MOLECULAR AND CELLULAR BIOLOGY, Aug. 1994, p. 5114–5122
0270-7306/94/$04.00+0

Vol. 14, No. 8

Simian Virus 40 Origin- and T-Antigen-Dependent DNA Replication with *Drosophila* Factors In Vitro

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Received 9 March 1994/Returned for modification 26 April 1994/Accepted 11 May 1994

DNA replication of double-stranded simian virus 40 (SV40) origin-containing plasmids, which has been previously thought to be a species-specific process that occurs only with factors derived from primate cells, is catalyzed with an extract derived from embryos of the fruit fly *Drosophila melanogaster*. This reaction is dependent upon both large T antigen, the SV40-encoded replication initiator protein and DNA helicase, and a functional T-antigen binding site at the origin of DNA replication. The efficiency of replication with extracts derived from *Drosophila* embryos is approximately 10% of that observed with extracts prepared from human 293 cells. This activity is not a unique property of embryonic extracts, as cytoplasmic extracts from *Drosophila* tissue culture cells also support T-antigen-mediated replication of SV40 DNA. By using highly purified proteins, DNA synthesis is initiated by *Drosophila* polymerase α-primase in a T-antigen-dependent manner in the presence of *Drosophila* replication protein A (RP-A; also known as single-stranded DNA-binding protein), but neither human RP-A nor *Escherichia coli* single-stranded DNA-binding protein could substitute for *Drosophila* RP-A. In reciprocal experiments, however, *Drosophila* RP-A was able to substitute for human RP-A in reactions carried out with human polymerase α-primase. These results collectively indicate that many of the specific functional interactions among T antigen, polymerase α-primase, and RP-A are conserved from primates to *Drosophila* species. Moreover, the observation that SV40 DNA replication can be performed with *Drosophila* factors provides a useful assay for the study of bidirectional DNA replication in *Drosophila* species in the context of a complete replication reaction.

DNA replication of the simian virus 40 (SV40) minichromosome is an important model system for the analysis of bidirectional double-stranded DNA replication in higher eukaryotes. Replication of the SV40 genome utilizes the host replication machinery in conjunction with a single virus-encoded protein, the large tumor antigen (T antigen). The development of a cell extract that is capable of replicating double-stranded DNA in the presence of an SV40 origin of replication has led to the identification of the components of the eukaryotic replication machinery and the characterization of their biochemical functions (see, for instance, references 9, 19, 37, 40, and 43).

SV40 DNA replication initiates within a unique, well-defined origin sequence and proceeds bidirectionally. Initiation of replication appears to proceed as follows. First, T antigen binds a specific sequence in the SV40 origin of replication and induces a structural distortion of the DNA in the presence of ATP. The helicase activity of T antigen then unwinds DNA at the origin in conjunction with a cellular single-stranded DNA-binding protein (which is variously termed RP-A [replication protein A], replication factor A, or human SSB [single-stranded DNA-binding protein]) that binds to the newly unwound stretches of single-stranded DNA. In the next step, T antigen recruits DNA polymerase α-primase to the DNA through specific protein-protein interactions to form an initiation complex. The primase subunit then synthesizes a short RNA primer on the lagging template strands, and this primer is extended by DNA polymerase α to form a short initiator DNA known as an Okazaki fragment. The resulting template-primer junction is recognized by a second multiprotein complex composed of replication factor C and proliferating cell nuclear antigen, both of which are accessory proteins for DNA polymerase δ. Binding of DNA polymerase δ to this complex then leads to a polymerase switching step, in which polymerase δ replaces polymerase α-primase as the leading-strand polymerase. Both polymerase α-primase and polymerase δ cooperate to synthesize Okazaki fragments on the lagging-strand template during the elongation phase of the replication reaction.

SV40 DNA replication has been considered to be highly species specific, as T-antigen-mediated replication can be re-created in vitro with extracts from human and monkey cells but not with extracts from mouse cells (see, for instance, references 23 and 28). This apparent primate specificity of replication is mediated by species-specific protein-protein interactions between T antigen and DNA polymerase α-primase which stimulate DNA polymerase α-primase activity (6, 7, 11–13, 26, 28, 34, 46). Specific interactions have also been observed between DNA polymerase α-primase and RP-A and are believed to be stimulatory (6, 14, 20, 26, 41). In addition, a species-specific interaction between T antigen and RP-A may function to overcome RP-A-mediated inhibition of DNA replication priming (6, 12, 26). It is therefore evident that T-antigen-mediated initiation of replication is dependent upon a network of functionally important interactions among T antigen, polymerase α-primase, and RP-A.

Extracts from *Drosophila* embryos that can support complementary-strand synthesis of single-stranded DNA into double-stranded DNA and assembly of the newly synthesized DNA into chromatin have been recently described (2, 18). In this

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study, we demonstrated that the *Drosophila* S-190 extract (18) is competent for T-antigen-mediated SV40 DNA replication. The initiation of DNA replication is the consequence of functional interactions among T antigen, *Drosophila* DNA polymerase α-primase, and *Drosophila* RP-A. We present these data and discuss the species specificity of SV40 DNA replication as well as the further application of these findings toward the purification and analysis of *Drosophila* proteins involved in DNA replication.

MATERIALS AND METHODS

Purification of proteins. SV40 T antigen was purified by immunoaffinity chromatography from Sf9 cells infected with a recombinant baculovirus as described previously (22, 33, 36). Topoisomerases I and II were prepared from calf thymus by slight modification of established procedures (25, 32). Human RP-A and polymerase α-primase were purified as described previously (26). *Drosophila* RP-A and polymerase α-primase were prepared as described by Mitsis et al. (27). *Escherichia coli* SSB was purchased from Pharmacia Biotech.

