Role for Cyclin A-dependent Kinase in DNA Replication in Human S Phase Cell Extracts*

(Received for publication, May 28, 1996, and in revised form, August 27, 1996)

Arun Foteder‡§, Dominique Cannella¶, Patrick Fitzgerald‡, Tristan Rousselle¶, Sunita Gupta¶, Marcel Doré¶, and Rati Foteder**

From the ‡Division of Molecular Biology, La Jolla Institute for Allergy and Immunology, La Jolla, California 92037, USA
the ¶Institut de Biologie Structurale, J.-P. Ebel, 41 Avenue des Martyrs, F-38027 Grenoble, Cedex 1, France and the
**CNRS UPR 9008, Centre de Recherches de Biochimie Macromoleculaire, B.P. 5051, 1919 route de Mende 34033
Montpellier Cedex, France

Cell cycle progression is regulated by cyclin-dependent kinases. Using in vitro replication of SV40 origin containing DNA as a model system, we have performed a detailed analysis of the dependence on cyclin-associated kinases of mammalian DNA replication. Complete immunodepletion of cyclin A from human S phase cell extracts decreases replication, and replication activity of cyclin A-depleted S phase extracts can subsequently be restored by the addition of purified CDK2-cyclin A kinase. Addition of cyclin A alone reconstitutes both kinase activity and DNA replication, whereas addition of cyclin E or cyclin B reconstitutes neither. We therefore conclude that reconstitution of DNA replication specifically correlates with an increase in kinase activity. By comparison, depletion of cyclin E from S phase cell extracts does not have any significant inhibitory effect on DNA replication. Moreover, specific p21mutant mutants that bind to CDK2-cyclin and inhibit both cyclin A and cyclin E kinase activities, but do not bind to proliferating cell nuclear antigen, inhibit DNA replication to the same extent as cyclin A depletion. Together, these results show that the kinase activity associated with cyclin A, but not with cyclin E, is primarily responsible for activating SV40 plasmid replication in mammalian S phase cell extracts. Finally, we present evidence that the cyclin-dependent kinase does not influence the assembly of initiation complexes but acts at a stage prior to elongation.

Replication of DNA is a strictly regulated event that occurs at a discrete period during the cell cycle. Cell cycle progression is regulated by distinct cyclin-dependent kinases that activate at different times in the cell cycle (reviewed in Ref. 1). In mammalian cells, cyclin E-dependent kinase is activated at the G1 to S phase transition, after the D type cyclins but prior to A type cyclins (reviewed in Ref. 2). Microinjection of either anti-cyclin A antibody (3, 4) or antisense cyclin A plasmid (3, 5) into exponentially growing cells prevents the entry of cells into S phase. Similarly, microinjection of anti-cyclin E antibody also prevents the entry of cells into S phase (6). However, unlike cyclin E-dependent kinase activity, the timing of activation of cyclin A-associated kinase activity in human cells coincides with the onset of DNA synthesis in S phase, which occurs several hours subsequent to the commitment to S phase (7–9). Cyclin A may therefore have a direct role in DNA replication in S phase.

Cyclin-dependent kinases have been implicated as inducers of DNA replication using systems for in vitro replication of DNA. p13suc1, the product of Schizosaccharomyces pombe suc1 gene, binds avidly to active forms of CDC2 and CDK2 (10–12). The removal of Cdk2 and Cdc2 proteins from an in vitro Xenopus egg extract replication system, using p13suc1 affinity matrices, has been shown to decrease its ability to replicate sperm DNA (13, 14). Specific depletion of Cdk2 protein, but not of Cdc2 protein from Xenopus egg extracts, has been shown to correlate with a decreased ability of these extracts to replicate sperm DNA (15). Recently, a similar inhibition of DNA replication was observed following depletion of cyclin E from Xenopus egg extracts (16).

In the SV40 in vitro replication system the inability of human G1 extracts to replicate SV40 origin containing DNA (17) can be overcome by the addition of cyclin A (18) or an active CDC2 kinase (19). Both cyclin A and Cdk2 are associated with SV40 origin containing DNA during replication in vitro (20). RPA, a cellular single-stranded DNA binding protein, and SV40 T antigen are both phosphorylated by Cdc2-associated kinase in vitro. Phosphorylation of bacterially expressed T antigen bycdc2 kinase increases its affinity for the SV40 origin of replication (21). However, the stimulation of DNA replication by CDC2 kinase in G1 cell extracts was shown not to be due to phosphorylation of T antigen by CDC2 kinase (18, 19). Instead, these studies suggested that phosphorylation of the 34-kDa subunit of RPA during G1 to S phase transition may contribute to the activation of origin unwinding and subsequent DNA replication. The functional significance of phosphorylation of the 34-kDa subunit of RPA by CDC2 kinase (19, 22) remains to be established, since mutation of the CDC2 consensus phosphorylation sites on RPA-34 has no effect on in vitro SV40 replication (23).

Although previous studies, discussed above, have implicated cdk-cyclins in DNA replication, such studies have not explicitly addressed whether it is the kinase activity itself which is important for DNA replication in S phase or if the cdk-cyclin plays a structural role in replication. Reconstitution of replication activity in G1 extracts by the addition of cyclin A or of Cdc2 kinase demonstrates a role for cdk-cyclins in activating events.
in G1, but it does not address whether the kinase is required during replication in S phase cell extracts (18, 19). Here we have used the SV40 in vitro replication system to examine the regulation of DNA replication by cyclin-associated kinases in S phase cell extracts, and we have specifically addressed whether cyclin-dependent kinase activity is important for DNA replication. Furthermore, since both cyclins A and E are present in S phase cell extracts, we have addressed whether the cyclin A or the cyclin E-dependent kinase has a specific function during DNA replication.

