JOURNAL OF VIROLOGY, Feb. 1976, p. 614-621

Vol. 17, No. 2
Printed in U.S.A.

Simian Virus 40 DNA Replication: Characterization of Gaps in the Termination Region

MICHAEL C. Y. CHEN, EDWARD BIRKENMEIER, AND NORMAN P. SALZMAN*

Laboratory of Biology of Viruses, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20014

Received for publication 3 September 1975

A class of precursor DNA (pDNA) II molecules has been identified as the immediate precursor of simian virus 40 DNA I. A pDNA II molecule contains a strand of newly synthesized DNA with an interruption located in the region where DNA synthesis terminates (4). These pDNA II molecules have been isolated and further characterized. They are converted to covalently closed structures (simian virus 40 DNA I) only when they are treated in vitro with both T4 DNA polymerase and *Escherichia coli* ligase. After in vitro repair of pDNA II with T4 DNA polymerase and nucleoside triphosphates, approximately 7 mol of α-[^{32}P]dATP is incorporated per mol of DNA II. Alkaline sucrose analysis of these gap-filled molecules, after they have been cleaved with *Eco* R_I restriction endonuclease, has demonstrated that gaps are specifically located in the termination region. α-[^{32}P]dATP is incorporated equally into the two labeled products that are generated by R_I cleavage of these molecules. This indicates the presence of gaps in both the newly synthesized plus and minus strands. Electrophoretic analysis of the gap-filled molecules, after they have been cleaved with endonuclease *Hind*, has shown that gaps are localized in *Hind* fragments G and B and to a minor degree in fragment J. pDNA II molecules have the following properties. There is a gap in the newly synthesized linear DNA strand contained in the pDNA II molecule. Nicked pDNA II molecules cannot be detected. The two molecules that arise by segregation contain gaps in both of the complementary strands. Based on the amount of α-[^{32}P]dATP incorporated and the rate of exonuclease III digestion of gap-filled molecules, it is estimated that the size of the gaps is between 22 and 73 nucleotides. Models for termination of DNA synthesis are proposed based on these findings.

Replicating cyclic DNA molecules go through a termination event that requires the segregation of duplicated molecules and preserves their circularity. However, to achieve segregation, it is necessary to nick one or both of the parental strands. In simian virus 40 (SV40) replication, the product of this segregation event is a circular DNA molecule, precursor DNA (pDNA) II, in which the newly synthesized DNA is present in a discontinuous strand (4). The discontinuity in the daughter strand of pDNA II is localized approximately 0.5 genome lengths from the initiation site of DNA synthesis (4). Since SV40 replication is bidirectional (10, 13), this discontinuity is located in the termination region of DNA synthesis and may be related to the segregation of progeny molecules.

In the present study, we have demonstrated that this discontinuity in the newly synthesized strand is a gap rather than a single, phosphodiester bond interruption. The gap has been localized to specific endonuclease *R·Hind* fragments, and its size has been determined.

MATERIALS AND METHODS

Isolation of pDNA II. CV-1 cells were grown in plastic petri dishes (150 mm, Falcon Plastics) in Eagle medium with 2 mM glutamine and 10% fetal calf serum. Confluent monolayers were infected with SV40 at an input multiplicity of 10 PFU/cell. At 32 h after infection, the medium was removed, and 8 ml of fresh medium containing 10 μCi of [^{3}H]thymidine (specific activity, 6.7 Ci/mmol) per ml was added for 10 min. At the end of the pulse-labeling period, viral DNA was selectively extracted by the 0.6% sodium dodecyl sulfate-1.0 M NaCl method of Hirt (5). The supernatant fluid from the Hirt precipitation was deproteinized with phenol and dialyzed against TES (0.01 M Tris, pH 7.02, 0.002 M EDTA, 0.05 M NaCl). Ethidium bromide and cesium chloride were added to the Hirt supernatant fluid to give a final density of 1.564 and an ethidium bromide concentration of 200 μg/ml. Gradients (30 ml) were centrifuged in a Beckman 60 titanium rotor at 42,000 rpm for 48 h at 4 C.

Tubes were punctured at the bottom, and fractions were collected. The distribution of the radioactive DNA was determined by counting samples in a toluene–Triton X-100–water scintillation fluid (6:3:1) containing 2,5-diphenyloxazole and 1,4-bis[2-(5-phenyloxazolyl)]benzene.

