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The *Saccharomyces cerevisiae* DNA Polymerase α Catalytic Subunit Interacts with Cdc68/Spt16 and with Pob3, a Protein Similar to an HMG1-Like Protein

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We have used DNA polymerase α affinity chromatography to identify factors involved in eukaryotic DNA replication in the yeast *Saccharomyces cerevisiae*. Two proteins that bound to the catalytic subunit of DNA polymerase α (Pol1 protein) are encoded by the essential genes *CDC68*/*SPT16* and *POB3*. The binding of both proteins was enhanced when extracts lacking a previously characterized polymerase binding protein, Ctf4, were used. This finding suggests that Cdc68 and Pob3 may compete with Ctf4 for binding to Pol1. Pol1 and Pob3 were coimmunoprecipitated from whole-cell extracts with antiserum directed against Cdc68, and Pol1 was immunoprecipitated from whole-cell extracts with antiserum directed against the amino terminus of Pob3, suggesting that these proteins may form a complex in vivo. *CDC68* also interacted genetically with *POL1* and *CTF4* mutations; the maximum permissive temperature of double mutants was lower than for any single mutant. Overexpression of Cdc68 in a *pol1* mutant strain dramatically decreased cell viability, consistent with the formation or modulation of an essential complex by these proteins in vivo. A mutation in *CDC68*/*SPT16* had previously been shown to cause pleiotropic effects on the regulation of transcription (J. A. Prendergrast et al., Genetics 124:81–90, 1990; E. A. Malone et al., Mol. Cell. Biol. 11:5710–5717, 1991; A. Rowley et al., Mol. Cell. Biol. 11:5718–5726, 1991), with a spectrum of phenotypes similar to those caused by mutations in the genes encoding histone proteins H2A and H2B (Malone et al., Mol. Cell. Biol. 11:5710–5717, 1991). We show that at the nonpermissive temperature, *cdc68-1* mutants arrest as unbudded cells with a 1C DNA content, consistent with a possible role for Cdc68 in the prereplicative stage of the cell cycle. The *cdc68-1* mutation caused elevated rates of chromosome fragment loss, a phenotype characteristic of genes whose native products are required for normal DNA metabolism. However, this mutation did not affect the rate of loss or recombination for two intact chromosomes, nor did it affect the retention of a low-copy-number plasmid. The previously uncharacterized Pob3 sequence has significant amino acid sequence similarity with an HMG1-like protein from vertebrates. Based on these results and because Cdc68 has been implicated as a regulator of chromatin structure, we postulate that polymerase α may interact with these proteins to gain access to its template or to origins of replication in vivo.

DNA replication is accomplished by large multiprotein complexes called replisomes, as demonstrated in studies using the replication machinery from *Escherichia coli* and its bacteriophages (39). The *E. coli* polymerase III holoenzyme is unusual in that it is stable enough to be purified intact, allowing its components to be identified (39). However, in most other systems the replisome is unstable, and components have been identified by using partial-function assays, genetic methods, in vitro complementation of extracts prepared from replication-defective mutants, or affinity chromatography (39, 73). Studies of the replication machinery used by the eukaryotic virus simian virus 40 (SV40) showed that functional units in eukaryotic replisomes are remarkably similar to those found in prokaryotic systems (73). Although the proteins typically have little similarity at the amino acid level, the need for polymerases, processivity clamps, clamp loading proteins, helicases, primases, nucleases, and single-stranded DNA binding proteins appears to be universal (29, 39, 73). Some physical interactions have been detected among components of the eukaryotic replication machinery (14, 18, 20, 53), suggesting that the construction of a complex structure composed of many replication proteins is also a feature conserved in eukaryotes.

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Although studies using SV40 have been extremely useful in identifying the core eukaryotic replication factors (~10 cellular factors plus the virally encoded helicase large T antigen) (44, 45, 74, 76, 82, 83), the regulation of viral DNA replication differs from the regulation of cellular DNA replication in several important ways. For example, while the SV40 genome can be replicated by using only polymerases α and δ (75, 78), cells require an additional replicative polymerase, polymerase ε (47, 56). SV40 also escapes the normal controls that restrict genomic replication to one round per cell cycle and tightly coordinate DNA replication with other events in the cell cycle (65). This finding suggests that there exist additional regulatory factors required for the proper replication and segregation of chromosomes that are not utilized by viral model systems.

Functional equivalents of all of the human proteins required for SV40 replication have been identified in *Saccharomyces cerevisiae*, and the phenotypes of cells containing mutations in the genes encoding these factors are consistent with the proposed roles for these proteins in cellular replication (3, 6, 14, 15, 23, 71, 86). The *S. cerevisiae* polymerase α holoenzyme is essential for viability (37, 63), and conditional mutations in the genes that encode the individual subunits of polymerase α cause defects in DNA replication under restrictive or semipermissive conditions (16, 25, 35, 37, 48, 63), indicating that polymerase α is part of the DNA replication complex in yeast. Polymerase α holoenzyme is a heterotetramer (62) consisting

of a 180-kDa catalytic subunit, Pol1 (8, 37), an 86-kDa subunit required for initiation, Pol12 (25), and two primase subunits, Pri1 (48 kDa) (68) and Pri2 (58 kDa) (24, 26). While these subunits form a stable complex (62), they do not copurify with other replication proteins, suggesting that the *S. cerevisiae* replisome is relatively unstable. However, eukaryotic polymerases have been isolated in larger complexes from mammalian cells by using gentle purification conditions (43, 50, 51). Pol1 protein can be purified separately from the other three subunits and retains catalytic activity.

Since the replisome is a large multiprotein complex, protein-protein contacts are likely to be important not only for the assembly and structural integrity of the complex but also for the regulation of enzymatic activity. We have used these interactions to determine the components of the replication complex by protein affinity chromatography (27, 28, 55, 81). Because of the high concentration of ligand binding sites, this method can be used to detect even weak interactions expected to form temporarily for regulating the properties of the replisome.

Previously, we had found that approximately six proteins from whole-cell extracts specifically bound Pol1 affinity matrices (55). One of these, Ctf4 (for chromosome transmission fidelity), was found to be nonessential but important for the proper replication, segregation, and/or repair of chromosomal DNA (40, 54, 55, 72). Cells lacking Ctf4 are predominantly large budded, have a single nucleus with a 2C DNA content at the neck of the bud, and have elevated rates of chromosome loss and recombination. Errors accumulated in *ctf4*Δ cells are monitored by the S-phase checkpoint gene *MEC1* (79) since *ctf4-Δ4 mec1-1* double mutant cultures have fewer viable cells than either single mutant (54). We interpreted these phenotypes to mean that although the bulk of DNA can be replicated in the absence of Ctf4, cells have difficulty proceeding through mitosis because the replicated DNA is of poor quality.

We have extended our studies to identify additional proteins that interact with Pol1 affinity matrices and have identified two additional binding proteins, Cdc68/Spt16 and Pob3 (Pol1 binding protein). Cdc68/Spt16 was previously identified as a global transcription regulator (52, 64). Pob3 has not been previously described.

CDC68 was found in a screen for genes required for Start; 80 to 90% of *cdc68-1* cells arrest as unbudded cells when shifted to 36°C for 3 h (64). Cdc68 is required for the proper expression of cyclins; *CLN1*, *CLN2*, and *CLN3* transcript levels are diminished in the *cdc68-1* mutant when cells are shifted to the nonpermissive temperature. However, reduction in the transcript level of cyclins cannot completely account for the essential role of Cdc68, since cells containing the *cdc68-1* mutation together with a hyperactive or hyperstable allele of *CLN2* proceed through Start but fail to complete a cell cycle (67). *SPT16* was found in a screen to identify factors that play a general role in the regulation of transcription as a suppressor of Ty insertion mutations near the *HIS4* and *LYS2* genes and was classified as histone-like because of its phenotypic similarity with *SPT11* (*HTA1*) and *SPT12* (*HTB1*) (the genes encoding histones H2A and H2B, respectively) (52). *SPT16* is essential for viability and is allelic with *CDC68* (52); we will refer to this gene as *CDC68*. In an attempt to decipher the function of Cdc68, a screen for extragenic suppressors of *cdc68-1* was conducted and a mutation in *SAN1* was identified (84). *san1-1* had previously been identified as a suppressor of *sir4*, a gene whose normal copy is required for chromatin-mediated transcriptional silencing at the silent mating-type loci (69). Because of the phenotypic similarity to histone gene mutations and the genetic association with genes involved in silencing, Cdc68 has been postulated to act in forming or regulating the properties of chromatin (52, 84).

The results reported here indicate that Cdc68, Pob3, and Pol1 interact with one another and that Cdc68 and Pob3 compete with Ctf4 for binding to Pol1. We propose that these interactions facilitate the ability of the polymerase to access its chromatin substrate during either initiation or elongation of replication.

MATERIALS AND METHODS

Media and strains. Synthetic medium (66), YEPD (66), and YM-1 (34) were prepared as described previously. All strains listed in Table 1 are congenic with strain A364a except as noted otherwise. The *cdc68-1* mutation (64) was provided by R. Singer and G. Johnston (Dalhousie University) in strain 21R and then backcrossed 10 times to A364a strains.

