Cdc7p–Dbf4p kinase binds to chromatin during S phase and is regulated by both the APC and the RAD53 checkpoint pathway

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Eukaryotic cells coordinate chromosome duplication by assembly of protein complexes at origins of DNA replication and by activation of cyclin-dependent kinase and Cdc7p–Dbf4p kinase. We show in *Saccharomyces cerevisiae* that although Cdc7p levels are constant during the cell division cycle, Dbf4p and Cdc7p–Dbf4p kinase activity fluctuate. Dbf4p binds to chromatin near the G₁/S-phase boundary well after binding of the minichromosome maintenance (Mcm) proteins, and it is stabilized at the non-permissive temperature in mutants of the anaphase-promoting complex, suggesting that Dbf4p is targeted for destruction by ubiquitin-mediated proteolysis. Arresting cells with hydroxyurea (HU) or with mutations in genes encoding DNA replication proteins results in a more stable, hyper-phosphorylated form of Dbf4p and an attenuated kinase activity. The Dbf4p phosphorylation in response to HU is RAD53 dependent. This suggests that an S-phase checkpoint function regulates Cdc7p–Dbf4p kinase activity. Cdc7p may also play a role in adapting from the checkpoint response since deletion of *CDC7* results in HU hypersensitivity. Recombinant Cdc7p–Dbf4p kinase was purified and both subunits were autophosphorylated. Cdc7p–Dbf4p efficiently phosphorylates several proteins that are required for the initiation of DNA replication, including five of the six Mcm proteins and the p180 subunit of DNA polymerase α -primase.

Keywords: Cdc7p–Dbf4p kinase/cell cycle/chromatin binding/DNA replication/*Saccharomyces cerevisiae*

Introduction

Precise regulation of the initiation of DNA replication is responsible for ensuring that the eukaryotic genome is duplicated exactly once per cell division cycle (Diffley, 1996; Stillman, 1996; Dutta and Bell, 1997; Piatti, 1997). The stepwise assembly of proteins at origins of DNA replication is a critical part of this regulation and is mediated by both Cdc6p levels and availability (Liang *et al.*, 1995; Piatti *et al.*, 1995) and by the presence of an active cyclin-dependent kinase (Cdk) (Dahmann *et al.*, 1995; Piatti *et al.*, 1996; Tanaka *et al.*, 1997). The origin recognition complex (ORC) is bound to origins of DNA replication at all stages of the cell cycle in budding yeast (Aparicio *et al.*, 1997; Liang and Stillman, 1997; Tanaka *et al.*, 1997), but Cdc6p is not recruited to origins until

late in M phase (Donovan *et al.*, 1997; Liang and Stillman, 1997). Cdc6p is required for the association of the six minichromosome maintenance (Mcm) proteins at origins to form a pre-replicative complex (pre-RC) (Detweiler and Li, 1997; Donovan *et al.*, 1997; Tanaka *et al.*, 1997), in a process that is perhaps analogous to the RFC-dependent loading of PCNA onto DNA (Perkins and Diffley, 1998; Neuwald *et al.*, 1999; Weinreich *et al.*, 1999).

The Cdc6p-dependent stage of the assembly reaction is inhibited by active Clb–Cdks (Dahmann *et al.*, 1995; Piatti *et al.*, 1996; Tanaka *et al.*, 1997). Since Cdc6p is synthesized only from late M phase until late G₁ (Piatti *et al.*, 1995), which corresponds to the period when the Clb–Cdks are inactive, pre-RCs can only assemble during this period of the cell cycle. An S-phase cyclin–Cdk (Cdc28p–Clb5p or Cdc28p–Clb6p) activity is required for the chromatin association of Cdc45p just prior to the actual start of DNA synthesis, suggesting that phosphorylation of Cdc45p itself or some other protein regulates the origin association of Cdc45p (Zou and Stillman, 1998). Cdc28p–Clb kinase also controls the temporal order of origin activation (Donaldson *et al.*, 1998b), and may also be required for other events leading to initiation and for DNA synthesis itself.

The Cdc7p kinase, first identified in budding yeast, is also required for entry into S phase at a very late stage (Hartwell, 1973; Patterson *et al.*, 1986; Hollingsworth and Sclafani, 1990; Yoon *et al.*, 1993; Bousset and Diffley, 1998). It is a member of the broad class of protein kinases including the cyclin-dependent kinases, but differs from the Cdks in that the activation loop and cyclin binding domains are altered (Hanks *et al.*, 1988; Morgan, 1995). Cdc7p is an ‘RD’ kinase (the catalytic site contains arginine and aspartic acid) (Johnson *et al.*, 1996) and as such may be regulated by phosphorylation or some other post-translational event. However, in contrast to several published reports (Patterson *et al.*, 1986; Buck *et al.*, 1991), it does not contain the conserved threonine in the activation loop that must be phosphorylated for full activity of the Cdks (e.g. see the alignment in Hanks *et al.*, 1988).

CDC7 transcript levels are reported to be constant throughout the cell cycle (Sclafani *et al.*, 1988), but a Cdc7p-associated H1 kinase activity is cell cycle regulated (Jackson *et al.*, 1993). It is not clear, however, whether this H1 kinase activity reflects the normal activity of the Cdc7p kinase.

Multi-copy suppressors of *cdc7* mutants identified the *DBF4* gene that encodes Dbf4p, a protein now known to be required for Cdc7p-mediated kinase activity (Kitada *et al.*, 1992). *DBF4* transcription is cell cycle regulated and the transcript abundance peaks near the G₁ to S-phase transition (Chapman and Johnston, 1989). Recently, orthologs of Cdc7p have been identified in *Schizosaccharomyces*

pombe (Masai *et al.*, 1995), mouse (Kim *et al.*, 1998), *Xenopus* and humans (Jiang and Hunter, 1997; Sato *et al.*, 1997; Hess *et al.*, 1998) and a Dbf4p ortholog has also been found in *S.pombe* (Brown and Kelly, 1998). Analyses of these orthologs suggest that Cdc7p–Dbf4p has a conserved role in the initiation of DNA replication.

Several lines of evidence indicate that Cdc7p–Dbf4p acts directly at origins of DNA replication and that the Mcm proteins may be one of the relevant targets of this kinase. *DBF4* was isolated in a one-hybrid screen for autonomously replicating sequence (ARS)-interacting factors (Dowell *et al.*, 1994). Interestingly, its interaction with origins was found to be independent of its ability to bind to Cdc7p. Also, two recent reports (Bousset and Diffley, 1998; Donaldson *et al.*, 1998a) suggest that Cdc7p activity is required to activate individual origins (rather than a global activation of S phase), since temperature-sensitive *cdc7* mutants demonstrate an inability to activate late rather than early origins at a semi-permissive temperature. Potential target(s) of Cdc7p that are required for the initiation of DNA replication were revealed through an analysis of a mutant in *Saccharomyces cerevisiae* that could bypass the requirement for *CDC7* or *DBF4* (Jackson *et al.*, 1993). The mutation was found to reside in *MCM5/CDC46* (Hardy *et al.*, 1997), a member of the Mcm family of six sequence-related proteins (Chong *et al.*, 1996). Since then, a number of reports have suggested that some Mcm proteins such as Mcm2 and Mcm3 are *in vitro* substrates of Cdc7p kinases from various sources (Lei *et al.*, 1997; Sato *et al.*, 1997; Brown and Kelly, 1998).

