J. Biochem. 120, 766-772 (1996)

Replication Protein-A Mediates the Association of Calf Thymus DNA Polymerase α-DNA Primase Complex with Guanine-Rich DNA Sequence[1]

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Received for publication, May 23, 1996

We have shown that calf thymus DNA polymerase α-DNA primase complex (polα-primase) preferentially binds to pyrimidine-rich sequences and initiates RNA primer synthesis [Suzuki, M. *et al.* (1993) *Biochemistry* 32, 12782-12792]. Here we tested the association of polα-primase with a guanine-rich DNA fragment (SVG, 30-mer) containing *in vivo* initiation sites of simian virus 40 DNA replication. While pyrimidine-rich fragment (CTPPS 1, 30-mer), that is a preferred sequence for calf thymus DNA primase, was well co-precipitated with polα-primase using anti-polα antibody, SVG was hardly precipitated under the same conditions. Competition studies in either gel-retardation assay or during *de novo* DNA synthesis by polα-primase demonstrated that the interaction of polα-primase with SVG was much weaker than that with CTPPS 1. On the other hand, replication protein-A (RP-A) could bind SVG, although less efficiently than CTPPS 1. After preincubation with RP-A, SVG could bind polα-primase that was immobilized on Sepharose beads. The simian virus 40 large T antigen also enhanced association of SVG to polα-primase, while *Escherichia coli* single-stranded DNA-binding protein did not. However, polα-primase, bound to SVG in the presence of RP-A, failed to synthesize RNA primers. When SVG was extended 10 nucleotides at its 5′-end, polα-primase synthesized trace amounts of RNA primers, and this activity was stimulated more than 10-fold by adding RP-A. These results suggest a new role for RP-A, *i.e.*, as a molecular tether that allows polα-primase to bind guanine-rich regions of DNA in order to initiate RNA primer synthesis.

Key words: DNA polymerase α-primase, DNA replication, replication protein A.

One of the basic rules of DNA replication is that DNA polymerase cannot start a chain and must rely on a priming device. DNA replication is usually initiated by synthesizing RNA primers, in either continuous synthesis on the leading strand or discontinuous synthesis on the lagging strand (*1*). In eukaryotic cells, the DNA primase subunit of DNA polymerase α-DNA primase complex (polα-primase) has been considered to be responsible for the synthesis of RNA primers (*2-4*) and both site-recognition and RNA synthesis are achieved by DNA primase subunit (primase) (*5, 6*). However, the DNA polymerase α (polα) moiety of the complex can modulate primase action, since binding of simian virus 40 large T-antigen (SV40T-Ag) to the polα moiety stimulates the primase activity in the complex (*7, 8*). Chain lengths of primers synthesized by polα-primase are around the decamer on a deoxyhomopolymer template, but can be as short as dinucleotide or tetranucleotide (*9*). However, RNA primers shorter than heptamer can hardly be used as primers for DNA synthesis by polα (*9-13*), so short primer synthesis may be abortive. Primase efficiently utilizes pyrimidine homopolymers as templates, such as poly(dT) (*14*), of which the minimum chain length is the hexamer for polα-primase (*9*). Studies have been performed using natural templates to determine the sequence specificity of priming sites (*6, 15-17*). It has been revealed that polα-primase preferentially recognizes a minimum priming unit consisting of a pyrimidine cluster of a hexamer or longer. This preference is rather stable irrespective of ribonucleoside triphosphate (NTP) concentrations in the reaction mixture (*5, 6*). Once polα-primase binds to priming sites, an enhanced NTP determines the initiation positions: it corresponds to the second nucleotide from the 5′-end of the primer RNA (*6, 15, 17*). Primase is known to complex with polα, and this complex will associate with other proteins such as SV40T-Ag (*8, 18-22*) and RP-A (*23*). These interactions may occur beyond animal species, since calf thymus polα-primase binds either SV40T-Ag

[1] A part of this work was supported by a Grant-in-Aid for Cancer Research and Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan.

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Abbreviations: polα-primase, DNA polymerase α-DNA primase; primase, DNA primase; SDS, sodium dodecylsulfate; PAGE, polyacrylamide-gel electrophoresis; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; NP-40, Nonidet P-40; SSB, single-stranded DNA-binding protein; SV40T-Ag, simian virus 40 large T-antigen; BSA, bovine serum albumin; RP-A, replication protein A.