We show that cyclin A is specifically required for efficient replication of DNA in S phase, since complete immunodepletion of cyclin A-associated kinase from S phase cell extracts leads to a decrease in the ability of these extracts to support DNA replication. Addition of either purified cyclin A-associated CDK2 kinase or of purified cyclin A alone, but not of cyclin E or cyclin B, fully restores replication activity in these depleted extracts. Furthermore, an increase in kinase activity is specifically required for the reconstitution of replication activity of cyclin A-depleted cell extracts.

We have also determined if DNA replication can be suppressed by specific interaction of p21Waf1/Cip1, an inhibitor of cyclin-dependent kinases (24–26), with Cdk-cyclin complexes. Since p21 protein also binds to and inhibits PCNA (27, 28), a cyclin-dependent kinases (24–26), with Cdk-cyclin complexes.

Finally, and most importantly, we show that the cyclin-dependant kinase is not required for the formation of initiation complexes but is rate-limiting at a stage before elongation.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Extracts**—Manca cells (a human Burkitt lymphoma line) were grown in spinner flasks with RPMI 1640 containing 5% calf bovine serum and 2 mM l-glutamine (29, 30). Exponentially growing lines (s) were grown in spinner flasks with RPMI 1640 containing 5% calf serum and 2 mM l-glutamine (29, 30). Exponentially growing cells were blocked with 2 mM hydroxyurea for 12 h to obtain Manca cells in S phase. Released cells were stained with propidium iodide and analyzed for DNA content by flow cytometric analysis. S-100 supernatants containing 100 mM NaCl were made from hypotonic lysates of Manca cells in S phase as described earlier (29, 30).

**Replication Reactions**—Replication reactions (31) were performed for 90 min at 37°C with S-200 S phase Manca cell extracts (100 μg) and with SV40 origin containing plasmids (150 ng) in the presence of 3 mM ATP to allow the formation of initiation complexes on DNA. In the second step, elongation was initiated by the addition of the remaining ribonucleoside triphosphates, ATP and dATP. At this time the reaction was given a pulse of [α-32P]dCTP for 30 s (33). The elongation reaction was limited during the pulse by the absence of dCTP. The pulse was terminated by adding 100-fold excess cold dCTP, and dCTP was added to the reaction to allow elongation during the chase. The reaction was then allowed to continue at 37°C. Aliquots were removed at various time points during the chase, and replication was terminated at each time point by adding an equal volume of 0.4% SDS and 100 mM EDTA. The reaction products were then digested with proteinase K at 37°C for 1 h. Finally, the samples were extracted with equal volume of chloroform-precipitated with ethanol, and resuspended in 50 mM NaOH, 1 mM EDTA, and then analyzed on a 1.2% agarose gel in 30 mM NaOH, 1 mM EDTA (34). After electrophoresis, the agarose gel was fixed in 10% trichloroacetic acid for 20 min followed by 15 min in 10% acetic acid, 12% methanol. The gel was then rinsed with distilled water, dried on DE81 paper (Whatmann), and autoradiographed.

**Immunoblotting**—Rabbit polyclonal antiserum raised against bacterially expressed human cyclin A and affinity purified antibodies, were kindly provided to us by M. Ohtsubo and J. Roberts (Fred Hutchinson Cancer Research Center, Seattle). The specificity of anti-cyclin A antibody has been confirmed using recombinant cyclin proteins.SPECIFICITY OF CYCLIN A ANTIBODY—The specificity of the cyclin A antibody was determined by immunoprecipitating cyclin A from S phase cell extracts and assaying for kinase activity (30). Rabbit polyclonal antisera was also raised against synthetic peptide corresponding to the C-terminal sequences of human CDK2 (CDVTKEVPHLRL) (30). Antiserum to CDK2 was affinity purified on a Sepharose 4B column containing covalently coupled peptide. Monoclonal antibody recognizing human PCNA was purchased from Coulter Immunology (Clone 19A2) and monoclonal antibody recognizing cyclin E was purchased from Oncogene.

**Immunodepletion of Cyclin A from S Phase Cell Extracts**—All the steps of immunodepletion were performed at 4°C. S phase Manca cell extract (25 mg of S-100 cell extract) was adjusted to 40 mM Hpes (pH 7.5), 8 mM MgCl2, 100 mM NaCl, 0.5% Nonidet P-40, 1 μg/ml each aprotinin and leupeptin (IP buffer) and loaded on a column containing 1 ml of packed protein A-Sepharose (Sigma) coupled to anti-cyclin A antibody. Extracts passed over Protein A-Sepharose coupled with non-immune rabbit immunoglobulin were similarly treated and used as controls. After 20 passages through the column, the flow-through comprising the depleted cell extract was collected, aliquoted, and stored at −70°C for further use in the replication assay. The columns were regenerated with 100 mM glycine HCl (pH 3.0) after use, washed with IP buffer without Nonidet P-40, and stored in the presence of 0.02% sodium azide.

**Protein Kinase Assays**—All the steps were performed at 4°C and the tubes were gently rocked during the incubations. 100 μg of S phase cell extract was adjusted to 40 mM Hpes, 8 mM MgCl2, 100 mM NaCl, 0.5% Nonidet P-40, 1 μg/ml each aprotinin and leupeptin (IP buffer) and added to 10 μl of packed Protein A-Sepharose (Sigma) that had been preincubated with anti-cyclin A antibody for 1 h and washed five times with IP buffer. After 1 h, Protein A-Sepharose containing the cyclin A immunocomplexes was washed three times with IP buffer and twice with kinase buffer (40 mM Hpes, 8 mM MgCl2). Kinase assays were performed in 15 μl of kinase reaction mixture containing 40 mM Hpes, 8 mM MgCl2, 166 μM ATP, 5 μCi of [γ-32P]ATP (DuPont NEN, 3,000 Ci/mmol), 4 μg of histone H-1 (Boehringer Mannheim), and 10 μl of packed Protein A-Sepharose as described earlier (30). After 20 min at 37°C, the reactions were stopped by adding SDS sample buffer. Two-fifths of the reactions were loaded on 12% SDS-polyacrylamide gels. The gels were stained with Coomassie Brilliant Blue, dried, and autoradiographed. For quantitation, the histone H-1 bands were excised from the gel and subjected to scintillation counting.

