981). A single transgenome molecule is usually found within each stable recipient. It thus appeared that rather than performing the lengthy process of ligating the *neo* gene to genomic Wg-1A DNA *in vitro*, in order to facilitate the isolation of the K12-complementing gene from transfectant cells, it may be possible to construct the required recombinant DNA molecules *in vivo*; if Wg-1A high-molecular-weight genomic DNA was cotransfected with pSV2neo, a transgenome consisting of Wg-1A DNA interspersed randomly with a significant number of *neo* sequences should form during the transfection process, and integrate into the K12 genome. Indeed, when Perucho *et al.* (1980) cotransfected mouse Ltk^- cells with 1 ng of the HSV thymidine kinase (tk) gene (a selectable marker which allows for the growth of tk^- cells in medium containing hypoxanthine, aminopterin and thymidine [HAT medium]), 1 μg of phage ØX174 DNA, and 20 μg of high-molecular-weight carrier salmon DNA per plate, analysis of a transfectant clone suggested that approximately 25 copies of the ØX174

DNA had become integrated. In addition, Pellicer *et al.* (1980) demonstrated that between 1 and 100 copies of the human growth hormone (hGH) gene became integrated in rat BRL tk^- cells cotransfected with 1 μg of the HSV tk gene, 1 μg of a plasmid bearing the hGH gene and 20 μg of high-molecular-weight carrier mouse Ltk^- DNA. Based on this evidence, if K12 was cotransfected with 1 μg of pSV2neo and 20 μg of high-molecular-weight Wg-1A genomic DNA, 25 - 100 copies (which represents a spacing of one copy of the *neo* gene per 10 - 40 kb of transgenome DNA) of the *neo* gene may be present in the integrated transgenome (pSV2neo is similar in size to ØX174 and the hGH-bearing plasmid, thus the ratio of DNA molecules present in the cotransfection mixture, and therefore the resulting copy numbers in the transgenome, should be comparable to those found in the studies of Perucho *et al.* (1980) and Pellicer *et al.* (1980). The K12-complementing gene may thus lie within 5 - 20 kb of a neighbouring *neo* sequence in temperature-resistant transfectants, which is close enough to allow for the cotransfer of both genes to secondary transfectants, and for their cloning within a single cosmid molecule. Superfluous *neo* sequences may therefore be eliminated by a second round of transfection of K12, this time in the absence of exogenously added *neo*, and pSV2neo screening of a cosmid library constructed from any temperature-resistant secondary transfectants should allow for the isolation and analysis of *neo*-bearing recombinant clones carrying the closely-linked K12-complementing gene.

Figure 3.54 Model of DNA-mediated gene transfer (adapted from Scangos *et al.*, 1981)



The initial step of this procedure had already been carried out i.e. K12 cells had been cotransfected with 1 μg of pSV2neo and 20 μg of Wg-1A high-molecular-weight

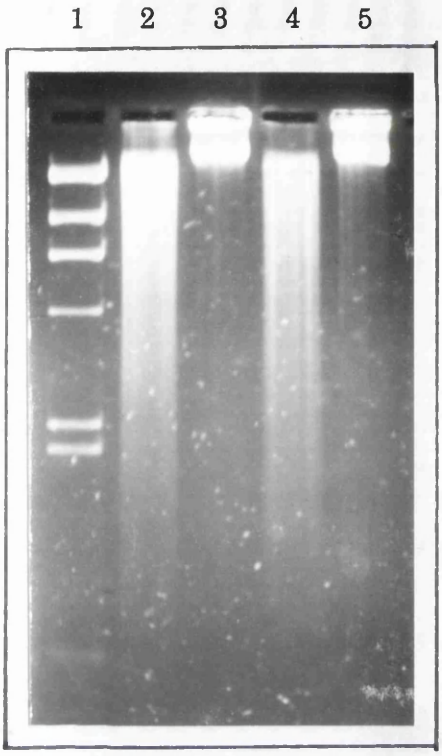
genomic DNA, to produce a total of 12 primary transfectant colonies on three separate plates. Each dish was trypsinized and the cells were replated and passaged; the colonies on each plate were pooled, since it seemed unlikely that they represented independent clones, and in any case it was not strictly necessary to maintain them as separate clones for the purposes of a second round of transfection, since each copy of the *neo* sequence and its associated K12-complementing gene would be likely to segregate into a single secondary transfectant cell. Cells from each transfectant-bearing plate were, however, maintained separately, since by necessity these must be independent clones, and the cell lines were named (according to the plates on which they arose) as primary transfectants 8, 11 and 12.

All three primary transfectant lines were used for a second round of transfection of K12, in order to maximize the likelihood of transferring the K12-complementing gene in close linkage with *neo*. High-molecular-weight DNA was prepared from the primary transfectants (Section 2.17; Fig. 3.56), and a number of 10 cm plates of K12, seeded at a concentration of 10^6 cells/dish, were each transfected with 20 μg of DNA from a single primary transfectant cell line, using the protocol outlined in Section 2.18.i. Duplicate control plates receiving no DNA were included in each experiment, in order to confirm the potency of 500 μg/ml G418. Twenty four hours after transfection, 500 μg/ml G418 was added to all dishes, and the medium was subsequently changed twice weekly. Plates were shifted to 40°C two weeks after the initial selection, at which time the number of colonies was counted by eye. Eleven plates were transfected with primary transfectant 8 DNA, and at the time of the temperature shift a total of 24 healthy colonies (1 - 4 per dish) was observed, eighteen primary transfectant 11 DNA-transfected plates each bore 0 - 9 G418-resistant colonies, with a total colony number of 20, and with primary transfectant 12 DNA, a total of 16 colonies was observed on five dishes (0 - 12 per plate). However, no colonies survived at 40°C on any plate.

The low frequency with which transfection to G418-resistance was obtained (approximately 1 in 10^6 cells) implies that the number of copies of the *neo* gene within each primary transfectant is low; calculations from the initial Wg-1A/pSV2neo cotransfection suggested that slightly less than a genome's worth of DNA should be successfully integrated on each plate, which, given the observed average number of G418-resistant colonies/dish obtained in the secondary transfections, implies a primary transfectant *neo* copy number of 1 - 3. If this value is correct, it seems unlikely that the K12-complementing gene would be in close enough proximity to a *neo* sequence for their cotransfer to a secondary transfectant, thus accounting for the lack of temperature-resistant secondary transfectants. Alternatively, it is possible that the primary transfectants may contain a large number of *neo* genes, but that a single

copy of this sequence within a secondary transfectant cell is insufficient for the accumulation of an adequate pool of neomycin phosphotransferase prior to selection (in previous cotransfections using the *neo* gene, several copies would be expected to be taken up by each primary transfectant cell). Only secondary transfectant cells in which more than one copy of the *neo* gene had become integrated, which may represent a small proportion of the total transfected population even if the copy number in primary transfectants was high, would therefore survive. If this is the case, then a delay in the time before the selective agent was applied may increase the number of transfectants detected or, alternatively, the primary transfectant DNA may be cotransfected with another selective marker e.g. the gene encoding xanthine-guanine phosphoribosyl transferase (the *gpt* gene) and selected on the basis of mycophenolic acid-resistance, prior to the construction of a cosmid library from temperature-resistant transfectants and screening with pSV2neo. It is also conceivable that a single copy of the *neo* gene within a secondary transfectant may be capable of providing adequate resistance to 500 μg/ml G418, but that the site of integration of the sequence may in some cases reduce its expression to below a certain required threshold level. Although this would not represent a problem in primary transfectant cells, where several copies of *neo* would be expected to be present along the length of the transgenome and it is therefore unlikely that all would be rendered insufficiently active, if each secondary transfectant carries only a single *neo* gene such a phenomenon may explain the low number of G418-resistant secondary transfectants obtained. In order to distinguish between these alternative hypotheses, the copy number of the *neo* gene within each primary transfectant was therefore determined (Section 3.23).

Figure 3.55 MINIGEL ELECTROPHORESIS OF K12 AND Wg-1A GENOMIC DNA



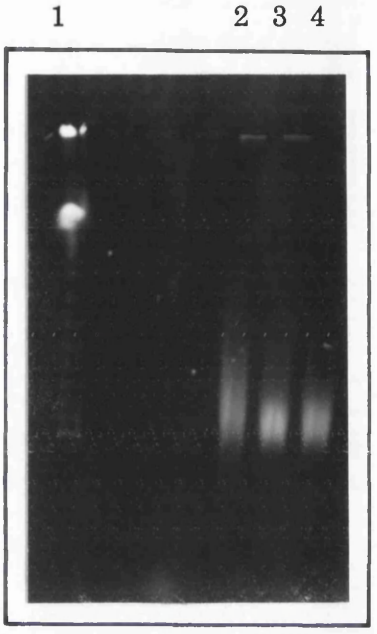
Track 1 - λ*Hin*d III (23.1, 9.4, 6.6, 4.4, 2.3, 2.0 and 0.6 kb)

Track 2 - *Xba* I/K12 DNA Track 3 - uncut K12 DNA

Track 4 - *Xba* I/Wg-1A DNA Track 5 - uncut Wg-1A DNA

Figure 3.56 PULSED-FIELD GEL ELECTROPHORESIS OF PRIMARY TRANSFECTANT GENOMIC DNA

(Pulse time = 90 secs, voltage = 3.6 V/cm, run time = 64 hours)



Track 1 - λ concatamers (multiples of approximately 50 kb)

Track 2 - uncut primary transfectant 8 Track 3 - uncut primary transfectant 11

Track 4 - uncut primary transfectant 12

3.23 DETERMINATION OF THE *neo* COPY NUMBER WITHIN PRIMARY TRANSFECTANT DNA

In order to determine the number of copies of the *neo* gene within the Wg-1A/pSV2neo primary transfectants of K12 from Section 3.22, genomic DNA from each of the three primary transfectant populations was digested to completion with a restriction enzyme which does not cut within pSV2neo (*Xba* I), subjected to agarose gel electrophoresis, transferred to nitrocellulose by Southern blotting, and probed with labelled pSV2neo. The number of bands observed represents the minimum number (two different fragments of equivalent sizes would appear as a single band) of copies of *neo* within the primary transfectant in question. Unfortunately, only a rough guide to the *neo* copy number could be obtained, since each primary transfectant population represented a pooled collection of up to five different colonies (although it seems unlikely that these were independent clones), thus the copy number calculated in such a manner may be a significant overestimate of the true value. However, this figure could still be used to calculate the approximate number of G418-resistant colonies which should, in theory, be obtained on the secondary transfection of K12 with primary transfectant DNA, and compared with the observed value, in order to provide an indication of whether the low frequencies of transfection to G418-resistance obtained in Section 3.22 could be explained by the presence of only a few copies of *neo* within the primary transfectant genome. If the primary transfectant *neo* copy number was found to be high, however, this may imply that secondary transfectants require the presence of more than one *neo* gene for survival in the presence of 500 μg/ml G418.

A number of methods for labelling and detection of the probe DNA were available. The sensitivity required was calculated as follows, assuming that 5 μg of each primary transfectant DNA was to be loaded for electrophoresis. The mammalian cell genome contains approximately $3x10^6$ kb (Winnaker, 1987) or $6.5x10^{-6}$ μg of DNA, thus 5 μg represents a total of $7.7x10^5$ copies of the genome, and each individual band on a probed blot will therefore contain $7.7x10^5$ identical molecules. Since *Xba* I, which does not cut within pSV2neo, was to be used for digestion of the DNA, the minimum fragment size detected by the probe could not be less than the full length of the pSV2neo molecule (5.6 kb). This plasmid has a molecular weight of $1.2x10^{-11}$ μg, thus if $7.7x10^5$ copies were found in each band, a total of 9.2 pg of DNA would be present. The probing system used therefore had to be capable of detecting quantities of DNA down to approximately 10 pg.

3.23.i Probing blots with the ECL system

The Amersham ECL (enhanced chemiluminescence) system was initially chosen, since it is claimed that this method can detect down to 1 pg of DNA on a Southern blot. Using this protocol, the probe DNA is labelled by covalent coupling to a modified form of horseradish peroxidase (Renz and Kurz, 1984). Detection of the probe is based on the ability of the attached enzyme to catalyze the oxidation of luminol, in the presence of hydrogen peroxide, to a derivative which emits light (Whitehead *et al.*, 1979). Phenolic compounds are incorporated into the detection solution, in order to enhance the degree of light emission (Thorpe *et al.*, 1985), and a permanent record of the luminescence so produced is provided by exposing the probed material to X-ray film, on which a dark spot will correspond to an area of light emission.

High-molecular-weight genomic DNA from the primary transfectants was prepared (Section 2.17), 5 μg of each was digested to completion with *Xba* I according to the protocol outlined in Section 2.20, and the samples were loaded on a 1% agarose gel. Control wells consisted of 1 μg of a λ*Hin*d III digest for sizing purposes, 5 μg of each undigested primary transfectant genomic DNA to confirm that cleavage of the digested samples was complete (a partial digest would provide an overestimate of the *neo* copy number since it would then be possible for a single *neo* gene to be represented in more than one fragment size), and 1 pg, 10 pg, 100 pg and 50 ng samples of *Eco* RI-linearized pSV2neo were also loaded in order to assess the sensitivity of the probing system. Electrophoresis, blotting, and probing of the blot with labelled *Eco* RI-digested pSV2neo were carried out as described in Sections 2.21.i, 2.22.i and 2.23 respectively. The detection signal was recorded on Fuji-RX film.

A photograph of the gel (Fig. 3.57) suggests that each primary transfectant DNA sample was digested to completion by *Xba* I, to produce a smear of fragments of differing sizes. However, only three extremely faint bands were detected on the X-ray film (data not shown), corresponding to cut *neo* in the 100 pg pSV2neo track, and to both cut and uncut *neo* in the 10 ng pSV2neo lane, whereas all four pSV2neo control tracks should have contained a sufficient quantity of DNA for detection. Since full transfer of the DNA to the nitrocellulose was confirmed by restaining of the gel following the blotting procedure, it appears likely that the labelling, hybridization or detection of the probe was not operating optimally. The low sensitivity obtained may have occurred for one of several reasons:

a. The recommended protocol suggests that a hybridization buffer volume of 0.125 ml/cm^2 of membrane is used, but this did not appear to cover the blot well, and was thus increased in subsequent experiments to 0.2 ml/cm^2.

b. The Amersham instruction manual recommends the application of Amersham Hyperfilm-ECL for detection of the signal, thus the use of Fuji-RX film may have decreased the sensitivity of the system.

c. The pSV2neo probe may not have labelled properly.

Each hypothesis could be tested by carrying out a dot blot. Using this method, small volumes of varying concentrations of pSV2neo may be spotted directly onto nitrocellulose, dried, and probed with labelled pSV2neo to provide a rapid and accurate assessment of the sensitivity of the detection system. Modifications may then be introduced into the hybridization and detection methods, and the subsequent sensitivities compared. The method initially used for dot blotting was a slight variation on that described by Kafatos *et al.* (1979). The DNA samples, denatured by incubation in 0.3 M NaOH at 65°C for one hour, were added to an equal volume of 2 M ammonium acetate and diluted down to the required concentration in 1 M ammonium acetate. 4 µl of each sample was spotted onto nitrocellulose, which had been pre-soaked in distilled water, followed by 1 M ammonium acetate. Once dry, the filter was subsequently washed in 6x SSC for 5 minutes, air-dried, and baked at 80°C for 2 hours. Samples of *Eco* RI-linearized pSV2neo were spotted onto two filters in aliquots containing 10 ng, 100 pg, 10 pg, 1 pg or 0.5 pg. A control sample of 1 M ammonium acetate was also applied to the nitrocellulose. Hybridization and detection was carried out as described in Section 2.23 (but with 0.2 ml of hybridization buffer/cm^2 membrane) using *Eco* RI-digested pSV2neo as the probe. The signal from one blot was detected by the application of Amersham Hyperfilm-ECL, and DuPont Cronex MRF-31 was used for the remaining filter. However, no signal was observed on either film, suggesting that the problem lay with either the dot blotting procedure itself, or with the labelling and hybridization of the probe.

In discussion with Amersham, they suggested that the post-hybridization primary wash buffer should not be autoclaved prior to use, since ammonia is produced from the urea within the solution, thus raising the pH to a level unacceptable for probe binding. Consequently, the dot blot was repeated using primary wash buffer made up in autoclaved water and, in addition, the dot blotting protocol was altered (Section 2.22.iii). 50 ng, 10 ng, 100 pg, 10 pg and 1 pg aliquots of *Eco* RI-linearized pSV2neo were applied to the nitrocellulose, and *Eco* R1-digested pSV2neo was used as the probe. A control spot consisting of 10x SSC was also included on the blot. The chemiluminescent signal was recorded on DuPont Cronex MRF-31 film. Figure 3.58 demonstrates that the 50 ng and 10 ng pSV2neo spots were now clearly visible, suggesting that the faulty primary wash buffer may indeed have been responsible for the lack of signal on the previous dot blot. However, this level of sensitivity was obviously inadequate for the required purpose of the detection of integrated *neo* on

Southern blots of primary transfectant genomic DNA, where only 10 pg of DNA may be present in the band of interest, and was much lower than Amersham's claims of a lower detection limit of 1 pg. In an attempt to increase the sensitivity of the system, further modifications of the probing method were therefore carried out, including the use of Hyperfilm-ECL, doubling of the probe concentration, and manipulation of the stringency of the hybridization conditions, but in no case did it prove possible to detect less than 10 ng of DNA on dot blots of pSV2neo. This method was therefore abandoned due to its poor degree of sensitivity in my hands.

