Proc. Natl. Acad. Sci. USA
Vol. 82, pp. 5710–5714, September 1985
Biochemistry

In vitro replication of duplex circular DNA containing the simian virus 40 DNA origin site

(large tumor antigen/DNA polymerase α/eukaryotic DNA replication/complex DNA structures)

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ABSTRACT **Extracts (0.2 M NaCl) of HeLa cells support replication of DNA containing simian virus 40 (SV40) origin in the presence of SV40 large tumor (T) antigen. The reaction leads to the accumulation of high molecular weight products that represent DNA containing one parental strand and one progeny strand as well as duplex molecules that contain both strands derived from the input deoxynucleoside triphosphates. The replication reaction is inhibited by aphidicolin and by camptothecin, two inhibitors known to inhibit eukaryotic DNA replication *in vivo*.**

Studies of eukaryotic DNA replication have focused on viral systems because of their well-defined structure and simplicity. Two systems have now been developed that support replication *in vitro*. One is the adenovirus system in which the initiation of replication occurs by a protein priming reaction (reviewed in ref. 1). The subsequent replication involves strand displacement concomitant with DNA elongation. Both strands of adenovirus DNA are initiated in the same manner and require the same proteins (2–4).

Recent studies (5–7) have shown that the replication of duplex circular DNA containing the simian virus 40 (SV40) DNA origin region can be carried out with cell-free extracts provided that the SV40 DNA-encoded protein, the large tumor (T) antigen, is present. Extracts, suitably fortified, support bidirectional replication starting from a fixed point on the genome, the origin region.

Using soluble extracts isolated from HeLa cells, we have confirmed and extended these findings. The replication of plasmid DNA containing the SV40 origin region has been efficiently carried out. The reaction is inhibited by agents known to interfere with replication of DNA *in vivo*; product analyses indicate that a substantial number of molecules undergo multiple rounds of DNA synthesis. The products formed are monomeric rings as well as complex structures differing from those observed by Varshavsky *et al.* (8) in their *in vivo* studies of SV40 DNA replication.

MATERIALS AND METHODS

Preparation of HeLa Cell Extracts. Suspension cultures of HeLa cells were maintained in Eagle's minimal essential medium for spinner cultures, containing 10% calf serum. Mid-log-phase cultures (10 liters, 5×10^5 cells per ml) were harvested by centrifugation and the cell pellet was washed with 200 ml of ice-cold phosphate-buffered saline (137 mM NaCl/2.7 mM KCl/10.6 mM Na_2HPO_4/1.4 mM NaH_2PO_4). The cells were then washed once with 100 ml of cold hypotonic buffer (20 mM Hepes, pH 7.5/5 mM KCl/1.5 mM $MgCl_2$/1 mM dithiothreitol) and resuspended in 30 ml of the same buffer. After swelling on ice for 10 min, the cells were disrupted by Dounce homogenization (20 strokes, B pestle). The lysate then was adjusted to 0.2 M NaCl and immediately centrifuged at 50,000 × *g* for 30 min. After dialysis for 3 hr against one change of buffer A (20 mM Hepes, pH 7.5/1 mM dithiothreitol/0.1 mM EDTA/10% (vol/vol) glycerol/50 mM NaCl), the extract was clarified by centrifugation at 50,000 × *g* for 30 min and stored in aliquots at −80°C.

DNA Preparations. Plasmids pBR322ΔEP [ori⁻, 2480 base pairs (bp)] and pSV01ΔEP (ori⁺, 2790 bp) were prepared from parent plasmids pBR322 and pSV01 [kindly provided by R. Tjian and containing the origin-bearing *Eco*RII G fragment of SV40 inserted into the *Eco*RI site of pBR322 (9)], by digestion with *Eco*RV and *Pvu* II and intramolecular ligation of the blunt ends. This resulted in removal of ≈1700 bp from the plasmid, including the tetracycline-resistance marker, and creation of a *Dpn* I (*Mbo* I) restriction site (shown as D* in Fig. 3*B*) at the junction of the *Eco*RV–*Pvu* II half-sites. These plasmids were maintained in *Escherichia coli* HB101.

