Simian virus 40 DNA replication in vitro
(eukaryotic DNA replication/simian virus 40 large tumor antigen/simian virus 40 origin of replication/Dpn I replication assay)

JOACHIM J. LI AND THOMAS J. KELLY
Department of Molecular Biology and Genetics, The Johns Hopkins University School of Medicine, Baltimore, MD 21205
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ABSTRACT Soluble extracts prepared from monkey cells (COS-1 or BSC-40) infected with simian virus 40 (SV40) catalyze the efficient replication of exogenously added plasmid DNA molecules containing the cloned SV40 origin of replication. Extracts prepared from uninfected monkey cells also support origin-dependent replication in vitro but only in the presence of added SV40 large tumor (T) antigen. Very little DNA synthesis is observed when the cloned viral origin contains a 4-base-pair deletion mutation known to abolish SV40 DNA replication in vivo or when the parental plasmid vector lacking SV40 sequence is employed as a template. The in vitro replication reaction proceeds via branched intermediates (6 structures) that resemble in vivo replication intermediates. Replication is sensitive to aphidicolin but relatively resistant to dideoxystymidine triphosphate. The product of the reaction consists of covalently closed circular DNA molecules that contain full-length daughter strands hydrogen bonded to the parental template. These observations support the conclusion that replication in the in vitro system closely resembles SV40 DNA replication in vivo. The system provides a biochemical assay for the replication activity of SV40 T antigen and should also facilitate the purification and functional characterization of cellular proteins involved in DNA replication.

The mechanisms involved in the replication of eukaryotic chromosomes are not yet understood. Animal viruses represent potentially useful model systems for studying such mechanisms (1). Our approach to the analysis of eukaryotic DNA replication has been to develop soluble cell-free systems that are capable of replicating exogenous viral DNA templates in vitro. We have previously described a cell-free system for adenovirus DNA replication (2, 3) that has proven useful for identifying replication proteins and for defining the mechanisms involved in initiation and elongation of adenovirus DNA chains (4, 5). In this report, we describe an analogous in vitro DNA replication system for the papovavirus, simian virus 40 (SV40).

SV40 appears to be an excellent model for a single chromosomal replicon. The viral genome is a 5.2-kilobase (kb) circular duplex minichromosome with a nucleoprotein structure analogous to cellular chromatin (6, 7). DNA replication is initiated within a unique origin on the viral chromosome and proceeds bidirectionally (8, 9). The initiation reaction requires a specific interaction between the origin sequence and a virus-encoded initiation protein, large tumor (T) antigen (10–13). The elongation of nascent SV40 DNA chains, mediated by cell-encoded replication proteins and takes place by mechanisms that closely resemble those that operate at chromosomal replication forks (14).

We have found that extracts prepared from monkey cells are capable of catalyzing the replication of DNA molecules containing the SV40 origin of replication. The replication of these templates is completely dependent on the presence of the SV40 T antigen. Moreover, the introduction of a 4-base-pair deletion mutation into the viral origin of replication greatly reduces the extent of in vitro DNA synthesis. In these and other respects, the DNA synthesis observed in the cell-free system closely resembles SV40 DNA replication in vivo.

MATERIALS AND METHODS

DNA Templates. The plasmid pKP45 was obtained from Keith Peden. It is derived from pBR322 by deletion of nucleotides 677–2364. The plasmid pLO was constructed by insertion of the HindIII/Sph I fragment of SV40 DNA (nucleotides 5171–128) between the HindIII and Sph I restriction sites in pKP45. The plasmid pLO-d4 was constructed in the same fashion except that the HindIII/Sph I fragment was derived from the plasmid 8-4, which contains a 4-base-pair deletion within the origin of SV40 DNA replication (15). Plasmids were propagated in Escherichia coli HB101 and plasmid DNA was purified by standard procedures (16).

Preparation of Cell Extracts and Purification of T Antigen. Monolayer cultures of COS-1 (17) or BSC-40 (18) cells were grown in 15-cm dishes at 37°C in Eagle's minimal essential medium supplemented with 10% fetal calf serum as described (18). Uninfected cells were harvested at 70–80% of confluence. Infected cells were harvested 39 hr after addition of SV40 cs1085 (19) at 20–40 plaque-forming units per cell to freshly confluent monolayers. The cells were washed twice with 10 ml of ice-cold hypotonic buffer (20 mM Hepes (pH 7.5), 5 mM KCl, 1.5 mM MgCl2, 1 mM dithiothreitol) and the excess buffer was removed. The swollen cells were then scraped into a Dounce homogenizer on ice, and the total volume was adjusted to 0.3 ml per dish. During the scraping procedure the cells lysed. The nuclei were disaggregated by 5–10 strokes in the Dounce homogenizer, and the lysate was incubated on ice for 30–60 min. After centrifugation at 10,000 × g for 10 min, the clarified lysate was stored at −70°C.

