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Genetic and Molecular Analysis of the *CDC68* Gene of *Saccharomyces cerevisiae.*

by

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Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy

at

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For my parents

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ABSTRACT

The cell cycle of the budding yeast *Saccharomyces cerevisiae* has been investigated through the study of conditional *cdc* mutations that specifically affect cell-cycle performance. Cells bearing the *cdc68-1* mutation are temperature-sensitive for the performance of the G1 regulatory event, Start. This study describes both genetic and molecular evidence that suggests that the *CDC68* gene plays a role in transcription. The abundance of transcripts from several unrelated genes has been found to decrease in *cdc68-1* mutant cells after transfer to the restrictive temperature while at least one transcript, from the *HSP82* gene, persists in an unusual fashion. Thus the *cdc68-1* mutation has both positive and negative effects on gene expression. Among transcripts that rapidly become depleted in *cdc68-1* mutant cells are those of the G1-cyclin genes *CLN1, CLN2* and *CLN3/WHI1/DAF1*, whose activity has been previously shown to be required for the performance of Start. The decreased abundance of cyclin transcripts in *cdc68-1* mutant cells, coupled with suppression of *cdc68-1* - mediated Start arrest by a hyperactive cyclin mutation, demonstrates that the *CDC68* gene affects Start through effects on cyclin gene expression.

The *CDC68* gene encodes a 1035 amino acid protein with a highly acidic and serine-rich carboxyl-terminus. Two temperature-sensitive mutations have been localized to the N-terminal third of the predicted Cdc68 protein. Surpisingly, however, this region of the *CDC68* open reading frame can be deleted with little effect on cell proliferation. Thus, removal of this region appears less deleterious than the presence of particular point mutations.

The pleiotropic effects of *cdc68* mutations, gene dosage effects, as well as structural and phenotypic similarities to other genes, suggest that the function of the Cdc68 protein may involve interaction with chromatin to influence transcription from a large number of promoters.

ABBREVIATIONS AND SYMBOLS

cM	centiMorgan
lab coll'n	laboratory collection
HMG	high mobility group
MCS	multiple cloning site
MPF	maturation-promoting factor
ORF	open reading frame
HSF	heat-shock transcription factor
RF	replicative form
RNAP	RNA polymerase
ssDNA	single stranded DNA
TCA	trichloroacetic acid

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I. INTRODUCTION

1. The eukaryotic cell cycle

The cell cycle is the process whereby one cell gives rise to two cells, each of which is genetically identical to the original cell. Failure to regulate cell proliferation can have deleterious consequences, such as cancer in multicellular organisms. Execution of the cell cycle requires the integration of biosynthetic processes that result in duplication of cellular constituents with other processes involved in the segregation of material between the two cells. In fact, Mitchison (1971) was one of the first to suggest that the cell cycle may actually be viewed as two ongoing cycles , the "DNA division cycle" and the "growth cycle". It is necessary that these two constituent cell cycles are coordinated to ensure balanced growth and to avoid the production of cells that are either too large (resulting from growth without division) or too small (resulting from division with insufficient growth).

The eukaryotic cell cycle is composed of four phases: S phase, in which DNA is replicated, followed by a gap, G2; then M phase, in which mitosis occurs, followed by another gap, G1. Cell-cycle regulation is accomplished at two positions within the cell cycle. The importance of each regulatory step varies between organisms, and between developmental stages of the same organism. For example, in embryonic cells of many organisms, including *Xenopus*, starfish and *Drosophila*, cell-cycle regulation is exerted prior to mitosis. Later in development, regulation shifts to the G1 interval (*e.g.* O'Farrell *et al.*, 1989; Murray and Kirschner, 1989). Despite these and many other differences, a unified view of the cell cycle has

recently begun to emerge. It is now clear that the proteins which are central to the regulation of cell proliferation have been widely conserved, and that, as a result, the discoveries made in one system often have implications for cell-cycle regulation as a whole.

This thesis describes the characterization of a gene, *CDC68*, which is necessary for cell proliferation in the budding yeast, *Saccharomyces cerevisiae*. Mutations in this gene cause cells to be temperature-sensitive for the completion of the major cell-cycle regulatory point, Start. To provide the context in which this work has been undertaken, this introduction will review the regulation of cell proliferation in eukaryotic cells. The experimental system used for these studies will be described as will previous approaches to cell-cycle analysis. Components of the *S. cerevisiae* cell-cycle regulatory machinery will also be discussed, followed by a consideration of how studies in several systems have recently converged to yield a unified view of cell-cycle regulation in eukaryotic cells. The final section of this introduction will detail the objectives of this study.

2. *Saccharomyces cerevisiae* as a model system for cell-cycle analysis

a) Life cycle, genetics and molecular biology

The unicellular budding yeast *S. cerevisiae* has been extensively utilized as a model system for the investigation of a wide variety of cellular processes, including regulation of cell proliferation. Several features of this organism's life cycle have contributed to its success in this respect. *S. cerevisiae* cells are easily propagated and have significantly shorter doubling times than those of many other eukaryotes. Genetic analysis is facilitated by the stable existence of haploid and diploid forms, so that

recessive mutations isolated in haploid cells can be analysed by complementation analysis in diploids. Diploid cells may then be sporulated, allowing analysis of the resulting meiotic products to determine genetic linkage relationships and to analyse genetic interactions (Mortimer and Hawthorne, 1969). The mode of reproduction of *S. cerevisiae* cells provides a particularly convenient morphological marker of cell-cycle progression: a cell begins the cell cycle with the emergence of a bud on its surface. This bud then grows in size as the cell cycle progresses and finally separates from the mother cell to become a new daughter cell.

Molecular genetic approaches are now highly developed in *S. cerevisiae*. High-frequency transformation, cloning by complementation, gene disruption and gene replacement are now routine procedures (reviewed in Struhl, 1983).

b) Regulation of cell proliferation in *S. cerevisiae*

Regulation of cell proliferation in *S. cerevisiae* occurs primarily at the G1 regulatory step termed Start. At Start, cells become committed to alternative developmental pathways, the selection of which is dependent upon the external environment and internal biosynthetic status of the cell. Under favourable conditions, a cell undertakes a new round of cell division which, once initiated, continues throughout all but the most extreme subsequent perturbation of the external environment. Under starvation conditions, haploid and diploid cells can enter a unique off-cycle state termed G_0, where they remain until nutrients become available. Diploid cells can, however, embark on an alternative meiotic pathway resulting in the production of four haploid spores which remain quiescent until the

return of favourable conditions. The remaining developmental alternative, available only to haploid cells, and then only in the presence of cells of the opposite mating type, leads to the production of stable diploid cells through the process of conjugation. The latter process is facilitated by the action of two small polypeptides known as mating pheromones: α-factor (secreted by *MAT*α cells) and **a**-factor (secreted by *MAT***a** cells), which act on cells of the opposite mating type to arrest proliferation at Start after completion of the ongoing round of cell division.

c) Coordination of growth and division at Start in *S. cerevisiae*

The DNA division cycle of *S. cerevisiae* consists of stage-specific events such as duplication and segregation of the spindle plaque, DNA replication, bud emergence and nuclear migration. The growth cycle refers to ongoing processes involved in mass accumulation. In experiments which led to the "critical size model" proposed by Johnston *et al.* (1977), it was demonstrated that coordination of the growth and DNA division sequence is accomplished through a critical size requirement exerted at Start, and that growth, rather than any particular stage-specific event in the DNA division sequence, is rate-limiting for cell-cycle progression.

It was recognized from the outset that cell size is merely an experimental parameter that correlates with the actual parameter(s) monitored by the cell. Several groups have speculated that the level of an unknown activator protein is monitored at Start (*e.g.*, Pardee *et al.*, 1978; Wheals, 1982). The studies described in later sections will indicate that the molecular basis for coordination between the cell-division sequence and cell growth is now close to being identified.

d) Cell-cycle analysis in *S. cerevisiae*

One approach to the study of a process of interest involves the identification of mutations that specifically inhibit that process. This mutational approach has been extensively used to study the cell cycle of *S. cerevisiae* by the isolation of conditional *cdc* (cell division cycle) mutations that cause cells to arrest proliferation at a particular point in the cell cycle (Hartwell *et al.*, 1970; 1973; Hartwell, 1974). Upwards of 70 such mutations have been identified (reviewed by Wheals, 1987). Analysis of such mutants led to a view of the cell cycle as a sequence of temporally coordinated events, each of which requires the action of specific gene products (Hartwell, 1974).

e) *S. cerevisiae* Start mutations

Among the large collection of *CDC* genes originally identified through mutation by Hartwell and his colleagues, only one, *CDC28*, has the characteristics expected of a gene which specifies a product central to the Start machinery. Determining the point at which the *cdc28-1* mutation exerts its deleterious effect on cell-cycle progression with respect to the α–factor sensitive step indicated that *cdc28-1* and α-factor interrupt the cell cycle at the same point (Hereford and Hartwell, 1974). However, in recognition of the fact that mutations which affect Start need not necessarily define products that function as part of the Start apparatus itself, Reed (1980) grouped Start mutations into two categories based on phenotypic considerations. Class I Start mutations are defined as those which cause cells to arrest at Start but allow continued mass accumulation and conjugation. The ability to conjugate is of significance for two reasons: only cells that

have not completed Start can conjugate and conjugation requires biosynthetic competence; mutations which severely compromise biosynthesis prevent the ability of cells to mate. *cdc28-1* is an example of a class I Start mutation; cells arrested by the *cdc28-1* mutation remain capable of conjugation (Reid and Hartwell, 1977). In contrast, Class II mutations are those which cause Start arrest accompanied by significant decreases in biosynthetic activity, and are likely to define genes that influence Start through underlying effects on growth-related processes. Subsequent isolation schemes have resulted in the identification of new Start mutations in genes including *CDC36*, *CDC37* and *CDC39* (Reed, 1980), *CDC60*, *CDC62*, *CDC63*, and *CDC64* (Bedard *et al.*, 1981), *CDC65*, *CDC67*, and the subject of this thesis, *CDC68* (Prendergast *et al.*, 1990). In general, the mutations identified have depended upon the isolation procedure employed. For example, the use of enrichment procedures based on survival under conditions otherwise likely to cause rapid cell death (such as inositol-less death or *cdc4*-mediated cell-cycle arrest), and a procedure based on mating competency, resulted in the identification of four new Start mutations but failed to identify any new alleles of previously identified Start genes (Bedard *et al.*, 1981).

Molecular analysis has demonstrated that Start mutations may be found in genes that function in a variety of processes. A number of Start genes have been found to encode products involved in the pheromone-response pathway. For example, *cdc36*- and *cdc39*-mediated Start arrest is probably due to mutational activation of this pathway (Connolly *et al.*, 1983; de Barros Lopes *et al.*, 1990). The *CDC36* gene encodes a product related both to Cdc4 (a yeast cell-cycle protein that functions after Start) and to *ets*, a

transformation-specific sequence of avian erythroblastosis virus E26 (Peterson *et al.*, 1984). The significance of this similarity is not yet clear since the mechanism by which *cdc36* mutations result in the activation of the mating pathway has not been determined. Not all mutations that affect Start have been identified solely on the basis of their *cdc* mutant phenotype. For example, conditional Start mutations such as those in the *SRM1* (Clark and Sprague, 1989) and *GPA1* (*SCG1*) genes (Jhang *et al.*, 1988;) were isolated using genetic strategies designed to identify further components of the mating-response pathway. The *GPA1*(*SCG1*) gene encodes the α subunit of the G protein implicated in the transduction of the pheromone signal (Nakafuku *et al.*, 1987; Miyajima *et al.*, 1987; Dietzel and Kurjan, 1987). Mutant forms of both *GPA1* and *SRM1* allow conjugation in the absence of mating-pheromone receptors (Jhang *et al.*, 1988; Clark and Sprague, 1989). Since arrest at Start is a prerequisite for conjugation, it is not surprising that mutations in these two genes should also be found to exhibit a G1-arrest phenotype, in addition to their effects on the mating pathway.

A second pathway in which Start mutations have been identified is the cAMP signal-transduction pathway. For example, *cdc35* Start mutations have been found to be alleles of the *CYR1* gene that encodes the enzyme adenylate cyclase (Boutelet *et al.*, 1985), while *cdc25* Start mutations define a gene that apparently encodes a regulatory component of the adenylate cyclase pathway (Camonis *et al.*, 1986; Broek *et al.*, 1987). Although the function of cAMP is thought to be limited to activation of cAMP-dependent protein kinase (protein kinase A) (Broach, 1991), some substrates of which are known (reviewed by Broach and Deschenes, 1990), it is not known how protein kinase A activity impinges upon cell-cycle regulation.

Mutations in translation factors have also been identified by their ability to cause cell-cycle arrest at Start. For example, *cdc65* mutations lie in an essential $tRNA^{Gln}$ gene (A. Rowley, L. Murray, N. Rowley, R. Singer and G. Johnston, unpublished data), while *cdc33* and *cdc63* mutations define factors thought to form part of the translation initiation machinery (Hanic-Joyce *et al.*, 1987; Brenner *et al.*, 1988). *CDC60*, in which mutations cause Start arrest, has recently been found to encode cytoplasmic leucyl-tRNA synthetase (J. Thevelein, personal communication). Mutations in two further tRNA synthetase genes, *ILS1* (isoleucyl-tRNA synthetase) and *MES1* (methionyl tRNA synthetase), can also cause regulated arrest at Start (Unger and Hartwell, 1976; Niederberger *et al.*, 1983). Again, it is not clear how mutations that affect translation cause specific cell-cycle arrest.

3. Universality of the cell-cycle regulatory machinery

a) The *S. cerevisiae CDC28* and *S. pombe* $cdc2^+$ genes encode conserved protein kinases

In addition to the fact that *cdc28* was one of the first Start mutations identified, a number of factors have resulted in the concentrated study of the *CDC28* gene. The demonstration by Beach *et al.* (1982) that the cloned *S. cerevisiae CDC28* gene complements a *Schizosaccharomyces pombe cdc2* temperature-sensitive mutation suggested that Cdc28-dependent functions are conserved in both organisms and, given that these two yeasts are only very distantly related, possibly in all organisms. This conclusion was later reaffirmed by the reciprocal demonstration that the $cdc2^+$ gene can complement a *cdc28* temperature-sensitive mutation (Booher and Beach, 1986). (For *S. pombe*, gene names are generally written in lower case, with a

superscript + to distinguish wild-type from mutant alleles). This finding was initially surprising because the cell cycle of *S. pombe*, unlike that of *S. cerevisiae*, is regulated primarily in the G2 phase, prior to the initiation of mitosis, although the cell cycle is also regulated at the *S. pombe* equivalent of Start. The *cdc2*$^+$ gene acts at both the G1 and G2 regulatory steps in the *S. pombe* cell cycle (Nurse and Bisset, 1981). This finding, in addition to the identification of *cdc2* mutant alleles that advance the timing of M-phase initiation (Nurse and Thuriaux, 1980), indicated that the *cdc2*$^+$ gene plays a central role in cell cycle regulation. Nucieotide sequencing demonstrated that the *cdc2*$^+$ and *CDC28* genes both encode 34-kDa proteins which have similarity with not only each other, but also with vertebrate protein kinases (Lorincz and Reed, 1984; Hindley and Phear, 1984). Both gene products have protein kinase activity *in vitro* (Reed *et al.*, 1985; Simanis and Nurse, 1986). These proteins are now generally referred to as p34^{cdc2} and p34^{cdc28}. The similarity between the *S. cerevisiae* and *S. pombe* p34^{cdc2} and p34^{cdc28} proteins led others to look for mammalian p34 homologues; Lee and Nurse (1987) identified a human gene by complementation of a temperature-sensitive *cdc2* mutation using a human cDNA library, whereas Draetta *et al.* (1987) identified a protein in HeLa cell extracts that was cross-reactive with antibodies that recognize p34^{cdc2} and p34^{cdc28} conserved epitopes. Since these earlier findings, p34-encoding genes have been identified in many other organisms.

b) *CDC28*, *cdc2*$^+$ and MPF

Recently the genetic analysis of the cell cycle, undertaken primarily in *S. cerevisiae* and *S. pombe*, has converged with biochemical analysis in

systems as diverse as frogs, starfish and clams. In 1988, Gautier *et al.* demonstrated that a M_r 32,000 component of a complex known as maturation promoting factor (MPF) purified from *Xenopus* oocytes is immunoprecipitated by antibodies raised against *S. pombe* p34^{cdc2}. MPF was originally defined as an activity from mature oocytes that (upon microinjection) caused immature oocytes (naturally arrested at the G2/M boundary prior to meiosis I) to advance into M phase, even in the absence of protein synthesis (Masui and Market, 1971; Smith and Ecker, 1971). Since these early studies it has become clear that, in addition to regulating meiotic M phase, MPF plays a role in mitotic induction in a wide variety of organisms. Purification of MPF proved problematic due to its instability but was eventually achieved by Lokha *et al.* (1988), who demonstrated that *Xenopus* MPF consists primarily of two proteins, one of M_r 32,000 and the other of M_r 45,000. Further evidence for the shared identity of the smaller MPF component and p34 was obtained by Dunphy *et al.* (1988), who found that MPF activity is retained on an affinity column containing p13, the product of the *S. pombe suc1*$^+$ gene. In fission yeast, p13^{suc1} is known to associate with p34^{cdc2} (Hayles *et al.*, 1986; Hindley *et al.*, 1987; Brizuela *et al.*, 1987). In fact, the affinity of p13^{suc1} for p34^{cdc2} has provided the basis for p34^{cdc2} purification schemes. *In vitro*, p13^{suc1} was found to associate with and to block the ability of MPF to cause *Xenopus* egg interphase extracts to enter a mitotic state. These data therefore suggest that mitotic controls in *Xenopus* and *S. pombe* are sufficiently similar to allow p13^{suc1} to function in *Xenopus* extracts, although a *Xenopus* homologue of p13^{suc1} has not yet been identified and the exact function of p13^{suc1} remains unclear.

MPF was originally identified using microinjection of oocytes. An alternate approach that has been used to identify cell-cycle regulatory proteins has been to search for those whose activity is periodic in the cell cycle. It is now clear that a well-described protein kinase identified using such an approach is the same as MPF. This "growth associated" or "M-phase-specific kinase" has been previously documented in a wide variety of eukaryotic systems including starfish, sea urchin, *Xenopus*, rat and human (reviewed in Arion *et al.*, 1988). The preferred *in vitro* substrate of this kinase is histone H1, and for this reason the kinase activity is also known as H1 kinase. The finding that purified MPF can phosphorylate histone H1 *in vitro* suggested that MPF and H1 kinase might be overlapping, if not the same, entity (Lokha *et al.*, 1988). Evidence that the H1 kinase is indeed the same as MPF comes from the finding that H1 kinase from starfish oocytes copurifies with a *cdc2*$^+$ homologue, as demonstrated by its reactivity with an anti-p34^{cdc2} antibody and binding to a p13^{suc1} affinity column (Arion *et al.*, 1988). Purified starfish M-phase-specific kinase has been shown to have MPF activity (Labbe *et al.*, 1989).

c) Regulators of p34-containing complexes

Genetic analysis in *S. pombe* has identified a network of regulatory genes whose products control the mitotic function of *cdc2*$^+$. One of these genes, *cdc25*$^+$, is a positive regulator of *cdc2*$^+$; the timing of mitosis is regulated by the level of *cdc25*$^+$ expression (Russell and Nurse, 1986), which varies through the cell cycle (Moreno *et al.*, 1990). The molecular mechanism by which the *cdc25*$^+$ gene product regulates p34^{cdc2} activity is not yet known. *wee1*$^+$ is a dose-dependent inhibitor of mitosis (Russell and

Nurse, 1987a), and is itself inhibited by *nim1*$^+$ (Russell and Nurse, 1987b). Both *wee1*$^+$ and *nim1*$^+$, like *cdc2*$^+$, share amino acid sequence similarities with serine/threonine protein kinases, suggesting that the induction of mitosis is, at least in part, regulated by a cascade of regulating phosphorylations. Strong evidence for the evolutionary conservation of at least some aspects of this regulatory system has been provided by the identification of homologues in other organisms, including *S. cerevisiae*. Homologues of the *S. pombe cdc25*$^+$ gene have been identified in *S. cerevisiae* as *MIH1* (Russell *et al.*, 1989), in HeLa cells as *CDC25* (Sadhu *et al.*, 1990) and in *Drosophila* as *string* (Edgar and O'Farrell, 1989). In *S. cerevisiae*, *MIH1* is not an essential gene but can abrogate the delay caused by the *S. pombe wee1*$^+$ mitotic inhibitor when the latter gene is expressed in *S. cerevisiae*. Thus, although it has not yet been determined that *S. cerevisiae* contains a homologue of the *S. pombe wee1*$^+$ gene, the responsiveness of the *S. cerevisiae* mitotic initiation machinery to *S. pombe wee1*$^+$, and the identification of a *cdc25*$^+$ homologue in *S. cerevisiae*, strongly suggest that mitotic controls are similar in these two yeasts. Another pair of conserved genes is *S. pombe suc1*$^+$ and *S. cerevisiae CKS1* (Hadwiger *et al.*, 1989a). *CKS1* and *suc1*$^+$ are both essential genes. *CKS1* was identified as a high-copy suppressor of a *cdc28* temperature-sensitive mutation, and the Cks1 protein, like its *S. pombe* counterpart, has been demonstrated to form a complex with p34^{cdc28}. Despite the clear indication that members of this mitotic pathway, first identified by genetic analysis in *S. pombe*, are conserved between divergent species at both the functional and structural level, the exact functions of these proteins are not clear.

d) Cyclins

One component of the complex known as MPF is p34, as described above. The second component is a cyclin (Draetta *et al.*, 1989; Gautier *et al.*, 1990). Cyclins were originally discovered in clam and sea urchin embryos as proteins that accumulate in periodic fashion, reaching a maximum level in early M phase (Evans *et al.* 1983). Cyclins are now known to exist in a wide variety of eukaryotic cells. Both G1 and G2 cyclins have been identified. Most G2 cyclins that have been identified to date belong to either A or B classes, depending on the time at which their accumulation peaks (A cyclins peak slightly earlier than B cyclins), and on their gel mobilities and nucleotide sequences. G1 cyclins have been studied primarily in *S. cerevisiae*. *S. cerevisiae* cyclins are considered separately in section 4 below.

A number of observations led to the conclusion that cyclin accumulation plays a role in mitosis. In early embryos of many species, including clams and sea urchins, the initiation of M phase requires synthesis of new proteins from mRNAs previously stored in the oocyte. Although the newly synthesized protein comprises only a very small proportion of the total protein (Davidson *et al.*, 1986), if protein synthesis is blocked zygotes arrest after DNA replication. Protein synthesis is also required for subsequent cycles, suggesting that regulating factors are destroyed or inactivated after each division.

The periodicity of cyclin accumulation suggests that these are the proteins whose synthesis is necessary for the completion of meiosis and/or induction of mitosis. This hypothesis was confirmed by the finding that when clam or sea urchin mRNAs are injected into frog oocytes (arrested at

the G2/M border of meiosis I), these oocytes are advanced into M phase (Swenson *et al.*, 1986; Pines and Hunt, 1987; Westendorf *et al.*, 1989). Similarly, in cell-free *Xenopus* extracts it has been demonstrated that the translation of cyclin mRNA is sufficient to cause nuclei to complete meiosis (Murray and Kirschner, 1989; Minshull *et al.*, 1989). These results suggest that synthesis of cyclin is the only synthetic requirement for induction of M phase in early frog embryos. In related experiments, Murray *et al.* (1989) demonstrated that a mutant sea urchin cyclin that retains the ability to activate MPF, but which is resistant to proteolysis, arrests cells in mitosis indicating that degradation of cyclin is necessary for exit from mitosis. Recent evidence suggests that degradation is mediated by the ubiquitin-conjugating system (Glotzer *et al.*, 1991) and may be governed by a negative feedback loop (Felix *et al.*, 1990).

The mechanism by which cyclin accumulation leads to MPF activation remains unclear. Mere association of cyclin with $p34^{cdc2}$ is insufficient to generate active MPF (Pines and Hunter, 1989; Pondaven *et al.*, 1990). Other post-translational events appear to be involved since even in the absence of continued protein synthesis after cyclin accumulation there is a lag period before $p34^{cdc2}$ activation occurs (Wagenaar, 1983; Picard *et al.*, 1985; Karsenti *et al.*, 1987). This lag period is also seen *in vitro* when excess cyclin is added to interphase extracts made from frog oocytes (Solomon *et al.*, 1990). Furthermore, cyclin accumulation does not appear to be rate-limiting for M-phase initiation in either somatic cells of *Drosophila* (Lehner and O'Farrell, 1989) or *S. pombe* (Moreno *et al.*, 1989). The current view is that cyclins are required, but are generally not rate-limiting, for the activation of MPF. Once activated, MPF remains active until its cyclin component is

destroyed. In its activated form, MPF brings about the major cellular changes associated with the initiation of mitosis by phosphorylation of appropriate substrates.

4. *S. cerevisiae* cyclins

a) G2 cyclins in *Saccharomyces cerevisiae*

It has only recently been established that *S. cerevisiae* contains cyclin genes that are necessary for the G2/M transition. The identification of these genes by Surana *et al.* (1991) and Ghiara *et al.* (1991) has partially clarified the mitotic role of *CDC28* in *S. cerevisiae*.

A mitotic role for the *CDC28* gene was first suggested by Piggot *et al.* (1982), who identified a temperature-sensitive allele of *CDC28* (*cdc28-1N*) that causes cells to arrest predominantly in the G2 phase of the cell cycle, unlike most other alleles (such as *cdc28-4* and *cdc28-13*) which cause G1 arrest. The phenotype of this mutation suggests that in *S. cerevisiae*, as in *S. pombe*, p34^{cdc28} is required for both the G1/S and G2/M transitions. This hypothesis was supported by the finding that the cloned *S. cerevisiae CDC28* gene fully complements *S. pombe cdc2* mutations, demonstrating that the G2 function of p34^{cdc28} has been conserved (Beach *et al.*, 1982).

Largely ignored until recently, the *cdc28-1N* allele has been exploited by Surana *et al.* (1991) using a genetic approach in which high-copy suppressors of this mutation were identified. In parallel with a strategy employing polymerase-chain-reaction amplification of genomic DNA, this approach has yielded four genes, *CLB1*, *CLB2*, *CLB3* and *CLB4*, that share amino-acid sequence similarity with cyclin B proteins from other organisms, in particular Cdc13 from *S. pombe*. *CLB1*, *CLB2* and *CLB4* in high-copy were

each demonstrated to suppress *cdc28-1N* but not *cdc28-4* temperature-sensitive mutations. Thus suppression by these cyclin genes is allele-specific and limited to suppression of the G2 defects of the *cdc28-1N* form of $p34^{cdc28}$. In an independent study, Ghiara *et al.* (1991) used the polymerase chain reaction to identify cyclin B homologues, one of which they have characterized and named *SCB1*. Comparison of the *SCB1* sequence (Ghiara *et al.*, 1991) with those of the *CLB* genes (Surana *et al.*, 1991) reveals that *SCB1* is the same as *CLB1*. Sequence comparisons between the *CLB* genes demonstrate that *CLB1* and *CLB2* are more closely related to each other than to *CLB3* and *CLB4* (Surana *et al.*, 1991). Levels of *CLB1* and *CLB2* transcripts were found to be periodic, reaching a maximum in late G2 (Surana *et al.*, 1991; Ghiara *et al*, 1991). In the case of *CLB1*, similar periodicity has been demonstrated at the protein level (Ghiara *et al.*, 1991). Neither *CLB1* or *CLB2* is an essential gene, but together they comprise an essential gene pair (Ghiara *et al*, 1991; Surana *et al.*, 1991): cells disrupted for both *CLB1* and *CLB2* germinate but eventually arrest at mitosis similar to *cdc28-1N* mutant cells. Surana *et al.* (1991) propose that a kinase composed of the *CDC28* and *CLB* gene products is required at mitosis, and that the primary defect in *cdc28-1N* mutant cells is the inability of the mutant p34 to interact with the *CLB* gene products. The latter hypothesis is supported by the finding that a *CLB2* gene disruption (which although non-lethal causes mitosis to be delayed) becomes lethal when present in combination with *cdc28-1N*, but not with *cdc28-4*. This suggests that the combination of the *cdc28-1N* mutation, which encodes a p34 protein already enfeebled in its interaction with G2 cyclins, with a *CLB2* gene disruption, results in insufficient levels of p34 - cyclin complex to initiate mitosis.

Biochemical evidence for the association of p34^{cdc28} and *SCB1* is provided by Ghiara *et al.* (1991), who demonstrate that H1-kinase activity is precipitated by anti-*SCB1* antibodies, and that kinase activity is dependent upon cell-cycle position (reaching a maximum in the budded interval), level of *CLB1* expression and p34^{cdc28}. Surana *et al.* (1991) find that H1 kinase activity bound by p13^{suc1} sepharose beads is greatly reduced in cells bearing a disrupted *CLB2* gene, consistent with Clb2 also comprising part of the H1 kinase.

Ghiara *et al.* (1991) show that an N-terminally truncated form of *CLB1* arrests cells at mitosis, presumably due to the cell's inability to degrade this mutant cyclin causing permanent activation of the mitotic form of the p34^{CDC28} kinase. This result is consistent with similar experiments in embryonic lysates of *Xenopus* (Murray *et al*, 1989). Thus, as is the case in other systems, degradation of G2 cyclins is necessary for exit from M-phase in *S. cerevisiae.*

b) A family of G1 cyclins in *S. cerevisiae*

In addition to a cyclin requirement for G2-to-M transition described above, recent studies, primarily in *S. cerevisiae,* have revealed a cyclin requirement for G1-to-S phase progression. This discovery stems from earlier studies in which a non-conditional dominant mutation, *WHI1-1,* was found to cause cells to initiate a new cell cycle at an unusually small cell size (Carter and Sudbury, 1980; Sudbury *et al.*, 1980). The *WHI1* gene was eventually cloned by two groups. Nash *et al.* (1988) determined that the *WHI1-1* mutation was tightly linked to *CDC24* on chromosome I. Transcription units surrounding *CDC24* had been previously mapped as

FUN genes (*F*unction *U*nknown *N*ow), one of which (*FUN10*) is now known to encode the *WHI1* gene. At the same time, Cross (1988) identified a dominant mutation, *DAF1-1*, that caused cells to become α-factor resistant. In addition to α-factor resistance, *DAF1-1* mutant cells were also defective in size control, suggesting that *DAF1-1* and *WHI1-1* were allelic. The *DAF1-1* mutant gene was cloned from a library of genomic DNA constructed from a *DAF1* mutant strain (Cross, 1988). The *DAF1* gene was also subsequently found to be within *FUN10*. Interestingly, deletion of the *WHI1* gene was found to have an effect opposite to either the *WHI1-1* or *DAF1-1* mutations: cells containing a disrupted *WHI1* gene exhibited an increase in cell volume and in the length of G1 (Nash *et al.*, 1988). Cells containing two copies of the *WHI1* gene behaved like *WHI1-1* mutant cells and grew at a small cell size and with a decreased G1 period. Cells with *WHI1-1* in increased copy number had a very short G1 period (Nash *et al.*, 1988). These results argue that *WHI1* is an activator of Start, although the viability of strains deleted for the *WHI1* gene indicate that other activators function in the absence of *WHI1*. The *WHI1-1* mutation is thought to encode a hyperactive Whi1 protein since cells bearing this mutation are advanced into the cell cycle at decreased cell size.

The finding that *WHI1* is not an essential gene was surprising, considering other evidence that indicated that the *WHI1* protein exerts rate-limiting control over the G1-to-S phase transition. When the wild-type *WHI1* gene was sequenced (Nash *et al.*, 1988) it was found to have limited amino-acid similarity with three cyclins: sea urchin cyclin (Pines and Hunt, 1987), clam cyclin A (Swenson *et al.*, 1986) and *S. pombe cdc13*$^{+}$ (Booher and Beach, 1988). It is now clear that *WHI1* is one of a family of related G1

cyclins in *S. cerevisiae*. Two other members of this family, *CLN1* and *CLN2*, have been detected as high-copy suppressors of temperature-sensitive mutations in the *CDC28* gene (Hadwiger *et al.*, 1989b). The predicted Cln1 and Cln2 proteins share amino-acid sequence similarity with each other and with a number of cyclins including clam cyclin A and, to a lesser extent, the *WHI1/DAF1* gene product, sea urchin cyclin B and *S. pombe cdc13*$^{+}$. This similarity is most evident in a region known as the "cyclin box". Disruption of either *CLN1* or *CLN2* is not a lethal event; even disruption of both *CLN1* and *CLN2* in a haploid cell leads only to a decrease in growth rate and aberrantly shaped cells. However, cells containing deletion of *CLN1*, *CLN2* and *DAF1/WHI1* are inviable, indicating that these three genes comprise a family of genes, any one of which can supply the function that is essential in *S. cerevisiae* (Richardson *et al.*, 1989). Placing any one of these three genes under the control of a regulated promoter in cells bearing *CLN1*, *CLN2* and *DAF1/WHI1* disruptions causes conditional G1 arrest, indicating that at least one member of this gene family is required for cell-cycle progression (Richardson *et al.*, 1989; Cross, 1990). On the basis of the relationship between these three genes, *WHI1/DAF1* has been renamed *CLN3* (Richardson *et al.*, 1989). *The DAF1-1* mutation has been renamed *CLN3-2* (Cross, 1990), and the *WHI1-1* mutation is known as *CLN3-1*.

During subcloning of the *CLN2* gene, an allele missing the carboxyl-terminal third of the coding region was produced. This truncated allele retains the ability to rescue *cdc28* temperature-sensitive mutations, but when introduced into wild-type cells causes a small-size phenotype similar to that seen in *CLN3-1* and *CLN3-2* mutant cells. Cells containing this dominant mutation, referred to as *CLN2-1*, spend little time in G1, are

moderately resistant to α-factor and do not undergo G1 arrest in response to nutrient starvation. The phenotype of the *CLN2-1* mutation suggests, as does the phenotype of *CLN3-1* and *CLN3-2*, that the yeast G1 cyclins are rate-limiting regulators of the cell cycle, and that these mutant genes encode hyperactive/hyperstable cyclin proteins that relieve normally rate-limiting constraints on cell-cycle progression, causing cells to become unresponsive to circumstances that would normally arrest cells in G1 (Nash *et al.*, 1988; Hadwiger *et al.*, 1989b). Cln2 protein has a half-life of less than 15 min when its synthesis from a regulated promoter is halted (Wittenberg *et al.*, 1990). Similar instability of the Cln1 polypeptide is suggested by the finding that cells arrest within one cell cycle when the transcription of *CLN1* under the control of a regulated promoter is turned off in cells that have no other intact G1 cyclin gene (Richardson *et al.*, 1989). Similar experiments suggest that Cln3 is likewise unstable (Cross, 1990). Both the *CLN3-1* and *CLN3-2* mutant genes contain stop codons that are also likely to result in the truncation of the carboxyl-terminal third of the Cln3 protein (Nash *et al.*, 1988; Cross, 1988). The carboxyl-terminal regions of Cln1, Cln2 and Cln3 contain what have been termed PEST sequences: sequences rich in proline, glutamate, threonine, serine and aspartate implicated in the targeting of certain proteins for degradation (Rogers *et al.*, 1986). PEST sequences were previously noted in clam cyclin A (Swenson *et al*, 1986). It is hypothesized that the removal of these PEST sequences results in the generation of a stabilized cyclin protein which, because of its increased stability, facilitates premature cell-cycle progression (Nash *et al.*, 1988; Hadwiger *et al.*, 1989b).

Extrapolation from the situation with mitotic cyclins predicts that G1 cyclins form a complex with $p34^{cdc28}$ (Hadwiger *et al.*, 1989b; Richardson

et al., 1989). Experimental support for an interaction between Cln2 and p34^{cdc28} comes from the observation that anti-Cln2 antibodies coprecipitate p34^{cdc28}, as measured by anti-p34^{cdc28} antibodies and H1-kinase activity (Wittenberg *et al.*, 1990). Additional support comes from the suppression of *cdc28* mutations by over-expression of *CLN* genes (Hadwiger *et al.*, 1989b); genetic interactions of this kind are often due to compensatory changes in interacting proteins.

c) Regulation of G1 cyclins in *S. cerevisiae*

The abundance of cyclins in oocytes and eggs of marine invertebrates is regulated primarily at the post-transcriptional level; stability varies greatly through the cell cycle (Evans *et al.*, 1983; Swenson *et al*, 1986; Westendorf *et al.*, 1989). Like cyclins in these invertebrate systems, the Cln2 protein of *S. cerevisiae* also oscillates in a cell-cycle dependent fashion: Cln2 protein has been shown to accumulate during late G1 and to disappear shortly thereafter. However, unlike the situation with marine invertebrate oocytes and eggs cyclins, similar oscillation of *CLN2* transcript levels was observed, suggesting that the rise and fall in Cln2 protein levels reflect underlying transcriptional regulation of the *CLN2* gene (Wittenberg *et al.*, 1990). *CLN1* transcript levels have also been found to oscillate during the cell cycle. In contrast, *CLN3* transcript levels vary little with cell-cycle position, suggesting that *CLN3* regulation occurs at a post-transcriptional level (Nash *et al.*, 1988; Wittenberg *et al.*, 1990).

Cells bearing *CLN2-1*, *CLN3-1* or *CLN3-2* mutations are impaired in many forms of G1 regulation, including arrest due to exposure to α-factor and nutrient starvation. Analysis of *CLN1*, *CLN2* and *CLN3* mRNA levels

demonstrates that *CLN1* and *CLN2* mRNA levels decrease following exposure to α-factor, suggesting that α-factor-mediated G1 arrest is accomplished by decreasing cyclin levels, an effect that can be abrogated by increased cyclin stability. Surprisingly, *CLN3* mRNA levels do not decrease, but actually exhibit a moderate increase upon α-factor treatment, indicating that if decreased levels of Cln3 are required for α-factor arrest, as predicted by the failure of cells bearing hyperactive *CLN3* alleles to arrest, then Cln3 must be regulated at a post-transcriptional level (Nash *et al.*, 1988; Wittenberg *et al.*, 1990). Recent evidence suggests that the abundance of each cyclin is regulated by a separate modulatory pathway, the combined actions of which result in G1 arrest of cells following α-factor exposure (Chang and Herskowitz, 1990). One component of this putative regulatory network is the recently identified gene *FAR1*, which appears to be responsible for down-regulation of *CLN2* expression following exposure of cells to α-factor (Chang and Herskowitz, 1990). Cells containing a null mutation in *FAR1* fail to arrest in response to α-factor treatment, although other features of the response, including transcriptional induction of genes such as *FUS1* (Trueheart *et al.*, 1987) and schmooing (a morphological change characteristic of cells responding to α-factor) are still observed. In cells deleted for the *CLN2* gene, a *FAR1* null mutation is suppressed such that cells arrest like wild-type cells in response to α-factor exposure, indicating that the only function of the *FAR1* gene in α-factor-mediated G1 arrest is to down-regulate *CLN2*. As predicted by this hypothesis, *CLN2* transcript levels do not decrease in response to α-factor treatment in *far1* mutant strains (Chang and Herskowitz, 1990).

A likely candidate for a regulator of Cln3 activity is the product of the *FUS3* gene (Elion *et al.*, 1990). *fus3* mutants, like *far1* mutants, fail to G1 arrest in response to α-factor treatment. Deletion of the *CLN3* gene restores α-factor-mediated arrest, indicating that at least one function of the *FUS3* gene is to lower effective Cln3 levels. The *FUS3* sequence suggests that the Fus3 protein may have protein kinase activity, and it has therefore been proposed that Fus3 inactivates Cln3 by phosphorylation. The mechanism by which *CLN1* mRNA levels are down-regulated is unclear.

Several groups have recently demonstrated that the regulation of cyclin gene expression is more complex than in the model proposed by Chang and Herskowitz (1990) which suggests that each cyclin is independently inactivated after α-factor treatment of cells. Dirick and Nasmyth (1991) and Cross and Tinkelenberg (1991) have demonstrated that cells containing *CLN3* hyperactive mutations (which are resistant to α-factor-mediated G1 arrest) fail to down-regulate *CLN1* and *CLN2* mRNA levels in response to treatment with α-factor, inconsistent with independent regulation of each cyclin. Furthermore, the persistence of *CLN2* transcripts under these circumstances actually contributes to the ability of cells containing hyperactive *CLN3* mutations to override G1 arrest, since *CLN3-1* mutant cells bearing a disrupted *CLN2* gene are considerably more α-factor sensitive than cells containing an intact *CLN2* gene (Dirick and Nasmyth, 1991). Cells containing hyperactive *CLN2* mutations (which cause cells to be partially α-factor resistant) also fail to down-regulate *CLN2* mRNA levels upon treatment with α-factor (Cross and Tinkelenberg, 1991). Thus, increased stability of either Cln2 or Cln3 results in persistent G1-cyclin expression following treatment with α-factor.

To explain these findings both Dirick and Nasmyth (1991) and Cross and Tinkelenberg (1991) propose the existence of a positive feedback loop in which G1-cyclin proteins positively regulate the expression of G1-cyclin genes. The mechanism by which the G1 cyclins exert positive regulation over their own mRNA accumulation is unclear, but is dependent upon Cdc28 activity: Dirick and Nasmyth (1991) demonstrate that *CLN1* and *CLN2* mRNA accumulation after release from either α-factor or stationary-phase arrest, or after synchronization by elutriation, is prevented in *cdc28-4* mutant cells at a restrictive temperature. Dirick and Nasmyth (1991) and Cross and Tinkelenberg (1991) speculate that positive feedback regulation might be accomplished by phosphorylation of *CLN1* and *CLN2* transcription factors by a complex containing $p34^{cdc28}$. Candidate transcription factors for such regulation include Swi4 and Swi6, first identified as factors required for Start-dependent activation of the *HO* endonuclease which controls mating-type switching (Stern *et al.*, 1984; Breeden and Nasmyth, 1987). The Swi4 and Swi6 transcription factors have now been shown to be required for the activity of all three G1 cyclins, an activity that accounts for the inviability of cells bearing disrupted *SWI4* and *SWI6* genes (Nasmyth and Dirick [in press] cited in North [1991]).

What is the physiological significance of this feedback loop? Cross and Trinkelenberg (1991) point out that the existence of such a feedback loop is consistent with the very steep rise in Cln2 protein that is observed at the G1/S boundary when synchronized wild-type cells are allowed to resume proliferation (Wittenberg *et al.*, 1990). Positive feedback resulting in such a sharp accumulation of cyclins might ensure that disparate cellular activities sensitive to cyclin accumulation are activated in a concerted fashion. An

analogous feedback loop has been proposed to account for the concerted activation of mitosis in the embryonic cell cycle (Solomon *et al.*, 1990). Other results raise questions concerning the importance of the *S. cerevisiae* feedback mechanism; Dirick and Nasmyth point out that *cln1 cln2 cln3* triple mutant cells bearing a *CLN2* gene under the control of a constitutive promoter can still proliferate, indicating either that Cln2 cell-cycle regulation is not absolutely required, or that Cln2, like Cln3, can be regulated at a post-transcriptional level.

5. Objectives

The objective of this study is to characterize, at the molecular level, the *CDC68* gene of *Saccharomyces cerevisiae*. The *CDC68* gene was first identified by a conditional mutation that affects cell proliferation: under permissive conditions *cdc68-1* mutant cells are unimpaired in cell proliferation, but upon transfer to a restrictive temperature, arrest within one cell cycle with an unbudded cell morphology. Previous studies in this laboratory have indicated that cell-cycle arrest caused by the *cdc68-1* mutation occurs at the same point in the cell cycle as that caused by the mating pheromone α-factor. This result indicates that the *cdc68-1* mutation causes conditional arrest at Start (Prendergast *et al.*, 1990). These same studies establish that *cdc68-1* mutant cells remain mating-competent and incorporate significant levels of precursors into RNA and protein under restrictive conditions. Thus *cdc68-1* mutant cells can be considered Class I Start mutants. Mutants of this type are thought to affect Start in a fairly direct manner.

As a first step to understanding the function of the Cdc68 protein the *CDC68* gene was cloned and sequenced. During the course of these studies, the *CDC68* gene was independently identified using a genetic approach designed to identify genes involved in transcription (Malone *et al.*, submitted). The relationship between structure and function of the *CDC68* gene in both cell-cycle regulation and transcription is been explored in this study. As discussed above, significant advances in the cell-cycle field made during the course of this study have identified components of the cell-cycle machinery. This recent progress in our understanding of the cell cycle allows analysis of how mutations in a gene involved in gene expression can affect cell proliferation. By determining the effects of *cdc68* mutations on the expression of those components of the cell-cycle apparatus that are rate-limiting for cell-cycle progression the molecular basis of cell-cycle arrest in *cdc68-1* mutant cells has been defined.

II. METHODS AND MATERIALS

1. Strains and plasmids

Yeast strains used in this study are listed in Table 1. Those used exclusively in mapping the *cdc68-1* mutation are listed separately in Table 2. *Escherichia coli* strains are listed in Table 3. Genetic nomenclature in *Saccharomyces cerevisiae* is as described by Sherman (1981). No convention for nomenclature of yeast proteins has yet been established: in this study protein names are written in ordinary text with only the first letter capitalized (*e.g.* Cdc68).

Cloning vectors YIp5, YEp24 and YEp352 have been previously described (Struhl, 1979; Botstein *et al.*, 1979; Hill *et al.*, 1986). Plasmid pUTX144 (kindly provided by D. Finkelstein) is a 2μ-based 16.2-kbp plasmid containing an in-frame *HSP82-lacZ* fusion gene and a *LEU2* gene for selection in *S. cerevisiae*. The *HSP82-lacZ* fusion gene of pUTX144 was constructed by the ligation *HSP82* sequences -334 to +282 (a *Hin*dIII-*Rsa*I fragment) (Farrelly and Finkelstein, 1984) to the *Sma*I site of a 6.2-kbp *Sma*I-*Sal*I fragment containing the *E. coli lacZ*, *lacY*, and *lacA* genes, isolated from plasmid pMC1403 (Casabadan *et al.*, 1980; Finkelstein and Strausberg, 1983; D. Finkelstein, personal communication).

