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The Product of the *Saccharomyces cerevisiae* Cell Cycle Gene *DBF2* Has Homology with Protein Kinases and Is Periodically Expressed in the Cell Cycle

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Several *Saccharomyces cerevisiae dbf* mutants defective in DNA synthesis have been described previously. In this paper, one of them, *dbf2*, is characterized in detail. The *DBF2* gene has been cloned and mapped, and its nucleotide sequence has been determined. This process has identified an open reading frame capable of encoding a protein of molecular weight 64,883 (561 amino acids). The deduced amino acid sequence contains all 11 conserved domains found in various protein kinases. *DBF2* was periodically expressed in the cell cycle at a time that clearly differed from the time of expression of either the histone H2A or DNA polymerase I gene. Its first function was completed very near to initiation of DNA synthesis. However, DNA synthesis in the mutant was only delayed at 37°C, and the cells blocked in nuclear division. Consistent with this finding, the execution point occurred about 1 h after DNA synthesis, and the nuclear morphology of the mutant at the restrictive temperature was that of cells blocked in late nuclear division. *DBF2* is therefore likely to encode a protein kinase that may function in initiation of DNA synthesis and also in late nuclear division.

In the budding yeast *Saccharomyces cerevisiae*, the events in the cell cycle from the beginning, START, to the initiation of DNA synthesis were originally defined by three cell cycle mutants. In order of function, these are *cdc28*, *cdc4*, and *cdc7* (for reviews, see references 25, 30, and 36). Recent molecular analysis of these genes suggests that progress through G1 up to and including initiation of DNA synthesis may be regulated by protein modification, specifically phosphorylation (22, 23). The *CDC28* gene product is a protein kinase (31), and *CDC4* has homology with the oncogene *ets* (29). In addition, *CDC7*, which functions very close to the beginning of DNA synthesis and is probably required directly in initiation (10), also encodes a protein kinase (1, 27). At present, nothing is known of the substrates of *CDC7* or its actual role in initiation, but it may be significant that at least one of the components of the yeast replication complex is phosphorylated and a protein kinase activity that is temperature sensitive in *cdc7* mutant strains is also found in this complex (14).

There are likely to be further essential G1 events, other than those defined by *cdc4* and *cdc7*, connected with S-phase control and initiation of DNA synthesis. With this in mind, we isolated new cell cycle mutants and screened them for defects in DNA synthesis under restrictive conditions (17). Four mutants, *dbf1* to *4*, were identified specifically as being defective in DNA synthesis, either in initiation or in DNA replication itself. *DBF1* is required for ongoing replication, and the gene has been cloned and sequenced, but its product has no homology with any known proteins in the data banks (manuscript in preparation). The *DBF4* gene acts between *cdc4* and *cdc7* (18); it has been cloned and sequenced (our unpublished result), and Northern (RNA) hybridization has shown that it is periodically expressed in the cell cycle (4). This expression occurs at the same point in the cell cycle, late G1, at which five DNA synthesis genes are expressed (19, 28, 38), although unlike these genes, *DBF4* itself is not required for ongoing DNA synthesis. It may, therefore, be involved in regulating initiation of DNA synthesis. *dbf3* mutations also do not affect ongoing DNA replication but may act shortly after initiation (18). *dbf2* was originally categorized as being defective in ongoing DNA replication (17); however, recent detailed analyses suggest that at the restrictive temperature, the mutant shows a partial defect in DNA synthesis (L. H. Johnston, unpublished result; this paper). To elucidate the function of *DBF2*, we have cloned the gene, and we show here that the amino acid sequence, deduced from the nucleotide sequence, has extensive homology with sequences of serine-threonine protein kinases. In addition, we show that levels of the *DBF2* transcript fluctuate periodically in the cell cycle. The first detectable defect in *dbf2* at the restrictive temperature is a delay in initiation of DNA synthesis, but *DBF2* also functions in the nuclear division cycle, possibly at the time of late nuclear division.

MATERIALS AND METHODS

Yeast strains. The *dbf2* mutant strains used were L119-7D (*MATα dbf2-1 ura3-52 trp1,2 ade1*) and L179-9C (*MATa dbf2-2 ade1 lys2 tyr1 his1,7 trp2 ura1*). Other strains were NCYC 239 (a prototorophic diploid) (41) and CG378 (*MATa ade5 leu2-3,2-112 trp1-289 ura3-52*; obtained from C. Giroux). The cell cycle mutants *cdc5*, *cdc9*, *cdc14*, and *cdc15* were all *MATa ade1 ade 2 ura1 his7 lys2 tyr1* and were obtained from L. H. Hartwell.

Media and general methods. YPD and YNB media have been described previously (37). Cell numbers were determined by use of a particle counter (Coulter Electronics, Dunstable, England). Yeast transformations were performed

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by using a modification (7) of the lithium acetate method (13).

Cloning of the *DBF2* gene. Twenty-three plasmids that complemented temperature-sensitive cell growth of *S. cerevisiae* L119-7D cells were isolated from a clone bank of *S. cerevisiae* chromosomal DNA in vector YRp12 (2). Seven plasmids with related inserts complemented more efficiently than did the remaining plasmids. This group contained the authentic *DBF2* gene, which was confirmed by integrative mapping (26). After restriction endonuclease mapping, Southern hybridization of chromosomal DNA showed that the insert DNA on the plasmid was not rearranged, and further subcloning was carried out to limit the DNA fragment that is able to complement the mutation by standard procedures.

Genetic mapping of *DBF2*. The gene was localized to chromosome VII by probing a Southern blot of yeast chromosomes separated by orthogonal field alternation gel electrophoresis (3). By choosing four markers on chromosome VII, standard genetic mapping was carried out as published previously (33).

Synchronization procedures. NCYC 239 cells were used for the feed-starve protocol of Williamson and Scopes (41). The elutriator-synchronized culture used here has been described previously (the 25°C culture in reference 38); CG378 and L179-9C were used for α-factor synchronization (19). For the latter procedure, cells were grown to mid-log phase (5×10^6 to 1×10^7 cells per ml) in either YPD or YNB medium, α-factor was added to a final concentration of 2 μg/ml, and incubation continued until budded cells had declined to 2 to 3% of the total cells (usually about 2 h at 25°C). The α-factor was removed by filtration and extensive washing, and the cells were then resuspended in fresh prewarmed (at 25°C) medium at 5×10^6 cells per ml.

Northern hybridization. Total RNA was extracted from yeast cells as previously described (37). A 5-μg sample of total RNA, denatured with glyoxal, was separated by agarose gel electrophoresis and transfer was to a GeneScreen hybridization membrane (Dupont, NEN Research Products, Boston, Mass.) as described previously (19, 37). Probes for RNA-DNA hybridization were internal fragments from the genes concerned, the *DBF2* probe being the 1.3-kilobase (kb) *Eco*RI fragment (Fig. 1). The other probes have been described previously (19, 37). DNA was labeled with [α-^{32}P]TTP (Dupont, NEN) to an approximate specific activity of 10^9 cpm/μg, using an oligolabeling protocol (37). After hybridization, blots were washed, dried, and autoradiographed at −70°C with X-ograph Hi-Speed-X intensifying screens. Several exposures were carried out for each experiment so that every RNA of interest was within the exposure range of the film. A Joyce-Loebl Chromoscan 3 densitometer was used for quantitation of RNA on the autoradiographs.

