

Protein-Protein Interactions of the Primase Subunits p58 and p48 with Simian Virus 40 T Antigen Are Required for Efficient Primer Synthesis in a Cell-free System*

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DNA polymerase α -primase (pol-prim, consisting of p180-p68-p58-p48), and primase p58-p48 (prim₂) synthesize short RNA primers on single-stranded DNA. In the SV40 DNA replication system, only pol-prim is able to start leading strand DNA replication that needs unwinding of double-stranded (ds) DNA prior to primer synthesis. At high concentrations, pol-prim and prim₂ indistinguishably reduce the unwinding of dsDNA by SV40 T antigen (Tag). RNA primer synthesis on ssDNA in the presence of replication protein A (RPA) and Tag has served as a model system to study the initiation of Okazaki fragments on the lagging strand *in vitro*. On ssDNA, Tag stimulates whereas RPA inhibits the initiation reaction of both enzymes. Tag reverses and even overcompensates the inhibition of primase by RPA. Physical binding of Tag to the primase subunits and RPA, respectively, is required for these activities. Each subunit of the primase complex, p58 and p48, performs physical contacts with Tag and RPA independently of p180 and p68. Using surface plasmon resonance, the dissociation constants of the Tag/pol-prim and Tag/primase interactions were $1.2 \cdot 10^{-8}$ M and $1.3 \cdot 10^{-8}$ M, respectively.

In eukaryotic cells the duplication of the genome is a highly accurate and tightly coordinated process (reviewed in Ref. 1). For each reaction a specific set of enzymes and accessory proteins is required to replicate chromosomal DNA (1–4). The first step in leading strand DNA replication is accomplished by the synthesis of oligoribonucleotides, called RNA primers, at the origin of DNA replication. This process is carried out by a special enzyme, DNA primase (5–7). The eukaryotic enzyme consists of two subunits, the catalytic subunit p48 and p58, which serves to stabilize the primase activity (8–13). In eukaryotic cells these two subunits assemble together with the catalytic DNA polymerase subunit, p180, and the p68 polypeptide into a heterotetrameric DNA polymerase α -primase complex (pol-prim)¹ (1–7, 14, 15).

The initiation of DNA replication requires the interaction of several proteins *in vivo* and *in vitro* (1). The start of DNA synthesis *de novo* occurs by two independent processes: the singular priming event on the leading strand at the origin and the multiple priming steps for Okazaki fragment synthesis on the lagging strand. Both tasks are carried out by the primase activity (1–3, 14–17). Two cell-free initiation reactions have served as model systems to investigate these processes, primer synthesis in the cell-free SV40 DNA replication system and primer synthesis on natural single-stranded (ss) DNA templates bound by replication protein A (RPA) (17, 18). Using these cell-free systems, it was shown that the initiation of SV40 DNA replication at the origin is species-specific and requires the activity of the p180 subunit of primate pol-prim (19–21). In contrast to these results, the initiation of Okazaki fragments during lagging strand DNA synthesis is not species-specific and the human as well as the bovine pol-prim can perform lagging strand initiation equally well (20). Moreover, the host-specific DNA replication of the mouse polyomavirus (PyV), which is closely related to SV40, is mediated by p48 of mouse pol-prim rather than by the p180 subunit as for the primate system (22). These results suggest that in eukaryotes the initiation of leading and lagging strand synthesis may be mechanistically distinct and regulated by different means. Indeed, in eukaryotic cells leading strand initiation at an origin of replication must occur once and only once per cell cycle to avoid re-replication of the genome (23, 24). In contrast to this setting, DNA synthesis on the lagging strand needs multiple initiations of Okazaki fragments (25).

The leading strand initiation of SV40 DNA replication *in vitro* requires double-stranded (ds) plasmid DNA with a viral origin of replication, the multifunctional viral Tag, the cellular topoisomerase I, and two cellular complexes, pol-prim and the eukaryotic ssDNA-binding protein, RPA (1, 3, 26, 27). Mutual protein-protein interactions between these proteins during primosome assembly and primer synthesis are well established and support the targeting of the proteins to the origin and the coordination of the enzymatic steps (3, 28–36). Both the p180 and p68 subunits of pol-prim have been implicated in Tag binding (28, 36). It is believed that these protein-protein interactions are important for the observed stimulation of both the primase and DNA polymerase activity by Tag as well as for the increased origin DNA binding activity of Tag in the presence of pol-prim (18, 37, 38). Complex formation between the p70 subunit of RPA and the heterodimeric primase consisting of p58 and p48 subunits has been demonstrated, and the p48 subunit

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¹ The abbreviations used are: pol-prim, DNA polymerase α -primase (consisting of p180-p68-p58-p48); prim₂, primase p58-p48; RPA, replication protein A; HRP, horseradish peroxidase; BSA, bovine serum

albumin; ss, single-stranded; ds, double-stranded; TBS/T, Tris-buffered saline with Tween 20; DTT, dithiothreitol; Tag, T antigen.

on its own seemed to be sufficient for this interaction (29, 39). Through these interactions RPA stimulates the polymerase activity and increases the processivity of pol-prim (40–43). RPA, especially its largest p70 subunit, is also required for DNA unwinding by Tag, but in this case RPA can be replaced by heterologous ssDNA-binding proteins, reflecting the fact that merely its ssDNA stabilizing activity is required in this process (27, 41, 44–51).

Natural ssDNA templates like M13 ssDNA are efficiently used by pol-prim and heterodimeric primase (10–15, 53, 54). Tag stimulates both the primase and DNA polymerase activities of pol-prim on these templates (18, 20, 32, 54). However, the primase activity of pol-prim is severely inhibited on M13 ssDNA that is bound by RPA, an inhibition that can be relieved by Tag (18, 20, 32). Therefore, it is thought that the coordinated oligoribonucleotide synthesis by pol-prim on M13 ssDNA in the presence of RPA and Tag *in vitro* resembles the initiation of Okazaki fragments on the lagging strand *in vivo*. To investigate the mechanism of Okazaki fragment synthesis on the lagging strand, we compared the ability of heterotetrameric pol-prim and heterodimeric primase (prim₂) to synthesize primers on natural ssDNA in the above mentioned model system *in vitro*. In contrast to the initiation at SV40 origins, prim₂ substitutes for the pol-prim in an assay containing ssDNA, Tag, and RPA. Physical interactions of Tag with primase and RPA are required for efficient oligoribonucleotide synthesis on the lagging strand in the cell-free system. Here, we show that the primase subunits p58-p48 directly contact the viral initiator protein Tag. Since only the four-subunit pol-prim can start DNA replication *de novo* at the SV40 origin of replication, these data suggest that the initiation of leading and lagging strand synthesis are mechanistically different.

MATERIALS AND METHODS

Proteins—SV40 T antigen, RPA, the primase (p58-p48, prim₂), and the DNA polymerase α -primase (pol-prim) complex (p180-p68-p58-p48) were purified from baculovirus-infected insect cells as described (22, 28, 39, 55, 56). In addition, RPA, prim₂, and the individual primase subunits p58 and p48 were bacterially expressed and purified as outlined before (13, 57). Five pmol of each protein were adsorbed to Strataclean resin, and the beads were applied to SDS-polyacrylamide gel electrophoresis (58). Proteins were stained by Coomassie Brilliant Blue. Human topoisomerase I expressed in yeast and purified as described by Lisby *et al.* (59) was a generous gift of M. Lisby (University of Århus, Århus, Denmark). Monoclonal antibodies PAb101 (60, 61), PAb220 (62), PAb204 (63), PAb414, and PAb419 (64) specific for Tag as well as the monoclonal antibodies 70A, 70B, and 70C (65) specific for RPA were purified by affinity chromatography (66). Rat monoclonal antibodies RAC-3D5 and RAC-4D9 against the p70 subunit of RPA and PRI-5G6 and PRI-8G10 against primase subunits p58 and p48, respectively, were produced according to standard procedures by using recombinant proteins for immunization and screening. A polyclonal rabbit serum specific for the three RPA subunits was produced by immunizing rabbits with a mix of subunits fused to maltose binding proteins (67). Polyclonal rabbit antisera against the pol-prim complex were obtained by immunizing rabbits (11).

