Simian Virus 40 DNA Replication in Isolated Replicating Viral Chromosomes

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Three subnuclear systems capable of continuing many aspects of simian virus 40 (SV40) DNA replication were characterized in an effort to define the minimum requirements for "normal" DNA replication in vitro. Nuclear extracts, prepared by incubating nuclei isolated from SV40-infected CV-1 cells in a hypotonic buffer to release both SV40 replicating and mature chromosomes, were either centrifuged to separate the total SV40 nucleoprotein complexes from the soluble nucleosol or fractionated on sucrose gradients to provide purified SV40 replicating chromosomes. With nuclear extracts, CV-1 cell cytosol stimulated total DNA synthesis, elongation of nascent DNA chains, maturation and joining of "Okazaki pieces," and the conversion of replicating viral DNA into covalently closed, superhelical DNA. Nucleoprotein complexes responded similarly, but frequently the response was reduced by 10 to 30%. In contrast, isolated replicating chromosomes in the presence of cytosol appeared only to complete and join Okazaki pieces already present on the template; without cytosol, Okazaki pieces incor-porated α-32P-labeled deoxynucleoside triphosphates but failed to join. Consequently, replicating chromosomes failed to extensively continue nascent DNA chain growth, and the conversion of viral replicating DNA into mature DNA was seven to eight times less than that observed in nuclear extracts. Addition of neither cytosol nor nucleosol corrected this problem. In the presence of cytosol, nonspecific endonuclease activity was not a problem in any of the three in vitro systems. Extensive purification of replicating chromosomes was limited by three as yet irreversible phenomena. First, replicating chromosomes isolated in a low-ionic-strength medium had a limited capability to continue DNA synthesis. Second, diluting either nuclear extracts or replicating chromosomes before incubation in vitro stimulated total DNA synthesis but was accompanied by the simultaneous appearance of small-molecular-weight nascent DNA not associated with intact viral DNA templates and a decrease in the synthesis of covalently closed viral DNA. Although this second phenomenon appeared similar to the first, template concentration alone could not account for the failure of purified replicating chromosomes to yield covalently closed DNA. Finally, preparation of nucleoprotein complexes in increasing concentrations of NaCl progressively decreased their ability to continue DNA replication. Exposure to 0.3 M NaCl removed one or more factors required for DNA synthesis which could be replaced by addition of cytosol. However, higher NaCl concentrations yielded nucleoprotein complexes that had relatively no endogenous DNA synthesis activity and that no longer responded to cytosol. These data demonstrate that continuation of endogenous DNA replication in vitro requires both the soluble cytosol fraction and a complex nucleoprotein template whose ability to continue DNA synthesis depends on its concentration and ionic environment during its preparation.

Subcellular systems that continue DNA replication initiated in vivo have provided several advantages over intact cells in the study of chromosome replication. Their lack of permeability barriers has permitted characterization of the requirements for DNA replication (12), elucidation of the role of RNA polymerase α (11, 22), and the identification and analysis of RNA primers (1, 2, 12, 19, 29). The fact that in vitro DNA replication systems are less efficient or defective relative to intact cells has allowed the accumulation of transient intermediates such as "Okazaki pieces" (10, 16, 20, 23, 28), specific

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classes of replicating DNA molecules, and termination intermediates (27). These individual components of chromosome replication, as well as enzymes, structural proteins, and other associated factors, can then be isolated and characterized, and their function can be deduced, through reconstitution experiments. This approach has been encouraged by the development of subnuclear systems that can complete replication of simian virus 40 (SV40) (13, 14, 26) or adenovirus (5, 17, 18, 31) DNA and by the discovery that chromatin assembly accompanies DNA replication in vitro (25).

SV40 as well as polyoma virus provide well-defined genomes that are particularly suited to studying the biochemical mechanisms involved in the replication of mammalian chromosomes. The nucleosome structure and histone composition (3, 7, 8, 15, 24, 30; E. R. Shelton, P. M. Wassarman, and M. L. DePamphilis, unpublished data) of viral chromatin replicating inside mammalian nuclei are strikingly similar to those of host chromatin. Because the single viral gene required for viral DNA and chromatin replication, gene A, is needed only to begin replication, the continuation of these processes must be carried out by cellular components (15). Beginning at a unique origin, viral DNA replication occurs bidirectionally through the synthesis of short pieces of nascent DNA with a mean length of 100 to 120 nucleotides which are then joined to the growing daughter strands (15). Covalently attached to the 5’ ends of these Okazaki pieces are uniquely sized RNA primers about 10 nucleotides long (1, 19, 29), which are excised during the DNA chain elongation process (2). Similar events occur during mammalian DNA replication (12, 28, 29). Replicating SV40 chromosomes, therefore, appear to be analogous to individual eucaryotic replicons.

By purification of SV40 chromosomes, we have tried to define the minimum conditions necessary for continuation of SV40 DNA replication. Five subcellular systems have been prepared from SV40-infected monkey cell cultures that continue replication of endogenous viral genomes. Cell lysates (9) and washed nuclei (10) contain insoluble forms of SV40 chromosomes, whereas nuclear extracts (26), nucleoprotein complexes, and replicating chromosomes represent relatively soluble systems (see Fig. 1). In the presence of a soluble cytosol fraction, all of these systems, except for isolated replicating chromosomes, faithfully convert SV40 replicating DNA molecules into mature forms of viral DNA. This paper describes the development of these soluble systems, their characteristics in the presence and absence of cytosol, and parameters that critically affect their ability to mimic DNA replication in intact cells.