Polymerase purification and affinity columns. Pol1 was overexpressed in strain 7311-2-4 containing the *GAL1-POL1* expression plasmid pTF59 as described previously (55) except that cells were grown in medium containing 3% glycerol and 2% lactate as the carbon source, induced overnight with 0.5% galactose, and harvested at an optical density at 600 nm (OD_{600}) of 0.6. This procedure allowed greater induction and stability of Pol1. About 15 mg of Pol1 protein purified from a 60-liter culture was attached to 10 ml of Affigel-10 matrix (Bio-Rad) as described previously (81). A control column was made by using similar amounts of human serum albumin as the ligand. This represents about a 30-fold increase in scale over amounts used in previous studies.

Preparation of extracts and affinity chromatography. 7208-12 or 7463-6-1 cells (Table 1) were grown to an OD_{600} of 1.0, harvested by centrifugation at 2,800 × *g* for 5 min, frozen in liquid nitrogen, and stored at −70°C. Whole-cell extracts for affinity chromatography were prepared as described previously (81). Affinity columns were equilibrated with buffer A (20 mM Tris-Cl [pH 7.5], 1 mM Na_2EDTA, 1 mM 2-mercaptoethanol, 50 mM KCl, 10% [wt/vol] glycerol, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 μg of leupeptin per ml, 0.7 μg of pepstatin per ml). Filtered extracts were loaded, columns were washed with 10 column volumes of buffer A, and bound proteins were eluted with buffer B (buffer A containing 400 mM KCl instead of 50 mM).

Identification of bound proteins. Proteins in eluates were concentrated in Centricon and Centriprep microconcentrators (Amicon) and fractionated on sodium dodecyl sulfate (SDS)–7.5% polyacrylamide gels (41). To visualize total bound protein, gels were stained with silver (2). To obtain sequence, proteins were transferred to ProBlott membranes (Applied Biosystems), stained with 0.1% Ponceau S in 1% acetic acid, destained in deionized water, excised, and digested with endoproteinase Lys-C (nonsequencing grade; Boehringer Mannheim) as described previously (21). Peptides generated from the limited proteinase digestion were recovered by reverse-phase high-pressure liquid chromatography on a C_{18} column in a gradient of 0 to 80% acetonitrile in 0.1% trifluoroacetic acid and sequenced with an Applied Biosystems 477A protein sequencer.

Immunoprecipitations. Cultures of strain 7382-3-4 (Table 1) were grown in rich medium to an OD_{600} of 0.7 and then collected by centrifugation at 2,000 × *g* for 5 min. Spheroplasts were prepared as described previously (19) and lysed in a buffer consisting of 50 mM Tris-Cl (pH 8.0), 1% Nonidet P-40, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 μg of leupeptin per ml, and 0.7 μg of pepstatin per ml with five passages in a Dounce homogenizer. After centrifugation at 16,000 × *g* for 10 min at 4°C, the supernatant was either precleared or used directly as noted. For preclearing, preimmune sera (50 μl/ml of extract) and protein A-Sepharose (a 1:1 slurry of beads in lysis buffer; 200 μl/ml of extract; Pierce) were added, and then the mixture was rotated for 1 h at 4°C. The protein A-Sepharose was removed by centrifugation, and the precleared extract was transferred to a fresh tube. Polyclonal antiserum against intact Pol1, Ctf4, or Cdc68 or antiserum against a peptide representing the 14 amino-terminal residues of Pob3 was added (50 μl/ml of extract), and the samples were incubated overnight on ice. Protein A-Sepharose was added, and the mixture was incubated as described above. The immunoprecipitates were collected by centrifugation, washed three times with 0.5 ml of lysis buffer, suspended in 2× SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer, and boiled for 5 min. Proteins were fractionated on SDS–7.5% polyacrylamide gels (41) and electrophoretically transferred to nitrocellulose. Membranes were blocked with 3% dry milk in Tris-buffered saline (TBS), incubated with the antisera indicated (1:1,000 dilutions) in blocking solution for one to several hours at room temperature (RT), washed with TBS, incubated with alkaline phosphatase-linked goat anti-rabbit immunoglobulin G (1:2,500 dilution; Bio-Rad) in blocking solution for 1 h at RT, washed with TBS, and developed with 4-nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate (each at 0.2 mg/ml; Boehringer Mannheim) in 100 mM Tris-Cl (pH 9.5)–100 mM NaCl–50 mM $MgCl_2$.

Temperature sensitivity analysis. Strains (Table 1) were grown to saturation in rich medium (YM-1), collected by centrifugation at 1,000 × *g* for 5 min, rinsed with deionized water, collected again, and resuspended in deionized water. Then 5 μl of 10-fold serial dilutions was applied to either YEPD or synthetic complete agar plates and incubated in water baths at the temperatures indicated.

TABLE 1. Strains used

Strain[a]	Genotype
Pol1 affinity chromatography	
7311-2-4	*MAT***a** *cdc17-1 leu2 ura3 can1 his7 pep4 prb1* pTF59 (*GAL1-POL1*; *LEU2*)
7208-12	*MAT***a**/α *ura3*/*ura3 trp1*/*trp1 his7*/*his7 can1*/*can1 pep4*/*pep4 prb1*/*prb1*
7463-6-1	*MAT*α *leu2 ura3 trp1 can1 ctf4-Δ4*(::*TRP1*) *pep4 prb1*
Immunoprecipitation, 7382-3-4	*MAT***a** *leu2 ura3 trp1 his7 can1 pep4 prb1*
Temperature sensitivity analysis and Cdc68 overexpression	
4053-5-4	*MAT*α *leu2 ura3 trp1 his7*
7376-9-2	*MAT***a** *leu2 ura3 his7 can1 cyh2 cdc17-1*
2034-1-3	*MAT*α *leu2 ura3 trp1 his7 cdc68-1*(::*URA3*)
2035-8-3	*MAT*α *leu2 ura3 trp1 his7 can1 cyh2 cdc17-1 cdc68-1*(::*URA3*)
7389-5-1	*MAT***a** *leu2 ura3 trp1 his7 cyh2 cdc17-1 ctf4-Δ4*(::*TRP1*)
2037-5-3	*MAT***a** *leu2 ura3 trp1 his7 cdc68-1*(::*URA3*) *ctf4-Δ4*(::*TRP1*)
2036-10-2	*MAT***a** *leu2 ura3 trp1 his3 cdc17-1 cdc68-1*(::*URA3*) *ctf4-Δ4*(::*TRP1*)
7372-1-4	*MAT*α *leu2 ura3 trp1 ctf4-Δ4*(::*TRP1*)
2037-7-2	*MAT*α *leu2 ura3 trp1 his7 cdc68-1*(::*URA3*) *ctf4-Δ4*(::*TRP1*)
7606-68-2	*MAT***a** *leu2 ura3 trp1 dna2-2*(::*LEU2*) *cdc68-1*(::*URA3*)
7360-5-3	*MAT***a** *leu2 ura3 trp1 his3 cdc17-2*
2037-7-4	*MAT*α *leu2 ura3 trp1 his3 cdc17-2 cdc68-1*(::*URA3*)
7604-17-5	*MAT***a** *leu2 ura3 trp1 his7 can1 pol1-17*
2143-1-2	*MAT*α *leu2 ura3 trp1 his7 pol1-17 cdc68-1*(::*URA3*)
7420-4-3	*MAT*α *leu2 ura3 trp1 his7 can1 cdc2-1*
2128-1-2	*MAT***a** *leu2 ura3 trp1 his7 cdc2-1 cdc68-1*(::*URA3*)
7604-9-2	*MAT*α *leu2 ura3 his7 can1 cdc9-1*
2130-9-2	*MAT***a** *leu2 ura3 trp1 his3 cdc9-1 cdc68-1*(::*URA3*)
7612-1-2	*MAT*α *leu2 ura3 trp1 his3 ade2 ade3 cdc6-1*
2110-7-4	*MAT*α *leu2 ura3 trp1 his7 can1 cdc6-1 cdc68-1*(::*URA3*)
Overexpression of Pol1 and Ctf4	
7373-4-4	*MAT***a** *leu2 ura3 trp1 his3*
2034-3-2	*MAT***a** *leu2 ura3 trp1 his3 cdc68-1*(::*URA3*)
Flow cytometry	
4053-5-2	ρ° *MAT***a** *leu2 ura3 trp1 his7*
2034-3-2	ρ° *MAT***a** *leu2 ura3 trp1 his3 cdc68-1*(::*URA3*)
Chromosome fragment loss	
7630-1-4	*MAT***a** *leu2 ura3 trp1 his3 can1 ade2 ade3*[CF:352; *LEU2*, *ade3-2p*]
2116-6-4	*MAT***a** *leu2 ura3 trp1 his3 ade2 ade3 cdc68-1*(::*URA3*) [CF:352; *LEU2*, *ade3-2p*]
Chromosome loss and recombination rates	
Chromosome V assay	
7862	*MAT***a**/α *leu2*/*leu2 ura3*/*ura3 trp1*/+ *his3*/+ +/*his7* +/*sap3 can1*/+ *hom3*/+
2106	*MAT***a**/α *leu2*/*leu2 ura3*/*ura3 trp1*/*trp1 his3*/+ +/*his7 can1*/+ *hom3*/+ *cdc68-1*(::*URA3*)/cdc68-1(*URA3*)
Chromosome III assay	
2113	*MAT***a**/α *leu2*/+ *ura3*/*ura3 trp1*/*trp1 ade3*/*ade3 his3*/*his3 can1*/+
2109	*MAT***a**/α *leu2*/+ *ura3*/*ura3 trp1*/*trp1 ade3*/*ade3 his3*/*his3 cdc68-1*(::*URA3*)/*cdc68-1*(::*URA3*)
Plasmid loss assay	
7468-4-2	*MAT***a** *leu2 ura3 trp1 ade2 ade3 his7* pDK243
2079-3-2	*MAT***a** *leu2 ura3 trp1 ade2 ade3 his7 can1 cdc68-1*(::*URA3*) pDK243
2088-5-1	*MAT*α *leu2 ura3 trp1 ade2 ade3 his3 can1 cdc68-1*(::*URA3*) pDK243
POB3 deletion analysis	
YPH49	*MAT***a**/α *ura3-52*/*ura3-52 lys2-801*/*lys2-801 ade2-101*/*ade2-101 trp1-Δ1*/*trp1-Δ1*
2021-3	*MAT***a**/α *ura3-52*/*ura3-52 lys2-801*/*lys2-801 ade2-101*/*ade2-101 trp1-Δ1*/*trp1-Δ1 pob3*Δ[::*TRP1*]/+
2041	*MAT***a**/α *ura3*/*ura3 leu2*/*leu2 trp1*/*trp1 his7*/+ *his3*/+ *pob3*Δ(::*TRP1*)/+

[a] All strains except those used for *POB3* deletion analysis are congenic with A364a.

