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In vitro replication of simian virus 40 DNA in a nucleoprotein complex

(subnuclear simian virus 40 DNA replication complexes/alkaline and neutral sucrose gradients/cesium chloride–ethidium bromide density gradients/gel electrophoresis)

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ABSTRACT **A simian virus 40 (SV40) nucleoprotein complex, extracted from nuclei isolated from a monkey cell line infected with SV40, continued DNA replication in the presence of a nuclear extract, cytosol, ATP, an ATP-regenerating system, and the four deoxyribonucleoside triphosphates. The DNA products of replication were also found as nucleoprotein complexes. Forty percent of the replicating viral DNA, labeled *in vivo*, was converted into covalently closed, superhelical DNA during incubation *in vitro*. Although the remaining labeled DNA was not converted into mature viral DNA, it was elongated to its full genome length. Failure to terminate replication successfully was not caused by endonuclease activity, since covalently closed DNA, labeled *in vivo*, was not damaged during incubation *in vitro*. When [α-^{32}P]dATP was present during the incubation, the label appeared first in replicating DNA and later in mature DNA; no unusual products were labeled *in vitro*. The covalently closed SV40 DNA made *in vitro* had the same superhelical density as viral DNA made *in vivo*. These data demonstrate that viral nucleoprotein complexes ("minichromosomes") are able to continue DNA replication outside of the nucleus.**

Nuclei, isolated from normal and virus-infected cells, have been used to explore the molecular mechanisms involved in DNA replication (1–3). Nuclei isolated from virus-infected cells can continue (4–7), and sometimes complete (8–10), viral DNA replication that was initiated *in vivo*. This has provided a means to manipulate environmental conditions, to fractionate and characterize cellular factors required for DNA replication, and to study the process of discontinuous DNA synthesis. However, despite their simplicity relative to intact cells, isolated nuclei systems are limited by their insolubility and restricted permeability when compared to systems that use purified bacteriophage DNA and isolated proteins (11–14). Recent attempts have been made to overcome this problem using subnuclear fragments from sensitized lymphocytes (15) and adenovirus-infected cells (16). However, characterization of the DNA products in these studies was insufficient to assess their relationship to *in vivo* replication.

We have now extracted simian virus 40 (SV40) nucleoprotein complexes from nuclei isolated from infected cells and have shown that the replicating viral DNA in these complexes can complete replication *in vitro*. The conditions were the same as those used for the continued replication of SV40 DNA in isolated nuclei (19), except that nuclei were replaced with a nuclear extract. This soluble system provides a new avenue for investigating replication of mammalian chromosomes.

Abbreviations: SV40, simian virus 40; SV40(I) DNA, SV40 covalently closed, superhelical DNA; SV40(II) DNA, SV40 double-stranded circular DNA containing an interruption of the phosphodiester bonds in one of the two strands; SV40(RI) DNA, SV40 DNA replicating intermediate containing a superhelical region of unreplicated DNA and two nonsuperhelical regions of newly replicated DNA (17 18); NaDodSO$_4$, sodium dodecyl sulfate; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N'*-tetraacetate; EDTA, (ethylenedinitrilo)tetraacetate.

MATERIALS AND METHODS

Virus and Cells. The small plaque SV40 strain Rh911 (20) was prepared by infection of MA-134 cells at multiplicities of 0.01 or less as previously described (10). The experiments described used a CV-1 monkey cell line obtained from P. Tegtmeyer. Cells were cultured as previously described (10).

Preparation of Viral DNA Standards. ^{32}P- and ^{3}H-Labeled SV40(I) and SV40(II) DNA were purified from SV40-infected cells as previously described (10).

Preparation of Cytosol. Cytosol was prepared from uninfected CV-1 cells as previously described (19), brought to 80% saturation with ammonium sulfate, and the precipitate was resuspended and dialyzed overnight against 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes), pH 7.8, 50 mM KCl, 0.5 mM $MgCl_2$, 0.5 mM dithiothreitol, and 0.4 M ethylene glycol. The cytosol fraction contained 10–15 mg of protein per ml and was stable for at least 2 months at −70°.