Purification of T Antigen. SV40 large T antigen was purified from Cos-1 cells (10) infected with SV40 cs1085 [kindly provided by D. Nathans (11)] at 10 plaque-forming units per cell. The purification included immunoaffinity chromatography according to the procedure of Simanis and Lane (12), using a monoclonal antibody against SV40 T antigen, PAb419 (13). T antigen was detected in column fractions by its ability to cause selective retention of ^{32}P-labeled DNA containing the SV40 origin of replication to nitrocellulose filters under the conditions used by Gronostajski *et al.* (14). Pooled T-antigen fractions were dialyzed against 10 mM Hepes, pH 7.5/1 mM dithiothreitol/5 mM NaCl/0.1 mM EDTA/50% glycerol/1 mM phenylmethylsulfonyl fluoride and stored at −20°C.

DNA Replication Assays. Reaction mixtures (50 μl) contained 30 mM Hepes (pH 7.5); 7 mM $MgCl_2$; 0.5 mM dithiothreitol; 4 mM ATP; 200 μM each CTP, GTP and UTP; 100 μM each of dATP, dGTP, and dTTP; 25 μM [α-^{32}P]dCTP (1–10 cpm/fmol, Amersham); 40 mM creatine phosphate; 1 μg of creatine kinase (Worthington); 0.3 μg of superhelical circular duplex (RFI) plasmid DNA; 300–400 μg (based on protein) of HeLa extract; and 0.6 μg of SV40 T antigen. The reaction mixtures were incubated at 37°C as indicated. Acid-insoluble radioactivity was measured as described (15). For analysis of products by gel electrophoresis, reactions were terminated by addition of 20 mM EDTA, 0.5% $NaDodSO_4$, and 20 μg of *E. coli* tRNA as carrier, followed by digestion with proteinase K (200 μg/ml; Worthington) at 37°C for 30 min. After phenol extraction, the products were precipitated with ethanol and electrophoresed in 1.5% agarose gels at 8 V/cm.

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Abbreviations: SV40, simian virus 40; T antigen, SV40-encoded large tumor antigen; RFI, superhelical circular duplex DNA; RFII, circular duplex DNA containing at least one single-strand break; RFIII, linear product formed from RFI DNA; RFI′, relaxed circular duplex DNA; bp, base pair(s).

Restriction Endonuclease Digestion. Restriction endonucleases were from New England Biolabs and were used according to the accompanying instructions, with the exception that incubation with *Dpn* I was carried out with 0.2 M NaCl; at NaCl concentrations less than 0.2 M, hemimethylated as well as doubly methylated DNA were cut by the enzyme (unpublished observation).

RESULTS

Preparation of HeLa Cytosolic Extracts and Requirements for Replication *in Vitro*. The extraction of HeLa cells under the conditions specified in *Materials and Methods* yielded extracts that were 3- to 5-fold more active than those prepared as described by Li and Kelly (6). These extracts catalyzed extensive incorporation of deoxynucleotides only in the presence of the SV40 T antigen and an SV40 ori^+ DNA (pSV01ΔEP). After a lag of 15 min (though not shown in Fig. 1*A*), DNA synthesis was linear for nearly 2 hr, after which the rate of incorporation declined.

Agarose gel electrophoresis (Fig. 1*B*) showed that the replication products included RFI, RFII (circular duplex DNA containing at least one single-strand break), intervening topoisomers, and discrete slower-migrating species. Prolonged autoradiography (not shown) revealed that labeled RFI, RFII, and topoisomers were formed even in the absence of T antigen or with ori^- DNA, possibly reflecting repair-type synthesis. The high molecular weight DNA species were formed only in the simultaneous presence of T antigen and ori^+ DNA.

