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Replication and Supercoiling of Simian Virus 40 DNA in Cell Extracts from Human Cells

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Soluble extracts prepared from the nucleus and cytoplasm of human 293 cells are capable of efficient replication and supercoiling of added DNA templates that contain the origin of simian virus 40 replication. Extracts prepared from human HeLa cells are less active than similarly prepared extracts from 293 cells for initiation and elongation of nascent DNA strands. DNA synthesis is dependent on addition of purified simian virus 40 tumor (T) antigen, which is isolated by immunoaffinity chromatography of extracts from cells infected with an adenovirus modified to produce large quantities of this protein. In the presence of T antigen and the cytoplasmic extract, replication initiates at the origin and continues bidirectionally. Initiation is completely dependent on functional origin sequences; a plasmid DNA containing an origin mutation known to affect DNA replication in vivo fails to replicate in vitro. Multiple rounds of DNA synthesis occur, as shown by the appearance of heavy-heavy, bromodeoxyuridine-labeled DNA products. The products of this reaction are resolved, but are relaxed, covalently closed DNA circles. Addition of a nuclear extract during DNA synthesis promotes the negative supercoiling of the replicated DNA molecules.

Since replication of DNA is a fundamental biological phenomenon, an understanding of the mechanism of DNA synthesis and the complex levels of control of this process is central to our understanding of how cells work. Although the mechanisms and some aspects of control of DNA synthesis in procaryotes have been elucidated (22, 23, 34), a similar understanding of DNA synthesis in eucaryotic cells is lacking. As with early work on procaryote DNA replication, initial studies of replication in eucaryotes have concentrated on small, autonomously replicating DNA molecules such as the DNA viruses. Some of these DNAs, such as papillomavirus (32) and Epstein-Barr virus DNAs (49), can exist in cells as stable extrachromosomal plasmids. Other studies have exploited the lytic DNA viruses of mammalian cells of which the best understood are the adenoviruses, primarily as a result of in vitro studies (6, 12, 37a). Although adenovirus has an atypical mechanism for initiation and elongation of DNA synthesis, these studies have led to the identification of some cellular proteins that are required for the replication of DNA in vitro.

The monkey virus simian virus 40 (SV40) contains a small (5,243-bp), double-stranded, circular genome with a single origin of DNA replication (reviewed in references 1 and 9). The virus genome encodes one protein that is required for replication of virus DNA, the SV40 large tumor (T) antigen (41). This protein is a multifunctional phosphoprotein that is required for DNA synthesis, control of RNA synthesis, and cell transformation. Four biochemical properties of the purified protein have been observed: an ATPase activity (7, 14, 44), a nucleotide-binding activity distinct from the ATPase-binding site (8), adenylation of the protein (5), and site-specific DNA binding to three sites that lie at the origin of DNA replication and early promoter region (reviewed in reference 43). The general characteristics of the mechanism of SV40 DNA replication have been elucidated in vivo (reviewed in reference 9), and these studies suggest that SV40 may replicate its DNA in a way similar to that of the chromosomes of the host cell. This may be true not only for initiation and elongation of DNA synthesis but also for the segregation of daughter DNA molecules after replication (39, 40). However, a detailed understanding of the mechanism and control of SV40 DNA synthesis must derive from studies of DNA replicated in vitro.

Two reports of cell-free systems for the replication of SV40 DNA have appeared (3, 26). Recently, Li and Kelly (26) demonstrated that cytoplasmic extracts prepared from SV40-infected or -uninfected monkey cells supplemented with purified SV40 T antigen can efficiently replicate DNA templates containing a functional origin of DNA replication. In this report, we have confirmed this finding by using similarly prepared cytoplasmic extracts from human 293 cells grown in suspension. We show that DNA synthesis proceeds bidirectionally from the origin of DNA replication and that multiple rounds of DNA replication occur. The products of this reaction are resolved into noncatenated, covalently closed but relaxed circular DNA. Addition of an extract prepared from the nuclei of cells promotes the negative supercoiling of the daughter DNA molecules.

MATERIALS AND METHODS

Cells. Human HeLa and 293 cells were propagated in suspension cultures in F13 medium (GIBCO Laboratories) containing 10% calf serum. The 293 cells are human embryo kidney cells that have been transformed with fragments of adenovirus type 5 (Ad5) DNA (17). The cell line, which grows in monolayer cultures, was adapted to growth in suspension and can be frozen in liquid nitrogen and then thawed and regrown. The doubling time is between 36 and 45 h.

Virus. The recombinant adenovirus Ad5SVR115 contains the coding region for the SV40 T antigen cloned into an Ad5 derivative that lacks the E1A and E1B gene regions. The T antigen mRNA is transcribed from a transposed major late promoter of Ad5 and also contains the late leader sequences I, II, and approximately one-half of III (29). A full description of this virus and its characterization will be published elsewhere. The virus was propagated in 293 cells.

Plasmid DNA. Plasmid pSV40 (previously called wild-type plasmid) and its mutant derivative p8-4 were described

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previously (16). Plasmids pSVOrigin plus (pSVO+) and pSVOrigin minus (pSVO−) were constructed by subcloning the 200-base-pair (bp) *Hin*dIII to *Sph*I fragments of pSV40 and p8-4, respectively, into pAT153 (46). Plasmid DNAs were propagated in *Escherichia coli* DH5 (D. Hanahan, personal communication) and purified by a modification of the procedure described by Mukhopadhyay and Mandal (35). Briefly, plasmid DNAs were amplified by treatment of the cells with chloramphenicol (170 μg/ml). The cells, in 200 ml of medium, were spun down, suspended in 3 ml of 50 mM NaCl—50 mM Tris-hydrochloride–5 mM EDTA–25% sucrose (pH 8.0), and chilled on ice. A solution (0.6 ml) containing 5 mg each of lysozyme and ethidium bromide (EtBr) per ml was added and left for a 5-min period, and then 1.2 ml of 0.25 M EDTA was added. After the mixture had been kept for a further 5 min on ice, 4.8 ml of lysis mixture (1% Brij 35, 0.4% sodium deoxycholate, 62.5 mM EDTA, 50 mM Tris-hydrochloride [pH 8.0]) was added, and the mixture was left on ice for 30 min. The extract was centrifuged for 25 min at 22,000 × *g* in a Beckman 50 Ti rotor, and the nucleic acid was precipitated from the supernatant for 1 h on ice by addition of polyethylene glycol to 10% (final concentration) and NaCl to 0.5 M (final concentration). The precipitate was collected by low-speed centrifugation and dissolved in 10 mM Tris-hydrochloride (pH 8.0)–1 mM EDTA and centrifuged to equilibrium in CsCl-EtBr gradients in a Beckman VTi65 rotor. The plasmid DNA was collected, the EtBr was removed by repeated extractions with isobutanol, and the DNA was dialyzed against 2,000 volumes of 10 mM Tris-hydrochloride (pH 8.0)–1 mM EDTA.

