Requirement of a Cytoplasmic Fraction for Synthesis of SV40 Deoxyribonucleic Acid in Isolated Nuclei

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About 50% of the SV40 DNA in the process of replication (SV40(RI) DNA) completed replication in lysates of infected BSC-1 cells by conversion to covalently closed, superhelical SV40 DNA (SV40(I) DNA). Fractionation of the lysate into nuclear and cytoplasmic components blocked 99% of the synthesis of SV40(I) DNA in the purified nuclei. The reconstituted system, made by adding back the cytoplasmic fraction before incubation at 30°C, completely restored the in vitro level of SV40(I) DNA synthesis. Preliminary characterization of the activity found in the cytoplasmic fraction suggested it was a soluble, heat-labile protein (or proteins) with a minimum molecular weight of about 30,000 and an active sulfhydryl group. The activity was present in both infected and uninfected monkey cells, and at a lower level in mouse, hamster, and human cell lines. Neither serum starvation nor cycloheximide treatment of cells diminished the activity in the cytoplasmic fraction. Purified cytoplasmic DNA polymerase from KB cells did not substitute for the cytoplasmic fraction which was required for elongation of newly synthesized DNA strands. In the absence of the cytoplasmic fraction, conversion of 4 S DNA into longer strands was inhibited, and SV40(RI) DNA appeared to be broken specifically at the replication forks.

In the accompanying paper (1) we demonstrated that a lysate from SV40-infected monkey cells can convert replicating SV40 DNA molecules (SV40(RI) DNA)† to covalently closed, superhelical DNA (SV40(I) DNA) in vitro. Further fractionation of this system revealed an absolute requirement for the cytoplasmic fraction. Purified nuclei in the presence of a buffered salt solution containing deoxynucleotides and an ATP regenerating system were unable to elongate further the daughter strands and make SV40(I) DNA. The reconstituted system, made by adding back the cytoplasmic fraction, was just as active as the unfractionated lysate. This system now provides a complementation assay for "cytoplasmic factors" required in SV40 DNA replication in vitro.

EXPERIMENTAL PROCEDURES

Virus and Cells

Growth of SV40 virus and established monkey kidney cell lines were described in the accompanying paper (1). Other cell lines used in this work were grown the same way. Established mouse cell lines, 3T3 and 3T6, were from Howard Green (2), and SV3T3, an SV40-transformed 3T3 cell line, was from George Todaro (3). Primary African green monkey kidney cells (AGMK) were purchased from Flow Laboratories. T22, an SV40-transformed monkey cell was obtained from Janet Butel (4). Hamster cell line BHK212 was obtained from Michael Stoker (5) and SV26, an SV40-transformed BHK cell line (6) was supplied by George Stark. David Sedwick provided the KB human cell line (8).

Analysis of DNA Synthesis in Vitro

Techniques for the preparation of lysates of SV40-infected cells, assay of SV40(I) DNA, neutral and alkaline sucrose gradient sedimentation, equilibrium centrifugation in CsCl-ethidium bromide density gradients, preparation of viral DNA standards, and the conditions for DNA synthesis in vitro are all described in the accompanying paper (1).

Preparation of Cytoplasmic Fractions

A lysate of uninfected, unlabeled cells (3 x 10⁶ cells/ml) was freshly prepared for each experiment. A low speed cytoplasmic fraction was the supernatant fraction obtained by sedimenting the nuclei at 1200 x g for 5 min at 2°C. The ability of this fraction to restore synthesis of SV40(I) DNA in purified nuclei was destroyed by freezing, but a high speed cytoplasmic fraction prepared by further sedimentation at 100,000 x g for 1 hour at 2°C and adjustment of the supernatant fraction to 25 mM KCl could be frozen in Dry Ice and ethanol and stored at −20°C for at least 2 weeks with no loss of activity. This fraction contained 4 to 6 μg of protein per ml (Lowry assay (7)).

