MOLECULAR AND CELLULAR BIOLOGY, July 1999, p. 4888–4896 Vol. 19, No. 7
0270-7306/99/$04.00+0

Cell Cycle Control of Cdc7p Kinase Activity through Regulation of Dbf4p Stability

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Received 10 November 1998/Returned for modification 17 December 1998/Accepted 8 April 1999

In *Saccharomyces cerevisiae*, the heteromeric kinase complex Cdc7p-Dbf4p plays a pivotal role at replication origins in triggering the initiation of DNA replication during the S phase. We have assayed the kinase activity of endogenous levels of Cdc7p kinase by using a likely physiological target, Mcm2p, as a substrate. Using this assay, we have confirmed that Cdc7p kinase activity fluctuates during the cell cycle; it is low in the G_1 phase, rises as cells enter the S phase, and remains high until cells complete mitosis. These changes in kinase activity cannot be accounted for by changes in the levels of the catalytic subunit Cdc7p, as these levels are constant during the cell cycle. However, the fluctuations in kinase activity do correlate with levels of the regulatory subunit Dbf4p. The regulation of Dbf4p levels can be attributed in part to increased degradation of the protein in G_1 cells. This G_1-phase instability is *cdc16* dependent, suggesting a role of the anaphase-promoting complex in the turnover of Dbf4p. Overexpression of Dbf4p in the G_1 phase can partially overcome this elevated turnover and lead to an increase in Cdc7p kinase activity. Thus, the regulation of Dbf4p levels through the control of Dbf4p degradation has an important role in the regulation of Cdc7p kinase activity during the cell cycle.

The initiation of DNA replication in *Saccharomyces cerevisiae* can be divided into at least two fundamental stages that are tightly coordinated with the cell cycle (reviewed in references 19, 21, and 64). In the first stage, as cells complete the M phase and enter the G_1 phase, initiation proteins are assembled at chromosomal origins (16), making them competent to initiate DNA replication (15, 50). At the core of this preinitiation complex is the origin recognition complex (ORC) (4), which is constitutively bound to origins throughout the cell cycle (3). The assembly is thought to involve the ordered recruitment of Cdc6, followed by a family of six minichromosome maintenance proteins (Mcm2p to Mcm7p) to the ORC-origin complex (3, 8, 59). Another initiation factor, Cdc45p (23, 30, 48, 69), is also thought to be recruited to the preinitiation complex at this time (3). Later, when cells pass through the G_1 commitment point Start and begin to enter the S phase, the preinitiation complex is thought to be activated by the action of at least two kinases: the cyclin-dependent kinase (CDK) Cdc28, in association with B-type cyclins called Clbs (53), and Cdc7 (reviewed in reference 55).

Cdc7p is a serine/threonine kinase (29, 66) that is essential for the initiation of DNA replication (7, 25, 49). In the absence of Cdc7p function, cells assemble preinitiation complexes at origins but cannot trigger them to initiate DNA synthesis (16) and hence arrest in the late G_1 phase. More recently, Cdc7p has been shown to be required throughout the S phase to trigger late origins as well as those that fire early, at the beginning of the S phase (5, 18). A point mutation in the putative ATP-binding site abolishes Cdc7p function, indicating that kinase activity is required for its function (7, 29). Homologs of *CDC7* have recently been identified and cloned for a number of organisms (28, 34, 37, 46, 51), including *Schizosaccharomyces pombe*, *Xenopus laevis*, mice, and humans, suggesting that the regulatory function of *CDC7* in replication initiation has been conserved in evolution.

CDC7 interacts genetically with a putative regulatory subunit encoded by *DBF4*. High-copy expression of either gene suppresses temperature-sensitive mutations in the other, and temperature-sensitive mutations of the two are synthetically lethal (41). Like *CDC7*, *DBF4* is essential for DNA replication, and cells lacking *DBF4* function have an arrest phenotype similar to that of cells lacking *CDC7* function (35). Moreover, Cdc7p and Dbf4p interact in a two-hybrid assay (33), and Dbf4p function is needed for Cdc7p kinase activity on histone H1 (33). Together, these data suggest that the active Cdc7p kinase required for replication initiation in vivo is a heteromeric Cdc7p-Dbf4p complex. Recent purification of such a complex from *S. pombe* supports this notion (6). A one-hybrid interaction of Dbf4p with the ARS1 origin suggests that Dbf4p may direct Cdc7p kinase to the origin (20). At the origin, the Cdc7p-Dbf4p complex may phosphorylate a key target(s) assembled in the preinitiation complex to trigger initiation in the S phase.

Leading candidates for the critical target(s) of the Cdc7p kinase include the Mcm family of proteins. The six proteins in this highly conserved family are each essential for DNA replication (11, 13, 22, 26, 27, 42, 45, 47, 58, 65) and have been shown to form heteromeric complexes containing all six proteins in several species (1, 61). Mcm proteins tightly associate with chromatin in the G_1 phase and gradually dissociate as the S phase proceeds (3, 38, 42, 59). In *S. cerevisiae*, the Mcm proteins are incorporated into preinitiation complexes at replication origins (3, 59) and are thought to play a key role in initiation. Genetic studies with budding yeast have established a connection between the replication functions of Cdc7p-Dbf4p and Mcm proteins. An allele of *mcm5* can suppress deletions of *CDC7* and *DBF4* (24), and an allele of *dbf4* can suppress a conditional allele of *mcm2* (44). Biochemical studies suggest that these proteins share a kinase-substrate relationship. Bud-

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TABLE 1. Yeast strains and plasmids

Strain or plasmid	Genotype	Source or reference
Strains		
YJO235	*MAT***a**	*CDC7-myc*$_9$ *leu2-3,112 ura3-52 trp1-289 bar1::LEU2*	This study
YJL310	*MAT***a**	*leu2-3,112 ura3-52 trp1-289 bar1::LEU2*	This study
YJO280	*MAT***a**	*CDC7-myc*$_9$ *leu2-3,112 ura3-52 trp1-289 bar1::LEU2 dbf4-1*	This study
GOY244	*MAT***a**	*ura3 trp1 can cdc16-1 pep4 prb1* + pYQ117	This study
GOY263	*MAT***a**	YJO235 + pYQ117	This study
GOY264	*MAT***a**	YJO235 + pYQ118	This study
237	*MAT***a**	*bar1 his6 ura3 trp1 leu2 cyh2 can1 ade1*	This study
271	*MAT***a**	*his6 bar1 trp1-289 can1 sap3 cdc16-1*	This study
Plasmids		
pYQ117	pYES2-His-HA	This study
pYQ118	pYES2-His-HA-DBF4	This study
pJO36	pRS306-CDC7-myc$_9$	This study
pEG.MCM2	GST-MCM2	44
pEG.MCM3	GST-MCM3	44
pEG.MCM4	GST-MCM4	44
pEG.MCM5	GST-MCM5	44
pEG.MCM6	GST-MCM6	44
pEG.MCM7	GST-MCM7	44

ding yeast Cdc7p-Dbf4p expressed and purified from baculovirus-infected cells phosphorylates fusions of Mcm2p, Mcm3p, Mcm4p, and Mcm6p with glutathione *S*-transferase (GST) (44). The fission yeast homolog Hsk1p-Dfp1p also has been purified and has been shown to phosphorylate a heteromeric Mcm complex purified from fission yeast and containing all six Mcm proteins (6). These data suggest the possibility that the Cdc7p-Dbf4p complex helps to trigger initiation at replication origins by phosphorylating one or more subunits of the Mcm complex in the preinitiation complex.

This putative role of Cdc7p kinase suggests that one potential way to control the onset of the S phase is to regulate Cdc7p kinase activity during the cell cycle. Previous studies monitoring the kinase activity of overexpressed Cdc7p protein on a nonphysiological substrate, calf thymus histone H1, have detected fluctuations of Cdc7p kinase activity during the cell cycle (33, 67). To examine the regulation of this activity in more detail, we have developed an assay that uses a potential physiological substrate, GST-Mcm2p, to monitor endogenous levels of Cdc7p kinase activity during the cell cycle. Using this assay, we confirm that Cdc7p kinase activity is periodic during the cell cycle. It is low during the G_1 phase and rises as cells pass the G_1 commitment point Start and enter the S phase. The activity remains high through the S phase and declines some time during mitosis. This periodicity is not due to fluctuations in Cdc7 protein levels, which remain constant during the cell cycle. Instead, levels of Cdc7p kinase activity correlate with Dbf4 protein levels. The changes in Dbf4p levels can be accounted for in part by changes in Dbf4p stability. The protein is most unstable in the G_1 phase, when steady-state levels are lowest. This G_1-phase turnover involves the anaphase-promoting complex (APC), which targets various cell cycle-regulated substrates for degradation by ubiquitination (reviewed in reference 62) and is dependent on the APC subunit Cdc16p. Overexpression of Dbf4p in the G_1 phase can partially counteract this high turnover, increasing Dbf4p levels and ectopically inducing Cdc7p kinase activity. These results suggest that the regulation of Dbf4p levels plays a significant role in the regulation of Cdc7 kinase activity during the cell cycle.

