MOLECULAR AND CELLULAR BIOLOGY, Dec. 1993, p. 7553–7565
0270-7306/93/127553-13$02.00/0

Vol. 13, No. 12

The *Saccharomyces cerevisiae* Cdc68 Transcription Activator Is Antagonized by San1, a Protein Implicated in Transcriptional Silencing

QUNLI XU,[1] GERALD C. JOHNSTON,[1]* AND RICHARD A. SINGER[2,3]

Departments of Microbiology and Immunology,[1] Biochemistry,[2] and Medicine,[3] Dalhousie University, Halifax, Nova Scotia, Canada B3H 4H7

Received 12 July 1993/Returned for modification 26 August 1993/Accepted 9 September 1993

The *CDC68* gene (also called *SPT16*) encodes a transcription factor for the expression of a diverse set of genes in the budding yeast *Saccharomyces cerevisiae*. To identify other proteins that are functionally related to the Cdc68 protein, we searched for genetic suppressors of a *cdc68* mutation. Four suppressor genes in which mutations reverse the temperature sensitivity imposed by the *cdc68-1* mutation were found. We show here that one of the suppressor genes is the previously reported *SAN1* gene; *san1* mutations were originally identified as suppressors of a *sir4* mutation, implicated in the chromatin-mediated transcriptional silencing of the two mating-type loci *HML* and *HMR*. Each *san1* mutation, including a *san1* null allele, reversed all aspects of the *cdc68* mutant phenotype. Conversely, increased copy number of the wild-type *SAN1* gene lowered the restrictive temperature for the *cdc68-1* mutation. Our findings suggest that the San1 protein antagonizes the transcriptional activator function of the Cdc68 protein. The identification of *san1* mutations as suppressors of *cdc68* mutations suggests a role for Cdc68 in chromatin structure.

For the budding yeast *Saccharomyces cerevisiae*, as for other eukaryotic cells, the initiation of transcription is a primary point for regulation of gene expression. RNA polymerase II and general transcription factors, including TATA-binding factor TFIID, constitute the basal transcription apparatus (reviewed in reference 56), but high-level gene expression requires transcription activators that bind to upstream activating sequences (25, 35). Eukaryotic transcription takes place in an environment where DNA is packaged into chromatin. There is considerable evidence that chromatin itself plays an important role in regulating transcription (10, 11, 14, 60). The fundamental component of chromatin is the nucleosome, consisting of histone octamers (two histone H2A-H2B dimers and one histone H3-H4 tetramer) around which DNA is wrapped into compact structures. For transcription initiation to occur, transcription factors must counteract the repressive effect of nucleosomes. In addition, maximum gene induction can require adaptor proteins (4, 13, 30) that facilitate interaction between the basal transcription machinery and sequence-specific regulatory factors. Conversely, regulated gene expression may also involve transcriptional repression. One example of transcriptional repression in *S. cerevisiae* is the silencing of the two cryptic mating-type loci, *HML* and *HMR* (29, 38, 50). A wide variety of effectors thus regulate the initiation of transcription.

The Cdc68 protein is a global transcription factor that regulates the expression of many genes (32, 52). This conclusion was derived in part from the effects of the temperature-sensitive *cdc68-1* mutation (48). This mutation blocks performance of the regulatory step, START, of the yeast cell cycle, presumably because of the decrease in G_1 cyclin gene expression required for START (52). The *cdc68-1* mutation also decreases transcription of many other genes, including the *ACT1* and *LEU2* genes and the *cdc68-1* gene itself. The diverse spectrum of genes that require Cdc68 function argues that the Cdc68 protein plays a general role in transcription.

The *CDC68* gene was also identified independently as an *SPT* gene, *SPT16*, by the ability of extra copies of *CDC68* to suppress the effects of solo-δ insertion mutations in the 5′ regions of the *HIS4* and *LYS2* genes (32). The δ insertions at these two loci have previously been shown to alter transcription and thereby cause a His^- or Lys^- phenotype (59, 66). All *spt* mutations that have been reported, including *cdc68/spt16* mutations, suppress these δ insertions by altering transcription initiation (8, 9, 32, 61). The identification of *CDC68* as an *SPT* gene provides further evidence that the Cdc68 protein plays an essential role in transcription.

In an effort to understand how the Cdc68 protein regulates transcription, we undertook a genetic suppressor approach to search for other proteins that affect Cdc68 function. We have identified four suppressor genes in which mutations can reverse the temperature sensitivity of the *cdc68-1* mutation. Here we report that one of these suppressor genes is the previously characterized *SAN1* gene (57). The original *san1* mutations were identified by the ability to reverse the nonmating phenotype of a *sir4* mutation that impairs the transcriptional repression of the two cryptic mating-type loci. We found that all *san1* mutations, including the *sir4*-suppressing *san1* alleles and a *san1*Δ::*URA3* null allele, suppress all the phenotypes imposed by the *cdc68-1* mutation. Conversely, overexpression of the wild-type *SAN1* gene lowers the restrictive temperature for the *cdc68-1* mutation. All of these findings lead us to conclude that the San1 protein antagonizes the Cdc68 transcription activation function; it is likely that San1 inhibits Cdc68 activity at the protein level.

MATERIALS AND METHODS

Strains and culture conditions. The *S. cerevisiae* strains used in this study are listed in Table 1. *Escherichia coli*

* Corresponding author. Electronic mail address: JOHNSTON@AC.DAL.CA.

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

Strain	Genotype or phenotype[a]	Source or reference[b]
21R	*MATa leu2-3,112 ura3-52 ade1*	27
68507A[c]	*MATα cdc68-1 ura3-52 ade⁻*	52
ARI68-7[c]	*MATa cdc68-1[URA3] leu2-3,112 ura3-52 ade⁻*	52
FY56	*MATα his4-912δ lys2-128δ ura3-52*	32
ACY12	*MATα cdc68-197 his4-912δ lys2-128δ ura3-52*	E.A.M.
FW232	*MATα spt2-150 his4-912δ ura3-52 ade2-1*	66
FY229	*MATa spt4-289 his4-912δ lys2-128δ ura3-52*	F.W.
FY300	*MATa spt5-194 his4-912δ lys2-128δ leu2Δ ura3-521*	61
FY137	*MATα spt6-140 his4-912δ lys2-128δ ura3-52*	F.W.
BM403	*MATa cdc68-197 his4-912δ lys2-128δ ura3-52 suc2ΔUAS*(−1900/−390)	E.A.M.
BM404	*MATα his4-912δ lys2-128δ ura3-52 suc2ΔUAS*(−1900/−390)	E.A.M.
BM64	*MATa/MATα cdc68-101::LEU2/CDC68 his4-912δ/his4-912δ lys2-128δ/lys2-128δ trp1/trp1 leu2-3,112/leu2-3,112 ura3-52/ura3-52*	E.A.M.
MCY863	*MATα ssn20-6 snf2-50 his4-539 ura3-52*	40
MCY1093	*MATa his4-539 lys2-801 ura3-52*	1
YRS934	*MATα san1::HIS3 his3Δ200 lys2-801 ura3-52 tyr1 ade2-101*	57
YRQ9344	*MATa cdc68-1 san1::HIS3 leu2-3,112 (his3Δ200?) ade⁻*	This study
YRS376	*MATa sir4 san1-2 his3-532 trp1-389am ura3-52*	57
XRS20-10c	*MATα san1-1 his4-580 leu2-1 trp5 ura3 (ura2-9,15,30?)*	57
XJB3-1B	*MATα met6*	YGSC
QX3[c]	*MATα cdc68-1 san1-3 ura3-52 ade⁻*	This study
QX170[c]	*MATα cdc68-1 san1-170 ura3-52 ade⁻*	This study
QX300	*MATa/MATα cdc68-1/CDC68 leu2-3,112/LEU2 ura3-52/ura3-52 ade1/ade⁻*	21R × 68507A
QX301[d]	*MATa/MATα cdc68-1/CDC68 san1Δ::URA3/SAN1 leu2-3,112/LEU2 ura3-52/ura3-52 ade1/ade⁻*	This study
QX401	*MATα cdc68-1 san1Δ::URA3 ura3-52 ade⁻*	QX301 segregant
QX402	*MATa cdc68-1 san1Δ::URA3 leu2-3,112 ura3-52*	QX301 segregant
QXN1	*MATa san1Δ::URA3 leu2-3,112 ura3-52*	QX301 segregant
SUX32[e]	*MATa cdc68-1 san1-3 his4-912δ lys2-128δ ura3-52 ade⁻*	This study
SUX1702[e]	*MATa cdc68-1 san1-170 his4-912δ lys2-128δ ura3-52 ade⁻*	This study
SUPA1701	*MATa cdc68-1 san1-170 leu2-3,112 ade1*	This study
IS170[f]	*MATa cdc68-1 san1-170 [SAN1 URA3] his4-912δ lys2-128δ ura3-52 ade⁻*	This study
IS170-1c	*MATa san1-170 [SAN1 URA3] his4-912δ ade⁻*	21R × IS170 segregant
IS170-1d	*MATα san1-170 [SAN1 URA3] his4-912δ ade⁻*	21R × IS170 segregant
JHY631	*MATa ade1 his3 leu2-3,112 trp1-1a ura3 cln2::LEU2*	C.W.
TS1[c]	*MATα cdc68-1 san1-201 ura3-52 ade⁻*	This study
EP25	*MATa cdc37-1 his6 ura1*	Lab coll'n
EP25a	*MATa cdc37-1 ura3-52*	EP25 × FY56 segregant
EP25d	*MATa cdc37-1 lys2-128δ ura3-52*	EP25 × FY56 segregant
ARM4R2	*MATa cdc37 lys4 prt3 trp1 tyr1 ura3-52*	Lab coll'n
LARM4-16B	*MATa ade8 trp4 ade1 arg4 cdc65-1 leu2 rna3 ura3*	Lab coll'n
X4119-15D	*MATα aro1B hom2 cdc8 cdc9 his1 lys11 gal2 trp4*	YGSC
X4119-3	*MATα aro1B hom2 ura3-52*	21R × X4119-15D segregant
DY1671	*MATa sin4::TRP1 ho::lacZ can1-100 his3-11 leu2-3,112 trp1-1 ura3-52 ade2-1 ade6*	23
DY1825	*MATa sin4::TRP1 ho::lacZ swi2::HIS3 his3 leu2 trp1 ura3-52 ade2 ade6*	23
DY131	*MATa ho::lacZ can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-52 ade2-1 ade6*	23
DY1725	*MATα sin4::TRP1 his3-Δ200 leu2-Δ1 lys2-801 trp1-Δ63 ura3-52*	23
DY882	*MATα leu2-Δ1 lys2-801am his3-Δ200 trp1-Δ63 ura3-52 ade2-101oc*	D.J.S.
DY1736	*MATa ho::lacZ swi2::HIS3 can1-100 leu2 his3 trp1 ura3 ade2 ade6*	D.J.S.

