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Initiation of simian virus 40 DNA replication *in vitro*: Pulse–chase experiments identify the first labeled species as topologically unwound

(SV40 tumor antigen/replication origin/DNA polymerases)

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ABSTRACT **A distinct unwound form of DNA containing the simian virus 40 (SV40) origin is produced in replication reactions carried out in mixtures containing crude fractions prepared from HeLa cells. This species, termed form U_R, comigrates on chloroquine-containing agarose gels with the upper part of the previously described heterogeneous highly unwound circular DNA, form U. As with form U, formation of form U_R is dependent upon the SV40 tumor (T) antigen. Pulse–chase experiments demonstrate that the first species to incorporate labeled deoxyribonucleotides comigrates with form U_R. Restriction analyses of the products of the pulse–chase experiments show that initiation occurs at the SV40 origin and then proceeds outward in a bidirectional manner. These experiments establish form U_R as the earliest detectable substrate for SV40 DNA replication and suggest that SV40 DNA replication initiates on an unwound species.**

Replication of simian virus 40 (SV40) DNA in permissive cells requires only a single viral-encoded polypeptide, the large tumor antigen (TAg) (1). All other proteins involved in SV40 replication are encoded by the host. SV40 *in vitro* replication systems have been developed (2–4) that are being used to identify the host proteins involved in SV40 replication. Initial studies established a role for the DNA polymerase α–DNA primase complex in SV40 DNA replication (2, 5) and indicated that this complex helps determine the host range for SV40 replication (5). The *in vitro* replication systems have also demonstrated that SV40 replication *in vitro* requires topoisomerase (topo) II, although topo I can substitute for certain activities (6). Furthermore, a three-subunit single-stranded DNA binding protein (SSB) has been shown to be essential for DNA synthesis; this protein was isolated from human cell extracts based solely on its ability to support SV40 DNA replication *in vitro* (7–9). Considerable evidence indicates that proliferating cell nuclear antigen is also required during SV40 DNA replication, especially for synthesis of leading strands (10–12). This has been interpreted as suggesting a role for DNA polymerase δ in this process. More recent studies have shown that a role played by proliferating cell nuclear antigen in this process is to overcome an inhibitor that blocks DNA synthesis by binding to ends of DNA (ref. 13 and unpublished work). Finally, using an *in vitro* replication system consisting of proteins purified from HeLa cells (HeLa SSB, DNA polymerase α–primase complex, topo II, and SV40 TAg), it has been demonstrated that a 44-kDa 5′ to 3′ exonuclease, DNA ligase, and RNase H are required for the synthesis of closed circular duplex products (7, 14).

Owing in part to techniques for producing large amounts of TAg (15, 16), a better understanding of the role played by TAg during initiation of replication has been achieved. In the absence of ATP, TAg binds to duplex DNA at two sites within the SV40 wild-type origin region (for review, see refs. 17 and 18). In the presence of ATP, the association of TAg with binding site II, the core origin, is stimulated up to 15-fold (19, 20). Binding of TAg to the core origin in the presence of ATP results in a complex nucleoprotein structure (21) in which TAg is assembled into two hexamers (22). The ATP-dependent TAg complex locally melts an 8-base-pair (bp) region on the early side of the core origin and changes the structure of the adenine-thymine tract on the late side (23). Moreover, when incubated in a reaction mixture containing a topo capable of removing positive supercoils and an SSB, TAg will further unwind SV40 origin-containing duplex DNAs (9, 24, 25). With circular duplex DNAs, the products of the unwinding reaction are heterogeneous highly unwound circular DNAs, termed form U. The ability of TAg to unwind DNA is the result of an intrinsic helicase activity (24, 26, 27) that translocates in the 3′ to 5′ direction (28, 29).

Indirect evidence that TAg-mediated unwinding is an essential step during SV40 replication has been provided (24, 30). However, a direct demonstration that replication initiates on an unwound DNA has not been presented. Moreover, assuming unwinding is essential, little is known about the extent of unwinding required to establish replication forks. To characterize the initiation of SV40 replication *in vitro*, we have used pulse–chase experiments to identify the initial topological species in which incorporated deoxyribonucleotides can be detected during initiation of DNA synthesis. In this report, we demonstrate that origin-specific DNA synthesis in cytoplasmic extracts of HeLa cells is detected first on a topological species whose migration on chloroquine-containing agarose gels is similar to form U.

