Temperature-Sensitive *cdc7* Mutations of *Saccharomyces cerevisiae* Are Suppressed by the *DBF4* Gene, Which Is Required for the G₁/S Cell Cycle Transition

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Manuscript received August 13, 1991

Accepted for publication December 20, 1991

ABSTRACT

When present on a multicopy plasmid, a gene from a *Saccharomyces cerevisiae* genomic library suppresses the temperature-sensitive *cdc7-1* mutation. The gene was identified as *DBF4*, which was previously isolated by complementation in *dbf4-1* mutant cells and is required for the G₁ → S phase progression of the cell cycle. *DBF4* has an open reading frame encoding 695 amino acid residues and the predicted molecular mass of the gene product is 80 kD. The suppression is allele-specific because a *CDC7* deletion is not suppressed by *DBF4*. Suppression is mitosis-specific and the sporulation defect of *cdc7* mutations is not suppressed by *DBF4*. Conversely, *CDC7* on a multicopy plasmid suppresses the *dbf4-1*, -2, -3 and -4 mutations but not *dbf4-5* and *DBF4* deletion mutations. Furthermore, *cdc7* mutations are incompatible with the temperature-sensitive *dbf4* mutations. These results suggest that the *CDC7* and *DBF4* polypeptides interact directly or indirectly to permit initiation of yeast chromosome replication.

INITIATION of chromosome replication in eukaryotes is tightly coordinated in the cell cycle. In the yeast *Saccharomyces cerevisiae*, several cell-division-cycle (*cdc*) mutations that block initiation of chromosome replication have been characterized [see CAMPBELL (1986), PRINGLE and HARTWELL (1981) and NEWLON (1988) for review], for example, *cdc28*, -4, -6 and -7. The execution points of the corresponding genes are unique during the cell cycle and the cell must progress through a conditional series of steps (*i.e.*, *CDC28* → *CDC4* → *CDC7*) before initiation of chromosome replication can occur (CAMPBELL 1986; PRINGLE and HARTWELL 1981; NEWLON 1988; HERFORD and HARTWELL 1974).

The *CDC28*, -4, -6 and -7 genes have been cloned and biochemical and genetic studies (LORINCZ and REED 1984; PATTERSON *et al.* 1986) have revealed that the *CDC28* and *CDC7* polypeptides have protein kinase activity, implicating protein phosphorylation in the G₁ → S phase progression of the cell cycle. The predicted amino acid sequence of the *CDC6* gene has a nucleoside triphosphate binding consensus sequence (LISZIEWICZ *et al.* 1988; ZHOU, HUANG and JONG 1989) and *CDC4* exhibits some amino acid sequence similarity to the *ets* oncogene (PETERSON *et al.* 1984). While considerable information is now available about these genes, their roles in replication and possible interactions among their gene products have not been

determined. The approach we have taken is to isolate suppressors of the *cdc* mutations, particularly *cdc7*, because the *CDC7* gene product is believed to be required at the last step prior to initiation of chromosome replication (CAMPBELL 1986; PRINGLE and HARTWELL 1981; NEWLON 1988). A gene suppressing *cdc7* mutations was isolated from a yeast genomic library constructed with a high copy number vector. Restriction mapping and complementation analyses with the isolated gene revealed that it is identical to *DBF4*, which we isolated independently (CHAPMAN and JOHNSTON 1989). *dbf4*, as well as *dbf1*, -2 and -3 mutants, are defective in DNA synthesis and exhibit a dumbbell terminal morphology at the restrictive temperature (JOHNSTON and THOMAS 1982a,b). The execution point of *DBF4* in the cell cycle was mapped after that of *CDC4*, but before that of *CDC7* (JOHNSTON and THOMAS 1982b). In this report, we describe the nucleotide sequence of *DBF4* and genetic analyses of *CDC7* and *DBF4*. Based on these data, we suggest that both gene products must interact to facilitate initiation of yeast chromosome replication.

MATERIALS AND METHODS

Strains and media: Yeast strains used in this study are listed in Table 1. KKY228 was constructed by gene replacement (ROTHSTEIN 1983) with the *cdc7-1* gene on pKK319. KKY701 (*dbf4::URA3/DBF4*) and KKY711 (*cdc7::LEU2/CDC7*) diploid strains were constructed by replacing the wild-type chromosomal gene with the *dbf4::URA3* gene on

