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Yeast Proteins That Modulate Cdc68 Transcriptional Activity

by

Qunli Xu

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at

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For my parents

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ABSTRACT

The Cdc68 protein is a global transcription factor in the yeast *Saccharomyces cerevisiae*. In an effort to gain in-depth understanding of the mechanism by which Cdc68 regulates transcription, a genetic suppressor approach was undertaken. Four suppressor genes were identified in which mutations alleviate the temperature sensitivity caused by the *cdc68-1* mutation. My gene cloning and nucleotide sequencing identified two of the suppressor genes as *SAN1* and *SUG1*. The San1 protein was previously uncovered by a genetic interaction with the Sir4 protein, which is itself involved in chromatin-mediated transcriptional repression. The Sug1 protein has been implicated in both transcriptional regulation and in proteolysis. The *san1* and *sug1* mutant alleles isolated here suppress pleiotropic effects of *cdc68-1*, but fail to compensate for the complete loss of Cdc68 function, suggesting that suppression is by restoration of Cdc68 activity. Several lines of evidence described here support the notion that both San1 and Sug1 antagonize Cdc68 function at the protein level. Although identified during the same suppressor screening, the San1 and Sug1 proteins apparently have different functions. Furthermore, the inhibitory effects of San1 and Sug1 are additive, suggesting that San1 and Sug1 modulate Cdc68 activity independently, most likely by different mechanisms.

Previous studies have raised the possibility that Cdc68 regulates transcription *via* chromatin. I pursued this line of investigation and uncovered additional links between Cdc68 and proteins known to modulate chromatin configuration. I also found that nucleosome density on plasmid DNA is dependent on Cdc68 activity. These and additional observations are all consistent with the model that the widespread transcriptional effects of Cdc68 are mediated through chromatin.

Independently from its effect on Cdc68 activity, the Sug1 protein plays an essential role in mitosis: *sug1-26* mutant cells arrest in the G2/M phase of the cell cycle with an undivided nucleus. To find out how Sug1 regulates mitosis, I determined interactions between Sug1 and several known mitotic regulators. Results of this study and of others suggest that Sug1 may mediate a proteolysis pathway that plays a pivotal role in several mitotic events.

ABBREVIATIONS AND SYMBOLS

bp	base pairs
CHX	cycloheximide
cM	centiMorgan
C-terminal	carboxyl-terminal
DAPI	4',6-diamidino-2-phenyl-indole
ddH_2O	distilled and de-ionized water
ECL	enhanced chemiluminescence
FACS	fluorescence activated cell sorting
h	hour
HU	hydroxyurea
kbp	kilobase pairs
min	minute
NPD	non-parental ditype
N-terminal	amino-terminal
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PD	parental ditype
rpm	revolutions per minute
sec	second
TAF	TBP-associated factors
TBP	TATA-binding protein
TT	tetratype

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I. INTRODUCTION

The Cdc68 protein function is required for the transcription of a number of physiologically unrelated *S. cerevisiae* genes. The *CDC68* gene was originally identified because of the cell-cycle defect conferred by the *cdc68-1* temperature-sensitive mutation (Prendergast et al., 1990). Further studies have indicated that the Cdc68 protein plays a primary role in transcription, and that Cdc68 impinges upon cell-cycle regulation by affecting expression of the three G1-cyclin genes *CLN1, CLN2,* and *CLN3* (Rowley et al., 1991). The major goals of this current investigation have been to further explore the function of Cdc68 in transcriptional regulation and to gain insight into the underlying mechanisms by which Cdc68 affects transcription. To provide a framework in which this thesis work has been undertaken, I review transcription initiation in eukaryotic cells in general, with emphasis on regulatory schemes that cells employ to control the level of gene expression. I also summarize our knowledge on the Cdc68 protein before my work was carried out. The final part of this introduction describes the rationale for the major experimental approach adopted for this study.

Section A. Transcriptional regulation

Control of gene expression plays a key role in various aspects of cellular and developmental processes, such as regulation of cell proliferation and determination of cell fate during development. Transcription initiation is believed to be the primary site for the regulation of gene expression in various eukaryotic cell types. Transcription in eukaryotes is a complicated process; the transcriptional state of a particular gene is determined by combinatorial DNA-protein and protein-protein interactions. In addition to the so-called basal initiation complex (comprised of RNA polymerase and general transcription factors)

that carries out the basal level of transcription, other proteins can influence gene expression by communicating with the basal initiation complex. Because in eukaryotes DNA, the template for transcription, exists in a compact chromatin structure, chromatin must be first remodeled to allow access by the transcription machinery. Thus, transcription initiation reflects a complex set of interactions in which proteins can either stimulate or repress gene expression at various levels: some proteins may influence transcription by affecting chromatin configuration, while others may affect the formation of basal initiation complexes.

The actual RNA synthesis during transcription is carried out by three distinct types of RNA polymerase: RNA polymerase I transcribes a single type of gene encoding the ribosomal RNA precursor; RNA polymerase II transcribes protein-coding genes and some snRNA (small nuclear RNA) genes; and RNA polymerase III transcribes genes coding for tRNA, 5S rRNA, and some snRNAs (reviewed in Young, 1991). Because research described here has focused mainly on transcription of protein-coding genes, this introduction concentrates on the RNA polymerase II-dependent transcription.

A.1. Transcription initiation by RNA polymerase II

Promoter structure. Transcription by RNA polymerase II is mediated by DNA sequences, termed promoters, typically located upstream of protein-coding regions (reviewed in Zawel and Reinberg, 1993). Promoter DNA elements can be classified into four functionally distinct groups. The binding site for RNA polymerase II (along with other proteins) and the transcription initiation site, together called the core promoter region, are required for the transcription of all protein-coding genes and serve as the nucleation site for the binding and assembly of the basal initiation complex. The core promoter in many cases contains a canonical TATA element. Two types of DNA sequences upstream from the core promoter, the upstream promoter element and enhancer (also called *UAS* in yeast), are target sites for transcription regulators, and largely determine the efficacy of

transcription initiation. An enhancer (or *UAS*) is capable of activating transcription in an orientation- and distance-independent manner (Guarente, 1988).

Basal initiation complex. *In vitro* biochemical studies have contributed significantly to our understanding of the transcription initiation process. Major components of transcription initiation have been purified and their functions have been tested by *in vitro* reconstitution experiments (for reviews see Zawel and Reinberg, 1993; Conaway and Conaway, 1993). In addition, genetic studies in yeast have led to the identification of proteins important for transcription initiation *in vivo*. Emerging from these biochemical and genetic studies is a universal mechanism for transcription initiation, which is highly conserved from yeast to humans (reviewed in Struhl, 1993).

The catalytic component of the transcription machinery for protein-coding genes is RNA polymerase II, a multi-subunit protein complex. RNA polymerase II itself, however, cannot initiate transcription accurately *in vitro*. Several additional protein factors (termed general transcription factors) are also required for accurate transcription initiation, including the TBP (TATA-binding protein), TFIIB, TFIIE, TFIIF, TFIIH and TFIIJ (reviewed in Zawel and Reinberg, 1993). In yeast, five general factors, a (TFIIE), b (TFIIH), d (TFIID), e (TFIIB), and g (TFIIF), are crucial for polymerase II to initiate transcription in a site-specific manner (Sayre et al., 1992). A stepwise model for the assembly of transcription initiation complexes onto a core promoter DNA has been proposed (reviewed in Zawel and Reinberg, 1993; Conaway and Conaway, 1993). As an initial step, TBP binds to the core promoter region (usually a TATA element) typically located from 30 to 120 nucleotides (depending on the cell type) upstream of the transcription initiation site. This TBP-DNA complex serves as the foundation for the sequential assembly of RNA polymerase II and other general transcription factors. An alternative model for the formation of the preinitiation complex was prompted by numerous findings that the RNA polymerase II is associated with one or several general transcription factors in solution prior to binding to promoter DNA (reviewed in Conaway and Conaway, 1993). Most

significantly, partially assembled initiation complexes (called RNA polymerase II holoenzyme) were purified from budding yeast cells (Koleske and Young, 1994; Kim et al., 1994; reviewed in Koleske and Young, 1995). One such holoenzyme is comprised of RNA polymerase II, factor b (TFIIH), factor e (TFIIB), factor g (TFIIF), and several proteins referred to as SRB proteins that were identified by virtue of functional interactions with the CTD (C-terminal domain) of RNA polymerase II (Nonet and Young, 1989; Thompson et al., 1993). This holoenzyme was able to initiate transcription accurately when TBP and factor a (TFIIE) were supplemented (Koleske and Young, 1994). Thus it is likely that RNA polymerase II can associate with some of the general transcription factors in solution, and then, as a pre-assembled complex, be recruited to a promoter *via* interactions with TBP. In any case, accurate transcription initiation requires RNA polymerase II and a common set of general transcription factors.

In *Drosophila* and human cells, purified TFIID complexes contain TBP and at least eight other proteins, which are called TAFs (TBP-associated factors; reviewed in Goodrich and Tjian, 1994). The major DNA-binding activity of the TFIID complex is contributed by the TBP subunit that binds directly to the core promoter region bearing a canonical TATA element. TAFs are dispensable for basal transcription but are required for transcriptional activation. Two major functions have been proposed for TAFs. TAFs may contribute to the selectivity of the TBP complex for promoter recognition by different RNA polymerases. TBP is a critical component for the transcription by all three RNA polymerases (reviewed in Hernandez, 1993), and different TBP-containing complexes have been identified that are specific for RNA polymerase I, II and III transcription (reviewed in Goodrich and Tjian, 1994). Since all RNA polymerase complexes share the common TBP component and differ only in TBP-associated proteins, TAFs may confer specificity to each TBP complex. In addition, TAFs have also been shown to play co-activator roles to mediate interactions between gene-specific activators and basal transcription complexes (Goodrich and Tjian, 1994).

TFIIB is encoded by a single gene and is essential for basal transcription *in vitro* (reviewed in Zawel and Reinberg, 1993). The TFIIB counterpart in the budding yeast *S. cerevisiae*, factor e, has been shown to play a pivotal role in the selection of transcription initiation sites *in vivo*: mutations in the factor e-coding gene *SUA7* alter transcription initiation sites (Pinto et al., 1992). Moreover, by swapping components of the basal initiation complex between *S. cerevisiae* and the fission yeast *S. pombe*, Li et al. (1994) have found that the accurate selection of transcription initiation sites is largely determined by factor e and RNA polymerase II; the *S. pombe* factor e and polymerase II can substitute for the *S. cerevisiae* counterparts for transcription, but selection of initiation sites follows the pattern common to *S. pombe*. In *S. pombe*, transcription initiation sites are generally about 30 nucleotides downstream from the TATA element, whereas in *S. cerevisiae*, initiation sites range from 40 to 120 nucleotides downstream from the TATA element (Struhl, 1987; Guarente, 1987). Therefore RNA polymerase II and factor e (TFIIB) appear to be responsible for accurate initiation site selection.

A.2. Transcriptional activation

In eukaryotic cells, expression of many genes is subjected to tight controls in response to various intra- or extra-cellular signals. Thus transcription in many cases involves other regulatory proteins in addition to RNA polymerase II and general transcription factors. Transcription of a specific gene can be either induced (activated) or decreased (repressed). Gene activation is achieved by the coordination of *cis*-acting DNA elements and *trans*-acting protein factors, termed activators, that bind to DNA in a sequence-specific manner. Other proteins can also potentiate transcription without directly binding to DNA, and effects of this class of activators are thought to be mediated through protein-protein interactions. Perhaps these non-DNA-binding factors are recruited to DNA by interactions with DNA-binding proteins. Thus, a number of proteins may engage in transcriptional activation.

In an effort to understand the mechanisms of transcriptional activation, extensive studies have been carried out to identify the structure-function relationships of transcription activators. A typical transcription activator contains at least two functional domains: a DNA-binding domain and an activation domain (reviewed in Ptashne, 1988; Mitchell and Tjian, 1989). The DNA-binding domain targets an activator to a specific promoter DNA sequence. Several DNA-binding motifs are defined by sequence comparisons, site-directed mutagenesis, and by crystallography studies. For example, these include the Zinc-finger motifs, helix-turn-helix motifs, bZIP motifs (Mitchell and Tjian, 1989; Schwabe and Rhodes, 1991). The activation domain of an activator is thought to increase the efficiency of transcription by communicating with the basal transcription machinery. Activation domains are generally categorized based on the most prevalent amino acid(s), such as the acidic activation domain, exemplified by the activation domains of Gal4 and Gcn4 in yeast (Ma and Ptashne, 1987; Hope and Struhl, 1986) and VP16 in mammalian virus (Triezenberg et al., 1988); the glutamine-rich domain, exemplified by that of the human transcription activator Sp1 (Courey and Tjian, 1988; Kadonaga et al., 1988); and the proline-rich domain, exemplified by the human transcription activator CTF/NF-1 (Mermod et al., 1989). However, there is controversy with regard to crucial features of activation domains: additional factors, for example hydrophobic amino acids within the activation domains, may also contribute to transcriptional activation (reviewed by Triezenberg, 1995). Currently the classification of activation domains is far from conclusive.

One function of transcription activators is to facilitate the assembly of the polymerase II-containing basal transcription machinery onto promoter DNA. By measuring the amount of general transcription factors assembled onto an immobilized DNA template *in vitro*, Choy and Green (1993) have demonstrated that several classes of transcription activators, including acidic, proline-rich and glutamine-rich activators, can affect multiple steps of the initiation complex assembly. Clearly, activators increase the affinity of TFIIB for DNA-bound TBP; this effect can be detected in the absence of TAF.

However, this increased recruitment of TFIIB is not the only role for activators: increased TFIIB dosage does not bypass the requirement for activators, suggesting that activators also function at additional steps after the entry of TFIIB, and activation in these latter steps requires TAFs. In summary, these studies suggest that activators can function during at least two steps of initiation complex assembly: a TAF-independent step to recruit TFIIB and a second TAF-dependent step after the entry of TFIIB.

The ability of an activator to stimulate transcription complex assembly was shown to be mediated by protein-protein interactions. In some cases, transcription activators can bind to several components of the basal initiation complex (including TBP, TFIIB, and TFIIH) *in vitro*. Most significantly, the VP16 activation domain has been shown to interact with all of the above components (Stringer et al., 1990; Lin et al., 1991; Goodrich et al., 1993; Xiao et al., 1994). Therefore multiple protein-protein interactions may be involved in gene activation, in which an activator may contact several components of the basal initiation complex simultaneously to achieve an optimal level of gene activation.

A.3. Co-activators, adaptors, or mediators

As described above, several components of the basal initiation complex are direct targets for certain transcription activators when tested *in vitro*. In other cases, additional proteins (called co-activators, adaptors, or mediators) may be required to mediate the interaction between an activator and the basal initiation complex, or to potentiate full activation. One of the early indications for the existence of such mediators came from *in vitro* transcription assays using the glutamine-rich activator Sp1 (Pugh and Tjian, 1990). In these assays, Sp1 can stimulate transcription in the presence of partially purified human or *Drosophila* TFIID (containing TBP and additional proteins) and other general transcription factors, but failed to activate transcription when recombinant TFIID (TBP) was used, suggesting that certain components in the TFIID fraction mediate activation by Sp1. These other proteins in the TFIID fraction are now known to be TAFs. Direct

interactions between activators and TAFs have been demonstrated in several cases. For example, the *Drosophila* dTAF110 interacts with the activation domain of Sp1 (Hoey et al., 1993). This interaction was shown to be functionally relevant, because a mutant form of Sp1 that fails to activate transcription also abrogates binding to dTAF110 (Gill et al., 1994). Similarly, another *Drosophila* TAF, dTAF40, was shown to interact with the acidic activation domain of VP16 (Goodrich et al., 1993). Thus TAFs of TFIID can function as co-activators to mediate the interactions between activators and basal transcription complexes.

Co-activators have also been found in yeast. Recently TBP was shown to exist in multi-protein complexes in yeast (Poon and Weil, 1993; Reese et al. 1994). A purified yeast TFIID complex shares similar features with its counterparts in higher eukaryotes, most significantly, two cloned components of the yeast TFIID complex are homologs of *Drosophila* TAFs (Reese et al., 1994). Another potential yeast TAF has been uncovered by protein sequence comparisons: the yeast *TSM1* gene shares 50% homology with dTAF150. Moreover, the Tsm1 protein was found to bind to the yeast TBP *in vivo*, suggesting that Tsm1 may function as a real TAF (Verrijzer et al., 1994). Another line of investigation shows that a mutation in the *SPT15* gene that codes for the yeast TBP can be suppressed by certain *spt3* alleles. Furthermore, a physical interaction between TBP and Spt3 was also detected (Eisenmann et al., 1992). It remains to be determined if Spt3 is an actual component of biochemically purified yeast TFIID complex.

Another class of co-activator or adaptor proteins has been identified by a different genetic approach in yeast. Overexpression of the Gal4-VP16 chimeric activator (a fusion between the Gal4 DNA-binding domain and the VP16 activation domain; Sadowski et al., 1988) is detrimental to yeast cells, and this toxicity is thought to result from sequestration of certain general transcription factors by an overwhelming amount of Gal4-VP16 (Berger et al., 1990). Mutations were isolated that can alleviate the toxicity caused by Gal4-VP16 overproduction, and it was reasoned that these mutant alleles were likely to define genes

coding for adaptor proteins that bridge the interaction between Gal4-VP16 and the basal initiation complex (Berger et al., 1992). Three adaptors identified in this way, Ada2, Ada3, and Gcn5 (Berger et al., 1992; Marcus et al., 1994), were found to be components of a common complex that potentiates transcriptional activation by some acidic activators (Horiuchi et al., 1995). An interaction between the acidic activation domain of VP16 and Ada2 has been detected (Silverman et al., 1994). No interaction between this trimeric Ada2-Ada3-Gcn5 complex and any general transcription factor has been reported, even though it is hypothesized that the bromodomain of Gcn5 may mediate such an interaction (Marcus et al., 1994). Based on the observation that an *ada2* mutation prevents transcriptional activation using naked DNA as a template (Berger et al., 1992), Horiuchi et al. (1994) proposed that the target of this trimeric adaptor complex is likely to be the basal transcription complex, as opposed to chromatin (see below).

Co-activator or mediator activity was also found to associate with RNA polymerase II. Two related forms of so-called RNA polymerase II holoenzyme were isolated that share a common set of proteins, including polymerase II itself and a mediator complex that binds to the C-terminal domain (CTD) of RNA polymerase II. The mediator complex contains at least 20 different proteins, including Gal11, Sug1 and several SRB proteins. Among these, Sug1 interacts with the Gal4 C-terminal activation domain and with TBP, implying a potential co-activator role for Sug1 (Swaffield et al., 1995). In addition, the activation domain of VP16 is also able to bind to the mediator complex (Hengartner et al., 1995). Thus, a number of proteins have been identified in both yeast and higher eukaryotes to facilitate the communication between transcription activators and the basal transcription machinery.

A.4. Transcriptional repression

In previous sections I have discussed transcriptional activation and how transcription activators communicate with the basal initiation complex. Another means to

regulate transcription is repression. Studies in recent years have established that repression is also commonly employed by eukaryotic cells to control gene expression (reviewed in Herschbach and Johnson, 1993).

The target of repression can be either activators or the basal transcription machinery. Gene repression can be achieved by preventing activation. In this case, repressive effects are mediated through activators. Repressor proteins can block activation by various means, for example by preventing nuclear import of transcription activators, by competing for target DNA sites shared with activators, or by restricting activity of activators. The latter case is exemplified by the effect of the Gal80 protein on the Gal4 activator in the budding yeast. Gal4 is required for the galactose induction of a group of genes (*GAL* genes) required for utilization of the carbon source galactose (reviewed in Johnston, 1987). All these *GAL* genes have the consensus Gal4 binding site (called UAS_G) upstream of their core promoters. Transcription of these *GAL* genes is largely determined by the type of carbon source available in the growth medium. Carbon source does not affect the DNA-binding capability of Gal4: the Gal4 activator binds constitutively to UAS_G in front of the *GAL* genes (Selleck and Major, 1987; Lohr, 1993). However, in the presence of glucose, Gal80 masks the Gal4 activation domain by a direct physical interaction and transcription of these *GAL* genes is turned off (Lue et al., 1987; Leuther and Johnston, 1992). Upon transfer of cells to galactose-containing (and glucose-free) medium, the Gal4 activation domain is relieved from Gal80 inhibition due to a conformational change of the Gal4-Gal80 complex (Leuther and Johnston, 1992) . Thus, repression by blocking the activity of a transcription activator provides an elegant scheme for cells to adapt to environmental signals, in this case, a change of nutrient.

Repression can also occur by preventing the assembly of the basal transcription complex on promoter DNA. The *MOT1* gene in budding yeast was originally identified by genetic studies because the *mot1-1* mutation increases basal transcription but has little effect on activated transcription (Davis et al., 1992). Convergent biochemical and genetic studies

indicate that Mot1 is the same as ADI, identified biochemically as an ATP-dependent inhibitor (Auble and Hahn, 1993; Auble et al., 1994). Mot1/ADI represses basal transcription by removing TBP from DNA and thereby preventing the assembly of RNA polymerase II and other general factors onto the core promoter (Auble et al., 1994). Perhaps other components of the basal initiation complex, in addition to TBP, may also be targets for transcriptional repressors.

A.5. Transcriptional regulation in the context of chromatin structure

In eukaryotic cells, the template for transcription is not naked DNA, but compacted chromatin. The basic unit of chromatin is called the nucleosome, a nucleoprotein complex in which DNA wraps around a histone octomer (2 copies of histones H2A, H2B, H3, and H4). It can be easily envisioned that nucleosomes constitute formidable obstacles for gene expression. Indeed, packaging of promoter DNA into nucleosomes has been shown to repress transcription *in vitro* (reviewed in Kornberg and Lorch, 1992).

Genetic studies in yeast have provided compelling *in vivo* evidence that chromatin structure affects gene expression. The *HTA1-HTB1* locus (encoding histones H2A and H2B) was identified as a high-copy suppressor of promoter inactivity that results from disruption by retrotransposon insertions, and this kind of suppression was found to be at the level of transcription initiation. Thus, changing the ratio of the histone pair H2A-H2B versus H3-H4 has an impact on transcription (Clark-Adams et al., 1988). Likewise, the role of histone H4 in gene expression was examined by Grunstein and his coworkers. They have deleted all genomic H4 loci so that the function of H4 was supplied only by a plasmid-borne H4 gene under the control of the *GAL1* promoter. Thus, by transfer of cells from galactose to glucose medium, effects of histone H4 depletion could be examined *in vivo*. This type of analysis has revealed that depletion of H4 causes increased basal transcription from the genes *GAL1*, *PHO5* and *CYC1* (Han and Grunstein, 1988; Han et al., 1988; Kim et al., 1988). These experiments established that histones inhibit gene

expression, and that removal of histones causes derepression. Less dramatic manipulations of histone H4 (for example, systematic deletion and site-directed mutagenesis) were also employed to investigate the function of different regions of the histone H4 protein. Complete depletion of the histone H4 is detrimental to yeast cells and so is the deletion of the hydrophobic core of H4 (reviewed in Paranjape et al., 1994). Although dispensable for cell growth, the N-terminus of histone H4 is also important: its complete removal or a modification by site-directed changes of acetylatable lysine residues prevented full activation of the *GAL1* and *PHO5* genes under inducible conditions (Durrin et al., 1991). These finding suggests that the N-terminal tail of H4 functions in activated transcription. Thus, these studies collectively indicate that histone H4 has both positive and negative effects on gene expression.

Nucleosomes are generally detected at specific positions along DNA *in vivo* (Simpson, 1991). One example is the yeast *PHO5* promoter. Expression of the *PHO5* gene is regulated by the availability of phosphate in the growth medium. Under repressive conditions (in the presence of phosphate), there is a cluster of nucleosomes precisely positioned on the *PHO5* promoter. Induction of the *PHO5* gene (upon phosphate depletion) is accompanied by disruption of four of the positioned nucleosomes (Almer and Höze, 1986; Almer et al., 1986). This type of nucleosome positioning plays a key role in the control of *PHO5* gene expression (reviewed in Felsenfeld, 1992). Nucleosome positioning can also be reconstituted *in vitro* using purified histones and short template DNA fragments (for example, see Archer et al., 1991; Piña et al., 1990). The location of nucleosomes is largely determined both by specific DNA sequences and by additional sequence-specific DNA-binding proteins (for example, see McPherson et al., 1993; Roth et al., 1992; Lee et al., 1993). Packaging of DNA into nucleosomes not only provides a simple means to repress transcription, but also contributes to gene activation: positioning of nucleosomes is likely to bring dispersed *cis*-acting DNA elements into proximity and to efficiently stimulate transcription (reviewed by Wolffe, 1994, and references therein). One

implication of these findings is that proteins that affect nucleosome positioning may have both positive and negative effects on transcription depending on the promoter context of a specific gene.

It is generally accepted that transcription activation accompanies changes of nucleosomal structure, a process called chromatin remodeling or reconfiguration. The detailed structural change of nucleosomes, such as whether nucleosomes are disassembled during transcription activation, is currently under debate (reviewed in Paranjape et al., 1994). Regardless of the actual alterations of nucleosomal structure, nucleosomes clearly provide a framework for regulated gene expression. In this regard, the functions of some transcription activators are likely to be two-fold: counteracting the repressive effect of nucleosome positioning at basal promoters and stimulating basal initiation complex formation. In all likelihood, proteins that affect nucleosome positioning, modification of histones, or other aspects of chromatin structure will have impacts on transcription.

A.6. Proteins that regulate transcription by remodeling chromatin

Since transcription takes place in the chromatin environment in eukaryotic cells, chromatin may provide a target for the action of transcription factors. Two independent lines of genetic studies have led to the identification of a common set of proteins in yeast that activate transcription by affecting chromatin. Studies on the expression of the glucose-repressible *SUC2* gene have identified several Snf proteins (Snf2, Snf5, and Snf6) required for *SUC2* transcription in the presence of sucrose and absence of glucose (reviewed in Winston and Carlson, 1992). In parallel, studies on mating-type switching and *HO* gene expression have identified ten *SWI* genes; among these, *SWI1, 2, 3* code for proteins with related functions and these proteins have been proposed to form a heteromeric complex (Peterson and Herskowitz, 1992). Gene cloning and nucleotide sequence comparisons revealed that *SWI2* was identical to *SNF2*. This led to the finding that the *SWI/SNF* genes in this group have similar effects on the expression of a common

set of genes, including *HO*, *SUC2*, *INO1*, and other glucose-repressible genes (reviewed in Winston and Carlson, 1992; Carlson and Laurent, 1994) Snf2, Snf5, or Snf6, when tethered to DNA by a heterologous DNA-binding domain, can activate transcription, suggesting that these Snf/Swi proteins function as transcription activators (Laurent et al., 1990, 1991).

Genetic data suggested that these five Snf/Swi proteins are functionally interdependent, and probably form a heteromeric complex (Laurent et al., 1991; Peterson and Herskowitz, 1992). This suggestion was confirmed by biochemical studies; a multi-subunit Snf/Swi complex has been purified from budding yeast cells and contains at least 10 different proteins, including Swi1, Snf2/Swi2, Swi3, Snf5, and Snf6 (Cairns et al., 1994; Côté et al., 1994).

The Snf/Swi complex is thought, for the following reasons, to counteract chromatin-mediated transcriptional repression. Deletion of *HTA1-HTB1* (one of the two gene pairs coding for histones H2A and H2B) suppresses the transcriptional defects caused by *snf2* or *snf5* (Hirschhorn et al., 1992). Mutations in the histone H3 coding gene *SIN2* can suppress the effects of *swi* mutations on *HO* expression (reviewed in Winston and Carlson, 1992). Most significantly, in *snf2* or *snf5* mutant cells nucleosome positioning at the *SUC2* promoter region is altered, and this change of nucleosome positioning is independent of transcription: a nontranscribable *SUC2* gene showed similar chromatin changes (Hirschhorn et al., 1992). This finding argues strongly that the Snf/Swi complex potentiates transcription by overcoming the repressive effect of chromatin structure. Recent biochemical isolation of the Swi/Snf complex from yeast cells allowed determination of the Swi/Snf complex activity in precisely defined *in vitro* systems. These *in vitro* studies augmented the *in vivo* results, suggesting that the Snf/Swi complex is directly responsible for a remodeling of chromatin structure that is required for transcriptional activation (Côté et al., 1994): the Swi/Snf complex can stimulate the binding of a Gal4 derivative to a chromatin template (containing the Gal4 target DNA site) in an ATP-dependent manner.

This increased nucleosome-binding activity is not dependent on the Gal4 activation domain. A Swi/Snf complex containing a mutant Swi2 subunit failed to stimulate nucleosome binding by the same Gal4 derivative. Recently, human Snf/Swi complex homologs (termed hSwi/Snf) have been partially purified. Similar to the yeast Snf/Swi complex, the hSwi/Snf complex was shown *in vitro* to alter histone-DNA contacts in the nucleosome. Moreover, hSwi/Snf complex facilitated the binding of several Gal4 derivatives to nucleosomal DNA (Kwon et al., 1994). Thus, it appears that eukaryotic cells employ a common mechanism to overcome the formidable repressive effects of nucleosomes on gene expression. Whether the Snf/Swi complex also functions in an activation step after remodeling of chromatin remains to be determined. Because the Swi/Snf complex only activates expression of a limited number of genes *in vivo*, complexes with similar activity may also exist for the expression of other genes.

In summary, eukaryotic cells employ various means to regulate gene expression in response to physiological or environmental signals. Transcription factors can function at different levels, from modulating chromatin configuration to influencing assembly of the basal transcription machinery.

A.7. Silencing of the *HM* loci in yeast

In *S. cerevisiae*, three loci on the chromosome III, *MAT*, *HML* and *HMR*, encode mating-type information. Although all three loci have identical promoter elements, only genes encoded by the *MAT* locus are expressed and determine the mating type of a haploid yeast cell. The *HML* and *HMR* loci are transcriptionally repressed and therefore called silent mating-type loci. This type of repression is region-specific: insertion of other genes next to either the *HML* or *HMR* locus results in transcriptional repression of inserted genes, whereas transposing mating-type genes away from the *HM* loci causes derepression (for review see Laurenson and Rine, 1992). Thus, the regions of chromosome at which *HM* loci reside are distinct from other chromosome regions.

Both *cis*-acting DNA elements (termed silencers) and *trans*-acting proteins have been identified for the repression of the *HM* loci. Proteins that are required for silencing include Abf1, Rap1, four Sir proteins (silent information regulators, including Sir1, Sir2, Sir3, and Sir4), Nat1, Ard1, and histones H3 and H4 (reviewed in Laurenson and Rine, 1992). Abf1 and Rap1 are DNA-binding proteins, and their binding sites have been found in silencers.

Results of numerous studies suggest that the transcriptional silencing at the *HM* loci is mediated through chromatin. Histone H4 is required for silencing: mutations that alter the N-terminal region of histone H4 were found to cause derepression of the *HM* loci (Kayne et al., 1988; Johnson et al., 1990; Park and Szostak, 1990). Secondly, genetic interactions were detected between Sir3 and histone H4, providing evidence that silencing involves histone (Johnson et al., 1990). Thirdly, a correlation between histone acetylation and silencing of the *HM* loci has been established, and Sir2 may affect histone acetylation (Braunstein et al., 1993). Finally, Sir3 and Sir4 were found to bind to histones H3 and H4 *in vitro* (Hecht et al., 1995). A model for silencing is proposed by Hecht et al. (1995). By virtue of the ability of Rap1 to bind to silencer DNA and by protein-protein interactions among Rap1, Sir3, Sir4, histone H3 and H4 (Moretti et al., 1994; Hecht et al., 1995), a special state of chromatin, similar to heterochromatin in higher eukaryotes, is created that prevents gene expression in the *HM* region. Thus, silencing of the *HM* loci provides an excellent example of how chromatin structure efficiently represses gene expression.

Section B. The Cdc68 protein in *S. cerevisiae*

B.1. The G1 (START) control in *S. cerevisiae*

For most eukaryotic cells, there are two major control points in the cell cycle: the G1/S and G2/M transitions. The basic machinery that controls these transitions is highly conserved from yeast to humans, although the relative importance of the two transition points may differ from one species to another. In the budding yeast *S. cerevisiae*, the G1

control is predominant and budding yeast cells make major decisions at a point in late G1 phase termed START (Pringle and Hartwell, 1981): at this cell-cycle stage, depending on environmental signals (for example, the availability of nutrients and mating pheromones), yeast cells can either become mating-competent (in the presence of the appropriate mating pheromone), or retreat from the mitotic cycle and enter a quiescent state called stationery phase, or alternatively, continue to proliferate.

Passage through START requires an active G1-specific Cdc28 kinase complex, comprised of a catalytic core Cdc28 kinase and a regulatory subunit, a G1-specific cyclin protein. Since the Cdc28 protein is expressed constitutively throughout the cell cycle, activation of the Cdc28 kinase for the performance of START is largely triggered by the association with a G1 cyclin. There are five potential G1 cyclins in the budding yeast, and among these the Cln1, Cln2, and Cln3 proteins have well-defined and partially overlapping G1 functions; cells can proliferate as long as one of these G1 cyclins is available, but arrest at the START when all three of these G1 cyclins are depleted (reviewed by Reed, 1992).

Cln3 is unique among cyclin proteins in that both the mRNA and protein of the *CLN3* gene exist at constant but low levels during the cell cycle (reviewed in Reed, 1992). In contrast, *CLN1* and *CLN2* mRNA and protein levels oscillate during the cell cycle, peaking in late G1 as expected for their G1 roles. This fluctuation of cyclin abundance is at least partially attributable to transcriptional regulation. Transcription of the *CLN1* and *CLN2* genes is activated by two transcription factors, Swi4 and Swi6, that form a heteromeric complex. The Swi4-Swi6 complex binds to a *cis*-acting DNA element (called the SCB box) located in the promoter regions of *CLN1* and *CLN2*. Interestingly, expression of *SWI4* is also cell-cycle dependent, and maximal accumulation of *SWI4* mRNA occurs in the G1 phase prior to the peak of *CLN1* and *CLN2* mRNAs. A positive-feedback mechanism has been proposed to account for the rapid induction of *CLN1* and *CLN2* expression and subsequent activation of START (reviewed in Nasmyth, 1993): expression of *SWI4* in early G1 induces transcription of the *CLN1* and *CLN2* genes,

which causes increased accumulation of Cln1 and Cln2 proteins and consequently activation of the G1-specific Cdc28 kinase activity. The Cdc28 kinase also stimulates transcription of *CLN1* and *CLN2* (perhaps by phosphorylation of Swi4) and results in even higher levels of Cln1- and Cln2-associated Cdc28 kinase activity.

B.2. Cdc68 is required for START

The *CDC68* gene was identified by virtue of the temperature-sensitive *cdc68-1* mutation. The *cdc68-1* mutant was isolated originally by a size-selection scheme that allowed enrichment of non-proliferating cells that maintained significant biosynthetic activity (Prendergast et al., 1990). Isolation of the *cdc68-1* mutant and subsequent gene cloning identified the wild-type *CDC68* gene (Prendergast et al., 1990; Rowley et al., 1991). The deduced amino acid sequence of the Cdc68 protein revealed a highly acidic C terminus, with a higher proportion of negatively charged residues than the activation domains of Gal4 or Gcn4 (Rowley et al., 1991). Nonetheless, the presence of the acidic stretch raised the possibility that the Cdc68 protein may be a transcription activator, required for the transcription of some genes that are critical for START. This hypothesis was validated by Rowley et al. (1991), who showed that transcription of three G1-cyclin genes, *CLN1*, *CLN2* and *CLN3*, was diminished in *cdc68-1* mutant cells at the restrictive temperature. Furthermore, ectopic expression of a hyperactive *CLN2-1* allele (coding for undegradable Cln2) allowed *cdc68-1* mutant cells to pass through START and arrest later in the cell cycle (Cdc68 is also required for the expression of other essential genes, see below). Therefore, failure of *cdc68-1* mutant cells to perform START is due to the lack of G1-cyclin expression.

B.3. Cdc68 has both positive and negative effects on transcription

Transcription of a number of other genes is also affected as a result of decreased Cdc68 activity, including *ACT1*, *LEU2*, and the *CDC68* gene itself. Therefore Cdc68 is

required for maintaining transcript abundance of apparently unrelated genes.

Cdc68 was also found to have negative effects on the transcription of at least two heat-shock genes, *HSP82* and *HSC82*. Upon a temperature shift from 23°C to 37°C, there is a transient induction of heat-shock gene transcription in *CDC68* wild-type cells, whereas in *cdc68-1* mutant cells, *HSP82* and *HSC82* mRNAs were induced during initial heat shock and remained at high levels during extended (5-h) incubation at 37°C (Rowley et al., 1991). This persistent expression of heat-shock genes in *cdc68-1* mutant cells suggests that the wild-type Cdc68 protein has a negative role on the transcription of heat-shock genes.

Cdc68 has also been found to affect the transcription from a *UAS*-less promoter. Deletion of the upstream activating sequences (*UAS*) of the *SUC2* gene abolishes transcription, and cells harboring the *suc2ΔUAS* allele can not produce invertase and consequently fail to grow in sucrose medium (Sarokin and Carlson, 1984). In addition, mutations in several *trans*-acting activators for *SUC2*, for example Snf2, Snf5, and Snf6 that presumably function through the *UAS*, also abrogate *SUC2* transcription (Neigeborn and Carlson, 1984). The *cdc68* mutations can bypass the requirement for the *UAS* for *SUC2* transcription and allow *snf2*, *snf5*, or *snf6* cells to grow in sucrose medium (Malone et al., 1991). These findings suggest that the wild-type Cdc68 protein represses transcription from the *UAS*-less *SUC2* promoter. Therefore the Cdc68 protein has pleiotropic effects on transcription.

B.4. The *SPT* genes

Ty elements are retrovirus-like transposons in yeast. The "long terminal repeats" (LTRs) of Ty are called δ sequences that contain functional promoters and terminators for transcription. Ty or solo-δ transposition into the 5'-region of a gene often abrogates the gene function, presumably because of interference by the transcription initiation or termination site within the δ sequence. For example, δ-insertions into the 5' regions of the

HIS4 and *LYS2* genes inactivate these two genes, and cells with these *his4-912δ* and *lys2-128δ* alleles can not grow without supplementary amino acids lysine and histidine (Winston et al., 1984; Clark-Adams and Winston, 1987). A number of so-called *SPT* (suppression of Ty) genes have been identified by virtue of the ability of either a mutated gene or increased gene dosage to suppress this His⁻ or Lys⁻ phenotype exhibited by *his4-912δ lys2-128δ* cells (reviewed in Winston, 1992). The rationale for this selection scheme is that these *spt* mutations that restore appropriate transcription from the *his4-912δ* or *lys2-128δ* promoter are likely to define genes that play a general role in transcriptional regulation.

All the characterized *spt* mutations suppress the effects of Ty or δ insertional mutations by affecting transcription initiation. The identified *SPT* genes can be categorized into several groups. One group includes *SPT3*, *SPT7*, *SPT8*, and *SPT15* (Eisenmann et al., 1989, 1992, 1994; Gansheroff et al., 1995). The *SPT15* gene encodes the general transcription factor TBP, and Spt3 is found physically associated with Spt15/TBP. Genetic interactions among *spt3*, *spt7*, *spt8*, and *spt15* mutations suggest that this group of Spt proteins, like TBP, may all affect some aspect of the basal initiation complex.

Another group of Spt proteins (the "histone group") is proposed to regulate transcription through chromatin structure (reviewed by Winston and Carlson, 1992). Two members of this "histone group" of *SPT* genes, *SPT11* and *SPT12*, encode histone H2A and H2B, which are building blocks of chromatin. Three other Spt proteins encoded by this group, Spt4, Spt5, and Spt6, are also implicated in chromatin structure (Swanson and Winston, 1992).

B.5. *CDC68* is an *SPT* gene

The *CDC68* gene was also identified as an *SPT* gene, *SPT16* (Malone et al., 1991; Rowley et al., 1991). Either decreased Cdc68 activity encoded by *cdc68* mutant alleles or increased *CDC68* gene dosage confers an Spt⁻ phenotype, and transcription initiation sites

are altered at the *lys2-128δ* promoter in *cdc68* mutant cells (Malone et al., 1991). These observations indicate that a primary role for Cdc68 is in transcription initiation. The Cdc68/Spt16 protein is structurally and functionally similar to the "histone group" of Spt proteins and provisionally has been assigned to this group (Swanson and Winston, 1992); like other members of this "histone group" of *SPT* genes, either mutation or increased copy number of *CDC68* causes a similar Spt$^-$ phenotype (Clark-Adams et al., 1988; Malone et al., 1991). Moreover, Cdc68 shares certain structural features with Spt5 and Spt6, with all three proteins having a region of high density of negatively charged amino acids (Rowley et al., 1991; Swanson et al., 1990, 1991). As described above, this group of Spt proteins includes two histones and Spt4, 5, 6 that are postulated also to be involved in chromatin structure. Thus, the identification of Cdc68 as Spt16 suggests that the effects of Cdc68 are also mediated through chromatin.

Section C. Genetic suppression

Genetic suppressor analysis is a broadly used approach to study functional interactions between proteins (Hartman and Roth, 1973). A prerequisite for this analysis is the availability of a mutant allele of the gene of interest. This kind of suppressor analysis normally begins with isolation of secondary mutations, so-called suppressor mutations, that alleviate the phenotype of the primary mutation. The secondary suppressor mutations can occur in the same gene as the primary mutation; these are called intragenic suppressors. Conversely, extragenic suppressors refer to suppressor mutations in genes other than the one harboring the primary mutation. Identification of extragenic suppressors can reveal other proteins that interact functionally with the protein of interest.

In principle, extragenic suppression can occur by several mechanisms. One class of suppressors may alleviate the phenotype of the original mutation by virtue of informational suppression. This class normally includes mutations in tRNA genes and mutations that alter the fidelity of the translational machinery, for example mutations in

ribosomal-protein genes. As a result of a mutant missense tRNA or a mutation that decreases translation fidelity, the mutant mRNA encoded by the original mutant gene may be misread so that functional protein is produced. This type of suppression, especially that caused by missense tRNAs, is expected to be allele-specific because only a specific codon can be misread and translated into the wild-type amino acid, but can suppress the same mutation in different genes, and is thus gene-nonspecific. A second class of suppressors may encode proteins that form a complex with the protein of interest. In this case, the primary mutation may alter conformation and abrogate activity of the complex, while a compensatory mutation in an interacting protein may restore the normal complex configuration and thereby restore wild-type function to the complex. This type of suppression is expected to be allele-specific in that only mutations that cause a compensatory secondary-structure change of the protein can restore the wild-type complex function. A third type of suppression is by boosting the activity or levels of the protein encoded by the primary mutant allele; these include mutations in proteins that antagonize the activity or repress expression of the protein of interest. This type of suppression should not display allele specificity in that any mutation that abolishes or decreases the inhibition of the protein of interest should allow suppression. Implicit in this type of suppression is the inability to suppress complete loss of function of the protein of interest. Suppression can also occur by other mechanisms in addition to those listed above. For example, in some cases fortuitous activation of an alternative pathway or eliminating the activity of a downstream target may also result in suppression.

The Cdc68 protein has been demonstrated to play a primary role in transcriptional regulation, a complicated process involving multiple protein-protein interactions. Identification of other proteins that functionally interact with Cdc68 should provide insight into the mechanism by which Cdc68 regulates transcription. Therefore a major part of this investigation involved the search for additional proteins that interact with Cdc68 by taking a genetic suppression approach.

II. MATERIALS AND METHODS

1. Strains and plasmids

The *Saccharomyces cerevisiae* strains used in this study are listed in Table 1, and plasmids used are listed in Table 2. The *Escherichia coli* strain DH5αF' (GIBCO BRL, Burlington, Ontario) was used to propagate plasmid DNA. For long-term storage, both yeast and *E. coli* strains were frozen at -70°C in 20% glycerol. For routine use, yeast strains were streaked on solid medium and kept at 4°C for up to six months, but *E. coli* strains were kept at 4°C only for up to one month.

2. Media and growth conditions

The YM1 rich medium (Hartwell, 1967) contained 1% Bacto-peptone, 0.5% Bacto-yeast extract, 1% succinic acid, 0.6% sodium hydroxide, and 0.67% bacto-yeast nitrogen base without amino acids (pH 5.8). The YNB synthetic minimal medium (Johnston et al., 1977) contained 1% succinic acid, 0.6% sodium hydroxide, and 0.67% bacto-yeast nitrogen base, with amino acids (40 μg/ml), ammonium sulfate (0.1%) and purine and pyrimidine bases (i. e., uracil and adenine, 20 μg/ml) added to satisfy auxotrophic requirements. Synthetic "dropout" media were YNB supplemented with ammonium sulfate (0.1%), amino acids (including arginine, aspartic acid, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, serine, tryptophan, threonine, tyrosine, and valine), adenine, and uracil, except for the one or several components indicated. For example, SC-Ura was YNB minimal medium plus all essential supplements except uracil, and was to select for strains harboring a *URA3*-marked plasmid. YM1, YNB, and synthetic "dropout" media were supplemented with a carbon source, such as glucose, galactose or sucrose (2%). Unless specified, glucose was used routinely to grow yeast cells. Whenever sucrose was used as the carbon source,

Table 1. *S. cerevisiae* strains used in this study.

Strain	Genotype or phenotype[a]	Source or reference
21R	*MAT***a** *leu2-3,112 ura3-52 ade1*	Lab collection
XJB3-1B	*MATα met6*	YGSC[b]
W303-1α	*MATα leu2-3,112 his3-11,15 trp1-1 ura3-52 can1-100 ade2-1*	Lab collection
68507A	*MATα cdc68-1 ura3-52* ade$^-$	Rowley et al., 1991[c]
FY56	*MATα his4-912δ lys2-128δ ura3-52*	Malone et al., 1991
FYARQ1	*MAT***a** *cdc68-1 his4-912δ lys2-128δ ura3-52*	Segregant of 68507A x FY56
ARI68-7	*MAT***a** *cdc68-1[URA3] leu2-3,112 ura3-52* ade$^-$	Rowley et al., 1991[c]
21R2	*MATα leu2-3,112 ura3-52*	Segregant of 21R x FY56
QXD2	*MAT***a**/*MATα cdc68-1[URA3]/CDC68 leu2-3,112/leu2-3,112 ura3-52/ura3-52* ade$^+$/ade$^-$	Mating of 21R2 x ARI68-7
L577	*MATα cdc68-197 his4-912δ lys2-128δ ura3-52*	E. A. Malone
FW232	*MATα spt2-150 his4-912δ ura3-52 ade2-1*	Winston et al., 1984
FY229	*MAT***a** *spt4-289 his4-912δ lys2-128δ ura3-52*	F. Winston
FY300	*MAT***a** *spt5-194 his4-912δ lys2-128δ ura3-52 leu2Δ*	Swanson et al., 1991
FY137	*MATα spt6-140 his4-912δ lys2-128δ ura3-52*	F. Winston
BM403	*MAT***a** *cdc68-197 his4-912δ lys2-128δ ura3-52 suc2ΔUAS(-1900/-390)*	E. A. Malone

Table 1. (cont.)

Strain	Genotype or phenotype[a]	Source or reference
BM404	*MATα his4-912δ lys2-128δ ura3-52 suc2ΔUAS(-1900/-390)*	E. A. Malone
BM64	*MATa/MATα his4-912δ/his4-912δ lys2-128δ/lys2-128δ cdc68-101::LEU2/CDC68 ura3-52/ura3-52 trp1/trp1 leu2-3,112/leu2-3,112*	E. A. Malone
QX3	*MATα cdc68-1 san1-3 ura3-52* ade⁻	This study[c]
QX170	*MATα cdc68-1 san1-170 ura3-52* ade⁻	This study[c]
TS1	*MATα cdc68-1 san1-201 ura3-52* ade⁻	This study[c]
QX300	*MATa/MATα cdc68-1/CDC68 leu2-3,112/LEU2 ura3-52/ura3-52 ade1/ade1*	Mating of 21R x 68507A
QX301	*MATa/MATα cdc68-1/CDC68 san1Δ::URA3/SAN1 leu2-3,112/LEU2 ura3-52/ura3-52 ade1/ade1*	This study[d]
QX401	*MATα cdc68-1 san1Δ::URA3 ura3-52* ade⁻	Segregant of QX301
QXN1	*MATa san1Δ::URA3 leu2-3,112 ura3-52*	Segregant of QX301
SUX32	*MATa cdc68-1 san1-3 his4-912δ lys2-128δ ura3-52* ade⁻	This study[e]
SUX1702	*MATa cdc68-1 san1-170 his4-912δ lys2-128δ ura3-52* ade⁻	This study[e]
SUPA1701	*MATa cdc68-1 san1-170 leu2-3,112 ade1*	This study
IS170	*MATa cdc68-1 san1-170[SAN1 URA3] his4-912δ lys2-128δ ura3-52* ade⁻	This study[f]
YRS934	*MATα san1::HIS3 his3Δ200 lys2-801 ura3-52 tyr1 ade2-101*	Schnell et al., 1989

Table 1. (cont.)