Replicating molecules of SV40 DNA that have nearly completed replication band at the same density as pDNA II in a dye-density gradient (4, 13). To obtain pDNA II free from late-replicating molecules, fractions in the pDNA II position ("light" band) were pooled. Ethidium bromide was extracted from the solution by isoamyl alcohol extraction, and the DNA solution was dialyzed and concentrated by pressure dialysis against TES. Aliquots (0.2 ml) of the DNA were then layered onto 12 ml of 5 to 30% neutral sucrose gradients in 0.05 M Tris (pH 7.5), 0.1 M NaCl, and 0.025 M EDTA. Samples were centrifuged in a Beckman SW41 rotor at 27,000 rpm for 16 h at 5 C. A well-resolved peak of DNA that sedimented in the 16S region was pooled and concentrated. These pDNA II molecules were repurified by a second cycle of centrifugation in a 5 to 30% neutral sucrose gradient as described above.

Analysis of pDNA II. ^{3}H-labeled pDNA II that sedimented at 16S in 5 to 30% neutral sucrose was dialyzed in 0.1 M Tris (pH 7.5) and 0.01 M $MgCl_2$ and then cleaved with 0.2 U of *Eco* R_1 endonuclease (Miles Laboratory) at 37 C for 1 h. The *Eco* R_1-cleaved pDNA II molecules and untreated pDNA II were analyzed in 12 ml of 10 to 30% alkaline sucrose gradients containing 0.7 M NaCl, 0.3 M NaOH, 0.01 M Tris, and 0.025 M EDTA. Centrifugation was at 10 C in a SW41 rotor at 40,000 rpm for 16 h.

In vitro repair of pDNA II. The "gap-filling" reaction was performed in a total volume of 100 μl that contained 0.4 μg of DNA, 6.6 mM $MgCl_2$, 10 mM dithiothreitol, 20 μM each of dGTP, dCTP, and dTTP, 11 μCi of α-[^{32}P]dATP (specific activity, 5.39 Ci/mmol), 10 mM Tris-hydrochloride, pH 8.0, 0.06 U of T4 DNA polymerase. The reaction mixture was incubated at 37 C for 2 h; the reaction was stopped by adding EDTA to a final concentration of 70 mM. Molecules reacted in this way are referred to as "gap filled." The gap-filled DNA was dialyzed in TES, and unincorporated α-[^{32}P]dATP was separated from gap-filled pDNA II by 5 to 30% neutral sucrose sedimentation. Part of the gap-filled pDNA II was dialyzed in 0.01 M Tris-hydrochloride (pH 8.0) and 1 mM EDTA and was then treated with *Escherichia coli* DNA ligase in a reaction mixture of 20 mM Tris-hydrochloride (pH 8.0), 1 mM NH_4Cl, 2.5 mM $MgCl_2$, 50 μg of bovine serum albumin per ml, 10 mM nicotinamide adenine dinucleotide phosphate, and 0.08 U of *E. coli* ligase (17). The reaction mixture was incubated at 30 C for 30 min, and the reaction was stopped by adding EDTA to a final concentration of 20 mM. Covalently closed DNA was separated from nicked DNA by isopycnic banding in ethidium bromide-cesium chloride as described above.

Conditions for alkaline sucrose analysis of pDNA II that has been treated with T4 polymerase have been described above.

Electrophoretic analysis of gap-filled pDNA II. The conditions for cleavage of gap-filled pDNA II with endonuclease *R·Hind* and electrophoretic analysis of the products have been described before (2).

Exonuclease III digestion. An enzyme excess, 180 U of exonuclease III from *E. coli*, was used to digest gap-filled pDNA II under conditions previously described (11), except that the incubation temperature was 12 C and β-mercaptoethanol was not included in the reaction mixture. Total volume of the reaction mixture was 160 μl, and 15-μl aliquots were removed at 1, 2, 3, and 4 min. Calf thymus DNA (0.6 mg/ml) was added as carrier, and the DNA in each 15-μl aliquot was precipitated in cold 5% trichloroacetic acid. After centrifugation, the acid-soluble counts contained in the supernatant fluid were measured.

