JOURNAL OF VIROLOGY, Oct. 1971, p. 478-490
Vol. 8, No. 4

Structure of Replicating Simian Virus 40 Deoxyribonucleic Acid Molecules[1]

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Received for publication 23 June 1971

Properties of replicating simian virus 40 (SV40) deoxyribonucleic acid (DNA) have been examined by sedimentation analysis and by direct observation during a lytic cycle of infection of African green monkey kidney cells. Two types of replicating DNA molecules were observed in the electron microscope. One was an open structure containing two branch points, three branches, and no free ends whose length measurements were consistent with those expected for replicating SV40 DNA molecules. A second species had the same features as the open structure, but in addition it contained a superhelix in the unreplicated portion of the molecule. Eighty to ninety per cent of the replicative intermediates (RI) were in this latter configuration, and length measurements of these molecules also were consistent with replicating SV40 DNA. Replicating DNA molecules with this configuration have not been described previously. RI, when examined in ethidium bromide-cesium chloride (EB-CsCl) isopycnic gradients, banded in a heterogeneous manner. A fraction of the RI banded at the same density as circular SV40 DNA containing one or more single-strand nicks (component II). The remaining radioactive RI banded at densities higher than that of component II, and material was present at all densities between that of supercoiled double-stranded DNA (component I) and component II. When RI that banded at different densities in EB-CsCl were examined in alkaline gradients, cosedimentation of parental DNA and newly replicated DNA did not occur. All newly replicated DNA sedimented more slowly than did intact single-stranded SV40 DNA, a finding that is inconsistent with the rolling circle model of DNA replication. An inverse correlation exists between the extent of replication of the SV40 DNA and the banding density in EB-CsCl. Under alkaline conditions, the parental DNA strands that were contained in the RI sedimented as covalently closed structures. The sedimentation rates in alkali of the covalently closed parental DNA decreased as replication progressed. Based on these observations, some possible models for replication of SV40 DNA are proposed.

The deoxyribonucleic acid (DNA) molecules of a number of viruses [ϕX174, λ, simian virus 40 (SV40), and polyoma] exist as covalently closed circles at some point in the intracellular viral life cycle (4, 7, 15, 26, 27). Recent studies (9, 16, 18, 19) indicate that the replication of these viral DNA molecules may have certain common features. In each of these cases, it is possible to isolate early replicative intermediates (RI) which are observed in the electron microscope to contain two forks, three branches, and no free ends. A second feature common to RI molecules is their adsorption and elution behavior on benzoylated-naphthoylated diethylaminoethyl (DEAE) cellulose columns (1, 12, 16, 18), which suggests that they contain limited single-stranded regions in the molecules.

Although there is agreement on these features, there have been conflicting reports concerning the association between newly made DNA and the parental DNA template. For phage λ, it has been reported that RI contain DNA strands longer than a complete viral DNA strand (12). These are postulated to arise by covalent linkage between one of the newly synthesized strands and an intact parental strand. Similar results have also been reported for ϕX174 (16). These data are consistent with the rolling-circle model for DNA replication as originally proposed by Gilbert and Dresser (8). However, these findings have not been confirmed in a second study of phage λ replication (25). In this study, newly replicated strands sedimented in alkaline gradients more slowly than did intact

[1] A preliminary report of these findings has been given at the American Society of Biological Chemists meeting, June 1971. Fed. Proc. 30:1177, 1971.

single-stranded viral DNA and were dissociated from parental DNA strands. These findings are not consistent with the rolling-circle model. Newly replicated strands shorter than intact single strands were also observed for RI of polyoma virus during alkaline sedimentation (2).

We have examined SV40 RI and have observed that, during alkaline sedimentation, newly replicated SV40 DNA is not covalently attached to the parental DNA strand. By electron microscopy of RI, we have observed structures similar to those described previously. However, we have also noted that almost all RI, in addition to two branch points and three branches, also contain a superhelical region in the unreplicated portion of the molecule. Alkaline sedimentation studies with RI have shown that the parental strands are present as covalently closed circles. These studies indicate that most of the SV40 replicating molecules isolated from infected cells do not have a swivel point in the unreplicated region. Based on these findings, a model for SV40 replication is proposed.

MATERIALS AND METHODS

Cells. African green monkey kidney (AGMK) cell monolayers were prepared from minced kidneys dispersed by trypsinization. Cells were refed 3 days after planting and became confluent by 6 days. Monolayers were inoculated with virus 1 to 10 days after they became confluent. There was no noticeable difference in the response of cells to virus infection during this period. The growth medium that was used for AGMK cells was Eagle's medium supplemented with 10% fetal calf serum (Industrial Biological Laboratories) and 2 mM glutamine.

Infection of AGMK cells with SV40 virus. A small plaque-forming SV40 strain obtained from K. Takemoto was used. Medium was removed from confluent AGMK cells contained in 60-mm plastic petri dishes (Falcon Plastics), and cells were infected by the addition of 0.2 ml of virus inoculum (100 to 150 plaque-forming units per cell). Adsorption was allowed to proceed for 2 hr at 37 C in a CO_2 incubator. Plates were gently rocked every 20 min during this period. At the end of the adsorption period (2 hr after infection), 5 ml of Eagle's medium containing 2% fetal calf serum and 2 mM glutamine was added to each plate.

Radioactive labeling of cells. Infected cultures were pulsed at 28 to 32 hr after the addition of virus, at which time there is active synthesis of viral DNA (18). Immediately before pulsing of the cells, the medium was removed by aspiration. A 2-ml amount of Eagle's medium containing ^{3}H-thymidine (50 to 100 μCi/ml, 20 to 25 mCi/μmole) was added after the medium was prewarmed to 37 C. At the end of the pulse period, the medium was removed, and the cell monolayer was washed twice with 10-ml portions of cold phosphate-buffered saline.

Separation of viral and cellular DNA. Viral DNA was separated from cellular DNA as described by Hirt (10). Monolayers that were pulsed and washed as described above were immediately lysed by addition of 0.5 ml of a solution containing 0.01 M tris(hydroxymethyl)-aminomethane (Tris), 0.01 M ethylenediaminetetraacetic acid (EDTA), 0.6% sodium dodecyl sulfate, *p*H 7.2. After standing for 15 min at room temperature, the contents were transferred to a test tube. One quarter volume of 5 M NaCl was added, and the tube was inverted gently 10 times to effect mixing and then placed at 4 C overnight. The insoluble material was pelleted by centrifugation at 4 C for 30 min at 14,000 rev/min in a Spinco SW 50.1 rotor. The supernatant fluid was dialyzed for at least 18 hr against 0.01 M Tris, 0.01 M EDTA (*p*H 7.2). The dialyzed supernatant fluid is referred to as a Hirt supernatant fluid.