Strain	Genotype or phenotype[a]	Source or reference
YRQ9344	*MAT***a** *cdc68-1 san1::HIS3 leu2-3,112 his3Δ200?* ade$^-$	This study
YRS376	*MAT***a** *sir4 san1-2 his3-532 trp1-389am ura3-52*	Schnell et al., 1989
XRS20-10c	*MATα san1-1 his4-580 leu2-1 trp5 ura3 (ura2-9,15,30?)*	Schnell et al., 1989
JHY631	*MAT***a** *ade1 his3 leu2-3,112 trp1-1a ura3 cln2::LEU2*	C. Wittenberg
DY1671	*MAT***a** *sin4::TRP1 ho::lacZ can1-100 his3-11 leu2-3,112 trp1-1 ura3-52 ade2-1 ade6*	Jiang and Stillman, 1992
DY1825	*MAT***a** *sin4::TRP1 ho::lacZ swi2::HIS3 his3 leu2 trp1 ura3-52 ade2 ade6*	Jiang and Stillman, 1992
DY131	*MAT***a** *ho::lacZ can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-52 ade2-1 ade6*	Jiang and Stillman, 1992
DY1725	*MATα sin4::TRP1 his3-Δ200 leu2-Δ1 lys2-801 trp1-Δ63 ura3-52*	Jiang and Stillman, 1992
DY882	*MATα leu2-Δ1 lys2-801am his3-Δ200 trp1-Δ63 ura3-52 ade2-101oc*	D. J. Stillman
DY1736	*MAT***a** *ho::lacZ swi2::HIS3 can1-100 leu2 his3 trp1 ura3 ade2 ade6*	D. J. Stillman
QXT20	*MATα cdc68-1 sug1-20 ura3-52* ade$^-$	This study[c]
QXT26	*MATα cdc68-1 sug1-26 ura3-52* ade$^-$	This study[c]
QXT25	*MATα cdc68-1 sug1-25 ura3-52* ade$^-$	This study[c]
QXT26-7	*MATα cdc68-1 sug1-26 san1Δ::URA3* ade$^-$	This study[g]
QX202	*MATα sug1-20 ura3-52* ade$^-$	Segregant of QXT20 x 21R

Table 1. (cont.)

Strain	Genotype or phenotype[a]	Source or reference
QX261	*MAT***a** *sug1-26 leu2-3,112 ura3-52 ade?*	Segregant of QXT26 x 21R
QX261-1d	*MAT***a** *sug1-26 his4-912δ lys2-128δ ura3-52*	Segregant of QX261 x FY56
QX2611	*MAT***a** *sug1-26 leu2-3,112 ura3-52 his3-11,15 trp1-1*	Segregant of QX261 x W303-1α
QX2612	*MATα sug1-26 leu2-3,112 his3-11,15 trp1-1 ura3-52* ade$^-$	Segregant of QX261x W303-1α
QX261-7	*MAT***a** *sug1-26 san1Δ::URA3 leu2-3,112 ura3-52 ade?*	This study[g]
SUXB261	*MATα cdc68-1 sug1-26 his4-912δ lys2-128δ ura3-52* ade$^-$	This study[e]
SUXB201	*MAT***a** *cdc68-1 sug1-20 his4-912δ lys2-128δ ura3-52* ade$^-$	This study[e]
XBI261	*MATα cdc68-1 sug1-26[SUG1 URA3] his4-912δ lys2-128δ ura3-52* ade$^-$	This study[h]
YJOZ	*MAT***a** *GAL1-10::LacZ gal4Δ gal80Δ his3 leu2-3,112 trp1 ura3-52 ade2-101*	Swaffield et al., 1992
YJOZS	*MAT***a** *sug1-1 GAL1-10::LacZ gal4Δ gal80Δ his3 leu2-3,112 trp1 ura3-52 ade2-101*	Swaffield et al., 1992
QX202-4d	*MAT? sug1-20 gal4Δ ura3-52* ade$^-$ *leu2? his3? trp1? gal80?*	Segregant of QX202 x YJOZS
CMY762	*MAT***a** *cim3-1 his3Δ-200 leu2ΔI ura3-52*	Ghislain et al., 1993
CMY762-1a	*MAT***a** *cim3-1 leu2* ade$^-$	Segregant of CMY762 x 21R
CMY762-1b	*MATα cim3-1 leu2 his3Δ-200* ade$^-$	Segregant of CMY762 x 21R

Table 1. (cont.)

Strain	Genotype or phenotype[a]	Source or reference
LY60	*MATα cdc68-11 ho::lacZ46 leu2-3,112 trp1-1 ura3-52 can1-100* ade⁻ met⁻	Lycan et al., 1994
QX6820	*MAT***a** *cdc68-11 leu2-3,112 trp1-1 ura3-52* ade⁻	Segregant of 21R x LY60
QX6810	*MAT***a** *cdc68-101::LEU2 his4-912δ lys2-128δ leu2-3,112 trp1Δ ura3-52* ((pXHA681))	This study[i]
QX6811	*MAT***a** *his4-912δ lys2-128δ leu2-3,112 cdc68-101::LEU2[CDC68-200N TRP1] trp1Δ ura3-52*	This study[j]
QX2614	*MAT***a** *sug1-26 leu2-3,112 cdc68-101::LEU2[CDC68-200N TRP1] trp1-1 ura3-52* ade⁻	Segregant of QX2612 x QX6811
QXT101	*MAT***a** *cdc68-1[cdc68-201N URA3] sug1-1 leu2-3,112 trp1-1 ura3-52* ade⁻	This study[k]
W303-S10SUG1 -HACDC68	*MAT***a** *CDC68[CDC68-200N TRP1] sug1Δ leu2-3,112 ura3-52 his3-11,15 trp1-1 ade2-1* ((pVT100U-S10SUG1))	J. C. Swaffield
QX6800	*MAT***a** *leu2-3, 112 ura3-52 cdc68-101::LEU2[CDC68-200N TRP1] sug1Δ his3-11,15 trp1-1 ade2-1* ((pVT100U-S10SUG1))	This study[l]
QX6801	*MAT? leu2-3,112 ura3-52 his3-11,15 trp1-1 ade2-1 sug1Δ ?* ((pVT100U-S10SUG1))	Segregant of QX6800 x FY56
CTY10-5d	*MAT***a** *gal4 gal80 leu2-3,112 his3-200 trp1-901 ade2 URA3::LexA-Gal1-LacZ*	C.-T. Chien and R. Sternglanz
MT72	*MAT***a** *cdc28-1N leu2-3,112 ura3 his3-11,15 trp1-1 can1-100 ade2-1*	M. Tyers
QX2628-1	*MAT? sug1-26 cdc28-1N leu2-3,112 ura3? his3-11,15 trp1-1* ade⁻	Segregant of MT72 x QX2612
H126-9-2	*MATα cdc15-2 ade1 ade2 ura1* leu⁻	Lab collection

Table 1. (cont.)

Strain	Genotype or phenotype[a]	Source or reference
QX2615-1	*MAT? sug1-26 cdc15-2* leu⁻ ade⁻ *ura? his3? trp1?*	Segregant of QX2611 x H126-9-2
CD105-1b	*MATα clb2::LEU2 leu2-3,112 ura3-1 his3-11,15 trp1-1 ade2-1*	B. Futcher
XSC2-1	*MAT? sug1-26 clb2::LEU2 leu2,3-112 ura3? his3-11,15 trp1-1* ade⁻	Segregant of QX2611 x CD105-1b
7859-7-4	*MAT***a** *rad9Δ::LEU2 leu2-3,112 ura3-52 trp1-289 his7*	Weinert and Hartwell, 1990
QX26110	*MAT? sug1-26 rad9Δ::LEU2 leu2-3,112 ura3-52 his? trp1? ade?*	Segregant of QX2612 x 7859-7-4
QX2029	*MAT? sug1-20 rad9Δ::LEU2 leu2-3,112 ura3-52 his? trp1? ade?*	Segregant of QX2021 x 7859-7-4
Strains used for mapping the *SAN1* gene.		
IS170-1c	*MAT***a** *san1-170[SAN1 URA3] his4-912δ* ade⁻	Segregant of 21R x IS170
IS170-1d	*MATα san1-170[SAN1 URA3] his4-912δ* ade⁻	Segregant of 21R x IS170
EP25	*MAT***a** *cdc37-1 his6 ura1*	Lab collection
EP25a	*MAT***a** *cdc37-1 ura3-52*	Segregant of EP25 x FY56
EP25d	*MAT***a** *cdc37-1 lys2-128δ ura3-52*	Segregant of EP25 x FY56
ARM4R2	*MAT***a** *cdc37 lys4 prt3 trp1 tyr1 ura3-52*	Lab collection
LARM4-16B	*MAT***a** *ade8 trp4 ade1 arg4 cdc65-1 leu2 rna3 ura3*	Lab collection

Table 1. (cont.)

Strain	Genotype or phenotype[a]	Source or reference
X4119-15D	*MATα aro1B hom2 cdc8 cdc9 his1 lys11 gal2 trp4*	YGSC
X4119-3	*MATα aro1B hom2 ura3-52*	Segregant of 21R x X4119-15D

[a] Gene designations in square brackets indicate plasmid-derived sequences integrated in single copy into the chromosome, and those in double-parentheses indicate episomal plasmids. Gene designations followed by question marks indicate the respective genotypes were not determined.

[b] YGSC, Yeast Genetic Stock Centre.

[c] Congenic with strain 21R.

[d] Constructed by transforming the linearized *san1Δ::URA3* fragment (from pLG7) into diploid strain QX300.

[e] A segregant from a cross between strains FY56 and 21R was backcrossed with strain 21R. A *his4-912δ lys2-128δ* segregant from this cross was mated with strains QX3, QX170, QXT20, and QXT26 to derive strains SUX32, SUX1702, SUXB201, and SUXB261, respectively.

[f] Constructed by the directed integration of plasmid pIBE3 into the *Cla*I site of the *san1-170* locus in strain SUX1702.

[g] Constructed by transforming the linearized *san1Δ::URA3* fragment (from pLG7) into strains QXT26 and QXT261 to generate QXT26-7 and QXT261-7, respectively.

[h] From directed integration of pIKE17 at the *sug1-26 Cla*I site in strain SUXB261.

[i] A segregant of BM64 transformed with plasmid pXHA681.

[j] From integration of pXHA682 into QX6810 at the chromosomal *cdc68-101::LEU2* locus and subsequent loss of pXHA681.

[k] A segregant from cross between YJOZS and QXT20 harboring pXIHA68-1.

[l] Constructed by replacing the wild-type *CDC68* gene of strain W303ΔS-HACDC68 by the *cdc68-101::LEU2* allele. (Gene replacement was verified by Southern blot analysis.)

Table 2. Plasmids used in this study.

Plasmid	Description
Vectors:	
pUC19	*E. coli* vector (Yanisch-Perron et al., 1985).
M13 mp18 and mp19	M13 phage vectors (Yanisch-Perron et al., 1985).
YEp352	*URA3*-marked high-copy yeast/*E. coli* shuttle vector (Hill et al., 1986).
YIp352	*URA3*-marked integrative yeast/*E. coli* shuttle vector (Hill et al., 1986).
pRS316	*URA3*-marked low-copy yeast/*E. coli* shuttle vector (Sikorski and Hieter, 1989).
pSH2-1	LexA DNA-binding domain plasmid, used for the two-hybrid interation assay (C.-T. Chien and R. Sternglanz)
pGAD3	Gal4 activation domain plasmid, used for the two-hybrid interaction assay (Chien et al., 1991)
CDC68-related:	
p68-Ba-1A	5.2-kbp *CDC68*-containing genomic insert in YEp352 at the *Bam*HI site (Rowley et al., 1991).
pDE-683	5.2-kbp *CDC68*-containing genomic insert in pRS316, between the *Kpn*I and *Xba*I sites (D. R. H. Evans).
pBM46	*cdc68-197* allele in pRS314 (*TRP1*-marked low-copy vector, Sikorski and Hieter, 1989) at the *Bam*HI site (E. A. Malone).
pBM46-4	The *cdc68-197* gene from pBM46 inserted into YEp352 at the *Bam*HI site.
YEpDE68-1	The *cdc68-1* allele recovered by gap-repair, inserted into YEp352 (D. R. H. Evans).
pXHA681	5.3-kbp HA-tagged *CDC68* gene (*CDC68-200N*) in YEp352 at the *Bam*HI site.
pXHA682	5.3-kbp HA-tagged *CDC68* gene from pXHA681, inserted into YIp352 as a *Xba*I-*Kpn*I fragment.
pXHA68-1	5.3-kbp HA-tagged *cdc68-1* allele (*cdc68-201N*) in YEp352 at the *Bam*HI site.

Table 2. (cont.)

Plasmid	Description
pXIHA68-1	5.3-kbp HA-tagged *cdc68-1* allele from pXHA68-1, inserted into YIp352 as a *Xba*I-*Kpn*I fragment.
pBD-CDC68	PCR-amplified *CDC68* ORF, fused in frame to the LexA DNA-binding domain sequence in pSH2-1 at the *Bam*HI site.
pBD-CDC68-Ec-3	The 3.0-kbp *Eco*RI restriction fragment of the *CDC68* gene (from nucleotide 476 to the downstream region of *CDC68* ORF; Rowley et al., 1991), fused in frame to the LexA DNA-binding domain sequence in pSH2-1 at the *Eco*RI site.
SAN1-related	
pTD33	11-kbp genomic insert containing the *SAN1* gene; recovered from a YCp50 (*URA3*-marked low-copy vector)-based yeast genomic library.
pBE3	3.5-kbp genomic insert containing the *SAN1* gene from pTD33, inserted into pRS316 as a *Bam*HI and *Eco*RI fragment.
pBB3	4-kbp genomic insert containing the *SAN1* gene from pTD33, cloned into pRS316 at the *Bam*HI site.
pBB2, pBE2, pCE1, and pCE2	Subclone plasmids derived from pTD33 (see Fig. 4), unable to complement *san1* mutations.
pEBE3	3.5-kbp *SAN1*-containing genomic insert from pBE3, inserted into YEp352 between the *Bam*HI and *Eco*RI sites.
pIBE3	3.5-kbp *SAN1* containing genomic insert from pBE3, inserted into YIp352 between the *Bam*HI and *Eco*RI sites.
pUC19-SAN1	3.5-kbp *SAN1*-containing genomic insert in pUC19 between the *Bam*HI and *Eco*RI sites.
pLG7 and pLG8	*san1Δ::URA3* null allele in pUC19 as a 2.6-kbp *Bam*HI-*Eco*RI fragment (see text for construction). These two plasmids differ in the orientation of the *URA3* gene with respect to that of *SAN1*.
SUG1-related	
pXB68	8-kbp genomic insert containing the *SUG1* gene, recovered from a YCp50-based yeast genomic library.
pXKE17	1.7-kbp *SUG1* genomic fragment from pXB68, inserted into pRS316 between the *Eco*RI and *Kpn*I sites.

Table 2. (cont.)

Plasmid	Description
pXES5	5-kbp *SUG1*-containing genomic insert from pXB68, inserted into pRS316 as an *Eco*RI-*Sst*I fragment.
pXKS4 and pXHH2	Subclone plasmids derived from pXB68 (see Fig. 12), unable to complement *sug1* mutations.
pEKE17	1.7-kbp *SUG1* genomic insert from pXKE17, cloned into YEp352 as an *Eco*RI-*Kpn*I fragment.
pIKE17	1.7-kbp *SUG1* genomic insert from pXKE17, cloned into YIp352 as an *Eco*RI-*Kpn*I fragment.
pUC19-SUG1	1.7-kbp *SUG1* genomic insert from pXKE17, inserted into pUC19 between the *Eco*RI and *Kpn*I sites.
pXBS23	*sug1::LEU2* disruption allele in pUC19 (see text for construction). A 3.4-kbp *sug1::LEU2*-bearing *Pvu*II-*Bam*HI fragment of this plasmid can be used for gene replacement.
pXBS24	*sug1::URA3* disruption allele in pUC19, constructed in a similar way as pXS23. A 2.9-kbp *san1::URA3* bearing *Eco*RI-*Kpn*I fragment of this plasmid can be used for gene replacement.
pAD-SUG1	PCR-amplified *SUG1* ORF, fused in frame to the Gal4 activation domain sequence in pGAD3 at the *Bam*HI site.
Miscellaneous:	
pSB32GAL4	3.6-kbp *GAL4* genomic insert between the *Bam*HI and *Hin*dIII sites in a *LEU2*-marked plasmid (Swaffield et al.,1992).
pSB32gal4D	Similar to pSB32GAL4 but containing the *gal4D* allele. (Swaffield et al., 1992)
pSWE1-14	3.5-kbp *SWE1* genomic insert in the *S. pombe* vector pIRT3 between the *Pst*I and *Bam*HI sites (Booher et al., 1993).
pSWE1-10g	The *swe1Δ::LEU2* null allele (the catalytic domain of *SWE1*, from nucleotide 1334 to 2318, was replaced with 2.2-kbp *LEU2* gene) in plasmid pUC19 as a 3.3-kbp *Hin*dIII/*Bam*HI fragment (Booher et al., 1993).
YEp352-SWE1	3.5-kbp *SWE1* genomic insert from pSWE1-14, inserted in YEp352 between the *Pst*I and *Bam*HI sites.
pMDMb143	2.7-kbp *SIC1* as an *Eco*RI-*Hin*dIII fragment in pGEM-4Z (Nugroho and Mendenhall, 1994).

Table 2. (cont.)

Plasmid	Description
pTN27-8	containing the *sic1Δ::URA3*-null allele, can be excised as a 3-kbp *Eco*RI-*Nco*I fragment for gene replacement (M. Mendenhall).
YEp352-SIC1	2.7-kbp *SIC1* gene from pbMDM143, cloned into YEp352 as an *Eco*RI-*Hin*dIII fragment.
pRR330	*rad9Δ::URA3* null allele in pUC18 as a 7.1-kbp *Eco*RI and *Sal*II fragment (Schiestl et al., 1989)
pMT422	*GAL1-CLB2* containing *LEU2*-marked episomal plasmid (M. Tyers).
pJH3-45-2-cln2::LEU2	4.7-kbp *cln2Δ::LEU2* fragment in YEp13 as a *Sal*I-*Hin*dIII fragment (C. Wittenberg).
pSH17-4	LexA-GAL4 activation domain fusion plasmid (C.-T.Chien and R. Sternglanz)

antimycin A (Sigma, St. Louis, MO) was added to a final concentration of 1 mg per liter to inhibit aerobic growth. The YEPD rich solid medium contained 2% glucose, 2% bactopeptone, 1% bacto-yeast extract, and 2% agar (Hartwell, 1967). 1.5% agar was added to the YNB minimal medium or synthetic "dropout" medium when used to make solid medium. 5-FOA medium was complete synthetic medium containing 1 mg of 5-fluoro-orotic acid (SCM Special Chemicals, Gainesville, FL) per liter and was used for the counterseletion of *URA3*-marked plasmids (Boeke et al., 1984). Sporulation medium contained 1% potassium acetate and 0.1% glucose. To assess expression of the bacterial *lacZ* gene in yeast cells, X-gal solid medium (synthetic "dropout" medium plus 0.1 M potassium phosphate, pH 6.9, 4 μg/ml X-gal, and 1.5% agar; C. Barnes, personal communication) was used.

E. coli strains were grown in 2xYT medium (Messing, 1983), containing 1.6% Bacto-tryptone, 1% Bacto-yeast extract, and 0.5% NaCl. Ampicillin was added to a final concentration of 75 μg/ml to select for the presence of Ampicillin-resistant plasmids. X-gal was added to a final concentration of 0.3 μg/ml to identify strains harboring *lacZ*-containing plasmids.

E. coli strains were routinely grown at 37°C either on solid medium (in an incubator) or in liquid medium (in a shaking water bath or on a rollerdrum). Yeast strains were normally grown at 23°C or 30°C. To assess temperature sensitivity, yeast strains were also incubated at a range of higher temperatures, from 33°C to 37°C.

3. Chemicals, molecular cloning enzymes, and antibodies

Chemicals were obtained from Sigma unless otherwise specified. Bacto-agar, Bacto-peptone, Bacto-yeast extract and Bacto-yeast nitrogen base were obtained from Difco Laboratories, (Detroit, MI).

Restriction enzymes and other modifying enzymes (including T4 DNA ligase, large fragment of DNA polymerase I [Klenow], Taq polymerase) were purchased from

GIBCO BRL (Burlington, Ontario) and New England Biolabs, Ltd. (Mississauga, Ontario). Alkaline phosphatase was purchased from Boehringer Mannheim (Laval, Quebéc).

Monoclonal rat anti-tubulin antibody (YOL1/34) was purchased from Sera-lab (Sussex, England). FITC-conjugated goat anti-rat IgG and anti-mouse IgG antibodies were purchased from Sigma. Monoclonal mouse anti-HA antibody (12CA5) was purchased from Boehringer Mannheim. Monoclonal mouse anti-T7 S10 antibody was purchased from Novagene (Madison, WI). Horseradish peroxidase-linked anti-mouse IgG, anti-rabbit IgG and anti-rat IgG antibodies were obtained from Amersham Canada Lt., (Oakville, Ontario). Sug1 antiserum was a gift from J. C. Swaffield and S. A. Johnston (Southwestern Medical Center, Dallas, TX).

4. Assessment of cellular parameters

a) Cell concentration

A 0.5 ml sample of yeast culture was fixed by the addition of 4.5 ml of 3.7% formalin. Cells were then sonicated for 5 s at 60% power with a Microson Sonicator (Heat System Ultrasonics, Farmingdale, NY) to break up cell clumps. Yeast cell concentrations were determined using an electronic particle counter (Coulter Electronics).

b) Budding index

The budding index, i. e., the percentage of unbudded (or budded) cells in a cell population, was determined by examining the morphology of at least 300 formalin-fixed and sonicated yeast cells using a phase-contrast microscope (Carl Zeiss, Germany).

5. Cell biological analysis

a) DAPI stain

Nuclear morphology of yeast cells was assessed by staining with 4',6-diamidino-2-phenyl-indole (DAPI; Williamson and Fennell, 1975). Yeast cells were grown to mid-log phase, and cells (5 ml) were washed in distilled H_2O and fixed in 70% ethanol for 1 h. Cells were washed again in distilled H_2O, sonicated briefly, and stained with DAPI (1 μg/ml). Nuclear morphology of DAPI-stained cells was examined using a microscope (Model 84489, Carl Zeiss) equipped with a fluorescent filter, and photographs were taken.

b) FACS analysis

FACS (fluorescence activated cell sorting) analysis was carried out essentially as described by Mann et al. (1992) to determine the DNA content of yeast cells. For this analysis, 1 ml of cell culture in mid-log phase (about 10^7 cells) was fixed in 70% ethanol for 1.5 h, and washed with 0.2 M Tris-HCl (pH 7.5). Cells were then resuspended in 1 ml 0.2 M Tris-HCl (pH 7.5) and treated with RNaseA (0.1%) for 2 h at 37°C, washed in PBS (phosphate-buffered saline) and stained with propidium iodide (5 μg in 0.1 ml PBS) for 20 min. After staining, cells were again washed in PBS and resuspended in 1 ml PBS containing 5 μg of propidium iodide. Samples were sonicated briefly to disrupt cell clumps and observed under a fluorescence microscope to ensure specific nuclear staining had taken place before running samples through a fluorescence-activated cell sorter.

c) Indirect immunofluorescence

For anti-tubulin staining, yeast cells were pretreated essentially as described (Pringle et al., 1991). Cells were fixed by adding formaldehyde directly to the culture medium to a final concentration of 3.7% (w/v) and incubated at room temperature for 2 h. Cell walls were permeabilized by treatment with glucunonidase and β-mercaptoethanol for 2 h. Monoclonal rat anti-tubulin antibody was used at a 1:20 dilution, and affinity-purified FITC-conjugated goat anti-rat IgG secondary antibody was used at a 1:600

dilution. Fluorescence photomicroscopy of stained cells was performed with a Nikon microphot FX equipped with epi-fluorescence.

To determine the intracellular location of the Cdc68 protein, a yeast strain harboring the HA-tagged Cdc68 protein was used for indirect immunofluorescence as described above. Anti-HA monoclonal antibody (12CA5) was used at a 1:100 dilution, and affinity-purified FITC-conjugated anti-mouse IgG secondary antibody was used at a 1:400 dilution.

d) Synchronization of yeast cell populations

Hydroxyurea (HU) was used to synchronize yeast cells in the S phase of the cell cycle. HU is an inhibitor of ribonucleotide reductase, an enzyme that maintains DNA precursor pools; upon HU treatment yeast cells that have not completed DNA replication arrest in the S phase of the cell cycle, while cells that have completed DNA synthesis continue through mitosis and become arrested in the subsequent S phase (Slater, 1973). Yeast cells were grown to a density of $5x10^6$ cells/ml and HU was added to a final concentration of 0.2 M. Incubation was continued in the presence of HU for 3 h at 23°C to achieve a synchronized S phase cell population. After HU treatment, cells were washed once in PBS and resuspended in fresh prewarmed YM1 medium for further incubation at the appropriate temperature. Samples were taken at hourly intervals and assessed for both budding index and cell number.

e) Evaluation of UV sensitivity

To assess UV sensitivity of mutant yeast strains, late log-phase or early stationary-phase yeast cells were diluted to a cell density of 10^6 cells/ml. A 10-µl sample of cell culture (about 10^4 cells) was spotted onto a YEPD plate. Cells were irradiated at 10 mjoule/cm^2 using a UV cross-linker (energy setting at 100; stratalinker, Stratagene, La Jolla, CA). Cells were then subjected to the same dose of UV irradiation 8-16 h later to

efficiently prevent recovery of UV-sensitive cells and incubated for 2-3 days at 23°C. UV sensitivity was assessed by the ability of UV-irradiated cells to form colonies.

6. Yeast genetic analysis

a) Construction of diploid strains

A diploid yeast strain was constructed by mixing two haploid strains of opposite mating type on a YEPD plate and incubating at 30°C for 4-12 h (Guthrie and Fink, 1991). Diploid cells were selected on YNB minimal medium by exploiting the decreased auxotrophic requirements of the resultant diploid compared to the haploid parents. In situations without useful auxotrophic markers, diploids were isolated by microscopic manipulation (Singer MK. III Micromanipulator, Singer Instrument Co. Ltd., Watchet, England) of zygotes to unpopulated areas of the agar surface.

b) Determination of mating type

A haploid yeast strain can be either one of two different mating types, *MAT***a** and *MATα*. To determine mating type, strain XJB3-1B (*MATα met6*) was mated with the uncharacterized haploid strain. All haploid strains constructed during this study are methionine prototrophs, so these diploids could be selected on YNB minimal medium. The ability to form diploids reveals that the two haploid strains have opposite mating types, and thus the tested haploid is *MAT***a**. Conversely, failure to form diploids suggests that the two haploids are of the same mating type and indicates that the tested haploid strain is *MATα*.

c) Dominance tests

To determine whether the effect of a *cdc68-1*-suppressor mutation is dominant or recessive with respect to the wild-type suppressor gene, dominance tests were carried out by mating each suppressed haploid strain with the *cdc68-1* strain ARI68-7; good growth

of the resultant diploid cells at the restrictive temperature indicated that the suppressor mutation overcame the effect of the wild-type suppressor gene and was therefore dominant. By the same token, poor growth indicated codominance, and no growth indicated recessiveness.

d) Sporulation of diploid cells

Under starvation conditions diploid yeast cells undergo a developmental process, termed sporulation, to produce four haploid spores. To induce sporulation, diploid cells were grown in YM1 medium to early log phase, washed twice in PBS, and incubated in liquid sporulation medium (1% potassium acetate and 0.1% glucose) for five days at 23°C. Adenine was added to the sporulation medium if the diploid had an adenine auxotrophy. Sporulation of diploid cells was monitored by the appearance of "diamond"-shaped asci under a phase-contrast microscope.

e) Tetrad analysis

To separate spores within a single ascus, sporulated diploid cells were washed three times in PBS and resuspended in 0.5 ml of PBS. Ascus walls were digested by the addition of 40 µl of β-glucuronidase (Sigma), and incubation on a roller drum for 1.5 h at 23°C. The digested samples were washed twice in PBS and resuspended in 5 ml of PBS. A loopful of digested sample was streaked on one edge of an agar slab and separation of the four spores of an ascus was carried out using a Singer MK. III Micromanipulator. The agar slab with separated spores was placed on YEPD solid medium and incubated at 23°C for 2-3 days until spores grew into colonies. These colonies were then patched onto fresh YEPD medium, and phenotypes were assessed by replica-plating to appropriate solid media. Recombination frequencies between two genetic markers were calculated according to Perkins (1949): distance between two markers (in cM) =

[(6NPD+TT)x100]/2(PD+NPD+TT). (PD, NPD, and TT stand for parental ditype, non-parental ditype and tetratype, respectively.)

f) Complementation tests

Complementation tests were used to determine the number of genes defined by the suppressor mutations isolated here. Complementation among the recessive suppressor mutations was assessed by scoring the phenotypes of diploid strains constructed from pairwise crosses of haploid suppressed strains. Maintenance of the suppressed phenotype (i. e. growth at 35°C) indicated that the two recessive suppressor mutations were unable to complement each other and are therefore most likely allelic. Alternatively, complementation resulted in temperature-sensitive diploids, suggesting that the two recessive suppressor mutations are in different genes.

g) Allelism tests

For co-dominant suppressor mutations, and also in cases of recessive mutations for which complementation results were ambiguous, allelism tests were performed. Diploid cells resulting from mating two independent suppressed haploid strains were sporulated; a virtual absence of temperature-sensitive haploid segregants suggested that the two suppressor mutations are tightly linked and thus most likely in the same gene. In contrast, the occurrence of approximately equal numbers of NPD (2 of the 4 spores are temperature-sensitive) and PD tetrads would suggest that the two suppressor mutations are unlinked to each other and therefore in different genes.

f) Mapping the *SAN1* gene

The *SAN1* gene was initially localized to an individual yeast chromosome by chromosome hybridization. The 3.5-kbp *Bam*HI-*Eco*RI genomic insert from plasmid pTD33 (harboring the *SAN1* gene, see Fig. 4) was labeled with [α-^{32}P]dCTP and used to

probe an *S. cerevisiae* chromosome blot (Clontech Laboratories, Palo Alto, CA) using standard Southern hybridization procedures (see below).

The position of the *SAN1* gene on chromosome IV was determined by tetrad analysis using *san1-170* strains in which the chromosomal *san1* locus was marked with an integrated *URA3* gene. Initially four genetic markers on chromosome IV were chosen; *cdc37*, *trp4*, and *ade8* on the left arm, and also the centromere-linked *trp1*. Two-factor crosses were performed, and as shown in Table 5 *san1* was unlinked to *trp1*, *trp4*, and *ade8*, but linked to *cdc37*. Subsequently, two more chromosome IV markers, *hom2* and *aro1*, were chosen that are close to *cdc37*. The *hom2* mutation confers a Met^- Thr^- phenotype, whereas the *aro1* mutation confers a Phe^- phenotype. A three-factor cross was performed by mating the *san1-170[URA3]* strain IS170-1c with a *hom2 aro1 ura3-52* strain, X4119-3. This linkage analysis positioned the *san1* allele relative to *hom2* and *aro1* on the right arm of chromosome IV.

7. Plasmid extraction and transformation

a) Plasmid extraction from *E. coli*

E. coli plasmid DNA was prepared by either rapid-boiling (Holmes and Quigley, 1981) or alkaline-lysis (Birnboim and Doly, 1979) methods. *E. coli* cells were grown overnight in 2xYT medium plus an antibiotic (e. g. ampicillin) to maintain plasmids. For the rapid-boiling method, cell pellets were resuspended in 200 µl of STET buffer (8% sucrose, 5% Triton X-100, 50 mM EDTA, and 50 mM Tris, pH 8) and lysed by boiling for 80 sec. The cell lysate was then spun down in a microcentrifuge for 10 min, and pellets were removed using toothpicks. The supernatant was extracted with phenol/chloroform/isoamyl alcohol (24:24:1) to remove proteins. The aqueous phase was transferred to a fresh tube, and DNA was precipitated in ethanol for 30 min at -20°C. DNA was pelleted by centrifugation in a microcentrifuge for 10 min. The pellet was

washed with 70% ethanol to remove salts, and air-dried for 30 min. The dried DNA pellet was dissolved in 40 µl of TE buffer (50 mM Tris-HCl and 10 mM EDTA, pH 8).

For the alkaline-lysis method, *E. coli* cell pellets were resuspended in 100 µl of TEG solution (25 mM Tris pH 8, 10 mM EDTA, and 50 mM glucose). Cells were lysed by the addition of 200 µl of freshly prepared alkaline buffer (0.2 M NaOH and 1% SDS) and incubated on ice for 5 min. 150 µl of chilled 3 M potassium acetate (pH 4.8) was added and lysates were vortexed gently for 10 sec, then incubated on ice for another 5 min and spun down in a microcentrifuge for 5 min at 4°C. The supernatant was transferred to a fresh tube and incubated with 4 µl of RNase A (10 mg/ml) for 15 min at 37°C. Phenol extraction and precipitation of DNA was carried out following the same procedure described for the rapid-boiling method.

Plasmid DNA concentration was determined by restriction enzyme digestion of 1 µl plasmid DNA and resolution by electrophoresis through an agarose gel containing ethidium bromide (0.5 µg/ml). The amount of plasmid DNA digested was approximated by comparison with 300 ng of *Hin*dIII-digested lambda DNA run in an adjacent lane.

b) Plasmid extraction from yeast

Yeast cells were broken by vortexing along with glass beads, and plasmid DNA was isolated essentially as described by Lorincz (1984). First yeast cells were spread on synthetic "dropout" medium to select for plasmid-bearing cells. A patch of stationary-phase cells (about 10 mm^2) was suspended in 200 µl of Solution A (100 mM NaCl, 10 mM Tris, pH 8, 1 mM EDTA, and 0.1 % SDS) in a microcentrifuge tube. Acid-washed glass beads were added to just below the meniscus. Cells were then broken by vortexing for 2 min. 200 µl of solution A and 400 µl of Tris-buffered phenol was then added to the mixture. After vortexing for a further 2-5 min, the sample was spun down in a microcentrifuge for 1 min. The aqueous phase was transferred to a fresh tube and extracted with phenol/chloroform/isoamyl alcohol (24:24:1). The aqueous phase was

again saved and precipitated by the addition of 1/10 vol. of sodium acetate and 2 vol. of 95% ethanol. After incubation at -20°C for 30 min, DNA was spun down in a microcentrifuge for 10 min, and the pellet was washed in cold 70% ethanol, dried and dissolved in 20 µl TE buffer. About 5 µl of a preparation of this sort was used to transform competent *E. coli* cells.

c) Transformation of *E. coli.* cells

Transformation of *E. coli* cells was accomplished by either $CaCl_2$ treatment or electroporation.

$CaCl_2$ transformation. *E. coli* cells competent for transformation were prepared essentially as described by Sambrook et al. (1989). Briefly, *E. coli* cells were grown to a density of $A_{600} = 0.7$, cells were chilled on ice and subsequent manipulations were performed at 4°C. Cells were harvested by centrifugation at 3,000 rpm for 10 min, washed once in 100 mM $CaCl_2$, resuspended in 100 mM $CaCl_2$ and incubated on ice in a refrigerator for 20 h before transformation. Portions of competent cells (in 20% glycerol) were also stored at -70°C for transformation at a later time.

For transformation, plasmid DNA was added to 50 µl of either freshly prepared or thawed frozen competent cells and the mixture was incubated for 30 min on ice. The DNA-cell mixture was then heat shocked for 45 sec in a 42°C water bath. After a 2-min incubation on ice, the DNA-cell mixture was incubated in 1 ml 2xYT broth at 37°C for 45 min before transfer to YT solid medium containing ampicillin.

For transformation using M13-derived phage DNA, DNA was mixed with 100 µl of thawed competent cells and left on ice for 40 min. Meanwhile, "top" agar (2xYT containing 0.6% agarose) was melted and a 3.5-ml portion was transferred to each tube incubated in a 52°C heat block. The DNA-cell mixture was transferred to 42°C for 3 min and then left on ice for 2 min. The mixture was transferred quickly to an agar tube,

mixed by vortexing, and poured onto prewarmed 2xYT solid medium. Phage plaques normally formed after 8 to 12 h at 37°C.

Electroporation. To achieve higher transformation efficiency, especially to introduce blunt-ended ligation products into *E. coli* cells, electroporation was used. *E. coli* cells competent for electroporation were prepared essentially as described (Ausubel et al., 1991). *E. coli* cells were grown up to an A_{600} of 0.5-0.8 and chilled on ice for 20 min. All subsequent steps were carried out at 4°C. Cells were harvested by centrifugation at 5,000 rpm for 10 min, and pellets were washed twice in ddH_2O (distilled and de-ionized H_2O) and once in chilled 10% glycerol and resuspended in 10% glycerol. After this treatment, cells were distributed to microcentrifuge tubes and stored at -70°C.

To introduce plasmid DNA into *E. coli* cells by electroporation, 50 µl (for the 0.1-mm electroporation cuvette) or 100 µl (for the 0.2-mm cuvette) of frozen electroporation-competent cells were thawed on ice, DNA was added and the mixture was transferred to a chilled electroporation cuvette (BioRad). The DNA-cell mixture was then electroporated using a Gene Pulser (BioRad) following the manufacturer's instructions. The time constant was generally between 4.2-4.5. 1 ml of 2xYT broth was added to the mixture immediately after electroporation, which was then incubated at 37°C for 45 min and spread on 2xYT plus ampicillin medium.

d) Transformation of yeast cells

Plasmid DNA was introduced into yeast cells by the spheroplast method (Hinnen, et al., 1978) with minor modifications, and also by the lithium-acetate method (Ito et al., 1983).

To achieve high transformation efficiency, especially when screening cells transformed with library DNA, plasmid DNA was introduced into yeast cells by the following spheroplast method. Cells were inoculated in 300 ml of YM1 medium and

grown to early log phase, harvested by centrifugation at 3,000 rpm for 10 min, and washed in ddH_2O. Cell pellets were resuspended in 1 M sorbitol, and cell walls were digested with β–glucuronidase (20 µl/ml) at 30°C for 1 h. Resultant spheroplasts were then washed once and resuspended in 1 M sorbitol. After incubation at room temperature for several hours, spheroplasts were then washed in SCT solution (1 M sorbitol, 10 mM $CaCl_2$, and 10 mM Tris-HCl, pH 7.5) and resuspended in SCT. 1-2 µg of DNA plus 50 µg of salmon sperm carrier DNA were added to a 300-µl portion of spheroplasts (in SCT). The mixture was incubated with 10 volumes of polyethylene glycol (PEG) solution (20% PEG, 20 mM Tris-HCl, pH 7.5, and 20 mM $CaCl_2$) for 10 min, pelleted and resuspended in 1 ml SCT, added to melted "regeneration" agar (kept at 52°C) and poured onto synthetic "dropout" medium.

For routine yeast transformation, the lithium acetate method was employed. About 10 ml of cell culture was grown to mid-log phase. Cells were pelleted, washed in ddH_2O and in lithium acetate solution (0.1 M lithium acetate, 10 mM Tris, pH 8.0, and 1 mM EDTA), resuspended in lithium acetate solution and incubated at 30°C for 1 h. 1-10 µg of DNA plus 50 µg of salmon sperm carrier DNA was added to a 100-µl lithium acetate-treated cell sample, and incubation was continued at 30°C for 30 min. 0.7 ml of PEG (40% PEG, 0.1 M lithium acetate, 10 mM Tris, pH 8.0, and 1 mM EDTA) was added to the cell-DNA mixture. After a 45 min incubation at 30°C, the mixture was transferred to 42°C for 5 min before being spread on synthetic "dropout" medium to select for transformants.

8. DNA manipulations

a) Restriction enzyme digestions

Restriction endonuclease digestion was used to characterize plasmid DNA or to generate restriction maps of DNA fragments. 20-µl reaction mixture contained 2 µl of appropriate restriction enzyme buffer (according to supplier's recommendations), 0.5-1

μg of DNA, and ddH_2O to make up the volume to 19 μl; 1-5 units (1 μl) of restriction enzyme was then added. The reaction was carried out in a 37°C heatblock for 1 h, except that for *Sma*I the digestion was done at 30°C and for *Bst*EII the reaction was at 65°C. To generate a refined restriction map of a gene or to retrieve a DNA fragment from a plasmid, double-restriction enzyme digestions were carried out, in which case two restriction enzymes were used at the same time to digest DNA. Digested products were analyzed by agarose gel electrophoresis as described below.

b) Ligation and plasmid constructions

Ligation of two DNA fragments was performed essentially as described by King and Blakesley (1986). In cases where the insert and vector had compatible cohesive ends, ligation reactions were carried out using T4-ligase and ligase buffer at 14°C for 4 h, or at 4°C overnight (whichever was convenient). Blunt-ended ligation was carried out at 30°C for 1 h. In cases where the insert and vector had incompatible cohesive ends, the ends were first made flush with Klenow enzyme in the presence of dNTP (including dATP, dTTP, dCTP and dGTP) (ends having 5'-overhangs) or in the absence of dNTP (ends having 3'-overhangs). Klenow-treated DNA was ethanol precipitated and redissolved in an appropriate amount of TE buffer, and the ligation reaction was carried out using blunt-ended ligation conditions. In most cases the vector DNA was treated with alkaline phosphatase to minimize vector religation.

c) Agarose gel electrophoresis of DNA

Agarose gel electrophoresis was performed essentially as described by Sambrook et al. (1989). Either TAE buffer (40 mM Tris-acetate, 1 mM EDTA) or TBE buffer (0.098 M Tris-borate, 0.098 M boric acid, 2 mM EDTA) and electrophoresis grade agarose (BRL) were used. Ethidium bromide (0.5 μg/ml) was added to melted agarose before casting the gel. 10X loading buffer (50% glycerol, 0.3% xylene cyanol, and 0.3%

bromophenol blue) was added to the DNA sample to a final concentration of 1X. Molecular size markers used were *Hin*dIII digested lambda DNA, a 100-bp DNA ladder, and a 1-kbp DNA ladder (BRL).

d) Gel purification of DNA fragments

To purify DNA fragments from agarose gels, a minimal portion of gel containing the desired DNA fragment (visualized by a UV transilluminator) was excised using a razor blade. DNA was purified using a Geneclean kit (BIO101, La Jolla, CA) following the manufacturer's instructions. The amount of DNA retained after purification was estimated by resolving a known quantity of purified DNA on an agarose gel along with molecular size markers.

e) Polymerase chain reaction (PCR)

PCR was used to amplify DNA or to introduce restriction enzyme recognition sites to the ends of DNA fragments. The PCR reaction mixture contained 50 mM KCl, 10 mM Tris (pH 9), 3 mM $MgCl_2$, 0.2 mM each of dATP, dTTP, dCTP, and dGTP, 50 pmol of primers, 50 ng of plasmid DNA or 1 μg of yeast genomic DNA, and 2 units of Taq polymerase (BRL). Samples were denatured at 94°C for 5 min, and 35 cycles of 1 min each at 94°C, 45°C, and 72°C were carried out in a thermal cycler (Perkin-Elmer/Cetus 9600, or Dia Med/COY tempcycler II). To complete the extension step of Taq polymerase, the final cycle was terminated by incubating at 72°C for 15 min. Conditions for the annealing and extension steps varied depending on the nature of the primers used and the size of the particular DNA sequence to be amplified. The products of PCR reactions were either purified directly using a Geneclean kit or a Wizard PCR Preps kit (Promega Corp., Madison, WI), or resolved electrophoretically on an agarose gel before purification.

f) Radiolabeling of DNA fragments

DNA fragments were labeled with [α-^{32}P]dCTP (Amersham) using either the random-primer labeling kit (Boehringer Mannheim, Germany) or the Radprime kit (BRL) following the manufacturers' instructions. Radiolabeled probes were processed through NICK columns (Pharmacia LKB, Uppsala, Sweden) to remove unincorporated isotope. DNA fragments used as probes for Southern and/or Northern hybridization are listed in Table 3.

9. Southern analysis

a) Preparation of yeast genomic DNA

Total yeast DNA was isolated essentially as described (Ausubel et al., 1991) with the modification that spheroplasts were prepared using a buffer containing 30 mM dithiothreitol (DTT) instead of β-mercaptoethanol. Briefly, yeast strains were inoculated into 5 ml of YM1 medium and grown on a rollerdrum to late log phase, and harvested by centrifugation. Cell pellets were resuspended in 0.5 ml of a sorbitol solution (0.9 M sorbitol, 0.1 M Tris, pH 8, and 0.1 M EDTA) containing 15 µl of 1 M DTT, and 50 µl of zymolyase or lyticase (0.3 mg/ml) was added. Cells were incubated on a rollerdrum at 37°C for 1 h to generate spheroplasts, which were then pelleted and resuspended in 0.5 ml of Tris-EDTA (50 mM Tris, pH 8 and 20 mM EDTA) and transferred to a microcentrifuge tube. 50 µl of 10% SDS was added and the contents were mixed by inversion and incubated for 20 min at 65°C to lyse spheroplasts. 200 µl of 5 M potassium acetate was added and mixed by inverting the tube. After a 40-min incubation on ice, the sample was spun down in a microcentrifuge for 3 min at room temperature. The supernatant was transferred to a fresh tube and mixed with 1 ml 95% ethanol to precipitate nucleic acids. After a 1-min centrifugation, the pellet was air dried and dissolved in 300 µl of TE buffer. The sample was then treated with RNase A for 1 h at 37°C and the DNA was precipitated by isopropanol and pelleted by a 1-min

Table 3. DNA probes used for Northern and Southern hybridization.

Gene	Description
ACT1	1-kbp *Hin*dIII-*Xho*I restriction fragment from pRS208 (Rowley et al., 1991).
CDC68	2.2-kbp internal *Hpa*I fragment of *CDC68* from p68-Ba-1A (Rowley et al., 1991)
CLB2	Linearized plasmid pCB1581 (B. Futcher).
CLB4	3.5-kbp *Eco*RV restriction fragment from pCLB4-NotI (B. Futcher).
CLN1	1.7-kbp *Nde*I-*Bam*HI restriction fragment from pRK-171-CLN1 (Rowley et al., 1991).
CLN2	1.8-kbp *Bam*HI restriction fragment from pUC19-CLN2 (C. Wittenberg).
CLN3	1.7-kbp *Eco*RI-*Xho*I restriction fragment from YRp-DAF1$^+$ (Rowley et al., 1991).
HTA1+ADK1	2.4-kbp *Sst*I restriction fragment from YIp5-TRT1 (M. Osley).
HTA1+HTB1+ADK1	linearized plasmid YIp5-TRT1 (M. Osley).
SAN1	3.5-kbp *Bam*HI-*Eco*RI restriction fragment from pBE3.
SIC1	2.7-kbp *Eco*RI-*Hin*dIII restriction fragment from pMDM143b (Nugroho and Mendenhall, 1994).
SUC2	1-kbp *Bam*HI-*Hin*dIII restriction fragment from pRB59 (F. Winston).
SUG1	1.7-kbp *Eco*RI-*Kpn*I restriction fragment from pXKE17.
SWE1	3.5-kbp *Pst*I-*Bam*HI restriction fragment from pSWE1-14 (Booher et al., 1993).
TUB2+YPT1	2-kb *Bam*HI restriction fragment from pBR1.129 (A. Wildeman).

centrifugation. The supernatant was removed and the pellet was resuspended in 50 µl of TE buffer. This method gives high-quality genomic DNA that is suitable for Southern analysis and PCR amplifications.

b) Southern analysis

Southern analysis was used to verify that a gene disruption or deletion had taken place after introducing an *in vitro* engineered version of a gene into the yeast genome. Restriction enzyme-digested yeast genomic DNA (about 1-10 µg) was resolved by agarose-gel electrophoresis (in the presence of ethidium bromide) as described above. DNA was transferred to Hybond nylon membrane (Amersham) by the method of Southern (1975). Briefly, after electrophoresis, the agarose gel was photographed on a UV transilluminator next to a ruler to facilitate size determination of restriction fragments after hybridization. The gel was then soaked in denaturation buffer (0.5 M NaOH and 1.5 M NaCl) for 2 x 20 min with constant shaking to denature double-stranded DNA. [Note: since the DNA fragments detected for this thesis work were all below 10 kbp in size, treatment with 0.25 M HCl (Southern, 1975) was omitted.]. The gel was then placed in neutralization buffer (0.5 M Tris-HCl, pH 7.5 and 1.5 M NaCl) for 2 x 20 min with constant shaking. After denaturation, DNA was transferred from the agarose gel to nylon membrane by the method of Southern (1975). The transfer buffer used was 20X SSC (3 M NaCl and 0.3 M sodium citrate). After transfer, the membrane was air-dried for 20 min, and DNA was cross-linked to the membrane using a UV-crosslinker (Model 2400, Stratagene).