Table 1. Yeast strains used in this study.

STRAIN	GENOTYPE[1]	SOURCE
21R	*MAT***a** *ura3-52 leu2-3,112 ade1*	lab coll'n[2]
Sc225	*MAT*α *ura3-52 leu2-3,112 ade1*	lab coll'n[3]
68507A	*MAT*α *cdc68-1 ura3-52 ade1* and/or *ade2*	C. B.[4]
ART68-1	*MAT***a** *cdc68-1 ura3-52 leu2-3,112 ade1* and/or *ade2*	C. B.[4]
L577	*MAT*α *spt16-197 his4-912*δ *lys2-128*δ *ura3-52*	E. A. M./F. W.[5]
FY56	*MAT*α *his4-912*δ *lys2-128*δ *ura3-52*	E. A. M./F. W.
BM64	*MAT***a**/*MAT*α *spt16-101::LEU2/SPT16 his4-912*δ*/his4-912*δ *lys2-128*δ */lys2-128*δ	E. A. M./F. W.
GCY24	*MAT***a** *CLN2-1 ade1 his3 leu2-3,112 trp1-1 ura3*	C. W.[6]
CLN-5A	*cdc68-1 ura3-52 ade1* and/or *ade2*	GCY24 x 68507A
CLN-5C	*cdc68-1 CLN2-1 ura3-52 leu2-3,112 ade1* and/or *ade2*	GCY24 x 68507A
JHY629	*MAT***a** *ade1 his3 leu2-3,112 trp1-1 ura3 cln1::URA3*	C. W.

[1]Square brackets indicate originally plasmid-borne sequences integrated, in single copy, into the chromosome. The symbol :: following the name of a gene indicates a disrupted copy of that gene, and is immediately followed by the designation of the sequences used to generate this disruption (for example, *cln1::URA3*, indicates that the *CLN1* gene has been disrupted by replacement with, or insertion of, *URA3* sequences).

[2]Laboratory collection, original source: J. E. Hopper (Johnston and Hopper, 1982)

[3]Original source: D. Botstein

[4]C. Barnes: Generated by backcrossing original *cdc68-1* isolate 3 times with GR2 then twice with 21R to achieve a transformable genetic background.

[5]E. A. Malone/F. Winston (Malone *et al.*, submitted)

[6]C. Wittenberg

Table 1 (continued).

BF338-2a whi1::URA3	*MAT*α *ade1 ura3 his3 cln3*::URA3	A. B. F.[7]
ZWU90-H2	*MAT***a** *ura3-52*::[*HSP90-lacZYA URA3*] *leu2-3,112 ade1*	C. B.[8]
ZWU90-C2	*MAT***a** *ura3-52*::[*HSC90-lacZYA URA3*] *leu2-3,112 ade1*	C. B.[9]
FP90-68	*cdc68-1 ura3-52*::[*HSP90-lacZYA URA3*] *leu2-3,112 ade1* and/or *ade2*	ZWU90-H2 X 68507A
FP90	*ura3-52*::[*HSP90-lacZYA URA3*] *leu2-3,112 ade1* and/or *ade2*	
FC90-68	*cdc68-1 ura3-52*::[*HSC90-lacZYA URA3*] *leu2-3,112 ade1* and/or *ade2*	ZWU90-C2 X68507A
FC90	*ura3-52*::[*HSC90-lacZYA URA3*] *leu2-3,112 ade1* and/or *ade2*	
PLD82α	*MAT*α can^r Ade$^-$ His$^-$ Trp$^-$ Ura$^-$ *hsp82*::*LEU2*	S. L.[10]
CLD82α	*MAT*α can^r Ade$^-$ His$^-$ Trp$^-$ Ura$^-$ *hsc82*::*LEU2*	S. L.

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[7]A. B. Futcher

[8]C. Barnes: Constructed by the directed integration of plasmid YIp144 into the SmaI site of the *ura3-52* locus in strain 21R. YIp144 is YIp5 containing a 6.8-kbp *Hin*dIII-*Sal*I *HSP82-lacZYA* fusion gene fragment in which sequences -334 to +282 of the *HSP82* gene (Farrelly and Finkelstein, 1984) are fused to codon 8 of *lacZ*. The fusion gene fragment was obtained from plasmid pUTX144 kindly provided by D. Finkelstein (see text).

[9]C. Barnes: Constructed by the directed integration of YIp232 as above. YIp232 is YIp5 containing a 7.4-kbp *Eco*RI-SalI *HSC82-lacZYA* fusion gene fragment in which sequences -600 (approximately) to +600 of the *HSC82* gene (Borkovich *et al.*, 1989) are fused in frame to *lacZ*. The fusion gene fragment was obtained from plasmid pUTX232 kindly provided by D. Finkelstein.

[10]S. Lindquist: Complete genotype of strains unavailable

Table 2. Yeast strains used in mapping the *cdc68-1* mutation.

STRAIN	GENOTYPE	SOURCE
19:3:4	*MAT***a** *mes1-1 ade1 his5 leu2 lys11 gal1 gal2*	lab coll'n[1]
GRX2-4B	*MAT*α *ade2 ade5 aro2 lys5 trp5 his6* and/or *his7 met13 cyh2*	lab coll'n
STX147-9B	*MAT***a** *ade5 arg4 his7 lys1 lys5 met13 trp1 trp5 tyr1 ura4 aro2 rad56 gal1 gal2 cyh2 cly8*	lab coll'n
S25k-13	*MAT*α *ade1 leu2-3,112 kex1::LEU2 ura3-52*	H. B.[2]
DP26A-1	*MAT*α *cdc68-1 leu2 rad52*	lab coll'n[3]
ADC26A1-1	*MAT***a** *cdc68-1 ade2 ade his5* and/or *his6*	lab coll'n[4]
ADC26A5-1	*MAT*α *cdc68-1 ade2 ura1*	lab coll'n
ARM7-2	*MAT***a** *cdc68-1 cyh2 ade5 ade2 his5* and/or *his6* and/or *his7*	ADC26A1-1 x GRX2-4B
ARM7-6	*MAT***a** *cdc68-1 ade5 cyh2 leu2-3,112 his5* and/or *his6* and/or *his7*	ARM7-2 x DP26A-1
A121-3A	*MAT***a** *ade5 pet8 lys1 leu2-3 his7 met14 ura3*	lab coll'n[5]
A121-3D	*MAT*α *ade5 pet8 lys1 leu2-3 his7 met14 ura3*	lab coll'n[5]

[1] Laboratory collection. Original source: Yeast Genetic Stock Centre
[2] H. Bussey (Dmochowska *et al.*, 1987)
[3] Constructed by D. Patel, this laboratory
[4] Constucted by D. Carruthers, this laboratory
[5] Original source: M. Culbertson

Table 3. *E. coli* strains used in this study

STRAIN	GENOTYPE	SOURCE
RR1	F⁻ *proA leuB6 thi lacY galK2, xyl5 mtl1 ara14 rpsL20 supE44 hsdS* λ^- *Str*^{r} $r_K^- m_K^-$	J. E. H.[1]
DH5α	F⁻ ϕ80d *lacZ*ΔM15 Δ(*lacZYA-argF*) U169 *rec*A1 *end*A1 *hsd*R17($r_K^- m_K^+$) *sup*E44 λ^- *thi*-1 *gyr*A96 *rel*A1	BRL[2]
DH5αF'	F' ϕ80d *lacZ*ΔM15 Δ(*lacZYA-argF*) U169 *rec*A1 *end*A1 *hsd*R17($r_K^- m_K^+$) *sup*E44 λ^- *thi*-1 *gyr*A *rel*A1	BRL[2]

[1] J. E. Hopper

[2] Bethesda Research Laboratories; derived from strain DH5 (Hanahan, 1985). The *lacZ*ΔM15 mutation (Gronenborn and Messing, 1978) allows identification of recombinants by screening for α-complementation (Ullman *et al.*, 1967) by pUC and M13 vectors bearing appropriate *lac* sequences. DH5αF' contains a stable F episome that allows infection by M13 phage but does not require selective conditions for F' maintenance.

2. Chemicals, enzymes and materials

All chemicals were purchased from Sigma Chemical Co., St Louis, MO, unless otherwise stated. Bacto-agar, Bacto-peptone, Bacto-yeast extract and Bacto-yeast nitrogen base were purchased from Difco Laboratories, Detroit, MI. The sources of enzymes and specialized reagents or equipment are given with the description of their use.

3. Media and growth conditions

a) Yeast growth media

YM1 rich medium (Hartwell, 1967) contained (per litre) 10 g succinic acid, 6 g sodium hydroxide, 10 g Bacto-peptone, 5 g Bacto-yeast extract, 6.7 g Bacto-yeast nitrogen base without amino acids and 20 g glucose. YEPD solid medium (Hartwell, 1967) contained (per litre) 20 g Bacto-peptone, 10 g Bacto-yeast extract, 20 g glucose and 20 g agar. The defined synthetic medium YNB (Johnston *et al.*, 1977) contained (per litre) 6.7 g Bacto-yeast nitrogen base without amino acids or ammonium sulphate, 10g succinic acid, 1 g ammonium sulphate, 6 g sodium hydroxide and 20 g glucose. YNB was routinely supplemented with adenine and uracil (20 µg/ml) in addition to tryptophan, tyrosine, lysine, arginine, histidine and leucine (40 µg/ml) to make YNB+10 medium (Barnes, 1989). Uracil or leucine supplements were omitted from YNB+10 to make YNB+9 media used for strains containing plasmids with *URA3* or *LEU2* selectable markers. The solid synthetic complete medium SC consisted of YNB with the following additions (per litre): 20 g Bacto-agar; 40 mg arginine, aspartic acid, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, serine, tryptophan, threonine, tyrosine and valine; 20 mg adenine and uracil. Drop-out media, used to

score auxotrophic requirements and to select transformants, consisted of SC medium lacking one of the supplements. YEPD plates spread with 0.2 ml of 0.75 mg/ml cycloheximide were used to score the *cyh2* mutation, which confers resistance to the inhibitor cycloheximide. Liquid sporulation medium (SPO) contained (per litre) 10 g potassium acetate and 1 g glucose. Regeneration agar used for plating spheroplasts after transformation consisted of (per litre): 6.7 g Bacto-yeast nitrogen base without amino acids, 110 g sorbitol, 20 g glucose, 20 g Bacto-agar, 2% YEPD (Hinnen *et al.*, 1978).

b) Bacterial growth media

E. coli strains were grown in 2X YT medium (Messing, 1983) which consisted of (per litre) 16 g Bacto-tryptone, 10 g Bacto-yeast extract and 5 g NaCl. Bacto-agar was added to make solid medium (20 g/l) and top agar (6 g/l). Ampicillin, for selection of plasmids bearing a resistance-conferring *bla* gene, was added to 40 μg/ml to both liquid and solid media after autoclaving.

c) Growth and storage conditions

S. cerevisiae and *E. coli* liquid cultures were routinely grown either in flasks on a gyratory shaker (New Brunswick Scientific Co., New Brunswick, NJ) or in test tubes on a roller drum (New Brunswick Scientific Co.) in temperature-controlled environmental rooms. Unless otherwise stated, yeast cultures were incubated at 23°C, the permissive temperature for all temperature-sensitive mutants used in these studies. *E. coli* cultures were grown at 37°C.

Stationary-phase yeast cultures were routinely stored on plates at 4°C for up to six months. *E. coli* cultures were similarly stored but for not longer than three weeks. For long-term storage, stationary-phase liquid cultures of both bacteria and yeast were frozen at -70°C following addition of glycerol to 25%.

4. Yeast culture manipulations

a) Determination of cell concentration

0.5-ml culture samples were fixed by the addition of 4.5 ml of 3:7 formalin:Diluton (BDH Chemicals, Toronto, Ontario) solution. Prior to counting, cells were sonicated for 5 s at 50% power with a Microson sonicator (Heat Systems Ultrasonics Inc., Farmingdale, NY) to break up clumps of cells. Finally, cells were diluted in Diluton and counted on a model ZM Coulter Particle Counter (Coulter Electronics, Mississauga, Ontario).

b) Determination of cell volume

The size distribution of cells from log-phase cultures was obtained using a Coulter Channelizer (Coulter Electronics). Cells were sonicated but not fixed prior to analysis. Median sizes were determined after calibration with latex bead standards (Coulter Electronics).

c) Determination of budding index

For *S. cerevisiae* bud morphology provides a convenient estimate of cell-cycle position; cells within the G1 interval are generally unbudded, while those in S, G2 and M are budded (Hartwell, 1974). The percentage of

cells in a population with an unbudded morphology was determined by scoring at least 200 fixed and sonicated cells using a phase-contrast microscope (Carl Zeiss, Oberkochen, West Germany).

5. Yeast genetic analysis

a) Construction of diploid cells

Haploid cells of opposite mating type were mixed on YEPD and incubated at 23°C for 24 h. Cells were then transferred to selective solid medium and incubated under selective conditions until single colonies of diploid cells formed.

b) Tetrad analysis

Tetrad analysis was performed to determine linkage relationships between genetic markers of interest, and in the construction of strains bearing required combinations of markers.

Diploid cells from a logarithmic-phase culture, derived from a single colony, were induced to sporulate by transfer to SPO medium after washing once in SPO to remove residual growth medium. After 4-7 days incubation at 23°C, asci walls were digested by treatment of cells from 1 ml sporulated culture, resuspended in an equal volume of PBS (containing per litre; 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4 and 0.24 g KH_2PO_4, adjusted to pH 7.4 with concentrated HCl), with 70 µl β-glucuronidase for approximately 45 min at 23°C. β-glucuronidase was removed by washing the cells twice with PBS before resuspending the mixture in 2 ml PBS and spreading a portion on an agar slab.

The four meiotic spore products from individual asci were separated and arranged on the agar slab using a Singer Mk. III Micromanipulator (Singer Instrument Co., Ltd., Watchet, England) and a phase-contrast microscope (Carl Zeiss). The agar slab was incubated at 23°C until the spores gave rise to colonies, which were then transferred to YEPD in an ordered arrangement and grown up a second time. Mutations were scored by replica-plating cells to suitable media incubated under appropriate conditions.

The distance in centiMorgans (cM) between two markers was determined using the equation of Perkins (1949);

$$X_p = [(6NPD + TT) \times 100] / 2 (PD + NPD + TT)$$

The above equation is appropriate only for map distances of less than 35 cM. However, larger values may be adjusted using the equation devised by Ma and Mortimer (1983);

$$X_e = (80.7 X_p - 0.833 X_p^2) / (83.3 - X_p)$$

Map distances of up to 100 cM are considered accurate when adjusted using this formula.

c) Complementation analysis

Recessive mutations in some cases could not be scored directly due to the presence of a second mutation with the same phenotype. Such mutations were scored by replica-plating haploid cells on YEPD and mating with tester strains bearing previously characterized mutations in the genes concerned. Mating each of the strains to be tested with each of the tester strains was accomplished by "cross-stamping" the tester strains using wooden tongue depressors. Plates were incubated for 24 h to allow diploid formation before replica-plating to selective media. Growth of the resultant

diploid cells indicated that the mutation under test was in a different complementation group to that of the tester strain.

6. Analysis of yeast proteins

a) Pulse-labeling with [^{35}S]methionine

Yeast cultures were grown overnight to a density of 2-4 x 10^6 cells/ml before a portion of the culture was transferred to 37°C and incubated further. 1-ml samples were removed at appropriate intervals and pulse-labeled for 10 min with 10 µCi [^{35}S]methionine (NEN Research Products, Boston, MA) in test-tubes equilibriated at either 23°C (time 0) or 37°C, as described by McAlister *et al.* (1979). Labelling was terminated by the addition of 1 ml of 40 mM sodium azide, 400 µg/ml cycloheximide solution and placing the sample on ice. After 5 min on ice the cells were pelleted and washed with 2 mM sodium azide. Cell pellets were stored at -20°C until required.

b) Sample preparation

Samples were prepared as described by McAlister *et al.* (1979). Briefly, cell pellets were thawed on ice in 200 µl SDS sample buffer (Laemmli, 1970: 62.5 mM Tris-HCl [pH 6.8], 2% SDS, 10% glycerol, 5% mercaptoethanol, 0.001% bromophenol blue). Cells were broken by 4 x 30 s cycles of vortexing after the addition of an equal volume of glass beads and 10 µl phenylmethyl-sulphonyl fluoride (10 mM solution in isopropanol). A further 200 µl of SDS sample buffer was added and the liquid was transferred to a micro-centrifuge tube which was then centrifuged at high speed to remove cellular debris, transferred to a further microcentrifuge tube and boiled for 3 min. Samples were stored at -20°C if not immediately required.

c) Determination of radioactivity incorporated into proteins

The amount of [^{35}S]methionine incorporated into protein was determined by trichloroacetic acid (TCA) precipitation as follows: 5 ml of chilled 5% TCA containing a 10-fold excess of unlabelled methionine was added to 10 µl of cell extract mixed with 10 µg of bovine serum albumin (BSA). Proteins were collected, after a 30 min incubation on ice, by filtration though a GF/C glass microfibre filter (Whatman International Ltd., Maidstone, England). The filter was washed 4 times with 5-ml volumes of chilled 5% TCA and twice with chilled 95% ethanol. Filters were air-dried and counted by liquid scintillation in 5 ml Omnifluor (NEN) toluene-based scintillation cocktail to determine incorporated radioactivity.

d) One dimensional polyacrylamide gel electrophoresis (1D SDS-PAGE)

Proteins were resolved by 1D SDS-PAGE in 8% gels as described (Laemmli, 1970) using a Protean dual slab cell (Bio-Rad, Richmond, CA). Equal amounts of TCA-precipitable radioactivity were loaded in each lane. Molecular weight standards were either from an Electrophoresis Calibration Kit containing proteins of 14,400-94,000 molecular weight (Pharmacia Inc., Piscataway, NJ), or from a Kit for Molecular Weights 30,000-200,000 (Sigma Chemical Co.), or Low Range SDS-PAGE Standards (M_r 97,400-14,400) (Bio-Rad). Gels were dried on a model 224 gel slab dryer (Bio-Rad) and exposed to Kodak X-OMAT AR film (Eastman Kodak Company, Rochester, NY).

7. Isolation of plasmid DNA

a) Isolation of plasmid DNA from *E. coli*

Small-scale preparations of plasmid DNA were performed using the rapid-boil procedure of Holmes and Quigley (1981).

Large-scale plasmid preparations were performed using a procedure similar to that of Birnboim and Doly (1979), as modified by Dr. Jason Hofman (Department of Biology, Dalhousie University). Cells from a 50-ml overnight *E. coli* culture were pelleted (5 min, 10,000 rpm) and resuspended in 25 ml TES (50 mM Tris-HCl [pH 8.0], 10 mM EDTA, 50 mM NaCl). The cells were then repelleted and suspended in 1.0 ml TEG (25 mM Tris-HCl [pH 8.0], 50 mM EDTA, 1% glucose). After the addition of 2.0 ml of freshly prepared 0.2 N NaOH/1% SDS, the mixture was shaken vigorously and placed on ice for 10 min before the further addition of 1.5 ml potassium acetate (pH 4.8). The mixture was then shaken until a granular precipitate formed. Following a further 5-min incubation on ice the precipitate was pelleted (10,000 rpm/10 min) and the supernatant was transferred to a fresh tube. Nucleic acids were then precipitated by the addition of 2 volumes of 95% ethanol and, after 10 min on ice and 5 min centrifugation at 10,000 rpm, the pellet was dissolved in 2.0 ml TE buffer (10 mM Tris-HCl, 1 mM EDTA). Large RNA molecules were removed by the addition of 1.0 ml 7.5 M ammonium acetate followed by at least 30 min of incubation on ice. After pelleting the RNA-containing precipitate (10 min/10,000 rpm), the supernatant was transferred to a fresh tube and remaining nucleic acids were precipitated by the addition of 2 volumes of ethanol as before. The resulting pellet, containing plasmid DNA, was vacuum dried and dissolved in 2.0 ml TE.

Residual RNA contamination was eliminated as follows: NaCl was added to 50 mM (25 µl of 5 M) followed by 2 µl RNAse T1 (100 U/µl) and 10 µl RNAse A (10 µg/µl). After a 15-min incubation at 55°C, a further 300 µl of 4 M NaCl was added and the mixture was cooled to room temperature. The solution was extracted with 1.0 ml phenol (saturated with Tris-HCl, pH 8.0) and 1.0 ml chloroform:isoamyl alcohol (24:1) and the aqueous phase was again precipitated by the addition of 2 volumes of 95% ethanol as above. The resulting pellet was drained, resuspended in 300 µl TE buffer and transferred to a microcentrifuge tube. The DNA was precipitated a further time by the addition of 150 µl of 7.5 M ammonium acetate and 900 µl 95% ethanol. The pellet was then resuspended in 300 µl TE and the DNA concentration calculated from the A_{260} of a 20-µl sample (Maniatis *et al.*, 1982). The DNA was again precipitated with 140 µl 7.5 M ammonium acetate and 840 µl ethanol, washed with 1.0 ml 80% ethanol and dissolved in TE at 500 ng/µl.

b) Isolation of plasmid DNA from yeast

Plasmids were extracted from yeast cells by vortexing with glass beads as described by Lorincz (1984).

8. Transformation

a) Yeast transformation

Yeast transformation was performed as described by Hinnen *et al.* (1978) with minor modifications. A logarithmic phase yeast culture was harvested by centrifugation, washed in 0.5 volume of H_2O and resuspended in 0.1 volume of 1 M sorbitol. Spheroplasts were prepared by treatment with

70 µl β-glucuronidase per 10 ml concentrated cells for 45 min, followed by 2 washes with 0.5 volume 1 M sorbitol and one wash with 0.2 volume SCT (1 M sorbitol, 10 mM $CaCl_2$, 10 mM Tris-HCl [pH7.5]). Spheroplasts were then resuspended in 0.01 original culture volume of SCT and plasmid DNA was added at 10-20 µg/ml. After a 10-min incubation at 23°C 10 volumes of 20% polyethylene glycol, 20 mM Tris-HCl (pH 7.5), 20 mM $CaCl_2$ were added and the mixture was incubated for a further 10 min before spheroplasts were gently pelleted and resuspended in 0.01 original culture volume of SCT. 0.3-ml aliquots were plated in 10 ml regeneration agar on selective solid medium.

b) Transformation of *E. coli*

Preparation of competent *E. coli* cells was by the $CaCl_2$ procedure, as described by Maniatis *et al.* (1982). 1 ml of an overnight *E. coli* culture was inoculated into 100 ml of 2X YT broth and grown to A_{600} = 0.65. The culture was chilled on ice for 10 min and the cells were pelleted in a refrigerated IEC centrifuge at 5000 rpm. The cell pellet was resuspended in half the culture volume of 50 mM $CaCl_2$, 10 mM Tris-HCl (pH 8.0) and chilled for 15 min on ice. Cells were then pelleted and finally resuspended in 1/15 original volume (for plasmid transformation) or 1/50 original volume (for M13 transformation) 50 mM $CaCl_2$, 10 mM Tris-HCl (pH 8.0), and were held at 4°C for 12-24 hours to increase transformation efficiency.

0.2-ml aliquots of competent cells were transformed with up to 40 ng plasmid DNA as described by Maniatis *et al.* (1982). Briefly, DNA was added to competent cells which were then held on ice for 30 min before being placed at 42°C for 2 min. Bacteria were allowed to recover for 1 hour at 37°C

after the addition of 1 ml 2X YT. The cells were then concentrated by centrifugation and resuspended in 2X YT (200 µl), and portions of this transformation mix were plated on selective medium. To detect inserts in the multiple cloning site (MCS) of plasmids conferring α-complementation (Ullman *et al.*, 1967) of the *E. coli lac*ZΔM15 mutation (Gronenborn and Messing, 1978) 40 µl of the indicator Xgal (2% solution in dimethylformamide) and 4 µl of the inducer IPTG (200 mg/ml solution) were spread on plates prior to plating transformation mixes.

Transformation of competent cells with M13 was by a similar procedure. Approximately 0.5 ng of RF DNA was added to 200 µl cells, which were plated immediately after heat-treatment as described by Messing (1983). The transformation mix was added, with 200 µl of an overnight culture of *E. coli* (strain DH5αF'), to 3 ml of molten YT top agar held at 50°C. This mixture was then quickly poured onto a pre-warmed YT plate. If required, 50 µl of Xgal and 10 µl of IPTG were added to the top agar prior to plating.

9. Routine analysis of nucleic acids

a) Restriction endonuclease digestion of DNA

Restriction endonucleases were purchased from BRL (Bethesda Research Laboratories, Gaithersburg, MD) and used with buffers provided according to the supplier's recommendations.

b) Agarose gel electrophoresis and DNA fragment purification

Agarose gel electrophoresis was performed as described by Maniatis *et al.* (1982) using TAE buffer (40 mM Tris-acetate, 1 mM EDTA) and

electrophoresis grade agarose (BRL). *Hin*dIII-digested lambda or *Hae*III-digested øX174 DNA fragments were used as size markers. Loading buffer consisted of 50% glycerol, 0.01% bromophenol blue and 0.01% xylene cyanol in TE buffer. DNA fragments excised from agarose gels were purified using a Geneclean kit (BIO 101, La Jolla, CA) according to the manufacturer's directions. SeaPlaque agarose (FMC BioProducts, Rockland, ME) was used for the separation of DNA fragments to be ligated directly in agarose (Struhl, 1985).

c) Ligation reactions

T4 DNA ligase was purchased from BRL and used with the buffer supplied. Ligations were performed as described by Sambrook *et al.* (1989), or in agarose as described by Struhl (1985).

10. Southern analysis

Yeast genomic DNA was extracted for Southern analysis as described by Rose *et al.* (1988). Restriction-endonuclease-digested DNA, resolved by agarose gel electrophoresis as described above, was transferred to GeneScreen nylon membranes purchased from NEN (NEN Research Products, Boston, MA) by the alkaline-transfer method of Reed and Mann (1985). Prior to transfer, gels were photographed next to a ruler to facilitate subsequent size determinations, and the lane containing size standards was cut off. Probes were radiolabelled with [α-^{32}P]dATP (NEN) using a random primed DNA labeling kit (Boehringer Mannheim GmbH, Mannheim, West Germany) following the manufacturer's directions. Incorporation was monitored by TCA precipitation (Maniatis *et al.*, 1982). Hybridization was performed as

recommended by the manufacturers of GeneScreen. Blots were washed as recommended by the manufacturers of GeneScreen under moderate stringency (60°C, 0.3 M NaCl, 60 mM Tris-HCl (pH 8.0), 2 mM EDTA (pH 8.0). Autoradiography was performed using Kodak X-OMAT AR film and Dupont Cronex Lightning-Plus intensifying screens (Dupont Co., Wilmington, DE).

11. Northern analysis

a) Isolation of yeast RNA

Yeast cultures were grown overnight to a density of 2-4 x 10^6 cells/ml before a portion of the culture was transferred to 37°C and incubated further. 25-40 ml samples were removed at appropriate intervals and cells were pelleted by centrifugation, resuspended in 1 ml LETS buffer without SDS (0.1 M $LiCl_2$, 10 mM EDTA, 10 mM Tris-HCl [pH 7.0]) and rapidly frozen in liquid N_2. Cells were stored at -70°C until processed further.

Prior to RNA extraction, cells were thawed on ice, pelleted by centrifugation and resuspended in 300 µl LETS buffer (as above with the addition of 1% SDS). RNA was extracted by lysing cells in the presence of glass beads and phenol:chloroform:isoamylalcohol, as described by Penn *et al.* (1984). The organic phase was re-extracted with 100 µl LETS buffer, and both aqueous phases were combined and extracted a second time before precipitation of the RNA with 2 volumes of 95% ethanol (Sripati and Warner, 1978). The RNA precipitate was then dissolved in 10-15 µl H_2O, quantitated spectrophotometrically (Maniatis *et al.*, 1982) and sample volumes were adjusted to equal concentration (usually 5 µg/µl) by the addition of H_2O to the more concentrated samples. RNA concentrations

were then determined a second time and loading, as described below, was based on these readings.

b) Electrophoresis of RNA through formaldehyde gels

Equal amounts of total RNA (15 or 20 µg per lane) were denatured and resolved by electrophoresis through a formaldehyde-containing agarose gel as described by Maniatis *et al.* (1982) with the following modifications: RNA samples were denatured at 65°C for 15 min instead of at 55°C, and the gel was cast with 0.375µg/ml ethidium bromide. A 0.24 - 9.5 kb RNA ladder (BRL) was used to provide size markers, which were cut off the gel prior to transfer. Gels were photographed on a UV transilluminator next to a ruler to assess the efficacy of loading (based on intensity of staining of rRNA by ethidium bromide) in addition to providing a record for subsequent size determinations.

c) Transfer of RNA to nylon membrane

Prior to transfer, RNA within the gel was partially hydrolysed to improve transfer efficiency, as described by Maniatis *et al.* (1982). Transfer was by capillary elution (as described by Sambrook *et al.*, 1989) to nylon membrane (GeneScreen, NEN).

After transfer, RNA was crosslinked to the membrane by UV irradiation using a model 2400 UV crosslinker (Stratagene, La Jolla, CA) according to the manufacturer's recommendations.

d) Filter hybridization

Northern blots were hybridized with restriction fragment probes, radiolabelled as described above, essentially as described by Thomas (1983) with minor modifications. Dextran sulphate was omitted from the hybridization buffer, making this the same as pre-hybridization buffer. 1% SDS was added to both buffers to reduce background associated with the use of nylon membranes. The SDS concentration in all washes was increased to 1%. Oligonucleotide probes were end-labelled with [γ^{32}P]dATP and T4 polynucleotide kinase as described by Sambrook *et al.* (1989). Hybridization with oligonucleotide probes was performed as described by Wallace and Miyada (1987). Autoradiography was as described for Southern blot analysis.

The following DNA restriction fragments were purified and labelled for use as probes: The *CLN1* and *CLN2* open reading frames were excised as 1.6 kbp *Nde*I-*Bam*HI fragments from inserts in pRK171, kindly provided by C. Wittenberg. The *CLN3* probe was a 1.6-kbp *Eco*RI-*Xho*I restriction fragment from a plasmid containing the *CLN3/DAF1/WHI1* gene (a gift from F. Cross). Specificity of the *CLN1* and *CLN3* probes was confirmed by hybridization to RNA isolated from strains JHY629 (provided by C. Wittenberg) and BF338-2a whi1::URA3 (provided by B. Futcher) deleted for *CLN1* and *CLN3*, respectively (data not shown). (Since *CLN1*, *CLN2* and *CLN3* encode functionally redundant products, disruption of even two of the three genes is not lethal [Richardson *et al.*, 1989]). The *ACT1* probe was a 1-kbp *Hin*dIII-*Xho*I restriction fragment from pRS208 (a gift from R. Storms). The *LEU2* probe was a 1.7-kbp *Hpa*I-*Acc*I fragment from YEp351 (provided by J. Hill. The *CDC68* probe used for the analysis illustrated in Fig. 22 was a 2.2-kbp *Hpa*I fragment from the *CDC68* open reading frame (ORF), excised

from pSC2-1. The *CDC68* probe used for the analysis illustrated in Fig. 15 was a 1.8-kbp *Cla*I-*Hpa*I fragment from the *CDC68* ORF excised from plasmid pSC2-1. The *lacZ* probe was a 6.2-kbp *Bam*HI-*Sal*I fragment from pUTX144 (provided by D. Finkelstein) and contained *lacZYA* sequences only.

The *HSP82* probe was an oligonucleotide of sequence 5'-CAAGGCCA-TGATGTTCTACC-3', complementary to nucleotides 2130-3149 of the *HSP82* coding sequence (Farrelly and Finkelstein, 1984). The *HSC82* probe was an oligonucleotide of sequence 5'-TGTTCTTAGCGAAGGCAGAG-3', complementary to nucleotides 1242 to 1261 of the *HSC82* ORF (Borkovich *et al.*, 1989).

12. DNA sequencing techniques

a) M13 cloning

All M13 cloning techniques were either as described by, or modified from, Messing (1983). M13 um20 and um21 (IBI, International Biotechnologies Inc.) were used with *E. coli* strain DH5αF' as a host.

Competent cells were prepared and transformed as described above (section 8b). Colourless plaques (generated by M13 phage that contain insert DNA) were picked with a sterile toothpick and transferred to 3 ml of 2X YT containing 10 µl of an overnight culture of untransformed DH5αF'. After 8 h incubation at 37°C the cells (containing the double-stranded replicative form [RF] of M13) were separated from the supernatant (containing M13 phage particles) by centrifugation. Cell pellets were stored at -20°C and supernatants at 4°C for later identification of recombinant M13 as described below.

b) Direct gel electrophoresis

Inserts of greater than approximately 200 bp retard the electrophoretic migration of single-stranded (ss) phage DNA. To screen potential recombinant phage and to estimate the extent of deletion achieved using the protocol described below, 20 µl of ssDNA-containing supernatant was mixed with 3 µl of loading buffer and 1 µl 2% SDS, thereby disrupting the protein coat of the M13 phage and releasing the ssDNA genome. This mixture was loaded directly onto a 0.7% agarose gel. Vector phage ssDNA was included as a standard.

c) The C-test

A restriction fragment may be inserted in two possible orientations when cloning into a single MCS restriction site in the RF form of M13. Since M13 phage always contains the (+) strand of DNA, the two possible orientations will be complementary in the insert region and can be made to hybridize; clones with the same insert orientation cannot. Two clones which hybridize in this manner migrate more slowly during electrophoresis, providing a means whereby alternate orientations can be identified.

Complementation tests (C-tests) were performed by mixing 20 µl of each of two supernatants together with 2 µl 2% SDS and 6 µl loading buffer. The mixture was heated at 65°C for 1 h and resolved on a 0.7% agarose gel at 10 V/cm for 30-60 min. Unheated controls were resolved in parallel.

d) Generation of ordered M13 deletions

Nested sets of deleted M13 clones for DNA sequencing were constructed using the procedure of Henikoff (1987), adapted by Dr. Jason Hofman (Department of Biology, Dalhousie University). This procedure takes advantage of the ability of *E. coli* ExoIII to uniformly digest DNA from a base-paired 3' end but not from a protruding 3' end. To facilitate this approach, M13 clones were constructed leaving a MCS restriction site generating a 5' overhang or blunt end (ExoIII-sensitive) next to the insert, and a MCS site generating a 3' overhang (ExoIII-resistant) next to vector sequences. The detailed protocol is described below.

RF DNA was prepared as follows: 50 ml of 2X YT was inoculated with 200 µl of an overnight culture of strain DH5αF' and incubated for 1 h prior to adding 200 µl phage stock and incubating for a further 6-12 h. After this time cells were pelleted and DNA extracted as described above (section 7a).

10 µg of RF DNA was doubly digested with appropriate restriction enzymes in RE buffer (50 mM Tris-HCl [pH 7.6], 50 mM KCl, 10 mM $MgCl_2$, 10 mM DTT) in a 25 µl volume. 0.5 µl (200 ng) was removed and checked for complete digestion by agarose gel electrophoresis. Where possible restriction enzymes were heat inactivated at 70°C for 10 min. 5 µl (2 µg) of the resulting linear RF was pre-incubated with 13 µl of H_2O at 37°C before adding 2 µl of ExoIII (20 U/ml; diluted and stored in 50 mM Tris-HCl [pH 8.0], 50 mM KCl, 1 mM DTT, 200 µg/ml bovine serum albumin, 50% glycerol). Samples were taken at timed intervals and pooled in a tube containing 6 µl mung bean nuclease buffer (250 mM sodium acetate [pH 5.0], 250 mM NaCl, 0.5 mM zinc acetate, 0.005% Triton X-100, 25% glycerol). The number and volume of samples taken was based on the size of the insert,

the rate of ExoIII deletion (400 nucleotides per minute under these conditions; J. Hofman, personal communication) and the desired spacing between deletions (usually 200 nucleotides).

The pooled products were mixed with 2 µl H_2O and 2 µl mung bean nuclease (10U/µl; diluted and stored in 10 mM sodium acetate [pH 5.0], 1 mM L-cysteine, 0.1mM zinc acetate, 0.001% Triton X-100, 50% glycerol) and incubated at 37°C for 10 min. After this time 1.5 µl of 1.0 M Tris base was added and the mung bean nuclease was inactivated by a 5-min incubation at 70°C. 5 µl of the deleted products were removed for agarose gel analysis, with linear M13 vector and linear starting RF as controls to check for complete coverage of the required range of deletions. The remaining products were blunt-ended using the 3' to 5' exonuclease and 5' to 3' polymerase activities of Klenow fragment, by adding 2.5 µl 100 mM $MgCl_2$, 5 µl 1 mM dNTPs, 11 µl H_2O, 2 U Klenow fragment and incubating for 20 min at room temperature. 5 µl of blunt-ended products were ligated overnight at 4°C in a 50 µl ligation containing 5 U T4 DNA ligase. Transformation of ligated products was as previously described using half the ligation mix per plate. Plaques were picked and the resultant deleted M13 phage were examined by direct gel electrophoresis as described above, using undeleted and vector phage supernatants as controls, to assess the extent of deletion of individual products.

e) Preparation of template ssDNA

Phage particles were precipitated in a 1.5 ml microcentrifuge tube containing 200 µl of 27% PEG-6000 in 3.3 M NaCl and filled with supernatant. After 60 min at 4°C phage were pelleted by centrifugation and

the pellet was resuspended in 300 µl TE buffer followed by phenol and phenol:chloroform extractions. The resulting phage DNA was then ethanol precipitated, dried, resuspended and quantitated by A_{260} determination.

f) Sequencing reactions

Chain-termination DNA-sequencing reactions (Sanger *et al.*, 1977) were performed with Sequenase, a genetically engineered variant of T7 DNA polymerase, according to the manufacturer's instructions and using reagents provided in the Sequenase Version 2.0 kit (USB, United States Biochemical Company, Cleveland, Ohio). Compression artefacts were resolved by substituting dGTP with 7-deaza-dGTP using a 7-deaza-dGTP reagent kit (USB) according to the manufacturer's instructions. All sequencing reactions were performed using [^{35}S]dATP (NEN).

Double-stranded DNA was sequenced using the following procedure from the laboratory of Dr. D. Theilman, University of British Columbia (D. Theilman, personal communication). DNA was prepared using the rapid-boil procedure previously described. Approximately 3 µg of DNA, in a 10 µl volume of TE or H_2O, was denatured by mixing with 1 µl of primer and 1.5 µl 2 M NaOH, 2 mM EDTA and heating to 85°C for 5 min. The denatured DNA was cooled on ice and precipitated with 1.3 µl 3 M sodium acetate and 35 µl absolute ethanol, and the pellet was washed with 150-200 µl 70% ethanol before drying at 55°C for 5 min. The denatured DNA pellet was resuspended in 2 µl 5X sequenase reaction buffer and 8 µl H_2O and heated at 37°C for 15 min. The remaining steps were carried out according to USB's instructions as for ssDNA sequencing.

g) Denaturing gel electrophoresis

Gels were poured and run using a Model S2 Sequencing Gel Electrophoresis System, wedge spacers and sharkstooth combs (BRL) according to manufacturer's recommendations. After electrophoresis, gels were soaked in 10% methanol, 10% acetic acid for 45 min to remove urea, rinsed briefly in water and dried on a Model 583 gel dryer (Bio-Rad Laboratories). Autoradiography was performed using Kodak XAR-5 film.

h) Computer analysis of sequence data

DNA sequence data were analyzed using the University of Wisconsin Genetics Computer Group (GCG) Sequence Analysis Software Package for VAX/VMS computers (Devereux *et al.*, 1984). DNA and protein sequence comparisons were performed using the FASTA Software Package (Pearson and Lipman, 1988). Both software packages were accessed through the Canada Scientific Numeric Database Service (CAN/SND) of the National Research Council. FASTA searches were also performed, by electronic mail, using facilities provided by GenBank. Additional sequence analysis was performed using the DNA Strider program for Apple Macintosh computers (Marck, 1988).

III. RESULTS

1. Cloning the *CDC68* gene by complementation

Saccharomyces cerevisiae cells bearing the *cdc68-1* mutation are temperature-sensitive for the performance of the G1 regulatory event, Start (Prendergast *et al.*, 1990). Clones containing the wild-type *CDC68* gene were therefore isolated from a *S. cerevisiae* genomic library by their ability to complement the temperature-sensitive phenotype of *cdc68-1* mutant cells. The library used consisted of 10-15 kbp fragments of yeast genomic DNA, partially digested with *Sau*3A, and cloned into the unique *Bam*HI site of the shuttle vector YEp24 (Carlson and Botstein, 1982). YEp24 contains both yeast and *Escherichia coli* origins of replication (the 2μ circle origin of replication from yeast and the *colE1* origin of replication from *E. coli*), an amp^r gene for selection in *E. coli*, and *URA3* for selection in *S. cerevisiae* (Botstein *et al.*, 1979). Strain ART68-1 (relevant genotype: *cdc68-1, ura3-52*) was transformed with the genomic library and transformants were incubated on selective medium at the restrictive temperature of 37°C.

DNA prepared from several independent Ura^+, temperature-resistant yeast transformants was used to transform *E. coli* strain RR1 to ampicillin resistance. The *Eco*RI restriction endonuclease digestion patterns of individual plasmids isolated from the resulting *E. coli* transformants were compared, and four clones containing common restriction fragments were chosen for further analysis. All four clones complemented the *cdc68-1* mutation when individually used to retransform strain ART68-1. Restriction mapping indicated that three of these clones, pLC3-2, pLC3-3 and

pLC3-4, were very similar (Fig. 1) The fourth, pLC2-2, contained a 1.8-kbp *Hin*dIII fragment common to pLC3-2, pLC3-3 and pLC3-4 but did not contain the adjacent 3.4-kbp *Hin*dIII fragment present in these plasmids. Overlap between pLC2-2 and the other three clones was confirmed by Southern analysis (data not shown). Thus, pLC2-2 contains the same complementing sequences as pLC3-2, pLC3-3 and pLC3-4. Assuming that the entire *CDC68* gene is required for complementation of the *cdc68-1* mutation, the overlap between pLC2-2, pLC3-2, pLC3-3 and pLC3-4 predicts that the maximum size of the *CDC68* gene is 4.5 kbp.

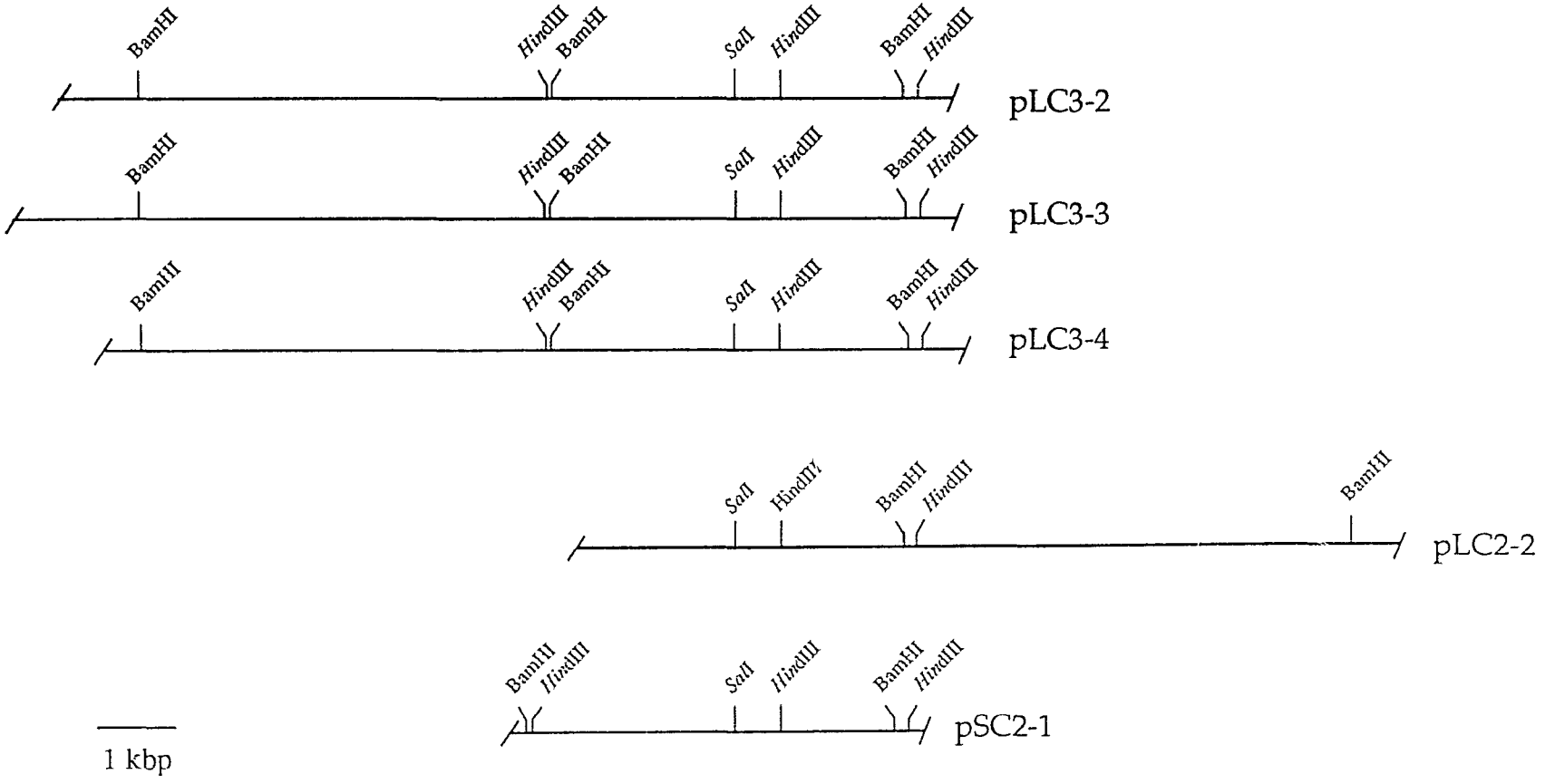
2. *Sau*3A subcloning of the *CDC68* gene

Plasmid pLC3-4 was chosen for further analysis. The insert of this plasmid is approximately 10.3 kbp and probably contains more than one gene since yeast genes are usually quite densely spaced. To further define the region of the insert containing the *CDC68* gene, plasmid pLC3-4 was subcloned by partial digestion with *Sau*3A followed by electrophoretic separation of the products and ligation of size-fractionated pools of fragments into the unique *Bam*HI site of YEp24. An appropriate concentration of *Sau*3A was determined from a preliminary optimization experiment. Pools of DNA fragments of 1-2, 2-3, 3-4, 4-5 and 5-6 kbp were excised from a 1% agarose gel, purified by phenol extraction and ligated into the *Bam*HI site of YEp24. Each pool of ligations was used to transform *E. coli* strain RR1, and transformation mixes were grown in liquid media containing ampicillin. Plasmid DNA prepared from each pool of *E. coli* transformants was used to transform yeast strain ART68-1 to uracil prototrophy. Ura^+ transformants were then screened for growth at 37°C.