Sedimentation Analysis of *In Vitro* DNA Replication Products. Viral DNA in the sodium dodecyl sulfate (NaDodSO$_4$) supernatant* was analyzed directly by neutral and alkaline sucrose gradient sedimentation and CsCl–ethidium bromide density equilibrium sedimentation as previously described (10). Nucleoprotein complexes were analyzed by sedimentation on linear 5–30% sucrose gradients in 10 mM Hepes, pH 7.8, 5 mM KCl, and 0.5 mM $MgCl_2$. Details are given in the figure legends.

Reagents. [α-^{32}P]dATP (50 Ci/mmol) was prepared by the method of Symons (22). All other nucleotides were purchased from P-L Biochemicals or New England Nuclear.

RESULTS

Preparation and characterization of SV40 nucleoprotein complexes

Two forms of SV40 nucleoprotein complexes, extracted from nuclei isolated from infected cells, were separated by sedimentation in a neutral sucrose gradient; SV40(RI) nucleoprotein contained only SV40(RI) DNA, whereas SV40(I) nucleoprotein contained predominately SV40(I) DNA. To prepare SV40(RI) nucleoprotein, SV40(RI) DNA was labeled *in vivo* by incubating infected cells for 3 min with 100 μCi/ml of [^{3}H]thymidine (20 Ci/mmol) at 36 hr after infection when the maximum

* *NaDodSO$_4$ supernatant* refers to DNA isolated by the method of Hirt (21), which precipitates cellular DNA in 0.6% NaDodSO$_4$ and 1.0 M NaCl, leaving viral DNA in the supernatant after centrifugation.

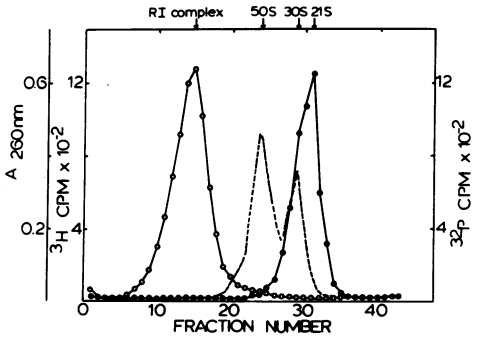

FIG. 1. Sedimentation analysis of SV40(RI) nucleoprotein complexes. Infected cells were labeled with [^{3}H]thymidine for 3 min at 37° and a nuclear extract was prepared containing SV40(RI) [^{3}H]DNA. The extract was layered on a linear 5–30% sucrose gradient in 10 mM Hepes, pH 7.8, 5 mM KCl, 0.5 mM $MgCl_2$, and centrifuged at 50,000 rpm in a Beckman SW50.1 rotor for 60 min at 4°. SV40(I) [^{32}P]DNA (21S) and *Escherichia coli* ribosomal subunits (50S and 30S) were included as markers. Fractions were collected from the bottom of the gradient. o——o, ^{3}H; ●——●, ^{32}P; - - - -, $A_{260\,nm}$.

rate of viral DNA synthesis occurs (10). Cells were then washed at 4° in a hypotonic medium (10), lysed in a Dounce homogenizer (10), and nuclei were recovered by sedimentation at 3000 × *g* for 5 min. Viral nucleoprotein complexes were extracted by resuspending the nuclei in 10 mM Hepes, pH 7.8, 5 mM KCl, 0.5 mM $MgCl_2$, and 0.5 mM dithiothreitol, and incubating for 1 hr at 4°. Nuclei were then removed by centrifugation at 8000 × *g* for 5 min. The supernatant, containing viral nucleoprotein complexes from 5 × 10^6 cells in 0.05 ml, was free of nuclei when viewed in a phase contrast microscope.

The extract contained approximately 50% of the total viral DNA that was originally present in the infected cells. At least 95% of the ^{3}H-labeled DNA found in the extract sedimented as a symmetrical 90S peak in a neutral sucrose gradient (Fig. 1). This material is referred to as SV40(RI) nucleoprotein because addition of either Pronase or $NaDodSO_4$ to the nuclear extract changed its sedimentation behavior to that of purified SV40(RI) DNA. When SV40 nucleoprotein complexes were isolated from nuclei containing SV40(I) [^{3}H]DNA, labeled *in vivo* from 28 to 36 hr after infection, 95% of the acid-precipitable label in the nuclear extract sedimented as a symmetrical 70S peak (see Fig. 3). DNA purified from the 70S material was 85% SV40(I) DNA and 15% SV40(II) DNA.