In this report, we describe the purification of recombinant Cdc7p–Dbf4p to near homogeneity and an analysis of Cdc7p–Dbf4p kinase activity and chromatin binding in yeast. Both subunits of the recombinant enzyme are autophosphorylated and this reaction requires catalytically active Cdc7p. This enzyme is not an efficient kinase of histone H1 or of several other good Cdk substrates, and therefore exhibits a novel substrate specificity different from the (S/T)PX(K/R) consensus sequence of some cyclin-dependent kinases (Nigg, 1993; Songyang *et al.*, 1994; Holmes and Solomon, 1996). The recombinant protein efficiently phosphorylates Mcm 2, 3, 4, 6 and 7, as well as the largest subunit of DNA polymerase α -primase (hereafter referred to as pol α -primase), all of which are phosphoproteins in yeast and other organisms (Nasheuer *et al.*, 1991; Kimura *et al.*, 1994; Foiani *et al.*, 1995; Todorov *et al.*, 1995; Hendrickson *et al.*, 1996; Lei *et al.*, 1997; this study). Cdc7p levels are constant throughout the cell cycle, but Dbf4p levels fluctuate, suggesting that Dbf4p is unstable or that its abundance is tightly regulated. Stabilization of kinase activity occurs in nocodazole-arrested cells or in *cdc16* and *cdc23* mutants, conditions that result in an inactive anaphase-promoting complex (APC), suggesting that Dbf4p is subject to ubiquitin-mediated degradation. Interestingly, Dbf4 is hyperphosphorylated upon treatment with hydroxyurea (HU) or upon inactivation of DNA replication enzymes and this phosphorylated kinase is less active than the unmodified Cdc7p–Dbf4p kinase. Since the checkpoint regulator *RAD53* is required for modification of Dbf4p in response to HU, Cdc7p–Dbf4p kinase may play a role in the response of yeast to DNA replication inhibitors. This is also suggested by the observation that deletion of *CDC7*

causes hypersensitivity to HU. We also show that Cdc7p–Dbf4p binds to chromatin after Mcm loading in G₁ and persists throughout S phase.

Results

Dbf4p and the chromatin association of Cdc7p–Dbf4p fluctuate during the cell cycle

A previous report had identified Dbf4p as a potential ARS-interacting factor, using a one-hybrid screen (Dowell *et al.*, 1994). It is therefore possible that Dbf4p is targeted directly to origins of DNA replication in yeast. To test the possibility that Cdc7p or Dbf4p might bind to chromatin, a yeast strain expressing Dbf4p that had been tagged with multiple Myc epitopes at its C-terminus (kindly provided by K.Nasmyth) was synchronized in anaphase (using a temperature-sensitive *dbf2-1* mutation) and released into the cell cycle at the permissive temperature. Time points following release from the *dbf2-1* block were taken and NP-40-permeabilized yeast spheroplasts were then fractionated into soluble and chromatin-containing fractions (Donovan *et al.*, 1997; Liang and Stillman, 1997). The Mcm protein, Mcm2p, and the ORC subunit Orc3p were used as controls (Liang and Stillman, 1997; Weinreich *et al.*, 1999). Cdc7p and Mcm2p were present at all times during the cell cycle in the soluble fraction (Figure 1B, also see Donovan *et al.*, 1997; Liang and Stillman, 1997). In contrast, Dbf4p levels were cell cycle regulated, with Dbf4p first appearing at 40 min after the release when ~50% of the cells had a G₁ DNA content (Figure 1A). Dbf4p then disappeared by 150 min when the majority of the cells had a G₂/M DNA content. We estimated that S phase began in this experiment 60 min after the mitotic release (Figure 1A).

Analysis of the chromatin fraction indicated that Mcm2p bound to chromatin between 20–30 min following the *dbf2-1* release (Figure 1B), in agreement with previous reports (Liang and Stillman, 1997; Weinreich *et al.*, 1999). This time corresponds to the time during the cell cycle when the pre-RC is formed. Dbf4p also bound to chromatin, but considerably later than Mcm2p (between 30 and 40 min following release), concomitant with its appearance in the cells. Cdc7p, in contrast, was present on the chromatin at all times during the cell division cycle (Figure 1B). The blot for the chromatin-bound Dbf4p represents a longer exposure relative to the blot for the soluble fraction of the protein and we estimated that only ~15–20% of the total Dbf4p was bound to chromatin. Orc3p was used as a control since it bound to chromatin throughout the cell cycle (Aparicio *et al.*, 1997; Liang and Stillman, 1997; Tanaka *et al.*, 1997). Since Dbf4p was bound to chromatin by 40 min and S phase does not begin until ~60 min, this suggested that the Cdc7p–Dbf4p kinase bound to chromatin before the initiation of S phase.

To estimate more accurately the timing of the association of Dbf4p with chromatin relative to S phase, cells were arrested in G₁ with α -factor and released into the cell cycle at 25°C. The majority of the cells entered S phase by 40 min following release from the α -factor block (Figure 2A). In the α -factor-arrested cells, Mcm2p was already bound to chromatin (Donovan *et al.*, 1997; Liang and Stillman, 1997). It released during S phase and then rebound before the next cell cycle began (Figure 2B).

Fig. 1. Dbf4-Myc₁₈p exhibits cell cycle binding to chromatin. YB515 (*MATa dbf2-1 DBF4-Myc₁₈-LEU2*) was arrested in late M phase at 37°C using the *dbf2-1* mutation and released into the cell cycle at 25°C. Time points were taken and cells were processed for DNA content by FACS analysis (A) or separated into soluble (Supernatant) and chromatin-containing (Pellet) fractions (Liang and Stillman, 1997), immunoblotted and probed for the indicated proteins (B). All the Orc3p protein was in the pellet fraction (not shown).

Dbf4p, however, bound to chromatin between 20 and 30 min following release, which was just before the cells had entered S phase, and unlike Mcm2p, Dbf4p remained on chromatin until the majority of the cells had completed S phase (Figure 2B). At this time, both soluble and chromatin-bound forms of Dbf4p were degraded. In the next cell cycle, Dbf4p rebound to chromatin with Mcm2p at 120 min but again, it remained associated with the chromatin longer than the Mcm protein. Orc3p and a significant fraction of Cdc7p were bound to chromatin throughout the cell cycle.

Both Cdc7p and Dbf4p were bound to chromatin and not simply part of an insoluble fraction since they were solubilized by a limited micrococcal nuclease digest (Liang and Stillman, 1997) and then repelleted with the polynucleosomes at 100 000 g (Figure 2C).