(This work was submitted in partial fulfillment of the doctoral dissertations of G. Oshiro and Y. Shellman at the University of Colorado. This work was also submitted in partial fulfillment of the doctoral dissertation of J. C. Owens at the University of California, San Francisco.)

MATERIALS AND METHODS

Media, strains, plasmids, and proteins. Yeast strains were grown in yeast extract-peptone (YEP) with 2% dextrose or in synthetic defined minimal medium supplemented with 2% dextrose or 2% raffinose. All strains were grown at 30°C unless otherwise indicated. All strains and plasmids used in this study are presented in Table 1. For analysis of the budding index, cells with a bud diameter less than 50% that of the mother cell were scored as small-budded cells, whereas those with a bud diameter greater than 50% that of the mother cell were scored as large-budded cells. In our strains, the appearance of small buds coincided with the onset of replication.

Plasmid pYQ117 was constructed by ligating two oligonucleotides, GAT CCT ACC CAT ACG ACG TCC CAG ACT ACG CG and AAT TCG CGT AGT CTG GGA CGT CGT ATG GGT AG, into the *Bam*HI-*Eco*RI sites of pYES2HisA (Invitrogen). Plasmid pYQ118 was constructed by ligating a 2.3-kbp *Sal*I-*Xba*I *DBF4* fragment into the *Xho*I-*Xba*I sites of pYQ117. The *Sal*I site was previously introduced 19 bp upstream of the initiation ATG codon of *DBF4* by in vitro mutagenesis with oligonucleotide TTG GTT CAT ATG TCG ACA GGA AAG ACG AAA ATG. All plasmid sequences were verified by direct sequence analysis.

Epitope-tagged *CDC7* was produced by first cloning a *Hin*dIII-*Hae*II genomic fragment of *CDC7* into the *Hin*dIII-*Sac*I sites of pRS306 (*Hae*II and *Sac*I were blunt) to form pTW004. A unique *Not*I restriction site was then introduced by inserting 9 nucleotides (GGC GGC CGC) immediately upstream of the termination codon of *CDC7* to produce plasmid pJO31. Three tandem repeats of a *Not*I fragment (a gift from A. Sil, University of California, San Francisco) carrying three direct repeats of the c-*myc* epitope were inserted into the *Not*I site in frame with the *CDC7* open reading frame of pJO31, resulting in plasmid pJO36. The nine-*myc*-tagged *CDC7* gene (*CDC7-myc*$_9$) was substituted for the wild-type copy of YJL310 by two-step gene replacement, resulting in strain YJO235. The desired replacement was confirmed by PCR analysis of genomic DNA.

The GST-Mcm fusion proteins were purified from yeast essentially as previously described (44). Briefly, plasmids expressing the various GST-Mcm fusion proteins under the control of the *GAL1* promoter (generously provided by B. Tye and Y. Kawasaki) were introduced into YJL312. After galactose induction for 4 h, extracts were made from these cells and incubated with glutathione-agarose beads for 1 to 2 h. The beads were loaded onto a column, washed with 10 volumes of wash buffer, and eluted with glutathione. The fractions were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), and the peak fractions were pooled and dialyzed. Proteins prepared this way were >95% pure. Some preparations contained a smaller GST fusion protein apparently derived from the full-length fusion protein.

Immunoprecipitation of Cdc7 from yeast cells. Fifty milliliters of log-phase cells was grown to an optical density at 600 nm of approximately 1.5, which is about 5×10^7 cells per ml. Cells were collected by centrifugation and transferred to 1.5-ml screw-cap tubes. Lysis buffer (50 mM Tris [pH 7.6], 1% Nonidet P-40, 5 mM EDTA, 100 mM NaCl, 4 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 μg of leupeptin per ml, 10 μg of aprotinin per ml, 2 mM benzamidine, 1 μg of

pepstatin A per ml) (0.5 ml) was added to the cell pellet along with 500 μl of 0.5-mm glass beads (Biospec Products, Bartlesville, Okla.). Cells were lysed by agitation in a Mini-Beadbeater-8 (Biospec Products) with three 1-min bursts interspersed by 1-min intervals on ice. The bottom of each tube was punctured by a needle, and the cell lysate was collected by spinning into a new Eppendorf tube. The lysates were then spun at 14,000 × *g* for 15 min at 4°C. The soluble protein was quantitated by a Bradford assay (Bio-Rad). Approximately 1 mg of protein was added to a solution containing 200 μl of lysis buffer, 40 μl of 1:1 protein G-Sepharose (Pharmacia) in lysis buffer, and 2 μl of 9E10 (anti-Myc) monoclonal antibody ascitic fluid. The reaction mixtures were incubated at 4°C with end-over-end rotation for 1 to 4 h. Immune complexes were washed three times with lysis buffer containing 0.5 M NaCl.

Cdc7 protein kinase assays. Anti-Myc immune complexes were washed once with kinase buffer (15 mM $MgCl_2$, 50 mM Tris [pH 7.6]) to remove detergents present in the lysis buffer. To these immunoprecipitates was added 30 μl of kinase buffer plus ATP (50 mM Tris, 15 mM $MgCl_2$, 10 μM ATP, 5 μCi of [γ-^{32}P]ATP [6,000 Ci/mmol]) and 1 μg of substrate. Reaction mixtures were incubated at 30°C for 20 min unless otherwise indicated, and reactions were stopped by adding 10 μl of 4× SDS sample buffer (33) and boiling for 5 min. The reaction components were resolved by SDS-PAGE and transferred to nitrocellulose, and phosphorylation was analyzed by either autoradiography or PhosphorImager analysis (Molecular Dynamics). Quantitation of Cdc7p-Myc9 (Cdc7p tagged with nine tandem repeats of the c-Myc epitope) and Dbf4p was performed by immunoblotting with anti-Myc monoclonal antibody ascitic fluid (9E10) and rabbit anti-Dbf4 polyclonal antibodies, respectively. The assay is linear with respect to the amount of Cdc7p immunoprecipitated in that more phosphorylation is observed when increasing amounts of Cdc7-Myc9 are immunoprecipitated.

Cell cycle synchrony. Cell cycle synchrony was accomplished with *MAT***a** *bar1* strains grown to the early log phase in YEP with 2% dextrose. Cultures were treated with α-factor (50 ng/ml) at 30°C for 2 h to allow cells to accumulate in the G_1 phase (>95% unbudded). Cells were released from the arrest by filtration through 0.8-μm-pore-size filters, washing with five culture volumes of fresh medium, and resuspension in fresh prewarmed medium. At 15-min intervals after the release, aliquots of cells were removed, washed with water, pelleted, and frozen in liquid nitrogen. The frozen cell pellets were used for Cdc7-Myc9-specific immunoprecipitation as described above. The culture was maintained in mid-exponential growth by monitoring the cell density and adding fresh prewarmed medium as necessary. Progression through the cell cycle was monitored by bud morphology and flow cytometry.

Cell cycle arrest by inhibitory drugs. Cultures were incubated in the presence of one of the following inhibitory drugs at 30°C for 2 h: 100 nM α-factor (G_1 phase), 10 mg of hydroxyurea (HU) per ml (S phase), or 10 μg of nocodazole per ml (G_2/M phase). Cells arrested by HU are blocked throughout the S phase, while cells released by α-factor into HU are arrested at the G_1/S-phase boundary (33). Cells were harvested for kinase assays as described above. For immunoblotting, cells were harvested by centrifugation, washed with one culture volume of SCE (33), resuspended in 1× SDS sample buffer to a final concentration of 2×10^8 cells/ml, and boiled for 5 min.

Analysis of Dbf4p stability. Cells were grown in YEP with 2% raffinose and arrested at specific points in the cell cycle by the use of inhibitory drugs as described above. The expression of *HA-DBF4* from the *GAL1* promoter was briefly induced by the addition of 2% galactose to the medium for 90 min. The expression of *HA-DBF4* was then shut off by the addition of 2% glucose to repress the *GAL1* promoter and cycloheximide (10 μg/ml) to inhibit protein synthesis. Samples were taken at 5-min intervals following the shutoff of Dbf4p expression, and protein extracts were prepared as described above. The protein samples were resolved by SDS-PAGE, transferred to nitrocellulose, and probed with antihemagglutinin (HA) monoclonal antibody ascitic fluid (12CA5) at 1:2,000.

APC inactivation experiment. Cells were first arrested in α-factor for 3 h at 22°C. Cells were then shifted to the restrictive temperature of 37°C, and aliquots were taken at 15-min intervals. Samples were prepared for immunoblot analysis as described above.

Dbf4p stabilization experiment. Cells were first arrested in 3 μM α-factor for 3 h at 22°C. Galactose (2%) was added to the cells to induce the ectopic expression of *HA-DBF4* driven by the *GAL1* promoter. Cells were incubated for 30 min at 22°C to allow the expression of *HA-DBF4*. Cells were then shifted to 36°C for an additional 30 min to allow for the inactivation of *cdc16*. The expression of *HA-DBF4* was then shut off by the addition of 2% glucose to repress the *GAL1* promoter and cycloheximide (10 μg/ml) to inhibit protein synthesis. Samples were taken at 5-min intervals following the shutoff of Dbf4p expression, and protein extracts were prepared as described above. The protein samples were resolved by SDS-PAGE, transferred to nitrocellulose, and probed with anti-HA monoclonal antibody ascitic fluid (12CA5) at 1:2,000.

