'PROCESS EFFECTIVENESS OF YEAST EXPRESSION VECTORS'

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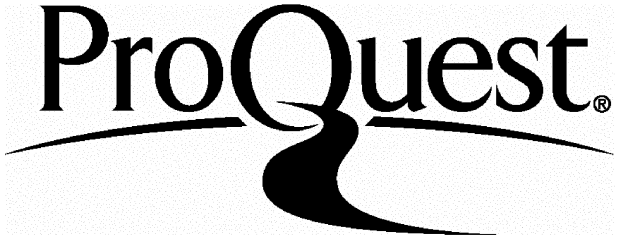

ABSTRACT

This thesis describes work on aspects of specific regulated *Saccharomyces cerevisiae* promoters, namely, the growth phase and medium dependent regulation of the yeast heat-shock promoter element or HSE (Chapter 3); the promoter of the polyubiquitin gene (Chapter 4) and the promoter of the mating factor alpha, *MFα1* gene (Chapter 5).

Chapter 3 describes the use of temperature upshift as a convenient way of inducing heterologous gene expression using a plasmid in which the regulatory elements from a yeast heat-shock promoter were present in a modified *CYC1* promoter *lacZ* fusion in place of the *CYC1* promoter regulatory region. Protein induction levels of up to 50 fold were seen on temperature upshift of a logarithmically growing yeast culture from 23°C to 39°C. Heat inducibility of the HSE was maximal at 39°C and lost at stationary phase of growth. The potential problem of protein degradation was tackled by using protease deficient strains which increased β-galactosidase accumulation 2-fold.

Studies with the yeast heat shock promoter *UBI4* (Chapter 4), chosen because of its potential use as a growth phase dependent promoter, showed that this promoter is primarily controlled (not as previously reported by intracellular cAMP levels) by carbon catabolite repression and this control is exerted probably through the *HAP 2/3/4* regulatory system and as such is the first gene for a non-mitochondrial component shown to be controlled by this system.

The yeast *MFα1* promoter was studied (Chapter 5). Results indicate that this promoter is regulated by the growth rate of the host cell, its activity rising as the cellular growth rate falls. Therefore maximum expression levels from this promoter can be achieved by growing cells at a low growth rate under respiratory conditions, a situation that will maximize both cell biomass and protein expression levels.

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ABBREVIATIONS

ADH	Alcohol dehydrogenase
BCY	Regulatory subunit of cAMP-dependent protein kinase
cAMP	Cyclic adenosine monophospate
CCR	Carbon catabolite repression
CCT1	Catalase T
CSF	Colony stimulating factor
CYC1	Isocytochrome C
CYR	Adenylate cyclase
D	Dilution rate
D_C	Critical dilution rate
D_R	Dilution rate at which maximum respiratory capacity of yeast is reached
EGF	Epidermal growth factor
GADPH	Glyceraldehyde-3-phosphate dehydrogenase
HSE	Heat shock element
HSF	Heat shock transcription factor
HSP	Heat shock protein
IFN	Interferon
IL2	Interleukin
LacZ	β-Galactosidase
MFα	Mating factor α
μmax	Maximum specific growth rate
PGK	Phosphoglycerate kinase
PHO5	Acid phosphatase
PI	Proinsulin
PRE	Pheromone response element
PYK	Pyruvate kinase
SD	Standard defined
SOD	Superoxide dismutase
TPK	Catalytic subunit of cAMP-dependent protein kinase
UBI	Ubiquitin

CHAPTER 1. INTRODUCTION

1.1 Summary of the use of *S. cerevisiae* as a host for heterologous gene expression

1.1.1 Benefits of *S. cerevisiae* as compared to other hosts

Advances in rDNA technology have enabled the expression of foreign proteins in a wide variety of host-vector systems, the most widely used hosts being *E. coli*, *S. cerevisiae*, insect cells and mammalian cells (Marino, 1989). It is becoming evident that there is no single host optimal for the production of all recombinant proteins. Because of this, the system that offers the most significant advantages for the production of a particular protein must often be identified by trial of a number of different expression systems. The final choice will depend on the quantity and purity of protein required, whether correct folding can be achieved *in vivo* or must occur *in vitro*, as well as the economics of the production process. In the commercial situation, the expression of the protein of interest will often be characterised in a range of host - vector combinations and only once this has been carried out can informed decisions can be made as to the host most suitable for the production of that protein (Marino, 1989).

The use of the yeast *S. cerevisiae* as a host for the production of heterologous proteins has expanded dramatically over the past decade (Martin & Scheinbach, 1989). There are several reasons for this, but its popularity mainly stems from the limitations encountered when using *E. coli* as a production host. Unlike *E. coli*, yeast has a secretion system resembling that found in mammalian cells (Scheckman & Novick, 1982; Kukurazinoka *et al.*, 1987). It can therefore perform many of the post translational modifications to the secreted protein such as disulphide bridge formation and glycosylation - leading to secretion of the protein in a bio-active form into the growth medium. Since yeast only secretes a small fraction of its proteins, secretion as a production route significantly aids further downstream processing. Also of significant importance in the field of downstream processing is the fact that yeasts have been used in the food industry for centuries and contain no detectable endotoxins. Purification protocols are less complex, and there is less problem in getting approval for the marketing of yeast - derived products from the regulatory authorities.

One of the first commercial applications of heterologous gene expression in yeast was the production of the hepatitis B vaccine (Scolnick *et al.*, 1984). Superoxide dismutase (SOD), α-1-antitrypsin, epidermal growth factor (EGF) and various colony stimulating factors (CSF's) produced in yeast are now in clinical trials (Goodey *et al.*, 1987).

1.1.2 Introduction of heterologous genes into *S. cerevisiae*

The production of recombinant proteins in yeast requires the gene of interest to be introduced into the cell via transformation (Hinnen, Hinks & Fink, 1978; Ito *et al.,* 1983). Yeast vectors fall into four main categories based on their mode of replication within yeast; Yeast Episomal plasmids, YEp vectors, utilise the yeast 2 μm plasmid DNA replication origin to replicate in yeast (Broach, 1981). Yeast Replicative plasmids, YRp vectors, utilise an ARS sequence (autonomous replication sequence) from chromosomal DNA (Stinchombe *et al.,* 1980). Yeast Centromeric plasmids; YCp vectors utilise either an ARS, or the 2 μm plasmid DNA replication origin in conjunction with a centromere sequence. Yeast Integrative plasmids; YIp vectors contain no replication sequence and are used to integrate foreign genes into the yeast chromosome by homologous DNA recombination (Orr-Weaver, Szostak & Rothstein, 1981). The two most commonly used vectors for industrial scale production of recombinant proteins in yeast are the YEp and YIp vector types.

VECTOR	YEp	YRp	YCp	YIp
Replicator	2 μm	ARS	ARS or 2μm + CEN	None
Transformation frequency *	10,000	10000	10000	10
Copies per cell	5-100	3-30	1	1-3
Stability	Depends on selection	Poor	Good	Good

Table 1.1. Vector types in *S. cerevisiae* (* Maximal efficiency per μg DNA)

1.1.3 Ensuring the stability of expression vectors in *S. cerevisiae*

The most commonly used system for ensuring that cells maintain a 2 μm based plasmid expression vector employs an auxotrophic host strain grown under conditions such that a marker gene on the plasmid is essential for the growth of the transformed cell. Of these nutritional marker genes, the most commonly used are *LEU2 TRP1* and *URA3* (Broach, 1981). Thus a *trp1* host transformed with a *TRP1* plasmid grown in medium minus tryptophan should ensure selection for plasmid bearing cells, plasmid free segregant cells arising from plasmid loss should suffer growth arrest.

However in many cases, the toxicity caused by the accumulation of high levels of the heterologous protein within the cell causes plasmid instability, manifested by plasmid rearrangement or complete loss of the plasmid. The expression cassette can also be lost without loss of the gene used for selection enabling the cells still to grow in the selective medium without expressing the heterologous gene (Belsham, Barker & Smith, 1986).

YEp vectors contain the 2µm replication origin which interacts with replication proteins often provided *in trans* by the endogenous 2µm plasmid (Broach *et al.*, 1981). YEp vectors can be maintained at high copy numbers; the use of the *LEU2* gene with a defective truncated promoter; *LEU2d*, allows copy numbers to rise to about 200 per cell in a *leu2* host because of the weak expression of the *LEU2* gene from the defective promoter (Beggs, 1978; Erhart & Hollenberg, 1983). One drawback of these 2µm vectors is that their stability is poor, especially under long term fermentation conditions (Futcher & Cox, 1986). This has variously been attributed to segregational bias between mother and daughter cell on division, especially marked at high growth rates and other factors as outlined by Tottrup & Carlsen, (1990). The stability of vectors containing the whole 2µm plasmid sequence has been shown to be much greater, and these may be of more use in long term industrial fermentations (Beggs, 1981).
Problems of vector stability are largely overcome using YIp transformants. YIp vectors are stably integrated into the host genome, allowing long term growth of the transformed cell in the absence of selection pressure (Stearns, Ma & Botstein, 1990). Such integrative transformants can often be grown in complex undefined media for many generations without showing any segregational instability unless the product is toxic to the cell (Zhy, Contreras & Fiers, 1986).

The choice of plasmid for heterologous gene expression is clearly of vital importance. The two most important factors to be taken into account are the copy number and stability of the expression cassette. Two micron based vectors are most suited to high level expression of a heterologous protein during a short fermenter run. For longer term fermentations such as fed-batch or continuous, the lowered stability of plasmid based expression will often lead to progressive reductions in yield and integrative vectors seem to be the most advisable.

1.1.4. Promoters employed for the expression of heterologous genes in *S. cerevisiae*

1.1.4.1. Constitutive promoters

Expression of heterologous proteins in yeast can be achieved by using promoters from the highly expressed glycolytic genes of *S. cerevisiae*. Initial vectors usually incorporated transcriptional promoters from genes such as the *PGK* and *GADPH* (Tuite *et al.*, 1983; Holland & Holland, 1979). Using these promoters to drive the expression of heterologous proteins can cause such proteins to accumulate to 2-5% total cell protein (Rosenberg, Barr & Najarian, 1984) or, in the few cases where the heterologous protein is extremely stable (eg. superoxide dismutase (SOD)) up to 50% total cell protein (Hallewell *et al.*, 1987). However, a major drawback of the use of strong constitutive promoters is that they are actively expressing protein right from the start of the fermentation, causing an energy drain to the cell and slowing down growth before the fermentation has reached the high biomass levels required for efficient and economic protein production (Fieschko *et al.*, 1987).

1.1.4.2 Regulated promoters

One potential way to overcome the problems mentioned above is to use regulated promoters (Martin & Scheinbach, 1989). These are turned on by physiological changes within the cell that often reflect changes to the growth medium or growth temperature. Utilizing a regulated promoter should allow the expression of a heterologous protein to be switched on when cells have obtained a high biomass, reducing the effect of product toxicity on growth rate and plasmid stability. A number of highly regulated systems have been developed for yeast. The promoter of the (*PHO5*) acid phosphatase gene is induced 200 fold by phosphate depletion from the growth medium and has been used for the regulated expression of a number of heterologous proteins (Kramer, DeChiara & Schaber, 1984). Promoters of genes involved in galactose metabolism (eg. *GAL1*) are glucose repressed and can be induced by up to 2000 fold by the removal of glucose and the addition of galactose to the growth medium (St John & Davis, 1981). Certain promoters are naturally switched on by the cell at defined stages of the fermentation without the need for medium manipulation. The *ADH2* promoter is an example of a catabolite repressed promoter induced 200 fold by growth associated glucose depletion from the growth medium and 1000 fold by transfer from 5% glucose to 3% ethanol (Yu, Donoviel & Young, 1989).

Temperature regulated expression of foreign genes may be of particular relevance to industrial scale fermentations as such systems are already of widespread use for the expression of heterologous proteins in *E. coli* (Caulcott & Rhodes, 1986). Cells may

be grown to a high cell density in the absence of expression the expression of the heterologous gene being induced by switching cells to the permissive temperature. This is discussed in greater detail in Section 4.

The choice of an expression system for a heterologous protein is dependent on the protein to be expressed. Proteins vary in their toxicity to the host and in their stability following expression. Certain stable, non-toxic proteins such as SOD and the p28 antigen (Loison, Vidal & Findell, 1989) can be constitutively overexpressed and accumulate intracellularly to high levels. For toxic proteins, constitutive overexpression is unsuitable and a regulated system must be implemented.

1.1.5 Post-translational modifications to heterologous proteins expressed in *S. cerevisiae*

1.1.5.1 Secretion of heterologous proteins from *S. cerevisiae*

An efficient way of overcoming many of the problems of protein toxicity is to secrete products to the surrounding growth medium. Secretion also offers several other advantages as during secretion sequence specific proteolytic cleavages occur often enabling the N-terminus to correspond to that of the mature protein (Kendall, Yamada & Bradshaw, 1990). Secretion may yield a properly folded product with the correct disulphide bond formation (Zsebo*et al.*,1986; Van Den Burgh*et al.*,1987; Jacobsen*et al.*,1989). Finally the passage of the protein through the secretory apparatus of the cell allows it to be glycosylated, although the glycosylation pattern of a protein made in yeast may not resemble that of the protein derived from natural sources (Kukurazinska, Bergh & Jackson, 1987). Most importantly secretion greatly aids subsequent purification of the protein. *S. cerevisiae* secretes less than 0.5% of its proteins into the growth medium enabling a high initial degree of product purity to be obtained if the heterologous protein is efficiently secreted from the cell.

Protein secretion in *S. cerevisiae* involves a complex series of events similar to those found in mammalian cells (Scheckman & Novick, 1982). First, proteins are translocated into the lumen of the endoplasmic reticulum (ER) by a hydrophobic signal sequence (Emr*et al.*,1983; Marten & Seo 1989), they pass through the ER where this sequence is usually removed and core glycosylation takes place. Then these are transported in transport vesicles into the golgi apparatus where outer chain oligosacharides are added, further proteolytic events occur and decisions with regard to final destination (vacuole, plasma membrane) are made (Seresiotiss & Bailey, 1987; Park, Seo & Lim, 1989). Glycosylated secretory proteins are then packaged into secretory vesicles which fuse with the plasma membrane of the growing daughter cell

with release of their contents (DaSilva & Bailey, 1989). Certain proteins are retained in the periplasmic space whereas others pass through the cell wall into the medium. The mechanism for passage through the cell wall is not as yet clear, although size, net charge and degree of glycosylation are all likely to affect final localization (Carlson & Botstein, 1982; Smith, Duncan & Moir, 1988).

Translation is initiated at an AUG methionine codon. In certain cases this methionine is cleaved off by cytoplasmic aminopeptidases but the extent of this removal depends on the proteins sequence content and level of expression. In many instances intracellularly expressed heterologous proteins retain this methionine, either due to overloading of the cleavage enzyme or due to the sequence context in the vicinity of the N terminus. The expressed protein may then prove to provoke an antigen response (when administered parenterally) if the N-terminal Met residue is not present on the natural protein. Secretion provides a route for creating authentic N termini as the heterologous protein can be designed to be derived from the cleavage of a larger precursor (Scorer, 1992).

Overexpression of normally disulphide bonded proteins within the cell cytoplasm has not been shown to cause inclusion body formation although the cytosol is essentially a reducing environment which will prevent or cause incorrect disulphide bond formation (Cousens *et al.,* 1987). Protein disulphide isomerase, located in the ER, catalyzes the accurate formation of these disulphide bonds as the protein traverses the secretory apparatus (Freedman, 1984), assisting the formation of a protein with native biological conformation.

Some heterologous proteins can be secreted from yeast using their homologous signal sequences. These include α-IFN (Hitzeman, 1983), mouse α-amylase (Thomsen, 1983) and bean phaseolin (Cramer*et al.,*1987). However, with the exception of the human serum albumin leader (Goodey*et al.,*1989) this process is inefficient and the processing of the signal sequence can be variable (Cabenzon, DeWilde & Herion, 1984). The signal peptides of the precursors of homologous yeast proteins such as acid phosphatase (Toda, 1976), invertase (DaSilva & Bailey, 1989) and prepro α-factor tend to give more efficient secretion of heterologous proteins into the culture medium. The most detailed studies of secretion signals and proteolytic processing sites have utilized yeast prepro α-factor (Julius, Schekman & Thorner, 1984). Successful secretion using the leader (pre) or leader-spacer (pre-pro) region of prepro α-factor (see 3.3.1) has been reported for endorphin & IFN (Bitter *et al.,* 1981), EGF (Brake *et al.,* 1984), IL2 (Miyajima *et al.,* 1985) and many others. In some cases it appears that the STE13 processing aminopeptidase (Section 1.3) may become overloaded as the spacer is incompletely processed to leave a Glu-Ala dipeptide attached to the amino

terminus of the secreted protein (Bitter *et al.,* 1981). Correctly processed hEGF can be made by removing this dipeptide sequence of the prepro leader (Brake *et al.,* 1984). This approach may not always work and may act to inhibit KEX2 (Section 1.3) cleavage (Stetler *et al.,* 1989). Evidence indicates that only the pre-sequence of the α-factor leader is necessary for correct processing, glycosylation and secretion of GM-CSF (Ernst *et al.,* 1988) and IFN-α (Piggott *et al.,* 1987). IFN-β, however, will only undergo secretion when preceeded by the entire pre-pro sequence (Piggott *et al.,* 1987), while hEGF and acid phosphatase are not secreted as efficiently with just the pre sequence as they are with the entire pre-pro sequence (Sihu & Bollon, 1987; Clements*et al.,*1991). These results indicate that the glycosylated pro-segment of the α-factor precursor is needed to direct some proteins into the proper secretory pathway but its function is, as yet, unclear.

1.1.5.2 Glycosylation of heterologous proteins

The glycosylation of a mammalian protein can effect both its serum half-life, as shown for EPO (Wojchowski, 1987), and its folding, solubility and antigenicity, as in the case of the hCG protein (Saira, 1985). Mammalian glycoproteins secreted from yeast are glycosylated, but not in the same way as the native cell would glycosylate them, as illustrated by influenza HA (Gething, 1985) and gp350 (Schultz, 1987).

Yeast's N-linked glycosylation system (Tanner, 1987) is restricted to the addition of high mannose type oligosacharides, although the recognition sequence for N-linked glycosylation (Asn-X-Ser/Thr), is identical to the Asn-X-Ser/Thr used by mammalian cells. Yeast is not capable of adding N-linked oligosaccharides of the complex type to the protein backbone, or trimming of the inner core residues. NMR spectroscopic analysis of yeast glycoproteins have shown that the basic core oligosacharide structure is identical to that found in mammalian systems with regard to both monosaccharide composition and linkage specificity. However, hyperglycosylation tends to occur, *S. cerevisiae* adding > 50 mannose residues to the core structure. Glycosylation heterogeneity also occurs, giving rise to secreted glycoproteins of different molecular weights. Because hyperglycosylation can effect a protein's biological activity and antigenicity, yeast may not be suitable for the synthesis of recombinant glycoproteins destined for parenteral use.

There are several ways of altering the glycosylation pattern of proteins secreted from yeast. One of the most interesting is the use of the *mmn9* mutant which lacks α1-6 mannosyl transferase, the enzyme responsible for the addition of outer chain mannose residues (Tsai, Frevert & Ballou, 1984; Hitzeman, Chen & Dowbenko, 1990). The *mmn9* mutant cannot add any outer chains or hyperglycosylate inner core residues, an example of its use being the expression of α1-antitrypsin (Moir & Dumais, 1987).

1.2.1 The growth characteristics of *S. cerevisiae*

1.2.1.1 Analysis of yeast growth in batch and fed-batch fermentations

The growth characteristics of yeast are quite complex and only recently has detailed analysis been available to provide insight into such phenomena as bi-phasic growth and catabolite derepression (Lievense & Lim, 1982).

Carbon, usually in the form of glucose, supplies the energy needs of the cell during growth. For glucose concentrations below 50-130 mg/l, *S. cerevisiae* oxidizes glucose (eqn. 1) with a maximum specific growth rate of 0.25-0.35 h^{-1}, a substrate yield of 0.5 grammes dry cell weight (DCW) per gramme glucose consumed, a respiratory quotient (RQ = ratio of CO_2 production to O_2 consumption) of 0.9 - 1.0 and an ATP yield of 16 - 28 per mole of glucose oxidised (Soumalainen, 1969).

(1) $C_6O_6H_{12} + 6O_2 = 6H_2O + 6CO_2$

At higher glucose concentrations in an aerobic environment, glucose is predominantly fermented despite the availability of oxygen (eqn. 2). The maximum specific growth rate is 0.15, RQ is greater than 1, ATP yield is 2 per mole glucose consumed and ethanol is produced.

(2) $C_6O_6H_{12} = 2C_2H_5OH + 2CO_2$

After exhaustion of glucose, under sufficient oxygen concentration, yeast oxidises the accumulated ethanol (eqn. 3), the maximum specific growth rate being 0.1 - 0.18 h^{-1}, yield of DCW 0.5g per gramme ethanol consumed, RQ is less than 1 and ATP yield is 6 - 11 per mole ethanol consumed (Von Meyenberg, 1969; Coppella & Dhurjati 1989).

(3) $C_2H_5OH + 3O_2 = 3H_2O + 2CO_2$

Most laboratory scale fermentations are carried out on mineral salts completely defined medium (usually consisting of glucose, yeast nitrogen base and the required amino acids for growth) to ensure plasmid stability, typically containing 20 g/l glucose (Sherman *et al.,* 1983). Because of the Crabtree effect, glucose is substantially fermented but with a nearly constant low level of oxidation resulting from incomplete glucose repression Such defined medium does not support appreciable growth on ethanol despite aeration (Coppella & Dhurjati, 1989a). Laboratory yeast strains growing on defined medium show two clear transitions to slower growth (Francois *et al.,* 1985; Boucherie, 1985).

The first transition occurs when only 50% of the available glucose has been fermented and appears to be in direct response to cAMP levels within the cell. Exhaustion of glucose coincides with maximal biomass and ethanol concentrations (Reiger, 1983).
Typical batch yeast fermentations on complex medium (typically consisting of carbon source, yeast extract and peptone) initially containing 20 g/l glucose do support an ethanol oxidation phase after glucose fermentation if oxygen supply is sufficient and ethanol levels are not so high as to inhibit respiration. After glucose exhaustion, a growth lag occurs (diauxic lag) resulting from the requirement to readjust enzyme levels and synthesise functional mitochondria in order to oxidise ethanol in the second phase of growth. Growth stops with ethanol exhaustion and cells arrest in stationary phase (Lievense & Lim, 1982).

During fermentative metabolism which is prevalent during batch growth, the biomass yield coefficient for glucose is low and ethanol is accumulated. This leads to inefficient utilization of the substrate and eventually to growth inhibition at very high glucose or ethanol concentrations. To obtain a high cell biomass without these drawbacks, the metabolism of glucose should be switched from fermentative to oxidative as the biomass yield coefficient for oxidation is higher and only small concentrations of ethanol are formed (Coppella & Dhurjati, 1989). To obtain oxidative metabolism it is necessary to keep the specific growth rate of the cell below 0.3 h^{-1} by controlling the feed rate of glucose so its concentration in the medium is kept at approximately 0.1 g/l. This type of growth control is known as fed batch and has the clear advantage over batch growth in that it enables cultures to reach a much higher biomass than cells grown in a conventional batch system (Hsieh *et al.*,1988; Fieshko *et al.*, 1989, Gu *et al.*, 1989).

1.2.1.2 Catabolite repression

Glucose represses the synthesis of many enzymes related to the utilisation of alternative carbon sources, a repression that operates mainly at the level of gene transcription. Recent research has highlighted two major features of this, the main regulatory system for carbohydrate metabolism in *S. cerevisiae* (Fraenkel, 1982; Entian, 1986; Gancedo, 1987). First of all, glucose does not need to be extensively metabolised to repress enzymes needed for the utilisation of alternative carbon sources. Secondly, cAMP is not the major controller of repression even though glucose acts to elevate cAMP levels
Mutations in a number of genes lead to defects in catabolite repression (Fraenkel, 1982; Gancedo & Gancedo, 1986; Gancedo, 1987). These are of two basic types; non-repressible mutants in which one or several enzymes are no longer repressed by

glucose, and non-derepressible mutants which lack the ability to derepress several enzymes in the absence of glucose. The spectrum of enzymes affected by each of these mutations in turn, and the epistasis relationships of the mutations provide strong evidence that "catabolite repression" is actually a number of different regulatory circuits. Rather than a single system for catabolite repression there are instead several systems, even though some of these regulatory circuits probably share certain trans-acting factors (Gancedo, 1987; 1992). Consistent with this model, no single mutation confers a global loss of glucose sensitivity that affects all enzymes normally subject to catabolite repression. Table 1.2 illustrates the different repression and derepression mutants that have been studied to date and the epistatic relationships between these mutants are illustrated in Table 1.3.

REPRESSION MUTANTS	DEREPRESSION MUTANTS
hex1	*snf1/cat1/ccr1*
hex2/reg1	*snf2*
cat80/grr1	*snf3*
cid1	*snf4/cat3*
cat4	*snf5*
cyc8/snn6	*snf6*
tup1	

Table 1.2 Carbon catabolite repression mutants of *S. cerevisiae*

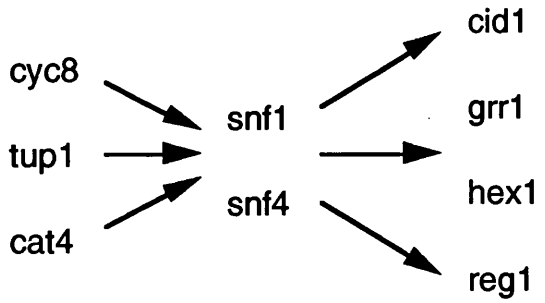


Table 1.3. Epistatic relationships between carbon catabolite repression mutants

Repression mutants

Most glucose repression mutants have been isolated by taking taking advantage of a selection system developed by Zimmerman and Scheel, (1977). Three complementation groups (*hex1*, *hex2* and *cat80*) had repression defects for invertase, maltase, malate dehydrogenase and respiratory enzymes. One of the hexokinase isoenzymes (PII, the HEX1 product) is crucial for glucose repression of maltase, invertase, malate dehydrogenase and respiratory enzymes (Entian, 1986). However it does not act in the repression of gluconeogenic enzymes. Hexokinase PII is itself expressed more highly in the presence of glucose, unlike the other hexokinase isoenzyme (PI) which is subject to glucose repression (Muratsubaki & Katsume, 1979; Fraenkel, 1982). The HEX2 gene probably encodes a nuclear protein and is allelic to *reg1* (Matsumoto *et al.*, 1983). The *cat80 (grr1)* gene (Entian & Zimmerman, 1980; Bailey & Woodward, 1984) may be required for the generation or transduction of the intracellular signal that causes glucose repression (Flick & Johnston, 1991). The role of the *cid1* (Neigeborn & Carlson, 1987) mutant is not yet known.

Tup1 and *cyc8* (Schamhart*et al.*,1975; Stark*et al.*,1980; Carlson *et al.*, 1984) are repression mutants that display the same array of phenotypes including calcium-dependent flocculation (Schamhart *et al.*, 1975), mating-type defects in MATα cells (Wickner, 1974; Rothstein & Sherman, 1980; Carlson *et al.*, 1984), nonsporulation of homozygous diploids (Schamhart *et al.*, 1975; Rothstein & Sherman, 1980), and derepression of many glucose-repressed enzymes. The diversity of phenotypes may arise from the involvement of *TUP1* and *CYC8* in general regulatory pathways as general repressors. The *TUP1* and *CYC8* genes are needed for the repression of respiratory enzymes in the presence of glucose, mutants in these genes cause depressed levels of many respiratory enzymes even in the presence of repressing concentrations of glucose. The *TUP1* and *CYC8* genes are also needed (in conjunction with the α2/MCM1 complex) for the repression of **a**-specific genes in α and **a**/α cells (Mukai *et al.*, 1991; Keleher *et al.*, 1992), Aberrant expression of α-specific genes cause growth defects in α and **a**/a cells such as self-shmooing (due to the production of **a**-factor) and inability to sporulate due to expression of the *RME1* gene (Mukai *et al.*, 1991).

Derepression mutants

Derepression mutants are unable to derepress the transcription of genes needed for the utilization of alternate carbon sources and have been identified by screening for cells unable to derepress invertase (Carlson *et al.*, 1981). The *SNF* (sucrose nonfermenting) genes fall into three main categories of functionally related genes: *SNF1* and *SNF4; SNF2, SNF5* and *SNF6* and *SNF3*. These groups are distinguishable on the basis of

phenotype and patterns of interaction with extragenic suppressors (Neigeborn *et al.*, 1986; Estruch & Carlson, 1990). Mutations in *SNF1* (also known as *CAT1* and *CCR1*) and *SNF4* (*CAT3*) prevent expression of many glucose repressible genes under derepressing conditions (Ciriacy, 1977, Carlson *et al.*, 1981; Entian & Zimmerman, 1982; Neigeborn and Carlson, 1984; Schuller & Entian, 1987). *SNF1* encodes a protein kinase (Celezna & Carlson, 1986). *SNF4* encodes a protein that is physically associated with the *SNF1* kinase and is required for maximal *SNF1* kinase activity (Celezna & Carlson 1989). *snf1* Mutant strains lose viability upon starvation, fail to accumulate glycogen in response to nutrient deprivation and are heat shock sensitive. The phenotypes of *snf1* mutants are those commonly associated with an overactivation of the adenylate cyclase pathway. Mutations in *CYR1* or *RAS2* which decrease the level of cAMP in the cell moderate the *snf1* phenotype. In contrast, the $RAS2^{val19}$ mutation, which increases intracellular cAMP, and the *bcy1* mutation which results in cAMP independent protein kinase A phosphorylation, accentuate the *snf1* phenotype (Thompson-Jaeger *et al.*, 1991). *SNF2*, *SNF5* and *SNF6* affect not only glucose repressible genes (Neigeborn & Carlson, 1986) but also expression of acid phosphatase (Abrams *et al.*, 1986), cell-type specific genes (Laurent *et al.*, 1990), and Ty elements (Happel *et al.*, 1991). Also *snf2* and *snf5* mutations cause constitutive expression of protease B; whereas a *snf1* mutation has no effect on the normal regulation of protease B (Moehle & Jones, 1990). These genes affect the expression of a variety of differentially regulated genes and it seems unlikely that they convey specific regulatory signals. Recent evidence implicates *SNF2* and *SNF5* in transcriptional activation: DNA-bound *LexA-SNF2* and *LexA-SNF5* fusion proteins activate transcription from a nearby promoter. The *SNF2*, SNF5 and SNF6 proteins appear to function interdependently because activation by *LexA-SNF2* is dependent on *SNF5* and *SNF6* (Laurent *et al.*, 1990, 1991). The *SNF5* gene is required for wild type levels of expression and correct repression of cell type specific genes; MATα *snf5* mutant strains aberrantly express the **a**-specific *BAR1* protease gene and express the *MFα1* gene at drastically reduced levels. For this reason, no halo was seen after plating MATα *snf5* cells on a MATα *sst1* tester lawn, partly due to the decreased expression of *MFα1* and partly due to the aberrant expression of the *BAR1* protease which would degrade the secreted α-factor (Laurent *et al.*, 1990). *SNF3* encodes a high-affinity glucose transporter (Bisson *et al.*, 1987; Celezna *et al.*, 1988), but does not effect invertase expression. Mutants were isolated because they were defective in growth on raffinose (Neigeborn *et al.*, 1986), which requires the ability to transport the low levels of fructose released by the action of secreted invertase.

1.2.1.3 cAMP and control of the mitotic cell cycle

The roles of cAMP in yeast have been unravelled largely through the analysis of mutants altered either in cAMP metabolism or in the protein phosphorylation mechanism that responds to cAMP levels (Matsumoto *et al.*, 1985). It transpires that yeast is like other eukaryotes in its possession of a crucial cAMP-activated protein kinase. It is through the action of this kinase in protein phosphorylation that cell proliferation, the mitosis-meiosis decision and a variety of cellular metabolic processes are controlled.

The pathways of cAMP synthesis and of cAMP-dependent protein phosphorylation are shown in Fig. 1.1. Nutrient status, by an unknown mechanism, activates the RAS protein through the *CDC25* product (Robinson *et al.*,1987), a protein that promotes exchange of GDP bound to RAS for free GTP. Stimulation of adenylate cyclase by RAS-GTP requires the product of the *SRV2* locus (Field *et al.*, 1990). The *IRA1* and *IRA2* genes are involved in negative regulation of *RAS2* (Tanaka *et al.*, 1990). cAMP is synthesised by the catalytic subunit of adenylate cyclase and broken down by phosphodiesterases, so that its levels reflect the balance of the actions of cyclase and phosphodiesterase. Once synthesised, cAMP can bind to the regulatory (*BCY*) subunits of the cAMP-dependent protein kinase, causing it to dissociate from the catalytic subunits (*TPK* gene products) of this kinase (Matsumoto *et al.*, 1982, Toda *et al.*, 1987). Activation of the TPK subunits accompanies this dissociation, the resultant protein phosphorylation stimulating cell proliferation and altering many metabolic activities. The extent of such cAMP-dependent protein phosphorylation is a balance between the activities of the protein kinase A and of protein phosphatases. A cAMP fall inactivates the protein kinase by promoting the association of its catalytic and regulatory subunits. Such inactivation is one of the signals for growth arrest in the G1 phase of the cell cycle. Very low levels of protein kinase A activity, present in cells with only the attenuated tpk^{w1} & tpk^{w2} subunits cause the prevention of catabolite repression (Mboyni *et al.*, 1990). Addition of glucose to depressed cells causes only a very transient cAMP increase (Mboyni *et al.*, 1990), hence the postulation of a catabolite repressed component in the RAS-cAMP pathway (Beullens*et al.*,1988) (Fig. 1.1). Also nitrogen stimulation of yeast occurs without cAMP increase, yet requires a low level of glucose (hence a free TPK subunit). It is thought therefore that TPK activity can be activated by nitrogen in a cAMP independent mechanism (Thevelein, 1991) (see Fig. 1.1)

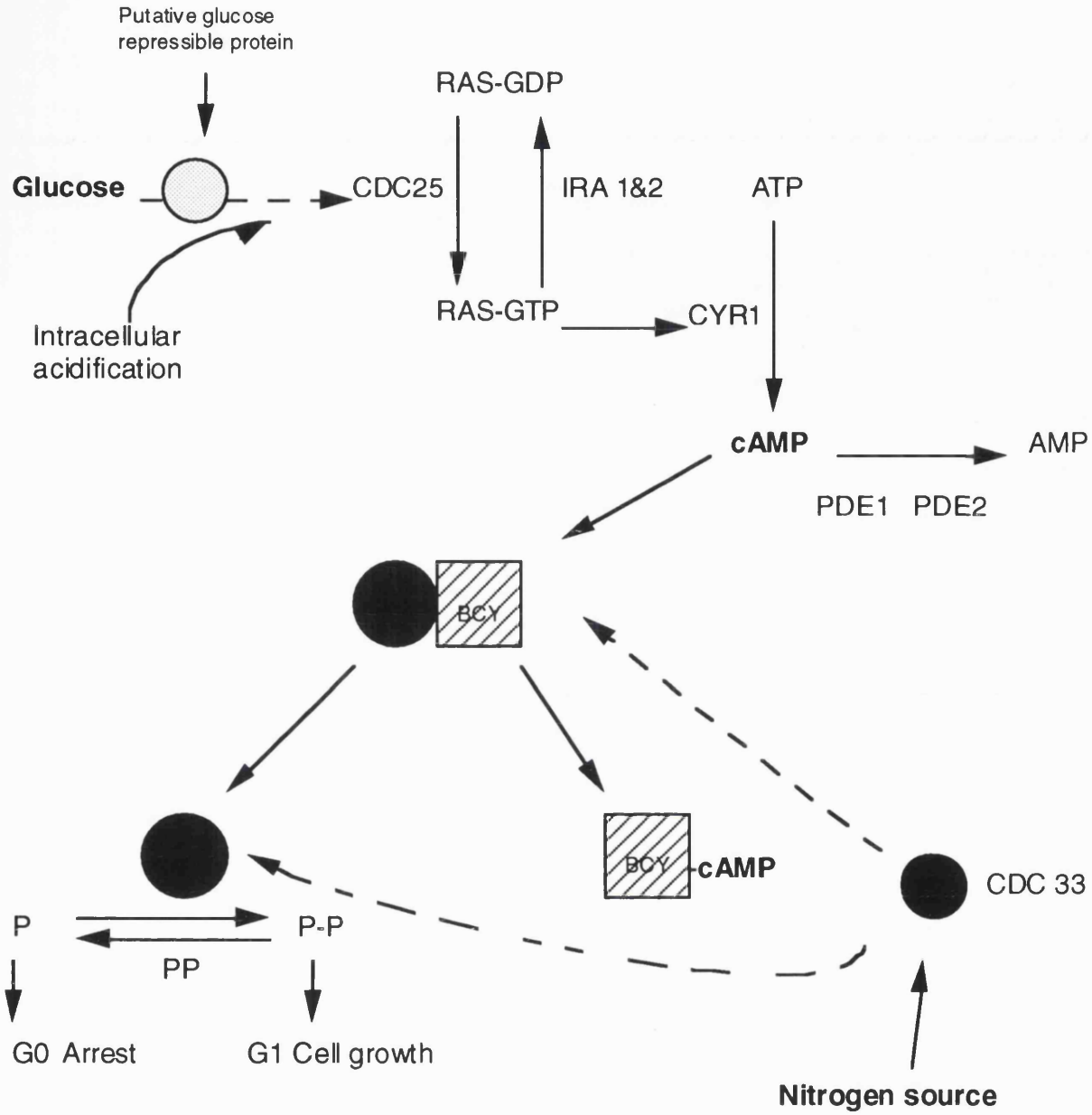


Figure 1.1. The RAS/cAMP pathway of *S. cerevisiae*

Among the major metabolic changes to ensue from falling cAMP levels are the commencement of storage carbohydrate (glycogen and trehalose) synthesis and decreases in fructose-2,6-bisphosphate (Francois*et al.*,1986). These do not occur in mutants that maintain high cAMP levels.

Evidence for a reciprocal nature between the RAS/cAMP pathway and nutrient status argues that it is not the only signalling pathway in operation. Strains expressing constitutive low-level protein kinase A activity (*bcy tpk*^{w} strains) respond normally to nutrient limitation even in absence of both *RAS* genes, *CDC25* or *CYR1* (Cameron *et al.*, 1988). Secondly, sporulation of diploids can proceed under high intracellular cAMP concentrations (Olempska-Beer & Freese, 1987). The main conclusion from this evidence is that the decision for the cell to enter G0 or G1 is based on the input from a number of different signalling pathways. If the input signal from the RAS/cAMP pathway were at one extreme or the other, due to its mutational activation or hyperactivation, the input from other signalling pathways would be drowned out and the cell would make cycle descisions based solely on the extreme signal from the RAS/cAMP pathway. Further evidence for the presence of a cAMP independent signalling pathway comes from the studies with the SCH9 protein kinase that, when over-expressed, can substitute for protein kinase A (Toda *et al.*, 1988) De-repression and constitutive expression of *ADH2* requires the activity of the SCH9 protein kinase and low levels of TPK activity, the action of these protein kinases being independent of each other (Denis & Audino, 1991).

1.2.1.4 Partial interdependence of the catabolite derepression and cAMP control systems of yeast

Glucose depletion at the end of batch fermentative growth causes release from catabolite repression. It also triggers a fall in cAMP, although most reported measurements of cAMP have been on defined medium batch fermentations where the cAMP decline clearly occurs in advance of glucose exhaustion (Francois*et al.*,1986). One study has been performed (measuring cAMP levels during growth) on a non-defined medium (Sachse, 1991) In addition aerobic cultures show respiratory adaptation as glucose falls below the threshold for respiratory repression. While glucose inhibits the synthesis of several enzymes in both *E. coli* and yeast, the mechanisms of this extensively-studied catabolite repression differ fundamentally in the two organisms. In *E. coli* it is mediated directly by decreases in cAMP in the presence of glucose, cAMP being necessary for the transcription of those genes sensitive to catabolite repression (Ullmann & Dauchin, 1983). Yeast differs markedly from *E. coli* in that glucose raises its cAMP levels (Eraso & Gancedo, 1985). Also much of the

catabolite repression in yeast does not operate through changes in cAMP since mutants unable to synthesize cAMP can still display catabolite repression (Matsumoto *et al.*, 1983; Eraso and Gancedo, 1984). Yeast mutants *tpk1*^{w1}, *tpk2*^{w1} and *tpk3*^{w1} contain reduced activity of cAMP-dependent protein kinase A. Addition of fermentable sugars, as opposed to non-fermentable carbon sources induce a permanent hyperaccumulation of cAMP. Investigation of mitochondrial respiration using the technique of *in vivo* ^{31}P nuclear magnetic resonance spectroscopy indicates that the *tpk1*^{w1} and the *tpk2*^{w1} mutant strains are defective in glucose repression (illustrated by a high level of ATP when these strains growing on glucose are resuspended in glucose free aerated buffer). These results indicate that a certain threshold level of TPK activity is needed for the repression of respiratory enzymes (Mboyni *et al.*, 1990).

1.2.2.1 Stationary phase: The adaptation of *S. cerevisiae* to starvation conditions

Lack of essential nutrients causes yeast cells to arrest in stationary phase. In this state (G_0) cells are primarily unbudded (Johnson, Pringle & Hartwell, 1977). Cells arrested outside G_0 at the time of deprivation complete the cell cycle before arresting (Shin, Uno & Ishikawa, 1987) and therefore intracellular reserves must supply most of the nutrients to complete division. G0 cells show a higher resistance to heat treatment (Schenberg-Frascino & Moustacchi, 1972) and cell wall degradative enzymes (Deutch & Parry, 1974), also drastic reductions in RNA and protein synthesis (Elliot & McLaughlin, 1978; Boucherie, 1985). Most of the changes at the approach to stationary phase are to enable cells to withstand extended periods of nutrient deprivation.
Cell cycle arrest is believed to be regulated primarily by decreased cAMP dependent protein kinase activity as explained in Section 2.1.3.
The fungal vacuole carries out the diverse functions of macromolecular degradation, metabolite storage and cytosolic ion and pH homeostatis (Klionsky, Hermann & Emr, 1990). The main protein constituents of the vacuole are the vacuolar hydrolases; proteinases A and B, carboxypeptidases Y and S, aminopeptidases, alkaline phosphatase and RNase. The levels of many vacuolar hydrolases vary with cell growth stage and nutrient supply, these enzymes being derepressed under conditions of limiting glucose and nitrogen and become most active as cells enter stationary phase (Bakalkin *et al.*, 1976; Lenney *et al.*, 1974). These general hydrolases have the function of proteolysis- the degradation and utilization of small peptides and sporulation associated protein degradation. Protein turnover ranges from 0.5-1% of total cell protein per hour when cells are grown on glucose to 2-3% per hour when cells are starved reflecting the induction of these hydrolases in stationary phase (Achstetter & Wolf 1985). During nutrient deprivation, the importance of vacuolar proteases is

underlined by the greatly reduced viability of strains lacking such enzymes during nitrogen starvation (Jones, 1991)

The vacuole is also the primary storage site for metabolites such as the basic amino acids (primarily arginine) and polyphosphate. Arginine is the amino acid with the highest nitrogen content which indicates the role of the vacuole as a nitrogen reserve. Arginine normally makes up 25 - 30 % of the amino acid pool (Durr *et al.*, 1979). Nitrogen starvation causes mobilization of this vacuolar arginine resulting in increased levels of cytosolic arginine (Kitamoto *et al.*, 1988). Polyphosphates are the only macromolecular anions in the vacuole and serve as a store of Pi (Indge, 1968).

The reserve carbohydrates glycogen and trehalose accumulate in cells starved for carbon, nitrogen, sulfur, phosphorous and energy, suggesting that the accumulation of these sugars is a general response to nutrient limitation. The pattern of their accumulation and utilization being compatible with their serving as an energy source during periods of starvation (Lillie & Pringle, 1980; Panek, 1963).

From the above evidence, cells under starvation conditions gain nitrogen from release of sequestered arginine from the vacuole and the nonspecific turnover of pre-existing proteins catalysed by starvation induced vacuolar hydrolases. mRNA turnover reduces the amount of non-essential message within the cell so energy is not wasted in the synthesis of functionally redundant proteins.The accumulation and breakdown of glycogen and trehalose supply the carbon requirements of the cell.

1.2.2.2 Gene regulation in stationary phase

Synthesis of 95% of proteins detected in log phase are repressed in stationary phase (Boucherie 1985). The mRNAs of a few stress-induced genes have been reported to increase in early stationary phase, these include *SSA3* (Boorstein & Craig, 1990), *HSP26* (Petko & Lindquist, 1986; Praekelt & Meacock, 1990), *HSP12* (Praekelt & Meacock, 1990), *UBI4* (Finley *et al.*, 1989) and *UBR5* (Steufert & Jentsch, 1990). The signalling system that directs *S. cerevisiae* into stationary phase includes signals from the RAS/cAMP pathway, among others. Falling cAMP levels at the approach to stationary phase is thought to be responsible for the induction of certain stress genes (*HSP26*, *SSA3*), through reduced activity of cAMP-dependent protein kinase A, whilst other stress genes are responding to catabolite repression (e.g. *UBI4*, Chapter 3).

