MOLECULAR AND CELLULAR BIOLOGY, June 1985, p. 1238–1246
0270-7306/85/061238-09$02.00/0

Vol. 5, No. 6

Simian Virus 40 DNA Replication In Vitro: Specificity of Initiation and Evidence for Bidirectional Replication

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Received 4 February 1985/Accepted 18 March 1985

We recently described a soluble cell-free system derived from monkey cells that is capable of replicating exogenous plasmid DNA molecules containing the simian virus 40 (SV40) origin of replication (J. J. Li, and T. J. Kelly, Proc. Natl. Acad. Sci. U.S.A. 81:6973–6977, 1984). Replication in the system is completely dependent upon the addition of the SV40 large T antigen. In this report we describe additional properties of the in vitro replication reaction. Extracts prepared from cells of several nonsimian species were tested for the ability to support origin-dependent replication in the presence of T antigen. The activities of extracts derived from human cell lines HeLa and 293 were approximately the same as those of monkey cell extracts. Chinese hamster ovary cell extracts also supported SV40 DNA replication in vitro, but the extent of replication was approximately 1% of that observed with human or monkey cell extracts. No replication activity was detectable in extracts derived from BALB/3T3 mouse cells. The ability of these extracts to support replication in vitro closely parallels the ability of the same cells to support replication in vivo. We also examined the ability of various DNA molecules containing sequences homologous to the SV40 origin to serve as templates in the cell-free system. Plasmids containing the origins of human papovaviruses BKV and JCV replicated with an efficiency 10 to 20% of that of plasmids containing the SV40 origin. Plasmids containing *Alu* repeat sequences (BLUR8) did not support detectable DNA replication in vitro. Circular DNA molecules were found to be the best templates for DNA replication in the cell-free system; however, linear DNA molecules containing the SV40 origin also replicated to a significant extent (10 to 20% of circular molecules). Finally, electron microscopy of replication intermediates demonstrated that the initiation of DNA synthesis in vitro takes place at a unique site corresponding to the in vivo origin and that replication is bidirectional. These findings provide further evidence that replication in the cell-free system faithfully mimics SV40 DNA replication in vivo.

Simian virus 40 (SV40) represents a simple model system for studying the mechanisms involved in DNA replication in eucaryotic cells (5, 12, 13). The SV40 genome is a double-stranded circular DNA molecule containing 5,243 base pairs (bp) (17, 42). In the nucleus of infected monkey cells the viral genome exists as a minichromosome with a nucleoprotein structure analogous to that of cellular chromatin (7, 25). Initiation of DNA synthesis takes place within a unique origin on the viral chromosome, and the elongation of nascent DNA chains proceeds bidirectionally (8, 16). The initiation reaction requires a specific interaction between the origin and a virus-encoded initiation protein, the T antigen (6, 39, 46, 49). Subsequent steps in the replication process (elongation and segregation) appear to require only cell-encoded proteins.

We recently described a soluble, cell-free SV40 DNA replication system (34). DNA replication in this system requires both an exogenous DNA template containing the wild-type SV40 origin of replication and the SV40 T antigen. The source of cellular replication proteins is a low ionic strength cytosol extract prepared from monkey cells. The system appears to carry out all of the steps involved in SV40 DNA replication, including initiation, chain elongation, and separation of sibling molecules. The intermediates in the in vitro reaction are branched circular molecules that closely resemble in vivo replication intermediates.

In this paper we report additional evidence for the biological relevance of the in vitro DNA replication system. Extracts prepared from monkey, human, or hamster cells (permissive or semipermissive for SV40 multiplication in vivo) are capable of supporting significant origin-dependent DNA synthesis in vitro, whereas extracts prepared from mouse cells (nonpermissive for SV40 DNA infection in vivo) are not. Circular DNA molecules are the most efficient templates for replication; however, linear DNA molecules containing the SV40 origin also support a low level of DNA replication that is dependent upon the presence of T antigen. The genomes of papovaviruses BKV and JCV, which are closely related to SV40, are capable of serving as templates for in vitro replication. The DNA of BLUR8, a plasmid containing a human *Alu* repeat sequence with partial homology to the SV40 origin (10, 31), is not an effective template. Finally, electron microscopic analysis of replication intermediates demonstrated that the initiation of DNA synthesis in vitro takes place at a unique site corresponding to the in vivo origin and that replication is bidirectional.

MATERIALS AND METHODS

DNA templates. Plasmids pJLO, pJLO-d4, and pKP45 were previously described in detail (34). Both pJLO and pJLO-d4 have the *Hin*dIII-*Sph*I fragment of SV40 DNA, containing the origin of replication, inserted between the corresponding restriction sites in pKP45. pJLO contains a wild-type origin fragment, whereas pJLO-d4 differs only in having a 4-bp deletion within the origin. Plasmids pSV5 and pSV5-d4 were constructed by Ron Wides. They are analo-

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gous to pJLO and pJLO-d4 but contain the entire SV40 genome inserted into the *Bam*HI site of pKP45. Plasmids pKP54 and pKP55 were obtained from Keith Peden. Both were derived from pBR322 by replacement of nucleotides 35 to 2456 by multiple restriction site polylinkers. pKP54 has a 27-bp polylinker *Sal*I-*Bam*HI-*Sma*I-*Kpn*I-*Xba*I oriented with *Sal*I closest to the *Hin*dIII site retained at nucleotide position 29. pKP55 has a 26-bp polylinker *Nco*I-*Sph*I-*Pvu*II-*Kpn*I-*Xba*I in the same orientation as above.

pBKO was constructed by inserting the origin-containing *Hin*dIII fragment of prototype BKV (19) into the *Hin*dIII site of pKP55. This fragment is equivalent to the 556-bp *Hin*dIII fragment spanning nucleotides 4945 to 348 in the Dunlop strain of BKV (45). It contains the putative BKV T-antigen-binding sites I and II and the 68-bp tandem repeat. pJCO was made by inserting the origin-containing *Nco*I fragment of prototype JCV (Mad-1 strain) (18) into the *Nco*I site of pKP55. This 331-bp insert contains the putative JCV T-antigen-binding sites I and II and the 98-bp tandem repeat. pAlu1, pAlu2, and pAlu3 contain the 265-bp human *Alu* repeat sequence cloned in BLUR8 (10). The sequence was isolated from a *Bam*HI digest of BLUR8 and inserted into the *Bam*HI site of pKP54. pAlu1, pAlu2, and pAlu3 contain, respectively, one, two, and three copies of the *Alu* sequence.