DNA templates. Plasmid pSV011 contains the SV40 origin of replication region from the *Hin*dIII to *Sph*I sites (nucleotides 5171 to 128) inserted into the *Hin*dIII and *Sph*I sites of pUC18 (31). Plasmid pSV011 ori$^-$ is identical to pSV011 except that it carries a defective origin of replication due to the p8-4 mutation in T-antigen binding site II (15). This mutation was generated by cleavage of the unique *Bgl*I site followed by treatment with *E. coli* DNA polymerase I and religation to create a new *Hpa*II site by the net deletion of 4 bp (SV40 positions 5239 to 5242).

Preparation of cell extracts. *Drosophila* S-190 extract was prepared as described previously (18). *Drosophila* Kc cell cytosolic extract was a gift of R. Kanaar and D. Rio. The extracts were made from cells grown in D-22 medium (13a) and was initially prepared as a 100,000 × *g* supernatant of the cytoplasmic fraction from cells hypotonically swelled and homogenized as described previously (10). To concentrate this extract further and to reduce the ionic strength, it was dialyzed against 25 mM Tris-HCl (pH 7.5)–25 mM NaCl–1 mM EDTA–0.01% (vol/vol) Nonidet P-40–10% (vol/vol) glycerol–20% (wt/vol) sucrose–1 mM dithiothreitol–0.5 mM phenylmethylsulfonyl fluoride prior to use. The human 293 cell extract was prepared as described previously (38). *Xenopus* cytoplasmic egg extract was a gift of M. Dasso and was prepared by the method of Newport (29).

Replication reactions with human 293, *Drosophila* embryonic, and *Xenopus* egg extracts. Reaction mixtures contained final concentrations of 30 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) (K^+) (pH 8.0), 0.5 mM dithiothreitol, 7 mM $MgCl_2$, 4 mM ATP, 200 μM each GTP, UTP, and CTP, 100 μM each dGTP, dTTP, and dCTP, 25 μM unlabeled dATP, 2 μg of plasmid DNA (either pSV011 or pSV011ori$^-$) per ml, 40 mM creatine phosphate, 20 μg of creatine phosphokinase per ml, 20 μCi of [α-^{32}P]dATP per ml, 45 μg of T antigen per ml, and 12 mg of extract (30 μl of a 20-mg/ml extract from human, *Drosophila*, or *Xenopus* cells) per ml in a final volume of 50 μl. The samples were prepared on ice and then incubated at 30°C for 1 h unless indicated otherwise. (When included in the reactions, aphidicolin [Sigma catalog no. A 0781], α-amanitin [Sigma catalog no. A 2263], and 5′-adenylylimidodiphosphate [Sigma catalog no. A 2647] were used.) The reactions were terminated by the addition of 50 mM EDTA (Na^+) (pH 8.0) (10 μl). RNase A (10 μl of 0.1 mg/ml) was added, and the samples were incubated at 37°C for 15 min. RNase A digestion was terminated by the addition of stop solution (20 mM EDTA [Na^+] [pH 8.0], 0.2 M NaCl, 1% [wt/vol] sodium dodecyl sulfate [SDS], 0.25 mg of glycogen per ml) (50 μl) and proteinase K (2 μl of 2.5 mg/ml). The samples were incubated at 37°C for 15 min and then extracted once with phenol-chloroform-isoamyl alcohol (25:24:1, vol/vol/vol), extracted once with chloroform-isoamyl alcohol (24:1, vol/vol), and precipitated with ethanol. The reaction products were resolved on a 1% (wt/vol) agarose gel in 1× Tris-borate-EDTA buffer and subjected to electrophoresis at 1 to 2 V/cm for 10 to 14 h at room temperature. Following electrophoresis, the gels were stained with ethidium bromide and photographed. The gels were fixed in a solution containing 15% (vol/vol) methanol and 15% (vol/vol) glacial acetic acid for 30 min, dried, and then subjected to autoradiography.

***Dpn*I digestion of newly replicated DNA.** DNA replication reactions were terminated by the addition of 50 mM EDTA (Na^+) (pH 8.0) (10 μl). RNase A (10 μl of 0.1 mg/ml) was added, and the samples were incubated at 37°C for 15 min. The RNase A digestion was terminated by the addition of stop solution (20 mM EDTA [Na^+] [pH 8.0], 0.2 M NaCl, 1% [wt/vol] SDS, 0.25 mg of glycogen per ml) (50 μl) and proteinase K (2 μl of 2.5 mg/ml). The samples were incubated at 37°C for 15 min, extracted twice with phenol-chloroform-isoamyl alcohol (25:24:1, vol/vol/vol), extracted once with chloroform-isoamyl alcohol (24:1, vol/vol), and precipitated with ethanol. The reaction products were digested with *Dpn*I (2 μl; 40 U; New England Biolabs) in a total volume of 30 μl at 37°C for 60 min. The resulting samples were then analyzed by electrophoresis in a 1% (wt/vol) agarose gel in Tris-borate buffer.