**MATERIALS AND METHODS**

**Abbreviations.** The following abbreviations are used throughout this paper: SV40(I) DNA, SV40 covalently closed, superhelical DNA; SV40(II) DNA, SV40 double-stranded circular DNA containing an interruption of the phosphodiester bonds in at least one of the two strands; SV40(III) DNA, SV40 double-stranded linear DNA one genome in length; SV40(RI) DNA, SV40 DNA replicating intermediates containing a superhelical region of unreplicated DNA and two nonsuperhelical regions of newly replicated DNA (15); HEPES, N-2-hydroxyethyl piperazine-N’-2-ethanesulfonic acid; and EGTA, ethyleneglycol-bis-(β-aminoethyl ether)-N,N’-tetraacetate.

**Virus and cells.** The small-plaque strain of SV40, Rh911, was prepared at a low multiplicity of infection (9). All experiments were done with a CV-1 cell line grown as previously described (2, 9).

**Preparation of nuclear extracts.** Routinely, 100-mm-diameter dishes confluent with CV-1 cells were infected with sufficient virus to provide the maximum rate of viral DNA synthesis at 36 h postinfection (about 40 PFU per cell) as previously described (9). Before isolating nuclei from infected cells, either SV40(I) or SV40(RI) DNA was radioactively labeled. To isolate nuclei from infected cells, either SV40(I) or SV40(II) DNA was labeled by incubating cells from 28 to 36 h after infection with either 2 μCi of [3H]thymidine per ml (52 Ci/mmol), or 5 μCi of [32P]thymidine per ml (57 mCi/mmol) at 37°C. Alternatively, SV40(II) DNA was labeled at 36 h after infection by removing the medium, incubating the cells for 3 min at 37°C in 0.5 ml of 20 mM Tris (pH 7.4), 137 mM NaCl, 5 mM KCl, 1 mM Na2HPO4, and 25 μCi of [3H]thymidine and then quenching the reaction by floating the dishes on ice water. The cells were then taken into a cold room, washed twice with 5 ml of ice-cold hypotonic buffer (10 mM HEPES [pH 7.8], 5 mM KCl, 0.5 mM MgCl2, and 0.5 mM dithiothreitol) and drained of excess liquid before scraping them off the dishes with a 1-inch-wide [ca. 2.54-cm-wide] rubber policeman. This broken cell suspension (about 0.2 ml per dish) was completely disrupted in a Dounce homogenizer with three strokes of tight-fitting pestle B. The nuclei were quickly separated from the cytoplasm by centrifugation at 3,000 × g for 5 min at 2°C and then taken up as a homogeneous suspension in 1 ml of hypotonic buffer with the aid of a vortex mixing device. The nuclei were transferred to a 1.5-ml Eppendorf centrifuge tube and incubated on ice for 1 to 2 h with occasional agitation to maintain a uniform suspension. The nuclei were then completely removed by centrifugation at 8,000 × g for 10 min at 2°C. The supernatant, containing about 5 μg of viral DNA in both SV40 replicating and mature chromosomes, was referred to as a nuclear extract.

About 50% of the SV40(II) DNA and 75% of the SV40(I) DNA present at this step was released in 10 min. Release of 90% of the SV40(I) DNA occurred within 30 min, although at least 60 min was required to recover 90% of the SV40(II) DNA. No cellular DNA was released (26).
Because viral chromosomes immediately began to leak out of the nuclei as soon as the cells were broken by the rubber policeman, as much as 40% of the total SV40(RI) DNA and 90% of the SV40(II) DNA may be found in the initial cytoplasmic fraction. This SV40(RI) was also capable of continuing DNA replication. The premature release of viral DNA was minimized by draining excess liquid from the dishes before removing cells to maintain a concentrated lysate. Addition of sucrose or KCl during the initial stages of nuclei isolation prevented release of viral chromatin, but subsequent resuspension of the nuclear pellet in hypotonic buffer did not release replicating chromosomes, although mature chromosomes were still slowly released.

Preparation of nucleoprotein complexes and nucleosol. SV40 nucleoprotein complexes were prepared from a nuclear extract by centrifuging it at 50,000 rpm for 50 min at 2°C in a Beckman SW50.1 rotor containing 0.8-ml tubes. These conditions allow complete recovery of both mature and replicating SV40 chromosomes. The sample was never diluted to occupy the centrifuge tube, but rather the size of the tube was chosen to fit the sample volume. The supernatant, referred to as nucleosol, was removed. The pellet, referred to as nucleoprotein complexes, was resuspended in 0.2 ml of hypotonic buffer by gently drawing it up and down with a capillary pipette and incubating it on ice for 1 to 2 h with occasional gentle agitation to achieve a homogeneous preparation (a Vortex mixing device was helpful).

Preparation of replicating chromosomes. SV40 replicating chromosomes were prepared by layering 0.2 ml of a nuclear extract over a 5 to 30% sucrose linear gradient in hypotonic buffer and centrifuging it in a Beckman SW50.1 rotor at 50,000 rpm for 50 min at 4°C. Fractions were collected from the bottom of the tube. SV40(RI) [3H]DNA and SV40(I) [35S]DNA were precipitated in 1 N HCl containing 0.5% sodium pyrophosphate and filtered through GF/C glass fiber paper. The filters were washed with 1 N HCl-0.5% sodium pyrophosphate followed by ethanol and then dried, placed in a toluene-based scintillation fluid, and analyzed in a liquid scintillation counter. The peak fractions containing SV40(RI) DNA were pooled and assayed directly as replicating chromosomes.

