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The Mouse DNA Polymerase α-Primase Subunit p48 Mediates Species-Specific Replication of Polyomavirus DNA In Vitro

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Mouse cell extracts support vigorous replication of polyomavirus (Py) DNA in vitro, while human cell extracts do not. However, the addition of purified mouse DNA polymerase α-primase to human cell extracts renders them permissive for Py DNA replication, suggesting that mouse polymerase α-primase determines the species specificity of Py DNA replication. We set out to identify the subunit of mouse polymerase α-primase that mediates this species specificity. To this end, we cloned and expressed cDNAs encoding all four subunits of mouse and human polymerase α-primase. Purified recombinant mouse polymerase α-primase and a hybrid DNA polymerase α-primase complex composed of human subunits p180 and p68 and mouse subunits p58 and p48 supported Py DNA replication in human cell extracts depleted of polymerase α-primase, suggesting that the primase heterodimer or one of its subunits controls host specificity. To determine whether both mouse primase subunits were required, recombinant hybrid polymerase α-primases containing only one mouse primase subunit, p48 or p58, together with three human subunits, were assayed for Py replication activity. Only the hybrid containing mouse p48 efficiently replicated Py DNA in depleted human cell extracts. Moreover, in a purified initiation assay containing Py T antigen, replication protein A (RP-A) and topoisomerase I, only the hybrid polymerase α-primase containing the mouse p48 subunit initiated primer synthesis on Py origin DNA. Together, these results indicate that the p48 subunit is primarily responsible for the species specificity of Py DNA replication in vitro. Specific physical association of Py T antigen with purified recombinant DNA polymerase α-primase, mouse DNA primase heterodimer, and mouse p48 suggested that direct interactions between Py T antigen and primase could play a role in species-specific initiation of Py replication.

The replication of the papovavirus minichromosome has been extensively utilized as a model for cellular DNA replication in vivo and in vitro. The replication of polyomavirus (Py) or simian virus 40 (SV40) DNA requires only one viral protein, the Py or SV40 large T antigen, and the corresponding viral origin of DNA replication, while the other replication proteins are supplied by the host (reviewed in references 7, 25, and 62). The T antigens of Py and SV40 share 36% sequence identity (reference 65 and references therein), possess an intrinsic helicase activity (41, 58, 61, 71), and bind to multiple copies of a pentanucleotide sequence (G[A/G]GGC) in the origin of replication (11, 52, 55). Despite these similarities, Py and SV40 DNA replicate in different hosts, Py DNA in mouse cells and SV40 DNA in primate cells (reviewed in reference 65).

The development of in vitro replication assays for papovaviral DNA has facilitated the identification and characterization of the cellular proteins required for replication in vitro (26, 31, 32, 41, 44, 63, 66, 69, 70, 74, 75). Detailed analysis of SV40 DNA replication has led to a model for initiation in which T antigen binds to the origin of replication, assembling a double hexamer that then unwinds the duplex DNA in the presence of ATP, the single-stranded DNA binding protein replication protein A (RP-A), and topoisomerase I or II (reference 3 and references therein). In the following step, DNA polymerase α-primase synthesizes RNA primers at the origin of replication which are subsequently elongated by DNA polymerase α (5, 6, 35, 40). Through a polymerase switch mechanism, DNA polymerase δ and the accessory proteins proliferating cell nuclear antigen (PCNA) and replication factor C (RF-C) are assembled on these initial DNA primers, forming the leading-strand replication complex (66–68, 74). Lagging-strand replication is mediated by DNA polymerase α-primase together with DNA polymerase δ and its accessory proteins (6, 12, 48, 49, 66–68, 70).

DNA polymerase α-primase was shown to be the major host factor responsible for species-specific replication of Py and SV40 DNA (41, 44). More detailed studies suggested that the addition of DNA primase, isolated after its dissociation from purified mouse DNA polymerase α-primase, enabled human S100 extracts to support replication of Py DNA (18). In contrast, replication of SV40 DNA in mouse cell extracts was shown to require both human polymerase α and human primase (44). Recently, however, *Drosophila* extracts were also found to support a low level of SV40 DNA replication in vitro in the absence of human DNA polymerase α-primase (27).

Physical interactions of SV40 T antigen with mammalian DNA polymerase α-primase can be detected by using both crude cell extracts and purified proteins (9, 14–16, 21–23, 56, 57, 59). However, these interactions are apparently not sufficient to explain the species specificity of SV40 DNA replication, since SV40 T antigen interacted physically and functionally with purified DNA polymerase α-primase from mouse and

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calf, as well as that from human cells (8, 15, 16, 23, 57). Only in the context of SV40 origin DNA was the human DNA polymerase α-primase specifically required, together with SV40 T antigen and mammalian RP-A, for primosome assembly and primer synthesis, indicating that species specificity of SV40 DNA replication is determined at the initiation step (36, 57).

DNA polymerase α-primase contains four subunits: p180, p68, p58, and p48 (reviewed in reference 73). The DNA polymerase activity resides in the p180 subunit, while the DNA primase activity requires the p58 and p48 subunits. We previously cloned and expressed cDNAs encoding the subunits of mouse and human DNA polymerase α-primase and showed that purified recombinant mouse DNA polymerase α-primase could restore Py DNA replication activity in mouse cell extracts depleted of DNA polymerase α-primase (60). To determine which of the mouse DNA polymerase α-primase subunits are required for host-specific replication of Py DNA, we overexpressed recombinant hybrid DNA polymerase α-primases containing subunits of the human and mouse enzymes and used these hybrids to investigate the replication of Py DNA in human cell extracts. Here, we present evidence that a purified hybrid DNA polymerase α-primase containing human p180, p68, and p58 and the mouse p48 subunit synthesized primers on Py origin DNA in a purified initiation system and efficiently supported Py DNA replication in DNA polymerase α-primase-depleted human cell extracts. We show that purified mouse DNA primase binds well to purified Py T antigen, while the p180 and p68 subunits interact more weakly with Py T antigen. We discuss a possible role for these interactions in the initiation of Py DNA replication.