Standard Conditions for Assaying Cytoplasmic Fractions

Two methods were used to prepare nuclei from SV40-infected cells that required the addition of a cytoplasmic fraction to convert SV40(RI) to SV40(I) DNA.

Triton X-100 Method—All steps were carried out at 2-4°C using an ice bath whenever possible. Cell lysates were prepared from infected...
cells that had their SV40(R1) DNA prelabeled with \[^{3}P\]thymidine for 4.5 min as described previously (1). Three or four strokes with the tight fitting Dounce pestle was sufficient to break 95% of the cells whereas excessive homogenization decreased the activity observed after combining isolated nuclei with a cytoplasmic fraction. The lysate was diluted 20-fold in 20 mM Hepes (pH 7.8), 50 mM KCl, 0.5 mM MgCl\(_2\), 0.5 mM dithiothreitol, 0.2 mM sucrose, and 0.01% Triton X-100 by inverting the tube several times to disperse the nuclei completely. Nuclei were then sedimented at 1200 x g for 5 min, the supernatant was decanted, and the pellet resuspended in the same diluent but without Triton X-100. Efficient resuspension was done by adding only one-fifth of the final volume then dispersing the pellet and vortexing for 3 s before adding the remaining diluent.

The nuclei were distributed among 5 ml of siliconized Corex tubes and again sedimented. The pellet, containing nuclei from about 2 x 10\(^6\) cells, was dispersed thoroughly, then resuspended in 0.05 ml of assay mix plus either 0.1 ml of hypotonic buffer or a cytoplasmic fraction. The hypotonic buffer contained 10 mM Hepes (pH 7.8), 0.5 mM KCl, 0.5 mM MgCl\(_2\), and 0.5 mM dithiothreitol, and standard assay mix is described in the accompanying paper (1). After incubation of the 0.2-ml sample for 1 hour at 30\(^\circ\), the amount of SV40(I) [\(^{3}P\)H]DNA relative to all noncovalently closed viral DNA was measured as described previously (1).

**Low Salt Method**—All procedures were identical with the Triton X-100 method except that the cell lysate was diluted 20-fold in 20 mM Hepes (pH 7.8), 5 mM KCl, 0.5 mM MgCl\(_2\), and 0.2 mM sucrose. The second wash contained 50 mM KCl and 0.5 mM MgCl\(_2\) as described in the Triton X-100 method.

**Enzymes**

*Escherichia coli* DNA polymerase I (8) was a gift from Arthur Kornew. KB cell cytoplasm and cytoplasmic DNA polymerase were gifts from David Sedwick (9). The high speed supernatant from a lysosome, EDTA, Brij-58, enzymes supplied by William Wickner. Three times recrystallized trypsin pretreated with tosyl-L-phenylalanyl chloromethyl ketone to inactivate chymotrypsin was purchased from Worthington.

### RESULTS

#### Requirement of Cytoplasmic Fraction for Conversion of SV40(R1) to SV40(I) DNA in Isolated Nuclei—Lysates of SV40-infected BSC-1 cells were capable of converting SV40(R1) DNA, prelabeled with \[^{3}P\]thymidine for 4.5 min in vivo, to SV40(I) DNA (1). Pelleting the nuclei did not impair their ability to carry out this conversion if the supernatant fraction and nuclei were recombined (Table I). If the pelleted nuclei were resuspended instead in hypotonic buffer (about two-thirds of the total lysate volume), the activity was only slightly diminished. However, if the lysate was first diluted 20-fold in a hypotonic buffer, the nuclei were about 18% as active as a cell lysate while those resuspended in the low speed cytoplasmic fraction contained 64% of the activity found in a cell lysate (Table I). Therefore, under certain conditions viral DNA synthesis in the nuclei was dependent upon addition of a soluble factor (or factors) in the cytoplasmic fraction.

