A soluble system has been developed that can initiate DNA replication de novo in simian virus 40 (SV40) chromatin isolated from virus-infected monkey cells as well as in circular plasmid DNA containing a functional SV40 origin of replication (ori). Initiation of DNA replication in SV40 chromatin required the soluble fraction from a high-salt nuclear extract of SV40-infected cells, a low-salt cytosol fraction, polyethylene glycol, and a buffered salt solution containing all four standard deoxyribonucleoside triphosphates. Purified SV40 large tumor antigen (T-ag) partially substituted for the high-salt nucleosol, and monoclonal antibodies directed against SV40 T-ag inhibited DNA replication. Replication began at ori and proceeded bidirectionally to generate replicating DNA intermediates in which the parental strands remained covalently closed, as observed in vivo. Partial inhibition of DNA synthesis by aphidicolin resulted in accumulation of newly initiated replicating intermediates in this system, a phenomenon not observed under conditions that supported completion of replication only. However, conditions that were optimal for initiation of replication repressed conversion of late-replicating intermediates into circular DNA monomers. Most surprising was the observation that p-n-butylyphenyl-dGTP, a potent and specific inhibitor of DNA polymerase-α, failed to inhibit replication of SV40 chromatin under conditions that completely inhibited replication of plasmid DNA containing the SV40 ori and either purified or endogenous DNA polymerase-α activity. In contrast, all of these DNA synthesis activities were inhibited equally by aphidicolin. Therefore, DNA replication in mammalian cells is carried out either by DNA polymerase-α that bears a unique association with chromatin or by a different enzyme such as DNA polymerase-δ.

Simian virus 40 (SV40) replicates in the nuclei of mammalian cells as a 5.2-kilobase pair circular chromosome whose

* This work was supported by grants from the National Cancer Institute and American Cancer Society. The costs of production of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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(Received for publication, February 3, 1987)
that chromosomes can initiate replication in vitro and suggest either that a more complex form of DNA polymerase-α is associated with replicating chromosomes or that a different enzyme, such as DNA polymerase-δ, is the replicative DNA polymerase.

**EXPERIMENTAL PROCEDURES**

**Materials and Standard Solutions**

Restriction endonucleases were obtained from New England Biolabs, Beverly, MA, with the exception of EcoRII. Yeast tRNA and EcoRII were obtained from Bethesda Research Laboratories. Adenosine-5'-tetraphosphate-5'-adenosine (lithium salt) was obtained from Boehringer Mannheim. All radioactive isotopes were from Du Pont-New England Nuclear. p-n-Butylnaphthyl-α-TTP was a gift from Dr. G. Wright (University of Massachusetts Medical School) (16), and HeLa cell DNA primase-DNA polymerase-α was a gift from Dr. E. Baril (Worcester Foundation for Experimental Biology, Shrewsbury, MA) (17). TS buffer contained 20 mM Tris-HCl (pH 7.4), 137 mM NaCl, 5 mM KCl, 1 mM CaCl₂, and 0.5 mM MgCl₂. TE buffer contains 10 mM Tris-HCl (pH 7.4) and 1 mM EDTA. Low-salt buffer contains 20 mM NaCl and 5 mM potassium acetate, 0.5 mM MgCl₂, and 0.5 mM dithiothreitol.

**Preparation of Cellular Fractions for Replication of SV40 Chromatin**

**Cell Lysate**—Ten 15-cm plates of 80% confluent CV-1 cells were infected with SV40 wt800 as described previously (10). At 36-38 h of postinfection, the medium was replaced with 1 ml of TS buffer, 10% calf serum, and 0.1% trypsin (GIBCO). 1.05-1.5 g (80% of the original) crude cell lysates were prepared in the same way, except that nuclei were resuspended with 3-4 strokes of pestle B. This cell lysate, containing swollen but fresh, nucleoprotein pellet contained 99% of the original SV40 DNA. The DNA was prepared in the same way, except that nuclei were resuspended in 1.3 ml of low-salt buffer and incubated on ice for 1 h. The resulting "cytosol" fraction consisted of about 2.5 ml containing 7-12 mg/ml protein but no detectable [3H]DNA. Following incubation the "nucleosol" supernatant (6-10 mg/ml protein) was washed twice in 0.6 M Tris-HCl (pH 7.5), 3.0 M NaCl, 10 mM EDTA and incubated for 1 h in a Beckman SW50.1 rotor. This "cytosol" fraction consisted of about 2.5 ml containing 7-12 mg/ml protein but no detectable [3H]DNA and was stored at −70 °C. All subsequent steps were carried out at 0-4 °C. Cells were washed twice with TS buffer containing 250 mM sucrose to prevent premature lysis of infected cells and then once with low-salt buffer. Excess buffer was removed by aspiration, and the cells were resuspended free with a rubber policeman, transferred to a Dounce homogenizer (Kontes, Vineland, NJ), and lysed completed with 3-4 strokes of pestle B. This cell lysate, containing swollen but intact nuclei, was either used directly for in vitro replication of endogenous SV40 viral chromatin (18) or fractionated further as described below.

**Cytosol and Nuclear Extract**—Nuclear extracts were prepared using a modification of the procedure of Su and DePamphilis (14). Cell lysate from 10 15-cm dishes was centrifuged at 1,200 × g for 5 min to remove nuclei, and the supernatant was centrifuged at 100,000 × g for 1 h in a Beckman Ti-50 rotor. This "cytosol" fraction consisted of about 2.5 ml containing 7-12 mg/ml protein but no detectable [3H]DNA and was stored at −70 °C. The 1,200 × g pellet, containing the nuclei, was resuspended in 1.3 ml of low-salt buffer and incubated on ice for 90 min with occasional agitation. Approximately 75% of the SV40 DNA was released from the nuclei. The nuclei were then sedimented at 8,000 × g for 10 min, and the resulting "low-salt nuclear extract" was stored at −70 °C. "High-salt nuclear extract" was prepared in the same way, except that nuclei were resuspended in low-salt buffer plus 500 mM potassium acetate.

