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Distribution of Replicating Simian Virus 40 DNA in Intact Cells and Its Maturation in Isolated Nuclei

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The maturation of replicating simian virus 40 (SV40) chromosomes into superhelical viral DNA monomers [SV40(I) DNA] was analyzed in both intact cells and isolated nuclei to investigate further the role of soluble cytosol factors in subcellular systems. Replicating intermediates [SV40(RI) DNA] were purified to avoid contamination by molecules broken at their replication forks, and the distribution of SV40(RI) DNA as a function of its extent of replication was analyzed by gel electrophoresis and electron microscopy. With virus-infected CV-1 cells, SV40(RI) DNA accumulated only when replication was 85 to 95% completed. These molecules [SV40(RI*) DNA] were two to three times more prevalent than an equivalent sample of early replicating DNA, consistent with a rate-limiting step in the separation of sibling chromosomes. Nuclei isolated from infected cells permitted normal maturation of SV40(RI) DNA into SV40(I) DNA when the preparation was supplemented with cytosol. However, in the absence of cytosol, the extent of DNA synthesis was diminished three- to fivefold (regardless of the addition of ribonucleotide triphosphates), with little change in the rate of synthesis during the first minute; also, the joining of Okazaki fragments to long nascent DNA was inhibited, and SV40(I) DNA was not formed. The fraction of short-nascent DNA chains that may have resulted from dUTP incorporation was insignificant in nuclei with or without cytosol. Pulse-chase experiments revealed that joining, but not initiation, of Okazaki fragments required cytosol. Cessation of DNA synthesis in nuclei without cytosol could be explained by an increased probability for cleavage of replication forks. These broken molecules masqueraded during gel electrophoresis of replicating DNA as a peak of 80% completed SV40(RI) DNA. Failure to convert SV40(RI*) DNA into SV40(I) DNA under these conditions could be explained by the requirement for cytosol to complete the gap-filling step in Okazaki fragment metabolism: circular monomers with their nascent DNA strands interrupted in the termination region [SV40(II*) DNA] accumulated with unjoined Okazaki fragments. Thus, separation of sibling chromosomes still occurred, but gaps remained in the terminal portions of their daughter DNA strands. These and other data support a central role for SV40(RI*) and SV40(II*) DNAs in the completion of viral DNA replication.

Simian virus 40 (SV40) and polyoma virus have provided relatively simple, but appropriate models for investigating mammalian chromosome replication (10, 13, 31; M. L. DePamphilis and P. M. Wassarman, *in Organization and Replication of Viral DNA*, in press). These viruses replicate as small circular chromosomes in the nuclei of their hosts and, with the exception of initiation of viral DNA replication, appear to rely solely on the host to carry out all subsequent steps in DNA replication and chromatin assembly. The final stages in replicon maturation, as well as the events at replication forks, appear to be the same for both viruses and cells since the topological problems in separating two sibling viral chromosomes are analogous to the merger of two adjacent replicons. The ability to rotate one DNA strand about the other is as restricted in an infinitely long linear DNA molecule as it is in a circular, covalently closed molecule.

Extensive analysis of the structure of viral chromosomes and their replication in lytically infected cells and subcellular systems derived from these cells has revealed a detailed picture of the sequence of molecular and enzymological events at native replication forks (11, 13, 14, 17, 22; DePamphilis and Wassarman, in press). However, the fundamental problem of separating sibling chromosomes as two replication forks

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advance toward one another remains a relative mystery. To elucidate this aspect of chromosome replication, the principle DNA intermediates, proteins, and other factors required to complete replication must be identified and characterized. Previously, data have been reported that support the accumulation of replicating DNA intermediates at about 90% replication (RI*) (6, 40, 45, 53), as a consequence of the arrest of replication forks in the genomic region where replication is terminated (54). These molecules can then separate into circular DNA one genome long containing a short gap in the nascent DNA strand within the termination region (II*) (9, 18, 33, 52). In isolated nuclei or nuclear extracts, termination of DNA replication, which results in the formation of circular, covalently closed, superhelical DNA (I), requires soluble proteins found in the cytosol fraction (16, 17, 47). In contrast to this view of replicon maturation, other data have been interpreted to support a completely uniform movement of replication forks (3–5, 36, 38), with separation of sibling chromosomes occurring via the formation and subsequent resolution of catenated dimers (48).

In this paper, we present additional data that support a central role for SV40(RI*) and SV40(II*) DNA in the maturation of replicating SV40 DNA to SV40(I) DNA (see Fig. 10). Furthermore, soluble factors found in cytosol from uninfected CV-1 cells are specifically required to complete the synthesis and joining of Okazaki fragments and to protect replicating intermediates [SV40(RI) DNA] from cleavage at replication forks. These observations alone can account for the failure of washed nuclei to synthesize SV40(I) DNA and for the cytosol requirement for extensive DNA synthesis in subcellular systems.

MATERIALS AND METHODS

Cells and virus. The growth and preparation of a CV-1 monkey cell line and a plaque-purified strain of SV40 (wt 800) have been described previously (29).

SV40 DNA synthesis in isolated nuclei. CV-1 cells which had just reached confluency in plastic dishes (diameter, 100 mm) were infected with SV40 at a multiplicity of 10 to 20 PFU/cell. SV40(I) and SV40(II) DNAs were radiolabeled by incubating the infected cells with [^{3}H]thymidine for 6 h immediately before isolation of nuclei (15). Alternatively, SV40(RI) DNA was radiolabled with [^{3}H]thymidine for 3.5 min when the rate of viral DNA replication reached a maximum at 36 h after infection (15). Nuclei were then isolated, suspended to a concentration of 10^7 nuclei per ml, and incubated under conditions that allowed DNA replication to continue (2). In some experiments cytosol was included during incubation (2, 16). α-^{32}P-labeled deoxyribonucleoside triphosphates (5 to 200 Ci/mmol) were present at a concentration of 10 μM. In pulse-chase experiments, a 100-fold excess of the appropriate unlabeled deoxyribonucleoside triphosphate containing an equimolar amount of $MgCl_2$ was added. DNA synthesis was terminated (2) by the method of Hirt (23), and viral DNA was extracted by using either 1 M NaCl or CsCl, depending on whether the DNA in the Hirt supernatant was to be analyzed directly by sedimentation in sucrose gradients or further purified in CsCl density equilibrium gradients (16).

Digestion of SV40(RI) DNA by S1 nuclease or *BglI* restriction endonuclease. SV40(RI) [^{3}H]DNA was synthesized by incubating infected cells with [^{3}H]thymidine for 30 min at 36 h postinfection (53) and then purified (54). The supernatant from a Hirt extract (23) was adjusted with CsCl to yield a density of 1.700 g/ml and centrifuged for 48 h at 18°C in a Beckman 60Ti rotor at 40,000 rpm to establish a density equilibrium gradient. SV40(RI) [^{3}H]DNA was then purified by chromatography on benzoylated-naphthylated DEAE-cellulose (BND-cellulose) and sedimented through a neutral sucrose gradient containing 1 m NaCl. In some experiments, purified SV40(RI) [^{3}H]DNA (1 μg) was either incubated with single-strand-specific S1 endonuclease (21) or digested with *BglI* restriction endonuclease under the conditions described by the supplier. Reactions were terminated in 12 mM EDTA. Samples from either reaction mixture were then deproteinized and prepared for either gel elecrophoresis or electron microscopy (54).

Analysis of SV40 DNA by gel electrophoresis. Native purified SV40(RI) [^{3}H]DNA labeled in whole cells and native purified SV40(RI) [^{32}P]DNA labeled in isolated nuclei were analyzed by electrophoresis in cylindrical 1.4% agarose gels (0.8 by 10 cm) (53). Before analysis, SV40(I) and SV40(II) [^{32}P]DNA or [^{3}H]DNA standards (15) were added to the samples. Gels were cut into 1.2-mm slices, digested overnight at 55°C in 10 ml of a toluene-based scintillation fluid containing 3% NCS (Amersham/Searle), and then analyzed in a liquid scintillation counter. Alternatively, agarose gels containing SV40(RI) [^{3}H]DNA isolated from intact cells were sliced longitudinally into equal halves (53). Each half was cut into 1.2-mm slices. One-half was analyzed as described above, whereas DNA from the other half was eluted electrophoretically by a modification of the method of Tabak and Flavell (50). Appropriate gel slices were pooled and placed in a 1-ml pipette tip with a glass wool plug. The tip containing the gel slices was inserted into another 1-ml pipette tip, which contained 0.2 ml of hydroxyapatite layered over 1.5 ml of Sephadex G-50, both equilibrated with electrophoresis buffer (40 mM Tris-hydrochloride, pH 7.6, 50 mM sodium acetate, 1 mM EDTA). Electroelution was then carried out at 100 V for 1.5 h, after which the column was removed and eluted with 1 M KPO_4 and the SV40 DNA was concentrated by centrifugation (54). The concentrated DNA (200 μl) was passed over a 5-ml Sephadex G-100 column to remove KPO_4, again concentrated by centrifugation, and finally precipitated after the addition of 0.25 M sodium acetate and 3 volumes of ethanol. This technique allowed the recovery of an average of 50% of the DNA in an undamaged form without the addition of carrier RNA or DNA.