RESULTS

In vitro repair of pDNA II. [^{3}H]thymidine pulse-labeled pDNA II was isolated from CV-1 cells and was labeled in vitro using α-[^{32}P]dATP, T4 polymerase, and *E. coli* polynucleotide ligase as described above. The ^{32}P-labeled reaction products were extensively dialyzed in TES and purified from the reaction mixture in a neutral sucrose gradient (Fig. 1). A single reaction product that sedimented at 16S was obtained. The incorporation of [^{32}P]-deoxyribonucleotides into pDNA II demonstrated that some or all of these molecules contain a gap. Covalently closed DNA I, which is formed by *E. coli* polynucleotide ligase under these conditions, has a zero superhelix density and sediments at 16S in neutral sucrose gradients (Fig. 1), as would pDNA II molecules that had not been ligated. To determine the extent of ligation, peak fractions from the 16S region were pooled and centrifuged to equilibrium in a cesium chloride-ethidium bromide density gradient. The results of this analytical gradient are illustrated in Fig. 2. In this gradient a bimodal distribution of both ^{32}P and ^{3}H is observed. The ^{32}P-labeled molecules of higher buoyant density correspond to covalently closed SV40 DNA I of zero superhelix density, which binds less ethidium bromide than supercoiled DNA I (Fig. 2). T4 polymerase and *E. coli* ligase converted 25% of the pDNA II molecules to SV40 DNA I. This indicates that at least some pDNA II molecules contain gaps bound by a 3′-hydroxyl group and a 5′-phosphorylated deoxyribonucleotide. Approximately 7 mol of α-[^{32}P]dATP are incorporated per mol of pDNA II. When pDNA II that has not been treated with T4 polymerase is treated with *E. coli* polynucleotide ligase, there is no formation of relaxed DNA I. This is consistent with the absence of nicked pDNA II molecules.

Alkaline sucrose analysis of gap-filled pDNA II. The linear and circular strands in

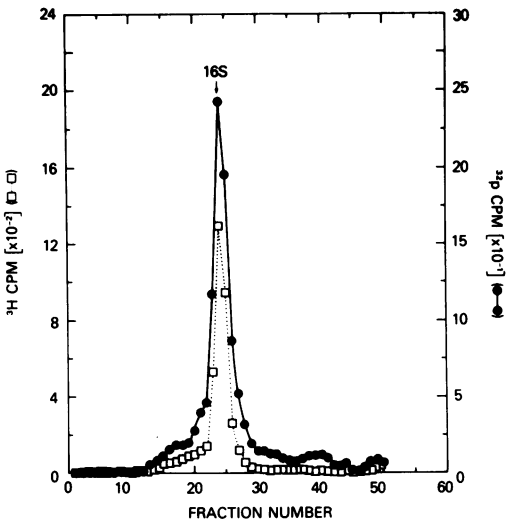

FIG. 1. *Preparative neutral sucrose gradient of ^{3}H-labeled pDNA II reacted with α-[^{32}P]dATP and T4 DNA polymerase. The gap in pDNA II was labeled with α-[^{32}P]dATP and treated with E. coli ligase as described in the text. The reaction mixture was extensively dialyzed in TES and centrifuged in a linear 5 to 30% neutral sucrose gradient containing 0.05 M Tris, 0.1 M NaCl, and 0.0025 M EDTA (pH 7.5). Centrifugation was performed in an SW41 rotor for 16 h at 27,000 rpm at 5 C. Fractions (0.22 ml) were collected from the bottom of the tube, and 10-μl aliquots of each fraction were counted in 10 ml of scintillation fluid in a Beckman scintillation counter. Sedimentation is from right to left.*

pDNA II can be separated by alkaline sucrose sedimentation. The linear strand sediments at 16S and the circular strand sediments at 18S. Gap-filled pDNA II that was analyzed by alkaline sucrose sedimentation is shown in Fig. 3. The newly synthesized ^{3}H-labeled DNA and ^{32}P-labeled gaps were exclusively in the linear 16S strand.

Since the linear 16S strand of pDNA II represents the full size of one strand of SV40 DNA, it is possible to localize the gaps in the newly synthesized strands of pDNA II (4). DNA replication is initiated at a specific site (3, 10, 15), and the replication is bidirectional (3, 10, 15). Thus, the termination site should be at approximately 0.5 genome lengths from the initiation site, provided both growing points move at equivalent rates until they meet. The R_I restriction endonuclease introduces one unique double-strand break into SV40 DNA I or II (8, 9). If the gaps in the pulse-labeled pDNA II are localized in a specific region, then cleavage of the newly synthesized strands by R_I endonuclease should yield two populations of labeled fragments that can be separated by alkaline sucrose sedimentation. Alkaline sucrose sedimentation analysis of gap-filled pDNA II, after cleavage by the R_I endonuclease, is shown in Fig. 4. There are two labeled fragments produced. The larger 15.3S fragment has a mass of about 1.43×10^6 daltons (14). The small fragment has an $s_{20,w}$ value of 8.6S and a