All plasmids were propagated as monomers in *Escherichia coli* DH1 (26). Plasmid DNAs were prepared by standard alkali lysis procedures (36). SV40 DNA (strain 776) was prepared from infected BSC-40 cells by the method of Hirt (29). All DNAs were purified by equilibrium banding in two CsCl gradients, and preparations consisted of ≥95% form I monomer DNA. The approximate sizes in kilobases (kb) of these DNAs are as follows: pKP45, 2.1; pJLO and pJLO-d4, 2.3; pSV5 and pSV5-d4, 7.9; pKP54 and pKP55, 2.0; pBKO, 2.6; pJCO, 2.3; pAlu1, 2.2; pAlu2, 2.5; pAlu3, 2.8.

Cells and extracts. Extracts from uninfected monolayer cultures of COS-1 (22), 293 (24), BALB/3T3, and Chinese hamster ovary (CHO) K1 (a proline auxotroph) cells were prepared as described previously for COS-1 cells (34). COS-1 and 293 cells were grown at 37°C in Eagle minimal essential medium supplemented with 10% fetal calf serum. BALB/3T3 and CHO K1 cells were maintained at 37°C in Dulbecco modified Eagle medium supplemented with 4.5 mg of D-glucose per ml and 10% fetal calf serum. Proline (11.5 μg/ml) was added for CHO K1 cells.

A modified extraction procedure was used for Spinner cultures. Spinner cultures of HeLa cells were grown at 37°C in Eagle minimal essential medium for suspension cultures supplemented with 5% horse serum. One liter of HeLa cells was harvested in mid-log phase at a density of 5×10^5 cells per ml by centrifugation at 500 to 1,000 × *g* for 5 min. The cell pellet was rapidly washed twice, first in 500 ml of ice-cold hypotonic buffer with 250 mM sucrose and then in 500 ml of ice-cold hypotonic buffer alone (20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; pH 7.5], 5 mM KCl, 1.5 mM $MgCl_2$, 1 mM dithiothreitol [DTT]). The washed cell pellet was suspended in hypotonic buffer at a density of 6×10^7 to 7×10^7 cells per ml and allowed to swell on ice for 15 min. The cells were then lysed with three to five strokes of a tightly fitting pestle in a Dounce homogenizer. After a 30- to 60-min incubation on ice the lysate was centrifuged at 10,000 × *g* for 10 min at 0°C. The clarified lysate was stored at −70°C. Replication activity was stable for at least 6 months.

Protein concentrations were measured by the method of Bradford (3) (Bio-Rad Laboratories, Richmond, Calif.) with bovine gamma globulin as a standard. Typical extract concentrations were 7 to 10 mg/ml for COS-1 and 3T3 cells and 15 to 20 mg/ml for 293, HeLa, and CHO cells.

T antigen was purified by immunoaffinity chromatography by a modification of the procedure of Dixon and Nathans (15). Eleven 850-cm^2 roller bottles of BSC-40 monkey kidney cells (4) were infected with mutant SV40 cs1085 (14), harvested, lysed, and clarified as described previously. The resulting cell extract was loaded onto a 1.5-ml PAb419 (27) immunoaffinity column at a rate of 10 ml/h. The column was washed with 10 ml of a solution containing 50 mM Tris (pH 8.0), 500 mM NaCl, 1 mM EDTA, 1 mM DTT, 10% glycerol, and 1% Nonidet P-40, followed by 10 ml of the same solution without Nonidet P-40 and then by 10 ml of the same solution at pH 9.0 without Nonidet P-40. The column was rapidly eluted with seven 1-ml fractions of elution buffer containing 20 mM Tris (pH 11), 500 mM NaCl, 1 mM EDTA, 1 mM DTT, and 10% glycerol. Fractions were collected directly into 0.2 ml of neutralizing buffer containing 500 mM Tris (pH 7.0), 1 mM EDTA, 1 mM DTT, and 10% glycerol. The elution was monitored by measuring the protein concentration as described above. Fractions containing T antigen were pooled and dialyzed against a solution containing 10 mM Tris (pH 8.0), 100 mM NaCl, 1 mM DTT, 1 mM EDTA, and 50% glycerol, before being stored at −20°C. The T-antigen replication activity was stable for at least 1 year. Typical yields were 5 mg of nearly homogeneous T antigen (by sodium dodecyl sulfate-polyacrylamide gel electrophoresis) at a concentration of 1 to 2 mg/ml.

In vitro replication conditions. The reactions were performed as described previously (34) with slight modifications. The volume of the reaction mixture was reduced to 50 μl. The concentration of [α-^{32}P]dCTP was lowered to 50 μM, and its specific activity was doubled to 6×10^3 to 12×10^3 dpm/pmol. The reaction also was changed to contain 60 ng of form I plasmid DNA and 2 μg of immunoaffinity-purified T antigen. All other components were maintained at the same concentrations. After a 4-h incubation at 37°C, the reaction was stopped and the products were isolated as described above. For the *Dpn*I assay (41) half of the deproteinized product was digested with 5 to 10 U of *Dpn*I (New England BioLabs, Inc., Beverly, Mass.) and 5 to 10 U of an enzyme that cut at a unique site in the plasmid under examination: pJLO, pJLO-d4, and pKP45 were digested with *Sal*I (Bethesda Research Laboratories, Inc., Gaithersburg, Md.); pBKO, pJCO, and pKP54 were digested with *Xba*I (Bethesda Research Laboratories); and pAlu1, pAlu2, pAlu3, and pKP55 were digested with *Pst*I (Bethesda Research Laboratories). All digestions were carried out in 50 μl of a solution containing 150 mM NaCl, 20 mM Tris-hydrochloride (pH 7.5), 10 mM $MgCl_2$, and 1 mM DTT at 37°C for 3 to 6 h. All digests were stopped by precipitation with ethanol.