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TABLE 1

Yeast strains

Strain	Genotype	Source
208	<i>MATa cdc7-1 ura3-52 leu2-3, 112</i>	PATTERSON <i>et al.</i> (1986)
136	<i>MATα cdc7-2 ura3-52 leu2-3, 112</i>	PATTERSON <i>et al.</i> (1986)
209	<i>MATa cdc7-3 ura3-52 leu2-3, 112</i>	PATTERSON <i>et al.</i> (1986)
142	<i>MATa cdc7-4 ura3-52 leu2-3, 112</i>	PATTERSON <i>et al.</i> (1986)
L128-2D	<i>MATa dbf4-1 ura3 trp1</i>	CHAPMAN and JOHNSTON (1989)
L128-1D	<i>MATa dbf4-1 leu2-3, -112 ura3-52</i>	From cross L128-2D with CG379 (DYKSTRA <i>et al.</i> 1991)
L202-1A	<i>MATa dbf4-2 ura3 leu2 trp1</i>	CHAPMAN and JOHNSTON (1989)
KKY228	<i>MATα cdc7-1 ura3-52 leu2 trp1-289</i>	This study
KKY401-10B	<i>MATα dbf4-1 ura3 trp1 leu2</i>	This study
KKY700	<i>MATa/MATα ura3-58/ura3-58 leu2-3, -112/leu2-3, -112 trp1- 289/trp1-289</i>	Our laboratory stock
KKY701	<i>MATa/MATα DBF4/dbf4::URA3 ura3-52/ura3-52 leu2-3, 112/ leu2-3, 112 trp1-289/trp1-289</i>	This study
KKY701-13AC	<i>MATα dbf4::URA3 ura3-52 leu2-3, 112 trp1-289 [pKK821]</i>	This study
KKY711	<i>MATa/MATα CDC7/cdc7::LEU2 ura3-52/ura3-52 leu2-3, 112/ leu2-3, 112 trp1-289/trp1-289</i>	This study
KKY724	<i>MATa/MATα CDC7/cdc7-2 dbf4-1/ DBF4 ura3/ura3 leu2/leu2</i>	136 × L128-1D
KKY725	<i>MATa/MATα cdc7-1/CDC7 DBF4/ dbf4-1 ura3/ura3 leu2/leu2</i>	208 × KKY401-10B
KKY727	<i>MATa/MATα cdc7-4/CDC7 DBF4/ dbf4-1 ura3/ura3 leu2/leu2</i>	142 × KKY401-10B
KKY743	<i>MATα dbf4-3 ura3 leu2 trp1</i>	This study
KKY744	<i>MATα dbf4-4 ura3 leu2 trp1</i>	This study
KKY745	<i>MATα dbf4-5 ura3 leu2 trp1</i>	This study

pKK716 and the *cdc7::LEU2* gene on pKK710, respectively. These constructions were verified by Southern blotting of chromosomal DNA digested with the appropriate restriction enzyme(s) followed by DNA-DNA hybridization. Yeast cells were grown in supplemented minimal medium or YPAD (SHERMAN, FINK and HICKS 1986). To isolate Ura⁻ yeast cells (BOEKE *et al.* 1987), 1 mg/ml of 5-fluoroorotic acid (FOA) (Sigma Biochemicals) and 20 µg/ml uracil were added in the medium. *Escherichia coli* strains DH5α (MANIATIS, FRITSCH and SAMBROOK 1982) and JM101 (MESSING, CREA and SEEBERG 1981) were used to prepare plasmids.

Plasmid DNA: The *S. cerevisiae* genomic library in the YEpl 213 vector (SHERMAN, FINK and HICKS 1986) has been described (HASEGAWA, SAKAI and SUGINO 1989). For subcloning both *CDC7* and *DBF4*, either YCplac22, YCplac33, YCplac111, YEplac112, YEplac195 or YEplac181 (GIETZ and SUGINO 1988) were used. pKK716 containing *dbf4::URA3* was constructed by replacing the 0.3-kb *Bgl*II fragment of *DBF4* (Figure 1) with the 1.1-kb *URA3* gene. pKK710 containing *cdc7::LEU2* was constructed by replacing the 0.4-kb *Eco*RV fragment of *CDC7* (PATTERSON *et al.* 1986) with the 2.2-kb *LEU2* gene. pUC18 containing the 3.3-kb *Bgl*II-*Eco*RI *cdc7-1* gene (pKK319) was identified from the library constructed with the strain 208 (*cdc7-1*) genomic DNA digested with *Bgl*II and *Eco*RI enzymes by colony hybridization to the 5'-³²P-labeled synthetic oligonucleotide probe corresponding to a part of *CDC7* (5'-ATG-ACAAGCAAAACGAAGAATATCGATGATATACCT^{3'}, from the nucleotide number 1 to 36 in the coding region) (PATTERSON *et al.* 1986). To recover plasmid DNA from

yeast cells, total DNA was extracted as described by SHERMAN, FINK and HICKS (1986) and used for *E. coli* transformation. In most cases, the electrophoration method (Bio-Rad Laboratories) was used for *E. coli* transformation.

DNA manipulations and genetic techniques: Cloning, preparing plasmid DNA from *E. coli* and Southern hybridization were performed as described (MANIATIS, FRITSCH and SAMBROOK 1982). DNA sequence analysis was carried out by the dideoxy chain-termination method (SANGER, NICKLEN and COULSON 1977) using double-stranded DNA and Sequenase (United States Biochemical) and both strands of the DNA were at least once sequenced. Yeast transformation was carried out by using lithium acetate methods (ITO *et al.* 1983), and other yeast genetic manipulations were as described by SHERMAN, FINK and HICKS (1986).

Isolation of new temperature-sensitive *dbf4* mutations: New temperature-sensitive alleles of *dbf4* mutation (*dbf4-3*, *-4* and *-5*) (Table 1) were isolated by the plasmid-shuffling method described previously (ARAKI *et al.* 1991). We do not know whether *dbf4-3* and *-4* are different from the alleles previously isolated (JOHNSTON and THOMAS 1982a,b), but *dbf4-5* is a new allele (see the text).

Other materials: A Multiprime labeling kit (Amersham) was used to label DNA probes. 5'-End of the synthetic oligonucleotide was labeled with [γ-³²P]ATP and T4 polynucleotide kinase as published elsewhere (MANIATIS, FRITSCH and SAMBROOK 1982). All restriction enzymes, *E. coli* Klenow DNA polymerase I, T4 polynucleotide kinase, T4 DNA ligase, and S1 nuclease were from IBI, New England BioLab, BRL, Boehringer Mannheim, Amersham,

FIGURE 1.—Restriction map of the gene suppressing *cdc7-1* and construction of a deletion mutant. (A) Each DNA fragment shown by a heavy line was cloned into the multicopy yeast vector YEplac 195 (Gietz and Sugino 1988) and introduced into KKY228 cells by transformation. The transformants were placed at 33° for 3 days and cell growth was examined. The lowest line shows the *DBF4* DNA fragment isolated by Chapman and Johnston (1989). (B). To construct a deletion/insertion mutation of *DBF4*, the 0.3-kb *Bgl*II fragment was replaced with 1.1-kb *Bam*HI *URA3* DNA. The open arrow indicates the ORF of *DBF4*.

or United States Biochemical and used as recommended by the supplier.