RESULTS

Cloning and genetic mapping of the *DBF2* gene. A DNA fragment that is able to complement the temperature-sensitive growth phenotype of the *dbf2* mutation was isolated from a yeast genomic library constructed in a multicopy yeast-*Escherichia coli* shuttle vector as described in Materials and Methods. This activity was localized to a 2.3-kb *Sal*I-*Bam*HI fragment (Fig. 1A). The identity of the clone was confirmed by integrative mapping (26), and classical genetic mapping showed that *dbf2* is linked to *ade6* (14 parental ditype [PD]:0 nonparental ditype [NPD]:5 tetratype [T]), *trp5* (5 PD:1 NPD:14T), and *leu1* (9 PD:0 NPD:24 T) on

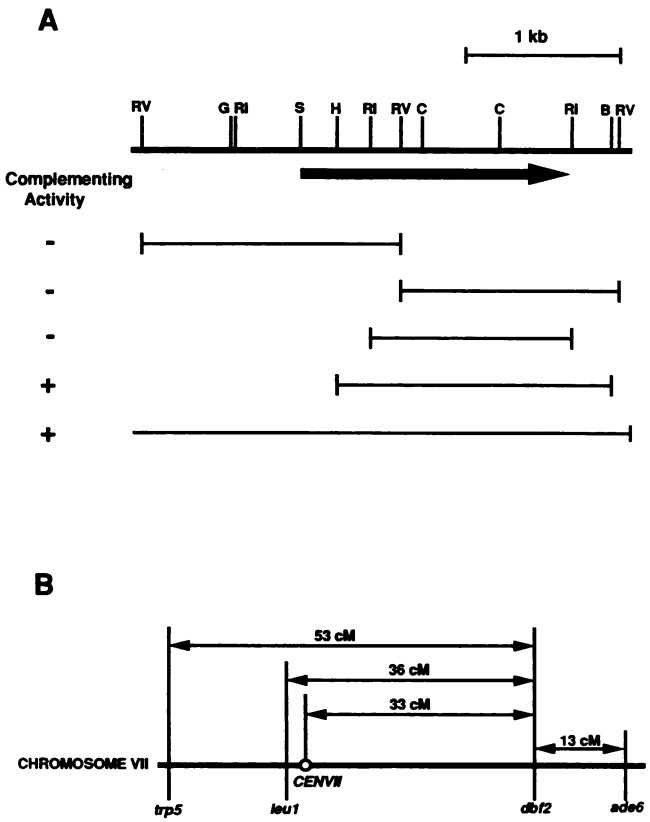

FIG. 1. Localization of *dbf2*-complementing ability, partial restriction map, and genetic map of *DBF2*. (A) The yeast DNA fragments represented by the lines drawn below the restriction map were inserted into plasmid YRp12, transformed into a *dbf2* mutant, and tested for the ability to rescue temperature sensitivity of the *dbf2* mutation. The arrow under the restriction map shows the ORF found from the nucleotide sequence (see Fig. 2). Abbreviations for restriction enzymes: B, *Bam*HI; C, *Cla*I; G, *Bgl*II; H, *Hin*dIII; RI; *Eco*RI; RV, *Eco*RV; S, *Sal*I. (B) Mapping of the *dbf2* mutation genetically, using standard procedures (33).

chromosome VII. Use of standard equations (24) places *dbf2* 13 centimorgans (cM) from *ade6*, 53 cM from *trp5*, and 36 cM from *leu1*, on the right arm of chromosome VII about 33 cM from the centromere (Fig. 1B). This analysis confirms that *DBF2* is a newly identified gene.

DNA and deduced amino acid sequence. The nucleotide sequence of about 2.3 kb of DNA that contained the *dbf2*-complementing activity was determined by the dideoxy sequencing method (32) (Fig. 2). In this region, one large open reading frame (ORF) was found which is capable of encoding a protein of 561 amino acids with a calculated molecular weight of 64,883. The predicted protein would be basic, and the estimated pI is about 9.0. Interestingly, subcloning had shown that a fragment extending from the *Hin*dIII site to the *Bam*HI site (Fig. 1A) had *dbf2*-complementing activity (transcription must presumably have initiated in flanking vector DNA); therefore, the N-terminal 75 amino acids of the protein seem to be nonessential for the activity.

When the deduced amino acid sequence of the *DBF2* gene product (Fig. 2) was compared with existing sequences in data banks, substantial homology was detected with protein kinases. This homology was not confined to the ATP-binding and phosphorylation sites but extended to all 11 domains