3.23.ii Use of ^{35}S-labelled pSV2neo for probing blots

The next approach to be investigated for the purpose of detection of the *neo* gene on blots of DNA was the use of ^{35}S-labelled pSV2neo probe and subsequent visualization of the signal by autoradiography. The sensitivity of the system was once again assessed by hybridization and detection of the labelled probe on a dot blot of pSV2neo. A comparative blot was also carried out using 123 DNA (kindly provided by Miss I. Lea, University College London), a cDNA of unknown function which is expressed at high levels in eukaryotic cells and is known to label to a high specific activity with ^{35}S (Miss I. Lea, personal communication). Preparation of 0.5 µg of labelled 123 DNA and *Eco RI*-digested pSV2neo was carried out as described in Section 2.24.i. The degree of incorporation of radioactivity was assessed by liquid scintillation counting on 1 µl aliquots of each sample (Table 3.87). The results demonstrate that no unincorporated ^{35}S-dATP appeared to remain after purification of the probe, since the total counts measured were less than those for nucleic acid alone (acid-insoluble counts). The acid-insoluble count values were used to calculate the specific activity, expressed as counts per minute (cpm)/µg DNA, for each probe. Although both DNA molecules were labelled to a reasonably high specific activity with ^{35}S, it is noticeable that pSV2neo incorporated significantly less radioactivity than 123. The linearization of pSV2neo by *Eco* RI digestion was halted by the addition of 10 mM EDTA, and it is possible that despite the fact that the DNA had been diluted approximately fourfold by the time of addition of the DNA polymerase I/DNase I solution, the presence of even such low concentrations of EDTA may inhibit the nick translation reaction by chelating out the required magnesium ions, suggesting therefore that it would have been advisable to terminate the linearization of pSV2neo by phenol/chloroform extraction rather than by the use of EDTA.

Two dot blots, bearing spots containing 50 ng, 10 ng, 100 pg, 10 pg or 1 pg of either 123 or *Eco* RI-linearized pSV2neo, were prepared according to the protocol outlined in Section 2.22.iii. Control spots consisting of 10x SSC were also included on

each blot. The filters were hybridized with approximately $2x10^6$ cpm of the appropriate denatured probe (Section 2.24.ii), and the signal was detected by autoradiography (Section 2.24.iii; Fig. 3.59). On the 123 DNA blot, the 50 ng dot was clearly visible, and 10 ng of DNA could be seen as a faint spot. For pSV2neo, the 50 and 10 ng spots were both detected well, and the 100 pg dot was weakly visible. Although this showed a slight improvement over the ECL probing system, where no spots below 10 ng were observed, the sensitivity still needed to be improved by a factor of tenfold if a 10 pg band on a Southern blot was to be visualized. The use of ^{32}P as the radioactive label, which shows a much higher specific activity than ^{35}S and is therefore significantly more sensitive when used for probing purposes, was considered, but as handling of this isotope is significantly more hazardous than of ^{35}S, an alternative non-radioactive labelling method, claimed to be as sensitive as ^{32}P, was first investigated.

3.23.iii Probing blots with the Boehringer Mannheim digoxigenin-based method

In the Boehringer Mannheim digoxigenin-based probing method, the nucleotide analogue digoxigenin-11-dUTP is incorporated into the probe DNA by the random primed-labelling technique (Feinberg and Vogelstein, 1983). Following hybridization to the target DNA, an alkaline phosphatase-conjugated anti-digoxigenin antibody is applied, which binds to the hybridized probe. The location of the antibody-antigen conjugate is subsequently visualized by the addition of nitroblue tetrazolium salt and bromochloroindolyl phosphate solutions, which are converted to an insoluble blue precipitate by the alkaline phosphatase. The results may be recorded photographically or simply photocopied, and Boehringer Mannheim claim that as little as 0.1 pg of DNA may be detected on a blot.

In order to confirm the manufacturer's claims on sensitivity, a number of preliminary blots were carried out. In the first, *Bam* HI-cleaved pSV2neo and linear pBR328, the latter molecule supplied in the probing kit, were labelled according to the manufacturer's protocol (Section 2.25.i), and spotted onto a dot blot (Section 2.22.iii) in aliquots containing 30, 10, 3, 1 or 0.3 pg of DNA. Spots containing the same quantities of pBR328 which had been supplied in an already labelled form were also placed on the blot, along with control dots consisting of 10x SSC alone. The DNA was subsequently fixed to the membrane by baking, and the blot was processed through the colour detection reaction (Section 2.25.iii). Figure 3.60 demonstrates that pSV2neo and pBR328 labelled as well as, if not better than the supplied labelled pBR328, and that the sensitivity of detection of labelled probe was extremely good; in the case of the pSV2neo and pBR328 samples labelled by myself,

0.3 pg of the DNA was clearly visible, and 1 pg of the supplied labelled pBR328 could be seen.

The sensitivity with which hybridized labelled pSV2neo and pBR328 molecules were detected was also determined. Two dot blots were prepared, one bearing spots of 30, 10, 3, 1 and 0.3 pg of *Bam* HI-linearized pSV2neo and a control dot of 10x SSC, and one with the same quantities of supplied unlabelled linear pBR328. Each blot was hybridized (Section 2.25.ii) with digoxigenin-labelled pSV2neo or the supplied labelled pBR328, as appropriate, and the signal was detected as outlined in Section 2.25.iii. As may be expected, the detection of the hybridized probe was slightly less efficient than that of labelled probe alone (Fig. 3.61), but the sensitivity remained high; 1 pg of pSV2neo and 3 pg of pBR328 were visible.

A final preliminary study was carried out in order to compare the efficiencies of vacuum and capillary blotting for the transfer of DNA from a gel to a nitrocellulose filter. Each side of a 1% agarose gel was loaded with 150, 30, 10, 3 and 1 pg of *Eco* RI-linearized pSV2neo and 0.5 μg of a λ*Hin*d III sample. Electrophoresis was carried out overnight (Section 2.21.i), and the following day the marker tracks were removed, stained in ethidium bromide and photographed. The gel was then cut in half, one side was subjected to vacuum blotting (Section 2.22.ii), and the DNA on the other half was transferred to nitrocellulose by capillary blotting (Section 2.22.i). Both filters were subsequently hybridized with digoxigenin-labelled *Bam* H1-cleaved pSV2neo (Section 2.25.ii), and colour detection was performed according to the protocol outlined in Section 2.25.iii. The results (Fig. 3.62) suggest that the use of capillary blotting resulted in the transfer of approximately tenfold more DNA to the nitrocellulose filter than that of vacuum blotting; 1 pg of pSV2neo was visible on the capillary blot, whereas the lower limit of detection on the vacuum blot was 10 pg of DNA. Since each side of the gel was restained with ethidium bromide after the blotting process was complete, and there appeared to be no untransferred DNA remaining on either, it appears that the use of a strong vacuum for transfer may force the DNA through the pores of the nitrocellulose to the other side of the filter, where it is lost in the fluid drained away by the blotting apparatus. Capillary blotting was thus used as the method of choice for DNA transfer in all further experiments.

Taken together, the results from the preliminary blotting studies suggested that the sensitivity of the digoxigenin method should be adequate for the detection of integrated *neo* on a Southern blot of primary transfectant genomic DNA, where only 10 pg of DNA may be present in the band of interest. However, on complete digestion of the primary transfectant DNA with *Xba* I, it is possible that a range of *neo*-containing fragment sizes, from a minimum of 5.6 kb up to several hundred kb,

may hybridize to the probe. Using conventional agarose gel electrophoresis, good resolution of individual bands cannot be maintained over such a wide range of sizes, thus separation of the genomic DNA was accomplished using pulsed-field gel electrophoresis (PFGE), where the application of an electric field which alternates in direction allows for the resolution of DNA fragments of a few thousand up to several million base pairs in length (see Gardiner, 1990 for review). In order to determine the optimum parameters for the separation of fragments over the size range required, test runs were performed using 1.2% agarose-NA gels loaded with 0.75 μg of λ*Hin*d III (this provides fragments of 23.1, 9.4, 6.6, 4.4, 2.3, 2.0, 0.56 and 0.13 kb), 0.5 μg of whole λ DNA (approximately 50 kb in size) and 1 μg of a λ concatamer sample (which gives bands of multiples of 50 kb), kindly supplied by Miss J. Woodall (University College London). Electrophoresis was carried out as described in Section 2.21.iii, and the buffer concentration, pulse time, voltage and run time were varied (see Fig. 3.63 for details). The use of 1x TBE-low EDTA gel running buffer, a pulse time of 10 seconds, a voltage of 8 V/cm, and a run time of 16.5 hours were found to give the optimum degree of separation over the size range of 4 - 200 kb.

10 μg of high-molecular-weight genomic DNA prepared from each primary transfectant cell line (Section 2.17) was digested to completion with *Xba* I (Section 2.20), and the samples were loaded onto a 1.2% agarose-NA gel in 1x TBE-low EDTA buffer. Control wells consisted of 10 μg of each undigested primary transfectant genomic DNA, in order to determine whether cleavage of the digested samples was complete, 30 pg of uncut pSV2neo and 5 and 30 pg of *Bam* HI-linearized pSV2neo, to confirm the sensitivity of the probing system, and 1 μg of whole λ DNA, 1.5 μg of λ concatamers and 0.75 μg of λ*Hin*d III for sizing purposes. Pulsed-field gel electrophoresis was carried out according to the protocol outlined in Section 2.21.iii, and the gel was stained and photographed on a transilluminator. Figure 3.64 suggests that size separation over the range of 4 - >150 kb was good, and that complete digestion of each primary transfectant DNA with *Xba* I had occurred. The DNA was subsequently transferred to nitrocellulose by capillary blotting (Section 2.22.i), and probing of the blot with digoxigenin-labelled *Bam* HI-linearized pSV2neo was performed as described in Section 2.25; the results are shown in Figure 3.65. The 30 pg uncut pSV2neo band gave a signal similar in intensity to that found in a preliminary blot using this method (Fig. 3.62), confirming that the probing system was operating at full sensitivity. However, the 30 pg band on the *Bam* HI-cleaved pSV2neo track could be only faintly seen and the 5 pg band was not visible, suggesting perhaps that incorrect quantities of these samples were loaded. In the uncut primary transfectant samples, a number of pSV2neo-hybridizing bands appeared in the primary transfectant 11 lane; it is

unclear what these may represent, since undigested genomic DNA tracks should contain a smear of random fragment sizes, thus any *neo*-containing molecules will be found not within discrete bands but spread over the entire continuum of fragments, in insignificant quantities at any particular place to give a detectable hybridization signal.

Within the *Xba* I-digested primary transfectant tracks, a number of bands hybridized to the pSV2neo probe, and the size of each fragment was calculated by reference to the migration of the λ markers (Fig 3.64 and Table 3.88). Since bands were observed in each digested sample, and all bands within a given lane should produce a signal of approximately equal intensity (an equivalent number of *neo* genes will be present in each band, and will therefore bind similar quantities of the probe irrespective of the total size of the target fragment), it appears likely that all pSV2neo-containing fragments were detected on the blot. The low pSV2neo copy number within each primary transfectant indicated by Figure 3.65 - two in primary transfectant 8, six in primary transfectant 11 and three in primary transfectant 12 (it seems likely that the two faint high-molecular-weight bands in the digested primary transfectant 12 track resulted from leakage from the primary transfectant 11 well, since they migrate to exactly the same position in each track) - appears to account for the poor efficiencies of transfection of K12 to G418-resistance obtained in the secondary transfection experiments carried out in Section 3.22. If it is assumed, using calculations from Section 3.22, that on secondary transfection of this cell line, approximately one genome's worth of primary transfectant DNA is taken up and integrated on a single transfectant plate, the calculated number of copies of the *neo* gene within the primary transfectant DNA suggests that only 2 - 6 G418-resistant colonies would be expected per dish, a figure compatible with the actual values obtained (on average, 1 - 3 colonies/plate). This implies that the poor transfection frequencies observed in Section 3.22 were simply a consequence of the low pSV2neo copy number in the primary transfectant genome, rather than being caused by a requirement for more then one *neo* gene within each secondary transfectant cell for survival in the presence of 500 μg/ml G418, as postulated in Section 3.22. The low primary transfectant pSV2neo copy number may also explain the inability to obtain G418-resistant secondary transfectants in Section 3.22 which were capable of growth at 40°C; given an average transgenome size of 1,000 kb (Perucho *et al.*, 1980), even in primary transfectant 11, where six pSV2neo sequences appear to be spread along the transgenome, it is unlikely that the K12-complementing gene of Wg-1A will be in close enough proximity to a *neo* gene for their cotransfer to a secondary transfectant (genomic DNA isolated from the primary transfectants was an average of 100 kb in size; see Fig. 3.56). It thus appeared that this method of *in vivo* linkage of Wg-1A

DNA to pSV2neo would be unlikely to result in the isolation of the gene of interest and an alternative route, incompatible with the time remaining, was therefore required.

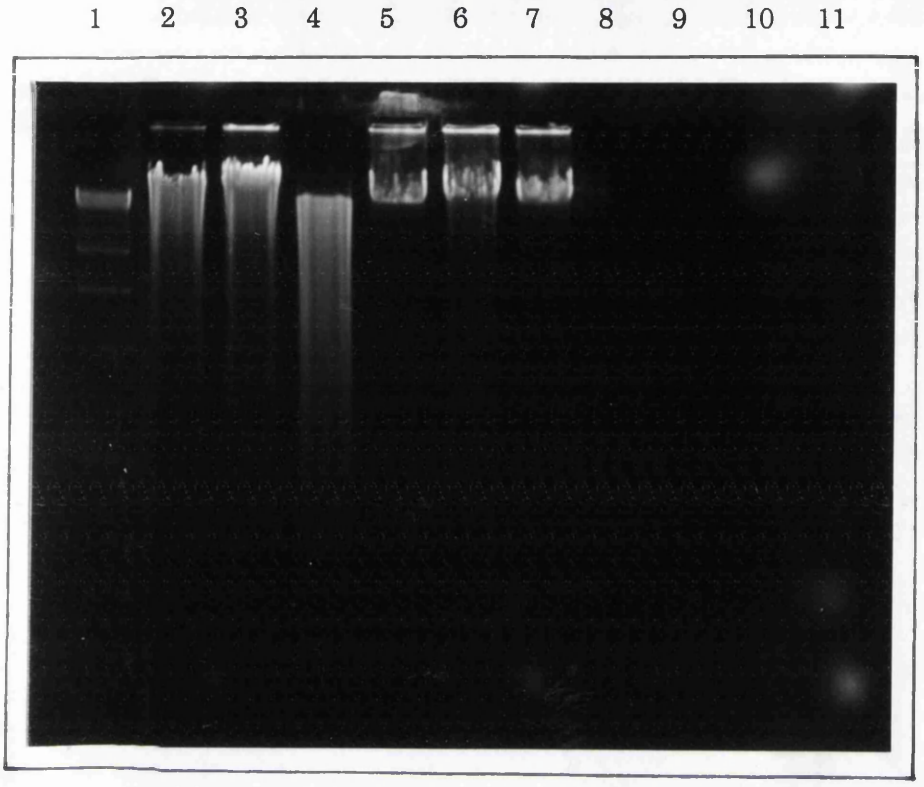
Table 3.87 LIQUID SCINTILLATION COUNTING OF ^{35}S-LABELLED PROBES

DNA	TOTAL COUNTS (cpm)	ACID-INSOLUBLE COUNTS (cpm)	TOTAL VOLUME OF PROBE (µl)	SPECIFIC ACTIVITY (cpm/µg DNA)
123	113,470.0	137,913.8	105	2.9×10^7
pSV2neo	26,844.8	34,909.6	125	8.7×10^6

Table 3.88 SIZE OF pSV2neo-HYBRIDIZING BANDS IN A SOUTHERN BLOT OF *Xba* I-DIGESTED PRIMARY TRANSFECTANT DNA

PRIMARY TRANSFECTANT	BAND SIZE (kb)
8	14, 20
11	9, 10, 16, 36, two at >100
12	14, 26, 42