RESULTS

Suppression of *cdc7-1* by a gene on a multicopy plasmid: Strain KKY228 (*cdc7-1*) is temperature-sensitive for growth on a YPAD plate above at 33°. To isolate multicopy suppressors which is able to rescue a temperature-sensitive *cdc7* mutant phenotype, *cdc7-1* cells were transformed with a yeast genomic DNA library in YEpl213 and incubated at 24° on Leu⁻ plates for two days and at 33° for 5 days. Among about 160,000 transformants grown at 24°, 18 also grew at 33°. When they were streaked on a fresh plate, two, T33 and T34, formed much smaller colonies at 33° than the others, but their colony size was uniform. Total DNA was extracted from the 18 transformant cells, electrophoresed, transferred to a nitrocellulose filter, and hybridized with radioactive DNA fragments of *CDC7* and pBR322. Plasmid DNA from all transformants except T33 and T34 hybridized with both fragments, indicating that they contained *CDC7*. Thus, they were not characterized further. DNA from T33 and T34 hybridized to ³²P-labeled pBR322 DNA but not to the radioactive *CDC7* DNA (data not shown), indicating that T33 and T34 cells harbored a plasmid containing a DNA insert other than *CDC7*.

Restriction enzyme analysis showed that the plasmids from both T33 and T34 cells possessed an identical 6.2-kb insert. Deletion analysis followed by re-

transformation using the plasmid DNA from T33 cells showed that an *Eco*RI-*Pst*I fragment was the minimum fragment able to suppress temperature-sensitive cell growth phenotype of *cdc7-1* mutation (Figure 1). The restriction map of this fragment with *Eco*RV, *Pst*I, *Sst*I, *Eco*RI, *Ava*I and *Cla*I was identical to that of *DBF4*, which was isolated by complementation of *dbf4-1* (Chapman and Johnston 1989). Furthermore, a partial nucleotide sequence of the *Eco*RI-*Pst*I fragment was identical with part of *DBF4* (Chapman and Johnston 1989). We therefore tested suppression of *cdc7-1* by *DBF4*. As shown in Table 2 and Figure 1, *DBF4* allowed temperature-sensitive *cdc7-1* cells to grow on either a Leu⁻ or YPAD plate at 33°. Furthermore, the plasmid (pKK616) from T33 cells complemented *dbf4-1* and *dbf4-2* as well as did the authentic *DBF4* gene (Chapman and Johnston 1989). We conclude from these results that the gene on either YEpl213 or YRp12 plasmid suppressing *cdc7-1* is *DBF4*. The suppression of *cdc7-1* by *DBF4* was temperature-dependent. The *cdc7-1* cells having *DBF4* formed much smaller colony at 35° than at 33° and the cells did not form colony at 37°. At those temperatures, however, *cdc7-1* cells containing authentic *CDC7* on a plasmid formed the same size of colony as wild-type cells.

To test the effect of gene dosage on the suppression of *cdc7-1*, *DBF4* was transferred to a single-copy plasmid containing a *CEN4* sequence. As shown in Figure 2, *cdc7-1* cells having *DBF4* on a *CEN4* plasmid did not form colony on a YPAD plate at 33°. Thus, the

TABLE 2

DBF4 on a multicopy plasmid suppresses *cdc7* mutations

Relevant genotype	Temperature (°C)	Plasmids		
		pKK616 (<i>DBF4</i>)	YEplac195	pKK709 (<i>CDC7</i>)
<i>cdc7-1</i>	33	+	—	++ ^b
	35	+/-	—	++
	37	—	—	++
<i>cdc7-2</i>	33	+	—	++
	35	+/-	—	++
	37	—	—	++
<i>cdc7-3</i>	33	+	+	++
	35	+/- ^c	+/- ^c	++
	37	—	—	++
<i>cdc7-4</i>	33	+	—	++
	35	+/-	—	++
	37	—	—	++
$\Delta cdc7^d$	25	—	—	++

KKY228 cells were transformed to Ura⁺ with pKK616 (*DBF4* in YEplac195), YEplac195, or pKK709 (*CDC7* in YEplac195). The resulting transformants were streaked on YPAD plates, incubated at the indicated temperatures for more than 3 days and their colony formation was examined.

^a + indicates that the cells formed colonies on the plate at the indicated temperature and — indicates that the cells did not form colonies at that temperature.

^b *cdc7* mutant cells harboring pKK709 formed much larger colonies than the cells harboring pKK616.

^c Although cells harboring either pKK616 or YEplac195 formed colonies at 35°, the colony size of cells containing pKK616 was much larger than that with YEplac195. None of the cells formed colonies at temperatures higher than 35°.

^d Due to the lethality of *cdc7Δ::LEU2*, the KKY711 diploid cells harboring pKK709 were sporulated and microdissected and a Ura⁺ Leu⁺ spore was isolated. This cell, KKY711-[pCDC7-URA3], was further transformed with pDBF4-TRP1 plasmid. The resulting cells were plated on FOA plates to test cell growth; no cells grew.

DBF4 copy number is important for suppression.