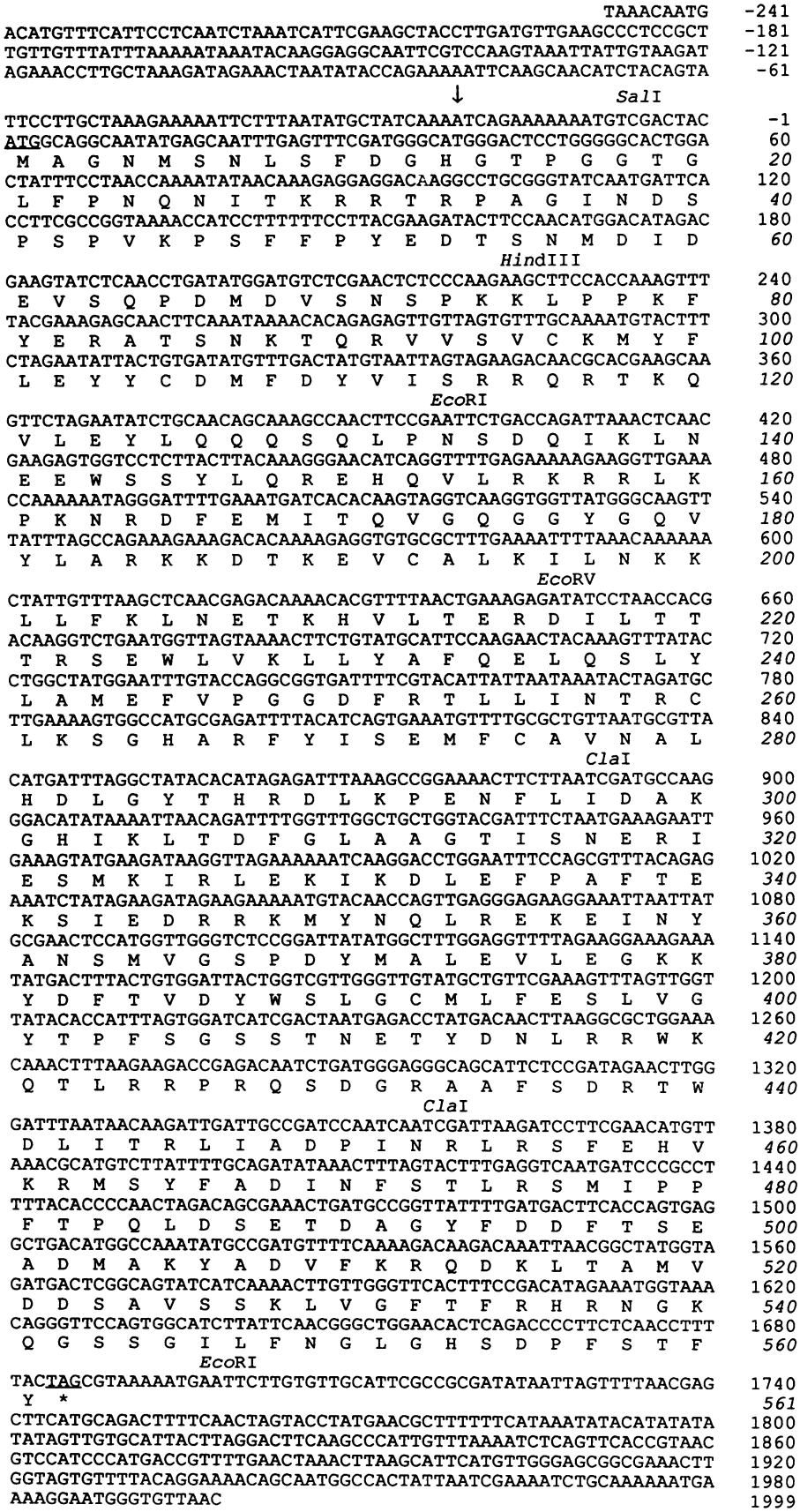

FIG. 2. Nucleotide and deduced amino acid sequences of *DBF2*. The DNA sequence of a 2,240-base-pair fragment that complemented the *dbf2* mutation was determined by the dideoxy-chain termination method (32). One large ORF is found in this sequence; the first nucleotide of the ORF has been numbered 1. Starting at nucleotide −93 is a possible initiation sequence TATACCA. The 5′ end of the mRNA (↓) has been located by primer extension (21), using the oligonucleotide 5′-AGCTTCTTGGGAGAGTTCGAGACATCCAT-3′, corresponding to the region from positions 227 to 199. The predicted amino acid sequence is shown in single-letter code.

FIG. 3. Comparison of the deduced amino acid sequence of the DBF2 protein (upper line) with the sequence of TPK1, a yeast catalytic subunit of cAPK. (lower line) (35). Only those TPK residues are shown which correspond to DBF2 amino acids at the same position or which show conservative changes. In every case, the size of the gap left in the TPK sequence equals the number of DBF2 residues above it. Numbers within the sequence refer to amino acids that have been omitted from the comparison. Bars between the sequences represent amino acids conserved in all protein kinases; roman numerals above the DBF2 sequence indicate the conserved domains found in these enzymes (8).

that are recognized in these proteins (8) (Fig. 3). All of the highly conserved protein kinase residues occur in the *DBF2* gene product sequence, and only in domain X is homology weak. This finding, however, is unremarkable, as this domain is rather divergent and shows few highly conserved amino acids (8). Most homology occurs with cyclic AMP-dependent protein kinase (cAPK), and Fig. 3 shows a comparison with TPK1, the catalytic subunit of *S. cerevisiae* cAPK (35). In view of these close structural similarities, it seems almost certain that DBF2 is a protein kinase.

The *DBF2* transcript was identified by probing a Northern blot of total yeast RNA with the *Eco*RI DNA fragment (Fig. 1 and 2). This analysis revealed a single transcript of about 1.8 kb. Allowing for an untranslated leader and the poly(A) tail, this result is consistent with the size of the gene.

The *DBF2* transcript is periodically expressed in the cell cycle. *DBF2* was first identified as a mutant with a defect in DNA synthesis (17). Since at least five DNA synthesis genes are coordinately regulated in the cell cycle (19, 38), the periodic expression of the *DBF2* mRNA was examined. Note that we use the term expression without any mechanistic implication; the expression observed may be occurring by transcriptional control or by changes in message stability. Cells were synchronized by using a feed-starve protocol (41), and as can be seen from the budding profile, a high degree of synchrony was obtained (Fig. 4A). To reduce the possibility of synchronization artifacts, more than one cell cycle was monitored. Total RNA was extracted from samples taken at intervals throughout the experiment, and transcript levels were assessed by Northern hybridization. Initially, transcript levels from histone H2A and the associated protein (10) were examined. Protein I is not cell cycle regulated and therefore serves as a loading control, although the levels increase slightly over an experiment of this sort (37). In this particular experiment there were some loading errors, but this fact did not obscure the expected sharp periodicity of histone H2A (12), which confirmed that the culture was highly synchronous (Fig. 4A). The DNA polymerase I gene is one of the group of five DNA synthesis genes that are coordinately regulated in the cell cycle (19, 38), and the level of its transcript also fluctuated sharply. Moreover, it preceded histone H2A gene expression (19) in each of the cycles so that the culture was physiologically normal. When the same blot was probed with *DBF2* DNA, it was immediately obvious that the gene was periodically expressed in the cell cycle. A large fluctuation in transcript levels occurred, leading to a peak before the onset of budding. Since DNA synthesis initiates at the time of budding in this system (40), *DBF2* must be expressed before DNA synthesis takes place. Furthermore, expression of *DBF2* also clearly preceded expression of *POL1*, which occurs in late G1 (19), confirming that *DBF2* is expressed early, at least in the first cell cycle. Later peaks in the *DBF2* transcript coincided with periods when budding in the culture was high, implying that in the subsequent cell cycles, *DBF2* expression may have been occurring in late G2/M phase. This finding could be misleading, since late in the cell cycle cytokinesis may be complete but not cell separation (it is often difficult to separate buds even by sonication); thus, apparently budded cells can be in G1. Perhaps more important, the peaks were exactly one cell cycle apart; therefore, by definition, expression must have occurred at the same point in each cell cycle. Consistent with this conclusion, *DBF2* expression preceded *POL1* expression by the same amount in each cycle.

Two other synchronization methods were also used to confirm that this result was not a synchronization artifact. First α-pheromone was used, which arrests a cells at or near START in G1; on release from the block, synchronous rounds of cell division take place (37). The budding profile of the culture and the levels of protein I and histone H2A gene transcripts showed that good synchrony was obtained over the two cycles examined (Fig. 4B). Expression of *DBF2* was clearly periodic, but the expected first peak after release from the α-factor arrest, comparable to that seen at 40 min in the feed-starve culture, was not observed. This result suggests that *DBF2* expression occurs either before the α-factor arrest point in the cell cycle or during incubation in α-factor; indeed, the transcript increased in amount during the incubation (Fig. 4B; see also below).

