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Sug1 Modulates Yeast Transcription Activation by Cdc68

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The Cdc68 protein is required for the transcription of a variety of genes in the yeast *Saccharomyces cerevisiae*. In a search for proteins involved in the activity of the Cdc68 protein, we identified four suppressor genes in which mutations reverse the temperature sensitivity caused by the *cdc68-1* allele. We report here the molecular characterization of mutations in one suppressor gene, the previously identified *SUG1* gene. The Sug1 protein has been implicated in both transcriptional regulation and proteolysis. *sug1* suppressor alleles reversed most aspects of the *cdc68-1* mutant phenotype but did not suppress the lethality of a *cdc68* null allele, indicating that *sug1* suppression is by restoration of Cdc68 activity. Our evidence suggests that suppression by *sug1* is unlikely to be due to increased stability of mutant Cdc68 protein, despite the observation that Sug1 affected proteolysis of mutant Cdc68. We report here that attenuated Sug1 activity strengthens mutant Cdc68 activity, whereas increased Sug1 activity further inhibits enfeebled Cdc68 activity, suggesting that Sug1 antagonizes the activator function of Cdc68 for transcription. Consistent with this hypothesis, we find that Sug1 represses transcription in vivo.

For eukaryotes, initiation of transcription of protein-coding genes is carried out by a multiprotein complex, termed the basal initiation complex, that consists of RNA polymerase II and other general transcription factors (for a review see reference 49). Additional proteins influence the efficiency of transcription initiation by interacting with the basal transcription complex either directly or indirectly. Because DNA exists in a compact chromatin structure in eukaryotic cells, chromatin must first be remodeled to allow access by basal initiation complexes and gene-specific transcription factors. Thus, transcription initiation reflects a complex set of interactions in which proteins can either potentiate or repress gene activation at various levels; some proteins may influence transcription by affecting chromatin configuration, whereas others may influence the assembly of basal initiation complexes.

The Cdc68 protein is required for the transcription of many genes in the yeast *Saccharomyces cerevisiae* (35). The *CDC68* gene was originally identified by the *cdc68-1* mutation, which causes temperature sensitivity for the performance of the G1/S cell cycle transition (32). Subsequent studies found that the G1 arrest phenotype of *cdc68-1* mutant cells is a consequence of diminished G1 cyclin gene expression needed for activation of the G1-specific $p34^{CDC28}$ kinase complex and for transit through G1 (35). Transcription of the *SWI4* gene (encoding a transcription activator for the *CLN1* and *CLN2* genes) also requires Cdc68 protein function, so that the effect of Cdc68 on the expression of *CLN1* and *CLN2* could well be mediated through Swi4 (28). In addition to *CLN1*, *CLN2*, *CLN3*, and *SWI4*, many other genes also require Cdc68 activity for expression, including *ACT1*, *LEU2*, and the *CDC68* gene itself (35). Additional observations support the notion that the Cdc68 protein affects transcription initiation. The *CDC68* gene was found to be identical to the *SPT16* gene; either overexpression of the wild-type *CDC68*/*SPT16* gene or *cdc68* mutant alleles can suppress the effects of solo-δ insertions in the 5′ regions of the *HIS4* and *LYS2* genes (8, 29). The δ sequence is the long terminal repeat of the Ty element, and δ insertions at the *HIS4* and *LYS2* loci alter normal transcription from these genes to cause a His^- or Lys^- phenotype (41, 45). Suppression of the effects of these δ insertions by a *cdc68* mutation was shown to result from altered transcription initiation (29), providing further evidence that Cdc68 participates in the transcription initiation process.

To expand our understanding of Cdc68 protein function, we isolated genetic suppressors of the temperature sensitivity caused by the *cdc68-1* mutation (46). We reasoned that this approach would enable us to identify proteins involved in the same transcriptional regulatory circuitry as the Cdc68 protein. Four suppressor genes were defined by this genetic approach, including the *SAN1* gene (46). Mutations in *SAN1* were also identified as suppressors of a *sir4* mutation that impairs the regional transcriptional silencing at the two cryptic mating-type loci, *HML* and *HMR* (38). The effects of Sir4 on transcriptional silencing are thought to be mediated through chromatin (7, 25, 30). We found that the absence of the San1 protein allows transcription in cells with enfeebled Cdc68 activity and conversely that overexpression of the *SAN1* gene further impairs the mutant activity encoded by the *cdc68-1* allele. Therefore, San1 antagonizes the positive effect of Cdc68 on transcription. Identification of *san1* mutations as suppressors of both *cdc68* and *sir4* is consistent with the suggestion that Cdc68 may function through remodeling chromatin structure.

Here we have characterized a second suppressor gene that turned out to be identical to the previously reported *SUG1* gene. *SUG1* was originally identified because a *sug1-1* mutation suppresses a *gal4D* mutation that prevents effective transcription activation by the Gal4 protein (42). The Sug1 protein has been uncovered by other unrelated studies and was inferred to have more than one function. Ghislain et al. (17) identified a *sug1* allele, which they termed *cim3-1*, during a screen for synthetic lethality using a cell cycle mutant. The *cim3-1* mutation (termed *sug1-3* here) affects degradation of ubiquitin conjugates, and it was shown that anti-Sug1 polyclonal antibodies react with a subunit of the purified *Drosophila* 26S protease, a large protein complex that mediates the degradation of ubi-

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TABLE 1. Yeast strains used in this study

Strain	Genotype and phenotype[a]	Source or reference
21R	*MAT***a** *leu2-3,112 ura3-52 ade1*	35
68507A[b]	*MAT*α *cdc68-1 ura3-52* Ade$^-$	35
ARI68-7[b]	*MAT***a** *cdc68-1*[*URA3*] *leu2-3,112 ura3-52* Ade$^-$	35
FY56	*MAT*α *his4-912*δ *lys2-128*δ *ura3-52*	29
FYARQ1	*MAT***a** *cdc68-1 his4-912*δ *lys2-128*δ *ura3-52*	Segregant of 68507A × FY56
21R2	*MAT*α *leu2-3,112 ura3-52*	Segregant of 21R × FY56
BM403	*MAT***a** *cdc68-197 his4-912*δ *lys2-128*δ *ura3-52 suc2*Δ*UAS*(−*1900*/−*390*)	46
BM404	*MAT***a** *his4-912*δ *lys2-128*δ *ura3-52 suc2*Δ*UAS*(−*1900*/−*390*)	46
BM64	*MAT***a**/*MAT*α *cdc68-101*::*LEU2*/*CDC68 his4-912*δ/*his4-912*δ *lys2-128*δ/*lys2-128*δ *leu2-3,112*/*leu2-3,112 trp1*/*trp1 ura3-52*/*ura3-52*	46
W303-1a	*MAT***a** *leu2-3,112 his3-11,15 trp1-1 ura3-52 can1-100 ade2-1*	
QX3[b]	*MAT*α *cdc68-1 san1-3 ura3-52* Ade$^-$	46
QXT20[b]	*MAT*α *cdc68-1 sug1-20 ura3-52* Ade$^-$	This study
QXT26[b]	*MAT*α *cdc68-1 sug1-26 ura3-52* Ade$^-$	This study
QXT25[b]	*MAT*α *cdc68-1 sug1-25 ura3-52* Ade$^-$	This study
QX202	*MAT*α *sug1-20 ura3-52* Ade$^-$	Segregant of 21R × QXT20
QX261	*MAT***a** *sug1-26 leu2-3,112 ura3-52 ade*?	Segregant of 21R × QXT26
QX2612	*MAT*α *sug1-26 leu2-3,112 his3-11,15 trp1-1 ura3-52* Ade$^-$	Segregant of W303-1a × QX261
QX261-1d	*MAT***a** *sug1-26 his4-912*δ *lys2-128*δ *ura3-52*	Segregant of QX261 × FY56
SUXB201[c]	*MAT***a** *cdc68-1 sug1-20 his4-912*δ *lys2-128*δ *ura3-52* Ade$^-$	This study
SUXB261[c]	*MAT*α *cdc68-1 sug1-26 his4-912*δ *lys2-128*δ *ura3-52* Ade$^-$	This study
XBI261[d]	*MAT*α *cdc68-1 sug1-26*[*SUG1 URA3*] *his4-912*δ *lys2-128*δ *ura3-52* Ade$^-$	This study
QXD2	*MAT***a**/*MAT*α *cdc68-1*[*URA3*]/*CDC68 leu2-3,112*/*leu2-3,112 ura3-52*/*ura3-52* Ade$^+$/Ade$^-$	21R2 × ARI68-7
YJOZ	*MAT***a** *GAL1-10*::*lacZ gal4*Δ *gal80*Δ *his3 leu2-3,112 trp1 ura3-52 ade2-101*	42
YJOZS	*MAT***a** *sug1-1 GAL1-10*::*lacZ gal4*Δ *gal80*Δ *his3 leu2-3,112 trp1 ura3-52 ade2-101*	42
QX202-4d	*MAT*? *sug1-20 gal4*Δ *ura3-52* Ade$^-$	Segregant of QX202 × YJOZS
CMY762	*MAT***a** *cim3-1 his3*Δ*-200 leu2*Δ*1 ura3-52*	17
LY60	*MAT*α *cdc68-11 ho*::*lacZ46 leu2-3,112 trp1-1 ura3-52 can1-100* Ade$^-$ Met$^-$	28
QX6820	*MAT***a** *cdc68-11 leu2-3,112 trp1-1 ura3-52* Ade$^-$	Segregant of 21R × LY60
QX6810[e]	*MAT***a** *cdc68-101*::*LEU2 his4-912*δ *lys2-128*δ *leu2,3-112 trp1*Δ *ura3-52* (pXHA681)	This study
QX6811[f]	*MAT***a** *cdc68-101*::*LEU2*[*CDC68-200N TRP1*] *his4-912*δ *lys2-128*δ *leu2-3,112 trp1*Δ *ura3-52*	This study
QX2614	*MAT***a** *sug1-26 cdc68-101*::*LEU2*[*CDC68-200N TRP1*] *leu2-3,112 trp1-1 ura3-52* Ade$^-$	Segregant of QX2612 × QX6811
QXT101[g]	*MAT***a** *cdc68-1*[*cdc68-201N URA3*] *sug1-1 leu2-3,112 trp1-1 ura3-52* Ade$^-$	This study

