

Assembly of a Complex Containing Cdc45p, Replication Protein A, and Mcm2p at Replication Origins Controlled by S-Phase Cyclin-Dependent Kinases and Cdc7p-Dbf4p Kinase

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In *Saccharomyces cerevisiae*, replication origins are activated with characteristic timing during S phase. S-phase cyclin-dependent kinases (S-CDKs) and Cdc7p-Dbf4p kinase are required for origin activation throughout S phase. The activation of S-CDKs leads to association of Cdc45p with chromatin, raising the possibility that Cdc45p defines the assembly of a new complex at each origin. Here we show that both Cdc45p and replication protein A (RPA) bind to Mcm2p at the G₁-S transition in an S-CDK-dependent manner. During S phase, Cdc45p associates with different replication origins at specific times. The origin associations of Cdc45p and RPA are mutually dependent, and both S-CDKs and Cdc7p-Dbf4p are required for efficient binding of Cdc45p to origins. These findings suggest that S-CDKs and Cdc7p-Dbf4p promote loading of Cdc45p and RPA onto a preformed prereplication complex at each origin with preprogrammed timing. The *ARS1* association of Mcm2p, but not that of the origin recognition complex, is diminished by disruption of the B2 element of *ARS1*, a potential origin DNA-unwinding element. Cdc45p is required for recruiting DNA polymerase α onto chromatin, and it associates with Mcm2p, RPA, and DNA polymerase ϵ only during S phase. These results suggest that the complex containing Cdc45p, RPA, and MCMs is involved in origin unwinding and assembly of replication forks at each origin.

In eukaryotic cells, DNA replication initiates from multiple origins scattered along chromosomes, making duplication of large genomes within a single cell cycle possible. Every replication origin fires once and only once with a characteristic timing during the S phase. Although the cell-cycle control of DNA replication has been gradually unveiled, how DNA replication is initiated at chromosomal origins remains largely unknown (reviewed in references 16, 21, 46, and 57).

Studies of *Saccharomyces cerevisiae* and other organisms have revealed that a prereplication complex (pre-RC) is assembled at each origin during late mitosis or early G₁ phase, the period when cyclin-dependent kinases (CDKs) are inactive (11, 12, 15, 40, 51, 58, 59). The origin recognition complex (ORC), Cdc6p, and the six minichromosome maintenance (MCM) proteins Mcm2p to -7p are present in the pre-RC (2, 19, 40, 55, 59, 63). To start DNA synthesis, S-phase CDKs (S-CDKs) and Cdc7p-Dbf4p kinase need to be activated at the G₁-S transition. Upon activation of these kinases, the pre-RC undergoes a transformation, and it is eventually converted to the post-RC and replication forks when initiation of DNA replication is completed (16, 21, 46, 57). To understand how DNA replication is initiated at chromosomal origins, we sought to uncover how the complexes at origins change during initiation and how they interact with origins. Furthermore, we pursued the mechanisms by which the activating kinases are involved in this transition.

In the well-characterized simian virus 40 (SV40) system, large T antigen is the origin recognition protein and also the helicase. Initiation of DNA replication from the SV40 origin

begins with local unwinding of the origin by cooperative action of large T antigen and replication protein A (RPA; reviewed in references 29 and 62). Subsequently, DNA polymerase α -primase is recruited to the unwound origin to synthesize the first Okazaki fragment, and then the DNA polymerase δ complex is loaded by a polymerase switching mechanism (62). Unlike the SV40 large T antigen, yeast ORC is not a helicase. Instead, as indicated by amino acid sequence composition and a study of three of the six human MCM proteins (32, 38), yeast MCMs might function as a helicase. The MCM proteins, but not RPA, are present at origins during G₁, and no origin firing can be detected until S-CDKs and Cdc7p-Dbf4p are activated at the G₁-S transition. Three recent studies have suggested that Cdc45p and RPA might mediate the activation of origins by the kinases (45, 60, 67). Cdc45p, a protein essential for initiation of DNA replication (26, 30, 49, 66), binds to MCMs and chromatin upon the activation of S-CDKs (45, 67). Association of RPA with origins depends on both S-CDKs and Dbf4p-dependent kinase (60). Moreover, Cdc45p is required for recruiting DNA polymerase α (Pol α) onto chromatin in *Xenopus* (45), and RPA is needed to recruit primase to origins in yeast (60).

The architecture of the complex at origins remains largely unknown. With the exception of the ORC, little is known about how the proteins at origins interact with origin DNA. A single leading-strand start site was recently identified at the chromosomal autonomously replicating sequence 1, (*ARS1*), a well-characterized yeast replication origin (6). This site is located between the binding site of the ORC (the A and B1 elements of *ARS1* [5, 52, 54]) and a potential DNA-unwinding element (the B2 element of *ARS1* [41]). It is likely that this site coincides with the site of initial unwinding at *ARS1* and/or the entry site for the replication machinery. However, the proteins associating with this region of *ARS1* have not been identified.

In this study, we show that Cdc45p, RPA, and Mcm2p start

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TABLE 1. *S. cerevisiae* strains used in this study

Strain	Genotype	Source
W303-1a	<i>MATa ade2-1 ura3-1 his3-1 trp1-1 leu2-3,112 can1-100</i>	R. Rothstein
YB0298	W303-1a; <i>cdc45-1</i>	This laboratory
YB0556	W303-1a; <i>cdc7-1</i>	This work
YB0557	W303-1a; <i>cdc7-4</i>	This work
YB0349	W303-1a; <i>dbf4-1</i>	This laboratory
YB0469	W303-1a; <i>cdc45::CDC45-HA3-LEU2</i>	This laboratory
YB0476	<i>MATα mcm2-1 cdc45::CDC45-HA3-LEU2</i>	This laboratory
YB0549	<i>MATa rfa2-2 cdc45::CDC45-HA3-LEU2</i>	This work
YB0472	W303-1a; <i>cdc7-1 cdc45::CDC45-HA3-LEU2</i>	This laboratory
YB0547	W303-1a; <i>cdc7-4 cdc45::CDC45-HA3-LEU2</i>	This work
YB0548	W303-1a; <i>dbf4-1 cdc45::CDC45-HA3-LEU2</i>	This work
YB0576	<i>MATa ars1T860G cdc45::CDC45-HA3-LEU2</i>	This work
YB0577	<i>MATa ars1::835-842 cdc45::CDC45-HA3-LEU2</i>	This work
YB0578	<i>MATa ars1::798-805 cdc45::CDC45-HA3-LEU2</i>	This work
YB0579	<i>MATa ars1::756,758 cdc45::CDC45-HA3-LEU2</i>	This work
YB0553	<i>MATa ura3::GAL1-SIC1ΔNT-URA3 cdc45::CDC45-HA3-LEU2</i>	This work
YB0477	W303-1a; <i>clb5::URA3 clb6::LEU2 cdc45::CDC45-HA3-LEU2</i>	This laboratory
OAY618	W303-1a; <i>pole::POLE-HA3-LEU2</i>	S. Bell
YB0550	W303-1a; <i>cdc45::CDC45-MYC3-LEU2 pole::POLE-HA3-LEU2</i>	This work
YB0551	<i>MATa mcm2-1 cdc45::CDC45-MYC3-LEU2 pole::POLE-HA3-LEU2</i>	This work
K6388	W303-1a; <i>leu2::DBF4-MYC18-LEU2</i>	K. Nasmyth
YB0552	W3031a; <i>cdc45-1 leu2::DBF4-MYC18-LEU2</i>	This work

to associate with each other at the onset of S phase. Cdc45p associates with different replication origins at specific times during S phase, and the origin associations of Cdc45p and RPA are mutually dependent. Both S-CDKs and Cdc7p-Dbf4p are required for efficient binding of Cdc45p to replication origins. Together, these findings suggest that S-CDKs and Cdc7p-Dbf4p promote formation of a complex containing Cdc45p, RPA, and the MCM proteins at each origin with preprogrammed timing. Association of Mcm2p with *ARS1* requires the potential unwinding element B2. Cdc45p is required for loading Pol α onto chromatin, and it associates with DNA polymerase ϵ (Pole) during S phase. We propose, therefore, that Cdc45p, RPA, and the MCM proteins collaborate to unwind origins and recruit other proteins to assemble replication elongation complexes.