Denatured SV40(RI) [^{3}H]DNA, with or without prior digestion by *BglI* restriction endonuclease, was fractionated by gel electrophoresis on 2% agarose slab gels (24 by 13 cm, with 0.15-cm spacers) after denaturation in glyoxal (41). Radioactivity was detected by

fluorography on preflashed Kodak type SB X-ray film (34).

SV40(II) [^{32}P]DNA synthesized in isolated nuclei was extracted and purified (52) before denaturation in glyoxal (41) and electrophoresis in 2% agarose slab gels (13 by 16 cm, with 0.15-cm spacers). Before denaturation, SV40(I,II) [^{3}H]DNA standards were added to the samples. The buffer was recirculated continuously between the upper and lower reservoirs. After electrophoresis, individual lanes were excised, and the top 10 cm of each lane was divided into 1.2-mm slices. The radioactivity in individual slices was measured as described above.

Sedimentation in neutral and alkaline sucrose gradients. Neutral and alkaline linear sucrose gradients were prepared in 1 M Na^+ (15). The details of this procedure are given in the figure legends.

Electron microscopy. DNA samples were prepared for electron microscopy by using either the aqueous procedure or the formamide procedure (12). Grids were rotary shadowed with platinum-palladium (80:20) and viewed with a Zeiss model EM10 electron microscope. Photographic images were projected onto a platform, and contour lengths were measured with a Hewlett-Packard model 9107A digitizer.

Materials. Restriction endonucleases were purchased from New England Biolabs, S1 nuclease was from Sigma Chemical Co., BND-cellulose was from either Gallard-Schlessinger or Boehringer Mannheim Corp., and [^{3}H]thymidine and [^{32}P]orthophosphoric acid were from New England Nuclear Corp. Glyoxal was purchased from Fisher Scientific Co. as a 40% aqueous solution and was deionized with type AG-501 resin (Bio-Rad Laboratories) just before use. α-^{32}P-labeled deoxyribonucleoside triphosphates were synthesized by the method of Symons (49), as modified by Rigby et al. (44).

RESULTS

Distribution of SV40(RI) DNA in intact cells and accumulation of SV40(RI*) DNA. In previous studies, SV40(RI) DNA was fractionated by gel electrophoresis as a linear function of its extent of replication, and the relative amounts of replicating DNA were determined throughout the gel (53). The nascent DNA chains in these replicating molecules either had been labeled uniformly in intact cells or had been pulse-labeled in isolated nuclei supplemented with cytosol. The results revealed a three- to fourfold accumulation of DNA that was 85 to 95% replicated [i.e., SV40(RI*) DNA] compared with an equivalent sample of DNA at early stages of replication. In addition, a smaller accumulation of DNA was observed at 80% replication. Although this second peak was not prominent in DNA labeled in whole cells, continued DNA replication in nuclei (53) or nuclear extracts (47) significantly increased its amount. Subsequently, the locations of replication forks in the termination region of SV40(RI) DNA from intact cells were mapped with respect to restriction endonuclease sites. This demonstrated that most forks were arrested when replication was 91% completed and that the two forks were separated by about 470 base pairs of unreplicated DNA centered at the expected termination site. However, although replication forks entering the termination region were arrested at several different DNA sites, none of the data appeared to account for an accumulation of replicating DNA at 80% completion. Other experiments suggested that DNA which appeared to accumulate at 80% replication may in fact have represented various forms of catenated dimers (48) or damaged replicating molecules (5, 38). Consequently, the steady-state distribution of SV40(RI) DNA in intact cells was reexamined.

SV40(RI) [^{3}H]DNA, containing uniformly labeled nascent DNA strands, was extracted from intact cells by the method of Hirt (23) and then purified by sedimentation to equilibrium in CsCl, followed by chromatography on BND-cellulose (53). This procedure avoided any loss of either very early or very late replicating molecules, but it did not exclude SV40(RI) DNA that may have been damaged. Therefore, a final sedimentation step was added to remove replicating molecules broken at one or both replication forks. Such molecules (16 to 20S) sediment slower than SV40(RI) DNA (21). An electron microscopic analysis of the purified replicating DNA confirmed the absence (<0.1%) of broken, rolling circle (3), and topologically relaxed (observed in the absence of ethidium bromide) forms of replicating intermediates (Table 1). The major contaminant was circular monomeric SV40 DNA, which was expected since the specific radioactivity of SV40(RI) [^{3}H]DNA prepared under these conditions was 8- to 10-fold higher than that of SV40(I) [^{3}H]DNA. Thus, although this purification procedure removed 90% of the ^{3}H-labeled monomeric DNA (Fig. 1A) (53), a significant amount of monomeric DNA remained. When both SV40(RI) and SV40(I) [^{3}H]DNAs had the same specific radioactivity, two cycles of BND-cellulose chromatography were required to remove all of the SV40(I) [^{3}H]DNA (data not shown).

Purified SV40(RI) [^{3}H]DNA was fractionated by electrophoresis in agarose gels (Fig. 1A) to display replicating viral DNA as a linear function of the extent of replication for molecules between 15 and 95% replicated (53). As previously observed, the SV40(I) and SV40(II) [^{32}P]DNA internal standards migrated as narrow, symmetrical peaks, whereas the SV40(RI) [^{3}H]DNA was broadly distributed from the position of SV40(I) DNA to the top of the gel. However, in contrast to our previous findings (53), there was no distinct peak of [^{3}H]DNA in the region of 80% replication in four independent experiments. Therefore, some of the replicating

TABLE 1. Electron microscopic analysis of replicating SV40 DNA purified from intact cells[a]

Molecules	Total no.
Replicating	
RI	288 (31)[b]
Dimer	5 (1)
Rolling circle	1 (0.1)
Monomer (I + II)	550 (60)
Dimer	
Circular	33 (4)
Catenated	5 (1)
Broken	
III	15 (2)
RI	0 (0)

[a] Purified SV40(RI) [^{3}H]DNA was prepared for electron microscopy by the aqueous technique (12) in the presence of ethidium bromide (1 μg/ml) in the hypophase and sample, which allowed positive identification of 90% of the molecules observed. Replicating molecules included SV40(RI) DNA, replicating dimers, and rolling circles with a tail longer than one genome. Monomers included SV40(I) and SV40(II) DNAs. Dimers included circular and catenated DNA circles. SV40(III) DNA and circles with a tail shorter than one genome were considered broken molecules. See the legend to Fig. 10 for definitions.

[b] Numbers in parentheses are percentages.

DNA that accumulated in the 80% replicated region of the gel may have been broken at one fork. In fact, the elecrophoretic behavior of such molecules agreed with this expectation (see Fig. 9).

The percent replication of SV40(RI) DNAs with different mobilities was determined by three techniques (Fig. 1B). Gels were cut into nine sections, and the DNA in each section was isolated. Previously (53), the size of the nascent [^{3}H]DNA was determined by sedimentation in alkaline sucrose gradients. In the experiment shown in Fig. 1A, part of the DNA was denatured in glyoxal and analyzed by gel electrophoresis (Fig. 2). Digestion of a separate fraction of DNA with *BglI* cleaved SV40 DNA at a single site within 35 base pairs of the origin of replication (39, 54) and generated [^{3}H]DNA chains that were 50% of the original length (Fig. 2). The average deviation from the mean for each band (8%) was the deviation expected from the size of the gel section from which the DNA was extracted. Thus, most of the [^{3}H]DNA in sections 1 to 8 represented intact bidirectionally replicating SV40(RI) [^{3}H]DNA. This conclusion was further documented by an electron microscopic analysis of the DNA extracted from each gel section in Fig. 1A (data not shown). The DNA in sections 1 to 9 contained intact replicating molecules whose average extent of replication as measured after digestion with *BglI* was in excellent agreement with the results of the other two analyses (Fig. 1B). These data confirmed our previous finding that SV40(RI) DNA can be fractionated by gel electrophoresis as a linear function of its extent of replication (53).

