The EMBO Journal Vol.17 No.19 pp.5699–5707, 1998

Xenopus Cdc45-dependent loading of DNA polymerase α onto chromatin under the control of S-phase cdk

Satoru Mimura and Haruhiko Takisawa[1]

Department of Biology, Graduate School of Science, Osaka University, Toyonaka, Osaka 560-0043, Japan

[1]Corresponding author
e-mail: takisawa@bio.sci.osaka-u.ac.jp

At the onset of S phase, chromosomal replication is initiated by the loading of DNA polymerase α onto replication origins. However, the molecular mechanisms for controlling the initiation are poorly understood. Using *Xenopus* egg extract, we report here the identification of a *Xenopus* homolog of Cdc45, a yeast protein essential for the initiation of replication, which is shown to be an essential molecule for the initiation of replication via the loading of DNA polymerase α onto chromatin. XCdc45, by physically interacting with the polymerase in the extract, became associated with chromatin only after nuclear formation. During S phase, XCdc45 co-localized with the polymerase in the nuclei, and the loading of the polymerase, which depended on endogenous XCdc45, was facilitated by exogenously added recombinant XCdc45. These findings, together with the apparent requirement of S-phase-cdk activity for the loading of XCdc45, suggest that XCdc45, under the control of S-phase cdk, plays a pivotal role in the loading of DNA polymerase α onto chromatin.

Keywords: DNA polymerase α/eukaryotic DNA replication/S-phase cdk/*Xenopus* Cdc45 homolog

Introduction

In eukaryotes, the replication of the genome is strictly controlled during cell-cycle progression. One of the fundamental questions about the control of replication is how replication is initiated at the onset of S phase. This question is intimately related to another question: how is re-replication prohibited until the end of M phase? Recent studies have revealed that the latter mechanism, the licensing of replication, is conserved in eukaryotes (Kubota *et al*., 1997; Thömmes *et al*., 1997; Tanaka *et al*., 1997; also reviewed in Blow, 1996). The replication-competent, licensed chromatin is in a pre-replicative state (Diffley *et al*., 1994), which is formed by sequential assembly of origin recognition complex (ORC), Cdc6 and minichromosome maintenance protein complex (MCM) onto chromatin (Coleman *et al*., 1996; Romanowski *et al*., 1996; Rowles *et al*., 1996; Donovan *et al*., 1997; Tanaka *et al*., 1997). The assembly of MCM on the chromatin apparently plays an essential role for the licensing of replication, i.e. MCM is dissociated from chromatin as it is replicated, and MCM is re-associated with chromatin only at the end of M phase (reviewed in Chong *et al*., 1996; Kearsey *et al*., 1996). In contrast, how the licensed chromatin in the pre-replicative state is activated into a replicative state at the onset of S phase is poorly understood. The replicative state is established by recruiting DNA polymerase α-primase onto the replication origins, but little is known about what is the essential component for recruiting the polymerase and how these processes are controlled by S-phase-promoting kinases such as cyclin-dependent kinases (cdks).

S-phase-promoting cdk has been proposed to play a dual role in controlling the initiation of chromosomal replication; one for triggering replication at the onset of S phase, and the other for preventing re-replication (Nasmyth, 1996; Stern and Nurse, 1996). Recent studies with budding yeast further suggest that S-phase-promoting cdk prevents re-replication by inhibiting the transition of replication origins to a pre-replicative state (Dahmann *et al*., 1995; Piatti *et al*., 1996). More recently, using formaldehyde cross-linking, it has been suggested that the cdk activity during S, G_2 and M phases prevents MCM loading onto the replication origins (Tanaka *et al*., 1997). Similar data has also been reported using the cell-free extract of *Xenopus* eggs, showing that high levels of S-phase cdk2 prevent association of Mcm3, but not ORC, with chromatin (Hua *et al*., 1997). These data suggest that S-phase-cdk activity modulates the state of chromatin at the onset of S phase; however, the molecular nature of the target of these kinases has not been clarified.

CDC45 of *Saccharomyces cerevisiae* was originally identified by screening cold-sensitive *cdc* mutants (Moir *et al*., 1982), which were subsequently shown to interact genetically with *MCM5* (*CDC46*) and *MCM7* (*CDC47*) (Hennessy *et al*., 1991). The cloning of *CDC45* reveals that it is not a member of MCM, but is essentially required for the initiation of chromosomal replication (Hopwood and Dalton, 1996; Hardy, 1997; Zou *et al*., 1997). In addition, Cdc45 interacts genetically with ORC (Hardy, 1997; Zou *et al*., 1997), and physically with MCM proteins (Hopwood and Dalton, 1996; Dalton and Hopwood, 1997; Zou and Stillman, 1998) and the yeast replication origins (Aparicio *et al*., 1997). Therefore, Cdc45 was initially assumed to be required for the formation of the pre-replicative complex (Hopwood and Dalton, 1996) or as a component of the pre-replicative complex (Aparicio *et al*., 1997), which has been formed before START. However, a recent study indicates that Cdc45 functions in late G_1, after START and prior to DNA synthesis (Owens *et al*., 1997). More recently, it has been shown that Cdc45 associates with chromatin after START, depending on Clb–Cdc28 kinase activity, thus suggesting that Cdc45 associates with the pre-replicative complex after activation of S-phase-promoting cdk (Zou and Stillman, 1998). All of these characteristics make Cdc45 a good candidate for

involvement in the activation of the licensed chromatin, in the pre-replicative state. However, its exact role in initiation of replication remains largely unknown. Here, we report the identification of a *Xenopus* Cdc45 homolog as an essential molecule for the loading of DNA polymerase α onto chromatin under the control of S-phase-cdk activity.

Results

Identification of a Xenopus Cdc45 homolog

A potential *Xenopus* Cdc45 homolog (XCdc45) was cloned as follows. We first identified a partial sequence of a putative human homolog (EST64671) by searching for the homolog of yeast Cdc45 in the EST database. Next we cloned a full-length human cDNA (DDBJ/EMBL/GenBank accession No. AF062495) by screening a HeLa cDNA library with the EST sequence as a probe. Finally, we isolated a putative *Xenopus* homolog (DDBJ/EMBL/GenBank accession No. AF062494) by screening a *Xenopus* oocyte cDNA library with the full-length human cDNA as a probe. The open reading frame of the *Xenopus* cDNA encoded a protein consisting of 567 amino acids with a calculated molecular mass of 65 kDa. The predicted amino acid sequence showed a strong homology to the putative human homolog (75% identity) and significant sequence identity with the yeast Cdc45 (~25%). Highly conserved regions among these three protein sequences (Figure 1A) were noted at locations along the entire length of the peptides. In addition, acidic patches (Hopwood and Dalton, 1996), consisting of ~50% of the acidic amino acids, and potential nuclear localization sequences located at the end of the patches (Hopwood and Dalton, 1996), were also found to be conserved among these proteins. These data suggest that the cloned *Xenopus* and human sequences are indeed putative homologs of the yeast Cdc45.