MATERIALS AND METHODS

Yeast strains and methods. All the yeast strains used in this study are listed in Table 1. The Cdc45HA3p and Cdc45myc3p in the corresponding strains were expressed from their endogenous promoters at the original chromosomal location and can support normal cell growth. The integrated *DBF4MYC18* in YB0552 was derived from the strain K6388 provided by K. Nasmyth (60). The *GAL1-SIC1ΔNT* in YB0553 was derived from YCS12 provided by J. Diffley (14). The integrated *POLEHA3* in YB0550 and YB0551 was derived from OAY618 provided by S. Bell (2). The integrated *ARS1* mutations were derived from the corresponding strains published by Marahrens and Stillman (44). In all the experiments involving temperature-sensitive mutant strains, α -factor arrest was performed at 25°C for 3 h. In the experiments using only non-temperature-sensitive strains (including the cold-sensitive *cdc45-1* strain), cells were arrested in α -factor at 30°C for 3 h. Expression of Sic1 Δ NTp was either induced in yeast extract-peptone medium with 2% galactose or remained uninduced in 2% raffinose.

Chromatin immunoprecipitations. Chromatin immunoprecipitations were conducted essentially as previously described (2). Cdc45HA3p, Mcm2p, Orc2p, Orc3p, and RPA p70 were immunoprecipitated by the monoclonal antibodies 12CA5, Mcm2-49, SB3, SB46, and a polyclonal rabbit anti-serum against RPA p70, respectively. All the PCR products were between 200 and 350 bp in size, and were separated on 2.3% agarose gels. The sequences of PCR primers used in this study are available upon request.

Chromatin binding analysis. Preparation and fractionation of cell lysates were performed as described previously (40) with several modifications. The incubation in prespheroplasting buffer was done on ice. Spheroplasts were washed three times with lysis buffer and lysed in the presence of 1% Triton X-100. The quality of fractionation was monitored by the separation of chromatin-bound Orc3p from the soluble protein cross-reacting with the SB3 antibody (anti-Orc3p [40]).

Immunoprecipitation and immunoblotting. Immunoprecipitations were carried out essentially as described previously (30) except that 150 Kunitz units of DNase I (Sigma) was added to the whole-cell lysates. Cdc45myc3p, Mcm2p, and RPA p70 were immunoprecipitated with monoclonal antibodies 9E10, Mcm2-49, and rabbit anti-serum against RPA p70, respectively. The antibodies used for immunoblotting were monoclonal 12CA5 (for PoleHA3p or Cdc45HA3p), 9E10 (for Cdc45myc3p or Dbf4myc3p), 6D2 (for Pol α p86), Mcm2-28, SB3 (for Orc3p), and polyclonal antisera against RPA p70 and p34.

RESULTS

Cdc45p associates with Mcm2p, RPA, and Pole in a cell cycle-regulated manner. The binding of Cdc45p to chromatin and the association of Cdc45p with the MCM proteins indicate the transformation from the pre-RC to a preinitiation complex (pre-IC [67]). To test whether Cdc45p and MCMs were part of a larger complex, we immunoprecipitated Cdc45p and Mcm2p, respectively, and analyzed the proteins coprecipitated with them (Fig. 1). Cells were treated with hydroxyurea (HU) to slow down fork movement and prolong the period in which the potential complex might be present. Because the potential complex was likely chromatin bound, DNase I was used to release the possible interacting proteins from chromatin. Immunoprecipitation of either myc-tagged Cdc45p or intact Mcm2p specifically brought down the hemagglutinin (HA)-tagged Pole catalytic subunit and intact RPA p70, the largest subunit of RPA (Fig. 1A and C and data not shown). Similarly, precipitation of RPA p70 brought down Cdc45p, Mcm2p, and Pole (Fig. 1B). Furthermore, Cdc45p was detected in the Pole immunoprecipitate (data not shown). These interactions were also observed in the absence of HU (Fig. 2 and data not shown). It is unlikely that these interactions were mediated by chromatin because several other chromatin-bound proteins were not detected in the immunoprecipitates (data not shown). The network of interactions among Cdc45p, Mcm2p, RPA, and Pole suggest that these proteins are present in a complex or multiple complexes in vivo. It should be noted that only small fractions of Pole and RPA coprecipitated with Cdc45p in these experiments. This could be due to the transient and/or unstable nature of the complex or the inefficiency of the co-immunoprecipitation procedure. When Mcm2p was precipitated from an extract derived from *mcm2-1* cells, hardly any

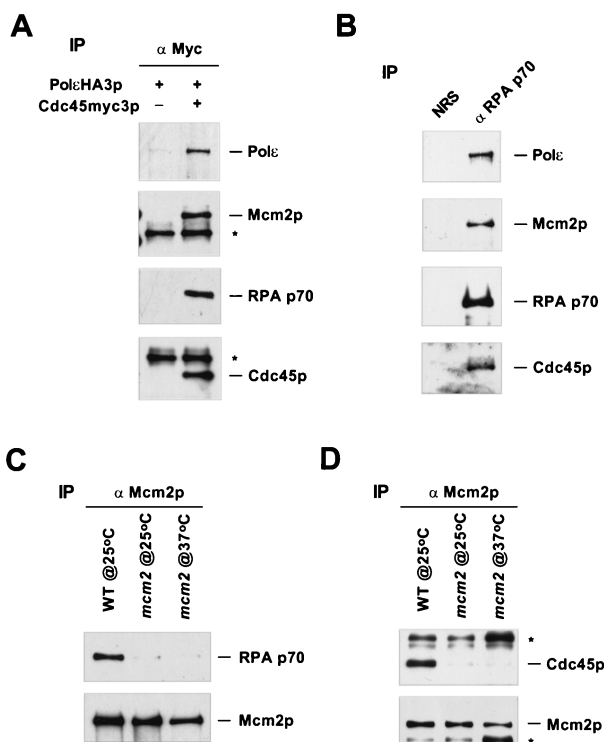


FIG. 1. Cdc45p associates with Mcm2p, Pole, and RPA p70 in vivo. (A) Mcm2p, PoleHA3p, and RPA p70 coprecipitate specifically with Cdc45myc3p. Cells expressing (+) both PoleHA3p and Cdc45myc3p (YB0550) or only PoleHA3p (OAY618) were arrested in S phase with 0.1 M HU for 2 h. Whole-cell extracts were prepared and subjected to immunoprecipitations (IP) with non-cross-linked anti-myc antibody. *, dimers of immunoglobulin G. (B) Mcm2p, PoleHA3p and Cdc45myc3p coprecipitate specifically with RPA p70. Whole-cell extract was prepared from S-phase wild-type (YB0550) cells. Immunoprecipitations were performed with either normal rabbit serum (NRS) or anti-RPA p70 antibody. (C) RPA p70 coprecipitates with Mcm2p only in wild-type but not in *mcm2-1* cell extracts. Wild-type (YB0550) (WT) and *mcm2-1* (YB0551) cells were arrested with HU for 2 h at the indicated temperatures. Immunoprecipitations were carried out with anti-Mcm2p antibody cross-linked to protein G. (D) Cdc45HA3p coprecipitates with Mcm2p only in wild-type but not in *mcm2-1* cell extracts. Wild-type (YB0469) and *mcm2-1* (YB0476) cells expressing Cdc45HA3p were arrested as in panel C, and immunoprecipitations were performed with non-cross-linked anti-Mcm2p antibody. Shown are immunoblots of the proteins present in the immunoprecipitation described above. The immunoblotting antibodies were against the proteins indicated at the right of each panel.

