MOLECULAR AND CELLULAR BIOLOGY, Nov. 1991, p. 5718–5726 Vol. 11, No. 11
0270-7306/91/115718-09$02.00/0
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CDC68, a Yeast Gene That Affects Regulation of Cell Proliferation and Transcription, Encodes a Protein with a Highly Acidic Carboxyl Terminus

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Received 5 July 1991/Accepted 27 August 1991

The cell cycle of the budding yeast *Saccharomyces cerevisiae* has been investigated through the study of conditional *cdc* mutations that specifically affect cell cycle performance. Cells bearing the *cdc68-1* mutation (J. A. Prendergast, L. E. Murray, A. Rowley, D. R. Carruthers, R. A. Singer, and G. C. Johnston, Genetics 124:81–90, 1990) are temperature sensitive for the performance of the G_1 regulatory event, START. Here we describe the *CDC68* gene and present evidence that the *CDC68* gene product functions in transcription. *CDC68* encodes a 1,035-amino-acid protein with a highly acidic and serine-rich carboxyl terminus. The abundance of transcripts from several unrelated genes is decreased in *cdc68-1* mutant cells after transfer to the restrictive temperature, while at least one transcript, from the *HSP82* gene, persists in an aberrant fashion. Thus, the *cdc68-1* mutation has both positive and negative effects on gene expression. Our findings complement those of Malone et al. (E. A. Malone, C. D. Clark, A. Chiang, and F. Winston, Mol. Cell. Biol. 11:5710–5717, 1991), who have independently identified the *CDC68* gene (as *SPT16*) as a transcriptional suppressor of δ-insertion mutations. Among transcripts that rapidly become depleted in *cdc68-1* mutant cells are those of the G_1 cyclin genes *CLN1*, *CLN2*, and *CLN3/WHI1/DAF1*, whose activity has been previously shown to be required for the performance of START. The decreased abundance of cyclin transcripts in *cdc68-1* mutant cells, coupled with the suppression of *cdc68-1*-mediated START arrest by the *CLN2-1* hyperactive allele of *CLN2*, shows that the *CDC68* gene affects START through cyclin gene expression.

Cell cycle regulation in the budding yeast *Saccharomyces cerevisiae* is exerted primarily at a step in G_1 termed START (19, 46). Components of the START regulatory machinery have been identified, in large part, as a result of investigations that exploit particular conditional mutations that affect the cell division cycle. One collection of cell division cycle (*cdc*) mutations defines the *CDC28* gene that encodes the *S. cerevisiae* homolog of the $p34^{cdc2}$ protein kinase, which has been shown to regulate cell cycle progression in a wide variety of eukaryotes (reviewed in references 29 and 42).

In *S. cerevisiae* the performance of START depends on the periodic activation of the p34 kinase upon association with one or more members of a family of proteins known as G_1 cyclins (47, 66). In addition, the performance of START is responsive to the biosynthetic status of the cell and, in the case of haploid cells, to the presence of mating pheromones. In a previous study we employed a novel gradient selection procedure to identify additional genes that affect START (45). In particular, we sought to identify mutations that affect START without significant impairment of biosynthetic activity; such mutations should define additional genes that impinge upon the START machinery in a fairly direct manner. This genetic approach yielded conditional START mutations in three previously uncharacterized genes. Cells bearing one of these mutations, *cdc68-1*, are temperature sensitive for the performance of START; *cdc68-1* mutant cells proliferate normally at 23°C but arrest proliferation as unbudded cells within one cell cycle after transfer to the restrictive temperature of 37°C. Arrested *cdc68-1* mutant cells retain significant biosynthetic capacity and remain mating competent for several hours (45). Here we describe the molecular characterization of the *CDC68* gene and present molecular and genetic evidence that the *CDC68* gene product plays a general role in transcription.

The *CDC68* gene has been independently identified (as *SPT16*) by its ability to suppress, in multiple copy number, certain δ-element insertion mutations that alter transcription of the *HIS4* and *LYS2* genes (32). The identification of the *CDC68* gene by this approach is consistent with a role for the Cdc68 protein in transcription.

MATERIALS AND METHODS

Strains and media. Yeast strains used in this study are listed in Table 1. Yeast cells were grown in YNB liquid defined medium (26) or in the complex rich medium YEPD as described elsewhere (18). Cell concentrations were determined with an electronic particle counter (Coulter Electronics, Inc.). Cell morphology was assessed by microscopic examination of at least 200 cells per sample. Nuclear morphology was assessed by staining with 4′,6-diamidino-2-phenylindole (Sigma Chemical Co.).

Recombinant DNA manipulations. Yeast transformation was performed by the spheroplast method described by Hinnen et al. (23). Recombinant DNA manipulations were carried out essentially as described by Maniatis et al. (33). Plasmid pSC2-1 containing the *CDC68* gene has been previously described (45), as have vectors YEp24 (3) and YEp352 (22). Plasmid pUTX144, kindly provided by D. Finkelstein, is a pBR322-based plasmid containing the *S. cerevisiae* 2μm origin of replication, the *LEU2* gene to allow selection in *leu2* mutant cells, and sequence from −334 to +282 of the

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

Strain	Genotype or phenotype	Source[a]
21R	*MAT***a** *adel leu2-3,112 ura3-52*	J.E.H.
ART68-1[b]	*MAT***a** *cdc68-1 ura3-52 leu2-3,112 ade*	This study
68507A[b]	*MATα cdc68-1 ura3-52 ade*	This study
FY56	*MATα his4-912δ lys2-128δ ura3-52*	E.A.M.
L577	*MATα spt16-197 lys2-128δ his4-912δ ura3-52*	E.A.M.
ZWU90-H2[c]	*MAT***a** *ura3-52::[HSP90-lacZYA URA3] leu2-3,112 adel*	C.B.
FP90-68[d]	*cdc68-1 ura3-52::[HSP90-lacZYA URA3] leu2-3,112 ade*	This study
FP90[d]	*ura3-52::[HSP90-lacZYA URA3] leu2-3,112 ade*	This study
GCY24	*MAT***a** *trp1-1::[CLN2-1 TRP1] adel leu2-3,112 ura3-52*	C.W.
PLD82α	*MATα* Can[r] Ade[−] His[−] Trp[−] Ura[−] *HSP82::LEU2*	S.L.
CLD82α	MATα Can[r] Ade[−] His[−] Trp[−] Ura[−] *HSC82::LEU2*	S.L.

[a] J.E.H., J. E. Hopper; E.A.M., E. A. Malone; C.B., C. Barnes; C.W., C. Wittenberg; S.L., S. Lindquist.

[b] Congenic with strain 21R.

[c] Constructed by the directed integration of plasmid YIp144 into the *SmaI* site of the *ura3-52* locus in strain 21R. YIp144 is YIp5 (57) containing a 6.8-kbp *HindIII-SalI HSP82-lacZYA* fusion gene fragment in which the sequence from −334 to +282 of the *HSP82* gene (12) is fused to codon 8 of *lacZ*. The fusion gene fragment was obtained from plasmid PUTX144, kindly provided by D. Finkelstein.

