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Origin and Direction of Simian Virus 40 Deoxyribonucleic Acid Replication

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Volume 10, no. 3, p. 484: replace abstract with the following:

Double-branched, circular, replicating deoxyribonucleic acid (DNA) molecules of simian virus 40 (SV40) have been cleaved by the R_I restriction endonuclease from *Escherichia coli*. This enzyme introduces one double-strand break in SV40 DNA, at a specific site. The site of cleavage in the replicating molecules was used in this study to position the origin and the two branch points. Radioactively labeled molecules fractionated according to their extent of replication were evaluated after cleavage by sedimentation analysis and electron microscopy. The results demonstrate that the R_I cleavage site is 33% of the genome length from the origin of replication and that both branch points are growing points. These data indicate that SV40 DNA replication is bidirectional and confirm other reports which have shown a unique origin of replication.

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Replicating deoxyribonucleic acid (DNA) molecules of simian virus 40 (SV40) are small, circular structures having two untwisted, replicated branches and one twisted, unreplicated branch (8, 14). The twisted or supercoiled configuration of the unreplicated branch is due to the presence of covalently closed template strands in the isolated molecules (8, 14). Two independent investigations have recently provided evidence for a specific initiation site for SV40 DNA replication. Using the restriction endonuclease from *Haemophilus influenzae* to fragment pulse-labeled replicating SV40 DNA, Nathans and Danna (11) were able to show a temporal gradient in the labeling of specific fragments. Studies on the initiation site carried out in our laboratory involved analysis of the rates of renaturation of different size categories of newly synthesized strands. The results demonstrated that the smallest newly synthesized strands were the least complex genetically and renatured most rapidly (17). Neither of these investigations were able to differentiate between unidirectional and bidirectional replication.

In another study (7), we examined replicating molecules of SV40 DNA which had been cleaved by the restriction endonuclease of *Escherichia coli* B. After cleavage the newly synthesized strands from partially replicated molecules dissociated from the template strands under nondenaturing conditions. Because of the possibility that the cleavage site was at a replication fork, a definitive analysis of the origin and direction of replication could not be made. In the present work replicating molecules fractionated on the basis of the extent of replication have been examined before and after cleavage by an *fi*$^+$ R factor restriction endonuclease from *E. coli*. From these results the origin and direction of SV40 DNA replication have been determined.

MATERIALS AND METHODS

The preparation of double-labeled replicating molecules of SV40 DNA from primary African green monkey kidney cells infected with SV40 has been described elsewhere (7, 14). Briefly, cells infected with SV40 are prelabeled with ^{14}C-thymidine and, following a chase period with unlabeled thymidine, are subsequently pulse-labeled with ^{3}H-thymidine. After extraction, the replicating viral DNA is fractionated into a "young" or partially replicated pool (less than 60% replicated) and a "mature" or nearly fully replicated pool (greater than 75% replicated) by banding the DNA in ethidium bromide-CsCl density gradients (7, 14).

The ~~replicative intermediate~~ (R_I) nuclease (4) was generously supplied by Herbert Boyer. The reaction mixture for the nuclease consisted of 0.01 to 0.05 μg of ^{14}C-labeled SV40 DNA component I or II (Comp. I, Comp. II) (7), 0.1 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride, *p*H 7.5, 5 mM $MgCl_2$, and 5 to 10 μliters of enzyme in 0.28 ml. Reactions were incubated at 23 C for 30 min and stopped by chilling to 0 C and then adding 10 μliters of 0.2 M ethylenediaminetetraacetic acid (EDTA) and 10 μliters of 1% Sarkosyl NL97 (Geigy). Ten microliters

of the purified enzyme solution was capable of converting 0.1 μg of SV40 DNA Comp. I or II to linear molecules (Comp. III) sedimenting at 14.5*S* in neutral sucrose gradients under the above conditions. In reaction mixtures containing replicating molecules, the DNA concentrations were too low to be measured and, therefore, the radioactivity of the labeled molecules was used for determining the amounts to be added to a given reaction mixture. In general, between 2,500 and 7,500 counts/min of ^{3}H-labeled replicating DNA molecules were added to a reaction mixture containing 10 μliters of the R_I nuclease solution.

