# Timed interactions between viral and cellular replication factors during the initiation of SV40 *in vitro* DNA replication

Poonam Taneja, Heinz-Peter Nasheuer, Hella Hartmann, Frank Grosse, Ellen Fanning, Klaus Weißhart



## SUPPLEMENTARY MATERIAL

### SUPPLEMENTARY METHODS

#### Enzymatic assays using origin containing plasmid DNA

All reactions contained in an end volume of 40 µl 30 mM Hepes-KOH pH 7.8; 7 mM MgAc; 1 mM EGTA; 0.5 mM DTT; 40 mM creatine-phosphate; 40 µg/µl creatine-kinase; 3.5 pmol replication-protein A (RPA, 0.116 µg/pmol); 6.25 pmol SV40 large T-antigen (Tag, 0.096 µg/pmol); 1.1 pmol human DNA polymerase α-primase (Pol, 0.354 µg/pmol) and 4 mM ATP.

To start assembly of Tag on the ori sequences (step 1) 0.1 pmol pUC-HS DNA (2 µg/pmol) containing the complete SV40 origin sequences were added, followed by 30 minutes incubation at 37 $^{o}$C. To induce the bidirectional unwinding reaction catalyzed by Tag (step 2) 1.2 pmol human Topoisomerase I (Topo, 0.1 µg/pmol) were supplied to the reaction to release the torsional stress created during unwinding. The sample was incubated for 60 minutes at 37 $^{o}$C. To trigger primer synthesis catalyzed by the primase subunit of Pol (step 3) 0.2 mM of each CTP, GTP and UTP were added and incubation continued for another 60 min. If the product was to be analyzed and quantified the concentration of CTP was reduced to 0.05 mM and the reaction was supplemented with 10 µCi $\alpha^{32}$P-CTP. To start primer extension by the polymerase subunit of Pol (step 4) 0.2 mM each dATP, dCTP dGTP and dTTP were added with a continuation of incubation for another 60 min. If the product was to be analyzed and quantified the concentrations of dCTP and dTTP were reduced to 0.05 mM and the reaction was supplemented with 10 µCi $\alpha^{32}$P-dCTP and $\alpha^{32}$P-dTTP. This step corresponds to the so-called mono-polymerase system, since Pol will synthesize both, the leading and the lagging strand in the reaction [1]. Finally, to obtain full replication (step 5) 190 µg

S100 extract prepared from human 239S cells and immunodepleted for RPA, Topo and Pol were added to the sample. Incubation continued than for an additional 90 minutes. This step corresponds to the cell-free SV40 *in vitro* DNA replication [2].

5 μl of radioactively labelled reactions were spotted on DE81 paper for quantification of incorporated labelled nucleotides (rNMPs or dNMPs) [3]. The rest of the reactions (exempt step 1) were stopped by adding EDTA, SDS and proteinase K to final concentrations of 20 mM, 0.65% and 1.7 μg/μl, respectively and a further 30 minutes of incubation. The sample was extracted once with phenol/chloroform and DNA was passed over a G-50 spin column (Boehringer / Mannheim) into TE buffer (10 mM Tris-HCl pH 8; 1 mM EDTA) to remove unincorporated nucleotides. DNA was than processed for gel electrophoretic analysis.

The reaction after the assembly step was supplied with 10 μl native 5 x loading buffer (50 mM Hepes-KOH pH 7.5; 10 mM EDTA; 25.% Ficoll 400; 0.1% bromophenolblue) and run on 0.8% agorose gels in TAE buffer (0.04M Tris-acetate, 0.002 M EDTA). DNA and DNA-protein complexes were Southern blotted onto a nitrocellulose-membrane [3]. Proteins were detected by immunodecoration using the enhanced chemiluminescence system (ECL, GE Healthcare) for detection.

DNA after the unwinding step was ethanol precipitated in the presence of 10 μg carrier glycogen and was re-dissolved in 20 μl non-denaturing loading buffer (10 mM Hepes-KOH pH 8; 10 mM EDTA, 2% Ficoll, 2% sucrose, 0.01% bromophenol blue, 0.1% SDS). One half of the sample was electrophoresed in a 1.5% agarose gel in TAE to resolve super-coiled (form I) and topoisomeric (form I') from unwound (form U) DNA. DNA was visualized by ethidium bromide staining and quantified by densitometric scanning.