MATERIALS AND METHODS

Protein manipulations. Protein concentration was determined by the Bradford method (4) by using a commercial reagent with bovine immunoglobulin G as a standard (Bio-Rad, Munich, Germany). Sodium dodecyl sulfate (SDS) gel electrophoresis was carried out as previously described (30) with prestained proteins or protein marker VI (both from Sigma, Deisenhofen, Germany) as molecular weight markers. After polyacrylamide gel electrophoresis, proteins were detected by Coomassie brilliant blue or silver staining (24). Protein renaturation was performed after SDS gel electrophoresis, and Western blotting (immunoblotting) was carried out as described previously (16, 60).

Protein purification. Expression and purification of primase proteins, DNA polymerase α-primase, p180, and p180-p68 complexes were performed as previously described (57, 60). Briefly, SF9X or High Five (Invitrogen, Heidelberg, Germany) insect cells (4×10^8 to 5×10^8) were infected with 10 PFU of each recombinant baculovirus per cell and incubated at 27°C for 44 to 48 h. DNA primase was purified from crude extracts by DEAE-Sephacel and phosphocellulose chromatography (60). The p58-p48 primase activity was 1,360 U/mg, while that of p48 was 370 U/mg (60). DNA polymerase α-primase was purified by phosphocellulose chromatography followed by immunoaffinity chromatography (57). Immobilized monoclonal antibody SJK 287-38 (64) was used to purify the DNA polymerase α-primase from human or mouse cells and the recombinant four-subunit mouse enzyme complex. SJK 237-71 (64) coupled to Sepharose 4B was used to purify recombinant DNA polymerase α-primase complexes containing human p180. DNA polymerase α assays and DNA primase assays were performed as previously described (45, 46, 60). The specific activities of the DNA polymerase α-primase complexes varied from 4,500 to 11,400 polymerase units/mg and from 250 to 1,670 primase units/mg. RP-A was purified as previously described (47). Topoisomerase I was a generous gift from I. Moarefi (38).

Py T antigen was expressed by using the baculovirus expression vector vEV51PyT (53). Plaque-purified vEV51PyT virus was amplified in SF9X cells and then used to infect High Five cells for expression. The cells were homogenized in lysis buffer (100 mM Tris-HCl [pH 9.0], 100 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM dithiothreitol, 0.5% Nonidet P-40, 1 μM leupeptin, 1% Trasylol), and Py T antigen was purified by immunoaffinity chromatography with monoclonal antibody F5 (51) coupled to Sepharose 4B essentially as described previously (41). Py T antigen was eluted with 20 mM Tris-HCl (pH 8.5)–0.5 M NaCl–1 mM EDTA–1 mM dithiothreitol–10% glycerol–50% ethylene glycol.

Preparation of S100 extracts and replication of Py DNA in vitro. S100 extracts were prepared from logarithmically growing human 293S cells as previously described (57). The cells were harvested by centrifugation and then washed twice with phosphate-buffered saline and once with hypotonic buffer. The cells were then 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 $11,000 \times g$. The supernatant was then adjusted to 100 mM NaCl and clarified by a second centrifugation at $100,000 \times g$ (S100 extract). Depletion of DNA polymerase α-primase from S100 extracts was performed essentially as previously described (44). The extracts were adjusted to 350 mM NaCl and incubated twice with SJK 132-20 monoclonal antibody (64) covalently coupled to Sepharose 4B (Pharmacia, Freiburg, Germany). The depleted and control extracts were dialyzed against 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid)-KOH (pH 7.5)–5 mM KCl–1.5 mM $MgCl_2$–100 mM NaCl–0.1 mM dithiothreitol–10% glycerol, frozen in liquid nitrogen, and stored at −80°C.

The replication of Py DNA in vitro was performed as previously described (57, 60). Briefly, the assay contained 1.2 μg of Py T antigen, 240 ng of pUC-Py1 DNA, and 200 μg of S100 or depleted S100 extract in 30 mM HEPES-KOH (pH 7.8)–0.5 mM dithiothreitol–7 mM magnesium acetate–1 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid] (pH 7.8)–4 mM ATP–0.3 mM (each) CTP, GTP, and UTP–0.1 mM (each) dATP and dGTP–0.05 mM (each) dCTP and dTTP–40 mM creatine phosphate–80 μg of creatine kinase per ml–5 μCi (each) of [α-^{32}P]dCTP and [α-^{32}P]dTTP (3,000 Ci/mmol). DNA polymerase α-primase purified from mouse cells or recombinant DNA polymerase α-primase complex was added as indicated. Comparisons between different polymerase-primase preparations were made by adjusting the amount so that reaction mixtures contained equal primase activity. The reactions were stopped by the addition of 0.7% SDS, 40 mM EDTA, and 0.2 mg of proteinase K per ml. The replication products were digested with *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. The total radioactivity was measured after spotting 5 μl of a 200-fold dilution of the replication assay mixture onto GF52 filters (Schleicher & Schüll, Dassel, Germany).

Initiation of replication of Py and SV40 DNA. Initiation reactions were performed as previously described (35, 40, 57) with slight modifications. The Py initiation reaction mixtures contained 0.25 μg of pUC-Py1 DNA, 1.6 μg of Py T antigen, 1 μg of RP-A, 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 μg of creatine kinase, 0.3 μg of topoisomerase I, 0.2 mg of bovine serum albumin (BSA) per ml, and 20 μCi of [α-^{32}P]CTP (3,000 Ci/mmol; ICN, Meckenheim, Germany). DNA polymerase α-primase purified from mammalian cells or recombinant DNA polymerase α-primase was added as indicated in the figure legends. The reaction products were precipitated, redissolved in 45% formamide–5 mM EDTA–0.09% xylene cyanol FF 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 to 800 V as previously described (57). The reaction products were visualized by autoradiography.

ELISAs. Enzyme-linked immunosorbent assays (ELISAs) were carried out under the buffer conditions used for in vitro replication, as described previously (57).