Purification of SV40 T antigen. Human 293 cells were infected with Ad5SVR115 virus at a multiplicity of infection of 5 PFU per cell and incubated at 37°C for 22 to 24 h. The cells were harvested by centrifugation, and the T antigen was extracted and purified exactly as described by Simanis and Lane (37a). Briefly, this procedure involves extracting the T antigen into the cytosol with Nonidet P-40 and preclearing the resulting extract by chromatography on a 1-ml protein A-Sepharose column. The flowthrough fraction was then passed over a 1-ml protein A-Sepharose column to which the anti-T monoclonal antibody PAB419 (previously called L19 [18]) had been covalently linked. The column was washed with a series of buffers and eluted as described previously (37a). Approximately 400 to 800 μg of pure SV40 T antigen (depending on the preparation) was obtained from 3×10^8 cells, and this protein was aliquoted and stored in buffer F [10 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) [pH 7.0], 1 mM dithiothreitol, 5 mM NaCl, 0.1 mM EDTA, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride] at −70°C. Repeated freezing and thawing reduces the activity of the protein. The protein concentration of the purified T antigen was determined by the method of Lowry et al. (31).

Preparation of cell extracts. The following procedure describes the isolation of nuclear and cytoplasmic extracts from 1 liter (5×10^5 to 6×10^5 cells per ml) of either 293 or HeLa cells grown in suspension. The cells were harvested by centrifugation and washed once with phosphate-buffered saline (8 g of NaCl per liter, 0.2 g of KCl per liter, 1.5 g of sodium phosphate [dibasic] per liter, 0.2 g of sodium phosphate [monobasic] per liter). The cells were washed again in 10 ml of ice-cold hypotonic buffer (20 mM *N*-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid [HEPES]-KOH [pH 7.5], 5 mM KCl, 1.5 mM $MgCl_2$, 0.1 mM dithiothreitol [26]), and finally resuspended in 5 ml of hypotonic buffer. After 10 min on ice, the cells were disrupted by repeated Dounce homogenization until more than 95% of the cells were broken (20 strokes of a B pestle), and the cells were left on ice for 30 min. The suspension was centrifuged (10,000 rpm in a Sorvall swing-out HB4 rotor for 10 min), and the supernatant was removed, aliquoted, and stored at −70°C (cytoplasmic extract). This procedure was adapted from the procedure described by Li and Kelly (26).

The pellet was suspended in 5 ml of 0.01 M $NaHCO_3$ (pH 8.0)–0.15 M NaCl and then centrifuged in the Sorvall SS34 rotor at 3,700 rpm for 10 min. The pellet was washed twice with 5 ml of 25 mM NaCl–8 mM EDTA (pH 8.0) and collected by centrifugation at 3,700 rpm for 5 min in the SS34 rotor. The pellet was washed three times with 10 mM Tris-hydrochloride (pH 8.0) and then collected by centrifugation (3,700 rpm for 5 min in the SS34 rotor). The material pelleting at this stage consisted primarily of proteins tightly bound to DNA. The pellet was then resuspended in 2.5 ml of 10 mM Tris-hydrochloride (pH 8.0) and disrupted by sonication with a Branson sonicator (microprobe, position 6; five times for 15 s each) until the solution was homogeneous. NaCl was added to a final concentration of 0.15 M, and the solution was centrifuged at 14,500 × *g* for 30 min at 4°C. Solid ammonium sulfate was slowly added to the supernatant to a final concentration of 25%, and the resulting solution was centrifuged at 13,000 × *g* for 30 min at 4°C. Solid ammonium sulfate was slowly added to this supernatant to a final concentration of 60%, and the precipitate was collected by centrifugation (13,000 × *g* for 30 min at 4°C). The pellet from this centrifugation was suspended in 3 ml of hypotonic buffer and then dialyzed for 12 h against 80 volumes of hypotonic buffer, with one change of buffer. The nuclear extract was then aliquoted and stored at −70°C.

The protein concentration of the cytoplasmic extract was approximately 7 mg/ml, and the concentration of the nuclear extract was approximately 0.7 mg/ml. Protein concentrations were determined by the method of Lowry et al. (31).

DNA synthesis. Reaction mixtures (50 μl) contained (final concentrations) 40 mM HEPES-KOH (pH 7.5), 8 mM $MgCl_2$, 0.5 mM dithiothreitol, 100 μM each dTTP, dGTP, and dCTP, 25 μM [α-^{32}P]dATP (ca. 1,000 cpm/pmol), 3mM ATP, 200 μM each CTP, UTP, and GTP, 40 mM creatine phosphate, and 1 μg creatine phosphokinase (rabbit muscle type I; Sigma Chemical Co.). Standard reaction mixtures also contained 0.3 μg of pSV40 DNA, 0.5 μg of T antigen, and 175 to 200 μg of 293 cell cytoplasmic extract. Nuclear extract was added where indicated. Reaction mixtures were incubated at 37°C for various times after being prepared on ice. DNA synthesis was very sensitive to the presence of $CaCl_2$ (6 mM $CaCl_2$ reduced DNA synthesis to background levels) and NaCl (50 mM NaCl reduced DNA synthesis by half, and 100 mM NaCl reduced DNA synthesis to background levels).

Analysis of replication products. Reactions were terminated by the addition of EDTA to 10 mM (final concentration). For the determination of the extent of DNA synthesis, samples were added to 50 μg of denatured, sheared calf thymus DNA as carrier and then mixed with 1 ml of 8% trichloracetic acid–1% sodium pyrophosphate for 15 min on ice. The precipitate was collected by filtration onto Whatman GF-C glass fiber filters and then washed four times with 10 ml of ice-cold trichloroacetic acid-sodium pyrophosphate and twice with ice-cold ethanol. The radioactivity was determined by liquid scintillation counting.

Samples for agarose gel electrophoresis were made 10 mM EDTA and 0.1% sodium dodecyl sulfate, pancreatic ribonuclease A was added to 20 μg/ml, and the mixtures were incubated for 15 min at 37°C. Protease XIII (final concentra-

tion, 1 mg/ml; Sigma) was added, and the samples were held for 1 h at 37°C and then extracted once with phenol. The DNA was separated from unincorporated nucleoside triphosphates by spin dialysis (24) with Sephadex G-50 in 1-ml syringes. The DNA was then extracted once with phenol and once with chloroform-isoamyl (24:1, vol/vol) and ethanol precipitated.

Agarose gel electrophoresis was done in Tris-borate-EDTA buffer (33), and chloroquine was added where indicated. High-resolution agarose gel electrophoresis was performed at 1 V/cm as described by Sundin and Varshavsky (41).

CsCl equilibrium gradient centrifugation of DNA samples labeled with bromodeoxyuridine (BUdR) and [^{32}P]dAMP was performed essentially as described by Li and Kelly (26). The marker LL (light-light) DNA was labeled by filling in the ends of *Eco*RI-digested pSV40 DNA with dATP and [*methyl*-^{3}H]dTTP by using the large fragment of *E. coli* DNA polymerase (Klenow fragment).