Overexpression of Cdc68, Pol1, and Ctf4. The *GAL1-CDC68* expression plasmid pJW9 was constructed by inserting the *CDC68* open reading frame (ORF) six nucleotides downstream of the *Bam*HI site in a *GAL1* promoter vector (pMTL-YCpLEU2). pTF59 and pTF69 were derived by inserting *POL1* (55) or *CTF4* (using a *Bam*HI linker inserted one nucleotide upstream of *CTF4*) ORF into the *Bam*HI site of YEp434 (49) such that each was placed under control of the *GAL1* promoter. These plasmids were transformed into the desired strains (Table 1), which were then grown to saturation under selection for the plasmids in synthetic medium containing 3% glycerol and 2% lactate as the carbon source. Dilutions were prepared as for the temperature sensitivity analysis, applied to plates containing 3% glycerol and 2% lactate in the absence or presence of 2% galactose, and incubated in water baths at the temperatures indicated.

Flow cytometry. Strains lacking mitochondrial genomes (ρ°) (Table 1) were obtained by growing cultures to saturation in the presence of 10 μg of ethidium bromide per ml and confirmed by hybridization analysis with a cytochrome *b* sequence and fluorescence microscopy. Cultures were grown to log phase in rich medium at RT, and aliquots were shifted to 36°C for 3 h in a shaking water bath. Cells were collected and prepared for analysis as described previously (60).

Chromosome loss and recombination rate assays. Chromosome fragment loss was assayed as described previously (59), using the chromosome III fragment CF352 in *CDC68* and *cdc68-1* haploid strains (Table 1). CF352 was maintained prior to analysis by leucine selection. For assays at 32°C, cultures were grown for 1 to 2 days at 32°C, sonicated, diluted, placed on YEPD prewarmed to 32°C, incubated at 32°C overnight, and then transferred to RT. For assays at 36°C, cultures were grown at RT to logarithmic phase, transferred to 36°C for 3 h, sonicated, diluted, placed onto YEPD, and incubated at RT.

Chromosome loss and recombination rates were determined in diploids by using a chromosome V assay (35), which monitors the *can1* and *hom3* mutations located on opposite arms of chromosome V, and a chromosome III assay (30), which monitors the *MAT***a** and *LEU2* markers located on opposite arms of chromosome III. Wild-type (WT) and *cdc68-1* diploid strains (Table 1) that were heterozygous for *can1* and *hom3* markers on chromosome V or heterozygous for *LEU2* on chromosome III were grown on defined media at either RT or 29°C (the maximum permissive temperature for *cdc68-1* homozygous diploids). For the chromosome V assay, cells that had undergone a loss or recombination event were selected on canavanine medium. These canavanine-resistant colonies were then tested for methionine prototrophy to distinguish recombinants (*HOM3* Met^+) from losses (*hom3* Met^-). For the chromosome III assay, cells that had undergone a loss or recombination event were selected for the ability to mate with a *MAT***a** *leu2* tester strain. Triploids were selected by complementation of mutant *ade* and *his* alleles. Losses (*leu2*/*leu2*/−) and recombinants (*leu2*/*leu2*/*LEU2*) were distinguished by testing leucine prototrophy. Fluctuation analysis was conducted for both assays by using the method of the median (42) to determine the rates of loss and recombination of each of these chromosomes.

Plasmid loss rates were determined in WT and *cdc68-1* haploids (Table 1) by using the single-copy plasmid pDK243 as described previously (36, 59).

Deletion of the *POB3* gene. Regions flanking the *POB3* gene were amplified by PCR. The amplified 5′ flanking region consisted of a 260-bp fragment terminating 8 bp before the initiating AUG; artificial *Kpn*I and *Xba*I sites were introduced during PCR amplification to the 5′ and 3′ ends, respectively, of this fragment. The amplified 3′ flanking region consisted of a 370-bp fragment terminating 1 bp after the terminating TAG; artificial *Eco*RI and *Kpn*I sites were introduced during PCR amplification to the 5′ and 3′ ends, respectively, of this fragment. The restriction sites noted were used to insert the fragments individually into YIplac204 (31), and the products were checked by nucleotide sequencing. A derivative with both fragments (pJW1) was then constructed, linearized with *Kpn*I, and used to transform diploid yeast cells by the lithium acetate method (66), removing all of the *POB3* gene from −13 to the stop codon.

RESULTS

Cdc68 and Pob3 bind specifically to Pol1, and their binding is enhanced in the absence of Ctf4. Approximately six proteins from a yeast whole-cell extract were observed to bind to an affinity matrix containing the polymerase α catalytic subunit (Pol1 protein) but not to a control matrix containing human serum albumin (Fig. 1, lanes 1 and 2). Previously, we found the main polymerase binding protein to be Ctf4, using this technique (55). Using a matrix with about 0.5 mg of Pol1 ligand, we were able to obtain enough Ctf4 for amino-terminal sequencing but could not obtain sequence from other polymerase binding proteins. Therefore, we increased the overexpression and the scale of the purification of Pol1 to generate a matrix containing about 15 mg of Pol1. Comparison of the proteins in eluates resolved by two-dimensional (2D) nonequilibrium pH gel electrophoresis (2D NEPHGE) (58) demonstrated that the elution profile from the larger-scale experiment did not vary significantly from that of the original experiments (data not shown). Furthermore, we detected the p48 primase subunit of the Polα/primase holoenzyme in Pol1 eluates by immunoblotting (data not shown), demonstrating that this technique can identify biologically relevant interactions.

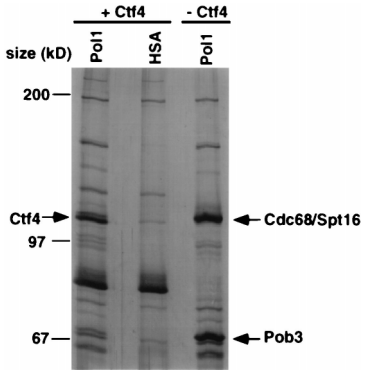

FIG. 1. The proteins in Pol1 affinity column eluates change when Ctf4 is removed from the extract. Whole-cell extracts were prepared for affinity chromatography and loaded onto two 10-ml columns, one containing ~15 mg of Pol1 and the other containing a similar amount of human serum albumin (HSA). The columns were washed with 10 column volumes of buffer A (50 mM KCl) and eluted with buffer B (400 mM KCl; see Materials and Methods). Fractions containing eluted proteins were pooled and concentrated, and ~1% of the total protein was fractionated in an SDS–7.5% polyacrylamide gel and stained with silver. + Ctf4 or − Ctf4 refers to extracts that were prepared from cells containing either a WT allele of *CTF4* (7208-12) or a deletion of this gene (7463-6-1), respectively. Size standards and major binding proteins migrated as indicated.

We attempted to obtain amino acid sequence from the 120-kDa protein migrating slightly more slowly than Ctf4 but repeatedly obtained sequence for the more abundant Ctf4 even when 2D NEPHGE was used to enhance separation. Therefore, since *CTF4* is not essential for viability, we prepared extracts for affinity chromatography from *ctf4-Δ4* cells in which the *CTF4* gene had been deleted. Surprisingly, the binding of two proteins to Pol1 was enhanced under these conditions (Fig. 1; compare lanes 1 and 3, 120 and 70 kDa). We interpret this result to mean that these proteins compete with Ctf4 for binding to Pol1, so that removing Ctf4 from the lysate caused an increase in the amount of the 120- and 70-kDa proteins bound.

Since Edman degradation of these two intact binding proteins did not produce a signal, we generated peptides by limited proteolysis. Two peptides generated from digestion of the 120-kDa protein indicated in Fig. 1 had the sequences NEEGEYTYLRL and IQDVQFYREASDM. A search of GenBank (61) revealed that these sequences match amino acids 584 to 594 and 755 to 767, respectively, of Cdc68/Spt16 (ORF YGL207w; GenBank accession no. M73533). Six peptides generated from the digestion of the 70-kDa protein indicated in Fig. 1 had the sequences NQGVIQLDGFSQD DYNLIK, SMAEAFYEELK, LQHRQIQRIVSLPK, THIVL SHVLK, GLTDRRVIVPGEYK, and YDQCAVSCSFK, which correspond to amino acids 72 to 90, 219 to 229, 277 to 290, 353 to 362, 363 to 376, and 379 to 389, respectively, encoded by ORF YML069w at positions 135500 to 137155 on *S. cerevisiae* chromosome XIII. Since this gene product had not been previously characterized, the 70-kDa protein was named Pob3.

