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The DNA-activated protein kinase is required for the phosphorylation of replication protein A during simian virus 40 DNA replication

GEORGE S. BRUSH*, CARL W. ANDERSON†, AND THOMAS J. KELLY*‡

*Department of Molecular Biology and Genetics, The Johns Hopkins University School of Medicine, Baltimore, MD 21205; and †Biology Department, Brookhaven National Laboratory, Upton, NY 11973

Contributed by Thomas J. Kelly, September 9, 1994

ABSTRACT **The 32-kDa subunit of replication protein A (RPA) is phosphorylated during the S phase of the cell cycle *in vivo* and during simian virus 40 DNA replication *in vitro*. To explore the functional significance of this modification, we purified a HeLa cell protein kinase that phosphorylates RPA in the presence of single-stranded DNA. By several criteria we identified the purified enzyme as a form of the DNA-activated protein kinase (DNA-PK), a previously described high molecular weight protein kinase that is capable of phosphorylating a number of nuclear DNA binding proteins. Phosphorylation of RPA by DNA-PK is stimulated by natural single-stranded DNAs but not by homopolymers lacking secondary structure. Studies with the simian virus 40 model system indicate that DNA-PK is required for DNA-replication-dependent RPA phosphorylation. Depletion of the kinase activity, however, has no effect on the extent of DNA replication *in vitro*. Our data support a model in which phosphorylation of RPA by DNA-PK is activated by formation of replication intermediates containing single- and double-stranded regions. This event may be involved in a signaling mechanism that coordinates DNA replication with the cell cycle.**

DNA replication in eukaryotic cells is a tightly regulated process. During the S phase of the cell cycle, DNA synthesis is initiated at multiple origins of replication distributed along the chromosomal DNA. Activation of each replication origin occurs once and only once, ensuring that exactly two copies of each segment of chromosomal DNA are produced during each cell cycle. The timing of initiation is also precisely controlled and is sensitive to both environmental and cellular factors. If DNA replication is blocked by inhibitors or the template is damaged by radiation or other factors, a signal is generated that delays cell cycle progression. This mechanism presumably functions to allow time for the cell to deal with the abnormal condition before attempting mitosis.

Direct biochemical studies on the regulation of chromosomal replication have been difficult because of the complexity of the cellular genome. However, simian virus 40 (SV40) has provided a useful approach to this problem, since viral DNA replication is almost completely dependent upon cellular replication machinery (1–3). Analysis of viral DNA replication *in vitro* has led to the identification of a number of cellular replication proteins, and several lines of evidence suggest that initiation of viral DNA replication may be regulated by mechanisms similar in at least some respects to those that control cellular DNA replication during the cell cycle (4).

One cellular replication protein identified by biochemical analysis of the SV40 system is replication protein A (RPA), a heterotrimeric single-stranded DNA (ssDNA)-binding protein that is required during both the initiation and elongation phases of DNA replication (5–7). The ssDNA binding activity of RPA resides in the 70-kDa subunit (RPA70) and is required for the unwinding of the duplex at the SV40 origin during initiation of DNA replication (8–10). The other two subunits, with molecular masses of 32 kDa and 14 kDa, have no known biochemical activities, but one or both of them are required for initiation of SV40 DNA replication, since the RPA70 subunit alone is not sufficient (9). The middle or 32-kDa subunit of RPA (RPA32) is phosphorylated in a cell-cycle-dependent fashion in both human and yeast cells (11). Phosphorylation of the subunit begins at the onset of the S phase of the cell cycle, indicating that it could play a role in regulating the replication process. Interestingly, RPA32 is also phosphorylated during the replication of SV40 DNA in human cell extracts *in vitro* (12). Phosphorylation in cell extracts is stimulated by the addition of M13 ssDNA, suggesting that the DNA-replication-dependent phosphorylation of RPA32 occurs after binding of RPA to the ssDNA formed as a consequence of the unwinding of the template (12). The phosphopeptide map of RPA32 phosphorylated in the presence of ssDNA is similar to that of RPA phosphorylated *in vivo*, indicating that the mechanisms of phosphorylation *in vitro* and *in vivo* are similar (12).

