The molecular analysis of two high copy number suppressors of a yeast cell cycle protein kinase mutant

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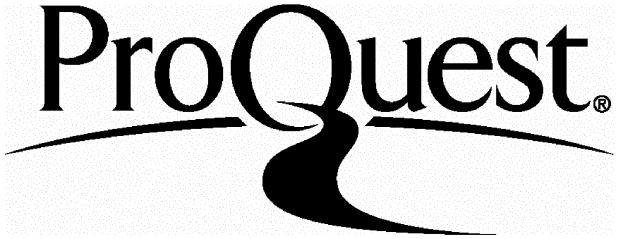

ABSTRACT

The *S. cerevisiae* protein kinase Dbf2 carries out an essential function in late mitosis, and its kinase activity is cell cycle regulated around anaphase/telophase. Originally it was identified as a temperature-sensitive cell cycle mutation, *dbf2*-ts, that is defective for S phase, the *dbf2* mutation causing a 40-minute delay in the initiation of DNA synthesis. However, subsequent analysis showed that the *dbf2* execution point is later in the cell cycle during nuclear division. Consistent with a late mitotic role for Dbf2, *dbf2* cells accumulate as large, swollen "dumbbells" with divided chromatin and fully extended spindles, when incubated at the restrictive temperature.

To further explore the physiological role of Dbf2, a *S. cerevisiae* high copy genomic library was screened for sequences that could rescue the *dbf2* temperature-sensitive phenotype. Five different suppressor genes were isolated, that were named pSDB21-25. Both pSDB24 and 25 are allele-specific, rescuing only one of the three *dbf2* alleles. The characterization of these two molecular suppressors forms the basis of the work described in this report.

SDB25 was sequenced and found to encode p40, a previously characterized substrate and inhibitor of Cdc28 kinase activity. Antibodies were raised against Sdb25 and used to show that Sdb25 is a phosphoprotein, and that Sdb25 has an associated kinase activity that is *CDC28*-dependent. Remarkably, Sdb25 transcript levels, protein levels and associated kinase activity are precisely cell cycle regulated, sharing a common peak in late mitosis. Moreover, Sdb25 protein levels and associated kinase activity are sharply upregulated at the peak of Dbf2 kinase activity in cells released from a *dbf2*-ts block. The Sdb25 protein then disappears around START in the next cell cycle. This indicates that *SDB25* function is confined to M/G1, and morphological analysis of *sdb25*Δ cells supports this conclusion.

The second allele-specific suppressor of *DBF2*, *SDB24*, is 2463 nucleotides long and is a newly identified yeast gene. *SDB24* is expressed under cell cycle control, and is maximally abundant in early S phase. Deletion of *SDB24* does not affect cell viability, although deletion of both *SDB24* and *DBF2* is lethal for the cell.

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ABBREVIATIONS

cdk	cyclin-dependent kinase
DAPI	4',6-diamidin-2-phenylindole dihydrochloride
FACS	Fluorescent-activated cell sorter
kD	kilodalton
kb	kilobase
LB	Luria broth
MCS	multiple cloning site
MPF	Maturating-promoting factor
PBS	Phosphate-buffered saline
ORF	open reading frame
ts	temperature-sensitive

CHAPTER ONE

INTRODUCTION

1.1 OVERVIEW OF THE EUKARYOTIC CELL CYCLE AND ITS CONTROL

The two defining events of the eukaryotic cell cycle are replication of chromosomes and segregation of the duplicated chromosomes into daughter cells. The periods when these events occur delimit two phases, S phase (DNA replication) and M phase (mitosis or cell division). The gaps between them are usually denoted G2 (following S phase and preceding M phase) and G1 (following M phase).

In all cells, except the early cleavage steps of mammalian embryos, there is co-ordination between cell growth and the initiation of S phase (Murray, 1987). This co-ordination occurs at a point in late G1 where, if conditions are favourable and the cell has reached the required size, it becomes committed to the mitotic cell cycle. Under unfavourable conditions, such as nutrient limitation, the cell will "pause" and enter stationary phase.

The initiation of M phase is also subject to a form of regulation that is common to all eukaryotic organisms (Nurse, 1990). In most cells entry into mitosis takes place when they attain a critical mass. In others, such as early embryonic cells, repeated division occurs with little growth and it is postulated that the period between successive M phases is determined by a timer or oscillator (Kirschner *et al.*, 1985). However, in all cells it is proposed that the onset of M phase is induced by activation of a member of the $p34^{cdc2}$

protein kinase family. Originally identified as the product of the *cdc2*$^{+}$ gene in the fission yeast *S. pombe*, functional homologues have been found in *S. cerevisiae*, frogs, starfish and mammals (Lewin, 1990). The current model postulates that mitosis is initiated after complex formation between p34^{cdc2} and an unstable protein called cyclin. Cyclins were initially discovered as proteins whose abundance oscillates dramatically during the cell cycle of early sea urchins and clam embryos (Evans, 1983), and are now divided into a number of classes based on sequence similarity and physiological function. Following formation of the complex, p34^{cdc2} is dephosphorylated and the kinase is activated. After the completion of nuclear division, p34 kinase activity decreases allowing exit from mitosis and entry into G1 (Ghiara *et al.*, 1991; Murray and Kirschner, 1989).

One further level of control links initiation of M phase with prior completion of S phase (Hartwell and Weinert, 1989). This ensures that M phase is delayed if chromosomes are damaged or not fully replicated. Control points of this sort, that monitor one event and inhibit a later event until the prior event has been completed, have been termed "checkpoints". The notion, and existence, of checkpoints reinforces the idea that the cell cycle is an ordered sequence of dependent events. In fact, by circumventing such dependency, control mechanisms can be identified that regulate cell cycle progression. A second checkpoint has also been proposed to function in *S. cerevisiae* that links the exit from mitosis to the successful completion of spindle assembly (Li and Murray, 1991).

(i) The central role of the Cdk kinase family in the cell cycle.

The Cdc2-related protein kinase family has come to occupy a central position in current understanding of control of the eukaryotic cell cycle, and has been referred to as the master regulator of cell cycle progression (Nasmyth, 1993). This viewpoint has

evolved over the last decade or so, as a result of convergence of work on the genetics of the yeast cell cycle and biochemical studies on both vertebrate and invertebrate organisms. One key, unifying, result was the discovery that the fission yeast *S. pombe* $cdc2^+$ gene product was homologous to the p34 subunit of *Xenopus* MPF (Maturating Promoting Factor) (Dunphy *et al.*, 1988; Gautier *et al.*, 1988). MPF was originally identified as an activity in frog eggs that causes maturation of frog oocytes (Masui and Markert, 1971). Subsequently, MPF was shown to be present also in mitotic mammalian cells (Sukara *et al.*, 1979). Biochemical purification of MPF to near homogeneity in several species then firmly established that it consists of an heterodimeric complex containing one Cdc2-like molecule and one cyclin. Both the kinase activity of the $Cdc2^+$ gene product of *S. pombe* and that of MPF fluctuates during the cell cycle in a similar manner, being maximal during M phase and low during interphase (Sinanis and Nurse, 1986; Moreno *et al.*, 1989). A specific connection between the $cdc2^+$ product and MPF activity was established when it was shown that the 13-kD product of the *S. pombe* gene, $suc1^+$, which interacts genetically with the $cdc2^+$ protein, will immunodeplete frog egg extracts of MPF activity (Dunphy *et al.*, 1988). The evolutionary conserved nature of the cell cycle was further strengthened with the demonstration that a *cdc2* human gene could functionally replace its yeast homologue in *S. pombe* (Lee and Nurse, 1987).

In both budding and fission yeast, a single Cdc2/Cdc28 kinase appears to be sufficient for transition through the major control points in the cell cycle. Higher eukaryotes, however, have more than one Cdc2 homologue, with the name Cdk (cyclin-dependent kinase) being used to describe the family of Cdc2-like kinases. Examples include the human Cdc2 (often referred to as Cdk1) and Cdk2 genes (Lee and Nurse, 1987; Elledge and Spottswood, 1991) the two Cdc2 homologues of *Xenopus* eggs, one

controlling the initiation of S phase, the other having a G2/M role (Dunphy *et al.*, 1988; Fang and Newport., 1991). Recently more Cdks have been isolated, including Cdk3, Cdk4 and Cdk5. Although the functions of these proteins is not known, it is believed that they may also regulate progression through particular phases of the cell cycle (Meyerson *et al.*, 1992). For example, Cdk4 is exclusively associated with human D-type cyclins, which are approximately equivalent to yeast Cln proteins

(ii) Substrates for cyclin-dependent kinases

To fully understand how Cdks regulate progression through the cell cycle it is necessary to identify *in vivo* substrates for their kinase activity. It has been proposed that a protein should be considered a relevant substrate of a Cdk if: (1) the protein is phosphorylated *in vitro* by the Cdk; (2) *in vivo* the protein undergoes appropriately cell cycle regulated phosphorylation; (3) the *in vivo* and *in vitro* phosphorylations occur on common sites; (4) these phosphorylation change the behaviour of the protein in an appropriate way (Nigg, 1993). So far, only three proposed substrates have met these four conditions; SV40 large T antigen (McVey *et al.*, 1993); lamin (Peter *et al.*, 1990, Luscher *et al.*, 1991); and the *S.cerevisiae* transcription factor Swi5 (Moll *et al.*, 1991). From various studies on the phosphorylation of proteins *in vitro*, using purified or partially purified $p34^{cdc2}$, a consensus sequence has been obtained that may identify potential substrates of the kinase. This sequence is Ser\Thr-Pro-X-Z (where X is a polar amino acid, and Z is generally a basic amino acid) (for review, see Moreno and Nurse 1990), although it is known that at least one other protein kinase can phosphorylate such sites (Vulliet *et al.*, 1989).

(iii) Inhibitors of Cdk activity

Related to Cdks, the acronym Cdki has been suggested to describe the class of proteins that negatively control, or inhibit, cyclin-dependent kinase activity (Nasmyth and Hunt, 1993). In mammalian cells at least four different Cdk inhibitors- p16, p21, p24 and p27- have been isolated. Of these, p21 has been best characterized, having been isolated independently by three different groups and variously called Cip1 (Harper *et al.*, 1993), Sdi1 (Noda *et al.*, 1994) and Waf1 (El-Deiry, 1993). This 21-kD protein, from human, is a potent inhibitor of various cyclin-Cdk complexes *in vitro*, and has been proposed to be a universal inhibitor of Cdks (Xiong *et al.*, 1993). Furthermore, several recent papers have indicated how the function of p21 may help to explain deregulated cell division, that is the hallmark of tumour growth, in terms of perturbations in the basic cell cycle machinery. In normal cells, Cdks exist predominantly in multiple quaternary complexes, each containing a Cdk, a cyclin, proliferating cell nuclear antigen (PCNA) and p21 (Xiong *et al.*, 1993). However, in many transformed cells, PCNA and p21 are absent from these complexes, suggesting that p21 may be involved in allowing Cdks to respond to signals which inhibit cell growth. Consistent with this, levels of p21 mRNA rise 10-20 fold when cells become senescent, and also rise as cells become quiescent. In normal cells, the mRNA and protein levels of p21 peak in late G1, and expression of the gene encoding p21 is under control of the p53 tumour-suppressor gene. P53 is a transcription factor, and mutations in the gene contribute to the development of up to 50% of all human cancers.

In contrast to p21, the Cdk inhibitor p16 binds only to one type of cyclin-dependent kinase complex, Cdk4. However, this negative cell cycle regulator is more directly implicated in tumour formation as homozygous deletions of p16 have been found at high frequencies in cell lines derived from tumours of lung, breast, brain, bone, skin,

bladder, kidney, ovary and lymphocyte (Kamb *et al.*, 1994). Of the other two Cdk inhibitors, p24 has homology to dual specificity phosphatases (Gyuris *et al.*, 1993), and p27 blocks the cell cycle in response to transforming growth factor β (Polyak *et al.*, 1994).

1.2 *S. CEREVISIAE* AS A MODEL ORGANISM FOR THE STUDY OF THE CELL CYCLE.

(i) The *S. cerevisiae* cell cycle

S. cerevisiae is a single celled ascomycete yeast which divides mitotically by budding. The onset of budding is approximately coincident with the beginning of DNA synthesis, and thus provides a morphological marker for position in the cell cycle. The control point in G1 at which environmental factors such as nutrient availability and the presence of mating pheromones is assessed is called START. In addition, provided nutrients and other factors are not limiting, START cannot be passed until a critical size has been attained. After passing START a cell is committed to the mitotic pathway; alternatively, undergo conjugation or arrest in stationary phase, or if conditions are appropriate the cell can enter the meiotic pathway.

During S phase a short mitotic spindle forms, a feature not common to all eukaryotic cell cycles. In fact, it has ben argued that S, G2 and M phases overlap in *S. cerevisiae* (Forsburg and Nurse, 1991) and the phases are not precisely defined cytologically. After completion of S phase the cell enters G2, the end of which is marked by the migration of the nucleus to the neck of the budded cell. M phase is characterized by the segregation of the replicated DNA into the two cells. In higher eukaryotes the main

sub-phases of mitosis, namely metaphase, anaphase and telophase, are clearly defined. However, in *S. cerevisiae* no visible condensation of chromosomes can be observed, nor is there any breakdown of the nuclear membrane. Another cell cycle characteristic that *S. cerevisiae* does not share with higher eukaryotes is the timing of the initiation of cell division. In most cells this is started during mitosis, or just after it has been completed. In budding yeast, the first stage in cell division is taken to be bud formation which occurs at the G1/S boundary. One consequence of this is that there is asymmetry of cell division, one of the products (the mother cell), being typically much larger than the other (the daughter cell) As a result, the large mother cell spends only a very short time in G1, requiring less time to overcome the size control required to traverse START (Hartwell and Unger, 1977).

(ii) Cell division cycle genes

In the *S. cerevisiae* cell cycle both haploid and diploid cells undergo mitosis, permitting the isolation of recessive mutations in haploids and their analysis by complementation in diploids. Using this method, over 50 genes have been identified that are required for progress through the cell cycle (Pringle and Hartwell, 1981). *Cdc* (Cell Division Cycle) mutants were originally isolated as conditional mutants which are temperature-sensitive (ts) for growth. Ts mutant strains have a wild-type phenotype at the permissive temperature, usually 25^{0}C, and arrest at the restrictive temperature, 36^{0}C or 37^{0}C. As originally defined, *cdc* mutants result in a defect in a particular stage-specific function of the cell cycle, as inferred from an identical cellular and nuclear morphology at 37^{0}C (Hartwell *et al.*, 1970), regardless of their stage at the time they were shifted from the permissive to the restrictive temperature. About 10% of a larger, general collection of

S. cerevisiae temperature-sensitive mutants were found to be *cdc* mutants, suggesting that there may be as many as 500 genes with stage-specific functions in the eukaryotic cell (Hartwell *et al.*, 1973). The fact that the number of *CDC* genes actually isolated is much less than 500 reflects the difficulty of obtaining temperature-sensitive alleles in many gene products, and the fact that many genes are present in redundant copies.

Two concepts important to understanding the function of *cdc* genes are execution point and terminal phenotype. The execution point is defined as the last point in the cell cycle for which the gene product is required. The terminal phenotype is the morphology characteristic of a particular *cdc* mutant strain when incubated at the restrictive temperature. From the analysis of terminal phenotype and execution point, *cdc* mutants can be segregated into groups which control different aspects of the cell cycle. *Cdc* mutants were isolated which effected the initiation of DNA synthesis, bud emergence, DNA synthesis, medial nuclear division, late nuclear division, cytokinesis and cell separation (Hartwell *et al.*, 1973). For example, the group of original *cdc* mutants which are thought to act at START are *cdc28*, *cdc37*, *cdc36* and *cdc35* (Pringle and Hartwell, 1981). Furthermore, by physiological analysis and ordering the terminal phenotypes of these *cdc* genes with respect to each other, and to the major landmarks in the cycle, a functional sequence map has been constructed (Wheals, 1987).

One of the main conclusions that has been drawn from this work is that there are only a few major rate-limiting steps in the cell cycle, and only when these are complete can other dependent events take place (Nurse and Bisset, 1981). Whether a gene product is involved in a rate-limiting step can be answered empirically, by determining whether speeding up the rate at which the product acts significantly advances progress through the cycle. For example, isolation of alleles of $cdc2^{+}$ that advance the onset of mitosis

suggested that this gene controls an important rate-limiting step in M phase (Draetta, 1990). Similarly, dominant alleles of the G1 cyclin *CLN3* cause cells to divide at a small size (Sudbery *et al.*, 1980), which can also be achieved by overexpression of the G1 cyclins (Richardson *et al.*, 1989). This mutant phenotype can be viewed as an advance of START in cycling populations, thus implying that the G1 cyclins are rate-limiting for execution of this control point.

1.3 PERIODIC EVENTS IN THE YEAST CELL CYCLE.

The cell cycle controls described earlier that involve $p34^{cdc2}$ are post-translational, involving both protein modification (i.e. phosphorylation/dephosphorylation) and degradation (of cyclins). It has also been proposed that transcriptional activation of both structural and regulatory genes under cell cycle control (also known as periodic expression) may be important for proper execution of cell cycle events (McKinney and Heintz, 1991; Johnston, 1990). Although most genes are expressed at roughly constant rates throughout the cell cycle, a small number have been found to fluctuate significantly in a cell cycle dependent manner (Breeden, 1988). In fact, a comprehensive search for genes which are periodically transcribed indicates that there may be up to 250 different cell cycle regulated transcripts (Price *et al.*, 1991). Periodic transcription has been found to occur in all phases of the cell cycle (reviewed in Johnston, 1992), and in many cases the time of expression correlates with the known function of the gene product. However, in no case so far has the cell-cycle regulated transcription of any gene proved to be vital for cell cycle progression.

(i) Regulation of the DNA synthesis genes

In *S. cerevisiae* over 15 genes involved in DNA replication are periodically expressed with a peak of expression at the G1/S boundary (reviewed in Johnston and Lowdnes, 1992; Merrill *et al.*, 1993). Some of these genes encode components of the replication machinery, e.g. *CDC9* (DNA ligase), *POL1* (DNA polymerase) and *CDC8* (Tymidylate kinase). Others encode proteins thought to participate in the initiation of S phase itself, such as *CDC6*. An examination of the promoter regions of this group of genes has found only a hexamer element, ACGCGT, (an *Mlu*I restriction site) as a common motif. This element has been called the *Mlu*I cell cycle box (MCB) (Verma *et al.*, 1991). Three synthetic copies of the MCB sequence were able to impart correct cell cycle expression to a *lac*Z reporter gene (Lowndes *et al.*, 1991). A transcription factor complex DSC1, which binds to MCBs in a periodic manner has also been identified. Two known components of the DSC1 complex are the Swi6 and Mbp1 proteins. Swi6 does not directly bind to MCB elements, but probably functions as a regulatory component of the DSC1 transcription factor complex (Lowndes *et al.*, 1992). Yeast strains deleted for either Swi6 or Mbp1 are viable but exhibit de-regulated expression of DNA synthesis genes (Koch *et al.*, 1993; Lowdnes *et al.*, 1992).

(ii) Regulation of the histone genes

The histones are also under tight cell cycle control, being expressed in S phase. Moreover this pattern of expression is essentially similar in all eukaryotic organisms (McKinney and Heintz, 1991). This periodic expression may be a means of confining histone production to S phase when they are required for chromatin synthesis. In *S. cerevisiae* there are four pairs of divergently transcribed histone genes. Promoter deletion

experiments have identified a 16bp sequence, GCGAAAAANTNNGAAC, which can confer cell cycle regulated transcription upon heterologous genes (Osley *et al.*, 1986). One pair of divergently expressed histone genes *H2A* and *H2B* have two copies of this sequence in their common promoter. Between the repeats is a negative control element, deletion of which results in over production of the transcripts (Osley *et al.*, 1986; Lycan *et al.*, 1987).

(iii) Regulation of the G1 cyclins

Another important group of genes with mRNA periodicity are the cyclins. In *S.cerevisiae* there are two groups of cyclins, the mitotic cyclins, encoded by the *CLB* genes, and the G1 cyclins, encoded by the *CLN* genes.

At present there are three well characterized G1 cyclins in budding yeast, *CLN1*, *CLN2* and *CLN3*. *CLN3*, originally called *WH1-1*, was originally isolated as a dominant mutation which caused cells to pass START at a reduced cell size (Sudbery *et al.*, 1980). *CLN1* and *CLN2* were isolated as high copy suppressors of the *cdc28-4* allele (Hadwiger *et al.*, 1989a). The three *CLN* genes constitute a genetically redundant gene family, which between them carry out an essential function in G1. If any two *CLN* genes are deleted the cells are still viable, however if all three are deleted the effect is lethal and the cell arrests in G1 (Richardson *et al.*, 1989). Two of the G1 cyclin genes *CLN1* and *CLN2* are maximally expressed in G1, while *CLN3* is constitutively expressed. Regulation of *CLN1* and *CLN2* transcription is controlled by the Swi4/Swi6 transcription factor complex. The *SWI4* and *SWI6* genes were isolated on the basis of their requirement for START-dependent transcription of the *HO* gene, which encodes a double-stranded DNA endonuclease that initiates mating type switching in homothallic yeast strains (Andrews

and Herskowitz, 1989).

The promoter element recognised by the Swi4/Swi6 complex is called SCB (Swi6 dependent cell cycle box), and consists of the sequence CACGAAAA. SCB elements are present in the promoter sequences of *CLN1*, *CLN2* and *HO* (Breeden and Nasmyth, 1987), and it has been shown that *CLN1* and *CLN2* transcript levels are substantially reduced in strains deleted for Swi6 (Lowndes *et al.*, 1992; Nasmyth and Dirick, 1991). The *CLN1* and *CLN2* protein products also fluctuate in abundance during the cell cycle, in a manner that correlates with the associated transcript levels, accumulating during G1 and being degraded during S phase (Wittenberg, 1990). The degradation of the G1 cyclins may be due to the PEST regions residing in all three Cln protein sequences. PEST regions are sequences rich in proline (P), serine (S) and threonine (T), and are thought to be signals for proteolysis (Rogers *et al.*, 1986). The dominant mutants of *CLN3* which confer small cell size are deleted for the C-terminal third of the protein which is rich in PEST residues. In fact, constitutive expression of any Cln leads to cells accelerating through START at a reduced cell size. Thus periodic expression of the G1 cyclins may serve to co-ordinate cell growth with the mitotic cell cycle.

A novel mechanism has been proposed to account for the control of *CLN1* and *CLN2*. This proposes that a positive feedback loop exists whereby activation of $p34^{CDC28}$ by Cln proteins directly promotes increased *CLN1* and *CLN2* mRNA accumulation (Cross and Tinkelenberg, 1991). Cdc28 is thought to activate *CLN1, CLN2* levels by phosphorylating the Swi6 component of the Swi4/Swi6 complex. *CLN3* expression is independent of the feedback loop and may function upstream of the other *CLN* genes, perhaps involved in their regulation (reviewed in Reed, 1991). In this regard, it has recently been shown that a burst of Cln3 activity is able to induce the transcription of

both SCB-related genes (*CLN1*, *CLN2*) and the DSC1-regulated genes (Tyers *et al.*, 1993).

Two other putative G1 cyclins, *HCS26* (Ogas *et al.*, 1991) and *ORFD* (Frohlich *et al.*, 1991), have unknown functions and are not capable of allowing the cell to progress into S phase in the absence of *CLN1*, *CLN2* and *CLN3*. *HCS26* was isolated as a high copy suppressor of a *swi4*-ts mutant, and like *CLN1* and *CLN2*, the *HCS26* promoter contains the SCB motif.

(iv) Regulation of Mitotic cyclins

In *S. cerevisiae* there are six mitotic cyclins, called *CLB1-CLB6*. *CLB1*, *CLB2* and *CLB4* were isolated as high copy suppressors of the *cdc28-1N* allele which causes G2 arrest, while *CLB3* was obtained by polymerase chain amplification (PCR) (Surana *et al.*, 1991). *CLB1* and *CLB2* are closely related at the level of protein sequence homology, as are *CLB3* and *CLB4*. Cells deleted for either *CLB1* or *CLB2* are viable, although deletion of both genes results in synthetic lethality. Of the two, *CLB2* seems to have the most important role in mitosis, as cells deficient in the gene exhibit a delay in the onset of M phase (Surana *et al.*, 1991). Both *CLB1* and *CLB2* are coordinately expressed in the G2/M phase of the cell cycle, transcript levels declining as cells exit from mitosis. Little is known about the transcription factors responsible for regulating *CLB* expression. However *CLB1* and *CLB2* regulation appears very similar to that of the *SWI5* gene, whose promoter requires the formation of a ternary complex containing the Mcm1 transcription factor (Lydall *et al.*, 1991). As with G1 cyclins, the Clb1 and Clb2 proteins are also periodically expressed in a similar pattern to the transcript levels. In higher eukaryotes it has been demonstrated that mitotic cyclins are degraded by the ubiquitin proteolytic pathway. Ubiquitin is a highly conserved protein which can be covalently linked through

isopeptide bonds to lysine residues in proteins. When proteins are multi-ubiquinated at a single site they become targeted for degradation. Mitotic cyclins are targeted to this pathway by an N-terminal consensus sequence called the destruction box (Glotzer *et al.*, 1991). In *S. cerevisiae* all four mitotic cyclins have a sequence at the amino terminus that fits this consensus (Richardson *et al.*, 1992).

A start has been made in understanding how the transition from *CLN* to *CLB* expression is regulated during the cell cycle, with the finding that *CLB1* and *CLB2* stimulate their own expression while repressing that of the *CLN*s (Amon *et al.*, 1993). This process is thought to be mediated by phosphorylation and inactivation of the Swi4 transcription factor, by a Clb-Cdc28 kinase complex.

Recently a new family of B-type cyclins, *CLB5* and *CLB6*, have been isolated and characterized (Epstein and Cross, 1992; Schwob and Nasmyth, 1993). The *CLB5* transcript peaks in abundance in G1, coincident with *CLN2*. Deletion of *CLB5* results in an extended S phase, and it is suggested that the gene may have a result in promoting the G1/S phase transition.

1.4 REGULATION OF THE *CDC28* PROTEIN KINASE IN THE CELL CYCLE

In the budding yeast *S. cerevisiae*, regulation of the activity of the $p34^{CDC28}$ protein kinase is central to ordered progression through the cell cycle (for a recent review, see Nasmyth, 1993). Activation of the kinase is required for traversal of START in G1, where commitment to a new round of cell division occurs, and for the initiation of mitosis at the G2/M boundary. In most eukaryotes, activation of the $p34^{cdc2}$ kinase at the G2/M boundary is due to dephosphorylation of the highly conserved Tyr15 residue of the Cdc2 protein.

However, dephosphorylation of the equivalent tyrosine residue, Tyr 19, in *S. cerevisiae* is not required for activation of the kinase (Amon *et al.*, 1992; Sorger *et al.*, 1992).

Although Cdc28 protein is present at a constant level throughout the cell cycle, its kinase activity, as measured by phosphorylation of the exogenous substrate histone H1, fluctuates periodically (Reed and Wittenberg, 1990). The control of Cdc28 function is, in part, effected by the formation of stage-specific, distinct, heteromeric $p34^{CDC28}$ kinase complexes, the monomeric form of the protein having little or no activity. At the two major control points, START and the G2/M interface, activation of Cdc28 requires its association with different members of the Cln/Clb cyclin families described above (for review, see Lew and Reed, 1992). Exit from mitosis may also require deactivation of the kinase, and concomitant destruction of the associated cyclin by proteolysis (Ghiara *et al.*, 1991). Although it has been shown that the minimum requirement for protein kinase activity is a heterodimer consisting of a cyclin and a Cdk catalytic subunit, it is clear that *in vivo* complexes are significantly larger than this minimum size. It is possible that properties such as substrate specificity, affinity and intracellular location may depend on components other than cyclins or Cdks.

Predating the identification of cyclins as regulatory subunits in $p34^{CDC28}$ heteromeric complexes, two other proteins were shown to be associated with the active form of the kinase. Cks1 is an 18-kD protein, isolated in a screen for high copy suppressors of the temperature sensitive *cdc28-1* mutation (Hadwiger *et al.*, 1989b), and homologous to the *S. pombe* Suc1 protein that interacts with Cdc2 (Hayles *et al.*, 1986). Furthermore, it was demonstrated that Cks1 co-precipitated with Cdc28, and associated with a 160-kD complex having Cdc28-dependent kinase activity. The *CKS1* gene is essential and *cks*-ts alleles impair the ability of cells to undergo both the G1/S phase and

G2/M phase-transitions of the cell cycle (Tang and Reed, 1993). The role of Cks1 may be to form specific multimeric complexes, or to localize Cdc28 properly in cellular compartments. Homologues have been found in all eukaryotes where they have been searched for (Richardson *et al.*, 1990), and all are able to complement a deletion of *CKS1* in yeast. Human cells contain two isoforms of Cks proteins: namely CksHs1 and CksHs2. The atomic crystal structure of CksHs2 has been determined at 2.1A resolution, and found to be a hexamer formed by the symmetric assembly of three interlocked dimers. This suggests that CksHs2 may participate in cell cycle control by acting as the hub for Cdk multimerization *in vivo* (Parge *et al.*, 1993). Another component of the 160-kD $p34^{CDC28}$ complex is a 40-kD protein, p40, that is also phosphorylated, in a Cdc28-specific, cell-cycle dependent manner (Reed *et al.*, 1985; Wittenberg and Reed, 1988; Reed *et al.*, 1990). Recently, using p40 purified from stationary-phase cells, the 40-kD protein has been shown to bind tightly to Cdc28, and to inhibit its kinase activity (Mendenhall, 1993). This has led to speculation that p40 may be a *S. cerevisiae* Cdk inhibitor (Nasmyth and Hunt, 1993; Hunter, 1993).

Another *S. cerevisiae* Cdk inhibitor is the 120-kD Far1 protein that has been demonstrated to bind to Cdc28 and inhibit its kinase activity (Peter *et al.*, 1993). Far1 is a non-essential gene, necessary for cell cycle arrest by α-factor (Chang and Herskowitz, 1990). Far1 is thought to arrest the cell cycle in G1 by binding to, and inhibiting, a Cln2-Cdc28 kinase complex. Far1 is phosphorylated *in vitro* by the Cln2-Cdc28 kinase complex, and *in vivo* in a *CDC28*-dependent manner (Peter *et al.*, 1993). Far1 protein levels are cell cycle regulated such that functional levels of Far1 accumulate only in pre-START G1 cells (McKinney *et al.*, 1993). As cell traverse START, Far1 is rapidly down-regulated, which may be a specific consequence of the activation of Cdc28. Interestingly,

cell cycle regulated expression of *FAR1* expression involves independent controls on *FAR1* transcription and protein accumulation, as cells in G2/M contain high levels of *FAR1* mRNA but low levels of Far1 protein (McKinney *et al.*, 1993). The authors speculate that the accumulation of *FAR1* transcript in G2/M is required to facilitate the rapid accumulation of Far1 protein when cells enter G1.

1.5 *S. CEREVISIAE* CDKS

Besides Cdc28, there are also two other Cdks in *S. cerevisiae*, *PHO85* (Toh-e *et al.*, 1988), and *KIN28* (Simon *et al.*, 1986). Pho85 is about 50% identical to Cdc28, and recent research indicates that it has a role not only in cell cycle progression but also in general cell metabolism (Kaffman *et al.*, 1994). Earlier genetic studies had identified *PHO85*, and another gene *PHO80*, as negative regulators of a phosphatase that provides yeast cells with a critical nutrient, inorganic phosphate (Gilliquet *et al.*, 1990). Kaffman and workers have found that *PHO80* has homology to GI cyclins, and is 33% identical to Hcs26 and OrfD in a region that is conserved in all cyclins. Furthermore, they found that Pho80 and Pho85 act as a cyclin-Cdk complex that phosphorylates a transcription factor which regulates the phosphatase expression. Thus the Cdk Pho85 appears to have two separate cyclin partners, indicating that it may play a role in integrating nutritional input with the cell cycle machinery.

The other *S. cerevisiae* Cdk, Kin28, is an essential serine-threonine kinase, 38% homologous to Cdc28. Multicopies of a novel, putative cyclin gene *CCL1*, are able to suppress the thermosensitivity of two *kin28*-ts mutants (Valay *et al.*, 1993), supporting the notion that Kin28 may also be a cyclin dependent kinase.

1.6 ANOTHER PROTEIN KINASE, *DBF2*, FUNCTIONS IN BOTH G1/S AND M/G1

The original search for *cdc* mutants in the period from START to the initiation of DNA synthesis identified three genes *CDC28*, *CDC7* and *CDC4* (Pringle and Hartwell, 1981). Both *CDC28* (Reed *et al.*, 1985) and *CDC7* (Bahman *et al.*, 1988) were subsequently found to be protein kinase homologues, while *CDC4* has homology with the oncogene *ets* (Peterson *et al.*, 1984). To identify further genes connected with S phase and its control a new screen was carried out for mutants defective in DNA synthesis (Johnston and Thomas, 1982). Such mutants are unable to progress through the cell cycle and arrest in G2 where they accumulate as large-budded cells or "dumbbells". Four dumbbell-forming mutants were isolated and identified specifically as being defective in DNA synthesis, either in initiation or replication itself.

Of these *dbf* genes, *DBF2* was subsequently sequenced and the gene product found to have all eleven conserved domains found in protein kinases (Johnston *et al.*, 1990). Further analysis of *dbf2* mutants showed that, despite being isolated on the basis of a defect in DNA synthesis, it in fact only underwent a 40 minute delay before continuing S-phase to finally block in late M-phase. It is also expressed periodically in the cell cycle, with a peak in late mitosis. Consistent with this, by execution point analysis it has recently been shown that Dbf2 carries out an essential function after the metaphase to anaphase transition (Toyn and Johnston, 1994). Although *DBF2* mutant alleles are conditionally lethal, strains with a deletion of *DBF2* are viable. This is explained by the presence of a structural and functional homologue, *DBF20*, which is over 80% identical to *DBF2* at the amino acid level (Toyn *et al.*, 1991). Either *DBF2* or *DBF20* can be deleted from haploid yeast strains, but deletion of both is lethal. However *DBF20* expression is not under cell cycle control. Recently the Dbf2/20 kinase activity has been

shown to be cell cycle regulated in a manner similar to the *DBF2* transcript (Toyn and Johnston, 1994). The kinase activity is specific for serine/threonine residues, and Dbf2 accounts for the bulk of the activity, with Dbf20 playing a minor role

1.7 MOLECULAR SUPPRESSORS OF *DBF2*

Given that *DBF2* is a cell cycle regulated protein kinase, it was decided that its physiological role should be further examined by attempting to find proteins with which it interacts, which might include possible substrates for its enzymatic activity. One method for achieving this, which has previously proved successful in yeast, is the isolation of high copy number molecular suppressors (Hayles *et al.*, 1986; Hadwiger *et al.*, 1989b; Wittenberg *et al.*, 1990; Adams *et al.*, 1989). In this approach a strain containing a conditional mutation in the gene of interest is transformed with a genomic library on a high copy number plasmid. Any colonies that now grow under restrictive conditions are assumed to be viable due to overexpression of a gene on the plasmid complementing, or suppressing, the mutation in the gene of interest. This method, also known as dosage suppression, assumes that increasing the intracellular concentration of one component of an interacting system can help stabilize the function of another, possibly by increasing the interactions or even by bypassing the defective function.

Screening of a *S. cerevisiae* genomic library led to the isolation of five dosage suppressors of *dbf2*, pSDB21-25. Three of these genes (*SDB21-23*) have subsequently been characterized, two of which turned out to be previously identified genes.

SDB21 is the sporulation gene *SPO12* which has been shown to be essential for meiosis (Malavasic and Elder, 1990). *SPO12* can suppress all three *dbf2*-ts alleles, even

when just one extra copy of *SPO12* is added to cells. However, suppression of *dbf2* mutants by *SPO12* requires *DBF20*. That is, *SPO12* can no longer suppress *dbf2*-ts alleles in strains deleted for *DBF20*. Detailed examination of the *SPO12* transcript shows that it is coordinately regulated with *DBF2*, and deletion of both *DBF2* and *SPO12* results in synthetic lethality (Parkes and Johnston, 1992). Furthermore, recent evidence indicates that *SPO12* may be a regulator of Dbf2 function (Toyn and Johnston, 1993). *SDB22* is *SIT4* (Parkes and Johnston, 1992) which encodes a protein highly similar to the catalytic subunit of mammalian type 1 and type 2A phosphatases. *SIT4* is required for the G1/S transition of the cell cycle (Sutton *et al.*, 1991), and for the normal accumulation of G1 cyclin RNA (Fernandez-Sarabia *et al.*, 1992). *SDB23* has only recently been identified and is believed to have homology to a human small ribonuclear protein.

Besides the five high copy suppressors of *dbf2* mutants isolated from a genomic library screen, three other genes have been shown to suppress alleles of *dbf2*. Two of these, *CDC5* and *CDC15,* encode protein kinases which are thought to function in M phase (Kitada *et al.*, 1993; Schweitzer and Phillipsen, 1991). The *CDC5* kinase may be a homologue of the Drosophila kinase encoded by the *polo* gene, which is activated during late anaphase to telophase (Fenton and Glover, 1993). The third high copy suppressor is *CDC14* which, like *SIT4*, encodes a protein phosphatase (Wan *et al.*, 1992). Interestingly, the cell cycle phenotypes of *cdc5*, *cdc14* and *cdc15* are all similar to *dbf2*. Moreover, Cdc28 kinase activity is high in all four mutants; *dbf2* (Toyn and Johnston, 1994), *cdc15* (Surana *et al.*, 1993), *cdc5* and *cdc14* (Toyn and Johnston, unpublished observations). One possibility is that all four genes are involved in a network of regulatory phosphorylations that occurs in late nuclear division. The purpose of this regulatory network could be to ensure the correct order of molecular events to ensure the exit from

M phase occurs at the appropriate time.

1.8 AIMS OF THE PROJECT

The other two suppressors of *dbf2*, pSDB24 and pSDB25, are allele-specific, rescuing only *dbf2-3* at the restrictive temperature. The characterization of these two genes form the basis of this project. Initially, the suppressor activity on the chromosomal fragments of pSDB24 and pSDB25 will be localized, enabling the relevant gene to be identified and then sequenced. Analysis of the transcript levels of both genes during the cell cycle will be undertaken, using synchronized cell cultures. If they are found to be periodically regulated this will provide an important indication that the genes have a cell cycle, phase-specific, function. Null alleles of each gene will be generated, through targeted knockout of each chromosomal gene by homologous recombination. This will allow determination of the importance of each gene for cell cycle progression, that is whether they are essential for completion of the cell cycle, or whether deleting either *SDB24* or *SDB25* results in a delay in a particular phase of the cell cycle. If either gene is non-essential, double deletions will be generated with that gene and the other suppressors, and also *DBF2/DBF20*. Any double knockout mutants that are inviable (synthetic lethals), will be evidence of genetic interactions between the two genes concerned.

Biochemical studies will also be undertaken to try and establish if there is a direct physical association between the suppressors and Dbf2. This will be done by firstly raising polyclonal antibodies against one, or both, suppressor proteins. Besides enabling immunoblotting, and coimmunoprecipitation experiments to be carried out, it will also be

possible to determine whether Sdb24, or Sdb25, has an associated kinase activity *in vitro*. The cell cycle levels of the proteins can also be determined and compared with their transcript levels.

From the results of the genetic, physiologieal and biochemical experiments proposed it is hoped that some insights may be gained into the function of both Sdb24 and Sdb25. The principal objective of these experiments being to further the understanding of the cell cycle role of the regulatory protein kinase Dbf2.

CHAPTER TWO

MATERIALS AND METHODS

2.1 BACTERIAL STRAINS

For routine bacteriological work, the strain used was *Eschericia coli* DH5α (F$^-$, *ϕ80dlacZΔMI5 recA1 endA1 gyrA96 thi-1 hsdR17*(r_k-,m_k+) *supE44 relA1 decR* (*lacZYA-argF*) *U169*). For transposon mutagenesis of pSDB25 the donor strains was MHI343 (F$^+$ *srl*::Tn10 *recA1 deoC*), and the recipient strain MHI755 (*srl*::Tn10 *recA1 rps3L thr-1 ara-14 leuB6 lacY1 (gpt-proA)62 supE44 tsx-33 galK2 hisG4 rfbD1 mgl-51 kdgKS1 xyl-5 mtl-1 argE3 thi-1*).

2.2 YEAST STRAINS

Details of yeast strains used in this study are given in Table 2.1.