Replicating chromosomes, containing only SV40(RI) DNA, sedimented at 90 ± 2S; mature chromosomes, containing 97% SV40(I) DNA and 3% SV40(II) DNA, sedimented at 70 ± 2S; and the non-associated materials remained at the top of the gradient (22, 26). Neither the sedimentation rates nor the symmetrical profiles of SV40 chromosomes were significantly altered by adjusting both the nuclear extract and sucrose gradient composition to either 5 mM EDTA, or to 2 mM EDTA, 0.2 M NaCl and 0.25% Triton X-100, conditions commonly employed in isolating SV40 chromatin (3, 7, 8, 24, 30).

Preparation of cytosol. Freshly prepared cytosol from uninfected CV-1 cells was obtained by centrifuging the cytoplasm (see above) at 100,000 x g for 1 h at 2°C. Protein content was 700 to 900 μg/ml as measured by a Lowry assay.

SV40 DNA replication in vitro. Viral DNA replication was routinely measured in a 100-μl volume containing 20 μl of either nuclear extract, nucleoprotein complexes, or replicating chromosomes, 70 μl of either hypotonic buffer or cytosol, and 10 μl of an assay mixture that brought the final concentrations of each component to 39 mM HEPES (pH 7.8), 24.5 mM KCl, 4.5 mM MgCl2, 1 mM EGTA, 0.45 mM dithiothreitol, 2 mM ATP, 5 mM phosphoenolpyruvate, 30 μg of pyruvate kinase per ml in glycerol, and 100 μM each of dATP, dTTP, dGTP, dCTP, and UTP. The mixture was incubated at 30°C for up to 1 h. The reaction was terminated by adding the mixture to a final concentration of 1 M NaCl and 0.5% sodium Sarkosyl at 0°C. Experiments involving α-32PdATP or α-32PdCTP (50 to 100 Ci/mmol) contained 20 μM dATP or dCTP in the reaction mixture.

Buoyant density of SV40 chromosomes. Peak fractions of SV40 chromosomes containing either SV40(RI) or SV40(I) DNA were pooled separately from analytical sucrose gradients and fixed at 4°C either in a final concentration of 5% neutralized glutaeraldehyde for 8 h or in 5% formaldehyde for 2 h and then in 5% glutaraldehyde overnight (8). Samples were layered onto gradients consisting of three layers of CsCl in hypotonic buffer (1.2, 1.4, and 1.5 g/cm3) and centrifuged in a Beckman SW50.1 rotor at 50,000 rpm for 6 h at 20°C. Fractions were collected from the bottom of the tube, and the density was determined from the refractive index. By using either procedure, replicating chromosomes had a density of 1.359 g/cm3, mature chromosomes had a density of 1.371 g/cm3, and virions had a density of 1.320 g/cm3. The density of SV40 virions was the same as previously reported (4). The ratio of protein to DNA may be estimated from the density of viral DNA, 1.701 (21), and protein, 1.295 (4), using the equation:

\[
\rho / \text{complex} = \left( \frac{(1 / \rho \text{ DNA})(1 - \% \text{ protein})}{100} \right) + \left( \frac{(1 / \text{ protein})}{100} \right)
\]

This calculation ignores differences in the hydration of protein and DNA in CsCl and differences between buoyant density and true partial specific volume.

Sedimentation analysis of SV40 DNA. After termination of DNA synthesis, 50 to 100 μl of the reaction mixtures was layered onto either neutral or alkaline sucrose gradients containing 5 to 20% sucrose in 10 mM Tris-hydrochloride (pH 7.8), 2 mM EDTA, and a final concentration of 1 M NaCl (9). Alkaline sucrose gradients also contained 0.5% Sarkosyl. Reaction mixtures (200 μl) containing replicating chromosomes were first terminated and then purified by passage through Sepharose 6B (Pharmacia, 0.5- by 15-cm column) equilibrated with 1 M NaCl and 0.5% sodium Sarkosyl before sedimentation analysis. Viral DNA appeared in the void volume. Neutral sucrose gradients were centrifuged in either a Beckman SW60 or SW50.1 rotor at 4°C for 3.25 h at 50,000 rpm. Alkaline sucrose gradients were centrifuged in the same rotors for either 2 or 6.5 h at 50,000 rpm, 4°C, depending on whether or not SV40(I) DNA (53S) as well as SV40(II) and SV40(RI) DNA (18S and smaller) was to be observed. In all cases, gradients were collected from the bottoms of the tubes, and acid-insoluble radioactivity was measured as described above.
RESULTS

Synthesis of SV40 DNA in vitro. Figure 1 outlines the preparation of three soluble systems, nuclear extracts, nucleoprotein complexes, and replicating chromosomes, from SV40-infected CV-1 cells and indicates the relationships between preparation of these systems and the insoluble cell lysates (9) and washed nuclei (10) systems. All details and comments are given above. The ability of the endogenous SV40(R1) DNA to continue DNA synthesis in the three subnuclear systems was compared under the standard reaction conditions described above (Fig. 2). In the absence of added cytosol, the amount of DNA synthesis in nuclear extracts and nucleoprotein complexes was comparable, whereas the more highly purified replicating chromosomes were about half as active. Addition of cytosol stimulated DNA synthesis in both the nuclear extract and nucleoprotein complexes 2- to 3-fold, whereas DNA synthesis in replicating chromosomes increased 6- to 10-fold. However, part of the newly synthesized DNA in replicating chromosomes was not found associated with SV40 DNA under nondenaturing conditions (see below). The addition of nucleosol made little or no difference in the amount of DNA synthesis in any of these three systems in either the presence or absence of cytosol. Both nuclear extracts and replicating chromosomes could be stored at 22°C for at least 30 min with no effect on the amount of DNA subsequently synthesized.

