DNA polymerase α-primase (pol-prim) is the only enzyme that can start DNA replication de novo. The 180-kDa (p180) and 68-kDa (p68) subunits of the human four-subunit enzyme are phosphorylated by Cyclin-dependent kinases (Cdks) in a cell cycle-dependent manner. Cyclin A-Cdk2 physically interacts with pol-prim and phosphorylates N-terminal amino acids of the p180 and the p68 subunits, leading to an inhibition of pol-prim in initiating cell-free SV40 DNA replication. Mutations of conserved putative Cdk phosphorylation sites in the N terminus of human p180 and p68 reduced their phosphorylation by Cyclin A-Cdk2 in vitro. In contrast to wild-type pol-prim these mutants were no longer inhibited by Cyclin A-Cdk2 in the initiation of viral DNA replication. Importantly, rather than inhibiting it, Cyclin A-Cdk2 stimulated the initiation activity of pol-prim containing a triple N-terminal alanine mutant of the p180 subunit. Together these results suggest that Cyclin A-Cdk2 executes both stimulatory and inhibitory effects on the activity of pol-prim in initiating DNA replication.

Eukaryotic DNA replication is tightly controlled and takes place during a restricted period of the cell cycle, the S phase (1, 2). S-phase-entry as well as cell cycle progression in general is triggered by the activity of conserved serine and threonine protein kinases, Cyclin-dependent kinases (Cdks) (3–10). The active kinase complex consists of a positive regulatory subunit called Cyclin and a catalytic subunit, the Cdk subunit (reviewed in Ref. 11). The minimal consensus recognition sequence for Cdks is serine or threonine followed by proline (18, 19). The Cdk2 activity appears later with the onset of DNA synthesis and then rises throughout S phase. In addition to Cdks, other protein-modifying factors such as protein phosphatase 2A (PP2A) and DBF4-CDC7 kinase (DDK) have essential functions in triggering S phase and in initiating chromosomal DNA replication (3, 4, 13).

Despite detailed knowledge of general cellular Cdk functions relatively little is known about their physiological substrates and how Cdks trigger initiation of eukaryotic DNA replication (8, 14). One of the Cdks targets is probably the replication factor DNA polymerase α-primase (pol-prim, (15–20)), which plays a key role in coordinating DNA replication, DNA repair, and cell cycle checkpoints (21). The enzyme complex is composed of four subunits: the p180 subunit contains the DNA polymerase activity, the p68 subunit is presumed to have regulatory functions, and p58 and p48 together comprise the primase with p48 carrying its catalytic center (reviewed in Refs. 21–24). Pol-prim becomes phosphorylated in a cell cycle-dependent manner on both its largest subunits, whereas cell cycle-dependent phosphorylation of p58 and p48 has not been observed. Phosphopeptide mapping of human p180 and p68 in vivo and in vitro suggested that a Cdk could be responsible for this modification (15, 17, 19). Functional studies with purified recombinant human pol-prim showed that its phosphorylation by Cyclin A-Cdk2 and Cyclin A-Cdk1 in vitro strongly inhibited its ability to initiate cell-free DNA replication on simian virus 40 (SV40) origin DNA, whereas on single-stranded DNA templates the primase and DNA polymerase activities were hardly affected (18, 19).

Duplication of the small genome of the SV40 depends on host replication factors and has led to a useful model system to study the molecular mechanism of eukaryotic cellular DNA replication. By using the cell-free SV40 system, which resembles the DNA replication of host chromosomes, a model for the initiation and elongation steps during eukaryotic DNA replication has been established (for reviews see Refs. 21, 24–26). During initiation of DNA replication SV40 T antigen (Tag) performs many of the functions attributed to the cellular prereplication complex and initiation proteins including ORC, CDC6, MCM2–7, Cdt1, and CDC45 (14, 26–35). These polypeptides are involved in origin binding, in loading of replication factors onto the chromatin, and in licensing of DNA for replication (14, 25, 28).

Genetic and biochemical experiments showed that cellular Cdks can both positively and negatively regulate the initiation of cellular DNA replication (5, 14). To investigate the effects of Cdk phosphorylation on pol-prim independently of its loading onto the origin by cellular factors, we used the SV40 model system in which the roles of these factors are essentially replaced by the multifunctional SV40 Tag (21, 26). Our findings show that Cyclin A-Cdk2 differentially controls SV40 origin-
dependent initiation activity of pol-prim. Pol-prim is maximally active when its p68 subunit is phosphorylated by Cyclin A-Cdk2 and the N terminus of p180 remains unmodified. Modification of both large subunits by Cyclin A-Cdk2 abolishes the initiation of leading strand DNA synthesis by pol-prim.

MATERIALS AND METHODS

**Cell Culture and Immunological Reagents**—High Five insect cells (Invitrogen) were grown in monolayers in TC100 medium supplemented with 10% fetal calf serum at 27 °C (36, 37). Hyridoma cells C160 (anti-human Cyclin A), SJRK237–71, and 2CT25 producing monoclonal antibodies against the p180 subunit of pol-prim (38–40) were grown as spinner cultures in RPMI 1640/Dulbeccos modified Eagles medium (1:1) supplemented with 10% fetal calf serum at 37 °C. Monoclonal antibodies 12CA5 against the hemagglutinin epitope were purchased from Roche Biochemicals (Mannheim, Germany). Monoclonal antibodies 2F6 recognizing p68 were a generous gift from S. Weiss (Strasbourg, France).