For preparation of SV40 T antigen, monolayer cultures of BSC-40 cells were infected with SV40 cs1085 (19) at 10–20 plaque-forming units per cell and incubated at 37°C for 50 hr. T antigen was purified by immunoaffinity chromatography (20) on a column containing monoclonal anti-T antibody (PAb419 = L19) (21). Greater than 95% of the product migrated as a single band during NaDodSO4/polyacrylamide gel electrophoresis.

Conditions for in Vitro DNA Synthesis. The standard replication reaction mixture contained (in 0.1 ml) 30 mM Hepes (pH 7.5), 7 mM MgCl2, 0.5 mM dithiothreitol, 100 μM [α-32P]dCTP (specific activity, 3000–6000 cpm/nmol), 100 μM dATP/dGTP/dTTP, 200 μM GTP/UTP/CTP, 4 mM ATP, 40 mM phosphocreatine, 10 μg of creatine phosphokinase (Sigma; stock solution, 2.5 mg/ml in 30% (v/vol) glycerol), 250 ng of form I plasmid DNA, and 60 μl of extract. Where indicated, 3.5 μg of immunoaffinity-purified T antigen was added.

Abbreviations: SV40, Simian virus 40; kb, kilobase(s); T antigen, SV40 large tumor antigen.
added to the reaction mixture. Reactions were incubated at 37°C for various periods and then stopped by adjusting the reaction mixtures to 15 mM EDTA, 200 µg of proteinase K/ml (EM Science, Gibbstown, NJ), 0.2% NaDodSO₄. After incubation for 20–30 min at 37°C, the solution was extracted once with phenol, desalted by gel filtration on superfine Sephadex G-50 (Pharmacia), and extracted once with chloroform, and the DNA was precipitated with ethanol. The deproteinized reaction was electrophoresed through 1.5% agarose gels. For the Dpn I assay (22), 50–60 ng of the deproteinized product was digested with 5–10 units of Dpn I (New England Biolabs) and 5–10 units of Sal I (Bethesda Research Laboratories) in 50 µl of 150 mM NaCl, 20 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 1 mM dithiothreitol at 37°C for 3–6 hr. For electron microscopy, the product was incubated with RNase A at 4 µg/ml (Calbiochem) for 5 min at 37°C. After phenol extraction, the DNA was precipitated with ethanol and dialyzed against 10 mM Tris-HCl (pH 8.0), 1 mM EDTA.

RESULTS

Replication in Vitro of DNA Templates Containing the SV40 Origin. In initial experiments, we studied the ability of soluble extracts of SV40-infected monkey cells to support DNA synthesis dependent on the viral origin of replication. The extracts were prepared from COS-1 or BSC-40 cells 39 hr after infection with SV40 cs1085, a viral mutant that overproduces wild-type T antigen (19). Two DNA templates were employed. Plasmid pJLO consists of nucleotides 5171–128 of the SV40 genome cloned in the vector pKP45, a derivative of pBR322. The viral DNA segment in this construct includes the complete wild-type SV40 origin of DNA replication (14). Plasmid pJLO-d4 is identical to pJLO except for the deletion of 4 base pairs at map positions 5239–5242 within the origin. This deletion is known to cause a severe defect in SV40 DNA replication in vivo (15). The extracts were incubated with each of the two plasmid templates, and the reaction products were analyzed by agarose gel electrophoresis. Since the plasmids are significantly smaller than the SV40 genome (2.3 kb vs. 5.2 kb), the products of replication of the exogenous DNA molecules were easily distinguished from the background of synthesis on the endogenous viral DNA templates contaminating the extracts.

As shown in Fig. 1, the extracts from both infected COS-1 and BSC-40 cells catalyzed extensive incorporation of radioactive precursor into pJLO DNA. The predominant reaction products migrated with the mobilities expected for the topological isomers of pJLO DNA, ranging from form I to relaxed circles. These radioactive products were not observed in significant amounts in reaction mixtures incubated in the absence of added DNA or in reaction mixtures incubated with pJLO-d4 DNA (Fig. 1). Thus, the observed incorporation was dependent on the presence of a template containing the wild-type SV40 origin of replication. DNA synthesis continued for at least 4 hr, at which time the extent of incorporation into pJLO DNA was typically 50–80 pmol of total nucleotide per standard reaction mixture. Since the reaction mixture contained extract derived from about 2 × 10⁶ cells, this corresponds to the synthesis of 2000–4000 pJLO molecules per cell equivalent of extract. Similar results were obtained with an extract from COS-1 cells infected with wild-type SV40 except that the extent of incorporation was lower (data not shown).