Optimum conditions for DNA synthesis were examined in the absence of added cytosol to minimize the contribution of endogenous pools. In nuclear extracts, the optimum pH ranged from 7 to 8, whereas the KCl concentration ranged from 25 to 90 mM and the MgCl₂ concentration ranged from 2 to 3 mM. As shown in Table 1, the more purified the SV40 chromosomes, the greater the requirement for added deoxyribonucleotides. In contrast, the reverse was true for added ribonucleotides. DNA synthesis was sensitive to N-ethylmaleimide as expected if DNA polymerase α was responsible for the majority of the DNA chain elongation (11, 22). Sucrose and Triton X-100, two reagents commonly employed in isolating SV40 chromosomes, had no effect on DNA synthesis in nuclear extracts or nucleoprotein complexes, but Triton X-100 reduced the activity of replicating chromosomes about 45%.

Structure of SV40 DNA synthesis in vitro. The fidelity of SV40 DNA replication in the three subnuclear systems was evaluated by analyzing the structure of DNA synthesized in vitro. Synthesis of SV40(I) DNA, for example, would suggest that the processes of DNA chain elongation and termination of replication occurred correctly. Table 2 summarizes the results of at least four independent experiments conducted in either the presence or absence of added cytosol. Cytosol always stimulated the synthesis of SV40(I) DNA. The fraction of SV40(I) DNA generally appeared larger when measured as the conversion of in vivo prelabeled

![Diagram](https://jvi.asm.org/content/50/1/56/F1.large.jpg)

**Fig. 1.** Diagrammatic representation of the preparation of the five subcellular systems used to study SV40 DNA replication and the cytosol and nucleosol fractions tested for complementation ability.
SV40 DNA replication in vitro

SV40(RI) [3H]DNA into SV40(I) [3H]DNA, as compared with the fraction of SV40(I) [32P]DNA synthesized in the presence of α-[32P]-labeled deoxynucleoside triphosphates (α-[32P]dNTP's) in vitro. This was expected because those SV40(RI) DNA molecules nearest termination when cells were lysed would contain the greatest amount of 3H label and would also have the highest probability of becoming SV40(I) DNA, whereas young SV40(RI) DNAs would incorporate the greatest amount of [32P]dNMP's but are

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

<table>
<thead>
<tr>
<th>Additions or deletions</th>
<th>Nuclear extract (pmol/tLg)</th>
<th>Nucleoprotein complexes (%)</th>
<th>Replicating chromosomes (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>-dT, dG, dC, dA</td>
<td>51</td>
<td>34</td>
<td>3</td>
</tr>
<tr>
<td>-dT, rA, rG, rC</td>
<td>82</td>
<td>85</td>
<td>100</td>
</tr>
<tr>
<td>+Tripton X-100, 0.5%</td>
<td>100</td>
<td>100</td>
<td>57</td>
</tr>
<tr>
<td>+Sucrose, 0.3 M</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>+N-ethylmaleimide, 5 mM</td>
<td>7</td>
<td>11</td>
<td>11</td>
</tr>
</tbody>
</table>

*Activity is expressed as the percentage of α-[32P]-dATP incorporated into SV40 DNA in 1 h at 30°C in the absence of added cytosol. The 100% values represent 22.7 pmol of dAMP incorporated per µg of viral DNA (nuclear extracts), 20 pmol/µg (nucleoprotein complexes), and 6.7 pmol/µg (replicating chromosomes). The three soluble DNA replication systems were prepared as described in the text.

**Table 2. Synthesis and degradation of SV40(I) DNA in vitro**

<table>
<thead>
<tr>
<th>Determination</th>
<th>SV40(I) [3H]DNA (%)</th>
<th>SV40(I) [32P]DNA (%)</th>
<th>% SV40(I) [32P]DNA, 60'</th>
<th>% SV40(I) [3H]DNA, 0'</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclear extract</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-C</td>
<td>7 ± 3</td>
<td>6 ± 3</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>+C</td>
<td>35 ± 3</td>
<td>29 ± 3</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Nucleoprotein complex</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-C</td>
<td>7 ± 1</td>
<td>5 ± 2</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>+C</td>
<td>30 ± 4</td>
<td>27 ± 3</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Replicating chromosomes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-C</td>
<td>2 ± 0.3</td>
<td>2 ± 0.3</td>
<td>0.6 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>+C</td>
<td>5 ± 0.6</td>
<td>7 ± 0.6</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>

*The fraction of SV40(I) DNA, in the presence (+C) or absence (−C) of cytosol, was determined either on alkaline sucrose gradients or by electrophoresis on agarose gels. The average error was calculated from at least four independent experiments.

* Pulse-chase experiments measured the fraction of SV40(RI) [3H]DNA, briefly labeled in vivo, that was converted into SV40(I) [3H]DNA after a 60-min incubation in vitro.

* The fraction of SV40(I) [32P]DNA present after incubation for 60 min in vitro with either α-[32P]dCTP or α-[32P]dATP.

* The ratio of the fraction of SV40(I) [3H]DNA, prelabeled for 8 h in vivo, present after a 60-min incubation in vitro to the fraction present initially at zero time.
were vulnerable, during constant action. Although before ally was the presence of nucleoprotein gradients sucrose labeled replicating DNA. For both nuclear extracts and nucleoprotein complexes in the presence and absence of cytosol, SV40 nucleoprotein complexes were similar to nuclear extracts in their ability to synthesize SV40(I) DNA, whereas isolated replicating chromosomes always made four to seven times less (Table 2 and Fig. 3 and 4).