**Nucleosol and SV40 Chromatin**—Nuclear extracts were fractionated by sedimentation in a Beckman SW60 Ti rotor at 300,000 × g for 1 h, and the "nucleosol" supernatant (6-10 mg/ml protein) was stored at −70 °C. High-salt nucleosol retained activity for about 2 months, at which time all cellular and viral fractions were prepared fresh. The nucleoprotein pellet contained 99% of the original [3H]DNA and was resuspended in one-fourth of its original volume in low-salt buffer, regardless of the ionic strength of the original extract buffer (11).

**Standard in Vitro DNA Replication Conditions**

Cytosol (15 µl), nuclear extract (8 µl, nucleotide mix (7 µl; 140 mM Hepes-HCl (pH 7.2), 50 mM phosphocreativate, 17 mM ATP, 83 µM each of CTP, GTP, UTP, dATP, and dGTP, 83 µM each of dCTP and dTTP, 425 mM sucrose, 1.5 µg (0.2 units/µg) of pyruvate kinase (60% glycerol, Boehringer Mannheim)), and polyethylene glycol mix (8 µl; 125 mM Hepes-HCl (pH 7.8), 32 mM MgCl₂, 6 mM EGTA, 1.5 mM ethylene glycol, 32% polyethylene glycol (PEG; 14,000 daltons, Aldrich)) were mixed in that order. [α-32P]dCTP and [α-32P]dTTP were present at concentrations of 3-30 µCi/nmol where the rate of incorporation of [α-32P]dNMP was proportional to the specific activity of nucleotide. Additional potassium acetate (4 µl of 1 M solution) was added when low salt nuclear extracts were used. The final reaction volume was 50 µl. Reaction mixtures were preincubated on ice for 15 min and then incubated at 30 °C for the times indicated. When nucleosol and SV40 chromatin were substituted for nucleosol extracts, 8 µl of nucleosol and 2 µl of chromatin were added per 50 µl of reaction. In vitro DNA replication conditions for fractionated cell lysates were the same as described by DePamphilis et al. (18).

Replication of plasmid DNAs (pSVori, pSVori6) was carried out under the same conditions as described above for SV40 chromatin in the presence of nuclear extracts. Plasmid pSVori was made by substituting nucleotides 28-582 of plasmid pML-1, a pBR322 derivative in which the initiation signal for replication at the late gene side of ori has been deleted (19), with the 212-bp HindIII-SphI segment of SV40 wt800 containing the origin of DNA replication (ori). Plasmid pSVori6A was pSVori but with a 5-bp deletion within or ori located at the Bglll site (6). In some cases (Fig. 7), plasmid DNA was incubated in the presence of uninfected CV-1 extracts, supplemented with purified SV40 large T-antigen under otherwise equivalent conditions.

The DNA products of the replication reaction were purified as described previously (6, 11). When replication was carried out in crude cell lysates or isolated nuclei, viral DNA was isolated by the method of Hirt (20).

**DNA Hybridization Assay**

Hybridization assays were done using circular, single-stranded M13mp7 DNA as in vitro products of SV40 chromatin replication (mSVori DNA). mSV01 and mSV02 contain complementary strands of the 311-bp SV40 BstN1 G fragment containing the ori region. mSV07 and mSV08 contain complementary strands of the 364-bp AccI-PstI fragment 1626-1988 bp to the late gene side of ori (10). Ten µg of either of these DNAs, or M13mp7 DNA alone, were denatured and adsorbed onto nitrocellulose discs (13.3 µm) using a "Dot-Blot" filtration manifold (Minifold SRC-97; Schleicher & Schuell) as described by Wirak et al. (21). Seventy-five percent of the DNA was retained by the nitrocellulose membrane, and adsorption was proportional to the amount of DNA added within a range of 1-15 µg of added DNA.

Individual discs (13 mm) were cut from the membrane and incubated in a prehybridization solution as previously described (21), but using a 5 × concentration of Denhardt's solution and 0.1 mg/ml salmon sperm DNA. SV40 [32P]DNA from the in vitro replication reactions was digested with either BstN1 endonuclease (ori) or AccI-PstI endonucleases (control), denatured at 100 °C, and chilled on ice. 5-50 ng of SV40 DNA (≥2000 cpm [H]) was added per disc; equal aliquots were also incubated with discs containing mp7 DNA alone. Hybridization reactions (115 µl) were carried out overnight at 68 °C in sealed microtiter dishes (3696; Costar, Cambridge, MA) under prehybridization conditions but substituting 0.3 mg/ml tRNA for salmon sperm DNA. Following hybridization the discs were washed twice in 0.6 M Tris-HCl (pH 7.5), 3.0 M NaCl, 10 mM EDTA at room temperature, once in 3 × standard saline citrate (SSC, Ref. 29). 0.2 M sodium dodecyl sulfate (SDS), and twice in 2 × SSC, 2.5% sodium dodecyl sulfate at 68 °C. The discs were dried, and radioactivity was determined by liquid scintillation.