RESULTS

Preparation of cell extracts. Cells growing in suspension rather than as monolayer cultures were preferable for development of cell extracts for DNA synthesis because of the large number of cells required for purification of replication factors. Monkey cells suffer from the limitation that they cannot easily be grown in suspension cultures. We therefore turned to human cell lines in our attempts to establish cell extracts that could support SV40 DNA replication. SV40 does not replicate its DNA in most human cells very efficiently. Recombinant DNA plasmids containing the origin of SV40 DNA replication and the gene for T antigen replicated efficiently when transfected into human 293 cells but not HeLa cells (E. D. Lewis and J. L. Manley, submitted for publication; J. S. Lebkowski, S. Clancy, and M. P. Calos, submitted for publication). Surprisingly, the amount of DNA synthesis was inversely correlated with the levels of SV40 T antigen that were synthesized in the transfected cells. Human 293 cells were derived from a population of primary human embryo kidney cells transformed with fragments of Ad5 DNA (17). These cells express the adenovirus early-region E1A and E1B genes present in an integrated form in chromosomal DNA (2). We therefore set out to use 293 cells for the preparation of soluble cell extracts for studies of SV40 DNA replication. This was facilitated by the recent development of Li and Kelly (26) of extracts from monkey cells that would support efficient SV40 DNA synthesis. The 293 cells were adapted to growth in suspension cultures and can be maintained in suspension for at least 9 months. These cells can be frozen in liquid nitrogen and then reestablished in suspension culture. Cytoplasmic extracts were prepared by hypotonic shock and Dounce homogenization (26), and the nuclear extract was prepared from isolated chromatin by methods described above.

Synthesis and purification of SV40 T antigen. Because we were preparing extracts from uninfected cells, it was necessary to obtain purified SV40 T antigen to reconstitute DNA synthesis in vitro. A recombinant virus, Ad5SVR115 was constructed as a nondefective vector that synthesizes large amounts of wild-type SV40 T antigen from mRNA made from the adenovirus major late promoter. The mRNA also contains, in the 5′ noncoding region, the first, second and one-half of the third "late leaders" as described by Logan and Shenk (29). The construction and characterization of this virus will be published elsewhere (Y. Gluzman, D. Scolnick, H. Reichl, and J. Harper, manuscript in preparation).

SV40 T antigen was purified to near homogeneity from Ad5SVR115-infected 293 cells by immunoaffinity chromatography as described by Simanis and Lane (37a) (Fig. 1A). Figure 1A, lane 1, shows the proteins obtained after extraction of Ad5SVR115-infected 293 cells; lane 2 shows the flowthrough from the immunoaffinity column, and lane 3 shows the protein eluted from the column. The T antigen is essentially pure, as judged by gel eletrophoresis; a minor product of 27,000 daltons that is visible on overloaded gels is a T antigen-related protein containing N-terminal sequences (data not shown). Increasing amounts of this purified SV40 T-antigen preparation were added to reaction mixtures containing an extract prepared from the cytoplasm of uninfected 293 cells as well as pSV40 as the template DNA (pSV40 contains the complete genome of SV40 cloned into the pMK16 *BglI*^{r} vector [16]). Very little DNA synthesis was observed in the absence of the T antigen, but addition of increasing amounts of the protein stimulated DNA synthesis (Fig. 1B). All subsequent experiments were performed with 0.5 μg of purified T antigen in 50 μl reactions. Although this is a suboptimal concentration, efficient DNA synthesis occurs (see below).

To test for authentic replication promoted by the T antigen, an assay that measures the state of methylation in DNA was used. This assay involves the use of methylated template DNAs grown in *dam*$^{+}$ *E. coli* (DH5; D. Hanahan, personal communication) and an enzyme, *Dpn*I, that will only digest DNA that is methylated on both strands. DNA that is replicated in vitro will become hemimethylated after one round of DNA synthesis and eventually unmethylated after two rounds of DNA synthesis. Thus, replicated DNA will be completely resistant to digestion by the *Dpn*I enzyme. For convenience, all DNA in the reaction is additionally digested with a single-cut enzyme (*Xho*I) to linearize the DNA. This method has been used to assay for SV40 DNA replication in vivo (37) and in vitro (26). Plasmid pSV40 DNA was added to reaction mixtures containing 293 cell cytoplasmic extract in the presence or absence of SV40 T antigen. After incubation for 2 h at 37°C, the DNA was isolated, digested with *Xho*I and *Dpn*I, and subjected to agarose gel electrophoresis (Fig. 1C). Both the autoradiogram of the ^{32}P-labeled products and the EtBr-stained gel show a *Dpn*I-resistant band comigrating with linear pSV40 DNA (form III) only when SV40 T antigen was present in the reaction (Fig. 1C, lanes 1 and 2). Moreover, the small *Dpn*I fragments from unreplicated DNA were not labeled with ^{32}P, demonstrating that little repair synthesis was occurring, even in the absence of T antigen (Fig. 1C). These results established that replication of SV40 DNA in extracts from 293 cells was T-antigen dependent.

293 versus HeLa cell extracts. As described above, plasmid DNAs containing the SV40 origin replicate in 293 cells but not in HeLa cells. It was therefore of interest to determine whether cell extracts prepared from HeLa cells were capable of supporting SV40 DNA synthesis as efficiently as were cell extracts from 293 cells. Cytoplasmic extracts were prepared from both 293 cells and HeLa cells and then used for DNA synthesis in reactions containing fixed amounts of T antigen (0.5 μg) and pSV40 DNA (0.3 μg). Figure 2A shows the level of DNA synthesis obtained when increasing amounts of either 293 or HeLa cell cytoplasmic extracts were used, and Fig. 2B shows a time course of DNA synthesis comparing equal amounts of HeLa and 293 cell extracts (210 μg of protein). In both cases, the amount of DNA synthesis was