It is possible that both Cdc68 and Pob3 compete independently with Ctf4 for binding to Pol1 or that they form a complex that competes. The increased binding does not appear to be due to an increase in the concentration of Cdc68 or Pob3

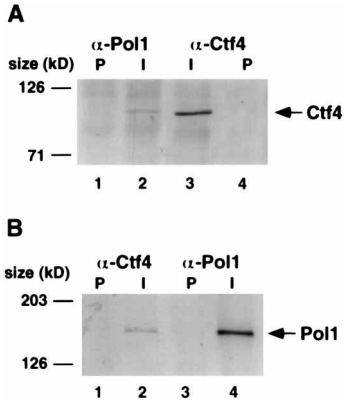

FIG. 2. Ctf4 and Pol1 coimmunoprecipitate. Proteins were immunoprecipitated from whole-cell extracts and visualized by immunoblotting as described in Materials and Methods. Each lane represents the protein immunoprecipitated from 5×10^7 cell equivalents. (A) Ctf4 was immunoprecipitated from precleared extracts by using preimmune (P) or immune (I) antiserum raised against Pol1 (lanes 1 and 2) or Ctf4 (lanes 4 and 3). (B) Pol1 was immunoprecipitated by using preimmune (P) or immune (I) antiserum raised against Ctf4 (lanes 1 and 2) or Pol1 (lanes 3 and 4).

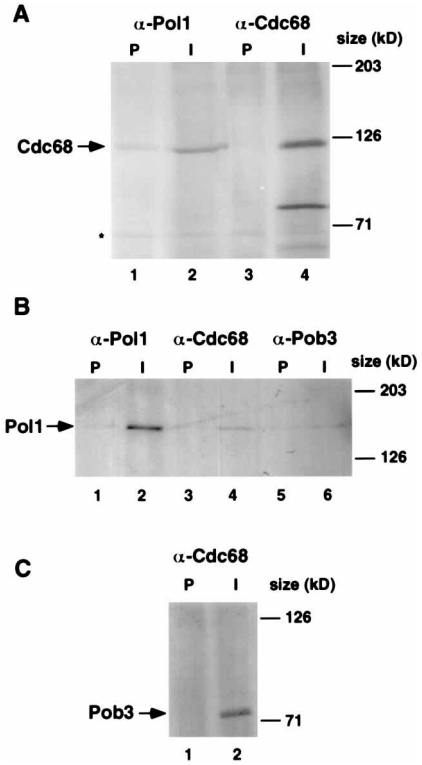

FIG. 3. Pol1 and Pob3 coimmunoprecipitate with Cdc68. Proteins were immunoprecipitated from whole-cell extracts and visualized by immunoblotting as described in Materials and Methods. Each lane represents the protein immunoprecipitated from 5×10^7 cell equivalents. (A) Cdc68 was immunoprecipitated from precleared extracts by using preimmune (P) or immune (I) antiserum raised against Pol1 (lanes 1 and 2) or Cdc68 (lanes 3 and 4). The asterisk marks a nonspecific cross-reacting protein that serves as an internal control for loading. (B) Pol1 was immunoprecipitated by using preimmune (P) or immune (I) antiserum raised against Pol1 (lanes 1 and 2), Cdc68 (lanes 3 and 4), or Pob3 (lanes 5 and 6). (C) Pob3 was immunoprecipitated by using preimmune (P) or immune (I) antiserum raised against Cdc68 (lanes 1 and 2).

protein, since the levels of both were similar in strains containing or lacking *CTF4* (data not shown). Since deletion of *CTF4* did not cause the level of Cdc68 or Pob3 protein to change, but it did increase the amount of Cdc68 and Pob3 bound to the Pol1 matrix, the Cdc68 and Pob3 interactions with Pol1 appear to be mutually exclusive with the Ctf4-Pol1 complex.

Interactions among Pol1, Ctf4, Cdc68, and Pob3 can also be detected by immunoprecipitation. Affinity chromatography allows detection of weak interactions because of the high concentration of binding sites on the matrix. To determine whether the proteins that bound to the Pol1 matrix could form complexes under conditions that more closely mimic the intracellular environment, we immunoprecipitated Pol1, Ctf4, Cdc68, and Pob3 proteins and then assayed for coprecipitation of other proteins. Ctf4 was coimmunoprecipitated from a whole-cell extract with Pol1, using an antibody against Pol1 (Fig. 2A, lane 2), and Pol1 was coimmunoprecipitated with Ctf4, using an antibody against Ctf4 (Fig. 2B, lane 2), while neither of the preimmune sera revealed any coprecipitation. The levels of the coprecipitating protein in each case were lower than the level of the primary target protein, suggesting that the interactions might not be stable under these conditions or that these proteins are not always associated with one another in a complex.

Cdc68 was coimmunoprecipitated from a whole-cell extract with Pol1, using an antibody against Pol1 (Fig. 3A, lane 2). A small amount of Cdc68 was also immunoprecipitated with the Pol1 preimmune serum (Fig. 3A, lane 1) but not with a second preimmune serum (Fig. 3A, lane 3). The coprecipitation of Cdc68 with Pol1 appears to be significant, since the amount of Cdc68 observed increased with the immune serum in several repeats of this experiment (Fig. 3A [compare lane 2 with lane 1] and data not shown), while a nonspecific cross-reacting protein (denoted by the asterisk) displayed equal intensity in all lanes, indicating uniform loading of the lanes. Furthermore, in the reciprocal experiment, Pol1 was also coimmunoprecipitated from a whole-cell extract with Cdc68, using an antibody against Cdc68 (Fig. 3B, lane 4). These data demonstrate that Cdc68 and Pol1 are associated in whole-cell lysates.

Pol1 was also immunoprecipitated from extracts by using antibodies raised against the amino terminus of Pob3 (Fig. 3B, lane 6), even though we could not detect any Pob3 in the immunoprecipitate in assays using the same antibodies. The anti-Pob3 antiserum did not cross-react directly with purified Pol1, as assayed by Western analysis (data not shown). One explanation for this result may be that the anti-Pob3 antiserum precipitates only a small amount of native Pob3 and is not sensitive enough to detect small quantities of Pob3 by Western analysis, whereas the anti-Pol1 antiserum is sensitive enough to detect the small amount of Pol1 associated with Pob3 in the immunoprecipitate. Pob3 was detected in immunoprecipitates in assays using an antibody against Cdc68 (Fig. 3C, lane 2); thus, the serum is capable of detecting Pob3 after SDS-PAGE, and Pob3 appears to associate with Cdc68 in whole-cell lysates.

***CDC68* interacts genetically with *POL1* and *CTF4*.** Cdc68 has previously been suggested to regulate chromatin structure (52, 67, 84), and Pob3 has sequence similarity with HMG1-like proteins (see below), which have been postulated to affect chromatin assembly (9, 10, 70). To determine whether the in vitro interaction of Cdc68 and Pob3 with Pol1 had any biological significance, we characterized mutations in these genes to determine whether they caused phenotypes consistent with an involvement of these proteins in the regulation of DNA replication.

A temperature-sensitive allele of *CDC68*, *cdc68-1*, was obtained (64) and crossed 10 times to the A364a background to

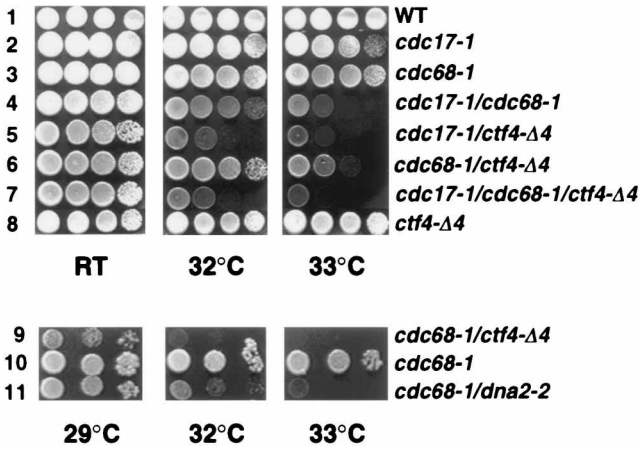

FIG. 4. The growth of strains containing various mutant combinations reveals interactions among *POL1*, *CDC68*, and *CTF4*. Strains (Table 1) were grown to saturation and washed, and 10-fold serial dilutions were spotted onto either YEPD (rows 1 to 8) or synthetic complete (rows 9 to 11) plates. Individual plates were then incubated either at RT or in water baths at the temperatures shown for several days. All strains are congenic with A364a (Table 1). Strain 2037-5-3 was used in row 6, and strain 2037-7-2 was used in row 9.

allow analysis of *cdc68-1* with other mutations under congenic conditions. Interactions between *CDC68* and other genes were investigated by analyzing the growth characteristics of cells containing various mutations.

cdc68-1 and *cdc17-1* (*CDC17* is allelic with *POL1*) cells are each temperature sensitive, displaying a maximum permissive temperature of 32 to 33°C on rich media (Fig. 4, rows 2 and 3). However, the maximum permissive temperature of cells containing both *cdc68-1* and *cdc17-1* mutations was decreased compared to either single mutant (Fig. 4; compare row 4 with rows 2 and 3), demonstrating that *cdc68* and *cdc17* interact genetically. These data are consistent with a physical interaction between Cdc68 and Pol1 in vivo, as was observed in vitro (Fig. 1 and 3).

cdc68-1 cells grow normally at temperatures at or below 32°C (Fig. 4, row 3), and *ctf4-Δ4* cells are not temperature sensitive (Fig. 4, row 8). However, combining the *ctf4-Δ4* mutation with *cdc68-1* caused a synthetic phenotype; the *cdc68-1 ctf4-Δ4* double mutant was less viable than either single mutant when strains were grown on rich media above 32°C (Fig. 4; compare row 6 with rows 3 and 8). Surprisingly, when these strains were grown on defined media, the effect of combining the *ctf4* null mutation with *cdc68-1* was more dramatic; the maximum permissive temperature of the *cdc68-1*/*ctf4-Δ4* double mutant was lower than that of *cdc68-1* alone (Fig. 4; compare rows 9 and 10). Thus, when nutrients are limiting, the absence of Ctf4 sharpens the transition characteristics of the temperature sensitivity of *cdc68-1* cells.