[a] Gene designations in brackets indicate plasmid-derived sequences integrated in single copy at the preceding chromosomal locus.
[b] E.A.M., E. A. Malone; D.J.S., D. J. Stillman; F.W., F. Winston; C.W., C. Wittenberg; Lab coll'n, laboratory collection; YGSC, Yeast Genetic Stock Center.
[c] Congenic with strain 21R.
[d] Constructed by transforming the linearized *san1Δ::URA3* fragment (from plasmid pLG7) into diploid strain QX300.
[e] A segregant from a cross between strains FY56 and 21R was backcrossed with strain 21R. A *his4-912δ lys2-128δ* segregant from this cross was mated with strains QX3 and QX170 to derive strains SUX32 and SUX1702, respectively.
[f] Constructed by the directed integration of plasmid pIBE3 into the *Cla*I site of the *san1-170* locus in strain SUX1702.

DH5αF′ (Bethesda Research Laboratories) was used to propagate plasmid DNA and was maintained in 2× YT broth (34). Ampicillin was added to a final concentration of 75 μg/ml to select for the presence of Amp^r plasmids. Yeast cells were grown in enriched YM1 medium (17) and defined YNB medium (26). The enriched medium YPSA contained 2% sucrose and 1 mg of antimycin A (Sigma, St. Louis, Mo.) per liter and was used to determine the ability of strains to utilize sucrose (32). 5-FOA medium, containing 1 mg of 5-fluoro-orotic acid (PCR, Gainesville, Fla.) per liter, was used to select cells that had lost the *URA3* plasmid (6).

Mutant isolation and genetic analysis. All suppressor mutations were isolated in the temperature-sensitive *cdc68-1* strain 68507A by selecting for spontaneous temperature-resistant revertants at the restrictive temperature of 35°C. Independent cultures of strain 68507A were grown in YM1 medium, and then 0.1 ml of each culture was spread on yeast extract-peptone-dextrose (YEPD) solid medium and incu-

bated at 35°C for 2 to 3 days. To ensure the independence of each suppressor mutation, only a single temperature-resistant colony from each 35°C plate was chosen for further assessment. The temperature resistance phenotype of each isolate was confirmed by replica plating.

Standard yeast genetic procedures were used (15). Dominance tests were performed by crossing each suppressor mutant with the *cdc68-1* tester strain ARI68-7; good growth of the resultant diploid at 35°C indicated that the suppressor mutation was dominant, poor growth indicated codominance, and no growth indicated recessiveness. Complementation among the recessive suppressor mutations was assessed by scoring the phenotypes of diploid strains constructed from pairwise crosses of haploid suppressed strains. Maintenance of the suppressed phenotype (growth at 35°C) indicated that the recessive suppressor mutations were not complemented and therefore allelic. Complementation resulted in temperature-sensitive diploids, suggesting that the two mutations are in different genes. In the case of codominant suppressors, and also in cases of recessive mutations for which complementation results were ambiguous, allelism tests were carried out. Diploid cells resulting from mating two independent haploid suppressed strains were sporulated; a virtual absence of temperature-sensitive haploid segregants showed that the two suppressor mutations are tightly linked and thus most likely in the same gene.

The Spt phenotype was scored at 30°C on synthetic complete (SC) medium lacking histidine or lysine (32).

Assessment of cellular parameters. Cell samples of 0.5 ml were fixed by the addition of 4.5 ml of 3:7 formalin-Celline (Fisher Scientific, Nepean, Ontario, Canada). Samples were then sonicated for 5 s at 60% power with a Microson Sonicator (Heat System Ultrasonics, Farmingdale, N.Y.) to break up cell clumps. Yeast cell concentrations were determined by using an electronic particle counter (Coulter Electronics). Cell morphology was determined by direct microscopic examination (17).

DNA manipulations and strain constructions. *E. coli* plasmid DNA was prepared by both rapid-boiling (21) and alkaline lysis (5) methods. Yeast plasmid DNA was extracted by vortexing cells with glass beads (31). Total yeast DNA preparation and Southern analysis were performed as described previously (3), with the modification that spheroplasts were prepared by using buffer containing 30 mM dithiothreitol instead of β-mercaptoethanol. DNA manipulations were carried out as described previously (3, 54).

Plasmid pRS316 (58) was used to subclone the 11-kb insert of pTD33, a plasmid that complemented *san1* suppressor mutations. Resulting subclone plasmids were then tested for the ability to restore the *cdc68-1* mutant phenotype by transforming the *cdc68-1 san1-170* strain SUX1702. Yeast transformation was performed by the spheroplast method (20) with minor modifications (52) and also by the lithium acetate method (22). Plasmids pEBE3, pBX3, pBS3, and pIBE3 were constructed by cloning a 3.5-kb *Bam*HI-*Eco*RI fragment harboring the *SAN1* gene (see Fig. 2) into a variety of vectors. Plasmid pEBE3 contains the 3.5-kb insert between the *Eco*RI and *Bam*HI sites of the high-copy-number vector YEp352 (19); pBX3 contains the same insert between the *Bam*HI and *Xho*I sites of pRS315 (58); pBS3 contains the insert between the *Bam*HI and *Sal*I sites of YEp351 (19); pIBE3 contains the insert between the *Eco*RI and *Bam*HI sites of the integrating vector YIp352 (19). For integration of cloned yeast DNA, pIBE3 was linearized at the unique *Cla*I site within the insert and transformed into strain SUX1702 to create transformant strain IS170. To assess dosage effects of a mutant *cdc68* gene, a 4.7-kb *Bam*HI fragment containing the *cdc68-197* allele from plasmid pBM46 (32; a gift from F. Winston and E. A. Malone) was cloned into YEp352, generating plasmid pBM46-4.

To construct a *san1* null allele, the 3.5-kb *Bam*HI-*Eco*RI fragment described above was cloned into pUC19 (70), and the 2-kb *Sac*I-*Eco*RV fragment within this insert, from the *SAN1* upstream region to position +1158 of the *SAN1* open reading frame, was replaced, by using blunt-end ligation, with a 1.1-kb *Sma*I-*Hin*dIII fragment containing the *URA3* gene from YEp24 (7); insertion of *URA3* in both orientations generated two versions of a *san1*Δ::*URA3* null allele in plasmids pLG7 and pLG8. In separate experiments, the genomic *SAN1* gene was replaced with each version of the *san1*Δ::*URA3* null allele by a one-step gene transplacement procedure (51). The 2.6-kb *Bam*HI-*Eco*RI fragments of plasmids pLG7 and pLG8, each carrying a *san1*Δ::*URA3* null allele, were used to replace a genomic *SAN1* gene by transforming cells of the diploid strain QX300 to uracil prototrophy. Southern hybridization verified that one genomic copy of the *SAN1* gene in the diploid transformant QX301 was replaced by a *san1* null allele. The effects of each *san1*Δ::*URA3* null allele were determined in haploid segregants; the two versions of the *san1*Δ::*URA3* null allele gave identical results.