Primer Synthesis Assays on M13 ssDNA—Primer-synthesis was carried out in an assay (40 μ l) containing 30 mM Hepes-KOH, pH 7.8, 7 mM MgAc, 0.1 mM EGTA, 1 mM DTT, 0.25 mg/ml BSA, 0.01 mg/ml creatine kinase, 40 mM creatine phosphate, 4 mM ATP, 0.2 mM each of GTP and UTP, 0.002 mM CTP, 2 μ l of [α -³²P]CTP (specific activity 3000 Ci/mmol, 10 μ Ci/ μ l; Amersham Pharmacia Biotech, Freiburg), 250 ng (0.76 nmol of nucleotides) of M13mp18 ssDNA template (Amersham Pharmacia Biotech), and, if not otherwise stated, 6 pmol of monomeric Tag (0.56 μ g) and 6 pmol of RPA (0.69 μ g). The reaction mixture was assembled on ice, and the reaction was started by the addition of pol-prim, and prim₂, respectively, as indicated. For antibody inhibition studies, 5 μ g of the respective antibody was added to the reaction before addition of 2.5 pmol of either pol-prim (0.88 μ g) or prim₂ (0.27 μ g), and the reaction was incubated 15 min on ice. After incubation for 1 h at 37 °C, one eighth of the reaction was used to estimate the amount of incorporated nucleotides by spotting the reaction mixture onto DE-81 paper (68). The

rest of the reaction was stopped by the addition of 6.4 μ l of S buffer (6.25 mg/ml tRNA, 62.5 mM MgCl₂, 5 M LiCl). The material was ethanol-precipitated and resuspended in 50 μ l of DL buffer (35% formamide, 8 mM EDTA, 0.005% bromphenol blue). The sample was heated at 65 °C for 30 min and stored at –20 °C. Prior to loading, the sample was heated for 2 min at 95 °C and applied to a 20% denaturing urea gel in TBE (89 mM Tris, 89 mM boric acid, 0.2 mM EDTA). The gel was run at 600 V until the bromphenol blue marker had migrated about two thirds into the gel. The wet gel was then exposed to an x-ray film at –80 °C using intensifier screens.

SV40 Initiation Assays—Standard initiation reactions (20) contained (in a 40- μ l reaction) 30 mM Hepes-KOH, pH 7.8, 7 mM MgAc, 0.1 mM EGTA, 0.5 mM DTT, 0.25 mg/ml BSA, 0.01 mg/ml creatine kinase, 40 mM creatine phosphate, 4 mM ATP, 0.2 mM each of GTP and UTP, 0.002 mM CTP, 2 μ l of [α -³²P]CTP (specific activity, 3000 Ci/mmol, 10 μ Ci/ μ l; Amersham Pharmacia Biotech), 200 ng of pUCHS DNA template, 100 ng of topoisomerase I, and, if not otherwise stated, 6 pmol of monomeric Tag (0.56 μ g), 6 pmol of RPA (0.69 μ g), pol-prim, and prim₂ as indicated. After incubation for 1 h at 37 °C, the samples were treated as described under "Primer Synthesis Assays on M13 ssDNA."

Unwinding Assays—Unwinding assays (end volume, 15 μ l) contained 30 mM Hepes-KOH, pH 7.8, 7 mM MgAc, 0.1 mM EGTA, 1 mM DTT, 0.25 mg/ml BSA, 0.01 mg/ml creatine kinase, 40 mM creatine phosphate, 4 mM ATP, 6 pmol of RPA, 6 pmol of Tag, the indicated amounts of pol-prim or prim₂, 10 fmol (approximately 20 ng) of *Xmn*I-restricted 5' end-labeled linear pUCHS DNA with blunt ends (fragment length, approximately 3000 base pairs) or 100 fmol of (approximately 200 ng) supercoiled pUCHS (69). The reaction was assembled on ice and started by addition of Tag. Then the reaction was incubated for 1 h at 37 °C. To stop the reaction, proteinase K, EDTA, and SDS were added to final concentrations of 0.3 mg/ml, 6 mM, and 0.3%, respectively, and the sample was incubated for another 1 h. Using linear DNA, 0.2 volume of 5 $\times$ loading buffer (30 mM Hepes-KOH, pH 7.8, 25% Ficoll 400, 0.1% bromphenol blue, 1% SDS) was added and the sample was applied to a 1% agarose gel in TAE. The gel was dried and exposed to x-ray films, and quantified by phosphorimaging.

Co-immunoprecipitation—Proteins were expressed either in insect cells with recombinant baculoviruses or in bacteria (20–22, 57). Cells were resuspended in one cell volume of I buffer (50 mM Hepes-KOH, pH 7.8, 150 mM NaCl, 0.5% Nonidet P-40). Insect cells were lysed by incubation for 10 min on ice, whereas bacterial cells were sonicated. After homogenizing the cells, debris were removed by centrifugation at 10,000 $\times g$. The protein concentrations of the extracts were determined with the Bio-Rad protein assay kit using BSA as the standard according to the manufacturer's protocol. Protein concentrations of insect cell extracts were 10–20 mg/ml, and 20–40 mg/ml for bacterial cell extracts. According to the efficiencies of expression, the following amounts of cell extracts were used: 50 μ g for Tag, 200 μ g for pol-prim, 200 or 50 μ g for the prim₂ expressed in insect or bacterial cells, respectively; and 500 or 50 μ g for RPA expressed in insect or bacterial cells, respectively (one fifth of these amounts was loaded on the SDS gel as input control). After mixing the extracts, the total volume was adjusted to 200 μ l with I buffer. In case of the baculovirus system, partner proteins were also co-expressed in insect cells. Approximately 200 μ g of cell extracts were used in the following steps. In some cases a benzoylase nuclease mixture (Merck) was added to the reaction at 0.05 units/ μ l to exclude indirect interaction between the proteins due to association with DNA. The extracts were supplemented with the respective monoclonal antibody (10 μ g) or 10 μ l of polyclonal serum specific for one of the interacting proteins. Then, 20 μ l of protein G-agarose beads (50% (v/v)) equilibrated in I buffer were added and the mixture rotated at 4 °C for 1 h. The beads were then centrifuged, and the supernatant was discarded. The beads were washed six times with 1 ml of I buffer. Finally, the beads were resuspended in 40 μ l of sample buffer and bound proteins separated by SDS-polyacrylamide gel electrophoresis. Proteins were blotted to nitrocellulose membranes and detected by ECL. To avoid cross-reaction with antibodies derived in mouse and rabbits used for immunoprecipitations, we applied monoclonal rat antibodies and a secondary horseradish peroxidase (HRP)-coupled anti-rat antibody preadsorbed to mouse and rabbit IgG (Dianova) to detect the proteins. In case of Tag, PAb419 was directly coupled to HRP according to the supplier's protocol (Roche Molecular Biochemicals) and used for detection.

Far Western Blots—35.4 μ g of pol-prim, 10 μ g of Tag, or 30 μ g of RPA (approximately 100 pmol of each protein) were incubated in L buffer (2.5% SDS, 2.5 mM Tris-HCl, pH 8, 100 mM DTT, 10% glycerol, 0.05% Pyronin T1) for 5 min at room temperature (28). 10 pmol of acetylated BSA (8 μ g; New England Biolabs), which served as a negative control, was treated the same way. Proteins were then separated by SDS-

polyacrylamide gel electrophoresis (28, 58). The gel was incubated for 1 h at room temperature in R buffer (50 mM Tris-HCl, pH 7.7, 20% glycerol) to renature the proteins. Then proteins were transferred to nitrocellulose filters in T buffer (10 mM NaHCO₃, 3 mM Na₂CO₃), and filters were blocked in TBS/T (10 mM Tris-HCl, pH 8, 150 mM NaCl, 0.1% Tween 20) supplemented with 5% nonfat milk powder for 1 h at room temperature. Filters were subsequently washed in TBS/T and incubated for 2 h at room temperature with the overlay protein (Tag, RPA, prim₂, or BSA) at a concentration of 10 µg/ml (Tag, RPA, and BSA) or 5 µg/ml prim₂ in TBS/T supplemented with 5% milk powder. The filters were washed three times for 5 min with TBS/T and incubated overnight with a primary polyclonal rabbit antibody specific for the overlay protein (1:2000 dilution) at 4 °C in TBS/T supplemented with 5% milk powder. In the case of BSA as the overlay protein a mixture of Tag- and RPA-specific (Fig. 8A) or pol-prim-specific (Fig. 8B) antibodies was used. On the following day, filters were washed three times for 5 min in TBS/T and incubated for 2 h at room temperature with HRP-conjugated (Dianova, 1:10,000) or alkaline phosphatase-conjugated (AP, Promega 1:3000) anti-rat secondary antibody in TBS/T. Bound antibody was detected by using an ECL kit (Amersham Pharmacia Biotech) or the ProtoBlot™ immunoscreening system (Promega) according to the suppliers' instructions.