T-Antigen Dependence of SV40 DNA Replication in Vitro. The only SV40 gene product known to be directly required for viral DNA replication is the viral initiation protein, T antigen. Therefore, we tested the ability of extracts from uninfected monkey cells to support DNA replication in the presence of purified T antigen. The extracts were prepared from subconfluent COS-1 or BSC-40 cells; T antigen was pu-

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**FIG. 1.** In vitro replication of plasmid DNA containing the SV40 origin of replication. DNA was synthesized in standard reaction mixtures containing extract from COS-1 or BSC-40 cells infected with SV40 cs1085. As indicated above each lane, the reaction mixtures were incubated for 1, 2, or 4 hr at 37°C in the absence of added DNA or in the presence of pJLO DNA (wild-type SV40 origin) or pJLO-d4 DNA (mutant SV40 origin). The radioactive reaction products were electrophoresed in a 1.5% agarose gel, and the gels were then autoradiographed. The arrow marks the position of the endogenous SV40 form I DNA. The bracket indicates the region of the gel occupied by the topological isomers of the plasmid templates pJLO and pJLO-d4.
rified by immunoaffinity chromatography using monoclonal anti-T antibody (20). The assay for DNA replication employed in these studies was somewhat more stringent (and sensitive) than that described above (22). The basis of the assay is the observation that the restriction endonuclease Dpn I cleaves DNA at specific sites (G-mA-T-C) that have been methylated at adenine residues on both strands. The input plasmid DNA templates (pKP45, pJLO, and pJLO-d4) were isolated from E. coli containing the required methylase activity (dam-) and, thus, are cleaved to small fragments by Dpn I digestion. The semiconservative replication of such templates in vitro would be expected to yield hemimethylated or unmethylated DNA molecules that are completely resistant to Dpn I digestion (23).

In the experiment shown in Fig. 2, the reaction products were digested to completion with Dpn I/Sal I and analyzed by gel electrophoresis. Since each of the input plasmid templates contains a single Sal I cleavage site, the completely replicated products should migrate as linear DNA molecules of 2.3 kb. In the absence of added T antigen, neither nor BSC-40 extracts supported DNA replication with any of the templates tested. In the presence of T antigen, both extracts catalyzed the replication of the template containing the wild-type origin of replication (pJLO). Most of the radioactive product migrated with the expected mobility for 2.3-kb linear DNA. However, 10–20% of the product was observed to migrate more slowly and may represent partially replicated molecules. As in the case of the infected-cell extracts described above, DNA synthesis continued for at least 4 hr. Under the same conditions, very little DNA synthesis was observed with the template containing the mutant SV40 origin (pJLO-d4) or the template lacking SV40 sequences entirely (pKP45). Based on densitometric analysis of the autoradiograms, the incorporation of radioactive precursor into pJLO DNA was more than 100-fold greater than the incorporation into pJLO-d4 or pKP45 DNA. The same samples were assayed for DNA replication by the method described in Fig. 1 and the data were consistent with those obtained by the Dpn I assay (data not shown). These results confirm the conclusion that replication in vitro is dependent on the viral origin of replication and, in addition, demonstrate the requirement for T antigen. The Dpn I resistance of the reaction product is consistent with the semiconservative replication of full-length DNA strands. More direct evidence on this point will be given below.

It was somewhat surprising that extracts of COS-1 cells did not catalyze significant replication of pJLO DNA in the absence of added T antigen because such cells are capable of supporting the replication of T-antigen-defective viral mutants in vivo (17). We assume that the concentration of active T antigen in our COS-1 extracts was too low to support a detectable level of initiation. However, in the presence of purified T antigen, COS-1 extracts were reproducibly more active in DNA replication than BSC-40 extracts. It should also be noted that although pKP45, pJLO, and pJLO-d4 lack the so-called "poison" sequences of pBR322 that appear to inhibit the replication of DNA molecules introduced into cultured cells (24), we have recently found that these sequences have no significant effect on DNA replication in vitro (data not shown).

