A role for the Cdc7 kinase regulatory subunit Dbf4p in the formation of initiation-competent origins of replication

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Using a reconstituted DNA replication assay from yeast, we demonstrate that two kinase complexes are essential for the promotion of replication in vitro. An active Clb/Cdc28 kinase complex, or its vertebrate equivalent, is required in trans to stimulate initiation in G1-phase nuclei, whereas the Dbf4/Cdc7 kinase complex must be provided by the template nuclei themselves. The regulatory subunit of Cdc7p, Dbf4p, accumulates during late G1 phase, becomes chromatin associated prior to Clb/Cdc28 activation, and assumes a punctate pattern of localization that is similar to, and dependent on, the origin recognition complex (ORC). The association of Dbf4p with a detergent-insoluble chromatin fraction in G1-phase nuclei requires ORC but not Cdc6p or Clb/Cdc28 kinase activity, and correlates with competence for initiation. We propose a model in which Dbf4p targets Cdc7p to the prereplication complex prior to the G1/S transition, by a pathway parallel to, but independent of, the Cdc6p-dependent recruitment of MCMs.

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Replication of the eukaryotic genome is achieved by DNA synthesis at bidirectional forks that initiate at multiple sites along the chromosome (Coverley and Laskey 1994). The initiation event is generally believed to be regulated by a two-step mechanism. First, during the G1 phase of the cell cycle an origin-associated complex assembles, rendering nuclei competent for initiation. Then, at the G1/S transition, a diffusible S-phase-promoting factor (SPF) triggers the events of origin unwinding and subsequent DNA synthesis [Rao and Johnson 1970]. In budding yeast, this assembly occurs at autonomous replication sequences (ARS), which bind the six-component origin recognition complex (ORC; for review, see Newlon 1997). All ARS elements function as replication origins on plasmids, although some are either inactive or late firing in their native chromosomal context (Brewer et al. 1993), due to local chromatin structure or possibly subnuclear context (Raghuraman et al. 1997).

The prereplicative complex (pre-RC) is a prerequisite for origin-specific initiation and requires the binding of factors whose assembly is inhibited by high Clb/Cdc28 activity [Diffley 1996; Pia...
S phase after the degradation of the Clb/Cdk inhibitor Sic1p (Schwob et al. 1994; Verma et al. 1997). Although Cdc28p interacts with several initiation factors and phosphorylates some of them in vitro (Jallepalli and Kelly 1997), it remains unresolved which, if any, of the pre-RC components is its critical substrate.

CDC7 encodes a 58-kD serine/threonine kinase that is conserved in Schizosaccharomyces pombe, Xenopus, and human cells (Bahman et al. 1988; Hollingsworth and Sclafani 1990; Yoon and Campbell 1991; Masai et al. 1995; Jiang and Hunter 1997; Sato et al. 1997). Its activity, as measured on an artificial substrate, peaks at the G1/S transition (Jackson et al. 1993), although CDC7 mRNA and protein levels remain constant throughout the cell cycle (Sclafani et al. 1988; Jackson et al. 1993). Because Cdc7p kinase activity depends on interaction with a regulatory subunit called Dbf4p (Dumbbell-forming), whose transcription is under cdc7 cycle control (Chapman and Johnston 1989), it has been proposed that the fluctuation of Dbf4p levels might result in the S-phase-specific activation of the kinase (Kitada et al. 1992; Jackson et al. 1993). In addition, Cdc7p is a Cdc28p target in vitro, although the physiological role of this phosphorylation is unclear (Yoon et al. 1993; Sclafani and Jackson 1994).

CDC7-deficient strains arrest immediately after Clb/Cdc28 activation and before the initiation of DNA replication (Hereford and Hartwell 1974; Hartwell 1976). However, two studies have recently challenged the original proposal that Cdc7p acts as a general regulator of the G1/S transition (Bousset and Diffley 1998; Donaldson et al. 1998a). Because late-firing origins are unable to initiate DNA replication in a cdc7-1 mutant that is shifted to restrictive temperature in early- to mid-S phase, it appears that Cdc7p activity is required throughout S phase and, in particular, to activate late-firing origins. Genetic evidence is consistent with the idea that the Dbf4p/Cdc7 complex interacts with origin-bound components (Dowell et al. 1994; Fox et al. 1995; Loo et al. 1995; Hardy 1996), and both biochemical and genetic data suggest that MCM proteins are its physiological substrates (Hardy et al. 1997; Lei et al. 1997). Because the domain of Dbf4p that interacts with an ARS-bound complex is separable from the domain that binds Cdc7p, Dbf4p may target the kinase subunit to origins to ensure modification of origin associated substrates, although no direct demonstration of this association has been shown (Dowell et al. 1994; Hardy and Pautz 1996). Furthermore, it is not known at the moment the kinase complex binds origins, whether it remains origin bound until replication initiates, or whether the association itself is regulated, particularly at late-firing origins.

To examine the kinase activities required for the initiation of DNA replication in vitro replication assays have been particularly useful [for review, see Pasero and Gasser 1998]. In a reconstituted vertebrate replication system, Laskey and colleagues have shown that Cyclin A/Cdk2 and Cyclin E/Cdk2 synergistically stimulate DNA replication in isolated G1-phase HeLa cell nuclei in the presence of a cytosolic extract (Krude et al. 1997). Similarly, replication assays based on Xenopus egg extracts indicate an essential role for Cdk activity [Adachi and Laemmli 1994; Hua and Newport 1998; Walter et al. 1998]. These results are consistent with yeast genetic studies that show that initiation requires a cyclin-dependent kinase (Schwob and Nasmyth 1993), yet none of these studies has assessed the role of the Dbf4/Cdc7 kinase in the promotion of DNA replication in vitro.

A recently developed replication system from yeast [Pasero et al. 1997] provides a unique opportunity to test the roles of CDC7 and CDC28 kinases in this event. Using extracts and nuclear templates from thermosensitive mutants, we confirm that a Clb/Cdk1 activity is not only essential, but is sufficient to promote the initiation of DNA replication in isolated G1-phase nuclei. Surprisingly, the presence of Dbf4/Cdc7 kinase is dispensable in the nuclear extract, but is absolutely required in cis within the G1-phase nuclei, to render the template initiation competent. We go on to show that a subpopulation of Dbf4p copurifies with an insoluble nuclear fraction and localizes to subnuclear foci that contain ORC in late G1. Unlike MCMs, Dbf4p requires ORC, but not Cdc6p, to associate with this Triton-insoluble chromatin fraction. Moreover, unlike Cdc45p [Zou and Stillman 1998], its association does not require activation of the Clb/Cdc28 kinase. Finally, in vitro data indicate that the chromatin-bound fraction of Dbf4p can be displaced by DNA replication, resulting in its rapid degradation.

The data presented here support a model in which Dbf4p is synthesized in G1 and targets the Cdc7 kinase to the pre-RC in a manner independent of Clb/Cdc28 kinase. This appears to provide a high concentration of active Cdc7 kinase at its critical site of action, and to impede degradation of its labile regulatory subunit, Dbf4p. The regulated synthesis and origin association of this Cdc7p cofactor thus provides a second level of control over pre-RC assembly, independent of the Clb/Cdc28 kinase-regulated association of Cdc6p and MCMs.

Results

Cdc28p, but not Cdc7p, is required in trans to activate DNA replication in isolated yeast nuclei

We have shown previously that nuclei isolated from yeast cells arrested in late G1 are able to initiate DNA replication in vitro in the presence of an S-phase nuclear extract, whereas G2 and M-phase nuclei cannot [Pasero et al. 1997]. Between 20% and 30% of the G1-phase template (usually 100 ng of genomic DNA) becomes fully substituted by BrdUTP, and is recovered after buoyant density gradient analysis as a heavy–light (HL) peak. To ensure that the template nuclei actually initiate replication during the in vitro replication assay, we use G1-phase nuclei from clb5clb6 deletion strains [designated clb5ΔΔ], which extends G1 phase by 30 min, such that pheromone-arrested cells do not progress to S phase during the spheroplasting step. We use three criteria to monitor that the template nuclei have not entered S
...to activate replication in G1-phase nuclei in vitro, leading us to suspect that Dbf4p and Cdc7p were being supplied by the template nuclei themselves. To test this hypothesis, we isolated G1-phase nuclei at permissive temperature from strains carrying thermosensitive alleles of these genes (cde7-1 and dbf4), and tested their ability to replicate at permissive and restrictive temperatures. Whereas all nuclei replicate with comparable efficiency at 23°C, the replication of mutant nuclei in the corresponding thermosensitive extract is significantly compromised at 35°C (Fig. 2B). In contrast, wild-type G1-phase nuclei in the same cde7-1 extract produce equivalent HL peaks at both temperatures (Figs. 1 and 2A).