RPA p70, Cdc45p, or Pole were brought down even at the permissive temperature (Fig. 1C and D and data not shown). Thus, the anti-Mcm2p antibody did not recognize RPA p70, Cdc45p, and Pole directly, and more importantly, functional Mcm2p was critical for the stability of the complex. Although an interaction between Cdc45p and Pol α was reported in *Xenopus* (45), we were unable to detect Pol α in the Cdc45p immunoprecipitate, perhaps because the interaction was very transient.

To determine how the interactions among Cdc45p, Mcm2p, RPA, and Pole were regulated during the cell cycle, we monitored these interactions across a complete cell cycle. Cells expressing both Cdc45myc3p and PoleHA3p were synchronously released from an α -factor block, and Cdc45myc3p was immunoprecipitated at 10- or 15-min intervals (Fig. 2A and B). All the proteins examined in this experiment, including Cdc45p, Mcm2p, Pole, and both the p70 and p34 subunits of RPA, were present at roughly constant levels throughout the cell cycle (data not shown). Cdc45myc3p was precipitated throughout the cell cycle, with a modest increase in S phase.

Mcm2p and both the p70 and p34 subunits of RPA coprecipitated with Cdc45p during early S phase, whereas Pole was brought down throughout S phase. Importantly, both the Cdc45p-Mcm2p and the Cdc45p-RPA interactions were established 20 to 30 min after release, concomitant with the onset of S phase. A slower-migrating form of RPA p34 was observed when cells reached early S phase, indicating the cell cycle-regulated phosphorylation of this protein. Nevertheless, both forms of RPA p34 were brought down by Cdc45p, suggesting that the phosphorylation of RPA p34 may not affect its association with Cdc45p. Mcm2p, Pole, and RPA p70 and p34 reappeared in the Cdc45p immunoprecipitate 120 min after release. It is likely that some cells had started the second S phase by that time.

Association of Mcm2p with Cdc45p and RPA requires active S-CDKs. Association of Cdc45p with chromatin requires activation of S-CDKs, indicating that S-CDKs regulate a key step of the assembly of the pre-IC (67). To test directly whether S-CDK activity was required for establishing the complex containing Cdc45p, RPA, and Mcm2p, we examined the effect of overexpression of Sic1p, an S-phase CDK inhibitor, on the interactions among these three proteins. Sic1 Δ NTP, a mutant of Sic1p that lacks the N-terminal 50 amino acids, is more stable than the wild-type Sic1p and is functional in inhibiting S-phase CDKs (14). Cells overexpressing Sic1 Δ NTP from the *GALI,10* promoter were synchronized in G₁ with α -factor and were released into either raffinose- or galactose-containing medium (Fig. 3). In raffinose-containing medium, Cdc45p and both p70 and p34 of RPA coprecipitated with Mcm2p as cells entering S phase (Fig. 3). In contrast, cells were stalled at the G₁-S boundary in galactose, and neither Cdc45p nor RPA coprecipitated with Mcm2p. Thus, Cdc45p and RPA could not interact with Mcm2p when S-CDKs were inactivated by overexpression of Sic1 Δ NTP.

Cdc45p and Mcm2p associate differently with replication origins. To analyze the distribution of the pre-IC on chromatin, we carried out a chromatin immunoprecipitation (CHIP) assay on Cdc45p. In this assay, proteins were cross-linked in vivo to chromatin with formaldehyde, the chromatin was then sheared, and the bound Cdc45p was immunoprecipitated. To detect DNA sequences that were associated with Cdc45p, specific primer pairs were used to analyze coprecipitated DNA by PCR.

A recent study using the CHIP assay suggested that Cdc45p was a component of the pre-RC in G₁ (2). However, we were unable to detect association of Cdc45p with chromatin or with MCMs at Start in G₁ (67). To determine whether Cdc45p was indeed a component of the pre-RC, we first compared the origin association of Cdc45p with that of Mcm2p, a known pre-RC component, by using CHIP assays. CHIP analyses of Cdc45HA3p and Mcm2p were performed in parallel with cells collected either at the α -factor block in G₁ or at the beginning of S phase (30 min after release from the α -factor block). Coprecipitated DNA fragments were amplified for *ARS1*, *ARS501*, *ARS301*, and a nonorigin control region (Fig. 4A). Both *ARS1* and *ARS501* are active origins, but *ARS1* fires early whereas *ARS501* fires late in S phase (24). *ARS301* only fires at low frequency (<15%) at its chromosomal location (20). No clear enrichment of Cdc45p was detected at these three origins in G₁ (Fig. 4A). In early S phase, Cdc45p was enriched at *ARS1* but not *ARS301* or *ARS501*. On the other hand, Mcm2p was found to associate with *ARS1*, *ARS301*, and *ARS501* in both G₁ and early S phase (Fig. 4A). In addition, Mcm2p associated with all three origins equally well. Thus, the association of Mcm2p with origins was temporally different from that of Cdc45p. Unlike Cdc45p, Mcm2p binding did not distinguish

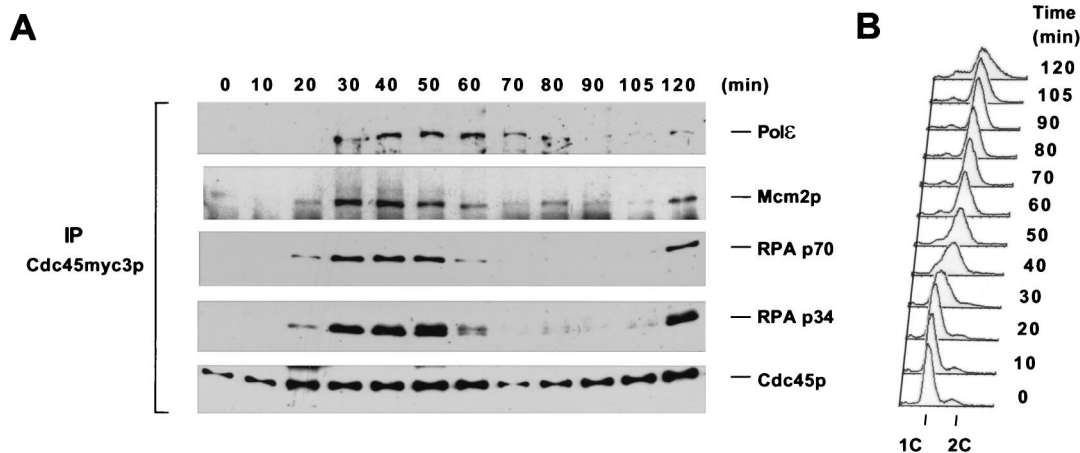


FIG. 2. Cdc45p interacts with Mcm2p, Pole, RPA p70, and p34 in a cell cycle-dependent manner. (A) Immunoprecipitation (IP) of Cdc45myc3p across the cell cycle. Wild-type cells expressing both PoleHA3p and Cdc45myc3p (YB0550) were synchronized in G₁ with α -factor and then released into yeast-peptone-dextrose medium at 25°C. The cells were collected at the indicated time points. Whole-cell extracts were prepared and subjected to immunoprecipitations with anti-myc antibody. (B) DNA content of the time point samples used in panel A.

early and late origins, nor did it differentiate between active and inactive origins. Nevertheless, Cdc45p and Mcm2p coexisted on *ARS1* in early S phase, suggesting that they might be present in a complex at this origin during initiation.

