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Initiation of simian virus 40 DNA replication *in vitro*: Large-tumor-antigen- and origin-dependent unwinding of the template

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ABSTRACT **Analysis of the kinetics of simian virus 40 (SV40) DNA replication *in vitro* demonstrated the existence of a slow presynthesis reaction that occurs prior to onset of extensive chain elongation and is dependent on a subset of the cellular proteins required for the complete replication reaction. When the presynthesis reaction is carried out in the presence of topoisomerase I, it is possible to detect extensive unwinding of the template DNA. This unwinding reaction is specific for templates that contain the wild-type SV40 origin of DNA replication and requires SV40 large tumor antigen (T antigen), ATP, and a protein fraction derived from HeLa cells. The required cellular protein may be a eukaryotic single-stranded-DNA-binding protein (SSB), since unwinding of the template is also observed when *Escherichia coli* SSB is substituted for the HeLa protein fraction. These observations suggest that during the initial stages of SV40 DNA replication, T antigen binds specifically to the viral origin and locally unwinds the DNA. This origin-dependent unwinding reaction is presumably a prerequisite for subsequent priming and elongation steps.**

Little is known about the mechanisms involved in chromosomal DNA replication in higher eukaryotic cells. As an approach to this problem, we have been studying the replication of the simple chromosome of a papovavirus, simian virus 40 (SV40) (1). We have previously described a cell-free system that is capable of replicating plasmid DNA molecules containing the SV40 origin of DNA replication (2, 3). A number of lines of evidence indicate that SV40 DNA replication in the cell-free system closely resembles SV40 DNA replication *in vivo* (2–8). Replication is completely dependent upon the virus-encoded replication protein, large tumor antigen (T antigen) and also requires proteins isolated from cells permissive for SV40 DNA replication *in vivo*. DNA synthesis is initiated within a single region of the viral genome corresponding to the *in vivo* origin of DNA replication. After initiation, DNA replication proceeds bidirectionally via branched circular intermediates that are identical in structure to *in vivo* replication intermediates.

The SV40 T antigen plays a central role in viral DNA replication (1, 9). Genetic and biochemical studies have shown that initiation of replication requires the binding of T antigen to a specific sequence element within the viral origin of DNA replication (10–15). Recently, it was demonstrated that T antigen possesses an intrinsic DNA helicase activity that is capable of unwinding duplex DNA segments at least 120 base pairs (bp) long in an ATP-dependent reaction (16). It was suggested that the T-antigen helicase activity might play an important role(s) in SV40 DNA replication, possibly during the elongation phase (16, 17).

In this paper we describe experiments aimed at defining the mechanism of initiation of SV40 DNA replication. Kinetic analysis of DNA replication in the cell-free system revealed the existence of a slow presynthesis reaction that occurs prior to the onset of extensive chain elongation. When the presynthesis reaction occurs in the presence of topoisomerase I, a highly underwound form of the template is generated. The formation of the underwound species is specific for templates that contain the wild-type SV40 origin of DNA replication and requires SV40 T antigen, a cellular protein fraction (cellular fraction I), and ATP. The *Escherichia coli* single-stranded-DNA-binding protein (SSB) can substitute for cellular fraction I in this reaction, suggesting that the major active cellular protein is a eukaryotic analogue of SSB. These data strongly suggest that both the DNA-binding and the helicase activities of T antigen play a direct role in the initiation of viral DNA replication. We propose that T antigen in the presence of an accessory cellular protein(s) binds specifically to the origin of replication and locally unwinds the DNA. This site-specific unwinding reaction may represent a critical step in generating a DNA structure that is suitable for RNA priming and subsequent chain elongation.

MATERIALS AND METHODS

Cellular Fractions. Cellular fractions were derived from cytoplasmic extract, which was made as described previously (4). The cytoplasmic extract was adjusted to the composition of buffer P (80 mM potassium phosphate/1 mM dithiothreitol/0.25 mM EDTA/0.25% inositol) and passed over a phosphocellulose column (7 mg of extract protein per ml of phosphocellulose) that was equilibrated in buffer P. The fraction of proteins that flowed through the column was designated cellular fraction I, and the fraction that was eluted by buffer P containing 500 mM KCl was designated cellular fraction II.