2.3 MEDIA AND CULTURAL CONDITIONS

(1) *E.coli*

E. coli strains were grown in Luria-Bertani broth (1% bacto-tryptone, 0.5% yeast extract, 1% NaCl pH 7.5) with the addition of 50μg/ml ampicillin and 50μg/ml methicillin from filter sterilised stock solutions for the selection of plasmids. Liquid cultures were grown in flasks at 37^{0}C with continuous shaking. For solid media, 1.5% Difco agar was added and the agar plates incubated at 37^{0}C in a constant temperature incubator. Strains

Table 2.1 Yeast strains

Strain	Relevant genotype	Source
L119-7D	MATα *dbf2-1 ade1 trp1-289 ura3-52*	Parkes and Johnston 1992
L181-6B	MATα *dbf2-2 leu2-3,112 trp1-289 ura3-52*	Toyn and Johnston, 1993
L182-7A	*MAT***a** *dbf2-3* ade1,5 leu2-3,112 trp1-289 ura3-52	This study
cdc28-1N	*MAT***a** *cdc28-1N ade2-1 leu2-3,112 trp1-1 ura3*	Kitada *et al.*, 1993
cdc28-13	*MAT***a** *cdc28-13 ade1 leu2-3,112 trp1 ura3Δ*	Kitada *et al.*, 1993
L153-6C	*MAT*α *cdc28-4 leu2 trp1 ura3 his7 tyr1*	This study
L195-6D	*MAT*α *cdc14 ade1 ura3 trp1*	This study
S7-4A	*MAT***a** *dbf2Δ::URA3 ade5 his7 leu2-3,112 trp1 ura3*	Toyn *et al.*, 1991
S2-2D	*MAT*α *dbf2Δ::LEU2 ade5 leu2 trp1*	Toyn *et al.*, 1991
CG378	*MAT***a** *ade5 leu2-3,112 trp1-289 ura3-52*	C. Giroux
CG379	*MAT*α *leu2-3,112 trp1-289 ura3-52 his7-2*	C. Giroux
J99	CG378 *dbf20Δ::TRP1*	Toyn *et al.*, 1991
V378	CG378 *spo12Δ::TRP1*	Parkes and Johnston, 1992
JD100	CG379 *sdb25Δ::TRP1*	This study
J149	*MAT***a** *dbf2-2 ade2-101 leu2 trp1 ura3-52*	Toyn and Johnston, 1993
LJ1	*dbf2-2/dbf2-2*	L181-6B x J149
J103-2A	*MAT*α *dbf20Δ::TRP1*	J99 x L181-6B
JD101	*MAT***a** *dbf2-3 dbf20Δ::TRP1*	L182-7A x J103-2A

were stored for up to several weeks at 4°C on LB agar (with ampicillin as required), but for long term storage, strains were grown to stationary phase in LB and 0.85ml of this culture was mixed with 0.15ml of sterile glycerol and stored at -70°C.

(ii) *S. cerevisiae*

For routine growth in rich media YEPD was used (1% Difco yeast extract, 2% Bacto-peptone and 2% glucose). The minimal medium used for selective growth of strains was Wickerham's basal (WB) (Wickerham, 1951), or yeast nitrogen base (YNB), plus 2% glucose with the appropriate amino acid supplements added at 40μg/ml (except for leucine which was added at 80μg/ml) from 10mg/ml sterile stock solutions. Liquid cultures were grown at 25°C, 30°C or 37°C with continuous shaking, as stated. For solid media, 2% New Zealand agar was added and the plates incubated at 25°C, 30°C or 37°C in a constant temperature incubator.

Yeast strains were stored for up to several weeks on YEPD or minimal agar plates at 4°C. For longer term storage, freshly grown cells were removed from agar plates and inoculated into 1ml of storage medium (1% yeast extract, 1% Bacto-peptone, 2% glucose and 25% glycerol) and stored at -70°C.

Diploids were incubated on potassium sporulation medium (KSM, 1% potassium acetate and 2% New Zealand agar) at 25°C or 30°C for two to seven days to permit sporulation to occur. Tetrads were dissected using an electronic Singer Micromanipulator after digestion with 5% glusalase (Sigma Biochemicals) at room temperature for 30 minutes.

2.4 STANDARD BUFFERS

TE 10mM Tris HCL, 1mM EDTA, pH8.0

TAE 40mM Tris base, 20mM glacial acetic acid, 1mM EDTA pH8.3

TBE 100mM Tris base, 100mM Boric acid, 0.05mM EDTA pH8.3

Saline 0.9% NaCl

2.5 ISOTOPES

Radiolabelled isotopes [γ-^{32}P] ATP (5000Ci/mol), [α-^{32}P] dTTP (3000Ci/mol) and [^{35}S] methionine (1200 Ci/mol), were obtained from Amersham (UK)

2.6 DNA/RNA TECHNIQUES

(i) Recovery of DNA fragments from agarose gels

Two methods were used for the extraction of DNA from agarose gels. Glass milk extraction was used to remove small fragments or amounts of DNA from TAE gels using the method of Vogelstein and Gillespie (1979). Electroelution was used to purify linearised vector or large amounts of DNA from TBE or TAE gels as described (Sambrook *et al.*, 1989).

(ii) DNA precipitation

DNA was precipitated from aqueous solution by adding 3M Sodium acetate to a final concentration of 0.3M, and three times the volume of Analar absolute alcohol, then cooling the sample to -20°C or -70°C for a minimum of 30 minutes. Precipitated DNA was pelleted by centrifugation at 15,000 r.p.m. for 20-30 minutes in a fixed angle Sorvall rotor. The dry DNA pellet was then dissolved in sterile distilled H_2O or 1xTE.

(iii) DNA ligations

Ligation of DNA fragments were carried out in a 20µl volume in 1x ligation buffer (50mM Tris-HCL pH7.8, 10mM $MgCl_2$, 50µg/ml BSA) with the addition of 1.0mM rATP, 20mM DTT and 0.4 units of T4 DNA ligase. Ligations were incubated at 25^0C for 5-16 hours before transformation into *E. coli.*

(iv) Flush ending of DNA fragments with T4 DNA polymerase

Flush ending of DNA fragments (0.2-5µg) was performed with T4 DNA polymerase (1-2 units) in a restriction endonuclease buffer with the addition of dNTPs to a final concentration of 2mM essentially as described (Sambrook *et al.*, 1989).

(v) Restriction endonucleases and DNA modifying enzymes

Restriction endonucleases were obtained from Bethesda Research Laboratories (BRL) and were incubated in the appropriate GIBCO-BRL restriction endonuclease buffer at the recommended temperature. Cloned Klenow polymerase 1, T4 DNA polymerase and T4 DNA ligase were obtained from Pharmacia and used with the recommended buffers. Molecular biology grade calf intestinal alkaline phosphatase was obtained from Boehringer Mannheim GmBH. T7 RNA polymerase and T3 RNA polymerase were obtained from Promega.

(vi) Agarose gel electrophoresis

Agarose gel electrophoresis was carried out in 0.8% w/v agarose gels with TAE electrophoresis buffer. DNA was loaded onto gels with 1/6 volume loading buffer (0.1% Bromophenol blue, 50% glycerol, 50mM EDTA) and run with a constant current of 50-

100mA. Gels were stained with 2μ/ml ethidium bromide for 20 minutes followed by destaining in water for 10 minutes. Gels were visualized using a Uniscience TF-20M transilluminator.

(vii) Southern hybridization

Samples (2-5μg) of genomic DNA were digested, electrophoresed through a 0.8% agarose gel and used for nucleotide hybridization using standard methodology (Sambrook *et al.*, 1989). Genescreen transfer membranes (New England Nuclear) were used, as described in the manufacturer's instructions.

(viii) Northern hybridization

Total RNA samples (5μg) were denatured in the presence of 0.5M deionised glyoxal, size separated by electrophoresis in a 1.5% agarose gel and transferred by capillary action to Genescreen membrane (New England Nuclear) as described in the manufacturer's instructions.

(ix) Hybridization and probing of blots

Further treatment of the Genescreen membrane, hybridisation (in the presence of Dextran Sulphate), washings and rehybridization were performed exactly as described in the manufacturer's instructions (New England Nuclear). To remove redundant bound probes from the Genescreen membranes, the membrane was incubated for 30 minutes at 60^{0}C in 96% deionised formamide, 10mM Tris-HCL pH 8.0, 10mM EDTA pH 8.0.

Probe DNA for hybridisation was labelled with ^{32}P-dTTP, using the oligolabelling protocol of Feinberg and Vogelstein (1983). Autoradiography was carried out at -70^{0}C

using Fuji RX X-ray film, or the supersensitive Kodak XAR-5 X-ray film in conjunction with X-ograph Hi-speed-X intensifying screens.

(x) DNA fragments used for oligolabelling

Probe	Fragment	Source
POLI	3.2kb *Hind*III-*Sal*I	Lucchini *et al.*, (1985)
H2A/Protein 1	2.3kb *Sst*I-*Sst*I	Hereford *et al.*, (1979)
ACTIN	563bp *Cla*I-*Cla*I	Gallwitz and Sures, (1980)
DBF2	1.3kb *Eco*RI-*Eco*RI	Johnston *et al.*, (1990)

(xi) DNA sequencing procedure

DNA sequence was determined by the dideoxy method (Sanger *et al.*, 1977) using a Sequenase DNA sequencing kit (US BIochemical Corporation). 2μg of supercoiled plasmid DNA in 20μl of 1xTE was denatured by the addition of 2M NaOH 2mM EDTA, left to stand at room temperature for 5 minutes and subsequently precipitated with 70% ethanol. The dried DNA pellet was redissolved in 1μl DNA primer to which 13.5μl reaction mix (containing 7μl H_2O, 2μl sequenase buffer, 0.5μl 0.1M DDT, 2μl 1xlabelling mix and 1μl [^{35}S]-TTP) was added and the mixture incubated at 37^{0}C for fifteen minutes to enable annealing of the primer to template DNA. 3μl of Sequenase enzyme, dilute one in eight with cold 1xTE, was added to the annealed primer-template mix and incubated at room temperature for a further ten minutes. 3.5μl of this labelled reaction mixture was then added to four tubes, each containing one of the dideoxynucleotides ddATP, ddTTP, ddCTP, ddGTP and further incubated in a 37^{0}C waterbath for ten minutes. The reaction was terminated by the addition of 4μl of stop buffer (95% Formamide, 20mM EDTA,

0.05% bromophenol blue, 0.05% xylene cyanol FF), denatured by heating to 90°C for 2 minutes and loaded onto a 6% polyacrylamide gel. Sequence data was analyzed by computer using the Wisconsin GCG programme.

(xii) Polyacrylamide gel electrophoresis

Gel electrophoresis of sequencing samples was performed in 6% polyacrylamide, 7M urea sequencing gel made from Sequagel stock solutions (National Diagnostics) in TBE running buffer. Electrophoresis was for 4-9 hours at a constant 60W power. Once electrophoresis was completed the gel plates were dismantled and the gel soaked for 15 minutes in a 10% methanol, 10% glacial acetic acid solution. The gel was then blotted dry, transferred to Whatmans 3MM paper and covered with Saran wrap. The gel was finally dried under vacuum and with heat on a Biorad gel drier for 10-15 minutes before autoradiography for 16-24 hours at room temperature using Fuji RX X-ray film.

(xiii) Primers used in DNA sequencing

For fragments of pSDB24 cloned into the multiple cloning site (MCS) of Bluescript the Universal primers M13 "-40" (5′-GTTTTCCCAGTCACGAC-3′), and M13 Reverse (5′-GTACCAGTATCGACAA-3′) were used. For sequencing of *sdb25*::Tn*1000*, the δ and γ primers specific to the ends of Tn*1000* were used. The δ primer (5′-AGGGGAACTGAGAGCTCTA-3′) is homologous to bases 86 to 68 of Tn*1000*, the γ primer (5′-CAGCTACAACATACGAAAG-3′) has its 3′ end 70bp from the terminus of Tn*1000*. All other sequencing primers were synthesized 17-mer oligonucleotides complementary to previously determined sequence.

2.7 *S. CEREVISIAE* TECHNIQUES

(i) Lithium acetate transformation

S. cerevisiae cells were transformed by the lithium acetate transformation protocol (Ito *et al.*, 1983). A 50ml culture was grown to $1x10^7$ cells/ml, harvested and washed twice in 10ml of 1x TE buffer. The cells were then harvested and resuspended in 5ml of LA (0.1M lithium acetate in 1x TE) solution and incubated at 30°C (or 25°C) for one hour with aeration. The cells were then harvested and resuspended in 5ml of LAG (0.1M lithium acetate, 15% glycerol in 1x TE) solution. 1-10μg of DNA was added to a 0.3ml aliquot of cells together with 5μg of "carrier" single stranded salmon sperm DNA. 0.7ml of 50% PEG 4000 (polyethylene glycol, Sigma Chemical Company) was then added and the cell suspension mixed by inversion before incubation at 30°C (or 25°C) for one hour. The cells were then heat shocked at 42°C for five minutes and 0.2ml aliquots of the transformation mixture directly plated onto selective media.

(ii) Small scale yeast plasmid preparations

Small amounts of yeast plasmids were prepared from 5ml yeast cultures by the method of Hoffman and Winston (1987). These plasmids were then transformed into *E. coli* for larger scale recovery and subsequent analysis.

(iii) Isolation of yeast genomic DNA

Yeast genomic DNA for Southern Hybridisation analysis was prepared from 10ml stationary phase yeast cultures as described by Hoffman and Winston (1987). Approximately 10-20μg of genomic DNA were obtained from each preparation.

(iv) Gene replacement

A null allele of *SDB25* was constructed by the one step gene disruption method (Rothstein, 1983). The 740-bp *Kpn*I-*Hpa*I fragment of *SDB25* was replaced by an 850-bp fragment containing the *TRP1* gene, this disruption being made in pSDB25. The DNA fragment containing *sdb25Δ::TRP1* was generated by *Nco*I-*Ssp*I digestion and used to transform the *trp1/trp1* diploid CG378/379. Selected diploid prototophs were determined for *SDB25* deletion by Southern blot analysis.

A null allele of *SDB24* was constructed by ligating the 5.6kb *Pst*I-*Sal*I fragment of pSDB24, containing *SDB24*, into the MCS of Bluescript. The internal 1.6 *Eco*RI-*Bgl*II fragment of *SDB24* was excised and replaced with the *URA3* gene. Subsequent steps were essentially the same as those for the *SDB25* null allele.

(v) Preparation of total RNA for Northern hybridisation analysis

10^8 yeast cells were harvested, washed in saline and frozen rapidly in dry ice. Total RNA was extracted using a hot phenol protocol described by (Aves *et al.*, 1985). RNA concentrations were determined, by measuring the A_{260}, and then diluted to 1mg/ml.

(vi) Culture synchronization methods

The most convenient method of synchronisation for *S. cerevisiae* cultures is by the arrest of the cell cycle by α-factor, a mating pheromone and the release of the cells into a new cell cycle following the removal of the α-factor so that the cells undergo a synchronous round of division. Haploid *Mata S. cerevisiae* strains were grown in rich medium to $5x10^6$ cells/ml at 25^0C. Alpha-factor was then added to a final concentration of 2.5 to 3.5μg/ml and the cells were then incubated for 3 hours. The α-factor was then

removed by rapid filtration and washing of the cells twice with saline and once with fresh media before finally resuspending in an equal volume of fresh media for their synchronous outgrowth.

Three other methods of synchronisation were utilized in this study: elutriation, feed-starve and a *dbf2* temperature shift. In all three cases other workers generated the synchronous culture, as indicated in the appropriate place in the relevant Figure Legend.

(vii) Determination of budding index and cell numbers

The budding index (the percentage of budded cells) was determined microscopically using a "Wild" microscope on the 100x objective lens under oil immersion. Approximately 200 cells were examined for each count.

The cell density of liquid cultures was determined with a Coulter (model ZM) particle counter. 1ml samples were gently sonicated to separate clumps of cells. The sample was then diluted 1:100 in filtered saline and counted.

(viii) Propidium iodide staining of yeast cells for Flow Cytometric analysis

Yeast cells were stained with propidium iodide for Flow Cytometric analysis using a modification of the protocol devised by Corliss and White (1981). Yeast cultures were gently sonicated before harvesting and then resuspended at 5x 10^7 cells/ml in 70% ethanol. The cells can then be stored at 4^0C for several weeks in this state. Immediately prior to analysis, 1ml of fixed cells were harvested, washed with 1ml of 50mM sodium citrate and resuspended in 1ml of staining solution (50mM sodium citrate, 100µg/ml RNaseA and 5µg/ml propidium iodide). The cells were then incubated for 2 hours at 37^0C in the dark. Flow cytometric analysis was performed within 24 hours of staining on a Becton

Dickenson Facstar with the assistance of C. J. Atkins. Analysis of the flow cytometric profiles was performed using the Facsplot programme developed by J. Green.

(ix) DAPI staining for fluorescent microscopy

$5x10^7$ cells were harvested, washed with sterile distilled water and fixed by resuspension in 1ml of 70% ethanol. The cells were left at room temperature for 20 minutes, before the addition of 0.5ml of sterile distilled water. The cells were then pelleted by centrifugation at 3000r.p.m. for 5 minutes and gently resuspended in 70% glycerol with 0.1μg/ml DAPI (4',6-diamidin-2-phenylindole dihydrochloride). DAPI stained nuclei were observed using x63 oil immersion objective of a 'Wild' Fluorescence Microscope with ultra violet illumination and the appropriate filter.

(x) Preparation of yeast protein extracts

Yeast total protein extracts were prepared by resuspending cells in cold lysis buffer (20mM Tris pH 7.4, 10mM EDTA, 100mM NaCl, 1% Triton X-100, 5% glycerol, 1mM each of NaF, β-glycerophosphate, Na_3VO_4, EGTA and sodium pyrophosphate). The following inhibitors were included during cell lysis: 5μg/ml each of leupeptin, pepstatin A, chymostatin, aprotinin (Sigma) and AEBSF (Calbiochem). For phosphatase treatment, cells were resuspended in phosphatase buffer (50mM Tris pH8.0, 10% glycerol), plus protease inhibitors as described above. Cells were broken by vortexing with acid-washed glass beads (4x30s bursts), after which the lysate was cleared by spinning for 5 minutes in a benchtop centrifuge. In any given experiment all samples were normalized to contain the same amount of protein (Lowry *et al.*, 1951). Extracts (approximately 100μg) used in the phosphatase treatment were incubated at 37°C for 30 min., with 1U of calf intestinal

alkaline phosphatase (Boehringer Mannheim) in 20µl of phosphatase buffer, or mock treated with 20µl of phosphatase buffer. When used, the phosphatase inhibitor β-glycerophosphate (50mM) was added to 20µl of phosphatase buffer.

2.8 *E. coli* TECHNIQUES

(i) Preparation of competent cells

Competent *E. coli* strains were prepared by the frozen storage protocol III of Hanahan (1985). Cells were inoculated from an overnight culture into 50ml SOB medium (2% Bacto peptone, 0.5% Bacto yeast extract, 10mM NaCl, 2.5mM KCl, 10mM $MgCl_2$, 10mM $MgSO_4$) and grown to an $O.D._{550}$ of 0.48. The cells were harvested and resuspended in 20ml filter-sterilised, ice-cold TFBI (30mM KOAc, 100mM RbCl, 10mM $CaCl_2$, 50mM $MnCl_2$, 15% glycerol (v/v), pH adjusted to 5.8 with 0.2M acetic acid) solution. The cells were left on ice for 45 minutes, harvested and resuspended in 2ml filter-sterilised TFBII (10mM MOPS (free acid), 75mM $CaCl_2$, 10mM RbCl, 15% glycerol (v/v), pH adjusted to 6.5 with KOH) solution.

(ii) Transformation

For transformation purposes, 10-20ng of plasmid DNA was added to 0.2ml of competent cells and incubated on ice for 45-60 minutes. The cells were then heat shocked at 42°C for 2 minutes, 0.8ml SOB plus 0.4% glucose was added, and the cells incubated at 37°C for 30-45 minutes before plating out onto LB agar with the appropriate antibiotic.

(iii) Small scale plasmid preparations

Small scale plasmid DNA preparations were made from transformed *E. coli* cells

by the Alkaline lysis protocol of Ish-Horowitz and Burke (1981). 3.0ml of cells were grown overnight in LB plus the appropriate antibiotic, harvested and resuspended in 0.1ml of solution I (50mM glucose, 25mM Tris-HCL pH8.0, 10mM EDTA pH8.0). 0.2ml of solution II (0.2M NaOH, 1% SDS) was added and the solutions gently mixed by inversion and left on ice for 5 minutes. Finally, 0.15ml of solution III (5M KOAc pH4.8) was added, mixed and the mixture left on ice for 5 minutes. The precipitated SDS-protein and cell debris was pelleted by spinning in a microfuge at 12,000 rpm for 3 minutes and the supernatant was removed. Plasmid DNA was precipitated by the addition of 2 volumes of absolute alcohol and incubating for 2-3 minutes at R/T, before pelleting the DNA in a microcentrifuge. The plasmid DNA was resuspended in 100µl of sterile 1xTE.

(iv) Large scale plasmid preparations

Large scale plasmid preparations were made using a larger scale version of the Alkaline lysis protocol described above. A 500ml LB culture with the appropriate antibiotics was grown overnight to stationary phase. The cells were harvested by centrifugation, resuspended in 25mls of Solution I plus 1mg/ml lysozyme and left on ice for 30 minutes. 40mls of freshly made solution II was then added, the cell suspension mixed well and left on ice for 5-10 minutes. 20mls of cold solution III was then added, mixed well and the cell lysis mixture left on ice for 15 minutes. The cell debris was removed by centrifugation at 8,000 rpm and 4^{0}C for 5 minutes. The supernatant was removed by filtering through gauze, 0.6 volumes of isopropanol was then added to precipitate the plasmid DNA and the DNA harvested by immediate centrifugation at 8,000 rpm as before. Plasmid DNA was purified by isopycnic centrifugation in $CsCl_2$ (0.8g/ml) density gradients in the presence of 1mg/ml ethidium bromide. Separation was performed

in a Beckman VTi65 vertical rotor, spun at 60,000 rpm for 4-6 hours at 20°C. The ethidium bromide was removed from the purified plasmid DNA by extraction with iso-amyl alcohol and the $CsCl_2$ removed by dialysis against 1xTE. Plasmid DNA was ethanol precipitated from solution, quantitated and stored at -20°C.

2.9 TRANSPOSON MUTAGENESIS OF pSDB25

i) Rationale

Tn*1000* is a part of the E.coli sex-factor which transposes readily and fairly randomly into plasmids such as pBR322, forming co-integrates. These can then be transferred to a recipient cell by conjugation with an F^- strain. Resolution of the co-integrated structure then yields the F-factor and the plasmid each with a single Tn*1000* insert (Sedgwick and Morgan, 1994).

ii) Generation of Tn*1000* insertions in pSDB25

E.coli strain MHI343 was transformed with pSDB25 and grown on LB plates containing ampicillin (50μg/ml) and methicillin (100μg/ml). After overnight growth at 37°C a single colony was picked and grown in 10ml of LB broth, containing ampicillin and methicillin. Simultaneously the recipient strain MH1755 was grown in 10ml of LB broth, containing ampicillin and streptomycin. After overnight growth, one ml of each culture was resuspended in separate flasks containing 100ml of LB broth, grown for 2-3 hours to midlog phase, spun down and resuspended in 5ml of LB. The two cultures were combined and poured into a prewarmed 3l flask and left standing at 37°C for two hours. A 10μl or 100μl aliquot of the mixed culture was then spread onto selective LB agar plates containing ampicillin, streptomycin and methicillin, and grown overnight at 37°C.

The colonies from 40 plates were harvested by washing twice with 2ml of TE, pooled and collected in two 50ml sterile tubes. The spun down pellets were resuspended in 5ml of TE, and the plasmid DNA extracted following a large-scale preparation.

iii) Selection of plasmids containing Tn*1000* insertions in *SDB25.*

The plasmid DNA preparation from (ii) can be considered as a library containing random Tn*1000* insertions in pSDB25. This was used to transform yeast strain L1827-A (*dbf2-3*), which was then grown on YEPD plates at 25^{0}C and replica-plated onto YEPD at 37^{0}C. Colonies which grew at 25^{0}C, but not at 37^{0}C, were then isolated as candidates for loss of suppressor function due to transposon insertion/disruption of *SDB25*. Plasmid extraction and restriction digest analysis could then determine the site of Tn*1000* insertion (i.e whether in the vector or in the cloned DNA insert).

2.10 PLASMIDS AND EPITOPE TAGGING

For routine cloning purposes, pBluescript KS or SK(-) plasmids (Stratagene, USA) were used. Plasmid YEpSDB25 was constructed by inserting the *Eco*RI-*Hind*III 1.8-kb fragment of pSDB25, containing *SDB25*, into the multiple cloning site of the high copy plasmid YEplac181 (Geitz and Sugino, 1988).

The *CDC28* gene was tagged by insertion of the HA1 epitope (from R. Deshaies and Dr. P. Sorge, Berkerley), which is recognized by the 12CA5 monoclonal antibody (Wilson *et al.*, 1984). The integrating plasmid pRD86 was used, which contains *URA*3 and the C-terminal part of the *CDC28* gene. The plasmid was linearized using the unique *Kpn*I site in the *CDC28* gene, before integrative transformation (Rothstein, 1983), resulting in one complete epitope-tagged *CDC28* gene, and one N-terminally truncated *CDC28* gene

in the yeast genome.

2.11 PREPARATION OF SDB25 ANTI-SERUM

The 2.1-kb *Kpn*I-*Bgl*II fragment of pSDB25 was subcloned into pRIT2 (Lowenadler *et al.*, 1986)) to give pRIT25 which contains a protein A-Sdb25 fusion gene having all but the first 13 amino acids residues of the Sdb25 polypeptide. The fusion protein was induced in *E.coli*, using an alkaline pH shift in the growth medium (Poindexter and Gayle, 1991). To purify the fusion protein, an *E. coli* colony, harbouring the pRIT25 plasmid, was grown overnight in 400ml of LB. Following induction of the recombinant fusion protein, a protein extract was prepared using a French Press. The extract was resuspended in 15ml of TST buffer (50mM Tris pH7.6, 150mM NaCl and 0.05% Tween) and loaded onto an pre-equilibrated IgG-Sepharose column (Pharmacia). The column was then washed with 20ml TST buffer, followed by a 2ml wash with 5mM NH_4Ac pH5.0. Elution of the sample was achieved with 0.5M HAc pH3.4.

2.12 IMMUNOBLOT ANALYSIS

For immunoblotting, cell extracts containing 100μg of total protein were separated on a 10% SDS-PAGE gel and transferred to nitrocellulose (0.45μm, Schleicher and Schuell) using a semi-dry blotting unit (Biorad) according to the manufacturers instructions. Nitrocellulose blots were blocked by overnight incubation in PBS/1% non-fat milk and then transferred to a 1:25 dilution of Sdb25 anti-serum for 2 hours, followed by sequential incubations with anti-rabbit IgG-conjugated biotin (1:1000), and streptavadin alkaline phosphatase (1:1000) (Sigma). All antibody incubations were done at room temperature with agitation, followed by 30 minute washes with several changes of

PBS/0.5% BSA buffer. Proteins that interacted with the antibodies were visualized using the chromogenic substrates BCIP/NBT, as previously described (Harlow and Lane, 1988).

2.13 PROTEIN KINASE ASSAYS

For Sdb25-associated kinase activity 1µl of Sdb25 anti-serum was added to 100µg of yeast extract and incubated for 1 hour on ice. 20µl of protein A beads (protein A-Sepharose CL-4B, Pharmacia) were then added, and incubation continued with mixing, for a further 30 minutes. The beads were then washed three times in 1ml of lysis buffer, followed by one wash in assay buffer (25mM MOPS pH 7.2, 60mM β-glycerophosphate, 15mM ρ-nitro-phenylphosphate, 15mM MgCl2, 5mM EGTA, 1mM dithiothreitol, 0.1mM Na_3VO_4, 10µmATP and 5µg/ml each of leupeptin, pepstatin A, chymostatin and aprontinin). The washed immunoprecipitate was then placed at 30°C and 10µl of assay buffer containing 1µCi of [γ^{32}P]-ATP and 5µg of calf thymus histone H1 (Sigma, type III-S) was added. Incubation was continued for thirty minutes and the reaction stopped by the addition of 15µl SDS-PAGE sample buffer, and heated for 3 minutes at 90°C prior to electrolysis.

For Cdc28 kinase assays 1µl (10µg of protein) of 12CA5 monoclonal antibody (ascites fluid, from Babco) was added to 100µg of yeast extract from strains containing the epitope-tagged Cdc28, subsequent steps being essentially the same as for Sdb25-associated kinase assays. Dbf2 kinase assays were as previously described (Toyn and Johnston, 1994), being essentially the same as Sdb25-associated and Cdc28 kinase assays. Incorporation of ^{32}P into histone H1 was quantitated by β-imaging using a Phosphor-Imager (Molecular Dynamics).

CHAPTER THREE

CHARACTERIZATION OF *SDB25*

The immediate background to this project lies in the attempted cloning of molecular suppressors of the budding yeast protein kinase mutant L182-7A (*dbf2-3 ura3*). This involved the screening of three separate *S. cerevisiae* genomic clone banks, which have varying sizes of *Sau*3A-partial chromosomal inserts ranging from 2 to 20 kb. The libraries were cloned into the multicopy vector YRp12 (*URA3 TRP1*), which results in an average of about seven copies of the plasmid per cell when transformed into yeast.

Yeast strain L182-7A was transformed with plasmid DNA from the three libraries, and URA^+ colonies selected by growing the transformants on minimal plates. Putative suppressors of *dbf2-3* were identified by replica-plating transformed colonies onto YEPD plates at 37^0C, the restrictive temperature for *dbf2*-ts mutants. From a total of about 95,000 transformants screened, 7 were isolated that were able to grow at the restrictive temperature (L.H. Johnston, unpublished). Restriction digest analysis of the plasmid DNA isolated from the 7 colonies, showed that they contained one of two separate chromosomal fragments. The two plasmids thus defined were named pSDB24 and pSDB25 (suppressor of *dbf2* 4 and 5). The localization and sequencing of the two corresponding genes, *SDB24* and *SDB25*, forms the immediate starting point of this project.

3.1 PLASMID pSDB25

Plasmid pSDB25 consists of a 3.75kb genomic DNA fragment, carrying the *dbf2-3* dosage suppressor gene *SDB25*, cloned into the *Bam*HI site of the multicopy vector YRp12. Prior to locating the gene, a basic restriction endonuclease map was constructed of the insert of pSDB25, using standard procedures (Figure 3.1A). This map was sufficient to show that pSDB25 contained a different genomic fragment to that of pSDB21-pSDB24.

3.2 LOCALIZATION OF THE CLONED *SDB25* GENE BY TRANSPOSON MUTAGENESIS

Given a genomic insert, one method of locating the gene of interest is by serial subcloning. This method was used to identify *SDB24*, as will be described in Chapter 5. However, to locate the suppressor gene in pSDB25 the Tn*1000* transposon strategy was employed as described in the Materials and Methods. In this approach, quasi-random Tn*1000* insertions are generated in pSDB25 within *E.coli*, which effectively results in a plasmid library, pSDB25::Tn*1000*. The sites of insertion of the transposon are quasi-random as there is a bias to AT richness or AT "valleys" in GC-rich DNA (Sedgwick and Morgan, 1994). The resulting plasmid pool is then screened for loss of suppressor activity by transforming pSDB25::Tn*1000* into *dbf2-3*, and identifying those colonies which now no longer grow at the restrictive temperature 37^{0}C. This enables identification of plasmids with Tn*1000* insertions in the *SDB25* gene. The generation of Tn*1000* insertions is rapid and has the advantage that as well as locating the suppressor gene on the pSDB25 genomic insert, it provides an immediate basis for obtaining the nucleotide sequence of the gene, as will be described below. This method was previously used to rapidly identify

Figure 3.1

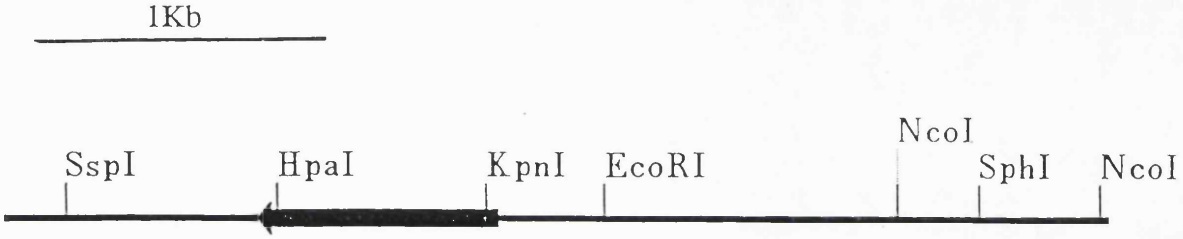
A. Partial restriction endonuclease map of the genomic insert of pSDB25

The principal restriction endonuclease sites of the 3.75kb genomic fragment of pSDB25 are shown. The black arrow indicates the ORF of *SDB25* as deduced from the nucleotide sequence.

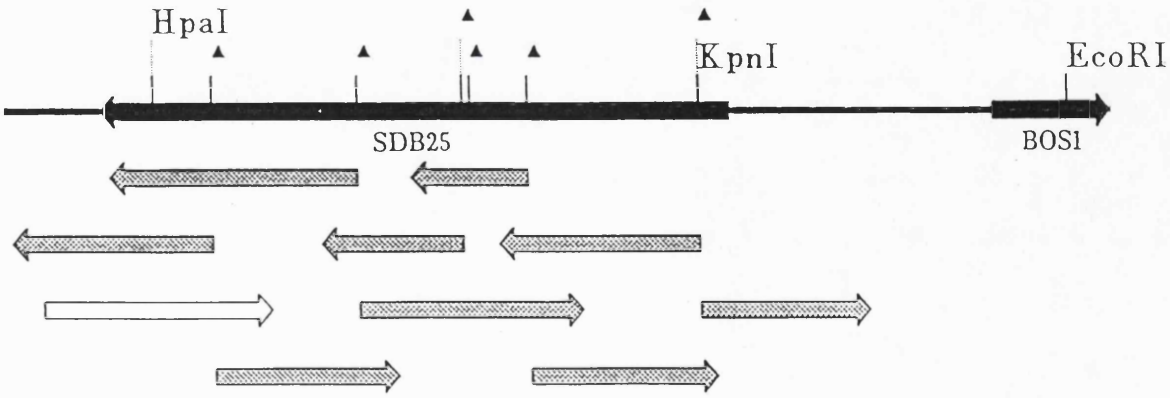
B. Insertion sites of Tn*1000* in *SDB25*

The black triangles indicate the insertion sites of Tn*1000* in *SDB25*, the exact locations of which were determined after sequencing. Shaded arrows indicate DNA sequence determined using the δ/γ primers, the open arrow indicates sequence determined using a synthesized primer. The sequence to the right of the *Kpn*I site has previously been determined, and is the upstream of the divergently expressed gene *BOSI*. The start, and direction, of the ORF of *BOSI* is indicated by the small black arrow.

A


1Kb
SspI
HpaI
KpnI
EcoRI
NcoI
SphI
NcoI

B


250bp
HpaI
KpnI
EcoRI
SDB25
BOS1

SDB22 (Parkes and Johnston, 1992), which was originally isolated as a genomic insert of approximately 22kb.

Plasmid pSDB25 was thus subjected to Tn*1000* transposon mutagenesis and the resulting library of randomly disrupted plasmids was transformed into L182-7A (*dbf2-3*). Of 200 colonies that grew at 25^{0}C, 6 failed to grow when replica-plated onto YEPD at 37^{0}C. From each of the six colonies exhibiting loss of suppressor function the plasmid was extracted and transformed into *E.coli.* Cells were grown overnight, and the DNA examined after small scale preparations following alkaline lysis of the cells. After a suitable restriction digest analysis it was found that each of the six plasmids harboured a Tn*1000* insertion in the chromosomal fragment of pSDB25. Furthermore, all six insertions were between the *Hpa*I and *Kpn*I sites (Figure 3.1B), thus defining, fairly precisely, the location of the *SDB25* gene.

3.3 SEQUENCING OF *SDB25*

The amino acid sequence of a protein, deduced from the nucleotide sequence of the gene, can prove informative in indicating homologies/similarities with other proteins and possibly provide some indication of function. One common method used to detect weak homologies between two proteins employs the computer search algorithm BLAST (Altschul *et al.*, 1990). This algorithm can be used to search sequence databanks, such as EMBL or GenBank, with the sequence of the protein of interest. A recent innovation has been the development of computer search methods to scan the rapidly expanding expressed sequence tag data base (dbEST), to find human open reading frames related to yeast protein sequences. ESTs are partial cDNAs sequences derived from clones that are

randomly selected from various human cDNA libraries. Such an approach was used to identify a human homologue of the budding yeast cell cycle gene *CDC27* (Tugendreich *et al.*, 1993). In addition, the database of functional domains, PROSITE, can also be searched to indicate possible important consensus sequences/motifs in the predicted protein sequence of the gene of interest.

Sequencing a gene requires the generation of a contiguous region of DNA sequence, which can be achieved by several methods. One rapid technique is primer "leapfrogging" in which oligonucleotide primers are synthesized complementary to the extremities of previously determined sequence, which can then be used to extend the sequence further, in both directions. Another common method is the subcloning of nested deletions generated by exonuclease digestion of the gene locus of interest. However, with pSDB25::Tn*1000* the transposon insertion sites can act as mobile bidirectional priming sites within the gene to be sequenced. This sequencing strategy has several advantages over more conventional approaches. Firstly, only two primers are needed for divergent sequencing reactions on opposite strands from any site of transposon insertion. Secondly, as mentioned above, transposon insertions are almost random, and are thus generated throughout the region to be sequenced. Thirdly, generation of Tn*1000* insertions is extremely rapid and the site and orientation of insertion can be quickly determined by restriction analysis of Tn*1000*-containing plasmids. For this reason the Tn*1000* method was chosen for sequencing *SDB25*.

3.4 SEQUENCING STRATEGY

DNA sequencing was carried out using the γ and δ primers specific to the

sequences located at either end of Tn*1000*, as described in the Materials and Methods. The precise site of the Tn*1000* insertion can also then be determined since when it transposes it duplicates five bases of the target DNA at the point of insertion (Reed *et al.*, 1979). Between 150 and 300bp of nucleotide sequence immediately flanking the ends of the transposon insertion was obtained from each plasmid. Using the six pSDB25::Tn*1000* plasmid preps as template DNA, a contiguous sequence of 931 nucleotides of DNA was obtained. In this sequence one large open reading frame was found which is capable of encoding a protein of 284 amino acids with a calculated molecular weight of 32,846 Da. (Figure 3.2). The predicted protein would be basic, and the estimated pI is about 8.9. When compared with existing DNA sequences in the EMBL database using the BLAST rapid search programme, it was found that the putative upstream of *SDB25* had already been sequenced, and was part of the upstream sequence of the divergently expressed gene *BOS1* (Shim, 1991). Comparison of the restriction map of the 1.1kb *BOS1* gene with that of the genomic insert of pSDB25, showed that *BOS1* is entirely contained within the insert. A search of the EST database, using the *SDB25* deduced amino acid sequence, indicates that, at present, there are no sequenced human cDNA homologues of *SDB25.*

3.5 DEDUCED AMINO ACID SEQUENCE OF SDB25

Searching the existing databanks suggests that *SDB25* is a newly identified gene. However, during the course of this work the identical gene has been independently isolated, named *SIC1*, and the encoded protein is the previously characterized p40 (M. Mendenhall, personal communication). As described in the Introduction, the initial identification of p40 was as an endogenous substrate, with an apparent molecular weight

Figure 3.2

Nucleotide sequence of *SDB25*

The first nucleotide of the ORF has been numbered one. Overlined is a potential promoter consensus sequence TATA (-107). The following restriction sites are indicated by underlining: *Kpn*I (38); *Hpa*I (786).