Isopycnic banding of DNA in EB-CsCl. Ethidium bromide, from Calbiochem, Los Angeles, Calif., and cesium chloride, from Henley & Co., Inc. (EB-CsCl), were added to the Hirt supernatant fluid to give a final density of 1.564 and an EB concentration of 200 μg/ml. Gradients (6 ml) were centrifuged in a Spinco no. 50 fixed-angle titanium rotor at 42,000 rev/min for 40 to 60 hr at 4 C. Tubes were punctured at the bottom, and fractions were collected. The distribution of the radioactive DNA was determined either directly by counting samples in toluene-Triton X-100-water (6:3:1) containing 4 g of (2,5-diphenyloxazole) and 50 mg of [1,4-bis-2-(5-phenyloxazolyl)benzene] per liter or by placing samples on Whatman filter paper discs and washing successively with cold 5% trichloroacetic acid, 95% ethanol, ethanol-ether (3:1), and finally ether. In the latter procedure, the dried discs were counted in vials containing 10 ml of liquifluor.

Velocity gradient fractionation of DNA. Neutral sucrose gradients were carried out by layering approximately 0.1-ml samples onto 11.6-ml 5 to 30% sucrose gradients in 0.05 M Tris, 0.1 M NaCl, 0.0025 M EDTA (*p*H 7.5). Samples were centrifuged at 40,000 rev/min in an SW 41 rotor at 10 C. Times of centrifugation varied and are noted for each experiment elsewhere in the text. Samples were collected and counted directly in liquid scintillation vials as described above.

Alkaline sucrose gradients were carried out as described for the neutral gradients, except that samples were layered onto 10 to 30% sucrose gradients containing 0.7 M NaCl, 0.3 M NaOH, 0.01 M Tris, 0.0025 M EDTA, and 0.015% Sarkosyl. Centrifugation was at 10 C in an SW 41 rotor at 40,000 rev/min.

Electron microscopy. The conditions for mounting DNA for electron microscopy were those described by Davis, Simon, and Davidson (6). The spreading solution contained 0.5 to 1.0 μg of DNA per ml, 0.1 mg of cytochrome *c* per ml, and 0.5 M NH_4 acetate. The hypophase was 0.25 M NH_4 acetate. The DNA was contrasted by staining with uranyl acetate. Electron micrographs were taken on Kodak Electron Image Plates with a Siemens Elmiskop 101 at magnifications from 3,000 to 9,000 with a 20-μm objective aperture and 40-kv accelerating voltage. Magnification was calibrated with a grating replica (E. F. Fullum, 54,864 lines/inch). The DNA molecules were projected onto a RAND tablet and traced with a stylus. Contour lengths were computed with a PDP-10 digital computer (Digital Equipment Corp.). The program for this computation was written by Robert Sproull.

RESULTS

Kinetics of uptake of ^{3}H-thymidine into replicating SV40 DNA molecules. ^{3}H-thymidine (50 μCi/ml) was added to replicate AGMK monolayer cultures 30 hr after they had been infected with SV40 virus. At 2.5, 5, 10, 20, and 60 min after addition of the labeled precursor, the cells were lysed and the Hirt supernatant fluid was obtained. It has been reported that the Hirt supernatant fluid contains all replicating SV40 DNA molecules (18). A Hirt supernatant fluid was also prepared from an uninfected cell monolayer that had been labeled for 60 min. Portions of the supernatant fluids were layered onto 5 to 20% sucrose gradients, and samples were centrifuged in an SW 50.1 rotor for 3 hr at 40,000 rev/min at 4 C (Fig. 1).

Sedimentation analysis of infected cultures pulsed for 2.5 and 5 min showed that newly synthesized viral DNA sedimented as a broad band with a 26*S* peak value. A shoulder at 21*S* was seen in a 10-min pulse, and by 20 min sedimentation peaks at 26 and 21*S* were seen. These results are similar to those reported by Levine et al. (18), who showed that the 26*S* SV40 DNA represents replicating viral DNA which is a precursor of SV40 component I(21*S*).

A comparison of the sedimentation patterns obtained for the infected and uninfected cultures pulsed for 60 min (Fig. 2) indicated that there was little labeled cellular DNA in the Hirt supernatant fluid of uninfected cells. In the infected culture, 21*S* viral DNA was present, and a small shoulder of more rapidly sedimenting material was seen at the position where replicating DNA sediments.

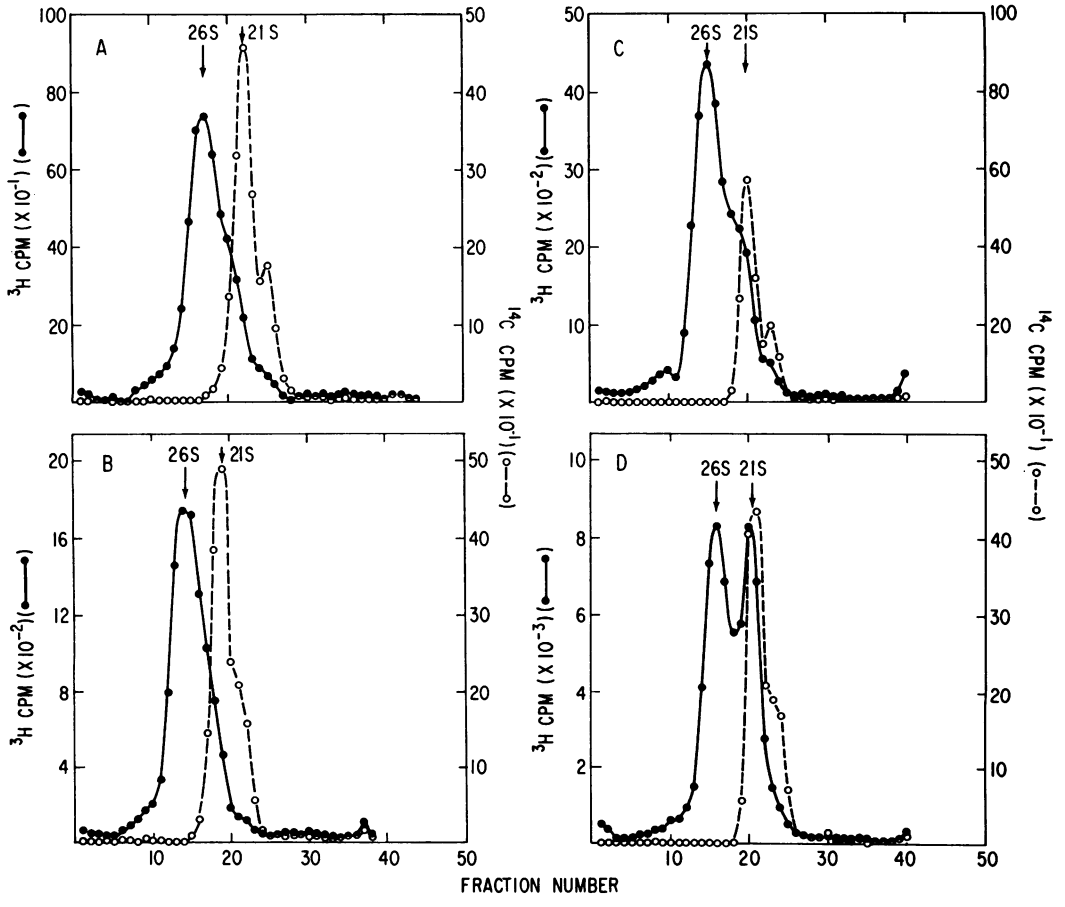

FIG. 1. *Velocity sedimentation in a 5 to 20% neutral sucrose gradient of Hirt supernatant fluid from SV40-infected African green monkey kidney cell monolayers pulsed with ^{3}H-thymidine for various lengths of time 30 hr after infection. A ^{14}C-thymidine SV40 DNA marker was cosedimented with each sample. Gradients were prepared and centrifuged as described by Levine et al. (18). (A) 2.5-min pulse, (B) 5-min pulse, (C) 10-min pulse, and (D) 20-min pulse. Sedimentation is from right to left.*