Ectopic expression of *DBF4*. Cells grown in YEP with 2% raffinose were arrested by α-factor, HU, or nocodazole as described above. After 2 h in the presence of the drug, 2% galactose or 2% glucose was added to the cells to either induce or repress the ectopic expression of *HA-DBF4* driven by the *GAL1* promoter. Extracts were prepared as described above for immunoprecipitations with anti-Myc antibodies, kinase assays, and immunoblots as described above. Parallel samples were taken for analysis by flow cytometry (48).

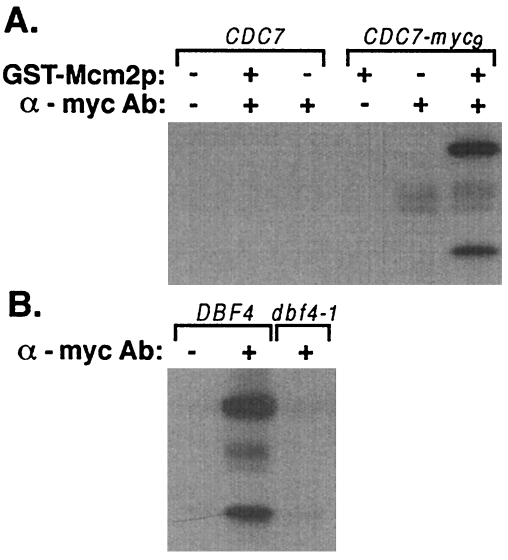

FIG. 1. Protein kinase assay of endogenous Cdc7p levels. (A) Immune complexes from asynchronous *CDC7* and *CDC7-myc*$_9$ cell lysates were assayed for kinase activity against GST-Mcm2p at 30°C, resolved by SDS-PAGE, and autoradiographed. Assays were done in the presence (+) or absence (−) of GST-Mcm2p or antibodies (Ab) against the Myc epitope. (B) Immune complexes from *CDC7-myc*$_9$ *DBF4* and *CDC7-myc*$_9$ *dbf4-1* lysates made from cells which were released at 22°C from an α-factor block into medium containing HU were assayed for kinase activity against GST-Mcm2p at 22°C, resolved by SDS-PAGE, and autoradiographed.

RESULTS

In order to investigate the regulation of Cdc7p in budding yeast cells, we constructed a strain (YJO235) in which nine tandem repeats of the c-Myc epitope (Myc9) were fused to the C terminus of the endogenous Cdc7 protein. The cells grew with the same kinetics and exhibited the same flow cytometry profile as cells of the congenic wild-type strain (YJL310) without the tag (data not shown), indicating that tagged Cdc7p was fully functional. The Myc9 tag allowed us to detect endogenous levels of Cdc7p by immunoblot analysis and to efficiently immunoprecipitate endogenous Cdc7p for kinase assays. GST-Mcm2p has been reported to be a substrate of budding yeast Cdc7p kinase purified from baculovirus-infected cells (44). Anti-Myc immunoprecipitates from YJO235 extracts were incubated with [γ-^{32}P]ATP, and GST-Mcm2p was affinity purified from yeast cells, subjected to SDS-PAGE, and autoradiographed. Both full-length GST-Mcm2p and a smaller breakdown product in the fusion protein preparation were radiolabeled (Fig. 1A). These phosphorylated products were not observed when immune complexes were prepared from extracts of an untagged strain (YJL310), when anti-Myc antibodies were omitted from the immunoprecipitation, or when purified GST-Mcm2p was not added to the kinase reaction (Fig. 1A). These results demonstrate that the phosphorylation of GST-Mcm2p observed in our kinase assay is dependent on Cdc7p kinase.

To further verify that we were assaying Cdc7p kinase activity, we tagged Cdc7p with Myc9 in a temperature-sensitive *dbf4-1* strain (YJO280). Dbf4p has previously been shown to be required to detect Cdc7p kinase activity in cells overexpressing Cdc7p with calf thymus histone H1 as a substrate (33). Cell lysates were made from *DBF4* and *dbf4-1* Myc9-tagged strains grown at the permissive temperature (22°C) and were assayed for Cdc7p kinase activity at 22°C. No phosphorylation of GST-

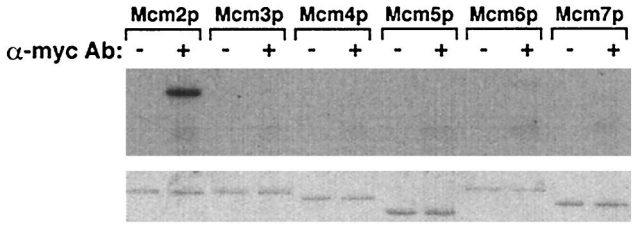

FIG. 2. Cdc7p kinase phosphorylates Mcm2p but not other Mcm proteins in vitro. (Upper panel) Immune complexes from asynchronous *CDC7-myc*$_9$ cell lysates prepared in the presence (+) or absence (−) of antibodies (Ab) against the Myc epitope were assayed for kinase activity against GST-Mcm2p, GST-Mcm3p, GST-Mcm4p, GST-Mcm5p, GST-Mcm6p, and GST-Mcm7p at 30°C, resolved by SDS-PAGE, and autoradiographed. (Lower panel) Coomassie blue stain of the same gel showing the GST fusions.

Mcm2p was observed in kinase assays performed with extracts of the *dbf4-1* strain (Fig. 1B). Thus, we conclude that we are specifically monitoring Cdc7p kinase activity in this assay.

Several fainter phosphorylated proteins migrating between the full-length GST-Mcm2p protein and its breakdown product were also generated in the kinase assay. These radiolabeled products were observed in the absence of added GST-Mcm2p but otherwise had the same dependencies as the phosphorylated GST-Mcm2p product. Because they comigrated with Cdc7p-Myc9 and Dbf4p, we suspect that they resulted from phosphorylation of either or both proteins by the Cdc7p-Dbf4p complex.

Genetic (24, 44) and biochemical (6, 44, 51) studies have suggested that the Mcm family of proteins is an in vivo target of Cdc7p kinase in the initiation of DNA replication. These proteins form a heteromeric complex (1, 61) that is incorporated into preinitiation complexes at replication origins (3, 59) before Cdc7p triggers origin firing. Exactly which, if any, of these proteins is phosphorylated by Cdc7p kinase in vivo is unknown. Budding yeast Cdc7p kinase purified from baculovirus-infected cells has been reported to phosphorylate GST-Mcm2p, GST-Mcm3p, GST-Mcm4p, and GST-Mcm6p but not GST-Mcm5p or GST-Mcm7p in vitro (44), raising the possibility that multiple subunits of the Mcm complex may be targeted by the Cdc7p kinase in vivo. We wanted to reexamine the question of Mcm substrate preference in vitro by using our Cdc7p kinase assay. Unlike the purified Cdc7p kinase assay discussed above, this assay examines endogenous Cdc7p kinase complexes formed in yeast cells and can be readily performed with mutant kinase complexes (in the *dbf4-1* strain background) to ensure that the assay is specific for Cdc7p kinase. We purified each of the Mcm proteins individually from yeast as GST fusion proteins by using the same expression constructs as those used in the baculovirus Cdc7p kinase experiments (44) and tested them as substrates for Cdc7p kinase. Only GST-Mcm2p was phosphorylated in this in vitro kinase assay (Fig. 2). GST-Mcm3p and GST-Mcm4p are capable of being phosphorylated by Cdc28p kinase complexes (data not shown), indicating that at least some of these substrates are capable of being recognized and phosphorylated by another kinase. In light of this substrate specificity, GST-Mcm2p was used as a substrate for Cdc7 kinase complexes in the remainder of this study.

To determine whether the activity of the endogenous Cdc7p kinase is cell cycle regulated, we used our assay to monitor Cdc7p kinase activity in synchronous cultures of cycling cells (Fig. 3). Previous studies have demonstrated that the Cdc7p level is constant but that kinase activity is periodic during the cell cycle; these studies examined the activity of overexpressed

