Evidence for a Cdc6p-independent mitotic resetting event involving DNA polymerase α

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Eukaryotic DNA replication is limited to once per cell cycle because cyclin-dependent kinases (cdks), which are required to fire origins, also prevent re-replication. Components of the replication apparatus, therefore, are ‘reset’ by cdk inactivation at the end of mitosis. In budding yeast, assembly of Cdc6p-dependent pre-replicative complexes (pre-RCs) at origins can only occur during G1 because it is blocked by cdk1 (Cdc28) together with B cyclins (Clbs). Here we describe a second, separate process which is also blocked by Cdc28/Clb kinase and, therefore, can only occur during G1; the recruitment of DNA polymerase α-primase (pol α) to chromatin. The recruitment of pol α to chromatin during G1 is independent of pre-RC formation since it cannot occur in the absence of Cdc6 protein. Paradoxically, overproduction of Cdc6p can drive both dephosphorylation and chromatin association of pol α. Overproduction of a mutant in which the N-terminus of Cdc6 has been deleted is unable to drive pol α chromatin binding. Since this mutant is still competent for pre-RC formation and DNA replication, we suggest that Cdc6p overproduction resets pol α chromatin binding by a mechanism which is independent of that used in pre-RC assembly.

Keywords: cell cycle/DNA polymerase α-primase/DNA replication/Saccharomyces cerevisiae

Introduction

Cell fusion experiments have shown that passage through mitosis is required to reset eukaryotic nuclei for the subsequent S phase (Rao and Johnson, 1970). Experiments in yeasts and other organisms have shown that resetting is driven by inactivation of the mitotic cyclin-dependent kinase (cdk) (Broek et al., 1991; Hayles et al., 1994; Moreno and Nurse, 1994; Dahmann et al., 1995; Itzhaki et al., 1997). In budding yeast, Cdc6p-dependent pre-replicative complexes (pre-RCs) assemble at origins at the end of mitosis, approximately when Cdc28/B cyclin (Clb) becomes inactivated (Diffley et al., 1994; Cocker et al., 1996; Liang and Stillman, 1997). Inactivation of Cdc28/Clb kinase is sufficient to drive pre-RC assembly (Dahmann et al., 1995), and pre-RCs cannot form after Cdc28/Clb kinase has been activated (Piatti et al., 1996). Pre-RCs contain a number of factors which are essential for initiating DNA replication, including the origin recognition complex (ORC), Cdc6p, Cdc45p and the MCM2–7 family of proteins (Diffley, 1996; Muzi-Falconi et al., 1996b; Nasmyth, 1996; Stillman, 1996; Wuarin and Nurse, 1996; Newlon, 1997). Therefore, the assembly of pre-RCs appears to be an essential step in mitotic resetting. However, at present it is not known if pre-RC assembly is the only target of cdk inhibition or whether other replication factors are also ‘reset’ by cdk inactivation, independently of pre-RCs.

Like components of the pre-RC, DNA polymerase α-primase (pol α) also plays an essential role in the initiation of DNA replication, synthesizing the RNA primers required to begin leading and lagging strand DNA synthesis (Foiani et al., 1997). Pol α is composed of four subunits. The DNA polymerase catalytic centre is located within the 180 kDa subunit (p180) (Plevani et al., 1985) while the DNA primase catalytic centre is located within the 48 kDa subunit (p48) (Santocanale et al., 1993). Although little is known about the activities of the two remaining subunits (p86 and p58), one (p86; also known as the B subunit) undergoes cell cycle-regulated phosphorylation to yield a form which migrates more slowly in SDS–PAGE (p91) (Foiani et al., 1995). This phosphorylated form first appears at the G1–S boundary and persists until the end of mitosis. Thus, it has been suggested that this subunit might play some regulatory role. This is supported by the results of reciprocal shift experiments which have shown that p91/p86 is required for a very early step in DNA replication (Foiani et al., 1994). Studies using cell-free systems which replicate viral DNA templates have shown that several subunits of the pα complex, including homologues of p180, p91/p86 and p48, specifically interact with viral initiator proteins (Domreiter et al., 1992, 1993; Collins et al., 1993; Fanning, 1994).