[d] Segregant from a cross between strains 68507A and ZWU90-H2.

HSP82 gene (12) fused in frame to codon 8 of the *Escherichia coli lacZ* gene.

Northern (RNA) analysis. Total RNA was extracted from cells (2×10^6 to 4×10^6/ml) by the method of Penn et al. (44). Oligonucleotide probes were end labelled with T4 polynucleotide kinase and [τ-^{32}P]dATP as described by Sambrook et al. (51). Hybridization with restriction fragment probes was essentially as described by Thomas (61). Hybridization with oligonucleotide probes was performed as described by Wallace and Miyada (62). The following DNA restriction fragments were purified and labelled for use as probes. The *CLN1* and *CLN2* open reading frames were excised as 1.6-kbp *NdeI-BamHI* fragments from inserts in pRK171, kindly provided by C. Wittenberg. The *CLN3* probe was a 1.7-kbp *EcoRI-XhoI* restriction fragment from a plasmid containing the *CLN3/WHI1/DAF1* gene (a gift from F. Cross). The *ACT1* probe was a 1-kbp *HindIII-XhoI* restriction fragment from pRS208 (a gift from R. Storms). The *LEU2* probe was a 1.7-kbp *HpaI-AccI* fragment from YEp351 (provided by J. Hill). The *CDC68* probe was a 2.2-kbp *HpaI* fragment from the *CDC68* open reading frame, excised from pSC2-1. The *lacZ* probe was a 6.2-kbp *BamHI-SalI* fragment from pUTX144 and contained *lacZYA* sequences only. The *HSP82* probe was an oligonucleotide of sequence 5′-CAAGGCCATGATGTTCTACC-3′ complementary to nucleotides 2130 to 2149 of the *HSP82* coding sequence (12). The specificity of this probe was confirmed by hybridization to RNA extracted from strains PLD82α and CLD82α deleted for *HSP82* and *HSC82*, respectively (kindly provided by S. Lindquist).

Radiolabeling, protein extraction, and gel electrophoresis. Pulse-labelling of cells (2×10^6 to 4×10^6/ml) with [^{35}S]methionine (NEN) and sample preparation were as previously described (36). Proteins from cell lysates containing equal amounts of radiolabelled trichloroacetic acid-precipitable material were resolved by one-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (27).

DNA sequencing. Overlapping restriction fragments from the cloned *CDC68* gene were subcloned into M13 um20 or um21 (International Biotechnologies Inc.), and nested sets of deletions were generated by the method of Henikoff (20). DNA sequencing (52) was performed with Sequenase version 2.0 (United States Biochemicals) and [^{35}S]dATP (NEN); both strands were completely sequenced. DNA sequence data were analyzed by using the University of Wisconsin Genetics Computer Group sequence analysis software (9).

Nucleotide sequence accession number. The GenBank accession number of the *CDC68* nucleotide sequence is M73533.

RESULTS

CDC68 **nucleotide sequence.** The wild-type *CDC68* gene was cloned previously by complementation of the recessive *cdc68-1* temperature-sensitive mutation (45). To localize the *CDC68* gene we subcloned into the high-copy-number vector YEp352 a number of restriction fragments from the insert of plasmid pSC2-1, the smallest complementing clone obtained in our earlier studies (45). The nucleotide sequence of a complementing fragment (a 5.2-kbp *BamHI* fragment; Fig. 1A) revealed a 3,105-bp open reading frame which we conclude specifies the *CDC68* gene product (Fig. 1B). The *CDC68* nucleotide sequence predicts a 1,035-amino-acid protein of 118,556 molecular weight. The predicted protein is highly charged, with 31.2% charged residues (14.2% basic and 17.0% acidic). Moreover, distribution of charged residues is different in different areas of the protein; the amino-terminal end of the *CDC68* gene product, from amino acid residues 1 to 451, is only moderately charged (26% overall, 12.2% acidic, and 13.8% basic), whereas the following region, encompassing amino acids 451 to 810, contains considerably more charged residues (37.2%, 15.8% acidic, and 21.4% basic). Particularly striking is the density of acidic amino acids at the carboxyl terminus; from residues 957 to 1021, 36 of 65 amino acids (55%) are either aspartic acid or glutamic acid, with a complete absence of basic amino acids (Fig. 1B). Of the remaining 29 residues within this acidic region, 12 (18%) are serine and 1 is threonine. Contained within this region are several consensus recognition sites for casein kinase II phosphorylation (34, 67), raising the possibility that the *CDC68* gene product is phosphorylated by casein kinase II in vivo.

CDC68 **and** ***SPT16*** **are the same gene.** The *cdc68-1* mutation was previously mapped to chromosome VII, between *ADE5* and *KEX1* (45). Subsequent genetic mapping of a temperature-sensitive mutation in *SPT16*, a gene isolated as a high-copy-number suppressor of δ-element insertions in *his4-912δ* and *lys2-128δ*, suggested that this mutation, *spt16-197*, is an allele of the *CDC68* gene (32). Upon further analysis, *cdc68-1* and *spt16-197* temperature-sensitive mutations failed to complement when combined in a heterozygous diploid and displayed tight linkage in tetrad analysis when this diploid was subsequently sporulated (32). Furthermore, transformation of cells containing the *spt16-197* temperature-sensitive mutation (strain L577) with the *CDC68* subclones described above gave the same pattern of complementation as in *cdc68-1* mutant cells (Fig. 1A). These genetic and molecular criteria establish that the *cdc68-1* and *spt16-197* mutations define the same gene.

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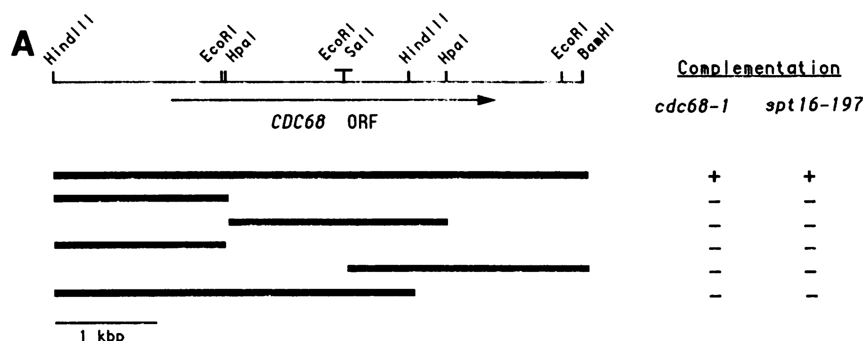