The fraction of SV40(RI) DNA that completed replication and the amount of SV40(I) DNA synthesized depended largely on the concentration and quality of the cytosol preparation. Although a cytosol preparation occasionally was contaminated with endonuclease activity, the fraction of SV40(I) [3H]DNA, labeled in vivo before preparation of nuclear extracts or nucleoprotein complexes, generally remained constant during the in vitro reactions both in the presence and absence of cytosol (Table 2). However, isolated replicating chromosomes were vulnerable, receiving about one broken phosphodiester bond per 25,000 nucleotides during the 1-h in vitro reaction. Addition of cytosol prevented this endonuclease activity, suggesting the presence of a nuclease inhibitor in the cytosol.

Elongation of SV40(RI) DNA chains in vitro. In the presence of cytosol, both nuclear extracts (26) and nucleoprotein complexes (Fig. 5) were able to continue elongation of the nascent [3H]DNA chains in SV40(RI) [3H]DNA that had been briefly labeled in vivo. Consequently, not only were significant amounts of SV40(I) DNA produced, but at least 80% of the [3H]DNA was sedimented to equilibrium in a cesium chloride-ethidium bromide density gradient (9), dialyzed overnight against 10 mM Tris-hydrochloride (pH 7.6) and 0.1 mM EDTA, and then a 100-μl sample was made 5% in glycerol and 0.025% in bromophenol blue before electrophoresis in a 15-cm, 1.4% (wt/vol) agarose gel (27). Electrophoresis was done at 3 V/cm for 17 h at 4°C in 40 mM Tris-hydrochloride (pH 7.6), 50 mM sodium acetate, and 1 mM EDTA and recirculated between the top and bottom reservoirs. The direction of electrophoresis was from left to right. Symbols: ○, [3H]DNA; ■, [32P]DNA. I, II, III, and RI show the positions of SV40(I), SV40(II), SV40(III), and SV40(RI) DNA, respectively.

Fig. 3. Synthesis of SV40(I) DNA by nucleoprotein complexes and replicating chromosomes. Nucleoprotein complexes (A) and replicating chromosomes (B) containing SV40(RI) [3H]DNA were prepared, and DNA synthesis was assayed in the standard reaction mixture containing both α-[32P]dCTP and cytosol as described in the text. The terminated reaction mixtures were then analyzed in alkaline sucrose gradients (2 h). Symbols: ○, [3H]DNA; ■, [32P]DNA. I and II indicate the positions of SV40(I) and SV40(II) DNA standards, respectively. Sedimentation was from right to left.

Fig. 4. Electrophoresis of SV40 DNA synthesized in nuclear extracts. A nuclear extract containing SV40(RI) [3H]DNA was incubated in the presence of α-[32P]dCTP and cytosol for 1 h in the standard reaction mixture. Viral DNA was sedimented to equilibrium in a cesium chloride-ethidium bromide density gradient (9), dialyzed overnight against 10 mM Tris-hydrochloride (pH 7.6) and 0.1 mM EDTA, and then a 100-μl sample was made 5% in glycerol and 0.025% in bromophenol blue before electrophoresis in a 15-cm, 1.4% (wt/vol) agarose gel (27). Electrophoresis was done at 3 V/cm for 17 h at 4°C in 40 mM Tris-hydrochloride (pH 7.6), 50 mM sodium acetate, and 1 mM EDTA and recirculated between the top and bottom reservoirs. The direction of electrophoresis was from left to right. Symbols: ○, [3H]DNA; ■, [32P]DNA. I, II, III, and RI show the positions of SV40(I), SV40(II), SV40(III), and SV40(RI) DNA, respectively.
mosomes, with or without cytosol present, the SV40(RI) [3H]DNA underwent a conformational change that was readily observed in neutral sucrose gradients (Fig. 7). This decrease in sedimentation was consistent either with synthesis of SV40(I) and SV40(II) DNA products or with relaxation of the intact, superhelical, unreplicated portion of the DNA by interruption of a single phosphodiester bond in front of a replication fork. Because extensive DNA chain elongation was not detected in either prelabeled [3H]DNA or in vitro-labeled [32P]DNA chains (see below), the second interpretation appeared correct. When α-[32P]dCTP was included in the in vitro reaction mixture, about 50 to 75% of the resulting [32P]DNA cosedimented with the SV40(RI) [3H]DNA that had been previously labeled in vivo. The remaining [32P]DNA was found as short pieces near the top of the neutral sucrose gradient. None of the [3H]DNA was ever found degraded. If a single phosphodiester bond interruption existed in front of a replication fork, the parental DNA template strands could easily reanneal, displacing 32P-labeled Okazaki pieces. Alternatively, the single-stranded DNA regions between Okazaki pieces may become the target of an endonuclease. Either event would release 32P-labeled Okazaki pieces without degrading the 3H-labeled DNA daughter strands. Neither mechanism is the result of nonspecific endonuclease activity which is suppressed in the presence of cytosol (Table 2).

Because a significant amount of in vitro-labeled [32P]DNA was not found associated with SV40(RI) DNA molecules, SV40(RI) [32P]DNA was first purified by gel filtration chromatog-
DNA elongation in the bulk of the daughter strands. This was consistent with the previous observation that prelabeled DNA daughter strands failed to elongate to full size (Fig. 6).