The efficiency of the assay was determined by hybridizing aliquots of between 2 to 250 ng of [5'-32P]-end-labeled (125 cpm/ng; Ref. 26) BstN1 restriction fragments of SV40 Form I DNA to discs containing mSV01 + mSV02 DNAs. The efficiency of annealing was 90%, and reproducibility between duplicate samples was within 4% (standard deviation/mean). Less than 6% of the [5'-32P]-DNA bound by mSV01 + mSV02 was retained by M13mp7 alone, and the amount of [5'-32P]-DNA bound was proportional to its concentration throughout the range tested (10-250 ng). Hybridization with mSV01 + mSV02 DNAs specifically removed the ori-containing BstN1 G fragment as shown by analysis of the [5'-32P]-DNA before and after hybridization by electrophoresis in 1.2% agarose gels.

**Electrophoresis**

Purified DNA products were analyzed by electrophoresis in 0.6% agarose in Tris-borate, EDTA buffer (TBE, Ref. 22). Where indicated, DNA products were separated by restriction endonuclease analysis under conditions suggested by Maniatis et al. (22) and fractionated by electrophoresis in 6% polyacrylamide in TBE. Radioactive DNA was visualized by fluorography using a Cronex (Du Pont). Relative amounts of DNA forms in the gel were estimated by densitometry (6).
**SV40 Chromatin Replication in Vitro**

**RESULTS**

Polyethylene Glycol Stimulates SV40 DNA Synthesis in Vitro—Su and DePamphilis (13, 14) showed that a low-salt extract of nuclei from SV40-infected CV-1 cells, free of cellular chromatin, allowed the endogenous SV40-replicating chromosomes to complete replication in vitro when supplemented with cytosol from either infected or uninfected CV-1 cells. However, this system was never observed to initiate DNA replication at ori. In an effort to correct this problem, a high-salt nuclear extract was substituted for the low-salt nuclear extract in the hope that it would contain higher concentrations of initiation factors such as large T-ag. Although this change stimulated total SV40 DNA synthesis about 50%, analysis of newly replicated DNA by electrophoresis, and its nascent (radiolabeled) DNA was localized in various forms of SV40 DNA has been described previously (25).

Remarkably, addition of 5% PEG to this high-salt nuclear extract stimulated SV40 DNA synthesis at least 4-fold and dramatically increased the amount of nascent DNA that appeared initially in newly initiated RI (Fig. 1, lane g). This RI migrated between Form I and II DNA during gel electrophoresis, and its nascent (radiolabeled) DNA was localized in the ori region (11, 25). The amount of radiolabel in early RI was more pronounced after 30 min (lane g) of incubation than after 10 min (lane f), suggesting that initiation of replication was delayed for at least 10 min. The difference in the DNA products produced in the presence and absence of PEG was most apparent when equal amounts of reaction mixture that had been incubated for the same time were compared directly (lanes k and l).

Addition of PEG also resulted in accumulation of replicating intermediates about 90% completed (RI*) at the expense of circular DNA monomer production (Fig. 1, lanes i and j), indicating that PEG interfered with termination of DNA replication. To confirm that RI was still converted into Form I and II in the presence of PEG, albeit less efficiently, a reaction mixture was incubated in the presence of [α-32P]dNTPs for 30 min ("pulse"), 500 μM unlabeled dNTPs were added and the incubation continued ("chase"). Radiolabel was no longer incorporated during the chase period, although prelabeled [3H]RI was converted into Form I and II (data not shown). After the 30 min pulse (0-min chase), a broad distribution of RI at various stages in its replication was apparent with some accumulation of RI* (Fig. 1, lane m). After a 30-min chase, early RI had completely disappeared, and several forms of middle-to-late RI were present (lane n), and after a 60-min chase (lane o) RI was converted into Form I and II. In fact, the distribution of 32P-radiolabel among the various forms of SV40 DNA after a 60-min chase (lane o) was equivalent to that observed after continuously labeling nascent DNA for 90 min (lane j). Thus, it appears that PEG stimulated initiation of SV40 DNA replication within the first 30 min but inhibited subsequent conversion of RI* into circular DNA monomers.

**Polyethylene Glycerol Stimulates DNA Synthesis Specifically in the Ori Region** —To determine whether or not PEG stimulated initiation of new rounds of DNA replication, newly synthesized SV40 [32P]DNA was annealed to unique segments of the SV40 genome that had been cloned into single-stranded M13 virion DNA. mSVO1 and mSV02 contain complementary strands of a 311-bp SV40 DNA restriction fragment that includes the 64-bp ori-core sequence as well as both 45-bp flanking ori- auxiliary sequences. Thus, hybridization of nascent DNA to mSVO1 + mSV02 measured the amount of DNA synthesis in ori. mSV07 and mSV08 contain the complementary strands of a 364-bp SV40 DNA restriction fragment centered about 1800 bp to the late gene side of ori. Hybridization of nascent DNA to mSV07 + mSV08 measured the amount of DNA synthesis at replication forks far removed from ori.

A high-salt nuclear extract supplemented with cytosol and 4–8% PEG stimulated DNA synthesis at least 7-fold in the ori region with only minor stimulation of DNA synthesis in late RI (control, Fig. 2). PEG preparations from 6,000 to 20,000 daltons in size were tested at concentrations of 1 and 5% and found to have the same amount of activity. Therefore, 14,000-dalton PEG was routinely used because its lower viscosity made it easier to handle. PEG did not stimulate initiation of SV40 chromatin replication in isolated nuclei from infected CV-1 cells supplemented with cytosol, a system that faithfully continues replication in SV40 RI (22). In fact, DNA synthesis in isolated nuclei was inhibited in proportion to PEG concentration. Polyvinyl alcohol also stimulated DNA synthesis in high-salt nuclear extracts at concentrations similar to those used with PEG. However, polyvinyl alcohol was more viscous than PEG, and some lots were inhibitory.