Two methods were developed for purifying nuclei that were dependent upon the cytoplasmic fraction for synthesis of SV40(I) DNA. Both of these methods (described in detail under "Experimental Procedures") produced nuclei with 1 to 6% of the activity observed in a cell lysate (Table I). Addition of a low speed cytoplasmic fraction from an equivalent number of cells resulted in a 5- to 7-fold increase in the actual amount of SV40(I) DNA synthesized; this corresponded to 85 to 100% of the activity observed in an unfractionated cell lysate.

Synthesis of SV40(I) DNA in isolated nuclei was prevented by washing nuclei in 20 mM Hepes (pH 7.8) and 0.5 mM dithiothreitol containing 5 mM KCl or less and no MgCl\(_2\). However, complete restoration of DNA synthesis was observed only when nuclei had been washed in 50 to 100 mM KCl.

#### Table 1

**Requirement for cytoplasmic fraction to convert SV40(R1) to SV40(I)**

| System                        | Per cent SV40(I) DNA at 0 min | Per cent SV40(I) DNA at 60 min |
|-------------------------------|--------------------------------|---------------------------------|
| Intact cells                  | 8                              | 58                              |
| Cell lysate                   | 8                              | 58                              |
| Nuclei + cytoplasm: pellet nuclei, resuspend in same supernatant | 8 | 58 |
| Nuclei: pellet nuclei, resuspend in buffer | 8 | 51 |
| Nuclei: dilute lysate, pellet nuclei, resuspend in buffer | 17 |
| Nuclei + cytoplasm: dilute lysate, pellet nuclei, resuspend in cytoplasm | 8 | 40 |
| Nuclei: Triton X-100 method   | 8                              | 9                               |
| Nuclei + cytoplasm: Triton X-100 method | 8 | 56 |
| Nuclei: low salt method       | 8                              | 11                              |
| Nuclei + cytoplasm: low salt method | 8 | 54 |

Therefore nuclei were first washed in a low ionic strength buffer followed by a high ionic strength buffer containing 0.5 mM MgCl\(_2\), a divalent cation which decreased nuclear aggregation and increased nuclear stability as judged by their ability to synthesize viral DNA after 2 to 3 hours at 0\(^\circ\). Addition of 0.5 mM CaCl\(_2\) to the final wash resulted in about 20% less activity in the reconstituted system, but addition of ethylene glycol bis(a-aminohethyl ether)-N,N′-tetraacetate (EGTA), a preferential Ca\(^{2+}\) chelator (11), made no difference in the degree of inactivation or reactivation of isolated nuclei. The requirement for a low ionic strength wash to remove the cytoplasmic fraction from nuclei could be circumvented by including as little as 0.01% of either Triton X-100 or Brij-58 in the wash solution. Nuclei treated in this manner required a second wash to remove excess detergent which depressed synthesis of SV40(I) DNA by increasing the amount of SV40(R1) that was converted to SV40(II). Including sucrose in the wash solutions resulted in a 10% increase in SV40(I) DNA made in the reconstituted system.

The amount of SV40(I) DNA synthesized in the reconstituted system was always proportional to the concentration of high speed cytoplasmic fraction added (0.6 to 6 mg of protein per ml; i.e. bovine serum albumin equivalents using Lowry method (7)). However, the proportionality constant relating the conversion of SV40(R1) DNA to SV40(I) DNA and the concentration of cytoplasm varied between 0.5 and 0.9 among different cytoplasmic preparations (Fig. 1). This might reflect a difference in the relative concentration of several active components. Whereas the high speed cytoplasmic fraction could never be diluted more than 10- to 15-fold, an unfractionated cell lysate diluted 40-fold with a proportional volume...
of assay mix and hypotonic buffer showed no decrease in SV40(I) synthesis (Fig. 1). Nuclei recovered from a lyse diluted 10-fold in this manner retained 60% of their capacity to make SV40(I) DNA. As indicated above, the soluble factor found in the cytoplasmic fraction is apparently associated with nuclei in the presence of KCl and MgCl₂ concentrations used in our standard assay.