FIG. 1. Restriction map, complementation analysis, and nucleotide sequence of the *CDC68* gene. (A) The restriction map of the 5.2-kbp complementing insert of pSC2-1 (45) is shown at the top. The position of the open reading frame (ORF) determined by nucleotide sequencing is indicated below. Thick bars indicate restriction fragments cloned into the high-copy-number vector YEp352 and tested for complementation of the *cdc68-1* and *spt16-197* temperature-sensitive mutations. (B) The nucleotide sequence of the *CDC68* gene is numbered from the presumptive ATG initiation codon of the open reading frame. The derived amino acid sequence is shown below and is also numbered from the initiator methionine. The most acidic region is underlined. The predicted polypeptide sequence of the *CDC68* gene was compared with sequences in the GenPept (release number 64.3), NBRF protein (release number 26), and Swiss-Prot (release number 17) protein data bases by using the sequence comparison program FASTA (43) and with sequences contained in GenBank (release number 67) and EMBL (release number 25) translated by using TFASTA (43). No significant similarities were found. However, similar DNA sequences were identified. The 3′ end of the *CHC1* (clathrin heavy chain) gene (28) extends from beyond the end of the sequenced region towards the 3′ end of the *CDC68* gene (to +4727). Furthermore, the upstream region of *URA2* (from nucleotides −169 to −838 [65]) is 98.8% identical to the reverse complement of the *CDC68* sequence from nucleotides −196 to +480. This finding was unexpected, because *cdc68-1* maps to chromosome VII (45) whereas *ura2* mutations map to chromosome X (39). The *CDC68* and *URA2* sequences diverge at a *Sau*3A site (at position −196 in the *CDC68* sequence and −169 in *URA2* [56]), suggesting that *CDC68* and *URA2* fragments were ligated during construction of the *Sau*3A partial-digest library of yeast genomic DNA used to clone the *URA2* gene (55).

***cdc68-1* and *spt16-197* mutations confer the same mutant phenotype.** *S. cerevisiae* cells containing the *cdc68-1* mutation have been shown previously to be defective in performance of the cell cycle regulatory step, START (45). Cells containing the *spt16-197* mutation (strain L577), when transferred from the permissive temperature (23°C) to the restrictive temperature (37°C), were also found to arrest proliferation promptly, within one cell cycle, with 80 to 90% unbudded cells. (For *S. cerevisiae*, bud morphology provides an estimate of cell cycle position; cells within the G_1 interval are unbudded [19]). Thus, like *cdc68-1* mutant cells, cells harboring the *spt16-197* mutation are conditionally defective in the G_1 interval.

The *SPT16* gene was identified by its ability, in multiple copy number, to suppress the δ insertions in *his4-912δ* and *lys2-128δ* (32). However, the *his4-912δ* and *lys2-128δ* mutations can also be suppressed by the *spt16-197* temperature-sensitive mutation (32). We therefore assessed the ability of the *cdc68-1* mutation to suppress *his4-912δ* and *lys2-128δ*. Analysis of meiotic segregants from a cross between strains ART68-1 (*cdc68-1*) and FY56 (*his4-912δ lys2-128δ*) demonstrated that the *cdc68-1* mutation, like *spt16-197*, can suppress *his4-912δ* and *lys2-128δ*. Thus, two independently isolated mutations in the *CDC68/SPT16* gene exhibit the same cell cycle and δ-element suppression phenotypes.

Cyclin transcript abundance is decreased in *cdc68-1* mutant cells. Recent investigations (8, 40, 48, 66) have revealed the involvement of a family of proteins, termed G_1 cyclins, in the activation of the START machinery. Since cells harboring the *cdc68-1* mutation are defective in the performance of START (45), we determined whether *cdc68-1*-mediated arrest involved altered expression of any of the three G_1 cyclin genes (8, 17, 40, 48, 66). To address this question, transcript levels of the three G_1 cyclin genes, *CLN1*, *CLN2*, and *CLN3* (previously designated *WHI1* [4, 40, 58] and *DAF1* [7]), were examined by Northern analysis (Fig. 2A). After transfer of *cdc68-1* mutant cells to the restrictive temperature, the levels of all three *CLN* transcripts rapidly and permanently decreased (Fig. 2A). A transient decrease in *CLN2* transcript abundance in wild-type cells after transfer to 37°C is coincident with a previously described transient accumulation of cells within the G_1 interval (50, 53). The rapid decrease in *CLN* mRNA abundance in *cdc68-1* mutant cells may be responsible for the START arrest phenotype exhibited by mutant cells at the restrictive temperature. Indeed, decreased cyclin expression has been previously demonstrated to cause a first-cycle G_1 arrest (8, 48).

The *CLN2-1* mutation abrogates the G_1 arrest of *cdc68-1* mutant cells. To test the possibility that the START arrest caused by the *cdc68-1* mutation is a consequence of decreased *CLN* gene expression, we examined the effect of *CLN2-1*, a hyperactive or hyperstable allele of the *CLN2* gene (17). The *CLN2-1* allele encodes a truncated Cln2 protein that is thought to exhibit increased stability due to the deletion of carboxyl-terminal PEST sequences implicated in protein degradation (49). The phenotype of cells containing the *CLN2-1* mutation, including decreased cell size at bud emergence, a shortened G_1 interval, moderate α-factor resistance, and failure to arrest at START in response to starvation, indicates that cyclin protein in these mutant cells is no longer rate limiting for cell cycle progression.

To assess the effect of increased cyclin stability on the START arrest caused by the *cdc68-1* mutation, we constructed a diploid strain heterozygous for both the *CLN2-1* and *cdc68-1* mutations. Temperature sensitivity segregated 2:2 in meiotic segregants from this cross, indicating that the *CLN2-1* mutation did not suppress the temperature sensitivity imposed by the *cdc68-1* mutation. Both *cdc68-1* and *cdc68-1 CLN2-1* segregants exhibited an approximately twofold increase in cell concentration after transfer to the restrictive temperature (Fig. 3A). However, the cell cycle behavior of *cdc68-1 CLN2-1* double-mutant cells was altered. Whereas *cdc68-1* segregants arrested proliferation as

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B

-747 AGATCTGTCACCCAGACCTCCGTTGGA

-720 ATATACAACAGACATTCCCGCCGTGTTTACTGACCCGTCTGTCATGGAACGATACTATTATACCTTGGATCGTCAGCAAAGCAATACTGATACGTCATGGCTGACACCACCCCAATTACC

-600 TCCTCAGTTGGAGAATGTTATCTTGAATAAATACTATGCCACGCAAGACCAGTTCAACGAGAACAACTCAGGCGCTCTGCCCATTCCGAACCATGTGGTGCTTAACCACTTGGTTACTAG

-480 TAGCATTAAGCACAATACACTTTGTGTAGCCTCCATCGTTCGATATAAACAGAAATATGTCACCCAGATTCTTTATACGCCCATAGAGTCCTCGTAATCCGGGATTCCTACTTTTTAAAG

-360 GCGTATTGTATATATTTTTGGCAGCTATGGCACACTAATTTCTATTTCCAATTTAGTGTAAGCAGAGATATAGAAGCATATAACATAATGATTTACTAATGGGCTCAGTACTCTCAATGC

-240 TTATTGTTATCACAAACCACGATACTAATACCCGGAAGTCGCGTGATCTTTTTTTTTTTCGGAAAATGAGAATCGCCACTTTTCTAACGAAAGAATATTGAAATCTTAATATTGGTGAGA