Prior to hybridization, the membrane was incubated in prehybridization solution for at least 4 h to block nonspecific binding; prehybridization solution contained 0.05 M sodium phosphate (pH 6.5), 5X SSC, 50% de-ionized formamide, Denhardt mix (0.02% Ficoll 400, 0.02% polyvinylpyrrolidone, and 0.02% BSA), 1% SDS and sheared denatured salmon sperm DNA (0.2 mg/ml). Hybridization with a ^{32}P-labeled DNA

probe was carried out in prehybridization solution in a 42°C incubator for about 12 h. After hybridization, the membrane was washed to remove non-specific radioactivity as following: 2 x 10 min in 2X SSC and 1% SDS solution at the room temperature and then the washes of increased stringency using 0.1X SSC and 0.1% SDS at 62°C for 2 x 20 min. The membrane was sealed in plastic and exposed to X-ray film (either Kodak X-ray film or Amersham hyperfilm). After an overnight exposure, the film was processed using an X-omat processor. Different exposures were routinely obtained.

10. Cloning wild-type suppressor genes by complementation

a) The *SCA68/SAN1* suppressor gene

The wild-type *SCA68/SAN1* gene was cloned by taking advantage of the recessive Spt^+ phenotype caused by the *cdc68-1 san1-170* genotype. A YCp50-based genomic DNA library was transformed into the *cdc68-1 san1-170 his4-912δ lys2-128δ ura3-52* recipient yeast strain SUX1702 by the spheroplast method. His^+ Lys^+ Ura^+ transformants were selected by plating transformed yeast cells on synthetic medium lacking histidine, lysine, and uracil. His^+ Lys^+ and Ura^+ transformants were then tested for temperature sensitivity at 35°C by replica-plating.

A yeast cell can normally accommodate more than one type of plasmid, so after transformation with library DNA, a single transformant may contain several different plasmids. To determine which plasmid was responsible for complementation of the suppressor mutation, yeast plasmids were segregated from each other by passage through *E. coli* cells: only one type of plasmid is present in each *E. coli* transformant cell. To this end, plasmids were recovered from several His^+ Lys^+ and Ura^+ yeast transformants and were transformed into *E. coli* cells. Plasmids were then extracted from ampicillin-resistant *E. coli* transformants and restriction enzyme digestions were carried out for these plasmids. Plasmids that gave distinct restriction maps were individually re-introduced into the original recipient yeast strain SUX1702 to determine which plasmid

produced the His^+, Lys^+ and temperature sensitivity phenotype. A single library plasmid, pTD33, was uncovered that complemented the *san1-170* suppressor phenotype.

b) The *SCB68/SUG1* suppressor gene

To isolate the wild-type *SCB68/SUG1* gene, YCp50 genomic-library DNA was transformed into the *cdc68-1 sug1-26 lys2-128δ ura3-52* strain SUXB261 by the lithium acetate procedure (Ito et al., 1983). Ura^+ Lys^+ transformants were selected on synthetic medium lacking uracil and lysine, and were further tested for complementation of the *sug1-26* mutation by assessing growth at 35°C; failure to proliferate at this restrictive temperature suggests that the plasmid complements the suppression caused by the *sug1-26* mutation. Three distinct complementing plasmids were recovered from independent transformants. Restriction fragments of the 8-kbp insert of one plasmid, pXB68, were subcloned into the pRS316 vector (Sikorski and Hieter, 1989). The resultant plasmids pXES5, pXKS4, pXHH21, and pXKE17 were then tested for the ability to complement the *sug1-26* suppressor mutation.

To confirm that the 1.7-kbp *Eco*RI-*Kpn*I complementing sequence of pXKE17 contained the wild-type suppressor gene, this insert was cloned into the integrating *URA3* vector YIp352 (Hill et al., 1986). The resulting plasmid pIKE17 was linearized at the unique *Cla*I site within the insert and transformed into the *cdc68-1 sug1-26 lys2-128δ ura3-52* strain SUXB261 to generate the transformed strain XBI261, which was then mated with the *cdc68-1 lys2-128δ ura3-52* strain FYARQ1. The resultant diploid was sporulated and, for the 14 tetrads dissected, the Ura^+ (due to the *URA3*-marked plasmid) and Ura^- phenotypes segregated in a 2:2 fashion, suggesting that the plasmid was integrated. The Lys^+ and Lys^- phenotypes segregated in a 4:0 fashion, indicating that the cloned DNA fragment had directed plasmid integration at the chromosomal suppressor locus. Plasmid integration at the homologous suppressor locus was confirmed by Southern analysis.

11. Gene replacement in yeast

a) Construction and assessment of *san1Δ::URA3* null alleles

The 3.5-kbp *SAN1*-containing fragment from plasmid pBE3 was cloned into pUC19 (Yanisch-Perron et al., 1985), and the 2-kbp *Sac*I-*Eco*RV fragment within the insert, from the upstream region to position +1158 of the *SAN1* open reading frame, was replaced with a 1.1-kbp *Sma*I-*Hin*dIII fragment containing the *URA3* gene from YEp24 (Botstein et al., 1979). Insertion of *URA3* in both orientations generated two versions of *san1Δ::URA3* null alleles in plasmid pLG7 and pLG8. In separate experiments the genomic *SAN1* gene was replaced with each version of the *san1* null allele by a one-step gene replacement procedure (Rothstein, 1983). The 2.6-kbp *Eco*RI-*Bam*HI linear fragments of pLG7 and pLG8, each carrying a *san1Δ::URA3* null allele, were introduced into the *SAN1/SAN1 cdc68-1/CDC68 ura3-52/ura3-52* diploid strain QX300 to replace a genomic wild-type *SAN1* gene with the *URA3*-marked *san1* null allele. Southern hybridization verified that one genomic copy of the *SAN1* gene in the diploid transformant QX301 was replaced by the *san1Δ::URA3* null allele (Fig. 5). Effects of *san1Δ::URA3* null alleles were determined in haploid segregants; the two versions of the *san1Δ::URA3* null allele gave identical results.

b) Construction of *sug1* disruption alleles

A *LEU2*-marked *sug1* disruption allele was constructed in the following way: the 1.7-kbp *Eco*RI-*Kpn*I fragment encompassing the *SUG1* gene was cloned into plasmid pUC19, and the *LEU2* gene (*Nar*I-*Hpa*I fragment) from YEp351 was inserted into the unique *Cla*I site (within the *SUG1* open reading frame) by blunt-end ligation, deriving plasmid pXBS23. The 3.4-kbp *Pvu*II-*Bam*HI fragment of pXBS23 bearing the *sug1::LEU2* allele was transformed into the diploid yeast strain QXD2. Southern analysis verified that one genomic copy of the *SUG1* gene in each diploid transformant had been replaced by the *sug1::LEU2* disruption allele. A *sug1::URA3* allele was

similarly constructed by inserting the *URA3* gene (a 1.2-kbp *Sma*I-*Hpa*I fragment from YEp352) at the *Cla*I site, and the resultant plasmid was named pXBS24.

12. Analysis of yeast RNA

a) Extraction of RNA

Total RNA was isolated from yeast cells to determine transcript abundance under different environmental conditions. All the glassware and plastic for RNA isolation were baked overnight in a 121°C oven. Solutions were made in baked glassware and autoclaved for 30 min.

Yeast cells were grown to 0.5-1x10^7 cells/ml and harvested by centrifugation at 3,000 rpm for 2 min. Total RNA isolation was performed essentially as described by Penn et al., (1984). Cell pellets were resuspended in LET buffer (0.1 M LiCl, 10 mM EDTA, and 10 mM Tris-HCl, pH 7.0), frozen immediately in liquid nitrogen, and stored at -70°C. Samples were thawed on ice, SDS was added to a final concentration of 1%, and yeast cells were broken by vortexing with acid-washed glass beads in the presence of water-saturated phenol/chloroform (pH 5). Vortexing was performed by 4 cycles of 30 sec of vortexing plus 30 sec of cooling on ice. This treatment was followed by a 5-min period of constant vortexing. Samples were centrifuged at 12,000 rpm for 2 min, and the aqueous supernatant was transferred to a fresh tube and extracted with phenol/chloroform for a second time. The aqueous phase was precipitated with 95% ethanol at -20° for at least 2 h and RNA was pelleted by centrifugation in a microcentrifuge for 30 min at 4°C. Pellets were washed with 70% ethanol, air-dried and dissolved in ddH_2O.

RNA concentration was determined by absorbence at 260 nm (A_{260}). The A_{260}/A_{280} ratio was also determined to assess the quality of RNA, with values normally ranging from 1.9 to 2.2.

b) Northern blot analysis

Total yeast RNA was resolved by electrophoresis through a formaldehyde-containing agarose gel essentially as described by Sambrook et al. (1989). Before casting a gel, the buffer chamber, gel tray and comb were all soaked in 1% SDS for at least 2 h, and rinsed thoroughly with ddH_2O to remove residual SDS. The gel contained (in 200 ml) 2.6 g of agarose, 20 ml of 10X MOPS buffer (0.4 M 3-[N-morpholino] propane-sulfonic acid, 0.1 M sodium acetate, pH 5.2, and 0.01 M EDTA, pH 8.0), 37 ml of formaldehyde, and 7.5 µl of 10 mg/ml ethidium bromide. Each RNA sample contained 20 µg of total RNA, 50% freshly de-ionized formamide, 17.5% formaldehyde and 1X MOPS. The RNA mixture was denatured for 15 min at 65°C, and chilled on ice for 5 min before electrophoresis in 1X MOPS running buffer at 2 V/cm. The gel was then photographed on a UV transilluminator to assess equal loading of RNA samples based on intensity of rRNAs. The gel was rinsed briefly in 2X SSC and Northern transfer was performed in a similar way as described for the Southern transfer. After transfer, RNA was UV cross-linked to the nylon membrane.

Hybridization of the RNA-containing membrane with radiolabeled probes was carried out by following a similar procedure as for Southern hybridization, except that the washing conditions were different. The RNA-containing membrane was washed 2 x 10 min in 2X SSC and 1% SDS followed by washing in 0.1X SSC and 1% SDS for 20 min at 50°C. After hybridization and autoradiography, the hybridized DNA probe was removed by placing the membrane in boiling 0.1X SSC and 1% SDS solution for 2 x 20 min. The membrane was stored wet at -20°C, and reused multiple times to visualize different RNAs.

13. Nucleotide sequencing

a) Sequencing of single-stranded DNA

To obtain nucleotide sequence information about the *SAN1* and *SUG1* suppressor genes, restriction fragments were cloned into the phage vectors M13 mp18 and M13 mp19 (Yanisch-Perron et al., 1985) using standard molecular cloning techniques as described above. To obtain *SAN1* sequence the DNA insert of pBE3 was transferred to M13 mp18 or M13 mp19: the 1.3-kbp *Hin*dIII-*Pst*I fragment was cloned into M13 mp19, and the 1.2-kbp *Sac*I-*Kpn*I fragment and the 3.5-kbp *Xba*I-*Sal*I fragment were cloned into M13 mp18. To obtain *SUG1* sequence the 1.7-kbp *Eco*RI-*Kpn*I fragment from pXKE17 was cloned into M13 mp19.

To isolate single-strand phage DNA, *E. coli* cells were first removed from the culture by centrifugation. 1 ml of the supernatant was transferred to a fresh microcentrifuge tube containing 300 µl of 20% PEG (8,000) and 3.5 M ammonium acetate, and single-strand phage DNA was precipitated by a 30-min incubation on ice followed by centrifugation in a microcentrifuge for 10 min. The pellet was resuspended in 150 µl of TE buffer by vortexing. After spinning in a microcentrifuge for 10 min to remove any insoluble contaminants, the phage DNA was extracted with phenol/chloroform and precipitated by ethanol. DNA was resuspended in 20 µl of ddH_2O, and quantified by determination of A_{260}. Normally 5-7 µl of single-strand DNA was used for each sequencing reaction.

Nucleotide sequence analysis was performed using the chain-termination sequencing method as described by Sanger et al. (1977). Sequencing reactions were carried out using the Sequenase version 2.0 kit according to the manufacturer's instructions (United States Biochemical Company, Cleveland, OH). [^{35}S]dATP was used for all sequencing reactions. After chain termination, sequencing reaction mixtures were resolved by denaturing polyacrylamide (6%) gel electrophoresis. The sequencing gel was then fixed in 10% methanol and 10% acetic acid for 30 min to remove urea, and dried on a gel dryer (Model 583, Bio-Rad) for 2 h. Sequence ladders were recorded by autoradiography using Kodak X-ray film.

b) Sequencing of double-stranded DNA

To sequence PCR-amplified mutant versions of the *SUG1* gene, the PCR amplified products were cloned into the pRS316 vector and double-stranded sequencing was carried out. Plasmid DNA was prepared using the alkaline lysis procedure, about 2 µg of plasmid DNA was denatured by incubating with 0.1 M NaOH, 0.05 M EDTA at 85°C for 5 min, and neutralized by adding 0.3 M sodium acetate (pH 4.8). Denatured DNA was ethanol-precipitated and re-dissolved in 7 µl of ddH_2O. 2 µl of 5X reaction buffer (from the Sequenase kit), and 1 µl of primer (25 ng) was added to the DNA. The annealing was achieved by an incubation at 37°C for 30 min followed by 10 min at room temperature. Sequencing reactions were carried out as described for sequencing single-strand DNA.

c) Computer analysis of nucleotide sequence data

Nucleotide sequence was recorded using the Seqspeak program for the Apple Macintosh computer (developed by Keith Conover, Dalhousie University, Halifax, Nova Scotia). Sequence analysis, including generating restriction maps and predicting possible open reading frames, was performed using the DNA Strider 1.1 program (Marck, 1988). Sequence comparison within the Genbank database was performed using the FASTA and BLAST programs (Pearson, 1990; Lipman et al., 1990).

14. Epitope tagging

a) Tagging the *CDC68* gene with an HA-epitope sequence

The HA epitope contains nine amino acids (YPYDVPDYA) from the influenza hemagglutinin, and three tandem HA epitopes were used here to increase sensitivity of the tagged protein to anti-HA antibody. A triple HA-epitope cassette (Field et al., 1988) was inserted into the *CDC68* open reading frame (ORF) at an N-terminal position in the

6th codon, at an engineered *Cla*I site (The *Cla*I-site-bearing *CDC68* gene is a gift from D. Evans). *Cla*I sites were added to both ends of the HA-epitope sequence by PCR amplification using plasmid pGTEP1 (a gift from B. Futcher) as the template. PCR primers used were 5'-GCATCGATGCACTGAGCAGCGTAATCTGGA, and 5'-CCATCGATGGCCGCATCTTTTACCCATACG (*Cla*I sites are underlined.). The PCR reaction was carried out as described above. The amplified DNA was gel-purified, digested with the restriction enzyme *Cla*I, and inserted into the 5' region of the *CDC68* gene at the *Cla*I site, generating plasmid pXHA681. Nucleotide sequencing of the ligation junctions verified that the epitope sequence was inserted in the correct orientation and reading frame. The HA-tagged *CDC68* gene, termed *CDC68-200N*, was subsequently cloned into the *TRP1*-marked integrating vector pRS304 (Sikorski and Hieter, 1989), generating plasmid pXHA682.

To determine whether HA-epitope addition affected Cdc68 protein function, plasmid pXHA681 was introduced into the *cdc68-101::LEU2/CDC68* diploid strain BM64 and the diploid transformant, BM641, was sporulated to assess the activity of high-copy *CDC68-200N*. Function of single-copy *CDC68-200N* was also assessed by a plasmid-shuffling procedure (Boeke et al., 1987). A Ura^+ Leu^+ meiotic segregant of BM641 (strain QX6810) was transformed with pXHA682, and plasmid pXHA681 was lost from this transformant by 5-FOA counterselection (Boeke et al., 1984). The resulting strain QX6811 contains the HA-tagged *CDC68* gene integrated at the genomic *cdc68-101::LEU2* disruption locus. A haploid *sug1-26 cdc68-101::LEU2 [CDC68-200N TRP1]* strain was constructed by mating strains QX6811 and QX261 (*sug1-26*) and selecting for a Leu^+ Trp^+ temperature-sensitive (*sug1-26*) segregant, strain QX2614.

b) Epitope-tagging the mutant Cdc68 polypeptide

The polypeptide encoded by the *cdc68-1* allele was epitope-tagged by a multi-step restriction-fragment swap. The *Kpn*I-*Eag*I restriction fragment of YEpDE68-1 (bearing

cdc68-1) was cloned into the bluescript vector pBSIIKS$^+$ (Stratagene) generating pKS68-1, and the 0.7-kbp *Spe*I fragment, which does not harbor the *cdc68-1* basepair substitution (D. R. H. Evans, personal communication), was replaced with the similar *Spe*I fragment of *CDC68-200N* (harboring the HA tag), generating pKSHA68-1. The HA-tagged *Kpn*I-*Eag*I fragment from pKSHA68-1 was then used to replace its counterpart in YEpDE68-1, generating pXHA68-1. The HA-tagged *cdc68-1* allele, *cdc68-201N*, was also cloned into the integrating vector YIp352 (Hill et al., 1986), generating pXIHA68-1, which was then linearized and transformed into strains 68507A (*cdc68-1*) and QXT26 (*cdc68-1 sug1-26*). The transformed QXT26 was then mated with strain YJOZS (*sug1-1*) and a *cdc68-1[cdc68-101N] sug1-1* segregant was chosen (strain QXT101).

15. Assessment of plasmid linking number

To determine whether the *cdc68-1* mutation affects plasmid linking number, the pRS316 vector was introduced into a *cdc68-1* strain 68507A and a congenic *CDC68* wild-type strain 21R. Total DNA was prepared using the spheroplast method and resolved on a 0.8% agarose gel containing 2.5 µg/ml chloroquine (Sigma; Han et al., 1987) without ethidium bromide. DNA was transferred to nylon membrane and hybridized with ^{32}P-labeled pRS316 DNA.

16. Analysis of yeast protein

a) Preparation of whole-cell extracts

S. cerevisiae cells were grown to early log phase and whole cell extracts were prepared at 4°C using glass beads essentially as described (Ausubel et al., 1991), except that cells were frozen in liquid nitrogen before lysis. Yeast cells were harvested by centrifugation, washed once in ddH_2O, resuspended in breaking buffer (20 mM Tris, pH 8.0, 10 mM $MgCl_2$, 1 mM EDTA, 5% glycerol, 1 mM DTT, 0.3 M ammonium sulfate, 100 mM NaCl), frozen immediately in liquid nitrogen, thawed on ice and broken by 8 x 1

min vortexing in an equal volume of acid-washed glass beads in a cold room. The protease inhibitors chymostatin, aprotinin, pepstatin A (2 μg/ml each), leupeptin (0.5 μg/ml), and PMSF (1 mM) were included in the extraction buffer. Supernatants were pooled and cleared by centrifugation at 12,000 rpm for 20 min, and protein concentrations were determined by the Bradford method (Bradford, 1976). Protein samples were boiled for 4 min immediately after mixing with appropriate volumes of 6X SDS sample buffer (0.35 M Tris pH 6.8, 36% glycerol, 10% SDS, 60 mM DTT, 0.012% bromophenol blue) to solubilize protein and to inactivate proteases. Samples were stored at -20°C.

To assess stability of Cdc68 wild-type and mutant proteins, yeast cells harboring HA-tagged Cdc68 protein were grown at 23°C to 10^7 cells/ml, 30-ml samples were removed at different times after the addition of cycloheximide (0.5 mg/ml) and transfer to 35°C (or continued incubation at 23°C). Cycloheximide was used to inhibit further protein synthesis so that protein levels detected by immunoblot analysis reflected the levels of preexisting proteins; the inhibition of protein synthesis was confirmed for each set of experiments.

b) SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Total cellular protein was resolved by molecular weight during one-dimensional gel electrophoresis under denaturing conditions (in the presence of 0.1% SDS) essentially as described (Ausubel et al., 1991), using the BioRad mini-gel apparatus. The separating gel (7.5% acrylamide) was prepared by combining 2.5 ml of 30% Acrylamide/Bis (40:1), 2.5 ml of 1.5 M Tris (pH 8.8), 0.1 ml of 10% SDS and 4.85 ml of ddH_2O and degassing this mixture for 10 min. 50 μl of 10% ammonium persulfate (freshly prepared) and 5 μl of TEMED was added to the degassed solution just before casting the gel. A layer of 0.1% SDS was applied to the top of the separating gel, which was allowed to set for 45 min. The SDS layer was then poured off, and a stacking gel (4% acrylamide) was cast on

top of the separating gel. For each set of experiments, equal amounts (between 5 to 20 µg) of protein extracts were loaded. The rainbow-color protein size markers (Amersham) were run side by side with protein samples to facilitate molecular weight determination. The SDS mini-gel was run at 200V for about 45 min until the bromophenol blue dye ran off the bottom of the gel.

c) Immunoblot analysis

After resolution by SDS-PAGE, proteins were transferred to PVDF (polyvinylidene difluoride) membrane for specific protein detection by immunological analysis. The protein gel was first equilibrated in Towbin transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol, 0.1% SDS, pH 8.3) for 20 min. The PVDF membrane was cut to the size of the gel and wet with methanol, followed by equilibration in Towbin buffer for 10 min. Protein transfer was carried out using a semi-dry trans-blot apparatus (BioRad) following the manufacturer's instructions.

Blots (protein bound PVDF membranes) were probed with specific monoclonal or polyclonal antibodies to identify antigenic proteins. The blots were immersed in 5% Blotto for 2 h at room temperature (with constant shaking) to fill protein binding sites with non-reactive protein; the 5% Blotto contained 5% skim-milk powder and 0.5% casein in TBST (10 mM Tris, pH 8.0, 0.88% NaCl, 0.1% Tween 20). Blots were then incubated with a primary antibody (diluted in 1% Blotto) for 45-60 min followed by washing 3 x 15 min in TBST buffer. For the anti-HA monoclonal antibody 12CA5 (1 µg/ml), the dilution was 1:10,000, and for the monoclonal anti-tubulin antibody, the dilution was 1:500. The Sug1 antiserum was used at 1:2000 dilution and the anti-T7 S10 antibody was used at a 1:10,000 dilution. The membrane was then incubated for 45 min with a peroxidase-conjugated secondary antibody specific for the primary antibody, used at a 1:20,000 dilution, and washed in TBST buffer for 3 x 15 min. These secondary

antibodies were identified using the ECL (enhanced chemiluminesence) system (Amersham), and results were recorded on Amersham hyper film.

17. Co-immunoprecipitation

To detect a possible physical interaction between the Cdc68 and Sug1 proteins, a co-immunoprecipitation procedure was adopted. A yeast strain (QX6800) was constructed in which the only functional *CDC68* and *SUG1* genes are fused to different epitope sequences: the *CDC68* gene was tagged with the HA-epitope sequence and the *SUG1* gene was tagged with the T7 S10 epitope sequence. Another strain, QX6801, constructed as a control strain, contains the T7 S10-tagged Sug1 but untagged Cdc68. Cells of strains QX6800 and QX6801 were grown at 23°C to early log phase and whole-cell extracts were prepared as described above. *Staphylococcus aureus* cells used to precipitate antigen-antibody complexes were pretreated by boiling in 3% SDS and 10% β-mercaptoethanol for 30 min; this treatment was found to decrease background significantly. Prior to immunoprecipitation, each extract was incubated with *S. aureus* cells at 4°C for 2 h to eliminate non-specific binding of proteins to protein A on the surface of *S. aureus* cells. These treated cell extracts were incubated with anti-HA polyclonal antibodies (BABCO, Berkeley, CA) on a 4°C shaker overnight to allow sufficient antigen-antibody interaction. *S. aureus* cells were then added to the antibody-extract mixture and incubated for another 2 h on a 4°C shaker. Cells were pelleted and washed three times with NET-0.05%NP buffer (150 mM NaCl, 5 mM EDTA, 50 mM Tris pH 7.4, and 0.05% Nonidet P40). After the final wash, SDS sample buffer was added to the *S. aureus* cell pellets and boiled for 5 min to release antibodies and antigens from the cell surface. Cells were then resuspended and repelleted in microcentrifuge for 1 min. Immunoprecipitates (supernatants containing solubilized antigens and antibodies) were subjected to SDS-PAGE and immunoblot analysis as described above.

18. The two-hybrid interaction assay

The two-hybrid interaction assay (Chien et al., 1991) was employed to detect a possible physical interaction between the Cdc68 and Sug1 proteins. *Bam*HI restriction sites were added to both ends of the complete *CDC68* ORF and complete *SUG1* ORF by PCR amplification. The amplified *CDC68* DNA was purified, digested with the restriction enzyme *Bam*HI, and fused in frame to the LexA DNA-binding domain sequence at the *Bam*HI site in plasmid pSH2-1, generating plasmid pBD-CDC68. Similarly, the amplified *SUG1* DNA was purified, digested with *Bam*HI, and fused in frame to the Gal4 activation domain sequence at the *Bam*HI site in plasmid pGAD3, generating plasmid pBD-SUG1. Plasmid pBD-CDC68 was introduced into a *cdc68-101::LEU2/CDC68* diploid strain. Spore viability was assessed after sporulation and tetrad analysis to determine if the LexA-Cdc68 fusion protein can provide sufficient Cdc68 activity to alleviate the lethality of *cdc68-101::LEU2* (Malone et al., 1991). Plasmid pAD-SUG1 was introduced into a *sug1-26* temperature sensitive strain to determine if the Gal4-Sug1 fusion protein can perform Sug1 function to allow *sug1-26* cells to grow at 37°C.

For the two-hybrid assay, several plasmid pairs, including pBD-CDC68 and pAD-SUG1, pSH2-1 and pAD-SUG1, pBD-CDC68 and pGAD3, were co-transformed into the yeast strain CTY10-5d that contains a *LexA-GAL1-LacZ* reporter gene (a fusion of *LexA* operator, *GAL1* basal promoter, and the *LacZ* gene). Transformants were grown in liquid SC-His, Leu medium (Synthetic medium lacking histidine and leucine) to maintain plasmids, and β-galactosidase assay was performed for permeabilized cells as described (Ausubel et al., 1991).

III. RESULTS

Section A. Genetic analysis of *cdc68-1* suppressor mutations

S. cerevisiae cells harboring the *cdc68-1* mutation are conditionally impaired for growth; at or above 35°C, *cdc68-1* mutant cells cease proliferation and arrest as large unbudded cells (Prendergast et al., 1990), a morphology characteristic of cells that have failed to perform the cell-cycle regulatory step termed START (Pringle and Hartwell, 1981). Studies described in this thesis were initiated by searching for suppressor mutations that alleviate the *cdc68-1* temperature sensitivity. Identification of suppressor genes and assessment of the interactions between Cdc68 and suppressor proteins will provide insight into the function of the Cdc68 protein itself. Data of this section appeared in Xu et al. (1993).

1. Mutations that suppress *cdc68-1* temperature sensitivity were identified.

Spontaneous mutations that allowed *cdc68-1* mutant cells to proliferate at the restrictive temperature of 35°C were isolated. Initially, cells of a yeast strain harboring the *cdc68-1* mutation were grown in several independent cultures to stationary phase, and a portion of each stationary-phase culture (about $5x10^7$ cells) was spread on YEPD solid medium and incubated at 35°C. After 2 to 3 days, between 50 to 100 colonies formed on each plate at 35°C. To avoid characterizing multiple isolates of the same mutational event, a single temperature-resistant colony from each stationary-phase culture was chosen as a candidate suppressed strain. Temperature resistance of these isolates was verified by replica-plating and incubation at 35°C. A total of 45 independent temperature-resistant mutants were chosen in this way for further genetic analysis.

To determine whether suppression was due to single or multiple mutations, cells of each independently derived temperature-resistant mutant were mated with cells of

another *cdc68-1* mutant strain, ARI68-7. The resulting diploid cells were sporulated, and meiotic segregants were examined for each sporulated diploid strain. For 35 of the 45 diploid strains, temperature sensitivity segregated in a 2:2 fashion, indicating that suppression of temperature sensitivity in these 35 suppressor strains is due to mutation of a single nuclear gene. For the remaining 10 diploid strains, temperature sensitivity segregated in a 3:1 or 4:0 fashion in some tetrads, indicating that suppression in these strains is due to more than one mutation. These multigenic suppressor strains were not studied further. In this way, I assembled a collection of 35 mutations that each suppresses the 35°C temperature sensitivity caused by the *cdc68-1* allele.

In general, suppressor mutations can occur within the mutant *cdc68* gene itself (intragenic suppressors) or in genes other than *cdc68* (extragenic suppressors). To address the question of whether suppressor mutations represented additional mutations at the *cdc68* locus or extragenic events, the segregation pattern of each suppressor mutation was compared with that of the *cdc68-1* allele. For this purpose, each suppressor mutant was mated with a yeast strain that carried a marker gene, the wild-type *URA3* gene, integrated next to the chromosomal *cdc68-1* locus. If a suppressor mutation is within the *cdc68-1* locus, the temperature-resistance phenotype should cosegregate with the unmarked *cdc68-1* allele (Ura^-). In other words, tetrads with parental ditypes (PD) should be seen more often than those with non-parental ditypes (NPD). On the other hand, equal numbers of PD and NPD tetrads would indicate that the suppressor mutation is unlinked to the *cdc68* locus and therefore defines an unlinked gene. This type of linkage analysis showed that 7 suppressor mutations most likely affect the *cdc68* locus, as demonstrated by a majority of PD tetrads compared to NPD tetrads. Although these intragenic mutations will be useful to the investigation of structure-function relationships of the Cdc68 protein, they were not pursued further in this study. For the remaining 28 independent suppressed strains, the suppressor mutation is unlinked to the *cdc68* locus. Thus, these 28 suppressor mutations are extragenic events, and were subjected to further

investigation. Thus, 28 extragenic suppressor mutations were isolated that each allowed *cdc68-1* mutant cells to proliferate at 35°C.

To assess dominance/recessiveness of the 28 extragenic suppressor mutations, diploid strains homozygous for the *cdc68-1* allele and heterozygous for suppressor mutations were constructed and growth of these diploid cells was examined at 35°C. This analysis showed that 8 suppressor mutations are recessive, because diploid cells heterozygous for a suppressor mutation in this group did not grow at 35°C; the remaining 20 mutations are codominant because intermediate levels of temperature resistance were observed (Table 4).

2. Four genes were defined.

To carry out its normal function the Cdc68 protein may interact with more than one protein. Therefore suppressor mutations may occur in different genes that each can compensate for the *cdc68-1* defect. To determine the number of genes represented by the suppressor mutations identified here, standard complementation tests were carried out for each recessive suppressor mutation, and allelism tests were performed for each codominant suppressor. For the 8 recessive suppressor mutations, diploid cells resulting from pairwise matings between haploid suppressor mutant strains were assessed for growth at the restrictive temperature of 35°C. Growth (suppression) indicates failure to complement and suggests that the two suppressor mutations reside in the same gene. On the other hand, failure to grow (no suppression) indicates complementation and suggests that the two recessive suppressor mutations are in different genes. For the 20 codominant suppressor mutations, diploid strains resulting from matings between two haploid suppressor strains were sporulated and meiotic segregants were assessed for growth at 35°C. Suppressor mutations that are allelic (in the same gene) will cause all meiotic products to be temperature resistant, whereas segregation of suppressor mutations that reside in different genes will give rise to temperature-sensitive segregants. These

complementation and allelelism tests assigned the 28 suppressor mutations to four genes, provisionally named *SCA68*, *SCB68*, *SCC68* and *SCD68* (Table 4). As shown below, the *SCA68* gene is the same as *SAN1* (Schnell et al., 1989), and *SCB68* is identical to *SUG1* (Swaffield et al., 1992). For clarity, the *SAN1* and *SUG1* gene designations are therefore used throughout this thesis. Thus, at least four genes can be altered to overcome the *cdc68-1* defect at the restrictive temperature of 35°C.

3. *cdc68-1* suppressors isolated at 37°C are all intragenic.

With one exception (see below), none of the extragenic suppressor mutations isolated at 35°C alleviates *cdc68-1* temperature sensitivity at a higher restrictive temperature of 37°C (Table 4). I therefore isolated suppressors of *cdc68-1* temperature sensitivity specifically at 37°C; five independent suppressor mutants were recovered. Genetic analyses similar to those described above were carried out for these suppressor mutants. Interestingly, all five suppressor mutations were shown to be intragenic revertants, changing the *cdc68-1* mutant gene itself. Consistent with the intragenic nature of the suppressor mutants, the temperature-resistance phenotype in each case was dominant. Temperature resistance of these suppressor mutants could be caused either by conversion of the *cdc68-1* allele to the wild-type *CDC68* gene, or by an additional compensatory mutation in the *cdc68-1* locus. The fact that suppressor mutations occurred at a lower frequency at 37°C than at 35°C, coupled with the finding that all the 37°C suppressor mutations are intragenic, suggest that defects caused by the *cdc68-1* mutation at 37°C are too detrimental to be compensated for by mutation in another gene (see below).

Table 4. Growth phenotypes of suppressor alleles

Suppressor gene	Number of alleles		Suppression of *cdc68-1*	
	recessive	co-dominant	35°C	37°C
SCA68/SAN1	2	18	+	-
SCB68/SUG1	4	2	+	-
SCC68	1	0	+	-
SCD68	1	0	+	-

Section B. Characterization of *SAN1* and interactions between Cdc68 and San1

Data presented in this section appeared in Xu et al. (1993).

1. Phenotypic characterization of *san1* suppressor mutants.

a) *san1* alleles allow different levels of suppression.

During the initial suppressor screening, 20 spontaneous mutations were identified in the *SCA68/SAN1* suppressor gene (Table 4). These 20 *san1* alleles suppressed the *cdc68-1* mutation to different degrees on solid medium (data not shown). To further compare levels of suppression caused by these *san1* alleles, two suppressed strains, QX3 and QX170, that displayed seemingly different levels of suppression were chosen to compare growth rates of cells in liquid medium. As shown in Fig. 1, at 35°C cells of strain QX3 (*san1-3 cdc68-1*) had a similar doubling time as that of wild-type cells, whereas cells of strain QX170 (*san1-170 cdc68-1*) had a much longer doubling time. Therefore, *san1-3* is a "strong" suppressor allele, whereas *san1-170* is a "weak" suppressor allele. For most studies described in this thesis, these two *san1* mutations were used to exemplify both strong and weak suppression.

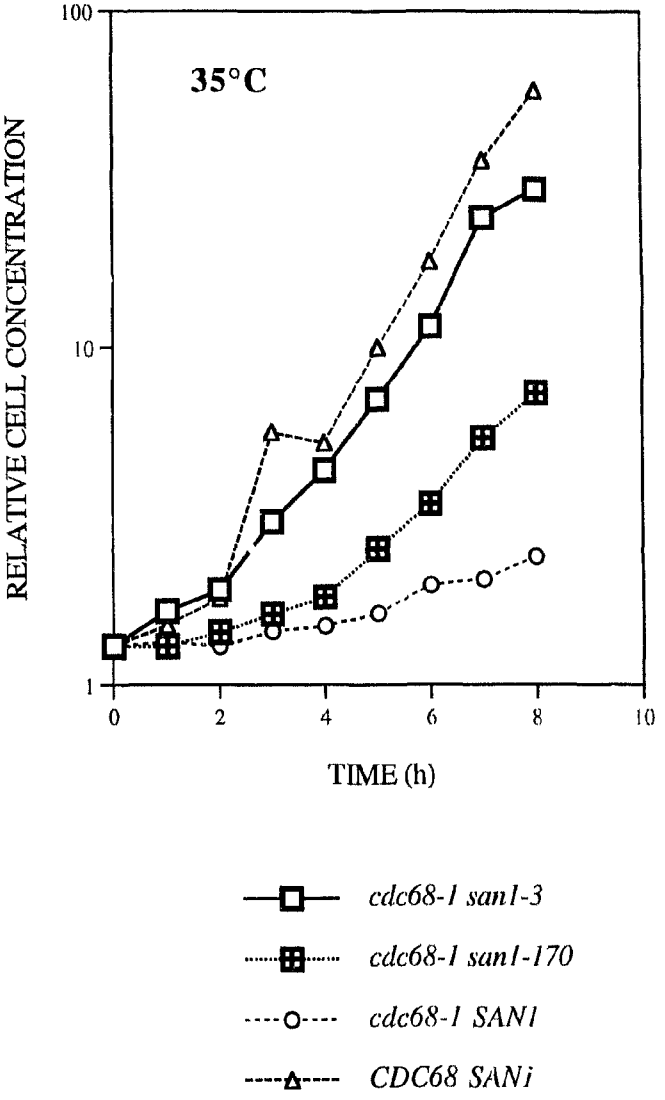
b) *san1* suppresses the *cdc68-197* mutation.

cdc68-197 is another temperature-sensitive allele generated by *in vitro* mutagenesis of the wild-type *CDC68* gene (Malone et al., 1991). To determine whether *san1* suppresses this *cdc68-197* allele, a *cdc68-1 san1-3* strain was mated to a *cdc68-197* strain and cells of the resultant diploid strain were sporulated. In each dissected ascus, temperature resistance and temperature sensitivity segregated in a 2:2 fashion, suggesting that *san1-3* suppresses both the *cdc68-1* and *cdc68-197* mutations (data not shown). By a similar analysis, the weak *san1-170* allele was also shown to be able to suppress the *cdc68-197* mutation. After the above experiments were conducted, related studies in our lab showed that *cdc68-1* and *cdc68-197* carry the same mutation that confers temperature sensitivity: both *cdc68-1* and *cdc68-197* alleles encode a Gly132-to-Asp substitution in

Figure 1. Different degrees of suppression by *san1* alleles.

Cells were grown in YM1 liquid medium to early-log phase and transferred to 35°C for further incubation. Samples were removed at hourly intervals for determination of cell concentration as described in the Materials and Methods. Yeast strains used were 21R (*CDC68 SAN1*), 68507A (*cdc68-1*), QX3 (*cdc68-1 san1-3*), and QX170 (*cdc68-1 san1-170*).

Figure 1


35°C
RELATIVE CELL CONCENTRATION
100
10
1
0
2
4
6
8
10
TIME (h)
cdc68-1 san1-3
cdc68-1 san1-170
cdc68-1 SAN1
CDC68 SAN1

the Cdc68 protein (D. R. H. Evans, personal communication). However, there may be a second modifying mutation within or linked to either the *cdc68-1* or *cdc68-197* locus, because phenotypically the *cdc68-197* appears to be less impaired than *cdc68-1*. In any case, *san1* suppressor mutations reverse temperature sensitivity of both the *cdc68-1* and *cdc68-197* alleles.

c) *san1* reverses the Spt⁻ phenotype caused by *cdc68*.

In addition to temperature sensitivity, the *cdc68-1* mutation also affects Ty-insertion or δ-insertion mutations. Ty elements are retrovirus-like transposons in *S. cerevisiae*. The long terminal repeat of a Ty element (termed δ sequence) carries its own promoter and terminator for transcription, and insertion of Ty or a solo-δ sequence in the 5' region of a yeast gene often alters the usage of transcription initiation or termination sites and results in defective gene function. Either increased copy number of the *CDC68* gene or a *cdc68* mutation can suppress the effects of δ insertions, a phenotype called Spt⁻ (Clark-Adams et al., 1988; Malone et al., 1991). δ insertions in the 5' regions of the *HIS4* and *LYS2* genes abolish normal transcription of these two genes so that cells bearing the *his4-912δ* and *lys2-128δ* insertional mutations cannot grow without added histidine and lysine (Winston et al., 1984; Clark-Adams and Winston, 1987). Both the *cdc68-1* and *cdc68-197* mutations confer an Spt⁻ phenotype and allow *his4-912δ lys2-128δ* cells to proliferate in the absence of supplementary histidine or lysine at a permissive temperature of 30°C (Malone et al., 1991; Rowley et al., 1991). To determine whether the *san1* suppressor mutations identified here reverse the Spt⁻ phenotype caused by a *cdc68* mutation, a *cdc68-1 his4-912δ lys2-128δ* mutant strain was mated with a *cdc68-1 san1* strain, and meiotic segregants were assessed for temperature sensitivity and growth in the absence of histidine or lysine. Results of this analysis indicated that both the *san1-3* and *san1-170* mutations can reverse the Spt⁻ phenotype caused by the *cdc68-1* mutation; in addition to being temperature-resistant at 35°C, *cdc68-1 san1 his4-912δ lys2-128δ* cells

did not grow in the absence of added histidine or lysine at 30°C (Fig. 2). In contrast, diploid cells homozygous for *his4-912δ, lys2-128δ* and *cdc68-1*, but heterozygous at the *san1* locus (*SAN1/san1*), were able to grow without added histidine and lysine, suggesting that for the Spt⁻ phenotype, as for temperature sensitivity, suppression by *san1* is also recessive. By a similar analysis, *san1* mutations were found to suppress the Spt⁻ phenotype conferred by the *cdc68-197* allele (data not shown).

Overexpression of the *CDC68* gene on a high-copy plasmid also confers an Spt⁻ phenotype (Clark-Adams et al., 1988). I therefore assessed the ability of *san1* suppressor mutations to reverse this Spt⁻ phenotype caused by increased *CDC68* copy number. A *his4-912δ lys2-128δ* strain was transformed with plasmid p68-Ba-1A (harboring the *CDC68* gene on a high-copy episomal plasmid). The resultant transformed strain was mated with a *cdc68-1 san1-170* strain and the diploid cells were sporulated. Because of the presence of the high-copy *CDC68* plasmid in each meiotic segregant, occurrence of His⁻ or Lys⁻ segregants would indicate that the *san1* mutation suppresses the Spt⁻ phenotype caused by increased *CDC68* gene dosage. Otherwise, every segregant should be His^+ and Lys^+ due to suppression caused by increased *CDC68* copy number. In 14 tetrads examined, there were His⁻ and Lys⁻ segregants detected, indicating that the *san1-170* allele reverses the Spt⁻ phenotype caused by overexpression of the wild-type *CDC68* gene. Thus, a *san1* suppressor mutation can reverse the Spt⁻ phenotype resulting from either a *cdc68* mutation or increased *CDC68* gene dosage.

d) *san1* mutations prevent the transcription of *suc2ΔUAS* that is allowed by a *cdc68* mutation.

The *cdc68-197* mutation was shown to cause another transcriptional alteration related to the expression of the *SUC2* gene required for sucrose fermentation. Cells carrying a *UAS*-less *suc2* gene (*suc2ΔUAS*) cannot transcribe this gene and hence can not

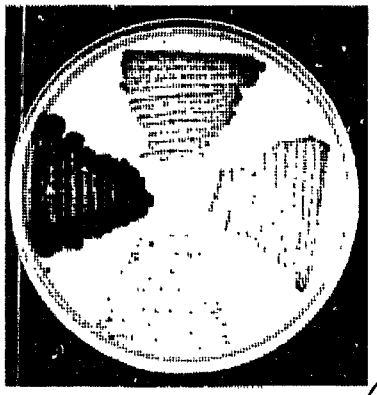
Figure 2. Suppression of temperature sensitivity and Spt$^-$ phenotype of *cdc68-1*.

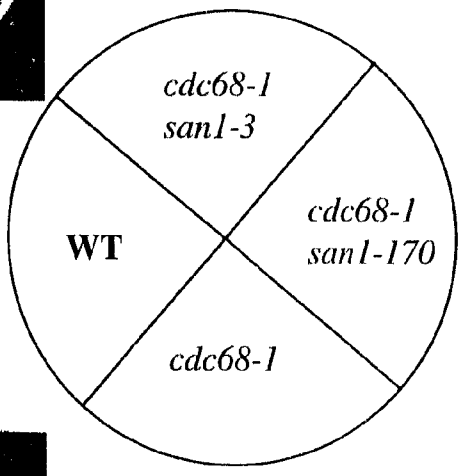
All strains harbor the *lys2-128δ* mutation. Cells were spread on YEPD solid medium, incubated at 23°C, and then replica-plated to YEPD medium for further incubation at 35°C (A) and to SC-Lys medium (SC medium lacking lysine) for further incubation at 30°C to assess Spt$^-$ phenotype (B). Strains used were FY56 (wild-type, WT), FYARQ1 (*cdc68-1*), QX3 (*cdc68-1 san1-3*), and QX170 (*cdc68-1 san1-170*).

Figure 2

A

YEPD 35°C





B

-LYS 30°C

grow in sucrose medium (Sarokin and Carlson, 1984). The *cdc68-197* mutation reverses this Suc$^-$ phenotype by allowing the *SUC2* gene to be transcribed in the absence of the *UAS* (Malone et al., 1991). By crossing a *cdc68-197 suc2ΔUAS* strain with a *cdc68-1* strain I found that, like *cdc68-197*, the *cdc68-1* allele also suppresses the effects of the *suc2ΔUAS* mutation, allowing *suc2ΔUAS* cells to utilize sucrose and display a Suc$^+$ phenotype. To determine whether *san1* suppressor mutations reverse this transcriptional effect of *cdc68*, a *cdc68-1 san1-170 SUC2* strain was mated with a *cdc68-197 SAN1 suc2ΔUAS* strain. Cells of the resultant diploid strain were sporulated and the phenotype of the meiotic products was assessed. Since each meiotic segregant harbors a *cdc68* mutation, all the spores should be able to grow in sucrose-based medium at 30°C (a permissive temperature for *cdc68* mutations) regardless of the nature of the *suc2* locus (either *SUC2* or *suc2ΔUAS*), unless the *san1-170* mutation can reverse the effect of the *cdc68-1* or *cdc68-197* mutation, in which case some segregants will once again become Suc$^-$. Of 10 tetrads dissected, 6 displayed a 3 Suc$^+$:1 Suc$^-$ segregation pattern, 3 were 2:2, and 1 was 4:0. Further genetic analysis confirmed that those Suc$^-$ segregants harbored the *san1-170* mutation. In a similar analysis, the "strong" suppressor allele, *san1-3*, was also shown to have an effect similar to that of the *san1-170* allele. Reversal of the Suc$^+$ phenotype by different *san1* alleles is shown in Fig. 3. Therefore *san1* mutations suppress the transcriptional alteration at the *suc2ΔUAS* locus that is caused by *cdc68*.

2. Cloning and identification of the *SAN1* suppressor gene.

a) *SAN1 (SCA68)* was cloned by complementation.

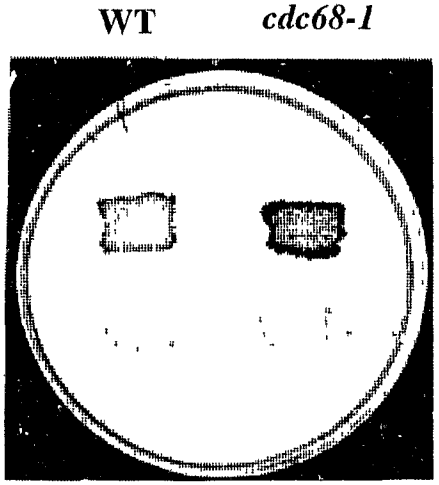
The *cdc68-1 san1* suppressed cells can proliferate at the restrictive temperature of 35°C, but the *san1* suppressor mutations are recessive, so that introduction of the wild-type suppressor gene was expected to complement the suppressor mutation and render

cells once again temperature sensitive. Therefore I expected that a direct selection to clone the suppressor gene cannot be carried out on the basis of temperature-dependent

Figure 3. Reversal by *san1* suppressor mutations of the Suc^+ phenotype caused by *cdc68*.