The predicted amino acid sequence is shown in single letter code.

```
      -130                  -110                  -90
GGCTTGTTGTTCGCGTCGCAACTTTTGATATTTATAAAAAAGAGAGAAGGCATATCCAAAG
       -70                   -50                   -30
GATAAGGTATTGTCTTTGTAAATCAATCAACCAAACCTCTACGGAATTTTGACCCTTGAA
       -10          1        10                    30
GCAGGGACTATTACACGAAAATGACTCCTTCCACCCCACCGAGGTCCAGAGGGACTAGGT
                    M  T  P  S  T  P  P  R  S  R  G  T  R  Y
        50                   70                    90
ACCTTGCGCAGCCTAGTGGCAATACTAGTTCTAGTGCCCTAATGCAAGGTCAAAAGACCC
  L  A  Q  P  S  G  N  T  S  S  S  A  L  M  Q  G  Q  K  T  P
       110                  130                   150
CCCAAAAGCCTTCACAGAACCTAGTCCCTGTCACTCCCTCAACAACTAAGTCTTTTAAAA
  Q  K  P  S  Q  N  L  V  P  V  T  P  S  T  T  K  S  F  K  N
       170                  190                   210
ATGCGCCATTATTAGCACCTCCCAATTCGAACATGGGTATGACCTCTCCATTTAATGGGC
  A  P  L  L  A  P  P  N  S  N  M  G  M  T  S  P  F  N  G  L
       230                  250                   270
TTACGTCTCCTCAACGCTCGCCGTTTCCAAAATCTTCAGTGAAGAGGACACTATTCCAAT
  T  S  P  Q  R  S  P  F  P  K  S  S  V  K  R  T  L  F  Q  F
       290                  310                   330
TTGAAAGTCATGATAATGGAACAGTAAGGGAAGAGCAGGAACCATTGGGTCGTGTAAATA
  E  S  H  D  N  G  T  V  R  E  E  Q  E  P  L  G  R  V  N  R
       350                  370                   390
GGATATTGTTTCCCACGCAGCAAAATGTGGATATAGATGCAGCAGAAGAAGAAGAAGAAG
  I  L  F  P  T  Q  Q  N  V  D  I  D  A  A  E  E  E  E  E  G
       410                  430                   450
GAGAAGTTCTTCTTCCCCCCAGCAGACCTACATCTGCCAGGCAGTTACATTTATCACTTG
  E  V  L  L  P  P  S  R  P  T  S  A  R  Q  L  H  L  S  L  E
       470                  490                   510
AAAGAGATGAGTTTGATCAGACACATAGAAAGAAGATTACTAAAGATGTACCTGGTACGC
  R  D  E  F  D  Q  T  H  R  K  K  I  T  K  D  V  P  G  T  P
       530                  550                   570
CCAGCGACAAGGTGATAACATTTGAATTGGCAAAAAATTGGAATAATAACTCTCCGAAAA
  S  D  K  V  I  T  F  E  L  A  K  N  W  N  N  N  S  P  K  N
       590                  610                   630
ATGACGCCAGGAGTCAAGAAAGTGAAGACGAGGAAGACATCATCATCAATCCAGTGCGGG
  D  A  R  S  Q  E  S  E  D  E  E  D  I  I  I  N  P  V  R  V
       650                  670                   690
TGGGTAAAAATCCCTTTGCATCAGATGAACTGGTCACTCAGGAAATTAGAAATGAACGTA
  G  K  N  P  F  A  S  D  E  L  V  T  Q  E  I  R  N  E  R  K
       710                  730                   750
AAAGGGCAATGTTGAGAGAAAACCCAGATATAGAAGACGTAATAACATATGTCAATAAGA
  R  A  M  L  R  E  N  P  D  I  E  D  V  I  T  Y  V  N  K  K
       770                  790                   810
AGGGAGAGGTGGTAGAGAAACGAAGGTTAACGGATGAAGAAAAGAGAAGATTCAAGCCAA
  G  E  V  V  E  K  R  R  L  T  D  E  E  K  R  R  F  K  P  K
       830                  850                   870
AGGCATTGTTTCAATCTAGGGATCAAGAGCATTGAAGAAdATGAAAAAAAAAAAGTTTCTG
  A  L  F  Q  S  R  D  Q  E  H  *
       890                  910                   930
GAACGATTATATTTTATTTACTTACTTATTCTACATTTATTTGCAAAGGGGC
```

of 40 kD, for a kinase activity associated with Cdc28 immune complexes (Reed *et al.*, 1985).

The Sdb25/Sic1 protein sequence is abundant in proline residues (9.5% of total amino acids), serine (8.8%), threonine (7.0%), acidic amino acids (14.8%) and basic amino acids (15.5%). The predicted Sdb25/Sic1 protein sequence also contains three PEST regions (amino-acids 37-49, 115-141 and 198-212) (Nugroho and Mendenhall, 1994). Such regions are defined as sequences rich in proline, glutamate, serine, threonine and aspartate, bounded by positively charged residues. As mentioned in the Introduction, it has been proposed that PEST sequences confer susceptibility to rapid intracellular proteolysis (Rogers *et al.*, 1986), raising the possibility that Sdb25 protein levels may fluctuate throughout the cell cycle. The range of PEST scores found in Sdb25/Sic1 is similar to that found in known short-lived proteins such as the p53 tumour suppressor and the oncogene v-*myb*.

Consistent with $p40^{SDB25}$ being a potential substrate of Cdc28 kinase, the Sdb25/Sic1 primary amino acid sequence has nine matches to the minimal $p34^{cdc2}$ phosphorylation consensus sequence (S/T-P), and one perfect match to the other proposed consensus motif for $cdc2^{+}$/Cdc28 phosphorylation S/T-P-X-Z (amino-acids 76-79).

3.6 pSDB25 DOES NOT SUPPRESS ANY *CDC28*-TS ALLELES

The finding that a dosage suppressor of the protein kinase *DBF2* is a component of a $p34^{CDC28}$ complex suggests that *SDB25* may be functioning in a pathway somehow linking the two enzymes *in vivo*. Previously it has been shown that overexpressing *DBF2* on a high copy plasmid did not suppress the *cdc28-4*, *cdc28-13* or *cdc28-1N* alleles

(Kitada *et al.*, 1993). The same three *cdc28*-ts alleles were also transformed with pSDB25 to see if overexpressing *SDB25* would suppress any of the *cdc28* temperature-sensitive mutations. However, no suppression was observed, nor did *CDC28* suppress any *dbf2*-ts alleles when overexpressed on the high copy plasmid YEp13. This would indicate that the two kinases may not be closely associated and/or they do not have significant overlapping substrate specificity.

3.7 pSDB25 REQUIRES *DBF20* TO SUPPRESS *dbf2-3*

Three other suppressors of *dbf2* have been previously described (Parkes and Johnston, 1992), but only one of these suppressors, *SDB21*, can rescue *dbf2-3* cells, and none exhibit clear allele-specific suppression. Suppression of *dbf2* mutants by *SDB21* (*SPO12*) requires *DBF20*, the homologue of *DBF2* (Toyn and Johnston, 1993). That is, overexpression of *SPO12* cannot suppress the *dbf2* mutant phenotype in cells deleted for *DBF20*. To examine whether *SDB25* has a similar requirement for *DBF20*, pSDB25 was introduced into yeast strain JD100 (*dbf2-3 dbf20*Δ::*TRP1*). As with *SPO12*, it was found that *SDB25* no longer suppresses the *dbf2*-ts phenotype in cells deleted for *DBF20*. The *SDB25* suppression of *dbf2-3* may therefore in some way recruit *DBF20* to complete the vital function carried out by the Dbf2/Dbf20 kinases.

3.8 EXAMINATION OF THE *SDB25* TRANSCRIPT THROUGHOUT THE CELL CYCLE

The period in the cell cycle where a transcript is maximally abundant may offer

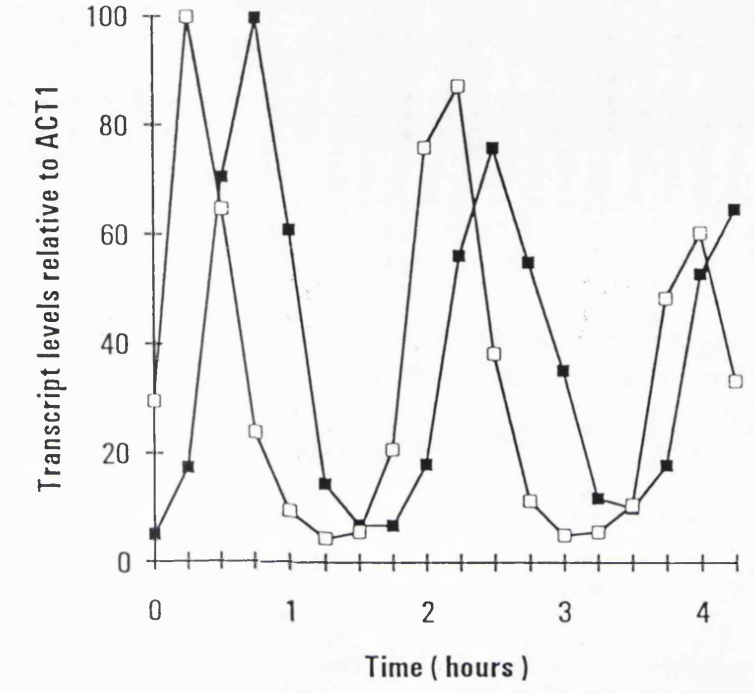
some insight into the function of a gene, for example the time of expression may be consistent with the time of function. As mentioned in the Introduction, the *DBF2* transcript is expressed under cell cycle control, with a peak of expression in M-phase (Johnston *et al.*, 1990), and it has recently been found that the its kinase activity is similarly regulated (Toyn and Johnston, 1994). Of the two molecular suppressors of *dbf2*-ts alleles previously characterized, *SDB22* (=*SIT4*) is constitutively expressed throughout the cell cycle. However, the other suppressor, *SDB21* (=*SPO12*) is coordinately regulated with *DBF2* in late mitosis (Parkes and Johnston, 1992).

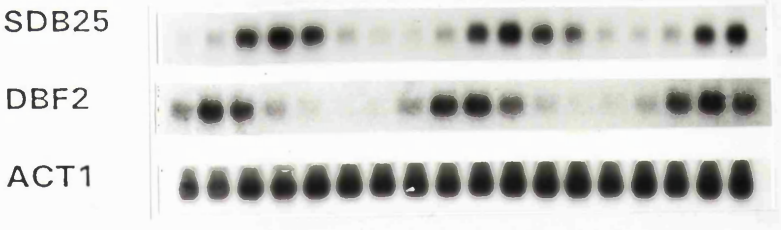
To investigate the expression of the *SDB25* gene during the cell cycle, Northern blot analysis was carried out on total RNA from synchronized cultures. Cells were synchronized by three distinct methods to reduce the possibility of artefactual results, and more than one cycle was monitored to ensure the normal pattern of expression was observed. The synchronization method which perturbs cell growth as little as possible is that of centrifugal elutriation, in which small G1 cells are obtained by elutriation, re-inoculated into fresh medium, and followed through two, or three, synchronous cell cycles. From a Northern blot prepared from elutriator-synchronized cells it was found that *SDB25* is strongly cell cycle regulated (Figure 3.3) with the *SDB25* transcript showing a more than 10-fold increase over background levels during the peak of expression. For comparison, the Northern blot was also probed with *DBF2*. In all three cell cycles *SDB25* peaks later than *DBF2* by 15-20 minutes, indicating that the peak of *SDB25* expression is very close to the M/G1 boundary. In this experiment the only indicator of cell cycle position measured was that of budding, which is only useful for correlating early events in the cell cycle. Thus it is not possible to tell whether *SDB25* is maximally abundant very late in the cell cycle, or very early in the next cycle.

Figure 3.3

Regulation of the *SDB25* transcript in cells synchronized by elutriation

The culture used in this experiment, and the budding profile, has been previously described (Kitada *et al.*, 1993). Just over two synchronous cycles were monitored, and RNA samples were analyzed by Northern hybridization using the indicated probes. RNA levels were quantitated by densitometry from suitably exposed autoradiograms, and normalized relative to Actin transcript levels. The peak value of *SDB25* and *DBF2* transcript levels were each given a value of 100 for graphical presentational purposes. □, *DBF2* transcript levels; ■, *SDB25* transcript levels.


100
80
60
40
20
0
Transcript levels relative to ACT1
0
1
2
3
4
Time (hours)


SDB25
DBF2
ACT1

In a second experiment, a Northern blot was prepared from cells synchronized by temperature arrest of a *dbf2*-ts mutant in late mitosis (Toyn and Johnston, 1994). On release form the block, the *SDB25* transcript is initially at approximately 25% of its peak value, which is reached in the next sample, taken 10 minutes later (Figure 3.4). Within 35 minutes *SDB25* has fallen to background levels, about the time when the percentage of budded cells is at a minimum. This confirms that *SDB25* is regulated later than *DBF2* in the cell cycle, and also suggests that maximal expression of *SDB25* may require a functional Dbf2 protein.

Finally a third Northern blot was probed with *SDB25*, using a synchronized culture prepared by the feed-starve protocol (Williamson and Scopes, 1963). Again, *SDB25* was found to be periodically expressed, although in this case *SDB25* appears to be only weakly transcribed in the first cycle following starvation (Figure 3.5).

3.9 GENERATION OF A YEAST STRAIN DELETED FOR *SDB25*

To examine the effect of deleting the chromosomal *SDB25* gene it is necessary to generate a strain in which the sequence of the *SDB25* gene is disrupted, or deleted, such that a non-functional gene is produced. A common method of achieving this is to transfer the gene to an appropriate vector, and replace this segment with a marker gene (Rothstein, 1983). It is desirable to delete as much of the gene as possible, since some truncated genes retain residual function. The disrupted, and marked, gene is then isolated on a linear fragment and transformed into a diploid strain wild-type for the gene of interest, but deficient for the marker gene. Targeted integration of the fragment into one of the chromosomes at the specific locus occurs by homologous recombination, and can be

Figure 3.4

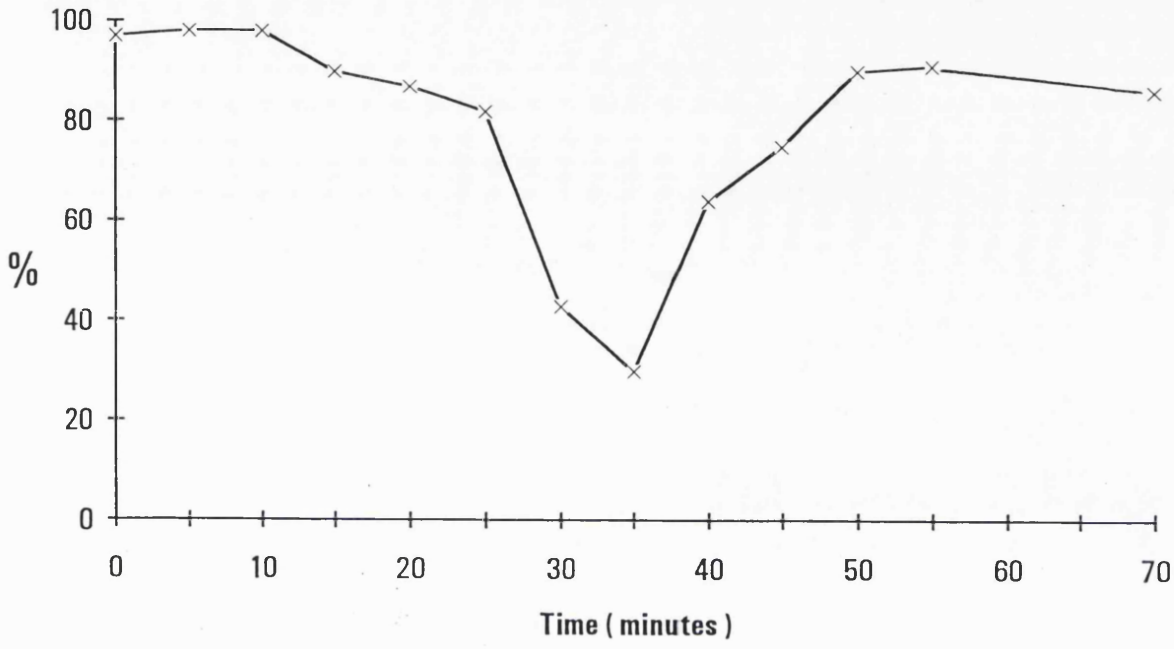
Regulation of the *SDB25* transcript in cells released from a *dbf2*-ts block

A culture of strain LJ1 (*dbf2-2*) was synchronized by a temperature shift, by holding at the restrictive temperature for 105 minutes. By this time greater than 95% of the cells had accumulated as dumbbells at the *dbf2* block point, and release from the block results in a very synchronous population of cells that rapidly re-enter the cell cycle. The first two cell samples were taken at 0 and 10 minutes after return to the permissive temperature. Subsequent cell samples were taken every 2 minutes, for just over one hour. RNA samples were analyzed by Northern hybridization using the indicated probes. *SDB25* transcript levels were quantitated by densitometry from a suitably exposed autoradiogram. (A) % budded cells. (B) □, *SDB25* transcript levels.

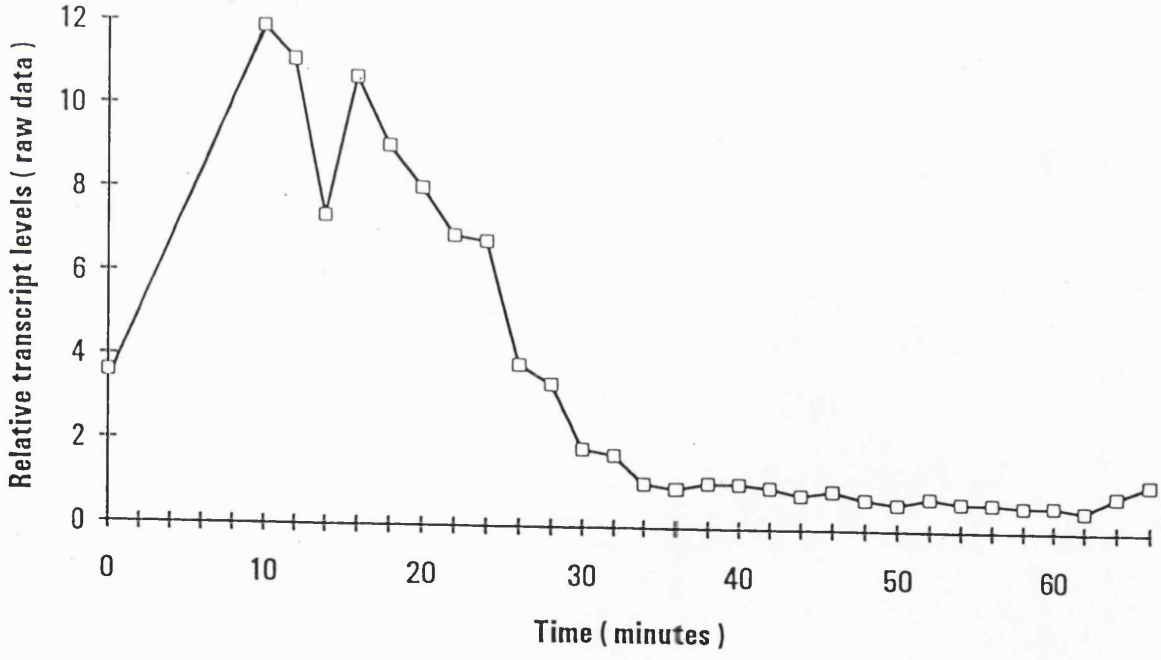
(C) Autoradiograms of *SDB25* and *ACT1*, which was used as a loading control. As can be seen from *ACT1* levels, the fourth sample was underloaded, hence the dip in *SDB25* RNA levels in (B).

Culture synchronization was carried out by L.H. Johnston, A.L. Johnson and J.H. Toyn.

A


100
80
60
%
40
20
0
0
10
20
30
40
50
60
70
Time (minutes)

B


12
10
8
6
4
2
0
Relative transcript levels (raw data)
0
10
20
30
40
50
60
Time (minutes)

C

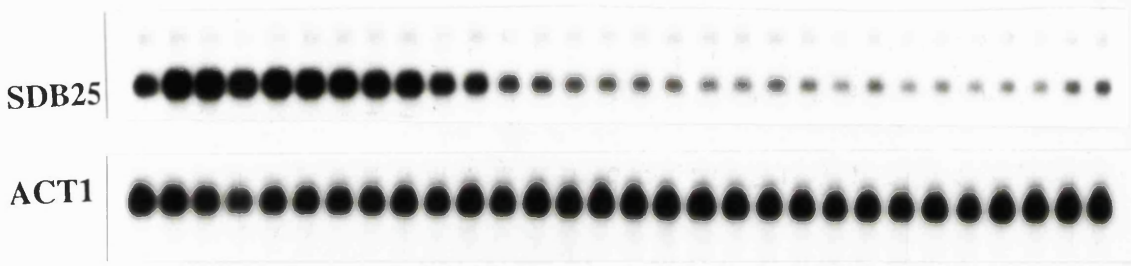
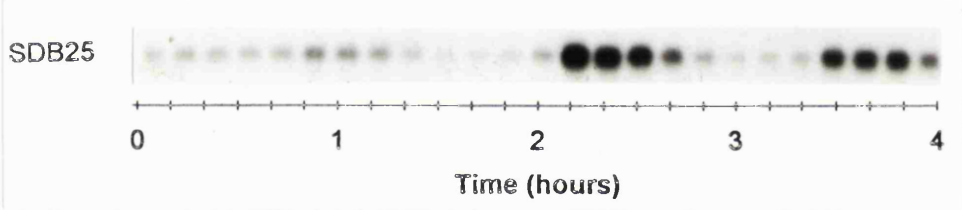

SDB25
ACT1

Figure 3.5

Regulation of the *SDB25* transcript in cells synchronized by feed-starve

The details of the culture used in this experiment have been described previously (Johnston *et al.*, 1990), and just over two synchronous cell cycles were monitored. The Northern blot used has previously been probed with *SPO12*, *DBF2* and various controls, (Parkes and Johnston, 1992).


SDB25
0
1
2
3
4
Time (hours)

selected for by the presence of the marker gene. Thus the diploid strain now contains one functional copy and one non-functional copy of the gene on separate chromosomes. Sporulation of the diploid and tetrad dissection of the haploid spores will then reveal if the disrupted gene is essential for viability. If the gene is essential for germination and growth then each tetrad should contain two viable and two non-viable spores. If non-essential, all four spores should be viable.

To generate an *sdb25* null allele (*sdb25*Δ), the internal 740bp *Kpn*I-*Hpa*I gene fragment was deleted from *SDB25* and replaced with *TRP1* (Figure 3.6). The 740bp *Kpn*I-*Hpa*I fragment represents approximately 90% of the *SDB25* ORF, thus the null allele constructed is effectively deleted for the entire *SDB25* gene. The *sdb25*Δ::*TRP1* gene was then isolated on a 3.1kb *Ssp*I-*Sph*I fragment and transformed into the diploid strain CG378/379. Integrants were selected on minimal media, and then streaked out for single colonies. To confirm replacement of *SDB25* with *TRP1*, genomic DNA from 24 single *TRP*$^+$ colonies was extracted, digested with *Hin*dIII, and subjected to Southern hybridization analysis. Probing with the 3.1kb *Ssp*I-*Sph*I fragment, spanning the *SDB25* gene, showed that 21 of the 24 colonies had a banding pattern consistent with a disruption of *SDB25* by *TRP1* (data not shown). As additional confirmation, three of the identified *sdb25*Δ::*TRP1* diploid colonies were used in a further Southern hybridization analysis (Figure 3.7). The appearance of the extra bands in *Eco*RI and *Eco*RV-digested *TRP1*$^+$ colonies confirming the disruption.

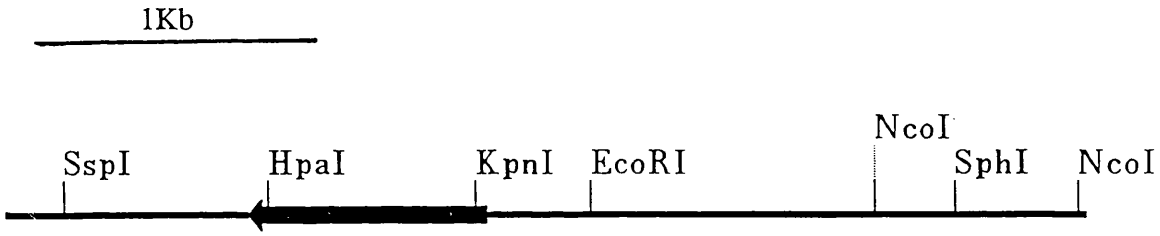
A single diploid from which one copy of *SDB25* had been deleted was sporulated, followed by dissection of 21 tetrads. All the tetrads resulted in four viable spore colonies. DNA was extracted from sixteen *TRP*$^+$ haploids spore colonies and used in a Southern hybridization analysis to confirm the *sdb25*::Δ*TRP1* genotype (Figure 3.8). Thus deleting

Figure 3.6

Strategy for deletion/disruption of the chromosomal *SDB25* gene

The internal 740bp *Kpn*I-*Hpa*I chromosomal gene fragment of *SDB25* was replaced with an 870bp fragment containing *TRP1*, flanked by sequence from the MCS of Bluescript. (A) Wild-type *SDB25* gene locus. (B) *sdb25*Δ:*TRP1* gene locus.

A


1Kb
SspI
HpaI
KpnI
EcoRI
NcoI
SphI
NcoI

B

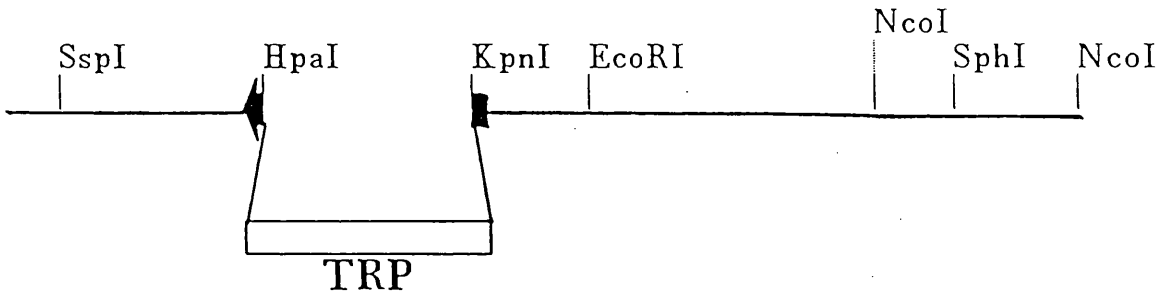

SspI
HpaI
KpnI
EcoRI
NcoI
SphI
NcoI
TRP

Figure 3.7

Southern hybridization confirming replacement of *SDB25* with *TRP1* in the diploid CG378/379

The probe used was made with the 3.1kb *Ssp*I-*Sph*I fragment spanning the *SDB25* gene. Genomic DNA from 3 single diploid *TRP1*$^+$ colonies was extracted and cut with *Eco*RI (lanes 6,7 and 8) and *Eco*RV (lanes 1,2 and 3). Disruption of *SDB25* with *TRP1* was predicted to introduce single *Hin*dIII, *Eco*RV sites, and a second *Eco*RI site into the *SDB25* locus. For comparison, wild-type CG378/379 genomic DNA was cut with *Eco*RI (lane 9), and *Eco*RV (lane 4). Also probed were wild-type uncut DNA (lane 10), and the 1kb DNA ladder (lane 11). The probe hybridized to the 1000bp and 500bp bands of the 1kb ladder.

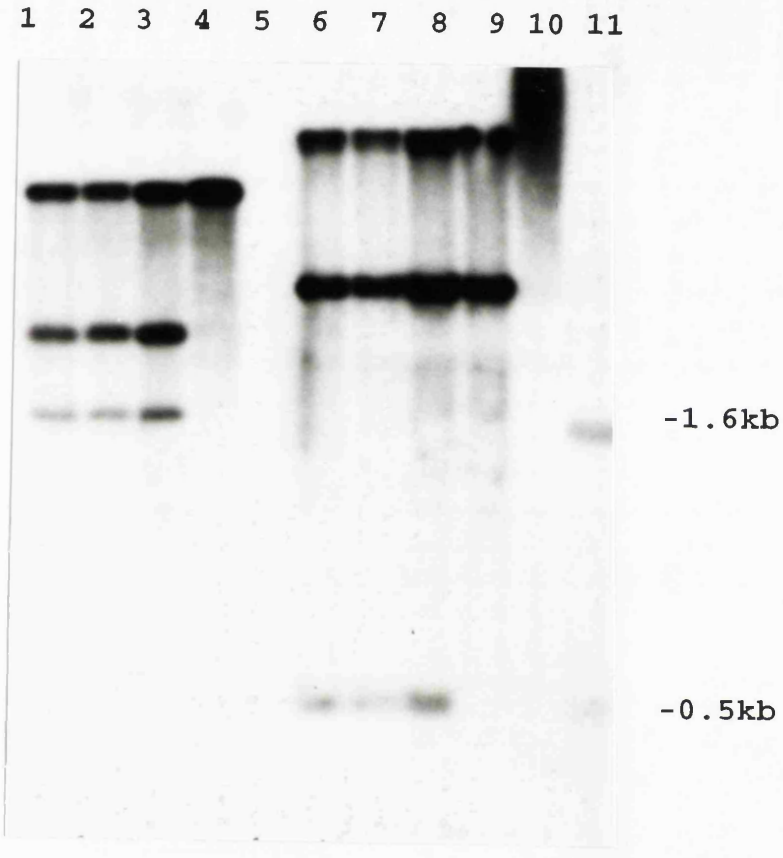
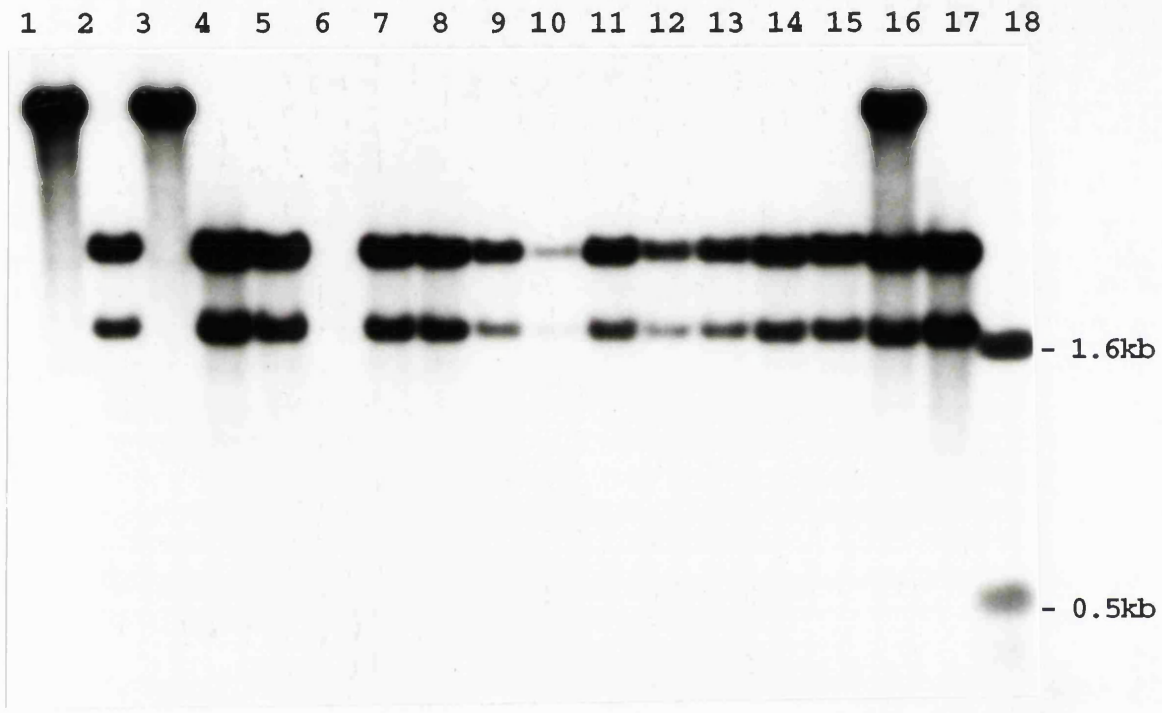

1 2 3 4 5 6 7 8 9 10 11
-1.6kb
-0.5kb

Figure 3.8

Southern hybridization confirming replacement of *SDB25* with *TRP1* in haploid cells

The probe used was made with the 3.1kb *Ssp*I-*Sph*I fragment spanning the *SDB25* gene. Genomic DNA from 16 single haploid *TRP1*$^+$ colonies was extracted and cut with *Eco*RV (lanes 2 to 17). For comparison, wild-type haploid CG378 genomic DNA was also cut with *Eco*RV (lane 1), and the 1kb DNA ladder was also probed (lane 18). The probe hybridized to the 1kb and 0.5kb bands of the 1kb ladder. Lane 3 has the same banding pattern as wild-type, suggesting it may be a *TRP*$^+$ revertant colony. Lane 16 may be a diploid colony, having a heterozygous deletion of *SDB25*.


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
- 1.6kb
- 0.5kb

SDB25 has no effect on cell viability, at least for cells growing under normal laboratory conditions.

3.10 MORPHOLOGICAL ANALYSIS OF CELLS DELETED FOR *SDB25*

Having determined that *sdb25Δ* cells are viable, it was decided to explore whether deletion of *SDB25* had any effect on progress through the cell cycle. To examine this in detail comparisons have to be made between *sdb25Δ::TRP1* cells and, ideally, cells which have an identical genotype, apart from deletion of the *SDB25* chromosomal gene. To achieve this, *SDB25* was deleted from the haploid strain CG379 to generate strain JD100 (CG379*sdb25Δ::TRP1*).

CG379 cells deleted for *SDB25* show no difference in growth rates, compared with an isogenic wild-type strain (doubling time of 140 minutes at 30^0C in rich media). However, microscopic analysis of *sdb25Δ* cells revealed a greater heterogeneity in cell size than with wild-type (Figure 3.9). Unusually, a significant proportion of cells were smaller than average wild-type cells, whilst others, some 6%, were considerably larger. In addition, the percentage of budded cells was much higher in *sdb25Δ* cells than in the control population (81% in *sdb25Δ* cells, 65% for wild-type). Furthermore, 23% of *sdb25Δ* cells were dumbbells, as compared to just 6% of wild-type cells.

3.11 FLOW CYTOMETRIC ANALYSIS OF CELLS DELETED FOR *SDB25*

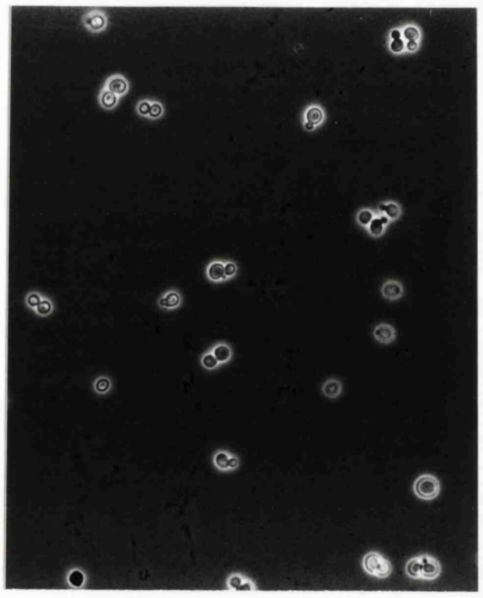
To further examine the effect of deleting *SDB25*, flow cytometric analysis (FACS) was carried out using asynchronous cultures in mid-logarithmic growth phase. Samples

Figure 3.9

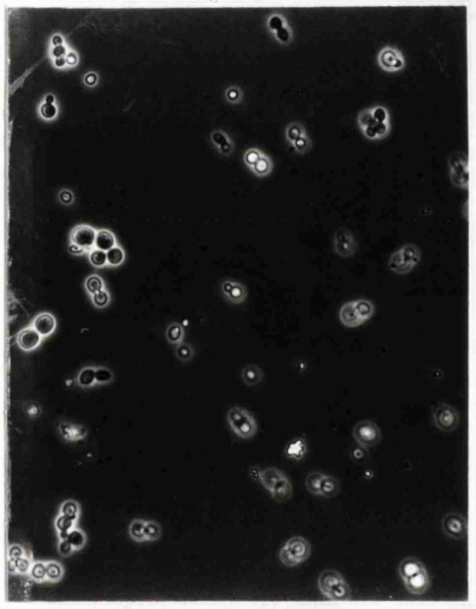
Morphology of *sdb25Δ* cells

Mid-log cells growing in YEPD were harvested, fixed and photographed using phase-contrast microscopy. The field of *sdb25Δ* cells shown was selected to illustrate the heterogeneity in cell size, rather than to represent the statistical distribution as described in the text.

WT



sdb25Δ



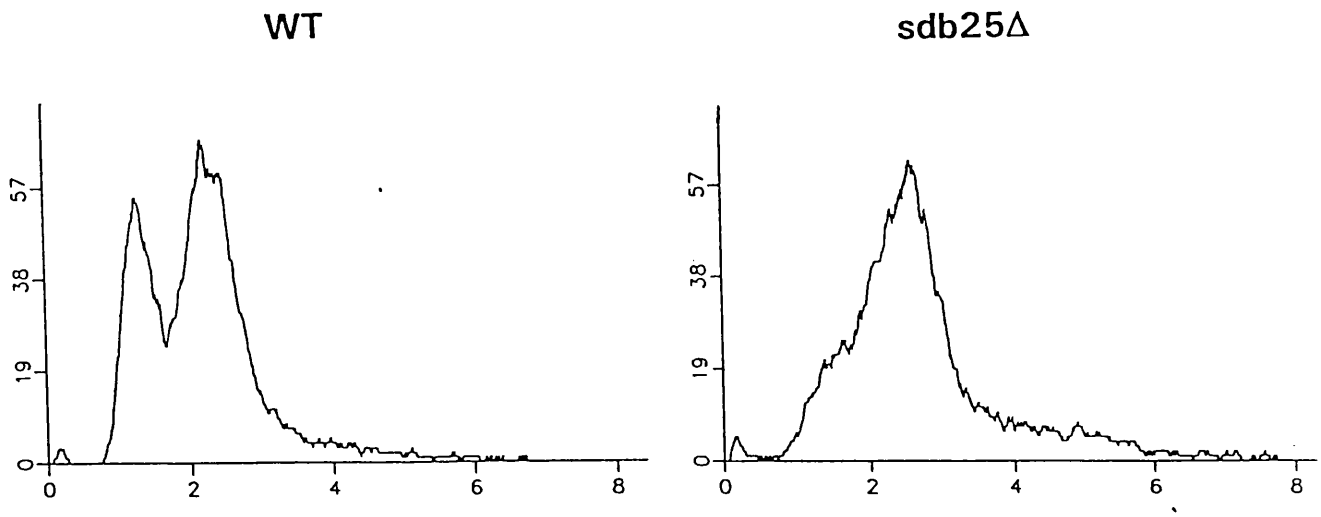
were taken of the isogenic wild-type strain CG379, and two separate cultures of CG379*sdb25Δ::TRP1* cells. Aliquots of cells were fixed in 70% ethanol, and nuclei stained with propidium iodide. As expected, FACS analysis of a wild-type strain gave two fairly sharp peaks when DNA content was plotted against cell number (Figure 3.10). The first peak corresponds to haploid cells in the G1 phase of the cell cycle, which have a single-chromosomal (1C) DNA content. The second peak corresponds to cells in G2/M when the chromosomes have been duplicated, but before cytokinesis has occurred. The trough between the two peaks denotes cells in S phase, when new DNA is being synthesized. By contrast, a mid-log phase culture of *sdb25Δ* cells consisted almost entirely of cells in S phase, or in G2/M with a 2C DNA content. There were very few cells with a 1C DNA content. Thus, FACS analysis indicates that deletion of *SDB25* results in cells spending less time in the G1 phase of the cell cycle. This is consistent with the increase in budded cells in an sdb25Δ culture as compared to wild-type, mentioned above. The onset of budding occurs at about the beginning of S phase, thus the reduction in unbudded cells as a result of deletion of *SDB25* implies the cells are spending less time in G1.

Interestingly, a similar FACS profile is observed when cells are deleted for *SPO12*, one of the other suppressors of *dbf2* (Parkes and Johnston, 1992). The authors interpret their finding as due to cells being unable to complete some function in G2 at the normal rate, in the absence of a functional Spo12 protein. This delay would result in an increase in a G2 peak in the *spo12Δ* FACS profile, relative to a wild-type control. Moreover, since cells spend more time in G2/M, and continue growth during this period, they exit M phase at a slightly increased size. As a consequence, *spo12Δ* cells arrive in G1 slightly larger than normal, and thus less time is required to achieve the critical size necessary to traverse START, so tending to reduce the time *spo12Δ* cells are in G1. In support of this

Figure 3.10

Flow cytometric analysis of *sdb25*Δ cells.

Mid-log cells growing in YEPD were, harvested, fixed and prepared for FACS analysis to measure the distribution of DNA content. Cells were prepared for flow cytometry as described in Materials and Methods. Horizontal axes: Fluorescence (Relative DNA content); Vertical axis: Relative Cell numbers. A total of 10,000 cells were observed for each sample.


WT
sdb25Δ
0
19
38
57
0
2
4
6
8

interpretation, forward scatter analysis, which gives a measure of cell size, shows that *spo12*Δ cells are, indeed, slightly larger than normal. However, in sharp contrast, forward scatter analysis of the *sdb25*Δ cells used in the FACS analysis shows that deletion of *SDB25* results in cells being significantly smaller on average than cells of the control strain (Figure 3.11). This phenotype is similar to that observed with mutants in the G1 cyclin *CLN3*, discussed in the Introduction. The small cell size associated with dominant *CLN3* alleles, is thought to be due to such cells overriding the size control operating in G1. Thus, analysis of *sdb25*Δ cells indicates that deletion of *SDB25* may have two distinct effects on cell cycle progression, a delay in G2/M and/or an acceleration through G1.