DNA replication with purified components. Reaction mixtures contained final concentrations of 30 mM HEPES (Na^+) (pH 8.0), 0.5 mM dithiothreitol, 7 mM $MgCl_2$, 4 mM ATP, 200 μM each GTP, UTP, and CTP, 100 μM each dGTP, TTP, and dCTP, 25 μM unlabeled dATP, 6 μg of pSV011 plasmid DNA per ml, 40 mM creatine phosphate, 0.1 mg of creatine phosphokinase per ml, 0.1 mg of acetylated bovine serum albumin per ml, 4 μg of topoisomerase I per ml, 1.8 μg of topoisomerase II per ml, 40 μCi of [α-^{32}P]dATP per ml, and the indicated amounts of polymerase α-primase, RP-A/SSB, and T antigen in a total volume of 10 μl. The reaction mixtures were incubated at 37°C for 1 h. A portion of the sample (5 μl) was removed to determine the incorporation of the radiolabeled nucleotide by spotting samples onto Whatman DE81 paper as described by Melendy and Stillman (26). The remainder of the reaction was terminated by the addition of a solution (45 μl) containing 0.22 mg of pronase per ml, 1.1% (wt/vol) SDS, and 11 mM EDTA, and the pronase digestion was carried out for 1 h at 37°C. Product DNA was extracted with phenol-chloroform-isoamyl alcohol (25:24:1, vol/vol/vol), precipitated with ethanol in the presence of glycogen (5 μg), washed with 75% (vol/vol) ethanol, and dried under a vacuum. The DNA was then analyzed by electrophoresis in alkaline 1% (wt/vol) agarose gels. DNA synthesis is indicated as picomoles of dAMP incorporated in the equivalent of a 50-μl assay volume.

DNA replication with Kc cell extracts. Buffer and template concentrations were identical to those used for the reactions with purified components. The reaction volume was 50 μl, and reaction mixtures were incubated at 37°C for 1 h. Reactions were stopped by addition of a solution (10 μl) containing 0.1 mg of pancreatic RNase A per ml and 50 mM EDTA. The RNase A digestion, which degrades endogenous RNA, was carried out at 37°C for 15 min. The RNase reactions were terminated by the addition of a solution (10 μl) containing 1 mg of pronase per ml, 5% (wt/vol) SDS, 10 mM Tris-HCl, pH 8.0, and 1 mM EDTA, and the samples were incubated at 37°C

for 1 h. DNA was extracted and precipitated as described for the reactions with purified components. The reaction products were analyzed on 1% (wt/vol) agarose gels in Tris-borate-EDTA buffer. DNA synthesis was quantitated as described for the reactions with purified components.

RESULTS

SV40 origin- and T-antigen-dependent DNA replication with *Drosophila* extracts. SV40 DNA replication in cytosolic extracts from human cells is accompanied by concomitant chromatin assembly in the presence of a crude nuclear extract or purified chromatin assembly factor I (CAF-I) (35, 36). To search for chromatin assembly activities in cell extracts from other organisms, an S-190 extract from *Drosophila* embryos (18; see also reference 2) was added to human cytosolic extract and SV40 T antigen, and replicated DNA was analyzed for evidence of chromatin assembly. Surprisingly, in a control reaction containing *Drosophila* extract without human cytosolic extract, DNA replication still occurred. On the basis of this initial observation, we further investigated the ability of the *Drosophila* factors to support SV40 replication.

Extracts derived from human 293 cells, *Xenopus* eggs, or *Drosophila* embryos were used in the replication of a double-stranded plasmid template (pSV011) that contains an SV40 origin of replication (31). Replication reactions were performed in either the presence or the absence of purified T antigen, and the products were analyzed on an agarose gel. As shown in Fig. 1A, both the human 293 cell extract and the *Drosophila* extract were capable of replicating double-stranded DNA in a T-antigen-dependent manner. In contrast, SV40 replication was not observed with the *Xenopus* egg extract, which was otherwise competent for replication of single-stranded DNA into double-stranded DNA (reference 21 and data not shown). Moreover, with the *Drosophila* S-190 extract, the replication products were a mixture of highly supercoiled DNA and nicked circular DNA, which suggested that the newly replicated DNA was assembled efficiently into chromatin in a process similar to that mediated by CAF-I (35, 36) (Fig. 1A, lanes 1 to 4).

Comparison of the quantity of DNA synthesized by equivalent amounts (in terms of mass of protein) of either *Drosophila* or human 293 cell extracts revealed that the human extract incorporated about 300 pmol of dAMP, while the *Drosophila* extract incorporated about 30 pmol of dAMP. Thus, the amount of replication that occurs with the *Drosophila* S-190 extract is approximately 10-fold lower than that obtained with the human 293 cell extracts but is only about 2-fold lower than the amount seen with HeLa cell extracts (38) and slightly greater than that observed with Chinese hamster ovary cell extracts (24). However, 293 cell extracts are unusually active compared with extracts from other human cells (38).

We also examined the ability of a cytoplasmic extract from *Drosophila* Kc cells to replicate double-stranded DNA templates containing an origin of SV40 replication in a T-antigen-dependent manner. We performed a series of replication reactions with increasing amounts of Kc cell extract in either the presence or the absence of T antigen. As depicted in Fig. 1B, the amount of DNA synthesis increased with the concentration of the Kc cell extract. It is therefore apparent that both *Drosophila* embryos and cultured cells are capable of replicating SV40 double-stranded DNA in a T-antigen-dependent manner similar to that observed with human cells.

To test the specificity of DNA replication with the *Drosophila* S-190 extract for a functional origin of replication, we carried out reactions with either wild-type or defective mutant versions of the SV40 origin of replication. In these experiments, we used a derivative of pSV011, termed pSV011ori$^-$, that contained a defective origin of replication due to a mutation in T-antigen binding site II (15). In the presence but not the absence of T antigen, SV40 DNA replication was observed with the pSV011 but not the pSV011ori$^-$ template with either the human 293 extract or the *Drosophila* S-190 extract (Fig. 2). Hence, the human and *Drosophila* factors displayed identical requirements for T antigen and a functional SV40 origin of replication.