Figure 1. Restriction maps of clones that complement the temperature-sensitive *cdc68-1* mutation.

Clones pLC3-2, pLC3-3, pLC3-4 and pLC2-2 were obtained from a yeast genomic DNA library on the basis of their ability to complement the *cdc68-1* temperature-sensitive mutation. The overlap between clones pLC3-4 and pLC2-2 has been confirmed by Southern analysis. Each insert contains additional *Hin*dIII sites that have not been mapped. pSC2-1 was derived from pLC3-4 by *Sau*3A subcloning (see text). Like the full-length clones, pSC2-1 fully complements the *cdc68-1* mutation.

Figure 1.


BamHI
HindIII
BamHI
SalI
HindIII
BamHI
HindIII
pLC3-2
pLC3-3
pLC3-4
pLC2-2
pSC2-1
1 kbp

Only transformation of ART68-1 with the 5-6-kbp insert pool resulted in the isolation of Ura^+ and temperature-resistant transformants. This finding suggests that the complete *CDC68* gene was not represented in the pools with smaller inserts, and that the *CDC68* gene is therefore relatively large.

As in the initial cloning of the *CDC*68 gene, DNA was prepared from Ura^+, temperature-resistant transformants and used to transform *E. coli* strain RR1. Individual plasmids were then retested for complementation of the *cdc68-1* mutation by transformation of strain ART68-1. The restriction map of pSC2-1, the smallest complementing clone identified using this approach, is shown in Fig 1. The growth kinetics presented in Fig. 2 demonstrate that pSC2-1, like pLC3-4, fully complements the *cdc68-1* temperature-sensitive mutation in cells of strain ART68-1. Furthermore, since the growth rates of *cdc68-1* mutant cells transformed with YEp24, pLC3-4 or pSC2-1 were similar under permissive conditions, the presence of the cloned sequences on a high-copy plasmid does not appear to be deleterious for cell proliferation.

3. Verification that the cloned sequences contain the *CDC68* gene

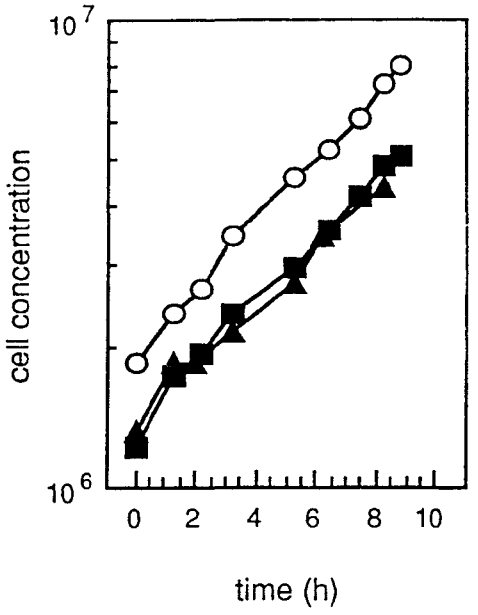
The plasmids described above were obtained from a library of yeast DNA contained in the 2μ-based vector YEp24. Because of the high copy number at which 2μ-based plasmids are typically maintained (between 10 and 40 copies per cell; Rose, 1987), it was possible that the cloned sequences did not contain the *CDC68* gene but a suppressor gene able to rescue the *cdc68-1* mutation by virtue of increased copy number rather than by true complementation. To confirm that this was not the case, the cloned sequences were tested for their ability to target integration, by homologous

Figure 2. Complementation of the *cdc68-1* mutation by the cloned *CDC68* gene.

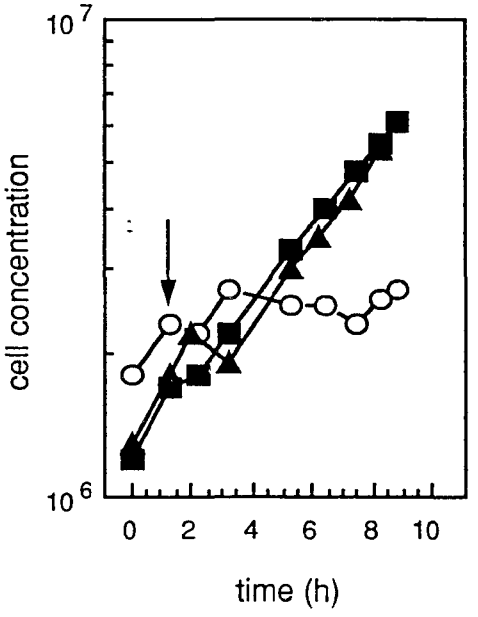
Strain ART68-1 (*cdc68-1 ura3-52*) was transformed with either YEp24 (vector control) (open circles), pLC3-4 (closed squares), or pSC2-1 (closed triangles). pLC3-4 and pSC2-1 each contain cloned sequences that complement the *cdc68-1* mutation. Actively dividing transformed cells growing in YNB+9 (lacking uracil for plasmid selection) at 23°C (A) were transferred to 37°C (B) at the time indicated by the arrow and incubated further.

Figure 2.

A


10^7
10^6
cell concentration
0 2 4 6 8 10
time (h)

B

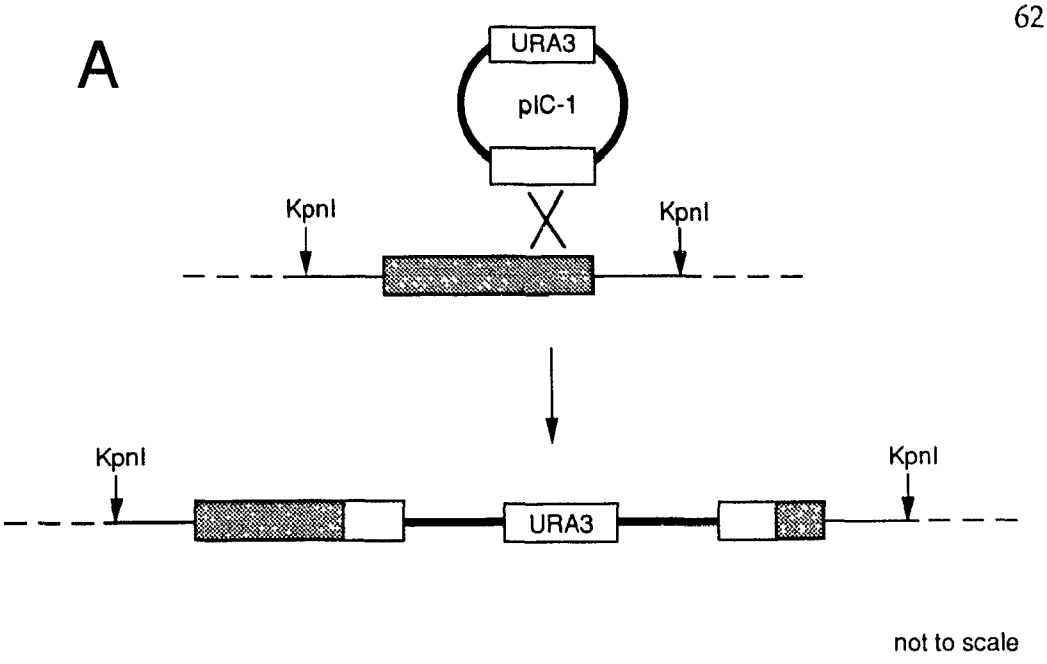

10^7
10^6
cell concentration
0 2 4 6 8 10
time (h)

recombination, of a plasmid-borne selectable marker to the *CDC68* chromosomal locus. The strategy used is illustrated in Fig. 3. The 1.8-kbp *Hin*dIII fragment common to all the complementing clones was cloned from pLC3-4 into the integrative vector YIp5 (Struhl *et al.*, 1979). YIp5 contains a *URA3* selectable marker but is incapable of autonomous replication in yeast cells and can therefore only replicate when integrated into the *S. cerevisiae* genome. The resulting plasmid, pIC-1, was used to transform strain ART68-1 (*cdc68-1 ura3-52*) to uracil prototrophy. Plasmid pIC-1 was not linearized to direct integration, therefore two classes of transformants were expected as a result of pIC-1 integration at two possible loci: *ura3-52*, or the chromosomal locus of the cloned sequences. Six Ura$^+$ transformants were obtained and all were temperature-sensitive, indicating that the 1.8-kbp *Hin*dIII fragment does not contain the entire *CDC68* gene or sequences that allow the reconstruction of a functional *CDC68* gene during the integration process (see Fig. 3). Each of the six transformants was crossed with strain Sc225 (*ura3-52*) to follow the genetic segregation of the integrated *URA3* marker with respect to the *cdc68-1* mutation. In 2 of the 6 transformants *URA3* segregated in a non-Mendelian fashion, indicating that the plasmid was not stably integrated. In a further three transformants uracil prototrophy segregated 2:2 in tetrads, as expected, but was not linked to the *cdc68-1* mutation. It is likely that in these transformants pIC-1 was integrated at the *ura3-52* locus. However, in the remaining transformant, ARI68-7, uracil prototrophy was linked to the *cdc68-1* mutation in all 26 tetrads examined. This result demonstrates that the insert of pIC-1 is capable of directing plasmid integration to the *cdc68-1* chromosomal locus. This insert must therefore originate from the *CDC68* genomic locus.

Figure 3. pIC-1 integration at the *cdc68-1* genomic locus.

(A) Schematic representation of pIC-1 integration at the *cdc68-1* genomic locus. Plasmid pIC-1 was constructed by the insertion of a 1.8-kbp *Hin*dIII fragment (lightly stippled bar), from the insert of plasmid pLC3-4, into the unique *Hin*dIII site of the integrative vector YIp5. The *URA3* marker of YIp5 is indicated; remaining plasmid sequences are shown as thick lines. The *cdc68-1* genomic locus (dark stippled bar) is flanked by neighbouring genomic sequences (thin lines) containing flanking *Kpn*I sites whose exact positions have not been determined. The insert of pIC-1 is shown aligned with its chromosomal homologue, and a single recombination event is indicated by a cross. The chromosome structure after recombination is shown below. The integration event resulting from integration leads to the duplication of part of the *cdc68-1* locus in the chromosome, increasing the size of the *Kpn*I fragment by the size of pIC-1. The result of this integration event is that the *URA3* selectable marker becomes linked to the *cdc68-1* chromosomal locus and these markers now segregate together in subsequent meiotic analysis.

(B) Southern analysis confirming the integration of pIC-1 at the *cdc68-1* genomic locus. Genomic DNA was isolated from wild-type strain 21R (lane 1) and integrant strain ARI68-7 (lane 2), and digested with *Kpn*I. The probe was radiolabelled plasmid pIC-1. The open arrow indicates the *Kpn*I fragment detected by *CDC68* sequences on pIC-1 prior to integration of pIC-1 at the *cdc68-1* chromosomal locus; the closed arrow after integration. The unmarked lower band, which is the same size in both the untransformed and transformed strains, is the *Kpn*I fragment detected by the *URA3* gene carried on plasmid pIC-1.



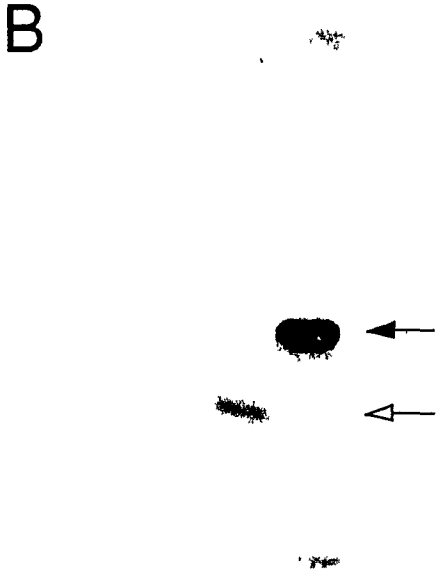


Figure 3.

Integration of plasmid pIC-1 at the chromosomal locus of the cloned sequences was confirmed by Southern analysis. Genomic DNA was isolated from the integrant strain, ARI68-7, and the wild-type strain 21R, and digested with *KpnI*, which does not cleave the integration plasmid pIC-1. A Southern blot prepared using this DNA was probed with radiolabelled pIC-1, which was expected to hybridize to at least two restriction fragments in the genomic DNA of both the integrant and wild-type strains. At least one fragment, detected by the *URA3* selectable marker on plasmid pIC-1, was expected to be of the same size in both integrant and wild-type strains. Another fragment, detected by the cloned insert sequences, was expected to be of greater length in strain ARI68-7 than in 21R as a result of the integration of pIC-1 (illustrated in Fig. 3). Fig. 3 shows that, as predicted, two fragments were detected in the DNA from each strain. One fragment was the same size in both strains and the other was of increased length in ARI68-7 as a result of the integration of plasmid pIC-1. The exact size of each fragment was not determined because the range of the standards used (*Hin*dIII-digested lambda DNA) was exceeded. These results confirm that the integration of plasmid pIC-1 into the genome of strain ARI68-7 is at the genomic locus of sequences carried on the plasmid. Taken with the genetic data described above, these results demonstrate that the insert of plasmid pIC-1 is derived from the *CDC68* genomic locus and that the *CDC68* gene has therefore been cloned.

4. Mapping the *cdc68-1* mutation

a) Mapping by hybridization

The development of pulse-field gel electrophoresis has facilitated the separation of intact yeast chromosomes, which range in size from 200 to 3000 kbp in length (Carle and Olson, 1984; 1985). One application of this advance is the rapid assignment of cloned yeast DNA sequences to a particular chromosome by hybridization to a Southern blot of separated whole chromosomes. This approach was used to identify the chromosome bearing the *CDC68* gene.

Radiolabelled plasmid pLC3-4 was used to probe a Southern blot of yeast chromosomes separated by CHEF (contour-clamped homogeneous electric field) electrophoresis (Chu *et al.*, 1986) prepared by L. C. Schalkwyk (Department of Biochemistry, Dalhousie University). Intact yeast chromosomes were prepared from strains AB972 (Sandmeyer and Olson, 1982), YNN281 (Carle and Olson, 1985), A364A (Hartwell, 1967) and DC04α (Broach and Hicks, 1980) which, because of their inherent chromosome-length polymorphisms, together facilitate resolution of fifteen *S. cerevisiae* chromosomes (Carle and Olson, 1985). Fig. 4 shows that two chromosomes were detected by pLC3-4. Hybridization of pLC3-4 to one of these, chromosome V, was expected as *URA3*, carried as a selectable marker on this plasmid, has been mapped previously to this chromosome (Mortimer and Hawthorne, 1966; Carle and Olson, 1985). The other chromosome to which pLC3-4 hybridized was chromosome VII. This chromosome was resolved as a single band only in DNA prepared from strain DC04α; in chromosome preparations from most other strains chromosome VII migrates as a doublet

Figure 4. Mapping the *CDC68* gene.

(A) Mapping by hybridization. The left hand panel shows a photograph of whole yeast chromosomes separated by CHEF (contour-clamped homogeneous electric field) electrophoresis. Individual chromosomes were identified from the electrophoretic karyotype described by Carle and Olson (1985). The right-hand panel shows a Southern blot prepared from the gel shown on the left, probed with radiolabelled pLC3-4. Hybridization to chromosome V was expected, as pLC3-4 contains a *URA3* selectable marker known to map to this chromosome. The remaining chromosome identified by sequences carried on pLC3-4, chromosome VII, is the chromosome from which the *CDC68* insert sequences were derived.

(B) Genetic mapping of the *cdc68-1* mutation. The markers shown indicate those that were tested for linkage with the *cdc68-1* mutation (except *chc1*, which was not tested directly).

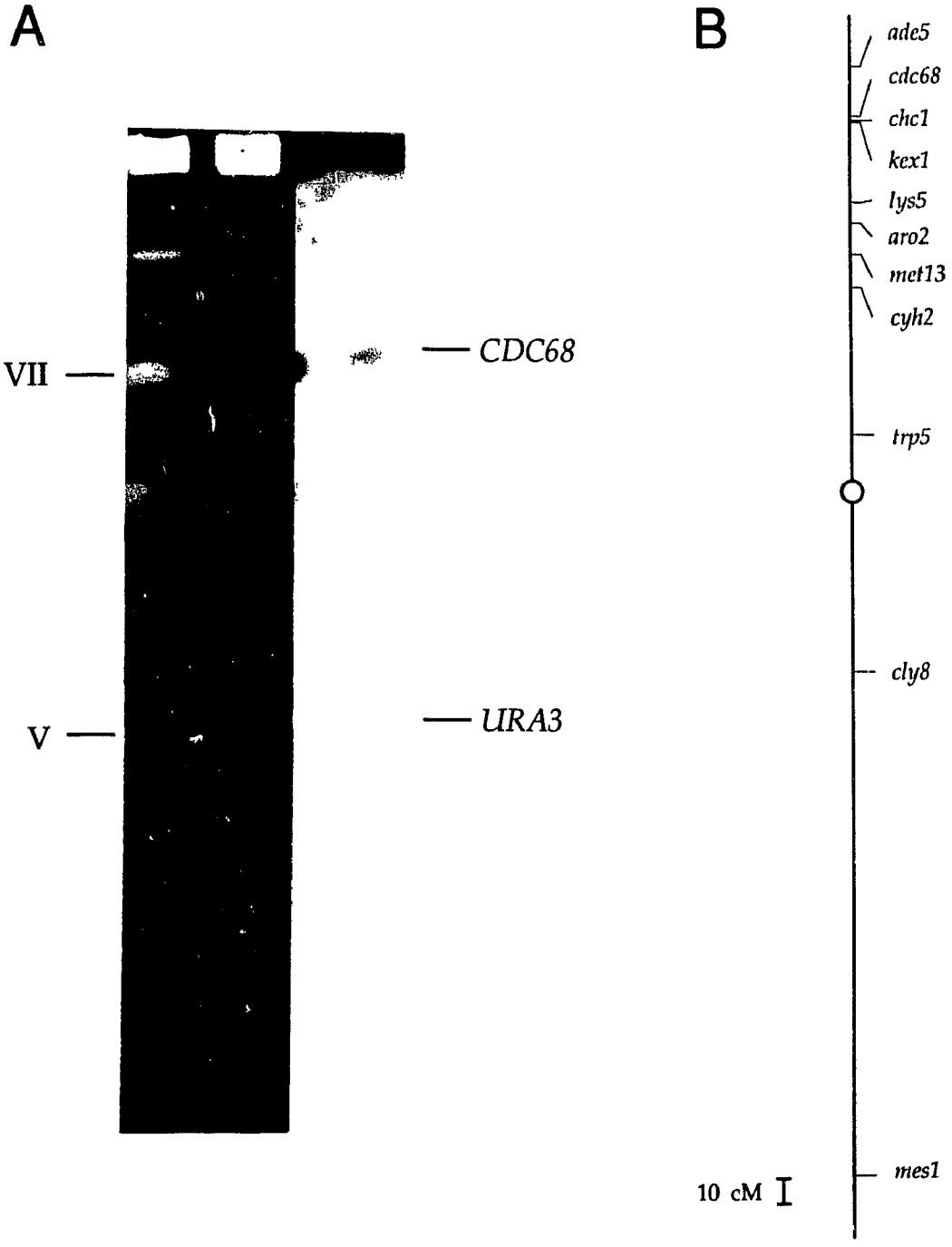

A
VII
V
CDC68
URA3
B
ade5
cdc68
chc1
kex1
lys5
aro2
met13
cyh2
trp5
cly8
mes1
10 cM

Figure 4.

with chromosome XV (Carle and Olson, 1985). Chromosome VII must therefore be the chromosome on which the *CDC68* gene is located.

b) Genetic mapping

The results described above localize the *CDC68* gene to chromosome VII. To order the *cdc68-1* mutation relative to other mutations on chromosome VII, and to determine whether *cdc68-1* was allelic to a previously mapped gene, genetic crosses between *cdc68-1* mutant strains and others bearing appropriate chromosome VII markers were performed. Resulting diploids were then sporulated and the spore products analysed by standard tetrad analysis (Mortimer and Hawthorne, 1969). Fig. 4 illustrates the genetic map of chromosome VII and the markers that were tested for linkage with the *cdc68-1* mutation; the strains containing these markers are listed in Table 2. The *cdc68-1* mutation was found to map to the left arm of chromosome VII, within 4 cM of the *KEX1* locus and 16 cM of *ade5* (Table 4).

The *KEX1* gene encodes a putative protease involved in killer-toxin and α-factor processing (Dmochowska *et al.*, 1987). *kex1* mutations are not easily scored by standard replica-plating assays; therefore strain Sc25k-13, in which the *KEX1* locus is disrupted by the replacement of most of the *KEX1* open reading frame with the yeast *LEU2* gene, was employed for this analysis. *KEX1* is not an essential gene, and it was therefore possible to use this *LEU2* disruption as a genetic marker of the *KEX1* locus in tetrad analysis. The replacement of *KEX1* sequences with *LEU2* increases the size of the *KEX1* locus by only 400 bp and does not therefore distort map-distance determinations made using this strain.

Table 4. Linkage between *cdc68-1* and chromosome VII markers.

DIPLOID	INTERVAL	ASCUS TYPE			DISTANCE (cM)
		PD	NPD	TT	
GRX2-4B x ADC26A1-1	*cdc68-1 - ade5*	19	0	9	16.1
	cdc68-1 - aro2	12	0	16	28.6
	cdc68-1 - lys5	11	0	17	30.4
	cdc68-1 - cyh2	5	0	21	*44.3
	cdc68-1 - met13	4	0	24	*47.7
	aro2 - lys5	28	0	0	0
	met13 - lys5	21	0	7	12.5
	aro2 - met13	21	0	7	12.5
	cyh2 - met13	18	0	8	15.4
	cyh2 - lys5	13	0	13	25.0
	cyh2 - aro2	13	0	13	25.0
	ade5 - lys5	7	1	20	*52.9
	ade5 - aro2	6	2	20	*72.2
	ade5 - cyh2	4	2	20	*83.1
Sc25k-13 x ARM7-6	*cdc68-1 - KEX1::LEU2*	59	0	5	3.9
	cdc68-1 - ade5	44	0	20	15.6
	cdc68-1 - cyh2	11	2	51	*57.3
	KEX1::LEU2 - ade5	41	0	23	18.0
	KEX1::LEU2 - cyh2	11	2	51	*57.3
	cyh2 - ade5	9	5	50	*86.1

The *CDC68* gene was mapped to chromosome VII by hybridization to a Southern blot of electrophoretically separated intact yeast chromosomes (Fig. 4). The *cdc68-1* mutation was then positioned relative to other chromosome VII markers by crossing *cdc68-1* mutant strains with mapping strains (listed in Table 2) bearing appropriate markers (Fig. 4) and analysing the resulting tetrads. PD, parental ditype; NPD, non-parental ditype; TT, tetratype. The distance in centiMorgans (cM) was calculated by the equation of

Perkins (1949); $X_p = [(TT + 6NPD) \times 100] / 2 (PD + NPD + TT)$. Distances over 35 cM were adjusted by the formula of Ma and Mortimer (1983); $X_e = (80.7 X_p - 0.833 X_p^2) / (83.3 - X_p)$. Values adjusted using this formula are indicated by *.

The average ratio of cM/kbp for the yeast genome is 0.34 (Mortimer *et al.*, 1989). Assuming this ratio is representative of that at the *ade5-cdc68-1-KEX1::LEU2* region, these map distances predict that the *CDC68* gene lies approximately 11.5 kbp away from *KEX1::LEU2* and 46 kbp from *ade5*. Comparison of the restriction map of the cloned *KEX1* gene (Dmochowska *et al.*, 1987) with that of *CDC68* did not reveal any overlap.

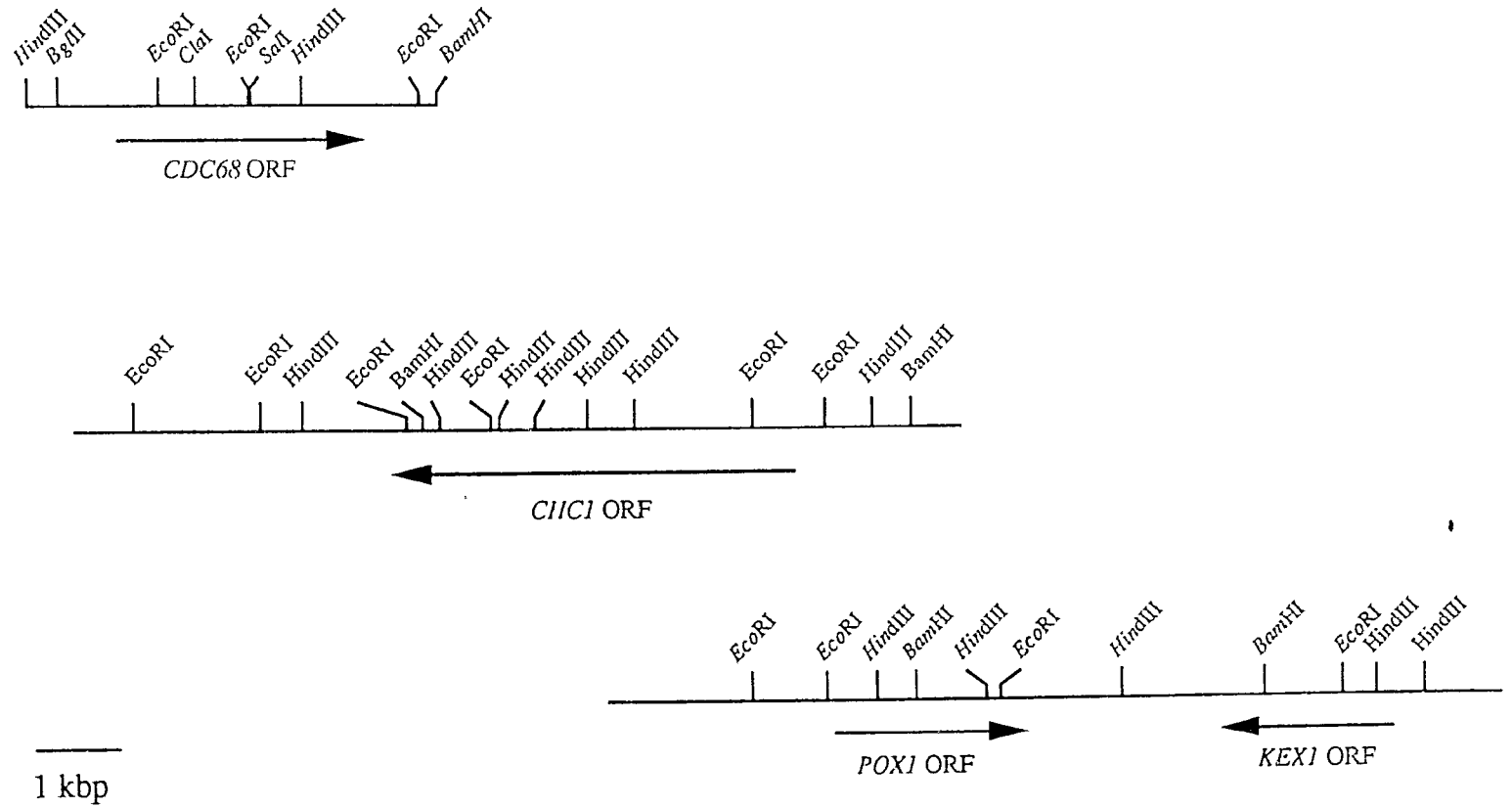
At the the time these data were obtained they did not suggest alleleism with any previously mapped gene. Subsequently the map position of the *CHC1* gene (encoding the clathrin heavy chain) was published independently by two groups: Payne *et al.* (1987) localized a *URA3* marker integrated immediately 3' to the *CHC1* gene to 23 cM from *ade5*; Lemmon *et al.* (1990) later reported distances of 2 cM between *CHC1* (marked by adjacent integration of a *LEU2* gene) and *KEX1*, and 16 cM between the marked *CHC1* locus and *ade5*. These data are similar to those obtained for the *cdc68-1* mutation. Comparison of the *CHC1* (Lemmon *et al.*, 1987; Payne *et al.*, 1987) and *CDC68* restriction maps showed that *CHC1* and *CDC68* are different genes, although their flanking sequences could be overlapped (Fig. 5). Overlap of the *KEX1* and *CHC1* restriction maps was previously noted by Lemmon *et al.* (1990).

During the course of these studies, independent mapping of the *spt16-197* temperature-sensitive mutation suggested that this mutation could be an allele of the *CDC68* gene. Further genetic analysis indicated that *spt16-197* and *cdc68-1* mutations fail to complement one another when combined in a heterozygous diploid, and exhibit tight linkage in tetrad analysis (Malone *et al.*, submitted). These genetic criteria therefore establish

Figure 5. Restriction maps of *CDC68*, *CHC1* and *KEX1*.

Overlap between the restriction maps of the *CHC1* and *KEX1* genes was previously noted by Lemmon *et al.* (1990). The *CHC1* restriction map and position of the coding region were adapted from the map of pSL6 described by Lemmon and Jones (1987), and from the *CHC1* gene sequence (Lemmon *et al.*, 1990). The *KEX1* and *POX1* restriction maps and open reading frame position were adapted from the map of pAD17 as determined by Dmochowska *et al.* (1990), and from the sequences of the *KEX1* and *POX1* genes (Dmochowska *et al.* 1987; 1990).

Figure 5.


HindIII
BglII
EcoRI
ClaI
EcoRI
SalI
HindIII
EcoRI
BamHI
CDC68 ORF
EcoRI
EcoRI
HindIII
EcoRI
BamHI
HindIII
EcoRI
HindIII
HindIII
HindIII
HindIII
EcoRI
EcoRI
HindIII
BamHI
CIIC1 ORF
EcoRI
EcoRI
HindIII
BamHI
HindIII
EcoRI
HindIII
BamHI
EcoRI
HindIII
HindIII
POX1 ORF
KEX1 ORF
1 kbp

that the *cdc68-1* and *spt16-197* mutations define the same gene. Further evidence for this conclusion, together with the characteristics of the *spt16-197* mutant phenotype, is discussed below.

5. *SPT16* and *CDC68* are the same gene

Genetic evidence described above indicates that the *spt16-197* mutation is allelic to *cdc68-1*. Comparison of the restriction maps of both cloned genes (this study; Malone *et al.*, submitted) reveals only slight differences and therefore supports the conclusion that *CDC68* and *SPT16* are the same gene. Consistent with this conclusion, transformation of strain L577 (relevant genotype: *spt16-197 ura3-52*) (kindly provided by E. A. Malone and F. Winston) with p68-Ba-1A (YEp352 containing the *CDC68* gene [see section 9 below]) demonstrates that the cloned *CDC68* gene complements the temperature sensitivity imposed by the *spt16-197* mutation. Similarly, the cloned *SPT16* gene complements the *cdc68-1* temperature-sensitive mutation (Malone *et al.*, submitted). Thus, on the basis of both genetic and physical evidence, *CDC68* and *SPT16* are the same gene.

6. Suppression of δ-insertion mutations by *SPT16* and *spt16-197*

The *SPT16* gene was originally isolated as a high-copy suppressor of *his4-912δ* and *lys2-128δ* mutations (*SPT16* is contained in the class 1 inserts described by Clark-Adams *et al.* [1988]). At the *his4-912δ* and *lys2-128δ* mutant loci, solo δ insertion elements interrupt 5' sequences of the *HIS4* or *LYS2* genes (Chaleff and Fink, 1980; Farabaugh and Fink, 1980; Simchen *et al.*, 1984; Clark-Adams and Winston, 1987). Solo δ elements are 300-bp insertion sequences found in approximately 100 copies in the *S. cerevisiae*

genome (Cameron *et al.*, 1979). These insertion sequences are thought to arise from the excision of a larger yeast transposable element, the Ty element. In Ty elements, direct repeats of so-called δ sequences flank internal Ty sequences; recombination between these direct repeats can result in the excision of most of the element leaving behind only a solo δ element (Farabaugh and Fink, 1980). The δ-insertion mutations in *his4-912δ* or *lys2-128δ* disrupt expression of downstream *HIS4* and *LYS2* coding sequences, rendering mutant cells unable to grow on media lacking histidine or lysine (Silverman and Fink, 1984; Clark-Adams and Winston, 1987). The presence of a wild-type *SPT16* gene in high copy permits sufficient expression, from the otherwise non-functional *his4-912δ* and *lys2-128δ* genes, to allow transformants to grow on media lacking histidine and lysine (Malone *et al.*, submitted).

In addition to the suppression of *lys2-128δ* and *his4-912δ* by increased *SPT16* copy number, the *spt16-197* mutation has also been found to suppress δ-insertion mutations when present either in single or multiple copy (Malone *et al.*, submitted). Suppression of δ-insertion mutations by *spt16-197* was previously documented at a temperature of 30°C, a permissive temperature for the *spt16-197* mutation. To determine the range of temperatures over which this mutation suppresses *his4-912δ* and *lys2-128δ*, the phenotype of isogenic strains L577 (*spt16-197 lys2-128δ his4-912δ*), FY56 (*lys2-128δ his4-912δ*) and FY56 transformed with p68-Ba-1A (containing a complete *CDC68* gene) was examined over a range of temperatures (Table 5). Suppression of *lys2-128δ* and *his4-912δ* by the *spt16-197* mutation was observed only in cells incubated at 30°C or above; below this temperature the phenotype of *spt16-197* mutant cells was indistinguishable from that of

Table 5. Suppression of *his4-912δ* and *lys2-128δ* caused by mutation or overexpression of the *CDC68/SPT16* gene.

TEMP.	MEDIUM	STRAIN			
		FY56 (*SPT16*)	L557 (*spt16-197*)	FY56 (+ *CDC68*)	L557 (+ *CDC68*)
14°C	YEPD	+	+	ND	ND
	C-lys	-	-	ND	ND
	C-his	-	-	ND	ND
23°C	YEPD	+	+	+	+
	C-lys	-	-	+	+
	C-his	-	-	+	+
29°C	YEPD	+	+	+	+
	C-lys	-	+/-	+	+
	C-his	-	-	+	+
30°C	YEPD	+	+	+	+
	C-lys	-	+	+	+
	C-his	-	+	+	+
37°C	YEPD	+	-	+	+

The wild-type FY56 (*his4-912δ, lys2-128δ*) and mutant L557 (*spt16-197 his4-912δ lys2-128δ*) strains were grown on rich medium at 23°C and replica-plated to media incubated at various temperatures as shown. FY56 (+ *CDC68*) and L557 (+ *CDC68*) refers to strains FY56 and L557 transformed with plasmid p68-Ba-1A (containing the entire *CDC68* gene cloned into the high-copy vector YEp352).

+ indicates growth; +/-, poor growth; -, no growth; and ND, not determined.

strain FY56 (containing a wild-type *SPT16* gene) on media lacking lysine and histidine. This was not the case with cells in which the *CDC68* gene was present in elevated copy; suppression of *his4-912δ* and *lys2-128δ* in this situation was observed at all temperatures examined. Presumably, only at temperatures of 30°C and above, is the *spt16-197* mutant gene product sufficiently enfeebled for the δ–suppression phenotype to be manifest.

7. The *cdc68-1* mutation can also suppress δ-insertion mutations

The *cdc68-1* and *spt16-197* mutations were, as described above, isolated independently using very different selection schemes. It was of interest, therefore, to determine whether the *cdc68-1* mutation can suppress δ-insertion mutations, and if so, whether the pattern of suppression is the same as that caused by the *spt16-197* mutation. To address this question, strain FY56 (*his4-912δ lys2-128δ*) was independently crossed with strains ART68-1 (*cdc68-1*) and ARI68-7 (containing a *URA3* gene integrated at the *cdc68-1* mutant locus; see section 3 above) . Segregation of *his4-912δ* and *lys2-128δ* was assessed at both 23°C and 30°C. Segregation of the *cdc68-1* mutation was assessed at 37°C. At 23°C, requirement for both histidine and lysine segregated independently with 2:2 Lys^+/Lys^- and His^+/His^- segregation, consistent with no δ-element suppression at this temperature. At 30°C, in both crosses, the frequency of His^- and Lys^- segregants was lower than at 23°C; furthermore, all segregants exhibiting a His^- phenotype at 23°C but a His^+ phenotype at 30°C contained the *cdc68-1* mutation. The same was true of segregants that became Lys^+ at 30°C, demonstrating that the *cdc68-1* mutation, like *spt16-197*, can suppress δ-insertion mutations. However, approximately 50% of *cdc68-1 his4-912δ* and *cdc68-1 lys2-128δ* segregants

unexpectedly exhibited a His$^-$ or Lys$^-$ phenotype at 30°C. This was also the case when strain L577 (*spt16-197 his4-912δ lys2-128δ*, isogenic with FY56) was crossed with 21R (*CDC68* but otherwise congenic with ART68-1 and ARI68-7), suggesting that the inconsistent suppression of *his4-912δ* and *lys2-128δ* mutations by the *cdc68-1* mutation is due to differences between the genetic backgrounds of strains used for this analysis. Backcrossing strains FY56 and 21R prior to crossing with the *cdc68-1* mutant strain described above has resolved these inconsistencies (Q. Xu, personal communication). Thus two independently identified *cdc68/spt16* mutations mediate similar suppression of *his4-912δ* and *lys2-128δ* δ–insertion mutations.

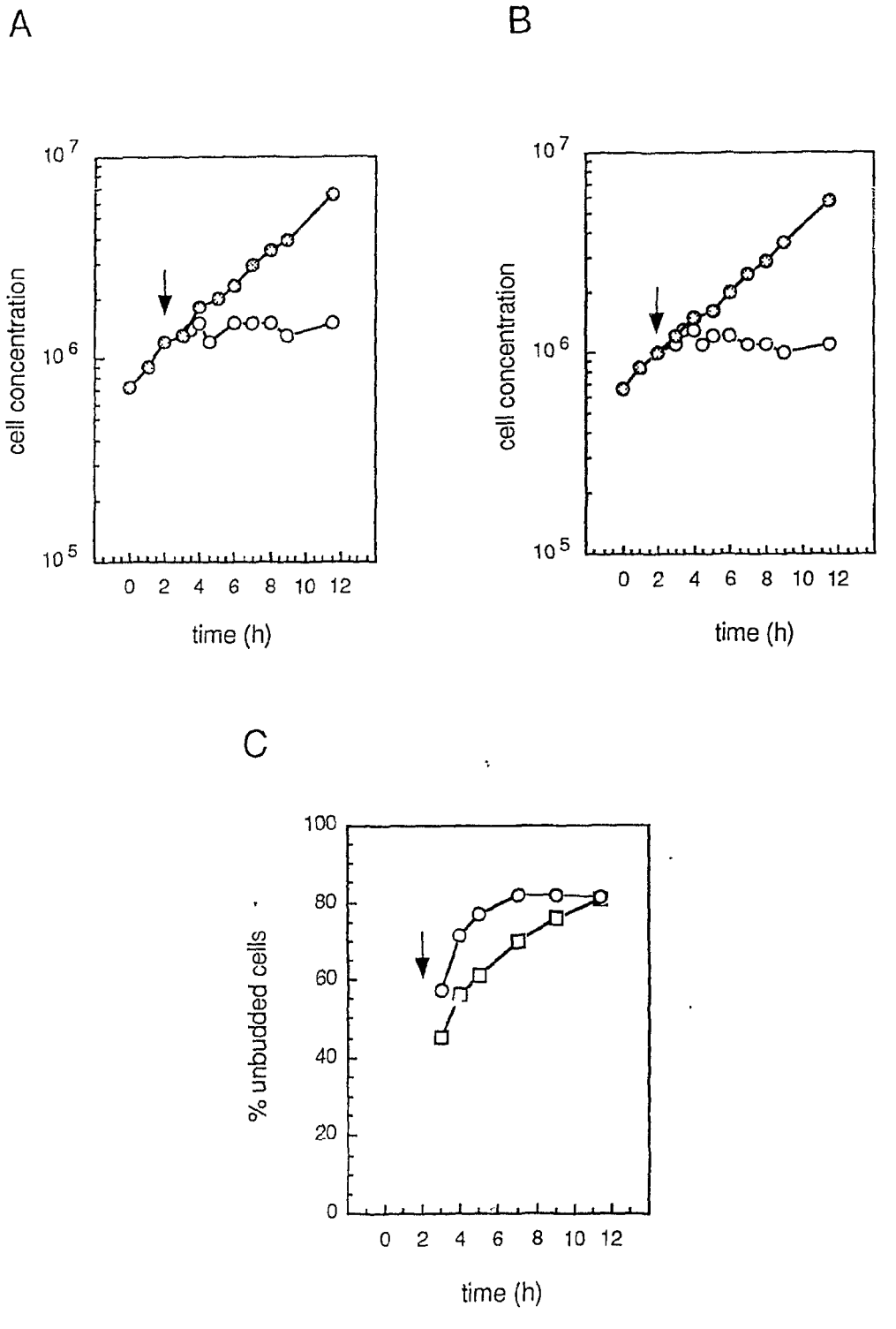
8. *spt16-197* mutant cells arrest in G1 at the restrictive temperature

S. cerevisiae cells containing the *cdc68-1* mutation are temperature-sensitive for the performance of the G1 regulatory event, Start. It was therefore of interest to determine whether, like *cdc68-1*, the *spt16-197* mutation caused cells to arrest within the G1 interval of the cell cycle after transfer to the restrictive temperature. Cells harbouring the *spt16-197* mutation (strain L577), when transferred from the permissive temperature (23°C) to the restrictive temperature (37°C), were found to arrest proliferation as unbudded cells, with less than a two-fold increase in cell concentration (Fig. 6). Thus, like *cdc68-1* mutant cells, cells bearing the *spt16-197* mutation are conditionally defective in one or more processes necessary for the completion of the G1 interval.

Figure 6. The *spt16-197* mutation causes cell-cycle arrest at Start.

Actively dividing cells of strain ART68-1 (*cdc68-1*) (A and C) and L577 (*spt16-197*) (B and C) growing in YNB+10 at 23°C were either transferred to 37°C at the time indicated by the arrow (open symbols), or incubated further at the permissive temperature (closed symbols). Cultures were monitored for both cell concentration (cells/ml) (A and B) and percentage of unbudded cells (C) as a measure of cell cycle position. In panel C, strain L577 is represented by squares, strain ART68-1 by circles.

Figure 6


A
B
C
cell concentration
10^7
10^6
10^5
0 2 4 6 8 10 12
time (h)
% unbudded cells
100
80
60
40
20
0

9. Subcloning the *CDC68* gene

The 10.3-kbp insert of yeast DNA contained in the fully complementing clone, pLC3-4, was reduced to approximately 5.2 kbp by *Sau*3A subcloning as described above. To further define the limits of the *CDC68* gene, a number of pSC2-1 insert restriction fragments were subcloned into the multiple cloning site of the 2μ-based shuttle vector YEp352. Each subclone was then tested for complemention of the *cdc68-1* and *spt16-197* mutations. Of the 6 plasmids constructed only one, p68-Ba-1A, containing a 5-kbp *Bam*HI restriction fragment, complemented either temperature-sensitive mutation (Fig. 7).