Replication was completely dependent on the presence of T antigen, Mg^{2+}, an ATP-regenerating system, deoxynucleoside triphosphates, and ori^+ DNA containing the SV40 origin region (Table 1). In the presence of creatine phosphate and creatine kinase, omission of ATP reduced incorporation by a factor of nearly 3, whereas the omission of UTP, GTP, and CTP reduced synthesis only 20%. Aphidicolin, a specific inhibitor of DNA polymerase α, markedly inhibited incorporation. Depleting extracts of DNA polymerase α activity (>90%) by use of polymerase α-specific monoclonal antibody columns (16) also effectively eliminated replication. Addition of purified DNA polymerase α-primase fractions (15) did not restore activity. However, crude nuclear extract (inactive by itself) partially activated (40–50%) the depleted fraction. Camptothecin, an inhibitor of eukaryotic DNA replication *in vivo* (17), inhibited incorporaion 50% at 10 μM, similar to *in vivo* observations; 60% inhibition was seen at 500 μM. Pancreatic DNase I (200 μg/ml) and RNase A (5 μg/ml) but not *E. coli* RNase H (0.5 unit) inhibited the reaction.

As shown by Li and Kelly (6), topoisomerase I-relaxed RFI (i.e., RFI′) DNA supported synthesis as efficiently as supercoiled RFI. In contrast, activity with linearized plasmid RFIII DNA was reduced 97%, as was found by Stillman and Gluzman (7). Replication was sensitive to salt (50% inhibition with 50 mM NaCl, KCl, or NH_4Cl).

Products formed in reactions described in Table 1 were analyzed by agarose gel electrophoresis (Fig. 2). In the absence of creatine phosphate and creatine kinase, incorporation into RFI, RFII, and topoisomers was seen even in the absence of T antigen; addition of T antigen did not alter this pattern. In the absence of ATP, synthesis of RFI, RFII, and topoisomers was more markedly inhibited (90%) than that of the high molecular weight species (60%). Finally, in the presence of camptothecin, the synthesis of the high molecular weight species was inhibited 80–90%, compared to 50–60% for the faster-migrating topoisomers.

Characterization of the High Molecular Weight Products. The labeled DNA products described above resulted from semiconservative replication, based on its sensitivity to restriction endonuclease *Dpn* I. Both *Dpn* I and *Mbo* I (see