To determine whether the *san1*Δ::*URA3* null allele conferred an Spt$^-$ phenotype, the 2.6-kb *Bam*HI-*Eco*RI fragment harboring the null allele was transformed into the *his4-912*δ *lys2-128*δ strain FY56, and Ura$^+$ transformants were tested for histidine and lysine auxotrophy at 23, 30, and 37°C. To assess interactions between a *san1* null allele and a *cdc68* disruption allele, cells of diploid strain BM64, with genotype *cdc68-101*::*LEU2*/*CDC68 SAN1*/*SAN1*, were transformed with the 2.6-kb *Bam*HI-*Eco*RI fragment containing a *san1*Δ::*URA3* construct, a Ura$^+$ transformant was sporulated, and spore viability was determined.

Disruption of the *CLN2* gene was achieved by transforming cells of strain SUPA1701 with a 4.7-kb *Sal*I-*Hin*dIII fragment that contains the *cln2*::*LEU2* allele (a gift from C. Wittenberg; see reference 16), to generate strain SUPA1701c. Replacement of the genomic *CLN2* gene by the *cln2*::*LEU2* allele in strain SUPA1701c was confirmed by Northern (RNA) analysis.

Northern analysis. Total RNA was isolated (46) from 0.5×10^7 to 1.0×10^7 cells grown in YM1 medium. Hybridization with various probes was carried out as described previously (63). The *SAN1* probe was a 3.5-kb *Eco*RI-*Bam*HI fragment from plasmid pBE3; the *TUB2*+*YPT1* probe was a 2-kb *Bam*HI fragment from plasmid pBR1.129 (a gift from A. Wildeman); the *HTA1*+*HTB1*+*ADK1* probe was made from linearized YIp5-TRT1 plasmid (a gift from M. Osley), and the *HTA1*+*ADK1* probe was a 2.4-kb *Sst*I fragment from the same YIp5-TRT1 plasmid; the other probes have been described elsewhere (52). DNA fragments were resolved by gel electrophoresis, purified from gel slices with Geneclean II (BIO 101, La Jolla, Calif.), and labeled with [α-^{32}P]dCTP (Amersham) by the random-primer (Boehringer, Mannheim, Germany) labeling method (12). Probes were purified by using NICK columns (Pharmacia) to remove unincorporated isotope.

Nucleotide sequence analysis. Restriction fragments were cloned into the vectors M13 mp18 and M13 mp19 (42), and nucleotide sequence was determined by the method of Sanger et al. (55) with a Sequenase kit, version 2.0 (U.S. Biochemical, Cleveland, Ohio). DNA and protein sequence comparison was done by using the FASTA software package

(47) and by inspection of the published *SAN1* gene sequence (57).

Mapping of the *SAN1* gene. The 3.5-kb *Bam*HI-*Eco*RI genomic insert from plasmid pTD33 was labeled with [α-^{32}P]dCTP and used to probe an *S. cerevisiae* chromosome blot (Clontech Laboratories, Palo Alto, Calif.). Linkage of the *san1* locus to markers on chromosome IV was determined by tetrad analysis using strains IS170-1c and IS170-1d. *san1* segregation was scored by a *URA3* marker integrated at the chromosomal *san1* locus.

RESULTS

Isolation of *cdc68-1* suppressor mutations. Spontaneous mutations which allow growth of cells harboring the *cdc68-1* mutation were isolated by incubating *cdc68-1* mutant cells on solid medium at the restrictive temperature of 35°C for 2 to 3 days. From each of the independently derived temperature-resistant colonies, cells were mated with tester cells of another *cdc68-1* mutant strain, ARI68-7, and the resultant diploid cells were sporulated. From 35 of these independent diploid strains, temperature sensitivity among tetrads segregated in a 2:2 fashion. Thus, the suppression of temperature sensitivity in each of these original *cdc68-1* mutant isolates was due to mutation within a single nuclear gene.

To determine whether suppressor mutations represent additional mutations at the *cdc68-1* locus or extragenic events, the segregation pattern of each suppressor mutation was compared with the segregation pattern of a *cdc68* allele, scored by a *URA3* marker integrated at the chromosomal *cdc68-1* locus. This analysis showed that in each of 28 independent suppressed strains, the suppressor mutation was unlinked to the *cdc68* locus. When these 28 extragenic suppressor mutations were tested for dominance relationships, 8 mutations were found to be recessive and the other 20 mutations were codominant, as indicated by intermediate levels of temperature resistance for diploids homozygous for *cdc68-1* and heterozygous for a suppressor mutation (data not shown).

To establish the number of genes represented by these suppressor mutations, we performed standard complementation tests for the recessive suppressor mutations and allelism tests for the codominant suppressor mutations. Diploid cells resulting from matings between haploids harboring recessive suppressor mutations and the *cdc68-1* allele were analyzed for growth at the restrictive temperature of 35°C; growth (suppression) indicated failure to complement and showed that the two recessive suppressor mutations reside in the same complementation group, whereas the failure to grow (no suppression) indicated complementation of the recessive suppressor mutations and suggested that the two suppressor mutations affected different genes. From diploid cells harboring codominant suppressor mutations, meiotic products were tested for growth at the restrictive temperature of 35°C. Suppressor mutations in the same gene caused all meiotic segregants to be temperature resistant at 35°C, whereas the segregation of suppressor mutations in different genes caused some meiotic segregants to be temperature sensitive. These complementation and allelism tests assigned the 28 extragenic suppressor mutations to four genes (Table 2). We show below that one of these genes is identical to the previously described *SAN1* gene (57), and therefore we have adopted the *SAN1* designation to describe this suppressor gene. Thus, at least four genes can be mutated to reverse the growth defect of *cdc68-1* mutant cells at the restrictive temperature of 35°C.

TABLE 2. Growth phenotypes of suppressor alleles

Suppressor gene	No. of alleles		Suppression of *cdc68-1*	
	Recessive	Codominant	35°C	37°C
SAN1	2	18	+	−
SCB68	4	2	+	−
SCC68	1	0	+	−
SCD68	1	0	+	−

Reversal of the Spt$^-$ phenotype of *cdc68* by *san1* suppressor mutations. In addition to temperature sensitivity, the *cdc68-1* mutation causes a phenotype called Spt$^-$ (suppression of Ty), which is the suppression of the histidine and lysine auxotrophies caused by δ insertion mutations in the 5′ regions of the *HIS4* and *LYS2* genes (8, 32). The *cdc68-1* mutation and another temperature-sensitive allele of *CDC68*, *cdc68-197* (also termed *spt16-197*), suppress these δ insertion mutations at a permissive temperature of 30°C (32, 52). Thus, *his4-912*δ *lys2-128*δ mutant cells harboring a *cdc68* mutation can proliferate at 30°C without added histidine and lysine. We determined that recessive *san1* mutations identified here also reversed the Spt$^-$ phenotype of a *cdc68* mutation, causing *san1 cdc68 his4-912*δ *lys2-128*δ cells to once again be His$^-$ and Lys$^-$ at 30°C (Fig. 1). In contrast, diploid cells homozygous for the *his4-912*δ, *lys2-128*δ, and *cdc68* mutations, but heterozygous at the *SAN1* locus (one mutant and one wild-type allele), were His$^+$ and Lys$^+$ at 30°C (data not shown), demonstrating that for the Spt$^-$ phenotype, suppression by *san1* is also recessive.

Increasing the gene dosage of *CDC68* (also termed *SPT16*) also produces an Spt$^-$ phenotype (32). We also tested the ability of *san1* mutations to suppress the Spt$^-$ phenotype caused by extra copies of the *CDC68* gene. For this analysis, the *his4-912*δ *lys2-128*δ strain FY56 was transformed with a *CDC68* gene carried on an episomal (high-copy-number) plasmid. The resultant transformed strain was mated with a *cdc68-1 san1-170* strain, and the diploid was sporulated. The occurrence of Ura$^+$ haploid segregants (harboring the high-copy-number *CDC68* plasmid) that were His$^-$ and Lys$^-$ at 30°C indicated that the *san1-170* mutation abolished the δ insertion suppression caused by increased *CDC68* gene dosage (otherwise, segregants that overexpressed the wild-type *CDC68* gene would be His$^+$ Lys$^+$ [Spt$^-$] as a result of

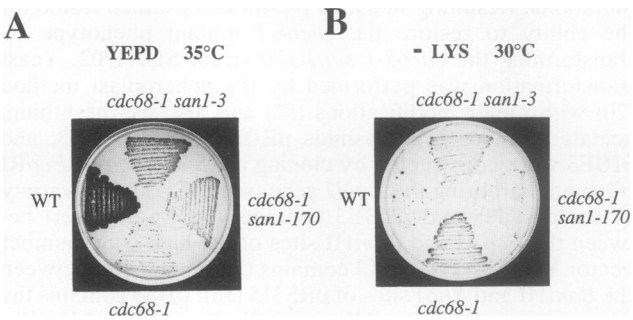

FIG. 1. Suppression of the temperature-sensitive and Spt$^-$ phenotypes of the *cdc68-1* mutation. All strains carry the *lys2-128*δ mutation. Cells were spread on YEPD solid medium, incubated at 23°C, and then replica plated to YEPD medium for further incubation at 35°C (A) and to SC-Lys medium (SC medium lacking lysine) for further incubation at 30°C (B). WT, wild type.