To examine whether the thermosensitivity of cde7-1 nuclei can be complemented in trans, we added a wild-type S-phase extract to the mutant template nuclei and again quantified replication by integration of the HL...
peak recovered after gradient centrifugation. In contrast to cdc28-4 nuclei (Fig. 2E), cdc7-deficient G1-phase nuclei could not be complemented by a wild-type S-phase extract (Fig. 2C,E). Western blots confirm the presence of Cdc7p and Dpb4p in nuclear extracts, although both proteins are relatively low in abundance [J.F-X. Difflley, pers. comm.; data not shown]. Furthermore, the Cdc7p-deficient nuclei were even more severely restricted for replication in cdc28-4 extracts at restrictive temperature (Fig. 2E). Taken together, this study of conditional mutant templates replicated in wild-type or mutant extracts leads us to conclude that Dpb4p/Cdc7 is required to promote DNA replication in vitro, yet it can only be effectively provided by the template nuclei. For Clb/Cdc28 kinase, the opposite is true: The deficiency in template nuclei is readily complemented by an S-phase kinase in the extract, and cdc28 deficiency in the extract cannot be compensated by use of Cdc28* template nuclei [Figs. 1 and 2E; data not shown].

Dbf4p levels in G1-phase nuclei correlate with competence for replication

Northern analysis indicates that DBF4 is transcribed in a cell cycle-dependent manner, as are many genes implicated in DNA replication (Chapman and Johnston 1989). Because transcriptional variation does not necessarily result in fluctuating protein levels, we monitored Dbf4 protein by a Western blot of total protein extracts from a synchronized culture that bears an epitope-tagged copy of DBF4 under its own promoter (GA-850; Fig. 3A). MATa cells were arrested in mid-G1 phase with α-factor, the pheromone was removed, and cells progressed synchronously through the cell cycle, as monitored by FACS analysis (Fig. 3B). The level of detectable Dbf4p drops significantly during α-factor arrest and subsequently increases, peaking as cells pass the G1/S boundary (30–45 min). Dbf4p levels decrease again as S phase is completed, and reach a minimum at the end of mitosis (75
wild-type G1-phase nuclei (GA-850) was performed as in Fig. 1A
initiation of DNA replication. A
The Dbf4p/Cdc7 complex is required in
ration. HL and LL are as in Fig. 1D. The increase in LL DNA
Profile after buoyant density gradient separation of the
extract from cells arrested at 37°C. Shown are profiles of the
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S-phase nuclei from
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S-phase extract, and dem-
ration in nuclei isolated from cells arrested by deple-
rtation correlates with the presence of Dbf4. Consistent
with this conclusion, we also observe low levels of rep-
lication proteins like Rpa2p or the Cdc28 kinase regula-
min after release, Fig. 3A]. In contrast, Cdc7p levels do not fluctuate significantly at any point in the cell cycle. These results were also observed in cells synchronized by temperature-shift protocols [data not shown].

To test whether the G1-phase synthesis of Dbf4p is required to render the template nuclei competent for initiation, we created a strain [GA-896] in which the genomic copy of DBF4 is under the control of the GAL1 UAS. This strain grows normally on galactose [see FACS profile in Fig. 3C], indicating that high DBF4 transcription rates are not detrimental to cell growth. When shifted to glucose-containing medium, Dbf4p expression is tightly repressed, and the cells accumulate at the G1/S transition with high Clb/Cdc28 kinase activity, but lacking Dbf4p and Cdc7 kinase activity [see FACS analysis, Fig. 3C; R. Nougarède and E. Schwob, unpubl.] This block is partially reversible, for if galactose is added after a 3 hr arrest on glucose, the cells that have not undergone a reductional anaphase replicate their DNA, resulting in a 2C DNA content [Fig. 3C]. This suggests that Dbf4p/Cdc7 kinase can execute its function even when synthesized after activation of the Clb5/Cdc28 kinase [further characterized in R. Nougarède and E. Schwob, unpubl.]

To isolate Dbf4p-depleted late G1-phase nuclei, we shifted GA-896 cells to glucose-containing medium for 2.5 hr, prior to nuclear isolation. As a control, G1-phase nuclei were isolated from an isogenic wild-type strain [GA-850] after the standard α-factor arrest and spheroplast formation [see Materials and Methods]. These wild-type G1-phase nuclei have low H1 kinase activity [Fig. 3D], yet contain Dbf4p and replicate efficiently when incubated in an S-phase extract [see Fig. 3E, quantified in D]. Dbf4p-depleted nuclei, on the other hand, support replication poorly: the peak of HL DNA is ~30% that recovered from control nuclei incubated in parallel. Importantly, unlike with wild-type G1-phase nuclei, this basal level cannot be stimulated by the addition of a wild-type S-phase extract [Fig. 3F, quantified in 3D], indicating that the competence of G1-phase nuclei for initiation correlates with the presence of Dbf4p. Consistent with this conclusion, we also observe low levels of replication in nuclei isolated from cells arrested by depletion of G1 cyclins [Pasero et al. 1997] and in nuclei isolated from cells exposed to α-factor for >90 min, in which Dbf4p levels drop [data not shown]. These results demonstrate a requirement for Dbf4p within the G1-phase template nuclei, possibly as a component of the origin-bound pre-RC [Bousset and Diffley 1998; Donaldson et al. 1998a].

\[DBf4p localizes to subnuclear foci in an ORC2-dependent manner\]

DNA replication occurs at discrete foci in yeast nuclei [Fig. 1C; Pasero et al. 1997], and immunostaining of Orc2p labels a similar subnuclear pattern that partially overlaps with newly synthesized DNA [Figs. 4C and 8A, below]. This punctate pattern of Orc2p stands in contrast to the diffuse staining observed for more abundant replication proteins like Rpa2p or the Cdc28 kinase regula-
Figure 3. Initiation of DNA replication in vitro depends on the presence of Dbf4p in the template G1 nuclei. (A) Dbf4p levels fluctuate during the cell cycle. A culture of GA-850 cells (carrying a Myc-tagged DBF4 gene) were arrested in G1 phase for 90 min with α-factor. Total protein extracts were prepared from the exponential culture (Exp), after α-factor arrest (αF) and every 15 min after release from the block. After separation by SDS-PAGE, fractions were blotted with either 9E10 Mab (Myc epitope), a polyclonal antibody against Cdc7p (gift of C. Mann, Institute of Atomic Energy Research, Saclay, France) or TAT1 Mab (anti-tubulin) as a loading control. (B) FACS analysis of the DNA content of GA-850 cells during the α-factor block-release experiment shown in A. (C) GA-896 cells that have a unique copy of DBF4 under the control of a galactose-inducible promoter, were grown exponentially on galactose-containing medium [YPGal] or arrested for 2, 3, 4, or 5 hr on glucose-containing medium [YPD]. DNA content analysis by FACS indicates arrest in G1 after 2 hr, and a peak at 0.5C corresponds to cells undergoing a reductional anaphase in the absence of Dbf4p after 3 hr on YPD. One fraction of the culture was shifted back to YPGal after 3 hr on YPD (YPD 3 hr, YPGal 2 hr) and FACS analysis shows that a significant proportion of the cells are able to enter S phase. (D) Nuclei were prepared from GA-850 cells arrested with α-factor (wt) or from GA-896 cells arrested at the G1/S transition due to a lack of Dbf4p expression after 2.5 hr on YPD (labeled Gal–DBF4). H1 kinase activity was monitored as described in Materials and Methods. We see low Clb/Cdc28 kinase activity in our G1-phase nuclei, and high kinase activity in the Dbf4p-depleted nuclei. Their ability to support semiconservative replication was monitored in standard reactions in a wild-type S-phase extract (GA-59; see F). (Dark gray bars) The integrated HL peak. (E,F) Profiles of the radioactivity recovered after buoyant density gradient analysis are shown for GA-850 G1 nuclei (E) or for Dbf4p-depleted nuclei (GA-896, F), incubated under standard conditions either with a wild-type S-phase extract (GA-59, •) or without added extract (○). HL and LL DNA migrate in fractions 2–5 and 8–10, respectively.
when compared with the isogenic Orc2+ strain [Fig. 4G,H]. Immunofluorescence of Orc1p in this same background also shows a partially diffuse staining at 23°C, and both Orc1p and Dbf4p patterns are very diffuse in the orc2-1 mutant at 37°C, although signals for both antigens are weaker (data not shown). Because ORC and origins are still able to function at permissive temperature in an orc2-1 mutant, and because Dbf4p is at least partially associated with a chromatin fraction at 23°C (see Fig. 5), we interpret the diffuse pattern of immunostaining as a demonstration that a delocalized subpopulation of Dbf4p masks a residual focal pattern.