The fractions of SV40(RI) [^{3}H]DNA at different stages of replication were calculated from both the distribution of [^{3}H]DNA after gel electrophoresis and an electron microscopic analysis. The total amount of ^{3}H label in sections 1 to 8 of Fig. 1A was divided by the average percent replication shown in Fig. 1B and the number of gel slices per section (53). In addition, a sample of purified SV40(RI) DNA was digested with *BglI* and analyzed by electron microscopy in order to determine directly the distribution of SV40(RI) DNA as a function of extent of replication (54). The results were in excellent agreement with our previously published data (Table 2). There was clearly a two- to threefold increase in molecules that were 85 to 95% replicated [i.e., SV40(RI*) DNA] compared with an equivalent sample of early SV40(RI) DNA.

Maturation of SV40(RI) DNA in isolated nuclei. Subcellular systems have been described that are capable of converting endogenous SV40(RI) DNA into SV40(I) DNA by a faithful continuation of bidirectional replication, as observed in intact cells (DePamphilis and Wassarman, in press). These systems consist primarily of either a cell lysate (15), nuclei plus cytosol (2, 16, 52), or a soluble nuclear extract plus cytosol (46, 47). The following data confirm and extend previous findings concerning the requirement for soluble factors found in cytosol for maturation of SV40(RI) DNA in isolated nuclei.

(i) Cytosol is required for extensive DNA synthesis. In the absence of cytosol, SV40 DNA synthesis in isolated nuclei was reduced three- to fivefold, whereas the initial rate of DNA synthesis was decreased only slightly (Fig. 3A). Both the extent of incorporation and the joining of Okazaki fragments to longer nascent DNA strands (see below) could be depressed further by washing the nuclei in concentrations of Triton X-100 greater than 0.02%. However, this also resulted in a corresponding loss of prelabeled viral DNA preferentially in the form of Okazaki fragments. It is noteworthy that washed nuclei from SV40-infected CV-1 cells (2, 52; this paper) produced less degraded replicating DNA than washed nuclei from BSC-1 cells (16), showing that both the preparation and the source of nuclei affect the results of in vitro experiments. However, the addition of cytosol always restored the amount of DNA synthesized per microgram of total DNA to its maximum level. Inclusion of rCTP, rGTP, and rUTP in the in vitro reaction mixture did not affect the initial

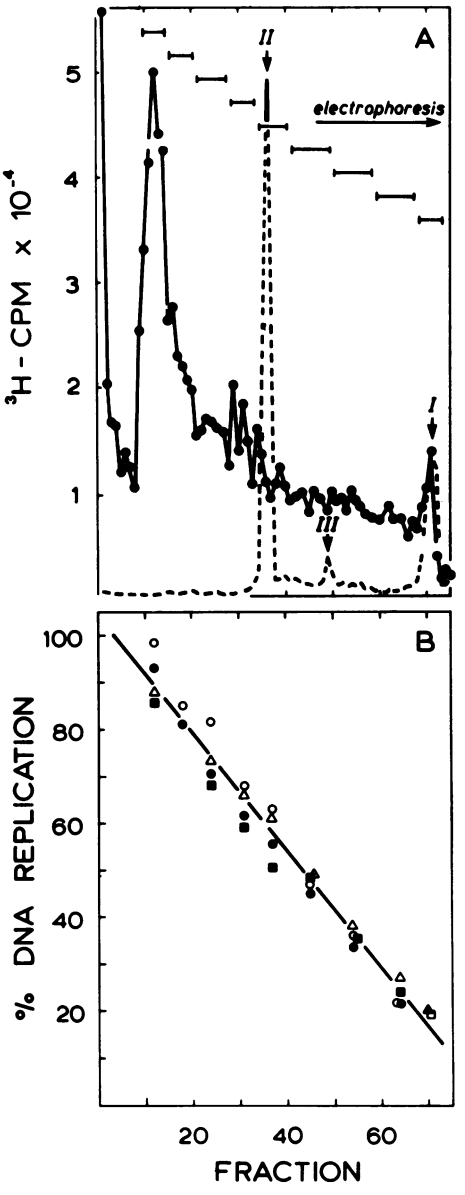

FIG. 1. Electophoretic analysis of the extent of replication in SV40(RI) [^{3}H]DNA from intact cells. (A) SV40(RI) [^{3}H]DNA was analyzed by agarose gel electrophoresis. The gel was cut longitudinally, and both halves were sliced into 1.2-mm sections. Slices from one half were used to measure the amount of radioactivity throughout the gel. Slices from the other half were pooled into nine sections (bars), from which the DNA was electroeluted. The solid line indicates SV40(RI) [^{3}H]DNA; the dashed line indicates SV40(I) and SV40(II) [^{32}P]DNA standards fractionated simultaneously in a separate gel. (B) Extent of replication for DNA extracted from each of the nine sections designated by the bars in (A), as determined by gel electrophoresis of the nascent [^{3}H]DNA either before (○) or after (●) *BglI* digestion (see Fig. 2) and by an electron microscopic analysis (■) of the SV40(RI) DNA after *BglI* digestion. In addition, the calibration curve previously determined by sedimentation of the [^{3}H]DNA in alkaline sucrose gradients (53) was plotted (△). The line is the result of at least-squares analysis of all of the data shown.

rate of DNA synthesis, but the extent of deoxyribonucleoside monophosphate incorporation was stimulated by approximately 20% regardless of whether cytosol was present (Fig. 3A).

(ii) Cytosol is required for completion of Okazaki fragments, not for their initiation. In the absence of cytosol, synthesis of SV40(I) DNA is reduced at least 20-fold (16). One likely reason for the increased dependence of SV40(I) DNA production on cytosol is the requirement for cytosol in the completion and joining of Okazaki fragments (1, 2); formation of RNA-DNA covalent junctions and the subsequent exision of these junctions are not affected significantly (2). To demonstrate further that the effect of cytosol on Okazaki fragment metabolism was primarily with completion and not with initiation, nuclei were isolated from SV40-infected cells and either immediately labeled with [α-^{32}P]dCTP or first incubated for 3.5 min with unlabeled dCTP before the radiolabel was added. Both preparations were then incubated in the presence of excess unlabeled dCTP to monitor the fate of ^{32}P-labeled Okazaki fragments. The size distribution of the nascent [^{32}P]DNA was analyzed by sedimentation in alkaline sucrose gradients, and the proportion of Okazaki fragments was calculated (1). These data showed that Okazaki fragments were initiated continually in the presence of cytosol and were joined to long nascent DNA strands (Fig. 4). The proportion of [^{32}P]DNA labeled from 3.5 to 5 min and found as Okazaki fragments (38%) was at least equivalent to the proportion labeled from 0 to 1.5 min of incubation (32%), and the rate of Okazaki fragment disappearance was the same (half-life, 3.2 min). Similarly, in the absence of cytosol, Okazaki fragments also appeared to be initiated continually; they represented 46 and 54% of the [^{32}P]DNA synthesized from 0 to 1.5 min and from 3.5 to 5 min, respectively. However, the rate of joining of these fragments to longer DNA strands was retarded severely (Fig. 4). This conclusion was based on the following two facts: the initial rate of DNA synthesis in nuclei, which is 100 ± 20 nucleotides per min at 30°C with cytosol (47; unpublished data on the time required to obtained uniformly labeled Okazaki fragments), was only slightly decreased without cytosol (Fig. 3A), and the average size of mature Okazaki fragments is 135 nucleotides (range, 40 to 290 nucleotides) (1). Thus, most of the Okazaki fragments present initially should have completed synthesis in 3.5 min. Therefore, although

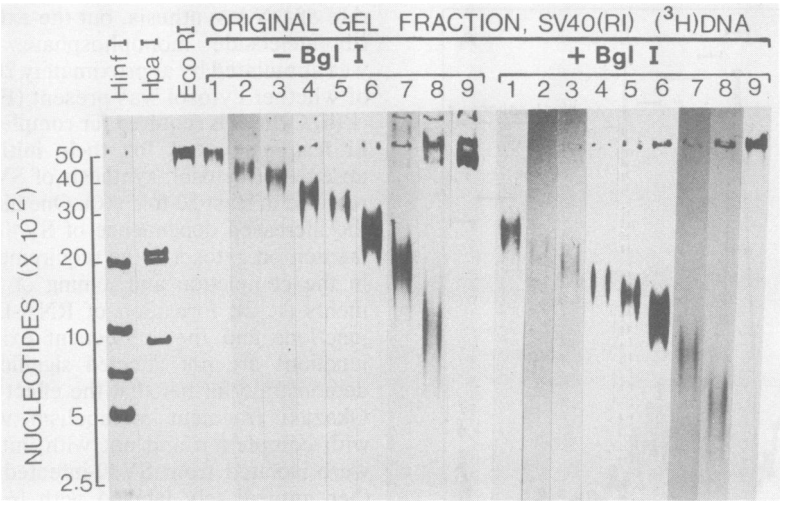

FIG. 2. Electrophoretic analysis of the size of nascent [^{3}H]DNA that was fractionated by gel electrophoresis. The SV40(RI) [^{3}H]DNAs from the nine sections of the gel in Fig. 1A were analyzed by electrophoresis in 2% agarose gels before and after digestion with *BglI* restriction endonuclease, and the [^{3}H]DNA was detected by autofluorography. Size standards were generated by digestion of SV40(I) [^{3}H]DNA with restriction endonucleases *Hin*fI, *Hpa*I, and *Eco*RI.

the fraction of Okazaki fragments observed may have been altered slightly by the incubation conditions, the requirement for cytosol in Okazaki fragment maturation is primarily at the gap-filling and ligation steps (1) and not at the initiation step.