Figure 3.57 GEL ELECTROPHORESIS OF PRIMARY TRANSFECTANT GENOMIC DNA FOR PROBING WITH THE ECL SYSTEM



Track 1 - λ*Hind* III (23.1, 9.4 and 6.6 kb)
Track 2 - *Xba* I/primary transfectant 8
Track 3 - *Xba* I/primary transfectant 11
Track 4 - *Xba* I/primary transfectant 12
Track 5 - uncut primary transfectant 8
Track 6 - uncut primary transfectant 11
Track 7 - uncut primary transfectant 12
Track 8 - 1 pg *Eco* RI/pSV2neo
Track 9 - 10 pg *Eco* RI/pSV2neo
Track 10 - 100 pg *Eco* RI/pSV2neo
Track 11- 10 ng *Eco* RI/pSV2neo

Figure 3.58 ECL DETECTION OF A DOT BLOT OF pSV2neo DNA WITH MODIFIED PRIMARY WASH BUFFER

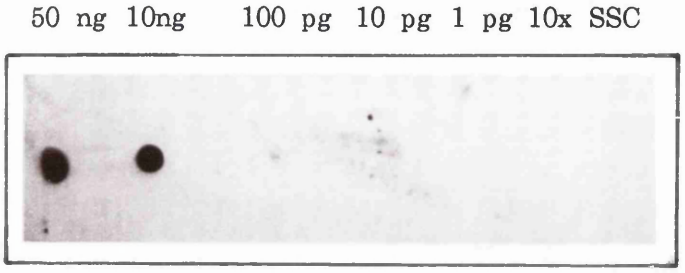


Figure 3.59 AUTORADIOGRAPHIC DETECTION OF DOT BLOTS OF pSV2neo AND 123 DNA PROBED WITH ^{35}S-LABELLED PROBES

a. pSV2neo

b. 123 DNA

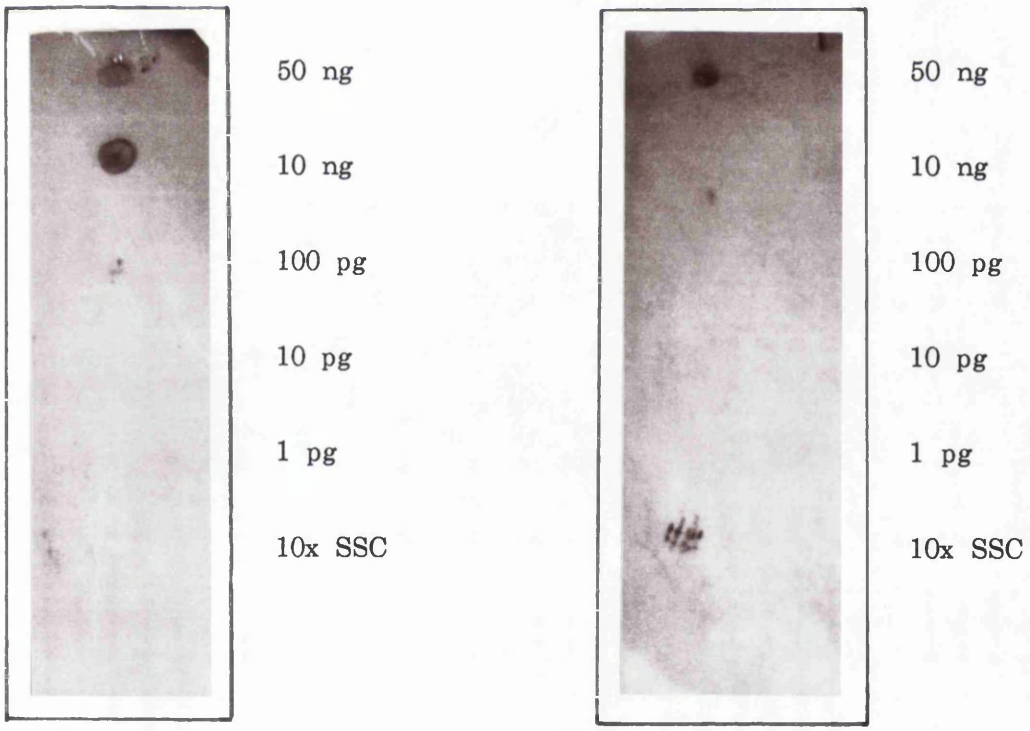


Figure 3.60 DETECTION OF DIGOXIGENIN-LABELLED DNA SAMPLES SPOTTED DIRECTLY ONTO NITROCELLULOSE

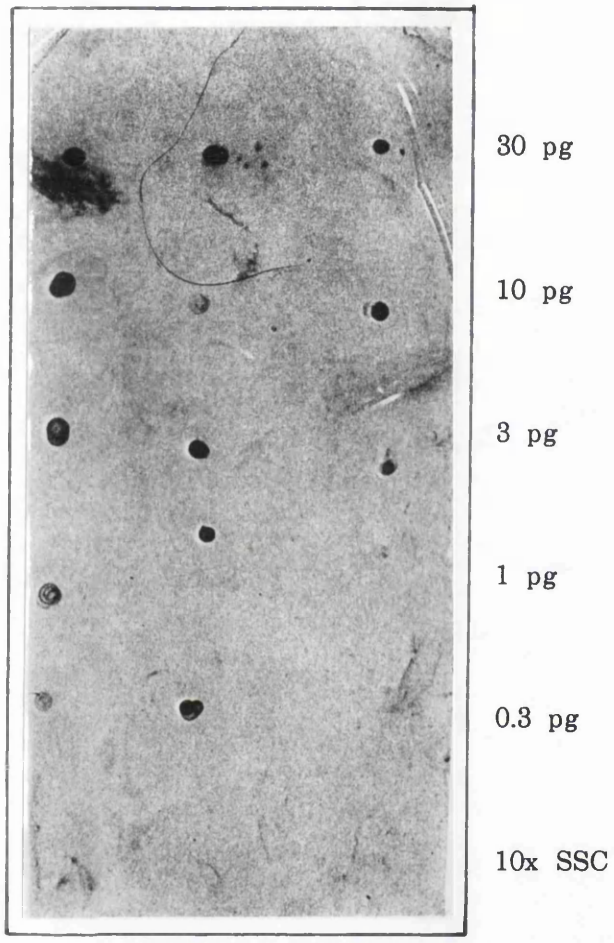


Figure 3.61 DETECTION OF DOT BLOTS OF pSV2neo AND pBR328 DNA PROBED WITH DIGOXIGENIN-LABELLED PROBES

a. pSV2neo

30 pg

10 pg

3 pg

1 pg

0.3 pg

10x SSC

b. pBR328

30 pg

10 pg

3 pg

1 pg

0.3 pg

10x SSC

Figure 3.62 COMPARISON OF THE EFFICIENCIES OF VACUUM AND CAPILLARY BLOTTING FOR THE TRANSFER OF DNA FROM A GEL TO A NITROCELLULOSE FILTER

a. Vacuum blotting

Eco RI/pSV2neo

150 pg 30 pg 10 pg 3 pg 1 pg

b. Capillary blotting

Eco RI/pSV2neo

150 pg 30 pg 10 pg 3 pg 1 pg

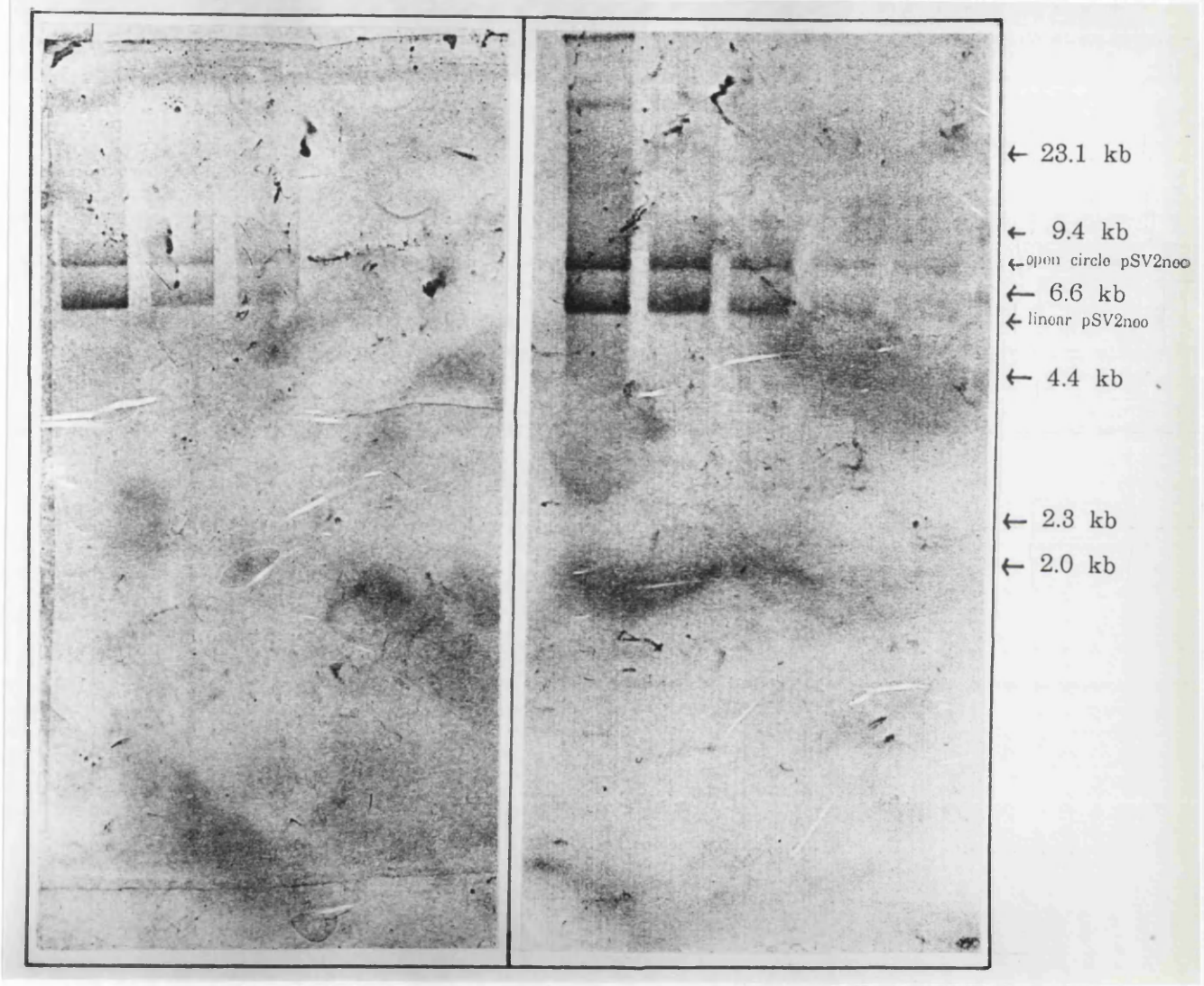
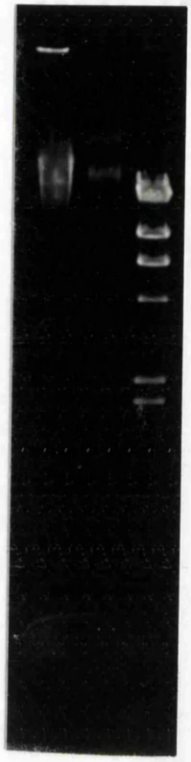


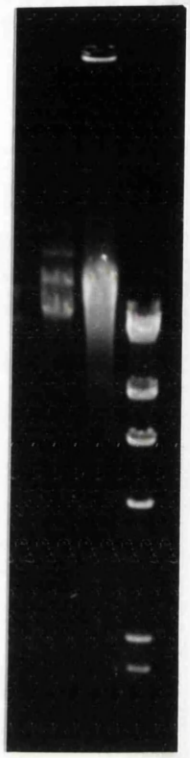
Figure 3.63 DETERMINATION OF THE OPTIMUM PARAMETERS FOR THE SIZE SEPARATION OF DNA MOLECULES BY PFGE

a.



Pulse time - 5 secs
Voltage - 6.8 V/cm
Current - 385 mA
Run time - 4.5 hrs
0.5x TBE low EDTA
Track 1 - whole λ
Track 2 - λ concatamer
Track 3 - λ*Hin*d III

b.



Pulse time - 10 secs
Voltage - 8 V/cm
Current - 408 mA
Run time - 6.5 hrs
1x TBE low EDTA
Track 1 - λ concatamer
Track 2 - whole λ
Track 3 - λ*Hin*d III

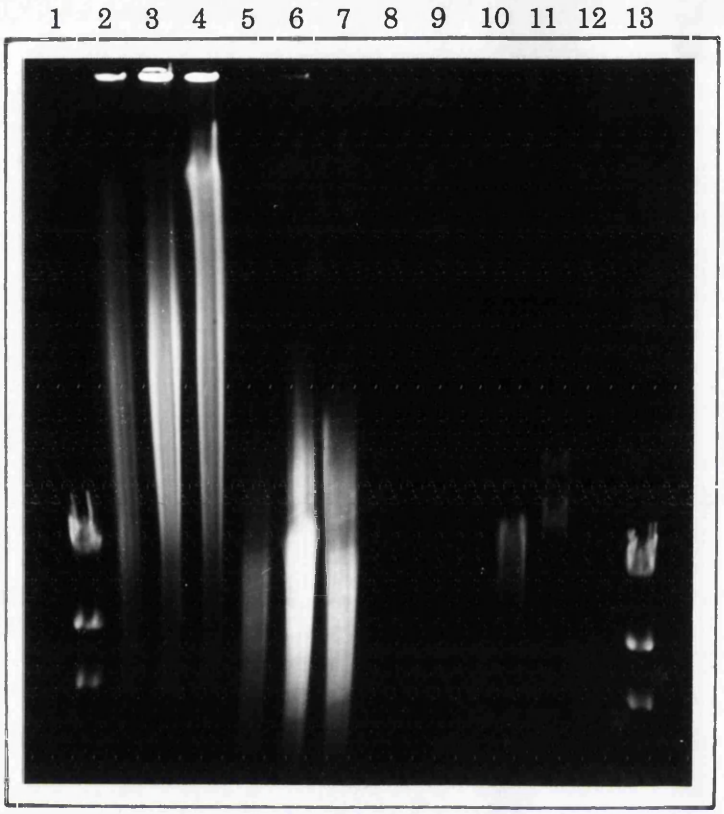
c.

Pulse time - 10 secs
Voltage - 10 V/cm
Current - 200 mA
Run time - 6.5 hrs
1x TBE low EDTA
Track 1 - λ concatamer
Track 2 - whole λ
Track 3 - λ*Hin*d III

d.

Pulse time - 10 secs
Voltage - 8 V/cm
Current - 288 mA
Run time - 16.5 hrs
1x TBE low EDTA
Track 1 - λ concatamer
Track 2 - whole λ
Track 3 - λ*Hin*d III

Figure 3.64 PFGE OF PRIMARY TRANSFECTANT GENOMIC DNA FOR PROBING WITH DIGOXIGENIN-LABELLED pSV2neo



Track 1 - λ*Hin*d III

Track 2 - uncut primary transfectant 8

Track 3 - uncut primary transfectant 11

Track 4 - uncut primary transfectant 12

Track 5 - *Xba* I/primary transfectant 8

Track 6 - *Xba* I/primary transfectant 11

Track 7 - *Xba* 1/primary transfectant 12

Track 8 - 30 pg uncut pSV2neo

Track 9 - 5 pg *Bam* HI/pSV2neo

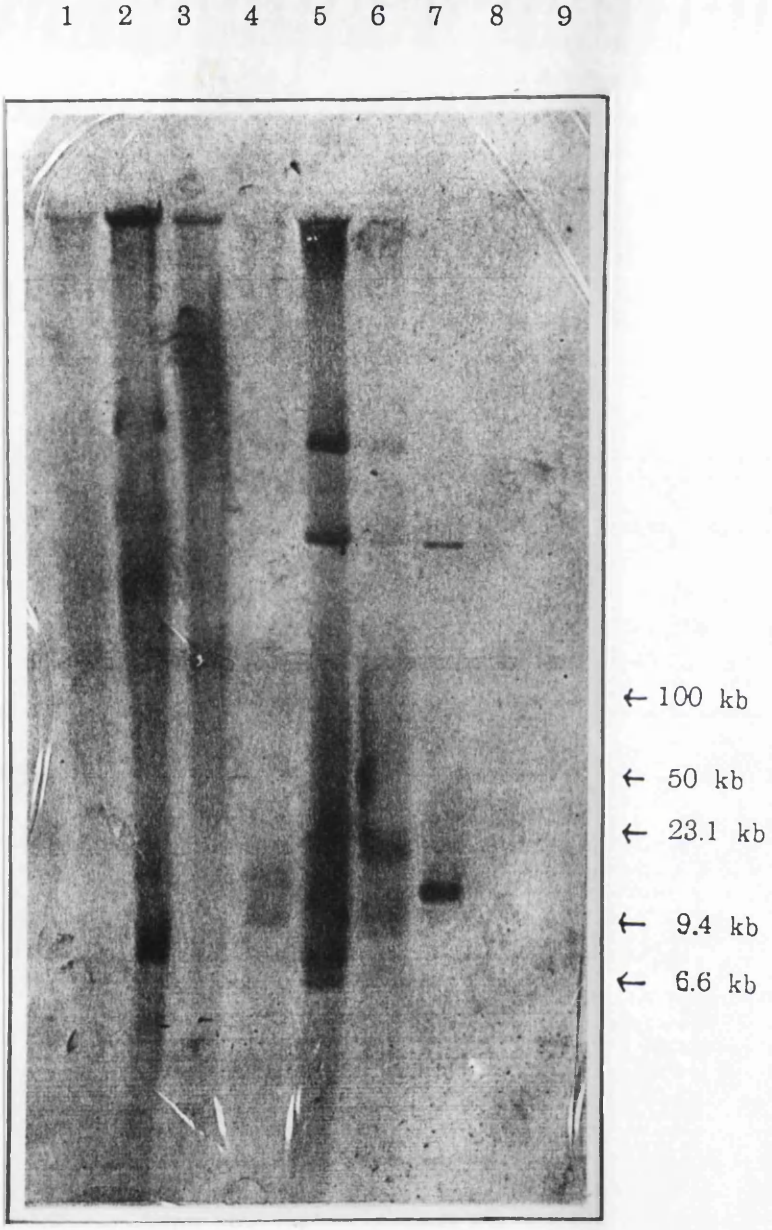
Track 10- 30 pg *Bam* HI/pSV2neo

Track 11- whole λ

Track 12- λ concatamers

Track 13- λ*Hin*d III

Figure 3.65 DIGOXIGENIN-BASED DETECTION OF A SOUTHERN BLOT OF PRIMARY TRANSFECTANT GENOMIC DNA PROBED WITH pSV2neo



Track 1 - uncut primary transfectant 8
Track 2 - uncut primary transfectant 11
Track 3 - uncut primary transfectant 12
Track 4 - *Xba* I/primary transfectant 8
Track 5 - *Xba* I/primary transfectant 11
Track 6 - *Xba* I/primary transfectant 12
Track 7 - 30 pg uncut pSV2neo
Track 8 - 5 pg *Bam* HI/pSV2neo
Track 9 - 30 pg *Bam* HI/pSV2neo