(*20*) or human RP-A (*24*).

The binding of polα-primase with template DNA is crucial in DNA replication, but its binding affinities are unequal among various sequences. In fact, calf thymus polα-primase itself shows much weaker affinity with purine-rich sequences than with pyrimidine-rich sequences (*5, 6*, and this study). To extend our understanding of the interaction of polα-primase with DNA templates, we synthesized DNA fragments named SVG and SVG-2, *i.e.*, dG-rich sequences in the vicinity of the core origin of SV40 DNA replication where two of the start sites of replication are located (*24*). These DNA fragments showed very weak interaction with calf thymus polα-primase. Taking advantage of this character, we examined whether polα-primase can bind to these DNA fragments in the presence of other DNA-binding proteins, *e.g.*, mammalian ssDNA binding protein (RP-A), or SV40T-Ag in order to synthesize RNA primers.

MATERIALS AND METHODS

Materials—Unlabeled ribonucleoside triphosphates (NTP) and deoxyribonucleoside triphosphates (dNTP) were purchased from Yamasa Shoyu (Chiba). Radioactive compounds were obtained from ICN Pharmaceuticals (CA, USA). Synthetic homopolymers, dT_{10}, $dT_{12\text{-}18}$, $dT_{19\text{-}24}$, and $dT_{25\text{-}30}$ were from Pharmacia (Uppsala, Sweden). All other reagents for enzyme assays were obtained from commercial sources. DNA fragments, CTPPS 1, SVG, and SVG-2 (Fig. 1), were chemically synthesized and purified by HPLC as described before (*6*).

Enzyme Purification—Four-subunit form of calf thymus polα-primase (*5*) and SV40T-Ag (*25, 26*) were obtained as described previously. RP-A was obtained as described (*27, 28*). In brief, the ssDNA cellulose fraction was loaded onto a Mono Q column (FPLC, Pharmacia) equilibrated with buffer BN (20 mM Tris-HCl, pH 7.5, 10% glycerol, 0.1 mM EDTA, 0.01% NP-40, 1 mM DTT). The column was washed with buffer BN extensively, then RP-A was eluted with a linear gradient from 0 to 0.5 M NaCl in the same buffer.

Immunoprecipitation—DNA fragments were labeled with ^{32}P at their 5′-ends using T4 polynucleotide kinase. One nanogram of ^{32}P-labeled DNA and 0.1 unit of polα-primase were incubated with anti-polα antibody (CL22-2-42B) (*29*) in TE buffer (10 mM Tris-HCl, pH 8.0, containing 1 mM EDTA) on ice for 15 min. Anti-mouse IgG antibody (MBL, Nagoya) was then added and the whole was incubated for 15 min. The mixture was centrifuged at 15,000 × *g*, then the pellet was washed twice with TE, supplemented with 0.1% SDS, and analyzed by SDS-PAGE (20%). The trapped ^{32}P-labeled DNA bound to polα-primase was quantified by NIH Image.

De Novo DNA Synthesis and Competition Analysis—The reaction mixture (25 μl) for DNA synthesis coupled with primer RNA synthesis (*de novo* DNA synthesis) contained 50 mM Tris-HCl (pH 7.5), 2 mM dithiothreitol (DTT), 5 mM $MgCl_2$, 50 μM each of dATP, dGTP, dTTP, 5 μM [α-^{32}P]dCTP, 2 mM ATP, 50 μM each of CTP, GTP, UTP, 4 μg/ml of synthetic DNA, and 0.1 unit of calf thymus polα-primase. For competition analyses, 0.1, 1, or 10 μg of synthetic DNA (1, 10, or 100-fold excess over DNA templates) were added to the standard reaction mixture. The mixtures were incubated for 60 min at 37°C, and the reaction products were concentrated by ethanol precipitation with 0.1 μg of bovine DNA, and then analyzed by PAGE (20%) in 8 M urea.

