Species specificity of Simian Virus 40 DNA replication in vitro requires multiple functions of human DNA polymerase α

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Abstract

Human cell extracts support the replication of Simian Virus 40 (SV40) DNA, while mouse cell extracts do not. Species specificity is determined at the level of initiation of DNA replication and it was previously found that this requires the large subunit, p180, of DNA polymerase α-primase to be of human origin. Furthermore, a functional interaction between SV40 large T antigen (TAg) and p180 is essential for viral DNA replication. In this study we determined that the N-terminal regions of human p180, which contain the TAg binding site, can be replaced with those of murine origin without losing the ability to support SV40 DNA replication in vitro. The same substitutions do not prevent SV40 TAg from stimulating the activity of DNA polymerase α-primase on ssDNA in the presence of replication protein A. Furthermore, biophysical studies show that the interactions of human and murine DNA polymerase α-primase with SV40 TAg are of a similar magnitude. These studies strongly suggest that requirement of SV40 DNA replication for human DNA polymerase α depends neither on the TAg binding site being of human origin nor on the strength of the binary interaction between SV40 TAg and DNA polymerase α-primase but rather on sequences in the C-terminal region of human p180.
Introduction

Polyomavirus DNA replication has been studied extensively owing to the ease with which viruses of this family, which include Simian Virus 40 (SV40) and Mouse Polyoma Virus (PyV), can be grown in cell culture and moreover to the availability of an in vitro replication system which allows detailed investigation of the individual factors that play a role in the replication process (1,2). Polyomavirus DNA replication has been found to be largely dependent upon factors involved in replication of the host DNA but does contribute one essential trans-acting factor known as large T-antigen (TAg) to the replication complex. TAg is responsible for recognition of the viral origin of replication at which it forms a double hexamer (3). In the presence of the single stranded DNA binding protein, RPA (replication protein A), and topoisomerase I, TAg proceeds to unwind the origin DNA and recruits the DNA polymerase α-primase heterotetramer which then initiates bidirectional DNA synthesis (4-11). DNA polymerase α-primase initiates DNA replication through the action of its smallest subunit, p48, which synthesises RNA primers that are subsequently elongated by the large subunit, p180 (12-15). The bulk of DNA synthesis is, however, carried out by a more processive enzyme complex consisting of DNA polymerase δ and its processivity factor PCNA (proliferating cell nuclear antigen) (16-18). The transition between DNA synthesis by DNA polymerases α and δ is mediated by the PCNA loading factor RF-C (replication factor C) (16,19). Throughout the viral replication cycle, TAg double hexamers are thought to act as the replicative helicase (20). For a more extensive account of the DNA replication process and its participating factors see (1,21-23).

SV40 and PyV are very similar with regard to DNA replication; their respective TAg s are 36% identical, have largely the same biochemical activities and recognise very similar pentanucleotide motifs at their replication origins which too exhibit great similarity (21). Nevertheless, both viruses show clear differences with respect to the host cells in which DNA replication can be initiated successfully. Indeed, this appears to be the basis for the
strict species specificity observed with SV40 and PyV. The former lytically infects only primate cells, the latter only murine cells (24). Whether this is a common factor in determination of species specificity of polyomaviruses in general remains to be seen but the similarity in replication mechanisms throughout this virus family would make it a likely possibility. Species specificity of replication can be reproduced in cell-free systems and this led to the demonstration that DNA polymerase $\alpha$-primase is the host factor which determines the species specificity of viral initiation of DNA replication of both SV40 and PyV (25-27). However, studies with hybrid DNA polymerase $\alpha$-primase complexes showed that the subunit requirement differs between these two viruses, PyV specifically needing p48 to be of murine origin and SV40 requiring a human p180 protein (28,29). In the case of PyV, further resolution was achieved with studies of DNA polymerase $\alpha$-primase complexes containing chimeric p48 subunits consisting of various combinations of human and murine sequences. It was concluded that a lesser conserved stretch of amino acids (residues 257-288) from the murine subunit was necessary for successful PyV DNA replication and it is likely that further study of the function of this region will shed light on the mechanism involved in this phenomenon (30,31). In this study we applied a similar approach in an attempt to determine which domains of human p180 are essential for initiation of SV40 DNA replication.