-120 AGAGGAATAAAAGTAGCTAAGAGAGTCACGGGACTAAAAGGTAAAAAGTTCAGCTATTCCAACTTTACTCCTTTCTGATATTGCACCTTTCGTGAACTGGCTTCGTGTATTTAAGTGAAT

1 M E E L N I D F D V F K K R I E L L Y S K Y N E F E G S P N S L L F V L G S S N
1 ATGGAAGAGCTGAATATTGATTTTGACGTATTTAAGAAAAGGATTGAATTGTTGTATTCCAAGTACAATGAGTTTGAAGGTTCTCCAAATTCGCTGCTGTTTGTTTTAGGTTCGTCCAAC

41 A E N P Y Q K T T I L H N W L L S Y E F P A T L I A L V P G K V I I I T S S A K
121 GCTGAAAACCCGTACCAGAAGACGACCATATTGCATAATTGGTTGCTAAGTTACGAATTTCCCGCTACTTTGATTGCATTAGTTCCCGGAAAGGTTATTATAATTACTAGTTCTGCCAAG

81 A K H L Q K A I D L F K D P E S K I T L E L W Q R N N K E P E L N K K L F D D V
241 GCCAAGCATTTACAAAAGGCAATTGATCTATTTAAAGACCCTGAAAGCAAAATTACGCTGGAATTGTGGCAAAGAAACAATAAAGAACCAGAACTCAATAAAAAGTTATTTGATGACGTT

121 I A L I N S A G K T V G I P E K D S Y Q G K F M T E W N P V W E A A V K E N E F
360 ATTGCCTTAATCAATAGCGCTGGTAAAACAGTGGGTATCCCTGAAAAGGACTCTTACCAAGGTAAATTTATGACTGAGTGGAACCCAGTATGGGAAGCGGCTGTGAAGGAGAACGAATTC

160 N V I D I S L G L S K V W E V K D V N E Q A F L S V S S K G S D K F M D L L S N
481 AATGTCATTGATATTTCGCTGGGTCTTTCTAAAGTTTGGGAAGTGAAGGATGTTAACGAACAAGCCTTCCTGTCCGTTTCAAGTAAGGGGTCGGACAAATTTATGGACCTTTTATCCAAT

201 E M V R A V D E E L K I T N A K L S D K I E N K I D D V K F L K Q L S P D L S A
601 GAAATGGTTCGGGCAGTTGACGAGGAGTTGAAAATCACCAATGCCAAGTTATCAGACAAAATTGAAAATAAAATTGATGATGTGAAATTTTTGAAGCAATTGAGTCCCGATTTAAGCGCA

241 L C P P N Y K F N F D L L D W T Y S P I I Q S G K K F D L R V S A R S T N D Q L
721 TTATGCCCACCAAACTACAAATTCAACTTTGATTTATTGGATTGGACCTATTCTCCAATCATTCAGTCCGGGAAAAAGTTTGATCTTAGGGTTTCTGCCCGTTCTACCAATGATCAGTTG

281 Y G N G C I L A S C G I R Y N N Y C S N I T R T F L I D P S E E M A N N Y D F L
841 TACGGCAACGGTTGTATTTTAGCTTCATGTGGTATTCGTTACAATAATTATTGTTCTAATATTACTAGGACCTTTTTGATCGATCCATCTGAAGAAATGGCCAACAACTACGATTTTCTA

321 L T L Q K E I V T N I L K P G R T P K E V Y E S V I E Y I E K T K P E L V P N F
960 TTAACTTTGCAAAAAGAAATTGTAACTAACATTCTGAAGCCCGGCCGTACACCAAAAGAAGTTTATGAATCAGTAATAGAATACATCGAAAAAACCAAACCTGAACTAGTGCCCAATTTC

361 T K N I G S L I G L E F R D S N F I L N V K N D Y R K I Q R G D C F N I S F G F
1081 ACCAAAAACATTGGTTCATTGATTGGTCTCGAATTTAGAGATTCTAACTTTATATTAAATGTTAAGAATGATTACCGTAAAATCCAACGCGGTGACTGCTTTAATATTTCGTTTGGTTTC

401 N N L K D S Q S A N N Y A L Q L A D T V Q I P L D E T E P P R F L T N Y T K A K
1201 AATAATCTGAAAGATTCTCAAAGTGCTAACAACTACGCTTTACAATTAGCTGATACGGTTCAAATTCCTCTCGATGAAACAGAGCCTCCACGCTTCTTAACAAATTACACCAAAGCCAAA

441 S Q I S F Y F N N E E E D N N K K K S S P A T K V P S K P D R N S K I L R T K L
1321 TCCCAGATATCATTTTATTTCAATAATGAAGAAGAAGACAATAATAAAAAGAAATCTTCACCTGCCACCAAAGTCCCTAGTAAACCAGACAGAAATTCCAAAATTTTAAGAACAAAGTTA

481 R G E A R G G A E D A Q K E Q I R K E N Q K K L H E K L E K N G L L R F S A A D
1441 CGTGGCGAAGCCCGTGGTGGTGCCGAAGACGCTCAAAAGGAGCAAATTCGTAAAGAAAACCAAAAGAAGCTACACGAAAAATTGGAAAAGAATGGATTACTCAGATTTAGTGCTGCTGAT

521 A N G P D S E P R Q Y F K K Y E S Y V R D S Q L P T N I R D L R I H V D W K S Q
1561 GCCAATGGCCCAGATAGTGAACCTCGTCAATACTTCAAAAAATATGAATCTTATGTTCGGGATTCACAACTACCAACCAATATTCGTGATCTAAGAATTCATGTCGACTGGAAAAGCCAA

561 T I I L P I Y G R P V P F H I N S Y K N G S K N E E G E Y T Y L R L N F N S P G
1681 ACAATTATTCTACCCATTTACGGTAGGCCAGTCCCATTTCATATAAATTCATATAAGAATGGTTCTAAAAACGAAGAAGGCGAATATACGTATTTACGTTTGAACTTTAATTCACCGGGA

601 S S G G I S K K V E E L P Y E E S A D N Q F V R S I T L R S K D G D R M S E T F
1801 TCTTCTGGTGGTATTTCTAAAAAAGTGGAGGAATTGCCGTATGAGGAATCAGCAGACAATCAATTTGTACGTTCGATTACACTAAGATCCAAAGATGGTGACCGCATGAGTGAAACCTTT

641 K Q I A D L K K E A T K R E Q E R K A L A D V V Q Q D K L I E N K T G R T K R L
1921 AAACAAATTGCAGATTTGAAAAAAGAAGCCACAAAGAGAGAGCAAGAACGTAAGGCGCTTGCTGATGTTGTTCAACAGGACAAATTGATTGAAAATAAAACTGGAAGAACGAAAAGACTG

