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Synthesis of Superhelical Simian Virus 40 Deoxyribonucleic Acid in Cell Lysates*

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In vivo-labeled SV40 replicating DNA molecules can be converted into covalently closed superhelical SV40 DNA (SV40(I)) using a lysate of SV40-infected monkey cells containing intact nuclei. Replication *in vitro* occurred at one-third the *in vivo* rate for 30 min at 30°. After 1 hour of incubation, about 54% of the replicating molecules had been converted to SV40(I), 5% to nicked, circular molecules (SV40(II)), 5% to covalently closed dimers; the remainder failed to complete replication although 75% of the prelabeled daughter strands had been elongated to one-genome length. Density labeling *in vitro* showed that all replicating molecules had participated during DNA synthesis *in vitro*. Velocity and equilibrium sedimentation analysis of pulse-chased and labeled DNA using radioactive and density labels suggested that SV40 DNA synthesis *in vitro* was a continuation of normal ongoing DNA synthesis. Initiation of new rounds of SV40 DNA replication was not detectable.

SV40 and polyoma are well suited as simple models and biological probes of the mechanism of DNA replication in mammalian cells. Following infection by either virus, host enzymes involved in cellular DNA replication are induced (1). This induction of host cell DNA synthesis (2, 3) as well as the initiation of viral DNA replication (4, 5) requires a viral gene function. Elongation and termination of viral DNA replication appear to be entirely dependent on cellular gene products. Therefore, by studying SV40 DNA replication one might learn more about the details of cellular DNA replication.

SV40 and polyoma DNA synthesis are currently the best understood examples of DNA synthesis in mammalian nuclei. SV40 DNA can be isolated from infected cells in five major forms; covalently closed superhelical circles (SV40(I),[1] 80%; relaxed circles containing a nick or gap in either strand (SV40(II)), 10%; circular replicating intermediates (SV40(RI)), 6%; linear molecules (SV40(L)), 3%; and covalently closed dimers (1%). SV40(RI) DNA contains a superhelical region of unreplicated parental DNA, two relaxed loops participating in DNA synthesis (9–11) and the parental strands remain covalently closed (12). Newly synthesized strands are not covalently attached to parental DNA and are never longer than one-genome length (9–10). Replication forks may contain regions of single-stranded DNA (10, 13). To separate template strands during replication, a repeated nicking and resealing of parental DNA must occur, perhaps using the "relaxing factor" reported in uninfected mouse nuclei (14, 39).

Replication of SV40 viral DNA begins at a unique point and proceeds bidirectionally at approximately equal rates (15–18). Chain elongation occurs discontinuously, apparently on both strands, through synthesis of short pieces 200 to 300 nucleotides long which are then joined into longer strands (19–22). In polyoma, viral DNA replicates semiconservatively (23) and the most newly synthesized DNA appears to contain RNA covalently attached to the 5′ terminus (12, 24).

The mechanism for separation of circular progeny molecules at the end of replication is unknown, but clearly one of the template strands must be broken and rejoined. The only apparent intermediate during segregation of SV40 DNA is a circular DNA containing a nick in the daughter strand close to the normal termination site (25). Errors during replication as well as recombination are a likely explanation of dimeric forms (26).

Since *in vitro* systems for SV40 DNA synthesis would allow greater control over biochemical parameters, purification, and characterization of DNA replication factors, and, most importantly, complementation of cell and virus mutants *in vitro*, we

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[1] Abbreviations used are: SV40(I) DNA, SV40 double-stranded covalently closed, circular, superhelical DNA; SV40(II) DNA, SV40 double-stranded circular DNA containing an interruption of the phosphodiester backbone in at least one of the two strands; SV40(L) DNA, SV40 double-stranded linear DNA; SV40(RI) DNA, SV40 DNA replicating intermediate; BrdUTP, bromodeoxyuridine triphosphate; SDS, sodium dodecyl sulfate; SDS supernatant and SDS pellet, DNA isolated by the method of Hirt (6) which precipitates cellular DNA in 0.6% SDS and 1 M NaCl leaving viral DNA in the supernatant following centrifugation. DNA-DNA hybridization studies showed that only 4% of the SV40(I) DNA is trapped in the pellet (P. Rigby, personal communication). Mitochondrial DNA also appears in the SDS supernatant but is labeled 0.1 to 1% as well as cellular or viral DNA (7, 46); Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.

have undertaken a study of SV40 DNA replication in nuclei from infected cells. Several cell-free systems have been described for the study of DNA replication in both uninfected and virus-infected mammalian cells (27–34). The purpose of this paper is to describe an *in vitro* system that converts SV40(RI) to the covalently closed, superhelical form SV40(I). An accompanying paper described how this system was used to detect and assay a cellular factor (or factors) required to convert SV40(RI) to SV40(I).

EXPERIMENTAL PROCEDURES

Cell Lines

CV1 cells obtained from S. Kit, MA-134 cells from J. Pagano, and BSC-1 cells from Flow Labs are established lines of African green monkey kidney cells. All were grown on plastic plates (Nunclon or Falcon) in Dulbecco-modified Eagle's Medium (Gibco) supplemented with 10% calf serum (Microbiological Associates), 500 units/ml of penicillin G, and 100 μg/ml of streptomycin sulfate in a CO_2 incubator at 37°.

Virus Stock

A plaque-purified isolate of the small plaque SV40 strain, Rh911 (35), was used in all experiments. Virus was grown on MA-134 cells by infecting at a multiplicity of 0.01 plaque-forming units per cell and harvesting the virus 10 to 12 days later as described by Estes *et al.* (36). Virus extracted from a polyethylene glycol precipitate was sterilized by shaking with 1 part $CHCl_3$ per 20 parts virus suspension for 10 min at 4°. The top layer was removed after centrifugation for 3 min at 3000 × *g*, adjusted to 10% calf serum, and frozen at −20° in 2-ml aliquots. Virus stocks were titered by plaque assay using CV-1 cells.

Preparation of Viral DNA

Viral DNA markers were prepared from infected BSC-1 or CV-1 cells (multiplicity of infection of 40) labeled 24 hours post-infection with either 50 μCi/ml, of $^{32}P_i$ (carrier free) in phosphate-free medium containing calf serum dialyzed against 0.15 M NaCl or 10 μCi/ml of [^{3}H]thymidine (20 Ci/mM) in normal medium. After 48 hours of infection, viral DNA was extracted by the method of Hirt (6). The sodium dodecyl sulfate supernatants were extracted twice with $CHCl_3$-isoamyl alcohol (24:1), and the DNA was precipitated with 5 volumes of ethanol at −20° overnight. The precipitate was collected by sedimentation at 23,000 rpm for 30 min at 0° in a Beckman SW 25.1 rotor, resuspended in 10 mM Tris, pH 7.8, 1 mM EDTA, and 0.1 M NaCl, then treated with pancreatic RNase (20 μg/ml) for 3 hours at 30° to digest RNA. SV40(I) and SV40(II) DNA were then isolated after neutral sucrose gradient sedimentation. With ^{32}P labeling, about equal amounts of SV40(I) and SV40(II) were recovered, whereas with ^{3}H labeling, 80 to 90% of the labeled DNA in the SDS supernatant was in SV40(I). SV40(I) DNA isolated in this manner was indistinguishable from SV40(I) DNA that had been purified further by equilibrium sedimentation in a CsCl-ethidium bromide density gradient.