Biomolecular Interaction Analysis—Association rate constants (k_{on}) and dissociation rate constants (k_{off}) for the interaction of SV40 Tag with four-subunit pol-prim or two-subunit prim₂ were determined by real-time biomolecular interaction analysis. Interaction analysis was performed using the BIAcore 2000 apparatus from BIAcore AB (Freiburg, Germany). Sensor chips CM5, surfactant P20 and the amine coupling kit were purchased from BIAcore AB. Antibodies were immobilized by amine coupling according to the supplier's protocol. For a final ligand immobilization yield of 1000 relative resonance units, about 1800 relative resonance units of the antibody was initially attached to the flow cell surface. The anti-primase monoclonal antibody PRI-5G6 (50 µg/ml) was loaded at flow rate of 5 µl/min in 0.03 M sodium acetate buffer, pH 5.0. The ligands, four-subunit pol-prim or prim₂, and the analyte, Tag, were microdialyzed against the binding buffer 20 mM HEPES-KOH, pH 7.5, containing 100 mM NaCl, and 0.005% P20 before use. Ligands were loaded with a concentration from 8 to 100 µg/ml and cross-linked to the antibody by using the amino coupling kit. For the studies, between 1500 and 10,000 relative resonance units of the ligands were immobilized. Binding studies were usually performed with 5–70 µg/ml Tag as an analyte at 25 °C in the presence of BSA and a flow rate of 40 µl/min. After recording of the association and dissociation phases, remaining non-cross-linked protein-protein contacts were dissociated by regenerating the flow cells with 0.1 M K₃PO₄, pH 12, for 30 s. A control cell contained antibody without loaded ligand to correct for nonspecific binding. Data were collected at 1 Hz and analyzed using the BIAevaluation program 3.0.

RESULTS

Primase Activities of pol-prim—Primase activities of baculovirus-expressed pol-prim tetramer (p180-p68-p58-p48), and of bacterially expressed prim₂ (p58-p48) as well as p48 on poly(dC) and poly(dT) have been presented previously (13, 21). To compare the initiation activities of the purified proteins (Fig. 1) on natural substrates, assays with natural ssDNA and SV40-origin containing dsDNA were performed (Fig. 2, A and B, respectively). The enzyme complexes prim₂ (Fig. 2A, lanes 1–4) and pol-prim (Fig. 2A, lanes 5–8) synthesized primers with product sizes ranging from 2 to 18 ribonucleotides. Using equal molar concentrations prim₂ had a more than 4-fold higher activity than the tetramer (Fig. 2A, compare lanes 1–4 with lanes 5–8). prim₂ expressed either in bacteria or in insect cells had comparable specific activities and the primase products had the same size distribution (data not shown), demonstrating that the activity is independent of the source that was used for purification. These data show that the recombinant prim₂ is highly active. Although it is not known whether there is any free primase in the cell, we use the heterodimeric primase complex to study in cell-free systems the functions of the primase subunits in the absence of both large subunits of pol-prim.

To analyze the initiation capability of the recombinant human proteins on dsDNA we used a plasmid containing the SV40 origin. prim₂ (Fig. 2B, lanes 1–5) or its constituent p58

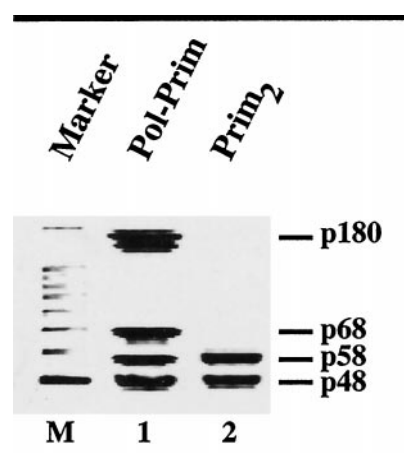


FIG. 1. Expression, purification, and enzymatic characterization of human DNA polymerase α -primase. 50 pmol of purified DNA polymerase α -primase tetramer (pol-prim) expressed in insect cells (lane 1), and primase dimer (prim₂, lane 2) expressed in *E. coli* were separated on a 10% SDS-polyacrylamide gel and stained with Coomassie Brilliant Blue. Microdensitometric scanning suggested that pol-prim contains in average a slight excess of p58 and p48 in comparison to p180 (Ref. 21, data not shown).

and p48 (data not shown) did not support the initiation reaction of SV40 DNA replication, whereas pol-prim in concert with RPA, Tag, and topoisomerase I efficiently initiated SV40 DNA replication (Fig. 2B, lanes 6–10). These results suggest that, in contrast to primer synthesis on a ssDNA template, initiation of DNA replication on SV40 origins requires all four subunits rather than the primase function alone. Since dsDNA is not the direct substrate for the priming reaction and requires the unwinding of DNA prior to initiation of leading strand replication, it is possible that prim₂ or pol-prim interfere with the unwinding step. Tag efficiently unwound dsDNA in the presence of RPA, and its activity was not significantly inhibited by low concentrations of pol-prim or prim₂ (Fig. 2C, compare lane 4 with lanes 5, 6, 9, and 10). However, high concentrations of pol-prim as well as prim₂ inhibited the unwinding activity of Tag (Fig. 2C, compare lane 4 with lanes 8 and 12; in the presence of 8 pmol of pol-prim or prim₂, the amount of unwound DNA was reduced by 45% and 35%, respectively). This inhibition was not due to buffer effects or contaminants since after heat inactivation of the proteins no inhibition could be detected (data not shown). However, inhibition of the unwinding reaction is most likely not the reason for the inactivity of prim₂ in the initiation reaction, since pol-prim inhibited the reaction to a greater extent (Fig. 2C, lanes 8 and 12). The amounts required to inhibit the unwinding reaction efficiently were higher (8 pmol; Fig. 2C, lanes 8 and 12) than those used to perform the initiation reaction (Fig. 2B; up to 4 pmol of pol-prim and prim₂ were used). Furthermore, the RNA polymerase activity of prim₂ was about 4 times more active than that of pol-prim when using natural ssDNA as a template (Fig. 2A). Therefore, the lack of enzyme activity cannot be the cause for the failure of prim₂ to synthesize the primers for leading strand DNA synthesis.

Influence of Tag and RPA on the Primase Activity—A stimulatory effect of T antigen on the primase activity of pol-prim has been reported (18, 20, 32, 37, 54). RPA inhibits the enzyme on M13ssDNA templates (18, 32). Indeed, we also determined a stimulation by Tag (Fig. 3A) and an inhibition by RPA (Fig. 4A) using M13mp18 ssDNA. The bacterially expressed prim₂ was very similarly influenced by Tag and RPA, and the behavior of prim₂ was indistinguishable from that of pol-prim (Figs. 3A and 4A). This interpretation is strengthened by the graphic presentations in Fig. 3B and 4B, where the primase activities of