These genes are induced in a temporally-orchestrated manner and are then repressed as cells age in stationary phase. The general repression of gene transcription in stationary phase occurs through a *TOP1*-dependent mechanism (Choder, 1991). *S. cerevisiae* has three known DNA topoisomerases: topoisomerase 1, topoisomerase 2 and topoisomerase 3 encoded by *TOP1* (Goto & Wang, 1985; Thrash *et al.*, 1985), *TOP2* (Goto & Wang, 1985) and *TOP3* (Wallis *et al.*, 1989), respectively. The function of *TOP3* is unknown. *TOP2* is an essential gene whose product is required for the separation of intertwined chromosomal DNA in mitosis and for the resolution of recombined chromosomes in meiosis 1 (Holm *et al.*, 1985; Rose *et al.*, 1990). *TOP1* is a non-essential gene (Goto & Wang, 1985; Thrash *et al.*, 1985) carrying the major activity that relaxes DNA supercoils. Efficient gene repression in stationary phase requires topoisomerase 1 activity. In *top1* mutants, repression occurs at a much slower rate in the case of all tested genes: *HSP26* induction in a wild type strain occurs in early stationary phase and its transcript can be detected for twelve hours into stationary phase. In a *top1* mutant, the *HSP26* transcript is still detectable after thirty hours. mRNA levels of a small subset of genes remains constant in all growth phases, including stationary phase, regardless of *TOP1* activity (Choder, 1991).

1.3.1 Cell interaction and control of cell type in *S. cerevisiae*

1.3.1.1 Control of cell type in *S. cerevisiae*

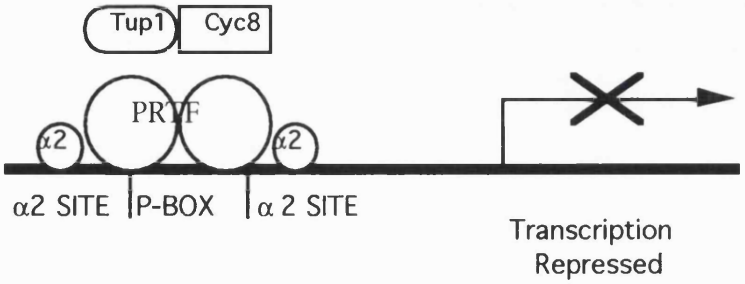
Laboratory *S. cerevisiae* normally exist as either **a** or α haploid cells or **a**/α diploid cells. The two haploid cell types elaborate mating-type specific functions; cell types can be distinguished by the basis of cell morphology, cell size and the expression of specific mating pheromones, receptors and cell aglutinins. **a** Cells secrete **a**-mating factor (Wilkinson & Pringle, 1974), α cells secrete α-mating factor (Bucking-Throm *et al.,* 1973). These peptide mating pheromones arrest cells of the opposite mating type in the G1 stage of the cell cycle in preparation for cell fusion to form the diploid **a**/α cell type. Diploid cells do not express or respond to mating factors but are able to sporulate in response to starvation. Haploid cells respond to starvation by entering G1 arrest. The regulator of cell type is the *MAT* locus. According to the a1-α2 hypothesis (Strathern *et al.,* 1981), the mating-type locus alleles *MATa* and *MATα* encode regulatory proteins that govern transcription of four gene sets: α-specific genes, **a**-specific genes, haploid specific genes and diploid specific genes.

α Cells:-

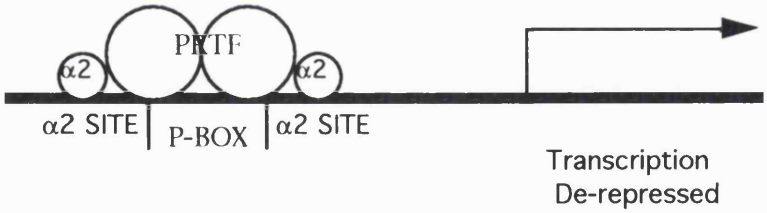
The products of the *MATα* locus, α1 and α2 are DNA binding proteins that serve to activate and repress, respectively, genes required to determine the α (Sprague *et al.,* 1983; Lipke *et al.,* 1989) or **a** cell type (Wilson & Herskowitz, 1984; Hartig *et al.,* 1986; Kronstad *et al.,* 1987; Michaelis & Hershkowitz, 1988). MATα1 works co-operatively with the product of the *MCM1* locus, PRTF (Bender & Sprague, 1987; Tan *et al.,*, 1988) to activate transcription of α-specific genes, binding to a common sequence motif found upstream of α-specific genes known as the PQ box (Bender & Sprague, 1987; Jarvis *et al.,* 1988; Inkouchi *et al.,* 1987). This 26bp sequence includes the binding sites for both MCM1 and MATα1. MCM1 can bind by itself to the P box (Bender & Sprague, 1987). MATα1 probably contacts the Q site, but only when PRTF is bound to the P box (Tan *et al.,* 1988; Jarvis *et al.,* 1989; Passmore *et al.,* 1988).

The repression of **a**-specific genes in a cells occurs by a complex mechanism that is still being elucidated. MATα2 (α2) is a sequence specific DNA binding protein that binds to α2 sites flanking the nearly symmetric P-box sequences of **a**-specific promoters in combination with the general transcription factor PRTF (Bender & Sprague, 1987; Tan *et al.,* 1988). The role of PRTF may be to assist the binding of α2 so it can bind efficiently to the operator, while α2 may mask the portion of PRTF that enables it to stimulate transcription. Occupancy of the operator with the α2-PRTF

complex however is not enough to bring about repression, since complete repression also requires the presence of the general repressors CYC8 and TUP1 which form a 'repressor complex' (Mukai *et al.*, 1991; Keleher *et al.*, 1992; Section 1.2.1.2) which then causes silencing of the **a**-specific promoter.



A **a** specific gene repression in α cells



B **a** specific gene expression in α Tup1, αCyc8 or αTup1 cyc8 mutants

Figure 1.2. Regulation of **a** genes in catabolite repression mutants

a Cells:-

The *MATa* allele encodes a single regulator, termed **a**1, this has no activity in haploid cells but in a/α diploids acts in combination with α2 to repress haploid genes. In **a** cells, **a** specific genes are expressed due to absence of the transcriptional repressor α2 and the presence of the general transcriptional activator PRTF which binds to the P-box of the **a**-specific promoter and activates transcription (Johnson & Herskowitz, 1985; Jarvis *et al.*, 1988; Keleher *et al.*, 1988). In-vitro DNA binding experiments reveal that PRTF can bind independently to the P-boxes of **a** specific genes since they are more nearly symmetric than their counterparts in the promoters of α-specific genes (Bender & Sprague, 1987; Keleher *et al.*, 1988; Tan *et al.*, 1988). α-Specific genes are not expressed due to absence of the transcriptional activator α1.

In addition to control by the *MAT* alleles, the transcription of **a**- and α- specific genes requires the products of some or all of the non-specific *STE* genes, *STE* 4,5,7,11 and 12 (Fields *et al.*, 1985, 1988)

a/α Diploids

The properties of **a**/α diploids arise from the combined expression of one MAT**a** product, **a**1, and one MATα product, α2. These proteins combine to form a transcriptional repressor **a**1-α2 that blocks expression of haploid specific genes, repression being exerted at an operator sequence found upstream of these genes (Goutte and Johnson, 1988). The activation of diploid specific genes is an indirect consequence of the action of **a**1-α2. Transcription of *IME1*, a diploid specific gene required for meiosis and sporulation (Kassir *et al.*, 1988), requires that the cell express **a**1-α2 and be subject to the appropriate nutrient starvation conditions. The function of the **a**1-α2 repressor complex in this case is to repress transcription of an inhibitor of meiosis, RME1 (Mitchell and Herskowitz, 1986), by binding to the *RME1* promoter (Goutte and Johnson, 1988).

1.3.2 The pheromone response

When **a** and α cells come into close contact they bind the peptide pheromone secreted by the cell of the opposite mating type. Pheromone binding to the cell surface receptor initiates signal transduction, terminating in G1 arrest (Marsh *et al.*, 1991). Cells of **a** mating type produce an α-factor receptor, STE2 and cells of α mating type produce an **a**-factor receptor, STE3. Both membrane bound receptors are coupled to the same heterotrimeric G protein, $G\alpha\beta\gamma$. The $G\alpha$ subunit normally has GDP bound. Stimulation of the receptor causes a switch to the GTP-bound state of $G\alpha$, which leads to release of $G\beta\gamma$. $G\beta\gamma$ then activates downstream components of the pathway: *STE5*, 7 and 11 and *FUS3/KSS1*, leading ultimately to phosphorylation of the transcriptional activator STE12. STE12 binds to the pheromone responsive elements (PRE's) of haploid specific genes its phosphorylation resulting in increased transcription of these genes and ultimately G1 arrest due to G1 cyclin inactivation (for review, see Fields, (1990)).

1.3.3.1 Mating factor α

α-Pheromone is encoded by two unlinked loci, *MFα1* and *MFα2* (Kurjan & Herskowitz, 1982; Singh*et al.*,1983). *MFα1* encodes four copies of the mature pheromone (Fig. 3), and is responsible for the majority of α-factor produced (Kurjan, 1985), MFα2 encodes two copies of α-factor. All but one of the six copies encode an identical tridecapeptide, Trp-His-Trp-Leu-Gln-Leu-Lys-Pro-Gly-Gln-Pro-Met-Tyr; the remaining copy contains two changes (Asn-4, Arg-7). The *MFα1* gene encodes a precursor of 165 amino acids, the amino terminal pre-region of the precursor contains a canonical twenty amino acid signal sequence, followed by a sixty amino acid pro-segment with three potential glycosylation sites (Figure 1.3). The C-terminal part of the precursor contains four copies of the mature α-factor sequence, linked in tandem array by short (6-8 residue) spacer peptides (Kurjan & Herskowitz, 1982). The hydrophobic N- terminus initiates translocation into the ER where core glycosylation occurs on the three N-linked sites in the pro-segment. The precursor is transported to the golgi apparatus where outer chain carbohydrate is added and proteolytic processing of the α-factor peptide repeats occurs. Four specific proteases are involved in the processing of this protein. Signal peptidase first removes the signal sequence. The *KEX 2* gene product, a Lys-Arg endoproteinase, cleaves the α-factor repeats from the precursor (Julius *et al.*, 1984). The *STE13* encoded diaminopeptidase removes the Glu-Ala, Asp-Ala or Val-Ala residues from the four excised peptides (Dmochowska *et al.*, 1987). These precursors are then trimmed by the *KEX1* gene product, exhibiting a

carboxypeptidase B type activity, to yield the mature tridecapeptide α-factor (Julius *et al.*, 1983).

1.3.3.2 Regulation of the *MFα1* promoter

MFα1 is transcriptionally regulated at two levels. First, it is only transcribed by MATα cells, and second, its transcription is induced by **a**-factor (Achstetter, 1989).

The promoter of the *MFα1* gene contains two UAS sequences to which the α1 protein binds in conjunction with the general transcription factor, PRTF, which is present in all three cell types (Flessel *et al.*, 1989; Inkouchi *et al.*, 1987). The role of α1 is to assist the binding of PRTF to these UAS sequences. The UAS can be divided into two parts. One part, termed the P box, is a degenerate version of a 16-bp symmetric diad. The second part is an adjacent 10-bp Q box (Jarvis *et al.*, 1988). PRTF binds efficiently to symmetric versions of the P box but poorly to the degenerate versions of the P box present in the promoter (Bender & Sprague, 1987; Tan *et al.*, 1988). However, co-operative binding with α1 allows PRTF to bind efficiently to the P box. In one view, α1 makes contact with the Q box and with PRTF, in another view α1 does not contact the Q box directly but causes a conformational change in PRTF that allows it to contact the Q box as well as the P box. In either case, the role of α1 is to recruit the general transcription factor PRTF to bind to UAS sequences that it cannot bind to alone. Thus, transcription of the *MFα1* gene is limited to α cells because only they contain α1 to facilitate transcriptional activation by PRTF. The role of the different elements of the PQ sequence in transcriptional control was investigated by synthesis of the *STE3* PQ sequence, Q sequence, P sequence and a perfectly symmetrical P sequence as oligonucleotides and their insertion into a plasmid borne *CYC1 -lacZ* hybrid gene deleted of its native UAS (Jarvis *et al.*, 1988). The PQ box directed significant levels of β-galactosidase activity only in a cells. The Q box was inactive in all three cell types, as was the *STE3* P box. However, the palindromic P box directed significant expression in all three cell types (Bender & Sprague, 1987; Jarvis *et al.*, 1988). Taken together, these results demonstrate that PRTF can bind to symmetrical P boxes and activate transcription in all three cell types. However, PRTF can only activate transcription from an imperfect P box if the UAS also contains an adjacent Q box and α1 is present (Jarvis *et al.*, 1988).

The structural gene for PRTF is *MCM1*. The *mcm1-1* allele was first isolated as a mutation that affected the maintenance of certain minichromosomes (Maine *et al.*, 1984). It was subsequently found that *mcm1-1* caused a mating defect in a cells and severely depressed transcription of α-specific genes. Moreover, deletion of *MCM1* is

lethal (Passmore *et al.*, 1988). The relationship between *MCM1* and PRTF was further qualified by the fact that antibodies raised against MCM1 peptides bound specifically to *STE3* UAS protein complexes (Jarvis *et al.*, 1989).

Several genes (*STE4, STE5, STE7, STE11 and STE12*) have been identified that are required for mating by haploid cells of both types (Hartwell, 1980; MacKay & Manney, 1974). Conditional mutations in these genes confer sterility at the restrictive temperature and failure to produce and respond to mating factor. The *STE12* gene product was found to be a positive regulator of *MFα1* and other α-specific genes by screening for mutants unable to express this gene (Fields & Herskowitz, 1985). Levels of mRNA of the three α-specific genes; *STE3*, *MFα1* and *MFα2* were all found to be severely reduced in a *ste12* mutant, as was RNA production from **a**-specific genes. Matα1 transcription was not found to be affected by the *ste12* mutation. Therefore Matα1 and STE12 proteins affect transcription through different mechanisms (Fields & Herskowitz,1985).

Null mutations in the *STE7* and *STE11* genes have also been found to depress **a** and α-specific mRNA's (Fields *et al.*, 1988). *STE3* and *STE6* transcripts were found to be identical in single and triple mutants arguing that the *STE7, STE11* and *STE12* gene products function in the same aspect of transcription rather than being required for independent steps of the process (Fields *et al.*, 1988). One possibility is that the *STE* gene products constitute a linear pathway, with one STE gene product being required to activate the next. Another possibility is that the STE proteins form a complex that aids in transcriptional activation. The finding that *STE7* and *STE11* encode protein kinases (Teauge *et al.*, 1986) provides evidence for phosphorylation dependent transcriptional activation/repression. However, α1 and PRTF have not yet been found to be phosphorylated.

The involvement of SNF5 protein (Section 1.2.1.2) in the regulation of the *MFα1* promoter was deduced from the fact that *snf5* mutants form homozygous diploids less readily than the corresponding wild type, suggesting that *SNF5* may effect cell type specific gene regulation (Laurent *et al.*, 1990). In a *snf5* mutant, *MFα1* transcript levels are less than 1/5th of wild type and α-factor is undetectable by the halo assay. SNF5 has been characterized as a glutamine and proline rich transcriptional activator although no DNA binding activity was detected

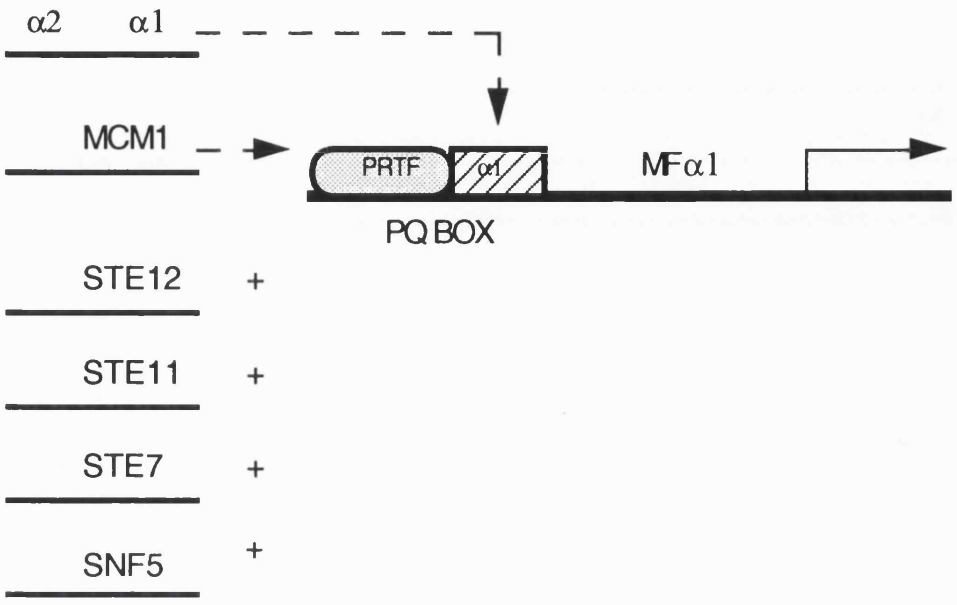


Figure 1.3 Model for the transcriptional activation of the *MFα1* promoter in α cells

1.3.3.3 *MFα1* Transcriptional regulation by the pheromone response

Another layer of regulation imposed on *MFα1* and other α-specific genes is their induction by **a**-factor treatment. Production of α-factor both at the mRNA and protein level was found to be induced after a two hour treatment of **a**-factor, de novo protein synthesis not being required for this response (Achstetter, 1989). Other treatments that cause G1 arrest such as nutritional starvation have been demonstrated not to activate *MFα1*, (see Chapter 5).

Transcriptional induction by pheromone appears to be mediated by the short sequence TGAAACA, the pheromone response element (PRE) (Kronstad *et al.*, 1987; Van Ardsell *et al.*, 1987). The transcription factor that binds to the PRE was identified as STE12 (Dolan *et al.*, 1989), which becomes rapidly phosphorylated upon pheromone treatment, the level of phosphorylation correlating with transcriptional activation (Song *et al.*, 1991). Six degenerate repeats of the consensus PRE are located in the *MFα1* promoter, however, MATα1 can also mediate gene activation by **a**-mating factor, this response being dependent on STE12 activity (Sengupta & Cochrane, 1991). Pheromone induction of the *MFα1* promoter could thus be due to either transcriptional activation by PRE-bound STE12 or through the PQ box via MATα1.

1.4.1 Production of heterologous proteins in *S. cerevisiae*

1.4.1.1 The use of constitutive promoters for the expression of heterologous proteins

The *PGK* (phosphoglycerate kinase) and *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) genes are two of the most efficiently expressed genes in yeast encoding about 5% of total cell protein and mRNA. The *PGK* promoter has been manipulated to produce high efficiency expression vectors which have been used for the expression of a number of different eukaryotic proteins. These include human interferons (Derynck *et al.*, 1983) and pro-urokinase (Turner *et al.*, 1991). The *PGK* gene is regulated to some degree by carbon source; levels are low during respiratory growth and higher during fermentative growth on glucose (Maitra & Lobo, 1971; Turner *et al.*, 1991). The levels of PGK promoter directed heterologous gene expression from multicopy vectors are lower than yields of the homologous protein PGK expressed from the same vector. This may be due to more rapid turn-over of heterologous proteins or the influence of codon usage on mRNA levels (Hitzeman *et al.*, 1982).

There are three GAPDH genes which are expressed to different extents (Holland & Holland, 1979). The most prevalent GAPDH protein is derived from the *TDH3* or *GAP491* gene (McAlister & Holland, 1985) and the promoter of this gene has been used for the high level constitutive expression of a number of proteins in yeast, including hepatitis B surface and core antigens (Bitter & Egan, 1984), human superoxide dismutase (Hallewell *et al.*, 1987) and epidermal growth factor (Urdea*et al.*, 1983). In at least two cases: human superoxide dismutase and core antigen, the levels of expression are more than 30% of soluble yeast proteins, comparable to levels seen in the best *E. coli* expression systems. To accumulate to the point where they comprise such a high percentage (15-50%) of total yeast protein these products must be both highly stable and relatively non-toxic to the host. The high stability of a protein like SOD can be exploited in the synthesis of an otherwise unstable protein. Human proinsulin (PI) is normally unstable when made intracellularly in *S.cerevisiae*, but cells can accumulate a SOD-PI fusion protein at high levels allowing subsequent purification of PI as a product of cyanogen bromide cleavage of the fusion protein (Cousens *et al.*, 1987; Tottrup & Carlsen, 1990). However reports of stable heterologous proteins being constitutively overexpressed so that they accumulate intracellularly to a high percentage of total yeast protein are few, indicating that many products will necessitate regulated expression, secretion to the medium or other refinements before stable high-level expression can be achieved.

1.4.2 The use of regulated promoters for heterologous gene expression

1.4.2.1 Using changes to medium composition to activate expression

The systems in most widespread use for inducing heterologous products in *S. cerevisiae* are those based on the *GAL1* and *PHO5* promoters. These require specific changes in growth medium composition to trigger expression. To achieve induction using the *GAL1* promoter it is necessary for glucose to have been depleted at the time that galactose is added (Matsumoto *et al.*, 1981; StJohn & Davis, 1981; Giniger *et al.*, 1985), unless *reg1* mutant strains are employed (Hovland *et al.*, 1989). To obtain *PHO5* promoter-directed induction the medium must be depleted of inorganic phosphate (Bajwa *et al.*, 1984). To effect these medium changes on large-scale fermentations can be inconvenient and present process engineering difficulties. Not only will these increase the costs of the fermentation plant, but they might increase risks of the engineering environment placing an additional stress on the cells just as they are being induced for heterologous protein production.

With *GAL1* promoter-directed expression one way to avoid the inhibitory effects of glucose is first to grow cells to a reasonably high density on low, nonrepressing levels of glucose; then to induce product formation by galactose addition. By this route the synthesis of 2g human IFN-γ per litre of glucose-limited yeast culture has been reported following galactose induction (Fieschko *et al.*, 1987). In contrast the continuous expression of the same IFN-γ gene from the constitutive *PGK* promoter resulted in the standard toxicity problems of poor cell growth, instability of the plasmid expression vector and low product yields (Fieschko *et al.*, 1987). Growth on nonrepressing levels of glucose is unnecessary in strains bearing the catabolite repression mutation (Section 2.1.2) *reg1*, since *reg1* abolishes the glucose inhibition of *GAL1* induction. Furthermore high induction in *reg1* strains is possible using galactose levels as low as 0.02% (Hovland *et al.*, 1989).

The *GAL1* promoter has the distinct advantage of giving high (>1000-fold) levels of induction and of being efficient when induced at maximum level (StJohn & Davis, 1981). One disadvantage for large-scale operations, partially overcome through use of *reg1* strains, is the relatively high cost of galactose. Another disadvantage, that the system can switch itself off as galactose becomes exhausted, can be avoided using mutants (e.g. *gal1* strains) that cannot metabolise galactose. An advantage *GAL1* has over most other yeast inducible promoters is that the latter usually display some constitutive expression and lower induction ratios.

Activity of *GAL1* depends on the action of a trans-activator protein (GAL4) bound to its UAS recognition sequences in the promoter DNA. This interaction is strongly

inhibited by glucose (Matsumoto *et al.*, 1981; StJohn & Davis, 1981; Giniger *et al.*, 1985). Promoter-bound GAL4 protein only activates transcription after the dissociation of another protein, GAL80, in response to binding of galactose by the latter.

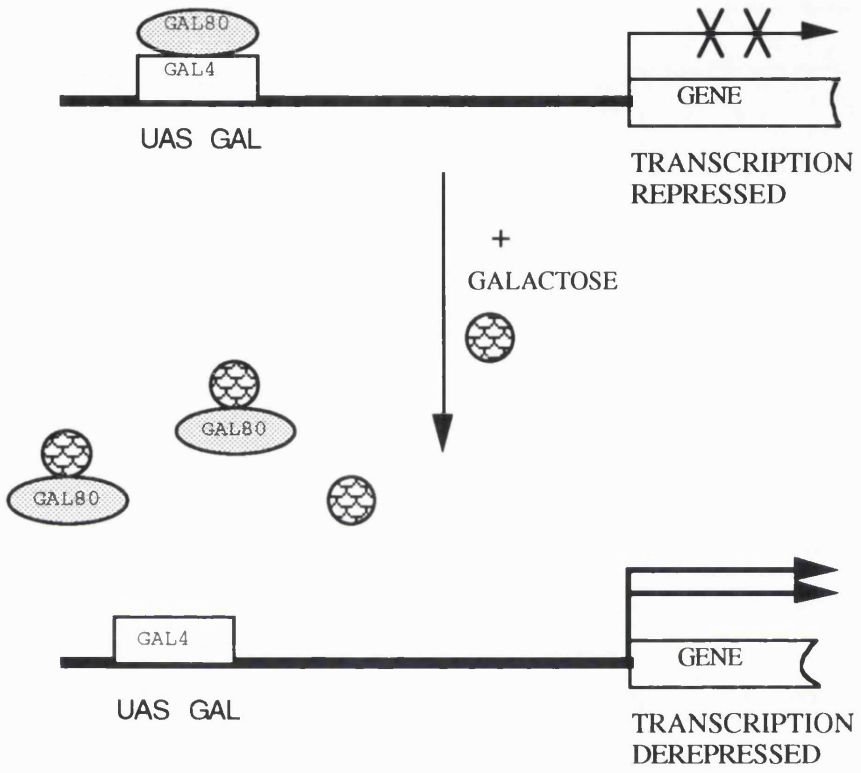


Figure 1.4. Schematic representation of *GAL1* promoter activation by galactose

One problem with using *GAL1* promoter induction systems for the high-level synthesis of heterologous proteins is that the levels of the GAL4 protein in the cell are normally rather low, insufficient for full induction of both the galactose utilisation enzymes and several copies of a heterologous gene under *GAL1* promoter control on a multicopy plasmid. This problem is not overcome merely by overexpressing the *GAL4* gene. Instead increasing levels of GAL4 protein causes the system to lose regulation because, through an insufficiency in GAL80 protein levels, GAL4 then activates transcription in the absence of galactose (Johnston & Hopper, 1982; Martin & Scheinbach, 1989). Schultz *et al.*, (1987) employed a strain with an extra integrated copy of *GAL4* under *GAL1* promoter control. As compared to a strain with just the normal *GAL4*, use of such a strain enabled GAL4 protein levels to increase 20-fold in response to galactose induction and provided tight galactose regulation together with a tenfold increased

expression of *GAL1* promoter-controlled genes on high copy number vectors (Schultz *et al.*, 1987).

Several laboratories have used the promoter of the *PHO5* repressible acid phosphatase gene to achieve regulated heterologous gene expression (Bajwa *et al.*, 1984; Kramer *et al.*, 1984; Rosenberg *et al.*, 1984; Lemontt *et al.*, 1985; Hsieh *et al.*, 1988). *PHO5* is transcriptionally repressed by inorganic phosphate, and the several positive (*PHO4, PHO2, PHO81*) and negative (*PHO80, PHO85*) regulatory genes controlling its action are very well characterised genetically (Oshima, 1982). It is not subject to quite such tight "off/on" regulation as *GAL1*, displaying a very low basal expression in the presence of phosphate even though it can be induced up to 200-fold by removal of inorganic phosphate. PHO5 promoter-directed IFN-γ1 production is about 200-fold higher on low phosphate medium than on high phosphate medium (Kramer *et al.*, 1984). Despite this fairly-tight regulation, product yields from the *PHO5* promoter appear to be only 5-10% of what is possible with strong constitutive promoters such as *PGK*, even when PHO5 is maximally induced (Kramer *et al.*, 1984).

1.4.2.2 Expression systems activated by temperature downshift.

Most yeast expression systems regulated by temperature exploit a ts mutational defect in a protein which is non-essential for growth but needed for the action of the promoter driving heterologous gene expression. Thus their operation is limited to use with the appropriate mutant strains. Cells are grown to high density at a temperature at which the ts mutation is expressed with the result that the heterologous gene is silent. Expression is then triggered by a decrease in temperature. One such system achieved temperature-regulated expression of IFN-γ1 in a *pho80, pho4ts* double mutant (Kramer *et al.*, 1984). The *PHO80* product is a repressor of acid phosphatase. Its inactivation in the *pho80* mutant leads to constitutive expression from the *PHO5* promoter, irrespective of inorganic phosphate levels. PHO4 encodes a positive regulator of acid phosphatases, *pho4* cells being incapable of acid phosphatase production and ts alleles (*pho4ts*) causing production only below a restrictive temperature. In a *pho80, pho4ts* strain, acid phosphatase production will occur only at temperatures permissive for PHO4 product function (24°C), not at 37°C when the positive regulator is absent. In such cells the PHO5 promoter-directed production of IFN-γ1 was induced 50-fold by a temperature downshift from 36°C to 24°C (Kramer *et al.*, 1984).

A second system based on temperature downshift has been described by Brake *et al.*, (1984) and Sledziewski *et al.*, (1988). To obtain inducible expression both groups

exploited the particular characteristics of *sir3ts* mutant. This expression was directed by either the *MATα2* promoter (Sledewski *et al.,* 1988), or the promoter of the *MFα1* gene (Brake *et al.,* 1984). Both promoters are active in haploid cells of appropriate mating type, but not in **a**\α diploids. Mutations in the *SIR* genes cause simultaneous Mat**a** and Matα expression (Herskowitz & Oshima, 1982) by preventing the normal SIR product repression of the normally-silent copies of mating-type information (*HMLa & HMRa*) not at the *MAT* expression locus. The result is that *sir-* haploid cells acquire a gene expression pattern usually found only in **a**\α diploids. *sir3ts* Cells make the MATα2 repressor from the *HMLa* locus, normally-silent in cells of mating type **a**, at 35°C but not at 25°C (Figure. 4). At the higher temperature lack of SIR3 function causes this *HMLa* locus to be active, leading to synthesis of MATα2 repressor. MATα2 protein is a repressor of **a**-specific genes in cells of a mating type. The alpha factor gene, *MFα1* is normally expressed in cells of a mating type, and repressed by the MATα2 repressor in haploid cells. It is also expressed in haploids of mating type **a** that inadvertently express MATα2, as in the sir3ts mutant at 37°C. *MFα1* is repressed by the MATα2 repressor in such cells at 37°C but induced 1000-fold by a shift-down to 24°C because this repressor is only made at the higher temperature (Herskowitz & Oshima, 1982). When the *MFα1* promoter was used for production of human epidermal growth factor (hEGF) in a *Matα, sir3ts* strain, this construct was subject to MATα2 repression at 36°C (Figure 1.5), but hEGF was induced >400-fold (from less than 10ng l^{-1} to 4mg l^{-1}) within several hours of a temperature downshift (Brake *et al.,* 1984). Potentially any gene can be made susceptible to MATα2 repression by placing the 31bp MATα2 protein binding sequence in its promoter. Multiple MATα2 binding sequences appeared to be necessary to achieve efficient MATα2 repression of β-galactosidase synthesis at 35°C in *sir3ts* cells (Sledziewski *et al.,* 1988).

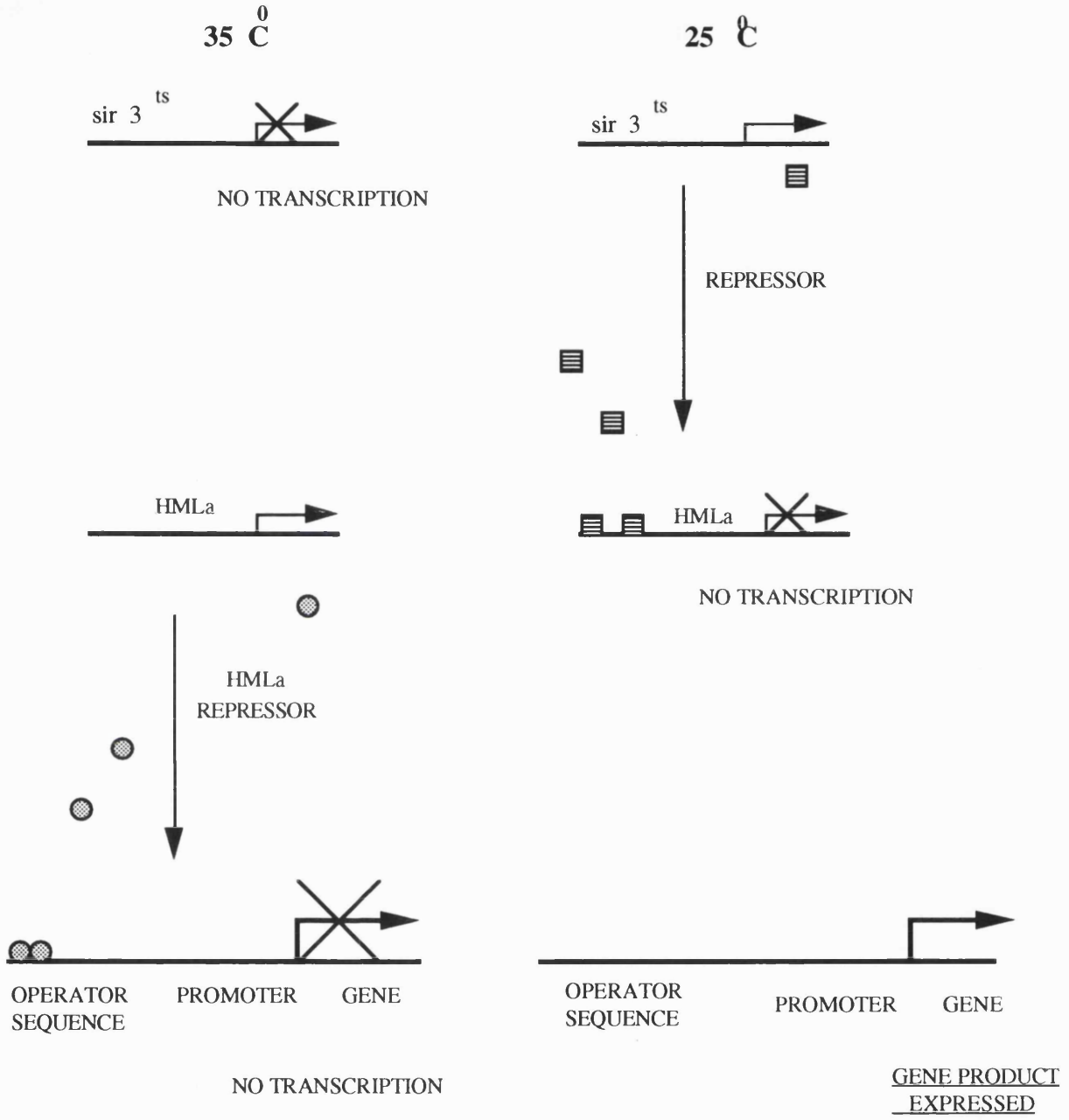


Figure 1.5. Heterologous gene regulation by temperature downshift in a *sir*^{ts} mutant

While these systems necessitating temperature downshift for induction are relatively straightforward to operate on a small scale, their large-scale operation is a very different matter. It is much easier to achieve rapid addition of thermal energy to a fermenter than rapid heat extraction. Also since yeast grows more slowly, with reduced biomass yield at temperatures greater than 33-35°C it is desirable to attain high biomass at temperatures slightly lower than this value (DaSilva & Bailey, 1989). For these two reasons temperature-regulated expression systems using a temperature upshift for

induction are more attractive than those using temperature downshift for large-scale operations.

1.4.2.3 Expression systems activated by temperature upshift.

ts Mutations that disrupt the interaction between the GAL80 and GAL4 proteins can be used as the basis of *GAL* promoter-based expression systems activated by temperature upshift (DaSilva & Bailey, 1989). A mutation in the *GAL80* gene could either prevent GAL80 repressor synthesis or result in a thermolabile repressor unable to bind GAL4 protein at high temperatures. Such a ts mutation would be recessive and upon upshift to nonpermissive temperatures allow expression from *GAL1* in the absence of galactose. Alternatively a ts mutation in *GAL4* could prevent the GAL4:GAL80 interaction at nonpermissive temperatures. It would be dominant, yet also cause galactose-independent *GAL1*-directed expression upon upshift to nonpermissive temperatures. Using ts mutations thought to be in *GAL80*, DaSilva and Bailey (1989) found comparatively-limited and rather slow induction of *GAL1*-directed ß-galactosidase synthesis in batch fermentations with 30-35°C upshift in the absence of galactose. Higher induction occurred with galactose addition in these cells maintained at 30°C. In cells cultivated at 35°C that were continuously expressing the ts mutation *lacZ* expression levels were similar to those obtained with galactose addition. Also at either 30°C or 35°C the ts mutation, when used together with galactose addition, reduced (as compared to an otherwise isogenic non-ts strain) the level of galactose required to achieve a given expression level (DaSilva & Bailey, 1989).
Utilisation of the heat shock element (HSE) sequences of heat-shock gene promoters to direct temperature dependent induction of heterologous gene expression is dealt with in Sections 1.5.1.1. and 1.5.1.4. and Chapter 3.

1.4.2.4 Catabolite and growth-regulated expression systems

A large number of promoters are subject to catabolite repression control, yet some are undoubtedly more suitable than others for inducing foreign gene expression in response to derepression. For example, the α-glucosidase (maltase) genes are subject to both glucose repression and maltose activation, yet their maltose\glucose induction ratios are less than 10-fold (Kopetski *et al.*, 1989). Induction levels greater than this are needed for most expression purposes. Fairly early on it was apparent that the gene for repressible alcohol dehydrogenase, *ADH2*, was derepressed at least 200-fold with the removal of glucose (Denis *et al.*, 1981). This has resulted both in extensive research on the *ADH2* promoter and the use of this promoter for the synthesis of commercial products.

The *ADH2* promoter is both efficiently derepressed as medium glucose is depleted and a fairly strong promoter (Beier & Young, 1982; Russell *et al.*, 1982). At least three biotechnology companies (Chiron (Barr *et al.*, 1987a,b,c; Cousens *et al.*, 1987; Sabin *et al.*, 1989), Immunex (Price *et al.*, 1987) and Nordisk Gentofte (Tottrup & Carlsen, 1990)) have used either the complete *ADH2* promoter or *ADH2* UAS elements inserted into other promoters to achieve strong induction of commercially valuable proteins after cultures have been grown to high biomass. Using a vector that also had the α-factor leader sequence so that products were secreted, exhaustion of the glucose in either complex or defined medium batch fermentations was found to coincide with induction of polypeptide hormone genes under *ADH2* promoter control (Price *et al.*, 1987). In complex medium cultures secretion started at diauxic lag and continued unabated through aerobic growth on ethanol until 50-20 mg l^{-1} recombinant protein (huGM-CSF or bovine interleukin-2) had accumulated in the culture supernatant by stationary phase. Replacement of the *ADH2* promoter with the weaker *MFα1* promoter led to 10-fold lower product levels in this system (Price *et al.*, 1987). One disadvantage of the *ADH2* promoter is that its regulation is lost when cells are grown under fed-batch respiratory conditions, acting under these conditions as a constitutive promoter (Tottrup & Carlson, 1990). Fermentation conditions used to facilitate regulated expression with the *ADH2* promoter therefore require batch growth on high concentrations of repressing sugar. One such example is the expression of glucose oxidase using the *ADH2-GADPH* hybrid promoter, with yields of 3 grams per liter glucose oxidase (De Baetselier *et al.*, 1991).

5.1. Yeast heat shock promoters and their application to heterologous gene expression

5.1.1. The heat shock response.

Yeast heat shock proteins are so-called because they are induced strongly when cultures are subjected to heat shock. Several of the same proteins are also induced at high cell densities and in transition phase defined medium culture (Boucherie, 1985). Levels of induction by heat shock decline as cells arrest growth in response to declining cAMP; also in cultures grown at temperatures above 30°C because basal heat shock gene expression levels start to rise above this temperature. In both heat stressed and transition phase cells heat shock protein induction reflects increased transcription of heat shock genes and it parallels marked physiological changes. Amongst these changes is an increase in thermotolerance, measured as the ability of cells to withstand brief periods at high, normally-lethal temperatures. It is unlikely that all heat shock proteins are involved in this increase in thermotolerance since the increase is unaffected by the inactivation of many yeast heat shock genes (Lindquist & Craig, 1988).
In heat-shocked yeast the transcription of heat shock genes is due to activation of a trans-activator protein (heat shock transcription factor (HSF)) bound to its recognition sequence (heat shock element (HSE)) within the DNA of heat shock gene promoters. In yeast HSF is a protein essential for viability (Sorger & Pelham, 1988; Weiderrecht*et al.*,1988) and is bound to HSEs both in unstressed and in heat shocked cells, becoming extensively phosphorylated upon activation (Sorger & Pelham, 1987; 1988, Figure 1.6). This is in contrast to higher organisms where heat shock activates HSF by promoting its binding to heat shock gene promoters (Sorger *et al.*, 1987).

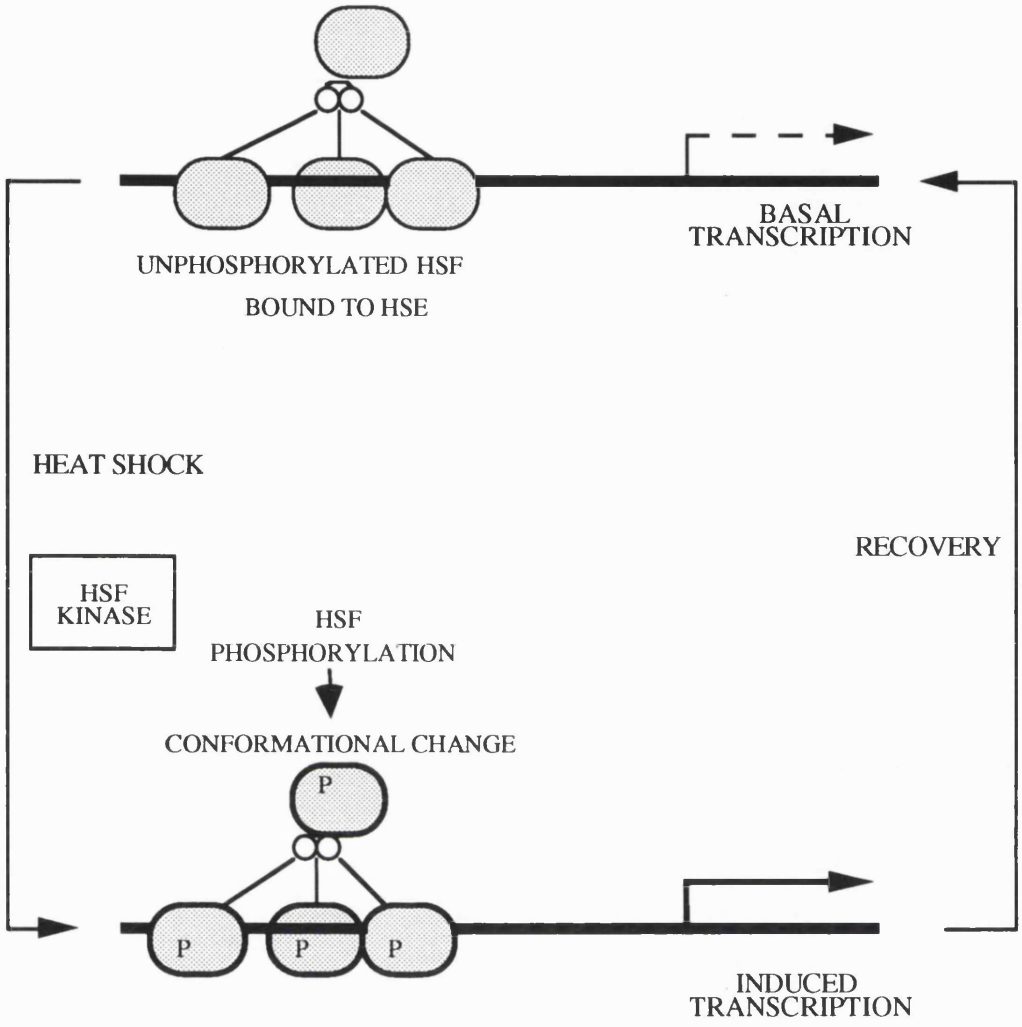


Figure 1.6. Model for the activation of HSF by heat shock

Heat shock elements are best described as contiguous arrays of variable numbers of the 5 bp sequence nGAAn arranged in alternating orientations, where n describes a less highly conserved nucleotide (Xiao & Lis, 1988; Sorger, 1991). At least two nGAAn units are needed for high affinity binding of mammalian or *Drosophila* HSF*in vitro* and these may be arranged either head to tail (nGAAnnTTCn) or tail to tail (nTTCnnGAAn) (Perasic *et al.,* 1989). HSF associates to form protein trimers in solution and when bound to DNA (Perasic *et al.,* 1989). However it is not clear whether *in vivo* it exists as trimeric, hexameric, or possibly larger complexes. It has yet to be proven exactly how both heat shock and a variety of potentially cytotoxic chemical agents are able to activate the heat shock response. They may operate by a common trigger, possibly the intracellular accumulation of aberrant or denatured protein (Pelham, 1986). Structurally abnormal proteins are substrates for, and regulators of the ubiquitination system for

protein turnover in yeast (Finley & Varshavsky, 1985; Finley *et al.,* 1987). An insufficiency of free ubiquitin for protein turnover may ensue from excessive protein damage caused by inducers of the heat shock response. This may be the reason that the *UBI4* gene for ubiquitin; also the *UBC4* and *UBC5* genes for ubiquitin ligases are activated by heat shock and required for viability under several stress conditions (Finley *et al.,* 1987; Seufert & Jentsch, 1990). Other heat shock proteins, notably those of the Hsp70 family, may act to limit denaturation damage by binding to the hydrophobic domains of partially unfolded proteins (Pelham, 1986).