Involvement of XCdc45 in DNA replication

To investigate the role of the putative XCdc45 in DNA replication, we expressed a His_6-tagged version of XCdc45 (XCdc45-His_6) in baculovirus-infected Sf-9 cells. The recombinant protein, purified with Ni-nitrilotriacetic acid (NTA) agarose, consisted of a single polypeptide with an apparent molecular mass of 66 kDa (Figure 1B). The purified XCdc45-His_6 protein and glutathione-*S*-transferase (GST)-fused C-terminal peptide were used as antigens to produce polyclonal antibodies. Both antibodies recognized a 63 kDa protein in S-phase egg extract (Figures 1C and 3A). For immunoprecipitation of endogenous XCdc45 from the extract, the antisera were further affinity purified with the recombinant protein. Figure 1B shows that the first immunoprecipitates of the extract with the purified anti-XCdc45 antibody yielded a specific protein with an apparent molecular mass of 63 kDa, slightly smaller than that of the recombinant protein which contained an additional tag. The immunoprecipitated 63 kDa protein was also recognized by antibodies against the full-length (Figure 3A) and C-terminal peptide (data not shown). These results indicate that the protein corresponding to the cloned *Xenopus* sequence is present in the egg extract.

With the anti-XCdc45 antibody, endogenous XCdc45 could be depleted from the extract almost completely (Figure 1C). We first examined whether or not XCdc45 is required for DNA replication in the extract. Upon depletion of XCdc45 from the extract, the replication activity was diminished to $<$10% of the mock-depleted extract (Figure 1D). In order to examine the nature of residual replication activity, total DNA was isolated from the extracts and subjected to alkali agarose gel electrophoresis (Figure 1E). The detection of replicated DNA with the incorporation of ^{32}P-labelled dCTP showed that nascent DNA was synthesized in the mock-depleted extract in the presence of a low concentration of aphidicolin (~80% inhibition of total DNA synthesis). In the XCdc45-depleted extract, no nascent DNA was synthesized, but a small amount of DNA with high molecular mass was produced. The high molecular DNA was also produced in the mock-depleted extract as a result of a complete round of replication (compare with Mahbubani *et al*., 1997). In accord with this, the polymerase activity measured with activated DNA was not significantly decreased upon XCdc45 depletion (data not shown). These results suggest that XCdc45 is required for the initiation stage of DNA replication in the extract.

In order to obtain more insight into the role of XCdc45, we examined the behavior of XCdc45 in the egg extract. Upon addition of sperm chromatin to the extract, ORC and MCM became associated with the chromatin within 10 min, before the nuclear structures had been assembled around the chromatin. However, XCdc45 only became associated with chromatin 30 min after the addition of chromatin; after the formation of nuclei. In the absence of membrane fractions of the extract (high speed supernatant; HSS), we detected neither the formation of nuclei nor the association of XCdc45 with the chromatin (Figure 2A), suggesting that nuclear formation is required for the assembly of XCdc45 onto chromatin. Essentially the same results were obtained by examining the chromatin fractions by immunoblotting (Figure 2B). XMcm6 became associated with chromatin before, or in the absence of, nuclear formation. As with the behavior of XCdc45, we found that XCdc45 is not required for the formation of licensed chromatin (Figure 2B). Examination of chromatin fractions prepared from mock- and XCdc45-depleted extracts demonstrated no difference in the association of XOrc1 and XMcm3 with the chromatin, while XCdc45 was only absent in the chromatin fraction prepared from the depleted extract. These results suggest that XCdc45 is not required for licensing chromatin, and that it is assembled onto the licensed chromatin only after nuclear formation. Consistent with this, recombinant XCdc45 could rescue the replication activity of the depleted extract when added after nuclear formation as well as before its formation (Figure 2C). These results indicate that recombinant XCdc45 could be functionally substituted for endogenous XCdc45 and that XCdc45 is required for the initiation process after the formation of nuclear structures.

Investigation into the interaction of XCdc45 with polymerase

Nuclear formation, which is essential for replication (Blow and Laskey, 1986; Newport, 1987), leads to the accumulation of various proteins required for replication. In order to understand the function of XCdc45, we looked for possible target proteins which physically interact with