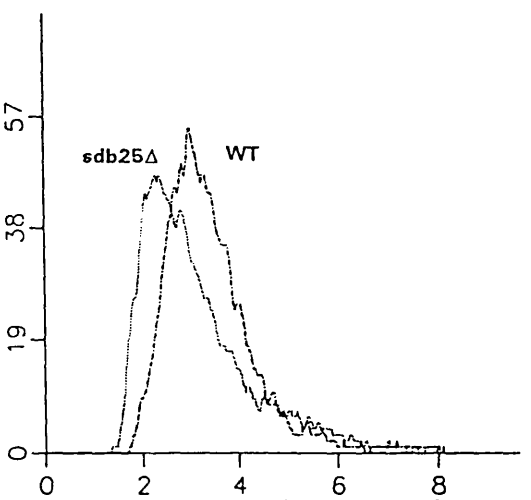
3.12 DELETION OF BOTH *SDB25* AND *DBF2* IS LETHAL

Although *DBF2* mutant alleles are conditionally lethal, yeast strains from which *DBF2* has been deleted are viable, this unusual state of affairs being due to the existence of the homologue *DBF20* (Toyn *et al.*, 1990). Given the allele-specific suppression of *dbf2-3* by pSDB25, suggesting a direct interaction between both genes, it is possible that synthetic lethality could occur when both genes are deleted. To examine this, a *MATa sdb25*Δ::*TRP1* strain was therefore crossed to a *MAT*α *dbf2*Δ::*URA3* strain, the resulting diploid was sporulated, and 35 asci were dissected and scored for the *URA3* and *TRP1* markers. Altogether 4 non-parental ditypes, 9 parental ditypes, and 22 tetratypes were obtained, from which it could be inferred that a total of 27 spores were deleted for both *SDB25* and *DBF2*. None of the double mutant spores formed colonies, but microscopic examination showed that all had germinated, and had subsequently ceased division, though a uniform terminal phenotype was not observed. Thus, *SDB25* has an essential function

Figure 3.11

Forward scatter analysis of *sdb25Δ* cells

Forward scatter analysis was carried out at the same time as FACS analysis, as an indicator of the relative cell size distribution of the two cultures. Horizontal axis: Forward scatter (Relative cell size); Vertical axis: Relative cell number. The reduction in the peak of *sdb25Δ* relative cell number, as compared to wild-type reflects the greater variance in size distribution in cells deleted for *SDB25*. Also note the slight elevation of the right-hand side of the *sdb25Δ* tracing above the wild-type tracing which probably reflects the small percentage of large cells in the *sdb25* culture.


sdb25Δ
WT
57
38
19
0
0
2
4
6
8

in a *dbf2*Δ background.

It was next examined whether there was a similar, essential requirement for *SDB25* in cells deleted for *DBF20*, the homologue of *DBF2*, or in cells deleted for *SPO12*. However, both *sdb25*Δ::*TRP1 dbf20*Δ::*TRP1* and *sdb25*Δ::*TRP1 spo12*Δ::*TRP1* double mutants are viable, at either 25°C or 37°C. There was also no difference in growth rates as compared to isogenic wild-type strains and no detectable morphology specific to the double delete strains.

3.13 PHYSICAL MAPPING OF *SDB25*

To physically map *SDB25*, the Riles-Olson ordered array of yeast genomic clones was screened by Southern blotting, using the internal *Kpn*I-H*pa*I *SDB25* gene fragment as a probe. The *SDB25* probe hybridized to clones 3688, 4673 and 6637 of the collection (Figure 3.12). This indicates that *SDB25* is mapped between *gal*2 and *spt*8 on the right arm of chromosome XII.

3.14 SUMMARY

One of the two allele-specific suppressors of the *S.cerevisiae* protein kinase mutant *dbf2* has been restriction mapped and sequenced. Initially no homology to the encoded protein was found, but during the course of this work it was discovered that the identical gene had been sequenced and named *SIC1*, for substrate/subunit inhibitor of cyclin-dependent kinases (Nugoroho and Mendenhall, 1994). The Sic1 protein is in fact the previously characterized p40, the tight-binding substrate and inhibitor of Cdc28 kinase

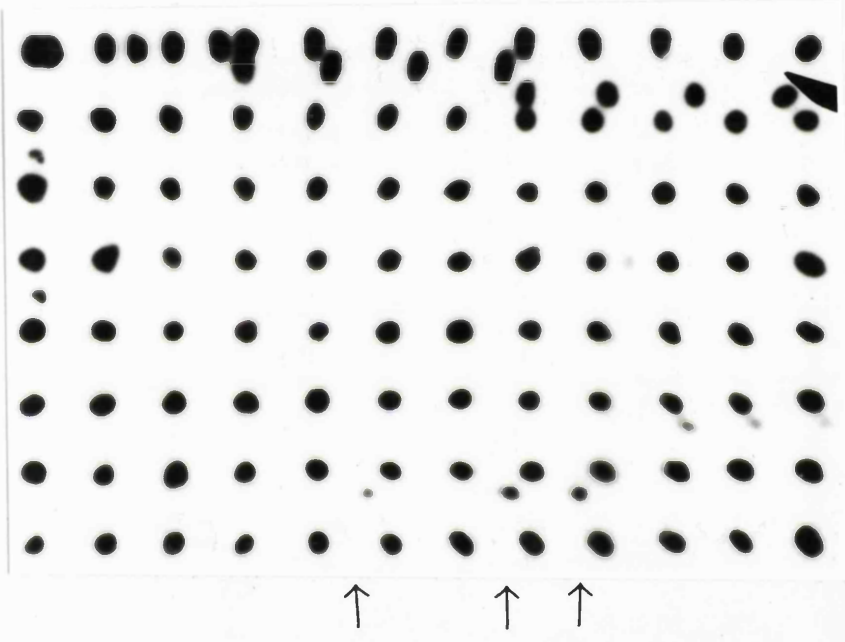
Figure 3.12

Physical map position of *SDB25*

The Riles-Olson collection consists of two hybridization membranes containing DNA from a set of overlapping, physically mapped, clones covering the entire genome of *S. cerevisiae* (Obtained from ATCC). The clones are arranged in rough chromosomal order on the membrane, in the form of 96 4x3 arrays. Each 4x3 array is associated with a "coordinate", the whole set of coordinates being the symmetrical 12x8 grid of large dots seen in the figure. The actual arrays are distributed symmetrically in the large "squares" made up of four neighbouring dots of the 12x8 grid.

To map a gene, a Southern hybridization is first carried out using a control (non-yeast DNA) probe. This hybridizes to the 12x8 grid points, and to 12 different, individual array points between rows 7 and 8 of the grid, as can be seen in the figure. The membrane is then screened with a probe from the gene to be mapped. Using the control probe hybridizations, the clones hybridizing to the gene probe can then be assigned to a specific position within a particular array, and the array itself can be assigned to particular coordinate. Using the software package provided, this information can than be correlated to a position on the physical map of the *S. cerevisiae* genome.

The collection was screened by Southern hybridization, using the internal *Kpn*I-*Hpa*I *SDB25* gene fragment as a probe. This hybridized specifically to the three clones indicated by the arrows. The other bands are a result of control probings, and previous screening using other gene probes.



activity. *SDB25/SIC1* has no significant homology to other recently identified Cdk inhibitors and, as yet, no human homologues of the gene have been found.

No genetic interactions between *SDB25* and *CDC28* were detected, pSDB25 did not suppress either the G1 ts-mutants *cdc28-4* or *cdc28-13*, or the G2 ts-mutant *cdc28-1N*. However, more evidence of genetic interactions between *SDB25* and *DBF2* was suggested with the finding that cells deleted for both *SDB25* and *DBF2* are inviable. Furthermore, it was found that suppression of *dbf2-3* by pSDB25 requires the presence of the *DBF2* structural and functional homologue *DBF20*.

Analysis of *sdb25*Δ cells revealed that deletion of the gene significantly perturbs progression through the cell cycle, although deletion of *SDB25* has no effect on growth rates. The preliminary examination carried out appears to indicate that deletion of *SDB25* may result in two distinct cell cycle effects: A delay in G2/M and/or an acceleration through G1. This suggests that the physiological role of *SDB25* is confined to M/G1, which was supported by a detailed examination of the *SDB25* transcript levels throughout the cell cycle. Using three different methods of synchrony, it was found that *SDB25* is strongly cell cycle regulated, with a peak of expression very close to the M/G1 transition point. Furthermore, it was found to be expressed slightly later than *DBF2*, suggesting that Sdb25 may function downstream of the dbf2 kinase.

CHAPTER FOUR

CHARACTERIZATION OF THE SDB25 PROTEIN

SDB25, a multicopy suppressor of a *dbf2*-ts mutant, was sequenced and characterized, as described in the Chapter Three. The results obtained raise several interesting questions about the physiological role of Sdb25, and its *in vivo* relationship to Dbf2. Firstly, where precisely in the cell cycle does Sdb25/p40 function? The early work on p40 suggested that it was confined to G1 (Mendenhall *et al.*, 1987), although several of the findings presented in the previous chapter strongly suggest p40/Sdb25 has an M phase role as well. Secondly, does Sdb25/p40 physiologically interact with both the Cdc28 and Dbf2 protein kinases? Thirdly, can the suppression of *dbf2-3* by pSDB25 be explained by the finding that Sdb25/p40 is a Cdk inhibitor *in vitro*? In an attempt to answer some of these questions it was decided to carry out biochemical experiments, which first of all required the generation of Sdb25-specific antibodies. With such antibodies the cell cycle levels of the Sdb25 protein could then be examined, and compared with the observed fluctuations in *SDB25* transcript levels. Protein kinase assays could also be carried out to see if Sdb25 associated with a kinase, and, if so, the identity of the catalytically active subunit possibly determined using known *cdc* protein kinase mutants. Finally, coimmunoprecipitation experiments could be undertaken to see if Sdb25 did indeed associate with Dbf2 and/or Cdc28.

4.1 GENERATION OF SDB25 ANTIBODIES

In order to detect the gene product of *SDB25*, polyclonal rabbit antibodies were generated against the full length Sdb25 protein, apart from the first 13 amino-acids (see Materials and Methods). To achieve this, a plasmid construct, pRIT25, was made which fused the gene for Staphyloccal protein A in frame to the coding sequence of Sdb25. Fusion with the protein A moiety has several advantages. Firstly, purification of the fusion protein is facilitated by IgG-affinity chromatography, resulting in a high degree of purification and good yield. Secondly, the gene fusion product usually does not precipitate in the host cell, thus avoiding the problem of solubilizing "inclusion bodies". Thirdly, the protein A moiety serves as a carrier protein during immunization, and may enhance the immune response (Lowenadler *et al.*, 1986).

The pRIT25 plasmid was transformed into *E. coli* and several single colonies were picked, and grown overnight to stationary phase, prior to inducing expression of the fusion construct. A common approach to controlling recombinant protein production in *E. coli* utilizes the λ_pl promoter-operator and the λ repressor. One example involves use of a temperature-sensitive λ repressor (λ-ts). When cells are shifted to the non-permissive temperature the λ-ts repressor is inactivated, which allows RNA polymerase to bind to the promoter and transcribe the gene of interest. However, there are several disadvantages to this approach, one being that the temperature shift results in the induction of many *E. coli* proteins, including proteases. Also, in some cases, growth at higher temperature results in lower amounts of properly folded proteins. To induce expression of the recombinant Sdb25-protein A fusion protein, an alkaline shift in the pH of the medium was undertaken by the addition of 5M NaoH. It has been shown that shifting the growth medium to pH 9 for two hours, after the cells have reached stationary phase, results in significant

recombinant protein synthesis (Poindexter and Gayle, 1991). Also, the amount of protein degradation products is significantly reduced. Employing this approach, protein extracts were prepared from 8 different *E.coli* colonies, carrying the pRIT25 plasmid, and used in an immunoblot. A strong, single band of approximately 61-kD, which is consistent with predicted molecular weight of the fusion protein, was detected in one lane (Figure 4.1). This colony was used for a large scale prep (400mls), from which a protein extract was prepared for purification of the fusion protein using an IgG-Sepharose column (Figure 4.2). Purified Sdb25-Protein A protein, approximately 15μgs, was dialysed overnight in PBS, and used to immunize three different rabbits. Following exsanguination, the anti-serum from each rabbit was collected and stored at -20^{0}C.

4.2 DETECTION OF THE SDB25 PROTEIN IN YEAST EXTRACTS

Having obtained Sdb25-Protein A polyclonal antibodies, it was determined whether they would specifically detect the native Sdb25 protein in yeast. Besides preparing protein extracts from the wild-type strain CG379, extracts were also prepared from a strain deleted for the *SDB25* gene, and from a stain carrying *SDB25* on a high copy plasmid. In an immunoblot of *S. cerevisiae* lysates from CG379, the Sdb25 polyclonal antibodies recognized two strong bands, of approximate molecular weights 46-kD and 40-kD (Figure 4.3A, lane 1). The 40-kD band is in fact a doublet, consisting of two very closely migrating bands of equal intensity. In lysates prepared from *sdb25Δ::TRP1* cells the 40-kD doublet band was absent, while the upper 46-kD band was of the same intensity as that in wild-type extracts (lane 2). This suggests that the 46-kD and that the Sdb25 protein is the 40-kD doublet. This was confirmed using extracts prepared from a strain carrying the

Figure 4.1

Immunoblot of Sdb25-protein A fusion protein

Protein extracts were prepared from eight *E. coli* colonies which had been induced to express the recombinant Sdb25-protein A fusion protein. Aliquots of each extract were loaded onto an SDS-PAGE gel, electrophoresed, and immunoblotted (lanes 11 to 18). In lane 14 a strong, single band of approximately 63-kD was detected, consistent with the predicted size of the Sdb25-Protein A fusion protein. Also probed as immunoblot controls, were protein A alone (lane 1) and a Dbf2-protein A fusion protein (lane 2). Lanes 3 to 10 are extracts prepared from *E. coli* colonies expressing an Sdb24-protein A fusion construct. Molecular weight markers are indicated in Kilodaltons.

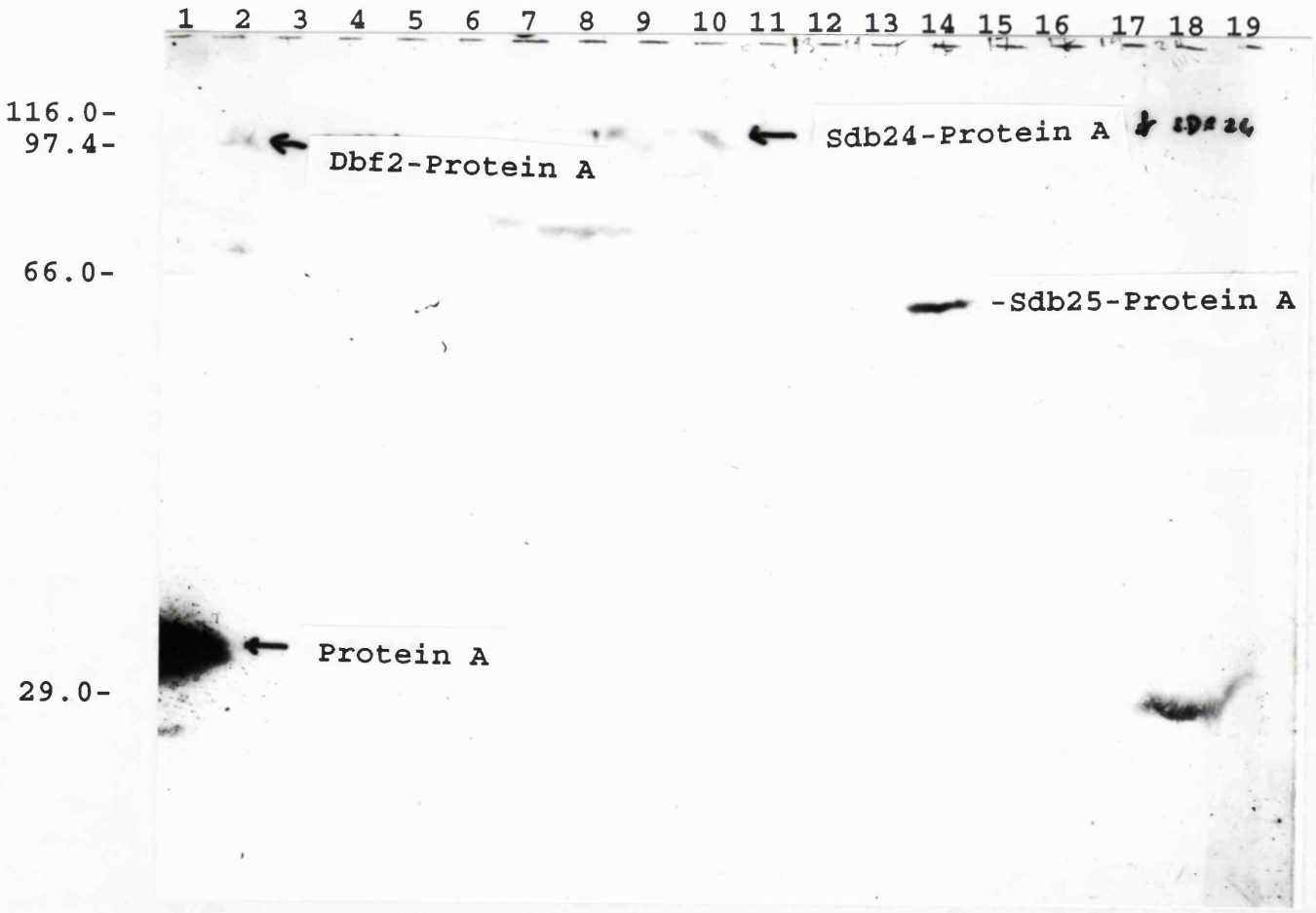

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
116.0-
97.4-
66.0-
29.0-
Dbf2-Protein A
Sdb24-Protein A
-Sdb25-Protein A
Protein A

Figure 4.2

Coomassie-stained SDS-PAGE gel of purified Sdb25-protein A fusion protein

A protein extract was made from a 400ml *E.coli* culture, harbouring the pRIT25 plasmid containing the Protein A-Sdb25 fusion protein construct. The extract was resuspended in 15mls of TST buffer and loaded onto a 1ml IgG column, as described in the Materials and Methods. The IgG-bound Protein A-Sdb25 fusion protein was eluted and 15 1ml fractions collected. Aliquots of the first six eluted fractions (lanes 7 to 2), the flowthrough from the initial loading of the protein extract (lanes 8 and 9) and the crude protein extract (lane 10), were loaded onto an SDS-PAGE gel. Following electrophoresis, the gel was stained with Coomassie Blue. Also loaded were molecular weight markers (lane 1), the sizes of which are shown in kilodaltons.

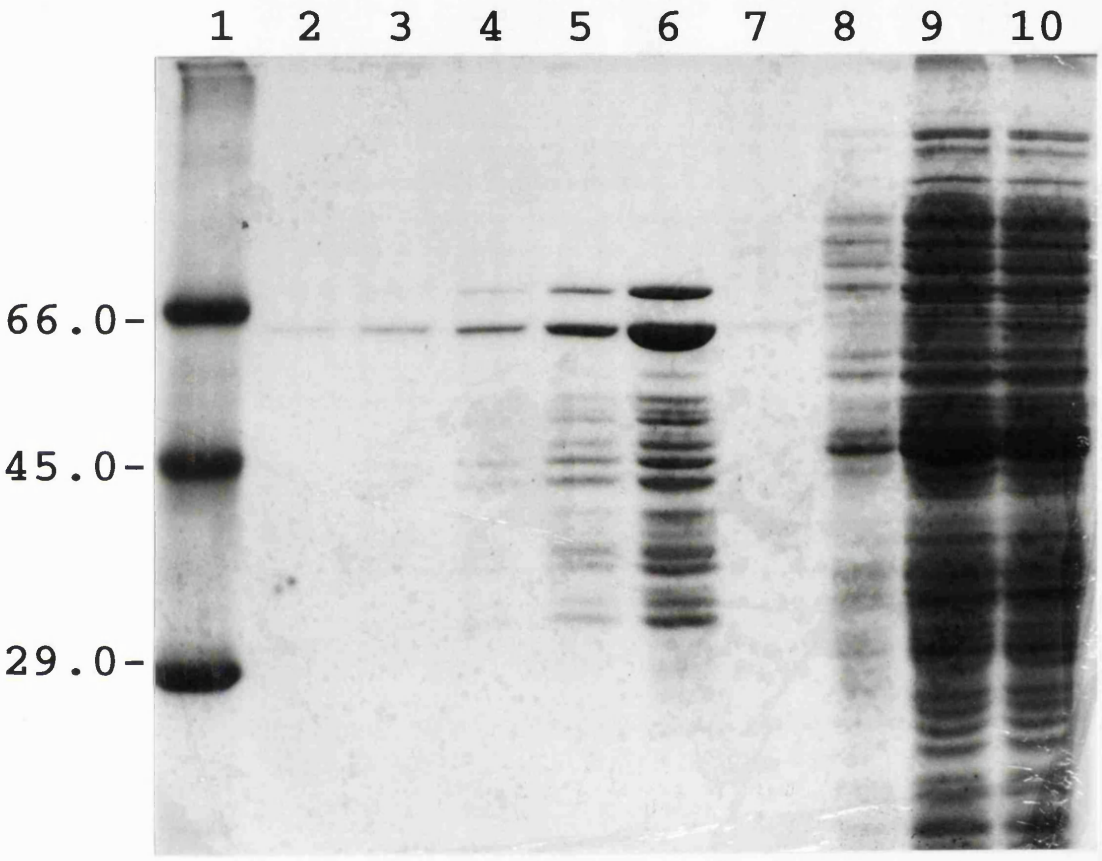

1 2 3 4 5 6 7 8 9 10
66.0-
45.0-
29.0-

Figure 4.3

Characterization of Sdb25 antibodies

A. Immunoblot of Sdb25 protein levels in wild-type and *SDB25* overexpressing strains

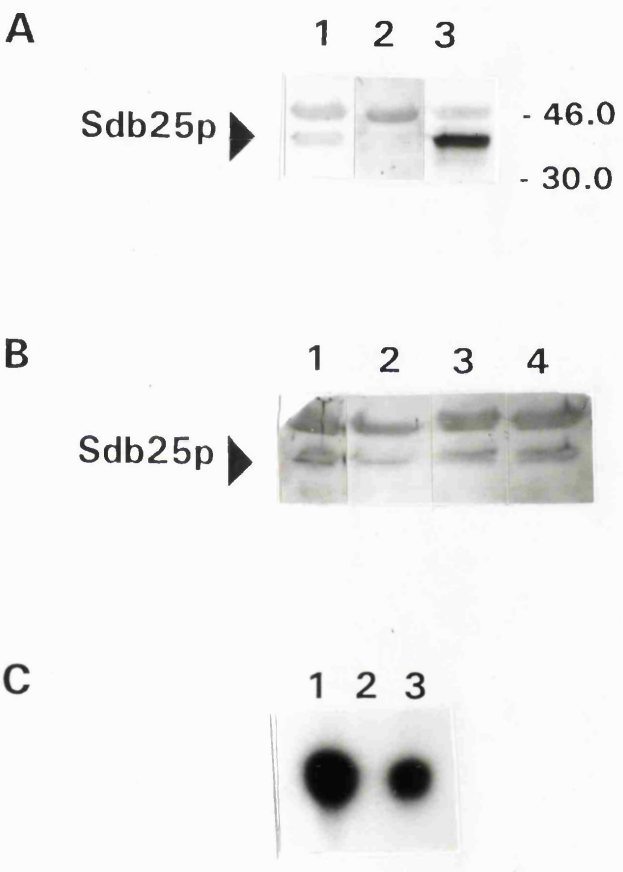
Protein extracts were electrophoresed on SDS-PAGE and immunoblotted using rabbit polyclonal anti-Sdb25 serum. Equivalent amounts of protein were loaded for each of the yeast lysates analyzed. Lane 1, CG379 (wild-type); lane 2, JD101 (sdb25Δ::*TRP1*); lane 3, CG379 carrying YEpSDB25. The band immediately above Sdb25 appeared in all immunoblots using Sdb25 antibodies, and served as an internal loading control. Molecular weight markers are given in kilodaltons.

B. Phosphorylation state of Sdb25

Extracts from CG379 cells for phosphatase treatment were prepared as described in Materials and Methods. Lane 1, untreated CG379 extract. In lanes 2 and 4 extracts were treated with calf intestinal phosphatase, and in lane 3 phosphatase buffer alone was used. In lane 4, 50mM β-glycerophosphate was added to the extract before addition of the phosphatase.

C. Immune complex histone H1 kinase assays using anti-Sdb25 serum

Cell lysates were immunoprecipitated with anti-Sdb25 serum and assayed for kinase activity, in the presence of γ-^{32}P-ATP, using histone H1 as substrate. Following termination of kinase reactions, samples were loaded onto a 10% polyacrylamide gel and subject to electrophoresis. Shown is the corresponding autoradiogram. Lane 1, CG379 (wild-type); lane 2, JD101 (sdb25Δ::*TRP1*); lane 3, CG379 carrying YEpSDB25.


A
1 2 3
Sdb25p
- 46.0
- 30.0
B
1 2 3 4
Sdb25p
C
1 2 3

highcopy plasmid YEpSDB25 (lane 3). In this case, the upper 46-kD band is of equal intensity to that in lanes 1 (wild-type) and 2 (*sdb25Δ*). However, the 40-kD band is now strongly enhanced, consistent with it being the highly overexpressed *SDB25* gene product. These results are consistent with *SDB25* encoding p40, and demonstrate that the Sdb25 protein migrates slightly anomalously with respect to its predicted molecular weight of 32.8 kD.

4.3 SDB25 IS A PHOSPHOPROTEIN

The observation that Sdb25 antibodies specifically recognize a polypeptide doublet in immunoblots suggest that the upper, more slowly migrating band may be a phosphorylated form of the Sdb25 protein. If this is the case, treatment of extracts with phosphatase before immunoblotting should result in the disappearance of the upper band. This technique has been used to show that several yeast cell cycle regulatory proteins are phosphoproteins, such as Far1 (McKinney *et al.*, 1993), Cln3 (Tyers *et al.*, 1993) and Dbf2 (Toyn and Johnston, 1994). In all three cases the protein has first been immunoprecipitated using the relevant antibody before the phosphatase treatment. However, using the Sdb25 antibodies, it was not possible, despite numerous attempts, to detect Sdb25 in an immunoblot using Sdb25 immunoprecipitates. Thus, to test whether Sdb25 was a phosphoprotein, extracts were prepared using a phosphatase buffer that allowed direct treatment with phosphatase. Treatment of crude cell lysates with phosphatase prior to immunoblotting resulted in the disappearance of the upper band (Figure 4.3B, lane 2). Addition of β-glycerophosphate, a competitive inhibitor of phosphatase, to the reaction, or treatment with reaction buffer alone did not result in the

disappearance of the more slowly migrating band (lanes 3 and 4). This demonstrates that Sdb25 is a phosphoprotein, the upper band in the doublet being the more phosphorylated form of the protein. However, in many immunoblots performed, no consistent pattern was observed with regards to the intensity of the two bands in the Sdb25 doublet. Thus no physiological conclusions are drawn from the fact that overexpressing *SDB25* resulted in both bands being equally enhanced in the immunoblot shown (Figure 4.3A, lane 3).

4.4 SDB25 HAS AN ASSOCIATED KINASE ACTIVITY

Using the Sdb25 antibodies, it was decided to see if there was a kinase activity associated with the Sdb25 protein. One reason for doing so is that pSDB25 is an allele-specific suppressor of *dbf2*-ts, suggesting that Sdb25 may be directly interacting with Dbf2 protein kinase. Thus one possibility is that Sdb25 may have an associated, *DBF2*-dependent kinase activity. Secondly, Sdb25 encodes p40, which as mentioned in the Introduction, is known to bind tightly to the Cdc28 protein kinase *in vitro*. This raises the alternative possibility that Sdb25 has an associated, *CDC28*-dependent kinase activity. First of all, to examine whether Sdb25 has an associated kinase activity immune complexes were prepared from *S. cerevisiae* lysates, using anti-Sdb25 antibodies, and assayed for histone H1 kinase activity in the presence of [γ-^{32}P]ATP. A histone H1 kinase activity was precipitated that cannot be detected in cells deleted for *SDB25* (Figure 4.3C, lane 1 and 2). Thus the kinase detected is associated with Sdb25 and does not bind non-specifically to the antibodies themselves. Interestingly, in immune complexes prepared from cells overexpressing *SDB25* the Sdb25-associated kinase activity is reduced relative to that observed in wild-type (lanes 1 and 3). This preliminary finding is consistent with

Sdb25 being an inhibitor of Cdc28 kinase activity, although confirmation of this would require much further work.

4.5 THE SDB25-ASSOCIATED KINASE ACTIVITY IS *CDC28*-DEPENDENT

Using preparations of Sdb25 immunoprecipitates from *cdc28* alleles, it was found that the Sdb25-associated kinase activity is almost totally destroyed in *cdc28-4* immunoprecipitates incubated at the non-permissive temperature (Figure 4.4). A similar, though not so dramatic, effect was observed using *cdc28-13* extracts. Sdb25 kinase activity was also assayed in immunoprecipitates prepared from *dbf2*Δ extracts, which have been shown to have a significantly reduced Dbf2 kinase activity (Toyn and Johnston, 1994). No significant reduction in Sdb25 -associated kinase was observed in either *dbf2*Δ or *dbf20*Δ immunoprecipitates, as compared to a wild-type control (Figure 4.5). The Sdb25-associated kinase activity thus exhibits defects only in *cdc28*-ts strains, which suggests that Cdc28 is the catalytic subunit in the Sdb25 kinase complex. In addition, there is a dramatic reduction in the Sdb25-associated kinase activity in extracts immuno-depleted of the Cdc28 protein (data not shown). Taken together with the finding that $p40^{SDB25}$ binds tightly to Cdc28 *in vitro* (Mendenhall 1993), this is convincing evidence that Sdb25 is an *in vivo* component of a Cdc28 kinase complex.

4.6 THE SDB25-ASSOCIATED KINASE ACTIVITY IS CELL CYCLE REGULATED

In view of the cell cycle regulated expression of *SDB25*, both Sdb25 protein levels and the associated kinase activity were examined in cells synchronized by α-factor, and

Figure 4.4

Sdb25-associated H1 kinase activity is *CDC28*-dependent

Cell lysates were immunoprecipitated with anti-Sdb25 serum and assayed for kinase activity, in the presence of γ-^{32}P-ATP, using histone H1 as substrate. Immunoprecipitates from the indicated strains were split in two and assayed for kinase activity at either 23^{0}C or 39^{0}C. Following termination of kinase reactions, samples were loaded onto a 10% polyacrylamide gel and subject to electrophoresis. A gel autoradiogram and corresponding phosphorimage quantitation is shown.

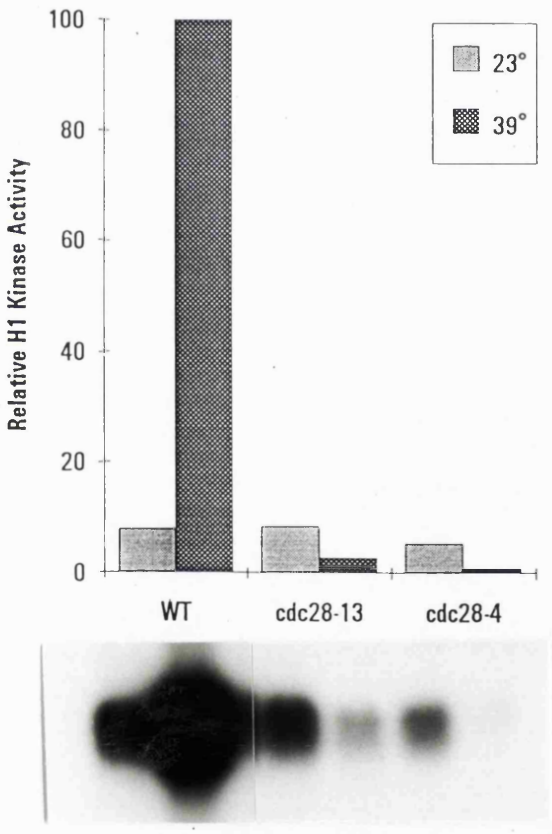
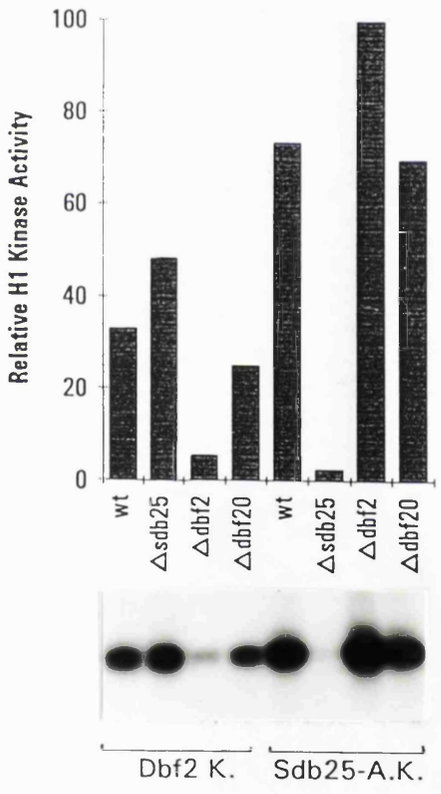

100
80
60
40
20
0
Relative H1 Kinase Activity
23°
39°
WT
cdc28-13
cdc28-4

Figure 4.5

Sdb25-associated H1 kinase activity is not *DBF2* or *DBF20*-dependent

Cell lysates, prepared from the indicated strains, were immunoprecipitated with anti-Sdb25 or anti-Dbf2 serum, and assayed for kinase activity, in the presence of γ-^{32}P-ATP, using histone H1 as substrate. Following termination of kinase reactions, samples were loaded onto a 10% polyacrylamide gel and subject to electrophoresis. A gel autoradiogram and corresponding phosphorimage quantitation is shown. The wild-type control strain used in the first and fourth lanes is CG379. Lanes 1 to 4: Dbf2 kinase activity; lanes 5 to 8: Sdb25-associated kinase activity.


Relative H1 Kinase Activity
100
80
60
40
20
0
wt
Δsdb25
Δdbf2
Δdbf20
wt
Δsdb25
Δdbf2
Δdbf20
Dbf2 K.
Sdb25-A.K.

compared with fluctuations in transcript levels. A strain carrying an epitope-tagged version of Cdc28 was used, so enabling total Cdc28 kinase activity to be measured as well. One reason for measuring total Cdc28 kinase activity throughout the cell cycle is that it can be used as a marker for the end of mitosis/ beginning of G1. Previous studies (Surana *et al.*, 1991, Wittenberg *et al.*, 1990) have shown that Cdc28 kinase activity declines to a minimum as cells exit M phase. As an additional, morphological, marker for late cell cycle events, samples of cells were stained with DAPI and scored for divided chromatin. If two clearly separated masses of stained chromatin were observed in budded cells this indicated that the cell had traversed the metaphase to anaphase transition and was in late M phase.

The Sdb25-associated kinase was found to be active for only a very short period during the cell cycle, at a time when many cells have fully divided chromatin, and total Cdc28 kinase activity is decreasing (Figure 4.6B). To rule out the possibility that this observed fluctuation might be influenced by the presence of an epitope-tagged version of Cdc28 a similar analysis was carried out in strain CG378, and obtained the same pattern of regulation of Sdb25-associated kinase activity (data not shown). Comparison of the fluctuations in the Sdb25-associated kinase activity and the *SDB25* transcript levels (Figure 4.6B and D) shows that both are maximal at approximately the same time, suggesting a causal relationship between both events.

4.7 SDB25 PROTEIN LEVELS ARE UPREGULATED IN LATE M/EARLY G1

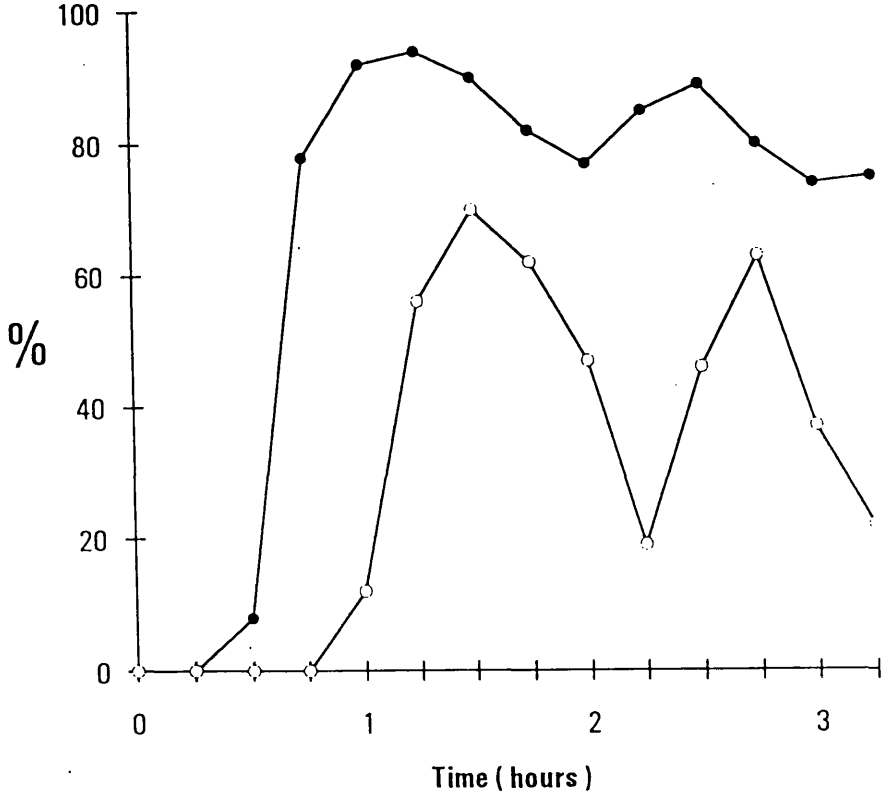
Following release from the α-factor block, the Sdb25 protein is evident for some 15 minutes up to about the time of budding (Figure 4.6C). This approximates to the

Figure 4.6

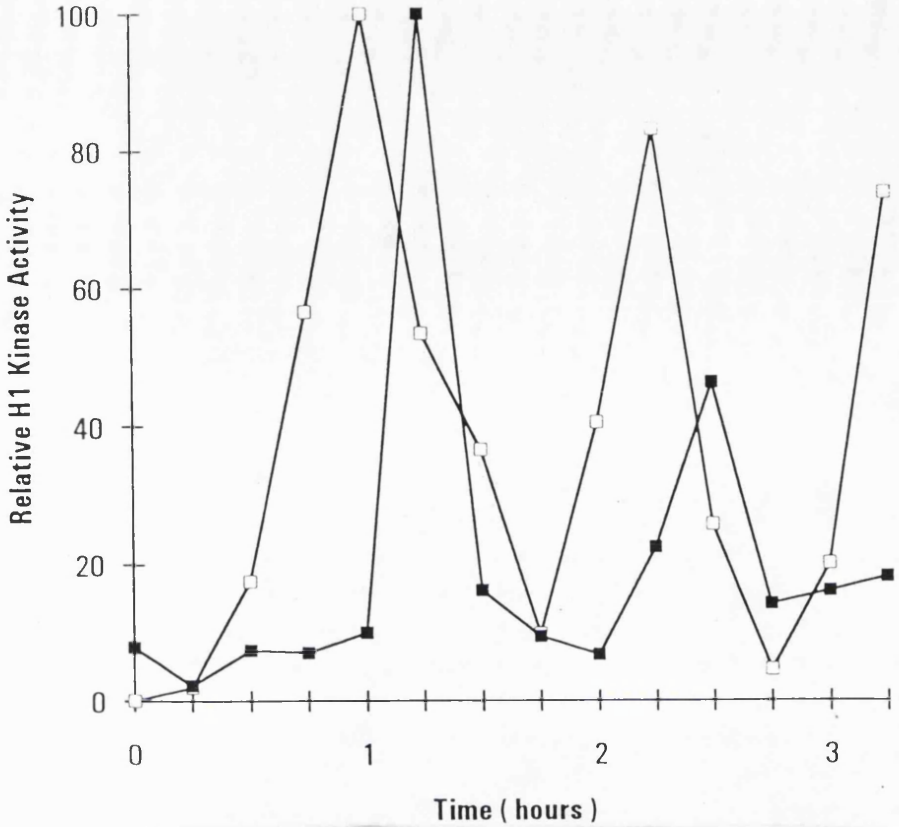
Coordinate cell cycle regulation of Sdb25 transcript levels, protein levels and associated kinase activity

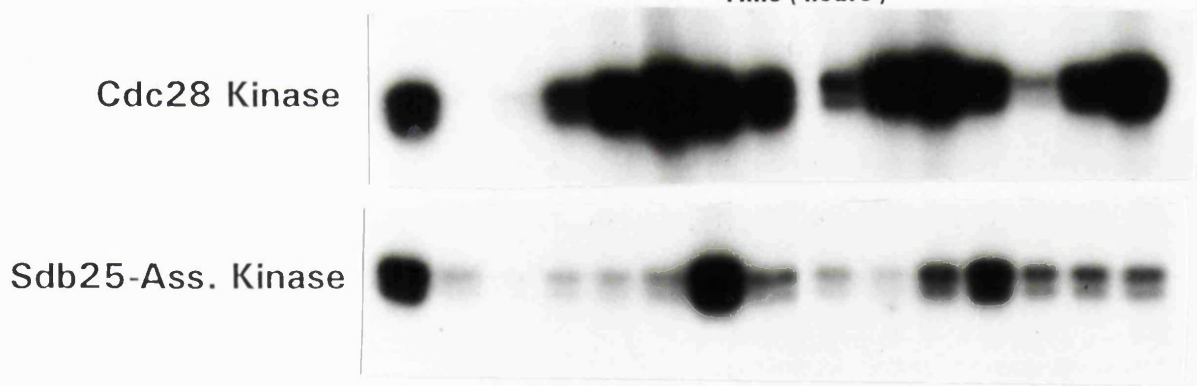
Cells were synchronized in G1 phase,using α-factor, as described in Materials and Methods. At the indicated time points following release from α-factor, samples were removed from the culture. (A) Morphological indicators of cell cycle position: ●, budded cells; ○, divided chromatin. (B) Immunoprecipitates prepared from protein samples were used for histone H1 kinase assays. Kinase assays were quantitated by phosphorimage analysis and normalized relative to the peak value in each series of assays. For purposes of graphical representation the peak value in each cell cycle assay was given a value of 100. □, Cdc28 kinase; ■, Sdb25-associated kinase. (C) Protein levels were analyzed by immunoblotting, using anti-Sdb25 antibodies. (D) RNA levels were analyzed by Northern blot hybridization with the indicated probes. The first lanes in panels (B), (C) and (D) are mid-log kinase activity, protein and transcript levels, respectively.