Initiation of DNA replication requires the interaction between various proteins at the origin of replication. Interactions between TAg and both RPA and DNA polymerase $\alpha$-primase have been shown to be important and SV40 TAg is known to bind the p180, p68, p58 and p48 subunits of DNA polymerase $\alpha$-primase independently (4,32-34). For human (H) p180 the site of interaction was mapped to an N-terminal stretch spanning residues 195 to 313 and it was shown that this interaction is required for the establishment of a functional initiation complex (35). This led us to investigate whether this interaction plays a part in the species specificity of SV40 DNA replication. To this end we constructed various chimeric p180 subunits which consist of regions derived from both the mouse and the human proteins. Using the baculovirus system these mutants
were expressed in complex with the other human DNA polymerase α-primase subunits and their activities were investigated in both a cell-free replication assay and in an initiation assay consisting of purified proteins only. In addition, we tested our mutant complexes for their ability to be stimulated by SV40 TAg in a system which assays coupled primer and DNA synthesis on single-stranded DNA (ssDNA) in the presence of RPA (32-34). We show that the N-terminal 488 amino acids of human p180, comprising the TAg binding domain, are not responsible for the species specificity of DNA replication nor for efficient stimulation of DNA polymerase activity by TAg on ssDNA. These results are supported by data obtained with surface plasmon resonance (BIAcore) which shows that SV40 TAg shows little difference in affinity for DNA polymerase α-primase from either human or murine origin.
Materials and Methods

Construction of baculoviruses expressing chimeric p180 proteins. For the construction of recombinant baculoviruses we used the Bac-to-Bac® system obtained from Invitrogen according to the manufacturer's protocol. Briefly, p180 wild-type and mutants were cloned into the plasmid pFastBac1® and this was introduced into the E. coli strain DH10Bac®. This strain harbours baculovirus DNA as a single copy F episome (Bacmid) as well as a plasmid encoding transposase. The insert in pFastBac1® is located on a Tn7 transposable element which can recombine in vivo with an acceptor site on the Bacmid resulting in recombinant baculovirus DNA for which there is a selection procedure. This DNA is recovered from the E. coli strain and transfected into Sf9 insect cells where it gives rise to recombinant baculoviruses expressing the desired protein.

pFB/Hp180 (36) was constructed by cloning an EcoRI-XbaI Hp180 cDNA fragment from pUC19/Hp180 into pFastBac1® digested with EcoRI-XbaI at the polylinker. pFB/Mp180 was constructed by cloning an Mp180 cDNA EcoRI-PstI fragment from pVL1393/Mp180 (37) into pFastBac1® digested with EcoRI-PstI. pFB/M257H and pFB/H257M were made by digesting pFB/Hp180 and pFB/Mp180 at the NcoI sites, located at the ATG start codon and internally, followed by reciprocal exchange and ligation of the resulting fragments. The orientation of the NcoI fragments in the resulting clones was checked by restriction analysis. pFB/H488M was created by digesting pFB/Hp180 and pFB/Mp180 with both EcoRI and PpuMI and ligating the 1513 bp fragment containing the 5'-end of the Hp180 gene onto the 3'-end of Mp180 in pFastBac. The reciprocal exchange resulting in pFB/M488H was made by first digesting pFB/Mp180 with EcoRI, followed by partial digestion with PpuMI and exchanging the resulting 1513 bp fragment with that of pFB/Hp180 fully digested with EcoRI and PpuMI.

To create pFB/H671M, the PflM I site at position 2011 in the Hp180 ORF (CCAAAGGCTTGG) was mutated by site directed mutagenesis to render it compatible
with the corresponding site in Mp180 (CCAAAACCTTG). The overlap extension PCR method (38) was used to introduce the mutation which is silent at the protein level. Modified pFB/Hp180 and pFB/Mp180 were then both fully digested with PflM I (both the Hp180 and Mp180 ORFs have additional PflM I sites at positions 809 and 3688, respectively, which are mutually incompatible and incompatible with the mutated site). pFB/H671M was produced by ligating the four resulting fragments. pFB/M1141H and pFB/H1141M were constructed by exchange of BamH I fragments from pFB/Hp180 and pFB/Mp180 using the internal site and the one located in the polylinker beyond the stop codon. pFB/M1395H and pFB/H1395M were produced by digesting pFB/Hp180 and pFB/Mp180 with EcoR I and AgeI, located before the start codon and internally at position 4196/4184 respectively, and exchanging the resulting fragments.

All constructs were transferred to baculovirus using the Bac-to-Bac® (Invitrogen) system, amplified in Sf9 insect cells and then used to infect High Five insect cells (ITC Biotechnology GmbH, Heidelberg) in order to test for expression of the desired proteins by Western Blotting.

**Proteins.** SV40 Tag, and the DNA polymerase α-primase complex (polymerase α-primase, p180-p68-p58-p48) were purified from baculovirus infected insect cells as described (28,37,39,40). Complexes containing hybrid p180 subunits were purified using monoclonal antibody SJK237-71 if they contained human sequence between amino acids 257 and 488 and otherwise monoclonal antibody SJK287-38 was used. During purification of hybrid complexes containg murine p68 the wash with 150 mM KCl was omitted.

RPA was bacterially expressed and purified as outlined before (41,42). Human topoisomerase I expressed in insect cells and purified as described by Søe et al. (43) was a generous gift of K. Søe, IMB, Jena. The monoclonal antibodies SJK237-71 and
SJK287-38 (44), specific for DNA polymerase α-primase, were purified by affinity chromatography (45).