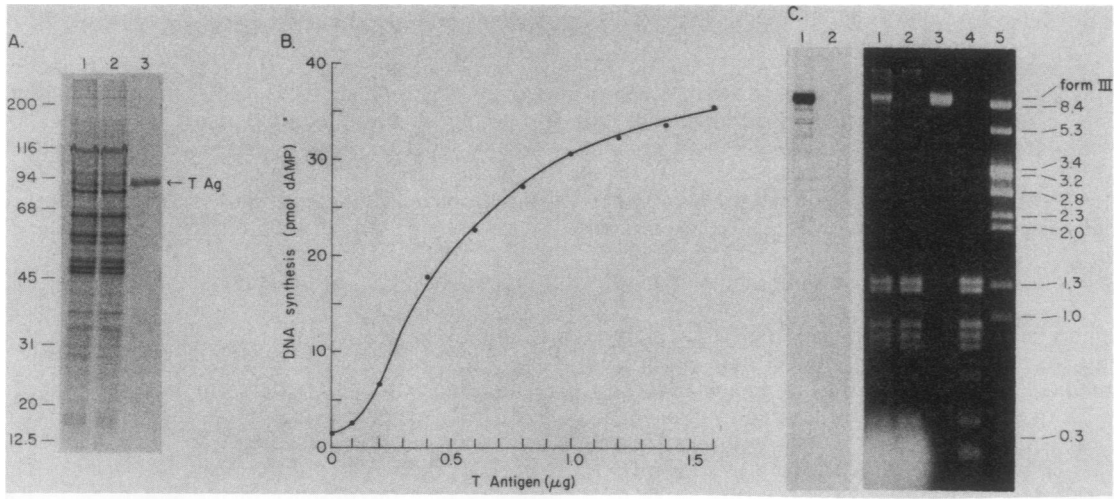

FIG. 1. Requirement of SV40 T antigen for DNA replication. (A) Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of protein fractions obtained during the purification of SV40 T antigen from Ad5SVR115-infected 293 cells (37a). Lane 1: crude cytoplasmic extract obtained after Nonidet P-40 extraction. Lane 2: The flowthrough fraction from the protein A-Sepharose-419 column. Lane 3: 1 μg of total protein from the final column eluate containing SV40 T antigen. The gel was stained with Coomassie brilliant blue, and molecular weight markers (10^3) are shown. (B) Stimulation of DNA synthesis by increasing amounts of purified SV40 T antigen. Standard 50-μl reaction mixtures containing 210 μg of 293 cell cytoplasmic extract and 0.3 μg of pSV40 DNA were incubated for 2 h at 37°C, and DNA synthesis was measured by incorporation of [^{32}P]dAMP. (C) *Dpn*I assay of replication products. Reactions containing 210 μg of 293 cell cytoplasmic extract and 0.3 μg of pSV40 in the presence (lanes 1) or absence (lanes 2) of added SV40 T antigen (0.5 μg) were incubated for 2 h at 37°C. The reaction products were isolated, digested with *Xho*I and *Dpn*I, and then subjected to agarose gel electrophoresis. The left-hand panels show an autoradiogram of the gel, and the right-hand panel shows an EtBr-stained pattern. Lane 3 contains *Xho*I-digested pSV40 DNA, (without *Dpn*I); lane 4 contains *Dpn*I- and *Xho*I-digested pSV40 DNA, and lane 5 contains Ad2 DNA digested with *Hin*dIII for markers. The numbers are kilobase pairs.

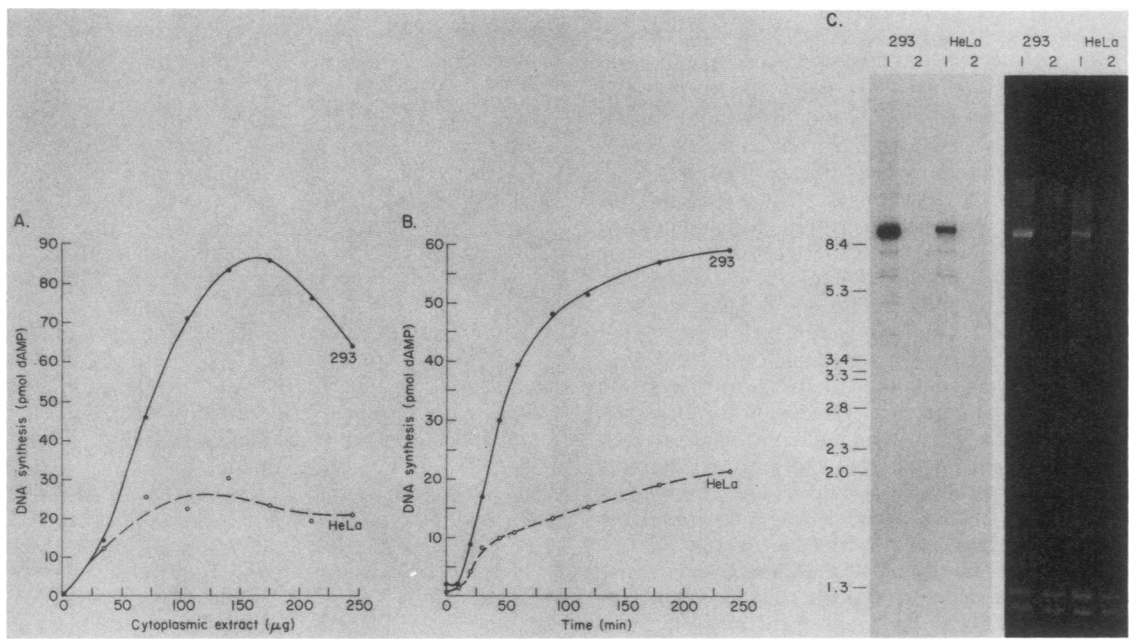

FIG. 2. DNA replication supported by cytoplasmic extracts from 293 and HeLa cells. (A) Stimulation of DNA synthesis by increasing amounts of 293 (——) or HeLa cell (----) cytoplasmic extracts in 50-μl reaction mixtures containing 0.5 μg of T antigen and 0.3 μg of pSV40 DNA. DNA synthesis was for 2 h at 37°C. (B) Time-dependent synthesis of DNA at 37°C in 50-μl reaction mixtures containing 210 μg of either 293 (——) or HeLa cell (----) cytoplasmic extracts, 0.5 μg of T antigen, and 0.3 μg of pSV40 DNA. Note the lag of approximately 10 min in the onset of DNA synthesis. (C) *Dpn*I assay of DNA replication products synthesized in 2 h at 37°C in 50-μl reaction mixtures containing 210 μg of 293 or HeLa cell cytoplasmic extracts (as indicated), 0.5 μg of T antigen, 0.3 μg of pSV40 DNA (lanes 1), or 0.3 μg of p8-4 DNA (lanes 2). Both the autoradiogram and the EtBr-stained gel are shown. The molecular weight markers (bp) (10^3) were fragments of Ad2 DNA produced by digestion with *Hin*dIII.