RESULTS

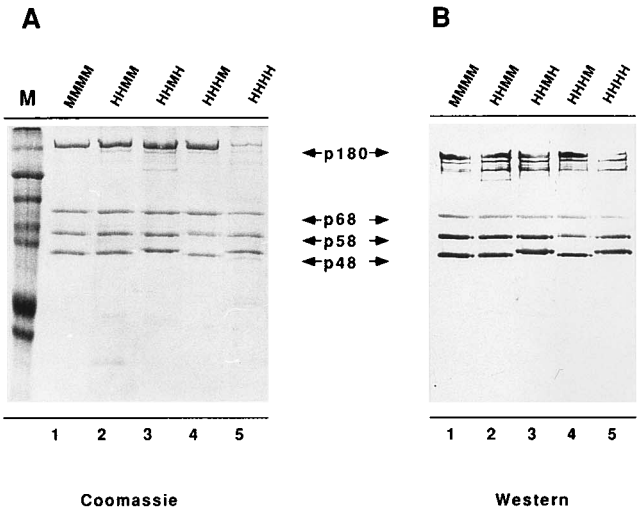
Expression and purification of recombinant DNA primase and DNA polymerase α-primase. To analyze the species specificity of Py DNA replication, the mouse and human DNA polymerase α-primases were expressed by using the baculovirus expression system. Tetrameric human-mouse hybrid complexes were produced by coinfection of insect cells with recombinant baculoviruses. The human p180 and p68 subunits of DNA polymerase α-primase were coexpressed with mouse p58 and p48 (HHMM). Hybrid complexes containing only the p58 mouse subunit (HHMH) and only the p48 mouse subunit (HHHM) were also produced. The DNA polymerase α-primases were purified by immunoaffinity chromatography with an immobilized antibody against the p180 subunit (Table 1). The specific activities of DNA polymerase and DNA primase varied from one preparation of each enzyme to another and with the time of storage after purification. Each preparation was assayed immediately before use in the experiments presented in this report; in the figures, the amount of each enzyme used is given in primase units, which were determined at the time the experiment was performed. All of the recombinant DNA polymerase α-primases showed similar subunit compositions (Fig. 1). Mouse p48 and the mouse p58-p48 primase dimer purified by ion-exchange chromatography had DNA primase specific activities of 370 and 1,360 U/mg, respectively, and DNA polymerase activities of background level (60). Their

TABLE 1. Yields and specific activities for purified, recombinant DNA polymerase α-primase complexes[a]

Complex[b]	Total activity (U)		Total protein (μg)	Sp act (U/mg)	
	DNA polymerase	Primase		DNA polymerase	Primase
Mouse DNA polα/prim (MMMM)	3,200	700	700	4,570	1,000
Hybrid DNA polα/prim (HHMM)	1,850	115	325	5,700	350
Hybrid DNA polα/prim (HHMH)	4,800	140	550	8,730	250
Hybrid DNA polα/prim (HHHM)	6,000	350	525	11,400	670
Human DNA polα/prim (HHHH)	1,300	250	150	8,670	1,670

[a] The yields and specific activities given are those of the peak fraction from a representative preparation of each enzyme complex, as measured directly after the purification.

[b] polα/prim, polymerase α-primase.



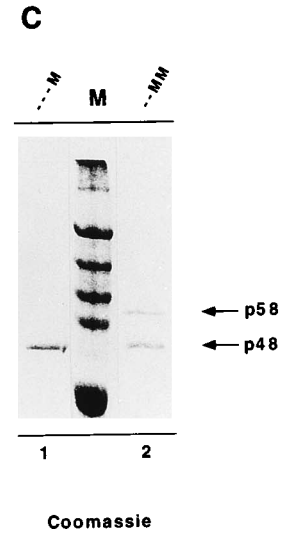


FIG. 1. SDS gel electrophoresis of purified recombinant DNA polymerase α-primase. Purified protein complexes (3 μg [A], 2 μg [B], or 1.5 μg [C]) were separated by SDS-polyacrylamide gel electrophoresis and stained with Coomassie brilliant blue (A and C) or detected by Western blot (B) with polyclonal antibodies against DNA polymerase α-primase (45). (A and B) Lane 1, mouse (M) tetramer; lane 2, hybrid tetramer composed of human (H) p180, human p68, mouse p58, and mouse p48; lane 3, hybrid tetramer composed of human p180, human p68, mouse p58, and human p48; lane 4, hybrid tetramer composed of human p180, human p68, human p58, and mouse p48; lane 5, human tetramer; lane M, marker proteins (Sigma). (C) Lane 1, mouse p48; lane 2, mouse p58-p48.

purity was assessed by electrophoresis in denaturing polyacrylamide gels and by Coomassie blue staining (Fig. 1C).

Replication of Py DNA in human S100 extracts supplemented with recombinant mouse DNA primase. The addition of purified mouse DNA polymerase α-primase to human cell extracts has been shown to permit replication of Py DNA in vitro (18, 39, 41, 57). One recent report also suggested that mouse primase by itself was sufficient to permit Py DNA replication in human cell extracts. To confirm this result, we wished to test whether the recombinant p58-p48 complex (60) could support Py DNA replication in human S100 extracts. The human cell extracts alone catalyzed only low incorporation of dNMP into Py DNA (approximately 12 pmol) (Fig. 2A), although control experiments showed that they did support efficient SV40 DNA replication (data not shown) (57). Furthermore, most of the newly synthesized Py DNA was not resistant to digestion with *Dpn*I, suggesting that the low incorporation was primarily due to repair synthesis (Fig. 2B, lanes 17 and 18). Consistent with previous reports (18, 39, 41, 57), the incorporation of radioactive dNMP increased 20- to 30-fold when the extracts were supplemented with DNA polymerase α-primase purified from mouse cells (Fig. 2A), and the newly synthesized Py DNA was resistant to digestion with *Dpn*I (Fig. 2B, lanes 19 and 20). The incorporation of dNMP into Py DNA by the human cell extracts supplemented with DNA polymerase α-primase purified from mouse cells was comparable to that of permissive mouse cell extracts (data not shown). Supplementation of the human cell extracts with the purified recombinant mouse p58-p48 primase permitted Py DNA replication (Fig. 2A). Py DNA replication increased with the amount of mouse primase added, reaching a level equivalent to that reached with authentic DNA polymerase α-primase purified from mouse cells (Fig. 2A). Furthermore, the amount of newly synthesized DNA resistant to digestion with *Dpn*I increased in parallel to the incorporation of labeled dNMP (Fig. 2B, lanes 1 to 8). These results confirm that DNA primase is a major determinant of the species specificity of Py DNA replication, as reported by Eki et al. (18).