As an approach to understanding the biological significance of RPA phosphorylation at S phase, we have purified the ssDNA-dependent RPA kinase from crude HeLa cell extracts. We have identified the purified enzyme as a form of the DNA-activated protein kinase (DNA-PK), a high molecular weight protein kinase that is capable of phosphorylating a number of nuclear DNA-binding proteins (13). In addition, we have demonstrated by immunodepletion experiments that the enzyme is required for the phosphorylation of RPA32 that occurs during SV40 DNA replication *in vitro*. Significantly, elimination of the replication-dependent phosphorylation of RPA32 has no effect on the extent of SV40 DNA synthesis. Our data are consistent with the hypothesis that phosphorylation of RPA32 occurs in response to the generation of partially duplex DNA structures during replication or repair and might play a role in signaling the presence of such structures to the cell.

MATERIALS AND METHODS

Extracts and Proteins. HeLa cytoplasmic extract was prepared by hypotonic lysis, as described (8, 14). Both RPA (5) and the ssDNA-activated RPA kinase were purified from this cellular fraction (see below). SV40 large tumor (T) antigen

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Abbreviations: SV40, simian virus 40; RPA, replication protein A; RPA70, 70-kDa subunit of RPA; RPA32, 32-kDa subunit of RPA; DNA-PK, DNA-activated protein kinase; DNA-PKc, catalytic subunit of DNA-PK; ssDNA, single-stranded DNA.

‡To whom reprint requests should be addressed.

was produced in Sf9 insect cells (15) and purified as described (16).

Antibodies and Western Blot Analysis. Monoclonal antibodies 71-9A (17) and 70-9 (11) directed against RPA32 and RPA70, respectively, were partially purified from hybridoma supernatants by ammonium sulfate precipitation. The hybridoma line expressing antibody 70-9 was the generous gift of Bruce Stillman (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY). Monoclonal antibodies N3H10 and N9C1 (18), directed against the 70- and 86-kDa subunits of Ku autoantigen, respectively, were kindly provided by Nancy Thompson and Richard Burgess (University of Wisconsin, Madison). Antiserum directed against DNA-PK was prepared from a rabbit immunized with a DNA-PK fusion protein. Purified monoclonal antibody 25-4 (19), directed against DNA-PK, was generously provided by Timothy Carter (St. John's University, Jamaica, NY). Immunoblot analysis was performed and immunocomplexes were visualized by the ECL detection method (Amersham).

SV40 DNA Replication and RPA Phosphorylation. Replication reactions (25 μl) were carried out as described (5). Reactions were terminated by adding 25 μl of 50 mM EDTA/2% (wt/vol) SDS, and aliquots of each reaction mixture were then analyzed for incorporation of radioactive dCMP by trichloroacetic acid precipitation and for RPA phosphorylation by Western blot analysis.

RPA Kinase Assays. Two methods were used to monitor ssDNA-activated RPA phosphorylation through the purification procedure. The Western blot assay takes advantage of the difference in mobility between phosphorylated and unphosphorylated RPA32 during denaturing gel electrophoresis (11, 12). Fraction samples were tested for activity in a standard reaction mixture (25 μl) containing 40 mM Hepes (pH 7.8), 7 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM ATP, M13mp18 ssDNA (2 μg/ml), and 11 μg of a phosphocellulose flow-through fraction containing RPA but devoid of kinase activity. The reactions were incubated for 10 min at 37°C, stopped with SDS/PAGE loading buffer, and analyzed on a Western blot.