**Mutagenesis of p180 cDNA**—Serine or threonine codons were exchanged for alanine codons by overlap extension polymerase chain reaction as described previously (Fig. 2A, Ref. 19). Double mutants (p180–2–X, S209A/T219A) and triple mutants (p180–3–X, T174A/S209A/T219A) of p180 as well as the quadruple mutant of p68 containing alanines at positions amino acids 141, 147, 152, and 156 (p68–4–X; Ref. 19) were constructed by successive repetition of this method. Each mutation was verified by DNA sequencing. Baculoviruses expressing mutant and wild-type p180 cDNAs were created using the Bac-To-Bac system (Life Technologies).

**Expression and Purification of Proteins**—To express human pol-prim in the presence or absence of Cyclin A-Cdk2, 3 × 10^9 High Five cells were coinfected with a multiplicity of infection of 5 for each recombinant baculovirus and incubated for 42 to 46 h at 27 °C. Cells were harvested and recombinant pol-prim was purified using a phosphocellulose step and an affinity step with a monoclonal antibody against human p180 (18, 41–43). The expression of p68–4–X and human PP2A core (PP2Acore) was carried out as described (16, 19). Replication protein A protein was produced and purified according to Ref. 44.

To test the activity of the recombinant kinase in the baculovirus co-expression system, 50 μg of lysates from baculovirus-infected cells were immunoprecipitated with the anti-hemagglutinin antibody 12CA5 (18). Phosphorylation of proteins was carried out as described (18).

**Purification of the Glutathione S-Transferase-suc1 (GST-suc1) Fusion Protein and Cyclin-Cdk Complexes**—The suc1 protein from *Schizosaccharomyces pombe* binds eukaryotic Cdks with high affinity and can be used to purify Cyclin-Cdks (45). Expression of GST-suc1 fusion protein in *Escherichia coli* (containing pEGX2T-suc1) was induced by addition of 1 mM isopropyl-1-thio-β-D-galactopyranoside at an A_600 of 1.0 in 50 ml of culture at 37 °C. The cells were collected by centrifugation (3,000 × g, 10 min) and washed twice with 20 ml STE (25 mM Tris/Cl, pH 8.0, 150 mM NaCl, and 1 mM EDTA). The cells were resuspended in 10 ml STE/ml cell pellet, adjusted to 50 μM leupeptin and 1% Trasylol® and sonicated on ice (5 min, maximum intensity). 1 mM phenylmethylsulfonyl fluoride was added, and the lysate was cleared by centrifugation (10,000 × g, 4 °C, 10 min). The supernatant was incubated rotating end over end for 1 h at 4 °C with 1 ml glutathione-agarose (Sigma). The affinity resin was then washed four times with 10 volumes of STE.

2 × 10^8 High Five cells were infected with baculoviruses encoding recombinant human Cyclin A and Cdk2 and harvested 42–46 h postinfection (18). The crude extracts were incubated with the GST-suc1-loaded agarose (equal volume of crude extract and agarose) to bind the Cdk. After column preparation, the proteins were eluted with 0.5 column volume of elution buffer (25 mM Tris/HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, pH 7.8, and 10 mM glutathione). The fractions were dialyzed overnight against 1 liter dialysis buffer (25 mM Tris/HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, and 10% glycerol).

**Coominunoprecipitation of Pol-prim and Cyclin-Cdk Complexes**—1 × 10^8 High Five cells were infected with baculoviruses encoding the four subunits of pol-prim, the corresponding Cyclin, and Cdk subunit (18, 42). 100 μl of crude extract was incubated with 10 μl of protein A-agarose coupledly against monoclonal antibodies and pre-equilibrated with insect cell lysis buffer. After binding (1 h, rotating end over end at 4 °C) the column material was washed with buffer (5 × 1 ml, 30 mM Hepes/KOH, pH 7.5, 25 mM KCl, 7 mM MgCl₂, 0.25% Inositol, 0.25 mM EDTA, 0.1% Nonidet P-40) and boiled in SDS sample buffer. The proteins were separated electrophoretically, transferred onto nitrocellulose, and probed with antibodies against pol-prim and Cyclin-Cdk subunits.

**Coimmunoprecipitation of DNA Polymerase α-Primase with Cyclin A-Cdk2**—Since earlier reports suggested a modification of human pol-prim by Cdk2 (17–19) we coexpressed pol-prim and various Cyclin-Cdk complexes in insect cells and performed coimmunoprecipitation experiments to test the physical interactions of these enzymes (Fig. 1, data not shown). Using the monoclonal antibody SJRK237–71 against the human p180 subunit we could precipitate Cyclin A-Cdk2 bound to pol-prim (Fig. 1, lane 5). However, when Cyclin A-Cdk2 was expressed without pol-prim and incubated with SJRK237–71 no Cdk complex was eluted (lane 6). The same interactions were determined with Cyclin E-Cdk2, Cyclin A-Cdk1, and Cyclin B-Cdk1 (data not shown).