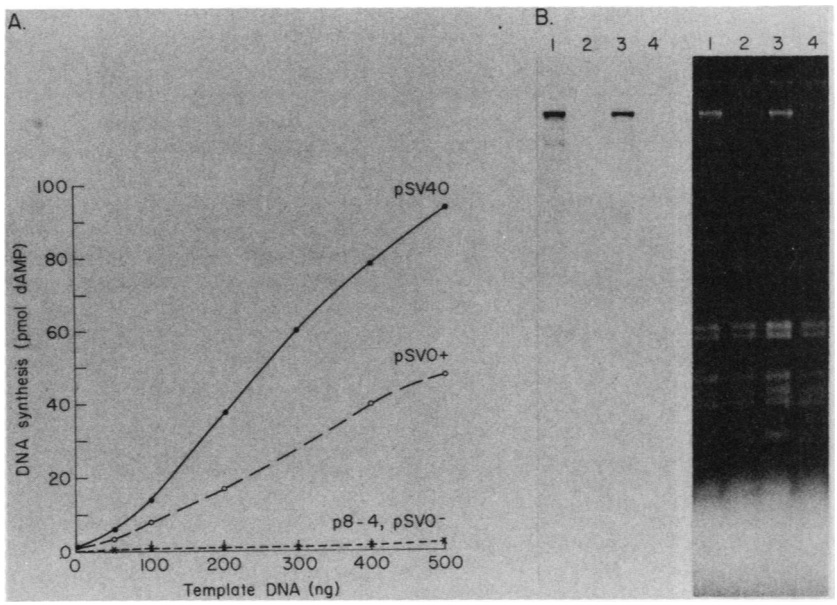

FIG. 3. Requirement for a functional origin of DNA replication. (A) Stimulation of DNA synthesis by increasing amounts of template DNA that contain wild-type origin sequences. Reaction mixtures (50 μl) contained 210 μg of 293 cell cytoplasmic extract, 0.5 μg of T antigen, and various amounts of pSV40, pSVO+, p8-4 or pSVO− DNA as shown. Replication was for 2 h at 37°C. pSV40 contains the entire SV40 genome cloned into a *BglI*^{R} derivative of pMK16. P8-4 is a *BglI*^{R} derivative of pSV40 and contains a 4-bp deletion in the origin sequences. pSVO+ and pSVO− are derivatives of pSV40 and p8-4, respectively, and contain only a 200-bp fragment (5,171 to 128 bp) of SV40 DNA that contains the minimal origin sequences. (B) *Dpn*I assay of reaction products obtained by incubating 0.3 μg of pSV40 (lanes 1, 3) or p8-4 (lanes 2, 4) in 50-μl reaction mixtures containing 210 μg of 293 cell cytoplasmic extract (lanes 1 and 2) or 293 cell cytoplasmic extract plus nuclear extract (lanes 3 and 4) and 0.5 μg of T antigen for 2 h at 37°C. The reaction products were digested with *Xho*I and *Dpn*I and subjected to agarose gel electrophoresis. Both the autoradiogram and the EtBr-stained gel are shown.

approximately fourfold less when HeLa extracts were used than when 293 cell extracts were used, and in other experiments the difference was up to eightfold (data not shown). Despite the reduced DNA synthesis observed when HeLa cell extracts were used, replication was authentic as judged by the *Dpn*I assay (Fig. 2C, lanes 1). Furthermore, replication in both 293 and HeLa cell extracts was dependent on the presence of functional origin sequences in the template DNA (Fig. 2C, lanes 2) (see below). The time course of DNA synthesis shown in Fig. 2B also demonstrates that a lag of approximately 10 min occurs before elongation of DNA replication. This effect is discussed below (see Fig. 4).

Dependence of replication on a functional origin. The experiments described above used a plasmid (pSV40) containing the entire SV40 genome cloned into the *BglI*^{r} pMK-16 vector. A derivative of pSV40, called p8-4, contains a 4-bp deletion in the T-antigen-binding site II which lies entirely within the minimal origin sequence, but is otherwise identical to pSV40. Only the pSV40 plasmid would support incorporation of label into DNA (Fig. 3A), and this replication was authentic as judged by the *Dpn*I assay (Fig. 3B). In the presence of either 293 cell cytoplasmic extracts (Fig. 3B, lanes 1 and 2) or 293 cell cytoplasmic extract plus 293 cell nuclear extract (lanes 3 and 4) (see below), only pSV40 yielded *Dpn*I-resistant linear DNA. In contrast, when p8-4 was used as template DNA, very little DNA synthesis was observed (Fig. 3A) and all the DNA was still sensitive to *Dpn*I (Fig. 3B, lanes 2 and 4). These results demonstrate the dependence of DNA synthesis in vitro on an intact T-antigen-binding site II, since the p8-4 deletion lies in the middle of this sequence.

DNA replication in vitro required only those SV40 DNA sequences derived from the region surrounding and containing the minimal origin sequence that had been defined by studies in vitro. Plasmids pSVO+ and pSVO− were obtained by subcloning the *Hin*dIII (5,171 bp)-to-*Sph*I (128 bp) 200-bp fragment from pSV40 or p8-4 into the pBR322 derivative pAT153. Only pSVO+, but not pSVO−, supported DNA synthesis in vitro. (Fig. 3A). The 200-bp *Hin*dIII-to-*Sph*I origin fragment present in pSVO+ does not contain the SV40 transcriptional enhancer sequence (21). Thus, unlike polyomavirus DNA synthesis in vivo (10), SV40 DNA replication in vitro does not appear to require enhancer sequences.

Bidirectional replication from the origin sequences. Although we had demonstrated that DNA synthesis in vitro was dependent on functional origin sequences, we sought to determine whether DNA synthesis started at these sequences and proceeded bidirectionally around the DNA or was unidirectional. Plasmid pSV40 DNA was labeled in vitro for short periods in reactions containing T antigen and 293 cell cytoplasmic extracts and then extracted and digested with the *Hin*dIII restriction enzyme. The resulting fragments were subjected to agarose gel electrophoresis (Fig. 4B), and the relative amount of label in each fragment was determined and corrected for fragment length (in bp). The results of this analysis are shown in Fig. 4A.

As noted above, a lag of approximately 10 min occurs before the onset of DNA synthesis (Fig. 2B). The pSV40 DNA labeled for 10 min in vitro shows weak preferential labeling of the *Hin*dIII-D and -E fragments and some labeling of the *Hin*dIII-A and -B fragments (Fig. 4B). Thus, during the first 10 min of incubation at 37°C, very little elongation of nascent DNA chains occurs. On the other hand, enhanced preferential labeling of the *Hin*dIII-D and -E fragments occurred in DNA labeled for 15 min, whereas the amount of label in the *Hin*dIII-A and -B fragments did not increase from that observed at 10 min. The SV40 origin of replication

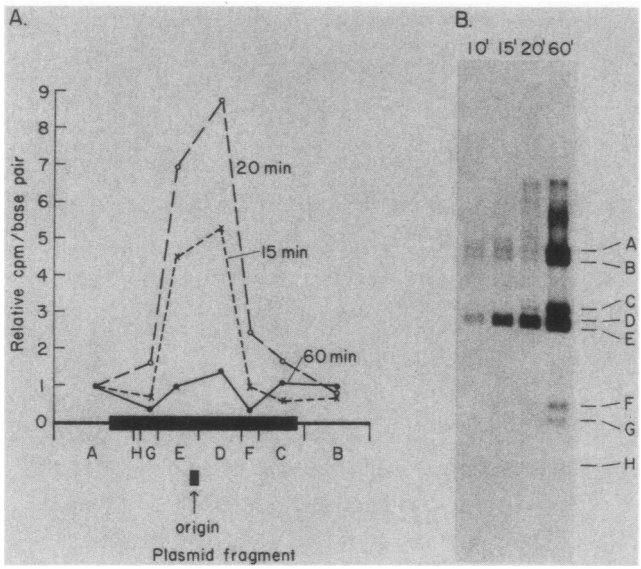

FIG. 4. Bidirectional replication from the SV40 origin. Reaction mixtures (50 μl) containing 210 μg of 293 cell cytoplasmic extract, 0.5 μg of T antigen, 0.3 μg of pSV40 DNA, and [α-^{32}P]dATP, dGTP, dCTP, and TTP (all 1,100 cpm/pmol) were incubated for 10, 15, 20, or 60 min at 37°C, and then the products were isolated and digested with *Hin*dIII. The fragments were separated by agarose gel electrophoresis (autoradiogram shown in part B), and the bands were located by EtBr staining of the gel. The bands were excised and counted, and the amount of label in each fragment was corrected for the number of bases present in each fragment. The graph (part A) shows the relative level of labeling for the 15-, 20-, and 60-min samples when the A fragment was given an arbitrary value of 1. A map of the fragments is shown (SV40 DNA shown by solid bar, and pMK16 sequences shown by thin line), together with the location of the origin region.

is located near the junction of the *Hin*dIII-D and E fragments (Fig. 4A). The fragments that first became labeled after 20 min were the *Hin*dIII-G, -F, and -C fragments (the small *Hin*dIII-H fragment was not included in this analysis). Finally, labeling of the *Hin*dIII-A and -B fragments was detected at the 60-min time point and the amount of labeling in all fragments relative to the fragment length was the same. This analysis demonstrated that DNA replication of pSV40 was bidirectional and started from the origin sequences. Furthermore, at this level of analysis, replication appeared to proceed in both directions from the origin with equal efficiency. These data do not exclude the possibility that replication proceeds in both directions from the origin but unidirectionally on individual molecules. However, Li and Kelly (27) have demonstrated by electronmicroscopy that bidirectional replication does occur in vitro on individual molecules.