Gel-Retardation Assay—One nanogram of ^{32}P-labeled DNA fragment and 0.2 unit of polα-primase, or 0.1 μg of RP-A, or 0.1 μg of SV40T-Ag was incubated on ice for 5 min in 50 mM Tris-HCl (pH 7.5) and 2 mM DTT. Various amounts of DNA competitors were added at the same time as ^{32}P-labeled DNA probe. To crosslink the complex between DNA fragments and polα-primase, 0.05% glutaraldehyde was added and the mixture was incubated at 37°C for 5 min. DNA-protein complex was resolved by PAGE (20%) and was quantified by NIH Image.

Polα-Primase-Conjugated Sepharose Column Chromatography—Polα-primase (100 μg) was covalently conjugated with Sepharose CL-4B (300 μl) as described (*30*). Two nanograms of the ^{32}P-labeled DNA probe and 0.1 μg of proteins, or aliquots of RP-A (Mono Q column fractions), were incubated on ice for 10 min and then applied to the enzyme-affinity column (300 μl) equilibrated with 2 ml of TE buffer containing 200 ng of unlabeled SVG and 50 mM NaCl. The column was washed with 2 ml of TE buffer containing 150 mM NaCl. Retained protein-DNA complex was eluted with 2 ml of TE buffer containing 500 mM NaCl and the amount of radioactivity was measured with a liquid scintillation counter.

Analysis of Reaction Products with SVG—Polα-primase (5 units) was preincubated with 0.5 μg of SVG-2 (40-mer) in the presence of various amounts of RP-A in 15 μl of 25 mM Tris-HCl (pH 7.5), 0.25 mM DTT, and 25% glycerol at 0°C for 40 min. It was mixed with primase reaction mixture (35 μl) containing 1.2 μM [α-^{32}P]CTP, 2 mM ATP, 0.1 mM GTP, 0.1 mM UTP, 4 mM $MgCl_2$, 20 mM KCl, 0.5 mM DTT, and 50 mM Tris-HCl (pH 7.5), and incubated for 30 min at 37°C. Then 4 dNTPs were added at the final concentration of 0.1 mM and the synthesized RNA primers were extended by incubation for another 30 min. The reaction was stopped by adding 3 μl of stop-solution and the mixture was analyzed on a sequencing gel as described previously (*6*).

RESULTS

Polα-Primase Binds to SVG Only Weakly—We synthesized SVG (30-mer) (Fig. 1) corresponding to a sequence in the vicinity of the core origin of SV40 DNA. The interaction between polα-primase and SVG was measured by two methods, *i.e.*, immunoprecipitation and competition in the primase reaction (Fig. 2). [^{32}P]CTPPS 1 was co-immunoprecipitated as a complex with polα-primase by anti-polα antibody. However, [^{32}P]SVG was not precipitated under the same conditions (Fig. 2A). Addition of SVG only weakly affected DNA synthesis coupled with RNA primer synthesis (*de novo* DNA synthesis) by polα-primase with the CTPPS 1 template (Fig. 2B) that normally gave reaction products initiated from positions 21, 22, and 23 (Figs. 1, 2, and Ref. *6*). In contrast, these reaction products were diminished by adding poly(dC) to the reaction mixture at equimolar concentration.

RP-A Binds to SVG—The interaction between SVG and RP-A, one of the components of the replication machinery for SV40 DNA (*31-33*), was studied by gel retardation analysis. The size of SVG or CTPPS 1 is 30-mer, which may

be long enough for binding with RP-A (*34*). In gel retardation analysis, formation of RP-A-[^{32}P]CTPPS 1 complex was inhibited by adding either unlabeled CTPPS 1 or SVG, although CTPPS 1 was 10 times more effective than SVG (Fig. 3, A and B). Likewise, binding of [^{32}P]SVG with RP-A was inhibited by either CTPPS 1 or SVG. The glutaraldehyde-crosslinking was not needed in the case of RP-A, while it was essential to stabilize the complex of polα-primase with the DNA fragment in the gel retardation analysis (see "MATERIALS AND METHODS"). Therefore, interaction between RP-A and ssDNA is much tighter than that with polα-primase. Furthermore, the complex between RP-A and DNA was stable in a buffer containing 800 mM NaCl, whereas that of polα-primase dissociated even in 50 mM NaCl (Fig. 4).