A



B

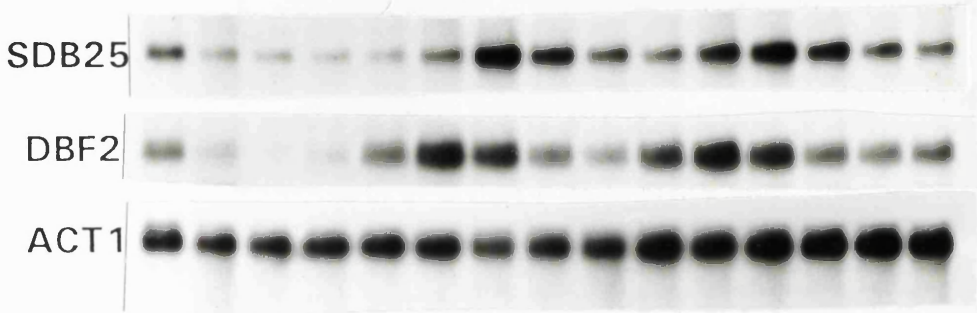

100
80
60
40
20
0
Relative H1 Kinase Activity
0
1
2
3
Time (hours)


Cdc28 Kinase
Sdb25-Ass. Kinase

C


Sdb25p

D


SDB25
DBF2
ACT1

beginning of S phase, so that Sdb25 clearly persists while cells are in G1. Interestingly, the Sdb25-associated kinase activity has disappeared at this time, so that the kinase is clearly subject to other, independent forms of control. The Sdb25 protein then reappears again at the same time-point where the peaks in the kinase activity and transcript levels occur, and persists for some 30 minutes (compare Figure 4.6B, C and D). Since the peak in Sdb25-associated kinase activity occurs in late mitosis (see Discussion), Sdb25 must only be present in the mitotic cell cycle for a brief period, from late M phase to shortly after START in the next cell cycle. To confirm this finding, protein extracts were prepared from temperature-sensitive cell cycle mutants that block in M phase or G1, when incubated at 37^{0}C. Sdb25 protein is present at very low levels in mutants arrested in late M phase (*dbf2* and *cdc14*), but accumulates in G1-arrested cells (*cdc28-4* and *cdc28-13*) (Figure 4.7). The observed fluctuations in the cell cycle of Sdb25 protein levels is consistent with the earlier finding indicating that p40/Sdb25 is an unstable protein (Mendenhall, 1993).

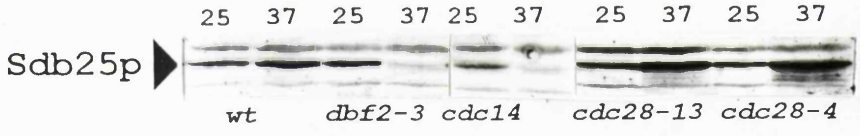
4.8 DBF2 AND SDB25-ASSOCIATED KINASES ARE SEQUENTIALLY ACTIVATED IN LATE MITOSIS

An experiment similar to that described above suggested that Sdb25 kinase activity appears to peak slightly later than the peak of Dbf2 kinase activity in cells synchronized by α-factor. To examine the timing of the two kinase activities more precisely, their relative fluctuations were measures in a *dbf2* culture synchronized by a temperature shift. The cells were held at the restrictive temperature, 37^{0}C, for 105 minutes, by which time both Dbf2 and Sdb25-associated kinase activities had fallen to a minimum. On return to

Figure 4.7

Sdb25 protein levels in cell cycle mutants arrested at 37^{0}C

Cultures of the indicated strains were grown to mid-log levels and then split into two. One half was incubated at the non-permissive temperature 37^{0}C for three hours, and the other grown at 25^{0}C for the same amount of time. Immunoblots were carried out using Sdb25 antibodies, equivalent amounts of protein being loaded in each lane.


25 37 25 37 25 37 25 37 25 37
Sdb25p
wt
dbf2-3
cdc14
cdc28-13
cdc28-4

the permissive temperature the cells re-entered the cell cycle synchronously and Dbf2 kinase activity rapidly reached a peak within 10 minutes, coincident with a sharp increase in Sdb25 protein levels. This was followed 5-10 minutes later by the peak of Sdb25-associated kinase activity, roughly coincident with the peak of Sdb25 protein levels (Figure 4.8). The Sdb25 protein was then degraded, returning to background levels 35 minutes after cells had been returned to 25^{0}C. Note that cells enlarge during the 37^{0}C holding, and consequently upon release to 25^{0}C the events of late M phase, G1 and START are compressed. On the basis of this experiment it is therefore difficult to determine the precise phase in which Sdb25 is degraded, but this data does show that activation of the Sdb25-associated kinase occurs after the Dbf2 kinase. Thus, both at the level of transcription and kinase activity, it appears that *SDB25* and *DBF2* are sequentially activated in late mitosis.

4.9 SUMMARY

Rabbit polyclonal antisera have been raised, and successfully used to detect the 40-kD Sdb25/p40 polypeptide in crude yeast extracts. Detection, by immunoblotting, required a 1:25 dilution of the Sdb25 anti-sera, indicative perhaps of the low abundance of the protein in yeast cells. Although Sdb25 was also shown to be a phosphoprotein, the physiological relevance of this finding is not clear. In the many immunoblots carried out the intensity of the more phosphorylated form varied in a seemingly random fashion. Thus it was not possible to determine, for example, whether Sdb25 phosphorylation was Cdc28 or Dbf2-dependent. Sdb25 is associated with a very strong kinase, which was shown to be clearly dependent on the presence of a functional Cdc28 protein kinase. This is in

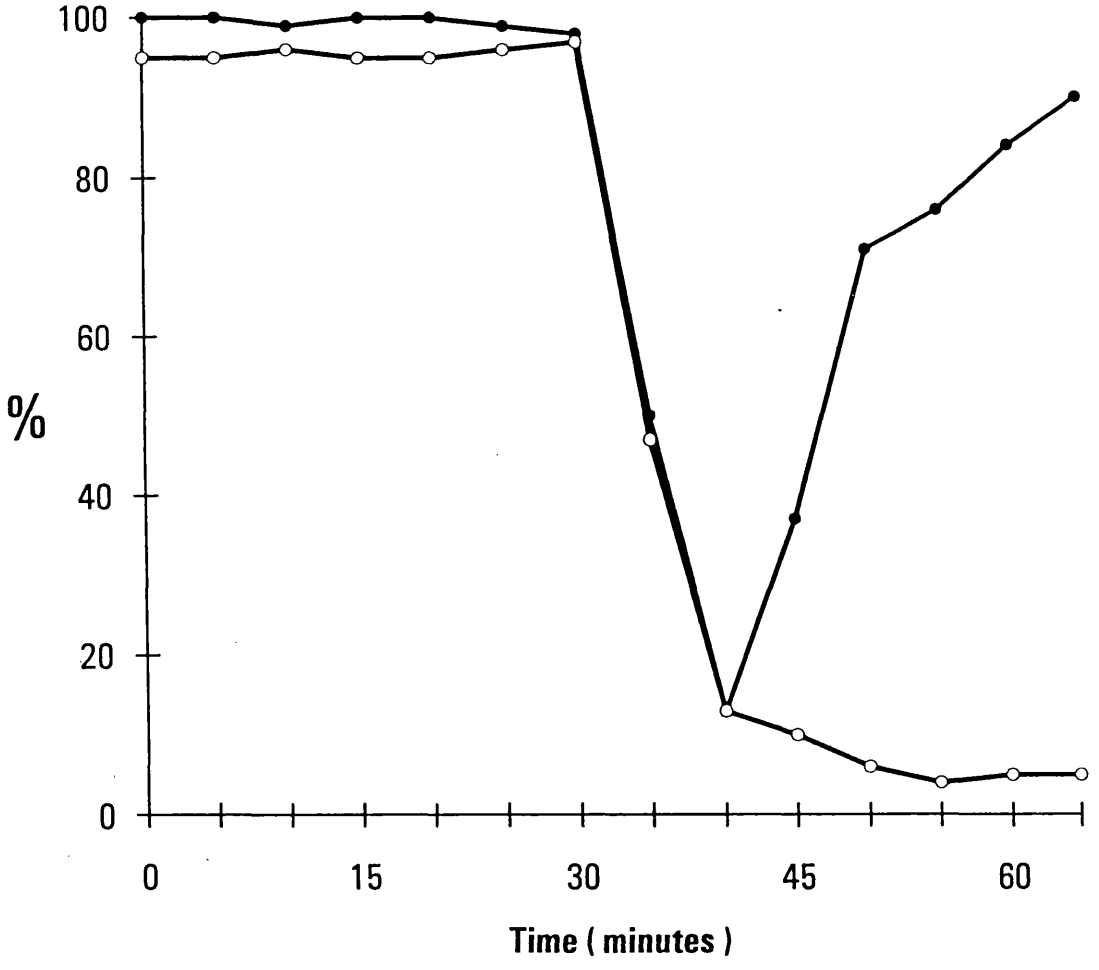
Figure 4.8

Sequential activation of Dbf2 and Sdb25-associated kinase

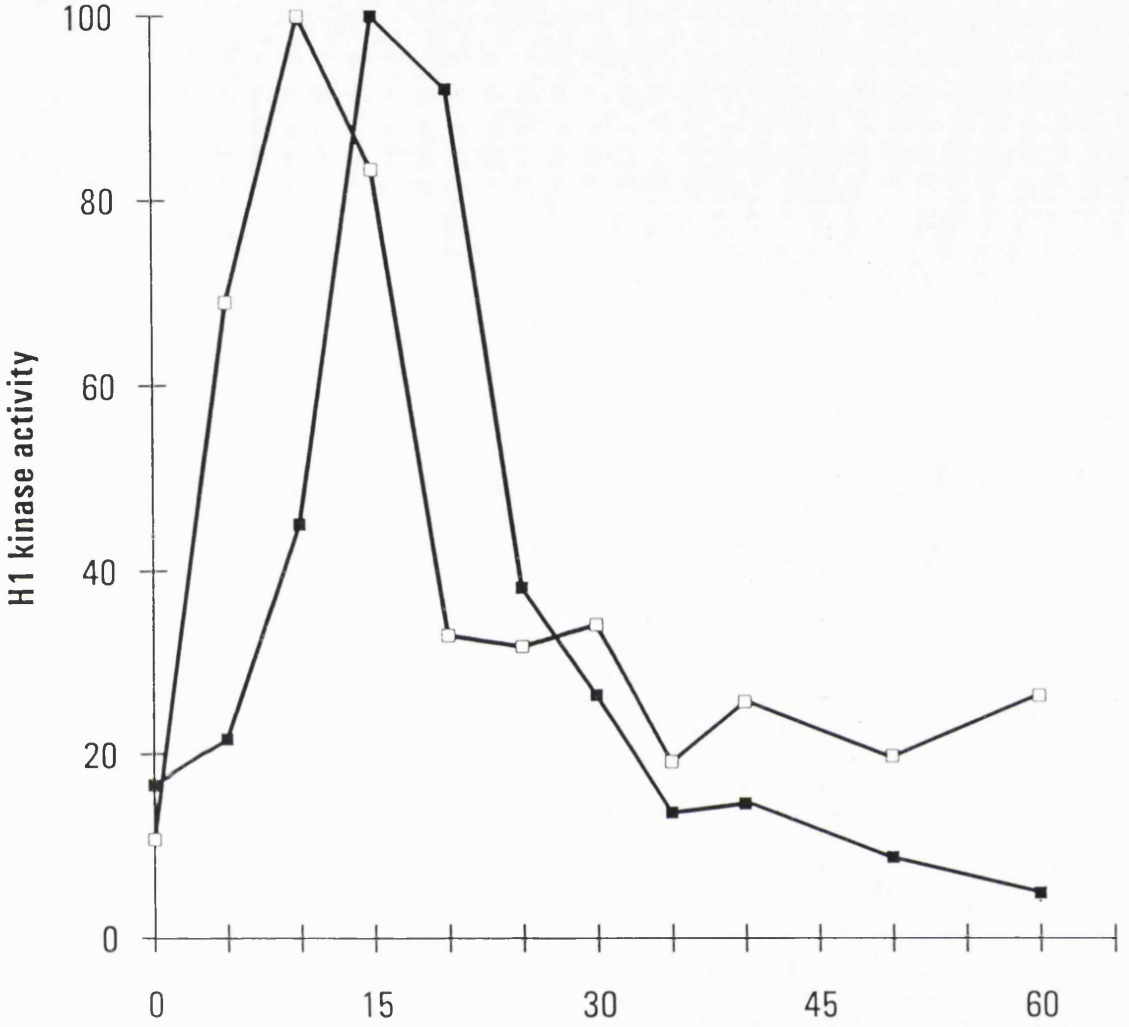
A culture of strain LJ1 (*dbf2-2*) was synchronized by a temperature shift of *dbf2-2* as described in Materials and Methods. Cell samples were taken every 5 minutes for just over one hour following return to the permissive temperature, and protein extracts were prepared. (A) Morphological indicators of cell cycle position: ●, budded cells; ○, divide chromatin. (B) Immunoprecipitates prepared from protein samples were used for kinase assays at the time points indicated. Kinase activities were quantitated and normalized as described in Figure 6. □, Dbf2 kinase; ■, Sdb25-associated kinase. Protein levels were analyzed by immunoblotting using anti-Sdb25 antibodies, the non-specific band appearing above the Sdb25 band serving as a loading control.

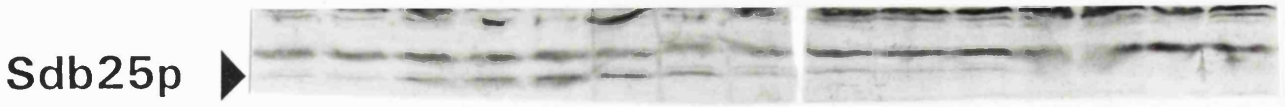
Culture synchronization was carried out by L.H. Johnston, A.L. Johnson and J.H. Toyn.

A



B


100
80
60
40
20
0
H1 kinase activity
0
15
30
45
60
Time (minutes)


Sdb25p

apparent contrast to earlier *in vitro* findings that p40/Sdb25 is a potent inhibitor of Cdc28 kinase activity (Mendenhall, 1993) (see Discussion).

Using a synchronized cell culture, a detailed examination was carried out on the relative fluctuations of Sdb25 transcript, protein and associated kinase activity levels. Sdb25 were found to fluctuate in a cyclin-like manner, being upregulated in late M phase/G1. This raises the question of whether the apparent instability of the Sdb25 protein is due to the presence of the three PEST regions in the Sdb25 protein sequence. Initial up-regulation of Sdb25 is coincident with maximal expression of *SDB25*, and the appearance of the Sdb25-associate kinase. Furthermore, from this cell cycle experiment it is possible to infer that maximal expression of *SDB25* occurs in late M phase, rather than in early G1. Thus *SDB25* is periodically regulated later in the cell cycle than any of the known cell cycle regulated genes, although the other *S.cerevisiae* Cdk inhibitor, Far1, appears to have a somewhat similar manner of regulation in mitotically dividing cells (McKinney *et al.*, 1993).

CHAPTER FIVE

CHARACTERIZATION OF *SDB24*

Plasmid pSDB24 consists of a 6.75kb *S.cerevisiae* genomic DNA fragment, cloned into the *Bam*HI site of the multicopy vector YRp12. Like pSDB25, it is an allele-specific suppressor of *dbf2*-ts mutants, rescuing only *dbf2-3* at the restrictive temperature 37^{0}C. Moreover, this suppression is as strong as that observed when *dbf2-3* is transformed with the wild-type *DBF2* gene in the multicopy plasmid YRp12 (Figure 5.1).

5.1 SUBCLONING AND LOCALIZATION OF *SDB24*

Initially a restriction map of the DNA fragment, carrying the suppressor gene *SDB24*, was constructed prior to testing subclones for complementing activity (Figure 5.2A). Subcloning was carried out by conventional means, and the complementing ability tested by transforming the DNA fragment into yeast strain L182-7A (*dbf2-3*) and assessing the ability to suppress the *dbf2*-ts phenotype at the restrictive temperature, 37^{0}C. This was done by growing the transformants at 25^{0}C on WB Agar plates, with the appropriate supplements added, and then replica plating onto YEPD at 37^{0}C. In each case the controls used were transformation with the YRp12 alone and transformation with YRp12*DBF2*. From the results of four subclones tested it was deduced that the 1.6kb *Xho*I- *Bgl*II genomic fragment of pSDB24 was necessary, but not sufficient, for suppression (Figure

Figure 5.1

pSDB24 is an allele-specific suppressor of *dbf2-3*

Yeast strains *dbf2-1*, *dbf2-2* and *dbf2-3* were transformed with pSDB24 and grown on selective plates for seven days at 25°C. For comparison, *dbf2-3* was also transformed with YRp12*DBF2* and grown under the same conditions. Single transformants were streaked out on YEPD plates, incubated for two days at 37°C, and then photographed as shown.

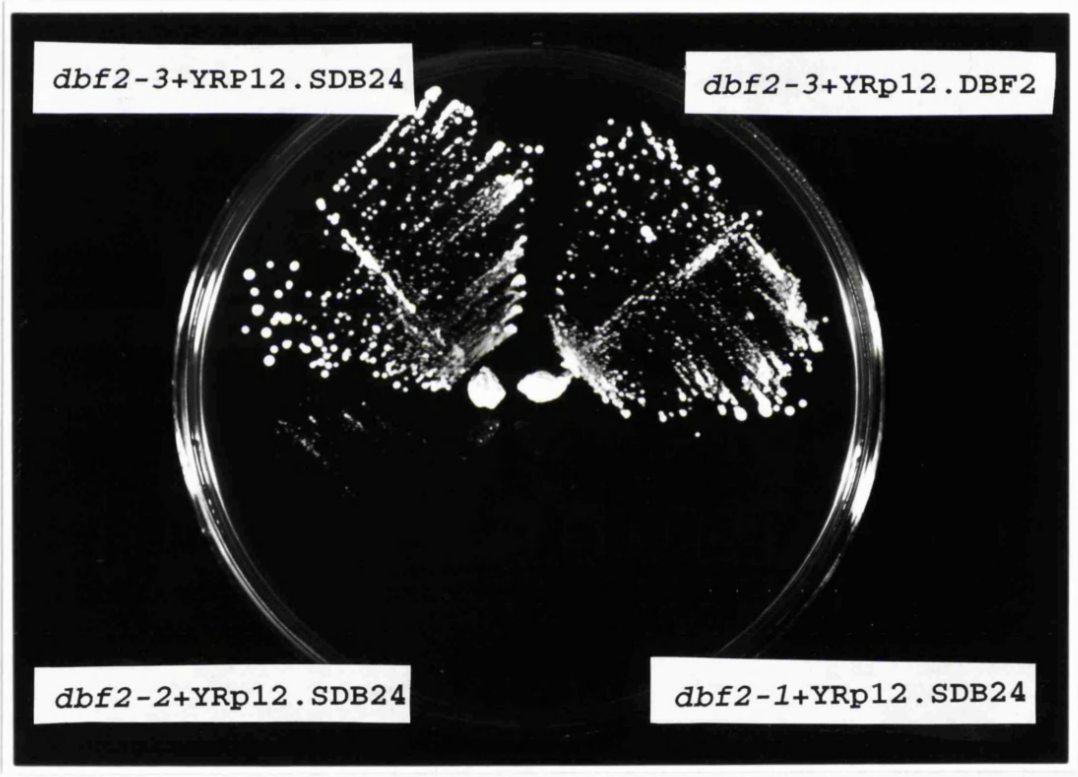

dbf2-3+YRP12.SDB24
dbf2-3+YRp12.DBF2
dbf2-2+YRp12.SDB24
dbf2-1+YRp12.SDB24

Figure 5.2

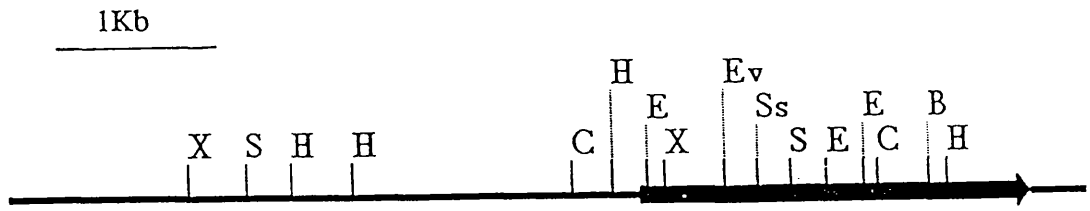
A. Partial restriction map of the 6.75kb genomic DNA fragment of pSDB24

The black arrow indicates the ORF of *SDB24* as determined from the nucleotide sequence. Abbreviations for restriction enzymes: B(*Bgl*II), C(*Cla*I), E(*Eco*RI), Ev(*Eco*RV), H(*Hin*dIII), S(*Sst*I), Ss(*Ssp*I), X(*Xho*I).

B. Localization of *dbf2-3* complementing activity

The bars represent restriction fragments either ligated into the MCS of the high copy number yeast shuttle vector pJF, or generated by excision, followed by religation, of part of the *SDB24* locus in YRp12.

A



B

Complementing activity	
–	
–	
–	
+	

*Xho*I-*Bgl*II 1.6kb

Deduced region having necessary, but not sufficient, complementing activity.

5.2B). This implied that *SDB24* was at least 1.6kb in length and spanned the *Xho*I-*Bgl*II region.

An estimation of the size of the *SDB24* transcript was made by probing a Northern blot of total RNA extracted from strain CG378. The probe used was the 1.1kb *Eco*RI fragment internal to the *Xho*I-*Bgl*II region. This hybridized to a single band of 2.5kb as measured against a ladder of RNA markers (data not shown).

5.2 SEQUENCING OF *SDB24*

To sequence *SDB24* it was decided to generate as many subclones as possible, centred on the 2.1kb *Hin*dIII fragment spanning the *Xho*I-*Bgl*II region, which could easily be ligated into the MCS of Bluescript. This allowed the M13 universal primers "-40" and "rev" to be used for all sequencing reactions. All other sequencing was done using synthetic DNA oligonucleotide primers complementary to a previously determined sequence. The complete sequencing strategy is shown in Figure 5.3. In this region one large open reading frame (ORF) was found capable of encoding a protein of 821 amino acids with a calculated molecular weight of 94,954 (Figure 5.4). The predicted protein would be acidic, with an estimated pI of about 4.7.

5.3 DEDUCED AMINO ACID SEQUENCE OF Sdb24

When compared with existing sequences in the databanks the *SDB24* amino acid sequence showed weak homology (19-20% identity, after the introduction of several gaps) with various cytoskeletal-related proteins, in particular myosin and tropomyosin. This

Figure 5.3

Sequencing strategy for *SDB24*

The shaded arrows indicate DNA sequence determined using the M13 universal primers Rev or "-40". The open arrows indicate sequence determined using synthesized primers. The *SDB24* ORF is represented by the black arrow.

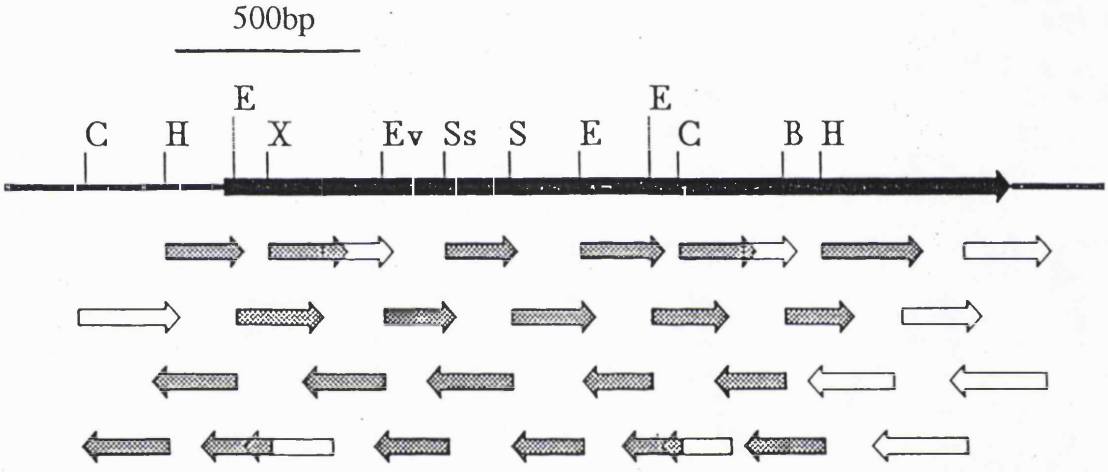

500bp
E
E
C
H
X
Ev
Ss
S
E
C
B
H

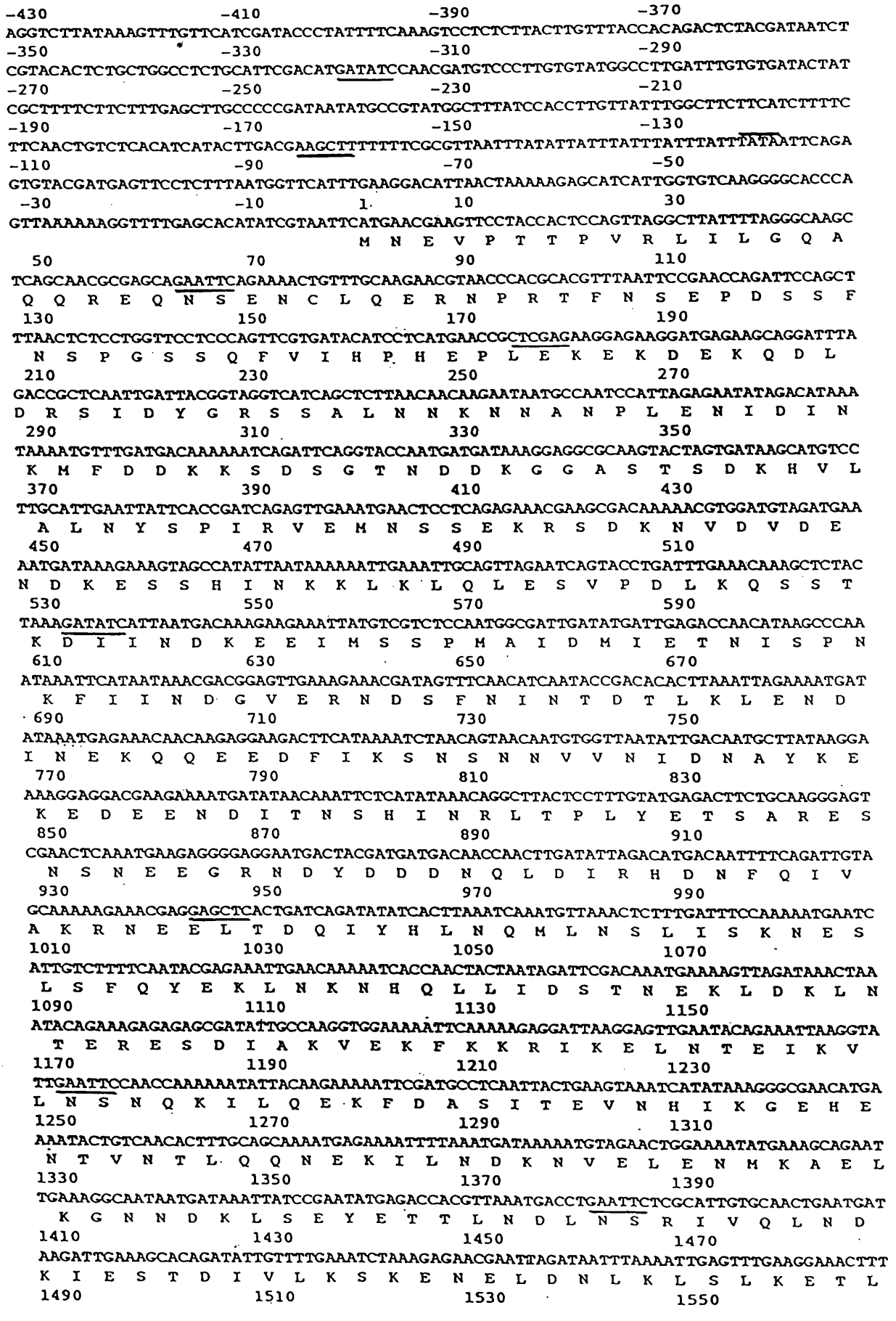
Figure 5.4

Nucleotide sequence of *SDB24*

The first nucleotide of the ORF has been numbered one. Overlined is a promoter consensus sequence TATA (-124). The following restriction sites are indicated by underlining:

*Bgl*II (1820); *Cla*I (-413), (1490); *Eco*RI (62), (1169), (1329); *Eco*RV (531); *Hin*dIII (-166), (1946); *Sst*I (942); *Xho*I (174).

The predicted amino acid sequence is shown in single letter code.


-430 -410 -390 -370
AGGTCTTATAAAGTTTGTTCATCGATACCCTATTTTCAAAGTCCTCTCTTACTTGTTTACCACAGACTCTACGATAATCT
-350 -330 -310 -290
CGTACACTCTGCTGGCCTCTGCATTCGACATGATATCCAACGATGTCCCTTGTGTATGGCCTTGATTTGTGTGATACTAT
-270 -250 -230 -210
CGCTTTTCTTCTTTGAGCTTGCCCCCGATAATATGCCGTATGGCTTTATCCACCTTGTTATTTGGCTTCTTCATCTTTTC
-190 -170 -150 -130
TTCAACTGTCTCACATCATACTTGACGAAGCTTTTTTTCGCGTTAATTTATATTATTTATTTATTTATAATTCAGA
-110 -90 -70 -50
GTGTACGATGAGTTCCTCTTTAATGGTTCATTTGAAGGACATTAACTAAAAAGAGCATCATTGGTGTCAAGGGGCACCCA
-30 -10 1 10 30
GTTAAAAAAGGTTTTGAGCACATATCGTAATTCATGAACGAAGTTCCTACCACTCCAGTTAGGCTTATTTTAGGGCAAGC
M N E V P T T P V R L I L G Q A
50 70 90 110
TCAGCAACGCGAGCAGAATTCAGAAAACTGTTTGCAAGAACGTAACCCACGCACGTTTAATTCCGAACCAGATTCCAGCT
Q Q R E Q N S E N C L Q E R N P R T F N S E P D S S F
130 150 170 190
TTAACTCTCCTGGTTCCTCCCAGTTCGTGATACATCCTCATGAACCGCTCGAGAAGGAGAAGGATGAGAAGCAGGATTTA
N S P G S S Q F V I H P H E P L E K E K D E K Q D L
210 230 250 270
GACCGCTCAATTGATTACGGTAGGTCATCAGCTCTTAACAACAAGAATAATGCCAATCCATTAGAGAATATAGACATAAA
D R S I D Y G R S S A L N N K N N A N P L E N I D I N
290 310 330 350
TAAAATGTTTGATGACAAAAAATCAGATTCAGGTACCAATGATGATAAAGGAGGCGCAAGTACTAGTGATAAGCATGTCC
K M F D D K K S D S G T N D D K G G A S T S D K H V L
370 390 410 430
TTGCATTGAATTATTCACCGATCAGAGTTGAAATGAACTCCTCAGAGAAACGAAGCGACAAAAACGTGGATGTAGATGAA
A L N Y S P I R V E M N S S E K R S D K N V D V D E
450 470 490 510
AATGATAAAGAAAGTAGCCATATTAATAAAAAATTGAAATTGCAGTTAGAATCAGTACCTGATTTGAAACAAAGCTCTAC
N D K E S S H I N K K L K L Q L E S V P D L K Q S S T
530 550 570 590
TAAAGATATCATTAATGACAAAGAAGAAATTATGTCGTCTCCAATGGCGATTGATATGATTGAGACCAACATAAGCCCAA
K D I I N D K E E I M S S P M A I D M I E T N I S P N
610 630 650 670
ATAAATTCATAATAAACGACGGAGTTGAAAGAAACGATAGTTTCAACATCAATACCGACACACTTAAATTAGAAAATGAT
K F I I N D G V E R N D S F N I N T D T L K L E N D
690 710 730 750
ATAAATGAGAAACAACAAGAGGAAGACTTCATAAAATCTAACAGTAACAATGTGGTTAATATTGACAATGCTTATAAGGA
I N E K Q Q E E D F I K S N S N N V V N I D N A Y K E
770 790 810 830
AAAGGAGGACGAAGAAAATGATATAACAAATTCTCATATAAACAGGCTTACTCCTTTGTATGAGACTTCTGCAAGGGAGT
K E D E E N D I T N S H I N R L T P L Y E T S A R E S
850 870 890 910
CGAACTCAAATGAAGAGGGGAGGAATGACTACGATGATGACAACCAACTTGATATTAGACATGACAATTTTCAGATTGTA
N S N E E G R N D Y D D D N Q L D I R H D N F Q I V
930 950 970 990
GCAAAAAGAAACGAGGAGCTCACTGATCAGATATATCACTTAAATCAAATGTTAAACTCTTTGATTTCCAAAAATGAATC
A K R N E E L T D Q I Y H L N Q M L N S L I S K N E S
1010 1030 1050 1070
ATTGTCTTTTCAATACGAGAAATTGAACAAAAATCACCAACTACTAATAGATTCGACAAATGAAAAGTTAGATAAACTAA
L S F Q Y E K L N K N H Q L L I D S T N E K L D K L N
1090 1110 1130 1150
ATACAGAAAGAGAGAGCGATATTGCCAAGGTGGAAAAATTCAAAAAGAGGATTAAGGAGTTGAATACAGAAATTAAGGTA
T E R E S D I A K V E K F K K R I K E L N T E I K V
1170 1190 1210 1230
TTGAATTCCAACCAAAAAATATTACAAGAAAAATTCGATGCCTCAATTACTGAAGTAAATCATATAAAGGGCGAACATGA
L N S N Q K I L Q E K F D A S I T E V N H I K G E H E
1250 1270 1290 1310
AAATACTGTCAACACTTTGCAGCAAAATGAGAAAATTTTAAATGATAAAAATGTAGAACTGGAAAATATGAAAGCAGAAT
N T V N T L Q Q N E K I L N D K N V E L E N M K A E L
1330 1350 1370 1390
TGAAAGGCAATAATGATAAATTATCCGAATATGAGACCACGTTAAATGACCTGAATTCTCGCATTGTGCAACTGAATGAT
K G N N D K L S E Y E T T L N D L N S R I V Q L N D
1410 1430 1450 1470
AAGATTGAAAGCACAGATATTGTTTTGAAATCTAAAGAGAACGAATTAGATAATTTAAAATTGAGTTTGAAGGAAACTTT
K I E S T D I V L K S K E N E L D N L K L S L K E T L
1490 1510 1530 1550

```
ATCGATTTCTAAGGACTTCAACGACTCTGACCTTATAGCACAAATTAATGAGCTAATATCTACAAAAAACAACTTGCAAC
 S  I  S  K  D  F  N  D  S  D  L  I  A  Q  I  N  E  L  I  S  T  K  N  N  L  Q  Q
1570                1590                1610                1630
AAAAAATGGATGATTTGAATAACCTGAACGACGATAATTTGAAGGTAGTACAGGATAAATTAATTAAAAATGAGGAGACG
  K  M  D  D  L  N  N  L  N  D  D  N  L  K  V  V  Q  D  K  L  I  K  N  E  E  T
1650                1670                1690                1710
CTAAAACTAAAAGAAGCTGAAATTGATTCATTGAATAGTGAAATGGACGAGCTAAAAAAAACAGATCACTACAAAAGATGA
L  K  L  K  E  A  E  I  D  S  L  N  S  E  M  D  E  L  K  K  Q  I  T  T  K  D  D
1730                1750                1770                1790
TGAATTTAAGATGTGGCAAAGTAAATACGAAACTGTCGAAGACGAAGCTAAGATACGCAATGCCGAAGTTACAGAGTTGA
 E  F  K  M  W  Q  S  K  Y  E  T  V  E  D  E  A  K  I  R  N  A  E  V  T  E  L  N
1810                1830                1850                1870
ACGGAGATATTGAAGATCTAAAGGAATCAAAATTGCATTTGGAAGAAACAATTACGGAACTAGAAAACAAAGTTCATAAA
  G  D  I  E  D  L  K  E  S  K  L  H  L  E  E  T  I  T  E  L  E  N  K  V  H  K
1890                1910                1930                1950
CTAGAAAATGAGTGTGAACTGGAGAAACAAAAATTCGAAAAAACTTCGCTAGAATTAGAAAGCTTACAACTAAAAAATAG
L  E  N  E  C  E  L  E  K  Q  K  F  E  K  T  S  L  E  L  E  S  L  Q  L  K  N  S
1970                1990                2010                2030
TAACATTCAGGCTGAGCACATCAAAGAATTGGAAAATCTTCATGAAAACCTTATTTCTCTTCAAAATGAATTGAAGATTT
 N  I  Q  A  E  H  I  K  E  L  E  N  L  H  E  N  L  I  S  L  Q  N  E  L  K  I  S
2050                2070                2090                2110
CATCCGACAGGATAACAACATTAACAAAAGAAAATGAAGTATTAATGGAACAAAATAATAATAACAATAATAGCGTTACT
  S  D  R  I  T  T  L  T  K  E  N  E  V  L  M  E  Q  N  N  N  N  N  N  S  V  T
2130                2150                2170                2190
TTAAGCAATGATCAAAAGGATAGAGATGATGAAAAAATTAAATCATTAGGAAAACAAGTACAAGATTGGAAAGAAAAGTA
L  S  N  D  Q  K  D  R  D  D  E  K  I  K  S  L  G  K  Q  V  Q  D  W  K  E  K  Y
2210                2230                2250                2270
TGAGGCCAAAGAAAAAAGATACAAATAAAAGGCTAAAGTTATTAGCAGAAGATTTGTATATCCAGTATTCATCCAAACACG
 E  A  K  E  K  D  T  N  K  R  L  K  L  L  A  E  D  L  Y  I  Q  Y  S  S  K  H  E
2290                2310                2330                2350
AACAAAAGGTCAAACTGTTGAAAAAAGGCTATGAAAACAAGTACCAAAATAAGTTTGATCAATTAAATTTAGAAAATAAA
  Q  K  V  K  L  L  K  K  G  Y  E  N  K  Y  Q  N  K  F  D  Q  L  N  L  E  N  K
2370                2390                2410                2430
ACCCTGTCAGAAGAAATCGAGCAATTGAACAAGCAGCTGTCTTCAGAAAGAGAAGAAAAGCAGGAGTTACTCAAGTTGTT
T  L  S  E  E  I  E  Q  L  N  K  Q  L  S  S  E  R  E  E  K  Q  E  L  L  K  L  L
2450                2470                2490
AGAAAATGAAAAAAAATAAACGCATTTTTTTTTTCTTTTGCATTTTATCTTTTCCCTT
 E  N  E  K  K  *
```

suggested that perhaps Sdb24 had the α-helical coiled-coil structure common to such proteins. As originally predicted for fibrous proteins, and shown by direct sequence analysis of skeletal muscle tropomyosin (Stone and Smillie, 1979), such coiled-coil structures can be divided into seven residue units. In this so called heptad repeat motif the first and fourth residues are hydrophobic, so promoting interactions between the two coils, and the remainder are largely hydrophillic.