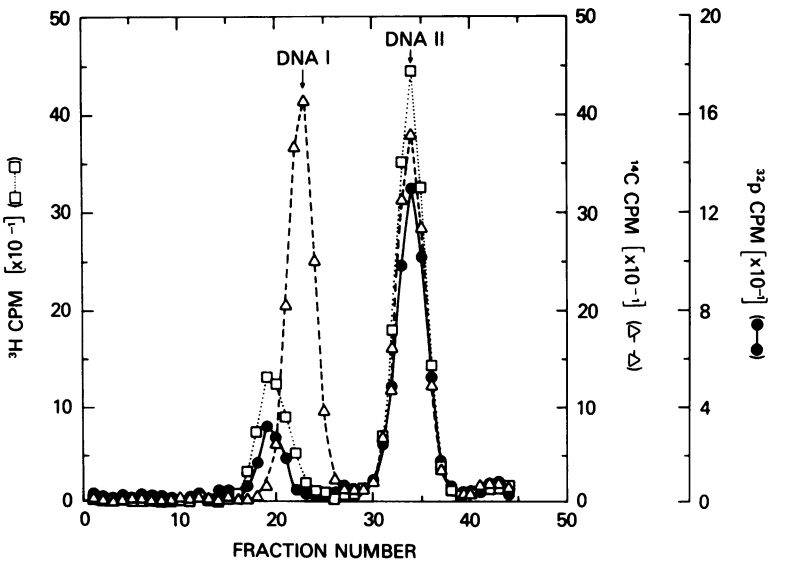

FIG. 2. *Dye-buoyant density analysis of in vitro repaired pDNA II. The peak fractions, which sedimented at 16S in Fig. 1, were pooled and dialyzed in TES and subjected to cesium chloride-ethidium bromide equilibrium centrifugation as described in the text. ^{14}C-labeled SV40 DNA I and DNA II isolated from virions were used as markers. The location of ^{14}C DNA I and ^{14}C DNA II are designated by the arrows. Density increases from right to left.*

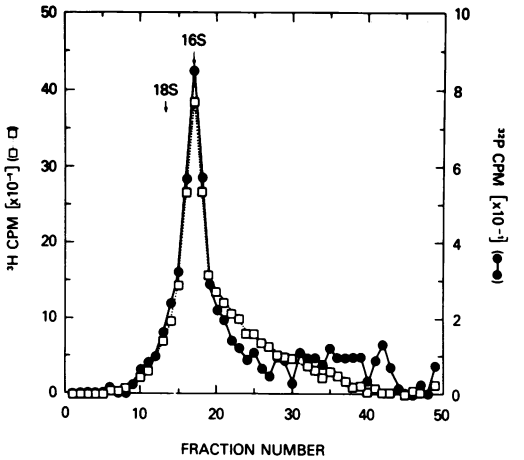

FIG. 3. *Alkaline sucrose velocity sedimentation analysis of T4 polymerase-treated pDNA II. pDNA II was repaired by T4 polymerase in the presence of α-[^{32}P]dATP as described in the text. The reaction mixture was extensively dialyzed in TES and centrifuged in a linear 5 to 30% neutral sucrose gradient as described in the legend to Fig. 1. 16S material was collected and dialyzed in 0.1 M Tris, pH 7.4, and then sedimented in a 10 to 30% alkaline sucrose gradient as described in the text. ^{14}C DNA II was added as a marker of the 18S and 16S positions.*

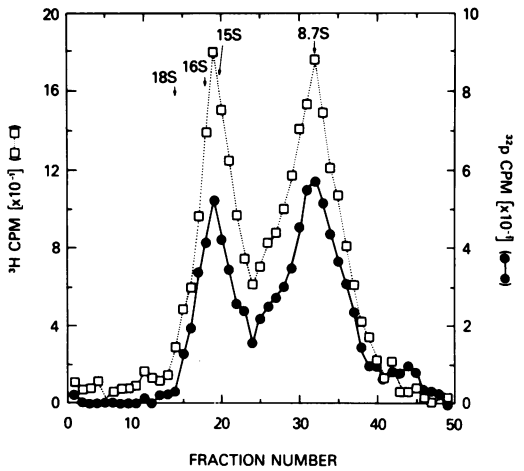

FIG. 4. *Alkaline sucrose sedimentation velocity analysis of the Eco R_I T4- polymerase-treated pDNA II. The experiment was essentially the same as described in the legend to Fig. 3, except the T4-polymerase-treated pDNA II was cleaved with R_I restriction enzyme before applying the sample onto an alkaline sucrose gradient.*