Growth and Infection of Cells for in Vitro DNA Synthesis

BSC-1 cells were generally used both because they gave the most active preparations and cell DNA synthesis is not stimulated by SV40 infection (37). Cells were seeded in 9-cm plastic dishes at a density of 5 × 10^5 cells per dish. The medium was changed 2 or 3 days later and the cells were infected for 1 hour on Day 4 or 5 at a multiplicity of 40 plaque-forming units of stock virus per cell in 0.6 ml of TS buffer (20 mM Tris-HCl, pH 7.6, 1 mM Na_2HPO_4, 5 mM KCl, 137 mM NaCl, 0.5 mM $MgCl_2$, and 0.9 mM $CaCl_2$) containing 5% calf serum. Fresh medium was added and incubation at 37° continued for 35 hours at which time cell lysates were prepared for *in vitro* DNA synthesis.

Standard Conditions for Preparation of Cell Lysates

Infected cells were labeled routinely with [^{3}H]thymidine (>15 Ci/mmol) prior to preparation of cell lysates. When *in vitro* DNA synthesis was to be monitored by incorporation of [α-^{32}P]dATP or dCTP, then the SV40(I) DNA pool was prelabeled by adding 50 μCi of [^{3}H]thymidine to 5 ml of medium 2 hours before the cell lysates were prepared. Alternatively, replicating forms already present *in vivo*, SV40(RI) DNA, were labeled by removing the medium, washing the cell monolayers with 10 ml of TD buffer (TS buffer without $MgCl_2$ and $CaCl_2$), and addition of 0.5 ml of TD buffer containing 100 μCi of [^{3}H]thymidine per ml and flotation of the dishes on a 37° water bath for 3.5 min. At this time 0.5 ml of TD buffer containing 4 mM EDTA and 1% trypsin was added. The plates were incubated an additional minute at 37° then floated on ice water to arrest further DNA synthesis. All subsequent steps were carried out between 2 and 4°, using ice baths whenever possible.

One milliliter of TD buffer was added to each dish and the cells were removed with the aid of a pipette, pooled, adjusted to 20% calf serum to inhibit the trypsin, then sedimented at 2000 × *g* for 3 min. The pellet was resuspended in 4 ml of hypotonic buffer per dish of cells supplemented with 0.2 M sucrose to prevent premature cell lysis and 0.1 mM thymidine to dilute residual [^{3}H]thymidine. Hypotonic buffer was 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, pH 7.8, 5 mM KCl, 0.5 mM $MgCl_2$, and 0.5 mM dithiothreitol. The cells were sedimented again and then resuspended in hypotonic buffer at about 2 × 10^7 cells per ml. The cells were allowed to swell for 8 min at 0° then lysed by four strokes of a tight fitting Dounce homogenizer (Kontes Glass Co., pestle B) and the extract used without further fractionation.

Standard Conditions for in Vitro DNA Synthesis

In a 5-ml centrifuge tube 0.15 ml of cell lysate was mixed with 0.05 ml of an assay mix at 2°. The assay mix contributed 0.20 M sucrose, 30 mM Hepes, pH 7.8, 40 mM KCl, 6 mM $MgCl_2$, 0.5 mM dithiothreitol, 4 mM ATP, 5 mM phosphoenolpyruvate, 29 μg of pyruvate kinase, and 0.2 mM each of dATP, dGTP, dCTP, and dTTP to the final concentrations. In addition, the hypotonic buffer contributes 15 mM Hepes, pH 7.8, 3.7 mM KCl, 0.37 mM $MgCl_2$, and 0.37 mM dithiothreitol. Where indicated labeled deoxynucleoside triphosphates were present at 0.02 mM.

The reaction was terminated after incubation for 1 hour at 30° by addition of 0.4 ml of 20 mM Tris, pH 7.8, 40 mM EDTA, and 1.2% SDS. When the preparation was completely solubilized, 0.4 ml of 2.5 M NaCl was added and the tubes stored at 4° for at least 8 hours to precipitate cellular DNA (6) which was then removed by sedimentation at 17,000 × *g* for 40 min. DNA found in the SDS supernatant was greater than 90% viral judged by sedimentation analysis of infected and uninfected cell lysates. This agrees with other reports where DNA-DNA hybridization assays were also performed (38).

Rapid Assays for Covalently Closed DNA

Two methods were routinely used to determine the fraction of SV40(I) DNA present in the SDS supernatants: sedimentation in alkaline sucrose gradients (9) and S1 nuclease digestion following heat denaturation of the DNA (40).

Alkaline Sucrose Sedimentation—SV40 [^{32}P]DNA containing about 50% SV40(I) and 50% SV40(II) DNA was added to the SDS supernatant of the samples to be analyzed to serve as an internal standard. An aliquot of the SDS supernatant was sedimented in an alkaline sucrose gradient as described below and two fractions collected; one containing SV40(I) DNA and the second containing SV40(II + L) DNA, nascent host DNA, and viral DNA released from replicating molecules. The labeled DNA in each fraction was precipitated with 25 μg of salmon sperm DNA by adding 10 ml of cold 1 N HCl containing 0.5% sodium pyrophosphate and collected on Whatman GF/C glass fiber filters, washed three times with 10-ml portions of HCl-pyrophosphate solution followed by 5 ml of ethanol, then dried, and counted in a toluene scintillator. The percentage of SV40(I) [^{3}H]DNA was corrected on the basis of the recovery of the SV40(I) [^{32}P]DNA added as the internal standard.

S1 Nuclease Assay—SV40 [^{32}P]DNA was added to the SDS supernatant fraction to serve as an internal standard and two 0.2-ml aliquots removed. The amount of acid-precipitable DNA was measured in one aliquot, and to the other was added: sonicated salmon sperm DNA (6 μl of 5 mg/ml), SDS (8 μl of 10% solution), and 0.186 ml of water to give final concentrations of 75 μg of DNA/ml, 0.2% SDS, and to dilute the NaCl present to 0.5 M. Samples were heated at 100° for 6 min to denature nicked forms of SV40 DNA (T_m of DNA containing 40% G + C is 95° in 0.5 M NaCl). Following rapid cooling in ice water, sodium acetate (0.2 ml of 0.6 M, pH 4.6), zinc acetate (0.14 ml of 0.05 M, pH 4.6), and 1.26 ml of water were added to give final concentrations of 60 mM sodium acetate, pH 4.6, 3.5 mM zinc acetate, and 0.1 M NaCl. Enough single strand-specific S1 nuclease (prepared by the method of Sutton (41)) was added to digest the single-stranded DNA in 5 to 10

min at 37°, although the incubation was generally for 25 min before precipitating the resistant DNA. Calculations of the fraction of SV40(I) DNA were normalized with respect to the recovery of the internal standard of [^{32}P]DNA.