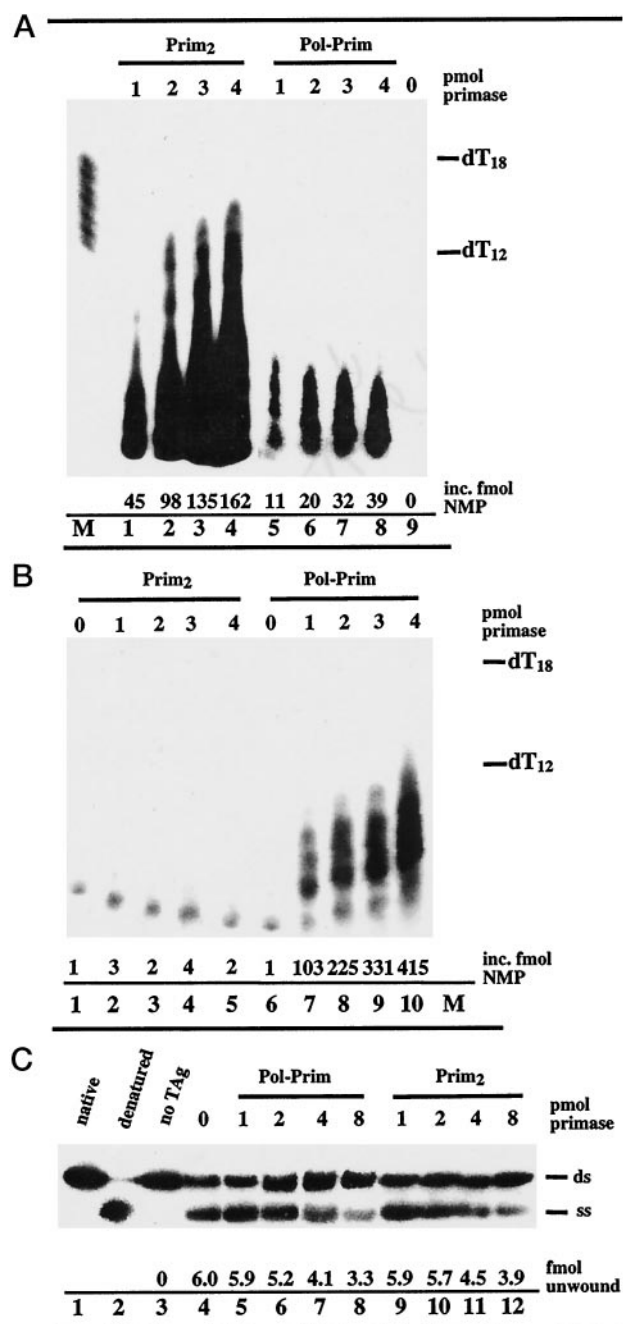


FIG. 2. RNA synthesis on single-stranded and double-strand DNA by DNA primase. The indicated amounts of pol-prim or prim₂ were tested using M13-mp18 ssDNA (panel A) or SV40 origin-containing pUCHS dsDNA (panel B). The activities in incorporated NMPs are presented below each panel. Markers (lane M) were included and their size is presented on the right. In the presence of 6 pmol of RPA and the indicated amounts of pol-prim or prim₂, the unwinding activity of Tag (6 pmol) was measured with 10 fmol of linear 5' end-labeled, blunt-ended pUCHS (panel C). Lanes 1 (input DNA) and 2 (heat-denatured DNA) were included in the gel for marking the positions of dsDNA and ssDNA, respectively. Lane 3 (no Tag) shows the negative control. The amount of unwound DNA is given below each panel (femtomoles of unwound DNA).

each complex were normalized to that activity determined in the absence of Tag and RPA.

On M13 ssDNA, Tag not only stimulated the primase activity on ssDNA but was also able to reverse the inhibition of primase by RPA (Fig. 5, A and B, lanes 1–5; summarized in Fig. 5C). In the presence of Tag and RPA, the activity of pol-prim with both Tag and RPA rose about 7–10 times over that determined with

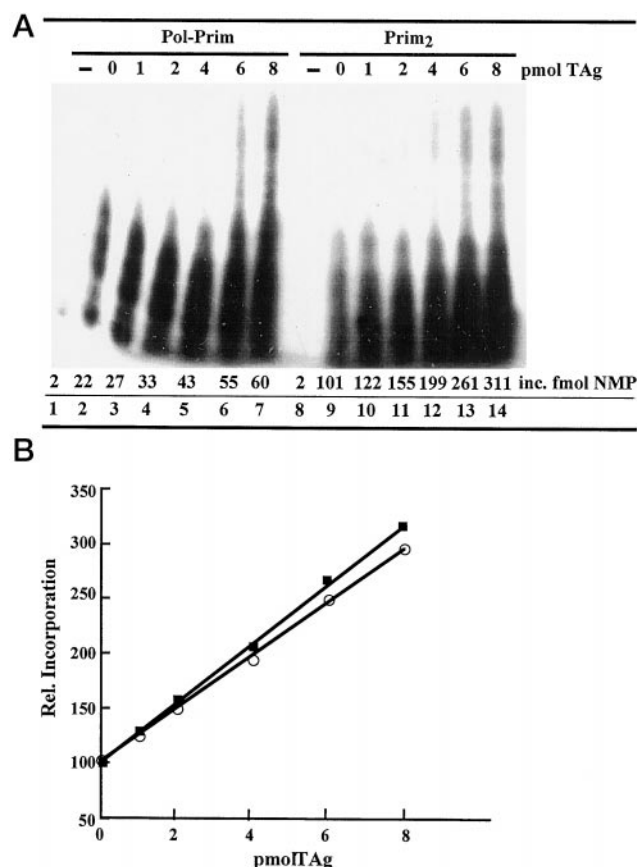


FIG. 3. Stimulation of primase activity. The primase activity of 2.5 pmol of pol-prim (A, lanes 2–7; one half of the reaction was loaded) and prim₂ (A, lanes 9–14; one tenth of the reaction was loaded) on ssDNA were determined in the presence of increasing amounts of Tag. Lanes 1 and 8 show labeled species observed without primase. The incorporated amount of radioactivity is shown beneath each lane. For the graphical presentation (B), the priming activities of pol-prim (○) and prim₂ (■) were normalized to their activities in the absence of Tag.

only RPA (Figs. 5C and 6, columns 2, 3, and 5). Thus, Tag was able to enhance the primase activity of pol-prim in the presence of RPA about 1.5 times over its activity on ssDNA alone. Again, prim₂ showed behavior similar to pol-prim and Tag stimulated the activity of prim₂ about 1.7 times in comparison with its activity on ssDNA alone and 7–11 times in comparison with that on ssDNA bound by RPA.

Influence of Monoclonal Antibodies against RPA on Primase Activity—The RPA-specific antibodies 70A, 70B, and 70C all inhibit SV40 DNA replication; however, the underlying mechanism is only understood for 70C (65). This antibody (70C) destabilizes the interaction of RPA with ssDNA, and as a consequence the T antigen-mediated unwinding step is blocked. To test whether the antibodies influence primer synthesis by pol-prim and prim₂, these antibodies were added to the assays. The primase activities of pol-prim and prim₂ were modulated to the same extents by Tag and RPA (Fig. 5 and Table I). A buffer control did not lead to any change in primer synthesis by pol-prim and prim₂ (lane 6, column 6). However, the presence of 70A significantly reduced RNA synthesis rates (lane 7, column 7). 70B behaved very similarly (data not shown). In contrast to these results, the inhibitory effect of RPA on primer synthesis was nearly reversed by antibody 70C (lane 8, column 8). None of the antibodies 70A, 70B, and 70C inhibited or stimulated the primase activity of pol-prim or prim₂ directly (data not shown).

Influence of Monoclonal Antibodies against Tag on Primase Activity—Antibodies PAb220 and PAb414 represent valuable

