The Yeast Protein Complex Containing Cdc68 and Pob3 Mediates Core-Promoter Repression Through the Cdc68 N-Terminal Domain

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ABSTRACT

Transcription of nuclear genes usually involves trans-activators, whereas repression is exerted by chromatin. For several genes the transcription mediated by trans-activators and the repression mediated by chromatin depend on the CP complex, a recently described abundant yeast nuclear complex of the Pob3 and Cdc68/ Spt16 proteins. We report that the N-terminal third of the Saccharomyces cerevisiae Cdc68 protein is dispensable for gene activation but necessary for the maintenance of chromatin repression. The absence of this 300-residue N-terminal domain also decreases the need for the Swi/Snf chromatin-remodeling complex in transcription and confers an Spt-like characteristic of chromatin alterations. The repression domain, and indeed the entire Cdc68 protein, is highly conserved, as shown by the sequence of the Cdc68 functional homolog from the yeast Kluyveromyces lactis and by database searches. The repression-defective (truncated) form of Cdc68 is stable but less active at high temperatures, whereas the known point-mutant form of Cdc68, encoded by three independent mutant alleles, alters the N-terminal repression domain and destabilizes the mutant protein.

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GENE expression in the eukaryotic nucleus takes place in the context of chromatin, a complex of DNA with proteins that play structural and regulatory roles. Transcription from promoter sequences by RNA polymerase II is stimulated by trans-activator proteins that become properly localized for this function through sequence-specific DNA binding; for many genes, trans-activator function is facilitated by chromatin-remodeling complexes such as the Swi/Snf complex (Peterson andTamkun 1995; reviewed in Kingston et al. 1996). Conversely, in the absence of trans-activators or their binding sites along DNA, promoter sequences are generally unable to serve as efficient sites for transcription, in large part due to the repressive effects exerted by the chromatin environment of the DNA (Kingston et al. 1996). Several protein complexes have been shown genetically to be needed for global gene activation and/or for chromatin-mediated repression; among these is the recently described “CP” complex (Brewster et al. 1998).

The CP complex of the budding yeast Saccharomyces cerevisiae, an abundant nuclear dimer of the Cdc68 and Pob3 proteins, facilitates transcription at a number of genes. This finding stems in part from the effects of cdc68-1, a temperature-sensitive mutation that impairs gene expression at a restrictive temperature because of an alteration in Cdc68 protein structure (Rowley et al. 1991). In cdc68-1 mutant cells many mRNAs become depleted, including some that encode essential proteins; this finding accounts for the essential nature of both components of the CP complex (Malone et al. 1991; Wittmeyer et al. 1997). The rates at which mRNAs disappear in cdc68 mutant cells correlate well with mRNA stabilities, suggesting that decreased mRNA abundance results from decreased transcription (Rowley et al. 1991).

The CP complex also mediates repression at promoters lacking DNA-bound trans-activators. For example, the effects of cdc68 mutations show that the CP complex not only facilitates transcription of the SUC2 and GAL1 genes (Q. Xu, unpublished observations), but is also necessary for the repression of these genes when their UAS sequences (trans-activator binding sites) are deleted (Malone et al. 1991; Prelich and Winston 1993; Xu et al. 1993; Lycan et al. 1994). Similarly, the CP complex maintains the HO gene in a transcriptionally inactive state when the Swi4-Swi6 transcription activator is absent (Lycan et al. 1994). The repressive effects of
CP necessitate the actions of the Swi/Snf complex for the expression of SUC2 (Malone et al. 1991). These findings indicate that the CP complex fosters chromatin repression as well as transcription. This dual role suggests that the CP complex may maintain chromatin in a configuration that facilitates proper gene regulation.

A chromatin role for the CP complex is also indicated by the effects of Cdc68 protein on the transcription of the reporter genes spt16-197 and sord-1288. Each of these mutant alleles is normally unable to express functional mRNA because of the presence of the Ty1 retrotransposon long terminal repeat (α-element) inserted within 5′ sequences. The transcriptionally active α-element, perhaps through a mechanism of promoter competition (Hirschman et al. 1988), changes the pattern of transcription (Clark-Adams and Winston 1987; Hirschman et al. 1988; Malone et al. 1991; Swanson et al. 1991; reviewed in Winston 1992; Winston and Carlson 1992). Functional transcription at these reporter genes can be restored either by a cdc68 mutation or by extra copies of the CDC68 gene (Malone et al. 1991; Rowley et al. 1991; Brewster et al. 1998). This effect, termed the Spt− phenotype (Winston 1992; Winston and Carlson 1992), has been used to identify genetically many components that affect chromatin structure, including the histones. Indeed, the genes encoding the histones and Cdc68 are grouped, by Spt− characteristics, in the same category of “SPT” genes; for several of these genes, mutations or increased gene dosage confer similar effects (Clark-Adams et al. 1988; Malone et al. 1991; Rowley et al. 1991; Hirschhorn et al. 1992; Winston 1992; Prelich and Winston 1993; Kruger et al. 1995; Santisteban et al. 1997). The P0B3 gene in increased dosage also causes changes in gene expression (Brewster et al. 1998). The similar effects brought about by altered activity or abundance for the CP components Cdc68 and Pob3 and by the histones are other indications that the CP complex is involved in chromatin structure and/or remodeling.

Here we report initial structure/function studies of the Cdc68 component of the CP complex (Rowley et al. 1991). As one approach we have cloned a functional homolog of the CDC68 gene from the yeast Kluyveromyces lactis and show that the polypeptide encoded by this K. lactis gene is homologous to S. cerevisiae Cdc68 along its entire length. Deletion studies show that the N-terminal 30% of Cdc68, a region highly conserved between these two yeast homologs, is not required for essential transcription functions. In contrast, this N-terminal domain is necessary for effective chromatin-mediated repression and is partially responsible for the need for the Swi/Snf chromatin-remodeling complex. The N-terminal region of this CP component therefore mediates chromatin repression, while transcriptional activation is facilitated by the rest of the CP complex.

MATERIALS AND METHODS

Strains and media: Yeast strains used in this study (Table 1) were grown as described (Rowley et al. 1991; Xu et al. 1993). Recombinant DNA manipulations were carried out by standard procedures (Sambrook et al. 1989).