Cdc45p associates with different replication origins in a temporally controlled manner. To investigate the cell-cycle

regulation of the association of Cdc45p with different origins, we carried out CHIP assays on Cdc45HA3p across the cell cycle. Wild-type cells were synchronized in G₁ with α -factor and then released into the cell cycle (Fig. 4C). The DNA fragments coprecipitated with Cdc45p were analyzed by PCRs using four pairs of primers that amplified either *ARS1* or one

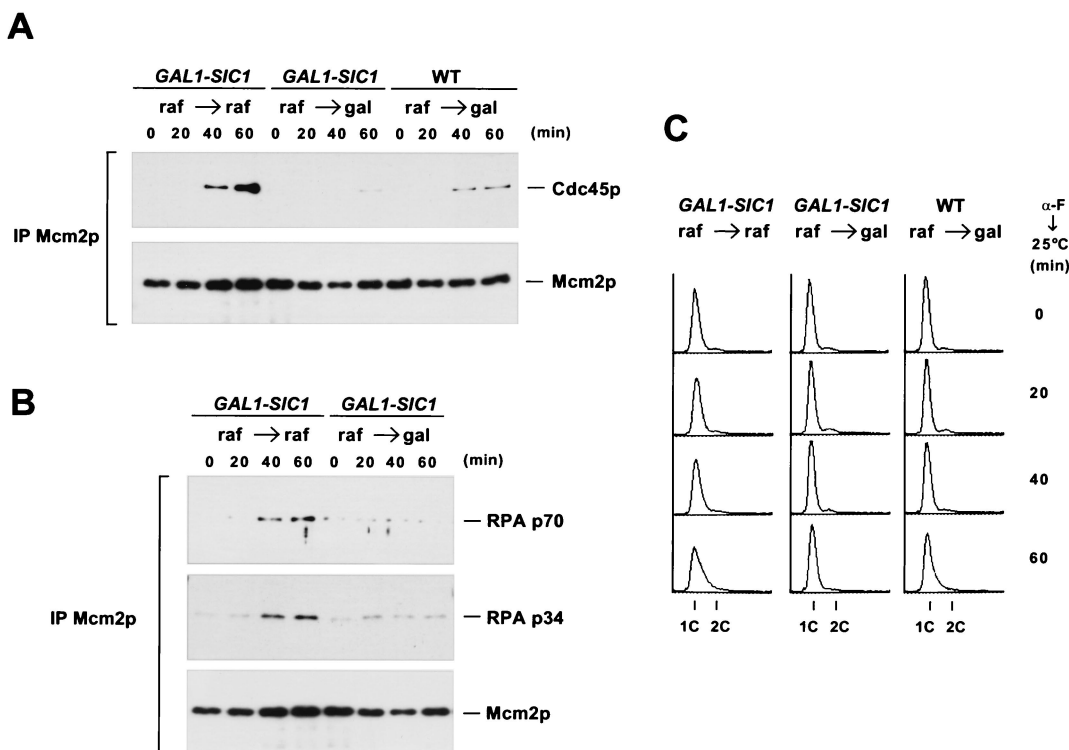


FIG. 3. S-CDK activity is required for the interactions among Cdc45p, Mcm2p, and RPA. (A) Overexpression of Sic1 Δ NTp prevents binding of Cdc45p to Mcm2p. Cells bearing *GAL1-SIC1 Δ NT* (YB0553) were arrested with α -factor in raffinose (raf)-containing medium and then released into medium containing either raffinose or galactose (gal) at 25°C. As a control, wild-type (WT) cells (YB0469) that do not carry *GAL1-SIC1 Δ NT* were also synchronized with α -factor in raffinose and released into galactose. Whole-cell extracts were prepared at the indicated time points and were immunoprecipitated with anti-Mcm2p antibody. Cdc45HA3p coprecipitated with Mcm2p was analyzed by immunoblotting. (B) Overexpression of Sic1 Δ NTp prevents binding of RPA p70 and p34 to Mcm2p. RPA p70 and p34 coprecipitated with Mcm2p in panel A were analyzed by immunoblotting. (C) DNA content of the samples used in panels A and B.

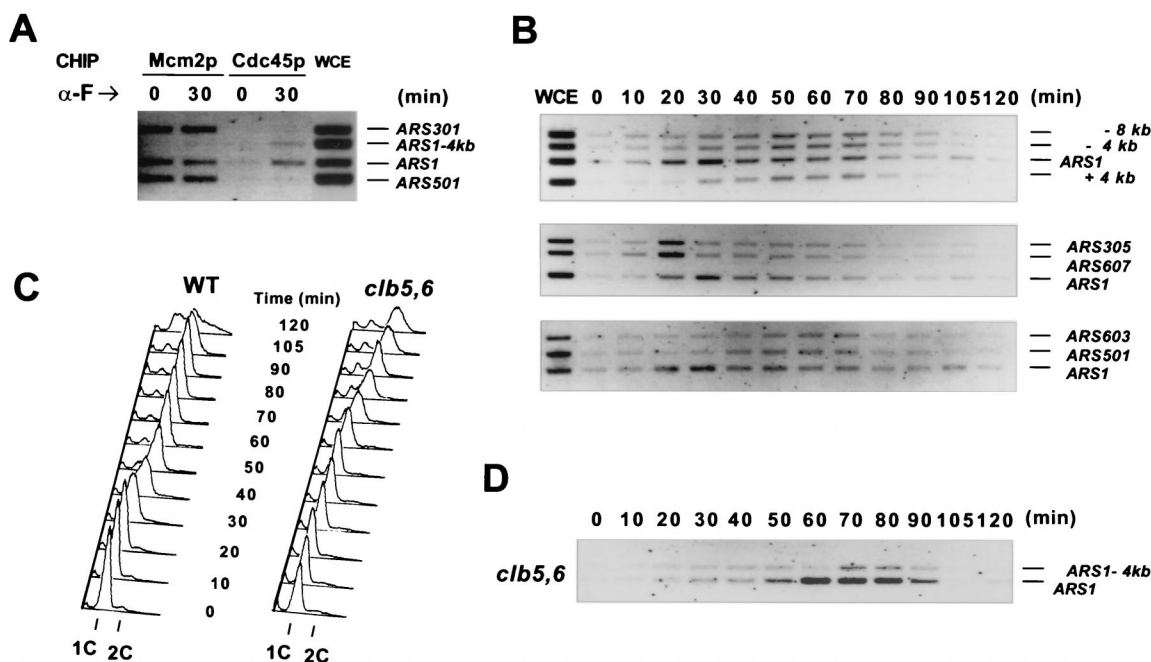


FIG. 4. Cell cycle regulation of association of Cdc45p with ARS-containing fragments. (A) Cdc45p and Mcm2p associate with origins differently in G_1 and early S phase. Wild-type (WT) cells expressing Cdc45HA3p (YB0469) were arrested in G_1 with α -factor and then released into yeast-peptone-dextrose medium at 25°C. The cells were collected at the α -factor block or 30 min after release. CHIP analyses of Mcm2p and Cdc45p were performed in parallel. WCE, input DNA prepared from the whole-cell extract; *ARS1-4kb*, a region 4 kb away from *ARS1* towards the left telomere. (B) Association of Cdc45p with different origins during the cell cycle. Wild-type cells were synchronized in G_1 with α -factor and then released into YPD at 25°C. CHIP analysis of Cdc45p was performed at each time point. +4 kb and -8 kb, regions on both sides of *ARS1* that are 4 or 8 kb away. (C) DNA content of the time point samples used in panels B and D. (D) Association of Cdc45p with *ARS1* is delayed in the absence of *CLB5* and *CLB6*. YB0477 (*clb5,6* Δ and *CDC45HA3*) cells were released from an α -factor block at 25°C. CHIP analysis of Cdc45p was performed as described above.

of the three flanking regions (Fig. 4B). In the α -factor-arrested cells, none of the four chromosomal loci was significantly enriched in the Cdc45p immunoprecipitate. Twenty minutes after release, Cdc45p began to associate with *ARS1* but not the surrounding regions. The specific enrichment of Cdc45p at *ARS1* was most evident 30 min after release, when the cells were clearly in early S phase. As the cells proceeded through S phase, the enrichment of Cdc45p at *ARS1* declined. Simultaneously, Cdc45p became associated with the regions flanking *ARS1*. When S phase was complete in most cells, Cdc45p could no longer be detected at *ARS1* and its flanking regions. Similar observations were also made in the cells synchronously released from a *dbf2* block in mitosis (data not shown). These results show that Cdc45p specifically associates with *ARS1* in early S phase rather than in G_1 as suggested by the earlier study (2). However, our data agree with the observation that at least some Cdc45p may move away from origins after initiation (2).