The heat shock response is normally transient. Upon temperature upshift to their maximum growth temperatures (normally about 39°C on fermentable carbon sources and 3-6°C lower than this on gluconeogenic substrates) most laboratory *S. cerevisiae* strains will synthesize heat shock proteins at high level for only 15-40 minutes. The heat shock response is then substantially switched off as cells resume a pattern of protein synthesis similar to that displayed prior to the heat shock, except with slightly higher basal levels of heat shock gene expression. With more severe shock, to about 3°C above maximum growth temperature, there is higher initial heat shock protein synthesis but this is followed by a steady inhibition of all protein synthesis over about one hour. This modulation of heat shock gene expression might prove a major obstacle to the use of heat shock induction of HSE sequences for sustained expression of a heterologous gene, as it will limit the expression period. Normally individual heat shock proteins accumulate to no more than 1-3% of total yeast cell protein after heat shock (Piper, P.W., unpublished data), so to achieve appreciable product levels a heterologous gene under HSE control should be be used on a high copy number vector. Since at least two heat shock genes (*HSP26* and *HSP82*) can be highly overexpressed after heat shock under the control of their natural promoters (Fitch, 1989; Cheng *et al.,* 1992), it is therefore unnecessary to increase HSF levels in order to obtain high levels of HSE-directed expression from multicopy plasmids. It might be possible to elevate such expression still further by preventing the normal switch-off of the heat shock response. The kinetics of this switch-off suggest the direct involvement of one or more of the induced heat shock proteins. This is the case in *E. coli* where modulation of the response is through the concerted action of two genes, *DNA K* and *RPO D*, both of which are expressed at higher levels in response to heat stress (Lindquist & Craig, 1988).

1.5.1.2 Transition phase activation of many heat shock genes is apparently in response to declining cAMP

As defined medium cultures adapt to slower growth at the start of transition phase, intracellular cAMP has fallen to its lowest levels and heat shock genes are undergoing activation. Evidence linking these two events comes from two lines of investigation; Firstly studies showing heat shock protein synthesis in transition phase cultures (Iida & Yahara, 1984a; Boucherie, 1985); also demonstrations that the *UBI4* heat shock-inducible ubiquitin gene is activated in response to depletion of cAMP (Tanaka *et al.*, 1987) and more highly expressed in stationary as compared to exponential cells (Finley*et al.*,1987). Secondly, studies using mutants altered in cAMP metabolism have revealed that many heat shock genes are controlled by cAMP levels (Shin *et al.*, 1986; Tanaka *et al.*, 1988; Boorstein & Craig, 1990; Praekelt & Meacock, 1990).

Strains unable to elevate cAMP in response to high glucose levels grow more slowly than wild-type on glucose and synthesize many heat shock proteins constitutively. Most studies that have demonstrated this have employed mutants defective in adenylate cyclase (*cyr1*). Several *cyr1* alleles have been used; notably the temperature-sensitive *cdc35* mutant (Iida & Yahara, 1985) (allelic with *cyr1*: Matsumoto *et al.*, 1984), a strain making a heat labile adenylate cyclase (*cyr1.2*: Shin *et al.*, 1987), and a mutant that has low cAMP levels through a Ty transposable element insertion within the *CYR1* gene promoter (*cyrT1*: Iida & Yahara, 1984; Iida, 1988). The *bcy1* mutation, a suppressor of *cyr1*, causes cAMP-independent, constitutive synthesis of protein kinase A (normally cAMP-dependent). *bcy1* Cells still synthesize certain heat shock proteins normally after heat shock (Cheng, L. unpublished data) but do not show normal arrest in the G1 phase of the cell cycle during nitrogen starvation or the usual elevations in thermotolerance with heat shock (Shin *et al.*, 1987). Decreases in levels of cAMP-dependent protein phosphorylation are therefore correlated with elevation of thermotolerance and the arrest of growth in the G1 phase of the cell cycle.

Although many heat shock genes are induced both in response to heat shock and a decline in cAMP, the mechanisms of their induction are clearly different in these two situations. Heat shock gene activation in heat shocked cells cannot be in response to a decline in cAMP since intracellular cAMP displays two-fold increases with 24-36°C (Boutelet *et al.*, 1985; Camonis *et al.*, 1986) or 25-40°C (Hirst, 1990) heat shocks. Also cAMP levels are 40% higher during growth at 35°C compared to growth at 25° (Matsumoto *et al.*, 1985) and are especially elevated by a shift to temperatures of 38°C and above (Hirst, 1990). Furthermore several heat shock genes are still capable of further activation by heat shock in *cyr1.1* (Tanaka *et al.*, 1988) and other mutants of low protein kinase A activity (L. Cheng and P.W.Piper, unpublished). Dramatic

evidence that cultures approaching stationary phase activate certain heat shock genes has been obtained by inserting the gene for Hsp26 (*HSP26*) into yeast on a high copy number plasmid. Hsp26 protein is virtually undetectable during exponential growth of this transformant yet overexpressed to >50% of total cell protein at stationary phase, the cells accumulate 45-55nm particles that are probably Hsp26 aggregates (Bentley, N. PhD dissertation, University of Kent). However not all yeast heat shock genes can be overexpressed to this extent when under the control of their natural promoters. *HSP82* on a high copy vector causes markedly altered growth regulation on defined medium (Hirst, 1990). There is no slowing of growth to mark the beginning of transition phase, or high synthesis of the *HSP82* product (Hsp90) as defined medium cultures of this transformant stop growing. Instead cultures grow to 2-3X the normal final cell density, and with the approach to stationary phase accumulate no glycogen and rapidly lose viability (Hirst, 1990). This is a phenotype characteristic of those mutants of cAMP metabolism that maintain high cAMP levels (Matsumoto *et al.*, 1985). The transformant with multiple *HSP82* gene copies has elevated Hsp90 levels during growth because of low constitutive expression of these *HSP82* genes, as well as elevated cAMP levels. The increase in Hsp90 is apparently sufficient to cause marked alterations to cAMP regulation (Hirst, 1990).

1.5.1.3 HSE-directed heterologous gene expression

Most of our knowledge of the sequence determinants of heat shock-directed transcription in yeast comes from results obtained with *lacZ* fusions. Brazzell & Ingolia (1984) fused the promoter of one of the genes for Hsp70 (YG100, since redesignated SSA1) to *lacZ* and showed that expression could be induced 50-fold with heat shock. The *YG100* promoter has since been shown to display low constitutive expression even though it has HSE sequences (Slater & Craig, 1987), so higher induction levels might be possible from more tightly regulated heat shock promoters. DNA containing the alternating repeats of the 5 base-pair consensus sequence nGAAn (Pelham, 1985; Tuite *et al.*, 1988) binds yeast HSF strongly *in vitro* (Sorger & Pelham, 1987). Also when used in place of the normal *CYC1* UAS in a *CYC1* promoter-*lacZ* fusion these sequences confer heat shock-inducible ß-galactosidase synthesis (Wei *et al.*, 1986; Sorger & Pelham, 1987). Sorger & Pelham (1987) found that 1, 2 or 4 repeats of the nGAAn HSE consensus all gave about 30-fold induction ratios with heat shock. Higher *lacZ* expression after heat shock was found with increased numbers of HSE repeats but, since there were also parallel increases in basal expression levels, induction ratios did not rise above 30-fold with more than two HSEs inserted in the disabled *CYC1* promoter. The findings of Wei *et al.*, (1986) were essentially similar to those of Sorger & Pelham (1987), except no induction was found with only a single copy of the

nGAAn HSE consensus. One suspects that greater than 30-fold *lacZ* induction could be obtained if the switch-off of the heat shock response could be prevented and a heat shock mRNA leader sequence was present on the induced mRNA. Instead HSE-directed *lacZ* expression normally plateaus within one hour after a 23-36°C heat shock (Wei *et al.*, 1986), due to the transience of the heat shock response.

Hirst (1990) has recently investigated whether increasing the number of HSEs driving expression of a human *IFN-α2* gene on a high copy number plasmid gives higher levels of expression after heat shock. This *IFN-α2* gene was inserted downstream of a modified *PGK* promoter, a promoter that had 1, 2 or 4 copies of the nGAAn HSE consensus in place of the normal *PGK* UAS element. Although transcription from these constructs was significant in the absence of heat shock, preventing induction ratios greater than about 20-fold, *IFN-α2* mRNA induction levels after heat shock were approximately proportional to the number of HSEs in the promoter (Hirst, 1990). Thus, there are probably advantages to having multiple HSEs in a heat shock expression vector.

1.5.1.4 A cAMP-responsive element in several heat shock promoters

The activation of many yeast heat shock genes with declining cAMP appears not to be under HSE control. Growth-dependent expression of the *HSE-lacZ* construct used by Sorger & Pelham (1987) in which *lacZ* is under the control of two nGAAn repeats has recently been studied. There were no growth phase-dependent changes in ß-galactosidase synthesis in either defined medium and complex medium batch cultures in the absence of heat shock (Tanaka*et al.*,1989). My own studies using a *HSE-lacZ* construct have also shown that the HSE is not responsible for growth phase dependent induction of the *lacZ* gene (Chapter 3). Thus growth-dependent activation of heat shock promoters is apparently due to a promoter element other than the HSE.

The stationary phase levels of the mRNAs of a few stress-induced genes have been reported to increase in early stationary phase, these include *SSA3* (Boorstein & Craig, 1990), *HSP26* (Petko & Lindquist, 1986; Praekelt & Meacock, 1990), *HSP12* (Praekelt & Meacock, 1990), *UBI4* (Finley*et al.*,1989) *UBR5* (Steufert & Jentsch, 1990) and *CTT1* (Bissinger*et al.*,1990). The expression of *SSA3* (Boorstein & Craig, 1990), HSP12 (Praekelt & Meacock, 1990), *UBI4* (Tanaka*et al.*,1988), *CYC7* (Pillar & Bradshaw, 1991) and *CTT1* (Bissinger*et al.*,1990) have been followed in

RAS/cAMP pathway mutants, the results showing that the transcription of these genes is to some extent induced by low protein kinase A activity.

1.6 Aims of research work

The aim of the research that follows is to define the utility of yeast heat shock and growth phase dependent promoters for the expression of heterologous proteins. The model heterologous protein used for these studies was β-galactosidase, an enzyme normally lacking in *S. cerevisiae*.

Two studies were conducted:-

Chapter 3 describes a study of the possible use of the heat shock element for inducing heterologous gene expression by temperature upshift involving:

- Identification of the temperature regimes that give maximal induction
- A study of the stability of β-galactosidase induced by heat-shock
- A study to identify other treatments that induce HSE-directed β-galactosidase expression
- A characterisation of the effect of host genetic background on induction and stability of β-galactosidase

Chapter 4-5 describe a study to investigate the use of growth phase dependent promoters for the regulated production of heterologous proteins, involving:-

- Construction of expression vectors containing growth phase dependent promoters
- Analysis of the regulation of these promoters using *lacZ* as a suitable reporter gene
- Identification of the conditions for maximal induction of β-galactosidase accumulation from such promoters

CHAPTER 2. MATERIALS AND METHODS

2.1 Materials

Standard Reagents :	AR grade supplied by BDH and Sigma
Microbiological media :	Supplied by Difco
	ampicillin and auxotrophic requirements from Sigma
Electrophoresis reagents :	Agarose from Sigma
Restriction enzymes :	Supplied by Promega, Cetus and Pharmacia
Probe labelling reagents :	Supplied by Amersham
Others :	DNase-free RNase from Sigma
	Zymolyase 20T from Seikagaku Kogyu Co.
	Lysozyme from Sigma
	[32P]dCTP from Amersham
	1-10 Phenanthroline from Sigma
	Paromomycin from Sigma
	4-Nitroquinoline-1-oxide from Sigma
	Cycloheximide from Sigma
	NA45 paper from Schleicher and Schull
	Hybond-N transfer membrane from Amersham

2.2.1 *E. coli* strains

DH5 *supE44 lacU169 (80lacZ M15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1*
JM101 *supE thi (lac-proAB)F' [traD36 proAB$^+$ lacI lacZΔ M15]*

2.2.2 *S. cerevisiae* strains

AM9-10A α *cyr1-2, bcy1*	Praekelt & Meacock, (1990)
AM180-2B a *bcy1, his7*	Praekelt & Meacock, (1990)
AM110-4C α *cyr1-2, leu1*	Praekelt & Meacock, (1990)
BWG1-7A a *ade1 his4 leu2 ura3*	L. Guarente, Dept. Of Biology, MIT, Cambridge, MA, USA
W32 a *ade1 his4 leu2 ura3 hap1-1*	L. Guarente, Dept. Of Biology, MIT, Cambridge, MA, USA
W1a *ade1 his4 leu2 ura3hap 2-1*	L. Guarente, Dept. Of Biology, MIT, Cambridge, MA, USA
JP40a *ade1 his4 leu2 ura3 hap3-1*	L. Guarente, Dept. Of Biology, MIT, Cambridge, MA, USA
SP1 a *TPK1 TPK2 TPK3 BCY1*	Mbyoni *et al.*, (1990)
RS13-58A-1 a tpk^{w1} *tpk2 tpk3 bcy1*	Mbyoni *et al.*, (1990)
RS13-7C-1 a *tpk1* $tpk2^{w1}$ *tpk3 bcy1*	Mbyoni *et al.*, (1990)
RTF3.1-1 a *tpk1 tpk2* $tpk3^{w1}$ *bcy1*	Mbyoni *et al.*, (1990)
CT3b a *leu2 trp1 ura3 pep4-3*	Wingfield & Dickinson, (1992)
CT3c a *leu2 trp1 ura3*	Wingfield & Dickinson, (1992)
SUB60 a *leu2 trp1 ura3 his3 lys2 ubi4 :: LEU2*	Finley *et al.*, (1987)
SUB62 a *leu2 trp1 ura3 his3 lys2*	Finley *et al.*, (1987)
ubc4 a *leu2 trp1 ura3 his3 lys2 ubc4 ::HIS3*	Jentch *et al.*, (1990)
ubc5 a *leu2 trp1 ura3 his3 lys2 ubc5 ::LEU2*	Jentch *et al.*, (1990)
PMY1 α *leu2 his3*	Meacock, P, University of Leicester, UK
PMY1.1 α *leu2 ura3 his3*	This study

2.2.3 Plasmids

Plasmid constructions are detailed in Section 2.4

2.3 Methods

2.3.1 *S. cerevisiae* methods

2.3.1.1 *S. cerevisiae* growth media

The recipes for growth media used in this study were obtained from Sherman *et al* ., (1983) and the Difco Manual.
All percentages are %(w/v), 2% bacto-agar was used for the preparation of solid media.

Auxotrophic requirements
Uracil : 20mg/l
L-histidine : 20 mg/l
L-tryptophan : 20mg/l
L-leucine : 30mg/l

YEPD :	1% yeast extract, 2% bacto-peptone, 2% glucose. Other complex media were used to the same recipe, but with alternate carbon sources
YEPG	2% galactose
YEPS	2% sucrose
YEPR	2% raffinose
YEPA	2% sodium acetate
YEPSS	2% sodium succinate
YNBG	0.67% yeast nitrogen base without amino acids, 2% glucose plus auxotrophic requirements
YNBGC	as YNBG with 2% cas-amino acids
Sporulation media	0.05% glucose, 0.01% yeast extract, 1% potassium acetate
Starvation media	YNB-AA was made up from its constituent parts substituting salts to make Nitrogen-free (N-free), Sulphur-free (S-free) and Phosphate-free (P-free) media as described below.

Composition of Bacto Yeast Nitrogen base W/O amino acids **Nitrogen Source**	
Ammonium Sulphate	5g- absent in N-free version
Vitamins	
Biotin	2mcg
Calcium Pantothenate	400mcg
Folic Acid	2mcg
Inositol	2000mcg
Niacin	400mcg
Aminobenzoic Acid	200mcg
Pyridoxine Hydrochloride	400mcg
Riboflavin	200mcg
Thiamine hydrochloride	400mcg
Compounds supplying trace elements	
Boric Acid	500mcg
Copper Sulphate	40mcg
Potassium Iodide	100mcg
Ferric chloride	200mcg
Manganese Sulphate	400mcg
Sodium Molybdate	200mcg
Zinc Sulphate	400mcg
Salts	N-free S-free P-free
Potassium Phosphate Monobasic	1g
Magnesium Sulphate	0.5g 1g
Sodium Chloride	0.1g
Calcium Chloride	0.1g
Ammonium Chloride	5g
Magnesium Chloride	0.5g
Potassium Sulphate	1g
Amount of final medium from 100g dehydrated medium	14.9 l

2.3.1.2 Yeast growth and monitoring of cell biomass

Cells were grown with rapid agitation to ensure aeration at the appropriate temperature in a media volume of 1/5th of the flask volume.
Three techniques were used to monitor cell biomass:
(1) OD_{550} readings were taken with appropriate dilution to ensure the turbidity of the culture obeyed the Beer-Lambert law
(2) OD_{550} readings were correlated to cell number by using an Improved Neubauer haemocytometer (Hawksley)
(3) OD_{550} readings were correlated to cell dry weight by removing 10 OD_{550} units of cells, filtering on filter paper and drying to a constant weight in a microwave oven.
Yeast strains were maintained as frozen stocks in 2xYEPD plus 15% glycerol at -70°C.

2.3.1.3 Chemostat Fermentation of *S. cerevisiae*

Chemostat experiments were carried out in a 2l series LH210 fermenter (LH Fermentation) used at a working volume of 1.3l. The defined medium (NG medium) used is detailed below, and is derived from that used by ICI Pharmaceuticals (Dr. Richard Ernill) for fed-batch culture, which is derived from that of Fieshko *et al.*, (1987)

VITAMINS	**CONCENTRATION (g/l)**
Inositol	0.5
Thiamine HCL	0.01
Calcium Pantothenate	0.05
Pyridoxine phosphate	0.014
Nicotinic acid	0.061
Biotin	0.001
Histidine	0.1
Leucine	0.1
Riboflavin	0.003

TRACE METAL	**CONCENTRATION (g/l)**
Ferric Chloride	27
Zinc Chloride	2
Calcium Chloride ($6H_2O$)	2
Sodium Molybdate	2
Calcium Chloride ($2H_2O$)	1
Copper Sulphate	1.9
Orthoboric Acid	0.5
Concentrated HCl	10%

ESSENTIAL SALTS	**CONCENTRATION (g/l)**
NH_4Cl	8
KH_2PO_4	3
$MgSO_4$	1
EDTA	0.4

CARBON SOURCE	**CONCENTRATION (g/l)**
Glucose	1%

2.3.1.4 Procedure for making NG medium

A 10l demijohn was used as a medium reservoir. for a 10l medium volume, the Essential Salts were added and the concentrated trace metal solution at a concentration of 7ml/l. 9l of ddH_2O was added to the vessel and the mixture was thoroughly stirred until all the particulate mattter had dissolved. The pH of the medium was then taken to 5 using 4M NaOH solution. The medium was then made up to 9.5l with ddH_2O and autoclaved for 40 minutes at 110 bar.

Glucose was autoclaved separately in 1l of distilled water.

The vitamins were filter sterilised through a Sartorius filter housing using a 2µm filter.

After the medium in the demijohn had cooled below 50°C, the glucose solution and the filter sterilised vitamin solution were added using sterile techniques. The resulting medium was allowed to cool to room temperature and kept at that temperature for two days prior to use to check for contamination. This was easily spotted as the initial crystal clear medium would turn opaque if any contamination occured.

2.3.1.4 Chemostat operation

A pH of 5.5 was maintained through the controlled addition of autoclaved 2.0M H_2SO_4 and 2M NH_4OH. The dissolved oxygen was maintained at a saturated concentration by feeding. The temperature was maintained at 28°C for all experiments. Growth medium was fed into the chemostat using Marston pumps set at the desired speed.

2.3.1.5 Chemostat theory and equations

A continous flow culture is characterised by its fractional rate of medium replacement or its *dilution rate* (D in units of h^{-1}). The dilution rate is defined as D = F/V, where F is the flow through the fermentation vessel and is measured in litres per hour F ($l\ h^{-1}$). The volume of the fermenter is designated as V and is measured in litres V(l).

Relating the medium inflow pump setting to the required dilution rate (D) is as follows: to set D to 0.2 in a 1.5l working volume chemostat

F=V*D F=1.5 * 0.2

F= 0.3 l/h

F= 5ml/min

At this high flow rate, 3.2mm bore tubing was used, which gives a maximum flow rate (PF) of 26ml/min when used with a Watson Marlow pump. To define the pump setting (PS):

PS= F/PF*100

PS= 5/26*100

PS=19

For lower dilution rates (0.03-0.1) where the flow rate into the fermenter is much slower, narrower bore tubing was used so as to work within the accurate calibration of the feed pump. For a dilution rate of 0.03 in the same 1.5l fermenter, 1.6mm bore tubing was used which gives a maximum flow rate (PF) of 7ml/min when used with the same pump.

F=VD - F=1.5*0.03

F= 0.045l/h

F= 0.75ml/min

PS= F/PF*100

PS= 0.75/7*100

PS= 10.7

A steady state was assumed when three volumes of medium had passed through the fermenter.

2.3.1.6 Yeast transformation

Yeast transformations were achieved using a modification of the lithium acetate method of Ito*et al.*,(1983).
Strains were grown to an OD_{550} of 1.0 in 100ml of YEPD, harvested and washed twice in 25ml of TEL buffer (10mM Tris HCl, 1mM EDTA) + 0.1M LiAc (pH 7.6), cells were then resuspended in 0.5ml TEL and shaken for 1 hour at 30°C. Transformation was achieved by adding 25µg denatured herring sperm DNA, 1µg plasmid DNA and 0.35ml of 40% PEG 4000 in TEL to 50ml competent cells, incubating at 30°C for 1 hour, heat shocking at 42°C for 5 minutes, then plating out cells on selective media.

2.3.1.7 Strain construction, tetrad dissection and random spore isolation

The two strains to be mated were grown on a YEPD plate overnight and then mixed with a sterile toothpick. After overnight incubation, cells were streaked onto selective plates and grown for two days. Cells from the selective plate were then transferred to sporulation plates and grown for four days, to check if cells had sporulated, they were examined under the microscope. A small amount of the sporulated culture was then incubated with a 1:40 dilution of filter sterilised Zymolyase 20T to dissolve the ascus wall.

Tetrad dissection
Tetrad dissection was carried out using a micromanipulator and the four individual spores from the ascus sac grown on YEPD plates.

Random sporulation
The ascospore digest was vortexed with an equal volume of sterile paraffin oil in an eppendorf and then spun at low speed in a microcentrifuge to partition the spores into the oil layer. This process was repeated four times to free the spores from vegetative cells. The spore suspension was then spread onto YEPD plates and incubated at 30°C till individual colonies appeared.

2.3.1.8 Heat shock treatment

To induce the heat shock response in yeast, liquid cultures were rapidly shifted from the stated temperature of growth (20-30°C) to the required heat shock temperature (35-45°C) and incubated for the appropriate time.

2.3.1.9 Identification of wildtype and *pep 4-3* mutants

This mutation causes deficiencies in carboxypeptidase Y, proteinase A, RNase and repressible alkaline phosphatase synthesis. The technique used was according to Jones*et al.*,(1982).
Cells were plated on YEPD plates and incubated for 2-3 days.
A mixture of 0.6% agar in ddH_2O (melted and held at 60°C) and 2ml of 3mg/ml N-acetyl-DL-phenylalanine-b-napthyl ester (dissolved in dimethyl formamide) was overlayed on the colonies and allowed to set.
4ml of a solution of 5mg/ml Fast Garnet B.C. in 0.1M Tris HCl was then poured over the surface of the overlay.
Once the colonies began to turn red the dye was poured off and the surface rinsed with ddH_2O .
Under these conditions, PEP4 colonies turn a much deeper red than *pep4-3*.

2.3.1.10 Isolation of petite mitochondrial mutants

The frequency with which *S. cerevisiae* cultures segregate spontaneous petite mutants is relatively high and levels of petites may reach as high as 1-2% with some strains.
Spontaneous petite mutants were isolated as small colonies from YNBG plates that were unable to grow on YEPA plates.

2.3.1.11 Isolation of *ura3* mutants

To isolate a *ura3*$^-$ derivative of PMY1, the technique of Boeke *et al.*, (1984) was used.
45ml of YNBG+2% agar was prepared, autoclaved and cooled to 60°C. To this, 5ml of a 1mg/ml filter sterilised solution of 5-fluoro-orotic acid in ddH_2O was added. Once the plates had set PMY1 was spread on top and the plates incubated at 28°C until *ura3*$^-$ colonies appeared.

2.3.2 Biochemical Assays

2.3.2.1 Protein assay

Bio-Rad protein assay reagent was diluted 1:5 using ddH_2O then filtered through Whatman1 filter paper. 1ml of assay reagent was added to a 1.5ml cuvette and 20µl of protein sample added, the mixture was shaken gently and left for 5 minutes at room temperature before assaying at OD_{595} against a blank of 1ml assay reagent plus 20µl ddH_2O.

A standard curve was prepared using the above technique with BSA as a standard protein, this curve was used to relate OD_{595} values to actual protein content in the sample.

2.3.2.2 β-Galactosidase assay

A defined number of OD_{550} units (1-5) were removed from the culture and harvested by centrifugation. Cells were resuspended in 0.5ml Z-Buffer (1.61% $Na_2HPO_4.7H_2O$, 0.55% $NaH_2PO_4.7H_2O$, 0.075% KCl 0.075%, 0.0246% $MgSO_4.7H_2O$, 0.27% b-meraptoethanol) then assayed by the crude assay or permeabilized cell techniques:-

Crude assay

Glass beads were added to the cell suspension and vortexed three times for 1 minute, holding the suspension on ice for 1 minute between vortexing. The cell slurry was then centrifuged for 1 minute and 0.2ml of the supernatant added to 0.3ml of Z-buffer. To initiate the assay, 0.2ml of ONPG (4mg/ml in Z-buffer) was added, and the mixture incubated at 28°C until it acquired a pale yellow colour.

The reaction was terminated by the addition of 0.5ml of 1M Na_2CO_3, and the time elapsed noted. The OD_{420} of the assay mixture was then read against a blank of ddH_2O.

The protein concentration in the extract was measured and the specific activity of the extract was expressed according to the equation: $\frac{OD_{420}}{\text{Protein (mg/ml)} \times \text{Time (minutes)}}$.

Specific activity is expressed as nmoles ONPG hydrolysed/minute/mg protein.

Permeabilized cell assay

60µl of chloroform and 40µl of 0.1% SDS were added to the cell suspension and it was vortexed for 10 seconds.
The assay mixture was then incubated at 28°C for 5 minutes before the reaction was initiated by the addition of 200µl of ONPG. After the mixture turned a pale yellow colour, the reaction was terminated by the addition of 0.5ml 1M Na2CO3, the time for the reaction was noted. The mixture was then centrifuged for 1 minute and the supernatant assayed at OD420nm against a blank of ddH_2O .
β-Galactosidase specific activity of the solution was expressed according to the equation: $\frac{OD_{420}}{OD550 \times \text{time (minutes)}}$

Specific activity is expressed as nmoles ONPG hydrolysed/minute/OD_{550} culture

2.3.2.3 Glucose assay

Glucose concentrations (mg/ml) in yeast fermentation broths were determined using the Sigma glucose assay kit.
The assay reagent was prepared by adding 20ml ddH_2O to the enzyme preparation, 1ml of the solution was added to a cuvette and 10ml of appropriately diluted sample added. The reaction was left for 5 minutes at room temperature and then an OD_{320} reading taken blanking the solution against 1ml of assay reagent plus 10ml ddH_2O.

2.3.2.4 Ethanol assay

Ethanol concentrations (%v/v) in yeast fermentation broths were determined using the Sigma ethanol assay kit.
10ml of clarified fermentation broth was added to 3ml of assay mixture. The reaction was left to proceed at room temperature for 5 minutes and the OD_{340} of the sample read against a blank of 3ml assay reagent plus 10ml ddH_2O.

2.3.2.5 Pheromone production assay (halo assay)

A simple petri plate assay (Sprague, 1991), was used to determine cell type and to test for pheromone production. The plate assay uses special tester strains that are hypersensitive to pheromone. The α-factor tester strain RC629 **a** *sst1* (*bar1*) lacks the barrier protease activity that degrades α-factor.

A master plate was prepared by making small patches of the strains to be tested on the surface of a YEPD plate. The plates were incubated at 30°C overnight.

10^5 cells of the pheromone tester strain were spread on the surface of a BBMB plate (YEPD plus 0.1M citrate, pH 4.5 and 0.03% (w/v) methylene blue, both added from concentrated stocks after autoclaving). The dye accents the halos and the low pH may increase the sensitivity of the assay.

The master plate was replica plated to the tester lawns and the halo's examined after growth for 1-2 days (incubation at room temperature gives larger halos than incubation at 30°C).

2.3.3 Recombinant DNA techniques

2.3.3.1 Restriction enzyme digests

Routinely, 1µg of plasmid DNA was digested with 5 units of the appropriate restriction enzyme for 1 hour at 37°C.

2.3.3.2 Polymerase chain reaction

In order to obtain the UBI4 promoter DNA, it was decided to use the PCR technique. The following primers were synthesized:
Primer A 5' GCAAGCTTAGAAAAGAAAGCAAAGCCTCCCACCACCAGCACTA-3'
Primer B 3' CATACTAGTTTAGTTAAAGTAAAGTGGGAGGGAGAGTTCGAGA-5'
Primer A is complementary to the 5' region of the UBI4 promoter , primer B is complementary to the promoter/protein junction (PCR reaction guideline's were given to me at I.C.I.).
100ng of yeast genomic DNA
10ng each primer
10ml 10x 15mM $MgCl_2$ buffer
100µm dNTPs
2 units Taq polymerase (Cetus)
Make up to 100µl with ddH_2O and add 2 drops mineral oil to prevent evaporation.
30 cycles: 94°C 1 minute, 65°C 2 minutes, 72°C 5 minutes.

2.3.3.3 Annealing of oligonucleotides

Oligonucleotides are synthesised as single stranded DNA. The concentration of oligonucleotides is given as an OD_{260} reading which relates as OD_{260} of 1 = 40µg ssDNA/ml. 2.5µg of each oligonucleotide were mixed in an eppendorf and made up to 200µl with ddH_2O . The eppendorf was placed in a boiling water bath which was taken off the heat source and allow to cool in bath for 3-4 hours. 50-100 fold excess oligonucleotide to vector (by weight) was used for ligation reactions.

2.3.3.5 Purification and precipitation of DNA

Plasmid DNA was purified by vortexing with an equal amount of phenol/chloroform/iso-amyl alcohol (25.24.1) and recovery of the top layer after centrifugation. Precipitation of DNA was achieved by adding 2 volumes of ethanol and 1/10 volume 5M NH_4Cl, the mixture was left on ice for 20 minutes and then spun for 30 minutes in a microcentrifuge. The pellet was washed with 70% ethanol to remove precipitated salt and resuspended in T.E. (pH7.6).

2.3.3.6 Gel electrophoresis of DNA

DNA restriction fragments were separated on 1% agarose gels containing 1xTAE (40mM Tris-Acetate, 1mM EDTA) and 0.1mg/ml ethidium bromide, run in 1xTAE buffer at a constant current of 30mA. Gels were viewed using a short wave U.V. transilluminator and photographed using Polaroid type 55 positive/negative film.

2.3.3.7 Recovery of DNA fragments from agarose gels

DNA fragments were recovered from agarose gels by a modification of the method of Young*et al.*,(1985).
Following electrophoresis a slit was cut in the gel below the fragment to be isolated and a piece of NA45 paper inserted into this slit. Electrophorsesis was then resumed allowing the fragment to run onto the paper. To remove the bound DNA from the paper it was placed in a solution of 0.2M NaCl and heated to 60°C for 15 minutes with gentle agitation. The paper was removed and the DNA recovered by extraction with phenol/chloroform/iso-amyl alcohol and precipitation with 2 volumes ethanol.

2.3.4.1 *E. coli* cell growth and culture conditions

E. coli were cultured in LB (1% bactotryptone, 0.5% yeast extract, 1% NaCl) at 37°C. Cultures for plasmid transformation and selection were grown in LB plus ampicillin to a final concentration of 100mg/l.

2.3.4.2 Preparation of competent *E. coli*

Competent *E. coli* were prepared according to Nishimura*et al.*,(1990).
A 50ml culture innoculated with 0.5ml of an overnight culture was grown with aeration in medium A (LB broth supplemented with 10mM $MgSO_47H_2O$ and 0.2% glucose) to

mid log. The cells were kept on ice for 10 minutes then pelleted at 1500g for 10 minutes at 4°C. Cells were resuspended in 0.5ml of ice-cold medium A , then 2.5ml of ice-cold storage solution B (LB broth plus 36% glycerol, 12% PEG (MW 7500)12mM $MgSO_47H_2O$; sterilized by filtration) was added and mixed gently. The competent cells were stored as aliquots of 0.1ml at -70°C.

2.3.4.3 *E. coli* transformation

Frozen competent cells were thawed on ice and 40µl pipetted into a chilled eppendorf tube.100ng of plasmid DNA was added per aliquot and the cells incubated on ice for 30 minutes. The cells were then heat-shocked at 42°C for 45 seconds and placed on ice for 2 minutes. 0.2ml S.O.C. (2% bacto-tryptone, 0.5% bacto-yeast extract, 0.05% NaCl, 20mM glucose) was added to the cells which were then incubated at 37°C for 1 hour, prior to plating on LB+AMP plates and incubating at 37°C overnight.

2.3.4.4 *E. coli* miniprep

The miniprep protocol was according to Maniatis*et al.*,(1982).

2.3.4.5 *E. coli* maxiprep

This technique was modified from Maniatis (1982).
E. coli were grown up overnight at 37°C in 400ml LB+AMP then pelletted at 3000g minutes. Cells were resuspended in 20ml lysis buffer A (0.45g glucose, 1.25ml Tris-Cl, 2ml 0.5M EDTA, 250mg lysozyme, 46.75ml H_2O) and left at room temperature for 20 minutes. 40ml lysis buffer B (0.2M NaOH, 1% SDS) were then added and the cells left on ice for 5 minutes. 20ml 5M Potassium acetate was then added and the cells shaken on ice for 30 minutes. Cell debris was pelletted by spinning the mixture at 6000 xg for 5 minutes at 4°C, and the supernatant clarified by straining through a layer of paper tissue. 50ml isopropanol was added and the solution mixed and left to stand at room temperature for 5 minutes. Plasmid DNA was precipitated by spinning at 8000 rpm for 10 minutes and the pellet resuspended in 15ml T.E. 30g of caesium chloride and 400µl of ethidium bromide were added and the DNA solution was transferred to a Beckman ultracentrifuge tube. DNA was purified by spinning in a Beckman 50Ti fixed angle rotor for 40 hours at 56K. The DNA band was removed from the gradient with a

hypodermic syringe. Ethidium bromide was removed by 3 extractions with isobutanol and the plasmid DNA precipitated with 1 volume isopropanol.

2.3.4.6 Quantitation of plasmid DNA

To determine the concentration of plasmid DNA in T.E., an OD_{260} reading was taken assuming that 1 OD_{260} unit is equal to 50mg/ml DNA.

2.3.5 Procedures for nucleic acid analysis

2.3.5.1 Isolation of yeast DNA

Yeast DNA was isolated by a method based on Challeff and Tatchell (1985).
30ml of culture were harvested at 5000 rpm at 4°C in a corex tube then washed with ice cold ddH_2O . 2 volumes of glass beads (BDH 40 mesh) were added to the pellet followed by 3ml extraction buffer (10mM Tris-HCl, 10mM EDTA, 1% SDS) and 3ml phenol. The tube was vortexed for 5 minutes, then the two phases separated by centrifugation at 7000 rpm. The aqueous phase was transferred to a fresh tube and 1/10 volume ammonium acetate and 2 volumes ethanol added. The nucleic acid was pelletted by centrifugation (7000 rpm for 20 minutes), washed with 70% ethanol and resuspended in T.E. and DNase free RNase was added to a concentration of 10µg/ml.

2.3.5.2 Isolation of yeast RNA

30 OD_{550} units of a culture were harvested by centrifugation at room temperature at 5000 rpm for 5 minutes. 8ml phenol, 1g glass beads (BDH 40 mesh) and 8ml RNA extraction buffer (10mM EDTA, 25mM Tris-HCl, 1% SDS) were added and the mixture vortexed for 2 minutes. The phases were separated by centrifugation at 6000 rpm for 8 minutes at 4°C in a corex tube and the aqueous phase transferred to a fresh corex tube. 1/10 volume 5M NH_4OAc and 2 volumes ethanol were added and the RNA precipitated at -20°C for 30 minutes. The RNA was pelletted by centrifugation at 12K for 30 minutes at 4°C in a corex tube and resuspended in 0.5ml DEPC treated ddH_2O (prepared by adding 0.5ml DEPC to 3l ddH_2O and mixing overnight by using a magnetic stirrer).

2.3.5.3 Southern blotting

Following electrophoresis, agarose gels were blotted by capilliary transfer of DNA as described in Maniatis*et al.*,(1982). The blotting membrane used was Hybond-N

(Amersham). Blotting was carried out overnight using 20XSSC (3M NaCl, 0.3M NaCitrate).

2.3.5.4 Northern blotting

Northern blotting of RNA was carried out according to Maniatis *et al.*, (1982) using the glyoxal/dimethyl sulfoxide protocol.

2.3.5.5.In vitro labelling of single stranded DNA probes

DNA probes were prepared using the Amersham Rapid Multiprime DNA labelling Kit. Probes were purified by spinning through a G-50 column (Maniatis (1982)).

2.3.5.6 Hybridization of probe DNA to membrane bound nucleic acid

Hybridization was according to the technique of Church and Gilbert (1981).
Membranes were dried at 80°C for 1 hour and then pre-hybridzed in a sealed bag with a solution of 7% SDS, 0.5M $NaHPO_4$, 1mM EDTA at 65°C for a minimum of 15 minutes. The probe was then added to the bag and the blot was hybridized at 65°C for a minimum of 12 hours. After hybridization, the blot was washed at room temperature for 15 minutes in 2XSSPE (17.5% NaCl, 2.8% NaH_2PO_4, 0.74% EDTA) 0.1% SDS, then at 65°C in 1XSSPE, 0.1% SDS for 15 minutes. All washes were carried out in duplicate with constant agitation. Membranes were heat sealed in plastic bags after the final wash to keep them damp.

2.3.5.7 Autoradiography

Membranes were exposed to Fuji X-Ray film at -70°C in an autoradiographic cassette (Protex) with an intensifying screen.

2.3.5.8 Removal of probe from membrane

Membranes were reprobed after probe removal which entailed washing membranes with boiling ddH_2O and shaking at 70°C for 30 minutes in ddH_2O , this process was then repeated.

2.4 Plasmids constructions

Plasmids were constructed using the integrative YIplac204 (Geitz and Sugino, 1988) as a backbone for the initial constructions. Each plasmid construction (PC) is illustrated in the following diagrams as PC1 to 16.

PC1 The standard pUC19 polylinker of YIplac204 was replaced with a polylinker with the following sites in order: Hind111, Spe1, EcoR1, Xba1, BamH1, Sal1, EcoR1 disruption, the resulting plasmid was called pDP387. This step enabled the promoters to be subsequently ligated between the Hind111 and EcoR1 sites, the lacZ gene ligated as an EcoR1-Xba1 fragment and the ADH terminator as a BamH1-Sal1 fragment.
PC2 The Sal1 site in the polylinker of pDP312 had to be replaced so the *ADH* terminator could be excised as a BamH1-Sal1 fragment. This was acheived by cutting pDP312 with BamH1 and Esp1 and ligating the polylinker BamH1, Aat1, Xho1, Esp1. This process replaces the Sal1 site with a unique Xho1 site creating pDP373.
PC3 pDP373 was digested with BamH1 and Sal1 to release the 300bp *ADHt* fragment.
PC4 Plasmid pOZ4 was the source of the *lacZ* gene. pOZ4 was cut with EcoR1 and Xba1, releasing the *lacZ* gene on a 3.5kb fragment.
PC5 pYES1.2 was digested with Hind111 and EcoR1 to release the *GAL1-10* promoter on a 449bp fragment.
PC6 pDP314 was digested with Xba1 to release the *MFα1* promoter on a 900bp Xba1 fragment.
PC7 pDP387 was digested with Spe1 and the *MFα1* promoter Xba1 fragment ligated into the Spe1 site. The orientation of the promoter was checked by a Pst1-Sal1 digest, a 600bp band was released showing the correct orientation this plasmid was called pDP388.
PC8 pDP387 was digested with EcoR1 and Xba1 and the lacZ gene ligated, creating pDP390.
PC9 pDP388 was digested with EcoR1 and Xba1 and the *lacZ* gene ligated, creating pDP391.
PC10 The *ADHt* was ligated to pDP390 digested with BamH1 and Sal1 to create pDP394.
PC11 The *ADHt* was ligated to pDP391 digested with BamH1 and Sal1 to create pDP395.
PC12 The 770bp *UBI4* promoter (*UBI4*p) Hind111-Spe1 fragment from PCR synthesis (2.3.3.2) was ligated into Hind111-Spe1 digested dephosporylated (2.3.3.3) pDP394 creating pDP398.

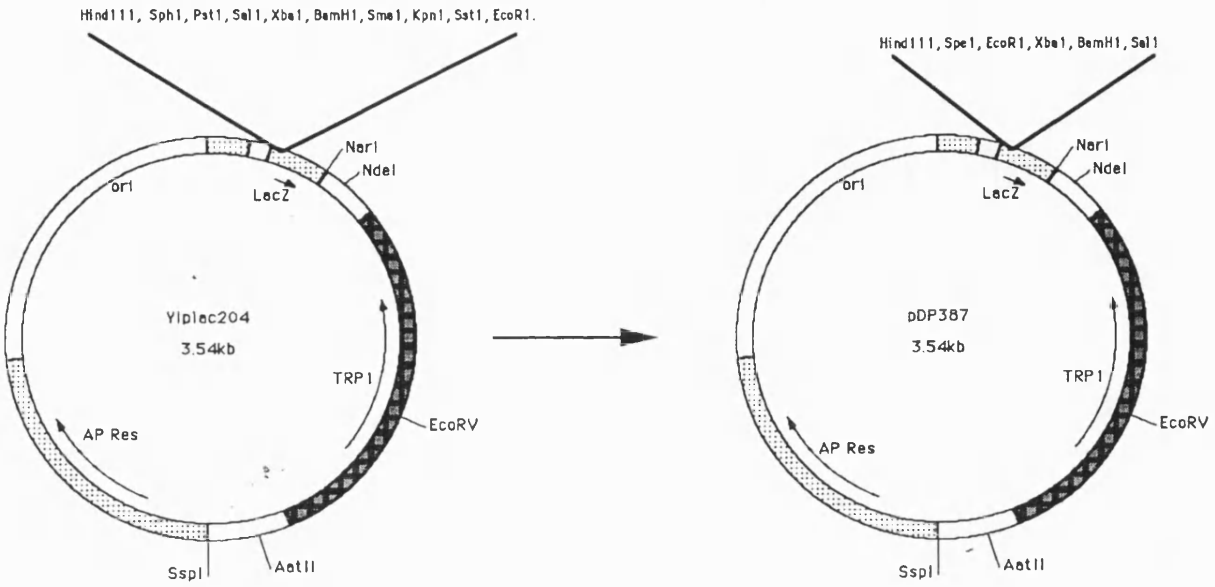
PC13 The 449bp *GAL1-10* promoter Hind111-EcoR1 fragment was ligated into Hind111-EcoR1 digested pDP394 creating pDP399.

Construction of final vectors

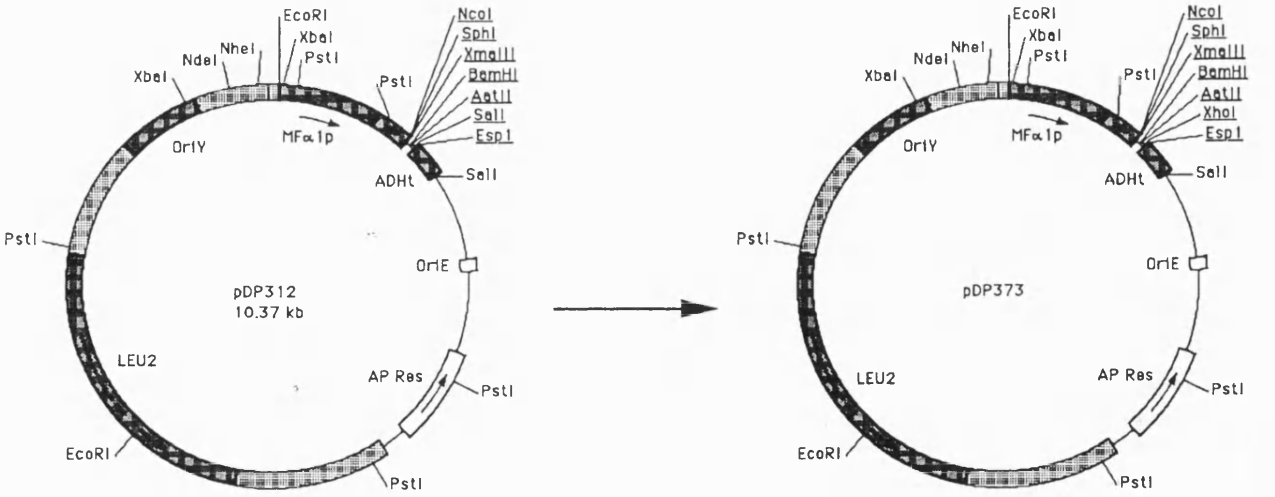
pDP395, pDP398 and pDP399 are completed integrating expression vectors, however, they use TRP1 selection and the required host (PMY1 *leu2 his3*)had not been made *trp*$^-$ at that time, therefore the expression cassettes had to be transferred to the *URA3* selection vector YIplac211 (they could not be used with the Leu selection vector YIplac128 because the unique linearising sites in *LEU2* EcoRV and Cla1, are also present in the *lacZ* gene).

PC14 The *MFα1p* cassette from pDP395 was ligated into Hind111-Sal1 digested YIplac211 creating pDP500.

PC15 The *UBI4p* cassette from pDP398 was ligated into Hind111-Sal1 digested YIplac211 creating pDP501.