mass of about 3.4 × 10^6 daltons (14). The specific fragmentation of the pulse-labeled strand in pDNA II demonstrates that the gaps exist at a specific site or are in a tightly clustered location on the SV40 genome. The small fragment formed after cleavage of the pulse-labeled pDNA II corresponds to approximately 19% of the mass of one strand of SV40 DNA, which is in good agreement with previous findings (4). Therefore, the gaps are separated from the R_I cleavage site by a distance of approximately 0.19 genome lengths. The almost equal amount of [^{32}P]deoxyribonucleotides present in the two peaks indicates that gaps are present both in plus and minus newly synthesized strands. The broadness of the 8.6*S* peak may be due to cleavage of gap-filled pDNA II molecules having gaps of different sizes and/or gaps located at varying distances from the R_I cleavage site.

Use of endonuclease R·Hind and polyacrylamide gel electrophoresis for localizing the gaps in pDNA II. Alkaline sucrose analysis revealed the gaps are located in the newly synthesized strands 0.19 genome lengths from the R_I site. In order to further localize the gaps, the DNA I that was generated from pDNA II by T4 polymerase and *E. coli* polynucleotide ligase was cleaved with endonuclease *R·Hind*, and the fragments were separated by polyacrylamide gel electrophoresis. Figure 5 shows that ^{32}P is primarily in fragments B and G but that a very low level is also present in fragment J.

If the gaps in pDNA II were located at an endonuclease *R·Hind* cleavage site, then the presence of a region of single-stranded DNA should block cleavage at that site, and a new fragment would be seen after electrophoretic analysis. To test this, ^{3}H-labeled pDNA II and uniformly ^{14}C-labeled DNA II were simultaneously cleaved by endonuclease *R·Hind* and co-electrophoresed in a polyacrylamide gel. The results are presented in Fig. 6. As is expected, the pulse-labeled ^{3}H SV40 DNA is heavily labeled in the fragments that are located approximately 0.5 genome lengths from the initiation site, near the termination site. All of the ^{3}H-labeled fragments comigrate with ^{14}C-labeled fragments. This demonstrates that the gaps are neither within any of the endonuclease *R·Hind* cleavage sites nor large enough to change the electrophoretic mobility of any fragments. The DNA I molecules produced by treatment of pDNA II with T4 polymerase and *E. coli* polynucleotide ligase and uniformly labeled ^{14}C SV40 DNA were also cleaved with endonuclease *R·Hind* and analyzed in polyacrylamide gel electrophoresis (Fig. 7). The ^{32}P label, which is incorporated in vitro into the gaps, appears in the fragments B, G, and J. Of the counts incorporated into these three fragments, the B and G fragments have approximately equal amounts of ^{32}P, which represents 93% of

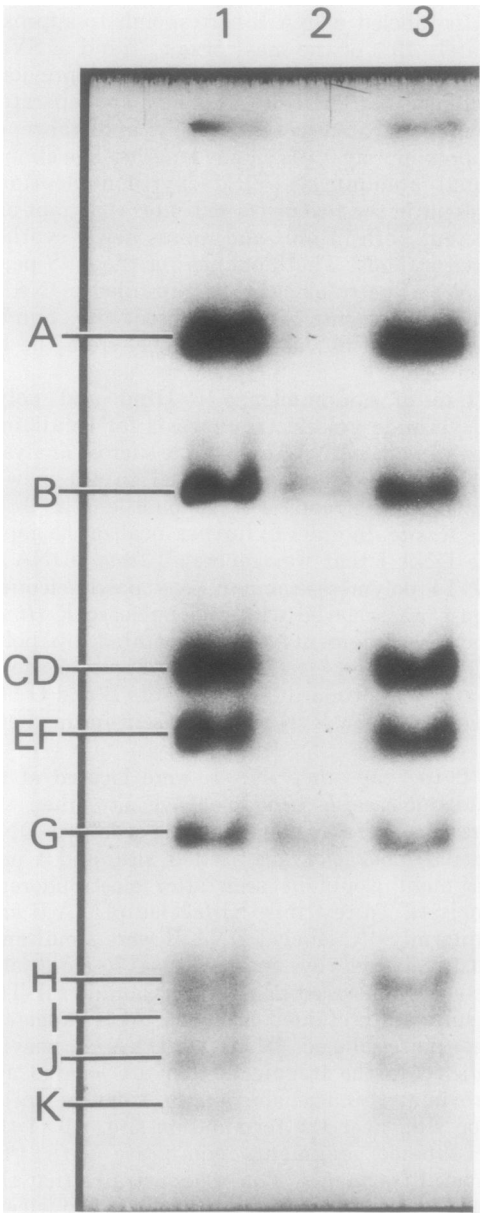

FIG. 5. *Electrophoretic analysis of DNA I that was formed from pDNA II by T4 polymerase and E. coli ligase and then digested with endonuclease R·Hind. The conditions for endonuclease R·Hind digestion of SV40 DNAs and polyacrylamide gel electrophoresis have been mentioned in the text. Columns 1 and 3 are samples of ^{32}P-labeled SV40 DNA I that have been cleaved with endonuclease R·Hind and that are used as a marker. Column 2 is the enzymatically generated DNA I that has been isolated (Fig. 2) and cleaved with endonuclease R·Hind.*