To determine whether the nuclear extract contained only viral DNA or was contaminated with cellular DNA, the total DNA was purified and then digested with *Hin*dIII restriction endonuclease. Following electrophoresis of the digest in 2.5% polyacrylamide gels and staining with ethidium bromide, all of the DNA was found in the six fragments expected from SV40 DNA (23).

Conditions for *in vitro* DNA synthesis

Conditions used for measuring DNA synthesis in nuclear extracts plus cytosol (Table 1) had previously been optimized with nuclei isolated from SV40-infected CV-1 cells reconstituted with cytosol from uninfected cells. Data obtained from these two systems were quite similar. None of the components in Table 1 appear to be stringently required because of contributions from endogenous pools. Cytosol stimulates the final level of incorporation in nuclear extracts 3-fold or more with little effect on the initial rate of synthesis (Fig. 2). After 1 hr in the presence of cytosol, about 12% of the total DNA had replicated, which is about the fraction of replicating molecules observed in polyoma-infected cells (8).

Table 1. Conditions for SV40 DNA synthesis *in vitro*

Reaction mixture	Viral DNA synthesis* (%)
Complete	100
– Cytosol	28
– ATP	47
– dCTP, dTTP, dGTP	30
– CTP, UTP, GTP	98
– Phospho*enol*pyruvate, pyruvate kinase	38
– Pyruvate kinase	90
– $MgCl_2$	29
– KCl	74
– EGTA	89

All DNA replication studies were carried in a 100 μl volume composed of 20 μl of nuclear extract containing SV40 nucleoprotein complexes, 70 μl of cytosol from uninfected monkey cells, and final concentrations of 46 mM Hepes, pH 7.8, 65 mM KCl, 4.5 mM $MgCl_2$, 1.0 mM ethylene glycol bis(β-aminoethyl ether)-*N,N'*-tetraacetate (EGTA), 0.45 mM dithiothreitol, 0.28 M ethylene glycol, 2 mM ATP, 5 mM phospho*enol*pyruvate, 30 μg/ml of pyruvate kinase, and 100 μM each dATP, dTTP, dGTP, dCTP, UTP, CTP, and GTP. The mixture was incubated at 30° for up to 60 min and the reaction was terminated by preparing a $NaDodSO_4$ supernatant fraction (10). Experiments using [α-^{32}P]dATP (50 Ci/mmol) contained 20 μM dATP.

* Activity is expressed as the percentage of [α-^{32}P]dATP incorporated into viral DNA in 60 min. The complete system incorporated 108 pmol of [α-^{32}P]dATP per μg of DNA in a $NaDodSO_4$ supernatant.

Replication of SV40(RI) in nucleoprotein complexes *in vitro*

Newly synthesized DNA was first associated with SV40(RI) nucleoprotein and later with SV40(I) nucleoprotein. After a 10 min incubation of the nuclear extract together with cytosol and the assay mixture containing [α-^{32}P]dATP, 95% of the incorporated label was found in SV40(RI) nucleoprotein (Fig. 3A). Continuation of the incubation for an additional 50 min resulted in the accumulation of 40% of the ^{32}P label in SV40(I) nucleoprotein (Fig. 3B), while the quantity of endogenous SV40(I) ^{3}H-labeled nucleoprotein previously labeled *in vivo* remained constant throughout the incubation. This indicates the absence of interference from endonuclease or protease activities. Furthermore, when the nuclear extract was derived from cells containing SV40(RI) DNA, previously labeled [^{3}H]thymidine *in vivo*, 45% of the ^{3}H-labeled SV40(RI) nucleoprotein was converted *in vitro* into SV40(I) nucleoprotein (data not shown). Therefore preexisting SV40 replicating nucleoprotein complexes continued some aspects of replication *in vitro*.