Results using either method were in excellent agreement. The average deviation of triplicates containing 50% SV40(I) DNA was ±1.5%.

Sedimentation Techniques

Routine analysis of the amount of SV40(I) DNA made *in vitro* was done directly on SDS supernatants as described above. To characterize the sedimentation behavior of newly synthesized DNA, the samples were dialyzed for 12 hours against two changes of 10 volumes of 10 mM Tris, pH 7.8, 1 mM EDTA, and 0.1 M NaCl before layering over sucrose gradients. Prior to equilibrium centrifugation in CsCl-ethidium bromide density gradients, the SDS supernatants were extracted twice with 2 volumes of $CHCl_3$-isoamyl alcohol (24:1) to remove protein.

DNA samples (0.1 ml) were layered on linear 5 to 20% sucrose gradients in 4.2 ml of polyallomer tubes and sedimented at 4° in a Beckman SW 56 rotor at 55,000 rpm for the indicated times. Neutral gradients contained 1 M NaCl, 1 mM EDTA, and 10 mM Tris, pH 7.4. Alkaline gradients contained 5 mM EDTA, 0.2 to 0.8 M NaOH (proportional to sucrose concentration), 0.8 to 0.2 M NaCl (to make the Na^+ concentration up to 1 M). Fractions of 3 to 10 drops were collected from the bottom through a 20-gauge needle onto 2.5-cm diameter Whatman No. 3MM paper discs, dried, and then washed in batches three times in cold 1 M HCl, 0.5% sodium pyrophosphate (5 to 10 ml per disc), and then twice in ethanol. After drying, the discs were counted in a toluene scintillator. Equilibrium density gradient centrifugation was performed either in a solution containing 10 mM Tris-HCl, pH 7.8, 1 mM EDTA, CsCl of final density 1.565 g/cc, and 400 μg of ethidium bromide per ml to separate SV40(I) DNA from other forms of DNA or in the same solution except with a final density of 1.700 g/cc and no ethidium bromide. Gradients (6 ml total volume) were formed during centrifugation in a Beckman 50 Ti rotor at 37,000 rpm for 55 hours at 4°. Fractions were collected from the bottom either in test tubes or on Whatman No. 3MM discs and washed as described above.

Reagents

[α-^{32}P]dATP, [α-^{32}P]dCTP, and [α-^{32}P]dGTP at specific activities of 50 to 80 Ci/μmol, were prepared by the method of Symons (42, 43). 5-Bromodeoxyuridine triphosphate was synthesized by the method of Chamberlin and Berg (44) and kindly donated by Klaus Geider. All other reagents were obtained from commercial sources.

Chromatography

Labeled nucleotides were separated on polyethyleneimine thin layer strips (Brinkmann Polygram CEL 300 PEI/UV 254) by ascending chromatography with fresh 0.4 M NH_4HCO_3.

RESULTS

Our preliminary studies on SV40 DNA synthesis in isolated nuclei provided two guidelines for this work. First, synthesis of SV40(I) DNA was more efficient in crude cell lysates than in purified nuclei, and second, that incorporation of radioactive deoxynucleoside triphosphates into DNA found in SDS supernatants was not a reliable indicator of SV40 DNA replication (45). Therefore, a crude cell lysate system was developed and evaluated entirely on its ability to convert replicating SV40 DNA (SV40(RI)) prelabeled *in vivo* into SV40(I) DNA *in vitro*. The following is a description of this system and an assessment of whether the conversion of SV40(RI) to SV40(I) DNA follows the same pattern of replication as occurs *in vivo*.

Conditions for DNA Synthesis in Cell Lysates—The rate of viral DNA synthesis in BSC-1 cells was about twice that observed in CV-1 or MA-134 cells and reached a maximum at 32 to 44 hours after infection. At that time greater than 95% of the cells were positive for T antigen (47). BSC-1 cells have the further advantages that SV40 infection does not induce cell DNA synthesis and the cells can be removed rapidly from the culture dishes by trypsinization following a 3.5-min pulse of [^{3}H]thymidine.

Three methods for lysing cells were compared. Cells were removed routinely by trypsinization, swelled in a hypotonic buffer, and then lysed in a Dounce homogenizer as described under "Experimental Procedures." In this way large numbers of cells could be collected and lysed just prior to use. An alternative method avoided trypsin by scraping osmotically swollen cells from their plates with a rubber policeman and then dispersing aggregated nuclei in a loose fitting Dounce homogenizer. This method gave equivalent results but was more time-consuming when large numbers of dishes were involved. A third approach involved trypsinization and lysis in 0.2% Triton X-100, Brij-58, or Nonidet P-40 detergents. These lysates produced about 50% less SV40(I) DNA compared to mechanically prepared lysates because some SV40(RI) was converted to SV40(II) DNA; however, endogenous SV40(I) DNA present before detergent was added was unaffected. Cell lysates prepared 32 to 42 hours after infection gave the maximum conversion of SV40(RI) to SV40(I) RNA, and were stable for at least 3 hours at 2°. At earlier times, *e.g.* 20 hours, conversion was only 70% as efficient.

Table I shows the conditions needed for optimal conversion of SV40(RI) to SV40(I) DNA. Because of contributions from the cell lysate, omission of any one component did not have a drastic effect on the reaction. The ATP concentration was optimal from 2 to 8 mM. At each ATP concentration, $MgCl_2$ was adjusted to 1 mM excess over the total added nucleotides. An excess over 1 mM free Mg^{2+} was inhibitory with $MgCl_2$ concentrations above 7 mM. KCl had a broad optimum between 30 and 80 mM (Fig. 1). The sulfate and ammonium ions were inhibitory even though the pH remained constant during the assay. Since pyruvate kinase was used as an $(NH_4)_2SO_4$ suspension, addition of more than 50 μg of enzyme

TABLE I

Requirements for SV40 DNA synthesis in vitro

The standard assay contained 45 mM Hepes, pH 7.8, 44 mM KCl, 6.4 mM $MgCl_2$, 0.2 M sucrose, 0.9 mM dithiothreitol, 4 mM ATP, 5 mM phosphoenolpyruvate, 100 μg/ml of pyruvate kinase, and 0.2 mM each of dATP, dGTP, dCTP, and dTTP. Activity refers to the conversion of prelabeled SV40(RI) to SV40(I) DNA measured as described under "Experimental Procedures." In the standard assay the percentage of [^{3}H]thymidine in SV40(I) DNA went from 8 to 55% in 1 hour at 30°. This conversion was defined as 100% activity.