[a] Gene designations in square brackets indicate plasmid-derived sequences integrated in a single copy into the chromosome, and the designation in parentheses indicates an episomal plasmid.
[b] Congenic with strain 21R.
[c] A segregant from a cross between strains FY56 and 21R was backcrossed with 21R. A *his4-912*δ *lys2-128*δ segregant from this cross was mated with QXT20 and QXT26 to derive strains SUXB201 and SUX261, respectively.
[d] From directed integration of pIKE17 at the *sug1-26 Cla*I site in strain SUXB261.
[e] A segregant of BM64 transformed with plasmid pXHA681.
[f] From integration of pXHA682 into QX6810 and subsequent loss of pXHA681.
[g] A segregant from a cross between YJOZS and QXT20 harboring pXIHA68-1.

quitinated proteins. These findings led to the proposal that the Sug1 protein is a component of the 26S protease and that a primary function of Sug1 is in proteolysis (17).

Other observations have led to the proposal that the Sug1 protein is directly involved in transcription. Sug1 was identified in a purified transcription mediator complex that, together with RNA polymerase II, forms the polymerase II holoenzyme (26). In addition, the Sug1 protein can bind to the activation domain of Gal4 and to the basal transcription factor TATA-binding protein (44). Swaffield et al. (44) suggested that Sug1 is not a subunit of the 26S protease and that other Sug1-like proteins exist in this large protease complex. Indeed, Sug1 belongs to a family of proteins that have a large region of homology, including an ATPase module (16). Members of this family of Sug1-like proteins, termed CAD (conserved, ATPase-containing domain) proteins (44), are involved in diverse functions such as transcriptional regulation (Sug1) (42), protein degradation (Cim5) (17), cell cycle control (Cdc48) (15), vesicle traffic (Sec18) (9), and organelle biogenesis (Pas1) (11).

Here we show that the Sug1 protein has a repressive effect on transcription. We find that Sug1 affects the degradation of mutant Cdc68 polypeptide, but further observations allow us to conclude that this proteolytic effect of Sug1 is indirect and does not entirely account for the suppression of the *cdc68-1* mutant phenotype. Instead we propose that the antagonistic effect of Sug1 on Cdc68 transcriptional activity is the cause for suppression.

MATERIALS AND METHODS

Strains and genetic methods. The yeast strains used in this study are listed in Table 1. Yeast cells were grown and plasmid DNA was propagated as described previously (46). Standard yeast genetic procedures were used for strain construction, sporulation, tetrad analysis, and other genetic manipulations (19).

Cloning the wild-type suppressor gene. To isolate the wild-type *SUG1* gene, YCp50 genomic-library DNA was transformed into the *cdc68-1 sug1-26 lys2-128*δ *ura3-52* strain SUXB261 by the lithium acetate procedure (24). Ura$^+$ Lys$^+$ transformants were selected and 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 (39), and the resultant plasmids, pXES5, pXKS4, pXHH21, and pXKE17 (see Fig. 3), were 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 (21). The resulting plasmid, pIKE17, was linearized at the unique *Cla*I site within the insert and transformed into the *cdc68-1 sug1-26 lys2-128*δ *ura3-52* strain SUXB261 to generate the transformed strain XBI261, which was then mated with the *cdc68-1 lys2-128*δ *ura3-52* strain FYARQ1, and the resultant diploid was sporulated. 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 (34). Plasmid integration at the homologous suppressor locus was confirmed by Southern analysis.

DNA manipulations and plasmid construction. *Escherichia coli* plasmid DNA was extracted by the alkaline lysis method (3). Transformation of plasmid DNA into *E. coli* cells was achieved by either the $CaCl_2$ method or electroporation (1). Yeast plasmid DNA was extracted as described previously (27). Preparation of total yeast DNA and Southern analysis were performed as described previously (1), with minor modifications (46). DNA manipulations were carried out essentially as described previously (1, 36).

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 (48), and the *LEU2* gene (*Nar*I-*Hpa*I fragment) from YEp351 (21) was inserted into the unique *Cla*I site (within the *SUG1* open reading frame) by blunt-end ligation. The resulting plasmid, pXS23, contains the disruption allele *sug1*::*LEU2*. The *Pvu*II-*Bam*HI linear fragment containing the *sug1*::*LEU2* allele was then 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 at the *Cla*I site.

Sequencing of *SUG1* and mutant *sug1* alleles. Restriction fragments of *SUG1* were cloned into the phage vector M13mp19 (31), and the nucleotide sequence was determined by the method of Sanger et al. (37) with the Sequenase kit, version 2.0 (U.S. Biochemical Corp., Cleveland, Ohio).

The *sug1* mutant alleles were amplified by PCR with genomic DNA from *cdc68-1 sug1* mutant strains as the template and the PCR primers 5′-GGGG TACCGTTATATCCTGTATA and 5′-GGAATTCGCTTTGGAAATGGCA (University of Calgary DNA Services, Calgary, Canada). The PCR mixture contained 50 mM KCl, 10 mM Tris (pH 8.4), 3 mM $MgCl_2$, 0.2 mM each deoxynucleoside triphosphate, 50 pmol of each primer, 1 μg of genomic template DNA, and 1 U of *Taq* polymerase (Bethesda Research Laboratories). Samples were denatured at 94°C for 5 min; 35 cycles of 1 min each at 94°C, 45°C, and 72°C were carried out in a thermal cycler (COY TempCycler II; DiaMed). To ensure completion of the extension step, the reaction was terminated by incubation at 72°C for 15 min. Amplified *sug1* mutant alleles were cloned into pRS316 by using the terminal *Eco*RI and *Kpn*I sites generated during PCR. The complete sequences of *sug1* mutant open reading frames (ORFs) were determined by a double-strand sequencing procedure with oligonucleotide primers corresponding to different regions of the *SUG1* ORF (gifts from J. C. Swaffield and S. A. Johnston). To verify that the G-to-C single-base-pair change detected for the *sug1-26* allele caused the temperature sensitivity, the *Spe*I-*Cla*I fragment of plasmid pIKE17 was replaced with the mutated version from the *sug1-26* plasmid pXsug1-26. The resultant plasmid, pXsug1-261 (harboring the G-to-C mutation), was linearized at the unique *Bst*XI site and transformed into a diploid strain heterozygous for the *sug1*::*LEU2* disruption. Ten diploid transformants were sporulated, and meiotic products were assessed for growth at 37°C.

Assessment of interactions between *cdc68*, *gal4D*, and *sug1* mutations. The *sug1-20* mutant strain QX202 was mated with the *gal4*Δ strain YJOZ (which has *GAL4* deleted) to generate strain QX202-4d, which was then transformed with the *gal4D* plasmid pSB32gal4D or the *GAL4* plasmid pSB32GAL4 and tested for growth on galactose medium.