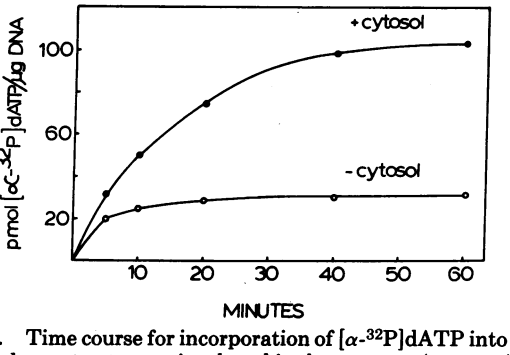

FIG. 2. Time course for incorporation of [α-^{32}P]dATP into viral DNA. Nuclear extracts were incubated in the presence (●——●) and absence (o——o) of cytosol. Conditions are described in the legend to Table 1.

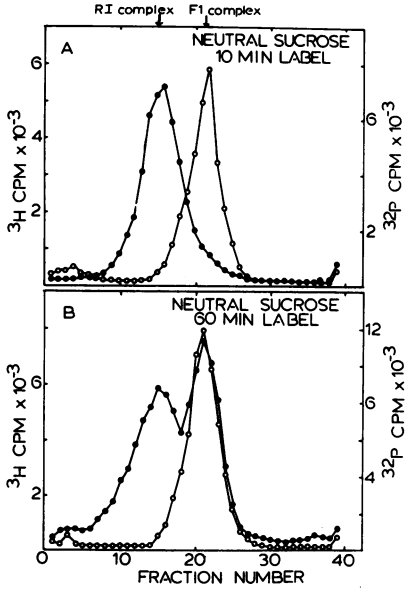

FIG. 3. Sedimentation analysis of SV40 nucleoprotein complexes after *in vitro* DNA synthesis. SV40(I) DNA was labeled *in vivo* with [^{3}H]thymidine (2 μCi/ml) for 8 hr and a nuclear extract was prepared that was then incubated with 2 μCi of [α-^{32}P]dATP as described in the legend to Table 1. The reaction was terminated by chilling to 0° after a 10 or 60 min incubation and layered directly onto linear 5–30% sucrose gradients in the buffer described in Fig. 1. The gradients were centrifuged at 50,000 rpm in a Beckman SW50.1 rotor for 35 min at 4°. Fractions were collected from the bottom of the tube. O——O, ^{3}H-labeled SV40(I) nucleoprotein complex; ●——●, nucleoprotein complex ^{32}P-labeled *in vitro*. (A) 10 min incubation; (B) 60 min incubation.