Dbf4p cofractionates with Orc2p in an insoluble chromatin fraction

In yeast, a simple chromatin-fractionation assay has been used to monitor the assembly of various components of the pre-RC [Donovan et al. 1997; Liang and Stillman 1997]. After the lysis of spheroplasts and a low-speed centrifugation step, a Triton-insoluble nuclear fraction retains between 5% and 10% of total cellular protein, but >95% of the genomic DNA [labeled P, Fig. 5A]. This so-called chromatin pellet can be fractionated further into soluble chromatin (Chr) and residual insoluble chromatin [here labeled Sc for scaffold] by digestion of the initial pellet with DNase I, followed by a rabbit serum specific for the HA epitope [Santa Cruz Biotechnology] and a DTAFCoupled goat anti-mouse secondary antibody. Staining patterns were mutually exclusive when both primary antibodies were used at once [see text]. [F] Merge of the signals in D and E, Dbf4p–Myc, and Orc1p–HA are in red and green, respectively, with the region of overlap in yellow. [G,H] Punctate pattern of Dbf4–Myc is altered in orc2-1 cells. Exponential cultures of GA-850 cells [ORC2, G] and GA-1027 cells [orc2-1, H] were grown at 23°C and fixed. Dbf4p–Myc was detected by immunofluorescence as described in A. Typical fields of cells are shown. The cell cycle fluctuation in Dbf4p levels can be observed in these nonsynchronous populations. Noteworthy is the decrease in the punctate appearance of the Dbf4p signal in the orc2-1 strain that is otherwise isogenic to GA-850. Bars, 2 µm.
a commercial anti-pore monoclonal antibody (mAb 414, Berkeley Antibody, Inc., see Rout and Blobel 1993) serve as loading controls, particularly as the middle band, Nup116, is almost equally represented in both soluble and bound fractions (Fig. 5D, see bars). Following the DNase I fractionation step, which releases DNA, histones, and most of the Mcm2 protein from the initial pellet, nearly all of Orc2p and ~50% of the Dbf4p are recovered in the insoluble scaffold fraction (see Fig. 5C). This partitioning is consistent with the proposal that even in a random population, a large fraction of the chromatin-associated Dbf4p is tightly bound to the ORC complex, because scaffold fraction contains <2% of the total cellular protein, but the vast majority of ORC. It is not known whether the Dbf4p that is released at this final step is associated with the Dbf4/CDC7 substrate Mcm2p, which, unlike ORC, is enriched in soluble chromatin (Fig. 5C, Chr).

Dbf4p requires ORC, but not Cdc6p, to bind chromatin

To examine whether the association of Dbf4p with the insoluble nuclear pellet requires an intact ORC complex, we performed the chromatin fractionation assay on synchronized cultures of the orc2-1 mutant and an isogenic Orc2+ strain (ORC2). In Xenopus extracts, as in yeast, it has been shown that the depletion or inactivation of ORC or Cdc6p precludes formation of a functional pre-RC (Diffl ey et al. 1994; Carpenter et al. 1996; Cocker et al. 1996; Coleman et al. 1996). Because a temperature shift in the orc2-1 mutant appears to disrupt the ORC
complex entirely (Santocanale and Diffley 1996), we first synchronized orc2-1 and wild-type cultures in G1 by pheromone arrest at 23°C, and then shifted a fraction of each culture to 37°C and released the cells from pheromone arrest. One hour after release, the mutant arrests at the G1/S boundary due to inactivation of Orc2p, whereas the ORC2 strain recovers from α-factor and progresses into S phase (see FACS analysis of arrested and released cultures, Fig. 5E). Cells arrested in early G1 at permissive temperature and in late G1 at nonpermissive temperature were fractionated into the soluble and insoluble nuclear fractions and probed for Dbf4p and nuclear pore. At 23°C, Dbf4p is efficiently retained in the insoluble pellet of both the mutant and wild-type strains, whereas after inactivation of Orc2p by a shift to 37°C, Dbf4p is almost entirely displaced to the soluble fraction (Fig. 5D; cf. P and S). The displacement is not due to the elevated temperature per se, because Dbf4p remains chromatin bound in the ORC2 background at 37°C, and is efficiently recovered in the chromatin fraction of cdc6-1 cells arrested at 37°C (Figs. 5D and 6B). Cdc6p, as expected, is released from the pellet in both mutant strains (data not shown). Quantitation of the amount of Dbf4p recovered in the insoluble nuclear pellet from three independent experiments is shown in Figure 5D, normalized to the recovery of the Nup116 band. Because the ORC2 strain progresses into S phase after removal of α-factor, it is not strictly comparable with the G1/S arrest of the orc2-1 strain, yet results presented below show that Dbf4p remains associated with the insoluble nuclear pellet throughout late G1 and S phase in Orc+ backgrounds (see Figs. 6 and 7). Thus, our results show that the association of newly synthesized Dbf4p with the insoluble pellet after recovery from pheromone arrest, is sensitive to the integrity of ORC. Because the level of Dbf4p is low in the α-factor-arrested cells used in panel D, we also tested exponentially growing wild-type and orc2-1 strains as controls for this experiment. Again, the distribution of Dbf4p between soluble and insoluble fractions was quantified and normalized to the nuclear pore signal. No temperature-induced release of Dbf4p is observed in wild-type cells, although the amount retained in the insoluble nuclear pellet drops by 80% when the orc2-1 strain is shifted to 37°C (data not shown).

Cdc6p and MCM loading are not essential for Dbf4p association with nuclear chromatin

 Whereas the ORC dependence shown in Figure 5 is consistent with the hypothesis that Dbf4p binds directly to the origin complex, Dbf4p might also associate through the MCM complex, because MCMs associate with the pre-RC prior to α-factor arrest and remain chromatin bound despite Cdc6p release (Donovan et al. 1997; Hua and Newport 1998). For this reason, we tested whether Cdc6p, and thereby MCM loading, is necessary for the association of Dbf4p with the insoluble nuclear fraction. The most efficient manner to prevent MCM loading is

Figure 6. Dbf4p does not require Cdc6p or MCM loading to associate with the insoluble nuclear fraction. (A) GA-1213 cells [cdc15, GAL–CDC6] grown on galactose were arrested in late M phase for 2 hr at 37°C. Half of the culture was maintained for another hour in the same medium at 37°C whereas 2% glucose was added to the other half to shut off the GAL–CDC6 promoter. Cells were then released from the cdc15 arrest in the presence (GAL–CDC6 ON) or the absence (GAL–CDC6 OFF) of Cdc6 function. Samples were taken at regular time intervals and the DNA content of the cells was analyzed by FACS. (B) Chromatin assays were performed on the samples described above and the distribution of Dbf4p and Mcm2p in the soluble and insoluble nuclear fractions was analyzed by Western blot. The amount of Dbf4p and Mcm2p in the insoluble nuclear fraction was determined relative to Swi6, which was used as a loading control. The percentage of budded cells is shown for the same time points. (C) GA-1026 cells grown at 25°C were arrested for 1 hr with 20 µg/ml nocodazole and were shifted to 37°C for another hour in the presence of nocodazole before being released from the arrest at 37°C. Samples were taken just prior to (Noc) and at regular time intervals after the release. Position of cells in the cell cycle was determined by FACS and the distribution of Dbf4p and Mcm2p in the insoluble nuclear fraction, relative to p53RNase, was analyzed by Western blot as described in Materials and Methods. The quantitation represents the mean value for two experiments. Relative Dbf4p levels for wild-type and cdc6-1 Noc samples are 160 and 220, respectively.
to deplete Cdc6p from cells as they pass from mitosis into G1. This can be achieved by placing the genomic CDC6 gene under control of the GAL1 UAS, which allows complete transcriptional repression by growth on glucose. This is done in a cdc15-1 strain, which permits synchronization in mid-metaphase by shifting to 37°C. A random culture on galactose was thus arrested prior to anaphase by a temperature shift, whereas half of the culture was switched to glucose medium for an additional hour. Release by a return to 23°C allowed the cells to progress synchronously into G1, either in the presence (galactose medium) or absence (glucose medium) of Cdc6p. FACS analysis confirmed both the efficiency of the arrest and the timing of G1.