A plasmid-shuffling procedure (5) was used to determine whether a *sug1* mutation can suppress the lethality caused by the *cdc68-101*::*LEU2* disruption allele. The *CDC68* plasmid p68-Ba-1A (35) was introduced into the *cdc68-101*::*LEU2*/*CDC68* diploid strain BM64. A Ura^+ Leu^+ segregant (harboring the *cdc68-101*::*LEU2* allele and kept alive by p68-Ba-1A) was mated with a *sug1-20* strain. After loss of p68-Ba-1A, the resultant diploid cells were sporulated and spore viability was determined.

Northern (RNA) analysis. Total yeast RNA was extracted and Northern blot hybridization was carried out as described previously (46). The *SUG1* probe was the 1.7-kbp *Eco*RI-*Kpn*I fragment from plasmid pXKE17. The *CDC68*, *CLN1*, *CLN2*, *CLN3*, *ACT1*, and *TUB2* probes (35, 46) were purified and labeled as described previously (46).

Epitope tagging of the Cdc68 polypeptide. A triple hemagglutinin (HA) epitope cassette (14) was inserted into the *CDC68* ORF at an N-terminal position (the sixth codon), at an engineered *Cla*I site (12). *Cla*I sites were added to both ends of the HA epitope-coding sequence by PCR amplification with plasmid pGTEP1 (a gift from B. Futcher) as the template. Primers were 5′-GCATC GATGCACTGAGCAGCGTAATCTGGA and 5′-CCATCGATGGCCGCAT CTTTTACCCATACG. The amplified DNA was gel purified, digested with *Cla*I, and inserted into the 5′ region of the *CDC68* gene at the *Cla*I site in episomal plasmid pX68c, generating plasmid pXHA681. Sequencing of the ligation junctions verified that the epitope was inserted in the correct orientation and reading frame. The HA-tagged *CDC68* gene, termed *CDC68-200N*, was cloned into the integrating *TRP1* vector pRS304 (39), generating plasmid pXHA682. To determine whether HA epitope addition affects Cdc68 protein function, plasmid pXHA681 was introduced into strain BM64 and the resultant transformant, BM641, was sporulated. A Ura^+ Leu^+ meiotic segregant harboring pXHA681 (strain QX6810) was then transformed with pXHA682, and pXHA681 was lost from this transformant by 5-fluoro-orotic acid counterselection (4). The resulting strain, QX6811, has a single copy of the HA-tagged *CDC68* gene integrated at the genomic *cdc68-101*::*LEU2* disruption allele. A haploid *sug1-26 cdc68-101*::*LEU2*[*CDC68-200N TRP1*] strain was constructed by mating QX6811 with QX261 and selecting for a Leu^+ Trp^+ temperature-sensitive (*sug1-26*) segregant (strain QX2614).

The polypeptide encoded by the *cdc68-1* allele was epitope tagged by a multistep restriction fragment swap. The *Kpn*I-*Eag*I restriction fragment of *cdc68-1* was cloned into the vector $pBSIIKS^+$ (Stratagene), generating pKS68-1, and the 0.7-kbp *Spe*I fragment, which does not harbor the *cdc68-1* base pair substitution (12), 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 the *cdc68-1* allele, generating pXHA68-1. The HA-tagged *cdc68-1* allele, *cdc68-201N*, was also cloned into the integrating vector YIp351 (21), 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*[*cdc68-201N*] *sug1-1* segregant was chosen (strain QXT101).

Indirect immunofluorescence. Yeast strain QX6811 was grown at 23°C to 5×10^6 cells per ml. Cells were then fixed by adding formaldehyde directly to the culture medium to a final concentration of 3.7% (wt/vol) and incubated at room temperature for 2 h. Cell walls were permeabilized by glucuronidase and β-mercaptoethanol treatment, and samples were prepared for indirect immunofluorescence as described previously (33). DAPI (4′,6-diamidino-2-phenylindole) was included in the mounting medium to visualize the nucleus. Monoclonal mouse anti-HA antibody 12CA5 (a gift from B. Futcher) was used at a 1:100 dilution and affinity-purified fluorescein isothiocyanate-conjugated anti-mouse immunoglobulin G secondary antibody (Sigma) was used at a 1:400 dilution. Fluorescence photomicroscopy of stained cells was performed with a Nikon microphot FX equipped with epifluorescence.

Protein extraction and immunoblot analysis. *S. cerevisiae* cells were grown to early log phase, and 30-ml samples were removed at intervals after transfer to 35°C and addition of cycloheximide (0.5 mg/ml). Cycloheximide was used to inhibit further protein synthesis so that protein levels detected by immunoblot analysis reflect stability of preexisting proteins; the inhibition of protein synthesis was confirmed for each set of experiments. Extracts were prepared with glass beads essentially as described elsewhere (1), except that cells were frozen in liquid nitrogen before breakage. The protease inhibitors chymostatin, aprotinin, pepstatin A (2 μg/ml each), leupeptin (0.5 μg/ml), and phenylmethylsulfonyl fluoride (1 mM) were included in the extraction buffer. Equal amounts of total protein (5 to 10 μg) (6) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis under denaturing conditions and then transferred to a polyvinylidene difluoride membrane (Bio-Rad) by using a semidry transfer system (Bio-Rad). Immunodetection was carried out with the enhanced chemiluminescence system (Amersham). Monoclonal anti-HA antibody 12CA5 (Boehringer Mannheim) was used as the primary antibody (at a dilution of 1:10,000) to detect HA-tagged Cdc68 wild-type and mutant proteins. Secondary horseradish peroxidase-linked anti-mouse immunoglobulin G antibody (Amersham) was used at a dilution of 1:15,000. A monoclonal rat antitubulin antibody (Sera-lab, Crawley Down, Sussex, England) was used at a 1:500 dilution to detect tubulin; secondary horseradish peroxidase-conjugated anti-rat immunoglobulin G antibody (Amersham) was used at a 1:10,000 dilution.

RESULTS

***sug1* mutations can suppress transcriptional defects caused by *cdc68-1*.** In an effort to elucidate the transcriptional activation mediated by Cdc68 we identified suppressor mutations that restore growth of *cdc68-1* mutant cells at the restrictive temperature of 35°C (46). One suppressor gene, originally termed *SCB68*, is characterized here. (Note: *SCB68* was our provisional gene designation, but we show below that *SCB68* is the *SUG1* gene [42], and we therefore use the *SUG1* gene designation throughout this report.) We reported previously that mutations in another suppressor gene, *SAN1*, identified during the same suppressor screen that isolated *sug1*/*scb68* mutations, ameliorate the transcriptional deficiencies imposed by the *cdc68-1* mutation (46). Both the *sug1-20* and *sug1-26* suppressor mutations that we report here also restore the transcription of Cdc68-dependent genes, including *ACT1* and the G1 cyclin genes *CLN1*, *CLN2*, and *CLN3* (Fig. 1A and data not