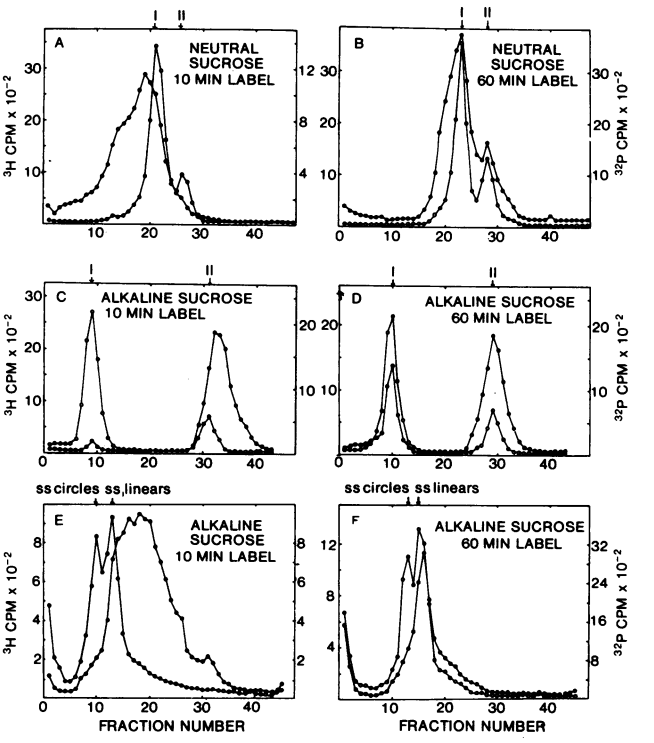

FIG. 4. Sedimentation analysis of SV40 DNA labeled during *in vitro* synthesis. A nuclear extract was prepared from infected cells incubated *in vivo* with [^{3}H]thymidine for 8 hr to label SV40(I) DNA. Viral DNA was extracted from the *in vitro* reaction mixture after a 10 or 60 min incubation in the presence of [α-^{32}P]dATP and analyzed on sucrose gradients. (A) and (B) Linear 5–20% neutral sucrose gradients, in 1 M NaCl, 10 mM Tris·HCl at pH 7.5 and 2 mM (ethylenedinitrilo)tetraacetate (EDTA), centrifuged at 50,000 rpm in an SW50.1 rotor for 3 hr at 4° to separate SV40(I) DNA (21S) and SV40(II) DNA (16S). (C) and (D) Linear 5–20% alkaline sucrose gradients in 0.7 M NaCl, 0.3 M NaOH, 2.5 mM EDTA, and 0.015% Sarkosyl, centrifuged at 50,000 rpm in an SW50.1 rotor for 2 hr at 4° to separate SV40(I) DNA (53S) from SV40(II) DNA (16–18S). (E) and (F) Linear 5–20% alkaline sucrose gradients centrifuged 6 hr to separate single-stranded (ss) circular (18S) and single-stranded linear (16S) DNA molecules. O——O, ^{3}H; ●——●, ^{32}P.

Characterization of viral DNA synthesized *in vitro*

To determine if *in vitro* DNA synthesis mimicked *in vivo* DNA synthesis, a nuclear extract was isolated from infected cells containing SV40(I) [^{3}H]DNA and incubated with cytosol and an assay mixture containing [α-^{32}P]dATP. A NaDodSO$_4$ supernatant fraction was prepared and the DNA was analyzed by neutral and alkaline sucrose gradient sedimentation. After a 10 min incubation, approximately 90% of the incorporated ^{32}P label was characteristic of SV40(RI) DNA (17, 18). This DNA sedimented between 22 S and 28 S in a neutral sucrose gradient (Fig. 4A) and, when denatured on an alkaline sucrose gradient (Fig. 4E), contained labeled daughter strands ranging from 4 S ("Okazaki pieces") to 16 S (genome length) in size. Only 4% of the ^{32}P label sedimented in an alkaline gradient as mature SV40(I) DNA (Fig. 4C).

In contrast, when the reaction mixture was incubated for 60 min, 32% of the incorporated ^{32}P label was found in SV40(I) DNA (Fig. 4D) and 28% appeared to be SV40(II) DNA (Fig. 4B). The remaining labeled DNA appeared to be SV40(RI) DNA that had almost completed replication. It sedimented in a neutral sucrose gradient slightly faster than SV40(I) DNA (Fig. 4B) and, when denatured on an alkaline sucrose gradient, contained nascent DNA predominantly one genome in length (Fig. 4F). Since there was no change in the ratio of SV40(I) to SV40(II) DNA previously labeled *in vivo*, contaminating endonuclease activity was absent. Therefore, replicating viral DNA is labeled first *in vitro* and is then converted into SV40(I) and SV40(II) DNA products.

Conversion of SV40(RI) DNA to SV40(I) DNA *in vitro*

To determine if the DNA synthesis observed *in vitro* represented a continuation of viral DNA replication that had been initiated *in vivo*, nuclear extracts were prepared from infected cells containing SV40(RI) [^{3}H]DNA and incubated with cytosol and unlabeled substrates. At the start of incubation, 90% of the ^{3}H-labeled viral DNA sedimented in a neutral sucrose gradient as a broad peak around 26 S (Fig. 5A) and contained labeled daughter strands sedimenting from 4 S to 16 S in an alkaline sucrose gradient (Fig. 5E). This is characteristic of SV40(RI) DNA labeled in this manner (10, 17, 18). Whereas at the beginning of the incubation only 4% of the ^{3}H-labeled DNA was SV40(I) DNA (Fig. 5C), after 60 min about 41% was SV40(I) DNA (Fig. 5D). Another 25% of the labeled viral DNA appeared to be SV40(II) DNA (Fig. 5B), whereas the remaining 34% was SV40(RI) DNA that had nearly completed replication; viral [^{3}H]DNA accumulated in the position of relaxed circular dimers (about 22 S) in a neutral gradient (Fig. 5B) and in the position of mature viral daughter strands in an alkaline gradient (Fig. 5F). These results are consistent with a continuation of normal viral DNA replication *in vitro*.