A histogram of the distribution of hydrophobic residues for amino acids 350-490 of Sdb24 strongly suggests that the protein forms a coiled-coil in this region (Figure 5.5). Consistent with this, there are no proline residues, a strong breaker of α-helices, in this segment of the protein. It is also interesting to note that Sdb24 has four examples of the leucine-zipper motif ($-L(x)_6L(x)_6L(x)_6L-$) originally proposed as a structure common to a class of DNA-binding proteins (Landschultz, 1988), but also predictive of a coiled-coil structure (O'Shea *et al.*, 1989). An extended example of this motif is embedded in the proposed coiled-coil region of Sdb24, and so reinforces the possibility that Sdb24 functions as a homodimer *in vivo*. The three other leucine zipper motifs begin at residues 316, 510 and 602.

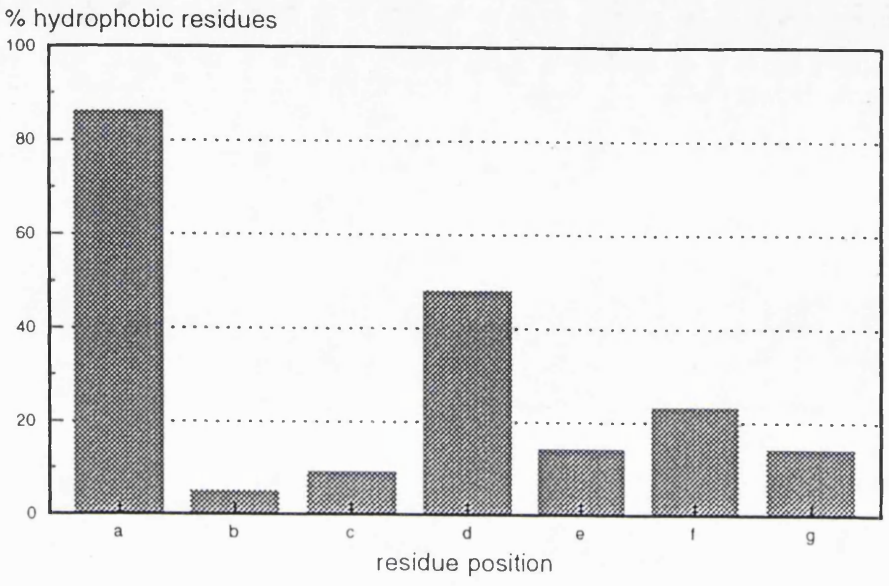
The predicted Sdb24 protein is highly charged, with 39.5% charged residues (16.3% basic and 23.2% acidic). Searching the PROSITE databank reveals that Sdb24 has potential phosphorylation sites for several different protein kinases. Of particular interest are the three copies of the Cdc28/Cdc2 phosphorylation motif S/T-P-X-K/R, all three residing at the N-terminal end of the protein, beginning at residues 7,128 and 202.

Figure 5.5

Region of heptapeptide repeats found in Sdb24

The numbers on the left indicate the residue number of the amino acids with respect to the protein sequence. The sequence is arranged in seven-residue repeats, with the positions a,b,c,d,e,f and g marked above the column. Leucine residues, defining the extended leucine-zipper motif, are shown in bold type. Alongside is a histogram showing the distribution of hydrophobic residues for this sequence.

	a	b	c	d	e	f	g
352	I	D	S	T	N	E	K
	L	D	K	L	N	T	E
	R	E	S	D	I	A	K
	V	E	K	F	K	K	R
380	I	K	E	L	N	T	E
	I	K	V	L	N	S	N
	Q	K	I	L	Q	E	K
	F	D	A	S	I	T	E
408	V	N	H	I	K	G	E
	H	E	N	T	V	N	T
	L	Q	Q	N	E	K	I
	L	N	D	K	N	V	E
436	L	E	N	M	K	A	E
	L	K	G	N	N	D	K
	L	S	E	Y	E	T	T
	L	N	D	L	N	S	R
464	I	V	Q	L	N	D	K
	I	E	S	T	D	I	V
	L	K	S	K	E	N	E
	L	D	N	L	K	L	S
492	L	K	E	T	L	S	I



5.4 EXAMINATION OF THE *SDB24* TRANSCRIPT THROUGHOUT THE CELL CYCLE

To determine the levels of *SDB24* mRNA in the cell cycle, a Northern blot was prepared from the elutriator-synchronized culture described in Chapter 3 (Section 3.8). The *SDB24* transcript levels clearly fluctuate in a cell cycle-dependent manner (Figure 5.6), with an approximately ten-fold increase in *SDB24* mRNA levels through the cell cycle. Correlation of the peak of *SDB24* expression and the budding curve indicates that *SDB24* appears to be maximally abundant early in the cell cycle around G1/S. To confirm this, the Northern blot was also probed with Histone *H2A*, which is known to have a broad peak of expression in S phase. Comparison of *SDB24* and *H2A* shows that *SDB24* peaks slightly earlier than *H2A* in both cell cycles. The Northern blot was also probed with *POL1*, one of the large group of genes that are coordinately regulated at the G1/S boundary. Comparison with *POL1* transcript levels shows that *SDB24* peaks some 15 minutes later in the cell cycle. Thus it appears that the *SDB24* transcript is maximally abundant early in S phase.

5.5 GENERATION OF AN *SDB24* NULL ALLELE

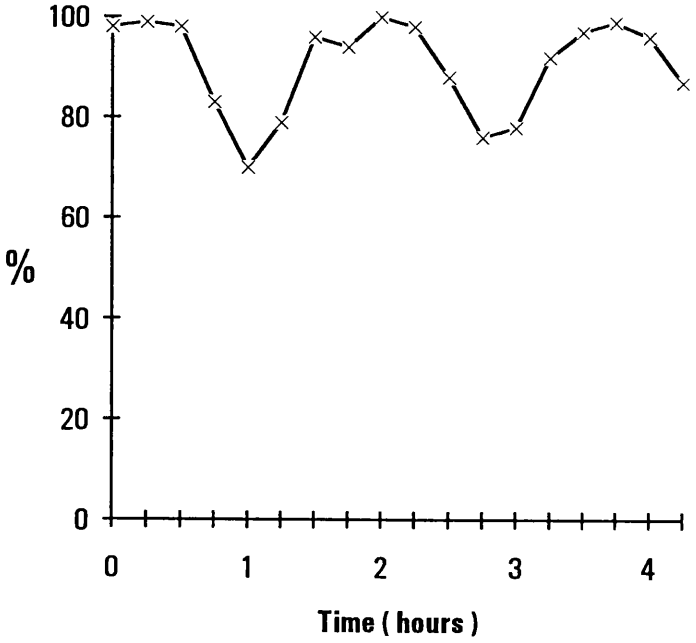
To examine the effect of deleting the chromosomal *SDB24* gene the same approach was adopted as that used to generate a yeast strain containing an *sdb25*Δ null allele (see Chapter 3, Section 3.9). The *BglII-Sal*I 5.6kb fragment, containing *SDB24* was excised from pSDB24 and ligated into the MCS of Bluescript. Following this, the internal 1.6 kb *Eco*RI-*Bgl*II fragment was removed and replaced with the *URA3* gene (Figure 5.7). This effectively results in deletion of the first 65% of the Sdb24 protein, and thus it is unlikely

Figure 5.6

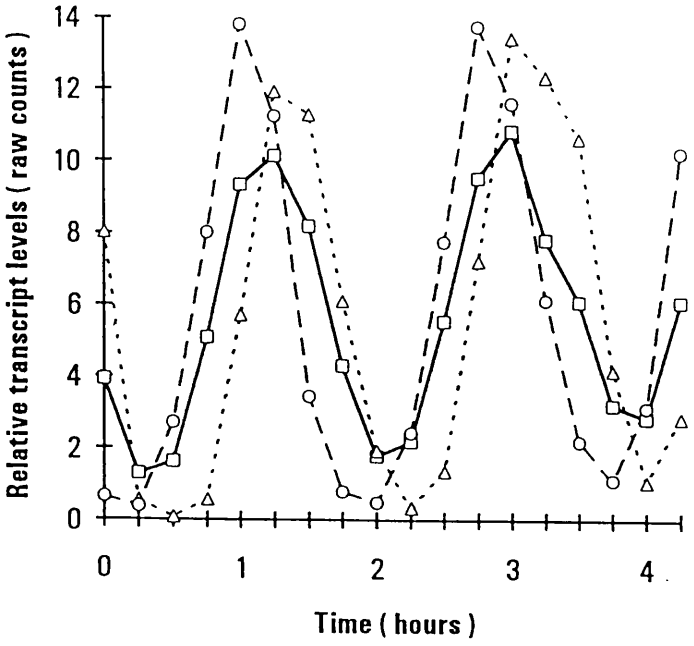
***SDB24* transcript levels in elutriator-synchronized cells**

The culture used in this experiment has been previously described (Kitada *et al.*,1993). Samples were taken every fifteen minutes for just over two cell cycles. RNA samples were analyzed by Northern hybridization using the indicated probes. RNA levels were quantitated by densitometry from suitably exposed autoradiograms. A. Percentage buds (from Kitada *et al.*, 1993). B. Comparison of the relative timing of *POLI*, *SDB24* and *H2A* transcripts. ○, *POL1*; □, *SDB24*; △, *H2A* C. Comparison of the relative timing of *DBF2* and *SDB24* transcripts. ◇, *DBF2*; ○, *SDB24* D. Autoradiograms of the transcript levels described in panels B and C.

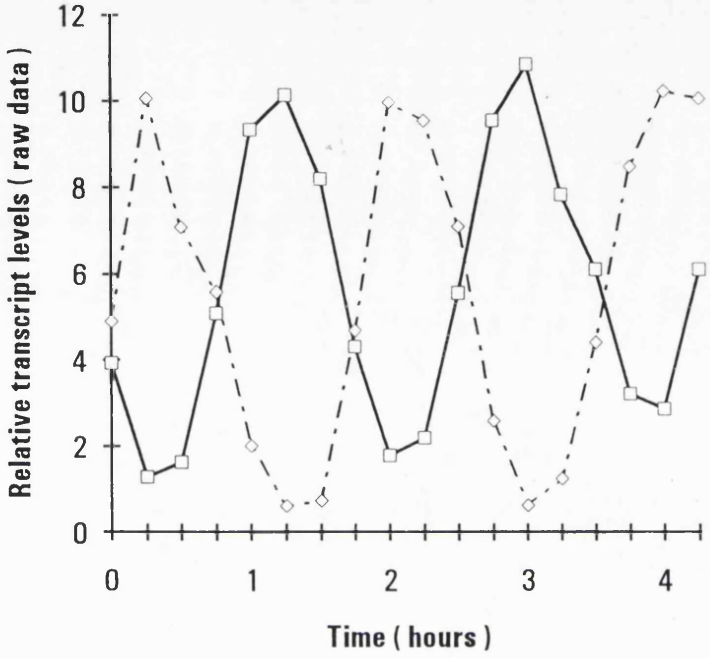
A


100
80
60
%
40
20
0
0
1
2
3
4
Time (hours)

B


14
12
10
8
6
4
2
0
Relative transcript levels (raw counts)
0
1
2
3
4
Time (hours)

C


Relative transcript levels (raw data)
12
10
8
6
4
2
0
0
1
2
3
4
Time (hours)

D

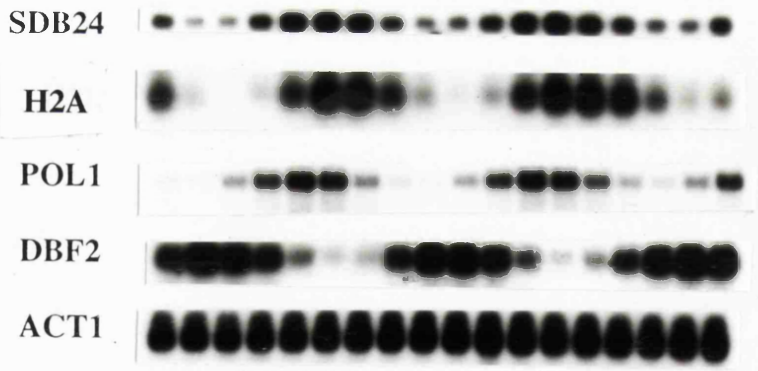
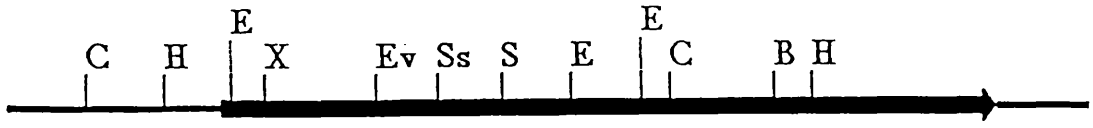

SDB24
H2A
POL1
DBF2
ACT1

Figure 5.7

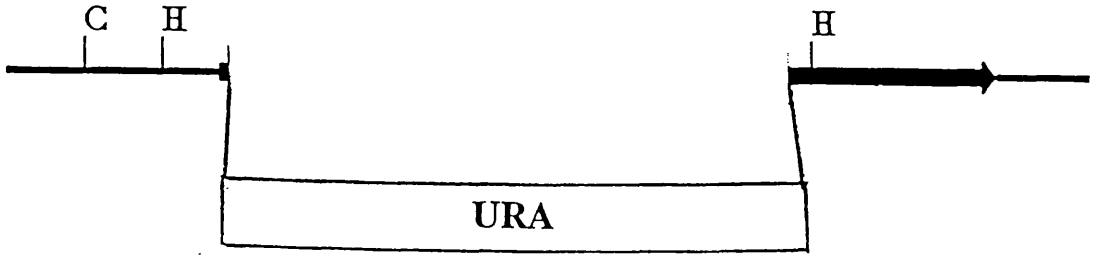
Strategy for deletion/disruption of the chromosomal *SDB24* gene

The internal 1.6kb *Eco*R1-*Bgl*11 gene fragment of *SDB24* was replaced with the *URA3* gene.



WT SDB24

Δsdb24::URA



that a truncated, functional, Sdb24 protein still remains. The *URA3* disrupted *SDB24* gene was then isolated as a linear fragment and used to transform the diploid strain CG378/379. To anticipate the possibility that *SDB24* is non-essential, the linear fragment containing *sdb24Δ::URA3* was also used to transform, separately, the haploid strain CG378. Integrants were selected on minimal media, lacking uracil. To confirm replacement of *SDB24* with *URA3*, genomic DNA from 9 single diploid URA^+ CG378/379 colonies, and 12 single haploid CG378 URA^+ colonies was extracted and used in a Southern hybridization analysis (Figure 5.8). This showed that all the colonies selected had a banding pattern consistent with *SDB24* being replaced by *URA3*, including the haploid transformants. Thus it would appear that the *SDB24* gene is dispensable. To confirm this, one of the URA^+ diploid colonies was sporulated and sixteen tetrads dissected. All four spores colonies from each tetrad germinated and formed colonies, and replica-plating onto selective plates showed that two colonies from each tetrad were URA^+ and two URA^-.

5.6 ANALYSIS OF CELLS DELETED FOR *SDB24*

As with *sdb25Δ* cells, *sdb24Δ* cells show no difference in growth rates as compared to an isogenic wild-type control strain. Similarly, deletion of *SDB24* results in an increase in budded cells comparable to that observed with deletion of *SDB25* (84% in *sdb24Δ* cells, 65% in wild-type). However, there is no significant increase in dumbbells in *sdb24Δ* cells, as there is in *sdb25Δ* cells.

FACS analysis of an *sdb24Δ* culture shows a significant reduction of cells with a 1C content as compared to a control strain (Figure 5.9A), again similar to the FACS profile of an *sdb25Δ* culture. However, the forward scatter analysis shows that deletion

Figure 5.8

Southern hybridization confirming replacement of *SDB24* with *URA3*

The probe used was made with the 2.1 *Hind*III fragment of *SDB24*. Genomic DNA from 9 single diploid (lanes 3 to 11), and 12 haploid (lanes 13 to 24), URA^+ colonies was extracted and cut with *Eco*R1. For comparison, wild-type CG378 genomic DNA was also cut with *Eco*R1 (lane 1), and the 1kb DNA ladder was loaded as a size marker (lanes 12 and 25). The probe hybridized to the 1kb and 0.5kb bands of the 1kb ladder. No DNA was loaded in lane 2.

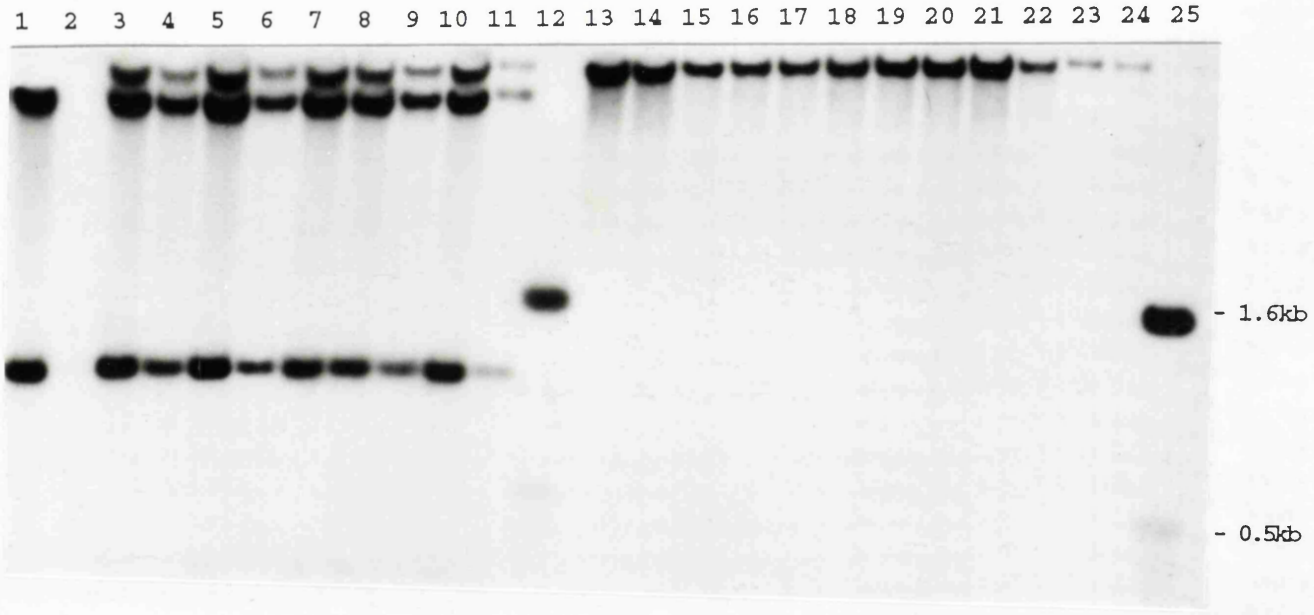

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25
- 1.6kb
- 0.5kb

Figure 5.9

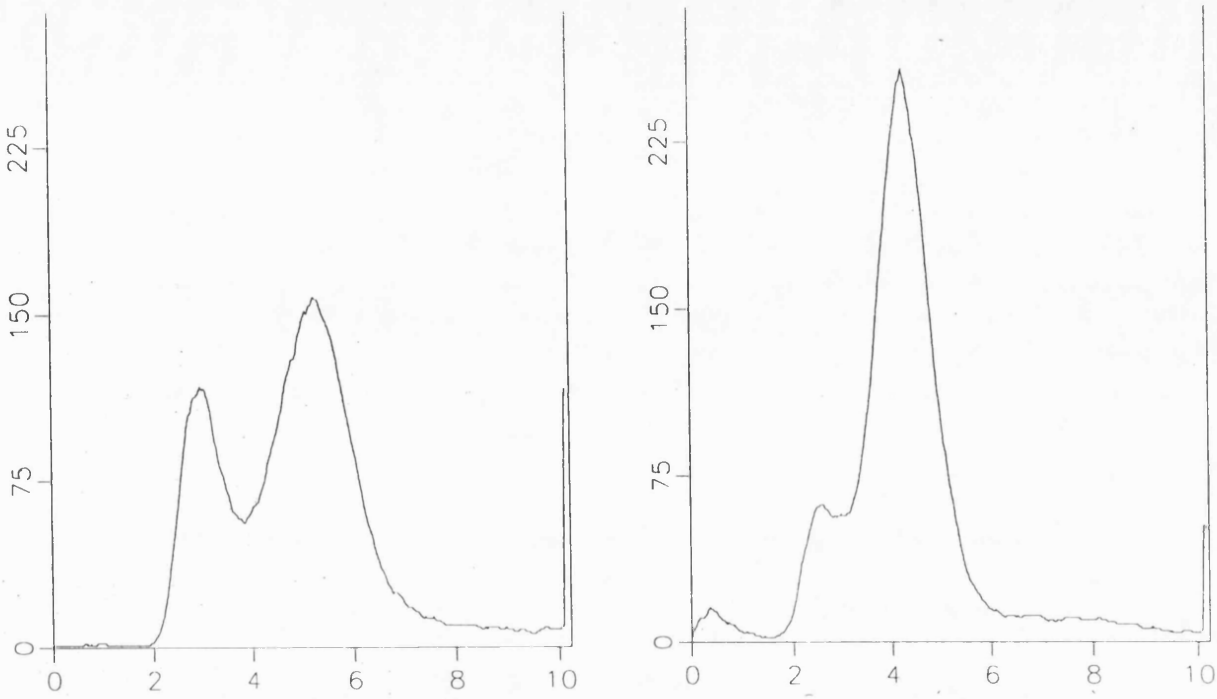
A. FACS analysis of cell deleted for *SDB24*

Mid-log cells growing in YEPD were harvested, fixed and prepared for FACS analysis to measure the distribution of DNA content. Cells were prepared for flow cytometry as described in Materials and Methods. Horizontal axes: Fluorescence (Relative DNA content); Vertical axis: Relative Cell numbers.

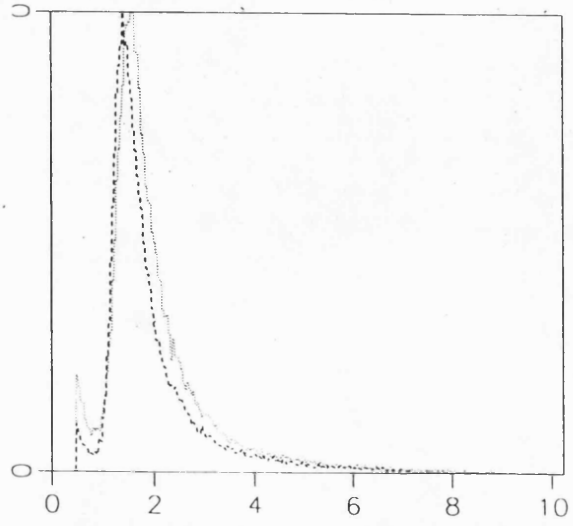
B. Forward scatter analysis of cells deleted for *SDB24*

Forward scatter analysis was carried out at the same time as FACS analysis, as an indicator of the relative cell size distribution of the two cultures. Horizontal axis: Forward scatter (Relative cell size); Vertical axis: Relative cell number.The lighter tracing, which has the peak slightly to the right, is the forward scatter plot of *sdb24*Δ cells, the darker tracing is the forward scatter plot of wild-type CG379 cells.

A



B



of *SDB24* results in cells being slightly larger, on average, than those of the control strain (Figure 5.9B). Thus, both morphological and FACS analysis of *sdb24*Δ cells indicate that deletion of *SDB24* perturbs progress through the cell cycle. Furthermore, this delay appears to be a delay in progress through some phase of the cycle after cells have completed G1.

5.7 CELLS DELETED FOR *SDB24* AND *DBF2* ARE INVIABLE

To examine whether cells deleted for *SDB24* and *DBF2* are inviable, strain CG378*sdb24*Δ::*URA3* was crossed to strain S2-2D*dbf2*Δ::*LEU2*. The resulting diploid was sporulated, 23 asci were dissected and scored for the *URA* and *LEU* markers. Altogether, 4 non-parental ditypes, 4 parental ditypes and 13 tetratypes were obtained, from which it could be inferred that a total of 18 spores were deleted for both *SDB24* and *DBF2*. Microscopic examination showed that all the double mutant spores had germinated, but subsequently ceased growing with a uniform dumbbell morphology. Thus both *SDB25* and *SDB24* have an essential function in a *dbf2*Δ background.

Next, it was tested whether synthetic lethality resulted when cells were deleted for *SDB24* and either of *DBF20*, *SDB25* or *SPO12*. However all pairwise combinations of double mutants involving *SDB24* are viable, and no phenotype was apparent that was specific to the double mutants.

5.8 SUMMARY

The *dbf2-3* suppressor gene residing on pSDB24 has been localized, sequenced and partially characterized. *SDB24* is a new yeast gene, and encodes a highly acidic protein of 95 kD. Cells deleted for *SDB24* are viable, and grow normally. However, progress through the cell cycle is perturbed, with a delay occurring some time after the initiation of S phase. In a strain deleted for *DBF2*, *SDB24* carries out an essential function, although this is not the case in any other mutant background tested, such as *dbf20*Δ, or *sdb25*Δ. Examination of *SDB24* transcript levels in synchronized cells reveals that the gene is periodically expressed, being maximally abundant in early S phase. No other *S. cerevisiae* cell cycle regulated gene is known to be regulated at precisely the same time, making *SDB24* unique in this respect.

CHAPTER SIX

DISCUSSION

With the objective of trying to further elucidate the physiological role of the protein kinase Dbf2 in regulation of the yeast cell cycle a screen was undertaken for high copy suppressors of *dbf2*-ts mutants. This work details the localization, sequencing and characterization of *SDB24* and *SDB25*, two of five *dbf2* suppressors that were isolated.

Of the two suppressors, more work was done on the characterization of *SDB25*, which extended to detailed biochemical experiments using Sdb25 antibodies. As a result of the genetic, physiological and biochemical experiments carried out, important advances have been made in understanding the role that Sdb25 may play in regulation of the budding yeast cell cycle.

6.1 *SDB25* AND *DBF2* MAY FUNCTION IN PARALLEL PATHWAYS IN LATE MITOSIS

Several lines of evidence suggest that Sdb25 has a role in regulating late mitotic events, and may act in a parallel pathway to Dbf2/20-dependent. Firstly, unlike previously identified suppressors of *dbf2*-ts mutants (Parkes and Johnston, 1992), *SDB25* is a strong allele-specific suppressor, when overexpressed on the multicopy plasmid YRp12. Furthermore, suppression by PSDB25 requires the presence of the Dbf2 structural and

functional homologue, Dbf20. One interpretation of this genetic data is that Sdb25 is directly interacting with one or both kinases, either in a complex or as a target substrate for the kinase activity. However, an equally valid explanation is that Sdb25 and Dbf2/20 are acting in parallel pathways, at the end of mitosis. Thus, when only slightly overexpressed on a multicopy plasmid, *SDB25* also requires Dbf20 to act in the alternative pathway, to allow completion of the cell cycle in the absence of a functional Dbf2 protein. From this, it might be anticipated that overexpressing *SDB25* to a sufficiently high level would result in cells bypassing the requirement for Dbf2/20 entirely. In fact, this was demonstrated in work carried out at the same time as this study by J. Toyn, who showed that YEpSDB25 can keep alive cells deleted for *DBF2/20.* (A YEp plasmid results in an, approximately four-fold increase in copy number per cell as compared to a YRp plasmid). Furthemore, YEpSDB25 can suppress all three alleles of *dbf2*, and also no longer requires the presence of *DBF20* to suppress *dbf2-3* The fact that *SDB25* has an essential function in a *dbf2*Δ background, *sdb25*Δ*dbf2*Δ cells being unable to grow, is consistent with this interpretation of parallel, partially redundant pathways. From this view, *sdb25*Δ*dbf2*Δ synthetic lethality arises because a component in both pathways is deleted, with the result that a vital cell function cannot be executed. This finding also supports the notion that *SDB25* has a role in the completion of essential mitotic events.

Further evidence for a late mitotic role for *SDB25* comes from examination of the *SDB25* transcript in synchronized cells. This shows that the gene is expressed under cell cycle regulation, with a peak of expression very close to the M/G1 boundary. Moreover, the tight correlation between the peaks of *SDB25* expression, Sdb25 protein levels and Sdb25-associated kinase activity demonstrates that transcriptional activation of *SDB25* is rapidly translated into a functional effect. This also suggests that the primary control over

Sdb25 function may be at the transcriptional level, rather than some post-translational effect, such as phosphorylation/dephosphorylation. Using two different methods of synchrony, Northern analysis also shows that the peak of *SDB25* expression occurs shortly after the peak of *DBF2* expression. Thus *SDB25* and *DBF2* are not coordinately, but rather sequentially regulated, which may be a transcriptional mechanism for ensuring that Sdb25 functions after Dbf2. The temporally close order of Dbf2 and Sdb25 function was also evident in cells released from a *dbf2*-ts block, where the Sdb25-associated kinase is activated immediately after the appearance of the Dbf2 kinase, following release from the block. However, neither the genetic interactions described above, nor the cell cycle analysis, prove anything conclusive about how the function of Sdb25 relates to the function of Dbf2. Using the available antibodies, co-immunoprecipitation experiments failed to demonstrate binding of Sdb25 and Dbf2, nor is an Sdb25-protein A fusion protein phosphorylated by the Dbf2 kinase.

6.2 *SDB25* IS INVOLVED IN REGULATION OF THE M/G1 TRANSITION

Irrespective of the precise physiological relationship between Dbf2 and Sdb25, production of Sdb25 in late M phase appears to result in the protein immediately binding to an active form of a Cdc28 kinase complex. Furthermore, cell cycle analysis indicates that Sdb25 associates with an active Cdc28 kinase complex for only a short time during the cell cycle. From an earlier, detailed examination of the cell cycle fluctuation of Cdc28 kinase activity using histone H1 as a substrate (Surana *et al.*, 1991), it was concluded that the peak of Cdc28 kinase activity occurs in G2/M, begins to decline on entry into anaphase, and reaches the lowest level on entry into G1. This is consistent with a similar

analysis (Reed and Wittenberg, 1990), which found that the peak of Cdc28 kinase activity occurs in mitosis, and that by the time mitosis has been completed the Cdc28 protein kinase has been inactivated. If these findings can be generalized, then Figure 4.6B would indicate that Sdb25 associates with an active Cdc28 kinase complex at, or more likely after, anaphase has been initiated, but before cells enter G1.

Mendenhall (1993), has shown that purified $p40^{SDB25}$ can inhibit *in vitro* Cdc28 kinase activity. Although no direct evidence is presented to support this conclusion it is interesting that the cell cycle analysis indicates that Sdb25 is present only when Cdc28 is down regulated across the M/G1 interface. Thus, although it was demonstrated that Sdb25 binds to an active kinase at the end of mitosis, its primary role in mitotic cells could well be involved with the maintenance of a deactivated Cdc28 kinase as cells traverse the M/G1 boundary. Furthermore, the demonstration that Sdb25 has an associated kinase activity that phosphorylates histone H1 *in vitro* is not incompatible with this suggestion. When assaying Sdb25-associated kinase activity what is being observed may be an already inhibited form of Cdc28 kinase activity. From Figure 4.7 it is clear that the rapid inactivation of the Sdb25-associated kinase at the end of mitosis is not due to destruction of Sdb25, which is present in G1 cells. In respect of this it would be of interest to determine whether binding of Sdb25 to a Cdc28 kinase complex is coincident with the appearance of Sdb25-associated kinase activity. However, using the available antibodies, attempts to coimmunoprecipitate p40/Sdb25 and Cdc28 failed, as previous workers have failed to do (Wittenberg and Reed, 1988).

Viewing Sdb25 as an inhibitor of Cdc28 kinase activity can help to explain the morphological observations concerning *sdb25Δ* cells, which indicate that deletion of *SDB25* results in two distinct cell cycle effects. The increase in budded cells and large

cells in an *sdb25Δ* culture indicate a G2/M delay. However, the overall smaller size and decrease in unbudded single cells both imply an acceleration through G1. Possibly, deletion of *SDB25* results in a phenotype that is a composite of a delay in late M phase, due to Cdc28 kinase activity taking longer to decay, and, independently, a speeding up of G1, due to Cdc28 kinase being up-regulated more rapidly as cells traverse START (see Figure 6.1B). Note that this interpretation of the *sdb25Δ* phenotype applies to a population of cells, the two partial morphological effects (large budded cells → small cells), being mutually exclusive when applied to successive cycles undergone by an individual cell. Furthermore, the combination of these two effects on the kinetics of growth rates could explain why the doubling time is essentially unaltered when *SDB25* is deleted.

6.3 A SPECULATIVE MODEL FOR THE CELL CYCLE ROLE OF SDB25/P40

In the first part of the project an initial characterization of a newly identified yeast gene, *SDB25*, is presented. *SDB25* was then found to encode p40, a previously characterized substrate and inhibitor of the Cdc28 kinase. It has been shown that Sdb25 has a function in late mitosis, most likely after the metaphase to anaphase transition. This is inferred from the timing of the appearance of the Sdb25-associated kinase complex, and from the fact that *SDB25* shows genetic interactions with *DBF2*, the function of which is required in late nuclear division (Toyn and Johnston, 1994). The demonstration that the Sdb25-associated kinase is *CDC28*-dependent suggests Sdb25 may function to regulate the activity of Cdc28 at the end of mitosis. A speculative model, outlining the cell cycle role of Sdb25, is shown in Figure 6.1A. Formally, formation of the Sdb25-associated, *CDC28*-dependent, kinase complex could have a positive role in promoting the M/G1

Figure 6.1

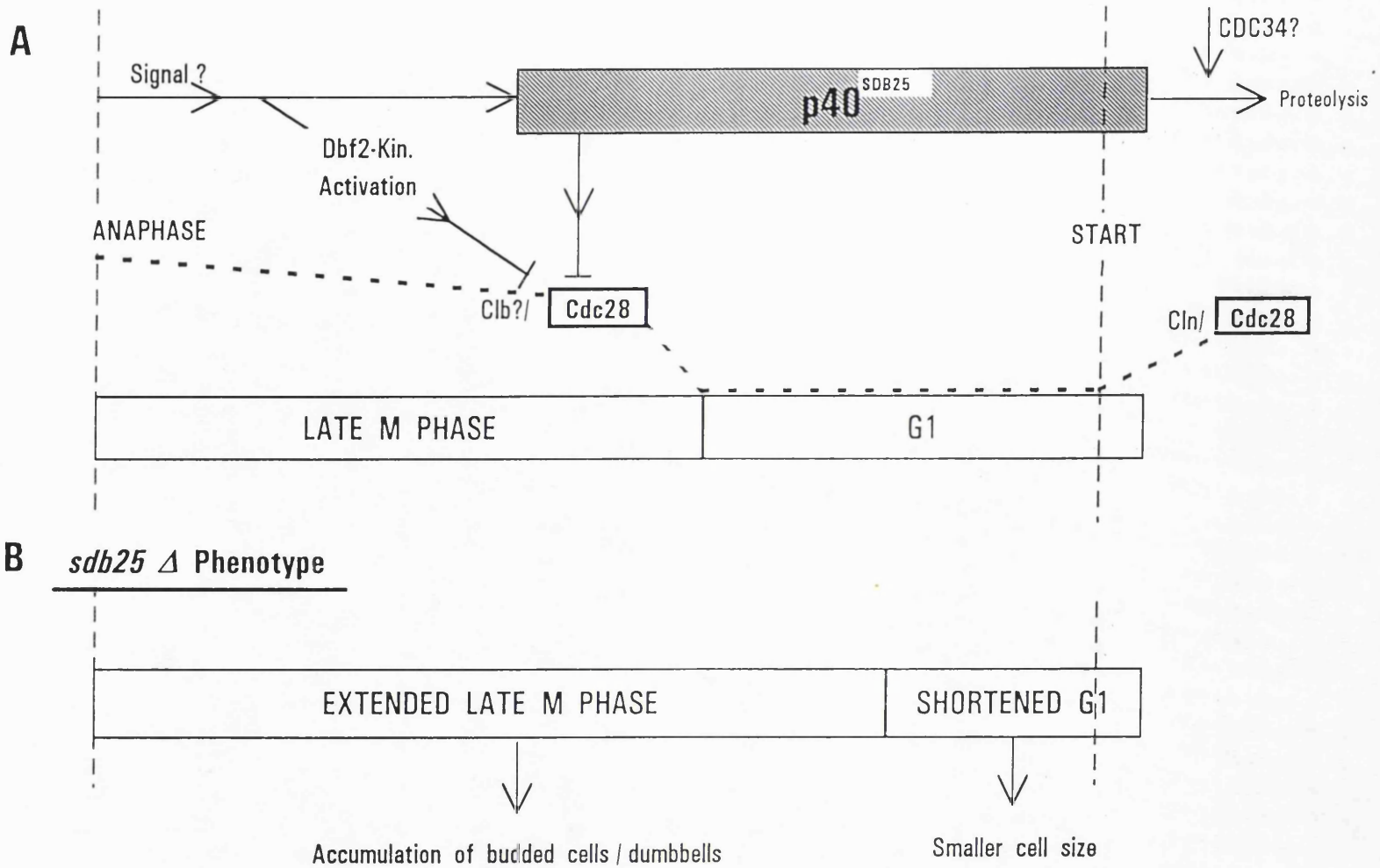
A. Speculative model for the role of Sdb25 in a late mitotic pathway

Dbf2 kinase is activated after the metaphase to anaphase transition (Toyn and Johnston, 1994), suggesting its activity may be regulated by a signal transduced via separation of sister chromatids. As Dbf2 kinase activity peaks, Sdb25 is rapidly up-regulated and binds to a *CDC28*-dependent kinase complex, at a time when total Cdc28 kinase activity is being down-regulated. As discussed in the text, our genetic analysis indicates that Sdb25 and Dbf2 act in parallel pathways, which may converge to help down-regulate Cdc28 at the end of mitosis. However, our data does not exclude the possibility that Sdb25 functions directly downstream in a Dbf2/20-dependent pathway. Once produced Sdb25 persists to approximately START of the next cell cycle, as depicted by the shaded box. The dashed line, linking the two Cdc28 boxes indicates the likely Cdc28 kinase activity as cells traverse M/G1. As for the role of Sdb25, at present we favour the interpretation that Sdb25 helps inactivate and maintain a down-regulated Cdc28 kinase activity, from late M phase to early G1. This may facilitate a rapid end to mitosis and a protraction of G1 prior to START. Sdb25 may not bind Cdc28 directly, rather it may physically associate with one of the Clb cyclins, the main regulators of mitotic Cdc28 kinase activity. As cells pass START in G1 Sdb25 is degraded, suggesting it may be targeted for destruction via the *CDC34* ubiquitin conjugating enzyme.

B. Consequences of deleting *SDB25*

On the basis of the model described above, the immediate consequence of deleting *SDB25* is to slow down the passage of cells through the end of mitosis. This would result

in the accumulation of budded cells and some enlargement in size as cells continue to grow, independently of the ongoing regulatory processes. Separately, deletion of *SDB25* may also result in some cells accelerating through pre-START G1, due to the restraining effect of Sdb25 being absent. This would result in such cells having a smaller size on average than wild-type.


A
Signal ?
p40 SDB25
CDC34?
Proteolysis
Dbf2-Kin.
Activation
ANAPHASE
START
Clb?/ Cdc28
Cln/ Cdc28
LATE M PHASE
G1
B
sdb25 Δ Phenotype
EXTENDED LATE M PHASE
SHORTENED G1
Accumulation of budded cells / dumbbells
Smaller cell size

transition, perhaps by altering the substrate-specificity of the kinase. As indicated in the model, the alternative view that Sdb25 may help to down-regulate Cdc28 activity at the end of mitosis, is favoured. This is consistent with the *in vitro* properties of $p40^{SDB25}$ being a potent inhibitor of Cdc28 kinase activity (Mendenhall, 1993), the morphology of *sdb25Δ* cells, and the timing of Sdb25 expression. Incidentally, if nutrients are limiting, instead of traversing START the cell of course moves into stationary phase in the next cell cycle, when Cdc28 is inactive. Significantly, Sdb25 is present only during these two phases, M/G1 (this work) and stationary (Mendenhall, 1993), strongly suggesting that its physiological role is to maintain an inactive form of Cdc28 prior to commitment to a new cell cycle.

This model also indicates the possible mechanism responsible for the cell cycle regulated degradation of Sdb25. In this respect, a parallel can be drawn with Far1 (McKinney *et al.*, 1993), a negative factor required for cell cycle arrest (Chang and Herskowitz, 1990). Like Sdb25, Far1 is a cell cycle regulated phosphoprotein (McKinney *et al.*, 1993), that has been shown to bind and inhibit the Cdc28 protein kinase (Peter *et al.*, 1993). Also, both proteins are degraded in G1, suggesting they both may be directed into a proteolytic pathway via the ubiquitin-conjugating enzyme *CDC34* (Goebl *et al.*, 1988). In fact, *CDC34*-mediated proteolysis has recently been proposed to account for the disappearance after START of Far1 (McKinney *et al.*, 1993). Perhaps future work will show that Sdb25 and Far1 are members of a group of cell cycle regulated Cdk inhibitor proteins that function to modulate the activity of Cdc28 in conjunction with the Clb/Cln cyclin family.