***cdc7Δ* mutation is not suppressed by *DBF4*:** We investigated whether the suppression of *cdc7-1* by *DBF4* is allele-specific. As shown in Table 2, all temperature-sensitive alleles of *cdc7* mutations (*cdc7-1*, 7-2, 7-3, and 7-4) were suppressed to varying degrees by *DBF4* on a multicopy plasmid. To test whether a *cdc7Δ* mutation is suppressed by *DBF4*, the heterozygous diploid strain KKY711 (*cdc7::LEU2/CDC7*) with the *DBF4* gene on a multicopy plasmid containing *URA3* (pKK616) was sporulated, tetrads were dissected, and the resulting spores were incubated at 24°. We obtained only two viable spores from each tetrad (total of 27 tetrads analyzed). All viable spores were Leu⁻ and 55% were Ura⁺. More than 90% of the inviable spores exhibited a dumbbell morphology on a YPAD plate as observed in all DNA replication mutants incubated at a nonpermissive temperature. In contrast, the same diploid strain with *CDC7* on a high copy number plasmid produced four viable spores in 65% of the dissected tetrads (total of 24 tetrads were analyzed), and these spores exhibited 2:2

cdc7-1

FIGURE 2.—*DBF4* on a multicopy plasmid suppresses the temperature-sensitive growth phenotype of *cdc7* mutations. KKY288 (*cdc7-1*) cells transformed by the indicated plasmid DNA were patched on Leu⁻ plates and incubated at 24° for 4 days. Then, the cells grown were replica-plated on a YPAD plate and incubated at 33° for 5 days and photographed. The pKK821 plasmid was constructed from the plasmid YCplac22 containing *CEN4* (GIETZ and SUGINO 1988) by inserting the *Bam*HI-*Pst*I fragment of *DBF4* (Figure 1). A and B represent two independent transformants of KKY228 with each plasmid.

Leu⁺:Leu⁻ segregation. These results clearly demonstrate that *DBF4* does not suppress the *CDC7* deletion mutation but can suppress all other *cdc7* temperature-sensitive alleles so far tested.

Mitosis-specific *cdc7* suppression by *DBF4*: It is known that *CDC7* is also required during meiosis, not for initiation of premeiotic DNA replication but for premeiotic homologous recombination (SCHILD and BYERS 1978). We therefore investigated whether *DBF4* suppresses sporulation defect phenotype of *cdc7* mutations at the restrictive temperatures. In contrast to the above results, the sporulation defect of *cdc7* mutations at the restriction temperatures was not suppressed by *DBF4* on a multicopy plasmid (Table 3). The failure of suppression by *DBF4* is not due to inability of expression of *DBF4* in meiosis, since *DBF4* is required for meiosis (Table 3) and it was confirmed that the *DBF4* gene complements sporulation defect of *dbf4-1* mutation (Table 3). We therefore conclude that suppression of *cdc7* mutations by *DBF4* is mitosis-specific.

Allele specific, mitotic suppression of *dbf4* by *CDC7*: As shown in Table 4, *CDC7* on a high copy number plasmid suppressed the *dbf4* mutations by an allele-specific manner. *CDC7* suppressed the temperature-sensitive *dbf4-1*, -2, -3 and -4 mutations but not the temperature-sensitive *dbf4-5* and *dbf4* deletion mutations. Furthermore, as observed with *DBF4* suppression of *cdc7* mutations, suppression by *CDC7* occurs only with a multicopy plasmid (data not shown). *dbf4* suppression by *CDC7* was mitotic specific like *cdc7* suppression by *DBF4* and *CDC7* could not suppress sporulation defect of *dbf4-1* mutation (Table 3).

Incompatibility of *dbf4* and *cdc7* mutations: To

TABLE 3

Suppression of meiotic defect of either *dbf4-1* or *cdc7-1* mutation

Relevant genotype	Temperature (°C)	Sporulation efficiency (% asci) with plasmid:		
		<i>DBF4</i> in YE _p	<i>CDC7</i> in YE _p	YE _p
<i>dbf4-1/dbf4-1</i>	25	40.2 ± 9.3	37.2 ± 2.1	32.2 ± 2.7
	35	25.8 ± 7.3	4.2 ± 1.6	8.7 ± 2.2
<i>cdc7-1/cdc7-1</i>	25	26.2 ± 1.4	25.7 ± 3.1	21.5 ± 5.0
	32	2.0 ± 0.7	13.9 ± 1.7	2.0 ± 1.2

Diploid cells L128-2D/KKY401-10B (*MAT α /MAT α dbf4-1/dbf4-1 ura3/ura3 trp1/trp1 LEU2/leu2*) and 208/KKY228 (*MAT α /MAT α cdc7-1/cdc7-1 ura3-52/ura3-52 leu2-3, -112/leu2 TRP1/trp1-289*) were transformed with either pKK709 (*CDC7*), pKK616 (*DBF4*) or YEplac195 and the transformants were grown in SPS medium to 5×10^7 cells/ml and the cells were resuspended to the acetate medium (SUGINO, NITISS and RESNICK 1988). Sporulation efficiency (% asci) was measured as published (SCHILD and BYERS 1978) at the indicated temperatures.

obtain a *cdc7-1 dbf4-1* double mutant, the diploid strain KKY725 (*ura3-52/ura3-52 dbf4-1/DBF4 CDC7/cdc7-1*) was sporulated at 24°. To avoid any possibility of incompatibility of *dbf4* with *cdc7* mutation, the strain was first transformed with pKK709 containing *CDC7* and *URA3* before sporulation. The resultant tetrads were dissected and incubated at 24°. Tetrads giving four viable Ura⁺ colonies were tested whether they are able to grow without the plasmid pKK709 (*CDC7*) by incubating on FOA plates at 24°. From 39 tetrads analyzed, 7, 22 and 10 tetrads exhibited 4, 3, and 2 spores viable, respectively. Each tetrad exhibiting 2 spores growing on a FOA plate was further examined by crossing with the strain carrying either *cdc7-1* or *dbf4-1* mutation. The two viable colonies on a FOA plate had neither *cdc7-1* nor *dbf4-1* mutation, whereas the two inviable colonies on a FOA plate had both *cdc7-1* and *dbf4-1* mutations. From this result, we conclude that a strain containing both *cdc7-1* and *dbf4-1* mutations is inviable without the *CDC7* gene. Similar results were obtained with combinations involving *dbf4-1* and *dbf4-2* with *cdc7-2*, *cdc7-3* or *cdc7-4*. Thus, *cdc7* and *dbf4* mutations are incompatible.