All strains harbor the *suc2ΔUAS(-1900/-390)* allele. Cells with indicated genotypes were patched onto YEPD solid medium, replica-plated to rich medium containing 2% sucrose and 0.1% antimycin A, and incubated at 33°C for two days. WT, wild-type.

Figure 3



growth. To circumvent this difficulty, the Spt phenotype was exploited to clone the wild-type suppressor gene by complementation. As described above, *san1* suppressor mutations reverse both the temperature sensitivity and the Spt$^-$ phenotype caused by the *cdc68-1* mutation so that cells of a *san1-170 cdc68-1 his4-912δ lys2-128δ ura3-52* strain (SUX1702) display a His$^-$ Lys$^-$ and temperature-resistant phenotype. This suppression is recessive, so that a transformant containing the wild-type suppressor gene should become His$^+$ Lys$^+$ at 30°C. A YCp50-based genomic library DNA was introduced into the recipient yeast strain SUX1702, and His$^+$ Lys$^+$ Ura$^+$ transformants were selected by plating transformed yeast cells on synthetic medium lacking histidine, lysine and uracil. The His$^+$ Lys$^+$ and Ura$^+$ transformants obtained in this way were also tested for temperature sensitivity at 35°C by replica-plating. From these initial transformants, a single plasmid pTD33 was recovered that, when reintroduced into the recipient yeast strain SUX1702, reproduced the His$^+$ Lys$^+$ Ura$^+$ and temperature-sensitive phenotype. Thus plasmid pTD33 contained sequences that completely relieved the suppression caused by *san1-170*.

b) The suppressor gene was localized by subcloning analysis.

Restriction enzyme mapping revealed that the insert carried on plasmid pTD33 was approximately 11 kbp. Because of the size of the insert, it is most likely that pTD33 contains more than one gene (yeast genes are normally between 1 and 5 kbp long without much intergenic space). To delimit the functional suppressor gene within the cloned DNA, the 11-kbp insert of pTD33 was subcloned into the centromere-based vector pRS316 (Fig. 4). These subclone plasmids were then transformed into strain SUX1702 to assess the ability to alleviate the suppression phenotype. As shown in Fig. 4, the 3.5-kbp *Eco*RI-*Bam*HI fragment in plasmid pBE3 was the smallest insert that complemented the *san1* (*sca68*) mutant phenotype.

c) The cloned DNA fragment contains the wild-type suppressor gene.

The presence of the wild-type version of the suppressor gene on the 3.5-kbp *Eco*RI-*Bam*HI fragment was verified by integrative transformation. For this, the 3.5-kbp putative suppressor gene was cloned into the integrating vector YIp352. The resultant plasmid pIBE3 was linearized at the unique *Cla*I site within the insert and transformed into a *cdc68-1 san1-170 ura3-52* strain. In yeast, a linearized fragment becomes integrated into the genome exclusively by homologous recombination. Cells of the resultant transformed strain IS170 (*cdc68-1 san1-170 [SAN1 URA3] ura3-52*) were mated with cells of a *cdc68-1 san1-170 ura3-52* strain; for 12 meiotic tetrads examined, the temperature-sensitivity (wild-type for *SAN1*) and Ura$^+$ (harboring the *URA3* marker) phenotypes cosegregated, verifying that the temperature sensitivity was due to the integrated plasmid. The transformed strain IS170 was also mated with a *cdc68-1 SAN1 ura3-52* strain. If the linearized fragment had integrated at the genomic *san1-170* locus, there should be no segregation of the *san1-170* allele from the integrated sequence, so that all meiotic segregants should be temperature sensitive (due to the presence of the *cdc68-1* mutation in all spores). Conversely, if the linearized fragment had integrated somewhere else in the genome at a site unlinked to the *san1-170* locus, there would be segregation between *san1-170* and the integrated sequence, and temperature-resistant segregants (*cdc68-1 san1-170*) would arise. For 16 tetrads derived from the above diploid, all segregants were temperature-sensitive, indicating that the complementing genomic sequence had indeed integrated at the chromosomal *san1* locus. Plasmid integration at the homologous chromosomal locus was confirmed by Southern analysis (data not shown). Thus the cloned genomic fragment contained the authentic suppressor gene.

d) The suppressor gene *SCA68* is identical to *SAN1*.

To obtain nucleotide sequence information about the cloned suppressor gene, the DNA insert of pBE3 was subcloned into the phage vectors M13 mp18 and M13 mp19 and subjected to nucleotide sequence analysis (see Materials and Methods). The derived nucleotide sequences and predicted amino acid sequences were sent to the Genbank and EMBL databases to search for homologous sequences (Pearson, 1990). The amino acid sequences encoded within the *Hind*III-*Kpn*I fragment (Fig. 4) were found to be identical to a region of the previously reported San1 protein (Schnell et al., 1989). By inspection of the published *SAN1* nucleotide sequence, I found that both the nucleotide sequence and the deduced amino acid sequence within the *Hind*III-*Pst*I fragment of the suppressor gene (Fig. 4) were identical to a region of the *SAN1* gene (nucleotides 1420 to 1870 within the open reading frame [ORF]; Schnell et al., 1989). A DNA sequence located within the *Sac*I-*Kpn*I fragment of the suppressor gene was also found to be identical to a region downstream of the *SAN1* ORF (nucleotides 2310 to 2668, Schnell et al., 1989; Fig. 4). (Nucleotide sequence derived from the *Sal*I end of the *Xba*I-*Sal*I subclone did not allow the identification of any significant homologous sequence from the databanks, presumably because this sequence is located outside of the *SAN1* gene.) The *SCA68* and *SAN1* genes also possess identical restriction maps. Thus, the *SCA68* gene is the same as *SAN1*.

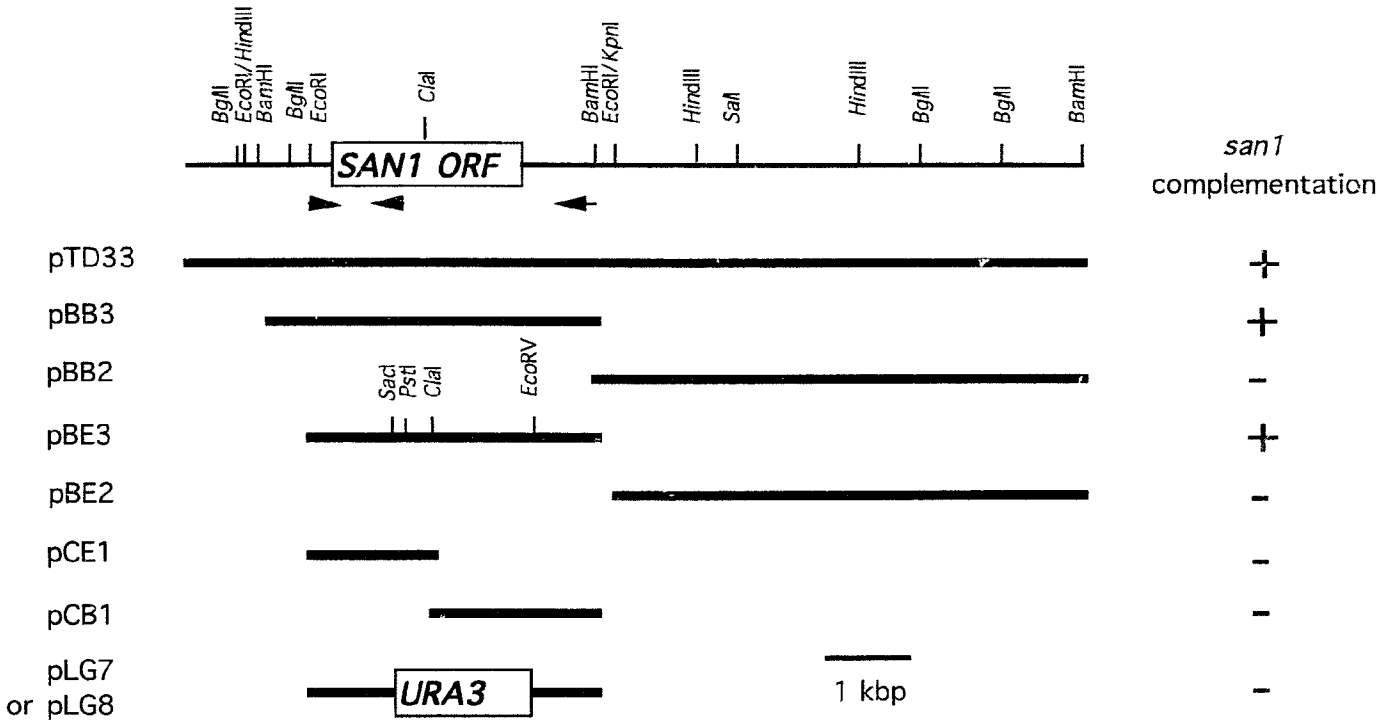
3. Features of the San1 protein.

A motif search for the San1 protein was carried out using the GCG (University of Wisconsin Genetics Computer Group) software (Devereux et al., 1984). This search was undertaken to identify regions in the San1 protein that correspond to the motifs defined in the PROSITE (Bairoch, 1990). The San1 protein has a well-defined nuclear localization signal (NLS), TKKRK (from amino acid 196 to 200); the proposed consensus sequence

Figure 4. Localization and identification of the *SAN1* gene.

Complementation by episomal plasmids, with genomic inserts as indicated, of the temperature-resistance and Spt^{-} phenotypes of a *san1 cdc68-1* mutant strain is indicated (+, complementation; -, no complementation). Open boxes show the approximate positions of open reading frames (ORF). Three small arrows below the restriction map indicate regions that were sequenced. Plasmid pTD33 was isolated from a centromere-based yeast genomic DNA library by its ability to complement a *sca68/san1* mutation. Plasmid pBB3, pBB2, pBE3, pBE2, pCE1, and pCB1 were derived by cloning DNA fragments (thick lines) into the low-copy pRS316 vector. Plasmid pLG7 and pLG8, with the *URA3* gene in opposite orientations, were constructed and used to disrupt the chromosomal *SAN1* gene as described in text.

Figure 4


BglII
EcoRI/HindIII
BamHI
BglII
EcoRI
ClaI
BamHI
EcoRI/KpnI
HindIII
SalI
HindIII
BglII
BglII
BamHI
SAN1 ORF
san1
complementation
pTD33
pBB3
pBB2
SacI
PstI
ClaI
EcoRV
pBE3
pBE2
pCE1
pCB1
pLG7
or pLG8
URA3
1 kbp
+
+
-
+
-
-
-
-

for a NLS is T/B-K-K-Z-K (B, basic residue; Z, pol.. residue; Gomez-Marquez and Segade, 1988). Protein import into the nucleus is highly selective, and the NLS is thought to be responsible for selective nuclear uptake of proteins. The existence of a NLS in the San1 protein is consistent with the fact that San1 interacts with both Cdc68 and Sir4 proteins involved in nuclear events such as transcriptional regulation and silencing of chromatin, respectively.

The San1 protein also has 19 putative casein kinase II phosphorylation sites (Kennelly and Krebs, 1991); San1 may be extensively phosphorylated *in vivo*. The functional significance of these casein kinase II phosphorylation sites is unclear at present.

4. Mapping of the *SAN1* gene.

To localize the *SAN1* gene in the *S. cerevisiae* genome, a radiolabeled 3.5-kbp *Bam*HI-*Eco*RI fragment encompassing the *SAN1* gene was used to probe a yeast chromosome blot. This hybridization procedure localized the *SAN1* gene to Chromosome IV (data not shown). To more accurately position the *SAN1* gene relative to other known genes on Chromosome IV, genetic crosses were carried out between strains containing the *URA3*-marked *san1* locus and strains containing other Chromosome IV markers. Initial genetic analysis of *san1* and markers *cdc37*, *trp4*, *ade8*, and *trp1* positioned *san1* on the right arm of chromosome IV near the *cdc37* locus. A three-factor cross was performed by using the *hom2* and *aro1* markers located in this region. The *URA3* marker integrated at the *san1* locus mapped midway between the *hom2* and *aro1* loci (Table 5). No gene has been previously reported to be in this region (Mortimer et al., 1992).

5. *san1* mutations that suppress the *sir4-9* mutation also reverse the *cdc68* phenotype.

Several addition.' *san1* mutations have been isolated during a different genetic

Table 5. Positioning *san1* on chromosome IV.

Genetic interval[a]	Number of tetrads			Map distance (centimorgans)[b]
	Parental ditype	Tetratype	Nonparental ditype	
san1 - *cdc37* (a)	22	3	0	6.0
san1 - *trp4* (b)	4	17	3	72.9
san1 - *ade8* (b)	5	15	4	81.2
san1 - *aro1* (c)	47	4	0	3.9
san1 - *hom2* (c)	47	4	0	3.9
aro1 - *hom2* (c)	47	8	0	7.8

[a] Tetrads were scored from the crosses EP25d x IS170-1d (a), LARM4-16B x IS170-1d (b), and X4119-3 x IS170-1c (c).

[b] Genetic map distances from the *URA3* gene integrated at the *san1* locus were calculated as specified by Mortimer and Schild (1985).

screen. These *san1* alleles were recovered as mutations that allowed *sir4-9* mutant cells that had lost mating capability to mate efficiently (Schnell et al., 1989). *SIR4* is one of the four *SIR* genes that are required for the establishment and maintenance of a silent state for the cryptic mating-type loci, *HML* and *HMR* (reviewed in Laurenson and Rine, 1992). I determined whether the *san1* alleles that were isolated by the ability to reverse the effects of the *sir4-9* mutation also suppress the *cdc68-1* mutation. Haploid strains harboring each of the three *sir4*-suppressing *san1* alleles (two point mutations, *san1-1* and *san1-2*, and one disruption allele, *san1::HIS3*) were mated with a *cdc68-1 his4-912δ lys2-128δ* mutant strain. The resultant diploids were sporulated, and examination of the meiotic segregants indicated that each of these three *san1* alleles suppresses both the temperature sensitivity and the Spt$^-$ phenotype caused by the *cdc68-1* mutation. The occurrence of 1:3 and 0:4 meiotic segregation patterns for temperature sensitivity suggested that these *san1* alleles suppress the effects of *cdc68-1*, because otherwise, temperature sensitivity should segregate in a 2:2 fashion due to the presence of the *cdc68-1* mutation in two spores of each ascus. In the case of the *san1::HIS3* disruption allele, the identification of *san1::HIS3* by its His$^+$ phenotype verified that this *san1* disruption allele was responsible for the suppression. Thus, the *san1* mutant alleles isolated by virtue of the ability to suppress the *sir4* phenotype have the same effects as the *san1* mutations identified here (Table 6).

I also found that *san3-1*, a mutation in a *SAN1*-related gene that was identified as a suppressor of the *sir4* phenotype (Schnell et al., 1989), failed to suppress the *cdc68-1* mutant phenotype. Thus, not all *sir4*-suppressor mutations were able to reverse *cdc68-1* defects: only *san1* mutations compensate for defects caused by the *cdc68-1* mutation.

6. *SAN1* is not essential.

Disruption of the *SAN1* gene by insertion of the *HIS3* gene into the *SAN1* open reading frame has no deleterious effect on cell proliferation (Schnell et al., 1989).

Nonetheless, proteins can consist of several separable domains that can function independently, so the possibility remained that the disrupted San1 protein retains some function. To resolve the question of whether *SAN1* is an essential gene I constructed a *san1* null allele, *san1Δ::URA3* as described in the Materials and Methods. The 2.6-kbp *Eco*RI-*Bam*HI fragment bearing the *san1Δ::URA3* null allele from plasmid pLG7 was used to replace one copy of the genomic *SAN1* gene in a *cdc68-1/CDC68* heterozygous diploid strain (Fig. 5). A diploid transformant (with deduced genotype *san1Δ::URA3/SAN1 cdc68-1/CDC68*) was sporulated, and all meiotic products were viable. Thus, the *SAN1* gene is indeed dispensable for cell viability in both *CDC68* and *cdc68-1* cells.

I found that the *san1Δ::URA3* null allele and the *san1::HIS3* disruption allele, like *san1* mutations, suppressed every aspect of the *cdc68-1* mutant phenotype (data not shown). All these observations suggest that *san1* suppression is a consequence of the decrease or loss of San1 function.

7. a *san1* mutation by itself has no detectable phenotype.

As shown above, *SAN1* is not an essential gene, and the absence of *SAN1* does not have any detectable effect on cell proliferation (data not shown). To determine whether a *san1* point mutation by itself impairs any cellular function, the *san1-3* mutation was segregated away from the *cdc68-1* mutation by mating a *cdc68-1 san1-3* strain with a *CDC68 SAN1* wild-type strain. Sporulation and tetrad analysis showed that all the meiotic segregants were viable at 23°C. At 35°C, there were at most two temperature-sensitive spores in each ascus, suggesting that only one mutation, *cdc68-1*, is able to confer temperature sensitivity. This finding shows that the *san1-3* mutation itself does not confer temperature sensitivity. For a tetrad displaying a 2:2 segregation pattern for temperature sensitivity at 35°C, the two temperature-sensitive segregants do not carry the *san1-3* mutation, because *san1-3* can suppress *cdc68-1* at this temperature. Thus, the

Figure 5. Disruption of the genomic *SAN1* gene.

(A) Schematic demonstration of replacement of the genomic *SAN1* gene by the *san1Δ::URA3* null allele. The 2.6-kbp *Bam*HI-*Eco*RI fragment of pLG7 harboring the *san1Δ::URA3* null allele was used to transform the *SAN1/SAN1* diploid strain QX300 to uracil prototrophy. Replacement of one genomic copy of the *SAN1* gene by the *san1Δ::URA3* null allele by homologous recombination is illustrated.

(B) Southern blot analysis verifying the replacement of one genomic copy of *SAN1* by the *san1Δ::URA3* allele. Genomic DNA isolated from diploid strain QX300 (*SAN1/SAN1*, lane 1) and from a Ura$^+$ transformant of QX300 (*san1Δ::URA3/SAN1*, lane 2) was digested with *Bam*HI and *Eco*RI, resolved electrophoretically through an agarose gel, and transferred to Nylon membrane. The Southern blot was probed with a radiolabeled 3.5-kbp *Bam*HI-*Eco*RI fragment harboring the *SAN1* gene. In addition to the 3.5-kbp wild-type *SAN1* genomic fragment (lanes 1 and 2), replacement of one genomic copy of the *SAN1* gene by the *san1Δ::URA3* null allele resulted in a smaller fragment (2.6 kbp, as illustrated above) in the diploid transformant (lane 2).

Figure 5

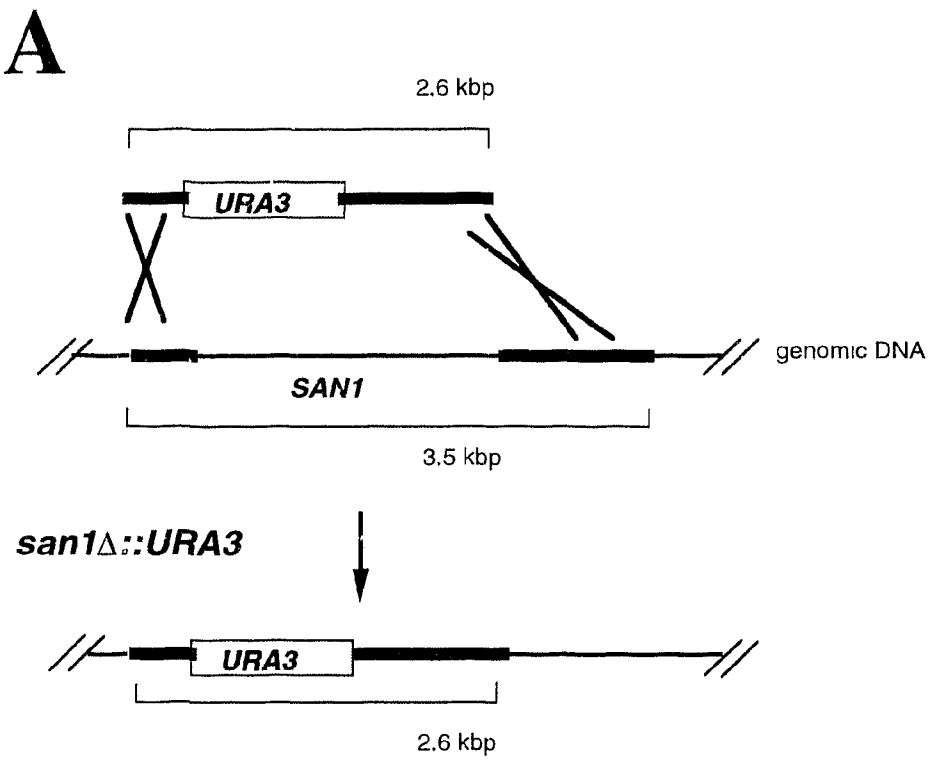

A
2.6 kbp
URA3
genomic DNA
SAN1
3.5 kbp
san1Δ::URA3
URA3
2.6 kbp
B
1 2
3 5 kbp
2.6 kbp

Table 6. Interactions between *san1* and *cdc68* mutations.

	Phenotype				
	Growth at 35°C			Suppression of *his4-912δ lys2-128δ*	Suppression of *suc2ΔUAS(-1900/-390)*
	cdc68 -1	*cdc68-197*	*cdc68-101::LEU2*	*cdc68-1*	*cdc68-1*
SAN1	-	-/+	dead	+	+
san1-3	++	++	dead	-	-
san1-170	+	+	ND	-	-
san1-1	++	++	ND	ND	ND
san1-2	++	++	ND	ND	ND
san1::HIS3	++	++	ND	ND	ND
san1Δ::URA3	++	++	dead	-	-

Growth of wild-type cells was scored as +++. ND = not determined.

remaining two temperature-resistant segregants harbor the *san1-3* mutation only. These *san1-3* mutant cells were found to proliferate at a rate indistinguishable from that of wild-type cells at both 23°C and 37°C (data not shown). Thus, neither a *san1* mutation nor complete removal of the *SAN1* gene has any detectable effect on cell proliferation.

8. *san1* mutant cells still need Cdc68 protein function.

CDC68 is an essential gene, and the *cdc68-101::LEU2* disruption mutation renders *SAN1* cells nonviable (Malone et al., 1991). To better understand the functional relationships between the Cdc68 and San1 proteins, I determined whether the *san1Δ::URA3* null mutation could suppress the complete loss of function of Cdc68. After replacing one genomic copy of the wild-type *SAN1* gene with the *san1Δ::URA3* null allele in diploid cells heterozygous for the *cdc68-101::LEU2* disruption allele, I sporulated the diploid transformant. For 10 tetrads dissected, spore viability segregated 2:2 in all cases, and all the viable spores were either Leu^- Ura^+ or Leu^- Ura^-, suggesting that spores with genotype *cdc68-101::LEU2 san1Δ::URA3* are inviable. Therefore the absence of San1 cannot compensate for the complete loss of Cdc68 function (Table 6). The *san1-3* mutation was also found unable to suppress the lethality caused by the *cdc68-101::LEU2* allele (A. Rowley, personal communication). These observations suggest that neither decreased San1 activity nor the absence of San1 can bypass the requirement for the Cdc68 protein for cell viability.

9. Transcription of G1 cyclin genes is restored in *cdc68-1 san1* cells.

Cells harboring the *cdc68-1* mutation are unable to perform START, and arrest in the G1 phase of the cell cycle (Prendergast et al., 1990). The inability of *cdc68-1* mutant cells to complete START is the consequence of decreased G1-cyclin gene expression (Rowley et al., 1991). The *san1* suppressor mutations allow *cdc68-1* mutant cells to proliferate at the restrictive temperature of 35°C: *san1* suppressed cells are therefore able

to complete START. To address the question of whether *san1* mutations suppress the START arrest of *cdc68-1* mutant cells by restoring G1-cyclin transcription or by bypassing the requirement for cyclin function, I determined mRNA abundance for the three G1-cyclin genes, *CLN1*, *CLN2*, and *CLN3*, in *cdc68-1* mutant cells and in *san1 cdc68-1* suppressed cells by Northern blot analysis. After transferring cells to 35°C there was, as expected, a rapid and dramatic decrease of mRNA abundance for *CLN1*, *CLN2*, and *CLN3* in *cdc68-1* mutant cells (Fig. 6; Rowley et al., 1991). However, in *san1-3 cdc68-1* suppressed cells, the mRNAs for all three G1-cyclin genes persisted at about the same levels as seen at 23°C (Fig. 6). Therefore the *san1-3* suppressor mutation restore normal mRNA abundance for these three G1-cyclin genes.

In weakly suppressed *san1-170 cdc68-1* cells the *CLN2* mRNA was abundant at 35°C and at about the same level as before the temperature shift, but the levels of *CLN1* and *CLN3* mRNAs were lower than those at 23°C (Fig. 6). These three G1-cyclin genes are functionally redundant; any one of the three *CLN* genes is sufficient to drive cells through START (reviewed in Reed, 1992). Therefore the weak *san1-170* mutation may suppress START inhibition in *cdc68-1* mutant cells by restoring significant expression of only one G1-cyclin gene, *CLN2*. To address this possibility I constructed *cdc68-1 san1-170* mutant strains lacking *CLN2* function. The resultant *cdc68-1 san1-170 cln2::LEU2* strains were still temperature resistant at 35°C, suggesting that the *san1-170* suppressor mutation restores sufficient function for at least one other G1 cyclin in addition to Cln2. Northern analysis showed that in the *cdc68 san1-170 cln2::LEU2* cells the levels of *CLN1* and *CLN3* mRNAs were about the same as those in isogenic *cdc68 san1-170 CLN2* cells (data not shown). These low levels of *CLN1* and/or *CLN3* expression may therefore be sufficient for cell proliferation. Thus, *san1* suppresses the START arrest of *cdc68-1* mutant cells by restoring G1-cyclin gene expression.

10. *san1* reverses other transcriptional alterations caused by the *cdc68-1* mutation.

In addition to decreasing G1-cyclin gene transcription, the *cdc68-1* mutation has other transcriptional effects. Enfeebled Cdc68 activity causes decreased transcription for the *ACT1* and *LEU2* genes and also for the *cdc68-1* gene itself (Rowley et al., 1991). I therefore determined whether *san1* mutations could restore transcription of the *ACT1* and *cdc68-1* genes in *cdc68-1* mutant cells. Transcript abundance was determined in two suppressor strains: one strain harbored the *san1-3* mutant allele, which allows *cdc68-1* mutant cells to proliferate almost as well as wild-type cells at 35°C, and the other strain contained the *san1-170* allele, which suppresses the *cdc68-1* phenotype less efficiently. Both the *san1-3* and *san1-170* mutations restored transcription of the *ACT1* and *cdc68-1* genes at 35°C (Fig. 6). I inferred that *san1* suppressor mutations also restore expression of the *LEU2* gene in *cdc68-1* mutant cells at 35°C, because at this temperature the suppressed cells proliferated in synthetic medium lacking leucine (data not shown). Therefore, *san1* suppressor mutations reverse those many transcriptional defects caused by the *cdc68-1* mutation at 35°C.

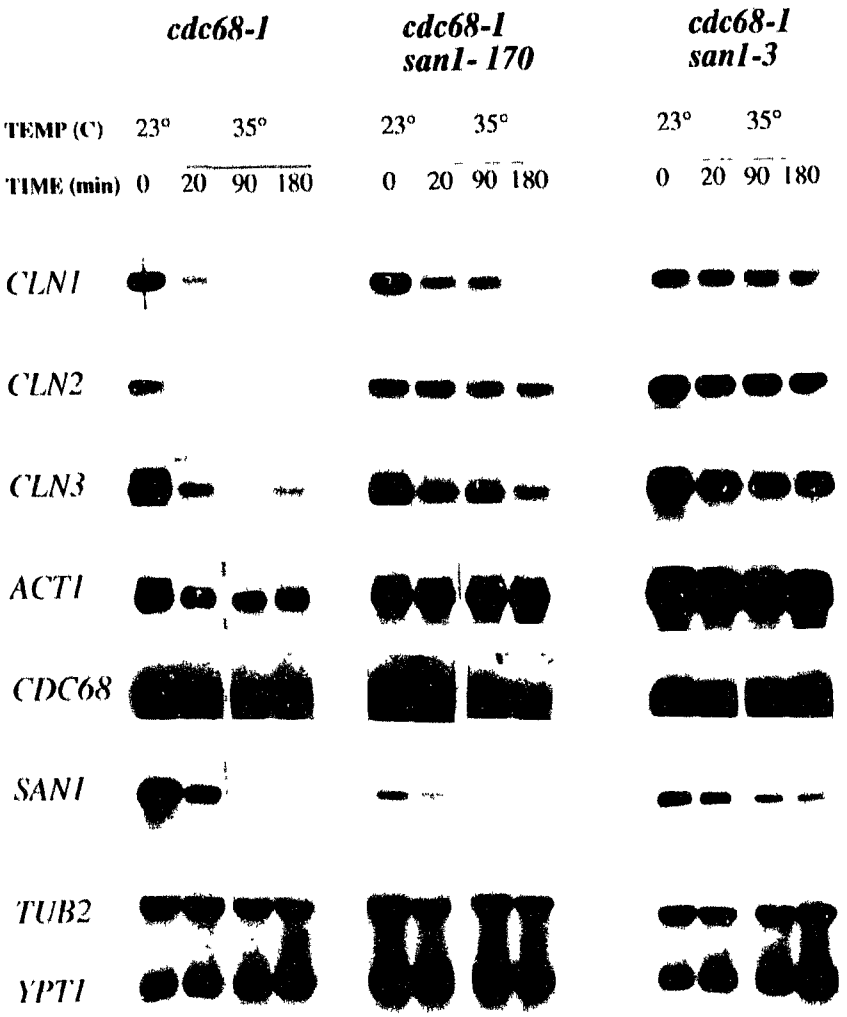
11. Cdc68 is required for the transcription of *SAN1*.

Given the fact that the Cdc68 protein is required for the expression of many unrelated yeast genes, I determined if Cdc68 also regulates transcription of the *SAN1* gene. To address this question, Northern blot analysis was carried out using the radio-labeled *SAN1* gene as a probe. As shown in Fig. 6, *SAN1* mRNA abundance decreased in *cdc68-1* mutant cells at the restrictive temperature of 35°C. This transcriptional defect of *cdc68-1* was again reversed by the *san1* suppressor mutations: transcription of the *san1* gene persisted in *san1 cdc68* double-mutant cells (Fig. 6). Thus, *SAN1* is another gene whose expression requires Cdc68 function.

Figure 6. mRNA levels in *cdc68-1* and *cdc68-1 san1* mutant cells.

Total yeast RNA was extracted from strains 68507A (*cdc68-1*), QX170 (*cdc68-1 san1-170*), and QX3 (*cdc68-1 san1-3*) growing at 23°C and also after incubation at 35°C for the indicated times. RNA was electrophoretically resolved on a formaldehyde-containing agarose gel and transferred to nylon membrane. The RNA blot was then probed separately with radiolabeled coding-sequence restriction fragments of the genes indicated (see Materials and Methods for details on probes and probe preparation). The mRNAs from the *TUB2* and *YPT1* genes, which are relatively unaffected by the *cdc68-1* mutation at 35°C, were used as controls for equal loading.

Figure 6


cdc68-1
cdc68-1 san1-170
cdc68-1 san1-3
TEMP (C) 23° 35° 23° 35° 23° 35°
TIME (min) 0 20 90 180 0 20 90 180 0 20 90 180
CLN1
CLN2
CLN3
ACT1
CDC68
SAN1
TUB2
YPT1

12. San1 antagonizes Cdc68 activity.

As described above, *san1* mutations have no detectable effect in cells harboring the wild-type *CDC68* gene and cause an observable phenotype only in *cdc68* mutant cells. Similarly, the *san1Δ::URA3* null allele, like the *san1* mutations identified here, suppresses the *cdc68-1* phenotype at 35°C. Thus, decreased San1 activity renders the *cdc68-1* gene more functional. To assess the effects of increased *SAN1* gene dosage, plasmids pEBE3 (carrying the *SAN1* gene on a high-copy episomal vector) and pBE3 (carrying *SAN1* on a low-copy centromeric vector) were introduced into *CDC68* wild-type and *cdc68-1* mutant cells. Similar to what I have found for decreased *SAN1* gene activity, increased *SAN1* copy number had no detectable effect in *CDC68* wild-type cells (data not shown). In *cdc68-1* mutant cells, however, increased *SAN1* gene dosage exacerbated the temperature sensitivity of these cells: at 33°C, which is a permissive temperature for *cdc68-1* mutant cells with normal *SAN1* gene dosage, transformants with additional copies of the *SAN1* gene (even those with only a few additional copies of *SAN1* on a centromere-based plasmid) were impaired (Fig. 7A). The growth rates of *cdc68-1* mutant cells were also affected by increased *SAN1* gene dosage. As shown in Fig. 7B, increased *SAN1* gene dosage had an inhibitory effect on proliferation of *cdc68-1* mutant cells even at the permissive temperature of 23°C, although this effect was not as profound as at 33°C, a temperature at which cells actually ceased proliferation. The growth kinetics shown in Fig. 7B also suggest that San1 inhibits the mutant *cdc68-1* gene activity in a dosage-dependent manner, in which higher *SAN1* gene dosage provided by pEBE3 had a stronger growth-inhibitory effect than the modest increase of *SAN1* gene dosage conferred by pBE3. The effects of altering *CDC68* and *SAN1* gene copy number are summarized in Table 7. The dosage effects of San1 suggests that San1 functions as a negative regulator of Cdc68 activity. This inhibition caused by increased *SAN1* gene dosage may indicate that the San1 protein restricts the expression of a gene (or genes) essential for cell proliferation, for example, the *ACT1* or *CDC68* gene, but does so

Figure 7. Inhibition of proliferation of *cdc68* mutant cells by overexpression of the *SAN1* gene.

(A) Cells of the *cdc68-1* mutant strain 68507A transformed with the low-copy *SAN1* plasmid pBE3, the high-copy *SAN1* plasmid pEBE3, or the high-copy vector YEp352 were grown at 23°C and replica plated to SC-Ura medium (SC medium lacking uracil) for further incubation at 30°C and 33°C.

(B) The same *cdc68-1* transformants harboring *SAN1* in low copy number (solid circles) or high copy number (solid triangles) or YEp352 vector alone (open squares), were grown in SC-Ura liquid medium at 23°C; at time zero, the cultures were split and a portion of each was transferred to 33°C for further incubation. Samples were removed at the times indicated for determination of cell concentration.

Figure 7

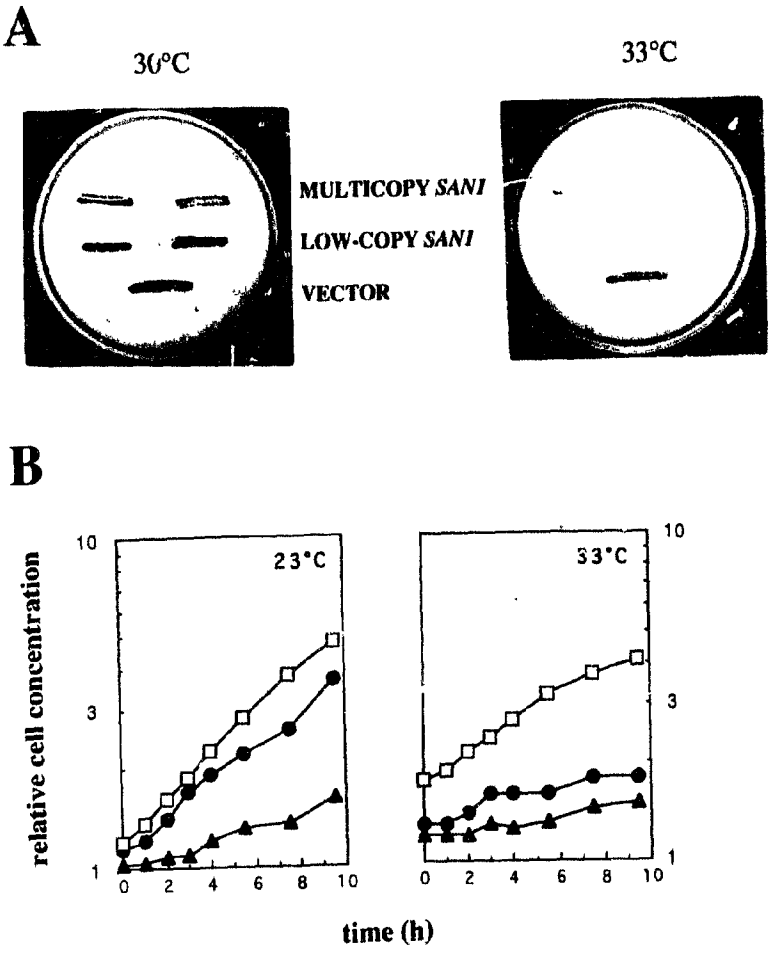

A
30°C
33°C
MULTICOPY SANI
LOW-COPY SANI
VECTOR
B
relative cell concentration
23°C
33°C
10
3
1
0 2 4 6 8 10
time (h)

Table 7. Dosage effects of *SAN1* and *CDC68* alleles.

Copy number		Growth			
SAN1	*CDC68* (or *cdc68*)	23°C	33°C	35°C	37°C
0	1 *CDC68*	+	+	+	+
1	1 *CDC68*	+	+	+	+
many	1 *CDC68*	+	+	+	+
0	1 *cdc68*	+	+	+	-
1	1 *cdc68*	+	+	-	-
many	1 *cdc68*	+	-	-	-
1	many *cdc68*	+	+	+	+
1	few *cdc68*	+	+	+/-	-
1	1 *cdc68*	+	+	-	-
1	0	dead	dead	dead	dead
0	0	dead	dead	dead	dead

effectively only when Cdc68 activity is enfeebled by mutation. Clearly, any inhibitory effect of San1 is weak and is masked by the potent activation function of the wild-type Cdc68 protein.

13. *SAN1* gene dosage does not affect transcript abundance.

I determined if changing San1 dosage causes any detectable effect on transcription in *CDC68* wild-type cells. Because genetic studies have revealed that San1 counteracts Cdc68 activity, those genes dependent on Cdc68 activity are good candidates to assess the effects of altered San1 dosage. Transcript abundance for *ACT1*, *CDC68*, and G1-cyclin genes was determined in *CDC68* wild-type cells with different amounts of San1: a *SAN1* wild-type strain, a *san1Δ::URA3* strain, and a strain carrying a high-copy *SAN1* plasmid (pEBE3). Overexpression of *SAN1* in cells harboring the pEBE3 plasmid was confirmed at the mRNA level (data not shown). As shown in Fig. 8, regardless of *SAN1* gene dosage there was no substantial change in mRNA abundance for any of the genes tested, either at 23°C or at 35°C. These Northern blot data lead to the conclusion that changes in *SAN1* gene dosage in a *CDC68* wild-type cell have no appreciable effect on transcription, and suggest that the inhibitory effect of San1 maybe mediated through other components, such as the Cdc68 protein.

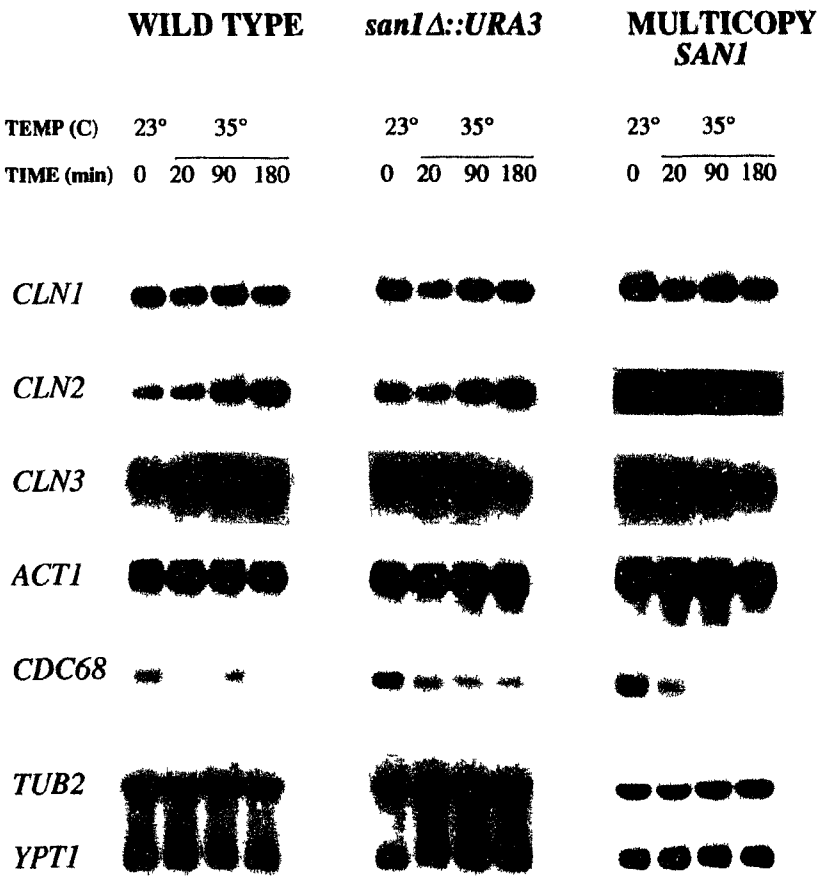
14. San1 does not affect the activities of other putative chromatin proteins.

A number of proteins have been suggested to regulate transcription by affecting chromatin structure, such as Spt2/Sin1 (an HMG-like protein; Kruger and Herskowitz, 1991), members of the histone group of Spt proteins, including Spt4, Spt5, and Spt6 (Clark-Adams et al., 1988; Malone et al., 1993; Swanson et al., 1991, 1992; Winston and Carlson, 1992), and a global transcription regulator, Sin4 (Jiang and Stillman, 1992). Deletion of the *SAN1* gene or extra copies of *SAN1* had no effect on the ability of an *spt2*, *spt4*, *spt5*, or *spt6* mutation to suppress δ-insertion mutations (the Spt$^-$ phenotype), and

Figure 8. mRNA abundance in cells with altered *SAN1* dosage.

Total yeast RNA was isolated from strains 21R (wild type), QXN1 (*san1::URA3*), and 21RH (strain 21R transformed with the multicopy *SAN1* plasmid pEBE3) that had been grown at 23°C or incubated at 35°C for the indicated times. RNA was electrophoretically separated on formaldehyde-containing agarose gel and transferred to nylon membrane. The RNA blot was then probed separately with radiolabeled coding-sequence restriction fragments for the genes indicated. All mRNAs, with exception of that from *CDC68*, were visualized from the same blots. The *TUB2* and *YPT1* mRNAs were used as internal loading controls.

Figure 8


WILD TYPE
san1Δ::URA3
MULTICOPY SAN1
TEMP (C) 23° 35° 23° 35° 23° 35°
TIME (min) 0 20 90 180 0 20 90 180 0 20 90 180
CLN1
CLN2
CLN3
ACT1
CDC68
TUB2
YPT1

changes in San1 activity did not suppress a *sin4* null mutation. Thus, it appears that San1 effects are mediated only through a limited number of proteins, including Cdc68 and Sir4.

15. Increased *cdc68* mutant gene dosage suppresses the *cdc68-1* mutation.

Transcription of the *cdc68-1* mutant gene was restored in *cdc68 san1* double-mutant cells at 35°C (Fig. 6). Thus decreased San1 activity may suppress the temperature sensitivity of *cdc68-1* mutant cells by allowing sufficient production of mutant Cdc68 protein. If so, then simply providing extra copies of a mutant *cdc68* gene should have the same consequence as removal of San1 to suppress temperature sensitivity caused by a *cdc68* mutation. This possibility was addressed by transforming a *cdc68-1* mutant strain with high-copy plasmids harboring either the *cdc68-1* allele or the *cdc68-197* allele. Increased gene dosage of either *cdc68-1* or the *cdc68-197*, similar to a *san1* mutation, allowed *cdc68-1* mutant cells to proliferate at the restrictive temperature (Fig. 9). Mutant *cdc68* alleles on high-copy plasmids did cause overproduction of mutant Cdc68 polypeptide (data not shown). Thus, polypeptides encoded by the *cdc68-1* and *cdc68-197* alleles still have residual activity at the restrictive temperature, and supplying cells with sufficient amounts of mutant Cdc68 protein suppresses the *cdc68-1* mutant phenotype. The fact that increased mutant *cdc68* gene dosage produces virtually the same effect as the inactivation of San1 protein is consistent with the conclusion that Cdc68 activity is inhibited by the San1 protein.

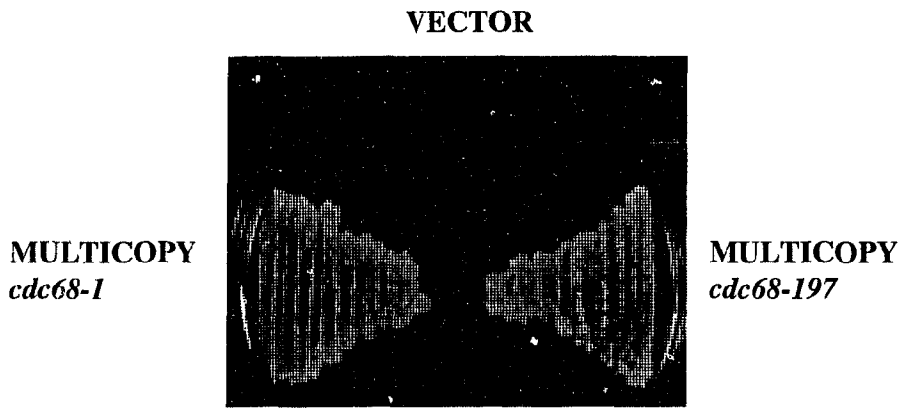
16. San1 does not function at the *CDC68* promoter.

San1 could impose a negative effect on Cdc68 activity at various levels. One possibility is that San1 may function at the *CDC68* promoter to affect the expression of the *CDC68* gene. To test this, a high-copy plasmid harboring only the *CDC68* promoter sequence was transformed into *cdc68-1* mutant cells. The resultant transformed cells

Figure 9. Effect of elevated gene dosage of *cdc68* mutant alleles.

Cells of the *cdc68-1* strain 68507A transformed with either the *cdc68-197* high-copy plasmid pBM46-4, the *cdc68-1* high-copy plasmid YEpDE68-1, or the YEp352 vector were patched onto SC-Ura solid medium (SC medium lacking uracil), incubated at 23°C, and then replica plated onto SC-Ura medium for incubation at 37°C for 2 days.

Figure 9



were still temperature sensitive, suggesting that extra copies of the *CDC68* promoter, which is affected by San1 inhibition, failed to titrate out the San1 inhibitor. This observation, coupled with the fact that overproduction of mutant Cdc68 protein titrates out the inhibitory effect of San1, suggest that San1 does not function directly at *CDC68* promoter, and most likely means that San1 antagonizes Cdc68 activity at the protein level.

17. The absence of San1 protein cannot suppress the effects of *cdc68-1* at 37°C.

Although all 20 *san1* suppressor mutations isolated in this study suppress the *cdc68-1* mutation at 35°C, none of them can alleviate temperature sensitivity at 37°C (Table 4). At this temperature, *cdc68-1 san1* double-mutant cells still arrest in the G1 phase of the cell cycle (about 70% unbudded) after a 4-h incubation, suggesting that inactivation of San1 cannot overcome the G1 arrest caused by severely impaired Cdc68 protein function at 37°C. Indeed, Northern blot analysis revealed that at 37°C none of the three G1-cyclin mRNAs persisted in *cdc68-1 san1* double-mutant cells (data not shown). The *san1Δ::URA3* null allele had the strongest suppressive effect among all the *san1* alleles yet did not suppress the *cdc68-1* mutation effectively at 37°C; at this temperature *san1Δ::URA3 cdc68-1* double-mutant cells grew only slowly in liquid medium and did not form colonies on solid medium. Nonetheless, the *cdc68* mutant gene products must still have residual activity at 37°C, because at this temperature extra copies of a *cdc68* mutant gene suppressed the growth defect of *cdc68-1* mutant cells, as described above (Fig. 9). It is noteworthy that one of the initial suppressor isolates, QXT1, harboring the *san1-201* allele did grow at 37°C, but when this *san1-201 cdc68-1* mutant strain was mated with another *cdc68-1* strain, temperature sensitivity at 37°C segregated in a 4:0 or 3:1 fashion for most tetrads, whereas suppression at 35°C segregated in a 2:2 fashion. These genetic data demonstrate that *san1-201* can suppress *cdc68-1* only at 35°C, and that the observed suppression at 37°C was most likely due to a multigenic effect: at least

one more mutation resides in the original QXT1 suppressor cells, that in conjunction with *san1-201* allows *cdc68-1* mutant cells to proliferate at 37°C. Therefore, other gene products may modulate Cdc68 activity at 37°C. In fact, in the absence of San1, *scb68/sug1* suppressor mutations that I identified can suppress the *cdc68-1* mutation at 37°C (see Section C).