In the third synchronous system, a culture was set up by using small cells isolated by elutriation (38). The synchrony obtained was rather poor, but the *DBF2* transcript was nonetheless clearly cell cycle regulated (Fig. 5). The one complete fluctuation evident had a peak at 3 h, compared with *POL1* peaks at 1 h 20 min and 4 h 20 min, which could be consistent with periodic expression of *DBF2* occurring in G2. However, it is difficult to rule out the possibility that the cells harvested from the rotor had already passed START, as occurred in one of our previous experiments (37). Thus, expression of *DBF2* in the first cycle may have been missed and in Fig. 5 only the expression associated with the second cycle is evident.

To further examine the expression of *DBF2*, α-pheromone-arrested cells were sampled at frequent intervals, and the level of *DBF2* was monitored (Fig. 6). The *DBF2* transcript increased some sixfold, unlike that of the histone H2A gene which showed the decline expected for a cell cycle-regulated message, or the control protein I gene, which remained constant. This result suggests that *DBF2* is expressed early in the cell cycle, at or near the α-factor arrest point.

On the other hand, when mutants that blocked in late

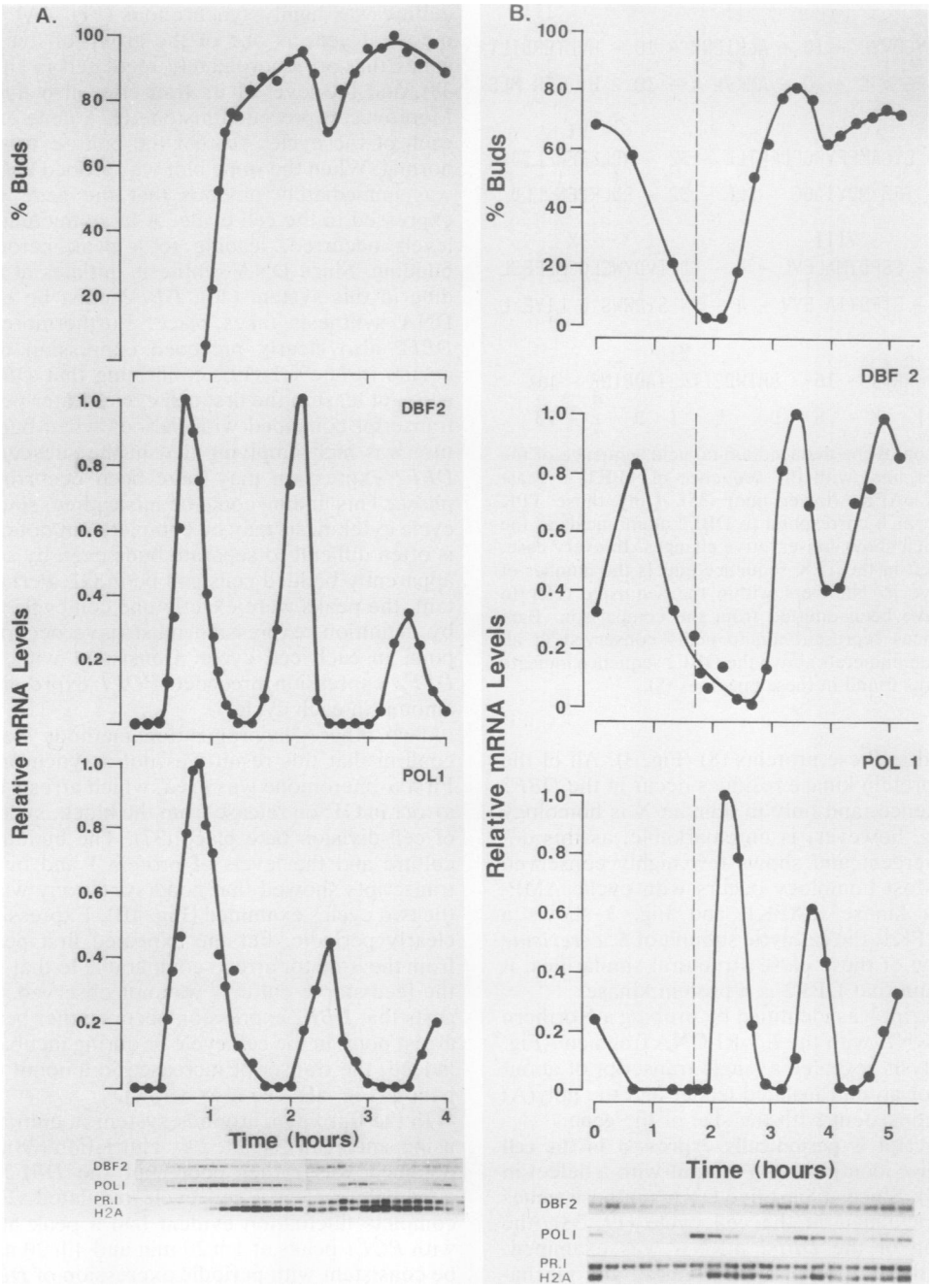

FIG. 4. Cell cycle regulation of the *DBF2* transcript in cultures synchronized by feed-starve (A) or by α-factor (B). Just over two synchronous cell cycles were monitored in each case; the top panels show budding in the cultures. Total RNA was extracted from samples, and Northern blots were prepared. After probing with ^{32}P-labeled DNA fragments, suitably exposed autoradiographs were quantitated by densitometry. The actual *DBF2* and *POL1* transcripts from these experiments, together with two control transcripts (histone H2A and protein I genes [12]), are shown at the bottom. The dashed line in panel B indicates the time of α-factor removal.

nuclear division were held at the restrictive temperature, the *DBF2* transcript increased in amount. For instance, with *cdc14* cells, the *DBF2* transcript increased in amount by some sixfold (Fig. 7). A similar induction, of some threefold, also was observed in two other cell cycle mutants, *cdc5* and *cdc15* (6, 30), which blocked late in the cycle (Fig. 7). The constant level of the *CDC36* message in these two mutants showed that no blotting artifacts had occurred (Fig. 7). This induction was not due to some general heat shock effect on cell cycle-regulated transcripts, since neither *CDC9* nor the histone H2A gene increased in amount under these conditions (Fig. 7). *DBF2* induction also seemed to be confined to mutants blocking late in the cell cycle, since it did not occur when the DNA ligase mutant *cdc9* was held at 37°C (Fig. 7). Finally, a wild-type strain was treated with either hydroxyurea, an inhibitor of DNA synthesis, or nocadazole, an antitubulin agent. Incubation in hydroxyurea for up to 5 h had little effect on levels of *DBF2*, although the cells were