Association of Polα-Primase with SVG in the Presence of RP-A—To measure the effect of RP-A on the interaction between SVG and polα-primase, we used a polα-primase-

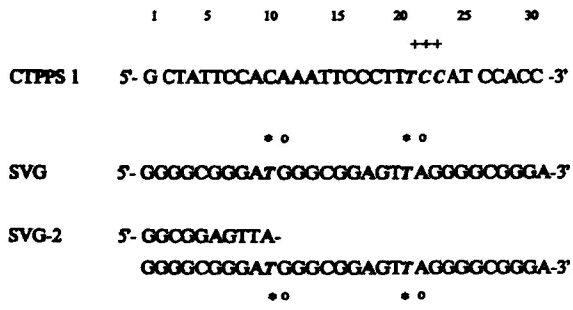

Fig. 1. **Sequences of templates, CTPPS 1, SVG, and SVG-2.** CTPPS 1 fragment (30-mer) is an efficient pyrimidine-rich template for *in vitro* priming, and was selected from a bovine genomic DNA library (*6*). SVG is a 30-mer corresponding to the sequence of nucleotide numbers 74-103 in the vicinity of the SV40 core origin (*15, 24*), which involves two initiation sites identified *in vivo*. SVG-2 is a 40-mer corresponding to the same site as SVG but with 10 extra nucleotides at the 5'-end (nucleotides 64-103). Crosses on CTPPS-1 indicate *in vitro* initiation positions (*6*). Asterisks and circles on SVG and SVG-2 indicate initiation positions *in vivo* and *in vitro*, respectively (*15, 36*). Numbers indicate nucleotide positions from the 5'-end of each fragment.

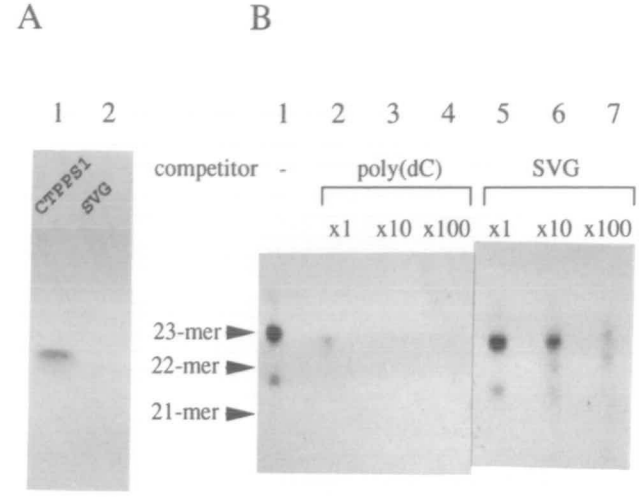

Fig. 2. **Interaction of polα-primase with DNA fragments.** (A) [^{32}P]CTPPS 1 (lane 1) or [^{32}P]SVG (lane 2) was incubated with polα-primase, and the enzyme-DNA complex was precipitated by anti-polα antibody as described in "MATERIALS AND METHODS." Pre-immune mouse serum did not precipitate CTPPS 1 or SVG (data not shown). (B) With competitor DNA, *de novo* DNA synthesis was performed as described in "MATERIALS AND METHODS." Competitors were added as follows: lane 1, no competitor; lanes 2 to 4, poly(dC); lanes 5 to 7, SVG. Amounts of DNA competitors (1, 10, or 100 times excess) are indicated in the figure. Product sizes are indicated on the left side (arrows).