Division potential conferred by amount of *DBF4* gene product: The amount of *DBF4* transcript fluctuates during the cell cycle and peaks at the G₁/S boundary similarly to other genes involved in DNA replication (JOHNSTON *et al.* 1990). This suggests that the amount of *DBF4* product also increases late in G₁ phase and that the newly synthesized *DBF4* protein is required for initiation of chromosome replication. To examine this possibility, the amount of *DBF4* product was estimated cytologically by examining division potential after removing *DBF4* from *dbf4::URA3* cells.

A *dbf4::URA3* strain carrying the *DBF4* gene on a plasmid containing *CEN4* was constructed (KKY701-13AC, Table 1). As previously described (SCLAFANI *et*

TABLE 4

CDC7 on a multicopy plasmid suppresses the temperature-sensitive *dbf4* mutations

Relevant genotype	Temperature (°C)	Plasmids		
		pKK602 (<i>CDC7</i>)	YE _p 213	pKK603 (<i>DBF4</i>)
<i>dbf4-1</i>	35	+	—	+
	37	+	—	+
<i>dbf4-2</i>	35	+	—	+
	37	+	—	+
<i>dbf4-3</i>	35	+	—	+
	37	+	—	+
<i>dbf4-4</i>	35	+	—	+
	37	+	—	+
<i>dbf4-5</i>	33	—	—	+
	35	—	—	+
	37	—	—	+
$\Delta dbf4^a$	25	—	—	+

KK401-10B (*dbf4-1*), L202-1A (*dbf4-2*), KKY743 (*dbf4-3*), KKY744 (*dbf4-4*) and KKY745 (*dbf4-5*) cells were transformed to Leu⁺ with either pKK602 (*CDC7* in YE_p213) (SHERMAN, FINK and HICKS 1986), YE_p213 or pKK603 (*DBF4* in YE_p213). The growth of the transformants was examined as in Table 2. + indicates that *dbf4* mutant cells grew at the indicated temperatures on both a YPAD and synthetic complete medium (SD) plates without leucine, while — indicates that the cells did not grow under these conditions.

^a Due to the lethality of *dbf4 Δ ::URA3*, KK701 diploid cells were transformed with plasmid pKK602 and sporulated and the resulting tetrads were dissected. Only 2 viable spores were obtained from each tetrads dissected (total 24 tetrads analyzed). On the other hand, KKY701 cells harboring pKK603 gave 4 viable spores from 12 (50%) tetrads out of 24 tetrads dissected. These exhibited 2:2 segregation for Ura⁺ and 4:0 for Leu⁺.

al. 1988), the cells were plated on YPAD plates and incubated at 24°. When a daughter cell emerged, it was separated from the mother cell by micromanipulation and incubated at 24°. Analysis of 642 such cell divisions revealed 11 loss divisions, that is, divisions in which either the mother or daughter cell failed to receive a plasmid molecule and yielded microcolonies containing an average of 4.7 (range 2–10) inviable cells. Most of these inviable cells exhibited a large dumbbell shape, typical of DNA replication mutants at a restrictive temperature (PRINGLE and HARTWELL 1981). At the time of cytokinesis, therefore, a cell which lost the plasmid either has sufficient *DBF4* protein to divide at least twice. Alternatively, a cell synthesizes enough *DBF4* protein from remaining mRNA although the protein turns over quickly. This result is significantly different from similar results obtained with *CDC7* (SCLAFANI *et al.* 1988), where at least 8 divisions occurred after loss of the plasmid.

***DBF4* is essential for mitotic cell growth:** Isolation of temperature-sensitive mutation does not ensure essentiality of its gene. Therefore, we tested whether *DBF4* is required for mitotic cell growth. To do this, one chromosomal *DBF4* gene in a diploid cell was disrupted as shown in Figure 1B. The resultant diploid