Section C. Functional relationships between Cdc68 and Sug1

As I described in Section A, the suppressor mutations identified here define four suppressor genes. One suppressor gene, *SCA68*, is identical to the *SAN1* gene. To reveal the identity of other suppressor genes and gain further insight into the Cdc68 protein function, I cloned a second suppressor gene, *SCB68*, which as I show below is the same as the previously reported *SUG1* gene (Swaffield et al., 1992). This section focuses on molecular characterization of the *SUG1* gene, and the functional interaction between Cdc68 and Sug1 at both genetic and protein levels. Data of this section are included in Xu et al. (submitted).

1. *sug1* mutations suppress transcriptional defects caused by *cdc68-1*.

During my initial suppressor-mutant screen, six mutant alleles were isolated in the *SCB68/SUG1* gene that allowed *cdc68-1* mutant cells to proliferate at the restrictive temperature of 35°C (Table 4). Two mutants, QXT20 (*cdc68-1 sug1-20*) and QXT26 (*cdc68-1 sug1-26*), were chosen for further study to represent suppressor mutations in this complementation group.

As shown above in Section B, mutations in the *SAN1* gene reverse transcriptional defects imposed by the *cdc68-1* mutation. I determined if this was also the case for *sug1* suppressor mutations. Total RNA was extracted from a *cdc68-1 sug1-20* strain and from a *cdc68-1* strain after cells were transferred to the restrictive temperature of 35°C. Northern blot analysis showed that transcription of the *cdc68-1* mutant gene was restored

in *cdc68-1 sug1-20* suppressed cells at 35°C (Fig. 10). As expected, transcription of other Cdc68-dependent genes, such as *ACT1* and the three G1-cyclin genes *CLN1*, *CLN2*, and *CLN3*, also persisted in *cdc68-1 sug1-20* cells at 35°C (Fig. 10). In a similar experiment, the *sug1-26* allele was also found to restore transcription of *cdc68-1* and other Cdc68-dependent genes in *cdc68-1* mutant cells at 35°C (data not shown). Thus it is likely that the *sug1* suppressor mutations suppress the temperature sensitivity of *cdc68-1* mutant cells because they restore the expression of Cdc68-dependent genes.

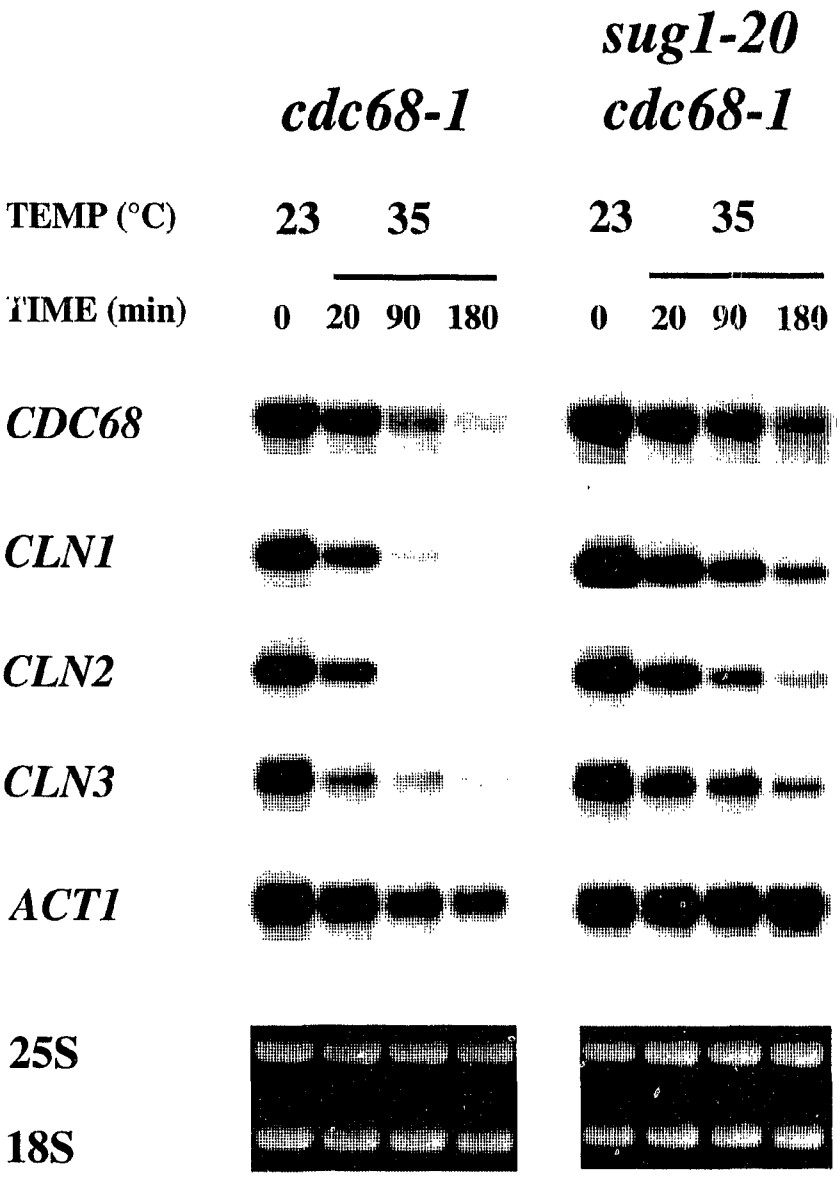
2. *sug1* mutations suppress other aspects of the *cdc68-1* phenotype.

The Cdc68 protein has been shown to affect transcription of a *UAS*-less *suc2* gene (Malone et al., 1991). A truncation of the *SUC2* upstream activation sequence, *ΔUAS (-1900/-390)*, abolishes transcription of the mutant *suc2* gene, and consequently cells harboring this *suc2ΔUAS* mutant allele cannot grow with sucrose as the sole carbon source (Sarokin and Carlson, 1984). Both the *cdc68-1* and *cdc68-197* mutations suppressed this Suc^- phenotype by allowing the *UAS*-less *suc2* gene to be transcribed, and as a result *cdc68 suc2ΔUAS* cells display a Suc^+ phenotype (Malone et al., 1991). I determined if *sug1* suppressor mutations affect this *suc2ΔUAS* transcription that is allowed by *cdc68*. A *sug1-26 cdc68-1* mutant strain was mated to a strain harboring the *cdc68-197* and *suc2ΔUAS* mutations. The resultant diploid cells were sporulated and phenotypes of meiotic segregants were tested both for temperature sensitivity and for growth on sucrose. In the absence of the *sug1-26* mutation, all the meiotic segregants would be expected to display a Suc^+ phenotype at 30°C due to the presence of a *cdc68* mutation in each meiotic segregant, and also because *cdc68 SUC2* cells show a Suc^+ phenotype at this temperature (data not shown). Occurrence of a Suc^- segregant would indicate that the *sug1-26* mutation suppresses the effects of a *cdc68* mutation that causes the Suc^+ phenotype. For 12 tetrads examined, not only did I detect Suc^- segregants, but each Suc^- segregant was also temperature-resistant at 35°C (data not shown), indicating

Figure 10. mRNA levels in *cdc68-1* and *cdc68-1 sug1-20* cells.

Total yeast RNA extracted from cells grown at 23°C and after incubation at 35°C for the indicated times was resolved electrophoretically on formaldehyde-containing agarose gel. RNA was then transferred to nylon membrane and the blot was probed with radiolabeled coding-sequence restriction fragments of the genes indicated. Strains used were 68507A (*cdc68-1*) and QXT20 (*cdc68-1 sug1-20*). Ethidium bromide stained 18S and 25S rRNAs served as internal loading controls.

Figure 10


cdc68-1
sug1-20 cdc68-1
TEMP (°C)
23
35
23
35
TIME (min)
0 20 90 180
0 20 90 180
CDC68
CLN1
CLN2
CLN3
ACT1
25S
18S

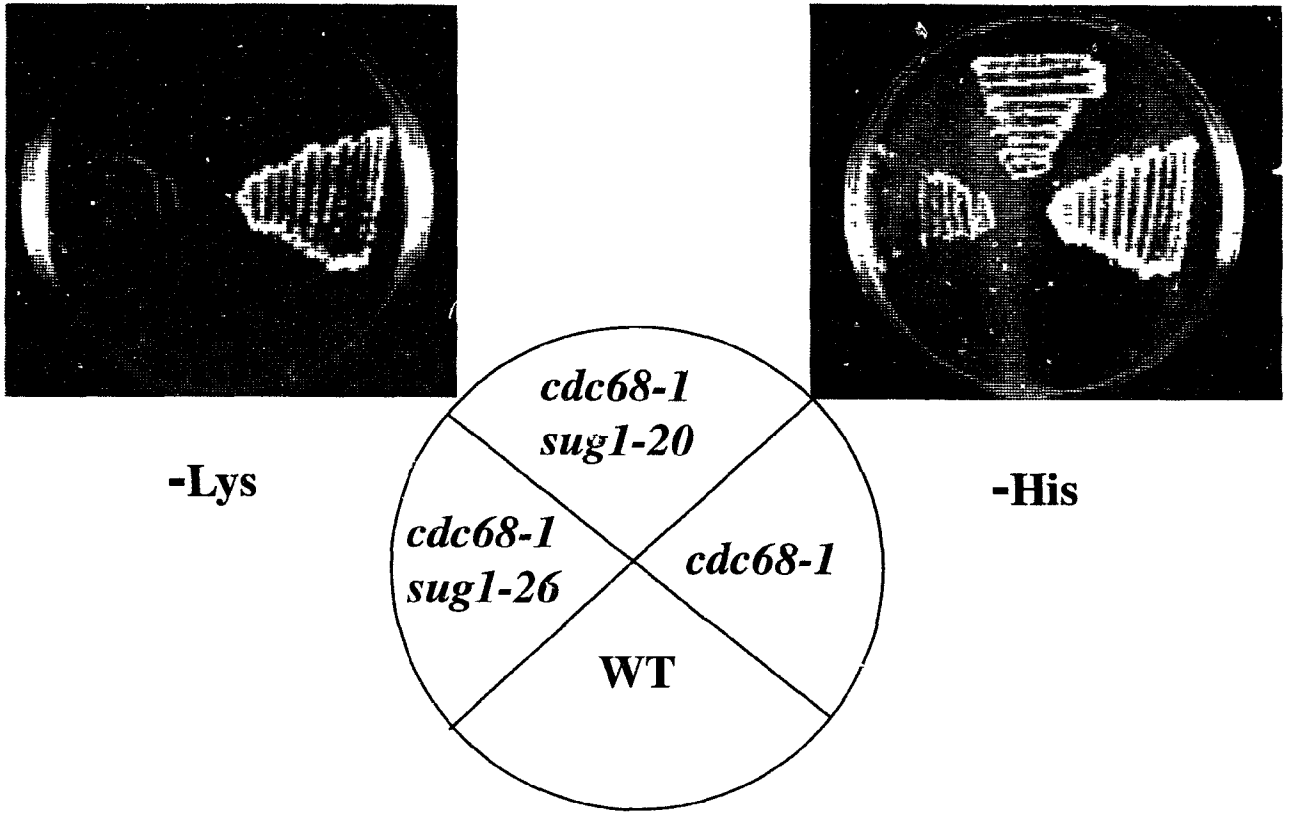
that these Suc$^-$ *cdc68* segregants also harbored the *sug1-26* mutation. Thus, the *sug1-26* mutation prevents the transcription of the *suc2ΔUAS* gene that is allowed by *cdc68-1*.

In addition to temperature sensitivity and increased transcription from the *ΔUAS*-less *SUC2* promoter, the *cdc68-1* mutation causes an Spt$^-$ phenotype (Rowley et al., 1991; Malone et al., 1991). Insertions of a Ty element or a solo-δ sequence in the 5' regions of the *HIS4* and *LYS2* gene change transcription from these two genes to cause a His$^-$ and Lys$^-$ phenotype (Clark-Adams and Winston, 1987; Winston et al., 1984). The *cdc68-1* mutation suppresses this kind of insertional mutation by restoring transcription at normal initiation sites and allows mutant cells to grow without added histidine and lysine, a phenotype termed Spt$^-$ (Malone et al., 1991). To determine whether *sug1* mutations suppress this Spt$^-$ phenotype caused by *cdc68-1*, a *cdc68-1 sug1-26* double-mutant strain was mated with a *cdc68-1 lys2-912δ his4-128δ* strain, and resultant diploid cells were sporulated. As expected, temperature sensitivity segregated in a 2:2 fashion among meiotic segregants. Occurrence of Lys$^-$ or His$^-$ segregants at 30°C would suggest that *sug1-26* suppresses the Spt$^-$ phenotype caused by *cdc68-1*, because otherwise every segregant should show a His$^+$ Lys$^+$ phenotype due to the effects of the *cdc68-1* mutation, regardless of the nature of the *his4* and *lys2* loci. Tetrad analysis showed that there were indeed Lys$^-$ segregants, and that every Lys$^-$ segregant was temperature-resistant at 35°C, suggesting that this Lys$^-$ phenotype was caused by the *sug1-26 cdc68-1 lys2-128δ* genotype. For the same 16 tetrads examined, however, there were no His$^-$ segregants at 30°C; segregants with relevant genotype of *cdc68-1 sug1-26 his4-912δ* were identified by inference from the phenotypes and these segregants were His$^+$ at 30°C. Therefore the *sug1-26* mutation partially suppresses the Spt$^-$ phenotype caused by the *cdc68-1* mutation in that *sug1-26* reverses the effect of *cdc68-1* at the *lys2-128δ* locus but not the *his4-912δ* locus (Fig. 11). A similar analysis showed that, like *sug1-26*, the *sug1-20* allele also partially reverses the Spt$^-$ phenotype caused by the *cdc68-1* mutation (Fig. 11).

Figure 11. Partial suppression by *sug1* of the *cdc68-1* Spt⁻ phenotype.

All strains harbor the *his4-912δ* and *lys2-128δ* mutations. Cells of strains FY56 (wild-type, WT), FYARQ1 (*cdc68-1*), SUXB201 (*sug1-20 cdc68-1*), and SUXB261 (*sug1-26 cdc68-1*) were grown on YEPD solid medium at 23°C, then replica-plated to SC-His and SC-Lys solid media (SC medium lacking either histidine or lysine) for further incubation at 30°C to assess Spt⁻ phenotype.

Figure 11



3. Molecular cloning and sequencing identify this suppressor gene as *SUG1*.

To clone the wild-type suppressor gene I took advantage of the observation that *scb68/sug1* suppressor mutations reverse some aspect of the Spt$^-$ phenotype caused by the *cdc68-1* mutation. A suppressed strain, SUX261, harboring the *sug1-26, cdc68-1, lys2-128δ,* and *ura3-52* mutations, displays a Lys$^-$ phenotype, whereas the same suppressed strain transformed with the wild-type *SUG1* gene would be expected to become phenotypically Lys$^+$ due to complementation of the *sug1-26* recessive mutation. A centromere-based yeast genomic library was used to transform strain SUXB261 and Lys$^+$ Ura$^+$ transformants were selected initially on synthetic medium lacking lysine and uracil. Transformants were then tested for temperature sensitivity at 35°C by replica-plating. The Lys$^+$ Ura$^+$ and temperature-sensitive transformants were candidates for cells harboring the wild-type suppressor gene.

Three library plasmids were identified that alleviated the Lys$^-$ phenotype of the recipient strain SUXB261. The insert of one plasmid, pXB68, was subcloned into the pRS316 vector, and subclone plasmids pXES5, pXHH2, pXSK4 and pXKE17 were tested for the ability to relieve the Lys$^-$ phenotype of strain SUXB261. This subcloning analysis localized the active sequence to a 1.7-kbp *Eco*RI-*Kpn*I restriction fragment (Fig. 12). Restriction enzyme digestion revealed that the other two candidate genomic clones contained the same overlapping 1.7-kbp *Eco*RI-*Kpn*I genomic fragment (data not shown).

I verified that the above 1.7-kbp *Eco*RI-*Kpn*I DNA sequence contained the wild-type version of the suppressor gene by integrative transformation (see Materials and Methods).

Partial nucleotide sequence analysis revealed that this cloned suppressor gene was identical to the previously reported *SUG1* gene (Swaffield et al., 1992; Goyer et al., 1992). The *SUG1* gene was identified by a different genetic screen: the *sug1-1* mutation was isolated by virtue of its ability to restore transcription activation in cells with a *gal4D* mutation, a mutant version of *GAL4* that encodes a Gal4 protein lacking part of the C-

terminal activation domain. Northern analysis using the radiolabeled 1.7-kbp *Eco*RI-*Kpn*I fragment detected a 1.4-kbp transcript, consistent with the size of the *SUG1* open reading frame (Fig. 12). The identity of the cloned suppressor gene as *SUG1* was further confirmed by the finding that the cloned suppressor gene complemented the effects of the *gal4D*-suppressing *sug1-1* allele (data not shown).

4. Sug1 has a repressive effect on transcription.

As described below in Section E, I found that in otherwise wild-type cells both the *sug1-20* and *sug1-26* alleles render yeast cells temperature-sensitive for proliferation. I therefore determined whether attenuating Sug1 activity by the transfer of *sug1* mutant cells to the restrictive temperature had any effect on transcription. Total RNA was extracted from *SUG1* wild-type and *sug1-26* mutant strains after transfer to the restrictive temperature of 37°C, and analyzed by Northern blots. As shown in Fig. 13A, there were modest increases in mRNA abundance for several genes: levels of *CDC68* mRNA were increased in *sug1-26* mutant cells at both 23°C and 37°C compared to those in wild-type cells, and mRNA levels for the *sug1-26* gene were also increased. Thus, decreased Sug1 activity (encoded by a recessive *sug1* allele) increases transcription, suggesting that Sug1 plays a negative role in transcription.

5. Sug1 does not affect all transcription that is mediated by Cdc68.

Both the *CDC68* and *SUG1* genes are essential for cell viability (Malone et al., 1991; Swaffield et al., 1992). *CDC68* is essential presumably because Cdc68 activity is required for the transcription of some essential genes, such as the *ACT1* gene. It is equally possible that Sug1 is essential because, in the absence of Sug1 activity, Cdc68-dependent genes cannot be transcribed. To determine whether this might be the cause for the essential nature of *SUG1*, transcription of a Cdc68-dependent gene, *ACT1*, was assessed in *sug1-26* mutant cells at 37°C, a temperature that impairs mutant Sug1

Figure 12. Restriction map and subcloning analysis of the *SCB68/SUG1* gene.

The ability of episomal plasmids harboring genomic inserts to complement the *sug1-26* mutation is indicated ("+" = complementation; "–" = no complementation). The arrowed line indicates the position and orientation of the open reading frame. Disruption of the *SUG1* gene by insertion of the *LEU2* marker gene is also shown. pXB68 was one of the library plasmids that complemented a *sca68/sug1* mutation. Plasmids pXES5, pXKS4, pXHH2, and pXKE17 were constructed by cloning DNA fragments (thick lines) into multiple cloning sites of the low-copy vector pRS316. Construction of pXS23 harboring the *sug1::LEU2* disruption allele is described in Materials and Methods.

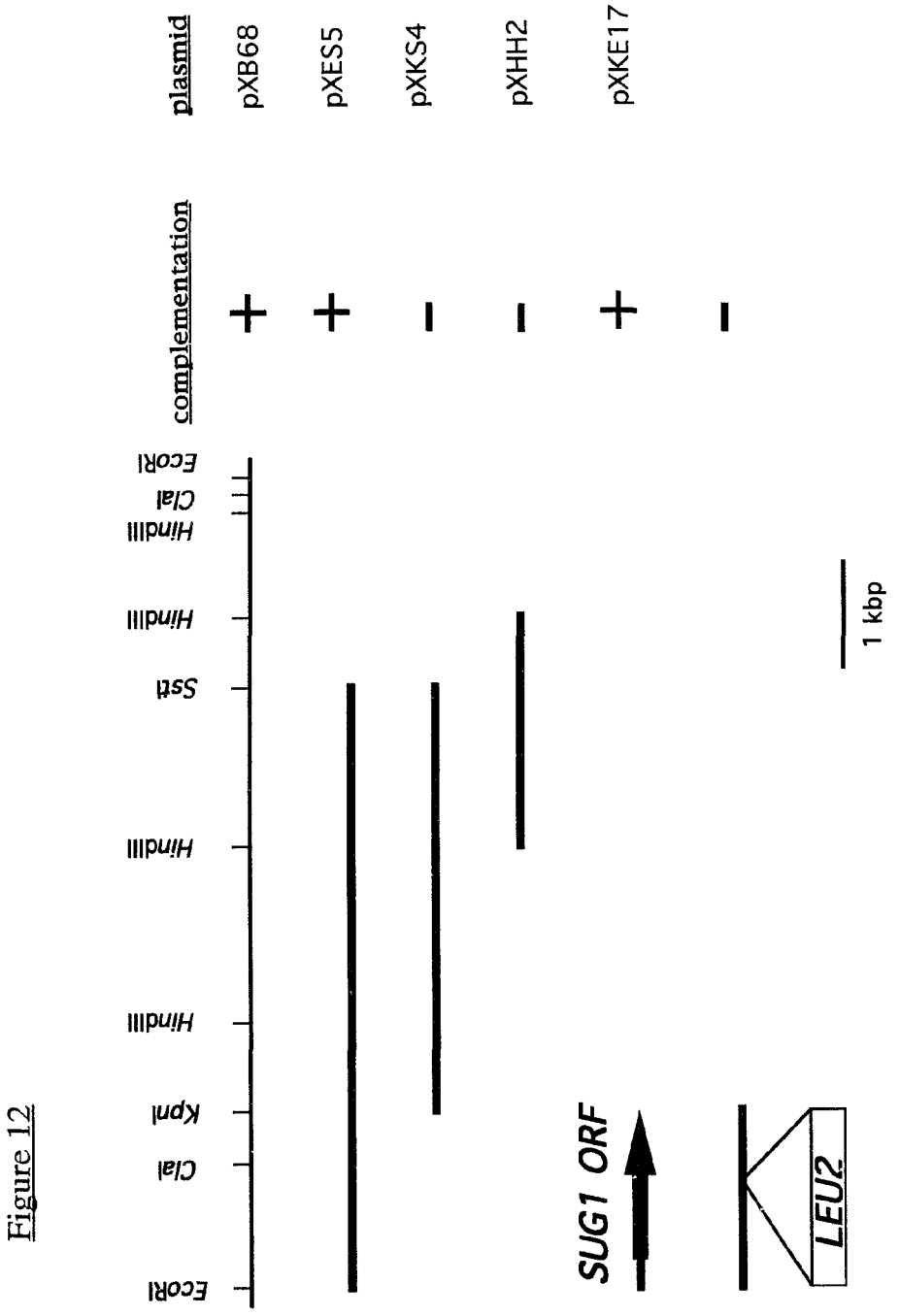

plasmid
complementation
pXB68
pXES5
pXKS4
pXHH2
pXKE17
+
+
-
-
+
-
EcoRI
ClaI
HindIII
HindIII
SstI
HindIII
HindIII
KpnI
ClaI
EcoRI
SUG1 ORF
LEU2
1 kbp

Figure 12

function. As shown in Fig. 13A, *ACT1* transcription persisted even when Sug1 activity was compromised, and there was no substantial change of *ACT1* mRNA levels even after a 5-h incubation at 37°C, at which point *sug1* mutant cells had already ceased proliferation (data not shown). Thus Sug1 does not affect all transcription that is mediated by Cdc68. The observation that there is no decrease in the transcription of Cdc68-dependent genes when Sug1 activity is virtually absent may suggest that Sug1 is not essential for the transcription mediated by Cdc68.

6. *sug1* can confer an Spt⁻ phenotype.

Because altered activity of Cdc68 and a number of other transcription regulators causes an Spt⁻ phenotype (Malone et al., 1991; Rowley et al., 1991), I determined whether the *sug1-26* allele produces an Spt⁻ phenotype. The *sug1-26* strain QX261 was mated with the *his4-912δ lys2-128δ* strain FY56, and the resultant diploid was sporulated. Four temperature-sensitive segregants that display a His⁻ Lys⁻ phenotype at 23°C (with the inferred genotype *sug1-26 his4-912δ lys2-128δ*) were tested for the Spt phenotype at 30°C. All four segregants failed to grow in lysine-free medium (data not shown), but grew slowly in medium lacking histidine (growth of one segregant, QX261-1d, is shown in Fig. 13B), suggesting that the *sug1-26* suppressor mutation by itself can confer a limited Spt⁻ phenotype.

7. Neither *cdc68* nor *sug1* mutations can compensate for the complete loss of gene function.

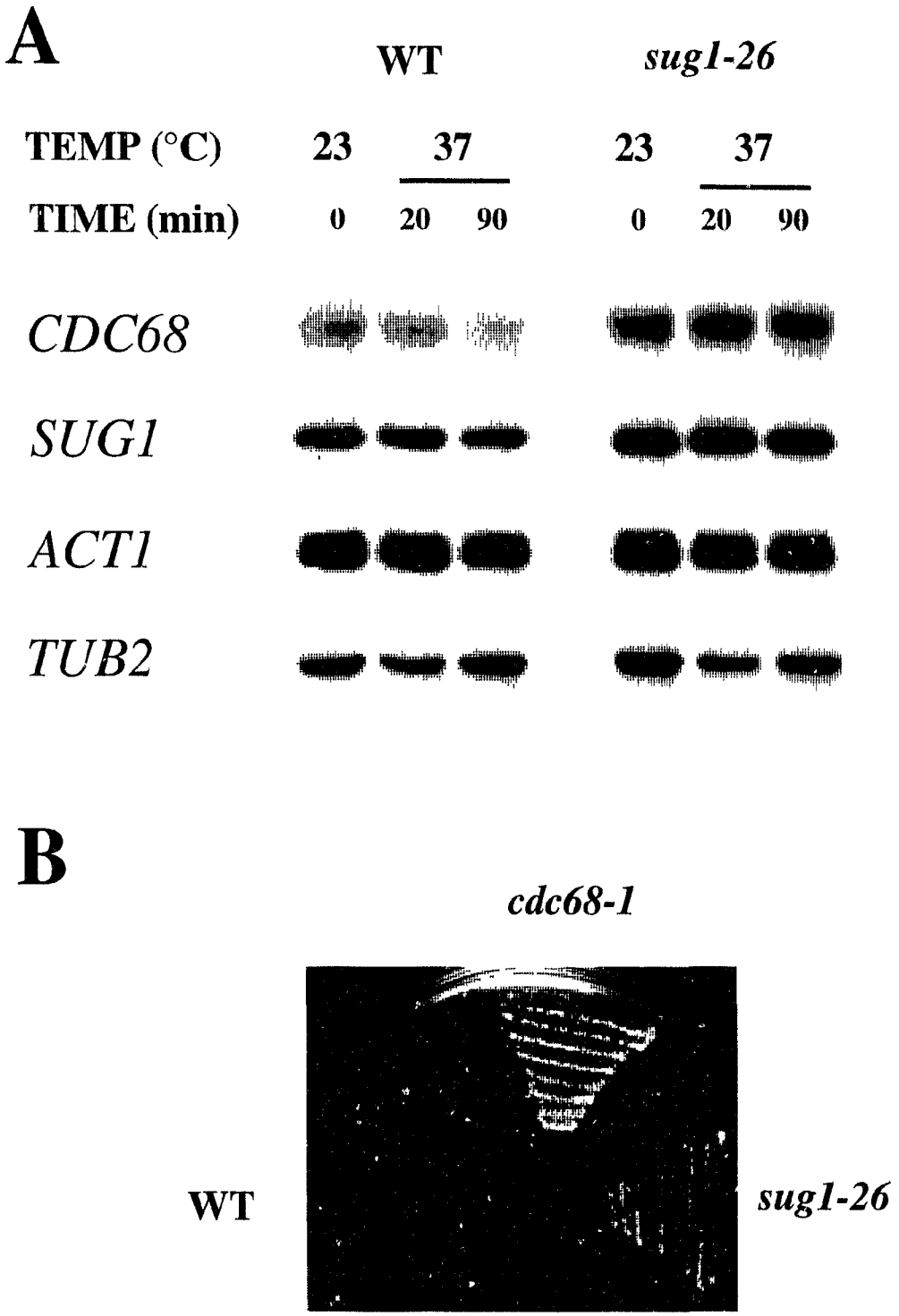
Disruption of the *CDC68* gene is a lethal event in haploid yeast cells (Malone et al, 1991). To address the question of whether the *sug1-26* mutation allows cells to bypass Cdc68 function, I determined the ability of *sug1-26* to suppress the lethality of the *cdc68* disruption allele *cdc68-101::LEU2*, using a plasmid-shuffling procedure (Boeke et al., 1987). Cells of diploid strain BM64 (*cdc68-101::LEU2/CDC68 ura3-52/ura3-52*) were

Figure 13. Transcriptional effects of Sug1.

(A) mRNA levels. Total RNA was extracted from cells of wild-type strain 21R and the *sug1-26* strain QX261 growing at 23°C and after incubation at the restrictive temperature of 37°C for the times indicated. RNA was resolved on formaldehyde-containing agarose gel, transferred to nylon membrane, and probed separately with radiolabeled DNA fragments for the genes indicated. *TUB2* mRNA was used as a loading control.

(B) Spt$^-$ phenotype. Cells of strain FY56 (*his4-912δ*), FYARQ1 (*cdc68-1 his4-912δ*), and QX261-d1 (*sug1-26 his4-912δ*) were grown on YEPD solid medium at 23°C, replica-plated to SC-His medium (SC medium lacking histidine) and incubated at 30°C to assess Spt$^-$ phenotype.

Figure 13


A
WT
sug1-26
TEMP (°C)
23
37
23
37
TIME (min)
0
20
90
0
20
90
CDC68
SUG1
ACT1
TUB2
B
cdc68-1
WT
sug1-26

transformed with the high-copy *CDC68* plasmid p68-Ba-1A, and a Ura$^+$ transformant was sporulated and tetrads were dissected. A Ura$^+$ Leu$^+$ segregant (harboring the *cdc68-101::LEU2* disruption allele and kept alive by the *CDC68* plasmid) was then mated with a *cdc68-1 sug1-26* strain, and the *URA3*-marked *CDC68* plasmid was lost from resultant diploid cells by 5-FOA counterselection (Boeke et al., 1984). Diploid cells that lost the *URA3 CDC68* plasmid were sporulated. For all the tetrads dissected there was a 2:2 segregation pattern for spore viability and all the viable spores were Leu$^-$, suggesting that no spore harboring the *cdc68* disruption allele was viable. Among the viable spores, about 50% were temperature sensitive, suggesting that half of the dead spores harbored the *sug1-26* mutation yet were still non-viable. A similar result was obtained using the *sug1-20* allele. Thus, neither the *sug1-20* nor the *sug1-26* mutation can overcome the lethality caused by disruption of the *CDC68* gene, and Cdc68 activity is still required for cell viability even in a *sug1* mutant background (Table 8). In view of the above Northern data that *sug1* restores transcription of the mutant *cdc68-1* gene, I conclude that *sug1* suppresses the deleterious effects of the *cdc68-1* mutation by restoring Cdc68 activity.

It was previously reported that *SUG1* is an essential gene (Swaffield et al., 1992). To determine whether the *cdc68-1* mutation could compensate for the complete loss of Sug1 function, a *sug1* disruption allele was constructed by inserting the *LEU2* gene into the *SUG1* ORF at the unique *Cla*I site. The resulting *sug1::LEU2* disruption allele was used to replace one genomic copy of the *SUG1* gene in a *cdc68-1/CDC68 SUG1/SUG1* diploid strain. Sporulation and tetrad analysis confirmed the previous report that *SUG1* is essential, and furthermore showed that Sug1 function is indispensable even in combination with the *cdc68-1* mutation (Table 8). The observation that the *SUG1* gene is essential even in *cdc68-1* mutant cells indicates that the recessive *sug1* suppressor alleles identified here cause only partial loss of Sug1 function.

I also determined the effect of a *cdc68 sug1* double disruption. A *sug1::URA3* disruption allele was constructed and used to replace one genomic copy of the *SUG1* gene

in a *cdc68-101::LEU2/CDC68 SUG1/SUG1* diploid strain. Sporulation and tetrad analysis revealed that the *cdc68 sug1* double disruption is lethal to haploid yeast cells (Table 8). Like spores harboring either a *cdc68* or a *sug1* single disruption, *cdc68-101::LEU2 sug1::URA3* spores failed to germinate. Although Sug1 and Cdc68 appear to play counteracting roles on transcription, their functions cannot be limited to overcoming the effects of each other: otherwise removal of both proteins would not affect cell viability. Thus the Cdc68 and Sug1 proteins have essential cellular functions in addition to antagonizing the activity of each other.

8. Interactions between *cdc68* and *sug1* are allele-specific.

To investigate the interactions between *cdc68* and *sug1* mutations, and thus possibly between Cdc68 and Sug1 proteins, I addressed the question of if *cdc68* and *sug1* display allele-specific interactions.

a) *sug1* suppressor mutations specifically affect the *cdc68-1* allele.

I first determined whether *sug1* mutations identified here as suppressors of the *cdc68-1* phenotype could reverse temperature sensitivity caused by other *cdc68* mutant alleles. The *cdc68-11* mutation was isolated by its ability to allow the *HO* gene to be expressed in the absence of the Swi4 or Swi6 activator, and this *cdc68-11* allele itself, like the *cdc68-1* allele, also causes temperature sensitivity and arrest in the G1 phase of the cell cycle (Lycan et al., 1994). The *cdc68-11* mutation was first crossed into the 21R genetic background and a temperature-sensitive *cdc68-11* segregant (QX6821) was then mated with the *sug1-20* strain QX2022 and the diploid (with relevant genotype *cdc68-11/CDC68 sug1-20/SUG1*)was sporulated. For a tetrad that displayed a 2:2 segregation pattern for temperature sensitivity at 37°C, the two temperature-resistant segregants should carry the wild-type *CDC68* and *SUG1* genes, because both *cdc68-11* and *sug1-20* causes temperature sensitivity at 37°C. The remaining two temperature-sensitive

segregants must therefore harbor both the *cdc68-11* and *sug1-20* mutations. The two temperature-sensitive spores identified at 37°C also failed to grow at 35°C, suggesting that the *sug1-20* mutation can not suppress the *cdc68-11* allele at this temperature. In a similar analysis, the *sug1-26* mutation was shown unable to suppress the *cdc68-11* allele. In a related study, Evans et al. (in preparation) constructed an N-terminal truncated *cdc68* allele, and this *cdc68-Δ922* allele, like *cdc68-1*, performs Cdc68 function at 23°C but confers temperature sensitivity at 35°C. The *sug1-26* mutation was found unable to suppress this *cdc68-Δ922* allele either (D. R. H. Evans, personal communication). Thus even though the three *cdc68* alleles tested all confer a similar temperature sensitivity, the *sug1* suppressors identified here can affect the temperature sensitivity of the *cdc68-1* allele only (Table 8).

b) *sug1* alleles identified by other means do not suppress *cdc68-1*.

I next addressed whether the ability to suppress the *cdc68-1* defect is unique to the *sug1* suppressor mutations isolated here. A *sug1* mutation was previously isolated by virtue of its ability to restore activator function to a mutant Gal4 protein encoded by the *gal4D* allele (Swaffield et al., 1992). I determined whether this *sug1-1* allele can suppress the *cdc68-1* temperature sensitivity. A heterozygous diploid strain (*cdc68-1/CDC68 sug1-1/SUG1*) was constructed and meiotic segregants were examined for growth at 35°C. (In preliminary experiments I determined that the *sug1-1* allele itself does not confer temperature sensitivity at 35°C) Each tetrad displayed a 2:2 segregation pattern for temperature sensitivity at 35°C; thus even though some temperature-sensitive spores showed some residual growth at 35°C (data not shown), the *sug1-1* mutation does not suppress *cdc68-1* temperature sensitivity efficiently (Table 8). I conclude from the above observation that the *sug1* alleles isolated by the ability to suppress the *cdc68-1* phenotype are different from the *sug1-1* allele that suppresses the *gal4D* phenotype.

During the course of this study, another *sug1* allele, originally named *cim3-1* (for clarity I will use the *sug1-3* designation in this thesis) was isolated because of its synthetic lethality in conjunction with the *cdc28-1N* mutation (Ghislain et al., 1993). I found that the *sug1-20* and *sug1-26* alleles shared some phenotypic similarity with *sug1-3*. For example, all three alleles cause temperature sensitivity, and at the restrictive temperature of 37°C mutant cells arrest in the G2/M phase of the cell cycle (Ghislain et al., 1993; Section E). I therefore determined whether the *sug1-3* allele also suppresses the *cdc68-1* mutant phenotype. The *sug1-3* mutation was first crossed into the strain 21R genetic background, and a temperature-sensitive segregant, CMY762-1 (*sug1-3*), was mated with the *cdc68-1 sug1-20* strain QXT20. Neither *sug1-3* nor *sug1-20* is temperature-sensitive at 35°C, so if *sug1-3* suppresses the *cdc68-1* mutation, every meiotic segregant should be temperature-resistant at 35°C regardless of the nature of the *cdc68* allele (whether *CDC68* or *cdc68-1*), due to presence of a *sug1* mutation. Phenotypic assessment of the meiotic products showed that there were temperature-sensitive segregants at 35°C. I infer from this observation that the *sug1-3* mutation does not suppress the *cdc68-1* phenotype (Table 8). Thus the interactions between *sug1* and *cdc68* are strictly allele-specific.

c) The ability of *sug1-26* to suppress *cdc68-1* can be overcome by either *sug1-1* or *sug1-3*.

In light of the above finding that Sug1 antagonizes Cdc68 activity, it is conceivable that the inability of the *sug1-1* and *sug1-3* alleles to suppress the *cdc68-1* mutation could be because these alleles encode too much residual Sug1 activity to alleviate the inhibition of Cdc68 function. I addressed this hypothesis using a genetic strategy, reasoning that if *sug1-1* and *sug1-3* have too much residual activity to relieve the *cdc68-1* transcription defect, then these mutations should be dominant over the *sug1-26* suppressing allele. To test this, I constructed diploid strains homozygous for the

cdc68-1 mutation but harboring different combinations of *sug1* alleles and assayed the resultant diploids for temperature sensitivity at 35°C. None of the three *sug1* alleles used here confer temperature sensitivity at 35°C. I found that *cdc68-1/cdc68-1 sug1-1/sug1-26* diploid cells failed to proliferate at 35°C, whereas *cdc68-1/cdc68-1 sug1-26/sug1-26* cells did proliferate (Table 9), indicating that the suppression by *sug1-26* was overwhelmed by the activity encoded by *sug1-1*. Similarly, *sug1-3* was also dominant to *sug1-26* (Table 9). These observations suggest that *sug1-1* and *sug1-3* alleles may encode too much residual Sug1 activity for *cdc68-1* suppression.

d) The *sug1-20* mutation does not suppress *gal4D*.

I also assessed the genetic interaction between *sug1-20* and *gal4D*. The *sug1-20* mutant strain QX202 was mated with the *GAL4*-deletion (*gal4Δ*) strain YJOZ to create strain QX202-4d (relevant genotype *sug1-20 gal4Δ leu2-3,112*). The *gal4D* plasmid pSB32gal4D was then transformed into strain QX202-4d; I found that Leu^+ transformants (harboring the plasmid-borne *gal4D* gene) failed to grow in galactose medium, whereas cells of strain QX202-4d transformed with pSB32GAL4 (carrying the wild-type *GAL4* gene) grew well under the same condition. Therefore the *sug1-20* allele does not suppress the *gal4D* phenotype (Table 8); suppression of the *gal4D* phenotype is unique to *sug1-1*. The *sug1-3* allele cannot suppress the *gal4D* defect either (J. C. Swaffield and S. A. Johnston, personal communication).

9. Molecular alterations are identified for *sug1* mutations.

As shown above, the *sug1* alleles isolated in this study as suppressors of the *cdc68-1* temperature sensitivity are phenotypically different from the *sug1-1* allele that suppresses *gal4D*. The *gal4D*-suppressing *sug1-1* allele encodes a Gly215-to-Asp substitution (J. C. Swaffield and S. A. Johnston, personal communication). I determined

Table 8. Allele-specific suppression* of *cdc68* and *gal4D* by *sug1*

	cdc68-1	*cdc68-11*	*cdc68-101::LEU2*	*gal4D*
sug1-20	++	-	dead	-
sug1-26	++	-	dead	ND
sug1-1	+/-	ND	ND	++
sug1-3 (cim3-1)	-	ND	ND	-
sug1::LEU2	dead	dead	dead	dead

* "++" = very good growth, "-" = no growth, "ND" = not determined.

The effect of *sug1* alleles on *gal4D* was assessed in galactose medium.

Table 9. *sug1* suppression in diploids

Genotype*	Growth at 35°C
sug1-26/sug1-26	+
sug1-1/sug1-26	-
sug1-1/sug1-1	-
sug1-3/sug1-26	-
sug1-3/sug1-3	-
SUG1/SUG1	-

* All diploid strains are homozygous for *cdc68-1*.

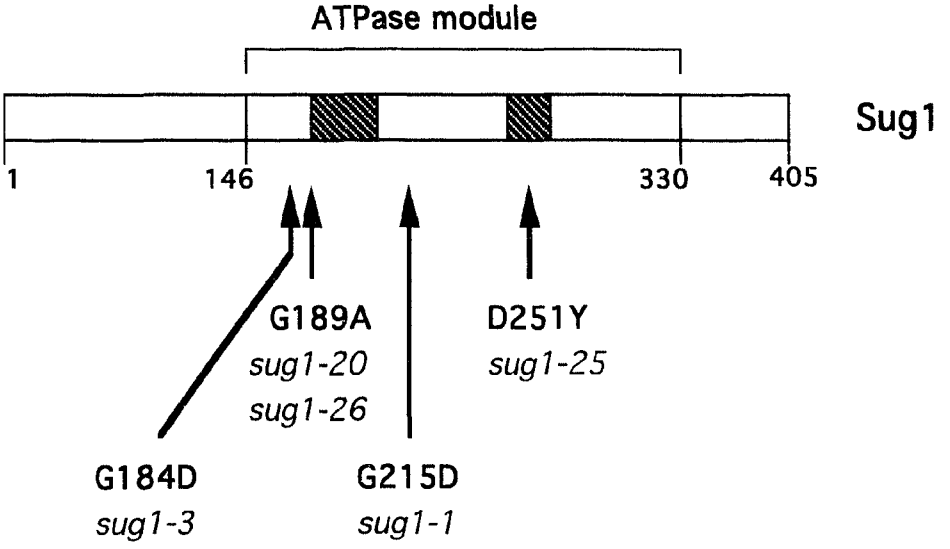
the molecular alteration in the *sug1-26* allele by using PCR to amplify the entire *sug1-26* mutant gene. Subsequent nucleotide sequence analysis revealed that the *sug1-26* mutation is a single base-pair substitution at nucleotide 568, causing a change of Gly189, an amino acid within a conserved ATP-binding motif of Sug1 (Swaffield et al., 1992), to Ala (Fig. 14). Although alanine also occurs in some NTP-binding sites at this position, all the Sug1-like proteins (also referred to as CAD proteins; Swaffield et al., 1995) contain glycine at this position. I also verified that this G189A alteration does in fact cause the *sug1-26* phenotype. For this, the 0.35-kbp *Spe*I-*Cla*I internal fragment of the wild-type *SUG1* gene was replaced with the mutant version from *sug1-26*, and this chimeric *sug1* mutant allele, named *sug1-261*, was cloned into the integrating vector YIp352. The resultant plasmid was linearized at the unique *Bst*XI site within the *sug1* ORF and used to transform a *SUG1/sug1::LEU2* heterozygous diploid strain. For some diploid transformants all four meiotic segregants were viable, indicating that the *sug1-261* plasmid had integrated at the *sug1::LEU2* disruption locus. Temperature sensitivity segregated in a 2:2 fashion among meiotic products of these diploid transformants, suggesting that the chimeric *sug1-261* allele (containing the G189A substitution), like *sug1-26* itself, allows cell viability but causes temperature sensitivity. Thus *sug1-1* and *sug1-26*, alleles that produce different phenotypes, have different molecular alterations.

I also amplified several other *sug1* alleles by PCR, among these two that were isolated as suppressors of the *cdc68-1* mutation, and another one, the *sug1-3* (*cim3*) allele that does not suppress *cdc68-1*. Nucleotide sequence analysis localized all the *sug1* mutations to the conserved ATPase module of Sug1 (Fig. 14). Although isolated independently, the *sug1-26* and *sug1-20* alleles have the same mutation that is responsible for suppression of the *cdc68-1* phenotype. It is noteworthy that *sug1* alleles that suppress the *cdc68-1* mutation all encode amino acid substitutions in the two highly conserved motifs shared by Sug1-like proteins (Dubiel et al., 1992; Fig. 14), whereas the *sug1-1* and

Figure 14. Sug1 mutant polypeptides.

Shaded boxes indicate the two highly conserved motifs of Sug1-like proteins (Dubiel et al., 1992). *sug1* mutant alleles were initially amplified by PCR using genomic DNA prepared from isogenic *sug1* mutant strains and cloned into the pRS316 vector. Nucleotide sequences were determined following a double-strand sequencing procedure as described in the Materials and Methods. The amino-acid alterations for different *sug1* alleles are indicated on top of allele names. For example, G189A indicates a change of Gly at position 189 to Ala.

Figure 14


ATPase module
Sug1
1
146
330
405
G189A
sug1-20
sug1-26
D251Y
sug1-25
G184D
sug1-3
G215D
sug1-1

sug1-3 mutations affect the amino acid composition of the ATPase module in regions outside of the two highly conserved motifs.

10. The *sug1-20* allele may stabilize ubiquitin-conjugates.

As an attempt to find out the mechanism by which *sug1* mutations suppress the *cdc68-1* defect, I initially addressed the possibility that Sug1 may modify Cdc68 activity at the posttranslational level, for example, by affecting stability of the Cdc68 protein. This idea was prompted by the findings of Ghislain et al (1993); they found that certain substrates for the ubiquitin degradation pathway are stabilized in *sug1-3* (*cim3-1*) mutant cells. As described above, *sug1-3* encodes a mutant protein different from those encoded by the *sug1* mutations identified here as suppressors of *cdc68-1*. I therefore determined whether *sug1* alleles identified as *cdc68-1* suppressors also affect protein stability by using plasmids encoding substrates for the ubiquitin degradation pathway. Plasmids harboring Ub-Pro-βgal, Ub-Arg-βgal, Ub-Leu-βgal, and Ub-Met-βgal (as control, Ghislain et al., 1993) were introduced into a *sug1-20* and a wild-type strain. By replica-plating to medium containing the chromogenic substrate X-gal I found that β-galactosidase levels were generally higher in *sug1-20* mutant cells compared to those in *SUG1* wild-type cells, as reflected by the intensity of the blue color (data not shown). The higher levels of β-galactosidase activity in *sug1* mutant cells can be a result of either increased gene expression or increased stability of these ubiquitin conjugates in *sug1-20* mutant cells. In light of the finding that the *sug1-3/cim3-1* allele stabilizes ubiquitin conjugates (Ghislain et al., 1993), the increased β-galactosidase activity seen here in *sug1-20* cells is most like a result of decreased degradation of ubiquitin conjugates.