Plasmids: The CDC68 and cdc68-197 plasmids pSC2-1, pBM13, and pBM46 have been described (Prendergast et al. 1990; Malone et al. 1991). The multicopy CDC68 plasmids p68-Ba-1A and YEpDE682 contain, respectively, the 5.2-kbp BamHI fragment and 4.75-kbp BgIII-BamHI fragment from pSC2-1 in the BamHI site of YEp352 (Hill et al. 1986). The low-copy CDC68 plasmid pDE683 contains the 5.2-kbp KpnI-XbaI fragment from p68-Ba-1A between the KpnI and XbaI sites of pRS316 (Sikorski and Hieter 1989). The gal1ΔUAS CEN LEU2 plasmid pAW638 was constructed by replacing the 2.6-kbp Xhol-SstI fragment of the SSA2-lacZ plasmid YCplp102 (Barnes 1998) with the 2.7-kbp SmaI-SstI fragment from the gal1ΔUAS-lacZ plasmid pJL638 (Li and Herskowitz 1993; a gift from M. Dobson).

Generation of nested deletions in the CDC68 gene: Unidirectional nested deletions in p68-Ba-1A were prepared using EcoRI and mung bean nucleases. The SstI and SmaI multiple cloning sites were used to generate 5′ deletions; for 3′ deletions the multiple cloning sites PstI and XbaI were used. All deleted clones were restriction mapped and most insert-vector junctions were sequenced.

Isolation of cdc68-1 mutant sequences by gap repair: YEpDE682 was cleaved with SpeI and BstEII, generating a 2.4-kbp gap spanning the region to which the cdc68-1 mutation was localized (see results), and then transformed into the cdc68-1 strain ART68-1. The recircularized, gap-repaired plasmid YEpDE684 recovered from a Ura+ transformant was verified by restriction mapping with EcoRI and sequenced through open reading frame (ORF) nucleotides 19–535 to identify the cdc68-1 mutation.

Confirmation of cdc68-1 and cdc68-197: The cdc68-197 (spt16-197) mutation was identified by sequencing a 1.2-kbp BgIII-EcoRI fragment from pBM46 (Malone et al. 1991). The equivalent fragment from pBM13, the unmutagenized precursor of pBM46, was also sequenced and found to be identical to that previously described (Rowley et al. 1991). The 2.1-kbp BamHI-ClaI fragment from pBM46 (containing the cdc68-197 sequence alteration) was used to replace the equivalent fragment of CDC68 in pDE683 and the resultant plasmid, pDE47, was tested after plasmid shuffling (Sikorski and Boeke 1991).

Plasmid shuffling: Test plasmids carrying the URA3 marker were introduced into cells of strain DE13a and Ura+ transformants were selected on medium lacking uracil. To determine if a URA3 plasmid could substitute for the resident CDC68 TRP1 plasmid, pBM13, trans-
formants were then grown to stationary phase in YM1 medium, spread on YEPD medium for colony formation, and replica-plated to selective medium lacking leucine (to confirm the presence of the chromosomal cdc68::LEU2 [spit16-101::LEU2] disruption allele) or tryptophan (to test for the presence of pBM13), or uracil.

In vitro mutagenesis and targeted deletion of Cdc68 coding sequences: Mutant alleles deleted for CDC68 ORF sequences but expressed from the native CDC68 promoter were constructed by in vitro mutagenesis. The 1.8-kbp SstI-EagI fragment from p68-8A-1A encompassing the promoter region and 5' end of the CDC68 ORF was first inserted into pBSII KS+ (Stratagene, La Jolla, CA) and a ClaI site was created at ORF nucleotides 16–21 by oligonucleotide-mediated site-directed mutagenesis (Kunkel et al. 1987), using single-stranded DNA (Russel et al. 1986) and Sequenase version 2.0 (United States Biochemical, Cleveland) for primer extension. The mutagenic primer, 5'-AGCTGAAATATCGATTTCGAC, contains a single mismatch to ORF nucleotide 18 and creates a ClaI restriction site. The desired product of the mutagenesis, pDE-68M, was identified by ClaI cleavage and DNA sequencing. To generate a 0.9-kbp in-frame deletion between the new ClaI site and a second ClaI site (ORF nucleotide 920), pDE-68M was cleaved with ClaI and recircularized by ligation at low concentration. The 0.9-kbp KpnI-EagI fragment containing this deletion was then used to replace the 1.8-kbp KpnI-EagI fragment of p68-8A-1A, generating the cdc68-D922 allele and YEpDE-MC4. The 3.75-kbp SstI-SphI cdc68-D922 fragment was moved to YEp351 to create YEpSH-D922. Low-copy cdc68-D922 plasmids pDE-MC41 and pDE-141 were constructed by inserting into pRS316 the YEpDE-MC4 4.3-kbp KpnI-XbaI fragment and the 3.8-kbp BglII-XbaI fragment, respectively.

An in-frame deletion of the Sall-Hpal fragment was constructed by cleaving pDE-MC41 with Sall and Hpal, filling in the 5'-protruding end of the Sall site with Klenow enzyme and recircularizing using T4 ligase, generating pDE-MC43, which contains both 5' and central in-frame ORF deletions. The 2.9-kbp EagI-XbaI fragment from p68-8A-1A was then replaced with the 1.8-kbp EagI-XbaI fragment from pDE-MC43 to generate YEpDE-MC43, which contains an in-frame deletion of the Sall-Hpal ORF fragment. Using the ClaI site at ORF nucleotide 923, an in-frame deletion of the ClaI-Sall ORF fragment was constructed by first inserting the 5.2-kbp KpnI-XbaI fragment from p68-8A-1A into pRS316. The