-360 TATCTCTTTCATCTAATTCCTCATTACCTTTTTTATTTTATTTCTTATCGGTCTTCGACGCCAAGTTCTTTTAAATATTTACCGCAGTAAGTAAATGGTTTTTTTTTTGGCAA
 -240 ACCGCTCTTAGTTCGATCTTTGCGAAAAAGAAAAATAAGAAATAACGATAATAACGGTAATGGCTCAGGCCAAGAAAAACAAATCAAGTTGCATAGTCTATACTACGCCAGG
 -120 TGTAGATTCCCTTAGATCTGAATTCGCTTCGAGGGC TAGTTATCTTCAGAAATCAAGGTTTGAATCTGAAAGGCATTGGAAATCTGCTTTTGGTTCATATTAGAAAAAGAAAGAAAA
 1 ATGGTTTCTCAACGAAATGATAAAGGTCCTCGTTAAAGGAAACAGATACCAACTGAAACATAATAATGGAATGCCGCATCAACAGCAGCGGGCCACTTGAATGTGTTTTCT
 M V S P L K M I R S P L K E T D T N L K H N N G I A A S T T A G H L N V F S 40
 121 AACGACAATAATTCGAACAATAACCACTGAATCCTTTCCGAAGAAAAGATCTCTTGAGCGCTCGAGCTCCACAGCAGCAGCATTGCGATGAAAAGAAAGGGCCAGAAATAGAA
 N D N N C N N N N T T E S F P K K R S L E R L E L Q Q Q Q H L H E K K R A R I E 80
 241 AGGGCCAGGCTATTGAAGCGCTGTCAAGTCAGCAAGGTACGGGTCTGAAAATGTCGAGCCAAAGGTTACGCTAAGGAATTCGTTGAATGGCAACAAATGGAAAAGATAATG
 R A R S I E G A V Q V S K G T G L K N V E P R V T P K E L L E W Q T N W K K I M 120
 361 AAAAGAGATTCTCGCATTACTTTGACATTACTGATGATGATAGATGATACATATAAAGTCCAAGATGGACAAACGAGAGATTATTGAAAAGAGGGTTTCTTACATTAATAACA
 K R D S R I Y F D I T D D V E M N T Y N K S K M D K R R D L L K R G F L T L N T 160
 481 CAAATAACTCAATTTTTGACACTACTGTCACAATAGTTATCACAAGAAAGAGGCTCTTGGAGAACAATATATTACTAAAAGATACCGACATTTTATCGAGAGCTAAAAAAGTACATG
 Q I T Q F F D T T V T I V I T R R R S V E N I Y L L K D T D I L S R A K K K Y M 200
 601 AAAGTTTGGAGTTACGAAAGGCTGCCAGATTTCGAAAATCTTGATGTTGATCTTGGATCATTTAGCAAGACTAAATCTGCTCTTTAGCTGCGCCACATTCCTCAATCTTCTACAC
 K V W S Y E K A A R F L K N L D V D L D H L S K T K S A S L A A P T L S N L L H 240
 721 AATGAAAATTTATGGAACACGGAATAGAGACCCGAGAATCAAGAGCGCATATTCCTACTCTTAAATCTCTATCTATACCTTTATGACCTATGCGAACTTGGGCCCCATAATA
 N E K L Y D P R T D R D P R T K R G D I H Y F K Y P H V Y L Y D L W Q T W A P I I 280
 841 ACTTTGGAATGGAACCTCAAGAACTAACAACCTAGAGCACTACCTTACCAATATTTGAAATAGGTTTCATCGGAAGATGCCCTTTTATAGGGGATAGGAATTTATGACGAAAGTTCT
 T L E W K P Q E L T N L D E L P I L K I G S F G R C P F I G D R N Y D E S S 320
 961 TATAAGCGCGTAGTAAAGAGATACTCGAGAGACAAAGCAAAATAATGCACTGCAACTTCGTGCTCTATTTCATATCATGCCGACACCTTACTGAATACGTCATCAGTTAATGAT
 Y K R V V K R Y S R D K A N K K Y A L Q L R A L E Q Y H A D T L L N T S S V N D 360
 1081 CAAACGAAAACTAATATTCATACCTCACACATGCAACGATTCTACCAAGAGCTTCAAAAAATGGATGCAAGAAAAGCAAAAAATTTTGAAGACCGAGTTAAAGAACCGGATGAT
 Q T K N L I F I P H T C N D S T K S F K K W M Q E K A K N F E K T E L K K T D D 400
 1201 AGCGCAGTTCAAGATTCGTAATGAACATGCTGACCAACCGATGAAACAAAGAGGCTCCGTTGAAAGAAAGAAAAAGAAATAGCGATCTATAGCAGAAATGCAATAGTACCA
 S A V Q D V R N E H A D Q T D E T K E P P L K E E K E N K R S I A E E S N K Y P 440
 1321 CAGCGAAAGAGCTGGCTGCCACACCAAACTAAACCATCCAGTATTAGCTACTTTTGCAGGCAAGAACTGAAGAAAGTCCCGGATGATTGTGCACTTTGAAAACAAAGTCAGCTCAG
 Q R K E L A A T P K L N H P V L A T F A R Q E T E E V P D D L C T L K T K S R Q 480
 1441 GCATTTGAAATCAAAGCAAGTGGTGCACATCAATCTAATGATGTTGCAACCTCTTTTGGCAATGTTTGGGCCCAACAGGCAAGCGCTAGTAAAGAACATGAAGTCATTAAAGTAGA
 A F T E I K A S G A H Q S N D V A T S F G N G L G P T R A S V M S K N K S L S R 520
 1561 CTAATGGTTGATAGAAAGCTGGAGTAAAGCAGACAAATGGAATAACAAAAATATACAGCACTATAGCAACTACTGCTGAAACATCAAGGAAATAGACACAGATAGATTTAAT
 L M V D R K L G V K Q T N G N N K N Y T A T I A T A E T S K E N R H R L D F N 560
 1681 GCTTGAAGAAAGACGAGCCCTTCGAAAGAGACGGGCAAGATAGTGTCTGACACTAGAACTAATAGAAAGCCCAAGAAATTCCTCAATAGGTAGCTACCAATCAGTCTCCGACAG
 A L K K D E A P S K E T G K D S A V H L E T N R K P Q N F P K V A T K S V A D 600
 1801 TCCAAAGTTCATAATGACATCAAGATAACAACACAGAAATCTCCAACGATCGAAGAAATCAACTTCCCAAAACGTCACCTTACATTTTAAAGCAGACAGCAGACAGCAGCGG
 S K V H N D I K I T T T E S P T A S K K S T S T N V T L H F N A Q T A Q T A Q P 640
 1921 GTGAAGAAAGAAACGGTAAAGATTCGGGATCTGTGAAAATTTGCTGTGAAAATACGAATCTTTAGAACACACATAGTTTTCGAGAACGATTGTCTTTCGCTGAAAACGATTTAAAT
 V K K E T V K N S G Y C E N C R V K Y E S L E Q H I V S E K H L S F A E N D L N 680
 2041 TTTGAGGCTATTGCTTAATTGAAATCTCAGATTTCAATATAGGACACGACGTAAGTGCAGTAGCTTTTGTAGTAAATCAAAATAGTATTGTTCCGTTTCCATTGCTGCTC
 F E A I D S L I E N L R F Q I * (695)
 2161 GGAACAAAAAGCTATCAAGCGCAATGTTATTGAATCACTTTTCATCTTCCCTTGTACTTTCTTGCTATTGACTTAACTCTTATTATCTGCTCATATATATCAATTCATATATAT
 2281 ACATATACATATATATATCATCTACCTTACCTATCATTAACCTATCTTGAACAAATATGATGAGTACCCACCGCGGTAAATGTGCCAGTTTCAATTTTACCGGATA