Different replication origins fire at characteristic times during S phase (23). We therefore examined the association of Cdc45p with origins that were known to have different timing of initiation in each S phase. The DNA fragments coprecipitated with Cdc45p in the cell cycle CHIP experiment were amplified for *ARS1*, *ARS305*, and *ARS607* (Fig. 4B). *ARS305* and *ARS607* are known to fire very early in S phase, even before initiation occurs at *ARS1* (25, 53, 65). Unlike the earlier study (3), we did not observe clear enrichment of Cdc45p at the two early-firing origins in cells arrested with α -factor. Only 10 min after release, weak association of Cdc45p with *ARS305* and *ARS607* was detected. By 20 min, Cdc45p was clearly bound to the two early origins. Cdc45p bound to these two early origins approximately 5 to 10 min before it bound to

ARS1, which correlated well with the timing of initiation at these origins (25, 53, 65). Similar analyses were also performed with two late origins, *ARS501* and *ARS603* (Fig. 4B). Cdc45p began to associate with these late origins 40 min after release, more than 10 min after it bound to *ARS1*. This is also in agreement with the timing of initiation at these loci (24, 25, 65). Persistent association of Cdc45p with *ARS1* and late origins were observed even in late S phase. This is possibly due to the rapid loss of cell cycle synchrony after cells have entered S phase. Together, these results show that Cdc45p binds to early-firing origins early and late-firing origins late in S phase, suggesting that binding of Cdc45p to each origin is concomitant with initiation at that origin.

Association of Cdc45p with *ARS1* requires S-CDK activity. Binding of Cdc45p to chromatin is delayed in *clb5,6* Δ mutant cells due to the lack of S-phase cyclins (67). To test whether the specific association of Cdc45p with *ARS1* was also postponed in the absence of *CLB5* and *CLB6*, we performed CHIP analysis on Cdc45p in the *clb5,6* Δ cells synchronously released from an α -factor block in G_1 (Fig. 4D). As indicated by the fluorescence-activated cell-sorting analyses, *clb5,6* Δ mutant cells entered S phase approximately 50 min after release, which was delayed by about 30 min relative to the wild type (Fig. 4C). Specific Cdc45p-*ARS1* association was first detected 50 min after release, concomitant with the apparent G_1 -S transition (Fig. 4C and D). Compared with that in the wild-type cells, binding of Cdc45p to *ARS1* in the *clb5,6* Δ cells was delayed for approximately 30 min. This is in good agreement with the delay of *ARS1* firing in the same mutant (18). Because only the cell cycle events relying on S-CDKs are delayed in the *clb5,6* Δ mutant, this result shows that the specific association of Cdc45p with *ARS1* requires S-CDK activity.

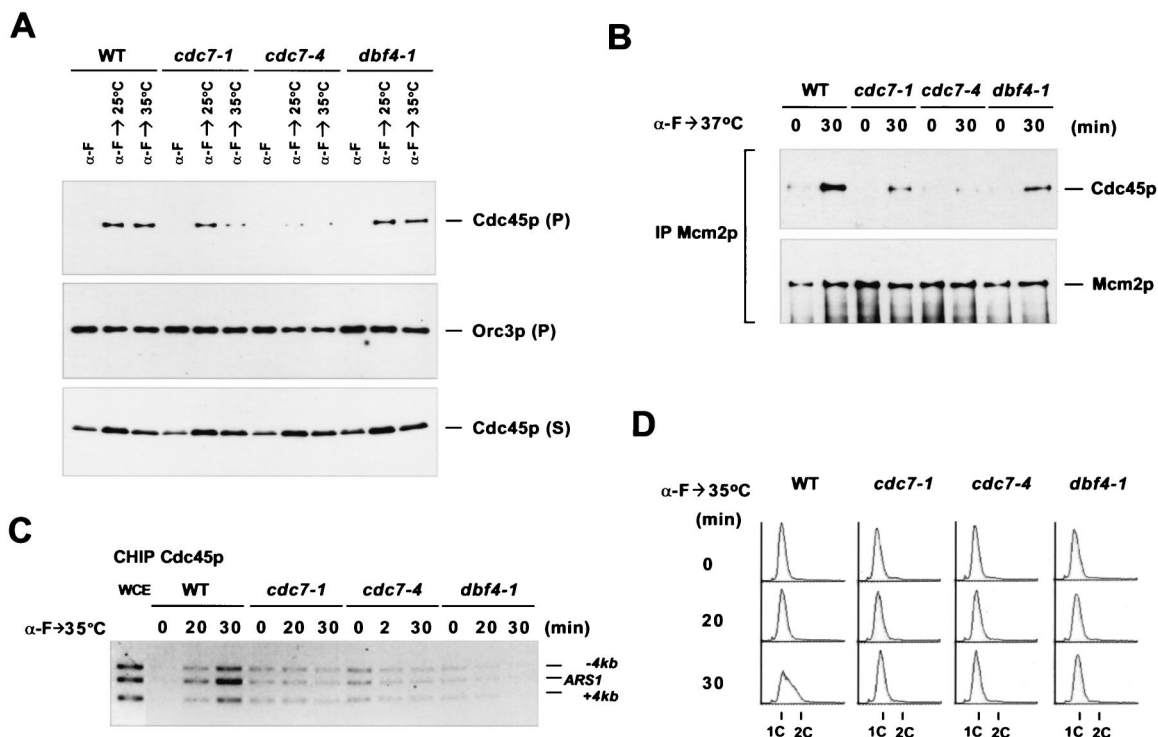


FIG. 5. Associations of Cdc45p with chromatin, Mcm2p, and *ARS1* are affected in *cdc7* and *dbf4* mutants. (A) Association of Cdc45p with chromatin is reduced in *cdc7* and *dbf4* mutants. Wild-type (YB0469) (WT), *cdc7-1* (YB0472), *cdc7-4* (YB0547), and *dbf4-1* (YB0548) cells expressing Cdc45HA3p were synchronized with α -factor and released at either 25 or 35°C. The cells were collected at the α -factor block or 30 min after release. Lysates were prepared and subjected to chromatin fractionation. Immunoblots of Cdc45p and Orc3p in the chromatin sediments (P) and Cdc45p in the supernatants (S) are shown. (B) Cdc45p-Mcm2p interaction is reduced in *cdc7* and *dbf4* mutants at the nonpermissive temperature. Cells were arrested as in panel A and released at 37°C. Whole-cell extracts (WCE) were prepared from the cells collected at the α -factor block or 30 min after release and subjected to immunoprecipitations (IP) using an anti-Mcm2p antibody. (C) Association of Cdc45p with *ARS1* is undetectable in *cdc7-1*, *cdc7-4*, and *dbf4-1* cells. Wild-type and mutant cells were synchronized with α -factor as in panel A and released at 35°C. The cells were collected at the indicated time points and analyzed by Cdc45p CHIP. (D) DNA content of the samples used in panel C.

Association of Cdc45p with chromatin, Mcm2p, and *ARS1* are affected in *cdc7* and *dbf4* mutants. Significant amounts of Cdc45p were detected on chromatin in the *cdc7-1* cells that were arrested by shifting asynchronously growing cultures to 37°C (67). However, two recent studies showed that Cdc7p-Dbf4p was important not only for entry into S phase but also for progression through S phase (7, 17). Therefore, inactivation of Cdc7p-Dbf4p in an asynchronous population might trap early S-phase cells in S phase, and the origins that had fired in these cells could be responsible for the detection of chromatin-bound Cdc45p. To assess this possibility, we examined the chromatin association of Cdc45p in the arrested *cdc7-1*, *cdc7-4*, and *dbf4-1* cells that had been synchronized in G₁ prior to the temperature shift (Fig. 5A). The mutants and the congenic wild-type cells were released from an α -factor block at either 25 or 35°C and were given 30 min to enter S phase or reach the arrest point at the G₁-S boundary (data not shown). At 25°C, *cdc7-4* cells entered S phase more slowly than wild-type, *cdc7-1*, and *dbf4-1* cells. The amount of Cdc45p on chromatin in *cdc7-4* cells was also smaller than that in the other three strains (Fig. 5A and data not shown). At 35°C, all three mutant strains were stalled at the G₁-S boundary 30 min after release from α -factor (data not shown). Chromatin-bound Cdc45p was hardly detectable in *cdc7-4* cells and was significantly reduced in *cdc7-1* cells, but the reduction of chromatin-bound Cdc45p in *dbf4-1* cells was very modest (Fig. 5A).