Here we show that, like components of pre-RCs such as the Mcm proteins, pol α undergoes a cell cycle-regulated association with chromatin. Like pre-RCs, pol α chromatin binding requires inactivation of Cdc28/Clb kinase. However, unlike pre-RCs, pol α chromatin association does not require Cdc6p. Thus, it appears to be distinct from pre-RC assembly as it is now known.

Results

Cell cycle-regulated association of DNA polymerase α-primase with chromatin

We have been interested in determining when and how pol α is first recruited to chromatin in the budding yeast.
Saccharomyces cerevisiae. To accomplish this, we have exploited an assay we previously developed to demonstrate Cdc6p-dependent loading of Mcm proteins onto chromatin in G1 (Donovan et al., 1997). In this assay, a whole-cell extract is fractionated into a chromatin fraction and a supernatant by centrifugation. This results in ~20-fold purification of chromatin. The presence of specific proteins in each fraction can then be determined by immunoblotting. To detect pol α, we used monoclonal antibodies to the p180 and B subunit (p91/p86) and, in some experiments, the p48 subunit was tagged with three epitopes from the influenza haemagglutinin gene (HA3).

Figure 1A shows that, consistent with earlier reports (Foiani et al., 1995), the levels of pol α subunits in whole-cell extracts do not change appreciably during the cell cycle, being similar in cells blocked in G2/M with nocodazole (Noc), in G1 with α-factor (α) or in S phase with hydroxyurea (HU). Chromatin binding experiments were performed as described in Materials and methods. The proteins present in the different fractions of the chromatin purification were examined by immunoblotting of SDS-PAGE: (WCE) whole-cell extract, (Sup) supernatant, (Ch) chromatin fraction. (B) Logarithmically growing cultures of cdc4 and cdc28 mutant strains were shifted to the restrictive temperature (37°C). Three hours after temperature shift, cells were collected and chromatin binding experiments were performed. (C) α-factor-arrested cells (PY26) were lysed as above. The extract was divided into two tubes and NaCl was added to final concentration of 250 mM to one sample (+H11001) before chromatin separation. (D) Extract prepared from α-factor-blocked cells was incubated on ice either without (–) or with (+) DNase I as described in Materials and methods before chromatin isolation through a sucrose cushion. Membranes were probed with anti-p180, anti-p86 (B subunit), 12CA5 (anti-HA for detecting tagged p48) and anti-Orc2p antibodies as indicated.

**Fig. 1.** Cell cycle-regulated chromatin binding of DNA polymerase α-primase. (A) CY835 (PRI1-3HA) cells were blocked either in G2/M with nocodazole (Noc), in G1 with α-factor (α) or in S phase with hydroxyurea (HU). Chromatin binding experiments were performed as described in Materials and methods. The proteins present in the different fractions of the chromatin purification were examined by immunoblotting of SDS-PAGE: (WCE) whole-cell extract, (Sup) supernatant, (Ch) chromatin fraction. (B) Logarithmically growing cultures of cdc4 and cdc28 mutant strains were shifted to the restrictive temperature (37°C). Three hours after temperature shift, cells were collected and chromatin binding experiments were performed. (C) α-factor-arrested cells (PY26) were lysed as above. The extract was divided into two tubes and NaCl was added to final concentration of 250 mM to one sample (+) before chromatin separation. (D) Extract prepared from α-factor-blocked cells was incubated on ice either without (–) or with (+) DNase I as described in Materials and methods before chromatin isolation through a sucrose cushion. Membranes were probed with anti-p180, anti-p86 (B subunit), 12CA5 (anti-HA for detecting tagged p48) and anti-Orc2p antibodies as indicated.