Both digested and undigested replication products were subjected to electrophoresis on 1.5% agarose gels. The gels were then dried and autoradiographed with XAR-5 X-ray film (Eastman Kodak Co., Rochester, N.Y.) at −70°C with a Dupont Lightning-Plus intensifier screen (E. I. du Pont de Nemours & Co., Inc., Wilmington, Del.). For quantitation of radioactive label incorporated during replication, segments containing the full-length linear *Dpn*I-resistant [^{32}P]DNA were located from the autoradiograph and excised from the dried gel. Another segment of identical size directly below the full-length linear DNA was also excised to correct for background counts in the lane. Gel segments were counted in Betafluor (National Diagnostics, Somerville, N.J.) scintillation fluid. The picomoles of total nucleotides incorporated

into the products within the gel segment were estimated by 4 × [radioactivity of gel segment (in counts per minute)]/[specific activity of [α-^{32}P]dCTP (in counts per minute per picomole)]. This number was doubled to obtain the level of incorporation in the entire reaction mixture.

Electron microscopy. For electron microscopy the specific activity of [α-^{32}P]dCTP was lowered to 300 dpm/pmol. The deproteinized products were incubated with RNase A (Calbiochem-Behring, La Jolla, Calif.) at 4 μg/ml for 5 min at 37°C. This reaction was terminated by adjusting the mixture to 0.2% sodium dodecyl sulfate–200 μg of proteinase K (EM Science, Gibbstown, N.J.) per ml. After incubation for 1 h at 37°C, the solution was extracted twice with phenol and once with chloroform. DNA was precipitated with ethanol, suspended, and dialyzed against 10 mM Tris-hydrochloride (pH 8.0) and 1 mM EDTA. When restriction endonuclease digestions were required, they were performed on the deproteinized products immediately before RNase A treatment. When pSV5 or pSV5-d4 was used as the template, the reaction product was digested with 10 U of *Kpn*I (Bethesda Research Laboratories) or 2 U of *Pvu*I (New England BioLabs). When SV40 DNA was used as the template, the reaction product was digested with 7 U of *Bgl*I (Bethesda Research Laboratories). All digests were carried out in 50-μl mixtures for 1 h at 37°C under conditions specified by the enzyme suppliers.

DNA was mounted for electron microscopy by the formamide method of Davis et al. (9).

RESULTS

Further characterization of the cell-free DNA replication system. (i) DNA replication in extracts derived from cells of different mammalian species. We previously reported the development of a cell-free system that catalyzes the replication of DNA templates containing the SV40 origin of DNA replication (34). The system consists of two components: a cytosol extract containing cellular replication proteins and purified SV40 T antigen. In the initial experiments, the active cellular extracts were obtained from African green monkey kidney cells (e.g., BSC-40, CV-1, or COS-1 cells), the normal permissive hosts for SV40. We have since tested extracts derived from several lines of nonsimian cells for their ability to support origin-dependent DNA replication in vitro in the presence of T antigen (Fig. 1).

DNA synthesis was detected and quantitated by the *Dpn*I assay described previously (34, 41). The assay depends on the fact that the *Dpn*I restriction enzyme cleaves DNA at specific sites that have been methylated at adenine residues on both strands. The input plasmid DNA templates are prepared in *E. coli* containing the appropriate methylase (*dam*$^+$) and thus are cleaved to small fragments by digestion with *Dpn*I. The semiconservative replication of such templates results in the production of hemimethylated or unmethylated DNA molecules which are completely resistant to *Dpn*I digestion. In the present experiment the reaction products were incubated with *Dpn*I and *Sal*I. Since the plasmid templates (pJLO and pJLO-d4) each contain a single *Sal*I site, newly replicated daughter plasmids are cleaved to unit-length linear molecules approximately 2.3 kb in size. Unreplicated plasmids, however, are cleaved at 13 sites by *Dpn*I, yielding fragments ranging in size from 8 to 701 bp. In this and subsequent experiments, the amount of *Dpn*I-resistant DNA synthesis was quantitated by excising the gel segment containing the unit-length linear product and measuring the radioactive incorporation by scintillation spectrometry.

Extracts derived from human cell lines HeLa and 293

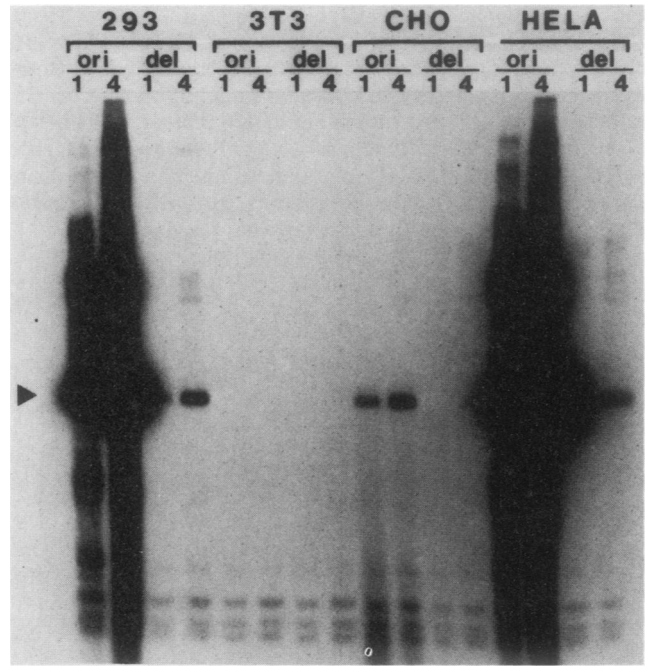

FIG. 1. In vitro replication in extracts from nonsimian cells. DNA was synthesized in replication reactions containing SV40 T antigen and extracts from uninfected 293, BALB/3T3, CHO, or HeLa cells. As indicated above each lane, the reactions were incubated for 1 or 4 h at 37°C in the presence of pJLO DNA (ori), which contains a wild-type SV40 origin, or pJLO-d4 (del), which contains a 4-bp deletion in the SV40 origin. The radioactive products were digested with *Dpn*I and *Sal*I before agarose gel electrophoresis and autoradiography (10-h exposure). The arrow marks the position of a full-length linear plasmid (pJLO or pJLO-d4). Autoradiography for shorter times reveals a single band at this position in the overexposed lanes. The low-molecular-weight fragments shared by all of the lanes across the bottom of the gel arise from complete *Dpn*I digestion of unreplicated plasmid. Most of the fragments from unreplicated plasmid have migrated off the bottom of the gel. After 4 h, typical levels of nucleotide incorporation into *Dpn*I-resistant DNA for the various combinations of extract and template are as follows: HeLa-pJLO, 100 to 300 pmol; HeLa-pJLOd4, 0.5 to 2 pmol; 293-pJLO, 100 to 300 pmol; CHO-pJLO, 0.5 to 2 pmol; CHO-pJLOd4, 0 pmol; 3T3-pJLO and 3T3-pJLOd4, 0 pmol; COS-1–pJLO, 100 to 300 pmol; COS-1–pJLOd4, 0.5 to 2 pmol; CV-1–pJLO, 100 to 300 pmol; CV-1–pJLOd4, 0.5 to 2 pmol.