**Phosphorylation of Distinct Sites of the p180 Subunit by Cyclin A-Cdk2**—In vitro phosphorylation of the p180 subunit by Cyclin A-Cdk2 was carried out as described (18). The p180 subunit of pol-prim contains several potential Cdk recognition sites, and to identify the parts of the p180 subunit that is phosphorylated by Cyclin A-Cdk2 overlapping p180-GST fusion proteins (49) were phosphorylated with purified Cyclin A-Cdk2 in vitro (Fig. 2B). This kinase modified the N-terminal p180 GST fusion protein, 1α (Fig. 2B, lane 4) but did not modify other regions of p180 or GST itself (lanes 5–8, data not shown). To narrow down the phosphorylation region the fusion protein 1α was split into smaller polypeptides A, B, and C (Fig. 2B). Only the peptides B and C, which contain the conserved residues Thr-174, Ser-209, and Thr-219, were modified (Fig. 2B, lanes 2, 3). These data indicate that Cyclin

![Figure](http://www.jbc.org/)

**RESULTS**

**Comininunoprecipitation of DNA Polymerase α-Primase with Cyclin A-Cdk2**—Since earlier reports suggested a modification of human pol-prim by Cdk2 (17–19) we coexpressed pol-prim and various Cyclin-Cdk complexes in insect cells and performed coimmunoprecipitation experiments to test the physical interactions of these enzymes (Fig. 1, data not shown). Using the monoclonal antibody SJRK237–71 against the human p180 subunit we could precipitate Cyclin A-Cdk2 bound to pol-prim (Fig. 1, lane 5). However, when Cyclin A-Cdk2 was expressed without pol-prim and incubated with SJRK237–71 no Cdk complex was eluted (lane 6). The same interactions were determined with Cyclin E-Cdk2, Cyclin A-Cdk1, and Cyclin B-Cdk1 (data not shown).

**Phosphorylation of Distinct Sites of the p180 Subunit by Purified Cyclin A-Cdk2 in Vitro**—Cdkks bind to and phosphorylate heterotetrameric pol-prim in vitro (Fig. 1 and Ref. 18). The p180 subunit of pol-prim contains several potential Cdk recognition sites, and to identify the parts of the p180 subunit that is phosphorylated by Cyclin A-Cdk2 overlapping p180-GST fusion proteins (49) were phosphorylated with purified Cyclin A-Cdk2 in vitro (Fig. 2B). This kinase modified the N-terminal p180 GST fusion protein, 1α (Fig. 2B, lane 4) but did not modify other regions of p180 or GST itself (lanes 5–8, data not shown). To narrow down the phosphorylation region the fusion protein 1α was split into smaller polypeptides A, B, and C (Fig. 2B). Only the peptides B and C, which contain the conserved residues Thr-174, Ser-209, and Thr-219, were modified (Fig. 2B, lanes 2, 3). These data indicate that Cyclin
A-Cdk2 preferentially phosphorylates the N terminus of the p180 subunit.

Since pol-prim associates with PP2A in vivo (16), we tested whether PP2A could dephosphorylate the phosphorylated p180 GST fusion proteins \(1/295\), B, and C in vitro. The radioactive label of these proteins was readily removed by PP2A (Fig. 2C, lanes 2, 4, 6). Furthermore, PP2A removed phosphate groups from the p68 subunit and coexpression of the PP2A core (an active enzyme complex consisting of the structural and catalytic subunits A and C, respectively) with pol-prim and Cyclin A-Cdk2 eliminated the phosphorylation-induced shift of the p68 subunit in baculovirus-infected cells (Fig. 2D, lane 3; Ref. 16). These data suggest that PP2A reverses the phosphorylation of the p180 and the p68 subunits by Cyclin A-dependent kinases.

Expression and Purification of Mutant DNA Polymerase \(\alpha\)-Primase—To reveal the contribution of single amino acids of the p180 subunit in the Cyclin A-Cdk2-mediated regulation of cell-free SV40 DNA initiation, the three conserved putative Cdk phosphorylation sites (Thr-174, Ser-209, and Thr-219) were changed either alone or in combination to alanine to yield the following mutant p180 subunits: T174A, S209A, S209A/T219A (p180\(^{-}\)2/H11003A) and T174A/S209A/T219A (p180\(^{-}\)3/H11003A). These mutants were coexpressed with the three smaller wild-type (wt) pol-prim subunits. Each mutant complex was coexpressed with or without Cyclin A-Cdk2 and purified to near homogeneity. All pol-prim complexes consisted of four subunits (Fig. 3A; data not shown) and had high specific primase and DNA polymerase activities comparable with those of the wt recombinant enzyme (data not shown), proving that all mutant pol-prim complexes are fully active in their basic replication functions. The p180 subunit of pol-prim is sensitive to proteolysis especially its N terminus (50). Since we modified