Reaction products after the primer synthesis step were precipitated in the presence of 0.8 M LiCl; 10 mM $MgCl_2$ and 10 μg carrier tRNA. The precipitate was dissolved in 20 μl denaturing loading buffer (35% formamide; 8 mM EDTA; 0.1% bromphenolblue; 0.1% xylene cyanol FF) for 30 min. at 65 $^{o}$C, heated for 3 min. at 95 $^{o}$C, and electrophoresed in 20% denaturing urea polyacrylamide gels in TBE (0.089 M Tris-borate, 0.089 M boric acid, 0.01 M EDTA) at 600 V until the bromphenol marker had migrated three quarters to the bottom of the gel. The gel was exposed wet to an X-ray film.

Reaction products after the primer extension reaction were ethanol precipitated in the presence of 10 μg carrier tRNA and dissolved in 20 μl alkaline loading buffer (50 mM

NaOH; 1mM EDTA; 5 % Ficoll 400; 0.025% bromcresol green). One half of each sample were separated in 1.5% agarose gels in circulating alkaline running buffer (50 mM NaOH; 1mM EDTA) for 10 hr at 150 mA in the cold. The gel was fixed in 10 % trichlor acetic acid, dried and exposed to X-ray films.

Reaction products after full replication were precipitated in the presence of 10 μg carrier tRNA, and suspended in 20 μl TE buffer. 5 μl aliquots were double digested in a 10 μl reaction with EcoRI to linearize the concatenated DNA and DpnI to remove any unreplicated template DNA. 2 μl 6 x native loading buffer (50 mM Hepes-KOH pH 7.5; 10 mM EDTA; 25.% Ficoll 400; 0.1% bromophenolblue) were added and the products were separated in a 0.8% agarose gel in TBE (89 mM Tris-borate, 89 mM boric acid). The gel was dried and exposed to X-ray films.

For inhibition studies antibodies or peptides were added 30 minutes before the single steps, which were activated by the addition of the specific missing factors.

**Protein-DNA interactions**

Gel retardation assays contained in a 10 μl mix 20 mM Hepes-KOH pH 7.8; 5 mM $MgCl_2$; 1 mM DTT; 0.1 mM EDTA; 0.083 pmol single-stranded M13mp18 DNA (2.4 μg/pmol) and 20 pmol of RPA (0.116 μg/pmol) [4]. 50 pmol antibodies (0.15 μg/pmol) were added as indicated. After 30 min. at 37 $^{o}$C, the sample was supplemented with 2 μl 6x native loading buffer (50 mM Hepes-KOH pH 7.5; 10 mM EDTA; 25.% Ficoll 400; 0.1% bromophenolblue) and electrophoresed in a 1% agarose gel in TAE buffer (0.04 M Tris-acetate; 0.002 M EDTA). The gel was stained with 0.5 μg/ml ethidiumbromide and DNA visualized on a UV-transilluminator.

**Origin DNA bidirectional unwinding assays**

Bidirectional unwinding reactions with super-coiled closed circular DNA contained in a total volume of 10 μl 0.1 pmol pUC-HS DNA (2 μg/pmol); 30 mM HEPES-KOH pH 7.9; 1 mM DTT; 7 mM MgAc; 4 mM ATP; 40 mM creatine phosphate; 0.1 mg/ml of creatine kinase; 0.25 mg/ml of BSA; 1.2 pmol human Topoisomerase I (Topo, 0.1 μg/pmol); and 3.5 pmol replication-protein A (RPA, 0.116 μg/pmol). To test unwinding of linear DNA the closed circular DNA was replaced by 10 fmol (approximately 20 ng) of XmnI-restricted 5´-labeled pUC-HS DNA and the Topo was omitted. The reaction was started by adding 6.25 pmol Tag (0.096 μg/pmol). After 1 h at 37 $^{o}$C, the reaction was stopped by adding proteinase K, EDTA,

and SDS to final concentrations of 0.3 mg/ml, 6 mM, and 0.3%, respectively. After incubation at 37 $^{o}$C for 30 min, DNA was ethanol precipitated, re-dissolved in 10 µl TE. 2 µl 6 x native loading buffer (50 mM Hepes-KOH pH 7.5; 10 mM EDTA; 25% Ficoll 400; 0.1% bromophenolblue) was added and samples electrophoresed in 1.5% agarose gels. The gel was stained with ethidiumbromide and photographed. Underwound form U DNA was quantified by densitometry. In the case of the linear DNA, the precipitation step was omitted and the reaction supplied with 2 µl 6 x native loading buffer. The sample was than applied to a 1% agarose gel in TAE. The gel was dried and exposed to X-ray films, and quantified by phosphoimaging.