**Protein manipulations.** Protein concentration was determined according to Bradford (46) using a commercial reagent with BSA as a standard. SDS gel electrophoresis was carried out as described (47) with 10 kDa ladders (Invitrogen) as molecular mass markers.

**Preparation of S100 extracts and replication of SV40 in vitro.** S100 extracts were prepared from logarithmically growing FM3A cells as previously described (29,37). Cells were harvested by centrifugation, then washed twice with PBS and once with hypotonic buffer. The cells were resuspended in hypotonic buffer, incubated for 10 min. on ice, and broken by 12 strokes in a Dounce homogenizer. The extracts were centrifuged at 4°C and 11,000 x g. The supernatant was then adjusted to 100 mM NaCl and clarified by a second centrifugation at 100,000 x g (S100 extract).

The replication of SV40 DNA *in vitro* was performed as previously described (28,37,39). Briefly, the assay contained 0.6 µg SV40 Tag, 250 ng of pUC-HS DNA (SV40 origin DNA (27)), and 200 µg S100 in 30 mM HEPES/NaOH (pH 7.8), 1 mM dithiothreitol, 7 mM magnesium acetate, 1 mM EGTA (pH 7.8), 4 mM ATP, 0.3 mM CTP, GTP, and UTP, 0.1 mM dATP and dGTP, 0.05 mM dCTP and dTTP, 40 mM creatine phosphate, and 80 µg/ml creatine kinase, and 5 µCi each of [α-32P]-dCTP and [α-32P]-dTTP (3000Ci/mmol, Amersham-Biosciences). DNA polymerase α-primase was added as indicated. The incorporation of radioactive dNMP was measured by acid-precipitation of DNA and scintillation counting. The total radioactivity was measured after spotting 5 µl of a 200-fold dilution of the replication assay onto GF52 filters (Schleicher & Schüll, Dassel, Germany).
Initiation of replication on SV40 DNA. Initiation reactions were performed essentially as previously described (12,28,39). Briefly, the SV40 initiation assay (40 µl) was assembled on ice and contained 0.25 µg of pUC-HS DNA (SV40 origin DNA), 0.6 µg of SV40 T antigen, and 0.5 µg of RPA, in 30 mM Heps-KOH (pH 7.8), 7 mM magnesium acetate, 1 mM EGTA, 1 mM dithiothreitol, 0.2 mM UTP, 0.2 mM GTP, 0.01 mM CTP, 4 mM ATP, 40 mM creatine phosphate, 1 µg of creatine kinase, 0.3 µg of topoisomerase I, 0.25 mg/ml heat treated BSA, and 20 µCi of [α-32P]CTP (3000 Ci/mmol, Amersham-Biosciences). Recombinant DNA polymerase α-primase was added as indicated in the figure legends. After incubation for 2 hr at 37°C 1/8th of the reaction mixture was used to estimate the amount of incorporated nucleotides by spotting it onto DE81 paper (48). The reaction products were precipitated with 0.8 M LiCl, 10 µg of sonicated salmon sperm DNA (Sigma), 10 mM MgCl2 and 120 µl of ethanol for 1 h on dry ice, washed twice with 75% ethanol-water, dried, redissolved in 45% formamide-5 mM EDTA-0.05% xylene cyanol FF-0.05% bromphenol blue at 65°C for 30 min., heated for 3 min. at 95°C, and electrophoresed in denaturing 20% polyacrylamide gels for 3 to 4 h at 600 V as described (27,28). The reaction products were visualised by autoradiography.

DNA synthesis on ϕX174 ssDNA with RPA and TAg. DNA synthesis was carried out according to Kautz et al. (30). Briefly, in an assay mixture (40 µl) containing 30 mM HEPES-KOH (pH 7.8); 7 mM MgAc; 0.1 mM EGTA; 1 mM dithiothreitol; 0.25 mg/ml BSA; 80 µg/ml creatine kinase; 40 mM creatine phosphate; 4 mM ATP; 0.2 mM (each) CTP, GTP and UTP; 0.1 mM (each) dATP, dGTP and dTTP; 0.002 mM dCTP; 0.5 µl of [α-32P]dCTP (specific activity, 3000 Ci/mmol; 10 µCi/µl; Amersham-Biosciences) and 250 ng (0.76 nmol of nucleotides) of ϕX174 ssDNA. Where appropriate this mixture was preincubated with 1 µg SV40 TAg for 2 min. on ice and then with 0.3–1.2 µg RPA for a further 5 min. on ice. The reaction was started by the addition of 100–400 ng (depending on specific activity) polymerase α-primase. After incubation for 90 min.
at 37°C, 10 µl of the reaction mixture was spotted onto GF-52 filters and precipitated in 10% trichloroacetic acid. The amount of DNA synthesis was measured by liquid scintillation counting.