SECTION 4

DISCUSSION

4.1 STUDIES ON THE VIABILITY OF K12 AT THE NPT

4.1.i The influence of sodium bicarbonate on the behaviour of K12 at the npt

When K12 was originally isolated in 1973 by Roscoe *et al.* (1973a), viability curve studies carried out shortly afterwards (Roscoe *et al.*, 1973b) showed that the loss of proliferative capacity began 15 - 20 hours after a shift to the nonpermissive temperature, and it was complete 20 hours later. However, when these experiments were repeated in the present study (Section 3.2.i), K12 now appeared to be capable of surviving for significantly longer periods of time (10 - 20 hours) at 40°C (Fig. 3.2). Time-lapse analyses (Section 3.2.ii) demonstrated that this prolonged viability was a consequence of the ability of the cells to undergo one or more extra cycles of division which were not observed in 1973. Whereas K12 had behaved as a classic non-leaky cell cycle mutant when first isolated, in that it was unable to pass the execution point of the mutation even once at 40°C, at present it seemed to have gained the capacity to pass this point once, or in some cases twice, at the npt, before finally halting. This implied that the reduction in activity of the gene product in question was a gradual process, and that the rate at which this loss occurred had altered since the original studies. It thus appeared that either the cell line or the conditions in which it was grown had changed in the intervening period. Since no original isolates of K12 were available for study, and the type of serum used to supplement the medium was shown to have little effect on the survival time of K12 at the npt (Figs. 3.2 and 3.3), attention was focussed on the influence of the medium composition on the time course of loss of viability of K12 at 40°C, in order to determine whether differences in formulation of the basic growth medium could account for the alteration in behaviour of this cell line over the years.

The first indication that the medium composition is indeed critical with regard to the behaviour of K12 at the npt was provided by the observation that K12 cells grown in 5% FCS MCDB-302 survived for approximately 8 hours longer at 40°C than those cultured in 5% FCS E4 low bic (Fig. 3.7). It seemed unlikely that this phenomenon occurred via a general effect on cell growth; although the use of MCDB-302 rather than E4 low bic did appear to influence the basic growth properties of K12 at 33°C, this was manifested as an increase in the growth rate (Section 3.5), which would be expected to accelerate, rather than slow down the loss of viability at

the npt, since the execution point would then be reached more rapidly. Experiments were thus concentrated on determining whether a particular component of the medium was responsible for the observed prolonged survival at 40°C, acting by a specific effect on the rate of loss of activity or heat stability of the K12 ts protein.

A comparison of the compositions of E4 low bic and MCDB-302 (Appendix III), together with a careful study of the exact protocol of the original viability curve experiments (Roscoe *et al.*, 1973b), suggested that variations in the level of sodium bicarbonate could potentially explain the differences in the behaviour of K12 exhibited in the two alternative media and, in addition, between the time of its isolation and the present study. Viability curves were carried out where the sodium bicarbonate level of both E4 and MCDB-302 was manipulated, and demonstrated that the rate of loss of viability of K12 at 40°C was directly correlated with the medium bicarbonate level, with a decrease in the $NaHCO_3$ concentration significantly prolonging the survival time of this cell line (Sections 3.6 and 3.7). The variation in the time course of loss of the proliferative capacity of K12 at the npt in the two media was shown to be accounted for almost entirely by the difference in their sodium bicarbonate levels, since an increase in the $NaHCO_3$ concentration of MCDB-302 from its usual value of 13.3 mM, to the E4 low bic concentration of 26 mM produced a viability curve virtually indistinguishable from that demonstrated in E4 low bic (Fig. 3.8). Differences in bicarbonate level could also help to explain the prolonged survival of K12 at the present time when compared with the 1973 results (Roscoe *et al.*, 1973b), as the original studies were carried out in 44 mM $NaHCO_3$ E4, whereas the current experiments used E4 containing 26 mM $NaHCO_3$. However, varying bicarbonate levels could not account entirely for the alteration in the behaviour of K12 over the years (see Sections 3.7 and 3.14), implying that other as yet unidentified factors, perhaps ionic medium components (see Section 4.1.iii) or changes in the cell line itself, may also be exerting an influence.

The general conclusion to be drawn from this set of experiments, therefore, is that the concentration of $NaHCO_3$ in the growth medium is critical with regard to the behaviour of K12 at 40°C, with a reduction in the bicarbonate level prolonging the survival of the cells at the npt. Sodium bicarbonate plays a dual role in tissue culture media, acting as both a buffer, to retain the pH at a level optimal for cell growth in the face of the acidifying influences of cell metabolism, and as a nutrient. It is conceivable that changes in either the pH or the nutritional quality of the medium may influence the behaviour of K12 *via* some general growth effect. One could argue that if low $NaHCO_3$ levels became nutritionally limiting, or provided pH values outside those optimal for cell growth (most fibroblasts perform best within the pH range of 7.0 - 7.7 [Ceccarini and Eagle, 1971; Rubin, 1971; 1973; Eagle, 1973]),

the cells would be protected for some time against death at the npt due to a reduced rate of cell cycling and a consequent increase in the time taken to reach the execution point of the mutation. However, the following observations militate against this hypothesis:

a. Media that use alternatives to bicarbonate, such as HEPES, as a buffer, incorporate $NaHCO_3$ at a concentration of 4 mM for its nutrient value alone. This figure presumably represents a threshold level above which growth is optimal for the majority of cell lines, and in most cases the bicarbonate concentrations used in the current viability curve studies were significantly above this value. Indeed, all levels of $NaHCO_3$ were chosen such that K12 cells gave an E.O.P. value and colony size at 33°C in each medium roughly equivalent to that seen in 5% FCS E4 low bic, the standard growth medium, thus it would seem improbable that the growth of K12 was in any way limited in these experiments.

b. In the course of the viability curve studies, colonies were detected which survived at 40°C for one week in 7 mM $NaHCO_3$ MCDB-302 (Section 3.7). These were large and healthy, and therefore appeared to be cycling normally, since a slow cycle time would be expected to manifest itself in a significantly smaller colony size over the time period studied.

c. At 33°C, colonies of K12 grown in 13.3 mM $NaHCO_3$ MCDB-302 were generally larger than those cultured in 26 mM $NaHCO_3$ E4 (Section 3.5), which seems to suggest that the cells actually divide more rapidly in the lower bicarbonate concentration. However, raising the MCDB-302 bicarbonate content from 13.3 to 26 mM, the E4 low bic level, did not alter the characteristic growth pattern of K12 in MCDB-302 (low E.O.P., large colony size) to that observed in E4 low bic (high E.O.P., small colony size) at 33°C (Section 3.8), thus it appears that differing $NaHCO_3$ levels are not responsible for the variations in the general growth properties of this cell line which are displayed in the two media. It therefore seems unlikely that changes in bicarbonate levels exert their effects on K12 at the npt by altering its overall cycle time.

d. Time-lapse studies on both random and mitotically-synchronized cultures (Section 3.14) demonstrated that the rate of mitoses remained constant for an increasing length of time at 40°C as the $NaHCO_3$ level of each medium was lowered (Tables 3.61 - 3.64), suggesting that the cells continued to cycle normally at the npt for longer at low bicarbonate concentrations, and had therefore gained the ability to pass the execution point of the mutation several times. Indeed, in cultures grown in 7 mM $NaHCO_3$ MCDB-302, a large number of cells were seen to divide four, and some even five times after the temperature shift (Table 3.62). In addition, calculation of average intermitotic times for K12 grown in the various media showed that growth

did not appear to be slower in the low bicarbonate media (Tables 3.61 - 3.64).

It thus appears unlikely that reduced sodium bicarbonate levels prolong the survival of K12 at 40°C *via* a general growth effect, but rather act in a more specific manner to slow down the loss of activity of the ts protein for sufficient time to allow for a few extra cell divisions. However, lowering the bicarbonate content of the medium cannot prevent the eventual halt of the cell at the execution point, since flow cytofluorometry was used to demonstrate that at the npt in all media, irrespective of the $NaHCO_3$ concentration, K12 continued to arrest in G1 (Section 3.15).

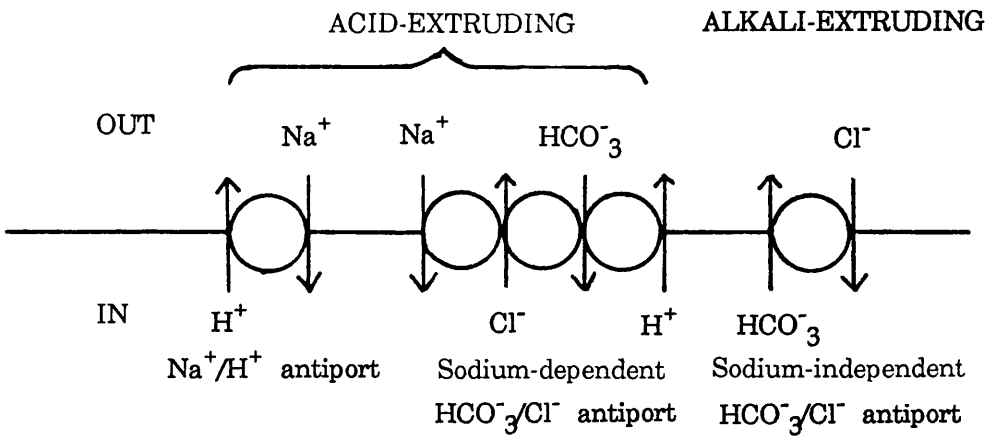
4.1.ii How may manipulations of the bicarbonate concentration affect the behaviour of K12?

4.1.iia Alterations in the internal pH of the cell - possible effects on the activity of the K12 ts protein

In order to shed light on the potential mechanism by which low $NaHCO_3$ concentrations could prolong the viability of K12 at the npt, it is necessary to consider the ways in which the alterations in extracellular bicarbonate will affect cellular processes. The most obvious result of such manipulations will be a significant lowering of the external pH as the bicarbonate level of the medium is reduced. In the viability curve (Section 3.7) and time-lapse (Section 3.14) studies, the pH of the media was varied over the pH range 6.91 - 7.97 as a consequence of the alterations in $NaHCO_3$ concentration (Section 3.10). However, this change in pH values is unlikely to be reflected by an increase or decrease of a similar magnitude in the pH_i of the cell, due to the existence of a number of homeostatic mechanisms which serve to maintain the internal pH of the eukaryotic cell at a very precise level, usually within the range of pH 7.0 - 7.4 (Madshus, 1988). Such strict regulation of the pH_i is required for the maintenance of an appropriate environment for cellular activities in the face of varying extracellular pH values, and a tendency to intracellular acidosis caused by the passive influx of acids or efflux of bases, and the metabolic generation of acid. In mammalian cells, pH_i regulation is mediated by several different mechanisms. In the short term, pH_i changes can be minimized to some extent by chemical buffering in the cytosol, the biochemical conversion of metabolic acids to non-acid compounds, and by the sequestering of protons in various organelles (Roos and Boron, 1981). However, in addition, most eukaryotic cells appear to possess two or more transmembrane ion transport systems, which accomplish active pH_i regulation, and allow the cells to maintain an internal pH at a steady-state

value more alkaline than that expected from purely passive proton distribution across the cell membrane. Three distinct mechanisms for active pH_i regulation in animal cells have been identified so far (Fig. 4.1), and their relative importance most likely varies between different cell types.

Figure 4.1 Ionic mechanisms involved in pH_i regulation in mammalian cells



There is general agreement that all animal cells possess an electroneutral Na^+/H^+ antiporter (see Boron, 1983 for review), which utilizes the large inward-directed Na^+ gradient generated by the Na^+/K^+-ATPase pump to drive protons from the cell in exchange for extracellular Na^+, and thus acts as an acid-extruder. The activity of this antiporter is pH-sensitive; at normal pH_i values (approximately 7.0 - 7.4), the Na^+/H^+ exchanger is relatively inactive, but as the pH_i falls below a certain 'threshold', the rate of Na^+/H^+ exchange is greatly stimulated by the presence of cytoplasmic protons (Frelin *et al.*, 1983; Moolenaar *et al.*, 1983), which have been proposed to act as allosteric activators of the antiporter (Aronson *et al.*, 1982). Thus, when the pH_i drops below the physiological level, the Na^+/H^+ exchanger is rapidly activated, and protons are driven out of the cell until the pH_i is normalized, at which point the exchange activity slows down and stabilizes again at the basal level. In this fashion, the antiporter acts as a 'pH sensor', allowing recovery of the cell from acid loads.

Initially, it was believed that Na^+/H^+ exchange was the primary homeostatic mechanism for pH_i regulation in mammalian cells (Vigne *et al.*, 1984; Mahnensmith and Aronson, 1985; Moolenaar, 1986). However; the studies leading to this conclusion were carried out in non-physiological conditions i.e. in the absence of sodium bicarbonate, and neglected the contributions made to pH_i regulation by two additional bicarbonate-dependent transport systems. The sodium-dependent HCO_3^-/Cl^- antiporter, which exchanges intracellular protons and chloride ions for external Na^+ and HCO_3^-

ions, driven by the inward-directed Na^+ gradient, is the major acid-extruding mechanism in many invertebrate cell systems (Thomas, 1977; Roos and Boron, 1981; Boron, 1983), and has also been shown to operate in a variety of mammalian cells in culture (L'Allemain *et al.*, 1985; Tonnessen *et al.*, 1987; Bierman *et al.*, 1988; Cassel *et al.*, 1990). In the presence of bicarbonate, the sodium-dependent HCO_3^-/Cl^- antiporter is relatively inactive at physiological pH_i values, but its rate of activity increases linearly as the pH_i falls (Boron *et al.*, 1979), reducing the intracellular concentration of free protons and thus restoring the pH_i to normal. A number of studies have indicated that in physiological bicarbonate-containing media, the sodium-dependent HCO_3^-/Cl^- antiporter, rather than the Na^+/H^+ exchanger, determines the value of the steady-state pH_i in various cell lines (Cassel *et al.*, 1985; L'Allemain *et al.*, 1985; Bierman *et al.*, 1988; Tonnessen *et al.*, 1990). In fact, in Vero cells it has been shown that Na^+/H^+ exchange appears to play a role in increasing the pH_i only after a substantial acidification of the cytosol (pH_i < 6.7), whereas the sodium-coupled HCO_3^-/Cl^- antiport is active at all pH_i values between 6.4 and 7.4, and therefore opposes acidification over a wide pH_i range (Tonnessen *et al.*, 1990).