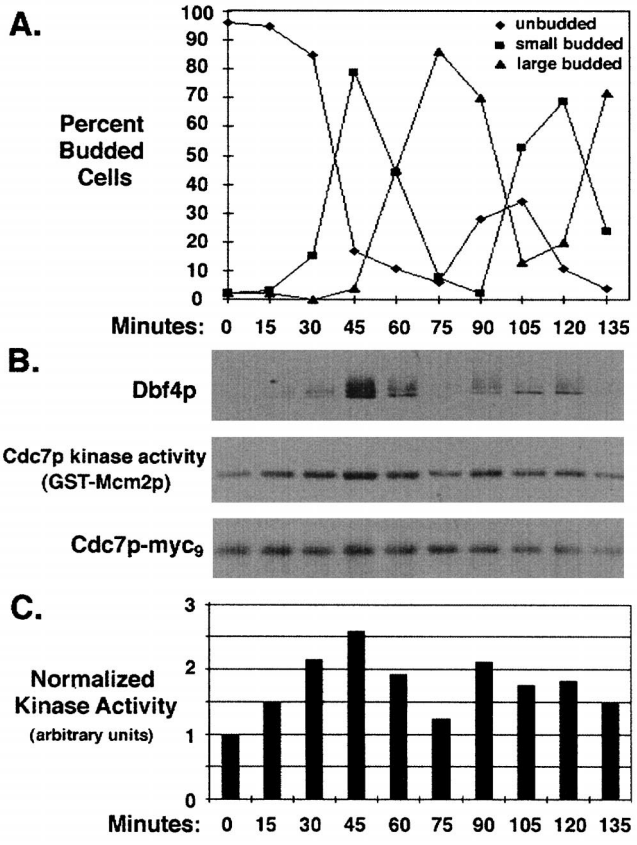

FIG. 3. Cdc7p kinase activity and Dbf4 protein levels fluctuate in cycling cells. *CDC7-myc*$_9$ cells were synchronously released from an α-factor arrest. (A) Cell synchrony was evaluated by determining the percentage of unbudded, small-budded, and large-budded cells. (B) Lysates were made at various times. Immune complexes were prepared and assayed for Cdc7p kinase activity against GST-Mcm2p at 30°C. Reactions were resolved by SDS-PAGE, transferred to nitrocellulose, immunoblotted with anti-Dbf4 antibodies (upper panel), developed by autoradiography to monitor GST-Mcm2p phosphorylation (middle panel), and reprobed with antibodies against the Myc epitope to detect Cdc7p in the immunoprecipitates (lower panel). (C) Cdc7p kinase activity was quantified on a PhosphorImager and normalized for the amount of Cdc7-Myc9 protein in each lane.

levels of Cdc7p with a very inefficient and nonphysiological substrate, histone H1 (33, 67). We synchronously released *CDC7-myc*$_9$ cells (YJO235) from α-factor-induced G_1-phase arrest and monitored them for nearly two cell cycles. Aliquots of cells were sampled at 15-min intervals and analyzed for Cdc7p kinase activity, Cdc7p levels, and Cdc7p-associated Dbf4p levels (Fig. 3B and C). Cell cycle position and cell synchrony were assessed by use of budding indices (Fig. 3A) and DNA content (data not shown). Cdc7p kinase activity starts off low in α-factor-arrested cells, begins to increase within 15 min after release, and peaks after 45 min. The peak of activity coincides with the onset of DNA replication, as suggested by the peak of small budded cells present at this time point; this finding was confirmed by flow cytometric analysis of DNA content (data not shown). At 75 min, when cells are undergoing mitosis and beginning to enter the next cell cycle, Cdc7p kinase activity drops back to starting levels. They increase again in the second cell cycle as cells begin to bud and replicate again, but the loss of synchrony makes it difficult to assess the precise timing of this second burst of activity. Taken as a whole, our data indicate that endogenous Cdc7p kinase

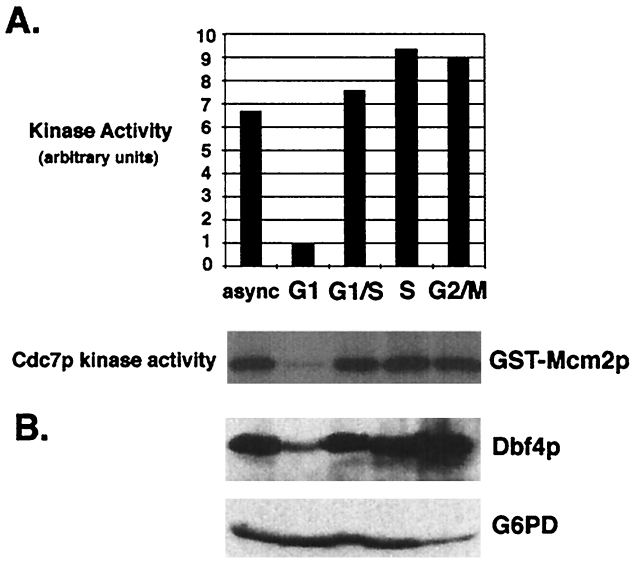

FIG. 4. Cdc7p kinase activity and Dbf4 protein levels in arrested cells. *CDC7-myc$_9$* cells were arrested with either α-factor (G_1), α-factor released into HU (G_1/S), HU (S), or nocodazole (G_2/M). (A) Lysates were used in kinase assays against GST-Mcm2p at 30°C. async, asynchronous cells. (B) In a separate experiment, total cellular Dbf4 protein levels were detected by immunoblotting with anti-Dbf4 antibodies (upper panel); blots were reprobed with anti-G6PD antibodies as a loading control (lower panel).

activity is cell cycle regulated, being low in G_1-phase cells and increasing as cells enter the S phase.

The fluctuation in Cdc7p kinase activity was not due to fluctuations in Cdc7p levels, as constant amounts of the protein were immunoprecipitated by anti-Myc antibodies (Fig. 3B, lower panel). In other, similar experiments, Cdc7p levels were shown to be constant with respect to those of a control protein, glucose-6-phosphate dehydrogenase (G6PD), analyzed on the same immunoblot (data not shown). Thus, the specific activity of Cdc7p kinase must change during the cell cycle. Interestingly, the changes in the specific activity of Cdc7p kinase corresponded to the changes in the amount of Dbf4p coimmunoprecipitating with Cdc7p (Fig. 3B, upper panel), which in turn mirrored the total levels of Dbf4p in the cells (data not shown). Given that Dbf4p is necessary for Cdc7p kinase activity on GST-Mcm2p, these results suggest that the cell cycle regulation of Dbf4p levels is responsible for the regulation of Cdc7p kinase activity.

The reduction (two- to threefold) in Cdc7 kinase activity seen in this experiment is not as great as that seen when Dbf4 is completely inactivated (Fig. 1). This is probably due to the efficiency of the synchrony. Therefore, we measured Cdc7p-Dbf4p complex levels in arrested cells blocked at a specific stage of the cell cycle. We made extracts from *CDC7-myc$_9$* cells (YJO235) arrested at various stages of the cell cycle, immunoprecipitated Cdc7p-Myc9, quantified kinase activity on GST-Mcm2p (Fig. 4A), and measured Dbf4p levels by immunoblot analysis (Fig. 4B). Cells arrested by α-factor in the G_1 phase before Start had low levels of Cdc7p kinase activity and small amounts of Cdc7p-associated Dbf4p. Cells released from this α-factor block into HU underwent arrest in the early S phase, at the G_1/S-phase boundary (33). Both Cdc7p kinase levels and Dbf4p levels were high at this point. They remained high in cells arrested throughout the S phase by HU and in cells arrested at the G_2/M phase by nocodazole. Thus, both Dbf4p association with Cdc7p and Cdc7p kinase activity are induced as cells pass Start and enter the S phase and decline some time during mitosis and entry into the next cell cycle.

Previous reports that the level of *DBF4* transcripts is cell cycle regulated have suggested that cell cycle regulation of Dbf4p levels could be due to transcriptional regulation (9). However, a recent genome-wide analysis of budding yeast mRNA transcript levels across the cell cycle failed to detect significant fluctuations in *DBF4* transcript levels (10, 57). We have also failed to detect cell cycle fluctuations in *DBF4* transcript levels by Northern analysis (reference 17 and data not shown). Thus, we conclude that *DBF4* transcript levels are constant during the cell cycle and cannot account for the changes in Dbf4p levels that we observed. Instead, the fluctuation in Dbf4p steady-state levels must be due to changes in the rate of Dbf4p translation and/or changes in the rate of Dbf4p degradation.

To examine the latter possibility, we analyzed the stability of Dbf4p at different stages of the cell cycle. Cells expressing Dbf4p under the control of the galactose-inducible *GAL1* promoter (GOY264) were shifted to medium containing glucose and cycloheximide to transcriptionally repress *DBF4* expression and to block new protein synthesis, respectively. Whole-cell extracts made every 5 min were separated by SDS-PAGE and immunoblotted with anti-HA antibodies. Dbf4p is less stable in cells arrested by α-factor (Fig. 5B) than in asynchronously growing cells (Fig. 5A). Consistent with its role in S-phase progression, Dbf4p is more stable in HU-arrested cells (Fig. 5C) and in cells arrested at the G_2/M phase by nocodazole (Fig. 5D). Thus, Dbf4p is an unstable protein during the whole-cell cycle, but it is especially unstable in the G_1 phase before Start, when its steady-state levels are lowest.

The regulation of Dbf4p stability during the cell cycle is reminiscent of the regulation of a number of proteins (e.g., Clb2p, Pds1p, and Ase1p) involved in mitosis (12, 36, 63). These proteins become highly unstable in mitosis and remain so until cells pass Start in the following G_1 phase. This instability is mediated by the APC, a large multisubunit complex that targets proteins for proteolysis by facilitating their polyubiquitination (reviewed in reference 62). The ability of the APC to polyubiquitinate proteins such as Clb2 is regulated during the cell cycle by a number of different cofactors (52, 56, 63). To determine if the APC is involved in destabilizing Dbf4p during the G_1 phase, we examined the steady-state levels of Dbf4p following inactivation of the APC. The APC can be conditionally inactivated by temperature-sensitive mutations in *CDC16*, which encodes one of the components of the APC (39, 68). We arrested *CDC16* and *cdc16-1* cells in the G_1 phase by using α-factor, shifted them to the restrictive temperature at time zero, and then collected samples every 15 min for analysis of Dbf4p levels on anti-Dbf4p immunoblots. If the APC is involved in the destruction of Dbf4p in the G_1 phase, inactivation of the APC should lead to an increase in the steady-state levels of Dbf4p. Such an increase was observed approximately 45 min after the shift to the restrictive temperature in *cdc16-1* but not *CDC16* cells (Fig. 6). Cells under these conditions remained in the G_1 phase throughout the course of the experiment, as shown by both flow cytometry and budding indices (data not shown). Others have found that *cdc16* mutant cells treated in a similar manner will eventually bud and enter the S phase, but only after a prolonged arrest (more than 2 h) (31).

