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Interaction of DNA polymerase α-primase with cellular replication protein A and SV40 T antigen

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The purified human single-stranded DNA binding protein, replication protein A (RP-A), forms specific complexes with purified SV40 large T antigen and with purified DNA polymerase α-primase, as shown by ELISA and a modified immunoblotting technique. RP-A associated efficiently with the isolated primase, as well as with intact polymerase α-primase. The 70 kDa subunit of RP-A was sufficient for association with polymerase α-primase. Purified SV40 large T antigen bound to intact RP-A and to polymerase-primase, but not to any of the separated subunits of RP-A or to the isolated primase. These results suggest that the specific protein-protein interactions between RP-A, polymerase-primase and T antigen may play a role in the initiation of SV40 DNA replication.

Key words: DNA polymerase α/initiation of DNA replication/protein-protein interactions/single-strand DNA binding protein/SV40 T antigen

Introduction

Many features of the replication of simian virus 40 (SV40) DNA in infected monkey cells resemble those observed for cellular DNA replication (reviewed by Stillman, 1989; Challberg and Kelly, 1989; Hay and Russell, 1989; Hurwitz *et al.*, 1990). Only a single viral protein, T antigen, is required for viral replication, rendering SV40 a useful model system to identify the cellular components of the replication machinery and to elucidate the sequence of events required to initiate specifically at an origin, elongate and complete replication. These studies have been greatly facilitated by a cell-free replication assay (Li and Kelly, 1984). Indeed, SV40 DNA replication reconstituted using purified proteins from human cells and T antigen was recently shown to correspond in many ways to viral DNA replication in the infected cell (Tsurimoto *et al.*, 1990; Weinberg *et al.*, 1990), suggesting that most of the essential cellular components are now known.

Control of SV40 DNA replication is realized primarily at the level of initiation at the viral origin (reviewed by Borowiec *et al.*, 1990). In the first step of initiation, a specifically phosphorylated subclass of T antigen binds to a palindromic sequence in the SV40 origin (reviewed by Prives, 1990). In the presence of ATP, a multimeric T antigen-nucleoprotein complex assembles at the origin, leading to structural distortion and unwinding of origin DNA sequences (Mastrangelo *et al.*, 1989). In concert with a cellular single-strand DNA binding protein (RP-A/RF-A/HSSB) and topoisomerase I or II, the DNA helicase activity of T antigen (Stahl *et al.*, 1986) promotes more extensive origin unwinding, forming a pre-initiation complex (Dean *et al.*, 1987; Wold *et al.*, 1987; Bullock *et al.*, 1989). In the next step, DNA polymerase α-primase is thought to interact with this complex, probably through direct, specific protein-protein contacts between T antigen and the large catalytic subunit of polymerase α (Smale and Tjian, 1986; Gough *et al.*, 1988; Gannon and Lane, 1987, 1990; Dornreiter *et al.*, 1990; Schneider *et al.*, 1991), resulting in an initiation complex (Tsurimoto *et al.*, 1990; Weinberg *et al.*, 1990). Once the initiation complex forms, primase synthesizes a short RNA primer on each template strand, which is extended by the polymerase activity of DNA polymerase α-primase. The template-primer junction can then be recognized by a second multiprotein complex composed of RF-C and PCNA, both accessory factors for DNA polymerase δ (Tsurimoto and Stillman, 1991a and references therein). Binding of DNA polymerase δ to this complex then leads to the assembly of the leading strand replication complex and replication forks (Tsurimoto and Stillman, 1991b). The ordered assembly of protein-DNA complexes at the SV40 origin is thus proposed to be the key step in coupling origin recognition and unwinding by T antigen to the actual synthesis of DNA.

In the present study, we have used highly purified T antigen, DNA polymerase α-primase and the single-strand DNA binding protein RP-A to demonstrate protein-protein interactions among these proteins, and to begin to define the molecular specificity of these interactions.

Results

Interaction of DNA polymerase α-primase with SV40 T antigen and RP-A

DNA polymerase α-primase was purified to near homogeneity from calf thymus and DNA primase was isolated from the polymerase-primase complex (Figure 1C; Dornreiter *et al.*, 1990). Human DNA polymerase α-primase was also purified from HeLa cell extract (not shown). SV40 large T antigen was isolated from insect cells infected by a recombinant baculovirus (Höss *et al.*, 1990) and human replication protein A (RP-A) was purified from HeLa cell extract (Figure 4A; Wold and Kelly, 1988; Erdile *et al.*, 1990). DNA polymerase α-primase was composed of four different polypeptides, a catalytic polymerase subunit designated p180, a p72 subunit of unknown function, and