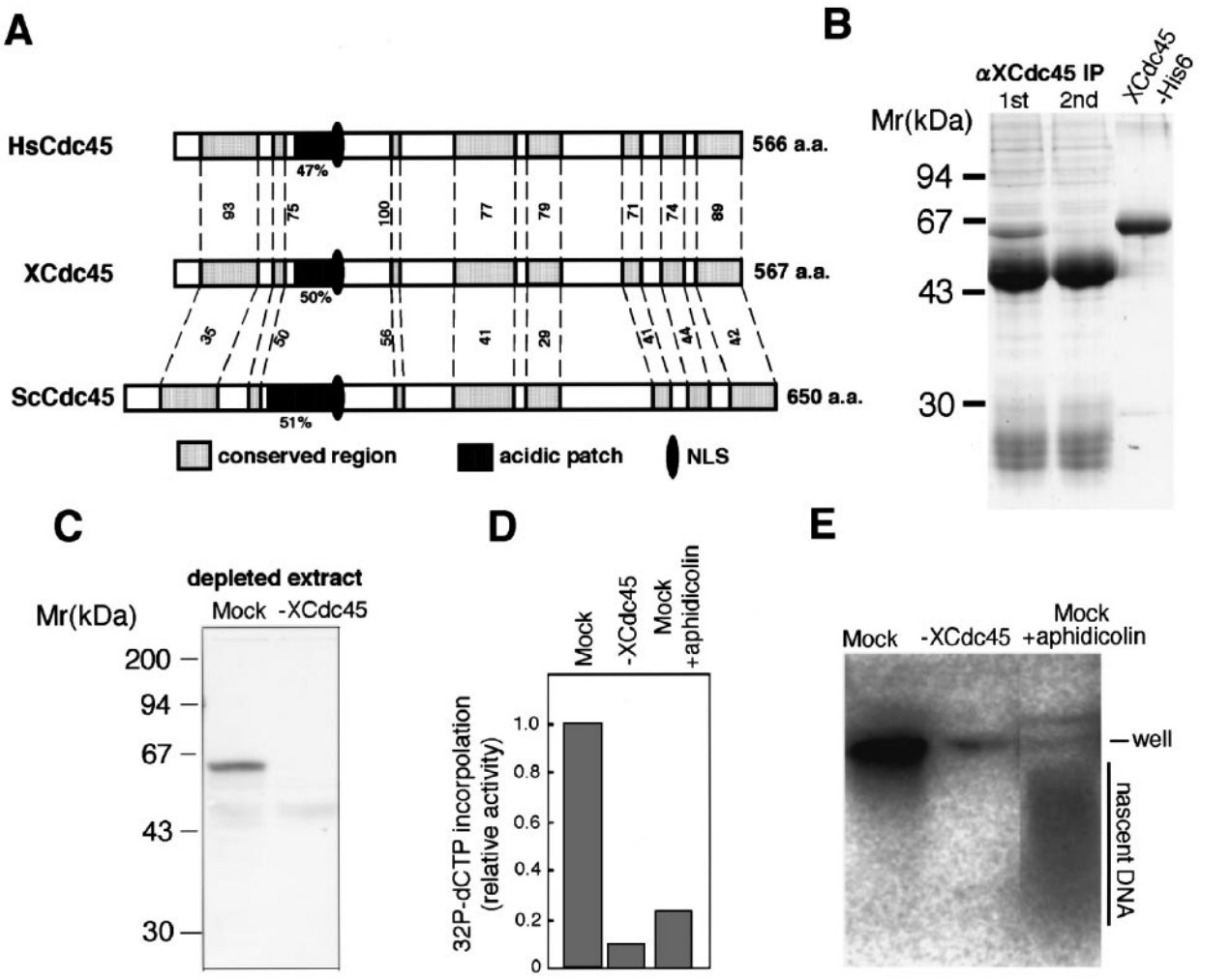

Fig. 1. Requirement of the *Xenopus* Cdc45 homolog for the replication of chromosomal DNA in cell-free extracts of *Xenopus* eggs. (**A**) The schematic structure of a putative *Xenopus* Cdc45 homolog compared with *S.cerevisiae* Cdc45 and a putative human homolog. Numerical values indicate the percent identity of amino acids in each conserved region between XCdc45 and the other proteins. The percentages of acidic amino acids are indicated just below the acidic patch region. (**B**) Expression of endogenous and recombinant XCdc45 protein. Endogenous protein was isolated by immunoprecipitating S-phase egg extract with anti-XCdc45 antibody bound to a protein A matrix. The first immunoprecipitates contained most of XCdc45 proteins. Recombinant XCdc45 protein tagged with His_6 at the C-terminus (XCdc45-His_6) was expressed in Sf9 cells and purified with Ni–NTA resin. Both samples were resolved by SDS–PAGE, and visualized by Coomassie Blue staining. (**C**) Immunodepletion of XCdc45 from S-phase egg extract. Extracts were treated with preimmune (mock) and anti-XCdc45 antibodies bound to protein A matrix, resolved by SDS–PAGE and immunoblotted with anti-XCdc45 C-terminal peptide antibody. (**D**) Inhibition of the replication activity of S-phase egg extract upon depletion of XCdc45. Sperm chromatin was incubated in mock, mock-plus 10 μg/ml aphidicolin and XCdc45-depleted extracts for 90 min at 23°C. Replication activity was monitored as the incorporation of ^{32}P-labelled dCTP into DNA. Relative values are given. (**E**) Requirement of XCdc45 for the initiation stage of DNA replication. Sperm chromatin was incubated in the extracts as in (D), and the total DNA was isolated and subjected to alkali agarose gel electrophoresis.

XCdc45 by examining the immunoprecipitates from the extract. Figure 3A shows that a small amount of DNA polymerase α was co-immunoprecipitated with XCdc45, but not the control and anti-XMcm3 immunoprecipitates. We also examined the possible association of XMcm7 and PCNA with XCdc45, but failed to detect a significant association (data not shown). It should also be noted that a very faint signal of XMcm5 was detected with anti-XCdc45 immunoprecipitates. This contrasts with the previous reports from budding yeast, which showed that the yeast Cdc45 forms a physical complex with Mcm5 and 7 in the cell extracts prepared from log-phase cultures (Hopwood and Dalton, 1996; Dalton and Hopwood, 1997). These conflicting results may be explained if XCdc45 can form a complex with XMcms only after nuclear formation, i.e. at the onset of S phase. A recent report with budding yeast has shown that Cdc45 physically interacts with Mcm2 during S phase (Zou and Stillman, 1998).

The possible association of XCdc45 with polymerase led us to speculate that XCdc45 is required for replication by physically interacting with the polymerase to recruit it onto replication origins. If this were the case, XCdc45 should be loaded onto the chromatin at the same time or prior to the loading of the polymerase. Immunoblotting of the chromatin fractions showed that the polymerase and XCdc45 became associated with chromatin at the same time, i.e. after the formation of the nuclei (Figure 3B). Figure 3C shows further that XCdc45 and the polymerase were co-localized in the nuclei throughout the progression of S phase. Both proteins precisely co-localized in every nuclei formed in the extract at the onset of replication (30 min). During the progression of S phase,

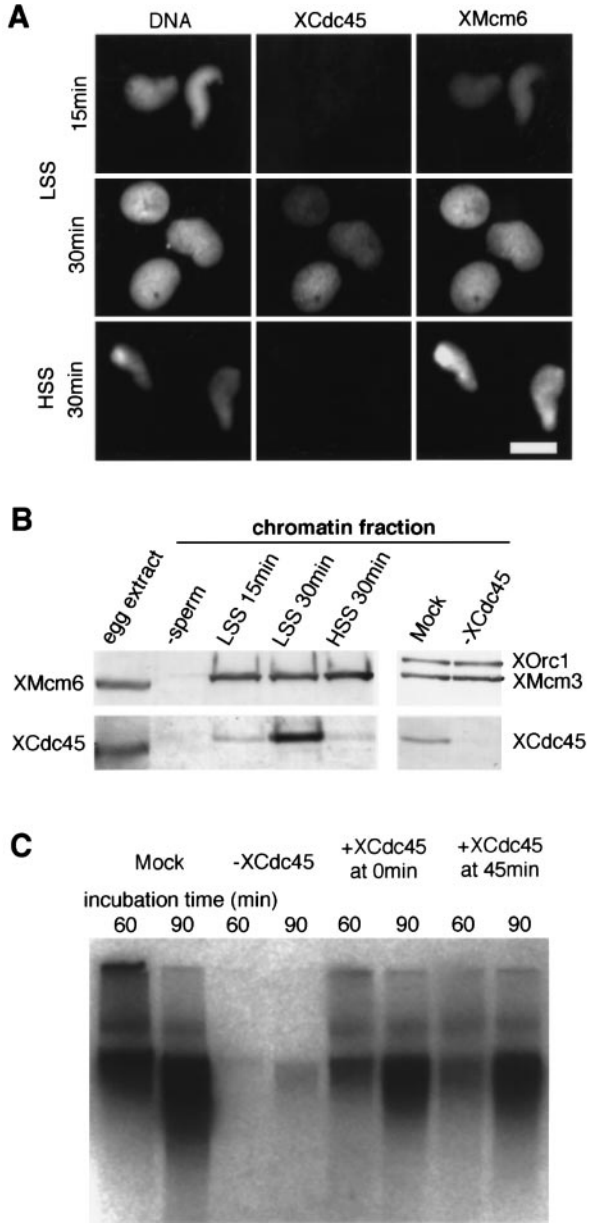

Fig. 2. Loading of XCdc45 onto chromatin after nuclear formation. (**A**) Immunofluorescent detection of XCdc45 and XMcm6 on chromatin with and without nuclear formation. Sperm chromatin was incubated in S-phase egg extract (low speed supernatant; LSS), and membrane-depleted S-phase extract (high speed supernatant; HSS) at 23°C for the indicated times, and the localization of XCdc45 and XMcm6 was visualized by rabbit anti-XCdc45 and mouse anti-XMcm6 antibodies followed by Cy2-labelled anti-rabbit and Texas red-labelled anti-mouse antibodies, respectively. DNA was visualized with Hoechst. Scale bar, 20 μm. (**B**) Loading of XCdc45 onto the chromatin after the assembly of licensed chromatin. Sperm chromatin was incubated in egg extracts as for (A). As a control (–sperm), sperm chromatin was omitted from the extract, and mock-, and XCdc45-depleted extracts were prepared as for Figure 1C. The chromatin fractions isolated from the extracts were resolved by SDS–PAGE, and visualized by immunoblotting with specific antibodies as indicated. (**C**) Rescue of the replication activity of XCdc45-depleted extract with recombinant XCdc45. Sperm chromatin was incubated in mock- and XCdc45-depleted extracts in the presence of [^{32}P]dCTP, and XCdc45-His_6 (20 ng/μl extract) was added to the XCdc45-depleted extracts before (0 min) or after nuclear formation (45 min). Sixty and 90 min after the addition of sperm chromatin, the replication reaction was stopped and DNA was isolated and subjected to agarose gel electrophoresis.

both signals in the nuclei decreased (60 min), and essentially no signal was detected 90 min after nuclear formation, when the replication was completed in most of the nuclei (compare with Figure 3D). Conspicuous co-localization of XCdc45 and the polymerase was also observed when the replication was inhibited by aphidicolin. Both signals in the nuclei were enhanced in the presence of aphidicolin (Figure 3C). This may suggest that the activation of origins in the absence of DNA synthesis facilitates the accumulation of both proteins onto the chromatin. In contrast to the polymerase, the XMcm6 signal in the nuclei was most intense at the onset of replication, and the signal rapidly decreased during replication (Figure 3D). The intensity of the XCdc45 signal in the nuclei increased after the start of replication (40 min), and its intensity was reciprocally related to that of XMcm6. These results showed that XCdc45 function is closely related to the loading of the polymerase onto chromatin.