MATERIALS AND METHODS

Preparation of Enzymes. TAg was immunoaffinity purified from COS-1 cells infected with SV40 cs1085 virus as described (22). HeLa SSB was isolated as reported (14).

DNA Unwinding and Replication Assays. The unwinding assay used to generate form U has been described (24). Replication reactions and the preparation of HeLa cell extracts have also been described (4).

Pulse–Chase Experiments. Reaction mixtures (60 ul) contained 7 mM $MgCl_2$, 0.5 mM dithiothreitol, 4 mM ATP, 40 mM creatine phosphate (di-Tris salt, pH 7.7), 1.4 μg of creatine kinase, 0.78 μg of supercoiled SV40 origin-containing pSV01ΔEP DNA, 1.25 μg of TAg, RNase A (0.42 μg/ml), and 30 μl of HeLa cell extract (13.8 mg/ml). Reaction mixtures were preincubated for 45 min at 37°C in the absence of TAg and then further incubated for 15 min after the addition of TAg. The 45-min preincubation without TAg

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Abbreviations: SV40, simian virus 40; TAg, SV40 tumor antigen; topo, topoisomerase; SSB, single-stranded DNA binding protein.

lowered the TAg-independent labeling of form II DNA (circular duplex DNA with at least one single-strand break). Reaction mixtures were pulse-labeled by the addition of 3.5 μl of a solution containing [α-^{32}P]dCTP (final concentration, 0.055 μM, 9000 cpm/fmol); dATP, dGTP, and dTTP (final concentration for each, 100 μM); and CTP, GTP, and UTP (final concentration for each, 200 μM) for 20 s at 37°C. The mixture was then chased with unlabeled dCTP (final concentration, 5.3 mM, which reduced the specific activity to 93 cpm/pmol) followed by incubation for various periods at 37°C. Although these reactions were performed in the presence of RNase A (0.42 μg/ml), the same results were obtained when RNase A (0.42 μg/ml) was added to the samples just prior to loading the gel.

All reactions were stopped by adding a solution of 15 mM EDTA, 2 μg of *Escherichia coli* tRNA, 0.3% NaDodSO$_4$, and 30 μg of proteinase K. At the end of each reaction, aliquots (6 μl) were withdrawn to monitor the amount of label incorporated: these measurements indicated that the incorporation that occurred during the chase period was <5% of the amount incorporated during the pulse period. All reactions were further incubated for 30 min at 37°C, diluted to 100 μl with 10 mM Tris·HCl, pH 7.8/1 mM EDTA, extracted with phenol/chloroform, and, after the addition of ammonium acetate to 2 M, precipitated by the addition of 2.5 vol of ethanol. After centrifugation, the pellets were washed with 80% (vol/vol) ethanol and dried.

Gel Electrophoresis and Autoradiography. DNA pellets were resuspended in 16 μl of 10 mM Tris·HCl, pH 7.8/1 mM EDTA. Aliquots (4 μl) were removed to determine the size of newly synthesized DNA by alkaline gel analyses (see below). Gel loading buffer (2 μl), containing 20% (vol/vol) Ficoll, 0.1 M EDTA, 0.25% bromophenol blue, and 0.25% xylene cyanol, was then added. Samples were electrophoresed through a 1.8% agarose gel containing chloroquine (1.5 μg/ml) and Tris/acetate/EDTA buffer (31) for 14 hr at 2.8 V/cm. DNA was visualized under ultraviolet illumination after soaking in 50 mM NaCl for 1 hr, staining in ethidium bromide (0.5 μg/ml) for 30 min, and destaining for 30 min in H_2O. Gels to be autoradiographed were then dried and exposed to Kodak X-OMAT AR film.

Alkaline/agarose gels (1.8%) were electrophoresed at 60 V for 15 hr, fixed in 8% (wt/vol) trichloroacetic acid, and dried (31).

Restriction Analyses of DNAs Labeled During Pulse–Chase Experiments. Reaction conditions were identical to those described for the pulse–chase experiments. DNA synthesis was initiated by the addition of rNTPs, [α-^{32}P]dCTP, and the other dNTPs (see above). Pulse labeling was carried out for 0.3, 1, 5, 15, or 30 min; incorporation of label was determined to be linear during this time period. To facilitate formation of duplex DNA that can be cleaved by restriction endonucleases, all reactions were chased with unlabeled dCTP for various times such that all pulse–chase reactions were conducted for 40 min. Reactions were stopped as described above and the DNA was subsequently cleaved with a combination of *Nco* I, *Hin*cII, *Hae* II, and *Pst* I restriction endonucleases. DNA fragments were separated on 6% polyacrylamide gels in TBE buffer (31). Lanes containing reaction mixtures with TAg were loaded with identical total amounts of radioactivity. For a given time point, lanes containing reaction mixtures without TAg were loaded with the same mass of DNA as lanes containing TAg.