Plasmid construction 1



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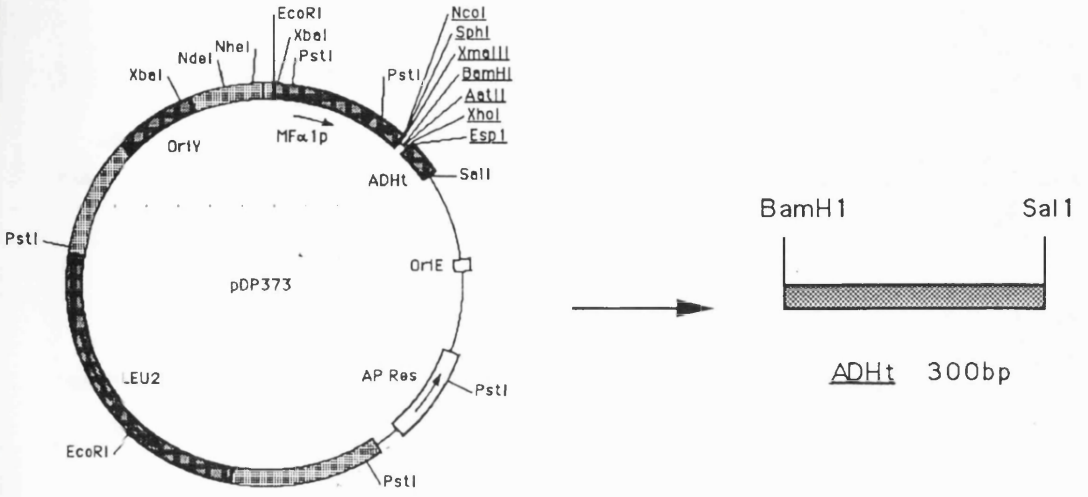

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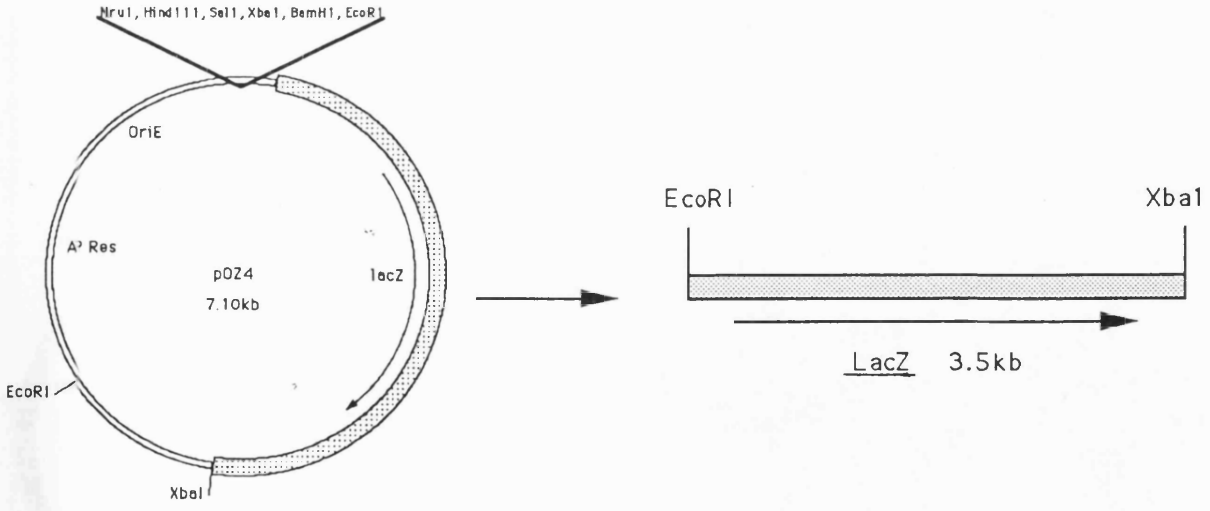

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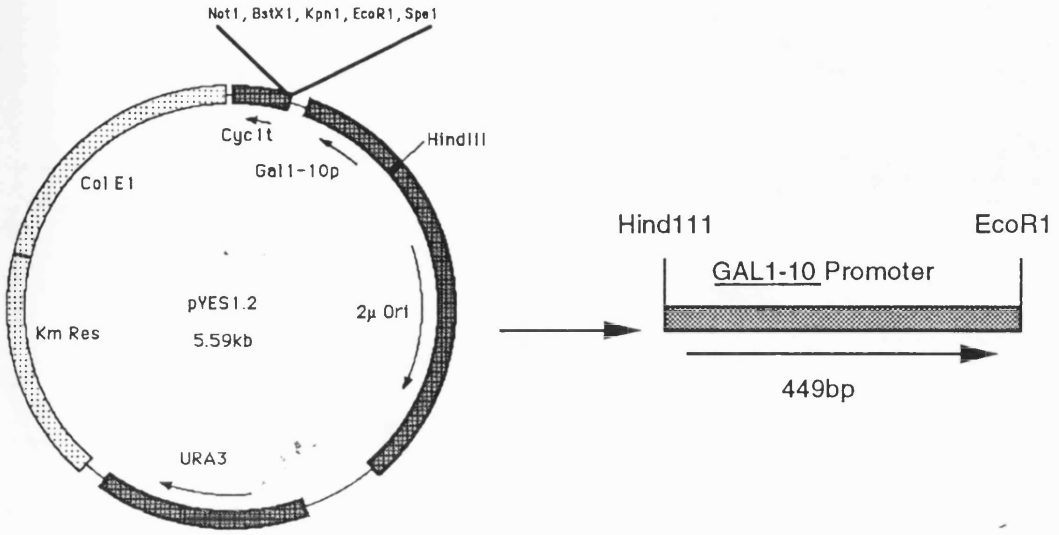

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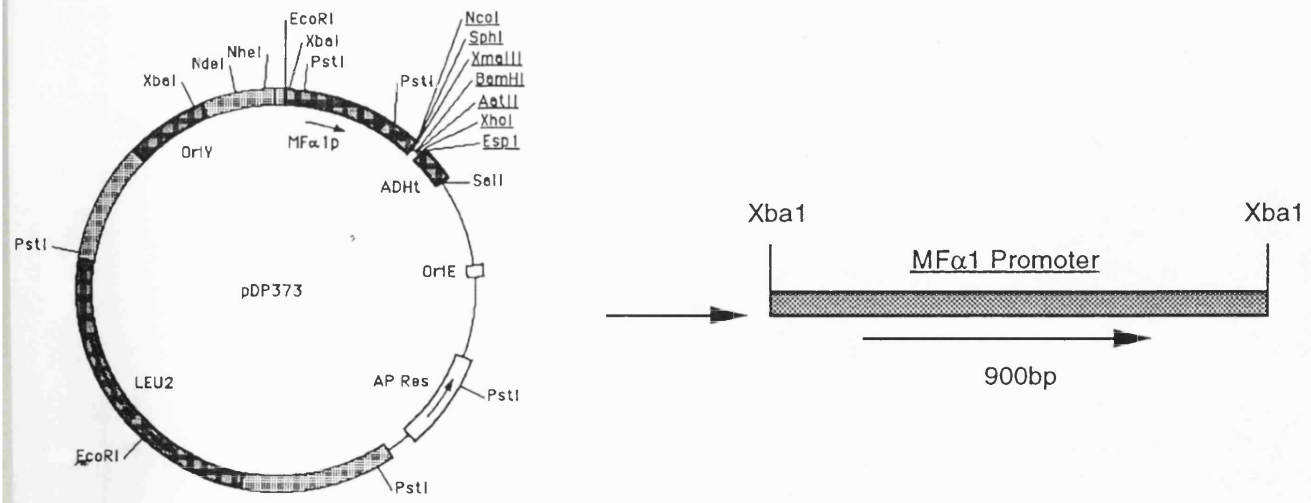

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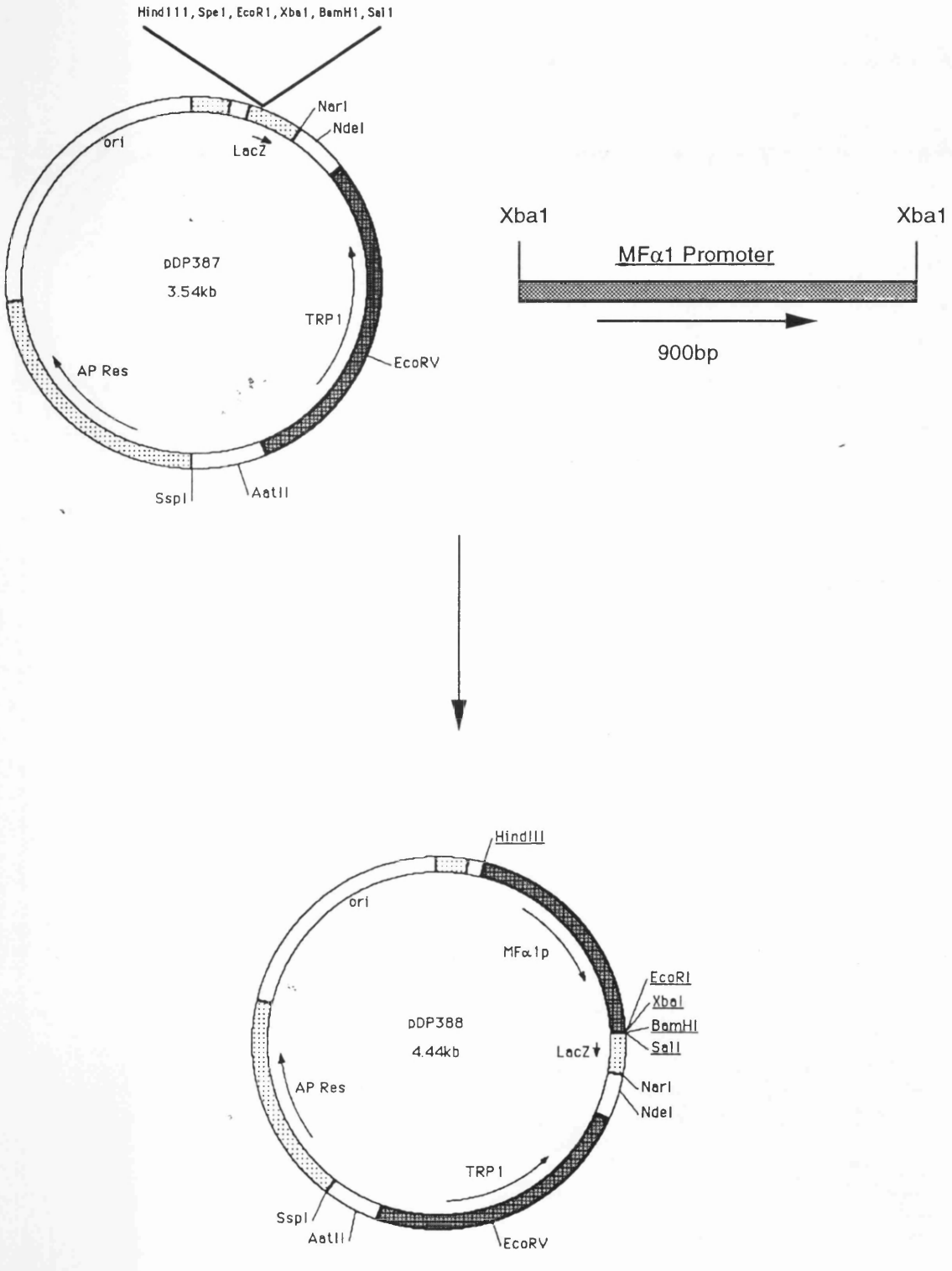

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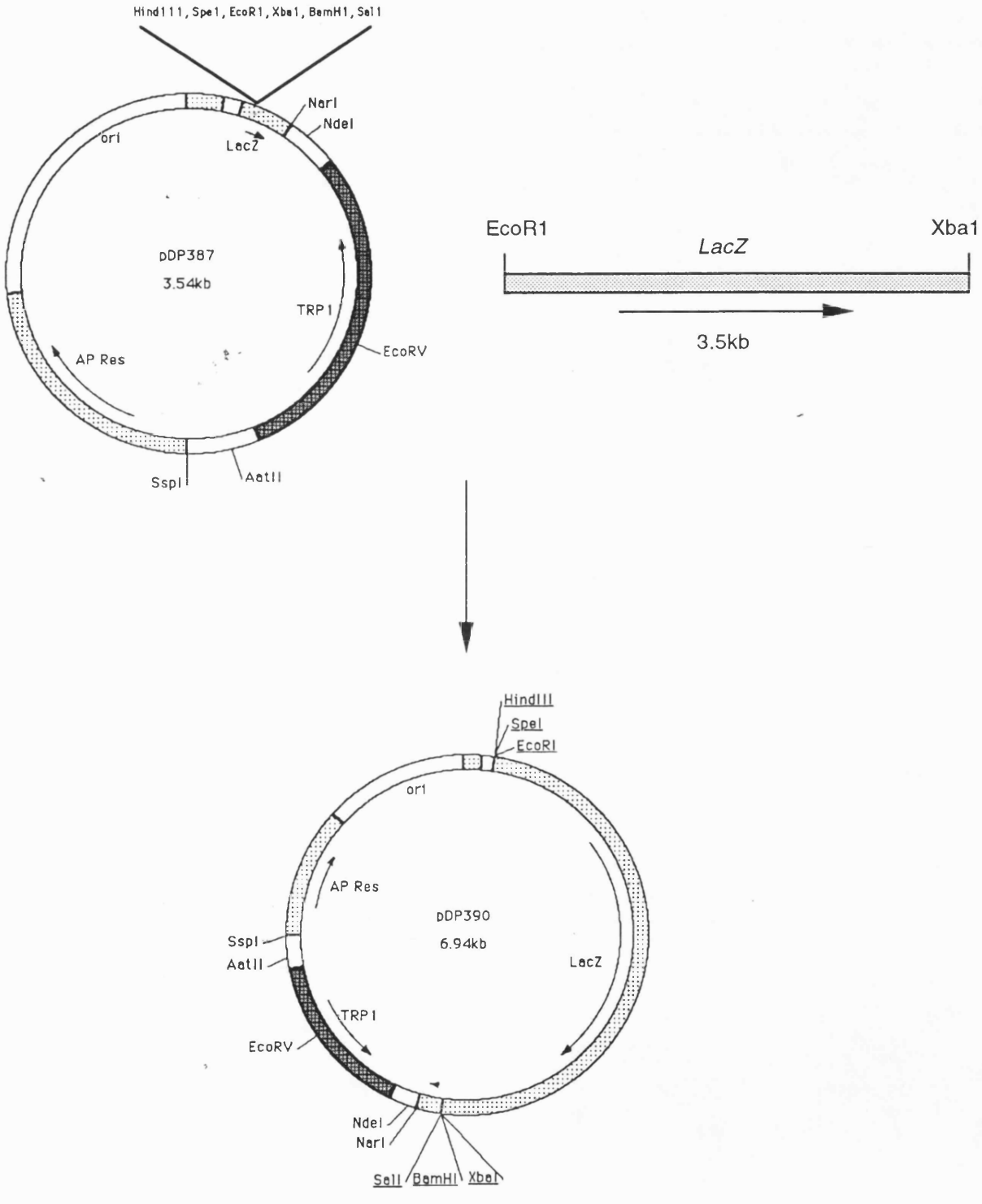

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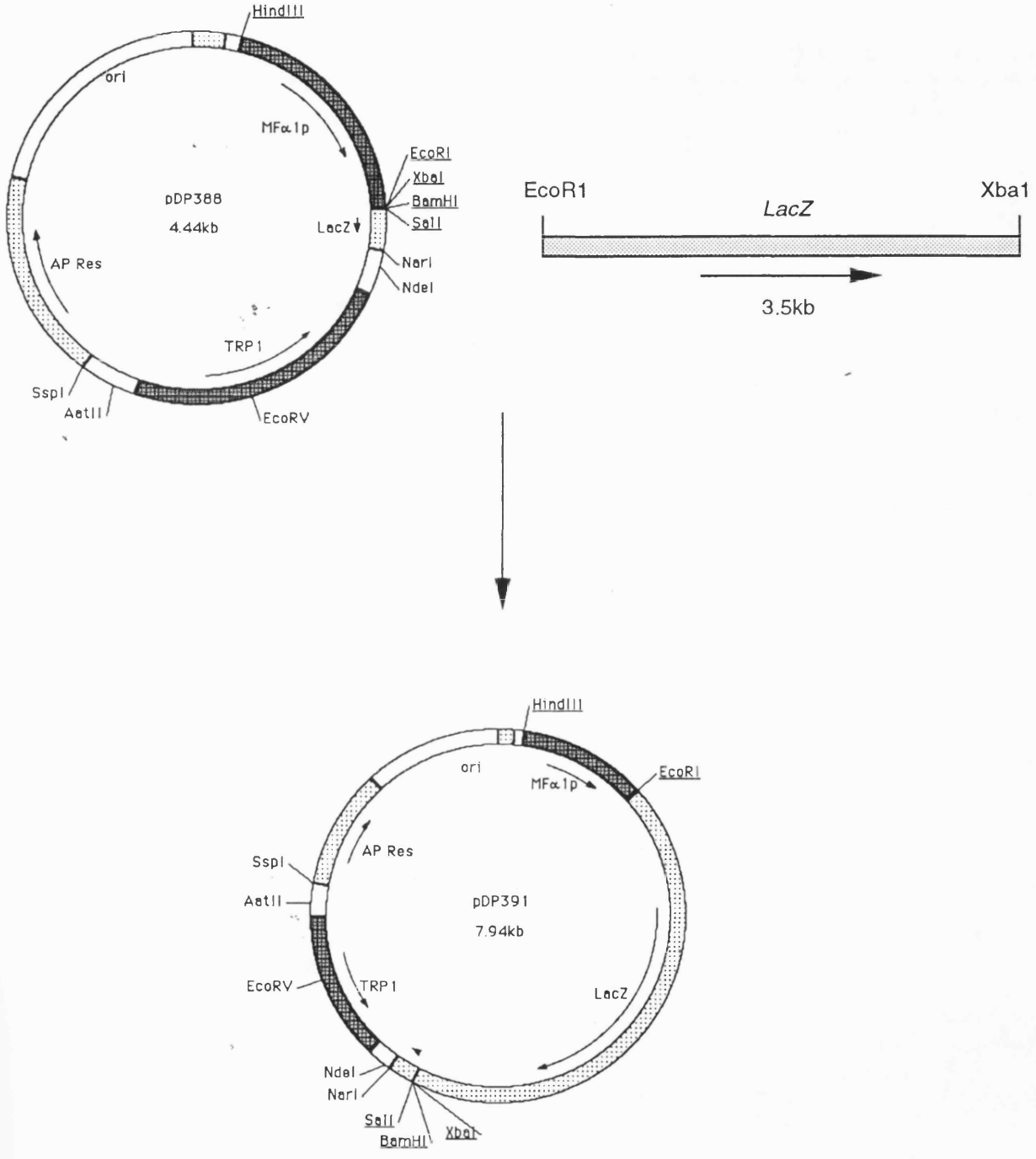

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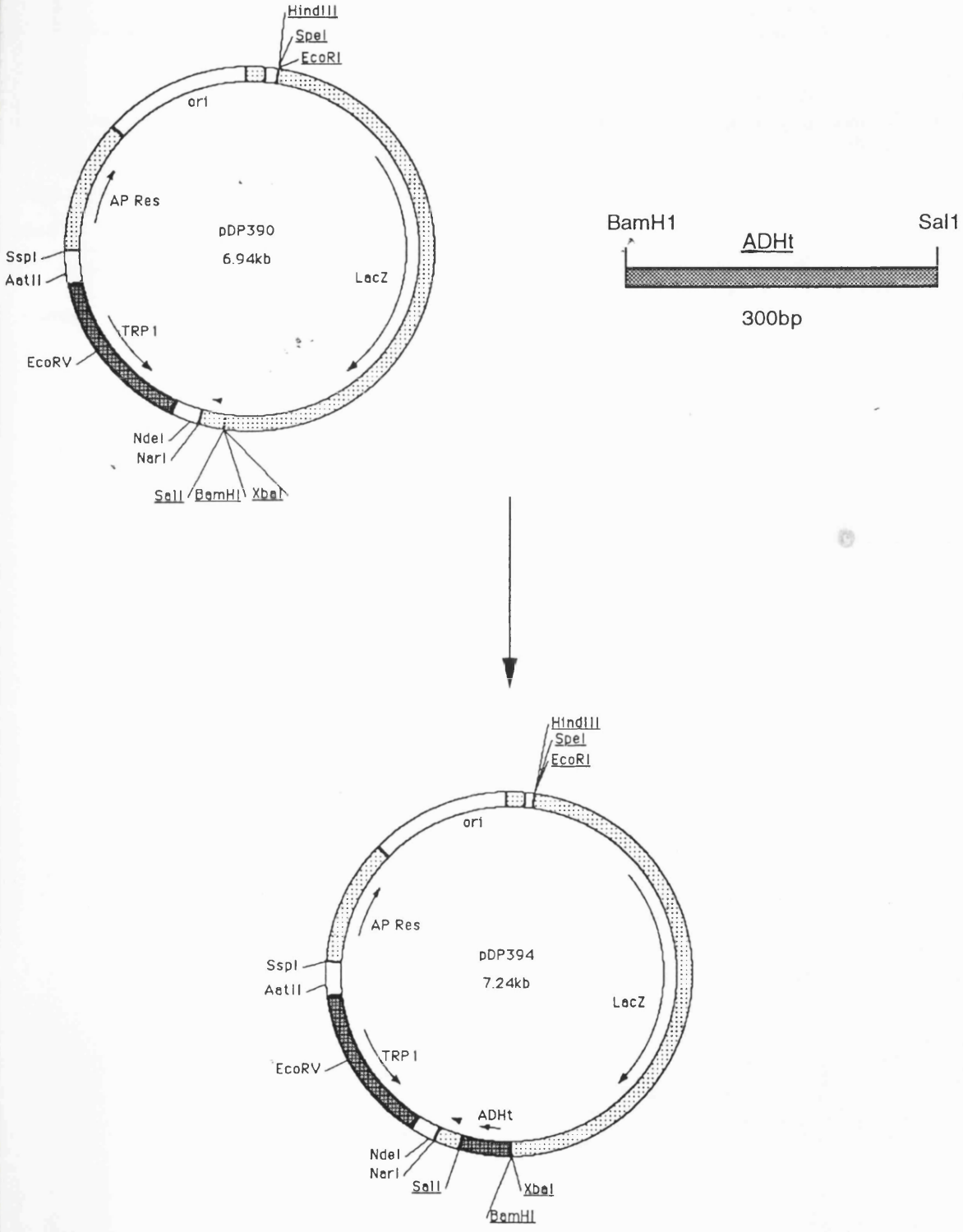
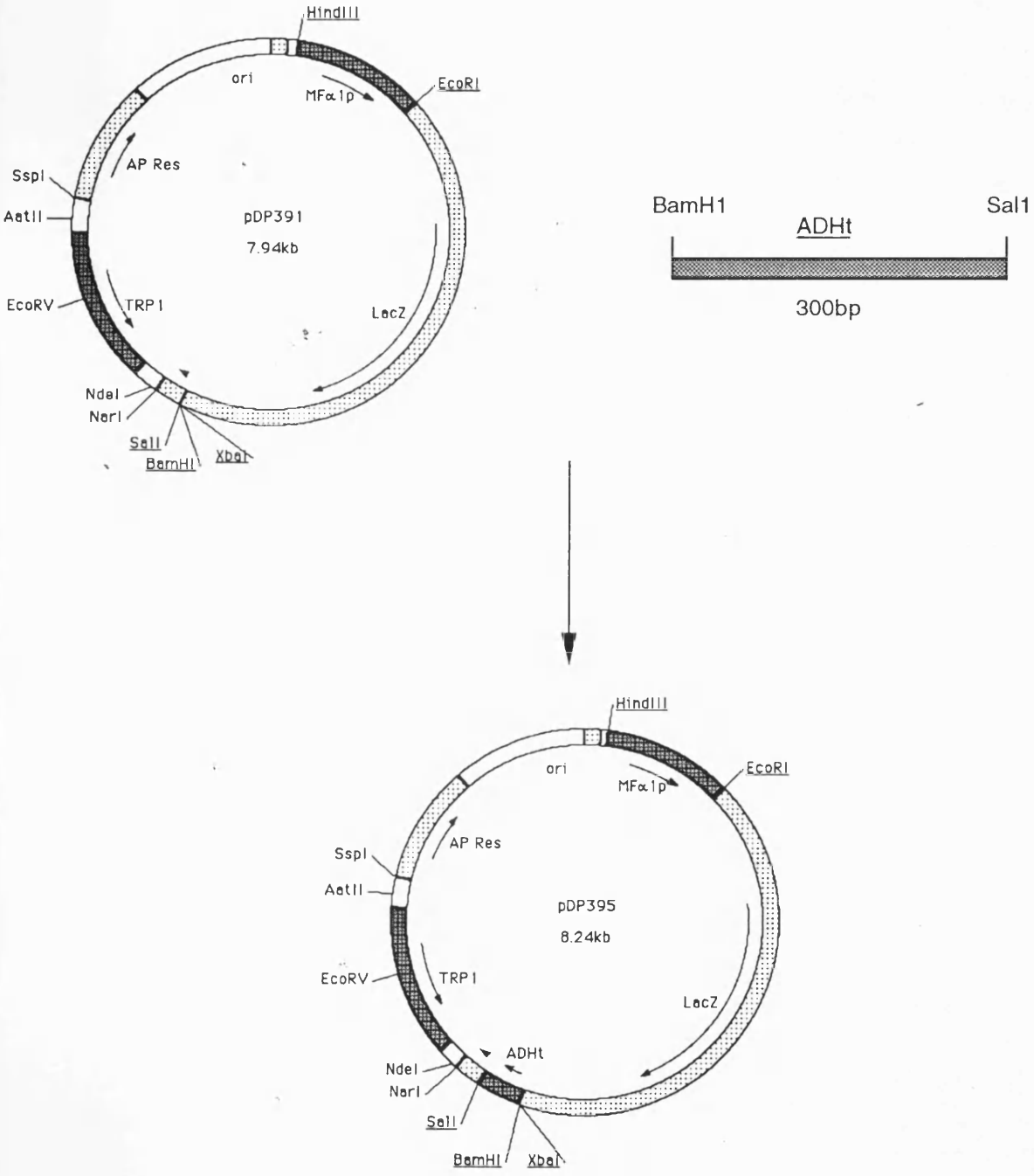


Plasmid construction 10



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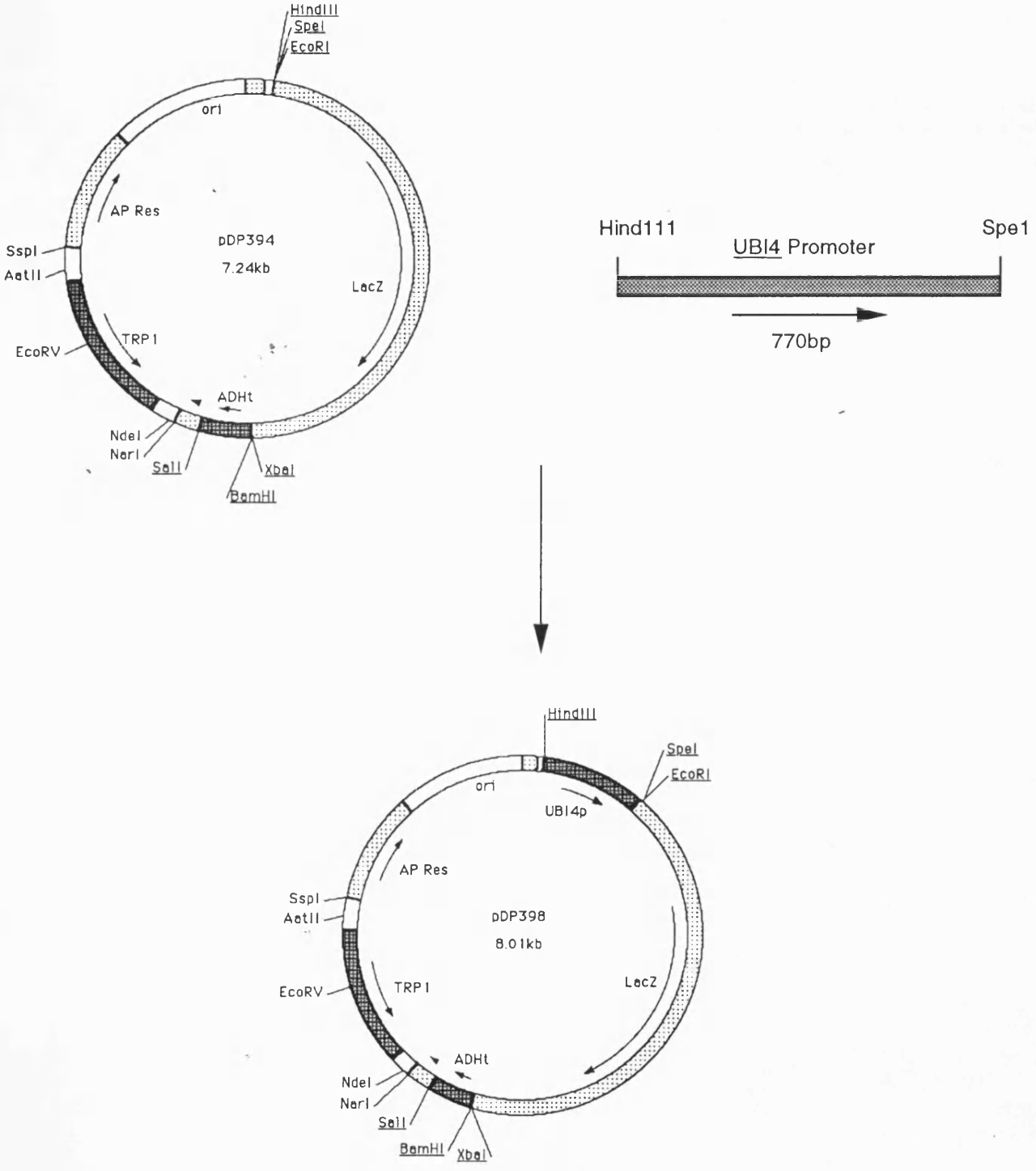
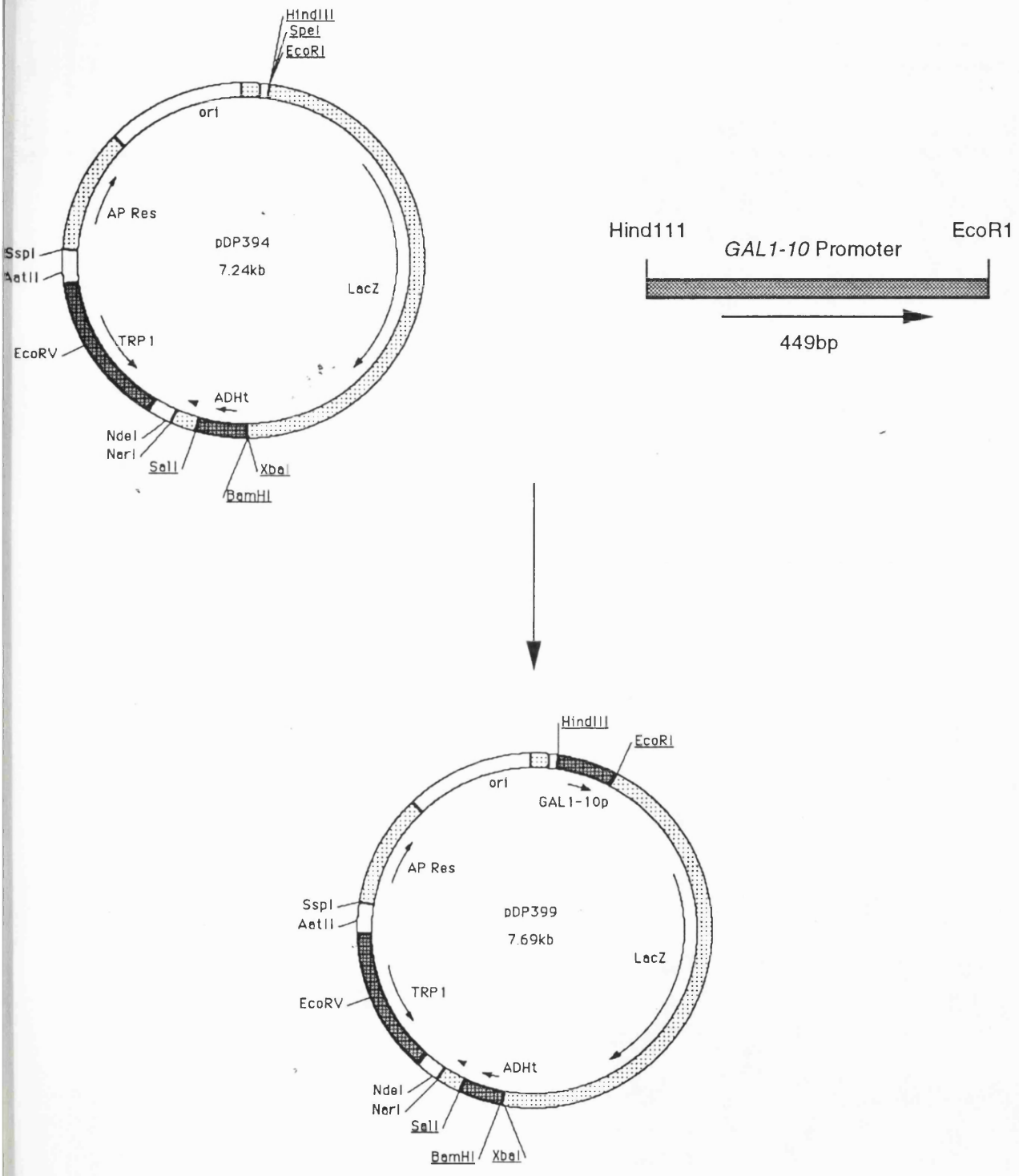
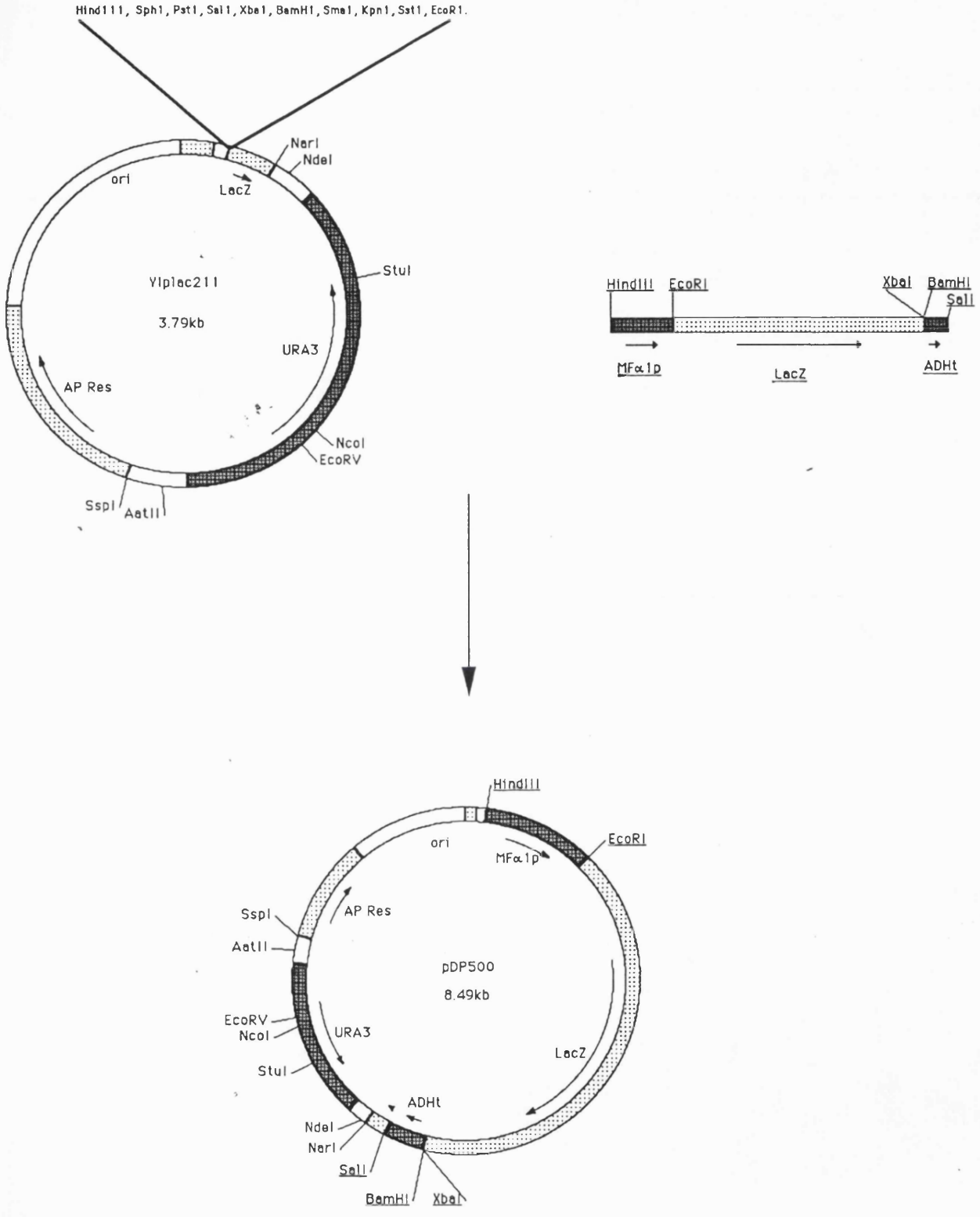


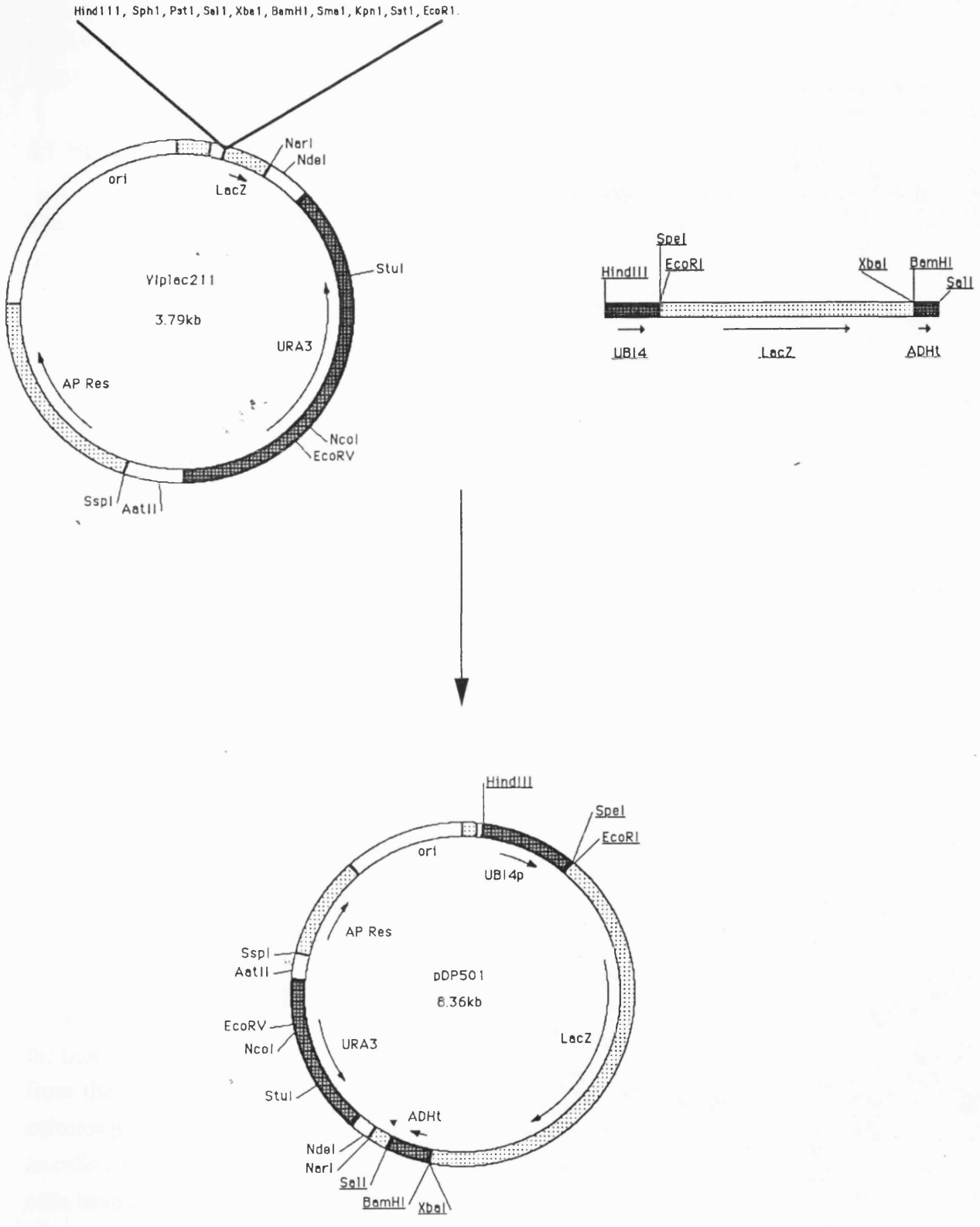
Plasmid construction 12



Plasmid construction 13



Plasmid construction 14



Plasmid construction 15

CHAPTER 3. HEAT-SHOCK ELEMENT DIRECTED HETEROLOGOUS GENE INDUCTION IN *SACCHAROMYCES CEREVISIAE*

3.1 Introduction

The constitutive over-expresssion of toxic proteins in *S. cerevisiae* can cause a variety of problems including slow growth, plasmid rearrangement or loss and cell lysis (Section 1.3.1). The technology of regulated promoters which was expected to circumvent this problem is often difficult to transfer from the laboratory scale to large-scale fermentations (Section 4.2). There is clearly a need for a larger number of yeast promoter systems which can be easily regulated both in laboratory and large scale fermentations. Temperature regulated induction appears to be an ideal way of conditionally expressing heterologous genes in yeast; cultures with temperature regulated expression units can be grown rapidly to high cell densities at temperatures at which the promoter is off, then synthesis of the heterologous protein can be induced by a simple temperature shift without the requirement of modification to medium composition. The main disadvantage of temperature shift induction might be the deleterious effect of the temperature change on the host cell or the expressed protein. Such side effects may include a lower specific growth rate and increased proteolysis. No heat-inducible proteases have been identified in *S. cerevisiae* in contrast to *E. coli* (Straus *et al.*, 1988) but hsp104 has homology to an *E. coli* protease (Parsell & Lindquist, 1992).

The activation of heat-shock genes by temperature upshift of yeast cultures (Section 1.5.1) indicates that the heat shock promoter regulatory elements (HSEs) could be used to direct heterologous gene induction in response to temperature upshift, the objective of this work was therefore to investigate HSE-directed induction of a model heterologous gene (*lacZ*) in response to temperature upshift. The results from studies reported in this chapter indicate induction ratios are low - no greater than 50 fold due to the transient nature of the heat shock response and an appreciable basal level expression from the HSE-*lacZ* construct. In batch fermentation, induction ratios are highest in cultures growing at low temperatures in early exponential growth and decrease rapidly as cells enter stationary phase. Therefore appreciable induction is only possible before cells have reached maximal biomass.

3.2 Results

3.2.1 Heat shock expression vector

The heat shock expression vector used in this study was constructed by Sorger & Pelham, (1987). It employs a disabled *CYC1* promoter into which has been inserted two consensus HSE sequences conferring heat shock inducible synthesis of the reporter gene *lacZ*. To do this, the plasmid pHSE2 illustrated in Figure 3.1 was used (Sorger & Pelham, 1987). This is a derivative of pLGA-178 (Guarente & Mason, 1983) with a disabled *CYC1* promoter. Insertion of two consensus HSE sequences into the BamHI-SalI site of the promoter confers heat-inducible expression of the reporter gene *lacZ* in response to temperature upshift (Sorger & Pelham, 1987). This *lacZ* gene under HSE control is referred to as HSE-*lacZ* in the ensuing sections. Also transformants of strains with pHSE2 are designated by a pHSE2 suffix to the strain (e.g. SUB60-pHSE2 is SUB60 transformed with pHSE2).

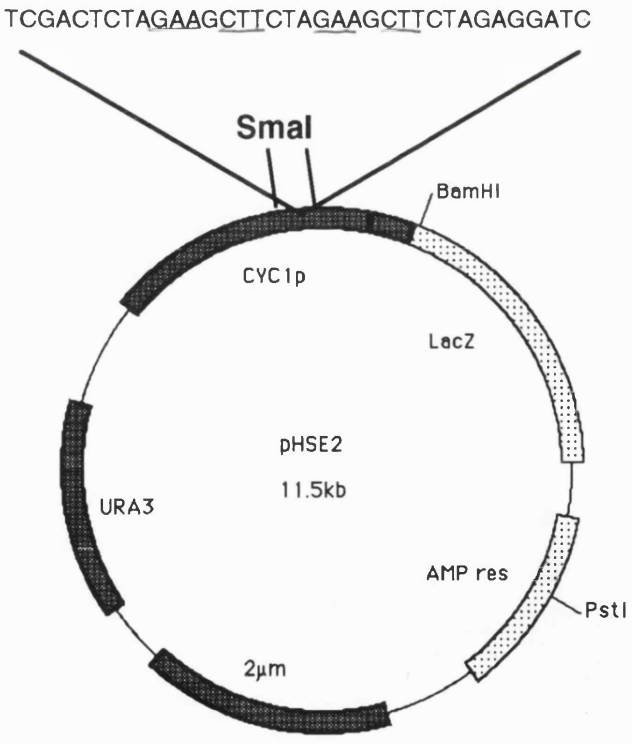


Figure 3.1 The plasmid pHSE2 (Sorger & Pelham, 1987). The HSE2 synthetic oligo was inserted as a Sal1-BamH1 fragment into the unique Xho1-Bgl11 sites of plasmid GA1695 (Ammerer *et al.*, 1986). The conserved nGAAn alternating repeats of the HSE consensus are underlined.