In the presence of PEG, newly synthesized ori region DNA accumulated relative to nascent DNA in RI at later stages of replication (Fig. 3A), consistent with initiation of SV40 DNA

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**Fig. 1. Stimulation of SV40 chromatin replication by PEG.**

A high-salt nuclear extract from SV40-infected CV-1 cells was supplemented with cytosol from the same cells and then incubated at 30 °C in the standard in vitro reaction mix containing [α-32P]dCTP and [α-32P]dTTTP ("Experimental Procedures") either in the presence (lanes f-j, l-o) or absence (lanes a-e and h) of 5% PEG. SV40 DNA was purified at the times indicated (pulse), and aliquots were fractionated by electrophoresis in a 6% agarose gel. Samples in lanes m-o were incubated in the presence of [α-32P]dCTP and [α-32P]dTTTP for 30 min, and then in the presence of an additional 500 μM each of unlabeled dCTP and dTTTP for the times indicated (chase). Equal amounts of [3P] were applied to lanes a-j, m-o. Equal volumes of the samples shown in lanes c and h were applied to lanes k and l, respectively. Relative DNA synthesis was total [32P]DNA divided by the amount observed after 10 min in the absence of PEG (lane a; 1.5 pmol dNMP/h/350 ng of DNA). I, II, III, and RI* indicate the positions of SV40 Form I, II, III, and late replicative intermediate DNA, respectively, based on DNA standards electrophoresed in parallel.
concentrations of PEG. Aliquots of [32P]DNA were hybridized to preparation of cell lysates. Each sample contained approximately control) DNA bound to nitrocellulose membranes ("Experimental 
tions described in Fig. 4). The amount of [32P]DNA synthesized prior to computing the 32P/3H ratio.

mSVO1 DNA, and the amount of radioactivity bound to these filters was subtracted from the amount bound to SV40 sequences to M13mp7 DNA. This was consistent with DNA synthesis occurring in pre-existing RI that were at all stages of replication with a bias toward late RI, consistent with previous observations that the in vivo pool of SV40 RI contains 2- to 3-fold more late RI than early RI (26, 27).

To determine whether or not new initiation events continued to occur in vitro, high-salt nuclear extracts supplemented with PEG were pulse-labeled with [α-32P]dNTPs for 20-min intervals from 0 to 80 min after beginning the incubation at 30°C (Fig. 3B). These data confirmed that the highest rate of ori synthesis occurred from 20 to 40 min after incubation began and that initiation continued to occur at decreasing rates as incubation continued. Similarly, the rate of DNA synthesis in late RI increased continuously as the in vitro reaction progressed, consistent with the appearance of RI late during the reaction (Fig. 1).

The observations made by hybridization of nascent DNA to unique segments of the SV40 genome were confirmed by measuring the relative amounts of DNA synthesis per base pair throughout the SV40 genome. Using the same in vitro conditions described above, [32P]DNA products from various times of incubation were digested with BstNI restriction endonuclease into 15 fragments that varied in length from 54 to 993 bp. In the presence of PEG, at least 65% of the newly synthesized DNA was in the ori region, even after 20 min of incubation (Fig. 4, HSN + PEG). After 45 min, [32P]DNA was distributed over 2000 bp of the genome centered around ori, and after 95 min, [32P]DNA was spread throughout the entire genome, coincident with the large accumulation of RI* and appearance of Form I DNA (Fig. 1, lanes f–j).

In contrast, a lysate of SV40-infected CV-1 cells capable only of continuing replication of RI that were initiated in vivo showed the opposite effect (Fig. 4, Cell Lysate – PEG). Newly synthesized DNA first appeared throughout the SV40 genome. After 1 h of incubation, newly synthesized DNA had accumulated as an increasing gradient beginning at ori and extending to the region containing the entire genome, coincident with the large accumulation of RI* and appearance of Form I DNA (Fig. 1, lanes c–j).

replication in vitro. The low level of DNA synthesis in the ori region observed in the absence of PEG may represent either initiation of DNA replication due to the presence of high-salt nuclear extract, or early RI that were initiated in vitro, but whose replication forks had not yet progressed outside of the 311-bp region containing ori. PEG stimulation of DNA synthesis at ori did not begin until 10–20 min of incubation, consistent with the appearance of early 32P-RI (Fig. 1). Stimulation of DNA synthesis at regions distant from ori (control, Fig. 3A) did not occur until 30–50 min of incubation as the newly initiated replication forks progressed around the genome. In the absence of PEG, DNA synthesis was more pronounced at replication forks in late RI (control) than at replication forks in ori. This was consistent with DNA synthesis occurring in pre-existing RI that were at all stages of replication with a bias toward late RI, consistent with previous observations that the in vivo pool of SV40 RI contains 2- to 3-fold more late RI than early RI (26, 27).

To determine whether or not new initiation events continued to occur in vitro, high-salt nuclear extracts supplemented with PEG were pulse-labeled with [α-32P]dNTPs for 20-min intervals from 0 to 80 min after beginning the incubation at 30°C (Fig. 3B). These data confirmed that the highest rate of ori synthesis occurred from 20 to 40 min after incubation began and that initiation continued to occur at decreasing rates as incubation continued. Similarly, the rate of DNA synthesis in late RI increased continuously as the in vitro reaction progressed, consistent with the appearance of RI late during the reaction (Fig. 1).
SV40 Chromatin Replication in Vitro