**Protein overlay assays (Far western blots)**

10 µg of proteins (Tag, Pol and RPA as well as MBP and BSA as negative controls) were incubated in 2.5% SDS; 2.5 mM Tris-HCl pH 8; 100 mM DTT; 10% glycerol and 0.05% Pyronin T1 for 5 min. at room temperature. Proteins were separated by sodiumdodecyl sulphate – polyacrylamid gel-electrophoresis (SDS-PAGE) [5]. The gel was incubated for 1 hr at room temperature in 50 mM Tris-HCl pH 7.5; 20% glycerol to re-nature proteins. Proteins were transferred to nitrocellulose (NC)-filters in transfer buffer (10 mM $NaHCO_3$; 3 mM $Na_2CO_3$) [6]. Filters were Ponceau S (Sigma Aldrich) stained and photographed. The single lanes were then cut in strips, which were placed in filter strip incubation trays. The strips were destained in phosphate buffered saline (PBS). Unspecific binding sites were blocked in 5 % non-fat milk powder (MP) in TBS (10 mM Tris-HCl pH 8; 150 mM NaCl) for 30 minutes. Filters were then washed in TBS and incubated with the soluble partner MBP-fusion (200 µg/ml) or replication protein (5 µg/ml) in TBS / 5% MP for 2 hr at room temperature. The filters were washed and incubated at 4 $^{o}$C overnight with primary antibodies, which included anti-his monoclonal antibodies for $H_6$-RPA (Quiagen), monoclonal antibody PAb 101 for Tag [7], monoclonal antibody SJK237-87 for Pol [8], and a polyclonal anti-MBP serum for MBP. All antibodies were non-inhibitory and hence non-disruptive to protein-protein interactions. Filters were washed and incubated for 2 hours at room temperature with an alkaline phosphatase coupled secondary antibody using the ProBlot$^{TM}$ system from Promega for detection.

## SUPPLEMENTARY FIGURES AND RESULTS

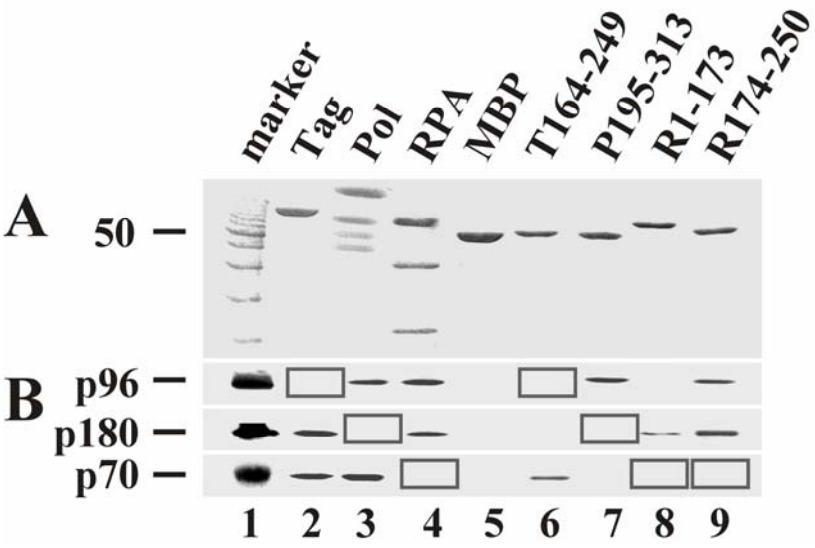


**Figure 1S: Characterization of peptides**

Panel A. Coomassie stained gel of ~20 pmol replication factors (lanes 2-4) and MBP-fusion proteins (lanes 5-9). The 50 kDa marker of the 10 kDa protein ladder (lane 1) is indicated.

Panel B. Co-precipitations of 25 pmol Tag, Pol and $H_6$-RPA were performed either with 25 pmol immobilised replication factors (lanes 2-4) or MBP-fusion peptides (lanes 5-9). Lane 1 contained $1/10^{th}$ of the input material. Proteins were detected by specific antibodies in western blots. The positions of Tag (p96), the p180 subunit of Pol and the p70 subunit of RPA are indicated. Boxed lanes contained no load.

**Results: Characterization of peptides**

Tag specific peptide T164-249, Pol specific peptide P195-313 and the RPA specific peptides R1-173 and R174-250 were expressed as maltose binding protein (MBP) fusions. All fusions were obtained in a soluble form and purified by affinity chromatography on amylose resin (Figure 1S, panel A). The binding capabilities of the peptides were evaluated in co-precipitation experiments (Figure 1S, panel B). Tag specific peptide T164-249 interacted exclusively with RPA, Pol specific peptide P195-313 solely with Tag and peptide R1-173 from RPA specifically with Pol (lanes 6-8). In contrast, each replication factor was able to co-precipitate both other factors (lanes 2-4). This was also the case for the second RPA derived peptide R174-250 that retained both Tag and Pol (lane 9). The fusion MBP part alone was not able to interact with any of the replication factors (lane 5). The peptides did not display specific activities associated with the intact proteins other than protein-protein interactions nor did they interfere with these activities (data not shown).