DNA Templates. Plasmids pOR.HSO, pOR.8-4, pOR.1097, pOR.41, pOR.43, pOR.S321, pOR.S312, and pKP55 have been described (2–4). pUC.HSO was constructed by insertion of the *Hin*dIII–*Sph* I origin-containing fragment of SV40 (nucleotides 5171–128) between the corresponding sites in pUC19. M13.HPO was constructed by insertion of the *Hin*dIII–*Pvu* II origin-containing fragment of SV40 (nucleotides 5171–272) between the *Hin*dIII and *Sma* I sites in bacteriophage vector M13mp9. pUC.HSOd4 and M13.HPOd4 were constructed in a similar fashion as the wild-type templates starting from the plasmid 8-4, which contains a 4-bp deletion within the SV40 origin of replication (18).

Presynthesis Reactions. The reactions were divided into two stages. The reaction mixture for the first (presynthetic) stage contained 30 mM Hepes/HCl (pH 7.5), 7 mM $MgCl_2$, 15 mM sodium phosphate (pH 7.5), 40 mM creatine phosphate, 0.5 mM dithiothreitol, 0.05% Nonidet P-40, 4 mM ATP, 2.5 μg of creatine kinase, 80–100 ng of DNA template,

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Abbreviations: SSB, single-stranded-DNA-binding protein; SV40, simian virus 40; T antigen, SV40 large tumor antigen; Ado*PP*[NH]*P*, adenosine 5′-[β,γ-imido]triphosphate; Ado*PPP*[S], adenosine 5′-[γ-thio]triphosphate.

1.8 μg of T antigen, and 90 μg of cellular fraction I (or 630 ng of SSB; gift of R. McMacken, Department of Biochemistry, John Hopkins University, Baltimore) in 25 μl. After this mixture was incubated for 20 min at 37°C, the second (synthetic) stage was begun by the addition of 25 μl of a solution containing 30 mM Hepes/HCl (pH 7.5); 7 mM $MgCl_2$; 15 mM sodium phosphate (pH 7.5); 40 mM creatine phosphate; 0.5 mM dithiothreitol; 0.05% Nonidet P-40; 4 mM ATP; 400 μM each CTP, GTP, and UTP; 200 μM each dATP, dGTP, and dTTP; 50 μM [α-^{32}P]dCTP (specific activity, 10,000–20,000 dpm/pmol); 2.5 μg of creatine kinase; 100 ng of topoisomerase I (gift of L. Liu, Department of Biological Chemistry, Johns Hopkins University, Baltimore); and 90 μg of cellular fraction II. The second-stage reaction mixture was incubated at 37°C for periods indicated in the figure legends. In experiments in which individual components were omitted from the first stage of the reaction, they were added to the second stage. When the products of the presynthesis reaction were examined for DNA unwinding, the reactions were stopped after the first stage by addition of *N*-lauroylsarcosine to 1% and EDTA to 12 mM. The products were purified as described (4).

RESULTS

Presynthesis Phase of SV40 DNA Replication. DNA replication in the cell-free system requires a template containing the SV40 origin of DNA replication, T antigen, and a cytoplasmic extract from cells permissive for SV40 DNA replication *in vivo* (2, 3). Under the standard reaction conditions, DNA replication displays biphasic kinetics (19, 20). During the first phase, which lasts about 15–20 min, little or no DNA synthesis is observed. During the second phase, the rate of DNA synthesis increases rapidly to a relatively constant level that is maintained for at least 2 hr. The initial lag in DNA synthesis can be eliminated if the complete reaction mixture is preincubated at 37°C in the absence of deoxynucleoside triphosphates (19, 20). These results suggest the existence of a slow presynthesis step that must occur before rapid chain elongation can take place.