**Biomolecular interaction analysis.** Interaction analysis was performed using the BIAcore 2000 apparatus from BIAcore AB (Freiburg; FRG) as previously described (34,49). Sensor chips CM5, surfactant P20 and the amine coupling kit were purchased from BIAcore AB. Antibodies were immobilised by amine coupling according to the supplier’s protocol. For a final ligand immobilisation yield of 1,000 relative resonance units (RU) about 1800 RU of the antibody was initially attached to the flow cell surface. The anti-polymerase α monoclonal antibody 2CT25 (50 µg/ml) were loaded at flow rate of 5 µl/min. in 0.03 M sodium acetate buffer, pH 5.0. The ligands, four subunit DNA polymerase α-primase, and the analyte, SV40 TAg, were microdialysed 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 8 to 100 µg/ml and crosslinked to the antibody by using the amino coupling kit. For the studies between 1,500 and 10,000 RU of the ligands were immobilised. Binding studies were usually performed with 5–70 µg/ml of SV40 TAg as an analyte at 25°C 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 non-specific binding. Data were collected at 1 Hz and analysed using the BIAevaluation programme 3.0.

**Results**

**Construction of hybrid p180 polypeptides.**

Human and murine p180 show 88% identity at the amino acid level and the corresponding similarity at the DNA level allowed us to construct exchange mutants between the two genes using conserved restriction sites (see Materials & Methods; Fig
1). Initially four mutants were constructed in which either part of or the entire TAg binding site was exchanged. The mutants were named H-X-M or M-X-H, where the first and last letters denote the origin (Human or Murine) of the N- and C-termini respectively and the number denotes the amino acid residue at the transition between human and murine sequences. These mutants were able to form complexes with the three smaller human DNA polymerase α-primase subunits (p48, p58 and p68) and furthermore, these complexes possessed both specific DNA polymerase and primase activities in the range of those of the wild-type, indicating that the exchanges had not disrupted the basic enzymatic functions of the various DNA polymerase α-primase complexes (Table 1, Fig 2). Indeed, all complexes could efficiently carry out coupled primer synthesis and elongation on ssDNA (data not shown).

The N-terminal 488 amino acids of Hp180 do not determine the species specificity of initiation of SV40 DNA replication.

The mutant protein complexes were compared with the the wild-type in both the cell-free DNA replication assay and in the initiation assay composed of purified proteins. As the various purified complexes show some variation in their specific enzymatic activities, equal levels of DNA polymerase units of each complex were compared in DNA replication assays. As shown in Fig. 3a, both (M257H)H3 and (M488H)H3 are active, albeit less so than H4, in the replication of DNA containing an SV40 replication origin to form a hemimethylated (Dpn I-resistant) product. In contrast, (H257M)H3 and (H488M)H3 are almost inactive. Therefore, the ability to replicate SV40 DNA appears not to be absolutely dependent upon the presence of an SV40 TAg binding site of human origin. This result was reproducible although absolute incorporation values varied between experiments due to the use of different batches of cell extracts and purified enzymes (TAg and RPA).

Although the specific DNA polymerase and primase activities of the various purified mutant and wild-type complexes are in the same range they do differ to some extent
To exclude the possibility that inactive protein present in purifications with low specific enzymatic activity is somehow acting as a (competitive) inhibitor in the replication assay we performed an experiment where replication inactive MH₃ was added to active (M257H)H₃. This did not result in any inhibition of SV40 replication activity (Fig. 3b).

Previous work demonstrated that the species-specific function of human p180 was executed at the initiation stage of DNA replication (27,29). To verify that this was again the case in these experiments the mutant complexes were tested in an initiation assay where the ability to form RNA primers at the replication origin is tested (Materials & Methods). In this case, equal levels of primase units were compared. Fig. 4 shows that the activity of the complexes in the DNA replication assay is reflected in the initiation assay. In conclusion, the region(s) of Hp180 which determine SV40 species specificity must be C-terminal of the TAg binding site.

Quantitation of the interactions between SV40 TAg and human DNA polymerase α-primase.

We used surface plasmon resonance to obtain quantitative data for the strength of interaction between SV40 TAg and human DNA polymerase α-primase, the p180-p68 subcomplex and p180 alone. The various proteins were immobilised with a monoclonal antibody, 2CT25, directed against p180 (50). SV40 TAg binds human p180 with an association constant \( K_A \approx 10^9 \text{ M}^{-1} \) (Table 2). Binding could only be detected if TAg was used as the analyte and was dependent upon the presence of Mg\(^{2+}\). The hetero-oligomeric complexes p180-p68 and p180-p68-p58-p48 did not display significantly greater binding constants than did p180 alone.