We and others have previously reported that the p48 subunit of mouse primase was active in primase assays in the absence of the p58 subunit, indicating that p48 is the catalytic subunit (1, 10, 20, 46, 54, 60). The p58 subunit lacked primase activity but stabilized the enzymatic function of p48. Therefore, we wished to determine whether the p48 mouse primase might be sufficient to support Py DNA replication in human cell extracts. When human S100 extracts were supplemented with purified recombinant p48 subunit alone, low levels of Py DNA replication were detected (Fig. 2A; Fig. 2B, lanes 9 to 16). The addition of 0.05 to 0.1 U of p48 consistently resulted in incorporation of radioactive dNMP that was above the background level with extract alone. However, when the concentration of mouse p48 was raised, dNMP incorporation did not increase in proportion (Fig. 2A). Analysis of radiolabeled DNA products by *Dpn*I digestion indicated a small increase in the level of *Dpn*I-resistant DNA from p48-supplemented reaction mixtures as compared with the products made in the unsupple-

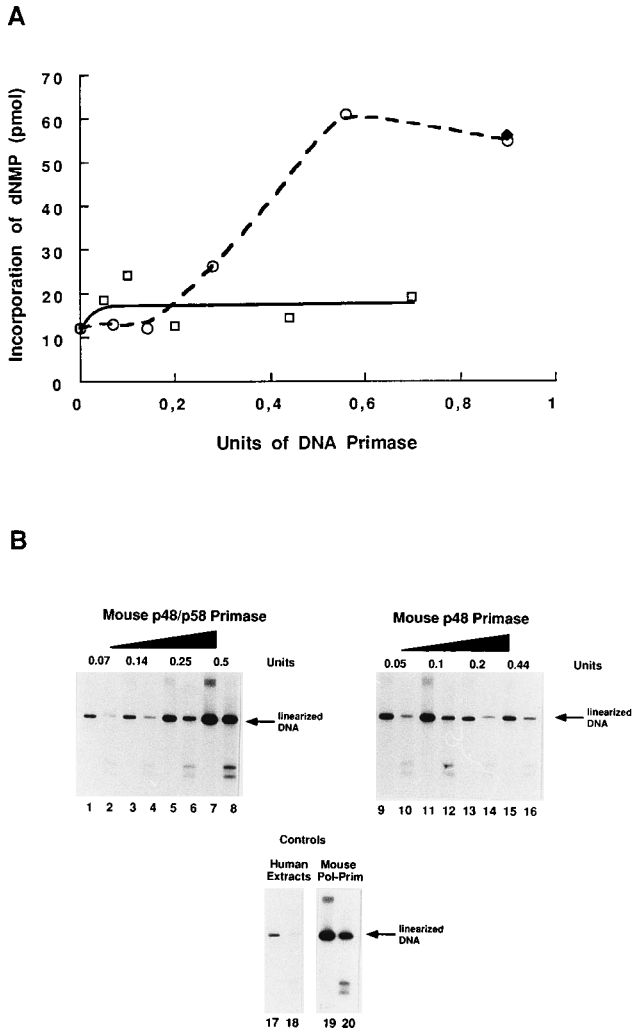


FIG. 2. Replication of Py DNA in vitro in human cell extracts supplemented with recombinant mouse p48 and mouse p58-p48 primase. (A) Py DNA replication was assayed in human 293S cell extracts supplemented with the indicated amounts of purified p48 (□) or p58-p48 (○). The black diamond (◆) indicates the incorporation obtained with human cell extracts supplemented with DNA polymerase α-primase purified from mouse cells. (B) Autoradiograms of the replication products in extracts supplemented with the indicated amounts of mouse p58-p48 (lanes 1 to 8) or mouse p48 (lanes 9 to 16). Controls show replication of Py DNA in human cell extracts alone (lanes 17 and 18) or in extracts supplemented with 0.9 U of polymerase α-primase purified from mouse cells (lanes 19 and 20). The replication products were digested with *Eco*RI alone (odd-numbered lanes) or *Eco*RI plus *Dpn*I (even-numbered lanes).

mented reaction mixtures (Fig. 2B, compare lanes 10, 12, 14, and 16 with lane 18). These results are consistent with the possibility that the mouse p48 subunit may mediate species-specific replication of Py DNA, but the low level of replication and the lack of concentration dependence suggested that mouse p58 could also be important.

Determination of the primase subunit involved in species-specific replication of Py DNA in vitro. To determine which of the mouse primase subunits were required for Py DNA replication, purified recombinant hybrid DNA polymerase α-primase complexes containing mouse primase subunits (Fig. 1) were tested for the ability to replicate Py DNA in human cell extracts depleted of DNA polymerase α-primase (Fig. 3). The depletion removed at least 90% of the DNA polymerase α-primase, as determined by Western blot analysis of the extracts before and after depletion (data not shown). Addition of the recombinant mouse tetrameric complex, which served as a positive control, permitted high levels of Py DNA replication in depleted human cell extracts (Fig. 3A). This result confirms and extends our previous finding that the recombinant mouse four-subunit complex restored Py DNA replication activity in mouse cell extracts depleted of DNA polymerase α-primase (60). The hybrid DNA polymerase α-primase complex containing human p180, human p68, and mouse primase subunits (HHMM) replicated Py DNA with an activity similar to that of the recombinant mouse DNA polymerase α-primase (MMMM) (Fig. 3A). In addition, a hybrid complex containing mouse p48 and human p180, p68, and p58 (HHHM) replicated Py DNA almost as efficiently as the recombinant mouse tetramer (Fig. 3C). To confirm that the incorporation of radiolabel measured with the hybrid enzymes containing mouse primase was due to replication, the newly synthesized DNA was linearized with *Eco*RI and digested with *Dpn*I (Fig. 3B and D). The amount of *Dpn*I-resistant DNA increased with the concentration of input DNA polymerase α-primase, indicating that the hybrids HHMM (Fig. 3B) and HHHM were nearly as active in Py DNA replication as the recombinant mouse DNA polymerase α-primase (Fig. 3D, compare lanes 11 to 16 with lanes 23 to 28). In contrast, the activities of the recombinant human DNA polymerase α-primase and the hybrid complex HHMH were significantly lower (Fig. 3C), and little or no *Dpn*I-resistant full-length DNA was detected (Fig. 3D, compare lanes 5 to 10 with lanes 17 to 22). The results demonstrate that the mouse primase subunits control the species specificity of Py DNA replication and that the p48 subunit is primarily responsible for this activity.