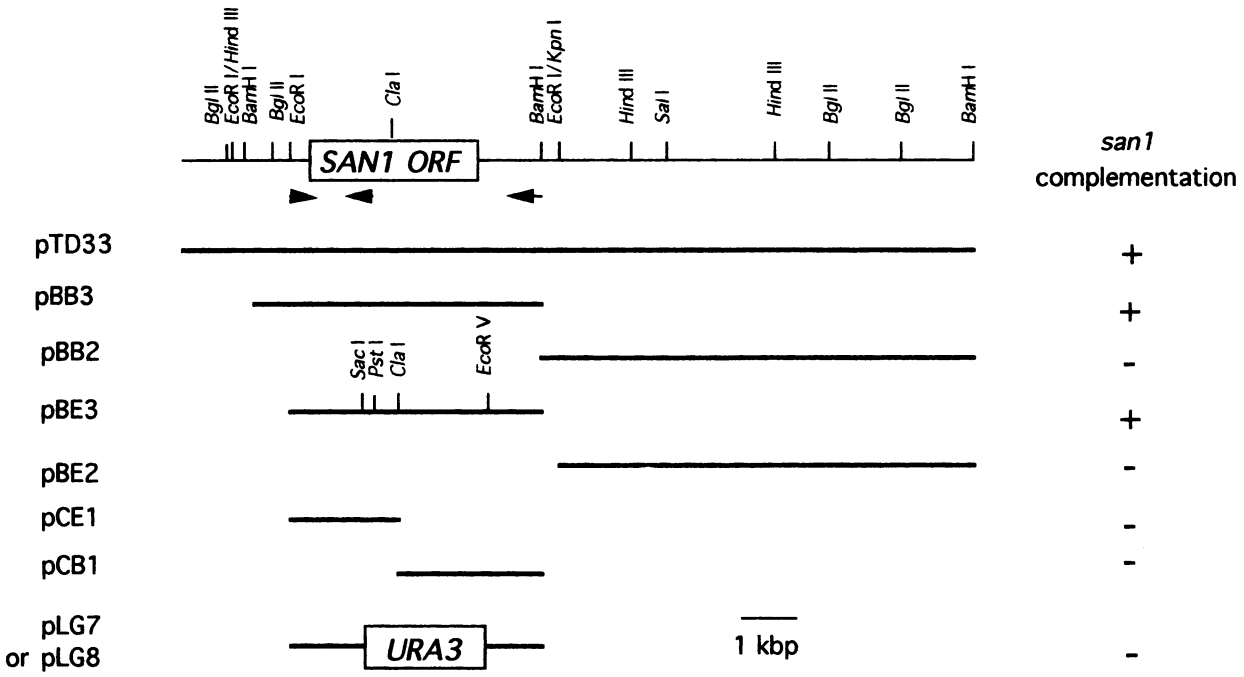

FIG. 2. Localization and identification of the *SAN1* gene. Complementation by episomal plasmids, with genomic inserts as indicated, of the temperature resistance and Spt$^+$ phenotypes of a *san1 cdc68-1* mutant strain is indicated (+, complementation; −, no complementation). Open boxes show the approximate positions of open reading frames (ORF). Three small arrows below the restriction map indicate regions that were sequenced. Plasmids pLG7 and pLG8, with the *URA3* gene in opposite orientations, were used to disrupt the chromosomal *SAN1* gene.

the high-copy-number suppression). Thus, a *san1* mutation can suppress the Spt$^-$ phenotype resulting from either *cdc68* mutations or increased copy number of the wild-type *CDC68* gene.

Molecular analysis identifies one suppressor as the *SAN1* gene. The wild-type suppressor gene was cloned by complementation of the recessive mutant phenotype. The starting strain, SUX1702, harbors the *cdc68-1*, *san1-170*, *lys2-128δ*, and *his4-912δ* mutations. Because of the ability of the *san1-170* suppressor mutation to reverse the Spt$^-$ phenotype of the *cdc68-1* mutation, these mutant cells were His$^-$ Lys$^-$ at 30°C. This effect was recessive, so that a transformant containing the wild-type *SAN1* gene would be His$^+$ Lys$^+$ at 30°C. In addition, this transformant would also be temperature sensitive at 35°C as a result of the loss of *cdc68* suppression. Transformants containing members of a YCp50-based yeast genomic library were therefore selected for histidine and lysine prototrophy at 30°C and then tested by replica plating for temperature sensitivity at 35°C. Plasmid pTD33, containing an 11-kb insert (Fig. 2), was recovered from two of the four transformants which were His$^+$ Lys$^+$ at 30°C and temperature sensitive at 35°C.

To delimit the active sequence of the cloned DNA, several subclones of plasmid pTD33 were tested (Fig. 2). The 3.5-kb *Eco*RI-*Bam*HI fragment in pBE3 was the smallest that complemented the *san1* mutant phenotypes. We confirmed that this fragment contained the wild-type suppressor gene by demonstrating that this genomic fragment can direct plasmid integration to the *san1* chromosomal locus by homologous recombination (see Materials and Methods). The resultant *cdc68-1 san1-170* integrant strain IS170 was mated with a *cdc68-1 san1-170 ura3-52* mutant strain; in 12 meiotic tetrads tested, the temperature sensitivity (wild type for *SAN1*) and Ura$^+$ (harboring the *URA3* marker) phenotypes cosegregated, verifying that the temperature sensitivity was due to the integrated plasmid. Strain IS170 was also crossed with a strain harboring the *cdc68-1* mutation; in 16 tetrads from this diploid, all of the segregants were temperature sensitive, indicating that the complementing genomic sequence had integrated at the chromosomal *san1* locus. Plasmid integration at the homologous chromosomal locus was confirmed by Southern analysis (data not shown). Thus, the cloned genomic fragment contains the wild-type *SAN1* gene.

Nucleotide sequence analysis of the *Hin*dIII-*Pst*I and *Sst*I-*Kpn*I fragments within the pBE3 genomic insert (Fig. 2) identified the cloned wild-type suppressor gene as *SAN1*: DNA and protein sequence comparisons showed that both the nucleotide sequence and the predicted amino acid sequence within the *Hin*dIII-*Pst*I fragment are identical to those from one region of the *SAN1* gene (nucleotides 1420 to 1870 within the open reading frame) (57). Also, a DNA sequence within the *Sst*I-*Kpn*I fragment was identical to a region downstream of the *SAN1* open reading frame (nucleotides 2310 to 2668). The identity of the cloned gene and *SAN1* was further substantiated by the identical restriction patterns exhibited by *SAN1* and our suppressor gene (Fig. 2).

A radiolabeled 3.5-kb *Bam*HI-*Eco*RI fragment encompassing the *SAN1* gene was used to probe a yeast chromosome blot, which localized *SAN1* to chromosome IV. To position *SAN1* relative to other genes on chromosome IV, we performed genetic crosses of strains containing a *URA3*-marked *san1* locus. After these studies mapped *san1* to the right arm of chromosome IV near the *cdc37* locus, a three-factor cross was performed by using the *hom2* and *aro1*

TABLE 3. Positioning *san1* on chromosome IV

Genetic interval[a]	No. of tetrads			Map distance (centimorgans)[b]
	Parental ditype	Tetratype	Nonparental ditype	
san1-cdc37 (a)	22	3	0	6.0
san1-trp4 (b)	4	17	3	72.9
san1-ade8 (b)	5	15	4	81.2
san1-aro1 (c)	47	4	0	3.9
san1-hom2 (c)	47	4	0	3.9
aro1-hom2 (c)	47	8	0	7.8

[a] Tetrads were scored from the crosses EP25d × IS170-1d (a), LARM4-16B × IS170-1d (b), and X4119-3 × IS170-1c (c).
[b] Genetic map distances from the *URA3* gene integrated at the *san1* locus were calculated as specified by Mortimer and Schild (37).

markers located in this region. The *URA3* marker integrated at *san1* mapped midway between the *hom2* and *aro1* loci (Table 3). No gene has been previously reported to be in this region (36).

***sir4*-suppressing *san1* alleles also suppress *cdc68*.** We assessed suppression of the *cdc68-1* mutation by the *san1* mutations that were originally recovered in a different way, as suppressors of a leaky *sir4* mutation (57). Haploid strains harboring each of the *sir4*-suppressing *san1* (*sir* antagonist) mutations (gifts from J. Rine) were mated with a *cdc68-1* mutant strain, and the resultant diploid cells were sporulated. Tetrad analysis showed that the point mutations *san1-1* and *san1-2* and a disruption allele, *san1*::*HIS3* (57), all suppressed both the temperature sensitivity and the Spt^- phenotype of the *cdc68-1* mutation (data not shown). The occurrence of 1:3 and 0:4 meiotic segregation patterns for temperature sensitivity suggested that *san1* suppressed *cdc68-1*; in the case of the *san1* disruption allele, the identification of *san1*::*HIS3* by its His^+ phenotype verified that the suppressing mutation was *san1*. Thus, the *san1* mutant alleles isolated by *sir4* suppression have the same effects as do the *cdc68*-suppressing *san1* alleles identified here.