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**Fig. 2. Phosphorylation of p180 by Cyclin A-Cdk2 in vitro.** A, the p180 subunit of four subunit pol-prim contains a number of putative Cdk phosphorylation sites (\(\text{S/T/P}\)), several of them are conserved from yeast to mammals (conserved sites are marked with bold lines and the amino acid number, non-conserved S/T/P sites are marked with fine lines). Some of these sites are found within or close to the Tag binding site of p180 (49). Regions containing the catalytic center and binding sites for the smaller subunits are also presented (56, 67). B, GST fusion proteins containing various regions of p180 are shown (49). The amino acids are indicated below each polypeptide. The polypeptide 1α was split into three overlapping regions A, B, and C. The polypeptides A, B, C, and 1α to 5α (panel B, lanes 1–8) were incubated with purified Cyclin A-Cdk2 and \(\text{[\gamma-32P]}\)ATP. The radioactive proteins were analyzed by SDS-polyacrylamide gel electrophoresis and x-ray autoradiography. The specific proteins are highlighted by arrows, whereas nonspecific bands are marked by an asterisk since they represent copurified bacterial proteins. C, these radioactive labeled proteins were incubated in buffer without (odd numbers) or with PP2A (even numbers). The arrows mark the phosphorylated polypeptides. D, all four subunits of pol-prim were coexpressed in insect cells in the absence of recombinant protein-modifying enzymes (lane 1), in the presence of recombinant Cyclin A-Cdk2 (lane 2), or with recombinant Cyclin A-Cdk2 plus PP2A-core (an enzyme complex consisting of the structural and catalytic subunits A and C, respectively, lane 3). After 45 h protein extracts were prepared and analyzed by Western blotting using the monoclonal antibody 2F6 recognizing human p68.
p180 by the alanine mutations we wondered whether this would destabilize the mutant proteins. However, we did not detect more than average degradation of the purified p180 mutants either by Coomassie-stained protein gels (Fig. 3A) or in Western blotting (data not shown). In addition, their specific enzyme activities were not changed in comparison to wt pol-prim. Therefore, we assume that the mutations did not dramatically change the three-dimensional structure of the polypeptide.

Phosphorylation of Mutant DNA Polymerase α-Primase in Vitro—Phosphopeptide maps of the p180 and p68 subunits phosphorylated by Cdk2 in vitro are similar to those of in vivo-phosphorylated subunits (17, 19). To determine whether the mutated phosphorylation sites of the p180 subunit are targets of Cyclin A-Cdk2, purified wt pol-prim and pol-prim complexes with a mutated p180 subunit were phosphorylated by Cyclin A-Cdk2. The single mutations, T174A and S209A, reduced the incorporation of radioactive phosphate to about 60% of the level of wt p180 (Fig. 3B, black columns 2–5). The double alanine mutants (p180–T174A and S209A) phosphorylated to about 30% of the wt level. The triple alanine mutants incorporated radioactive phosphate to about 10% of the wt level. The amounts of primase products were quantified by a PhosphorImager and normalized to the initiation activity of wt pol-prim produced in the presence of recombinant Cyclin A-Cdk2 (panel A, column 1), or with (even numbers) recombinant Cyclin A-Cdk2 (columns 2 and 3), or both (column 4). The enzyme complexes were produced without (odd numbers) or with (even numbers) recombinant Cyclin A-Cdk2. Columns 3–10, mutant pol-prim containing p180–T174A (columns 3 and 4), p180–S209A (columns 5 and 6), p180–2xA (columns 7 and 8), and p180–3xA (columns 9 and 10). The amounts of primase products were quantified by a PhosphorImager and normalized to the initiation activity of wt pol-prim produced in the presence of recombinant Cyclin A-Cdk2 (panel A, column 1, or panel B, column 2). Each value is the mean of at least four experiments. The error bars indicate standard deviations.

FIG. 3. Purification and in vitro phosphorylation of DNA polymerase α-primase with mutated p180 subunits. A shows a Coomassie Brilliant Blue-stained SDS gel of purified recombinant human pol-prim complexes (H4). Insect cells were coinfected with four baculoviruses encoding either four wt pol-prim subunits (lane 1) or the indicated p180 mutant (see “Materials and Methods”) and wt smaller subunits (lanes 2–5). Apparent molecular masses of the proteins are marked on the right (note that the enzyme preparation shown in lane 1 is not identical to the preparation used in the experiments since it showed a partial loss of the primase subunits). B, mutant and wt pol-prim complexes were adjusted to equal p180 amounts, phosphorylated in vitro by purified Cyclin A-Cdk2, and separated by SDS-polyacrylamide gel electrophoresis. The incorporation of radioactive phosphate was quantified with a PhosphorImager. The black columns represent incorporation into the p180 subunits, the relative incorporation levels into p68 are shown in gray columns. The amount of radioactive phosphate in wt p180 was set to 100% (black column 1). The relative incorporations of mutant protein complexes are presented in columns 2–5.