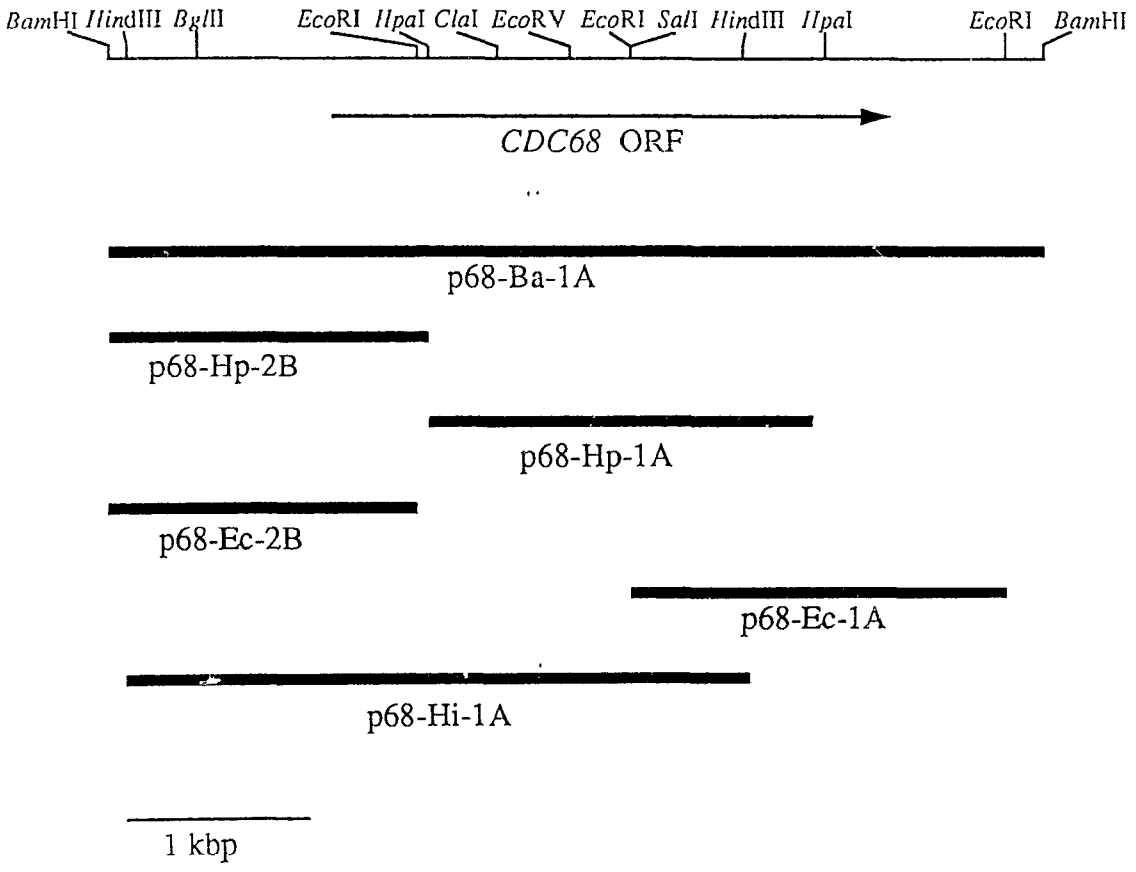
Three of the subclones, although unable to complement either of the temperature-sensitive mutations, gave rise to abnormally frequent "revertant" colonies when transformants were replica-plated to the restrictive temperature (Fig. 8). These three clones all contain sequences to the left of the leftmost *Eco*RI restriction site as presented in Fig. 7. The high frequency of revertant colony formation in these transformants is most easily explained by plasmid insert sequences containing the wild-type sequences corresponding to the *cdc68-1* and *spt16-197* mutations. "Revertant" colony formation might therefore result from reciprocal recombination between insert and genome sequences such that, in a minority of the cells replica-plated to the restrictive temperature, a fully wild-type *CDC68* gene is reconstructed. This hypothesis was tested by determining whether, as this hypothesis predicts, the ability of cells from such temperature-resistant colonies to proliferate at the restrictive temperature is no longer dependent upon the presence of the plasmid with which the cells were originally transformed. Cells from temperature-

Figure 7. Subclone analysis of the *CDC68* gene.

The restriction map shown is that of pSC2-1, the smallest subclone generated by *Sau*3A subcloning of complementing plasmid pLC3-4. The position of the *CDC68* open reading frame (ORF) was determined by subsequent nucleotide sequencing. The wide bars represent restriction fragments of the pSC2-1 insert that were subcloned into the multiple cloning site of YEp352. Each restriction fragment was excised from pSC2-1 with a single restriction endonuclease. p68-Hp-2B and p68-Ec-2B contain inserts which extend to the left of the pSC2-1 insert, to *Hpa*I or *Eco*RI restriction sites within YEp24 vector sequences.

Each subclone was tested for complementation of the *cdc68-1* and *spt16-197* temperature-sensitive mutations in strains ART68-1 and L577, respectively. The results are shown on the right of the figure. Complemention is shown as +, no complementation as -. R indicates the occurrence of numerous small "revertant" colonies within the background of a temperature-sensitive replica-plated streak, as shown in Fig. 8.

Figure 7.



	Complementation	
	cdc68-1	*spt16-197*
p68-Ba-1A	+	+
p68-Hp-2B	R	R
p68-Hp-1A	-	-
p68-Ec-2B	R	R
p68-Ec-1A	-	-
p68-Hi-1A	R	R

Figure 8. Complementation of *cdc68-1* and *spt16-197* by *CDC68* subclones.

Strains ART68-1 (*cdc68-1 ura3-52*) and L577 (*spt16-197 ura3-52*) were transformed with the subclones shown in Figure 7. Transformants of each strain are shown on a separate plate. Four independent transformants were streaked on selective medium (C-ura) and incubated at 23°C before replica-plating to rich medium incubated at 37°C. The order of transformants on each plate is as follows: 1, p68-Ec-1A; 2, p68-Ec-2B; 3, p68-Hp-1A; 4, p68-Hp-2B; 5, p68-Sa-1A; 6, p68-Hi-1A; and 7, p68-Ba-1A.

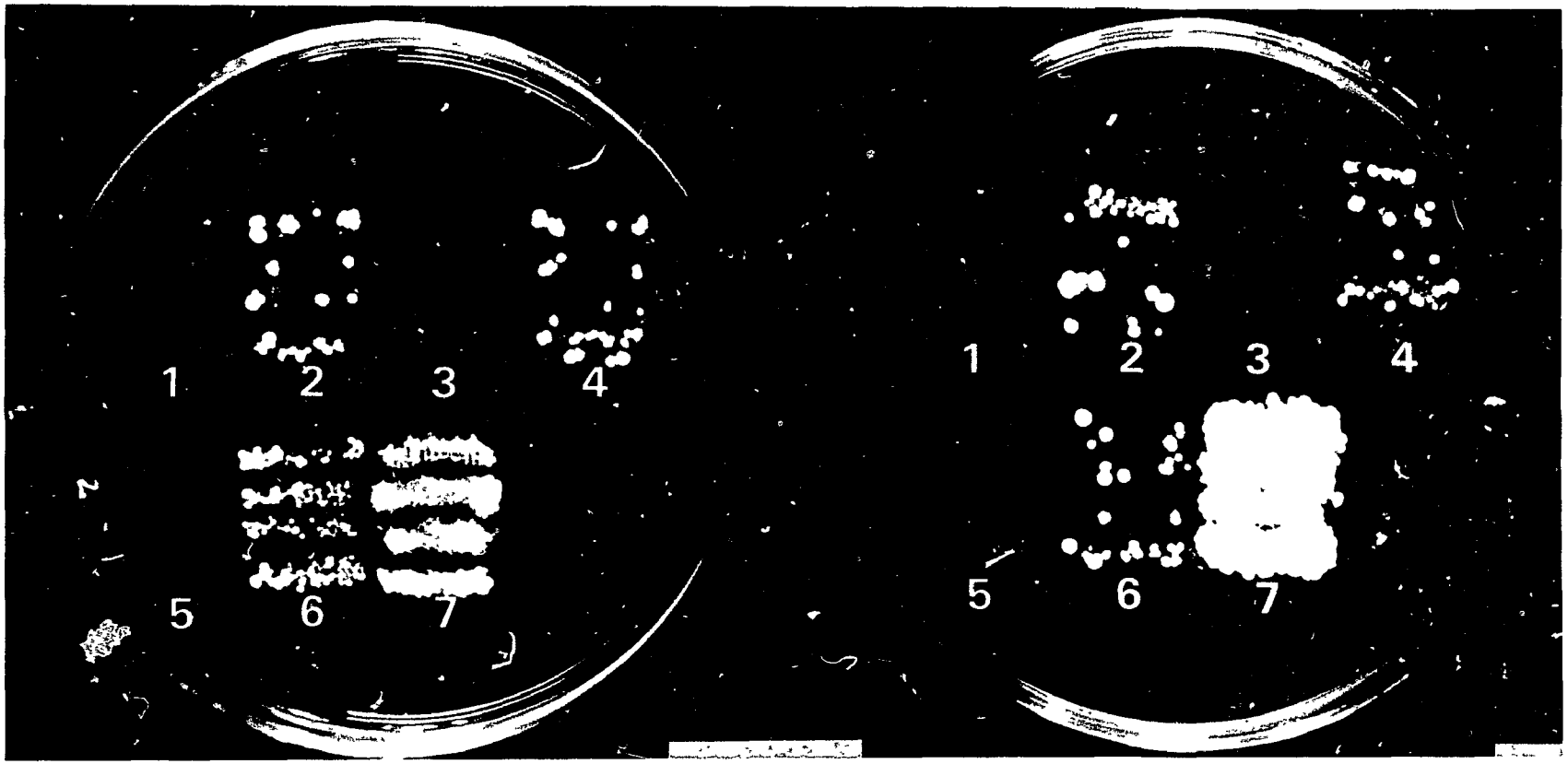


Figure 8.

resistant "revertant" colonies formed following transformation with plasmids p68-Hi-1A, p68-Ec-2B and p68-Hp-2B (Fig. 7) were grown to stationary phase in medium containing 5-fluoroorotic acid (5-FOA). Growth on 5-FOA provides selection for loss of plasmid-borne *URA3* genes (Boeke *et al.*, 1987), *URA3* being the selectable marker carried on vector YEp352. Colonies formed on rich medium following 5-FOA selection were then replica-plated to rich medium incubated at 37°C and to medium lacking uracil. In all three cases some of the colonies that grew at the restrictive temperature were unable to grow on uracil-deficient medium. These results therefore establish that the temperature resistance of the "revertant" cells has become independent of the plasmid with which they were transformed. Plasmids p68-Hi-1A, p68-Ec-2B and p68-Hp-2B must therefore contain wild-type sequences capable of reverting the *cdc68-1* and *spt16-197* mutations, indicating that both mutations lie to the left of the leftmost *Eco*RI site as shown in Fig. 7.

10. Determination of the *CDC68* gene sequence

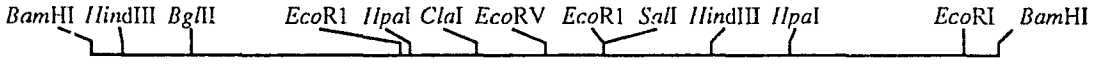
The analysis described above indicated that the *CDC68* gene must occupy most of the 5.0-kbp insert of plasmid p68-Ba-1A. A sequencing strategy was therefore devised in which p68-Ba-1A insert DNA could be cloned into M13 sequencing vectors with the minimum number of overlapping clones (Fig. 9). Unidirectionally deleted templates suitable for sequencing were prepared by ExoIII/mung bean nuclease digestion (see Methods and Materials). The DNA sequence of the *CDC68* gene and flanking regions, together with the amino acid sequence predicted by the *CDC68* open reading frame, is shown in Fig. 10.

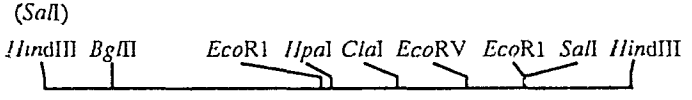
Figure 9. A strategy for sequencing the *CDC68* gene.

The upper restriction map is that of the complementing subclone p68-Ba-1A. The lower restriction map is that of subclone p68-Hi-1A. M13 clones E-3, E-4, E-20 and E-22 were constructed by cloning *Eco*RI-digested p68-Ba-1A fragments into the unique *Eco*RI site of M13 um20. M13 clones SS-4, SS-9 and SS-32 were constructed by cloning of p68-Hi-1A *Sal*1-*Hpa*1 restriction fragments into *Sal*I-*Sma*I digested M13 um20 (SS-4 and SS-9) or M13 um21 (SS-32). The *Sal*I site in brackets is an adjacent site in YEp352 vector sequences. Each M13 clone is shown as a line underneath the restriction map of the clone from which it was constructed. The arrowheads point away from the M13 primer-binding site and therefore indicate the direction in which the clone was sequenced.

Each clone (except SS-4) was deleted using an ExoIII/mung bean nuclease protocol to facilitate sequencing of the entire insert (see Methods and Materials). The *Eco*RI clones E-3, E-4, E-20 and E-21 were digested with *Bam*HI (ExoIII sensitive) and *Pst*I (ExoIII resistant) prior to deletion. SS-9 was digested with *Sal*I (ExoIII sensitive) and *Apa*I (ExoIII resistant), SS-32 was digested with *Asp*718 (an isoschizomer of *Kpn*I and ExoIII sensitive) and *Sst*I (ExoIII resistant). Clone SS-4, which spans the *Eco*RI site between clones E-20 and E-22 and clones E-3 and E-4, was constructed to confirm that the inserts of these clones are indeed contiguous in the *CDC68* gene; SS-4 was not deleted, only a limited amount of sequence was obtained from this clone. This cloning and deletion strategy allowed determination of the complete *CDC68* nucleotide sequence, in addition to 747 bp and 647 bp of upstream and downstream sequence, respectively, from seven original clones obtained as the products of two ligation reactions.

Figure 9.





CLONE	FRAGMENT	VECTOR
E-3	*Eco*RI-*Eco*RI	um20
E-4	*Eco*RI-*Eco*RI	um20
E-20	*Eco*RI-*Eco*RI	um20
E-22	*Eco*RI-*Eco*RI	um20
SS-9	*Hpa*I-*Sal*I	um20
SS-32	*Sal*I-*Hpa*I	um21
SS-4	*Sal*I-*Hpa*I	um20

1 kbp

Figure 10. The nucleotide sequence of the *CDC68* gene.

The nucleotide sequence of the *CDC68* gene is numbered from the presumptive ATG initiation codon of the open reading frame. The derived amino acid sequence is shown below and is numbered from the initiator methionine. The most acidic region is underlined. The GenBank accession number of the *CDC68* nucleotide sequence is M73533.

Figure 10.

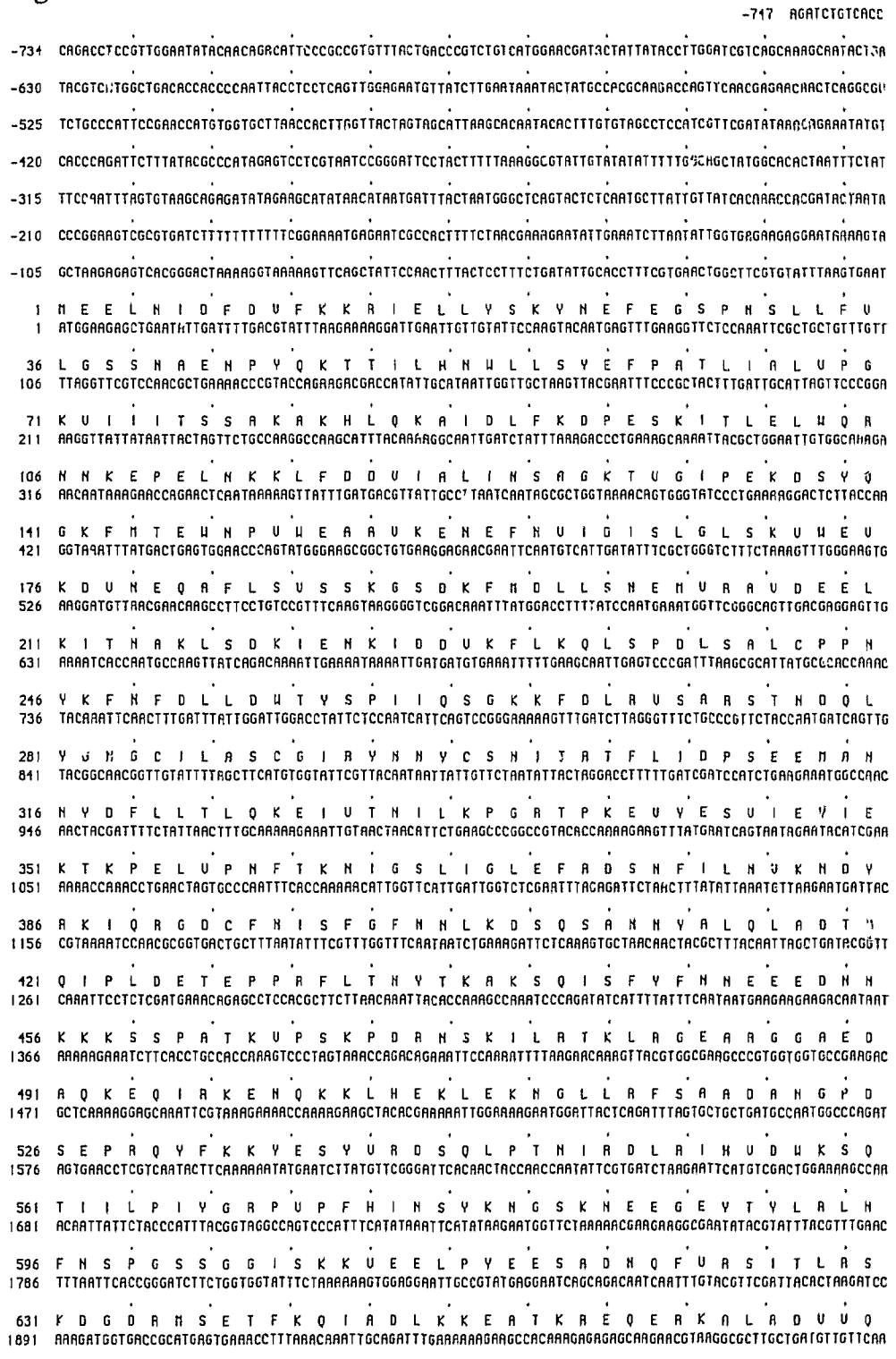
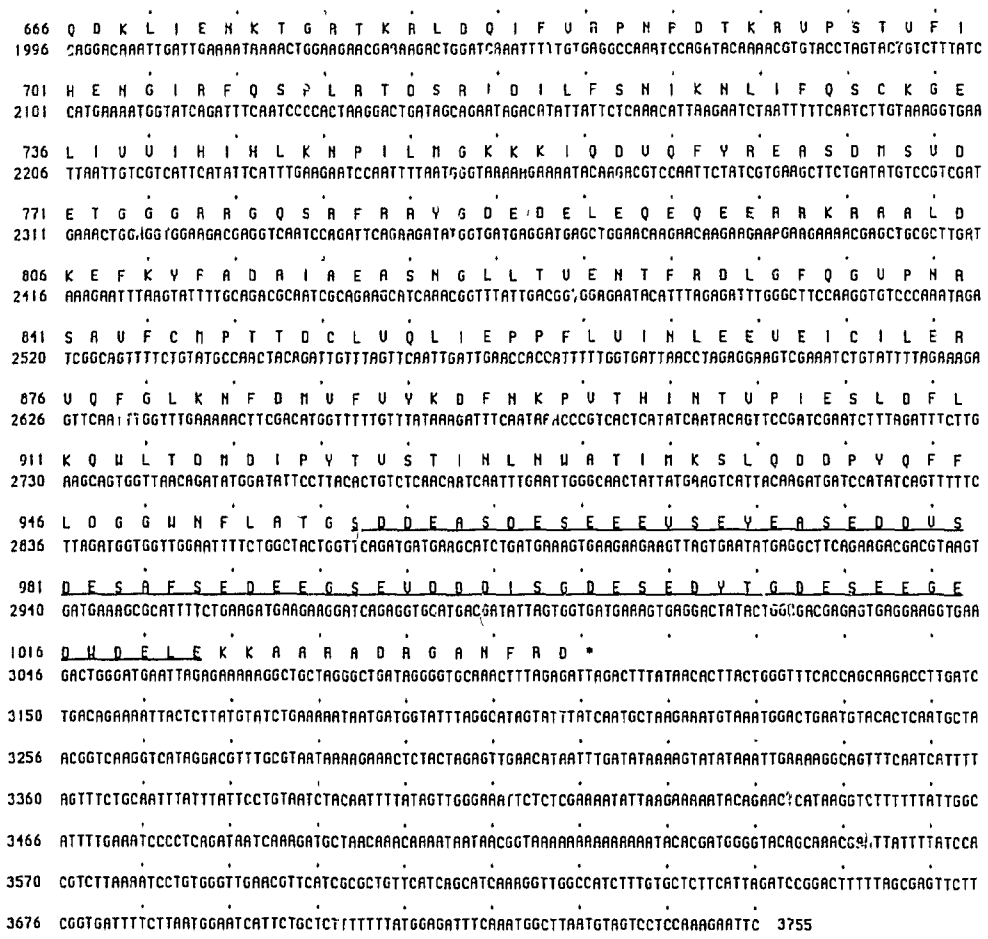

-717 AGATCTGTCACC
-734 CAGACCTCCGTTGGAATATACAACAGACATTCCCGCCGTGTTTACTGACCCGTCTGTCATGGAACGATACTATTATACCTTGGATCGTCAGCAAAGCAATACTGA
-630 TACGTCTTGGCTGACACCACCCCAATTACCTCCTCAGTTGGAGAATGTTATCTTGAATAAATACTATGCCACCGCAAGACCAGTTCAACGAGAACAACTCAGGCGT
-525 TCTGCCCATTCCGAACCATGTGGTGCTTAACCACTTAGTTACTAGTAGCATTAAGCACAATACACTTTGTGTAGCCTCCATCGTTCGATATAAACAGAAATATGT
-420 CACCCAGATTCTTTATACGCCCATAGAGTCCTCGTAATCCGGGATTCCTACTTTTTAAAGGCGTATTGTATATATTTTTGTCHGCTATGGCACACTAATTTCTAT
-315 TTCCAATTTAGTGTAAGCAGAGATATAGAAGCATATAACATAATGATTTACTAATGGGCTCAGTACTCTCAATGCTTATTGTTATCACAAACCACGATACTAATA
-210 CCCGGAAGTCGCGTGATCTTTTTTTTTTCGGAAAATGAGAATCGCCACTTTTCTAACGAAAGAATATTGAAATCTTAATATTGGTGAGAAGAGGAATAAAAGTA
-105 GCTAAGAGAGTCACGGGACTAAAAGGTAAAAAGTTCAGCTATTCCAACTTTACTCCTTTCTGATATTGCACCTTTCGTGAACTGGCTTCGTGTATTTAAGTGAAT
1 M E E L N I D F D V F K K R I E L L Y S K Y N E F E G S P N S L L F V
1 ATGGAAGAGCTGAATATTGATTTTGACGTATTTAAGAAAAGGATTGAATTGTTGTATTCCAAGTACAATGAGTTTGAAGGTTCTCCAAATTCGCTGCTGTTTGTT
36 L G S S N A E N P Y Q K T T I L H N W L L S Y E F P A T L I A L V P G
106 TTAGGTTCGTCCAACGCTGAAAACCCGTACCAGAAGACGACCATATTGCATAATTGGTTGCTAAGTTACGAATTTCCCGCTACTTTGATTGCATTAGTTCCCGGA
71 K V I I I T S S A K A K H L Q K A I D L F K D P E S K I T L E L W Q R
211 AAGGTTATTATAATTACTAGTTCTGCCAAGGCCAAGCATTTACAAAAGGCAATTGATCTATTTAAAGACCCTGAAAGCAAAATTACGCTGGAATTGTGGCAWAGA
106 N N K E P E L N K K L F D D V I A L I N S A G K T V G I P E K D S Y Q
316 AACAATAAAGAACCAGAACTCAATAAAAAGTTATTTGATGACGTTATTGCCTTAATCAATAGCGCTGGTAAAACAGTGGGTATCCCTGAAAAGGACTCTTACCAA
141 G K F M T E W N P V W E A A V K E N E F N V I G I S L G L S K V W E V
421 GGTAAATTTATGACTGAGTGGAACCCAGTATGGGAAGCGGCTGTGAAGGAGAACGAATTCAATGTCATTGATATTTCGCTGGGTCTTTCTAAAGTTTGGGAAGTG
176 K D V N E Q A F L S V S S K G S D K F M D L L S N E M V R A V D E E L
526 AAGGATGTTAACGAACAAGCCTTCCTGTCCGTTTCAAGTAAGGGGTCGGACAAATTTATGGACCTTTTATCCAATGAAATGGTTCGGGCAGTTGACGAGGAGTTG
211 K I T N A K L S D K I E N K I D D V K F L K Q L S P D L S A L C P P N
631 AAAATCACCAATGCCAAGTTATCAGACAAAATTGAAAATAAAATTGATGATGTGAAATTTTTGAAGCAATTGAGTCCCGATTTAAGCGCATTATGCGCACCAAAC
246 Y K F N F D L L D W T Y S P I I Q S G K K F D L R V S A R S T N D Q L
736 TACAAATTCAACTTTGATTTATTGGATTGGACCTATTCTCCAATCATTCAGTCCGGGAAAAAGTTTGATCTTAGGGTTTCTGCCCGTTCTACCAATGATCAGTTG
281 Y G M G C I L A S C G I R Y N N Y C S N I I A T F L I D P S E E M A N
841 TACGGCAACGGTTGTATTTTAGCTTCATGTGGTATTCGTTACAATAATTATTGTTCTAATATTACTAGGACCTTTTTGATCGATCCATCTGAAGAAATGGCCAAC
316 N Y D F L L T L Q K E I V T N I L K P G R T P K E V Y E S V I E Y I E
946 AACTACGATTTTCTATTAACTTTGCAAAAAGAAATTGTAACTAACATTCTGAAGCCCGGCCGTACACCAAAAGAAGTTTATGAATCAGTAATAGAATACATCGAA
351 K T K P E L V P N F T K N I G S L I G L E F R D S N F I L N V K N D Y
1051 AAAACCAAACCTGAACTAGTGCCCAATTTCACCAAAAACATTGGTTCATTGATTGGTCTCGAATTTACAGATTCTAACTTTATATTAAATGTTAAGAATGATTAC
386 R K I Q R G D C F N I S F G F N N L K D S Q S A N N Y A L Q L A D T Y
1156 CGTAAAATCCAACGCGGTGACTGCTTTAATATTTCGTTTGGTTTCAATAATCTGAAAGATTCTCAAAGTGCTAACAACTACGCTTTACAATTAGCTGATACGGTT
421 Q I P L D E T E P P R F L T N Y T K A K S Q I S F Y F N N E E E D N N
1261 CAAATTCCTCTCGATGAAACAGAGCCTCCACGCTTCTTAACAAATTACACCAAAGCCAAATCCCAGATATCATTTTATTTCAATAATGAAGAAGAAGACAATAAT
456 K K K S S P A T K V P S K P D R N S K I L R T K L R G E A R G G A E D
1366 AAAAAGAAATCTTCACCTGCCACCAAAGTCCCTAGTAAACCAGACAGAAATTCCAAAATTTTAAGAACAAAGTTACGTGGCGAAGCCCGTGGTGGTGCCGAAGAC
491 A Q K E Q I R K E N Q K K L H E K L E K N G L L R F S A A D A N G P D
1471 GCTCAAAAGGAGCAAATTCGTAAAGAAAACCAAAAGAAGCTACACGAAAAATTGGAAAAGAATGGATTACTCAGATTTAGTGCTGCTGATGCCAATGGCCCAGAT
526 S E P R Q Y F K K Y E S Y V R D S Q L P T N I R D L R I N V D W K S Q
1576 AGTGAACCTCGTCAATACTTCAAAAAATATGAATCTTATGTTCGGGATTCACAACTACCAACCAATATTCGTGATCTAAGAATTCATGTCGACTGGAAAAGCCAA
561 T I I L P I Y G R P V P F H I N S Y K N G S K N E E G E Y T Y L R L N
1681 ACAATTATTCTACCCATTTACGGTAGGCCAGTCCCATTTCATATAAATTCATATAAGAATGGTTCTAAAAACGAAGAAGGCGAATATACGTATTTACGTTTGAAC
596 F N S P G S S G G I S K K V E E L P Y E E S A D N Q F V R S I T L R S
1786 TTTAATTCACCGGGATCTTCTGGTGGTATTTCTAAAAAAGTGGAGGAATTGCCGTATGAGGAATCAGCAGACAATCAATTTGTACGTTCGATTACACTAAGATCC
631 K D G D R M S E T F K Q I A D L K K E A T K A E Q E R K A L A D V V Q
1891 AAAGATGGTGACCGCATGAGTGAAACCTTTAAACAAATTGCAGATTTGAAAAAAGAAGCCACAAAGAGAGAGCAAGAACGTAAGGCGCTTGCTGATGTTGTTCAA

Figure 10 (Cont.)



11. Analysis of the *CDC68* nucleotide sequence

The largest open reading frame within the sequenced region is 3105 bp long and has the potential to encode a 1035 amino acid protein. The position of this open reading frame is consistent with the subclone data presented in Fig. 7 and must therefore encode the Cdc68 protein.

The ATG codon designated +1 in Fig. 10 almost certainly corresponds to the translation start site, since the next downstream methionine codon is located considerably further downstream at nucleotide +429. It is unlikely that a gene would be preceded by such a long region of untranslated open reading frame. Comparison of the context of the ATG codon at +1 with the proposed consensus for yeast initiation codons (Hamilton *et al.*, 1987; Cigan and Donahue, 1987) shows that the A at -3, found in 75% of yeast translational initiation regions, is conserved in the *CDC68* gene. The G residue at +4 in the *CDC68* initiation region is somewhat more unusual for yeast, although preferred in higher eukaryotes, but was still found in 24% of yeast translational initiation regions examined by Cigan and Donahue (1987). Thus, the context of the putative *CDC68* initiation codon is consistent with those of other yeast genes.

The DNA sequence upstream of the *CDC68* ORF is moderately AT-rich (62%), consistent with the nature of sequences found upstream of most yeast protein-coding genes. Closest matches to the TATAA consensus sequence, at which TATA-binding factor TFIID may recognize the DNA template, are located at -180 (TAATA) and -12 (TATTTA) in 5' *CDC68* sequence. In yeast genes, TATA boxes are generally found between 40 and 120 bp upstream of the mRNA initiation site (Struhl, 1989), therefore the second of these TATA-like sequences is probably too close to the *CDC68* ORF

to serve as the TFIID binding site. Also upstream of the putative *CDC68* ORF is an 11-bp poly(dA) sequence. For several yeast genes poly(dA) sequences can serve as the upstream promoter elements required for transcription (Struhl, 1989).

12. Analysis of the predicted *CDC68* gene product

The *CDC68* nucleotide sequence predicts a 1035 amino acid protein of 118,556 Dalton molecular mass. The amino acid composition of the putative Cdc68 protein is listed in Table 6. Overall the predicted protein is highly charged (31.2% charged residues), with 14.2% basic (lysine, arginine and histidine) and 17.0% acidic (aspartic acid, glutamic acid) residues. The predicted isoelectric point of the protein is 4.8. The distribution of charged residues is, however, not random. The distribution of charged residues is illustrated in diagrammatic form in Fig. 11. While the amino-terminal residues from 1 to 451 are moderately charged (26% overall, 12.2% acidic, 13.8% basic), the following sequences, comprising residues 451 to 810, are considerably more charge-dense (37.2%, 15.8% acidic, 21.4% basic). Particularly striking is the density of negatively charged amino acids at the carboxyl-terminal region. Of the 65 residues from amino acid 957 to amino acid 1021, 36 (55%) are acidic and none are basic. Of the remaining 29 residues 12 (18.5%) are serine and 1 is threonine. Contained within this acidic region are numerous consensus recognition sites for casein kinase II phosphorylation (Kuenzel *et al.*, 1987; Marchiori *et al.*, 1988) raising the possibility that the *CDC68* gene product may be phosphorylated by casein kinase II *in vivo*. The physiological significance of casein kinase II-

Table 6. Amino acid usage in the predicted *CDC68* gene product

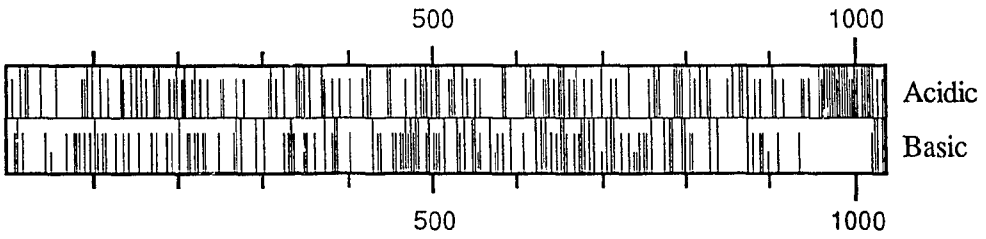
	amino acid	n[1]	n(%)[2]	MW[3]	MW(%)[4]
A	alanine	50	4.8	3551	3.0
C	cysteine	9	0.9	927	0.8
D	aspartic acid	80	7.7	9202	7.8
E	glutamic acid	96	9.3	12388	10.4
F	phenylalanine	55	5.3	8088	6.8
G	glycine	50	4.8	2851	2.4
H	histidine	9	0.9	1233	1.0
I	isoleucine	69	6.7	7802	6.6
K	lysine	87	8.4	11144	9.4
L	leucine	90	8.7	10177	8.6
M	methionine	12	1.2	1572	1.3
N	asparagine	70	6.8	7983	6.7
P	proline	44	4.3	4270	3.6
Q	glutamine	39	3.8	4994	4.2
R	arginine	51	4.9	7961	6.7
S	serine	80	7.7	6962	5.9
T	threonine	47	4.5	4749	4.0
V	valine	53	5.1	5250	4.4
W	tryptophan	11	1.1	2046	1.7
Y	tyrosine	33	3.2	5381	4.5

[1]number of residues
[2]number of residues as a percentage of the total
[3]molecular weight
[4]molecular weight as a percentage of the total

Figure 11. Distribution of charged amino acids in the predicted Cdc68 protein sequence.

The upper lane shows the position in the *CDC68* ORF of acidic amino acids glutamic acid (long vertical bars), aspartic acid (intermediate-length bars). The lower lane indicates the position of basic amino acids: histidine (short bars); lysine (intermediate-length bars) and arginine (long bars). This figure was generated using the sequence analysis programme Strider (Marck, 1988).

Figure 11.



mediated phosphorylation is indicated by the finding that the genes that encode the two subunits of casein kinase II are essential for viability in *S. cerevisiae* (Padmanabha *et al.*, 1990).

The codon usage in the *CDC68* open reading frame is listed in Table 7. The codon bias index, calculated as described by Bennetzen and Hall (1982), is 0.19 (on a scale of 0 to 1). This value predicts that the *CDC68* gene is expressed at a relatively low level. (For comparison *ADH1*, a highly expressed gene encoding alcohol dehydrogenase, has a codon bias index value of 0.92 whereas, the the lower value of 0.15 for the gene encoding iso-2-cytochrome c gene correlates well with the lower levels of this protein in the cell).

13. Database searches with the *CDC68* DNA and predicted amino acid sequences

The *CDC68* DNA and translated amino acid sequences were compared to sequences contained in 4 databases to determine whether *CDC68* is related to any other previously sequenced gene.

Comparison of DNA sequences is often too insensitive to detect similarities between genuinely related sequences for two reasons: the redundant nature of the genetic code and the high probability that unrelated sequences will appear similar due to the limited number of units, A, T, G and C, which comprise DNA sequences. Nevertheless, nucleotide sequence comparisons do reveal sequence identities and for this reason, the nucleotide sequence of the *CDC68* gene and flanking regions was compared with all DNA sequences contained in both GenBank (release number 65) and EMBL (release number 24) databases using the sequence-comparison

Table 7. Codon usage in the predicted *CDC68* gene product

codon	amino acid		number	codon	amino acid		number
TTT	phe	F	38	TCT	ser	S	22
TTC	phe	F	17	TCC	ser	S	11
TTA	leu	L	31	TCA	ser	S	18
TTG	leu	L	27	TCG	ser	S	7
TAT	tyr	Y	18	TGT	cys	C	7
TAC	tyr	Y	15	TGC	cys	C	2
TAA	OCH	Z	-	TGA	OPA	Z	-
TAG	AMB	Z	1	TGG	trp	W	11
CTT	leu	L	5	CCT	pro	P	10
CTC	leu	L	4	CCC	pro	P	7
CTA	leu	L	12	CCA	pro	P	23
CTG	leu	L	11	CCG	pro	P	4
CAT	his	H	8	CGT	arg	R	14
CAC	his	H	1	CGC	arg	R	3
CAA	gln	Q	32	CGA	arg	R	2
CAG	gln	Q	7	CGG	arg	R	2
ATT	ile	I	46	ACT	thr	T	16
ATC	ile	I	14	ACC	thr	T	11
ATA	ile	I	9	ACA	thr	T	14
ATG	met	M	12	ACG	thr	T	6
AAT	asn	N	46	AGT	ser	S	16
AAC	asn	N	24	AGC	ser	S	6
AAA	lys	K	54	AGA	arg	R	22
AAG	lys	K	33	AGG	arg	R	8
GTT	val	V	23	GCT	ala	A	19
GTC	val	V	13	GCC	ala	A	13
GTA	val	V	7	GCA	ala	A	15
GTG	val	V	10	GCG	ala	A	3
GAT	asp	D	54	GGT	gly	G	34
GAC	asp	D	26	GGC	gly	G	7
GAA	glu	E	75	GGA	gly	G	7
GAG	glu	E	21	GGG	gly	G	2

programme FASTA (Pearson and Lipman, 1988).

The following protein sequence databases were searched with the *CDC68* predicted amino acid sequence also using FASTA: GenPept (release number 64.3), NBRF protein (release number 26) and Swiss-Prot (release number 17). The amino acid sequence was further compared with the translation (three forward reading frames) of sequences contained in GenBank (release number 66) and EMBL (release number 25) using TFASTA (Pearson and Lipman, 1988).

The FASTA sequence comparison program (Pearson and Lipman, 1988; Pearson, 1990) uses a series of steps to characterize sequence similarity at either the DNA or amino acid level. In FASTA, the balance between selectivity and sensitivity is set by the KTUP value, which determines how many adjacent residues must be identical in two test sequences before those sequences are considered in the subsequent steps of the program. A high KTUP value demands that several adjacent residues are identical in two sequences and thus specifies a selective search with limited sensitivity, whereas a low KTUP specifies a sensitive but not very selective search. Despite liberal search parameters (KTUP=4 in the DNA database searches, KTUP=2 in the NBRF and TFASTA searches, and KTUP=1 in the GenPept and Swiss-Prot searches) no biologically significant similarity was detected between *CDC68* and any other gene.

14. Overlap between *CDC68* and *URA2*

Although the database searches described above identified no biologically relevant sequence similarities, an apparently artifactual similarity was repeatedly identified in these searches. Sequences from the

upstream regions of the *S. cerevisiae URA2* gene (Souciet *et al.* 1989), were found to share 98.8% identity with the reverse complement of the *CDC68* nucleotide sequence over a 669-nucleotide overlap (Fig. 12). The overlap of *CDC68* and *URA2* upstream sequences falls partly within *CDC68* coding sequences; at the amino acid level the translated *URA2* upstream sequence was 99.4% identical to the predicted amino acid sequence of the *CDC68* gene over a 158 amino acid length.

The identification of sequences practically identical to the *CDC68* gene upstream of *URA2* was surprising, as *cdc68* and *ura2* mutations have been previously mapped to different chromosomes: *cdc68-1* and *spt16-197* have been independently mapped to chromosome VII (this study; Malone *et al.*, submitted), while *ura2* mutations map to chromosome X (Mortimer and Hawthorne, 1975). These mapping data are therefore inconsistent with physical linkage of the *CDC68* and *URA2* loci.

CDC68 and *URA2* were both cloned by complementation using independently constructed libraries (this study; Souciet *et al.*, 1982). In the alignment of the reverse complement of the *CDC68* sequence and the *URA2* sequence it is immediately evident that the sequences diverge at the sequence GATC (Fig. 12) which corresponds to a recognition site for *Sau*3A, the restriction enzyme used to partially digest yeast genomic DNA for construction of both libraries. During construction of the library from which *URA2* was cloned it is likely that two *Sau*3A fragments, one originating from chromosome X and the other from chromosome VII, were inadvertently joined together generating the hybrid clone sequenced by Souciet *et al.* (1989).

Figure 12. Overlap between *CDC68* and sequences cloned upstream of the *S. cerevisiae URA2* gene.

The alignment of the reverse complement of the *CDC68* sequence and sequnces found upstream of the cloned *URA2* gene is shown. The non-coding strand of the *CDC68* sequence is numbered according to the coordinates of the coding (messenger-identical) strand shown in Fig. 10. The *URA2* sequence is numbered as described by Souciet *et al.* (1989). *CDC68* open reading frame sequences are overlined. The *URA2* initiation codon is found downstream of the alignment with *CDC68* sequences and is not shown in this figure. The amino acid substitutions predicted by two of the discrepancies between the *URA2* and *CDC68* sequences are shown above.

Figure 12.

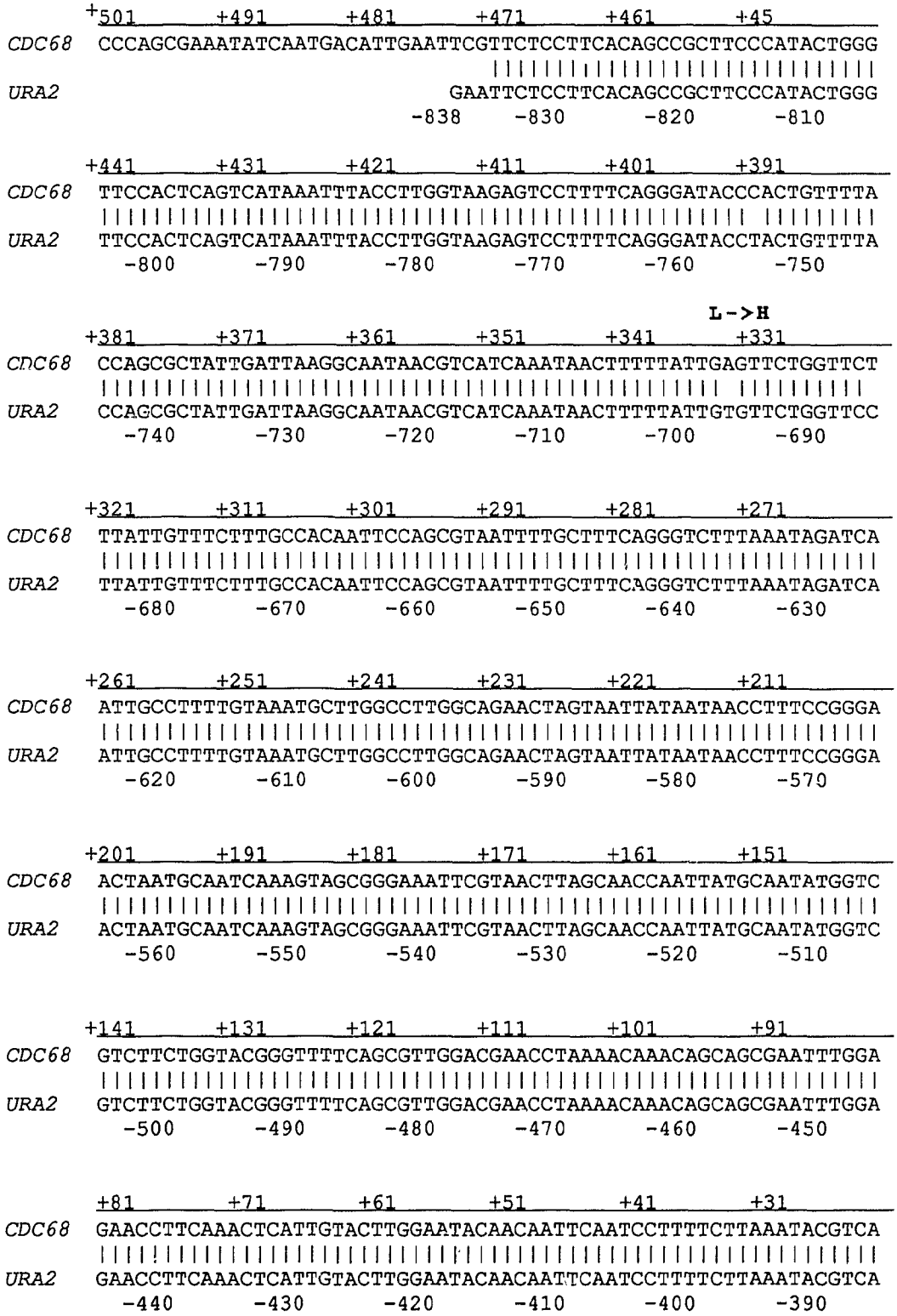
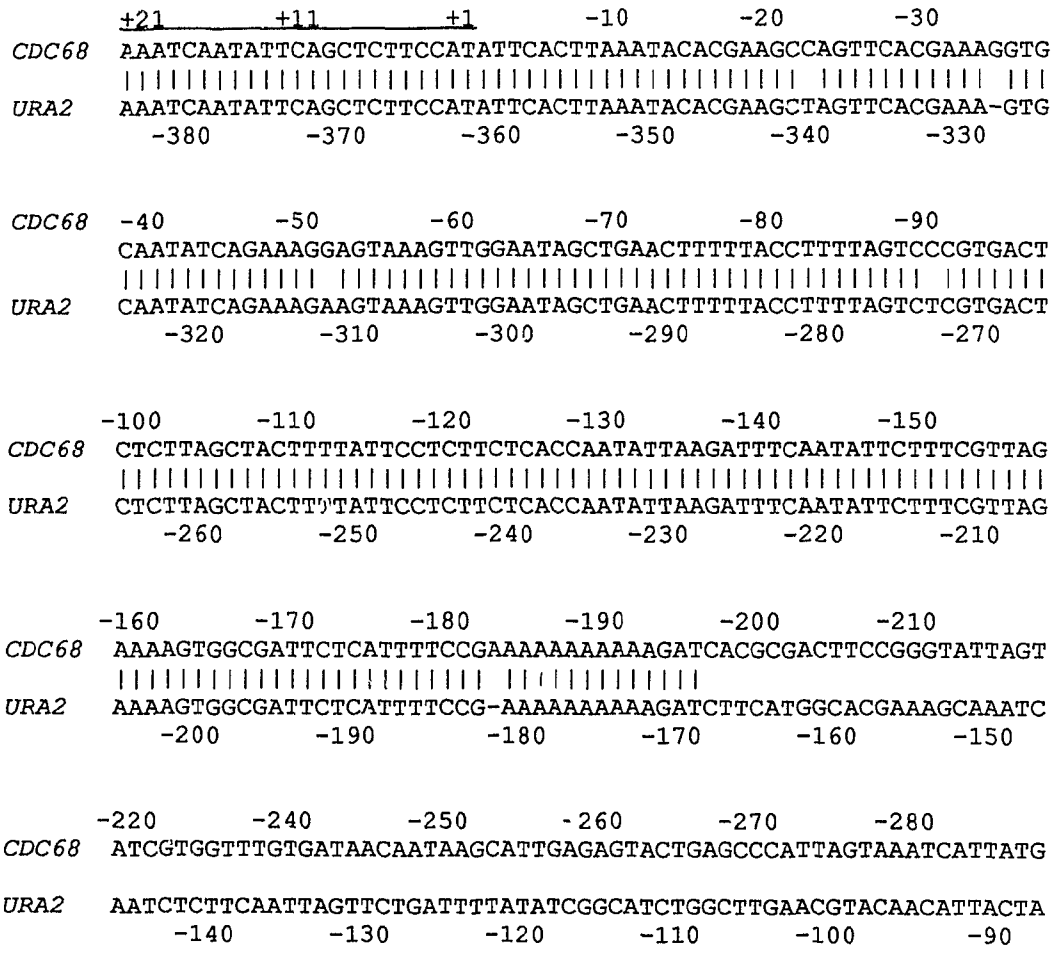

+501 +491 +481 +471 +461 +45
CDC68 CCCAGCGAAATATCAATGACATTGAATTCGTTCTCCTTCACAGCCGCTTCCCATACTGGG
URA2 GAATTCTCCTTCACAGCCGCTTCCCATACTGGG
-838 -830 -820 -810
+441 +431 +421 +411 +401 +391
CDC68 TTCCACTCAGTCATAAATTTACCTTGGTAAGAGTCCTTTTCAGGGATACCCACTGTTTTA
URA2 TTCCACTCAGTCATAAATTTACCTTGGTAAGAGTCCTTTTCAGGGATACCTACTGTTTTA
-800 -790 -780 -770 -760 -750
L->H
+381 +371 +361 +351 +341 +331
CDC68 CCAGCGCTATTGATTAAGGCAATAACGTCATCAAATAACTTTTTATTGAGTTCTGGTTCT
URA2 CCAGCGCTATTGATTAAGGCAATAACGTCATCAAATAACTTTTTATTGTGTTCTGGTTCC
-740 -730 -720 -710 -700 -690
+321 +311 +301 +291 +281 +271
CDC68 TTATTGTTTCTTTGCCACAATTCCAGCGTAATTTTGCTTTCAGGGTCTTTAAATAGATCA
URA2 TTATTGTTTCTTTGCCACAATTCCAGCGTAATTTTGCTTTCAGGGTCTTTAAATAGATCA
-680 -670 -660 -650 -640 -630
+261 +251 +241 +231 +221 +211
CDC68 ATTGCCTTTTGTAAATGCTTGGCCTTGGCAGAACTAGTAATTATAATAACCTTTCCGGGA
URA2 ATTGCCTTTTGTAAATGCTTGGCCTTGGCAGAACTAGTAATTATAATAACCTTTCCGGGA
-620 -610 -600 -590 -580 -570
+201 +191 +181 +171 +161 +151
CDC68 ACTAATGCAATCAAAGTAGCGGGAAATTCGTAACTTAGCAACCAATTATGCAATATGGTC
URA2 ACTAATGCAATCAAAGTAGCGGGAAATTCGTAACTTAGCAACCAATTATGCAATATGGTC
-560 -550 -540 -530 -520 -510
+141 +131 +121 +111 +101 +91
CDC68 GTCTTCTGGTACGGGTTTTCAGCGTTGGACGAACCTAAAACAAACAGCAGCGAATTTGGA
URA2 GTCTTCTGGTACGGGTTTTCAGCGTTGGACGAACCTAAAACAAACAGCAGCGAATTTGGA
-500 -490 -480 -470 -460 -450
+81 +71 +61 +51 +41 +31
CDC68 GAACCTTCAAACTCATTGTACTTGGAATACAACAATTCAATCCTTTTCTTAAATACGTCA
URA2 GAACCTTCAAACTCATTGTACTTGGAATACAACAATTCAATCCTTTTCTTAAATACGTCA
-440 -430 -420 -410 -400 -390

Figure 12. (Cont.)