In addition to the sodium-coupled HCO_3^-/Cl^- antiporter, a number of cell lines have also been shown to possess a sodium-independent HCO_3^-/Cl^- exchanger (Olsnes *et al.*, 1987a; Tonnessen *et al.*, 1987; Cassel *et al.*, 1990; Lin *et al.*, 1990). This uses an inward-directed chloride gradient to drive out bicarbonate ions and thus, in contrast to the previous two pH_i-regulatory mechanisms, acts to extrude alkali, and therefore to allow recovery from alkaline loads by decreasing the pH of the cytosol. The functioning of this antiport is strongly activated with increasing pH_i above a threshold level of pH 7.1 (Olsnes *et al.*, 1986; 1987a; 1987b; Tonnessen *et al.*, 1990), possibly by a similar mechanism to that proposed for the Na^+/H^+ antiporter (Aronson *et al.*, 1982). It thus appears that in the majority of cells cultured in bicarbonate-containing media, the steady-state pH_i value is the result of a balance between the alkalinizing activity of the sodium-coupled HCO_3^-/Cl^- exchanger and the acidifying influence of the sodium-independent HCO_3^-/Cl^- antiporter, and that Na^+/H^+ exchange plays a role only when the pH_i is substantially acidified.

In K12, all or part of this complex system of pH_i-regulatory mechanisms would be expected to act to resist the changes in the pH_i imposed by the alterations in pH_{ex}, brought about as a consequence of the manipulations of the bicarbonate level of the media, in the viability curve (Section 3.7) and time-lapse (Section 3.14) experiments of the present study. It was therefore unclear to what extent these changes in pH_{ex} would be reflected inside the cell. However, preliminary measurements of the internal pH of K12 at 40°C in media of varying sodium bicarbonate concentrations (Table 3.76), although somewhat imprecise, suggested that altering the $NaHCO_3$ level of the

medium did indeed significantly influence the pH_i of the cell, thus it remains a possibility that bicarbonate may exert its effects on the viability of K12 at the npt by altering the intracellular pH of this cell line.

Table 4.1 Cellular functions shown to be influenced by intracellular pH changes

FUNCTION	REFERENCE
Protein synthetic rates	Grainger *et al.*, 1979; Winkler and Steinhardt, 1981
Gap junction conductivity	Spray *et al.*, 1982
Endocytosis	Hwang *et al.*, 1986; Cosson *et al.*, 1989
Glycolysis	Triveda and Danforth, 1966; Fidelman *et al.*, 1982
Gluconeogenesis	Kashwagura *et al.*, 1984
Cell motility	Taylor, 1962; Rubin, 1971
Contraction of cytoplasm	Taylor, 1962
Contractility of actin/myosin	Condeelis and Taylor, 1977
Microtubule disassembly	Regula *et al.*, 1981
Actin filament assembly	Begg and Rebhun, 1979
Membrane permeability to ions and low-molecular-weight compounds	Oxender and Christensen, 1963; Heinz, 1967; Lacko *et al.*, 1972; Bannai and Kitamura, 1981; Forster and Lloyd, 1987
Mucopolysaccharide accumulation and distribution	Lie *et al.*, 1972; Kittlick *et al.*, 1976
Lysosome distribution	Heuser, 1989
Thermotolerance	Holahan and Dewey, 1986; Miyakoshi *et al.*, 1986
Nuclear lamin distribution	Jost *et al.*, 1986
Calcium mobilization	Maly *et al.*, 1990

Variations in the internal pH would be expected to have a pervasive influence on the cell, since a host of diverse cellular functions have been shown to be markedly pH-sensitive (Table 4.1). In almost every example, a lower pH_i value is associated with a reduced cellular activity or efficiency of the process in question suggesting, at first sight, that acidification of the external pH by a decrease in the bicarbonate level may prolong the survival of K12 at the npt by a generalized overall reduction in cellular fitness for growth. However, as discussed above (Section 4.1.i), it seems

likely that bicarbonate acts in a more specific manner on the K12 ts protein itself. Nonetheless, until the precise nature of the defective molecule is elucidated, the possibility that specific alterations in one or more of the pH-sensitive processes listed above may affect the activity or stability of this protein, and thus indirectly increase the viability of K12 at 40°C, should not be ruled out.

It seems likely that, as postulated in Section 1.12, a threshold level of the K12 ts protein is required in order to pass the execution point of the mutation, and that the cell fails to attain this critical amount at the npt, due to the structural collapse or loss of activity of the protein in question, and thus halts at this stage of the cell cycle. Alterations in pH are known to influence the conformation and stability of proteins, through an effect on the degree of ionization of amino acid side chains, and therefore on the ionic interactions that help to determine the shape of the molecule as a whole. If the pK of residues located so as to affect the conformational properties of the protein lie within the physiological pH_i range, changes in their ionization state brought about by even small variations in the pH_i may thus have significant effects on the 3D structure of the molecule. It is therefore conceivable that the low pH provided by reduced medium bicarbonate concentrations may allow the K12 ts protein to adopt a conformation that increases its heat stability, and thus slows down its collapse at the npt such that the execution point may be passed several times before the amount of active protein falls below the required threshold. It is also possible that the protein defective in K12 carries out its function as an oligomer, and that the reduction in its activity at the npt is due to the thermal collapse of its subunit associations. Since the same pH-sensitive non-covalent ionic forces that are involved in intrachain structure are also responsible for holding protein subunits together, alterations in the pH_i may increase the thermal stability of the subunit interactions, and therefore prolong the activity of the protein. The slowing down of the collapse of the protein may also be achieved indirectly, with a low pH_i perhaps promoting the association of the molecule with other cellular components, which may protect against its denaturation.

Alternatively, it is possible that a reduced pH may increase the expression or activity of the K12 ts protein without affecting its heat stability. This would temporarily compensate for the loss of the functional molecule, which is caused by a gradual denaturation or reduction in activity at the npt, by raising the initial total activity of the protein, thus a longer period of time would elapse before this activity falls below the threshold value. The activity of a large number of enzymes is known to be pH-sensitive. If the pK of an enzyme is within the physiological pH_i range, even minor changes in pH_i around these values will markedly affect its activity. The increase in activity in response to such small alterations in the pH can be quite

dramatic, as is the case with the highly pH-sensitive rate-limiting enzyme of glycolysis, phosphofructokinase, where raising the pH from 7.1 to 7.2 enhances its activity by nearly twentyfold (Trivedi and Danforth, 1966). This increase in enzyme activity as the pH is altered may be due to a change in the conformation of the entire molecule, which improves its catalytic properties, or it may act in a more specific manner on the degree of ionization of particular residues in the active site or on the substrate, and thus affect their interaction. Alternatively, changes in pH may increase the availability or binding of a cofactor to the reaction, thus there are many means by which a low pH may directly increase the activity of the K12 ts protein. However, it is also possible that rather than a direct effect on the defective K12 protein itself, reduced pH levels may influence the interaction, level or activity of a pH-sensitive effector of this molecule, which in turn controls the activity or expression of the defective protein, and can therefore also compensate transiently for the reduced protein levels.

The overall conclusion, thus, is that low external pH levels may simply prolong the viability of K12 at the npt by increasing the stability or activity of the ts protein, or of an associated controlling molecule, such that a required threshold level of activity is maintained for an extended period.

4.1.iib Alterations in the internal pH of the cell - effects on the K12 cell cycle

One could also postulate that the K12 G1 block is caused not by a mutation in a cell cycle protein, but is a consequence of a defect in one of the cell's pH_i-regulatory ion transport systems, such that the expression of the mutation leads to a gradual raising of the pH_i at the npt. It may be that a particular cell cycle protein, required to pass the K12 execution point, has a pH optimum at acidic values, thus such an increase in the pH_i could cause the activity of this molecule to fall below a threshold level and therefore arrest the cell at this point. A lowering of the bicarbonate concentration, and therefore of the pH_{ex} of the medium would be expected to acidify the cytosol, and may thus temporarily offset the increase in the pH_i caused by the faulty ion pump, and consequently allow for the prolonged survival of the cell line at 40°C.

A large number of studies do indeed suggest that a critical pH_i value is required in G1 before commitment to DNA synthesis can occur. However, all the available evidence points towards the need for an alkaline pH_i for cell cycle progression. It has been demonstrated in many mammalian cell lines that activation of amiloride-sensitive Na^+/H^+ exchange, and a consequent rise in the pH_i of 0.1 - 0.2 pH units, is an early response to the stimulation of quiescent cells by a variety of mitogens

(Moolenaar *et al.*, 1981; 1983; Schuldiner and Rozengurt, 1982; Burns and Rozengurt, 1983; Cassel *et al.*, 1983; L'Allemain *et al.*, 1984a; 1984b). Activation of the Na^+/H^+ antiporter is thought to result from an alkaline shift in the pH_i sensitivity of the exchanger (Moolenaar *et al.*, 1983; Paris and Pouyssegur, 1984; Grinstein *et al.*, 1985a), which may be brought about by a conformational change in the molecule which increases its apparent affinity for cytoplasmic protons. Protein kinase C has been implicated, either directly or indirectly, in this modification process (Moolenaar *et al.*, 1984; Grinstein *et al.*, 1985b; Vara *et al.*, 1985). Initially, it was widely believed that this cytoplasmic alkalinization acted as a trigger for the initiation of DNA synthesis, based on a number of lines of evidence:

1. An increase in the pH_i is a common early response in many situations where proliferation is initiated, ranging from the fertilization of sea urchin eggs (Johnson *et al.*, 1976; Whitaker and Steinhardt, 1982) to growth factor addition to mammalian cells in culture (see Moolenaar, 1986 for review).
2. Treatment of cells with amiloride, which inhibits the Na^+/H^+ exchanger (Johnson *et al.*, 1976; Aickin and Thomas, 1977) and thus also the pH_i increase, was shown to prevent the initiation of mitogen-induced DNA synthesis (Moolenaar *et al.*, 1981; Boonstra *et al.*, 1983; L'Allemain *et al.*, 1984a).
3. PS120, a mutant Chinese hamster cell line lacking a functional Na^+/H^+ exchanger (Pouyssegur *et al.*, 1984) fails both to increase its internal pH_i and to reinitiate DNA synthesis in response to mitogens at neutral and acidic pH_{ex} values (L'Allemain *et al.*, 1984b; Pouyssegur *et al.*, 1985).

These results were taken to imply that an obligatory increase in pH_i in G1 acted as a mitogenic signal, triggering the process of DNA replication. However, further investigation of this proposal cast doubt on each of these lines of evidence. The main fault with the majority of these studies was that they were conducted in the absence of bicarbonate i.e. under non-physiological conditions. When $NaHCO_3$ was added to each system, the results were seen to be very different:

1. Physiological levels of bicarbonate suppress the mitogen-induced alkalinization in a range of different cells without abolishing mitogenesis (Cassel *et al.*, 1985; Bierman *et al.*, 1988; Ganz *et al.*, 1988; Szwergold *et al.*, 1989). In addition, the stimulation of Na^+/H^+ exchange and the resultant increase in pH_i was shown to be neither obligatory for the induction of DNA synthesis, since even in the absence of bicarbonate a number of growth factors elicit proliferation without raising the pH of the cytoplasm (Cook *et al.*, 1988; Gelfand *et al.*, 1988; Hesketh *et al.*, 1988), nor sufficient e.g. EGF stimulates an increase in the pH_i in A431 cells via Na^+/H^+ exchange, but does not induce mitogenesis (Rothenberg *et al.*, 1983).
2. In the presence of bicarbonate, amiloride no longer inhibits mitogen-induced

proliferation in a range of cell types (Besterman *et al.*, 1984; L'Allemain *et al.*, 1984a; 1984b; 1985; Mills *et al.*, 1985; 1986; Cassel *et al.*, 1987).

3. PS120 cells show normal proliferation on serum stimulation if physiological $NaHCO_3$ is included in the medium (L'Allemain *et al.*, 1984b; 1985; Pouyssegur *et al.*, 1985), or if the pH_i is raised above a threshold level of pH 7.2 by manipulation of the pH_{ex} (Pouyssegur *et al.*, 1985), despite the lack of a functional Na^+/H^+ exchanger.

Experiments carried out in the absence of bicarbonate neglected the contribution of the Na^+-dependent HCO_3^-/Cl^- exchanger to the determination of the steady-state pH_i of the cell. Measurements of the pH_i of mammalian cells suggest that it is maintained at a significantly higher level in the presence of $NaHCO_3$ than in its absence, due to the activity of this antiporter (Cassel *et al.*, 1985; Szwergold *et al.*, 1989). In view of this fact, these studies taken together suggest that rather than an alkaline transient playing a causal role in the triggering of mitosis, a threshold level of pH_i of approximately 7.1 - 7.2 (Pouyssegur *et al.*, 1985; Cassel *et al.*, 1987) is merely required as a permissive factor for the initiation of DNA synthesis. In the presence of bicarbonate, the pH_i of the cell is already at or above this threshold due to the activity of the Na^+-dependent HCO_3^-/Cl^- exchanger. In its absence, however, this antiporter is no longer operative and the pH_i is reduced, thus stimulation of the Na^+/H^+ exchanger is required to raise the internal pH before entry into S phase can occur. It therefore appears that commitment to DNA replication requires the establishment of a permissive alkaline pH_i, perhaps simply to stimulate general metabolism and to provide an adequate rate of the pH-sensitive processes of glycolysis and protein synthesis to allow for progression through the cell cycle.

Given this association between an alkaline pH_i in G1 and an increased proliferative capacity, it therefore appears unlikely that the K12 G1 block could be caused by a defective ion pump raising the pH_i at the npt, and indeed measurements of the pH_i of K12 and Wg-1A (Section 3.16), although somewhat unreliable, suggest that no alkalinization of the K12 cytoplasm above the wild-type level occurs at 40°C. This further strengthens the hypothesis that any effect of alterations in the pH of the medium on the viability of K12 at the npt are exerted via a specific influence on the activity or stability of the ts protein itself.

4.1.iic Changes in the nutritional quality of the medium

If the alterations in the pH of the media caused by varying the $NaHCO_3$ level are solely responsible for the observed effects on the viability of K12 at the npt, then MCDB-302 and E4 media with equivalent pH values would be expected to produce similar viability curves. However, it was demonstrated that this was not the

case (Section 3.12), and in fact there appeared to be a better correlation between the concentration of bicarbonate and the rate of loss of viability, with an increased $NaHCO_3$ level accelerating death, than between the pH and the survival time. In addition to its role as a buffer, sodium bicarbonate also serves as a nutrient, acting as a substrate for a number of essential biosynthetic reactions, including the synthesis of indispensable metabolic intermediates such as purines, pyrimidines and long-chain fatty acids (Ham, 1981). It is unclear, however, how variations in the level of such a nutrient could exert a specific effect on the activity or stability of the K12 ts protein. In addition, when viability curve studies were carried out on K12 grown in MCDB-302 and E4 of equivalent $NaHCO_3$ levels, a given bicarbonate concentration did not produce identical viability curves in each of the two media (Fig. 3.9), suggesting that $NaHCO_3$ is not acting on the behaviour of K12 solely *via* its nutrient function either. Unfortunately, due to the apparent obligatory requirement of K12 for sodium bicarbonate, demonstrated in Section 3.11, the pH of the medium may not be manipulated independently of the $NaHCO_3$ concentration, and the differential effects of its nutrient and buffering roles cannot therefore be dissociated. However, it seems clear that the influence of bicarbonate on the viability of K12 at the npt is not simply attributable either to changes in pH of the medium or to variations in the nutritional status of the cell, but that a more complex situation exists. It appears likely that the two functions of bicarbonate each play a role in combination. This alone could explain why viability curves in media with equivalent pH values or $NaHCO_3$ levels do not give similar results - in MCDB-302 and E4 media of identical pH values, the nutritional effects of bicarbonate on the behaviour of K12 at 40°C (and thus the viability curves produced) would differ, since the bicarbonate concentrations would not be the same (Fig. 3.13). A similar situation would exist with MCDB-302 and E4 media with equivalent $NaHCO_3$ concentrations, where the pH of the media would be different. Alternatively, it is possible that other medium components, which differ in concentration between E4 and MCDB-302, may also be involved in determining the rate of loss of viability of K12 at 40°C, and that these influences are superimposed on the effects of the bicarbonate variations. This is supported by the observation that cells grown in E4 consistently lose viability more rapidly than those cultured in MCDB-302 with either an equivalent $NaHCO_3$ concentration (Fig. 3.9) or an identical pH (Fig. 3.15).

4.1.iii Effects of ions on the behaviour of K12 at the npt

When the compositions of both media were compared (Appendix III), it was found that a number of ions differed markedly in concentration between MCDB-302 and

E4. In order to determine whether variations in the level of any of these ions could influence the viability of K12 at the npt, the concentration of each was brought separately to equivalence in the two media, and viability curves were carried out (Section 3.13). Although increased levels of five of the ions (namely VO_3^-, SeO_3^{2-}, Zn^{2+}, Cu^{2+} and Mn^{2+}) appeared to prolong the survival of K12 at 40°C when their concentrations were manipulated individually, most likely through general growth promoting or inhibiting effects, this ability was lost when they were altered simultaneously. It thus appears that although some of the ionic components of MCDB-302 and E4 can influence the behaviour of this cell line at the npt when their levels are altered in isolation, it is unclear whether the particular combination of ionic concentrations present is responsible for the observed difference in behaviour of K12 at 40°C in the two media with either equivalent pH values or $NaHCO_3$ levels, since interactions between them seem to alter their effects. In order to conclusively determine whether this is the case, the concentrations of all the ions of one medium should be simultaneously brought to equivalence with those of the other, and the effects on the viability of K12 at the npt compared. It is also possible that some other constituent which varies in concentration between E4 and MCDB-302, such as a vitamin or an amino acid, causes the variations in the rate of loss of viability of K12 in the two media, and this should be further investigated. Indeed, the component responsible for the increased colony size and lower E.O.P. observed when K12 is grown at 33°C in MCDB-302 rather than in E4 low bic (Sections 3.5 and 3.8) has yet to be identified, and it is conceivable that this factor also influences the behaviour of the cell line at the npt.