Table 1 summarizes other features of the in vitro replication reaction. Relaxed circular pJLO DNA, generated by incubation with Hela topoisomerase I, supports replication nearly to the same extent as form I pJLO DNA. Linear pJLO DNA, generated by cleavage with Pst I, also supports replication but is significantly less effective as a template than the circular forms of pJLO DNA. The incorporation observed with linear pJLO DNA is not simply a result of recircularization during incubation because the radioactive product is linear. Replication is strongly dependent on ATP and Mg<sup>2+</sup>-. Less dependence on the other ribonucleoside triphosphates or on the deoxyribonucleoside triphosphates was observed, presumably because the extracts contain significant concentrations of precursors. The reaction is almost completely inhibited by aphidicolin at 7 μg/ml but is resistant to inhibition by α-amanitin and dideoxythymidine triphosphate. This pattern of response to inhibitors suggests that SV40 DNA replication in vitro is mediated by DNA polymerase α, as it is in vivo (25).

**Evidence for Semiconservative DNA Replication.** To rule out the possibility that the in vitro DNA synthesis represented repair synthesis at nicks or gaps in the template molecules, pJLO DNA was synthesized in reaction mixtures containing 3-bromodeoxyuridine triphosphate in place of dTTP and analyzed by equilibrium centrifugation in CsCl (Fig. 3). Under neutral conditions, essentially all of the newly synthesized molecules banded in a narrow zone near the density expected for hybrid DNA. Under alkaline conditions the same product banded near the density expected for pJLO strands fully substituted with bromouracil. For both gradients the calculated extent of replacement of thymine with bromouracil residues in the newly synthesized DNA was ≈80%. It seems likely that replacement was not complete because of the presence of endogenous dTTP in the extracts.
Table 1. Requirements for SV40 DNA synthesis in vitro

<table>
<thead>
<tr>
<th>Reaction mixture and template</th>
<th>Relative activity</th>
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<tbody>
<tr>
<td>Complete + form I or III* pJLO-d4</td>
<td>100</td>
</tr>
<tr>
<td>- CTP, GTP, UTP</td>
<td>77</td>
</tr>
<tr>
<td>- ATP</td>
<td>17</td>
</tr>
<tr>
<td>- Phosphocreatine and creatine</td>
<td>14</td>
</tr>
<tr>
<td>phosphokinase</td>
<td></td>
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<tr>
<td>- MgCl2</td>
<td>0.5</td>
</tr>
<tr>
<td>- dATP, dGTP, dTTP</td>
<td>57</td>
</tr>
<tr>
<td>+ Aphidicolin</td>
<td>40 µg/ml</td>
</tr>
<tr>
<td></td>
<td>7 µg/ml</td>
</tr>
<tr>
<td>+ dTTP/dTTP</td>
<td>100 µM/20 µM</td>
</tr>
<tr>
<td></td>
<td>400 µM/20 µM</td>
</tr>
<tr>
<td>+ α-Amanitin at 250 µg/ml</td>
<td>94</td>
</tr>
<tr>
<td>Complete - template</td>
<td>0.5</td>
</tr>
<tr>
<td>Complete + relaxed pJLO</td>
<td>72</td>
</tr>
<tr>
<td>Complete + form III* pJLO</td>
<td>18</td>
</tr>
<tr>
<td>Complete + form I or III* pJLO-d4</td>
<td>2</td>
</tr>
</tbody>
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Standard reaction mixtures incubated for 4 hr contained either extract from SV40 cts1085-infected COS-1 cells or extract from uninfected COS-1 cells supplemented with purified T antigen (Tag). After electrophoresis of the samples and autoradiography of the dried gel, the regions containing the topological isomers of the plasmid templates were excised, and 32P incorporation was quantitated by scintillation counting in Aquasol (New England Nuclear). In each case, the amount of 32P incorporated by the complete reaction mixture was normalized to 100. The amount of DNA synthesized in the complete reaction was typically 50–80 pmol for infected extracts and 20–40 pmol for uninfected extracts plus T antigen. These results have been confirmed by the Dpn I assay.

 strokes DNA incubated with HeLa topoisomerase I (a gift of Leroy Liu).

 strokes DNA linearized with Pst I, which places the origin of SV40 one-third of the distance from one end.

These data indicate that DNA molecules containing the wild-type SV40 origin of replication support the semiconservative synthesis of complete DNA strands that are hydrogen-bonded, but not covalently linked, to the template.