An essential requirement of XCdc45 for the loading of the polymerase is shown in Figure 4A. Upon depletion of XCdc45 from the extract, neither the loading of the polymerase nor the initiation of replication was observed (see Figure 1D). In contrast, the loading of Mcm proteins onto the chromatin occurred normally in the depleted extracts (Figure 2B). These results suggest that XCdc45 is required for the loading of the polymerase on the licensed, pre-replicative chromatin, which has been assembled before nuclear formation. Data with recombinant XCdc45 provided further support for the idea that XCdc45 is intimately involved in the loading of the polymerase. Figure 4B shows that the addition of an excess of recombinant XCdc45 (the concentration of endogenous XCdc45 was estimated to be about 10 ng/μl extract), accelerated the association of the polymerase with chromatin in parallel with the rapid association of the recombinant XCdc45 on chromatin. In addition, the rapid association of the recombinant XCdc45 apparently suppressed the association of endogenous XCdc45 on the chromatin (Figure 4C), suggesting competition for the same site. Therefore, the acceleration of the polymerase loading with the recombinant protein suggests that XCdc45 is essential for the rate determining step in the polymerase loading.

S-phase cdk-dependent loading of XCdc45 onto chromatin

The next question is how the loading of XCdc45 onto the chromatin might be regulated. Since nuclear formation has been shown to facilitate the accumulation of S-phase cdk into the nuclei (Hua *et al*., 1997), we examined whether the cdk activity is required for the loading of XCdc45. With *Xenopus* egg extracts, it has been established that p21(Cip1), inhibits replication through the inhibition of S-phase-cdk activity in the extract (Strausfeld *et al*., 1994; Chen *et al*., 1995; Jackson *et al*., 1995). The same concentration of p21 sufficient to inhibit replication also inhibited the loading of both XCdc45 and the polymerase onto the chromatin (Figure 5A). Immunoblotting of chromatin fractions confirmed further that the loading of XOrc1 and XMcm3 was not affected by p21 (Figure 5A), consistent with the fact that both proteins were loaded before the formation of nuclei. This observation is in

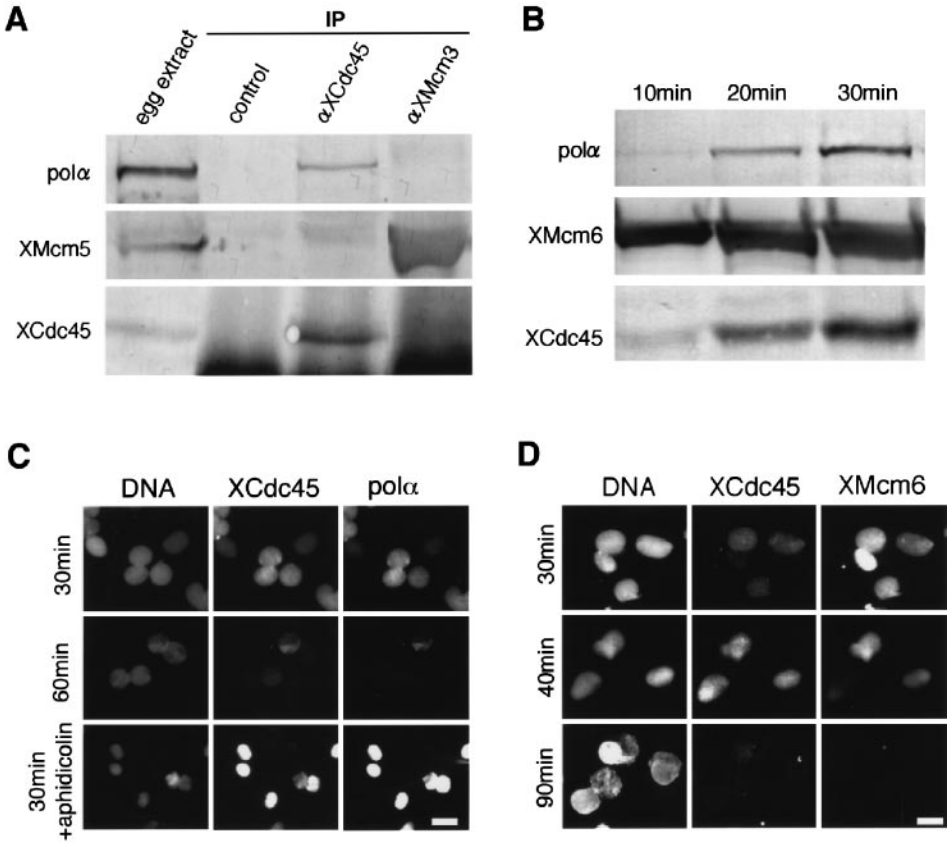

Fig. 3. Identical behavior of XCdc45 and DNA polymerase α throughout the S phase. (**A**) Co-immunoprecipitation of DNA polymerase and XCdc45 in S-phase egg extract with anti-XCdc45 antibody. The egg extract was incubated with preimmune, anti-XCdc45 and anti-XMcm3 antibodies bound to a protein A matrix. The egg extract (1 μl) and immunoprecipitates from 50 μl extracts were resolved by SDS–PAGE and immunoblotted with anti-DNA polymerase α, anti-XMcm5 and anti-XCdc45 antibodies. (**B**) Simultaneous loading of XCdc45 and polymerase onto chromatin during nuclear formation. Sperm chromatin was incubated in the egg extract at 23°C for the indicated times and the chromatin fraction was isolated, resolved with SDS–PAGE, and immunoblotted with anti-DNA polymerase α, anti-XMcm6 and anti-XCdc45 antibodies. (**C**) Co-localization of XCdc45 and the polymerase in replicating nuclei. Sperm chromatin was incubated in the egg extract for 30 and 60 min at 23°C in the presence and absence of 10 μg/ml aphidicolin, and the samples were treated with Triton X-100 followed by fixation with formaldehyde, and processed for immunofluorescence. Scale bar, 20 μm. (**D**) Different behavior of XCdc45 and XMcm6 in replicating nuclei. Sperm chromatin was incubated in the egg extract for 30, 40 and 90 min, and the samples were treated and processed as for (C). Scale bar, 20 μm.

agreement with the previous finding that the pre-replicative complex is formed in the absence of the S-phase-cdk activity (Dahmann *et al*., 1995; Hua *et al*., 1997; Tanaka *et al*., 1997). In order to confirm that the effect of p21 is due to the inhibition of cdk activity, we next prepared an extract depleted of Xcdk2, an essential component for the initiation of replication in the extract (Fang and Newport, 1991; Jackson *et al*., 1995). In order to do this, we used suc1-conjugated agarose, which was successfully used to deplete Xcdc2 and Xcdk2 from the extract (Blow and Nurse, 1990; Fang and Newport, 1991; Strausfeld *et al*., 1996). Upon treating the extract with suc1 agarose, Xcdk2 was almost completely depleted from the extract, while a small amount of Xcdc2 remained in the extract (Figure 5B), and both the loading of XCdc45 and the polymerase onto the chromatin were severely inhibited (Figure 5C). All these results indicate that S-phase cdk is required for the loading of XCdc45, and eventually for the loading of the polymerase onto the pre-replicative chromatin.