Cdc7p-Dbf4p levels and kinase activity fluctuate during the cell cycle

Cells in which the endogenous *CDC7* gene was replaced by *3HA-CDC7* were arrested in G₁ phase (with α -factor), S phase (with HU) or M phase (with nocodazole) and then cell extracts were immunoprecipitated using the 12CA5 monoclonal antibody that recognized the HA epitope. A portion of the immunoprecipitates were transferred to a membrane and probed for Cdc7p and Dbf4p using antibodies against these proteins (Figure 3A). Another portion was assayed for kinase activity following addition of recombinant Mcm7p (Figure 3B). Even though Cdc7p was clearly present, Dbf4p protein was

not co-immunoprecipitated in the α -factor-arrested cells (Figure 3A, lane 3), probably due to the low levels of Dbf4p in these cells (see Figure 2). Upon HU treatment, Dbf4p remained bound to Cdc7p, but underwent a shift to a lower mobility compared with the Dbf4p from asynchronous cells (Figure 3A, lane 4 versus lane 2). Similarly, in nocodazole-arrested cells, Cdc7p-bound Dbf4p migrated in two different forms (Figure 3A, lane 5). The mobility of the two Dbf4p bands in nocodazole-arrested cells was similar to the mobility seen with the native and autophosphorylated forms of Dbf4p purified from *Sf9* cells (Figures 3A, lane 5, and 6A, see below). Compared with the complex from asynchronously growing cells, the amount of Dbf4p co-precipitated with Cdc7p was increased greatly when cells were arrested with either HU or nocodazole.

Cdc7p-associated kinase activity was detected in immunoprecipitates from asynchronously growing cells and from cells that had been arrested with HU or nocodazole, but was barely detected from cells arrested with α -factor. The kinase immunoprecipitated from nocodazole-arrested cells was much more active than the enzyme from the HU-arrested cells, even though the amounts of the Cdc7p-Dbf4p protein complex were similar (compare lanes 2, 4 and 5 of Figure 3A and B). An unknown ~180 kDa protein immunoprecipitated by the 12CA5 antibody was also phosphorylated *in vitro* in these immunoprecipitates (Figure 3B).

As noted above, Dbf4p was shifted to a slower-mobility form after treatment of cells with HU (Figure 3C, compare

Fig. 2. Dbf4p binds to chromatin prior to S phase. K6388 (K.Nasmyth, *MATa DBF4-Myc₁₈-LEU2*) was arrested in G₁ phase using α -factor and released into the cell cycle at 25°C in the absence of α -factor. Time points were taken and processed as in Figure 1. (A) DNA content as determined by FACS analysis. (B) Proteins in either the soluble (supernatant) or chromatin-bound (pellet) fractions. Only the pellet fractions for Orc3p and Mcm2p are shown. (C) Cdc7p and Dbf4p are released from the chromatin fraction in a soluble form following a limited micrococcal nuclease digestion (Liang and Stillman, 1997) (Lo Sup) and then pelleted after spinning at 100 000 g (Hi Pel). This is shown for Cdc7p in the pellet fraction from α -factor-arrested cells (α) and for both Cdc7p and Dbf4p from exponentially growing cells (asy). Micrococcal nuclease digestion was done at 25°C for the (asy) samples, since Dbf4p was unstable at 37°C in the pellet fraction.

lanes 1 and 2). This was due to protein phosphorylation, since treatment with phosphatase increased the mobility of Dbf4p (Figure 3C, lanes 3 and 4). A similar shift in the mobility of Dbf4p and decrease in kinase activity occurred when cells were arrested at the non-permissive temperature (37°C) in S phase using temperature-sensitive mutants in either *cdc17* (encoding polymerase α) or *cdc9* (encoding DNA ligase) (Figure 4C and D, lanes 5 and 6 compared with lane 1).

To test whether this modification depended on known checkpoint functions, we compared Dbf4p phosphorylation in response to HU in both wild-type and *rad53-1* strains. In the wild type, Dbf4p was phosphorylated in response to treatment with HU within 1 h, as seen by the shift to slower mobility (Figure 3E). This did not occur in the *rad53-1* strain even after 2 h of exposure to HU. Similar data has been obtained by J. Diffley and colleagues (personal communication). Taken together, these data suggest that conditions which arrest DNA synthesis and trigger the S-phase checkpoint (Elledge, 1996) result in the *RAD53*-dependent phosphorylation of Dbf4p and a decrease in its associated kinase activity (Figure 3D).

A series of temperature-sensitive mutations that result

in cell cycle arrest were crossed into the strain expressing 3HA-Cdc7p and Cdc7p-Dbf4p was immunoprecipitated using 12CA5 at the permissive and restrictive temperatures for growth. The immunoprecipitates were probed for the presence of Cdc7p, Dbf4p (Figure 4A and C) and the associated kinase activity (Figure 4B and D). At the permissive temperature, recovery of Cdc7p-Dbf4p and kinase activity was relatively constant except for a slight decrease in the *dbf4-1* mutant (Figure 4A and B, lane 2) and stabilization in the *cdc9-1* and *cdc23-1* mutants (Figure 4A and B, lanes 6 and 7). In contrast, at the restrictive temperature, recovery of Cdc7p-Dbf4p complex was low from mutants that arrested the cell cycle in early G₁ (Figure 4C and D, lanes 2 and 3), but was high in mutants that arrested the cell cycle during late G₁ (*cdc4*) or S phase (*cdc9* or *cdc17*) (Figure 4C, lanes 4, 5 and 6). Dbf4p showed a decrease in mobility in the *cdc17-1* and *cdc9-1* mutants similar to that observed in HU-arrested cells and the kinase activity was ~2.5-fold lower in the S-phase-arrested cells compared with the wild-type asynchronous cells (Figure 4D, lane 1 versus lanes 5 and 6; Figure 3D; and see Discussion). In addition, Cdc7p-Dbf4p complex and the associated kinase activity was

Fig. 3. The amount and activity of Cdc7p-Dbf4p fluctuate at different cell cycle arrest points. Bead-beaten whole cell extracts from YB516 (*MATa 3HA-CDC7 TRP1*) were prepared from asynchronous cells, or cells arrested in G₁ (α -factor), S (HU) or G₂/M (nocodazole) phase and immunoprecipitated using the 12CA5 antibody. The immunoprecipitates were blotted to a membrane and probed for the presence of Cdc7p and Dbf4p (A) or assayed for kinase activity following the addition of 50 ng of Mcm7p (B). (C) Immunoprecipitates of 3HA-Cdc7p from asynchronous (lane 1) or HU-arrested cell extracts (lanes 2–4) were immunoblotted and probed for Cdc7p and Dbf4p after treatment with potato acid phosphatase in the absence (lane 3) or presence (lane 4) of phosphatase inhibitors. (D) The immunoprecipitated Cdc7p-Dbf4p kinase activity using exogenous Mcm7p substrate was quantified (average of two experiments and normalized for the amount of Dbf4p and Cdc7p present in each immunoprecipitate), and expressed relative to the level present in wild-type cells at 25°C (left) or wild-type cells at 37°C (right). (E) Early exponential YB530 (WT) and YB531 (*rad53-1*) strains containing *3HA-CDC7* were incubated with HU (0.1 M) for 1 and 2 h, then immunoprecipitated using 12CA5 antibody and blotted for Cdc7p and Dbf4p.

abundant in G₂/M phase-arrested cells using the *cdc23-1* mutant. In contrast, little kinase was recovered from the additional mutants that arrested later in G₂/M phase (Figure 4C and D, lanes 7–10).