### TABLE 1

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype and phenotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>21R</td>
<td>MAT a ura3-52 leu2-3,112 ade1</td>
<td>Rowl ey et al. (1991)</td>
</tr>
<tr>
<td>68507A</td>
<td>MAT a cdc68-1 ura3-52 Ade-</td>
<td>Rowl ey et al. (1991)</td>
</tr>
<tr>
<td>ART68-1</td>
<td>MAT a cdc68-1 ura3-52 leu2-3,112 ade2</td>
<td>Rowl ey et al. (1991)</td>
</tr>
<tr>
<td>LS77</td>
<td>MAT a cdc68-197 his4-9126 lys2-1286 ura3-52</td>
<td>Malone et al. (1991)</td>
</tr>
<tr>
<td>LY60</td>
<td>MAT a cdc68-11 HO::iaaZ46 ura3 leu2-3,112 trp1-1 can1-100 Ade- Met-</td>
<td>Lycan et al. (1994)</td>
</tr>
<tr>
<td>FY56</td>
<td>MAT a his4-9126 lys2-1286 ura3-52</td>
<td>Malone et al. (1991)</td>
</tr>
<tr>
<td>BM64</td>
<td>MAT a/ MAT a cdc68-101::LEU2/+ his4-9126 his4-9126 lys2-1286/lys2-1286 ura3-52 ura3-52 leu2-3,112 trp1-1 trp1-1</td>
<td>Malone et al. (1991)</td>
</tr>
<tr>
<td>DE13a</td>
<td>MAT a cdc68-101::LEU2 his4-9126 lys2-1286 ura3-52 leu2-3,112 trp1-1 pBM13(TRP1 CDC68)</td>
<td>BM64 segregant harboring pBM13</td>
</tr>
<tr>
<td>DE48B</td>
<td>BM64 with cdc68-101::LEU2 replaced by cdc68-D922</td>
<td>This study</td>
</tr>
<tr>
<td>DE48B-17a</td>
<td>MAT a his4-9126 lys2-1286 ura3-52 leu2-3,112 trp1-1</td>
<td>DE4B segregant</td>
</tr>
<tr>
<td>DE48B-17b</td>
<td>MAT a cdc68-922 his4-9126 lys2-1286 ura3-52 leu2-3,112 trp1-1</td>
<td>DE4B segregant</td>
</tr>
<tr>
<td>DE48B-17c</td>
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<td>DE4B segregant</td>
</tr>
<tr>
<td>FY711</td>
<td>MAT a san1::UTA3 his4-9126 lys2-1286 ura3-52</td>
<td>F. Winston</td>
</tr>
<tr>
<td>BM400</td>
<td>MAT a his4-9126 lys2-1286 ura3-52</td>
<td>E. A. Malone</td>
</tr>
<tr>
<td>BM403</td>
<td>MAT a his4-9126 lys2-1286 ura3-52</td>
<td>E. A. Malone</td>
</tr>
<tr>
<td>AR168-7</td>
<td>MAT a cdc68-1[URA3] ura3-52 leu2-3,112 ade2</td>
<td>Rowl ey et al. (1991)</td>
</tr>
<tr>
<td>QX3</td>
<td>MAT a cdc68-1 san1-3 ura3-52 ade2</td>
<td>Xu et al. (1993)</td>
</tr>
<tr>
<td>DE815-2d</td>
<td>MAT a cdc68-1[URA3] san1-3 ura3-52 leu2-3,112 ade2</td>
<td>AR168-7 X QX3 segregant</td>
</tr>
<tr>
<td>DE81-2b</td>
<td>MAT a cdc68-D922 san1-3 his4-9126 lys2-1286 trp1-1 Ade-</td>
<td>DE815-2d X DE48B-17b segregant</td>
</tr>
<tr>
<td>DE81-6c</td>
<td>MAT a cdc68-D922 san1-3 lys2-1286 ura3-52 leu2-3,112</td>
<td>DE815-2d X DE48B-17b segregant</td>
</tr>
<tr>
<td>QXN1</td>
<td>MAT a san1::URA3 ura3-52 leu2-3,112 Ade-</td>
<td>Xu et al. (1993)</td>
</tr>
<tr>
<td>DE1N-15d</td>
<td>MAT a cdc68-D922 san1::URA3 lys2-1286 ura3-52</td>
<td>QXN1 X DE81-6c segregant</td>
</tr>
</tbody>
</table>

* Square brackets enclose plasmid-derived sequences integrated in single copy. cdc68-D922 genotype confirmed by Southern analysis.
resultant plasmid, pDE-683, was then cleaved with Clal and Sall, the 5’-protruding ends were made flush using Klenow enzyme and the DNA was circularized using T4 ligase, generating pDE-CS8, whose 4.5-kbp Kpnl-Xbal fragment was transferred to YEp352 to generate plasmid YEpDE-CS8. Similarly, using the Clal site created at ORF nucleotide 17 an in-frame Clal-Sall deletion was constructed in plasmid QX681, creating plasmid QX681-ΔCS. Plasmid p68d915-1035 was constructed by inserting an XbaI double-stranded oligonucleotide 5’-CTA GTCTAGACTAG (New England Biolabs, Beverly, MA) into the Hpal site of CDC68 in p68-Ba-1A, a truncated ORF in which a single phenylalanine (only) is encoded following W913.

**Frameshift mutations:** A +2 frameshift mutation was generated by cleaving pDE-683 (prepared from the dam- Escherichia coli strain BW58) with Clal, filling in 5’ protruding ends with Klenow enzyme and ligating at low concentration, generating pDE-921. The resultant polypeptide contains the C-terminal extension AIHLK-KWPITTIFY following I307. The 5.2-kbp Kpnl-Xbal fragment of pDE-921 was then transferred to YEp352, generating plasmid YEpDE-921. A similar +2 frameshift mutation was introduced at the 5’ end of the CDC68 ORF in pDE-68M that had been prepared from the dam+ E. coli strain DH5α to block cleavage at the Clal site at nucleotide position 923, generating YEpDE-45. Both frameshift mutations were verified by sequencing.

**Nucleotide sequencing:** Nucleotide sequencing was carried out using Sequenase version 2.0 (U.S. Biochemicals, Cleveland, OH) and [35S]dATP (New England Nuclear, Boston). The junctions between deleted inserts and the YEp352 vector in plasmids of the p68-Exo1 series were sequenced directly using the M13-40 primer, while insert-vector junctions for plasmids of the p68-Exo2 series were sequenced using Reverse Primer. Plasmid DNA was sequenced directly (Mierendorf and Pfeffer 1987).