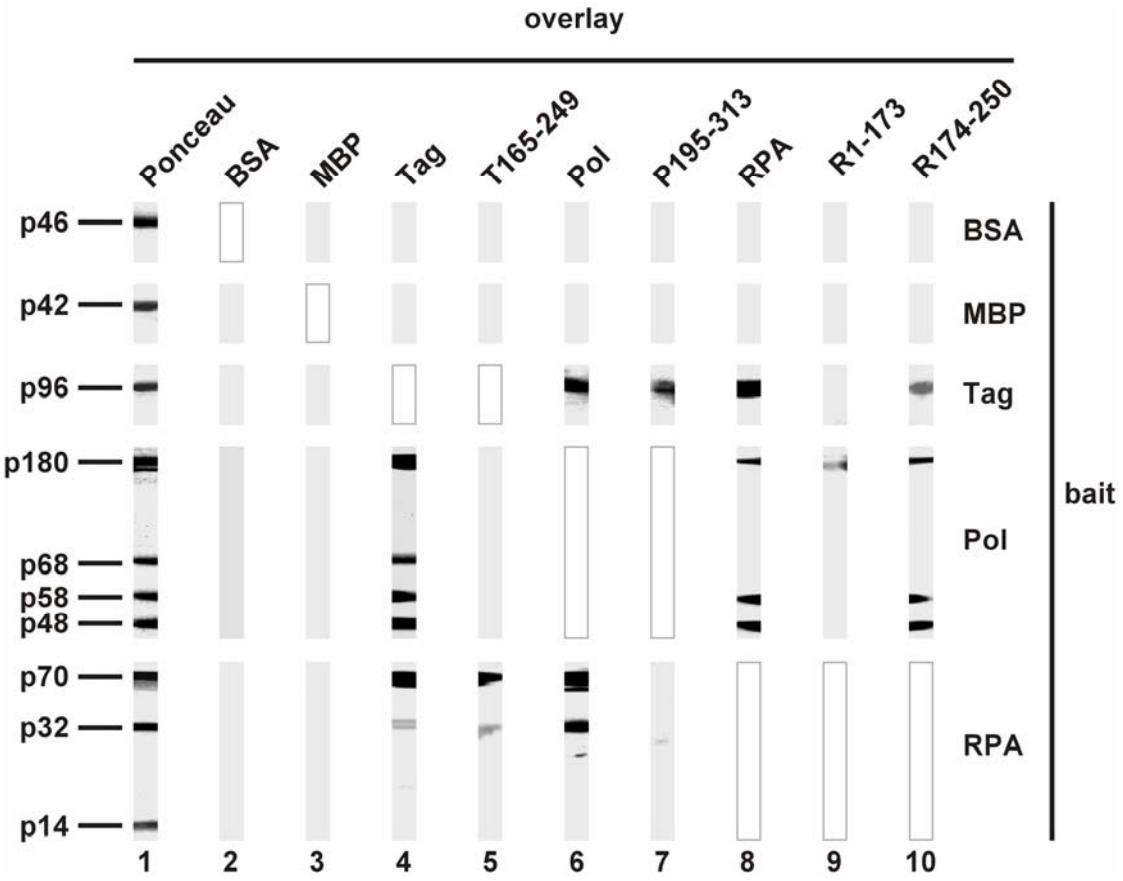


**Figure 2S: Subunit specificity of peptides**

BSA, MBP (both negative controls), Tag, Pol and RPA were immobilised onto nitrocellulose filter strips. The strips with these bait proteins were either stained with Ponceau to visualize the single polypetides (lane 1) or were incubated with the indicated overlay polypeptides (lanes 2-10). Bound proteins were detected by immunostaining using a color substrate that was turned into an insoluble chromogenic precipitate. The following antibodies were used for detection: a mixture of anti Tag, anti Pol, anti his-tag (specific for the histidin-tag of $H_6$-RPA) and anti MBP, whereby exactly those antibodies were omitted, which were specific for the bait protein (lane 2); anti Tag (lane 4); anti Pol (lane 6); anti his-tag (lane 8); anti MBP (lanes 3, 5, 7, 9 and 10). Boxed lanes indicate combinations that were omitted. Molecular weights of the single subunits are indicated.