Dbf4p (but not Cdc7p) was also stabilized in the *cdc16-1* and *cdc23-1* mutants arrested in G₁ phase using α -factor (when the APC was active) both at the permissive and restrictive temperature (Figure 4E). Thus, this stabilization is independent of the cell cycle stage. Since *CDC16* and *CDC23* encode subunits of the APC (Peters *et al.*, 1996) and the stability of Dbf4p (but not Cdc7p) during the cell cycle mimics that of other APC substrates (Figure 1B), these data argue that Dbf4p is a substrate of the APC. Therefore it is possible that Dbf4p is targeted for degrad-

ation by ubiquitin-mediated proteolysis during the period from anaphase to late G₁ (King *et al.*, 1996).

CDC7 is required for timely entry into S phase and the normal response to HU

Since Dbf4p and Cdc7p-Dbf4p kinase activity are specifically modified in response to S-phase arrest (compare Figure 3A and B, lanes 2 and 4), perhaps Cdc7p kinase activity plays a role in the checkpoint arrest mediated by HU. A P83L change in *MCM5* (termed *mcm5^{bob1}*) is able to bypass the requirement for both *CDC7* and *DBF4*. We introduced this mutation into W303 together with a deletion of *CDC7* to obtain a congenic series of strains and first examined the time to enter S phase from a G₁ arrest by fluorescence activated cell sorting (FACS) analysis (Figure 5A). The *mcm5^{bob1}* strain has a delayed entry into S phase (~40 min after release from α -factor arrest) compared with the wild-type strain (~30 min). Furthermore, deletion of *CDC7* in the *mcm5^{bob1}* background greatly delays entry into S phase to between 50 and 60 min following the G₁ release. This is consistent both with the slower growth phenotype of this strain and the normal *CDC7* requirement for entry into S phase.

Interestingly, deletion of *CDC7* results in hypersensitivity to HU in the *mcm5^{bob1}* background (Figure 5B). This is dependent on the absence of *CDC7*, since introduction of the wild-type *CDC7* on a CEN-ARS plasmid reverses the HU sensitivity (not shown). The HU hypersensitivity is not equivalent to a loss of the checkpoint response since the strain lacking *CDC7* exhibits only a modest reduction in viability compared with the >1000-fold loss of viability for a congenic *rad53-1* strain (Figure 5C). Also, the Δ *cdc7 mcm5^{bob1}* strain shows a profile similar to the wild type by FACS analysis following α -factor arrest and release into HU (not shown). The HU hypersensitive phenotype is thus more consistent with an inability of the strain to adapt to a HU arrest and continue growth in the presence of HU, as occurs with the wild-type and *mcm5^{bob1}* strains (Figure 5C).

Purification of Cdc7p-Dbf4p

To begin an analysis of the targets of Cdc7p-Dbf4p kinase in replication initiation, we purified the kinase. To facilitate purification, Cdc7p was tagged at its N-terminus with a single HA epitope followed by six histidine residues. This altered Cdc7p was completely functional in yeast and exhibited similar properties to the untagged protein (data not shown). Recombinant Cdc7p-Dbf4p was purified from baculovirus-infected Sf9 cells and its activity was compared with that of a catalytically inactive form of Cdc7p-Dbf4p, containing a mutation in Cdc7p that changes an essential aspartic acid 163 to an alanine. Figure 6A shows a silver-stained gel of the purified wild-type kinase and the purified inactive form of Cdc7p-Dbf4p. In the wild type, but not the mutant kinase preparation, the Dbf4p subunit exhibited two distinct electrophoretic mobilities, suggesting a post-translational modification (lane 1). However, the catalytic site mutation did not prevent association of Cdc7p with the Dbf4p subunit (lane 2). A kinase assay using the purified enzymes (Figure 6B, lane 1) revealed that both Cdc7p and Dbf4p were phosphorylated upon incubation with [γ -³²P]ATP, but that this phosphorylation did not occur with the cata-

Fig. 4. Cdc7p-Dbf4p is stabilized in *cdc17-1*, *cdc9-1* and *cdc23-1* mutants. *dbf2-1*, *dbf4-1* and various *cdc* mutations were crossed into YB516 (WT) to give YB517 (*dbf4-1*), YB518 (*cdc28-4*), YB519 (*cdc4-1*), YB520 (*cdc17-1*), YB521 (*cdc9-1*), YB522 (*cdc23-1*), YB523 (*cdc15-2*), YB524 (*dbf2-1*), YB525 (*cdc5-1*) all containing *3HA-CDC7*. Following incubation at the permissive (A and B) or the restrictive temperature (C and D), bead-beaten whole cell extracts were prepared, immunoprecipitated using 12CA5, and a portion of the immunoprecipitate was blotted and probed for Cdc7p and Dbf4p (A and C); or autoradiographed after another portion was assayed for kinase activity in the presence of 50 ng Mcm7p (B and D). Notice that at 37°C, although Cdc7p-Dbf4p was stabilized in *cdc17-1*, *cdc9-1* and *cdc23-1* mutants, the kinase activities are different (D, lanes 5–7). (E) Equivalent total cell extracts of K6388 (*MATa DBF4-Myc₁₈-LEU2*), YB526 (K6388 *cdc16-1*) and YB527 (K6388 *cdc23-1*) were probed using Cdc7 and 9E10 antibodies. Cells were arrested in G₁ phase using α -factor and then incubated 1 h further at 25 or 37°C still in the presence of α -factor.

lytically inactive kinase (lane 2). Treatment with phosphatase removed the slower-migrating form of Dbf4p and also slightly increased the mobility of the Cdc7p subunit (Figure 6C). Taken together, these results indicated that the recombinant protein was an active kinase that autophosphorylated on both the Cdc7p and Dbf4p subunits.

pol α -primase and Mcm proteins are substrates of the Cdc7p-Dbf4p kinase

In preliminary experiments, the Cdc7p-Dbf4p kinase did not efficiently phosphorylate several proteins, including histone H1 (data not shown). Many proteins are known to be required for the initiation of DNA replication in eukaryotes and as such, are potential substrates of the Cdc7p-Dbf4p kinase. Therefore, all available proteins were screened for their ability to act as substrates for the purified enzyme. While purified ORC (containing six subunits), Cdc6p and replication protein A (RPA) (containing three subunits) were not efficient substrates (data

not shown), five members of the Mcm protein family and the largest subunit of pol α -primase were phosphorylated. Figure 7A shows a silver-stained gel of all six Mcm proteins purified individually from *Sf9* cells using recombinant baculoviruses (M.Akiyama, M.Weinreich and B.Stillman, manuscript in preparation). Kinase assays using the six Mcm family members and either wild-type Cdc7p-Dbf4p or the inactive kinase are shown in Figure 7B. The use of the mutant kinase as a negative control demonstrated that phosphorylation of the Mcm proteins was mediated by the Cdc7p-Dbf4p kinase and not by contaminating kinases in either the Mcm protein or the Cdc7p-Dbf4p samples. All of the Mcm proteins, with the exception of Mcm5p, were substrates of Cdc7p-Dbf4p (which differs from a previous report by Lei *et al.*, 1997).