We additionally investigated the effects of various poly-

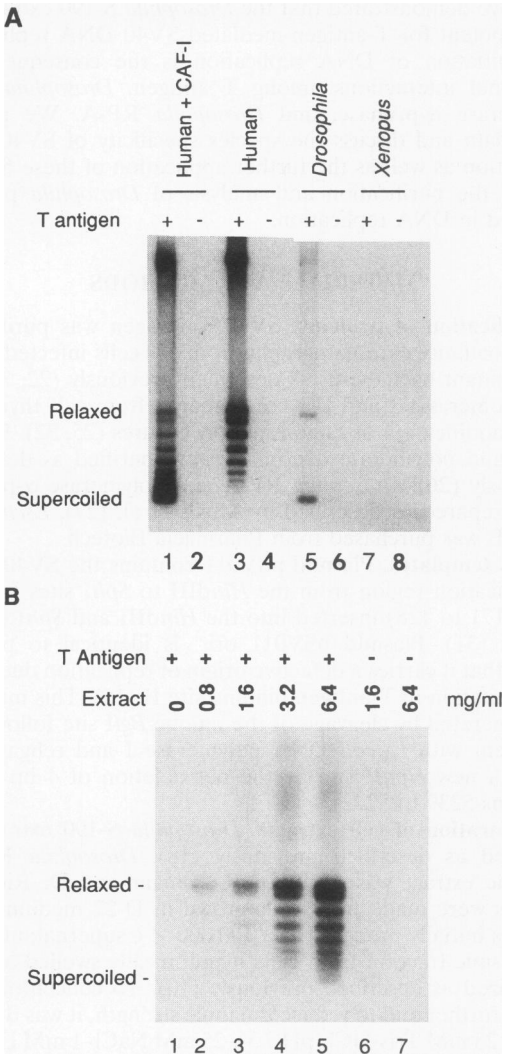

FIG. 1. Replication of double-stranded DNA templates in vitro with human, *Drosophila*, and *Xenopus* extracts. (A) Double-stranded pSV011 DNA (2 μg/ml) was used as a template in replication reactions with extracts from human 293 cells, *Drosophila* embryos, or *Xenopus* eggs. Recombinant T antigen (45 μg/ml) was added to the reactions shown in lanes 1, 3, 5, and 7. Reaction mixtures in lanes 1 and 2 also contained purified human CAF-I (1.4 μg/ml). (B) T-antigen-dependent DNA synthesis catalyzed by a cytosolic extract from *Drosophila* Kc tissue culture cells. Reactions shown in lanes 1 to 5 were performed with increasing concentrations of extract in the presence of T antigen (84 μg/ml). Lanes 6 and 7 represent reaction mixtures that contained two different concentrations of extract in the absence of T antigen. The reactions in lanes 1 to 7 generated 0.31, 1.08, 2.39, 3.76, 4.72, 0.45, and 0.64 pmol of incorporated dAMP, respectively.

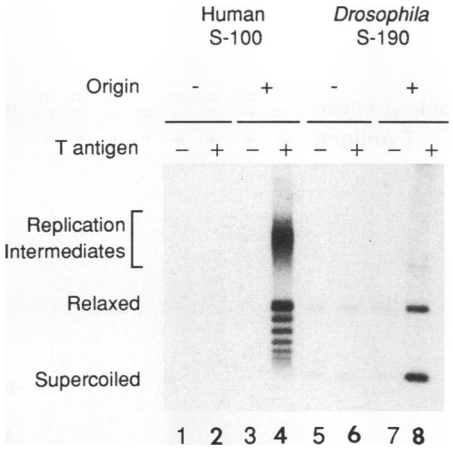

FIG. 2. Both T antigen and a functional SV40 origin of replication are required for the replication of double-stranded DNA in vitro. Reactions in lanes 1 to 4 were performed with human 293 cytoplasmic extract, while the reactions in lanes 5 to 8 were carried out with *Drosophila* embryo extract. pSV011ori$^-$, which contains a defective mutant version of the SV40 origin of replication, was used as a template in lanes 1, 2, 5, and 6. pSV011, which contains a wild-type origin sequence, was used in the reactions shown in lanes 3, 4, 7, and 8. Purified T antigen (15-μg/ml final concentration) was included in the reactions shown in lanes 2, 4, 6, and 8. The positions of supercoiled DNA and relaxed (or nicked circular) DNA are indicated. The location of replication intermediates is denoted by a bracket.

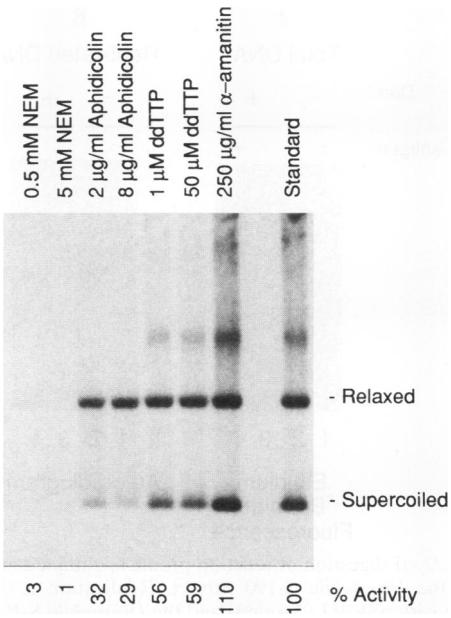

FIG. 3. Effects of polymerase inhibitors on DNA replication by the *Drosophila* S-190 extract. Complete replication reactions were carried out either in the absence of inhibitors (Standard) or in the presence of the indicated concentrations of polymerase inhibitors. The numbers below the lanes indicate the relative amounts of replication. In this experiment, 100% represents 40 pmol of dAMP incorporated into the newly synthesized DNA. NEM, *N*-ethylmaleimide.

merase inhibitors upon the efficiency of DNA replication by the *Drosophila* factors. As shown in Fig. 3, replication is strongly inhibited by *N*-ethylmaleimide (at either 0.5 or 5 mM) and is insensitive to α-amanitin at a concentration (250 μg/ml) that inhibits both RNA polymerases II and III. DNA replication is weakly inhibited (about threefold inhibition of total replication products) by aphidicolin (at either 2 or 8 μg/ml). With this reagent, however, there was stronger inhibition of the synthesis of supercoiled DNA than that of relaxed (or nicked circular) DNA. (In addition, the degree of inhibition by aphidicolin varied with different preparations of the reagent [data not shown].) Finally, there was slight inhibition of replication by ddTTP (at either 1 or 50 μM). Collectively, these data are consistent with the observed properties of *Drosophila* DNA polymerases α (1, 8, 17) and δ (5, 30); it is therefore likely that the observed DNA synthesis was carried out by DNA polymerase α and/or δ.