Fig. 4. Bidirectional DNA replication from ori. Replication of SV40 chromatin was carried out in high-salt nuclear extracts (HSNE + PEG) as described in Fig. 1 (lanes f-j) for 20 min (solid area), 45 min (medium-shaded area), or 95 min (lightly shaded area). Cell lysates containing SV40 chromatin (Cell Lysate − PEG), "Experimental Procedures," were incubated under the same reaction conditions as high-salt nuclear extracts except that PEG was not added and incubation times were for 5 min (solid area) or 60 min (lightly shaded area). [32P]DNA was purified, digested with BstNI restriction endonuclease, and the products fractionated by electrophoresis in a 6% polyacrylamide gel. [32P]DNA fragments were identified by autoradiography, and the relative amounts of DNA synthesis quantitated by densitometry of the x-ray film. Several film exposures per gel were analyzed to ensure a linear range in film response. The area under each peak was divided by the number of base pairs (bp) in that fragment to compensate for differences in fragment length, and the relative amount of DNA synthesis/base pair in each fragment was normalized relative to the ori-containing fragment. The genomic locations of BstNI cleavage sites in SV40 wt800 are indicated on the abscissa. Fragments containing ori and the termination region (ter) are indicated.

localized within a 600–700-bp region centered at ori (11). Furthermore, hybridization of the [32P]DNA in Fig. 5, lane d, to ori and control sequences confirmed the amplification of ori-specific DNA (data not shown). No accumulation of early RI was observed when PEG was omitted from the in vitro reaction (Fig. 5, lanes a and b).

T-Antigen is Required for Initiation of SV40 Chromosome Replication in Vitro—Addition of PAB419, a monoclonal antibody directed against SV40 T-ag (24), to the in vitro system resulted in a 90% reduction in DNA synthesis and a marked decrease in the fraction of nascent DNA in RI (Fig. 5, lanes e and f). The remaining prominent [32P]DNA species was RI*, consistent with a requirement for T-ag in the initiation of DNA replication, but not in the elongation of DNA. In order to examine the initiation of DNA replication specifically, sufficient aphidicolin was added to the in vitro system to inhibit DNA synthesis by 50% and thereby cause an accumulation of early RI (11). This accumulation of early RI molecules was specifically inhibited by addition of increasing amounts of PAB419, while the small amount of RI* remains unaffected (Fig. 5, lanes g–l). Addition of either control IgG (Fig. 5, lanes m–r) or nonimmune media from the parental NS-1 myeloma cell line (data not shown) had no effect on SV40 chromatin replication. PAB419 immunoprecipitated T-ag and reacted with T-ag in an enzyme-linked immunoadsorption assay, whereas control mouse IgG did not (data not shown).

Initiation of SV40 chromatin replication in vitro required a high-salt nuclear extract of SV40-infected CV-1 cells, but addition of 200 ng or more of immunopurified SV40 T-ag increased nucleotide incorporation 2-fold (Table 1). Since addition of T-ag also doubled the amount of newly synthesized DNA that accumulated as early RI in the presence of aphidicolin, as well as increased labeling of BstNI fragment G (data not shown), addition of purified T-ag further stimulated initiation of SV40 chromatin replication in vitro. Apparently, high-salt nuclear extracts are already rich in T-ag. This was demonstrated by the fact that low-salt nuclear extracts supplemented with PEG did not initiate SV40 chromatin replication unless immunopurified T-ag was added (Fig. 3 in Ref. 11). Taken together, these data demonstrate that T-ag is required for initiation of SV40 chromatin replication in vitro.

SV40 Ori is Required for Initiation of DNA Replication in Vitro—To determine whether or not the conditions that promoted T-ag-dependent DNA synthesis at ori in SV40 chromatin depended on a functional ori sequence, plasmid DNA containing either a functional (pSVori) or nonfunctional (pSVori62) SV40 ori sequence was incubated under a variety of in vitro conditions. Replication was measured by converting all DNA products to linear molecules through cleavage at the single EcoRI site, and then digesting with DpnI (6). Plasmid DNA propagated in dam + Escherichia coli is methylated at DpnI cleavage sites which allows them to be cut by DpnI. DNA that undergoes one or more rounds of replication in mammalian cells, which lack this methylase, becomes resistant to cleavage by DpnI. Thus, the amount of plasmid length Form III [32P]DNA is proportional to the fraction of DNA that replicated in vitro.

Addition of PEG to a low-salt nuclear extract of SV40-infected CV-1 cells stimulated plasmid DNA synthesis 2.2-fold to a final rate of 3.3 pmol of dNMPs/h/250 ng of plasmid DNA (Fig. 6, lanes a and b). This stimulation was dependent upon SV40 ori; pSVori62 did not replicate (lane c). Addition of high-salt nucleosol plus PEG stimulated ori-dependent plasmid DNA replication at a rate of 5 pmol of dNMPs/h/250 ng of plasmid DNA (lanes e–g). Replication of endogenous...
SV40 chromatin was also evident. The amount of SV40 Form III and EcoRI-cleaved RI \(^{32}\)P\(\text{DNA}\) evident in Fig. 6 was greatly increased by high-salt nucleosol and PEG, consistent with the experiments described above. These results demonstrated that in vitro conditions necessary for initiation of replication in SV40 chromatin utilized a functional SV40 ori.

To compare ori-dependent plasmid DNA replication under the conditions described above with results reported by others (4–8), psVori replication was carried out in the absence of endogenous SV40 chromatin by preparing extracts from uninfected CV-1 cells and supplementing them with purified T-ag, psVori DNA replication exhibited a 10- to 20-min delay (Fig. 7, lanes a–c), as previously reported (29, 30), followed by linear incorporation of \(\Delta^{32}\)PdNMPs for at least 3 h. At 20 min, intact newly synthesized psVori DNA migrated slightly slower than psVori Form II DNA, the position expected for topologically relaxed RI (lane c), and then increased in size with continuing incubation (lanes d–i). However, RI* was not evident and appeared to be supplanted by high molecular weight \(\Delta^{32}\)P\(\text{DNA}\) that remained at the top of the gel. This material was the major product of plasmid DNA replication and consisted either of knots of many catenated circular monomers or of concatenated linear monomers linked head-to-tail because digestion of total \(\Delta^{32}\)P\(\text{DNA}\) with EcoRI restriction endonuclease, which cuts psVori at a single site, produced linear monomers (Form III) as the major product (lanes p, r, t, v, and x). Since this Form III \(\Delta^{32}\)P\(\text{DNA}\) was resistant to cleavage by DpnI, it had been replicated in vitro.