**Results: Subunit specificity of peptides**

T165-249 interacted with the two larger subunits of RPA as did full length Tag, but showed no reactivity to any of the Pol subunits unlike the latter, which was positive for all of them (Figure 2S, lanes 4 and 5). Binding to the p32 subunit was relatively week for both Tag and the Tag specific peptide. The binding of T164-249 to both RPA subunits is in line with NMR studies showing that sequences within the OBD of Tag contacted both p32 and p70 [9, 10]. Like Pol peptide P195-313 interacted with Tag [11], but unlike the former did not interact with the p70 and p32 subunits (lanes 6 and 7). R174-250 and RPA interacted with Tag and the three Pol subunits p180, p58 and p48. In contrast R1-173 interacted exclusively and weakly with p180 (lanes 8-10). The fact that R1-173 can inhibit the Pol-RPA interaction even in the presence of a stronger binding site comprising amino acids 174-250 of p70 suggests that amino acids 1-173 of p70 might serve as the initial contact site for Pol. None of the peptides and replication proteins interacted with immobilized BSA or MBP, nor did any of the employed sera cross-react with any of the subunits for which they were not specific (lanes 2 and 3).

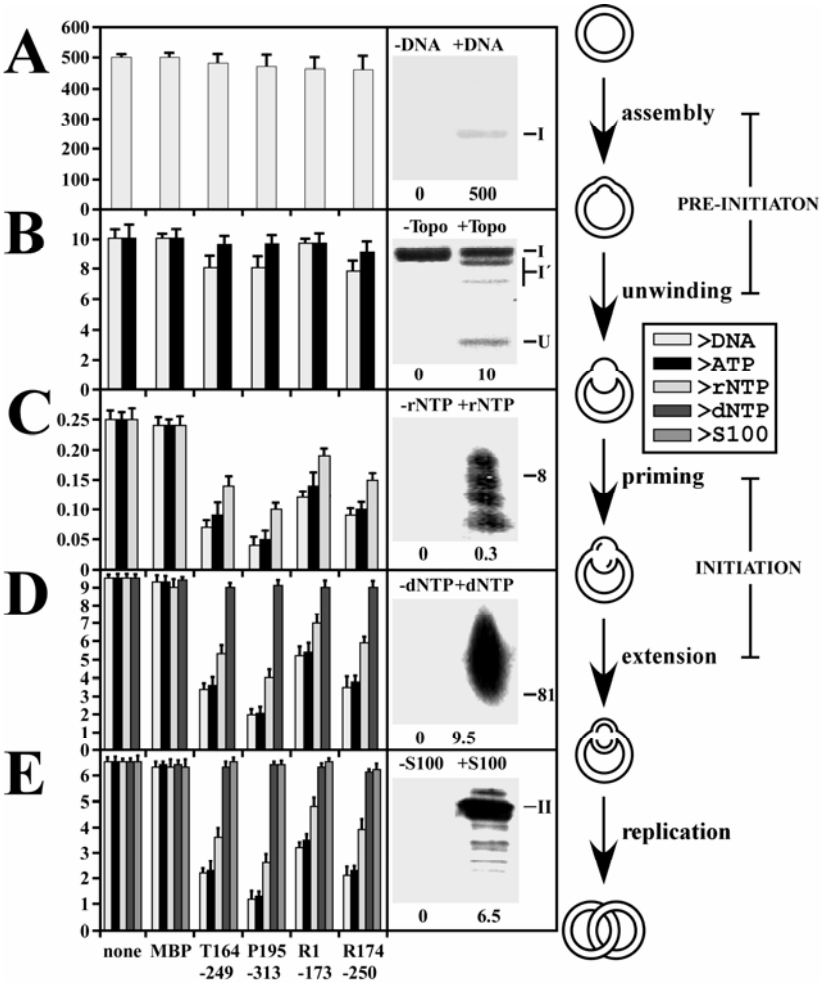


**Figure 3S: Influence of peptides on different steps during SV40 *in vitro* DNA replication**

Assembly (A), unwinding (B), primer synthesis (C), primer extension (D) and in vitro replication (E) reactions were sequentially performed by adding DNA, Topo, rNTPs, dNTPs and S100 extracts to depleted reactions, respectively. Peptides were pre-incubated with the reaction prior to the addition of a specific factor, which is identified after the ">" sign in the legend. Pre-initiation and initiation phases are indicated. The DNA templates and products are represented by symbols and include from top to bottom super-coiled, distorted super-coiled, under-wound, primed, primer extended and concatenated plasmid DNAs. The products of each reaction are displayed in the right panels in the absence (lane 1) and presence (lane 2) of the respective missing factor. Numbers below each lane indicate the quantity of products formed. Positions of super-coiled DNA (form I), topoisomers (form I`), under-wound DNA (form U), linear DNA (form II) as well as oligo $dT_8$ and an 81 nt fragment markers are indicated. The column diagrams on the left show the quantities of each product formed in the absence and presence of the indicated peptides, which include fmol bound Tag (A), fmol under-wound DNA (B), pmol incorporated rNMPs (C) and pmol incorporated dNMPs (D and E).