**Replacement of the chromosomal CDC68 gene with cdc68-Δ922:** The cdc68-Δ922 allele was integrated into the chromosome by a modification of the two-step gene-replacement method (Sherer and Davis 1979). The URA3 plasmid pDE-1410, comprising the 3.8-kbp BglII-Xbal cdc68-Δ922 fragment in pRS306 (Sikorski and Hieter 1989), was cleaved at a unique Hpal site in cdc68-Δ922 sequences and transformed into the cdc68::Δ922 diploid strain BM64. Ura+ transformants were patched onto medium containing 5-fluoroorotic acid and Ura+ recombinants were tested for leucine auxotrophy. Ura- Leu- recombinants were selected as candidate strains in which the pDE-1410 DNA had initially integrated at the chromosomal cdc68::ΔLEU2 locus and from which recombination had excised cdc68::ΔLEU2 sequences. Strains in which replacement of cdc68::ΔLEU2 by the cdc68-Δ922 allele had occurred were identified by Southern analysis of BamHI-digested genomic DNA and one such strain, DE4B, was selected for further study.

**Cloning the K. lactis CDC68 homolog:** Genomic sequences from the budding yeast K. lactis that complement the temperature sensitivity of cdc68-1 were identified in a low-copy (CEN) genomic library (Starke and Milner 1989); the active region was localized by subcloning and sequenced on both strands. The ORF so identified is named KlCDC68 (accession number U48701). The suc2ΔUAS probe was from pRB58 (Carlson and Botstein 1982; a gift from F. Winston), while the HTA1+HTB1+ADK1 probe was as described (Xu et al. 1993).

**Protein stability assay:** Cells were inhibited with cycloheximide and extracts were prepared, resolved electrophoretically, and analyzed by immunoblot as described (Xu et al. 1995), using polyclonal anti-Cdc68 antibodies (Brewster et al. 1998).

**RESULTS**

**Deletions of the CDC68 gene:** Many proteins consist of multiple domains, which in some cases can function independently. This modular feature of proteins is particularly evident for proteins involved in transcription (Frankel and Kim 1991). To assess potential domains within the Cdc68 component of the CP complex we constructed deletions of the CDC68 gene using ExoIII exonuclease and tested these deleted clones for the restoration of high-temperature growth of temperature-sensitive cdc68 mutant cells. These studies (Figure 1A) suggested that C-terminal coding sequences of Cdc68 are essential for CP function. A more striking observation was that six of the clones deleted for 5’ flanking and N-terminal coding sequences alleviated the temperature sensitivity of cdc68 mutations, suggesting that these plasmids supply essential Cdc68 function (Figure 1A).

The complementation by N-terminally deleted Cdc68 plasmids caused us to determine if the CDC68 ORF inferred from the nucleotide sequence accurately predicts the Cdc68 protein. Two frameshift mutations, one 6 codons downstream of the 5’ ATG of the ORF and the other at codon 308, each abolished gene function (Figure 1B). These findings suggest that Cdc68 is in fact encoded by the entire ORF. The sizes of CDC68 mRNA (3.2 kb; Rowley et al. 1991) and the Cdc68 protein itself (Brewster et al. 1998) are entirely consistent with this conclusion.

**An internally deleted Cdc68 polypeptide lacking N-terminal sequences supplies essential function:** In the six functional but 5’-deleted clones described above the CDC68 promoter and the first ATG of the ORF are missing. (The truncated genes are presumably expressed from a fortuitous vector promoter.) To generate truncated forms of Cdc68 that are expressed from the
authentic CDC68 promoter, and thus with known structure and expression, we made in-frame deletions within the CDC68 ORF. One of these deletion alleles, termed cdc68-Δ922 (Figure 1B, plasmid YEfpDE-MC4), is missing nucleotides 19 to 921 of the ORF (up to the CiaI site), more than those removed by the largest functional 5' deletion generated by ExoIII digestion (Figure 1A, p68-Exo2-4). The cdc68-Δ922 ORF contains the first 5 CDC68 codons (including the putative translation initiation codon) fused in-frame to codon 307.

A plasmid-borne cdc68-Δ922 allele alleviated the 37° temperature sensitivity of cdc68-1 and cdc68-197 mutant cells (Figure 1B); this effect was also seen for cdc68-Δ922 on a low-copy CEN vector (data not shown). The cdc68-Δ922 allele could also support the growth of cells lacking a functional CDC68 chromosomal locus. To show this we used the diploid strain BM64, in which one CDC68 homolog is disrupted by a LEU2 insertion and thus unable to support growth (Malone et al. 1991; Rowley et al. 1991), and which may as a consequence contain Cdc68 fragments synthesized from intact N-terminal sequences of cdc68::LEU2. We therefore showed that cdc68-Δ922 is functional in the absence of any other CDC68 sequences. The cdc68::LEU2 allele in diploid strain BM64 was replaced with the cdc68-Δ922 allele, generating the cdc68-Δ922/CDC68 strain DE4B. Sporulation of this diploid showed that in each of the 20 tetrads analyzed all four spores were viable; spore germination and mitotic growth can thus be supported entirely by the Cdc68-Δ922 polypeptide. In liquid culture at 22° the growth rate of cdc68-Δ922 mutant segregants was the same as that of CDC68 wild-type cells (data not shown). In these cdc68-Δ922 mutant cells the truncated Cdc68 protein is present at normal abundance, as shown by immunoblot analysis of cdc68-Δ922 and CDC68 cell extracts (data not shown), and is still associated with the Pob3 protein in the CP complex (Brewster et al. 1998). Therefore the N-terminally deleted polypeptide encoded by the cdc68-Δ922 mutant allele provides all Cdc68 functions necessary for the transcription of essential genes (Rowley et al. 1991).

**N-terminal sequences are conserved in K. lactis**

Cdc68: The growth of cdc68-Δ922 mutant cells raised questions concerning the importance of the N-terminal portion of the Cdc68 polypeptide and suggested that a...
The Cdc68 N-terminal domain affects transcription—The Spt\(^2\) effect: The his4-912 and lys2-1288 mutant alleles cause histidine and lysine auxotrophy, respectively, because the \(\alpha\) sequence inserted in the 5' region of each gene alters the pattern of transcription (Winston 1992; Winston and Carlson 1992). Both increased Cdc68 activity through increased gene dosage and decreased activity caused by temperature-sensitive cdc68 mutations can affect transcription and restore histidine and/or lysine prototrophy to his4-912 lys2-1288 mutant cells (the Spt\(^2\) phenotype; Malone et al. 1991; Rowley et al. 1991; Brewer et al. 1998).