3.2.2 Investigation of heterologous gene induction via HSE activation

Experiments were designed to investigate how *lacZ* induction in response to temperature upshift is influenced by: (1) Growth state and growth temperature prior to heat shock; (2) Magnitude of temperature upshift and final temperature of heat-shock; (3) The kinetics of *lacZ* induction in response to temperature upshift; and (4) The stability of the reporter enzyme β-galactosidase at the induction temperature.

3.2.2.1 The effect of growth stage on HSE-*lacZ* induction

Figure 3.2 shows how the growth stage of a culture of SUB62-pHSE2 prior to heat shock affects HSE-*lacZ* induction. The highest induction ratios were seen in exponentially growing cultures (doubling time =3hr), as cells entered transition phase, induction levels decreased dramatically reaching only two-fold induction in stationary phase. Stationary phase cells regain their capacity for maximal induction within two hours if diluted 20 fold in fresh medium (not shown)

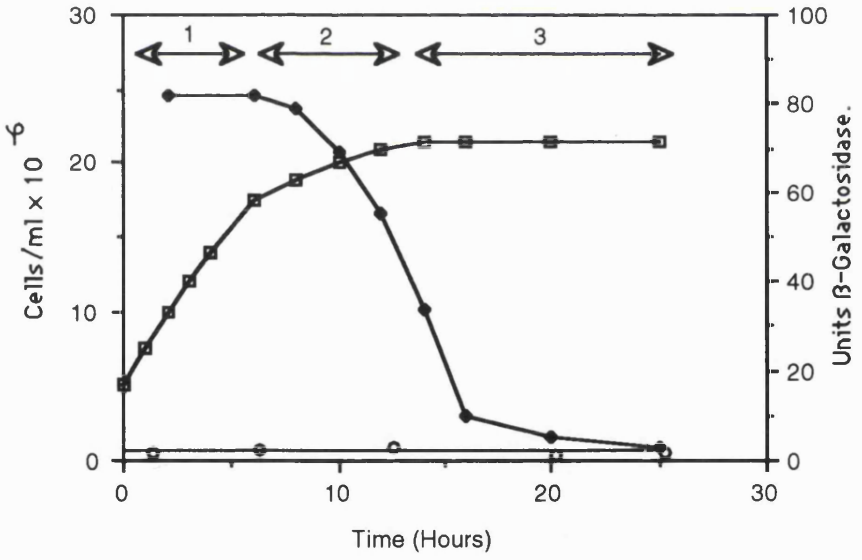


Figure 3.2 Batch fermentation of SUB62-pHSE2 in 23°C YNBG medium. 1,2 and 3 approximate to the exponential, transition and stationary phases of cell growth (□) respectively. β-galactosidase activity expressed as in Section 2.3.2.2 is shown both before (○) and 1 h after (◆) temperature upshift to 39°C. This figure shows the progressive decline of the heat inducibility of the HSE-*lacZ* construct as cells enter transition and stationary phase.

3.2.2.2 The optimal growth and heat-shock temperatures for HSE-*lacZ* induction

With SUB62-pHSE2 in logarithmic growth at 23°C, only a narrow range of heat-shock temperatures gave high *lacZ* induction. Induction was maximal at 39°C, 37°C giving half the maximal induction, Temperatures above 39°C gave lower induction ratios and appreciable killing of the cells.

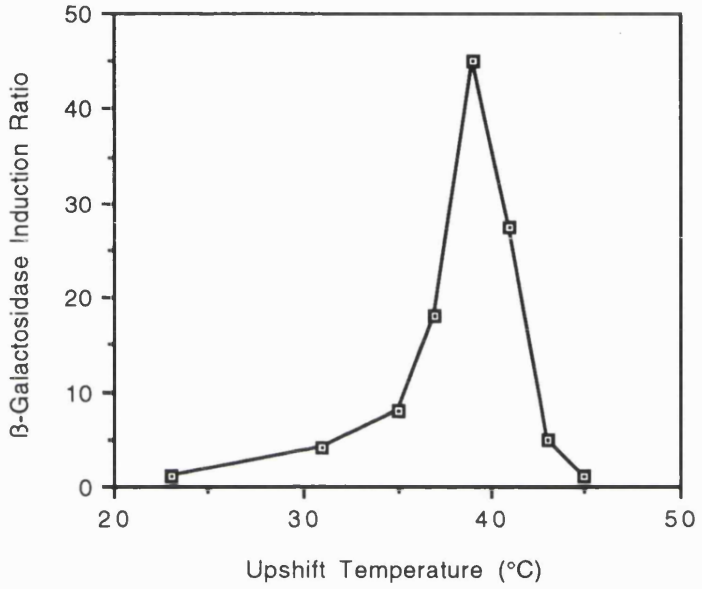


Figure 3.3. The importance of final upshift temperature on induction. Using SUB62-pHSE2 in exponential growth at 23°C, β-galactosidase levels were measured 1 h after shift to the temperature indicated.

3.2.2.3 Effect of growth temperature on HSE-*lacZ* induction

Growth temperature was found to be extremely important in determining levels of induction after heat shock. Using SUB62-pHSE2, basal β-galactosidase levels were found to be relatively constant during growth at temperatures of 20-30°C, increasing above 30°C as basal expression from the HSE-*lacZ* construct rose (Figure 3.4). In contrast, HSE-*lacZ* induction after heat shock was strongly growth temperature dependent, induction levels declining as growth temperature rose. Induction was found to be highest for cells grown at 18-20°C prior to temperature upshift to 39°C (Figure 3.4).

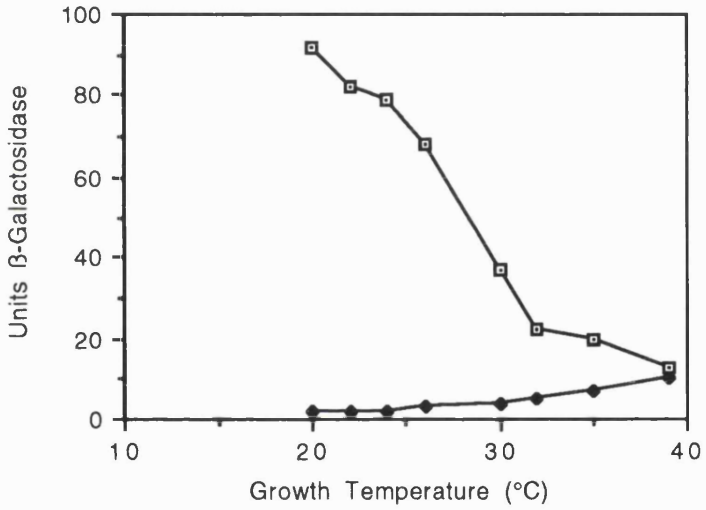


Figure 3.4. Variations in HSE-*lacZ* heat inducibility using SUB62-pHSE2 grown at different temperatures. β-galactosidase levels were measured before (◆) and after (□) temperature upshift to 39°C.

3.2.2.3 Kinetics of HSE-*lacZ* induction

The initial kinetics of HSE-*lacZ* induction, measured as β-galactosidase accumulation were determined for SUB62-pHSE2. *lacZ* gene expression was induced in exponentially growing cultures at 23°C by raising culture temperature to 39°C. Figure 3.5 illustrates the initial induction kinetics, maximal β-galactosidase activity was found 60-120 minutes after temperature upshift, representing an induction of about 30 fold. After two hours β-galactosidase levels start to drop. This decline probably being due to "dilution out" of the relatively stable β-galactosidase as cells continue to divide at 39°C and this decline was not seen with cells treated with cycloheximide (not shown).

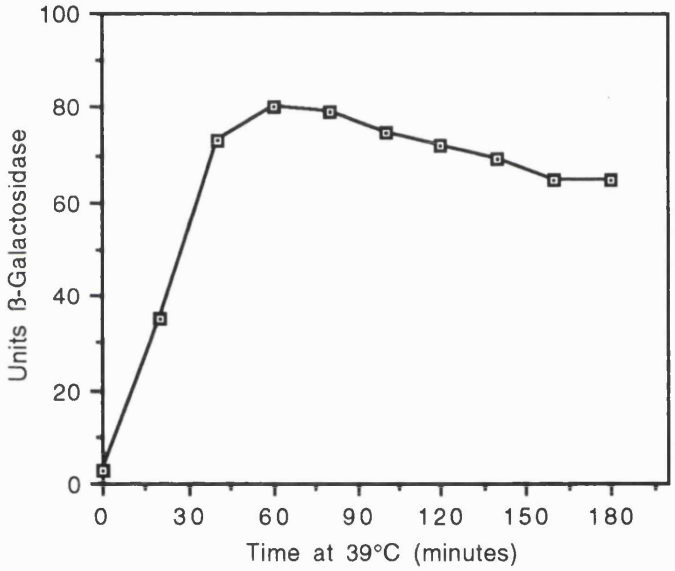


Figure 3.5. Initial kinetics of induction of β-galactosidase activity in SUB62-pHSE2 after temperature upshift from 23°C to 39°C.

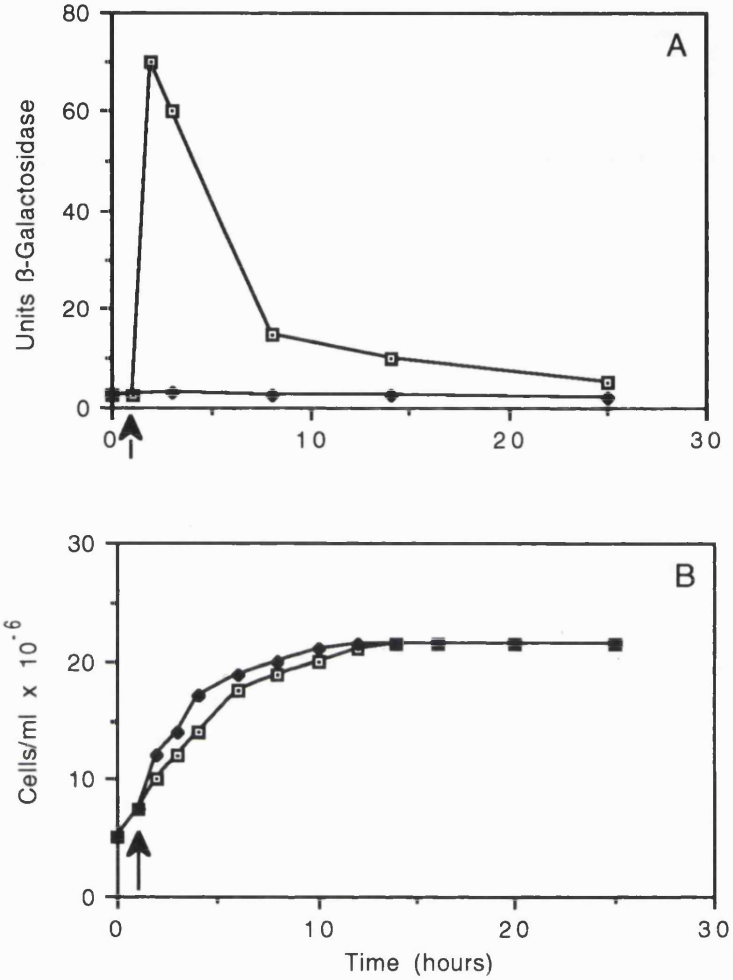


Figure 3.6 Long term induction kinetics of β-galactosidase activity in SUB62-pHSE2 with and without temperature upshift from 23°C to 39°C during cell culture. Cells were grown at 23°C in YNBG (Figure 3.6B), at the time indicated by the arrow, half the culture was shifted to 39°C (□) and the rest kept at 23°C (◆) Figure 3.6A shows the corresponding change in β-galactosidase activity.

Long term induction kinetics illustrated in Figures 3.6 A and B demonstrate that continued incubation at 39°C does not cause any further induction of HSE-*lacZ* activity and after 20 hours intracellular β-galactosidase levels have declined to 2 fold that of the levels prior to temperature upshift.

3.2.3 Effects of yeast genetic background on HSE-*lacZ* expression.

3.2.3.1 Effect of the *rho* mutation on HSE-*lacZ* expression

To determine how respiratory deficiency affects HSE-*lacZ* expression spontaneous *rho-* mutants were isolated from SUB62-pHSE2 by the technique outlined in Section 2.3.1.10. Both the basal and the heat shock-induced β-galactosidase of these *rho-* cells were approximately 2-fold higher than in the *rho+* parents (not shown). The *rho-* mutation did not therefore greatly enhance intracellular accumulation of β-galactosidase. The effects of *rho-* may be more dramatic on the synthesis of secreted products since it increases human lysozyme secretion by yeast up to tenfold, especially at stationary phase (Kaisho *et al.*, 1989).

3.2.3.2 Effect of mutations in the ubiquitination system in HSE-*lacZ* induction

Heat shock activates certain genes of the ubiquitination system for intracellular protein turnover. These include *UBI4* (encoding polyubiquitin; Finley *et al.*, 1987); also *UBC4* and *UBC5* (encoding the ubiquitin ligases which join ubiquitin to substrates destined for proteolysis; Seufert & Jentsch, 1990). Although β-galactosidase synthesised in the cytosol of yeast normally has a long half-life (>20h) (Varshavsky *et al.*, 1988), when made at heat shock temperatures it could conceivably turn over more rapidly due to simultaneous activation of the ubiquitination pathway. *ubi4* Expression hosts might therefore give higher and\or more prolonged synthesis. We compared β-galactosidase induction in SUB60-pHSE2 and SUB62-pHSE2, two transformants that are isogenic but for a *LEU2* gene disruption of the *UBI4* locus in SUB60-pHSE2 (Table 2.1) (Finley *et al.*, 1987). The *ubi4* strain did not give appreciably higher or more prolonged HSE-*lacZ* expression as illustrated in Figure 3.7. Should β-galactosidase synthesised at heat shock temperatures be subject to appreciable degradation by the ubiquitination pathway, reductions in ubiquitin ligase activity might also increase its stability. Two such ligases, UBC4 and UBC5, are involved in degradation of abnormal proteins (Seufert & Jentsch, 1990). Preventing the expression of either UBC4 or UBC5 might therefore conceivably increase heat-induced HSE-*lacZ* expression. This was not found, the use of *ubc5* or *ubc4* expression hosts having no major effect on heat shock induction of β-galactosidase (Figure 3.7). This is consistent with earlier findings that turnover of long-lived and short-lived normal proteins (unlike proteolysis of abnormal canavanyl-peptides) is barely affected by *ubc4* or *ubc5* as single mutations (Seufert & Jentsch, 1990).

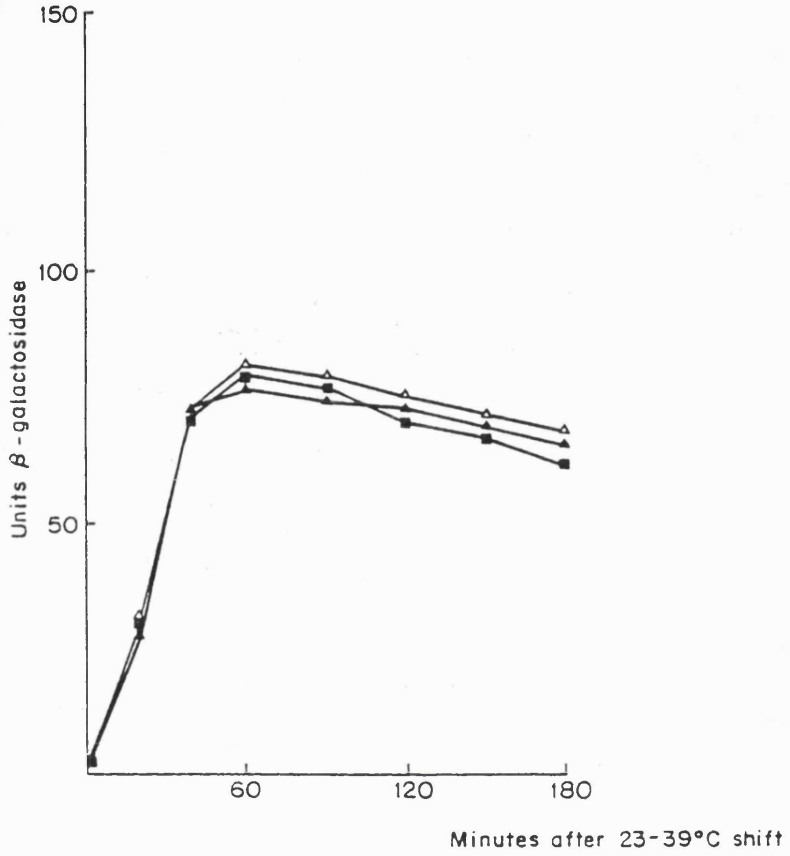


Figure 3 7 Heat-shock induction of β-galactosidase in SUB62-pHSE2 (■), SUB60-pHSE2 (△), ubc5-pHSE2 (▲).

3.2.3.3. Effects of protease mutants on *HSE-lacZ* expression

The highly-selective protein turnover catalysed by the ubiquitination system (Ciechanover & Schwartz, 1989) contrasts with the nonspecific proteolysis catalysed by the vacuolar proteases. *pep4.3*, a defect in the PRA1 gene, reduces the activity of several vacuolar hydrolases by over 90% (Jones, 1982). *pep4.3* Expression strains are therefore in widespread use to minimise the breakdown of products made in yeast during their recovery. Basal and heat-induced HSE-*lacZ* expression were both approximately two-fold higher in a *pep4.3* strain as compared to a wild-type strain of the same parentage (Figure 3.8). These effects appeared not to be due to any higher instability of β-galactosidase in crude cell extracts from wild-type cells (the rates of inactivation of this enzyme in crude extracts from CT3b-pHSE2 and CT3c-pHSE2 were similar irrespective of the presence or absence of protease inhibitors; data not shown). β-galactosidase increases due to *pep4-3* have also been found by Wingfield and Dickinson (1990; 1992) for *lacZ* expressed from the complete *CYC1* promoter. They have also concluded this is due to *pep4-3* increasing β-galactosidase during growth, rather than any artefact of the procedure for making cell extracts prior to enzyme assay.

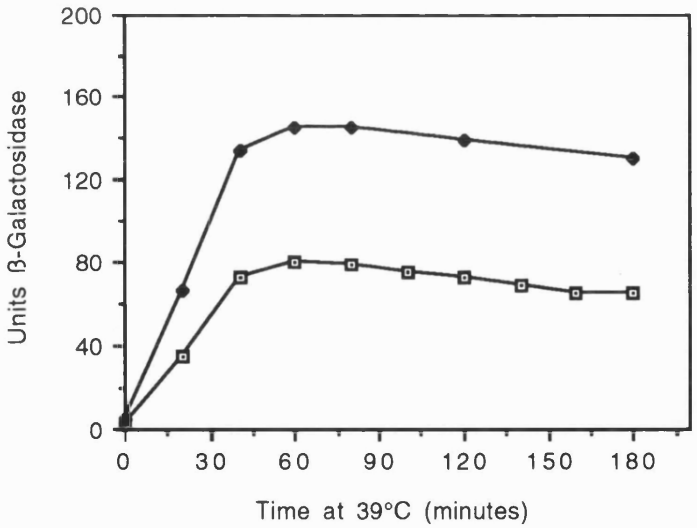


Figure 3.8. Heat shock induction of β-galactosidase in 6T3b-pHSE2 - the *pep4.3* strain (◆) and 6T3c-pHSE2 - the PRA1 strain (□). Cultures were in exponential growth at 23°C prior to heat-shock to 39°C for the indicated times. CT3b and CT3c were derived as single spores from the same tetrad (J. Wingfield, personal communication).

3.2.3.4 Stability of β-galactosidase at the optimal temperature for HSE-lacZ induction

A criticism frequently levelled at the use of temperature upshift for regulated expression of heterologous proteins is the increased possibility of proteolysis of the expressed protein at the upshift temperature.

To investigate whether increased proteolysis is apparent at the optimum induction temperature for HSE-*lacZ* activity, exponentially growing cultures of the transformants 6T3b-pHSE2 and 6T3c-pHSE2 were induced at 39°C. After 1h at 39°C, cycloheximide was added to arrest protein synthesis and hence cell division. The results from this experiment, shown in Figure 3.9, illustrate the stability of β-galactosidase at 39°C in the absence of protein synthesis in the wild type parent 6T3c and the otherwise isogenic *pep4-3* strain, 6T3b.

In both 6T3b and 6T3c it is evident that no degradation of β-galactosidase occurs at the upshift temperature, as the S.A. values stay constant during the 2h incubation period in the presence of cycloheximide. This result also indicates that the decrease in β-galactosidase activity at 39°C in the absence of cycloheximide is due to the 'dilution out' effect as cells continue to divide at the elevated temperature. Although the *pep4-3* mutation affects the amount of accumulated β-galactosidase both before and after heat shock, the *PRA1+* strain does not exhibit temperature dependant degradation of the accumulated β-galactosidase.

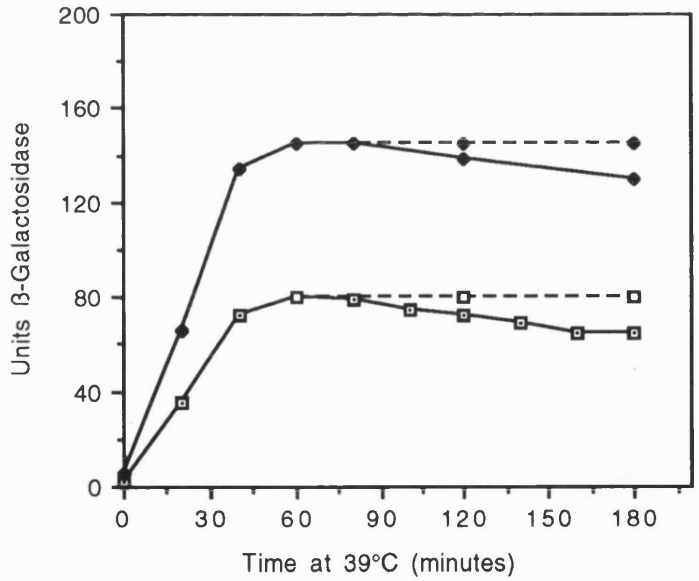


Figure 3.9. Induction kinetics of β-galactosidase accumulation in the strains 6T3b-pHSE2 (◆) and 6T3c-pHSE2 (□) in the presence(- - -) and absence (——) of cycloheximide (added after 1h incubation at 39°C).

3.2.4 HSE-*lacZ* induction by other inducers of the heat-shock response (HSR)

A striking feature of the HSR is its induction by a multiplicity of environmental and chemical stress agents (Ananthan *et al.*, 1985; Lindquist & Craig, 1988). A number of known inducers of the HSR and other agents known to alter gene expression patterns (dimethyl-sulphoxide and salt stress) were tested to see if they gave appreciable or more prolonged HSE-*lacZ* induction.

Agent	**Fold β-galactosidase induction**
Heat shock to 39°C	30X[a]
Ethanol 8% (v/v)	2X[a]
Methanol 8% (v/v)	20X[a]
Dimethylsulfoxide 10% (w/v)	nil[b]
Canavanine 1mg/ml	nil[b]
NaCl 1.7M	nil[b]
Paromomycin 1mg/ml	2X[c]
4-Nitroquinoline-1-oxide 1mg/ml	2X[b]

Table 3.1. HSE-*lacZ* induction by various inducers of the stress response. Induction was measured either 1h (a) 1-4h (b) or 5h (c) after the addition of each agent to SUB62-pHSE2 cells in exponential growth at 23°C, the fraction of cells surviving being greater than 98% in all cases.

From the data displayed in Table 3.1, it is evident that, with the exception of methanol, all agents give only very slight induction or no induction at all. Since the addition of sub-lethal concentrations of methanol may provide an easily-executed alternative to temperature upshift for achieving HSE-*lacZ* induction, its action was studied in more detail.

3.2.6 Induction of HSE-*lacZ* activity by methanol

S. cerevisiae cells will survive a brief exposure to remarkably high concentrations of methanol, although extensive killing occurs above a threshold concentration of 18-20% (Table 3.2). They are appreciably less tolerant of ethanol, although it is a natural fermentation product. This finding is consistent with the general tendency for the alkanol tolerance of yeast to decrease with increasing hydrocarbon chain length and lipid-buffer partition coefficients of alkanols (Leao & Van Uden, 1985).

Methanol concentration (v/v)	**Fraction of cells surviving**
4%	100%
8%	100%
10%	100%
14%	95%
16%	92%
18%	90%
20%	1.5%

Table 3.2. Survival following a one hour treatment of an exponential culture of strain SUB62 with varying concentrations of methanol.

Although HSE-*lacZ* activity is not induced to a great extent by 8% ethanol, a concentration known to induce many heat shock proteins in yeast (Craig & Lindquist, 1988)(Table 3.1) it is strongly induced by sublethal (8%) methanol concentration. Optimal induction (about 15 fold) was with the addition of methanol to a concentration of 10% (v/v) to cultures in exponential growth (Figure 3.10A). The induction kinetics of this response being similar to the kinetics of induction by temperature upshift and are illustrated in Figure 3.10C. Induction of HSE-*lacZ* activity by methanol treatment shows the same growth-phase dependancy as induction by temperature upshift, induction ratios being highest during exponential growth (Figure 3.10B).

To confirm that methanol induction of HSE-*lacZ* activity was directed by the HSE and not another sequence within the *CYC1* promoter, a derivative of pHSE2 was made which lacked the HSE sequences. This was achieved by digesting pHSE2 (Figure 3.1) with SmaI to remove the HSE sequences and then religating the plasmid to form p178. p178 was then transformed into SUB62 and tested for heat-shock and methanol inducibility. SUB62-p178 showed no induction under either of these conditions (not shown).

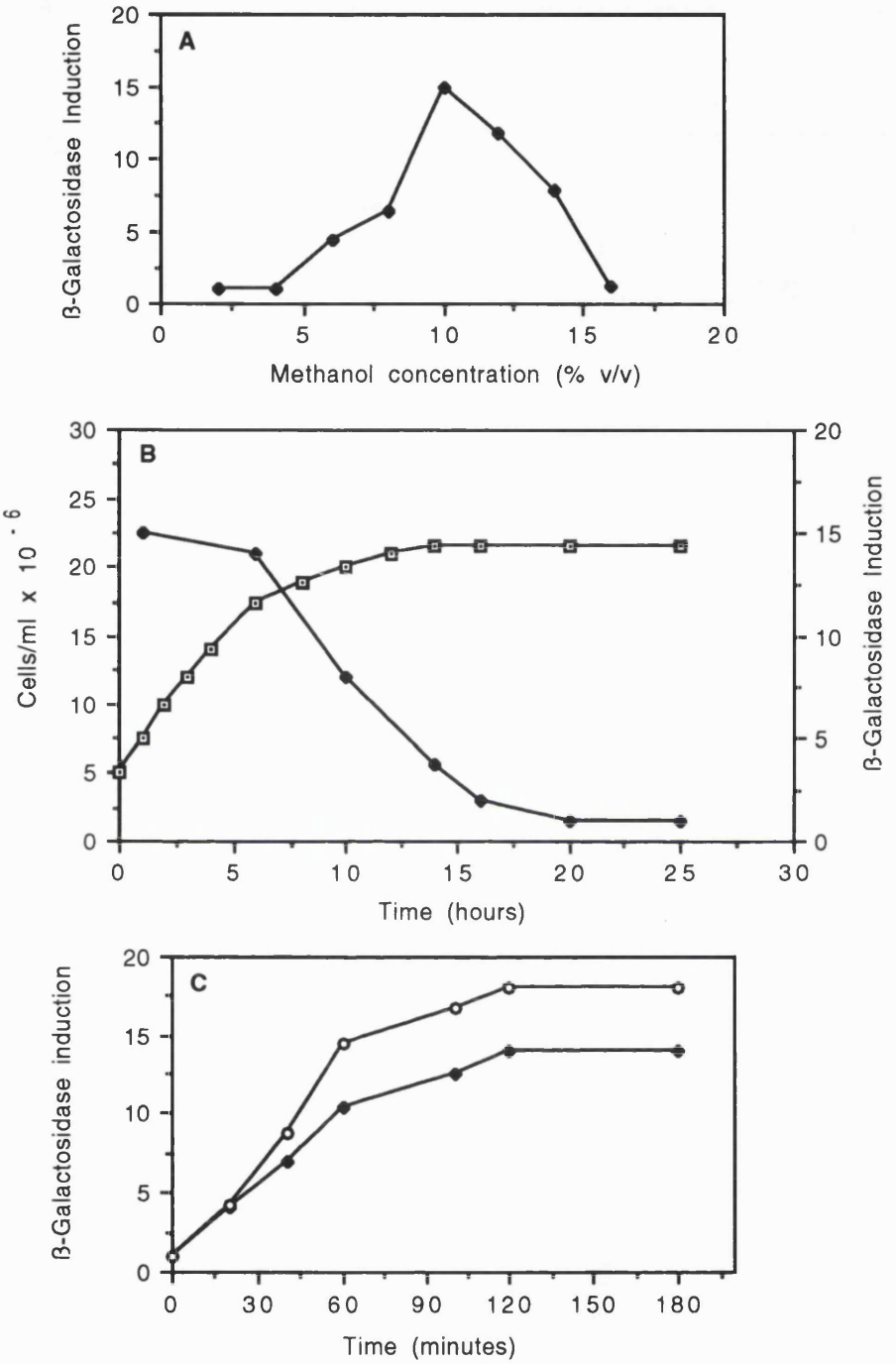


Figure 3.10 **A-C**. HSE-*lacZ* induction in SUB62-pHSE2 in response to methanol. β-galactosidase activity (◆) is given as fold induction over basal activity of 2.7 units. **A** Induction 1 hour after the addition of different concentrations of methanol. **B** Induction 1 hour after the addition of 10% methanol at different stages of cell growth (□) on YNBG. **C** Kinetics of induction by treatment of exponentially growing cells at 25°C with 10% methanol (◆) or by temperature upshift to 39°C (○).

3.2.7 Regulation of heat shock transcription factor

In heat-shocked yeast the transcription of heat shock genes is due to activation of a trans-activator protein (heat shock transcription factor (HSF)) bound to its recognition sequence (heat shock element (HSE)) within the DNA of heat shock gene promoters. In yeast HSF is a protein essential for viability (Weiderrecht *et al.*, 1988) and is bound to HSEs both in unstressed and in heat shocked cells, becoming extensively phosphorylated upon activation (Sorger & Pelham, 1987; 1988) To investigate the phenomenon of growth-phase dependent heterologous gene induction from the HSE, regulation of the *HSTF* gene was studied. mRNA levels of HSTF were studied during a normal growth curve of the strain SUB62 grown on YNBG at 28°C. RNA samples were isolated during all stages of the growth curve and blotted, the resulting Northern blot was probed with the EcoRI fragment of the plasmid HSFL YCp50 (Wiedderecht *et al.*, 1988) (Figure 3.11).

Figure 3.11 demonstrates that *HSTF* mRNA is synthesised at a moderate level during rapid exponential growth, but is not detectable after cells have entered stationary phase. This result was expected due to lack of HSE directed heat inducibility of *lacZ* in stationary phase (Figure 3.2) and the downregulation of transcripton of most genes in stationary phase (see Section 1.2.2.1). Since HSTF is a protein essential for cell viability, the HSTF accumulated during cell growth must be required for cell viability in stationary phase.

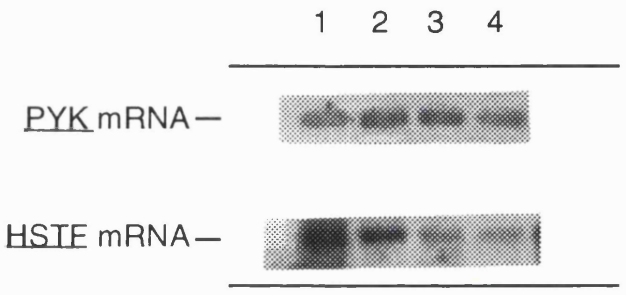


Figure 3.11. HSTF mRNA levels during growth of SUB62 on YNBG.
RNA samples were isolated at 1.- early exponential (OD550 = 0.5)
2.- late exponential (OD550 = 5), 3.- entry to stationary (OD550 = 7)
and 4. - three hours into stationary phase. PYK was used as a loading control.

3.3 Discussion

The potential use of HSEs in directing regulated heterologous gene expression in response to temperature upshift was investigated by studying HSE-*lacZ* induction from the plasmid pHSE2 under a variety of growth conditions and host backgrounds.

The results obtained can be summarised as follows;

1. Cells have to be grown at low temperatures - 20-23°C - to ensure low basal HSE activity and maximum levels of β-galactosidase induction (Figure 3.4).
2. Cells have to be subjected to a large temperature increase (23-39°C) to obtain maximal induction of HSE-*lacZ* activity at 39°C (Figure 3.3).
3. Induction levels are limited by the transience of the heat shock response (Figure 3.5).
4. HSE-*lacZ* induction in response to temperature upshift is only appreciable during exponential growth (Figure 3.2).
5. pHSE2 can direct temperature regulated expression of β-galactosidase in all *S. cerevisiae* host strains tested.

Each of these points is discussed in more detail below;

- 1. Basal expression from the HSE-*lacZ* construct was seen under all growth conditions, it could be minimized by growing cells at low temperatures 20-23°C. This basal expression is dependent on the number of HSE sequences present within the *CYC1* promoter (Sorger & Pelham, 1987) and increases with the number of HSEs present.presumably reflecting the basal activity level of HSE bound HSTF. This phenomena is the opposite of the results from the MATα2 regulated system (Sledziewski *et al.*, 1988) where increasing the number of MATα2 operators in the expression vector decreases basal expression without significantly altering induced expression levels. Cultures have therefore to be grown at a low temperature for maximum HSE inducibility, the resulting lower growth rate necessitating longer fermentation times.

- 2. The large temperature increase the cells are subjected to in order to obtain maximal HSE-*lacZ* activation may be detrimental to the cells physiology, although no evidence could be obtained for increased proteolysis at these higher temperatures.

- 3. The transience of the heat shock response and the basal expression cause maximal induction levels to be low; the maximal induction possible with this system was 40 fold. However, most other temperature regulated expression systems also

only show low induction ratios in the 30-70 fold range (Kramer *et al.*, 1984; Sledziewski *et al.*, 1988; Da Silva & Bailey, 1989) with the noteable exception of Brake *et al.*, (1984) using the *MFα1* promoter in combination with a *sir3-8^{ts}* strain to achieve 2x10^5-fold more h-EGF at 24°C than at 36°C. The different mutant host strains tested had little effect on induction ratios.

- 4. Maximal HSE-*lacZ* induction in response to temperature upshift can only be obtained with cells in exponential growth, therefore cells have to be induced before they have reached maximal cell density. This problem could be overcome to a certain extent by growing cells in fed-batch mode where they can reach a much higher cell density during exponential growth.

- 5. Since HSTF is an essential protein, the HSE regulated system can be used in all *S.cerevisiae* host strains, this is an advantage over all the other temperature regulated systems available which have to be used in defined mutant hosts.

- 6 The system has been shown to work with normal temperature regulation at high gene copy numbers. Studies have shown induced HSP90 levels can reach 30-40% total cell protein within 1 hour of a 39°C upshift (Cheng *et al.*, 1992). Many other expression systems (e.g. ADH2, GAL1), lose control of regulation at high promoter copy levels due to titration out of regulatory *trans*-acting factors (Johnston & Hopper, 1982: Schultz *et al.*, 1987).

In conclusion, in comparison to the temperature regulated systems discussed, the HSE system gives similiar induction ratios, the transience of the heat-shock response only allowing a short induction period. The two main drawbacks to the system are (1) the low growth rate obtained at the sub-optimal growth temperatures needed to minimize basal expression; and (2) maximal induction is only possible when cells are at a relatively low biomass in exponential growth. However, it may be useful in conjunction with high plasmid copy numbers, when a substantial amount of product can accumulate over a short induction period (Cheng *et al.*, 1992).

The data presented in this chapter have been published as two papers:-

1. Kirk, N. & Piper, P.W. "The Determinants of Heat-Shock Element-Directed *lacZ* Expression in *Saccharomyces cerevisiae* ", Yeast 7, 539-546

2. Kirk, N. & Piper, P.W. "Methanol as a convenient inducer of heat-shock element directed heterologous gene expression in yeast", Biotechnology Letters 13, 465-470

CHAPTER 4. ON THE REGULATION OF THE YEAST *UBI4* PROMOTER AND ITS SUITABILITY FOR THE CONDITIONAL EXPRESSION OF HETEROLOGOUS PROTEINS

4.1 Introduction

The yeast *UBI4* gene is reported to be transcriptionally regulated by nutrients via the cAMP pathway (Tanaka *et al.,* 1988; 1989) and by heat-shock (Finley *et al.,* 1987). Under conditions of high intracellular cAMP, expression of the *UBI4* gene is basal, however, under starvation conditions and in mutants with low levels of cAMP, *UBI4* is reported to be induced, presumably to provide ubiquitin to cells under these stressful conditions (Tanaka *et al.,* 1988). This conditional expression of the yeast *UBI4* gene under conditions of low cAMP indicated that it might be used for the regulated expression of toxic heterologous proteins in *S. cerevisiae.* In theory, the synthesis of a heterologous protein driven by the *UBI4* promoter would be subject to the same type of regulation that controls the native *UBI4* protein and hence would only be synthesised when cAMP levels fall as cells enter stationary phase due to nutrient limitation. This might be advantageous for the expression of proteins whose expression are deleterious to the cell in that their expression would only be induced when cells enter stationary phase - thus separating biomass formation from heterologous gene expression. Secondly, the induction of the *UBI4* gene in stationary phase occurs without any change being applied to the fermentation conditions, thus simplifying operations.

4.2 Results

4.2.1 Construction of vectors and strains to study *UBI4* regulation

4.2.1.1 PCR synthesis of the *UBI4* promoter

The promoter sequence of the *UBI4* gene was constructed by PCR directed synthesis. Oligonucleotides complementary to the 5' and 3' ends of the promoter (Ozkaynak *et al.*, 1987) were constructed by ICI Pharmaceuticals and used to synthesise the whole promoter and 5' non-coding region from -767 bp to 0 bp. The synthesised promoter was bounded by HindIII and SpeI restriction sites. The promoter was cloned into the yeast/*E. coli* shuttle vector YipLac204b (Geitz & Sugino, 1989)and 4 positive clones were sequenced by ICI Pharmaceuticals. The sequence of the clone picked for further studies is shown in Figure 4.1. It matches the sequence reported by Ozkaynak *et al.*, (1987).

McEntee sequence

ATACGCACGAATATGCCCGATTCTCTTTGGATCCCCTGGCCGAAGAAGACAGTGAAGATACGCCCGGCGT

GGCGGGTGGAATAAGCAAGCTCTCATTTAGACCCAAGTCTTCAAATAACCCGTTCAGATGATTTTAGAGT

UP1 UAS

CTTCCTTGACCAATCATCTTATTCGCGCAGGGCAACCCATGATAGGAAATGTCCCTTTAAACGAACTGTG

GGAAATCCTGCAAAAGTGATGAAAGTGACGTTTTATGAAAAGAAAGTGAAAAAGTTCTTGTTTGACAATA

CTT1 Seq. HSE

TTCTATTCCGAAAAGTCTTTGTGGTCAGTATTGTTTCTAGAACGTTCTAGAATAATCCTGGATAAACCAA

TTTCGGTACCAAAAAAAAAAATAGCCGCCCAGTGGAGCATCACACACCGTACATCCGGCCACACACGGTG

GTTACCCCACTTCGTTTCTTTTTTCAGGGGCGATGCCACTTATCAGTTGTTTTTAGAATTCAGTGTCATT

TGAGGGCGGTTCCTCCTTTTGGGGGTATATATAGAGAGGCTCCGGGTTTTGCCACCTTTGAATTCGCCTG

TATA box

CTTATCTTTCTTCTTCCGAAAGTGCTACTTCAGAAAGAGCAAGAACTGTACGGATAAGGATAAGTATATC

Putative RNA start site

TTCTATCTACTACAATACGACAAGAACTCTCGAACTCTCCCTCCCACTTTACTTTAACTAATAGATTATG

ubiquitin

Figure 4.1A. Sequence of the *UBI4* promoter with relevant regulatory sequences underlined.