DNA replication time course and products with *Drosophila* factors. Studies of the time course of DNA replication with human factors revealed a lag period of about 10 min before DNA replication was observed (38, 39, 42, 47). This lag is believed to reflect the requirement for T antigen, RP-A, and topoisomerase I to form a complex at the origin of replication prior to the initiation of DNA synthesis. A time course experiment with the *Drosophila* factors also revealed a slow presynthesis step, but with a slightly longer lag period of approximately 20 to 25 min (Fig. 4). The increased lag phase observed in *Drosophila* extract may reflect the slower formation of complexes between T antigen and *Drosophila* proteins. Alternatively, the longer lag phase could be due to the presence of histones in the *Drosophila* extract that may inhibit the binding of T antigen at the origin of replication (see, for example, reference 4). Consistent with either hypothesis is the observation that reactions with the *Drosophila* extracts accumulate fewer slowly migrating replication intermediates compared with reactions with the human extract (Fig. 2; compare lanes 4 and 8), which suggests that in the *Drosophila* extracts, the elongation phase of the reaction is fast relative to the earlier steps leading to the formation of the intermediates.

The nature of the replication products was further examined by using a *Dpn*I digestion assay (23). This assay is based on the observation that restriction enzyme *Dpn*I cleaves only DNA in which both strands are methylated. Therefore, unreplicated DNA produced in *dam*$^+$ *E. coli* is cleaved, whereas products generated by semiconservative DNA replication in vitro are resistant to *Dpn*I digestion. DNA replication reactions were carried out with the *Drosophila* S-190 extract under conditions in which about 7 to 10% of the DNA undergoes replication, and the samples were subsequently digested with *Dpn*I. Anal-

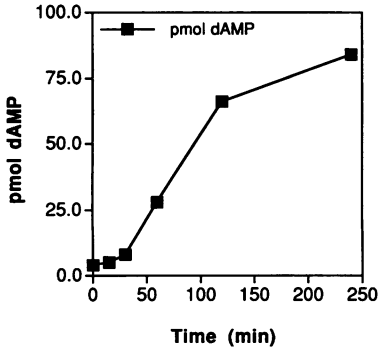

FIG. 4. Time dependence of DNA synthesis with the *Drosophila* embryonic extract. The incorporation of dAMP in replication reactions performed with *Drosophila* embryonic extracts is presented as a function of time.

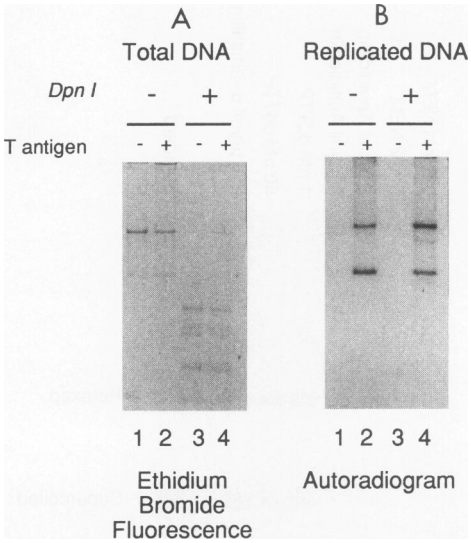

FIG. 5. *Dpn*I digestion of reaction products obtained after replication with the *Drosophila* S-190 extract. Replication reactions were carried out with pSV011 (2 μg/ml) and the *Drosophila* S-190 extract in either the presence (lanes 1 and 3) or the absence (lanes 2 and 4) of T antigen (45 μg/ml). The reaction products shown in lanes 3 and 4 were digested with *Dpn*I, and lanes 1 and 2 are the corresponding reaction products that were not treated with *Dpn*I. (A) Ethidium bromide-stained fluorograph of the agarose gel; (B) autoradiogram of the same gel.

ysis of the bulk of the DNA by agarose gel electrophoresis and ethidium bromide staining revealed cleavage of the majority of the pSV011 DNA into several small fragments (Fig. 5A). In contrast, the newly replicated DNA, which was radiolabeled by incorporation of [α-^{32}P]dATP, was nearly completely resistant to digestion by *Dpn*I, as detected by autoradiography (Fig. 5B). It is therefore unlikely that the observed incorporation of radiolabeled dAMP into pSV011 was due to the action of DNA repair enzymes, as all of the *Dpn*I sites in the radiolabeled DNA had become resistant to digestion by *Dpn*I. Hence, these results are consistent with semiconservative replication of the pSV011 template by the *Drosophila* replication machinery.

Initiation of SV40 replication with purified *Drosophila* RP-A and polymerase α-primase. To examine interactions among T antigen, polymerase α-primase, and RP-A under well-defined conditions, we used minimal, replication initiation reactions with the three factors purified to near homogeneity (16, 40, 45, 46). This monopolymerase replication reaction includes T antigen, polymerase α-primase, RP-A, topoisomerases I and II, and template DNA and results in the synthesis of single-stranded DNA segments that predominantly range in length from 100 to 1,000 nucleotides. This reaction appears to reflect the early steps in the initiation of replication prior to the involvement of replication factor C, proliferating cell nuclear antigen, and DNA polymerase δ but is also somewhat artificial because at low levels of RP-A and in the absence of replication factor C and proliferating cell nuclear antigen, polymerase α is able to reprime on the 3′ ends of DNA products that it has just completed and therefore can synthesize the leading strand in the absence of polymerase δ (42, 43). In our studies, we sought to use this polymerase α-dependent replication reaction to investigate specific functional interactions among T antigen, RP-A, and polymerase α-primase that are believed to be responsible for the species specificity of SV40 replication.