catalyzed efficient replication of plasmid pJLO, which consists of nucleotides 5172 to 128 of the SV40 genome cloned in the vector pKP45, a derivative of pBR322 (Fig. 1). The viral DNA in this construct contains the complete wild-type origin of DNA replication (34). Almost all (85 to 95%) of the incorporated radioactivity was present in fully *Dpn*I-resistant DNA. Under standard reaction conditions the extent of DNA synthesis observed with HeLa and 293 extracts (100 to 300 pmol) was approximately the same as that observed routinely with extracts from monkey cells. Neither extract catalyzed significant DNA synthesis in the absence of T antigen (Fig. 2A). When pJLO-d4 DNA was substituted for pJLO DNA, the extent of DNA synthesis was reduced 150- to 200-fold. Plasmid pJLO-d4 is identical to pJLO except for a deletion of 4 bp at map positions 5239 to 5242 within the origin. This deletion is known to abolish DNA replication in vivo (23).

Extracts of CHO cells were also capable of supporting

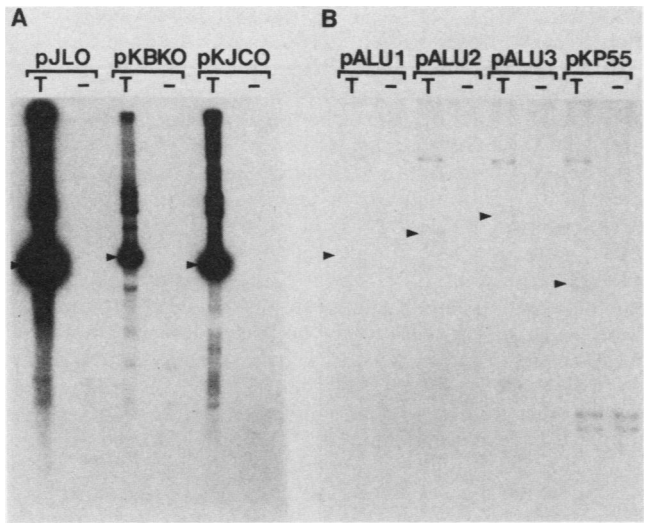

FIG. 2. In vitro replication of sequences homologous to the SV40 origin. Reaction mixtures containing uninfected HeLa cell extracts were incubated in the presence of various DNA templates for 4 h at 37°C with (T) or without (−) SV40 T antigen as indicated above each lane. The radioactive product DNAs were linearized and digested with *Dpn*I as described in the text. The products were subjected to electrophoresis in 1.5% agarose gels and then autoradiographed. The arrows indicate the positions of full-length linear plasmid for each template. (A) Exposure for 1 h. *Dpn*I-resistant DNA synthesis in the presence of pJLO (SV40 origin), pBKO (BKV origin), and pJCO (JCV origin). The respective levels of nucleotide incorporation in the presence of T antigen were 160, 14, and 46 pmol. In the absence of T antigen there was no measurable incorporation into *Dpn*I-resistant DNA. (B) Exposure for 24 h. *Dpn*I-resistant DNA synthesis in the presence of pAlu1, pAlu2, pAlu3 (one, two, and three copies, respectively, of human Alu repeat sequence from BLUR8), and pKP55 (parent vector).

origin-dependent DNA synthesis in the presence of purified T antigen (Fig. 1). However, the extent of synthesis was less than 1% of that observed with primate extracts. Extracts derived from BALB/3T3 cells were totally inactive in origin-dependent DNA replication. These results provide additional evidence for the biological relevance of the cell-free replication system, since the ability of the various extracts to catalyze replication in vitro parallels the ability of the same cells to support viral DNA replication in vivo (see below).

(ii) Replication of templates containing sequences homologous to the SV40 origin. The putative origins of replication of human papovaviruses BKV and JCV share considerable nucleotide sequence homology with the SV40 origin. We therefore examined the ability of plasmids containing the BKV and JCV origins to serve as templates for DNA replication in vitro (Fig. 2A). Both plasmids (pBKO and pJCO) supported significant DNA replication as determined by the *Dpn*I assay, but the extent of replication was 5- to 10-fold less than that observed with a similar plasmid containing the SV40 origin (pJLO). In both cases replication was completely dependent on the presence of SV40 T antigen. Although the data (Fig. 2A) were obtained with extracts derived from human (HeLa) cells, extracts from monkey (COS-1) cells also supported replication of pBKO and pJCO, albeit at a 5- to 10-fold lower efficiency than that of HeLa extracts (data not shown).

We also studied the ability of the human *Alu* family of interspersed repeat sequences to serve as templates for DNA replication in vitro. Such repeats are known to contain sequences that are homologous to papovavirus origins of DNA replication (31). Figure 2B shows replication assays performed with plasmids containing one, two, or three copies of the *Alu* repeat found in BLUR8 (10). None of these templates supported significant replication in HeLa cell extracts in either the presence or absence of SV40 T antigen. Long exposures of the autoradiograms revealed a slight stimulation of DNA synthesis by the addition of T antigen, but the same effect was also seen with a control template (pKP55) which contains only vector sequences. Plasmids containing the *Alu* repeats also failed to support DNA synthesis in monkey cell extracts supplemented (or unsupplemented) with T antigen (data not shown).