In the absence of added cytosol, existing Okazaki pieces were labeled with α-[32P]dATP, but the distribution of this label between long and short pieces of DNA (Fig. 8A, E, and I) and the ability of "Okazaki pieces" to join to daughter strands (Fig. 8B, F, and J) depended on the amount of contaminating cytosol. Replicating chromosomes, the most purified system, completely failed to join Okazaki pieces in the absence of cytosol (Fig. 8J). Although nuclear extracts and, to a lesser extent, nucleoprotein complexes contained sufficient residual cytosol activity to join pulse-labeled Okazaki pieces, the bulk of the daughter DNA chains did not continue the elongation process (Fig. 8B and F).

**Effect of DNA template concentration on DNA replication.** The concentration of the DNA template in each of the three subnuclear systems was critically important in determining both the total amount of DNA synthesis and the fraction of SV40(I) DNA observed. When nuclear extracts were first diluted with hypotonic buffer and then assayed in the presence of cytosol, the total amount of [32P]DNA synthesized in vitro increased dramatically while a concomitant decrease occurred in the fraction SV40(II) [3H]DNA that was converted into SV40(I) [3H]DNA (Fig. 9). When the SV40 [32P]DNA was analyzed in alkaline sucrose gradients, not only was the fraction of SV40(II) [32P]DNA found to decrease with increasing dilution, but the increase in total [32P]DNA found was accounted for by the appearance of small pieces of DNA about the size of Okazaki pieces (Fig. 10). These short DNA chains were not associated with SV40(II) DNA in neutral sucrose gradients. This effect of template concentration was only observed upon addition of cytosol; in the absence of cytosol little change was observed in the amount of [32P]DNA synthesized in vitro. The effect of template dilution was irreversible. A nuclear extract was diluted 50-fold with hypotonic buffer before isolating the nucleoprotein complexes, and the nucleoprotein complexes were then resuspended to the original volume of the undiluted nuclear extract. DNA replication in this preparation behaved the same as in the diluted nuclear extract (Fig. 10D).

Considering that the initial concentration of SV40 DNA in preparations of replicating chromosomes was generally three to four times less than in nuclear extracts, the ability of replicating chromosomes to synthesize [32P]DNA in vitro followed the same dilution curve as nuclear extracts (Fig. 9). However, the failure of replicating

**Fig. 7.** Conformational changes in SV40(II) DNA contained in isolated replicating chromosomes during incubation in vitro. Replicating chromosomes containing SV40(II) [3H]DNA, labeled for 3 min in vivo, were prepared as described in the text and assayed as described in the legend to Fig. 5. The terminated reaction mixture, however, was then analyzed by sedimentation in neutral sucrose gradients (right to left) with SV40(I) and SV40(II) [32P]DNA internal standards. Symbols: ○, [3H]-DNA; ●, [32P]DNA.

Discontinuous DNA synthesis. In each of the three subnuclear systems, DNA chain elongation appeared to occur discontinuously on at least one side of the replication fork (Fig. 8). DNA synthesized in the first 2 min in vitro appeared in alkaline sucrose gradients either as short pieces of 4 to 5S nascent DNA (Okazaki pieces) or as a broad distribution of longer DNA chains up to 16S, the length of mature SV40 DNA. The Okazaki pieces were then joined to the growing daughter DNA chains upon continued incubation in the presence of cytosol. However, unlike nuclear extracts (Fig. 8D) and nucleoprotein complexes (Fig. 8H), isolated replicating chromosomes (Fig. 8L) failed to continue

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chromosomes to synthesize an equivalent amount of SV40(I) DNA could not be accounted for by template concentration alone (Fig. 9).

Reconstitution of salt-depleted nucleoprotein complexes with cytosol. Nuclear extracts were treated with increasing concentrations of NaCl to determine whether the viral chromosomes could be further purified without

FIG. 8. Discontinuous DNA synthesis in the three subnuclear systems. Nuclear extracts and nucleoprotein complexes were isolated containing 90% SV40(I) and 10% SV40(II) [3H]DNA. Replicating chromosomes were isolated containing SV40(III) [3H]DNA. The three preparations were incubated in the standard reaction mixture containing 10 μM α-[32P]dATP with or without cytosol. After a 2-min pulse-labeling period, a 67-fold excess of unlabeled dATP was added, and the reaction continued for 58 min. The ratio of [32P]DNA/[3H]DNA remained essentially constant during this “chase” period. Assays were terminated either at the end of the 2-min pulse or at the end of the 58-min chase and analyzed on alkaline sucrose gradients (6.5 h). The results with nuclear extracts are shown in (A) to (D), nucleoprotein complexes in (E) to (H) and replicating chromosomes in (I) to (L). (A), (E), and (I) show the results of a 2-min pulse in the absence of cytosol, whereas (B), (F), and (J) show the results of the 58-min chase. (C), (G), and (K) show the results of a 2-min pulse in the presence of cytosol, whereas (D), (H), and (L) show the results of the 58-min chase. Symbols: ————, [3H]DNA; ————, [32P]DNA. The minimum percentage of Okazaki pieces was calculated as the area of the 4 to 5S [32P]DNA peak above the extrapolated background for short nascent DNA chains. The counts-per-minute value of (e) on the Y axis for 32P is also given below. Sedimentation was from right to left. Percentage of Okazaki pieces: (A), 33; (B), 0; (C), 36; (D), 0; (E), 61; (F), 13; (G), 33; (H), 0; (I), 50; (J), 42; (K), 44; (L), 0. Counts per minute of 32P: (e): (A), 5.5 × 10^3; (B), 7 × 10^3; (C), 3.5 × 10^3; (D), 5.1 × 10^3; (E), 10.2 × 10^3; (F) 5.6 × 10^3; (G), 7.2 × 10^3; (H), 8.2 × 10^3; (I), 7.8 × 10^3; (J), 5 × 10^3; (K), 11.4 × 10^3; (L), 6.8 × 10^3.
either changing their concentration or sedimenting them through sucrose gradients and to what degree the expected loss of DNA replication activity could be reconstituted by addition of cytosol. Nuclear extracts containing prelabeled SV40(RI) [3H]DNA were adjusted to the NaCl concentration indicated in Table 3 and incubated for 20 min on ice before preparing nucleoprotein complexes. The nucleoprotein complexes were then incubated for 1 h in the standard reaction mixture containing α-[32P]dCTP, with and without cytosol. The relative amounts of DNA synthesis were calculated from the ratio of [32P]DNA/[3H]DNA and from the conversion of SV40(RI) [3H]DNA into SV40(I) [3H]-DNA, determined by sedimentation in alkaline sucrose gradients.