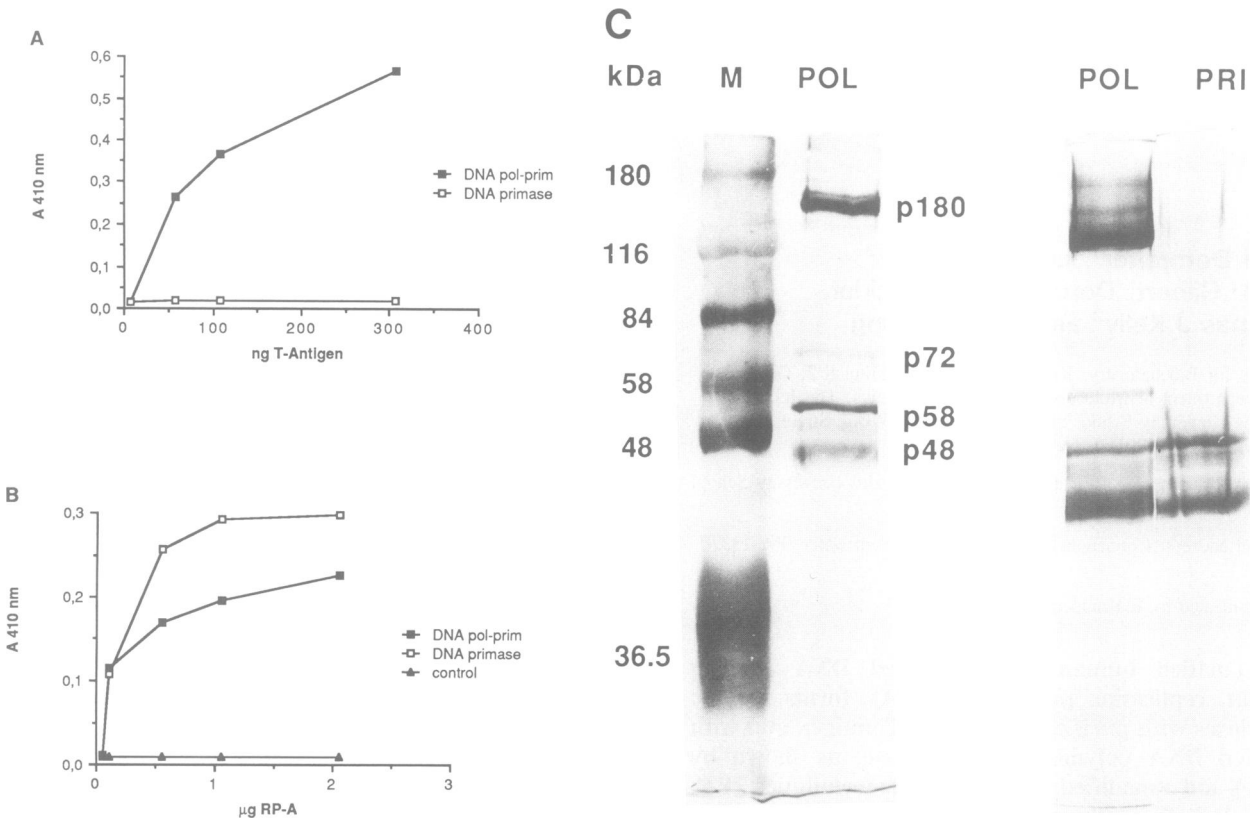

Fig. 1. Interactions of calf thymus DNA polymerase α−primase with SV40 T antigen and RP-A. (**A**) Purified DNA polymerase α−primase or primase, as indicated, were immobilized on ELISA plates. SV40 T antigen was added in the indicated amounts and incubated for 1 h. After washing, bound T antigen was detected using peroxidase-coupled monoclonal anti-T antibody Pab 419 and a chromogenic substrate. (**B**) Purified polymerase α−primase, primase or bovine serum albumin (control) were immobilized on ELISA plates, as indicated. Increasing amounts of purified human RP-A were added, as indicated, and incubated for 1 h. After washing, bound RP-A was detected using polyclonal rabbit antiserum to RP-A, followed by peroxidase-coupled anti-rabbit antibody and a chromogenic substrate. (**C**) Purified DNA polymerase α−primase (3 µg) (POL) and purified DNA primase (1 µg) (PRI) were electrophoresed in denaturing PAGE (Laemmli, 1970) and silver stained (Heukeshoven and Dernick, 1988). This preparation of primase was used for the experiments in (A) and (B) and in Figure 4C. M, prestained marker proteins (Sigma).

the p58 and p48 subunits that together constitute the primase activity (reviewed by Lehman and Kaguni, 1989; Wang *et al.*, 1989). RP-A was composed of three subunits, a 70 kDa subunit that has single-strand DNA binding activity, and subunits of 32 kDa and 14 kDa whose functions remain unknown (Fairman and Stillman, 1988; Wold and Kelly, 1988; Wold *et al.*, 1989; Kenny *et al.*, 1990; Erdile *et al.*, 1991; D.von Winkler, unpublished data).

The interaction of polymerase α−primase with T antigen and human RP-A was assayed by using the purified proteins in an enzyme-linked immunosorbent assay (ELISA). The solid phase was either calf thymus DNA polymerase−primase or the isolated primase. Soluble T antigen was added in increasing concentrations and bound T was detected using a monoclonal anti-T−antibody coupled to horseradish peroxidase. T antigen bound specifically to the polymerase−primase in a dose-dependent manner, but binding to the isolated primase was not detectable (Figure 1A). These results are consistent with our recent observation that T antigen complexes directly and specifically with DNA polymerase α−primase from calf thymus or human cells and that the p180 large catalytic subunit of polymerase α is sufficient for complex formation (Dornreiter *et al.*, 1990).

In Figure 1B, increasing concentrations of soluble human RP-A were incubated with the polymerase or primase solid phase and bound RP-A was measured using a polyclonal antibody against RP-A and peroxidase-conjugated second antibody. RP-A formed complexes with the isolated primase, as well as with the intact polymerase−primase, but not with a control protein, bovine serum albumin. Similar results were obtained with RP-A purified from calf thymus (I.U.Gilbert, D.von Winkler and E.Fanning, unpublished data). These results suggest that RP-A interacts specifically with DNA primase, both in an isolated form and in association with the other polymerase−primase subunits p72 and p180.