Species-specific initiation of Py DNA replication is determined by mouse p48. Schneider et al. (57) reported that initiation of DNA replication at the SV40 origin was the species-specific step in replication of SV40 DNA. This result raised the question of whether the species specificity of Py DNA replication was also controlled at the level of initiation and whether hybrid DNA polymerase α-primase containing a single mouse primase subunit would catalyze primer synthesis in an initiation assay containing only purified proteins. Initiation of primer synthesis on a double-stranded Py origin DNA template was observed in the presence of Py T antigen, topoisomerase I, the mammalian single-stranded DNA-binding protein RP-A, and mouse DNA polymerase α-primase (57). Consistent with this result, DNA polymerase α-primase purified from mouse cells efficiently catalyzed primer synthesis on Py origin DNA (Fig. 4A, lanes 1 to 4), while DNA polymerase α-primase purified from human cells did not (Fig. 4A, lanes 9 to 12). This result demonstrates that initiation of Py DNA replication with purified proteins specifically requires the mouse DNA polymerase α-primase.

To determine which of the mouse DNA polymerase α-primase subunits is required for primer synthesis on Py origin DNA, the recombinant hybrid enzymes were tested by using the initiation assay (Fig. 4). Two different complexes containing the mouse p48 subunit, HHMM and HHHM, stimulated primer synthesis in a concentration-dependent manner (Fig. 4A, lanes 5 to 8 and 4B, lanes 2 to 4), although the activity was lower than with the recombinant mouse tetramer (Fig. 4B, lanes 8 to 10). As little as 0.6 U of either hybrid complex was sufficient to synthesize more oligoribonucleotides on Py origin DNA than were detected with human DNA polymerase α-primase, which served as a negative control (Fig. 4B, compare lane 3 with lane 1). In contrast, the HHMH DNA polymerase α-primase did not synthesize oligoribonucleotides on double-stranded Py DNA even at the highest concentration added

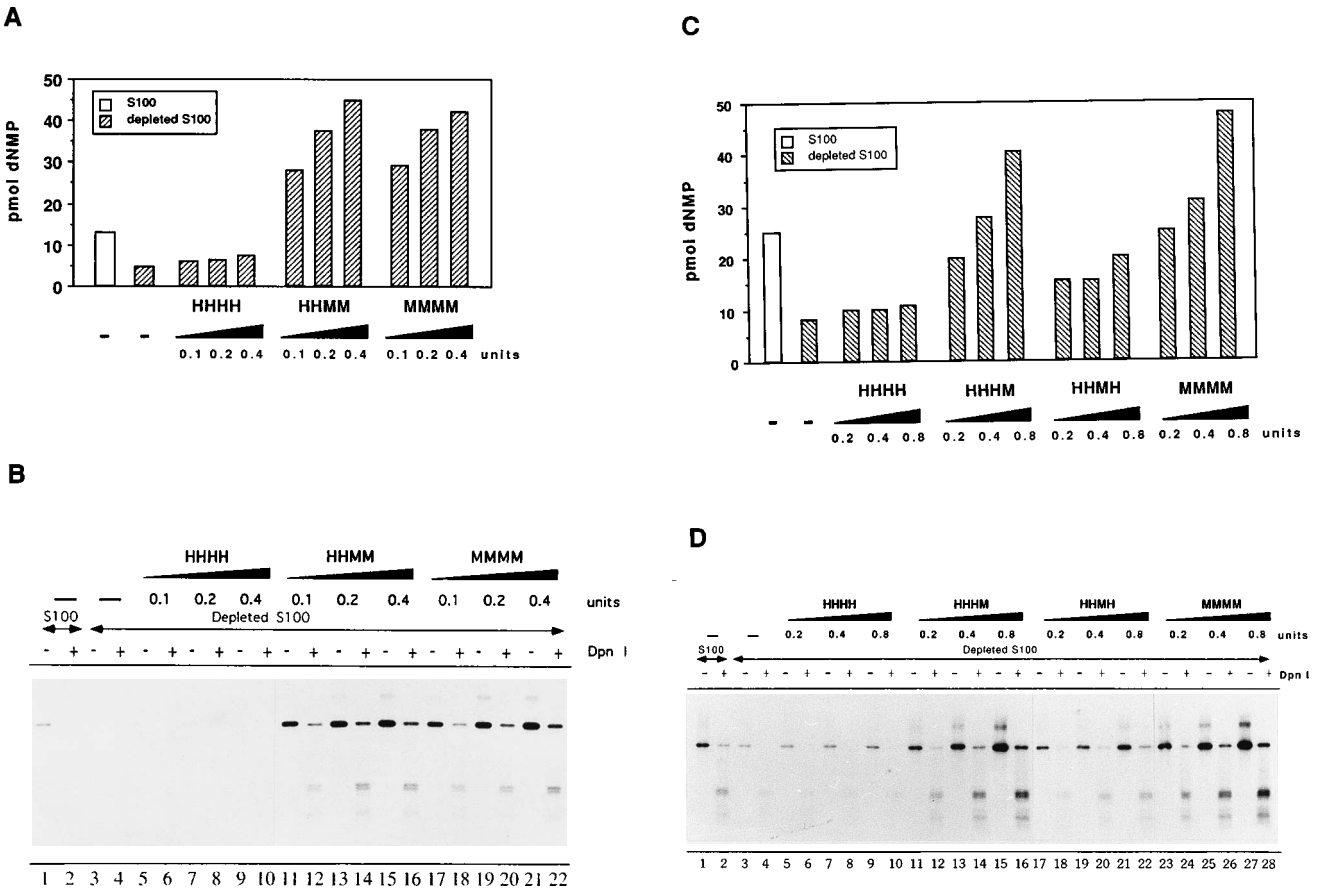


FIG. 3. Replication of Py DNA in vitro in polymerase α-primase-depleted human cell extracts by recombinant hybrid DNA polymerase α-primase complexes. (A and C) Py DNA replication was assayed in human 293S cell extracts depleted of polymerase α-primase and supplemented with increasing amounts of purified polymerase α-primase as measured in primase units. Incorporation into acid-precipitable products is indicated. (B and D) Analysis of the replication products of the reactions whose results are shown in panels A and C. (B) Lanes 1 and 2, undepleted 293S cell extracts; lanes 3 and 4, depleted 293S cell extracts; lanes 5 to 10, depleted 293S cell extracts supplemented with HHHH; lanes 11 to 16, depleted 293S cell extracts supplemented with HHMM; lanes 17 to 22, depleted 293S cell extracts supplemented with MMMM. (D) Lanes 1 and 2, undepleted 293S cell extracts; lanes 3 and 4, depleted 293S cell extracts; lanes 5 to 10, depleted 293S cell extracts supplemented with HHHH; lanes 11 to 16, depleted 293S cell extracts supplemented with HHHM; lanes 17 to 22, depleted 293S cell extracts supplemented with HHMH; lanes 23 to 28, depleted 293S cell extracts supplemented with MMMM. The reaction products were digested with *Eco*RI alone (odd-numbered lanes) or *Eco*RI plus *Dpn*I (even-numbered lanes).