FIG. 4. Initiation activity of mutant pol-prim is not inhibited by Cyclin A-Cdk2. Wild-type or pol-prim complexes containing mutant p180 subunits were produced in insect cells. The purified enzyme complexes (0.2 primase units) were used to initiate SV40 DNA replication in vitro. A, wt pol-prim was coexpressed either alone (column 1) or in the presence of either recombinant PP2A core (column 2), recombinant Cyclin A-Cdk2 (column 3), or both (column 4). B, the enzyme complexes were produced without (odd numbers) or with (even numbers) recombinant Cyclin A-Cdk2. Columns 1 and 2, wt pol-prim, columns 3–10, mutant pol-prim containing p180–T174A (columns 3 and 4), p180–S209A (columns 5 and 6), p180–2xA (columns 7 and 8), and p180–3xA (columns 9 and 10). The amounts of primase products were quantified by a PhosphorImager and normalized to the initiation activity of wt pol-prim produced in the presence of recombinant Cyclin A-Cdk2 (panel A, column 1) and wt pol-prim (panel B, column 2), arbitrarily set to 1. Each value is the mean of at least four experiments. The error bars indicate standard deviations.
and determined the SV40 initiation activity of the purified enzyme complex (Fig. 4A). Pol-prim coexpressed with PP2A core had a reduced initiation activity on DNA containing an SV40 origin (Fig. 4A, compare column 1 and 2), whereas the initiation activity of Cyclin A-Cdk2-phosphorylated enzyme complex was nearly completely abrogated (Fig. 4A, column 3). Coexpression of pol-prim with Cyclin A-Cdk2 and PP2A core did not only abolish the phosphorylation-induced shift of p68 (Fig. 2D) but enabled pol-prim to synthesize primers in the SV40 origin of replication (Fig. 4A, column 4) with a similar activity as the untreated enzyme (Fig. 4A, column 1).

To investigate whether the mutant pol-prim complexes are still inhibited by Cyclin A-Cdk2 in initiating cell-free SV40 DNA replication (Fig. 4A, 18), SV40 initiation assays were performed with our pol-prim mutants modified by Cyclin A-Cdk2 and pol-prim expressed without this kinase, and then the initiation products were quantified (Fig. 4B). The mutant enzyme complexes containing the p180-T174A, p180-S209A, or p180–2×A subunits, which were not coexpressed with Cyclin A-Cdk2, were less active in origin-dependent initiation than wt pol-prim (Fig. 4B, compare column 1 with columns 3, 5, and 7). In contrast to the inhibition seen with wt pol-prim, Cyclin A-Cdk2 only slightly inhibited the origin-dependent initiation of these mutants (compare columns 1 and 2 (80% inhibition of wt pol-prim); and columns 3, 5, and 7 with 4, 6, and 8, respectively, which showed about 15% less incorporation after modification of mutant enzyme complexes by Cyclin A-Cdk2)). These results revealed that the phosphorylated enzyme complexes H4(p180-T174A), H4(p180-S209A), and H4(p180–2×A) were three to four times more active than phosphorylated wt pol-prim.

Compared with these mutant enzymes p180–3×A pol-prim had a significantly higher initiation activity (Fig. 4B, column 9). As phosphorylation of wt pol-prim by Cyclin A-Cdk2 results in an inhibition of initiation activity (column 2), it was surprising that p180–3×A pol-prim showed an increased initiation activity upon modification (compare column 9 and 10). These results indicate that phosphorylation of the N terminus of the p180 subunit contributes to the regulation of cell-free SV40 DNA replication. Extending earlier findings that Cyclin A-Cdk2 inhibits pol-prim in SV40 origin-dependent initiation (18, 19) these results showed that Cyclin A-Cdk2 stimulates origin-dependent initiation when pol-prim becomes modified on residues other than Thr-174, Ser-209, and Thr-219 of the p180 subunit. Since these amino acids seem to be the only targets of Cyclin A-Cdk2 in the p180 subunit (Figs. 2B and 3B) and since the p180 and p68 subunits are the only known substrates of Cdkks within pol-prim (17–18), it appears that phosphorylation of p68 probably stimulates SV40 initiation activity of pol-prim in vitro.

Phosphorylation of p68 Is Required for Stimulation and Inhibition of the Origin-Dependent Initiation Reaction—To test the hypothesis that in the presence of a nonphosphorylated N terminus of p180 Cyclin A-Cdk2-mediated modification of the p68 subunit stimulates the SV40-initiation we used a mutant in which the four putative Cdk phosphorylation sites Ser-141, Ser-147, Ser-152, and Thr-156 were mutated to alanine (p68–4×A) (19). A pol-prim complex containing the p68–4×A subunit and wt p180 with and without coexpressing Cyclin A-Cdk2 was purified. The mutant enzyme complex was indistinguishable in its basic enzymatic functions and subunit composition from the recombinant wt enzyme (data not shown). Mutation of these residues reduced phosphorylation of the p68 subunit by Cyclin A-Cdk2 in vitro, and the incorporation of radioactive phosphate reached only 40% of that of wt p68, whereas the capability of Cyclin A-Cdk2 to phosphorylate wt p180 of