The interaction of RP-A with DNA polymerase α−primase was detected using polymerase α−primase from calf thymus; however, calf thymus polymerase−primase is unable to replicate SV40 DNA efficiently in the cell-free system (Murakami *et al.*, 1986; Schneider *et al.*, 1992). Thus, if the association of polymerase α−primase with RP-A and T antigen plays a functional role in SV40 DNA replication, one would expect to find similar protein−protein interactions between the human replication proteins. This prediction was tested in an ELISA using purified human RP-A immobilized in the solid phase (Figure 2). Bovine serum albumin was the control solid phase. Increasing amounts of purified human DNA polymerase α−primase were incubated with the solid phases and bound polymerase α−primase was detected with a monoclonal antibody specific for polymerase−primase and peroxidase-coupled second antibody. The amount of RP-A-bound polymerase−primase

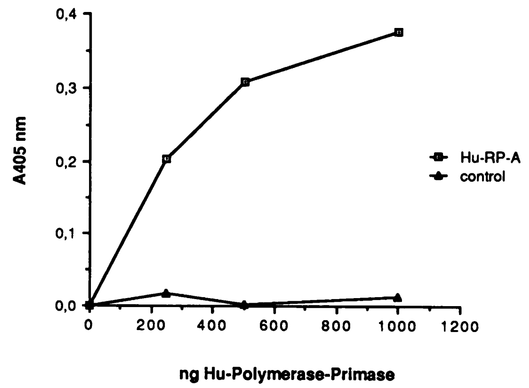

Fig. 2. Interaction of human RP-A with human DNA polymerase α – primase. Purified human RP-A or bovine serum albumin (BSA) were immobilized on ELISA plates. Purified HeLa DNA polymerase α – primase was added in the indicated amounts and incubated for 1 h. After washing, bound polymerase – primase was detected using a monoclonal antibody 2CT25 directed against polymerase – primase (I.Dornreiter, S.Dehde and E.Fanning, in preparation), followed by peroxidase-coupled anti-mouse antibody and a chromogenic substrate.

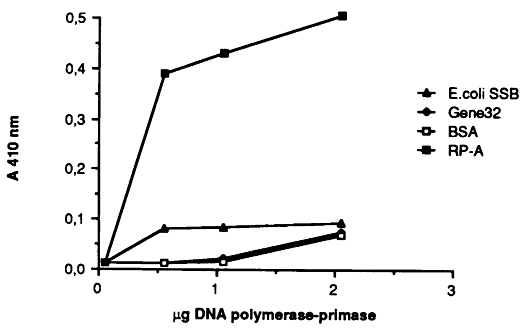

Fig. 3. Specificity of RP-A association with DNA polymerase α – primase. Bacterial SSB (Pharmacia, Freiburg), bacteriophage T4 gene32 protein (Life Technologies, Eggenstein), bovine serum albumin (BSA) and purified HeLa RP-A were immobilized on ELISA plates. Purified calf thymus DNA polymerase – primase was added in the indicated amounts and incubated for 1 h. After washing, bound polymerase – primase was detected with polyclonal rabbit antibody against DNA polymerase – primase, peroxidase-coupled goat anti-rabbit antibody and a chromogenic substrate.

increased in a concentration-dependent fashion, confirming a specific interaction between human RP-A and human polymerase – primase.

Specificity of complex formation between DNA polymerase α – primase and RP-A

In the initial unwinding of the SV40 origin of DNA replication, human RP-A can be replaced without loss of function by heterologous single-stranded DNA binding proteins such as *Escherichia coli* SSB, adenovirus DNA binding protein, herpes simplex ICP8 and yeast RP-A (Wold *et al.*, 1987; Dean *et al.*, 1987; Brill and Stillman, 1989; Kenny *et al.*, 1989; Virshup and Kelly, 1989). However, these proteins were unable to substitute for human RP-A in the cell-free replication of SV40 DNA (Kenny *et al.*, 1989; Brill and Stillman, 1989), suggesting that human RP-A is specifically required at one or more steps later in the replication process. If the physical association of DNA polymerase α – primase with human RP-A (Figures 1B and 2) is specific, this interaction could account, at least in part, for the specific requirement for human RP-A. Thus, the specificity of the interaction between DNA polymerase – primase and RP-A was further tested by inverting the ELISA. Purified human RP-A and prokaryotic single-stranded DNA binding proteins, *E.coli* SSB and T4 gene32 protein, were used as solid phase and DNA polymerase – primase as the soluble phase (Figure 3). Serum albumin served as a control solid phase. Binding of polymerase – primase to RP-A increased with increasing concentration, confirming a specific interaction between these two proteins. A comparatively weak interaction was observed between bacterial SSB and polymerase – primase, while no significant association of polymerase – primase with gene32 protein or serum albumin was detected.

Subunit specificity of RP-A binding to polymerase α – primase

The subunit specificity of the interaction between DNA polymerase – primase and human RP-A was then investigated using a modified immunoblotting method (Dornreiter *et al.*, 1990). The subunits of RP-A were separated by electrophoresis in denaturing polyacrylamide gels. *E.coli* single-strand DNA binding protein, T4 gene32 protein and serum albumin were electrophoresed in a similar manner (Figure 4A). The proteins were renatured in the gel, transferred to nitrocellulose by electroblotting and then incubated with purified DNA polymerase α – primase. Figure 4B demonstrates that polymerase – primase associated with the 70 kDa band of RP-A, but the smaller RP-A subunits did not interact with polymerase – primase. Polymerase – primase failed to bind to serum albumin and to gene32 protein. Unexpectedly strong binding of polymerase – primase to *E.coli* SSB was also detected, qualitatively confirming the result shown in Figure 3. Interestingly, a search for sequence homology between bacterial SSB (Sancar *et al.*, 1981; Chase *et al.*, 1984) and 70 kDa human RP-A (Erdile *et al.*, 1991) revealed a short region with matches at 16 of 37 residues.