Multiple rounds of semiconservative DNA replication. Plasmid pSV40 DNA was labeled in vitro with [α-^{32}P]dAMP and BUdR monophosphate, digested with the *Sma*I restriction endonuclease to linearize the DNA, and then subjected to equilibrium centrifugation in both neutral and alkaline CsCl gradients. The ^{32}P-labeled DNA formed two peaks after neutral gradient separation (Fig. 5A), one corresponding to light-heavy, intermediate-density DNA and a second corresponding to heavy-heavy DNA. Of the ^{32}P-labeled DNA, 12% banded in the heavy-heavy BUdR-labeled position, which corresponds to 35% of all the input DNA replicating, assuming that light-heavy DNA reenters the DNA template pool and is replicated again with the same probability as unlabeled DNA. Based on the level of incorporation of dAMP into DNA, between 30 and 45% of the input DNA is replicated under these conditions when calculated as the number of nucleotides incorporated relative to the number of nucleotides in added template DNA. However, these experiments were done with suboptimal amounts of T antigen (0.5 μg). In experiments in which the amount of T antigen is increased, up to 70% of added template DNA can be replicated in extracts from 293 cell cytosol (data not shown).

The DNA synthesized in vitro and labeled with [^{32}P]dAMP and BUdR banded as a single peak in alkaline CsCl gradients at the position expected for fully substituted DNA (Fig. 5B). Very little DNA banded in the region between heavy- and light-density DNA, indicating that the daughter DNA molecules were not covalently attached to the added template DNA. Identical data (not shown) have been obtained with DNA labeled with BUdR in the presence of the nuclear extract (see below), where all the products of replication are covalently closed circular DNA. Since this DNA is of monomer length, these results suggest that DNA synthesis is semiconservative and that replication proceeds via Cairns θ structures rather than by rolling-circle intermediates. The presence of heavy-heavy density DNA in the neutral CsCl gradient demonstrates that multiple rounds of initiation of DNA synthesis occur in vitro.

Inactivity of linear DNA templates. Table 1 indicates that efficient DNA replication of pSV40 in reactions containing T antigen and 293 cell cytoplasmic extracts required $MgCl_2$, ATP, deoxynucleoside triphosphates, and an energy-regenerating system (creatine phosphate and creatine phosphokinase). Replication in these crude extracts did not require ribonucleoside triphosphates (other than ATP), but it is possible that the extracts contain sufficient amounts of these precursors or that deoxynucleoside triphosphates can substitute. DNA synthesis was also completely sensitive to the drug aphidicolin, a specific inhibitor of human DNA polymerase α (30, 48). This result is similar to that published by Li and Kelly (26).

In a separate experiment, the pSV40 template DNA was

TABLE 1. Requirements for SV40 DNA replication[a]

Expt no.	Component omitted or added	Relative DNA synthesis
1	None	100
	− $MgCl_2$	4
	− ATP	33
	− CTP, UTP, GTP	103
	− ATP CTP UTP GTP	17
	− dGTP, dCTP, dTTP	29
	− CP, CPK	8
	+ Aphidicolin, 10 μg/ml	7
	+ Aphidicolin, 100 μg/ml	4
2	Complete	100
	− pSV40, + p8-4	3
	− pSV40, + *Bam*HI-linearized pSV40	5
	− pSV40, + *Bam*HI-linearized p8-4	3

[a] Complete reaction mixtures (50 μl) contained final concentrations of 40 mM HEPES-KOH (pH 7.5); 8 mM $MgCl_2$; 0.5 mM dithiotheitol; 100 μM each of dTTP, dGTP, dCTP, and 25 μM [α-^{32}P]dATP (ca. 1,000 cpm/pmol); 3 mM ATP; 200 μM each GTP, CTP, UTP; 40 mM creatine phosphate (CP); 1 μg creatine phosphokinase (CPK); 300 ng of pSV40 DNA; 210 μg of 293 cell cytoplasmic extract, and 0.5 μg of T antigen. Reaction mixtures were incubated for 2 h at 37°C, and the amount of dAMP incorporated was determined by trichloroacetic acid precipitation and normalized to 100% for the complete reaction.

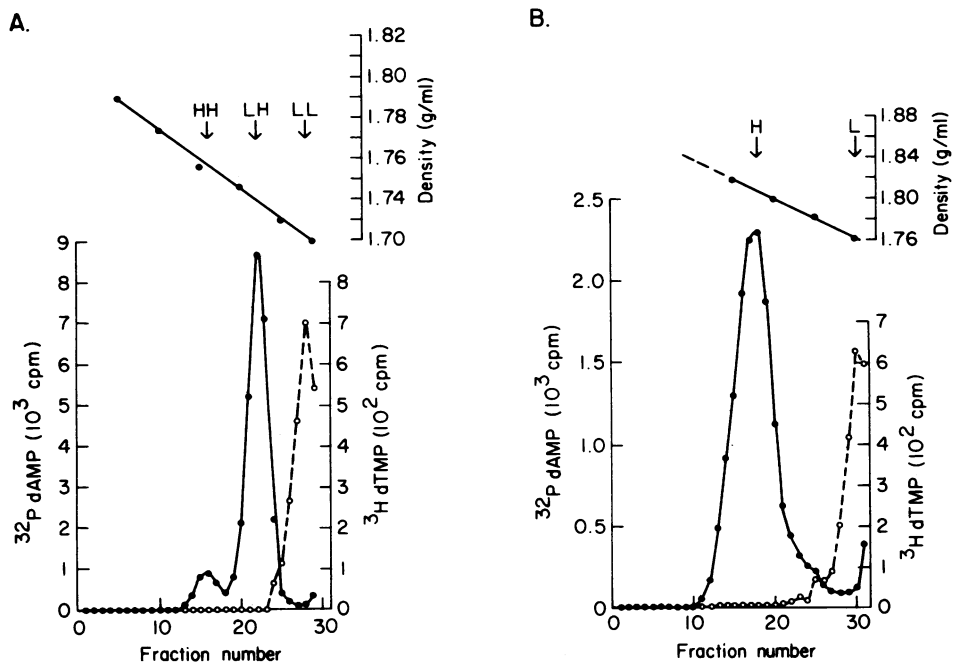

FIG. 5. Multiple rounds of semiconservative DNA replication. Reaction mixtures (50 μl) containing 210 μg of 293 cell cytoplasmic extract, 0.5 μg of T antigen 0.3 μg of pSV40, 100 μM BUdR triphosphate instead of TTP, and [α-^{32}P]dATP were incubated for 2 h at 37°C. The reaction products were isolated, digested with *Sma*I, and subjected to equilibrium CsCl gradient centrifugation for 24 h under neutral (A) or alkaline (B) conditions. [^{3}H]dTMP-labeled pSV40 DNA marker was included (---). Fractions were collected, the radioactivity in a portion of each fraction was determined, and the density was determined by refractometry.

replaced by p8-4 DNA or by *Bam*HI-linearized pSV40 and p8-4 DNAs. None of these template DNAs could support DNA synthesis, and when either *Bam*HI- or *Hin*dIII-linearized pSV40 DNA fragments were used as template DNAs, the low level of incorporation of label into DNA was not specific for the origin-containing fragment (data not shown). These results demonstrate that circular DNA is necessary for DNA synthesis and that supercoiling or torsional strain in the DNA may be a requirement. This is in contrast to results recently reported when extracts from monkey cells were used (26).