Preparation of nucleoprotein complexes in NaCl concentrations up to 0.3 M progressively reduced their ability to continue DNA replication without changing their sedimentation profile (Table 3). DNA synthesis in the presence of cytosol also showed a parallel decline, which suggested that an increasing fraction of the chromosomes was inactivated by the salt treatment. However, nucleoprotein complexes treated with 0 to 0.2 M NaCl were stimulated 2- to 3-fold by cytosol, whereas chromosomes exposed to 0.3 to 0.4 M NaCl were stimulated about 20-fold by cytosol. Apparently, the higher NaCl concentration removed one or more components from the template complex that could be replaced by...


**Table 3. Reconstitution of salt-depleted nucleoprotein complexes with cytosol**

<table>
<thead>
<tr>
<th>NaCl (M)</th>
<th>Recovery of [3H]DNA (%)</th>
<th>Replicating chromosomess (S)</th>
<th>SV40 DNA synthesis (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>[32P]DNA/[3H]DNA</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>-C  +C -C  +C</td>
</tr>
<tr>
<td>0</td>
<td>100</td>
<td>90 ± 2</td>
<td>50 100 24 100</td>
</tr>
<tr>
<td>0.05</td>
<td>43</td>
<td>96</td>
<td>29 88</td>
</tr>
<tr>
<td>0.10</td>
<td>36</td>
<td>87</td>
<td>31 77</td>
</tr>
<tr>
<td>0.20</td>
<td>24</td>
<td>82</td>
<td>9 57</td>
</tr>
<tr>
<td>0.30</td>
<td>4</td>
<td>73</td>
<td>4 43</td>
</tr>
<tr>
<td>0.40</td>
<td>1</td>
<td>27</td>
<td>&lt;1 22</td>
</tr>
<tr>
<td>0.60</td>
<td>100</td>
<td>50</td>
<td>2 5 &lt;1 12</td>
</tr>
<tr>
<td>0.80</td>
<td>80</td>
<td>42</td>
<td>6 &lt;1 12</td>
</tr>
<tr>
<td>1.0</td>
<td>57</td>
<td>30</td>
<td>7 &lt;1 10</td>
</tr>
<tr>
<td>2.0</td>
<td>30</td>
<td>23</td>
<td>6 &lt;1 13</td>
</tr>
</tbody>
</table>

* Nuclear extracts containing SV40(RI) [3H]DNA were treated with varying NaCl concentrations. The nucleoprotein complexes were recovered and assayed for DNA synthesis in the presence of α-[32P]dCTP either in the absence (–C) or presence (+C) of cytosol. See text for details. The sedimentation value was determined in sucrose gradients containing the same NaCl concentration as the sample. Internal sedimentation standards were SV40 DNA and 50S and 30S Escherichia coli ribosomal subunits fixed with 1% glutaraldehyde. In these experiments, 32% SV40(I) [3H]DNA and 490 pmol of α-[32P]dCMP per assay were defined as 100% responses.

addition of cytosol.

Treatment of nucleoprotein complexes with 0.6 to 2.0 M NaCl resulted in the complete loss of endogenous DNA synthesis with a concomitant decrease in sedimentation presumably due to the loss of chromosomal proteins (Table 3). The ratio of protein to DNA, estimated from buoyant density measurements (see above), was 80/20% in replicating chromosomes, 76/24% in mature chromosomes and 92/8% in virions (data not shown). Such a high content of protein in both types of chromosomes suggests that proteins other than histones are associated with the DNA. Addition of cytosol to these samples restored about 6% of the original amount of α-[32P]dCTP incorporation and about 12% of the original amount of SV40(I) [3H]DNA synthesis. Synthesis of [32P]DNA would be expected from the limited action of DNA polymerase on the 3'-OH termini of nascent DNA chains. However, the surprising appearance of SV40(I) [3H]DNA could not be explained by a simple repair processing of preexisting SV40(I) DNA. Cytosol factors apparently catalyzed the completion of replication either of those replicating DNA molecules poised near termination (27) or, more likely, in those SV40(II) DNA termination intermediates containing a small gap in the termination region (6). Cytosol contains both DNA polymerase α and DNA ligase activities (B. Karas, unpublished data).