Like other cdc68 mutations, cdc68-\(\Delta922\) confers an Spt\(^2\) phenotype; lys2-1288 cdc68::LEU2 haploid cells harboring a low-copy cdc68-\(\Delta922\) plasmid were Lys\(^+\), whereas the same recipient cells harboring a low-copy Cdc68 plasmid were not (Figure 3). Similarly, for each of 20 tetrads from a cdc68-\(\Delta922\)/CDC68 diploid that was complemented for both his4-912 and lys2-1288, the Spt\(^2\) phenotype of His\(^1\)/Lys\(^1\) growth segregated 2:2 and co-
segregated with the temperature sensitivity that we show below is due to cdc68-\(\Delta 922\) (data not shown). Thus the Cdc68-\(\Delta 922\) polypeptide alters transcription at the his4-9126 and lys2-1286 loci. This alteration was seen not only at 30\(^\circ\) as found for other cdc68 mutant alleles (Malone et al. 1991; Rowley et al. 1991), but also at 22\(^\circ\). At all temperatures the effects of cdc68-\(\Delta 922\) were recessive to those of the wild-type CDC68 gene (data not shown). Thus the CP complex containing the Cdc68-\(\Delta 922\) protein has a constitutively decreased activity.

**The Cdc68 N-terminal domain helps bring about the need for the Swi/Snf complex:** Chromatin can be remodeled by a large protein complex termed the Swi/Snf complex (reviewed in Peterson and Tamkun 1995). This complex can be isolated from yeast and mammalian cells as a distinct multiprotein assembly (Cairns et al. 1994; Kwon et al. 1994; Peterson et al. 1994). In cells containing structurally normal CP complex the Swi/Snf complex facilitates the expression of several genes. For example, mutations that disable the Swi2/ Snf2 or Snf5 components of the Swi/Snf complex prevent effective SUC2 expression and cause a Suc\(^+\) phenotype (Abrams et al. 1986; Hirschhorn et al. 1992). However, Suc\(^+\) growth by swi2 mutant cells and snf5 mutant cells is restored at 30\(^\circ\) by the cdc68-197 mutation (Malone et al. 1991), suggesting that Swi/Snf chromatin-remodeling activity can be bypassed to some extent by altering the CP complex.

The Swi/Snf complex is also less important for gene expression in cdc68-\(\Delta 922\) mutant cells. We showed this genetically by crossing a cdc68-\(\Delta 922\) strain with a snf5::URA3 strain and analyzing the growth of meiotic segregants genetically by crossing a cdc68-\(\Delta 922\) snf5::URA3 strain and analyzing the growth of meiotic segregants (Malone et al. 1991). All temperature-sensitive (cdc68-\(\Delta 922\)) segregated 2:2 for the 23 tetrads tested, whereas Suc\(^+\) segregants were in excess (in 14 tetrads Suc\(^+\):Suc\(^-\) segregated 3:1, and 6 others were 4:0), indicating that some segregants were Suc\(^+\) due to increased expression of suc2\(\Delta UAS\). All temperature-sensitive (cdc68-\(\Delta 922\)) segregants were Suc\(^+\) (even though the CDC68 and SUC2 genetic loci assort independently), indicating that cdc68-\(\Delta 922\) can mediate this increased suc2\(\Delta UAS\) expression. Suc\(^+\) growth was seen not only at 30\(^\circ\), as found for cdc68-197 (Malone et al. 1991), but also at 22\(^\circ\). Increased cdc68-\(\Delta 922\) gene dosage did not restore suc2\(\Delta UAS\) repression in cdc68-\(\Delta 922\) mutant cells, and cells remained Suc\(^+\) (data not shown). Thus the CP complex lacking the Cdc68 N-terminal domain is unable to maintain effective chromatin repression.

We also measured cdc68-\(\Delta 922\) effects on expression from another core promoter lacking trans-activation sequences, that of the GAL1 gene deleted for UAS\(_{GAL}\). Transcription from this gal1\(\Delta UAS\) promoter was monitored as \(\beta\)-galactosidase activity expressed by a gal1\(\Delta UAS\)-lacZ reporter gene on a centromeric plasmid. This \(\beta\)-galactosidase activity was greater in cdc68-\(\Delta 922\) mutant cells than in CDC68 cells (0.7 Miller units for cdc68-\(\Delta 922\) mutant cells growing on rafinoose medium at 23\(^\circ\) vs. <0.1 Miller units for CDC68 cells under the same growth conditions). Therefore, for at least two core promoters (lacking trans-activation) the CP complex lacking the Cdc68 N-terminal domain is unable to maintain full repression, suggesting that the CP complex has a general role in repression.

**Core-promoter repression by the CP complex is not mediated through histone gene expression:** Transcriptional repression at suc2\(\Delta UAS\) and other loci is maintained by normal histone abundance and stoichiometry, as shown by the fact that altered histone gene expression can activate suc2\(\Delta UAS\) (Clark-Adams et al. 1988). The CP complex facilitates expression of the HTA1-HTB1 gene pair encoding histones H2A and H2B, respectively (Xu et al. 1993), raising the possibility that impaired repression shown above for suc2\(\Delta UAS\) may result indirectly from cdc68 effects on histone gene expression. We therefore determined if the activation of suc2\(\Delta UAS\) in cdc68 mutant cells depends on histone gene expression. For these experiments we used the cdc68-197 gene, whose activity can be rapidly decreased by temperature shift. Populations of actively growing cells were aligned...
at the G2/M cell-cycle boundary by treatment with nocodazole (Jacobs et al. 1988), a situation that allows virtually no histone gene expression (Figure 4). After the cells had become aligned at the nocodazole blockpoint, the blocked culture was transferred to 37°C for further incubation. Northern analysis showed that this 37°C incubation resulted in a significant increase in suc2ΔUAS mRNA levels within 30 min of transfer in cdc68-197 mutant cells, while there was no increase in CDC68 cells similarly treated (Figure 4A). During the 37°C incubation in this protocol histone mRNA levels remained low (Figure 4B), consistent with the absence of cdc68 effects on the timing of histone gene expression (Xu et al. 1993).

These findings suggest that altered histone stoichiometry through altered histone biosynthesis may not be necessary for the activation of the suc2ΔUAS core promoter by modified CP-complex function.