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                 123                                                        157
E.coli SSB        GGNI GGGQPQG  GWGQPQQ  PQGGNQFSGGAQSRPQQ
                  :    ::    :      :  ::::    :          :    :  ::::
human RP-A 70     GVKI GNPVPYNEGLGQPQVAPPAPAA  SPAASSRPQPQ
                  109                                                       145
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This region of *E.coli* SSB is thought to interact with the bacterial replication machinery (Chase *et al.*, 1984), suggesting that this region of RP-A may be involved in complex formation with DNA polymerase α – primase. However, it should be noted that because the strength of the interactions between partially renatured proteins as detected in the blotting assay may be quite different from that of the native proteins employed for ELISAs, the blots should not be interpreted quantitatively.

To confirm the subunit specificity of polymerase – primase binding to RP-A, a purified human 70 kDa RP-A subunit expressed in bacteria carrying a recombinant plasmid was tested in an ELISA using either intact polymerase – primase or isolated primase as the solid phase (Figure 4C). Serum albumin served as a control solid phase (not shown). The recombinant 70 kDa subunit associated with the intact polymerase α – primase, as well as with isolated primase, in a concentration-dependent manner. These results indicate that polymerase – primase can bind to the 70 kDa subunit of RP-A in the absence of the smaller subunits and provide additional evidence for the molecular specificity of the interaction between polymerase α-primase and RP-A.

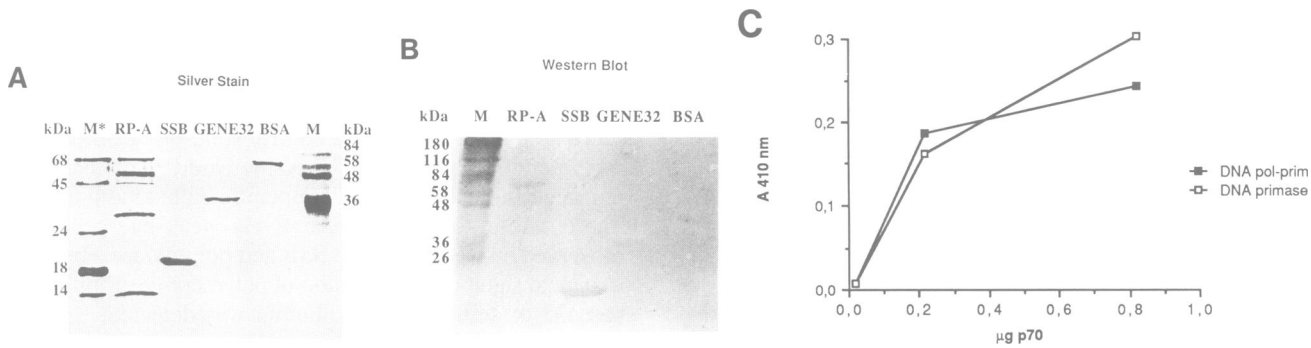

Fig. 4. Subunit specificity of RP-A interaction with DNA polymerase α−primase. (**A**) Purified human RP-A (1.6 µg), bacterial SSB (0.5 µg), T4 gene32 protein (0.5 µg) and bovine serum albumin (BSA) (0.5 µg) were electrophoresed in a 13.7% denaturing polyacrylamide gel and silver stained (Heukeshoven and Dernick, 1988). M, prestained marker proteins; M*, unstained marker proteins (Dalton Mark VI, Sigma, Munich). (**B**) Purified human RP-A (5 µg), bacterial SSB (5 µg), T4 gene32 protein (5 µg), and BSA (5 µg) were electrophoresed in a 13.7% denaturing polyacrylamide gel, renatured in the gel and transferred to a nitrocellulose filter. The filter was incubated with purified calf thymus DNA polymerase α-primase (8 µg/ml TBS), washed, and then incubated with 15 ml polyclonal rabbit antibody against DNA polymerase α−primase (1:500 in TBS), followed by an alkaline phosphatase-based detection system. M, prestained marker proteins. (**C**) Purified calf thymus DNA polymerase α−primase and DNA primase, as indicated, were immobilized on ELISA plates. The purified recombinant 70 kDa subunit of human RP-A was added in the indicated amounts and incubated for 1 h. Bound 70 kDa subunit was detected using polyclonal rabbit anti-RP-A antibody (1:2000), peroxidase-conjugated second antibody and a chromogenic substrate.

Specific interaction of SV40 T antigen with RP-A

The protein−protein interactions of DNA polymerase α−primase with T antigen and RP-A raised the question whether T antigen and RP-A might also interact with each other. To address this question, purified T antigen immobilized in ELISA plates was incubated with increasing concentrations of human RP-A. Serum albumin was used as a control solid phase. Binding of RP-A to serum albumin remained at background levels, whereas binding to T antigen increased as a function of concentration, indicating a specific interaction between these two proteins (Figure 5A).

The molecular specificity of this interaction was further investigated using purified T antigen as the solid phase, and human RP-A or the purified recombinant 70 kDa subunit of human RP-A as the soluble phase (Figure 5B). The intact RP-A bound to T antigen in a concentration-dependent manner, as expected, but the isolated 70 kDa subunit failed to interact with T antigen. Thus, T antigen may recognize one of the smaller subunits of RP-A. Alternatively, it may require an RP-A complex containing multiple subunits, or eucaryote-specific modifications of RP-A (Eki and Hurwitz, 1991).