RESULTS

A Distinct Topological Form Appears upon Replication of pSV01ΔEP DNA in HeLa Cell Extracts. Since DNA unwinding is expected to play a role in DNA replication, we investigated whether TAg-dependent unwinding could be detected in DNA replication reactions. The topological isomers formed at various times during the replication of pSV01ΔEP by HeLa cell extracts, after deproteinization and electrophoresis on a chloroquine-containing agarose gel, are shown in Fig. 1*A*, lanes 2–9. The heterogenous unwound topological species, termed form U DNA (24), generated by incubating pSV01ΔEP DNA with TAg, topo I, ATP, and HeLa SSB, is shown in Fig. 1*A*, lane 10, and noted by the arrow. As shown, a distinct topological species, comigrating with the upper limit of the form U distribution and slightly above the 2027-bp linear size marker, was formed in the replication reactions. Formation of this species, which we term form U_R (R, replication), was TAg-dependent (Fig. 1*A*, lane 9). Moreover, inspection of Fig. 1*A* indicated that, like form U, the mobility of form U_R on a chloroquine (1.5 μg/ml) gel is greater than form I DNA (superhelical circular duplex DNA). This indicates that form U_R is more unwound than form I DNA. This was shown for form U (25) and demonstrated by topo I and S1 nuclease studies of purified form U_R (data not shown).

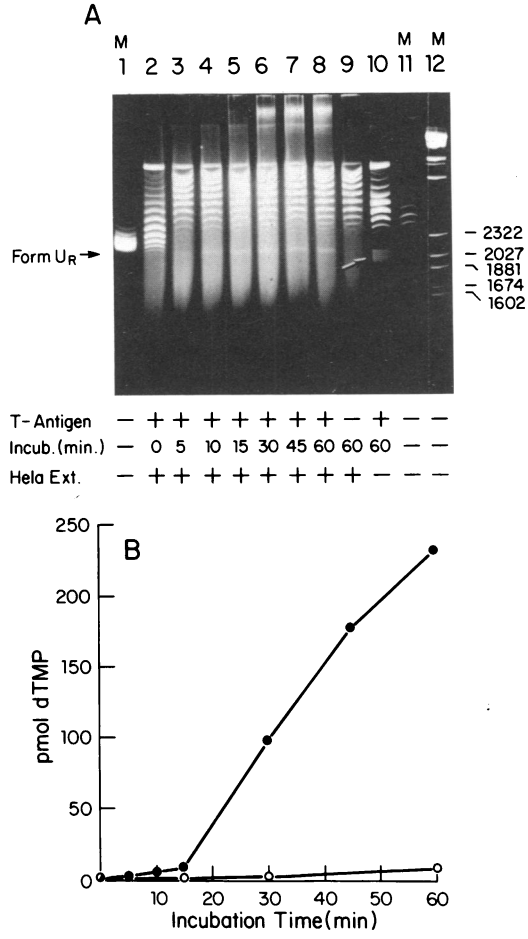

FIG. 1. Rate of replication and appearance of topological isomers by HeLa extracts. (*A*) Replication mixtures, containing TAg, pSV01ΔEP DNA, HeLa cytoplasmic extracts, ATP, and an ATP-regenerating system, were incubated for the times indicated. The topological isomers present in the reaction mixtures were analyzed on an agarose gel containing chloroquine (1.5 μg/ml, lanes 2–8). The topological isomers generated after 60 min of incubation in crude extracts in the absence of TAg are also shown (lane 9). Markers (lanes M) include form I (lane 1) and relaxed pSV01ΔEP DNA (lane 11); lane 10 contains form U DNA, generated with TAg, HeLa SSB, and topo I (24); lane 12 contains restriction fragment size markers (in bp), resulting from cleavage of bacteriophage λ DNA with *Hin*dIII and *Ava* I. The positions of form U and form U_R are indicated by arrows. −, Absence of a reaction component; +, presence of a reaction component. (*B*) The extent of incorporation of [^{3}H]dTMP at the various time points presented in *A*. ●, With TAg; ○, without TAg.