The Cdc68 N-terminal domain is altered by temperature-sensitive point mutations: In cdc68-1 and cdc68-197 cells certain noncomplementing plasmids harboring only fragments of the CDC68 gene gave rise to temperature-resistant papillae (Figure 1) consisting of cells that remained temperature-resistant after plasmid loss (data not shown). Thus a functional CDC68 gene results from recombination between the mutant chromosomal locus and plasmid-borne wild-type DNA. Plasmids with this effect all contain sequences upstream of the EcoRI site within codon 159, localizing mutations responsible for the temperature sensitivity of cdc68-1 and cdc68-197 to the 5' portion of the gene.

Nucleotide sequencing showed that the cdc68-1 and cdc68-197 mutant alleles each have only one sequence alteration within the ORF upstream of the codon-159 EcoRI site, a G-to-A substitution at nucleotide 395 causing a glycine-to-aspartate substitution at residue 132 (G132D). This mutation generates an EcoRV site; Southern analysis revealed that both the cdc68-1 strain ART68-1 and the cdc68-197 strain L577 (distinguishable by auxotrophies) contain this site, which was absent from wild-type genomic DNA (data not shown). A fragment from cdc68-197 encoding the G132D substitution was used to replace the homologous wild-type fragment in a low-copy CDC68 plasmid; the resultant recombinant plasmid was shown, by plasmid shuffling in a cdc68::LEU2 strain, to confer the temperature sensitivity and Spt- phenotype of cdc68-197 (data not shown). We conclude that the cdc68-1 and cdc68-197 mutant effects are due to a G132D substitution in CDC68.

Restriction analysis showed that another allele, cdc68-11 (Lycan et al. 1994), also contains the EcoRV site diagnostic of the G132D substitution, while the parent strain in which cdc68-11 was isolated is missing this EcoRV site (data not shown). Thus three independently isolated cdc68 mutant alleles each encode the same amino acid substitution in the N-terminal region.

CP complex lacking the Cdc68 N-terminal domain has temperature-sensitive function: Although the Cdc68-Δ922 protein can supply all essential CP functions, cdc68-Δ922 mutant cells grew poorly at 37°C (Figure 5). Diploid cells heterozygous for cdc68-Δ922 were temperature resistant, indicating that the temperature sensitivity caused by cdc68-Δ922 is recessive. This finding indicates decreased Cdc68-Δ922 protein function at elevated temperatures.

The presence in cdc68-Δ922 cells of plasmid YEDE-921, encoding the N-terminal 307 residues (only) of Cdc68 (the same segment missing from Cdc68-Δ922), had no effect on temperature sensitivity (data not shown). On the other hand, 37°C growth is restored by increased gene dosage: cdc68-Δ922 mutant cells harbor-
The San1 protein (not other effects: the indicated temperatures.

San1 mediates cdc68-Δ922 temperature sensitivity but not other effects: The San1 protein (Schell et al. 1989) can antagonize CP activity; san1 mutations alleviate the temperature sensitivity (at 35°C) and reverse the Spt- phenotype of cdc68 point-mutant cells (Xu et al. 1993). To determine the effects of san1 mutations in cdc68-Δ922 mutant cells, a strain containing a cdc68-1 allele identifiable by a URA3 gene integrated downstream (cdc68-1::URA3; Rowley et al. 1991) and san1-3, a mutant allele that alleviates cdc68-1 temperature sensitivity, was crossed with a cdc68-Δ922 SAN1 strain, and the diploid was sporulated. In the 12 tetrads tested, 2:2 segregation was seen for Ura+ (cdc68-1::URA3) and for temperature resistance; some Ura- (cdc68-Δ922) segregants were temperature resistant. Thus the temperature sensitivity of both cdc68-1 and cdc68-Δ922 is alleviated by san1-3. Southern analysis and transformation with a low-copy SAN1 plasmid confirmed that the temperature resistance of the cdc68-Δ922 cells is due to san1-3 (data not shown). The san1Δ::URA3 deletion/ replacement allele (Xu et al. 1993) also alleviated cdc68-Δ922 temperature sensitivity (data not shown). San1 therefore antagonizes high-temperature function of the CP complex lacking the Cdc68 N-terminal domain.

Figure 5.—The cdc68-Δ922 mutant allele supports growth in a temperature-sensitive and dosage-dependent manner. Cells of the cdc68-Δ922 strain DE4B-17c harboring either the multicopy cdc68-Δ922 plasmid YEpSH Δ922 or the control vector YEp351 were spread on solid medium and incubated at the indicated temperatures.

At 33°C a low-copy SAN1 plasmid markedly inhibited the growth of cdc68-Δ922 mutant cells (Figure 6A), with no effect on wild-type CDC68 cells as previously noted (Xu et al. 1993). Increased SAN1 gene dosage therefore exacerbates the temperature sensitivity of cdc68-Δ922 mutant cells, as it does for cdc68 point-mutant cells (Xu et al. 1993). SAN1 gene dosage had negligible effect in cdc68-Δ922 mutant cells at 22°C (Figure 6A), a finding analogous to that for cdc68-1 or cdc68-197 mutant cells at a permissive temperature (Xu et al. 1993).

San1 function did not affect the cdc68-Δ922 Spt- phenotype: cdc68-Δ922 lys2-1286 cells containing either san1-3 or san1Δ::URA3 grew on medium lacking lysine (Figure 6B). This finding contrasts with the effective suppression of the Spt- phenotype of cdc68-1 and cdc68-197 mutant cells by san1 mutations (Xu et al. 1993). Eliminating San1 protein also fails to restore chromatin repression to cdc68-Δ922 mutant cells san1Δ::URA3 did not alter the Suc+ growth of cdc68-Δ922 suc2ΔUAS mutant cells (data not shown), whereas san1 cdc68-1 suc2ΔUAS triple-mutant cells are Suc- due to san1 suppression (Xu et al. 1993). Thus the mutant effects of cdc68-Δ922 cannot be completely alleviated by eliminating San1 function, suggesting that the Spt- and repression effects and the temperature sensitivity of cdc68-Δ922 mutant cells may reflect different aspects of CP-complex function.