Analysis of the cleavage products by velocity gradient centrifugation in neutral and alkaline sucrose as well as by electron microscopy using an aqueous technique has already been described (7, 14).

RESULTS

Experimental design. Bacterial restriction nucleases have been shown both by genetic and biochemical tests to be highly specific with regard to their sites of cleavage in bihelical DNA (1, 3, 4). The R_I nuclease creates one double-strand break in SV40 DNA component I or II (SV40 DNA Comp. I, II, and III are supercoiled, relaxed circular, and linear forms of SV40 DNA, respectively) as determined in this work, presumably at a specific site. If the cleavage site of the R_I nuclease is at a specific nucleotide sequence in replicating SV40 DNA, then analysis of the cleavage products should locate the origin as well as indicate the direction of replication. Two methods of analysis of the cleaved replicating DNA have been employed. (i) Double-labeled replicating molecules containing ^{14}C-thymidylate in the template strands and ^{3}H-thymidylate in the newly synthesized strands were examined before and after cleavage by neutral and alkaline velocity gradient centrifugation. If the site of cleavage is distinct from the initiation site, then replication must proceed beyond the cleavage site before a change in size of the newly synthesized DNA can be detected by alkaline sucrose sedimentation. Specific size categories of fragments of the newly synthesized strands formed after cleavage of the almost fully replicated molecules would be expected if the sites for initiation of DNA synthesis and cleavage by the R_I nuclease are specific. From these data the location of the cleavage site can be related to the termination point of replication. (ii) Cleavage of replicating molecules of SV40 DNA at a single location at some distance from the origin should produce molecules whose morphology can be distinguished by electron microscopy. In "young" or partially replicated molecules in which the cleavage site has not yet been replicated, the cleavage products would contain an internal loop or circular region composed of the two replicated branches to which, at opposite branch points, are attached the linear branches or tails (created by cleavage in the unreplicated region of the molecules). Measurements of the lengths of the two linear branches can differentiate unidirectional from bidirectional replication. In unidirectional replication, one of the linear branches would be invariable in length whereas the other would vary as a function of the extent of replication. For bidirectional replication, both branches would be variable in length in molecules which were replicated to different extents.

Cleavage of fractionated, double-labeled replicating molecules. The "young" or partially replicated molecules were examined by sedimentation analysis to determine whether the R_I cleavage site was replicated early after initiation of synthesis. In Fig. 1 a composite of graphs is shown, with the neutral sucrose results on the left and alkaline sucrose profiles on the right. Before cleavage (Fig. 1A) the replicating molecules sediment through neutral sucrose at 25 to 26*S* as expected. The molecules prelabeled with ^{14}C-thymidylate consisted of replicating molecules which cosediment with the ^{3}H-labeled molecules and DNA Comp. I and II (Fig. 1A). The purification techniques did not completely remove the ^{14}C-labeled DNA Comp. I and II from the replicating molecules. After cleavage by the R_I nuclease (Fig. 1B), the double-labeled replicating molecules sediment at about 20*S*, or slightly slower than the DNA Comp. I reference. The prelabeled DNA Comp. I and II in the preparation of replicating molecules are converted after cleavage to Comp. III (Fig. 1B) which sediments with an S value of 14.5. This finding that the newly synthesized strands remain associated with the template strands after cleavage is in contrast with the cleavage of replicating molecules by the *E. coli* B restriction nuclease (7). After cleavage by the B enzyme, the newly synthesized strands separated from the cleaved template strands.