**Fig. 5.** The subunits p180 and p68 cooperate to regulate the initiation of SV40 DNA replication. A, the p68 subunit of four subunit pol-prim contains a number of putative Cdk phosphorylation sites, some of them are conserved from yeast to mammals (conserved sites are marked with bold lines and the amino acid number, non conserved (S/T/P) sites are marked with fine lines). Several of these sites are found within or close to the Tag binding site of p68 (68). Wild-type (columns 1) and mutant pol-prim complex containing p68–4×A (columns 2) were adjusted to equal p180 amounts, incubated with Cyclin A-Cdk2 in the presence of [γ-32P]ATP, and separated by SDS-polyacrylamide gel electrophoresis. Incorporation of radioactive phosphate was quantified with a PhosphorImager. The black columns represent incorporation into the p180 subunits, the relative incorporation levels into p68 are shown in gray columns. The amount of radioactive phosphate in wt p180 was set to 100% (black column 1). B, pol-prim complexes containing mutant p180 or p68 were produced in insect cells with (odd numbers) or without (even numbers) recombinant Cyclin A-Cdk2. The purified enzyme complexes were used to initiate SV40 DNA replication in vitro. Columns 1 and 2, wt pol-prim; columns 3-8, mutant pol-prim containing p68–4×A (columns 3 and 4), p180–3×A (columns 5 and 6), and p180–3×A plus p68–4×A (columns 7 and 8), respectively. The primase products were quantified by a PhosphorImager. The amounts normalized to the initiation activity of wt pol-prim produced with recombinant Cyclin A-Cdk2 (column 2, arbitrarily set to 1). The mean values and standard deviation of at least four experiments are presented.

H4(p68–4×A) remained unchanged (Fig. 5A; (19)). In the SV40 initiation assay the mutant enzyme complex H4(p68–4×A) displayed a reduced activity in comparison with wt pol-prim.
(Fig. 5B, columns 1 and 3) and Cyclin A-Cdk2 inhibited the activity of this mutant pol-prim by only 30% (Fig. 5B, columns 3 and 4). This confirms the previous report that modification of the p68 subunit is needed for the inhibition of cell-free SV40 replication (19).

To test whether modification of the p68 subunit by Cyclin A-Cdk2 can also stimulate the initiation reaction, we compared the enzyme complex H4(p180–3×A) with pol-prim H4(p180–3×A,p68–4×A). These mutant enzyme complexes were also indistinguishable in their basic enzymatic functions and subunit composition from the recombinant wt enzyme (data not shown). Comparing both enzyme complexes in the initiation of cell-free SV40 DNA replication H4(p180–3×A) showed high origin-dependent initiation activity that was stimulated by Cyclin A-Cdk2 as shown before (Fig. 5B, columns 5 and 6). However, the enzyme complex H4(p180–3×A,p68–4×A) was no longer stimulated through modification by Cyclin A-Cdk2 (Fig. 5B, lanes 7 and 8). Moreover its activity was not influenced by coexpression of Cyclin A-Cdk2 at all. These findings suggested that phosphorylation of the p68 subunit by Cyclin A-Cdk2 is necessary for stimulation of pol-prim in origin-dependent initiation activity in vitro.

In summary these results show that the p180 and p68 subunits cooperate to control the origin-dependent initiation activity of pol-prim in vitro. When pol-prim is phosphorylated at the residues Ser-141–Thr-156 of the p68 subunit and the N terminus of the p180 subunit remains unmodified, the initiation activity of pol-prim is stimulated (Fig. 5B, lane 6). However, when both the p180 and the p68 subunits are phosphorylated by Cyclin A-Cdk2 the origin-dependent initiation activity of pol-prim is turned off (Fig. 5B, lane 2). Comparing both states of pol-prim, we observed an 8-fold difference in activity between inhibition and stimulation of pol-prim by Cyclin A-Cdk2 in the SV40 system (Fig. 5B, compare lanes 2 and 6).

**DISCUSSION**

Eukaryotic chromosomal DNA replication is tightly regulated to replicate the genome once per cell cycle with high fidelity and to ensure the stability of genomic information (2, 51). SV40 DNA replication in vitro is also tightly controlled and does not start before the host cells have entered S phase. Therefore, understanding the regulation of SV40 DNA replication and the way(s) in which this virus overcomes cellular regulatory pathways will advance the elucidation of host cell cycle control mechanisms (52, 53).