As previously demonstrated (54), deletion of the *CTF4* gene in *cdc17-1* cells decreased the maximum permissive temperature of *cdc17-1* cells (Fig. 4; compare rows 5 and 2), consistent with an interaction between Ctf4 and Pol1 proteins that stabilizes Pol1 in vivo. Surprisingly, *cdc17-1 cdc68-1 ctf4-Δ4* triple mutant cells were no more temperature sensitive than those of the most severe double mutant, the *cdc17-1 ctf4-Δ4* strain (Fig. 4; compare rows 7 and 5).

DNA2 is an essential gene which encodes a DNA helicase (11, 12). *dna2-2* was previously identified in a screen for mutants that are synthetically lethal with *ctf4-Δ4* (26a). *dna2-2* cells are not temperature sensitive (26a). Combining the *dna2-2* mutation with *cdc68-1* also caused a synthetic phenotype; the *cdc68-1 dna2-2* double mutant was less viable than *cdc68-1* cells when strains were grown on defined media at elevated temperatures (Fig. 4; compare rows 11 and 10). Curiously, a temperature-sensitive allele of *DNA2*, *dna2-1*, did not interact genetically with *cdc68-1* (data not shown), demonstrating allele specificity for this interaction.

The interaction between *CDC68* and *POL1* is allele specific. To assess the specificity of the genetic interactions with *cdc68-1*, we tested the temperature sensitivity of a set of double mutations of various *cdc* mutants with *cdc68-1*. In addition to interacting with *cdc17-1*, *cdc68-1* also interacts genetically with both *cdc17-2* and *pol1-17*, two other temperature-sensitive alleles of Pol1. Each of these mutations displays a decrease in viability with *cdc68-1* relative to the single mutant at 30°C or above (Fig. 5), but the effect is subtle compared to that of *cdc17-1*. There is no genetic interaction between *cdc68-1* and *cdc2-1*, an allele of the gene encoding the catalytic subunit of polymerase δ (Fig. 5), demonstrating that *cdc68-1* does not interact with all polymerases. We found subtle interactions between *cdc68-1* and *cdc9* (DNA ligase) at RT and 26°C, *cdc6* (required for replication initiation) at 30°C or above (Fig. 5), and *cdc4* (required for the G_1/S transition [data not shown]) (7). Each of these mutations displays a decrease in viability with *cdc68-1* relative to the single mutant. No genetic interaction was seen between *cdc68-1* and *cdc7* (encoding a protein kinase required for G_1/S transition), *cdc16* (encoding a component of the anaphase promoting complex), or *cdc25* (encoding adenylate cyclase) (data not shown) (7).

In summary, the genetic interactions detected with *cdc68-1* demonstrate allele specificity for mutants of *DNA2* and polymerase α (the interaction with *cdc17-1* is stronger than that with *cdc17-2* or *pol1-17*) and are so far confined to a subset of those *cdc* mutants whose native products have been demonstrated to be involved in DNA replication.

Overexpression of Cdc68 decreases the viability of *cdc17-1* and *cdc17-2* cells. Expression of *CDC68* was placed under the

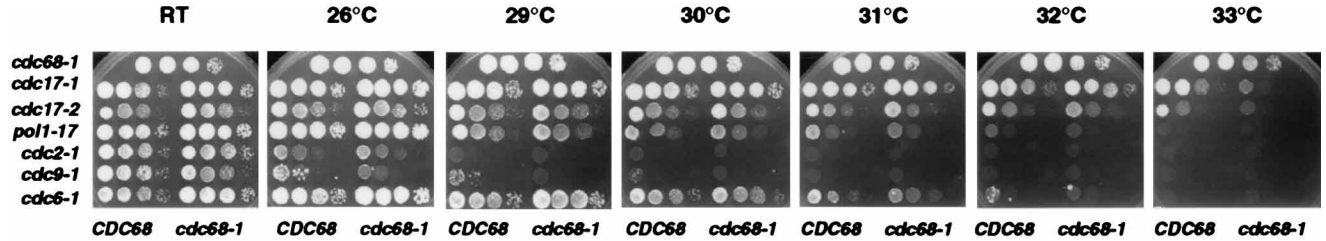

FIG. 5. The interaction between *cdc68-1* and *cdc17-1* is allele specific. Strains (Table 1) were tested on YEPD plates as described in Materials and Methods. Individual plates were then incubated either at RT or in water baths at the temperatures shown.

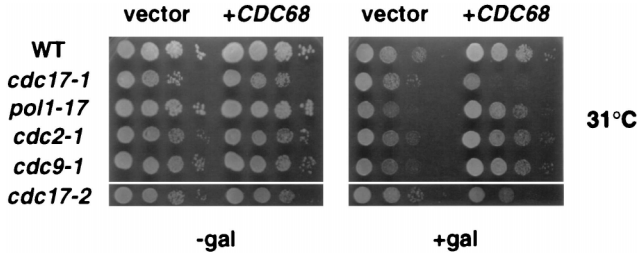

FIG. 6. Overexpression of Cdc68 decreases the viability of *cdc17-1* cells. Transformed strains (Table 1) were grown to saturation under leucine selection and washed, and 10-fold serial dilutions were spotted onto synthetic plates lacking leucine and containing 3% glycerol and 2% lactate in the absence or presence of 2% galactose (−gal or +gal). Individual plates were then incubated in water baths at the temperatures shown for several days. Note that growth under these conditions appears to stabilize these *cdc* mutant strains at elevated temperatures compared to growth on glucose-containing media. In a parallel experiment using liquid cultures grown under these same conditions, the level of Cdc68 protein increased upon induction relative to the uninduced control as assayed by immunoblotting (data not shown).

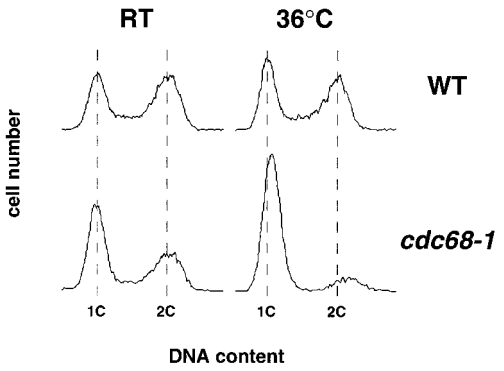

FIG. 8. Flow cytometry analysis reveals that *cdc68-1* cells arrest with a 1C DNA content. ρ° strains of WT and *cdc68-1* cells were grown to log phase at RT, and aliquots of each culture were shifted to 36°C for 3 h. DNA content was determined as described in Materials and Methods.

control of the *GAL1* promoter, and this construct was transformed into normal and *cdc* mutant strains. Overexpression of Cdc68 in a *cdc17-1* strain decreased the viability of cells relative to cells containing the vector only (Fig. 6; note that the temperature sensitivity of *cdc68* and *cdc17* mutations, like that of several other *cdc* mutations, is partially or completely suppressed by growth on glycerol-lactate medium). Overexpressing Cdc68 also decreased cell viability in a *cdc17-2* mutant strain, though less dramatically than for the *cdc17-1* strain. However, overexpression of Cdc68 did not have an adverse effect on viability in a WT strain or in a variety of other *cdc* mutants. In fact, it appears as though *pol1-17*, *cdc2-1*, and *cdc9-1* strains were slightly healthier under these growth conditions when Cdc68 was overexpressed (Fig. 6). Therefore, the detrimental effect of overexpressing Cdc68 on cell viability appears to be limited to strains with a *cdc17-1* mutation or, to a lesser extent, a *cdc17-2* mutation. These same alleles of *POL1* also displayed the strongest genetic interactions with *cdc68-1* (Fig. 5). These results are consistent with formation of a complex between Cdc68 and Pol1 proteins in vivo, such that overexpression of one member of the complex disrupts a critical stoichiometry required for proper formation or function of the complex.

Overexpression of Pol1 or Ctf4 decreases the viability of *cdc68-1* cells. *POL1* and *CTF4* expression plasmids under the control of the *GAL1* promoter were transformed into *CDC68* and *cdc68-1* strains. Both WT and *cdc68-1* cells had decreased viability when either Pol1 or Ctf4 was overexpressed (Fig. 7). However, the effect was more severe in *cdc68-1* cells, and the relative difference between these two strains was most dramatic at temperatures above 30°C (Fig. 7). Therefore, even though overexpression of either of these proteins makes WT cells relatively unhealthy, their overexpression in *cdc68-1* cells severely diminishes viability. These results are consistent with Pol1 and Ctf4 forming and/or regulating the formation of a complex with Cdc68 that is critical for cell viability.