Quantitative kinase assays with purified enzyme fractions employed a radioassay. Standard reaction mixtures (25 μl) contained 40 mM Hepes (pH 7.8), 7 mM $MgCl_2$, 1 mM dithiothreitol, 50 μM ATP, 5 μCi of [γ-^{32}P]ATP (650 Ci/mmol; 1 Ci = 37 GBq; ICN), DNA (2 μg/ml), and 300 ng of purified RPA. Samples were incubated for 10 min at 37°C, and the reactions were stopped and prepared for SDS/PAGE as in the Western blot assay. After electrophoresis, the gels were dried and subjected to autoradiography and PhosphorImager analysis for quantitation.

Purification of ssDNA-Activated RPA Kinase. HeLa cytoplasmic S100 fraction (360 mg) was loaded onto a 2.5 × 9.3 cm phosphocellulose (Whatman P-11) column and a 240-ml 0.1–1.0 M NaCl gradient was applied in buffer A [25 mM Hepes, pH 7.8/1 mM EDTA/1 mM EGTA/0.02% Tween 20/10% (vol/vol) glycerol/1 mM dithiothreitol]. The active fractions (≈0.35 M NaCl) were dialyzed vs. buffer A to ≈0.08 M NaCl and loaded onto a 1.5 × 2.5 cm DEAE-Sepharose (Pharmacia) column. A 22-ml 0.08–0.38 M NaCl gradient was applied in buffer A, and activity was recovered at ≈0.15 M NaCl. The active fractions were diluted in buffer A and loaded onto a 1-ml heparin-agarose (Sigma) column. Protein was eluted with a 10-ml 0.1–0.6 M NaCl gradient in buffer A, and the active fractions (≈0.4 M NaCl) were dialyzed vs. buffer A containing 0.1 M NaCl and loaded onto a 1-ml Mono S column (FPLC; Pharmacia). A 20-ml 0.1–0.5 M NaCl gradient was applied in buffer A, and activity was recovered at ≈0.2 M NaCl. The active fractions were dialyzed vs. buffer B [same as buffer A with Tris·HCl(pH 7.8) replacing Hepes (pH 7.8)] containing 0.1 M NaCl, and ≈40% of the activity was loaded onto a 1-ml Mono Q column (FPLC; Pharmacia). Protein was eluted with a 20-ml 0.1–0.5 M NaCl gradient in buffer B, and the enzyme fractions (≈0.25 M NaCl) were dialyzed vs. buffer A containing 0.1 M NaCl, frozen with liquid nitrogen, and stored at −70°C.

Immunodepletions. Purified IgG was bound to protein A-Sepharose (Pharmacia) and mixed with HeLa S100 extract at 4°C. The beads were then pelleted by centrifugation, and the supernatants were assayed as described above. For the study on ssDNA-dependent RPA phosphorylation, 2 mg of S100 was treated with 100 μg of either anti-DNA-PK or nonimmune polyclonal IgG bound to 50 μl of beads in a final volume of 0.25 ml for 2 hr. For the study on replication-dependent RPA phosphorylation, 1.1 mg of S100 was treated with 50 μg of either anti-DNA-PK (25-4) or anti-Ku (N3H10) IgG bound to 10 μl of beads in a final volume of 0.21 ml for 3 hr.

RESULTS

Purification of RPA Kinase. Previous studies have demonstrated that RPA32 is phosphorylated during the S phase of the cell cycle *in vivo* (11) and during SV40 DNA replication *in vitro* (12). In both cases, phosphorylation is accompanied by significant reduction in the mobility of RPA32 in SDS/PAGE. As shown in Fig. 1*A*, the phosphorylation of RPA32 in the SV40 model system is dependent upon both T antigen and a functional SV40 origin of replication. We observed phosphorylation of RPA32 in the presence of either HeLa cytoplasmic extract or an S100 fraction. The S100 exhibits significantly less background phosphorylation in the absence of DNA replication than the cytoplasmic extract (compare the second and sixth lanes from the left in Fig. 1*A*). This is likely due to the absence of contaminating DNA fragments that can activate RPA phosphorylation in a replication-independent fashion (see below). At least three RPA32 species of reduced mobility are generated during SV40 DNA