The rate of incorporation of [^{3}H]dTMP during the DNA replication reactions described in Fig. 1*A* is shown in Fig. 1*B*. Comparison of Fig. 1*A* with Fig. 1*B* indicated that initiation of replication occurred shortly after form U_R was detected.

Form U_R^* Is the Earliest Labeled Product Detected During Pulse–Chase Experiments in HeLa Extracts. When a complete replication reaction mixture containing ATP but lacking dNTPs and the other rNTPs was incubated for 60 min at 37°C (45 min without TAg and 15 min in the presence of TAg), form U_R was generated (Fig. 2*A*, lane 5). The mobility of form U_R generated under these conditions was the same as form U_R formed during DNA synthesis in the presence of dNTPs (slightly above the 2027-bp size marker: compare Figs. 1*A* and 2*A*). When the preincubated reaction mixtures were pulse-labeled with rNTPs, dNTPs, and [α-^{32}P]dCTP for 20 s, the only labeled TAg-dependent species migrated slightly above form U_R (Fig. 2 *A* and *B*, lanes 6). Form II DNA was also labeled but this reaction was TAg-independent (Fig. 2 *A* and *B*, lanes 2). When preincubated mixtures were pulse-labeled for 5 s instead of 20 s, the labeled species comigrated with form U_R (Fig. 2 *C*, lane 3). These data suggest that form U_R is a precursor to the earliest detectable labeled forms that comigrate with form U_R; we term such labeled DNAs form U_R^*.

When pulse-labeled products were chased for various periods with an excess of unlabeled dCTP, the labeled species matured from form U_R^* into higher molecular weight forms (Fig. 2*B*, lanes 7–11); these presumably include Cairns structures and catenated dimers (32). Moreover, after chasing for 30 min, the products of the pulse–chase reaction included covalently closed forms that comigrated with relaxed and form I DNAs (Fig. 2*B*, lane 10). Fig. 2*B*, lane 3, demonstrates that monomer products were not detected in the absence of TAg. The progression of labeled forms from form U_R^* to monomer-size relaxed and form I DNAs demonstrates that newly initiated DNA chains matured into the expected products of *bona fide* SV40 replication. Finally, we have observed that the level of form U_R present during the reactions shown in Figs. 1 and 2 is consistent with its being at a steady-state level of synthesis.

It is interesting to note that the rate of product formation was considerably slower than the time (1 min) required to detect form I DNA during similar pulse–chase experiments in prokaryotic replication systems, such as in pBR322 (33). Also, with a 20-s pulse, the labeled form U_R^* species were not detected at low concentration of aphidicolin and at the same low amount of N^2-[*p*-(*n*-butyl)phenyl]deoxyguanosine 5′-triphosphate (10 μM, data not shown). This indicates that in addition to TAg, formation of the labeled form U_R^* species is dependent on DNA polymerases α and, possibly, δ (34).

An aliquot of each reaction mixture was analyzed on an alkaline/1.8% agarose gel to determine the size of the DNA synthesized during a given pulse–chase experiment (Fig. 2*D*). The DNA formed during the initial 20-s pulse (Fig. 2*D*, lane 6) had a size distribution centered between the 185- and 322-nucleotide size markers, similar to the size of Okazaki fragments. After a 1-min chase, the products were extended