(Fig. 4B, lanes 5 to 7). These results support the interpretation that the p48 subunit is sufficient to determine the species specificity of Py DNA replication.

However, the results presented above do not rule out the possibility that the HHMH DNA polymerase α-primase complex could be inactive in Py replication because of some subtle defect in the assembly of this particular hybrid enzyme complex. To address this possibility, we reasoned that if the p48 subunit also controls the host specificity of SV40 DNA replication, the HHMH polymerase α-primase should be able to initiate SV40 DNA replication. Therefore, the hybrid DNA polymerase α-primase complexes were tested for primer synthesis activity on SV40 origin DNA. Recombinant human DNA polymerase α-primase served as a positive control (Fig. 5, lanes 8 to 10). In contrast to the results in the Py initiation assay, the HHMH DNA polymerase α-primase complex was clearly active in the SV40 initiation assay (Fig. 5, lanes 5 to 7). Conversely, the HHHM enzyme did not synthesize RNA primers on SV40 origin DNA (Fig. 5, compare lanes 2 to 4 with lane 1). These results confirm that the p48 subunit determines species-specific replication of Py DNA and suggest that it may participate in species-specific control of SV40 DNA replication as well.

Protein-protein interactions between Py T antigen and DNA polymerase α-primase subunits. SV40 T antigen has been shown to interact physically with purified DNA polymerase α-primase and with the isolated p180 and p68 subunits (8, 9, 14–16). Despite the fact that the physical interactions of SV40 T antigen with DNA polymerase α-primase are not species specific (23, 57), they are thought to play an important role in the initiation of SV40 replication by recruiting DNA polymerase α-primase to the origin, thereby orienting DNA primase to facilitate synthesis of the first primers (8, 9, 14–16, 34, 39, 41, 42, 57). Py T antigen might be expected to play a similar role in the initiation of Py DNA replication, since it binds specifically to mouse DNA polymerase α-primase in crude extracts (22, 39, 57). However, its physical interactions with purified DNA polymerase α-primase and with the individual subunits have not been investigated. To address this issue, equal masses of purified mouse DNA polymerase α-primase, p180, p180-p68 complexes, primase dimer, and p48 were immobilized in the wells of an ELISA plate. Serum albumin-coated wells were used as a negative control. Soluble purified Py T antigen was added to each well and incubated for 1 h. After washing, bound Py T antigen was detected by using a mouse monoclonal antibody against Py T antigen, followed by

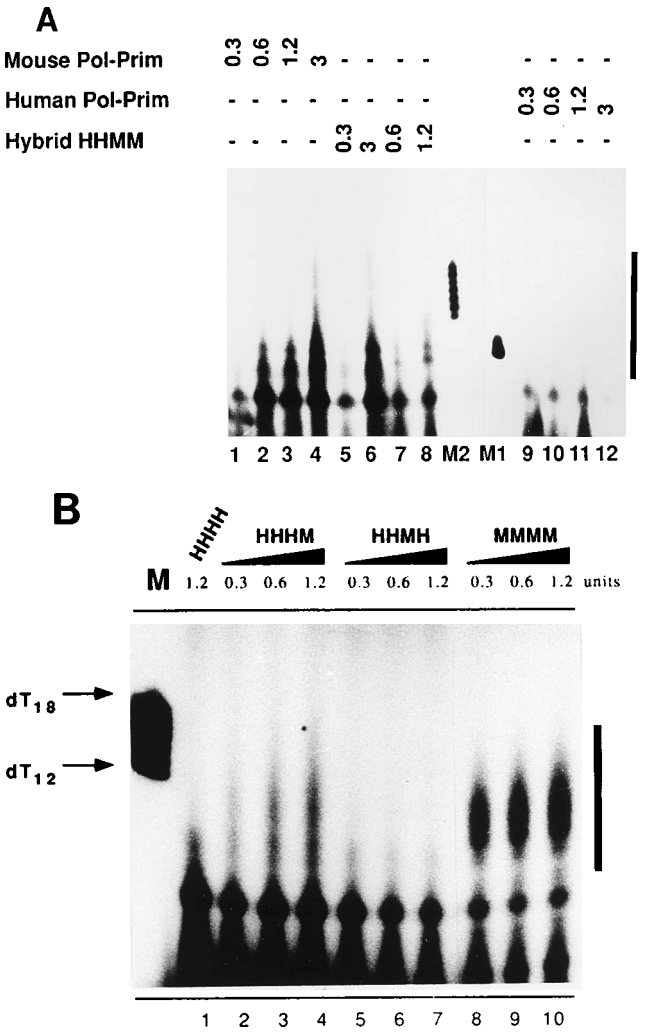


FIG. 4. Species-specific initiation of Py DNA replication by hybrid DNA polymerase α-primase complexes. The purified protein complexes (0.3 to 1.2 primase units) were tested for primer synthesis activity by using a template containing the Py origin. (A) Lanes 1 to 4, polymerase α-primase purified from mouse cells; lanes 5 to 8, recombinant HHMM; lanes 9 to 12, polymerase α-primase purified from human cells. M1, 5′-end-labeled oligo(dT)$_8$; M2, 5′-end-labeled oligo(dT)$_{12-18}$ ladder. The primase products were analyzed by electrophoresis in denaturing polyacrylamide gels and by autoradiography. (B) Lane M, 5′-end-labeled oligo(dT)$_{12-18}$ ladder. The vertical bars at the right indicate the primase products. The labeled species present in lanes containing human polymerase α-primase were also observed in reactions without polymerase α-primase.