Conditions	Activity
	%
Complete standard assay	100
− ATP	60
− dATP, dGTP, dCTP, dTTP	30
− Phosphoenolpyruvate and pyruvate kinase	55
− Pyruvate kinase	80
− Phosphoenolpyruvate	72
− Sucrose	70
− KCl	60
+ EDTA (2 mM)	67
+ EDTA (5 mM)	5
+ $CaCl_2$ (0.5 mM)	80
+ Spermine (5 mM)	19
+ Spermidine (5 mM)	23
+ *N*-Ethylmaleimide (5 mM)	<1
+ *p*-Chloromercuribenzoic acid (5 mM)	<1
+ UTP, GTP, CTP (50 μM each)	100

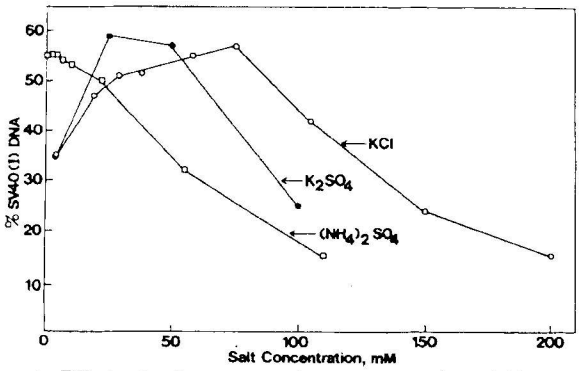


FIG. 1. Effect of salt concentration on conversion of SV40(RI) to SV40(I) DNA. Using the standard assay described under "Experimental Procedures," the amount of KCl present was varied. K_2SO_4 was then substituted for KCl. $(NH_4)_2SO_4$ was added to the standard assay containing 44 mM KCl. The percentage of [^{3}H]thymidine in SV40(I) DNA before incubation *in vitro* was 9%.

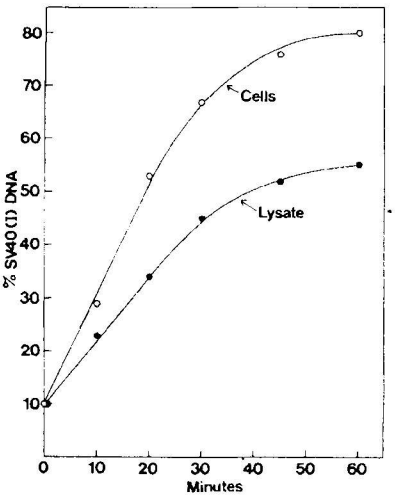


FIG. 2. Time course for conversion of SV40(RI) to SV40(I) DNA at 30°. Infected cells were pulse-labeled for 4 min with [^{3}H]thymidine as described under "Experimental Procedures." Lysates were prepared and assayed in the standard way. The remaining cell monolayers were washed twice with cold TS buffer containing 500 μM thymidine then covered with 10 ml of TS buffer containing 5% calf serum and 100 μM thymidine. The dishes were floated on a 30° water bath. Reactions were stopped by adding 1 ml of 0.6% SDS, 20 mM EDTA, and 10 mM Tris (pH 7.8) to a 100-cm dish of cells, and the SDS supernatant prepared and analyzed for the fraction of [^{3}H]thymidine in SV40(I) DNA. The same conversion of SV40(RI) to SV40(I) DNA was obtained with cell monolayers covered with 10 ml of Dulbecco's Modified Eagle's Medium plus 10% calf serum and incubated at 37° for 1 hour with 6% CO_2.

per ml also inhibited DNA synthesis. Even with added ATP, the presence of an ATP regenerating system composed of phosphoenolpyruvate and pyruvate kinase stimulated formation of SV40(I) DNA. Increasing the deoxyribonucleotide concentrations above 200 μM did not increase the rate of SV40(I) DNA synthesis.

In a typical experiment the amount of [^{3}H]thymidine in SV40(I) DNA varied from 7 to 10% of the total acid-insoluble label at the start of incubation and reached a maximum of 50 to 60% by 1 hour at 30° (Fig. 2). The initial rate of SV40(I) DNA synthesis *in vitro* was approximately 60% of that observed *in vivo* during a pulse chase at the same temperature. Based on initial rates of synthesis *in vivo*, about 50% of the SV40(RI) was converted to SV40(I) DNA in 16 min; one round of replication *in vivo* required 30 min at 30°.

The period of DNA synthesis *in vitro* was not increased by adding more of the assay components after 40 min of incubation at 30° although sufficient nucleotides were still present at this time to incorporate [α-^{32}P]dGTP into added DNase-treated salmon sperm DNA. The fate of deoxynucleotides in the complete assay system was examined directly using [α-^{32}P]dGTP. After 60 min at 30°, 20% of the remaining unincorporated label chromatographed as dGMP, 35% as dGDP, and 45% as dGTP. In contrast, when 2 mM ATP was present without a regenerating system the initial rate of dGTP disappearance was 3-fold faster and resulted in 80% dGMP, 15% dGDP, and 5% dGTP after 1-hour incubation. This suggests that one function of ATP and an ATP regenerating system was to maintain the nucleotide pools.

Efforts to improve the synthetic capacity of a cell lysate by increasing enzyme stability included addition of bovine serum albumin, an acidic protein (1 to 12 mg/ml), cytochrome *c*, a basic protein (1 to 12 mg/ml), and polymers such as Ficoll (10 mg/ml), polyethylene glycol 6000 (10 mg/ml), dextran 500 (10 mg/ml), and glycerol (0.1 to 1.0 M) in place of sucrose. These agents either had no effect or caused a 10 to 20% inhibition at the highest concentrations tested. Small amounts of ionic polymers such as dextran sulfate (0.5 mg/ml) and DEAE-dextran (0.5 mg/ml) resulted in 75% inhibition.

Some nuclear enzymes may have been lost or diluted in the preparation of lysates. However, a cell lysate could be diluted with the assay mix as much as 40-fold with no decrease in the amount of SV40(I) DNA synthesis. In addition, supplementation of the lysates with *Escherichia coli* DNA polymerase I (10 units (48)) alone or together with *E. coli* DNA ligase (2 units (49)) and NAD (30 μM) did not increase the amount of SV40(I) DNA made. Although heparin and *E. coli* DNA polymerase I stimulated DNA synthesis in isolated rat liver nuclei 10- to 20-fold (50), we found that heparin (100 μg/ml) alone inhibited formation of SV40(I) DNA by 90%, while addition of both heparin and DNA polymerase I showed no difference from the control lysate.