***san1* mutant cells still need Cdc68 protein function.** The growth kinetics of representative mutant strains showed that different alleles of *san1* suppress the *cdc68-1* mutation to different degrees (data not shown). For example, the *san1-3* allele allowed *cdc68-1* mutant cells to proliferate as rapidly as wild-type cells at 35°C, with a doubling time of 1.5 h; the *san1-170* allele suppressed the *cdc68-1* mutation less efficiently, allowing a doubling time of 2.5 h for *cdc68-1 san1-170* double-mutant cells at 35°C. These observations suggest that *san1* alleles have different degrees of residual function.

Disruption of the *SAN1* gene by insertion within the *SAN1* open reading frame (the *san1*::*HIS3* mutation) has no deleterious effects on cell viability (57). Nonetheless, proteins can consist of several domains that are able to function independently, so the possibility remained that the disrupted San1 protein retains some function. We therefore constructed a *san1* null allele, *san1*Δ::*URA3*, by replacing the *Sac*I-*Eco*RV internal restriction fragment of a plasmid-borne *SAN1* gene (see Materials and Methods) with the *URA3* gene (Fig. 2). An *Eco*RI-*Bam*HI linear fragment harboring this *san1*Δ::*URA3* null allele was then transformed into cells of the Ura^- diploid strain QX300, with genotype *SAN1/SAN1 cdc68-1/CDC68*, to replace a resident *SAN1* locus with the *URA3*-marked *san1* null allele. Substitution of one of the genomic copies of *SAN1* by the null allele was confirmed by Southern analysis (data not shown). The Ura^+ diploid transformant QX301 was sporulated, and all meiotic products were viable, showing that the *SAN1* gene is dispensable for cell viability.

The *san1*Δ::*URA3* null allele and the *san1*::*HIS3* disruption allele have the same suppression phenotypes as do the other *san1* mutant alleles (data not shown). In addition, the effects of another temperature-sensitive allele of the *CDC68* gene, *cdc68-197*, were also suppressed by the *san1* null allele, as well as by the *san1-3* and *san1-170* mutations (data not shown). All of these observations (summarized in Table 4) imply that *san1* suppression is simply a consequence of decreased San1 function.

To better understand the functional relationship between the San1 and Cdc68 proteins, we determined whether the *san1*Δ::*URA3* null mutation could suppress a *cdc68* disruption allele; this allele, *cdc68-101*::*LEU2*, renders haploid *SAN1* cells nonviable (32). After replacing one genomic copy of the wild-type *SAN1* gene with the *san1*Δ::*URA3* null allele in diploid cells heterozygous for the *cdc68-101*::*LEU2* disruption, we sporulated the diploid transformant. For 10 tetrads dissected, spore viability was 2:2 in all cases, and all of the viable spores were either Leu^- Ura^+ or Leu^- Ura^-. The fact that no Leu^+ spores (harboring the *cdc68* disruption allele) were obtained suggests that a *cdc68* disruption *san1* null double-mutant cell is not viable. In addition, we used a plasmid loss procedure to show that the *san1-3* mutation also does not suppress the *cdc68-101*::*LEU2* allele; *cdc68-101*::*LEU2 san1-3* double-mutant cells could not lose a *URA3*-marked *CDC68* plasmid (53). These observations suggest that neither decreased San1 protein function nor the absence of the San1 protein can bypass the requirement for the Cdc68

TABLE 4. Interactions between *san1* and *cdc68* mutations

Gene	Phenotype of *san1 cdc68* double mutant[a]				
	Growth at 35°C			Suppression of *his4-912δ lys2-128δ* by *cdc68-1*	Suppression of *suc2ΔUAS* (−1900/−390) by *cdc68-1*
	cdc68-1	*cdc68-197*	*cdc68-101*::*LEU2*		
SAN1	−	−/+	Dead	+	+
san1-3	++	++	Dead	−	−
san1-170	+	+	ND	−	−
san1-1	++	++	ND	ND	ND
san1-2	++	++	ND	ND	ND
san1::*HIS3*	++	++	ND	ND	ND
*san1*Δ::*URA3*	++	++	Dead	−	−

[a] Growth of wild-type cells was scored as +++. ND, not determined.

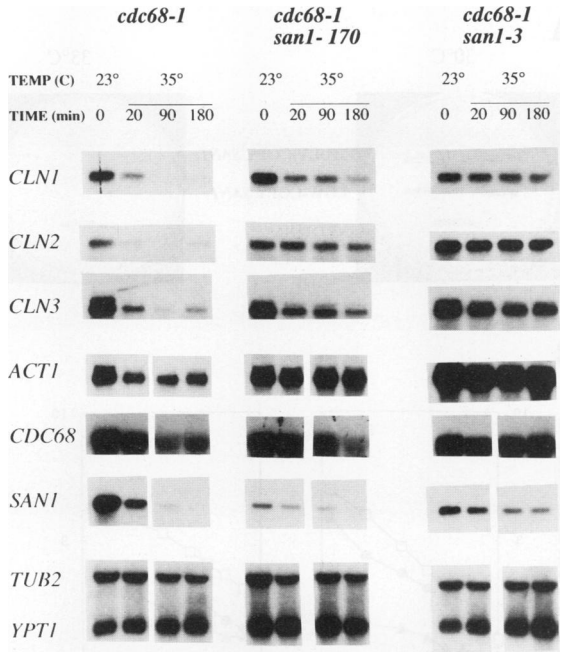

FIG. 3. Transcript levels in *cdc68-1* and *cdc68-1 san1* mutant cells. Total yeast RNA was extracted from strains 68507A (*cdc68-1 SAN1*), QX170 (*cdc68-1 san1-170*), and QX3 (*cdc68-1 san1-3*) growing at 23°C and after incubation at 35°C for the indicated times. Transcripts from the *TUB2* and *YPT1* genes, which are relatively unaffected by the *cdc68-1* mutation at 35°C, were used as loading controls.

protein for cell viability. Therefore, either San1 acts upstream of Cdc68 or these two proteins function at the same stage.

***san1* mutations restore G_1 cyclin gene transcription in *cdc68-1* mutant cells.** Cells harboring the *cdc68-1* allele are unable to perform START, the regulatory step for cell proliferation, presumably as a result of the decreased G_1 cyclin transcription that they experience (52) (Fig. 3). We therefore assessed the ability of *san1* mutations to restore expression of three G_1 cyclin genes, *CLN1*, *CLN2*, and *CLN3*, in *cdc68-1* mutant cells. After transfer of *san1-3 cdc68-1* double-mutant cells to the restrictive temperature of 35°C, transcripts for all three G_1 cyclin genes persisted at about the same level as found at 23°C (Fig. 3). Therefore, the *san1-3* suppressor mutation restores normal transcript abundance for these three G_1 cyclin genes.

Although in weakly suppressed *san1-170 cdc68-1* cells the *CLN2* transcript was abundant at 35°C and at about the same level as before the temperature shift, the levels of *CLN1* and *CLN3* transcripts were lower than those at 23°C. These three *CLN* genes are functionally redundant; any one of the three G_1 cyclin genes is sufficient for cell proliferation (49). Therefore, it is possible that the weak *san1-170* mutation suppresses the START inhibition in *cdc68-1* mutant cells by restoring significant expression of only one G_1 cyclin gene, *CLN2*. We therefore constructed cells lacking *CLN2* function by one-step gene replacement and also by genetic crosses. The resultant *cdc68-1 san1-170 cln2::LEU2* strains were still temperature resistant at 35°C, suggesting that the *san1-170* suppressor mutation must restore sufficient function of at least one other G_1 cyclin in addition to Cln2. Northern analysis showed that in the *cln2 cdc68 san1* cells, the levels of *CLN1* and *CLN3* transcripts were about the same as those in isogenic *CLN2 cdc68-1 san1-170* cells (data not shown). These low levels of *CLN1* and/or *CLN3* expression may therefore be sufficient for cell proliferation.

***san1* mutations reverse other transcriptional effects of the *cdc68-1* mutation at 35°C.** In addition to decreasing G_1 cyclin expression, the *cdc68-1* mutation has other transcriptional effects. Impaired Cdc68 activity causes decreased transcript abundance for the *ACT1* and *LEU2* genes and for the *cdc68* gene itself (52). We therefore determined the effects of the *san1* alleles on transcript abundances for the *ACT1* and *cdc68-1* genes. We examined gene expression in two strains; one strain harbors the *san1-3* allele, which allows *cdc68-1* mutant cells to proliferate almost as well as wild-type cells at 35°C, and the other strain bears the *san1-170* allele, which suppresses the *cdc68-1* mutation less effectively. Both the *san1-3* and *san1-170* mutations restored transcription of the *ACT1* and *cdc68-1* genes at the restrictive temperature of 35°C (Fig. 3). We infer that *san1* mutations also restore expression of the *LEU2* gene in *cdc68-1* mutant cells at 35°C, because suppressed cells proliferated in synthetic medium without leucine (data not shown). Therefore, *san1* suppressor mutations reversed many transcriptional defects caused by the *cdc68-1* mutation at 35°C.

