

## Species-Specific Replication of Simian Virus 40 DNA In Vitro Requires the p180 Subunit of Human DNA Polymerase $\alpha$ -Primase

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**Human cell extracts efficiently support replication of simian virus 40 (SV40) DNA in vitro, while mouse cell extracts do not. Since human DNA polymerase  $\alpha$ -primase is the major species-specific factor, we set out to determine the subunit(s) of DNA polymerase  $\alpha$ -primase required for this species specificity. Recombinant human, mouse, and hybrid human-mouse DNA polymerase  $\alpha$ -primase complexes were expressed with baculovirus vectors and purified. All of the recombinant DNA polymerase  $\alpha$ -primases showed enzymatic activity and efficiently synthesized the complementary strand on an M13 single-stranded DNA template. The human DNA polymerase  $\alpha$ -primase (four subunits [HHHH]) and the hybrid DNA polymerase  $\alpha$ -primase HHMM (two human subunits and two mouse subunits), containing human p180 and p68 and mouse primase, initiated SV40 DNA replication in a purified system. The human and the HHMM complex efficiently replicated SV40 DNA in mouse extracts from which DNA polymerase  $\alpha$ -primase was deleted, while MMMM and the MMHH complex did not. To determine whether the human p180 or p68 subunit was required for SV40 DNA replication, hybrid complexes containing only one human subunit, p180 or p68, together with three mouse subunits (HHMM and MHMM) or three human subunits and one mouse subunit (MHHH and HMHH) were tested for SV40 DNA replication activity. The hybrid complexes HHMM and HMHH synthesized oligoribonucleotides in the SV40 initiation assay with purified proteins and replicated SV40 DNA in depleted mouse extracts. In contrast, the hybrid complexes containing mouse p180 were inactive in both assays. We conclude that the human p180 subunit determines host-specific replication of SV40 DNA in vitro.**

Replication of the DNA of the papovaviruses simian virus 40 (SV40) and polyomavirus (Py) has served as an excellent model system for eukaryotic DNA replication, since it requires only one viral protein, the multifunctional large T antigen, while the other replication proteins are supplied by the host (8, 26, 28, 31, 36, 61, 65). Use of the in vitro SV40 DNA replication system allowed the identification and purification of essential cellular replication proteins (8, 28, 37, 65, 72, 73). Reconstitution of complete SV40 DNA replication requires 10 cellular proteins or protein complexes (28, 72, 73).

SV40 and Py use similar strategies to replicate in their hosts. Nevertheless, SV40 propagates only in primate cells, while Py multiplies only in mouse cells (67). Their core origins of DNA replication determine the host specificity of viral DNA replication in vivo, while the flanking auxiliary regions of the origin are not involved in species specificity (1). Biochemical studies of SV40 and Py DNA replication revealed that the host specificity could be reproduced in vitro and that DNA polymerase  $\alpha$ -primase was the species-specific factor in both viral DNA replication systems (4, 21, 37, 44, 46, 48, 58, 59). In the pres-

ence of human DNA polymerase  $\alpha$ -primase, mouse S100 extracts supported replication of SV40 DNA in vitro and mouse DNA polymerase  $\alpha$ -primase allowed Py DNA replication in human cell extracts (4, 21, 44, 46, 48, 58, 59). With purified proteins, the initiation of DNA replication was determined to be the species-specific reaction in both viral systems (4, 58).

Detailed studies of partial reactions have led to a model for initiation of SV40 and Py DNA replication (13, 14, 64, 69, 74, 75, 78). First, the viral T antigen recognizes specific origin DNA sequences and forms double hexamers in an ATP-dependent manner (13, 14, 40). Then, T antigen's intrinsic helicase activity unwinds duplex DNA in the presence of RP-A, the eukaryotic single-stranded DNA (ssDNA)-binding protein. Topoisomerase I is required to release topological stress introduced during unwinding, but it is not part of the initiation complex. In the subsequent step, DNA polymerase  $\alpha$ -primase synthesizes the primer RNA molecules on the origin of DNA replication, and DNA polymerase  $\alpha$  elongates them (24, 29, 76, 77). Then, this RNA-DNA molecule is recognized by DNA replication factor C (RF-C) and proliferating cell nuclear antigen, facilitating the assembly of the leading-strand DNA replication complex containing in addition DNA polymerase  $\delta$  for synthesizing the leading strand (23, 34, 35, 55, 68–71, 76). In the reconstituted SV40 DNA replication system, the Okazaki fragments on the lagging strand are also synthesized by DNA polymerase  $\alpha$ -primase and the leading-strand complex (72, 73, 76). A third DNA polymerase may also be involved in the lagging-strand synthesis (6, 52, 53, 68, 72, 73).

During the initiation reaction, the recruitment of the cellular initiation proteins to the viral origin of replication depends on a network of essential protein-protein interactions of the viral

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T antigen with DNA polymerase  $\alpha$ -primase and RP-A (16, 25, 56). The DNA polymerase  $\alpha$ -primase interacts directly with Py and SV40 T antigen (4, 20, 27, 44, 58, 60). Detailed biochemical studies showed that the viral large T antigens bind independently to the p180, p68, and p48 subunits (4, 10, 20). However, these physical interactions of DNA polymerase  $\alpha$ -primase do not seem to be sufficient to control the species specificity of papovavirus DNA replication, since purified Py and SV40 T antigen interact equally well with purified DNA polymerase  $\alpha$ -primase from different mammalian organisms (4, 5, 58). The DNA polymerase  $\alpha$ -primase interacts physically with RP-A; the isolated p70 subunit of RP-A was shown to bind to DNA polymerase  $\alpha$ -primase and primase heterodimer immobilized in the sandwich enzyme-linked immunosorbent assay, and purified RP-A complex in solution interacted with p48 immobilized on a blot (19, 51). The functional relevance of these protein-protein interactions has been demonstrated by biochemical and genetic methods (10, 32, 38, 39, 42, 58, 59, 71). At low concentrations, RP-A stimulated DNA polymerase activity on synthetic templates, but high concentrations of RP-A inhibited enzyme activities (71). The functional interaction of the three initiation components was also detected in DNA synthesis on M13 ssDNA coated with mammalian RP-A by DNA polymerase  $\alpha$ -primase, since T antigen could stimulate DNA polymerase  $\alpha$ -primase on this template (10, 42, 58). For this reaction, the physical binding of SV40 T antigen to mammalian RP-A seems to be required, since the failure of yeast RP-A to support both reactions correlated with its inability to bind to SV40 T antigen in vitro (42).

To identify the subunit(s) of DNA polymerase  $\alpha$ -primase responsible for species specificity in SV40 DNA replication, we purified hybrid human-mouse DNA polymerase  $\alpha$ -primase complexes and tested them in *in vitro* SV40 DNA replication assays. Here, we present evidence that all of the recombinant hybrid human-mouse enzyme complexes were functional, since they synthesized DNA on an M13 ssDNA template. The hybrid complex HHMM, containing the human p180 and p68 and mouse p58 and p48 subunits, efficiently supported replication of SV40 DNA in vitro. Detailed studies showed that human p180-DNA polymerase  $\alpha$  determined the species specificity of SV40 DNA replication in a purified system and in crude replication extracts.

## MATERIALS AND METHODS

**Protein manipulations.** Protein concentrations were determined according to the method described by Bradford (3) with a commercial reagent with bovine immunoglobulin G as a standard (Bio-Rad, Munich, Germany). Sodium dodecyl sulfate (SDS)-gel electrophoresis was carried out as described elsewhere (33) with prestained molecular weight marker proteins (Sigma, Deisenhofen, Germany). After polyacrylamide gel electrophoresis, proteins were detected either by staining with Coomassie brilliant blue or by Western blot (immunoblot) analysis as described previously (63). The amount of each DNA polymerase  $\alpha$ -primase subunit was determined by microdensitometry of the corresponding Coomassie brilliant blue-stained protein band with a scanner from Cybertech (Berlin, Germany).