Viral DNA Synthesis in Isolated Nuclei in Absence of Cytoplasmic Fraction—Although this paper only presents the data obtained with Triton X-100-prepared nuclei, the same experiments performed on nuclei prepared using the low salt method gave essentially identical results. SV40(RI) DNA, prelabeled in vivo with [³H]thymidine, was not converted into nuclei in the presence of KCl and MgCl₂ concentrations used in SV40(1) synthesis (Fig. 1). Nuclei recovered from a lysate diluted lo-fold in this manner retained 60% of their capacity to of assay mix and hypotonic buffer showed no decrease in SV40(I) synthesis (Fig. 1). Nuclei recovered from a lyse diluted 10-fold in this manner retained 60% of their capacity to make SV40(I) DNA. As indicated above, the soluble factor found in the cytoplasmic fraction is apparently associated with nuclei in the presence of KCl and MgCl₂ concentrations used in our standard assay.

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Fig. 2 (left). Sedimentation in alkaline sucrose gradients of prelabeled SV40 DNA incubated in vitro. A, nuclei from infected BSC-1 cells were prelabeled in vivo with [\(^{3}H\)]thymidine for 4.5 min (1) then purified by the Triton X-100 method and incubated for 1 hour at 30° in the absence of the cytoplasmic fraction under standard assay conditions. The SDS supernatant was prepared and 0.1 ml layered on the gradient. [\(^{32}P\)]SV40(I) and [\(^{32}P\)]SV40(II) DNA, indicated as I and II respectively, were added to the sample just prior to centrifugation. B is the same as A except that nuclei were incubated in the presence of the added low speed cytoplasmic fraction. Gradients were layered with an 0.1 ml of sample and centrifuged in a Beckman SW 56 rotor, 4°, 2 hours at 55,000 rpm and collected from the bottom. O--O, [\(^{3}H\)]; O-O, *T. Sedimentation is from right to left.

Fig. 3 (center). Sedimentation in neutral sucrose gradients of prelabeled SV40 DNA incubated in vitro. A, purified nuclei incubated in the absence of the cytoplasmic fraction as described in Fig. 2. B, nuclei incubated with the added low speed cytoplasmic fraction. Gradients remained constant throughout the in vitro incubation, no significant endonucleolytic activity was present in the reconstituted system.

Although DNA synthesis in a reconstituted system mimicked that observed in a cell lysate, the reconstituted system often incorporated more in the in vitro label (Fig. 5). This difference generally appeared as 3 to 7 S material on a neutral sucrose gradient (Fig. 8B) although the amount varied among experiments. The sample analyzed in Figs. 6B and 7B had about 5% 7 S DNA; in Fig. 5 about 10%; and in Fig. 8B about 25%. Because of the erratic appearance of this anomalous DNA, the conversion of prelabeled SV40(RI) to SV40(I) [\(^{3}H\)]DNA was always used as the criterion for optimal conditions in vitro.

The amount of cellular DNA in the SDS supernatant was estimated from the amount of [\(^{32}P\)]DNA found when using nuclei from mock infected cells and, with nuclei from infected cells, the fraction of viral DNA was determined by DNA-DNA hybridization (15). In the reconstituted system, about 10 to 15% of the \(^{32}P\) label was in cellular DNA whereas with purified nuclei incubated alone about 30% of the labeled DNA was cellular. The labeled cellular DNA was distributed throughout a neutral sucrose gradient with about 25% concentrated in the 3 to 7 S region.

Fig. 4 (right). Separation of single strand circular, linear, and 4 S SV40 DNA in alkaline sucrose gradients. Infected cells were labeled at 36 hours post-infection for 30 s at 25° with 0.5 ml of \(^{3}H\)thymidine (50 Ci/mmol, 100 \(\mu\)Ci/ml) per 100 mm diameter dish. Nuclei were then purified and incubated in the standard assay mix without the cytoplasmic fraction for 0 min (A), 60 min (B), and with the high speed cytoplasmic fraction for 60 min (C). Viral DNA from four dishes (A and B) or 12 dishes (C) was precipitated in 70% ethanol at -20°, resuspended in 0.1 ml of TEN buffer (1) and layered on a sucrose gradient. SV40(II) [\(^{32}P\)]DNA was added as a standard. Centrifugation was performed in a Beckman SW 41 rotor at 40,000 rpm, 10° for 20 hours. Gradients were collected from the bottom. O--O, [\(^{3}H\]; O-O, *T.