Cells were fractionated by spheroplasting and Triton-lysis as described above (see Fig. 5A), and the association of Dbf4p and Mcm2p with the insoluble nuclear fraction (P) was monitored by Western blot. In this case, Swi6p serves as the loading control, for it remains chromatin associated independent of the pre-RC. When Cdc6p is present (ON), Mcm2p associates rapidly with the chromatin fraction peaking between 30 and 45 min after release, whereas Dbf4p loads more slowly, peaking at 60-min postrelease (Fig. 6B). When no Cdc6p is present (OFF), Mcm2p does not bind the insoluble nuclear fraction, whereas Dbf4p association is, proportionately, even more efficient in the absence of Cdc6p (Fig. 6B). A drop in the level of bound Dbf4p at 30-min postrelease is seen in both the Cdc6+ and Cdc6-depleted cells, and may reflect a point in late telophase/early G1, at which time Dbf4p is specifically depleted from the chromatin fraction. These results clearly indicate that MCM complex loading is not a prerequisite for the association of Dbf4p with the chromatin pellet.

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Further evidence that Dbf4p loading does not require pre-RC formation was obtained by use of a cdc6-1 temperature-sensitive strain. Again, cells were synchronized in mitosis at permissive temperature, and then released from the nocodazole block at restrictive temperature. The cdc6-1 cells accumulate at the G1/S boundary, whereas the isogenic CDC6 strain progresses through G1 and into S phase (see FACS analysis in Fig. 6C). The distribution of Dbf4p and Mcm2p between the soluble fraction and the insoluble nuclear pellet was determined by Western blot as described above, with an abundant cellular RNase (p55, Karwan et al. 1990) to normalize recovery. Again, we observe that Mcm2p loading is entirely dependent on a functional Cdc6p, whereas Dbf4p associates with the chromatin pellet in a Cdc6p-independent fashion (Fig. 6C).

**Association of Dbf4p with chromatin fluctuates through the cell cycle, but is independent of Clb/Cdc28 kinase**

Although ORC association with the insoluble nuclear fraction is constant through the cell cycle (Fig. 7A), the association of Cdc6p, MCMs, and Cdc45p with the pre-RC is carefully controlled. Moreover, in the case of these latter three components, Clb/Cdc28 kinase activity plays an important regulatory role, either preventing (for Cdc6p and MCMs) or promoting (for Cdc45p) their assembly at origins. To examine the cell cycle and Cdc28 kinase dependence of the association of Dbf4p with this insoluble nuclear fraction, we have fractionated wild-type cells carrying a Myc-tagged Dbf4p as they progress synchronously through the cell cycle following an α-factor arrest (see Fig. 7B for FACS analysis of the culture). Identical samples were probed with antibodies recognizing the Myc-tagged Dbf4p, Mcm2p, Orc2p, and tubulin (Fig. 7A). As noted above, the total amount of Dbf4p present in the cell drops significantly on α-factor arrest, and fluctuates during the cell cycle, peaking in early S phase. Consistently, Dbf4p is most highly represented in the chromatin-bound fraction at the G1/S transition (see 30-min time point), and appears to be displaced gradually throughout S phase, reaching a minimum at mitosis (70–80 min postrelease, Fig. 7A). Mcm2p, on the other hand, loads prior to α-factor arrest, remains associated during the arrest/release protocol, and then becomes rapidly displaced as DNA replication proceeds (40–50 min postrelease). Quantitation of the bound fractions of Dbf4p and Mcm2p through this synchronous cell cycle reveals asynchrony in the association and loss of Dbf4p and Mcm2p from the pellet fraction: Mcm2p association and release precedes that of Dbf4p by 15–30 min (Fig. 7C). Although the fluctuation of Dbf4p in the insoluble nuclear chromatin closely follows variations in total Dbf4p levels, we note that during S phase the bound fraction of Dbf4p is significantly more stable than the unbound fraction (40–60 min postrelease, Fig. 7A). Thus, together with the fact that soluble Dbf4p is more readily degraded in vitro (Fig. 8), it appears that the association of Dbf4p with nuclear chromatin contributes toward its stabilization.

It is well established that the assembly of the pre-RC is prevented by high Clb/Cdc28 kinase activity (Tanaka et al. 1997), whereas the loading of Cdc45p at origins was recently shown to require Clb/Cdc28 kinase activation (Zou and Stillman 1998). Because the association of Dbf4p with the insoluble nuclear fraction peaks near to the G1/S transition, it was important to test whether this binding also requires an active S-phase Cdk kinase. To this end, we analyzed the distribution of Dbf4p between the insoluble and soluble fractions, in cells that express a nondegradable form of the Clb/Cdc28 inhibitor Sic1p under the control of the GAL1 UAS (GA-980). Exponentially growing GA-980 cells were synchronized with α-factor and released into galactose medium, which induces SIC1 and thus arrests cells at the G1/S transition with no Clb/Cdc28 activity (see scheme and FACS profiles, Fig. 7D,E). As shown above, the Dbf4p level is very low in α-factor-blocked cells, yet a significant fraction of the remaining protein cofractionates with ORC (see P, Fig. 7F,G). As cells traverse late G1 and arrest with high Sic1p levels (Sic1), Dbf4p levels increase, and ~10% of the cellular Dbf4p is tightly associated with the insoluble nuclear fraction. This is true despite a complete absence of Clb/Cdc28 activity (Fig. 7F; see also Duncker et al. 1999). Thus, association of Dbf4p occurs independently of Clb/Cdk activation.

To examine the behavior of Dbf4p following the activation of Clb/Cdc28 kinase, a part of the α-factor-arrested culture was released and allowed to progress into S phase. A third aliquot was arrested in S phase by the addition of the ribonucleotide reductase inhibitor, hydroxyurea (HU). All cultures were subjected to the identical fractionation protocol, and probed for Dbf4p, Mcm2p, and the RNase p55. We observe an increase in the association of Dbf4p with the insoluble nuclear fraction in S phase, although this is compromised when cells are arrested with HU (Fig. 7G,H). The association of Mcm2p with the insoluble fraction, on the other hand, does not vary significantly under these conditions. Although both the synchronous S-phase population and the HU-arrested population have high Clb/Cdc28 kinase activity, the HU arrest additionally provokes a cell cycle checkpoint response that activates Mec1 and Rad53 kinases (cell cycle stages are confirmed by FACS analysis; Fig. 7H). Because it has been shown that HU induces a Rad53p-mediated phosphorylation of Dbf4p (Boussau and Difflay 1998; Santocanale and Diffley 1998), we examined whether this modification might be responsible for the release of Dbf4p from the insoluble nuclear pellet when cells are exposed to HU. An HU arrest experiment was therefore performed with the mec2-1 allele of RAD53, which fails to activate Rad53 kinase activity in response to HU (Weinert et al. 1994). Fractionation into the insoluble nuclear pellet and supernatant shows that under these conditions Dbf4p remains chromatin associated (Fig. 7G, lane 5), consistent with the proposal that Rad53, but not Cdc28 kinase, controls the association of Dbf4p with the replication origin complex. In conclu-
Dbf4p is displaced from subnuclear foci by replication fork movement in vitro