Discussion

In the present study we have identified *Xenopus* Cdc45 homolog as an essential molecule for the initiation of chromosomal replication in the extract of *Xenopus* eggs. XCdc45 became associated with chromatin only after nuclear formation, and it could rescue the replication activity of the XCdc45-depleted extract after nuclear formation. This contrasts with the behavior of XMCM, XCdc6 and XORC, which assemble with chromatin before nuclear formation (Coleman *et al*., 1996; Romanowski *et al*., 1996; Rowles *et al*., 1996). In addition, XMCM and XCdc6 are unable to rescue the replication activity of the depleted extracts after nuclear formation (Coleman *et al*., 1996; Kubota *et al*., 1997). These data suggest that XCdc45 is not involved in the licensing reaction observed with the extract, and in accordance with this, XCdc45 is not required for the loading of XMCM onto chromatin. Therefore, it is reasonable to assume that XCdc45 is not a component of the licensed chromatin in a pre-replicative state. Absence of physical interaction between XCdc45 and XMCM in the egg extract further suggests that XCdc45 interacts with XMCM only after nuclear formation. Actually, the inhibition of replication activity with aphidicolin led to the co-localization of XCdc45 and XMCM in the nuclei, since both proteins appeared to be uniformly distributed on the chromatin in the absence of replication activity. Preliminary experiments indicated the

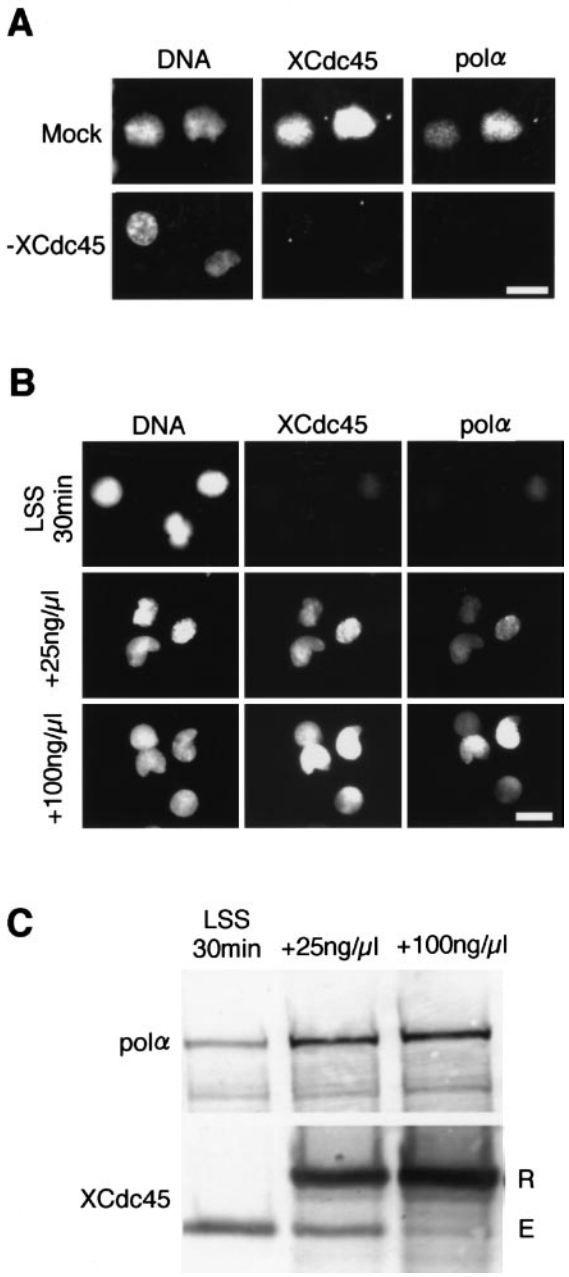

Fig. 4. Essential role of XCdc45 for the loading of DNA polymerase α onto the chromatin. (**A**) Requirement of XCdc45 for the loading of polymerase onto chromatin. Sperm chromatin was incubated in the mock- and XCdc45-depleted extracts for 40 min at 23°C, and the samples were processed for immunofluorescence as for Figure 3C. Scale bar, 20 μm. (**B** and **C**) Stimulation of the loading of the polymerase onto chromatin by recombinant XCdc45. Sperm chromatin was incubated in S-phase egg extract with 0, 25 and 100 ng recombinant XCdc45-His_6 per μl extract for 30 min at 23°C, and samples were processed for immunofluorescence (B), and immunoblotting of the chromatin fraction (C). R and E denote recombinant and endogenous XCdc45, respectively. Scale bar, 20 μm.

physical association of XMCM and XCdc45 under such conditions (data not shown). In consideration of recent data on Cdc45 (Zou and Stillman, 1998), we would like to propose that XCdc45 interacts with XMCM on the licensed chromatin after nuclear formation, that is, at the onset of S phase. The present finding that XCdc45 is required for the loading of DNA polymerase α reinforced the view that XCdc45 plays a pivotal role in the activation of the licensed chromatin.

Previous studies of the simian virus 40 (SV40) DNA replication system (Challberg and Kelly, 1989; Stillman, 1989; Borowiec *et al*., 1990; Waga and Stillman, 1994) provide us with a good framework for understanding the molecular mechanisms for the initiation of replication in eukaryotes. In the SV40 system, SV40 large T antigen plays crucial roles in the initiation (Fanning and Knippers, 1992). It has three distinct functions: first to recognize origins, secondly to unwind the origin DNA, and thirdly to recruit the polymerase onto the origins. Recent studies with eukaryotic chromosome replication reveals that the first function is provided by ORC, a conserved initiator protein for the assembly of pre-replicative complex (Stillman, 1996; Dutta and Bell, 1997). The second function may be provided by the MCM 4-6-7 complex, which has recently been reported as having DNA helicase activity (Ishimi, 1997). The present study suggests that the third function requires a Cdc45 homolog as an essential component. This notion is further supported by recent studies concerning the physical association of Cdc45 with replicating origins of the yeast *S.cerevisiae* (Aparicio *et al*., 1997). Although the dependence of the polymerase loading on XCdc45 did not support the direct role of XCdc45 on the polymerase loading, the physical association of XCdc45 and the polymerase, together with the close relationship between the loading of XCdc45 and the polymerase, suggests that XCdc45 mediates the loading of the polymerase, possibly as a component of a putative loading protein of the polymerase.