681 D Q I F V R P N P D T K R V P S T V F I H E N G I R F Q S P L R T D S R I D I L
2041 GATCAAATTTTTGTGAGGCCAAATCCAGATACAAAACGTGTACCTAGTACTGTCTTTATCCATGAAAATGGTATCAGATTTCAATCCCCACTAAGGACTGATAGCAGAATAGACATATTA

721 F S N I K N L I F Q S C K G E L I V V I H I H L K N P I L M G K K K I Q D V Q F
2161 TTCTCAAACATTAAGAATCTAATTTTTCAATCTTGTAAAGGTGAATTAATTGTCGTCATTCATATTCATTTGAAGAATCCAATTTTAATGGGTAAAAAGAAAATACAAGACGTCCAATTC

761 Y R E A S D M S V D E T G G G R R G Q S R F R R Y G D E D E L E Q E Q E E R R K
2281 TATCGTGAAGCTTCTGATATGTCCGTCGATGAAACTGGAGGTGGAAGACGAGGTCAATCCAGATTCAGAAGATATGGTGATGAGGATGAGCTGGAACAAGAACAAGAAGAAAGAAGAAAA

801 R A A L D K E F K Y F A D A I A E A S N G L L T V E N T F R D L G F Q G V P N R
2401 CGAGCTGCGCTTGATAAAGAATTTAAGTATTTTGCAGACGCAATCGCAGAAGCATCAAACGGTTTATTGACGGTGGAGAATACATTTAGAGATTTGGGCTTCCAAGGTGTCCCAAATAGA

841 S A V F C M P T T D C L V Q L I E P P F L V I N L E E V E I C I L E R V Q F G L
2521 TCGGCAGTTTTCTGTATGCCAACTACAGATTGTTTAGTTCAATTGATTGAACCACCATTTTTGGTGATTAACCTAGAGGAAGTCGAAATCTGTATTTTAGAAAGAGTTCAATTTGGTTTG

881 K N F D M V F V Y K D F N K P V T H I N T V P I E S L D F L K Q W L T D M D I P
2641 AAAAACTTCGACATGGTTTTTGTTTATAAAGATTTCAATAAACCCGTCACTCATATCAATACAGTTCCGATCGAATCTTTAGATTTCTTGAAGCAGTGGTTAACAGATATGGATATTCCT

921 Y T V S T I N L N W A T I M K S L Q D D P Y Q F F L D G G W N F L A T G S D D E
2761 TACACTGTCTCAACAATCAATTTGAATTGGGCAACTATTATGAAGTCATTACAAGATGATCCATATCAGTTTTTCTTAGATGGTGGTTGGAATTTTCTGGCTACTGGTTCAGATGATGAA

961 A S D E S E E E V S E Y E A S E D D V S D E S A F S E D E E G S E V D D D I S G
2881 GCATCTGATGAAAGTGAAGAAGAAGTTAGTGAATATGAGGCTTCAGAAGACGACGTAAGTGATGAAAGCGCATTTTCTGAAGATGAAGAAGGATCAGAGGTGGATGACGATATTAGTGGT

1001 D E S E D Y T G D E S E E G E D W D E L E K K A A R A D R G A M F R D *
3001 GATGAAAGTGAGGACTATACTGGCGACGAGAGTGAGGAAGGTGAAGACTGGGATGAATTAGAGAAAAAGGCTGCTAGGGCTGATAGGGGTGCAATGTTTAGAGATTAGACTTTATAACAC

3121 TTACTGGGTTTCACCAGCAAGACCTTGATCTGACAGAAAATTACTCTTATGTATCTGAAAAATAATGATGGTATTTAGGCATAGTATTTATCAATGCTAAGAAATGTAAATGGACTGAAT

3241 GTACACTCAATGCTAACGGTCAAGGTCATAGGACGTTTGCGTAATAAAAGAAACTCTACTAGAGTTGAACATAATTTGATATAAAAGTATATAAATTGAAAAGGCAGTTTCAATCATTTT

3361 AGTTTCTGCAATTTATTTATTCCTGTAATCTACAATTTTATAGTTGGGAAATTCTCTCGAAAATATTAAGAAAAATACAGAACTCATAAGGTCTTTTTTATTGGCATTTTGAAATCCCCT

3481 CAGATAATCAAAGATGCTAACAAACAAAATAATAACGGTAAAAAAAAAAAAAATACACGATGGGGTACAGCAAACGAATTATTTTATCCACGTCTTAAAATCCTGTGGGTTGAACGTTCA

3601 TCGCGCTGTTCATCAGCATCAAAGGTTGGCCATCTTTGTGCTCTTCATTAGATCCGGACTTTTTAGCGAGTTCTTCGGTGATTTTCTTAATGGAATCATTCTGCTCTTTTTTTATGGAGA

3721 TTTCAAATGGCTTAATGTAGTCCTCCAAAGAATTC 3755

FIG. 1—*Continued.*

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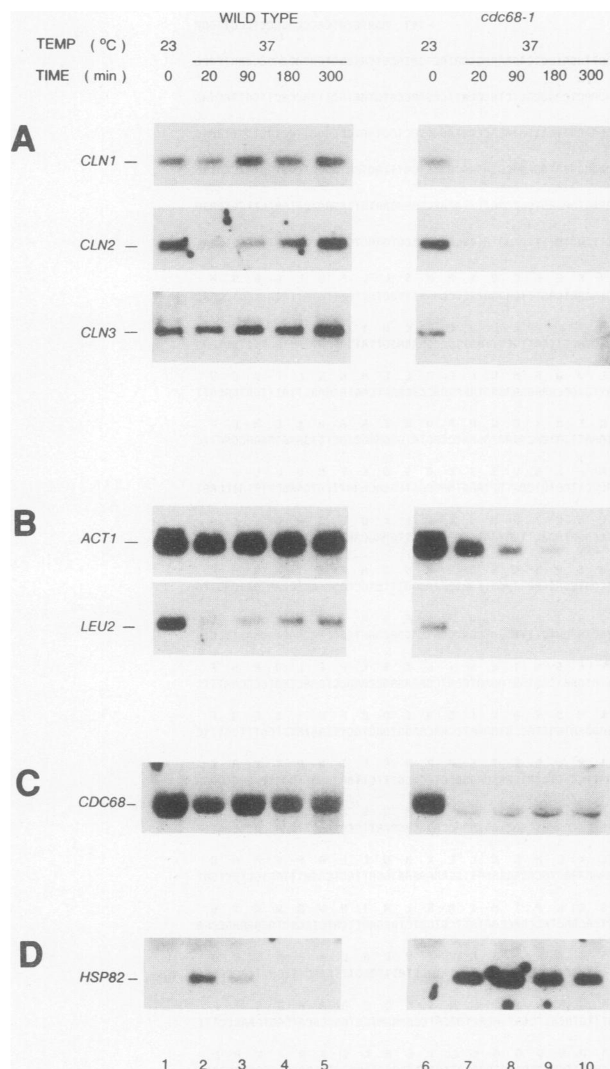

FIG. 2. Northern analysis of transcript abundance in wild-type and *cdc68-1* mutant cells. Total RNA was extracted from strain FP90 (*CDC68*) (panels A to C, lanes 1 to 5) or FP90-68 (*cdc68-1*) (panels A to C, lanes 6 to 10) grown at 23°C (lanes 1 and 6) or for the indicated times after transfer to 37°C (lanes 2 to 5 and 7 to 10). Probes were purified coding-sequence restriction fragments (see Methods and Materials). (D) Total RNA was extracted from strain 21R (*CDC68*) (lanes 1 to 5) or ART68-1 (*cdc68-1*) (lanes 6 to 10) grown at 23°C (lanes 1 and 6) or for 20 min (lanes 2 and 7), 90 min (lanes 3 and 8), 200 min (lanes 4 and 9), or 300 min (lanes 5 and 10) after transfer to 37°C. A synthetic oligonucleotide *HSP82* probe was used (see Methods and Materials).