RNA synthesis may be required to initiate discontinuous elongation of viral DNA (24). However, addition of ribonucleoside triphosphates (Table I) did not stimulate production of SV40(I) DNA. Rifampicin (20 to 200 μg/ml), a specific inhibitor of *E. coli* RNA polymerase (51), showed no inhibition of either incorporation of [α-^{32}P]dATP into viral DNA or conversion of SV40(RI) to SV40(I) DNA. A rifampicin derivative, AF/ABDP (20 μg/ml), reported to inhibit gene amplification in *Xenopus* (51–53) and *in vitro* synthesis of φX174 parental RF,[2] also had no effect. About 80% of the inhibition observed at higher drug concentrations could be accounted for by the residual dimethylsulfoxide used to dissolve the inhibitors.

Since proteolytic enzymes in the lysate could prevent further *in vitro* DNA synthesis, both toluene sulfonyl fluoride (0.01 to 1 mM), a general inhibitor of serine proteases, and tosyl-L-phenylalanyl chloromethyl ketone (0.01 to 0.1 mM), a specific inhibitor of chymotrypsin, were added but these had no effect on either viral DNA synthesis or formation of SV40(I) DNA *in vitro*.

Characterization of Viral DNA Replication Products Made in Vitro—SV40(RI) DNA was labeled with [^{3}H]thymidine in intact cells in order to analyze its conversion into SV40(I) DNA in a cell lysate. Following a 4.5-min pulse of [^{3}H]thymidine, only 8% of the ^{3}H label found in the SDS supernatant was in

[2] W. Wicker, personal communication.

SV40(I) DNA; the remainder sedimented between 18 S and 4 S in an alkaline sucrose gradient (Fig. 3*A*). About 85% of the viral [^{3}H]DNA sedimented at 26 S in a neutral sucrose gradient, the position characteristic of SV40(RI) DNA (9), while about 5% was SV40(II) DNA (Fig. 4*A*). Moreover, about 80% of the viral ^{3}H[DNA] banded at a buoyant density expected of SV40(RI) DNA in a CsCl-ethidium bromide gradient (Fig. 5*A*), *i.e.* intermediate between that of SV40(I) and SV40(II) DNA (9). When uninfected cells were labeled and processed like infected cells, only 2% as much labeled DNA appeared in the SDS supernatant and it was generally found distributed in the bottom third of the neutral sucrose gradients as previously reported (9).

After incubation of the pulse-labeled infected cell lysate for 1 hour at 30° under standard conditions, the amount of ^{3}H label in SV40(I) DNA increased to 55% as judged by the amount of

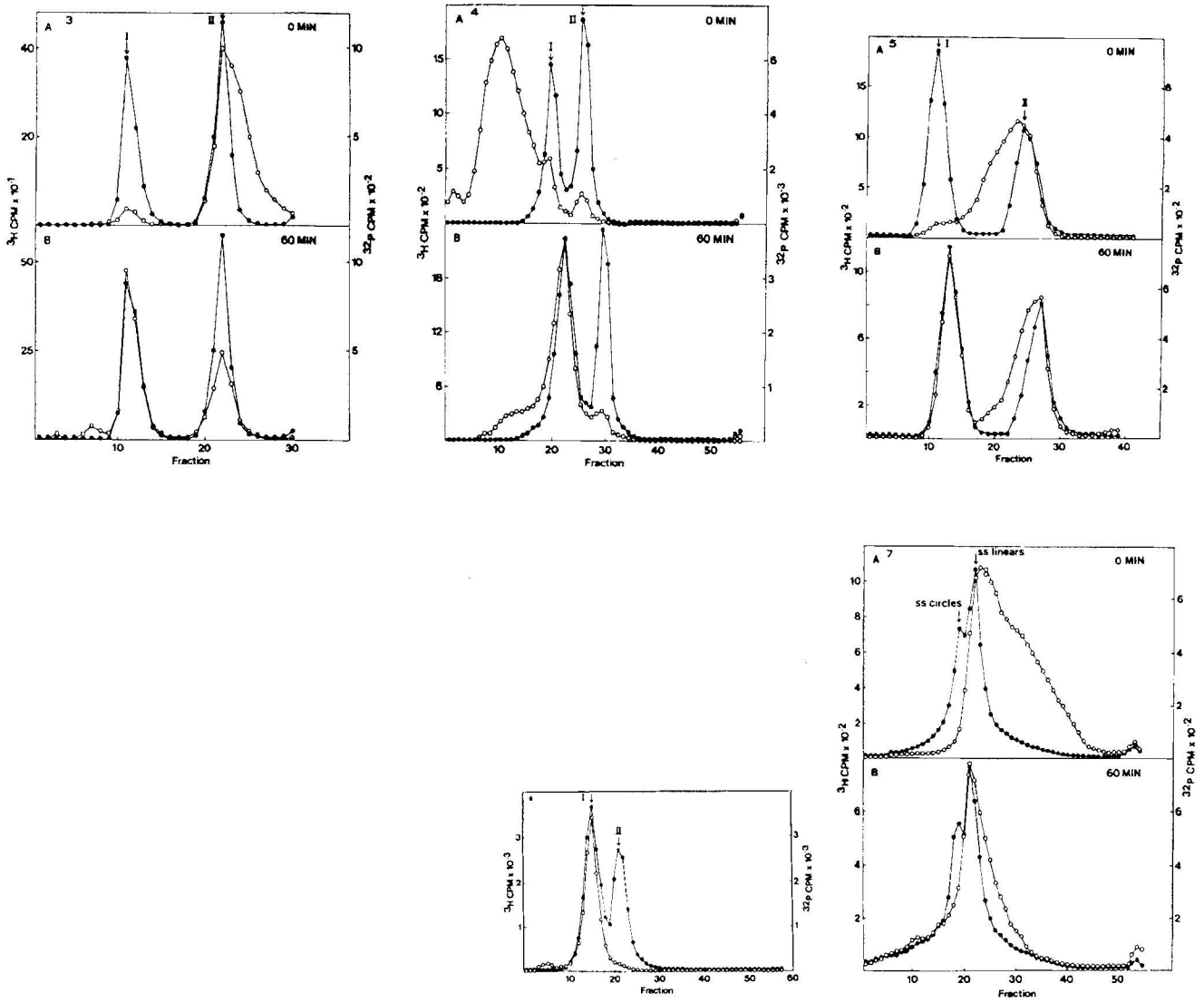


FIG. 3 (*upper left*). Alkaline sucrose gradient sedimentation of SV40 DNA synthesized *in vitro*. BSC-1 cells infected with SV40 were incubated for 4 min at 37° with [^{3}H]thymidine to predominantly label SV40(RI) DNA. Cell lysates were prepared and incubated *in vitro* for 9 or 60 min as described under "Experimental Procedures." Alkaline sucrose gradients were overlaid with 0.1 ml of the SDS supernatant fraction dialyzed against 10 mM Tris (pH 7.8), 1 mM EDTA, and 0.1 M NaCl. A ^{32}P-labeled SV40 DNA standard containing both SV40(I) and SV40(II) DNA was then added to the sample and the gradient centrifuged in a Beckman SW 56 rotor at 55,000 rpm, 4°, for 2 hours. Fractions were collected from the bottom of the gradient. O——O, ^{3}H; ●——●, ^{32}P; *I*, SV40(I) DNA; *II*, SV40(II) DNA; covalently closed dimers appear in Fractions 6 to 8 after 50 min of incubation *in vitro*.