Interactions of DNA polymerase α−primase with RP-A and T antigen are not mediated by contaminating DNA

DNA polymerase α−primase from calf thymus and from human cells appears to associate with RP-A and T antigen in a direct and highly specific manner (Figures 1−4; Dornreiter *et al.*, 1990). Moreover, native RP-A appears to interact with T antigen (Figure 5). However, all three proteins have DNA binding activity. If the purified proteins were contaminated by significant amounts of DNA during extraction of the cells or chromatography on DNA affinity columns, one could argue that the observed interactions between the proteins in ELISAs could be due to their association with the same tightly bound DNA fragment rather than to protein−protein interactions.

To assess this possibility, the ELISAs were repeated in the presence of an amount of DNase I sufficient to hydrolyze 100 ng of double-stranded or denatured plasmid DNA

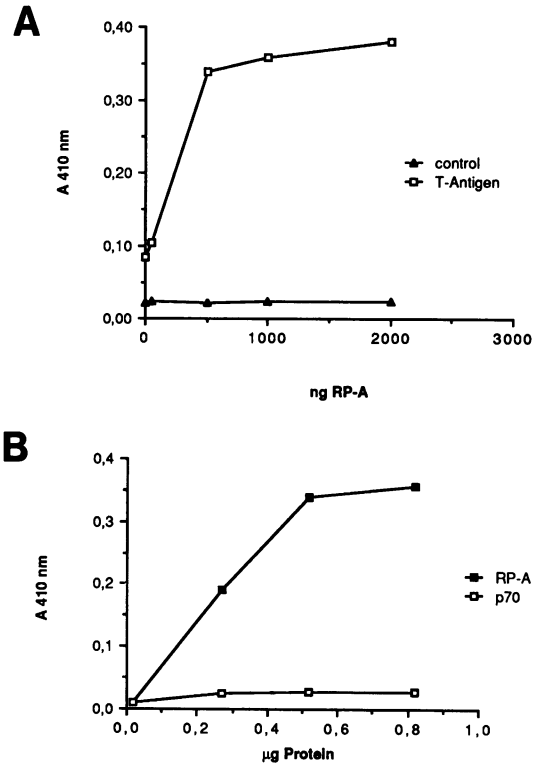

Fig. 5. Interaction of SV40 T antigen with RP-A. (**A**) SV40 T antigen or bovine serum albumin (control) was fixed in ELISA plates (1 µg per well). Human RP-A was added in the indicated amounts and incubated for 1 h. After washing, bound RP-A was detected with polyclonal rabbit antibody against human RP-A (1:2000), peroxidase-coupled goat anti-rabbit antibody and a chromogenic substrate. (**B**) Purified T antigen fixed on ELISA plates (1 µg per well) incubated with the indicated amounts of purified human RP-A or the recombinant 70 kDa subunit of human RP-A for 1 h. Bound RP-A and 70 kDa subunit were detected using polyclonal rabbit anti-RP-A (1:2000), peroxidase-conjugated goat anti-rabbit antibody and a chromogenic substrate.

(Figure 6A). Complex formation between DNA polymerase α−primase and either T antigen or RP-A detected in the presence of DNase I was equivalent to that observed without