FIGURE 3.—Nucleotide sequence of the *DBF4* gene. The translation is shown in the single-letter code below the nucleotide sequence. The numbers at the left count from the first base of the first ATG of the *DBF4* ORF. The numbers at the right are the amino acid residue numbers of the ORF. The putative regulatory sequence ACGCGT at nucleotide -235 is underlined.

strain KKY701 (*dbf4::URA3/DBF4*) was sporulated and microdissected, and the spores were incubated at 24°. In each tetrad dissected (total 24 tetrads analyzed), only two spores grew and both were Ura⁻. The inviable spores exhibited the same dumbbell shape as did *dbf4-1* and *dbf4-2* cells incubated at the nonpermissive temperature. This result indicates that *DBF4* is essential for mitotic cell growth.

***DBF4* nucleotide sequence:** To obtain a knowledge of the *DBF4* gene product, the nucleotide sequence of the *EcoRI-PstI* fragment derived from pKK616 (Figure 1) was determined by the dideoxy-termination method. It revealed one large open reading frame capable of encoding 695 amino acids with a predicted molecular weight of 79,768 (Figure 3). Computer analysis showed that *DBF4* lacks the conserved amino acid sequences found in protein kinases. However, using FASTA on the UWCG program revealed weak amino acid sequence similarity to the yeast *SIR4* gene (MARSHALL *et al.* 1987) (similarity 37.7%, identity 21.2%) and CAD protein (similarity 43%, identity 20.8%), although the biological significance of these similarities is not yet clear. The predicted pI of the gene product is about 9.4; thus, it might bind to DNA. The sequence -ACGCGT- (a *MluI* restriction enzyme recognition sequence) was found about 240 bp upstream from the first ATG in the open reading frame

(ORF). It has been postulated that this and related sequences regulate the expression of various DNA replication genes in a cell cycle-dependent manner (PIZZAGALLI *et al.* 1988). In fact, our previous studies (CHAPMAN and JOHNSTON 1989) showed that the levels of *DBF4* transcript fluctuate during the cell cycle with those of other DNA replication genes (JOHNSTON *et al.* 1990).

DISCUSSION

The *CDC7* gene encodes a protein kinase (PATTERSON *et al.* 1986; BAHMAN *et al.* 1988; HOLLINGSWORTH and SCLAFANI 1990) and its cell cycle execution point is just before S-phase. Beyond this point, protein synthesis is no longer required for initiation of chromosome replication (HEREFORD and HARTWELL 1974; PRINGLE and HARTWELL 1981; CAMPBELL 1986; NEWLON 1988). It is, however, not known whether the *CDC7* protein directly participates in the initiation of chromosome replication. Moreover, the substrates for the *CDC7* protein kinase are not known. In order to facilitate our understanding of the regulatory mechanism controlling the initiation of chromosome replication, we used a multicopy plasmid to isolate a gene that suppresses the *cdc7* mutation. This gene is identical to *DBF4*, which we previously isolated by complementation of *dbf4-1*. The *dbf4* mutants were

identified by their dumbbell-shaped terminal morphology, which is characteristic of many DNA replication mutants, and by their defect in DNA synthesis at the restrictive temperature (JOHNSTON and THOMAS 1982a,b). Suppression of *cdc7* by *DBF4* is allele specific: a *cdc7::LEU2* deletion mutation was not suppressed by *DBF4*, suggesting that *DBF4* on a multicopy plasmid is not able to bypass the *CDC7* function. Furthermore, this suppression is specific to mitotic cells, because the sporulation defect of *cdc7* mutants (SCHILD and BYERS 1978) was not suppressed. Conversely, *CDC7* on a multicopy plasmid also suppressed the temperature-sensitive *dbf4-1*, -2, -3 and -4 mutations, but not the temperature-sensitive *dbf4-5* and *dbf4::URA3* deletion mutations. All temperature-sensitive *cdc7* mutations (*cdc7-1*, -2, -3 and -4) so far tested were incompatible with the temperature-sensitive *dbf4-1* and *dbf4-2*. These data suggest that the *CDC7* product directly interacts with and forms a complex with the *DBF4* product to facilitate initiation of chromosome replication. Because the *DBF4* gene lacks amino acid sequence similarity to any known protein kinases (Figure 2), it is unlikely that it is another protein kinase able to substitute for the *CDC7* function. Instead, like the *CLN1*, 2 and 3 gene functions which interact directly with the *CDC28* protein and activate the protein kinase activity (RICHARDSON *et al.* 1989; WITTENBERG, SUGIMOTO and REED 1990), the *DBF4* protein may act as either an activator or a modulator of the *CDC7* product. However, other possibilities are equally likely. One of these is that the *DBF4* gene product is a substrate of the *CDC7* protein kinase. Another possibility is that the *CDC7* and *DBF4* proteins may act in a common biochemical pathway. To distinguish between these possibilities, antibodies against either the *CDC7* or *DBF4* gene product may be useful.