**Protein purification.** Expression and purification of DNA polymerase  $\alpha$ -primase were performed as previously described (4, 58, 63). The DNA polymerase  $\alpha$ -primase complexes were purified by immunoaffinity chromatography with monoclonal antibodies SJK237-71 and SJK287-38, which recognize the p180 subunit (66). DNA polymerase  $\alpha$  assays and DNA primase assays were performed as previously described (49, 50, 63). The specific activities of the recombinant DNA polymerase  $\alpha$ -primase complexes varied from 3,000 to 6,000 DNA polymerase U per mg and from 1,650 to 3,100 primase U per mg (Table 1).

Bovine RP-A was purified as previously described (51). Topoisomerase I was a generous gift from I. Moarefi (43). SV40 T antigen was expressed in SF9X cells (63) infected with recombinant baculovirus and was purified by immunoaffinity chromatography as previously described (43). Py T antigen was expressed by using the baculovirus expression vector vEV51PyT (57) and was purified according to the method described by Brückner et al. (4).

**DNA replication of M13 ssDNA.** The DNA replication of M13 ssDNA was

TABLE 1. Purified, recombinant DNA polymerase  $\alpha$ -primase complexes

Complex	Total activity (U)		Amt of total protein (mg)	Sp act (U/mg)	
	DNA polymerase $\alpha$	Primase		DNA polymerase $\alpha$	Primase
HHHH	1,800	500	0.30	6,000	1,670
HMHH	1,000	580	0.35	2,860	1,660
MHHH	1,250	805	0.26	4,810	3,100
MMHH	900	850	0.30	3,000	2,830
HMMM	635	300	0.18	3,530	1,670
MHMM	975	700	0.21	4,640	3,330
HHMM	940	600	0.22	4,270	2,730
MMMM	1,550	1,200	0.40	3,870	3,000

carried out with a reaction mixture containing 66 ng of M13 ssDNA (Pharmacia, Freiburg, Germany), 20 mM Tris-acetate (pH 7.3), 5 mM magnesium acetate, 20 mM potassium acetate, 1 mM dithiothreitol, 0.1 mg of bovine serum albumin (BSA) per ml, 1 mM ATP, 0.1 mM CTP, GTP, UTP, dATP, dCTP, dGTP, and 0.1 mM [ $\alpha$ - $^{32}$ P]dCTP (100 cpm/pmol). Radioactive nucleotides were purchased from ICN, Meckenheim, Germany; the unlabeled nucleotides were ultrapure grade from Pharmacia. Comparisons between different DNA polymerase  $\alpha$ -primase preparations were made by adding 0.2 U of primase per assay. The incorporation of dCMP was determined by acid precipitation of DNA and scintillation counting.

**Preparation of S100 extracts and viral DNA replication in vitro.** S100 extracts were prepared from logarithmically growing mouse FM3A cells as previously described (4, 58). SV40 DNA was replicated *in vitro* as described elsewhere, with minor modifications (58). Briefly, the SV40 assay mixture (60  $\mu$ l) contained 0.6  $\mu$ g of SV40 T antigen, 0.25  $\mu$ g of SV40 origin DNA (pUC-HS), 0.6  $\mu$ g of bovine RP-A, 300  $\mu$ g of S100 or depleted S100 extract from FM3A cells in 30 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES)-KOH (pH 7.8)–0.5 mM dithiothreitol–7 mM magnesium acetate–1 mM ethylene glycol-bis( $\beta$ -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (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–80  $\mu$ g of creatine kinase per ml–5  $\mu$ Ci each of [ $\alpha$ - $^{32}$ P]dCTP and [ $\alpha$ - $^{32}$ P]dTTP (3,000 Ci/mmol). Comparisons between different DNA polymerase  $\alpha$ -primase preparations were made by adjusting the amounts of enzyme added to equal the primase activities as indicated. Primase activities were determined when the replication assays were performed.

The reactions were stopped by the addition of SDS, EDTA, and proteinase K so that the final concentrations were 0.7% SDS, 40 mM EDTA, and 0.2 mg of proteinase K per ml. After extraction with phenol-chloroform, 15  $\mu$ g of yeast tRNA (Sigma) was added to the solution, which was adjusted to 0.6 M ammonium acetate, and the DNA was precipitated by the addition of 300  $\mu$ l of ethanol. The DNA synthesis products (10% of the total amount) were digested with 10 U each of *Eco*RI and *Dpn*I and then analyzed by agarose gel electrophoresis and autoradiography. The incorporation of radioactive deoxynucleoside monophosphate (dNMP) was measured by acid precipitation of DNA and scintillation counting.

**Initiation of replication on SV40 and Py DNA.** Initiation reactions were performed essentially as previously described (4, 41, 45, 58). Briefly, the SV40 initiation assay mixture (40  $\mu$ l) was assembled on ice and contained 0.25  $\mu$ g of pUC-HS DNA (SV40 origin DNA), 0.6  $\mu$ g of SV40 T antigen, and 0.5  $\mu$ g of RP-A in 30 mM HEPES-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  $\mu$ g of creatine kinase–0.3  $\mu$ g of topoisomerase I–0.2 mg of heat-treated BSA per ml, and 20  $\mu$ Ci of [ $\alpha$ - $^{32}$ P]CTP (3,000 Ci/mmol; ICN). Recombinant DNA polymerase  $\alpha$ -primase was added as indicated in the figure legends. Py initiation reactions (40- $\mu$ l mixtures) were carried out as described above, but the mixtures contained 0.25  $\mu$ g of pUC-Py1 DNA (Py origin DNA [58]), 1.6  $\mu$ g of Py T antigen, and 1  $\mu$ g of RP-A.

The reaction products were precipitated with 0.8 M LiCl, 10  $\mu$ g of sonicated salmon sperm DNA (Sigma), and 120  $\mu$ l of ethanol for 15 min on dry ice; washed twice with 75% ethanol-water; dried; redissolved in 45% formamide–5 mM EDTA–0.09% xylene cyanol FF–0.09% bromophenol 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 elsewhere (4, 58). The reaction products were visualized by autoradiography.

## RESULTS

**Recombinant human-mouse hybrid DNA polymerase  $\alpha$ -primase complexes efficiently synthesize DNA on M13 ssDNA.** To study the species specificity of SV40 and Py DNA replication, we purified recombinant hybrid DNA polymerase  $\alpha$ -primase