The present study further shows that the loading of XCdc45 onto chromatin is under control of S-phase cdk. An essential role for cdk in the regulation of cell-cycle progression has been established (Nasmyth, 1996; Stern and Nurse, 1996). However, the target of the kinase at the onset of S phase has not been identified. Here, we have shown that the loading of XCdc45 is apparently controlled by S-phase-cdk activity, possibly by cdk2 in the extract. It is therefore possible that factors involved in its loading are good candidates for bona fide substrates of the cdk. During the preparation of the manuscript, similar data has been reported with budding yeast, showing that the association of Cdc45 with chromatin depends on the activity of S-phase cdk (Zou and Stillman, 1998). Their report together with ours suggest that the function of S-phase cdk is conserved from the yeast to higher eukaryotes. Further studies on the mechanisms of the activation of XCdc45 will give us a better understanding of how S-phase cdk controls replication in eukaryotes.

Materials and methods

Cloning and sequencing of cDNAs

A partial sequence of potential human Cdc45 homolog (EST64671) was identified by searching for homology with the yeast *S.cerevisiae* Cdc45 using the EST database, and the fragment of EST64671 was obtained by amplifying cDNA of HeLa cells by PCR using a 5′ primer (TCCGATTTCCGCAAAGAG) and a 3′ primer (AATATAGTGTCTTCATCAGG). The amplified cDNA fragment was used as a probe to clone a full-length human cDNA from a lambda ZAPII-derived cDNA library of HeLa cells mRNAs (Stratagene), which was then used to clone a *Xenopus* homolog by screening a lambda ZAPII-derived cDNA library of *Xenopus* oocyte mRNAs (Kubota *et al*., 1995). From 12.5×10^4 plaques, we isolated two clones for human Cdc45, and one clone for XCDC45. Each

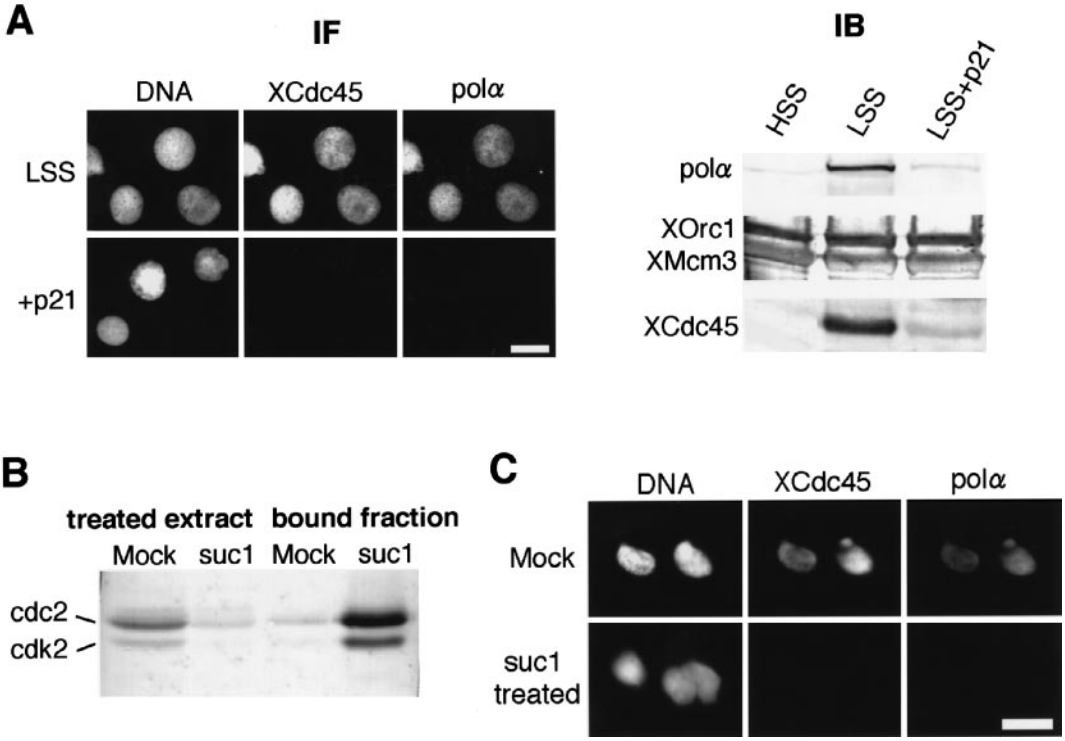

Fig. 5. Requirement of S-phase-cdk activity for loading of XCdc45 onto chromatin. (**A**) Inhibition of the loading of XCdc45 and DNA polymerase α onto chromatin by the cdk inhibitor, p21. Sperm chromatin was incubated in S-phase egg extract in the presence and absence of 300 nM p21 for 30 min (immunofluorescence, IF) and 45 min (immunoblotting, IB) at 23°C, and the samples were processed for immunofluorescence with anti-XCdc45 and anti-DNA polymerase α antibodies (IF), and for immunoblotting of the chromatin fraction with anti-polymerase, -XOrc1, -XMcm3 and -XCdc45 antibodies (IB). Scale bar, 20 μm. (**B**) Depletion of cdks from S-phase egg extract with suc1 agarose. The egg extracts were treated with mock- and suc1-conjugated agarose, and the treated extract together with the proteins bound to the agarose beads were resolved by SDS–PAGE and immunoblotted with anti-PSTAIR antibody. (**C**) Absence of the loading of XCdc45 and the polymerase onto chromatin following depletion of cdks. Sperm chromatin was incubated in the egg extracts treated with mock- and sucl agarose for 30 min at 23°C, and XCdc45 and the polymerase were detected by immunofluorescence. Scale bar, 20 μm.

clone was sequenced for both strands with an automatic DNA sequencer (ABI,373A).

Expression of XCdc45 protein

To prepare a full-length recombinant XCdc45 with His_6 at the C-terminus (XCdc45-His6), a 5′ primer (AATTCTAGAATGTTTGTGAGTGATCT-CCG) and a 3′ primer (TGCGGATCCGTGATGATGATGATGATGTG-ACAATAGAGAAATAAGAGC) were used to amplify XCDC45 cDNA. PCR mixtures (50 μl) contained template (10 ng), primers (50 pmol each), LA-Taq DNA polymerase (TAKARA), and the buffer supplied by the manufacturer. The reactions were cycled 30× as follows: 94°C 1 min; 50°C 1 min; and 72°C 3 min. The product was subcloned into pFastBac1 (Gibco-BRL), and a recombinant baculovirus XCdc45-His_6 was obtained by standard procedures (Gibco-BRL). Sf9 cell lysates containing XCdc45-His_6 were prepared and immediately applied onto a Ni-NTA column (Qiagen) with purification according to the manufacturers' instructions. Protein concentrations were determined using a protein assay kit (Bio-Rad). To prepare a GST fused C-terminal XCdc45 peptide, the *Eco*RI–*Xho*I fragment of XCDC45 cDNA (~400 bp) was subcloned into pGEX 4T-2 (Pharmacia). The recombinant protein, expressed in *Escherichia coli* (lon^- strain), was purified as described earlier (Kubota *et al*., 1995).