Eight differences were detected within the alignment of the *URA2*-associated sequences with the reverse complement of *CDC68*. Three of these lie within the complement of *CDC68* coding sequences. The first, at the beginning of the alignment, is a 4-bp deletion in the *URA2*-associated sequence. Comparison of the two sequences in this region suggests that this discrepancy is the result of a sequencing error in the *URA2* upstream sequence (see Fig. 12). The other two coding-sequence differences are 1-bp substitutions which would result in the substitution of a histidine for a leucine at amino acid 112 and only a silent change at amino acid 132 in the Cdc68 protein. These differences may reflect natural polymorphism between the strains from which library sequences were derived. The remaining five differences are found in sequences upstream of both genes and, again, may be a result of natural polymorphism.

15. Identification of consensus recognition sites in the predicted Cdc68 amino acid sequence

Molecular characterization of increasing numbers of genes and the proteins that these genes encode is facilitating increasingly significant correlation of structure and function. In a number of cases particular amino acid sequence "motifs" necessary for recognition by sequence-specific modifying enzymes such as protein kinases have been identified. Searching new amino acid sequences for such previously identified motifs, although time-consuming, has the potential to identify sites at which biologically significant modifications or interactions may take place. The recent compilation of consensus sequences for enzyme recognition and certain types of sequence-specific interactions in the form of a database (PROSITE;

Bairoch, 1990), has increased the speed at which a new sequence can be scanned for such consensus motifs.

The Cdc68 predicted amino acid sequence was compared to all the sites and recognition patterns contained in PROSITE (Table 8). This analysis suggests that the Cdc68 protein could be multiply phosphorylated by several different protein kinases. In addition to computer-assisted inspection of the predicted Cdc68 protein sequence, manual comparison revealed the presence of motifs that meet sequence criteria for phosphorylation by the p34^{cdc2} protein kinase (reviewed by Pines and Hunter, 1990) and are also included in Table 8.

The results of this analysis must however be interpreted with caution since, while consensus sites specify a sequence prerequisite for certain modifications or interactions, such modifications do not necessarily occur at every position at which that sequence is located. Conversely, there is often sufficient flexibility in site recognition that searching for a particular consensus will fail to identify biologically important recognition sites. Nevertheless, as more becomes known about the Cdc68 protein, this analysis will become increasingly significant.

16. Deletion analysis of the *CDC68* gene

a) Partially deleted *CDC68* genes can complement *cdc68* mutations.

In the course of determining the limits of the *CDC68* gene a series of deletions in plasmid p68-Ba-1A was prepared, using *Exo*III exonuclease. Deleted clones were tested for complementation of *cdc68* temperature-sensitive mutations. These results provide interesting insight into the relationship between structure and function of the *CDC68* gene.

Table 8. Consensus recognition sites for post-translational modification in the Cdc68 protein sequence.

Modification	Position	Modification	Position
protein kinase C phosphorylation	78->81 187->190 191->194 218->221 263->266 272->275 337->340 577->580 606->609 627->630 639->642 651->654 674->677 677->680 691->694 731->734 828->831	casein kinase II phosphorylation	39->43 275->279 337->341 344->348 352->356 467->471 517->521 582->586 651->655 715->719 768->772 828->832 915->919 936->940 955->959 957->961 965->969 970->974 975->979 986->990 992->996 999->1003 1007->1011
cAMP-dependent protein kinase phosphorylation	456->460		
tyrosine kinase phosphorylation	12->20 274->282 583->590 607->615 753->762 754->762 935->943	$p34^{cdc2}$ kinase phosphorylation	337->339 709->712

Two series of unidirectional deletions were prepared in plasmid p68-Ba-1A, using MCS sites on either side of the insert to generate *Exo*III-resistant and sensitive sites, facilitating deletion of the insert from both directions. Sequential samples were taken from each reaction to ensure that a series of nested deletions was obtained. Deleted plasmids isolated from individual *E. coli* transformants were screened by digestion with *Pvu*II, which cuts on both sides of the MCS in vector YEp352 but not within the insert of plasmid p68-Ba-1A, to determine the extent of insert deletion in each clone. A selection of these clones were restriction-mapped tested for complementation of the *cdc68-1* and *spt16-197* temperature-sensitive mutations in strains ART68-1 and L577, respectively (Fig. 13).

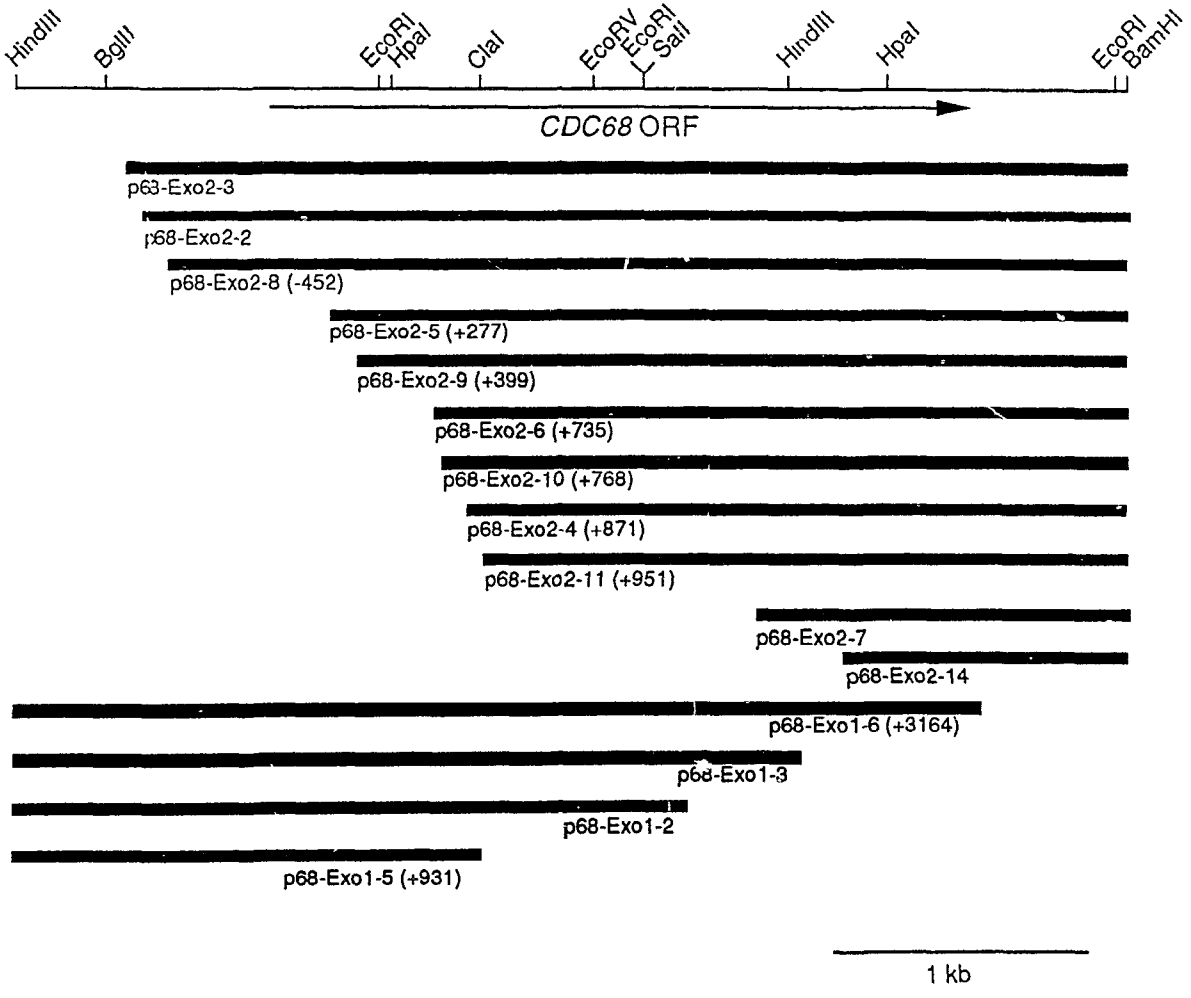
The most striking observation was that six clones deleted for 5'-flanking and N-terminal sequences of the *CDC68* ORF still complement the *cdc68-1* and *spt16-197* mutations. The precise endpoints of these deletions have been confirmed by plasmid sequencing and are indicated in brackets below the appropriate line (Fig. 13). Although the extent of growth seen in cells replica-plated to the restrictive temperature was not equal for all six clones, growth did not correlate with the extent of deletion as shown in Figs. 13 and 14. Complementation by these deleted clones might suggest that the first ATG of the *CDC68* ORF is not used normally in the cell. However, two lines of evidence argue against this possibility. Firstly, as described above (section 9), both the *cdc68-1* and *spt16-197* mutations have been localized upstream of the *Eco*RI site at +475 on the *CDC68* ORF. Four of the six deleted, but complementing, clones are deleted beyond +475. Secondly, Malone *et al.* (submitted) have demonstrated, and it has been confirmed in

Figure 13. Deletion analysis of the *CDC68* gene.

Two series of deletions of the *CDC68* gene were prepared in plasmid p68-Ba-1A: a 3'-to-5' series (series 1) and a 5'-to-3' series (series 2). ExoIII-resistant ends were generated by digestion of plasmid p68-Ba-1A with *Pst*I (series 1) and *Sst*I (series 2); ExoIII-sensitive ends were generated by digestion with *Xba*1 (series 1) and *Sma*I (series 2) (see Methods and Materials for further details). The upper line illustrates the restriction map of the p68-Ba-1A insert and the position of the *CDC68* ORF as determined by nucleotide sequencing. Solid bars below the restriction map indicate insert sequences remaining in each of the deleted clones. For those clones in which the insert-vector junctions have been sequenced, the first nucleotide of the *CDC68* ORF remaining is specified under the line.

Deleted clones were tested for complementation of the *cdc68-1* and *spt16-197* mutations in strains ART68-1 (*cdc68-1 ura3-52*) and L577 (*spt16-197 ura3-52*). Growth of each transformant is indicated as + (some growth at 37°C) or ++ (growth equal to that conferred by p68-Exo2-8, which contains the entire *CDC68* open reading frame). R indicates the formation of "revertant" colonies, as described previously (Figs. 7 and 8). Deleted clones were also tested for the ability to suppress the *his4-912δ* and *lys2-128δ* mutations in strain FY56: +, indicates suppression; -, no suppression. ND, not determined.

Figure 13.



	Complementation		Suppression
	cdc68-1	*spt16 197*	*his4-912δ*
p63-Exo2-3	++	ND	ND
p68-Exo2-2	++	ND	ND
p68-Exo2-8 (-452)	++	++	++
p68-Exo2-5 (+277)	+	+	-
p68-Exo2-9 (+399)	+	+	-
p68-Exo2-6 (+735)	++	++	-
p68-Exo2-10 (+768)	++	++	-
p68-Exo2-4 (+871)	++	++	-
p68-Exo2-11 (+951)	+	+	-
p68-Exo2-7	-	-	ND
p68-Exo2-14	-	-	ND
p68-Exo1-6 (+3164)	+	+	ND
p68-Exo1-3	R	R	ND
p68-Exo1-2	R	R	ND
p68-Exo1-5 (+931)	R	R	ND

Figure 14. Complementation of the *cdc68-1* and *spt16-197* temperature-sensitive mutations by deleted *CDC68* clones.

Strains ART68-1, L577 and FY56 were transformed with plasmids p68-Exo2-10 (Δ+768) (1); p68-Exo2-4 (Δ+871) (2); p68-Exo2-11 (Δ+951) (3);.Ec-4D (Δ+871R, containing the insert of plasmid p68-Exo2-4 but in reverse orientation; see text) (4); p68-Exo2-8 (Δ-452) (5); p68-Exo2-5 (Δ+277) (6); p68-Exo2-9 (Δ+399) (7); and p68-Exo2-6) (8). Transformed strains were grown at 23°C on selective medium (C-ura) before replica-plating to either rich medium subsequently incubated for 3 days at the restrictive temperature of 37°C (ART68-1 and L577) or to selective medium (C-his) subsequently incubated at 30°C for 4 days (L577 and FY56).

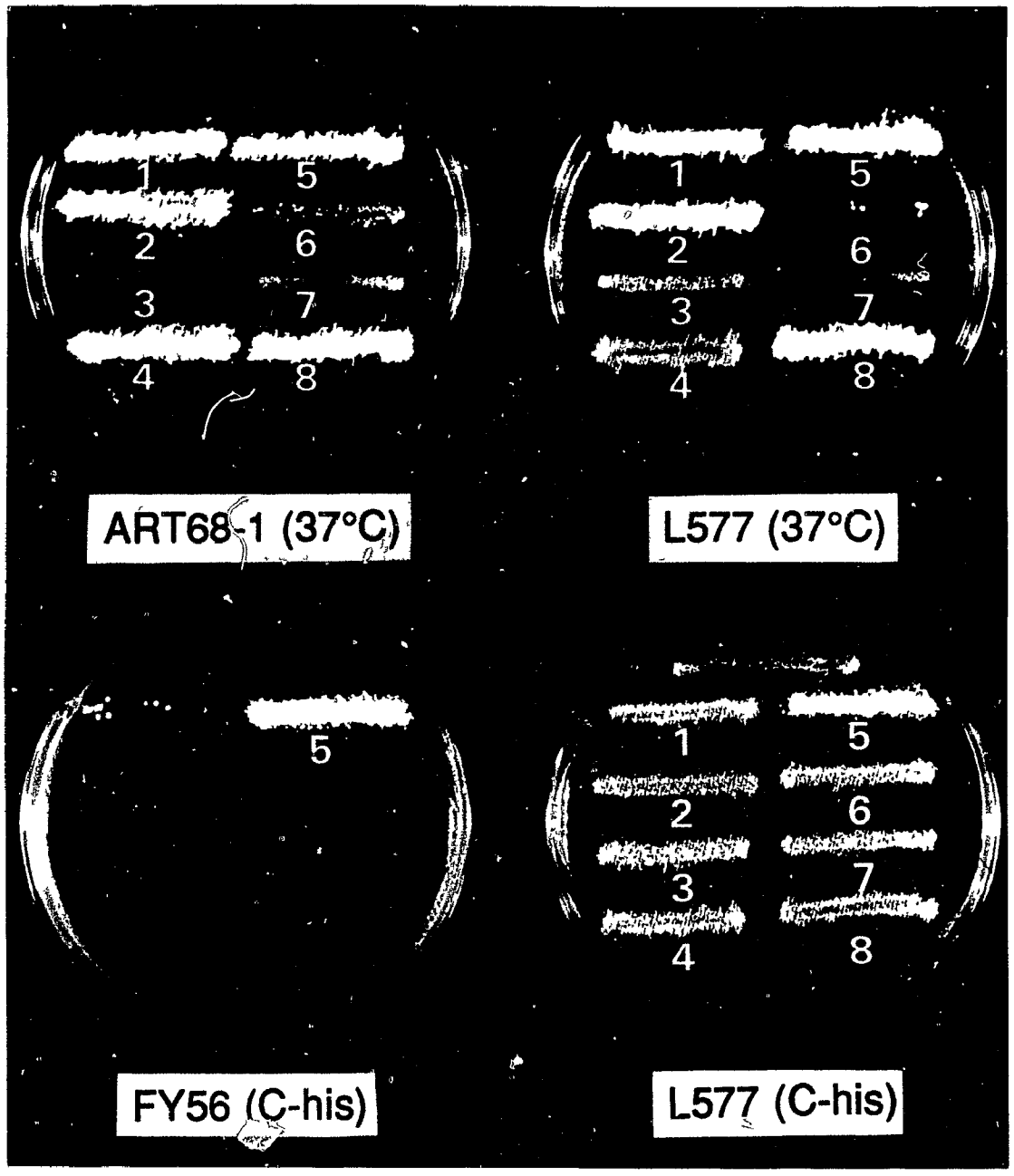

1
5
2
6
3
7
4
8
ART68-1 (37°C)
1
5
2
6
3
7
4
8
L577 (37°C)
5
FY56 (C-his)
1
5
2
6
3
7
4
8
L577 (C-his)

Figure 14.

this study, that the *CDC68* gene on a high-copy vector suppresses δ insertions in *his4-912*δ and *lys2-128*δ. Although able to complement the temperature-sensitive *cdc68-1* and *spt16-197*mutations, none of the six N-terminally deleted clones suppressed *his4-912*δ and *lys2-128*δ and cannot therefore contain the entire *CDC68* gene. This latter finding indicates that, although the N terminus of the *CDC68* gene product is apparently dispensable for complementation of the *cdc68-1* and *spt16-197* mutations, it is essential for δ-element suppression under these circumstances. It is curious that the *cdc68-1* and *spt16-197* mutations cause cells to arrest at the restrictive temperature but can be complemented by versions of the gene which lack that region in which the mutations reside. Thus, a mutation in the N-terminus of the Cdc68 protein is apparently more deleterious than deletion of this entire region.

b) Transcription initiation in partially deleted *CDC68* clones.

In each of the six clones described above the *CDC68* promoter and initiation codon were completely deleted. It is not known, therefore, what sequences serve as a promoter for remaining *CDC68* coding sequences, and where transcription and translation initiate to allow their expression. The promoter could be located either within adjacent vector sequences which might fortuitously function as a promoter in *S. cerevisiae*, or within the *CDC68* sequence itself. In the latter situation this internal promoter might be cryptic, only revealed when upstream sequences are removed. In an attempt to distinguish between these possibilities the insert of one of the above clones, p68-Exo2-4, was recloned in the reverse orientation with respect to adjacent vector sequences. This was accomplished by partially

digesting p68-Exo2-4 with *Eco*RI, excising the smallest partial product from a preparative gel and ligating it into the *Eco*RI site of YEp352. The p68-Exo2-4 *Eco*RI fragment that was recloned retains only 6 bp of adjacent vector sequences from the original orientation (between the *Eco*RI and *Sst*I MCS sites). Recombinant clones prepared from resulting *E. coli* transformants were then screened by restriction endonuclease digestion for clones in which the insert had been cloned in the reverse orientation. One such clone, P68-Ec-4D, transformed into strains ART68-1 (*cdc68-1*), L577 (*spt16-197*) and FY56 (*his4-912δ*, *lys2-128δ*), complemented both temperature-sensitive mutations but, as with p68-Exo2-4, did not suppress *his4-912δ* and *lys2-128δ* δ–insertion mutations. Thus, complementation was not orientation-dependent, at least in this one example, indicating that either fortuitous promoters exist in vector sequences on both sides of the MCS, or that a promoter is located within the remaining *CDC68* sequence.

Transcription from a single internal promoter might be expected to generate a transcript that is the same size in each of the transformants, despite the different extents to which the cloned *CDC68* sequences have been deleted. In contrast, transcripts originating within upstream vector sequences might be expected to decrease in size as the *CDC68* ORF is successively deleted. To distinguish between these two situations, total RNA was isolated from strain ART68-1 (*cdc68-1*) and from ART68-1 transformed with eight of the deleted clones including one, p68-Exo2-8, containing the entire *CDC68* ORF. Transcripts from these deleted clones were examined by northern analysis, as shown in Fig. 15. The probe used was a 1.8-kbp *Hpa*I-*Cla*I fragment common to the *CDC68* ORFs remaining in each of the deleted clones. The transcript patterns seen in the transformants

Figure 15. Northern analysis of *cdc68-1* mutant cells transformed with plasmid-borne *CDC68* deletions.

Total RNA was extracted from 23°C log-phase cultures of strain ART68-1 (*cdc68-1*) and from ART68-1 transformed with plasmids containing deleted versions of the *CDC68* gene. Transformants were grown in YNB+9 medium (without uracil) to provide selection for the plasmids, which contain a *URA3* selectable marker. YNB+9 medium was supplemented with uracil for growth of the untransformed strain ART68-1. The probe was a 1.8-kbp *Hpa*I-*Cla*I fragment from the *CDC68* ORF. Use of this probe allowed comparison of relative transcript intensity, since this restriction fragment was intact in each of the clones analysed here. Lane 1, untransformed; lane2, p68-Exo2-8 (Δ-452); lane3, p68-Exo2-5 (Δ+277); lane4, p68-Exo2-9 (+Δ399); lane5, p68-Exo2-6 (Δ+735); lane 6, p68-Exo2-10 (Δ+768); lane 7, p68-Exo2-4 (Δ+871); lane 8, p68-Ec-4D (Δ+871R [insert of plasmid p68-Exo2-4 in reverse orientation; see text]); lane 9, p68-Exo2-11 (Δ+951).

Size markers shown on the left were provided by an RNA ladder (see Materials and Methods). The positions of the rRNA bands (25s, 3.4 kb; 18s, 1.8 kb [Klootwijk and Planta, 1989]) are indicated on the right.

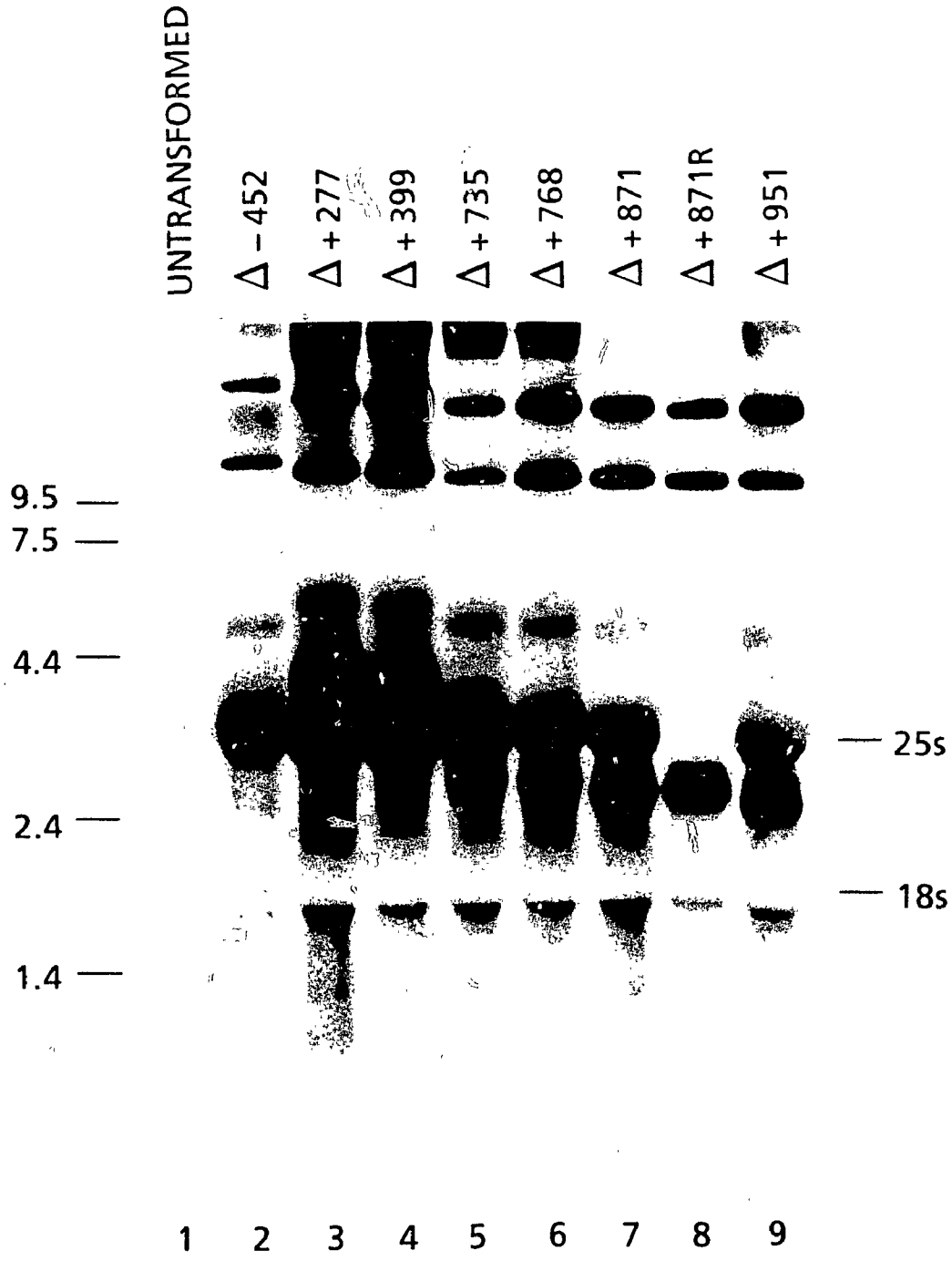

UNTRANSFORMED
Δ −452
Δ +277
Δ +399
Δ +735
Δ +768
Δ +871
Δ +871R
Δ +951
9.5
7.5
4.4
2.4
1.4
25s
18s
1
2
3
4
5
6
7
8
9

Figure 15.

were complex. In comparison to the endogenous *CDC68* transcript in recipient strain ART68-1 (Fig. 15, lane 1), the intensities of the numerous plasmid-derived transcripts were significantly higher (Fig. 15, lanes 2-9). The transformants contained no visible transcript of constant size (the endogenous transcript from the genomic *cdc68-1* locus is barely visible in this analysis), but several that decreased in size with successively larger deletions. These data therefore suggest that plasmid sequences are acting as fortuitous promoters in this context. Without further analysis, however, it cannot be concluded that transcripts from such promoters are those from which truncated Cdc68 proteins are translated. In addition, it is also not possible to identify, without further analysis, where each of the transcripts initiate and terminate, and therefore from which plasmid sequences these transcripts are initiated.

The transcript pattern in ART68-1 cells transformed with p68-Ec-4D (which contains the same insert as p68-Exo2-4, but in reverse orientation) was less complicated than in the other series-2 clones (Fig. 15, lane 8). In p68-Ec-4D, upstream vector DNA includes pUC18 sequences (including MCS sequences [53 bp], *lacZ* sequences [in the opposite orientation to the *CDC68* ORF; 161 bp], sequences of pBR322 origin [48 bp]) and *S. cerevisiae* sequences from the *URA3* locus (*URA3* is in the same orientation as the *CDC68* ORF). The size of the major p68-Ec-4D transcript (approximately 2.8 kb) suggests that this transcript might be initiated from within sequences 3' to the *URA3* gene, assuming that the transcript terminates at the same site as the wild-type *CDC68* transcript. The size of this p68-Ec-4D transcript is not consistent with readthrough from the *URA3* promoter.

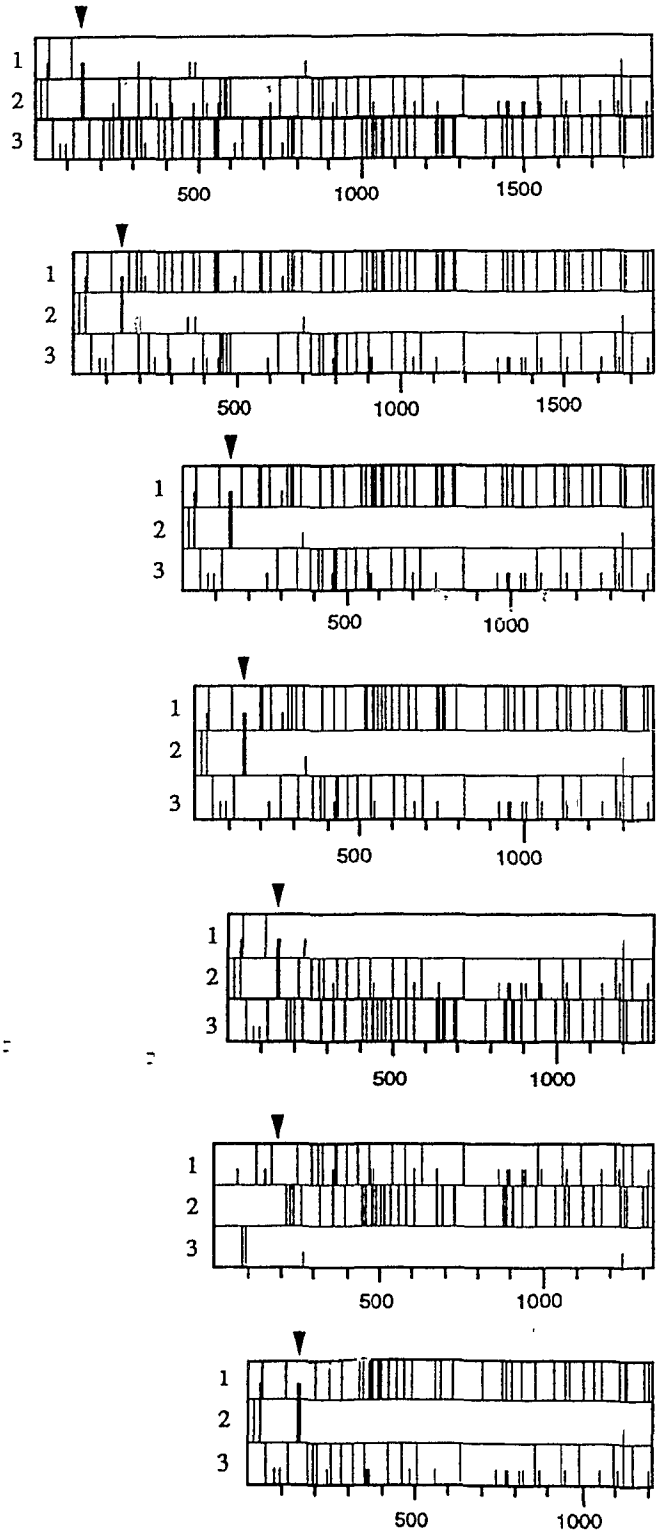
c) Translation initiation in partially deleted *CDC68* clones.

From the above analysis it is not possible to conclude what sequences provide promoter function for the partially deleted *CDC68* genes. A further question concerns where translation initiates on these deleted templates. To address this question, sequences from the region surrounding the vector-*CDC68* ORF junction of each of the deleted but complementing clones were analysed for the distribution of ATG initiation codons using the sequence-analysis program Strider (Marck, 1988). Fig. 16 illustrates that, with the exception of p68-Exo2-11 (which complements only weakly), in-frame ATG codons, that could serve as translation initiation codons for the N-terminally deleted Cdc68 gene products, are present in the 5' regions of the *CDC68* ORF remaining in each of the constructs. Previous analysis of many yeast genes has demonstrated that in most cases, the 5' proximal mRNA AUG codon is used for translation initiation (Cigan and Donahue, 1987) consistent with the scanning hypothesis proposed by Kozak (1978). Thus, any upstream AUG codons present in the mRNA from the deleted *CDC68* constructs would be expected to significantly reduce the initiation of translation at downstream in-frame sites. Analysis of alternate reading frames reveals several out-of-frame initiation and termination codons. Thus, if the Cdc68 deleted proteins are translated from mRNAs that originate within upstream vector sequences, the efficiency of translation from these mRNAs is probably very low. Nevertheless, if transcript levels are high, as Fig. 15 suggests that they may be, then even inefficient translation initiation at an appropriate codon may lead to the production of sufficient protein to complement the *cdc68-1* and *spt16-197* mutations.

Figure 16. Reading-frame analysis of deleted *CDC68* clones.

Possible open reading frames in seven clones deleted for *CDC68* promoter and N-terminal coding sequences (series-2 deleted clones) were analysed using DNA Strider (Marck, 1988). In this representation, full-length vertical bars in a reading frame indicate stop codons (TAA, TGA and TAG) and half bars indicate ATG codons. Only the three reading frames in the same orientation as the *CDC68* ORF are considered. The sequences used for this analysis include the 162 bp of YEp352 vector sequence (deduced from the complete YEp352 DNA sequence, kindly provided by J. Hill) that precede the vector - *CDC68* ORF junction in p68-Exo2-8 (Δ-452), p68-Exo2-5 (Δ+277), p68-Exo2-9 (+Δ399), p68-Exo2-6 (Δ+735), p68-Exo2-10 (Δ+768), p68-Exo2-4 (Δ+871) and p68-Exo2-11 (Δ+951). In p68-Ec-4D (Δ+871R) the 200 bp of YEp352 vector sequence that precede the vector - *CDC68* ORF junction are included. The vector - *CDC68* ORF junctions, as determined by nucleotide sequencing, are indicated by arrows (note that the vector - insert junction of all the clones except p68-Ec-4D immediately follows a pair of stop codons contained in one reading frame of preceding YEp352 sequences). The extent of complementation of the *cdc68-1* and *spt16-197* temperature-sensitive mutations exhibited by these deleted clones is indicated on the right, and is the same as that described in Fig. 14.

Figure 16.


Clone
Complementation
1
2
3
500
1000
1500
p68-Exo2-5
(Δ+277)
+
p68-Exo2-9
(Δ+399)
+
p68-Exo2-6
(Δ+735)
++
p68-Exo2-10
(Δ+768)
++
p68-Exo2-4
(Δ+871)
++
p68-Ec-4D
(Δ+871R)
++
p68-Exo2-11
(Δ+971)
+

It is interesting to note that in each of the partially deleted constructs that give the strongest complementation (p68-Exo2-6, p68-Exo2-10 and p68-Exo2-4, and p68-Ec-4D) the first in-frame ATG codon internal to *CDC68* sequences in that at +936 in the *CDC68* ORF (see Fig. 16). Other clones, which complement less well, are either deleted beyond this codon or retain other upstream in-frame ATG codons. Perhaps translation initiation at the +936 codon produces an optimally deleted gene product. Alternatively, perhaps the superior complementation conferred by p68-Exo2-6, p68-Exo2-10, p68-Exo2-4 and p68-Ec-4D is due to more efficient expression of the deleted gene products which they encode. The *CDC68* nucleotide sequence upstream of the +936 ATG is strikingly AT rich, a characteristic feature of yeast promoter regions. For example, of the 66 nucleotides between the *CDC68* - vector junction and the +936 ATG in p68-Exo2-4, 46 nucleotides (70%) are either A or T. Furthermore this region contains a number of TATA-like elements which might serve as transcription initiation signals. Although no transcripts with sizes consistent with initiation internal to *CDC68* sequences were visible in the northern analysis illustrated in Fig. 15, relatively minor transcripts that are not visible might nevertheless be the transcripts from which complementing protein is produced. Determination of the sizes of the proteins produced from these deleted *CDC68* clones would be informative in determining from what site translation is initiated. Primer extension or S1 nuclease analysis could be used to address the question of whether sequences internal to the *CDC68* ORF provide promoter function in the context of the deleted clones analysed here.

d) Partially deleted *CDC68* clones can complement a disruption in the *CDC68* gene.

As described above, six *CDC68* clones deleted for N-terminal *CDC68* sequences retain the ability to complement the *cdc68-1* and *spt16-197* mutations but cannot suppress δ-insertion mutations. The *CDC68* has been disrupted and found to be an essential gene (Malone *et al.*, submitted). Since the truncated *CDC68* genes share some but not all properties of the full-length *CDC68* gene it was of interest to determine whether plasmid-borne truncated *CDC68* genes could rescue the lethality of a *CDC68* disruption. Diploid strain BM64 (kindly provided by E. A. Malone) contains one functional copy of the *CDC68* gene and one copy disrupted by the insertion of the *S. cerevisiae LEU2* gene into the *Sal*I site within the *CDC68* open reading frame (Fig. 10). The disrupting *LEU2* gene is the only functional *LEU2* gene in BM64 cells, therefore all Leu^+ cells must contain the *spt16::101* disruption. Sporulation of strain BM64 gives rise to a maximum of only two viable spores, both of which are Leu^-, indicating that each of the non-viable segregants are those to which the *spt16::101* disruption segregated (Malone *et al.*, submitted).

Preliminary analysis indicates that at least two of the partially deleted *CDC68* clones can rescue the lethality of the *spt16::101* disruption. Clones p68-Exo2-5 and p68-Ec-4D (which each strongly complement temperature-sensitive *cdc68* mutations) when transformed into diploid BM64 allowed LEU^+ spores to be recovered after sporulation. Although spore viability was poor in this analysis, approximately 50% of viable segregants were LEU^+, as expected.

17. *CDC68* is a unique gene

To ascertain whether the *S. cerevisiae* genome contains sequences similar to the *CDC68* gene, a Southern blot of genomic DNA from strain 21R was probed with M13 clones E-4 (data not shown) and E-22, which contain 1.2-kbp and 2.1-kbp *Eco*RI restriction-fragment inserts from the cloned *CDC68* gene (Fig. 9). Genomic DNA was digested with a collection of restriction enzymes, as shown in Fig. 17. Plasmid pSC2-1 (which contains the entire *CDC68* gene) was digested with four of the same enzymes: *Eco*RI, *Hpa*I, *Hin*dIII and *Bam*HI.

In all cases the E-4 and E-22 probes detected only the expected restriction fragments (see Fig. 17 legend), indicating that it is unlikely that the *S. cerevisiae* genome contains sequence with significant similarity to the *CDC68* gene. Should a similar sequence exist, its restriction map would have to be identical to that of the *CDC68* gene and flanking sequences. Thus the *CDC68* gene is unique in the genome of *S. cerevisiae.*

As expected, both the E-4 and E-22 probes detected common restriction fragments in genomic and plasmid DNA digested with *Eco*RI, *Hpa*I and *Hin*dIII. Unexpectedly however, the single *Bam*HI fragment detected by both probes E-22 and E-4 in genomic DNA was slightly smaller than that detected in the plasmid pSC2-1. The possibility that this discrepancy was due to a restriction-fragment-length polymorphism between strains 21R and the strain from which library DNA was obtained was ruled out when the sizes of the *Bam*H1 fragments of pSC2-1 and pLC3-4 (the plasmid from which pSC2-1 was derived) were compared: pLC3-4, like strain 21R genomic DNA, was found to contain a *Bam*HI fragment that was slightly smaller than the equivalent band in pSC2-1. Furthermore, the cloned *SPT16* sequences also

Figure 17. Southern analysis of *CDC68* genomic sequences.

Genomic DNA was isolated from strain 21R and digested with *Hin*dIII, *Bam*HI, *Hpa*I, *Eco*RI, *Bgl*II, *Kpn*I, *Cla*I, and *Sal*I as shown. Plasmid pSC2-1 (containing the entire *CDC68* gene; see Fig. 1) was also digested with the first four of these enzymes and blotted from the same agarose gel as the genomic DNA. The autoradiograph shown was obtained using M13 clone E-22 as a probe. E-22 contains a 2.1-kbp insert comprising sequences +1656 to +3755 of the *CDC68* ORF (see Fig. 10) and is expected only to hybridize to *CDC68* insert sequences in pSC2-1, and not to the YEp24 vector.

As expected, single restriction fragments were detected by E-22 in genomic and pSC2-1 DNA digested with all the enzymes except *Hin*dIII and *Hpa*I, which each have one site within the probe and are therefore expected to hybridize to two restriction fragments. All pSC2-1 restriction fragments comigrated with their genomic equivalents except for the *Bam*HI fragment, which migrated more slowly (see text).

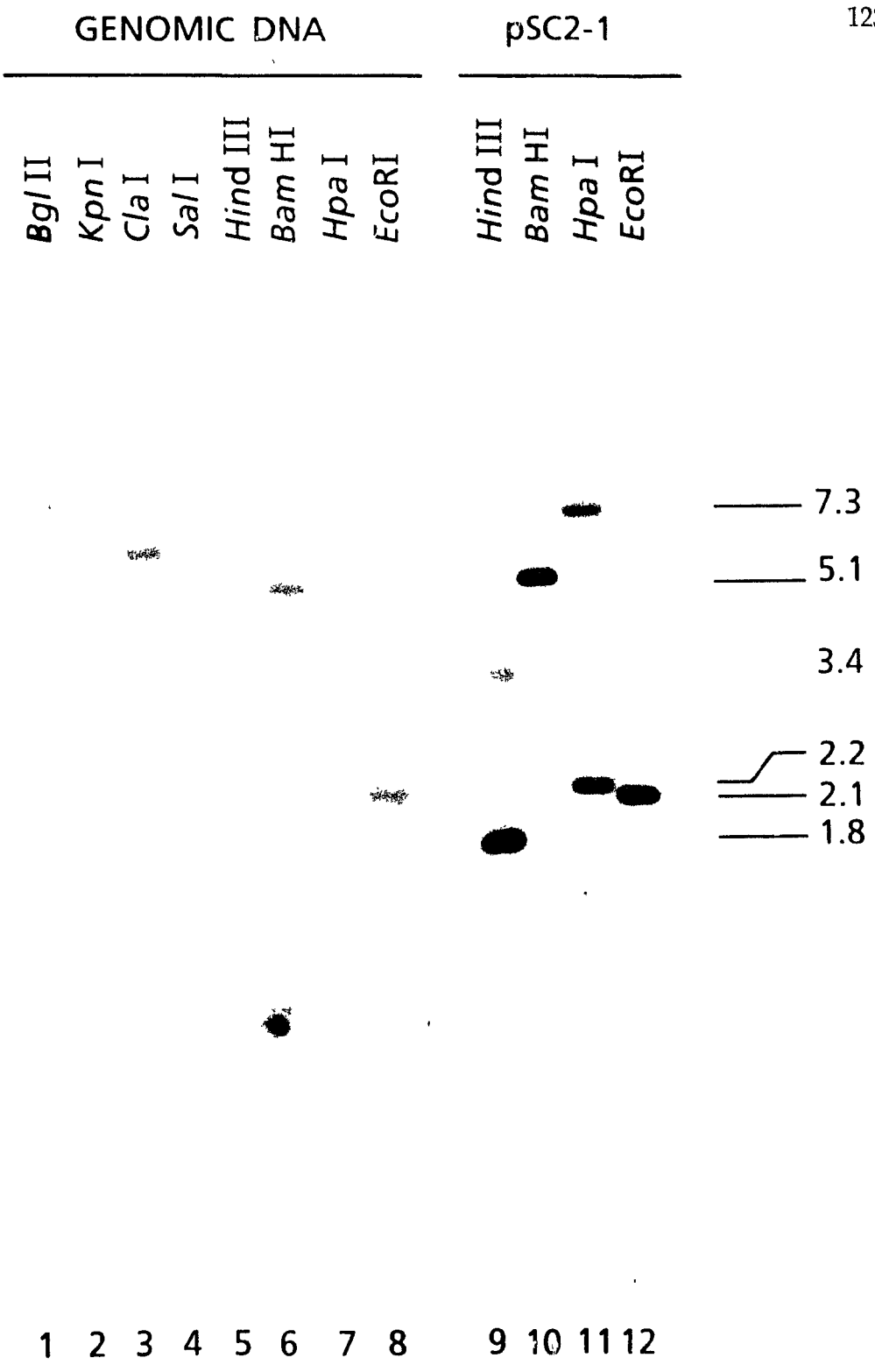

GENOMIC DNA
pSC2-1
Bgl II
Kpn I
Cla I
Sal I
Hind III
Bam HI
Hpa I
EcoRI
Hind III
Bam HI
Hpa I
EcoRI
7.3
5.1
3.4
2.2
2.1
1.8
1 2 3 4 5 6 7 8 9 10 11 12

Figure 17.

contain a slightly smaller *Bam*HI fragment (Malone *et al.*, submitted). In addition, the *Hin*dIII site upstream of the *CDC68* gene in plasmid pSC2-1 is absent in the cloned *SPT16* sequences suggesting that the order of *Bam*HI and *Hin*dIII sites in pSC2-1 is reversed. Thus, although most restriction fragments are common to both plasmid and genomic sequences, pSC2-1 appears to have suffered a cloning artefact during the *Sau*3A subcloning procedure by which it was generated from pLC3-4.