An alternate explanation for the persistence of Okazaki fragments in the absence of cytosol could be the incorporation of endogenous dUTP into nascent DNA and its subsequent excision by uracil *N*-glycosylase, which generates short nascent DNA strands similar to genuine Okazaki

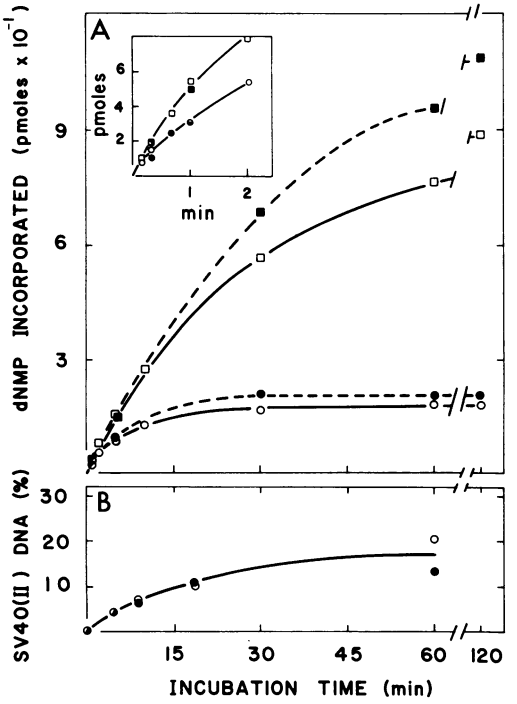

FIG. 3. Time course for SV40 DNA synthesis in nuclei with and without cytosol present. (A) SV40-infected CV-1 cells were incubated with [^{3}H]thymidine for 6 h to label SV40(I,II) DNA. Nuclei were then isolated and incubated with (□ and ■) or without (○ and ●) cytosol in the presence of [α-^{32}P]dCTP and [α-^{32}P]dATP. Viral DNA was extracted selectively and assayed for acid-precipitable radioactivity (2). [^{32}P]DNA was normalized to the [^{3}H]DNA in each sample to correct for variations in the number of nuclei per assay. Results were expressed as picomoles of [^{32}P]DNA synthesized as a function of time of incubation in vitro. ATP was always present at a concentration of 2 mM. In some cases, CTP, GTP, and UTP were also present at a concentration of 50 μM (● and ■). (Inset) Expanded scale to show initial rates of deoxyribonucleotide incorporation. dNMP, Deoxyribonucleoside monophosphate. (B) Fraction of SV40(II) DNA generated during incubation of washed nuclei (no cytosol present) as determined by sedimentation of viral DNA in neutral sucrose gradients (see Fig. 6). Symbols: ●, fraction of SV40(II) [^{3}H]DNA produced from SV40(RI) [^{3}H]DNA prelabeled for 3.5 min with [^{3}H]thymidine in intact cells [these values were corrected for the 4% of the label in SV40(II) DNA at the start of the in vitro incubation (see Fig. 6A).]; ○, SV40(II) [^{32}P]DNA labeled during the in vitro incubation in (A).

TABLE 2. Steady-state distribution of replicating SV40 DNA from intact cells

Purification step	No. of expt	Analysis	% of SV40(RI) DNA that replicated an average of:							90%/30% ratio
			30%	40%	50%	60%	70%	80%	90%	
Chromatography (BND-cellulose)	6[a]	Gel	10	10	10	11	13	18	28	2.8
Sedimentation (sucrose)	4	Gel	12	12	11	13	11	16	26	2.2
	1[b]	EM	12	12	13	12	12	14	25	2.1

[a] The data for the distribution of SV40(RI) DNA after BND-cellulose chromatography are from Tapper and DePamphilis (53) after the omission of molecules that were <25% replicated.

[b] The electron microscopic (EM) analysis included 313 molecules cleaved by *BglI*. The standard error of the mean was about 10% of the value shown.

fragments (8, 20, 56). However, under the conditions used in our experiments, this artifact was not observed. The inclusion of either dUTP or excess dTTP in the reaction mixture had little effect on the size or relative number of nascent SV40 DNA strands when nuclei were incubated with cytosol (Fig. 5D to F). Apparently, the ratio of dTTP to dUTP is maintained at a high level by the deoxyuridine triphosphatase present in cytosol (56). Similarly, the addition of excess dTTP had no effect on nuclei incubated without cytosol (Fig. 5A and C). Only when 10 μM dUTP was included in the incubation mixture was there a discernible increase in the relative amount of short nascent DNA strands and a decrease in the size of the strands (Fig. 5B). Apparently, washed nuclei also contained sufficient deoxyuridine triphosphatase activity to prevent significant incorporation of any endogenous dUTP.

(iii) Separation of sibling molecules in SV40(RI*) DNA yields SV40(II*) DNA. In the absence of cytosol, nuclei containing SV40(RI) DNA labeled either for 3.5 min with [^{3}H]thymidine in intact cells (Fig. 6A) or for 5 min with [α-^{32}P]dCTP in washed nuclei (Fig. 6D) allowed a limited continuation of DNA replication (Fig. 3A) that converted 12 to 20% of the radiolabel into SV40(II) DNA (Fig. 6B and E). The time course of SV40(II) DNA formation was the same as that observed for [α-^{32}P]dCTP incorporation (Fig. 3B), which is consistent with its being a product of replication. This SV40(II) DNA did not result from random endonuclease activity since none of the SV40(I) [^{3}H]DNA prelabeled in intact cells was converted to SV40(II) [^{3}H]DNA (Fig. 6D and E). SV40(II) DNA was also generated in nuclei incubated with cytosol (Fig. 6C and F), but some of this DNA may have resulted from the endonuclease activity observed under these conditions (Fig. 6D and F).

The SV40(II) [^{32}P]DNA synthesised in vitro was purified as previously described (52) and then analyzed under denaturing conditions (Fig. 7) in order to determine whether its structure was consistent with its proposed role in SV40 DNA replication (see Fig. 10). Topologically relaxed circular monomers containing a short gap in the nascent DNA chain within the termination region [SV40(II*) DNA] have been identified in intact cells as transient intermediates in bidirectional DNA replication, resulting from separation of sibling molecules in SV40(RI) DNA (9, 18, 52). Thus, after denaturation of SV40(II*) [^{32}P]DNA synthesized in vitro, the label should have been found exclusively in linear single-stranded DNA chains one genome long. In fact, after a 10-min incubation of nuclei plus cytosol, 87% of the label in SV40(II) [^{32}P]DNA was in the expected linear DNA (Fig. 7C); by 60 min, 77% of the ^{32}P label was still found in single-stranded linear DNA (Fig. 7D). This result could not be explained by a single randomly placed endonucleolytic cleavage of newly synthesized SV40(I) DNA because this

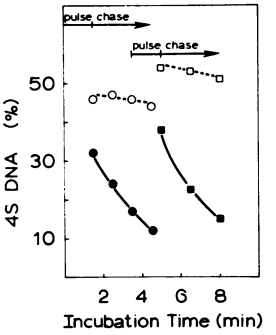

FIG. 4. Steady-state synthesis of Okazaki fragments during SV40 DNA replication in nuclei with and without cytosol. Nuclei isolated from SV40-infected CV-1 cells were incubated with [α-^{32}P]dCTP (pulse) either from 0 to 1.5 min (○ and ●) or from 3.5 to 5.0 min (□ and ■) in the presence (● and ■) or in the absence (○ and □) of cytosol. At the end of the radiolabeling periods, a 100-fold excess of unlabeled dCTP was added, and the incubation was continued (chase). Before the labeling period from 3.5 to 5.0 min, a 20 μM concentration of each deoxyribonucleoside triphosphate was present to allow DNA replication to proceed. At the indicated times, SV40(RI) [^{32}P]DNA was isolated, and the proportion of ^{32}P label in Okazaki fragments was determined by sedimentation in alkaline sucrose gradients (2).