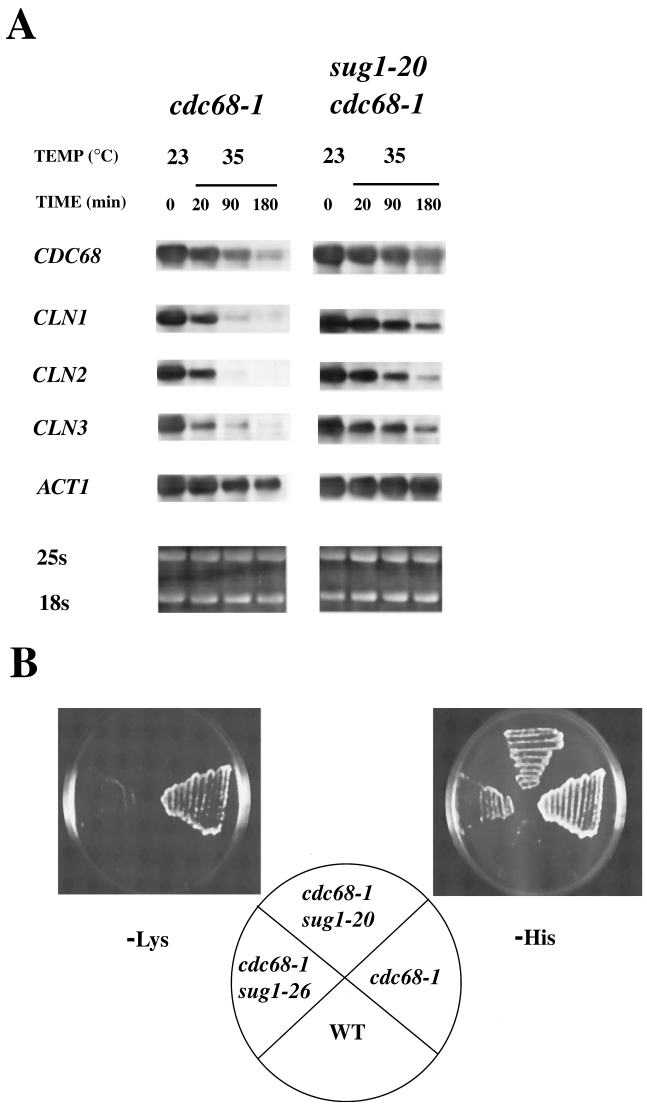

FIG. 1. *sug1* suppression abrogates transcriptional alterations caused by *cdc68-1*. (A) mRNA levels in *cdc68-1* (strain 68507A) and *cdc68-1 sug1-20* (strain QXT20) mutant cells. Total yeast RNA was extracted from cells growing at 23°C and after incubation at 35°C for the indicated times. Ethidium bromide-stained rRNAs serve as loading controls. (B) *sug1* partially suppresses the Spt$^-$ phenotype of *cdc68-1*. Cells of strains FY56 (wild type [WT]), FYARQ1 (*cdc68-1*), SUXB201 (*cdc68-1 sug1-20*), and SUXB261 (*cdc68-1 sug1-26*) were incubated on yeast extract-peptone-dextrose solid medium at 23°C and then replica plated to synthetic complete medium lacking either histidine (−His) or lysine (−Lys) for further incubation at 30°C. All strains carry the *his4-912*δ and *lys2-128*δ alleles. Images were processed with Adobe Photoshop (version 2.5.1) for the Macintosh computer.

shown). (Although the *sug1* suppressor mutations restored expression of the *CLN* genes, we noted that *CLN* mRNA levels in suppressed cells were lower than those in wild-type cells [Fig. 1A]. This feature of *sug1* suppression is most likely related to other effects of the *sug1* mutations isolated here; at high temperatures the *sug1-20* and *sug1-26* mutations also cause cells to pause in the G2/M interval of the cell cycle [47], during which time there is decreased *CLN1* and *CLN2* gene expression.) Moreover, transcription of the *cdc68-1* mutant gene itself was restored in *cdc68-1 sug1* double-mutant cells (Fig. 1A). Thus, it is likely that the *sug1* mutations identified here suppress the temperature sensitivity of *cdc68-1* mutant cells because they restore the transcription of Cdc68-dependent genes.

***sug1* mutations can reverse the effect of *cdc68-1* on *SUC2* transcription.** The Cdc68 protein has been shown to affect transcription of an upstream activation sequence (UAS)-less *SUC2* gene, the *suc2*Δ*UAS*(*−1900*/*−390*) allele. Deletion of the UAS of the *SUC2* gene abolishes transcription, and consequently cells harboring this *suc2*Δ*UAS* mutant allele cannot grow with sucrose as the sole carbon source. *cdc68* mutations suppress this Suc$^-$ phenotype by allowing the UAS-less *suc2* gene to be transcribed, and as a result *cdc68 suc2*Δ*UAS* cells display a Suc$^+$ phenotype (29). We determined if *sug1* suppressor mutations affect this *suc2*Δ*UAS* transcription that is allowed by the *cdc68-1* mutation. A *sug1-26 cdc68-1* mutant strain (that is Suc$^+$) was mated to a strain harboring both *cdc68* and *suc2*Δ*UAS* mutations, and meiotic segregants were tested both for temperature sensitivity and for growth on sucrose. In the absence of the *sug1-26* mutation, all segregants would be expected to display a Suc$^+$ phenotype at 30°C because of 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 Suc$^-$ segregants would indicate that the *sug1-26* mutation suppresses the effects of a *cdc68* mutation that cause the Suc$^+$ phenotype. Not only did we detect Suc$^-$ segregants, but also each Suc$^-$ segregant was temperature resistant at 35°C (data not shown), indicating that these Suc$^-$ *cdc68* segregants also harbored the *sug1-26* mutation. Thus, *sug1* mutations prevent the transcription of *suc2*Δ*UAS* that is allowed by *cdc68-1*.

***sug1* mutations reverse the Spt$^-$ phenotype caused by the *cdc68-1* mutation.** In addition to temperature sensitivity and increased transcription from the *suc2*Δ*UAS* promoter, the *cdc68-1* mutation causes an Spt$^-$ phenotype (29, 35). Insertion of a solo-δ sequence in the 5′ regions of the *HIS4* and *LYS2* genes alters transcription from these genes to cause a His$^-$ and Lys$^-$ phenotype (41, 45). The *cdc68-1* mutation suppresses the transcriptional alterations imposed by these δ insertions and allows mutant cells to grow without added lysine and histidine, a phenotype termed Spt$^-$ (29, 35). By mating suppressed *cdc68-1 sug1* cells with *cdc68-1 SUG1 his4-912*δ *lys2-128*δ cells and assessing the meiotic segregants, we found that both the *sug1-20* and *sug1-26* suppressor mutations reversed *cdc68-1* effects at the *lys2* locus but not at the *his4* locus: *cdc68-1 sug1 his4-912*δ *lys2-128*δ mutant cells displayed a His$^+$ Lys$^-$ phenotype (Fig. 1B). Thus, *sug1* suppressor mutations can partially reverse the Spt$^-$ phenotype caused by the *cdc68-1* mutation.

Molecular cloning and sequencing identify this suppressor gene as *SUG1*. To isolate the wild-type suppressor gene we took advantage of the observation that suppressor mutations reverse the Spt$^-$ phenotype caused by *cdc68-1*. A strain harboring the *sug1-26*, *cdc68-1*, and *lys2-128*δ mutations displays a Lys$^-$ phenotype, whereas the same strain transformed with a wild-type *SUG1* gene is expected to become phenotypically Lys$^+$ because of complementation of the recessive *sug1-26* mutation. From a centromere-based yeast genomic library, three genomic inserts that complemented the Lys$^-$ phenotype of a *sug1-26 cdc68-1 lys2-128*δ recipient strain were isolated. Subcloning one of the genomic inserts localized the complementing sequence to a 1.7-kbp *Eco*RI-*Kpn*I restriction fragment (Fig. 2); the other two complementing genomic clones contained the same *Eco*RI-*Kpn*I fragment (data not shown). We verified that this restriction fragment contained the wild-type version of the suppressor gene by integrative transformation (see Materials and Methods).

Partial nucleotide sequence analysis revealed that the cloned suppressor gene was identical to the *SUG1* gene (18, 42).

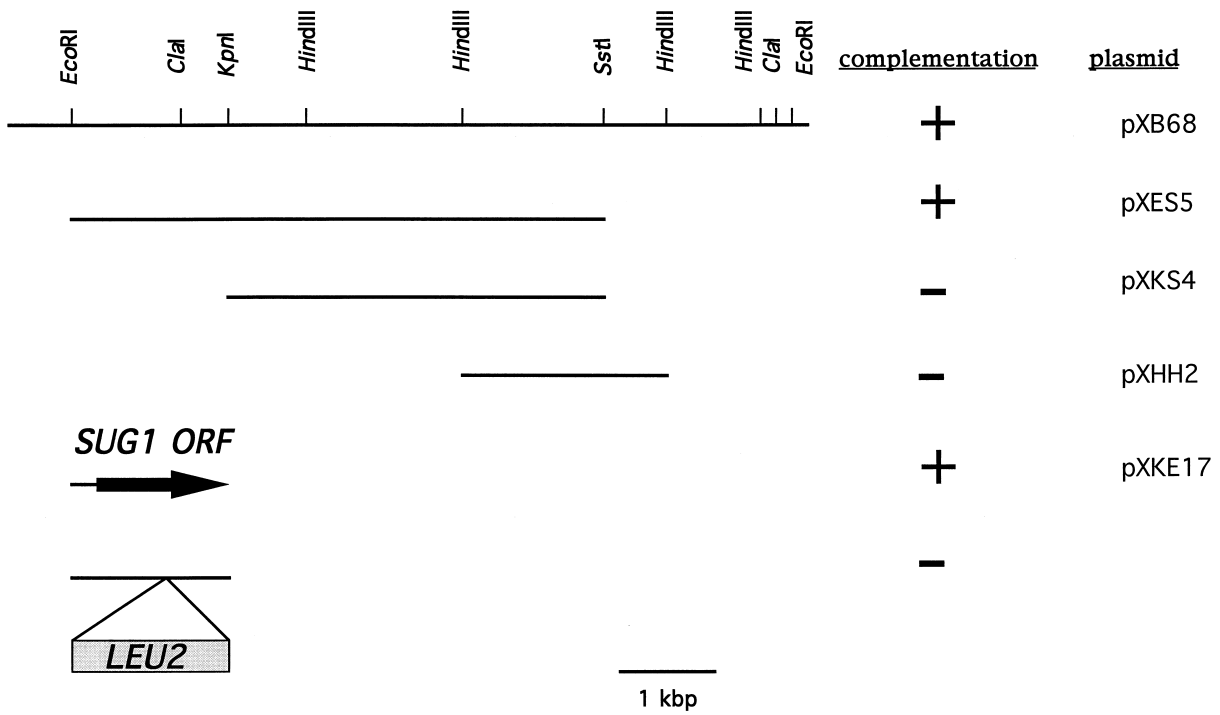

FIG. 2. The cloned suppressor gene is *SUG1*. Complementation of the temperature resistance and Spt$^+$ phenotypes of a *sug1 cdc68-1* mutant strain (SUXB261) by episomal plasmids containing genomic inserts is indicated (+, complementation; −, no complementation). The arrowed line indicates the position and orientation of the open reading frame. Disruption of the *SUG1* gene by insertion of the *LEU2* marker gene is also shown.