We also analyzed the stability of Dbf4p after Cdc16p inactivation (Fig. 7). Mutant *cdc16-1* cells expressing Dbf4p under the control of the *GAL1* promoter (GOY244) were first arrested in the G_1 phase by α-factor. The cells were shifted to glucose-cycloheximide medium to inhibit further Dbf4p expression, as in the experiment shown in Fig. 5. Dbf4p was then

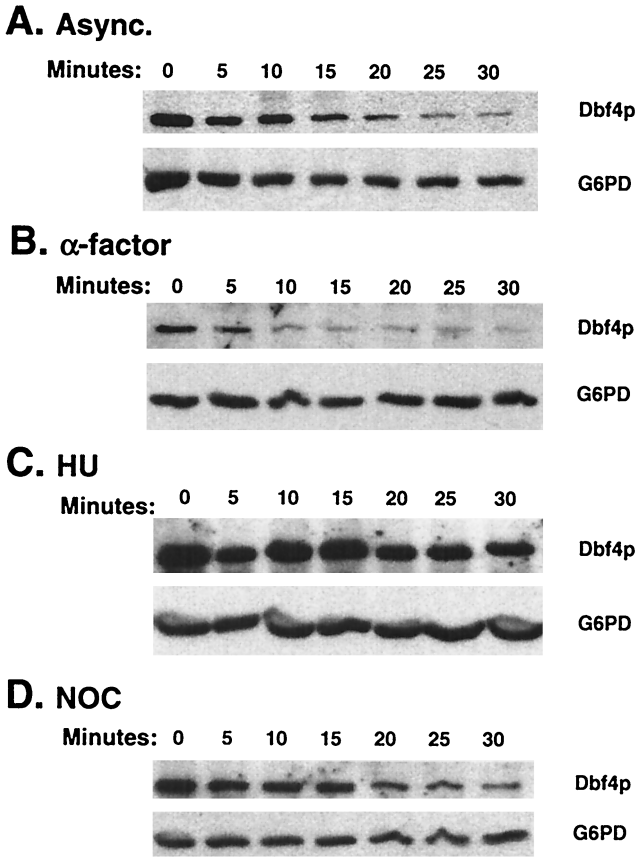

FIG. 5. Dbf4 protein stability changes at different points in the cell cycle. Cells containing a galactose-inducible *HA-DBF4* plasmid (GOY264) were grown in the presence of raffinose and treated with no drug (A), α-factor (B), HU (C), or nocodazole (NOC) (D) for 2 h. HA-Dbf4p was induced for 90 min by the addition of galactose. At time zero (0 min), glucose and cyclohexamide were added to repress HA-DBF4 transcription and block protein synthesis, respectively. Lysates were made at the indicated times, and protein levels were analyzed by immunoblotting with antibodies against the HA epitope. Blots were reprobed with anti-G6PD antibodies as a loading control. Async., asynchronous cells.

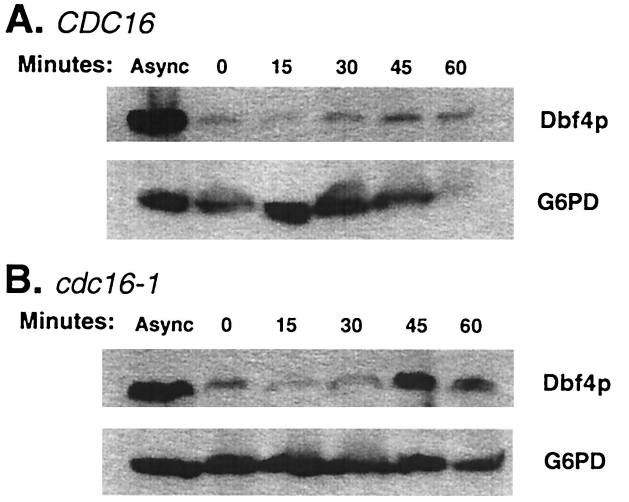

FIG. 6. Dbf4p degradation in the G_1 phase is dependent upon *CDC16*. *CDC16* (A) and *cdc16-1* (B) cells were arrested in the presence of α-factor for 3 h at 22°C. At time zero (0 min), cultures were shifted to 36°C, and lysates were made at the indicated times. Dbf4 protein levels were analyzed by immunoblotting with anti-Dbf4 antibodies. Blots were reprobed with anti-G6PD antibodies as a loading control. Async, asynchronous cells.

analyzed by immunoblotting (Fig. 7). In contrast to the results seen for wild-type cells (Fig. 5), Dbf4p was more stable at the restrictive temperature, at which Cdc16p is inactivated. Wild-type cells given the same treatment showed the same instability of Dbf4p as that shown in Fig. 5 (data not shown). These data indicate that the APC is required for the degradation of Dbf4p in G_1-phase cells and raise the possibility that regulation of the APC ubiquitination of Dbf4p may be responsible for the regulation of Dbf4p stability during the cell cycle.

Although reduced steady-state levels of Dbf4p can clearly account for the low levels of Cdc7p kinase activity observed in the G_1 phase, cells could also use additional controls to suppress kinase activity. If Dbf4p levels are the primary determinant of Cdc7p kinase activity, raising the steady-state levels of Dbf4p in the G_1 phase should lead to the ectopic induction of Cdc7p kinase activity. On the other hand, if other controls are used to keep Cdc7p kinase activity in check, Cdc7p kinase activity should remain low. In order to raise the steady-state levels of Dbf4p in the G_1 phase, we overexpressed *DBF4* under the control of the *GAL1* promoter, hoping that the increased rate of Dbf4p synthesis arising from elevated transcript levels would counteract the high rate of Dbf4p degradation during this stage of the cell cycle. As shown in Fig. 8C, the overexpression of *DBF4* in the G_1 phase led to both increased association of Dbf4p with Cdc7p and increased Cdc7p kinase activity. Flow cytometry and budding indices verified that the cells remained arrested in the G_1 phase throughout the course of the experiment (data not shown). Apparently the overexpression could not completely overcome the instability of Dbf4p in the G_1 phase, as the amount of Dbf4p associated with Cdc7p was smaller than that seen for HU-arrested cells expressing endogenous levels of Dbf4p. Accordingly, Cdc7p kinase activity was proportionally lower, correlating with the lower level of Dbf4p associated with the kinase. These results argue that Dbf4p levels are the primary determinant of Cdc7p kinase activity.

Given the importance of the Cdc7-Dbf4 complex in S-phase regulation, we investigated whether the ectopic production of Dbf4p perturbs the cell cycle. Cells of strain GOY264, in which *HA-DBF4* is overexpressed under the control of the galactose-inducible *GAL1* promoter, were grown under either inducing or repressing conditions. As a control, the same strain with the plasmid vector (GOY263) was treated similarly. Flow cytometry showed that Dbf4p overexpression leads to a decrease in

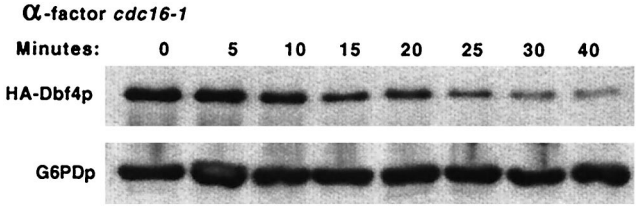

FIG. 7. Dbf4 protein stability increases in the absence of Cdc16p function. *cdc16-1* cells were arrested in the presence of α-factor for 3 h at 22°C. HA-Dbf4p was induced by the addition of galactose, and cells were incubated at 22°C for 30 min. Cells were then shifted to 36°C and incubated for 30 min. At time zero (0 min), glucose and cycloheximide were added to repress HA-DBF4 transcription and block protein synthesis, respectively. Lysates were made at the indicated times, and protein levels were analyzed by immunoblotting with antibodies against the HA epitope. Blots were reprobed with anti-G6PD antibodies as a loading control.