(iii) Replication of linear DNA molecules. We previously reported evidence that linear pJLO DNA molecules can support DNA synthesis in vitro (34). We since extended this observation to other DNA templates and carried out additional characterization of the reaction. Figure 3 shows an analysis of the products of replication reactions containing linear pJLO, SV40, or pSV5 DNA molecules as templates. A sample of pJLO DNA was digested with *Ban*I which cleaves

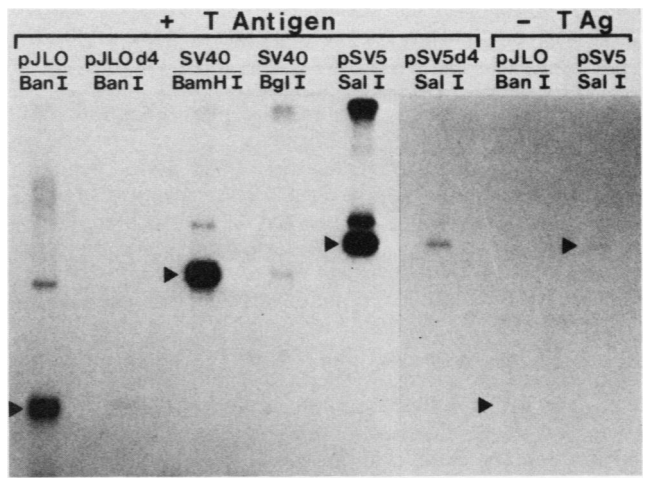

FIG. 3. In vitro replication of linear templates. Reaction mixtures containing uninfected COS extracts were incubated for 4 h at 37°C with (+ T antigen) or without (− T ag) SV40 T antigen in the presence of different linear templates. Indicated above each lane are the templates and enzymes used to linearize them. pJLO and pSV5 contain the wild-type SV40 origin of replication. pJLO-d4 and pSV5-d4 differ only in having a 4-bp deletion within the origin. These molecules were cleaved at restriction sites several kb from the origin region. SV40 was cut either opposite its origin (*Bam*HI) or within its origin (*Bgl*I). All preparations of linear templates had no detectable circular forms. The reaction products were subjected to electrophoresis on the same 1.5% agarose gel and autoradiographed with the same exposure (3 h). Only samples that were not digested with *Dpn*I after the reaction are shown. (Digestion with *Dpn*I did not significantly alter the result.) The arrows point to the positions of the full-length linear templates. In the presence of T antigen, the level of nucleotide incorporation into these linear DNAs were: pJLO/*Ban*I, 2 pmol; pJLO-d4/*Ban*I, 0.2 pmol; SV40/*Bam*HI, 9 pmol; SV40/*Bgl*I, 0.4 pmol; pSV5/*Sal*I, 11 pmol; pSV5-d4/*Sal*I, 0.2 pmol. In the absence of T antigen, the level of nucleotide incorporation was 0.1 pmol. When form I pJLO was used as the template, the level of nucleotide incorporation into monomer circles was 12 pmol, whereas total incorporation into all product forms was 100 pmol. The radioactive products that lie directly above the linear DNAs migrate at the position of form II templates. They may represent some low level of recircularization during the reaction.

at a single site within the vector sequences, opposite the SV40 origin. The resulting linear molecules supported DNA replication in the presence, but not the absence, of SV40 T antigen. The extent of incorporation of radioactive precursor into linear products was about 10 to 20% of the incorporation into monomer circles when form I pJLO DNA was used as the template. Since the major product of the reaction had the electrophoretic mobility expected of full-length linear plasmid DNA, we conclude that the observed DNA replication is not a result of recircularization during incubation. (We also note that when relaxed or supercoiled circular DNA is used as template, no linear DNA is seen among the products.) Similar results were obtained with plasmid pSV5, which contains the entire SV40 genome cloned at the *Bam*HI site of vector pKP45. Linear pSV5 DNA molecules were generated by digestion with *Sal*I which cleaves within the vector sequences.

To verify that the replication of linear templates, like that of circular templates, is dependent on the presence of the SV40 origin, we also tested the ability of linear pJLO-d4 and pSV5-d4 DNA molecules to support DNA synthesis in vitro. Both of these molecules contain a 4-bp deletion mutation in the origin (see above), and both were found to be relatively ineffective as templates in the cell-free replication system (Fig. 3). Similarly, when SV40 DNA isolated from infected cells was cleaved at the *Bam*HI site, opposite the origin, the resulting linear molecules supported significant DNA synthesis in vitro; however, when the same DNA was digested with *Bgl*I, which cleaves within the origin, replication activity was almost completely abolished.

Electron microscopy of replicative intermediates. (i) Structure of intermediates. Standard replication reaction mixtures containing extract from uninfected COS-1 cells, purified SV40 T antigen, and pSV5 DNA were incubated for 30 to 60 min at 37°C, and the DNA was isolated as described above. Electron microscopic examination revealed the presence of two types of branched DNA molecules in addition to the input pSV5 DNA and the small amount of cellular DNA that contaminated the extracts. The first type of branched molecule, observed at a frequency of 1 to 3% of the input DNA molecules, contained two branch points, three branches, and no free ends (theta structure) (Fig. 4A to C). Length measurements of pSV5 theta structures demonstrated that two of the branches were equal in length (replicated segments) and that the sum of the lengths of one of these branches and the third branch (unreplicated segment) was equal to the total length of the pSV5 genome (7.9 kb). Thus, these molecules are identical in structure to SV40 replicative intermediates previously characterized in in vivo studies (30, 44) and to the pJLO theta structures observed in our initial in vitro study (34). The second type of branched molecule, observed at approximately the same frequency as theta structures, consisted of a circular pSV5 genome with a single branch (sigma structure; Fig. 4J and K). The lengths of the branches in such molecules varied but were generally smaller than those of the pSV5 genome. (In samples prepared from reaction mixtures incubated for longer periods of time, sigma structures were more frequent and the lengths of the branches were commonly much longer than that of one pSV5 genome.) It is possible that these molecules are derived from theta structures as a consequence of the action of nucleases present in the extract. Sigma structures have previously been observed in studies of SV40 DNA replication in vivo, but at low frequency (37, 47). Neither type of branched molecule was observed in significant numbers (<0.2%) in control reaction mixtures that lacked SV40 T antigen or in reaction mixtures that contained pKP45 (vector) DNA in place of pSV5 DNA.