Preliminary Characterization of Active Cytoplasmic Fraction

The cytoplasmic fraction was prepared from several cell lines and tested for the presence of the activity which restores the conversion of SV40(RI) to SV40(I) DNA in nuclei purified from SV40-infected BSC-1 cells (Table II). Since the activities from uninfected and infected BSC-1 cells were equivalent, a viral gene product was not responsible for the observed activity. The cytoplasmic fraction from other monkey cell lines was fully active whereas the same fractions from the human, mouse, and hamster cell lines tested were 75 to 25% as active as that from an equivalent number of BSC-1 cells. One surprising result was the very low activity found in two SV40-transformed cell lines, T22 and SV3T3. However, the significance of this difference must await further studies on the efficiency of the isolation procedure and stability of the various cytoplasmic fractions.

The requirement for the cytoplasmic fraction was not met by gelatin (0.2 to 2 mg/ml), bovine serum albumin (0.2 to 2 mg/ml), cytochrome c (0.2 to 2 mg/ml), or a mixture containing 120 \(\mu\)M each of ATP, UTP, CTP, and GTP. Furthermore, neither the high speed supernatant fraction from an Escherichia coli lysate (10) which supports conversion of a single strand circular M13 parental DNA to a double strand replicative form, nor E. coli DNA polymerase I (8), nor the cytoplasmic DNA polymerase from KB cells (9) exhibited any activity.
However, both a freshly prepared cytoplasmic fraction from KB cells and the cytoplasmic extract from which the KB cell DNA polymerase was purified (9) contained 75% as much cytoplasmic factor activity as did BSC-1 cell cytoplasm. Either the cytoplasmic polymerase is not the active agent or it requires additional factors.

The cytoplasmic fraction from cells that were not actively synthesizing DNA was also active. DNA synthesis in mammalian cells can be arrested by holding a confluent monolayer in low concentrations of serum (16, 17). Confluent monolayers of BSC-1 cells were kept in Dulbecco's Modified Eagle's medium plus 0.1% calf serum for 4 days at which time autoradiographic analysis of a [3H]thymidine pulse showed that only 1% of the cells were active in DNA synthesis. The monolayers were then infected with SV40 for 1 hour at a multiplicity of 40 and further incubated in the same medium. Cell lysates from these virus-infected, serum-starved cells incorporated about 2.5 times less [3H]thymidine into viral DNA during a 4-min pulse than did a lysate from cells grown in the usual manner.

**TABLE II**

Activity of cytoplasmic fractions from several cell lines

A low speed cytoplasmic fraction was prepared from each cell line as described under “Experimental Procedures” and assayed for its ability to support SV40(II) DNA formation when incubated with nuclei (low salt method) from infected BSC-1 cells. Activity was measured as the conversion of prelabeled SV40(II) [3H]DNA to SV40(II) [3H]DNA relative to that observed using BSC-1 cell cytoplasm.

| Cell line         | Cell type | Activity % |
|-------------------|-----------|------------|
| BSC-1, infected   | Monkey    | 100        |
| BSC-1, uninfected | Monkey    | 110        |
| CVI, uninfected   | Monkey    | 100        |
| AGMK, uninfected  | Monkey    | 90         |
| T22, uninfected   | SV40-transformed monkey | 2 |
| KB                | Human     | 75         |
| 3T3               | Mouse     | 65         |
| 3T6               | Mouse     | 45         |
| SV3T3             | SV40-transformed mouse | 5 |
| BHK              | Hamster   | 25         |
| SV28             | SV40-transformed hamster | 28 |