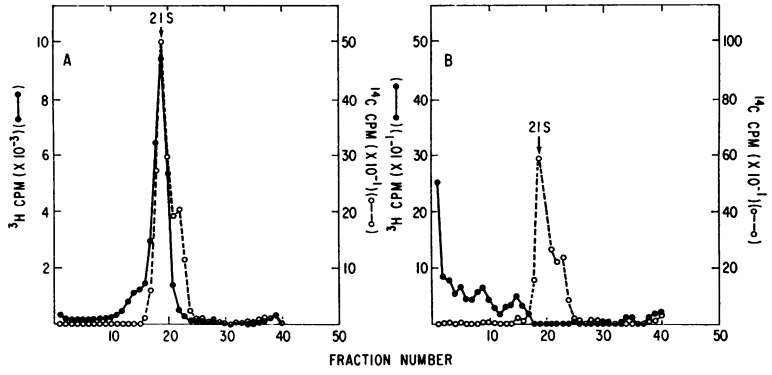

FIG. 2. *Velocity sedimentation in neutral sucrose of Hirt supernatant fluid from SV40-infected and uninfected African green monkey kidney cell monolayers. An uninfected culture and a 30-hr infected culture were exposed to 10 μCi of ^{3}H-thymidine per ml for 1 hr. Hirt supernatant fluids were prepared from each culture, and samples were sedimented in a 5 to 20% neutral sucrose gradient as described in Fig. 1. (A) Infected culture, (B) uninfected culture.*

Electron microscopy of replicating SV40 DNA molecules. Monolayers of AGMK cells were pulse-labeled for 5 min with ^{3}H-thymidine 29 hr after SV40 infection. The intracellular DNA contained in the Hirt supernatant fluid was centrifuged to equilibrium in EB-CsCl (20). The peak of radioactive replicating DNA was collected and, after removal of EB (5), was centrifuged through a neutral sucrose gradient. A sedimentation profile similar to that shown in Fig. 1B was obtained. The zone of radioactive DNA was divided at the peak into a leading half (L) and a trailing half (T). Both halves were concentrated and examined in the electron microscope.

Both pool L and pool T contained five distinct molecular species. Three of these species, long linear DNA (probably of host origin) and components I and II of SV40, were present in small amounts and presumably represented contaminants. The fourth species was an open structure containing two branch points, three branches, and no free ends (Fig. 3A and B). Length measurements of a number of these molecules revealed that two of the branches were of similar length. When the average length of these two branches was added to the length of the third branch, the sum was approximately equal to the length of component II of SV40. Similar structures already have been described for the replicating molecules of SV40, polyoma, lambda, φX174, *Escherichia coli*, and mitochondrial DNA (3, 9, 14, 16, 18, 19). The fifth species was a molecule that has not been previously described (Fig. 3C–H). This molecule was similar to the open structure of Fig. 3A and B in that it contained two branch points, three branches, and no visible ends; however, one of the branches was twisted on itself to form a superhelix. This structure was observed about 4 to 5 times as frequently as the open replicating structure.

Length measurements were carried out on molecules that contained a superhelical branch to determine whether their dimensions were consistent with those expected for replicating molecules. The various DNA segments measured in each molecule are shown in the idealized drawing of Fig. 4B. The lengths of branches L1 and L2 (the two which did not contain the superhelical region) were approximately equal (Fig. 5). In over 70% of the cases the difference in length between L1 and L2 was less than 10%. The total length (L1 + L2 + L3) of these molecules was between one and two times the length of mature SV40 DNA molecules (1.5 to 3.2 μm). (In a separate experiment, the mean length of 70 molecules of SV40 component II was 1.52 ± 0.10 μm.) When the average length of L1 and L2 was added to the length of L3 (Fig. 6), the sum was approximately equal to the length of mature SV40 DNA molecules (1.51 ± 0.14 μm for pool L and 1.45 ± 0.14 μm for pool T). These data agree with the expectations for replicating molecules. In addition, the results indicate that the superhelix is in the unreplicated portion of these molecules.

The extent of replication of the twisted replicating molecules was estimated by the equation: (L1 + L2)/(L1 + L2 + 2L3). Very "young" replicating forms (<20% replicated) were not apparent in either pool L or pool T (Fig. 7). Most of the molecules in pool L were more than 50% replicated, whereas the molecules of pool T were more evenly distributed. It should be noted that 50 to 60% of the molecules on these grids were too tangled to be measured unambiguously. Therefore it is not possible to state with assurance that the observed bias against molecules which were less