The data presented above, and the reversibility of the Gal–DBF4 arrest [Fig. 3C], show that the ORC-dependent association of Dbf4p with chromatin is not regulated like that of Cdc6p or MCMs. However, we do observe a gradual loss of Dbf4p from the chromatin pellet during S phase in vivo [Fig. 7A,C]. This is confirmed by an absence of immunoreactive Dbf4p foci in late G1- and M-phase cells [Fig. 4A]. To test whether this is due to the displacement of Dbf4p during DNA replication, we have followed the fate of epitope-tagged Dbf4p by confocal microscopy and chromatin-binding assays during initiation and subsequent elongation steps in vitro. Wild-type G1 phase nuclei expressing Dbf4–Myc [GA-850], were isolated and incubated in a wild-type GA-59 S-phase extract. DNA synthesis was monitored by the incorporation of DIG–dUTP and subsequent immunodetection [green signals in Fig. 8A,B], revealing foci that overlap partially with the persistent foci of Orc2p staining [red; for details, see Fig. 8, legend]. In nuclei that have not yet incorporated DIG–dUTP, we see clear Dbf4p foci (Fig. 8B). However, as nuclei synthesize increasing amounts of DNA, Dbf4p staining is lost (the parallel detection of newly synthesized DNA and Dbf4p are superimposed in the color panel of Fig. 8B). This series of images represents nuclei at different stages of replication in vitro, demonstrating the progressive loss of the punctate Dbf4p pattern (red), as genomic DNA replication proceeds [DIG–dUTP, green].

To confirm that Dbf4p is released from the replicating nuclei and not simply masked, we have fractionated the nuclei by Triton X-100 extraction either before or after replication in the S-phase nuclear extract. As shown above, Dbf4p is present in the wild-type G1-phase nuclei prior to replication, and a significant fraction is tightly

sion, although the amount of Dbf4p that is associated with the insoluble nuclear fraction varies through the cell cycle, it appears to neither require nor be impeded by Cdc28 kinase activity.

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associated with the chromatin pellet [Fig. 8C, labeled G1n, S, and P]. When the same fractions are probed for Rpa2p, we find Rpa2p entirely soluble, because the initiation of DNA replication has not yet occurred [Adachi and Laemmli 1994; Pasero et al. 1997; Tanaka and Nasmyth 1998]. After a 90-min replication reaction in an S-phase extract, the Triton extraction was repeated, and both pellet and soluble fractions were probed for Dbf4p and Rpa2p. Now only a trace of Dbf4p is recovered from either fraction, although the full complement of Rpa2p is present, again primarily in the supernatant [Fig. 8C, labeled –aph]. The small fraction of insoluble Rpa2p probably represents the fraction engaged at replication forks, because it is not present when aphidicolin is added [Fig. 8C, cf. –aph and +aph, S, and P]. The apparent release or degradation of Dbf4p might either reflect nonspecific proteinolyisis, the action of the Clb/Cdc28 kinase in the extract, or a specific displacement due to the act of replication. When the identical incubation is performed in the presence of aphidicolin, which blocks DNA polymerase but not Cdc28p activity, Dbf4p remains associated with the insoluble nuclear fraction and is largely resistant to degradation [Fig. 8C, +aph, S, and P]. The fact that the soluble fraction of Dbf4p is degraded in the presence of aphidicolin, whereas the insoluble fraction remains bound and stable, suggests that one of the functions of Dbf4p’s association with origin complexes, may be to stabilize this highly labile protein. Although we have not monitored the turnover of Dbf4p in intact cells, our in vitro results suggest that active DNA synthesis, rather than active Clb/Cdc28 kinase, provokes Dbf4p release from chromatin and enhances its instability.

Discussion

Cell fusion experiments performed nearly 30 years ago showed that S-phase cytosol provides a diffusible substance that can activate DNA replication in G1-phase, but not in G2-phase nuclei [Rao and Johnson 1970]. This led to two proposals; first, that nuclei can assume at least two states, one in which they are competent to initiate DNA replication and another in which they are not, and second, that a trans-acting, S-phase promoting factor triggers initiation. Although these phenomena could be reconstituted subsequently in replication assays based on Xenopus egg extracts [for review, see Coverley and Laskey 1994; Blow 1996], the identification of the genes and proteins that participate in these events was primarily achieved through the genetic analysis of the G1/S transition in yeast. The components of the pre-RC [ORC, Cdc6p, MCMs, and Cdc45p] were identified and demonstrated genetically to be essential for the initiation of DNA synthesis in yeast. Moreover, mutations in two universally conserved Ser/Thr kinases, Cdc28p and Cdc7p, and their unstable regulatory subunits, arrest or delay passage from G1 to S phase [Hartwell et al. 1974; Schwob and Nasmyth 1996; Jallepalli and Kelly 1997]. Recently it was shown that inactivation of CDC7 in mid-S phase also impairs initiation at late-firing origins, suggesting that this kinase does not only act at the G1/S transition [Bousset and Diffley 1998; Donaldson et al. 1998a]. To explain this observation it was proposed that the Dbf4/Cdc7 kinase acts locally at origins to promote initiation. In this paper we confirm with an in vitro replication assay that the mode of action of Dbf4/Cdc7 kinase is distinctly different from that of Clb5/Cdc28, which behaves as a diffusible SPF. More importantly, we have confirmed biochemically that Dbf4p, the Cdc7p regulatory subunit, associates with prereplicative chromatin in an ORC-dependent manner. Its binding, which peaks in late G1 phase, is nonetheless independent of Cdc6 and MCM complex loading. Finally, we show that the presence of Dbf4p in G1-phase nuclei correlates with the potential of these templates for the initiation of DNA synthesis in vitro.

S- or M-phase cyclin/Cdk1 complexes have SPF activity

Using extracts and template nuclei from mutant yeast strains, we find that either an S-phase or mitotic B-type cyclin/Cdc28 complex, or its vertebrate equivalent, is necessary to stimulate semiconservative DNA replication in G1-phase nuclei in vitro. The addition of kinase alone is sufficient to stimulate replication slightly, although another component of the nuclear extract is limiting for maximal replication efficiency [Fig. 1]. Our results from in vitro replication are fully consistent with the timing of Clb5p and Clb6p expression and genetic evidence implicating the Clb5,6/Cdc28 kinase in the promotion of replication in vivo [Schwob and Nasmyth 1993, 1996, Donaldson et al. 1998b]. However, it appears that yeast is rather permissive as to the nature of the Cdk that can function to initiate DNA replication. In a G1-phase extract with high Sic1p levels, we find that purified Xenopus CycB/Cdc2 kinase stimulates replication more efficiently than the purified Clb5/Cdc28 kinase [data not shown]. This may either indicate a need for both the Clb5 and Clb6/Cdc28 complexes [Donaldson et al. 1998b], or reflect inhibition of the exogenously added Clb5p/Cdc28 by the high levels of Sic1p. Although the synthesis of Dbf4p also peaks in late G1 and early S phase, the presence of active Dbf4/Cdc7 kinase is not required in trans to activate replication in wild-type G1-phase nuclei. Dbf4/Cdc7 is therefore not, formally speaking, a component of SPF.

Dbf4p renders G1-phase nuclei competent for activation by Clb/Cdc28

Unlike Cdc7p, which maintains a constant level through the cell cycle, Dbf4p is a highly unstable protein, particularly when cells are blocked by an α-factor arrest in early G1 [Fig. 3]. Using nuclei from a synchronized culture in which we can shut off DBF4 expression, we show that the competence of a late G1-phase nucleus to support DNA replication in vitro correlates with the presence of this factor. This is true even when a wild-type S-phase extract is added to promote replication. In sup-
port of this conclusion, we show that the conditional inactivation of either Cdc7p or Dbf4p compromises the replicative ability of G₁-phase templates, whereas cdc28− deficient G₁-phase nuclei replicate efficiently in Cdk1-containing extracts.