Electron Microscopy of Replicative Intermediates. A standard replication reaction mixture containing extract from uninfected COS-1 cells, purified SV40 T antigen, and pJLO DNA was incubated for 30 min at 37°C and examined by electron microscopy. Circular molecules containing two forks and no free ends (θ structures) were observed at a frequency of ~2% of the pJLO DNA molecules. Two examples are shown in Fig. 4. Length measurements showed that the structural features of these molecules were identical to those of SV40 replicative intermediates previously characterized in vivo studies (27, 28). In particular, two of the branches in each θ structure were equal in length and presumably represented replicated segments of the pJLO genome. Although the lengths of the replicated segments varied from molecule to molecule, in each case the sum of the lengths of one of the replicated segments and the unreplicated segment was equal to the total length of the pJLO genome. The extent of replication of the observed molecules ranged from 20% to 90%. In addition to θ structures, replication reaction mixtures also contained circular molecules with a single branch, as well as some more complex branched structures. It seems likely that the singly branched molecules were generated from θ structures in which one of the forks encountered a nick in a template strand. Such molecules were more frequent in reaction mixtures incubated for longer periods of time. θ structures and other branched molecules were not observed in significant numbers (<0.2%) in control reaction mixtures that contained pKP45 or pJLO-d4 DNA as template. A complete description of the electron microscopic data will be presented elsewhere.

**DISCUSSION**

In this communication, we have described a soluble enzyme system from monkey cells that carries out the replication of exogenous DNA templates containing the SV40 origin of DNA replication. Several lines of evidence indicate that the DNA synthesis observed in this cell-free system closely resembles SV40 DNA replication in vivo. The system is completely dependent on the presence of the SV40 T antigen. Extracts prepared from COS-1 or BSC-40 cells infected with

**Fig. 3.** Isopycnic centrifugation of DNA synthesized in vitro. DNA was synthesized in a reaction mixture containing extract from SV40 cts1085-infected COS-1 cells, pJLO DNA, and BrdUTP in place of dTTP. After incubation for 3 hr, the 32P-labeled product was deproteinized and electrophoresed in 1.5% agarose gel. The region of the gel containing the circular forms of pJLO DNA was excised and the DNA was eluted. The purified pJLO [32P]DNA (α) was linearized by digestion with EcoRI and mixed with linear marker pJLO [3H]DNA (β). The mixture was centrifuged at 30,000 rpm for 70 hr at 25°C in an SW50.1 rotor. (A) Centrifugation under neutral conditions (10 mM Tris-HCl, pH 7.5/1 mM EDTA, p = 1.745 g/ml in a total volume of 3 ml). (B) Centrifugation under alkaline conditions (150 mM K2PO4, pH 12.5/1.5 mM EDTA, p = 1.76 g/ml in a total volume of 3 ml). The arrows mark the positions for doubly substituted (HH), hybrid (HL), and unsubstituted (LL) pJLO duplexes and substituted (H) and unsubstituted (LL) pJLO single strands. X, Fraction density.

**Fig. 4.** Electron microscopy of in vitro replication intermediates. DNA was mounted for electron microscopy by the method of Davis et al. (26). The extent of replication of molecule A is ~20% and that of molecule B is ~90%. (Bar = 0.2 μm.)
SV40 cts1085 contain sufficient endogenous T antigen to support DNA replication in vitro. However, extracts from uninfected COS-1 or BSC-40 cells are inactive unless exogenous T antigen is added. Since the preparation of T antigen used in these studies was purified to near homogeneity, we conclude that the T antigen is the only viral gene product required for SV40 DNA replication in vitro. The system also exhibits a requirement for the SV40 origin of replication. Very little DNA synthesis was observed with a template lacking the viral origin (pKF45 DNA) or a template containing an origin mutation known to abolish SV40 DNA replication in vitro (pJLO-d4). With the template containing the origin (pJLO), replication was maximal with closed circular DNA. However, linear pJLO DNA also supported a low level of DNA synthesis, suggesting that superhelical tension is not absolutely required for replication in vitro. Electron microscopic observations showed that branched circular molecules (θ structures) identical in structure to in vivo replication intermediates are generated during the course of the in vitro replication reaction. The presence of these intermediates strongly suggests that DNA replication in the in vitro system proceeds bidirectionally from the viral origin, although this has not yet been demonstrated directly. Aphidicolin, an inhibitor of DNA polymerase α, inhibits in vitro replication at concentrations that have a similar effect on replication in vivo. Conversely, 2′, 3′-dideoxythimidine triphosphate, an inhibitor of DNA polymerase α, proceeds with the in vitro reaction.

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