**Immunoblotting**—Proteins were resolved on 12% SDS-polyacrylamide gels and immunoblotted as described earlier (30). The nitrocellulose filters were processed for ECL (enhanced chemiluminescence system; Pharmacia Biotech Inc.) Western blot procedure as instructed by the suppliers.

**Generation of Recombinant Full-length p21 and Deletion Mutants of p21**—The full-length p21 cDNA was generated by reverse transcription of Jurkat poly(A) RNA, followed by polymerase chain reaction (PCR) using two sets of nested p21-specific primers. Sequence analysis revealed that the PCR product was identical in sequence to the published human p21 cDNA (35). The deletion mutants of p21 were generated by

---

1 The abbreviations used are: PCNA, proliferating cell nuclear antecedent; GST, glutathione S-transferase; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; RPA, replication factor A.
RESULTS

Cyclin A Is Required for Efficient In Vitro DNA Replication—
Cyclin A was immunodepleted from S phase cell extracts using a cyclin A-specific antibody, under conditions that were compatible with subsequent use of the depleted cell extract for replication assay, as described under “Experimental Procedures.” Following depletion, there was no detectable cyclin A in the depleted extract (Fig. 1). Control S phase extracts treated identically with nonimmune rabbit antibody completely retain cyclin A (Fig. 1). Cyclin A has been reported to be in a quaternary complex with proliferating nuclear cell antigen (PCNA), p21, and Cdk2 in nontransformed cells (43). We therefore determined if depletion of cyclin A resulted in a loss of PCNA that could consequently affect the replication activity of the extracts. Fig. 1 shows that the level of PCNA was not significantly different following depletion of cyclin A from S phase cell extracts, in comparison to control extracts (Fig. 1).

Depletion of cyclin A from S phase extracts results in complete loss of cyclin A-associated H-1 kinase activity (Fig. 1), although the amount of Cdk2 protein does not change significantly after depletion (Fig. 1). There is a substantial loss of Cdk2-associated kinase activity in cyclin A-depleted extracts (Fig. 1). This result suggests that the active form of Cdk2 in S phase extracts is predominantly associated with cyclin A and constitutes a small fraction of the total Cdk2. In keeping with this result, the kinase activity associated with cyclin E in these cell extracts was 10-fold lower than that associated with cyclin A or Cdk2 (data not shown).

DNA replication in control extracts treated with nonimmune rabbit antibody is largely completed by 90 min (data not shown). The extent of DNA synthesis in cyclin A-depleted S phase cell extracts was therefore compared with control extracts at the onset of replication, and the amount of DNA synthesized was determined at 90 min. Replication in the control extract is shown for reference. The cyclin A-associated kinase activity in control extracts and in cyclin A-depleted extracts is 240 and 10.3 pmol/20 min/18 µl, respectively. These values were obtained by immunoprecipitating cyclin A from control and cyclin A-depleted extracts using an anti-cyclin A antibody. The cyclin A immunoprecipitates were then tested for their ability to phosphorylate histone H-1. The products of kinase reactions were run on SDS-PAGE, and the histone H-1 bands were subsequently excised and counted.

DNA replication is shown in cyclin A-depleted and control S phase cell extracts from four independently obtained S phase cell extracts, each subsequently immunodepleted.

Preparation of Cyclin A-CDK2 and Cyclin B-Cdk2 Kinase—Cyclin B-Cdk2 kinase was purified to apparent homogeneity from maturing starfish oocytes as described previously (37). Recombinant cyclin A-CDK2 kinase was prepared from human cyclin A and GST-CDK2, both produced in E. coli and purified as described in Lorea et al. (36). Highly purified CAR (Cdk Activating Kinase), purified to the Mono S step from starfish oocytes (39), was used to stoichiometrically phosphorylate GST-Cdk2 in a mixture containing 0.33 mM ATP, 16.6 µM MgCl₂, 30 µg/ml GST-cdk2, and 100 µg/ml cyclin A in 10 mM Tris (pH 7.5). The GST-CDK2-cyclin A kinase was then purified using glutathione affinity matrix by standard procedures.

Purification of GST-Cyclins—GST-cyclin A (40), GST-cyclin B (41), and GST-cyclin E (42) were produced in bacteria and purified by identical procedures using a glutathione affinity matrix as described (36).
the inhibition is not due to dephosphorylation of previously phosphorylated proteins required for replication.

Complete depletion of cyclin A from S phase extracts did not lead to total inhibition of DNA replication. To test if cyclin E kinase activity may be responsible for activating the residual levels of DNA replication, cyclin E was immunodepleted from S phase extracts. When these extracts were tested for replication activity, there was only a 10–15% reduction of DNA replication compared with control extracts. Similarly, immunodepletion of both cyclin A and cyclin E from S phase extracts results in only an additional 10–15% inhibition of DNA replication, compared with extracts depleted of cyclin A alone. Depletion of cyclin E therefore does not have a significant inhibitory effect on DNA replication.

**Cyclin A Restores DNA Replication and Cyclin A-associated Kinase Activity in Cyclin A-depleted Cell Extracts**—To demonstrate that the inhibition of DNA replication is indeed due to depletion of cyclin A and not due to depletion of another protein associated with cyclin A, we tested the ability of CDK2-associated cyclin A kinase to restore DNA replication in cyclin A-depleted cell extracts. Fig. 3 shows that purified cyclin A-CDK2 kinase fully rescues DNA replication in depleted extracts and brings replication to the level observed in control cell extracts. In contrast, no more than 1–2 pmol of dCMP are incorporated in a control experiment, where addition of cyclin A is performed in the absence of T antigen. This low level of incorporation probably represents repair.