Reconstitution experiments indicated that the presynthesis reaction requires only a subset of the cellular proteins that are required for the complete replication reaction. We have previously shown (19) that the cytoplasmic extract can be separated into two fractions (here called cellular fractions I and II) by column chromatography on phosphocellulose. While both of these fractions are absolutely required for DNA replication *in vitro*, only cellular fraction I is required for the presynthetic reaction. When the DNA template, T antigen, cellular fraction I, and ATP were preincubated at 37°C for 20 min prior to addition of cellular fraction II and deoxynucleoside triphosphates, the initial lag in DNA synthesis was virtually abolished (Fig. 1). Analysis of the requirements for this effect revealed that complete removal of the lag phase was dependent upon the presence of DNA, T antigen, and cellular fraction I during the preincubation; however, a smaller, but significant, reduction in the length of the lag was observed when the protein components were preincubated in the absence of DNA. The lag phase was not removed when the order of addition of the cellular fractions was reversed or when the preincubation was carried out at 0°C instead of 37°C (data not shown). When ATP was omitted from the preincubation the lag was not removed, and the rate of subsequent DNA synthesis was significantly reduced. We conclude from these results that cellular fraction I contains a factor(s) that is involved in the initial stages of the SV40 DNA replication reaction. Cellular fraction II does not contain factors required during the presynthesis reaction but does contain factors required for extensive DNA synthesis, including DNA polymerase α and primase activities (19). The role of T antigen and ATP in the presynthesis reaction is explored further in subsequent sections of this paper.

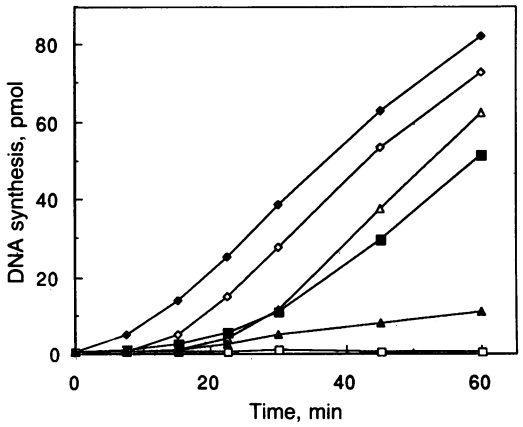

FIG. 1. Presynthesis phase of SV40 DNA replication. The complete presynthesis reaction mixture contained cellular fraction I, SV40 T antigen, and a plasmid template (pUC.HSO) containing the SV40 origin of replication. After preincubation for 20 min at 37°C, the reactions were supplemented with cellular fraction II, deoxynucleoside triphosphates, and components omitted from the preincubation. Incubation was continued at 37°C, and DNA synthesis was monitored by measuring the incorporation of [α-^{32}P]dCMP by trichloroacetic acid precipitation. ◆, Complete reaction mixture; △, T antigen omitted from preincubation; ■, cellular fraction I omitted from preincubation; ◇, DNA omitted from preincubation; ▲, ATP and creatine kinase omitted from preincubation; □, cellular fraction II omitted from second incubation.

T Antigen Unwinds Duplex DNA. Stahl *et al.* (16) have shown that T antigen has an intrinsic helicase activity, dependent upon ATP and Mg^{2+}, that is capable of unwinding a duplex segment formed by the hybridization of an oligonucleotide to a long single strand. We have found that under the same conditions, purified T antigen can unwind DNA molecules that are completely duplex. Covalently closed circular DNA molecules were incubated at 37°C with T antigen, ATP, and topoisomerase I as described (16), and the reaction products were analyzed by agarose gel electrophoresis (Fig. 2*A*). The supercoiled input DNA was initially relaxed due to the action of topoisomerase I, but upon further incubation faster-migrating topoisomers with increased numbers of superhelical turns reappeared. The generation of these forms was completely dependent upon the presence of T antigen and ATP. Moreover, no accumulation of faster-migrating topoisomers was observed when adenosine 5′-[β,γ-imido]triphosphate (Ado*PP*[NH]*P*) or adenosine 5′-[γ-thio]triphosphate(Ado*PPP*[S]) was substituted for ATP, suggesting that ATP hydrolysis was required for their formation. The reaction showed no dependence upon the SV40 origin of DNA replication. For example, rapidly migrating topoisomers were generated with the replication-defective plasmid pUC.-HSOd4, which contains a 4-bp deletion in the SV40 origin, or with pUC19, which contains no SV40 sequences. With all DNA molecules tested, the reaction was very sensitive to salt and could be completely inhibited by 100 mM NaCl.