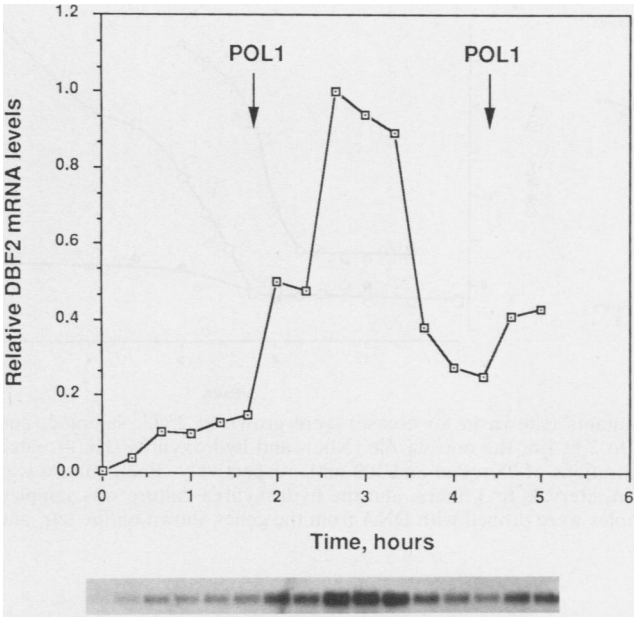

FIG. 5. Cell cycle regulation of the *DBF2* transcript in a culture synchronized by elutriation. The synchronous culture and the RNA blot derived from it have been described previously (25°C culture in Fig. 3 of reference 38). The blot was characterized by probing with the protein I and histone H2A genes, *CDC8*, *CDC9*, and *CDC21* (Fig. 3 in reference 38), and *POL1* (Fig. 2 in reference 19). Arrows indicate positions of the *POL1* peaks in the two cell cycles monitored on the culture (19).

affected by the drug, since the histone H2A gene transcript declined sharply (Fig. 7). In nocadazole-treated cultures the histone H2A gene transcript again declined, but in sharp contrast, the *DBF2* transcript increased in amount by some eightfold after 3 h of incubation (Fig. 7). Thus, *DBF2* does appear to be induced when cells are blocked late in the cell cycle, possibly around late nuclear division.

The first *DBF2* function occurs shortly before S phase. The *dbf2* mutants were initially identified as having a defect in

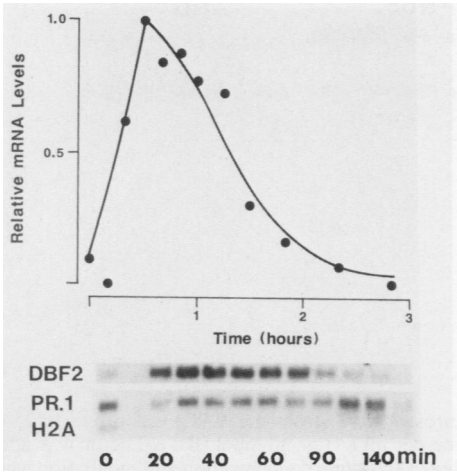

FIG. 6. *DBF2* transcript profile in cells held in α-pheromone. α-Factor was added to a mid-log-phase culture of strain CG378, and samples were removed at various times and prepared for Northern hybridization. See legend to Fig. 4 for further details.

ongoing DNA synthesis (17). However, in view of the probable protein kinase activity of DBF2, the effect of the mutants on DNA synthesis was reinvestigated. In contrast to the previous results, *DBF2* was found to have no immediate effect on DNA synthesis at the restrictive temperature, a discrepancy which may in part be explained by the ready suppression of all of the alleles of *dbf2* (unpublished observations). The defect that we now detect is no more than a delay in initiation of S phase. This was detected by monitoring DNA synthesis at either 25 or 37°C in *dbf2* cultures synchronized with α-factor. In the case of a mutant that is deficient in initiation of DNA replication, such as *cdc7*, no DNA synthesis occurs at the restrictive temperature after release from α-factor arrest (11, 18), since α-factor blocks at or near the beginning of the cell cycle and the cells do not progress through S phase (30). With *dbf2* mutants, however, when cells were transferred to 37°C immediately after release from α-factor, DNA synthesis did occur, but there was a 40-min delay compared with either *dbf2* mutant cells incubated at 25°C (Fig. 8A) or wild-type cells incubated at 25 or 37°C (data not shown). The control *dbf2* culture left at 25°C after α-factor release started DNA synthesis at approximately 20 min (Fig. 8A). When part of this culture was transferred to 37°C at 20 min after α-factor release, there was again a delay of about 40 min before the start of DNA synthesis (Fig. 8A). By 30 min in the control culture, DNA synthesis had started, and upon transfer to 37°C, cells continued DNA synthesis without any delay (Fig. 8A), presumably since some cells had traversed the point at which *DBF2* was required. (Incidentally, this absence of a delay in DNA synthesis shows that the 40-min delay was not simply a result of heat shock.) However, α-factor synchrony is not perfect, and by 30 min a proportion of the cells had not yet passed the point at which *DBF2* acted; these still required the 40 min delay before beginning DNA synthesis. Since DNA synthesis lasts for only 20 to 30 min in individual cells (34, 39), there was a brief cessation of DNA synthesis in the culture (Fig. 8A). Similar kinetics were obtained when cells were shifted to 37°C at 40 min, but by 60 min the entire population has passed the *DBF2* point, and DNA synthesis was completed without further interruption. Therefore, we conclude that *DBF2* acts in G1 and completes its function very close to the beginning of DNA synthesis.

The cell cycle block in *dbf2* mutants is not associated with a defect in DNA synthesis. Despite seeming to complete DNA synthesis at 37°C, *dbf2* mutant cells still blocked in the cell cycle and did not divide at the restrictive temperature, suggesting a defect in some other aspect of the cell cycle. On the other hand, a subtle defect in DNA synthesis, such as failure to join replication intermediates (Okazaki fragments or completed replicons), can lead to a block in mitosis, as occurs with the DNA ligase mutant *cdc9* (16). However, alkaline sucrose gradient sedimentation experiments (20) revealed no defect of this sort in *dbf2* cells at the restrictive temperature (data not shown). Alternatively, a failure to synthesize its entire genome at 37°C could also result in a mitotic block, as happens with *cdc2* (5). To establish whether any defect at all in DNA synthesis could account for the cell cycle arrest in *dbf2*, the time of DNA synthesis at 25°C in the α-factor-synchronized culture was compared with the time when cells acquired the ability to divide at 37°C. At intervals, samples of the synchronized culture at 25°C were transferred to 37°C, and cell numbers were then determined after a suitable incubation at 37°C (Fig. 8B). Cells transferred to 37°C up to 1 h 45 min after α-factor release failed to divide. In this culture, DNA synthesis