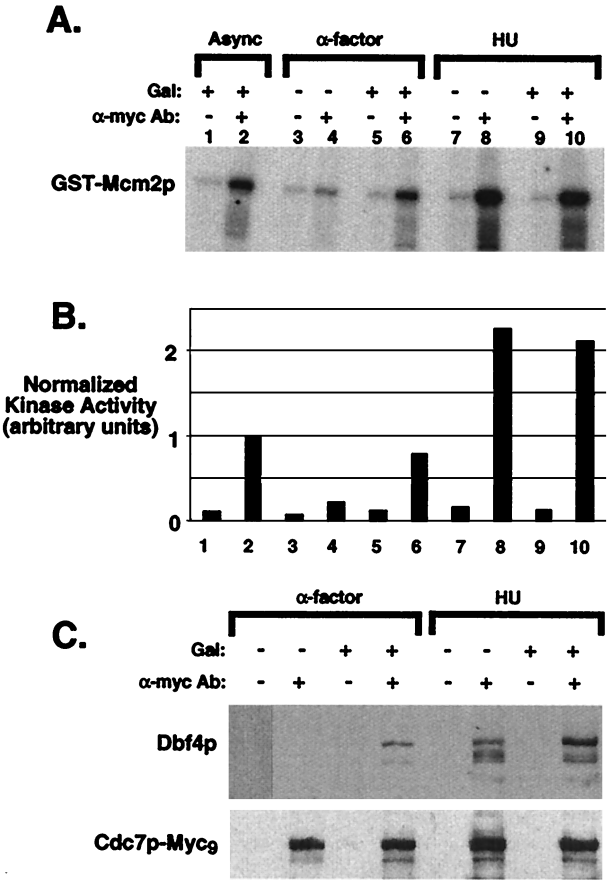

FIG. 8. Ectopic expression of Dbf4p is sufficient to induce Cdc7p kinase activity in the G_1 phase. *CDC7-myc*$_9$ cells containing a galactose-inducible *HA-DBF4* plasmid were arrested with α-factor or HU for 2 h. Dbf4p was either induced (+ Gal) or repressed (− Gal) for 90 min. (A) Lysates were used for kinase assays against GST-Mcm2p, resolved by SDS-PAGE, and autoradiographed. Async, asynchronous cells; Ab, antibodies. (B) Kinase reactions were quantitated on a PhosphorImager and normalized for the Cdc7-Myc9 level in each lane as described in the legend to Fig. 3. (C) Dbf4p levels in the anti-Myc immune complexes were detected by immunoblotting with anti-Dbf4 antibodies (upper panel) and reprobed with antibodies against the Myc epitope to detect Cdc7p in the immunoprecipitates (lower panel).

the G_1-phase population, consistent with a slight acceleration in the G_1/S-phase transition (data not shown). A similar subtle phenotype is also seen with the *mcm5-bob1* mutant (24). These results demonstrate that the ectopic expression of Dbf4p perturbs the cell cycle (24).

DISCUSSION

The initiation of DNA synthesis at eukaryotic replication origins is tightly controlled during the cell cycle. We have shown that the activity of Cdc7p kinase, which is essential for triggering this initiation, is itself cell cycle regulated. In these experiments, we monitored the activity of endogenous Cdc7p kinase on a candidate in vivo target, Mcm2p. Our results confirm and extend previous reports that the activity of overproduced Cdc7p as assayed on a nonphysiological substrate, histone H1, is cell cycle regulated (33, 67). We observed that endogenous Cdc7p kinase activity is low in the G_1 phase before Start; during this period, origins are primed for initiation (15, 50) by the assembly of preinitiation complexes in a Cdc7p-independent manner (16). Cdc7p activity increases dramatically after passage through Start during entry into the S phase, as would be expected for an activity required to activate primed origins. The activity remains high through the S phase and the beginning of mitosis and then drops to G_1-phase levels during mitosis and the transition into the next cell cycle. By coupling the induction of Cdc7p kinase activity to Start in each cell cycle, this regulation could help ensure that replication initiation is dependent on passage through Start.

Our investigation of the basis for this regulation revealed that changes in the activity of Cdc7p kinase correlate with changes in the level of its regulatory subunit, Dbf4p. Dbf4p protein levels are low in the early G_1 phase before Start, rise as cells enter the S phase, and decline sometime during mitosis and the transition into the next cell cycle. In contrast, endogenous levels of Cdc7p protein remain constant throughout the cell cycle. Disrupting Dbf4p regulation by forced expression of Dbf4p in G_1-phase-arrested cells leads to the ectopic induction of Cdc7p kinase activity. These results indicate that the regulation of Dbf4p protein levels plays a key and possibly primary role in the regulation of Cdc7p kinase activity. Furthermore, the ectopic induction of Cdc7p kinase activity in G_1-phase-arrested cells strongly argues that the S-phase-promoting CDKs (Cdc28p/Clb5p and Cdc28p/Clb6p), which are not active at this arrest, are not required for the activation of Cdc7p kinase. This notion is consistent with the observation that mutation of all CDK consensus phosphorylation sites in Cdc7p has no effect on *CDC7* function in the cells (see reference 55). Since the S-phase-promoting CDKs conversely can be induced in the absence of Cdc7p (54), we conclude that the two kinases required to initiate DNA synthesis at replication origins can be activated independently of each other.

Contrary to previous reports (9), a genome-wide analysis of mRNA regulation during the cell cycle (10, 57) and our own analysis of *DBF4* mRNA levels (17) indicate that *DBF4* transcript levels are relatively constant during the cell cycle. Thus, changes in Dbf4 protein levels must be due to changes in the rates of Dbf4p translation and/or Dbf4 degradation. We have monitored Dbf4p degradation at different points in the cell cycle and have shown that changes in Dbf4 protein stability can account, in part, for the observed changes in Dbf4p steady-state levels. The protein is relatively unstable throughout the cell cycle, but its half-life is particularly short in G_1-phase-arrested cells, when Dbf4 levels are lowest. (Since we have not measured Dbf4 synthesis rates, we cannot rule out changes in these rates also contributing to Dbf4 periodicity.)

The regulation of Cdc7p kinase activity thus appears to be determined in significant measure by the regulation of Dbf4p degradation. We have shown that Dbf4p degradation in the G_1 phase is dependent on Cdc16p, a component of the APC, which targets proteins for proteolysis by facilitating their polyubiquitination (reviewed in reference 62). By targeting the degradation of a number of mitotic proteins in a cell-cycle-regulated fashion, the APC ensures that key mitotic events are executed in their proper order. For example, APC-mediated polyubiquitination of the B-type cyclin Clb2p is activated at the end of mitosis and remains activated until Cln kinase activity is induced at Start (2). It remains to be seen whether APC-mediated ubiquitination of Dbf4p is also regulated during the cell cycle. If so, it would be interesting to determine how this ubiquitination is regulated. Inhibition of APC-mediated ubiquitination of Dbf4p by Cln kinases, for example, could help explain why Dbf4p induction and Cdc7p kinase activity are dependent on passage through Start.

Dbf4p contains a number of potential destruction boxes (reviewed in reference 62), most notably a region rich in four amino acids, PEST, at the C terminus and six RXXL motifs.

We have found that deletion of the PEST sequence at the C terminus (amino acids 570 to 695) does not change the stability of Dbf4p or its function, as it can complement a deletion of *dbf4* (data not shown). Further investigation is required to determine the function of the RXXL motifs in the regulation of Dbf4p stability.

A multitude of genetic interactions between *CDC7* and *DBF4* have strongly suggested that their products are physically associated in a heteromeric complex in vivo (33, 40, 44). Purification of the homologous kinase activity from *S. pombe* provided the first direct evidence that these proteins physically associate in a kinase complex in the cells (6). Our coimmunoprecipitation data confirm that this physical association also occurs in *S. cerevisiae* and demonstrate that this association is determined primarily by the level of Dbf4p protein in the cells. Cdc7p is not associated with Dbf4p in the early G_1 phase but becomes associated as Dbf4p accumulates in the S phase or if Dbf4p is ectopically expressed in G_1-phase cells.

Because Dbf4p is required for Cdc7p kinase activity on exogenous substrates, the relationship between these two proteins has been likened to that of a cyclin and its CDK (55). Our observation that Dbf4p is periodically associated with Cdc7p during the cell cycle further extends this analogy. Unlike CDKs, however, which have no detectable kinase activity in the absence of cyclin association, purified Hsk1p, the *S. pombe* homolog of Cdc7p, has intrinsic kinase activity in the absence of the Dbf4p homolog, Dfp1p (6). This activity is manifested as autophosphorylation of monomeric Hsk1p, and it has been suggested that the role of Dfp1p is to direct this activity toward exogenous substrates. However, we were not able to detect autophosphorylation of Cdc7p kinase that was immunoprecipitated from G_1-phase extracts devoid of Dbf4p. Biochemical analysis of purified Cdc7p and Dbf4p will be needed to resolve whether Cdc7p from *S. cerevisiae* has intrinsic kinase activity which is then properly targeted by Dbf4p or whether Cdc7p is completely dependent on Dbf4p for kinase activity. Regardless of the answer, the critical initiation function of Cdc7p presumably involves the phosphorylation of exogenous targets and hence is almost certainly dependent on Dbf4p.