To resolve this discrepancy, sequences upstream of the *CDC68* gene in plasmids pSC2-1 and pLC3-4 were compared using direct plasmid sequencing with oligonucleotides synthesized for this purpose (see Methods and Materials). The pSC2-1 and pLC3-4 sequences were found to diverge at a *Bgl*II site (also a *Sau*3A site) located at position -747 upstream of the *CDC68* open reading frame. The origin of the sequences upstream of this site in pSC2-1 is not known, but is probably another region of the original clone, pLC3-4. To avoid confusion due to this minor cloning artefact, only sequence downstream of position -747 are shown in Fig. 10. This minor artefact is unlikely to be of significance for any of the experiments described in this thesis.

18. Cyclin transcript abundance in *cdc68-1* mutant cells

Since cells bearing the *cdc68-1* mutation are temperature-sensitive for the performance of Start (Prendergast *et al.*, 1990) it was of interest to determine whether *cdc68-1*-mediated cell-cycle arrest involves altered expression of any of the three G1-cyclin genes that have been shown to regulate the G1 to S phase progression in *S. cerevisiae* (Nash *et al.*, 1988;

Hadwiger *et al.*, 1989; Richardson *et al.*, 1989; Wittenberg *et al.*, 1990; Cross, 1990). To address this question, transcript levels of the three genes, *CLN1*, *CLN2* and *CLN3*, were examined in wild-type and *cdc68-1* mutant cells by northern analysis (Fig. 18). Transcripts of approximately 2.0 kb were detected by *CLN1* and *CLN2* probes in total RNA isolated from both wild-type and mutant cells incubated at the permissive temperature, consistent with the observations of Wittenberg *et al.* (1990) (Fig. 18, lanes 1 and 6). A slightly larger transcript was detected using a *CLN3* probe, as expected from the larger size of the *CLN3* open reading frame (Nash *et al.*, 1988; Cross, 1988). After transfer of *cdc68-1* mutant cells to the restrictive temperature the levels of all three *CLN* transcripts rapidly and permanently decreased (Fig. 18). In contrast, *CLN* transcripts in wild-type cells persisted after transfer to 37°C, although *CLN2* transcript levels exhibited a transient decrease in response to the sudden increase in temperature. The transient decrease in *CLN2* transcript abundance in wild-type cells after transfer to 37°C is coincident with a previously described transient accumulation of cells within the G1 interval (Singer and Johnston, 1979; A. Rowley and G. C. Johnston, manuscript in preparation). The rapid decrease in *CLN* mRNA abundance in *cdc68-1* mutant cells may be responsible for the *cdc68-1*-mediated Start-arrest phenotype. Indeed, decreased cyclin expression has been previously demonstrated to cause a first-cycle G1 arrest (Richardson *et al.*, 1989; Cross, 1990).

Figure 18. Northern analysis of cyclin transcripts in wild-type and *cdc68-1* mutant cells.

Total RNA was extracted from strains FC90 (*CDC68*) (lanes 1-5) and FC90-68 (*cdc68-1*) (lanes 6-10) grown at 23°C (lanes 1 and 6), or at 20 min (lanes 2 and 7), 90 min (lanes 3 and 8), 180 min (lanes 4 and 9) and 300 min (lanes 5 and 10) after transfer to 37°C. The *CLN1* and *CLN2* probes were 1.6-kbp *Nde*I-*Bam*HI purified coding-sequence restriction fragments. The *CLN3* probe was a 1.7-kbp *Eco*RI-*Xho*I purified coding-sequence restriction fragment (see Methods and Materials for further details on probe preparation).

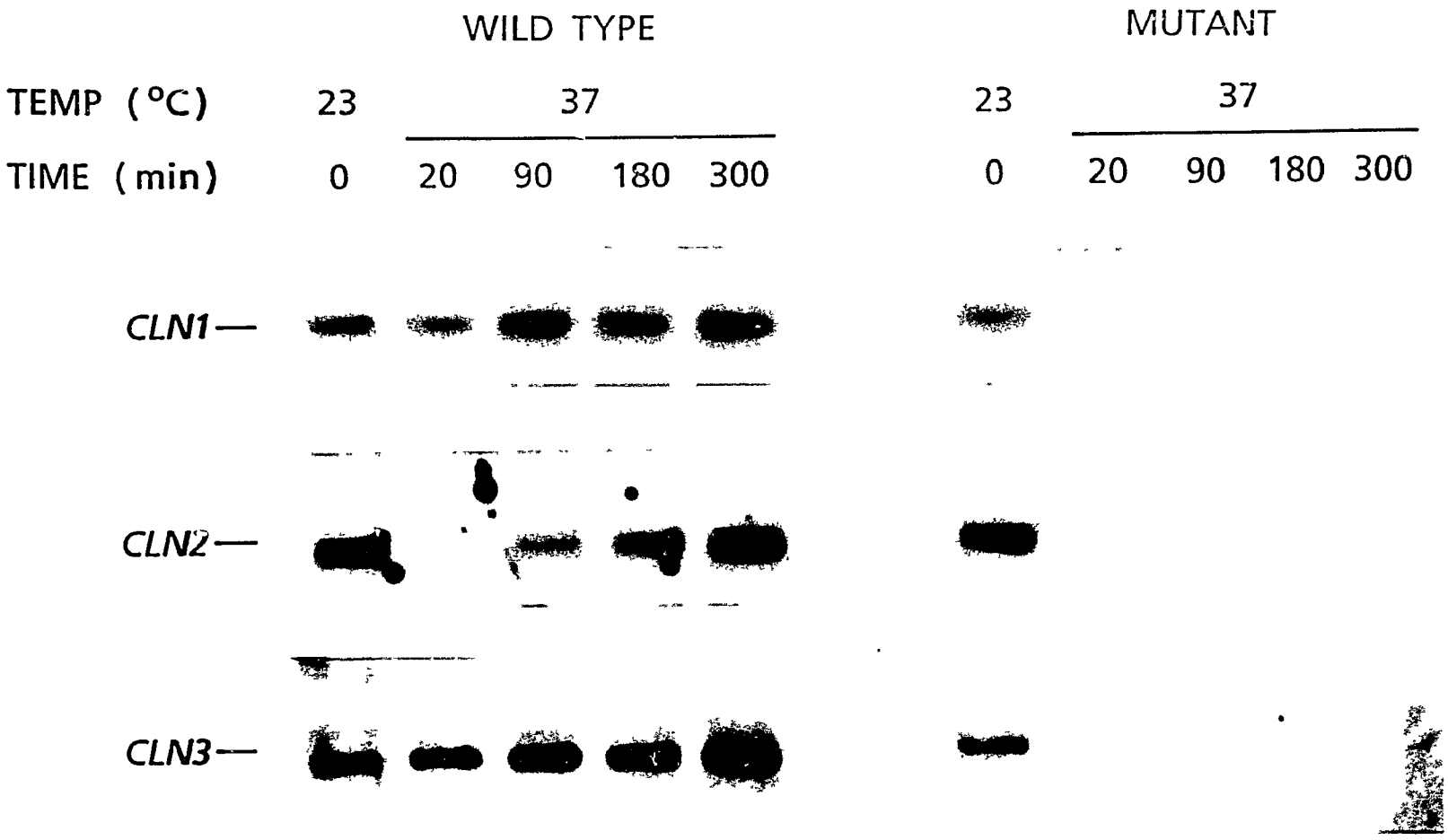

WILD TYPE
MUTANT
TEMP (°C)
23
37
23
37
TIME (min)
0
20
90
180
300
0
20
90
180
300
CLN1
CLN2
CLN3
1
2
3
4
5
6
7
8
9
10

Figure 18.

19. Interaction of *cdc68-1* and *CLN2-1*

The results described above suggest that Start arrest of *cdc68-1* mutant cells at the restrictive temperature may be a consequence of decreased *CLN* gene expression. To test this hypothesis, the ability of *CLN2-1*, a hyperactive/hyperstable allele of the *CLN2* gene (Hadwiger *et al.*, 1989), to suppress *cdc68-1*-mediated Start arrest was examined. The *CLN2-1* allele encodes a truncated Cln2 protein that is assumed to be of increased stability due to the deletion of carboxyl-terminal PEST sequences implicated in protein degradation (Rogers *et al.*, 1986). The phenotype of cells containing the *CLN2-1* mutation; small cell size, shortened G1 interval, moderate α-factor resistance and failure to arrest at Start in response to starvation, indicates that, in the presence of the *CLN2-1* allele, cyclin protein is no longer rate-limiting for cell-cycle progression.

A diploid strain heterozygous for both the *cdc68-1* and *CLN2-1* mutations was constructed by crossing strain GCY24 (*CLN2-1*) with 68507A (*cdc68-1*). Temperature sensitivity segregated 2:2 in meiotic segregants from this diploid, indicating that the *CLN2-1* mutation does not suppress temperature sensitivity imposed by the *cdc68-1* mutation. The *CLN2-1* mutation was scored on the basis of budded stationary-phase cell morphology and small cell size (Hadwiger *et al.*, 1989). Both *cdc68-1* and *cdc68-1 CLN2-1* segregants underwent an approximately two-fold increase in cell number after transfer to 37°C (Fig. 19). However, cell-cycle behaviour was altered in *cdc68-1 CLN2-1* double-mutant cells; whereas *cdc68-1* mutant cells arrested as primarily unbudded cells, *cdc68-1 CLN2-1* double-mutant cells arrested as mostly budded cells that had initiated, but not completed, an additional cell cycle (Fig. 19). DAPI (diaminophenylindole) staining of DNA

Figure 19. Interaction of *cdc68-1* and *CLN2-1*.

Actively dividing cells bearing either the *cdc68-1* mutation (strain CLN-5A; open circles) or both *cdc68-1* and *CLN2-1* mutations (strain CLN-5C; closed circles), growing in YM-1 rich medium at 23°C, were transferred to 37°C at time zero. Cultures were monitored for cell concentration (A) and percentage of unbudded cells (B) as a measure of cell-cycle position. *cdc68-1* (C) and *cdc68-1 CLN2-1* (D) mutant cells were photographed after 3 h of incubation at 37°C. Results shown are typical of all temperature-sensitive segregants from a cross between strains GCY24 and 68507A.

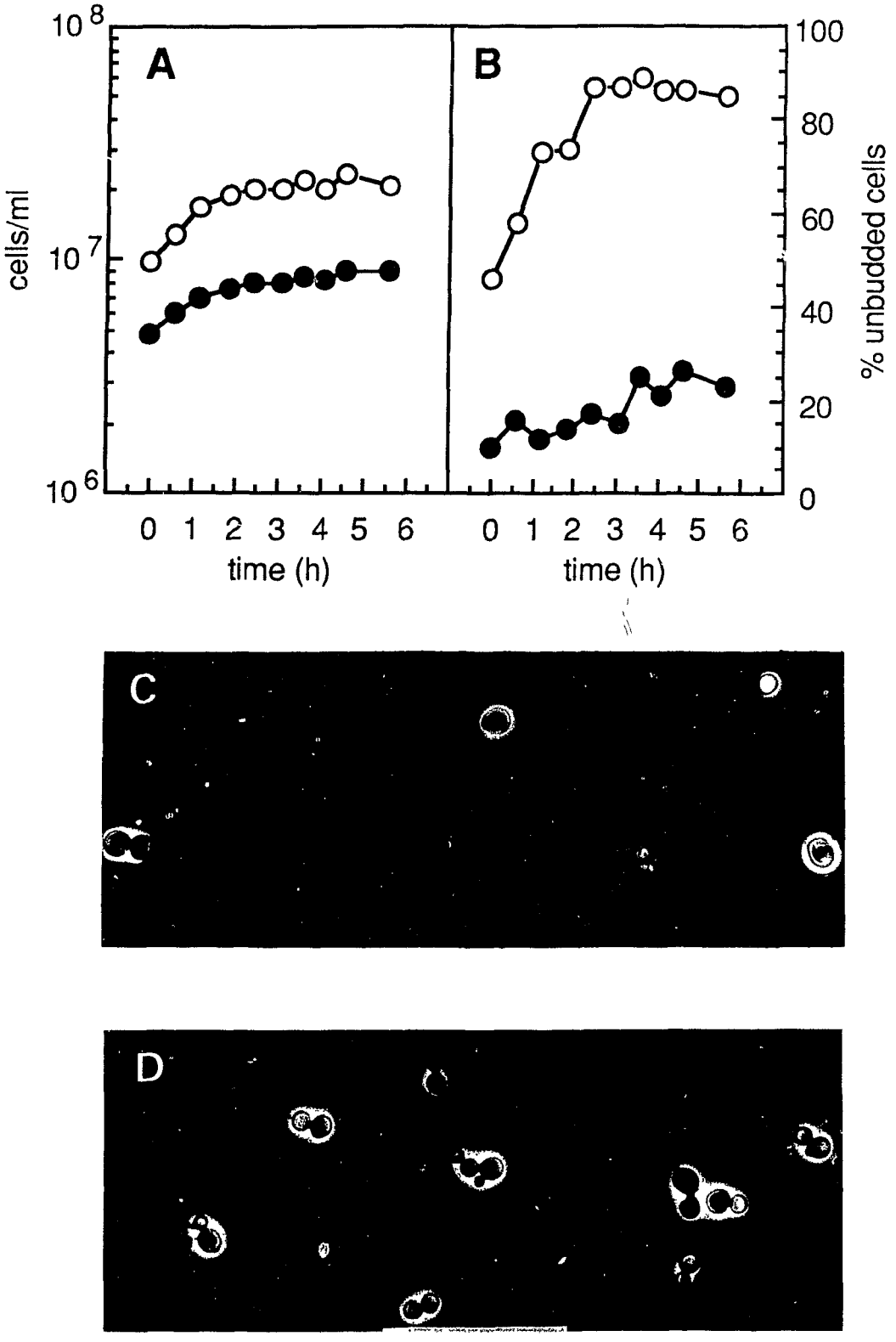

A
B
C
D
cells/ml
10^8
10^7
10^6
0 1 2 3 4 5 6
time (h)
0 1 2 3 4 5 6
time (h)
100
80
60
40
20
0
% unbudded cells

Figure 19.

demonstrated that, while *cdc68-1*-mediated arrest was characterized by the presence of unbudded cells containing single nuclei, *cdc68-1 CLN2-1* arrested cells displayed nuclear morphologies typical of all stages of the cell cycle (data not shown). Thus *cdc68-1 CLN2-1* double-mutant cells perform Start after transfer to the restrictive temperature but are unable to complete the newly initiated cell cycle and so arrest in a random fashion. The ability of the *CLN2-1* mutation to suppress the Start arrest of *cdc68-1* mutant cells, and the *cdc68-1* - mediated decreases in cyclin-transcript abundance, strongly suggest that Start arrest of *cdc68-1* mutant cells at the restrictive temperature is a consequence of cyclin limitation.

20. Suppression of *cdc68-1*-mediated cell cycle arrest by the CLN2-1 mutation does not involve positive feedback on cyclin gene expression

During the course of these studies Cross and Trinkelenberg (1991) and Dirick and Nasmyth (1991) reported the existence of a positive feedback loop that contributes to the periodic accumulation of the G1-cyclin transcripts. This finding challenges the simple explanation that suppression of *cdc68-1*-mediated cell cycle arrest by the *CLN2-1* mutation is due solely to prolonged activation of the Start machinery by the hyperstable *CLN2-1*-encoded Cln2 protein. Suppression by *CLN2-1* could also be due to secondary effects on the transcription of *CLN1*, *CLN2*, or *CLN3* which might allow cells to initiate a new cell cycle even in the absence of functional Cdc68 protein. Indeed, the ability of hyperactive *CLN3* mutations to overcome α-factor-mediated cell-cycle arrest appears not only to be due to effects on Cln3 protein stability but also due to indirect effects on *CLN2* transcription (Dirick and Nasmyth, 1991).

To distinguish between these models, total RNA was isolated from *cdc68-1* mutant cells and from cells containing both the *cdc68-1* and *CLN2-1* mutations. Fig. 20 demonstrates that the behaviour of G1 cyclin transcripts is the same in *cdc68-1 CLN2-1* double mutant cells as in cells containing only the *cdc68* mutation. Thus, *CLN2-1*-mediated suppression of the concerted cell-cycle arrest caused by the *cdc68-1* mutation is not the result of *CLN2-1*-mediated stimulation of cyclin gene transcription, but is due to effects on cell-cycle progression caused by a Cln2 protein of increased stability.

21. Abundance of other transcripts in *cdc68-1* mutant cells

To further define the effects of the *cdc68-1* mutation on gene expression, the levels of two additional transcripts were determined by northern analysis using *ACT1* and *LEU2* probes. Transcripts detected by *ACT1* and *LEU2* probes, like those of the three cyclin genes, decreased in abundance in *cdc68-1* mutant cells after transfer to the restrictive temperature (Fig. 21). Even at the permissive temperature the *LEU2* transcript was present at a lower level in *cdc68-1* mutant cells (Fig. 21). Both strains used in this analysis (FC90 [*CDC68*] and FC90-68 [*cdc68-1*]) contain a *leu2-3,112* mutation. This *LEU2* mutant allele contains a double frameshift mutation which is unlikely to affect transcription of this mutant gene (Hinnen *et al.*, 1978). Although the expression of the *LEU2* gene is subject to leucine repression (Martinez-Arias *et al.*, 1984), in this analysis both wild-type and *cdc68-1* mutant strains were grown in identical leucine-containing media.

Figure 20. Northern analysis of G1-cyclin transcripts in *cdc68-1 CLN2-1* double-mutant cells.

Total RNA was extracted from strains CLN-5A (*cdc68-1*) (lanes 1-5) and CLN-5C (*cdc68-1 CLN2-1*) (lanes 6-10) grown in YM-1 rich medium at 23°C (lanes 1 and 6), or at 20 min (lanes 2 and 7), 40 min (lanes 3 and 8), 60 min (lanes 4 and 9) and 90 min (lanes 5 and 10) after transfer to 37°C. The cyclin probes were as described for Fig. 18. The *ACT1* probe was a 1-kbp *Hin*dIII-*Xho*I purified coding-sequence restriction fragment. *CLN2* and *ACT1* transcripts were detected using a mixture of two probes; the amount of *ACT1* probe used was adjusted to compensate for the greater abundance of *ACT1* mRNA. Note the change in time scale between this figure and Fig. 18.

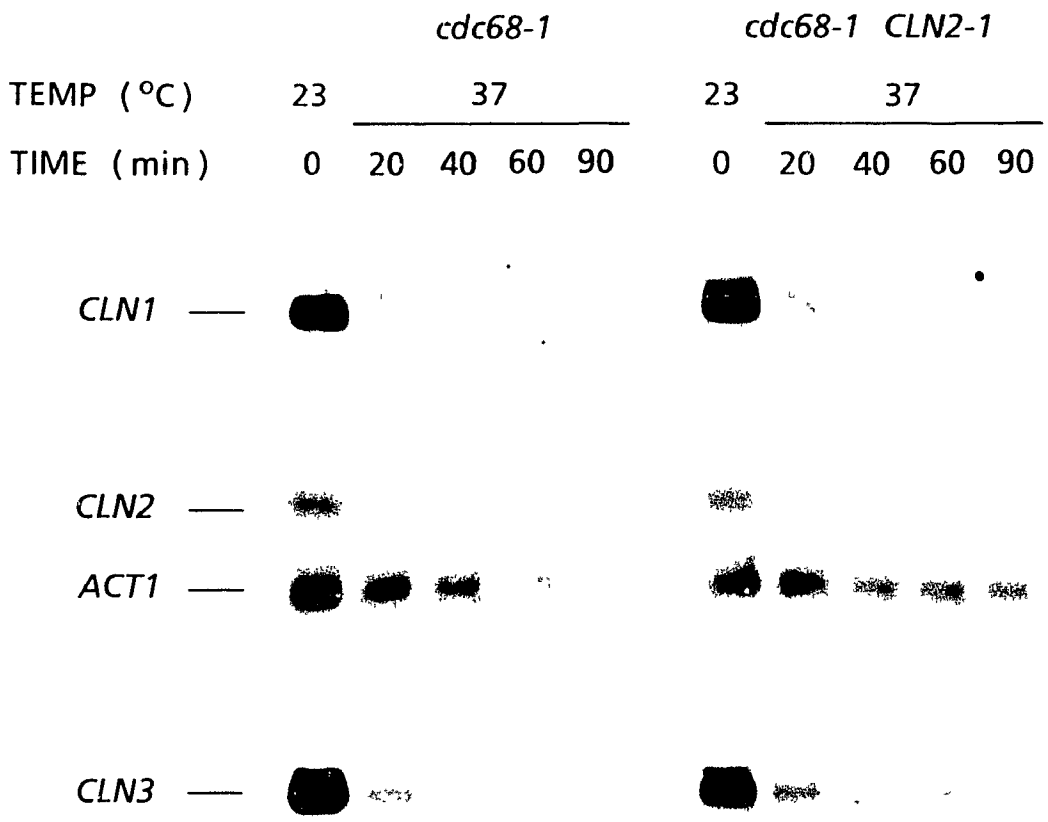

cdc68-1
cdc68-1 CLN2-1
TEMP (°C)
23
37
23
37
TIME (min)
0
20
40
60
90
0
20
40
60
90
CLN1
CLN2
ACT1
CLN3

Figure 20.

Figure 21. Northern analysis of *ACT1* and *LEU2* transcripts in wild-type and *cdc68-1* mutant cells.

Total RNA was extracted from strains FC90 (*CDC68*) (lanes 1-5) and FC90-68 (*cdc68-1*) (lanes 6-10) grown at 23°C (lanes 1 and 6), or at 20 min (lanes 2 and 7), 90 min (lanes 3 and 8), 180 min (lanes 4 and 9) and 300 min (lanes 5 and 10) after transfer to 37°C. The blots used were the same as those probed in Fig. 18, stripped and rehybridized. The *ACT1* probe was a 1-kbp *Hin*dIII-*Xho*I purified coding-sequence restriction fragment. The *LEU2* probe was a 1.7-kbp *Hpa*I-*Acc*I purified coding-sequence restriction fragment (see Methods and Materials for further details on probe preparation).

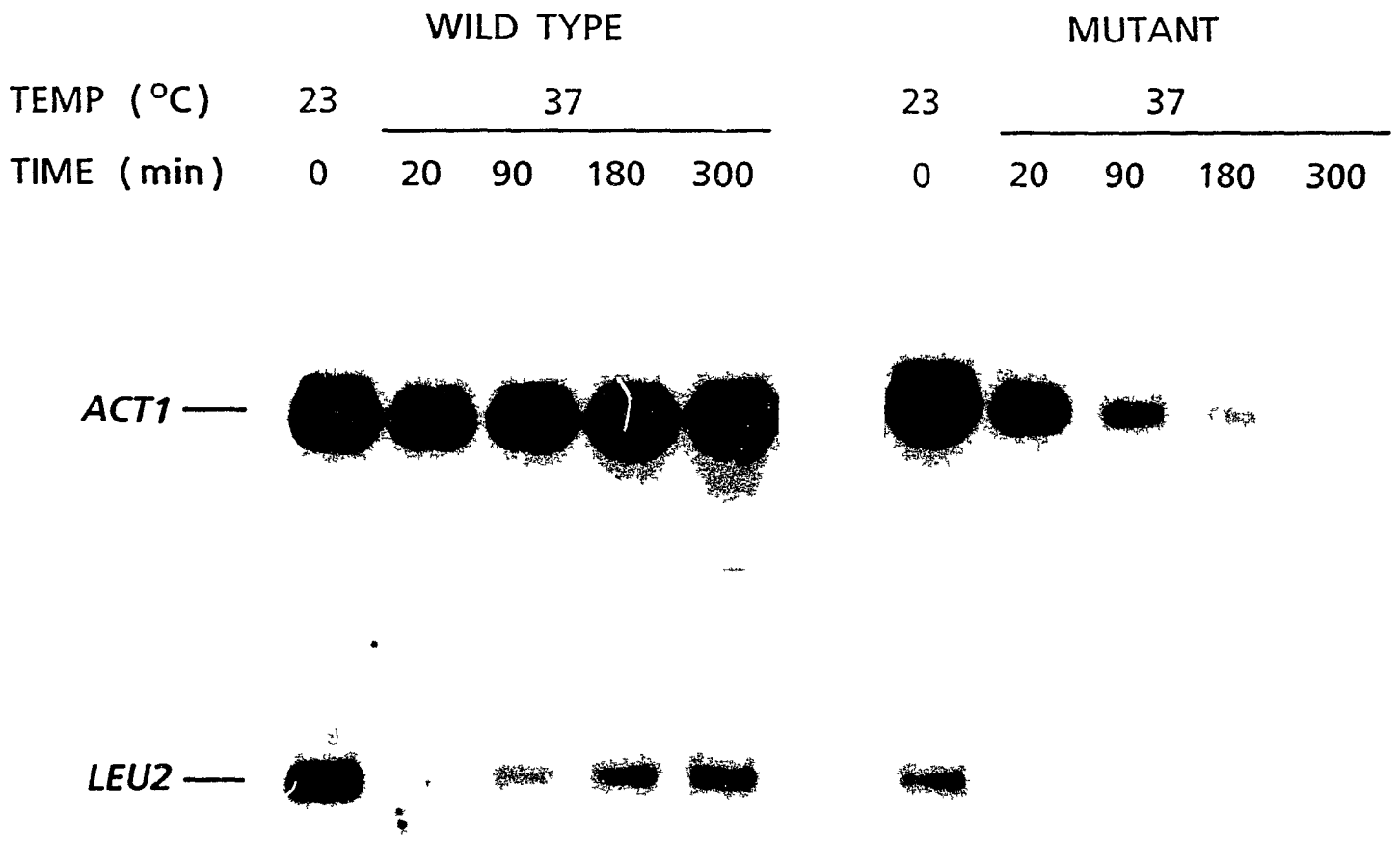

WILD TYPE
MUTANT
TEMP (°C)
23
37
23
37
TIME (min)
0
20
90
180
300
0
20
90
180
300
ACT1
LEU2
1
2
3
4
5
6
7
8
9
10

Figure 21.

The decreased abundance of *ACT1*, *LEU2* and cyclin transcripts seen here could result from either decreased transcription in *cdc68-1* mutant cells following transfer to the restrictive temperature, increased mRNA turnover, or a combination of both of these effects. The identification of *CDC68* as *SPT16* suggests that these effects are manifest at the transcriptional level. Indeed, Malone *et al.* (submitted) have demonstrated that transcription at *lys2-128δ* is altered in *spt16-197* mutant cells incubated at the restrictive temperature. The rapidity of change in mRNA levels in this analysis suggests that mutant Cdc68 protein is rapidly inactivated under these restrictive conditions. Even the more gradual effect seen on *ACT1* transcript levels is consistent with an inhibition of transcription, since *ACT1* mRNA has a relatively long half-life (approximately 30 min; Herrick *et al.*, 1990) and is therefore expected to persist even after disruption of normal transcription initiation. Indeed, a similar pattern of *ACT1* mRNA decay has been observed in cells containing the *rpb1-1* mutation (a temperature-sensitive mutation in the *RPB1* gene encoding the largest subunit of RNAP II) (Herrick *et al.*, 1990; Minvielle-Sebastia *et al.*, 1991).

22. *CDC68* transcript abundance in *cdc68-1* mutant cells

A northern blot identical to that used in the previous sections was probed with a radiolabelled 2.2-kbp *Hpa*I fragment containing sequences internal to the *CDC68* open reading frame (see Fig. 10) to confirm that the size of this open reading frame is consistent with the size of the *CDC68* transcript. Fig. 22 (lanes 1 and 6) shows that 3.2-kb transcripts were detected in both wild-type and *cdc68-1* mutant cells grown at 23°C, consistent with the 3105-bp *CDC68* open reading frame (Fig. 10). Unexpectedly, the abundance of

Figure 22. Northern analysis of the *CDC68* transcript in wild-type and *cdc68-1* mutant cells.

Total RNA was extracted from strains FC90 (*CDC68*) (lanes 1-5) and FC90-68 (*cdc68-1*) (lanes 6-10) grown at 23°C (lanes 1 and 6), or at 20 min (lanes 2 and 7), 90 min (lanes 3 and 8), 180 min (lanes 4 and 9) and 300 min (lanes 5 and 10) after transfer to 37°C. The blot was that used previously (see Figs. 18 and 21). The probe was a 2.2-kbp purified *CDC68* coding-sequence *Hpa*I restriction fragment (see Methods and Materials for further details on probe preparation).

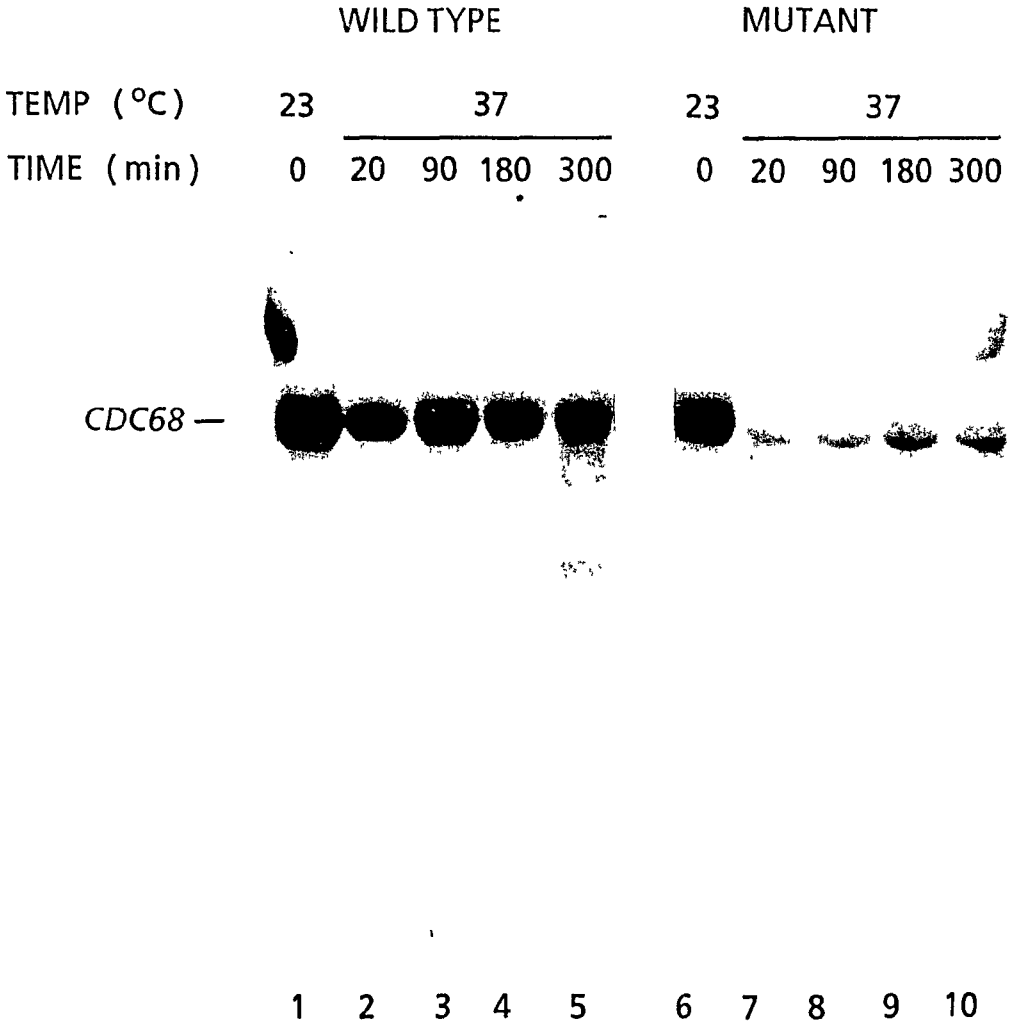

WILD TYPE
MUTANT
TEMP (°C)
23
37
23
37
TIME (min)
0
20
90
180
300
0
20
90
180
300
CDC68 —
1
2
3
4
5
6
7
8
9
10

Figure 22.

this 3.2-kb transcript decreased to barely detectable levels within 20 min after transfer of *cdc68-1* mutant cells to 37°C (Fig. 22 lanes 6-10) although transcript levels were unaffected in wild-type cells (Fig. 22 lanes 1-5). These results suggest that either the *cdc68-1* mutation lies in the *CDC68* promoter or that the *CDC68* gene product is required for transcription of its own gene.

23. The *CDC68* gene is required for a normal heat-shock response

Previous studies have demonstrated that significant levels of protein and RNA synthesis are maintained in *cdc68-1* mutant cells at the restrictive temperature, despite the deleterious effects on the expression of the individual genes described here (Prendergast *et al.*, 1990). Furthermore, additional studies which have examined the patterns of proteins synthesized in conditional cell-cycle mutants transferred to restrictive temperatures indicate that *cdc68-1* mutant cells persistently synthesize a 90-kDa protein that migrates with the Hsp82 heat-shock protein. This phenotype was unique among those mutants examined (Barnes, 1989). The effect on this protein is opposite to the effects on the expression of *ACT1*, *LEU2* and the G1 cyclin genes described above and indicates that the *cdc68-1* mutation may have both positive and negative effects on gene expression. To reexamine the effects of the *cdc68-1* mutation on the synthesis of proteins at the restrictive temperature, total cellular proteins were extracted and resolved by 1D-SDS PAGE following brief pulse-labelling of cells with [^{35}S]methionine. The use of a short labelling time (10 min) argues that any differences seen between wild-type and mutant cells reflect altered synthesis rather than differential protein stability. The pattern of proteins synthesized under these conditions is shown in Fig. 23. As expected, wild-type cells

Figure 23. Proteins synthesized in wild-type and *cdc68-1* mutant cells.

Actively proliferating cells of strain 21R (*CDC68*) (lanes 1-3), ART68-1 (*cdc68-1*) (lanes 4-6), ART68-1 transformed with YEp24 (lanes 7-9) and ART68-1 transformed with pSC2-1 (containing a wild-type *CDC68* gene) (lanes 10-12) were pulse-labelled with [^{35}S]methionine during growth at 23°C (lanes 1, 4, 7 and 10), and at 15 min (lanes 2, 5, 8 and 11) or 240 min (lanes 3, 6, 9 and 12) after transfer from 23°C to 37°C. Cells were grown in YNB+9 medium(without methionine). Equal amounts of radiolabelled TCA-precipitable material were resolved by 1-D SDS-PAGE. The migration of molecular-weight markers (in kDa) is given on the left. Migration of the Hsp82/Hsc82 doublet is as indicated by the arrow on the right.

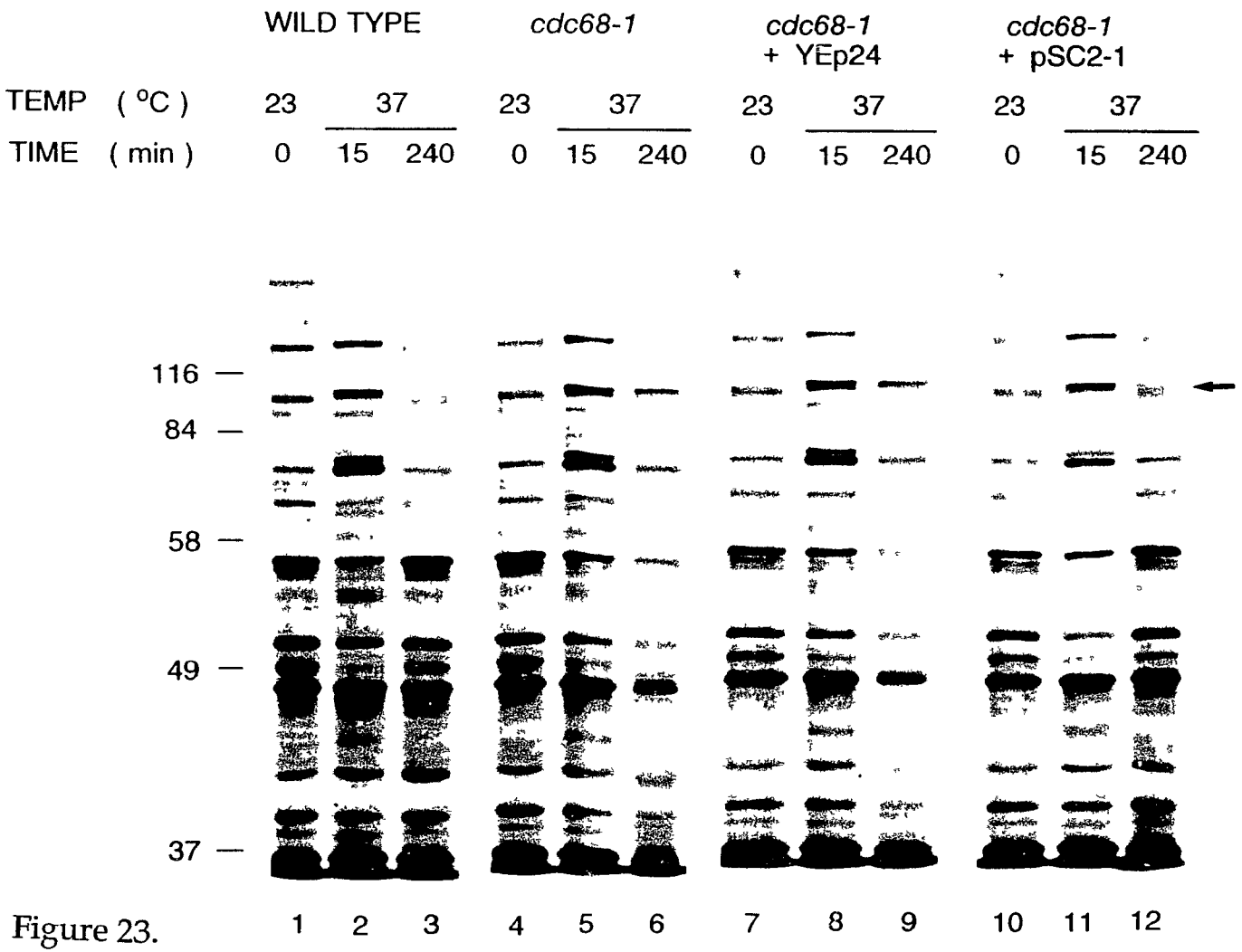

WILD TYPE
cdc68-1
cdc68-1 + YEp24
cdc68-1 + pSC2-1
TEMP (°C)
23
37
TIME (min)
0
15
240
116
84
58
49
37
1
2
3
4
5
6
7
8
9
10
11
12

Figure 23.

responded to transfer from 23°C to 37°C by transiently increasing the synthesis of previously characterized heat-shock proteins (reviewed in Lindquist and Craig, 1988) while decreasing the synthesis of other proteins. The pattern of newly synthesized proteins extracted from *cdc68-1* mutant cells showed a number of differences (Fig. 23, lanes 4-6); the synthesis of several proteins decreased after transfer to 37°C but, in contrast to the situation in wild-type cells, remained low even after 240 min at the restrictive temperature (compare lanes 1-3 with 4-6). Conversely, at least one protein whose synthesis increased after transfer to 37°C continued to be synthesized at an elevated level 240 min after transfer to the restrictive temperature, unlike in wild-type cells where this protein returned to its previous level of synthesis. The apparent molecular weight (approximately 90 kDa) and heat-shock induction of this latter protein suggested that it was the heat-shock protein Hsp82, the product of the *HSP82* gene (Finkelstein and Strausberg, 1983; Farrelly and Finkelstein, 1984; Borkovich *et al.*, 1989). A virtually wild-type pattern of labelled proteins was restored by the introduction of a wild-type *CDC68* gene contained in the high-copy vector YEp24 (Fig. 23, lanes 10-12), but not by YEp24 itself (Fig. 23, lanes 7-9). These results suggest that the *CDC68* gene is required for the appropriate synthesis of many proteins whose synthesis is normally either induced or decreased in response to the stress of heat shock.

24. A Hsp82-lacZ fusion protein is persistently synthesized in *cdc68-1* mutant cells

As described above, one of the more marked differences between the patterns of newly synthesized proteins in wild-type and in *cdc68-1* mutant

cells was evident in a band of approximately 90 kDa molecular weight. A polypeptide of this molecular weight was transiently synthesized in wild-type cells in response to a shift from 23°C to 37°C, whereas in *cdc68-1* mutant cells synthesis of this protein persisted (Fig. 23). The molecular weight and induction characteristics of this polypeptide suggested it to be Hsp82, the product of the *HSP82* gene. The *HSP82* gene is known to be expressed at a relatively low basal level at the initial incubation temperature used here (23°C) and to be transiently induced upon transfer of cells from 23°C to 37°C. This induction has been shown to be mediated primarily at the level of transcription (McAlister and Finkelstein, 1980; Finkelstein *et al.*, 1982). The effect on what was tentatively identified as Hsp82 represents a further aspect of the *cdc68-1* mutant phenotype; persistent high-level expression of at least one heat-shock gene. To pursue this observation it was first necessary to confirm the identity of the 90-kDa protein, and show that *HSP82* expression is altered in *cdc68-1* mutant cells. For this purpose a 2μ-based plasmid carrying an *HSP82-lacZ* fusion gene (plasmid pUTX144, kindly provided by D. Finkelstein) was introduced into *cdc68-1* mutant cells. In this fusion gene, *HSP82* promoter sequences have been fused in-frame to codon 8 of the *lacZ* gene from *E. coli*. Previous studies have demonstrated that expression of *lacZ* when fused to *HSP82* sequences accurately reflects regulation of the endogenous *HSP82* gene (Finkelstein and Strausberg, 1983). Examination of the pattern of newly synthesized proteins in *cdc68-1* mutant cells containing this plasmid (Fig. 24) showed that the fusion gene directs the synthesis of a new 116-kDa polypeptide in a manner which parallels that of the 90-kDa polypeptide in Fig. 23. No polypeptide of this molecular weight was

Figure 24. Synthesis of an Hsp82-lacZ fusion protein in *cdc68-1* mutant cells.

Actively proliferating cells of strain ART68-1 (*cdc68-1*) transformed with pUTX144 were pulse-labelled with [^{35}S]methionine during growth at 23°C (lane 1), and at 15 min (lane 2), 150 min (lane 3), 240 min (lane 4), 300 min (lane 5) and 360 min (lane 6) after transfer from 23°C to 37°C. Cells were grown in YNB+9 medium (without methionine). Equal amounts of radiolabelled TCA-precipitable material were resolved by 1-D SDS-PAGE. The migration of molecular-weight markers (in kDa) is given on the left. Migration of the Hsp82 and Hsp82-lacZ fusion proteins is as indicated by the arrows on the right.

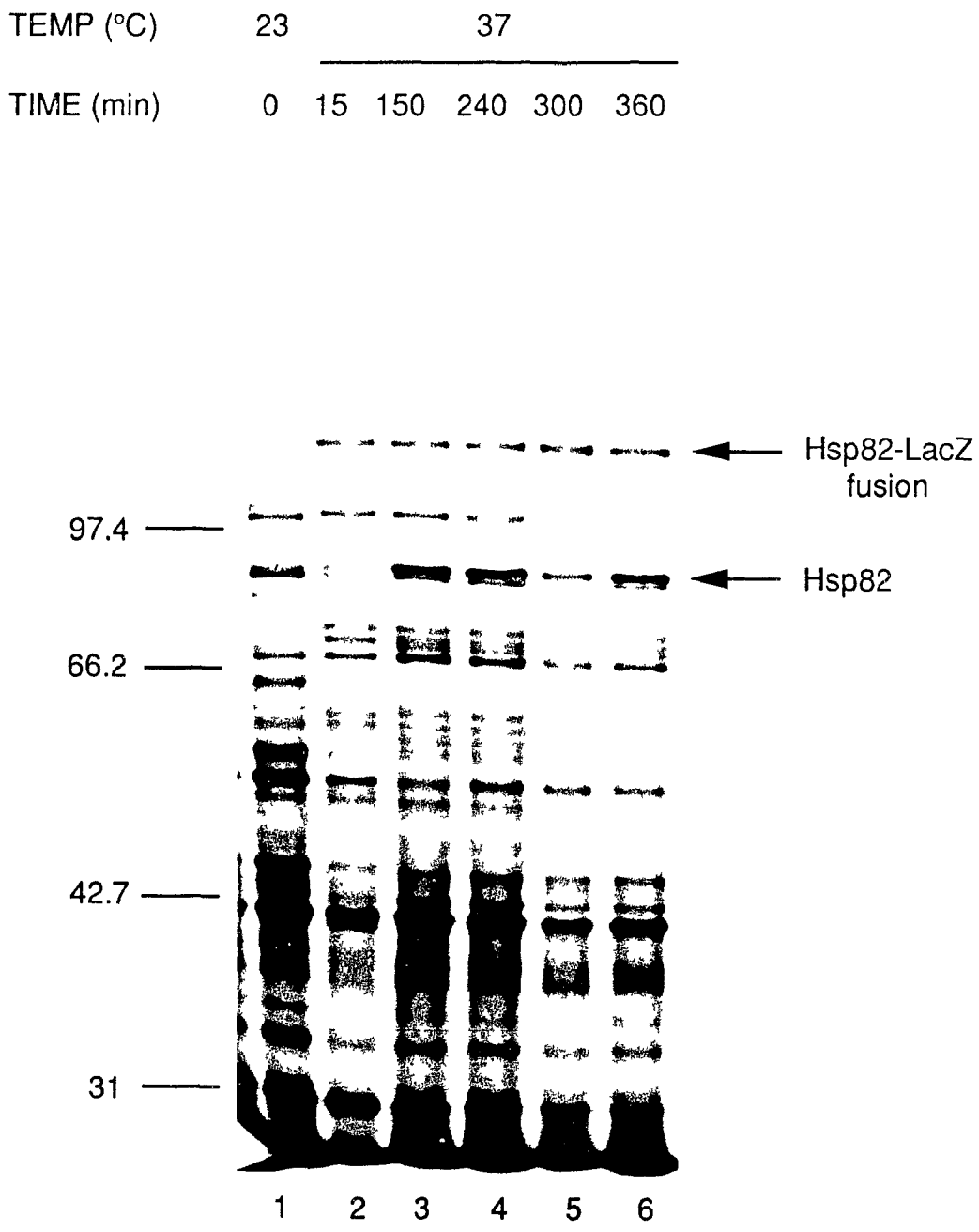

TEMP (°C)
23
37
TIME (min)
0
15
150
240
300
360
Hsp82-LacZ fusion
Hsp82
97.4
66.2
42.7
31
1
2
3
4
5
6

Figure 24.

observed in untransformed cells (Fig. 23). This analysis therefore supports the identification of the 90 kDa polypeptide as the product of the *HSP82* gene and confirms that expression from the *HSP82* promoter is altered in *cdc68-1* mutant cells.