A one-hybrid assay indicated that Dbf4p can interact with a complex that recognizes the ARS consensus (Dowell et al. 1994), and suggested that at least part of the regulatory function of Dbf4p might be to target Cdc7p to origins. Consistent with this hypothesis, immunolocalization of Dbf4p reveals ORC-like foci in late G₁ and early S phase, and this punctate distribution is compromised in orc2-1 mutant strains. Using techniques optimized by the Diffley and Stillman laboratories (Donovan et al. 1997; Liang and Stillman 1997), we show that a subpopulation of Dbf4p copurifies with Orc2p in an insoluble nuclear fraction and furthermore, like ORC, resists solubilization by DNase I digestion. Importantly, the association of Dbf4p with the chromatin fraction in late G₁ requires an intact ORC complex, but not Cdc6p or MCM-bound proteins. Although we cannot rule out that a subfraction of Dbf4p can bind the assembled MCM complex, our data clearly define a Cdc6/MCM-independent association that requires intact ORC.

Two pathways dependent on unstable proteins, prepare origins for initiation

It remains an open question as to what are the minimal components required to form a replication-competent origin complex. Clearly, the association of the MCM complex with chromatin and the pre-RC requires the presence of both ORC and Cdc6p (Diffley et al. 1994; Carpenter et al. 1996; Cocker et al. 1996; Coleman et al. 1996). Cdc6p is a highly labile protein, which has to be synthesized in late G₁ following an α-factor block or other events that deplete Cdc6p from the cell. Its ability to load MCM proteins is inhibited by high Clb/Cdc28 kinase activity (Piatti et al. 1995, 1996). We propose that association of Dbf4p with chromatin and its potential targeting of Cdc7p to the pre-RC defines a second pathway necessary for creation of an initiation competent state at origins in G₁ (see Fig. 9). In contrast to MCMs, Dbf4p recruitment is clearly independent of Cdc6p, and can occur either in the absence or presence of Clb/Cdc28 activity. Like Cdc6p, Dbf4p is highly labile, and is depleted in stationary phase and pheromone-blocked cells. Unlike Cdc6p, however, part of Dbf4p persists in the insoluble nuclear fraction throughout much of S phase. We propose that two steps are required to form an initiation-competent nucleus: Cdc7p loading through Dbf4p and MCM loading through Cdc6p. Both pathways converge on the MCM complex: The ORC–Cdc6p–MCM pathway assembles MCMs at origins, and the ORC–Dbf4p pathway is likely to target the Cdc7p kinase to its critical target, Mcm2p (Lei et al. 1997). This model provides a mechanism to restrict Dbf4/Cdc7 kinase action to those MCMs assembled at origins.

Is the origin-targeting function of Dbf4p essential?

Previous studies have shown that deletion of the Dbf4p amino terminus renders the protein nonfunctional (Dowell et al. 1994). This deletion removed not only the ARS-targeting domain, but also the only NLS in the protein, thus its inactivity may have reflected the lack of nuclear localization. In a preliminary study, we have overexpressed a series of subdomains of the Dbf4p amino terminus, to see if we could saturate Dbf4p-binding sites at origins. Constructs encoding the full origin-targeting domain cause cell growth arrest when induced, although in liquid culture this is manifest only when cells are recovering from stationary phase or from α-factor arrest (P. Pasero and B.P. Duncker, data not shown). This shows that overexpression of the Dbf4N-terminus is not lethal per se, and suggests that competition for origin targeting is most successful when the endogenous Dbf4p levels drop, and the targeting of Cdc7p must be achieved de novo.

The best evidence that the targeting of Dbf4p through
ORC may be a regulated event, is demonstrated by the release of Dbf4p from the insoluble nuclear fraction in cells arrested in S phase by HU. This displacement is Rad53p dependent, and results in a highly stabilized, soluble form of Dbf4p. Dbf4p also appears to be displaced from chromatin during DNA synthesis in vitro, although in this case the displaced Dbf4p is rapidly degraded. Thus, the Rad53p modification of Dbf4p (Santocanale and Diffley 1998; J.F. Diffley, pers. comm.) may serve to stabilize the released factor, whereas release of Dbf4p during a normal S phase may serve to deplete the nuclear pool of Dbf4p to prevent reformation of an active pre-RC. Because the amount of active Cdc7p kinase appears to be regulated primarily through the level of its labile Dbf4p cofactor, a targeting and stabilization of the complex by its association with origins may be essential for achieving a critical concentration of the kinase at its essential sites of action. Such a mechanism is particularly relevant in view of the low abundance and relatively low affinity shown by Cdc7p for its substrates in vitro (Jack-
verson et al. 1993; Dixon and Campbell 1997). Future stud-
ies will address the critical question of how Dbf4p synthesis and degradation are controlled, because we predict that these will be closely linked to proper cell cycle progression and recovery from a resting or G0 state.

Materials and methods

Yeast strains and synchronization

The strains used in this study are listed in Table 1. All are in the same genetic background, and were backcrossed to W303, ex-
cept GA-59, GA-85, and GA-161. We have seen no indication that the nuclear replication assay is background dependent.

Synchronization of wild-type cells (GA-59; GA-850) in G1 was achieved by incubating at 30°C or 23°C until spheroplasts appear round and re-

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with 4% paraformaldehyde, are spotted on a microscope slide and processed for immunodetection as described elsewhere (Gotta et al. 1996). FITC-coupled anti-DIG F(ab)2 fragments or a monoclonal antibody directed against DIG are used to detect DIG–dUTP incorporation as described in Braguglia et al. (1998). Genomic DNA is stained with ethidium bromide. Confocal microscopy was performed with a Zeiss Laser Scanning Microscope 410 with a 63× Plan-Apo-chromat objective (1.4 oil). Immunofluorescence of nuclear proteins was performed as described (Gotta et al. 2000). Chromatin association assays are performed as described previously (Donovan et al. 1997; Liang and Stillman 1997), except that cells were fixed prior to spheroplasting by optical microscopy and should be completed within 2 min.

### Table 1. Strains used in this study

| Code   | Description                                                                 |
|--------|-----------------------------------------------------------------------------|
| GA-59  | MATa, leu2, trp1, ura3-52, gal2, prb1-1122, pcr1-407, pep4::URA3              |
| GA-85  | MATa, bar1, his6, leu2-2, -112, trp1-289, ura3-52, cdc7-1 (formerly RM1-3A from B. Brewer, University of Washington, Seattle) |
| GA-112 | MATa, ade2-11, can1-100, his3-11, -15, leu2-3, -112, trpl-1, ura3, GAL, psi, pep4::URA3, cdc28-4 (formerly K1719 from K. Nasmyth) |
| GA-161 | MATa, his7, ura1, cdc6-1 (L. Hartwell, FHCl, Seattle, WA)                     |
| GA-366 | MATa, ade2-1, ade3, can1-100, his3-11, -15, leu2-3, -112, trpl-1, ura3, GAL, psi, cdc7-1 (formerly K2032 from K. Nasmyth) |
| GA-453 | MATa, ade2-1, can1-100, his3-11, -15, leu2-3, -112, trpl-1, GAL, psi*, ura3:ADH–HA–CLB5/URA3 (formerly K3819 from K. Nasmyth) |
| GA-719 | MATa, ade2-1, ade3, can1-100, his3-11, -15, leu2-3, -112, trpl-1, ura3, GAL, psi*, cln1::hisG, cdc2::del, cln3::GAL–CLN3/URA3, CLA1(S3D1) (also called E79) |
| GA-769 | MATa, ade2-11, can1-100, his3-11, -15, leu2-3, -112, trpl-1, ura3, GAL, psi*, clb5::URA3, clb6::LEU2 (also called E145) |
| GA-849 | MATa, can1-11, ura3-52, dna5-1 (dbf4), [also called E458]                     |
| GA-850 | MATa, ade2-1, can1-100, his3-11, -15, leu2-3, -112, trpl-1, ura3, GAL, psi*, dbf4::DBF4–MyC18/LEU2 (formerly K6388 from K. Nasmyth) |
| GA-851 | MATa, ade2-1, can1-100, his3-11, -15, leu2-3, -112, trpl-1, ura3, GAL, psi*, cdc4-1 (also called E197) |
| GA-893 | MATa, ade2-1, can1-100, his3-11, -15, leu2-3, -112, trpl-1, ura3, GAL, psi*, orc2::ORC2–MyC9/LEU2 (formerly K6447 from K. Nasmyth) |
| GA-896 | MATa, ade2-1, can1-100, his3-11, -15, leu2-3, -112, trpl-1, ura3, GAL, dbf4::HIS3, URA3::GAL–DBF4 (also called E633) |
| GA-980 | MATa, ade2-1, can1-100, his3-11, -15, leu2-3, -112, trpl-1, GAL, psi*, GAL1–SICT5V,T33A, S76A, HA1::URA3, dbf4::DBF4–myC18/LEU2 (also called E623) |
| GA-1026 | MATa, DBF4–myC18/LEU2, cdc6-1 (derived from GA-850)                     |
| GA-1027 | MATa, DBF4–myC18/LEU2, orc2-1 (derived from GA-850)                     |
| GA-1123 | GA-850 x GA-1124                                                        |
| GA-1124 | MATa, ade2-1, can1-100, his3-11, -15, leu2-3,112, trpl-1, ura3, orc1::ORC1–HA3/URA3 |
| GA-1212 | MATa, DBF4–myC18/LEU2, mec2-1 (derived from GA-850)                     |
| GA-1213 | MATa, leu2, cdc6::HisG DBF4–MyC18/LEU2, cdc15, ura3::URA3 6ALubiCDC6 (derived from GA-850 and K5032 from S. Piatti, University of Milan, Italy) |