4.1.iv Conclusions and further work

In summary, it appears that low bicarbonate levels can significantly prolong the viability of K12 at the npt by a combination of its effects on both the pH and the nutritional quality of the medium, but that additional medium components may also play a role. Several lines of evidence suggest that rather than affecting the general growth properties of the cells, this is achieved by a specific influence on the K12 ts protein, with a reduced $NaHCO_3$ concentration increasing its heat stability or activity. If this is the case, then low bicarbonate levels, in addition to postponing the cell cycle block, would also be expected to delay the onset of the other pleiotropic manifestations of the loss of function of the ts protein i.e. the glycosylation defect and overproduction of the GRPs (see Section 1.12), which appear to be causally unconnected with the cell cycle block (Feige and Scheffler, 1987). These defects can therefore be used as biochemical markers to investigate whether the loss of activity

of the aberrant protein is indeed slowed down under such conditions. The effects of the manipulation of the medium bicarbonate concentration on the time course of the expression of either the defect in glycosylation (monitored by the rate of ^{3}H-glucosamine uptake [Melero, 1981]), or GRP overproduction (visible as enhanced bands on SDS-polyacrylamide gel electrophoresis of cell protein extracts [Melero and Fincham, 1978]), should thus provide evidence for the specific action of sodium bicarbonate on the activity or stability of the ts molecule. However, it is conceivable that reducing the $NaHCO_3$ concentration may prolong the survival of K12 at the npt by increasing the interaction of the ts protein with a molecule with which it normally works in concert, or by raising the activity of the latter molecule. If the associated molecule is only required for the cell cycle function of the protein defective in K12, low bicarbonate levels would then have no effect on the expression of the glycosylation defect or on GRP overproduction, thus a lack of alteration in the time course of expression of these defects cannot be taken as evidence to rule out a specific influence on the K12 ts protein. It seems that ultimately, a full understanding of how sodium bicarbonate exerts its effects on the viability of K12 at the npt must await the cloning of the defective gene and the characterization of its protein product.

It would also be of considerable interest to determine whether the influence of bicarbonate concentration on the behaviour of K12 at the npt is an isolated case, or represents a widespread phenomenon among other ts cell cycle mutants, as this appears to be the first documented evidence of such an effect. If a range of cdc mutants is shown to exhibit similar behaviour, this may provide evidence for the pH-sensitivity of the activity of a number of cell cycle proteins, and thus add to our understanding of the mechanism by which passage through the cell cycle is regulated. If this were the case, the observed results would also have implications for the isolation of ts cdc mutants. The general procedure currently followed consists of the incubation of a mutagenized population at the npt for two or three generation period equivalents, with the incorporation into the medium of an agent lethal for dividing cells (Basilico, 1978). Once the wild-type cells have succumbed to the toxin, the population is returned to the permissive temperature to allow for recovery of the ts cells. Given that even a relatively small reduction in the $NaHCO_3$ concentration of the medium can significantly prolong the active division of K12 at the npt (Section 3.14), the use of low bicarbonate media, such as MCDB-302, may result in the loss of many potentially ts cells during the isolation procedure, due to their continued cycling for several generation period equivalents at the npt and consequent sensitivity to the lethal agent. It would thus seem wise to use media containing reasonably high $NaHCO_3$ levels in future isolations of temperature-sensitive cell cycle mutants.

4.2 REVERSION OF K12

4.2.i Isolation of revertants of K12 in 7 mM $NaHCO_3$ MCDB-302 and proposed mechanism by which they arise

K12 differs from the majority of ts mammalian cell cycle mutants as it displays an unusually low spontaneous reversion frequency (< 1 in 60 million) when incubated under non-selective conditions (Roscoe *et al.*, 1973a), although revertants may be obtained at a frequency of approximately 1 in 10^5 - 10^6 following ethyl methane sulphonate mutagenization (Roscoe *et al.*, 1973a; Tenner *et al.*, 1976), and a single spontaneous revertant has been isolated following incubation at the npt (Scharff *et al.*, 1982). Perhaps the most interesting and unexpected finding of this study, therefore, was that in addition to significantly prolonging the survival of K12 at the npt, the use of 7 mM $NaHCO_3$ MCDB-302 as growth medium caused the emergence of a population of revertant cells, at a relatively high frequency, which appear to have undergone a distinct genetic change allowing for their continuous growth at 40°C (Section 3.17). The precise nature of the genetic alteration is unclear. Since the revertants do not display fully wt behaviour (Tables 3.78 - 3.80), it seems unlikely that a direct back-mutation of the original defect is responsible. It could be argued that a mutation in a gene elsewhere in the genome has occurred, which allows the cell cycle block caused by the ts protein at 40°C to be bypassed to some extent. However, the influence of the medium bicarbonate level on both the survival time of K12 and the E.O.P. of the revertants at the npt strongly implicates the ts protein in determining both processes. It is also possible that the reversion may involve a change in a region of the gene defective in K12 distinct from that of the original mutation, which results in an increase in the stability or activity of the protein product. It is difficult, however, to see how this proposed mechanism could account for the high frequency with which this reversion occurs. It seems more likely, given the observed reversion frequency of approximately 1 in 6,000 cells, that the use of 7 mM $NaHCO_3$ MCDB-302 at 40°C selects for the growth of cells which have undergone amplification of the K12 defective gene.

Gene amplification is thought to occur spontaneously in tumour cells and permanent cell lines at a frequency within the range of 10^{-4} - 10^{-7} events/cell/generation (Stark and Wahl, 1984). The initial amplification event has been proposed to arise from the random duplication of a gene by unequal crossover or disproportionate replication, which may occur constantly in cultured cells in any region of the genome (Schimke *et al.*, 1978). Duplications are generally unstable (Anderson *et al.*, 1976), and are thus comparatively rare, and pass undetected unless

the cells are placed under a selection pressure which confers a growth advantage on cells with the increased gene dosage. Further amplification may then occur by similar mechanisms to the original duplication.

There are a growing number of examples of ts mutations which have been reverted by the process of gene amplification (Wang, 1976; Fuscoe *et al.*, 1983; Cirullo and Wasmuth, 1984; Greco *et al.*, 1987). It would appear that providing the ts mutation does not totally inactivate the corresponding gene product, overexpression of the defective protein may be able to compensate to some extent for its heat instability. Fuscoe *et al.* (1983) showed that the amplification of the hypoxanthine-guanine phosphoribosyl transferase (HGPRT) gene was responsible for the reversion of the ts HGPRT Chinese hamster mutant RJK526 following growth in selective medium. Although total enzyme activity was raised by two- to thirteenfold in different revertant lines at the pt, this activity was reduced at the npt, demonstrating that the restoration of the temperature-resistant phenotype was not due to increased thermal stability of the protein. Rather, the cumulative residual activity of the defective enzyme at the npt was sufficient, due to its overproduction, to allow for growth. Of particular interest to the current study are the examples of ts mammalian cell cycle mutants shown to revert to a temperature-resistant phenotype through the process of gene amplification. Wang (1976) found that when the ts655 hamster cell line, which blocks in prophase at the npt, was incubated at the npt, 1 in 500 cells revert to ts^+ growth. The reversion was shown to correlate with the possession of an elevated number of chromosomes, and thus presumably with a higher copy number of the ts gene, a process akin to amplification. A more direct demonstration of gene amplification as a causative agent in the reversion of a cdc mutant was provided by Greco *et al.* (1987), who demonstrated that a temperature-resistant revertant of the BHK (baby hamster kidney) ts11 line, a G1 cell cycle mutant, had amplified the defective gene by a factor of more than tenfold. It would thus appear a reasonable proposition that the amplification of the K12 gene may account for the growth of the revertants isolated in 7 mM $NaHCO_3$ MCDB-302.

If the K12 ts protein shows residual activity at the npt, then the presence of many copies of the gene may provide the cell with such large amounts of the defective protein product that the resulting cumulative level of activity allows the required threshold for passage through the execution point to be just attained in 26 mM $NaHCO_3$ MCDB-302, and therefore for growth to occur at 40°C, albeit with a low E.O.P. (Table 3.80). The increase in the E.O.P. of the cells observed at the npt when the bicarbonate level was lowered to 7 mM may be accounted for if it is postulated that the use of 7 mM $NaHCO_3$ can increase the activity of the protein, by a similar process to that which prolongs the survival of K12 at the npt, in

which case the threshold level might then be exceeded by a significant degree, and as a consequence growth would be improved. The presence of abnormally large quantities of this protein in its fully active form at 33°C may be detrimental to the cell (see also Section 3.19, point b.), which would account for the reduced growth of the revertants observed at the pt (Table 3.80). In MCDB-302, the E.O.P. of the revertants decreased as the $NaHCO_3$ level was lowered at the pt, suggesting perhaps that low bicarbonate concentrations act, directly or indirectly, to raise the activity of the ts protein irrespective of the temperature, rather than simply increasing the heat stability of the defective form, and the prolonged viability of K12 at the npt in media with a reduced $NaHCO_3$ content would therefore appear to be accounted for by an increase in the initial total activity of the ts protein. It thus seems that the mutation in K12 may define a cell cycle protein whose activity is sensitive to the pH and nutritional quality of the medium, perhaps permitting the cell to respond to environmental cues. However, contrary to the abundant evidence implying the requirement for an alkaline pH and adequate nutrients in G1 for transition to the S phase (see Sections 1.2 and 4.1.iib), it would appear that the activity of this G1-controlling molecule increases as the pH and nutritional quality of the medium is lowered (although whether the magnitude of such a change in activity is of any significance in the wt cell has yet to be determined). Further investigation of the nature and role of the protein in question is clearly required in order to resolve this paradox. The use of E4 rather than MCDB-302 allowed for only poor growth of the revertant cells, even when the bicarbonate level was low (Table 3.80) suggesting, as was found in the studies on the viability of K12 at the npt, that an additional component, which differs in concentration between the two media, may also influence the activity of the ts protein, perhaps inhibiting this activity in cells grown in E4 so that the threshold level is harder to attain.

Since amplification has been demonstrated to occur randomly at a low frequency within cell populations (Stark and Wahl, 1984), it seems likely that the observed reversion may occur spontaneously within the stock culture of K12 cells maintained at 33°C, but since the revertants are unable to grow in the usual medium, 26 mM $NaHCO_3$ E4 (Table 3.80), their presence is never detected at 40°C. Culturing of the cells in 7 mM $NaHCO_3$ MCDB-302 at the npt may select for this pre-existing population, by providing conditions that enhance the growth of the revertants, and thus allow for their survival. However, if this is the case, it is unclear why no revertants were isolated in 26 mM $NaHCO_3$ MCDB-302 at the npt (Table 3.77) despite the fact that revertant cells are capable of growth in this medium, albeit with a lower E.O.P. than when 7 mM $NaHCO_3$ is used (Table 3.80). This suggests either that revertant populations do indeed arise in this medium, at a decreased

frequency due to their reduced fitness, but that the number of cells studied was insufficient for their detection, or that the use of 7 mM $NaHCO_3$ MCDB-302 at 40°C actually induces the required amplification in some unknown manner. If the medium bicarbonate concentration is found to influence the viability of a number of cdc mutants at the npt, it may be of interest to determine whether the emergence of temperature-resistant revertants in low bicarbonate media is a generalized phenomenon among mammalian cell cycle mutants, or is specific to K12 alone, and may perhaps, in addition, shed light on the mechanism by which this occurs.

It may be of relevance to the reversion process that during the course of the time-lapse studies (Section 3.14), it was noted that when either 7 mM $NaHCO_3$ MCDB-302 or 13.3 mM $NaHCO_3$ E4 was used as the growth medium at the npt, a number of mitotic cells divided into three daughters, only one or two of which subsequently divided again. This situation was not observed when the cells were cultured in media with higher bicarbonate levels. It is possible that the proper regulation of chromosome partitioning breaks down in K12 cells at 40°C under these conditions, and that the revertant cells which are isolated in 7 mM $NaHCO_3$ MCDB-302 may be daughters of such a division which have received an extra chromosome, that bearing the K12 ts gene. The survival of these cells at 40°C, and their behaviour in the different media and at the two incubation temperatures, could then be accounted for by similar mechanisms to those proposed for gene amplification; an increased copy number of the gene, combined with the raising of the activity of the protein by the use of low bicarbonate MCDB-302, may allow for good growth and division at the npt.

4.2.ii Future studies on revertant cells

Detection of amplified regions of the genome may be possible in metaphase chromosome spreads. Three cytogenetic markers of gene amplification have been characterized in transformed cells: double minute (DM) chromosomes, homogeneously staining regions (HSRs) and episomes (Cowell, 1982; Wahl, 1989). The amplified DNA may be integrated into the chromosomes, where it replicates and segregates normally, and appears as an HSR, a region in the chromosome which fails to show the usual differential G-banding pattern in metaphase spreads. Alternatively, the amplified genes may exist in extrachromosomal structures, DM chromosomes, which are detected in metaphase spreads as small, paired dot-like chromosomes. DM chromosomes lack centromeres, and thus segregate randomly at cell division and are lost in the absence of selective pressure (Wahl, 1989). A third class of amplification structures has also been described in several different cultured mammalian cell lines, namely

submicroscopic circular DNA molecules ranging from 100 - 750 kb in size, termed episomes (Carroll *et al.*, 1987; Maurer *et al.*, 1987; Von Hoff *et al.*, 1988; Wahl, 1989), which have been proposed to represent the precursors of DM chromosomes (S.M. Carroll *et al.*, 1988; Von Hoff *et al.*, 1988; 1990; Wahl, 1989). It would thus be of interest to examine metaphase spreads of the K12 revertants for the presence of such cytogenetic markers of gene expansion, in order to determine whether amplification has occurred. However, the absence of such structures does not necessarily preclude the existence of amplified regions, since amplified genes may integrate into new chromosomal sites without generating an obvious HSR (Milbrandt *et al.*, 1981), perhaps indicating that the increase in copy number in such cases is small.

If the degree of amplification is large, it may also be possible to visualize discrete bands against the background smear on restriction endonuclease digestion and agarose gel electrophoresis of genomic DNA from the revertant lines, as was shown with a methotrexate-resistant CHO cell line, which had amplified the dihydrofolate reductase gene by a factor of approximately 500 (Milbrandt *et al.*, 1981). Alternatively, if such examinations of the DNA of the K12 revertants fail to demonstrate any obvious amplification of the genome, it may instead be possible to detect overproduction of the protein encoded by the gene in question. This may be visualized by comparison of protein extracts from K12 and the revertant cell line by 2D SDS-polyacrylamide gel electrophoresis, where an excess of this protein would appear as an increase in intensity of a single spot in the revertants when compared with the K12 pattern. However, a direct demonstration of amplification may have to await the cloning of the K12 defective gene. Once a probe for the gene of interest becomes available, it should be possible to detect an increase in its copy number in the revertants by its hybridization to a Southern blot of a restriction digest of genomic DNA, where the amplified fragments would produce more intense bands in the revertant genome than in K12. The probe could also be used to quantitate the levels of mRNA transcribed from this gene in the two lines, to provide further evidence for gene amplification.

The use of such a probe could also shed light on whether reversion had occurred through the amplification of the gene encoding the K12 ts protein itself, rather than of a compensatory function. Preliminary investigation of this question may, however, be carried out in the absence of a cloned probe. If, as suggested, the observed reversion occurred by the amplification of the K12 ts gene to a level that produces sufficient residual activity of the protein product at the npt to allow the cell to cycle, it would be expected that the glycosylation defect and overproduction of the GRPs, also produced as a consequence of the loss of activity of the ts protein at 40°C (see Section 1.12), would be less severe in the revertants when compared to

K12 at the npt. Studies on ^{3}H-glucosamine uptake and GRP expression in the revertants may therefore provide evidence for the selective amplification of the K12 ts gene. However, although it seems unlikely that any compensatory function would play a role in these additional manifestations of the K12 defect, particularly since they appear to be causally unrelated to the cell cycle block (Feige and Scheffler, 1987), this does remain a possibility, thus a definitive investigation of the underlying basis of the observed reversion of K12 in 7 mM $NaHCO_3$ MCDB-302 at 40°C must await the cloning of the defective gene.