**DISCUSSION**

The DNA replication characteristics of each of the five subcellular systems studied so far (Fig. 1) are summarized in Table 4. Cytosol from either uninfected or infected CV-1 cells was the only one of the three soluble cellular fractions that complemented the ability of endogenous SV40 chromosomes to continue DNA replication in any of these systems. Added to washed nuclei or nucleoprotein complexes, cytosol stimulated total DNA synthesis, elongation of nascent DNA chains, maturation and joining of Okazaki pieces, and the conversion of SV40(RI) DNA into SV40(I) and (II) DNA products. Nuclear extracts responded similarly except that sufficient cytosol contaminated the preparations to allow the initial population of Okazaki pieces to complete synthesis and join to the growing nascent DNA chains without addition of cytosol. In the presence of added cytosol, these three in vitro DNA replication systems were essentially as effective as cell lysates in continuing viral DNA replication; initiation of replication in new genomes does not appear to occur (9).

In contrast to these systems, purified replicating chromosomes in the presence of cytosol appeared only to complete and join Okazaki pieces already present on the DNA templates. They failed to significantly extend nascent DNA chains either prelabeled in vivo with [3H]thymidine or labeled in vitro with α-[32P]dCTP. Consequently, seven to eight times less prelabeled SV40(RI) DNA was converted into SV40(I) DNA in vitro. Note that although Fig. 8K and L give the appearance of extensive DNA chain growth, these data can be readily interpreted as the joining of 32P-labeled Okazaki pieces to preexisting nascent DNA chains of all

**Table 4. Properties of in vitro SV40 DNA replication systems**

<table>
<thead>
<tr>
<th>System</th>
<th>Synthesis of 4S DNA</th>
<th>Joining of 4S DNA</th>
<th>Elongation of SV40 DNA chains</th>
<th>Synthesis of (II) DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell lysates</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Washed nuclei</td>
<td>+</td>
<td>(C)</td>
<td>(C)</td>
<td>(C)</td>
</tr>
<tr>
<td>Nuclear extracts</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Nucleoprotein complexes</td>
<td>+</td>
<td>+</td>
<td>(C)</td>
<td>(C)</td>
</tr>
<tr>
<td>Replicating chromosomes</td>
<td>+</td>
<td>(C)</td>
<td>(C)</td>
<td>(C)</td>
</tr>
</tbody>
</table>

*+, Maximum response obtained; (C), maximum response was only obtained in the presence of cytosol; -(C), minimum response was obtained even in the presence of cytosol and nucleosol.
sizes. These data are particularly deceptive because more replicating DNA molecules exist at later stages of replication than at earlier ones (27). In the absence of cytosol, Okazaki pieces were still labeled with α-[32P]dCTP in vitro, but completely failed to join to the growing nascent DNA chains, clearly demonstrating the requirement of cytosol factors at this stage in DNA elongation. However, the addition of either cytosol, nucleosol, nor the unassociated material remaining at the top of the sucrose gradients restored the ability of isolated replicating chromosomes to continue extensive DNA replication. Replicating chromosomes isolated either in glycerol gradients or on Sepharose 4B columns or in the presence of ATP gave the same results.

Purified replicating chromosomes have difficulty either in initiating new Okazaki pieces or in preventing their loss from the replication fork or in both. The failure of ribonucleoside triphosphates to stimulate DNA synthesis (Table 1) suggests the former, whereas the failure of about 50% of the in vitro-labeled DNA to be associated with SV40(RI) DNA which underwent a conformational change (Fig. 7) suggests the latter. This problem, which occurred in both the presence and absence of cytosol, could not be explained by nonspecific endonuclease activity which was effectively suppressed in the presence of cytosol (Table 2).

Both purification and dilution of SV40 chromosomal templates in these subnuclear systems had four effects on subsequent DNA replication in the presence of cytosol: (i) total DNA synthesis was increased, (ii) much of the DNA synthesized in vitro was found as short pieces not associated with large-molecular-weight SV40 DNA, (iii) elongation of nascent DNA chains was limited, and (iv) synthesis of SV40(I) DNA was decreased. A similar dilution of cell lysates had no effect on their ability to replicate viral DNA (9); the viral chromosomes in this system remained concentrated inside the nuclei. Because we were unable to reverse these phenomena by either reconstituting the chromosomes or complementing them with a soluble fraction, a chromosomal component may have dissociated, allowing an unfavorable conformational change in the nucleoprotein template. Consistent with a less compact conformation, both mature and replicating viral chromosomes sedimented at about 70 and 60 S, respectively, after diluting nuclear extracts in hypotonic buffer.

Attempts to selectively extract chromosomal proteins in increasing concentrations of NaCl led to a progressive decrease in the ability of nucleoprotein complexes to continue DNA replication (Table 3). Changes in the composition and conformation of replicating chromosomes resulting from either purification or dilution may impose a limitation on how well in vitro systems can be simplified and still retain their capacity to continue "normal" DNA replication. Because detection of the phenomena described above relied on the use of a well-defined viral genome, they could go unrecognized in studies dealing with less-well-defined mammalian chromatin preparations. Furthermore, the limitations encountered in purifying active replicating chromosomes suggest that attempts to accurately reconstruct the "native" nuclear replication machinery from DNA and proteins will be difficult.

Edenberg et al. (13, 14) have developed similar subnuclear systems from SV40-infected monkey cells beginning with the preparation of "chromatin bodies" that appear to be functionally equivalent to our washed nuclei. However, their "soluble system" made about 10 times less SV40(I) DNA in the presence of a cytoplasmic fraction than either the nuclear extracts or nucleoprotein complexes. Although the authors concluded that extensive DNA chain elongation occurred in purified replicating chromosomes, their results are similar to ours and could also be interpreted as simply the completion and joining of Okazaki fragments.

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