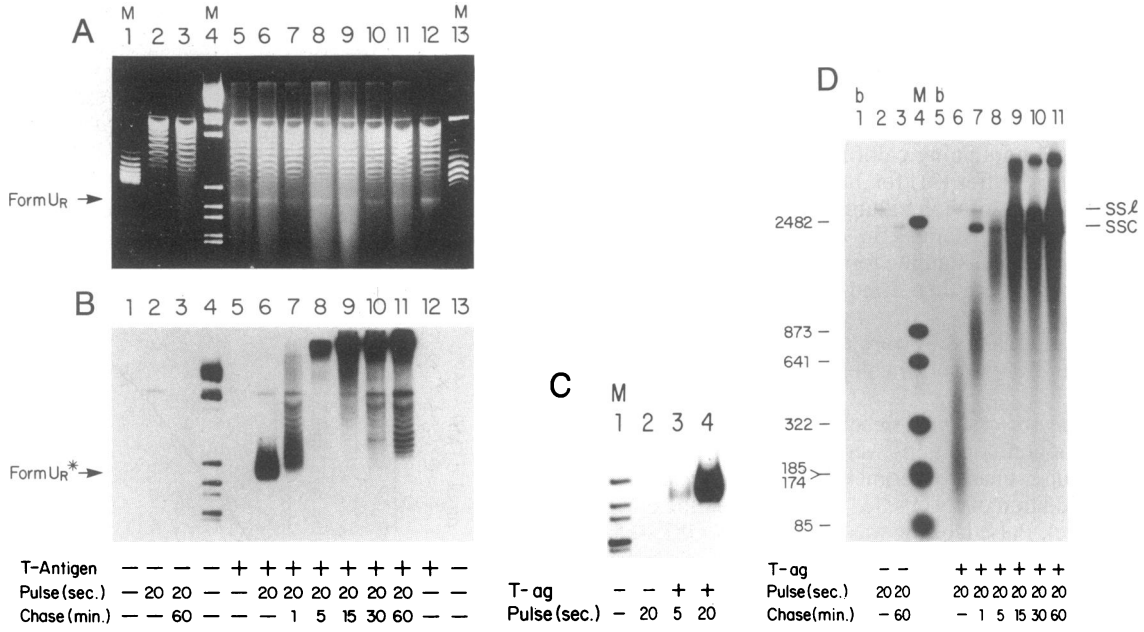

FIG. 2. Determination of the initial labeled topological form in cytoplasmic extracts after pulse–chase labeling. Reaction mixtures were pulse-labeled for 20 s and, where indicated, the pulse was followed by a chase period with unlabeled dCTP. The labeled products were electrophoresed on an agarose gel containing chloroquine, photographed after staining with ethidium bromide (*A*) and then autoradiographed (*B* and *C*). Aliquots from these reaction mixtures were electrophoresed on an alkaline/agarose gel to determine the size of the newly synthesized DNA (*D*). (*A*) Form I DNA, the input DNA used in these reactions, is in lane 1 and topo I-relaxed DNA is shown in lane 13. The distribution of topoisomers found in reaction mixtures lacking TAg, after a 20-s pulse (lane 2) or a 20-s pulse and a 60-min chase (lane 3) are also shown. The topological isomers present after 15 min of preincubation in the presence of TAg, followed by a 20-s pulse and a chase of various lengths of time are presented in lanes 6–11. The reaction presented in lane 12 was incubated at 37°C for 120 min without rNTPs or dNTPs; this reaction demonstrated that form U_R was stable during the chase period. The distribution of topoisomers after 15 min of preincubation with TAg, just prior to pulse addition, is shown in lane 5. The restriction fragment size markers, lane 4, are the same as those described in the legend to Fig. 1. (*B*) Autoradiogram of the gel described in *A*. Each lane was described above. Size markers are the same as those described above, labeled at their 3′ termini with Klenow fragment of DNA polymerase I and [^{32}P]dNTPs. (*C*) Autoradiogram of a gel in which reaction mixtures were pulse-labeled for either 5 s or 20 s. Markers (M) are the same as described in Fig. 1*A*. (*D*) The size of products synthesized after the pulse and chase periods determined by alkaline/agarose gel electrophoresis. Aliquots (25%) of the samples loaded in *A* were loaded in *D*. The positions of single-stranded linear (ssl) and single-strand circular (ssc) DNAs are indicated. Lanes without DNA, blank lanes (b), are also indicated. +, presence of TAg; −, absnece of TAg or pulse/chase. Markers (M) were prepared by first cleaving pSV01ΔEP with *Eco*RI. The ends of the two resulting fragments (2482 and 322 bp) were 3′-end-labeled with the Klenow fragment and [α-^{32}P]dATP. One aliquot of the two labeled fragments was cleaved with *Alu* I to generate labeled fragments of 18, 37, 185, and 641 bp. A second aliquot was digested with *Hin*dIII and *Bgl* I, resulting in labeled fragments that were 31, 85, 174, and 873 bp long. Aliquots from the *Eco*RI, *Alu* I, and *Hin*dIII–*Bgl* I digests were then pooled.

into a species whose distribution was centered around the 873-nucleotide size marker. After a 5-min chase, the upper part of the newly formed DNA distribution included DNA that migrated with the 2482-nucleotide size marker. Since pSV01ΔEP is 2804 bp long, this indicates that some of the newly synthesized DNA was nearly full length. With longer chase periods, much of the newly synthesized DNA appeared to be full length. However, some of the DNA had a mobility that suggests it was greater than unit length. The cause of this is unknown, but we have not excluded the possibility that replication at later time points includes rolling-circle synthesis.