DNA replication was reconstituted by the addition of exogenous cyclin A-CDK2 kinase (final specific activity 98.2 pmol/20 min/18 µl) to cyclin A-depleted extracts. The kinase activity of cyclin A-depleted extracts is 10.3 pmol/20 min/18 µl. The addition of kinase activity that fully restores replication is less than the activity measured in control extracts (240 pmol/20 min/18 µl). These results indicate that replication can be fully reconstituted by kinase activity that is substantially below control levels.

We also tested whether purified GST-cyclin A alone could rescue DNA replication activity of cyclin A-depleted cell extracts. Fig. 4A shows that the inhibition of DNA replication was fully rescued by addition of cyclin A at a concentration of 80 nM. Furthermore, the addition of cyclin A to cell extracts depleted of cyclin A restores cyclin A-associated H-1 kinase activity (Fig. 4B). DNA replication in cyclin A-depleted extracts is fully restored by kinase activity (88 pmol/20 min/18 µl) which is 2.3-fold lower than that in control extracts. The increase in kinase activity associated with cyclin A (Fig. 4B) correlates with an increase in CDK2-associated kinase activity (Fig. 4C) and probably reflects the association of added cyclin A with endogenous CDK2. This conclusion is supported by the observation that the kinase activity associated with CDC2 remains unchanged upon the addition of GST-cyclin A (Fig. 4C).

The ability to restore replication in cyclin A-depleted cell extracts was specific for cyclin A addition, since cyclin E or cyclin B could not rescue replication (Fig. 5A) or restore kinase activity (Fig. 5B) in these cell extracts. The failure of cyclin E to restore replication in cyclin A-depleted extracts is not due to an intrinsic inability of this cyclin E to form an active kinase. Both the cyclin E and cyclin B used for these experiments exhibited H-1 kinase activity when incubated with insect cell extracts that contained baculovirus expressed human CDK2 and CDC2, respectively. Together, these results imply that reconstitution of DNA replication in cyclin A-depleted extracts requires an
increase in cyclin-associated kinase activity and that cyclins E and B specifically fail to form an active kinase in S phase mammalian cell extracts. It is important to note that all GST-cyclins were prepared by identical methods.

To address whether a cyclin A-associated kinase activity, and not cyclin A alone, is specifically required for reconstitution of replication, we tested if cyclin B-Cdc2 kinase could rescue replication in cyclin A-depleted extracts. Fig. 5 shows that purified cyclin B-Cdc2 kinase rescues DNA replication of cyclin A-depleted cell extracts. These results suggest that the inhibition of DNA replication in cyclin A-depleted cell extracts is due to the loss of kinase activity, as it can be rescued by a homologous protein kinase which has similar in vitro substrate specificities (reviewed in Ref. 44). Fig. 5C also shows that cyclin B-Cdc2 kinase and cyclin A-CDK2 kinase are both equally effective in restoring DNA replication in cyclin A-depleted extracts.

p21 Mutants That Bind Cdk-Cyclin Inhibit DNA Replication—Having demonstrated a role for cyclin A in DNA replication, we wanted to examine the effect of p21 in suppressing Cdk-cyclin A and E-dependent activation of DNA replication. The full-length p21 binds to both Cdk-cyclin and to PCNA. Addition of full-length p21 inhibits DNA replication in the SV40 replication system (27, 28) and in Xenopus egg extracts (45–47). In order to distinguish the effect on DNA replication due to p21 binding to cdk-cyclin as opposed to PCNA, we have generated a panel of deletion mutants of p21 as GST-p21 fusion proteins (36). The characteristics of these mutants are summarized in Fig. 6. Full-length p21 (containing residues 1–164) and p21 deletion mutants containing N-terminal residues 1–132, 1–103, or 1–82 bind CDK2, cyclin A, cyclin E, and inhibit both cyclin A and cyclin E kinase activities. These p21 deletion mutants do not bind PCNA. In contrast, the C-termi-

---

**FIG. 5.** Cyclin and cyclin-dependent kinase requirement for restoration of DNA replication. A, cyclin E addition does not restore replication activity of cyclin A-depleted S phase cell extracts. Purified GST-cyclin A, or GST-cyclin E, or GST-cyclin B was added to replication reactions containing 100 μg of cyclin A-depleted cell extracts at a final concentration of 80 nM. DNA synthesis was measured at 90 min. B, cyclin E addition fails to restore kinase activity of cyclin A-depleted S phase cell extracts. Either purified GST-cyclin E or GST-cyclin B was added to reactions containing 100 μg of cell extract that had been depleted of cyclin A. For assay, either cyclin E or cyclin B were then immunoprecipitated using specific antibodies, and these cyclin immunoprecipitates were then tested for their ability to phosphorylate histone H-1. For reference we show the cyclin-associated histone H-1 kinase activity remaining in the cyclin A-depleted cell extract. C, addition of a homologous kinase restores replication activity of cyclin A-depleted cell extract. Approximately 83 and 136 pmol/20 min/18 μl of purified cyclin B-Cdc2 kinase or cyclin A-CDK2 kinase were added back to cyclin A-depleted cell extracts at the onset of replication, and the amount of DNA synthesized was determined at 90 min. Percent of control DNA synthesis in cyclin A-depleted extracts without addition of kinase is also shown as a control (No addition). All values were measured as a percent of control undepleted extracts.

---

**FIG. 6.** Deletion mutants of p21. Top, schematic representation of the p21 mutants used in this study. Bottom, the abilities of these p21 mutants to bind Cdk-cyclin and PCNA are summarized, and the kinase inhibitory characteristics of these mutants are also listed. Wild type p21 (1–164) and p21 mutants (1–132), (1–103), and (1–82) inhibit both cyclin A-cdk2 and cyclin E-cdk2 kinase activity (36).

---

**Cyclin A in DNA Replication**

31631
We determined the comparative abilities of full-length GST-p21 or mutant GST-p21 proteins with T antigen to SV40 replication reactions. After addition the reactions were continued for 90 min. The values (pmol of dCMP incorporated) are plotted as % DNA replication relative to values obtained in control assays without added p21. DNA synthesis in control assays without added p21 was 39 ± 2.3 pmol of dCMP incorporated per 90 min/50-μl reaction. B, replication in cyclin A-depleted extracts is not inhibited by p21 mutant-(1–82) which binds cdk-cyclin. p21 mutant-(1–82) was added at a final concentration of 4 μM to replication reactions. DNA synthesis at 90 min is shown.