11. An epitope-tagged Cdc68 protein.

Results in the previous sections of this chapter have established that Sug1 and Cdc68 interact genetically. To further investigate the interaction between Cdc68 and

Sug1, but at the protein level, I tagged the Cdc68 protein with an epitope. The epitope-tagging method was chosen in this case to ensure high specificity; the Cdc68 protein has a highly charged C-terminal region (Rowley et al., 1991) so that polyclonal antibodies raised against Cdc68 may cross-react with other proteins with similar negatively charged regions, especially acidic transcription activators.

The Cdc68 polypeptide was tagged with a triple HA-epitope (Field et al., 1988) in the N-terminal region (see Materials and Methods). This HA-tagged *CDC68* allele is designated *CDC68-200N*. HA-epitope addition to the Cdc68 polypeptide did not have any detectable effect on cell growth, and the *CDC68-200N* allele alleviated the temperature sensitivity caused by the *cdc68-1* mutation. Moreover, a single copy of the *CDC68-200N* gene integrated into the genome complemented the lethality caused by disruption of the *CDC68* gene: *CDC68-200N cdc68-101::LEU2* cells proliferate as efficiently as wild-type cells at all temperatures (data not shown). As a more sensitive test for Cdc68 function I determined the Spt phenotype for the HA-tagged *CDC68* gene. As expected, the *CDC68-200N* gene on a high-copy plasmid, like *CDC68* itself, caused an Spt$^-$ phenotype. However, unlike the wild-type *CDC68* gene, *CDC68-200N* in single copy also caused a weak Spt$^-$ phenotype (data not shown), suggesting that the *CDC68-200N* allele produces a modified Cdc68 polypeptide with slightly altered activity compared to that of the wild-type Cdc68 protein. Nonetheless, the HA-tagged Cdc68 protein has appreciable Cdc68 function.

12. The wild-type Cdc68 protein is stable.

The ability of *sug1* mutations to stabilize ubiquitin conjugates prompted an assessment of the ability of Sug1 to affect the stability of the Cdc68 protein. I therefore compared Cdc68 stability in wild-type and *sug1* mutant cells. Cells of wild-type and *sug1-26* strains (both having the HA-tagged *CDC68-200N* allele integrated in single copy next to the *cdc68-101::LEU2* disruption allele) were grown at 23°C, transferred to 35°C

and treated with cycloheximide to prevent further protein synthesis. In the absence of new protein synthesis (due to the inhibition caused by cycloheximide), protein levels detected by immunoblot analysis should reflect the stability of preexisting Cdc68 protein. As shown in Fig. 15, the HA-tagged wild-type Cdc68 protein was stable, and the *sug1-26* mutation had no detectable effect on Cdc68 abundance. I conclude that the stability of wild-type Cdc68 is unaffected by Sug1 activity.

13. *sug1* suppressor mutations decrease the degradation of mutant Cdc68 protein.

To detect the mutant polypeptide encoded by the *cdc68-1* allele, I added the HA-epitope sequence to the *cdc68-1* gene by a restriction-fragment swapping: the tagged *cdc68-1* gene is designated *cdc68-201N*. Epitope addition did not affect the mutant Cdc68 protein function appreciably, because a single copy of the *cdc68-201N* gene did not alter the temperature sensitivity of *cdc68-1* mutant cells, and on a high-copy plasmid the *cdc68-201N* gene suppressed the temperature sensitivity of *cdc68-1* mutant cells, as expected (see Section B).

A single copy of the HA-tagged *cdc68-1* gene (*cdc68-201N*) was introduced into *cdc68-1 SUG1* and *cdc68-1 sug1-26* strains. Stability of the mutant Cdc68 protein at 35°C was assessed as above by using cycloheximide to inhibit further protein synthesis. Under these conditions, the HA-tagged mutant Cdc68 protein was extremely labile in *SUG1* wild-type cells, whereas in *sug1* mutant cells the mutant Cdc68 protein was more stable, although not as stable as the wild-type Cdc68 protein: a significant degradation of mutant protein was detected within a 2-h period (Fig. 16). Thus, the *sug1* suppressor mutation somehow renders the mutant Cdc68 protein more stable.

The *cdc68-1* mutant cells behave virtually as wild-type cells at 23°C; I therefore asked whether the instability of the mutant protein was temperature-dependent. To this end, stability of the Cdc68 mutant polypeptide was determined at 23°C by following the same cycloheximide-treatment procedure. As shown in Fig. 17, the Cdc68 mutant

protein was unstable even at 23°C, but the rate of degradation was slower than at 35°C. The *sug1* mutations again rendered the Cdc68 mutant protein more stable even at the permissive temperature. Thus the mutant protein encoded by the *cdc68-1* allele is inherently labile, with higher rates of degradation at the restrictive temperature, and the *sug1* suppressor mutations are able to decrease degradation of the mutant Cdc68 protein but only to a limited degree.

14. Suppression is unlikely attributable to stabilization of the mutant Cdc68 protein.

As shown above, not all *sug1* mutations suppress the *cdc68-1* mutation. I was therefore able to determine if *sug1* suppression of *cdc68-1* temperature sensitivity is a consequence of increased stability of the mutant Cdc68 protein. As shown in Fig. 16, the *sug1-3* allele that does not suppress the temperature sensitivity of *cdc68-1* stabilized mutant Cdc68 protein to an even greater degree than the suppressing *sug1-26* allele. In addition, the *san1-3* mutation that suppress *cdc68-1* defects efficiently (see Section B) had no appreciable effect on the mutant Cdc68-1 protein stability (data not shown), suggesting that stabilization of the mutant Cdc68-1 protein may not be required for restoring Cdc68 activity. I conclude from this observation that stabilization of the mutant Cdc68 protein by a *sug1* mutation does not account for suppression of the *cdc68-1* mutant phenotype.

15. Sug1 does not coimmunoprecipitate with the HA-tagged Cdc68 protein.

The allele-specific interactions between *cdc68* and *sug1* alleles may suggest a direct physical contact between the Cdc68 and Sug1 proteins. I addressed this question by determining whether the Sug1 protein can be precipitated by the anti-HA antibody from extracts of yeast cells containing HA-tagged Cdc68 protein. In this experiment the

Figure 15. Stability of wild-type Cdc68 protein.

Cells of the *SUG1* strain QX6811 and the *sug1-26* strain QX2614 (both containing the HA-tagged wild-type Cdc68 protein) were grown at 23°C. At time zero, cycloheximide (CHX) was added to prevent further protein synthesis, and cells were transferred to 35°C for further incubation. Extracts were prepared at the indicated times and resolved by SDS-PAGE. Protein was then transferred to PVDF membrane and the blot was probed with anti-HA monoclonal antibody to detect the HA-tagged Cdc68 protein. The levels of tubulin, detected using an anti-tubulin antibody, were employed as loading controls.

Figure 15

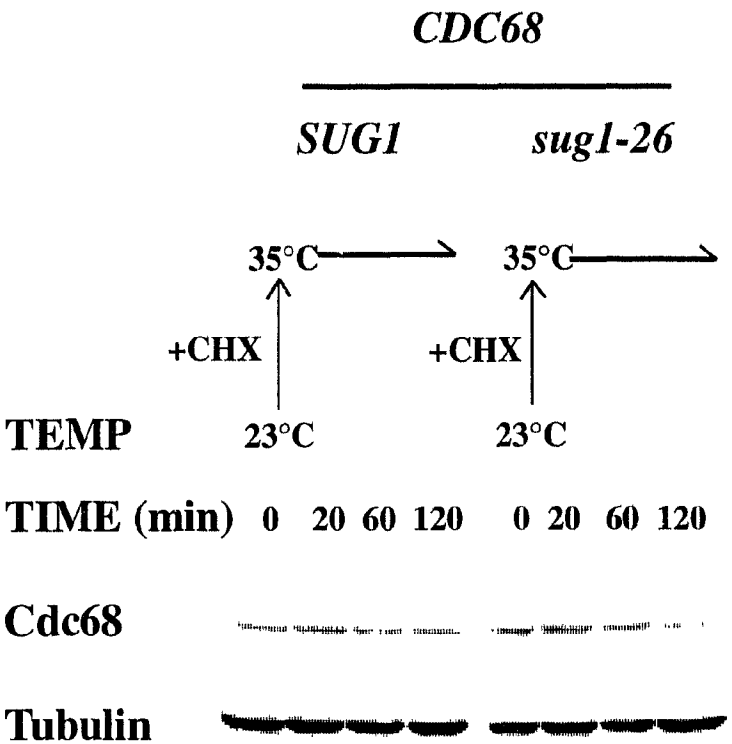
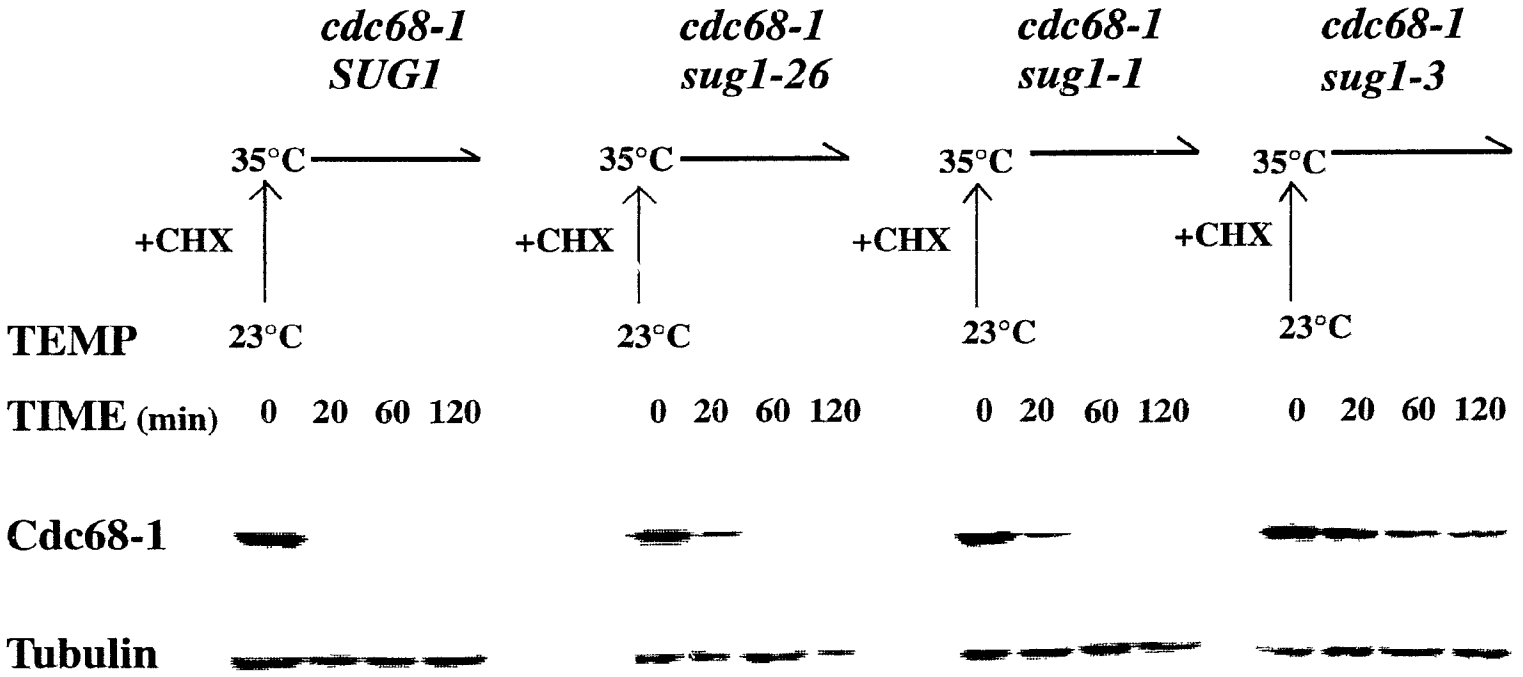

CDC68
SUG1
sug1-26
35°C
35°C
+CHX
+CHX
TEMP
23°C
23°C
TIME (min) 0 20 60 120 0 20 60 120
Cdc68
Tubulin

Fig. 16. Effects of Sug1 activity on mutant Cdc68 protein stability.

Cells of *SUG1 cdc68-1*, *sug1-26 cdc68-1*, and *sug1-1 cdc68-1*, and *sug1-3 cdc68-1* strains (all bearing an integrated HA-tagged *cdc68-1* gene) were incubated at 23°C, and extracts were prepared at indicated times after the addition of cycloheximide and incubation at 35°C. After resolution by SDS-PAGE, protein was transferred to PVDF membrane and probed with anti-HA antibody to detect the HA-tagged Cdc68-1 mutant protein. Levels of tubulin, detected by an anti-tubulin antibody, served as loading controls.

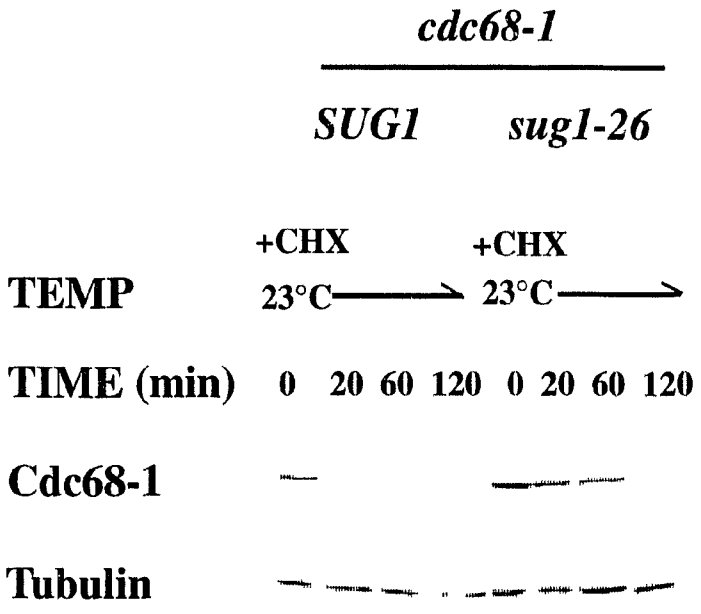
Figure 16


cdc68-1
SUG1
cdc68-1
sug1-26
cdc68-1
sug1-1
cdc68-1
sug1-3
35°C
+CHX
23°C
TEMP
TIME (min)
0 20 60 120
Cdc68-1
Tubulin

17. Intrinsically labile mutant Cdc68 protein.

Cells of *SUG1 cdc68-1* and *sug1-26 cdc68-1* strains (both bearing an integrated HA-tagged *cdc68-1* gene) were incubated at 23°C. Extracts were prepared at different time intervals after the addition of cycloheximide and continuing incubation at 23°C. Protein was separated by SDS-PAGE and transferred to PVDF membrane. The HA-tagged Cdc68-1 protein was detected using anti-HA antibody, and tubulin levels were employed as loading controls.

Figure 17


cdc68-1
SUG1
sug1-26
+CHX
+CHX
TEMP
23°C
23°C
TIME (min)
0 20 60 120 0 20 60 120
Cdc68-1
Tubulin

Sug1 protein was tagged with the phage T7 S10 epitope to ensure specific detection of the Sug1 protein (Swaffield et al., 1995). As shown in Fig. 18, a portion of HA-tagged Cdc68 protein was precipitable with polyclonal anti-HA antibodies, but the S10-tagged Sug1 protein was not precipitated by anti-HA antibodies. Because of the presence of significant amounts of HA-tagged Cdc68 protein that could not be precipitated by the anti-HA antibodies even when antibodies were in excess (data not shown), the possibility remains that this nonprecipitable portion of Cdc68 may interact with Sug1. Nonetheless, this experiment indicates that the Sug1 protein does not interact with the fraction of Cdc68 protein that is precipitable by the anti-HA antibodies.

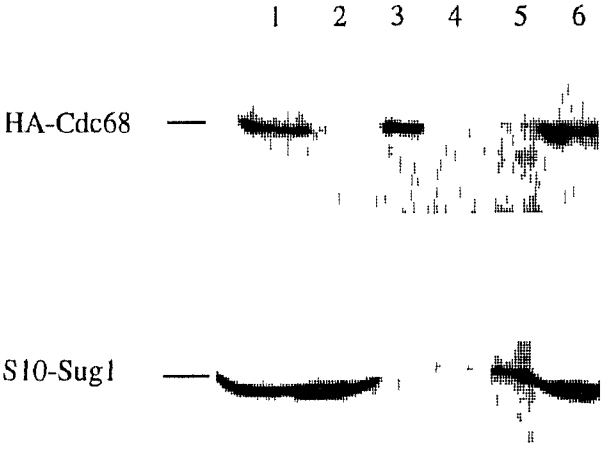
16. Sug1 does not interact with Cdc68 by the two-hybrid assay.

The above coimmunoprecipitation experiment is not conclusive and therefore I took a second approach, the two-hybrid assay (Chien et al., 1991). This procedure has been widely used to identify protein-protein interactions in various systems. This interaction assay is based on the fact that transcription activators are modular and have separable DNA-binding and activation domains that can function in *trans* to potentiate transcription. For this experiment the entire *CDC68* ORF was amplified by PCR and fused to the LexA DNA-binding domain in plasmid pSH2-1. The resultant fusion plasmid, pBD-CDC68, was found to provide wild-type Cdc68 function, which demonstrates that the LexA-Cdc68 polypeptide is properly expressed. Moreover, the LexA-Cdc68 fusion protein does not activate transcription by itself (see Section D) and can therefore be used for the two-hybrid assay. The entire *SUG1* ORF was also PCR-amplified and fused to the Gal4 activation domain in plasmid pGAD3. The Sug1-Gal4 fusion plasmid, pAD-SUG1, provides wild-type Sug1 function: plasmid pAD-SUG1 complemented the *sug1-20* temperature-sensitive mutation, whereas *SUG1* fused in reverse orientation to the Gal4 activation-domain ORF did not, suggesting that Sug1-Gal4 is expressed as a fusion protein. The fusion plasmids pBD-CDC68 and pAD-SUG1

Figure 18. S10-tagged Sug1 does not co-immunoprecipitate with the HA-tagged Cdc68 protein.

Whole cell extracts from strain QX6800 (containing both the S10-tagged Sug1 protein and the HA-tagged Cdc68 protein) and strain QX6801 (containing the S10-tagged Sug1 protein but untagged Cdc68 protein) were subjected to immunoprecipitation using polyclonal anti-HA antibodies as described in the text. Extracts or immunoprecipitates were then resolved by SDS-PAGE, transferred to PVDF membrane, and probed separately with monoclonal antibody against HA (to detect Cdc68) or against S10 (to detect Sug1). Lane 1, QX6800 extract; lane 2, QX6801 extract; lane 3, immunoprecipitate of QX6800 extract (from 5X extract used in lane 1); lane 4, immunoprecipitate of QX6801 extract (from 5X extract used in lane 2); lane 5, *S. aureus* cells alone; lane 6, the supernatant of QX6800 extract after immunoprecipitation (from 2X extract used in lane 1).

Figure 18



were introduced into yeast strain CTY10-5D that harbors a LexA operator-*lacZ* fusion reporter construct. As shown in Table 10, there was no appreciable increase in β-galactosidase expression from the reporter construct in cells co-transformed with pBD-CDC68 and pAD-SUG1 compared to cells with either pBD-CDC68 or pAD-SUG1 alone. Thus, results of this two-hybrid assay do not suggest a physical interaction between the Cdc68 and Sug1 proteins.

17. Sug1 and San1 modulate Cdc68 activity independently.

Both *san1* and *sug1* mutations were isolated from the same suppressor screen by their ability to suppress the temperature sensitivity caused by the *cdc68-1* mutation. Several lines of evidence suggest that the Sug1 and San1 proteins have different functions. The *SUG1* gene is essential whereas *SAN1* is dispensable for cell viability. Secondly, as described below (Section E) the *sug1-26* mutant allele exhibits a conditional nuclear-division defect, whereas neither a *san1* mutation nor a *san1*-null allele has a detectable phenotype in an otherwise wild-type strain. Thirdly, enfeebled Sug1 activity increases stability of mutant Cdc68 protein, albeit perhaps indirectly (see Discussion), while I found that the *san1-3* mutation had no effect on the mutant Cdc68 protein stability. Nonetheless, both of them counteract Cdc68 activity: either loss of San1 function or partial loss of Sug1 function suppresses the *cdc68-1* phenotype at the restrictive temperature of 35°C. To address the question of whether San1 and Sug1 proteins participate in the same process to modulate Cdc68 activity, I removed the *SAN1* gene from the *cdc68-1 sug1-26* suppressed strain QXT26. As shown in Fig. 19A, when both the *SUG1* and *SAN1* genes were mutated the *cdc68-1* mutant cells could proliferate at 37°C, a temperature at which neither a *san1* nor *sug1* single mutation suppresses the *cdc68-1* phenotype efficiently. This observation is perplexing, given the facts that the *sug1-26* allele by itself causes temperature sensitivity at 37°C (see Section E), and the *san1Δ::URA3* null allele cannot suppress the temperature sensitivity of *sug1-26*

Table 10. Sug1 does not interact with Cdc68 by the two-hybrid assay*

Plasmid	β-galactosidase activity (U)
pBD-CDC68 + pGAD3	0.048
pBD-CDC68 + pAD-SUG1	0.038
pSH2-1 + pADSUG1	0.031
pSH17-4	974

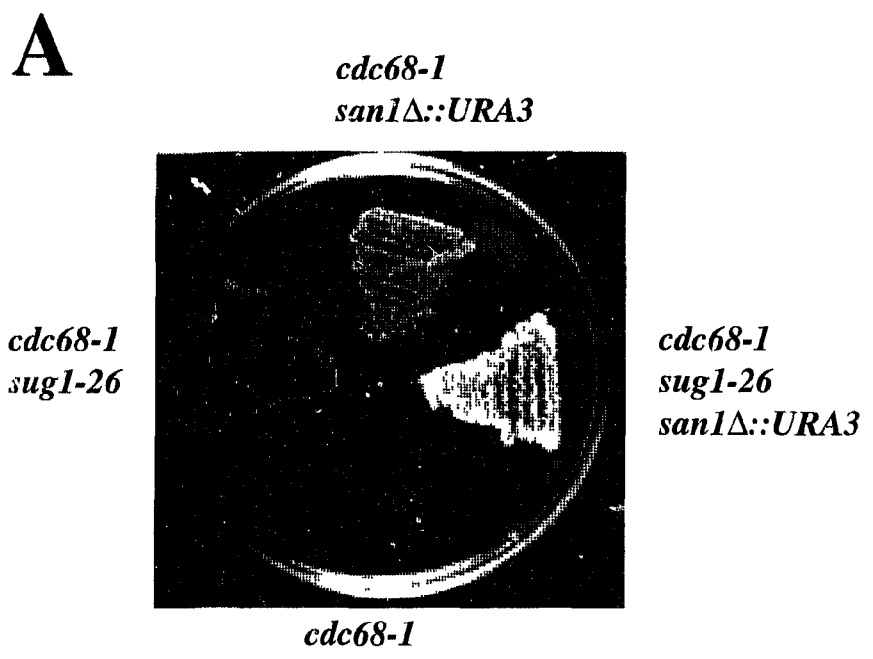
* Strain CTY10-5D was transformed and grown under selective conditions to maintain plasmids. β-galatosidase assays were performed for permeabilized cells as described in the Materials and Methods. For each combination of plasmids, 2-4 independent transformants were used, and variation of β-galactosidase activity was found to be less than 20% (data not shown).

Figure 19. *sug1* and *san1* suppress *cdc68-1* independently.

(A) *cdc68-1 sug1-26 san1Δ::URA3* triple-mutant cells grow at 37°C. Cells of strains QX401 (*cdc68-1 san1Δ::URA3*), QXT26 (*cdc68-1 sug1-26*), QXT26-7 (*cdc68-1 sug1-26 san1Δ::URA3*), and 68507A (*cdc68-1*), were incubated on YEPD solid medium at 23°C and replica-plated to YEPD for further incubation at 37°C as shown. Strain QXT26-7 was constructed by transforming QXT26 with the 2.6-kbp *san1Δ::URA3* *Bam*HI-*Eco*RI fragment of pLG7.

(B) the *san1Δ::URA3* null allele does not suppress the temperature sensitivity of *sug1-26*. Growth at 37°C was assessed as described in (A). Strains used were QXT26, QXT26-7 (see above), QX261 (*sug1-26*), and QX261-7 (*sug1-26 san1Δ::URA3*). QX261-7 was constructed similarly as for QXT26-7, except that the 2.6-kbp *san1Δ::URA3* DNA fragment was transformed into QX261.

Figure 19


A
cdc68-1
san1Δ::URA3
cdc68-1
sug1-26
cdc68-1
sug1-26
san1Δ::URA3
cdc68-1
B
sug1-26
san1Δ::URA3
sug1-26
cdc68-1
sug1-26
san1Δ::URA3
cdc68-1
sug1-26

(Fig. 19B). At 37°C, *cdc68-1 san1Δ::URA3 sug1-26* triple-mutant cells somehow manage to proliferate. In any case, this finding that Sug1 and San1 have additive effects on Cdc68 activity suggests that these two suppressor proteins modulate the Cdc68 activity independently of each other, most likely through different pathways.

Section D. The role of Cdc68 in transcription

As described in previous sections of this thesis, one approach to gain further insight into the Cdc68 protein function is to identify and characterize other proteins that are involved in the transcriptional regulation mediated by Cdc68. In this section, I report related studies focusing on the Cdc68 protein itself and the major issue addressed here is how Cdc68 regulates transcription.

1. The Cdc68 protein resides in the nucleus.

I initially determined the subcellular localization of the Cdc68 protein. Based on the fact that Cdc68 regulates transcription initiation, it is conceivable that Cdc68 is in the nucleus. To verify this prediction, the intracellular location of Cdc68 was determined by indirect immunofluorescence using a yeast strain in which the only functional Cdc68 protein is tagged with HA epitope. As shown in Fig. 20, the Cdc68 protein is indeed located in the nucleus.

2. Cdc68 is implicated in promoter usage.

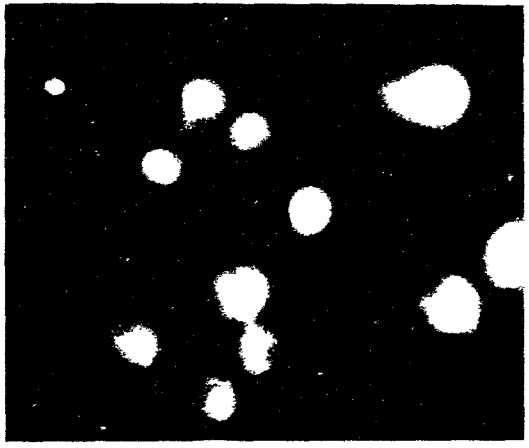
The *cdc68-1* and *cdc68-197* mutations were found to affect promoter competition at *his4* and *lys2* loci harboring δ-insertions (Malone et al., 1991). During the course of this study, I noted that transcription initiation from several wild-type promoters was altered in *cdc68-1* mutant cells at the restrictive temperature. As shown in Fig. 21, a second shorter mRNA appeared for the *SUG1*, *SWE1*, and *CLB4* genes in *cdc68-1* cells at 35°C, whereas in *sug1-26 cdc68-1* suppressed cells there was only one mRNA species for

Figure 20. Nuclear localization of the Cdc68 protein.

QX6811 cells that harbor an integrated HA-tagged *CDC68* gene were grown in YM1 liquid medium to early log phase, fixed in formaldehyde and permeabilized using glucuronidase and β-mercaptoethanol, and subjected to indirect immunofluorescence using anti-HA monoclonal antibody 12CA5 and FITC-conjugated anti-mouse IgG. DAPI was included in the mounting medium to reveal nuclei.

Figure 20

Cdc68 protein

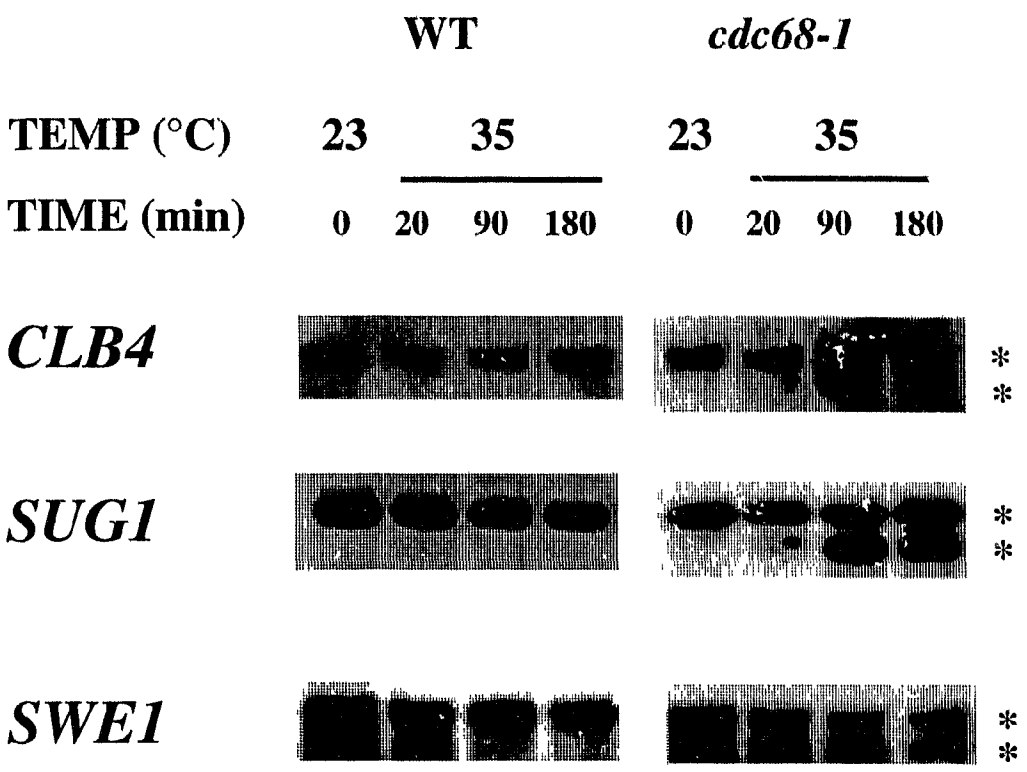


Nuclear stain

Figure 21. Cdc68 affects transcription initiation-site selection.

Total yeast RNA was extracted from strains 21R (wild type) and 68507A (*cdc68-1*) growing at 23°C and also after incubation at 35°C. RNA was electrophoretically resolved on formaldehyde-containing agarose gel, transferred to nylon membrane, and probed separately with radiolabeled restriction fragments containing coding sequences for the *CLB4*, *SUG1*, and *SWE1* genes.

Figure 21


WT
cdc68-1
TEMP (°C)
23
35
23
35
TIME (min)
0
20
90
180
0
20
90
180
CLB4
SUG1
SWE1
*
*
*
*
*
*

each of the above three genes, suggesting that the occurrence of the shorter mRNA is due to enfeebled Cdc68 activity. In general, a shorter mRNA species may result from an internal transcription initiation or a premature termination. Since Cdc68 was shown to affect transcription initiation at the *his4-912δ* and *lys2-128δ* loci, it is most likely that the second mRNA detected for the *SUG1*, *SWE1* and *CLB4* genes is a consequence of altered transcription initiation. Thus, Cdc68 may also affect transcription initiation-site selection for wild-type promoters.

3. Cdc68 is an activator of histone gene expression.

Either increased gene dosage of the *CDC68* gene or a *cdc68* mutation confers an Spt$^-$ phenotype (Malone et al., 1991; Rowley et al., 1991). This effect of altered Cdc68 activity resembles that resulting from altered histone gene dosage (Clark-Adams et al., 1988). It is therefore possible that the Spt$^-$ phenotype caused by altering Cdc68 activity is mediated through changes in histone gene expression. I investigated histone gene expression by determining mRNA abundance for the *HTA1/HTB1* locus, which is one of the two gene pairs encoding histones H2A and H2B. As shown in Fig. 22A, transcription of the *HTA1* and *HTB1* genes at this locus decreased in *cdc68-1* mutant cells at the restrictive temperature of 35°C, and the *san1::HIS3* disruption allele reversed this effect. It is formally possible that the decrease in *HTA1/HTB1* transcription is a secondary effect of G1 arrest caused by *cdc68-1* because histone genes are not expressed in G1 phase of the cell cycle (Osley, 1991); however, the rapidity of the decrease in *HTA1/HTB1* mRNAs argues against this explanation: the mRNA level for *HTA1/HTB1* was significantly lower (compared to that in *cdc68 san1* suppressed cells) even 20 min after transfer *cdc68-1* cells to 35°C, at which point G1 arrest had not occurred. Thus, it is most likely that Cdc68 has a direct effect on the expression of histone genes.

Histone gene expression is periodic in the yeast cell cycle, with maximal synthesis of histone mRNAs during DNA replication (for review see Osley, 1991). Several genes

have been implicated in the regulation of histone gene expression; mutations in these regulatory genes allow histone genes to be transcribed even when DNA replication is inhibited by hydroxyurea treatment (Osley and Lycan, 1987; Xu et al, 1992). *CDC68* is unlikely to be a member of this class of histone regulatory genes, because altered Cdc68 activity (either by mutation or by increased gene dosage) did not allow *HTA1* transcription after DNA replication was blocked by hydroxyurea treatment (Fig. 22B). Thus my Northern data suggest that the Cdc68 protein activates at least some histone gene expression without apparent influence on the S-phase regulation of histone gene transcription.

4. The *cdc68-1* mutation changes plasmid linking number.

I addressed the possibility that Cdc68 regulates transcription by affecting chromatin structure. This was done by determining if the *cdc68-1* mutation affects plasmid linking number. For a closed circular plasmid, the assembly of each nucleosome introduces one superhelical turn, also called linking number; therefore the linking number of a plasmid reflects nucleosome density on the plasmid DNA (Worcel et al., 1981). The low-copy plasmid pRS316 was introduced into *cdc68-1* and wild-type cells by transformation, and total DNA was prepared from transformants growing at 30°C, and resolved by agarose gel electrophoresis in the presence of the intercalating chemical chloroquine. Hybridization of the DNA blot using radiolabeled pRS316 DNA showed that at a permissive temperature of 30°C there was a significant increase of plasmid linking number in *cdc68-1* mutant cells, as reflected by an increased length of the DNA ladder (Fig. 23). Thus the Cdc68 protein indeed affects plasmid linking number; decreased Cdc68 activity causes an increase in nucleosome density on a plasmid. Without further experiments, this observation does not answer the question of whether the change of nucleosome assembly is a cause or an effect of altered transcription activity

Figure 22. Cdc68 affects expression of histone genes.

(A) Total RNA was extracted from yeast strains 68507A (*cdc68-1*) and YRQ9344 (*cdc68-1 san1::HIS3*) grown at 23°C and also after incubation at 35°C for the indicated times. RNA was electrophoretically resolved on a formaldehyde-containing agarose gel, transferred to nylon membrane, and probed with a radiolabeled DNA fragment containing coding sequences for *HTA1/HTB1* and *ADK1* (a gene next to *HTA1* coding for adenylate kinase).

(B) Determination of the Hir phenotype. Northern analysis of *HTA1* and *ADK1* (used as internal control) mRNA abundance from cells grown in the absence (-) or presence (+) of hydroxyurea (HU) for 30 min at 30°C. Strains used were 21R (wild type[WT]), 68507A (*cdc68-1*), and 21R transformed with multicopy *CDC68* plasmid pSC2-1.

Figure 22

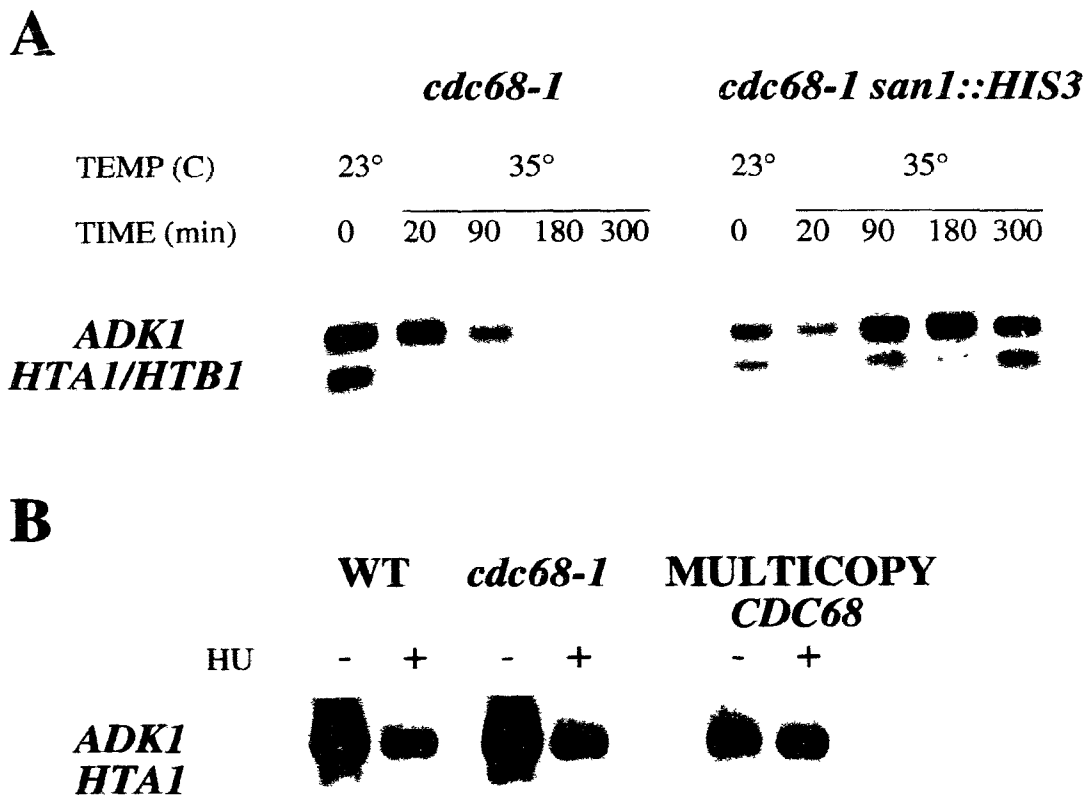
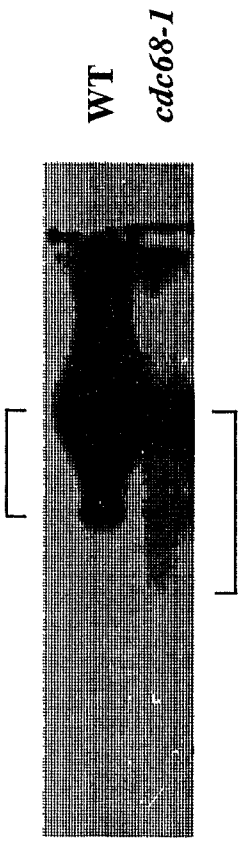

A
cdc68-1
cdc68-1 san1::HIS3
TEMP (C)
23°
35°
23°
35°
TIME (min)
0
20
90
180
300
0
20
90
180
300
ADK1
HTA1/HTB1
B
WT
cdc68-1
MULTICOPY
CDC68
HU
-
+
-
+
-
+
ADK1
HTA1

Figure 23. The *cdc68-1* mutation affects plasmid linking number.

The *cdc68-1* strain 68507A and a congenic wild-type strain 21R were transformed with the pRS316 vector and grown in SC-Ura liquid medium (SC medium lacking uracil) at 30°C. Total DNA was isolated (after first converting cells to spheroplasts), resolved on a chloroquine-containing agarose gel, transferred to a nylon membrane, and probed with radiolabeled pRS316 DNA. The length of ladders (brackets) reflects relative plasmid linking number.

Figure 23



in *cdc68-1* cells. Considering that the increase in plasmid linking number caused by *cdc68-1* was detected at 30°C, a temperature at which no significant decrease in transcriptional activity is apparent, it is likely that Cdc68 affects nucleosomal structure and thereby regulates transcription.

5. Cdc68 is required for *SUC2* expression.

The Cdc68 protein has been shown to have a global effect on gene transcription, and Cdc68 also affects expression from the *UAS*-less *SUC2* gene. I determined whether transcription from the intact *SUC2* promoter also requires Cdc68 activity. This question was addressed by examining the temperature dependence of the growth of *cdc68-1* mutant cells in sucrose-containing medium. Although *cdc68-1* mutant cells can grow, albeit slowly, in glucose-containing medium at 34°C, I found that the same mutant cells failed to proliferate in sucrose-containing medium at 34°C (Fig. 24A). This temperature-sensitive growth in sucrose medium was accompanied by diminished *SUC2* gene transcription at the restrictive temperature; at 34°C there was a marked decrease in *SUC2* transcription in *cdc68-1* mutant cells (Fig. 24B). Thus, wild-type Cdc68 activity is required for activation of the wild-type *SUC2* promoter, but inhibits transcription from the *UAS*-less *SUC2* promoter (Malone et al., 1991). Furthermore, both the *sug1* and *san1* suppressor mutations restored *SUC2* transcription in *cdc68-1* mutant cells (Fig. 24B), and suppressed the temperature sensitivity (at 34°C) on sucrose (Fig. 24A). Thus, the responsiveness of *SUC2* to Cdc68, Sug1, and San1 resembles that of other genes.

6. Overexpression of *CDC68* bypasses the requirement of Swi2 for *HO* expression.

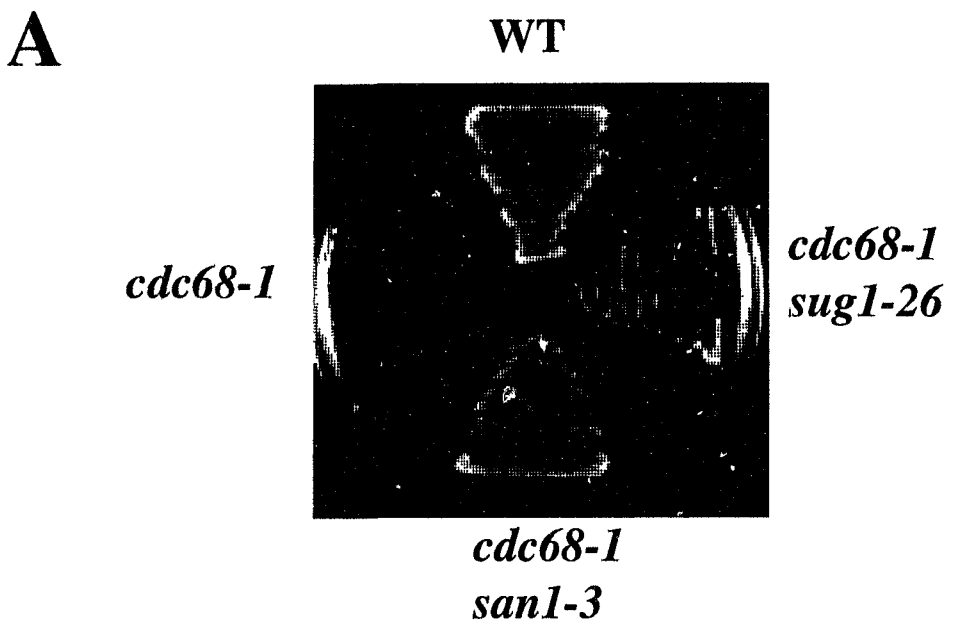
The Cdc68 protein has widespread transcriptional effects and influences the nucleosomal structure of a test plasmid (Fig. 23); thus it is likely that Cdc68 may impinge on transcription by affecting chromatin structure. This suggests in turn that

Figure 24. Cdc68 activates *SUC2* transcription.

(A) Growth of cells on sucrose medium. Cells were grown on YEPD solid medium at 23°C and replica-plated to sucrose medium (containing antimycin A to prevent aerobic growth) for further incubation at 34°C. Strains used were 21R (wild-type), 68507A (*cdc68-1*), QX3 (*cdc68-1 san1-3*), and QXT26 (*cdc68-1 sug1-26*).

(B) Northern analysis. Cells of the four strains described in (A) were grown at 23°C in sucrose-containing liquid medium to early log phase and transferred to 34°C for further incubation. Total RNA was prepared from samples taken at the times indicated, resolved on formaldehyde-containing agarose gel, transferred to nylon membrane, and probed for *SUC2* mRNA and *TUB2* mRNA (as loading control).

Figure 24


A
WT
cdc68-1
cdc68-1
sug1-26
cdc68-1
san1-3
B
WT
cdc68-1
cdc68-1
sug1-20
cdc68-1
san1-3
TEMP (°C) 23 34 23 34 23 34 23 34
TIME (min) 0 20 90 0 20 90 0 20 90 0 20 90
SUC2
TUB2

Cdc68 function may be related to that of the Snf/Swi complex that activates transcription by remodeling chromatin structure (for review see Winston and Carlson, 1992). Indeed, a genetic interaction between Cdc68 and components of the Snf/Swi complex has been observed: mutations in the *CDC68* gene suppress defects in the Snf2 or Snf5 protein, two components of the Snf/Swi complex, and thereby allow the *SUC2* gene to be transcribed in *snf2* or *snf5* mutant cells (Malone et al., 1991). I determined the effect of overexpression of *CDC68* in a *swi2 (snf2)*-disruption strain by assessing the expression of an *HO::lacZ* fusion gene (The Snf/Swi complex activates *HO* transcription). As shown in Fig. 25, although having little effect on *HO* expression in *SWI2* wild-type cells, elevated *CDC68* gene dosage significantly increased *HO* gene expression in *swi2::HIS3* mutant cells. Thus, increased Cdc68 gene dosage can bypass the requirement of Swi2 for the activation of *HO* gene expression, suggesting that Cdc68 functions either downstream of the Swi/Snf complex or in a parallel and alternative pathway.

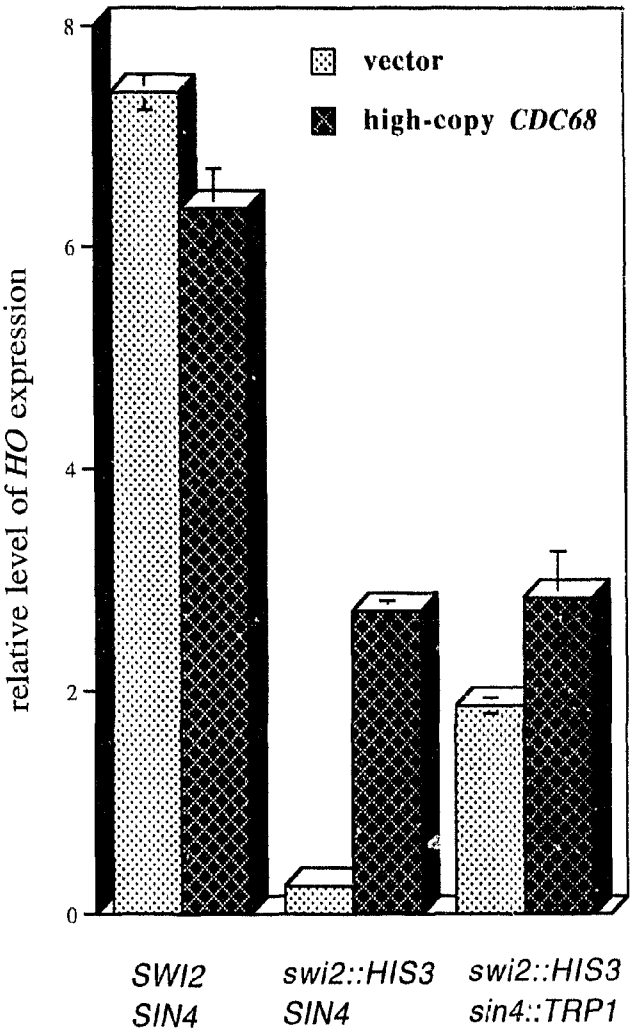
The Sin4 protein is a negative regulator of *HO*, and the *sin4::TRP1* disruption allele suppresses *swi* mutations, including a *swi2* disruption (Jiang and Stillman, 1992). I found that the effect of high-copy *CDC68* is comparable to that of the *sin4::TRP1* disruption allele, and furthermore that the suppression of *swi2::HIS3* by high-copy *CDC68* and *sin4::TRP1* is not additive; Increased *CDC68* dosage did not cause further increase in *HO* gene expression in *swi2::HIS3 sin4::TRP1* cells (Fig. 25). This lack of an additive effect may suggest that both the Cdc68 and Sin4 proteins function in the same pathway to affect *HO* gene expression.