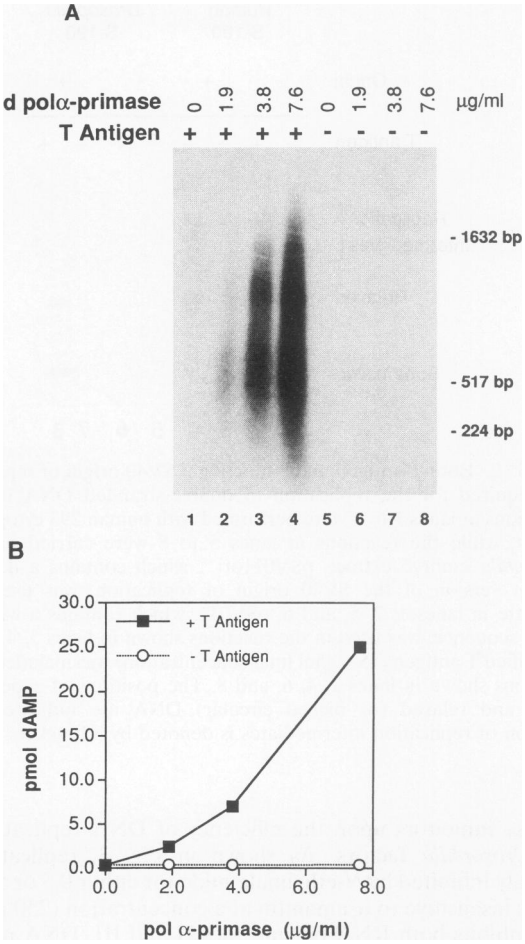

FIG. 6. T-antigen-dependent DNA synthesis by *Drosophila* polymerase α-primase in the presence of *Drosophila* RP-A. (A) Analysis of replication products by alkaline agarose gel electrophoresis. DNA replication reactions contained 10 μg of *Drosophila* RP-A per ml and *Drosophila* polymerase α (d polα)-primase at the indicated final concentrations. T antigen was either added at a final concentration of 84 μg/ml (lanes 1 to 4) or omitted (lanes 5 to 8). (B) Quantitation of DNA synthesis. The incorporation of dAMP is shown as a function of the concentration of polymerase α-primase.

By using highly purified *Drosophila* polymerase α-primase, *Drosophila* RP-A, recombinant T antigen, and calf thymus topoisomerases I and II, we investigated the initiation of DNA synthesis from an SV40 replication origin. As shown in Fig. 6, initiation of replication with the purified *Drosophila* factors was dependent upon T antigen. In the presence of a fixed amount of topoisomerases I and II, T antigen, and *Drosophila* RP-A, increasing the amount of *Drosophila* polymerase α-primase resulted in a corresponding increase in the amount of DNA replication. In addition, replication with the purified *Drosophila* factors was dependent upon the presence of a functional origin of replication (data not shown). Hence, *Drosophila* polymerase α-primase and RP-A can productively function with T antigen in the purified, minimal replication initiation reaction.

***Drosophila* RP-A can substitute for human RP-A for the initiation of SV40 DNA replication.** To determine whether the specificity of replication could be ascribed to the *Drosophila* factors involved in the initiation of replication, we compared