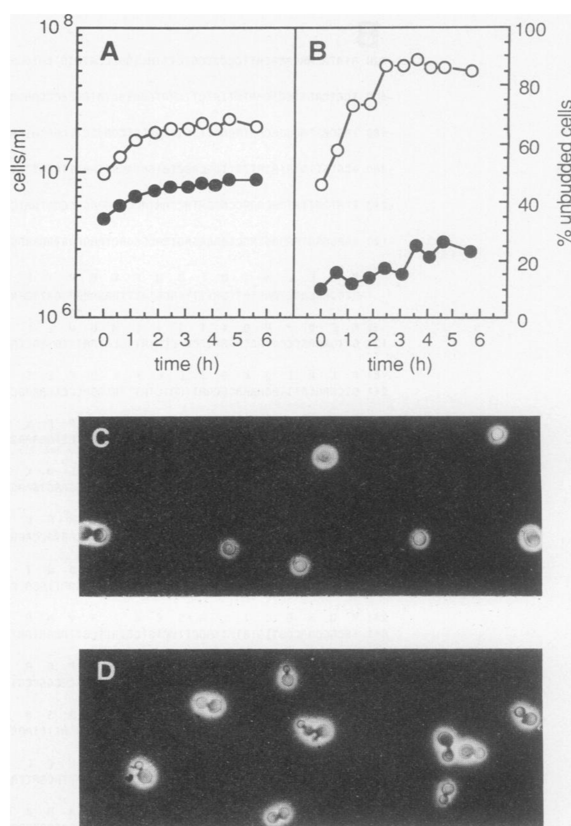

FIG. 3. Suppression of *cdc68-1*-mediated START arrest by the *CLN2-1* mutation. Actively dividing cells harboring either the *cdc68-1* mutation alone (open circles) or both *cdc68-1* and *CLN2-1* mutations (closed circles) were transferred from 23 to 37°C. Cultures were monitored for cell concentration (A) and percentage of unbudded cells (B) as a measure of cell cycle position. *cdc68-1 CLN2* (C) and *cdc68-1 CLN2-1* (D) mutant cells were photographed after 3 h of incubation at the restrictive temperature. Results shown are typical of all of the tested segregants from a cross between strains GCY24 and 68507A.

unbudded cells unable to initiate a subsequent cell cycle, *cdc68-1 CLN2-1* double-mutant segregants arrested proliferation primarily as budded cells that had initiated but failed to complete an additional cell cycle (Fig. 3B and C). The *cdc68-1 CLN2-1* double-mutant cells arrested proliferation with nuclear morphologies typical of all stages of the cell cycle (data not shown). Thus, *cdc68-1 CLN2-1* double-mutant cells perform START but do not complete the new cell cycle. The ability of the *CLN2-1* mutation to suppress the START arrest of *cdc68-1* mutant cells, combined with the *cdc68-1*-mediated decreases in *CLN* transcript levels, led us to conclude that START arrest of *cdc68-1* mutant cells at the restrictive temperature is a consequence of cyclin limitation.

Abundance of other transcripts is decreased in *cdc68-1* mutant cells. To further define the effects of the *cdc68-1* mutation on gene expression, we examined the levels of other transcripts in *cdc68-1* mutant cells. Transcripts from the *ACT1* and *LEU2* genes, like those from the three G_1 cyclin genes, decreased in abundance after transfer of *cdc68-1* mutant cells to the restrictive temperature (Fig. 2B). Even at the permissive temperature the *LEU2* transcript was present at lower levels in *cdc68-1* mutant cells (Fig. 2B).

The identification of *CDC68* as an *SPT* gene suggests that the decreased transcript levels in *cdc68-1* mutant cells are brought about by decreased transcription. The rapidity of change in *CLN* and *LEU2* transcript abundance after transfer of *cdc68-1* mutant cells to 37°C suggests that functional Cdc68 protein is rapidly depleted under these restrictive conditions. Even the more gradual effect on *ACT1* transcript levels is consistent with an inhibition of transcription, since *ACT1* mRNA has a relatively long half-life (approximately 30 min [21]) and is therefore expected to persist in the absence of further transcription.

***CDC68* transcript abundance is decreased in *cdc68-1* mutant**

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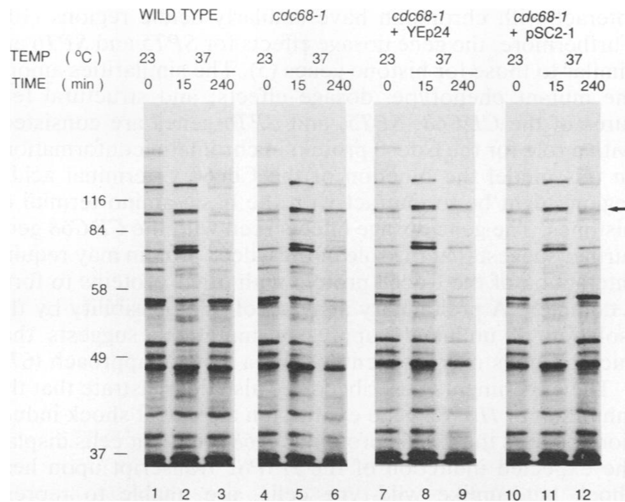

FIG. 4. Proteins synthesized in wild-type and *cdc68-1* mutant cells. Actively proliferating cells of strains 21R (*CDC68*) (lanes 1 to 3), ART68-1 (*cdc68-1*) (lanes 4 to 6), ART68-1 transformed with YEp24 (lanes 7 to 9), and ART68-1 transformed with pSC2-1 (containing a wild-type *CDC68* gene) (lanes 10 to 12) were pulse-labelled with [^{35}S]methionine during growth at 23°C (lanes 1, 4, 7, and 10) or for the indicated times after transfer to 37°C. Equal amounts of radiolabelled trichloroacetic acid-precipitable material were resolved by one-dimensional SDS-PAGE. Migration of standards is indicated on the left. The arrow identifies Hsp82.

cells. A 3.2-kb transcript was detected in both wild-type and *cdc68-1* mutant cells grown at 23°C by using a restriction fragment internal to the *CDC68* open reading frame as a probe (Fig. 2C, lanes 1 and 6). This transcript size is consistent with the 3,105-bp *CDC68* open reading frame shown in Fig. 1. Unexpectedly, the abundance of the 3.2-kb transcript decreased to barely detectable levels in cells containing the *cdc68-1* mutation within 20 min of transfer to 37°C (Fig. 2C, lanes 6 to 10). This finding suggests that functional Cdc68 protein is required for transcription of the *CDC68* gene itself.