**Fig. 5.** Incorporation of [α-32P]dATP and [α-32P]dCTP (1) into viral DNA in vitro. Infected cells were labeled in vivo for 2 hours with 5 μCi of [H]thymidine per ml, 20 Ci/mmol, before cell lysates and Triton X-100-purified nuclei were prepared and incubated in our standard assay (1) at 30°C. αT incorporation into total acid-insoluble material was measured in SDS supernatants. The 32P incorporation was normalized with respect to the 3H label in order to correct for variations in the amount of DNA per assay. Results are shown for a cell lysate (O--O), purified nuclei (O--O), and nuclei in the presence of the low speed cytoplasmic fraction (O--O).

**Fig. 6 (left).** Sedimentation in alkaline sucrose gradients of SV40 DNA labeled in vitro. Nuclei were purified by the Triton X-100 method from infected cells prelabeled for 1 hour with [3H]thymidine and then incubated in our standard assay containing [α-32P]dATP and [α-32P]dCTP for 1 hour at 30°C either without the cytoplasmic fraction (A) or with the low speed cytoplasmic fraction (B). The 3H label shows the positions of SV40(II) and SV40(II) DNA. Gradients were centrifuged in a Beckman SW 50.1 rotor for 2 hours at 55,000 rpm, 4°C, and collected from the bottom. O--O, 3H; O--O, 32P.

**Fig. 7 (center).** Sedimentation in alkaline sucrose gradients of SV40 DNA labeled in vitro. The methods are the same as described in Fig. 6 except gradients were centrifuged 6.5 hours in order to separate single-stranded circles (ss circles) from single-stranded genome length lines (ss lines); their positions are marked by 3H label. A shows purified nuclei and B is purified nuclei incubated with the low speed cytoplasmic fraction. O--O, 3H; O--O, 32P.

**Fig. 8 (right).** Sedimentation in neutral sucrose gradients of SV40 DNA labeled in vitro. The methods are the same as described in Fig. 5 except neutral gradients were centrifuged for 3.25 hours. 3H label shows the position of SV40(II) and SV40(II) DNA. A shows purified nuclei and B is nuclei incubated with the low speed cytoplasmic fraction. O--O, 3H; O--O, 32P.
However, in vitro conversion of prelabeled SV40(RI) to SV40(I) DNA was the same in lysates from both starved and normal cells. Furthermore, the cytoplasmic fractions from uninfected and infected, serum-starved cells were equally capable of supporting synthesis of SV40(I) DNA in isolated nuclei from normally grown cells.

Treatment of SV40-infected cells with cycloheximide inhibits viral DNA replication (18, 19), but the level of activity in the cytoplasmic fraction did not diminish. Exposure of infected BSC-1 cells to 20 μg/ml of cycloheximide (19) between 33 and 36 hours after infection reduced the rate of viral DNA synthesis in vivo to 15% of its normal value. (Cells were incubated for 1 hour in 50 μCi of [3H]thymidine, 10 ml of complete medium, 27°C, 6% CO₂.) The low speed cytoplasmic fraction, isolated from infected cells that had been treated with cycloheximide, was equivalent to the same fraction from untreated cells in both its ability to support the in vitro conversion of SV40(RI) to SV40(I) DNA and the incorporation of [α-3P]dATP, dCTP into viral DNA. However, the cytoplasmic fraction from untreated cells was not able to stimulate incorporation of labeled deoxynucleotides into viral DNA in isolated nuclei from cycloheximide-treated cells.