SUG1 was isolated previously by complementation of the *sug1-1* mutation that restores transactivation function to the product of the mutant *gal4D* gene, a truncated Gal4 protein lacking the C-terminal activation domain (42). The identity of our suppressor gene as *SUG1* was further confirmed by the fact that our cloned suppressor gene complemented the *sug1-1* allele and reversed the *sug1-1* suppression of the *gal4D* mutation (data not shown). Northern analysis with the radiolabeled 1.7-kbp *Eco*RI-*Kpn*I fragment detected a 1.4-kb transcript, consistent with the size of the *SUG1* open reading frame (data not shown).

Overexpression of *SUG1* exacerbates *cdc68-1* temperature sensitivity. Decreased Sug1 activity encoded by recessive *sug1* mutant alleles suppressed temperature sensitivity caused by *cdc68-1*, suggesting that the wild-type Sug1 protein has a negative effect on mutant Cdc68 activity. To further test the antagonistic effect of Sug1 on Cdc68 activity, we overproduced the Sug1 protein. Initially we expressed the *SUG1* gene using a high-copy-number plasmid but found that the Sug1 protein did not accumulate to a high level (data not shown), probably because Sug1 represses transcription of its own gene (see below). Therefore, we used the plasmid pVT100U-S10SUG1 (44), which contains the *SUG1* gene under the control of the *ADH1* promoter. Immunoblot analysis verified that expression of *SUG1* from the *ADH1* promoter indeed caused at least sixfold overproduction of the Sug1 protein (Fig. 3A). Overproduction of Sug1 did not affect the growth of *CDC68* wild-type cells (Fig. 3B), but we found that increased amounts of the Sug1 protein exacerbated *cdc68-1* defects. At 33°C, which is a permissive temperature for *cdc68-1* mutant cells with normal levels of Sug1, *cdc68-1* mutant cells with high levels of Sug1 became temperature sensitive (Fig. 3B). On the basis of the effects both of enfeebled Sug1 activity and of overproduction of the Sug1 protein, we conclude that Sug1 antagonizes Cdc68 activity.

***sug1* suppressor mutations alter transcription.** In a related study we found that the *sug1-26* allele renders cells temperature sensitive for growth (47). Thus, Sug1 has an important function and has in fact been suggested to be a component of the proteasome and the transcription mediator complex (17, 26). We therefore tested our *sug1* alleles for transcriptional effects. As shown in Fig. 4A, both at 23°C and after transfer of *sug1* mutant cells to the restrictive temperature of 37°C there was a modest increase in mRNA abundance for several genes: levels of *CDC68* mRNA were increased approximately twofold in *sug1* mutant cells at both 23 and 37°C, compared with levels in wild-type cells, and mRNA abundance for the *sug1-26* gene itself was also increased approximately twofold. On the other hand, mRNA abundance for one *CDC68*-dependent gene, *ACT1*, was unaffected by the *sug1-26* mutation. There was no appreciable change of *ACT1* mRNA levels even after 5 h of incubation at 37°C, at which point *sug1-26* mutant cells had already ceased proliferation (47). Thus, Sug1 does not affect all transcription that is mediated by Cdc68.

Because altered activity of Cdc68 and a number of other transcription regulators causes an Spt$^-$ phenotype, we determined whether the *sug1-26* allele itself results in an Spt$^-$ phenotype. Cells harboring the *lys2-128*δ mutation failed to grow in lysine-free medium regardless of the presence of the *sug1-26* mutation (data not shown), but *sug1-26 his4-912*δ cells could grow, albeit slowly, in medium lacking histidine (Fig. 4B). This growth indicates that the *sug1-26* suppressor mutation can confer a limited Spt$^-$ phenotype.

Neither *cdc68* nor *sug1* mutations can compensate for a complete loss of gene function. Disruption of the *CDC68* gene is lethal in haploid yeast cells (29). We determined if the *sug1-26* allele was able to suppress the lethality of a *cdc68* disruption mutation (see Materials and Methods) and found that *sug1-26* did not restore viability to *cdc68-101*::*LEU2* cells (Table 2).

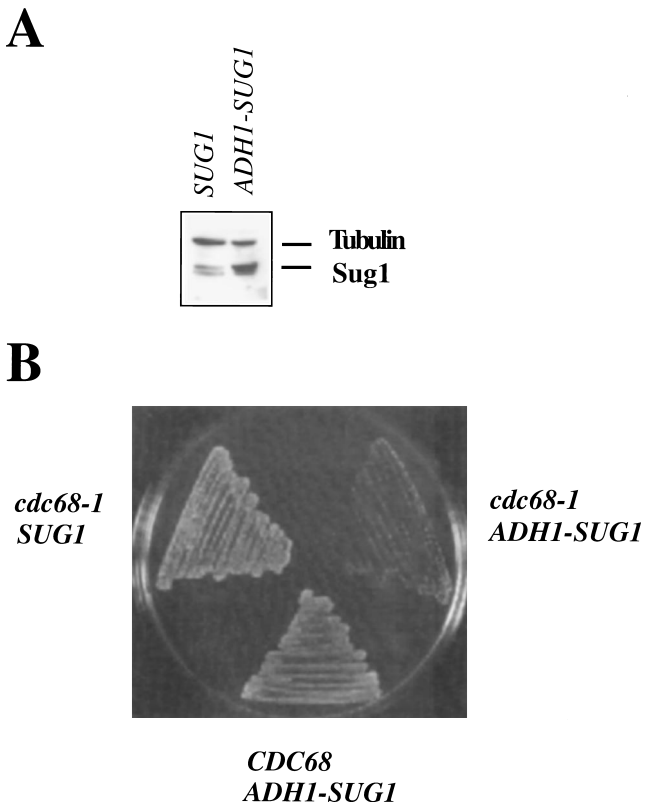

FIG. 3. Overproduction of Sug1 inhibits the proliferation of *cdc68-1* mutant cells. (A) Protein levels. Extracts were prepared from a yeast strain containing a wild-type *SUG1* gene and a strain harboring the *SUG1* gene under the control of the *ADH1* promoter. Tubulin was used as a control for equal loading. (B) Cells of a *cdc68-1* mutant strain (68507A), a *cdc68-1* mutant strain (68507A) harboring the *ADH1-SUG1* plasmid, and a *CDC68* wild-type strain (21R) harboring the *ADH1-SUG1* plasmid were replica plated for incubation at 33°C as shown. Images were processed as described for Fig. 1.

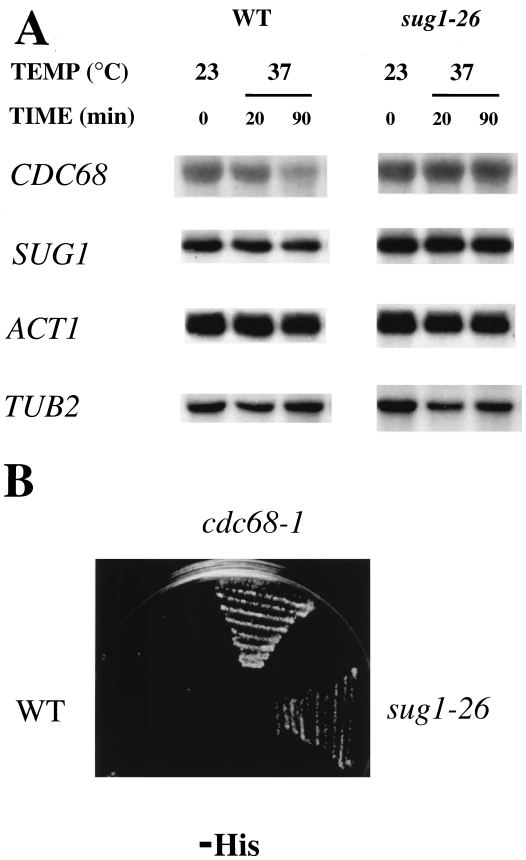

FIG. 4. Sug1 affects transcription. (A) mRNA levels. Total RNA was extracted from cells of strain 21R (wild type [WT]) and strain QX261 (*sug1-26*) growing at 23°C and after incubation at the restrictive temperature of 37°C for the times indicated. *TUB2* mRNA was used as a loading control. (B) Spt$^-$ phenotype. Cells of strains FY56 (WT), FYARQ1 (*cdc68-1*), and QX261-1d (*sug1-26*) were replica plated to synthetic complete medium lacking histidine for incubation at 30°C. All strains harbor the *his4-912*δ mutation. Images were processed as described for Fig. 1.