The *cdc68* mutations cause another transcriptional alteration related to *SUC2* gene expression and sucrose fermentation. Cells that carry a mutant *suc2ΔUAS* allele (deleted for the *SUC2* upstream activating sequence) cannot transcribe *SUC2* and hence cannot catabolize sucrose. The *cdc68-1* and *cdc68-197* mutations reverse this Suc^- phenotype by allowing transcription of the *suc2ΔUAS* allele, thereby permitting mutant cells to grow on sucrose (32; data not shown). To determine whether *san1* suppressor mutations reverse this transcriptional effect of *cdc68* mutations, a diploid strain with the genotype *cdc68-1/cdc68-197 suc2Δ UAS/SUC2 san1-170/SAN1* was constructed, and phenotypes of meiotic products were determined. Since every meiotic segregant carries a *cdc68* mutant allele and either the *SUC2* gene that is unaffected by a *cdc68* mutation or the *suc2ΔUAS* allele that is transcribed in *cdc68* mutant cells, the occurrence of Suc^- segregants indicates that the *san1-170* suppressor allele prevents *suc2ΔUAS* transcription in *cdc68* mutant cells. Of 10 complete tetrads, 6 displayed 3 Suc^+:1 Suc^- segregation patterns, three were 2:2, and one was 4:0. Further genetic analysis confirmed that Suc^- segregants harbored the *san1-170* allele. In a similar analysis, the *san1-3* allele was shown to have the same effect as the *san1-170* allele did. Reversal of this Suc^+ phenotype by different *san1* alleles is shown in Fig. 4. Thus, *san1* mutations also reverse the *cdc68* effect on *suc2ΔUAS* expression.

Cdc68 is an activator of histone gene expression. Either increased gene dosage of the *CDC68* gene or a *cdc68* mutation confers an Spt^- phenotype (32, 52). This effect of altered Cdc68 activity resembles that resulting from altered histone gene dosage (8). It is therefore possible that the Spt^- phenotype that is imposed by altering *CDC68* gene dosage is mediated through changes in histone gene expression. We investigated histone gene expression by determining transcript abundance for the *HTA1/HTB1* locus, which is one of the two gene pairs encoding histones H2A and H2B. As shown in Fig. 5A, transcription of the *HTA1* and *HTB1* genes at this locus decreased in *cdc68-1* mutant cells at the restrictive temperature of 35°C, and the *san1::HIS3* disruption allele reversed this effect. It is likely that an altered histone stoichiometry thereby causes the Spt^- phenotype under conditions of altered Cdc68 activity.

Expression of histone genes is periodic in the yeast cell

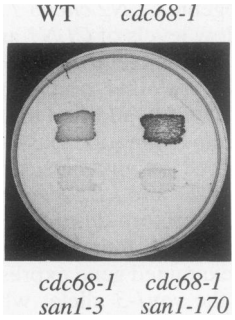

FIG. 4. Reversal by *san1* suppressor mutations of the Suc$^+$ phenotype caused by a *cdc68* mutation. Strains harboring the *suc2ΔUAS*(−1900/−390) allele were patched onto YEPD solid medium, replica plated to enriched medium containing 2% sucrose and 0.1% antimycin A, and incubated at 33°C. WT, wild type.

cycle, with maximal synthesis of histone mRNAs during DNA replication (for a review, see reference 43). Several genes have been implicated in the regulation of histone gene expression; mutations in these regulatory genes allow histone genes to be transcribed even when DNA replication is inhibited by hydroxyurea treatment (44, 68). *CDC68* is unlikely to be a member of this class of histone regulatory genes, because altered Cdc68 activity (either by mutation or by increased gene dosage) did not allow *HTA1* transcription after DNA replication was blocked by hydroxyurea treatment (Fig. 5B). Thus, our Northern data suggest that the Cdc68 protein activates at least some histone gene expression without apparent influence on the S-phase regulation of histone gene transcription.

Transcription of the *SAN1* gene is regulated by Cdc68 and San1. Because Cdc68 can activate the expression of many diverse genes, we determined whether Cdc68 also activated transcription of the *SAN1* gene. As shown in Fig. 3, the

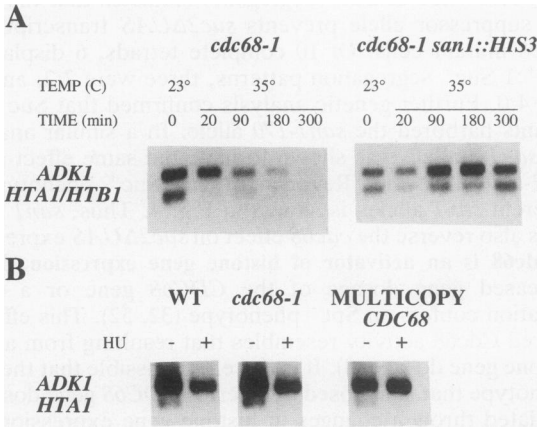

FIG. 5. The San1 and Cdc68 proteins regulate histone gene expression. (A) Total RNA was extracted from yeast strains 68507A (*cdc68-1*) and YRQ9344 (*cdc68-1 san1::HIS3*) as described in the legend to Fig. 3. The RNA blot was probed for *HTA1*, *HTB1*, and *ADK1* (adenylate kinase, a gene next to *HTA1*). (B) Determination of the Hir phenotype. Northern analysis of *HTA1* and *ADK1* (used as internal control) transcript abundance from cells grown in the absence (−) or presence (+) of hydroxyurea (HU) for 30 min at 30°C. Strains used were 21R (wild type [WT]), 68507A (*cdc68-1*), and 21R transformed with high-copy-number *CDC68* plasmid pSC2-1.

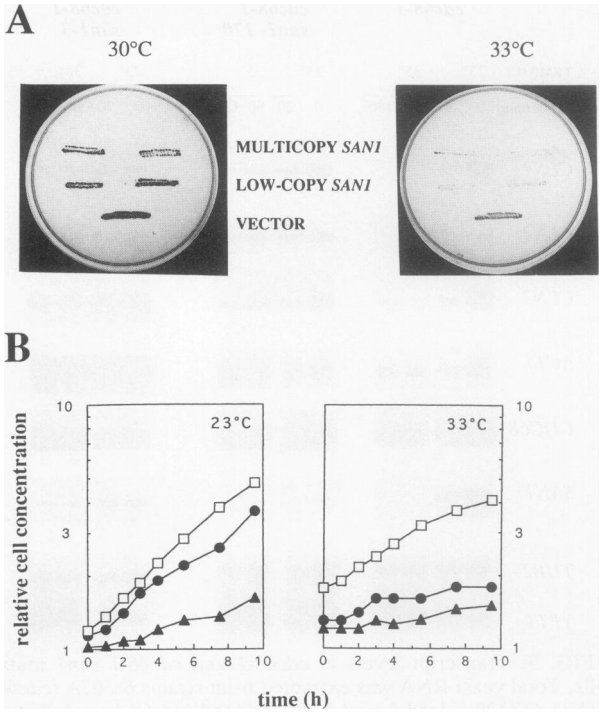

FIG. 6. Overexpression of the *SAN1* gene inhibits the proliferation of *cdc68* mutant cells. (A) Cells of the *cdc68-1* mutant strain 68507A transformed with the low-copy-number *SAN1* plasmid pBE3, the high-copy-number *SAN1* plasmid pEBE3, or the high-copy-number vector YEp352 were replica plated to SC-Ura medium and incubated at 30 and 33°C. (B) The same *cdc68* transformants harboring *SAN1* in low copy number (solid circles) or high copy number (solid triangles) or vector alone (open squares) were grown in SC-Ura liquid medium at 23°C; at time zero, cultures were split and a portion was transferred to 33°C for further incubation.

cdc68-1 mutation decreased *SAN1* transcript abundance at 35°C, whereas *san1* mutations reversed this transcriptional defect. *SAN1* is therefore one of the many genes activated by Cdc68. We conclude that San1 and Cdc68 antagonize each other for the expression of many genes, including the *SAN1* and *CDC68* genes themselves.