Interactions of SV40 TAg with both human and murine DNA polymerase α-primase are of the same order of magnitude.

Human DNA polymerase α-primase (H₄), murine DNA polymerase α-primase (M₄) and a hybrid enzyme complex (H₂M₂) consisting of the human two large subunits
combined with the murine primase subunits were immobilised on a BIAcore chip with the antibody 2CT25 and purified SV40 TAg was used as the analyte. Table 3 shows that the $K_A$ values for H4 are approximately 2 to 3 times as great as those for M4. Variations in the experimental conditions such as changing the temperature over the range of 25°C–37°C, the addition of 1 mM ATP or changes in the buffer system (HEPES or Tris-acetate) did not alter the relative binding of human and murine DNA polymerase $\alpha$ to SV40 TAg nor did the use of a different monoclonal antibody for immobilisation (data not shown).

**Construction of further p180 exchange mutants.**

So far we had mapped the region of human p180 necessary for SV40 DNA replication as being C-terminal of amino acid residue 488. As an attempt to define this region more precisely we created further reciprocal exchange mutants using conserved BamH I (aa 1141) and Age I (aa 1395) sites and a partially conserved PflM I (aa 671) site. The exchange mutants formed complexes with the human p68, p58 and p48 subunits which were active both in DNA polymerase assays on activated DNA and in primase assays (Fig. 2; Table 1). One mutant, M671H, proved to be unstable for unknown reasons and could not be purified. Fig. 5 shows the results of in vitro replication assays using DNA polymerase $\alpha$-primase complexes containing mutant subunits created from pairs of reciprocal exchanges. All complexes are partially active but the presence of murine sequences within the C-terminal 974 amino acids of p180 is deleterious to SV40 replication ([H1141M]H$_3$, [H1395M]H$_3$, [M1395H]H$_3$ and [H671M]H$_3$ [see Fig. 6A, columns 11 and 12]). Furthermore, the human C-terminal 321 amino acids contribute significantly towards SV40 replication activity but are insufficient for full activity ([M1141H]H$_3$). The exchanges made do not allow the clear definition of a single domain within p180 responsible for the species specificity of SV40 DNA replication, rather various sequences C-terminal of amino acid 488 contribute to activity either severally or in combination.
Influence of the murine p68 subunit on the SV40 replication activity of hybrid complexes.

The region of p180 which is necessary for SV40 replication encompasses the p68 binding site which has been shown to be located between residues 1275 and 1462 ([51] our unpublished data). Furthermore, binding of p68 to p180 induces a conformational change in the latter polypeptide ([51]). This prompted us to investigate whether the lack of activity of complexes containing a murine p180 C-terminus stems from a sub-optimal interaction of the mutant p180 subunit with human p68. Therefore, complexes were purified containing murine p68 and we investigated whether its presence could suppress the low replication activity of some of the mutant complexes. Fig. 6a shows that this is, to some extent, the case. The activities of the (H/Mp180)MH2 complexes are reproducibly higher than those of the corresponding (H/Mp180)H3 complexes. H671M complexes (lanes 11–14) show little effect of the p68 substitution, nevertheless this minor effect was reproducible with several independently purified batches of these complexes. Substitution of Mp68 for Hp68 in the human polymerase α-primase complex containing wild-type Hp180 did not enhance activity (Fig. 6b; [29]), indicating that a murine p68 binding site on p180 is required for the stimulatory effect of murine p68.

Interaction of SV40 TAg, RPA and DNA polymerase α-primase on ssDNA.

So far we have demonstrated species specificity of SV40 replication only on double-stranded DNA containing an SV40 origin of replication. A functional interaction between SV40 TAg, RPA and DNA polymerase α-primase has also been shown to occur on natural single-stranded DNA. Low concentrations of RPA inhibit the activity of DNA polymerase α-primase on M13 ssDNA and this inhibition can be relieved by the addition of SV40 TAg ([27,33,34,52,53]). We investigated how our hybrid DNA polymerase α-primase complexes would behave in this system in order to draw
conclusions concerning the requirements for a functional interaction between p180 and
SV40 TAg.