Pol α -primase is a four-subunit enzyme that has been shown to undergo phosphorylation on the two largest subunits in a cell cycle-regulated manner (Nasheuer *et al.*,

Fig. 5. *CDC7* is required for timely entry into S phase and for the normal response to HU arrest. (A) FACS analysis of W303 *Mata*, YB528 (W303 *MATa mcm5^{bob1}*) and YB529 (W303 *MATa mcm5^{bob1} $\Delta cdc7::HIS3$*) of both asynchronous cultures and after G_1 arrest (αF) and release at $30^\circ C$ at the indicated time points. The *mcm5^{bob1}* allele is designated bob1 in the figure. (B) The same strains were streaked onto YPD or YPD containing 0.1 M HU and photographed after 3 days at $30^\circ C$. (C) These strains were grown to early exponential phase in YPD, HU was added to 0.2 M and cultures were assayed for viable colonies on YPD at $30^\circ C$ (average of two experiments). The *rad53-1* strain is YB533 (W303 *MATa*, *rad53-1*).

1991; Foiani *et al.*, 1995). The p180 subunit encodes the DNA polymerase activity, while p86 is an accessory subunit and the DNA primase complex is composed of the p58 and p48 subunits (Foiani *et al.*, 1997). The kinase responsible for phosphorylating p86 in human cells is most likely the mitotic kinase cdc2–cyclin B (Nasheuer *et al.*, 1991). Similarly, in *S.cerevisiae*, this phosphoryl-

ation has been shown to require Cdc28p but not Cdc7p activity (Foiani *et al.*, 1995). However, the identity of the kinase(s) that phosphorylates p180 is not known with certainty but may also include cdc2–cyclin B (Nasheuer *et al.*, 1991).

To test possible phosphorylation of the polymerase by Cdc7p–Dbf4p, the four-subunit pol α -primase holoenzyme

Fig. 6. Cdc7p and Dbf4p are phosphoproteins. (A) Silver-stained SDS-polyacrylamide gel showing 100 ng of purified Cdc7p-Dbf4p and Cdc7^{M1}/Dbf4p. HA-Cdc7p-Dbf4p wild type (WT) and the catalytically inactive mutant (M1) were purified as described in Materials and methods. (B) Kinase reaction indicating that the wild-type but not the mutant Cdc7p-Dbf4p becomes phosphorylated on both subunits. (C) Immunoblot probed with antibodies against Cdc7p and Dbf4p showing that both proteins are phosphorylated. Treatment of the purified protein (lane 1) with potato acid phosphatase (lane 2) results in increased mobility of both subunits, but not in the presence of phosphatase inhibitors (lane 3).

or a complex lacking the p180 subunit was produced by co-infection of *Sf9* cells with baculoviruses expressing individual subunits of the enzyme (C.Mirzayan, C.Liang and B.Stillman, manuscript in preparation). A monoclonal antibody against p180 (Plevani *et al.*, 1985) was then used to immunoprecipitate these proteins from *Sf9* cell lysates and phosphorylation of the subunits using purified Cdc7p-Dbf4p was examined. The p180 antibody co-immunoprecipitated the four subunits of pol α -primase only when p180 was co-expressed with the other three subunits (Figure 7C, lane 2; Plevani *et al.*, 1985). Immunoblotting revealed that the 91 kDa band (labeled p86-P in Figure 7C) was in fact p86, probably representing phosphorylation of this subunit in *Sf9* cells. The immunoprecipitated proteins were then used as substrates for the purified Cdc7p-Dbf4p kinase (Figure 7D). There was no contaminating kinase present in the immunoprecipitated proteins (Figure 7D, lane 3), but addition of Cdc7p-Dbf4p (Figure 7D, lane 4) resulted in efficient phosphorylation of p180. The p180 subunit of pol α -primase was also specifically phosphorylated by Cdc7p-Dbf4p when immunoprecipitates of the polymerase from yeast were used as a substrate (not shown).

Fig. 7. Mcm proteins and pol α -primase are substrates of Cdc7p-Dbf4p. (A) Silver-stained gel showing ~100 ng of each purified Mcm protein. (B) These proteins were incubated in kinase buffer with equivalent amounts of wild-type (lanes 1–6) or catalytically inactive (lanes 7–12) Cdc7p-Dbf4p, separated in an SDS-10% polyacrylamide gel and autoradiographed. (C) A Coomassie Blue-stained gel showing a p180 immunoprecipitate from *Sf9* cells expressing the indicated subunits of pol α -primase. p86-P indicates a phosphorylated form of p86. (D) An autoradiograph of kinase reactions performed with the p180 immunoprecipitates after addition of purified Cdc7p-Dbf4p. Lane 2 is the p180 immunoprecipitate from *Sf9* cells expressing only the three small subunits of pol α -primase.

Fig. 8. Mcm4p, Mcm6p and Mcm7p are phosphoproteins in yeast. (A) An autoradiograph of 12CA5 immunoprecipitates from the parental strain, W303-1A (lane 1) or from strains containing tagged Mcm4-HA3p (OAY535; Aparicio *et al.*, 1997) (lane 2), Mcm6-HA3p (YB532) (lane 3) and Mcm7-HA3p (OAY534; Aparicio *et al.*, 1997) (lane 4) metabolically labeled with $^{32}\text{P}_i$. (B) An immunoblot of these same immunoprecipitates probed using a peptide antibody against a highly conserved region of the Mcm proteins (Hu *et al.*, 1993).

Mcm4p, Mcm6p and Mcm7p are phosphoproteins in yeast

Mcm2p and Mcm3p have been shown to be phosphoproteins in yeast (Young and Tye, 1997; M.Akiyama and B.Stillman, unpublished). Since Mcm4p, Mcm6p and Mcm7p were also phosphorylated by the purified Cdc7p-Dbf4p kinase, we determined whether these Mcm proteins were also phosphoproteins in *S.cerevisiae*. Mcm4p, Mcm6p and Mcm7p, each tagged with a triple HA epitope, were immunoprecipitated using the 12CA5 monoclonal antibody following *in vivo* labeling of yeast with inorganic phosphate (Figure 8A). Lane 1 shows the phosphoproteins non-specifically precipitated in the parental, untagged strain and lanes 2–4 show the phosphoproteins immunoprecipitated from strains expressing Mcm4-HA3p, Mcm6-HA3p and Mcm7-HA3p, respectively. These proteins have the correct molecular mass for the corresponding Mcm proteins. An immunoblot of these immunoprecipitates using an antibody directed against the conserved region of the Mcm proteins (Hu *et al.*, 1993) confirmed that these phosphoproteins were indeed Mcm proteins (Figure 8B).

Genetic interactions among CDC7, DBF4, MCM2,3,5,7, CDC17 and PRI2

If phosphorylation of pol α -primase or the Mcm proteins by Cdc7p-Dbf4p kinase affects their activity, mutants in these putative substrates in combination with *cdc7* or *dbf4* mutants might exhibit synthetic lethality or reduced growth rates. To test these possibilities, all mutations were backcrossed at least four times into the W303 background from their respective sources. Double mutants were then constructed and tested for genetic interactions. It was not possible to recover the original *cdc54-1* cold-sensitive mutation (encoding Mcm4p) in the W303 background and no mutation in *MCM6* has yet been described. At least 20 tetrads were examined for each cross and Figure 9 summarizes the growth properties of the double mutants. No combination was found to exhibit synthetic lethality (i.e. non-parental di-type tetrads were recovered near the

Fig. 9. Genetic interactions among genes encoding Cdc7p, Dbf4p, the Mcm proteins and Pol α -primase. The indicated alleles were crossed into the W303 background, mated with each other and the resulting haploid, double mutant progeny were examined for growth properties at permissive (25°C), semi-permissive (27 or 30°C) and restrictive temperatures (37°C) for the single mutants. A dashed line indicates no compromised growth at the permissive or semi-permissive temperature. A double arrow indicates that the double mutants exhibited slow growth at 25°C and little or no growth at 27 or 30°C. A 'P' indicates that the gene product is phosphorylated by Cdc7p-Dbf4p *in vitro*.

expected frequency) when all mutations were present in the same genetic background. This contrasts with a previously published report of synthetic lethality between *mcm2-1* and *dbf4-1* using different strain backgrounds (Lei *et al.*, 1997).