Cyclin A in DNA Replication

Fig. 7. Inhibition of SV40 origin-dependent DNA replication by p21 mutants that bind cdk-cyclin. A, varying amounts of full-length GST-p21 or mutant GST-p21 proteins were added with T antigen to SV40 replication reactions. After addition the reactions were continued for 90 min. The values (pmol of dCMP incorporated) are plotted as % DNA replication relative to values obtained in control assays without added p21. DNA synthesis in control assays without added p21 was 39 ± 2.3 pmol of dCMP incorporated per 90 min/50-μl reaction. B, replication in cyclin A-depleted extracts is not inhibited by p21 mutant-(1–82) which binds cdk-cyclin. p21 mutant-(1–82) was added at a final concentration of 4 μM to replication reactions. DNA synthesis at 90 min is shown.

We determined the comparative abilities of full-length p21 and of our panel of p21 deletion mutants to inhibit in vitro replication of SV40 origin containing plasmids. Full-length p21, which binds to both Cdk-cyclin and PCNA, effectively inhibits DNA replication (Fig. 7A), whereas GST control protein is not inhibitory. When added at initiation of replication, p21 mutants containing residues 1–132, 1–103, or 1–82, which bind Cdk-cyclins and inhibit Cdk2-cyclin A and E kinase activities, but do not bind PCNA, inhibit DNA replication (Fig. 7A). These results thus demonstrate that the cdk-cyclin binding domain of p21 alone can inhibit DNA replication. This conclusion is supported by the demonstration that p21 mutant (1–82), which binds cdk-cyclin and inhibits kinase activity, does not further inhibit DNA replication in cyclin A-depleted extract (Fig. 7B). These findings are consistent with our results (Fig. 2) in which depletion of cyclin A from S phase extracts inhibited DNA replication to the same extent. In addition, these results are consistent with our observation that depletion of cyclin E from S phase extracts does not have a significant inhibitory effect on DNA replication.

Effect of Cyclin-dependent Kinase at Different Stages of Replication—DNA replication can be divided into two phases, initiation and elongation. Initiation of DNA replication at the SV40 origin involves multiple steps. First, SV40 T antigen binds to the sequences within the core origin leading to structural distortion and local untwisting of the DNA (for a review see Ref. 48). Subsequently, the DNA helicase property of T antigen leads to unwinding of the origin in a process that requires ATP, RPA (a single-stranded DNA binding protein), and topoisomerase I (49–52). Finally, DNA polymerase α-prime complex associates with the unwound origin.3 These events lead to the synthesis of RNA primers that are used for subsequent leading and lagging strand DNA replication during the elongation phase (for review see Ref. 53). Kinetic analysis of SV40 replication in vitro reveals that a lag of 10–15 min precedes the start of DNA synthesis (see Fig. 9A) (32, 52, 54, 55). This lag represents the time required for SV40 T antigen to bind to the origin and form a complex with RPA in the initiation phase, resulting in origin unwinding (32, 52).

To determine whether cyclin-dependent kinase activity is required for the initiation or the elongation phase of DNA replication, we have used a two-step replication assay. In the first step, plasmid DNA containing the SV40 origin of replication is incubated for 30 min under conditions that only allow initiation to occur (29). For this purpose the start of DNA synthesis is prevented by the omission of deoxyribonucleoside and ribonucleoside triphosphates, whereas ATP is present to enable T antigen-dependent DNA unwinding and formation of the initiation complex (32). Under these conditions, no measurable DNA synthesis occurs. In the second step, DNA synthesis is initiated by addition of deoxyribonucleoside and ribonucleoside triphosphates to preincubated reactions.

Here we have used the two-step replication assay to address whether the addition of cyclin A to extracts depleted of cyclin A was necessary during the initiation phase of DNA replication. Fig. 8A shows that cyclin A is equally effective in reconstituting replication if added at the beginning of the initiation phase or at the induction of the elongation phase. However, cyclin A failed to restore replication in cyclin A-depleted extracts when added 5 min after the elongation step was induced (Fig. 8B).

We have described p21 mutants that inhibit Cdk2-cyclin A and E kinase activities and suppress DNA synthesis when added at initiation of replication (Fig. 7). Using the same two-step replication assay, we tested whether these mutants might also exert a specific effect during DNA elongation. We find that p21 mutants (containing residues 1–132, 1–103, or 1–82) that bind and inhibit Cdk2-cyclin A and E kinases, but do not bind PCNA, fail to inhibit DNA synthesis when added during elongation (Fig. 8C). In contrast, p21 mutant 76–164, which only binds PCNA, effectively inhibits DNA replication in the elongation phase. As expected, full-length p21, which binds both cdk-cyclins and PCNA, also inhibits the DNA elongation phase (Fig. 8C).

These results suggest that while cyclin-dependent kinase activates elongation phase (Fig. 8A), the addition of cyclin A to

---

2 A. Fotedar, D. Cannella, P. Fitzgerald, T. Rousselle, and R. Fotedar, unpublished observations.

3 Cannella, D., Roberts, J., and Fotedar, R. (1996) Chromosome, in press.
depleted extracts or the addition of p21 to normal extracts is without effect once elongation has begun.