Oscillation of phosphorylation is involved in various regulatory pathways controlling cellular processes (54). In the present report we have focused on the effects of cell cycle-dependent phosphorylation of DNA polymerase α-primase, the key enzyme for the initiation of eukaryotic DNA replication (21, 22). To gain a more detailed understanding of how Cyclin A-Cdk2 affects the ability of pol-prim to initiate DNA replication, we mapped the phosphorylation sites of this kinase on the p180 subunit and generated mutants of the polypeptide in which conserved amino acids of potential Cdk phosphorylation sites were altered. We then assayed the functional effects of different phosphorylation patterns by Cyclin A-Cdk2 using pol-prim complexes with mutant p180 subunits in combination with a mutant p68 subunit containing an altered cluster of putative Cdk recognition sites. SV40 has served as a model system to study eukaryotic DNA replication since the viral initiator protein Tag carries out specific physical interactions with host replication proteins (24, 55). Among these the binding of pol-prim to Tag is crucial for the initiation of viral DNA replication (49), and modification of pol-prim by Cyclin A-Cdk2 was recently shown to abolish its binding to Tag. In contrast, unphosphorylated pol-prim interacts with Tag (16). The interaction of pol-prim and the SV40 Tag is most likely controlled by the phosphorylation of the p180 and p68 subunits of pol-prim since PP2A and mutation of putative Cdk recognition sites in p180 and p68 to alanine allowed the binding of SV40 Tag to pol-prim after modification of the cellular initiation enzyme complex by Cyclin A-Cdk2 (16).

To analyze the regulatory phosphorylation of pol-prim by Cyclin A-Cdk2 and its functional consequences we used the cell-free SV40 DNA initiation assay (Figs. 4 and 5). The presented and previous data suggested that Cyclin A-dependent kinases and Cyclin E-Cdk2 phosphorylate multiple amino acids in the N terminus of the p180 subunit (amino acids 174–219) and the p68 subunit (amino acids 141–156) (Figs. 2, 3B, and 5A; Ref. 19). Our findings showed that p180 subunit is most likely only phosphorylated at its N-terminal residues Thr-174, Ser-209, and Thr-219 since their mutation to alanine reduced the phosphorylation by Cyclin A-Cdk2 to background levels. Various experiments indicated that Cyclin A-Cdk2 probably phosphorylates three amino acids within a p68 region containing the four potential Cdk recognition sites Ser-141, Ser-147, Ser-152, and Thr-156, whereas three additional sites of p68, putatively Thr-115, Thr-127, Thr-130, Thr-241, Ser-396, or Thr-534, are most likely also modified (Fig. 5A; Ref. 19). The exchange of serines and threonines into alanine probably did not alter the conformation of the proteins since the mutant enzyme complexes were recognized by Cyclin A-Cdk2 (Figs. 3 and 5) and were active in the cell-free SV40 DNA replication (Figs. 4 and 5), and SV40 Tag still bound to the protein complex containing p180–3×A together with p68–4×A (16). The large subunit of pol-prim is highly sensitive to proteolysis (37, 42, 50, 56) but SDS gel electrophoresis and Western blotting experiments revealed that degradation of p180 was not above average (Fig. 3A; data not shown).

Since the mutation of the p180 subunit did not affect the phosphorylation of the p68 subunit and vice versa (Figs. 3B and 5A), we could independently examine the functional effects of the modification of each subunit. We show here that single alanine mutations (T174A or S209A) and double alanine mutations (S209A/T219A) of p180 prevent the inhibition of pol-prim by Cyclin A-Cdk2 (Fig. 4B). This suggests that phosphorylation of the three amino acids Thr-174, Ser-209, and Thr-219 is necessary for the Cyclin A-Cdk2-mediated inhibition of the origin-dependent initiation activity of pol-prim. In agreement with an earlier report, origin-dependent initiation activity of pol-prim containing the quadruple alanine-mutated p68 (p68–4×A) was not inhibited by Cyclin A-Cdk2 (Fig. 5B; Ref. 19) suggesting that phosphorylation of these amino acids (Ser-141, Ser-147, Ser-152, and Thr-156) contributes to the regulation of pol-prim. However, pol-prim complexes with p68 mutants in which single sites were mutated (S141A, S147A, S152A, or T156A, respectively) are still inhibited by Cyclin A-Cdk2 phosphorylation, and their initiation activity is reduced to a level comparable with that of Cyclin A-Cdk2-modified wt pol-prim (57). These findings indicate that the p180 as well as the p68 subunit need to be phosphorylated to inhibit the initiation activity of pol-prim. Since recently published results showed that Cyclin A-Cdk2 but not Cyclin E-Cdk2 abrogated the binding of pol-prim to SV40 Tag (16), we assume that the interactions of the cellular and viral replication factor are regulated by these residues within pol-prim.