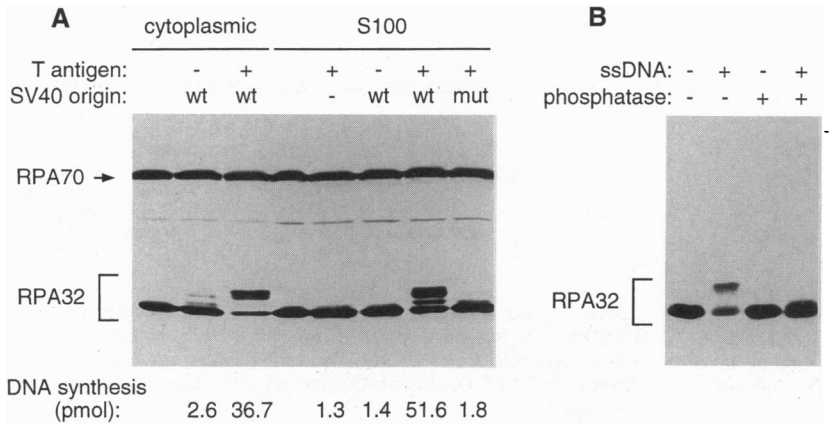

FIG. 1. Phosphorylation of RPA in HeLa cell extracts. (*A*) DNA replication-dependent phosphorylation. Western blot of RPA32 and RPA70 from cytoplasmic and S100 fractions after incubation under the indicated replication conditions. Reactions contained no DNA (−) or plasmid DNA with a wild-type (wt) or mutant (mut) SV40 origin. Starting extracts are shown in the first and fourth lanes from the left. (*B*) ssDNA-dependent RPA phosphorylation. Western blot of cytoplasmic RPA32 after incubation with (+) or without (−) M13 ssDNA (12). The indicated samples were treated with 10 units of calf intestinal alkaline phosphatase.

replication, suggesting the presence of multiple phosphorylation sites.

Fotedar and Roberts (12) reported that M13 ssDNA can activate the phosphorylation of RPA32 in extracts of Manca (human) cells and suggested that the reaction requires the binding of RPA to ssDNA. Fig. 1*B* shows the dependence of RPA32 phosphorylation upon M13 ssDNA in replication extracts derived from HeLa cells. These data confirm the previously reported observations (12) that the reduction in mobility of the RPA32 subunit is absolutely dependent upon the addition of M13 ssDNA and that the effect is reversed by treatment with a phosphatase. Although it is possible that the action of more than one kinase could result in a reduction in RPA mobility, the simplest hypothesis is that a common enzyme is responsible for the S-phase phosphorylation of RPA32 *in vivo* and the phosphorylation induced *in vitro* by SV40 DNA replication or M13 ssDNA. Therefore, we used a ssDNA-dependent kinase assay to purify an RPA kinase from HeLa cell extracts.

Fractionation of HeLa S100 through five chromatographic steps yielded a highly purified ssDNA-activated RPA kinase activity. The major polypeptide coeluting with enzyme activity from the final Mono Q column had an apparent molecular mass of >200 kDa (Fig. 2). The large size of this polypeptide and its chromatographic behavior were similar to those of DNA-PK, a previously described protein kinase of unknown function that is capable of phosphorylating a number of nuclear DNA binding proteins *in vitro* (13, 19, 20). Therefore, we surveyed the active fractions for the presence of this protein kinase by immunoblot analysis. The active >200-kDa polypeptide was found to be strongly reactive with an antiserum raised against the catalytic subunit of DNA-PK (DNA-PKc) (Fig. 2*A*). We conclude that the ssDNA-activated RPA kinase is similar, if not identical, to the 350-kDa DNA-PK polypeptide.