\textit{Inhibition of Cdc28/Clb kinase drives association of DNA polymerase α-primase with chromatin}

The experiments shown in Figure 1A and B show that association of pol α with chromatin correlates with the cell cycle-regulated dephosphorylation of the B subunit (p91/p86). That is, only the hypophosphorylated form, p86, was found associated with chromatin. This point was examined further in Figure 2. First, we were interested in determining whether the correlation between pol α hypophosphorylation and chromatin association could be
sufficiently high levels of Sic1p (Dahmann et al., 1995) of the GAL1,10 promoter were required to generate G2/M. Because Sic1p is highly unstable during G2/M, five integrated copies of the wild-type (Raf) or 90 min after galactose induction. as above and chromatin binding assays were performed just before anti-p86 and anti-9E10 antibodies. (C) The same strains were treated as above and chromatin binding assays were performed just before (Raf) or 90 min after galactose induction.

seen during an unperturbed cell cycle. Figure 2A shows that, in an asynchronous population, both the hypo- and hyperphosphorylated forms of the pol α B subunit are present at significant levels. In this experiment, as in the previous experiments, however, only the hypophosphorylated form of pol α was found associated with chromatin. Therefore, the inability of the hyperphosphorylated form of pol α to associate with chromatin is not simply a consequence of cell cycle arrest.

It has been shown previously that the hyperphosphorylation of pol α begins at the onset of S phase and the hyperphosphorylated form of pol α remains present until the end of mitosis (Foiani et al., 1995). Since this timing is consistent with the possibility that Cdc28 might be involved, we sought to determine whether Cdc28, together with the Clbs, might act as inhibitors of pol α chromatin association. Previous experiments have shown that over-expression of the Cdc28/Clb inhibitor, Sic1, can drive inappropriate pre-RC assembly in cells blocked in G2/M (Dahmann et al., 1995). We have used a similar approach to test whether inactivation of Cdc28/Clb kinase could also drive association of pol α with chromatin in G2/M. Because Sic1p is highly unstable during G2/M, five integrated copies of the wild-type SIC1 gene under control of the GAL1,10 promoter were required to generate sufficiently high levels of Sic1p (Dahmann et al., 1995).

We have found that Sic1p is stabilized significantly by deleting its N-terminal 50 amino acids (L.S.Drury and J.F.X.Diffley, data not shown). Thus, a strain containing a single copy of this stable mutant under the control of the GAL1,10 promoter is sufficient to drive pre-RC assembly and re-replication during G2 (E.A.Noton and J.F.X.Diffley, unpublished data). We have used this strain for the experiments in Figure 2B and C. In the experiment shown in Figure 2B, cells grown in raffinose-containing medium were first blocked in G2/M in medium containing nocodazole and raffinose and then transferred to fresh medium containing nocodazole and galactose. Figure 2B (upper panel) shows that, in a wild-type strain, pol α remained in its hyperphosphorylated form in nocodazole-blocked cells after transfer to galactose-containing medium. However, in a strain expressing the stable form of Sic1p from the GAL1,10 promoter (Figure 2B, lower panel), p91 was quantitatively converted to the hypophosphorylated, faster migrating p86 form after transfer to galactose-containing medium (Figure 2B, upper panel). Figure 2C shows that neither p180 nor the hyperphosphorylated p91 is bound to chromatin during G2/M in wild-type cells in either raffinose- or galactose-containing medium (Figure 2C, lanes 1–6). As in G1-blocked cells, a significant fraction of both p180 and the hypophosphorylated p86 form of the B subunit were found associated with chromatin after Sic1p induction (Figure 2C, lanes 10–12). Therefore, the overexpression of Sic1 in cells blocked in G2/M drives dephosphorylation of the B subunit and the association of pol α with chromatin.

**DNA primase subunits are required for DNA polymerase α-prime chromatin association**

Several subunits of pol α previously have been shown to interact with viral initiator proteins (Dormreiter et al., 1992, 1993; Collins et al., 1993). We have, therefore, begun to examine the role of individual subunits in pol α chromatin binding. Figure 3 shows that mutants in either of the DNA primase subunits (pri1 and pri2 encode p48 and p58, respectively) are defective in recruitment of p180 and B subunit to chromatin. In this experiment, cells were first blocked in G2/M (Noc), raised to the restrictive temperature for the primase mutants (37°C) and then
released into G₁ (α factor) at the restrictive temperature. Under these conditions, both p180 and B subunit become associated with chromatin in wild-type cells while chromatin association of these proteins is lost in pri2 or pri1 mutants at the restrictive temperature. Thus, the primase subunits appear to play a key role in the cell cycle-regulated association of pol α with chromatin. We have attempted similar experiments with several pol12 mutants (the POL12 gene encodes the B subunit). To date, however, we have been unable to demonstrate any role for B subunit in chromatin association (C.Desdouets, M.Ferrari, C.Santocanale and J.F.X.Diffley, data not shown).