To verify that the accumulation of faster-migrating topoisomers was due to unwinding of the DNA, the reaction products were analyzed by electrophoresis in agarose gels containing chloroquine, an intercalating agent that produces a decrease in the twist of DNA (21) (Fig. 2*B*). Control plasmids that had been exposed to topoisomerase I alone migrated more rapidly in the presence of 6 μM chloroquine due to the introduction of positive superhelical turns in compensation for the decrease in twist. Plasmids that had been exposed to T antigen, topoisomerase I, and ATP migrated more slowly than the control molecules, indicating that they had a lower linking number. Thus, our data show

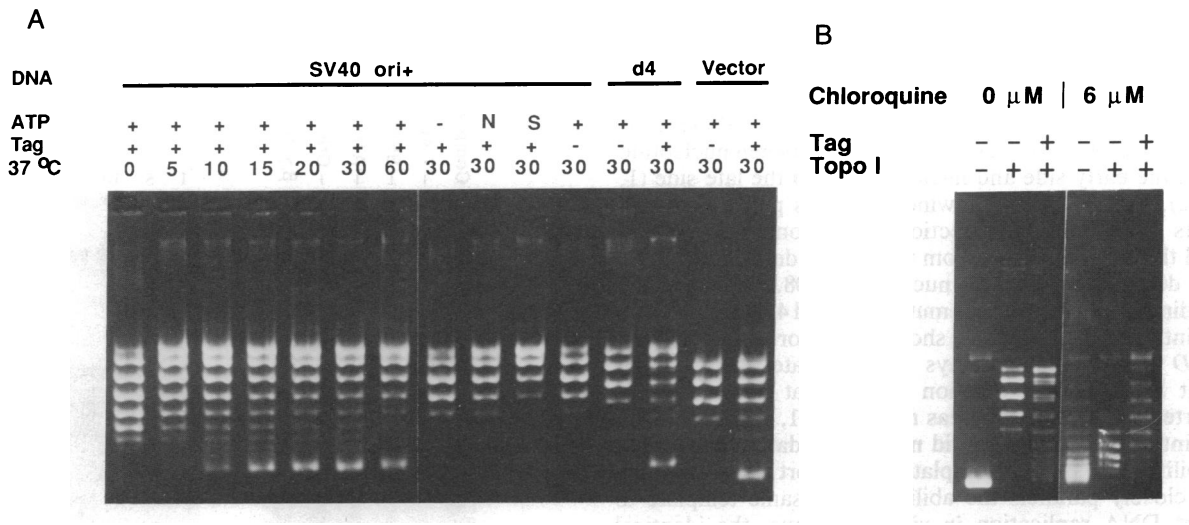

FIG. 2. T antigen unwinds duplex DNA. (*A*) Unwinding reaction mixtures (10 μl) contained 20 mM Tris (pH 7.5), 10 mM $MgCl_2$, 0.5 mM dithiothreitol, 100 μg of bovine serum albumin per ml, 100 ng of DNA, and 100 ng of topoisomerase I. The reaction mixtures were preincubated for 10 min at 37°C to allow the topoisomerase I to relax the DNA. T antigen (Tag, 0.9 μg) was then added and the incubations were continued at 37°C for the indicated times. The reactions were terminated by the addition of *N*-lauroylsarcosine and EDTA to 1% and 12 mM, respectively. The products were electrophoresed in 1.3% agarose gels (in 100 mM Tris base/91 mM boric acid/0.1 mM EDTA) and visualized by staining with ethidium bromide. Lanes marked SV40 ori+ contained pUC.HSO DNA; lanes marked d4 contained pUC.HSOd4 DNA; and lanes marked Vector contained pUC19 DNA. Letters N and S indicate that ATP was replaced by Ado*PP*[NH]*P* or Ado*PPP*[S], respectively. Numbers refer to time of incubation in minutes. (*B*) Products of standard unwinding reactions with pUC.HSO DNA were analyzed in 1.3% gels as described by Shure *et al.* (21). The two gels shown contained either 0 μM or 6 μM chloroquine as indicated. Reaction mixtures contained topoisomerase I and T antigen (Tag) as indicated.