**Results: Influence of peptides on different steps during SV40 *in vitro* DNA replication**

We have tested the influence of the peptides when administered at different time points into replication reactions on various activities: To this end a step-wise reaction scheme was used by depleting the reaction for certain factors and adding them back gradually (see Figure 2S). The first step was the assembly of Tag onto the origin sequences, which was triggered by the addition of an origin containing super-coiled plasmid DNA into the assay. This resulted in a Tag-DNA complex that was analyzed by immunoblotting for the presence of Tag (panel A). RPA and Pol were only seen in trace amounts associated with Tag, indicating only a weak association of these factors that might not resist the electrophoretic conditions (data not shown). Peptides had virtually no influence on the binding efficiency of Tag. The resulting distorted super-coiled plasmid DNA could be used efficiently in the second step, the bidirectional unwinding reaction catalyzed by Tag. This step was started by the addition of Topo, which is required to release torsional stress induced in the plamid DNA during unwinding (panel B). The assembly and initial unwinding are referred to as the pre-synthesis or pre-initiation phase [12, 13]. All peptides (exempt R1-173) showed only a slight inhibitory influence on the unwinding reaction being more effective if administered before the assembly step. The product of this reaction is an under-wound DNA. This product was used as the template for step 3, the primer synthesis reaction, which was catalyzed by Pol after addition of missing rNTPs (panel C). All peptides inhibited the reaction most efficiently if they were added to the assay before the assembly or unwinding steps. If added before the primer synthesis reaction, inhibition was reduced. The primed DNA with primers 5-10 nucleotides (nt) in length served as the template for primer extension (step 4) on both strands by Pol (mono-polymerase system), which was triggered by the addition of dNTPs (panel D). Primer extensions were for around 50 to 250 nt in length. As before, the extension reaction was most efficiently inhibited, if peptides were present in the assay prior to the assembly and unwinding steps. Intermediate inhibition was seen when antibodies were added before primer synthesis. Interference capability was lost for all peptides if they were administered right before primer extension. The primer synthesis and initial extension reactions are referred to as the initiation phase. The primer extended DNA template was finally used in step 5 for full replication by the addition of cellular S100 extracts. As was the case before, strong, intermediate and no inhibition was seen for peptides present before assembly / unwinding, primer synthesis and primer extension, respectively. There was also no influence exerted by peptides if they were added directly before the cellular extracts.