Kinetin fractionation assays

Chromatin association assays are performed as described previously (Donovan et al. 1997; Liang and Stillman 1997), with the following modifications. After spheroplasting, cells are washed once in 20 ml of YPD/1.1 M Sorbitol, 0.5 mM PMSF and three times in 0.25X A buffer (20 mM Tris-HCl at pH 7.4, 20 mM KCl, 2 mM EDTA-KOH, 0.05 mM spermine, 0.125 mM spermidine), supplemented with 1 M sorbitol, 1% thioglycol, 1% trislyzol, and 0.5 mM PMSF. The pellet of spheroplasts (~4.10^8 cells) is then resuspended in 1 ml of the same buffer on ice, but containing 0.4 M sorbitol, 1% Triton X-100, 300 µg/ml benzanidine, 1 µg/ml pepstatin A, 2 µg/ml antipain, 0.5 µg/ml leupeptin, 100 µg/ml TPCK, and 50 µg/ml TLCK. Lysis is monitored by optical microscopy and should be completed within 2 min. The insoluble nuclear fraction is recovered by centrifugation in an Eppendorf centrifuge for 15 min at 13,500 rpm at 4°C and, unless otherwise stated, is resuspended in one-tenth of the initial volume lysis buffer without Triton X-100. DNase I digestion of the insoluble nuclear pellet was performed for 20 min on ice with 30 µg/ml DNase I and 5 mM MgCl2. The residual scaffold fraction is recovered by centrifugation in an Eppendorf centrifuge for 10 min at 13500 rpm at 4°C. Total protein concentration was determined for all fractions by a Bradford assay. Fractionation of nuclei is performed as for spheroplasts.

Western blots were performed by luminescence detection (ECL, Amersharn) and multiple exposures of films were scanned and quantitated with the Aida 2.0 software (Raytest). Rabbit anti-Cdc6p was raised against a GST–Cdc6p fusion protein. Other antibodies used are mAb 9E10 [anti-Myc], mAb 12CA5 [anti-HA], mAb TAT1 [anti-tubulin, gift of K. Gull, University of Manchester, UK], mAb 414 [Berkeley Antibodies, CA], and the immune sera, anti-Orc2p [JAB12, gift of J. Difley, ICRF, South Mimmsa, UK], anti-Mcm2p [YN-19, Santa Cruz Antibodies, CA], anti-Rpa2p [gift of S. Brill, UMDNJ, Nutley, NJ], anti-p55Rnase [Karwan et al. 1990, gift of R. Karwan], and anti-Swi6p [gift of K. Nasmyth, IMP, Vienna, Austria].
the basis of a standard curve of histone H1 phosphorylation. 1 unit is equivalent to the activity in 20 μg of the GA-161 mitotic nuclear extract [Duncker et al. 1999].

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References
Adachi, Y. and U.K. Laemmli. 1994. Study of the cell cycle-dependent assembly of the DNA pre-replication centres in Xenopus egg extracts. EMBO J. 13: 4153–4164.
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.
Bahman, M., V. Buck, A. White, and J. Rosamond. 1988. Characterisation of the CDC7 gene product of Saccharomyces cerevisiae as a protein kinase needed for the initiation of mitotic DNA synthesis. Biochim. Biophys. Acta 951: 335–343.
Blow, J.J. 1996. Chromosome replication in Xenopus egg extracts. In Eukaryotic DNA replication (ed. J.J. Blow), pp. 143–165. IRL Press, Oxford, UK.
Boussau, K. and J.F. Diffley. 1998. The Cdc7 protein kinase is required for origin firing during S phase. Genes & Dev. 12: 480–490.
Braguglia, D., P. Heun, P. Pasero, B.P. Duncker, and S.M. Gasser. 1998. Semi-conservative replication in yeast nuclear extracts requires Dna2 helicase and supercoiled template. J. Mol. Biol. 281: 631–649.
Brewer, B.J., J.D. Diller, K.L. Friedman, K.M. Raghuaman, and W.L. Fangman. 1993. The topography of chromosome replication in yeast. Cold Spring Harb. Symp. Quant. Biol. 58: 425–434.
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.
Chapman, J.W. and L.H. Johnston. 1989. The yeast gene, DBF4, essential for entry into S phase is cell cycle regulated. Exp. Cell. Res. 180: 419–428.
Cocker, J.H., S. Piatti, C. Santocanale, K. Nasmyth, and J.F. Diffley. 1996. An essential role for the Cdc6 protein in forming the pre-replicative complexes of budding yeast. Nature 379: 180–182.
Coleman, T.R., P.B. Carpenter, and W.G. Dunphy. 1996. The Xenopus Cdc6 protein is essential for the initiation of a single round of DNA replication in cell-free extracts. Cell 87: 53–63.
Coverley, D. and R.A. Laskey. 1994. Regulation of eukaryotic DNA replication. Annu. Rev. Biochem. 63: 745–776.
Diffley, J.F. 1996. Once and only once upon a time: Specifying and regulating origins of DNA replication in eukaryotic cells. Genes & Dev. 10: 2819–2830.
Diffley, J.F., 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.
Dixon, W.J. and J.L. Campbell. 1997. Preparation of active Cdc7/Dbf4 kinase from yeast cells. Methods Enzymol. 283: 390–397.
Donaldson, A.D., W.L. Fangman, and B.J. Brewer. 1998a. Cdc7 is required throughout the yeast S phase to activate replication origins. Genes & Dev. 12: 491–501.
Donaldson, A.D., M.K. Raghuraman, K.L. Friedman, F.R. Cross, B.J. Brewer, and W.L. Fangman. 1998b. CLB5-dependent activation of late replication origins in S. cerevisiae. Mol. Cell 2: 173–182.
Donovan, S., J. Harwood, L.S. Drury, and J.F. Diffley. 1997. Cdc6p-dependent loading of Mcm proteins onto pre-replicative chromatin in budding yeast. Proc. Natl. Acad. Sci. 94: 5611–5616.
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.
Duncker, B.P., P. Pasero, D. Braguglia, P. Heun, M. Weinreich, and S.M. Gasser. 1999. Cyclin B-cdk1 kinase stimulates ORC- and Cdc6-independent steps of semiconservative plasmid replication in yeast nuclear extracts. Mol. Cell. Biol. 19: 1226–1241.
Dutta, A. and S.P. Bell. 1997. Initiation of DNA replication in eukaryotic cells. Annu. Rev. Cell Dev. Biol. 13: 293–332.
Fox, C.A., S. Loo, A. Dillin, and J. Rine. 1995. The origin recognition complex has essential functions in transcriptional silencing and chromosomal replication. Genes & Dev. 9: 911–924.
Gotta, M., T. Laroche, A. Formenton, L. Maillet, H. Scherthan, and S.M. Gasser. 1996. The clustering of telomeres and co-localization with Rap1, Sir3, and Sir4 proteins in wild-type Saccharomyces cerevisiae. J. Cell Biol. 134: 1349–1363.
Hardy, C.F. 1996. Characterization of an essential Orc2p-associated factor that plays a role in DNA replication. Mol. Cell. Biol. 16: 1832–1841.
Hardy, C.F. and A. Pautz. 1996. A novel role for Cdc5p in DNA replication. Mol. Cell. Biol. 16: 6775–6782.
Hardy, C.F., O. Dryga, S. Seeamet, P.M. Pahl, and R.A. Sclafani. 1997. mcm5/cdc46-bob1 bypasses the requirement for the S phase activator Cdc7p. Proc. Natl. Acad. Sci. 94: 3151–3155.
Hartwell, L.H. 1976. Sequential function of gene products relative to DNA synthesis in the yeast cell cycle. J. Mol. Biol. 104: 803–817.
Hartwell, L.H., J. Culotti, J.R. Pringle, and B.J. Reid. 1974. Genetic control of the cell division cycle in yeast. Science 183: 46–51.
Hennessy, K.M., A. Lee, E. Chen, and D. Botstein. 1991. A group of interacting yeast DNA replication genes. Genes & Dev. 5: 958–969.
Hereford, L.M. and L.H. Hartwell. 1974. Sequential gene function in the initiation of Saccharomyces cerevisiae DNA synthesis. J. Mol. Biol. 84: 445–461.
Hollingsworth, R.E. Jr. and R.A. Sclafani. 1990. DNA metabolism gene CDC7 from yeast encodes a serine (threonine) pro-
tein kinase. Proc. Natl. Acad. Sci. 87: 6272–6276.