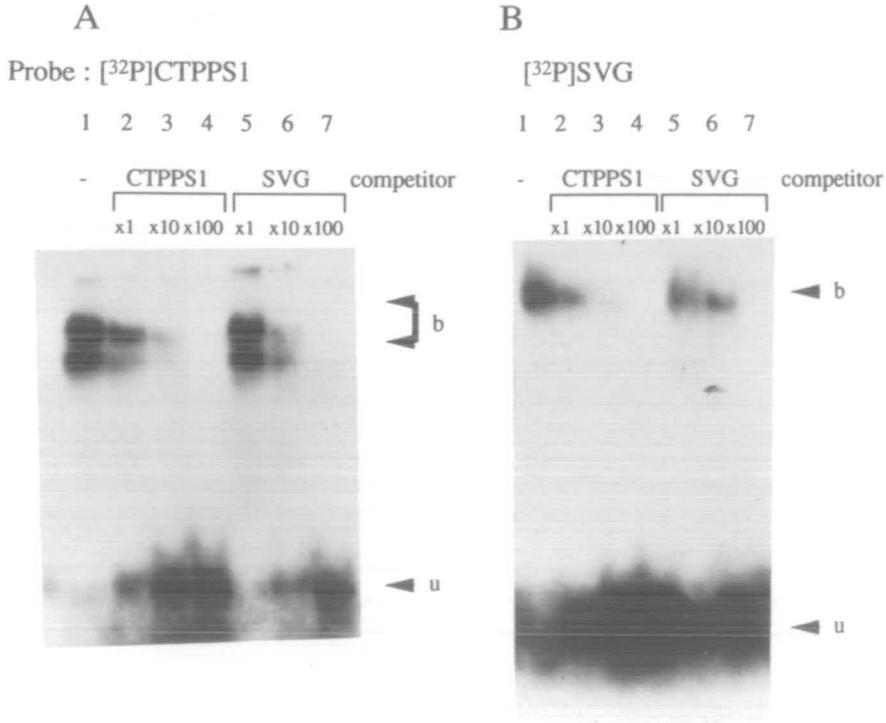

Fig. 3. **Interaction of RP-A with DNA fragments.** (A) RP-A-[^{32}P]CTPPS 1 complex was detected by gel-retardation assay as described in "MATERIALS AND METHODS." Competitors were added as follows: lane 1, no competitor: lanes 2 to 4, CTPPS 1; lanes 5 to 7, SVG. The amounts of competitors (1, 10, or 100 times excess) are indicated in the figure. u, unbound DNA; b, DNA-protein complex. (B) RP-A-[^{32}P]SVG complex was detected by gel-retardation assay. Competitors were added as follows: lane 1, no competitor; lanes 2 to 4, CTPPS 1; lanes 5 to 7, SVG. The amounts of competitors (1, 10, or 100 times excess) are indicated. u, unbound DNA fragments; b, protein-DNA complex.

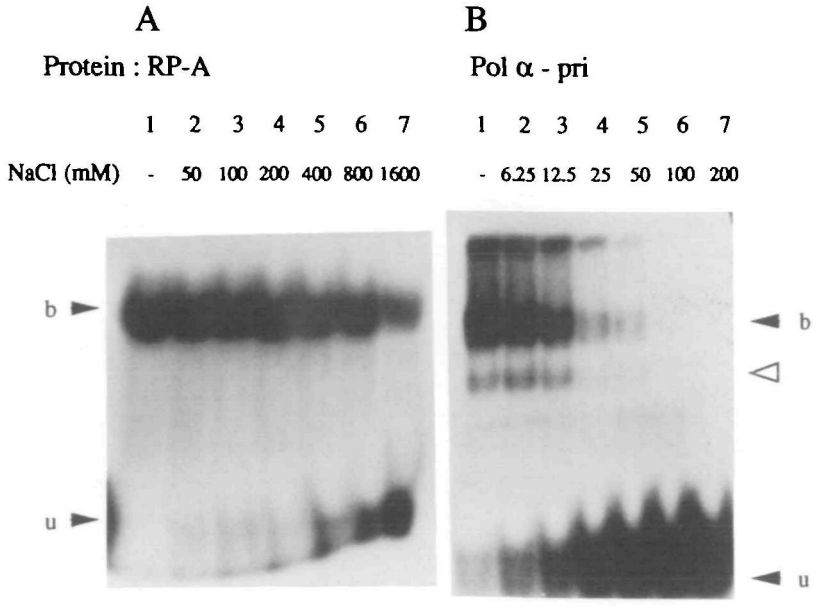

Fig. 4. **Effects of salt on the stability of protein-DNA complexes.** RP-A (panel A) and polα-primase (panel B) were incubated with [^{32}P]CTPPS 1 in the presence of NaCl at concentrations of 0 to 1,600 mM and gel-retardation analyses were performed as described in "MATERIALS AND METHODS." u, unbound DNA fragments; b, protein-DNA complex. The open arrowhead in panel B indicates 'free primase'-DNA complex (*5, 38*).