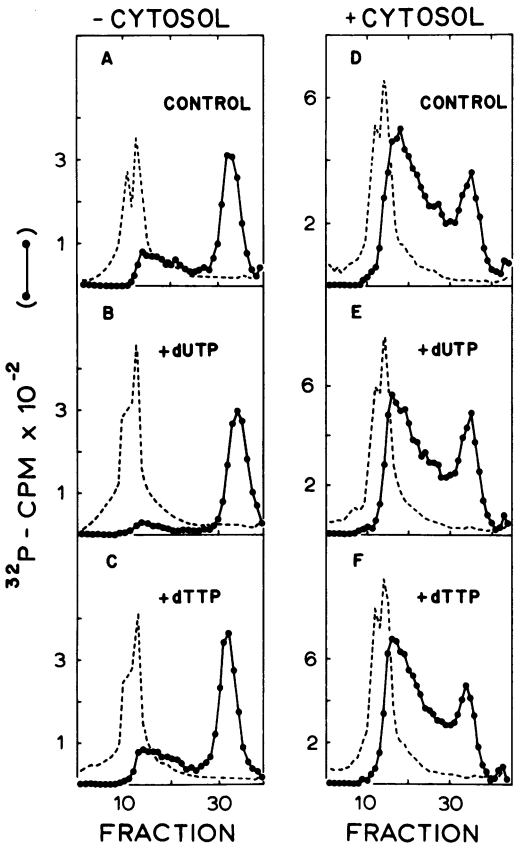

FIG. 5. Effect of dUTP on SV40 DNA replication. Nuclei isolated from SV40-infected CV-1 cells were labeled with [α-^{32}P]dGTP for 1.5 min either in the presence (D through F) or absence (A through C) of cytosol. The controls contained 20 μM dTTP (A and D). In addition, two samples contained 10 μM dUTP (B and E), and two samples contained 200 μM dTTP (C and F). SV40(RI) DNA was extracted, purified on preparative neutral sucrose gradients, and analyzed by sedimentation in alkaline sucrose gradients (2). SV40(II) [^{3}H]DNA was included as a marker (dashed line). Sedimentation was from right to left.

would have generated equal amounts of ^{32}P label in both single-stranded circles and linear molecules. Even after a 1-h incubation, only 20% of the prelabeled SV40(I) [^{3}H]DNA was converted to SV40(II) [^{3}H]DNA (Fig. 6F), demonstrating a maximum of one nick per molecule. SV40(II) DNA containing more than one randomly placed nick showed a slight preference for single-stranded linear molecules over circles [note the DNase I-prepared SV40(II) DNA standard in Fig. 7C and D].

Further evidence that SV40(II*) DNA represents an intermediate in the maturation of SV40(RI) DNA was obtained by an analysis of SV40(II) DNA generated in nuclei without cytosol. SV40(II) [^{32}P]DNA synthesized either in washed nuclei or in nuclei plus cytosol had less than 10% of the label in single-stranded circles (Fig. 7A and B). However, in the absence of cytosol, SV40(II) [^{32}P]DNA synthesized in the first 10 min also contained about 40% of the label in Okazaki fragments, whereas incubation in the presence of cytosol gave SV40(II) [^{32}P]DNA with only full-length strands (Fig. 7A). Similar results were obtained after a 1-h incubation (Fig. 7B). A previous analysis of SV40(II) [^{32}P]DNA synthesized in washed nuclei showed that only the termination region was labeled (52). Therefore, when the joining of Okazaki fragments was inhibited by the absence of cytosol, some of the SV40(RI) DNA close to completion [SV40(RI*) DNA] still underwent separation to generate SV40(II*) DNA containing Okazaki fragments in the termination region.

(iv) Cytosol is required for stabilization of SV40(RI) DNA. A previous analysis (46, 53) of the fate of SV40(RI) DNA in nuclei supplemented with cytosol demonstrated that the majority of the molecules that did not complete replication accumulated when they were 90% replicated [i.e., SV40(RI*) DNA]. The fate of SV40(RI) DNA in the absence of cytosol was still not known. Although the addition of cytosol to nuclei before incubation at 30°C completely restored the ability of nuclei to continue SV40 DNA replication, later additions of cytosol with or without added deoxyribonucleoside triphosphates progressively reduced its effectiveness; by 40 min, stimulation was only marginal. This observation implied that one or more factors required for replication in isolated nuclei (including the replicating DNA) were inactivated during incubation.

A requirement for cytosol for stabilization of SV40(RI) DNA was demonstrated by incubating nuclei with [α-^{32}P]dCTP for 45 s, followed by a chase period of up to 1 h in excess unlabeled dCTP; then the SV40(RI) [^{32}P]DNA was purified, and its extent of replication was analyzed by gel electrophoresis. The initial distribution of SV40(RI) [^{32}P]DNA (Fig. 8A) was indistinguishable from the distribution obtained in the presence of cytosol (53). After 15 min, when DNA synthesis was essentially completed (Fig. 3A), a prominent peak was observed at 90% replication [SV40(RI*) DNA], and a more heterogeneous peak was observed at 80% replication; the remaining [^{32}P]DNA was distributed throughout the lower region of the gel (Fig. 8B). However, further incubation produced changes in the conformation of [^{32}P]DNA, even though DNA synthesis had stopped. By 60 min, the sharp peak of SV40(RI*) DNA that migrated near SV40 relaxed circular dimers had decreased 40%, and there was a concomitant increase in the size and