Fig. 7a shows the effect of RPA and SV40 TAg on the activity of human DNA
polymerase α-primase in a coupled priming and DNA synthesis assay on unprimed
single-stranded ϕX174 DNA. Increasing amounts of RPA reduced DNA synthesis by
DNA polymerase α-primase to about 22% (columns 2 and 5). Addition of 1 µg of TAg
completely reversed this inhibition (columns 6 and 9). We performed this assay with our
hybrid DNA polymerase α-primase complexes and found that all complexes efficiently
synthesised DNA in the absence of RPA (see also Table 1) and that all were inhibited
by the addition of RPA. However, the ability of TAg to reverse this inhibition varied
significantly between the complexes. Fig. 7b shows the maximal stimulation achieved
with each pair of reciprocal exchange mutants. Firstly, we observe that a complex
containing a murine p180 subunit, MH3, fails almost entirely to be stimulated by SV40
TAg and secondly that there exists among all complexes a qualitative correlation
between those that show a strong TAg mediated stimulation in this assay and those that
function well in the SV40 DNA replication assay on double-stranded DNA carrying an
SV40 origin of replication (Fig. 3 and 5). However, a difference between the two assays
is seen in the effect of murine p68. The complex (H1395M)MH2, which shows much
greater activity than (H1395M)H3 in the SV40 DNA replication assay (Fig. 6a, columns
15 to 18), is not subject to greater stimulation by SV40 TAg on ssDNA (Fig. 7b). This
probably reflects differences in the interactions between DNA polymerase α-primase
and SV40 TAg in the initiation complex and the simpler complex on ssDNA studied
here.
Discussion

Initiation of SV40 replication requires the formation of an active quaternary complex between DNA polymerase α-primase, TAg, RPA and the viral origin of replication. DNA polymerase α-primase interacts directly with each of the three other components of the complex and any of these interactions could, in principle, form the basis of the observed species specificity of DNA replication (4,5,34). However, the functional cooperation of RPA and DNA polymerase α-primase from mammalian origin has been shown not to be species-specific (27,29,54). Therefore, we concentrated on the functional interactions between SV40 TAg and DNA polymerase α-primase and, more specifically, on the p180 subunit known to control SV40 species specificity (29).

Since residues 295–313 of human p180 are involved in binding the viral TAg, and since the N-terminus of the protein exhibits slightly reduced conservation between the human and murine p180 subunits (35,37), we constructed mutants in which parts of the p180 N-termini were reciprocally exchanged between the human and murine counterparts. Although all mutants were capable of forming enzymatically active DNA polymerase α-primase (Fig. 2; Table 1), only those enzymes carrying sequences, which lie C-terminal of human amino acid residue 488, were capable of initiating DNA replication at an SV40 origin of replication (Figs. 3 and 4). This was a surprising result as it indicates that the interaction of p180 with SV40 TAg is not responsible for species specificity. However, this agrees with quantitative surface plasmon resonance data which show that SV40 TAg binds human DNA polymerase α-primase only two- to three-fold more strongly than the replication-incompetent murine analogue (Table 3). Since a ten-fold increase in the concentration of murine enzyme complex does not allow murine cell extracts to replicate SV40 DNA (data not shown), the measured difference in binding constants for the interaction of SV40 TAg with human versus murine proteins is insufficient to explain species specificity. Therefore, the mapped N-terminal TAg binding site of p180 (35) may be the primary site of interaction between the proteins; on the other hand, formation
of an active initiation complex may require additional interactions involving other regions of the DNA polymerase \(\alpha\)-primase. This notion has some experimental support as an N-terminal fragment of p180 can competitively inhibit formation of the initiation complex and thus DNA replication only during the first 15 min of the reaction (35). This indicates that the establishment of an active initiation complex may be a dynamic process where interactions of the p180 N-terminus may play an important role only during the initial recruitment of DNA polymerase \(\alpha\)-primase to the complex. The existence of an SV40 TAg binding site at the N-terminus of the murine protein has not been excluded by this study. This putative murine binding site may compensate for the absence of the human counterpart in our hybrid p180. Possibly secondary interaction sites, which only occur in a quaternary initiation complex and which are consequently not detected by binary interaction studies, may contribute to the initiation reaction.

Further exchange mutants indicate that the substitution of C-terminal p180 sequences with those of murine origin, especially beyond amino acid 1141, has an inhibitory effect on SV40 replication. This part of the polypeptide contains the DNA binding domain necessary for interaction with the replication template but is also involved in interactions with the p68 subunit, which has no known catalytic activity but induces a conformational change into p180 (1,2,51,55). We investigated whether the inhibitory effect of the murine p180 C-terminus on SV40 replication could be (in part) the consequence of a sub-optimal interaction between murine p180 and human p68. Substitution of murine p68 for its human counterpart shows that the interactions between p180 and p68 appear to contribute to species specificity to some extent, perhaps by changing the conformation of p180. Alternatively, since p68 itself binds SV40 TAg (32,34,56), active complex formation may require a particular coordination between the interactions of p180 and p68 with TAg, which might be disturbed when the two subunits are derived from different species. Abundant evidence exists that p68 acts as a regulator of DNA polymerase \(\alpha\)-primase (36,55-58) but the suppression of Mp180 C-terminus-induced inhibition of SV40 DNA replication by Mp68 is only partial and is not evident in
complexes such as (H671M)MH2 and M2H2 (Fig. 6a; 29). It appears therefore that multiple functions contribute to the species specific requirement for human p180 and that the sequences involved are spread over a large part of the p180 protein. This situation differs from that found in polyomavirus DNA replication, where a short defined stretch of Mp48 controls the species specificity and biochemical data in conjunction with protein structure predictions gave some insight into possible functions for this region (30). With p180 the situation is more complicated and therefore detailed conclusions concerning the mechanism underlying species specificity will very likely require resolution of the three dimensional structure of the initiation complex.