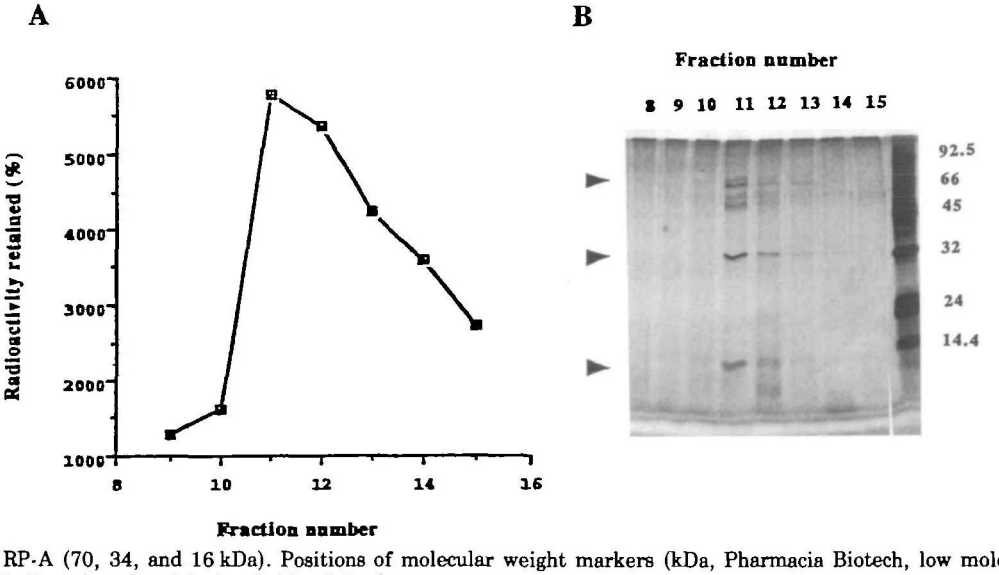

Fig. 5. **Formation of ternary complex of RP-A, [^{32}P]SVG, and polα-primase.** RP-A fractions eluted from a Mono Q column ("MATERIALS AND METHODS") were used. (A) Fifty microliters of each fraction from the Mono Q column was incubated with [^{32}P]SVG on ice for 10 min, diluted twice with TE buffer, and then applied to a polα-primase-conjugated Sepharose column. Retained radioactivity (cpm) was measured as described in "MATERIALS AND METHODS." (B) An aliquot of each fraction eluted from the Mono Q column was subjected to SDS-PAGE and peptide bands were visualized by silver staining. Arrowheads indicate the positions of 3 subunits of RP-A (70, 34, and 16 kDa). Positions of molecular weight markers (kDa, Pharmacia Biotech, low molecular weight protein markers) are indicated on the right-hand side of the figure.

conjugated Sepharose column ("MATERIALS AND METHODS"). As shown in Fig. 5, aliquots of RP-A fractions from the Mono Q column (the final purification step) were mixed with the [^{32}P]SVG and applied to the polα-primase-conjugated column. The radioactivities retained on the column coincided well with the RP-A contents in Mono Q fractions (Fig. 5, A and B). These results suggest that polα-primase associates with SVG with the aid of RP-A. The enhancement of association of polα-primase with SVG may be specific to RP-A, because another ssDNA-binding protein, *E. coli SSB*, could not support the association (Fig. 6, column 3), although *E. coli SSB* was reported to be able to interact with calf polα-primase (*20*). On the other hand, SV40T-Ag also stimulated the binding of polα-primase with [^{3}H]SVG (efficiency, 33% of RP-A). When both RP-A and SV40T-Ag were added together, the retained radioactivity was at a similar level to that with RP-A alone (data not shown). These results suggest that RP-A enhances the affinity of polα-primase to SVG by protein-protein interaction.