that highly purified T-antigen preparations contain an activity that is capable of mediating the unwinding of a fully duplex DNA molecule. Given the findings of Stahl *et al.* (16), we assume that this activity is intrinsic to the T-antigen molecule itself, but further work will be required to verify this directly.

Unwinding of the DNA Template During the Presynthesis Reaction. To detect unwinding of the DNA template during the presynthesis reaction, we added topoisomerase I to the reaction mixture. This modification had no detectable effect on the kinetics of DNA synthesis following the preincubation but ensured that unwinding would be manifested by a change in the linking number of the template. In contrast to the results described in the preceding section of this paper, we found that T antigen and ATP were not sufficient to induce extensive unwinding under the conditions employed for the presynthesis reaction (Fig. 3). Presumably this was due to the relatively high ionic strength of the reaction mixture. However, when the complete presynthesis reaction mixture, containing T antigen, cellular fraction I, and ATP, was incubated at 37°C we observed the appearance of a new species of DNA with a mobility similar to that of marker form I DNA (asterisk in Fig. 3). This species first appeared at about 10 min and accumulated rapidly between 10 and 30 min, reaching a final level equivalent to about 1–5% of the input DNA. Formation of the rapidly migrating species was dependent upon T antigen, ATP, and cellular fraction I. The requirement for ATP could not be fulfilled by nonhydrolyzable ATP analogues such as Ado*PP*[NH]*P*, Ado*PPP*[S], or adenosine 5′-[β,γ-methylene]triphosphate (Ado*PP*[CH_2]*P*). The formation of the rapidly migrating species was absolutely specific for templates containing the wild-type SV40 origin of DNA replication. For example, a mutant template containing a 4-bp deletion in the viral origin (pOR.8-4) did not support this reaction (Fig. 3*A*).