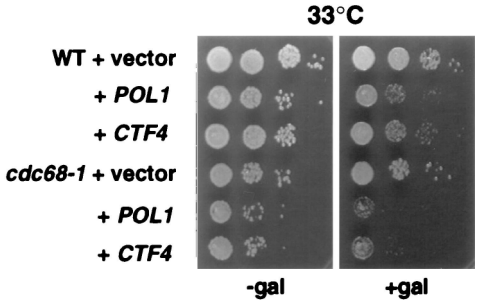

FIG. 7. Overexpression of either Pol1 or Ctf4 decreases the viability of *cdc68-1* cells. Effects of overexpression were tested as for Fig. 6.

***cdc68-1* cells have a 1C DNA content at the nonpermissive temperature.** Prendergrast et al. have noted that 80 to 90% of *cdc68-1* cells arrest with an unbudded cell morphology when shifted to 36°C for 3 h (64), consistent with Cdc68 having a role in G_1. While bud emergence occurs concomitantly with the onset of DNA replication (S phase) in some yeast strains, these two processes are not coupled in all yeast strains, and even when they are coupled in a WT strain, they can become uncoupled with the introduction of certain mutations. To determine whether *cdc68-1* cells had initiated DNA replication at the nonpermissive temperature, we examined the DNA content of these cells by using flow cytometry.

ρ° isolates (lacking mitochondrial DNA) of WT and *cdc68-1* cells were obtained, grown to logarithmic phase, and shifted to the nonpermissive temperature for 3 h. Flow cytometry revealed that 80 to 90% of *cdc68-1* cells contained a 1C DNA content after incubation at a nonpermissive temperature, consistent with a function for Cdc68 in the prereplicative stage (G_1) of the cell cycle (Fig. 8). (The slight increase in fluorescence intensity above 1C with *cdc68-1* cells at 36°C is probably not due to cells initiating DNA synthesis, since this was seen even when α-factor [G_1]-synchronized cells were shifted to 36°C for 3 h whether they were maintained at or released from the α-factor block [data not shown]). Since it has been reported that *cdc68-1* cells arrest in G_1 because of a lack of G_1 cyclin expression (67), it is possible that the 1C DNA content is due to cyclin depletion. These cells have also been reported to bypass the G_1 morphological arrest in the presence of a hyperstable G_1 cyclin, Cln2-1 (32), although the cells remain temperature sensitive and fail to complete another cell cycle (67). We constructed strains with the *cdc68-1* mutation and the hyperstable *cln2-1* allele and found that this had little effect on the DNA content at the arrest point, suggesting that the 1C arrest is not simply due to a failure to induce cyclin production (data not shown).

The majority of *cdc* mutant alleles whose products function in replication, including mutations in *POL1*, typically arrest at nonpermissive temperatures as large-budded cells containing a single nucleus with a 2C DNA content (15, 57), although a few

TABLE 2. *cdc68-1* increases the rate of chromosome fragment loss but does not affect the rate of loss and recombination of an intact chromosome or the stability of a CEN plasmid

Assay	Genotype	Temp	Events	Loss rate	Relative loss rate[a]	Relative recombination rate[a]
Chromosome fragment	WT	RT	2/9,430	2.0×10^{-4}		
	cdc68-1		2/8,340	2.4×10^{-4}	1	
	WT	32°C	5/25,285	2.0×10^{-4}		
	cdc68-1		27/24,586	1.1×10^{-3}	5.5	
	WT	36°C	1/6,882	1.5×10^{-4}		
	cdc68-1		9/7,029	1.3×10^{-3}	8.7	
Intact chromosome						
Chromosome V	WT	RT			1.0	1.0
	cdc68-1				0.3	0.5
	WT	29°C			1.8	2.0
	cdc68-1				2.0	1.3
Chromosome III	WT	29°C			1.0	1.0
	cdc68-1				0.3	0.3
Centromere plasmid	WT	RT	7/697	1.0%		
	cdc68-1		8/849	0.9%		
	WT	36°C	23/1,130	2.0%		
	cdc68-1		16/685	2.3%		

[a] WT rates (assigned a value of 1.0) were 1.2×10^{-5} loss and 2.4×10^{-5} recombination events/mitosis for the chromosome V assay and 3.8×10^{-6} loss and 6.6×10^{-6} recombination events/mitosis for the chromosome III assay. Note that twofold changes are within the range of normal variation.

alleles, including *pol1-17* arrest with a 1C DNA content (13). Apparently, most defects in replication functions allow the genome to be mostly if not completely copied but block progression through mitosis, although severe blocks prevent replication entirely. By this assay, Cdc68 appears to act before elongation. The remaining 10 to 20% of the *cdc68-1* cells that appear to arrest with a 2C DNA content at 36°C (Fig. 8) are actually G_1 doublets that were not separated by sonication, since staining with the DNA-specific dye 4′,6-diamidino-2-phenylindole (DAPI) revealed that the majority (80 to 90%) of these large-budded cells had two distinct nuclei (data not shown).

***cdc68-1* affects the stability of a chromosome fragment but does not affect the stability of intact chromosomes or a CEN plasmid.** As previously demonstrated by Hartwell and Smith using a chromosome V-based assay, cells containing temperature-sensitive alleles of *cdc* genes whose native products function in DNA metabolism have elevated rates of chromosome loss and recombination when the cells are grown under semipermissive conditions (35). We therefore measured the rate of chromosome loss for *cdc68-1* cells, initially by measuring the loss rate of an extraneous copy of a chromosome III fragment in otherwise haploid cells and subsequently measuring chromosome loss and recombination rates, using intact chromosomes V and III in diploids. The chromosome fragment assay measures the rate of loss of a 155-kbp telocentric fragment of chromosome III (CF352) that is marked with *LEU2* for selection and *ade3-2p* for colony color development (59). Cells that are *ade2 ade3* and have maintained CF352 are red, whereas cells that lose CF352 are white. The loss rate of this fragment is obtained by examining only the events which occurred during the first cell division after plating cells under nonselective conditions (the number of half-sectored colonies/total is then the same as the rate of events/mitosis). CF352 was lost from WT cells at a rate of 2×10^{-4} events/mitosis (Table 2). Thus, this fragment is not as stable as an intact chromosome in WT cells (10^{-5} losses/mitosis [Table 2]), yet it is significantly more stable than a centromeric (CEN) plasmid (10^{-2} losses/mitosis [Table 2]). The loss rate of this chromosome fragment in *cdc68-1* cells was six- or ninefold higher than in WT cells incubated at 32 or 36°C, respectively (Table 2).

While the unusual telocentric fragment of chromosome III was destabilized by the *cdc68-1* mutation, when chromosome loss and recombination rates were measured at 29°C (the maximum permissive temperature for a *cdc68-1* homozygous diploid) in assays based on intact versions of either chromosome V or chromosome III, the rates of chromosome loss and recombination for *cdc68-1* and WT strains were not significantly different (Table 2).

Previously, we demonstrated that *ctf4*-Δ cells have about 10-fold-higher chromosome loss and recombination rates compared to WT cells when measured in the chromosome V assay (54). The *ctf4*-Δ cells display a much higher rate of loss in the chromosome fragment assay (27% loss/mitosis), and this high rate of loss appeared to be enhanced in the *ctf4*-Δ *cdc68-1* double mutant, although quantitation of such high rates is difficult (data not shown). We therefore assayed *ctf4*-Δ *cdc68-1* double mutant cells at 29°C to determine if the rate of chromosome loss and recombination were further increased by *cdc68-1*, using the chromosome V assay. However, the chromosome loss and recombination rates (data not shown) were the same as for *ctf4*-Δ cells (54).

One possible explanation for the different effects of the *cdc68-1* mutation on the loss of intact and fragmented chromosomes is that the results could reflect a size dependence or an effect seen only in cases of inherent instability. The size of the chromosome fragment is 155 kb (about one-half of the size of intact chromosome III and one-fourth of the size of chromosome V). We therefore examined the loss rate of a small, circular minichromosome by using the CEN/autonomously replicating sequence plasmid pDK243 (14 kb), which has a higher loss rate than the chromosome fragment in WT cells (59). However, the loss rate of this single-copy plasmid in *cdc68-1* cells was the same as in WT cells (Table 2).

The 70-kDa Pol1 binding protein, Pob3, has amino acid similarity with an HMG1-like protein from various organisms.