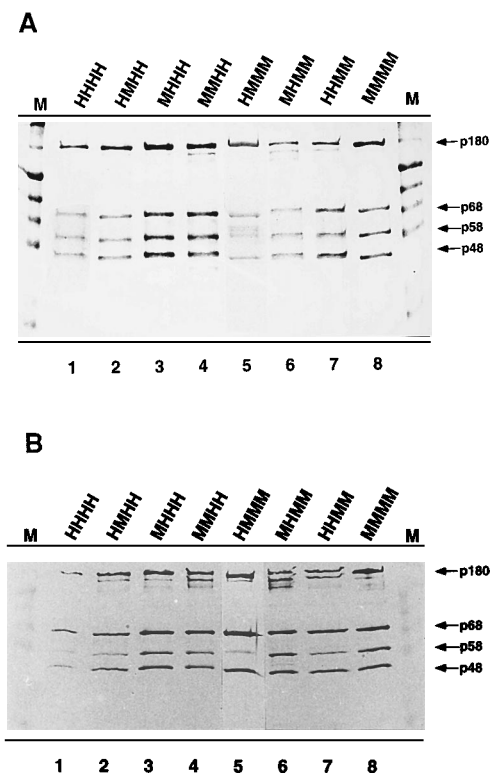


FIG. 1. Purified hybrid human-mouse DNA polymerase  $\alpha$ -primase complexes. (A) A 3- $\mu$ g amount of each enzyme complex analyzed by SDS-gel electrophoresis and then by Coomassie brilliant blue staining. (B) A 1.5- $\mu$ g amount of each DNA polymerase  $\alpha$ -primase complex separated by SDS-gel electrophoresis and then analyzed by Western blotting with polyclonal antiserum against bovine DNA polymerase  $\alpha$ -primase. Lanes: 1, four subunit human DNA polymerase  $\alpha$ -primase (HHHH); 2, hybrid enzyme complex containing human p180, mouse p68, human p58, and human p48 (HMHH); 3, hybrid enzyme complex containing mouse p180, human p68, human p58, and human p48 (MHHH); 4, hybrid enzyme complex containing mouse p180, mouse p68, human p58, and human p48 (MMHH); 5, hybrid enzyme complex containing human p180, mouse p68, mouse p58, and mouse p48 (HMMM); 6, hybrid enzyme complex containing mouse p180, human p68, mouse p58, and mouse p48 (MHMM); 7, hybrid enzyme complex containing human p180, human p68, mouse p58, and mouse p48 (HHMM); 8, four-subunit mouse enzyme complex (MMMM); M, prestained, high-molecular-weight marker. The HMMM preparation was analyzed separately from the other enzymes.

complexes containing subunits of mouse and human origin from baculovirus-infected insect cells and studied their activity in viral DNA replication *in vitro*. Most of the recombinant DNA polymerase  $\alpha$ -primase complexes were purified to near homogeneity in high yields (Fig. 1A and B; Table 1). These purified complexes were named after the sources of their subunits in order of decreasing molecular weight (first position, p180 the DNA polymerase catalytic subunit; second position, p68; third position, p58; and fourth position, the catalytic primase subunit p48). The purified enzyme complexes showed different degrees of proteolytic degradation of the p180 subunit, but they had similar specific DNA polymerase activities (Fig. 1A and B; Table 1).

Only the hybrid enzyme HMMM, which contains the human subunit p180 and the three smaller mouse subunits p68, p58, and p48, was difficult to purify. In five independent attempts, HMMM complexes consistently contained lower amounts of the primase p58 subunit (Fig. 1A; compare lane 5 with lanes 1 to 4 and 6 to 8), although the immunoaffinity column for purifying HMMM was less stringently washed than those for purifying the other enzymes. The subunit composition of the

HMMM complex that was determined by microdensitometry of a Coomassie brilliant blue-stained SDS gel was 1:1.8:1:2.3 ([p180]:[p68]:[p58]:[p48]), while for comparison the average subunit composition of the other recombinant DNA polymerase  $\alpha$ -primase complexes was 1:1.5:2.2:2.6. By Western blotting, all four subunits were detectable in the HMMM complex, as in the other purified DNA polymerase  $\alpha$ -primase complexes (Fig. 1B). However, the HMMM complex contained two polypeptides that ran slightly more slowly than the p58 subunit in SDS gels. These proteins were most likely contaminating insect proteins, since they were not recognized by the polyclonal antiserum produced against bovine DNA polymerase  $\alpha$ -primase (Fig. 1B, lane 5). The specific DNA polymerase activity and DNA primase activity of the HMMM complex were comparable to those of the other recombinant enzymes (Table 1).

To study whether the primase and DNA polymerase activities of the recombinant enzyme complexes acted in a coordinated manner during DNA synthesis, we tested their ability to synthesize DNA on an M13 ssDNA template. All of the purified enzymes replicated M13 ssDNA (Fig. 2). The incorporation varied from 25 to 81 pmol of dCMP, although the amounts of DNA polymerase  $\alpha$ -primase used in the assay were adjusted to contain equal primase activities. The four-subunit mouse enzyme, HMHH, and the HHMM DNA polymerase  $\alpha$ -primase showed the highest replication activity (Fig. 2). The HMMM complex efficiently synthesized DNA on an M13 ssDNA template, and the incorporation of dCMP (32 pmol) was comparable to that of the recombinant human DNA polymerase  $\alpha$ -primase (25 pmol; Fig. 2).

**The hybrid DNA polymerase  $\alpha$ -primase HHMM initiates SV40 DNA replication in a species-specific manner.** The species specificity of Py DNA replication is mediated by mouse primase (4, 21). Initial results from initiation assays of SV40 origin DNA *in vitro* suggested that the human primase may also be involved in the species specificity of SV40 DNA replication. The HHMM complex synthesized oligoribonucleotides on SV40 origin DNA in a system containing only purified proteins, while HHHM did not (4).

First, we compared the initiation activity of a hybrid HHMM

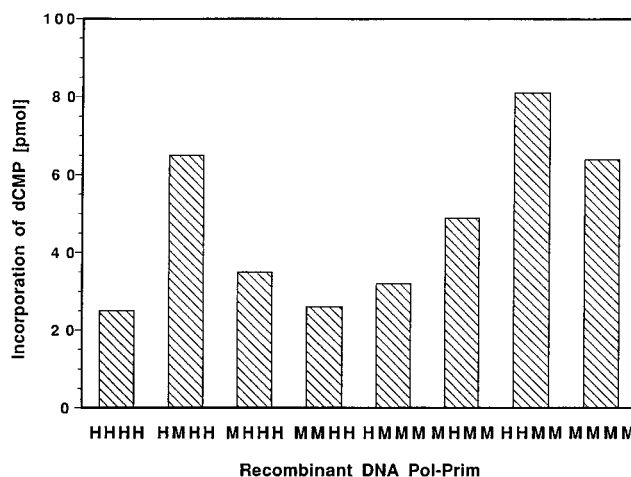


FIG. 2. DNA synthesis on M13 ssDNA template by hybrid DNA polymerase  $\alpha$ -primase complexes. The recombinant four-subunit human, mouse, and hybrid DNA polymerase  $\alpha$ -primases (0.2 U of primase per incubation) were incubated with M13mp18 ssDNA (30  $\mu$ M) in the presence of 20 mM potassium acetate and four deoxynucleotides and four ribonucleotides (Materials and Methods). After 60 min, incorporation of acid-precipitable dCMP in M13 DNA by DNA polymerase  $\alpha$ -primase was determined.