Cdc68 activity is therefore still required for cell proliferation even when Sug1 activity is impaired. In view of our Northern data (Fig. 1A) showing that *sug1* restores expression of the mutant *cdc68-1* gene, we conclude that *sug1* suppresses the defects of *cdc68-1* mutant cells by restoring Cdc68 activity.

SUG1 is also an essential gene (42). To determine if Sug1 activity is required in *cdc68-1* mutant cells, we disrupted the *SUG1* gene (see Materials and Methods). 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 report that *SUG1* is essential and furthermore showed that Sug1 function is indispensable even in combination with the *cdc68-1* mutation at both 23 and 37°C (Table 2). The observation that the *SUG1* gene is essential even in *cdc68-1* mutant cells indicates that the *sug1* suppressor alleles identified here cause only partial loss of Sug1 function.

We 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 was lethal to the haploid yeast cell. 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 have counteracting effects on transcription, their functions cannot be limited to overcoming the effects of each other: otherwise, removal of both proteins should not affect cell viability. Thus, the Cdc68 and Sug1 proteins have essential cellular functions in addition to antagonizing the activity of each other.

Interactions between *cdc68* and *sug1* are allele specific. Suppression by our *sug1* mutations was found to be specific for the *cdc68-1* allele, because the *sug1-20* allele failed to reverse the temperature sensitivity caused by another *cdc68* mutant allele, *cdc68-11*, that was isolated in a different genetic screen (28).

To investigate in more detail the interaction between *cdc68*

TABLE 2. Allele-specific suppression of *cdc68* and *gal4D* by *sug1*

sug1 allele	Growth by cells carrying[a]:			
	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

[a] The effect of *sug1* alleles on *gal4D* was assessed in galactose medium. ++, very good growth; ±, poor growth; −, no growth; ND, not determined.

TABLE 3. *sug1* suppression in diploids

Genotype[a]	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	–

[a] All diploid strains are homozygous for *cdc68-1*.

and *sug1* mutations, and thus perhaps between Cdc68 and Sug1 proteins, we assessed whether the *sug1-1* allele, isolated as a suppressor of *gal4D* (42), could also suppress the *cdc68-1* mutation. A heterozygous diploid strain (*cdc68-1/CDC68 sug1-1/SUG1*) was constructed, and meiotic segregants were examined for growth at 35°C (the *sug1-1* allele itself does not confer temperature sensitivity at 35°C). Each meiotic tetrad displayed a 2:2 segregation pattern for temperature sensitivity at 35°C (some temperature-sensitive segregants showed some residual growth at 35°C). Thus, the *sug1-1* mutation does not suppress *cdc68-1* temperature sensitivity efficiently (Table 2). We conclude from these observations that our *sug1* alleles, isolated by the ability to suppress the *cdc68-1* phenotype, are different from the *sug1-1* allele that suppresses the *gal4D* phenotype. Another *sug1* allele (called *cim3*) has also been identified, in this case by a genetic interaction with the *cdc28-1N* mutation (17). We found that this *sug1* mutant allele, *sug1-3* (*cim3-1*), also did not suppress the *cdc68-1* phenotype. Thus, suppression of the *cdc68-1* phenotype is a feature of only certain *sug1* alleles.

In light of our finding that Sug1 antagonizes Cdc68 activity, it is possible that the reason for the inability of the *sug1-1* and *sug1-3* alleles to suppress the *cdc68-1* mutation could be that these alleles encode too much residual Sug1 activity to alleviate the inhibition of Cdc68 function. We 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 suppressing *sug1-26* allele. To test this, we 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 to *CDC68* wild-type strains at 35°C. We 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, 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 3). These observations suggest that *sug1-1* and *sug1-3* alleles may encode too much residual Sug1 activity for *cdc68-1* suppression.

We also assessed the genetic interaction between our *sug1* alleles (that suppress *cdc68-1*) and the *gal4D* mutation that can be suppressed by *sug1-1*. We found that our *sug1-20* allele failed to suppress the *gal4D* phenotype; others have found that *sug1-3* does not suppress the *gal4D* phenotype (43). Thus, *gal4D* and *sug1* mutations display allele-specific interactions, but in this case suppression is not correlated with apparent residual Sug1 activity.

Molecular alterations in *sug1* alleles. We show above that *sug1* alleles that suppress the *cdc68-1* mutant phenotype are different from the *sug1-1* allele, which suppresses the *gal4D* phenotype. The *gal4D*-suppressing *sug1-1* allele encodes a Gly-215–to–Asp substitution in the Sug1 protein (43). We determined the molecular alteration in the *sug1-26* allele identified here by using PCR to amplify the entire *sug1-26* mutant gene. Subsequent nucleotide sequence analysis revealed that the *sug1-26* mutation was a single-base-pair substitution at nucleotide 568, causing a change of Gly-189, in a conserved ATPase motif of Sug1 (42), to Ala. We then verified that this G189A substitution caused the *sug1-26* mutant 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 (encoding the G189A substitution), like *sug1-26* itself, allows cell viability but causes temperature sensitivity. Thus, *sug1* alleles that produce different phenotypes have different molecular alterations.

We amplified several other *sug1* alleles by PCR, including 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 located all the *sug1* mutations to the conserved ATPase module of Sug1 (Fig. 5). Although isolated independently, the *sug1-26* and *sug1-20* alleles have the G189A mutation, which 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 ATPase signature motifs (Fig. 5), whereas the *sug1-1* and *sug1-3* mutations affect the amino acid sequence of the ATPase module in regions outside the two highly conserved motifs.

An epitope-tagged Cdc68 protein. To study Cdc68 at the protein level, we tagged the *CDC68* gene with sequences encoding a triple-HA epitope (see Materials and Methods). This HA-tagged *CDC68* allele is designated *CDC68-200N*. The addition of HA to the Cdc68 polypeptide did not have any detectable effect on cell growth, and the *CDC68-200N* allele reversed the temperature sensitivity caused by the *cdc68-1* mutation. Moreover, a single copy of the *CDC68-200N* gene integrated into the genome complemented the lethal phenotype of a *cdc68* disruption mutation: *CDC68-200N cdc68-101*::*LEU2* cells proliferated as efficiently as wild-type cells at all

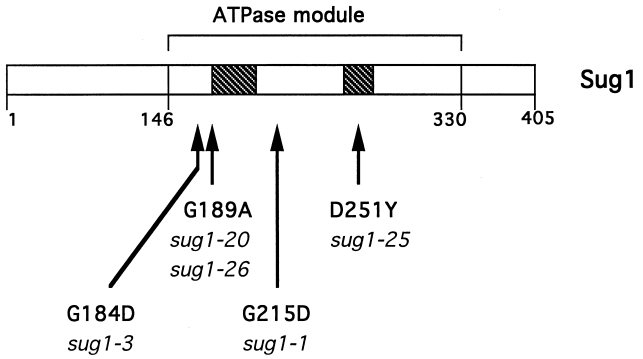

FIG. 5. Sug1 mutant proteins. Shaded boxes indicate the two highly conserved ATPase signature motifs. The amino acid alterations for different *sug1* alleles are indicated.