4.3 ISOLATION OF THE GENE DEFINED BY THE K12 MUTATION

4.3.i Attempted cloning of the gene defective in K12

In order to elucidate the nature of the defective gene product in K12, and thus to understand its role in the normal cell cycle, attempts were made to clone the corresponding gene by transfection with various human gene libraries. Initial experiments using the Okayama-Berg λcDNA expression library (Okayama and Berg, 1985) gave transfection frequencies too low (at best, approximately 0.01%) to allow for isolation of the K12-complementing gene (Section 3.18). The use of a modified transfection protocol (Chen and Okayama, 1987) and the plasmid form of the library (Okayama and Berg, 1983) resulted in the greatly increased transfection efficiency of roughly 30% (Section 3.19). However, although five temperature-resistant colonies were isolated from a total of 10^7 transfected cells (a frequency suggesting that the gene defective in K12 is present as a single copy in the genome), these survived for only nine days at the npt before viability was lost. Since untransfected cells generally lose viability within three days under these conditions (Table 3.61), it appeared likely that these colonies contained the gene of interest, allowing for the observed prolonged survival, but that the complementing gene was unable to support continued survival at the npt. It is possible that pCD is unstable in this cell line and was lost, a phenomenon noted by Alder *et al.* (1989) in their attempts to complement the defect in several hamster ts cell cycle mutants using this library. Alternatively, it may be that the use of the SV40 early region promoter for the regulation of the human insert resulted in the inappropriate expression of the gene in question. It thus appeared that although a human homologue to the gene defective in K12 seems to exist, it may not be isolated using either of the Okayama-Berg cDNA libraries.

In order to eliminate the potential problems associated with the expression of the human gene from a heterologous promoter, the use of human genomic DNA, which would allow each gene to retain its own promoter, was next considered. Transfection

of K12 with human DNA, followed by the construction of a cosmid library from the DNA of cells that grew at the npt and screening for human-specific sequences e.g. by the use of the BLUR-8 *Alu* probe (Rubin *et al.*, 1980), should allow for the isolation of the K12-complementing gene. However, two rounds of transfection are necessary to exclude irrelevant human DNA, and preliminary work of this kind (Dr. D.H. Roscoe, unpublished results) demonstrated an inability to transfer the gene of interest to secondary transfectants. In order to enrich for the K12-complementing gene, and thus to eliminate the requirement for a second round of transfection, K12-human hybrid cells were produced (Section 3.20), in the hope that much of the superfluous human DNA would be lost by isolating cells which retain only the human chromosome complementing the K12 mutation, i.e. chromosome 14 (Ming *et al.*, 1979). However, after seven months of culture of the hybrid cells, many other human chromosomes remained, thus this protocol was deemed impractical for the given purpose.

The next approach taken was the use of an Epstein-Barr virus-based human genomic cosmid library (Kioussis *et al.*, 1987), where each insert was expressed from its own promoter. On transfection of K12 with this library (Section 3.21), a similar pattern of results to those found with the Okayama-Berg plasmid cDNA library was observed i.e. several colonies survived for a few weeks at the npt, but subsequently died. This lent further support to the idea that the K12-complementing gene is present in each of these libraries, but even if its expression is regulated by its natural promoter, although allowing for extended growth at 40°C, it cannot support indefinite survival. It thus appears that the human homologue of the defective gene in K12 may be inappropriately expressed in the Chinese hamster background, perhaps due to inherent differences in the expression pattern produced by the hamster and human promoters, or to an inability of hamster regulatory molecules to interact with the heterologous promoter. Alternatively, it is possible that the human gene product requires interaction with an accessory human molecule to carry out its function, for which the Chinese hamster homologue is unable to substitute.

To avoid the potential difficulties implied in the utilization of a heterologous gene to complement the ts defect, K12 was subsequently cotransfected with the selective marker pSV2neo and genomic DNA from the wild-type parent, Wg-1A (Section 3.22). The frequencies of transfection to temperature-resistance were again consistent with the idea that the K12-complementing gene is a single copy gene. However, in order to allow for the detection of the gene of interest in the otherwise identical Chinese hamster background of the transfectants, a necessary prerequisite for its recovery, its tagging with an adjacent physical marker, such as the *neo* gene, was required. In vitro ligation of the *neo* gene to Wg-1A genomic DNA would have been a time-

consuming process, thus an infrequently used method of in vivo linkage was attempted. This took advantage of the observation that when high-molecular-weight DNA is cotransfected with a plasmid-borne selectable marker, the DNA molecules are degraded and ligated to produce a large concatamer or transgenome of approximately 1000 kb (Perucho *et al.*, 1980; Scangos *et al.*, 1981), consisting of plasmid sequences randomly interspersed with non-selectable DNA, which subsequently integrates into the chromosomes and is stably maintained (Scangos *et al.*, 1981). It was hoped that on cotransfection of K12 with Wg-1A genomic DNA and pSV2neo, a copy of the *neo* gene would lie in close enough proximity to the K12-complementing gene in primary transfectants, due to transgenome formation, that on a second round of transfection, this time in the absence of exogenous pSV2neo, they would be transferred together. The construction of a cosmid library from a temperature-resistant secondary transfectant and screening for the adjacent *neo* gene should then allow for the isolation of the clone of interest. However, on secondary transfection with DNA from the Wg-1A/pSV2neo cotransfectants, only a low frequency of transfection to G418-resistance was obtained (1 in 10^6 cells), and no temperature-resistant clones were isolated. Probing of an *Xba* I digest of primary transfectant genomic DNA with pSV2neo (Section 3.23) suggested that this was a consequence of a low copy number of the *neo* gene in the transfectant DNA (2 - 6 copies/transfectant). Given an average transgenome size of 1000 kb (Perucho *et al.*, 1980), even if the maximum copy number of six pSV2neo sequences were spread along the transgenome, it seemed unlikely that the K12-complementing gene would be close enough to a *neo* gene for their cotransfer to a secondary transfectant (genomic DNA isolated from the primary transfectants was an average of 100 kb in size - see Fig. 3.56), thus accounting for the observed failure to isolate any temperature-resistant clones on the second round of transfection. This method therefore seemed unlikely to result in the isolation of the gene of interest.

4.3.ii Further work on cloning the K12 defective gene

Any future attempts to clone the defective gene in K12 could take a number of different routes:

1. Wg-1A genomic DNA could be ligated to a selective marker in vitro and used for two rounds of transfection. A cosmid library constructed from temperature-resistant secondary transfectants would then be screened for the presence of the selective marker, which should be adjacent to the gene of interest and therefore allow for its isolation and further analysis. This approach was applied successfully by Westerveld *et al.* (1984) to isolate the human repair gene *ERCC1* complementing the repair defect

in a CHO mutant cell line, using HeLa genomic DNA and the *gpt* gene as the marker. However, recent evidence suggests that both *neo* and *gpt* can act negatively in *cis* on the transcriptional activity of eukaryotic promoters (Artelt *et al.*, 1991). Of particular interest to the current study is the observation that the insertion of the *neo* gene into plasmids containing a reporter gene resulted in a five- to tenfold decrease of expression from a proximal SV40 early promoter, independent of the insertion site or orientation. Since the Okayama-Berg plasmid cDNA library uses the SV40 early promoter for the expression of the human insert, this may have contributed to the inability of the pCD molecule containing the human K12-complementing gene to support the continued the survival of K12 at the npt (Section 3.19); the combination of the use of a heterologous human gene, which may show reduced activity in the Chinese hamster background, and the repression of its expression by *neo* may have reduced the amount of activity of the required gene product to an inadequate level, allowing for only limited viability. The greater distance separating the *neo* gene and the K12-complementing gene in the Wg-1A/pSV2neo cotransfections (Section 3.22), coupled with the expected compatibility of gene products, could account for the lack of problems in isolating temperature-resistant clones from such transfections. In view of the silencing effect of the *neo* and *gpt* genes on adjacent promoters, it would thus appear wise to use an alternative selective marker for ligation to the Wg-1A genomic DNA; the *pac* gene of *Streptomyces alboniger*, which confers resistance to puromycin (Vara *et al.*, 1986) and does not seem to influence proximal promoters (Artelt *et al.*, 1991), would appear to be a suitable candidate.

2. High-molecular-weight genomic rat DNA may be used for transfection of K12, reducing the likelihood of problems with incompatible gene products due to the close relationship of the two species. The required clone would then be recovered from a cosmid library constructed from secondary transfectants by screening with a probe which hybridizes to rat-specific repetitive sequences.

3. Two rounds of transfection of K12 with a Chinese hamster genomic cosmid shuttle library, followed by packaging of the DNA from temperature-resistant secondary transfectants with a λ packaging extract, and infection of *E. coli* with the recombinant phage, should allow for the isolation of the gene of interest.

Whatever the method used for its isolation, once a K12-complementing clone has been obtained, the insert should be restriction-mapped and reduced to its minimum effective size by digestion. The DNA sequence of the gene, and thus the putative sequence of its protein product, could then be determined, and used to screen for potential homologies with known cell cycle or other genes, which may help to elucidate its role in the cell cycle. In addition, the gene may be expressed as a

glutathione S-transferase fusion protein in a baculovirus expression system (Luckow and Summers, 1988; Maeda, 1989), purified and used to raise an antibody, which may then be employed for the isolation of the native polypeptide by immunoprecipitation from K12 and Wg-1A cell extracts for the further analysis of its characteristics. The cloned probe could also be used to investigate a number of the unanswered questions raised by this current study:

a. A cosmid library of K12 genomic DNA could be constructed and screened with the probe, in order to isolate the defective form of the gene. Sequencing of this gene and comparison with the wild-type sequence would identify the cause of the heat instability of the gene product in K12.

b. The probe may be used to detect whether the observed reversion of K12 in 7mM $NaHCO_3$ MCDB-302 (Section 3.17) is indeed due to the amplification of the defective gene (see Section 4.2.ii).

c. If the ts protein was isolated from K12 and its role in the cell cycle elucidated, the influence of pH on its activity and heat stability could be investigated, and may help to explain the observed effects of variations in medium bicarbonate concentration on the viability of this cell line at the npt.

d. The human homologue of the gene defective in K12 could be isolated from the Okayama-Berg plasmid cDNA and Kioussis Epstein-Barr virus-based genomic human libraries by screening with the hamster or rat clone, and subsequently transfected into K12. It may be that the human genes prove unable to support the continued survival of K12 at the npt, confirming that the human homologue of this particular cell cycle gene is unable to function in a hamster background. If this proves to be the case, this will enhance the interest of the K12 gene, since the majority of the cell cycle genes studied to date have been shown to be functionally conserved across great species barriers, and will thus justify further analysis of the role of the gene product in question in the cell cycle.

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APPENDIX I

SUPPLIERS OF CHEMICALS AND CONSUMABLES

Anglian Biotechnology Ltd Restriction enzymes (*Eco* R1, *Hind* III, *Xba* I), λ DNA.

β-LAB Bacto-yeast extract.

BDH Ltd Bromophenol blue, caesium chloride, chloroform, DMSO, 8-hydroxyquinoline, isoamyl alcohol, isopropanol, PEG 1500, PEG 6000, potassium chloride, sodium acetate, sodium bicarbonate, SDS, Triton X-100.

Calbiochem BCECF-AM, BCECF-free acid, hygromycin B, propidium iodide.

Comak Chemicals Ltd Di-sodium hydrogen phosphate, potassium dihydrogen phosphate, sodium dihydrogen phosphate, sucrose.

Difco Bacto-tryptone.

DuPont Ltd ^{35}S-dATP.

Falcon 10 cm plastic tissue culture dishes, 25 cm^2 plastic tissue culture flasks, 15 ml polystyrene screw-cap conical tubes, 50 ml polypropylene screw-cap conical tubes.

Fisons plc Boric acid, EDTA, glucose, glycerol, magnesium chloride, magnesium sulphate, maltose, phenol, urea.

FSA Laboratory Supplies Hydrochloric acid, sodium hydroxide.

Gibco BRL G418.

Hopkin and Williams Ltd Calcium chloride.

Imperial Cancer Research Fund E4 low bic, glutamine, trypsin.

Imperial Laboratories Ltd CS, E4 no bic, FCS, MEM.

May and Baker Glacial acetic acid, sodium chloride, sodium citrate, trichloroacetic acid.

Nunc 1.8 ml cryotubes, 6 cm plastic tissue culture dishes.

Oxoid Nutrient agar, nutrient broth.

Park Scientific Ltd Dithiothreitol.

Penbritin Ampicillin.

Pharmacia Agarose-NA.

Schleicher and Schuell 0.45 μm pore size nitrocellulose

Sherman Chemicals Ltd Potassium hydroxide.

Sigma Chemical Company Ltd Agarose type II-low EEO, BES, chloramphenicol, choline chloride, diaminopimelic acid, ethidium bromide, gelatin, HEPES, lysozyme, MCDB-302 medium (1x powder), nigericin, ouabain, pancreatic DNase, PBSA tablets, proteinase K, RNase A, salmon sperm DNA, Sephadex-G50, sodium N-lauroyl sarcosine, streptomycin sulphate, thymidine, Trizma base.

Sterilin 10 cm petri dishes.

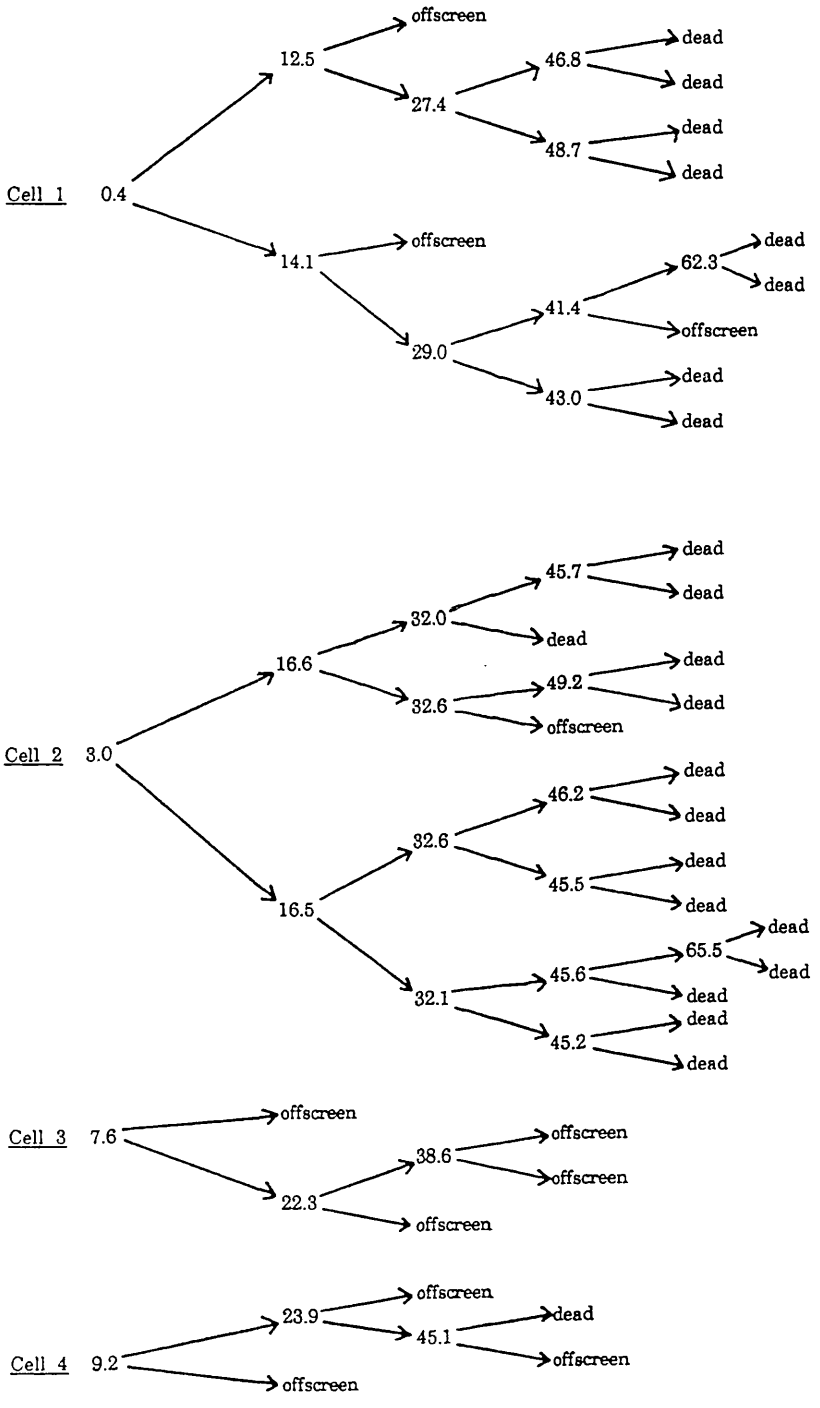
Whatman 3MM filter paper

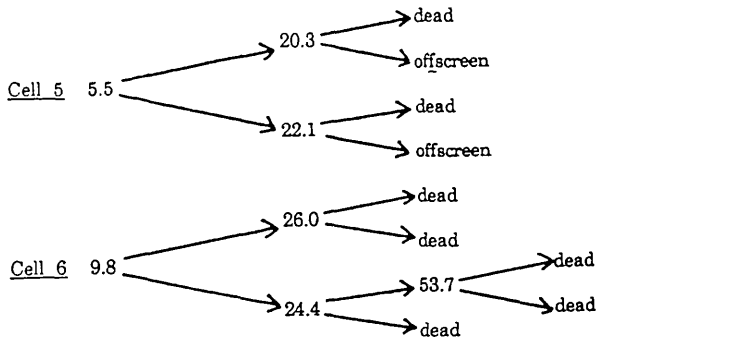
Pluronic F-127 and carboxy-SNARF-1/AM acetate were kind gifts from Dr. S. Bolsover (University College London).