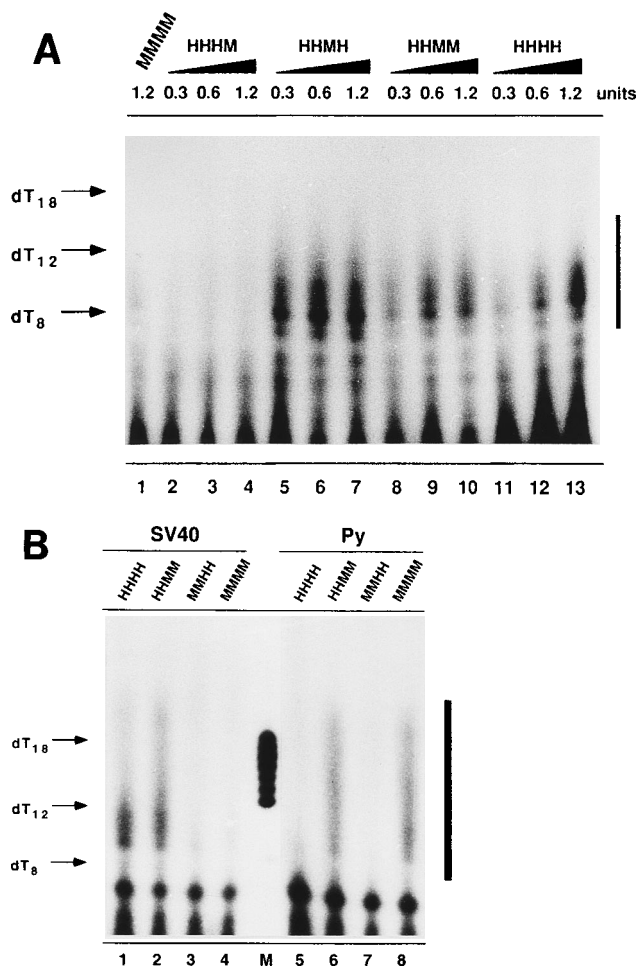


FIG. 3. Initiation of SV40 and Py DNA replication by recombinant DNA polymerase  $\alpha$ -primase. (A) The SV40 initiation activities of increasing amounts of HHHM (lanes 2 to 4), HHMH (lanes 5 to 7), and HHMM (lanes 8 to 10), determined by primase units, compared with the activity of recombinant human DNA polymerase  $\alpha$ -primase (HHHH [lanes 11 to 13]). The recombinant mouse DNA polymerase  $\alpha$ -primase, served as a negative control (MMMM [lane 1]). (B) SV40 initiation assays (lanes 1 to 4) and Py initiation reactions (lanes 5 to 8) were performed with 1.2 U of primase activity of DNA polymerase  $\alpha$ -primase to synthesize RNA primers on the viral origin of DNA replication. Lane M, 5'-end-labeled oligo(dT)<sub>8</sub> and oligo(dT)<sub>12-18</sub>. The marker lengths are indicated at the left by arrows. The bar at the right side of the figure marks the primase products.

complex to those of the HHHH, HHMH, HHHM, and MMMM complexes. The initiation reaction was dependent on a viral origin, viral T antigen, and DNA polymerase  $\alpha$ -primase (data not shown). The HHMM and HHMH complexes were as active as or slightly more active than human DNA polymerase  $\alpha$ -primase (Fig. 3A; compare lanes 8 and 9 and lanes 5 and 6 with lanes 11 to 13), while the mouse DNA polymerase  $\alpha$ -primase and HHHM complex were unable to initiate SV40 DNA replication (Fig. 3A, lanes 1 to 4). Two preparations each of HHMH and HHHM and three independently expressed and purified HHMM complexes gave similar results (data not shown). To address the question of whether initiation of SV40 DNA replication requires human primase subunits, the purified MMHH complex was tested in the initiation assay. The positive control, human DNA polymerase  $\alpha$ -primase, and the hybrid HHMM complex were active in SV40 initiation, while mouse DNA polymerase  $\alpha$ -primase was not (Fig. 3B, lanes 1,

2, and 4, respectively). In contrast to the recombinant human HHHH and the hybrid HHMM DNA polymerase  $\alpha$ -primases, the hybrid complex MMHH did not initiate SV40 DNA replication in vitro (Fig. 3B; compare lane 3 with lanes 1 and 2), and the radioactive material observed in Fig. 3B, lane 3 (MMHH complex) was identical to that in Fig. 3B, lane 4 (mouse DNA polymerase  $\alpha$ -primase). These results suggested that the human primase subunits were not the main determinant of species specificity in the SV40 system and that the two large subunits of human DNA polymerase  $\alpha$ -primase controlled the species specificity of initiation of SV40 DNA replication.

To confirm and extend earlier results that mouse primase is required for initiation of Py DNA replication (4), we used the Py system as a control and tested the hybrid DNA polymerase  $\alpha$ -primase complexes in Py initiation. The mouse DNA polymerase  $\alpha$ -primase and the HHMM complex initiated Py DNA replication to similar extents (Fig. 3B, lanes 6 and 8), while human DNA polymerase  $\alpha$ -primase and the MMHH complex were inactive in Py initiation (Fig. 3B, lane 5 and 7), despite the high specific primase and DNA polymerase activities of MMHH and its activity in M13 DNA replication (Table 1; Fig. 2). These data show that the MMHH complex was inactive in both viral initiation reactions in vitro, while the HHMM complex initiated both SV40 and Py DNA replication.

**SV40 DNA replication in vitro by hybrid DNA polymerase  $\alpha$ -primase HHMM.** The results of the initiation assays raised the question of whether human p180 and p68 were also responsible for the species specificity of SV40 DNA replication in vitro in mouse extracts.

In preparation for these experiments, we optimized the SV40 DNA replication assay conditions in mouse extracts. The addition of human DNA polymerase  $\alpha$ -primase supported DNA synthesis of a plasmid DNA (Fig. 4) containing an SV40 origin in a T-antigen-dependent fashion (data not shown). The incorporation of radioactive dNMP into the SV40 DNA was about threefold of that observed with mouse DNA polymerase  $\alpha$ -primase (Fig. 4A; compare the bar without RP-A [left half] with the bar without RP-A [right half]). However, the newly synthesized SV40 DNA was not resistant against digestion with *DpnI* (Fig. 4B, lane 2), although these depleted mouse S100 extracts efficiently replicated Py DNA in vitro in the presence of mouse DNA polymerase  $\alpha$ -primase (data not shown). To achieve efficient SV40 DNA replication in mouse cell extracts, additional bovine RP-A, topoisomerase I, or SV40 T antigen was titrated into the replication assay mixture (data not shown). Only the addition of RP-A increased the incorporation of dNMPs and generated *DpnI*-resistant replication products, which were readily detected even at the lowest RP-A concentration (Fig. 4B, lane 4). In contrast, RP-A did not stimulate SV40 DNA synthesis by mouse DNA polymerase  $\alpha$ -primase (Fig. 4A and B, lanes 11 to 20). These data suggested either that the FM3A extracts prepared following standard procedures (44, 46, 58) contained too little RP-A for SV40 DNA replication in vitro or that the endogenous mouse RP-A was only partially active in SV40 DNA replication. Therefore, all DNA replication mixtures used to study SV40 DNA replication with FM3A cell extracts were subsequently supplemented with bovine RP-A.

However, even in the presence of bovine RP-A, a significant fraction of the SV40 DNA replication products was only 400 to 1,500 bp in size, smaller than the 3,210 bp of full-length pUC-HS plasmid (Fig. 4B, lanes 1 to 10). Although we do not understand the reasons for the incomplete replication, the synthesis of these products was dependent on human DNA polymerase  $\alpha$ -primase (Fig. 4B; compare lanes 1 to 10 with

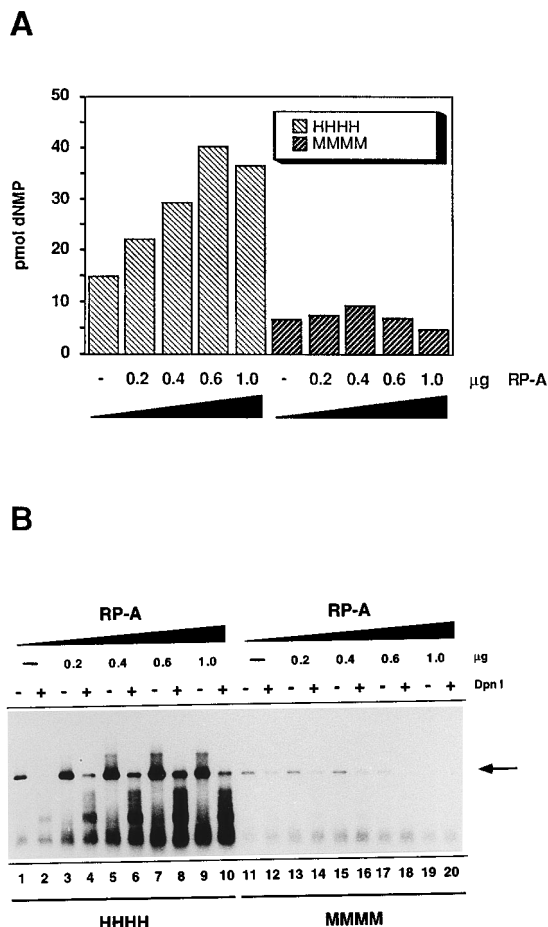


FIG. 4. Stimulation of SV40 DNA replication in vitro by RP-A. Mouse S100 extracts depleted of DNA polymerase  $\alpha$ -primase were supplemented with increasing amounts of bovine RP-A (0.2, 0.4, 0.6, and 1  $\mu$ g), and SV40 DNA replication in vitro was assayed in the presence of recombinant human (HHHH) or mouse (MMMM) DNA polymerase  $\alpha$ -primase (0.8 U of primase in each assay). (A) Incorporation of dNMPs into DNA containing the SV40 origin in the presence of human (left half) or mouse (right half) recombinant DNA polymerase  $\alpha$ -primase was measured by acid precipitation. (B) Analysis of products synthesized in the presence of human (HHHH) or mouse (MMMM) DNA polymerase  $\alpha$ -primase by digestion with *DpnI*. Equal amounts of reaction products were linearized with 10 U of *EcoRI* (odd-numbered lanes) or digested with 10 U each of *EcoRI* and *DpnI* (even-numbered lanes) for 4 h under conditions suggested by the manufacturer. The arrow at the right side of the figure points to the linearized DNA synthesis products.