A preliminary characterization of the activity in the cytoplasmic fraction suggests that it is a soluble protein having a molecular weight greater than 30,000 and containing a reactive sulfhydryl group (Table III). Centrifugation at 100,000 × g for 1 hour at 4°C did not sediment the activity required for conversion of SV40(RI) to SV40(I) DNA in isolated nuclei. The activity in the high speed cytoplasmic fraction was inactivated by trypsin, heating at 80°C, N-ethylmaleimide, and p-chloromercuribenzoate. Dialysis for 6 hours at 4°C against a 100-fold excess of 10 mM Hepes (pH 7.8), 50 mM KCl, 0.5 mM MC₂₄, and 0.5 mM dithiothreitol did not reduce the activity. The high speed fraction was concentrated 5-fold by filtration with an Amicon UM-2 filter before eluting from a Sephadex G-50 column with hypotonic buffer containing 25 mM KCl. The column was calibrated previously with a mixture of β-galactosidase, pig myoglobin, and Na₂HPO₄ markers. The activity was found in the void volume suggesting that it had a molecular weight of at least 30,000. The high speed cytoplasmic fraction could be frozen in Dry Ice and ethanol and stored at −20°C for at least 2 weeks without significant loss in activity.

**DISCUSSION**

Nuclei purified from SV40-infected monkey cell cultures require a soluble factor (or factors) found in the cytoplasmic fraction in order to convert SV40(RI) to SV40(I) DNA. Although this activity was found in the cytoplasmic fraction, it presumably is associated with the nucleus in the intact cell as suggested by the fact that a cell lysate can be diluted under our standard assay conditions without a decrease in the synthesis of SV40(I) DNA (Fig. 1). However, this factor is not bound tightly to DNA since it was completely removed by washing nuclei in a low ionic strength buffer. The effectiveness of nonionic detergents in purifying nuclei further suggests that increasing the permeability of the outer nuclear membrane may be important in releasing all the residual factor (21).

Although purified nuclei incubated in the absence of the cytoplasmic fraction did not synthesize SV40(I) DNA, some viral DNA synthesis did occur. This synthesis, which was completed within 10 min, accounted for only one-third as much labeled DNA in the SDS supernatant as found in the reconstituted system. Correction for the relative amounts of labeled cellular DNA present disclosed a 6-fold decrease in viral DNA synthesis. Of the in vitro labeled DNA found in the SDS supernatant, 20% was in SV40(RI) and 80% was in DNA equivalent in size to the 4 S DNA shown to be precursors in SV40 DNA replication (Fig. 4 and Ref. 12). However, 80% of the 4 S DNA prelabeled in vivo was joined to nascent daughter strands during incubation in vitro although their size did not increase to the length of SV40 DNA. Therefore, in the absence of the cytoplasmic fraction, nuclei permit the joining of relatively complete 4 S DNA to daughter strands, but further elongation is inhibited and newly made 4 S DNA labeled in vitro accumulates. The cytoplasmic fraction may be involved in the synthesis of DNA in the gaps created by discontinuous synthesis, or the excision of RNA primers and subsequent joining of DNA pieces. The rapid initial rate of DNA synthesis (Fig. 5) suggests that synthesis of 4 S pieces previously initiated in vivo is unaffected by removal of the cytoplasmic fraction.

The cytoplasmic fraction may also be involved in preventing the breakdown of replicating molecules. SV40(RI) DNA, prelabeled in vivo, changed its sedimentation behavior in neutral sucrose gradients from a broad peak at 26 S to three peaks at 22 S, 20 S, and 17 S (Fig. 3A). Only the 22 S peak contained DNA synthesized in vitro and was apparently SV40(RI) at an

| Conditions                  | Activity % |
|-----------------------------|------------|
| Low speed cytoplasm         | 100        |
| High speed cytoplasm (HSC)  | 100        |
| Trypsin treatment           |            |
| Cell lysate + trypsin        | 4          |
| Cell lysate + trypsin inhibitor and trypsin | 94 |
| HSC + trypsin then inhibitor | 8          |
| Heat treatment              |            |
| HSC (35 min at 24°C)        | 100        |
| HSC (5 min at 80°C)         | 0          |
| NEM treatment               |            |
| Cell lysate + NEM           | 0          |
| Cell lysate + NEM and DTT   | 94         |
| HSC + NEM then DTT          | 10         |
| PCMB treatment              |            |
| Cell lysate + PCMB          | 0          |
| Cell lysate + PCMB and BME  | 65         |
| HSC + PCMB then BME         | 15         |
| HSC + BME                   | 85         |