The suppression of the *swi2::HIS3* disruption allele by elevated *CDC68* gene dosage is unlikely to be a secondary effect of increased transcription of another gene, because no increase in transcript abundance of any gene has been detected when the *CDC68* gene is overexpressed (Fig. 26). Thus, most likely in a direct way, elevated expression of the *CDC68* gene compensates for the impaired Snf/Swi complex.

Figure 25. Elevated *CDC68* gene dosage allows *HO* expression in the absence of *SWI2*.

All strains contain an *HO-LacZ* reporter gene. Cells were grown in liquid SC-Ura medium (SC medium lacking uracil) at 30°C to log phase. Expression of the *HO* gene was monitored by measuring β-galactosidase activity for permeabilized cells. Strains used were DY131 (*SWI2 SIN4*), DY1736 (*swi2::HIS3 SIN4*), and DY1825 (*swi2::HIS3 sin4::TRP1*), each harboring either the YEp352 vector or the high-copy *CDC68* plasmid p68-Ba-1A.

Figure 25


relative level of *HO* expression
8
6
4
2
0
vector
high-copy *CDC68*
SWI2
SIN4
swi2::HIS3
SIN4
swi2::HIS3
sin4::TRP1

7. Cdc68 does not function as an activator.

The deduced protein sequence of Cdc68 does not contain any identifiable DNA-binding domains (Rowley et al., 1991). This does not in any way contradict the suggestion that Cdc68 functions as a transcription activator, because Cdc68 may gain access to DNA with the aid of a DNA-binding protein. To address the question of whether Cdc68 can function as a transcription activator, the entire *CDC68* ORF was fused to the LexA DNA-binding domain. This fusion protein performs wild-type Cdc68 function because the chimeric gene expressing the LexA-Cdc68 fusion protein complemented the *cdc68-101::LEU2* disruption mutation at all temperatures. The plasmid-borne chimeric *LexA-CDC68* gene was introduced into strain CTY10-5D containing a LexA operator-LacZ fusion reporter gene. Results of β-galactosidase assays indicated that this LexA-Cdc68 fusion protein cannot activate transcription (Table 11). Similarly, an N-terminally truncated Cdc68 protein, Cdc68-Δ157, although providing Cdc68 activity when fused to the LexA-DNA binding domain (plasmid pBD-CDC68-Ec-3), does not function as an activator either (Table 11). Thus, Cdc68 does not function as a transcription activator when tethered to DNA by a heterologous DNA-binding domain.

In a separate experiment, I have found that overexpression of the *CDC68* gene from a high-copy plasmid does not increase transcription of Cdc68-dependent genes (Fig. 26). Taken together these observations suggest that although Cdc68 is required for the expression of many genes, Cdc68 appears not to activate transcription in a similar manner as conventional transcription activators.

Section E. Sug1 is required for mitosis.

In previous sections of this thesis, I have described the identification and analysis of two suppressor genes, *SAN1* and *SUG1*, that encode proteins antagonizing Cdc68 function. In this chapter I report studies aimed at investigating another aspect of the Sug1 protein function. During the course of this thesis work I independently observed that

Table 11. LexA-Cdc68 does not activate transcription*

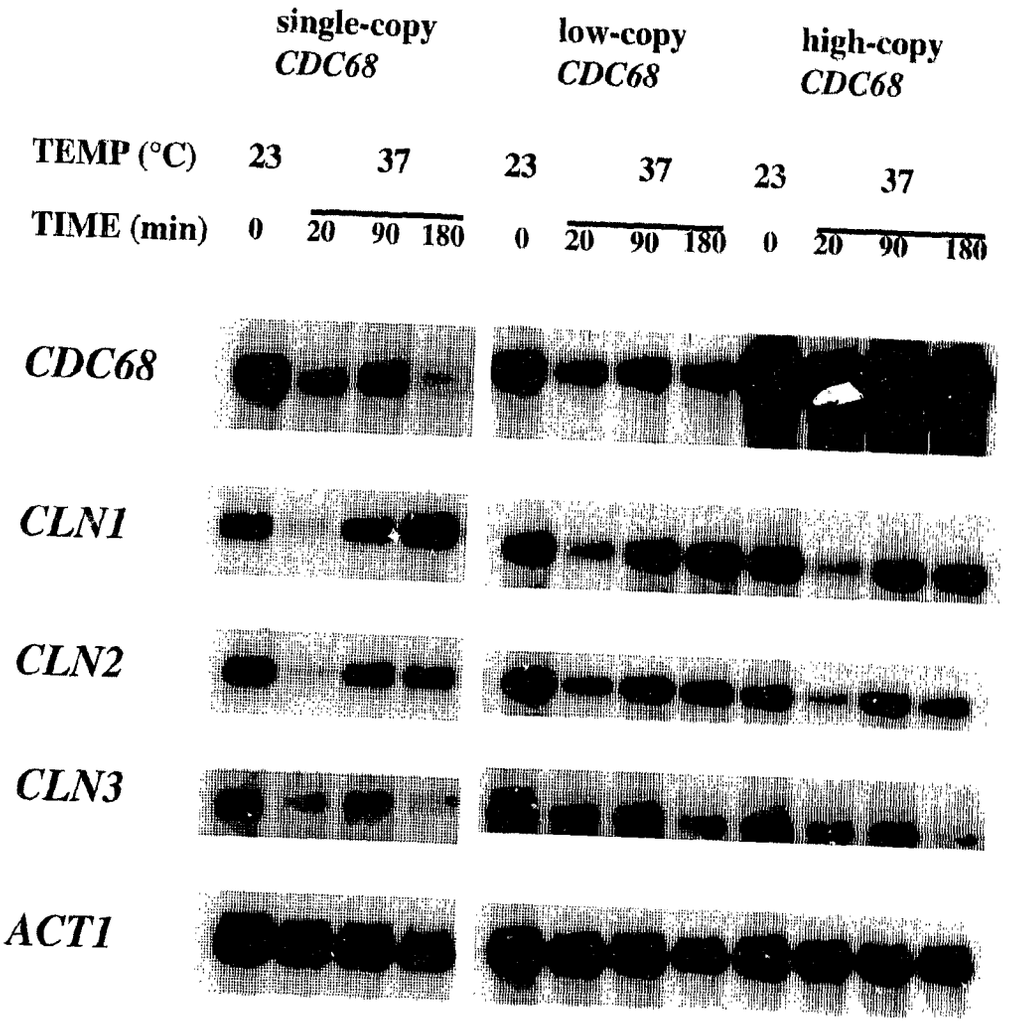
plasmid	β-galactosidase activity (U)	activation
pSH2-1	0.049	-
pBD-CDC68	0.057	-
pBD-CDC68-Ec-3	0.040	-
pSH17-4	974	+++

* CTY10-5D transformants were grown under selective conditions to maintain plasmids. β-galactosidase activity was determined for 2-4 transformants in each case, and variation was less than 15% (data not shown).

Figure 26. Increased *CDC68* gene dosage does not affect expression of Cdc68-dependent genes.

Total yeast RNA was extracted from cells grown in SC-Ura liquid medium (SC medium lacking uracil) at 23°C and also after incubation at 37°C for the times indicated. Strains used were 21R harboring the YEp352 vector (single-copy *CDC68*), 21R harboring pDE-683 (low-copy *CDC68*), and 21R harboring p68-Ba-1A (high-copy *CDC68*). Equal amount of RNA for each sample was electrophoretically resolved on formaldehyde-containing agarose gels, transferred to nylon membranes, and probed with radiolabeled DNA fragments for the genes indicated. The decrease of mRNA levels for *CLN1*, *CLN2*, and *CLN3* after 20 min of incubation at 37°C was a heat-shock response (Rowley et al., 1993).

Figure 26


single-copy CDC68
low-copy CDC68
high-copy CDC68
TEMP (°C)
23
37
23
37
23
37
TIME (min)
0
20
90
180
0
20
90
180
0
20
90
180
CDC68
CLN1
CLN2
CLN3
ACT1

Sug1 is required for the progression through the G2/M phase of the cell cycle. This finding is consistent with the report of Ghislain et al. (1993). In an effort to understand this mitotic requirement for Sug1, I have determined genetic interactions between Sug1 and several known mitotic regulators.

1. *sug1-26* mutant cells are temperature-sensitive for proliferation.

The *SUG1* gene was identified here because certain *sug1* mutations can restore activity to an enfeebled Cdc68 transcription factor. To characterize these *sug1* suppressor alleles I determined whether *sug1* mutations have any effect on cell proliferation in a *CDC68* wild-type strain. A strain harboring only the *sug1-26* mutation was constructed by mating a *cdc68-1 sug1-26* strain with a wild-type strain, followed by sporulation of the resultant diploid strain. For some tetrads temperature sensitivity at 37°C segregated in a 4:0 fashion, suggesting that the *sug1-26* mutation itself can cause temperature sensitivity. For one tetrad that gave a 4:0 segregation pattern for temperature sensitivity, each meiotic segregant was mated with a *cdc68-1* mutant strain to determine which segregants harbored the *cdc68-1* mutation: *cdc68-1/cdc68-1* diploids are temperature-sensitive, whereas *cdc68-1/CDC68 sug1-26/SUG1* diploids are temperature-resistant. Segregants that by this test did not contain *cdc68-1* were deemed to harbor the *sug1-26* mutation; growth kinetics of one *sug1-26* segregant are shown in Fig. 27A. The above inference was confirmed by the finding that the cloned wild-type *SUG1* gene complemented the temperature sensitivity caused by those *sug1-26* segregants (Fig. 27B). Thus, the *sug1-26* allele confers temperature sensitivity in the presence of the wild-type Cdc68 protein.

2. *sug1-26* cells arrest in the G2/M phase of the cell cycle.

Cdc68 activity is required for the performance of START, so that at the restrictive temperature *cdc68-1* mutant cells arrest cell proliferation in the G1 phase of the cell cycle

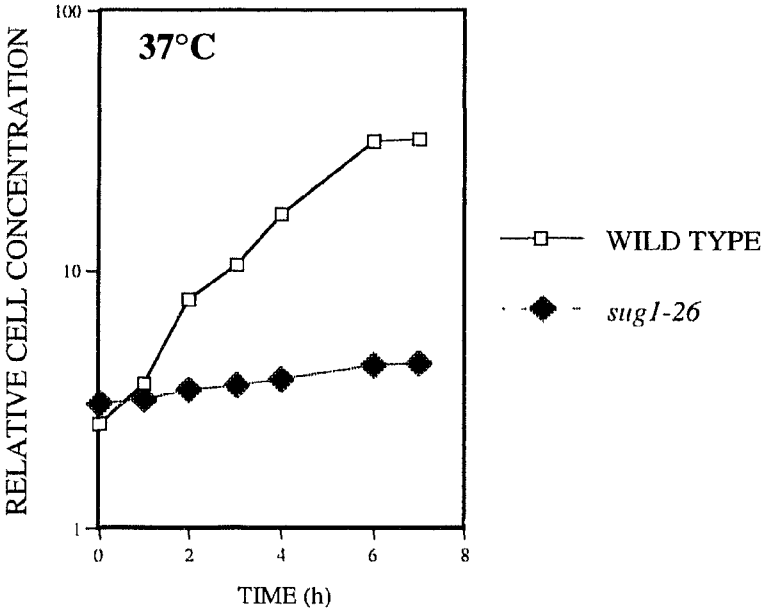
Figure 27. *sug1-26* cells are temperature-sensitive for proliferation.

(A) Growth kinetics of *sug1-26* and wild-type cells at 37°C. Cells were grown in YM1 liquid medium to early log phase and transferred to 37°C for further incubation. At intervals, samples were taken for determination of cell concentration.

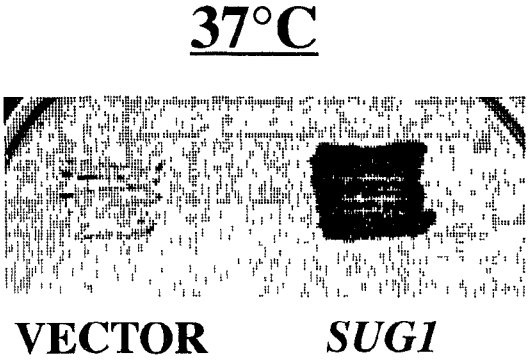
(B) Complementation of the temperature sensitivity by *SUG1*. The temperature sensitive *sug1-26* strain used above was transformed with either the *SUG1* low-copy plasmid pXKE17 or the pRS316 vector. Transformants were grown on SC-Ura medium (SC lacking uracil) at 23°C and replica-plated to SC-Ura medium for incubation at 37°C as shown.

Figure 27

A


37°C
RELATIVE CELL CONCENTRATION
100
10
1
0
2
4
6
8
TIME (h)
WILD TYPE
sug1-26

B


37°C
VECTOR
SUG1

(Prendergast et al., 1990). Because the Sug1 and Cdc68 proteins are related functionally, I determined if temperature-sensitive *sug1-26* mutant cells arrest at a specific stage of the cell cycle. *sug1-26* mutant cells were grown at 23°C and transferred to the restrictive temperature of 37°C to impair mutant Sug1 protein function. After a 3-h incubation at 37°C, the budding index of *sug1-26* mutant cells was determined by light microscopy. The budding morphology of *S. cerevisiae* cells can be used as an indication of cell cycle position. A budding yeast cell remains unbudded in the G1 phase, and a small bud emerges at the beginning of the S phase and gradually enlarges as the cell progresses through the S, G2 and M phases of the cell cycle; at the end of M phase the bud separates from the mother cell. Approximately 75% of *sug1-26* mutant cells ceased proliferation at 37°C as large-budded cells, characteristic of G2/M phase cells. DAPI staining of arrested *sug1-26* cells revealed that most of them contained a single nucleus at or within the neck of the bud (Fig. 28). I also noted that the *sug1-26* mutation caused a similar, albeit less severe, cell-cycle defect even at the permissive temperature of 23°C; at this temperature there was a higher proportion of *sug1* mutant cells undergoing nuclear division compared to a congenic wild-type strain (data not shown).

FACS analysis was used to determine the DNA content of arrested *sug1-26* mutant cells. As shown in Fig. 29, most haploid *sug1-26* mutant cells arrested with a 2N DNA content at 37°C, indicating that the large-budded *sug1-26* mutant cells cease proliferation after DNA replication.

The morphology of *sug1-26* mutant cells suggests that mutant cells become arrested during mitosis. Because microtubule distribution and spindle morphology can also give an indication of cell cycle position, I examined spindle morphology of arrested *sug1-26* mutant cells by indirect immunofluorescence. As shown in Fig. 30, most *sug1-26* mutant cells were found to arrest with elongated spindles at 37°C, whereas under the same conditions *SUG1* wild-type cells displayed various spindle morphologies,

Figure 28. *sug1-26* cells arrest at the stage of nuclear division.

Cells of strains 21R (wild type) and QX261 (*sug1-26*) were grown in YM1 liquid medium to early log phase and transferred to 37°C for further incubation. After 3-h of incubation at 37°C, a portion of cells was fixed in 70 % ethanol and stained with DAPI (1 μg/ml). Nuclei of DAPI-stained cells were visualized using a microscope equipped with a fluorescence filter.

Figure 28

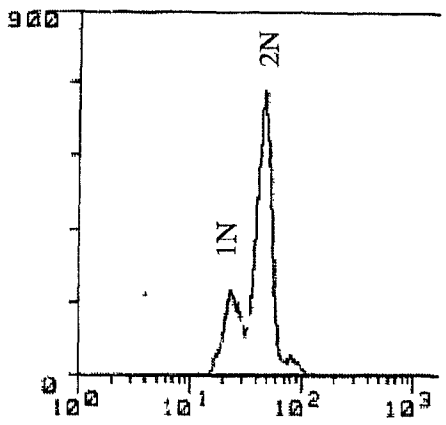
sug1-26

WILD TYPE

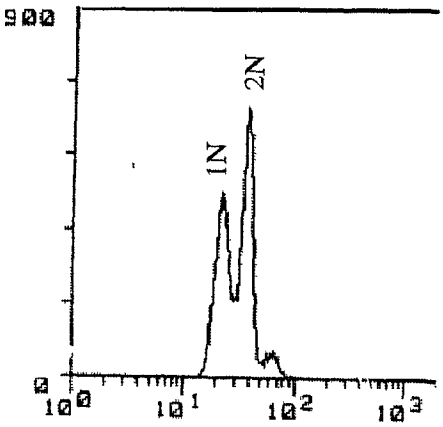
Figure 29. FACS analysis of *sug1-26* mutant cells.

Cells of haploid yeast strains 21R (wild-type, WT) and QX261 (*sug1-26*) were grown in YM1 medium at 23° to early log phase and transferred to 37°C for further incubation. After 3-h of incubation at 37°C, about 10^7 cells were fixed in 70% ethanol, treated with RNase, stained with propidium iodide (5%), and processed through a fluorescence-activated c .ll sorter (FACS). The X-axis is intensity of fluorescence; the first peak corresponds to 1N DNA content, and the second peak corresponds to 2N DNA content. The Y-axis is relative cell number; about 10^5 cells were processed for each strain.

Figure 29



sug1-26

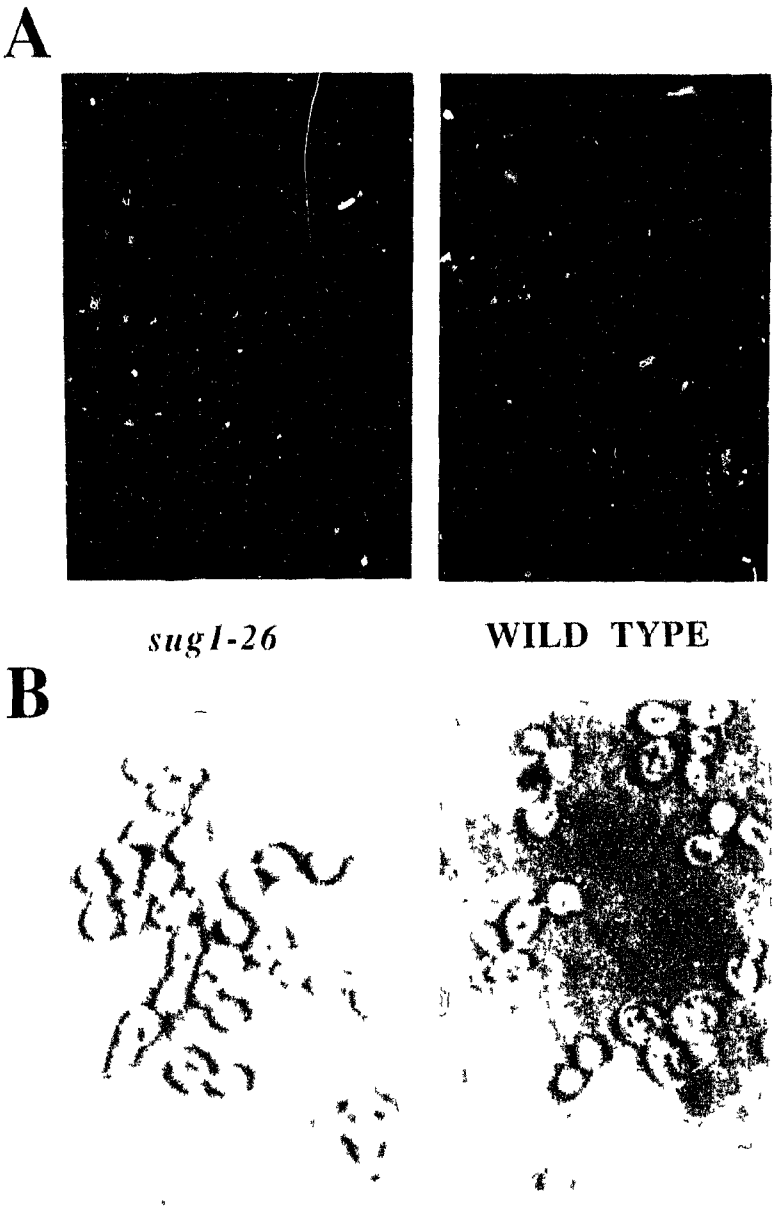


WT

Figure 30. *sug1-26* cells arrest with elongated spindles.

Cells of strains 21R (wild type) and QX261 (*sug1-26*) were grown in YM1 liquid medium at 23°C to early log phase and transferred to 37°C for further incubation. After 3-h of incubation at 37°C, cells were fixed in formalin (3.7%), permeabilized and subjected to indirect immunofluorescence analysis. Anti-tubulin antibody and FITC-conjugated secondary anti-IgG antibody were used to visualized spindles under a fluorescence microscope (A). Photographs were also taken without the fluorescence filter to visualize cells (B).

Figure 30



characteristic of cells in all stages of the cell cycle. The existence of elongated spindles in *sug1-26* mutant cells further demonstrates that *sug1-26* cells arrest in mitosis.

3. *cdc68-1* does not affect the mitotic defect caused by *sug1-26*.

sug1 suppressor mutations identified here can alleviate the *cdc68-1* defect at 35°C, but not at 37°C. I therefore determined the terminal arrest phenotype of *sug1-26 cdc68-1* double-mutant cells at 37°C by microscopic examination. The *sug1-26 cdc68-1* double-mutant cells arrested as large-budded cells, resembling *sug1-26* single-mutant cells, suggesting that the *cdc68-1* mutation cannot alleviate the mitotic defect caused by the *sug1-26* mutation.

4. *sug1* mutations show synthetic enhancement with the *cdc28-1N* allele.

Ghislain et al. (1993) reported the isolation of a *sug1* allele, *sug1-3 (cim3-1)*, by a synthetic lethal screen with the *cdc28-1N* allele. The *cdc28-1N* mutation specifically arrests the cell cycle at the G2/M transition at the restrictive temperature (Piggott, et al., 1982; Surana et al., 1991). The *sug1-26* allele exhibits a related although slightly different phenotype from that of *sug1-3*: *sug1-26* cells arrest with elongated spindles (Fig. 30) whereas *sug1-3* cells arrest with short spindles (Ghislain et al., 1993). In addition, *sug1-26* and *sug1-3* have different molecular defects (see Section C). I therefore assessed the genetic interaction between *sug1-26* and *cdc28-1N*. A diploid strain was constructed heterozygous for *cdc28-1N* and *sug1-26*. Assessment of phenotypes of meiotic segregants revealed that *sug1-26 cdc28-1N* double-mutant cells are more defective compared to either *sug1-26* or *cdc28-1N* single-mutant cells: *sug1-26 cdc28-1N* cells grew very slowly at 23°C, and displayed an abnormal morphology with extremely extended buds (5X the normal size). A majority of these cells displayed multiple buds and the cells themselves were elongated (data not shown). Moreover, *sug1-26 cdc28-1N* double mutant cells could not grow at 30°C, a temperature permissive for

both *sug1-5* and *cdc28-1N* cells (Fig. 31A). Therefore *sug1-26* does indeed interact genetically with the *cdc28-1N* mutation, and *sug1-26* and *sug1-3* have a similar effect with respect to genetic interactions with the *cdc28-1N* mutation that affects mitosis.

5. Altered Sug1 activity does not affect mitosis through the Rad9 checkpoint control.

In many eukaryotic organisms there exists a G2 checkpoint control to ensure that mitosis occurs only after DNA replication is completed and any DNA damage is repaired. In the budding yeast, the Rad9 protein is a key component in the DNA-damage checkpoint control: cells with defective Rad9 function proceed through mitosis even when DNA is damaged (for review see Hartwell and Weinert, 1989). I determined if the cell-cycle arrest seen in *sug1-26* mutant cells reflects inappropriate activation of the Rad9 checkpoint. I disrupted the *RAD9* gene in a *sug1-26* mutant strain (QX2611) by introducing by transformation the *Eco*RI-*Sal*I fragment (containing *rad9Δ::URA3*) from plasmid pRR330. In a separate experiment, I also crossed the *sug1-26* or *sug1-20* mutation into a *rad9Δ::LEU2* deletion strain. In each case, the absence of Rad9 activity did not alter the temperature sensitivity caused by the *sug1* mutations (Fig. 32A), and *rad9Δ sug1-26* double-mutant cells still arrested in the G2/M phase of the cell cycle. Thus, the *sug1*-mediated mitotic arrest is not a consequence of activation of the Rad9 checkpoint control.

6. Decreased Sug1 activity relieves UV sensitivity caused by defective Rad9 checkpoint control.

I also determined if the *sug1-26* mutation could suppress the UV sensitivity caused by deletion of the *RAD9* gene. Cells harboring combinations of *rad9Δ*, *RAD9*, *sug1-26* (or *sug1-20*), and *SUG1* alleles were exposed to UV irradiation as described in Materials and Methods. As shown in Fig. 32B, at the permissive temperature, either the

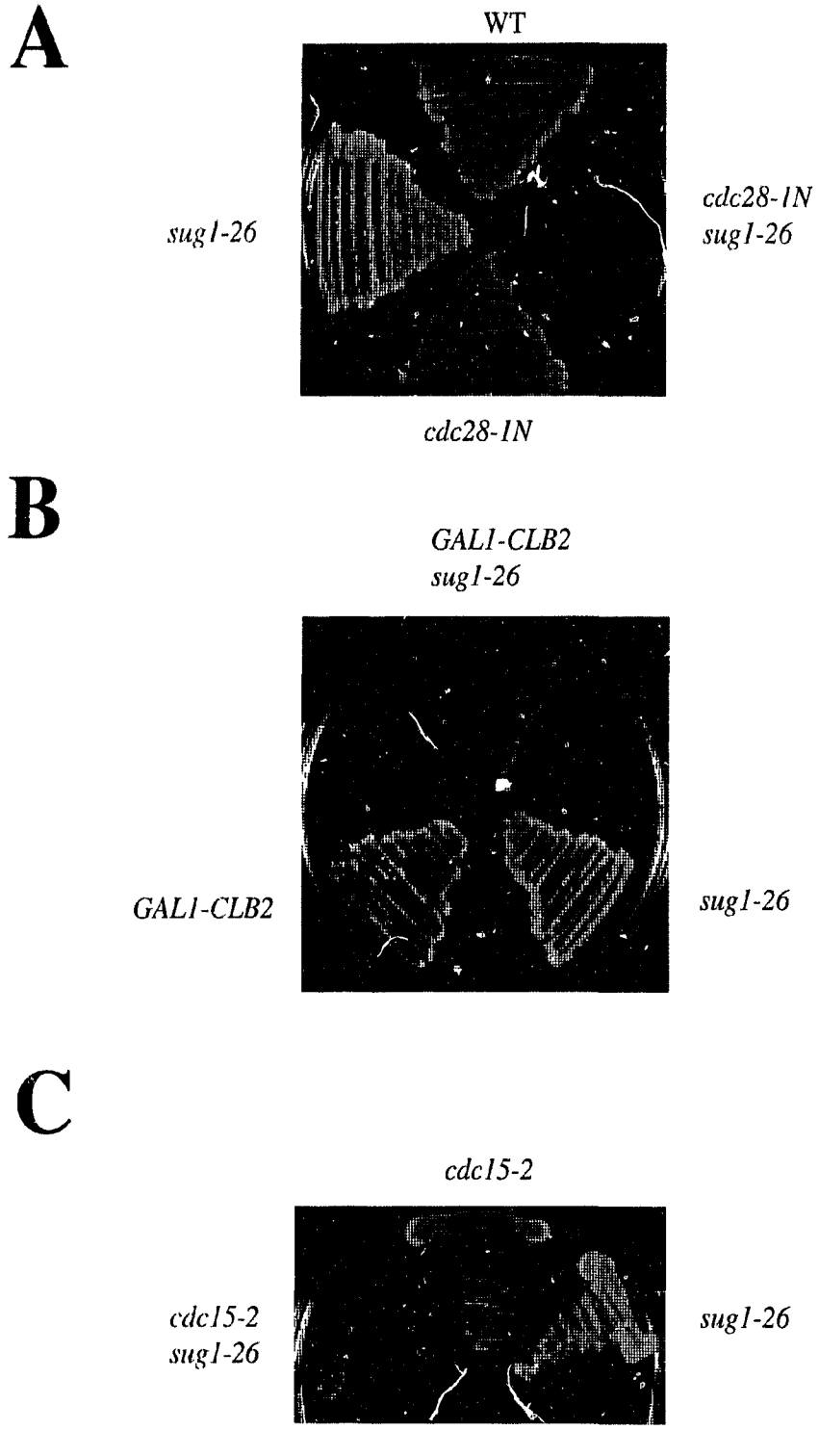
Figure 31. Synthetic enhancement between *sug1* and other mitotic perturbations.

(A) *sug1-26* and *cdc28-1N*. Cells of strains 21R (wild type), QX2612 (*sug1-26*), MT72 (*cdc28-1N*), and QX2628-1 (*cdc28-1N sug1-26*) were grown on YEPD solid medium at 23°C and replica-plated to YEPD medium for further incubation at 30°C as shown.

(B) *sug1-26* and elevated expression of *CLB2*. Cells were grown on SC-Ura galactose solid medium (SC medium lacking uracil and with galactose as the carbon source) at 23°C and replica-plated to SC-Ura galactose medium for further incubation at 33°C as shown. Strains used were 21R harboring the *GAL1-CLB2* plasmid pMT422 (*GAL1-CLB2*), QX2612 harboring pMT422 (*GAL1-CLB2 sug1-26*), and QX2612 harboring the vector YEp352 (*sug1-26*).

(C) *sug1-26* and *cdc15-2*. Cells of strains QX2612 (*sug1-26*), H126-9-2 (*cdc15-2*), and QX2615-1 (*cdc15-2 sug1-26*) were grown on YEPD solid medium and incubated at 33°C as shown. All strains grew well at 23°C (data not shown).

Figure 31


A
WT
sug1-26
cdc28-1N
sug1-26
cdc28-1N
B
GAL1-CLB2
sug1-26
GAL1-CLB2
sug1-26
C
cdc15-2
cdc15-2
sug1-26
sug1-26

sug1-26 or *sug1-20* allele partially relieved UV sensitivity caused by *rad9Δ*: *sug1-26 rad9Δ* and *sug1-20 rad9Δ* cells displayed an intermediate level of UV sensitivity compared to *RAD9* wild-type and *SUG1 rad9Δ* cells. (*sug1* mutant cells were not UV sensitive.) The radiation sensitivity caused by a *rad9* deletion is presumably due to the lack of sufficient time (normally provided by a Rad9-activated cell-cycle delay) for repair of damaged DNA before the onset of mitosis. It is conceivable that an extended G2/M phase seen in *sug1* mutant cells allows sufficient time for cells to repair radiation-induced DNA damage before cell division even in the absence of the Rad9 checkpoint activity. Analogously, extension of G2/M phase using a microtubule-destabilizing drug was found to relieve UV sensitivity caused by a *rad9* mutation (Weinert and Hartwell, 1988).

7. G2/M arrest of *sug1* mutant cells is not dependent on the Swe1 mitotic inhibitor.

The Swe1 protein in the budding yeast is a homolog of the fission yeast mitotic inhibitor Wee1 (Booher et al., 1993). Overexpression of the *SWE1* gene blocks cells in G2, suggesting that Swe1 functions as a mitotic inhibitor (Booher et al., 1993). I therefore addressed the possibility that *sug1-26* causes mitotic blockage by upregulating the Swe1 mitotic inhibitor. Because the Sug1 protein has been found to inhibit transcription of some genes, I determined *SWE1* mRNA levels in *sug1-26* cells. As shown in Fig. 33, however, there was no increase in *SWE1* mRNA levels in *sug1-26* cells compared to those in *SUG1* wild-type cells, suggesting that Sug1 does not activate mitosis by inhibiting expression of the *SWE1* gene. In a separate experiment the *SWE1* gene was deleted in *sug1-26* mutant cells, but absence of the *SWE1* gene failed to alleviate the temperature sensitivity of *sug1-26* mutant cells: *sug1-26 swe1Δ* double-mutant cells still arrested in the G2/M phase with undivided nucleus at 37°C (data not shown). Even overexpression of the *SWE1* gene using a high-copy plasmid did not change the *sug1-26* nuclear-division blockage. Thus, there is no detectable genetic interaction between Sug1 and Swe1.

Figure 32. *sug1-26* relieves UV sensitivity caused by *rad9Δ*.

(A) Growth of cells at 37°C. Cells of strains QX2612 (*sug1-26*), 7859-7-4 (*rad9Δ*), QX26110 (*sug1-26 rad9Δ*), and QX2029 (*sug1-20 rad9Δ*) were grown on YEPD solid medium at 23°C and replica-plated to YEPD for further incubation at 37°C as shown.

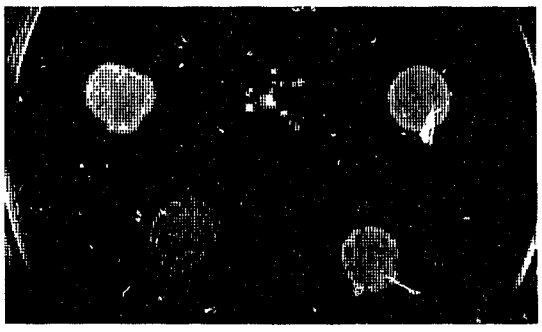
(B) UV sensitivity. About 10^4 cells (10 µl of 10^6 cells/ml liquid culture) were spotted on YEPD solid medium and incubated at 23°C after two exposures to radiation (10 mjoules/cm^2) from a UV crosslinker. The photograph was taken 3 days after UV irradiation.

Figure 32

A


sug1-26
rad9Δ
sug1-26
sug1-20
rad9Δ
rad9Δ

B


sug1-26
rad9Δ
WT
sug1-26
rad9Δ
sug1-20
rad9Δ

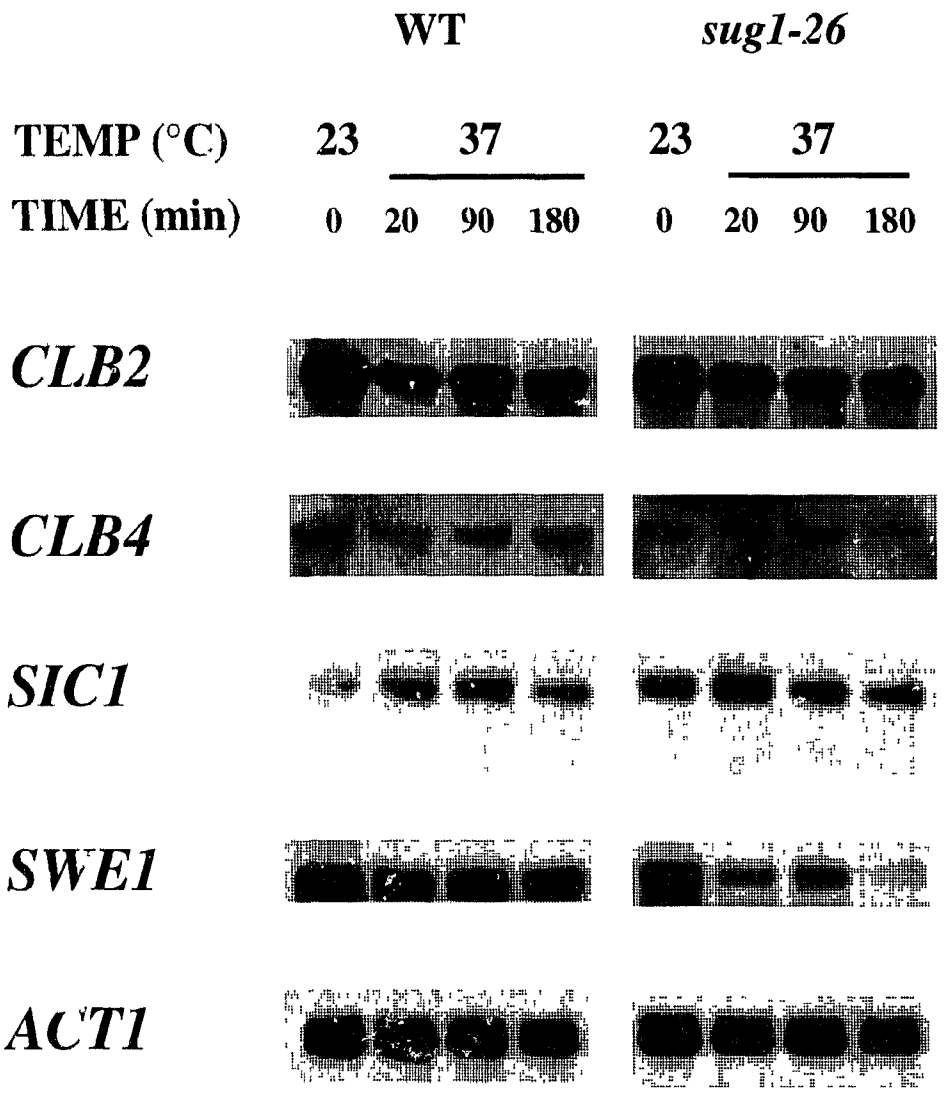
8. Sug1 does not function through Sic1 to regulate G2/M.

Recently, so-called CDIs (CDK inhibitors) have been identified that restrict the activity of cyclin-dependent kinases (CDKs) by directly binding to the CDKs (for review, see Peter and Herskowitz, 1994). Sic1 is a CDI in *S. cerevisiae* that inhibits the Clb5,6-associated Cdc28 protein-kinase activity required for the initiation of S phase (Schwob et al., 1994). In addition, Sic1 has been shown to play a role in the progression through mitosis: deletion of *SIC1* was found to cause both an extended M phase and an abbreviated G1 phase (Nugroho and Mendenhall, 1994), and furthermore *SIC1* (also called *SDB25*) was identified as a multicopy suppressor of *dbf2* that is defective at completing mitosis (Donovan et al., 1994). I determined if there is any functional interaction between Sug1 and Sic1. Northern blot analysis showed that there was a modest increase in *SIC1* mRNA abundance in *sug1-26* mutant cells (Fig. 33). This increase in *SIC1* expression may, however, be irrelevant to the G2/M arrest phenotype of *sug1-26*, because expression of the *SIC1* gene to a much higher level (from the potent *GAL1* promoter) in *SUG1* cells does not block cell division (Nugroho and Mendenhall, 1994). Analogously to what I found for the *sug1-26* mutation, deletion of the *SIC1* gene causes an increased percentage of M phase cells (Donovan et al., 1994). I therefore determined if *sic1Δ* and *sug1-26* mutations interact. The *SIC1* gene was replaced by a *sic1Δ::URA3* deletion allele in *sug1-26* cells, and this change was shown to have little impact on the terminal phenotype of *sug1-26* cells. When transferred to 37°C, about 75% of *sic1Δ sug1-26* cells arrested promptly as large-budded cells, similar to *sug1-26* cells. The absence of additive effects between *sug1-26* and *sic1Δ* mutations may suggest that Sug1 and Sic1 are functionally unrelated. Similarly, increased expression of the *SIC1* gene from the high-copy vector YEp352 also did not change the phenotype of *sug1-26*. Thus, the mitotic effects of Sug1 and Sic1 are probably unrelated.

Figure 33. Effects of Sug1 on the expression of cell cycle regulators.

Total RNA was extracted from strains 21R (wild type) and QX261 (*sug1-26*) grown at 23°C and also after incubation at 37°C for the indicated times. RNA was resolved on a formaldehyde-containing agarose gel, transferred to nylon membrane, and probed separately with radiolabeled DNA fragments of the genes indicated. Levels of *ACT1* mRNA were used as loading controls.

Figure 33


WT
sug1-26
TEMP (°C)
23
37
23
37
TIME (min)
0
20
90
180
0
20
90
180
CLB2
CLB4
SIC1
SWE1
ACT1

9. Genetic interactions between Sug1 and Clb2.

Clearly Sug1 plays an important function in some aspect of mitosis, as indicated by the terminal phenotype of *sug1* mutant cells as well as the synthetic enhancement between *sug1* and *cdc28-1N*. The above observations, however, rule out the possibility that Sug1 functions through the Rad9 checkpoint, the Swe1 mitotic inhibitor, or the CDK inhibitor Sic1. In addition to these mitotic regulators, the B-type cyclins are also important for progression through mitosis because they regulate the mitotic Cdc28 kinase activity (Surana et al., 1991; Ghiara et al., 1991). Since Sug1 has been shown to be a component of the transcription mediator complex (Kim et al., 1994) and has negative effects on the transcription of certain genes in yeast (see Section C), I determined whether Sug1 affects transcription of B-type cyclin genes required for mitosis. As shown in Fig. 33, there was no significant change in mRNA abundance for *CLB4*, and only a modest increase in *CLB2* mRNA levels in *sug1* mutant cells. This increase probably reflects a bias in cell-cycle distribution because there is a larger proportion of a *sug1-26* mutant cell population in the G2/M phase, where B-type cyclins are expressed. Thus, the mitotic block caused by *sug1-26* is not due to decreased B-type cyclin gene expression.

The Sug1 protein is also implicated in protein turnover (Ghislain et al., 1993). Regulated degradation of proteins plays a pivotal role in cell-cycle progression: for instance, degradation of Clbs has been shown to be critical to the completion of mitosis (Surana et al., 1993). There are indications that the mitotic blockage caused by *sug1-3* may be a consequence of decreased proteolysis (Ghislain et al., 1993); *sug1-3* was also found to impair ubiquitin-mediated protein degradation. Moreover, *sug1-3* mutant cells contain high levels of Clb2 and Clb3 proteins and, perhaps as a result, mutant cells also contain a high level of Clb-associated Cdc28 kinase activity (Ghislain et al., 1993). Taken together these findings may suggest that inability of *sug1-3* cells to complete mitosis is a consequence of elevated Cdc28 kinase activity caused by decreased degradation of Clb proteins. Similar to *sug1-3*, *sug1-26* may also cause higher Clb

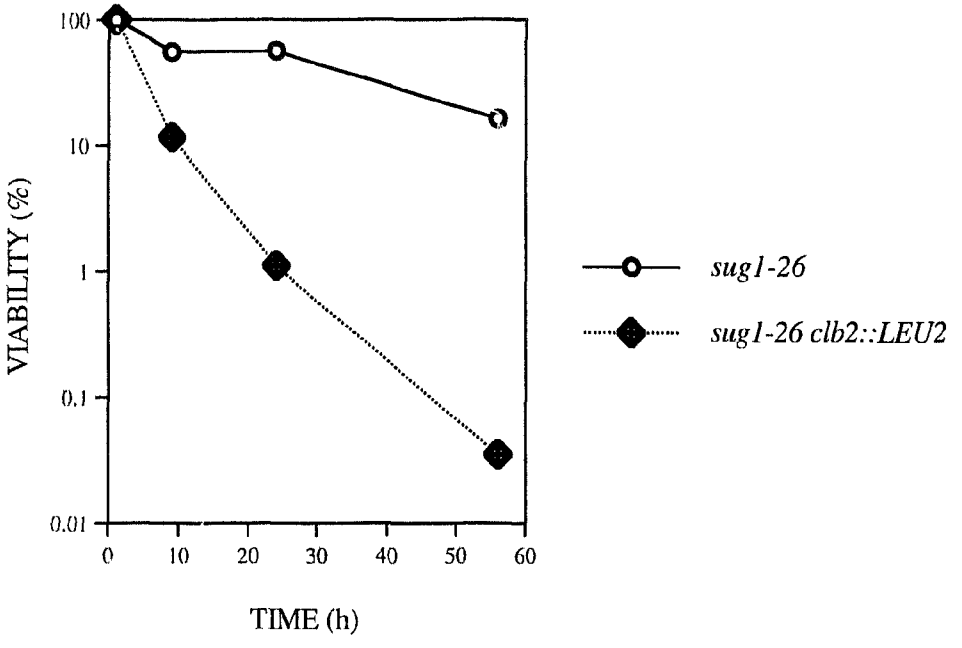
protein levels by affecting proteolysis: *sug1-26* stabilizes certain proteins, and thus probably also affects proteolysis (Section C). If *sug1-26*-mediated mitotic arrest is caused by elevated Clb2 accumulation, further increased Clb2 levels by overexpression of *CLB2* may exacerbate the *sug1-26* mitotic defect. The effect of overexpression of *CLB2* under the control of the *GAL1* promoter was therefore determined. The *GAL1-CLB2* plasmid pMT422 was introduced into *sug1-26* and *SUG1* wild-type strains, and transformants were grown on glucose medium and replica-plated to medium containing galactose to induce expression from the *GAL1* promoter. *sug1-26* cells harboring the *GAL1-CLB2* gene failed to grow in galactose medium at 33°C, a permissive temperature for the *sug1-26* mutation and also for *SUG1* wild-type cells containing the same *GAL1-CLB2* gene (Fig. 31B). Thus, overexpression of *CLB2* indeed exacerbates the mitotic defect caused by *sug1-26*.

Gene deletion experiments suggest that *CLB2* encodes the most important form among the yeast B-type cyclins (Surana et al., 1991; Ghiara et al., 1991). However, even though the above results implicate Clb2 in the *sug1-26* mitotic block, deletion of the *CLB2* gene did not alleviate the mitotic arrest caused by *sug1-26* and had little effect on the *sug1-26* terminal phenotype: *sug1-26 clb2Δ* double-mutant cells still arrested with a single nucleus at or within the neck of the bud (data not shown). This finding suggests that the inability of *sug1-26* cells to complete mitosis is not attributable solely to high levels of Clb proteins, because in this case removal of the predominant Clb2 should relieve *sug1-26*-mediated mitotic blockage. On the other hand, in contrast to *sug1-26* or *clb2Δ* cells, *sug1-26 clb2Δ* double-mutant cells lost viability significantly (Fig. 34). One interpretation for the loss of viability of *sug1-26 clb2Δ* double-mutant cells is that decreased Clb abundance in these *sug1-26* cells, due to deletion of the *CLB2* gene, allows some mitotic activity to proceed, with irreversible deleterious consequences.

Figure 34. *sug1-26 clb2::LEU2* double-mutant cells lose viability at 37°C.

Actively proliferating cells of strains QX2611 (*sug1-26*) and XSC2-1 (*sug1-26 clb2::LEU2*) were grown in liquid YM1 medium at 23°C, and transferred to 37°C for further incubation. Samples were taken at times indicated for determination of cell concentration, and were also diluted and spread evenly on YEPD solid medium and incubated at 23°C to assess the number of viable cells. Percentage of viable cells (viability) was calculated accordingly.

Figure 34


100
10
1
0.1
0.01
VIABILITY (%)
0
10
20
30
40
50
60
TIME (h)
sug1-26
sug1-26 clb2::LEU2

10. *sug1* and *cdc15* show synthetic enhancement.

The Cdc15 protein is required for late mitotic events (Pringle and Hartwell, 1981). Like a *sug1* mutation, a *cdc15* mutation also causes mutant cells to arrest late in mitosis with high levels of Clb-associated Cdc28 activity, as reflected by high histone-H1 kinase activity (Surana et al., 1993), and for this reason the Cdc15 protein is thought to be involved in the destruction of Clb-associated Cdc28 kinase activity to allow exit from mitosis (Surana et al., 1993). To determine if there is any genetic interaction between *sug1-26* and a *cdc15* mutation, a *sug1-26 cdc15-2* double-mutant strain was constructed and assessed for growth over a range of temperatures. The *sug1-26 cdc15-2* double-mutant cells ceased proliferation at 33°C, a temperature permissive for both the *sug1-26* and *cdc15-2* single-mutant cells (Fig. 3!C). Thus, maintaining a high level of Clb-associated Cdc28 protein-kinase activity as a consequence of impaired Cdc15 activity, like elevated expression of the *CLB2* gene, exacerbates the *sug1-26* defect. It is noteworthy that while both a *cdc15* mutation and high-level expression of the *CLB2* gene (using multiple copies of a *GAL1-CLB2* plasmid) cause telophase arrest (two nuclei, in mother and bud, and an elongated spindle apparatus; Surana et al., 1993), *sug1-26 cdc15-2* double-mutant cells arrest before this point, with the nuclear morphology of *sug1-26* cells (the nucleus in the bud neck; data not shown). These findings suggest that *sug1-26* has additional defects that inhibit mitosis at a stage earlier than that reached by cells with excess mitotic Cdc28 kinase activity (see DISCUSSION).