As shown in Figure 3, lanes 10–12 and 16–18, neither the pri1 nor pri2 mutations affect the dephosphorylation of p91. However, although p91 becomes dephosphorylated during G₁, neither p180 nor p86 associates with chromatin. Therefore, the dephosphorylation of p91 is not sufficient for its association with chromatin. These results suggest that the association of pol α with chromatin is a specific event which occurs, at least in part, through one or both of the primase subunits and is not simply an electrostatic interaction due to the phosphorylation state of the B subunit.

**DNA polymerase α-primase chromatin association does not require Cdc6p**

The timing of pol α association with chromatin is very similar to that of the Mcm proteins (Aparicio et al., 1997; Donovan et al., 1997; Liang and Stillman, 1997; Tanaka et al., 1997). We were interested, therefore, in determining whether pol α is a component of pre-RCs. Pre-RC formation, as defined by either (i) genomic footprinting (Cocker et al., 1996; Santocanale and Diffley, 1996), (ii) loading of ~50% of the total Mcm protein onto chromatin (Donovan et al., 1997) or (iii) association of Mcm proteins with origins using chromatin immunoprecipitation (Aparicio et al., 1997; Tanaka et al., 1997), is absolutely dependent upon de novo synthesis of Cdc6p. In Figure 4, we have asked whether pol α association with chromatin is also dependent on Cdc6p. To accomplish this, we used a strain in which the sole copy of the CDC6 gene was grown in YP-galactose and arrested in G2/M with nocodazole. When cells were blocked, the culture was divided into two and incubated in either YP-galactose or YP-glucose; in both cases, cells were released from the G2 block into G₁ (α-factor). Cdc6p, Mcm7p (Cdc47p) and pol α (p180 and B subunit) chromatin binding was examined in the G2/M block (Noc) and in G₁ with (α-Gal) or without (α-Glu) Cdc6p expression. (B) Cells from (A) were released from the α factor block. Samples for FACS analysis were collected during the nocodazole and α factor blocks, and at different times (minutes) after the release from the α factor block.

![Fig. 4. Pol α binding to chromatin does not require Cdc6p.](image)

(A) (YCD2) cells carrying a Gal–CDC6 fusion as the only source of Cdc6p protein were grown in YP-galactose and arrested in G2/M with nocodazole. When cells were blocked, the culture was divided into two and incubated in either YP-galactose or YP-glucose; in both cases, cells were released from the G2 block into G₁ (α-factor). Cdc6p, Mcm7p (Cdc47p) and pol α (p180 and B subunit) chromatin binding was examined in the G2/M block (Noc) and in G₁ with (α-Gal) or without (α-Glu) Cdc6p expression. (B) Cells from (A) were released from the α factor block. Samples for FACS analysis were collected during the nocodazole and α factor blocks, and at different times (minutes) after the release from the α factor block.

**Cdc6p overproduction can drive chromatin association of DNA polymerase α-primase**

Together, the experiments described above indicate that, in budding yeast, there are at least two separate aspects of the replication machinery which require Cdc28/Cln inactivation at the end of mitosis for resetting: Cdc6p-dependent pre-RC assembly and Cdc6p-independent pol α chromatin binding. At face value, this is difficult to reconcile with experiments showing that overexpression of the Cdc6p homologue (cdc18) in fission yeast is sufficient to induce re-replication (Nishitani and Nurse, 1995; Muzi-Falconi et al., 1996a). We therefore decided to test whether overproduction of Cdc6p could promote chromatin binding of pol α even though this process is normally independent of Cdc6p. Cdc6p cannot accumulate...
DNA polymerase α in Cdc6p-independent mitotic resetting