Aliquots of psVori DNA were digested with MboI to ascertain the amount of reinitiation that occurred in the same

### Table I

**Optimal conditions for initiation of SV40 chromatin replication**

| Parameter                      | Concentration | DNA synthesis |
|--------------------------------|---------------|---------------|
| Complete system                | 100%          | 100%          |
| (high-salt nuclear extract)    |               |               |
| - CTP, GTP, UTP                | 100%          |               |
| - PEG                          | 96%           |               |
| - Cytoseol                     | 50%           |               |
| - Nucleosol                    | 26%           |               |
| - SV40 chromatin               | 13%           |               |
| + SV40 chromatin               | 100%          |               |
| + SV40 chromatin               | 100%          |               |
| + psVori DNA                   | 100%          |               |
| Mg\(^{2+}\)                    | 0 mM          | 14            |
|                                | 2 mM          | 60            |
|                                | 5 mM          | 100           |
|                                | 10 mM         | 28            |
| K\(^+\)                        | 90 mM         | 77            |
|                                | 150 mM        | 33            |
|                                | 220 mM        | 33            |
| + anti-T-ag (PAb419)           | 5.0 μg        | 11            |
| + T-ag                         | 0.2 μg        | 195           |
|                                | 0.3 μg        | 231           |
|                                | 0.6 μg        | 220           |
| + ApA                          | 0.1–100 μM    | 100           |
| + α-amanitin                   | 50 μM         | 100           |
| + ddTTP/dTTP                   | 5 μM          | 94            |
|                                | 50 μM         | 87            |
| + Aphidicolin                  | 5 μM          | 50            |
|                                | 20 μM         | 10            |
| + p-n-Butylphenyl-dGTP         | 114 μM        | 50            |
|                                | 1 mM          | 8             |

plasmid molecules during in vitro replication. MboI cuts the same site recognized by DpnI, but MboI cuts only the unmethylated form while DpnI cuts only the fully methylated form. Thus the appearance of MboI digestion fragments after 2 h of incubation (Fig. 7, lane u) and their subsequent accumulation (lanes w and y) revealed that a substantial fraction
of the DNA had undergone at least two rounds of DNA replication in vitro.

Parameters that Affect Initiation of SV40 Chromatin Replication in Vitro—Parameters that affected efficiency of SV40 chromatin replication under conditions that promoted initiation of DNA synthesis at ori are summarized in Table I. DNA synthesis depended on the presence of cytosol, nucleosol, PEG, and SV40 chromatin. Although the rate of DNA synthesis per microgram of DNA decreased with increasing chromatin concentration, 350 ng of chromatin DNA was used routinely because the total amount of DNA synthesis per assay was greatest. The optimal Mg2+ concentration was 5 mM, which represented about 1 mM uncomplexed Mg2+. Little effect of [Mg2+] was observed on elongation or replication of sequences 1800 bp distal from ori (i.e. mSV07 + mSV08). The optimal K+ concentration was 90 mM; initiation was more sensitive than elongation to ionic strength. Acetate was used to adjust the salt concentration since Cl− inhibits DNA polymerase-α (31).

Adenosine-5′-tetraphosphate-5′-adenosine (Ap4A) binds to DNA polymerase-α and can serve as a primer for template-dependent DNA synthesis (32) by a complex form of this enzyme (33). Furthermore, the intracellular concentration of Ap4A rises from 0.1 to 1 mM uncomplexed Ap4A when quiescent cells are stimulated to divide, and Ap4A levels rise as high as 10 μM in cells arrested in S-phase (34). However, Ap4A neither increased nor decreased the level of SV40 chromatin replication in vitro.

α-Amanitin concentrations sufficient to inhibit RNA polymerase II and III had no effect on SV40 chromatin replication in vitro. Similarly, omission of CTP, GTP, and UTP did not affect viral chromatin replication. These data suggest that transcription is not required to initiate SV40 DNA replication. Dideoxynucleoside triphosphates (ddTTP) can selectively inhibit DNA polymerases-β and -γ without inhibiting DNA polymerases-α and -δ (35-37). ddTTP/ddTTP ratios that inhibit DNA polymerases-β and -γ did not inhibit SV40 chromatin replication. Aphidicolin, a specific inhibitor of DNA polymerases-α and -δ (16, 28), did inhibit SV40 chromatin replication to the same extent that it inhibited DNA polymerase-α (Fig. 8B). These data support an exclusive role for DNA polymerase-α or -δ in replication of SV40 chromosomes.

To determine whether the stimulatory factor associated with high-salt nuclear extracts was associated with the chromatin or with the nucleosol component, nuclear extracts were prepared under either high-salt or low-salt conditions. SV40 chromatin was separated from nucleosol, and individual components were recombined (Table II). The amount of DNA synthesis originally observed in unfractionated nuclear extracts was reconstituted when the same two fractions were recombined. Total DNA synthesis was 5-fold better in the high-salt nuclear extract than in the low-salt nuclear extract, and only the high-salt nuclear extract specifically stimulated DNA synthesis at ori. These properties were due solely to one or more factors found in high-salt nucleosol; high-salt nucleosol combined with SV40 chromatin from either high-salt or low-salt nuclear extracts stimulated initiation of SV40 chromatin replication whereas low-salt extracts were inactive regardless of which SV40 chromatin fraction was used as substrate. In all instances, recovery of initiation activity was confirmed through analysis of [3P]DNA fractionated by electrophoresis in agarose gels.