peroxidase-conjugated rabbit anti-mouse antibody and a chromogenic substrate. The results revealed efficient binding of Py T antigen to the isolated primase and to p48 (Fig. 6). The intact DNA polymerase α-primase bound less Py T antigen than did p48 or primase dimer. This is probably due to the fact that the wells coated with DNA polymerase α-primase contain a lower molar amount of intact polymerase α-primase than those coated with an equal mass of p48 or primase dimer. Py T antigen associated less efficiently with mouse p180 and the mouse p180-p68 complex than with p48 or the complexes containing p48. However, the molar amounts of immobilized p180 and p180-p68 complex in each well were presumably greater than those of immobilized DNA polymerase α-primase complex. Taking these data together, we conclude that Py T antigen interacts directly with the p48 subunit of DNA primase and

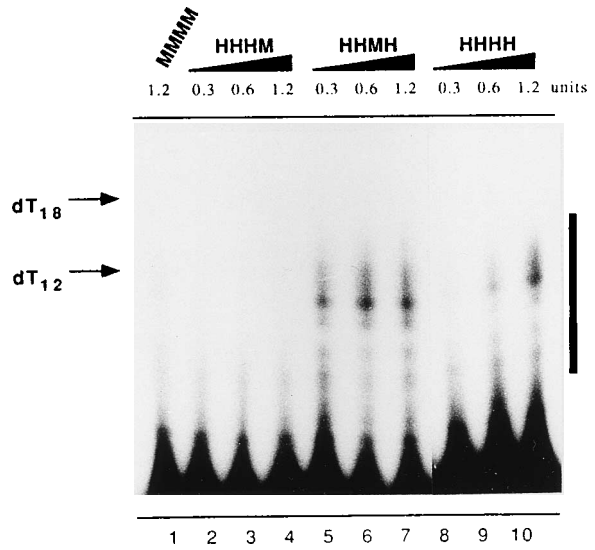


FIG. 5. Initiation of SV40 DNA replication by hybrid DNA polymerase α-primases. Purified polymerase-primase complexes (0.3 to 1.2 primase units) were tested for primer synthesis activity by using a plasmid DNA template containing the SV40 origin as described previously (57). The primase products were analyzed by electrophoresis in denaturing polyacrylamide gels and by autoradiography. The positions of 5′-end-labeled oligo(dT)$_{12}$ and oligo(dT)$_{18}$ are indicated by arrows. The vertical bar at the right indicates the primase products.

may associate more strongly with p48 and primase than with the two larger subunits.

DISCUSSION

DNA primase controls species-specific replication of Py DNA in vitro. On the basis of work from cell-free systems that reproduce the species specificity of SV40 and Py DNA replication, DNA polymerase α-primase has been shown to be primarily responsible for this species specificity (18, 39, 41, 44, 57). Here, we report that a recombinant mouse primase is

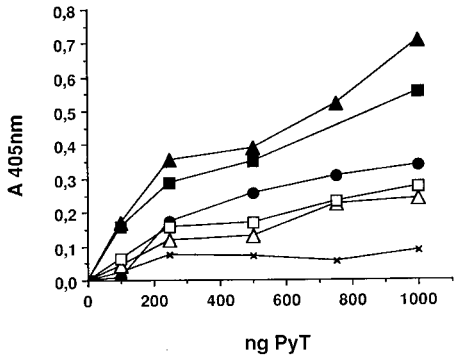


FIG. 6. Physical interactions of Py T antigen with mouse DNA polymerase α-primase and its subunits. Purified recombinant mouse DNA polymerase α-primase (●), p180 (△), p180-p68 complexes (□), DNA primase (■), and p48 (▲) were immobilized on ELISA plates (0.5 μg per well) and blocked with 3% BSA. BSA alone (×) was used as a control solid phase. Purified Py T antigen was added in the indicated amounts and incubated for 1 h. After washing, bound T antigen was detected by using monoclonal anti-T antibody F5 (2 μg per well), followed by peroxidase-coupled rabbit anti-mouse antibody (Dakopatts, Hamburg, Germany) (1:200) and a chromogenic substrate. As an additional control, Py T antigen immobilized on ELISA plates was incubated with the peroxidase-coupled second antibody and substrate in parallel; only a background signal was detected (not shown).

capable of supporting Py DNA replication in human cell extracts (Fig. 2). These data confirm and extend previous results suggesting that mouse primase determines the species specificity of Py DNA replication in vitro (18). In these earlier studies, mouse DNA primase was prepared by dissociating it from the purified DNA polymerase α-primase complex. Consequently, it is difficult to be certain that the primase did not contain traces of residual mouse DNA polymerase α that could contribute to Py DNA replication. In our studies, the mouse DNA primase was purified from baculovirus-infected insect cells in the complete absence of the other two mouse DNA polymerase α-primase subunits, confirming that the DNA primase subunits were sufficient for the species-specific control of Py DNA replication in vitro in human cell extracts.

The activity of the mouse primase subunits in undepleted human cell extracts suggests that the mouse subunits are capable of interacting functionally with human DNA polymerase α or human DNA polymerase α-primase. Currently, we do not understand the mechanism of this interaction. All of the recombinant DNA polymerase α-primase complexes could be isolated with similar yields and subunit compositions (Fig. 1; Table 1), implying that the complexes are stable and do not dissociate significantly during purification even under the fairly harsh conditions required to elute the complex from immunoaffinity columns. It is possible that the added mouse primase subunits partially displace the human primase subunits from endogenous preformed complexes simply because primase is present in excess. Alternatively, it may be that the mouse primase subunits associate with free p180 and p68 present in the human cell extracts to form an active hybrid DNA polymerase α-primase complex. Two other possibilities are that the subunits exchange during the replication process and that free mouse primase synthesizes primers at the origin without associating with the larger subunits. Experiments to address these questions are in progress.

Here, we report that the addition of the p48 subunit to crude human cell extracts supported low levels of Py DNA replication, although the replication activity of p48 was not concentration dependent and the level of replication was very low compared with that obtained with the mouse p58-p48 dimer (Fig. 2). This result raised the question of whether p48 might be responsible for the species specificity. Its low activity could be due to inefficient complex formation between mouse p48 and human DNA polymerase α-primase in the S100 cell extract. Alternatively, there may be only a small amount of free p58 available in the extract to associate with the mouse p48 subunit, and once this is bound, further addition of p48 may have no effect. The primase activity of p48 may also be unstable under replication conditions unless it associates with p58 (60).