Superhelicity of SV40(I) DNA synthesized *in vitro*

Covalently closed DNA synthesized *in vitro* from SV40(RI) [^{3}H]DNA that had been previously labeled *in vivo* was indistinguishable from SV40(I) DNA labeled *in vivo*. This conclusion is based on density equilibrium sedimentation of DNA in a

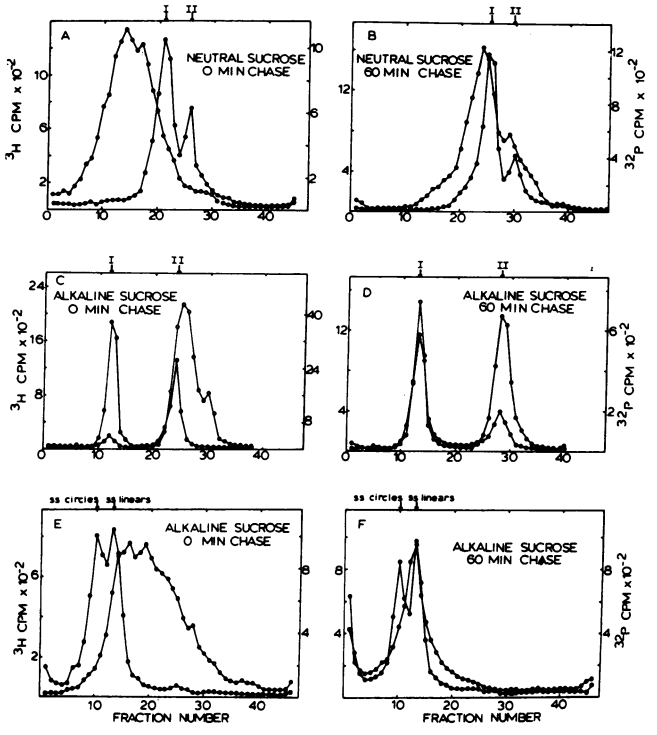

FIG. 5. Sedimentation analysis of SV40 DNA previously labeled *in vivo* and incubated with nuclear extracts. A nuclear extract was prepared from infected cells containing SV40(RI) [^{3}H]DNA and incubated *in vitro* in the absence of labeled substrate, as described in the legend to Table 1. DNA was extracted before and after a 60 min incubation and analyzed on sucrose gradients. SV40(I) [^{32}P]DNA and SV40(II) [^{32}P]DNA were added to the samples just prior to centrifugation. (A) and (B) neutral sucrose gradients; (C) and (D) alkaline sucrose gradients centrifuged for 2 hr; (E) and (F) alkaline sucrose gradients centrifuged for 6 hr. See Fig. 4 for details. O——O, ^{3}H; ●——●, ^{32}P.

CsCl–ethidium bromide gradient (Fig. 6), and electrophoresis in an agarose gel (Fig. 7). Identical results were obtained with DNA labeled *in vitro* with [α-^{32}P]dATP. Therefore SV40(I) DNA synthesized *in vitro* has the same number of superhelical turns as its *in vivo* counterpart.

DISCUSSION

The *in vitro* replication of SV40(RI) DNA in nucleoprotein complexes was strikingly similar to the continued replication of SV40 DNA in isolated nuclei (10, 19). Both systems required cytosol to convert 30–50% of the labeled DNA into mature covalently closed, superhelical DNA (ref. 19; R. T. Su and M. L. DePamphilis, unpublished results) and to stimulate total DNA synthesis approximately 3- to 4-fold (19). Although DNA synthesis was stimulated by ATP, an ATP-regenerating system, and the four deoxyribonucleoside triphosphates, addition of the other three ribonucleoside triphosphates had little or no effect. Both systems followed a similar time course, elongating the nascent DNA associated with replicating molecules to its full genome length. However, in contrast to nuclear extracts, nucleoprotein complexes isolated from neutral sucrose gradients synthesized DNA poorly, even in the presence of cytosol (data not shown). A soluble nucleoplasmic fraction is apparently required in place of nuclei. The completed elongation of daughter strands previously labeled *in vivo*, the synthesis of SV40(I) DNA, and the absence of any detectable interference of endonuclease strongly suggest that *in vitro* DNA synthesis was not the result of a repair type of reaction. Instead, the data from