Recent studies have indicated that the Ku autoantigen can act as a component of DNA-PK to target the enzyme to certain protein substrates (21–23). Therefore, the active fractions were probed for both the 70- and 86-kDa subunits of Ku. As shown in Fig. 2*A*, the peak fraction appears devoid of Ku, as does the purified RPA (data not shown). Western blot analysis indicated that DNA-PKc and Ku were resolved at the final step (Mono Q) of the purification with Ku eluting at a slightly higher salt concentration than DNA-PKc. These results suggest that Ku is not a necessary component of the ssDNA-activated RPA kinase *in vitro;* however, it should be noted that *in vivo* conditions may require the presence of this protein.

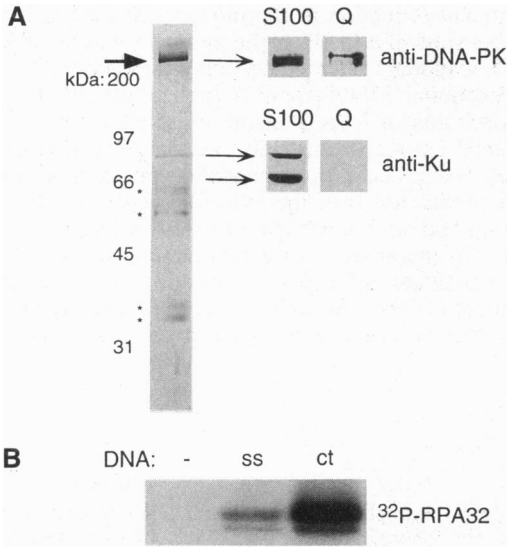

FIG. 2. Analysis of purified DNA-activated RPA kinase. (*A*) SDS/PAGE of the peak Mono Q fraction (150 ng) stained with silver. The position of DNA-PKc is marked by the arrow, and the asterisks indicate artifact bands contributed by the sample buffer. Western blots from the indicated regions of the peak Mono Q fraction (150 ng) in comparison with HeLa S100 (10 μg) are also shown to the right. (*B*) RPA kinase activity of the peak Mono Q fraction (30 ng) in the absence of DNA (−) or in the presence of M13 ssDNA (ss) or activated calf thymus DNA (ct).

DNA-PK Is Required for DNA Replication-Dependent Phosphorylation of RPA. The data described above indicate that purified DNA-PKc can phosphorylate RPA32 in the presence of ssDNA. To verify that the enzyme is responsible for the ssDNA-activated phosphorylation of RPA in crude extracts, we carried out immunodepletion experiments. DNA-PK was depleted from HeLa cell S100 fraction by incubation with anti-DNA-PK IgG coupled to protein A-Sepharose beads. The depleted extract exhibited greatly reduced ssDNA-activated RPA kinase activity that was partially restored by addition of purified DNA-PKc (Fig. 3). The RPA kinase activity of a control extract, incubated with nonimmune antibody, was essentially unchanged from that of an untreated S100 extract. The small residual RPA kinase activity in the depleted extract was probably due to incomplete removal of DNA-PK, although we cannot rule out the possibility of a low level of RPA phosphorylation by a distinct enzyme. We conclude that DNA-PK is the major ssDNA-activated RPA kinase in S100 extracts.

Similar immunodepletion experiments were performed to determine whether DNA-PK is responsible for phosphorylation of RPA during SV40 DNA replication. When HeLa S100 extracts depleted of DNA-PK were incubated under standard replication conditions with T antigen and a plasmid containing the viral origin of DNA replication, no phosphorylation of RPA32 was observed (Fig. 4*A*, lane 4 vs. lane 2). DNA-replication-dependent RPA phosphorylation was partially restored by addition of purified DNA-PKc (Fig. 4*A*, lane 6). These data are entirely consistent with the results of the RPA kinase assays (Fig. 3) and indicate that the DNA-replication-dependent phosphorylation of RPA32 is also mediated by DNA-PK. In the same experiments, depletion of the Ku antigen from the HeLa S100 extracts had no apparent effect on RPA phosphorylation. However, examination of the depleted extracts by Western blot analysis indicated that we were able to remove only 80–90% of the Ku antigen (data not shown). Thus, we cannot be certain at present whether or not Ku plays a role in RPA phosphorylation during DNA replication.