To pursue the potential role of p48 in the species specificity of Py DNA replication, we separately expressed each of the mouse primase subunits with three subunits of human DNA polymerase α-primase. We found that hybrid DNA polymerase α-primase complexes containing mouse p48 and either mouse or human p58 were active in the replication of Py DNA, but complexes containing human p48 and mouse p58 were not (Fig. 3). Moreover, the HHHM complex was active in the initiation of Py DNA replication, but a complex containing HHMH was not. Conversely, the HHHM did not synthesize oligoribonucleotides from the SV40 origin of replication, while the HHMH complex did. Although these data do not rule out a contribution of the mouse p180, p68, or p58 subunit to the species specificity of replication, they argue strongly that p48 is the major determinant of the species specificity of Py DNA replication and suggest that it may also play a role in controlling the species specificity of SV40 DNA replication.

The data presented here do not directly address the question of whether a single human subunit would impair the Py DNA replication activity of a polymerase α-primase containing three murine subunits. If p48 is, in fact, the sole subunit controlling the species specificity of Py DNA replication, one would expect that a hybrid enzyme containing the human p180, p68, or p58 subunit should replicate Py DNA as well as the MMMM enzyme. Preliminary results indicate that HMMM and MHMM replicated Py DNA as well as MMMM, whereas MMHH was inactive. These results support the interpretation that primase or one of its subunits is the sole determinant of species specificity of Py DNA replication, but not all combinations of murine and human subunits have been tested.

Superficially, our results on SV40 initiation might appear to contradict the results of Murakami et al. (44) who showed that both human polymerase α and human primase were required for the synthesis of SV40 DNA in mouse extracts. However, hybrid human-mouse primase dimers, such as those discussed here, were not tested in the previous work. Moreover, the subunits controlling the species specificity of SV40 and Py DNA replication need not be identical. Although a hybrid polymerase-primase complex containing mouse p58 and human p48 was active in SV40 initiation assays, the role of the two larger subunits in species-specific replication of SV40 DNA has not yet been addressed. Clearly, it will be of interest to determine whether either of these human subunits is required in addition to human p48 to replicate SV40 DNA in vitro.

Interaction of Py T antigen with DNA primase. Our data demonstrate a direct physical interaction between purified Py T antigen and purified mouse DNA primase. The association of Py T antigen with DNA primase was somewhat unexpected, since SV40 T antigen appeared to be unable to associate with purified DNA primase (15, 16). However, it should be noted that in those experiments, the DNA primase was either denatured and partially renatured on blots (16) or isolated after dissociation from purified calf thymus DNA polymerase α-primase (15). Although the dissociated primase was enzymatically active, its activity in SV40 DNA replication was not tested (15). The possible influence of the purification method or the species of origin of the primase on its ability to associate with Py and SV40 T antigens is currently under investigation.

Several examples of other DNA helicases that interact with a cognate DNA primase in DNA replication have been reported. The *Escherichia coli* helicases DnaB and PriA interact with the primase DnaG to form primosomes active in *E. coli oriC* and ϕX174 DNA replication (reference 29 and references therein; reference 65a). The herpes simplex virus-encoded UL5 and UL52 proteins associate as a complex that has both helicase and primase activities (13). The T7 DNA helicase and primase not only form a complex but are encoded by the same gene (reference 37 and references therein). The helicase and primase encoded by bacteriophage T4 genes 41 and 61 interact functionally but have not been shown to exist in a complex (reviewed in reference 50). By analogy, we anticipate that the association of Py T antigen with DNA primase may play a role in Py DNA replication.

How does p48 primase determine the species-specific initiation of Py DNA replication? As in the initiation of SV40 DNA replication, binding of a Py T antigen double hexamer to the Py origin of replication causes the destabilization of duplex DNA and local unwinding of the origin, followed by binding of mammalian RP-A (17, 34, 71, 72). The next step in initiation probably involves the association of DNA polymerase α-primase,

followed by the synthesis of the first set of primers. This step has been shown to be the species-specific step in both SV40 and Py DNA replication (57) (Fig. 4 and 5). Our results demonstrate that primer synthesis during initiation of Py DNA replication requires the mouse p48 subunit and that the human subunit cannot substitute for it, raising the question of how the p48 subunit mediates this species specificity.

The p48 subunit carries out multiple functions in eucaryotic DNA replication. It binds to single-stranded DNA (10) and appears to be the catalytic subunit of DNA primase (1, 10, 20, 46, 54, 60). The p48 subunit associates specifically with the p58 primase subunit (10, 60) and interacts genetically (33) and physically (15, 47) with RP-A, probably with the p70 subunit of RP-A (15, 33, 47), as well as with Py T antigen (Fig. 6). Many of these protein-protein interactions have been shown to be essential for primosome assembly and initiation of DNA replication (8, 14, 15, 19, 28, 33, 36, 40, 56, 57).

These observations lead us to suggest that the role of p48 in controlling the species-specific initiation of viral replication could lie in a step during recognition by DNA polymerase α-primase of the single-stranded origin DNA complexed with RP-A or during primer synthesis. The core origins of replication of Py and SV40 DNA are structurally similar and each contains four binding sites for T antigen, yet they are essential elements in determining the species specificity of viral replication (2). The T antigen-binding site II in the SV40 core origin differs from the equivalent region in the Py core origin, in that the central pentanucleotide sites of SV40 binding site II are separated by one nucleotide, while the central pentanucleotide sites of the corresponding Py DNA binding site overlap. This spatial difference may require slightly different orientations of DNA polymerase α-primase in the preinitiation complex on the two origins. One possible mechanism to adjust the orientation of DNA polymerase α-primase is suggested by the observation that Py T antigen associates with DNA polymerase α-primase predominantly through the primase. We speculate that in the context of the preinitiation complex on Py origin DNA, DNA polymerase α-primase containing the human p48 primase subunit either cannot bind to or cannot utilize single-stranded origin template DNA coated by RP-A, whereas DNA polymerase α-primase containing mouse p48 can do so. The species specificity of replication could also be determined by p48 at an earlier step in the initiation reaction. For example, DNA polymerase α-primase has been reported to play a role in promoting the assembly of T antigen complexes on the origin or in facilitating the unwinding of the duplex origin DNA (43). It is possible that species-specific interactions between origin-bound T antigen, RP-A, and the p48 subunit of DNA polymerase α-primase are involved in these preinitiation reactions. Additional experiments to explore these possibilities are in progress.

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The first three authors contributed equally to this work.

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