Cyclin-dependent Kinase Is Not Required to Assemble Initiation Complexes—The stimulation of DNA replication in cyclin A-depleted extracts by the addition of cyclin A at the onset of elongation suggests that cyclin A-associated kinase activity is not essential for the assembly of an initiation complex (Fig. 8A). An initiation complex consisting of SV40 T antigen and RPA forms at the origin during the initiation phase. Cyclin-dependent kinases phosphorylate bacterially expressed SV40 T antigen, resulting in the binding of T antigen to site II ori DNA (the minimal origin sequence) (21, 56). The SV40 T antigen that we used in our experiments was purified from insect cells infected with the baculovirus vector and was therefore already maximally phosphorylated on the Cdc2/Cdk2 phosphorylation site, Thr-124 (57). It is therefore not likely that cyclin-dependent kinases activate T antigen. It is also unlikely that RPA function is affected by cyclin-dependent kinase. Although the 34-kDa subunit of RPA is a substrate for both the G1 or S phase cyclin-dependent kinases \textit{in vitro} (19), mutation of the two consensus CDC2 phosphorylation sites in RPA-34 has no effect \textit{in vitro} DNA replication (58).

It should be noted that using the \textit{in vitro} SV40 replication system, we have previously shown that phosphorylation of RPA-34 results from the binding of RPA to single-stranded DNA generated at the origin during initiation of replication (29). This phosphorylation of RPA-34 is not dependent on cyclin-associated kinase activity as it occurs normally in both p13 \textit{suc1}-depleted cell extracts (29) and in cyclin A-depleted cell extracts (data not shown). These results imply that the single-stranded DNA binding function of RPA is not altered by the lack of cyclin-dependent phosphorylation in S phase cell extracts. The kinase responsible for the phosphorylation of DNA-associated RPA-34 has been identified as DNA-dependent protein kinase (59).

It therefore appears unlikely that cyclin-dependent kinase affects either T antigen or RPA function in the \textit{in vitro} system. The kinase therefore may be required to either activate the assembled initiation complex or to stimulate elongation. If this model is correct, addition of cyclin A at the time of elongation will have an immediate stimulatory effect on elongation, since assembly of initiation complexes leads to a lag of 15 min preceding the start of DNA synthesis (Fig. 9A) (32, 52, 54, 55). If cyclin A were instead stimulating the formation of new initiation complexes, there would be a lag before elongation is stimulated. Fig. 9B shows that addition of cyclin A to cyclin A-depleted extracts at elongation has an immediate stimulatory effect on DNA elongation.

Taken together, these results suggest that cyclin-dependent kinase activity is not required to assemble initiation complexes but that it may play a role in elongation.

Cyclin A in DNA Replication

**FIG. 8.** Cyclin-dependent kinase activity is required for an activation step prior to DNA elongation. \textit{A,} to determine the ability of GST-cyclin A to restore DNA synthesis in cyclin A-depleted S phase extracts at different phases of DNA replication, the experiment was performed in two steps (see “Experimental Procedures”). For this, SV40 T antigen was preincubated with S phase cell extracts and SV40 origin containing DNA in the presence of ATP to allow the formation of initiation complexes on DNA. Elongation was then initiated by the addition of ribonucleoside triphosphates and deoxyribonucleoside triphosphates. The amount of DNA synthesis was measured after 30 min of elongation. \textit{B,} GST-cyclin A was added to reactions containing cyclin A-depleted S phase extracts either at the onset of initiation, or at the onset of elongation, or 5 min after elongation had started. Values are expressed as percent of control replication. \textit{C,} mutant p21 proteins that bind CDK2-cyclin but do not bind PCNA fail to inhibit the elongation process of SV40 origin-dependent DNA replication. To determine the effect of p21 on the elongation phase of DNA replication, the experiment was performed in two steps as above. After 10 min of elongation, either GST-p21 or GST-p21 mutant proteins or GST control protein was added at a final concentration of 4 μM, and the reactions were allowed to continue for an additional 20 min (total time of elongation = 30 min). DNA synthesis (pmol of dCMP incorporated per 50-μl reaction) was measured at 30 min in control reactions and in reactions containing the added protein. The amount of DNA synthesis measured after 10 min of elongation in control reactions indicates the level of replication obtained at the time when p21 or GST control proteins are added. Data are plotted as the mean ± the standard deviation for three separate experiments.
To further determine if the depletion of cyclin A affects elongation rates, we undertook pulse-chase experiments (Fig. 10C). DNA elongation was initiated by the addition of ribonucleoside triphosphates dTTP and dATP to preincubated reactions and pulsed with \( [\alpha-\text{P}]dCTP \). After 30 s, dGTP was added, and the elongation products were chased with excess unlabeled dCTP. As expected, fewer elongation products are formed at each time point in cyclin A-depleted extracts. To compare the chase of low molecular weight elongation products into the high molecular weight fraction in the two extracts at equivalent density, the autoradiographs were exposed for different times as indicated in the figure (Fig. 10C). After 1 min the two reactions were not distinguishable, suggesting that the dominant effect of cyclin A depletion was before elongation started.

**DISCUSSION**

Cyclin-associated Kinase Activity Restores DNA Replication in Cyclin A-depleted S Phase Cell Extracts—We have now demonstrated that efficient DNA replication is restored upon addition of cyclin A to extracts depleted of cyclin A and that this reconstitution is accompanied by an increase in cyclin A-associated kinase activity (Fig. 4). The parallel increase in CDK2-associated kinase activity probably reflects the association of added cyclin A with endogenous CDK2. The active form of CDK2 in S phase extracts, which constitutes a small fraction of the total CDK2 (Fig. 1), is predominantly associated with cyclin A. We have further demonstrated that the ability to restore replication in cyclin A-depleted S phase cell extracts correlates with an increase in cyclin-dependent kinase activity as the addition of cyclin E or cyclin B cannot restore kinase activity or replication in these cell extracts (Fig. 5, A and B). Cyclin-dependent kinase activity per se is permissive for reconstitution of replication in the *in vitro* system, since addition of an active cyclin B-Cdc2 kinase fully restores replication (Fig. 5C). In the previous studies, replication of p13*sucl*-depleted (13) or Cdk2-depleted (15) *Xenopus* egg extracts was shown to be restored by the addition of proteins from the p13*sucl*-bound fraction. However, in those studies, as well as in the recent report by Jackson et al. (16), restoration of kinase activity in the depleted extracts was not determined.