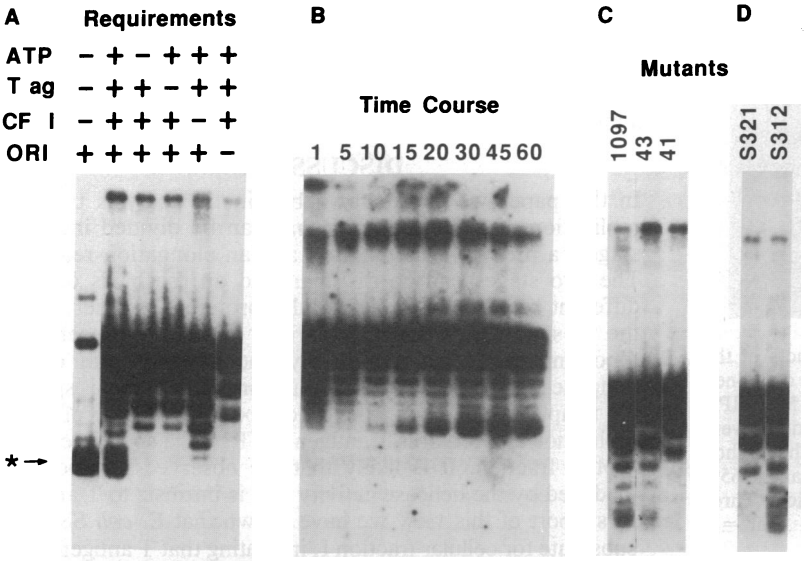

FIG. 3. Specific DNA unwinding during the presynthesis reaction. Standard presynthesis reaction mixtures supplemented with 100 ng of topoisomerase I were incubated at 37°C. Reaction products were subjected to agarose gel electrophoresis as in Fig. 2 and visualized by hybridization with ^{32}P-labeled (random-primer extended) pKP55 DNA. (*A*) Requirements for the reaction. DNA templates were pOR.HSO (ori+) or pOR.8-4 (ori−). Tag, T antigen; CFI, cellular fraction I. Asterisk indicates position of the unwound species of DNA. (*B*) Time course of the reaction with pUC.HSO as template. Numbers refer to time of incubation in minutes. (*C*) Unwinding of templates with deletion mutations on the early side of the origin of replication. Mutant templates contained a segment of the SV40 genome from nucleotide 5171 to 135 withthe following deletions: nucleotides 5178–5208 (pOR.1097), nucleotides 5177–5214 (pOR.41), and nucleotides 5177–5217 (pOR.43). (*D*) Unwinding of templates with deletion mutations on the late side of the origin of replication. Mutant templates contained segments of the SV40 genome from nucleotide 5171 to nucleotide 25 (pOR.S321) or from nucleotide 5171 to nucleotide 34 (pOR.S312).

To define further the sequence requirements for the unwinding reaction, we studied several mutant templates with deletions that extend toward the minimal origin from either the early or late sides. Previous work had established that the minimal segment of the SV40 genome that is sufficient for DNA replication *in vivo* or *in vitro* maps between nucleotide 5211 on the early side and nucleotide 31 on the late side (1, 4, 6, 22). Fig. 3*C* shows unwinding assays performed with mutants containing unidirectional deletions that extend toward the minimal origin from the early side. Mutant 1097, with a deletion endpoint at nucleotide 5208, supported the unwinding reaction, whereas mutants 43 and 41, with deletion endpoints at 5214 and 5217, showed little or no unwinding. Fig. 3*D* shows similar assays with the late-side mutants. Mutant S312, with a deletion endpoint at nucleotide 34, supported unwinding, whereas mutant S321, with a deletion endpoint at nucleotide 25, did not. These data indicate that the ability of a mutant template to support the unwinding assay closely parallels the ability of the same template to support DNA replication *in vitro* (4). Thus, the identical segment of the viral genome is required for both processes.

The identity of the rapidly migrating species as a highly underwound form of the template was verified by electron microscopy (23). When the products of a presynthesis reaction containing the 7.5-kbp plasmid M13.HPO were mounted for microscopy in the presence of normally subdenaturing concentrations of formamide (65%), approximately 2% (50/2500) of the molecules showed extensive single-stranded regions (Fig. 4). No such molecules (0/1000) were observed in control reactions containing the mutant template M13.HPOd4, which has a 4-bp deletion in the minimal origin. The extent of unwinding in 60 M13.HPO molecules chosen at random ranged from 6% to 34%. The average extent of unwinding corresponded to about 850 bp.