Dosage effects of *SAN1* and *CDC68* on transcription. The *san1* suppressor alleles had no marked effect on the growth rate of cells harboring the wild-type *CDC68* gene and caused an observable phenotype only for cells containing a *cdc68* mutation. Similarly, decreased *SAN1* gene dosage resulting from gene replacement by the *san1Δ::URA3* null allele, like the *san1* mutations described above, suppressed the phenotype of the *cdc68-1* mutation at 35°C. To assess the effects of increased *SAN1* gene dosage, plasmids pEBE3, carrying the *SAN1* gene on a high-copy-number episomal vector, and pBE3, bearing *SAN1* on a low-copy-number centromeric vector, were transformed into wild-type and *cdc68-1* mutant cells. Increased *SAN1* gene dosage had no detectable effects in wild-type cells (data not shown) but actually exaggerated the temperature sensitivity of *cdc68* mutant cells. At 33°C, which is a permissive temperature for *cdc68-1* mutant cells with normal *SAN1* gene dosage, transformants with additional copies of the *SAN1* gene (even those with only a few additional copies of *SAN1* on a *CEN*-based plasmid) were inhibited (Fig. 6A). The growth rates of *cdc68-1* mutant cells carrying extra copies of *SAN1* were also affected. As shown

TABLE 5. Dosage effects of *SAN1* and *CDC68* alleles

Copy no.		Growth			
SAN1	*CDC68* or *cdc68*	23°C	33°C	35°C	37°C
0	1 *CDC68*	+	+	+	+
1	1 *CDC68*	+	+	+	+
Many	1 *CDC68*	+	+	+	+
0	1 *cdc68*	+	+	+	−
1	1 *cdc68*	+	+	−	−
Many	1 *cdc68*	+	−	−	−
1	Many *cdc68*	+	+	+	+
1	Few *cdc68*	+	+	+/−	−
1	1 *cdc68*	+	+	−	−
1	0	Dead	Dead	Dead	Dead
0	0	Dead	Dead	Dead	Dead

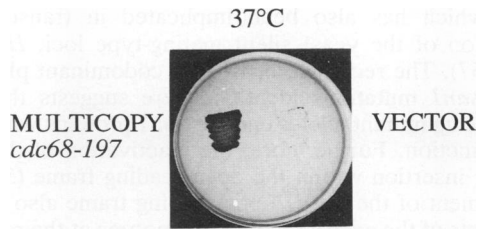

FIG. 7. Increasing *cdc68* mutant gene dosage overcomes Sanl inhibition. Cells of the *cdc68-1* mutant strain 68507A transformed with the multicopy *cdc68-197* plasmid pBM46-4 or the vector were patched onto SC-Ura solid medium, incubated at room temperature, and then replica plated onto SC-Ura medium and incubated at 37°C.

in Fig. 6B, increased *SAN1* gene dosage slowed the growth of *cdc68-1* mutant cells even at 23°C, although not as dramatically as seen at 33°C, a temperature at which cells actually ceased proliferation. This inhibition caused by increased *SAN1* gene dosage indicates that the Sanl protein inhibits the expression of a gene essential for cell proliferation but does so effectively only when Cdc68 activity is attenuated by mutation. Clearly, any inhibitory effect of Sanl is weak and is masked by the potent wild-type Cdc68 transcription activator. The effects of altering *CDC68* and *SAN1* gene copy number are summarized in Table 5.

A negative regulator like Sanl could function directly by repressing transcription of target genes or indirectly through effects on transcription factors like the Cdc68 protein. If Sanl directly represses gene expression, this negative effect should be independent of Cdc68 function. In this case, we might detect transcriptional alterations from changing the *SAN1* gene dosage in *CDC68* wild-type cells. Because our suppressor analysis reveals that the Cdc68 and Sanl proteins are functionally antagonistic, those genes regulated by Cdc68 are good candidates to assess the effects of altered Sanl content. We chose to determine the transcript abundances for *ACT1*, *CDC68*, and G_1 cyclin genes in *sanl*Δ::*URA3 CDC68* cells and in *CDC68* cells carrying a high-copy-number *SAN1* plasmid. Overexpression of *SAN1* in cells harboring the high-copy-number *SAN1* plasmid was confirmed at the transcript level (data not shown). Regardless of *SAN1* gene dosage, there were no substantial changes in mRNA abundance for any of the assessed genes, either at room temperature or at 35°C (data not shown). These Northern blot data lead us to conclude that changes in *SAN1* gene dosage in *CDC68* wild-type cells have no dramatic effects on transcription and suggest that other components mediate Sanl inhibition of transcription.

Transcription of the *cdc68-1* mutant gene at 35°C was restored in *cdc68 sanl* double-mutant cells (Fig. 3). This observation raised the possibility that decreased Sanl activity suppresses the temperature sensitivity of *cdc68* mutant cells by allowing relatively normal production of mutant Cdc68 protein. If so, then extra copies of a mutant *cdc68* gene in *cdc68 SAN1* mutant cells might also allow the production of enough mutant Cdc68 protein to activate transcription. We addressed this possibility directly by transforming a *cdc68-1* mutant strain with high-copy-number plasmids harboring either the *cdc68-197* mutant gene or just the *CDC68* promoter (without the open reading frame). The *cdc68-197* transformants were temperature resistant at 35°C (data not shown). Thus, supplementing cells with more mutant Cdc68 protein suppressed the *cdc68* mutation. In contrast, extra copies of the *CDC68* promoter, which is affected by Sanl inhibition, had no effect. These observations suggest that Sanl can be titrated out by the Cdc68 protein but not by the availability of protein-binding sites along *CDC68* promoter DNA. The fact that increased mutant *cdc68* gene dosage produces virtually the same effect as the inactivation of Sanl protein further supports the conclusion that Cdc68 activity is inhibited by the Sanl protein. Extra Cdc68 protein (even the mutant form) can overcome the Sanl inhibitor and restore Cdc68 function.

Absence of the Sanl protein cannot suppress the *cdc68-1* mutation at 37°C. Although all of the 20 *sanl* suppressor mutations isolated in this study suppressed the *cdc68-1* mutation at 35°C, none reversed the phenotype of the *cdc68-1* mutation at the higher restrictive temperature of 37°C. At this temperature, *cdc68-1 sanl* double-mutant cells became arrested in the cell cycle as large unbudded cells (60 to 70% unbudded) after a 4-h incubation, suggesting that inactivation of Sanl cannot reverse the G_1 arrest caused by the *cdc68-1* mutation at 37°C. Indeed, our Northern data revealed that at 37°C, *sanl* mutations did not restore transcription of any of the three G_1 cyclin genes studied here (data not shown). The *sanl*Δ::*URA3* null allele has the strongest suppressive effect among all of the *sanl* alleles yet does not effectively suppress the *cdc68-1* mutation at 37°C; at this temperature, *sanl*Δ::*URA3 cdc68-1* double-mutant cells grew slowly in liquid medium and did not form colonies on solid medium. Nevertheless, the *cdc68* mutant gene products must have residual activity even at 37°C, because at this temperature, extra copies of the *cdc68-197* allele allowed growth (Fig. 7). It is noteworthy that the initial suppressor isolate harboring the *sanl-201* allele did grow at 37°C, but genetic analysis showed this stronger suppression to be due to a multigenic effect, caused by the interaction of additional mutations with *sanl-201* and *cdc68-1*. Therefore, other gene products may modulate Cdc68 activity at 37°C. In fact, we have found that in the absence of *SAN1*, the *scb68* suppressor mutations suppress the *cdc68-1* mutation at 37°C (data not shown). Therefore, it is likely that Sanl and other suppressor proteins act synergically to antagonize Cdc68 activity.

DISCUSSION

Four genes in which mutations can suppress the growth defect caused by the conditional *cdc68-1* mutation have been identified. One of these suppressor genes has been characterized in detail. Partial nucleotide sequence analysis reveals that this suppressor gene is the previously described *SAN1*

gene, which has also been implicated in transcriptional repression of the yeast silent mating-type loci, *HML* and *HMR* (57). The recessive or weakly codominant phenotype of the *san1* mutations identified here suggests that these suppressing mutant alleles encode San1 proteins with diminished function. Furthermore, the inactivation of the *SAN1* gene by insertion within the open reading frame (57) or by replacement of the *SAN1* open reading frame also reverses all aspects of the *cdc68-1* mutant phenotype at the restrictive temperature of 35°C. Therefore, one role of the San1 protein is to inhibit Cdc68 protein function.

The Cdc68 gene product activates the cell cycle regulatory step START (48); *cdc68-1* mutant cells arrest predominantly at START after transfer to the restrictive temperature. Performance of START requires new synthesis of G_1 cyclin proteins (16, 39, 67). The failure of *cdc68* mutant cells to perform START is therefore not unexpected, because Cdc68 activity is essential for transcription of G_1 cyclin genes (52). Any mutation that suppresses the conditional growth defect of *cdc68* mutant cells may therefore restore at least some G_1 cyclin function, since G_1 cyclin production is rate limiting for START (16, 49). Our Northern analysis verified this prediction: decreased *SAN1* gene function (either by mutation or by removal of the *SAN1* gene) restored G_1 cyclin transcription and allowed the performance of START by *cdc68-1* mutant cells at the restrictive temperature of 35°C.

In addition to restoring G_1 cyclin gene transcription, the *san1* suppressor mutations reversed other transcriptional alterations caused by the *cdc68-1* mutation at 35°C, including the increased expression of the *suc2ΔUAS* allele and the decreased transcription of many other genes, including *ACT1*, *LEU2*, *cdc68*, and *SAN1* itself. The fact that loss of function of the San1 protein restores the Cdc68 transcription activation function suggests that San1 inhibits Cdc68 activity.