The leading candidates for Cdc7p kinase targets are the Mcm proteins. The Mcm proteins form heteromeric complexes (1, 14, 43, 61) that appear to load onto origins during the assembly of preinitiation complexes (3, 59) and possibly relocalize to replication forks following initiation (3). Indirect evidence for a potential role of these proteins in origin unwinding during initiation has been reported (60). Moreover, helicase activity has been detected in an Mcm4p-Mcm6p-Mcm7p subcomplex from humans (32), raising the possibility that the Mcm proteins facilitate fork movement during replication elongation. The idea that these proteins may be important substrates for the Cdc7p-Dbf4p complex is supported by observations of genetic interactions between components of this complex and the Mcm family of proteins in budding yeast (24, 44). A biochemical connection was first established by reports that human Cdc7p immune complexes phosphorylate bacterially expressed human Mcm2p and Mcm3p (51) and that budding yeast Cdc7p-Dbf4p purified from baculovirus-infected cells phosphorylates GST-Mcm2p, GST-Mcm3p, GST-Mcm4p, and GST-Mcm6p purified from yeast cells (44). We found that Cdc7p kinase immunoprecipitated from yeast cells also phosphorylates GST-Mcm2p, but we did not observe phosphorylation of any of the other GST-Mcm proteins. Although this discrepancy could be due to differences in protein preparation or assay conditions, our observations are consistent with the report that purified Hsk1p-Dfp1p preferentially phosphorylates Mcm2p when presented with a heteromeric Mcm complex containing all six Mcm proteins (6). Analysis of Mcm phosphorylation in vivo will be needed to determine whether these proteins are indeed important targets of the Cdc7p-Dbf4p complex in the initiation of DNA replication and, if so, exactly which Mcm proteins are phosphorylated. Nonetheless, given the report that human Mcm2p inhibits the helicase activity of a human Mcm4p-Mcm6-Mcm7p subcomplex (32), it is tempting to speculate that Cdc7p phosphorylation of Mcm2p triggers initiation by neutralizing the inhibitory activity of Mcm2p and unleashing the unwinding activity of a heteromeric Mcm complex.

ACKNOWLEDGMENTS

G.O. and J.C.O. contributed equally to the work reported in this paper.

We thank Bik Tye, Yasuo Kawasaki, and Akio Sugino for strains and plasmids and Tom Wang for help in plasmid constructions. University of Colorado Cancer Center Core facilities were used for DNA sequencing, flow cytometry, and monoclonal antibody production (supported by PHS grant P30 CA46934).

This work was supported by PHS grant GM35078 awarded to R.A.S. J.C.O. is supported by an Achievement Rewards for College Scientists Foundation graduate fellowship and by a National Institutes of Health training grant. J.J.L. is a Searle Scholar and a Rita Allen Foundation Scholar.

REFERENCES

1. **Adachi, Y., J. Usukura, and M. Yanagida.** 1997. A globular complex formation by Nda1 and the other five members of the MCM protein family in fission yeast. Genes Cells **2:**467–479.
2. **Amon, A., S. Irniger, and K. Nasmyth.** 1994. Closing the cell cycle circle in yeast: G2 cyclin proteolysis initiated at mitosis persists until the activation of G1 cyclins in the next cycle. Cell **77:**1037–1050.
3. **Aparicio, O. M., D. M. Weinstein, and S. P. Bell.** 1997. Components and dynamics of DNA replication complexes in S. cerevisiae: redistribution of MCM proteins and Cdc45p during S phase. Cell **91:**59–69.
4. **Bell, S. P., and B. Stillman.** 1992. ATP-dependent recognition of eukaryotic origins of DNA replication by a multiprotein complex. Nature **357:**128–134.
5. **Bousset, K., and J. F. Diffley.** 1998. The Cdc7 protein kinase is required for origin firing during S phase. Genes Dev. **12:**480–490.
6. **Brown, G. W., and T. J. Kelly.** 1998. Purification of Hsk1, a minichromosome maintenance protein kinase from fission yeast. J. Biol. Chem. **273:**22083–22090.
7. **Buck, V., A. White, and J. Rosamond.** 1991. *CDC7* protein kinase activity is required for mitosis and meiosis in *Saccharomyces cerevisiae*. Mol. Gen. Genet. **227:**452–457.
8. **Carpenter, P. B., P. R. Mueller, and W. G. Dunphy.** 1996. Role for a Xenopus Orc2-related protein in controlling DNA replication. Nature **379:**357–360.
9. **Chapman, J. W., and L. H. Johnson.** 1989. The yeast gene, *DBF4*, essential for entry into S phase is cell cycle regulated. Exp. Cell Res. **180:**419–428.
10. **Cho, R. J., M. J. Campbell, E. A. Winzeler, L. Steinmetz, A. Conway, L. Wodicka, T. G. Wolfsberg, A. E. Gabrielian, D. Landsman, D. J. Lockhart, and R. W. Davis.** 1998. A genome-wide transcriptional analysis of the mitotic cell cycle. Mol. Cell **2:**65–73.
11. **Chong, J. P., H. M. Mahbubani, C. Y. Khoo, and J. J. Blow.** 1995. Purification of an MCM-containing complex as a component of the DNA replication licensing system. Nature **375:**418–421.
12. **Cohen-Fix, O., and D. Koshland.** 1997. The metaphase-to-anaphase transition: avoiding a mid-life crisis. Curr. Opin. Cell. Biol. **9:**800–806.
13. **Coxon, A., K. Maundrell, and S. E. Kearsey.** 1992. Fission yeast cdc21+ belongs to a family of proteins involved in an early step of chromosome replication. Nucleic Acids Res. **20:**5571–5577.
14. **Dalton, S., and B. Hopwood.** 1997. Characterization of Cdc47p-minichromosome maintenance complexes in *Saccharomyces cerevisiae*: identification of Cdc45p as a subunit. Mol. Cell. Biol. **17:**5867–5875.
15. **Detweiler, C. S., and J. J. Li.** 1997. Cdc6p establishes and maintains a state of replication competence during G1 phase. J. Cell Sci. **110:**753–763.
16. **Diffley, J. F. X., J. H. Cocker, S. J. Dowell, and A. Rowley.** 1994. Two steps in the assembly of complexes at yeast replication origins in vivo. Cell **78:**303–316.
17. **Dohrmann, P. R., and R. A. Sclafani.** 1998. Personal communication.
18. **Donaldson, A. D., W. L. Fangman, and B. J. Brewer.** 1998. Cdc7 is required throughout the yeast S phase to activate replication origins. Genes Dev. **12:**491–501.
19. **Donovan, S., and J. F. Diffley.** 1996. Replication origins in eukaryotes. Curr. Opin. Genet. Dev. **6:**203–207.