Labeling of Form U_R^* Is Origin Specific. As shown in Fig. 2, TAg-dependent DNA synthesis was detected first in form U_R^*. To determine if the newly synthesized DNA was localized to the origin of replication, the site of DNA synthesized during pulse–chase reactions was mapped using restriction endonucleases.

A restriction map of pSV01ΔEP DNA showing the sites of cleavage of the relevant restriction endonucleases is shown in Fig. 3*B*. The position of the SV40 origin of replication is indicated as are the sizes of the fragments produced by digestion of this DNA with *Hin*cII, *Nco* I, *Hae* II, and *Pst* I. Fig. 3*A*, lane 1, shows the distribution of label in the restriction fragments after a 0.3-min pulse. This reaction was chased with unlabeled dCTP to facilitate formation of duplex DNA and subsequent cleavage with the restriction endonucleases. It is clear that pSV01ΔEP was labeled primarily in the origin region during the 0.3-min pulse. A densitometric trace of this lane indicated that >92% of the label was contained in the two origin-containing fragments. As demonstrated for all of the fragments, Fig. 3*A*, lane 2, shows that, in the absence of TAg, labeled fragments were not detected. After synthesis for 1 min, most of the label was still distributed in the two origin fragments. However, label was also detected in the 370- and 296-bp fragments. After 5 min of synthesis, label was detected in all of the fragments. It is clear from Fig. 3 that the percentage of label in the nonorigin-containing fragments continued to increase with time. From this data it is apparent that labeling of pSV01ΔEP DNA initiates at the origin and proceeds outward in a bidirectional manner.

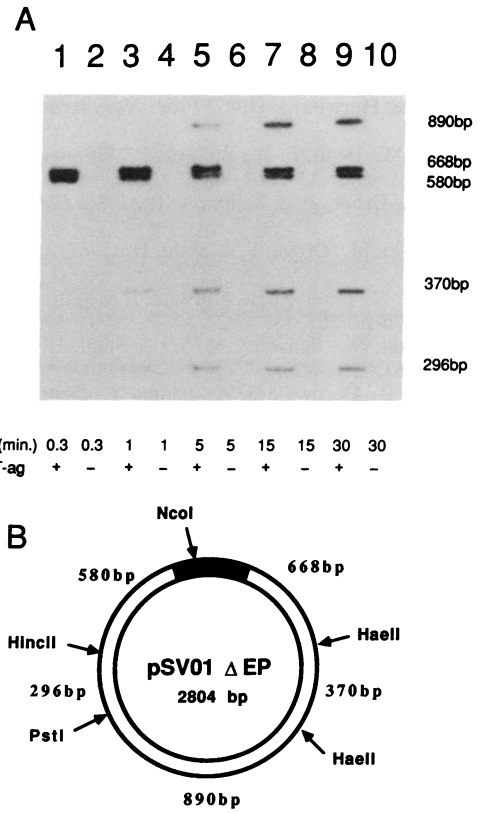

FIG. 3. Restriction endonuclease analyses of DNAs labeled during pulse–chase experiments. (*A*) After pulse-labeling for the indicated times and a chase period such that all pulse–chase reactions were carried out for 40 min, DNAs were cleaved with *Nco* I, *Hin*cII, *Hae* II, and *Pst* I. The sizes of the fragments resulting from cleavage of pSV01ΔEP with these restriction endonucleases are indicated to the right of the figure. The DNA fragments were separated on a 6% polyacrylamide gel. +, TAg (T-ag) present; −, TAg absent. (*B*) A restriction map of pSV01ΔEP is shown with the positions of the relevant restriction sites and the sizes of the fragments produced. The solid area represents the 322-bp SV40 origin-containing fragment cloned into this plasmid.

DISCUSSION

Previous experiments with TAg, topo I, *E. coli* SSB, and plasmids containing point mutations in the SV40 core origin indicated that unwinding of SV40 origin-containing DNA plays an essential role in replication (30). Moreover, it was demonstrated that a plasmid containing the SV40 origin deletion mutation 6-1 (35) was not unwound *in vitro* (24). This mutant, which contains a 6-bp deletion around the *Bgl* I site in the SV40 core origin, is unable to replicate in cell lines permissive for SV40 replication. Additional evidence for a role for unwinding in SV40 replication has been reported (9). Furthermore, the mobility of certain early replication intermediates observed by others suggests that they are related to unwinding events. Prelich and Stillman (10) suggested that an early replication intermediate was related to form U.