Fig. 5. Overexpression of full-length Cdc6p causes dephosphorylation and chromatin association of pol α. (A) MTY668 (cdc4-1), YLD16 (cdc4-1 Gal-Cdc6) and YLD20 (cdc4-1 Gal-Cdc6ΔNT) cells were grown in YP-raffinose and arrested in G2 with nocodazole at the permissive temperature. Cells were then transferred to YP-galactose containing nocodazole at 37°C. Aliquots of the cultures were withdrawn at the indicated times and protein extracts analysed by immunoblot using anti-p86 and anti-Cdc6p antibodies. (B) The same strains were treated as above and chromatin binding assays were performed just before (Raf) or 90 min after galactose induction (Gal 37°C).

to high levels in G2/M because it is degraded rapidly by the Cdc4/34/53 pathway (Drury et al., 1997). We therefore examined the effect of overproducing Cdc6p in cells blocked in G2/M with nocodazole after inactivation of this degradation pathway using a cdc4 temperature-sensitive mutant. Figure 5A (lanes 6–10) shows that overproduction of Cdc6p under these conditions drives the dephosphorylation of B subunit. Figure 5B (lanes 10–12) shows that ~50% of this dephosphorylated B subunit becomes associated with chromatin. This level is similar to that seen during G1 (Figure 1).

The N-terminus of Cdc6p is required to target it for degradation via interactions with Cdc4p (Piatti et al., 1995; Drury et al., 1997). This region has also been shown to interact with and inhibit Cdc28/Clb kinase (Elsasser et al., 1996). We therefore examined whether removal of this domain, which is not required for DNA replication (Drury et al., 1997), affected its ability to promote pol α chromatin association when overexpressed. Figure 5A (lanes 11–15) and B (lanes 16–18) shows that overproduction of the truncated Cdc6 causes neither dephosphorylation nor chromatin association of p91/p86. Therefore, overexpression of Cdc6p causes dephosphorylation and chromatin association of pol α by a mechanism which is not required for normal Cdc6p function.

Discussion

There is now considerable experimental support for the idea that the assembly of Cdc6p-dependent pre-RCs at replication origins is crucial in limiting the initiation of DNA replication to once per cell cycle at each origin. Most notably, pre-RCs are unable to form in the presence of Cdc28/Clb kinase. This provides an explanation for

why the inactivation of Cdc28/Clb kinase at the end of mitosis plays a key role in resetting the replication apparatus. While this is, theoretically, sufficient to explain how re-replication is blocked in each cell cycle, there is little evidence that this is the only mechanism blocking re-replication. Here we have provided evidence for a separate pathway that is also involved in resetting the replication apparatus in mitosis: the recruitment of pol α to chromatin.

After origins are unwound, short RNA oligonucleotides must be synthesized to act as primers for the DNA polymerases which initiate both leading and lagging strand DNA synthesis (Sugino, 1995). Therefore, the recruitment of DNA primase to pre-initiation complexes is likely to be an early event in DNA replication. In eukaryotes, DNA primase is tightly associated with one of the three replicative DNA polymerases, DNA polymerase α. This suggests that, after the synthesis of the RNA primer, initial elongation is likely to be carried out by the DNA polymerase α component of the complex. After this, the bulk of DNA synthesis, particularly on the leading strand, is likely to be carried out by one or both of the other replicative DNA polymerases, DNA polymerase δ and ε. However, pol α is still required during elongation to synthesize the RNA primers and at least part of the DNA in each Okazaki fragment on the lagging strand.

The experiments presented here show that pol α undergoes periodic association with chromatin only during G1 and (early) S phase (HU) of the cell cycle. This interaction is mediated, at least in part, by the primase subunits of the complex. At present, we do not know whether pol α interacts with chromatin only near replication origins or throughout the genome. If the former is true, it is possible that chromatin association is involved
in the initiation reaction. However, if the latter is true, it is possible that chromatin association may play a role in general lagging strand synthesis.