Phosphorylation of RPA Does Not Affect DNA Replication *in Vitro*. An important question is whether RPA phosphoryla-

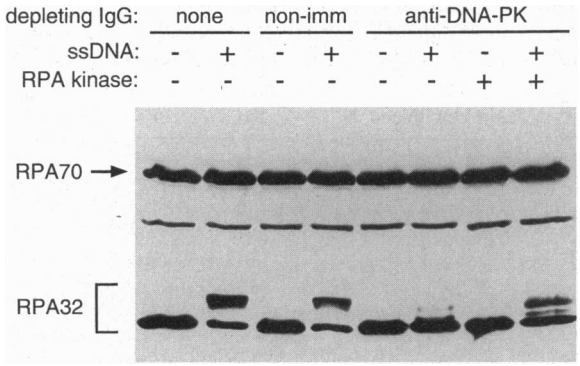

FIG. 3. RPA kinase activity of immunodepleted extract. DNA-PK was immunodepleted from HeLa S100, and RPA kinase activity was measured by the Western blot assay. Purified RPA kinase (≈15 ng of peak Mono Q fraction) was added to the indicated reactions (+).

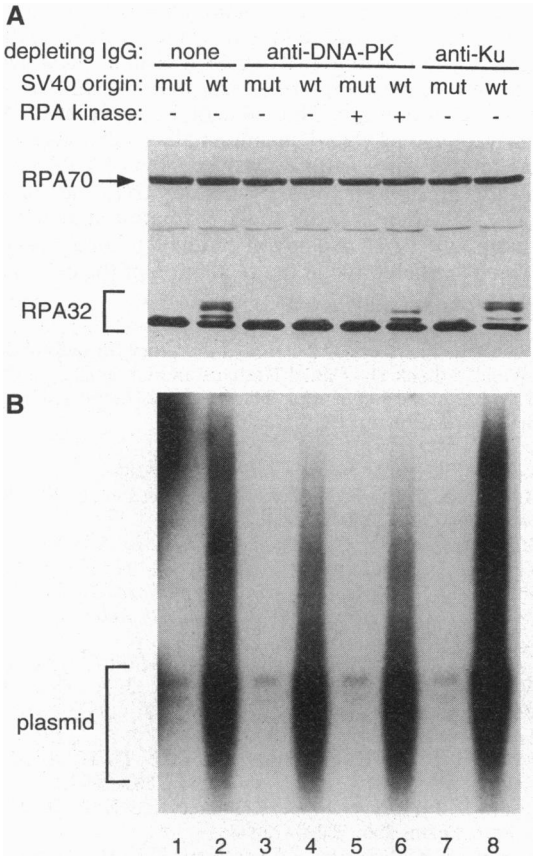

FIG. 4. Replication-dependent RPA kinase activity of immunodepleted extract. HeLa S100 was immunodepleted with either anti-DNA-PK or anti-Ku and assayed for replication-dependent RPA phosphorylation and DNA synthesis. Replication reactions contained plasmid DNA with either mutant (mut) or wild-type (wt) SV40 origin. Purified RPA kinase (30 ng of peak Mono Q fraction) was added to the indicated reactions (+). (*A*) Western blot of RPA32 and RPA70 after the replication reactions. (*B*) Autoradiogram of replication products.

tion plays a direct role in regulating DNA replication. To assess the effect of RPA phosphorylation on SV40 DNA replication *in vitro*, we compared the replication activities of extracts depleted of DNA-PK with those of undepleted control extracts (Fig. 4*B*). The overall extent of DNA replication in depleted extracts was the same as in undepleted extracts even though no phosphorylation of RPA occurred. Moreover, the distribution of replication products after agarose gel electrophoresis was unaffected by immunodepletion of DNA-PK (Fig. 4*B*). These results were confirmed by immunodepleting S100 extract with another antibody directed against DNA-PK (data not shown). Thus, DNA-PK-mediated phosphorylation of RPA is not required for the initiation of DNA synthesis or for the elongation of DNA chains.