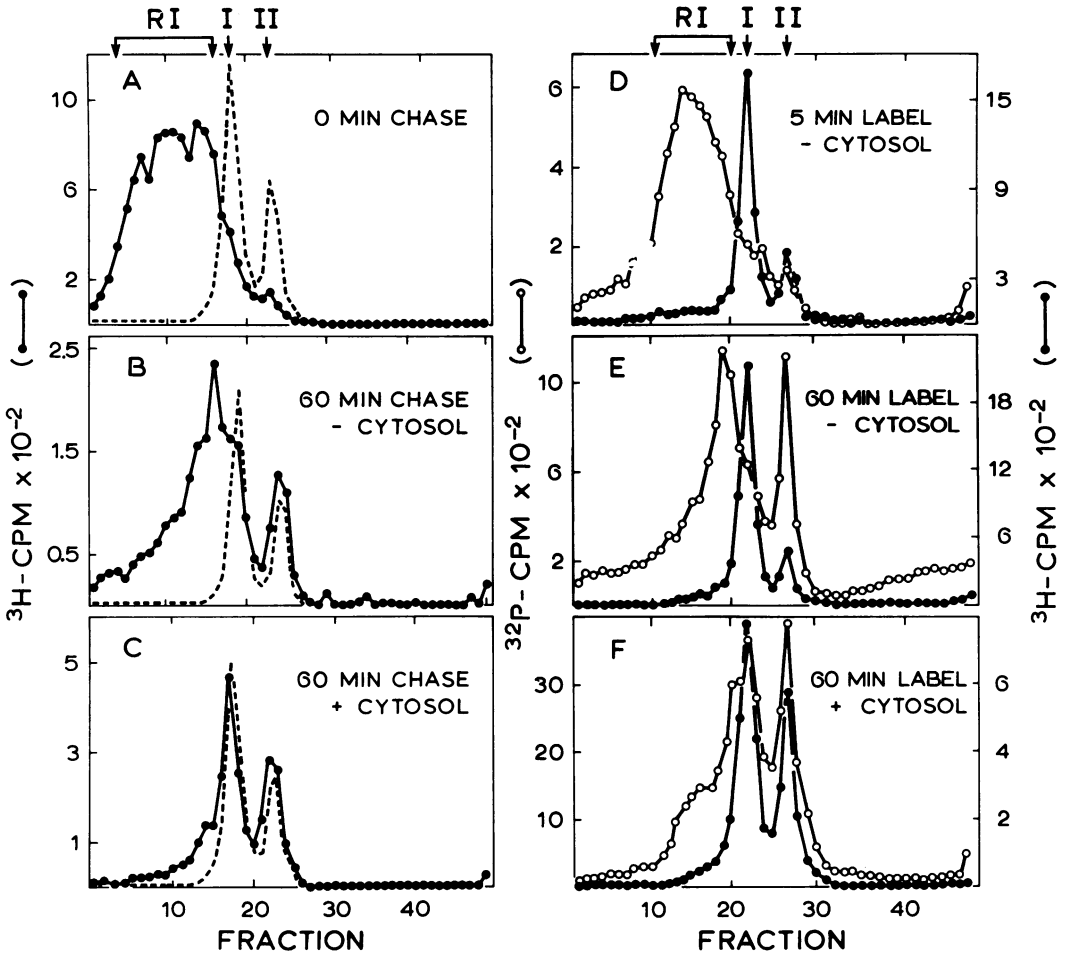

FIG. 6. Products of SV40 DNA replication analyzed by sedimentation in neutral sucrose gradients. SV40 DNA in infected CV-1 cells was radiolabeled with [^{3}H]thymidine for either 3.5 min to label SV40(RI) DNA or 6 h to label SV40(I,II) DNA. Nuclei were then isolated and incubated with or without cytosol in the presence or absence of [α-^{32}P]dCTP and [α-^{32}P]dATP. Viral DNA was extracted from nuclei by the method of Hirt (23) and was sedimented in neutral 5 to 20% sucrose gradients by using a Beckman SW60 rotor for 3.25 h at 55,000 rpm and 4°C. SV40(I) and SV40(II) [^{32}P]DNA standards (dashed lines) were added just before sedimentation. The direction of sedimentation was from right to left. (A) Viral DNA prelabeled in vivo for 3.5 min. (B) Viral DNA prelabeled in vivo for 3.5 min and then incubated in vitro without cytosol for 60 min. (C) Viral DNA prelabeled in vivo for 3.5 min and then incubated in vitro with cytosol for 60 min. (D) Viral DNA prelabeled in vivo for 6 h and then incubated in vitro for 5 min without cytosol. (E) Viral DNA prelabeled in vivo for 6 h and then labeled in vitro for 60 min without cytosol. (F) Viral DNA prelabeled in vivo for 6 h and then labeled in vitro for 60 min with cytosol. Symbols: ●, SV40 [^{3}H]DNA prelabeled in intact cells (in vivo); ○, SV40 [^{32}P]DNA labeled in nuclei (in vitro).

homogeneity of material at 80% replication and decrease in material migrating as "early" replicating DNA (Fig. 8C). Analogous conformational changes in SV40(RI) DNA were also apparent when the less sensitive method of sedimentation analysis was used (Fig. 6A, B, D, and E; data not shown). Furthermore, when DNA synthesis was inhibited 95% by the addition of cytosine 1-β-D-arabinoside-5'-triphosphate after the 45-s radiolabeling period, the same conformational changes in SV40(RI) [^{32}P]DNA were observed in neutral sucrose gradients (data not shown). An electron microscopic analysis of total SV40(RI) DNA isolated from nuclei incubated for 1 h either with or without cytosol revealed some circular viral DNA molecules with a tail; these were most prevalent in nuclei incubated without cytosol. Therefore, in the absence of cytosol, some SV40(RI) DNA completed replication, and some was damaged. The conformational changes were consistent with either topologically relaxed replicating DNA or molecules

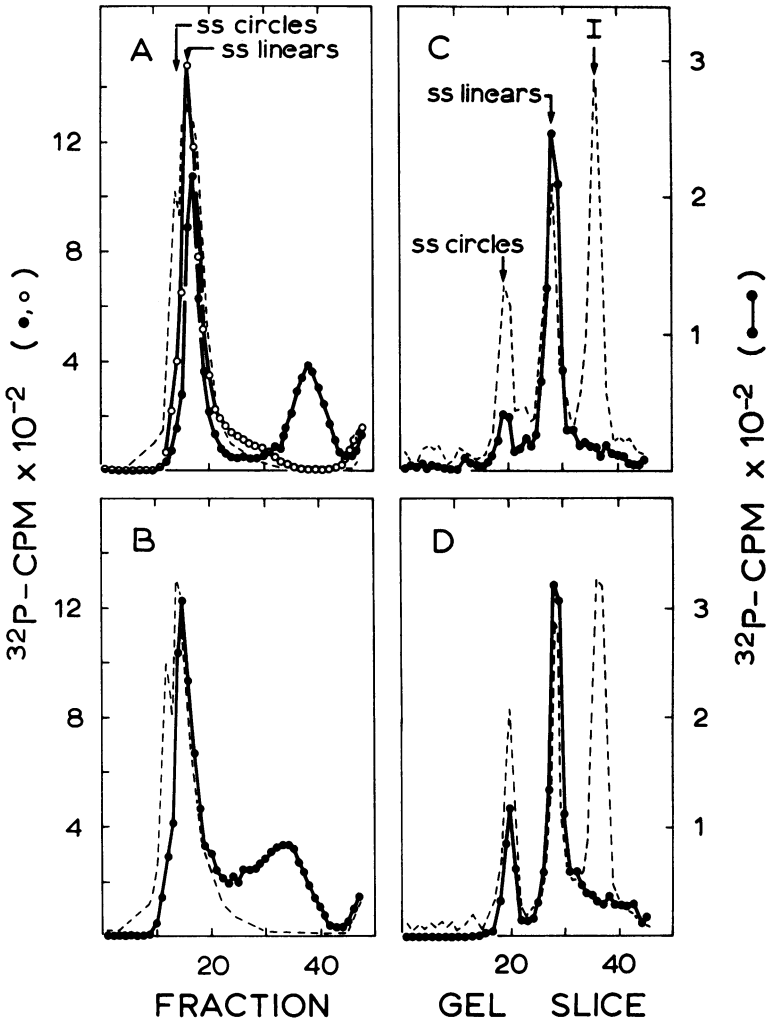

FIG. 7. Structure of SV40(II) [^{32}P]DNA synthesized in washed nuclei with and without cytosol. Nuclei were isolated from SV40-infected CV-1 cells and incubated with [α-^{32}P]dCTP in the presence or absence of cytosol. SV40(II) [^{32}P]DNA was purified from the nuclei after either 10 min (A and C) or 60 min (B and D) of incubation and was analyzed either by sedimentation in alkaline 5 to 20% sucrose gradients (A and B) with a Beckman SW60 rotor at 56,000 rpm for 6.5 h at 4°C or by electrophoresis in a 2% agarose gel after denaturation in glyoxal (C and D). SV40(II) [^{32}P]DNA synthesized in the presence of cytosol was analyzed in a separate sucrose gradient (A). SV40(II) [^{3}H]DNA standards (dashed lines) were prepared by DNase I treatment of SV40(I) [^{3}H]DNA and were added to each sample before analysis. Sedimentation was from right to left, and electrophoresis was from left to right. (A and B) Symbols: ●, without cytosol; ○, with cytosol. (C and D) Symbol: ●, with cytosol. ss, Single-stranded.

cleaved at one replication fork. The stability of endogenous SV40(I) [^{3}H]DNA under these conditions implied the absence of double-stranded DNA breaks.

The behavior of relaxed replicating DNA was determined by analyzing purified SV40(RI) DNA treated with *Escherichia coli* topoisomerase I. Relaxed SV40(RI) [^{3}H]DNA that was labeled for 30 min in cells sedimented just ahead of SV40(I) DNA (about 22S) and migrated in gels with a profile similar to that of native SV40(RI) DNA (Fig. 1A), except that the profile was compressed between SV40(II) DNA and relaxed circular dimers, with the bulk of the ^{3}H label near the circular dimers (46; data not shown). Relaxation of SV40(RI) DNA did not generate a distinct peak in the 80% replicated region of the gel.