25. Transcription from the *HSP82* promoter in *cdc68-1* mutant cells

To examine the characteristics of *HSP82* mRNA induction in *cdc68-1* mutant cells, the abundance of transcripts from the *HSP82-lacZ* fusion gene described above, in this case integrated in single copy at the *ura3-52* locus, was examined by northern blot analysis. Use of this fusion, with a *lacZ*-specific probe, allowed unambiguous identification of transcripts originating from an *HSP82* promoter while avoiding the necessity to distinguish between transcripts from *HSP82* and those from the closely related, but differentially regulated, *HSC82* gene (Borkovich *et al.*, 1989). Several transcripts were detected in both *cdc68-1* and wild-type cells at 23°C using a *lacZ* probe (Fig 25, lanes 2 and 7). Subsequent rehybridization using a radiolabelled probe specific for vector sequences immediately upstream of the *HSP82* promoter region (vector-specific probe, Fig. 28) detected three of these transcripts (Fig. 27, lane 1) indicating that these latter transcripts are not under the regulation of the *HSP82* promoter. The origin and behaviour of these additional transcripts are discussed in section 26.

The most abundant of the *lacZ*-specific transcripts, a 3.5-kb transcript, was regulated in a heat-shock-dependent fashion in wild-type cells (Fig. 25, lanes 2-6). Upon shift from 23 to 37°C this transcript became considerably more abundant (compare Fig. 25, lanes 2 and 3), but after 90 min the transcript level had decreased to virtually pre-shift levels as expected for a

Figure 25. Northern analysis of *HSP82-lacZ* fusion transcripts.

Total RNA was extracted from strains FP90 (*CDC68 HSP82-lacZ*) (lanes 1-6) and FP90-68 (*cdc68-1 HSP82-lacZ*) (lanes 7-11) grown in YNB+10 medium at 23°C (lanes 1, 2 and 7), or at 20 min (lanes 3 and 8), 90 min (lanes 4 and 9), 180 min (lanes 5 and 10) and 300 min (lanes 6 and 11) after transfer to 37°C. Lanes 2-11 represent the first use of the blot subsequently used for the experiment shown in Fig. 27. Lane 1 is the same as lane 1 in Fig. 27. The probe for lane 1 was a purified vector-specific restriction fragment. The probe for lanes 2-11 was a 6.2-kbp *Bam*HI-*Sal*I purified restriction fragment containing only *lacZYA* sequences (see Methods and Materials for further details on probe preparation).

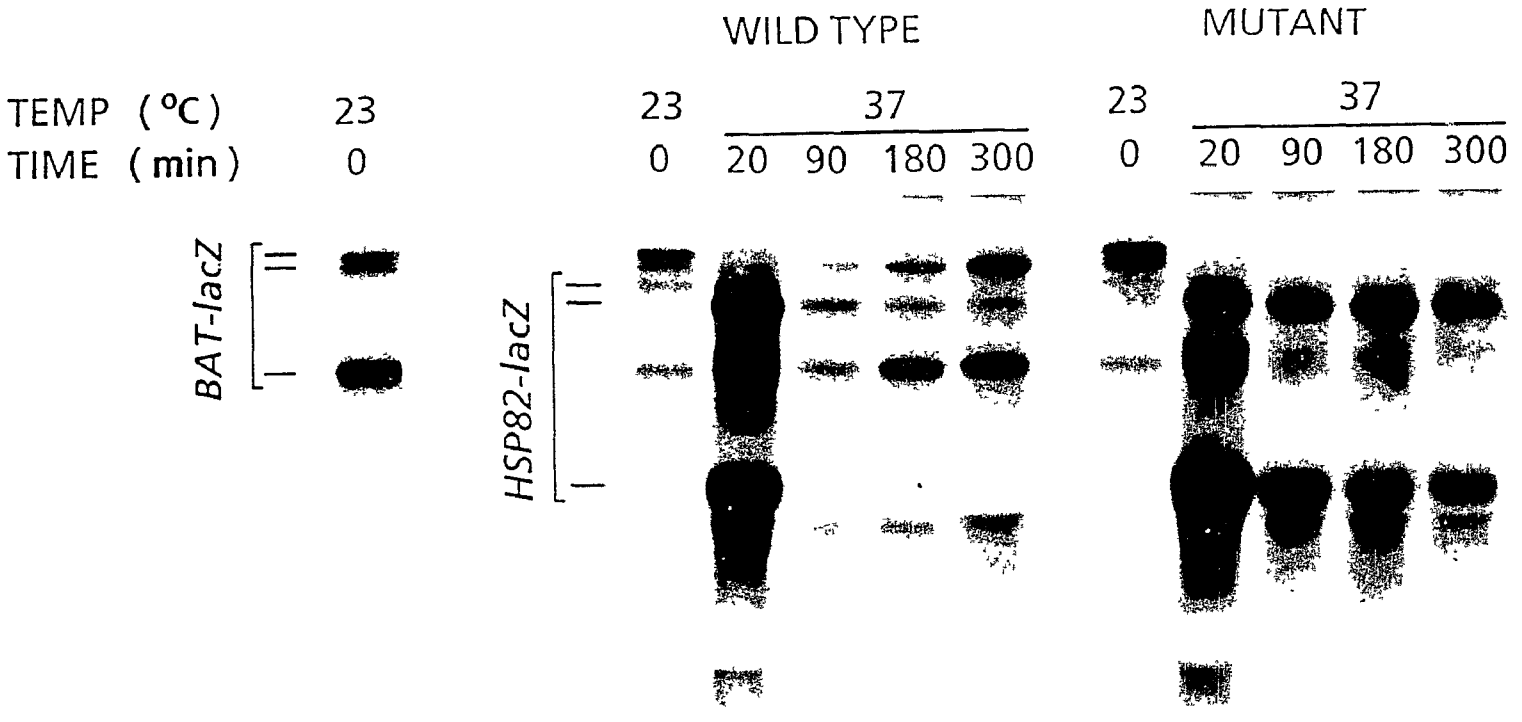

WILD TYPE
MUTANT
TEMP (°C)
TIME (min)
23
0
23
37
0
20
90
180
300
23
37
0
20
90
180
300
BAT-lacZ
HSP82-lacZ
1
2
3
4
5
6
7
8
9
10
11

Figure 25.

transcript under the control of the *HSP82* promoter (Fig. 25 lane 4). The size of this transcript is consistent with initiation at the HSP82 promoter and termination just 3' to the *lacZ* gene. Previous work in this laboratory has utilized analogous constructs in which 70-kDa heat-shock gene sequences (*SSA1* and *SSA2*) were fused to *lacZ* and integrated at the same chromosomal locus (*ura 3-52*). In these earlier studies, as here, three transcripts were detected with an identical *lacZ* probe (Barnes *et al.*, in press). All three transcripts originated from the heat-shock gene promoter used in the fusion but terminated at 3 discrete downstream sites in a temperature-dependent manner. As in this study, the major site of termination was 3' to *lacZ*. Termination at this site has also been observed by others (Clements *et al.*, 1988). In both these previous studies, termination was not 100% efficient and readthrough transcripts were readily detected. Two additional transcripts specifically detected by the *lacZ* probe in this study are probably the result of similar readthrough. The sizes of these transcripts (7.7 kb and 8.8 kb) are consistent with initiation within *HSP82* sequences, at the same site as the 3.5-kb transcript, and with termination at two previously described termination regions: one upstream of the *URA3* gene (Yarger *et al.*, 1986) and a second approximately 1 kb downstream, 3' to *URA3* coding sequences (Buckholz and Cooper, 1983). The integrated sequences and transcripts derived from these sequences are schematically represented in Fig. 26.

Despite the complexity of transcription patterns seen here it is clear that the three transcripts that originate from the *HSP82* promoter, and which collectively represent the activity of this promoter, respond in both wild-type and *cdc68-1* mutant cells to the imposition of heat-shock

Figure 26. Schematic representation of transcripts produced from an integrated *Hsp82-lacZ* fusion gene.

Integration of plasmid YIp144 was accomplished as described in Methods and Materials. The probes used in northern analysis in Figs. 25 and 26 are shown. Transcripts detected by these probes are also shown.

Figure 26.

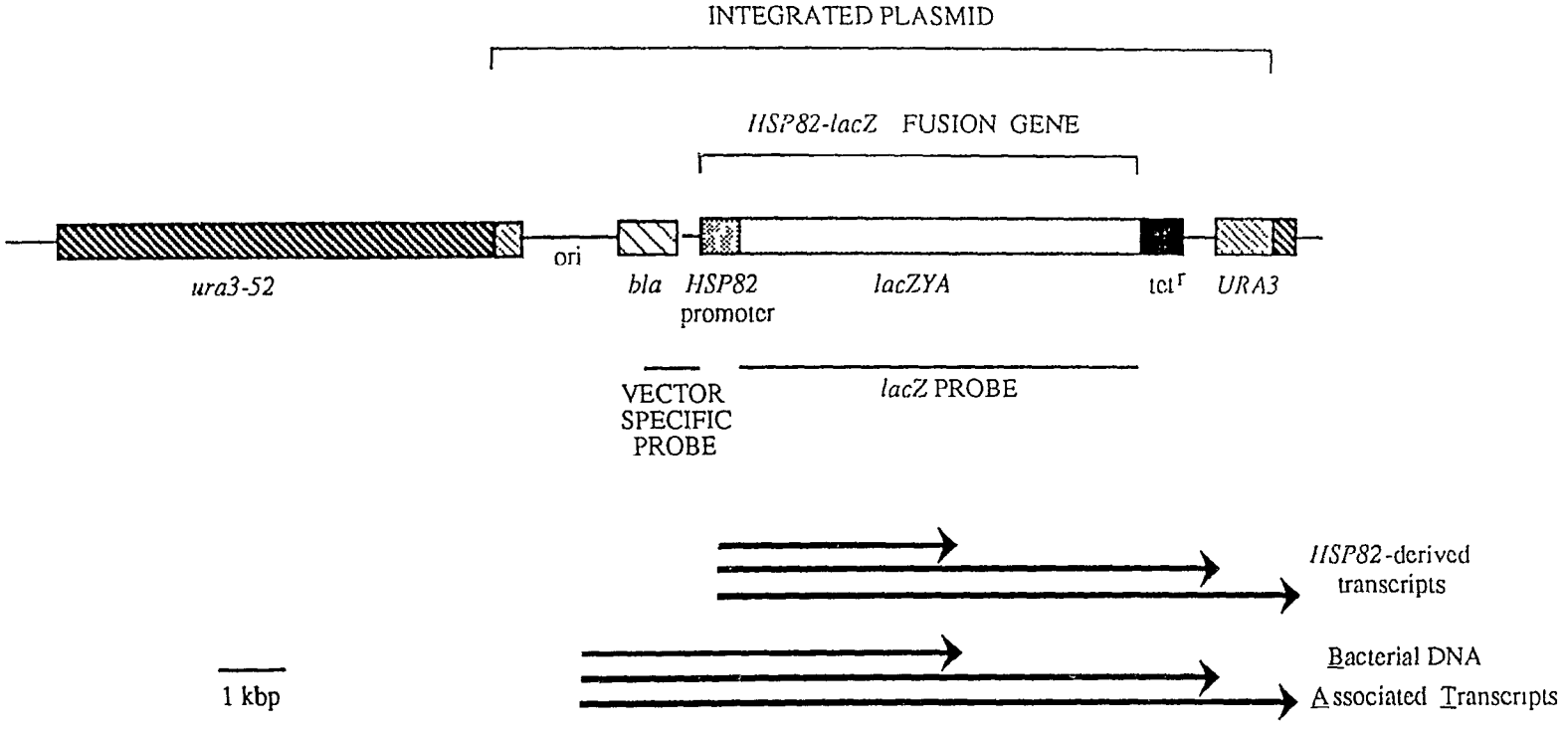

INTEGRATED PLASMID
HSP82-lacZ FUSION GENE
ori
ura3-52
bla
HSP82
promoter
lacZYA
tetr
URA3
VECTOR
SPECIFIC
PROBE
lacZ PROBE
HSP82-derived
transcripts
Bacterial DNA
Associated Transcripts
1 kbp

Figure 27. Northern analysis of transcripts initiating within vector sequences.

The blot was that used previously in Fig. 25, stripped and reprobed with a vector-specific restriction fragment (see Methods and Materials).

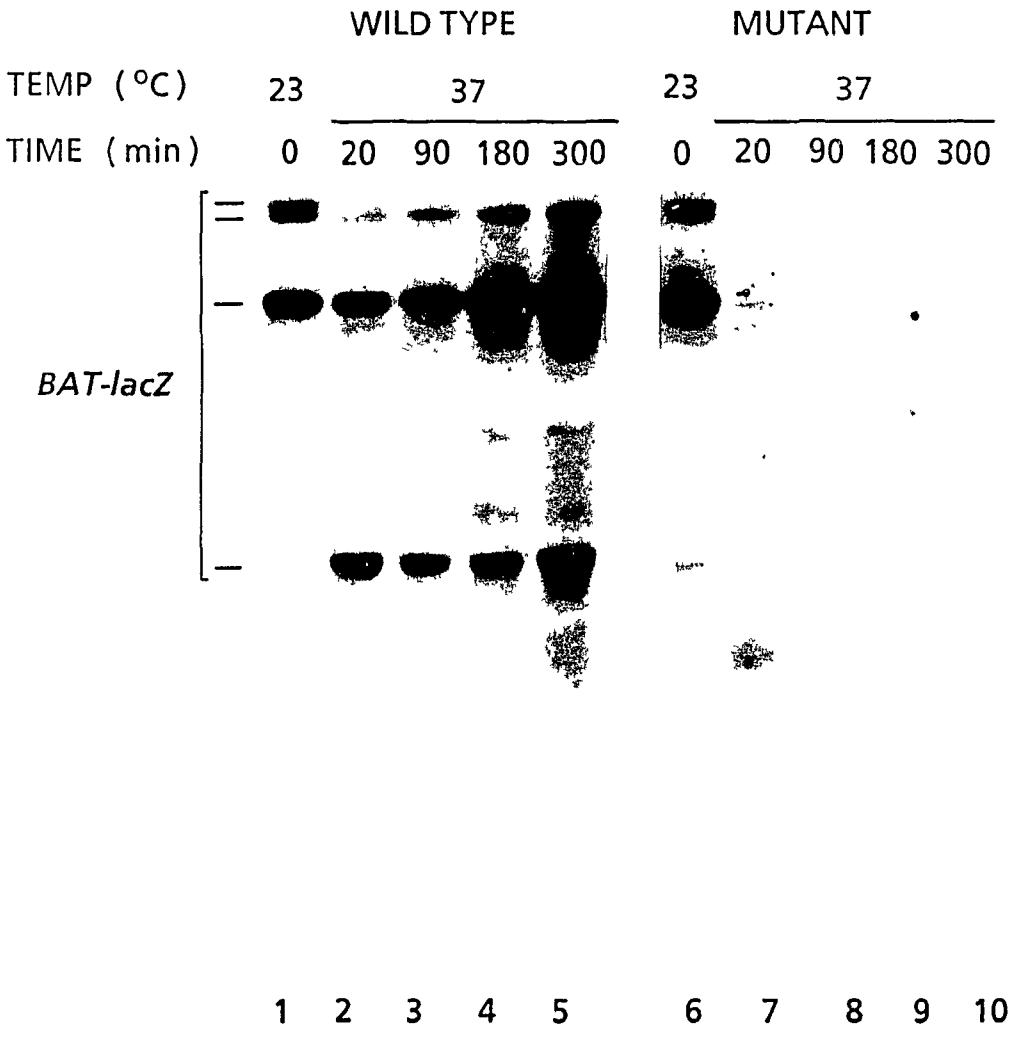

WILD TYPE
MUTANT
TEMP (°C)
23
37
23
37
TIME (min)
0
20
90
180
300
0
20
90
180
300
BAT-lacZ
1
2
3
4
5
6
7
8
9
10

Figure 27.

conditions. As expected, upon shift from 23°C to 37°C these mRNAs became considerably more abundant (compare Fig. 25, lanes 2 and 3, and lane 7 with 8). By 90 min after shift to 37°C, transcript levels in wild-type cells decreased, as described earlier, to virtually pre-shift levels (Fig. 25, lane 4). In comparison, at this time, and up to 5 hours after shift to 37°C, mRNA levels in *cdc68-1* mutant cells were lower than the peak level of abundance at 20 min after shift but significantly higher than in wild-type cells (compare lanes 4-6 with 9-11). Thus, the continued presence of significant levels of fusion transcript in *cdc68-1* mutant cells is consistent with the continued synthesis of the 90-kDa protein in Fig. 23. The persistent synthesis of this protein almost certainly reflects persistent transcription of the *HSP82* gene, since a fusion gene under the control of the *HSP82* promoter encodes transcripts that are present at elevated levels at this time.

26. A fortuitous promoter is also affected in *cdc68-1* mutant cells.

Section 25 above describes three transcripts which probably initiate within *HSP82* sequences. A further three transcripts detected by the *lacZ* probe were also detected by a probe specific for sequences upstream of the *HSP82-lacZ* fusion gene (Fig. 26; Fig. 27, lane 1). The sizes of these transcripts are 5.7 kb, 10 kb and 11 kb, although the sizes of the two largest transcripts are only approximate since they fall outside the range of the RNA standards used. Each of these transcripts is approximately 2.2 to 2.3 kb larger than those which originate within *HSP82* sequences, suggesting that these longer transcripts initiate 2.2 to 2.3 kb upstream of the *HSP82* initiation site and terminate at the sites described above (Figs. 26 and 27). The sequences upstream of the *HSP82-lacZ* fusion gene are YIp5 vector sequences derived

from pBR322. A previous study by Marczynski and Jaehning (1985), in which a transcription map of the yeast centromere plasmid YCp19 was compiled, demonstrated that sequences close to the pBR322 origin of replication act as a major site of transcription initiation in yeast cells. They termed transcripts originating within this region BAT (for Bacterial DNA Associated Transcripts. The structure of the construct used in this study is similar to YCp19 (both have YIp5 as a precursor), and it is therefore probable that the three transcripts described here initiate at the same site as transcripts identified by Marczynski and Jaehning (1985).

In contrast to the three transcripts from *HSP82* sequences, the putative BAT-lacZ transcripts were present in much lower abundance in *cdc68-1* mutant cells after transfer to the restrictive temperature (Fig. 27). As early as 20 min after shift, BAT-lacZ transcripts were barely detectable. In this respect, these transcripts behave like those from the cyclin and *LEU2* genes, suggesting that transcription from a fortuitous vector promoter is dependent upon functional *CDC68* gene product and that these transcripts are normally short-lived.

27. *HSC82* promoter activity is altered in *cdc68-1* mutant cells

S. cerevisiae contains a second gene, *HSC82*, closely related to *HSP82*. Although the Hsp82 and Hsc82 proteins are 97% similar at the amino acid level, the *HSP82* and *HSC82* genes are regulated quite differently: *HSP82* is expressed at only a low level at 23°C but is markedly induced upon heat shock; whereas *HSC82* exhibits higher basal expression but is induced to a lesser extent upon heat shock (Borkovich *et al.*, 1989). Comparison of *HSP82* and *HSC82* upstream sequences has shown that the two genes have in

common a heat-shock consensus element despite their differing response to the imposition of heat-shock conditions (Borkovich *et al.*, 1989). Given the interesting differences between the regulation of *HSP82* and *HSC82*, despite the presence of common regulatory elements, it was of interest to examine the effects of the *cdc68-1* mutation on the *HSC82* promoter. This was done using an *HSC82-lacZ* fusion similar to the *HSP82-lacZ* fusion described in the previous section. Three transcripts were detected by the *lacZ*-specific probe (Fig. 28). The sizes of these transcripts (3.8 kb, 7.9 kb and 9.0 kb) were larger than those detected from the *HSP82-lacZ* fusion, consistent with the greater length of heat-shock gene coding sequences included in the *HSC82-lacZ* fusion gene. As predicted, transcripts from the *HSC82-lacZ* fusion were more readily detectable at 23°C than those from the *HSP82-lacZ* fusion (Figs. 26, lanes 2 and 7, Fig. 28 lanes 1 and 6) and were induced when cells were shifted to 37°C (Fig. 28). Interestingly, fusion transcripts in *cdc68-1* mutant cells were present at higher abundance after prolonged incubation at 37°C than in wild-type cells (Fig. 28). Thus, despite differences in regulation under heat-shock conditions, transcripts from both *HSP82* and *HSC82* promoters appear to be affected by the *cdc68-1* mutation.

28. Endogenous *HSP82* and *HSC82* transcript abundance in wild-type and *cdc68-1* mutant cells

To examine more directly the *cdc68-1*-mediated effects on *HSP82* and *HSC82* gene expression, *HSP82* and *HSC82* transcript levels were assessed by northern analysis using oligonucleotide probes designed to discriminate between these two closely related transcripts (see Materials and Methods). Specificty of these probes under the conditions used was confirmed by

Figure 28. Northern analysis of *HSC82-lacZ* fusion transcripts.

Total RNA was extracted from strains FC90 (*CDC68 HSC82-lacZ*) (lanes 1-5) and FC90-68 (*cdc68-1 HSC82-lacZ*) (lanes 6-10) grown in YNB+10 medium at 23°C (lanes 1 and 6), or at 20 min (lanes 2 and 7), 90 min (lanes 3 and 8), 180 min (lanes 4 and 9) and 300 min (lanes 5 and 10) after transfer to 37°C. The probe was a 6.2-kbp *Bam*HI-*Sal*I purified restriction fragment containing only *lacZYA* sequences, the same as that used for the analysis shown in Figs. 24 and 25 (see Methods and Materials).

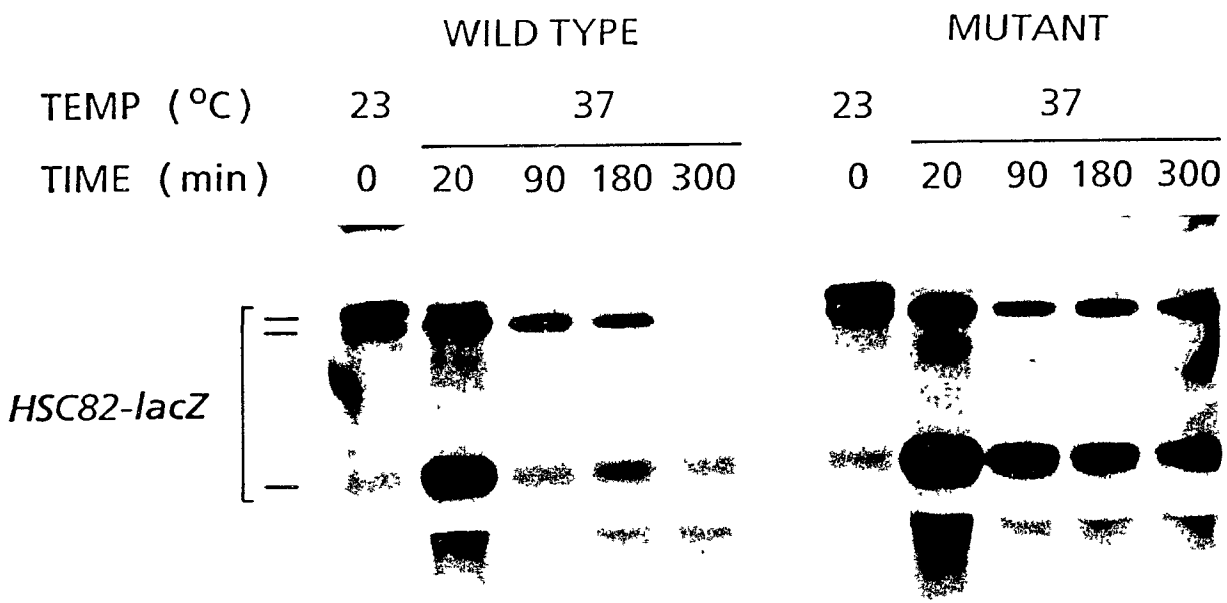


Figure 28.

1 2 3 4 5 6 7 8 9 10

hybridization to RNA isolated from strains disrupted for each member of this two-gene family (Fig. 29). Transcripts of approximately 2.4 kb were detected with each of the probes, consistent with the sizes of the two open reading frames and previous mapping of the *HSP82* transcript (Farrelly and Finkelstein, 1984; Borkovich *et al.*, 1989).

As expected, in wild-type cells (strain 21R), *HSP82* transcript levels increased following transfer from 23°C to 37°C but returned to pre-shift levels within 200 min (Fig. 30, lanes 1-5). In contrast, in *cdc68-1* mutant cells (strain ART68-1), not only did *HSP82* transcripts persist but the pattern of induction was altered; transcript levels reached a maximum at 90 min, rather than at 20 min as in wild-type cells (Fig. 30, lanes 6-10). In addition, the level of *HSP82* induction appeared higher. This is a somewhat different pattern than that observed using the *HSP82-lacZ* fusion described above, where fusion transcript levels reached a maximum at 20 min after transfer to 37°C in both wild-type and *cdc68-1* mutant cells (Fig. 25). Nevertheless, in both cases it is clear that in *cdc68-1* mutant cells, both fusion and endogenous *HSP82*-regulated transcripts are persistently synthesized, in contrast to the transient synthesis observed in wild-type cells. The difference between fusion and endogenous *HSP82*-regulated transcript abundance may reflect differences in the stability of these transcripts.

Comparison of *HSC82* transcript levels under these conditions also revealed interesting differences between wild-type and *cdc68-1* mutant cells. In this analysis, pre-shift *HSC82* transcript levels were considerably higher in *cdc68-1* mutant cells than in wild-type. For comparison, the intensity of ethidium bromide-staining of rRNA bands, photographed in the gel prior to

Figure 29. Specificity of *HSP82* and *HSC82* oligonucleotide probes

Total RNA was extracted from strains ART68-1 (*HSP82 HSC82 cdc68-1*) (lane 1), PLD82α (*CDC68 HSP82::LEU2 HSC82*) (lane 2), CLD82α (*CDC68 HSP82 HSC82::LEU2*) (lane 3), and 21R (*CDC68 HSP82 HSC82*) (lane 4). The probes were specific synthetic oligonucleotides complementary to *HSP82* or *HSC82* mRNAs (see Methods and Materials).

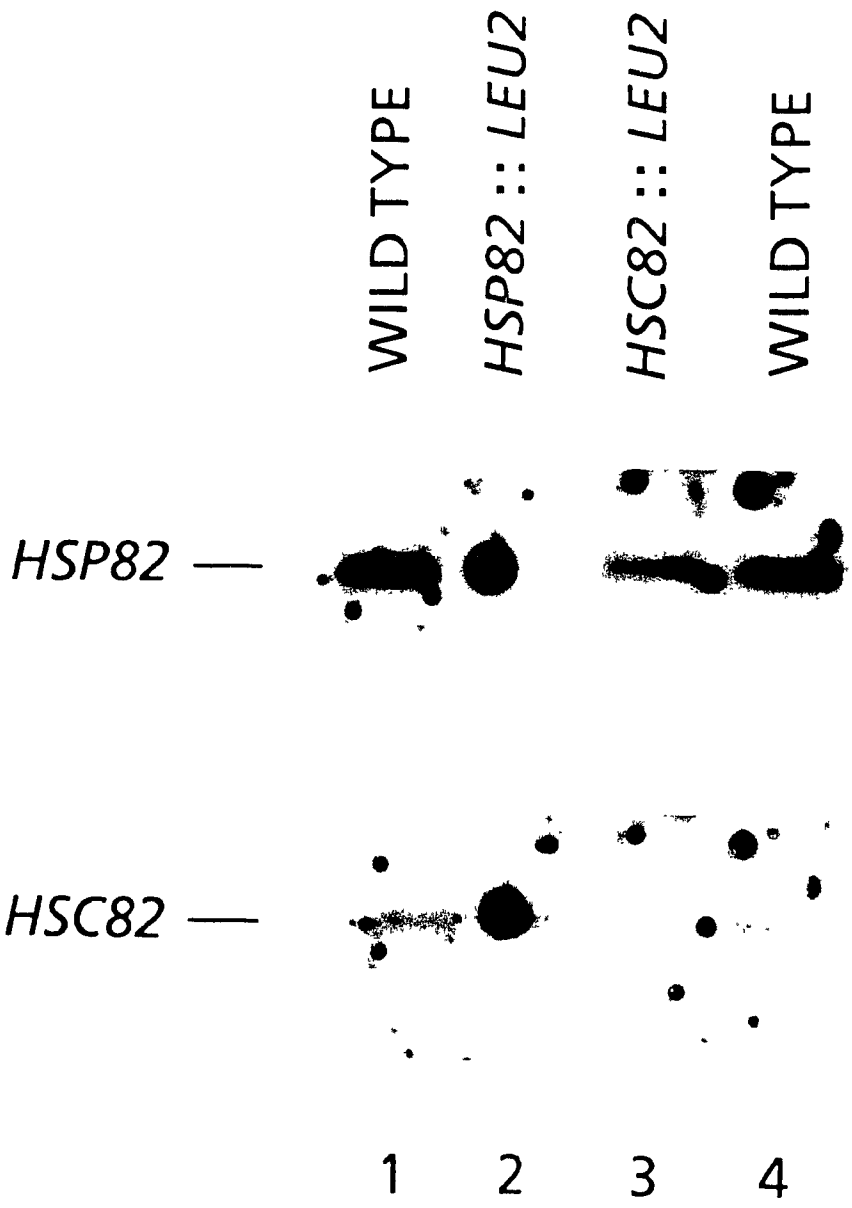


Figure 29.

Figure 30. Northern analysis of endogenous *HSP82* and *HSC82* transcripts.

Total RNA was extracted from strains 21R (*CDC68*) (lanes 1-5) and ART68-1 (*cdc68-1*) (lanes 6-10) grown at 23°C (lanes 1 and 6), or at 20 min (lanes 2 and 7), 90 min (lanes 3 and 8), 200 min (lanes 4 and 9) and 300 min (lanes 5 and 10) after transfer to 37°C. The probes were specific synthetic oligonucleotides complementary to *HSP82* or *HSC82* mRNAs (see Methods and Materials). The lower panel shows the ethidium-bromide-stained gel used to prepare the blot used in this experiment.

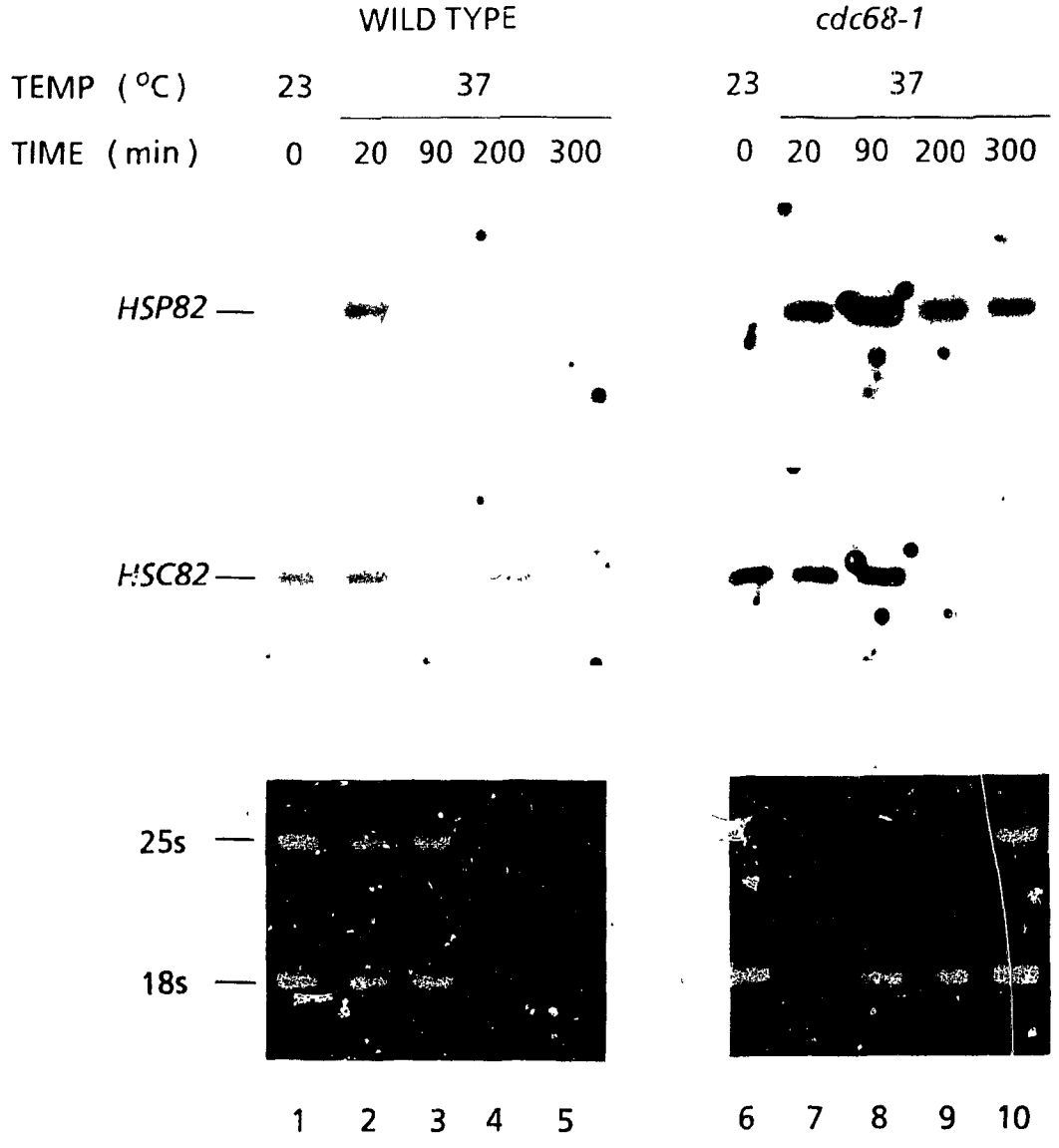

WILD TYPE
cdc68-1
TEMP (°C)
23
37
23
37
TIME (min)
0
20
90
200
300
0
20
90
200
300
HSP82
HSC82
25s
18s
1
2
3
4
5
6
7
8
9
10

Figure 30.

transfer, confirms that the increased abundance of *HSC82* transcripts is not due to inconsistent loading (Fig. 30, lanes 1 and 6). This is therefore the second gene whose expression has been found to be altered in *cdc68-1* mutant cells at the permissive temperature of 23°C. However, whereas *HSC82* expression appears greater under these circumstances, the expression of the other affected gene, *LEU2*, is decreased (Fig. 21). Despite the differences between *HSC82* transcript levels at 23°C, transcript levels in both wild-type and *cdc68-1* mutant cells exhibited a moderate increase in response to transfer to 37°C. Furthermore, in both wild-type and mutant cells *HSC82* mRNA levels decreased to low levels by 200 min after transfer to 37°C. Thus, the overall pattern of *HSC82* expression appears similar in wild-type and *cdc68-1* mutant cells although absolute transcript levels appear higher in *cdc68-1* mutant cells, at least until several hours after transfer to the restrictive temperature.

29. Effects on gene expression are common to other *cdc68* mutant alleles

The cell-cycle and δ-element-suppression phenotypes caused by the *cdc68-1* temperature-sensitive mutation are indistinguishable from those caused by the *spt16-197* mutation, despite the different approaches by which these two mutations were identified. It was of interest, therefore, to determine whether the effects on heat-shock gene expression seen in *cdc68-1* mutant cells are also a feature of the *spt16-197* mutant phenotype. The patterns of newly synthesized proteins in strains L577 (*spt16-197*) and FY56 (*SPT16*) were compared. As shown in Fig. 31, the *spt16-197* mutation caused changes in the pattern of labelled proteins similar to those changes seen in *cdc68-1* mutant cells (Fig. 23). These changes include persistent synthesis of

Hsp82. Therefore the effect on heat-shock gene expression is an additional feature common to the *cdc68-1/spt16-197* mutant phenotype.

Figure 31. Proteins synthesized in wild-type and *spt16-197* mutant cells.

Actively proliferating cells of strains FY56 (*SPT16*) (lanes 1-8) and L577 (*spt16-197*) (lanes 9-16) were pulse-labelled with [^{35}S]methionine before (lanes 1 and 9) and at 0.5, 1, 2, 3, 4, 5 and 6 h after transfer from 23°C to 37°C. Cells were grown in YNB+9 medium (without methionine). Equal amounts of radiolabelled TCA-precipitable material were resolved by 1-D SDS-PAGE in lanes 1-8 and in lanes 9-16. The migration of molecular-weight markers (in kDa) is given on the left. The arrow on the right indicates the position of the Hsp82 protein.

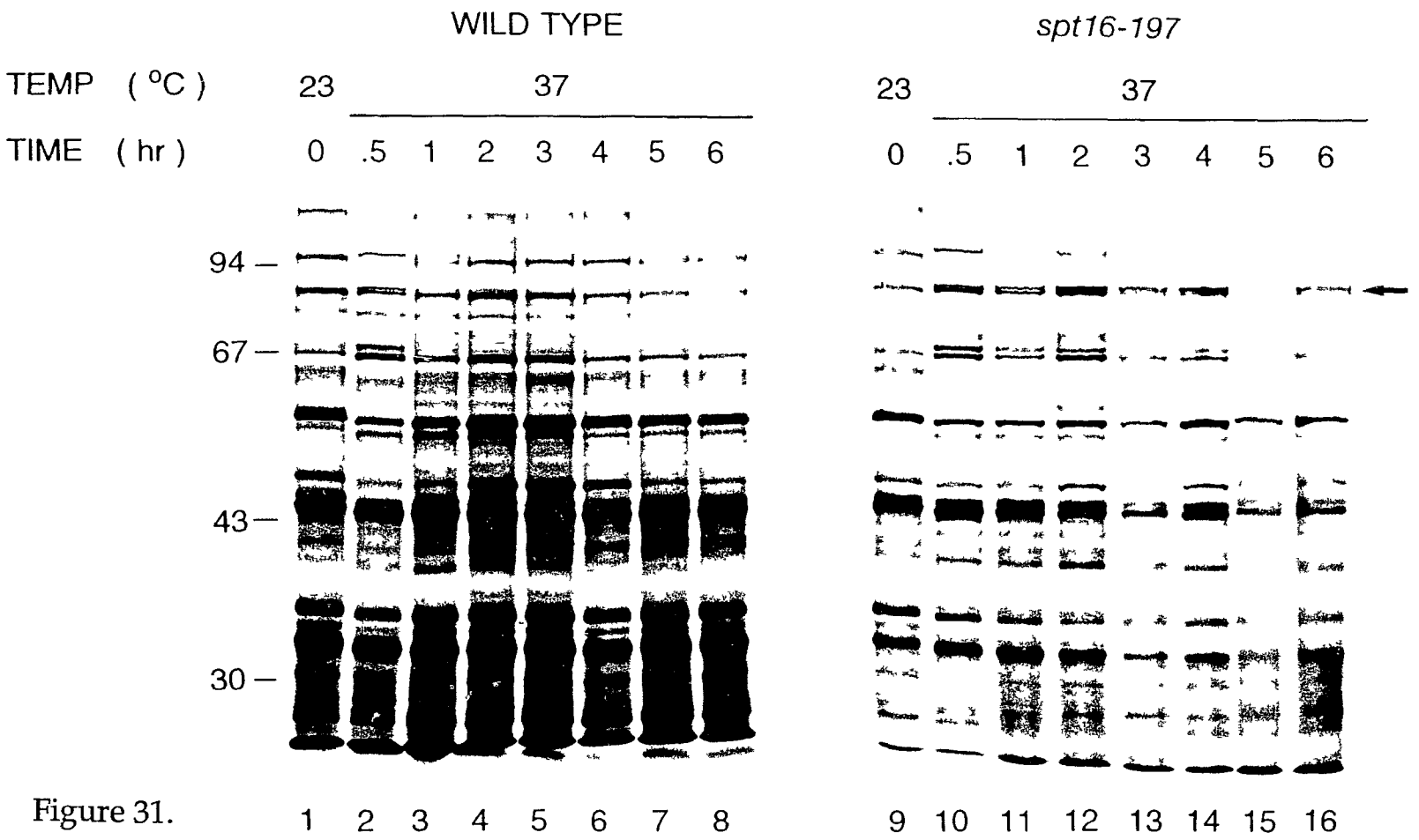

WILD TYPE
spt16-197
TEMP (°C)
23
37
23
37
TIME (hr)
0 .5 1 2 3 4 5 6
0 .5 1 2 3 4 5 6
94
67
43
30
1 2 3 4 5 6 7 8
9 10 11 12 13 14 15 16

Figure 31.

IV. DISCUSSION

1. The *CDC68* gene plays a general role in transcription

The *CDC68* gene was first identified by a conditional mutation that caused cells to become temperature-sensitive for the performance of the G1 regulatory event, Start (Prendergast *et al.*, 1990). In the present study, additional aspects of the *cdc68* mutant phenotype have been identified. The *cdc68-1* mutation affects the abundance of transcripts from apparently unrelated genes including *ACT1*, *LEU2*, the G1-cyclin genes, and at least one heat-shock gene, *HSP82*. While most of these transcripts are present at decreased abundance in *cdc68-1* mutant cells after transfer to the restrictive temperature, the *HSP82* transcript is present at increased levels. Thus, the *cdc68-1* mutation has both positive and negative effects on the expression of an unexpectedly wide variety of genes.

During the course of this study, the *CDC68* gene was identified independently (as *SPT16*) as a high-copy suppressor of *his4-912δ* and *lys2-128δ* mutations (Malone *et al.*, submitted). In the *his4-912δ* and *lys2-128δ* mutations δ-element insertions interrupt the 5' non-coding or coding sequences of the *HIS4* and *LYS2* genes, disrupting the expression of downstream *HIS4* or *LYS2* coding sequences. As a result, these mutations confer His^- or Lys^- phenotypes. *CDC68/SPT16* is one of a collection of *trans*-acting *SPT* (suppressor of Ty insertion) genes that suppress certain combinations of solo δ- or Ty-insertion mutations when present either in mutant form or in elevated copy number. Suppression of Ty and solo δ insertion mutations by previously characterized *SPT* genes has been found

to result from the restoration of some degree of functional transcription at otherwise non-functional mutant genes, and has therefore provided an effective genetic selection scheme for the identification of genes involved in transcription (Winston *et al.*, 1984; Silverman and Fink, 1984; Winston *et al.*, 1987; Clark-Adams and Winston, 1987; Clark-Adams *et al.*, 1988; Fassier and Winston, 1988; Eisenmann *et al.*, 1989; Swanson *et al.*, 1991). Indeed, a number of *SPT* genes have been shown to define factors directly involved in transcription. For example, *SPT15* has been found to encode the TATA-binding factor, TFIID (Eisenmann *et al.*, 1989), while *SPT13* (Fassler and Winston, 1988; 1989) is the same as *GAL11*, identified as a transcriptional activator of *GAL4*-regulated genes and subsequently demonstrated to affect the expression of several others (Nogi and Fukasawa, 1980; Suzuki *et al.*, 1988; Nishizawa *et al.*, 1990; Himmelfarb *et al.*, 1990).

The pleiotropic effects on gene expression shown here to be caused by the *cdc68-1* mutation, and the independent identification of the *CDC68* gene by the δ-suppression approach, strongly suggest that the *CDC68* gene product plays a general role in transcription. Temperature-sensitive *cdc68* mutations therefore provide a link between transcription and cell-cycle regulation since mutations in the *CDC68* gene also cause concerted cell-cycle arrest at the restrictive temperature. This discussion will first focus on the role of the Cdc68 protein in transcription. Subsequent sections will consider the relationship between structure and function in the Cdc68 protein. Finally, the manner in which mutations in the *CDC68/SPT16* gene influence cell cycle regulation and the heat-shock response will be discussed.