DNA Cofactor Requirement for RPA Phosphorylation by DNA-PKc. The nucleic acid requirement for DNA-PKc-catalyzed RPA phosphorylation was investigated by comparing the ability of various DNA structures to stimulate the reaction (Table 1). In addition to activated calf thymus DNA, which is a heterogenous mixture of DNA structures, M13 ssDNA and linear duplex DNA stimulated the RPA kinase activity of the purified enzyme. However, supercoiled plasmid [pUC.HSO (24)] and single-stranded homopolymers [poly(dT) and poly(dA)] were ineffective as cofactors. These results suggest that duplex DNA ends are important for activity, as has been reported for the DNA-PK-catalyzed

Table 1. Nucleic acid cofactor specificity of DNA-activated RPA kinase

DNA added	P_i incorporated, pmol
None	0.08
M13 ssDNA	0.44
Activated calf thymus DNA	2.60
Supercoiled pUC.HSO	0.05
Linear pUC.HSO	0.98
Poly(dT)	0.06
Poly(dA)	0.06
Poly(dT)/linear pUC.HSO	0.10

RPA kinase activity was measured with purified enzyme (30 ng) and the indicated nucleic acid(s) (each at 1 μg/ml). The level of [^{32}P]RPA was determined relative to a reaction containing activated calf thymus DNA and no RPA.

phosphorylation of other protein substrates (13, 23). The data also indicate that secondary structure within the M13 ssDNA molecule is likely to be necessary for activity, perhaps serving the same function as duplex ends. DNA homopolymers lacking such structure not only fail to activate RPA phosphorylation but actually inhibit phosphorylation induced by active cofactors, possibly by sequestering bound RPA (Table 1). We have observed that DNA with both double- and single-stranded characteristics is more effective than duplex DNA under certain conditions (data not shown). Transitions between double- and single-stranded DNA would occur naturally at a replication fork and may play an important role in stimulating phosphorylation of RPA during DNA replication.

DISCUSSION

Previous studies in both yeast and mammalian cells have established that phosphorylation of RPA32 begins at the transition from G_1 to S phase and continues until mitosis and cell division (11). Consistent with these *in vivo* studies, Fotedar and Roberts (12) demonstrated that RPA32 is phosphorylated *in vitro* during DNA replication in the SV40 cell-free system. Based upon the observation that phosphorylation is also promoted by ssDNA, it was suggested (12) that the DNA-replication-dependent phosphorylation of RPA32 occurs after the unwinding of origin-containing duplex DNA. Similar results were reported by Fang and Newport (25) who studied phosphorylation of RPA32 in cycling extracts of *Xenopus* eggs. In such extracts, phosphorylation of RPA32 is dependent upon entry into S phase, which is in turn dependent upon the activity of the cdk2 protein kinase.

Since the S-phase-dependent phosphorylation of RPA32 may play a role in regulating DNA replication, it is of considerable interest to identify the protein kinase responsible for this activity. It has been shown that RPA32 is a substrate *in vitro* for the mitotic protein kinases cdc2-cyclin B and cdc2-cyclin A (26, 27). However, analysis of the kinase activities present in cycling *Xenopus* extracts indicates that neither kinase is a good candidate for the activity that phosphorylates RPA32 during S phase (25). Consistent with this conclusion is the observation that in mammalian extracts depletion of cdc2 kinases with p13-Sepharose does not abolish the DNA-replication-dependent phosphorylation of RPA32 in the SV40 system (12). Although there is strong evidence for the involvement of kinases of the cdk2 family in regulating entry into S phase, there is no evidence that these enzymes phosphorylate RPA32 directly. Depletion of cdk2 kinase activity from *Xenopus* extracts does not prevent the phosphorylation of RPA32 in the presence of ssDNA (25). Moreover, RPA does not appear to be a substrate for cdk2 kinases *in vitro* (27).