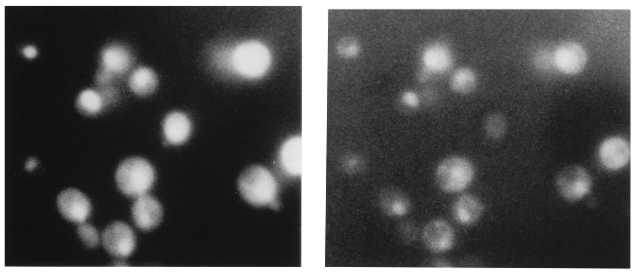

FIG. 6. Nuclear localization of the Cdc68 protein. Cells containing HA-tagged Cdc68 were subjected to indirect immunofluorescence. DAPI was added to the mounting medium to reveal nuclei.

temperatures (data not shown). As a more sensitive test for Cdc68 function we determined the Spt phenotype of the HA-tagged *CDC68* gene. As expected, the *CDC68-200N* gene on a high-copy-number plasmid, like *CDC68* itself, caused an Spt^- phenotype. However, unlike the wild-type *CDC68* gene, a single copy of *CDC68-200N* caused a weak Spt^- phenotype (data not shown), suggesting that the *CDC68-200N* allele produces a modified Cdc68 polypeptide with slightly altered activity compared with the wild-type Cdc68 protein. Nonetheless, the HA-tagged Cdc68 protein has appreciable Cdc68 function.

Nuclear localization of the Cdc68 protein. The Sug1 protein has been localized to the nucleus (42). Since Cdc68 regulates transcription and *cdc68* mutations display allele-specific interactions with *sug1* mutations, it is reasonable that the Cdc68 protein is also in the nucleus. We determined the intracellular location of Cdc68 by indirect immunofluorescence using a yeast strain in which the only functional *CDC68* gene was *CDC68-200N*, encoding an HA-tagged Cdc68 protein. As shown in Fig. 6, this Cdc68 protein is indeed located in the nucleus.

The wild-type Cdc68 protein is stable. Ghislain et al. (17) have reported that the Sug1 protein (which they called Cim3) mediates protein degradation, in part because certain ubiquitin-conjugated fusion proteins are stabilized in *sug1* (*cim3*) mutant cells. We determined whether the *sug1* mutations identified here as *cdc68-1* suppressor mutations also affect protein stability. For this analysis we employed plasmids encoding substrates for the ubiquitin degradation pathway. Plasmids Ub-Pro-βgal, Ub-Arg-βgal, Ub-Leu-βgal, and Ub-Met-βgal (as a control construct) (17) were introduced into wild-type and *sug1-20* mutant cells. We found that β-galactosidase levels from each of these constructs were at least threefold higher in *sug1* mutant cells than in wild-type cells, as indicated by standard β-galactosidase assays (1) (data not shown). In light of the finding that the *sug1-3* (*cim3-1*) allele also stabilizes ubiquitin conjugates (17), we infer that the increased β-galactosidase activities seen here in *sug1-20* cells are most likely a result of decreased degradation of ubiquitin conjugates.

The ability of *sug1* mutations to stabilize ubiquitin conjugates prompted us to ask if Sug1 affects the stability of the Cdc68 protein. We 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 a single copy next to the *cdc68-101*::*LEU2* disruption allele) were grown at 23°C, transferred to 35°C to impair mutant Sug1 activity, and treated with cycloheximide to prevent further protein synthesis. We reasoned that 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. 7A, wild-type Cdc68 was stable, and the *sug1-26* mutation had no detectable effect on Cdc68 abundance. We conclude that the stability of wild-type Cdc68 is unaffected by Sug1 activity.

***sug1* suppressor mutations decrease the degradation of mutant Cdc68 protein.** The polypeptide encoded by the *cdc68-1* allele was tagged with the HA epitope sequence by a restriction fragment swap; the tagged *cdc68-1* gene is designated *cdc68-201N*. Epitope addition did not affect the mutant 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-number plasmid the *cdc68-201N* gene suppressed the temperature sensitivity of *cdc68* mutant cells, as does *cdc68-1* (46).

A single copy of the HA-tagged *cdc68-1* gene was introduced into *cdc68-1 sug1-26* and *cdc68-1 SUG1* strains. Cells were then transferred to 35°C, and protein extracts were prepared following the addition of cycloheximide to prevent further protein synthesis (the same procedure as the one used to determine stability of the wild-type Cdc68 protein). Under these conditions, the mutant Cdc68-1 polypeptide was extremely unstable in *SUG1* (wild-type) cells but more stable in *sug1-26* mutant cells (Fig. 7C). Thus, the *sug1-26* suppressor mutation slows the degradation of the mutant Cdc68-1 protein.

We also assessed the stability of mutant Cdc68 protein at the permissive temperature of 23°C following the same cycloheximide treatment protocol. As shown in Fig. 7B, the mutant protein was unstable even at 23°C, but with a lower rate of degradation than at 35°C (Fig. 7C), and the *sug1-26* suppressor mutation again provided limited stability to the mutant Cdc68-1 protein. Thus, the mutant Cdc68 protein is inherently unstable, and its degradation is mediated by Sug1 activity.

Suppression by *sug1* mutations is not entirely due to stabilization of mutant Cdc68 protein. As shown above, not all *sug1* mutations suppress the effects of the *cdc68-1* mutation. We were therefore able to determine if *sug1* suppression of *cdc68-1* temperature sensitivity was a consequence of increased stability of the mutant Cdc68 protein. Although the *sug1-1* allele increased mutant Cdc68 protein stability only modestly, the other nonsuppressing allele studied here, *sug1-3*, significantly increased stability of the mutant Cdc68 protein; the half-life of mutant Cdc68 protein was estimated to be 50 min in *sug1-3* cells but less than 10 min in *SUG1* wild-type cells (Fig. 7C). The *sug1-3* allele that does not suppress *cdc68-1* temperature sensitivity therefore stabilizes the mutant Cdc68 protein to a greater degree than does the suppressing *sug1-26* allele. We conclude from these observations that stabilization of the mutant Cdc68 protein by a *sug1* mutation does not entirely account for suppression of the *cdc68-1* mutant phenotype.

DISCUSSION

The Cdc68 protein mediates transcription of a diverse set of genes (35). To further explore the role of Cdc68, we identified suppressor genes in which mutations suppress the *cdc68-1* temperature sensitivity at the restrictive temperature of 35°C (46). Here we report our study of one suppressor gene that we show to be the *SUG1* gene. The *SUG1* gene was originally identified by a *sug1-1* mutation that restores transactivation function to a mutant Gal4 protein encoded by the *gal4D* allele (42). Sug1 has also been identified recently by other laboratories (17, 26), and strikingly divergent functions have been proposed for this protein. Sug1 is thought to play a direct role in transcriptional regulation (26, 42, 44) but has also been suggested to be part of a protein degradation complex called the 26S protease (17).