lanes 11 to 20), T antigen, and the SV40 origin of replication (data not shown), and they were not observed in parallel replication experiments using Py DNA in mouse FM3A cell extracts or SV40 DNA in human 293S cell extracts (data not shown).

After optimizing the assay conditions, the human DNA polymerase  $\alpha$ -primase efficiently supported the replication of SV40 DNA in DNA polymerase  $\alpha$ -primase-depleted mouse extracts, and the incorporation of dNMPs rose 20- to 40-fold above the background upon addition of human DNA polymerase  $\alpha$ -primase (Fig. 5A). *DpnI*-resistant replication products were detected even with the lowest concentration of human DNA polymerase  $\alpha$ -primase (Fig. 5B, lanes 6, 8, and 10). The negative control, mouse DNA polymerase  $\alpha$ -primase, did not support SV40 DNA replication in the depleted mouse extracts, and no *DpnI*-resistant replication products were detectable (Fig. 5A and B, lanes 24, 26, and 28). The hybrid DNA poly-

merase  $\alpha$ -primase HHMM was as active as the human DNA polymerase  $\alpha$ -primase in the replication of SV40 DNA (Fig. 5A); the amounts of *DpnI*-resistant replication products synthesized by the HHMM complex (Fig. 5B, lanes 12, 14, and 16) were comparable to those of the human DNA polymerase  $\alpha$ -primase (Fig. 5B, lanes 6, 8, and 10), confirming the results obtained by the initiation assay.

In contrast with these results, the hybrid enzyme MMHH showed little or no SV40 replication activity in depleted mouse extracts (Fig. 5A and B, lanes 17 to 22), and its activity was comparable to that of the negative control (Fig. 5A and B, lanes 23 to 28).

**Human p180 DNA polymerase  $\alpha$  is essential for SV40 DNA replication in vitro.** The activity of DNA polymerase  $\alpha$ -primase containing only human p180 and p68 suggested that one or both of these subunits could be essential for SV40 DNA replication in vitro. To pursue this question, hybrid DNA polymerase  $\alpha$ -primase complexes containing only one mouse subunit (either p180 or p68 [MHHH and HMHH]) or only one human subunit (either p180 or p68 [HHMM and MHMM]) were purified to near homogeneity from insect cells coinfecting with recombinant baculoviruses (Fig. 1; Table 1).

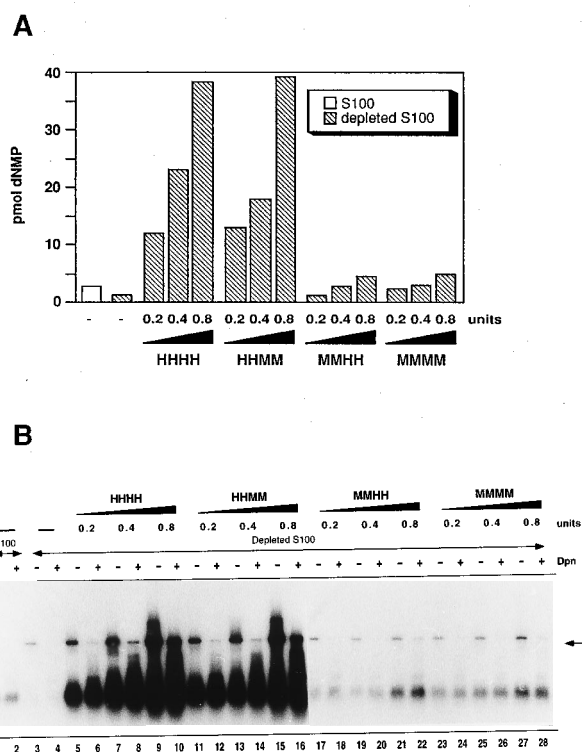


FIG. 5. Species-specific replication of SV40 DNA by hybrid DNA polymerase  $\alpha$ -primase (HHMM). Increasing amounts (0.2, 0.4, and 0.8 U of primase as indicated) of recombinant human (HHHH), mouse (MMMM), or hybrid DNA polymerase  $\alpha$ -primase (HHMM or MMHH) were added to depleted S100 mouse FM3A extracts supplemented with 0.6  $\mu$ g of RP-A. (A) Incorporation of dNMPs into SV40 DNA. The addition of recombinant DNA polymerase  $\alpha$ -primase was as indicated. (B) DNA synthesis products from panel A were analyzed for complete DNA replication by digestion with 10 U each of *EcoRI* and *DpnI* (even-numbered lanes). In parallel, the products were linearized with 10 U of *EcoRI* (odd-numbered lanes). Lanes: 1 and 2, undepleted mouse extracts; 3 and 4, depleted mouse extracts; 5 to 10, depleted mouse extracts plus human DNA polymerase  $\alpha$ -primase (HHHH); 11 to 16, depleted mouse extracts plus hybrid complex HHMM; 17 to 22, depleted mouse extracts plus hybrid complex MMHH; 23 to 28, depleted mouse extracts plus mouse enzyme (MMMM). The arrow at the right side of the figure indicates the linearized DNA synthesis products.

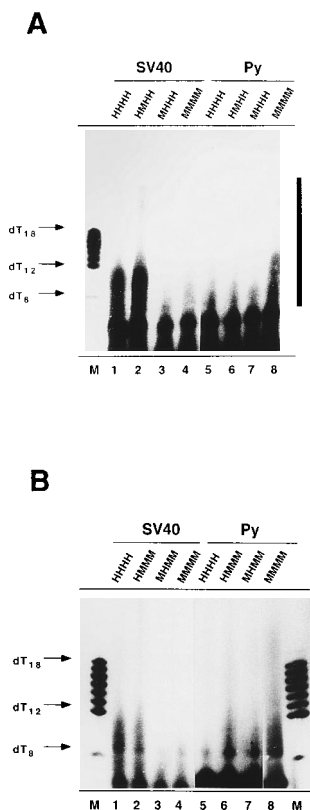


FIG. 6. The species specificity of initiation of SV40 DNA replication is controlled by human p180 DNA polymerase  $\alpha$ . Each recombinant DNA polymerase  $\alpha$ -primase (1.2 U of primase activity) was tested in the SV40 (lanes 1 to 4) and in the Py initiation reaction (lanes 5 to 8). The indicated DNA polymerase  $\alpha$ -primases were used in the initiation reaction. Lane M, 5'-end-labeled oligo(dT)<sub>8</sub> and oligo(dT)<sub>12-18</sub> markers as indicated at the left by arrows. The bar at the right marks the initiation products.