Our evidence suggests that this association with chromatin is regulated, either directly or indirectly, by Cdc28/Clb kinase. The most important observation we have made in this regard is that inactivation of Cdc28/Clb kinase during G2/M by Sic1 overproduction promotes efficient association of pol α with chromatin. This provides strong evidence that Cdc28/Clb kinase acts, either directly or indirectly, as an inhibitor of pol α chromatin association. Since the association of pol α with chromatin appears to occur before Start (α factor and cdc28 mutant) but does not require Cdc6p, it defines a second component or pathway in DNA replication which is reset in mitosis by inactivation of Cdc28/Clb kinase.

Although there is a very strong correlation between the phosphorylation state of the B subunit and its chromatin association, our experiments with primase mutants have shown that dephosphorylation of the B subunit is not sufficient to promote chromatin association. Aside from the correlation with phosphorylation state, we have no direct evidence indicating that the B subunit plays a role in pol α chromatin association. In fact, preliminary experiments suggest that p48 can still associate with chromatin in pol12 (B subunit) mutants (C.Desdouets, M.Ferrari, C.Santocanale and J.F.X.Diffley, unpublished results). Since there is no evidence that either of the primase subunits are phosphoproteins in vivo, it is possible that Cdc28/Clb kinase blocks association of pol α with chromatin, not by phosphorylating pol α itself, but by phosphorylating some other protein, such as the putative target for interaction in chromatin. Further experiments are required to identify this target.

Somewhat surprisingly, pol α is still in the hypophosphorylated form and is bound to chromatin in cells blocked early in S phase with HU. This result contrasts with the previously published result showing that the B subunit is phosphorylated in HU-blocked cells (Foiani et al., 1995). It is likely that the critical difference between our experiments is that we have used a higher concentration of HU (0.2 versus 0.08 M). In a separate series of experiments, we have found that concentrations of HU up to 0.2 M do not arrest fork progression but, instead, slow fork progression in a dose-dependent manner (C.Santocanale and J.F.X.Diffley, unpublished data). Thus, it is likely that our conditions simply cause an earlier S-phase block. Still, this result is somewhat surprising since Cdc28 kinase activity has been reported to remain high in HU-blocked cells (Stueland et al., 1993). We are currently examining the possibility that the maintenance of pol α chromatin binding is dependent upon the S phase checkpoint genes. While Cdc28/Clb kinase may be unable to cause phosphorylation of pol α in HU-blocked cells, it still can act to block pre-RC formation since overproduction of Sic1 in HU-blocked cells drives the re-assembly of pre-RCs at early firing origins (E.A.Noton and J.F.X.Diffley, unpublished data).

Finally, we have shown that, although pol α chromatin binding does not require Cdc6p, the overproduction of Cdc6p can drive inappropriate dephosphorylation and chromatin association of pol α. Previous experiments have suggested that Cdc6p is composed of at least two distinct domains. The N-terminal 47 amino acids of Cdc6 interact with both Cdc4p and with Cdc28/Clb kinase and are required for the Cdc4-dependent proteolysis of Cdc6p at the end of G1 (Elsasser et al., 1996; Drury et al., 1997). However, this domain is not required for the essential function of Cdc6p in DNA replication. Cells containing only the N-terminally truncated Cdc6p still form pre-RCs only during G1 and do not show any evidence of re-replication. This truncated molecule can perform all of the essential functions of Cdc6p since it can complement a cdc6 null mutant (Drury et al., 1997). The physiological significance of this N-terminal domain remains unclear; however, in this study we show that it is essential to drive pol α chromatin binding during G2/M when Cdc6p is overexpressed.