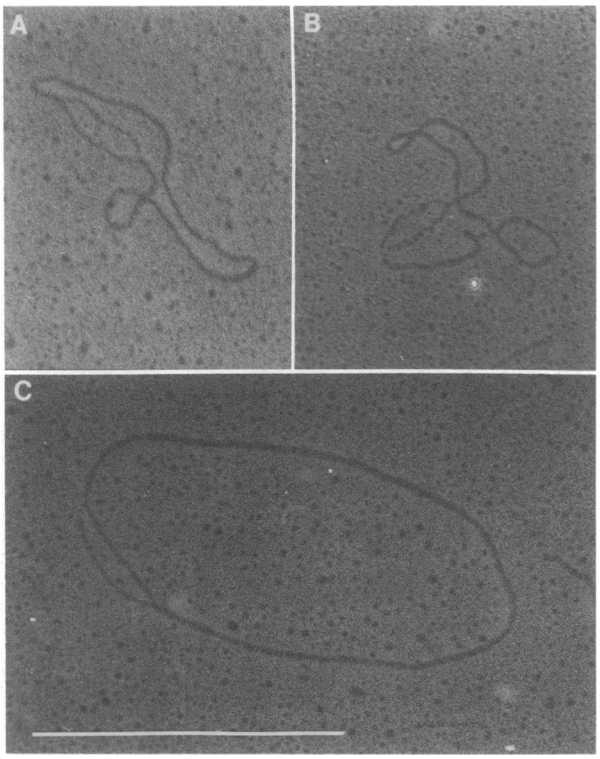

FIG. 4. Electron micrographs of the unwound products of the presynthesis reaction. The presynthesis reaction mixture contained T antigen, ATP, topoisomerase I, cellular fraction I, and M13.HPO DNA. After incubation at 37°C for 25 min, the reaction products were isolated as described (3) and mounted for microscopy by the method of Davis and Hyman (23). The spreading solution contained 65% formamide and the hypophase contained 35% formamide. Three representative partially unwound molecules are shown. (Bar = 1 μm.)

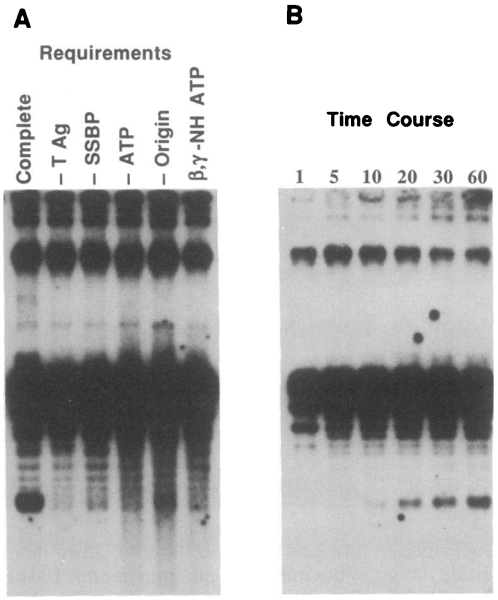

FIG. 5. Substitution of *E. coli* SSB for HeLa cellular fraction I. The complete presynthesis reaction mixtures containing T antigen, ATP, plasmid DNA template, topoisomerase I, and *E. coli* SSB in place of cellular fraction I were incubated at 37°C for 25 min and analyzed as in Fig. 3. (*A*) Requirements of the reaction. The indicated components were omitted from the reaction mixture. The DNA template was the plasmid pUC.HSO, except for the reaction corresponding to the lane marked -Origin, which contained pUC.HSOd4 as template. The reaction analyzed in the lane marked β,γ-NH ATP contained the nonhydrolyzable ATP analogue Ado*PP*[NH]*P* in place of ATP. (*B*) Time course of the reaction with pUC.HSO DNA as template. Numbers refer to time of incubation in minutes.

Substitution of *E. coli* SSB for Cellular Fraction I. During analysis of the protein requirements for the unwinding reaction, we found that extensive unwinding occurred when *E. coli* SSB was substituted for cellular fraction I (Fig. 5). The protein and cofactor requirements for unwinding in the presence of SSB were otherwise identical to those for reactions carried out with cellular fraction I (Fig. 5*A*), and the time course of unwinding was similar (Fig. 5*B*). Moreover, unwinding occurred preferentially with templates containing the wild-type SV40 origin of replication. We also found that *E. coli* SSB was capable of stimulating DNA replication in reactions carried out in the absence of cellular factor I, although the extent of DNA synthesis was much less than that observed with cellular fraction I (data not shown). These results suggest that one of the replication proteins in cellular fraction I that is important in the unwinding reaction may be a eukaryotic analogue of SSB.

DISCUSSION

In this paper we have shown by kinetic analysis that the replication of SV40 DNA *in vitro* can be divided into two stages: a presynthesis reaction and an elongation reaction. The two stages can be distinguished on the basis of their different requirements for cellular proteins. Further, when the presynthesis reaction is carried out in the presence of topoisomerase I, an extensively underwound form of the template DNA is generated. This unwinding reaction is specific for templates containing the wild-type SV40 origin of DNA replication and requires T antigen, ATP, and a specific cellular protein fraction. It is likely that the observed unwinding is mediated by the helicase activity that is intrinsic to T antigen. In support of this view we have shown that *E. coli* SSB can substitute for cellular fraction I, indicating that T antigen alone

is capable of providing all of the catalytic activities necessary for origin-specific DNA unwinding. The mechanism of T-antigen-mediated unwinding presumably involves direct melting of duplex DNA coupled to ATP hydrolysis, although other possible mechanisms cannot be ruled out.