The alkaline sucrose sedimentation was used to evaluate a possible change in size of newly synthesized strands as a consequence of cleavage and also to demonstrate that one break had been produced in each template strand. In Fig. 1C and D it is evident that there is no appreciable change in size of the newly synthesized strands (^{3}H-labeled) after cleavage. This indicates that the cleavage site has not been replicated in these molecules which are between 30 and 55% replicated (based on the size of the newly synthesized strands). All of the ^{14}C-labeled DNA was converted to linear, 16*S* strands after cleavage, indicating that one double-strand break had been made in each molecule. Before cleavage the co-

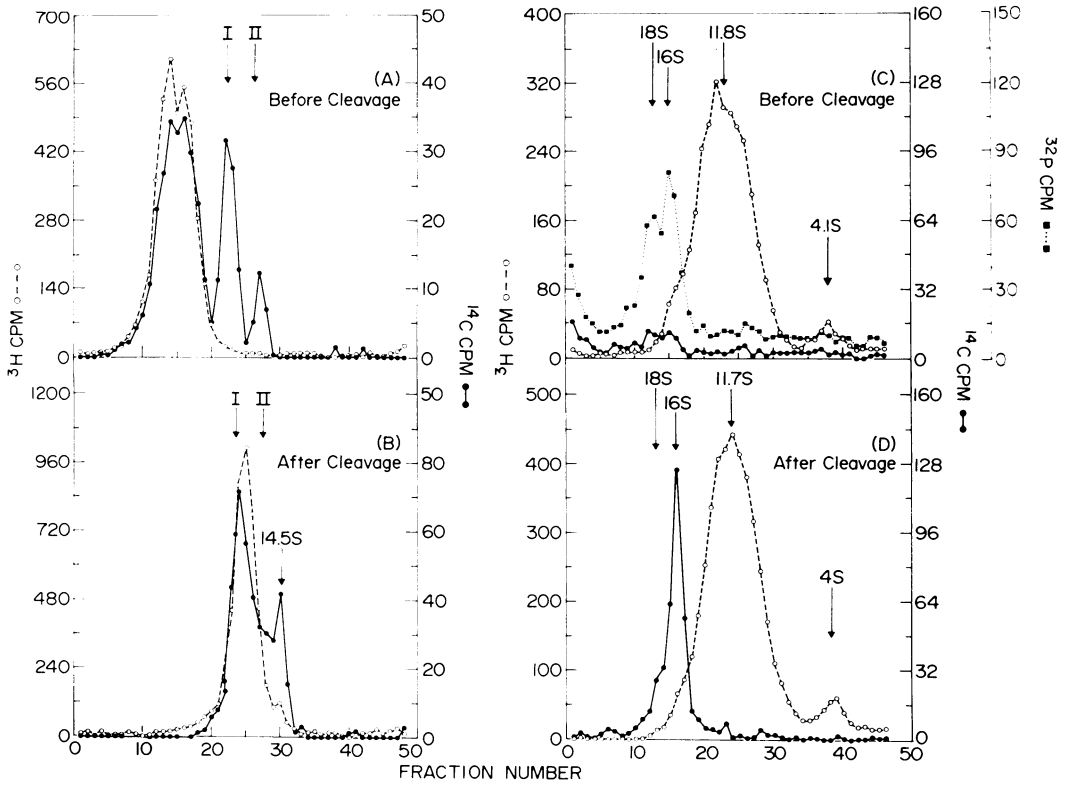

FIG. 1. *Sedimentation analysis of "young" replicating molecules before and after the R_I nuclease treatment. R_I molecules labeled with ^{14}C-thymidylate in their template strands and ^{3}H-thymidylate in their newly synthesized strands were incubated with the R_I restriction enzyme as described in Materials and Methods. Two reaction mixtures were prepared, with the enzyme absent from the control (A and C) and present in the other (B and D). After termination of the reaction, 2 ng of ^{32}P-labeled SV40 DNA (7) containing both the closed circular form (Comp. I) and the open circular form (Comp. II) was added to each sample. The reaction mixtures were divided into two equal parts and analyzed by both neutral (A and B) and alkaline (C and D) sucrose rate zonal centrifugation (7, 14). The concentration of NaCl in the neutral gradients shown here and in Fig. 2 was 0.1 M, whereas the alkaline gradients here and in Fig. 3 contained 0.7 M NaCl and 0.3 M NaOH. The direction of sedimentation in these and other sucrose gradients in this paper is from right to left, and the recovery of labeled DNA was greater than 90% in each. The positions of ^{32}P-labeled DNA Comp. I (21S) and II (16S) are designated by the arrows in (A) and (B). The separated circular (18S) and linear (16S) strands of ^{32}P-labeled DNA Comp. II are also designated by arrows in (C) and (D).*