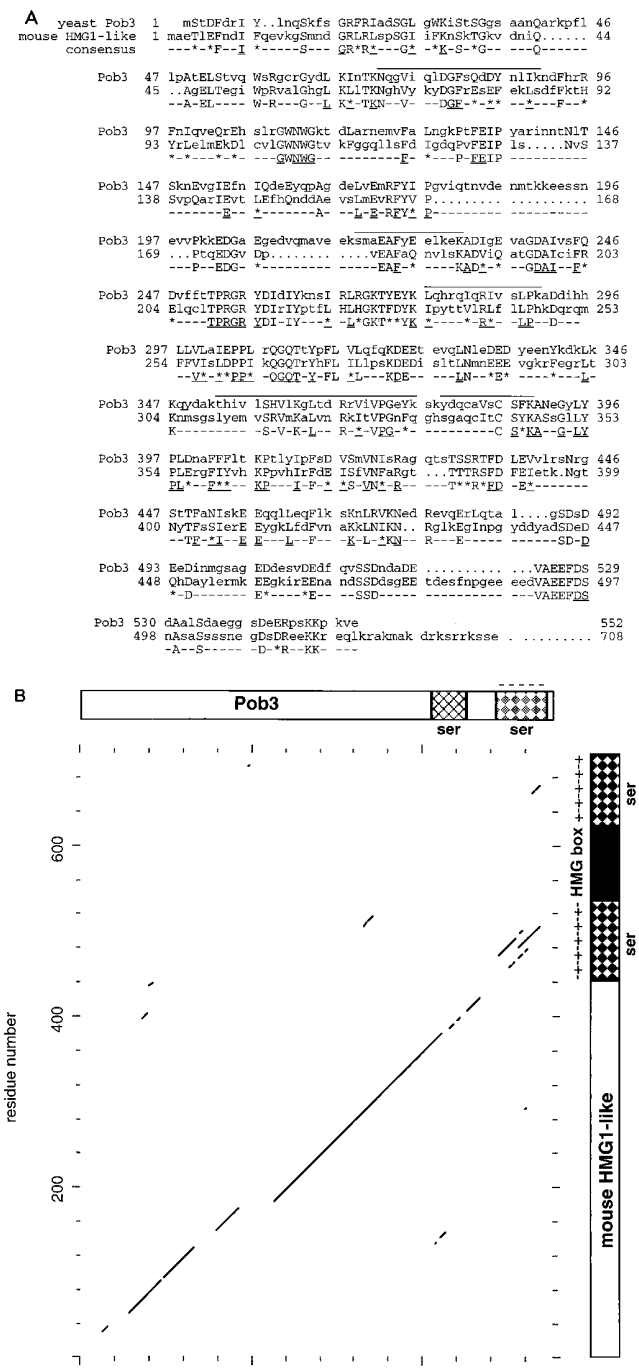

FIG. 9. *S. cerevisiae* Pob3 has amino acid identity with an HMG1-like protein from a variety of organisms. (A) A sequence alignment of *S. cerevisiae* Pob3 and the mouse HMG1-related protein was performed by using the Genetics Computer Group program (29a). Amino acids that are identical or similar between the two proteins are capitalized. A consensus sequence was established by comparing yeast Pob3 with the HMG1-like protein from seven organisms: mouse (70), human (10), chicken (77), rat (77), *Drosophila* (9), *Arabidopsis* (85), and periwinkle. (The chicken and rat HMG1-like sequences are from cDNA clones that appear to be partial clones, and so the chicken and rat sequences do not begin in the alignment until residues 39 and 145, respectively, of the mouse HMG1-like protein.) Amino acids that are identical or similar between yeast Pob3 and the HMG1-like protein from vertebrates only are shown as capital letters or asterisks, respectively, in the consensus; those residues that are conserved in Pob3 and all of the HMG1-like proteins are underlined in the consensus. Peptides recovered from limited proteolysis of the 70-kDa protein that were sequenced during the determination of the identity of Pob3 in Pol1 affinity matrix elutions are overlined in the Pob3 sequence. (B) A dot matrix plot of yeast Pob3 and the mouse HMG1-like protein was generated by using the Genetics Computer Group program with the following parameters: substitution matrix = BLOSSOM 62, window = 30, and stringency = 28. Rectangular representations of Pob3 and mouse HMG1-like protein are shown to the top and right, respectively of the dot matrix plot. Subdomains of each protein are boxed in the following manner: serine rich (cross-hatched), serine rich and acidic (gray/white checkerboard), serine rich and mixed charge (black/white checkerboard), and showing 50% identity with the B box from mouse HMG1 (black).

The sequences of all six peptides generated by limited proteolysis of the 70-kDa Pol1 binding protein were identical to the sequence encoded in an uncharacterized ORF on chromosome XIII. The size of the protein encoded in this ORF is predicted to be 70 kDa, consistent with the mobility of the polymerase binding protein observed by SDS-PAGE. Since this gene had not been previously characterized, we named it Pob3. A BLAST search of the GenBank database (61) revealed that Pob3 is similar to an HMG1-like protein found in a wide variety of organisms. *S. cerevisiae* Pob3 is most similar to the mouse HMG1-like protein (70), having 35% identity and 50% similarity over a region of ~500 amino acids spanning the entire length of Pob3 and the amino-terminal three-quarters of the HMG1-like protein (Fig. 9). A consensus sequence was established by comparing Pob3 with the HMG1-like proteins from mouse (70), human (10), chicken (77), rat (77), *Drosophila* (9), *Arabidopsis* (85), and periwinkle (Fig. 9A). Pob3 had 33% identity and 45% similarity with the HMG1-like protein from vertebrates but only 15% identity and 20% similarity with all of the HMG1-like proteins when the fly and plant protein sequences were included in the alignment.

The functions of these HMG1-like proteins are not known, although they have been implicated as components of chromatin. The mouse (T160) (70) and human (SSRP1) (10) HMG1-like proteins were identified through their binding to structurally altered forms of DNA. These proteins are called HMG1-like because they have ~50% identity within a region of 80 amino acids near their carboxy termini with the HMG B box of the abundant chromatin protein, HMG1 (22, 80). However, the similarity between Pob3 and the HMG1-like proteins diverges at this point; there is no HMG box or any other known DNA binding motif found in Pob3.

One striking feature of Pob3 is its acidic, serine-rich carboxy terminus; of the 57 residues from 489 to 546, 44% are acidic and 18% are serine (Fig. 9A). This same feature is found in the Cdc68 protein: of the 65 amino acids from 957 to 1021, 55% are acidic and 18% are serine (67).

Genetic analysis of *POB3* demonstrates that it is essential for viability. We deleted one copy of the *POB3* gene from a diploid strain and replaced it with a vector containing the *TRP1* gene. This strain was sporulated, and tetrads were dissected, resulting in two viable spores without the *TRP1* marker and two inviable spores, demonstrating that *POB3* is essential for viability. Of the 93 tetrads examined, 56 segregated 2:2 for viability and 37 had only one viable spore (presumably because of an overall poor spore viability). The inviable microcolonies (112 in total) were dissected and examined for cell morphology. All of these contained 1 to 10 cells, with an average of 4 cells, suggesting that the Pob3 protein is stable enough to be passed on to progeny for about two to four generations before protein levels drop below a critical threshold level required for viability, or that two to four divisions can occur in the absence of Pob3. The 468 total cells that were examined possessed the

following morphologies: 33% were unbudded, 7% were small budded, 23% were large budded, and 37% were large or multiply budded and pleiomorphic.

DISCUSSION

DNA replication in prokaryotes is catalyzed by large multiprotein complexes, and the available evidence suggests that eukaryotes have a similar organization (39, 73). Studies of the replication of the eukaryotic virus SV40 revealed the core replication machinery in mammalian cells (44, 45, 74, 76, 82, 83), and equivalent proteins have been identified in yeasts (3, 6, 14–16, 23, 37, 63, 71, 86). The replication of SV40 DNA is exempt from several types of control that are necessary to produce accurate copies of the cellular genome (65). We suspect that the genomic replisome interacts with other proteins to acquire the activities that execute this regulation and have therefore used protein affinity chromatography with a component of the replication complex, Pol1, which is the catalytic subunit of DNA polymerase α, to identify more peripheral or temporary components of the replisome. This strategy allows the identification of relatively weak interactions due to the high concentration of binding sites on an affinity matrix (27, 81).

In previous studies, we found that the most abundant protein identified by using this technique to be Ctf4 (55). While the function of this protein in DNA metabolism remains under investigation, cells lacking this factor have several defects in chromosome metabolism, apparently reflecting inefficient DNA replication or repair (54). The genetic evidence supports a role for this polymerase binding protein in promoting fidelity of chromosome duplication (40, 54, 72), providing confidence that the approach of affinity chromatography can provide useful and unique information about the architecture of the eukaryotic replication complex. We have now extended these studies to identify other polymerase binding proteins.

We find that Cdc68 and Pob3 proteins bind specifically to Pol1 and that the binding of both proteins was enhanced when Ctf4 was absent from extracts. This finding suggests that Cdc68 and/or Pob3 compete with Ctf4 for binding to the polymerase. Since the amount of Cdc68 and Pob3 that bound to a Pol1 matrix increased when Ctf4 was absent even though the total amount of these proteins was unchanged in *ctf4-Δ4* extracts compared to WT cells, it seems unlikely that Cdc68/Pob3 and Ctf4 bind simultaneously to the same molecule of Pol1. Also, Pol1 coimmunoprecipitated with either Cdc68 or Ctf4 from whole-cell lysates, but no interaction was observed between Cdc68 and Ctf4 with this technique, suggesting that the interactions between Cdc68-Pol1 and Ctf4-Pol1 may be mutually exclusive.

Because Cdc68 and Pob3 both show enhanced binding to Pol1 in the absence of Ctf4, it is possible that these two proteins form a complex, of which only one member contacts Pol1. Consistent with this possibility, Pob3 was coimmunoprecipitated with Cdc68 from whole-cell extracts. Furthermore, the 120-kDa Cdc68 protein elutes from a size exclusion matrix at a position corresponding to a size of 500 to 600 kDa (reference 5 and our unpublished results), also consistent with its inclusion in a complex. However, we do not yet know whether Pob3 is a component of this complex.