Combination of *cdc7-4* or *dbf4-1* with *cdc46-1* (encoding Mcm5p) or with *pri2-1* (encoding the primase subunit p58) yielded no interaction by testing growth at 25°C or at intermediate permissive temperatures for the single mutants. Neither Mcm5p nor the p58 subunit of primase were substrates of Cdc7p-Dbf4p *in vitro* (Figure 6). In contrast, *mcm2-1*, *mcm3-1*, *cdc47-1* (encoding Mcm7p) and *cdc17-1* (encoding polymerase α) all exhibited reduced growth rates at 25 and 27°C, and no growth at 27°C for *cdc47-1* when combined with *cdc7-4* or *dbf4-1*. All of these genes encode substrates of Cdc7p-Dbf4p kinase *in vitro*, suggesting that these proteins might be regulated by Cdc7p-Dbf4p *in vivo*. Definitive proof that these proteins are relevant *in vivo* substrates of Cdc7p-Dbf4p will require identification of the sites of phosphorylation *in vivo* and mutation of these sites to residues that could not be phosphorylated. It may well be that combinations of mutants may be necessary to determine the role of the phosphorylation in regulating DNA replication.

Discussion

The initiation of DNA replication is a complex process requiring the ordered assembly of numerous factors at origins of DNA replication with precise timing during the cell division cycle (Diffley, 1996; Stillman, 1996; Dutta and Bell, 1997; Piatti, 1997). It is known that Clb-Cdk activity prevents assembly of pre-replicative complexes at origins at all times during the cell cycle (Dahmann *et al.*, 1995; Tanaka *et al.*, 1997). However, Clb-Cdk activity is required in late G₁ for entry into S phase (Schwob *et al.*, 1994). This may reflect the Clb-Cdk dependence for Cdc45p loading onto chromatin (Zou and Stillman, 1998), but it is not known if this is the only requirement for this activity in the initiation of DNA replication. It is also not known what the relevant targets

for Cdc7p–Dbf4p in initiation are, and how phosphorylation of those targets affects the initiation process.

CDC7 and *DBF4* are required very late in G_1 and are part of the last genetically defined steps before the initiation of DNA replication (Hereford and Hartwell, 1974; Johnston and Thomas, 1982). Reciprocal shift experiments with *cdc45* and *cdc7* mutants indicate that these two steps are genetically interdependent (Owens *et al.*, 1997). It has further been suggested that Cdc7p–Dbf4p is required for initiation at individual origins, rather than acting in a global sense for progression into S phase. This was based on the inefficient firing of origins in late, versus early S phase seen with some *cdc7* mutants at the semi-permissive temperature (Bousset and Diffley, 1998; Donaldson *et al.*, 1998a). Additional evidence for the involvement of Cdc7p–Dbf4p directly at origins of replication came from the isolation of *DBF4* as an ARS-interacting factor in a ‘one-hybrid’ genetic screen (Dowell *et al.*, 1994) and the identification of a mutation in *MCM5* that could bypass the requirement for *CDC7* (Hardy *et al.*, 1997). It is known that members of the Mcm family are loaded directly at or near ARS elements prior to the initiation of S phase (Aparicio *et al.*, 1997; Liang and Stillman, 1997; Tanaka *et al.*, 1997). Furthermore, the interaction between Cdc45p and the Mcm proteins occurs in a cell-cycle-regulated manner, after activation of Clb/Cdc28 kinase (Zou and Stillman, 1998). However, none of these genetic observations presents compelling evidence that Cdc7p–Dbf4p acts directly at origins.

In an effort to understand why the Cdc7p–Dbf4p kinase is required for the initiation of DNA replication in *S. cerevisiae*, we have purified the recombinant kinase from *Sf9* cells and have undertaken a cell cycle analysis of its activity and chromatin binding properties. A number of proteins that are required for initiation were screened as possible Cdc7p–Dbf4p substrates and five of the Mcm proteins (with the notable exception of Mcm5p) and the p180 subunit of pol α -primase were found to be efficient substrates. Given the genetic interactions described above, it seems possible that phosphorylation of these polypeptides will be significant. However, conclusive proof will require it to be determined whether these proteins are *in vivo* substrates of Cdc7p–Dbf4p, mapping the sites of phosphorylation, and then whether mutation of those sites results in a phenotype. Both subunits of Cdc7p–Dbf4p become autophosphorylated and this might play some role in regulating its kinase activity or the instability of Dbf4p (King *et al.*, 1996).

The Mcm proteins are attractive targets for Cdc7p–Dbf4p, since a mutation in *CDC46/MCM5* can alleviate the requirement for Cdc7p kinase (Hardy *et al.*, 1997). This P83L mutation removes a proline in Mcm5p and this is likely to alter the conformation of Mcm5p. It is tempting to speculate that a Mcm complex containing this Mcm5p mutation is in an ‘activated’ conformation that no longer requires phosphorylation of the other Mcm proteins by Cdc7p–Dbf4p. The Mcm proteins are assembled at or near ARS elements from late M phase to early G_1 . Following initiation, the Mcm proteins gradually dissociate from chromatin (Todorov *et al.*, 1995; Thommes *et al.*, 1997) and it has been suggested that they move away from origins as replication proceeds (Aparicio *et al.*, 1997). Although the Mcm proteins become associated

with chromatin and ARS elements in early G_1 (Aparicio *et al.*, 1997; Donovan *et al.*, 1997; Liang and Stillman, 1997; Tanaka *et al.*, 1997) and this association is independent of Cdc7p–Dbf4p, phosphorylation of the Mcm proteins by Cdc7p–Dbf4p could trigger an activity of the Mcm complex, an association with other factors, or a remodeling of interactions between the Mcm proteins themselves. It has been shown that Cdk phosphorylation of Mcm proteins from human and *Xenopus* can prevent their chromatin association (Fujita *et al.*, 1997, 1998) and the overall phosphorylation of some Mcm proteins increases with passage through interphase (see Chong *et al.*, 1996 and references therein). The pre-replicative and pre-initiation complexes that assemble at origins must do so through stable protein–protein interactions. Subsequently, these interactions must somehow be weakened or altered for duplex unwinding and clearance of proteins such as the Mcm proteins or DNA polymerases that were initially recruited to origins of DNA replication (Diffley, 1996; Stillman, 1996) which may be accomplished by protein phosphorylation.