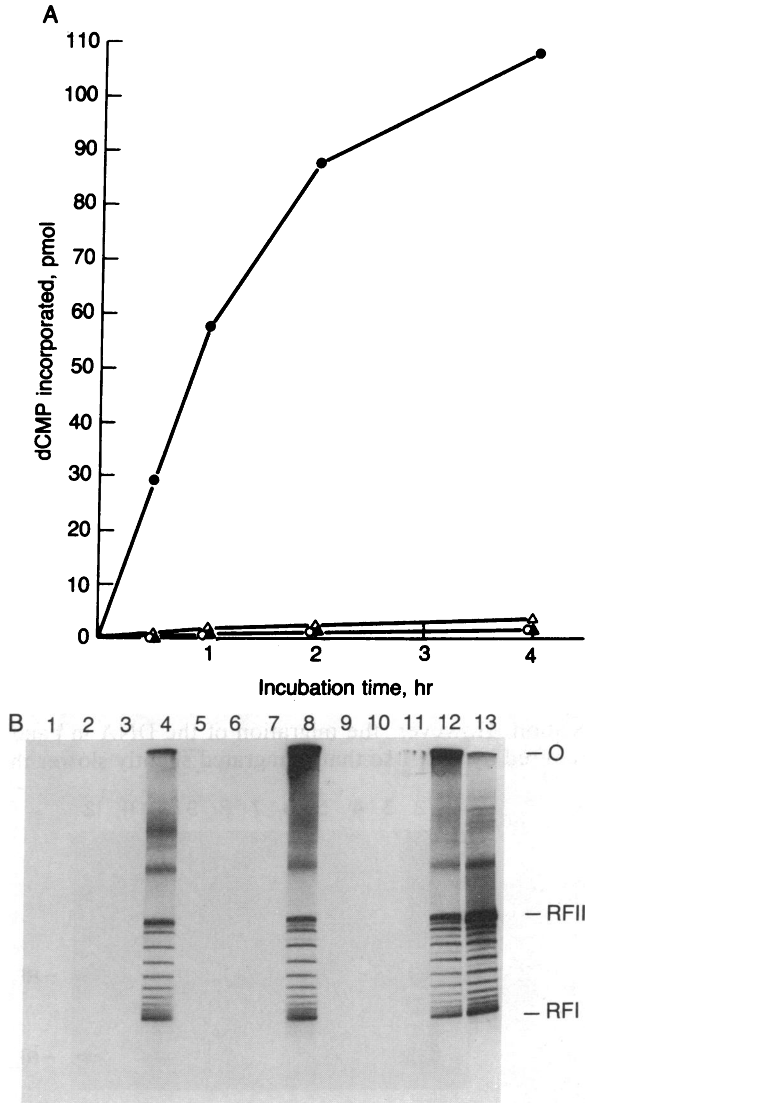

FIG. 1. (*A*) Time course of replication of ori^+ DNA (○, ●) or ori^- DNA (△, ▲) in presence (solid symbols) or absence (open symbols) of 0.6 μg of SV40 T antigen. Reaction conditions were as described in *Materials and Methods*. At indicated times, aliquots were assayed for acid-insoluble radioactivity. (*B*) Agarose (1.5%) gel electrophoresis of reaction products after 1 (lanes 1–4), 2 (lanes 5–8), 4 (lanes 9–12), or 24 hr (lane 13) of incubation. Reaction mixtures containing ori^- DNA without (lanes 1, 5, and 9) or with (lanes 2, 6, and 10) SV40 T antigen or containing ori^+ DNA without (lanes 3, 7, and 11) or with (lanes 4, 8, 12, and 13) SV40 T antigen were incubated and processed for electrophoresis as described in *Materials and Methods*. RFI and RFII indicate positions of pSV01ΔEP DNA markers run in parallel and visualized with ethidium bromide. O, origin of electrophoresis.

below) act at the sequence $\frac{\text{-G-A-T-C-}}{\text{-C-T-A-G-}}$, the site of action of the *E. coli* Dam methylase (18). *Dpn* I will cut at this site if both adenine residues are methylated, whereas *Mbo* I will cut only if neither adenine is methylated. The ori^+ plasmid was isolated from a dam^+ strain of *E. coli* (HB101) and is efficiently digested by *Dpn* I.

We routinely observed four bands of high molecular weight products, labeled A, B, C, and D, in addition to supercoiled (form I), and nicked (form II) product monomer circles (Fig. 3*A*). Products isolated after various periods of DNA synthesis were resistant to digestion with *Dpn* I (i.e., no small DNA fragments were produced by the enzyme). This suggests that all of the products are either hemimethylated or unmethylated and are therefore the products of semiconservative

Table 1. Requirements for replication of pSV01ΔEP DNA

Component omitted or added (+)	dNMPs incorporated, pmol/2 hr
None	620
T antigen	10
DNA	<4
DNA + ori⁻ DNA	8
DNA + topoisomerase I-treated ori⁺ DNA	516
DNA + *Pst* I RFIII of ori⁺ DNA	16
ATP	214
Creatine phosphate and creatine kinase	56
dATP, dGTP, dTTP and CTP, UTP, GTP	19
dATP, dGTP, dTTP	19
CTP, UTP, GTP	496
+ Aphidicolin 100 μM	9
400 μM	7
+ Camptothecin 100 μM	360
500 μM	260

Reaction mixtures (50 μl) were as described in *Materials and Methods*, with 0.3 μg of pSV01ΔEP(DNA) or 0.3 μg of pBR322ΔEP (ori⁻ DNA), 380 μg of HeLa extract protein and 25 μM [α-^{32}P]dCTP (2000 cpm/pmol).

replication. However, the migration of the DNA in band B was altered by *Dpn* I so that it migrated slightly slower than