the ^{32}P counts. About 7% of the ^{32}P counts are in the J fragment. Since 7 mol of [^{32}P]dATP are incorporated per mol of pDNA II, it was calculated that the minimum gap size is approximately 22 nucleotides. The ^{32}P label incorporated into the gap should be sensitive to exonuclease III digestion, and the time required to remove this label should be a function of the gap size. From the rate of digestion of ^{32}P labeled pDNA II by an excess of exonuclease III, it was calculated that the maximum gap size is 73 nucleotides (Table 1).

DISCUSSION

During SV40 DNA replication, the template strands remain covalently closed (13). Production of two daughter molecules from a late replicative intermediate requires segregation of the closed template strands. These daughter molecules have been characterized as open circular DNA II (pDNA II) molecules with a discontinuity in the newly synthesized DNA strands. The discontinuity was located in the termination region, approximately 0.5 genome lengths from the initiation site of DNA replication (4). The present data demonstrate that this discontinuity is a gap of 22 to 73 nucleotides, rather than a single phosphodiester bond interruption. Thus, segregation can occur before completion of DNA synthesis. The action of both T4 polymerase and *E. coli* ligase are required to effect the in vitro formation of covalently closed molecules. Previous data had demonstrated that both of these enzymes were required for in vitro repair of "gap circles" synthesized in the presence of hydroxyurea (7).

Location of the gap in specific endonuclease *R·Hind* fragments revealed that 93% of the ^{32}P that was incorporated into the gaps was almost equally divided between *R·Hind* fragments B and G. Both of these fragments are in the region approximately 0.5 genome lengths from the initiation site, where termination is expected to occur (Fig. 6). Three models can be proposed to explain the equal distribution of isotope between B and G fragments.

The first model proposes a precisely located gap that maps at the same position in the two daughter molecules that arise by segregation. Since the amount of isotope in the B and G fragments is equal, this model requires the gap to be positioned at the BG junction. However, a single-stranded region of DNA at the BG cleavage site in pDNA II would prevent cleavage at this site. Since endonuclease *R·Hind* cleavage of pDNA II produces B and G fragments, this model can be excluded.

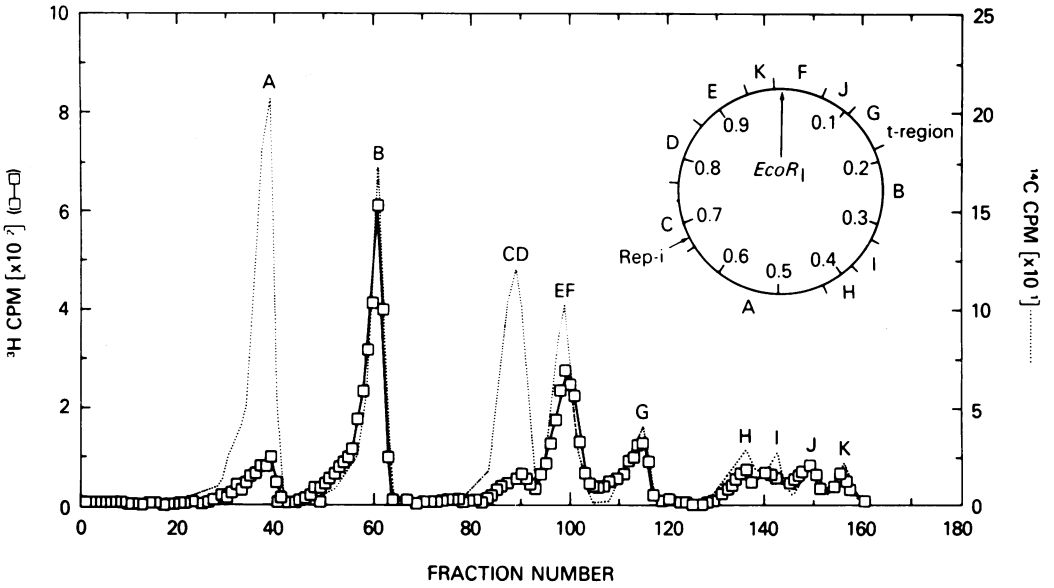

FIG. 6. *Polyacrylamide gel electrophoresis of endonuclease R·Hind fragments produced from ^{3}H pulse-labeled pDNA II and ^{14}C-labeled DNA II isolated from SV40 virions. The condition for electrophoresis has been mentioned in the text. Migration is from left to right. In the upper right corner, a physical map of the SV 40 genome is shown (10).*