Kinase Activity Associated with Cyclin A but Not Cyclin E Activates DNA Replication in Human S Phase Cell Extracts—Complete depletion of cyclin A from S phase cell extracts does not lead to total inhibition of DNA replication *in vitro* but only inhibits replication approximately 50%. These results are in accord with partial inhibition of replication reported by others (13, 15) following total depletion of Cdk2. Although cyclin E-associated kinase activity appears during G1, before the cyclin A-dependent kinase activity is apparent (60, 61), our results suggest that the failure to completely inhibit DNA replication by depletion of cyclin A does not result from a significant contribution of cyclin E to the replication process. Our evidence that cyclin E does not substantially contribute to DNA replication in mammalian cells is the following. DNA replication in the presence of p21 mutants that effectively bind and inhibit both cyclin A and cyclin E kinase activities was similar to that observed in extracts depleted of cyclin A alone. Furthermore, the depletion of both cyclin E and cyclin A from S phase cell extracts results in only slightly more (10–15%) inhibition of DNA replication than with cyclin A depletion. While both GST-cyclin A and GST-cyclin E exhibit comparable ability to activate baculovirus expressed CDK2, exogenous cyclin E fails to form an active kinase in S phase cell extracts and thus fails to rescue replication in depleted extracts, whereas addition of cyclin A effects a rescue. Taken together, these results suggest that cyclin A must be the predominant cyclin which stimulates

---

**FIG. 9.** Cyclin-dependent kinase activity is required to activate assembled initiation complexes. A, replication reactions were performed with 150 ng of SV40 origin containing DNA and 100 μg of control cell extracts. DNA synthesis was then measured at the indicated times. A lag of 15 min precedes the start of DNA synthesis. B, replication reactions were performed in two steps. GST-cyclin A was added to reactions containing cyclin A-depleted S phase extracts after initiation, at the onset of elongation (filled squares). DNA synthesis was then measured at the indicated times during the elongation phase. The data shown represent the means for three separate experiments. Standard deviation of the data shown do not exceed 10%. Elongation in cyclin A-depleted extracts, treated identically, is shown for comparison (open squares).

In cyclin A-depleted extracts, DNA elongation was started by the addition of ribonucleoside and deoxyribonucleoside triphosphates and \( [\alpha-\text{P}]dCTP \) to preincubated reactions. A comparison of the replication elongation products at various times during the reaction reveals that fewer elongation products are formed at each time point in cyclin A-depleted extracts as compared with control extracts (Fig. 10A). This experiment also shows that when comparing cyclin A-depleted extracts with control extracts, there is no difference in the size of the elongation products at each of the several time points. The final product of replication, covalently closed circular DNA (34, 52), is seen in both control and cyclin A-depleted extracts. Quantitation of the replication products formed at various times shows lower levels of replication in cyclin A-depleted extracts as compared with control extracts (Fig. 10B). This result is consistent with the conclusion that fewer elongation complexes are activated in cyclin A-depleted extracts.
FIG. 10. Effect of cyclin A on DNA elongation. A, DNA elongation in cyclin A-depleted and control extracts was determined by performing the experiment in two steps (see “Experimental Procedures”). In the first step, SV40 T antigen was preincubated with cell extracts and SV40 origin containing DNA in the presence of ATP to allow the formation of initiation complexes on DNA. After initiation complexes were formed on DNA, elongation was initiated by the addition of ribonucleoside and deoxyribonucleoside triphosphates and \( [\alpha^{32}\text{P}]\)dCTP. Aliquots were removed at the indicated time points (15 s to 5 min), and DNA was isolated and analyzed on a 1.2% alkaline agarose gel. Covalently closed circular DNA (ccc) and single-stranded linear (ssl) elongation full-length products are indicated. Molecular weight markers (in kilobases) were estimated from the position of \( ^{32}\text{P}-\text{labeled HindIII}-\text{digested DNA run in parallel. B, quantitation of replication products by trichloroacetic acid precipitation at various times (15 s to 15 min) during the reaction. C, pulse-chase analysis of DNA elongation rates in cyclin A-depleted and control extracts. This experiment was performed in two steps as in A with the following modification. After initiation complexes were formed on DNA, elongation was initiated by the addition of ribonucleoside triphosphates, dTTP and dATP. After a 30-s pulse with \([\alpha^{32}\text{P}]\)dCTP, dGTP and 100-fold excess cold dCTP were added to the reaction to initiate the chase. Aliquots were removed at the indicated time points during the chase, and DNA was isolated and analyzed on a 1.2% alkaline agarose gel. To compare the chase of low molecular weight elongation products into the high molecular weight fraction in the two extracts at equivalent density, the autoradiographs were exposed for different times as indicated.
DNA replication during S phase in mammalian cells. Cyclin E may contribute to a small extent, consistent with the low levels of cyclin E-dependent kinase activity that we detect in these extracts.

Thus, unlike the results recently reported in the Xenopus system (16), where depletion of cyclin E was shown to inhibit DNA replication, we find no evidence that cyclin E is required for mammalian DNA replication in the SV40 replication system. In Xenopus egg extracts, cyclin A is predominantly associated with Cdc2 rather than Cdk2 (15, 62, 63), whereas Cdk2 is associated with cyclin E (16). Drosophila embryos containing a loss of function mutation in the cyclin E gene arrest in G1 of cell cycle 17 (64). This is the first embryonic cell cycle that exhibits a G1 phase. Xenopus egg extracts thus act as a model system for embryonic cell cycles, like those of early Drosophila embryos, and lack a G1 phase. It is possible that in both embryonic systems and somatic cells cyclin E may fulfill an early role, such as assembly of initiation foci as proposed by Jackson et al. (16), and that cyclin A may act at a step after formation of initiation complexes, as we find in mammalian cells. It should be noted that in cyclin E-depleted Xenopus oocyte extracts, cyclin A or cyclin E were equally effective in reconstituting DNA replication (16). We have found a similar capacity for cyclins to substitute, since replication in cyclin A-depleted cell extracts can be rescued by either cyclin A-CDK2 kinase (Fig. 3) or cyclin B-Cdc2 kinase (Fig. 5C).