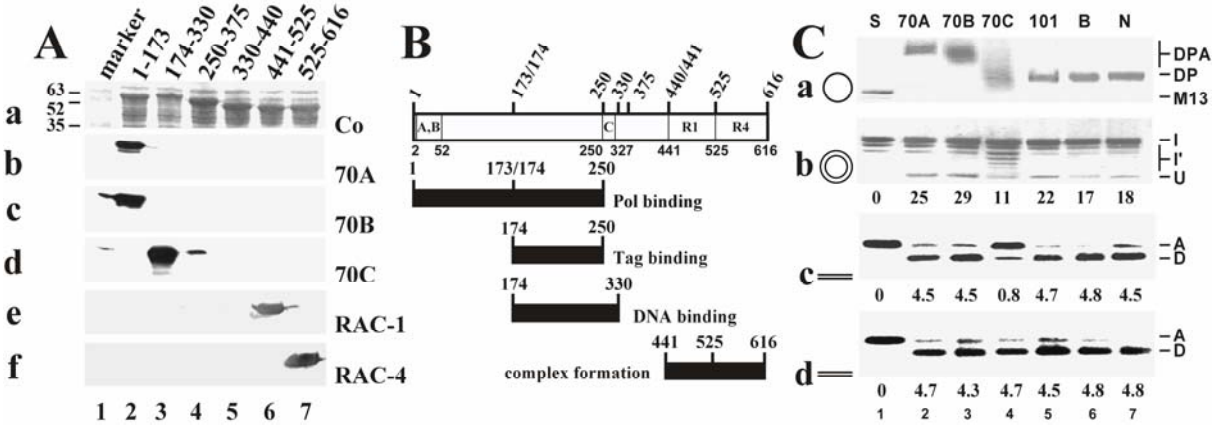


**Figure 4S: Characterization of monoclonal antibodies specific for the p70 subunit of RPA.**

Panel A. Reactivity of antibodies to p70 sequences. Crude extracts containing expressed MBP fusion proteins with the indicated sequences of p70 were separated by SDS-polyacrylamide gel electrophoresis and analyzed by Coomassie staining (a). The positions of pre-stained markers in lane 1 are indicated in kDa. Parallel samples were transferred to nitrocellulose filters and reacted with the indicated monoclonal antibodies (b-f) using the enhanced chemiluminescence system for detection. Only the relevant parts of the gels and filters are shown.

Panel B. Domain structure of p70. The top diagram schematically depicts the p70 subunit as an open box with numbers referring to amino acids. The localization of the binding regions for the antibodies (A, B and C for 70A, 70B and 70C; R1 and R4 for RAC-1 and RAC-4) are indicated within the box with the respective border amino acids below. Structural and functional domains are depicted as black boxes with extensions defined in amino acids. The function of each region is indicated.

Panel C. Influence of antibodies on DNA binding to a closed circular M13mp18ssDNA substrate (symbolized as a circle) in gel retardation (a). RPA was incubated with the ssDNA in the presence of the indicated antibodies. Shifted DNA was analysed by agarose gel electrophoresis and staining the gel with ethidium bromide. Positions of free DNA (M13), shifted DNA-RPA complexes (DP) and super-shifted DNA-RPA-antibody complexes (DPA) are indicated. Bidirectional unwinding using a closed circular super-coiled plasmid, symbolized as a double circle (b), or linearized double stranded DNA, symbolized by two parallel lines (c, d), each containing the SV40 origin sequences, as the DNA substrates. Antibodies were added simultaneously with RPA (b), after addition of RPA (d) or were pre-incubated with RPA (c). 70A, 70B, 70C are RPA specific antibodies; 101 is Tag specific. The pmol of unwound (b) or fmol of displaced (c, d) DNA are quantified below each lane. The positions of the under-wound form (form U), topoisomers (form I'), relaxed form (form I), annealed (A) and displaced (D) DNA strands are indicated. B, buffer; N, water; S, substrate without RPA.

**Results: Characterization of monoclonal antibodies specific for the p70 subunit of RPA**

Of the five monoclonal antibodies used in this study and specific for the p70 subunit of RPA two, 70A and 70B, bound within the N-terminal Pol interacting domain (amino acids 1-173) and one, 70C, within the C-terminal boundary of the DNA binding domain A (amino acids 174-330) (see supplementary Figure 3S, panels A and B) [14]. The epitopes of the other two, RAC-1 and RAC-4, were located within C-terminal sequences, which are needed for complex formation with the two smaller subunits [15]. 70A, 70B, RAC-1 and RAC-4 did not interfere with the binding of RPA to ssDNA and RPA-DNA complexes were supershifted in their presence (supplementary Figure 3S, panel C). 70C, on the other hand, prevented the formation of a stable tertiary complex between RPA, DNA and antibodies. This was manifested by a smear in gel retardation assays that indicates the disassembly of protein-DNA complexes during gel migration [14]. The bidirectional unwinding of a super-coiled SV40 origin-containing plasmid catalyzed by Tag and Topo was reduced in the presence of 70C, whereas 70A and B had virtually no effect on the unwinding process and extents of unwinding comparable to those of the positive controls were achieved. When 70C was pre-incubated with RPA prior to the addition of the DNA, inhibition was enhanced (data not shown). Using a labeled double-stranded DNA fragment bearing origin sequences in the middle as the substrate, a profound inhibition of unwinding in the presence of 70C but not 70A and B was observed when the antibodies were pre-incubated with RPA prior to the addition of the DNA substrate. Such an inhibition was not noticed for 70C when the pre-incubation phase was omitted.