McEntee sequence - Kobayashi & McEntee, (1993)

UP1 UAS - Guarente *et al.*, (1984)

CTT1 Sequence - Marchler *et al.*, (1993) EMBO J. 12, 1997-2003

HSE - Ozkaynak *et al.*, (1987)

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AGCTTAGAAAAGAAAGCAAAGCCTCCCACCACCAGCACTAGCTTAGATTTCCAGAGACAACGGACAAGGAATACGCACGA      80

ATATGCCCGATTCTCTTTGGATCCCCTGGCCGAAGAAGACAGTGAAGATACGCCCGGCGTGGCGGGTGGAATAAGCAAGC     160

TCTCATTTAGACCCAAGTCTTCAAATAACCCGTTCAGATGATTTTAGAGTCTTCCTTGACCAATCATCTTATTCGCGCAG     240

GGCAACCCATGATAGGAAATGTCCCTTTAAACGAACTGTGGGAAATCCTGCAAAAGTGATGAAAGTGACGTTTTATGAAA     320

AGAAAGTGAAAAAGTTCTTGTTTGACAATATTCTATTCCGAAAAGTCTTTGTGGTCAGTATTGTTTCTAGAACGTTCTAG     400

AATAATCCTGGATAAACCAATTTCGGTACCAAAAAAAAAAAATAGCCGCCCAGTGGAGCATCACACACCGTACATCCGGCC     480

ACACACGGTGGTTACCCCACTTCGTTTCTTTTTTCAGGGGCGATGCCACTTATCAGTTGTTTTTAGAATTCAGTGTCATT     560

TGAGGGCGGTTCCTCCTTTTGGGGGTATATATAGAGAGGCTCCGGGTTTTGCCACCTTTGAATTCGCCTGCTTATCTTTC     640

TTCTTCCGAAAGTGCTACTTCAGAAAGAGCAAGAACTGTACGGATAAGGATAAGTATATCTTCTATCTACTACAATACGA     720

CAAGAACTCTCGAACTCTCCCTCCCACTTTACTTTAACTAATAGATT ATG CAG ATT TTC GTC AAG ACT TTG      791
                                                Met Gln Ile Phe Val Lys Thr Leu        8
```

Figure 4.1B Sequenced UBI4 Promoter as reported in Ozkaynak *et al.*, (1987)

4.2.1.2 Construction of the *UBI4-lacZ* expression vector pDP501

The clone illustrated in Figure 4.1 was digested with HindIII and SpeI to remove the sequenced *UBI4* promoter and the fragment was then cloned into pDP394 to create pDP398 (Section 2.4), a plasmid with a *UBI4* promoter-*lacZ* gene fusion. The expression cassette containing the *UBI4* promoter, *lacZ* gene and *ADHt* from pDP398 was excised by digestion with HindIII and SalI and inserted into YipLac211 (Geitz & Sugino, 1989) to create pDP501 as illustrated in Figure 4.2. pDP501 is an integrating yeast expression vector that, when linearized with the restriction enzyme NcoI which cuts in the *URA3* region of the vector (illustrated in Figure 4.3) can transform *ura3*$^{-}$ *S. cerevisiae* to *URA3*$^{+}$ by integration into the homologous *URA3* region of the hosts chromosome. The process of integration makes the transformed strain very stable even in the absence of a selection process, allowing the transformed cells to be grown on a variety of complex substrates without loss of the insert.

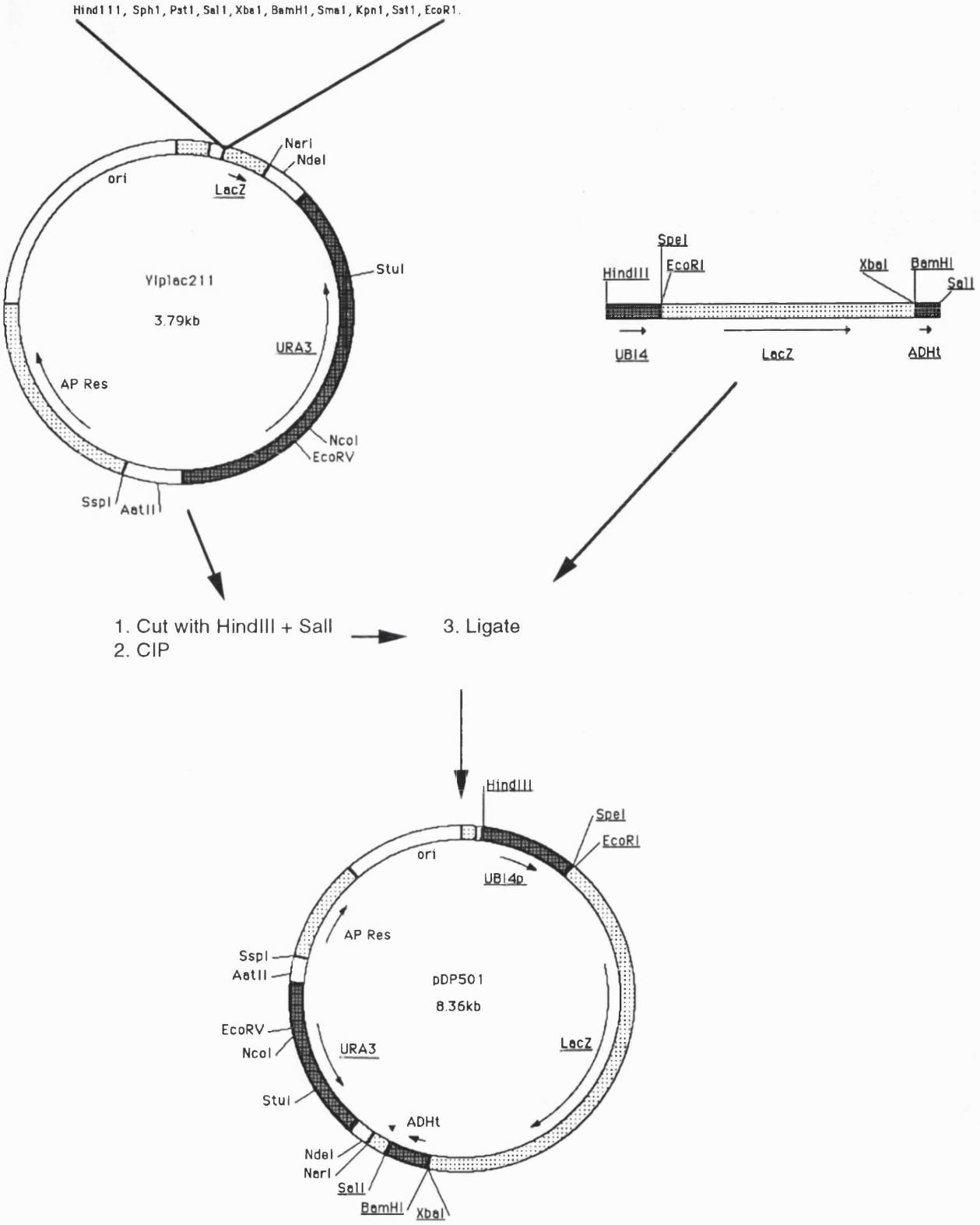


Figure 4.2. Construction of expression vector pDP501

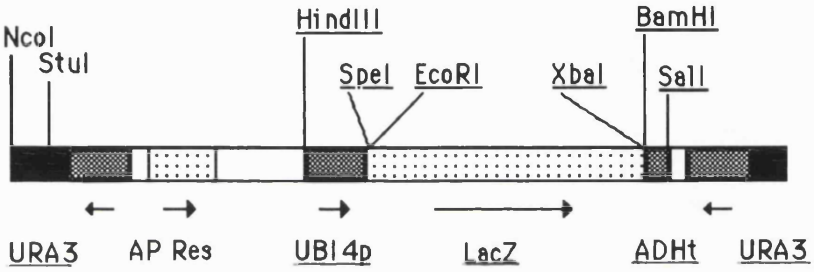


Figure 4.3. pDP501 vector linearised with NcoI

4.2.1.3 Construction of expression host PMY1.1

The expression host PMY1 (*his4 leu2*) was chosen for this study since it had been previously characterised by ICI Pharmaceuticals as a strain able to grow to high cell density in fermenters and as one able to secrete heterologous products. Since PMY1 is *URA3*$^{+}$, transformants using the *URA3* based plasmid pDP501 could not be selected. PMY1 was therefore made *ura3*$^{-}$ by 5-fluoro-orotic acid technique (Boeke *et al.*, 1984). Uracil auxotrophs selected for by this method were plated for growth on selective plates containing histidine and leucine plus and minus uracil. A positive clone (PMY1.1) that could only grow on selective media plus histidine, leucine and uracil was selected and its growth characteristics compared to the parent strain PMY1. As illustrated in Table 4.1, PMY1.1 had the same growth rate as its parent PMY1 in both YNBG and YEPD and obtained the same final cell densities indicating that the selection procedure had not affected the tested growth characteristics of the strain.

STRAIN	μMAX (YNBG)	μMAX (YEPD)	OD550 (YNBG)	OD550 (YEPD)
PMY1	0.23 h^{-1}	0.36 h^{-1}	9.3	65.2
PMY1.1	0.23 h^{-1}	0.36 h^{-1}	9.1	67.3

Table 4.1 Growth characteristics of PMY1 and PMY1.1

The strain PMY1.1 was then transformed with NcoI cut pDP501 and transformants selected on YNBG plates plus histidine and leucine. The transformant selected for detailed study was named PMY/501.

4.2.2 Analysis of the response of the *UBI4-lacZ* gene to different chemical stress agents

4.2.2.1 Induction in response to the DNA damaging agent 4-nitroquinoline-1-oxide (4-NQO).

The *UBI4* gene has been shown to be induced by the action of DNA damaging agents (Treger *et al.*, 1988). On exposure of *S. cerevisiae* to the DNA damaging agents 4-NQO and N-methyl-N'-nitro-N-nitrosoguanadine for 30-60 minutes, *UBI4* mRNA was reported to be induced strongly. This induction was dependent on the growth stage of the culture prior to addition of the inducing agent. At high cell densities induction of *UBI4* mRNA was not as pronounced as at low cell densities since levels of the mRNA were already high. In order to check that the *UBI4-lacZ* gene was regulated in the same manner as the homologous *UBI4* gene, the experiment of Treger *et al.*, (1988) was repeated using strain PMY/501. The results indicated that the pDP501 construct was regulated in the same manner as the homologous *UBI4* gene (Figure 4.4)

The blot was probed with radiolabelled *lacZ* and *UBI4* probes. The resultant autoradiograph, Figure 4.4, indicates that the *lacZ* gene is induced by both heat-shock and 4-NQO in the same manner as the homologous *UBI4* gene. The smearing associated with the *lacZ* probed track was found with both probes used (3.5kb EcoRI-XbaI fragment and 236 bp DdeI fragment) and in all Northern blots probed.

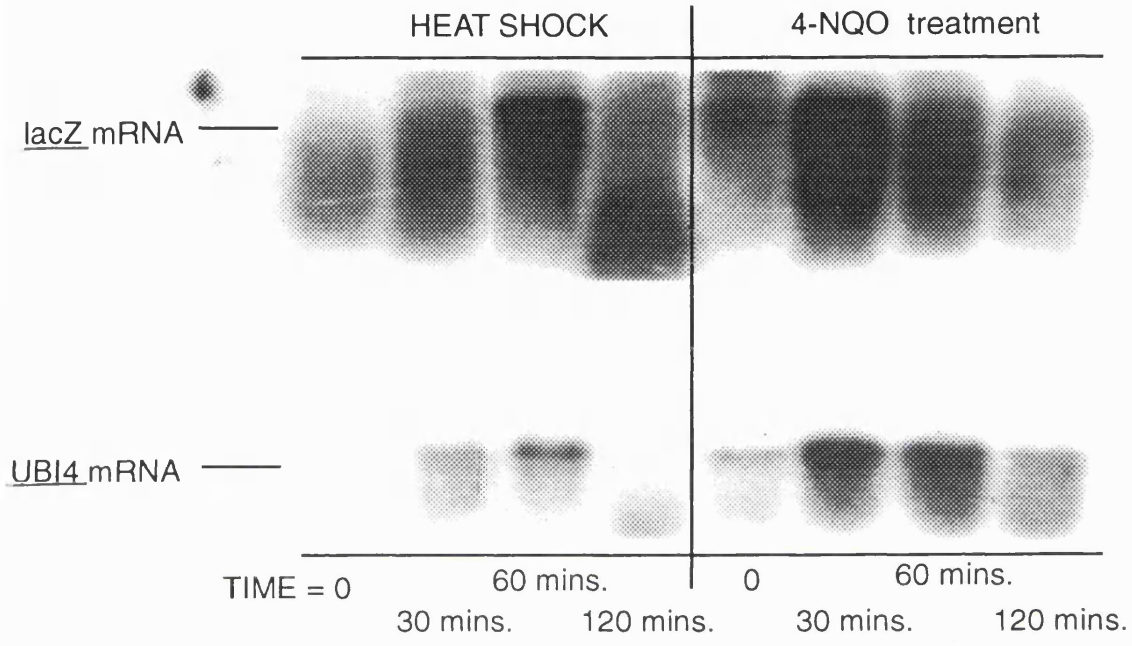


Figure 4.4. Induction of lacZ and UBI4 mRNA by heat-shock and 4-NQO treatment. PMY/501 was grown at 23°C to mid-log (OD 550 = 0.5) in YEPD and the culture split in two. One half of the culture was subjected to a 39°C heat-shock. To the second half of the culture, 1ug/ml of 4-NQO was added. RNA was extracted at time zero and after 30 minutes, 60 minutes and 2 hours.

4.2.2.2 Is the *UBI4* promoter induced by peroxide stress?

Reactive oxygen species (free radicals) such as hydrogen peroxide - H_2O_2 and the superoxide anion O_2^- are known to cause damage to many cell components, causing lipid peroxidation, DNA lesions and protein oxidation (Imlay *et al.*, 1988; Dean & Simpson, 1989). In mammals, such free radicals are implicated in the ageing process, particularly as the cause of arthritis (Schrenk *et al.*, 1991). The cell naturally produces enzymes that scavenge hydrogen peroxide, free radicals (SOD and catalase) and vitamins C and E, uric acid and glutathione act as non enzymatic protectors (for a review of the role of free radicals in disease see Matson-Jack *et al.*, 1992).

In order to study the relationship between the oxidative stress response and the heat-shock response, the response of the strain PMY/501 to hydrogen peroxide was examined. PMY/501 was grown to mid log in non selective medium and then split in two. To one culture, 0.2mM of H_2O_2 was added and samples taken every 30 minutes for two hours. The second culture was used as a control. As can be seen from Figure 4.5, H_2O_2 treatment does not induce *UBI4* mRNA. In fact, after 30 minutes treatment with H_2O_2 the *UBI4* mRNA levels start to decrease, even though the treated cells are still dividing at the same rate as the control culture.

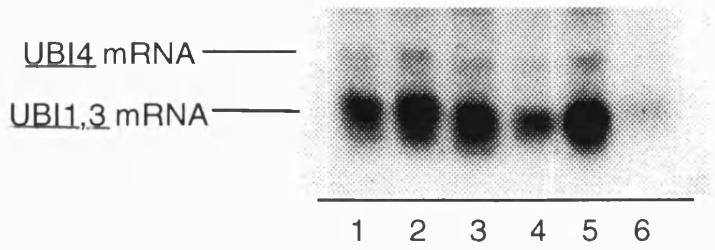


Figure 4.5. Northern blot of yeast strain PMY/501, grown to mid-log (OD 550 = 1) in YEPD. The culture was split into two and one half was made 0.2mM with hydrogen peroxide. The other half of the culture was used as a control. Lanes 1, 3 and 5; control culture at time = 0, 30 minutes and 90 minutes respectively. Lanes 2,4 and 6; test culture at time = 0, 30 minutes and 90 minutes respectively.

4.2.2.5 Comparison of HSE-lacZ induction by different stress agents

Table 4.2 illustrates β-galactosidase accumulation directed by the *UBI4-lacZ* construct in response to exposure with the different stress agents tested. It is evident that heat shock and 4-NQO produce the greatest induction of the promoter, although the increase in β-galactosidase levels after exposure to these stress agents does not match the levels of induced mRNA (discussed in more detail in Section 4.2.4.1). It is evident that of all the tested stress agents, 4-NQO and HS treatment induce HSE-lacZ synthesis by the greatest magnitude.

Stress Agent	**β-galactosidase SA** (Units as in Section 2.3.2.2)
Mid-log growth on YEPD	7.3
4-NQO (Section 4.2.2.1)	31.1
Heat-shock (Section 4.2.2.1)	22.4
H_2O_2 (Section 4.2.2.2)	8.1

Table 4.2. β-galactosidase accumulation directed by the *UBI4-lacZ* construct after exposure of cells to stress agents.

4.2.3 Regulation of the *UBI4* promoter during growth on different carbon sources

4.2.3.1 Analysis of *UBI4-lacZ* regulation during growth on complex and defined media.

To characterise the regulation of the *UBI4-lacZ* construct, accumulation of β-galactosidase driven by the *UBI4* promoter was analysed during batch growth curves of the transformant PMY/501 on YNBG and YEPD media. Throughout growth, samples were taken and assayed for β-galactosidase activity by the permeabilised cell assay as outlined in Section 2.3.2.2. The PMY/501 growth curve on YNBG is illustrated in Figure 4.6. Exponential cell growth is mirrored by an increase in β-galactosidase specific activity until the cells enter stationary phase due to glucose exhaustion. The rate of increase in β-galactosidase activity starts declining, becoming negative 4-8 hours into stationary phase, presumably due to the proteolytic degradation of β-galactosidase by proteases induced in stationary phase. No major induction of β-galactosidase specific activity could be detected as cells entered stationary phase. No second phase of growth due to respiration of ethanol was detected in the YNBG culture presumably reflecting a limitation in nutrients essential for respiratory growth.
The results of batch fermentation using YEPD as the growth medium are illustrated in Figure 4.7. The growth pattern of PMY/501 on YEPD showed the classic two phases of growth; initial fermentative growth on glucose followed by diauxic shift when respiratory enzymes are derepressed as a consequence of glucose exhaustion, followed by a period of respiratory growth on the ethanol produced during fermentation of glucose. The kinetics of β-galactosidase accumulation during growth on YEPD are initially similar to those on YNBG with β-galactosidase specific activity increasing with cell density at a constant rate. However, after diauxic shift the rate of β-galactosidase specific activity increase is greater than during the initial fermentative growth. When these results are displayed as specific activity versus OD550 as in Figure 4.8, it can be seen that β-galactosidase accumulates in greater amounts during fermentative growth on YNBG as compared to YEPD, possibly indicating a nutrient limitation in YNBG.
The conclusion made from this preliminary data was that the *UBI4* promoter is not as tightly regulated as was indicated by the literature and that its induction in stationary phase may be due to glucose derepression rather than a consequence of low cAMP.

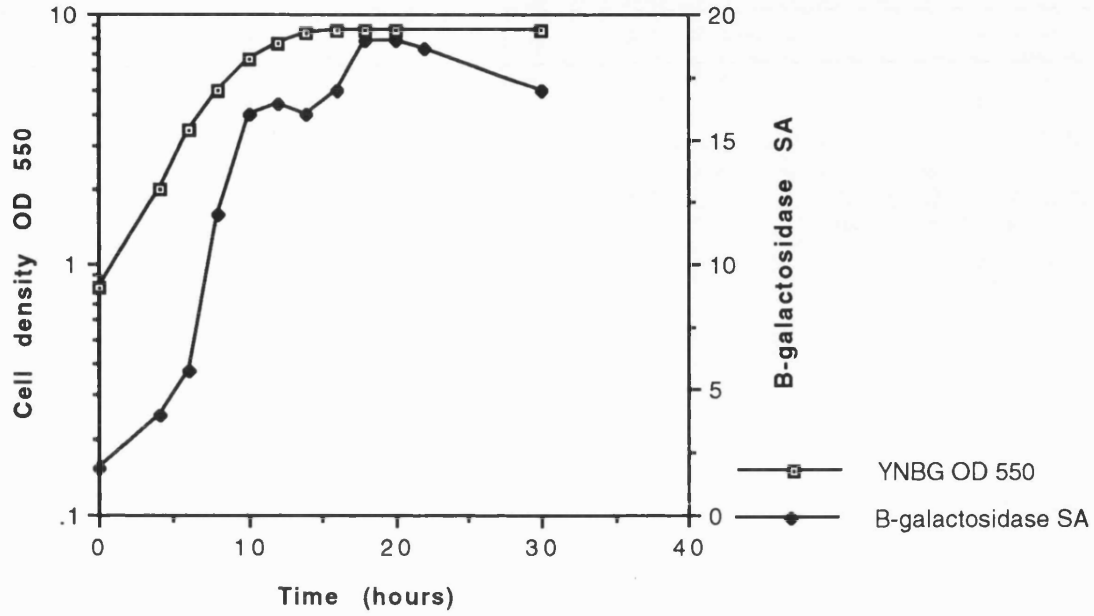


Figure 4.6. Growth characteristics and β-galactosidase accumulation in PMY/501 grown on YNBG with vigorous shaking at 28°C.

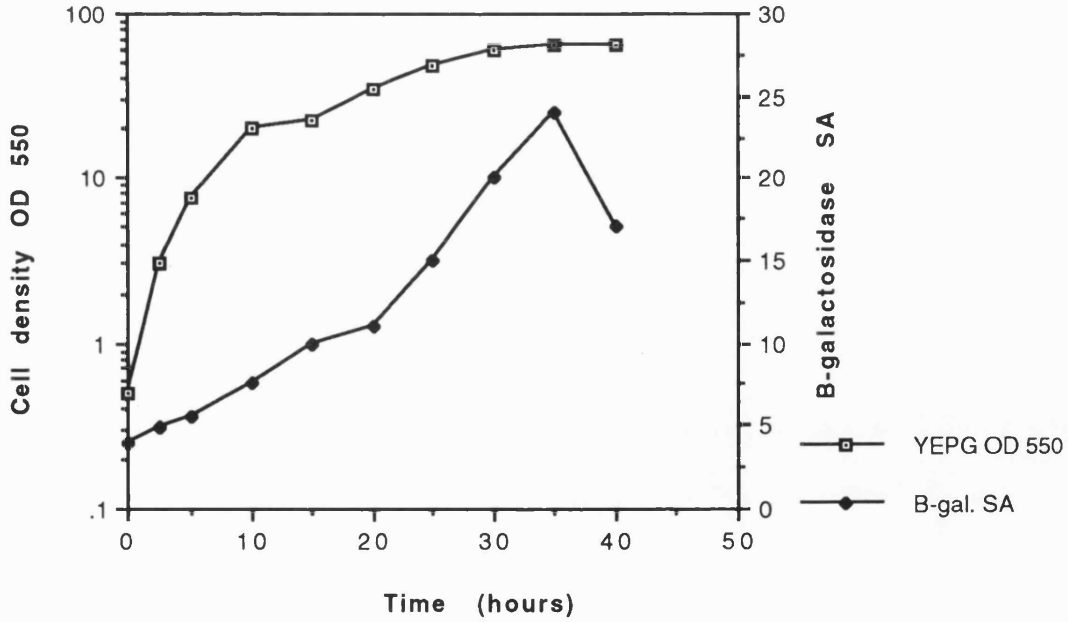


Figure 4.7. Growth characteristics and β-galactosidase accumulation in the PMY/501 transformant grown on YEPD with vigorous shaking at 28°C.

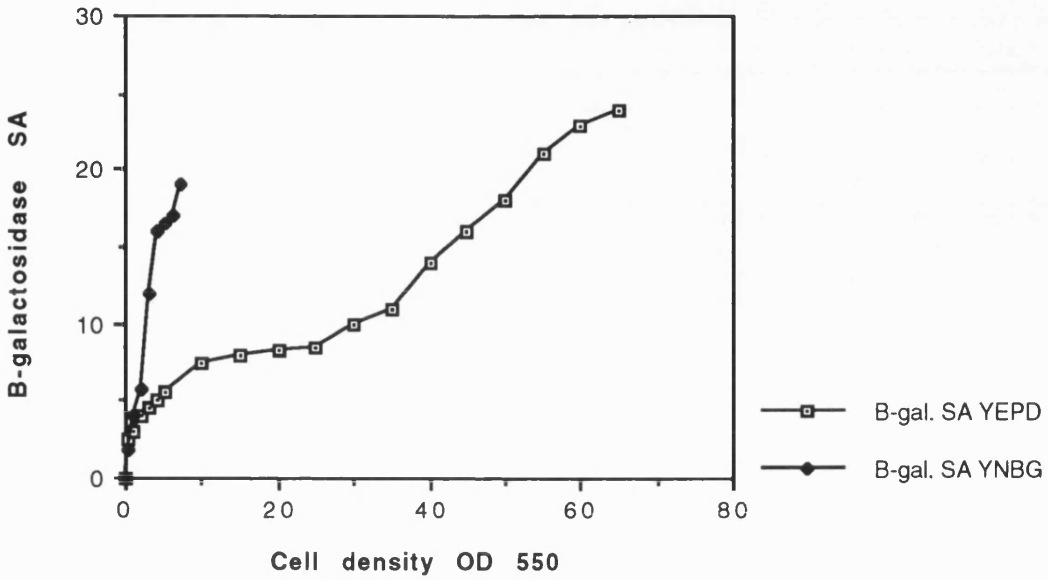


Figure 4.8. Rate of β-galactosidase accumulation in strain PMY/501 grown in YNBG and YEPD.

4.2.3.2 *UBI4-lacZ* expression on repressing and non-repressing carbon sources.

To study the possible influence of glucose repression on the activity of the *UBI4* promoter, β-galactosidase accumulation was monitored during growth of PMY/501 on YEPD (glucose), YEPR (raffinose) and YEPA (acetate). Glucose is a purely repressive carbon source that supports largely fermentative growth at the concentration used for batch experiments (2%), raffinose is a non-repressing carbon source which *S. cerevisiae* will ferment, while acetate is a purely respiratory carbon source.

Growth curves on these media are illustrated in Figure 4.10. They illustrate typical diauxic growth on YEPD, monauxic growth on YEPA where only respiration occurs and no evidence of a diauxic shift during growth on raffinose due to the fact that respiratory enzymes are not repressed on this carbon source. The rate of increase of β-galactosidase specific activity during growth on these three carbon sources is illustrated in Figure 4.11. On acetate, β-galactosidase specific activity increases at a constant rate until the growth arrest occurs, on raffinose the increase in β-galactosidase specific activity is approximately constant until stationary phase, however on glucose the rate of increase in β-galactosidase specific activity is similar, but increases after diauxic shift when cells are respiring as in Figure 4.8. Figure 4.11B illustrates that β-galactosidase accumulation is greater on non-repressing carbon sources than on repressing carbon sources. Figure 4.12 shows Northern analysis of *UBI4* mRNA accumulation on the same three different carbon sources. It is evident that *UBI4* mRNA accumulation when cells are grown on YEPD follows two stages. During initial fermentative growth, *UBI4* mRNA levels are basal. After diauxic shift, when glucose is fully depleted from the culture medium, an induction of *UBI4* mRNA is obvious. During growth on raffinose and acetate, *UBI4* mRNA levels are constant throughout the fermentation and no induction at the end of growth (on depletion of carbon source) is seen.

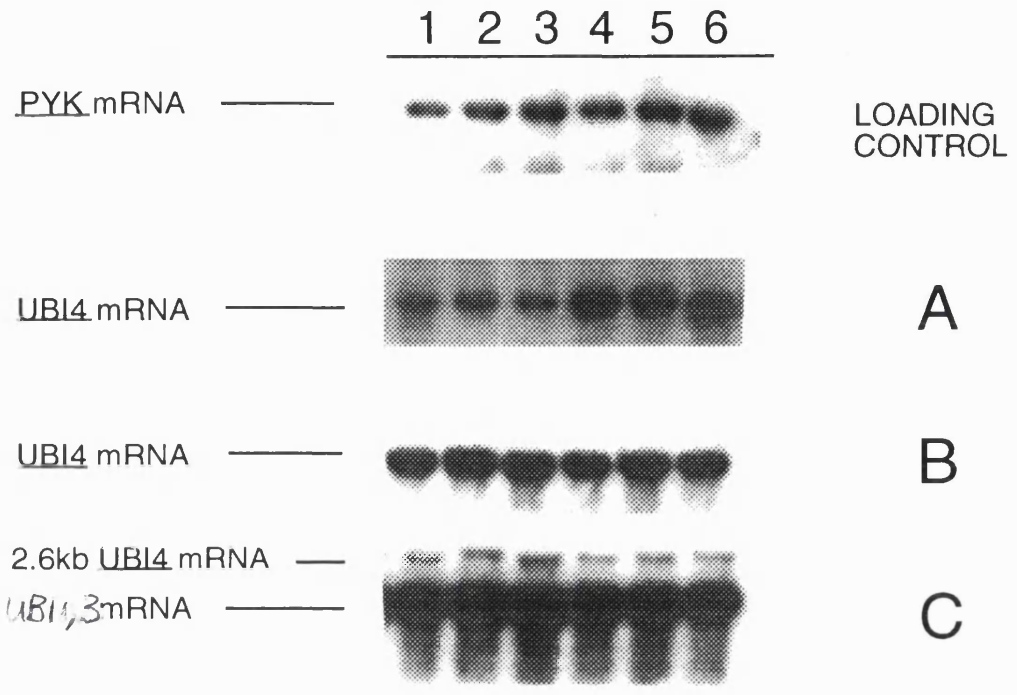


Figure 4.12. Growth curve mRNA probed with a radiolabelled UBI4 probe. Cells were grown at 28°C with vigorous agitation, 5 µg of RNA were blotted as in Section 2.3.5.4. A = YEPD RNA samples isolated at OD 550 values of 1 = 0.5, 2 = 2.0, 3 = 10.0, 4 = 22.0, 5 = 35.0 and 6 = 39.0. B = YEPR RNA samples isolated at OD 550's 1 = 0.5, 2 = 2.3, 3 = 12.0, 4 = 23.0, 5 = 36.0 and 6 = 37.0. C = YEPA RNA samples isolated at OD 550's 1 = 0.5, 2 = 2.0, 3 = 7.8, 4 = 15.0, 5 = 21.0 and 6 = 34.0. Loading control lane (duplicate samples from the YEPD growth curve) probed with a PYK probe.

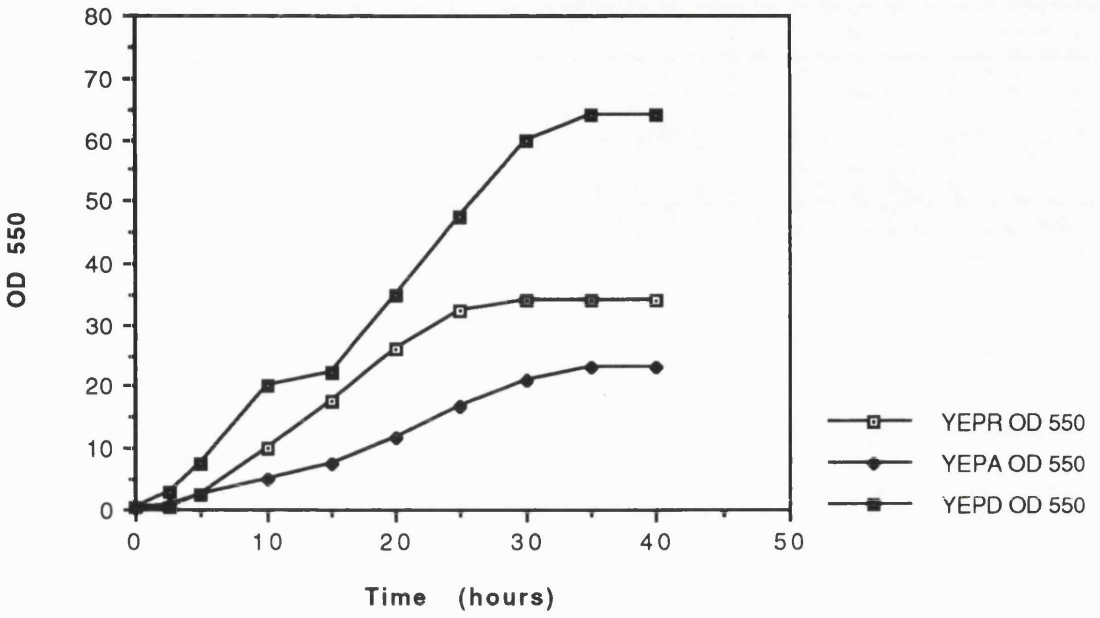


Figure 4.10. Growth curves of PMY/501 grown in YEP medium with glucose, raffinose and acetate as carbon sources.

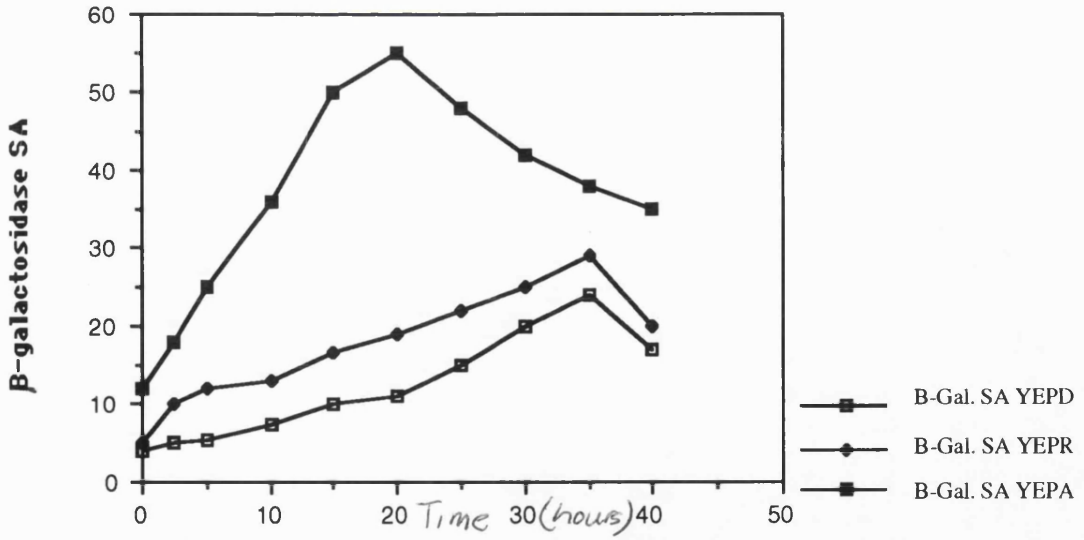


Figure 4.11A. Rate of β-galactosidase accumulation in PMY/501 grown in YEP medium with glucose, raffinose and acetate as carbon sources

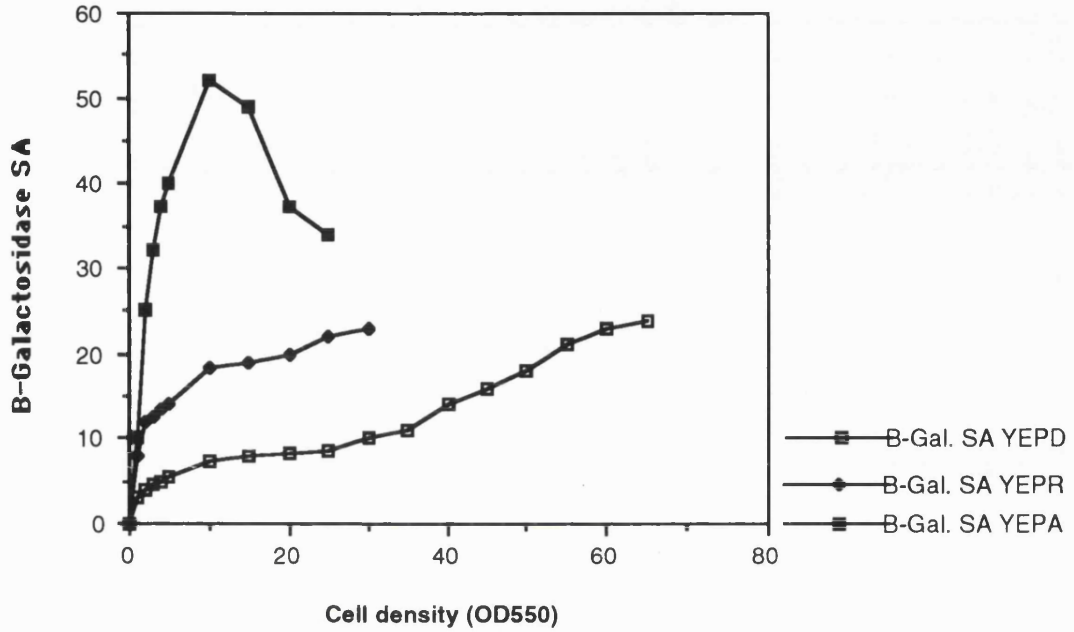


Figure 4.11B. β-galactosidase accumulation in PMY/501 grown in YEP medium with glucose, raffinose and acetate as carbon sources versus cell density (OD550)

4.2.3.3 Induction of the *UBI4* gene in response to glucose starvation.

The results gained so far have indicated that the *UBI4* gene may be regulated by carbon catabolite repression. In order to investigate this possibility further, an experiment was designed to investigate whether the *UBI4* transcript was induced by glucose starvation.

PMY/501 was grown to mid-log (OD_{550}=1) in 900mls YEPD medium. The cells were then divided into three equal portions and spun down. One third of the culture was resuspended in the YEPD, one third resuspended in YEP (-glucose) and one third resuspended in YNB (-glucose). Samples were removed and processed for mRNA analysis at time = 0, 30, 60 and 120 minutes after medium transfer. The mRNA's were run on a formaldehyde gel and probed with the *UBI4* probe. Control samples were probed with a radiolabelled PYK probe.

The results from this experiment, illustrated in Figure 4.13, indicate that the process of resuspension and transfer did not induce the *UBI4* gene (tracks 1-4) and that *UBI4* was not induced in the YEPD medium during the course of the experiment. Moderate induction of the *UBI4* message and shutdown of the *UBI1,3* message can be seen in tracks 5-8 (YEP - glucose). What is most apparent is the induction of the *UBI4* message in tracks 9-11 (YNB-glucose), this induction is stronger than on transfer to

YEP - glucose medium and may indicate that either the YEP minus glucose medium retains a small amount of fermentable carbon source that slightly represses induction of the *UBI4* message (yeast extract contains a small amount of glucose) or that the strong induction evident in the YNB-glucose medium is due to both: glucose derepression and induction due to nutritional limitation in the defined YNB medium.

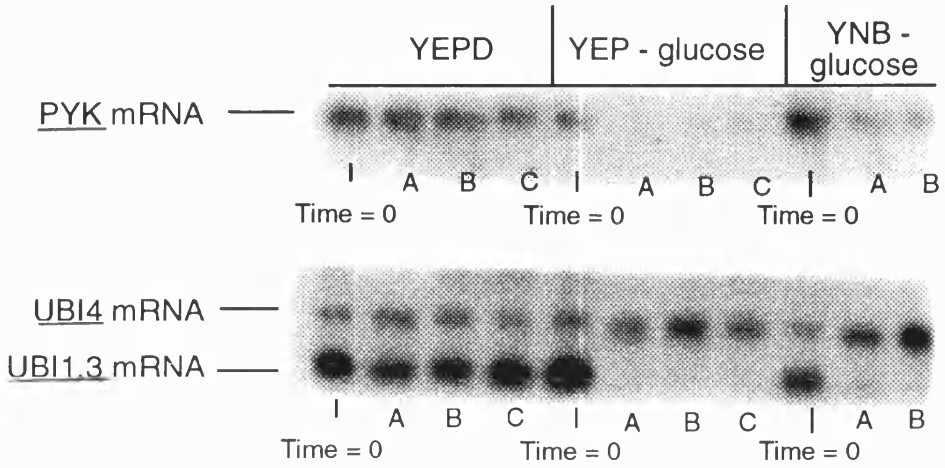


Figure 4.13. Induction of UBI4 mRNA in response to glucose starvation.
PMY/501 was grown to mid-log (OD 550 = 1) and split into three. The cells in each sample were pelleted, then resuspended in original medium (YEPD), YEPD - glucose and YNB - glucose. RNA samples were isolated at time = 0, then after A = 30 minutes, B = 60 minutes and C = 2 hours. Loading control used is PYK.
The PYK message only shows clearly in the YEPD sample, it's levels declining rapidly on glucose starvation.

4.2.3.4 On the possible control of *UBI4* promoter activity by growth rate

The results so far have indicated that glucose repression may affect the activity of the *UBI4* promoter, however, the increased accumulation of β-galactosidase on respiratory and non-repressing carbon sources could be interpreted as the effects of growth rate such that as growth rate declines the accumulation of β-galactosidase increases. To investigate this possibility, an experiment was designed that could demonstrate whether growth rate was involved; PMY/500 was grown on YNBG and YNBG + 1% casamino acids. This supplement of casamino acids will effectively increase the growth rate of the cells without changing the carbon source. Figure 4.14A illustrates the growth behaviour of PMY/501 on these media. What is most obvious is that the casamino acids supplement increases growth rate during initial fermentative growth from a μmax of 0.22 on YNBG to a μmax of 0.29 on YNBGC. It also allows the cells to respire the accumulated ethanol from the initial fermentative growth phase, enabling a subsequent respiratory growth phase during which cells grow to the densities achieved in YEPD.
If growth rate were a major controller of *UBI4* promoter activity, one would have expected to see a lower level of β-galactosidase accumulation during growth on YNGBC due to the higher growth rate, however the pattern of β-galactosidase accumulation during initial fermentative growth is practically identical in both media (Figure 4.14B), a result illustrating that growth rate has little effect on *UBI4* promoter activity.

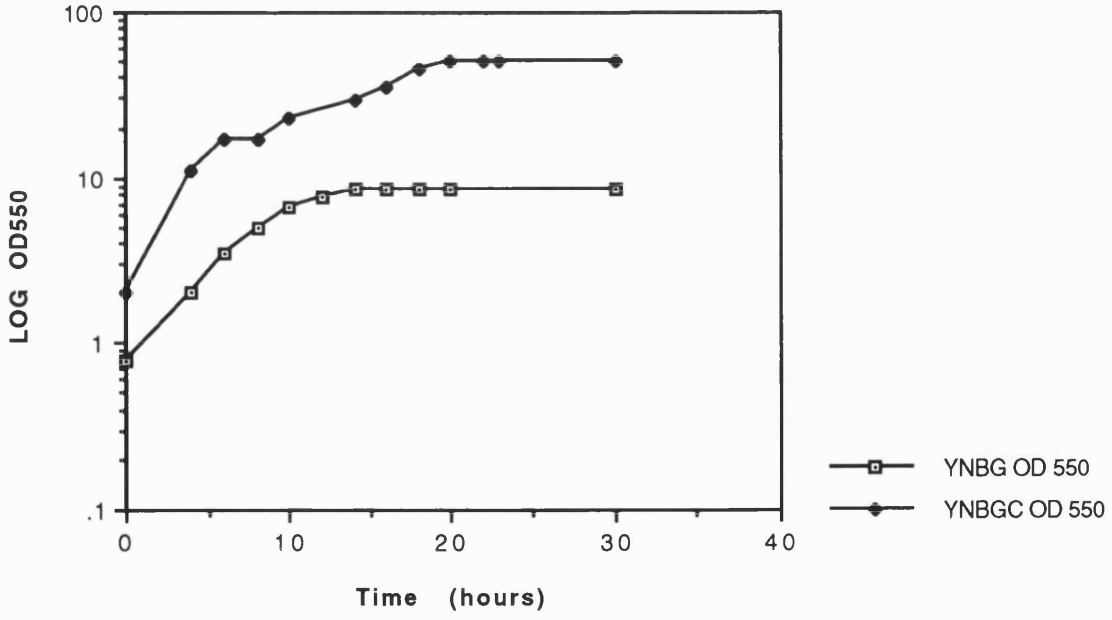


Figure 4.14A. Growth curves of PMY/501 grown in YNBG and YNBG plus 1% casamino acids (YNBGC).

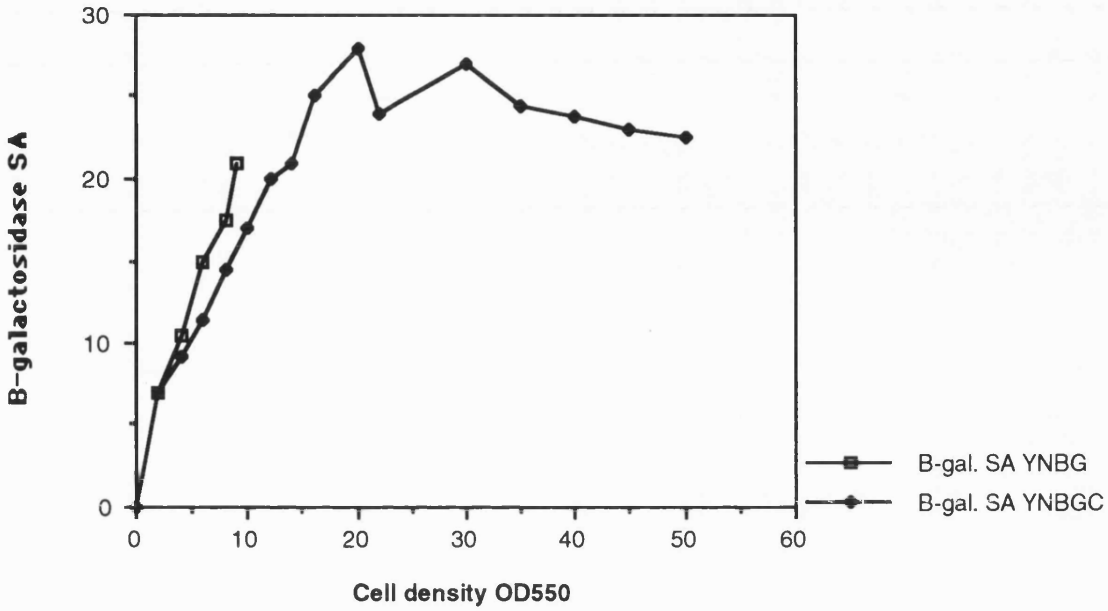


Figure 4.14B. Accumulation of β-galactosidase from the PMY/501 transformant grown in YNBG and YNBGC.

4.2.3.5 Control of the *UBI4* promoter by carbon catabolite repression probably operates through the action of the HAP regulatory proteins.

Through analysis of the nucleotide sequence of the *UBI4* promoter we identified a sequence at -673 to -666 (relative to the ATG) (underlined in Figure 4.1) with direct homology to the *CYC1* UAS2 motif.

Consensus HAP2/HAP3/HAP4 binding site	5' - TNA/GTTGGT - 3'
UBI4 match	-673 - TGATTGGT - 666

This motif is required for derepression of the *CYC1* gene on respiratory carbon sources (Guarente *et al.*, 1984). It is also found in a number of genes for mitochondrial components (Zaman *et al.*, 1992) causing their induction during catabolite repression when cells are forming functional mitochondria and becoming competent in respiration. In the case of the *CYC1* gene, the HAP2-4 proteins form a trimeric complex which binds to the *CYC1* UAS2 activating transcription about thirty-fold when cells are shifted from a fermentable to a non-fermentable carbon source (Guarente *et al.*, 1984). Mutations in HAP2, HAP3 or HAP4 are pleiotropic resulting in an inability of cells to grow on non-fermentable carbon sources.
So far, this UAS element has only been found in yeast genes involved in respiration (Gancedo, 1992; Zaman *et al.*, 1992). The presence of such an element in the *UBI4* gene would therefore be a novel finding. To test the hypothesis that the *UBI4* promoter is controlled by the carbon catabolite repression pathway, regulation of the *UBI4* gene was studied in the well characterised hap mutant strains:

BWG1-7A a *ade1 his4 leu2 ura3*
W32 a *ade1 his4 leu2 ura3 hap1-1*
W1 a *ade1 his4 leu2 ura3 hap 2-1*

Strains BWG1-7A, W32 and W1were grown in YEPD at 28^0C with vigorous aeration. Samples were taken at mid-exponential and stationary phase of growth for RNA analysis. Figure 4.15 demonstrates that *UBI4* mRNA levels during exponential growth are not affected by the *hap* mutation. As cells enter stationary phase due to glucose depletion the induction of *UBI4* mRNA illustrated in the isogenic parent strain BWG1-7A is not detectable in either the *hap1* or *hap2* mutants. It is therefore evident that the HAP gene product transcription factors are needed for induction of *UBI4* mRNA as cells undergo glucose derepression.

The absence of *UBI4* mRNA induction as the *hap1* strain W32 entered stationary phase is not expected since, the HAP1 transcription factor binds to the UAS1 sequence and is responsible for the basal expression of *CYC1* on glucose and transcriptional activation due to heme whose biosynthesis requires oxygen. This indicates that the *UBI4* gene may also be regulated by heme, the sensor of oxygen availability in yeast (Pillar & Bradshaw, 1991).

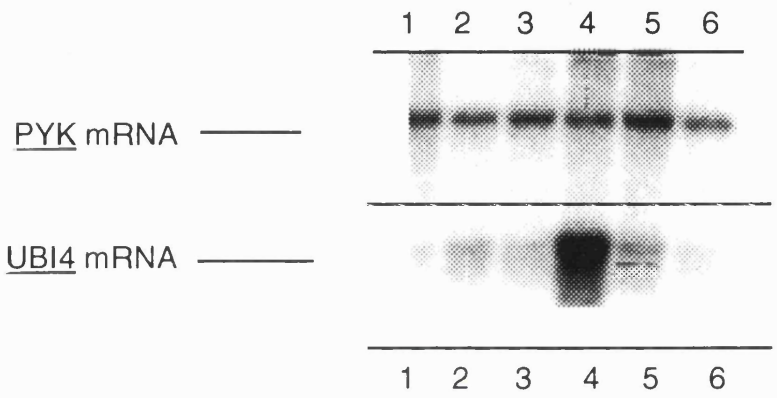


Figure 4.15. Northern blot of RNA samples from BWG1-7a (WT), W32 (hap1-1) and W1 (hap2-1) grown on YEPD. Exponential phase samples were harvested at an OD 550 = 1 and stationary phase samples harvested 2 hours after medium glucose levels reached zero. 1 = BWG1-7a exponential, 4 = BWG1-7a stationary. 2 = W32 exponential, 5 = W32 stationary. 3 = W1 exponential, 6 = W1 stationary. Loading control used was PYK.

4.2.4 On the regulation of the *UBI4* gene by the RAS/cAMP pathway

4.2.4.1 Induction of *UBI4-lacZ* activity in response to nutrient starvation

Induction of stress related protein synthesis in response to nutrient starvation has been explored in detail with the catalase T gene (Bissinger *et al.*, 1989). Starvation of exponentially growing cells for sulphur or glucose has been shown to cause a dramatic reduction in intracellular cAMP levels (Van der Platt, 1974, Shin *et al.*, 1987). Starvation of cells for nitrogen or phosphate (in the presence of glucose) has been shown not to affect cAMP levels (Boy-Marcotte *et al.*, 1987; Francois *et al.*, 1988). The following experiments were designed in order to ascertain whether the *UBI4* promoter could be induced to a much greater extent after the depletion of a key nutrient in the cells growth media. Since preliminary results in Section 4.2.3 had suggested that induction of *UBI4-lacZ* and *UBI4* mRNA when cells entered stationary phase was more likely to be due to catabolite derepression rather than an effect of low protein kinase A activity, the regulation of the *UBI4-lacZ* construct was studied under conditions of nutrient starvation that reduced intracellular cAMP and starvation conditions that left cAMP levels unaffected.

The response of the *UBI4-lacZ* construct was analysed using SD medium as described in Section 2.3.1.1. PMY/501 cells were grown to mid-exponential phase (OD_{550} = 1) in YNBG then transferred to either SD + glucose (SD+G), SD-glucose (SD-G), SD-nitrogen (SD-N), SD-sulphur (SD-S) or SD-phosphate (SD-P). The resulting changes in β-galactosidase specific activity are illustrated in Table 4.3.

MEDIA	β-galactosidase S.A. at TIME = 0	β-galactosidase S.A. at 1 hour	β-galactosidase S.A. at 3 hours	β-galactosidase S.A. at 6 hours
SD + G	4	12	15	17
SD - G	4	20	23	23
SD - N	4	30	33	33
SD - S	4	27	32	32
SD - P	4	21	21	25

Table 4.3. Induction of β-galactosidase in PMY/501 in response to different nutrient starvation regimes.

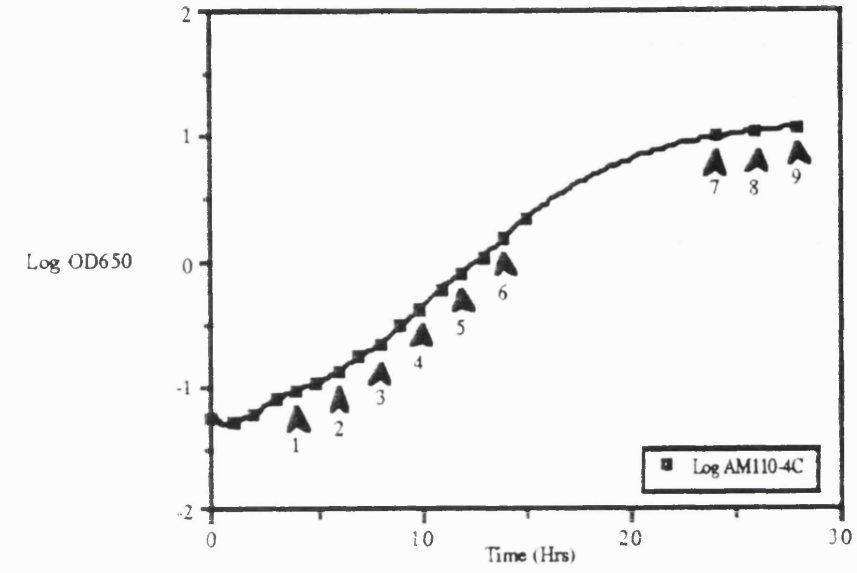
The results show that all tested starvation conditions caused β-galactosidase specific activity to rise approximately 1.5-2 fold with respect to the control culture, indicating moderate induction of the promoter by all these treatments. Corresponding mRNA samples when probed with a labelled *UBI4* probe showed similar pattern of induction of *UBI4* mRNA under these conditions, although the levels of mRNA induction were greater than corresponding β-galactosidase induction in Table 4.2, perhaps indicating constraints in *lacZ* mRNA translation (data not shown). Similar discrepancies have been found between *lacZ* mRNA levels and β-galactosidase levels by groups studying the *CTT1* promoter (Bissinger *et al.*, 1989) and the CYC7 promoter (Pillar & Bradshaw, 1991).

4.2.4.2 Regulation of the *UBI4* gene in cAMP mutants.

The results in Section 4.2.3 have indicated that the *UBI4* promoter is not primarily under the control of the RAS/cAMP pathway. To confirm this, *UBI4* gene regulation was studied in mutants of the RAS/cAMP pathway using the strains AM-180-2B and AM-410-4C. AM-180-2B has a *bcy1* mutation. This is in the regulatory subunit (BCY) of the protein kinase A complex and causes cAMP-independent, constitutive activation of protein kinase A, mimicking high cAMP levels (Matsumoto *et al.*, 1985). AM-410-4C contains a *cyr1-2* mutation which attenuates adenylase cyclase activity, causing cells to have minimal cAMP levels and hence low protein kinase A activity.

Blotted RNA samples from YEPD growth curves of these mutants were obtained from Richard Hather at the Leicester Biocenter. Corresponding growth curves are illustrated in Figure 4.16. The RNA blots of both strains illustrate the pattern of *UBI4* mRNA accumulation associated with wild-type strains; mRNA levels are basal during initial fermentative growth on glucose and then increase after diauxic shift when glucose has been fully utilised. If the *UBI4* promoter was under negative regulation by protein kinase A, one would have expected basal low levels of *UBI4* mRNA throughout all stages of growth for the AM-180-2B (*bcy1*) mutant, while the cyr1-2 mutant would have shown high induced levels of *UBI4* mRNA irrespective of growth stage. Figure 4.17 illustrates that HSP26, a heat shock protein also normally induced in stationary phase (Petko & Lindquist, 1986; Praekelt & Meacock, 1990) is constitutively expressed during fermentative growth of the *cyr1-2* strain and therefore subject to negative regulation by protein kinase A, although it is further induced in stationary phase in both *cyr1-2* and *bcy1* mutants.