Mutations in the *CDC68* gene cause abnormal synthesis of heat shock proteins. Previous studies have demonstrated that significant levels of protein and RNA synthesis are maintained in *cdc68-1* mutant cells at the restrictive temperature (45), suggesting that the *cdc68-1* mutation is not deleterious to the expression of all genes. Therefore, we compared the patterns of proteins synthesized in wild-type and *cdc68* mutant cells to identify other effects of *cdc68* mutations. Total cellular proteins were extracted and resolved by one-dimensional SDS-PAGE following brief exposure of cells to [^{35}S]methionine. The use of a short labelling period (10 min) to label proteins argues that any differences seen between wild-type and mutant cells reflect altered protein synthesis rather than altered protein stability. The pattern of proteins synthesized in wild-type cells before and after transfer to 37°C is shown in Fig. 4 (lanes 1 to 3). As expected, wild-type cells responded to increased temperature by transiently inducing the synthesis of heat shock proteins (reviewed in reference 30) while decreasing the synthesis of others. The pattern of newly synthesized proteins extracted either from *cdc68-1* (Fig. 4, lanes 4 to 6) or from *spt16-197* (data not shown) mutant cells showed a number of differences: the synthesis of many proteins decreased after transfer to 37°C but, in contrast to the situation in wild-type cells, remained at a low level even after 240 min at the elevated temperature (compare lanes 1 to 3 with lanes 4 to 6). Conversely, at least one protein whose synthesis was transiently increased after transfer to 37°C continued to be synthesized at an elevated level in both *cdc68-1* and *spt16-197* mutant cells even 240 min after transfer to the restrictive temperature (Fig. 4 and data not shown). We tentatively identified this protein as Hsp82, the product of the *HSP82* gene (2, 12, 14), on the basis of its apparent molecular mass (approximately 90 kDa) and induction upon heat shock. A virtually wild-type pattern of labelled proteins was restored by the introduction of a wild-type *CDC68* gene (45) on the 2μm-based vector YEp24 (Fig. 4, lanes 10 to 12) but not by YEp24 itself (Fig. 4, lanes 7 to 9). These results suggest that *CDC68* gene function is required for the appropriate synthesis of many proteins following transfer to 37°C, proteins whose synthesis is either induced (like Hsp82) or decreased in response to the stress of heat shock.

The *HSP82* gene is expressed at low basal levels at 23°C but is rapidly and transiently induced upon transfer of cells from 23 to 37°C. This induction has been shown to be mediated primarily at the level of transcription (16, 35). The effect on expression of what we tentatively identified as Hsp82 represents a further aspect of the *cdc68-1* mutant phenotype: persistent high-level expression of at least one heat shock gene. To pursue this observation, we first confirmed that gene expression under the regulation of *HSP82* sequences is altered in *cdc68-1* mutant cells. For this purpose we transformed cells with a plasmid-borne fusion gene in which *HSP82* sequences are fused to codon 8 of the *lacZ* gene from *E. coli*. Previous studies have demonstrated that expression of *lacZ* when fused to *HSP82* sequences accurately reflects regulation of the endogenous *HSP82* gene (15).

The pattern of newly synthesized proteins in transformants showed that the fusion gene in wild-type and *cdc68-1* mutant cells directs the synthesis of a new 116-kDa polypeptide in a manner which parallels that of the 90-kDa polypeptide in Fig. 4 (data not shown). These data supported the identification of the 90-kDa polypeptide as the product of the *HSP82* gene.

Abundance of *HSP82* transcripts is increased in *cdc68-1* mutant cells. To examine more directly the *cdc68-1*-mediated effects on *HSP82* gene expression, we determined the abundance of *HSP82* mRNA in both wild-type and *cdc68-1* mutant cells transferred from 23 to 37°C. For this determination we had to take into consideration that in addition to *HSP82*, *S. cerevisiae* contains a second closely related gene, *HSC82*. *HSP82* and *HSC82* are regulated differently: *HSP82* is expressed at a low level at 23°C but is induced upon heat shock, whereas *HSC82* exhibits higher basal expression and is induced to a lesser extent upon heat shock (2). To distinguish between the *HSP82* and *HSC82* transcripts, an *HSP82*-specific oligonucleotide probe was used; the specificity of this probe was confirmed by Northern hybridization to RNA extracted from strains harboring disrupted copies of each member of this two-gene family (for details, see Methods and Materials). Transcripts of approximately 2.4 kb were detected by this probe in RNA extracted from both wild-type and mutant cells (Fig. 2D), consistent with the size of the *HSP82* open reading frame (12). *HSP82* mRNA levels increased, as expected, in both wild-type and *cdc68-1* mutant cells in response to the imposition of heat shock conditions. However, in *cdc68-1* mutant cells, *HSP82* mRNA abundance remained elevated compared with that in wild-type cells for as long as 5 h after transfer to the

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restrictive temperature (Fig. 2D, compare lanes 1 to 5 with lanes 6 to 10). Similar results were obtained when the abundance of transcripts from the *HSP82-lacZ* fusion gene described above, in this case integrated in single copy at the *ura3-52* locus, was determined by Northern blot analysis (data not shown).

The continued presence of significant levels of *HSP82* transcript in *cdc68-1* mutant cells parallels the continued synthesis of the 90-kDa protein in Fig. 4. The persistent synthesis of this protein probably reflects persistent transcription of the *HSP82* gene, since we have shown that transcripts from the endogenous *HSP82* gene and both transcripts and protein from a heterologous fusion gene under the control of the *HSP82* promoter are all present at elevated levels at this time.

DISCUSSION

The studies described here extend our previous work in which we identified a new temperature-sensitive START mutation, *cdc68-1*, and cloned the wild-type *CDC68* gene (45). Both genetic and molecular evidence suggest that the product of the *CDC68* gene plays a general role in transcription. The Cdc68 protein is required for continued transcription of a variety of otherwise unrelated genes, including the genes encoding three yeast G_1 cyclins, and is required to repress *HSP82* expression after induction upon heat shock. Thus, the *CDC68* gene product has both positive and negative effects on gene expression.