(ii) Origin and direction of DNA replication. To determine the site(s) in the pSV5 genome at which DNA synthesis begins and to define the directionality of replication, we analyzed the structure of replicative intermediates after digestion with restriction endonucleases. When DNA isolated from reaction mixtures identical to those described above was digested with *Kpn*I, linear molecules with a branch point at each end were observed (Fig. 4D to F). Since pSV5 DNA contains a single *Kpn*I cleavage site, such molecules represented the expected products of digestion of theta structures. We also observed linear molecules with one branch point (Fig. 4L) which were presumably produced by *Kpn*I cleavage of sigma structures. Figure 5A summarizes length measurements of 40 molecules with two branch points. For each molecule the shortest distance from a replication fork to the *Kpn*I site is plotted against the extent of replication. Nearly all of the data points fall close to a straight line, indicating that in vitro replication proceeds from a unique origin. The slope of the line is 0.50, which demonstrates that the average rate of fork movement is one-half of the total rate of replication, i.e., in vitro replication is bidirectional. Finally, the location of the in vitro origin can be deduced from the value of the x-intercept (0.11). This value indicates that the *Kpn*I site is duplicated after about 11% of the pSV5 DNA molecule has been replicated. Since replication is bidirectional, it follows that the origin used in vitro is approximately 5.5 map units from the *Kpn*I site. This corresponds almost precisely with the location of the in vivo SV40 origin (13) cloned in pSV5 (3 to 4 map units from the *Kpn*I site). This result was confirmed by analysis of a similar preparation of replicative intermediates that was digested with *Pvu*I, an enzyme that cleaves pSV5 DNA at a site 46 map units from the in vivo SV40 origin. The products of *Pvu*I cleavage of theta structures were predominantly linear molecules, each containing an internal replication bubble (Fig. 4G to I). When the shortest distance from a replication fork to the *Pvu*I site was plotted against the extent of replication (Fig. 5B), a straight line with a slope of -0.54 was obtained. Extrapolation of the line to zero extent of replication indicated that the in vitro replication of pSV5 was initiated at a point approximately 47 map units from the *Pvu*I site, in close proximity to the in vivo SV40 origin.

(iii) Synchrony of fork movement. To assess the degree of synchrony in the movement of the two forks during bidirectional replication, we analyzed reaction mixtures containing SV40 DNA as the template. After 30 to 60 min of incubation the replicative intermediates were isolated and cleaved with *Bgl*I. Since *Bgl*I cleaves SV40 DNA at a site within the origin of replication, the expected products of digestion are linear molecules with a branch point at each end (Fig. 5A inset). In such molecules the average distance of each branch point from the cleavage site provides a measure of the distance that each replication fork has moved from the origin of replication. This data is plotted as a histogram of the ratio of the distance moved by the slower fork (b) to the distance moved by the faster fork (a) (Fig. 6). The data demonstrate that the degree of synchrony in the movement of the two forks can vary considerably from one molecule to another. Although the rate of movement of the two forks is similar for the majority of molecules, a minority display substantial degrees of asynchrony. In about 25% of the molecules one fork had moved at least twice as far as the other.

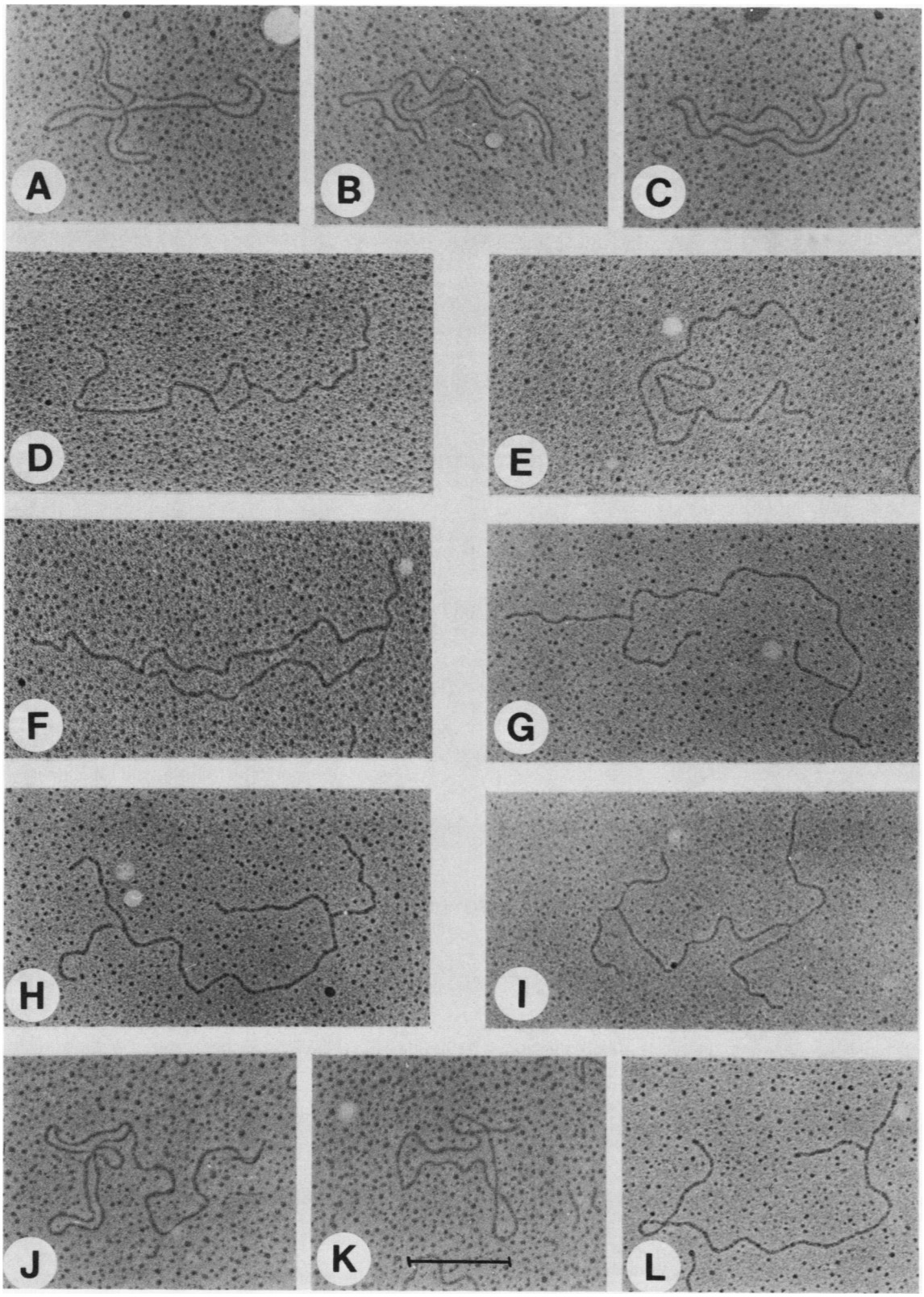

FIG. 4. Electron microscopy of cut and uncut in vitro replication intermediates. The 7.9-kb plasmid pSV5 (wild-type SV40 origin) was incubated for 30 min at 37°C in the presence of uninfected COS extracts and T antigen. Bar, 0.5 μm. A, B, and C, Uncut theta structure replication intermediates; D, E, and F, *Kpn*I-digested theta structure replication intermediates; G, H, and I, *Pvu*I-digested theta structure replication intermediates; J and K, uncut sigma structure replication intermediates; L, *Kpn*I-digested sigma structure replication intermediates (*Y* structures).