Cdc68 and Pob3 bind to Pol1 on an affinity column, but do these interactions reflect an important interaction among these proteins in vivo? Pol1 coimmunoprecipitated with Cdc68 and immunoprecipitated with an anti-Pob3 antibody from whole-cell lysates, which is consistent with these proteins directly contacting Pol1 in cells. Furthermore, double-mutant combinations of *cdc68*, *cdc17*, and *ctf4* mutations cause more severe phenotypes than do any of the single mutations. The effect is not general, since combinations of these mutations with several other mutations cause subtle, if any, changes. The presence of a normal binding partner and formation of a complex could be especially important for stabilizing temperature-sensitive protein products against increases in temperature. We therefore interpret the decreased temperature resistance observed to mean that Ctf4 and Cdc68 bind to and stabilize Pol1 in vivo.

In addition to interactions observed between mutated genes, overexpression of normal Cdc68 in a *cdc17-1* or a *cdc17-2* mutant decreased cell viability. Overexpression of Pol1 or Ctf4 in a *cdc68-1* mutant also decreased cell viability more dramatically than when either of these proteins was overexpressed in a WT strain. One possible explanation for this is that Cdc68 and Pol1 are components of a complex and that increasing the levels of one member in the presence of a mutant form of another member disrupts a critical stoichiometry that is necessary for optimal function of the complex. Another possibility is that the increased levels of Cdc68 sequester Pol1, thereby preventing an association with another protein, such as Ctf4, that may be required for a different function. Although either of these explanations for overexpression of a protein in a mutant strain could be applied here, we favor the latter since Cdc68 and Ctf4 appear to compete for binding to Pol1.

We were unable to verify a physical interaction between Cdc68-Pol1 and Ctf4-Pol1 by using a two-hybrid assay (not shown). While this does not support an interaction, given the uncertainties inherent in observing activation with this method, lack of a signal cannot be interpreted to suggest that no interaction occurs. The results from affinity chromatography, immunoprecipitation, mutant interaction, and overexpression experiments all strongly suggest that interactions between Cdc68 and Pol1 and between Ctf4 and Pol1 exist in vivo.

Additional evidence in favor of a role for Cdc68 in DNA metabolism comes from the observation that cells with the *cdc68-1* mutation have elevated rates of chromosome fragment loss relative to that of WT cells when assayed either at the semipermissive temperature or after a brief incubation at a nonpermissive temperature, suggesting a role for Cdc68 in chromosome transmission. Observation of this destabilization was restricted to an assay involving loss of a telocentric chromosome III fragment. No change in chromosome loss or recombination rates was observed in *cdc68-1* cells in assays using intact chromosomes. In the chromosome fragment assay, loss events arise as a 1n+1 cell becomes euploid (1n), while in the other assays a diploid becomes aneuploid (2n to 2n−1). The chromosome fragment assay also uses an unusual telocentric fragment that is less stable than intact chromosomes. It is possible that this assay is simply more sensitive than assays using intact chromosomes or that it reveals a feature of chromosome stability that is available only to intact metacentric chromosomes. Since plasmids are also less stable than chromosomes, we tested the ability of *cdc68-1* cells to maintain a single-copy plasmid. To our surprise, its stability was normal. This finding indicates that some feature of the telocentric fragment of chromosome III is uniquely prone to instability when cells are limited for Cdc68 function. While deletion of *CTF4* did affect the stability of intact chromosomes, it also had a more dramatic effect on the stability of the chromosome fragment: the loss rate of 27% observed was much higher than the rate for chromosome V and could not be similar to the rate for other chromosomes since this would lead to severely impaired viability.

Since *CDC68* was initially isolated in screens that suggest that it is a regulator of general transcription in the same class of elements as the histones (52), we determined whether the

density of nucleosomes on a single-copy plasmid was altered in a *cdc68-1* mutant, using 2D chloroquine gel electrophoresis. Alterations in the levels of histones H2B (*SPT12*) and H4 affected this property (33, 38), but no change in nucleosome density was detected in plasmids from *cdc68-1* cells compared to that of the WT at 32°C (data not shown). This assay detects the number of nucleosomes present on the plasmid in vivo, and so this result suggests that chromatin with a normal number of nucleosomes is formed on plasmids in *cdc68-1* mutant cells.

Mutations in *POL1* and some other genes involved in replication cause cells to have elongated telomeres (1, 17). To determine whether Cdc68 alters the role of Pol1 in telomere metabolism, we measured telomere length in *cdc68-1* cells alone and in combination with *pol1* mutants. *cdc68-1* did not significantly affect telomere length in these tests (data not shown).

Deletion of *POB3* was lethal, and attempts to obtain a conditional lethal allele by using the plasmid shuffle technique revealed that the *POB3* sequence becomes rearranged in *E. coli*, and so similar experiments to assess its role cannot be conducted yet. However, Pob3 has some interesting similarities to Cdc68. The binding of both proteins to Pol1 is enhanced in the absence of Ctf4, suggesting a competition between one of these proteins with Ctf4 for binding to the polymerase. Both genes are essential, and both proteins have serine-rich, acidic carboxy-terminal tails (Ctf4 does not have these features). Singer et al. (70a) found that a high-copy-number plasmid containing the *CDC68* ORF with a stop codon inserted 45 codons upstream of this region (corresponding to amino acid position 912) fails to complement the temperature sensitivity of either *cdc68-1* cells or a *cdc68* null mutant, suggesting that the carboxyl terminus is required for the essential function of the Cdc68 protein (although it has not been established whether the carboxyl terminus is merely required for proper folding and/or stabilization of the Cdc68 protein product). Pob3 was coimmunoprecipitated with Cdc68 and Pol1 was immunoprecipitated from whole-cell lysates by using an antiserum against the amino terminus of Pob3, consistent with these proteins forming a complex in the cell.

Pob3 has a striking sequence identity with a vertebrate HMG1-like protein (10, 70, 77). Because of their ubiquity and sequence conservation, HMG1-like proteins have been proposed to play fundamental roles in chromatin structure, DNA replication, and transcription (9, 10, 70). The function of the HMG1-like protein is not yet known; however, the mouse (70) and human (10) HMG1-like proteins were identified because of their ability to bind structurally altered forms of DNA. The DNA binding domain of the mouse HMG1-like protein was mapped to the HMG box (70). However, Pob3 does not contain an HMG box. Therefore, the one link between the structure and function of these HMG1-like proteins is missing in the Pob3 sequence. One possibility is that Pob3 is a functional equivalent of these HMG1-like proteins but that Pob3 interacts with DNA indirectly through another protein. Another possibility is that Pob3 is functionally distinct from these HMG1-like proteins; note that their carboxy termini are unique. Clearly, elucidation of the essential function of Pob3 and the biological significance of the interaction between Pob3 and Pol1 await the acquisition of a conditional mutation of the *POB3* gene.

Why does Pol1 interact with Cdc68 and Pob3? One model is that DNA polymerase α must be recruited to origins, or to a chromatin template, and that Cdc68 and Pob3 facilitate this process. While the details of this reaction are obscure, at some point Pol1 must be recruited to the replication complex. It is known that the origin recognition complex binds to autonomously replicating sequence elements to promote the assembly of the replisome (4, 46); perhaps this complex is built on chromatin whose properties are partially determined by Cdc68 or Pob3. Initiation of DNA replication shares features and components with initiation of transcription, and so it is possible that proteins that affect chromatin composition or function are used in both processes. We suggest that Pol1 might be recruited to origins through interaction with Cdc68 and Pob3. Alternatively, Cdc68 and Pob3 could be general chromatin proteins, and Pol1 binds to them to remain in the vicinity of its substrate. Note that Cdc68 and Pob3 could be general chromatin proteins and still not alter the density of nucleosomes.

Why do Cdc68/Pob3 and Ctf4 appear to compete for binding to Pol1? Polymerase α appears to have two distinct roles in replication. It must be assembled into an initiation complex at origins, possibly before the origin actually functions, and it must be a part of the elongation complex during S phase. Conceivably Pol1 associates with various proteins in a cell cycle-dependent manner to orchestrate this switch. Cells with the *cdc68-1* mutation arrest in G_1 with a 1C DNA content, whereas cells lacking Ctf4 delay in G_2 with a 2C DNA content. Perhaps Pol1 associates initially with Cdc68 to start replication and later with Ctf4 to facilitate the switch between initiation and elongation. This would be consistent with the phenotype of a *CTF4* deletion (54); loss of the efficiency of switching would cause problems with coordination but would allow replication to be completed. This is also consistent with the observation that both Ctf4 and Cdc68 appear to compete for binding to Pol1 in a mutually exclusive manner and with the fact that the triple mutant is no more temperature sensitive than the most severe double mutant. If Pol1-Cdc68 and Pol1-Ctf4 complexes act to perform independent essential functions, and both interactions stabilize mutant Pol1 molecules, then destabilizing both complexes would be no worse than destabilizing only one.

This model predicts that initiation of replication should be inefficient in a *cdc68-1* mutant but that elongation should be normal. This would explain the lack of a recombination phenotype in the *cdc68-1* cells even when chromosome fragment loss was increased but leaves the plasmid stability enigmatic unless linearity or overall size are important for the *CDC68* requirement. Perhaps the single origin on a small element is sufficient to provide a high density of origins per DNA sequence, while the chromosome III fragment has a low density. The interactions between Pol1 and its binding proteins and the efficiency of initiation are currently under investigation to test these predictions.

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