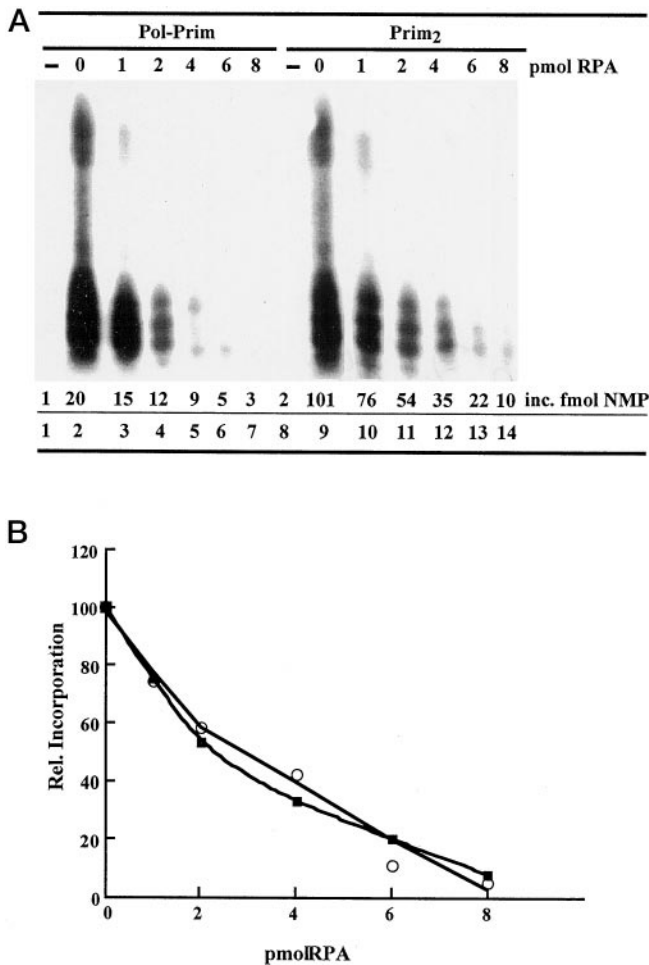


FIG. 4. Inhibition of primase by RPA. Primer synthesis by 2.5 pmol of pol-prim (A, lanes 2–7; one half of the reaction was loaded) or prim₂ (A, lanes 9–14; one tenth of the reaction was loaded) was inhibited on ssDNA by increasing amounts of RPA. In the graphical presentation of these data (B), the priming activity of pol-prim (○) and prim₂ (■) are corrected to their respective activities in the absence of RPA. Lanes 1 and 8 show labeled species observed without primase. The incorporated amount of radioactivity is presented beneath each lane.

tools to study the interaction of Tag with RPA or pol-prim. PAb220 was shown previously to specifically block the interaction of Tag with RPA, but has only minor effects on the unwinding capability of Tag, whereas PAb414 blocks complex formation with pol-prim and also inhibits the unwinding function of Tag to a great extent (28, 69). In the M13-ssDNA primer synthesis assays, an unwinding step is not required and hence cannot contribute to any effects on primase activity by these antibodies. Therefore, we suspected that on ssDNA all stimulatory or inhibitory effects exerted by the antibodies are due to a disruption of essential protein-protein interactions. The antibody PAb204, on the other hand, essentially blocked all replicatory functions of Tag. Therefore, it served as a positive control for inhibition, whereas PAb419 and PAb101 proved to be non-inhibitory in any respect and hence represented negative controls (69). None of these antibodies against Tag directly inhibited or stimulated the primase activity of either pol-prim or prim₂ (data not shown).

To determine the functional relevance of these specific protein-protein contacts, the stimulatory activity of Tag on pol-prim and prim₂ was tested with or without five Tag region-specific antibodies in the absence and presence of RPA (Fig. 6, columns 6–11 and 12–17, respectively). In the absence of RPA, the stimulatory effect of Tag exerted on the primase activities

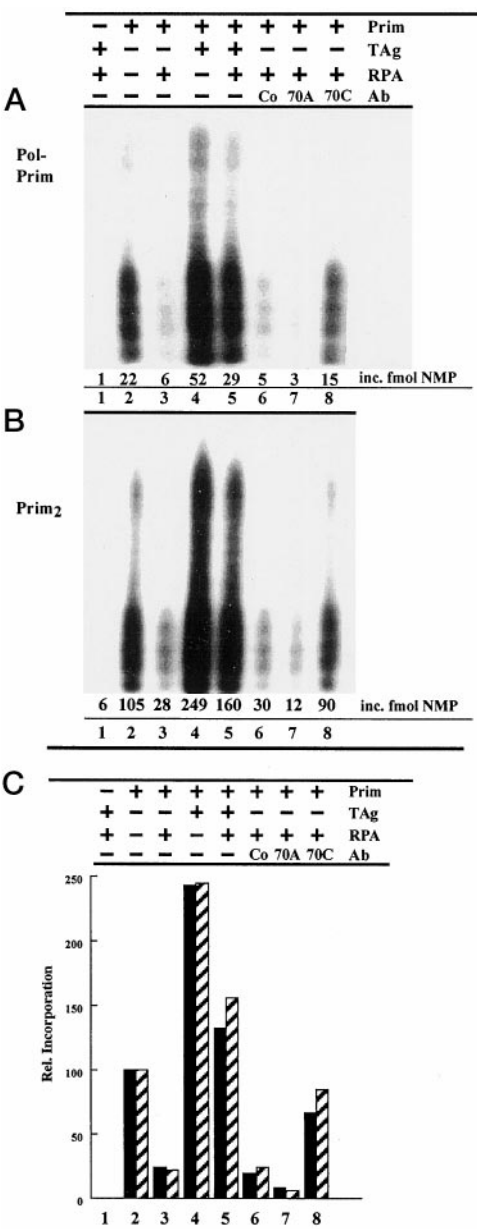


FIG. 5. Influence of monoclonal antibodies on the inhibitory effect of RPA. Primer formation by four-subunit pol-prim (A; one half of the reaction was loaded) and the two-subunit primase (B; one-tenth of the reaction was loaded) on ssDNA were tested under various conditions. The graphical evaluation of these data is presented in panel C (the priming activities of pol-prim (black columns) and prim₂ (hatched columns) are corrected to the respective activities in the absence of RPA and Tag). Lane and column 1, negative control without primase; lane and column 2, enzyme activity on ssDNA; lane and column 3, priming activity in the presence of RPA; lane and column 4, enzyme activity in the presence of Tag; lane and column 5, enzyme activity in the presence of Tag and RPA; lanes and columns 6–8, enzyme activity in the presence of RPA, and dialysis buffer (Co), 5 μ g of antibody 70A or 70C, respectively. The incorporated amount of radioactivity is shown underneath each lane.

of both pol-prim and prim₂ was influenced neither by buffer nor by the antibodies PAb419, PAb220, and PAb101 (Fig. 6, compare columns 2, 6–8, and 11). In contrast, the stimulation was reduced by the antibodies PAb204 and PAb414 to levels close to that without Tag (Fig. 6, columns 9 and 10). These findings strongly suggest that the physical interactions between Tag and prim₂ are necessary for the observed stimulatory effects of Tag.

In the presence of RPA, buffer as well as PAb419 and PAb101 showed no major effects on the primase activities of both en-

TABLE I

Inhibitory and stimulatory effects of RPA and TAG on the primase activity of pol-prim and prim₂ in the presence of monoclonal antibodies ND, not determined; RPA and DNA binding of Tag, region of Tag that interacts with DNA and RPA.

Region of recognition ^a	Antibodies						
	PAb419	PAb220	PAb204	PAb414	PAb101	70A	70C
	N terminus of SV40 Tag (aa 1–82)	RPA and DNA binding of Tag (aa 130–246)	Central part of Tag (aa 453–469)	C terminus half of Tag (aa 367–708)	C terminus of Tag (aa 512–708)	N terminus of RPA p70	DNA binding of RPA p70
Inhibition by RPA						Increased	Reversed
Stimulation by TAG	Yes	Yes	No	No	Yes	ND	ND
Stimulation with TAG and RPA	Yes	No	No	No	Yes	ND	ND
Immunoprecipitation of TAG	Yes	Yes	Yes	Yes	Yes		
Immunoprecipitation of RPA						Yes	Yes
Co-immunoprecipitation of pol-prim	Yes	Yes	No	No	Yes	No	No
Co-immunoprecipitation of RPA	Yes	No	No	Yes	Yes		

^a For references, see Refs. 37, 41, 48, 65, and 69.