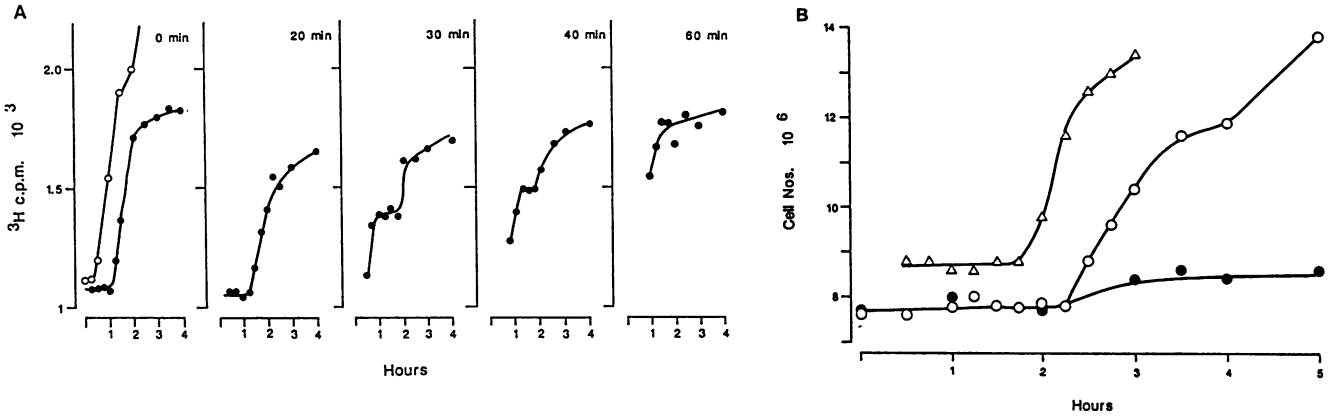

FIG. 7. *DBF2* transcript levels in G2-blocked cells. The four *cdc* mutants (shown in lowercase) were grown at 25°C, sampled, and transferred to 37°C, and further samples were taken at 40-min intervals to 2 h. For the nocadazole (Noc)- and hydroxyurea (HU)-treated cultures, the drugs were added to mid-log cells of CG378 to final concentrations of 25 μg/ml and 100 mM, respectively. Each culture was sampled immediately; the nocadazole culture was then sampled at 45-min intervals to 3 hours, and the hydroxyurea culture was sampled hourly to 3 h and then again at 5 h. Northern blots prepared from the samples were probed with DNA from the genes shown on the left, and the resulting autoradiographs are presented.

started at 20 min (Fig. 8A); in individual cells, DNA synthesis is of 20- to 30-min duration (34, 39). Thus, the first cells will have completed DNA synthesis by 50 min at the latest. Yet these cells were able to divide at 37°C only after incubation for 1 h 45 min at 25°C, or about 60 min after completing DNA synthesis. It is therefore unlikely that any defect in DNA synthesis contributed to the cell cycle block in *dbf2* mutants, although it is difficult to entirely eliminate the possibility that some replicons were completed only very late in the cycle even at 25°C.

***dbf2* nuclear morphology at 37°C.** *S. cerevisiae* mutants defective in DNA replication typically form large, swollen pairs of daughter cells (dumbbells) at the restrictive temperature, with the nucleus located in the isthmus between the two daughter cells (for reviews, see references 9 and 30). *dbf2* mutant cells form this cellular morphology (17), but when the cells incubated at 37°C were stained with Giemsa, about 70% of cells appeared to contain two separate nuclei, one in each daughter (Fig. 9). Careful focusing showed that in at least half of them, a very thin bridge of stained material connected the two nuclei, a morphology described previously as due to a defect in late nuclear division (6). Among the remaining 30% of cells, some showed a single nucleus in one of the cells, whereas in others there were two nuclei in one cell (examples can be seen in Fig. 9).

DISCUSSION

The *DBF2* gene has been cloned, sequenced, mapped, and shown to be periodically expressed in the cell cycle. This analysis has shown it to be a newly identified gene, and although its precise role is not yet clear, its product has striking homology with protein kinases. This homology extends to all 11 domains recognized in these enzymes, and all of the highly conserved individual amino acids that occur in various of the domains (8) are also found in DBF2 (Fig. 3).

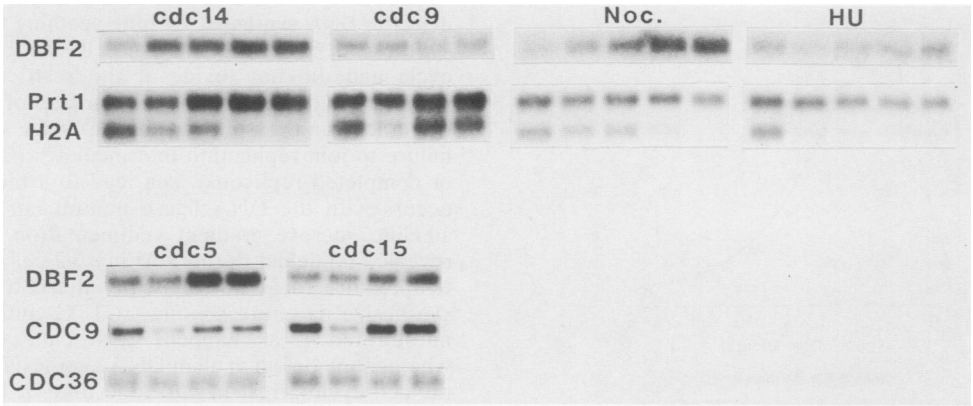

FIG. 8. DNA synthesis (A) and cell division (B) in α-factor-synchronized cultures. A *dbf2* strain (L179-9C) was grown at 25°C for about 10 generations to mid-log phase in YPD containing either 0.37 MBq of [^{3}H]adenine per ml (A) or no label (B). α-Factor was added for 2 h; after its removal, cells were resuspended in fresh YPD. For panel A, label was added to the same concentration and incubation was continued at 25°C (○), with samples being transferred to 37°C at the times shown (●). Duplicate samples were placed in an alkaline stop mix and processed for scintillation counting as described previously (17). For panel B, part of the culture was immediately placed at 37°C (●), and the remainder was incubated at 25°C (○); cell numbers were monitored regularly in both cultures. In addition, samples were transferred at intervals from the 25°C culture to 37°C, and cell numbers were determined after an additional 4 h of incubation (△).

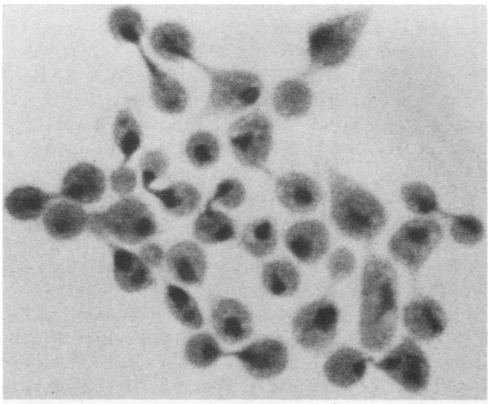

FIG. 9. Nuclear morphology of *dbf2* at 37°C. L179-9C cells were treated with α-factor for 2 h at 25°C, washed, and resuspended in fresh medium at 37°C. After the equivalent of two generations of incubation, cells were fixed in 1% Formalin and stained with Giemsa before being photographed.