Antibodies

Polyclonal rabbit antisera against recombinant XCdc45 proteins were kindly generated by Dr K.Tamai (MBL). Each antiserum was further purified with recombinant full-length XCdc45-His6 protein immobilized on Affi-Gel 15 (Bio-Rad). The affinity purified antibodies were dialyzed against 0.1 M NaCl, 20 mM HEPES–KOH (pH 7.5) containing 0.02% NaN_3, concentrated and stored at 4°C. Anti-chick DNA polymerase α monoclonal antibody (6-1E) was generously provided by Dr A.Matsukage (Aichi Cancer Research Center). The antibody specifically recognized a *Xenopus* egg protein with an apparent molecular mass of 200 kDa, and also the 200 kDa *Xenopus* DNA polymerase α expressed in Sf9 cells (unpublished data). Polyclonal rabbit antisera against XOrc1 was generously provided by Drs A.Rowles and J.J.Blow (ICRF Clare Hall), and it was further purified with recombinant XOrc1 immobilized on Affi-Gel 10 (Bio-Rad). Anti-PSTAIR monoclonal antibody was generously provided by Dr M.Yamashita (Hokkaido University). Characterization of other antibodies was as described previously (Kubota *et al*., 1997).

Preparation of egg extracts, immunoprecipitates and chromatin fractions

S-phase egg extract (LSS), and membrane-depleted S-phase egg extract (HSS) were prepared from activated *Xenopus* eggs (Kubota *et al*., 1993). To prepare samples for immunoprecipitation, 100 μl of LSS were incubated with 10 μg preimmune and anti-XCdc45 antibodies bound to 20 μl of Affi-Prep Protein A matrix (Bio-Rad) for 15 min at 0°C. Proteins bound to the matrix were recovered and washed with EB (100 mM KCl, 2.5 mM $MgCl_2$ and 50 mM HEPES–KOH pH 7.5) three times and the obtained immunoprecipitates were solubilized with SDS–PAGE sample buffer. To prepare immunodepleted extracts, 100 μl of LSS were treated three times with the same composition of the matrix as for the immunoprecipitation. To prepare Xcdc2- and Xcdk2-depleted extract, 100 μl of LSS were treated two times with 30 μl of control agarose, and suc1-conjugated agarose (5 mg sucl protein/ml agarose, Oncogene Research Products) for 15 min at 0°C. To prepare chromatin fractions, sperm chromatin (4000 sperm head/μl extract) was incubated with the treated extracts for appropriate times at 23°C, and 100 μl samples were diluted with 900 μl of ice cold EB containing 0.25% Triton X-100. After 1 min incubation at 0°C, the chromatin fractions were isolated from the extracts, and their identity of the chromatin fraction was verified by immunofluorescence. Digestion with micrococcal nuclease led to release of XOrc1 and XCdc45 from the chromatin fractions, but not nuclear lamin (unpublished data).

Assay for DNA replication

DNA replication activity was measured as the incorporation of [α-^{32}P]dCTP (from 0.7–3.5 kBq/μl extract) into sperm DNA according to the method described by Dasso and Newport (1990). The reaction

was initiated by the addition of sperm chromatin to the extract at a concentration of 2000 sperm heads/μl of extract. After incubation at 23°C for an appropriate time, the samples (5 μl) were removed and added to the same volume of a stop buffer consisting of 0.25 mg/ml proteinase K, 8 mM EDTA, 80 mM Tris–HCl (pH 8.0), 0.13% (w/v) phosphoric acid, 10% (w/v) Ficoll, 5% (w/v) SDS, 0.2% (w/v) bromophenol blue. After incubation at 37°C for 1 h, the samples were applied to agarose gels and subsequently autoradiographed with BAS 2000 (Fuji Film). For alkali agarose gel electrophoresis, the reaction was stopped by adding a stop buffer consisting of 20 mM Tris–HCl (pH 8.0), 200 mM NaCl, 5 mM EDTA, 0.5% SDS, 2 μg/ml RNase A and 200 μg/ml Proteinase K (Mahbubani *et al*., 1997). After further incubation for 30 min at 37°C, DNA was extracted sequentially with phenol–chloroform and chloroform, then precipitated with ethanol. Pellets were resuspended in alkali gel loading buffer (50 mM NaOH, 1 mM EDTA, 1.25% Ficoll, 0.0125% bromophenol blue). Alkaline gels were prepared by adding 1% (w/v) agarose to 50 mM NaOH and 1 mM EDTA, and run at 2 V/cm in alkali gel running buffer (30 mM NaOH, 1 mM EDTA). After electrophoresis, gels were fixed in 7% trichloroacetic acid, dried and autoradiographed.

Immunofluorescence microscopy

Samples for immunofluorescence microscopy was prepared as follows. Sperm chromatin (2000 sperm head/μl extract) were incubated in egg extracts for the indicated times at 23°C and 10 μl samples were diluted with 90 μl of ice cold EB containing 0.25% Triton X-100 and fixed by adding 10 μl of 37% formaldehyde 1 min after the dilution. The fixed samples were processed for the immunofluorescent observation as described previously (Kubota *et al*., 1997).

Acknowledgements

We are greatly indebted to Hisao Masukata and Hiroyuki Araki for initiating this project. We thank J.Julian Blow and Yumiko Kubota for helpful comments,Takashi Oku and Toshiki Tsurimoto for GST-p21 protein, Katsuyuki Tamai (MBL), Akio Matsukage, Alison Rowles, J.Julian Blow and Masakane Yamashita for antibodies, and Fumio Hanaoka for helpful suggestions on baculovirus expression system. S.M. was supported by JSPS Research Fellowships for Young Scientists. This research was supported in part by Grants-in-Aid for Scientific Research on Priority Areas and for Scientific Research (B) from the Ministry of Education, Science, Sports and Culture of Japan.

References

Aparicio,O.M., Weinstein,D.M. and Bell,S.P. (1997) Components and dynamics of DNA replication complexes in *S.cerevisiae*: redistribution of MCM proteins and Cdc45p during S phase. *Cell*, **91**, 59–69.

Blow,J.J. (1996) Chromosome replication in *Xenopus* egg extracts. In Blow,J.J. (ed.), *Eukaryotic DNA Replication*. IRL Press, New York, pp. 143–164.

Blow,J.J. and Laskey,R.A. (1986) Initiation of DNA replication in nuclei and purified DNA by a cell-free extract of *Xenopus* eggs. *Cell*, **47**, 577–587.

Blow,J.J. and Nurse,P. (1990) A cdc2-like protein is involved in the initiation of DNA replication in *Xenopus* egg extracts. *Cell*, **62**, 855–862.

Borowiec,J.A., Dean,F.B., Bullock,P.A. and Hurwitz,J. (1990) Binding and unwinding—how T antigen engages the SV40 origin of DNA replication. *Cell*, **60**, 181–184.

Challberg,M.D. and Kelly,T.J. (1989) Animal virus DNA replication. *Annu. Rev. Biochem.*, **58**, 671–717.

Chen,J., Jackson,P.K., Kirschner,M.W. and Dutta,A. (1995) Separate domains of p21 involved in the inhibition of Cdk kinase and PCNA. *Nature*, **374**, 386–388.

Chong,J.P.J., Thömmes,P. and Blow,J.J. (1996) The role of MCM/P1 proteins in the licensing of DNA replication. *Trends Biochem. Sci.*, **12**, 102–106.

Coleman,T.R., Carpenter,P.B. and Dunphy,W.G. (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.

Dahmann,C., Diffley,J.F. and Nasmyth,K.A. (1995) S phase-promoting cyclin dependent kinases prevent re-replication by inhibiting the transition of replication origins to a pre-replicative state. *Curr. Biol.*, **5**, 1257–1269.