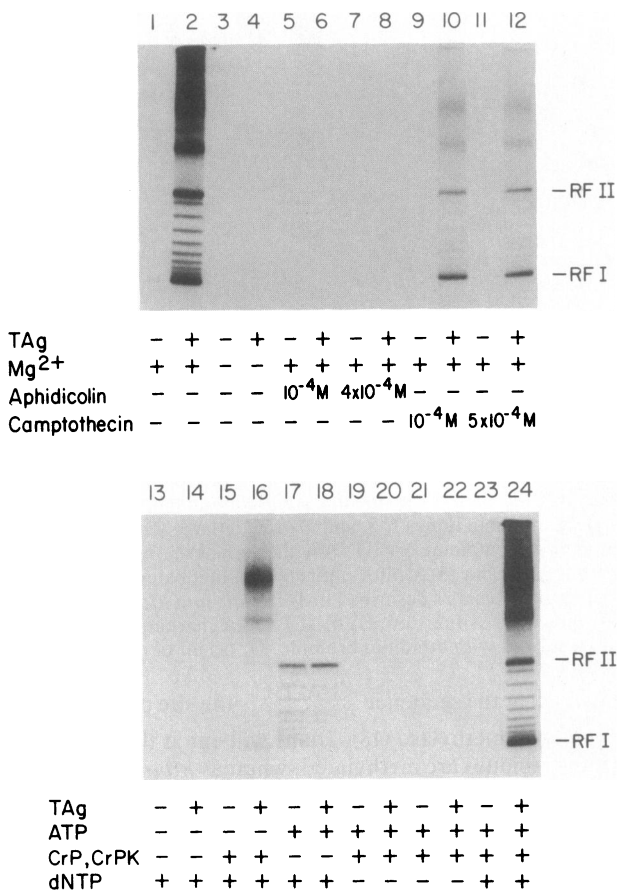

FIG. 2. Requirements for replication of ori⁺ DNA *in vitro* by HeLa cell extracts. Reaction mixtures were as described in Table 1 with additions or deletions as indicated below each lane. Products visualized in lanes 1 and 2 were isolated from complete reaction mixtures without and with T antigen (TAg) respectively. Incubation was for 2 hr at 37°C. CrP, creatine phosphate; CrPK, creatine kinase.

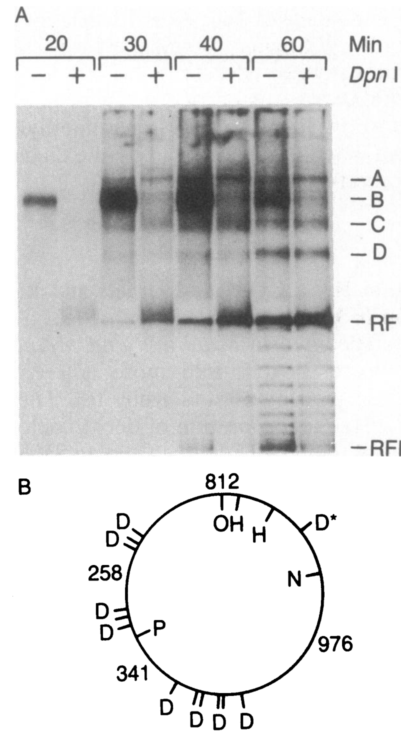

FIG. 3. (*A*) Analysis of replication products with restriction endonuclease *Dpn* I. A 150-μl reaction was preincubated at 30°C for 20 min in the absence of both [α-^{32}P]dCTP and T antigen but included all four unlabeled dNTPs. This reduced the background of T antigen-independent nucleotide incorporation by 80%. After the addition of 1.8 μg of T antigen and [α-^{32}P]dCTP, 25-μl aliquots were removed at 0 min, 10 min, and the times indicated above the lanes. No incorporation was observed until 20 min due to the 15-min lag in initiation. Products were electrophoresed after incubation either with (+) or without (−) restriction endonuclease *Dpn* I. An autoradiogram of the dried gel is shown. High molecular weight products are indicated by A, B, C, and D, and supercoiled (RFI) and nicked (RFII) ori⁺ DNA are also labeled. (*B*) A restriction map of pSV01ΔEP is presented, showing *Dpn* I/*Mbo* I (D), *Hin*dIII (H), *Pst* I (P), *Nde* I (N) and replication origin (O) sites. The sizes of the four largest *Dpn* I/*Mbo* I fragments are indicated in bp. The asterisk indicates the *Dpn* I site created at the junction of the ends brought together by the deletion of the *Eco*RV–*Pvu* II fragment of pBR322.

the monomer form II (Fig. 3*A*, lanes 1 and 2). The DNA in band B was the first replication product to appear and it contained some unreplicated *Dpn* I sites. This species may consist of theta replicative forms (19).