Moreover, the spacing between the domains in DBF2 is almost identical to that found in protein kinases except for an insert of 43 amino acids between domains VII and VIII. The *S. cerevisiae* cell cycle protein kinase, CDC7, similarly has a large but unrelated insert at this position (1, 27). These structural similarities suggest very strongly that DBF2 is itself a protein kinase. The sequences Asp-Leu-Lys-Pro-Glu-Asn in domain VI and Gly-Ser-X-X-Tyr-X-Ala-Leu-Glu (X being any amino acid) in domain VIII further suggest that it is a serine-threonine protein kinase (8).

As judged by the extent of homology in the stretches of sequence between each of the conserved domains, DBF2 is not very closely related to any other known protein kinases. There is, however, some homology with the catalytic subunit of cAPKs. For instance, within the region of the protein containing the 11 conserved domains (but excluding the 43-amino-acid insertion), DBF2 has 51% homology with TPK1 (Fig. 3), an *S. cerevisiae* cAPK (35). Similar levels of homology are found with both bovine and murine cAPKs. By comparison, the homology with CDC7 and CDC28 is no more than 28% and essentially confined to the conserved domains. Much of the homology with cAPKs occurs within particular motifs common to these enzymes, but it seems unlikely that DBF2 is itself a cAPK. *TPK1* is part of a three-gene family, members of which show more than 75% amino acid homology in their C-terminal regions and more than 50% with the bovine enzyme (35). *DBF2* may therefore have diverged from a common ancestor gene (8).

A single amino acid change from Lys to Thr (A-to-C change at nucleotide 584 in Fig. 2) in domain II resulted in total loss of *dbf2*-complementing activity of the isolated *DBF2* gene (S. L. Eberly, H. Araki, and A. Sugino, unpublished result), suggesting that the phosphorylation activity of *DBF2* directly participates in its function in vivo.

DBF2 may be unique among the known yeast protein kinases in being periodically expressed in the cell cycle. Three different methods of synchronizing cells have been used, and all show clearly that the level of the *DBF2* transcript fluctuates sharply in the cell cycle. It is expressed at a time different from the time of expression of either the histone H2A gene or *POL1*, one of the group of five coordinately expressed DNA synthesis genes (19, 38), suggesting that it is on a separate regulatory circuit. However, the precise point at which it is expressed in the cell cycle is difficult to assess.

The data from the feed-starve synchrony (Fig. 4A) suggest quite strongly that *DBF2* is expressed in G1 before expression of *POL1*. Similarly, the expression while cells are being incubated in α-factor (Fig. 6) also strongly supports this interpretation. On the other hand, the single fluctuation observed with the elutriation culture (Fig. 5), together with the relative positions of the *POL1* peaks, suggests that in this experiment it may be expressed in G2/M phase. The increase in message level that occurs in cells blocked late in the cycle by use of appropriate mutants or nocadazole (Fig. 7) also may indicate that the cell cycle expression occurs during mitosis.

In exponential-phase cells, the time difference between late mitosis and START of the next cycle is probably no more than 5 to 10 min. Moreover, START can be experimentally manipulated so that it occurs in the preceding cycle in dividing cells (15). Conceivably, therefore, the underlying physiological event (or signal) that ultimately controls *DBF2* expression may occur in either G2/M or near START, depending on how the cells are manipulated. We favor the interpretation that it normally occurs near START, but a detailed molecular analysis will be necessary to prove this conclusively.

Another interesting question is how this periodic expression relates to the probable role of *DBF2* in the cell cycle. Since the first defect detected in *dbf2* mutants at 37°C is a delay in DNA synthesis, the periodic expression may be associated with a role in controlling S phase. Note that the delay in DNA synthesis at 37°C, rather than a total block, does not mean that *DBF2* is necessarily participating in a nonessential function but simply that its role can in some way be bypassed. In this respect, it may be significant that we have recently isolated a DBF2 homolog that shows about 95% amino acid homology with DBF2 in the region that has so far been sequenced (Johnston, unpublished observation).

Despite the delay in DNA synthesis at 37°C, *DBF2* seems to have a late execution point, about 1 h after completion of DNA synthesis in individual cells. Consistent with this finding, the nuclear morphology of *dbf2* cells at 37°C is not characteristic of DNA synthesis mutants but rather of mutants blocked in nuclear division, possibly late nuclear division. Furthermore, the increase in the *DBF2* transcript when cells are blocked in late nuclear division could be an induction phenomenon reflecting an increased requirement for the *DBF2* product under these conditions, again pointing to a role in late nuclear division. Hence, *DBF2* may act twice in the cell cycle, once before S phase and again in nuclear division.

In budding yeast cells, however, the nuclear division cycle begins very early, with spindle pole body separation occurring at about the time of S-phase initiation. Hence, an alternative interpretation of *DBF2* function is that it first acts in the nuclear division cycle at around this stage, leading to the DNA synthesis delay at 37°C as a secondary consequence. It then may function a second time in late nuclear division or even act continuously or intermittently throughout the nuclear division cycle. At present these two interpretations cannot be reconciled, but monitoring the actual levels of the DBF2 protein and the kinase activity in these experiments may help resolve this question; such experiments are under way. Equally important is the identification and analysis of possible substrates for the putative DBF2 protein kinase, and a number of molecular and genetic suppressors have been identified.