The HMHH and MHMM complexes were tested for activity in the initiation reaction mixture containing only purified proteins. While the HMHH complex, containing mouse p68, synthesized RNA primers on SV40 origin DNA as efficiently as human DNA polymerase  $\alpha$ -primase (Fig. 6A; compare lanes 1 and 2), the MHMM complex, containing mouse p180, was unable to initiate SV40 DNA replication in vitro (Fig. 6A; compare lane 3 with lanes 1 and 4). Neither hybrid complex could initiate Py DNA replication in assays performed in parallel (Fig. 6A; compare lanes 6 and 7 with lanes 5 and 8). These data indicate that mouse p68 did not detectably impair initiation of SV40 DNA replication yet mouse p180 poisoned it.

These initiation data suggested that the HMMM complex, containing only human p180 and the three small mouse subunits, initiates SV40 DNA replication in vitro, while MHMM, containing human p68 and three mouse subunits, does not. To test this prediction, both enzyme complexes were used in the initiation reaction. The HMMM complex synthesized oligoribonucleotides on SV40 plasmid DNA with approximately the same activity as the human DNA polymerase  $\alpha$ -primase that served as a positive control (Fig. 6B; compare lanes 2 and 1). The complex MHMM, containing human p68, did not initiate SV40 DNA replication in vitro (Fig. 6B, lane 3). Both HMMM and MHMM synthesized RNA primers on Py origin DNA (Fig. 6B, lanes 5, 6, and 7), in agreement with the requirement for mouse primase in the Py initiation reaction (4, 21).

Taken together with the results from Fig. 3, these data in-

dicate that human p180 was sufficient to determine the species specificity of SV40 initiation in vitro and that mouse p180 prevented the initiation of SV40 DNA replication in vitro. Hybrid complexes composed of mouse p180 and human primase were unable to initiate both SV40 and Py DNA replication.

To confirm and extend these results, the hybrid DNA polymerase  $\alpha$ -primases containing only one mouse or one human subunit and three subunits from the other species were then tested in the crude SV40 DNA replication system in vitro. The HMHH complex replicated SV40 DNA in vitro (Fig. 7A and B, lanes 11 to 16); the incorporation of dNMP was about 30-fold above background, which is similar to the level observed with human DNA polymerase  $\alpha$ -primase. In contrast, the MHMM complex was essentially inactive, catalyzing incorporation of only 1.4-fold background levels even at the highest concentrations of enzyme complex (Fig. 7A and B, lanes 17 to 22). To study the requirements for SV40 DNA replication further, we tested the activities of hybrid complexes HMMM and MHMM. The HMMM complex was active in the replication of SV40 DNA, catalyzing dNMP incorporation about 15-fold above background level and generating *DpnI*-resistant replication products even at the lowest concentration of enzyme complex (Fig. 7C and D, lanes 11 to 16). In contrast, MHMM could not replicate SV40 DNA; the incorporation of dNMP was not above the negative control value obtained with mouse DNA polymerase  $\alpha$ -primase (Fig. 7C and D; compare lanes 17 to 22 with lanes 23 to 28).

The results obtained from the SV40 DNA replication in crude extracts and from the initiation reaction in a purified system are in good agreement and indicate that p180 controls the species specificity of SV40 DNA replication in vitro.

## DISCUSSION

**The human p180 DNA polymerase  $\alpha$  subunit mediates species-specific SV40 DNA replication in vitro.** The papovaviruses SV40 and Py propagate in different hosts. SV40 multiplies in primate cells, and Py multiplies in mouse cells (67). Since the DNA polymerase  $\alpha$ -primase was the major factor controlling species specificity in SV40 and Py DNA replication in vitro (4, 21, 44, 46, 48, 58), we purified recombinant hybrid human-mouse DNA polymerase  $\alpha$ -primase complexes (Table 1; Fig. 1 and 2) to determine which subunits are required for species-specific replication of SV40 DNA in vitro.

The similarities of the Py and SV40 DNA replication, the observation that the mouse primase mediates species specificity of Py DNA replication in vitro, and the finding that the mouse p48 primase subunit in the HHHM complex poisoned the SV40 initiation reaction (Fig. 3A) (4, 21, 46, 48) suggested that human primase also controls the host specificity of SV40 DNA replication. However, we found that the recombinant HHMM complex efficiently initiated SV40 and Py DNA replication in the purified initiation system, while MMHH did not (Fig. 3A and B). These findings indicate that the human p180 and/or p68 subunit(s) mediates the species specificity of SV40 DNA replication (summarized in Table 2). The inability of the HHHM complex to initiate SV40 DNA replication could be due to some subtle disturbance of interactions between mouse p48 and human p58 or to special requirements for primase conformation during the assembly of the primosome at the SV40 origin of replication that cannot be achieved by the hybrid primase Hp58-Mp48. This perturbation might remain unrecognized in Py DNA replication, since mouse p48 controls the species specificity of Py DNA replication in vitro. Another possible explanation for the behavior of HHHM is that p48 is



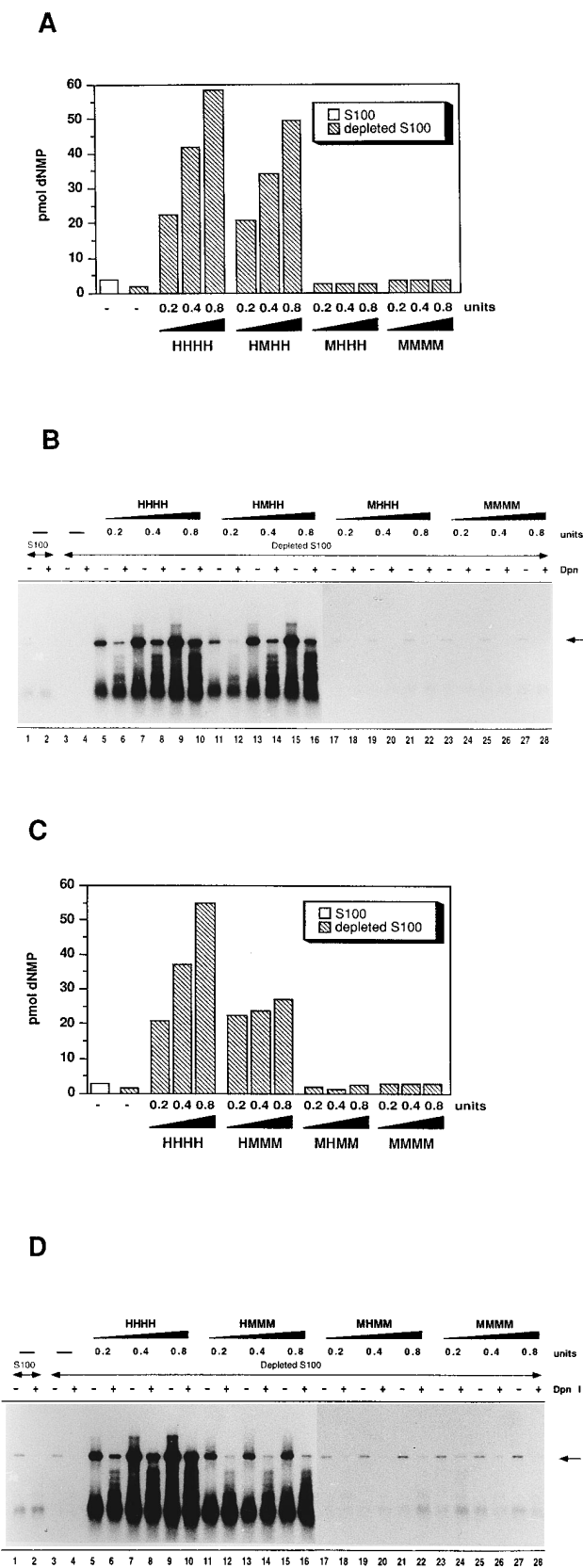


FIG. 7. The human p180 subunit of DNA polymerase  $\alpha$ -primase is responsible for species-specific SV40 DNA replication in vitro in cell extracts. The activities of recombinant hybrid DNA polymerase  $\alpha$ -primase complexes (HMHH, MHHH, HMHH, and MHMM) in SV40 DNA replication in vitro in

involved in the determination of species specificity of SV40 DNA replication in a restricted way, which is manifested only in the presence of human p180 and/or p68, since human p48 is inactive in the MMHH complex even in the presence of human p58 (Fig. 3B).