APPENDIX II

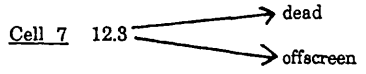
EXAMPLE OF TIME-LAPSE DATA

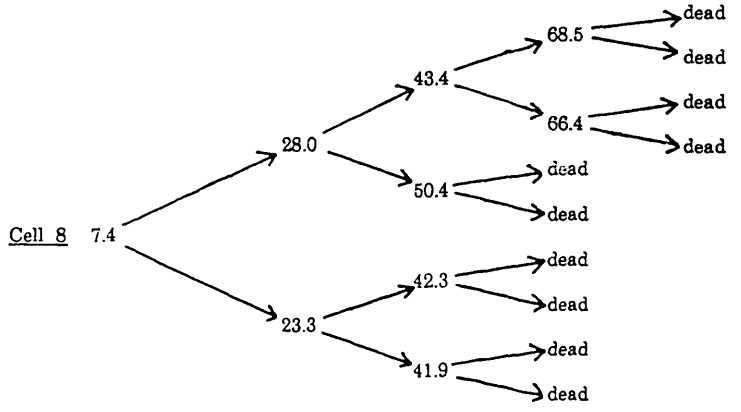
a. 'Family tree' of division times (in hours) for K12 cells grown in 5% FCS E4 low bic at 33°/40°C and recorded by time-lapse cinematography



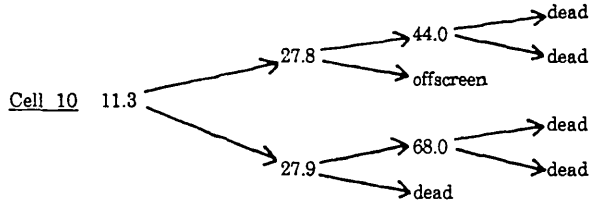

Cell 5
5.5
20.3
dead
offscreen
22.1
dead
offscreen

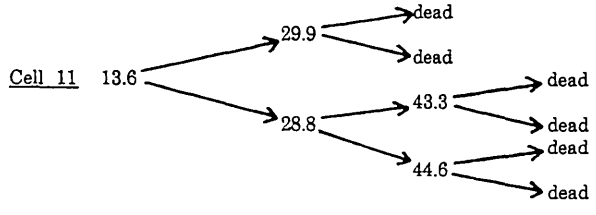
Cell 6
9.8
26.0
dead
dead
24.4
53.7
dead
dead
dead

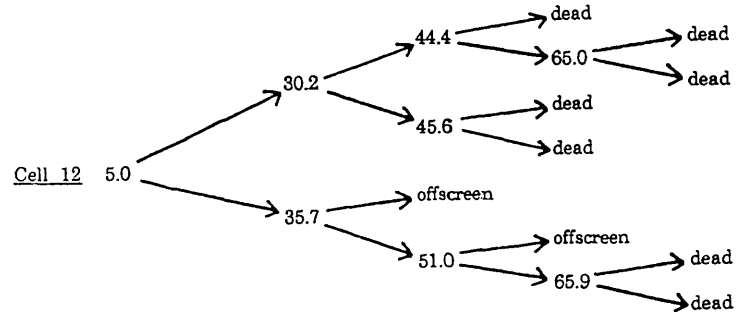

Cell 7
12.3
dead
offscreen

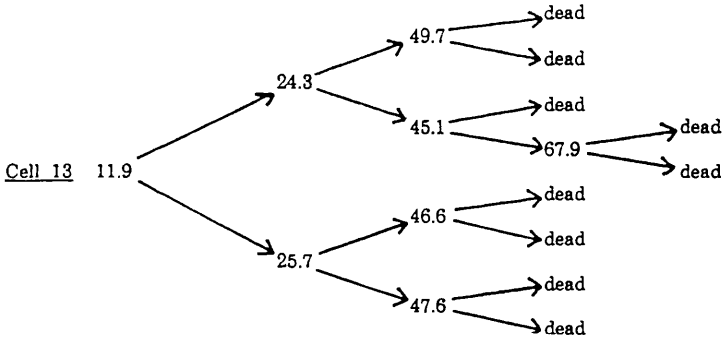

Cell 8
7.4
28.0
43.4
68.5
dead
dead
66.4
dead
dead
50.4
dead
dead
23.3
42.3
dead
dead
41.9
dead
dead

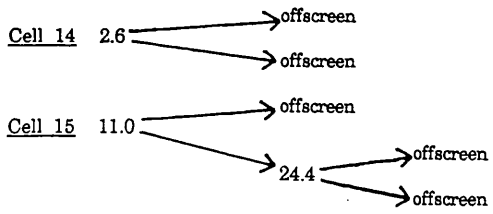

Cell 9
14.6
offscreen
offscreen

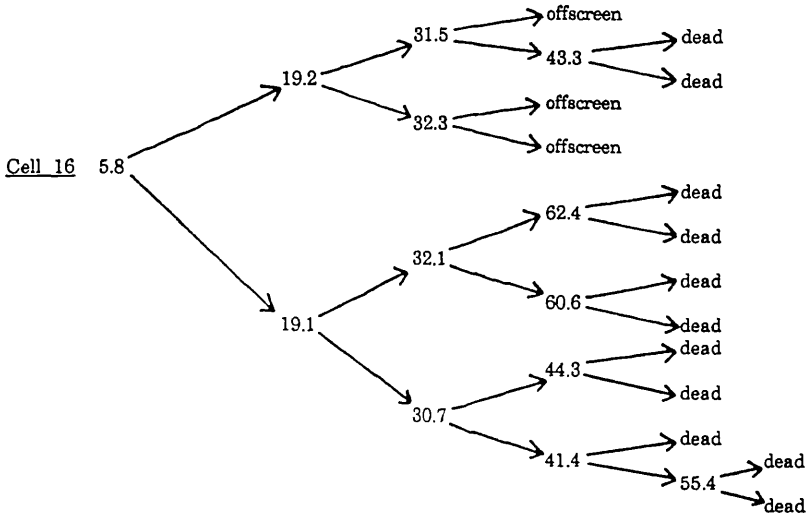

Cell 10
11.3
27.8
44.0
dead
dead
offscreen
27.9
68.0
dead
dead
dead

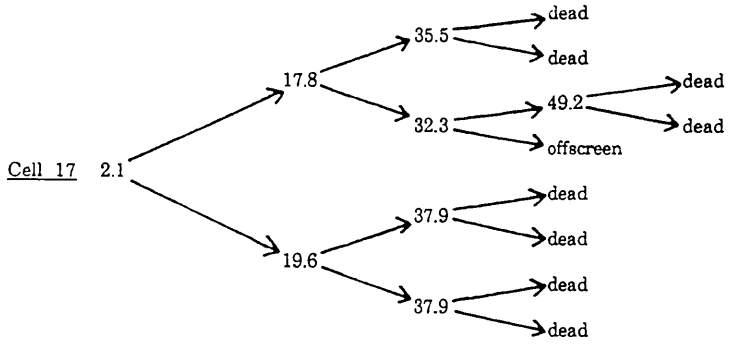

Cell 11
13.6
29.9
dead
dead
28.8
43.3
dead
dead
44.6
dead
dead

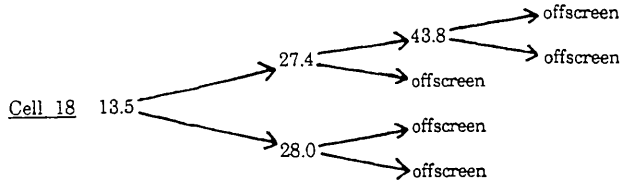

Cell 12
5.0
30.2
44.4
dead
65.0
dead
dead
45.6
dead
dead
35.7
offscreen
51.0
offscreen
65.9
dead
dead

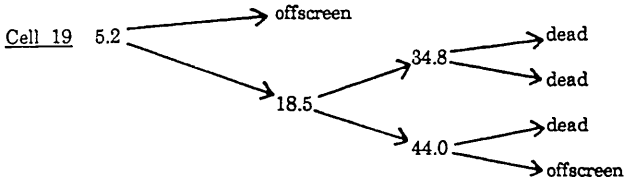

Cell 13
11.9
24.3
25.7
49.7
45.1
46.6
47.6
67.9
dead

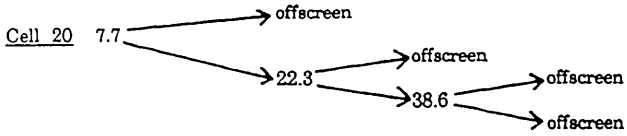

Cell 14
2.6
offscreen
Cell 15
11.0
24.4

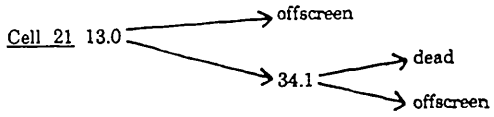

Cell 16
5.8
19.2
19.1
31.5
32.3
43.3
32.1
30.7
62.4
60.6
44.3
41.4
55.4
offscreen
dead


Cell 17
2.1
17.8
19.6
35.5
32.3
37.9
49.2
dead
offscreen


Cell 18
13.5
27.4
43.8
offscreen
offscreen
offscreen
28.0
offscreen
offscreen


Cell 19
5.2
offscreen
18.5
34.8
dead
dead
44.0
dead
offscreen


Cell 20
7.7
offscreen
22.3
offscreen
38.6
offscreen
offscreen


Cell 21
13.0
offscreen
34.1
dead
offscreen

b. Data for cumulative mitosis plot taken from 'family tree'

Time of division	Mitosis	Cumulative mitoses	Time of division	Mitosis	Cumulative mitoses
0.4	1	1	32.3	1	57
2.1	1	2	32.3	1	58
2.6	1	3	32.6	1	59
3.0	1	4	32.6	1	60
5.0	1	5	34.1	1	61
5.2	1	6	34.8	1	62
5.5	1	7	35.5	1	63
5.8	1	8	35.7	1	64
7.4	1	9	37.9	1	65
7.6	1	10	37.9	1	66
7.7	1	11	38.6	1	67
9.2	1	12	38.6	1	68
9.8	1	13	41.4	1	69
11.0	1	14	41.4	1	70
11.3	1	15	41.9	1	71
11.9	1	16	42.3	1	72
12.3	1	17	43.0	1	73
12.5	1	18	43.3	1	74
13.0	1	19	43.3	1	75
13.5	1	20	43.4	1	76
13.6	1	21	43.8	1	77
14.1	1	22	44.0	1	78
14.6	1	23	44.0	1	79
16.5	1	24	44.3	1	80
16.6	1	25	44.4	1	81
17.8	1	26	44.6	1	82
18.5	1	27	45.1	1	83
19.1	1	28	45.1	1	84
19.2	1	29	45.2	1	85
19.6	1	30	45.5	1	86
20.3	1	31	45.6	1	87
22.1	1	32	45.6	1	88
22.3	1	33	45.7	1	88
22.3	1	34	46.2	1	89
23.3	1	35	46.6	1	90
23.9	1	36	46.8	1	91
24.3	1	37	47.6	1	92
24.4	1	38	48.7	1	93
24.4	1	39	49.2	1	94
25.7	1	40	49.2	1	95
26.0	1	41	49.7	1	96
27.4	1	42	50.4	1	97
27.4	1	43	51.0	1	98
27.8	1	44	53.7	1	99
27.9	1	45	55.4	1	100
28.0	1	46	60.6	1	101
28.0	1	47	62.3	1	102
28.8	1	48	62.4	1	103
29.0	1	49	65.0	1	104
29.9	1	50	65.5	1	105
30.2	1	51	65.9	1	106
30.7	1	52	66.4	1	107
31.5	1	53	67.9	1	108
32.0	1	54	68.0	1	109
32.1	1	55	68.5	1	110
32.1	1	56			

c. Data for intermitotic time plot taken from 'family tree'

Time of division	Intermitotic time	Time of division	Intermitotic time
0.4	12.1	27.4	16.4
	13.7	27.4	19.4
2.1	15.7		21.3
	17.5	27.8	16.2
3.0	13.5	27.9	40.1
	13.6	28.0	15.4
5.0	25.2		22.4
	30.7	28.8	14.5
5.2	13.3		15.8
5.5	14.8	29.0	12.4
	16.6		14.0
5.8	13.3	30.2	14.2
	13.4		15.4
7.4	15.9	30.7	10.7
	20.6		13.6
7.6	14.7	31.5	11.8
7.7	14.6	32.0	13.7
9.2	14.7	32.1	28.5
9.8	14.6		30.3
	16.2	32.1	13.1
11.0	13.4		13.5
11.3	16.5	32.3	16.9
	16.6	32.6	12.9
11.9	12.4		13.6
	13.8	32.6	16.6
12.5	14.9	35.7	15.3
13.0	21.1	41.4	14.0
13.5	13.9	41.4	20.9
	14.5	43.4	23.0
13.6	15.2		25.1
	16.3	44.4	20.6
14.1	14.9	45.1	22.8
16.5	15.6	45.6	19.9
	16.1	51.0	14.9
16.6	15.4		
	16.0		
17.8	14.5		
	17.7		
18.5	16.3		
	25.5		
19.1	11.6		
	13.0		
19.2	12.3		
	13.1		
19.6	18.3		
	18.3		
22.3	16.3		
22.3	16.3		
23.3	18.6		
	19.0		
23.9	21.2		
24.3	20.8		
	25.4		
24.4	29.3		
25.7	20.9		
	21.9		

APPENDIX III

COMPOSITION OF MCDB-302 AND E4 LOW BIC MEDIA

	CONCENTRATION (mg/l)	
COMPONENT	**MCDB-302**	**E4 LOW BIC**
L-Alanine	8.91	0
L-Arginine.HCl	210.7	84
L-Asparagine.H_2O	15.01	0
L-Aspartic acid	13.31	0
L-Cysteine.HCl.H_2O	17.56	48
L-Glutamic acid	14.71	0
L-Glutamine	438.6	584
Glycine	7.51	30
L-Histidine.HCl.H_2O	20.96	42
L-Isoleucine	3.94	104.8
L-Leucine	13.12	104.8
L-Lysine.HCl	36.54	146.2
L-Methionine	4.48	30
L-Phenylalanine	4.96	66
L-Proline	34.53	0
L-Serine	10.51	42
L-Threonine	11.91	95.2
L-Tryptophan	2.04	16
L-Tyrosine	0	72.4
L-Tyrosine.2Na	7.84	0
L-Valine	11.72	93.6
d-Biotin	7.33×10^{-3}	0
D-Pantothenic acid.Ca	0.238	4
Choline chloride	13.96	4
Folic acid	1.324	4
Inositol	0	7
Myo-Inositol	18.02	0

	CONCENTRATION (mg/l)	
COMPONENT	MCDB-302	E4 LOW BIC
Niacinimide	0.0366	0
Nicotinamide	0	4
Pyridoxine.HCl	0.0617	4
Riboflavin	0.0376	4
Thiamine.HCl	0.337	4
Vitamin B_{12}	1.36	0
Linoleic acid	0.0841	0
DL-α-Lipoic acid	0.206	0
Putrescine.2HCl	0.161	0
Dextrose	0	4500
D-Glucose	1801.6	0
Phenol red.Na	1.242	15.02
Sodium pyruvate	110.1	110
Streptomycin	0	100
Penicillin	0	10^5 units
n-Butyl-p-hydroxybenzoate	0	0.2
$CaCl_2.2H_2O$	88.21	265
$Fe(NO_3)_3.9H_2O$	0	0.1
KCl	223.65	400
$MgCl_2.6H_2O$	122	0
$MgSO_4.7H_2O$	0	200
$NaHCO_3$	1117.6	2200
NaCl	7599	6400
Na_2HPO_4	141.98	0
$NaH_2PO_4.2H_2O$	0	140
$CuSO_4.5H_2O$	2.497×10^{-3}	0
$FeSO_4.7H_2O$	0.834	0
$MnSO_4.5H_2O$	2.41×10^{-4}	0
$[NH_4]_6Mo_7O_{24}.4H_2O$	0.01236	0
Na_2SeO_3	7.896×10^{-4}	0
NH_4VO_3	1.17×10^{-3}	0
$ZnSO_4.7H_2O$	0.8626	0