We also examined the Cdc45p-Mcm2p association in the arrested *cdc7-1*, *cdc7-4*, and *dbf4-1* cells. Wild-type and mutant cells were first synchronized with α -factor and then released at

37°C so that all mutant cells were arrested at the G₁-S boundary (Fig. 5B). Compared to that in the wild-type cells, the Cdc45p-Mcm2p interaction was reduced to various extents in the three mutants (Fig. 5B). *cdc7-4*, which entered S phase more slowly than the other strains even at 25°C, exhibited the most dramatic reduction of Cdc45p-Mcm2p interaction at 37°C. Overall, the reduction of Cdc45p-Mcm2p interaction in the *cdc7* and *dbf4* mutants correlated well with the reduction of chromatin-bound Cdc45p in these mutants (Fig. 5A and B and data not shown). These results suggest that Cdc7p-Dbf4p is important, if not essential, for the association of Cdc45p with Mcm2p and chromatin.

To investigate directly the effect of inactivation of Cdc7p-Dbf4p on origin association of Cdc45p, we performed CHIP assays on Cdc45p in *cdc7-1*, *cdc7-4*, and *dbf4-1* mutant cells. Wild-type and mutant cells were synchronized with α -factor and released at 35°C (Fig. 5C and D). Cdc45p specifically bound to *ARS1* when the wild-type cells reached early S phase. In contrast, no enrichment of Cdc45p could be detected at *ARS1* in all three mutants arrested at the G₁-S boundary (Fig. 5C). Mcm2p, on the other hand, remained bound to *ARS1* in the arrested *cdc7* and *dbf4* cells (data not shown), indicating that *ARS1* did not fire efficiently. Thus, Cdc7p-Dbf4p appeared to be essential for the Cdc45p-*ARS1* interaction.

***ARS1* associations of Cdc45p and RPA are mutually dependent.** Both Cdc45p and RPA require S-CDKs and Cdc7p-Dbf4p kinase to associate with replication origins (Fig. 4 and 5) (60). We therefore investigated whether there was an interdependence between Cdc45p and RPA for their binding to ori-

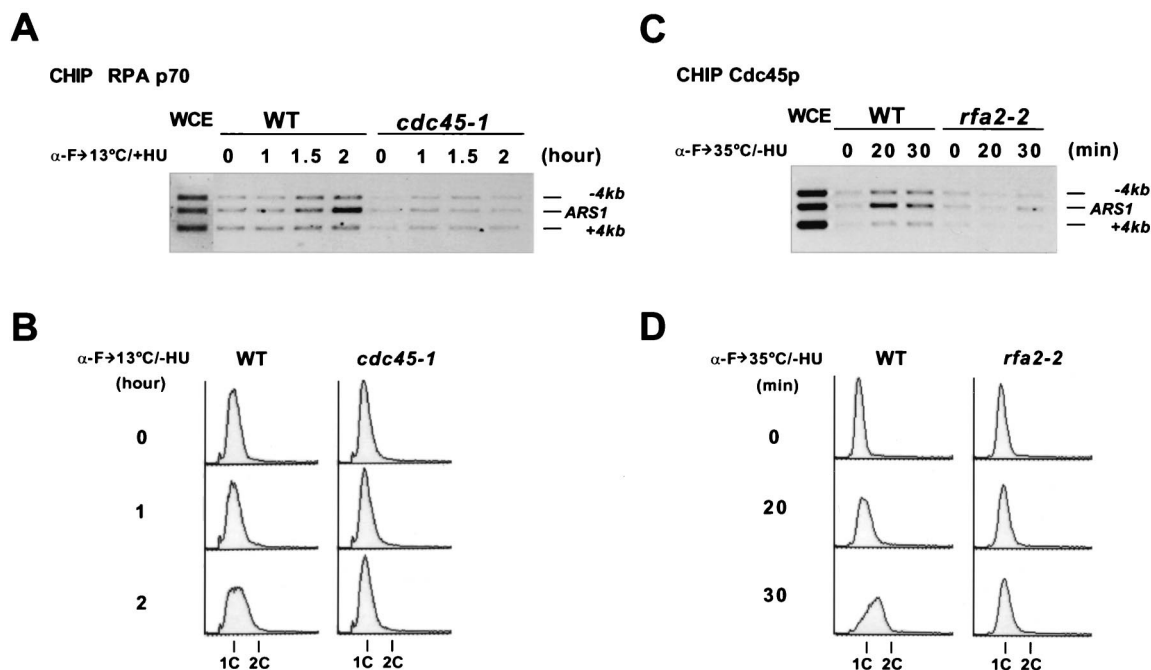


FIG. 6. Origin associations of Cdc45p and RPA are mutually dependent. (A) *ARS1* association of RPA p70 requires Cdc45p. Wild-type (WT) and *cdc45-1* (YB0298) cells were synchronized in G_1 with α -factor and released into medium containing 0.1 M HU at 13°C. CHIP analysis of RPA p70 was performed at the indicated time points. (B) DNA content of the cells blocked and released as in panel A but in the absence of HU. (C) *ARS1* association of Cdc45p requires RPA p34. Wild-type and *rfa2-2* (YB0549) cells were synchronized in G_1 with α -factor and released at 35°C in the absence of HU. CHIP analysis of Cdc45p was performed at the indicated time points. (D) DNA content of the samples used in panel C. WCE, input DNA from whole-cell extract.

gins. A cold-sensitive *cdc45-1* mutant and the congenic wild-type cells were synchronized with α -factor and released at 13°C, at which temperature *cdc45-1* cells were stalled at the G_1 -S boundary (Fig. 6B). To detect origin association of RPA in these cells, we carried out CHIP analysis with an antibody against RPA p70. Within 2 h after release at 13°C, wild-type cells had entered S phase and specific association of RPA p70 with *ARS1* was detected (Fig. 6A). In contrast, RPA p70 did not bind to *ARS1* in the *cdc45-1* cells stalled at the G_1 -S boundary. To determine whether RPA was required for loading Cdc45p onto origins, we synchronized wild-type and *rfa2-2* (a temperature-sensitive mutant of RPA p34) cells with α -factor and then released them at 35°C (Fig. 6D). Cdc45p bound to *ARS1* in the wild-type cells 20 min after release, but it did not do so in the *rfa2-2* cells stalled at the G_1 -S boundary (Fig. 6C). Hence, the *ARS1* association of Cdc45p and RPA were interdependent, indicating that Cdc45p and RPA were recruited to this origin at roughly the same time. Since RPA is required for origin unwinding in the SV40 system, these results raise the possibility that the complex containing Cdc45p, RPA, and the MCM proteins is involved in unwinding the origins.

Cdc45p is required for loading Pol α p86 but not Dbf4p onto chromatin. Consistent with the idea that the pre-IC is involved in origin unwinding, it was shown in *Xenopus* that Cdc45p was required for association of Pol α with chromatin (45). To investigate whether such a mechanism was conserved in budding yeast, we analyzed chromatin association of Pol α in the cold-sensitive *cdc45-1* mutant and wild-type cells released from an α -factor block at 13°C (Fig. 7B). As previously described, cell lysates were fractionated into supernatants and chromatin-enriched sediments (Fig. 7A). In the α -factor-arrested cells, Pol α p86 was virtually undetectable on chromatin. As wild-type cells entered S phase, the level of chromatin-bound Pol α p86 increased. However, such an increase was hardly detectable in

the *cdc45-1* cells stalled at the G_1 -S boundary. Thus, association of Pol α with chromatin requires Cdc45p function in budding yeast.