LITERATURE CITED

1. **Bahman, M., V. Buck, A. White, and J. Rosamond.** 1988. Characterization of the *CDC7* gene product of *Saccharomyces cerevisiae* as a protein kinase needed for initiation of mitotic DNA synthesis. Biochim. Biophys. Acta **951**:335–343.
2. **Barker, D. G., and L. H. Johnston.** 1983. *Saccharomyces cerevisae cdc9*, a structural gene for yeast DNA ligase which complements *Schizosaccharomyces pombe cdc17*. Eur. J. Biochem. **134**:315–319.
3. **Carle, G. F., and M. V. Olson.** 1984. Separation of chromosomal DNA molecules from yeast by orthogonal-field-alternation gel electrophoresis. Nucleic Acids Res. **12**:5647–5664.
4. **Chapman, J. W., and L. H. Johnston.** 1989. The yeast gene, *DBF4*, essential for entry into S phase is cell cycle regulated. Exp. Cell Res. **180**:419–428.
5. **Conrad, M. N., and C. S. Newlon.** 1983. *Saccharomyces cerevisiae cdc2* mutants fail to replicate approximately one third of their nuclear genome. Mol. Cell. Biol. **3**:1000–1012.
6. **Culotti, J., and L. H. Hartwell.** 1971. Genetic control of the cell division cycle in yeast. III. Seven genes controlling nuclear division. Exp. Cell Res. **67**:389–401.
7. **Gietz, R. D., and A. Sugino.** 1988. New yeast-*Escherichia coli* shuttle vectors constructed *in vitro* mutagenized yeast genes lacking six-base pair restriction sites. Gene **74**:527–534.
8. **Hanks, S. K., A. M. Quinn, and T. Hunter.** 1988. The protein kinase family: conserved features and deduced phylogeny of the catalytic domains. Science **241**:42–52.
9. **Hartwell, L. H.** 1974. *Saccharomyces cerevisiae* cell cycle. Bacteriol. Rev. **38**:164–203.
10. **Hereford, L., K. Fahrner, J. Woolford, M. Rosbash, and D. B. Kaback.** 1979. Isolation of yeast histone genes H2A and H2B. Cell **18**:1261–1271.
11. **Hereford, L. M., and L. H. Hartwell.** 1974. Sequential gene function in the initiation of *S. cerevisiae* DNA synthesis. J. Mol. Biol. **84**:445–461.
12. **Hereford, L., M. A. Osley, J. R. Ludwig, and C. S. McLaughlin.** 1981. Cell cycle regulation of yeast histone mRNA. Cell **24**: 367–375.
13. **Ito, H., Y. Fukuda, K. Murata, and A. Kimura.** 1983. Transformation of intact yeast cells treated with alkali cations. J. Bacteriol. **153**:163–168.
14. **Jazwinski, S. M.** 1988. *CDC7*-dependent protein kinase activity in yeast replicative-complex preparations. Proc. Natl. Acad. Sci. USA **85**:2101–2105.
15. **Johnston, G. C., and R. A. Singer.** 1983. Growth and the cell cycle of the yeast *Saccharomyces cerevisiae*. 1. Slowing S phase or nuclear division decreases the G1 cell cycle period. Exp. Cell Res. **149**:1–13.
16. **Johnston, L. H., and K. Nasmyth.** 1978. *Saccharomyces cerevisiae* cell cycle mutant *cdc9* is defective in DNA ligase. Nature (London) **274**:891–893.
17. **Johnston, L. H., and A. P. M. Thomas.** 1982. The isolation of new DNA synthesis mutants in the yeast *Saccharomyces cerevisiae*. Mol. Gen. Genet. **186**:439–444.
18. **Johnston, L. H., and A. P. M. Thomas.** 1982. A further two mutants defective in initiation of S phase in the yeast *Saccharomyces cerevisae*. Mol. Gen. Genet. **186**:445–448.
19. **Johnston, L. H., J. H. M. White, A. L. Johnson, G. Lucchini, and P. Plevani.** 1987. The yeast DNA polymerase I transcript is regulated in both the mitotic cell cycle and in meiosis and is also induced after DNA damage. Nucleic Acids Res. **15**:5017–5029.
20. **Johnston, L. H., and D. H. Williamson.** 1978. An alkaline sucrose gradient analysis of the mechanism of nuclear DNA synthesis in the yeast *Saccharomyces cerevisiae*. Mol. Gen. Genet. **164**:217–225.
21. **Krainer, A. R., T. Maniatis, B. Ruskin, and M. R. Green.** 1984. Normal and mutant human β-globin pre-mRNAs are faithfully and efficiently spliced *in vitro*. Cell **36**:993–1005.
22. **Lee, M., and P. Nurse.** 1988. Cell cycle control genes in fussion yeast and mammalian cells. Trends Genet. **4**:284–290.
23. **Mendenhall, M. D., H. E. Richardson, and S. I. Reed.** 1988. Dominant negative protein kinase mutations that confer a G1 arrest phenotype. Proc. Natl. Acad. Sci. USA **85**:4426–4430.
24. **Mortimer, R. K., and D. Schild.** 1980. Genetic map of *Saccharomyces cerevisiae*. Microbiol. Rev. **44**:519–571.
25. **Newlon, C.** 1988. Yeast chromosome replication and segregation. Microbiol. Rev. **52**:568–601.
26. **Orr-Weaver, T. L., J. W. Szostak, and R. J. Rothstein.** 1981. Yeast transformation: a model system for the study of recombination. Proc. Natl. Acad. Sci. USA **78**:6354–6358.
27. **Patterson, M., R. A. Sclafani, W. L. Fangman, and J. Rosamond.** 1986. Molecular characterization of cell cycle gene *CDC7* from *Saccharomyces cerevisiae*. Mol. Cell. Biol. **6**:1590–1598.
28. **Peterson, T. A., L. Praleash, S. Prakash, M. A. Osley, and S. I. Reed.** 1985. Regulation of *CDC9*, the *Saccharomyces cerevisiae* gene that encodes DNA ligase. Mol. Cell. Biol. **5**:226–235.
29. **Peterson, T. A., J. Yochem, B. Byers, M. F. Nunn, P. H. Duesberg, R. F. Doolittle, and S. I. Reed.** 1984. A relationship between the yeast cell cycle genes *CDC4* and *CDC36* and the *ets* sequence of oncogenic virus E26. Nature (London) **309**:556–558.
30. **Pringle, J. R., and L. H. Hartwell.** 1981. The *Saccharomyces cerevisiae* cell cycle, p. 97–142. *In* J. N. Strathern, E. W. Jones, and J. R. Broach (ed.), The molecular biology of the yeast *Saccharomyces*: life cycle and inheritance. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
31. **Reed, S. I., J. A. Hadwiger, and A. T. Lorincz.** 1985. Protein kinase activity associated with the product of the yeast cell division cycle gene *CDC28*. Proc. Natl. Acad. Sci. USA **82**: 4055–4059.
32. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
33. **Sherman, F., G. R. Fink, and J. B. Hicks.** 1986. Laboratory course manual for methods in yeast genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
34. **Slater, M. L., S. O. Sharron, and J. J. Gart.** 1977. Cell cycle of *S. cerevisiae* in populations growing at different rates. Proc. Natl. Acad. Sci. USA **74**:3850–3854.
35. **Toda, T., S. Cameron, P. Sass, M. Zoller, and M. Wigler.** 1987. Three different genes in *S. cerevisiae* encode the catalytic subunits of the cAMP dependent protein kinase. Cell **50**:277–287.
36. **Wheals, A. E.** 1987. Biology of the cell cycle in yeasts, p. 283–390. *In* A. Rose (ed.), The yeasts, vol. 1. Academic Press, Inc. (London), Ltd., London.
37. **White, J. H. M., D. G. Barker, P. Nurse, and L. H. Johnston.** 1986. Periodic transcription as a means of regulating gene expression during the cell cycle: contrasting mode of expression of DNA ligase genes in budding and fission yeast. EMBO J. **5**:1705–1709.
38. **White, J. H. M., S. R. Green, D. G. Barker, L. B. Dumas, and L. H. Johnston.** 1987. The *CDC8* transcript is cell cycle regulated in yeast and is expressed coordinately with *CDC9* and *CDC21* at a point preceding histone transcription. Exp. Cell Res. **171**:223–231.
39. **Williamson, D. H.** 1965. The timing of DNA synthesis in the cell cycle of *Saccharomyces cerevisiae*. J. Cell Biol. **25**:517–528.
40. **Williamson, D. H.** 1973. Replication of the nuclear genome in yeast does not require concominant protein synthesis. Biochem. Biophys. Res. Commun. **52**:731–740.
41. **Williamson, D. H., and A. W. Scopes.** 1962. A rapid method for synchronizing division in the yeast *Saccharomyces cerevisiae*. Nature (London) **193**:256–257.