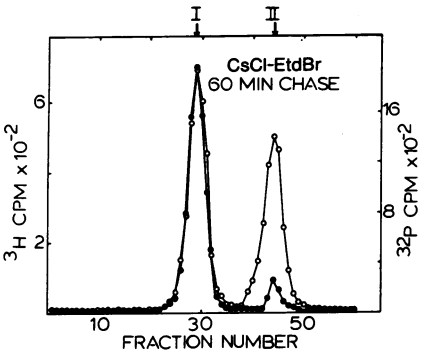

FIG. 6. CsCl–ethidium bromide density equilibrium gradient centrifugation of SV40(I) DNA synthesized *in vitro* from SV40(RI) [^{3}H]DNA labeled *in vivo*. Fractions numbered 12–35 in Fig. 5B were pooled and dialyzed against 10 mM Tris, pH 7.5, and 1 mM EDTA for 8 hr at 4°. Solid CsCl was added to a final density of 1.56 g/cm^3, the solution was adjusted to 200 μg/ml of ethidium bromide, and a gradient was formed in a Beckman 50Ti rotor at 38,000 rpm for 48 hr at 18°. Fractions were collected from the bottom of the tube. O——O, SV40 [^{3}H]DNA from the *in vitro* incubation; ●——●, SV40(I) [^{32}P]DNA and SV40(II) [^{32}P]DNA added as markers.

both systems are most readily interpreted as a normal continuation of DNA replication that was already initiated in the intact cell. However, a slow rate of initiation *in vitro* would be difficult to detect against a high background of ongoing DNA synthesis.

The method for isolating viral nucleoprotein complexes described here represents a significant departure from the methods commonly employed (25–27), which involve higher salt concentrations, EDTA in place of $MgCl_2$, and a nonionic detergent. Viral nucleoprotein complexes isolated by this latter procedure have substantially lower sedimentation constants, are not well resolved into replicating and mature viral nucleoproteins, and appear to be defective in replicating DNA (R. T. Su and M. L. DePamphilis, unpublished results). However, they have been shown to contain a subunit structure similar to mammalian chromatin (refs. 28 and 29; E. Shelton, P. Wassarman, and M. L. DePamphilis, unpublished results) and contain all the histones with the possible exception of H1 (30).

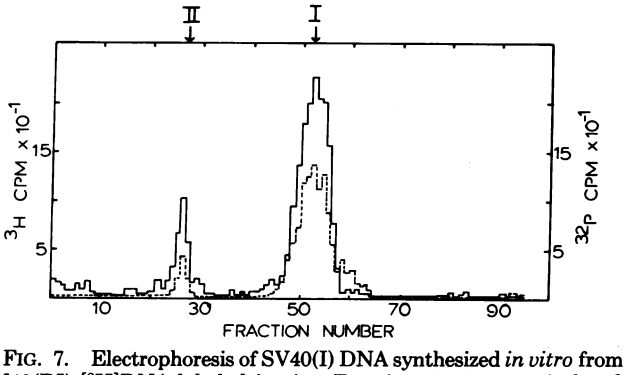

FIG. 7. Electrophoresis of SV40(I) DNA synthesized *in vitro* from SV40(RI) [^{3}H]DNA labeled *in vivo*. Fractions 25–34 were isolated from a CsCl–ethidium bromide gradient as described in Fig. 6. Ethidium bromide was removed with Dowex-50 and the sample was dialyzed and concentrated as previously described (10). Electrophoresis of SV40 DNA was carried out in 10 cm long 1.4% agarose gels (24) for 10 hr at 5 mA per gel at room temperature. Gels were stained with 0.5 μg/ml of ethidium bromide, sliced into 1 mm fractions, dissolved in 3% Nuclear Chicago Solubilizer in a toluene-based scintillation fluid, incubated at 55° overnight, and the radioactivity was measured in a scintillation counter. Solid line [^{32}P]DNA, broken line is [^{3}H]DNA isolated from the *in vitro* incubation. Electrophoresis was from left to right.

In this respect, the replicating SV40 nucleoprotein complex can be considered a unique form of chromatin that can complete replication outside the nucleus, an observation consistent with autoradiographic data suggesting that DNA synthesis is not associated with the nuclear membrane (1).

We expect that this SV40 nucleoprotein replicating complex will allow the complete fractionation and subsequent reconstitution of factors required for mammalian DNA replication and provide further insight to the relationship between DNA replication and chromatin structure.

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