In addition to a species-specific effect of p180 on initiation of replication at the double-stranded SV40 origin we describe a similar effect on TAg-mediated stimulation of DNA polymerase α-primase on RPA-coated ssDNA. Although this system is not strictly species-specific and the mechanism behind stimulation of primer synthesis by TAg is not entirely understood, it requires specific interactions of TAg with multiple sites on several or all RPA and DNA polymerase α-primase subunits (27,30,53). Substitution of Mp180 for Hp180 prevents stimulation by SV40 TAg. The regions of Mp180 that inhibit this stimulation appear largely to coincide with those that prevent initiation of SV40 DNA replication (Fig. 3, 4, 5 and 7b). Since all tested DNA polymerase α-primase complexes are inhibited to comparable degrees by RPA, it appears that differences in the interactions of the DNA polymerase α-primase hybrids with TAg affect the levels of stimulation. Notwithstanding the fact that the protein-DNA complex under study here differs fundamentally from the initiation complex, we note the importance of the presence of human C-terminal regions for TAg-mediated stimulation and, moreover, the lack of a simple correlation between effective stimulation and the presence of the mapped N-terminal TAg binding site (35). This reinforces our conclusion that this region is not sufficient for the functional interactions of DNA polymerase α-primase with TAg and for this reason probably is not the determinant of SV40 species specificity.
References


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Figure legends

Fig. 1. Wild-type and mutant polymerase α polypeptides used in this study. Shaded and non-shaded regions are of human and murine origin, respectively. For details see Materials and Methods. At the top the sites of reported interaction of Hp180 with SV40 TAg, and with p68 are indicated (35,51). Roman numerals I–VI indicate regions of conservation among DNA polymerases of the B family (59). The TAg binding site is indicated in each construct as a box. Conserved restriction sites used for construction of the hybrids are shown at the corresponding fusion points in the protein.

Fig. 2. SDS-polyacrylamide gel electrophoresis of indicated purified DNA polymerase α-primase complexes consisting of human or hybrid p180 subunits and human p68, p58 and p48. Approximately 4 µg of each protein complex was loaded and the gel was stained with Coomassie Brilliant Blue. The additional protein band of approximately 55 kDa in lane 5 is probably the large chain of murine IgG eluting from the antibody resin.

Fig. 3. A. In vitro SV40 DNA replication assay with 0.25, 0.5 and 1.0 DNA polymerase units of the indicated DNA polymerase α-primase complexes. Enzyme activities were determined beforehand with a DNA polymerase assay on activated calf thymus DNA. Pairs of reciprocal exchange mutants are separated by vertical dashed lines. Standard deviations are indicated as error bars. B. In vitro SV40 DNA replication assay with 0.5 DNA polymerase units of (M257H)H3 in the absence (Column 2) and in the presence of increasing amounts of MH3 (0.25, 0.5 and 1.0 DNA polymerase).
Fig. 4. Autoradiogram of an in vitro SV40 DNA replication initiation assay with 0.2 primase units of various DNA polymerase α-primase complexes. Specific primase activities were determined beforehand with a primase assay on poly (dT). Lanes 1 and 2: control reaction with DNA polymerase α-primase (H4) lacking TAg and TAg but lacking DNA polymerase α-primase, respectively; Lanes 3 and 4: human (H4) and murine DNA polymerase α-primase (M4), respectively; lanes 5 to 9: the hybrid complexes MH3 (murine p180 (H257M)H3 in complex with three small human subunits), (M257H)H3, (H488M)H3, and (M488H)H3 (chimerical human-murine p180, for explanation see Fig. 1 together with three small human subunits), respectively. The approximate sizes of the reaction products are indicated on the right in nucleotides (nt).

Fig. 5. In vitro SV40 DNA replication assay with 0.5, 1.0 and 2.0 DNA polymerase units of the indicated DNA polymerase α-primase complexes. The activities shown are the average from three experiments performed with identical batches of cell extracts and RPA. Specific DNA polymerase activities were determined beforehand with a DNA polymerase assay on activated calf thymus DNA.