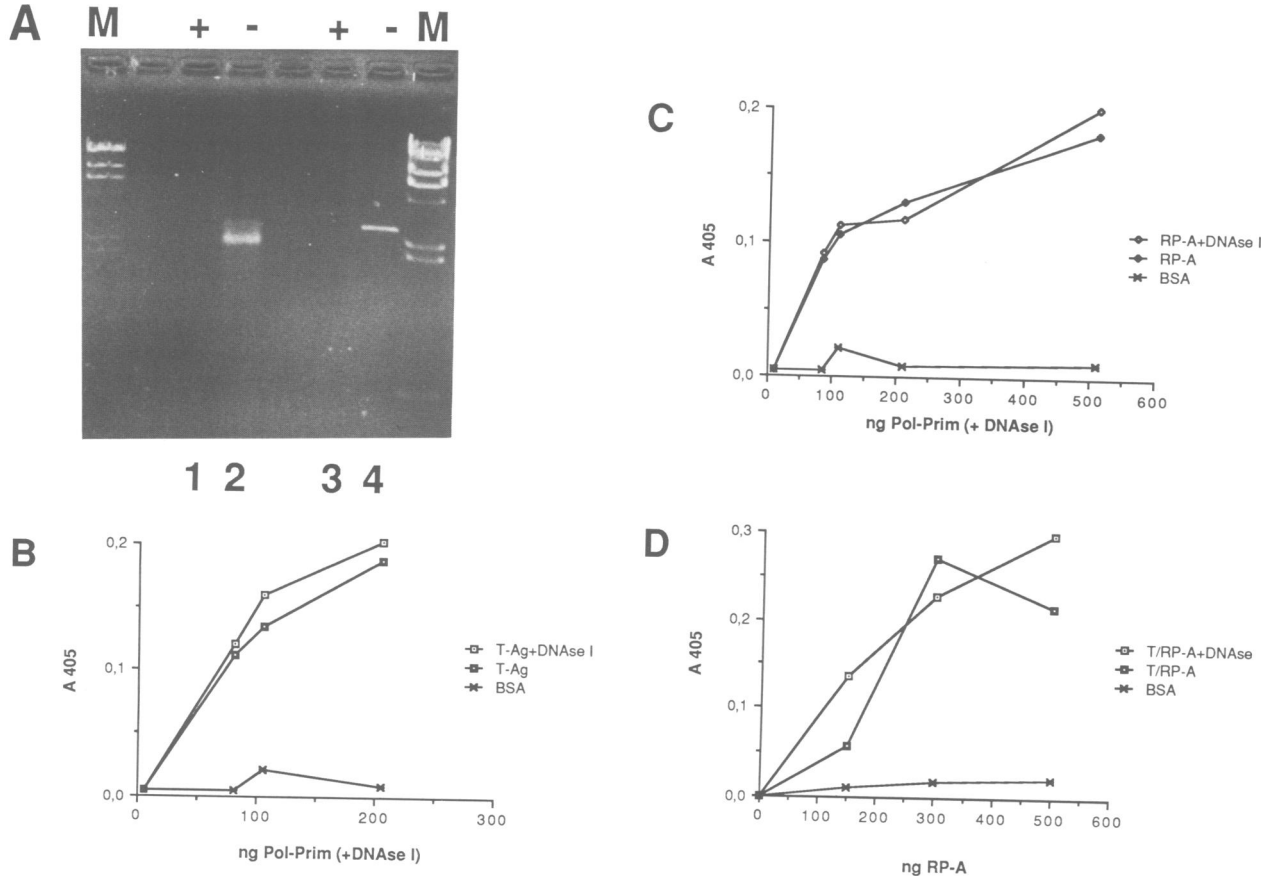

Fig. 6. Protein–protein interactions in the presence of DNase I. (**A**) 100 ng of double-standed linearized pUC18 DNA (lanes 3 and 4) was incubated for 1 h at room temperature with (+) or without (−) 25 ng DNase I (238 U/μg, Pharmacia, Freiburg) in PBS containing 10 mM $MgCl_2$. 1 μg of heat-denatured linearized pUC18 DNA (lanes 1 and 2) was incubated for 1 h at room temperature with (+) or without (−) 250 ng DNase I in PBS, 10 mM $MgCl_2$. Half of each sample was analyzed by electrophoresis in a 1% agarose gel and stained with ethidium bromide. M, marker DNA fragments. (**B** and **C**) Samples (1 μg) of SV40 T antigen (A), human RP-A (B) or bovine serum albumin (BSA) as a control in 50 μl PBS containing 10 mM $MgCl_2$ with or without 25 ng DNase I were immobilized in microtiter plates. The indicated amount of calf thymus DNA polymerase α–primase in 50 μl PBS containing 10 mM $MgCl_2$ and 25 ng DNase I was added and incubated for 1 h. After washing, bound polymerase–primase was detected with a monoclonal antibody 2CT25 against polymerase–primase (I.Dornreiter, S.Dehde and E.Fanning, in preparation) (1 μg per well), peroxidase-coupled rabbit anti-mouse antibody and a chromogenic substrate. (**D**) Samples (1 μg) of SV40 T antigen or BSA in 50 μl PBS containing 10 mM $MgCl_2$ and 25 ng DNase I were immobilized in microtiter plates. The indicated amounts of human RP-A in 50 μl PBS, 10 mM $MgCl_2$ with or without 25 ng DNase I were added and incubated for 1 h. After washing, bound RP-A was detected with polyclonal anti-RPA antibody, peroxidase-coupled second antibody and a chromogenic substrate.

DNase I treatment (Figure 6B and C). Thus, the results confirm that specific protein–protein interactions, rather than protein–DNA interactions, are responsible for the association of DNA polymerase–primase with RP-A and T antigen.

To address the question whether the interaction between RP-A and T antigen could be mediated by DNA bound to either protein, T antigen was immobilized in microtiter plates in the presence of DNase I sufficient to digest 100 ng of plasmid DNA. The indicated amounts of soluble human RP-A were added with or without DNase I. Figure 6D shows that binding of RP-A to T antigen was not significantly decreased in the presence of nuclease, implying that the association between these two proteins is mediated by protein–protein interactions.

Discussion

The initiation of SV40 DNA replication can occur in the presence of three purified proteins: viral T antigen and two cellular factors, DNA polymerase α–primase, and RP-A (Ishimi *et al.*, 1988; Tsurimoto *et al.*, 1990; Weinberg *et al.*, 1990; Matsumoto *et al.*, 1990). Specific protein–protein interactions between T antigen and DNA polymerase α–primase in crude cell extracts have been documented by a number of investigators (Smale and Tjian, 1986; Gough *et al.*, 1988; Gannon and Lane, 1987, 1990) and were recently confirmed using highly purified proteins (Dornreiter *et al.*, 1990; Schneider *et al.*, 1992). The experiments described here document for the first time that similar interactions also occur between DNA polymerase α–primase and RP-A and between T antigen and RP-A. The interactions between DNA polymerase α–primase and RP-A have been localized to the primase subunits of DNA polymerase α–primase and the 70 kDa subunit of RP-A. The interactions of polymerase α–primase with T antigen and RP-A, as well those of RP-A with T antigen, appear to be mediated by protein–protein contacts.