To analyze whether the human p180 or p68 subunit is required for SV40 DNA replication in vitro, additional hybrid human-murine DNA polymerase  $\alpha$ -primases were tested in SV40 initiation (Tables 1 and 2). Earlier findings showed that both the p180 and p68 subunits interacted with SV40 T antigen (11, 20). Since the amino acid sequences of human and mouse p68 subunits are significantly less highly conserved than those of p180 subunits (63), the p68 subunit seemed to be a good candidate for controlling species specificity. However, human and mouse p68 subunits had no detectable influence on the species specificity of SV40 and Py initiation of DNA replication (Table 2). These results suggested that human p180 controls the species specificity of SV40 DNA replication. This hypothesis is supported by the finding that none of the DNA polymerase  $\alpha$ -primase complexes containing mouse p180 (MMMM, MHMM, MMHH, and MHHH) catalyzed initiation of SV40 DNA replication (summarized in Table 2). Furthermore, we showed that the HMHH complex containing only human p180 efficiently initiated SV40 DNA replication (Fig. 6B, lane 2), demonstrating that human p180 mediates species-specific initiation of SV40 DNA replication.

To study the replication of SV40 DNA in vitro in mouse S100 FM3A extracts, we had to add purified bovine RP-A to DNA polymerase  $\alpha$ -primase-depleted extracts from mouse FM3A cells (Fig. 4), although these extracts contained sufficient RP-A, the eukaryotic ssDNA-binding protein, to support Py DNA replication in vitro (data not shown). This finding could mean that mouse RP-A is not as active in SV40 DNA replication as human RP-A or that SV40 DNA replication requires higher concentrations of RP-A than Py DNA replication. Experiments to test these possibilities are currently under way. A recent report that *Escherichia coli* ssDNA-binding protein (SSB) stimulates Py DNA replication in FM3A extracts and that the addition of *E. coli* SSB did not alter the dependence of Py DNA replication on mouse DNA polymerase  $\alpha$ -primase supports the view that cytosolic FM3A extracts contain less RP-A than is required for optimal DNA replication (22). Our results show that the species specificity of SV40 DNA replication was maintained despite the addition of bovine or human RP-A to FM3A extracts, since the human DNA polymerase  $\alpha$ -primase supported SV40 DNA replication in vitro in the supplemented extracts, while mouse DNA poly-

DNA polymerase  $\alpha$ -primase-depleted mouse FM3A cell extracts (depleted S100) supplemented with 0.6  $\mu$ g of RP-A were tested (A to D). HHHH and MMMM served as positive and negative controls, respectively. (A and C) Incorporation of dNMPs into SV40 DNA in the presence of increasing amounts of recombinant enzymes (0.2, 0.4, and 0.8 primase U) as indicated; (B) DNA synthesis products on SV40 origin-containing DNA (A) were linearized with 10 U of *Eco*RI (odd-numbered lanes) or were analyzed by digestion with 10 U each of *Eco*RI and *Dpn*I (even-numbered lanes). Lanes: 1 and 2, undepleted mouse extracts; 3 and 4, depleted mouse extracts; 5 to 10, depleted mouse extracts plus human DNA polymerase  $\alpha$ -primase (HHHH); 11 to 16, depleted extracts supplemented with hybrid complex (HMHH); 17 to 22, depleted extracts supplemented with hybrid complex (MHMM); 23 to 28, depleted extracts supplemented with hybrid complex (MMMM). (D) Analysis of DNA synthesis products of the reactions plotted in panel C. Lanes: 1 and 2, undepleted mouse extracts; 3 and 4, depleted mouse extracts; 5 to 10, depleted mouse extracts plus human DNA polymerase  $\alpha$ -primase (HHHH); 11 to 16, depleted extracts supplemented with hybrid complex (HMHH); 17 to 22, depleted extracts supplemented with hybrid complex (MHMM); 23 to 28, depleted extracts supplemented with hybrid complex (MMMM). The arrow at the right indicates the linearized DNA synthesis products.

merase  $\alpha$ -primase did not (Fig. 4A and B; data not shown). Nevertheless, under these conditions a significant fraction of the SV40 DNA replication products was 400 to 1,500 bp in size (Fig. 4B, lanes 1 to 10). These short SV40 replication products were observed in mouse extracts that were supplemented with either human or bovine RP-A and required SV40 T antigen and the SV40 origin of replication but were not observed in replication of Py DNA in mouse FM3A cell extracts or SV40 DNA replication in human 293S cell extracts (data not shown). The small products might be identical to those determined in vitro after the addition of SV40 DNA to carcinogen-treated, nonpermissive CO60 cells, since the size distribution and the requirements for synthesis of the small products were comparable (2, 9).

The results from the SV40 initiation assays were confirmed by those obtained from assays of SV40 DNA replication in the depleted mouse cell extracts (summarized in Table 2). The HHMM complex catalyzed SV40 DNA replication in vitro. At low concentrations, the HHMM complex was reproducibly nearly as active as HHHH, and *DpnI*-resistant replication products were detectable (Fig. 7C and D, lanes 12); at high concentrations, the HHMM complex was less active than HHHH (Fig. 7C). Since HHMM was nearly as active as HHHH in the initiation reaction (Fig. 6B), the partial reduction in replication activity of HHMM might be due to the reduction in activity of HHMM during elongation of the newly synthesized primers.

Our data are not consistent with previously reported findings that an enzyme complex reconstituted from biochemically purified human DNA polymerase  $\alpha$ -core complex (p180 plus p68) and mouse primase (p58 plus p48) was unable to replicate SV40 DNA in vitro, while the complex reconstituted from human DNA polymerase  $\alpha$ -core complex and human primase was active (48). In contrast to these earlier results, the hybrid recombinant DNA polymerase  $\alpha$ -primase, HHMM, was as active as the human DNA polymerase  $\alpha$ -primase in SV40 DNA replication (Fig. 5; Table 2). One possible explanation for this apparent contradiction is that the recombinant HHMM enzyme was assembled in vivo by coexpressing all four subunits in insect cells and was then purified as a complex, whereas the earlier study was performed with in vitro-reconstituted enzyme. Perhaps the reconstituted hybrid complex was less stable than the reconstituted human one or the complex was assembled in an inactive form. Further work would be necessary to examine this and other possibilities.

In summary, the functional assays with a purified and a crude system indicate that the human p180 subunit is essential for species-specific SV40 DNA replication in vitro and that the human p68 subunit and p58 primase subunit do not mediate the species specificity of SV40 DNA replication (Table 2). The catalytic primase subunit p48 appears to play a secondary role in controlling the species specificity of SV40 DNA replication in vitro, since the mouse p48 of HHHM can abolish SV40 initiation; however, this phenotype can be rescued by coexpressing the second mouse primase subunit. In addition, the findings that hybrid complexes containing human primase cannot initiate and replicate SV40 DNA in the presence of mouse p180, but that human p180 does so, even in the presence of three mouse subunits, suggest the priority of human p180 in determining the species specificity of SV40 DNA replication in vitro. These results show for the first time that DNA polymerase  $\alpha$ -primase may control the species specificity of SV40 and Py DNA replication by different mechanisms.