Primer RNA Synthesis on Extended SVG Sequence in the Presence of RP-A—With SVG (30-mer) as the template, we could not detect synthesis of RNA primer by polα-primase in the presence of RP-A (data not shown), under conditions where polα-primase associates with SVG (Fig. 6). When SVG was extended by adding 10 nucleotides at its 5′-end, however, this fragment (SVG-2, 40-mer; see Fig. 1) supported a small amount of incorporation of [^{32}P]ATP by polα-primase into RNA in the presence of all RNA and DNA precursors (Fig. 7, lane 1). This priming activity was stimulated more than 10-fold by the addition of RP-A (Fig. 7, lanes 2-6). The stimulation was observed when the molar

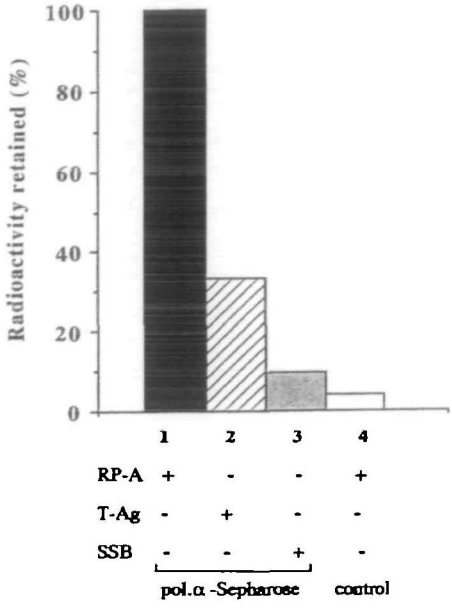

Fig. 6. **Association of SVG with polα-primase in the presence of DNA-binding proteins.** The [^{32}P]SVG was preincubated with either RP-A, SV40T-Ag, *E. coli* SSB, or BSA, and applied to a polα-primase-conjugated Sepharose column. Radioactivity retained on the column was measured as described in "MATERIALS AND METHODS." Background radioactivity, measured with BSA and [^{32}P]SVG, was subtracted, and retained radioactivity is shown as percent of that with RP-A (100%). Experimental conditions are shown under the figure. pol.α, polα-primase-conjugated Sepharose; control, Sepharose only.

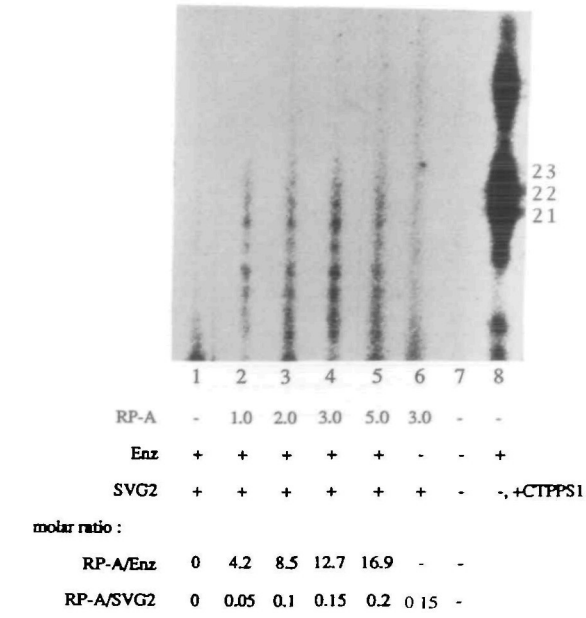

Fig. 7. **Stimulation of primer RNA synthesis with SVG-2 template by RP-A.** The reaction was performed using SVG-2 (40-mer) as a template, which had been preincubated with various amounts of RP-A (expressed as μl of 120 μg protein/ml) and reaction products were analyzed by electrophoresis on sequencing gel as described in "MATERIALS AND METHODS." Sizes of products were measured by comparison with sequencing ladders run in parallel. The molar ratios between RP-A and polα-primase, and those between RP-A and DNA template are shown at the bottom of the figure.

ratio of RP-A/polα-primase was 4.2 or higher, and the maximum stimulation was obtained at the molar ratio of 13. The molar ratio of RP-A/SVG-2 showing stimulation was 0.02 or higher, and maximum stimulation was seen at 0.15. The reaction products, [^{32}P]RNA primers, elongated further by the successive DNA synthesis, were detected as ladders ranging from 22-mer and shorter upon gel-electrophoresis. At present, 22-mer and shorter products can not be correlated with any of the *in vivo* initiation sites on this sequence (Fig. 1). The products seen as ladders could be due to highly distributive elongation by polα on this dG-rich sequence. It should also be noted that the amount of reaction products with SVG-2 template in the presence of RP-A was still less than 1/20 of that with pyrimidine-rich CTPPS 1 (Fig. 7, lane 8).