valently closed template strands sediment between 36 and 48S (14) under alkaline conditions and, therefore, do not appear in the gradient (Fig. 1C). The small amounts of pulse-labeled strands which sediment at 4S (Fig. 1C and D) have been shown in another investigation (Fareed and Salzman, Nature [London], *in press*) to be intermediates in SV40 DNA chain growth and are not affected by cleavage.

Double-labeled replicating molecules which were greater than 75% replicated were next examined before and after the R_I nuclease reaction. Figure 2A and B illustrate the neutral sucrose gradient profiles of the molecules. Before cleavage, the replicating molecules sediment at 26 to 27S with good correspondence of the two labels. After cleavage (Fig. 2B), the "mature" replicating molecules are converted to structures sedimenting at about 20S as was seen with the cleaved "young" molecules. Again the template strands remain associated with the newly synthesized strands while the ^{14}C-labeled DNA Comp. I and II are converted to Comp. III.

Since the mature molecules were quantitatively broken by the nuclease, the newly synthesized strands were expected to be fragmented. The alkaline sucrose analysis of these molecules demonstrates before cleavage (Fig. 3A) that the ^{3}H-labeled newly synthesized strands sediment close to the 16S marker. The size calculated for

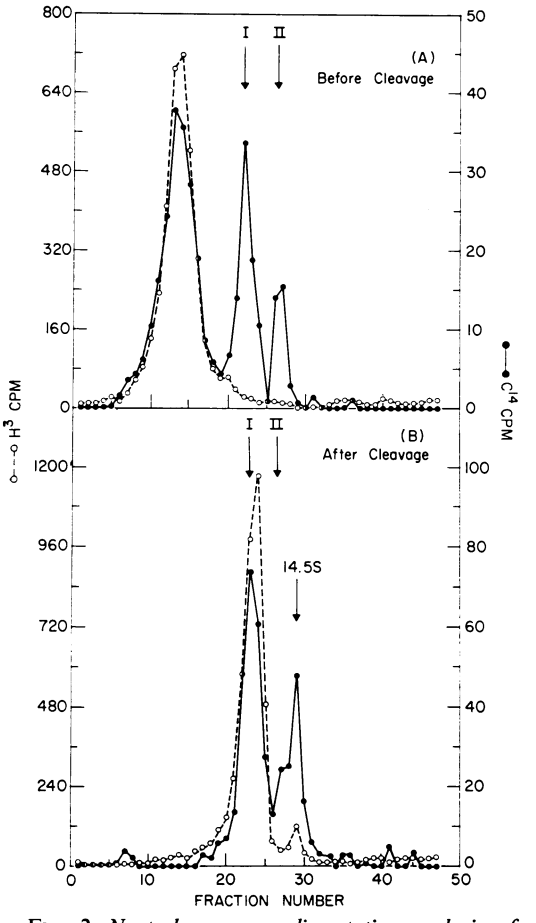

FIG. 2. *Neutral sucrose sedimentation analysis of "mature" replicating molecules before and after cleavage by the R_I nuclease. A population of replicating molecules in which replication was nearly completed was treated with the R_I restriction nuclease and analyzed by neutral sucrose centrifugation through linear gradients. Sample (A) was incubated in the absence of the R_I enzyme whereas sample (B) was treated with the enzyme (see Materials and Methods). Half of each reaction mixture was analyzed in these gradients, and the remainder of each was analyzed by alkaline sedimentation shown in Fig. 3. The sedimentation references were the same as those mentioned in the legend to Fig. 1.*