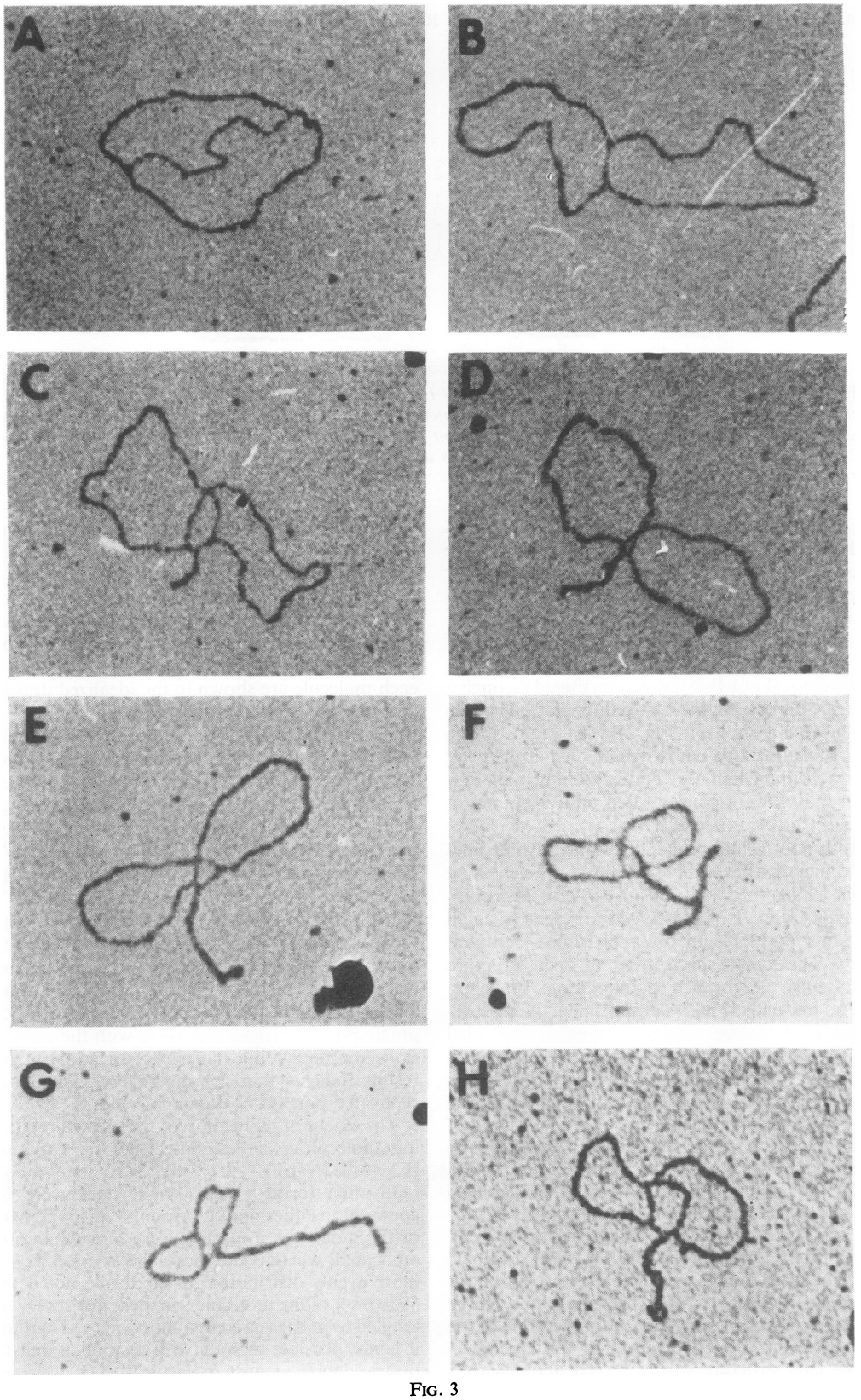

FIG. 3

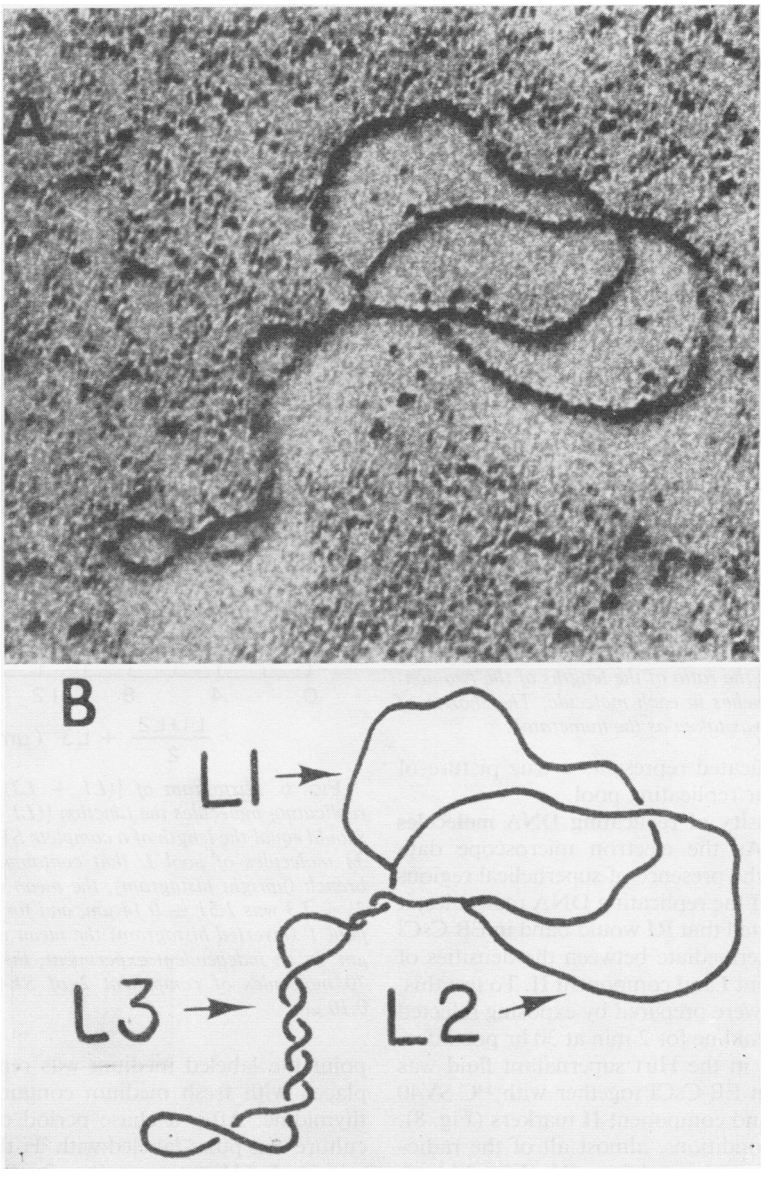

FIG. 4. *Twisted SV40 replicating molecule. The electron micrograph (A) was obtained by a modification of the technique described in the text. (The hypophase was distilled water and the DNA was contrasted by shadowing from one direction with 80% platinum-20% paladium.) In some regions the individual strands which comprise the superhelical branch can be seen. Magnification is 1.5 × 10⁵. In an interpretive drawing of the molecule (B), the branches of the molecules measured are indicated. The two branches that were not superhelical were designated L1 and L2. The superhelical branch was designated L3. Most measurements were carried out on stained molecules as described for Fig. 3. The duplex length of L3 was estimated by measuring its linear length and multiplying by 2.*

FIG. 3. *Replicating SV40 DNA molecules. DNA was mounted for electron microscopy as described by Davis, Simon, and Davidson (aqueous technique) and stained with uranyl acetate. Molecules A and B are "open" replicating molecules. Molecules C to H are "twisted" replicating molecules. Twisted replicating molecules are four to five times as frequent as open replicating molecules. Both types of molecules contain two forks, three branches, and no visible ends. However, in molecules C to H one of the branches is superhelical. The superhelical branch is so tightly twisted that in stained preparations it was not usually possible to distinguish the individual DNA duplexes. With other techniques of mounting the DNA for electron microscopy, the individual duplexes could be seen (Fig. 4A). Magnification is 5.6 × 10⁴.*

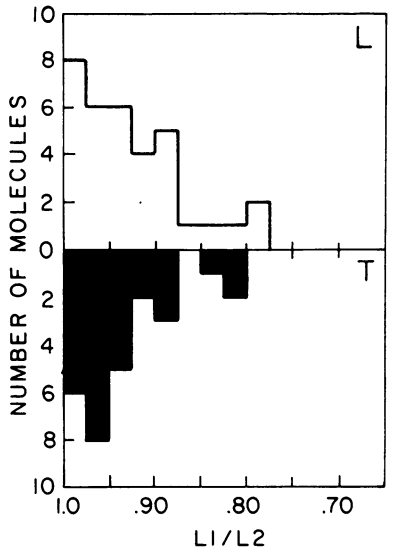

FIG. 5. *Histogram of L1/L2. The molecules containing a superhelical branch in pool L (upright histogram) and pool T (inverted histogram) were measured according to the scheme given in Fig. 4B. The abscissa of this histogram is the ratio of the lengths of the two nonsuperhelical branches in each molecule. The shorter of the two lengths was taken as the numerator.*