Ty and solo δ-insertion mutations have provided an effective selection scheme for the identification of a variety of *trans*-acting proteins, many involved directly in the regulation of transcription (5, 11, 13, 63, 64). The independent identification of the *CDC68* (*SPT16*) gene by this suppressor approach supports the argument that the altered transcript levels that we have observed in *cdc68-1* mutant cells are the result of transcriptional changes. Indeed, in the accompanying report Malone et al. (32) demonstrate that a temperature-sensitive *CDC68* allele, *spt16-197*, alters transcription initiation at the *lys2-128δ* locus. Further support for a transcriptional role for the Cdc68 protein comes from the finding that mutation of the *CDC68/SPT16* gene allows derepression of the glucose-repressible gene *SUC2* in the absence of upstream regulatory sequences and at least partially suppresses a null mutation in *SNF2*, a gene required for the expression of several diversely regulated genes, including *SUC2* (32).

The predicted amino acid sequence of the Cdc68 protein contains an extremely acidic carboxyl-terminal region which is also rich in serine residues. Acidic regions necessary for transcriptional activation have been identified in a number of transcriptional activators, including *GCN4* (24) and *GAL4* (31). The Cdc68 protein may function as an acidic activator for transcription, a role that may be facilitated by an as-yet-unidentified DNA-binding domain within the Cdc68 protein or by association of the Cdc68 protein with other sequence-specific DNA-binding factors.

Malone et al. (32) have demonstrated that both overexpression (by increased copy number) and mutation of the *CDC68/SPT16* gene result in suppression of δ-element insertion mutations. In this respect the *CDC68/SPT16* gene is similar to the *SPT5* (60) and *SPT6/SSN20/CRE2* genes (6, 59). Both *SPT5* and *SPT6* encode essential nuclear proteins with extremely acidic N termini and have been suggested to influence gene expression through a function in chromatin assembly or modification. Indeed, other proteins thought to interact with chromatin have similarly acidic regions (10). Furthermore, the gene dosage effects for *SPT5* and *SPT6* are similar to those for histone genes (5). The similarities among the mutant phenotype, dosage effects, and structural features of the *CDC68*, *SPT5*, and *SPT6* genes are consistent with a role for the Cdc68 protein in chromatin conformation. In this model the function of the Cdc68 C-terminal acidic region might be to interact with the basic amino termini of histones. The gene dosage effects seen with the *CDC68* gene further suggest that the role of the Cdc68 protein may require interaction of the Cdc68 protein with other proteins to form a complex. A preliminary analysis of this possibility by the isolation of unlinked suppressor mutations suggests that such proteins may be identified by a genetic approach (67).

The experiments described here also demonstrate that the inhibition of *HSP82* gene expression after heat shock induction requires the Cdc68 protein; *cdc68-1* mutant cells display the expected induction of the *HSP82* transcript upon heat shock but, unlike wild-type cells, are unable to repress *HSP82* gene expression and, as a result, exhibit prolonged *HSP82* expression after heat shock. This prolonged expression is not simply a result of cell cycle blockage at START; other START mutations do not show this phenotype (1). The prolonged *HSP82* expression therefore results from *cdc68* effects at the *HSP82* locus. Transcriptional regulation of *HSP82* is complex. Recent experiments by McDaniel et al. (37) have demonstrated that the TATA-proximal heat shock element is required for basal expression of the *HSP82* gene, implying that heat shock transcription factor is involved in basal-level expression of *HSP82* as well as induction by heat shock. Recent analyses (25, 41, 54) of the heat shock transcription factor have identified physically separable activation domains that are regulated by conformational changes and perhaps also by interactions with other proteins. The Cdc68 protein may be one such protein that affects heat shock transcription factor activity.

An additional transcript that exhibited decreased abundance in *cdc68-1* mutant cells transferred to the restrictive temperature was the *CDC68* transcript itself. This result suggests that transcription of the *CDC68* gene requires its own gene product, the Cdc68 protein. Although unusual, this situation is unlikely to be unique, since other genes encoding components of the basic transcription machinery such as RNA polymerase II subunits and transcription initiation factors are thought to be transcribed by complexes of proteins which must include their own gene products.

In addition to effects on transcript abundance, mutations in the *CDC68* gene affect proliferation and bring about a regulated arrest at the cell cycle regulatory step, START. Recent investigations have revealed that the performance of START involves periodic activation of a protein kinase referred to as $p34^{cdc28}$ (38, 65). In turn, activation of the $p34^{cdc28}$ protein kinase requires periodic accumulation of G_1 cyclin proteins, the products of three genes, *CLN1*, *CLN2*, and *CLN3* (47, 66). Use of heterologous promoters to prevent cyclin gene expression leads to a rapid, first-cycle arrest of proliferation with cells blocked at the START event (8, 48). Conversely, the introduction of hyperactive or hyperstable alleles of *CLN2* or *CLN3* prevents cell cycle arrest at START (7, 17, 40). The physiological state of cells arrested at START as a result of cyclin depletion resembles the phenotype of arrested *cdc68-1* mutant cells; both arrest conditions allow continued cell growth and mating competency (7, 45). Under these arrest conditions, *cdc68-1* mutant cells contain dramatically decreased levels of all three *CLN* transcripts.

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The findings described above provide a mechanism through which effects on transcription can result in START arrest. Under nonpermissive conditions, the short half-lives of the cyclin proteins (8, 48, 66), coupled to an immediate *cdc68-1*-mediated decrease in *CLN* transcript levels, preclude accumulation of sufficient cyclin proteins to activate START. Such cells thus become incapable of initiating a new cell cycle. We conclude that the Cdc68 protein is required for continued cyclin gene expression and that in the absence of an active Cdc68 protein, cyclin levels are insufficient to activate START. The mechanism of *cdc68-1*-mediated inhibition of cyclin gene expression is not yet established. However, an inappropriate activation of the mating-response pathway is unlikely, because the effects on cyclin transcript levels in *cdc68-1* mutant cells do not resemble the pattern of transcript abundance observed after α-factor treatment. Upon activation of the mating-response pathway by α-factor, only *CLN1* and *CLN2* transcript levels decrease (66) and those of the *CLN3* gene actually exhibit a moderate increase (40).

The *CLN2-1* hyperactive allele alleviates *cdc68-1*-mediated START arrest, presumably by providing a more stable Cln2 protein. However, double-mutant cells harboring both the *cdc68-1* and *CLN2-1* mutations remain temperature sensitive for cell proliferation. Assessment of nuclear morphology showed that the cell cycle does not become blocked at any particular position. Therefore, the continued temperature sensitivity of *cdc68-1 CLN2-1* double-mutant cells reflects additional effects of the *cdc68-1* mutation on gene expression, but not on that of genes whose expression is rate limiting in the post-START cell cycle.

ACKNOWLEDGMENTS

We thank E. A. Malone and F. Winston for generous communication of unpublished data, helpful discussions, and sharing of materials. We also thank C. Wittenberg, F. Cross, B. Futcher, S. Lindquist, D. Finkelstein, R. Storms, and J. Hill for kindly supplying us with plasmids and strains. We thank C. Barnes for the initial observation that *cdc68-1* mutant cells exhibit an altered heat shock response.

This work was supported by the Medical Research Council of Canada. A.R. was the recipient of an MRC studentship and an Izaak Walton Killam Memorial Scholarship. G.C.J. is a senior research scientist of the National Cancer Institute of Canada.

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