Phosphorylation of the large subunit of pol α -primase might also affect its activity or interactions with other proteins at origins. The p180 subunit is the catalytic subunit of the DNA polymerase and it is necessary for synthesis of the short Okazaki fragments that are later completed by the more processive polymerases δ and ϵ (Waga and Stillman, 1998). Phosphorylation of p180 is known to occur before and during S phase, so the timing of its phosphorylation suggests that it might be relevant to initiation (Nasheuer *et al.*, 1991). Recently, it has been shown that there is an inverse correlation between Clb–Cdk activity and the binding of pol α -primase to chromatin (Desdouets *et al.*, 1998), suggesting that protein phosphorylation ultimately prevents the association of pol α -primase with the chromatin. Once bound at origins of replication, phosphorylation by another protein kinase, Cdc7p–Dbf4p, might allow the initiation of DNA synthesis.

We showed that Dbf4p fluctuates during the cell cycle, accumulating during S phase, consistent with the reported fluctuation in Cdc7p–Dbf4p kinase activity from cells containing a multi-copy *CDC7* plasmid (Jackson *et al.*, 1993). Recent reports have also shown that Dbf4p levels fluctuate during the cell cycle (Cheng *et al.*, 1999; Oshiro *et al.*, 1999). Dbf4p protein is absent in late M phase and accumulates in G_1 before DNA replication has begun, while Cdc7p is relatively constant during the cell cycle. An examination of Cdc7p–Dbf4p levels and kinase activity at different *cdc* arrest points has also suggested how the kinase might be regulated. The finding that Dbf4p is stabilized in APC mutants (during both a G_2/M and a G_1 arrest) but not other mutants with a G_2/M arrest point strongly suggests that it is targeted for destruction by ubiquitin-mediated proteolysis (King *et al.*, 1996). Its pattern of expression during the cell cycle supports this idea. Dbf4p also contains potential matches to the destruction box sequence at amino acids 10–18 (RSPLKETDT) and 62–70 (RLELQQQH) (Yamano *et al.*, 1998 and references therein).

Interestingly, Cdc7p–Dbf4p is stabilized, and Dbf4p is shifted to a slower-mobility form due to direct protein phosphorylation, by treatments that trigger an S-phase

arrest and the DNA damage checkpoint (Elledge, 1996). The modification of Dbf4p is also associated with a decrease in the Cdc7p–Dbf4p kinase activity. The 2.5-fold decrease in kinase activity that we observe could also be an underestimate of the inhibitory effect since we are comparing an arrested culture to an asynchronous one. Also, partial dephosphorylation of Dbf4p might occur during extract preparation. In addition, phosphorylation of Dbf4p might alter other properties of Cdc7p–Dbf4p, such as its interaction with specific substrates or its chromatin binding ability.

The Dbf4p phosphorylation that occurs in response to HU is dependent on the known checkpoint gene, *RAD53*, and might be involved in preventing activation of new origins when cells are arrested before or during S phase due to DNA damage. The modified Dbf4p kinase may also recognize novel substrates or be prevented from accessing its normal substrates. A recent report has demonstrated genetic and physical links between Dbf4p and Rad53p, again suggesting that Rad53p regulates DNA replication via the Cdc7p–Dbf4p kinase (Dohrmann *et al.*, 1999). Since deletion of *CDC7* results in hypersensitivity to HU but not a dramatic loss of cell viability in the presence of the *mcm5^{bob1}* allele, it is also possible that *CDC7* is required for the normal recovery or adaptation from the HU-induced S-phase arrest. This could occur by Cdc7p–Dbf4p activating stalled DNA replication forks or activating origins that have not fired. Cdc7p kinase may also be involved in the adaptation to the S-phase checkpoint response by directly targeting proteins in this pathway. A failure to phosphorylate these proteins could result in a highly inefficient recovery from the HU arrest. In this regard, a potential Cdc7p kinase target is Sml1p, a negative regulator of ribonucleotide reductase, which is itself inhibited in response to HU (Zhao *et al.*, 1998).

The inactive Cdc7p subunit was bound to chromatin at all times during the cell cycle. In contrast, the Dbf4p subunit was only associated with chromatin at certain times. Thus we propose that the binding of Dbf4p might determine which origins become activated. In addition, the binding of Dbf4p to the chromatin-bound Cdc7p would form an active Cdc7p–Dbf4p kinase complex that could promote initiation at that origin. A ‘one-hybrid’ interaction assay suggested that Dbf4p associates with proteins that are assembled at ARS elements (Dowell *et al.*, 1994). Thus, the Dbf4p–chromatin association we detect might represent binding near individual origins. A recent report, however, failed to find any evidence for a Dbf4p interaction at several origins using a chromatin co-immunoprecipitation technique (Tanaka and Nasmyth, 1998). One possible explanation for this is that only a small fraction of Dbf4p was bound to chromatin during S phase, perhaps reflecting a transient association of the kinase with any individual origin. Thus, the ability to detect Dbf4p binding to chromatin might require the association of the kinase with a large number of origins. Also, the experiments to look at Dbf4p–origin interactions (Tanaka and Nasmyth, 1998) were performed in the presence of HU (a condition that alters Dbf4p).

Although direct recruitment is certainly consistent with the requirement for Cdc7p–Dbf4p kinase activity in the initiation of DNA replication, we cannot rule out the possibility that the association of Cdc7p–Dbf4p with

chromatin may also reflect binding to chromatin at many other loci. For example, it is possible that the active Cdc7p–Dbf4p kinase complex might associate with assembled DNA replication proteins at DNA replication forks and continually modulate chain elongation during S phase.

The ability to purify large amounts of active Cdc7p–Dbf4p should allow further biochemical characterization of this kinase, such as the determination of a consensus phosphorylation site, the pathway for modification of Dbf4p in response to DNA synthesis arrest, and whether phosphorylation affects the biochemical activity of the Mcm proteins and of pol α -primase. Furthermore, the availability of large amounts of the purified kinase complex and other initiation proteins will allow reconstitution of the process of initiation of DNA replication *in vitro*.

Materials and methods

Strains and viruses

Cell cycle mutants were backcrossed at least four times into the W303 background (Thomas and Rothstein, 1989). *CDC7* was tagged with three HA epitopes at its N-terminus and used to replace *CDC7* at its endogenous locus by homologous recombination into YB514 [*MATa ade2-101 lys2-801 leu2 Δ -1 his3 Δ -200 ura3-52 TRP1 cdc7 Δ ::HIS3*, pMW219 (*CDC7, URA3*)]. K6388 (*MATa DBF4-Myc₁₈-LEU2*) was a gift from K.Nasmyth. YB528 (W303 *Mata mcm5^{bob1}*) and YB529 (W303 *MATa mcm5^{bob1} Δ cdc7::HIS3*) were obtained by backcrossing the *bob1 Δ cdc7::HIS3* alleles from P211 (Hardy *et al.*, 1997) four times into W303. Recombinant baculoviruses expressing *DBF4*, *CDC7*, *HAHIS₆-CDC7* and *HAHIS₆-CDC7^{M1}* were constructed using the BaculoGold system (PharMingen) and identified using polyclonal rabbit sera raised against GST–Dbf4p and GST–Cdc7p. Yeast strains containing the tagged *CDC7* gene grew normally and complemented the *cdc7* disruption strain, YB514, in a plasmid shuffle assay. *CDC7^{M1}*, containing a D162A mutation in the catalytic aspartic acid, was made using site-directed mutagenesis (an adjacent 1163L mutation introduced a novel *ApaI* site) and this mutant plasmid [pMW222 (*cdc7^{M1}, LEU2*)] failed to complement YB514, unlike the parental wild-type plasmid pMW221.