**Function of DNA polymerase  $\alpha$ -primase in the species specificity of SV40 and Py DNA replication.** Several possible mechanisms can be proposed for the control of the species specific-

TABLE 2. Species-specific replication of SV40 and Py DNA in vitro<sup>a</sup>

Recombinant DNA polymerase $\alpha$ -primase	Initiation of replication		DNA replication		Single subunit exchanged
	SV40	Py	SV40	Py	
HHHH	+++	-	+++	- <sup>b</sup>	
HMHH	+++	-	+++	ND	Mouse p68
MHHH	-	-	-	ND	Mouse p180
MMHH	-	-	-	- <sup>c</sup>	
HMMM	+++	++	++	ND	Human p180
MHMM	-	++	-	ND	Human p68
HHMM	+++	+++	+++	+++ <sup>b</sup>	
MMMM	-	+++	-	+++ <sup>b</sup>	
HHMH	+++	- <sup>b</sup>	ND	+/- <sup>b</sup>	Mouse p58
HHHM	-	++ <sup>b</sup>	ND	++ <sup>b</sup>	Mouse p48

<sup>a</sup> +++, optimal initiation or DNA replication; ++, initiation or DNA replication products easily detected; +/-, some replication products detected; -, no initiation products or no DNA replication detectable; ND, not determined.

<sup>b</sup> Data from Brückner et al. (4).

<sup>c</sup> Data from Stadlbauer (62).

ity of SV40 and Py DNA replication by DNA polymerase  $\alpha$ -primase. One explanation is that the formation of a quaternary initiation complex on the origin of DNA replication is species specific. Biochemical studies showed that T antigen interacted independently with p180, p68, and p48 subunits of DNA polymerase  $\alpha$ -primase (4, 5, 11, 17, 19, 20). The p48 subunit of DNA polymerase  $\alpha$ -primase also physically binds to RP-A (51). The participation of RP-A in the species-specific reaction is also suggested by the finding that *Drosophila* DNA polymerase  $\alpha$ -primase synthesized DNA in SV40 T antigen- and SV40 origin-dependent fashion in the presence of *Drosophila* RP-A, but not human RP-A (30). In addition, several monoclonal antibodies against p180 or primase disturb the physical binding of DNA polymerase  $\alpha$ -primase to RP-A, as well as to SV40 T antigen (18). These antibodies inhibited neither DNA polymerase nor primase activities but abolished SV40 DNA replication in vitro. These findings suggested that RP-A- and T antigen-binding sites in the DNA polymerase  $\alpha$ -primase complex are essential for SV40 DNA replication in vitro and might reside close together in the native protein complexes.

The subunits of the DNA polymerase  $\alpha$ -primase are highly conserved, since 88, 80, 89, and 90% of the amino acids are identical between human and murine p180, p68, p58, and p48, respectively (63). In addition to exchanges in amino acids, human p180 protein has a six-amino-acid deletion at position 8, an insertion of two amino acids at positions 316 and 317, and an insertion of one amino acid at position 1410 in comparison with the mouse protein (Fig. 8A). Interestingly, the SV40 T antigen-binding site of human p180 was mapped to residues 195 to 313 of human p180, which is close to the two-amino-acid insertion (Fig. 8A) (17). Although p48 is the most highly conserved subunit of the DNA polymerase  $\alpha$ -primase complex, human p48 contains two insertions, one additional amino acid at position 281, and two inserted amino acids at positions 375 and 376 (in comparison with mouse p48) (Fig. 8B). The species specificity of physical protein-protein interactions was not observed with purified protein complexes in the absence of viral origin DNA (5, 58). However, these experiments are unlikely to mimic the quaternary initiation complex on the origin of DNA replication, so that the quality of the interaction between the proteins within the quaternary initiation complex might differ from the simple binding of purified proteins to one



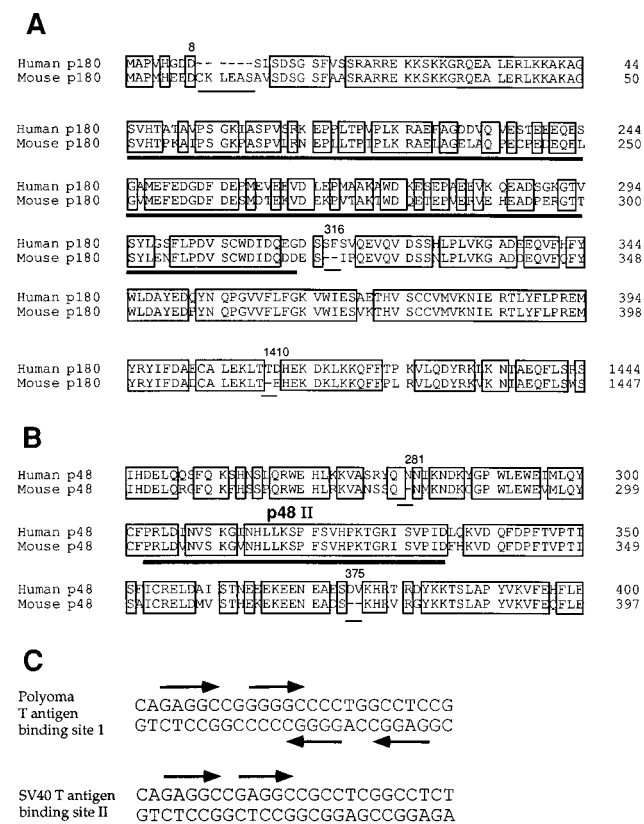


FIG. 8. Sequence comparisons. In the amino acid comparisons of p180 (amino acids 1 to 44/50, 194/200 to 394/398, and 1395/1399 to 1444/1447) (A) and p48 (amino acids 250 to 400/397) (B), the putative SV40 T antigen-binding site of human p180 (amino acids 194 to 313) and the conserved region II of p48 (amino acids 303 to 335) are indicated by bold lines (17, 63). The deletions (human p180 [after amino acid 8]) and insertions (human p180 [positions 316, 317, and 1410] and human p48 [positions 281, 375, and 376]) are underlined with thin lines. Boxed regions show identical amino acids of the proteins. (C) The T antigen-binding site I of the Py origin and the T antigen-binding site II of the SV40 origin are presented (12, 26). The T antigen recognition elements G(A/G)GGC are marked by arrows.

another. Therefore, the species-specific amino acid deletion and insertions might disturb the physical assembly only of a quaternary complex consisting of T antigen, DNA polymerase  $\alpha$ -primase, RP-A, and origin DNA, leaving the simple protein-protein interactions and DNA-binding domains of p180 and p48 untouched.

In a second model, assembly of the initiation proteins on the DNA, the unwinding of origin DNA, and primer synthesis are coupled (47). DNA polymerase  $\alpha$ -primase complexes containing the mouse p180 subunit could inhibit or fail to promote unwinding of SV40 origin DNA, while those containing human p48 could impair unwinding of the Py origin.

A third possibility is that the differences in the SV40 and Py core origin DNA sequences explain the species specificity. Although they show a high sequence homology to each other, the SV40 and Py core DNA replication origins determine species specificity on the DNA level in vivo (1). Both core origins contain a dyad symmetry element, SV40 T antigen binding site II and Py binding site 1, consisting of four T antigen recognition sites, G(A/G)GGC (Fig. 8B) (12, 26). However, these recognition sites are differently arranged in each origin; the G(A/G)GGC elements are separated by one nucleotide in the SV40 origin, while there are two nucleotides between the Py

G(A/G)GGC elements, and in the center of Py binding site 1, the two inverted G(A/G)GGC elements overlap (Fig. 8B). These or other distinctions between the two origins might direct the mouse primase in the presence of Py T antigen to recognize and utilize specific Py origin template DNA sequences, while the DNA polymerase  $\alpha$ -primase containing human p180 with SV40 T antigen does the equivalent on the SV40 origin (7, 8, 15, 28, 54, 64, 74, 75). Therefore, each initiation complex would be spatially unique, and the conformation of interactions between the viral T antigens and the host DNA polymerase  $\alpha$ -primase are optimally adapted to each other, such that an initiation complex assembled with an inappropriate DNA polymerase  $\alpha$ -primase would be unable to synthesize primers. Studies to test these models are currently under way.

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