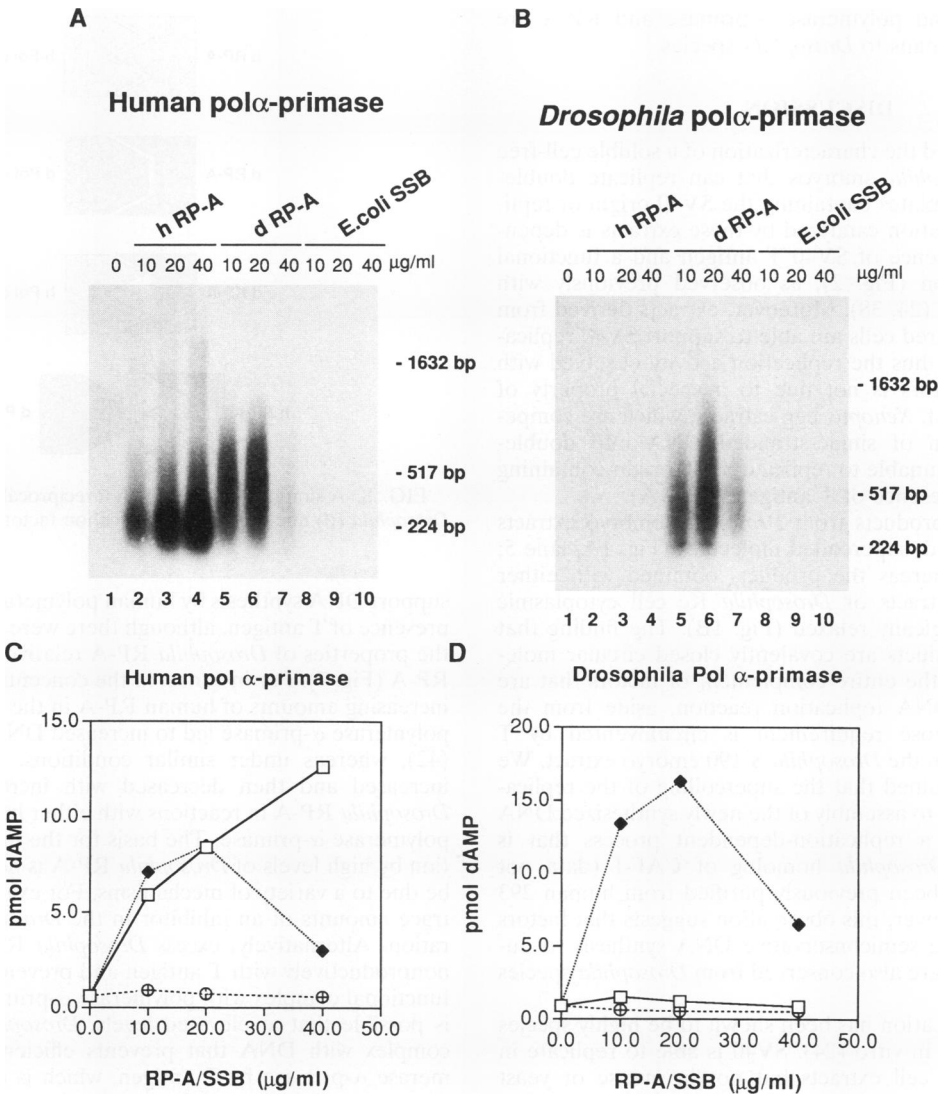

FIG. 7. Species specificity of functional interactions between polymerase α-primase and RP-A/SSB. (A) Analysis of replication products by alkaline agarose gel electrophoresis. DNA replication reaction mixtures contained T antigen (84 μg/ml), human polymerase α (polα)-primase (40 μg/ml), and other components as described in Materials and Methods. Reactions were performed with the concentrations of human (h) and *Drosophila* (d) RP-A and *E. coli* SSB indicated above the lanes. (B) DNA replication reactions were carried out as for panel A except that *Drosophila* polymerase α-primase (7.6 μg/ml) was used instead of its human counterpart. Note that the concentration of human polymerase α-primase used in panel A (40 μg/ml) was higher than that of the *Drosophila* polymerase α-primase in panel B (7.6 μg/ml). These concentrations were chosen to give equivalent amounts of polymerase activity, as determined by the method described by Melendy and Stillman (26). When equivalent concentrations of human versus *Drosophila* polymerase α-primase were used under the same conditions, the results were identical except that the amount of replication with the human polymerase was lower than that observed with the *Drosophila* polymerase (data not shown). (C) Plot of incorporation of dAMP as a function of RP-A/SSB with human polymerase α-primase. (D) Plot of incorporation of dAMP as a function of RP-A/SSB with *Drosophila* polymerase α-primase. Symbols for panels C and D: □, human RP-A; ◆, *Drosophila* RP-A; ⊕, *E. coli* SSB.

the abilities of human and *Drosophila* polymerase α-primase to initiate replication with human RP-A, *Drosophila* RP-A, or *E. coli* SSB (Fig. 7). With human polymerase α-primase, it was found that *Drosophila* RP-A could partially substitute for its human homolog, whereas *E. coli* SSB was unable to initiate replication with purified human polymerase α-primase (Fig. 7A and C). Moreover, replication with human polymerase α-primase and *Drosophila* RP-A was dependent upon both T antigen and an origin of replication (data not shown), as observed with the complete set of factors present in the crude S-190 extract (Fig. 2). When the complementary experiments were performed with *Drosophila* polymerase α-primase, low levels of *Drosophila* RP-A were able to stimulate DNA synthesis, although at higher levels of *Drosophila* RP-A, replication activity was reduced. Under identical conditions, however, neither human RP-A nor *E. coli* SSB was able to substitute for *Drosophila* RP-A (Fig. 7B and D). Hence, *Drosophila* RP-A can substitute for human RP-A in reactions containing human polymerase α-primase, whereas in contrast, human RP-A cannot substitute for *Drosophila* RP-A in reactions containing *Drosophila* polymerase α-primase. These findings indicate that a significant proportion of the specific interactions between

SV40 T antigen and polymerase α-primase and RP-A are conserved from humans to *Drosophila* species.

DISCUSSION

We have described the characterization of a soluble cell-free system from *Drosophila* embryos that can replicate double-stranded DNA templates containing the SV40 origin of replication. DNA replication catalyzed by these extracts is dependent upon the presence of SV40 T antigen and a functional origin of replication (Fig. 2), as observed previously with human cell extracts (24, 38). Moreover, extracts derived from *Drosophila* Kc cultured cells are able to support SV40 replication (Fig. 1B), and thus the replication activity observed with the *Drosophila* factors is not due to a special property of embryos. In contrast, *Xenopus* egg extracts, which are competent for replication of single-stranded DNA into double-stranded DNA, are unable to replicate SV40 origin-containing templates in the presence of T antigen (Fig. 1A).

The replication products from *Drosophila* embryo extracts are covalently closed, supercoiled molecules (Fig. 1A, lane 5; Fig. 2, lane 7), whereas the products obtained with either human 293 cell extracts or *Drosophila* Kc cell cytoplasmic extracts are topologically relaxed (Fig. 1B). The finding that the replication products are covalently closed circular molecules suggests that the entire complement of factors that are required for the DNA replication reaction, aside from the cellular factors whose requirement is circumvented by T antigen, is present in the *Drosophila* S-190 embryo extract. We have further determined that the supercoiling of the replication products is due to assembly of the newly synthesized DNA into chromatin in a replication-dependent process that is mediated by the *Drosophila* homolog of CAF-I (data not shown), which has been previously purified from human 293 cells (35, 36). Moreover, this observation suggests that factors that couple ongoing semiconservative DNA synthesis to nucleosome assembly are also conserved from *Drosophila* species to humans.