6.4 *SDB24* MAY FUNCTION IN EARLY S PHASE

The second *dbf2* suppressor worked on, *SDB24*, has also been sequenced and found to be a newly identified yeast gene. The nucleotide sequence of *SDB24* is approximately 2.5kb in length and the gene encodes a protein with a predicted molecular weight of about 95 kD. Analysis of the Sdb24 amino-acid sequence reveals it has several interesting motifs, which may be of relevance to the physiological role of Sdb24. The leucine-zipper motif, four copies of which occur in the Sdb24 amino acid sequence, has been found in several DNA-binding proteins. However, in such regulatory molecules, preceding the leucine-zipper are two highly conserved, and precisely positioned, basic residues (N-$(X)_7$-R-$(X)_9$-Leucine-Zipper) (Landschultz *et al.*, 1988). This composite structure, known as bZIP is not found anywhere in Sdb24. However, there are examples of transcriptional activators which have the leucine-zipper motif without the accompanying basic residues (Thomas *et al.*, 1992). Another feature of the Sdb24 amino acid sequence which also, at first glance, suggests the possibilty that Sdb24 is involved in transcriptional activation is the highly acidic composition of the protein. Acidic regions necessary for transcriptional activation have been found in a number of transcriptional activators, including *GCN4* (Hope and Struhl, 1986) and *GAL4* (Ma and Ptashne, 1987). However, although the overall number of acidic residues in Sdb24 is high, inspection of the Sdb24 amino acid sequence indicates that the acidic residues are fairly evenly distributed throughout the entire protein, and there are no concentrated regions of acidic residues. Thus, analysis of the predicted amino acid sequence of Sdb24 does not strongly support the possibility that it may function as a transcriptional activator, although this cannot be discounted. Another feature of the Sdb24 protein is the putative coiled-coil region in the central region of the amino acid sequence. This might imply that Sdb24 functions as a

dimer *in vivo*, a possibility that could be tested using the two-hybrid system (Fields and Song, 1989). Finally, the occurrence of three potential Cdc28/Cdc2 phosphorylation sites near the N-terminal of the Sdb24 sequence suggest that perhaps the physiological function of Sdb24 is partly regulated by post-translational modification via the Cdc28 kinase.

SDB24 is expressed under cell cycle control, although the peak of expression is much earlier in the cell cycle than that of *DBF2*. Comparison with the transcript levels of one of the DNA synthesis genes (*POL1*), and the Histone *H2B*, indicates that the peak of *SDB24* expression occurs in early S phase. With both *DBF2* and *SDB25* the timing of transcriptional expression has been shown to correlate quite well with a physiologically important function of each protein. The *DBF2* mRNA peaks slightly in advance of activation of the Dbf2 kinase (Toyn and Johnston, 1994), and the authors speculate that the cell-cycle regulated expression of *DBF2* contributes to a post-translational mechanism required for maximal activation of the Dbf2 kinase in M phase. With *SDB25* the peak of transcription correlates more tightly with the appearance of the Sdb25-associated kinase in late mitosis (this study). Thus, the finding that *SDB24* is maximally abundant in early S phase may signify that Sdb24 carries out an important physiological role in that phase of the cell cycle. One attractive possibility is that *SDB24* may be involved in transcriptional activation of *DBF2,* which appears to be initially upregulated at the peak of *SDB24* expression. However, the fact that PSDB24 is an allele-specific suppressor of *dbf*2-ts is harder to explain from this viewpoint. Nevertheless the possibility is worth testing, perhaps by examining the cell cycle levels of *DBF2* in a synchronized cell culture, using a strain deleted for *SDB24.*

Cell deleted for *SDB24* are viable, which suggests that there may be homologues of *SDB24* which can carry out its physiological role in the absence of a functional Sdb24

protein. However, probing a Southern Blot for the *SDB24* gene at low stringency did not result in the appearance of any new bands. Thus, although the existence of *SDB24* homologues cannot be ruled out, it may be that the viability of *SDB24*-deleted cells is due to the function of Sdb24 being somehow bypassed.

As with *SDB25*-deleted cells, an *sdb24Δ* strain shows no difference in doubling time as compared to an isogenic wild-type control strain. Morphological analysis of an *sdb24Δ* strain reveals a significant increase in budded cells in an asynchronous mid-log population, as compared to a wild-type control. From this it can be inferred that deletion of *SDB24* results in a delay in progression through the cell cycle beginning at some point after the initiation os S phase, which is approximately equivalent with the onset of budding. However, unlike *SDB25*-deleted cells, deletion of *SDB24* did not result in a noticeable increase in the appearance of dumbbells. FACS analysis of a culture of *sdb24Δ* cells shows a similar profile to that of an *sdb25Δ* culture. That is, very few of the cells are in the G1 phase of the cell cycle in an *SDB24*-deleted strain, as compared to a wild-type control. However, the corresponding forward scatter analysis shows that *sdb24Δ* cells are slightly larger on average. By contrast, deletion of *SDB25* was seen to result in cells that are significantly smaller on average than those of a wild-type population. As explained above, this may be because *SDB25* also has a G1 role, perhaps involved in inhibition of the Cdc28 kinase at the beginning of the cell cycle. Clearly then, although similar, there are important differences in the effect on cell cycle progression in *SDB24*-deleted cells as compared to *SDB25*-deleted cells. A more precise understanding of the cell cycle delays would be obtained by morphological analysis of synchronized *SDB24* and *SDB25*-deleted cells.

Further evidence that *SDB24* and *DBF2* interact genetically comes from the finding

that *sdb24Δdbf2Δ* cells are inviable. In addition, all the cells which can be inferred to be deleted for both *SDB24* and *DBF2* arrest as single dumbbells, the identical morphology to *dbf2*-ts cells arrested at the restrictive temperature. In contrast, there is no uniform morphology associated with *sdb25Δdbf2Δ* cells, although they are also inviable. Interestingly, the three suppressors of *dbf2-3*, *SPO12*, *SDB24* and *SDB25*, all have an essential function in a dbf2Δ background, as cells deleted for *DBF2* and any one of the three are inviable. However, no synthetic lethality arises from deleting any two of the three suppressors. Thus, it appears that although all three suppressors interact in some way with *DBF2*, there is no interactions between the three suppressors themselves. This finding may reflect the diversity of cell cycle processes that the Dbf2 protein kinase is involved in. In respect of this, it is worth recalling that beside carrying out an essential function in late M phase (Toyn and Johnston, 1992) there is also strong evidence that Dbf2 has a G1 role as well. This is inferred from the observation of an S phase delay in a *dbf2*-ts mutant (Johnston *et al.*, 1990), and the finding that *dbf2*-ts is suppressed by the protein phosphatase *SIT4*, which is required for the G1/S transition.

FUTURE WORK

The genomic screen for multicopy suppressors of *dbf2* mutants has resulted in the isolation of five genes, *SDB21-SDB25* (Parkes and Johnston, 1992; this study). All five genes have been characterized to varying degrees, and the relationships between *DBF2* and *SDB21*(=*SPO12*), and *DBF2* and *SDB25* have been explored in detail. As yet, none of the suppressors have been demonstrated to physically interact with Dbf2, although efforts to coimmunoprecpitate Spo12 and Dbf2 are being pursued, given that Spo12 may be a regulator of Dbf2 function (Toyn and Johnston, 1993). The results presented in this work concerning Sdb25, imply that perhaps the function of Dbf2 in late mitosis is to inactivate the Cdc28 kinase and so allow exit from mitosis. Whether this is accomplished via a pathway connecting Dbf2, Sdb25 and Cdc28 has yet to be determined. As discussed earlier, it may be that Sdb25 is a *dbf2* suppressor because it can accomplish the essential Dbf2/20 mitotic function, although its function acts in a separate, but parallel, pathway. However, the possibility that Dbf2 is involved in transcriptional regulation of *SDB25*, which may be the main physiological determinant of Sdb25 function, has not been ruled out. The findings concerning *SDB24* presented in Chapter Five, may reflect the G1 role of Dbf2. Currently, an epitope-tagged version of Sdb24 is being prepared with a view to testing whether Sdb24 has an associated kinase, and/or whether it binds to Dbf2/20. As discussed earlier, the other avenue being explored with regards to the *in vivo* relationship between Sdb24 and Dbf2 is to examine whether *SDB24* is involved in transcriptional activation of the protein kinase.

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APPENDIX

Reprint of publication

P40^{SDB25}, a putative CDK inhibitor, has a role in the M/G_1 transition in *Saccharomyces cerevisiae*

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P40^{SDB25}, a putative CDK inhibitor, has a role in the M/G_1 transition in *Saccharomyces cerevisiae*

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The *Saccharomyces cerevisiae* protein kinase Dbf2 carries out an essential function in late mitosis, and its kinase activity is cell-cycle regulated around anaphase/telophase. We have isolated *SDB25*, a high copy suppressor of temperature-sensitive *dbf2* mutants, and genetic analysis suggests that the two proteins may function in parallel pathways in late mitosis. *SDB25* encodes p40, a previously characterized substrate and potent inhibitor of Cdc28 kinase activity. Sdb25 is a phosphoprotein, and Sdb25 immunoprecipitates have a histone H1 kinase activity that is *CDC28*-dependent. Remarkably, Sdb25 transcript levels, protein levels, and associated kinase activity are precisely cell-cycle regulated, sharing a common peak in late mitosis. Moreover, Sdb25 protein levels and associated kinase activity are sharply up-regulated at the peak of Dbf2 kinase activity in cells released from a *dbf2* ts block. The Sdb25 protein then disappears around Start in the next cell cycle. This indicates that *SDB25* function is confined to M/G_1, and morphological analysis of *sdb25Δ* cells supports this conclusion. Our data suggest that Sdb25 functions in a pathway in late mitosis leading to the down-regulation of Cdc28 kinase activity as cells traverse the M/G_1 boundary.

[*Key Words*: Cell cycle; late mitosis; CDK inhibitor; *S. cerevisiae*; pathway]

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In the budding yeast *Saccaromyces cerevisiae*, regulation of the activity of the p34^{CDC28} protein kinase is central to ordered progression through the cell cycle (for review, see Nasmyth 1993). Activation of the kinase is required for traversal of Start in G_1, where commitment to a new round of cell division occurs, and for the initiation of mitosis at the G_2/M boundary. Although Cdc28 is present at a constant level throughout the cell cycle, its histone H1 kinase activity fluctuates periodically (Reed and Wittenberg 1990). The control of Cdc28 function is partly affected by the formation of stage-specific, distinct, heteromeric p34^{CDC28} kinase complexes, with the monomeric form of the protein having little or no activity. At the two major control points, Start and the G_2/M interface, activation of Cdc28 requires its association with different members of a group of unstable proteins, called cyclins (for review, see Lew and Reed 1992). Exit from mitosis may also require deactivation of the kinase, and concomitant destruction of the associated cyclin by proteolysis (Ghiara et al. 1991).

Predating the identification of cyclins as regulatory subunits in p34^{CDC28} heteromeric complexes, two other proteins were shown to be associated with the active form of the kinase. Cks1 is an 18-kD protein that was isolated in a screen for high-copy suppressors of the temperature-sensitive *cdc28-1* mutation (Hadwiger et al. 1989a) and is homologous to the *Schizosaccharomyces pombe* suc1$^+$ protein that interacts with cdc2$^+$ (Hayles et al. 1986). Furthermore, it was demonstrated that Cks1 coprecipitated with Cdc28 and associated with a 160-kD complex with Cdc28-dependent kinase activity. Another component of this p34^{CDC28} complex is a 40-kD protein, p40, that is also phosphorylated in vitro, in a Cdc28-specific, cell-cycle-dependent manner (Reed et al. 1985; Wittenberg and Reed 1988; Reed and Wittenberg 1990). Recently, by use of p40 purified from stationary-phase cells, the 40-kD protein has been shown to bind tightly to Cdc28 and to inhibit its kinase activity (Mendenhall 1993). This finding has led to speculation that p40 may function as a cyclin-dependent kinase (CDK) inhibitor (Hunter 1993; Nasmyth and Hunt 1993). A number of CDK inhibitors have recently been discovered that inhibit, by binding stoichiometrically, the kinase activity of a range of CDK complexes (for review, see Pines 1994).

Another protein kinase that has also been implicated in control of the mitotic cell cycle is Dbf2. Initially identified in a screen for mutants defective in S phase, further analysis revealed that in *dbf2* mutant cells, DNA synthesis is delayed, but the cells finally block in mitosis (Johnston et al. 1990). At the restrictive temperature, *dbf2* mutants arrest in late nuclear division with a uniform morphology resembling a dumbbell consisting of large, budded, single cells. Although *dbf2* thermosensi-

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tive (*dbf2*-ts) mutants are lethal, deletion of the gene does not affect cell viability, there being a *DBF2* functional and structural homolog, *DBF20* (Toyn et al. 1991). Either gene can be deleted, but the *dbf2*Δ*dbf20*Δ double mutant is inviable, indicating that both genes have a common, essential function. Unlike *CDC28*, the *DBF2* transcript is expressed under cell-cycle control, with a peak of expression in M phase, whereas *DBF20* is constitutively expressed. Dbf2 kinase activity is cell-cycle regulated in a manner similar to the *DBF2* transcript, although the Dbf2 protein itself is present throughout the cell cycle (Toyn and Johnston 1994). Moreover, the late mitotic genes *CDC5* and *CDC15* are high-copy suppressors of *dbf2* mutations (Kitada et al. 1993), so that Dbf2 may be part of a cell-cycle regulatory network in late mitosis.

In addition to *CDC5* and *CDC15*, several high-copy suppressors of *dbf2* mutants have been isolated from a genomic library, two of which were previously identified genes, *SIT4* and *SPO12* (Parkes and Johnston 1992). *SIT4* has homology to type-2 protein phosphatases (Arndt et al. 1989) and is required for the G_1/S transition (Sutton et al. 1991) and for the normal accumulation of G_1 cyclin RNA (Fernandez-Sarabia et al. 1992). *SPO12* has been implicated in meiosis, mutations in the gene leading to aberrant ascus formation, and is not essential for mitotic growth (Malavasic and Elder 1990). Detailed examination of the *SPO12* transcript shows that it is coordinately regulated with *DBF2*, and recent evidence indicates that *SPO12* may be a regulator of Dbf2 function (Toyn and Johnston 1993).

In this paper we report on another high-copy suppressor of *dbf2*, *SDB25*, which encodes the putative CDK inhibitor protein p40 described above. The cell cycle abundance of Sdb25 transcript and protein levels fluctuate in a cyclin-like manner, and up-regulation of Sdb25 protein levels is confined to late M/early G_1. Coincident with its initial production, Sdb25 associates with a *CDC28*-dependent kinase in late mitosis, although the Sdb25 protein persists until after cells have passed Start in the next cell cycle. Our cell cycle analysis is consistent with Sdb25 being involved in inactivation of Cdc28 kinase as cells traverse the M/G_1 boundary. Moreover, we have found that cells deleted for *SDB25* have a morphology that supports this interpretation of Sdb25 as a negative regulator of Cdc28 kinase activity. The functional relationship between *SDB25* and *DBF2* is not clear, although analysis of the genetic interactions between the two genes indicates that they may function in parallel, partially redundant, pathways in late mitosis. One possibility is that both pathways converge to help down-regulate Cdc28 kinase activity at the end of mitosis.

Results

SDB25 *suppression of* dbf2-*ts alleles is copy-number dependent*

Yeast genomic libraries in the multicopy vector YRp12 were transformed into strain L182-7A (*dbf2-3 ura3*), and transformants were screened for plasmids capable of suppressing the *dbf2* ts phenotype. Restriction mapping of all suppressing plasmids indicated that they contained one of two different regions of chromosomal DNA. These two genomic fragments were named *SDB24* and *SDB25* (suppressor of *dbf2* numbers *4* and *5*), respectively). This paper reports on the sequencing and characterization of *SDB25*.

To determine whether the suppression of the *dbf2* phenotype by overexpression of *SDB25* is specific for the *dbf2-3* allele, we transformed strains L119-7D (*dbf2-1 ura3*) and L181-6B (*dbf2-2 ura3*) with YRpSDB25 (YRp12-*SDB25*). None of the resulting *URA*$^+$ transformants were observed to grow at 37°C, although expression of *SDB25* from YRp12 allowed growth of *dbf2-3* at 37°C, which is comparable to a wild type. *SDB25* is therefore an allele-specific suppressor, at least when overexpressed on the multicopy plasmid YRp12. Of the other suppressors of *dbf2* (Parkes and Johnston 1992), *SDB21* (*SPO12*) has been studied in most detail, and suppression by *SPO12* requires *DBF20*, the homolog of *DBF2* (Toyn and Johnston 1993). To examine whether *SDB25* has a similar requirement for *DBF20*, YRpSDB25 was introduced into yeast strain JD101 (*dbf2-3 dbf20*Δ::*TRP1*). As with *SPO12*, we find that YRpSDB25 no longer suppresses the *dbf2*-ts phenotype in cells deleted for *DBF20*. The YRpSDB25 suppression of *dbf2-3* may therefore in some way allow *DBF20* to complete the vital function carried out by these two kinases.

We also tested whether *SDB25* was an allele-specific suppressor when overexpressed on the high-copy plasmid YEp24. Surprisingly, we found that YEpSDB25 can suppress all three alleles of *dbf2*-ts, when incubated at 37°C and that the suppression is no longer *DBF20*-dependent. Furthermore, YEpSDB25 can keep alive cells deleted for both *DBF2* and *DBF20*, *dbf2*Δ*dbf20*Δ cells normally being inviable (Fig. 1). Thus, the essential function carried out by the Dbf2/20 kinase can be also be executed by overproducing Sdb25 protein to a sufficient level.

SDB25 *encodes a known component of a p34*^{CDC28} *kinase complex*

The *SDB25* gene was located on the library plasmid by transposon mutagenesis, all insertions that destroyed the suppressor activity lying between the *Kpn*I and *Hpa*I sites on the restriction map (Fig. 2A). The DNA sequence of a 1-kb region around these sites was determined, and one large open reading frame (ORF) of 852 nucleotides was found (Fig. 2B). The predicted molecular mass of Sdb25 is 32.2 kD, and the protein is basic with an estimated pI of 8.9.

Searching the data banks revealed that there was perfect identity between DNA sequence upstream of the *SDB25* gene locus and that of the membrane protein Bos1 gene locus (Shim et al. 1991), indicating that the genetic map position of *SDB25* is adjacent to *BOS1*. Southern blot analysis of *S. cerevisiae* chromosomes separated by pulse-field gel electrophoresis shows that

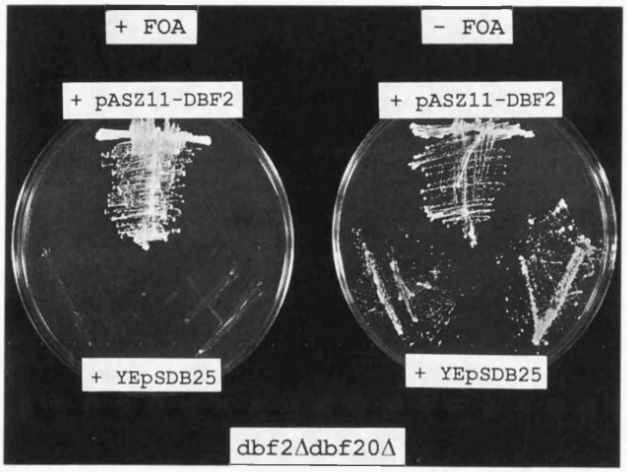


Figure 1. YEpSDB25 rescues the lethality of *dbf2Δdbf20Δ*. Strain J211-2C (*dbf2Δdbf20Δ*) containing the plasmid pASZ11–*DBF2* was transformed with the plasmid YEpSDB25. Two separate red sectors, corresponding to colonies that had lost the pASZ11–*DBF2* plasmid, were picked and streaked out onto plates containing FOA (*left* plate), or one without FOA (*right* plate). As a control a white colony was also streaked out (*upper* position). Cells from the red sectors were very clumpy; hence, the heavy inoculum visible on the *left* plate. The plates were incubated at 25°C for 3 days and then photographed.

SDB25 is physically mapped on chromosome *XII* (data not shown). Therefore, the *SDB25* locus flanks the divergently expressed gene *BOS1* on chromosome *XII*.

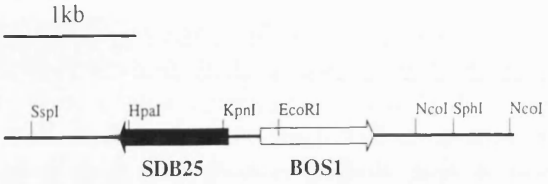
Searching the existing data banks suggests that *SDB25* is a newly identified gene. However, the identical gene has been independently isolated, named *SIC1*, and the encoded protein is p40 (M. Mendenhall, pers. comm.). The initial identification of p40 was as an endogenous substrate, with an apparent molecular mass of 40 kD, for a kinase activity associated with Cdc28 immune complexes (Reed et al. 1985). Consistent with $p40^{SDB25}$ being a substrate of Cdc28 kinase, we find that the Sdb25 primary amino acid sequence has one perfect match to the proposed consensus motif for cdc2$^+$/Cdc28 phosphorylation site S/T-P-*X*-*Z*, (where *X* is a polar amino acid, and *Z* is generally a basic amino acid) (Fig. 2B) (for review, see Moreno and Nurse 1990).

The finding that a dosage suppressor of the protein kinase *DBF2* is a component of a $p34^{CDC28}$ complex suggests that *SDB25* may be functioning in a pathway somehow linking the two enzymes in vivo. Previously it has been shown that overexpressing *DBF2* on a high-copy plasmid did not suppress the *cdc28-4*, *cdc28-13*, or *cdc28-1N* alleles (Kitada et al. 1993). We found that high-copy *SDB25* also failed to suppress a number of *cdc28* alleles including *cdc28-4*, *cdc28-13*, and *cdc28-1N*, and *CDC28* did not suppress any *dbf2*-ts alleles when overexpressed on the high-copy plasmid YEp13 (J. Donovan et al., unpubl.). This would indicate that the two kinases may not be closely associated and/or they do not have significant overlapping substrate specificity.

Cells deleted for SDB25 *are viable but have an aberrant size distribution*

To examine whether *SDB25* has a function in cell-cycle

A



B

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      -130                -110                -90
GGCTTGTTGTTCGCGTCGCAACTTTTGATATTTATAAAAAGAGAGAAGGCATATCCAAAG
       -70                 -50                 -30
GATAAGGTATTGTCTTTGTAAATCAATCAACCAAACCTCTACGGAAATTTTGACCCTTGAA
       -10          1      10                  30
GCAGGGACTATTACACGAAAATGACTCCTTCCACCCCACCGAGGTCCAGAGGGACTAGGT
                    M  T  P  S  T  P  P  R  S  R  G  T  R  Y
        50                  70                  90
ACCTTGCGCAGCCTAGTGGCAATACTAGTTCTAGTGCCCTAATGCAAGGTCAAAAGACCC
  L  A  Q  P  S  G  N  T  S  S  S  A  L  M  Q  G  Q  K  T  P
       110                 130                 150
CCCAAAAGCCTTCACAGAACCTAGTCCCTGTCACTCCCTCAACAACTAAGTCTTTTAAAA
  Q  K  P  S  Q  N  L  V  P  V  T  P  S  T  T  K  S  F  K  N
       170                 190                 210
ATGCGCCATTATTAGCACCTCCCAATTCGAACATGGGTATGACCTCTCCATTTAATGGGC
  A  P  L  L  A  P  P  N  S  N  M  G  M  T  S  P  F  N  G  L
       230                 250                 270
TTACGTCTCCTCAACGCTCGCCGTTTCCAAAATCTTCAGTGAAGAGGACACTATTCCAAT
  T  S  P  Q  R  S  P  F  P  K  S  S  V  K  R  T  L  F  Q  F
       290                 310                 330
TTGAAAGTCATGATAATGGAACAGTAAGGGAAGAGCAGGAACCATTGGGTCGTGTAAATA
  E  S  H  D  N  G  T  V  R  E  E  Q  E  P  L  G  R  V  N  R
       350                 370                 390
GGATATTGTTTCCCACGCAGCAAAATGTGGATATAGATGCAGCAGAAGAAGAAGAAGAAG
  I  L  F  P  T  Q  Q  N  V  D  I  D  A  A  E  E  E  E  E  G
       410                 430                 450
GAGAAGTTCTTCTTCCCCCCAGCAGACCTACATCTGCCAGGCAGTTACATTTATCACTTG
  E  V  L  L  P  P  S  R  P  T  S  A  R  Q  L  H  L  S  L  E
       470                 490                 510
AAAGAGATGAGTTTGATCAGACACATAGAAAGAAGATTACTAAAGATGTACCTGGTACGC
  R  D  E  F  D  Q  T  H  R  K  K  I  T  K  D  V  P  G  T  P
       530                 550                 570
CCAGCGACAAGGTGATAACATTTGAATTGGCAAAAAAATTGGAATAATAACTCTCCGAAAA
  S  D  K  V  I  T  F  E  L  A  K  N  W  N  N  N  S  P  K  N
       590                 610                 630
ATGACGCCAGGAGTCAAGAAAGTGAAGACGAGGAAGACATCATCATCAATCCAGTGCGGG
  D  A  R  S  Q  E  S  E  D  E  E  D  I  I  I  N  P  V  R  V
       650                 670                 690
TGGGTAAAAATCCCTTTGCATCAGATGAACTGGTCACTCAGGAAATTAGAAATGAACGTA
  G  K  N  P  F  A  S  D  E  L  V  T  Q  E  I  R  N  E  R  K
       710                 730                 750
AAAGGGCAATGTTGAGAGAAAACCCAGATATAGAAGACGTAATAACATATGTCAATAAGA
  R  A  M  L  R  E  N  P  D  I  E  D  V  I  T  Y  V  N  K  K
       770                 790                 810
AGGGAGAGGTGGTAGAGAAACGAAGGTTAACGGATGAAGAAAAGAGAAGATTCAAGCCAA
  G  E  V  V  E  K  R  R  L  T  D  E  E  K  R  R  F  K  P  K
       830                 850                 870
AGGCATTGTTTCAATCTAGGGATCAAGAGCATTGAAGAAdATGAAAAAAAAAAGTTTCTG
  A  L  F  Q  S  R  D  Q  E  H  *
       890                 910                 930
GAACGATTATATTTTATTTACTTACTTATTCTACATTTATTTGCAAAGGGGC
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Figure 2. Restriction map and nucleotide sequence of the *SDB25* gene. (*A*) Partial restriction map of the 3.75-kb genomic fragment of YRpSDB25. The solid arrow indicates the ORF of *SDB25* as deduced from the nucleotide sequence. The sequence to the *right* of the *Kpn*I site has been determined previously, and is the upstream of the divergently expressed gene *BOS1*. The ORF of *BOS1* is indicated by the open arrow. (*B*) Nucleotide and predicted amino acid sequence of the *SDB25* gene. Underlined are the restriction sites *Kpn*I (38) and *Hpa*I (786), used in constructing the *SDB25* null allele. Enclosed in the box is the putative cdc2$^+$/Cdc28 phosphorylation site. The predicted amino acid sequence is shown in single-letter code.

progression, a null allele was created by disrupting the chromosomal copy of a wild-type strain. The *KpnI–HpaI* 750-bp fragment, representing ~90% of the *SDB25* ORF, was deleted, and the *TRP1* gene inserted in a plasmid construct that was used to replace one copy of *SDB25* in a diploid. Sporulation of this diploid strain, followed by dissection of 21 tetrads, resulted in four viable spore colonies from each tetrad. Southern hybridization was performed on genomic DNA from a number of *TRP1* spores to confirm that the wild-type *SDB25* gene was replaced with the *sdb25Δ::TRP1* deletion allele (data not shown). Thus, haploids deleted for *SDB25* are viable.

CG379 cells deleted for *SDB25* show no difference in growth rates, compared with an isogenic wild-type strain (doubling time of 140 min at 30°C in rich media). However, microscopic analysis of *sdb25Δ* cells revealed a greater heterogeneity in cell size than with wild type. Unusually, a significant proportion of cells were smaller than average wild-type cells, whereas others, some 6%, were considerably larger (Fig. 3A). In addition, the percentage of budded cells was much higher in *sdb25Δ* cells than in the wild-type population being 81% in *sdb25Δ* cells compared with 65% for wild type. FACS analysis is consistent with these observations. A mid-log phase culture of *sdb25Δ* cells consisted almost entirely of cells in S phase, or in G_2/M with a 2C DNA content (Fig. 3B). There were very few cells with a 1C DNA content. The corresponding forward scatter analysis also confirmed our microscopic observations that *sdb25Δ* cells are significantly smaller on average than cells of the control strain (Fig. 3C). One interpretation of these results is that deletion of *SDB25* may have two distinct effects on cell-cycle progression, a delay in G_2/M and/or an acceleration through G_1.

SDB25 *has an essential function in cells deleted for* DBF2

The genetic data described above suggested that *DBF2* and *SDB25* might interact in vivo. If this were so, it

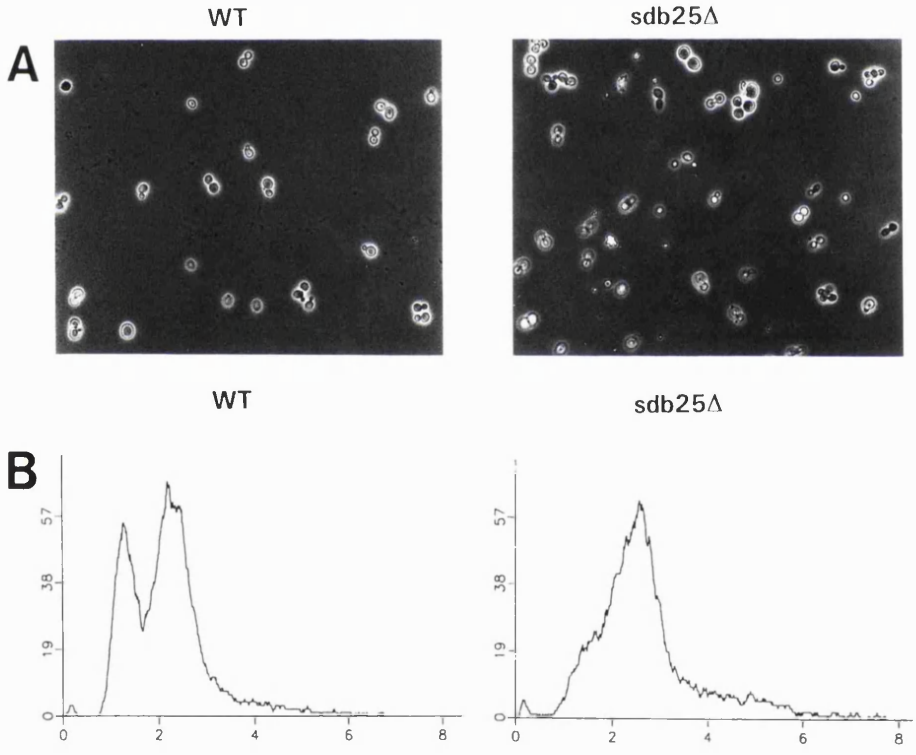

Figure 3. Morphological and FACS analysis of *sdb25Δ* cells. (*A*) Mid-log cells in YEPD were harvested, fixed, and photographed by use of phase-contrast microscopy. The field of *sdb25Δ* cells shown was selected to illustrate the heterogeneity in cell size, rather than to represent the statistical distribution as described in the text. (*B*) Aliquots of fixed cells were also used for FACS analysis, to measure the distribution of DNA content. Cells were prepared for flow cytometry as described in Materials and methods. Horizontal axes, fluorescence (relative DNA content); vertical axis, relative cell numbers. (*C*) Forward scatter analysis was carried out at the same time as FACS analysis, as an indicator of the relative cell size distribution of the two cultures. Horizontal axis, forward scatter (relative cell size); vertical axis, relative cell number. The reduction in the peak of *sdb25Δ* relative cell number, as compared with wild type reflects the greater variance in size distribution in cells deleted for *SDB25*. Also note the slight elevation of the *right*-hand side of the *sdb25Δ* tracing above the wild-type tracing, which probably reflects the small percentage of large cells in the *sdb25* culture.

might be anticipated that cells deleted for both *SDB25* and *DBF2* would be severely perturbed, or inviable. A *MAT***a** *sdb25*Δ::*TRP1* was therefore crossed to a *MAT*α*dbf2*Δ::*URA3* strain, the resulting diploid was sporulated, and 35 asci were dissected and scored for the *URA3* and *TRP1* markers. Altogether 4 nonparental ditypes, 9 parental ditypes, and 22 tetratypes were obtained, from which it could be inferred that a total of 27 spores were deleted for both *SDB25* and *DBF2*. None of the double mutant spores formed colonies, but microscopic examination showed that all had germinated and had subsequently ceased division, though a uniform terminal phenotype was not observed. Thus, *SDB25* has an essential function in a *dbf2*Δ background. To show that this apparent synthetic lethality was not simply a defect in outgrowth of *sdb25*Δ*bf2*Δ double delete spores, we transformed a *ura3*/*ura3* diploid heterozygous for *sdb25*Δ::*TRP1* and *dbf2*Δ::*LEU2* with YEpSDB25(*URA3*). The diploid was then sporulated and *URA*$^+$ *spore clones isolated, which were deleted for both SDB25* and *DBF2*. All such strains failed to grow on agar containing 5-fluoro-orotic acid (FOA), which is toxic in the presence of the *URA3* gene. Thus, these strains require the YEpSDB25 plasmid for viability, confirming that the *sdb25*Δ*dbf2*Δ double delete is lethal. In contrast, there was no essential requirement for *SDB25* in cells deleted for *DBF20* or in cells deleted for *SPO12*.

SDB25 *is expressed under cell-cycle control after* DBF2 *in late mitosis*

To investigate the expression of the *SDB25* gene during the cell cycle, Northern blot analysis was carried out on total RNA from synchronized cultures. Cells were synchronized by three distinct methods to reduce the possibility of artifactual results, and more than one cycle was monitored to ensure that the normal pattern of expression was observed. All of the data obtained indicated that *SDB25* is strongly cell-cycle regulated. For instance, in cells synchronized by elutriation the *SDB25* transcript showed more than a 10-fold increase over background levels during the peak of expression (Fig. 4). Comparison with the *DBF2* mRNA shows that *SDB25* peaks later in the cell cycle, very close to the M/G_1 boundary. This was true in all Northern experiments, with *DBF2* preceding *SDB25* expression, suggesting that Sdb25 acts after Dbf2. Consistent with this, in cells synchronized by temperature arrest of a *dbf2*-ts mutant in late mitosis, the *SDB25* transcript appears almost immediately after the release from the block (data not shown).

Sdb25 is a phosphoprotein

To detect the gene product of *SDB25*, antibodies were generated against the full-length Sdb25 protein, apart from the first 13 amino acids (see Materials and methods). In an immunoblot of *S. cerevisiae* lysates, these antibodies recognized a polypeptide doublet with an apparent molecular mass of 40 kD (Fig. 5A). Both bands were absent when lysates were prepared from

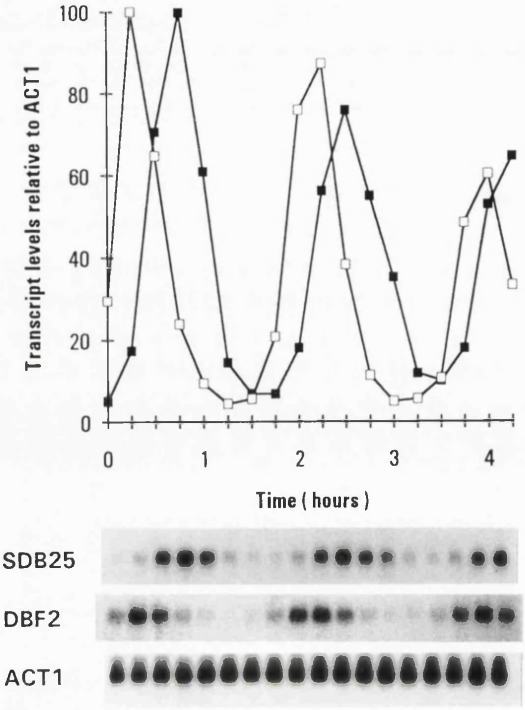

Figure 4. Regulation of the *SDB25* transcript in cells synchronized by elutriation. The culture used in this experiment has been described previously (Kitada et al. 1993). RNA samples were analyzed by Northern hybridization with the indicated probes. RNA levels were quantitated by densitometry from suitably exposed autoradiographs and normalized relative to actin transcript levels. The peak value of *SDB25* and *DBF2* transcript levels were each given a value of 100 for graphical presentational purposes. (□) *DBF2* transcript levels; (■) *SDB25* transcript levels.

*sdb25*Δ::*TRP1* cells and equally enhanced in cells carrying YEplacSDB25 (lanes 2,3). These results are consistent with *SDB25* encoding p40 and demonstrate that the Sdb25 protein migrates slightly anomalously with respect to its predicted molecular mass of 32.2 kD. Treatment of crude cell lysates with phosphatase prior to immunoblotting resulted in the disappearance of the upper band (Fig. 5B, lane 2). Addition of β-glycerophosphate, a competitive inhibitor of phosphatase, to the reaction or treatment with reaction buffer alone did not result in the disappearance of the more slowly migrating band (lanes 3,4). This demonstrates that Sdb25 is a phosphoprotein, the upper band in the doublet being the more phosphorylated form of the protein. However, in many immunoblots performed, no consistent pattern was observed with regard to the intensity of the two bands in the Sdb25 doublet. Thus, we do not draw any physiological conclusions from the fact that overexpressing *SDB25* resulted in both bands being equally enhanced in the immunoblot shown (Fig. 5A).

Sdb25 has an associated kinase activity that is CDC28 *dependent*

Immune complexes were prepared from *S. cerevisiae* lysates and assayed for histone H1 kinase activity in the

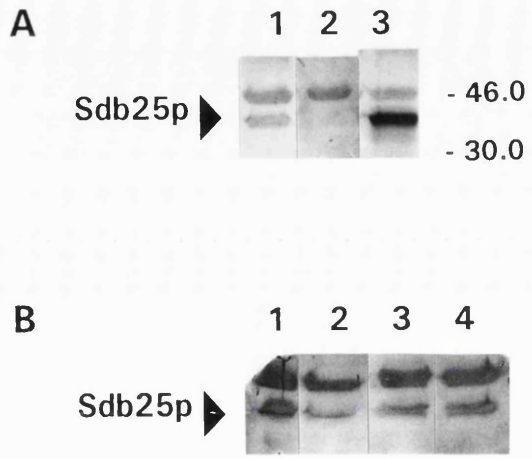

Figure 5. Characterization of Sdb25 antibodies. (*A*) Sdb25 protein levels in wild-type and *SDB25* overexpressing strains. (Lane *1*) CG379 (wild type); (lane *2*) JD100 (*sdb25*Δ::*TRP1*); (lane *3*) CG379 carrying YEplacSDB25. Protein extracts were subjected to SDS-PAGE and immunoblotted with rabbit polyclonal anti-Sdb25 serum. Equivalent amounts of protein were loaded for each of the yeast lysates analyzed. The band immediately above Sdb25 appeared in all immunoblots with Sdb25 antibodies and served as an internal loading control. Molecular mass are given in kilodaltons. (*B*) Phosphorylation state of Sdb25. Extracts from CG379 cells for phosphatase treatment were prepared as described in Materials and methods. (Lane *1*) Untreated CG379 extract. In lanes *2* and *4* extracts were treated with calf intestinal phosphatase; in lane *3* phosphatase buffer alone was used, in lane *4*, 50 mM β-glycerophosphate was added to the extract before addition of the phosphatase.

presence of [γ-^{32}P]ATP. Anti-Sdb25 antibodies precipitate a histone H1 kinase activity that cannot be detected in cells deleted for *SDB25* (Fig. 6B, lanes 5,6). Thus, the kinase detected is associated with Sdb25 and does not bind directly to the antibodies. By use of preparations of Sdb25 immunoprecipitates from *cdc28* alleles, we found that the Sdb25-associated kinase activity is almost totally destroyed in *cdc28-4* immunoprecipitates incubated at the nonpermissive temperature (Fig. 6A). A similar, though not so dramatic, effect was observed with *cdc28-13* extracts. We also tested Sdb25 kinase activity in immunoprecipitates prepared from *dbf2*Δ extracts, which have been shown to have a significantly reduced Dbf2 kinase activity (Toyn and Johnston 1994). No significant reduction in Sdb25-associated kinase was observed in either *dbf2*Δ or *dbf20*Δ immunoprecipitates, as compared with a wild-type control (Fig. 6B). The Sdb25-associated kinase activity thus exhibits defects only in *cdc28* temperature-sensitive strains, which suggests that Cdc28 is the catalytic subunit in the Sdb25 kinase complex. To confirm this result, we carried out kinase assays in extracts depleted of the Cdc28 protein and found a dramatic reduction in Sdb25-associated kinase activity (data not shown). Taken together with the recent finding that p40^{SDB25} binds tightly to Cdc28 in vitro (Mendenhall 1993), we regard this as convincing evidence that Sdb25 is an in vivo component of a Cdc28 kinase complex.