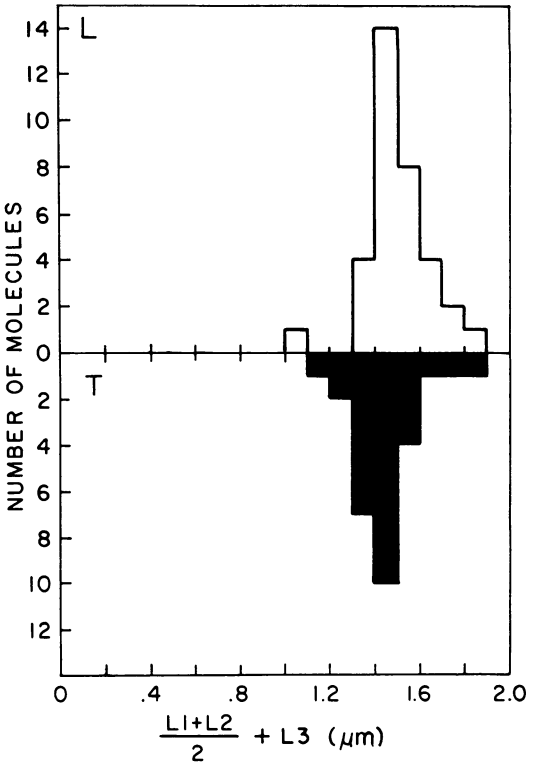

FIG. 6. *Histogram of [(L1 + L2)/2] + L3. For replicating molecules the function [(L1 + L2)/2] + L3 should equal the length of a complete SV40 genome. For 34 molecules of pool L that contained a superhelical branch (upright histogram), the mean of [(L1 + L2)/2] + L3 was 1.51 ± 0.14 μm, and for 27 molecules of pool T (inverted histogram) the mean was 1.45 ± 0.14 μm. In an independent experiment, the mean length of 70 molecules of component 2 of SV40 was 1.52 ± 0.10 μm.*

than 20% replicated represents a true picture of the intracellular replicating pool.

Buoyant density of replicating DNA molecules in EB-CsCl. As the electron microscope data demonstrated the presence of superhelical regions in 80 to 90% of the replicating DNA molecules, it might be expected that RI would band in EB-CsCl at densities intermediate between the densities of SV40 component I and component II. To test this, ^{3}H-labeled RI were prepared by exposing infected cells to ^{3}H-thymidine for 2 min at 30 hr postinfection. ^{3}H-DNA in the Hirt supernatant fluid was then banded in EB-CsCl together with ^{14}C-SV40 component I and component II markers (Fig. 8). Under these conditions, almost all of the radioactivity was incorporated into RI (Fig. 1A) although traces of newly synthesized SV40 component I were observed. The radioactively labeled replicating DNA molecules were seen to band in a heterogeneous manner. A fraction of the RI banded at the same density as that of component II. The remaining radioactive RI banded at densities higher than that of component II, and material at all densities between that of components I and II was present. To further characterize the replicating molecules and to define the basis for the heterogeneous banding pattern in EB-CsCl, the following experiment was carried out. Beginning 25 hr after infection, AGMK cells were exposed to ^{14}C-thymidine for 2 hr, at which point the labeled medium was removed and replaced with fresh medium containing unlabeled thymidine. After a chase period of 1.25 hr, the culture was pulse-labeled with ^{3}H-thymidine for 5 min, and a Hirt supernatant fluid was prepared. With this labeling schedule, it was expected that most of the ^{14}C-thymidine would be incorporated into progeny SV40 molecules. If some of these progeny molecules then reentered the replicating pool, a fraction of the replicating molecules would contain ^{14}C in the parental strands. In contrast, the tritium label would be contained exclusively in the newly replicated DNA strands. Figure 9 shows the distribution of ^{3}H and ^{14}C after preparative EB-CsCl equilibrium density-gradient centrifugation. The replicating molecules were divided into four pools of DNA (average densities: 1.548, pool A; 1.555, pool B; 1.562, pool C; and 1.570, pool D).

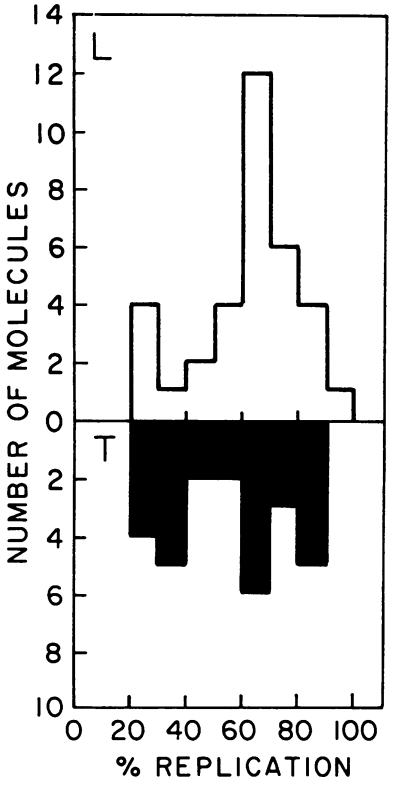

FIG. 7. *Histogram of percentage of replication. The percentage of replication of each twisted replicating molecule was estimated by using the equation: per cent replication = 100 × [(L1 + L2)/(L1 + L2 + 2L3)]. Upright histogram, molecules of pool L; inverted histogram, molecules of pool T.*

Sedimentation properties of newly synthesized ^{3}H-DNA. The four pools of RI described above were extracted with isopropanol to remove the EB and layered on 10 to 30% alkaline sucrose gradients with a ^{14}C-SV40 component II sedimentation marker. The sedimentation patterns of the four pools of newly replicated ^{3}H-DNA are shown in Fig. 10. Two properties of RI were clearly demonstrated by this experiment. The first was that in all four pools essentially all of the newly synthesized DNA sedimented more slowly than the single-stranded linear 16*S* marker. The RI examined in this experiment contain ^{14}C-DNA parental strands. The alkaline sedimentation properties of the ^{14}C-labeled parental strands were determined in a separate experiment. The parental ^{14}C-DNA sedimented at 36 to 47*S* under these conditions (see Fig. 12); therefore cosedimentation of ^{14}C-DNA and ^{3}H-DNA was not observed. Our findings clearly are not in accord with a rolling-circle model of DNA replication.

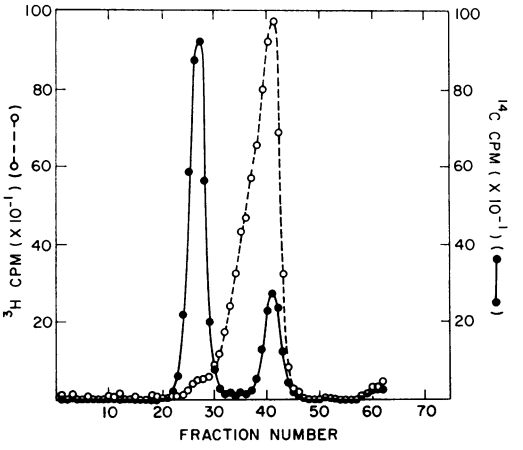

FIG. 8. *EB-CsCl isopycnic banding of DNA contained in a Hirt supernatant fluid. At 30 hr after infection with SV40, an African green monkey kidney cell monolayer was pulsed for 2 min with medium containing 50 μCi of ^{3}H-thymidine/ml. The Hirt supernatant fluid was prepared and dialyzed. A sample of this fluid and purified ^{14}C-SV40 DNA marker were centrifuged to equilibrium in an EB-CsCl gradient (volume, 6 ml; ethidium bromide, 200 μg/ml; CsCl density, 1.564). Fractions were collected directly into scintillation vials and counted.*

The second property observed was that the buoyant density of RI in EB-CsCl depends on their extent of replication. The relationship between buoyant density and the size of newly synthesized strands contained in the replicating molecules is shown in Table 1. Those replicating molecules that banded close to the density of SV40 component II (pool A) contained molecules that had almost completed their replication cycle. By contrast, replicating molecules that banded close to component I in EB-CsCl (pool D) were, on the average, only 23% replicated.