2. The *CDC68* nucleotide sequence: implications for *CDC68* gene product function.

A particularly striking feature of the predicted Cdc68 protein is the dense cluster of acidic residues at the C-terminus; over a 65 amino acid region, from residues 957 to 1021, 36 of 65 amino acids (55%) are either aspartic or glutamic acid. Basic residues are completely absent from this region. This acidic region is also notably rich in serine residues (12 of 65, or 18%). This structural feature of the Cdc68 sequence is reminiscent of acidic transcriptional activators, and of a more diverse group of proteins thought to affect transcription through a role in chromatin conformation. Sequence considerations alone are insufficient to place Cdc68 in either of these categories of previously identified proteins, but these sequence similarities suggest that certain aspects of Cdc68 protein function may be related to the functions of these other proteins, as described below.

a) Similarity with acidic transcriptional activators

The C-terminal acidic region of the Cdc68 protein is reminiscent of acidic stretches found in many transcriptional activators including those encoded by the yeast *GAL4* (Ma and Ptashne, 1987a) and *GCN4* genes (Hope and Struhl, 1986). In *GAL4* and *GCN4*, as with several other transcriptional activators from a variety of systems, large parts of the encoded protein appear to be dispensable. Furthermore, sequences which activate transcription are separable from sequences that bind DNA, and these activating sequences control transcription when fused to either their own or to heterologous DNA-binding regions (provided that appropriate binding sites are present within the promoter of the target gene) (Brent and Ptashne,

1985; Keegan *et al.*, 1986; Hope and Struhl, 1986). Eukaryotic transcription factors are thus composed of combinations of functional domains that can be exchanged with remarkable flexibility. The similarity of the Cdc68 amino acid sequence to features of such modular transcription factors is considered below.

i) Acidic transcriptional activation domains

Deletion and domain-swapping experiments have revealed that the minimal sequences of *GCN4* and *GAL4* required to activate transcription share no sequence similarity, but fall within regions of each gene that specify appreciable acidic content, suggesting that it is the acidity of these regions that is necessary for transcriptional activation (Hope and Struhl, 1986; Ma and Ptashne, 1987a). Consistent with this hypothesis, many mutations in *GAL4* which result in increased transcriptional activation increase the net negative charge of the Gal4 activation domain (Gill and Ptashne, 1987). The finding that even acidic sequences from *E. coli* can function as "acidic activators" when fused to an appropriate DNA-binding domain further supports the hypothesis that acidic regions play a role in transcriptional activation (Ma and Ptashne, 1987b). The mechanism by which acidic regions activate transcription is unknown: some studies suggest that such regions directly contact the TATA-binding factor, TFIID (Stringer *et al.*, 1990; Ingles *et al.*, 1991), or a second component of the transcription initiation apparatus, TFIIB (Lin and Green, 1991), while others suggest that acidic activators contact the initiation complex through intermediate proteins termed mediators, adaptors or coactivators (Berger *et al.*, 1990; Kelleher *et al.*, 1990; Pugh and Tjian, 1990; Flanagan *et al.*, 1991).

Several lines of evidence suggest that acidic content is not the only important feature of an acidic activation domain. For example, Cress and Triezenberg (1991) have demonstrated, by extensive mutagenesis, that derivatives of the viral acidic activator VP16 which contain the same negative charge mediate different levels of transcriptional activation in transient-transfection assays. Additional structural features that have been implicated in transcriptional activation by acidic domains include amphipathic α-helices and specific arrangements of basic residues.

The involvement of α-helices in transcriptional activation is suggested by the observation that several acidic activation regions contain amino acid sequences with potential to form short amphipathic structures bearing acidic and hydrophobic residues on opposite sides of an α-helix. It has therefore been proposed that it is this type of structure, rather than overall negative charge, that determines the activity of an acidic activator region (Ptashne, 1988). Support for this view is provided by a study in which a 15-amino-acid peptide with potential to form an amphipathic α-helix was shown to activate transcription when fused to the DNA-binding domain of *GAL4*, while a sequence containing the same residues but in a scrambled order (and therefore unlikely to form an α-helix), was unable to activate transcription (Giniger and Ptashne, 1987). However, while amphipathic α–helical structure may be important in some cases it is apparently not universally required, as shown by Cress and Triezenberg (1991) who found that inserting helix-breaking proline residues into the putative amphipathic α-helix of VP16 had negligible effect on transcriptional activation. These same studies also failed to reveal any correlation between transcriptional activation and predicted amphipathic character of mutant activation

sequences. Furthermore, one of the two acidic activating regions of the *GAL4* gene product is unlikely to form an α-helical structure, as is at least one of the *E. coli* acidic activating sequences identified by Ma and Ptashne (1987b).

The study of Cress and Triezenberg (1991), while arguing against the importance of amphipathic α-helices, reveals other considerations important for transcriptional activation. Cress and Triezenberg (1991) suggest that the position and sequence context of bulky hydrophobic residues may be a critical feature of the activation domains of a number of transcriptional activators, including those of the acidic activator class.

Although the acidic region of the Cdc68 protein is similar to those of acidic transcriptional activators it also differs from them in several respects. Firstly, most well-characterized acidic activation regions are less acidic than that at the C-terminus of the Cdc68 protein. For example, the minimal activating portion of Gcn4 falls within a segment of 60 amino acids that is predicted to contain only 18 acidic and 2 basic residues (Hope and Struhl, 1986), compared with the most acidic stretch of Cdc68 which contains 36 acidic, and no basic amino acids, within a 65 amino-acid stretch. In addition, the predicted Cdc68 protein contains no additional structural features implicated in transcriptional activation. Although secondary structure analysis (Chou and Fasman, 1978) reveals that the acidic region of the predicted Cdc68 protein has the potential to form an α-helix, the order of residues is inconsistent with amphipathic character. Similarly, while the acidic region of the Cdc68 protein does contain hydrophobic residues, their positions do not correspond with those of the hydrophobic residues in the activation regions aligned by Cress and Triezenberg (1991). Thus, significant

differences are apparent between the Cdc68 C-terminal acidic region and the transcriptional activation domains of proteins such as Gal4, Gcn4 and VP16, suggesting that Cdc68 may function in transcription in a manner distinct from these other transcriptional activators.

While the acidic region of the Cdc68 protein shows only limited similarity to those of transcriptional activators of RNAP II-dependent gene expression, the similarity of the *CDC68* gene product to an RNAP I transcription factor, human transcription factor, hUBF (human upstream binding factor), is worthy of discussion at this point. The hUBF nucleotide sequence predicts a protein of 89 kDa which exhibits an acidic C-terminus in which 64% of the terminal 89 amino acids are either glutamic or aspartic acid (Jantzen *et al.*, 1990). As in Cdc68, this region contains a high proportion (25%) of serine residues and contains consensus recognition sites for casein kinase II phosphorylation. Jantzen *et al.* (1990) have proposed that this acidic region mediates interaction with the highly positively charged surface of hSL1, a second factor which, like hUBF, is indispensable for RNA polymerase I transcription *in vitro* (Lerned *et al.*, 1985). hSL1 does not have DNA-binding activity, but forms part of an initiation complex by interaction with hUBF (Bell, 1988). By analogy, the Cdc68 protein may interact with a positively charged protein to form a transcription-promoting complex.

ii) DNA-binding domains of transcriptional activators

As described above, many transcriptional activators contain sequences that bind DNA. Inspection of the predicted Cdc68 sequence has failed to reveal any recognizable DNA-binding motif. The absence of such a motif is, however, not inconsistent with the hypothesis that the Cdc68 protein plays a

direct role in transcription. For example, the Cdc68 protein might not bind DNA itself, but might do so by association with other proteins. Precedent for this situation is seen in the case of the viral transcriptional activator VP16, which itself has no DNA-binding activity (Marsden *et al.*, 1987) but binds DNA through association with host-cell DNA-binding proteins such as Oct1 (McKnight *et al.*, 1987; Triezenberg *et al.*, 1988a, 1988b; O'Hare and Goding, 1988; Preston *et al.*, 1988; Gerster and Roeder, 1988). Similarly, Adenovirus E1a binds DNA by associating with the DNA-binding protein ATF2 (Liu and Green, 1990). A related situation is exemplified by the yeast *HAP4* gene product, which forms a DNA-binding complex with the products of the *HAP2* and *HAP3* genes at the *CYC1* promoter in *S. cerevisiae.* None of the components of this DNA-binding complex exhibit DNA-binding activity individually (Forsburg and Guarente, 1989). It is also possible, of course, that the *CDC68* gene product contains a previously undescribed DNA-binding domain.

b) Similarity with proteins thought to interact with chromatin

Acidic regions are also found in a diverse group of nuclear proteins, including high mobility group (HMG) proteins, nucleoplasmin (the most abundant protein in the *Xenopus* oocyte nucleus; Laskey and Earnshaw, 1980) and nucleolin (Lapeyre *et al.*, 1987). The acidic regions of these proteins are of various lengths, but in general contain a higher density of acidic residues than do the acidic activation regions of proteins such as Gal4 and Gcn4 which directly activate transcription. In most cases the *in vivo* roles of these highly acidic proteins have not been clearly defined and the functions of their various acidic regions remain speculative. Nevertheless,

it has been suggested that the acidic regions of proteins such as these may play an important role in mediating protein-protein and protein-DNA interactions within the nucleus, through which they may play a role in transcription. In particular some of these proteins have been suggested to interact *in vivo* with chromatin, and in some cases have been shown to bind to core histones (reviewed by Earnshaw, 1987).

In eukaryotic chromatin, 146-bp lengths of DNA are wrapped approximately 1.8 times around nucleosome core particles which each consist of an octamer of histone subunits (two molecules each of histones H2A, H2B, H3 and H4). In higher eukaryotes, histone H1 mediates the further compaction of this structure into a higher order chromatin fibre; in yeast however, histone H1 has not been identified and chromatin does not appear to be further folded to any significant extent. Considerable evidence correlates altered chromatin structure with transcriptionally active regions of the chromosome and suggests therefore that chromatin structure must be modified in order to allow efficient transcription (reviewed by Elgin, 1988; Gross and Garrard, 1988; Grunstein, 1990). Such altered structure includes: sensitivity to nucleases, increased acetylation of core histones, histone ubiquitination, and association with non-histone proteins including HMG proteins. In *S. cerevisiae*, alteration of core histone subunit stoichiometry leads to altered gene expression (Clark-Adams *et al.*, 1988) demonstrating that gene expression is sensitive to chromatin structure. In addition, a growing body of *in vitro* evidence confirms that the presence of nucleosomes on promoter sequences is inhibitory for transcription. Several groups have demonstrated that *in vitro* assembly of nucleosomes competes with the assembly of active transcription complexes and that these

inhibitory effects can be overcome by the preincubation of templates with the TATA binding factor, TFIID (Matsui, 1987; Lorch *et al.*, 1987; Workman and Roeder, 1987; Knezetic *et al.*, 1987), or by the addition of certain transcriptional activators (Workman *et al.*, 1990; 1991).

It has been proposed that the function of some nuclear proteins which contain extremely acidic regions is to facilitate transcription by alteration of chromatin structure. Indeed both HMG1 and HMG2 have been demonstrated to stimulate the initiation of transcription by RNA polymerase II and RNA polymerase III *in vitro*, even when transcription is placed in competition with nucleosomes (Tremethick and Molloy, 1986). Numerous mechanisms through which acidic regions could influence chromatin structure have been suggested, chiefly based upon the interaction of acidic sequences with the positively charged N-termini of core histones.

One mechanism whereby acidic regions could modify chromatin structure to favour transcription is at the level of nucleosome disassembly; such a role has been postulated for *Xenopus* nucleoplasmin based on limited *in vitro* evidence (Laskey and Earnshaw, 1980). A further explanation is that acidic regions, through weak electrostatic interactions with histones, serve to bring DNA-binding proteins (or complexes) closer to DNA, increasing the frequency of interaction between DNA-binding proteins (or complexes) and their target DNA sequences (Earnshaw, 1987). Alternatively acidic regions could act as a "reservoir" onto which histones are temporarily displaced while proteins such as RNA polymerase, occupy DNA sites on which the histones are usually present. This process might facilitate transcription by providing a "storage site" for temporarily displaced histones (Earnshaw, 1987).

All of the above explanations could be consistent with the effects on gene expression seen in *cdc68* mutant cells and in cells containing multiple copies of the wild-type *CDC68* gene. Perturbation of any of the above mechanisms might result in anomalous chromatin conformation leading to alterations in transcription by RNA polymerase II. Such alterations in chromatin conformation might not necessarily have the same effects on gene expression at all loci. Indeed, in experiments which deplete *S. cerevisiae* cells of nucleosomes, transcription from several promoters (*HIS3, TRP1*) was found to be unaffected (Han *et al.*, 1988) while transcription from the *PHO5, CYC1, GAL1,* and *CUP1* promoters was activated (Han and Grunstein, 1988; Grunstein, 1990). These findings could explain why mutations in the *CDC68* gene (or increased copy) have different effects on different genes. To investigate the relationship between the *cdc68* mutant phenotype and nucleosome-mediated transcriptional repression it will be of interest to compare chromatin structure in wild-type and *cdc68* mutant cells.

c) *CDC68* is similar to other *SPT* genes

The amino acid sequences specified by the *SPT5* (Swanson *et al.*, 1991) and *SPT6* (Swanson *et al.*, 1990) genes of *S. cerevisiae* both contain extensive N-terminal acidic stretches, analogous to the C-terminal acidic region in the predicted Cdc68 protein. The Spt5, Spt6, and Cdc68 acidic regions are similar both in size and in density of negatively charged residues; one stretch of 81 amino acids in Spt5 is 60% acidic, whereas in Spt6, 50% of the the first 70 residues are acidic (compared with 55% acidic residues in a 65 amino acid stretch in the Cdc68 protein). However, Spt5 differs from Spt6 and Cdc68 by

containing uninterrupted stretches of acidic residues (of 11 and 20 residues); in contrast, in Spt6 there are no stretches of acidic amino acids longer than 7 residues, and in Cdc68 none greater than 4 residues in length. Both Spt5 and Spt6, like Cdc68, are large proteins (116 and 168 kDa, respectively) without recognizable DNA-binding motifs and contain potential casein kinase II phosphorylation sites, as does Cdc68.

The similarity between these structural features in the Spt5, Spt6 and Cdc68 amino acid sequences suggests that the functions of these gene products may be related. Genetic evidence supports this idea. The *SPT5* and *SPT6* genes were first identified by mutations that suppress *his4-912δ* and *lys2-128δ* δ-insertion mutations (Winston *et al.*, 1984; Fassler and Winston, 1988). Like *CDC68/SPT16*, *SPT5* and *SPT6* have been demonstrated to suppress these δ-insertion mutations when carried on high-copy plasmids (Clark-Adams and Winston, 1987; Swanson *et al.*, 1991). Similarly, temperature-sensitive mutations in *CDC68/SPT16* suppress δ-insertion mutations (Malone *et al.*, submitted; this study). Unlike *CDC68* however, null mutations in both *SPT5* and *SPT6* are dominant for suppression of δ-insertion mutations; diploids homozygous for *his4-912δ* and *lys2-128δ* but which contain one wild-type and one disrupted copy of either *SPT5* or *SPT6* exhibit His^+, Lys^+ phenotypes. Null mutations in the *CDC68* gene are recessive for suppression of δ-insertion mutations (Malone *et al.*, submitted). The dosage effects of *SPT5* and *SPT6* have led to the suggestion that the Spt5 and Spt6 proteins may function as part of a complex; disruption of stoichiometry in this putative complex leading to altered transcription (Clark-Adams and Winston, 1987; Swanson *et al.*, 1991). By analogy, Cdc68 may also function in a complex whose function is dependent upon proper

stoichiometry of its components. Preliminary analysis of this possibility by the isolation of unlinked suppressor mutations suggests that other components of such a complex may be identified using a genetic approach (Q. Xu, T. Parsons, and A. Rowley, unpublished observations). Additionally, the suppression caused by *CDC68*, *SPT5* and *SPT6* is also similar to that caused by the histone genes, *HTA1* and *HTB2* (Clark-Adams *et al.*, 1988), suggesting that *CDC68*, *SPT5* and *SPT6* gene function may be related to chromatin structure.

In addition to suppression of *his4-912δ* and *lys2-128δ*, the *spt16-197* mutation has been shown to suppress both *cis*- and *trans*-acting mutations which disrupt the expression of *SUC2*, a gene necessary for growth on carbon sources such as sucrose and raffinose. *spt16-197* at least partially suppresses deletions of *SUC2* regulatory sequences and mutations in *SNF2*, *SNF5* and *SNF6* (Neigeborn and Carlson, 1984; Abrams *et al.*, 1986; Laurent *et al.*, 1990; Estruch and Carlson, 1990; Laurent *et al.*, 1991), which encode *trans*-acting regulatory proteins required for *SUC2* expression (Malone *et al.*, submitted). *SNF2* is also required for transcription of Ty elements (Happel *et al.*, 1991); this requirement is also suppressed by the *spt16-197* mutation (Malone *et al.*, submitted). Thus *cdc68/spt16* mutations can restore functional transcription to a non-functional promoter whether such lack of function is the result of a promoter mutation or the absence of a *trans*-acting regulatory factor. Similar phenotypes have been described for *spt6* mutations (Neigeborn *et al.*, 1986, 1987). These phenotypic similarities further suggest that the *SPT5*, *SPT6*, and *CDC68/SPT16* genes encode functionally related proteins that affect the expression of a variety of genes. However *SPT5*, *SPT6* and *CDC68/SPT16* cannot encode proteins that perform identical functions since

all three genes are essential for viability (Clark-Adams and Winston, 1987; Malone *et al.*, submitted; Swanson *et al.*, 1991). Furthermore, genetic evidence suggests that the protein encoded by *CDC68/SPT16* functions in a manner distinct from proteins encoded by *SPT5* and *SPT6* (discussed in Malone *et al.*, submitted). This genetic evidence suggests that the Spt5 and Spt6 proteins may form a complex with the product of the *SPT4* gene (Winston *et al.*, 1984; Swanson *et al.*, 1991). Thus, while Cdc68/Spt16 may function in a complex, this is likely to be distinct from, although possibly related to, the putative Spt5/Spt6/Spt4 complex.

In summary, structural features of the Cdc68 amino acid sequence and characteristics of the *cdc68/spt16* mutant phenotype, coupled with effects caused by *CDC68* elevated copy number, suggest that the Cdc68 protein plays a novel role in transcription. This role may be related to chromatin structure and probably involves interaction with other proteins. The function of the Cdc68 protein may be related to, although distinct from, those of Spt5 and Spt6.

3. Structure and function in the Cdc68 gene product

The two temperature-sensitive *cdc68/spt16* mutations that have been identified have similar pleiotropic phenotypes: both *spt16-197* and *cdc68-1* suppress *his4-912δ* and *lys128δ* insertion mutations, both mutations confer G1 arrest at the restrictive temperature (Prendergast *et al.*, 1990; Malone *et al.*, submitted; this study) and both similarly affect *HSP82* expression. This functional similarity is consistent with structural analysis, which has localized both mutations to the 5' region of the *CDC68* gene (5' to nucleotide +471 of the *CDC68* ORF) by transformation with subclones. These mutations

are assumed to lie within Cdc68 N-terminal coding sequences, although nucleotide sequencing will be necessary to confirm that this is indeed the case.

Surprisingly, the first 971 nucleotides of the *CDC68* ORF were found to be dispensable for complementation of both the *cdc68-1* and *spt16-197* mutations when deleted from a *CDC68* gene carried on a high-copy vector. In this respect, temperature-sensitive mutations in the 5' end of the *CDC68* gene appear to be more damaging than removal of this entire region. One explanation for this finding is that the *cdc68-1* and *spt16-197* mutations do not themselves fall within critical regions of the Cdc68 protein, but that the presence of these mutations precludes appropriate function of downstream domains. For example, the mutations could alter protein structure. Perhaps appropriate structure is possible in the complete absence of N-terminal sequences.

Transcripts from the deleted *CDC68* genes have not been mapped (all *CDC68* promoter sequences were deleted in this analysis) and it is not clear whether vector sequences, or sequences internal to the *CDC68* ORF, provide promoter sequences from which these deleted genes are expressed. Furthermore, reading-frame analysis of the deleted clones does not clearly indicate where translation of the truncated Cdc68 proteins begins. It is probable, however, that translation initiation will be inefficient due to the abundance of short open reading frames present upstream of suitable in-frame initiation codons in most of the deleted clones. Because translation invariably begins at the AUG codon closest to the 5' end of the mRNA in yeast (Cigan and Donahue, 1987), as predicted by the scanning model (Kozak, 1978), upstream open reading frames of this kind are thought to seriously

affect translation initiation at downstream AUG codons due to the inefficiency with which the translation machinery reinitiates translation on the same template. Furthermore, significant levels of translation initiation at non-AUG codons have not been demonstrated in yeast, despite two studies which have addressed this issue (Clements *et al.*, 1988; Donahue and Cigan, 1988).

Preliminary analysis demonstrates that levels of *CDC68*-hybridizable transcripts are significantly higher in cells transformed with these deleted constructs than in cells containing only the endogenous *CDC68* gene. These partially deleted genes may be capable of complementing *cdc68* mutations because of increased levels of expression compared to that of the endogenous *CDC68* gene. Even if production of a truncated Cdc68 protein is inefficient, sufficient protein to complement the *cdc68-1* and *spt16-197* temperature-sensitive mutations might still be produced. It would be of interest to determine whether these deleted constructs complement the temperature sensitivity of *cdc68-1* or *spt16-197* mutant cells when expressed at normal levels, for example under the control of the *CDC68* promoter. This might determine whether complementation of *cdc68* mutations by these deleted clones is due solely to over-expression or whether the deleted Cdc68 proteins can complement *cdc68* mutations at normal, physiological, levels.

Although able to complement the temperature sensitivity of *cdc68-1* and *spt16-197* mutant cells, none of the deleted clones that produced N-terminal truncated Cdc68 proteins suppressed *his4-912δ* or *lys2-128δ* mutations when present in elevated copy number. Thus, the N-terminus of the Cdc68 protein is essential for suppression of δ-insertion mutations, but

not for complementation of temperature-sensitivity. In this respect, suppression of *his4-912δ* or *lys2-128δ* is perhaps a more sensitive assay of Cdc68 gene product function than is complementation of *cdc68* temperature-sensitive mutations. Alternatively, perhaps suppression of *his4-912δ* or *lys2-128δ* is sensitive to a different aspect of Cdc68 gene-product function than is proliferation; for example, suppression of *his4-912δ* or *lys2-128δ* may require interactions between N-terminal Cdc68 sequences and other proteins involved in transcription. Such protein interactions would obviously be precluded by N-terminal Cdc68 deletions.

Although only a limited number of C-terminal deletions have been characterized, all deletions that extend into the *CDC68* ORF have been found to abrogate the suppression of δ-insertion mutations and the complementation of both the *cdc68-1* and *spt16-197* temperature-sensitive mutations. The failure of these deleted clones to complement the *cdc68-1* and *spt16-197* mutations or suppress δ-insertion mutations is probably a direct result of the removal of C-terminal amino acid residues. However, each of these deletions removes all the *CDC68* translation and transcription termination signals, which might in itself have a profound effect on gene-product stability and/or function. A more defined analysis of the C-terminus of the predicted Cdc68 protein, by site-directed mutagenesis, will be informative about the function of this region.

4. The significance of phosphorylation consensus recognition sites in the predicted Cdc68 amino acid sequence

Examination of the predicted Cdc68 amino acid sequence has identified numerous consensus recognition sites for several protein kinases.

It is likely that at least some of these sites are phosphorylated *in vivo*. In particular, the density of serine residues and casein kinase II consensus phosphorylation sites in the acidic region of the *CDC68* gene product suggests that this region of the Cdc68 protein may be phosphorylated by casein kinase II *in vivo*. The significance of this observation is however uncertain as the physiological role of casein kinase II remains unclear.

Phosphorylation increasingly appears to be a means by which the activities of proteins which function in transcription are modulated. A growing list of transcriptional activators, including Gal4 (Mylin *et al.*, 1989, 1990), heat-shock transcription factor (HSF) (Sorger et al., 1987; Sorger and Pelham, 1988), and Ste12 (Song *et al.*, 1991) have been demonstrated to become phosphorylated in response to an environmental signal, and such phosphorylation has been correlated with increased potency as a transcriptional activator. Phosphorylation might regulate the effectiveness of a transcriptional activator at a number of levels. One suggestion is that regulated phosphorylation of activation regions might alter activity by reversible contribution of negative charge (Sorger *et al.*, 1987; Sorger and Pelham, 1988). Such modification might affect the Cdc68 protein whether it functions as a conventional transcriptional activator such as Gal4 or Gcn4 or, in a more general manner, through chromatin interactions facilitated by C-terminal acidic sequences.

Other mechanisms by which phosphorylation might alter the activity of transcription factors include modification of a DNA-binding region, facilitating a conformational change or by affecting interactions with other regulatory proteins (discussed in Song *et al.*, 1991). The latter suggestion is a particularly intriguing possibility, since in the case of the Cdc68 protein,

interaction with other proteins, possibly in the form of a complex as discussed above, may be necessary for normal function. A further mechanism by which phosphorylation has been postulated to modulate the activity of a transcription factor is by regulating entry into the nucleus. Cell-cycle-specific phosphorylation has been proposed to facilitate the cell-cycle-regulated entry of the Swi5 transcription factor into the nucleus, where Swi5 directs the cell-cycle-regulated transcription of the *HO* gene required for mating-type switching (Nasmyth *et al.*, 1990).

The identification within the Cdc68 protein sequence of potential $p34^{cdc2}$ phosphorylation sites raises the possibility that the activity of Cdc68 may be subject to cell-cycle regulation as a result of phosphorylation at specific stages of the cell cycle. This is an interesting possibility, especially in light of recent findings concerning the cell-cycle regulation of the G1-cyclin genes, whose expression has been shown here to be dependent upon functional Cdc68 protein: cell-cycle-regulated phosphorylation of Cdc68 might contribute to the cell-cycle regulation of the G1 cyclin genes. It has recently been demonstrated that the products of the G1-cyclin genes *CLN1*, *CLN2* and *CLN3* (*DAF1/WHI1*) contribute in a positive manner to the expression of the *CLN1* and *CLN2* genes themselves (Dirick and Nasmyth, 1991; Cross and Trinkelenberg, 1991). Transcription of *CLN1* and *CLN2* was found to depend upon the presence of at least one functional cyclin gene and upon *CDC28*. The mechanism by which the G1 cyclins exert positive regulation over their own mRNA accumulation is unknown, but it has been speculated that this might be accomplished by phosphorylation of *CLN1* and *CLN2* transcription factors by a complex containing $p34^{cdc28}$ and one or more G1 cyclins. Perhaps Cdc68 is such a transcription factor. The

existence of this positive feedback loop is consistent with observations in embryonic systems, where mitosis is initiated in a highly concerted fashion (Solomon *et al.*, 1990).

A number of proteins with highly acidic regions, which are thought to interact with chromatin, have also been identified as putative p34^{cdc2} substrates (reviewed by Pines and Hunter, 1990). It is not known by what mechanism p34^{cdc2}-mediated phosphorylation might regulate the activity of these proteins, which include nucleolin and some HMG proteins, but since Cdc68 resembles these proteins in both structural and functional respects as described above, this finding may have implications for the elucidation of Cdc68 function.

A further transcription factor suggested to be phosphorylated by p34^{cdc2}, and thereby potentially regulated in a cell-cycle-dependent manner, is the largest subunit of RNAP II. The largest RNAP II subunit from many species contains an unusual C-terminal repeat structure comprising tandem copies of a seven amino acid consensus sequence. The function of this structure is unknown (reviewed by Corden, 1990). A protein kinase capable of phosphorylating the C-terminal conserved repeats of the mouse RNAP II large subunit (a modification associated with active transcription) contains the mouse homologue of p34^{cdc2} (Cisek and Corden, 1989). In yeast a related kinase appears to be involved (Lee and Greenleaf, 1989; Cordon, 1990). The significance of p34^{cdc2}-mediated phosphorylation of the RNAP II C-terminal repeats is so far unresolved. Repeats similar to those contained in RNAP II are found in Spt5, but the predicted Cdc68 protein contains no such motifs.

To further investigate the possibilities discussed above it will be of interest to examine the phosphorylation state of the Cdc68 protein. This

could, for example, be accomplished by ^{32}P-radiolabelling of cells bearing an "epitope-tagged" (Munro and Pelham, 1987; Field *et al.*, 1988) *CDC68* gene, followed by immunoprecipitation using an antibody known to recognise the specific epitope tag used; this approach avoids the necessity of raising antibodies and purifying Cdc68 protein. The phosphorylation state of the immunoprecipitated tagged protein could then be examined by autoradiography of electrophoretically separated protein. Such analysis might reveal regulated phosphorylation of the Cdc68 protein. An alternative approach could be to assess the effects of altering (by site-directed mutagenesis) potential phosphorylation sites identified in this study.

5. The Cdc68 gene product is required for transcription of the *CDC68* gene

One of several transcripts present at decreased abundance in *cdc68-1* mutant cells transferred to the restrictive temperature was the *cdc68-1* transcript itself. This result suggests that wild-type Cdc68 gene product is required for transcription of the *CDC68* gene. This situation is not unusual, since genes encoding components of the basic transcription apparatus are transcribed by protein complexes that in many cases include the products encoded by these same genes. Indeed, transcripts from the yeast *RPB1* and *RPB2* genes (which encode the two largest subunits of RNA polymerase II) decrease in *rpb1* temperature-sensitive mutant cells transferred to a restrictive temperature as one would expect (Nonet *et al.*, 1987).

Certain transcription factors which function at a more restricted group of RNAP II-dependent promoters may also be dependent upon their own gene product for transcription. For example, the *ABF1* gene, which encodes a DNA-binding protein implicated in the transcription of several genes

including ribosomal-protein genes, nuclear genes encoding mitochondrial proteins and glycolytic-enzyme genes, contains several sequences that are closely related to the consensus for Abf1 binding in its own 5' non-coding region (Halfter *et al.*, 1989). This finding suggests that *ABF1* gene expression may be autoregulated. The significance of Abf1 binding to putative binding sites upstream of the *ABF1* coding sequence has not yet been evaluated *in vivo*. However, one of the Abf1 consensus sequences found upstream of the *ABF1* gene has been shown to bind Abf1 protein *in vitro*. Furthermore, Abf1 binding to this sequence was abolished by the introduction of a point mutation into the consensus binding site.

The requirement of a gene product for the expression of its own gene provides an opportunity for gene regulation. For example, *ABF1* gene expression might be modulated by differential occupation of upstream Abf1 binding sites and possible differential interactions with accessory factors (Halfter *et al.*, 1989). Perhaps *CDC68* gene expression too is regulated through interaction of the Cdc68 protein with *CDC68* promoter sequences, possibly involving other proteins, in response to altered growth conditions. Autoregulation has been demonstrated in several *Drosophila* homeobox genes whose expression is both spatially regulated and required throughout development. In the case of both the *Deformed* (*Dfd*) and *Ultrabithorax* (*Ubx*) genes it is thought that requirement of their own gene product for efficient expression (termed autoregulation in these studies) provides a mechanism whereby the genes continue to be expressed in the appropriate region of the embryo after original positional cues are lost as development proceeds (Kuziora and McGinnis, 1988; Bienz and Temml, 1988). Autoregulation of the *Fushi tarazu* (*ftz*) gene appears to provide a

mechanism whereby gene expression is amplified in the appropriate area of the embryo (Hiromi and Gehring, 1987). It is not clear what implications this developmental autoregulation may have in the yeast system.

6. *cdc68* mutations cause cell-cycle arrest at Start

a) How does mutation of a transcription factor cause cell-cycle arrest at Start?

In addition to the transcriptional effects described above, mutations in the *CDC68* gene bring about a regulated arrest at the cell-cycle regulatory step, Start. What is the basis for arrest at this specific stage of the cell cycle? One might expect that mutational inactivation of a factor necessary for the transcription of a wide variety of genes would cause cells to arrest relatively quickly, resulting in a population of cells arrested randomly at all stages of the cell cycle. Such a random cell-cycle arrest is characteristic of several different temperature-sensitive mutations in the *RPO21* (*RPB1*) gene encoding the largest subunit of RNA polymerase II (Himmelfarb *et al.*, 1987; Nonet *et al.*, 1987). In contrast to this situation, in which many essential cellular processes apparently become limiting, the results presented here suggest that concerted arrest of *cdc68-1* mutant cells is a result of the cell's acute sensitivity to the levels of particular unstable gene products, the G1 cyclins, whose production, at the transcriptional level, is dependent upon the presence of active Cdc68 protein.

It is now clear that the performance of Start involves periodic activation of a protein kinase encoded by the *CDC28* gene, referred to as $p34^{cdc28}$ (Mendenhall *et al.*, 1987; Wittenberg and Reed, 1988). In turn, activation of the $p34^{cdc28}$ protein kinase requires periodic accumulation of

G1-cyclin proteins, the products of three genes, *CLN1*, *CLN2*, and *CLN3*. Utilization of heterologous promoters to prevent cyclin-gene expression leads to a rapid, first-cycle arrest of proliferation with cells blocked at the Start event (Richardson *et al.*, 1989; Cross, 1990). Moreover, the introduction of hyperactive/hyperstable alleles of *CLN2* or *CLN3* prevents cell-cycle arrest at Start (Nash *et al.*, 1988; Cross, 1988; Hadwiger *et al.*, 1989). The physiological state of cells displaying a cyclin-mediated Start arrest resembles the phenotype of arrested *cdc68-1* mutant cells: both arrest conditions allow continued cell growth and mating competency (Prendergast *et al.*, 1990; Cross, 1990). In addition, *cdc68-1* mutant cells transferred to the restrictive temperature display dramatically decreased levels of all three *CLN* transcripts, and introduction of the *CLN2-1* hyperactive allele alleviates the *cdc68-1*-mediated Start arrest, presumably by providing a more stable Cln2 protein. Taken together, these findings provide a mechanism through which effects on transcription can result in Start arrest.

The Cdc68 protein is required for expression of the G1-cyclin genes. Under restrictive conditions, the short half-lives of the cyclin proteins (Cross, 1990; Richardson *et al.*, 1989; Wittenberg *et al.*, 1990) coupled to an immediate *cdc68-1* - mediated decrease in *CLN* transcript levels, precludes accumulation of sufficient cyclin proteins to activate the Start machinery. Such cells become incapable of initiating a new cell cycle. An alternative mechanism whereby the *cdc68-1* mutation could cause Start arrest involves inappropriate activation of the mating-response pathway. This eventuality is, however, unlikely because the effects on cyclin transcript levels in *cdc68-1* mutant cells do not resemble the pattern of transcript abundance observed after α-factor treatment. Upon activation of the mating-response pathway by

α-factor, only *CLN1* and *CLN2* transcript levels decrease (Wittenberg *et al.*, 1990) and those of the *CLN3* gene actually exhibit a moderate increase (Nash *et al.*, 1988). In contrast, in *cdc68-1* mutant cells, transcript levels from all three G1-cyclin genes rapidly decrease upon transfer to the restrictive temperature. Inappropriate activation of the mating pathway does not therefore appear to be the cause of cell cycle arrest in *cdc68-1* mutant cells; rather it is the requirement of Cdc68 protein for continued cyclin gene expression that, in the absence of an active Cdc68 protein, leads to decreased cyclin levels which are insufficient to activate Start.

b) Why do *cdc68-1 CLN2-1* mutant cells cease proliferation?

Cells harbouring both the *cdc68-1* and *CLN2-1* mutations remain temperature-sensitive. This continued temperature sensitivity probably reflects additional effects of the *cdc68-1* mutation on gene expression. Mutant cells are able to initiate a new cell cycle in the presence of the *CLN2-1* mutation but fail to complete the newly initiated cell-division process. Although such double-mutant cells eventually stop proliferating, arrested cells do not become impaired at a uniform, post-Start event in the cell cycle. Thus, continued temperature-sensitivity of *cdc68-1 CLN2-1* mutant cells is not due to deleterious effects on the expression of genes that encode products which are normally rate-limiting in the post-Start cell cycle. Rather, *cdc68-1 CLN2-1* double-mutant cells arrest proliferation due to depletion of products necessary for ongoing growth-related processes.

Previous studies have demonstrated that *cdc68-1* mutant cells continue to enlarge for some time after transfer to the restrictive temperature, indicating that, at least initially, many growth related processes

are relatively unaffected by the *cdc68-1* mutation (Prendergast *et al.*, 1990). Nevertheless, growth-related processes begin to be affected within one doubling time. Measurements of [^{14}C]uracil and [^{3}H]histidine incorporation indicate that, by 2 hours after transfer to the restrictive temperature, levels of precursor incorporation into RNA and protein have fallen to 50% of pre-shift levels in *cdc68-1* mutant cells and continue to decrease as cells are incubated further (Prendergast *et al.*, 1990). Reduced levels of protein synthesis are a predictable consequence of the deleterious effects on transcription of many genes observed in this study. In fact, the results of this analysis reveal little change in protein synthesis until 1 hour after transfer to the restrictive temperature, suggesting that decreased levels of protein synthesis are a consequence of the underlying transcriptional defect. Incorporation of the [^{14}C]uracil precursor mostly reflects the rate of rRNA transcription, since rRNAs are the most abundant RNA species in the cell. Two explanations can be invoked to explain the effect, albeit delayed in comparison to the effects observed on RNAP II-dependent transcription, on precursor incorporation into rRNA. Decreased rRNA transcription could occur as a consequence of necessary transcription-factor depletion. Alternatively, rRNA transcription could be down-regulated by the stringent response, a cellular reaction to starvation or to conditions of decreased protein synthesis, whose primary consequence is the decreased synthesis of rRNA and ribosomal-protein mRNAs (Gross and Pogo, 1974; Warner and Gorenstein, 1978). These explanations have previously been invoked to account for delayed effects on rRNA transcription in cells containing a temperature-sensitive mutation in the *RPB1* gene after transfer to the restrictive temperature (Nonet *et al.*, 1987).

7. *cdc68* mutant cells exhibit altered *HSP82* regulation.

In a previous study, the patterns of proteins synthesized in response to the imposition of heat-shock conditions were examined in a collection of cell-cycle mutants using the same pulse-labeling procedure utilized here (Barnes, 1989). These mutants contained defects in both uncharacterized and previously described genes that function in a variety of cellular pathways. Of those examined, only the *cdc68-1* mutation caused persistent synthesis of a protein identified as Hsp82 on the basis of its molecular weight and induction upon heat shock. The current study has demonstrated that Hsp82 is indeed persistently synthesized in *cdc68* mutant cells following transfer to 37°C, that a relatively normal pattern of expression is restored by the introduction of a plasmid-borne *CDC68* gene, and that persistent Hsp82 synthesis is a result of persistent expression of the *HSP82* gene.

The persistent expression of *HSP82* in *cdc68-1* mutant cells transferred to the restrictive temperature is not simply a result of cell-cycle blockage. As described above, only *cdc68-1* mutant cells displayed this phenotype, even though other mutants that arrest at various points in the cell cycle, including Start, were examined. The persistent *HSP82* expression in *cdc68-1* mutant cells probably results from a more direct effect on *HSP82* promoter activity.

Transcriptional regulation of the *HSP82* gene is complex. Recent experiments by McDaniel *et al.* (1989) have demonstrated that the TATA-proximal heat-shock element, recognized by heat shock transcription factor (HSF), is required for basal expression of the *HSP82* gene, implying that HSF is involved in basal level expression of *HSP82* as well as induction by heat-shock. Recent analysis of the heat shock transcription factor (HSF) (Sorger,

1990; Nieto-Sotelo *et al.*, 1990; Jakobson and Pelham, 1991) has revealed physically separable transcriptional activation domains that may independently mediate the "classical" (*i.e.* transient) heat-shock response that occurs at many heat-shock-responsive promoters (including that of *HSP82*) and the more sustained induction that occurs at other promoters, such as that of the *SSA1* gene (Slater and Craig, 1987; Park and Craig, 1989). Sustained activation following heat shock appears to be mediated by a C-terminal activator domain of HSF that is normally masked by N-terminal HSF sequences under non-heat-shock conditions and after a transient heat-shock response has been mounted. How such intramolecular repression is accomplished is unclear. Conformational changes (perhaps influenced by the phosphorylation state of HSF) and interaction with other proteins have both been suggested to regulate this intramolecular repression. In particular, it has been suggested that heat-shock proteins themselves, in particular Hsp70, may negatively regulate the heat-shock response by acting as repressors of HSF activity (Morimoto *et al.*, 1990; Sorger, 1991; Jakobson and Pelham, 1991). Thus under normal conditions, where only low levels of heat-shock proteins are required, HSF activity would be repressed by free heat-shock proteins. However, after the imposition of heat-shock conditions, when the demand for heat-shock proteins is greater, repression of HSF would be temporarily alleviated until heat-shock protein levels were again sufficient to reestablish repression of HSF.

The persistent expression of *HSP82* in *cdc68* mutant cells following transfer to the high temperature resembles the phenotype of cells in which HSF N-terminal sequences, that ordinarily repress the C-terminal activation domain, are deleted. In such cells, expression from transient-response

promoters is rendered constitutive (Sorger, 1990). The similarity between these two situations suggests that HSF cannot be repressed in *cdc68* mutant cells after the transient phase of the heat-shock response. In *S. cerevisiae,* HSF is bound to DNA both before and after heat shock (Sorger *et al.*, 1987; Jakobson and Pelham, 1988; Sorger and Pelham, 1988); thus activation and repression of HSF must occur in the context of a promoter region that is complexed within chromatin. Chromatin structure of the *HSP82* gene exhibits a "half-nucleosomal" DNase I cleavage periodicity, termed "split nucleosome structure", when *HSP82* is transcribed even at low basal levels. In contrast, DNase I sensitivity of nontranscribed *HSP82* exhibits periodicity consistent with DNA wound around whole nucleosomes (Lee and Garrard, 1991). Thus, transcription of the *HSP82* gene, like most other genes, is correlated with altered chromatin structure. Failure to reestablish HSF repression in *cdc68-1* mutant cells could be due to *cdc68-1*-mediated changes in chromatin structure at the *HSP82* promoter that influence the activity of bound HSF. Testing this hypothesis will require direct examination of chromatin structure at the *HSP82* promoter in *cdc68* mutant cells. An alternate possibility is that, if HSF is repressed by one or more *trans*-acting proteins (which may include heat-shock proteins), the expression of genes encoding such regulatory proteins might be dependent upon functional Cdc68 protein. Failure to synthesize such proteins after transfer of *cdc68* mutant cells to 37°C would thus render the heat-shock response constitutive. Evaluation of this hypothesis will require identification of these putative repressor proteins.

8. Summary

In this study, the *CDC68* gene of *S. cerevisiae* has been characterized at the molecular level and the *cdc68* mutant phenotype has been investigated. The *CDC68* gene was first identified by the effects of the *cdc68-1* mutation on the cell-cycle regulatory point Start. In addition to effects on cell proliferation, the *cdc68-1* mutation has widespread effects on gene expression: many transcripts are present at decreased abundance in *cdc68-1* mutant cells after transfer to the restrictive temperature although one, from the *HSP82* gene, is present at increased levels. Thus, the *cdc68-1* mutation has both positive and negative effects on gene expression. The *CDC68* gene has been independently identified as a high-copy suppressor of certain δ–insertion mutations, an approach which further suggests a role for the *CDC68* gene in transcription.

The wild-type *CDC68* gene was cloned by complementation of the *cdc68-1* temperature-sensitive mutation. The *CDC68* gene contains a 3105-bp open reading frame and has the potential to encode a 1035 amino acid protein of 118.6 kDa The C-terminus of the predicted Cdc68 protein is particularly rich in acidic amino acids and contains numerous consensus recognition sites for casein kinase II phosphorylation. The acidic region of the Cdc68 protein is also similar to regions in the Spt5, Spt6 and hUBF predicted proteins, suggesting that the function of Cdc68 may be related to the functions of these other proteins. Genetic evidence further supports a relationship between Cdc68, Spt5 and Spt6 gene-product function. Genetic evidence also suggests that this function may be related to chromatin structure.

The relationship between structure and function of the *CDC68* gene

has been examined. Two *cdc68* temperature-sensitive mutations have been localized to the 5' end of the *CDC68* gene. Surprisingly, deletion of upstream non-coding sequences and the first 971 base pairs of the *CDC68* open reading frame (which includes the region to which *cdc68* mutations have been localized) has little effect on complementation of the *cdc68-1* and *spt16-197* temperature-sensitive mutations when these deleted *CDC68* genes are carried on a high-copy vector. Furthermore, these deleted clones can apparently rescue an otherwise lethal deletion of the *CDC68* gene. Unlike the full-length *CDC68* gene, these deleted clones do not suppress either the *his4-912δ* or *lys2-128δ* mutations, indicating that such deleted *CDC68* genes cannot fulfill all the functions of the wild-type *CDC68* gene.

The molecular basis of the cell-cycle arrest phenotype of *cdc68-1* mutant cells has been determined. Upon transfer to restrictive conditions, transcript levels from all three G1 cyclin genes rapidly decrease in *cdc68-1* mutant cells. That this decrease is responsible for the regulated arrest of *cdc68-1* mutant cells is established by the suppression of specific cell-cycle arrest by the *CLN2-1* mutation, a hyperactive allele of the *CLN2* gene which is thought to encode a Cln2 protein of increased stability. *cdc68-1 CLN2-1* double mutant cells do however arrest randomly within the cell cycle, within one doubling time of transfer to the restrictive temperature, presumably due to depletion of other (non-regulatory) gene products.

Future directions will include the identification of extragenic suppressors to identify proteins that interact with the Cdc68 protein. Preliminary indications are that extragenic suppressor mutations that allow *cdc68-1* mutant cells to grow at the restrictive temperature are easily selected and can be cloned by complementation. A parallel approach will be to

isolate high-copy suppressors of the *cdc68-1* mutation. Further studies of the interesting relationship between structure and function of the *CDC68* gene will include sequencing the *cdc68-1* and *spt16-197* mutations, and the determination of the subcellular localization and phosphorylation state of the Cdc68 protein, possibly by generating an epitope-tagged Cdc68 protein. The influence of the Cdc68 C-terminal acidic region on cell proliferation and gene expression will also be examined. Finally, the relationship between Cdc68 and other transcription factors, particularly those such as Swi4 and Swi6 that that have been implicated in the expression of the G1-cyclin genes, will be examined.

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