These results were confirmed and extended by using two-dimensional gel electrophoresis or gel-purified preparations of products A, B, C, D, and monomer forms I and II (data not shown). Treatment of the DNA with restriction enzymes *Pst* I and *Nde* I (see Fig. 3*B*) yielded the expected two fragments in all cases, and these fragments were *Dpn* I-resistant. However, treatment of bands A and B with *Hin*dIII yielded no 2594-bp linear DNA and only 10% of the label contained within bands C and D yielded this product; the remainder of the DNA migrated as a heterogeneous population slower than the linear form. *Hin*dIII treatment of forms I and II resulted in their quantitative conversion to the 2594-bp linear structure. Thus, we conclude that very little of the high molecular weight material consists of catenated DNA rings. The product forms A, B, C, and D are substantially but not completely replicated, but the unreplicated DNA segment is located at random sites around the circle, its position probably determined by the relative rates of movement of the two replication forks.

Multiple Rounds of Replication. As shown in Table 1, more than 600 pmol of deoxynucleoside monophosphate was

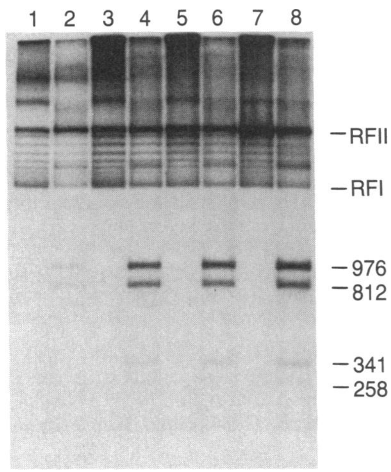

FIG. 4. *Mbo* I digestion of replication products. ori$^+$ DNA (0.3 μg) was incubated with HeLa cell extract (350 μg of protein) plus 0.6 μg of T antigen for 1 (lanes 1 and 2), 2 (lanes 3 and 4), 4 (lanes 5 and 6), or 24 hr (lanes 7 and 8), followed by protease digestion, phenol extraction, and ethanol-precipitation as described in *Materials and Methods*. After the DNA precipitate was dissolved in 50 mM Tris Cl, pH 8.0/10 mM $MgCl_2$/1 mM dithiothreitol/50 mM NaCl/ bovine serum albumin (100 μg/ml), it was incubated for 1 hr at 37°C without (lanes 1, 3, 5, and 7) or with 5 units of *Mbo* I (lanes 2, 4, 6, and 8) and electrophoresed in a 1.5% agarose gel. The positions of *Mbo* I bands, indicated at right (sizes in bp) were determined from ethidium bromide-stained, *Dpn* I-digested ori$^+$ DNA run simultaneously with the above digests.