FIG. 4 (*upper center*). Neutral sucrose gradient sedimentation of SV40 DNA synthesized *in vitro*. Procedure was that described in Fig. 3 except gradients were centrifuged for 3.25 hours. O——O, ^{3}H; ●——●, ^{32}P; *I*, SV40(I) DNA; *II*, SV40(II) DNA.

FIG. 5 (*upper right*). CsCl-ethidium bromide density equilibrium gradient of SV40 DNA synthesized *in vitro*. Procedure was that described in Fig. 3 except that the sample was centrifuged to equilibrium in CsCl-containing ethidium bromide as described under "Experimental Procedures." O——O, ^{3}H; ●——●, ^{32}P; *I*, SV40(I) DNA; *II*, SV40(II) DNA.

FIG. 6 (*lower left*). Neutral sucrose gradient sedimentation of SV40 DNA removed from the denser band of Fig. 5. SV40(I) [^{3}H]DNA taken from a 60-min pulse-chase experiment such as described in Fig. 3 was isolated from the lower band in CsCl-ethidium bromide gradient such as described in Fig. 5 and then sedimented through a neutral sucrose gradient. A ^{32}P-labeled standard SV40 DNA sample containing both SV40(I) and SV40(II) DNA was added to the sample after it was layered on the gradient. O——O, ^{3}H; ●——●, ^{32}P; *I*, SV40(I) DNA; *II*, SV40(II) DNA. Dimeric DNA is found in Fractions 4 to 7.

FIG. 7 (*lower right*). Separation of single-stranded circular and single-stranded linear viral DNA. The same procedure described in Fig. 3 was followed except that an alkaline sucrose gradient was centrifuged for 6.5 hours. A ^{32}P-labeled SV40(II) DNA standard was added to the sample after it was layered on the gradient. O——O, ^{3}H; ●——●, ^{32}P.

SV40(I) DNA observed in either alkaline gradient sedimentation (Fig. 3*B*) or CsCl-ethidium bromide equilibrium centrifugation (Fig. 5*B*); more than 93% of the SV40(I) DNA isolated from density equilibrium gradients also behaved as SV40(I) DNA on neutral (Fig. 6) and alkaline sucrose gradients (data not shown). The small discrepancy occurred during handling the DNA since an SV40(I) [^{32}P]DNA internal standard was damaged to the same extent. Thus, SV40(I) DNA produced *in vitro* was indistinguishable from SV40(I) DNA synthesized *in vivo*.

Following this 1-hour incubation *in vitro*, about 55% of the ^{3}H label was identified as SV40(I) DNA, 10% as SV40(II), 5% as covalently closed dimers, and the remaining 30% as SV40(RI). SV40(II) DNA was identified on neutral sedimentation gradients (Fig. 4*B*), while covalently closed dimeric DNA was detected in alkaline sedimentation gradients (Fig. 3*B*), and neutral sedimentation gradients of covalently closed DNA taken from CsCl-ethidium bromide gradients (Fig. 6). The remaining ^{3}H-labeled viral DNA was very likely SV40(RI) DNA because it sedimented faster than the SV40(I) peak in neutral sucrose gradients (Fig. 4*B*) and banded at an intermediate density in CsCl-ethidium bromide gradients (Fig. 5*B*). These may be molecules which were initiated *in vivo* but failed to complete replication *in vitro*. Some of the apparently unfinished SV40(RI) DNA may also be nicked circular dimeric DNA which would sediment at about 21.5 S at pH 7 in 1 M NaCl and have a buoyant density in CsCl-ethidium bromide gradients identical with SV40(II) DNA.

Although the data presented in Figs. 3 to 6 show the nature and extent of the change in viral DNA after 1 hour of incubation, the same analysis with samples taken earlier during the incubation confirmed the progressive conversion of prelabeled SV40(RI) to SV40(I) DNA expected from Fig. 2. With time, pre-existing daughter strands are elongated; at the beginning about 67% of the SV40(RI) [^{3}H]DNA was in DNA strands shorter than one SV40 length (Fig. 7*A*), but after a 1-hour incubation at 30°, 75% of the ^{3}H label was in strands of SV40 length (Fig. 7*B*).

Incorporation of Labeled Nucleotides into SV40 DNA—The incorporation of [α-^{32}P]dATP and [α-^{32}P]dCTP was used to characterize intermediates in the conversion of SV40(RI) to SV40(I) DNA *in vitro*. Lysates from infected cells, prelabeled for 1 hour with [^{3}H]thymidine, were prepared and incubated with the α-^{32}P-labeled deoxynucleoside triphosphates. About 90% of the ^{3}H label appeared in SV40(I) DNA which was neither degraded nor nicked during *in vitro* incubation with ^{32}P-labeled substrates since the ratio of SV40(I) to SV40(II) DNA remained unchanged (Fig. 8). During the incubation the ^{32}P label was incorporated about equally into the SDS supernatant and the SDS pellet.

Following a 10-min incubation, about 90% of the ^{32}P label in the SDS supernatant was in SV40(RI) DNA (Fig. 8*A*), and about 5% was in SV40(I) DNA as judged by the S1 nuclease assay. Fifty minutes later, 35% of the ^{32}P in the SDS supernatant was in SV40(I) DNA and about 5% sedimented as covalently closed dimers (Fig. 9). The remaining ^{32}P label was in SV40(RI) DNA which had only been partially completed (Fig. 8*B*). Occasionally some of the ^{32}P label (<25%) was found to sediment between 3 S and 7 S (Fig. 8), but there was never a peak of ^{3}H label from the prelabeled SV40(RI) [^{3}H]DNA in this region of a neutral sucrose gradient. The significance of this is discussed later.