The Sdb25-associated kinase complex is tightly cell-cycle regulated in late mitosis

In view of the cell-cycle-regulated expression of *SDB25*, both Sdb25 protein levels and the associated kinase activity were examined in cells synchronized by α-factor and compared with fluctuations in transcript levels. A

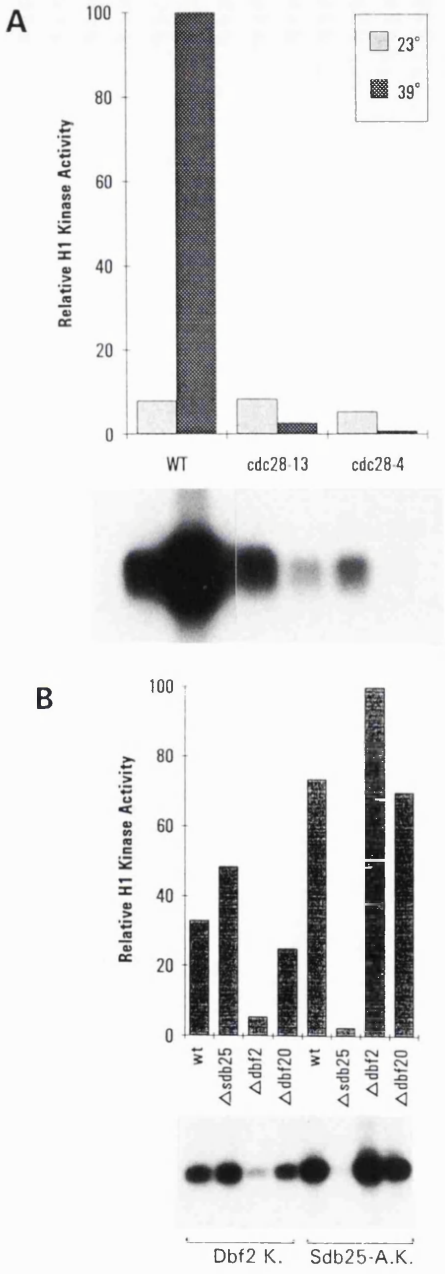

Figure 6. (*A*) Sdb25-associated H1 kinase activity is *CDC28*-dependent. Cell lysates were immunoprecipitated with anti-Sdb25 serum and assayed for kinase activity with histone H1. Immunoprecipitates from the indicated strains were split in two and assayed for kinase activity in vitro at either 23°C or 39°C. A gel autoradiograph and corresponding PhosphorImage quantitation are shown. (*B*) Sdb25-associated H1 kinase activity is not *DBF2* or *DBF20*-dependent. Cell lysates were immunoprecipitated with either anti-Dbf2, or anti-Sdb25, serum and assayed for histone H1 kinase activity. Immunoprecipitates from the indicated strains were assayed for either Dbf2 kinase activity or Sdb25-associated kinase activity. The wild-type control strain used in lanes *1* and *4* is CG379.

strain carrying an epitope-tagged version of Cdc28 was used, enabling total Cdc28 kinase activity to be measured. The Sdb25-associated kinase was found to be active for only a very short period during the cell cycle, at a time when many cells have fully divided chromatin and total Cdc28 kinase activity is decreasing (Fig. 7B). To rule out the possibility that this observed fluctuation might be influenced by the presence of an epitope-tagged version of Cdc28, we carried out a similar analysis in strain CG378 and obtained the same pattern of regulation of Sdb25-associated kinase activity (data not shown). Comparison of the fluctuations in the Sdb25-associated kinase activity and the *SDB25* transcript levels (Fig. 7B,D) shows that both are maximal at approximately the same time, suggesting a causal relationship between both events.

Sdb25 protein levels are up-regulated in late M/early G_1 phase

Following release from the α-factor block, the Sdb25 protein is evident for some 15 min up to about the time of budding (Fig. 7C). This approximates to the beginning of S phase, so that Sdb25 clearly persists while cells are in G_1. Interestingly, the Sdb25-associated kinase activity has disappeared at this time, so that the kinase is clearly subject to other, independent forms of control. The Sdb25 protein then reappears again at the same time point where the peaks in the kinase activity and transcript levels occur and persists for some 30 min (cf. Fig. 7, B, C, and D). Because the peak in Sdb25-associated kinase activity occurs in late mitosis (see Discussion), Sdb25 must only be present in the mitotic cell cycle for a brief period, from late M phase to shortly after Start in the next cell cycle. To confirm this finding we used temperature-sensitive cell cycle mutants that block in M phase or G_1, when incubated at 37°C. Sdb25 protein is present at very low levels in mutants arrested in late M phase (*dbf2* and *cdc14*) but accumulates in G_1-arrested cells (*cdc28-4* and *cdc28-13*) (Fig. 8). The observed fluctuations in the cell cycle of Sdb25 protein levels are consistent with the earlier finding indicating that p40/Sdb25 is an unstable protein (Mendenhall 1993). Interestingly, analysis of the coding region of *SDB25/SIC1* shows it has three regions with significant PEST scores (amino acids 37–49, 115–141, and 198–212) (Nugroho and Mendenhall 1994). Such regions, rich in proline (P), serine (S), and threonine (T), are present in all G_1 cyclins (Nash et al. 1988; Hadwiger et al. 1989b) and are thought to be signals for proteolysis (Rogers et al. 1986).

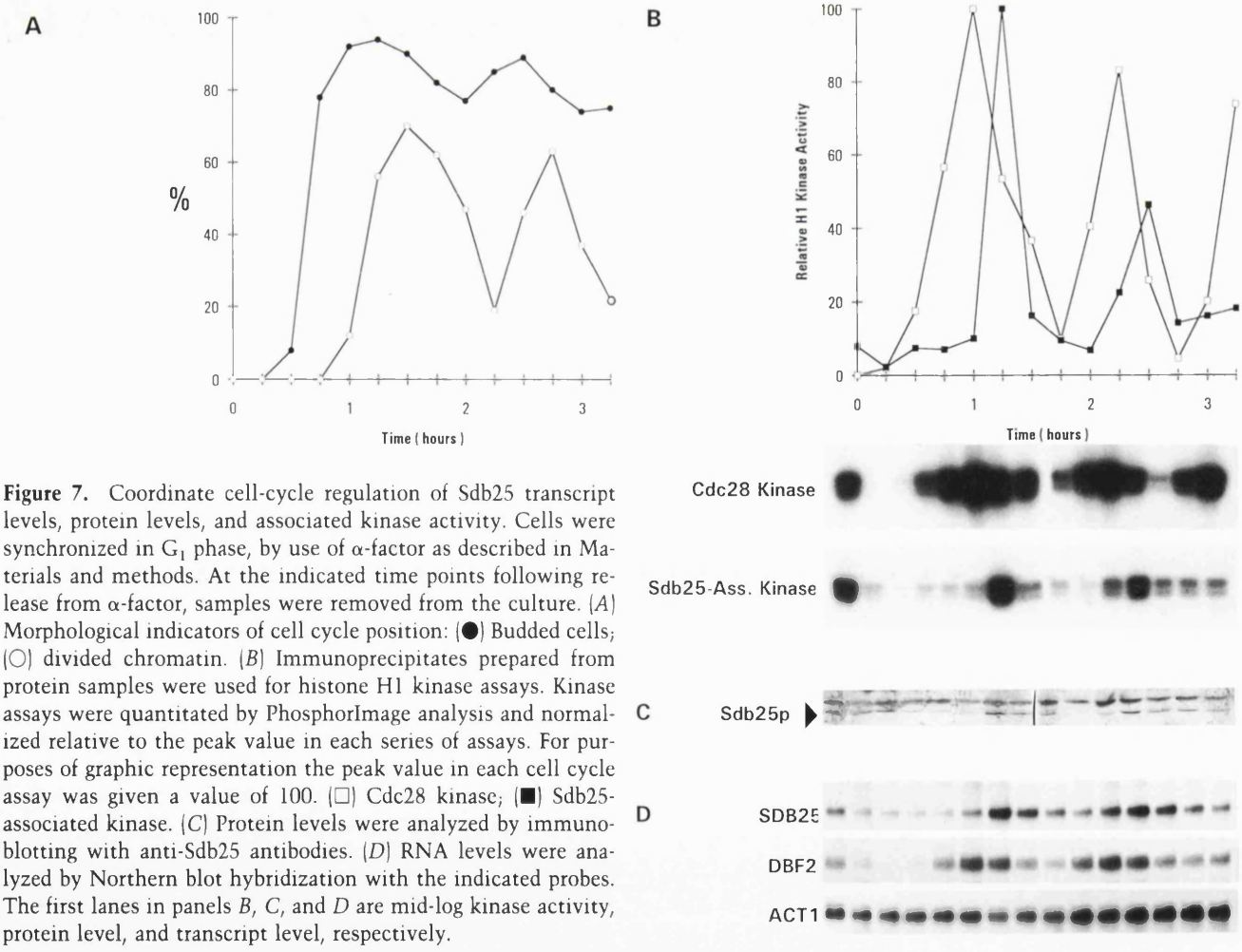

Figure 7. Coordinate cell-cycle regulation of Sdb25 transcript levels, protein levels, and associated kinase activity. Cells were synchronized in G_1 phase, by use of α-factor as described in Materials and methods. At the indicated time points following release from α-factor, samples were removed from the culture. (*A*) Morphological indicators of cell cycle position: (●) Budded cells; (○) divided chromatin. (*B*) Immunoprecipitates prepared from protein samples were used for histone H1 kinase assays. Kinase assays were quantitated by PhosphorImage analysis and normalized relative to the peak value in each series of assays. For purposes of graphic representation the peak value in each cell cycle assay was given a value of 100. (□) Cdc28 kinase; (■) Sdb25-associated kinase. (*C*) Protein levels were analyzed by immunoblotting with anti-Sdb25 antibodies. (*D*) RNA levels were analyzed by Northern blot hybridization with the indicated probes. The first lanes in panels *B*, *C*, and *D* are mid-log kinase activity, protein level, and transcript level, respectively.

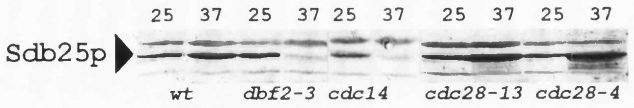

Figure 8. Sdb25 protein levels in cell cycle mutants arrested at 37°C. Cultures of the indicated strains were grown to mid-log levels and then split into two. One half was incubated at the nonpermissive temperature 37°C for 3 hr, and the other was grown at 25°C for the same amount of time. Immunoblots were carried out with Sdb25 antibodies, with equivalent amounts of protein being loaded in each lane.

Dbf2 and Sdb25-associated kinases are sequentially activated in late mitosis

Having established the timing of Sdb25 function, we then sought to determine whether activation of Sdb25-associated kinase occurred after activation of the Dbf2 kinase. An experiment similar to that described above suggested that Sdb25 kinase activity appears to peak slightly later than the peak of Dbf2 kinase activity in cells synchronized by α-factor. To examine the relative timing of the two kinase activities more precisely, *dbf2* cells were held at 37°C, the restrictive temperature, for 105 min. By this time >95% of the cells had fully divided chromatin, indicative of an anaphase/telophase arrest point, and both Dbf2 and Sdb25-associated kinase activities had fallen to a minimum. On return to the permissive temperature, Dbf2 kinase activity rapidly reached a peak within 10 min, coincident with a sharp increase in Sdb25 protein levels. This was followed 5–10 min later by the peak of Sdb25-associated kinase activity, roughly coincident with the peak of Sdb25 protein levels (Fig. 9B). The Sdb25 protein was then degraded, returning to background levels 35 min after cells had been returned to 25°C. Note that cells enlarge during the 37°C holding, and consequently, on release to 25°C, the events of late M phase, G_1, and Start are compressed. On the basis of this experiment it is therefore difficult to determine the precise phase in which Sdb25 is degraded, but these data show that activation of the Sdb25-associated kinase occurs after the Dbf2 kinase. Thus, both at the level of transcription (Fig. 4) and kinase activity, it appears that *SDB25* and *DBF2* are sequentially activated in late mitosis.

Discussion

With the objective of trying to further elucidate the physiological role of the protein kinase Dbf2 in regulation of the yeast cell cycle, we undertook a screen for high-copy suppressors of *dbf2*-ts mutants. This paper reports on the cloning, sequencing, and characterization of *SDB25*, one of five *dbf2* suppressors that were isolated.

Our genetic data indicate that suppression of *dbf2*-ts alleles by overexpression of *SDB25* varies with the copy

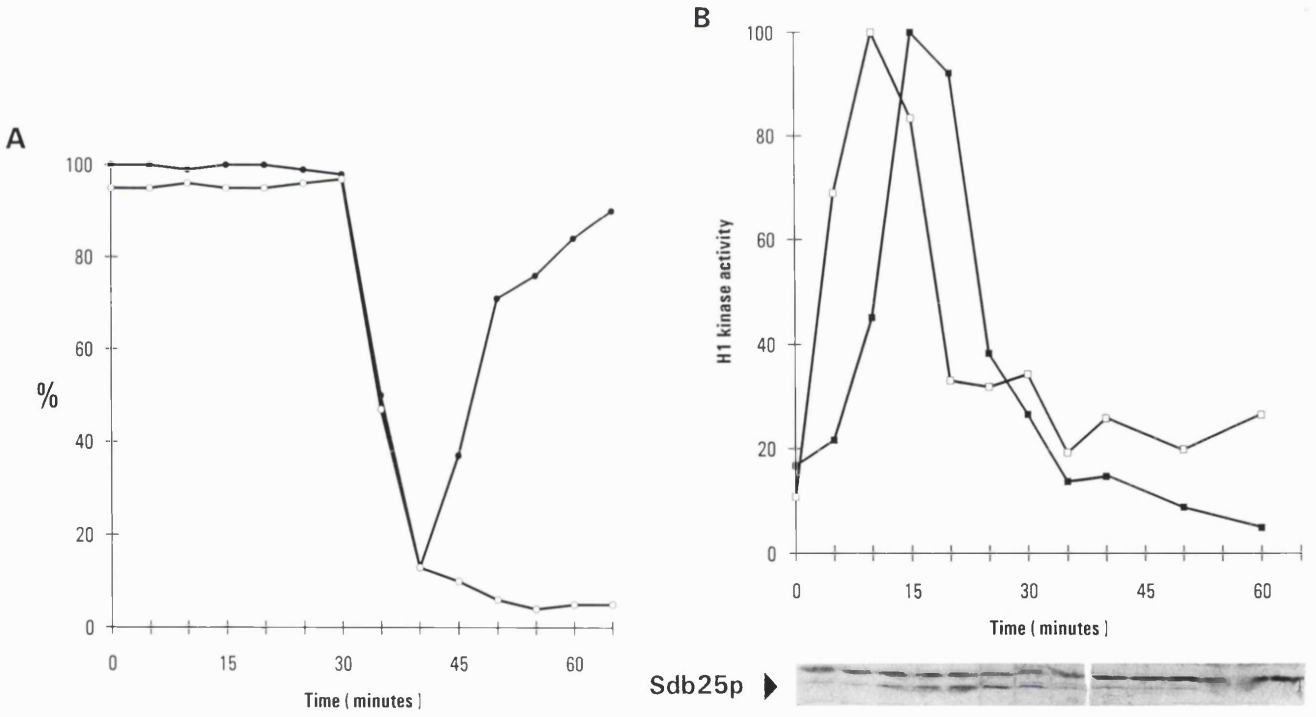

Figure 9. Sequential activation of Dbf2 and Sdb25-associated kinase. A culture of strain LJ1 (*dbf2-2*) was synchronized by a temperature shift of *dbf2-2* as described in Materials and methods. Cell samples were taken every 5 min for just over 1 hr following return to the permissive temperature, and protein extracts were prepared. (*A*) Morphological indicators of cell cycle position: (●) Budded cells; (○) divide chromatin. (*B*) Immunoprecipitates prepared from protein samples were used for kinase assays at the time points indicated. Kinase activities were quantitated and normalized as described in Fig. 6. (□) Dbf2 kinase; (■) Sdb25-associated kinase. Protein levels were analyzed by immunoblotting with anti-Sdb25 antibodies, with the nonspecific band appearing above the Sdb25 band serving as a loading control.

number of the plasmid used. That is, YRpSDB25 is an allele-specific suppressor of *dbf2-3*, whereas the higher copy number YEpSDB25 suppresses all three *dbf2*-ts alleles. Furthermore, suppression of *dbf2-3* by YRpSDB25 requires the presence of the Dbf2 structural and functional homolog, Dbf20. But more significantly, introducing YEpSDB25 into cells actually bypasses the normally essential requirement of Dbf2 and Dbf20 for completion of the cell cycle. One straightforward explanation of these data is that Sdb25 and Dbf2/20 are acting in parallel pathways, at the end of mitosis. Thus, when only slightly overexpressed, *SDB25* also requires Dbf20 to act in the alternative pathway, to allow completion of the cell cycle in the absence of a functional Dbf2 protein. However, when highly overexpressed, *SDB25*, alone, allows completion of the essential cell-cycle function common to the two pathways. The fact that *SDB25* has an essential function in a *dbf2*Δ background, *sdb25*Δ*dbf2*Δ cells being unable to grow, is consistent with this interpretation of parallel, partially redundant pathways. From this view, *sdb25*Δ*dbf2*Δ synthetic lethality arises because a component in both pathways is deleted, with the result that a vital cell function cannot be executed. This finding also supports the notion that *SDB25* has a role in the completion of essential mitotic events.

Further evidence for a late mitotic role for *SDB25* comes from examination of the *SDB25* transcript in synchronized cells. This shows that the gene is expressed under cell-cycle regulation, with a peak of expression very close to the M/G_1 boundary. Moreover, the tight correlation between the peaks of *SDB25* expression, Sdb25 protein levels, and Sdb25-associated kinase activity demonstrates that transcriptional activation of *SDB25* is rapidly translated into a functional effect. This also suggests that the primary control over Sdb25 function may be at the transcriptional level, rather than some post-translational effect, such as phosphorylation/dephosphorylation. With two different methods of synchrony, Northern analysis also shows that the peak of *SDB25* expression occurs shortly after the peak of *DBF2* expression. Thus *SDB25* and *DBF2* are not coordinately, but rather sequentially regulated, which may be a transcriptional mechanism for ensuring that Sdb25 functions after Dbf2. The temporally close order of Dbf2 and Sdb25 function is also demonstrated in cells released from a *dbf2*-thermosensitive block, where the Sdb25-associated kinase is activated immediately after the appearance of the Dbf2 kinase, following release from the block. However, neither the genetic interactions described above nor our cell cycle analysis prove anything conclusive about how the function of Sdb25 relates to the function of Dbf2. Using the available antibodies we were unable to demonstrate coimmunoprecipitation of Sdb25 and Dbf2, nor is an Sdb25–protein A fusion protein phosphorylated by the Dbf2 kinase.

Irrespective of the precise physiological relationship between Dbf2 and Sdb25, production of Sdb25 in late M phase appears to result in the protein immediately binding to an active form of a Cdc28 kinase complex. Furthermore, our cell cycle analysis indicates that Sdb25 associates with an active Cdc28 kinase complex for only a short time during the cell cycle. From an earlier, detailed examination of the cell cycle fluctuation of Cdc28 kinase activity with histone H1 as a substrate (Surana et al. 1991), it was concluded that the peak of Cdc28 kinase activity occurs in G_2/M, begins to decline on entry into anaphase, and reaches the lowest level on entry into G_1. This is consistent with a similar analysis (Reed and Wittenberg 1990), which found that the peak of Cdc28 kinase activity occurs in mitosis, and that by the time mitosis has been completed the Cdc28 protein kinase has been inactivated. If these findings can be generalized, then our own analysis (Fig. 7B) would indicate that Sdb25 associates with an active Cdc28 kinase complex at, or more likely after, anaphase has been initiated, but before cells enter G_1.

Mendenhall (1993), has shown recently that purified $p40^{SDB25}$ can inhibit in vitro Cdc28 kinase activity. Although we have no direct evidence to support this conclusion, it is interesting that our cell cycle analysis indicates that Sdb25 is present only when Cdc28 is down-regulated across the M/G_1 interface. Thus, although we have shown that Sdb25 binds to an active kinase at the end of mitosis, its primary role in mitotic cells could well be involved with the maintenance of a deactivated Cdc28 kinase as cells traverse the M/G_1 boundary. Furthermore, the demonstration that Sdb25 has an associated kinase activity that phosphorylates histone H1 in vitro is not incompatible with this suggestion. When assaying Sdb25-associated kinase activity we may be detecting an already inhibited form of Cdc28 kinase activity. From Figure 7 it is clear that the rapid inactivation of the Sdb25-associated kinase at the end of mitosis is not attributable to destruction of Sdb25, which is present in G_1 cells. In respect of this, it would be of interest to determine whether binding of Sdb25 to a Cdc28 kinase complex is coincident with the appearance of Sdb25-associated kinase activity. However, with the available antibodies, we have yet to determine coimmunoprecipitation of p40/Sdb25 and Cdc28, as previous workers have failed to do (Wittenberg and Reed 1988).

Viewing Sdb25 as an inhibitor of Cdc28 kinase activity can help to explain our morphological observations concerning *sdb25*Δ cells, which indicate that deletion of *SDB25* results in two distinct cell-cycle effects. The increase in budded cells and large cells in an *sdb25*Δ culture indicate a G_2/M delay. However, the overall smaller size and decrease in unbudded single cells both imply an acceleration through G_1. Possibly, deletion of *SDB25* results in a phenotype that is a composite of a delay in late M phase, because Cdc28 kinase activity takes longer to decay, and, independently, G_1 speeds up, because Cdc28 kinase is up-regulated more rapidly as cells traverse Start (see Fig. 10B). Note that this interpretation of the *sdb25*Δ phenotype applies to a population of cells, the two partial morphological effects (large budded cells → small cells), being mutually exclusive when applied to successive cycles undergone by an individual cell. Furthermore, the combination of these two effects on the ki-

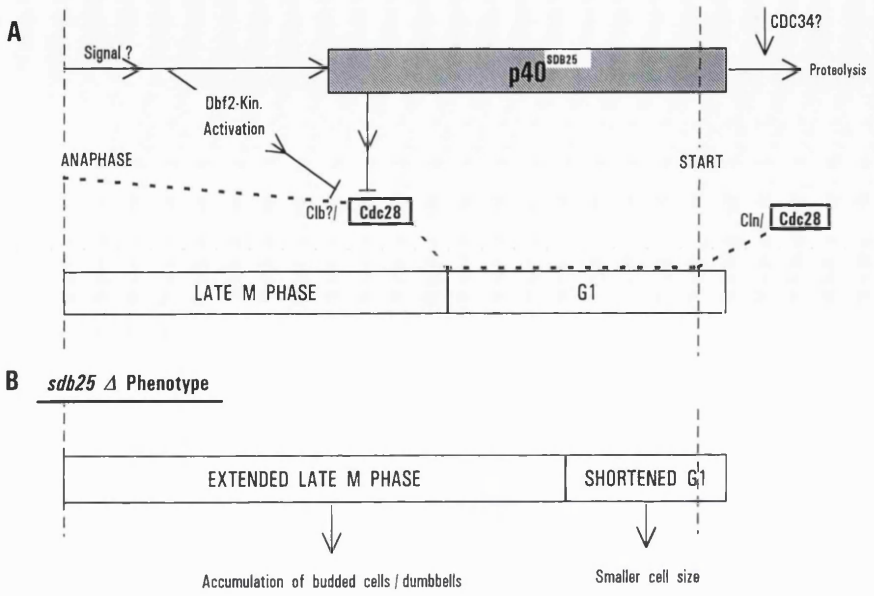

Figure 10. Speculative model for the role of Sdb25 in a late mitotic pathway. (*A*) Dbf2 kinase is activated after the metaphase to anaphase transition (Toyn and Johnston 1994), suggesting that its activity may be regulated by a signal transduced via separation of sister chromatids. As Dbf2 kinase activity peaks, Sdb25 is rapidly up-regulated and binds to a *CDC28*-dependent kinase complex, at a time when total Cdc28 kinase activity is being down-regulated. As discussed in the text, our genetic analysis indicates that Sdb25 and Dbf2 act in parallel pathways, which may converge to help down-regulate Cdc28 at the end of mitosis. However, our data do not exclude the possibility that Sdb25 functions directly downstream in a Dbf2/20-dependent pathway. Once produced Sdb25 persists to approximately Start of the next cell cycle, as depicted by the shaded box. The broken line linking the two Cdc28 boxes indicates the likely Cdc28 kinase activity as cells traverse M/G$_1$. As for the role of Sdb25, at present we favor the interpretation that Sdb25 helps inactivate and maintain a down-regulated Cdc28 kinase activity, from late M phase to early G$_1$. This may facilitate a rapid end to mitosis and a protraction of G$_1$ prior to Start. Sdb25 may not bind Cdc28 directly; rather it may physically associate with one of the Clb cyclins, the main regulators of mitotic Cdc28 kinase activity. As cells pass Start in G$_1$ Sdb25 is degraded, suggesting it may be targeted for destruction via the *CDC34* ubiquitin conjugating enzyme. (*B*) Consequences of deleting *SDB25*. On the basis of the model described above, the immediate consequence of deleting *SDB25* is to slow down the passage of cells through the end of mitosis. This would result in the accumulation of budded cells and some enlargement in size as cells continue to grow independently of the ongoing regulatory processes. Separately, deletion of *SDB25* may also result in some cells accelerating through pre-Start G$_1$, because of the restraining effect of Sdb25 being absent. This would result in such cells having a smaller size on average than wild type.

netics of growth rates could explain why the doubling time is essentially unaltered when *SDB25* is deleted.

In this paper we have presented an initial characterization of a newly identified yeast gene, *SDB25*, which encodes a previously characterized substrate and inhibitor of the Cdc28 kinase. We have shown that Sdb25 has a function in late mitosis, most likely after the metaphase to anaphase transition. This is inferred from the timing of the appearance of the Sdb25-associated kinase complex and from the fact that Sdb25 shows genetic interactions with Dbf2, the function of which is required in late nuclear division (Toyn and Johnston 1994). Our demonstration that the Sdb25-associated kinase is *CDC28*-dependent suggests Sdb25 may function to regulate the activity of Cdc28 at the end of mitosis. A speculative model, outlining the cell cycle role of Sdb25, is shown in Figure 10A. Formally, formation of the Sdb25-associated, *CDC28*-dependent, kinase complex could have a positive role in promoting the M/G$_1$ transition, perhaps by altering the substrate specificity of the kinase. As indicated in the model, we favor an alternative view that Sdb25 may help to down-regulate Cdc28 activity at the end of mitosis. This is consistent with the in vitro properties of p40^{SDB25} being a potent inhibitor of Cdc28 kinase activity (Mendenhall 1993), the morphology of *sdb25*Δ cells, and the timing of Sdb25 expression. Incidentally, if nutrients are limiting, instead of traversing Start, the cell moves into stationary phase in the next cell cycle, when Cdc28 is inactive. Significantly, Sdb25 is present only during these two phases, M/G$_1$ (our results) and stationary (Mendenhall 1993), strongly suggesting that its physiological role is to maintain an inactive form of Cdc28 prior to commitment to a new cell cycle.

This model also indicates our thinking about the possible mechanism responsible for the cell-cycle regulated degradation of Sdb25. In this respect, a parallel can be drawn with Far1 (McKinney et al. 1993), a negative factor required for cell-cycle arrest (Chang and Herskowitz 1990). Like Sdb25, Far1 is a cell-cycle-regulated phosphoprotein (McKinney et al. 1993) that has been shown to bind and inhibit the Cdc28 protein kinase (Peter et al. 1993). Also, both proteins are degraded in G$_1$, suggesting that they both may be directed into a proteolytic pathway via the ubiquitin-conjugating enzyme *CDC34* (Goebl et al. 1988). *CDC34*-mediated proteolysis has been proposed recently to account for the disappearance of Far1 after Start (McKinney et al. 1993). Perhaps future work will show that Sdb25 and Far1 are members of a group of cell-cycle-regulated CDK inhibitor proteins that function to modulate the activity of Cdc28 in conjunction with the Clb/Cln cyclin family.

Materials and methods

Culture conditions and general techniques

The relevant genotypes of *S. cerevisiae* strains used in this study

are listed in Table 1. Cells were grown in YEPD (1% Difco yeast extract, 2% Difco Bacto-peptone, and 2% glucose) except when selecting for plasmids; in these cases, they were grown in 0.67% YNB (Difco), 2% glucose, with the appropriate supplemented amino acids. Selection of *ura3* cells that had lost a *URA3* plasmid was accomplished by plating the cells on media containing FOA (Boeke et al. 1984). Yeast transformations were performed by a modification (Gietz and Sugino 1988) of the lithium acetate method (Ito et al. 1983). The incubation temperature for yeast cultures was 30°C, except for thermosensitive strains, which were grown at the permissive temperature of 25°C, or a restrictive temperature of 37°C. Cell numbers were determined by use of a particle counter (Coulter Electronics, Dunstable, England).

Construction of the *dbf2*Δdbf20Δ strain containing YEpSDB25 was as follows: Strain J211-2C has the genotype *dbf2Δdbf20Δ ade2 ura3* and is kept alive by the plasmid pASZ11–DBF2 (*CEN ARS ADE2 DBF2*), resulting in white colonies. The plasmid YEpSDB25 was introduced into this strain, and red sectors, corresponding to clones that had lost the pASZ11-DBF2 plasmid, were picked.

All DNA manipulations were as described previously (Maniatis et al. 1982). The dideoxy chain termination method (Sanger et al. 1977) and double-stranded DNA were used for DNA sequence analysis, using the Sequenase version 2.0 sequencing kit (U.S. Biochemical).

Identification of SDB25

SDB25 was isolated as a single clone with an insert of ~3.75-kb from a *S. cerevisiae* genomic library ligated into the multiple-copy plasmid YRp12 (Barker and Johnston 1983). To locate the suppressor gene within the genomic fragment, plasmid YRpSDB25 (YRp12 carrying the 3.75-kb insert) was subject to Tn*1000* mutagenesis in *Escherichia coli* (Sedgwick and Morgan 1994), and the resulting library of randomly disrupted plasmids was transformed into yeast strain L182-7A (*dbf2-3*). Subsequent replica-plating of the transformants onto YEPD at 37°C and 25°C indicated those plasmids where suppressor function had been disrupted. The transposon sites were located by restriction analysis of the extracted plasmids, and DNA sequencing analysis was carried out using primers specific to the γ, the δ, and γ sequences of Tn*1000*.

Synchronization procedures

The elutriator–synchronized cultures used here have been described previously (Kitada et al. 1993). Synchronous cultures of *MAT***a** cells were produced by the α-factor method (Johnston et al. 1990). Synchronous cultures of *dbf2-2* cells were produced by a temperature-shift method (Creanor and Toyn 1994). Briefly, a log phase culture of diploid strain LJ1 (homozygous for *dbf2-2*), growing at 25°C in YEPD, was transferred to 37°C for 105 min. The arrested cells were then rapidly cooled to 25°C, the permissive temperature, and cell samples were taken.

Cytological methods

To visualize nuclei, cells were stained with 4,6-diamidino-2-phenylindole (DAPI) (Shero et al. 1991) and observed with a Zeiss Axiophot microscope. The DNA content of individual cells was measured using a FACStar Plus (Becton Dickinson) flow cytometer. Cells were prepared for flow cytometry as described previously (Parkes and Johnston 1992). Photomicrographs of cells were taken with a phase-contrast microscope with a 40× objective.

Gene replacement, epitope tagging, and plasmids

A null allele of *SDB25* was constructed by the one-step gene disruption method (Rothstein 1983). The 750-bp *Kpn*I–*Hpa*I fragment of *SDB25* was replaced by an 850-bp fragment containing the *TRP1* gene, this disruption being made in YRpSDB25. The DNA fragment containing *sdb25Δ::TRP1* was generated by *Nco*I–*Ssp*I digestion and used to transform the *trp1/trp1*1 diploid CG378/379 (Table 1). Selected diploid prototophs were determined for *SDB25* deletion by Southern blot analysis. Plasmid YEplacSDB25 was constructed by insertion of the *Eco*RI–*Hin*dIII 1.8-kb fragment of YRpSDB25, containing *SDB25*, into the

Table 1. *Yeast strains used in this study*

Strain	Relevant genotype	Source
L119-7D	*MAT*α *dbf2-1 ade1 trp1-289 ura3-52*	Parkes and Johnston (1992)
L181-6B	*MAT*α *dbf2-2 leu2-3,112 trp1-289 ura3-52*	Toyn and Johnston (1993)
L182-7A	*MAT***a** *dbf2-3 ade1,5 leu2-3,112 trp1-289 ura3-52*	this study
cdc28-1N	*MAT***a** *cdc28-1N ade2-1 leu2-3,112 trp1-1 ura3*	Kitada et al. (1993)
cdc28-13	*MAT***a** *cdc28-13 ade1 leu2-3,112 trp1 ura3Δ*	Kitada et al. (1993)
L153-6C	*MAT*α *cdc28-4 leu2 trp1 ura3 his7 tyr1*	this study
L195-6D	*MAT*α *cdc14 ade1 ura3 trp1*	this study
S7-4A	*MAT***a** *dbf2Δ::URA3 ade5 his7 leu2-3,112 trp1 ura3*	Toyn et al. (1991)
S2-2D	*MAT*α *dbf2Δ::LEU2 ade5 leu2 trp1*	Toyn et al. (1991)
CG378	*MAT***a** *ade5 leu2-3,112 trp1-289 ura3-52*	C. Giroux (Wayne State University, Detroit, MI)
CG379	*MAT*α *leu2-3,112 trp1-289 ura3-52 his7-2*	C. Giroux
J99	CG378 *dbf20Δ::TRP1*	Toyn et al. (1991)
V378	CG378 *spo12Δ::TRP1*	Parkes and Johnston (1992)
JD100	CG379 *sdb25Δ::TRP1*	this study
J149	*MAT***a** *dbf2-2 ade2-101 leu2 trp1 ura3-52*	Toyn and Johnston (1993)
LJ1	*dbf2-2/dbf2-2*	L181-6B × J149
J103-2A	*MAT*α *dbf20Δ::TRP1*	J99 × L181-6B
JD101	*MAT***a** *dbf2-3 dbf20Δ::TRP1*	L182-7A × J103-2A
J211-2C	*MAT***a** *ade2-101 ura3 can1 dbf2Δ::LEU2 dbf20Δ::TRP* + pASZ11-*DBF2*	Toyn and Johnston (1993)

multiple cloning site of the high-copy plasmid YEplac181 (Geitz and Sugino 1988). Plasmid YEpSDB25 contains the *SDB25* gene cloned into the high-copy plasmid YEp24. The plasmid pASZ11-DBF2 has been described previously (Toyn and Johnston 1993).

The *CDC28* gene was tagged by insertion of the HA1 epitope (from R. Deshaies and Dr. P. Sorge, University of California, Berkeley), which is recognized by the 12CA5 monoclonal antibody (Wilson et al. 1984). The integrating plasmid pRD86 was used, which contains *URA3* and the carboxy-terminal part of the *CDC28* gene. The plasmid was linearized using the unique *KpnI* site in the *CDC28* gene, before integrative transformation (Rothstein 1983), resulting in one complete epitope-tagged *CDC28* gene, and one amino-terminally truncated *CDC28* gene in the yeast genome.

Northern and Southern blot analysis

Total RNA was extracted from cells as described previously (White et al. 1986). A 5-μg sample of total RNA, denatured with glyoxal, was separated on a 1.2% agarose gel, transferred to a GeneScreen hybridization membrane (Dupont, NEN Research Products, Boston, MA). Probes for RNA–DNA hybridization were internal fragments from the genes concerned, with the *SDB25* probe being the 750-bp *KpnI–HpaI* fragment. DNA was labeled with [α-^{32}P]TTP by use of an oligo-labeling protocol (White et al. 1986). RNA levels were quantitated with a Personal Densitometer PD-130 (Molecular Dynamics).

Yeast genomic DNA was extracted by the method of Hoffman and Winston (1987). The *SspI–NcoI* 3.1-kb fragment of the *SDB25* locus was labeled as for Northern blotting and used as a probe for Southern blot analysis.

Preparation of Sdb25 antiserum

The 2.1-kb *KpnI–BglII* fragment of YRpSDB25 was subcloned into pRIT2 (Lowenadler et al. 1986) to give pJ100, which contains a protein A–Sdb25 fusion gene having all but the first 13 amino acid residues of the Sdb25 polypeptide. The fusion protein was induced in *E. coli*, purified using an IgG–Sepharose column (Pharmacia), and injected into rabbits.

Preparation of yeast extracts and immunoblot analysis

Yeast total protein extracts were prepared by resuspending cells in cold lysis buffer (20 mM Tris at pH 7.4, 10 mM EDTA, 100 mM NaCl, 1% Triton X-100, 5% glycerol, 1 mM each of NaF, β-glycerophosphate, Na_3VO_4, EGTA, and sodium pyrophosphate). The following inhibitors were included during cell lysis: 5 μg/ml each of leupeptin, pepstatin A, chymostatin, aprotinin (Sigma), and AEBSF (Calbiochem). For phosphatase treatment, cells were resuspended in phosphatase buffer (50 mM Tris at pH 8.0, 10% glycerol) plus protease inhibitors as described above. Cells were broken by vortexing with acid-washed glass beads (4 × 30 sec bursts), after which the lysate was cleared by spinning for 5 min in a benchtop centrifuge. In any given experiment all samples were normalized to contain the same amount of protein (Lowry et al. 1951). Extracts (~100 μg) used in the phosphatase treatment were incubated at 37°C for 30 min, with 1 unit of calf intestinal alkaline phosphatase (Boehringer Mannheim) in 20 μl of phosphatase buffer, or mock-treated with 20 μl of phosphatase buffer. When used, the phosphatase inhibitor β-glycerophosphate (50 mM) was added to 20 μl of phosphatase buffer.

For immunoblotting, cell extracts containing 100 μg of total protein were separated on a 10% SDS–polyacrylamide gel and transferred to nitrocellulose (0.45 μm, Schleicher & Schuell) using a semidry blotting unit (Bio-Rad) according to the manufacturer's instructions. Nitrocellulose blots were blocked by overnight incubation in PBS/1% nonfat milk and transferred to a 1:25 dilution of Sdb25 antiserum for 2 hr, followed by sequential incubations with antirabbit IgG-conjugated biotin (1:1000), and strepavidin alkaline phosphatase (1:1000) (Sigma). All antibody incubations were done at room temperature with agitation, followed by 30-min washes with several changes of PBS/0.5% BSA buffer. Proteins that interacted with the antibodies were visualized using the chromogenic substrates BCIP/NBT, as described previously (Harlow and Lane 1988).

Protein kinase assays

For Sdb25-associated kinase activity, 1 μl of Sdb25 antiserum was added to 100 μg of yeast extract and incubated for 1 hr on ice. Protein A beads (20 μl of protein A–Sepharose CL-4B; Pharmacia) were then added, and incubation continued with mixing for an additional 30 min. The beads were then washed three times in 1 ml of lysis buffer, followed by one wash in assay buffer (25 mM MOPS at pH 7.2, 60 mM β-glycerophosphate, 15 mM ρh-nitro-phenylphosphate, 15 mM $MgCl_2$, 5 mM EGTA, 1 mM dithiothreitol, 0.1 mM Na_3VO_4, 10 μm ATP and 5 μg/ml each of leupeptin, pepstatin A, chymostatin, and aprontinin). The washed immunoprecipitate was then placed at 30°C and 10 μl of assay buffer containing 1 μCi of [γ-^{32}P]ATP, and 5 μg of calf thymus histone H1 (Sigma, type III-S) was added. Incubation was continued for 30 min, and the reaction stopped by the addition of 15 μl of SDS–polyacrylamide gel sample buffer and heating for 3 min at 90°C prior to electrophoresis.

For Cdc28 kinase assays, 1 μl (10 μg of protein) of Mab 12CA5 (ascites fluid, from Babco) was added to 100 μg of yeast extract from strains containing the epitope-tagged Cdc28, with subsequent steps being essentially the same as for Sdb25-associated kinase assays. Dbf2 kinase assays were as described previously (Toyn and Johnston 1994), being essentially the same as Sdb25-associated and Cdc28 kinase assays. Incorporation of ^{32}P into histone H1 was quantitated by β-imaging using a PhosphorImager (Molecular Dynamics).

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Note added in proof

The sequence data described in this paper have been submitted to the EMBL data library under accession number X78309.

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