Introduction of negative supercoils into replicated DNA. The results presented above demonstrated that replication proceeds bidirectionally from the origin in the presence of T antigen and extracts from 293 cell cytosol. The product of this reaction migrated on an agarose gel as full-length linear DNA when it was digested with a single-cut restriction enzyme (*Xho*I; Fig. 1 through 3). Similarly, the product of DNA synthesis in reactions containing T antigen and extracts from 293 cell cytoplasm and nucleus migrated as form III linear DNA after digestion with *Xho*I and *Dpn*I, followed by agarose gel electrophoresis (Fig. 3, lane 3). The absence of substantial amounts of labeled species migrating more slowly than form III DNA suggests that there were few partially replicated molecules that contain a branch. Moreover, addition of the nuclear extract to the reaction mixtures did not induce any repair synthesis reactions on the origin-defective p8-4 plasmid (Fig. 3, lane 4). However, addition of the nuclear extract to replication reaction mixtures containing template DNAs with a functional origin significantly changed the topological structure of the replicated product.

Either pSV40 or pSVO+ was used as template for DNA replication in reaction mixtures containing T antigen, 293 cell cytoplasmic extract, and various amounts of nuclear extract. Figure 6 shows the products of DNA synthesis that had been deproteinized and then analyzed by high-resolution agarose gel electrophoresis as described by Sundin and Varshavsky (39). In the absence of the nuclear extract, the majority of reaction products migrate as a series of topoisomers that migrate from slightly slower than to slightly faster than the form II marker DNA for each plasmid, depending on the isomeric form. A series of other products, which are probably replication intermediates and catenated circles, migrated between the origin of the gel and the position of the form II marker DNA. Some of these forms were resolved into topoisomers, which was more evident for the pSVO+ replication products (migrating between the 8.4- and 5.3-kilobase linear markers). It is most likely that these forms are similar to the replication termination intermediates observed in SV40-infected monkey cells by Sundin and Varshavsky (39, 40). A more detailed analysis of these products is beyond the scope of this report.

Addition of increasing amounts of the nuclear extract to the replication reactions caused a shift in the isomeric forms of the replication products until all forms migrated slightly more slowly than the negatively supercoiled form I marker DNA isolated from *E. coli*. The nuclear extract also contains an inhibitor of DNA synthesis (Fig. 6, bottom). The activity in the nuclear extract that alters the structure of the replication products is probably present in the cytoplasmic extracts in low amounts, since some topoisomers are seen even in the absence of nuclear extract. When the products of DNA synthesis that were made in reactions containing only T antigen and cytoplasmic extract were isolated, they could not be converted to the faster-migrating species when incubated with the nuclear extract alone (data not shown). This indicates that factors from both the nuclear and cytoplasmic extracts are required to produce the faster-migrating species.

Figure 7 shows the products of DNA synthesis after electrophoretic separation in agarose gels containing 0, 3,

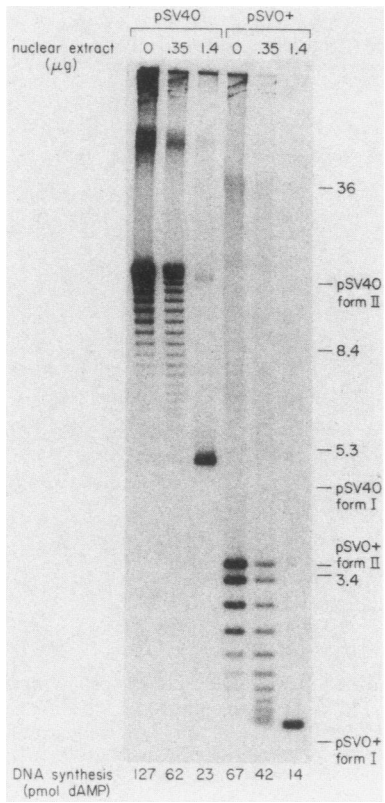

FIG. 6. High-resolution agarose gel electrophoresis of replication products. Reaction mixtures (50 μl) containing 210 μg of 293 cell cytoplasmic extract, 0.5 μg of T antigen, 0.3 μg of either pSV40 or pSVO+ as shown, and either 0, 0.35, or 1.4 μg of nuclear extract protein (as shown) were incubated for 2 h at 37°C. The reaction products were deproteinized and subjected to high-resolution agarose gel electrophoresis as described by Sundin and Varsharsky (39). The gel was run at 1 V/cm for 48 h, stained with EtBr to visualize the marker DNAs and then dried and autoradiographed. The extent of DNA synthesis (in picomoles of dAMP incorporated) is shown below each lane.

and 15 μM chloroquine. Chloroquine intercalates into DNA and unwinds the double helix, introducing positive superhelical twists into covalently closed DNA. It can be seen that the replication products from reactions that do not contain the nuclear extract migrate relatively faster in the gel (compared with the linear DNA markers) as the concentration of chloroquine is increased, suggesting that these products are relaxed, covalently closed circles. On the other hand, the products of reactions containing the nuclear extract initially migrate more slowly with increasing chloroquine concentrations, indicating that they are negatively supercoiled. The level of negative supercoiling is lower than that observed with the supercoiled plasmid marker produced in *E. coli* cells (data not shown), which probably explains why the negatively supercoiled product obtained in vitro migrates more slowly than the bacterially supercoiled DNA (Fig. 6).

These data demonstrate that the products produced in the absence of nuclear extract were covalently closed, relaxed DNA that could be positively supercoiled in the presence of chloroquine. Independent confirmation of this conclusion was obtained when these products banded in CsCl gradients that contained EtBr at the position of supercoiled DNA (data not shown). The relaxed, covalently closed circular DNA must also be decatenated, which suggests that the cytoplasmic extracts contain factors that can resolve the intertwined replication products. Finally, we conclude that the replication products made in the presence of the nuclear extract are negatively supercoiled and that factors present in both the cytoplasm and the nucleus are required for this process.