sug1 mutations that suppress cdc68-1 do not alleviate cdc68-Δ922 temperature sensitivity: The temperature sensitivity caused by cdc68-1 can also be alleviated by sug1 mutations (Xu et al. 1995). The Sug1 protein is part of the 26S proteasome complex for ubiquitin-targeted
protein degradation (Ghislain et al. 1993; Rubin et al. 1996), and a sug1 mutation identified by cdc68-1 suppression has other effects consistent with impaired protein degradation (Q. Xu, G. C. Johnston and R. A. Singer, unpublished results). Therefore protein degradation is implicated in this suppression. Tetrad analysis showed that sug1 mutant alleles that alleviate the temperature sensitivity of cdc68-1 do not affect the temperature sensitivity of cdc68-Δ922 (data not shown). In fact, a sug1 mutant allele (Ghislain et al. 1993) that does not affect cdc68-1 (Xu et al. 1995) further impairs the growth of cdc68-Δ922 mutant cells at 35°, so that sug1-3 cdc68-Δ922 double-mutant cells showed less growth than single-mutant segregants (data not shown). Similarly, sug1 mutant alleles did not alter the gene-dosage effects of cdc68-Δ922 (data not shown). These observations suggest that protein degradation may not be an important feature of cdc68-Δ922 temperature sensitivity.  

Polypeptide stability: Cdc68 is a stable protein, while that encoded by cdc68-1 is inherently unstable and is degraded more rapidly at high temperature (Xu et al. 1995). To assess directly the stability of the Cdc68-Δ922 mutant protein, cycloheximide was added to growing cells to halt new protein synthesis and the abundance of preexisting Cdc68 protein was determined over time by immunoblotting with polyclonal antiserum (Xu et al. 1995). In this assay the Cdc68-Δ922 polypeptide was stable at 35° and unaffected by san1-3 (Figure 7). Thus neither cdc68-Δ922 temperature sensitivity nor its alleviation by san1-3 is accounted for by altered abundance or stability of the Cdc68-Δ922 polypeptide. In contrast, the unstable Cdc68-1 polypeptide was indeed stabilized by the san1-3 suppressor mutation (Figure 7), suggesting that decreased protein degradation may contribute to the effects of san1-3.  

DISCUSSION  

The CP complex is necessary both for gene activation by trans-activators and for repression at core promoters (Brewster et al. 1998). These functions are revealed by the effects of mutations that affect the Cdc68 component of the CP complex. A transfer of cdc68 mutant cells to 37° causes a rapid inhibition of transcription for a wide spectrum of genes, indicating that the CP complex facilitates transcription (Rowley et al. 1991; Xu et al. 1993). At 30°, however, cdc68 mutations have little effect on gene activation but allow transcription from core promoters (lacking trans-activators or their binding sites) that are otherwise subject to the repressive effects of chromatin (Malone et al. 1991; Prelich and Winston 1993; Xu et al. 1993; Lycan et al. 1994). Until now the relationship between these positive and negative activities of the Cdc68 protein and the CP complex has not been investigated; we show here using the newly created cdc68-Δ922 mutant allele that only a portion of the Cdc68 protein is needed for the transcription function of the CP complex and that this activation domain can be distinguished structurally from a Cdc68 domain that is necessary for CP-mediated repression.  

The cdc68 point mutation: Previous evidence for Cdc68 function was provided by the effects of three independently derived temperature-sensitive cdc68 mutations (Malone et al. 1991; Rowley et al. 1991; Lycan et al. 1994). Remarkably, each of these mutant alleles, as shown here, contains the same base pair substitution that is sufficient for temperature sensitivity. This mutation destabilizes the mutant Cdc68 polypeptide (Xu et al. 1995; Figure 7); thus a decrease in Cdc68 protein abundance may be sufficient to compromise CP-mediated chromatin repression and gene activation. This suggestion is supported by two findings. First, mutations that enfeeble the Sug1 protein, a component of the nuclear 26S proteasome that mediates targeted protein degradation (Rubin et al. 1996), can alleviate the effects of the cdc68 point mutation on chromatin repression and cell growth (Xu et al. 1995); indeed, a suppressing sug1 mutation stabilizes the mutant Cdc68 polypeptide (Xu et al. 1995) and exerts other effects consistent with decreased protein degradation (Q. Xu, G. C. Johnston et al. 1996).
and R. A. Singer, unpublished results). Second, gene dosage studies show that the Cdc68-197 mutant protein retains function even at 37°C, suggesting that much of the effects of the cdc68 point mutation, including impaired core-promoter repression, may be due to inadequate Cdc68 mutant protein (and CP complex) abundance (Xu et al. 1993). The localization of the point mutations to the N-terminal portion of the Cdc68 ORF may be indicative of a surface of the Cdc68 protein that can be sensitive for ubiquitin-mediated protein degradation. Alternatively, the unexpected identity of the three independent temperature-sensitive mutations may reflect specificity in N-terminal functions, an idea consistent with the conservation of the mutated glycine-132 in the Cdc68 polypeptide of K. lactis (Figure 2). In any event, the complicating factor of polypeptide degradation makes the effects of these cdc68 point mutations difficult to interpret mechanistically.

The Cdc68-Δ922 allele is also temperature sensitive for function, but in this case Cdc68 polypeptide proteolysis is not a factor, for we show that the Cdc68-Δ922 polypeptide is stable under our assay conditions. The N-terminal portion of Cdc68 may facilitate the 37°C function of the C-terminal part of Cdc68 represented by the Cdc68-Δ922 protein, perhaps by providing structural cues for polypeptide folding. Alternatively, the Cdc68-Δ922 protein, with residues 1–5 of the full-length protein grafted onto residue 307, may be dysfunctional at high temperatures due to a misfolding of these juxtaposed sequences. Regardless, at low growth temperatures the stability of the Cdc68-Δ922 protein allows the functions of the N-terminal and C-terminal portions of the Cdc68 protein to be deduced from effects on gene expression.

N-terminal Cdc68 sequences in the CP complex facilitate chromatin-mediated repression: Promoter sequences do not support transcription in the absence of trans-activator proteins or the DNA sequences that localize such trans-activators. The lack of promoter activity under these conditions is seen in vivo, and also in vitro for promoters complexed with histones and other proteins in the form of chromatin (reviewed in Kingston et al. 1996). Chromatin thus has a generally repressive effect on transcription. Maintenance of this chromatin-mediated repression depends on the CP complex (Malone et al. 1991; Prêlitch and Winston 1993; Xu et al. 1993; Lycan et al. 1994), and we show here that the N-terminal 300 residues of the Cdc68 component of CP are especially important for this repression.