IV. DISCUSSION

1. Functional Interactions between Cdc68 and San1

Four suppressor genes were identified in which mutations alleviate the *cdc68-1* temperature sensitivity at 35°C. Cloning and nucleotide sequence analysis showed that one suppressor gene, *SCA68*, is the same as *SAN1*, a gene identified previously during a different genetic screen; *san1* alleles were isolated as suppressors of a *sir4* mutation that abolishes transcription repression at the two silent mating type loci, *HML* and *HMR* (Schnell et al., 1989). I found that eliminating San1 activity reversed all aspects of the *cdc68-1* mutant phenotype. On the other hand, overexpression of the *SAN1* gene using a high-copy plasmid further decreased the weak activity encoded by *cdc68-1*. Therefore, one role of the San1 protein is to inhibit Cdc68 function.

1a) San1 antagonizes Cdc68 activity in a dose-dependent manner

All the 20 *san1* alleles identified here as suppressors of *cdc68-1* are either recessive or weakly codominant. This feature of *san1* mutations suggests that these mutant alleles encode San1 protein with diminished function. Consistent with this suggestion, inactivation of the *SAN1* gene by insertion within the open reading frame (Schnell et al., 1989) or by removal of the *SAN1* gene, like *san1* mutant alleles isolated here, also reverses every aspect of the *cdc68-1* mutant phenotype tested. The fact that loss of function of the San1 protein restores activity to *cdc68-1*-coded mutant protein suggests that the wild-type San1 protein inhibits Cdc68 activity.

The dosage effects of San1 and mutant Cdc68 protein on cell proliferation provide further evidence that the San1 protein counteracts Cdc68 activity (Table 7). For example, my initial genetic tests of dominance/recessiveness were carried out in diploid cells, and in this situation most of the *san1* suppressor alleles were codominant: growth of diploid cells

with the genotype *cdc68/cdc68 san1/SAN1* was better than that of *cdc68/cdc68 SAN1/SAN1* homozygous diploid cells, although not as good as that of wild-type diploids. However, in haploid *cdc68 san1* cells, all of the codominant *san1* alleles were recessive to the wild-type *SAN1* gene on a low-copy centromeric plasmid. A yeast cell may maintain several copies of a centromeric plasmid, and this difference between a *cdc68 san1* haploid cell harboring a low-copy *SAN1* gene and *cdc68/cdc68 san1/SAN1* diploid cells may be due to a modest elevation of *SAN1* gene expression. On the other hand, this difference may also reflect the ability of two copies of the mutationally compromised *cdc68* gene (in diploid cells) to provide increased Cdc68 activity and to overcome the San1 inhibitory function. The suggestion that increased copy number of the mutant *cdc68* gene can supply additional Cdc68 activity is a reasonable one because elevated gene dosage of the *cdc68-1* allele (on a high-copy plasmid) actually overcomes San1 inhibition completely: *cdc68-1* mutant cells harboring a high-copy *cdc68-1* plasmid proliferate at the otherwise restrictive temperature (Fig. 9). This effect of increased *cdc68-1* mutant gene dosage also implies that the *cdc68-1* allele used here still retains some, albeit weaker, Cdc68 activity. Removal of the San1 protein alleviates its inhibitory effect and renders mutant Cdc68 protein more active.

As a converse example of the effects of altered gene dosage, overexpression of the wild-type *SAN1* gene lowers the restrictive temperature for the *cdc68-1* mutation: *cdc68-1* mutant cells with increased San1 activity were temperature-sensitive at 33°C, which is a permissive temperature for *cdc68-1* mutant cells with normal *SAN1* gene dosage (Fig. 7). The antagonistic effects of San1 and Cdc68 proteins therefore show tight stoichiometry.

Modulation of Cdc68 function by San1 is most evident in cells with enfeebled Cdc68 activity. For *CDC68* wild-type cells, neither increased nor decreased *SAN1* gene dosage, or even complete loss of San1 function, causes any appreciable effect on cell proliferation, and there is no substantial change in transcript abundance for genes activated by Cdc68 (Fig. 8). Thus, San1 is a weak inhibitor for Cdc68.

1b) Biological roles for San1

Insertion of the *HIS3* gene into the *SAN1* open reading frame has no deleterious effect on cell proliferation (Schnell et al., 1989). Similarly, removal of the majority of the *SAN1* ORF to create the *san1Δ::URA3* null allele also does not have any detectable effect on cell growth and division. Thus, *SAN1* is not an essential gene. There are two explanations to account for the apparent lack of phenotype of the *san1* null allele. Another protein may share an overlapping function with San1 so that this putative San1-like protein can provide San1 function even when San1 is absent. There are a number of cases in yeast in which two or more proteins function in a redundant manner, so that loss of only one protein does not have a profound effect. For example, the Clb1 and Clb2 proteins carry out overlapping functions to activate the mitotic-specific Cdc28 kinase; the absence of either one of these Clb proteins does not have significant effects on cell proliferation (for review see Nasmyth, 1993). In this regard, one of the suppressor genes identified here, *SCC68*, may encode an activity related to San1, because *SAN1* even on a low-copy plasmid fully suppresses the *scc68-1* phenotype.

Although other San1-like activity may be present in yeast, San1 function may simply be dispensable under normal growth conditions, and become important only under certain environmental or physiological conditions. One circumstance revealed by this study in which San1 has significant effects is the presence of an enfeebled transcription activator encoded by *cdc68-1*: in this situation, San1 inhibits transcription activation by Cdc68. San1 has also been implicated in the transcriptional repression of the two silent mating-type loci *HML* and *HMR*; mutation in the *SIR4* gene abrogates silencing to allow the mating information to be expressed from these *HM* loci (Rine and Herskowitz, 1987). Removal of San1 maintains the two *HM* loci in a repressed state even in *sir4* mutant cells (Schnell et al., 1989). Likewise, overexpression of *SAN1* was found to cause a partial derepression of the *HMR* locus (Schnell et al., 1989), suggesting that San1 plays a role in this

transcriptional repression process. Therefore, even though yeast cells proliferate well in the absence of San1, the San1 protein plays a role in modulating gene expression.

San1 is unlikely to play a global role affecting transcription, but may do so individually through proteins such as Cdc68. A number of proteins, like Cdc68, have been implicated in global transcriptional regulation: Spt2/Sin1 (an HMG-like protein; Kruger and Herskowitz, 1991), several members of the histone group of the Spt proteins; including Spt4, Spt5, and Spt6 (for review see Winston and Carlson, 1992), and Sin4 (a global transcription regulator; Jiang and Stillman, 1992). These proteins have been postulated to regulate transcription by remodeling chromatin structure. Removal of the *SAN1* gene or extra copies of *SAN1* did not affect the ability of an *spt2*, *spt4*, spt5, or *spt6* mutation to suppress δ-insertion mutations (the Spt$^-$ phenotype), and altered San1 activity did not suppress the *sin4* null phenotype, arguing against functional interactions between San1 and these Spt and Sin proteins. A conclusion from these observations is that San1 effects are mediated only through a limited number of proteins, including Cdc68.

1c) Models for San1 and Cdc68 interaction, and impact on transcription

Although allowing *cdc68-1* cells to proliferate under otherwise restrictive conditions, removal of the San1 inhibitor does not suppress the lethality caused by a *cdc68*-disruption allele. The finding that loss of San1 inhibition can not bypass the requirement for Cdc68 function indicates that San1 operates upstream of the Cdc68 protein. Two models for San1 function are consistent with this absolute requirement for Cdc68 function, as well as with the gene-dosage effects seen in *cdc68-1* mutant cells. San1 and Cdc68 could function independently to repress (San1) and to activate (Cdc68) gene expression. In this model, the target of San1 would ultimately be a *cis*-acting negative regulatory element. Normally, the weak repressive effects of San1 would be overwhelmed by the strong activation function provided by the Cdc68 protein, even when more San1 repressor is available due to increased *SAN1* gene dosage. Only when the Cdc68 activator is enfeebled

by mutation would repression by San1 become evident. The *SAN1* gene-dosage effects would suggest weak binding by San1, which would be improved at higher San1 protein levels. In a second model, the primary inhibitory target of San1 activity would be the Cdc68 protein itself. Any effects of San1 would only be obvious with mutant forms of the Cdc68 protein. Although San1 could modify Cdc68 in a number of ways, I found that San1 does not affect stability of the mutant Cdc68 polypeptide: in *san1 cdc68-1* suppressed cells there was no appreciable increase in stability of the mutant Cdc68 polypeptide. Thus, San1 must restrict Cdc68 activity by other means, perhaps by interactions in a protein complex containing both San1 and Cdc68. This latter model is reminiscent of the direct interaction between the yeast Gal4 transcription activator and its inhibitor Gal80, which is affected by *GAL4* and *GAL80* gene dosage (Hashimoto et al., 1983; Johnston and Hopper, 1982; Nogi et al., 1984).

The San1 protein inhibits the expression of many genes that Cdc68 activates, including the *CDC68* gene itself. We show that expression of a *cdc68* mutant allele, encoding a polypeptide with enfeebled Cdc68 activity, is decreased in *SAN1 cdc68* mutant cells due to San1 inhibition (Fig. 6). This interruption of the positive-feedback loop for Cdc68 synthesis (Rowley et al., 1991) suggests that the widespread transcriptional effects of San1 could all stem from San1 inhibition only at the *CDC68* promoter, thereby curtailing the ongoing Cdc68 synthesis needed for the activation of other genes. Implicit in this model is a short half-life for the mutant Cdc68 protein, with continual resynthesis needed for gene activation. I found that the mutant polypeptide encoded by *cdc68-1* is indeed labile, and especially so at the restrictive temperature (Fig. 16, 17). However, the inability of increased copies of the *CDC68* promoter to titrate the San1 protein and to relieve inhibition argues against this model. Therefore, I favor an alternative model in which San1 also functions at other promoters activated by Cdc68, perhaps as part of a protein complex as mentioned above.

It is noteworthy that Cdc68 activity is also required for the transcription of the

SAN1 gene. Upon transfer of *cdc68-1* mutant cells to the restrictive temperature, transcription of the *SAN1* gene also decreases (Fig. 6), suggesting that San1 inhibition of the mutant Cdc68 protein persists in the absence of continuing *SAN1* synthesis. Of course transcript abundance does not necessarily reflect protein levels: according to the N-end rule for ubiquitin-mediated protein degradation (reviewed by Varshavsky, 1992), San1 is likely to be a stable protein with a half-life of more than 20 h. Therefore, at the restrictive temperature the stable San1 protein would most likely persist to antagonize the residual Cdc68 activity (conferred by a labile Cdc68 mutant protein) and inhibit transcription.

2. Functional interactions between Cdc68 and Sug1

The *SUG1* gene was originally identified based on a genetic interaction between the Gal4 transcription activator and the Sug1 protein; the *sug1-1* mutation was isolated by virtue of its ability to restore transactivation function to a mutant Gal4 protein encoded by the *gal4D* allele that is defective for activation (Swaffield et al., 1992). Sug1 has also been identified recently in several other laboratories (Ghislain et al., 1993; Kim et al., 1994), and strikingly divergent functions have been proposed for this protein. Sug1 is thought to play a direct role in transcriptional regulation (Swaffield et al., 1992; Kim et al., 1994; Swaffield et al., 1995), but has also been suggested to be part of the 26S protease, executing ubiquitin-mediated protein degradation (Ghislain et al., 1993). Results of my study show that Sug1 influences the transcriptional activation mediated by Cdc68, an acidic protein that has widespread effects on transcription. Assessment of the phenotype of *sug1* mutations indicates that Sug1 plays a negative role in transcription, but not through effects on Cdc68 proteolysis.

2a) A primary role for Sug1 in transcription

Two related types of RNA polymerase II holoenzyme have been purified from budding yeast cells (Koleske and Young, 1994; Kim et al., 1994). One holoenzyme is

comprised of RNA polymerase II and a multi-protein complex, called the mediator complex. Polyclonal antibodies against Sug1 recognize a subunit of this mediator complex, suggesting that Sug1 is a component of the RNA polymerase II holoenzyme (Kim et al., 1994). A direct role for Sug1 in transcription is further shown by the finding that *in vitro* Sug1 can bind to the Gal4 activation domain and to the general transcription factor TBP (Swaffield et al., 1995). Results in this thesis provide *in vivo* evidence that Sug1 influences transcription. Mutant Sug1 activity conferred by the *sug1-26* allele causes an Spt$^-$ phenotype (Fig. 13B), generally interpreted to indicate involvement in some aspect of transcription initiation. Indeed, a variety of genes identified by an Spt$^-$ phenotype encode proteins that play important roles in transcription, such as the TATA-binding protein TBP (encoded by the *SPT15* gene; Eisenmann et al., 1989; Hahn et al., 1989) and the Gal11/Spt13 protein (Fassler and Winston, 1989). However, some effects of Sug1 on transcription may be indirect: increased *CDC68/SPT16* copy number itself can cause an Spt$^-$ phenotype, so it is conceivable that the Spt$^-$ phenotype caused by *sug1-26* maybe a consequence of increased *CDC68* gene expression.

The Sug1 protein is found in the same transcription mediator complex as Gal11 (Kim et al., 1994), a protein that can stimulate transcription when tethered to DNA by a DNA-binding domain (Himmelfarb et al., 1990). Similarly, the Ada2 and Ada3 proteins, members of another class of mediator/adaptor proteins that are thought to bridge certain acidic activators and the basal transcription complex (Berger et al., 1992), can also activate transcription when fused to a DNA-binding domain (Horiuchi et al., 1995; Silverman et al., 1994). In contrast, the Sug1 protein does not provide such activator function when fused to a heterologous DNA-binding domain (Swaffield et al., 1992). Indeed, I found here that the Sug1 protein plays a repressive role in the transcription of some genes, including *CDC68* and the *SUG1* gene itself (Fig. 13A). Thus Sug1 appears to have a negative role in transcription with distinct activity compared to the Gal11, Ada2, and Ada3 proteins.

2b) Sug1 and proteolysis

The Sug1 protein influences protein degradation. Ghislain et al. (1993) showed that the *sug1-3* allele stabilizes ubiquitinated substrates of the 26S protease, and I found that *sug1-26* increases the stability of an intrinsically labile mutant Cdc68 polypeptide (Fig. 16, 17). However, the mechanism of Sug1 action in this proteolysis is not clear, and may be indirect. In spite of the observation that polyclonal anti-Sug1 antibodies cross-react with the *Drosophila* 26S protease (Ghislain et al., 1993), Swaffield et al. (1995) have shown that epitope-tagged Sug1 and the 26S protease are not in the same fraction of a gel-filtration column, and that the epitope-tagged Sug1 does not co-immunoprecipitate with the 26S protease. These findings suggest that Sug1 may not be a component of the 26S protease. Thus, the influence of Sug1 on proteolysis may be a secondary effect of Sug1 on the expression of components of the ubiquitin-mediated degradation pathway. In any case, stabilization of mutant Cdc68 polypeptide cannot account for the suppression of *cdc68-1* by the *sug1-26* allele: the *sug1-3* allele that does not suppress *cdc68-1* temperature sensitivity increased the stability of the Cdc68 mutant polypeptide to a greater degree than that caused by the *sug1-26* suppressor allele (Fig. 16). Thus a modest increase in mutant Cdc68 protein stability is not sufficient to restore Cdc68 activity. Moreover, the *san1-3* mutation that restores function to the mutant Cdc68 protein has little effect on protein stability, indicating that an increase in mutant Cdc68 protein stability is not a prerequisite for the restoration of Cdc68 function. Therefore, increased stability of the mutant Cdc68 protein is neither sufficient nor necessary to restore Cdc68 protein function. Despite increased stability of mutant Cdc68 polypeptide detected in *sug1-26* cells, another mechanism must be responsible for the restoration of Cdc68 activity.

2c) Allele-specific interactions between *cdc68* and *sug1*

Genetic studies revealed allele-specific interactions between *cdc68* and *sug1* mutant

alleles (Table 8). The *sug1* alleles, *sug1-1* and *sug1-3*, that were isolated for different reasons, did not suppress the temperature sensitivity of *cdc68-1*. On the other hand, the *sug1* mutations isolated here by the ability to suppress *cdc68-1* failed to suppress other temperature-sensitive *cdc68* alleles, including *cdc68-11* (Lycan et al., 1994) and *cdc68-Δ922*, a truncated version of the *CDC68* gene (D. R. H. Evans, personal communication). This allele-specific interaction may reflect a physical contact between the Cdc68 and Sug1 proteins. However, I have been unable to detect co-immunoprecipitation of epitope-tagged Cdc68 and Sug1 proteins from yeast cell extracts (Fig. 18), and results of a two-hybrid interaction assay also do not support a physical interaction between the Cdc68 and Sug1 proteins (Table 10). Therefore the observed allele-specific interactions may reflect relative activities of different *cdc68* and *sug1* alleles, and may not necessarily imply any direct physical contact. For example, non-suppressing *sug1-1* and *sug1-3* alleles may retain too much residual Sug1 activity for effective Cdc68 function. Similarly, the inability of *sug1-20* to suppress other *cdc68* alleles could indicate that these *cdc68* alleles encode proteins with too little residual activity to be restored by *sug1-20*. Without a functional assay for Sug1 activity I was unable to compare the activity of different *sug1* alleles biochemically. Nonetheless, genetic data suggest that the *sug1-1* and *sug1-3* alleles may indeed retain too much residual activity: the non-suppression by *sug1-1* and *sug1-3* is dominant over the *cdc68-1* suppression by the *sug1-26* allele (Table 9). Likewise, temperature sensitivity conferred by *cdc68-11* appears to be more severe than that of *cdc68-1*. Taken together, these observations indicate that the allele-specific interactions detected here most likely reflect different levels of residual activity conferred by various *sug1* and *cdc68* alleles.

2d) Antagonistic effect of Sug1 on Cdc68 function

The *sug1* suppressor alleles, exemplified by *sug1-20*, reverse most aspects of the *cdc68-1* phenotype except in one case. It appears that the *cdc68-1* effect at the *his4-912δ* locus is not reversed by the *sug1-20* allele: like *cdc68-1 his4-912δ* cells, *sug1-20 cdc68-1*

his4-912δ cells still display a His^+ phenotype (Spt^-). However, it is conceivable that *sug1-20* also suppresses the *cdc68-1* effect in this case, and that the Spt^- phenotype is caused by the *sug1-20* mutation instead, because the *sug1-20* allele by itself causes an Spt^- phenotype at the *his4-912δ* locus: *sug1-20 his4-912δ* cells show a His^+ phenotype. Thus, where phenotypes can be interpreted, the *sug1* suppressor mutations reverse the *cdc68-1* effect in each case.

Most significantly, the *sug1* suppressor mutations restore transcription of the *cdc68-1* gene itself (Fig. 10). This observation, coupled with the finding that *sug1-20* does not suppress the lethality caused by complete loss of function of Cdc68, suggests that *sug1-20* does not suppress *cdc68* defects by bypassing the requirement for Cdc68. Instead, *sug1-20* restores sufficient Cdc68 activity to support growth. Indeed, mutant polypeptide encoded by the *cdc68-1* allele still has residual activity at the restrictive temperature, because overexpression of *cdc68-1* can substitute for wild-type Cdc68 protein function. An inference from the recessive nature of the *sug1-20* allele is that enfeebled Sug1 activity allows this mutant Cdc68 protein to be more active, suggesting that Sug1 inhibits transcription activation by Cdc68.

2e) Sug1 modulates Cdc68 activity at the protein level

The *CDC68* gene is autoactivated in that the Cdc68 protein activates transcription of its own gene (Rowley et al., 1991). Based on the observation that Sug1 inhibits *CDC68* transcription, one simple model for the suppression of *cdc68-1* by a *sug1* mutation is that enfeebled Sug1 activity relieves repression only at the *cdc68-1* promoter, thereby allowing *cdc68-1 sug1* cells to produce enough mutant Cdc68 protein to activate transcription from other Cdc68-dependent promoters. However, there was no evidence for titration of Sug1 effects: increased dosage of the *CDC68* promoter region failed to alleviate the *cdc68-1* defect in growth, whereas either overexpression of the entire mutant *cdc68-1* gene, or weakening Sug1 activity by mutation, allows *cdc68-1* mutant cells to proliferate at 35°C.

Furthermore, Cdc68-dependent genes respond differently to altered Sug1 activity: in *sug1-26* cells, mRNA levels for *CDC68* and *SUG1* are increased whereas those for *ACT1* are not affected (Fig. 10). These findings suggest that the antagonistic effect of Sug1 is not solely due to repression at the *CDC68* promoter. Thus, I favor a model in which Sug1 counteracts Cdc68 activity at the protein level, by a mechanism other than affecting protein stability (see above).

3. Sug1 and San1 modulate Cdc68 activity independently

Despite the facts that *san1* and *sug1* mutations were uncovered during the same mutational suppressor screening, and that both San1 and Sug1 antagonize Cdc68 protein function, these two proteins have different functions. *SAN1* is not an essential gene, whereas *SUG1* is essential for cell viability. Secondly, loss of San1 function has no detectable impact on transcription in otherwise wild-type cells, while Sug1 represses transcription of some genes and the *sug1-26* mutation displays an Spt$^-$ phenotype. Thirdly, San1 does not affect stability of the mutant Cdc68 polypeptide whereas Sug1 facilitates degradation of mutant Cdc68 protein, although the mechanism through which Sug1 mediates this proteolysis is unknown. Thus, it is very likely that San1 and Sug1 modulate Cdc68 function differently. Consistent with this hypothesis, the San1 and Sug1 proteins have an additive effect on Cdc68 activity: neither *san1* nor *sug1* can suppress *cdc68-1* temperature sensitivity at 37°C, whereas *san1 sug1 cdc68-1* triple-mutant cells can proliferate at this high restrictive temperature (Fig. 19A). The ability of triple-mutant cells to proliferate at 37°C is surprising, considering that the *sug1-26* allele itself confers temperature sensitivity at 37°C and that *san1* does not alleviate *sug1-26* temperature sensitivity at this temperature (Fig. 19B). Nonetheless, *san1* and *sug1* together provide more effective suppression of *cdc68-1*. These observations suggest that San1 and Sug1 function independently to restrict Cdc68 activity, most likely through different pathways.

In a related study, Evans et al. (in preparation) have found that an N-terminally

truncated version of the Cdc68 protein encoded by the *cdc68-Δ922* allele is subjected to San1 inhibition: *san1* mutations suppress the temperature-sensitive growth defect of *cdc68-Δ922*, and increased *SAN1* gene dosage renders *cdc68-Δ922* mutant cells more defective. The inhibitory effect of San1 on the N-terminally truncated Cdc68 protein indicates that San1 functions through the remaining C-terminal portion of the Cdc68 protein. In contrast, the *sug1* alleles identified as suppressors of *cdc68-1* do not relieve the defects caused by *cdc68-Δ922*, although the *cdc68-Δ922* appears to encode at least as much residual Cdc68 activity as *cdc68-1*. These observations suggest that the N-terminally truncated Cdc68 protein may have lost the ability to respond to Sug1 inhibition, perhaps because the antagonistic effect of Sug1 is mediated through the N-terminal portion of Cdc68 that is missing in the truncated protein. Thus, the independence of San1 and Sug1 inhibition with respect to Cdc68 activity may be due to the fact that San1 and Sug1 function through distinct portions of the Cdc68 protein.

4. Transcriptional regulation by Cdc68

4a) Cdc68 is implicated in transcription initiation

The Cdc68 protein was found to affect transcription of genes in which 5' regions have been interrupted by insertion of a Ty element, a type of retrovirus-like transposon in yeast. The presence of a Ty element or of a Ty long terminal repeat (a δ element) in the 5' region of a gene often inactivates gene function. For example, δ insertions into the 5' regions of the *HIS4* and *LYS2* genes (creating mutant alleles *his4-912δ* and *lys2-128δ*) alter the transcription patterns of these genes, and consequently *his4-912δ lys2-128δ* cells cannot express the *HIS4* and *LYS2* genes properly and are His^- and Lys^- (Winston et al., 1984; Clark-Adams and Winston, 1987). *cdc68* mutations can overcome the effect of this type of δ insertion by altering transcription initiation (or promoter usage) to allow the synthesis of functional *HIS4* and *LYS2* gene products, and as a result *cdc68 his4-912δ lys2-128δ* cells are His^+ and Lys^+ (a phenotype called Spt^-; Malone et al., 1991).

In addition to influencing transcription initiation at genes bearing δ sequences, Cdc68 may also affect promoter usage of genes in their natural promoter context. During the course of this study I noticed that, in *cdc68-1* cells at the restrictive temperature, a second shorter transcript became evident for the *CLB4*, *SWE1*, and *SUG1* genes (Fig. 21). Although primer-extension experiments have not been performed to rule out the possibility that the second transcript derived from altered transcription termination, I infer from the *cdc68-1* effect at the *his4-912δ* and *lys2-128δ* loci that the two transcripts observed for *CLB4*, *SWE1*, and *SUG1* most likely originate from different sites of transcription initiation. It has been demonstrated that, at least in yeast, accurate transcription initiation is largely determined by RNA polymerase II and the general transcription factor TFIIB (Li et al., 1994). The yeast TFIIB is encoded by the *SUA7* gene, and in *sua7* mutant cells transcription for several genes tested initiates from multiple sites. In this situation it appears that *sua7* mutations change the distance between TATA elements and initiation sites, and initiation from the new sites may still employ the same TATA elements as that from the normal sites (Pinto et al., 1992). Thus TFIIB does not affect the choice of TATA element. In contrast, in *cdc68-1* cells the putative second transcription initiation site appears to be more than 100 nucleotides downstream from the primary one. For *S. cerevisiae* genes, transcription initiation sites are normally located between 40 to 120 nucleotides downstream of the TATA element; the second initiation site for the *CLB4*, *SWE1*, and *SUG1* is thus at least 140 nucleotide downstream from the primary TATA element. Therefore it is unlikely that in *cdc68-1* cells both transcription initiation events share the same TATA element. Instead, the second transcription initiation event may employ a cryptic downstream promoter that becomes active in *cdc68-1* cells. Similarly, the alternative transcription initiation sites for the *his4-912δ* and *lys2-1128δ* genes are also some distance away. Thus, this assumed altered promoter preference can not be explained by Cdc68 affecting TFIIB function. A more reasonable interpretation is that changed transcription initiation is caused by altered nucleosome positioning (see

below), so that an otherwise nucleosome-repressed promoter becomes active for transcription in *cdc68-1* mutant cells.

4b) Cdc68 is an activator for histone gene expression

Upon transfer of *cdc68-1* cells to the restrictive temperature, there is a rapid and significant decrease in transcription from the *HTA1-HTB1* locus, one of the two gene pairs coding for histones H2A and H2B (Fig. 22A). It is reasonable to suspect that at least part of this decrease in histone gene expression may be a secondary effect of *cdc68-1*-mediated G1 arrest, because histone genes are only transcribed during a short window of time within S phase (reviewed by Osley, 1991). However, the *HTA1-HTB1* mRNA levels are already significantly lower compared to those in wild-type cells after incubation at 35°C for only 20 min (Fig. 22A), long before significant G1 accumulation occurs. This rapid decrease in *HTA1-HTB1* transcription in *cdc68-1* mutant cells suggests that the Cdc68 protein plays a more direct role in the expression of histone genes. This result is not surprising, since transcription of a majority (if not all) mRNA genes tested so far is affected by the *cdc68-1* mutation to some degree.

Altered histone levels affect transcription (Clark-Adams et al., 1988; Han and Grunstein, 1988; Durrin et al., 1991). The observation that Cdc68 activates histone gene transcription therefore raises the interesting possibility that some transcriptional effects of Cdc68 may be secondary and stem from altered histone gene expression. Altering the stoichiometry of histones H2A and H2B versus H3 and H4, similar to altering *CDC68* gene dosage, causes an Spt$^-$ phenotype (Clark-Adams et al., 1988). Thus the Spt$^-$ phenotype caused by *cdc68-1* may be an indirect effect of altered histone gene expression. Similarly, the persistent expression of heat-shock genes *HSP82* and *HSC82* in *cdc68-1* cells may also be related to the altered heat-shock response caused by deletion of the *HTA1-HTB1* locus (Rowley et al., 1991; Norris and Osley, 1987). Thus, the aberrant heat-shock response of *cdc68-1* mutant cells may be a secondary effect of decreased

HTA1-HTB1 expression.

A decrease in histone gene expression is unlikely to account for all *cdc68-1*-mediated transcriptional alterations, especially the transcription activation caused by *cdc68-1*. After transfer of *cdc68-1* mutant cells to the restrictive temperature, there is a rapid decrease in transcript abundance for many genes (Rowley et al., 1991), an effect too rapid to be explained by altered expression of proteins as stable as the histones. Moreover, histone synthesis normally occurs during S phase when cells replicate their DNA, so that changes in the abundance of histones resulting from decreased histone gene transcription would only be seen after S phase, whereas the rapidity of the transcriptional effects on other genes argues that decreased transcription imposed by the *cdc68-1* mutation probably occurs in all cells regardless of cell-cycle stage, and is therefore unlikely to be solely mediated by altered histone gene transcription. Furthermore, at the restrictive temperature *cdc68-1* mutant cells show a first-cycle inhibition of cell proliferation (Prendergast et al, 1990), suggesting that for many cells the *cdc68-1* inhibition of G1-cyclin gene transcription is imposed before another round of DNA replication. Thus, while some effects of Cdc68 may be an indirect consequence of altered histone levels, the Cdc68 protein nonetheless has a more direct impact on transcriptional activation.

4c) Does Cdc68 regulate transcription by affecting chromatin structure?

Previous studies have established that Cdc68 functions in transcription initiation (Rowley et al., 1991; Clark-Adams et al., 1988; Malone et al., 1991). The widespread transcriptional effects of Cdc68 is inconsistent with the possibility that Cdc68 acts as a gene-specific transcription factor. A global transcription regulator like Cdc68 can function at various levels in the transcription initiation process. Any model to explain the action of Cdc68 must take into consideration the following features. Cdc68 activity is required for the transcription of a diverse set of apparently unrelated genes; when Cdc68 activity is compromised by a mutation, transcription of a number of unrelated genes decreases

drastically (Rowley et al., 1991). Secondly, Cdc68 has both positive and negative effects on transcription. For example, *cdc68* mutations allow the *SUC2* gene to be expressed in the absence of the *UAS* (Malone et al., 1991), and also allow *HO* gene expression in the absence of the Swi4 activator (Lycan et al., 1994); in these situations the wild-type Cdc68 protein prevents transcription. On the other hand, Cdc68 clearly activates transcription of a number of genes. Thirdly, increased wild-type *CDC68* gene dosage in certain cases resembles the effect of the *cdc68-1* mutation: either overexpression of the wild-type *CDC68* gene or the *cdc68-1* allele causes a similar Spt$^-$ phenotype (Clark-Adams et al., 1988; Rowley et al., 1991; Malone et al., 1991). Finally, Cdc68 does not activate transcription when fused to a heterologous DNA-binding domain (Table 11), and in addition, overexpression of the *CDC68* gene using a high-copy plasmid does not further increase transcription of Cdc68-dependent genes (Fig. 26). These observations suggest that Cdc68 is not a conventional transcription activator.

The promoter regions of many yeast genes are assembled into nucleosomes. Thus, the initial step of transcription is to create a chromatin environment that allows TBP and other DNA-binding transcription factors to bind to their respective target DNA sites on nucleosomes. Proteins that affect nucleosome positioning or another aspect of chromatin are deemed to affect transcription efficiency. A number of such proteins have been identified. For example, the Snf/Swi complex is thought to exert its transcriptional role at this initial step by assisting the binding of transcription activators to their respective target sites on nucleosomes (see INTRODUCTION). The Cdc68 protein may also function at this step of transcription initiation by affecting nucleosome positioning or chromatin structure. Alternatively, Cdc68 may engage in a step after passing the nucleosome obstacle, for example to promote the interaction between gene-specific activators and the transcription initiation complex. The evidence we have so far favors the idea that Cdc68 is involved in some aspect of chromatin remodeling, but does not rule out an alternative chromatin-independent scenario.

The Cdc68 protein shares functional and structural similarities with a number of global transcription factors that are thought to mediate their effects *via* chromatin. Based on phenotypic effects, several *SPT* genes are categorized into the "histone group", among these the *SPT11* and *SPT12* genes that encode histone H2A and H2B, respectively (Clark-Adams et al., 1988). Three other members encode Spt4, Spt5 and Spt6 that are also implicated in chromatin structure (Swanson and Winston, 1992). The *CDC68/SPT16* gene has been assigned to this group because, like other members of this class, either overexpression of the wild-type *CDC68* gene or a *cdc68* mutation causes a similar Spt$^-$ phenotype (Clark-Adams et al., 1988; Malone et al., 1991; Swanson and Winston, 1992). Moreover, Cdc68 shares certain structural feature with Spt5 and Spt6: all three proteins have a highly negatively charged region (Rowley et al., 1991; Swanson et al., 1990, 1991), more acidic than the activation domain of acidic transcription activators such as Gal4 and Gcn4 (Ma and Ptashne, 1987; Hope and Struhl, 1986). The widespread transcriptional effects of Cdc68, like those of other members of this group of Spt proteins, may therefore be mediated through chromatin. Genetic interactions between *cdc68* and two *snf* mutations are consistent with this notion. A *cdc68* mutation can suppress the *snf2* and *snf5* defect in the *SUC2* expression (Malone et al., 1991). Snf2 (the same as Swi2) and Snf5 are components of the large Swi/Snf complex that activates the transcription of several sets of yeast genes (reviewed in Winston and Carlson, 1992; Carlson and Laurent, 1994). Studies *in vitro* suggest that the Swi/Snf complex potentiates transcription activation by assisting the binding of gene-specific activators such as Gal4 to nucleosomal DNA (Côté et al., 1994). The additional finding that overexpression of Cdc68 can bypass the requirement of Snf2/Swi2 for the expression of the *HO* gene once again suggests that Cdc68 function is related to that of the Snf/Swi complex (Fig. 25). Thus, the structural similarity and functional interactions between Cdc68 and other proteins that are thought to affect chromatin structure argue that Cdc68 may play a similar role in modulating chromatin.

The opposing effects of Cdc68 on the intact versus the *UAS*-less *SUC2* promoter are also consistent with Cdc68 being a chromatin protein. Previous studies showed that at a permissive temperature a *cdc68* mutation allows transcription from a *SUC2* basal promoter lacking the *UAS* (Malone et al., 1991). In this study, I found that the Cdc68 protein activates transcription from the intact *SUC2* promoter: there was a significant decrease in *SUC2* transcription in *cdc68-1* cells at the restrictive temperature (Fig. 24). It is possible that the repressive effect of Cdc68 on the *UAS*-less *SUC2* basal promoter is indirect and mediated through histones; as I show here, transcription from at least one histone gene locus, *HTA1-HTB1,* is affected by the *cdc68-1* mutation (Fig. 22A). Moreover, deletion of the *HTA1-HTB1* locus suppresses defects on *SUC2* expression caused by *snf2*. *snf5*, or *snf6*, suggesting that decreased histone H2A and H2B levels can allow *SUC2* expression in the absence of activators (Hirschhorn et al., 1992). Thus, it may be that altered histone levels as a result of enfeebled Cdc68 activity relieve repression of the *UAS*-less *SUC2* promoter. An equally reasonable model, similar to the one proposed by Lycan et al. (1994), is that Cdc68 may be a chromatin structural protein. In the absence of Cdc68, nucleosome positioning at the *UAS*-less *SUC2* promoter region may be altered in a way that renders the DNA more accessible to the basal transcription complex. Thus, transcription initiation can take place even without the aid of activator(s). Cdc68 may also be a chromatin component that facilitates the binding of gene-specific transcription activators to their target DNA sites on nucleosomes. In the absence of a *UAS*, transcription is independent of a gene specific activator(s) and therefore does not require Cdc68 activity. In this regard, Cdc68 maybe a chromatin protein that not only plays an architectural role to pack DNA into nucleosomes, but most importantly, it is also required for gene activation. In this respect, the Cdc68 protein may communicate with the basal transcription complex via the Sug1 protein, as suggested by the functional interaction between Cdc68 and Sug1 revealed by this investigation, although Cdc68 and Sug1 may not physically interact with each other.

It is worth noting that some functions of Cdc68 may be analogous to those of histones. For example, histone H4 has been shown to play both positive and negative roles in gene activation: removal of histone H4 increases basal transcription from the *CYC1*, *GAL1*, and *PHO5* promoters (Han and Grunstein, 1988), whereas deletion of the N-terminal tail of histone H4 decreases the activated transcription from the *GAL1* and *PHO5* promoters upon induction (Durrin et al., 1991). Similarly, mutational analysis of a histone H2A gene suggests that H2A is also required for transcription activation (Hirschhorn et al., 1995). Because of the highly acidic nature of the Cdc68 protein, Cdc68 may physically interact with basic histones.

The San1 protein has been implicated in chromatin effects. In addition to inhibiting Cdc68 transcriptional activity, the San1 protein is also involved in the regulation of the *HML* and *HMR* loci (Schnell et al., 1989), containing transcriptionally silent copies of the yeast mating-type genes. Maintenance of the repressed state of the *HM* loci requires, among other proteins, Sir3, Sir4, and histones H3 and H4 (reviewed in Laurenson and Rine, 1992). Several lines of evidence suggests that chromatin structure plays an important role in repression of the *HM* loci. Mutations in genes coding for histone H3 and H4 were found to cause derepression of the *HM* loci (Aparicio et al., 1991; Johnson et al., 1990; Park and Szoska, 1990), and *sir3* mutations can restore silencing that is abolished by mutated histone H4, providing a genetic link between silencing and histones (Johnson et al., 1990). Most recently, Hecht et al. (1995) have demonstrated that the Sir3 and Sir4 proteins can bind to the N-terminal regions of histone H3 and H4 *in vitro*, further strengthening the notion that silencing is the result of a special state of chromatin (heterochromatin). The genetic interactions between *san1* and *sir4* and between *san1* and *cdc68* may suggest that, like Sir4, San1 and Cdc68 are also involved in some aspect of chromatin structure. It is of course equally possible that San1 may modify both Sir4 and Cdc68 by other means, for example, as a modifying enzyme.

Like chromosomal DNA, plasmid DNA is also assembled into chromatin in

eukaryotic cells. The number of nucleosomes on a plasmid can be monitored by measuring plasmid linking number (the number of supercoils), because each nucleosome causes one superhelical turn (Worcel et al., 1981). I found that even at a permissive temperature in *cdc68-1* mutant cells there was an increase in plasmid linking number (Fig. 23), which is an indication of increased nucleosome assembly. This observation suggests that the number of nucleosomes assembled onto plasmid DNA depends on Cdc68 activity. However, one needs to be cautious when interpreting this finding, because even at the permissive temperature the *cdc68-1* mutant protein does not exhibit fully wild-type Cdc68 activity. The plasmid linking number experiment does not address the causal relationship between chromatin structure and transcription activity in *cdc68-1* mutant cells. In any event, chromatin structure is altered in *cdc68-1* mutant cells.

In summary, all the previous findings and the current investigation regarding Cdc68 function are consistent with the hypothesis that Cdc68 regulates transcription by affecting nucleosome positioning or chromatin configuration. However, alternative models also can explain the transcriptional role of Cdc68. Nonetheless, the available data provide justification to pursue the interaction between Cdc68 and chromatin. Examination of nucleosome positioning at Cdc68-dependent promoters in cells with altered Cdc68 activity, and establishment of a casual relationship between any change of nucleosome positioning and transcription activity, will ultimately determine if Cdc68 impinges upon gene expression by affecting chromatin configuration.

5. Sug1 is implicated in mitotic control

During the investigation of functional interactions between Cdc68 and Sug1 I found that the *sug1-26* allele by itself confers temperature sensitivity at or above 37°C (Fig. 27). Further studies showed that *sug1-26* cells arrest at a specific stage of the cell cycle at the restrictive temperature, with between 75 to 80% of mutant cells displaying a large-budded

terminal morphology characteristic of cells arrested in the G2/M phase of the cell cycle. FACS analysis showed that these *sug1-26* haploid cells have a 2N DNA content (Fig. 29), indicating that cells have completed (or almost completed) DNA replication. DAPI staining revealed that a *sug1-26* cell has a single undivided nucleus either at or stretched through the bud neck (Fig. 28), an indication of failure to execute nuclear division. Furthermore, anti-tubulin immunofluorescence revealed the existence of elongated spindles in arrested *sug1-26* cells (Fig. 30), a feature of mitotic cells. All these observations suggest that *sug1-26* mutant cells cease proliferation during the G2/M phase of the cell cycle under restrictive conditions.

In general, a G2/M cell-cycle block can result from an actual defect in a G2/M event (for example, chromosome separation), or alternatively from a defect in some early cell-cycle event (for example, DNA replication). A DNA replication defect or damaged DNA can trigger checkpoints that ensure that mitosis takes place only after DNA replication is complete and any DNA lesion is repaired (for reviews see Hartwell and Weinert, 1989; Murray, 1992). Cells monitor the status of DNA replication and genome integrity by using two overlapping checkpoints: the S phase checkpoint that arrests cells in S phase when DNA replication is inhibited (for example, by hydroxyurea), and the G2 checkpoint that causes a G2 arrest when a DNA lesion occurs either as a result of incomplete DNA replication or DNA damage (Weinert et al., 1993). The observation that the majority of *sug1-26* haploid cells have a 2N DNA content rules out the possibility of an activation of the S-phase checkpoint. A functional G2-checkpoint requires the *RAD9* gene product (Weinert and Hartwell, 1988, 1990). For example, a temperature-sensitive mutation in the *CDC9* gene (encoding DNA ligase) arrests cells in G2 phase in the presence of functional Rad9 protein; however, *cdc9 rad9Δ* double-mutant cells fail to arrest and die rapidly (Johnston and Nasmyth, 1978), suggesting that the DNA lesions caused by *cdc9* can be detected by the Rad9-dependent G2 checkpoint and *cdc9* cells can therefore arrest promptly before the onset of mitosis. If the Sug1 protein functions in a late stage of DNA

replication, any DNA lesion caused by the *sug1-26* allele should be detected by the Rad9 checkpoint. In this case, deletion of the *RAD9* gene may suppress the G2/M arrest phenotype of *sug1-26* mutant cells and *sug1-26 rad9Δ* double mutant cells are expected to lose viability. However *sug1-26* cells arrest in G2/M phase regardless of Rad9 activity, and deletion of *RAD9* does not affect the viability of *sug1-26* cells. These results indicate that the cell-cycle arrest caused by *sug1-26* is not dependent on Rad9, and argues strongly that Sug1 does not function in DNA replication or other aspects of DNA metabolism. Thus Sug1 most likely participates in mitosis.

During the course of this thesis work, Ghislain et al. (1993) reported a similar phenotype for a different *sug1* allele, *sug1-3* (also called *cim3-1*). This *sug1-3* allele causes lethality in conjunction with the cell-cycle mutation *cdc28-1N* that specifically affects the G2/M phase transition (Piggott et al., 1982; Surana et al., 1991). There are several interesting features of the G2/M arrest caused by *sug1-3*. Cell-cycle arrest in this case is accompanied by high levels of Clb2 and Clb3 proteins and, perhaps as a result, elevated levels of histone H1 kinase activity (Ghislain et al., 1993). These observations suggest that *sug1-3* cells retain an active mitosis-specific Cdc28 kinase. In addition, *sug1-3* cells have defect in protein degradation through the ubiquitin pathway. Thus the failure of *sug1-3* cells to complete mitosis may be due to insufficient proteolysis (Ghislain et al., 1993).

The *sug1-26* allele identified here shares a similar but not identical terminal phenotype with *sug1-3*. Both *sug1-26* and *sug1-3* cells display a large-budded terminal morphology and haploid cells have 2N DNA content, suggesting that they arrest during G2/M phase. However, while a portion of *sug1-26* cells have an extended nucleus and elongated spindles, *sug1-3* cells all arrest with the nucleus at the bud neck and with short spindles (Fig. 28, 30; Ghislain et al., 1993). This subtle difference in terminal phenotype could be due to leakiness of the *sug1-26* mutation, or alternatively could suggest that *sug1-26* cells are defective at a later cell-cycle stage than *sug1-3* cells. In any case, both *sug1-26*

and *sug1-3* mutant cells share a similar G2/M arrest phenotype at 37°C. Similarly, I found that *sug1-26* also affects protein degradation, for the stability of certain protein is increased in *sug1-26* cells (Fig. 16, 17). Furthermore, like *sug1-3*, *sug1-26* also shows synthetic enhancement in combination with *cdc28-1N* (Fig. 31A). Thus, both *sug1-26* and *sug1-3* alleles may arrest cells in mitosis due to a similar defect.

Based on the known properties of the Sug1 protein, two models can be proposed to account for the mitotic requirement for Sug1. Because Sug1 has been demonstrated to regulate transcription (Swaffield et al., 1992; 1995; Kim et al., 1994), the mitotic role of Sug1 is most likely a secondary effect of Sug1 on the expression of a mitotic regulator. I found that Sug1 represses transcription in some cases (Fig. 13A), so it is reasonable to propose that Sug1 restricts the expression of a mitotic inhibitor; altered Sug1 activity in *sug1-26* cells alleviates inhibition and allows the putative mitotic inhibitor to be expressed at an elevated level to block mitosis. One mitotic inhibitor is the Swe1 protein, a Wee1 homolog in the budding yeast; overproduction of Swe1 from the *GAL1* promoter was found to block cells in G2/M phase (Booher et al., 1993). However, the expression level of *SWE1* was not elevated in *sug1-26* cells (Fig. 33) and deletion of *SWE1* did not influence the *sug1-26* terminal phenotype, suggesting that the Sug1 effect is not mediated by Swe1. So far I do not know the identity of the hypothetical mitotic inhibitor (if it exits). A mutational suppressor approach will be informative here, because loss of function of such a mitotic inhibitor should alleviate the mitotic defect of *sug1-26* cells.

An alternative model can be envisioned based upon the observation that Sug1 is implicated in protein degradation through the ubiquitin pathway. As proposed by Ghislain et al. (1993), the failure of *sug1* mutant cells to complete mitosis may be a consequence of a defect in protein degradation critical for mitosis. Protein degradation plays a pivotal role during mitosis. For example, downregulation of mitotic Cdc28 kinase activity through active degradation of Clbs was found to be essential for the exit from mitosis (Surana et al., 1993). Although there was increased accumulation of Clb proteins in *sug1-3* cells, the

defect of *sug1* cannot be attributable solely to stabilization of Clbs. The terminal phenotypes of both *sug1-3* and *sug1-26* cells indicate that mutant cells arrest before or during the process of nuclear division, stages at which the active degradation of Clbs is not required (Surana et al., 1993). Furthermore, ectopic overproduction of B-type cyclins, or of an undegradable cyclin derivative, blocks yeast cells in telophase with completely separated nuclei (Surana et al., 1993). Thus the nuclear morphology of *sug1* mutant cells implies that, in addition to Clbs, degradation of some other proteins (presumably those that keep sister chromatids together) is also affected by *sug1-26*. Indeed, a proteolytic requirement for chromosome segregation is suggested by studies using *Xenopus* egg extracts (Holloway et al., 1993). Inhibition of polyubiquitination delays entry into anaphase, suggesting that proteolysis through the ubiquitin pathway is required for chromosome segregation. Furthermore, this proteolysis for chromosome segregation may employ the same set of proteins as the degradation of cyclin B, because overexpression of a cyclin B fragment containing the destruction box, presumably as a competitor for the proteolytic machinery, also delays chromosome segregation (Holloway et al., 1993). Recent studies in budding yeast have found a set of molecules required for both nuclear division and Clb degradation (Irniger et al., 1995). The elevated Clb protein levels in arrested *sug1* mutant cells may be an indication of an incompetent protein degradation machinery. Despite the fact that the precise function of Sug1 in proteolysis remains controversial (Ghislain et al., 1993; Swaffield et al., 1995), the failure of *sug1* mutant cells to complete mitosis can nonetheless be explained by a defect in proteolysis.

V. APPENDIX

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Ms. Linda M. Illig
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Dear Ms. Illig,

I am writing to ask for permission to use the data that I reported in the paper "The *Saccharomyces cerevisiae* Cdc68 transcription activator is antagonized by San1, a protein implicated in transcriptional silencing" that appeared in MOLECULAR AND CELLULAR BIOLOGY vol. 13, p. 7553-7565. I wish to include these data in my Ph.D. thesis. In addition, I have recently submitted another manuscript entitled "Sug1, a yeast transcription mediator, modulates transcription activation by Cdc68" to MOLECULAR AND CELLULAR BIOLOGY. If it is accepted for publication, I also wish to ask for permission to use data of this paper for my Ph.D. thesis.

Thanks for your attention.

Yours sincerely,

Qunli Xu

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