It is not clear whether DNA as extensively underwound as that observed in the presynthesis reaction is a normal intermediate in DNA replication. Accumulation of the highly underwound form may in part be due to the uncoupling of the early steps in replication from chain elongation. Nevertheless, the specificity of the unwinding reaction, as well as the identity of the requirements for the unwinding and the presynthesis reactions, strongly suggests that a more limited unwinding of the template is an important early step in SV40 DNA replication. All our data are consistent with the hypothesis that initiation of SV40 DNA replication involves the specific binding of T antigen to the origin, followed by T-antigen-mediated melting of the DNA at the origin region. The formation of such a partially single-stranded structure is presumably essential for subsequent RNA-priming and chain-elongation steps. This hypothesis is consistent with previous genetic and biochemical studies indicating that both the specific DNA-binding activity and the ATPase activity of T antigen are required for SV40 DNA replication (24–26). In addition, the origin-dependent unwinding that occurs during the presynthesis phase of SV40 DNA replication is remarkably similar to the events that have been observed during the prepriming phases of two prokaryotic DNA-replication systems (27, 28), suggesting that there may be a high degree of evolutionary conservation of the fundamental mechanisms of initiation. Our data do not rule out a role for T antigen in the steps that occur after the initiation of replication. It seems quite possible that the helicase activity of T antigen could function to unwind the parental strands ahead of the replication forks, as previously suggested (16, 17).

As was mentioned above, we also observed origin-specific DNA unwinding when purified *E. coli* SSB was substituted for cellular fraction I. It seems likely that the function of SSB in this reaction is to stabilize newly formed single-stranded regions generated by the helicase activity of T antigen. Thus, the active factor contributed to the unwinding reaction by cellular fraction I may be a eukaryotic analogue of SSB. This possibility can be verified directly by purifying and characterizing this factor. Our data do not bear on the question of whether additional proteins are required during the presynthesis reaction to prepare for rapid chain elongation. This is clearly a possibility, since efficient reconstitution of DNA replication *in vitro* cannot be obtained by simply substituting *E. coli* SSB for cellular fraction I. Moreover, we have reproducibly observed some reduction in the initial lag in DNA synthesis when T antigen and cellular fraction I are preincubated in the absence of the template. This observation suggests the possibility that specific protein–protein interactions or protein-modification reactions may occur during the presynthesis reaction. Thus, while T-antigen-mediated unwinding may represent a crucial step in the initiation process, there may well be other essential steps.

At low salt concentrations, T antigen is capable of unwinding duplex DNA in a reaction that is dependent upon ATP but displays no sequence specificity. T antigen has significant nonspecific affinity for DNA under these conditions (29), so it is likely that the protein binds to random sites in the DNA and induces unwinding. Although binding itself could result in unwinding of the DNA, the requirement for ATP hydrolysis suggests that the observed unwinding is due to an active process. Under these low ionic strength conditions, T antigen may also bind to single-stranded DNA (30), thus stabilizing unwound regions in a manner analogous to SSB.

T antigen has two biochemical properties that are not shared by any of the known prokaryotic helicases (31). First, T antigen does not require a single-stranded region adjacent to a duplex region in order to initiate unwinding. Second, T antigen recognizes a specific nucleotide sequence and thus appears capable of acting as a site-specific helicase. In the *E. coli oriC* system, three bacterial proteins are required for origin-specific DNA unwinding in addition to SSB and topoisomerase (gyrase) (27). The DnaA protein binds to specific sequence elements in the origin region, the DnaB protein provides helicase activity, and the DnaC protein facilitates the binding of the DnaB helicase to the DnaA–origin complex. T antigen appears to combine in a single protein the functions performed by several prokaryotic proteins.

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