Hua, X.H. and J. Newport. 1998. Identification of a preinitiation step in DNA replication that is independent of Origin Recognition Complex and Cdc6, but dependent on Cdk2. J. Cell Biol. 140: 271–281.

Jackson, A.L., P.M. Pahl, K. Harrison, J. Rosamond, and R.A. Sclafani. 1993. Cell cycle regulation of the yeast Cdc6 protein kinase by association with the Dbf4 protein. Mol. Cell. Biol. 13: 2899–2908.

Jallepalli, P.V. and T.J. Kelly. 1997. Cyclin-dependent kinase and initiation at eukaryotic origins: A replication switch? Curr. Opin. Cell Biol. 9: 358–363.

Jiang, W. and T. Hunter. 1997. Identification and characterization of a human protein kinase related to budding yeast Cdc7p. Proc. Natl. Acad. Sci. 94: 14320–14325.

Karwan, R.M., T. Laroche, U. Wintersberger, S.M. Gasser, and M. Binder. 1990. Ribonuclease H(70) is a component of the yeast nuclear scaffold. J. Cell Sci. 96: 451–459.

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 cell cycle transition. Genetics 131: 21–29.

Krude, T., M. Jackman, J. Pines, and R.A. Laskey. 1997. Cyclin/Cdk-dependent initiation of DNA replication in a human cell-free system. Cell 88: 109–119.

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.

Liang, C. and B. Stillman. 1997. Persistent initiation of DNA replication and chromatin-bound MCM proteins during the cell cycle in cdc6 mutants. Genes & Dev. 11: 3375–3386.

Liang, C., M. Weinreich, and B. Stillman. 1995. ORC and Cdc6p interact and determine the frequency of initiation of DNA replication in the cell. Cell 81: 667–676.

Loo, S., C.A. Fox, J. Rine, R. Kobayashi, B. Stillman, and S. Bell. 1995. The origin recognition complex in silencing, cell cycle progression, and DNA replication. Mol. Biol. Cell. 6: 741–756.

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.

Moreno, S., J. Hayles, and P. Nurse. 1989. Regulation of p34cdc2 protein kinase during mitosis. Cell 58: 361–372.

Newlon, C.S. 1997. Putting it all together: Building a repli- cative complex. Cell 91: 711–720.

Pasero, P. and S.M. Gasser. 1998. New systems for replicating DNA in vitro. Curr. Opin. Cell Biol. 10: 304–310.

Pasero, P., D. Braguglia, and S.M. Gasser. 1997. ORC-dependent and origin-specific initiation of DNA replication at defined foci in isolated yeast nuclei. Genes & Dev. 11: 1504–1518.

Pasero, P., B.P. Duncker, and S.M. Gasser. 1999. In vitro DNA replication in yeast nuclear extracts. Methods, Companion Methods Enzymol. [In press].

Piatti, S., C. Lengauer, and K. Nasmyth. 1995. Cdc6 is an unstable protein whose de novo synthesis in G1 is important for the onset of S phase and for preventing a ‘reductional’ anaphase in the budding yeast Saccharomyces cerevisiae. EMBO J. 14: 3788–3799.

Piatti, S., T. Bohn, 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.

Raghuaman, M.K., B.J. Brewer, and W.L. Fangman. 1997. Cell cycle-dependent establishment of a late replication program. Science 276: 806–809.

Rao, P.N. and R.T. Johnson. 1970. Mammalian cell fusion: Studies on the regulation of DNA synthesis and mitosis. Nature 225: 159–164.

Rose, M.D., F. Winston, and P. Hieter. 1990. Methods in yeast genetics Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Rout, M.P. and G. Blobel. 1993. Isolation of the yeast nuclear pore complex. J. Cell Biol. 123: 771–783.

Santocanale, C. and J.F. Diffley. 1996. ORC- and Cdc6-dependent complexes at active and inactive chromosomal replication origins in S. cerevisiae. EMBO J. 15: 6671–6691.

———. 1998. Mccl- and Rad53-dependent checkpoint controls late-firing origins of DNA replication. Nature 395: 615–618.

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: 4430–4431.

Schwob, E. and K. Nasmyth. 1993. CLB5 and CLB6, a new pair of B cyclins involved in DNA replication in Saccharomyces cerevisiae. Genetics & Dev. 7: 1160–1175.

———. 1996. Cell cycle control of DNA replication in Saccharomyces cerevisiae. In Eukaryotic DNA replication [ed. J.J. Blow], pp. 165–197. IRL Press Oxford, UK.

Schwob, E., T. Bohn, 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.

Sclafani, R.A. and A.L. Jackson. 1994. Cdc7 protein kinase for DNA metabolism comes of age. Mol. Microbiol. 11: 805–810.

Sclafani, R.A., M. Patterson, J. Rosamond, and W.L. Fangman. 1988. Differential regulation of the yeast CDC7 gene during mitosis and meiosis. Mol. Cell. Biol. 8: 293–300.

Shen, S.H., P. Chretien, L. Bastien, and S.N. Silliaty. 1991. Primary sequence of the glucanase gene from Oerskovia xanthinolytica. Expression and purification of the enzyme from Escherichia coli. J. Biol. Chem. 266: 1058–1063.

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.

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.

Verma, R., R.M. Feldman, and R.J. Deshaies. 1997. SIC1 is ubiquitinilated in vitro by a pathway that requires CDC4, CDC34, and cyclin/CDK activities. Mol. Biol. Cell 8: 1427–1437.

Walter, J., L. Sun, and J. Newport. 1998. Regulated chromosomal DNA replication in the absence of a nucleus. Mol. Cell 1: 519–529.

Weinert, T., G.L. Kiser, and L.H. Hartwell. 1994. Mitotic checkpoint genes in budding yeast and the dependence of mitosis on DNA replication and repair. Genes & Dev. 8: 652–665.

Yan, H., S. Gibson, and B.K. Tye. 1991. Mcm2 and Mcm3, two proteins important for ARS activity, are related in structure and function. Genes & Dev. 5: 944–957.

Yoon, H.J. and J.L. Campbell. 1991. The CDC7 protein of S. cerevisiae is a phosphoprotein that contains protein kinase activity. Proc. Natl. Acad. Sci. 88: 3574–3578.

Yoon, H.J., S. Loo, and J.L. Campbell. 1993. Regulation of S. cerevisiae CDC7 function during the cell cycle. Mol. Biol. Cell. 4: 195–208.

Zou, L. and B. Stillman. 1998. Formation of a preinitiation complex by S-phase cyclin CDK-dependent loading of Cdc45p onto chromatin. Science 280: 593–596.