strands sedimenting at 14.9*S* is 1.35×10^6 daltons as compared to 1.6×10^6 daltons for the 16*S* reference strands. A small amount of the precursor 4*S* DNA is also present in these samples. After cleavage though (Fig. 3B), new species are created. The large strands of newly synthesized DNA are reduced slightly in size with a narrowing of the band width, and a new fragment sedimenting at 7.3*S* is detected. The size calculated (16) for the small fragment is 225,000 daltons or about 14% of the mass of one intact strand of SV40 DNA. The finding of such a small fragment liberated in the cleaved, "mature" replicating molecules indicates that the R_I cleavage site is near to the termination point for replication.

We have previously characterized the role of DNA Comp. II in viral DNA replication (Fareed and Salzman, Fed. Proc. **31**:442, 1972) and shown that the newly synthesized DNA is located

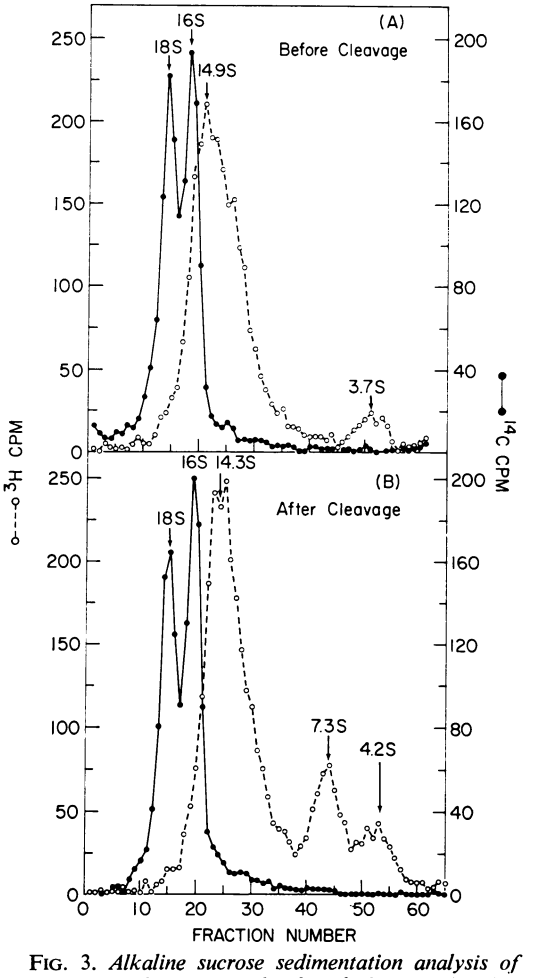

FIG. 3. *Alkaline sucrose sedimentation analysis of "mature" replicating molecules before and after cleavage by the R_I nuclease. The samples from the reaction mixtures described in the legend to Fig. 2 were layered onto 10 to 30% linear alkaline sucrose gradients, and centrifugation analysis was carried out as described previously (7, 14). The location of the ^{14}C-labeled template strands in these gradients has been masked by the addition of 0.4 μg of ^{14}C-labeled DNA Comp. II (7) which provides the 18S and 16S sedimentation references. In a separate alkaline sucrose analysis on a similar sample (not shown here), ^{14}C-labeled template strands sedimented at 16S after cleavage, as was observed in Fig. 1(D) for the template strands of the cleaved, "young" molecules.*

in the interrupted strands. In another study (Fareed, McKerlie, and Salzman, *unpublished data*), pulse-labeled DNA Comp. II was cleaved by the R_I nuclease, and the resulting small fragment of the newly synthesized strands corresponded to 17.5% of the mass of one strand. This result is probably a more accurate estimate of the position of the R_I cleavage site in relation to the termination point since the 16*S* strand in the pulse-labeled DNA Comp. II represents the complete size of one SV40 DNA strand.