When the same four pools obtained from EB-CsCl were examined by neutral velocity sedimentation, the ^{3}H-labeled material in each case sedimented with a peak of 26*S*. The sedimentation pattern was not related in any apparent way to the extent of replication. This was not surprising in view of the fact that the neutral sedimentation velocity of RI depends on both total mass and number of superhelical turns contained. As replication proceeds, the mass of RI increases, but the number of superhelical turns decreases.

Sedimentation properties of ^{14}C-parental strands. The electron microscope data and buoyant density data suggested the possibility that both parental strands were covalently closed circles. To test this possibility, the sedimentation rate of the parental strands of SV40 replicating DNA was examined under alkaline conditions.

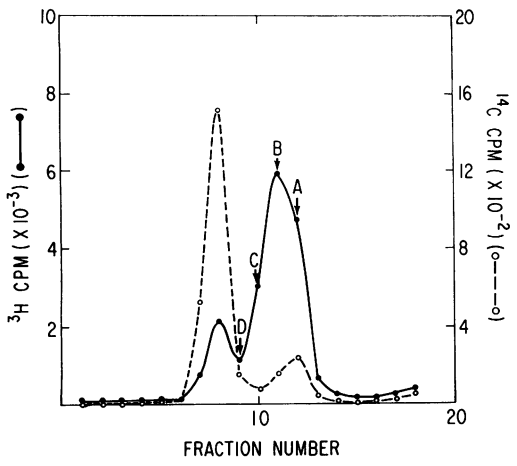

FIG. 9. *Preparative isopycnic banding of ^{14}C-thymidine and ^{3}H-thymidine (double-labeled) Hirt supernatant fluid in EB-CsCl. As described in the text, SV40-infected African green monkey kidney cell monolayers were pulsed for 2 hr with ^{14}C-thymidine, chased with cold thymidine, and pulsed for 5 min with ^{3}H-thymidine. Hirt supernatant fluids were prepared and banded in CsCl (final density, 1.564) and EB (200 μg/ml). Samples were centrifuged at 4 C for 40 hr at 42,000 rev/min in a no. 50 fixed-angle titanium rotor. The contents of each tube were collected in 10-drop fractions. A 20-μliter amount of each fraction was placed on a filter-paper disc, washed with 5% trichloroacetic acid, ethanol, ethanol-ether, and ether, and counted after addition of 10 ml of liquofluor.*

Parental strands of replicating SV40 DNA molecules were labeled with ^{14}C as described above. As expected with a 2-hr ^{14}C-labeling period, most of the ^{14}C-labeled DNA was found in the region of the EB-CsCl gradient corresponding to component I of SV40 (Fig. 9). However, a small but significant portion of the ^{14}C-labeled DNA banded at lower densities. When various fractions from the EB-CsCl gradient were sedimented through neutral sucrose gradients, the profiles of ^{14}C-labeled material were rather complex and were not coincident with the profiles of ^{3}H-labeled material (Fig. 11). This lack of coincidence resulted at least in part from the presence of contaminating ^{14}C-labeled host DNA. To reduce the contribution of host contamination, only the ^{14}C-labeled DNA that cosedimented with the peak of ^{3}H-labeled DNA (26*S*) was used for the alkaline sedimentation analysis. The ^{14}C-labeled DNA sedimented much more rapidly in alkali (36*S* to 47*S*) than would be expected for linear (16*S*) or circular (18*S*) single-stranded SV40 DNA, although slightly slower than component I of SV40 (53*S*, Fig. 12). The rapid sedimentation rate of the ^{14}C-labeled DNA is consistent with the idea that the parental strands are covalently closed circles. The fact that this material sediments somewhat more slowly than SV40 component I can be explained by postulating that the topological winding number of the parental strands is less than that of SV40 component I. It is reasonable to expect that, as the degree of replication increases, the topological winding number of the parental strands decreases. Indeed the data (Fig. 12) show that the sedimentation rate of the ^{14}C-labeled DNA is inversely correlated with its extent of replication. Material taken from the lower density portion of the EB-CsCl gradients (pool A, Fig. 9), which corresponded to the most highly replicated molecules, had a sedimentation coefficient of 36*S*. On the other hand, material taken from the higher density portion of the gradient (pool C), corresponding to the least replicated molecules, had a sedimentation coefficient of 47*S*. Material taken from the region of intermediate density had an intermediate sedimentation coefficient (44*S*).

DISCUSSION

The characteristics of the RI of SV40 and the data that support its structure as proposed in Fig. 13 are as follows.

Newly replicated SV40 DNA strands are not covalently linked to the parental DNA strands. When RI-containing parental ^{14}C-DNA and newly replicated ^{3}H-DNA are sedimented in alkaline gradients, cosedimentation of ^{3}H-DNA and ^{14}C-DNA was not observed (see Fig. 10 and 12). The absence of a covalent link between parental DNA and newly replicated DNA excludes the rolling-circle model of DNA replication. In alkaline gradients, all newly replicated DNA sedimented more slowly than 16*S* linear single-stranded DNA. In addition to excluding covalent linkage of newly replicated DNA to parental DNA strands, this latter finding also establishes the absence of a covalent link between the two newly replicated strands at either of the two forks. Such a covalent link would generate newly replicated single-stranded DNA larger than 16*S* in molecules that were more than 50% replicated.

The parental DNA strands in the replicative intermediate are covalently closed. Three types of experiments support this finding. First is the finding that 80 to 90% of the replicating molecules examined by electron microscopy contained a superhelical region. In the molecules that were measured, the superhelical region was restricted to the unreplicated portion of the molecule. The reason for this is not understood. A structure in which the two daughter duplexes are wound about