High-salt nuclear extracts contain at least one initiation factor in addition to T-ag that is required for initiation of SV40 chromatin replication. This was demonstrated by addition of saturating amounts of purified T-ag to a low-salt nuclear extract (100 ng of T-ag/50 μl of reaction). These conditions never yielded more than 56% of the maximum replication rate observed with high-salt nuclear extracts saturated with T-ag (Tables I and II); additional T-ag did not further increase the rates of viral DNA synthesis. However, addition of high-salt nucleosol to the low-salt nucleosol supplemented with a saturating amount of T-ag increased the
rate of DNA synthesis a maximum of 2-fold (Table II). This was equivalent to the maximum levels obtained with high-salt nuclear extracts saturated with T-ag (Table I). Therefore, one or more soluble factors, in addition to T-ag, are eluted from nuclei by extraction with high-salt buffer.

Is DNA Polymerase-α Involved in SV40 Chromatin Replication?—DNA polymerase-α and DNA polymerase-β exhibit similar sensitivities to aphidicolin, but α-polymerase is 1000 times more sensitive than β-polymerase to inhibition by p-n-butylyphenyl-DGTP (BuPDGTP; 15). Therefore, BuPDGTP should be useful in identifying which DNA polymerase is required for DNA replication. The sensitivity of DNA polymerase-α to this BuPDGTP was confirmed using α-polymerase purified from either CV-1 (38) or HeLa cells (17) on DNase I-activated DNA. Both enzymes were inhibited 50% by 0.1 μM BuPDGTP in a standard α-polymerase assay (130 μM dGTP; 39). However, inhibition of SV40 chromatin replication in high-salt nuclear extracts containing PEG required a 1000-fold higher concentration of BuPDGTP (114 μM) to inhibit 50% of the total SV40 DNA synthesis (Fig. 5A). Analysis of the DNA synthesized under these conditions by agarose gel electrophoresis did not reveal an accumulation of SV40 early T-ag RI previously observed with aphidicolin (data not shown). When the same activated DNA substrate was added either to a high-salt nuclear extract or high-salt nucleosol, 50% of the DNA synthesis was inhibited by 4 μM BuPDGTP, demonstrating that the unexpectedly high concentration of BuPDGTP required to inhibit SV40 chromatin replication was not due to lability of the BuPDGTP in the presence of the cellular extracts. Furthermore, purified α-polymerase was inhibited 50% by 1.4 μM BuPDGTP when assayed under the same conditions used to initiate SV40 chromatin replication in vitro except that cellular and viral fractions were absent to avoid measuring endogenous DNA polymerase activities. These results reveal either the presence of a novel form of DNA polymerase-α that is associated specifically with replicating chromosomes, or the presence of a unique replicative enzyme, such as DNA polymerase-β (16, 40), that is resistant to BuPDGTP but sensitive to aphidicolin. ρSVori replication in high-salt nucleosol plus PEG was also tested for its sensitivity to BuPDGTP. Remarkably, ρSVori replication was reduced 50% by 15 μM BuPDGTP, intermediate between the sensitivity of SV40 chromatin and DNA polymerase-α. The sensitivity of DNA polymerase-α, ρSVori replication, and SV40 chromatin replication to aphidicolin was essentially the same under all conditions; 5–10 μM aphidicolin inhibited dNMP incorporation 50% (Fig. 8B), as previously reported for SV40 DNA replication in isolated nuclei and nuclear extracts (39).

**DISCUSSION**

De novo initiation of DNA replication in viral chromosomes isolated from infected cells was made evident by comparing the properties of subcellular systems that do not initiate replication with the properties of one that does and by comparing the replication of chromosomes with that of purified plasmid DNA. Incubation of SV40 chromatin in a high-salt nucleosol from virus-infected cells, a cytosol fraction, polyethylene glycol, and a buffered salts solution containing all four dNTPs resulted in a rate of DNA synthesis that was 7- to 12-fold higher in a 311-bp DNA restriction fragment containing ori than at an equivalent length of DNA that was located about 1800 bp from ori (Figs. 2–4). Since ori constitutes a minimum of 64 bp (ori-core) and a maximum of about 155 bp (ori-core plus flanking auxiliary sequences (1, 2)), from 21 to 50% of the DNA synthesis in this fragment resided within ori itself. This observation was confirmed by the appearance of early RI with the same electrophoretic mobility that is observed in vivo (slower than Form I DNA but faster than Form II DNA, Fig. 1). Therefore, these newly initiated molecules contained covalently closed parental DNA strands that, following DNA purification, expressed the superhelical turns imposed by their organization into nucleosomes (1, 3). DNA replication proceeded bidirectionally from ori in either the presence (11) or absence (Fig. 4, top panel) of aphidicolin, but accumulation of early RI was most easily observed in the absence of aphidicolin which rapidly suppressed DNA synthesis proceeding beyond the ori-region (Fig. 5 and Ref. 11). However, when either the PEG or high-salt nuclear extract were omitted from the in vitro reaction, little or no preference for DNA synthesis at ori was observed, no discrete population of early RI was observed either in the presence or absence of aphidicolin, and newly synthesized DNA was predominantly located in middle-to-late RI that had undergone bidirectional replication against ori (Fig. 1–4 and Ref. 11). These data were consistent with continued elongation of RI that were initiated in vivo in the absence of new initiation events in vitro (3, 13, 14, 18). Therefore, PEG and high-salt nuclear extract appeared to specifically stimulate a low-salt cytoplasmic extract to initiate new rounds of DNA replication.