The conversion of SV40(RI) to SV40(I) DNA *in vitro* was 30

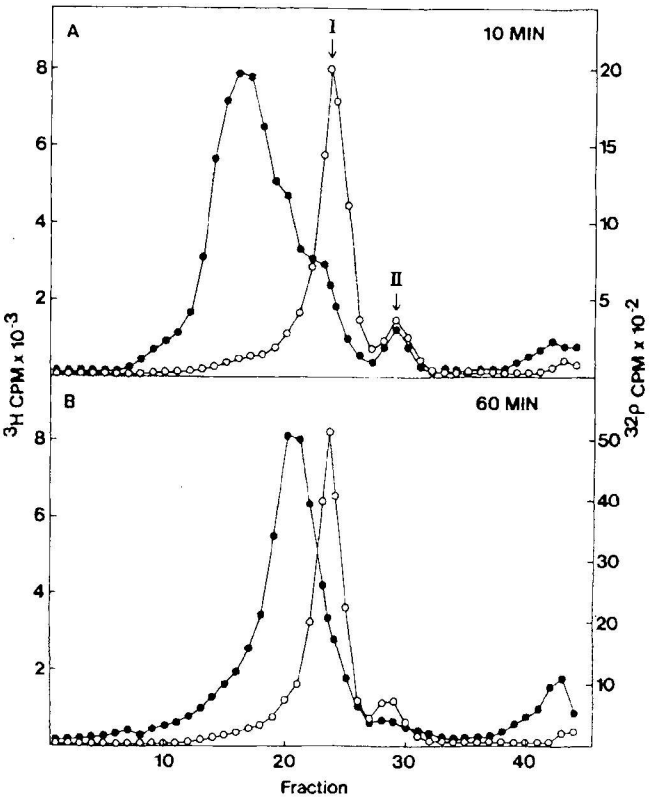
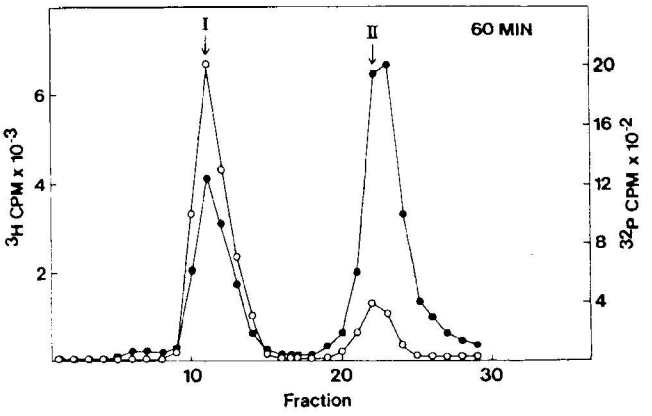


FIG. 8. Neutral sucrose gradient sedimentation of SV40 DNA labeled during *in vitro* synthesis. Cell lysates were prepared from BSC-1 cells infected with SV40 and incubated 1 hour at 37° with [^{3}H]thymidine to label predominantly SV40(I) DNA and then incubated *in vitro* for 10 or 60 min in the presence of [α-^{32}P]dATP and [α-^{32}P]dCTP. The SDS supernatant fraction was dialyzed, 0.1 ml layered over a neutral sucrose gradient, and centrifuged in a Beckman SW 56 rotor, 4°, 55,000 rpm for 3.25 hours. O——O, ^{3}H; ●——●, ^{32}P; *I*, SV40(I) DNA; *II*, SV40(II) DNA.

to 40% less efficient when measured with ^{32}P-nucleotides than with ^{3}H-prelabeled SV40(RI). This was expected because those SV40(RI) molecules which had only just begun replication would incorporate the greatest amount of [α-^{32}P]dATP and those closest to completion of replication would contain the greatest amount of ^{3}H label and also have the highest probability of becoming SV40(I) DNA. Note that 70% of the ^{32}P-labeled single strand, linear viral DNA that had not entered SV40(I) by 1 hour had elongated to complete SV40 DNA length (Fig. 10). Results determined by both labeling techniques were consistent with the conclusion that most growing DNA chains proceed to completion *in vitro* as they would have *in vivo* and about 50% of the nearly completed SV40(RI) continue onto SV40(I) DNA.

DNA Synthesis in Presence of BrdUTP—The previous experiments indicate that SV40 DNA molecules that had already begun replication *in vivo* continue to be replicated *in vitro*. A similar conclusion was obtained by density labeling. Cell lysates were prepared from SV40-infected cells that had been labeled in two ways. The first was labeled *in vivo* with [^{3}H]thymidine for 1 hour at 37° and the second for 4 min. In the first, 90% of the ^{3}H label was in SV40(I) DNA whereas in the second 85% of the ^{3}H label was in SV40(RI) DNA. These lysates were prepared and incubated for 30 min under the standard conditions (see "Experimental Procedures") except that BrdUTP (10 mM) replaced dTTP in the reaction mixture and the temperature was 37°. As expected, the density of



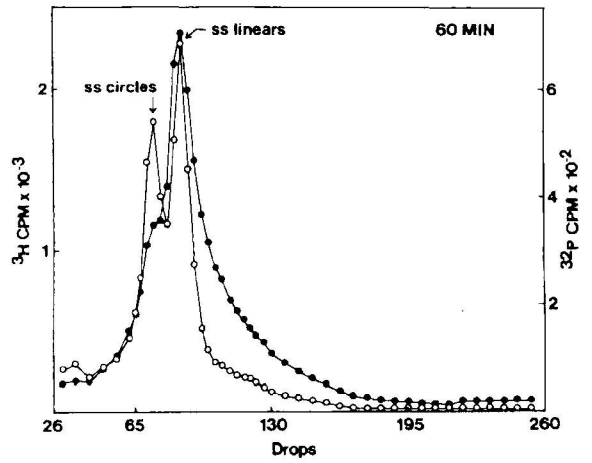


FIG. 9 (*top*). Isolation of covalently closed viral DNA labeled during *in vitro* synthesis. Procedure was the same as described in Fig. 8 except sedimentation was in an alkaline sucrose gradient. O——O, ^{3}H; ●——●, ^{32}P; *I*, SV40(I) DNA; *II*, SV40(II) DNA. Covalently closed dimers appear in Fractions 6 to 7. The gradient was centrifuged for 2 hours as in Fig. 3.

FIG. 10 (*bottom*). Length of linear single-stranded viral DNA synthesized in the presence of [α-^{32}P]dATP and [α-^{32}P]dCTP. Procedure was the same as described in Fig. 8. The alkaline gradient was centrifuged for 6.5 hours as in Fig. 7. O——O, ^{3}H; ●——●, ^{32}P.

prelabeled SV40(I) DNA shows no density shift after the incubation but about 90% of the prelabeled SV40(RI) DNA sedimented as a symmetrical peak to a higher buoyant density (a shift in density of 10 mg/ml) (Fig. 11). Therefore, essentially all of the SV40(RI) DNA molecules that had initiated *in vivo* were elongated *in vitro*.