Cell cycle analysis

The chromatin binding assay, FACS analysis of DNA content, yeast media and cell synchronization were as described previously (Weinreich *et al.*, 1999).

Purification of Cdc7p–Dbf4p protein kinase

To purify *HAHIS₆-Cdc7p–Dbf4p*, 20 175-mm² flasks containing a total of $\sim 4.4 \times 10^8$ *Sf9* cells were infected using a multiplicity of infection (m.o.i.) = 10 for each virus. After 48 h at 27°C, cells were harvested, washed once with PBS, resuspended in 10 ml hypotonic buffer [20 mM HEPES–KOH pH 7.5, 5 mM KCl, 1.5 mM MgCl₂, 1 mM dithiothreitol (DTT)] containing protease and phosphatase inhibitors and disrupted by Dounce homogenization 20 times using a B-pestle. All subsequent steps were performed at 4°C. The lysate was incubated on ice for 20 min and then nuclei were collected by centrifuging at 12 000 g for 10 min. Nuclei were resuspended in 10 ml of H₂O.1 buffer (50 mM HEPES–KOH pH 7.5, 100 mM NaCl, 0.02% NP-40, 1 mM DTT, 1 mM EDTA and EGTA, 10% glycerol), lysed by the addition of 12.5% ammonium sulfate (final) and then centrifuged at 100 000 g for 60 min. The soluble protein was precipitated using 45% ammonium sulfate (final) and resuspended in 10–20 ml of H₂O.0 buffer (H buffer containing no NaCl) until the conductivity was equivalent to that of H₂O.1. This material was applied to a 5 ml SP-Sepharose column (Pharmacia) in H₂O.1, washed with 8 vol of H₂O.1 buffer and eluted with 15 ml of H₂O.25 buffer. The H₂O.25 fraction was brought to 400 mM NaCl and then incubated overnight with ~ 200 μ l (bed volume) of 12CA5-coupled protein A–Sepharose. The resin was washed three times with 10 ml H₂O.4 and then eluted twice using 1.0 ml H₂O.4 containing 1 mg/ml 12CA5 peptide (YPYDVPDYA). The eluates were pooled, dialyzed against H₂O.1 and frozen in liquid N₂.

Purification of the Mcm proteins

The purification of Mcm2p, Mcm3p and Mcm5p will be described elsewhere (M.Akiyama, M.Weinreich and B.Stillman, in preparation). Mcm4p was tagged at its C-terminus using a T7 epitope (Novagen) followed by six histidine residues. Mcm6p and Mcm7p were each tagged at the N-terminus using the HAHIS₆ tag. Each derivative efficiently complemented a yeast strain containing a deletion of the corresponding MCM gene, although the HAHIS₆-MCM6 strain grew more slowly than the wild type. These genes were used to construct recombinant baculoviruses, and separate Sf9 cell extracts from $\sim 2.2 \times 10^8$ cells were made as above following m.o.i. = 10 for each individual Mcm virus. Each protein was purified similarly. After Dounce homogenization and centrifugation, the nuclei were discarded. The supernatant for Mcm4-T7HIS₆p was applied to a 5 ml SP-Sepharose column in H/0.05. After washing, the protein was eluted with a 40 ml gradient from 0.05 to 0.5 M NaCl in H buffer. Mcm4-T7HIS₆p peaked in fractions containing 0.22 M NaCl. The HAHIS₆-Mcm6p and -Mcm7p supernatants were applied to a Q-Sepharose column (5 ml, Pharmacia) in H/0.1 and then eluted with a 40 ml gradient from 0.1 to 0.4 M NaCl in H buffer. Mcm6p peaked in fractions containing 0.33 M NaCl and Mcm7p at 0.2 M NaCl. The eluates were brought to 0.5 M NaCl (Mcm4-T7HIS₆p) and 0.4 M NaCl (HAHIS₆-Mcm6p and -Mcm7p) and incubated overnight with either $\sim 250 \mu\text{l}$ (bed volume) T7-Tag-agarose (Novagen) for Mcm4-T7HIS₆p or 12CA5-protein A-Sepharose for HAHIS₆-Mcm6p and -Mcm7p. After washing the resin three times with 10 ml of the same buffer, the HA-tagged proteins were eluted as above. Mcm4-T7HIS₆p was eluted twice using 1.0 ml of 0.1 M citric acid pH 2.2 and immediately neutralized with 150 μl 2 M Tris-HCl pH 10.4. All proteins were dialyzed and stored as above.

Protein kinase immunoprecipitation and assays

Kinase assays typically contained 20 ng HAHIS₆-Cdc7p-Dbf4p and were carried out at 30°C for 15 min in 10 μl kinase buffer (50 mM Tris-HCl pH 7.5, 10 mM MgCl₂, 100 μM ATP, 10 μCi [γ -³²P]ATP, 1 mM DTT). For immunoprecipitations, cells were grown at 25°C to early logarithmic phase and either kept at 25°C or arrested at 37°C for 3 h. Equivalent OD₆₀₀ units were collected, washed and lysed by bead beating, resulting in equivalent amounts of total protein in the extracts. 3HA-Cdc7p was immunoprecipitated from bead-beaten cell extracts in 20 mM Tris pH 7.5, 0.5% NP-40, 1 mM EDTA and 300 mM NaCl for 2–3 h using 12CA5-protein A-Sepharose. After washing extensively, the immunoprecipitate was split into two. One-half was separated on an SDS–10% polyacrylamide gel, blotted and probed with rabbit sera against GST-Cdc7 (1:5000) and GST-Dbf4 (1:2000). The other half was washed once with 50 mM Tris-HCl pH 7.5, 10 mM MgCl₂, incubated for 15 min at 30°C in 10 μl kinase buffer containing purified Mcm7p, separated on an SDS–10% polyacrylamide gel and autoradiographed.

Phosphate labeling in vivo

In vivo labeling was performed on 50 ml of yeast growing in YPD media lacking inorganic phosphate (YPD-P) at an OD₆₀₀ of ~ 0.8 . Cells were collected, resuspended in 10 ml fresh YPD-P containing 5 mCi ³²P-labeled organic phosphate (³²P_i) and labeled for 60 min at 30°C. Cells were washed extensively and extracts were immunoprecipitated as for 3HA-Cdc7p.

Acknowledgements

We would like to thank A.Verreault for helpful comments on the manuscript, L.Thelander for discussions about Sml1p, J.Diffley for communicating results prior to publication, M.Akiyama for purified Mcm2p, Mcm3p and Mcm5p during the initial phase of this work, S.Bell, C.Hardy, L.Hartwell, K.Nasmyth and J.Scott for yeast strains, R.Knippers and P.Plevani for antibodies, J.Duffy for preparation of the figures, M.Coronesi for FACS analysis and P.Wendel for technical assistance. This work was supported by a grant from the National Institutes of Health (GM45436 to B.S.). M.W. was supported by a fellowship from the Cancer Research Fund of the Damon Runyon-Walter Winchell Foundation (DRG-1241) and is a Special Fellow of the Leukemia Society.

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Received January 11, 1999; revised June 28, 1999;
accepted August 2, 1999