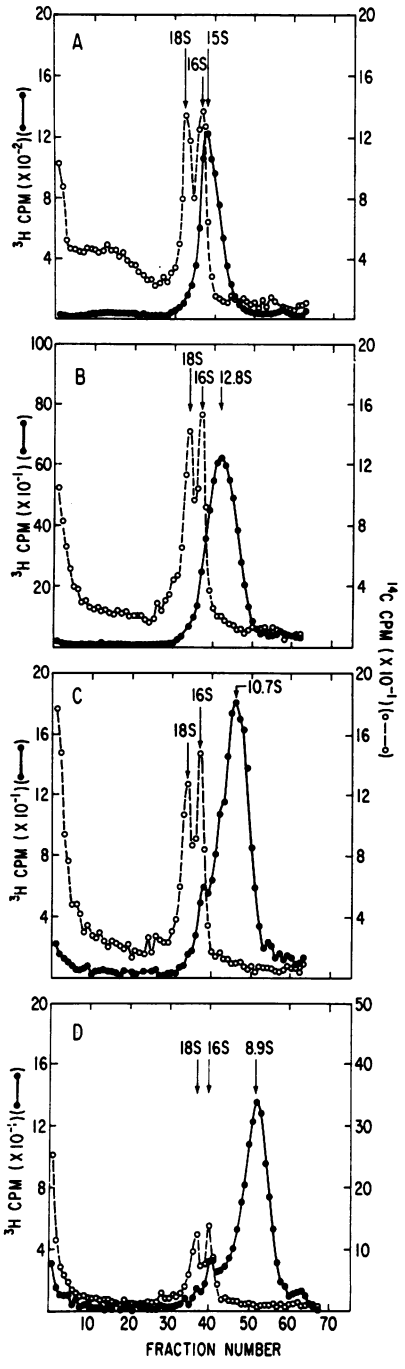

FIG. 10. *Velocity sedimentation in alkaline sucrose of fractions A, B, C, and D indicated in Fig. 9. Each fraction was extracted three times with CsCl-saturated isopropanol to remove the ethidium bromide; dialyzed against 0.01 M Tris, 0.01 M EDTA (pH 7.2) buffer; and concentrated. A sample was layered onto an 11.6-ml 10 to 30% alkaline sucrose gradient and sedimented at 10 C for 13 hr at 40,000 rev/min in an SW 41 rotor. Fractions were collected directly into scintillation vials, neutralized by the addition of two drops of glacial acetic acid, and counted. Sedimentation is from right to left. The ^{14}C counts per minute reflect not only the small amount of ^{14}C present in the double-labeled sample but also a purified ^{14}C-SV40 DNA that was added to each gradient as a marker.*

TABLE 1. *Relationship of percentage of replication of SV40 RI to density at which it bands in EB-CsCl*

Fraction	Density[a]	Sedimentation coefficient	Mol wt[b]	Replication (%)
A	1.548	15.0	1.28×10^6	85.1
B	1.555	12.8	0.859×10^6	57.2
C	1.562	10.7	0.549×10^6	36.6
D	1.570	8.9	0.346×10^6	23.1

[a] Densities of SV40 components I and II were 1.580 and 1.550, respectively.

[b] Molecular weights were calculated from the sedimentation coefficients by the equation of Studier (24). A sedimentation coefficient of 16.0*S* and a molecular weight of 1.5×10^6 daltons were used for linear single-stranded SV40 DNA.

one another might be expected to be as stable as the structures actually observed. It is possible that the surface forces in the protein monolayer during preparation of the sample for electron microscopy may preferentially pull the two daughter segments apart and confine the superhelix to the unreplicated portion. An alternative possibility is that molecules in which the two daughter duplexes are wound about one another are more likely to be tangled and, therefore, are not scored. A second finding that supports a covalent structure in RI was the heterogeneous banding of RI in EB-CsCl. Covalently closed SV40 DNA component I binds less EB than does nicked component II and bands at a higher density. A large fraction of the RI banded at densities intermediate between that of SV40 component I and SV40 component II. The heterogeneous banding in EB also was observed when RI contained in the Hirt supernatant fluid was extracted with phenol or was treated with Pronase before isopycnic banding. This would exclude the possibility that the superhelical structure is maintained by a protein. The electron microscopy finding that replicating molecules contain superhelical segments of various lengths is consistent with the heterogeneous distribution of replicating molecules in EB-CsCl.

A third line of evidence that establishes the covalently closed structure of RI lies in the sedimentation properties of RI in neutral and alkaline gradients. When RI containing parental ^{14}C-DNA

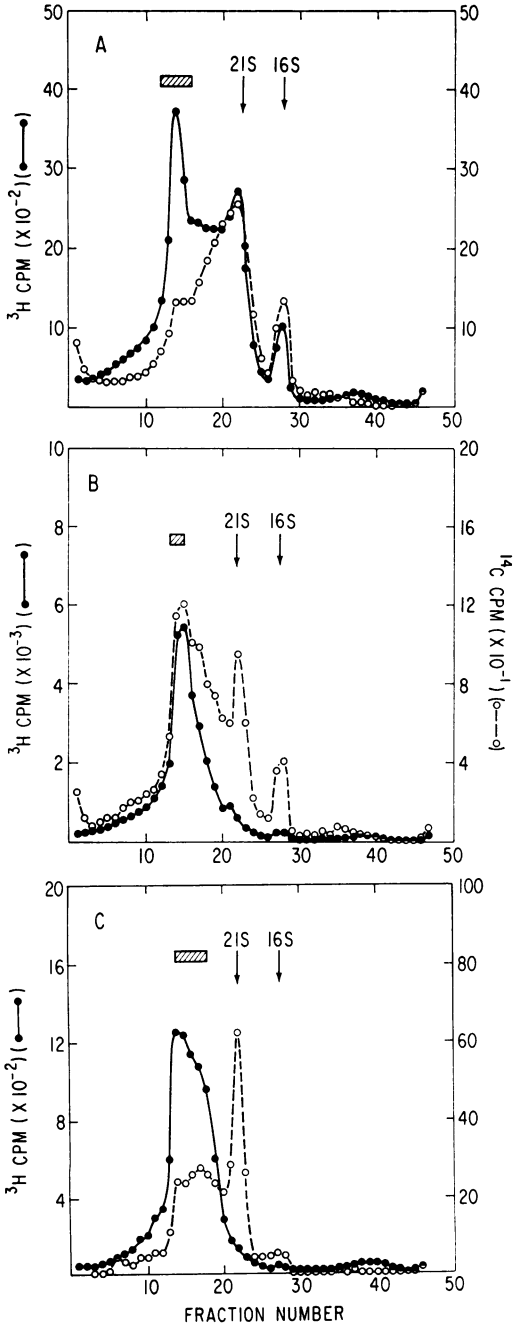

FIG. 11. *Velocity sedimentation in neutral sucrose of fractions A, B, and C indicated in Fig. 9. Each fraction was extracted as described for Fig. 10. The sample was layered onto an 11.6-ml 5 to 30% neutral sucrose gradient and sedimented at 10 C for 7 hr at 40,000 rev/min in an SW 41 rotor. The tubes were tapped, 0.25-ml fractions were collected, and samples were counted. Sedimentation is from right to left.*

and newly replicated ^{3}H-DNA was examined in neutral sucrose gradients, it sedimented in a heterogeneous manner with a peak at 26*S*. Under alkaline conditions, the 26*S* RI released parental