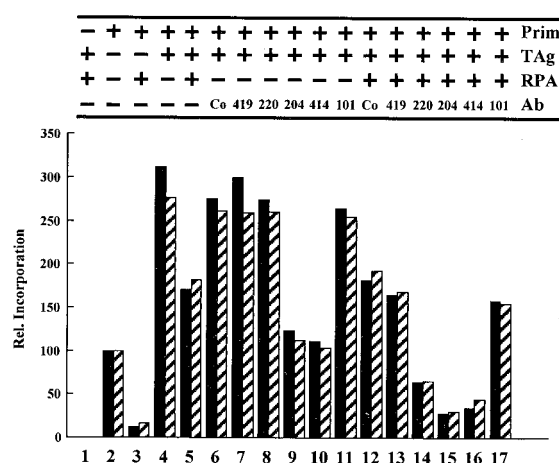


FIG. 6. Influence of monoclonal antibodies on the stimulatory activity of T antigen. Primase activity of 2.5 pmol of pol-prim (black columns) and prim₂ (hatched columns) was determined on ssDNA in the presence of Tag with or without RPA (the priming activities of pol-prim and the primase dimer were normalized to the activity in the absence of RPA and Tag; column 2). Column 1, negative control without primase; column 2, enzyme activity on ssDNA; column 3, enzyme activity in the presence of RPA; column 4, enzyme activity in the presence of Tag; column 5, enzyme activity in the presence of Tag and RPA; column 6, enzyme activity in the presence of Tag and dialysis buffer (Co), columns 7–11, enzyme activity in the presence of Tag and 5 μ g of antibody PAb419, PAb220, PAb204, PAb414, and PAb101, respectively; column 12, enzyme activity in the presence of Tag, RPA, and dialysis buffer (Co); columns 13–17, enzyme activity in the presence of Tag, RPA, and 5 μ g of antibody PAb419, PAb220, PAb204, PAb414, and PAb101, respectively.

zyme complexes, and Tag efficiently reversed the inhibitory activity of RPA (Fig. 6, columns 3, 12, 13, and 17). However, a significantly lower stimulation by Tag was noticed with PAb220 than that with PAb419 and PAb101 (Fig. 6, compare column 14 with columns 13 and 17). This observation is consistent with previous results indicating that PAb220 disrupts the interaction of Tag with RPA (28, 69), suggesting that Tag alleviates the inhibitory influence of RPA by directly contacting this protein. The addition of PAb204 and PAb414 led to a strong decrease in Tag's ability to stimulate primase, activity resulting in activities that were close to those determined in the presence of RPA and in the absence of Tag (Fig. 6, compare columns 3, 15, and 16).

Influence of Monoclonal Antibodies on Complex Formation between TAG, RPA, and pol-prim—After showing that these antibodies interfere with the primer formation on ssDNA, we wanted to determine the mechanism of these activities of Tag and RPA. Therefore, we investigated the influence of these antibodies on the protein-protein interactions of Tag, RPA, pol-prim, and prim₂ by co-immunoprecipitation experiments

(Figs. 7 and 8). Since the RPA-specific antibodies 70A and 70B interact with the most N-terminal region of RPA70, which is important for the interaction with pol-prim, and 70C binds within the DNA binding domain of RPA70, which is part of the binding site for pol-prim and Tag (41, 48), we reasoned that these antibodies might also interfere with the complex formation of RPA. We show here (Fig. 7) that 70A, 70B, and 70C abolished the ability of RPA to form complexes with both pol-prim (lanes 2–4) and prim₂ (lanes 7–9). In contrast, a rabbit polyclonal serum raised against all three RPA subunits coprecipitated significant amounts of pol-prim as well as prim₂ (lanes 5 and 10).

From the five Tag-specific antibodies tested only PAb204 and PAb414 did not allow the coprecipitation of pol-prim with Tag (Fig. 8, lanes 2–6). prim₂ behaved comparably to pol-prim since it was not coprecipitated with Tag by both PAb204 and PAb414 (Fig. 8, lanes 8–12). The antibody PAb220, which did not interfere with the stimulation of primase activities by Tag in the absence of RPA, efficiently coprecipitated both pol-prim and prim₂ with Tag (Fig. 8, lanes 3 and 9). In addition to the co-immunoprecipitation of Tag with pol-prim or prim₂, the ability of these antibodies to disrupt the interaction of Tag and RPA was studied. The antibodies PAb220 and PAb204 prevented the coprecipitation of RPA with Tag, whereas the antibodies PAb419, PAb414, and PAb101 efficiently precipitated their specific antigen Tag together with RPA (Fig. 8, lanes 15 and 16 and lanes 14, 17, and 18, respectively). These results showed that PAb204 disrupted the pol-prim-Tag as well as the RPA-Tag interactions. In contrast to these findings, the antibodies PAb220 and PAb414 specifically abolished the interactions of Tag with only RPA and the primase complexes, respectively. Therefore, these studies suggest that the stimulatory effect of Tag on ssDNA requires protein-protein contacts between primase and Tag. Furthermore, the binding of Tag with RPA is essential to reverse the inhibition of primase by RPA. Additionally, proteins coexpressed in insect cells behaved the same way in co-immunoprecipitation experiments (data not shown).

Mapping of pol-prim Subunits Involved in Protein-Protein Interactions with Tag and RPA—To visualize the subunits of pol-prim interacting with Tag and RPA we performed protein overlay assays. These assays demonstrated that both primase subunits, p48 and p58, of pol-prim bind independently to Tag in addition to reported interactions of Tag with p180 and p68, which were also determined in our experiments (Fig. 9A, lane 5; Refs. 28 and 36). Furthermore, p180, p58, and p48 of pol-prim directly contacted RPA whereas in previous reports only p48 bound to RPA (Fig. 9A, lane 3; Ref. 39). The observed interactions were specific and not observed with, e.g., BSA (Fig. 9A, lanes 4 and 6) and were not due to cross-reactivities of the Tag- and RPA-specific antibodies with any subunit of pol-prim

(Fig. 9A, lane 7). Direct interactions of primase with Tag and an RPA subunit were also observed protein in overlay assays with Tag and RPA as immobilized proteins and prim₂ in the soluble phase (Fig. 9B). prim₂ independently bound to Tag and the p70 subunit of RPA, whereas it did not bind to BSA (Fig. 9B, lane 1) and the primase-specific antibody did not recognize these proteins (Fig. 9B, lane 2). These data show that Tag and primase independently bind to each other and that primase exclusively binds to the large subunit of RPA. For comparison, Coomassie Brilliant Blue stains of the proteins that were used in the immobilized phase and of the primase are presented in Fig. 9, panels A (lanes 1 and 2) and B (lane 3), and Fig. 1 (lane 2), respectively.

To quantify the interactions of Tag with both the primase subunits and pol-prim, we performed biomolecular interaction analysis, a method that is based on the physical principle of surface plasmon resonance studies. After coupling of prim₂ to

the chip by using various chemical reagents, the protein no longer bound to Tag. Therefore, tetramer and dimer were immobilized with the aid of the non-neutralizing monoclonal antibody PRI-8G10 against the p58 primase subunit. Binding of Tag to immobilized four-subunit pol-prim and primase dimer could be described by the simple bimolecular mechanism $A + B = AB$. After determining the kinetic constants k_{on} and k_{off} according to standard protocols, the dissociation constants of Tag binding to four-subunit pol-prim and primase dimer were calculated as $1.2 \cdot 10^{-8} \pm 2.1 \cdot 10^{-8}$ mol/liter and $1.3 \cdot 10^{-8} \pm 1.5 \cdot 10^{-8}$ mol/liter, respectively (summarized in Table II). These results support the data obtained by the protein overlay and immunoprecipitation assays.

DISCUSSION

The initiation of DNA replication requires an RNA-synthesizing primase activity (5, 6, 15). In eukaryotes the mechanism and regulation of primer formation is still poorly understood. Tag, RPA, and pol-prim are all involved in oligoribonucleotide synthesis on both the leading and lagging strand. However, it is still unclear whether the priming reactions on each strand occur by different mechanisms and whether they are independently regulated (7).

The four-subunit pol-prim, prim₂ containing p58-p48, and the catalytic subunit p48 efficiently synthesize oligoribonucleotides on ssDNA templates (Figs. 1 and 2A; data not shown; Refs. 13 and 55), but at an origin of replication the complete pol-prim complex is required for primer formation (Fig. 2B; data not shown; Ref. 54). On natural ssDNA bound by RPA, pol-prim needed SV40 Tag for efficient primer synthesis (Figs. 5 and 6; Refs. 18, 20, and 32). Since the p180 and p68 subunits interact with Tag and the primase subunits bind to RPA (28, 29, 36), it was generally thought that these protein-protein interactions are essential to perform the initiation reaction on both the leading and lagging strand. The interaction of Tag with the p180 subunit seemed to be necessary at the preinitiation stage probably targeting the pol-prim to and positioning it within the primosome (30). The Tag-p180-p68 contacts might also be relevant for the stimulation of Tag binding to the SV40 origin of replication and the inhibition of the DNA unwinding activity of Tag (38, 70). However, the data presented here showed that concentrations of pol-prim and prim₂, which were higher than those use in the initiation assays, inhibited the origin-dependent unwinding of dsDNA by Tag (Fig. 2, B and C).