Specific protein–protein interactions of replicative single-strand DNA binding proteins with their cognate DNA polymerase, primase and helicase are a general feature in prokaryotic DNA replication systems, such as bacteriophages T4 (Cha and Alberts, 1988) and T7 (Huber *et al.*, 1988). The T4 gene32 protein, for example, interacts specifically

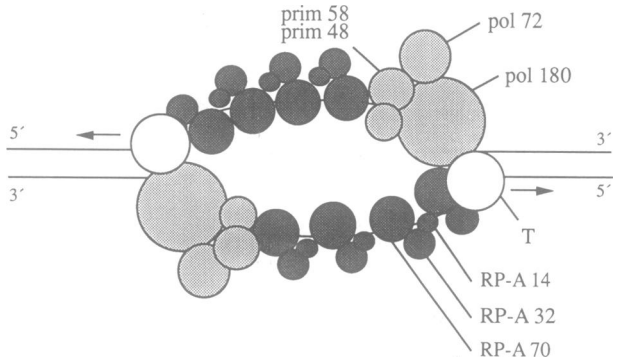

Fig. 7. Model for an initiation complex at the SV40 origin of DNA replication. A multiprotein complex composed of RP-A, DNA polymerase α−primase and T antigen, probably as a hexamer (Mastrangelo *et al.*, 1989), is assembled at the origin. We suggest that T antigen directs DNA polymerase α−primase to bind on the opposite strand, while RP-A is bound to the same strand as T antigen. The complex would thus be poised to begin synthesis of the initial primers.

with six of the seven T4 replication proteins, as shown genetically and biochemically, attesting to its multifunctional nature. Multiple functions have also been ascribed to human RP-A, including single-strand DNA binding and a concentration-dependent stimulation of DNA polymerase α−primase and DNA polymerase δ (Tsurimoto and Stillman, 1989, 1991b; Kenny *et al.*, 1989, 1990). Our findings that RP-A associates specifically with DNA polymerase−primase extend the similarities between RP-A and gene32 protein, suggesting that the protein−protein interactions reported here are functionally relevant in SV40 DNA replication.

Indeed, there is a body of evidence suggesting that the physical interactions among the three proteins involved in the initiation of SV40 DNA replication are functionally significant. The interaction between T antigen and DNA polymerase α−primase appears to have functional consequences since T antigen can stimulate the activity of human DNA polymerase α−primase on an M13 single-stranded template (Collins and Kelly, 1991). T antigen binds to DNA polymerase α−primase purified from human cells and calf thymus (Dornreiter *et al.*, 1990), and to murine polymerase−primase in crude extracts (Gough *et al.*, 1988). However, its affinity for purified human polymerase−primase is 5- to 10-fold greater than for purified calf polymerase−primase (Dornreiter *et al.*, 1990). The species specificity of the interaction between these two proteins (Dornreiter *et al.*, 1990; Schneider *et al.*, 1992) may explain why the SV40 replication activity of polymerase α−primase-depleted human extracts could be restored by the addition of purified human DNA polymerase α−primase, but not polymerase−primase purified from mouse or calf thymus (Murakami *et al.*, 1986; Schneider *et al.*, 1992).

The functional significance of interactions between RP-A and DNA polymerase α−primase is suggested by the ability of human RP-A to stimulate the activity of human DNA polymerase α−primase on the artificial template poly(dA): oligo(dT) (Kenny *et al.*, 1989; Erdile *et al.*, 1991). The specificity of this stimulation, i.e. the inability of either prokaryotic or viral SSBs or even the homologous *Saccharomyces cerevisiae* RP-A to substitute for the human proteins (Kenny *et al.*, 1989; Erdile *et al.*, 1991) argues that the stimulation may require specific protein−protein interactions between RP-A and DNA polymerase α−primase. This idea is further supported by the observation that the 70 kDa subunit of human RP-A, which we have shown to be sufficient to interact with DNA polymerase α−primase, is able to replace the complete human RP-A in stimulation of DNA polymerase α−primase on poly(dA): oligo(dT) (Erdile *et al.*, 1991). However, the functional interaction between RP-A and polymerase−primase is not strictly species-specific, since purified RP-A from calf thymus will substitute for human RP-A in SV40 DNA replication (L.Erdile, unpublished data; Schneider *et al.*, 1992).

The functional importance of the interactions between DNA polymerase α−primase, T antigen and RP-A is supported by experiments carried out on an artificial fork template. Human RP-A, the recombinant human 70 kDa subunit, and *E.coli* SSB inhibit the activity of DNA polymerase α−primase on an artificial fork template; however, the three proteins differ in that the inhibition produced by RP-A, but not by the 70 kDa subunit or *E.coli* SSB, can be overcome by the addition of T antigen (Collins *et al.*, 1992). These interactions involving all three replication proteins are likely to be quite complex and may well involve more subtle interactions including the other subunits of DNA polymerase α−primase and RP-A, as well as the DNA template. Such interactions could explain the inability of the 70 kDa subunit to replace whole RP-A in DNA replication (Erdile *et al.*, 1991) and the inhibition of replication produced by monoclonal antibodies against the 32 kDa subunit of RP-A (Erdile *et al.*, 1990; Kenny *et al.*, 1990).

The fact that the three proteins involved in the initiation of SV40 DNA replication can interact suggests that a complex containing all three of these proteins may act to promote initiation. Multiprotein complexes have been shown to carry out the initiation of DNA replication in *E.coli* (reviewed by Bramhill and Kornberg, 1988) and bacteriophage λ (Alfano and McMacken, 1989a,b; Dodson *et al.*, 1989). Once assembled, these prokaryotic initiation complexes proceed to become part of the multiprotein replication complexes at the replication forks. The interactions reported here for T antigen, RP-A and DNA polymerase α−primase may thus be functionally relevant for initiation of SV40 replication. Support for this idea is provided by a recent study demonstrating that purified human polymerase α−primase, T antigen and RP-A are specifically required for the initial synthesis of primers at the SV40 origin in the absence of DNA synthesis; RP-A could not be substituted by heterologous SSB proteins (Matsumoto *et al.*, 1990).