Surprisingly, pol-prim containing the triple alanine mutation p180–3×A had an increased origin-dependent SV40 initiation activity, which was even stimulated by Cyclin A-Cdk2 (Fig. 4B). This stimulation depends on modification of the p68 region residues 141–156 since a pol-prim lacking these putative phosphorylation sites (H4(p180–3×A,p68–4×A)) is no longer stimulated upon phosphorylation and in fact did not...
respond to Cyclin A-Cdk2 phosphorylation at all, either positively or negatively (Fig. 5B). This view that phosphorylation influences the initiation activity of pol-prim is supported by the findings that the enzyme complex H4(p180–3×A, p68–4×A) binds SV40 Tag as well as wt pol-prim, and in contrast to wt pol-prim, its interaction with Tag is not diminished by Cyclin A-Cdk2 (16). In addition, recently published findings showed that Cyclin A-Cdk2 did not influence the complex formation of the phosphorylation-sensitive monoclonal antibody HP180—12 with H4(p180–3×A, p68–4×A), whereas Cyclin A-Cdk2-modified wt pol-prim was not recognized by this antibody (16). These results underline that the binding of an antibody to pol-prim is not affected by these mutations and that phosphorylation of pol-prim regulates its ability to interact with other proteins. Furthermore, coexpression of PP2A as well as introducing the four alanines S141A, S147A, S152A, and T156A reduce the phosphorylation-dependent shift of p68 (Fig. 2D, Ref. 19)) and prevent the Cyclin A-Cdk2-dependent inhibition of the SV40 initiation reaction (Figs. 4A and 5B; Ref. 16). However, alternatively the binding of Cyclin A-Cdk2 to pol-prim rather than its modification might control the initiation activity of the enzyme. This explanation is probably not applicable since the kinase still binds to mutated pol-prim complexes as seen by the phosphorylation of other non-mutated sites within the complex. Importantly, these interactions of Cyclin A-Cdk2 and various mutant pol-prim complexes do not determine whether a specific enzyme complex is inhibited, stimulated, or not responding (Figs. 3B, 4B, 5A, and 5B; Refs. 16, 57).

The biochemical data presented here suggest that the initiation activity of pol-prim is regulated in multiple ways. Cyclin A-Cdk2 can both stimulate the activity of pol-prim to initiate origin-dependent DNA replication and inhibit it depending on distinct phosphorylation events. This notion fits well with the findings that Cdkks can stimulate as well as inhibit origin-dependent DNA replication (7, 8) and with data reporting the regulation of other proteins such as SV40 Tag and Cdkks by phosphorylation (10, 11, 20, 52, 55). Activation and inactivation of these enzymes are also mediated by different phosphorylation events. Modification of specific sites, such as Thr-161 of Cdk1, is required for the kinase activity, whereas modification of Thr-14 and Tyr-15 inhibits the kinase activity. Phosphorylation of SV40 Tag at Thr-124 is required for SV40 DNA replication, whereas Tag phosphorylated at Ser-120 and Ser-123 is replication-inactive.

Together the presented data extend our understanding of the molecular mechanism of regulation of pol-prim by Cdkks in that not only p68 plays a role as a regulatory subunit but also p180 displays control functions during the initiation of DNA replication and that both subunits can act independently of one another. It is possible that the regulation we see in the SV40 system has been conserved throughout evolution. It has been reported that in Saccharomyces cerevisiae the second largest subunit of pol-prim becomes phosphorylated early in S phase. The authors speculate that this phosphorylation probably regulates DNA replication, but the mechanism has not been determined (58, 59). The finding in yeast is consistent with our data that pol-prim modified by Cyclin A-Cdk2 at the p68 subunit but with an unmodified p180 N terminus is maximally active to initiate DNA replication in vivo. Furthermore, recent findings of Nguyen et al. (30) showed that the coordinated regulation of CDC6, the ORC, and the MCM complex as well as of some yet undetermined factors is required to control the DNA replication in S. cerevisiae. These data are consistent with the view that pol-prim might be involved in the regulation of DNA replication of this yeast.

The removal of inhibitory phosphate groups is an essential condition for viral and most likely also for cellular DNA replication, and recent evidence suggests that PP2A performs this task during cellular DNA replication (13, 16, 20, 60). Our data that pol-prim with an unphosphorylated p180 N terminus could play a role in vivo are supported by the finding that PP2A binds in vitro to the p180-N terminus in the region of residues Thr-174 to Thr-219 (data not shown). One possible mechanism is that PP2A binds to and dephosphorylates this region to prevent a premature inhibitory phosphorylation of pol-prim by Cdkks before leading strand initiation at chromosomal origins occurs. These findings have tempted us to speculate about the coordination and cooperation of Cdkks and PP2A. In eukaryotic cells Cdkks, such as Cyclin A-dependent kinases, and Cyclin E-Cdk2 in higher eukaryotes and CLB-CDC28 kinases in S. cerevisiae interact with replication factors such as pol-prim, ORC, CDC6, and MCM proteins and phosphorylate them (16, 19, 30, 59–65). However, the modification by Cyclin A-dependant kinases inhibits the origin-dependent initiation activity of pol-prim as well as the helicase activity of the MCM 4, 6, 7 complex (Fig. 4, (16, 18, 62, 63)). Therefore, an additional factor or several proteins such as Cdt1 and PP2A (13, 33, 35) are required to prevent Cdk-dependent inhibition at specific sites and to balance the phosphorylation state of these replication factors to allow optimal initiation of DNA replication. In this model, PP2A binds to replication factors such as pol-prim and dephosphorylates them (Fig. 2, C and D, (16)). This S phase promoting activity is then changed by switching off PP2A, e.g. by methylating or by destabilizing the PP2A complex (16, 66). Thus, the inhibitory phosphorylation of pol-prim and that of other replication factors is no longer prevented and reinitiation of DNA replication is abrogated.

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Multiple Phosphorylation Sites of DNA Polymerase α-Primase Cooperate to Regulate the Initiation of DNA Replication in Vitro

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