The activation of Cdc7p kinase, which is essential for S-phase entry, is achieved at least in part by stabilization of Dbf4p and its association with chromatin at the G_1 -S transition (10, 33, 48, 63). Having noticed that Cdc45p and Dbf4p bound to chromatin with similar timing, we investigated whether Cdc45p played a role in loading Dbf4p onto chromatin. After the release from an α -factor block at 13°C, Dbf4p bound to chromatin in both wild-type and *cdc45-1* cells with similar kinetics (Fig. 7A). Therefore, the *cdc45-1* allele is not defective for loading Dbf4p onto chromatin, even though it is incompetent to recruit RPA and Pol α .

The B2 element of *ARS1* is involved in association with Mcm2p. The association of the ORC with *ARS1* requires the A and B1 but not the B2 element of the origin (5, 52, 54). Disruptions of either A or all three B elements abolished association of Mcm7p with *ARS1* (59). However, none of these studies identified the proteins that associate with B2, the potential DNA-unwinding element. If the complex containing Cdc45p, RPA, and Mcm2p were involved in origin unwinding, then one would predict that it should be present in close proximity to B2. To test this possibility, we performed CHIP analyses on Mcm2p with cells in which the chromosomal *ARS1* was mutated at either A or one of the three B elements (44). The association of Mcm2p with *ARS1* was abolished in the A⁻ mutant, just like the association of the ORC (Fig. 8). In the B1⁻ mutant, although no ORC was detected at the mutant origin, a weak Mcm2p-*ARS1* association was observed by CHIP (Fig. 8). Unlike mutations of the essential A element, disruption of the B1 element only weakened but did not abolish the origin activity of *ARS1* (43). It is likely that the ORC could transiently associate with the B1⁻ *ARS1* and recruit

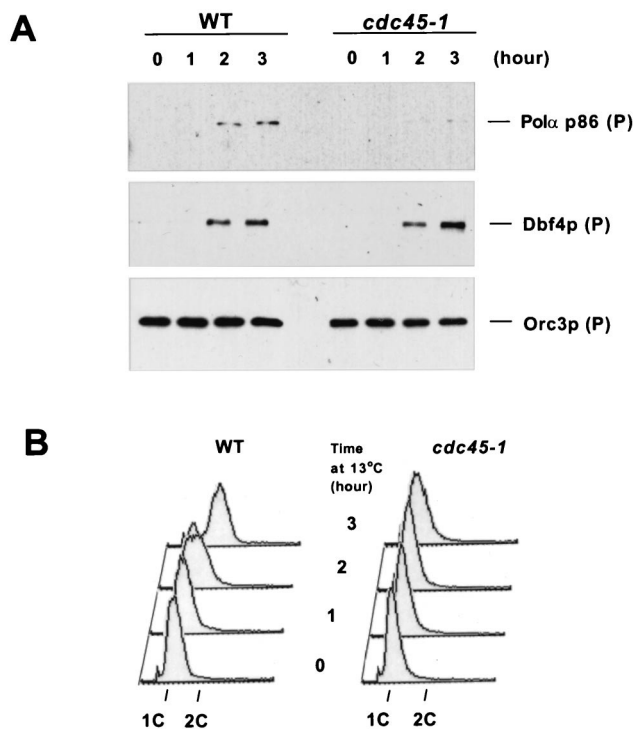


FIG. 7. Cdc45p is required for loading Pol α p86 but not Dbf4p onto chromatin. (A) Wild-type (K6388) (WT) and *cdc45-1* (YB0552) cells were synchronized in G₁ with α -factor and released at 13°C. Cells were collected at the indicated time points and were processed for chromatin fractionation. The chromatin-sediment fractions (P) were analyzed by immunoblotting. (B) DNA content of the samples used in panel A.

some MCM proteins to this mutant origin. Interestingly, disruption of the B2 element weakened the association of Mcm2p with *ARS1* but not the association of the ORC with this mutant origin (Fig. 8). Therefore, although the B2 element of *ARS1* is not required for ORC binding, it is involved in the stable association with Mcm2p and perhaps Cdc45p and RPA.

DISCUSSION

In budding yeast, initiation of DNA replication is accomplished by a number of sequential transitions at origins. The first transition is to establish the pre-RC at replication origins in late mitosis or early G₁, when CDKs are inactive (11, 12, 19, 40, 51, 58, 59). During late G₁ and S phases, a series of less-understood transitions occur at individual origins, and DNA replication is launched from different origins at specific times. Two of the critical events that take place during the transitions at each origin are the unwinding of origin DNA and the assembly of replication elongation complexes. Accompanying these events, the pre-RC at each origin undergoes a transformation and is eventually converted to the post-RC and replication forks. Here we have uncovered a number of events that are part of this transformation, thereby shedding light on the mechanisms of origin unwinding and replication fork assembly at eukaryotic chromosomal origins.

The transformation of the pre-RC was first detected through the disappearance of Cdc6p from chromatin and origins at the G₁-S transition (11, 31, 59, 63). At about the same time, Cdc45p, RPA, and Mcm2p start to associate with each other in vivo (Fig. 1 to 3) (13, 30, 67). CHIP experiments show that both Cdc45p and RPA begin to associate with early-firing origins at

this moment of the cell cycle (Fig. 4 and 5) (60). Mcm2p, a component of the pre-RC and one subunit of the MCM complex, is also present at origins at this time (Fig. 4) (2, 59). It was shown that Cdc45p required Mcm2p to associate with chromatin and that RPA depended on Mcm5p to bind to origins (60, 67). We show here that the origin associations of Cdc45p and RPA are mutually dependent. Together, these findings suggest that Cdc45p and RPA bind to the MCM proteins at origins at the onset of S phase and that together they form the pre-IC. Interestingly, we find that binding of Cdc45p to replication origins is temporally controlled. Cdc45p binds to early-firing origins early and late-firing origins late in S phase, and the timing of Cdc45p binding at each origin correlates well with the timing of initiation at that origin. Since RPA associated with early- but not late-firing origins in the presence of HU, it was postulated that the timing of RPA binding differed between early- and late-firing origins (60). Given that Cdc45p and RPA are both essential for initiation and that their binding to each origin coincides with firing of the origin, the formation of the pre-IC is likely a key step for origin activation.

Unlike the formation of the pre-RC in late mitosis or early G₁, the later transitions at origins require activation by CDKs and Cdc7p-Dbf4p. The opposite requirement for CDKs ensures that these transitions always occur after the pre-RC is formed. Several recent studies suggested that S-CDKs and Cdc7p-Dbf4p might function at origins (7, 17, 18). Although a number of proteins present in the pre-RC appear to be substrates of CDKs or Cdc7p in yeast or other organisms (8, 22, 34, 35, 39, 42, 50, 56, 64), how these phosphorylations trigger the transitions remains to be addressed. We have previously shown that Cdc45p was associated with chromatin upon activation of S-CDKs, suggesting that Cdc45p could play an important role in mediating the origin activation by S-CDKs (67). Here we show that S-CDKs are indeed required for the association of Cdc45p with *ARS1* and most likely other origins in a temporally specific manner. Furthermore, S-CDK activity is needed for establishing the interactions among Cdc45p, RPA, and Mcm2p. In addition to S-CDKs, Cdc7p-Dbf4p is also required for efficient binding of Cdc45p to *ARS1*. Likewise, binding of RPA to origins also requires S-CDKs and Dbf4p-dependent kinase (60). Therefore, formation of the pre-IC at individual origins may be the key event during origin activation that is controlled by S-CDKs and Cdc7p-Dbf4p. Because inactivation of Cdc7p-Dbf4p in early S phase leads to silencing of late-firing origins, Cdc7p-Dbf4p probably executes its function at individual origins in a temporally controlled manner (7, 17). The requirement for Cdc7p-Dbf4p in loading Cdc45p and

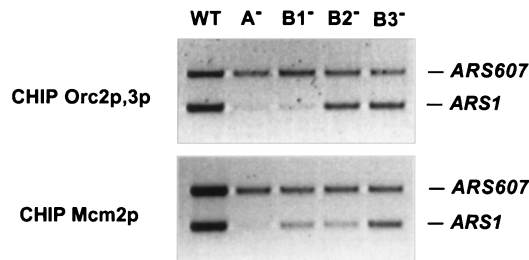


FIG. 8. The B2 element of *ARS1* is involved in association with Mcm2p but not ORC. The Cdc45HA3p-expressing cells with the chromosomal *ARS1* mutated in either the A (YB0576), B1 (YB0577), B2 (YB0578), or B3 (YB0579) element were analyzed by CHIP assays. Asynchronously growing wild-type (WT) and *ARS1* mutant cells were subjected to CHIP analysis with antibodies against Orc2p-Orc3p (antibodies against Orc2p and Orc3p were mixed and used together) and Mcm2p, respectively.