To determine whether DNA synthesis *in vitro* was consistent with semiconservative replication, lysates from cells prelabeled *in vivo* with [^{3}H]thymidine for 1 hour were incubated for 30 min at 37° in the presence of [α-^{32}P]dGTP and BrdUTP. The newly incorporated ^{32}P label had a mean density 20 mg/ml greater than SV40(I) [^{3}H]DNA (Fig. 12*A*). Since complete replacement of dTMP by BrdUMP in poly[d(A-T)] results in an increase in the buoyant density of 200 mg/ml (54), SV40 DNA containing 59% A+T would be expected to shift its density (200)(0.59)/2 or 59 mg/ml higher after one complete round of replication. Under these conditions (see below), about 50% of one round of replication occurred on the average during the 30-min incubation *in vitro*; about 20% of the ^{32}P label was in SV40(I) DNA.

After sonication of the DNA analyzed in Fig. 12*A* into pieces

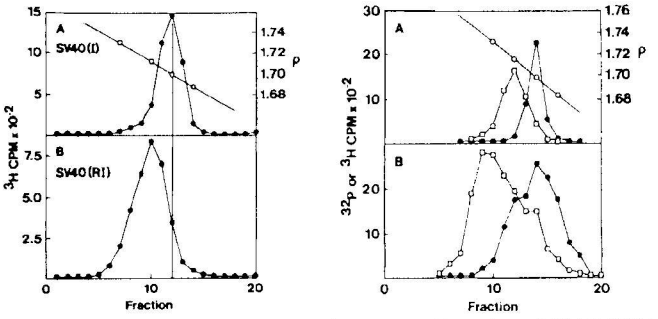


FIG. 11 (*left*). CsCl density gradient centrifugation of SV40 DNA synthesized in the presence of BrdUTP. Lysates from SV40-infected cells were exposed to [^{3}H]thymidine for either 1 hour to label SV40(I) DNA (*A*) or 4 min to label SV40(RI) (*B*) and then incubated with BrdUTP in the absence of dTTP. ●——●, ^{3}H; O——O, density.

FIG. 12 (*right*). CsCl density gradient centrifugation of SV40 DNA synthesized in the presence of BrdUTP and [α^{32}P]dGTP. SV40-infected cells were prelabeled with [^{3}H]thymidine for 1 hour to label SV40(I) DNA before the lysates were incubated *in vitro* with BrdUTP and [α-^{32}P]dGTP in the absence of dTTP (*A*). *Panel B* is the same material after sonication to an average chain length of 700 nucleotides. ●——●, ^{3}H; O——O, ^{32}P; □——□, density.

about 700 nucleotides long, the bulk of the ^{32}P label banded in a neutral CsCl gradient at a density 40 mg/cc greater than the DNA labeled with [^{3}H]thymidine *in vivo* (Fig. 12*B*). This shift corresponds to a replacement of 70% of the dTMP residues by bromouracil. Complete replacement would not be expected because of the endogenous nucleotide pools (about 10 μM (2)) present in the cell lysate.

DISCUSSION

The results show that a lysate of SV40-infected cells can convert endogenous replicative intermediate, SV40(RI) DNA, to covalently closed, superhelical SV40 DNA (SV40(I)). The reaction requires both the nuclei and the cytoplasmic fraction; each fraction is inactive by itself but synthetic activity is restored when the system is reconstituted (45). An evaluation of the significance of other requirements (Table I) awaits a more purified system.

SV40(RI) DNA, initiated and prelabeled *in vivo*, is converted to SV40(I) DNA under our conditions at about 30% the rate and to about 70% the extent as occurs *in vivo*; the reaction is complete after 60 min (Fig. 2). Since incorporation of [α-^{32}P]dATP into viral DNA follows a similar time course (45), it appears that viral DNA synthesis *in vitro* ceases when the pre-existing SV40(RI) DNA templates have been consumed.

The incomplete conversion of SV40(RI) to SV40(I) DNA may result from faulty termination and segregation of progeny SV40(I) DNA molecules. Most of the label remaining in nicked viral DNA after 60 min is at least one genome in length (Figs. 7*B* and 10), indicating that the remaining SV40(RI) DNA is arrested late in replication. An important fact is that cessation of *in vitro* DNA synthesis is not the result of degradation of either SV40(RI) or SV40(I) DNA.

Since our assays score, specifically, for covalently closed DNA derived only from SV40(RI), prelabeled *in vivo*, elongation of previously initiated strands and covalent closure of newly formed circular DNA must have occurred *in vitro*. The SV40(I) DNA formed *in vitro* is indistinguishable from SV40(I) prepared *in vivo* as judged by sedimentation analysis in neutral and alkaline sucrose gradients and equilibrium centrifugation in CsCl-ethidium bromide density gradients (Figs. 3 to 6).

With labeled deoxynucleoside triphosphate precursors, the radioactivity first enters SV40(RI) DNA and then appears in SV40(I) DNA (Figs. 8 and 9). As with pulse-chase experiments *in vivo*, viral daughter strands are elongated *in vitro* until they are of viral genome length (Figs. 7 and 10). With BrdUTP as a density label, it was clear that virtually all SV40(RI) DNA participates in DNA synthesis *in vitro* and that synthesis occurs by a semiconservative mechanism (Figs. 11 and 12). These observations of SV40 DNA replication *in vitro* are identical with those made by ourselves (Figs. 6 to 8) and others (9, 10) of the process *in vivo* and suggest that normal chain elongation, termination, and segregation can occur in a cell-free extract.

The newly formed DNA sedimenting at 7 S in neutral sedimentation gradients (Fig. 8*B*) in some experiments was the only indication of a possible artifact. This material, which never comprised more than 25% of the incorporated ^{32}P label, did not contain any of the prelabeled viral strands. Similar observations have been reported in SV40 (22) and polyoma (8) DNA replication following inhibition by hydroxyurea or FdUrd. This phenomenon may involve displacement of short newly synthesized DNA strands from the replicating fork by retarding DNA synthesis in the gaps created by the process of discontinuous DNA replication (8, 22). Conditions that retard the progress of DNA replication may promote a successful competition between reannealing and further separation of template strands at the replicating fork.

A serious but intriguing limitation of this system is the inability to initiate new rounds of SV40(I) DNA replication. DNA synthesized in the presence of BrdUTP did not accumulate at a density indicative of BrdUTP in both strands. SV40(I) DNA prelabeled *in vivo* did not shift to SV40(RI) after incubation *in vitro*. Additional work is needed to determine the reasons for this defect.

Polyoma DNA replication has also been studied in cell lysates of polyoma virus-infected mouse cells (33). In this instance the reaction was monitored by incorporation of labeled nucleotides into the viral DNA, but strikingly similar conclusions were reported. Although synthesis of polyoma (32), adenovirus (34), and SV40 (31) DNA in purified nuclei was consistently and markedly lower than in the cell lysates, the process was, nevertheless, an extension of ongoing DNA replication; here, too, initiation has not been detected.

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