The behavior of replicating DNA cleaved at one or both forks was determined by analyzing purified SV40(RI) [^{3}H]DNA treated with single-strand-specific S1 endonuclease. Cleavage at

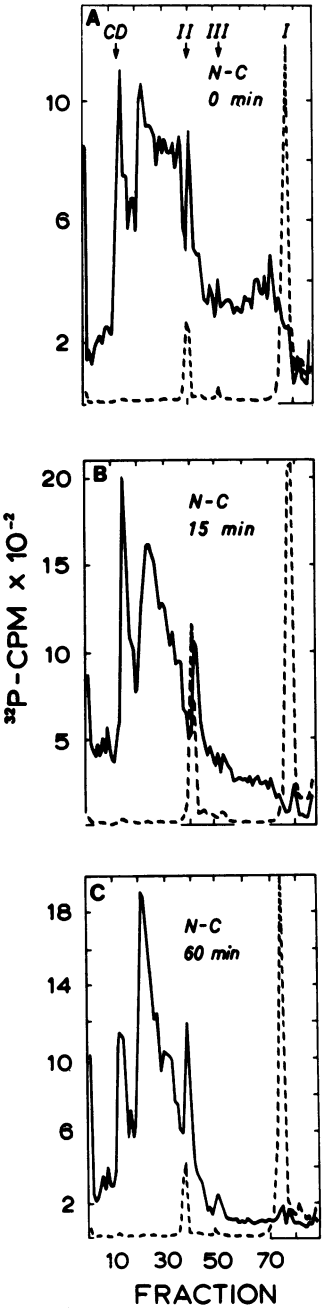

FIG. 8. Electrophoretic analysis of SV40(RI) DNA replicating in washed nuclei without cytosol. Nuclei in the absence of cytosol (N-C) were incubated for 45 s with [α-^{32}P]dCTP (A). Incubation was continued with a 100-fold excess of unlabeled dCTP for 15 min (B) and 60 min (C). SV40(RI) [^{32}P]DNA was purified, combined with SV40(I) and SV40(II) [^{3}H]DNA standards, and then analyzed by electrophoresis in 1.4% agarose gels. The positions of relaxed SV40 circular dimers (CD) and full-length SV40 linear DNA (III) are indicated. Solid lines, ^{32}P; dashed lines, ^{3}H.

one replication fork rapidly generated circular molecules that had a tail and sedimented at about 19S, closely behind SV40(I) DNA (21). Additional cuts, recognized by the release of Okazaki fragments, generated a heterogeneous population of linear molecules longer than one genome and SV40(II) DNA (21). Gel electrophoresis revealed that SV40(RI*) DNA was cleaved readily by S1 endonuclease, generating a distinct peak equivalent to 80% replication and a second peak near SV40(II) DNA (Fig. 9A and B). Further digestion produced a heterogeneous peak from SV40(II) DNA to SV40(III) DNA (Fig. 9C). An electron microscopic analysis confirmed the appearance of circles with tails in the 0.5-U S1 reaction and the absence of such circles in the 5-U S1 sample, which was consistent with our assumption that these molecules migrated in the 80% replicated region of the gel. These results were also consistent with the previous observation (Fig. 1) that purification of SV40(RI) DNA by sedimentation removed broken molecules that migrated during gel electrophoresis as 80% replicated. Therefore, the disappearance of SV40(RI*) DNA in isolated nuclei after cessation of DNA synthesis (Fig. 8C) most likely resulted from cleavage of single-stranded DNA at one replication fork. The requirement for cytosol to achieve extensive DNA synthesis (Fig. 3A) probably involved stabilization of native SV40(RI) DNA.

DISCUSSION

The data presented in this paper support a central role for SV40(RI*) DNA and SV40(II*) DNA in the maturation of replicating SV40 DNA into SV40(I) DNA. Our data also demonstrate that soluble factors found in the cytosol fraction of uninfected CV-1 cells are required primarily for completion of Okazaki fragment synthesis and stabilization of SV40(RI) DNA; these two parameters can account for the failure to synthesize SV40(I) DNA in washed nuclei. Based on these and other observations, we summarized the pathways involved in SV40 DNA replication in Fig. 10, which shows only those aspects germane to this paper.

Elongation of nascent DNA strands results in the accumulation of SV40(RI*) DNA. SV40 DNA synthesis is predominantly, if not exclusively, continuous on the forward arms of replication forks, where the direction of synthesis is the same as the direction of fork movement, and discontinuous (via repeated initiation of Okazaki fragments) on retrograde arms, where synthesis proceeds opposite to fork movement (11, 30, 42). In isolated nuclei, cytosol must be present for completion of Okazaki fragment synthesis (gap-filling) and subsequent ligation of Okazaki

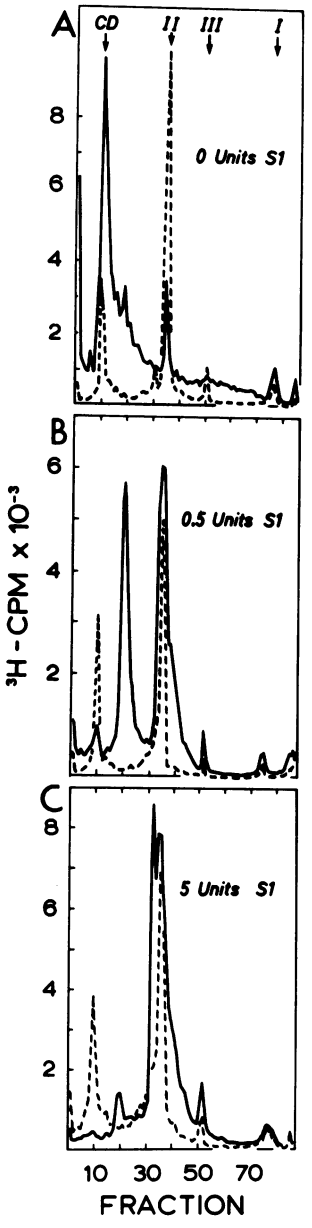

FIG. 9. Electrophoretic analysis of SV40(RI) DNA after digestion with S1 nuclease. SV40(RI) [^{3}H]DNA was incubated with either 0, 0.5, or 5.0 U of single-strand-specific S1 endonuclease (23). The reaction products were combined with a mixture of ^{32}P-labeled SV40(I), SV40(II), SV40(III) and relaxed circular dimers (CD) before electrophoresis, as described in the legend to Fig. 8. Solid lines, ^{3}H; dashed lines, ^{32}P.

fragments to the 5′ ends of long growing DNA strands (Fig. 4) or excision of RNA primers (2). The cytosol activity is a protein(s) (16) that can be purified free of DNA polymerase α and DNA ligase activities, both of which are present in washed nuclei (unpublished data). Detailed analyses of the synthesis and excision of RNA primers, the initiation of Okazaki fragments, the involvement of DNA polymerase α, and the structure of chromatin at replication forks have been presented elsewhere (11, 13, 15, 17).

Elongation of nascent DNA chains on SV40(RI) DNA proceeds bidirectionally until it is 85 to 95% completed. At this point, replicating DNA accumulates [SV40(RI*) DNA] to a level two to three times greater than the level observed for SV40(RI) DNA at earlier stages in replication. This accumulation has been demonstrated by both electrophoretic and electron microscopic analyses of purified SV40(RI) DNA containing nascent DNA strands uniformly labeled in intact cells (Table 2) (53). The SV40(RI) DNA was purified by three different procedures, and the agarose gels were standardized in three separate ways (Fig. 1B). SV40(RI*) DNA was also demonstrated by electrophoretic fractionation of SV40(RI) DNA pulse-labeled in isolated nuclei plus cytosol (53) and sedimentation analysis of SV40(RI) DNA labeled in nuclear extracts plus cytosol (46; Tapper, unpublished data). SV40 catenated and circular dimers also migrate as bands during gel electrophoresis (48), but they were not present in SV40(RI) DNA prepared by BND-cellulose chromatography (Table 1). Previous reports that replicating SV40 (35, 40) and polyoma virus (6) DNAs accumulated late during replication apparently overestimated the extent of accumulation three- to eightfold because of difficulty in identifying replicating molecules by electron microscopy (6, 35) and by not separating late SV40(RI) DNA in dye density gradients from SV40(II*) and dimeric DNA (40), which are labeled rapidly during replication (see below).

An analysis of the genomic positions of 3′ ends of long nascent DNA strands on SV40(RI) DNA demonstrated that DNA replication forks were arrested at preferred DNA sites in the termination region (54). Most forks were arrested when bidirectional DNA replication was 91% completed, and the two forks were separated by about 470 base pairs of unreplicated DNA, centered at the expected termination site. This is clearly consistent with the observed accumulation of SV40(RI) DNA when 90 ± 2% completed.