Dalton,S. and Hopwood,B. (1997) Characterization of Cdc47p-minichromosome maintenance complexes in *Saccharomyces cerevisiae*: identification of Cdc45p as a subunit. *Mol. Cell. Biol.*, **17**, 5867–5875.

Dasso,M. and Newport,J.W. (1990) Completion of DNA replication is monitored by a feedback system that controls the initiation of mitosis *in vitro*: studies in *Xenopus*. *Cell*, **61**, 811–823.

Diffley,J.F.X., Cocker,J.H., Dowell,S.J. and Rowley,A. (1994) Two steps in the assembly of complexes at yeast replication origins *in vivo*. *Cell*, **78**, 303–316.

Donovan,S., Harwood,J., Drury,L.S. and Diffley,J.F.X. (1997) Cdc6p-dependent loading of Mcm proteins onto pre-replicative chromatin in budding yeast. *Proc. Natl Acad. Sci. USA*, **94**, 5611–5616.

Dutta,A. and Bell,S.P. (1997) Initiation of DNA replication in eukaryotic cells. *Annu. Rev. Cell. Dev. Biol.*, **13**, 293–332.

Fang,F. and Newport,J. (1991) Evidence that the G_1–S and G_2–M transitions are controlled by different cdc2 proteins in higher eukaryotes. *Cell*, **66**, 731–742.

Fanning,E. and Knippers,R. (1992) Structure and function of Simian virus 40 large tumor antigen. *Annu. Rev. Biochem.*, **61**, 55–85.

Hardy,C.F. (1997) Identification of Cdc45p, an essential factor required for DNA replication. *Gene*, **187**, 239–246.

Hennessy,K.M., Lee,A., Chen,E. and Botstein,D. (1991) A group of interacting yeast DNA replication genes. *Genes Dev.*, **5**, 958–969.

Hopwood,B. and Dalton,S. (1996) Cdc45p assembles into a complex with Cdc46p/Mcm5p, is required for minichromosome maintenance and is essential for chromosomal DNA replication. *Proc. Natl Acad. Sci. USA*, **93**, 12309–12314.

Hua,X.H., Yan,H. and Newport,J. (1997) A role for cdk2 kinase in negatively regulating DNA replication during S phase of the cell cycle. *J. Cell Biol.*, **137**, 183–192.

Jackson,P.K., Chevalier,S., Philippe,M. and Kirschner,M.W. (1995) Early events in DNA replication requires cyclin E and are blocked by $p21^{CIP1}$. *J. Cell Biol.*, **130**, 755–769.

Ishimi,Y. (1997) A DNA helicase activity is associated with an Mcm4,-6 and -7 protein complex. *J. Biol. Chem.*, **272**, 24508–24513.

Kearsey,S.E., Maiorano,D., Holmes,E.C. and Todorov,I.T. (1996) The role of MCM proteins in the cell cycle control of genome duplication. *BioEssays*, **18**, 183–190.

Kubota,Y. and Takisawa,H. (1993) Determination of initiation of DNA replication before and after nuclear formation in *Xenopus* egg cell free extracts. *J. Cell Biol.*, **123**, 1321–1331.

Kubota,Y., Mimura,S., Nishimoto,S., Takisawa,H. and Nojima,H. (1995) Identification of the yeast MCM3-related protein as a component of *Xenopus* DNA replication licensing factor. *Cell*, **81**, 601–609.

Kubota,Y., Mimura,S., Nishimoto,S., Masuda,T., Nojima,H. and Takisawa,H. (1997) Licensing of DNA replication by a multi-protein complex of MCM/P1 proteins in *Xenopus* eggs. *EMBO J.*, **16**, 3320–3331.

Mahbubani,H.M., Chong,J.P., Chevalier,S., Thömmes,P. and Blow,J.J. (1997) Cell cycle regulation of the replication licensing system: involvement of a cdk-dependent inhibitor. *J. Cell Biol.*, **136**, 125–135.

Moir,D., Stewart,S.E., Osmond,B.C. and Botstein,D. (1982) Cold-sensitive cell-division-cycle mutants of yeast: properties and pseudoreversion studies. *Genetics*, **100**, 547–563.

Nasmyth,K. (1996) Control of S phase. In DePamphilis,M.L. (ed.), *DNA Replication of Eukaryotic Cells*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, pp. 331–386.

Newport,J. (1987) Nuclear reconstitution *in vitro*: stages of assembly around protein-free DNA. *Cell*, **48**, 205–217.

Owens,J.C., Detweiler,C.S. and Li,J.J. (1997) *CDC45* is required in conjunction with *CDC7/DBF4* to trigger the initiation of DNA replication. *Proc. Natl Acad. Sci. USA*, **94**, 12521–12526.

Piatti,S., Bohm,T., Cocker,J.H., Diffley,J.F. and Nasmyth,K. (1996) Activation of S-phase-promoting CDKs in late G_1 defines a 'point of no return' after which Cdc6 synthesis cannot promote DNA replication in yeast. *Genes Dev.*, **10**, 1516–1531.

Romanowski,P., Madine,M.A., Rowles,A., Blow,J.J. and Laskey,R.A. (1996) The *Xenopus* origin recognition complex is essential for DNA replication and mcm binding to chromatin. *Curr. Biol.*, **6**, 1416–1425.

Rowles,A., Chong,J.P.J., Brown,L., Howell,M., Evan,G.I. and Blow,J.J. (1996) Interaction between the origin recognition complex and the replication licensing system in *Xenopus*. *Cell*, **87**, 287–296.

Stern,B. and Nurse,P. (1996) A quantitative model for the cdc2 control of S phase and mitosis in fission yeast. *Trends Genet.*, **12**, 345–350.

Stillman,B. (1989) Initiation of eukaryotic DNA replication *in vitro*. *Annu. Rev. Cell Biol.*, **5**, 197–245.

Stillman,B. (1996) Cell cycle control of DNA replication. *Science*, **274**, 1659–1664.

Strausfeld,U.P., Howell,M., Rempel,R., Maller,J.L., Hunt,T. and Blow,J.J. (1994) Cip1 blocks the initiation of DNA replication in *Xenopus* extracts by inhibition of cyclin-dependent kinases. *Curr. Biol.*, **4**, 876–883.

Strausfeld,U.P., Howell,M., Descombes,P., Chevalier,S., Rempel,R., Adamczewski,J., Maller,J.L., Hunt,T. and Blow,J.J. (1996) Both cyclin A and cyclin E have S-phase promoting (SPF) activity in *Xenopus* egg extracts. *J. Cell Sci.*, **109**, 1555–1563.

Tanaka,T., Knapp,D. and Nasmyth,K. (1997) Loading of an Mcm protein onto DNA replication origins is regulated by Cdc6p and CDKs. *Cell*, **90**, 649–660.

Thömmes,P., Kubota,Y., Takisawa,H. and Blow,J.J. (1997) The RLF-M component of the replication licensing system forms complexes containing all six MCM/P1 polypeptides. *EMBO J.*, **16**, 3312–3319.

Waga,S. and Stillman,B. (1994) Anatomy of a DNA replication fork revealed by reconstitution of SV40 DNA replication *in vitro*. *Nature*, **369**, 207–212.

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

Zou,L., Mitchell,J. and Stillman,B. (1997) CDC45, a novel yeast gene that functions with the origin recognition complex and Mcm proteins in initiation of DNA replication. *Mol. Cell. Biol.*, **17**, 553–563.

Received June 15, 1998; revised July 29, 1998; accepted August 11, 1998