RPA onto origins perhaps contributes to formation of the pre-IC at each origin, with a characteristic timing during S phase.

We noticed that although the Cdc45p-*ARS1* association was not detected by CHIP in the arrested *cdc7* and *dbf4* cells, binding of Cdc45p to Mcm2p and chromatin was only weakened but not abolished (Fig. 5). Unlike chromatin fractionation and Mcm2p coimmunoprecipitation, CHIP analysis examines the enrichment of Cdc45p at a single replication origin. It is possible that in the arrested *cdc7* and *dbf4* cells, Cdc45p associated with origins other than *ARS1* and/or even nonorigin sequences. Alternatively, when Cdc7p-Dbf4p activity was limited, the enrichment of Cdc45p at each origin might not be sufficient to be detected by CHIP. When combined, even the inefficient binding of Cdc45p to a number of origins could give detectable amounts of chromatin-bound Cdc45p and Cdc45p-Mcm2p interaction. Since we have shown that Cdc45p could interact weakly with Mcm2p off the chromatin (67), Cdc7p-Dbf4p might be required for recruiting Cdc45p to origins and establishing a more stable Cdc45p-MCM interaction.

The conclusion of Aparicio et al. (2) that Cdc45p is a component of the pre-RC must be reassessed. We did not find Cdc45p on chromatin until the S-CDKs were activated (67), and here we show that Cdc45p associates with individual origins in a temporally regulated manner during S phase. Indeed, Aparicio et al. (3) agree with this result for most origins, except for the very early origins. We find that, even at the earliest origins, Cdc45p is loaded after the MCM proteins are loaded, the latter step being the one that best defines pre-RC formation and which even occurs upon exit from mitosis (15). Furthermore, since we have used CHIP and direct chromatin binding, as well as coimmunoprecipitation with MCM proteins, we suggest that the differences are unlikely to be due to different methods (as suggested by Aparicio et al. [3]). Nevertheless, we agree that Cdc45p is required for Pol α loading and that it might move away from origins after initiation.

How does the pre-IC interact with origins? Disruption of the B2 element of *ARS1* diminished its origin activity but did not affect ORC-*ARS1* binding (Fig. 8) (43, 52). In contrast, we found that the Mcm2p-*ARS1* association was weakened in the B2⁻ mutant, suggesting that the B2 element might be specifically involved in the association with the MCMs. Since only the 8-bp linker substitution in the B2 element, but not the single-base-pair changes in this region, affected the origin activity of *ARS1* (43), the B2 element is likely a structural element rather than a sequence-specific binding site for proteins. Indeed, it has been suggested that the B2 element is a DNA-unwinding element (41), and it is very close to the site of initiation of leading-strand DNA replication (6). Therefore, we suggest that the MCMs preferentially associate with the easily unwound sequences (such as the B2 element of *ARS1*) adjacent to the ORC binding sites. The low helical stability and the position relative to the ORC binding site, but not the actual DNA sequence, might be important for this association. Furthermore, Cdc45p and RPA may be recruited to these regions by the MCMs to form the pre-ICs.

What, then, is the function of the pre-IC at origins? In the SV40 system, RPA binds to the origin that is locally "melted" by T antigen, and then it stimulates the helicase activity of T antigen and stabilizes the unwound origin (29). Subsequently, T antigen, RPA, and the unwound origin recruit Pol α -primase to form a complex called the primosome (62). At chromosomal origins, the MCM proteins might function as a T-antigen-like helicase. A helicase activity was reported to be associated with a complex of human Mcm4p, Mcm6p, and Mcm7p (32). Six fission yeast MCM proteins form a globular complex in vivo

(1). Our data suggest that the MCM complex might associate with the B2 element of *ARS1*, a potential DNA-unwinding element (41). However, unlike SV40 T antigen, which can "melt" the origin by itself, it is unlikely that MCM proteins can unwind origins in G₁ when they are present in the pre-RC. If the MCM complex functions as a helicase, then its activity might need to be stimulated by S-CDKs and Cdc7p-Dbf4p at the G₁-S transition. Together with the findings by Tanaka and Nasmyth (60), our results show that S-CDKs and Cdc7p-Dbf4p promote the binding of Cdc45p and RPA to the MCM proteins at origins. From genetic evidence, both Cdc7p-Dbf4p and Cdc45p have been implicated in the activation of MCMs. A mutant allele of *MCM5* called *bob1* can suppress the lethality caused by deletions of either *CDC7* or *DBF4* (27, 33). The loss of Cdc45p function in the *cdc45-1* mutant can be bypassed by *cdc46-1* and *cdc47-1*, two mutant alleles of *MCM5* and *MCM7* (28). Furthermore, RPA can stimulate the helicase activity of T antigen in the SV40 system, indicating that it might also be involved in the activation of MCMs in yeast (29). Therefore, it is plausible that Cdc45p and RPA collaborate to activate the MCM helicase and/or to increase its processivity. Consistent with their potential roles in origin unwinding, Cdc45p is required for loading Pol α onto chromatin, and RPA is needed for recruiting primase to origins (Fig. 7) (45, 60).

The function of Cdc45p may not be restricted to origin unwinding and assembly of replication forks. We find that Cdc45p associates with Mcm2p, RPA, and Pole during S phase. Unlike Pol α and Pol δ , Pole is not required for in vitro replication of SV40 DNA (62). Consistent with this, it was recently shown that Pole polymerase activity was not essential in yeast (37). Nevertheless, the carboxyl terminus of Pole is essential for viability, and it may function as a sensor of stalled replication forks (47). Two recent studies using CHIP analysis suggested that Cdc45p, RPA, MCMs, and Pole might move away from origins after initiation (2, 60). Moreover, *Xenopus* Cdc45p was colocalized with Pol α throughout S phase (45). Together, these findings argue that Cdc45p, RPA, and MCMs move with replication forks. Cdc45p, together with RPA, may continue to stimulate MCM helicase activity during replication elongation, or it may play an important structural role at replication forks. In addition to chromosomal replication, Pole and RPA are also implicated in the repair of UV-induced DNA damage (9, 61). Furthermore, *DPB11*, a gene that interacts genetically with both *POL2* (encoding Pole) and *CDC45*, is involved in the S-phase-induced checkpoint arrest of entry into mitosis (4, 36). It will be interesting to test whether Cdc45p plays a role in nucleotide excision repair and replication-induced checkpoints.

Our results suggest that S-CDKs and Cdc7p-Dbf4p control the formation of the pre-IC at origins. However, it is still unclear how phosphorylation is involved in this process. Identification of the key substrates of the kinases will be crucial for solving the puzzle. Our findings also present us with new challenges. Are Cdc45p, RPA, and MCMs sufficient for origin unwinding? Does the formation of the pre-IC require an initial melting of origins? If so, then what melts the origins? Extensive biochemical investigations will be essential to address these questions.

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