DISCUSSION

As with replication of SV40 DNA in cell extracts prepared from monkey cells (26, 27), soluble extracts prepared from adenovirus-transformed human embryo kidney cells are capable of supporting complete and efficient replication of SV40 DNA. Replication is dependent on the virus-encoded T antigen and proceeds bidirectionally from the SV40 origin sequences. A significant lag occurs between the time the reactions are first placed at 37°C (from 0°C) and the onset of DNA synthesis. The lag can be overcome by preincubating the template DNA at 37°C with 293 cell cytoplasmic extract and T antigen before addition of the deoxynucleoside triphosphates (R. Guggenheimer and B. Stillman, unpublished observations). A similar lag period has been observed before elongation of DNA synthesis from the *E. coli oriC* origin of replication in vitro (13) and appears to be due to the interactions between proteins and origin sequences for primer formation or preparation of the template DNA for initiation. It is thus likely that a number of preinitiation events occur at the origin of SV40 DNA replication that eventually lead to formation of the primer for DNA synthesis. Since linear DNA is inactive as a template, it is possible that these events require torsional strain in the DNA. The mechanism of initiation of SV40 DNA synthesis is not known, but potential start sites for leading-strand replication in vivo have been determined (19). Tseng and Ahlem (45) demonstrated that the DNA primase from mouse tissue can

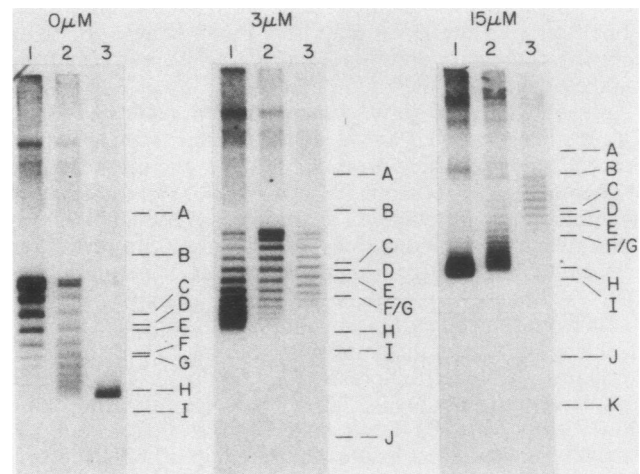

FIG. 7. Analysis of reaction products by agarose gel electrophoresis in the presence of chloroquine. Reaction mixtures were as described in the legend to Fig. 6, and the reaction products were deproteinized and divided into three aliquots. The samples were then subjected to agarose gel electrophoresis at 2 V/cm for 12 h in Tris-borate-EDTA buffer containing 0, 3, and 15 μM chloroquine. The amount of nuclear extract added was as follows: lanes 1, 0 μg; lanes 2, 0.35 μg; and lanes 3, 1.4 μg. The gels were stained with EtBr to locate the marker DNA bands (Ad2 DNA digested with *Hin*dIII) and then dried and autoradiographed.

specifically interact with SV40 origin sequences, but the relationship between this reaction and authentic initiation is not clear.

Initiation of DNA synthesis in extracts prepared from human cells (this report) or monkey cells (26, 27) is dependent on functional origin sequences, since a deletion of 4 bp within the origin eliminates DNA synthesis in vitro, as it does in vivo (16). The sequences surrounding the origin of SV40 DNA replication also contain the early and late promoters for transcription of virus DNA, a transcription enhancer region, and binding sites for two specific, double-stranded DNA binding proteins. One of these, the SV40 T antigen, binds to three sites in this region (42, 43) and the other, factor SPI, is required for transcription in vitro (11). The requirement for SV40 T antigen for initiation of DNA synthesis is well documented (see reference 9 for review). Clearly, DNA replication in extracts from human cells (this report) or monkey cells (26) requires the SV40 T antigen. The development of cell extracts prepared from suspension cultures of cells will expedite the identification of the cellular factors required for initiation and elongation of DNA synthesis.

SV40 DNA replication occurs efficiently in monkey cells, but is very inefficient in most cell lines derived from other species. It is well known that monkey cells can provide a permissivity factor in *trans* to nonpermissive nuclei when monkey cells are fused to cells incapable of supporting SV40 DNA synthesis (reviewed in reference 9). Most human cells are incapable of suppporting replication of SV40 DNA; however, human 293 cells can support very efficient DNA synthesis (Lewis and Manley, submitted for publication; Lebkowski et al., submitted for publication). In contrast, human HeLa cells do not support SV40 DNA replication very well. The different levels of SV40 DNA replication observed in cell extracts prepared from these cell lines are qualitatively similar to the amount of replication found in vivo. Presumably, the 293 cells contain a factor(s) that stimulates DNA synthesis, and candidates are the adenovirus E1A and E1B gene products that are produced in 293 cells and which could stimulate DNA synthesis directly or indirectly. Althernatively, HeLa cells could contain an inhibitor of SV40 DNA replication. We are currently attempting to determine whether factors present in 293 cell extracts can stimulate SV40 DNA synthesis in HeLa cell extracts. Furthermore, we have demonstrated that extracts prepared from the mouse cell line C127 fail to support SV40 DNA replication (unpublished observations), which may provide an assay to purify and identify permissivity factors from 293 cells. Similar results were obtained by Li and Kelly (27).

Finally, it is of considerable interest that the products of DNA synthesis made in the absence of the nuclear extract are covalently closed, relaxed circles, and that on addition of an extract derived from the nucleus of the cells, the DNA becomes negatively supercoiled in a reaction that requires both cytoplasmic and nuclear factors. The termination of DNA synthesis and resolution of the replicated DNA molecules can occur in cytoplasmic extracts, although there may be some unresolved catenanes present (Fig. 6; also see reference 39). The negative supercoiling of replication products presumably requires a topoisomerase activity to alter the isomeric form of the DNA. However, other factors must be involved, since neither the purified topoisomerase I nor topoisomerase II from eucaryotes can supercoil DNA (see reference 28 for a review). This is in contrast to the situation in procaryotes, where a type II topoisomerase (DNA gyrase) can introduce negative supercoils into DNA (28) and is required for the enzymatic replication of *E. coli oriC*-containing plasmids (20). We must assume that in the 293 cell extracts other factors must combine with a topoisomerase to yield negatively supercoiled DNA. It will be important to determine whether the supercoiling of SV40 replication products is a result of their being arranged into ordered structures such as nucleosomes, or whether a DNA gyrase-like activity has been found in these cell extracts. Circular DNA can be supercoiled and assembled into chromatin in extracts prepared from *Xenopus* eggs and oocytes (4, 15, 25) or *Drosophila melanogaster* embryos (36), but the relationship between these reactions and those reported here is not clear. We are currently purifying the nuclear factor(s) in an attempt to identify the mechanism of DNA supercoiling in these extracts from human cells. This reaction may have relevance to the regulation of gene transcription by torsional strain in eucaryotic DNA (see reference 47 for a discussion).

It is expected that exploitation of this system for replication of SV40 DNA will lead to the identification of cellular DNA replication factors as well as the elucidation of the mechanism of initiation, elongation, and termination of DNA synthesis, as well as the mechanism of resolution of DNA molecules. Ultimately, the complex regulation of DNA synthesis and the relationship between DNA replication and transcription from the SV40 origin-promoter region may be understood. Since the replication of SV40 DNA only requires one virus encoded protein and is similar in mechanism to eucaryote chromosome replication, insight into the regulation of cellular DNA synthesis is an ultimate goal.

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