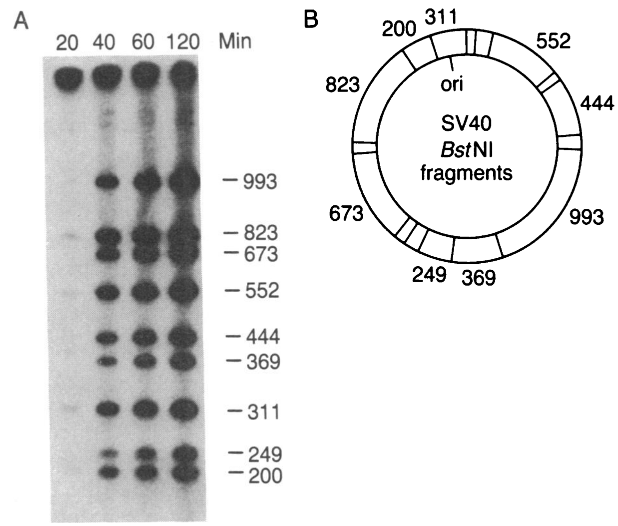

FIG. 5. Bidirectional replication starting from the SV40 origin. A 300-μl reaction mixture was preincubated at 30°C for 20 min in the absence of both [α-^{32}P]dCTP and T antigen but in the presence of all four unlabeled dNTPs. Replication was initiated by the addition of 3.6 μg of T antigen and [α-^{32}P]dCTP. Aliquots (50 μl) were removed from a 300-μl reaction mixture at 0 min, 10 min, and the times indicated. No incorporation was observed until 20 min due to the 15 min lag in DNA synthesis. (*A*) Reaction products were isolated, treated with restriction endonuclease *Bst*NI, and electrophoresed in a 5% acrylamide gel in 0.1 M Tris borate (pH 8.5) plus 2 mM EDTA and then subjected to autoradiography. The sizes of *Bst*NI restriction fragments are indicated at right in bp. (*B*) A map of the SV40 *Bst*NI restriction fragments with the replication origin indicated within the 311-bp fragment.

incorporated in reactions containing 300 ng of DNA, corresponding to roughly 70% net synthesis. To determine the fraction of this replication that was due to reinitiation and replication of daughter molecules, reaction products were isolated at intervals during incubation and digested with the restriction endonuclease *Mbo* I. Since *Mbo* I sites of input DNA or molecules that have undergone only one round of replication would be fully or hemimethylated, respectively (see above), they would be resistant to cleavage by *Mbo* I; any *Mbo* I-sensitive material may result from two or more rounds of replication. Labeled *Mbo* I bands were observed after 1 hr incubation (Fig. 4, lane 2), and they accumulated with longer times (Fig. 4, lanes 4, 6, and 8). Quantitation of the radioactivity present in the *Mbo* I bands and in the higher molecular weight material remaining after digestion showed that (i) after 4 hr, ≈20% of the total replicated material was *Mbo* I-sensitive; (*ii*) material migrating slower than RFII was more sensitive to *Mbo* I cleavage than RFI, RFII, and topoisomers; and (*iii*) the extent of labeling of origin-containing (812 bp) and origin-distal (341 bp) *Mbo* I fragments was the same, after normalization for length, indicating efficient and extensive replication after second-round initiation events. Furthermore, of the high molecular weight species at 1 hr, only band D was affected by *Mbo* I treatment (it almost completely disappeared), and at longer times, all of the high molecular weight bands were reduced by *Mbo* I treatment.

Replication Is Bidirectional from the Origin. *In vitro* replication of SV40 RFI DNA was allowed to proceed for various times, and the products were digested with *Bst*NI. Only a subset of fragments was labeled after 20 min of reaction, the 823-, 552-, 311-, and 200-bp fragments (Fig. 5*A*). These are the fragments nearest the SV40 replication origin, in the 311-bp fragment, and their detection indicates that replication initiates at the origin and proceeds in both directions. It is possible that these results reflect a site-specific initiation reaction followed by a random unidirectional fork movement on each DNA molecule. However, electron micrographs of replicating molecules, obtained by Li and Kelly (6), showed bidirectional fork movement on individual DNA molecules.

DISCUSSION

The results presented here agree with those reported by Li and Kelly (6). Extracts (0.2 M NaCl) of lysed HeLa cells yield cell-free preparations that replicate circular DNA containing the SV40 origin in the presence of T antigen. This reaction also requires Mg^{2+}, ATP, an ATP-regenerating system, and the four deoxynucleoside triphosphates and is marginally stimulated by the addition of UTP, GTP, and CTP. The reaction leads to the accumulation of complex forms of DNA that migrate more slowly in agarose gels than do RFI and RFII. Cleavage of the products with *Dpn* I indicated that virtually all the labeled duplex DNA contained one strand totally devoid of methylated bases; cleavage with *Mbo* I markedly reduced the high molecular weight complex selectively and indicated that up to 10% of the total labeled DNA molecules had undergone rounds of synthesis yielding strands devoid of methylated bases.