DISCUSSION

SV40 DNA replication in vitro requires cellular proteins in addition to the viral large T antigen. In our original studies (34) we demonstrated that cytosol extracts of African green monkey kidney cells, the usual hosts for SV40, can provide all of the necessary cellular proteins. In the present study we show that similar extracts derived from other cells that are permissive for SV40 replication in vivo will also support DNA replication in vitro. In the presence of T antigen, extracts of human cells (HeLa and 293 cells) are quite comparable in replication activity to extracts of monkey cells. Although human cells are generally classified as semipermissive for SV40 infection (50), recent in vivo studies provided evidence that the low yield of progeny virions in human cells is not due to a defect in viral DNA replication (40). Indeed, direct measurement of the rates of initiation and chain elongation in these studies suggests that viral DNA replication is approximately as efficient in human fibroblasts as in monkey cells. We also detected a small but significant level of origin-dependent, T-antigen-dependent DNA replication with extracts derived from CHO cells. It recently was shown that CHO cells support SV40 DNA replication in vivo when the template is introduced by DNA transfection methods (F. LaBella and H. L. Ozer, Virus Res., in press). The efficiency of replication in vivo, like that in vitro, is very low.

We have been unable to detect any significant origin-specific DNA replication in mouse cell extracts supplemented with SV40 T antigen. Mouse cells are completely nonpermissive for SV40 in vivo; although T antigen is expressed, viral DNA synthesis does not occur and progeny virions are not produced (21, 28). Cell fusion experiments suggest that the inability of SV40 DNA to replicate in mouse cells is due to a deficiency in one or more cellular factors required for viral DNA synthesis (2, 32, 51). If this hypothesis proves correct, it may be possible to exploit the cell-free replication system to identify and purify such permissive factors.

Plasmid DNA molecules containing the origins of replication of human papovaviruses BKV and JCV are capable of

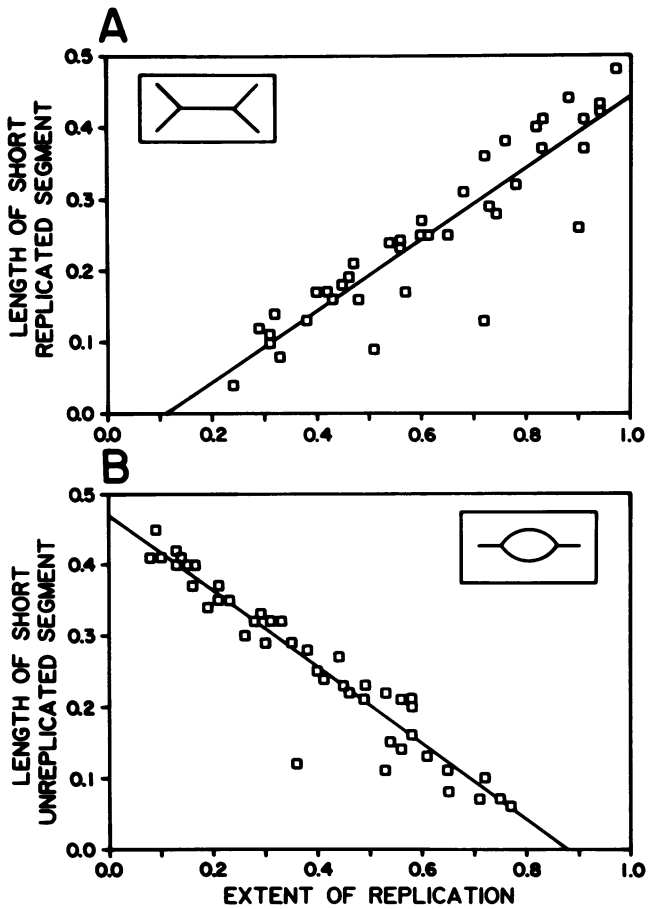

FIG. 5. Origin and direction of DNA replication in vitro. The 7.9-kb plasmid pSV5, which contains the wild-type SV40 origin, was incubated for 30 min at 37°C in the presence of extract from COS-1 cells and SV40 T antigen. The replication products were digested with *Kpn*I (A) or *Pvu*I (B), enzymes that cleave at unique sites within the plasmid. The resulting molecules were mounted for electron microscopy by the formamide method of Davis et al. (9). Replication intermediates containing two branch points were photographed and measured. All lengths are expressed in pSV5 map units (the pSV5 genome contains 100 map units). (A) Cleavage of theta structures with *Kpn*I yielded linear molecules with a branch point at each end (see the inset). The lengths of the two branches at each end were averaged to obtain an estimate of the distance of each replication fork from the *Kpn*I restriction site. The shortest such distance was plotted against the extent of replication. The line, generated by linear regression analysis, has an x-intercept of 0.11 and a slope of 0.50. The *Kpn*I site is 0.04 map units from the in vivo SV40 origin cloned in pSV5. (B) Cleavage of theta structures with *Pvu*I yielded almost exclusively linear molecules with internal replication bubbles (see the inset). For each molecule the shortest distance from a replication fork to the *Pvu*I site was plotted against the extent of replication. The line, generated by linear regression analysis, has an x-intercept of 0.47 and a slope of -0.54. The *Pvu*I site is 0.46 map units from the in vivo SV40 origin cloned in pSV5.