In vitro DNA replication in SV40 chromatin also required T-ag which was generally provided by the high-salt nucleosol from infected cells. Replication was inhibited either by omitting this fraction or by addition of a monoclonal antibody directed against SV40 T-ag (Fig. 5). Addition of purified T-ag restored most of the activity (11). Several monoclonal antibodies directed against different T-ag domains, including PAb419, inhibited DNA synthesis at ori without inhibiting DNA synthesis at regions distal to ori. Although it was not possible to select the ori sequence in endogenous viral chromosomes, it was possible to show that ori was required to replicate purified plasmid DNA under the same conditions used to replicate viral chromatin (Fig. 6). Since plasmid DNA replication, like chromatin replication, required SV40 T-ag (6), initiation of chromatin replication must also require a functional ori. Furthermore, both ori-dependent plasmid replication and SV40 chromatin replication continued to replicate in vitro for at least 3 h at similar rates of DNA synthesis (Table I), whereas subcellular systems that simply continue replication of pre-initiated RI expire after 30–60 min (3, 13, 14, 18). Plasmid DNA became Mbol sensitive during this period and therefore had reinitiated replication (Fig. 7, lanes j–y) which resulted in accumulation of newly replicated DNA larger than RI* (Fig. 7, lanes a–t). Similarly, chromatin continued to undergo DNA synthesis at ori (Fig. 3B) which also resulted in accumulation of high molecular weight material (Fig. 1, lanes j–o). This high molecular weight DNA was not observed under in vitro conditions that did not permit initiation of replication (Fig. 1, lanes a–e). Taken together, the above results suggest that both plasmid DNA and endogenous SV40 chromatin underwent multiple rounds of SV40 ori-dependent, T-ag-dependent DNA replication in vitro.

Four significant differences were observed between in vitro replication of chromatin and plasmid DNA. First, initiation of DNA replication in plasmids occurred on non-nucleosomal DNA, because newly initiated plasmid DNA in this (Fig. 7) and other in vitro systems (29, 41) migrated with or slower than Form II DNA, as expected for RI with topologically relaxed parental DNA strands. Some superhelical DNA (Form

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1 R. S. Decker, M. Yamaguchi, R. Possenti, M. K. Bradley, and M. L. DeFamphilis, unpublished results.

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I) was produced, consistent with assembly of nascent DNA into nucleosomes (42), but most of the DNA product was high molecular weight material that may or may not have been assembled into nucleosomes (Fig. 7). In fact, other in vitro conditions that are optimal for assembling newly replicated DNA into nucleosomes are inhibitory for initiation of SV40 DNA replication (41, 42). Second, conditions that were optimal for initiation of DNA replication were suboptimal for separating sibling molecules, although plasmid DNA replication was more efficient at completing replication and producing Form I and II DNA than was chromatin replication. PEG specifically stimulated initiation of replication but inhibited completion of replication. PEG inhibited replication of endogenous SV40 chromatin in isolated nuclei where initiation of replication did not occur. Under conditions where initiation did occur, the high molecular weight DNA produced was converted into genomic length monomers upon cleavage at a single restriction endonuclease site (Figs. 1, 6, 7 and Refs. 6, 11). Therefore, this material had completed replication but was SV40 chromatin replication (Fig. 8) even though both were equally as sensitive to inhibition by aphidicolin (Fig. 8B), consistent with a recent report that DNA replication in permeabilized human fibroblasts is about 500 times more resistant to BuPDGTP than is purified DNA polymerase-α (43). Therefore, either plasmid DNA replication is deficient in one or more cofactors that modify the behavior of DNA polymerase-α, or plasmid replication utilizes a mixture of DNA polymerases-α and -δ, while chromatin replication utilizes DNA polymerase-δ exclusively (16, 40).

Previously published data supporting DNA polymerase-α as the enzyme solely responsible for DNA synthesis during SV40, polyoma virus, and mammalian chromosome replication (1–3) are also consistent with DNA polymerase-δ (16, 37, 40, 44–46). First, with the exception of BuPDGTP (15, 16, 40), DNA polymerases-α and -δ are remarkably similar in their sensitivities to inhibitors. Second, in virtually all of the studies attempting to correlate polymerase-α activity with DNA replication activity, polymerase-δ activity was not considered. Third, the most direct evidence that DNA polymerase-α is involved in SV40 DNA replication comes from elimination of polymerase-α activity in cellular extracts either by treatment with N-ethylmaleimide (39) or by passing them over immobilized monoclonal antibody SJK-287 directed against polymerase-α (7) and then demonstrating that only purified polymerase-α will restore activity. However, SJK-287 partially cross-reacts with polymerase-δ (44), and the absence of polymerase-δ in the preparations of polymerase-α was not established. Furthermore, the difference between DNA replication in SV40 ori-containing plasmids and SV40 chromatin in sensitivity to BuPDGTP (Fig. 8) indicates that polymerase-δ may specifically initiate and carry out replication only when the DNA substrate is organized into a unique chromatin structure. Thus, it is possible that DNA polymerase-δ is the replicative enzyme in vivo. Alternatively, the sensitivity of polymerase-α to BuPDGTP may depend on the structure of the DNA template. This notion is reflected in the decreasing sensitivity of DNA synthesis to BuPDGTP on activated DNA, plasmid DNA, and chromatin (Fig. 8), as well as the fact that utilization of dNTP substrates varies 200-fold depending on whether polymerase-α is assayed on single-stranded DNA, double-stranded DNA, or as "replicase" activity in isolated nuclei (47). A more detailed comparison of the properties of DNA polymerases-α and -δ will be needed to resolve this question.

Acknowledgments—We are indebted to Beth Weiner for providing T-ag and for her assistance with plasmid DNA replication assays.

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