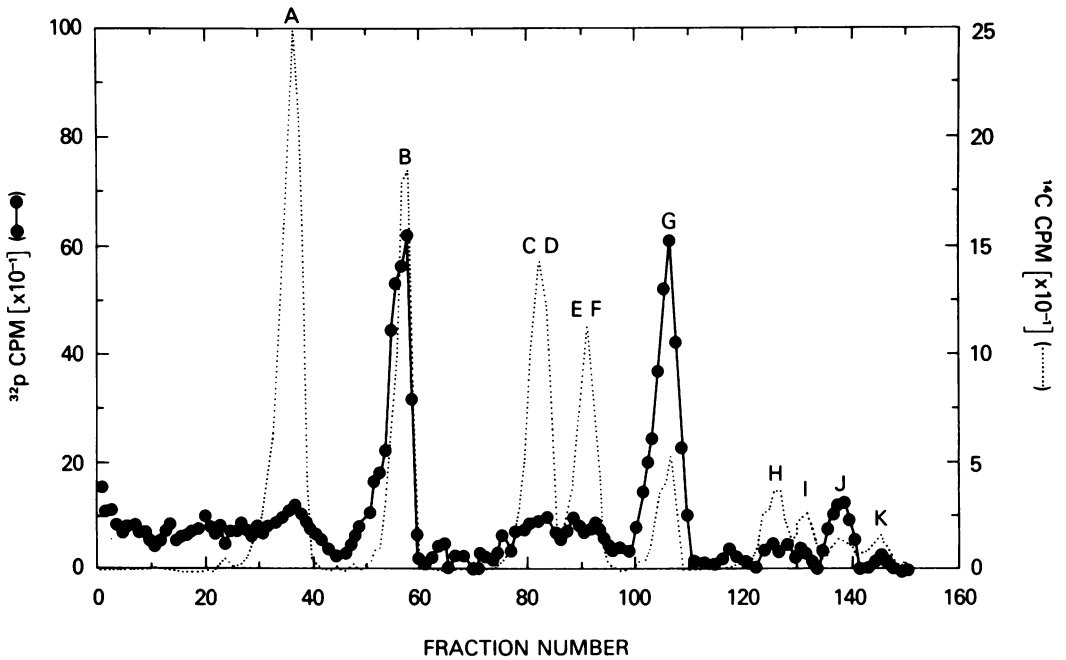

FIG. 7. *Polyacrylamide gel electrophoresis of endonuclease R·Hind fragments produced from DNA I generated in vitro from pDNA II by treatment with T4 polymerase and E. coli ligase and ^{14}C-labeled DNA II isolated from SV40 virions. DNA I that was isolated from the high-buoyant-density region (Fig. 2), together with ^{14}C-labeled DNA II isolated from SV40 virions, was cleaved with endonuclease R·Hind and analyzed in polyacrylamide gel electrophoresis. The distribution of ^{3}H pulse label is the same as that shown in Fig. 5 and, therefore, it is not shown in this figure. The conditions for electrophoresis are the same as described in the legend to Fig. 6.*

TABLE 1. *Rate of exonuclease III digestion of gap-filled pDNA II*[a]

Incubation time (min)	% ^{32}P acid soluble	% Available ^{3}H acid soluble	Gap size in nucleotides
1	47	0.69	80
2	70	0.84	66
3	75	0.97	71
4	77	1.06	76

[a] ^{32}P gap-filled pDNA II (average gap size = 73) and uniformly ^{3}H-labeled, randomly nicked DNA II were digested together with an excess of exonuclease III as described in the text. Acid-soluble counts were measured after 1, 2, 3, and 4 min of incubation at 12 C. The percentage of available ^{3}H (^{3}H in nicked strands as determined by an alkaline sucrose gradient) made acid soluble was multiplied by 5,500 (number of nucleotides in one SV40 DNA strand) and then divided by the percentage of ^{32}P made acid soluble to obtain the gap size.