Figure 4.17 illustrates that the primary regulation of the *UBI4* promoter is not by protein kinase A, the results from the nutrient starvation experiments may indicate a role for a cAMP independent nutrient signalling pathway (Bissinger *et al.*, 1989) and that the induction of *UBI4* promoter activity in stationary phase is due primarily to release from carbon catabolite repression.



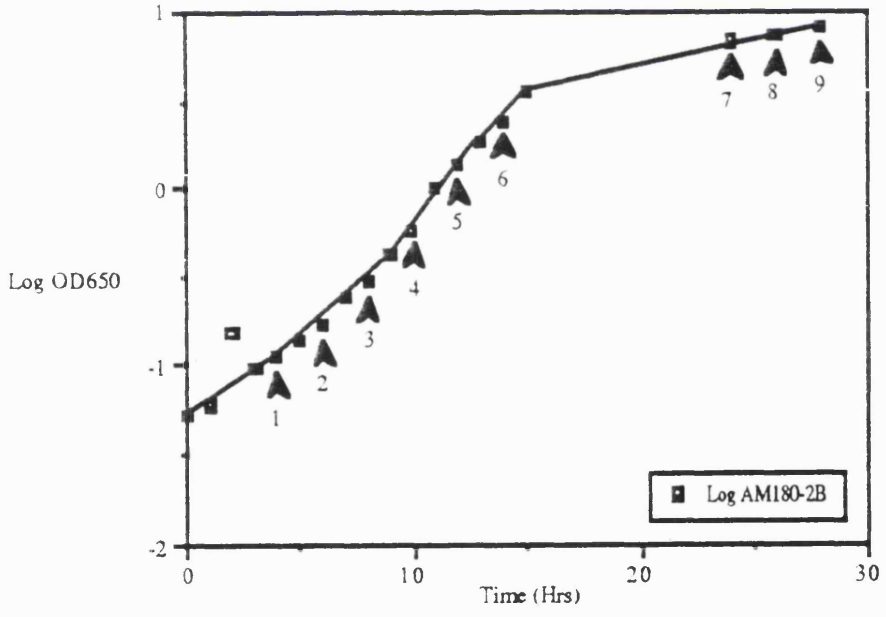


Figure 4.16. Growth curves of AM180-2B (*bcy1*, *his7*) and AM110-4C (*cyr1-2*, *leu1*) grown on YEPD. RNA samples isolated at the indicated times were used for Northern blotting, results illustrated in Figure 4.17.

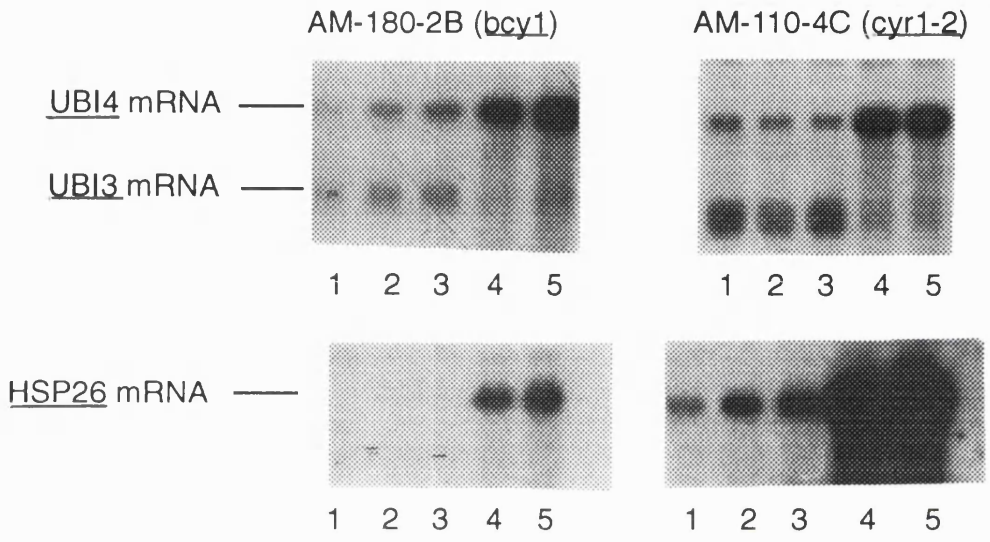


Figure 4.17. Regulation of UBI4 and HSP26 in mutants of the RAS/cAMP pathway. Strains AM110-4C (cyr 1-2) and AM180-2B (bcy1, his7) were grown in YEPD at 28°C with vigorous shaking and samples isolated at mid-log (samples 1-3, Figure 4.16) and after glucose depletion (samples 4 &5, Figure 4.16).

4.2.4.3 Regulation of the *UBI4* gene in *tpk* mutant strains in which very low protein kinase A activity causes a deficiency in carbon catabolite repression.

Experiments carried out by Mboyni *et al.*, (1990) have indicated that mutations in the TPK genes affect carbon catabolite repression in *S. cerevisiae*. Their results show that strains with a single, active but attenuated subunit, designated $tpk1^{w1}$ or $tpk2^{w1}$ mutations (but not $tpk3^{w1}$ mutations) reduce protein kinase A levels to such a low level as to cause deficiency in carbon catabolite repression, illustrated by their ability to form mitochondria under conditions where normally repressing amounts of glucose are present in the growth medium. tpk^{w1} Strains were acquired that additionally contained the *bcy1* mutation, so that their limited protein kinase A activity was cAMP-independent and constitutive. These strains were chosen to try to prove that carbon catabolite repression was more dominant in regulating the *UBI4* gene than any effect of the RAS-cAMP pathway.

These strains were grown with vigorous agitation in YEPD medium to exponential phase and then RNA isolated and processed as in Section 2.3.5.4.

The northern blot from this experiment (Figure 4.18) indicates that in the WT and $tpk3^{w1}$ *bcy1* strains, *UBI4* mRNA levels are basal in exponential phase. The *bcy1* mutation alone, as shown in Figure 4.18, does not reduce *UBI4* mRNA levels as one would expect if *UBI4* was under negative regulation by protein kinase A. However, the $tpk1^{w1}$ *bcy1* and $tpk2^{w1}$ *bcy1* strains, defective in glucose repression, however, show an increased level of *UBI4* message during exponential growth as compared to the WT, consistent with the theory that *UBI4* is predominantly under the control of carbon catabolite repression. The $tpk1^{w1}$ *bcy1* mutation also had the effect of repressing the *UBI3* message. Glucose derepressed conditions have already been shown to shut off expression of the *UBI3* message (see Figure 4.13).

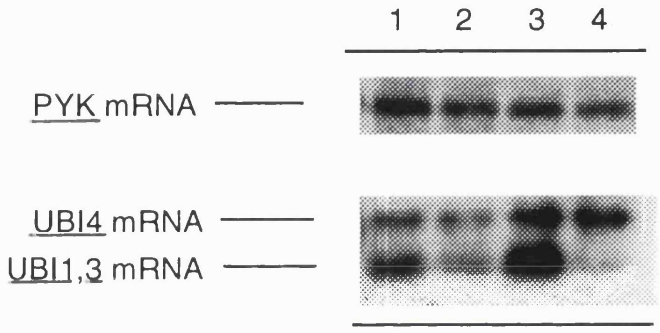


Figure 4.18. Northern blot of tpk mutant strains isolated in mid-log growth (OD 550 = 1) on YEPD. Lane 1 = SP1 (WT). Lane 2 = RTF3.1-1 (tpk3, bcy1). Lane 3 = Rs13-7c-1 (tpk2, bcy1). Lane 4 = RS13-58A-1 (tpk1, bcy1).

4.3 Discussion

4.3.1 On the suitability of the *UBI4* promoter for the regulated expression of heterologous proteins in *S. cerevisiae*

The relatively high basal level of *UBI4*-promoter activity during early exponential growth on YNBG and YNBGC media was not expected from the literature reports on the *UBI4* gene. The data from Finley *et al.*, (1987) show the *UBI4* transcript to be practically absent from log phase cells and greatly induced in stationary phase. Data from Tanaka *et al.*, (1988) showed accumulation of the *UBI4* transcript only after depletion of intracellular cAMP from a *cyr1-1/cyr1-1* mutant, these results indicating that the *UBI4* gene was primarily regulated by intracellular cAMP levels and that the promoter was induced in stationary phase by the fall in cAMP. Results with the *UBI4-lacZ* gene of PMY/501 showed high level accumulation of β-galactosidase directed by the *UBI4* promoter during all stages of growth (Figure 4.6-4.11)which demonstrated that the *UBI4* promoter was unsuitable for regulated expression of heterologous proteins in *S. cerevisiae* due to its lax regulation when compared to the well characterised promoters such as *GAL1-10* and *ADH2* (Section 1.1.3.2). The contradiction between the data presented here and the paper by Finley *et al.*, (1987) arise partly from the fact that the log samples of the Finley *et al.* study were from very early log growth in YEPD and the 'stationary phase' samples were actually samples from cells in diauxic shift that were unable to respire due to insufficient aeration. I suspect that if a more comprehensive northern blot study had been presented it would have resembled my data more closely.

4.3.2 Is the *UBI4* promoter controlled by the RAS/cAMP pathway?

The results from the northern blots of the two mutants AM180-2B and AM110-4C (Figure 4.17) would indicate that the *UBI4* promoter is not strongly affected by intracellular cAMP levels since *UBI4* mRNA accumulation in these mutants was more or less identical to that observed in wild type cells. A repeat of the *cyr1-1/cyr1-1* experiment done by Tanaka *et al.*, (1988) would also be needed to disprove the cAMP hypothesis. In their study, this *cyr1-1/cyr1-1* mutant (which requires exogenous cAMP for growth) was deprived of cAMP and *UBI4* induction observed. However, the resultant decrease in protein kinase A activity will also cause release from carbon catabolite repression (Mboyni *et al.*, 1990) so the interpretation Tanaka *et al.*, (1988) gave for the observed *UBI4* induction must be questioned. These results were further underscored by a poster presentation at the 16th International Conference on Yeast

Genetics from the lab of Varshavsky that also found no evidence for control of the *UBI4* gene by the RAS/cAMP pathway.

Results from the nutrient starvation experiments in Table 4.2 would tend to indicate that the promoter could be controlled by a cAMP independent nutrient signalling pathway (Cameron *et al.*, 1989) since these results seem to mimic, in part, the behaviour of the *CTTI* promoter (Bissinger *et al.*, 1989) which has been proved to be under nitrogen starvation control. To unequivocally establish any role of cAMP in the regulation of the *UBI4* promoter would necessitate a complete characterisation of a promoter deletion series .

4.3.3 Is the *UBI4* promoter controlled by carbon catabolite repression?

Most of the results from this chapter indicate that the *UBI4* gene is regulated by carbon catabolite repression. The main findings are listed below:

- Both the accumulation of β-galactosidase from a *UBI4-lacZ* fusion and *UBI4* mRNA levels are higher on respiratory as compared to fermentative carbon sources. This is not due to the differences in growth rate on these media.

- *UBI4* mRNA levels are low when cells are fermenting then increase when cells are derepressed and start to respire. This pattern of mRNA accumulation is identical to that of the *CYC1* promoter which is under carbon catabolite repression control.

- *UBI4* mRNA is induced on transfer of an exponential YEPD culture to medium minus glucose.

- HAP transcription factors are needed for induction of *UBI4* in stationary phase (Figure 4.15).

- Mutations (*tpk1*^{w1} and *tpk2*^{w1}) that make cells insensitive to carbon catabolite repression also increase *UBI4* mRNA levels on normally repressing carbon sources (Figure 4.18).

4.4 Conclusions

Results in this chapter indicate that growth phase dependent regulation of the *UBI4* gene is primarily by carbon catabolite repression. Regulation by a cAMP dependent signalling pathway has not been proven. There appears to be a degree of basal activity of the *UBI4* promoter that could prevent this being a tightly-regulated promoter suitable for heterologous protein production. The exact mechanism for this basal activity has not been clarified although it appears to operate through a cAMP independent mechanism.

4.5 Further studies to elucidate the regulation of the *UBI4* gene

- A complete promoter deletion series of the *UBI4* promoter will need to be created. With this, the elements responsible for regulation could be defined to specific DNA sequences, which could then be studied further by site-directed mutagenesis.

- The deletion series should be studied with respect to regulation by stress agents, carbon source, nitrogen and haem (oxygen).

This work was presented as a poster at the 16th International Conference on Yeast Genetics in Vienna.

CHAPTER 5. ON THE REGULATION OF THE *MFα1* PROMOTER IN *S. CEREVISIAE*

5.1 Introduction

The *MFα1* promoter was chosen as a control promoter for parallel experiments during the study of *UBI4* regulation due to the fact that it is widely used in heterologous gene expression studies. *MFα1* expression is transcriptionally regulated at two levels (see Section 1.3.3.2). First, it is only transcribed by MATα cells, and second, its transcription is induced by **a**-factor (Achstetter, 1989). The promoter of the *MFα1* gene contains two UAS sequences to which the α1 protein (an α cell specific protein) binds in conjunction with the general transcription factor, PRTF, which is present in all three cell types (Flessel *et al.*, 1989; Inkouchi *et al.*, 1987). The role of α1 is to assist the binding of PRTF to these UAS sequences in order to activate transcription.

The secretion of α-factor by α type cells causes start B arrest in **a** type cells and enables mating to occur to form the diploid **a**/α cell type. Start B, the pheromone arrest site in the cell cycle, differs from the nutrient limitation arrest site start A (G0 arrest) in that cell growth is not blocked. Progression past the pheromone arrest site requires activity of the CDC28 kinase, while progression over start site A requires a low basal level of protein kinase A. Addition of mating factor α to **a** cells does not affect levels of intracellular cAMP, hence causing arrest by a different signalling mechanism from the RAS/cAMP signalling pathway (Grandori *et al.*, 1990).

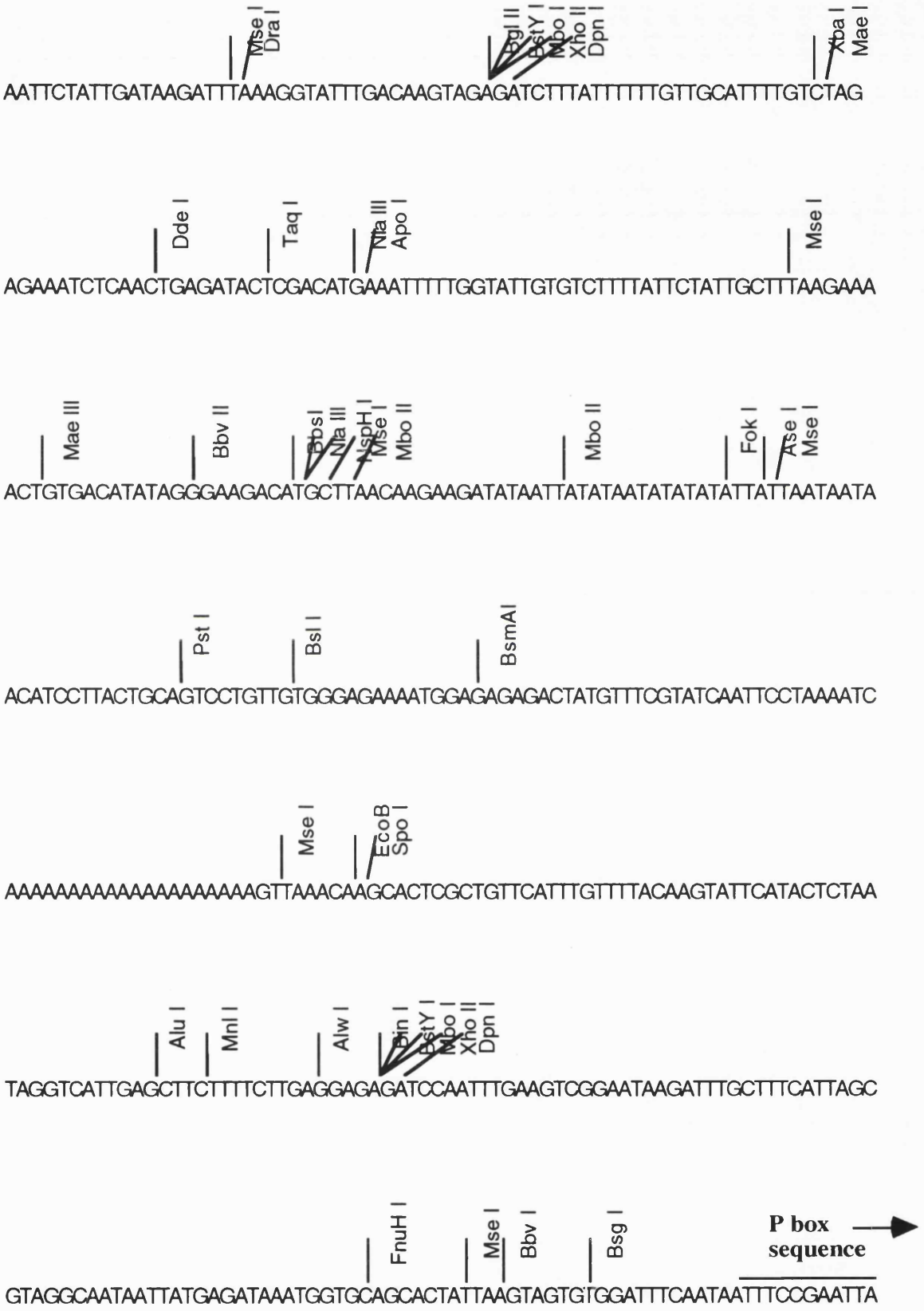
Several studies have given evidence that the *MFα1* promoter, as well as being regulated by cell type, is also in some way regulated by growth phase. Studies have shown that the promoter is more active during late logarithmic fermentative and respiratory growth rather than during early to mid-exponential growth (Ernst, 1986; Gu *et al.*, 1991; Park *et al.*, 1989). The promoter leader sequences of the pre-pro α-factor gene (*MFα1*) are often used to direct recombinant protein secretion from yeast. There are indications in the literature that secretion directed by the *MFα1* promoter among others, may be more efficient under certain conditions (for a general review see De Nobel & Barnett, (1991)). Studies by Ernst, (1989) show that the rate of secretion of somatomedin-C is maximal during logarithmic growth in batch fermentations. It is not clear, however, if this is due to more efficient transcription from the *MFα1* promoter or an increased capacity for secretion in general. Studies on the *MFα1* promoter have largely concentrated on its cell type regulation (for a general review see Sprague *et al.*, (1983)), and little is known of how its activity changes during cell growth.

Knowledge of the physiological conditions giving maximal *MFα1* promoter activity, also optimal activity of the secretory apparatus, would improve the productivity of yeast as a host for secreted products. We therefore constructed a strain with an integrated *MFα1-lacZ* gene so as to investigate the effects of different media and carbon sources on *MFα1* promoter activity. This reporter construct will cause the synthesis of β-galactosidase as an intracellular product, giving results that should be a direct reflection of promoter activity, unaffected by the efficiency of the secretory apparatus.

5.2 Results

5.2.1 Construction of an *MFα1-lacZ* expression vector

Construction of the *MFα1-lacZ* expression vector is outlined in Section 2.4. The sequence of the *MFα1* promoter used for this study is illustrated in Figure 5.1. The plasmid used to carry out the work in this chapter is illustrated in Figure 5.2. Integration of the NcoI cut pDP500 into the PMY1.1 genome provided the transformant designated PMY/500.


Mse I
Dra I
Bgl II
DrdY I
Nbo I
Xho II
Dpn I
Xba I
Mae I
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Dde I
Taq I
Nla III
Apo I
Mse I
AGAAATCTCAACTGAGATACTCGACATGAAATTTTTGGTATTGTGTCTTTTATTCTATTGCTTTAAGAAA
Mae III
Bbv II
Bbs I
Nla III
NspH I
Mse I
Mbo II
Mbo II
Fok I
Ase I
Mse I
ACTGTGACATATAGGGAAGACATGCTTAACAAGAAGATATAATTATATAATATATATATTATTAATAATA
Pst I
Bsl I
BsmAI
ACATCCTTACTGCAGTCCTGTTGTGGGAGAAAATGGAGAGAGACTATGTTTCGTATCAATTCCTAAAATC
Mse I
EcoB
Spo I
AAAAAAAAAAAAAAAAAAAAAGTTAAACAAGCACTCGCTGTTCATTTGTTTTACAAGTATTCATACTCTAA
Alu I
Mnl I
Alw I
Bin I
DrdY I
Nbo I
Xho II
Dpn I
TAGGTCATTGAGCTTCTTTTCTTGAGGAGAGATCCAATTTGAAGTCGGAATAAGATTTGCTTTCATTAGC
FnuH I
Mse I
Bbv I
Bsg I
P box
sequence
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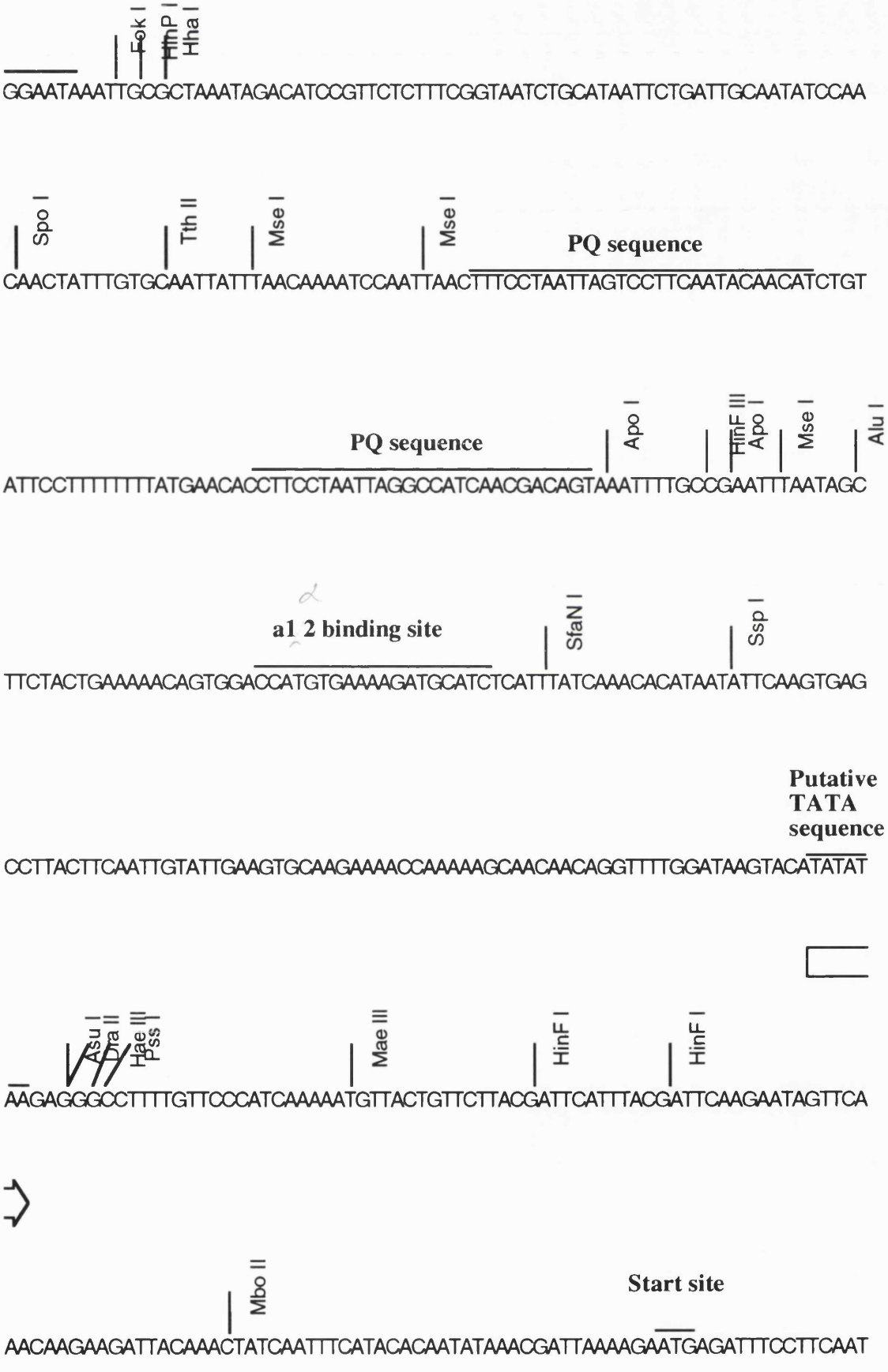
Fok I
Hha I
GGAATAAATTGCGCTAAATAGACATCCGTTCTCTTTCGGTAATCTGCATAATTCTGATTGCAATATCCAA
Spo I
Tth II
Mse I
Mse I
PQ sequence
CAACTATTTGTGCAATTATTTAACAAAATCCAATTAACTTTCCTAATTAGTCCTTCAATACAACATCTGT
PQ sequence
Apo I
HinF III
Apo I
Mse I
Alu I
ATTCCTTTTTTTTATGAACACCTTCCTAATTAGGCCATCAACGACAGTAAATTTTGCCGAATTTAATAGC
a1 2 binding site
SfaN I
Ssp I
TTCTACTGAAAAACAGTGGACCATGTGAAAAGATGCATCTCATTTATCAAACACATAATATTCAAGTGAG
Putative
TATA
sequence
CCTTACTTCAATTGTATTGAAGTGCAAGAAAACCAAAAAGCAACAACAGGTTTTGGATAAGTACATATAT
Asu I
Dra II
Hae III
Pss I
Mae III
HinF I
HinF I
AAGAGGGCCTTTTGTTCCCATCAAAAATGTTACTGTTCTTACGATTCATTTACGATTCAAGAATAGTTCA
Mbo II
Start site
AACAAGAAGATTACAAACTATCAATTTCATACACAATATAAACGATTAAAAGAATGAGATTTCCTTCAAT
MF-alpha-1

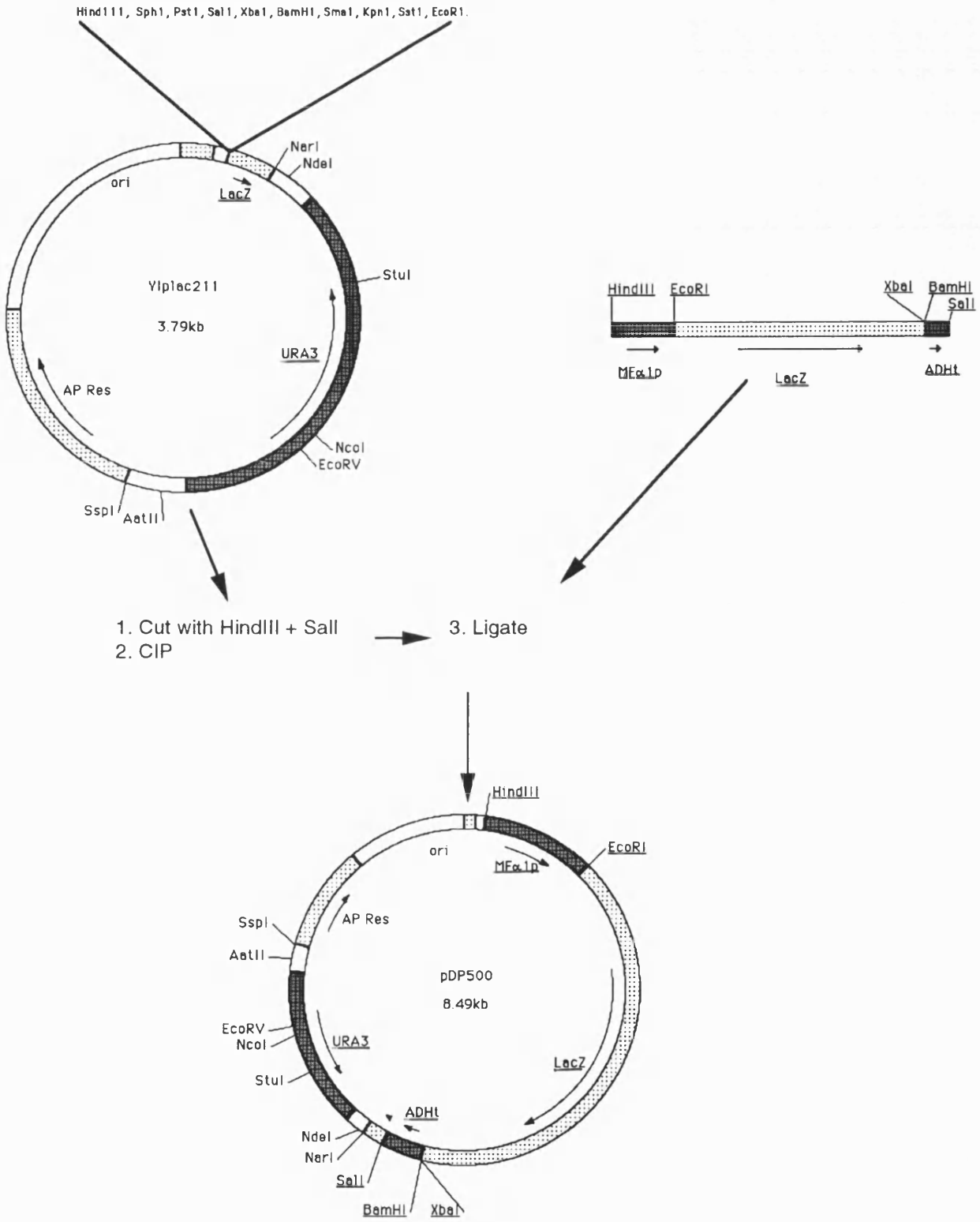

Figure 5.2. Construction of expression vector pDP500

5.2.2 *MFα1* promoter activity during batch fermentation

5.2.2.1 *MFα1* promoter activity during batch fermentation on minimal media plus glucose.

PMY/500 was grown with vigorous shaking at 28°C in batch culture using YNBG medium. Throughout growth, samples were taken and assayed for β-galactosidase activity and mRNA samples extracted. As can be seen from Figure 5.3, cell growth in YNBG is predominantly fermentative, with maximal biomass being attained when glucose is fully utilized and no subsequent growth on the excreted ethanol even under aerated conditions. As illustrated in Figure 5.4, β-galactosidase specific activity increased at an approximately constant rate during exponential growth reflecting β-galactosidase accumulation within the cell, reaching a maximum as growth starts to slow and before cells enter stationary phase and then declining, presumably due to proteolytic breakdown of the accumulated β-galactosidase. The corresponding plot of β-galactosidase volumetric productivity (units of β-galactosidase accumulated per ml of cells) versus time (Figure 5.5) indicates that the *MFα1* promoter directs the accumulation of β-galactosidase at an approximate constant rate during logarithmic growth. A Northern blot of mRNA samples probed with a *lacZ* probe show the same smearing as was found in Chapter 4 (Figure 4.4). A probe for the *MFα1* mRNA species was employed in Northern analysis but appeared to bind to several messages in the cell and also to messages in **a** type cells, indicating that it was not specific. These initial results suggest that the *MFα1* promoter is turned on constitutively through out growth and is not induced, but may be even downregulated at late logarithmic and entry to stationary phases of growth.

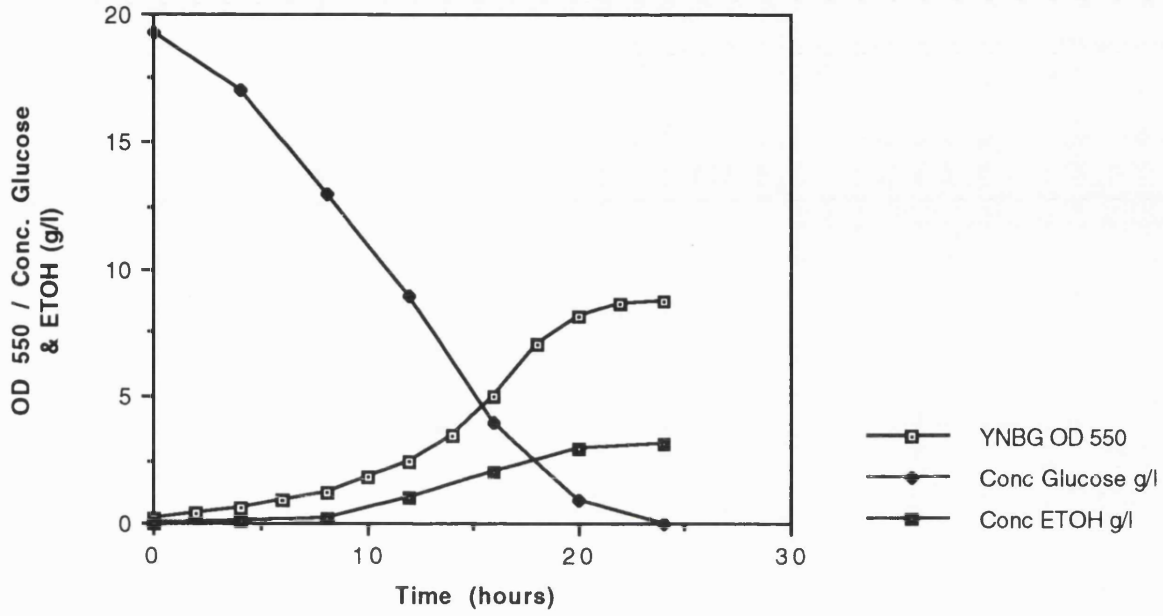

Figure 5.3 Growth characteristics of the strain PMY/500 on YNBG medium

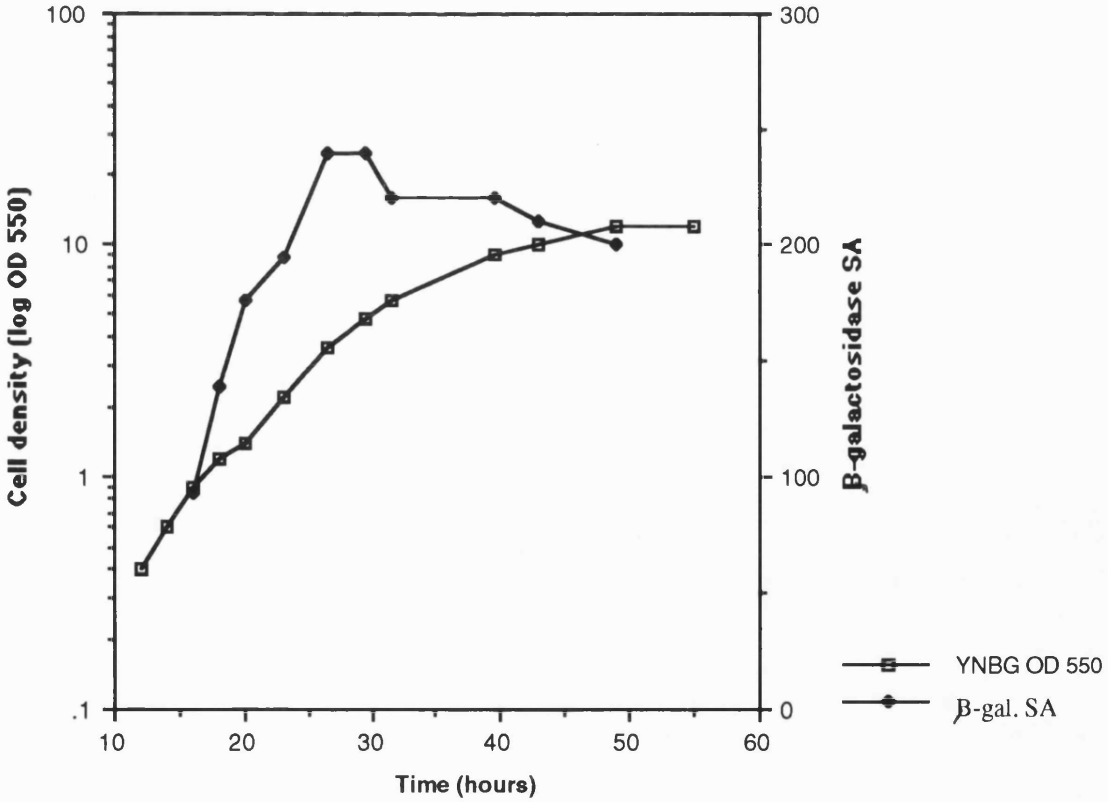

Figure 5.4. Accumulation of β-galactosidase directed by the *MFα1* promoter in PMY/500 growing on YNBG medium

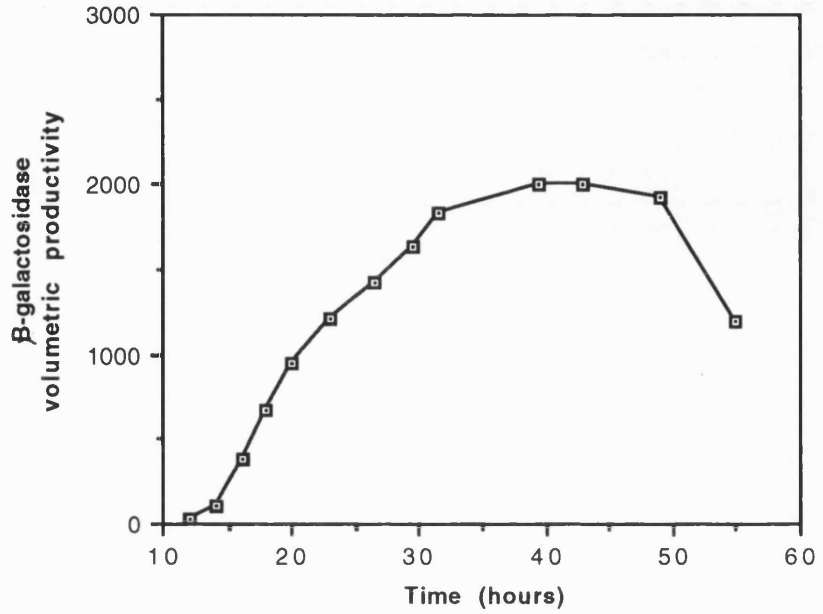

Figure 5.5. PMY/500 volumetric productivity (units β-galactosidase per ml cells) during on YNBG medium.

5.2.2.2 *MFα1* promoter activity during growth on a enriched YNBG medium.

In an effort to create a minimal medium that would support respiratory growth, additional vitamin and mineral supplements were added to the YNBG. YNBG was supplemented with the following extra vitamins and minerals (per litre): d-biotin 0.1 mg; Ca-pantothenate 20 mg; myo-inositol 40mg; pyridoxine-HCl 10 mg; thiamine-HCl 40 mg; $CuSO_4.5H_2O$ 1.6 mg; $FeSo_4.7H_2O$ 16 mg; $MnSO_4.H_2O$ 8 mg; $ZnSO_4.7H_2O$ 6 mg (as outlined in materials and methods, Section 2.3.1.1). Although this new medium supported a slightly higher cell growth rate and cell density as shown in Figure 5.6, very little utilization of the accumulated ethanol was found. As illustrated in Figure 5.7, the pattern of β-galactosidase accumulation during growth on YNBG+VM is similiar to that of the original media. However, two definite differences were noticed;

- Breakdown of the accumulated β-galactosidase in cells grown in the supplemented media is less pronounced as cells approach stationary phase. This indicates that the supplements provide a nutrient source for the cells after glucose exhaustion and hence less proteolysis of intracellular proteins occurs

- β-galactosidase volumetric productivity is slightly lower during growth on the YNBG+VM as compared to growth on YNBG. This result could indicate that the promoter is either more active under poor nutritional conditions or that it's activity is in some way controlled by growth rate.

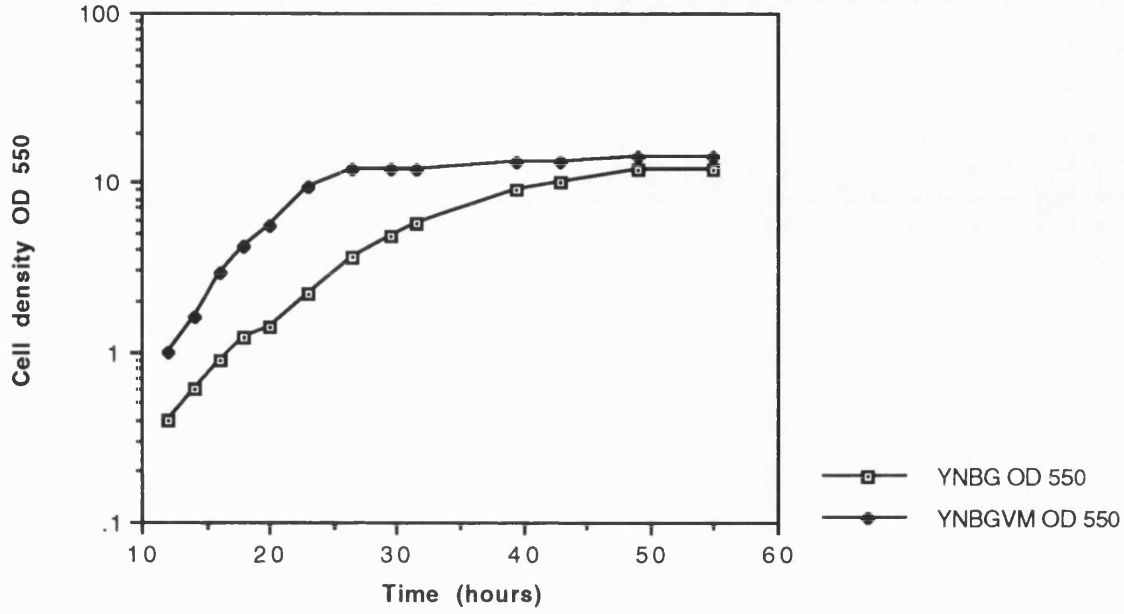

Figure 5.6. Comparison of PMY/500 growth on YNBG and YNBGVM

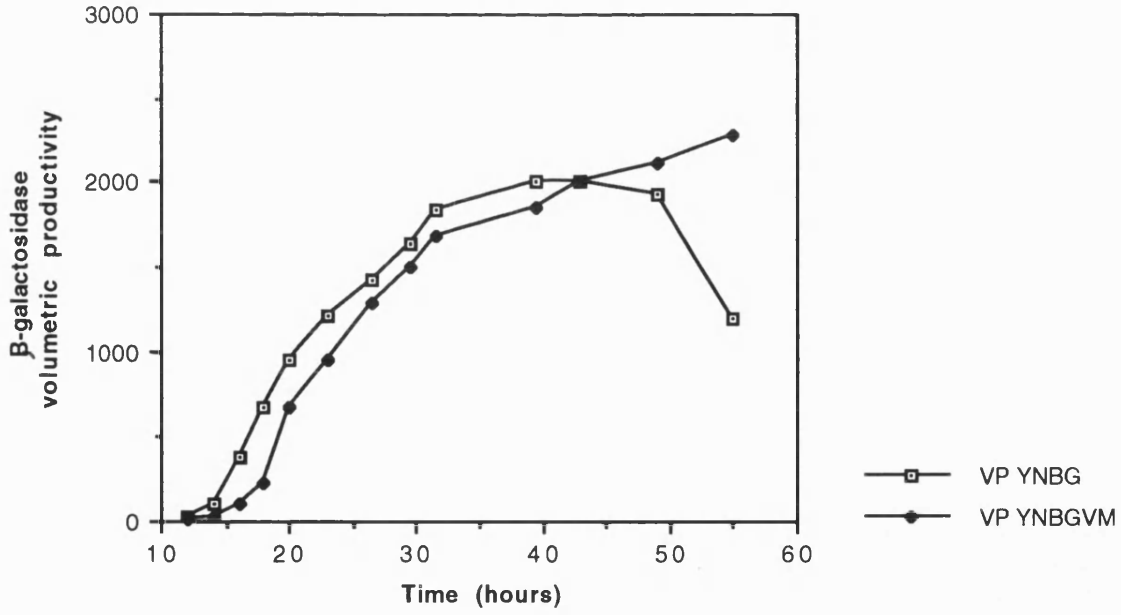

Figure 5.7. Comparison of β-galactosidase volumetric productivity in cells grown on YNBG and YNBGVM.

5.2.3 On the possible nutrient control of the *MFα1* promoter.

This results described so far may indicate that the *MFα1* promoter is under some sort of nutritional control and is downregulated under conditions of nutrient limitation. Subsequent studies using either complete SD+2% glucose or SD media limited in carbon, nitrogen and sulphur (see Section 2.3.1.1) showed little difference in β-galactosidase accumulation after nutrient limitation (Table 5.1).

Time after transfer (mins).	**CONTROL** β-galactosidase SA	**SD-glucose** β-galactosidase SA	**SD-nitrogen** β-galactosidase SA	**SD-sulphur** β-galactosidase SA
0	35	38	37	33
30	40	33	33	30
60	47	31	30	27
120	52	41	33	25

Table 5.1 β-galactosidase accumulation under different nutrient starvation conditions.

5.2.4 Comparison of *MFα1* promoter directed β-galactosidase accumulation on different carbon sources in complex medium

Due to the fact that YNBG+VM supported a higher growth rate than standard YNBG (Section 5.2.2.2) we investigated if growth rate was a major controlling factor on the activity of the *MFα1* promoter using casamino acid supplemented YNB medium (YNBC, Section 2.3.1.1) and the carbon sources glucose (YNBCG), raffinose (YNBCR) and acetate (YNBCA). These supported respective μmax values, with the strain PMY/500, of 0.35, 0.25 and 0.14. As can be seen from Figure 5.8, the same pattern of β-galactosidase accumulation occurs in each of these media. However, for the three carbon sources, β-galactosidase volumetric productivity rises as the cellular growth rate falls indicating that, for the *MFα1* promoter, growth rate may play a part in the activity of the promoter.(If the promoter were responding to carbon catabolite repression we would expect a major difference in β-galactosidase accumulation between YNBCG and YNBCA, which is not observed (Figure 5.8)).

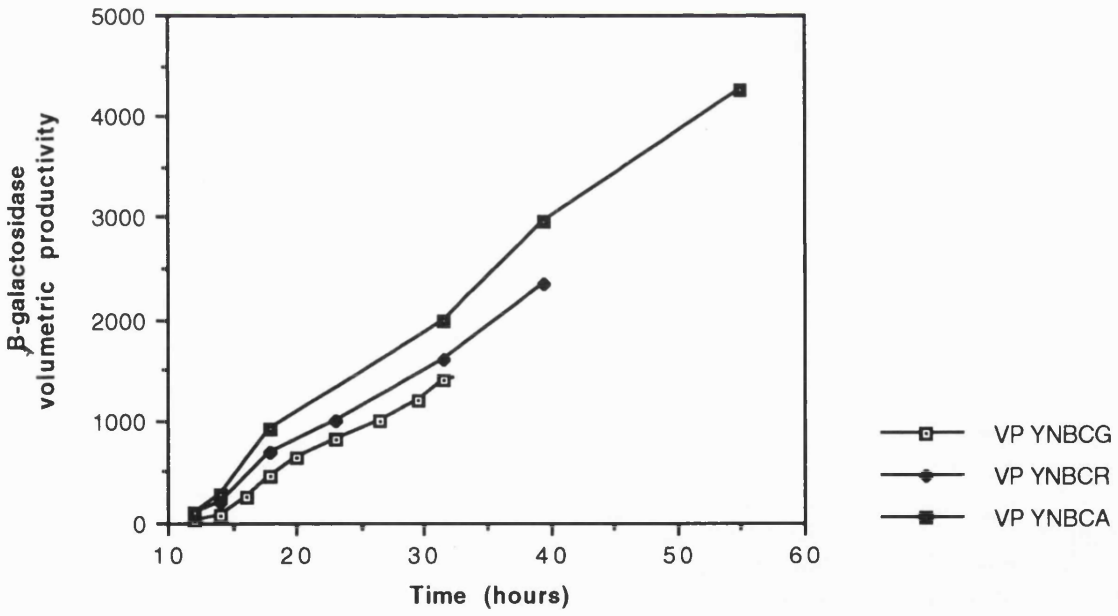

Figure 5.8.. β-galactosidase volumetric productivity of PMY/500 grown on the carbon sources glucose, raffinose and acetate.

5.2.5 Chemostat culure of PMY/500

In order to define if growth rate of the host cell is a major determining factor of *MFα1* promoter activity in *S. cerevisiae* continuous culture was used. The growth of *S. cerevisiae* in chemostat culture can be divided into three regimes as illustrated in Figure 5.9. Under low dilution rates (growth rates) area A of Figure 5.9, the concentration of glucose in the chemostat medium is not high enough to repress respiratory enzymes, and the cells consume the carbon source via respiration. A transition state occurs when the dilution rate is increased to a point where the concentration of glucose in the medium is just high enough to start to repress respiration. In this state, growth is very unsteady as the glucose is both oxidatively and fermentatively metabolised (area B of Figure 5.9). Cells in the state are regarded as unstable and are very sensitive to changes in their external envoironment, reacting with sudden and long lasting changes in their metabolism. *S. cerevisiae* strain CBS 8066 undergoes a transition phase at growth rates between 0.30 h-1 and 0.38 h-1 (Postma *et al.*, 1989). Any change in the dilution rates in this state by more than 0.02h-1 causes it to enter fermentative metabolism after which an extensive period of time requiring many residence times is required before the cells enter a steady state again (Postma *et al.*, 1989). As the dilution rate of the culture increases beyond this point, respiratory enzymes are repressed almost totally and the cells ferment, producing the energy rich compound ethanol, and the biomass yield of the culture drops (area C of Figure 5.9).

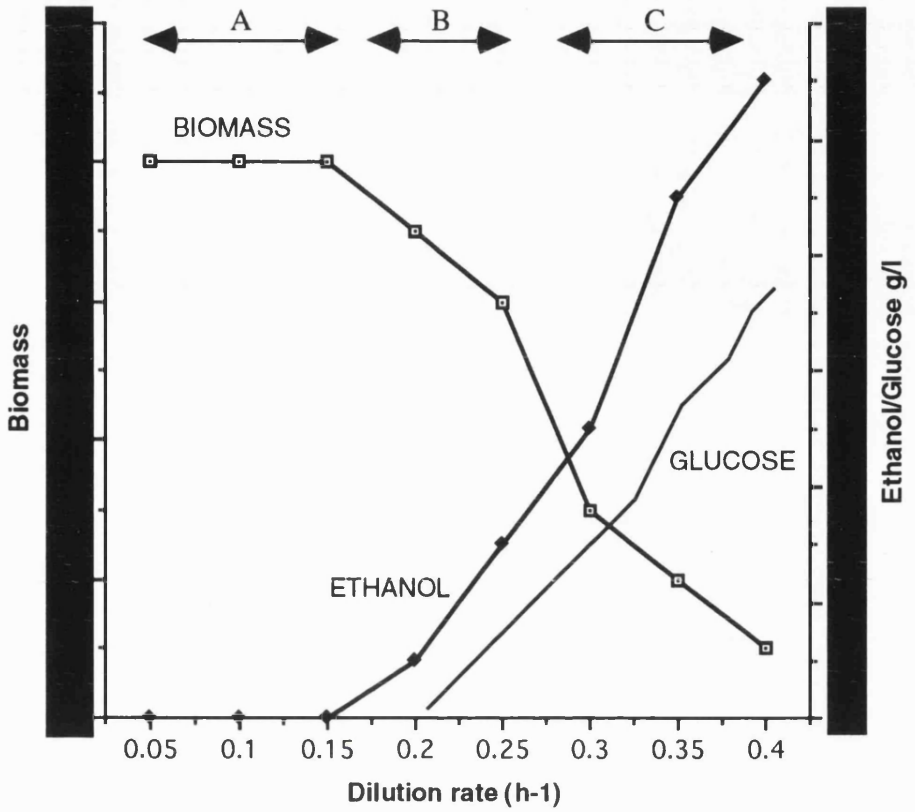

Figure 5.9 Chemostat growth of *S. cerevisiae* (adapted from Postma *et al.*, 1989)

5.2.5. Establishing chemostat conditions for PMY/500

5.2.5.1 Chemostat growth medium

It was decided to use a defined medium for the chemostat experiments in order to simplify the analysis of results. Due to the problems associated with *S. cerevisiae* respiration on a defined medium, several defined media were chosen from the literature and tested to see if they could support respiratory growth.

A defined medium optimised for yeast growth (Fiechter *et al.*, 1981) and a medium used by Bruinenberg *et al.*, (1983) were originally chosen and made up according to the recipes. To test if these media could support oxidative growth the media were prepared with 2% ethanol as a carbon source. Vigorously aerated shake flask cultures were tested for cell growth. However, the strain PMY/500 and another tested strain, DBY746, could not grow in these media. The medium finally chosen for continuous culture of the strain PMY/500 is based on a recipe from Fieshko *et al.*, (1987) which was used for fed-batch high cell density cultivation of *S. cerevisiae*. The medium as optimised by R. Ernhill at ICI Pharmaceuticals was adapted for this study. Several problems were encountered when making up this medium. The main problem was the fact that the original ammonium sulphate salt would not dissolve in the medium. This was then changed to an ammonium choloride salt that showed better solubility, the final composition being as detailed in Table 5.2

VITAMINS	CONCENTRATION (g/l)
Inositol	0.5
Thiamine HCL	0.01
Calcium Pantothenate	0.05
Pyridoxine phosphate	0.014
Nicotinic acid	0.061
Biotin	0.001
Histidine	0.1
Leucine	0.1
Riboflavin	0.003

TRACE METAL	CONCENTRATION (g/l)
Ferric Chloride	27
Zinc Chloride	2
Calcium Chloride (6H2O)	2
Sodium Molybdate	2
Calcium Chloride (2H2O)	1
Copper Sulphate	1.9
Orthoboric Acid	0.5
Concentrated HCl	10%

ESSENTIAL SALTS	CONCENTRATION (g/l)
NH4Cl	8
KH2PO4	3
MgSO4	1
EDTA	0.4

CARBON SOURCE	CONCENTRATION (%)
Glucose	1%

AUXOTROPHIC SUPPLEMENTS	CONCENTRATION (g/l)
Histidine	0.1
Leucine	0.1

Table 5.2 Composition of chemostat medium stock solutions

For detailed protocol for making up this medium, see Section 2.3.1.4.

5.2.5.2 Chemostat culture parameters.

The parameters of temperature, pH and dissolved oxygen were largely decided from previous experiments. Temperature was maintained at 28^{o}C, pH at 5.5 and the aeration rate and stirrer speed were maintained at 1vvm and 1300 rpm respectively. No antifoam was used as the medium tended not to froth even at high aeration rates and stirrer speeds.

5.2.5.3 Cell growth characteristics over chemostat range

Figure 5.10 illustrates the growth characteristics of PMY/500 over the range of dilution rates (DR) used. The behaviour of the strain is broadly in agreement with the published data for *S. cerevisiae* (von Meyenberg, 1969; Rieger *et al.,* 1983). The apparent μmax of this strain in defined medium is 0.33 h^{-1} (the observed value for Dc, the critical dilution rate that the cells have washed out at). These values are low compared to the usual μmax for wild type *S. cerevisiae* of 0.47 h^{-1} (Reiger *et al.,* 1983) but are in agreement with the μmax values reported for most genetically engineered yeast strains (Zubriggen *et al.,* 1988; Gopal *et al.,* 1989 Beretta *et al.,* 1991). Glucose starts to accumulate in the culture medium at a value of about 2/3 of the Dc, compared to the results of Rieger *et al.,* (1983) where glucose starts to accumulate at 3/4 of the Dc value.

The biomass yield at or below the DR of 0.125 was 0.47 g/g, as expected for purely respirative growth on glucose. Above this dilution rate, ethanol started to accumulate in the medium and the biomass yield factor dropped dramatically as the cells began oxidoreductive growth. A recently published, experimentally derived model (Sonnleitner & Kappeli; 1986) has explained this physiological response in terms of a limited respiratory capacity, DR corresponding to saturation of the respiratory machinery of the cell and the onset of fermentative metabolism.

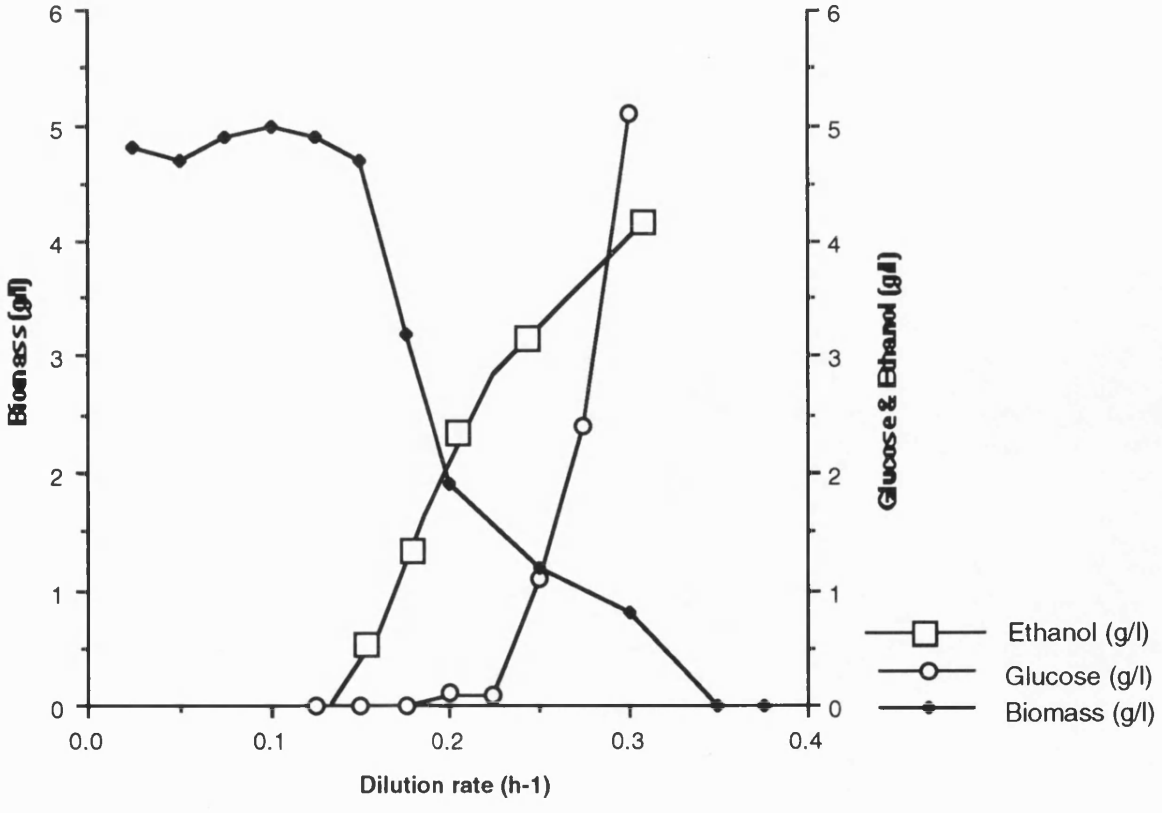

Figure 5.10. Chemostat culture of PMY/500

5.2.5.4 Influence of growth rate on β-galactosidase accumulation in PMY/500 under continuous culture

Figure 5.11 demonstrates the accumulation of β-galactosidase in PMY/500 over the dilution rates studied. The influence of growth rate on β-galactosidase accumulation forms a two stage pattern. Initially at low dilution (growth) rates, β-galactosidase specific activity increases up to fourfold as the growth rate increases. This can be explained by the principal of 'maintenance energy' (Bugeja *et al.*, 1982). At these low dilution rates, the concentration of carbon source in the medium is low enough that all the synthetic capacity of the cell is devoted to maintenance and growth rather than in the production of a functionally redundant protein (β-galactosidase). As the dilution rate increases and more carbon source becomes available to the cell, this extra energy can be chanelled into making β-galactosidase.