The previously reported accumulation of replicating DNA at about 80% completion (53) most likely resulted from contamination of the purified SV40(RI) DNA with replicating molecules cleaved at one replication fork by a single-stranded DNA interruption (Fig. 9). This peak was not present when the purification procedure was modified to exclude these molecules (Fig. 1). Fractured replicating molecules were more evident with subcellular systems, such as nuclei

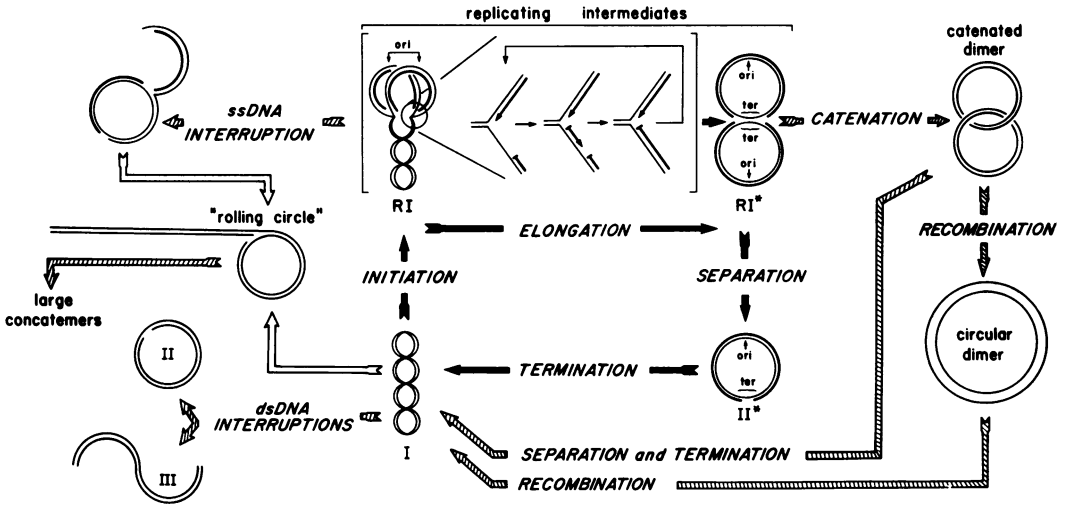

FIG. 10. Major forms of SV40 DNA and their proposed relationships to DNA replication. The primary pathways for SV40 DNA replication are indicated by the broad solid arrows, the secondary pathways are indicated by the cross-hatched arrows, and hypothetical pathways are indicated by the open arrows. Nascent DNA strands are bolder than template strands. ori indicates the unique origin of replication, and ter indicates the normal termination region. SV40(I) DNA is covalently closed, superhelical, circular monomeric DNA. This is the mature form of viral DNA extracted from virions. SV40(II) DNA is circular DNA monomers containing an interruption in either of the two strands; SV40(II*) DNA contains an interruption only in the nascent DNA strand within the termination region. SV40(III) DNA is linear DNA monomers. SV40(RI) DNA is circular replicating intermediates in which the two newly replicated regions are topologically relaxed and the unreplicated region contains superhelical turns; SV40(RI*) DNA is an accumulation of replicating intermediates at 91% completion. Catenated dimers contain two interwound circular monomers in which neither, one, or both are covalently closed and superhelical; the number of interwinds can also vary. Circular dimers consist of two concatenated monomers; larger circular and linear oligomeric concatemers are also found. Rolling circles are circular monomers with a linear duplex DNA tail attached that is longer than one genome; circular monomers with shorter tails can result from SV40(RI) DNA broken at one replication fork. A detailed discussion of these pathways has been presented elsewhere (DePamphilis and Wassarman, in press). ss, Single-stranded; ds, double-stranded.

(53) and nuclear extracts (47) supplemented with cytosol, than with intact cells (53). In the absence of cytosol (Fig. 8), the 80% replicated peak containing fractured replicating DNA became the major fraction of replicating DNA (Fig. 8); the striking accumulation of this DNA coincided with the cessation of DNA synthesis. Cytosol factors apparently prevent cleavage at replication forks that result in broken molecules unable to continue replication. The amount of replicating DNA observed at 80% completion also depended on the incubation conditions (53) and whether the fate of replicating molecules was followed by a pulse-chase (53) or a continuous labeling protocol (47) (Fig. 8). In principle, replicating DNA fractured at one fork could continue replication as rolling circles. Therefore, inactivation of the cytosol component that stabilizes replication forks late during the course of viral replication could result in conversion of SV40(RI) DNA into rolling circles (3, 37, 43).

Separation of sibling DNA molecules produces a transient intermediate, SV40(II*) DNA. The existence of replicating intermediates that accumulate at 91% completion implies that SV40(RI*) DNA represents a rate-limiting step in the separation of sibling molecules. In intact cells (9, 18, 33), nuclei plus cytosol (52), and nuclei without cytosol (52; this report), the major product of separation is SV40(II*) DNA. SV40(II*) DNA contains labeled deoxyribonucleotides solely in the nascent (linear) strand (18) (Fig. 7), and its ends are located in the termination region (9, 18, 33) and are separated by a gap of about 50 nucleotides (9). In cells, SV40(II*) DNA appears as rapidly as SV40(RI) DNA disappears and SV40(I) DNA is synthesized (18). In nuclei incubated with cytosol, the appearance of radiolabeled deoxyribonucleotides in the physical map of SV40(II*) DNA is consistent with the role of a transient intermediate in the formation of SV40(I) DNA (52). With washed nuclei alone, SV40(RI*) DNA, which contained 18 to 30% of an in vivo or in vitro pulse-label (52) (Fig. 8), was apparently converted into SV40(II*) DNA (52) that contained ra-

diolabeled Okazaki fragments in addition to essentially a full-length linear nascent DNA strand (Fig. 7). Therefore, ligation of Okazaki fragments is not required for separation of sibling molecules, and the nascent DNA strand is interrupted only at the position where DNA synthesis last occurred, the termination region.

Separation of sibling molecules does not require a unique DNA site (7, 32), but this event may be promoted at preferred DNA sites that arrest bidirectional replication when it is approximately 91% completed (54). Forks were also arrested at other locations, such that the center of the termination region defined by DNA arrest sites varied by ±450 base pairs (54). An analysis of the locations of these sites and the movement of forks during replication suggested that the variability in the location of the 50-nucleotide gap in SV40(II*) DNA (9) results from asynchronous arrival of replication forks and the accumulation of SV40(RI*) DNA whenever two forks are separated by about 500 base pairs (54). After separation, DNA synthesis may continue rapidly until the polymerase approaches the 5′ ends of nascent DNA located at arrest sites utilized by forks that entered the termination region from the opposite direction. SV40(II*) DNA containing a short gap accumulates because gap-filling, as demonstrated in Okazaki fragment metabolism (1), is slow relative to DNA synthesis. Like the completion of Okazaki fragments (1), this final gap-filling step requires cytosol factors in vitro.

An alternate mechanism for the separation of sibling molecules is through formation of catenated dimers. Catenated dimers could be separated either by a topoisomerase (24) or by intramolecular recombination to first generate circular concatenated dimers and then SV40(I) DNA (55). Circular concatenated dimers and higher oligomers (26, 37), as well as catenated dimers with one or both rings topologically relaxed (48), have been identified in SV40 lytic infections; the two rings are interwound from one to seven times (48). Circular dimers consist of monomeric units in a head-to-tail tandem arrangement (19, 43). Covalently closed catenated and circular dimers are labeled rapidly during the period of maximum DNA synthesis (28, 48). Since 95% of the dimers formed in mixed infections with two mutants were homodimers, they must have been products of replication rather than intermolecular recombination (19). About one-half of the pulse-labeled covalently closed dimers were catenated, and one-half were circular; the catenated dimers disappeared with a half-life of 3.7 h generating circular dimers and monomers (28). More recently (48), it was found that pulse-labeled covalently closed catenated dimers disappear completely by 1.3 h, similar to the time required for a 90% turnover in SV40(RI) DNA. However, this result was based on an analysis of isolated SV40 chromosomes which were enriched for catenated dimers (10 to 20%) (48, 51) relative to a Hirt extract (1 to 2%) (19, 27, 28). Since the disappearance of dimers was not correlated quantitatively with an equivalent increase in monomeric DNA, dimeric chromosomes may have changed conformation to a less extractable form.

Catenated dimers appear to result from the failure of SV40(RI*) DNA to undergo normal separation into SV40(II*) DNA. SV40 DNA synthesized in the presence of cycloheximide, an inhibitor of DNA replication, contains three- to fourfold more dimeric DNA than normal DNA (27, 28). Similarly, dimeric and oligomeric viral DNAs increase 10-fold by 70 h postinfection, when the rate of DNA synthesis decreases and cells show cytopathic effects (19, 37). Finally, infection of cells with dimeric DNA generates predominantly dimeric DNA as a product, showing that cells do not convert dimeric DNA into monomers rapidly (25). If catenation were the normal cellular mechanism for separation of sibling chromosomes, then sister chromatids would become interwound about once every 30 to 50 μm, requiring a topoisomerase surveillance mechanism to unlock the chromatids before mitosis. Thus, one advantage of arresting the process of two approaching replication forks at preferred DNA sites might be to promote separation of sibling molecules before they become interwound. However, if separation fails, a topoisomerase can still unlock the two molecules.

SV40(I) DNA can also be generated by intramolecular recombination. Since catenated dimers are radiolabeled more rapidly and decay more quickly than circular dimers, circular dimers could result from a homologous recombination event with catenated dimers or SV40(RI) (19, 28). Infection of cells with SV40 circular dimers constructed with DNA from two different mutants demonstrated that monomers can be generated by homologous recombination (55).

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