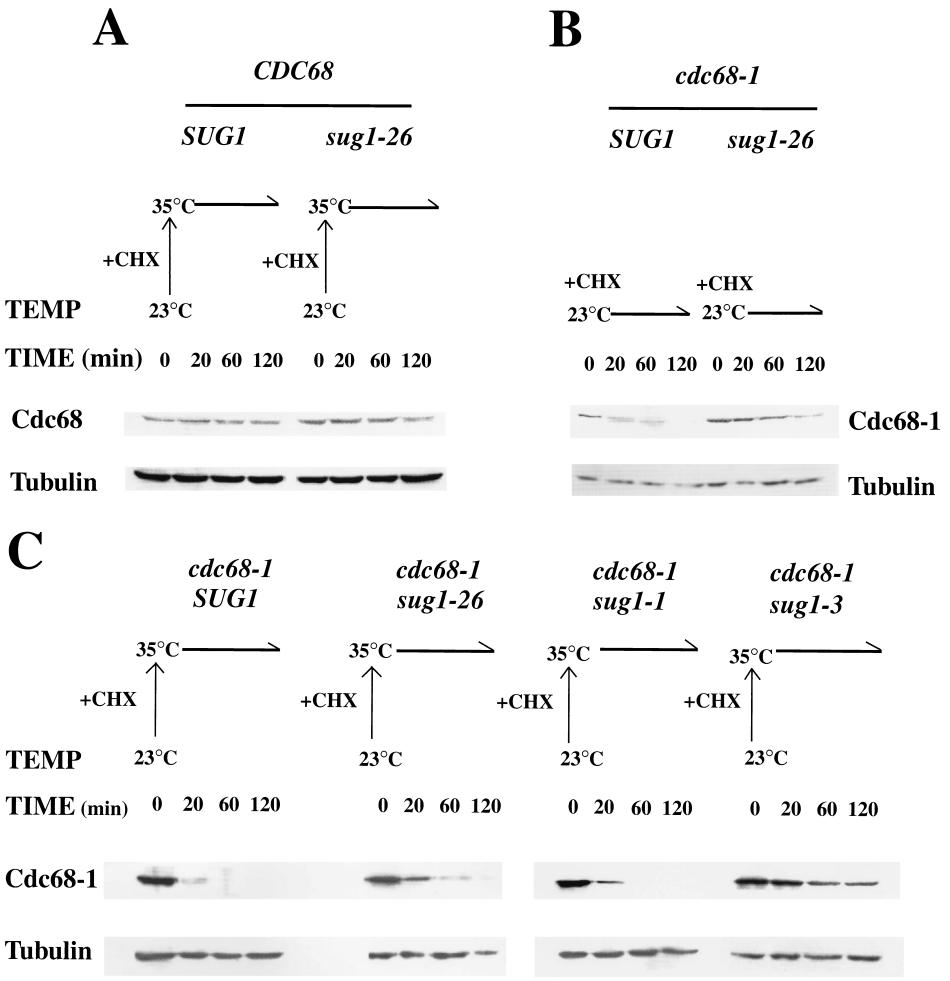

FIG. 7. Stability of wild-type and mutant Cdc68 polypeptides. (A) Cells of *SUG1* and *sug1-26* strains (containing the HA-tagged wild-type Cdc68 protein) were grown at 23°C. Cycloheximide (CHX) was added to block further protein synthesis, and cells were shifted to 35°C for further incubation. Extracts were prepared at the indicated times. (B) Cells of *SUG1* and *sug1-26* strains (both containing the HA-tagged mutant Cdc68-1 polypeptide) were incubated at 23°C, and extracts were prepared at the indicated times after the addition of cycloheximide. (C) Extracts were prepared from *cdc68-1* cells harboring wild-type *SUG1* or *sug1* mutant alleles growing at 23°C and also after the addition of cycloheximide and incubation at 35°C for the indicated times. Each strain contained the HA-tagged Cdc68-1 polypeptide. For all the immunoblots, tubulin was used as loading control. Images were processed as described for Fig. 1.

Our investigations have revealed that Sug1 influences the transcription activation mediated by Cdc68, an acidic protein that has widespread effects on transcription. Our assessment of the phenotype of *sug1* mutations indicates that Sug1 plays a negative role in transcription, but not necessarily through effects on Cdc68 protein degradation.

Sug1 plays a role in transcription. A transcription mediator complex that activates both basal and activated transcription in vitro has been purified from budding yeast, and polyclonal antibodies against Sug1 recognize one subunit of this complex. This observation has led to the proposal that Sug1 is a component of the transcription mediator complex (26). A direct role for Sug1 in transcription is supported by the finding that Sug1 can bind to the Gal4 activation domain and to the basal factor TATA-binding protein (44). Here we provide in vivo evidence for a transcriptional role for Sug1 by showing that the *sug1-26* mutation displays an Spt$^-$ phenotype (Fig. 4B), generally interpreted as 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 (encoded by the *SPT15* gene) (10, 20) and the Gal11/Spt13 protein (13). We note that some effects of Sug1 on transcription may be indirect: increased *CDC68*/*SPT16* copy number itself can cause an Spt$^-$ phenotype (8), so that the Spt$^-$ phenotype caused by *sug1-26* may be a consequence of increased *CDC68* gene expression (Fig. 4A).

The Sug1 protein is found in the same mediator complex as Gal11 (26), which can stimulate transcription when tethered to DNA by a DNA-binding domain (22). Similarly, the Ada2 and Ada3 proteins, members of another class of mediator and adaptor proteins that are thought to bridge certain acidic activators and the basal transcription complex (2), can also activate transcription when fused to a DNA-binding domain (23, 40). However, the Sug1 protein does not provide such activator function when fused to a DNA-binding domain (42). Indeed, we found that the Sug1 protein plays a repressive role in the transcription of some genes, including *CDC68* and the *SUG1* gene itself (Fig. 4A). Thus, Sug1 appears to have an activity distinct from that of the Gal11, Ada2, and Ada3 proteins, with a negative role in transcription.

Sug1 affects mutant Cdc68 protein stability. The Sug1 protein influences proteolysis. Ghislain et al. (17) showed that the *sug1-3* mutation stabilizes ubiquitinated substrates of the 26S

protease, and we found that enfeebled Sug1 activity (encoded by the *sug1-26* allele) stabilizes the intrinsically labile mutant form of the Cdc68 polypeptide (Fig. 7B and C). However, despite the observation that polyclonal anti-Sug1 antibodies cross-react with the *Drosophila* 26S protease (17), Swaffield et al. (44) indicated that Sug1 and the 26S protease are not in the same fraction of a sizing column and that the Sug1 protein does not coimmunoprecipitate with the 26S protease. In any case, stabilization of mutant Cdc68 polypeptide cannot entirely account for the suppression of *cdc68-1* caused by the *sug1-26* mutation: the *sug1-3* allele, which does not suppress *cdc68-1* temperature sensitivity, increased the stability of the Cdc68 mutant polypeptide to a greater degree than the *sug1-26* suppressor allele (Fig. 7C). Our data do not indicate if stabilization is necessary for suppression. In any case, despite increased stability of the mutant Cdc68 polypeptide, another mechanism must be involved in the restoration of Cdc68 activity by altered Sug1.

Sug1 antagonizes Cdc68 activity. The *sug1* suppressor alleles, exemplified by *sug1-20*, reverse most aspects of the *cdc68-1* phenotype, including transcriptional alterations. Most significantly, the *sug1* suppressor mutations restore transcription of the *cdc68-1* gene itself. This observation, coupled with the finding that *sug1-20* does not suppress the lethality of the loss-of-function allele *cdc68-101*::*LEU2*, suggests that *sug1-20* does not suppress *cdc68* defects by bypassing the need for Cdc68. Instead, *sug1-20* restores enough Cdc68 activity to support growth. Indeed, the mutant polypeptide encoded by the *cdc68-1* allele still has residual activity at the restrictive temperature (46). We infer from the recessive nature of the *sug1-20* mutation that enfeebled Sug1 activity allows this mutant Cdc68 polypeptide to be more active. Likewise, overproduction of the Sug1 protein, presumably conferring higher Sug1 activity, further exacerbates the weak Cdc68 activity encoded by the *cdc68-1* allele (Fig. 3). Thus, Sug1 functions in an antagonistic manner to modulate Cdc68 activity.

The Cdc68 protein activates transcription of the *CDC68* gene itself (35). Therefore, 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, we find no evidence for titration of Sug1 effects: increased copy number of the *CDC68* promoter region fails to alleviate the *cdc68-1* defect in growth, whereas either overexpression of the entire mutant *cdc68* gene or weakening Sug1 activity by mutation allows *cdc68-1* mutant cells to proliferate at 35°C (46). Furthermore, Cdc68-dependent genes respond differently to altered Sug1 activity (Fig. 4A). These findings suggest that the antagonistic effects of Sug1 are not solely due to repression at the *CDC68* promoter. Thus, we prefer a model in which Sug1 counteracts Cdc68 activity at the protein level.

The *sug1* alleles that suppress the *cdc68-1* mutation all encode amino acid substitutions in the two highly conserved ATPase signature motifs (Fig. 5) (16). The putative ATPase activity of Sug1 may influence the ability of the Sug1 protein to affect Cdc68 function. Decreased ATPase activity caused by the recessive *sug1* suppressor mutations would attenuate Sug1 activity and thereby potentiate the activity of Cdc68.

***sug1* suppression is allele specific.** Our genetic studies reveal allele-specific interactions between *cdc68* and *sug1* mutations (Table 2). Neither *sug1-1* nor *sug1-3* could suppress the temperature sensitivity caused by *cdc68-1*. On the other hand, the *sug1* mutations isolated by their ability to suppress *cdc68-1* failed to suppress temperature sensitivity caused by other *cdc68* alleles, including *cdc68-11* (28) and *cdc68-Δ922*, a truncated version of the *CDC68* gene (12). This allele-specific interaction may reflect a physical contact between the Cdc68 and Sug1 proteins. However, we have been unable to detect coimmunoprecipitation of HA-tagged Cdc68 and Sug1 from yeast cell extracts, and results of a two-hybrid interaction assay also do not support a physical interaction between the Cdc68 and Sug1 proteins (47). Therefore, the observed allele-specific interactions may simply reflect relative activities of different *cdc68* and *sug1* alleles and may not indicate any direct physical interaction. For example, nonsuppressing *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 we have not been able to determine the activities of different *sug1* alleles biochemically. Nonetheless, our genetic data suggest that the *sug1-1* and *sug1-3* alleles may indeed have too much residual Sug1 activity: the nonsuppression by *sug1-1* and *sug1-3* is dominant over the *cdc68-1* suppression by the *sug1-26* allele (Table 3). The allele-specific interactions detected here therefore probably reflect different levels of residual activity conferred by different *sug1* alleles.

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