We thus propose that these proteins assemble at the SV40 origin of replication in the presence of ATP to form a specific initiation complex. Figure 7 depicts schematically the specific contacts between T antigen and DNA polymerase−primase, and RP-A and DNA primase, as reported here and by Dornreiter *et al.* (1990). In our model, origin unwinding by T antigen is coupled to the synthesis and elongation of primers by the association of polymerase α−primase with T antigen and RP-A. These interactions are proposed to orient polymerase−primase properly for the initiation of DNA synthesis. Once the first nascent strand is synthesized, the assembly of the complete replication machinery can occur and elongation at both forks can ensue (Tsurimoto *et al.*, 1990; Tsurimoto and Stillman, 1991a,b; Weinberg *et al.*, 1990; Matsumoto *et al.*, 1990).

Based on the similarities between the replication of SV40 DNA and the replication of chromosomal DNA, we suspect

that the interactions between T antigen and DNA polymerase α–primase may mimic those made by putative cellular initiator proteins. Once the domains of DNA polymerase α–primase involved in interacting with T antigen are better defined, it might be possible to use peptides derived from those domains as affinity reagents to identify and purify the cellular initiator proteins.

Materials and methods

Protein purification

Calf thymus DNA polymerase α and primase were purified by immunoaffinity chromatography (Nasheuer and Grosse, 1987) with the modifications described (Dornreiter *et al.*, 1990). Protein concentrations were determined spectrophotometrically, assuming that A_{280} = 1.0 corresponds to 1 mg/ml of protein (Warburg and Christian, 1941). The specific activity of purified polymerase α was 18.000–22.000 units/mg, depending on the individual preparation, at a concentration of 1–1.7 mg/ml. Human DNA polymerase–primase was purified from HeLa cell extract by immunoaffinity chromatography as described (Dornreiter *et al.*, 1990) except that the heparin–Sepharose step was omitted and a non-specific transferrin–bovine serum albumin–Sepharose affinity column (Takada-Takayama *et al.*, 1990) preceded the immunoaffinity column (C.Schneider, unpublished data).

SV40 T antigen expressed by a recombinant baculovirus 941T (Lanford, 1988) in Sf 9 insect cells was purified by immunoaffinity chromatography as described (Dornreiter *et al.*, 1990; Höss *et al.*, 1990). T antigen concentration was determined spectrophotometrically (Gill and von Hippel, 1989).

Human RP-A was isolated from HeLa cells as described (Wold and Kelly, 1988) with minor modifications (Erdile *et al.*, 1990). Expression and purification of the recombinant 70 kDa subunit of human RP-A from *E.coli* (1.3 M NaSCN eluate from Affigel-Blue) is described elsewhere (Erdile *et al.*, 1991). The quality of purified proteins was monitored by silver staining (Heukeshoven and Dernick, 1988) after electrophoresis in denaturing polyacrylamide gels (Laemmli, 1970).

Antibodies

Monoclonal antibody Pab 419 against SV40 T antigen (Harlow *et al.*, 1981) and its purification (Dornreiter *et al.*, 1990) were described previously. Polyclonal rabbit antibody against DNA polymerase α–primase was a generous gift from Frank Grosse and Heinz-Peter Nasheuer. Polyclonal rabbit antibody raised against human RP-A was described previously (Virshup *et al.*, 1990). This antibody specifically recognizes the 70 and 32 kDa subunits of both human and calf thymus RP-A in immunoblots, and reacts with RP-A from both species in ELISA (not shown). Monoclonal antibody 2CT25 against polymerase α–primase will be described elsewhere (I.Dornreiter, S.Dehde and E.Fanning, in preparation).

Enzyme-linked immunosorbent assay

ELISAs were carried out in 96-well plastic plates (Immuno Maxi Sorb, Nunc, Wiesbaden) exactly as described previously (Dornreiter *et al.*, 1990). Briefly, wells were coated for 1 h with excess (1 μg) purified DNA polymerase α–primase or another purified protein as indicated in the figure legends, and then blocked with bovine serum albumin (A-7030, Sigma, Munich) in phosphate-buffered saline (PBS). Purified T antigen or another purified protein, as indicated in the figure legends, was added in a volume of 50 μl of PBS, incubated for 1 h at room temperature, and then washed with PBS. Detection of bound T antigen was accomplished with horseradish peroxidase-conjugated Pab 419 monoclonal antibody, and the chromogenic substrate 2,2-azino-bis-(3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS) (Sigma, Munich), as described previously (Dornreiter *et al.*, 1990). After 15–20 min, the resulting green colour was quantified spectrophotometrically at 405 or 410 nm as indicated on the ordinates. Detection of bound RP-A was carried out with polyclonal rabbit antibody against RP-A (diluted 1:2000), peroxidase-conjugated goat anti-rabbit antibody (1:100) (Dakopatts, Hamburg) and ABTS.

Immunoblotting

Purified proteins, as indicated in the figure legends, were denatured at room temperature in a 4- to 5-fold volume of sample buffer as described (Miskimins *et al.*, 1985), electrophoresed in 10 or 13.7% denaturing polyacrylamide minigels (0.5 mm thick) (Laemmli, 1970) and renatured in the gel in 50 mM Tris pH 7.4, 20% glycerol for 1 h at room temperature before electrophoretic transfer to nitrocellulose filters in Na-carbonate buffer overnight with cooling as described (Dunn, 1986). The filter was blocked with 3% non-fat dry milk powder in Tris-buffered saline (TBS), washed in TBS and incubated with a second purified protein, as indicated in the figure legends, followed by polyclonal antibodies directed against the second protein and an alkaline phosphatase-based commercial detection system (Promega, Heidelberg).

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