The N-terminal Domain of p21 Both Binder Cdk2-Cyclins and Inhibits DNA Replication—The full-length p21 binds to both Cdk-cyclin kinase (24–26, 65, 66) and to PCNA (27, 28, 65, 67, 68). Our mapping of cdk2 (36) and PCNA binding domains in p21 is in agreement with that of others who have also mapped the cdk binding (65–67, 69) and the PCNA binding domain (65–67) to the N- and the C-terminal regions of p21, respectively.

We have shown that p21 mutants that inhibit both CDK2-cyclin A and -cyclin E kinase activity but do not bind PCNA suppress DNA replication when added during the initiation step but do inhibit DNA replication during elongation. In contrast, the DNA elongation step is inhibited by a p21 mutant that does not bind Cdk-cyclins but does bind PCNA. These results are strengthened by our independent determination that depletion of cyclin A from S phase cell extracts yields a similar inhibition. The 50% inhibition of DNA replication that we observe on addition of the N terminus of p21 (which binds to CDK2-cyclin complex) in the SV40 in vitro replication system contrasts with the complete inhibition obtained in the Xenopus in vitro replication system (65). One explanation for the difference may be that in Xenopus egg extracts progression from mitosis to S phase occurs in the absence of a G1 phase. Thus, the Xenopus system cannot distinguish the activating steps that would occur during G1 in somatic cells from events that would occur specifically in S phase.

Our results, showing that the N terminus of p21 inhibits SV40 in vitro replication, contrast with the lack of inhibition obtained with a similar construct in the same system by Chen et al. (65). One explanation for this discrepancy may be that Chen et al. (65) used cell extracts from exponentially growing cells, whereas we used S phase cell extracts. Extracts from exponentially growing cells contain cyclin B kinase activity that cannot be effectively inhibited by the N-terminal domain of p21 (24). Such cyclin B kinase activity may be sufficient to allow replication to occur in the presence of the N-terminal domain of p21.

How Does Cyclin-dependent Kinase Activity Facilitate Replication?—We have made the important demonstration that cyclin-dependent kinase is rate-limiting for an event prior to elongation (Fig. 10). The appearance of elongation products varying in length from 500 to 2000 base pairs within 2 min in both control and cyclin A-depleted extracts (Fig. 10) is consistent with this conclusion. The addition of cyclin A to cyclin A-depleted extracts at the onset of elongation has an immediate stimulatory effect on DNA elongation (Fig. 9B), demonstrating that cyclin A-associated kinase activity is not essential for the assembly of an initiation complex.

We have previously shown that cyclin A is associated with replicating DNA (20). Here we have shown that cyclin A is required at elongation (Fig. 10). Interestingly, however, once elongation has initiated, neither the addition of cyclin A to depleted extracts (Fig. 8B) nor of p21 to control extracts (Fig. 8C) can influence elongation. These results may be interpreted as showing that the site for binding cyclin A in the elongation complex as well as the p21 binding site on cyclin A are both inaccessible once elongation has commenced.

Subsequent to the formation of a T antigen-RPA complex at the origin, DNA polymerase α-primase complex associates with the origin, and this leads to formation of RNA primers that are used for the synthesis of the first nascent strands at the origin of replication (for review see Ref. 53). Next, in a complex series of events the replication machinery switches from DNA polymerase α to δ in a process requiring PCNA and the cellular replication factor C (see Ref. 53). Replication proteins involved in these latter steps have the proposed consensus cdk phosphorylation motif, S*/T*/PXX, with X being a polar amino acid and Z a basic amino acid (70). Specifically, the catalytic subunit of DNA polymerase α (p180) has 10 consensus cdk phosphorylation sites that may serve a regulatory function during replication (71), and DNA polymerase δ contains six consensus cdk phosphorylation sites (72). Although DNA polymerase δ is phosphorylated during S phase (72), it is unknown whether it is phosphorylated at these cdk phosphorylation sites. In cycling cells, PCNA is unphosphorylated in G1 and in mitosis but becomes phosphorylated in S phase, and this phosphorylated form appears to be associated with chromatin (73). It is therefore conceivable that the activities of DNA polymerase α, DNA polymerase δ, or PCNA are regulated by cyclin-dependent kinase phosphorylation and that phosphorylation of these proteins represents the Cdk-dependent activation step that we have observed.

It is clear from the present study that cyclin A-dependent kinases play a role in replication, but the targets of cyclin-dependent kinase at the replication origin remain to be established. The pathway of activation may either involve phosphorylation of replication proteins or phosphorylation of downstream protein kinases. Through these mechanisms activation of DNA replication can be coupled to cell cycle progression.

Acknowledgments—We thank Motoaki Ohtsubo and Jim Roberts (Fred Hutchinson Cancer Research Center) for providing antibody to cyclin A and the GST-cyclin E plasmid, Helen Piwinka-Worms (Harvard Medical School) for GST-cyclin A plasmid, and Mark Solomon (Yale University) for GST-cyclin B plasmid. We thank Sheila Erickson for technical assistance in the early part of this work, Claudia Gruss (Universität Konstanz) for useful advice, and Giovanni Maga (Universität Zürich–Irchel) for providing the protocol for alkaline gels. We thank Jim Roberts for his generous and useful advice and Robert L. Margolis for critically reading the manuscript.

REFERENCES
1. Reed, S. I. (1992) Annu. Rev. Cell Biol. 8, 529–561
2. Fines, J. (1993) Biochim. Biophys. Acta 1191, 921–925
3. Girard, F., Strausfeld, U., Fernandez, A., and Lamb, N. J. C. (1991) Cell 67, 1169–1179
4. Pagano, M., Peiper, R., Verde, F., Ansorge, W., and Draetta, G. (1992) EMBO J. 11, 961–971