We have demonstrated that form U_R, a species that comigrates with the upper limit of the form U distribution and whose appearance is TAg-dependent, is formed in replication reaction mixtures containing crude extracts. Since form U_R is produced in otherwise complete reaction mixtures lacking dNTPs, formation of this species does not depend on DNA replication; its appearance is more likely a prerequisite for DNA synthesis. With pulse–chase experiments, we have also shown that in crude extracts the first detectable labeled species, form U_R^*, comigrates with form U_R suggesting a precursor–product relationship. Nevertheless, since we cannot exclude the possibility of topological changes during the 5-s or 20-s pulse periods, we cannot state unequivocally that form U_R is the initial substrate for replication. However, since form U_R comigrates with form U_R^* and since labeling is origin-specific, form U_R is certainly an early intermediate.

It is apparent from studies of other prokaryotic and eukaryotic replication systems that origin-specific unwinding is a critical step during initiation of replication. Origin-specific unwinding has been shown to be an essential step during initiation of bacteriophage λ DNA replication (36). This reaction requires λ proteins O and P, along with host proteins DnaB, DnaJ, DnaK, and SSB; unwinding is probably catalyzed by the helicase activity of DnaB. DnaB has also been shown to play a key role in the unwinding of plasmid templates containing the origin of the *E. coli* chromosome (37). In addition to DnaB, unwinding in the *E. coli* system requires DnaA, DnaC, gyrase, SSB, and ATP and is stimulated by the protein HU. It has been shown (38) that DnaB is guided to the origin after DnaA protein recognizes and opens the 13-mer sequences. Moreover, it has been reported (39) that a yeast *ARS* element requires an additional region that predisposes DNA to unwind. Finally, it has been proposed that prior to entry into the S phase during the mammalian cell cycle, a factor is produced that is required for unwinding of the SV40 origin (40).

At present, we are uncertain of the steps required to generate form U_R. In complete reaction mixtures lacking only

rNTPs and dNTPs, form U_R was detected in less than 15 min. These reaction conditions have been shown (41) to eliminate a lag that normally precedes replication. Presumably, form U_R production is a reflection of local melting of the origin region by TAg and subsequent initiation complex formation; the specificity of the unwinding event for the origin is suggested by our restriction endonuclease studies. We have demonstrated that form U_R comigrates with the upper part of the form U distribution. Studies of form U by electron microscopy revealed that it consists of a distribution of molecules extending from partially unwound to fully unwound species (25). Thus, it is possible that, owing to local unwinding at the origin region, form U_R comigrates with the partially unwound portion of form U. An indication that form U_R is locally unwound was suggested by experiments in which excess purified HeLa SSB was added with crude extracts to reaction mixtures lacking rNTPs and dNTPs; there was a broadening downward of the form U_R species such that it begins to resemble form U (data not shown). The linking number of the form U_R species formed after a 15-min preincubation with TAg is presently unknown. However, once determined this information will enable us to more accurately estimate the size of the single-strand bubble induced by the initiation complex.

Studies of unwinding events in crude extracts will enable a more complete understanding of the events that govern initiation of replication. For example, we have found that even after 45 min of preincubation with TAg, pulse labeling of form U_R is still localized to the origin region. This suggests that in crude extracts of HeLa cells, in the absence of rNTPs and dNTPs, a high percentage of the replication complexes are maintained at the origin. Moreover, we have found that pSV01ΔEP will unwind in mouse FM3A extracts in an SV40 TAg-dependent manner. This demonstrates that SV40 DNA replication in mouse extracts is blocked at a point subsequent to unwinding (data not shown).

Preliminary studies of replication of pSV01ΔEP in a purified system containing TAg, HeLa SSB, topo I, polymerase α–primase complex, a 5′ to 3′ exonuclease, DNA ligase, and RNase H (14) indicated that the topoisomers formed are similar to those in the crude system. Moreover, in the purified system a species comigrating with form U_R^* was also the first detectable species labeled (data not shown). However, the efficiency of labeling and elongation in the purified system is less than that in the crude system. These results indicate that certain factor(s) necessary for efficient initiation of replication are missing in the purified system.

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