DISCUSSION

Recently we have shown that the frequency of initiation of *de novo* DNA synthesis by polα-primase correlates well to the binding affinity of primase to initiation sites, by analysis using a pyrimidine-rich active template (CTPPS 1) and its base-substituted derivatives (*6*). While poly(dT) and poly(dC) are active templates for RNA primer synthesis by primase, poly(dA) and poly(dG) are inactive as templates and do not bind to polα-primase. In the present study, the possible interaction of polα-primase with guanine-rich sequences was studied using SVG, corresponding to a sequence that contains two start sites for primer RNA in *in vivo* and *in vitro* SV40 DNA replication (*24, 36*). It has been shown that these *in vivo* start sites are entirely inactive *in vitro* using purified polα-primase (*15*). It is conceivable, therefore, that other factor(s) might be required for primase action at dG-rich initiation sequences. Among protein factors involved in eukaryotic DNA replication, RP-A and SV40T-Ag are of special interest because of their affinities for both polα-primase and DNA (*8, 18, 23*). Here we used a highly purified calf thymus polα-primase which resembles that from primates with respect to its template preference (*5, 6, 15*) and affinity for both SV40T-Ag and RP-A (*8, 18-20, 23*). Although RP-A tightly binds pyrimidine-rich sequences, it also binds purine-rich sequences (*35*).

Using a column of polα-primase-conjugated Sepharose, we showed that SVG could bind to polα-primase after preincubation with either RP-A or SV40T-Ag (Fig. 5). Nevertheless, purified polα-primase failed to synthesize RNA primer on SVG (30-mer) in the presence of RP-A or SV40T-Ag. The lack of template activity of SVG could be due to its size (30-mer) because the enzyme complex including RP-A must be larger than polα-primase alone. In this context, we extended SVG by adding 10 nucleotides at its 5′-end (SVG-2, 40-mer). With this new template, a trace amount of RNA synthesis was detected without RP-A. Template activity of SVG-2 for synthesis of RNA primer was stimulated more than 10-fold by RP-A (Fig. 7). The molar ratio of RP-A/polα-primase was 4.2 and higher when stimulation was observed. In contrast, the molar ratio of RP-A/template DNA under the same conditions was much lower: 0.02 and higher (Fig. 7). This stoichiometry of stimulation suggests that stimulation by RP-A is mainly

caused by protein-protein interaction with polα-primase, rather than by binding to template DNA. The sizes of products are expected to be 20-mer and 30-mer, if the initiation occurs at *in vivo* initiation sites (Fig. 1). At present, however, it is still uncertain where the initiation occurred, because the reaction products did not show a discrete band but were distributed as ladders of 22-mer and shorter. Nevertheless, these results suggest that RP-A is involved in the initiation of RNA primer synthesis as a molecular tether in forming the complex of polα-primase with initiation sequences rich in deoxyguanylate. The amount of primer RNA synthesized on SVG-2 with the optimal concentration of RP-A was still far less than that with CTPPS 1 measured under the same conditions. It is also suggested, therefore, that some other unknown factor is required to increase the efficiency of initiation to the levels at pyrimidine-rich initiation sites.

It has been reported that RP-A is required for SV40T-Ag-dependent unwinding of the origin region of SV40 DNA at an early step of replication (*32, 33*). In the elongation step, polα can overcome error-prone pause-sites with the help of RP-A, resulting in higher fidelity of elongation (*28, 37*). These results strongly suggest that RP-A is involved not only in initiation but also in elongation of DNA replication by interacting with both DNA and polα-primase to modulate its functions.

We thank Ms. T. Tomita, S. Furumoto, and M. Takahashi for their excellent technical assistance, Dr. C. Simbulan for advice on the manuscript. We also thank Dr. M. Fry in Technion-Israel Institute of Technology for critical discussions about the structure of DNA.

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