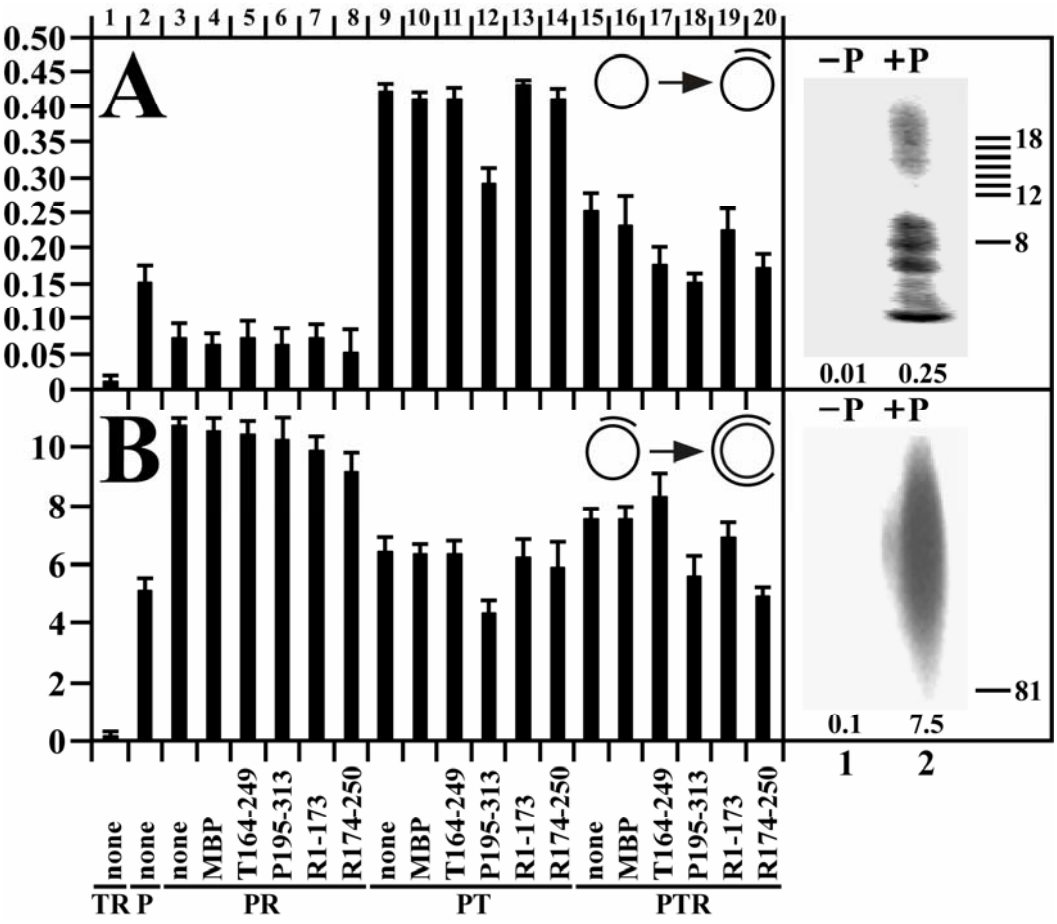


**Figure 5S: Influence of peptides on enzymatic activities using natural single-stranded DNA templates.**

Primer synthesis (panel A) and primer elongation (panel B) reactions were carried out using an unprimed or primed single stranded circular DNA, respectively, as the templates. Products are shown on the right panels in the absence (-P, lane 1) or presence (+P, lane 2) of Pol, with all other replication factors provided. The positions of oligo $dT_8$, oligo $dT_{12\text{-}18}$, and an 81 nt fragment markers are indicated. The column diagrams and numbers below each lane display the incorporated pmol of rNMPs (panel A) and dNMPs (panel B). Reaction templates and products are represented by symbols in the column diagrams. The presence of any of the replication proteins Tag, Pol and RPA are indicated by “T”, “P” and “R”, respectively. Peptides were added as indicated.

**Results: Influence of peptides on enzymatic activities using natural single-stranded DNA templates**

Using ssM13mp18 DNA as the template, none of the peptides showed any pronounced influence on the inhibition exerted by RPA on the primer synthesis activity of Pol (Figure 4S, panel A, lanes 3-8). The stimulation of Tag on Pol's priming activity was exclusively reduced by P195-313, which disrupts the Tag-Pol interaction (lanes 9-14). Three of the peptides T164-249, P195-313 and R174-259 reduced the inhibition relief Tag exerted on the primer synthesis reaction in the presence of RPA (lanes 15-20). Using a primed ssM13mp18 DNA template both R1-183 and R174-250 slightly reduced the stimulatory influence of RPA on the polymerase activity (Figure 4S, panel B, lanes 3-8). On the other hand, P195-313 exclusively reduced the stimulation Tag exerted on the polymerase activity (lanes 9-14). In the presence of all replication factors T164-249 stimulated the extension reaction, whereas P195-313, R1-173 and R174-250 peptides reduced incorporation levels (lanes 15-20).

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