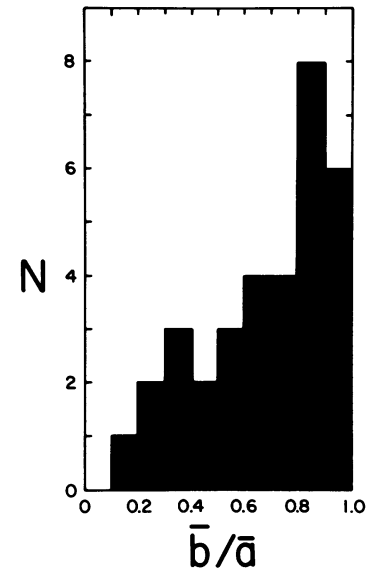

FIG. 6. Relative fork movements during in vitro replication. SV40 DNA was incubated for 30 min at 37°C in the presence of uninfected COS extracts and T antigen. The replication products were cut at the unique *Bgl*I site within the origin of replication. Almost all theta structures were converted to doubly forked structures (Fig. 5A inset). At each fork, the lengths of the two branches were averaged to give the length of the replicated segment attached to that fork. The ratio of the length of the shorter replicated segment ($\bar{b}$) over the length of the longer replicated segment ($\bar{a}$) was calculated for each of 33 molecules. A histogram of the $\bar{b}/\bar{a}$ ratios is shown. N, Number of molecules.

supporting DNA replication in primate cell extracts supplemented with SV40 T antigen. This observation is perhaps not surprising in view of the high degree of nucleotide sequence conservation in the origins (and T-antigen genes) of these viruses (17, 18, 42, 45). Previous studies provided evidence for the functional similarity of the T antigens of SV40 and BKV. SV40 T antigen binds specifically to the BKV origin of replication (43). Moreover, human embryonic kidney cells immortalized by an origin-defective mutant DNA of SV40 can support the replication of plasmids containing only the putative BKV origin (35). (Curiously, in this same study the replication of JCV was not detectable.) Finally, the DNA replication defect of SV40 tsA mutants at the nonpermissive temperature can be overcome by superinfection with BKV (33, 38).

The human *Alu* interspersed repeat sequence in plasmid BLUR8 does not support DNA replication in vitro in either the presence or absence of SV40 T antigen. The BLUR8 repeat contains some sequence elements that are homologous to the SV40 origin, including specific pentanucleotide sequences that have been implicated in the interaction of SV40 T antigen with the origin (10, 11). However, the homology is not extensive, and methylation protection experiments with the gene encoding human 7SL RNA, which shares extensive homology with the *Alu* family consensus sequence, revealed only weak binding of SV40 T antigen (52). Attempts to detect replication of BLUR8 plasmids in vivo after introduction into COS-7 cells also have been unsuccessful (R. Tjian and R. Myers, personal communication). Our data are in direct contrast to a previous report that BLUR8 plasmids are capable of T-antigen-dependent replication in vitro (1). The reason for this discrepancy is not clear.

Circular DNA is the most effective template for DNA replication in the cell-free system, but it is clear that linear DNA also supports DNA replication. It is unlikely that the latter reaction represents repair synthesis or some other nonreplicative process, since the replication of linear templates is dependent on the presence of both T antigen and a wild-type SV40 origin. It is not yet clear why the template efficiencies of circular and linear templates are different. The efficiency of initiation or the rate of chain elongation may be lower for linear molecules than for circular molecules. In contrast, explanations of a more trivial nature are also possible. For example, linear templates might be more easily damaged or destroyed by nucleases in the extract. In any case, the ability of linear molecules to support substantial DNA replication in vitro suggests that it is not essential that the template be able to maintain superhelical tension. This result is in contrast to a number of studies in procaryotic systems in which replication of circular DNA templates appears to require negative supercoiling (20).

Electron microscopic analysis of in vitro reaction mixtures revealed the presence of branched molecules (theta structures) identical in structure to in vivo replicative intermediates. We also observed sigma structures in significant numbers. At early time points (45 min), sigma structures are roughly equal in number to the theta structures; at later times (3 to 4 h), they accumulate to become the predominant branched species. Although the origin of sigma structures is not yet clear, their existence requires the presence of both a wild-type SV40 origin and T antigen. It seems most likely that they arise from theta structures by release of one arm of a replication fork, perhaps as a consequence of the activity of nucleases in the reaction mixture. Since the average length of the branches in sigma structures increases with the time of incubation, it is probable that DNA synthesis continues by a rolling-circle mechanism. However, we cannot yet rule out the possibility that sigma structures and theta structures are generated by distinct initiation mechanisms. Rolling circles have been observed in the population of in vivo replicative intermediates isolated from infected cells, but their numbers are generally small (37, 47). The increased frequency of such structures in subcellular replication systems, such as isolated nuclei or replication complex preparations from SV40-infected cells, has been noted previously (47).

Analysis of in vitro replicative intermediates cleaved with restriction endonucleases suggests that replication begins at a unique site corresponding to the in vivo origin and demonstrates that replication is bidirectional. When averaged over the population of replicative intermediates the rate of movement of the two replication forks is approximately the same; however, in individual molecules the progression of the two forks can be quite asynchronous. Similar results have been obtained in studies of in vivo replicative intermediates (48). These findings support the view that chain elongation at one fork is not rigidly coupled to chain elongation at the other.

In this and a previous communication (34) we described the properties of a cell-free system that is capable of replicating exogenous templates containing the SV40 origin of replication. By all of the tests that we applied to date, the DNA replication that occurs in vitro closely resembles SV40 DNA replication in vivo. Thus, it seems likely that the system will be a useful model for studying eucaryotic replication mechanisms.

ACKNOWLEDGMENTS

We appreciate the gift of plasmids pKP54 and pKP55 from K. Peden and plasmids pSV5 and pSV5-d4 from Ron Wides. We are indebted to M. A. Bolanowski for confirming the sequence of pAlu1. We thank D. Rawlins, J. Sherley, P. Rosenfeld, M. Wold, and H. Ozer for stimulating discussions and M. A. Bolanowski, E. O'Neill, and J. Kreidberg for carefully reviewing the manuscript. We also thank C. Danevic for excellent technical assistance and M. C. Bolanowski for preparing the manuscript.

This work was supported by research grant CA16519 from the National Cancer Institute. J.J.L. is a trainee of the Medical Scientist Training Program (GM07309).

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