The relief of chromatin repression at several promoters can be mediated by the Swi/Snf complex, a multisubunit protein assembly with nucleosome-reorganizing activity in vivo (Hirschhorn et al. 1992; reviewed in Peterson and Tamkun 1995; Kingston et al. 1996). We show here that one function of the Swi/Snf complex is to counteract the repressive effect of the Cdc68 N-terminal domain. The effects of the Cdc68 N-terminal domain—maintaining repression at core promoters and necessitating Swi/Snf function to relieve chromatin repression—may be manifestations of the same repressive function of the CP complex.

The chromatin-repression effects of cdc68 mutations are reminiscent of those caused by impaired histone function (Prêlitch and Winston 1993; Krüger et al. 1995; Lefant et al. 1996; Santisteban et al. 1997; Wechsler et al. 1997). Certain histone H4 mutations that alter interaction with the histone H2A-H2B dimer mimic the effects of cdc68-Δ922, including the different degrees of SU2 and INO1 expression in cells impaired for Swi/Snf function (Santisteban et al. 1997). These similarities suggest that the nucleosome may be a downstream effector of some aspects of CP function.

Transcription in the absence of localized trans-activators or under conditions of impaired Swi/Snf function can be brought about by alterations in histone stoichiometry through decreased histone-gene dosage (Clark-Adams et al. 1988; Hirschhorn et al. 1992). Altered histone stoichiometry may also be caused by the cdc68-1 mutation, which affects expression of the HTA1-HTB1 gene set encoding histones H2A and H2B (Xu et al. 1993). It is therefore significant that we find, for cells arrested at a cell-cycle position that precludes histone gene induction, that the suc2ΔUAS gene is activated promptly after the impairment of CP function. This finding indicates that an altered histone stoichiometry through aberrant histone gene expression is not necessary for core-promoter activity in cdc68 mutant cells, and points to a more direct role for the CP complex in the maintenance of a repressive chromatin structure. The abundant nature of the CP complex (Brewster et al. 1998) is consistent with this suggestion.

Chromatin repression is mediated by several other proteins, including the Spt5 and Spt6 proteins. These proteins have effects on the his4-912 and lys2-128 reporter genes and can be mutated to activate core promoters and decrease the need for Swi/Snf activity (Neijbörn et al. 1986, 1987; Clark-Adams and Winston 1987; Swanson and Winston 1992; Prêlitch and Winston 1993). Recent studies indicate a coordinate role for Spt5 and Spt6 in the elongation phase of transcription (Haytes et al. 1998; Wada et al. 1998). Mutations affecting the Spt6 protein can alter chromatin structure, and Spt6 physically interacts with histone H3 (Bortvin and Winston 1996). Genetic evidence suggests that these Spt proteins and CP have overlapping but distinct functions (Malone et al. 1991), while biochemical characterization suggests that the CP complex does not contain these Spt proteins (Wittmeyer and Formosa 1997; Brewster et al. 1998).

C-terminal Cdc68 sequences supply all essential functions, probably for gene activation: The Cdc68-Δ922 protein, although constitutively impaired for chromatin repression, is sufficient for cell viability. Among the essential functions supplied by the C-terminal 70% of Cdc68 is the facilitation of trans-activation, as evidenced
by rapid declines in mRNA abundance for a variety of genes when the function and abundance of the Cdc68-1 mutant protein is decreased (Xu et al. 1995; Figure 7). The deletion studies illustrated in Figure 1 show that several subregions of the essential Cdc68 C-terminal domain are themselves essential. Notable among these are the C-terminal 122 residues, which include a highly acidic region similar to that of the other CP component Pob3 (Wittmeyer and Formosa 1997) and analogous to the acidic regions of the Spt5 and Spt6 proteins (Swanson et al. 1990, 1991).

The eukaryotic CP complex: There is homology along the entire lengths of the Cdc68 polypeptides from S. cerevisiae and the yeast K. lactis, including the N-terminal repression domain (Figure 2). Moreover, a database search, including the EST database containing animal and plant partial cDNA sequences, yielded several cDNA or cDNA fragments encoding polypeptides structurally related to yeast Cdc68 protein (Figure 8). Similarities are found not only for the acidic C terminus and the region most highly conserved between the two yeast Cdc68 proteins, but also along the N-terminal repression domain as defined here. Even the glycine at position 132 that is substituted in the three Cdc68 point-mutant alleles is conserved in cDNA-encoded polypeptides related to this region. These similarities, plus the existence of proteins structurally related to the Cdc68 partner protein Pob3 in the CP complex (Wittmeyer and Formosa 1997), suggest that the CP complex is found across the eukaryote kingdom, an inference consistent with the essential nature of both Cdc68 and Pob3 in yeast.

The relationship between the activation and repression effects of the CP complex is not clear. Despite these opposing effects the abundant CP complex may have a single function, imposing an orderly structure upon chromatin. This view hypothesizes that chromatin can be altered to a degree that actually inhibits transcription, and that this situation develops when CP function is grossly insufficient. Thus the relief of core-promoter repression and the inhibition of trans-activation, seen under different situations of altered CP function, might be manifestations of different degrees of chromatin mal-function. This view is consistent with the effects of certain histone H4 mutations that affect interactions within the nucleosome; these mutations at one growth temperature bypass the need for trans-activators and alter chromatin structure at some promoters, but under more severe conditions inhibit the expression of several genes (Sanquist et al. 1997). Alternatively, the CP complex may be bifunctional, maintaining chromatin repression but allowing trans-activator proteins to stimulate transcription.

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LITERATURE CITED


Figure 8.—Cdc68 schematic, showing the acidic domain (hatched), the dispensable N terminus (white), the G→D mutation in cdc68-1, cdc68-11, and cdc68-197, and the region of maximal identity between the S. cerevisiae and K. lactis forms of Cdc68. Horizontal bars denote regions of similarity between Cdc68, the Drosophila dre4 protein (fly), and the polypeptides encoded by EST partial cDNAs from Caenorhabditis elegans (worm), human, rice, and Arabidopsis sources.