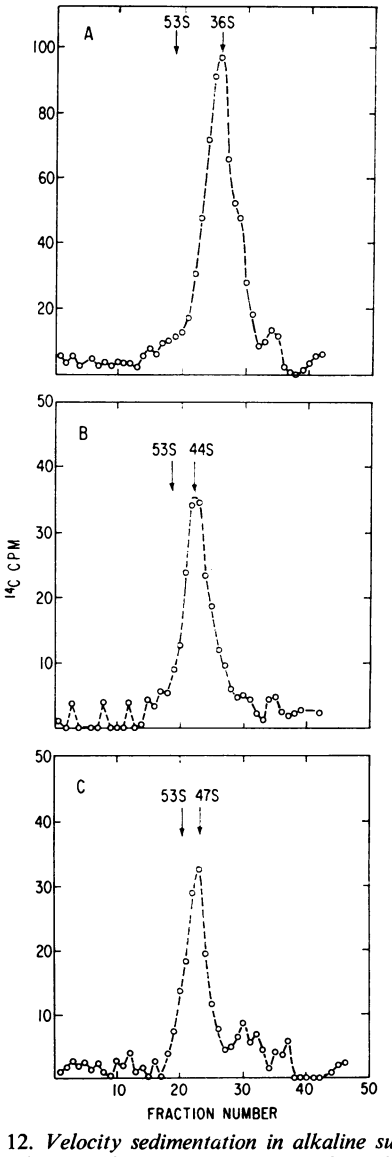

FIG. 12. *Velocity sedimentation in alkaline sucrose of pooled fractions from gradients A, B, and C indicated in Fig. 11. Each pool was dialyzed against 0.01 M Tris, 0.01 M EDTA (pH 7.2) buffer; concentrated to 0.05 ml; layered onto an 11.6-ml 10 to 30% alkaline sucrose gradient; and sedimented at 10 C for 5 hr at 40,000 rev/min in an SW 41 rotor. The tubes were tapped, and seven-drop fractions were collected directly into scintillation vials, neutralized by the addition of three drops of glacial acetic acid, and counted. The 53S arrow indicates the position of a ^{3}H-thymidine SV40 component I marker that was cosedimented with the sample. Sedimentation is from right to left.*

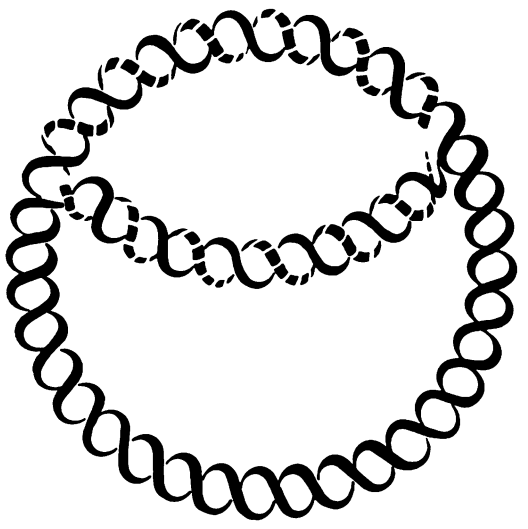

FIG. 13. *Diagrammatic representation of replicating SV40 DNA. The salient features of the molecule are: (i) both parental DNA strands (solid lines) are covalently closed, and (ii) the two newly synthesized DNA strands (broken lines) are not covalently linked to the parental DNA nor are they linked together.*

DNA molecules with sedimentation coefficients of 36 to 47*S*. Parental DNA that sedimented at 16 or 18*S* was not observed.

The position at which a molecule banded in EB-CsCl (Fig. 9) was correlated with the degree of replication of the molecule. The extent of replication of RI was calculated from the size of the newly replicated DNA strands, which were measured in alkaline sucrose gradients. RI banding at the density at which SV40 component II bands (1.548) contained newly replicated DNA strands of molecular weight 1.28×10^6 (85% replicated); RI banding at a density of 1.57, close to the density at which component I bands (in EB-CsCl), contained newly replicated single-stranded DNA of molecular weight 3.46×10^5 (23% replicated). Molecules banding at densities intermediate between these two values contained newly replicated DNA of intermediate molecular weights (Table 1).

Initiation of replication of the *E. coli* genome occurs at a specific site (17), as does that replication of phage λ and P2 (22, 23). If initiation of replication of SV40 also occurs at a specific site on the viral genome, then the fragments that we have isolated by alkaline sedimentation should represent unique portions of the total genome. Such fragments will be of value in locating the regions in the SV40 genome that are involved in hybridization of viral DNA with those species of viral messenger ribonucleic acid that are present in SV40 transformed and in lytically infected cells. The newly replicated DNA contained in RI that is fractionated and sized by alkaline sedimentation is assumed to represent equal quantities of the complementary strands. However, this has not been established.

It was most surprising to find that the parental strands of most SV40 replicating molecules are covalently closed circles. This fact presents certain conceptual problems. During replication of a covalently closed molecule, the process of unwinding the parental duplex would necessarily introduce superhelical turns into the molecule. As replication proceeds, the introduction of these turns would make it progressively more difficult (and eventually impossible) to unwind the parental strands. To resolve this difficulty it is necessary to postulate the existence of a "swivel" in the unreplicated portion of the molecule. The present observation that very few SV40 replicating molecules contain such a swivel strongly suggests that the swivel is present only intermittently during replication. This concept of an intermittent swivel is consistent with our interpretation of the sedimentation properties of the parental strands. If the swivel were introduced at fairly frequent intervals during replication, then the winding number of the parental strands would be expected to be inversely related to the degree of replication.

In agreement with Levine et al. (18), we have observed that progeny SV40 molecules (component I) are not detectable in significant amounts until 5 to 10 min after the initiation of a pulse of ^{3}H-thymidine. Huberman (11) reported that animal cell DNA is replicated at approximately 2 μm/min. We have observed that SV40 has a length of about 1.5 μm; therefore its synthesis should be complete in less than 1 min. Levine et al. suggested that there may be a rate-limiting step late in the replication cycle which accounts for this discrepancy. In support of this hypothesis, they reported that 75% of SV40 replicating molecules have almost completed replication (18). Our own data, however, do not confirm this observation. The necessity to introduce a swivel at frequent intervals during replication may account for the slow production of progeny molecules, but it would not cause an accumulation of molecules at a specific stage of replication.

Our data do not bear on the question of the chemical nature of the swivel. However, the simplest possible swivel is produced by the introduction of a single-strand break. The intermittent swivel then could be envisioned as the alternate action of a nicking endonuclease and ligase. If the swivel were present only very briefly (a small fraction of the lifetime of the RI), then most RI

isolated from a cell at any given moment would not contain a swivel. The temporal relationship between the process of active DNA synthesis and the swiveling process could take one of several forms. For example, synthesis and swiveling could occur alternately, in which case synthesis could continue until the number of superhelical turns in the molecule became so great that the parental strands could be unwound no further. At this point replication would cease until there was an endonucleolytic cleavage of one of the parental strands in the unreplicated portion of the molecule. This cleavage would permit relaxation of the RI with removal of the superhelical turns. The break would be sealed by a ligase (21), and the whole process would repeat until replication was complete. An advantage of this model is that the temporal separation of synthesis and swiveling reduces the probability of replication through a nick. (Replication through a nick could cause the complete and premature detachment of one of the daughter duplexes.) On an alternative model, active DNA synthesis would occur only when the swivel is present. One of the parental strands would be cleaved in the unreplicated portion of the molecule, and DNA replication could proceed to just short of the swivel point. Ligase then would repair the break, but replication could not proceed until the endonuclease created another swivel ahead of the growing point. The latter mechanism is similar to one that has been proposed by Tomizawa (25).

ACKNOWLEDGMENTS

We thank Robert Sproull and William Mohler, Division of Computer Research and Technology, National Institutes of Health, for valuable assistance.

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