Fig. 6. In vitro SV40 DNA replication assay with DNA polymerase α-primase complexes. In the following experiments we compare the hybrid enzyme complexes containing all small subunits p68, p58 and p48 from human origin or containing the human primase subunits p58 as well as p48 but murine p68 (indicated by MH2). Specific DNA polymerase activities were determined beforehand with a DNA polymerase assay on activated calf thymus DNA. A. 0.5 and 1.0 DNA polymerase units of the indicated DNA polymerase α-primase complexes were added to murine cell extracts. Pairs of complexes differing in the nature of p68 are separated by vertical dashed lines. Standard deviations are indicated as error bars. B. In vitro SV40 DNA replication assay with 0.5 and 1.0 DNA polymerase units of human (H4) and two independent purifications of hybrid DNA polymerase α-primase, HMH2, consisting of murine p68,
human p180, p58 and p48. The mean of the incorporation values obtained with these purifications are shown and standard deviations are indicated as error bars.

**Fig. 7. A.** Stimulation of human DNA polymerase α-primase (H4) by TAg on ϕX174 ssDNA in the presence of RPA. 200 ng of human DNA polymerase α-primase H4 was added to the reaction mixture in the presence or absence of 1 µg SV40 TAg and increasing amounts of RPA (0, 0.6, 0.9 and 1.2 µg) as indicated. **B.** A summary of the results of experiments as in panel A performed with the human DNA polymerase α-primase H4 and the chimerical enzyme complexes MH3 (with murine p180 and the three small human subunits), (M257H)H3, (H257M)H3, (M488H)H3, (H488M)H3, (M1141H)H3, (H1141M)H3, (M1395H)H3, (H1395M)H3 and (H1395M)MH2 (with chimerical human-murine p180, for explanation see Fig. 1, and three small human subunits indicated as H3, or murine p68 together with human p58 as well as p48 indicated as MH2). The activity of each complex in the presence of 1.2 µg of RPA was set at an arbitrary value of 100% (horizontal dotted line). The degree of stimulation of DNA synthesis in the presence of 1.2 µg of RPA and 1 µg of SV40 TAg (this was the maximal stimulation obtained in each case) is shown for each complex as the mean value from three or more assays, standard deviations are indicated as error bars. Pairs of reciprocal exchange mutants are separated by vertical dashed lines.
Table 1. Specific DNA polymerase and primase activities of DNA polymerase α-primase complexes used in this study.

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<tr>
<td>H4</td>
<td>179</td>
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<td>M4</td>
<td>104</td>
<td>4400</td>
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<td>1700</td>
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<td>800</td>
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<tr>
<td>(H1395M)H3</td>
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<td>600</td>
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Table 2. Interactions between SV40 TAg and human DNA polymerase α-primase. Standard deviations were calculated from multiple experiments.

<table>
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<th>Ligand</th>
<th>k_{on} \left(10^5 \text{ M}^{-1}\text{s}^{-1}\right)</th>
<th>k_{off} \left(10^{-4} \text{ s}^{-1}\right)</th>
<th>K_A \left(10^9 \text{ M}^{-1}\right)</th>
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<tr>
<td>Hp180</td>
<td>1.6 ± 1.0</td>
<td>1.6 ± 0.4</td>
<td>1.0 ± 0.9</td>
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<td>Hp180•Hp68</td>
<td>2.4 ± 1.8</td>
<td>1.6 ± 0.6</td>
<td>1.5 ± 1.7</td>
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<tr>
<td>H4</td>
<td>2.3 ± 1.4</td>
<td>2.2 ± 1.1</td>
<td>1.0 ± 1.1</td>
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</tbody>
</table>

Table 3. Species specific interactions between SV40 TAg and human or murine DNA polymerase α-primase. Standard deviations were calculated from multiple experiments.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>k_{on} \left(10^5 \text{ M}^{-1}\text{s}^{-1}\right)</th>
<th>k_{off} \left(10^{-4} \text{ s}^{-1}\right)</th>
<th>K_A \left(10^8 \text{ M}^{-1}\right)</th>
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</thead>
<tbody>
<tr>
<td>H4</td>
<td>2.3 ± 1.4</td>
<td>2.2 ± 1.1</td>
<td>10 ± 1.1</td>
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<tr>
<td>H_2M_2</td>
<td>2.2 ± 1.3</td>
<td>4.8 ± 2.9</td>
<td>4.6 ± 5.5</td>
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<td>M4</td>
<td>1.4 ± 1.0</td>
<td>3.3 ± 0.4</td>
<td>4.2 ± 3.5</td>
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</tbody>
</table>
Smith et al., Figure 1
Smith et al., Figure 2
Smith et al., Figure 3
Incorporation (pmol dNMP)
Smith et al., Figure 4

-Tag
-Pol-Prim
H4
M4
MH3
(H257M)H3
(M257H)H3
(H488M)H3
(M488H)H3
Smith et al., Figure 5
Smith et al., Figure 6
Smith et al., Figure 6
Smith et al., Figure 7
Smith et al., Figure 7
Species specificity of Simian virus 40 DNA replication in vitro requires multiple functions of human DNA polymerase alpha
Richard W.P. Smith, Claudia Steffen, Frank Grosse and Heinz P. Nasheuer

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