The lower growth rates at which β-galactosidase accumulation is increasing (ranging from a doubling time of 28 hours to 6 hours, D_R = 0.025 - 0.125) do not correspond to any growth rates tested in shake flask scale. This is because the doubling times are too low to maintain in a shake flask except by dropping the growth temperature of the cell, which would in turn affect other dynamics of protein synthesis. As the D_R increases above 0.2 h^{-1} (a doubling time of 3.5 hours), accumulation of β-galactosidase begins to decrease. This pattern of decreasing accumulation of β-galactosidase as the growth rate increases is as was observed under shake flask culture (e.g. Figures 5.7, 5.8), and occurs at the same range of doubling times as was found in shake flask culture with different carbon sources (Figure 5.8).

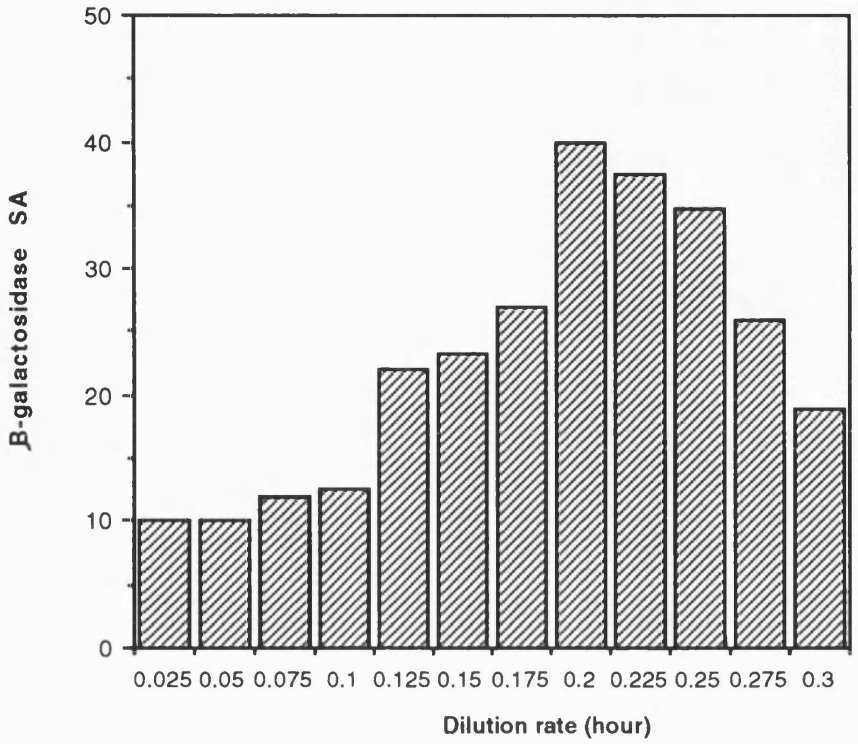

Figure 5.11. Influence of growth rate on accumulation of β-galactosidase across the dilution rates studied.

5.2.5.5 Stability of plasmid pDP500 during continuous culture

It has been described that plasmid stability changes with growth rate in continuous culture. In selective media, DiBiasio & Sardonini (1986) have demonstrated that a host/plasmid construct was more stable at higher rather than lower growth rates. Kleinman *et al.*, (1986) have also found that the stability of the plasmid pJDB248 in *S. cerevisiae* strain S150-2B increased with increasing growth rate. In non-selective envoironments, Impoolsup *et al.*, (1989) have found that the stability of the plasmid pLG69Z in the strain YN124 was found to decrease with increasing dilution rates. For these studies, an integrant was used so the *MFα1-lacZ* constuct should be stable. To ensure that the pattern of β-galactosidase accumulation during chemostat culture was not caused by any instability effects (ie loss of integrated pDP500 sequences from the cell), plasmid stability was monitored by replica plating cells from the chemostat onto selective medium and complex (non-selective) medium and comparing the numbers on each medium type. The results, illustrated in Figure 5.12, indicate that the plasmid was stable at all dilution rates and only a very small proportion of cells lost the plasmid during growth.

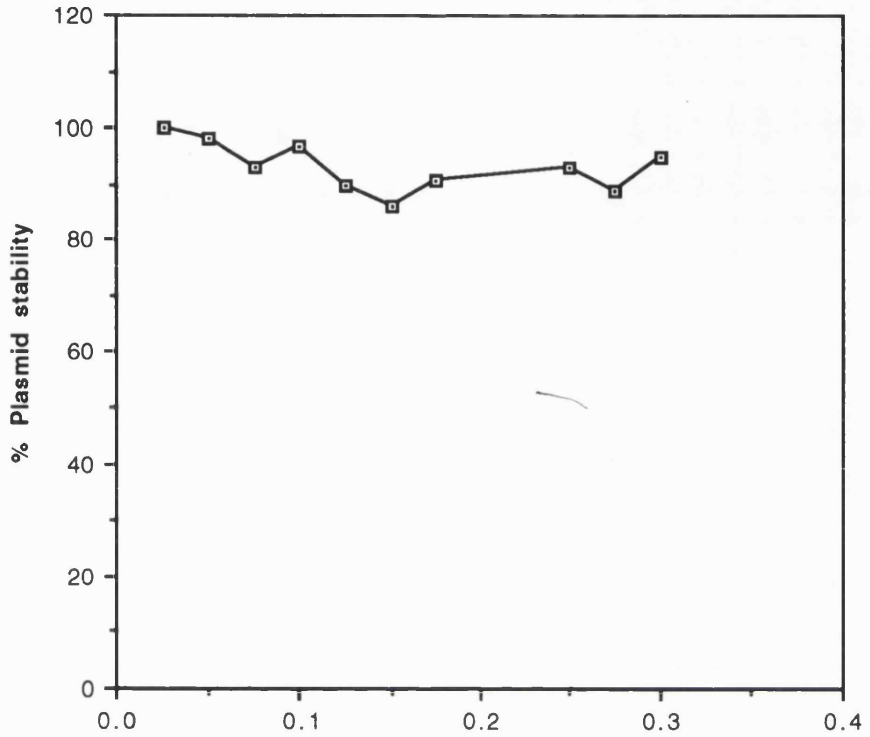

Figure 5.12. Influence of growth rate on accumulation of β-galactosidase across the dilution rates studied.

5.3 Discussion

This chapter studied the regulation of the *MFα1* promoter in the yeast *S. cerevisiae*. Growth rate regulation of this promoter has not been reported before. It was the aim of this work to identify:

1: Whether the *MFα1* promoter was in any way growth phase regulated as certain reports in the literature have suggested (Ernst, 1986; Gu *et al.*, 1991; Park *et al.*, 1989).

2: To characterise the fermentation conditions giving maximal promoter activity using the accumulation of intracellular β-galactosidase driven by the *MFα1* promoter as an indication of promoter activity.

The aims have in most ways been met. Despite the fact that no accurate measurement of mRNA levels driven by the promoter could be ascertained due to the points mentioned in 5.2.2.1, the accumulation of a reporter gene product is a convenient and reliable means to measure promoter activity.

The conclusions of this work are as follows:

- By extrapolation from data on the accumulation of β-galactosidase, we can say that the *MFα1* promoter is not regulated during growth, but is constitutive. No change in the rate of accumulation of β-galactosidase can be seen during growth on YNBG.
- The *MFα1* promoter appears to be negatively regulated by growth rate such that as the growth rate of the culture falls, down to a doubling time of 3.5 hours, the accumulation of β-galactosidase from the *MFα1* promoter rises. This has been demonstrated both in batch culture and in a controlled chemostat culture.
- With further decreases in growth rate, promoter activity (as measured by accumulation of β-galactosidase) declines, as shown by chemostat study (Figure 5.10).
- Using the appropriate controls, the phenomenon of growth rate controlled *MFα1* promoter activity has been shown not to be an artefact caused by differences in pH during the experiments or differences in plasmid stability at different growth rates.

- This study has shown that heterologous gene expression using the *MFα1* promoter can be optimised for productivity by carefully controlling the growth rate of the host cell. This result of growth rate controlled activity of the *MFα1* promoter is novel and has wide implications, especially for industrial labs producing valuable therapeutic proteins, where fermentation productivity is a key economic factor of the production process.

CHAPTER 6. DISCUSSION

6.1 Introduction

The studies carried out for this thesis were aimed at defining in more detail the regulation of *S. cerevisiae*heat-shock and growth phase dependent promoters and to assess their potential use for the regulated expression of heterologous proteins in yeast. Research described in Chapter 3 has identified optimal conditions for the use of HSE's for the regulated expression of *E. coli* β-galactosidase in yeast and identified that methanol is a novel inducer of *HSE-lacZ* expression in yeast. Studies on the *UBI4* promoter (Chapter 4), a growth-phase dependent promoter, have identified that it is regulated by carbon catabolite repression. No definitive evidence could be found that the *UBI4* promoter is controlled by the RAS/cAMP pathway, as previously reported, (Tanaka *et al.*, 1990). Studies aimed at characterising the growth-phase regulation of the *S. cerevisiae MFα1* promoter and at optimising conditions for *MFα1* promoter directed expression of *lacZ* (Chapter 5) have found that although the promoter is constitutively active during cell growth, continuous culture experiments have identified that it is regulated by the growth rate of the host cell.

6.2 *HSE-lacZ* regulation in *S. cerevisiae*

The activation of heat shock genes by temperature upshift (Section 1.5 and Chapter 3) suggests that they could be of use to induce heterologous genes in yeast. The heat shock expression vector used for this study (pHSE2; Sorger & Pelham, 1987), employs a disabled *CYC1* promoter into which has been inserted two consensus HSE sequences conferring heat shock inducible expression of the reporter gene *lacZ* (Section 3.2.1). Comprehensive studies were carried out utilising this vector in a variety of host strains to identify the optimal conditions for HSE-directed *lacZ* induction.

The use of different temperature upshifts as a means to induce *lacZ* gene expression from this vector underlined the need for carefully controlled culture conditions for optimal induction. Cells have to be grown at low temperatures - 20-23°C - to ensure low basal HSE activity and maximum levels of β-galactosidase induction at 39°C (Figure 3.4). Cells have to be subjected to a large temperature increase (23-39°C) to obtain maximal induction of *HSE-lacZ* activity at 39°C (Figure 3.3). Induction levels are limited by the transience of the heat shock response (Figure 3.5). *HSE-lacZ* induction in response to temperature upshift is only appreciable during exponential growth (Figure 3.2). Other inducers of the heat shock response tested (dimethyl

sulphoxide, canavanine, NaCl, paromomycin and 4-nitroquinoline-1-oxide) caused minimal or no induction of *HSE-lacZ* activity. It was found that methanol was an effective inducer of *HSE-lacZ* activity (Section 3.2.6, Kirk & Piper, 1991b) a finding previously unreported. The levels of heat shock transcription factor (HSF) mRNA, the transcription factor responsible for activation of heat shock genes in response to temperature upshift (Section 1.5) were studied in order to see why induction of *HSE-lacZ* activity was only appreciable during mid-log growth. The findings (Section 3.2.7) indicate that HSF is down regulated on entry to stationary phase, a possible reason for minimal induction of *HSE-lacZ* activity during this period of growth. Mutant host strains were studied in order to identify if any defined mutations would improve *HSE-lacZ* induction in response to stress agents. The findings (Section 3.2.3) indicate that mutations in the ubiquitination system have little effect on induction of *HSE-lacZ* activity (Section 3.2.3.2). In contrast. the *pep4-3* mutation was found to increase both basal and induced levels of *HSE-lacZ* activity, but it had no effect on the magnitude of induction (Section 3.2.3.3).

Chapter 3 therefore provides a comprehensive guide, from data utilising a *HSE-lacZ* fusion as a model system, for heterologous gene expression in yeast directed by the HSE regulatory element. Optimal conditions for the use of the HSE element include growth at low temperatures (23-25^{o}C) to minimise basal expression, from the promoter, then heat-shock to relatively high temperatures (37-39 C) to fully induce expression. Utilisation of a *pep4-3* host strains causes a marked improvement in the accumulation of β-galactosidase under these conditions. Similar levels of induction can be found with methanol induction of the *HSE-lacZ* fusion, however, since methanol is a flammable solvent, its use in industrial settings would be limited.

6.3 *UBI4* promoter regulation in response to chemical stress agents

In order to study the regulation and potential use of a growth-phase dependent promoter for the conditional expression of heterologous proteins, the *UBI4* promoter was chosen (Section 4). The yeast *UBI4* gene is reported to be transcriptionally regulated by nutrients via the cAMP pathway (Tanaka*et al.*,1988; 1989) and by heat-shock (Finley*et al.*,1987). Under conditions of high intracellular cAMP, expression of the *UBI4* gene is basal, however, under starvation conditions and in mutants with low levels of cAMP, *UBI4* is reported to be induced, presumably to provide ubiquitin to cells under these stressful conditions (Tanaka*et al.*,1988). This conditional expression of the yeast *UBI4* gene indicated that it might be used for the regulated expression of toxic heterologous proteins in *S. cerevisiaie*. An expression vector was constructed

(pDP501) that contained a *UBI4* promoter-*lacZ* fusion (Section 4.2.1.2). This expression vector was designed as an integrating vector in order to allow the transformed cells to grow on a variety of complex media without appreciable plasmid loss. Initial studies looked at induction of *UBI4-lacZ* activity in response to different chemical stress agents (Section 4.2.2). Appreciable induction of *HSE-lacZ* activity and *UBI4* mRNA was found on treatment with the DNA damaging agent 4-nitroquinoline-1-oxide, confirming the results of Treger*et al.*,(1988). No induction was seen on treatment with H_2O_2, the major product of SOD - catalysed dismutation of oxidative free radicals.

6.4 *UBI4* promoter regulation during growth, a control exerted by carbon catabolite repression

Studies aimed at characterising the growth phase regulation of the *UBI4* promoter (Section 4.2.3) showed no induction of *UBI4-lacZ* activity either on entry to stationary phase or during prolonged maintenance in stationary phase when cells were grown on YNBG (Section 4.2.3.1). However, when the same experiment was performed using a medium that allows a respiratory growth phase (YEPD), the rate of accumulation of β-galactosidase from the *UBI4-lacZ* fusion increased during the second, respiratory, phase of growth on YEPD medium (Figure 4.11B). These initial results indicated that the *UBI4* promoter, rather than being controlled by the RAS/cAMP pathway, may be under control of carbon catabolite repression. This would explain why no induction of *UBI4-lacZ* activity could be observed during entry to stationary phase on YNBG. Induction should have occurred in response to the decline in intracellular cAMP if the promoter were under negative regulation by cAMP dependent protein kinase A activity). It would also explain why the rate of accumulation and levels of β-galactosidase were higher during respiratory growth (Figures 4.6-4.8). *UBI4-lacZ* activity was then studied during growth on repressing and non-repressing carbon sources (4.2.3.2). Both *UBI4* mRNA and *UBI4-lacZ* activity levels indicated that the activity of the *UBI4* promoter is constitutive during growth on a single carbon source, not induced on entry to stationary phase, and higher on non-repressing carbon sources (acetate and raffinose) as compared to repressing carbon sources (glucose) (Figures 4.10-4.12). These results provided further evidence that the *UBI4* promoter was controlled primarily by carbon catabolite repression rather than the RAS/cAMP pathway. The promoter sequence of the *UBI4* gene was then studied to see if any sequences were present with homology to known sequences involved in carbon catabolite repression (Section 4.2.3.5). A sequencewas identified at -673 to -666bp in the *UBI4* promoter that bore exact homology to the *CYC1* UAS2 required for

derepression of the *CYC1* gene on respiratory carbon sources (Guarente*et al.*,1984). Transcription factor (HAP2-4) binding to the UAS2 sequence activate transcription about thirty-fold when cells are shifted from a fermentable to a non-fermentable carbon source (Guarente*et al.*,1984). Since this UAS element has so far only been found in yeast genes involved in mitochondrial functions (Gancedo, 1992; Zaman*et al.*,1992). The presence of such an element in the *UBI4* gene would therefore be a novel finding. To test the hypothesis that the *UBI4* promoter is controlled by the HAP carbon catabolite repression pathway, regulation of the *UBI4* gene was studied in the well characterised *hap1.1* and *hap2.1* mutant strains and the wild-type parent (Section 4.2.3.5). As cells enter transition phase due to glucose depletion in YEPD medium, it is evident that both the *hap1.1* and *hap2.1* mutant strains do not allow derepression of the *UBI4* promoter as compared to the WT. This indicates that *HAP*-encoded transcription factors are required for derepression of *UBI4* in response to glucose depletion in YEPD.

6.5 The role of the RAS/cAMP pathway in the regulation of the *UBI4* promoter

The role of the RAS/cAMP pathway in regulation of the *UBI4* promoter was studied using strains of high and low protein kinase A activity (AM-180-2B and AM-410-4C) respectively. Figure 4.17 illustrates that regulation of the *UBI4* gene is normal (as compared to WT) in these strains, the mutations in the cAMP pathway having no effect on induction with glucose depletion. This is further evidence that induction of *UBI4* is in response to carbon catabolite repression rather than declining cAMP levels.

Experiments carried out by Mboyni*et al.*,(1990) have indicated that mutations in the TPK genes can affect carbon catabolite repression in *S. cerevisiae* (Section 1.2.1.4 & Section 4.2.4.3). Their results show that strains with a single, active but attenuated subunit, the $tpk1^{w1}$ or $tpk2^{w1}$ (but not $tpk3^{w1}$ mutations) reduce protein kinase A levels to such a low level as to cause deficiency in carbon catabolite repression, illustrated by their ability to form mitochondria under conditions where normally repressing amounts of glucose are present in the growth medium. tpk^{w1} Strains were acquired that additionally contained the *bcy1* mutation, so that their limited protein kinase A activity was cAMP-independent and constitutive. These strains were chosen to try to prove that carbon catabolite repression was more dominant in regulating the *UBI4* gene than any effect of the RAS-cAMP pathway. Results illustrated in Figure 4.18 indicate that these strains defective in carbon catabolite repression due to low levels of protein kinase A activity show increased basal levels of *UBI4* transcript. This result may indicate why Tanaka*et al.*,(1988) came to the conclusion that the *UBI4* promoter

was regulated by the RAS/cAMP pathway. Their experiments were carried out in a *cyr1/cyr1* strain that requires exogenous cAMP this strain showing *UBI4* induction on starvation for cAMP. The interpretation that this shows cAMP regulation must be questioned however, because the decline in protein kinase A activity to very low levels that will result from starving the cells for cAMP will also cause a release from carbon catabolite repression.

Nutrient starvation causes induction of several heat shock proteins including *CTT1* (Bissinger*et al.*,1989) and the secreted heat-shock protein *HSP150* (Russo*et al.*,1993). Starvation of exponentially growing cells for sulphur or glucose has been shown to cause a dramatic reduction in intracellular cAMP levels (Van der Platt, 1974, Shin*et al.*,1987). Starvation of cells for nitrogen or phosphate (in the presence of glucose) has been shown not to affect cAMP levels (Boy-Marcotte*et al.*,1987; Francois*et al.*,1988) although it may inactivate protein kinase a in a cAMP-independent manner (Thevelein, 1992). The *UBI4--lacZ* fusion was found to be induced 4-5 fold with all of these starvation conditions (Table 4.2) which would tend to indicate that the promoter could not be solely controlled by cAMP levels. Although these starvation results seem to mimic, in part, the behaviour of the *CTT1* promoter (Bissinger *et al.*, 1989) which is also under nitrogen starvation and heat-shock control. A direct influence of protein kinase A activity on *CTT1* promoter activity is clearly demonstrable (Weiser*et al.*,1991; Marchler *et al.*, 1992) whereas for *UBI4* promoter activity, no such effect is demonstrable (this study).

6.6 On the optimal conditions for expression of heterologous products from the *UBI4* promoter

My studies have indicated that the *UBI4* promoter is controlled by carbon catabolite repression, as such it is more active during respiratory growth as compared to fermentative growth. This result is of importance as the trend in industrial labs is towards respiratory, fed-batch fermentation. Results indicating that the *UBI4* promoter has a significant level of basal expression in unstressed cultures (Section 4.2.3) rules out it's use for the expression of toxic heterologous proteins in *S. cerevisiae*. The *UBI4* promoter could however be used for the expression of non-toxic heterologous proteins employed as a constitutive promoter under fed-batch conditions.

6.7 Conclusion to experimental results on the regulation of *UBI4*

This study on the regulation of the *UBI4* promoter, discussed in Section 4, constitutes a novel and illuminating body of data that provides new insight into *S. cerevisiae* gene regulation. These experimental findings indicate that growth phase dependent regulation of the *UBI4* gene is primarily by carbon catabolite repression. Regulation by a cAMP dependent signalling pathway has not been proven. The basal activity of the *UBI4* promoter prevents it being a tightly-regulated promoter suitable for heterologous protein production. The exact mechanism for this basal activity has not been clarified although it appears to operate through cAMP independent mechanisms.

The reason for regulation of the *UBI4* gene by carbon catabolite repression is illuminated by the studies of Elliot & Futcher, (1993) which show that some of the properties of stationary phase cells do not require residence in stationary phase. Slow growing cells are more heat shock resistant than faster growing cells particularly when the cells are respiring. Resistance to heat shock and other stress treatments requires synthesis of the heat shock protein *HSP104*, which is also expressed at higher levels during respirative as compared to fermentative growth (Sanchez*et al.*,1992). It is possible that the *UBI4* protein may be required in the same manner as HSP104 for resistance to the increased stress of respiratory growth.

6.8 Regulation of the *MFα1* promoter

Certain published papers have indicated that the *MFα1* promoter could be growth phase regulated, being maximally activated as yeast cells enter stationary phase (Section 5.1, Ernst, 1986; Gu *et al.*, 1991; Park *et al.*, 1989). In order to test this hypothesis, a fusion vector was constructed as in Section 5.2.1. This vector, designated pDP500, contained an *MFα1* promoter-*lacZ* fusion without the *MFα1* secretory signals, thus the promoter activity could be assessed directly, unaffected by the efficiency of the secretory apparatus.

6.9 *MFα1-lacZ* fusion activity during batch fermentation

Section 5.2.2.1 describes the regulation of the *MFα1-lacZ* fusion during growth. Results obtained (Figures 5.3-5.4) indicate that the promoter is constitutive and apparently down regulated on entry to stationary phase. Investigations in Section 5.2.2.2 indicated that the activity of the *MFα1-lacZ* fusion, however, was dependent

on the composition of the growth medium. YNBG + VM (Section 2.3.1.1 and Section 5.2.2.2) was found to support a higher growth rate, but lower β-galactosidase accumulation, as compared to the standard YNBG. This result could be interpreted in either of two ways; either the additional nutrients in the YNBG + VM growth medium repressed the activity of the *MFα1* promoter indicating nutrient regulation, or that the *MFα1* promoter was subject to growth-rate control with its activity dropping as growth rate increases. The experiments in Section 5.2.3 showed no pronounced effects on *MFα1* promoter activity as a result of glucose, nitrogen or sulphur starvation, indicating no nutrient regulation.

6.10 *MFα1-lacZ* activity and the role of growth rate

To initially test the hypothesis that the *MFα1* promoter was growth rate dependent an experiment was designed in which cellular growth rate was altered by utilising different carbon sources (Section 5.2.4). This experiment, although limited by the fact that the carbon sources used altered the carbon catabolite repression state of the cell, did show that *MFα1-lacZ* fusion activity rose with growth rate. The ideal experimental tool for altering cellular growth rate without affecting catabolite repression is the chemostat (Postma *et al.*, 1989). Chemostat culture of PMY/500 (Section 5.2.5) showed that the promoter was growth rate controlled in the same manner as in shake flask experiments. The *MFα1* promoter appears to be negatively regulated by growth rate such that as the growth rate of the culture falls, down to a doubling time of 3.5 hours, the accumulation of β-galactosidase from the *MFα1* promoter rises (Figure 5.11).

6.11 On the relevance of the results regarding *MFα1* promoter activity

There has been no comprehensive study on the activity of the *MFα1* promoter during growth, experimental results on the activity of the *MFα1-lacZ* fusion (summarised in Section 5.3) are therefore of great value to the biotechnology industry since many companies, including Chiron (Barr *et al.*, 1987a,; Cousens *et al.*, 1987; Sabin *et al.*, 1989) and Immunex (Price *et al.*, 1987) are utilising the *MFα1* promoter for the expression of heterologous proteins. These results provide new insight into the regulation of the *MFα1* promoter during growth. Experimental results indicating control of promoter activity by growth rate provide vital evidence that can be used in the optimisation of fermentation conditions for maximum expression of a heterologous protein from the *MFα1* promoter.

6.12 Further studies on the heat-shock response of yeast and the relation between the HSE and the CCCCT 'McEntee' sequence

Further studies on the relationship between the well characterised HSE and the CCCCT sequence (Kobayashi *et al.,* 1990; Kobayashi *et al.,* 1993) and their contribution to transcriptional activation by heat-shock would clarify the HSR of *S. cerevisiae*. A CCCCT sequence is present in the *UBI4* promoter (Figure 4.1) it would be valuable to identify the contribution it makes to transcriptional activation of the *UBI4* gene in response to heat-shock.

6.13 Possible further studies on the regulation and utilisation of stress and growth rate dependent promoters in *S. cerevisiae*

A complete series of *UBI4* promoter deletions is necessary to ultimately characterise the upstream activating sequences present. This work is being carried out in the lab by Richard Watt. Elements that may be present, in view of the experimental results in Section 4 include a HAP 2/3/4 binding element. Considering the function of the ubiquitination system in yeast, an element that activates transcription in response to the presence of unfolded proteins within the cell (the UPR element of Kohno *et al.,* 1993) might be expected. However, *UBI4* is not induced by tunicamycin, a drug which causes incorrectly folded, unglycosylated proteins to accumulate in the lumen of the ER (R. Watt, unpublished). It is unlikely therefore that *UBI4* has a UPR element such as that found in *KAR2* (Kohno *et al.,* 1993).

The results of Marchler *et al.,* (1993) indicate that the catalase T gene has a single sequence present at bp -382 to -325, designated the STRE element, that is responsible for activation of the promoter by all tested stresses including nitrogen starvation, cAMP starvation, heat shock and oxidative stress. This element is homologous to that found in the *CYC7* (Pillar & Bradshaw, 1991), *HSP12* (Praekelt & Meacock, 1990) and *SSA3* (Boorstein & Craig, 1990) genes, which are all regulated by heat shock and nutritional stress indicating a common sequence element responsible for stress induced activation of these promoters. An exact sequence match to the STRE element does not exist in the *UBI4* promoter, indicating that the regulation of the *UBI4* promoter in response to stress may be by a different mechanism than that for the *CTT1*, *HSP12*, *CYC7* and *SSA3* genes.

It would also be worthwhile to analyze the resistance of *UBI4* mutants to different stress agents during respiratory growth as compared to WT to see if the results followed those of Sanchez *et al.*, (1992), using *hsp104* mutant cells.

Now that the optimal growth rates for *MFα1-lacZ* fusion activity have been defined, the next step would be to see what effect altering growth rate has on a secreted protein using the *MFα1* promoter under continuous culture conditions.

6.14 Which promoter is optimal for regulated expression of heterologous proteins in *S. cerevisiae*

Results from Chapters 4 and 5 indicate that neither the *UBI4* or *MFα1* are true growth-phase regulated promoters, both being essentially constitutive during batch growth with the *UBI4* promoter only being induced 3 to 4 fold after carbon catabolite derepression. The *GAL* and *ADH2* promoters seem to be the most ideal for truly regulated expression of heterologous proteins with induction ratios of over 100 fold. The *GAL* promoter is completely represssed by glucose and induced by galactose depletion and glucose addition, this control being very tight (StJohn & Davis 1981). The regulation of the *ADH2* promoter is not so well controlled with significant basal expression under conditions of maximal cell productivity (fed-batch) (Tottrup & Carlson, 1990).

CHAPTER 7. REFERENCES

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