20. **Dowell, S. J., P. Romanowski, and J. F. Diffley.** 1994. Interaction of Dbf4, the Cdc7 protein kinase regulatory subunit, with yeast replication origins in vivo. Science **265:**1243–1246.
21. **Dutta, A., and S. P. Bell.** 1997. Initiation of DNA replication in eukaryotic cells. Annu. Rev. Cell Dev. Biol. **13:**293–332.
22. **Forsburg, S. L., and P. Nurse.** 1994. The fission yeast *cdc19+* gene encodes a member of the MCM family of replication proteins. J. Cell Sci. **107:**2779–2788.
23. **Hardy, C. F.** 1997. Identification of Cdc45p, an essential factor required for DNA replication. Gene **187:**239–246.
24. **Hardy, C. F. J., O. Dryga, S. Seematter, P. M. B. Pahl, and R. A. Sclafani.** 1997. *mcm5/cdc46-bob1* bypasses the requirement for the S phase activator Cdc7p. Proc. Natl. Acad. Sci. USA **94:**3151–3155.
25. **Hartwell, L. H., R. K. Mortimer, J. Culotti, and M. Culotti.** 1973. Genetic control of the cell division cycle in yeast. V. Genetic analysis of *cdc* mutants. Genetics **74:**267–286.
26. **Hennessy, K. M., C. D. Clark, and D. Botstein.** 1990. Subcellular localization of yeast *CDC46* varies with the cell cycle. Genes Dev. **4:**2252–2263.
27. **Hennessy, K. M., A. Lee, E. Chen, and D. Botstein.** 1991. A group of interacting yeast DNA replication genes. Genes Dev. **5:**958–969.
28. **Hess, G. F., R. F. Drong, K. L. Weiland, J. L. Slightom, R. A. Sclafani, and R. E. Hollingsworth.** 1998. A human homolog of the yeast *CDC7* gene is overexpressed in some tumors and transformed cell lines. Gene **211:**133–140.
29. **Hollingsworth, R. E., and R. A. Sclafani.** 1990. DNA metabolism gene *CDC7* from yeast encodes a serine(threonine) protein kinase. Proc. Natl. Acad. Sci. USA **87:**6272–6276.
30. **Hopwood, B., and S. Dalton.** 1996. Cdc45p assembles into a complex with Cdc46p/Mcm5p, is required for minichromosome maintenance, and is essential for chromosomal DNA replication. Proc. Natl. Acad. Sci. USA **93:**12309–12314.
31. **Irniger, S., and K. Nasmyth.** 1997. The anaphase-promoting complex is required in G1 arrested yeast cells to inhibit B-type cyclin accumulation and to prevent uncontrolled entry into S-phase. J. Cell Sci. **110:**1523–1531.
32. **Ishimi, Y.** 1997. A DNA helicase activity is associated with an MCM4, -6, and -7 protein complex. J. Biol. Chem. **272:**24508–24513.
33. **Jackson, A. L., P. M. Pahl, K. Harrison, J. Rosamond, and R. A. Sclafani.** 1993. Cell cycle regulation of the yeast Cdc7 protein kinase by association with the Dbf4 protein. Mol. Cell. Biol. **13:**2899–2908.
34. **Jiang, W., and T. Hunter.** 1997. Identification and characterization of a human protein kinase related to budding yeast Cdc7p. Proc. Natl. Acad. Sci. USA **94:**14320–14325.
35. **Johnston, L. H., and A. P. Thomas.** 1982. A further two mutants defective in initiation of the S phase in the yeast *Saccharomyces cerevisiae*. Mol. Gen. Genet. **186:**445–448.
36. **Juang, Y. L., J. Huang, J. M. Peters, M. E. McLaughlin, C. Y. Tai, and D. Pellman.** 1997. APC-mediated proteolysis of Ase1 and the morphogenesis of the mitotic spindle. Science **275:**1311–1314.
37. **Kim, J. M., N. Sato, M. Yamada, K. Arai, and H. Masai.** 1998. Growth regulation of the expression of mouse cDNA and gene encoding a serine/threonine kinase related to *Saccharomyces cerevisiae CDC7* essential for G1/S transition. Structure, chromosomal localization, and expression of mouse gene for *S. cerevisiae* Cdc7-related kinase. J. Biol. Chem. **273:**23248–23257.
38. **Kimura, H., N. Nozaki, and K. Sugimoto.** 1994. DNA polymerase alpha associated protein P1, a murine homolog of yeast *MCM3*, changes its intranuclear distribution during the DNA synthetic period. EMBO J. **13:**4311–4320.
39. **King, R. W., J. M. Peters, S. Tugendreich, M. Rolfe, P. Hieter, and M. W. Kirschner.** 1995. A 20S complex containing *CDC27* and *CDC16* catalyzes the mitosis-specific conjugation of ubiquitin to cyclin B. Cell **81:**279–288.
40. **Kitada, K., A. L. Johnson, L. H. Johnston, and A. Sugino.** 1993. A multicopy suppressor gene of the *Saccharomyces cerevisiae* G_1 cell cycle mutant gene *dbf4* encodes a protein kinase and is identified as *CDC5*. Mol. Cell. Biol. **13:**4445–4457.
41. **Kitada, K., L. H. Johnston, T. Sugino, and A. Sugino.** 1992. Temperature-sensitive *cdc7* mutations of *Saccharomyces cerevisiae* are suppressed by the *DBF4* gene, which is required for the G1/S transition. Genetics **131:**21–29.
42. **Kubota, Y., S. Mimura, S. Nishimoto, H. Takisawa, and H. Nojima.** 1995. Identification of the yeast MCM3-related protein as a component of Xenopus DNA replication licensing factor. Cell **81:**601–609.
43. **Lei, M., Y. Kawasaki, and B. K. Tye.** 1996. Physical interactions among Mcm proteins and effects of Mcm dosage on DNA replication in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **16:**5081–5090.
44. **Lei, M., Y. Kawasaki, M. R. Young, M. Kihara, A. Sugino, and B. K. Tye.** 1997. Mcm2 is a target of regulation by Cdc7-Dbf4 during the initiation of DNA synthesis. Genes Dev. **11:**3365–3374.
45. **Madine, M. A., C. Y. Khoo, A. D. Mills, and R. A. Laskey.** 1995. MCM3 complex required for cell cycle regulation of DNA replication in vertebrate cells. Nature **375:**421–424.
46. **Masai, H., T. Miyake, and K. Arai.** 1995. *hsk1+*, a *Schizosaccharomyces pombe* gene related to *Saccharomyces cerevisiae CDC7*, is required for chromosomal replication. EMBO J. **14:**3094–3104.
47. **Miyake, S., N. Okishio, I. Samejima, Y. Hiraoka, T. Toda, I. Saitoh, and M. Yanagida.** 1993. Fission yeast genes *nda1+* and *nda4+*, mutations of which lead to S-phase block, chromatin alteration and Ca2+ suppression, are members of the *CDC46/MCM2* family. Mol. Biol. Cell **4:**1003–1015.
48. **Owens, J. C., C. S. Detweiler, and J. J. Li.** 1997. *CDC45* is required in conjunction with *CDC7/DBF4* to trigger the initiation of DNA replication. Proc. Natl. Acad. Sci. USA **94:**12521–12526.
49. **Patterson, M., R. A. Sclafani, W. L. Fangman, and J. Rosamond.** 1986. Molecular characterization of cell cycle cene *CDC7* from *Saccharomyces cerevisiae*. Mol. Cell. Biol. **6:**1590–1598.
50. **Piatti, S., T. Bohm, J. H. Cocker, J. F. Diffley, and K. Nasmyth.** 1996. Activation of S-phase-promoting CDKs in late G1 defines a "point of no return" after which Cdc6 synthesis cannot promote DNA replication in yeast. Genes Dev. **10:**1516–1531.
51. **Sato, N., K. Arai, and H. Masai.** 1997. Human and Xenopus cDNAs encoding budding yeast Cdc7-related kinases: in vitro phosphorylation of MCM subunits by a putative human homologue of Cdc7. EMBO J. **16:**4340–4351.
52. **Schwab, M., A. S. Lutum, and W. Seufert.** 1997. Yeast Hct1 is a regulator of Clb2 cyclin proteolysis. Cell **90:**683–693.
53. **Schwob, E., T. Bohm, M. D. Mendenhall, and K. Nasmyth.** 1994. The B-type cyclin kinase inhibitor p40SIC1 controls the G1 to S transition in S. cerevisiae. Cell **79:**233–244.
54. **Schwob, E., and K. Nasmyth.** 1993. *CLB5* and *CLB6*, a new pair of B cyclins involved in DNA replication in *Saccharomyces cerevisiae*. Genes Dev. **7:**1160–1175.
55. **Sclafani, R. A., and A. L. Jackson.** 1994. Cdc7 protein kinase for DNA metabolism comes of age. Mol. Microbiol. **11:**805–810.
56. **Shirayama, M., W. Zachariae, R. Ciosk, and K. Nasmyth.** 1998. The Polo-like kinase Cdc5p and the WD-repeat protein Cdc20p/fizzy are regulators and substrates of the anaphase promoting complex in Saccharomyces cerevisiae. EMBO J. **17:**1336–1349.
57. **Spellman, P. T., G. Sherlock, M. Q. Zhang, V. R. Iyer, K. Anders, M. B. Eisen, P. O. Brown, D. Botstein, and B. Futcher.** 1998. Comprehensive identification of the cell cycle regulated genes of the yeast *Saccharomyces cerevisiae* by microarray hybridization. Mol. Biol. Cell **9:**3273–3297.
58. **Takahashi, K., H. Yamada, and M. Yanagida.** 1994. Fission yeast minichromosome loss mutants *mis* cause lethal aneuploidy and replication abnormality. Mol. Biol. Cell **5:**1145–1158.
59. **Tanaka, T., D. Knapp, and K. Nasmyth.** 1997. Loading of an Mcm protein onto DNA replication origins is regulated by Cdc6p and CDKs. Cell **90:**649–660.
60. **Tanaka, T., and K. Nasmyth.** 1998. Association of RPA with chromosomal replication origins requires an Mcm protein, and is regulated by Rad53, and cyclin- and Dbf4-dependent kinases. EMBO J. **17:**5182–5191.
61. **Thommes, P., Y. Kubota, H. Takisawa, and J. J. Blow.** 1997. The RLF-M component of the replication licensing system forms complexes containing all six MCM/P1 polypeptides. EMBO J. **16:**3312–3319.
62. **Townsley, F. M., and J. V. Ruderman.** 1998. Proteolytic ratchets that control progression through mitosis. Trends Cell Biol. **8:**238–244.
63. **Visintin, R., S. Prinz, and A. Amon.** 1997. *CDC20* and *CDH1*: a family of substrate-specific activators of APC-dependent proteolysis. Science **278:**460–463.
64. **Wang, T. A., and J. J. Li.** 1995. Eukaryotic DNA replication. Curr. Opin. Cell. Biol. **7:**414–420.
65. **Yan, H., A. M. Merchant, and B. K. Tye.** 1993. Cell cycle-regulated nuclear localization of MCM2 and MCM3, which are required for the initiation of DNA synthesis at chromosomal replication origins in yeast. Genes Dev. **7:**2149–2160.
66. **Yoon, H.-J., and J. L. Campbell.** 1991. The *CDC7* protein of *Saccharomyces cerevisiae* is a phosphoprotein that contains protein kinase activity. Proc. Natl. Acad. Sci. USA **88:**3574–3578.
67. **Yoon, H. J., S. Loo, and J. L. Campbell.** 1993. Regulation of *Saccharomyces cerevisiae CDC7* function during the cell cycle. Mol. Biol. Cell **4:**195–208.
68. **Zachariae, W., T. H. Shing, M. Galova, B. Obermaier, and K. Nasmyth.** 1996. Identification of subunits of the anaphase-promoting complex of *Saccharomyces cerevisiae*. Science **274:**1201–1204.
69. **Zou, L., J. Mitchell, and B. Stillman.** 1997. *CDC45*, a novel yeast gene that functions with the origin recognition complex and Mcm proteins in initiation of DNA replication. Mol. Cell. Biol. **17:**553–563.