Circular structures (RFI, RFI′, and RFII) supported DNA synthesis but RFIII was a poor template. It is not clear whether this finding reflects a requirement for circular DNA or is due to differences in nuclease susceptibility of these DNAs. The *in vitro* synthesis of DNA was blocked by pancreatic DNase as well as by RNase A. High concentrations of DNase (200 μg/ml) were required to reduce incorporation substantially, suggesting that the DNA may be complexed with proteins, such as histones, that protect DNA from nuclease attack. The inhibition of the reaction by RNase A remains to be explained.

The inhibition of the reaction with aphidicolin suggests that DNA polymerase α is the enzyme responsible for the incorporation of deoxynucleotides. Extracts depleted of their DNA polymerase α activity did not support DNA synthesis. The addition of purified DNA polymerase α-primase preparations (15) did not activate these depleted fractions. Elution of the material adsorbed to the antibody with triethylamine (pH 10.8) buffer did not yield fractions capable of activating the depleted fraction. However, the addition of low levels of competent crude extracts, which by themselves were totally inactive, activated depleted fractions.

The reaction was inhibited by camptothecin, a drug known to inhibit chromosomal DNA replication *in vivo* (17). This drug inhibits the action of topoisomerases I and II (unpublished observations) in a manner analogous to that found with the epipodophyllotoxin derivatives specific for topoisomerase II (ref. 20 and unpublished results). The selective inhibition of the formation of higher molecular weight products suggests an important role for topoisomerase I and/or topoisomerase II in the accumulation of the complex molecules.

Catenated DNA molecules were not detected among the products of ori^+ DNA replication though such intermediates were expected (8). Since a substantial amount of progeny monomer rings were formed, it is possible that the crude extracts efficiently decatenated any multiply linked rings that were produced. We have shown that the DNA molecules synthesized were the products of semiconservative replication. However, it is possible that some of the products may represent abortive rather than bona fide intermediates in the formation of progeny rings. Further analysis will be necessary to determine their precise structure and role in replication.

Fractionation of the cytosol has yielded multiple components that must be combined to support ori^+, T-antigen-dependent DNA synthesis. Some fractions catalyze extensive deoxynucleoside monophosphate incorporation into RFI, RFII, and monomeric topoisomers in the absence of T antigen. The addition of fractions totally devoid of activity inhibited the T-antigen-independent synthesis of the above products and restored the T-antigen-dependent synthesis of DNA. All fractions were heat-labile, suggesting that proteins are essential for the selective ori^+ DNA replication pathway.

The complexity of the bidirectional synthesis of DNA has been well documented by studies carried out with the *oriC* replication system of *E. coli* (21–24). The initiation reaction in this system depends upon the binding of the DnaA protein to the origin site, coupled to the action of gyrase, binding protein, RNA polymerase, and other components. The DnaA protein and the SV40 T antigen behave in an analogous fashion in that they both bind to origin sites (21–25). There is good reason to believe that the bidirectional replication of SV40 DNA will be as complex as the bacterial replication system.

Since SV40 DNA replication is dependent upon only one viral coded protein, the T antigen, it should provide a means to identify and characterize host proteins essential for bidirectional DNA replication.

Note Added in Proof. Electron microscopy of the high molecular weight DNA products (bands A–D in Fig. 3), performed in collaboration with Dr. M. Hsu of Rockefeller University, reveled predominantly theta replicative-form molecules (19) and rolling-circle structures. Few catenated rings were observed.

We are indebted to Dr. M. Bradley of the Sidney Farber Center for the gift of the PAb419 hybridoma cell line and for helpful discussions. These studies benefitted from the expert technical assistance of Mrs. C. Turk and N. Belgado. This work was supported by National Institutes of Health Grant 5-R01-GM13344-19. C.R.W. is supported by a Damon Runyon–Walter Winchell Cancer Fund Fellowship (DRG-867).

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