Transcriptional regulation of *CDC7* is very different from that of *DBF4*; the levels of *DBF4* transcript fluctuate in the cell cycle and peak at the G₁/S boundary (CHAPMAN and JOHNSTON 1989), as do other DNA replication-related gene transcripts (JOHNSTON *et al.* 1990). On the other hand, the levels of *CDC7* mRNA are constant throughout the cell cycle (SCLAFANI *et al.* 1988). Although not yet proven, it seems likely that the levels of both the *CDC7* and *DBF4* proteins reflect the levels of their transcripts. If that is so, the *CDC7* product, which is synthesized throughout the cell cycle, may be relatively stable and able to function for several cell generations (SCLAFANI *et al.* 1988) before the protein is either diluted or inactivated. On the other hand, the amount of *DBF4* product is only enough for one or two additional cell generations (this study).

Because new protein synthesis is still required before initiation of DNA replication after release from

the *dbf4* arrest, the execution point of *DBF4* was mapped to a time in the cell cycle earlier than that of *CDC7* (JOHNSTON and THOMAS 1982b). This result, however, may now be interpreted differently from that offered previously (JOHNSTON and THOMAS 1982b): the amount of *DBF4* protein is small, is likely to be turned over rapidly during incubation at the restrictive temperature and newly synthesized *DBF4* protein is thus required for the progression of the cell cycle after shift-down to the permissive temperature in *dbf4* cells. Therefore, the levels of *DBF4* protein may be of primary importance for the progression of the cell cycle through G₁ to S phase and may stimulate this progression by modulating the *CDC7* protein kinase activity. BAHMAN *et al.* (1988) have shown that the *CDC7* polypeptide made by *in vitro* translation has a protein kinase activity. On the other hand, the other group has observed that the polypeptide expressed in *E. coli* does not have a protein kinase activity (YOON and CAMPBELL 1991). Nonetheless, it will be interesting to learn whether the *DBF4* polypeptide further activates the *CDC7* protein kinase made *in vitro* or in *E. coli*.

Other explanations for the suppression of *cdc7* mutations by *DBF4* are equally plausible. One is that overproduction of *DBF4* protein nonspecifically stabilizes the temperature-sensitive *cdc7* protein, which thus becoming less temperature-sensitive. This, however, is less likely because *dbf4-1* on a multicopy plasmid failed to suppress *cdc7* mutations (our unpublished results). Furthermore, a 4-bp insertion at the *AccIII* site of *DBF4* (nucleotide number 2375 in Figure 3) generates a frameshift mutation producing a mutant gene (designated *dbf4-6*) encoding a polypeptide of 652 amino acids (predicted molecular weight 74,780) lacking about 40 residues at the C terminus of the *DBF4* polypeptide. This mutant allele can complement all temperature-sensitive *dbf4* mutations. However, it neither complemented the *dbf4::URA3* mutation nor suppressed temperature-sensitive *cdc7* mutations (our unpublished results). This suggests that the C-terminal region of the *DBF4* polypeptide is essential for suppression of *cdc7* mutations but is not essential for *DBF4* function when the full-size polypeptide generated by temperature-sensitive *dbf4* mutations is present. It also suggests that the *DBF4* protein is multimeric inside the cell.

dbf4 mutation was tentatively mapped at 46 cM from the centromere on the left arm of chromosome IV (CHAPMAN and JOHNSTON 1989). In that region of chromosome, another gene *CDC2*, which encodes a catalytic subunit of DNA polymerase III (δ), is located. We have tried to map more precisely *dbf4* mutation using *cdc2* and *rad55* mutations. However, we were not able to see any significant linkage between *cdc2* and *dbf4* [5 parental ditype (PD), 9 nonparental ditype

(NPD) and 31 tetratypes (T) spores]. Contrary to the previous data (CHAPMAN and JOHNSTON 1989), we observed a significant linkage between *rad55* and *dbf4* (35 PD, 2 NPD and 25 T) (K. KITADA and A. SUGINO, unpublished results). Therefore, it is likely that *DBF4* is located on the right arm of chromosome IV, instead of the left arm. This result may explain why two wild-type spores and two double-mutant spores (*cdc7 dbf4*) were unexpectedly predominant in the cross between *cdc7* and *dbf4* mutations, when we studied the incompatibility of *cdc7* and *dbf4* mutations.

In the past, attempts have been made to isolate extragenic suppressors of *cdc7* mutations after mutagenesis of *cdc7* cells (our unpublished results). So far, no *dbf4* mutation has been identified, although other mutations have been obtained (our unpublished results). This might be due to incompatibility of *dbf4* with *cdc7* mutations.

As previously demonstrated (HAYLES *et al.* 1986; HADWIGER *et al.* 1989), the approach used in this study is powerful and may be used to identify new genes which interact with any particular gene. More importantly, this is the first case providing genetical evidence of a possible direct interaction between the *CDC7* and other known cell cycle gene products. It also provides a clue to understanding how initiation of chromosome replication is regulated during the cell cycle. We hope to identify other genes whose proteins directly interact with the *DBF4* polypeptide, using the temperature-sensitive *dbf4* mutations.

After this manuscript had been submitted, we were informed that *DBF4* is allelic to *DNA52* whose mutations were isolated by other group (DUMAS *et al.* 1982) by sequence comparison and the cell cycle phenotype in *dna52-1* was the same as in *dbf4-1* (SOLOMON *et al.* 1992).

We thank MICHAEL A. RESNICK, ALAN MORRISON and NOEL LOWNDES for their critical readings of this manuscript. K.K. especially appreciates H. ARAKI's advice on yeast genetics.

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Communicating editor: M. CARLSON