**Table III**

Characteristics of activity in cytoplasmic fraction

The cytoplasmicfrac ions' activity was measured by conversion of in vivo labeled SV40(RI) [3H]DNA to SV40(I) [3H]DNA in vitro. Trypsin treatment: 300 μg/ml of trypsin were included in a cell lysate with and without 600 μg/ml of soybean trypsin inhibitor. The high speed cytoplasmic fraction (HSC) was treated with trypsin for 30 min at 24°C, then with trypsin inhibitor for 5 min before incubation with nuclei.

Heat treatment: HSC was incubated at either 24°C for 35 min or 80°C for 5 min. N-ethylmaleimide (NEM) treatment: a cell lysate was incubated with 10 mM NEM with and without 12 mM dithiotohreitol (DTT). HSC was incubated with NEM for 5 min at 24°C then DTT was added for 5 min before assaying for cytoplasmic factor activity. p-Chloromercuribenzoate (PCMB) treatment was the same as NEM treatment except 5 mM PCMB and 25 mM β-mercaptoethanol (BME) were used. Since PCMB reacts reversibly with sulfhydryl groups, it was not completely inactivated by excess BME. BME alone is sufficient to convert some SV40(I) to SV40(II) DNA (20).
advanced stage in replication. One hypothesis is that SV40(RI) DNA in the absence of the cytoplasmic fraction was subjected to single strand endonucleolytic degradation at its replication forks. Assuming one single-stranded template region at each fork and on opposite sides (22), the postulated nucleocapsid would produce relaxed circular molecules with a tail (the 20 S material), linear molecules greater than one genome in length (the 17 S material), and release short pieces of DNA only where the newly made 4 S pieces had not yet been joined to the growing daughter strand. Treatment of polyoma RI with *Neurospora crassa* single strand-specific endonuclease partially confirms these predictions (24). Note that the prelabeled daughter strands in SV40(RI) DNA were not degraded (Fig. 3A). The cytoplasmic fraction may contain an inhibitor of a single strand-specific endonuclease which prevents degradation of the single strand regions exposed during discontinuous DNA replication.

An alternate hypothesis is that the template strands of replicating molecules are nicked, causing them to sediment more slowly in neutral sucrose gradients and release newly made DNA at the replication forks by a strand displacement mechanism before it can be joined to the daughter strands. Such a phenomenon may result from the general inhibition of DNA synthesis is suggested by *in vivo* studies with hydroxyurea (25, 26) and 5-fluorodeoxyuridine (27).

Addition of the cytoplasmic fraction to purified nuclei completely restores the nuclei’s ability to complete replication of SV40(RI). The reconstituted system behaves essentially the same as an unfraccionated cell lysate; a lysate converts about 55% of the SV40(RI) to SV40(1) at about one-third the rate found *in vivo* (1). The active factors in the cytoplasmic fraction have not been identified although they are found in uninfected cells and one of them is a heat-labile protein with an active sulfhydryl group. Cytoplasmic DNA polymerase could not replace this activity.

The function and properties of the cytoplasmic fraction described in this paper are similar to those of a cytoplasmic fraction in *in vitro* systems. Viral DNA replication in isolated nuclei provides a way to overcome this problem since SV40 and polyoma DNA molecules have been well characterized and portions of their normal *in vivo* replication process (14, 22, 38) have been duplicated in isolated nuclei (39, 40) and cell lysates (1, 41). Results with Triton X-100 isolated nuclei from SV40 infected African Green monkey kidney cells (40) are consistent with our results obtained in the absence of the cytoplasmic fraction. These *in vitro* systems should permit a functional analysis of the various factors involved.

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