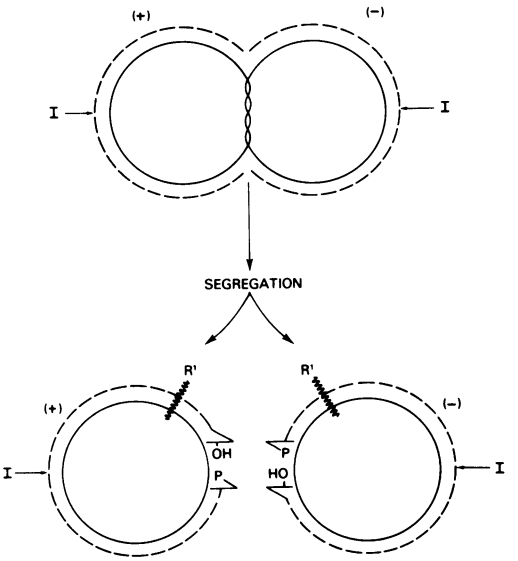

FIG. 8. *Schematic diagram of the segregation event. Replicating molecules segregate to form two daughter molecules in which the newly synthesized strands (plus or minus) have a gap in the termination region 0.5 genome lengths from the initiation site I. Labeling of the gap in both daughter molecules at the 3'-OH end with T4 polymerase and α-[^{32}P]dATP produces one 8.7S labeled fragment and one 15.3S labeled fragment after Eco R_I cleavage and subsequent analysis in alkaline sucrose gradients.*

A second possibility is that the gap position is precisely located but that the gap is at different sites in the two daughter molecules that arise by segregation. R_I cleavage of T4 polymerase-treated pDNA II showed equal distribution of ^{32}P in the 15.3S and 8.7S peaks, indicating that both of the complementary daughter molecules have a gap (Fig. 8). This finding is consistent with a model in which there is a gap in one newly synthesized strand in the B fragment and that in the complementary newly synthesized strand the gap exists in the G fragment.

A third model seems most consistent with the available data. It proposes that the gap is not in a specific location but is able to vary in location, depending on where the two replicating forks are positioned at the time of segregation. Studies of the replication of SV40 deletion mutant DNAs have shown that the site of termination is not determined by a specific nucleotide sequence but occurs where the two replication forks meet (C. Lai and D. Nathan, personal communication). In this model, we assume there is a Gaussian distribution for the location of the gap, with a mean at the junction of the B and G fragments. It is for this reason that we have designated the BG junction as a termination region rather than a termination site. Based on frequency with which ^{32}P is found in the J fragment (0.07) and using a maximum gap size of 73 nucleotides, it can be calculated that 85% of the pDNA II molecules would be cleaved at the *R·Hind* site at the BG junction. A fragment corresponding to a combined fragment of B and G and containing approximately 15% of the amount of radioactivity as that contained in the separated B and G fragments has not been seen in gels. This quantity of DNA may be difficult to detect, and it is difficult to predict how a combined BG cleavage product that contains a single-stranded gap would migrate in the acrylamide gels.

The relation between the gaps and the segregation of template strands remains unclear. We have shown that DNA replication is not complete at the time of segregation, raising the possibility that molecular events occurring during DNA replication may in themselves permit segregation. During SV40 DNA replication, it has been shown that the parental strands unwind and that the topological winding number, α, decreases as replication proceeds. Thus, a process similar to nicking, unwinding, and sealing of parental strands occurs during DNA replication. A relaxing protein capable of untwisting superhelical DNA has been purified from nuclei of mouse embryo cells (1) and is also present in nuclei of SV40 infected BSC-1 and CV-1 monkey kidney cells (1, 6; Chen, Birkenmeier, and Salzman, unpublished data). This activity could momentarily interrupt one or both template strands, unwind them allowing segregation, and then reseal the strands to maintain the circular integrity of the pDNA II molecules. The relaxing protein purified from cul-

tured mouse and human cells relaxes PM2 DNA in vitro, and, once the relaxation process begins, it appears that the protein is not released until complete relaxation is achieved (16). The location of the gaps could represent the areas at which the relaxation protein binds to the supercoiled DNA molecule throughout the cycle of replication. The binding of this protein in the termination region may be facilitated by unpaired regions of DNA located in the B and G fragments (12).

ACKNOWLEDGMENTS

We are indebted to Nancy Nossal (T4 DNA polymerase), Martin Gellert (DNA ligase), Reed Wickner (exonuclease III), and Walter Keller for providing the enzymes used in this study, and to Nanette Tahner and Marilyn Thoren for their invaluable technical assistance during the course of this work.

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