SV40 DNA replication has been shown to be highly species specific in vivo and in vitro (24). SV40 is able to replicate in simian and human cell extracts but not in mouse or yeast extracts. There is a large body of evidence suggesting that specific protein-protein interactions among T antigen, DNA polymerase α-primase, and RP-A are necessary for replication initiation (3, 6, 7, 11–14, 20, 26, 28, 34, 41, 42). Specifically, the lack of SV40 origin-dependent DNA replication in mouse cell extracts has been attributed to the absence of a functional interaction between T antigen and mouse polymerase α-primase (28). With the yeast factors, the inability to support SV40 replication is at least in part due to the blockage of T-antigen-mediated loading of polymerase α-primase onto DNA templates by yeast RP-A (26).

The observation that extracts from *Drosophila* embryos and cells were capable of supporting SV40 DNA replication prompted us to study the specificity of these protein-protein interactions by using a defined system composed of purified topoisomerases I and II, recombinant T antigen, RP-A, and polymerase α-primase. The results presented in Fig. 6 demonstrate the ability of the *Drosophila* proteins to interact productively with T antigen and initiate replication. From an evolutionary point of view, it is surprising that T antigen forms a functional initiation complex with *Drosophila* polymerase α-primase and RP-A but not with the homologous mouse proteins.

In reactions with mixed *Drosophila* and human factors, we observed that either *Drosophila* or human RP-A is able to support DNA synthesis by human polymerase α-primase in the presence of T antigen, although there were some differences in the properties of *Drosophila* RP-A relative to those of human RP-A (Fig. 7). For instance, in the concentration range tested, increasing amounts of human RP-A in the presence of human polymerase α-primase led to increased DNA synthesis (Fig. 7) (42), whereas under similar conditions, replication activity increased and then decreased with increasing amounts of *Drosophila* RP-A in reactions with either human or *Drosophila* polymerase α-primase. The basis for the inhibition of replication by high levels of *Drosophila* RP-A is not known but could be due to a variety of mechanisms. For example, there may be trace amounts of an inhibitor in the *Drosophila* RP-A preparation. Alternatively, excess *Drosophila* RP-A could interact nonproductively with T antigen and prevent it from forming a functional complex with polymerase α-primase. More likely, it is possible that at elevated levels, *Drosophila* RP-A forms a complex with DNA that prevents efficient loading of polymerase α-primase by T antigen, which is consistent with the aggregation of *Drosophila* RP-A≅DNA complexes at high concentrations (26a).

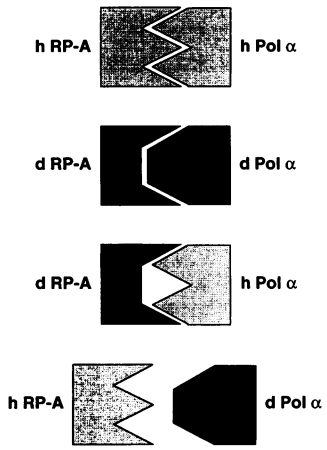

FIG. 8. A simple model for the nonreciprocal interactions between *Drosophila* (d) and human (h) replication factors. Pol, polymerase.

The observation that *Drosophila* RP-A can substitute for human RP-A in replication reactions carried out with human polymerase α-primase indicates that RP-A function is closely conserved from *Drosophila* species to humans, and it was therefore somewhat intriguing that reciprocal experiments revealed that human RP-A is not able to substitute for *Drosophila* RP-A in reactions performed with *Drosophila* polymerase α-primase (Fig. 7). Given the complexity of the replication process, in which each of the proteins interacts with multiple other factors, it may not, perhaps, be surprising to find such nonreciprocal effects. Offhand, it seems reasonable to speculate that the basis for the nonreciprocal interactions is related not to the intrinsic functional properties of the factors, as they are conserved from *Drosophila* species to humans, but rather to a nonessential difference in the three-dimensional structure of the factors. For instance, while *Drosophila* RP-A may be able to interact with human polymerase α-primase, steric hindrance may prevent the reciprocal interaction, as illustrated in Fig. 8. Further analysis of this nonreciprocal relationship between the *Drosophila* and human versions of the replication factors may lead to a better understanding of the interactions among RP-A, polymerase α-primase, and T antigen.

In summary, the data presented in this report suggest that many of the functional interactions among polymerase α-primase, RP-A, and T antigen have been conserved between *Drosophila* species and humans. This conservation of function of a fundamental biological process from *Drosophila* species to humans is reminiscent of that seen with basal RNA polymerase II transcription factors (44). In the future, the availability of the genes encoding *Drosophila* replication factors should lead to a better understanding of the protein-protein interactions that are involved in the process of initiation complex formation at the origin. Moreover, the observation that *Drosophila* factors can functionally substitute for human factors in the SV40 replication system provides an assay that could be exploited for the purification and functional characterization of *Drosophila* replication factors in the context of a complete replication reaction. The results from these experiments could then be used in conjunction with the genetic tools available in *Drosophila* species for a combined biochemical and genetic approach to the study of metazoan DNA replication and chromatin assembly.

ACKNOWLEDGMENTS

We thank Tom Melendy, Rong Li, Mike Pazin, Beth Blackwood, Namrita Dhillon, Lucy DeVito, Deborah Frank, and Bruno Zimm for critical reading of the manuscript. We are grateful to Mary Dasso for the *Xenopus* extract, Tom Melendy for human polymerase α-primase and RP-A, Roland Kanaar and Don Rio for Kc cell extract, and Laurie Kaguni for useful discussion regarding *Drosophila* DNA polymerases.

J.T.K. is a Presidential Faculty Fellow. This work was supported by grants to J.T.K. from the National Institutes of Health, the National Science Foundation, and the Council for Tobacco Research and by grant CA13106 to B.W.S. from the National Institutes of Health. P.D.K. is a Smith Kline Beecham Fellow of the Life Sciences Research Foundation.

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