The mechanism by which overproduction of Cdc6p drives pol α chromatin binding is not certain. The fact that the B subunit becomes dephosphorylated together with the fact that Cdc28/Clb kinase blocks pol α chromatin binding suggests that overproduction of Cdc6p leads to a decrease in Cdc28/Clb kinase activity in vivo. Because the N-terminus of Cdc6p interacts with both Cdc4p and Cdc28p (Elsasser et al., 1996; Drury et al., 1997), Cdc28/Clb inhibition may occur via direct interaction with Cdc6. Alternatively, it may be due to inhibition of the Cdc4/34/53 pathway which stabilizes a cdk inhibitor such as Sic1 which, in turn, inhibits Cdc28/Clb kinase. Regardless, this may help to reconcile experiments in budding and fission yeasts. If such a pathway also exists in fission yeast, it is possible that overexpression of cdc18 not only overcomes direct inhibition of pre-RC formation by cdc2 (Jallepalli et al., 1997; Lopez-Girona et al., 1998), but also, by either directly or indirectly inhibiting cdc2 kinase, aids in the rebinding of pol α to chromatin. This is supported by the fact that cdc18 overproduction leads to a detectable decrease in the levels of cdc2-associated kinase activity (Nishitani and Nurse, 1995), although it is also important to consider that overproduction of cdc18 or Cdc6p might inhibit cdc2/Cdc28p in vivo but might not interact tightly enough to survive the immunoprecipitation conditions required to assay for kinase activity.

It is likely that there are other targets involved in resetting the replication apparatus. For example, the 34 kDa subunit of the single-stranded DNA-binding protein RPA also undergoes cell cycle-regulated phosphorylation (Din et al., 1990; Dutta and Stillman, 1992). Preliminary experiments suggest that chromatin association of RPA is not regulated (C.Desdouets and J.F.X.Diffley, data not shown); however, phosphorylation may affect some other aspect of RPA function. The localization of RPA by immunofluorescence to pre-replication foci in Xenopus nuclei has been shown previously to be cell cycle regulated (Adachi and Laemmli, 1992). Moreover, the formation of these pre-replication foci is inhibited by mitotic cdk's (Adachi and Laemmli, 1994). The localization of RPA to pre-replication foci occurs in the absence of Xenopus Cdc6 protein (Coleman et al., 1996), suggesting that this, like pol α chromatin binding, is a separate process which is inhibited by mitotic cdk activity. Thus, because of its importance, the block to re-replication may occur through many targets of Cdc28/Clb kinase and it will not be until we identify all of these targets that we will truly understand how DNA replication is limited to once per cell cycle.
Materials and methods

Strains and media
The different strains used (Table I) were grown in YP (1% yeast extract, Difco; 2% bacto-peptone, Difco) containing either glucose (YPD), raffinose (YP-Raf) or galactose (YP-Gal) at 2%. Cell cycle blocks with nocodazole, α factor and HU were as previously described (Diffley et al., 1996; Donovan et al., 1997).

Chromatin binding assay
The procedure for chromatin purification is based on the method described by Donovan et al. (1997) with the following modification: after spheroplasting, the regrowth step was omitted and, after washing with lysis buffer, cells were lysed immediately by addition of Triton X-100 as previously described. For DNase I treatment, cell extracts were incubated for 15 min on ice with 230 U of DNase I (Sigma, D7291). Lysates subsequently were separated into a supernatant and pellet fraction as previously described (Donovan et al., 1997) except that samples were overlaid onto a 30% sucrose cushion (0.1 ml) prior to centrifugation.

Immunoblotting
For the immunoblot analysis described herein, proteins were transferred to Hybond ECL membrane and blocked with 5% dry milk in Tris-buffered saline containing 0.1% Tween-20. Detection of pol α was performed using monoclonal antibodies: 24D9 (anti-p180, dilution 1:10 000 of ascites fluid) (Brooke and Dumas, 1991), 6D2 (anti-p86, dilution 1:10 000 of ascites fluid) (Brooke and Dumas, 1991) and 24D9 (anti-p180, dilution 1:10 000 of ascites fluid) (Foiani et al., 1997). mAb 9H85 (anti-Cdc6p) was used at 5 μg/ml. JAB12 polyclonal antibody was used with JAB12 (Sigma), and immunoreactive bands were visualized with enhanced chemiluminescence (ECL, Amersham) according to the manufacturer’s instructions.

FACS analysis
Samples for flow cytometric analysis were collected and processed as described (Santocanale et al., 1995) and analysed using a Becton Dickinson FACSScan.

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