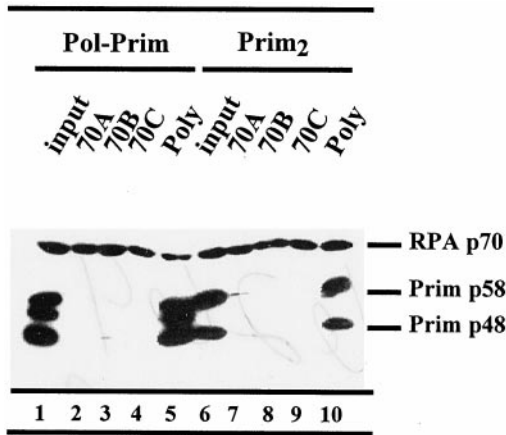


FIG. 7. Co-immunoprecipitations of pol-prim or primase with RPA using monoclonal antibodies against RPA. Lane 1, input proteins (pol-prim and Tag); lanes 2–5, immunoprecipitations of pol-prim and RPA with 70A, 70B, 70C, and polyclonal antiserum against RPA, respectively; lane 6, input proteins (prim₂ and RPA), lanes 7–10, immunoprecipitations of prim₂ and RPA with 70A, 70B, 70C, and polyclonal antiserum against RPA, respectively. After SDS gel electrophoresis and transfer to nitrocellulose, the p70 subunit of RPA and the p58 and p48 of pol-prim were detected with rat monoclonal antibodies and visualized with HRP-conjugated anti-rat antibodies and ECL. In lanes 1 and 5, the protein band running slightly faster than p58 is most likely a frequently detected proteolysis product of p58.

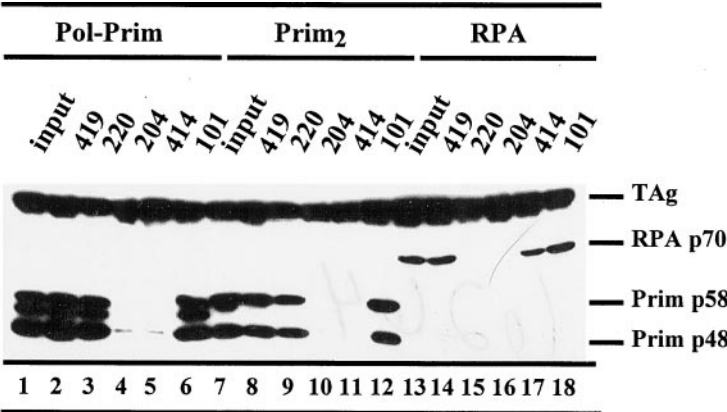


FIG. 8. Co-immunoprecipitations of pol-prim, primase, or RPA with Tag using monoclonal antibodies against Tag. Lane 1, input proteins (pol-prim and Tag); lanes 2–6, immunoprecipitations of pol-prim and Tag with PAb419, PAb220, PAb204, PAb414, and PAb101, respectively; lane 7, input proteins (prim₂ and Tag); lanes 8–12, immunoprecipitations of two-subunit primase and Tag with PAb419, PAb220, PAb204, PAb414, and PAb101, respectively; lane 13, input proteins (RPA and Tag); lanes 14–18, immunoprecipitations of RPA and Tag with PAb419, PAb220, PAb204, PAb414, and PAb101, respectively. After SDS gel electrophoresis and transfer to nitrocellulose, the p70 subunit of RPA and the p58 and p48 of pol-prim were detected with rat monoclonal antibodies and visualized with HRP-conjugated anti-rat antibodies and ECL. The antibody PAb419 to detect Tag was directly coupled to HRP. In lanes 1–3 and 6, the protein band running slightly faster than p58 is most likely a frequently detected proteolysis product of p58.

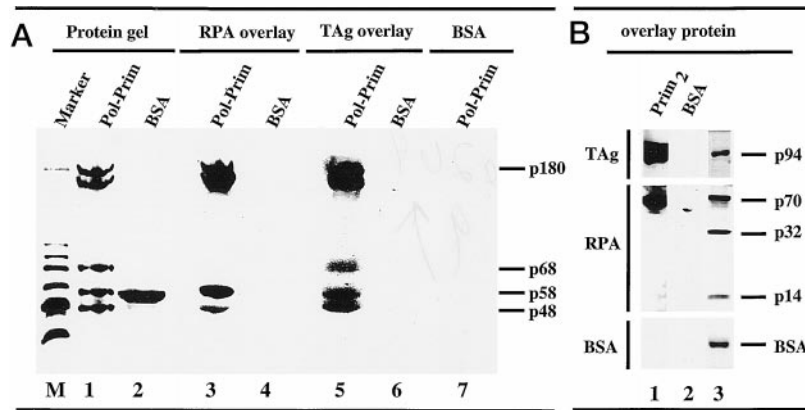


FIG. 9. **Protein overlay assays of replication proteins.** A, pol-prim and BSA were subjected to SDS-gel electrophoresis, renatured in the gel, and transferred to nitrocellulose. After saturation of nonspecific binding sites with 5% milk powder in TBS/T, the blot was developed with RPA (10 μ g/ml, lanes 3 and 4), or Tag (10 μ g/ml, lanes 5 and 6), as overlay proteins and monoclonal antibodies specific for these proteins. As a control BSA (10 μ g/ml, lane 7) was used as an overlay protein and a mixture of monoclonal antibodies against Tag and RPA. Lanes 1 and 2, pol-prim and BSA, respectively, stained with Coomassie Brilliant Blue; lanes 3, 5, and 7, 35 μ g of pol-prim; lanes 4 and 6, 10 μ g of BSA. B, Tag, RPA, or BSA were subjected to SDS-gel electrophoresis and the proteins were treated as described in panel A. The blots were then developed with prim₂ (5 μ g/ml) as an overlay protein and polyclonal antibodies specific for primase. Lane 1, 10 μ g of Tag, 30 μ g of RPA, or 8 μ g of BSA were treated as described above, incubated with prim₂, and primase-specific antisera; lane 2, 10 μ g of Tag, or 30 μ g of RPA was used, incubated with BSA, and primase-specific antisera; lane 3, Coomassie Brilliant Blue-stained protein gel with Tag (2 μ g), RPA (4 μ g), and BSA (1 μ g), respectively.

TABLE II

Interaction between Tag and immobilized human primase dimer or four-subunit DNA polymerase α -primase

Number of measurements: $n = 8$.

	Association rate constant k_{on}	Dissociation rate constant k_{off}	Dissociation constant K_D
	liters/mol $\cdot$ s	1/s	mol/liter
p58-p48 primase	$3.7 \pm 2.2 \cdot 10^6$	$4.8 \pm 2.4 \cdot 10^{-2}$	$1.3 \pm 1.5 \cdot 10^{-8}$
pol-prim	$3.5 \pm 3.2 \cdot 10^6$	$4.2 \pm 1.4 \cdot 10^{-2}$	$1.2 \pm 2.1 \cdot 10^{-8}$

Therefore, the inability of prim₂ to synthesize RNA primers in an origin-dependent reaction, which is coupled with an unwinding step, is most likely not due to an inhibitory effect or a lack of stimulation of the unwinding step by prim₂, since prim₂ and pol-prim behave similarly in this assay. The inhibitory effect of pol-prim on the unwinding activity of Tag has already been shown (70). Although in our hands the unwinding activity of Tag was quite effective (approximately 60% unwinding of a 3000-base pair fragment (Fig. 2C) in contrast to 33% unwinding of a 2200-base pair fragment (Ref. 70)), we had to use about 10 times more protein than in previously published studies to achieve similar effects. In contrast to their results (70), prim₂, although slightly less effective than pol-prim, inhibited the unwinding reaction. The possible reasons for these apparent contradictions are discussed below.