We have purified the ssDNA-dependent RPA kinase present in SV40 replication extracts to near homogeneity. The purified activity was identified as DNA-PKc by several criteria, including chromatographic behavior, cofactor requirements, and reactivity with specific anti-DNA-PKc antibodies. Depletion experiments demonstrated conclusively that DNA-PK is required for the DNA-replication-dependent phosphorylation of RPA32 observed in the SV40 DNA replication system. We propose that the same enzyme is responsible for the S-phase-dependent phosphorylation of RPA32 in mammalian cells. Although further work will be required to verify this possibility, it is consistent with previous reports indicating that the phosphopeptide map of RPA32 phosphorylated in the presence of ssDNA is similar to that of RPA phosphorylated *in vivo* (12).

The DNA-PK is an abundant protein kinase with several *in vitro* DNA-binding substrates (13). The enzyme is activated by linear double-stranded DNA (13, 19, 20) and by DNA molecules containing single- to double-strand transitions (28). Our finding that M13 ssDNA molecules can induce the phosphorylation of RPA32 by purified DNA-PKc was somewhat surprising since ssDNA was not effective as a cofactor with previously studied substrates (19, 20). It seems likely that RPA is an effective substrate for the enzyme only when it is bound to ssDNA. However, binding of the substrate to ssDNA, while probably necessary, is clearly not sufficient, since DNA homopolymers do not function as effective cofactors. We suspect that naturally occurring ssDNA molecules that have extensive secondary structure, such as M13 ssDNA, may also provide duplex regions required to activate enzyme activity. Thus, a reasonable model is that efficient phosphorylation of RPA32 occurs when enzyme and substrate are bound to the same DNA molecule. Such a colocalization mechanism has been described for the phosphorylation of the transcription factors p53 and Sp1 by DNA-PK (23, 29). We have also observed that linear duplex DNA, but not circular duplex DNA, can serve as a cofactor for RPA32 phosphorylation at relatively high concentrations of purified DNA-PKc. The colocalization model may apply in this case as well, since RPA can unwind duplex DNA under certain conditions (30). Unwinding of the ends of linear duplex molecules would allow the binding of RPA and enzyme to the same double-stranded DNA molecule.

We have observed that the extent of SV40 DNA replication is unaffected by depletion of DNA-PK. Since the depletion effectively prevents DNA-replication-dependent phosphorylation of RPA32, our data demonstrate that the efficiency of SV40 DNA replication *in vitro* is independent of the phosphorylation state of RPA32. Thus, at least in the cell-free SV40 system, phosphorylation of RPA32 is a consequence of DNA replication, not a requirement for DNA replication. While this conclusion does not rule out possible effects of RPA phosphorylation on DNA replication *in vivo*, it raises the possibility that phosphorylation of RPA32 does not affect the efficiency of DNA replication directly but plays some other regulatory role, perhaps in coordinating DNA replication with other cell cycle events. It is known that interruption of DNA replication by inhibitors or conditional mutations results in a delay of cell-cycle progression. This fact implies the existence of a cellular mechanism for sensing that DNA replication is ongoing. One reasonable speculation is that phosphorylation of RPA32 is part of such a sensing/signal transduction mechanism. Replication intermediates contain both ssDNA and single- to double-strand transitions and would be expected to support efficient phosphorylation of RPA32 by DNA-PK. The phosphorylated form of RPA could then function as part of a signaling pathway that prevents cell-cycle progression while such intermediates are present. In this scenario, RPA functions as a receptor that is phosphorylated when bound by a ssDNA ligand. It should be noted in this context that cell-cycle delay is also a consequence of DNA damage. Thus, it is of interest that RPA32 is efficiently phosphorylated in mammalian cells exposed to ionizing and UV irradiation, and the sites of RPA32 phosphorylation are apparently the same as those utilized in S phase (31, 32). Interestingly, the γ-irradiation-induced phosphorylation of RPA32 is delayed in ataxia telangectasia cells (31), which are defective in the regulation of the cell cycle in response to DNA damage.

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