The dosage effects of San1 and mutant Cdc68 protein on cell growth (Table 5) provide further evidence that the San1 protein counteracts Cdc68 activity. For example, our initial genetic tests of dominance/recessiveness were carried out in diploid cells, and in this situation, most of the *san1* suppressor alleles were codominant: growth of diploid cells with the genotype *cdc68/cdc68 san1/SAN1* was better than that of *cdc68/cdc68 SAN1/SAN1* homozygous diploid cells (although not as good as that of wild-type diploids). However, in haploid *cdc68 san1* double-mutant cells, all of the codominant *san1* alleles were recessive to the wild-type *SAN1* gene on a low-copy-number centromeric plasmid. This difference between *cdc68 san1* haploid *SAN1* transformants and *cdc68/cdc68 san1/SAN1* diploid cells suggests that two copies of the mutationally impaired *cdc68* gene (in diploid cells) provide increased Cdc68 activity to counteract San1 inhibitory function (Table 5). Similarly, more copies of a mutant *cdc68* gene (on a high-copy-number plasmid) overcome San1 inhibition. These effects of increased *cdc68* gene dosage show that the *cdc68* mutations used here simply weaken the Cdc68 transcriptional activator function. Conversely, we also found that overexpression of the wild-type *SAN1* gene lowered the restrictive temperature for the *cdc68-1* mutation: *cdc68-1* mutant cells with increased San1 activity were temperature sensitive at 33°C, which is a permissive temperature for *cdc68-1* mutant cells with normal *SAN1* gene dosage. The antagonistic effects of San1 and Cdc68 proteins therefore show tight stoichiometry.

The effects of altered stoichiometry for the antagonistic San1 and Cdc68 proteins are most evident in *cdc68* mutant cells enfeebled for Cdc68 function. For *CDC68* wild-type cells, neither increased nor decreased *SAN1* gene dosage, or even complete loss of San1 function, caused any significant phenotypic alteration, and there was no substantial change in transcript abundance for genes activated by Cdc68. We conclude that San1 is a weak inhibitor.

The loss of San1 inhibition cannot bypass the requirement for Cdc68 function; this finding implies that San1 operates upstream of the Cdc68 protein. Two models for San1 function are consistent with this absolute requirement for Cdc68 function, as well as with the gene dosage effects seen in *cdc68* mutant cells. San1 and Cdc68 could function independently to repress (San1) and to activate (Cdc68) gene expression. In this model, the target of San1 would ultimately be a *cis*-acting negative regulatory element. Normally, the weak repressive effects of San1 would be overwhelmed by the strong activation function provided by the Cdc68 protein, even when more San1 repressor is available due to increased *SAN1* gene dosage. Only when the Cdc68 activator is enfeebled by mutation would repression by San1 protein be evident. The *SAN1* gene dosage effects would suggest weak binding by San1, which would be improved at higher San1 protein levels. In the second model, the primary inhibitory target of San1 activity would be the Cdc68 protein itself. If San1 covalently modifies Cdc68, this modification would significantly inhibit only mutant forms of the Cdc68 protein. As pointed out previously (57), San1 could play a role in the stability of mutant proteins so that the mutant Cdc68 protein, rendered unstable in a *SAN1* cell, becomes more abundant in the absence of San1 activity. Alternatively, San1 could exert its effects as part of a protein complex containing both San1 and Cdc68. This model is reminiscent of the direct interaction between the yeast Gal4 transcription activator and its inhibitor Gal80, which is affected by *GAL4* and *GAL80* gene dosage (18, 27, 41).

The San1 protein inhibits the expression of many genes that Cdc68 activates, including the *CDC68* gene itself. We show that expression of a *cdc68* mutant allele, encoding a mutant Cdc68 protein with enfeebled activity, is decreased in *SAN1 cdc68* mutant cells due to San1 inhibition. This interruption of the positive-feedback loop for Cdc68 synthesis (52) suggests that the widespread transcriptional effects of San1 could all stem from San1 inhibition only at the *CDC68* promoter, thereby curtailing the ongoing Cdc68 synthesis that may be needed for the activation of other genes. Implicit in this model is a short half-life for the Cdc68 protein, with continual resynthesis needed for gene activation. However, the inability of increased copies of the *CDC68* promoter to titrate out San1 inhibition argues against this model. Therefore, we favor an alternative model in which San1 works at all promoters activated by Cdc68, perhaps as part of a protein complex as discussed above.

It is noteworthy that after transferring *cdc68-1* mutant cells to the restrictive temperature, transcription of the *SAN1* gene is also decreased, suggesting that San1 inhibition of the mutant Cdc68 protein persists in the absence of continuing *SAN1* transcription. Of course, transcript abundance does not necessarily reflect protein levels: according to the N-end rule for ubiquitin-mediated protein degradation (reviewed in reference 64), San1 is likely to be a stable protein with a half-life of more than 20 h. At the restrictive temperature, the presumably stable San1 protein would most likely persist to antagonize the mutant Cdc68 activity and inhibit transcription.

There is increasing evidence that global transcription factors such as Cdc68 regulate transcription through chromatin structure. Both the Snf/Swi group of transcription

factors and the histone group of Spt proteins have been postulated to play a general role in transcription through the remodeling of chromatin structure (reviewed in reference 65). Two members of this histone group of *SPT* genes, *SPT11* and *SPT12*, encode histone H2A and H2B, while three other Spt proteins encoded by this group, Spt4, Spt5, and Spt6, are also implicated in chromatin structure (62). On the basis of Spt phenotype, the *CDC68* gene has been assigned to this histone group of *SPT* genes (32, 62). The widespread transcriptional effects of Cdc68 may therefore be mediated through chromatin structure. However, while other members of this histone group of Spt proteins have been proposed to maintain a repressed state of chromatin (65), the Cdc68 protein probably functions differently, to create a chromatin structure that favors transcription initiation, because Cdc68 function is required for the activation of many genes. Perhaps the C-terminal acidic region of the Cdc68 protein (52) interacts with basic histone proteins to facilitate the destabilization or removal of nucleosomes before transcription initiation.

The San1 protein has also been implicated in chromatin effects. In addition to inhibiting Cdc68 transcriptional activity, the San1 protein participates in the transcriptional repression of the *HML* and *HMR* loci (57), containing transcriptionally silent copies of the yeast mating-type genes. Several experiments have suggested that chromatin structure plays an important role in repression of *HML* and *HMR* (2, 24, 38, 45). The establishment and maintenance of the repressed state is brought about by four *SIR* gene products, including Sir4 (50), so the identification of *san1* mutant alleles by virtue of their *sir4* suppression argues that San1 plays a role (through modulating other protein function, including that of Cdc68) in chromatin structure. Thus, interactions of Cdc68 and San1 and of Sir4 and San1 may remodel chromatin structure.

Several other proteins have been implicated in widespread transcriptional regulation: Spt2/Sin1 (an HMG1-like protein [28]), members of the histone group of Spt proteins, including Spt4, Spt5, and Spt6 (8, 33, 61, 62, 65), and a global transcription regulator, Sin4 (23), have been postulated to regulate transcription by remodeling chromatin structure. Deletion of the *SAN1* gene or extra copies of *SAN1* had no effect on the ability of an *spt2*, *spt4*, *spt5*, or *spt6* mutation to suppress δ insertion mutations (the Spt$^-$ phenotype), and changes in San1 activity did not suppress a *sin4* null mutation (69). We conclude that San1 effects are mediated only through a limited number of proteins, including Cdc68.

Despite the general activation function for the Cdc68 protein, Cdc68 activity exerts negative effects on the δ insertion alleles at the *HIS4* and *LYS2* loci (32). This effect is likely indirect, as a consequence of histone gene expression. Altered histone gene expression has been shown to cause an Spt$^-$ phenotype, presumably through effects on chromatin structure (8). Indeed, we found that transcription of at least some of the histone genes was impaired by the *cdc68-1* mutations. This effect on histone gene expression may therefore be related to the Spt$^-$ phenotype conferred by *cdc68* mutation. In keeping with this model, the *san1* suppressor mutations restored histone gene transcription and also suppressed the Spt$^-$ phenotype imposed by *cdc68* mutations.

A decrease in histone gene expression is unlikely to account for the transcriptional alterations caused by the *cdc68-1* mutation at 35°C. After transfer of *cdc68* mutant cells to this restrictive temperature, there is a rapid decrease in transcript abundance for many genes, an effect too rapid to be explained by altered expression of stable proteins like histones. Moreover, histone synthesis normally occurs at the time of DNA replication, so that changes in the abundance of histones resulting from decreased histone gene transcription would only be seen after S phase, whereas the rapidity of the transcriptional effects argues that decreased transcription imposed by the *cdc68-1* mutation probably occurs in all cells and is therefore unlikely to be mediated by altered histone gene transcription. Furthermore, *cdc68* mutant cells show a rapid first-cycle inhibition of cell proliferation, suggesting that for many cells, the *cdc68-1* inhibition of G_1 cyclin gene transcription is imposed before another round of DNA replication. Therefore, Cdc68 protein has a more direct effect on transcriptional activation compared with any transcriptional effects that result from altered histone gene expression.

ACKNOWLEDGMENTS

We thank M. Carlson, E. A. Malone, M. Osley, J. Rine, D. Stillman, A. Wildeman, F. Winston, and C. Wittenberg for kindly supplying strains and/or plasmids. We also thank F. Winston and members of the Dalhousie yeast group for helpful discussions.

This work was supported by the Medical Research Council of Canada. Q.X. is the recipient of an Edward F. Crease Memorial Graduate Studentship in Cancer Research. G.C.J. is a Terry Fox Cancer Research Scientist of the National Cancer Institute of Canada, supported by funds from the Terry Fox run.

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