The requirements of protein-protein interactions following the initiation of DNA replication at the SV40 origin are quite complex. It was reported that the addition of pol α was required to allow primase to initiate on ssDNA bound by RPA (54). Furthermore, pol-prim was shown to interact directly with the p70 subunit of RPA and two different binding sites on p70 have been mapped for pol-prim (29, 41). Additionally, an interaction of pol-prim with p32 and/or p14 of RPA was reported (41). One site at the N terminus of p70 was responsible for the stimulation of DNA polymerase activity; the other, located within the major DNA binding region of p70, is necessary to increase DNA polymerase processivity (41). However, RPA has been shown to interact with prim₂ and subsequently an interaction with the p48 subunit was recorded (29, 39). These reported interactions seem to explain all requirements for the initiation of leading and lagging strand DNA synthesis (1).

To study the mechanism of initiation of Okazaki fragments

on the lagging strand, we investigated the priming activity of pol-prim and prim₂ on natural ssDNA in the presence of RPA and Tag. This assay system circumvents the divergent influences of pol-prim on Tag during origin binding and subsequent DNA unwinding steps (38, 69, 70). Both pol-prim and prim₂ behaved very similarly on natural ssDNA templates. They were both stimulated by Tag (Fig. 3) and inhibited by RPA (Fig. 4). The inhibition of primase by RPA was reversed by Tag, and there was no difference whether the four- or two-subunit primase complex was used (Fig. 5, A and B, lanes 1-5; Figs. 5C and 6, columns 1-5). These results confirmed the functional interactions of pol-prim with the replication proteins Tag and RPA described earlier (18, 20, 32). Moreover, they show that these functions can be fully assigned to the two primase subunits of pol-prim (summarized in Fig. 10). However, a previous report detected only a very weak stimulation of a human prim₂ by Tag on RPA-bound ssDNA (54). This apparent contradiction to our results might be explained by the source of the primase. In the report presented here, recombinant proteins produced and purified in high quantities were used, whereas the prim₂ used in the earlier report were produced by disrupting the pol-prim complex with ethylene glycol. It was described previously by the same laboratory that the separation of the DNA polymerase α and prim₂ with ethylene glycol leads to a rapid inactivation of the primase activity (71). In addition, the amounts of Tag used in these assays are very important, since high concentrations of Tag reproducibly inhibited the initiation activity of pol-prim and prim₂ (data not shown).

The findings that prim₂ is sufficient for the initiation of Okazaki fragments in a cell-free model system led to the question whether protein-protein interactions were required and whether p58 and p48 directly bound to Tag. The stimulation and the inhibition of the priming reaction as well as the reversal of the inhibition required multiple contacts of Tag to prim₂ and RPA. This interpretation is supported by several experiments. A monoclonal antibody (PAb414) against Tag prevented the stimulation of primase activity (Fig. 6) and hindered its interaction with pol-prim and prim₂ (Fig. 8, summarized in Table I; Refs. 29 and 69). In contrast, the addition of the monoclonal antibody PAb220 exclusively disrupts interactions of Tag with RPA (Table I; Ref. 69) and no longer allowed Tag to reverse the inhibition of primase by RPA (Fig. 6). The RPA-specific antibody 70A, which interferes with the binding of RPA

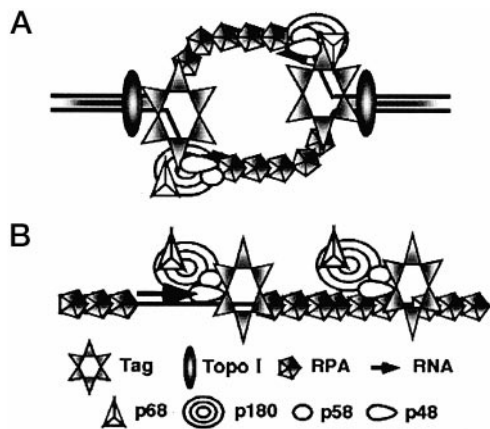


FIG. 10. Protein-protein interactions during primer synthesis. A, all four subunits of pol-prim are needed to start DNA replication of the leading strand in an SV40 DNA replication system that requires a coordinated unwinding and initiation reaction. B, the two smallest subunits of pol-prim, the primase subunits p58 and p48, are necessary and sufficient to initiate the synthesis of Okazaki fragments in a model system for lagging strand DNA replication.

to prim₂ and pol-prim, led to a decreased primase activity compared with the reaction that only contained primase complexes and RPA (Figs. 5 and 7, Table I). The overall inhibition on the M13 template is most likely due to the binding of RPA to and thereby blocking access of the primase to the template ssDNA, since RPA prefers to bind to pyrimidine-rich sequences, which primase also preferentially utilizes for its initiation activity. The antibody 70C, which destabilizes ssDNA binding of RPA (37), corroborates this conclusion, because its titration into the primer assay led to the reversion of the inhibitory activity during primer synthesis.

Protein-protein interactions between the three initiation proteins Tag, pol-prim, and RPA have been shown by four different assays, including protein overlay, modified enzyme-linked immunosorbent assay, fluorescence quenching, and co-immunoprecipitations with pol-prim, separated subcomplexes, and in a few cases using expressed subunits (22, 28–30, 32, 36, 41, 72, 73). For the first time we demonstrate here direct physical contacts between the primase subunits and SV40 Tag by co-immunoprecipitations (Fig. 8), and far Western experiments (Fig. 9). The surface plasmon resonance studies supported our findings that primase directly binds to Tag and that this association does not require either the p180 or the p68 subunits of pol-prim (Table II). The calculated dissociation constants for the interactions of Tag with pol-prim and prim₂, respectively, were very similar and were 2-fold higher than the calculated constant for the interactions of Tag and the tumor suppressor protein p53 (74). Additionally, the protein overlay experiments allowed us to determine that both p58 and p48 independently contributed to the binding of Tag. The results that the eukaryotic cellular primase physically binds to the replicative helicase SV40 Tag parallels the findings determined in herpes simplex virus 1, mouse polyomavirus, bacteriophages, and *Escherichia coli* (5, 22). Therefore, the protein-protein interactions presented here are most likely also required for DNA replication in the cell.

Although direct interactions between Tag and either of the primase subunits had not been detected before, fluorescence quenching experiments indirectly suggested that one or both subunits might contribute to overall Tag binding (29, 73). The failure to detect these interactions in previous reports (28, 29, 39) might be due to several reasons. In our present investigation, all proteins were from human origin (13, 21, 22, 55, 57). In contrast, earlier investigations used prim₂ that was purified

from non-permissive cells, i.e. from calf thymus tissue or other mammalian organisms (28, 29, 39). Furthermore, all proteins used in this study were recombinant proteins. Thus, highly concentrated proteins could be used in the far Western blot experiments which is important for obtaining reproducible results (data not shown). Additionally, protein interaction sites can be masked depending on the immobilization technique. This view was supported by our surface plasmon resonance experiments, which showed that the interaction of primase and Tag could exclusively be detected when the prim₂ was immobilized via monoclonal antibodies. When the prim₂ was directly immobilized with amino or thiol couplings, binding of primase and Tag was no longer detectable (data not shown). Similar findings were reported earlier when the physical interactions of RPA with p53 were analyzed (74).

In summary, in addition to the two large subunits p180 and p68 of pol-prim, both primase subunits p58 and p48 bind to Tag. These newly identified contacts of prim₂ and Tag were essential for efficient initiation of DNA synthesis on the lagging strand. Furthermore, the initiation of leading and lagging strand replication is more complicated than earlier models supposed. Both processes require different mechanisms and protein-protein interactions to start DNA replication on a given strand (summarized in Fig. 10). Hereby, primer synthesis on the lagging strand, but not at the origin of replication, can apparently be carried out without Tag binding to the two large subunits of pol-prim (Fig. 10). This interpretation is supported by the earlier finding that the addition of the Tag binding site within the N terminus of p180/DNA polymerase α inhibited SV40 DNA replication *in vitro* only during the so-called lag period of the assay. Later, when the leading and lagging strand are replicated and the priming of the Okazaki fragments on the lagging strand is the only task of the primase, the addition of the polypeptide could no longer inhibit or reduce the incorporation of dNMPs (30). Our interpretation, that the initiation reactions on the leading and lagging strand require in part dissimilar protein-protein interactions and that the mechanisms of both processes might differ (Fig. 10), raises the question whether the initiation of leading and lagging strand replication are controlled by different mechanisms. With the recombinant replication proteins in hand and with the known regulation of leading strand initiation by cyclin-dependent kinases (52, 75, 76), this question can now be addressed.

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