Electron microscopy of the cleavage products. In order to orient the site of cleavage in relation to the growing point(s), molecules which were replicated to different extents were examined in the electron microscope after cleavage. The population selected was from 5 to 70% replicated. These partially replicated molecules are broken in the unreplicated region as determined by the sedimentation studies already presented. Therefore, if replication is unidirectional from a fixed initiation site, the cleavage site should be approximately 17% of the genome length from the initiation site. Furthermore, the termination site would be adjacent to, or 360° from, the origin. In this model, one of the two linear branches formed in the looped molecules should maintain this distance between its free end and the branch point (initiation site). In bidirectional replication, the cleavage site should correspond to a site approximately 17% of the genome length from the termination point. Since the termination point is 180° from the initiation site in the bidirectional model, the cleavage site could be at one of two locations, 119° or 241°, both 17% of the genome length from the termination point. In this model the two linear branches in the cleaved molecules should decrease in length with increasing extents of replication.

Representative replicating molecules cleaved by the R_I nuclease are shown in Fig. 4. The molecules are oriented such that the short linear branches are on the left. Two hundred and twenty molecules examined by electron microscopy conformed with the structural features of those shown in Fig. 4. The majority of these molecules did contain one short linear branch and one longer linear branch as seen in the micrographs in Fig. 4. However, when the lengths of the short branches were compared in molecules which were replicated to different extents, a progressive decrease in length was observed as the extent of replication increased (Fig. 4 and 5).

Length measurements were made on the 220 molecules having this double-branched, looped configuration with one linear branch or tail at each branch point. The subparts measured (inset to Fig. 5) were the length of the short branch (L1), the lengths of the replicated segments (L2 and L3), and the length of the long branch (L4). The total length of the molecule was calculated from the sum of the two linear branches (L1 and L4) plus the average of the replicated segments ([L2 + L3]/2). This determination (1.52 $\pm$ 0.05 μm) corresponded quite well to previous length measurements of SV40 DNA Comp. II (14). The ratio of L2 to L3 was 1.02 $\pm$ 0.08 and in good agreement with the prediction that the lengths of the two replicated segments be equal. To distinguish between the unidirectional and the bidirectional models for SV40 DNA replication, the length of the short branch (L1) was plotted as a percentage of the total length of an individual molecule versus the extent that replication had proceeded in that molecule (calculated from the average of L2 and L3 divided by the total length). It can be seen (Fig. 5) that L1 decreases in the majority of molecules in a linear fashion with increasing extents of replication. Therefore, these molecules undergo bidirectional replication. The slope for the line representing the length of the longer, linear branch (L4) as a function of the extent of replication is the same as that shown in Fig. 5. This is consistent with both growing points moving at the same rates in a symmetrical fashion in the majority of replicating molecules. The small number of measurements which do not fall close to the straight line in Fig. 5 come from molecules in which replication is asymmetric. In these molecules, one of the two growing points must have moved more rapidly than the other. The bulk of the determinations in Fig. 5 fall very close to a straight line which intercepts the abscissa at 33% of the genome length. This value is the distance between the cleavage site and the origin of the replication in the bidirectional model. In accord with this finding are the sedimentation data on the cleaved, pulse-labeled DNA Comp. II which demonstrated that the cleavage site was about 17% of the genome length from the termination point (50% of the genome length from the origin in this model). The small fragment produced in the newly synthesized strands in the cleaved, mature, but nonsegregated replicating molecules is 14% of the genome length. The accumulation of these molecules after pulse-labeling may indicate a delay or rate-limiting step in replication at that point (94% replicated) while daughter molecules separate. Immediately after segregation, gaps (less than 6% of the genome length in size) may exist in the interrupted circular daughter molecules, and these would be closed rapidly by the action of DNA polymerase(s) and ligase.

Only about 3% of the 250 molecules examined exhibited two terminal branches similar to the

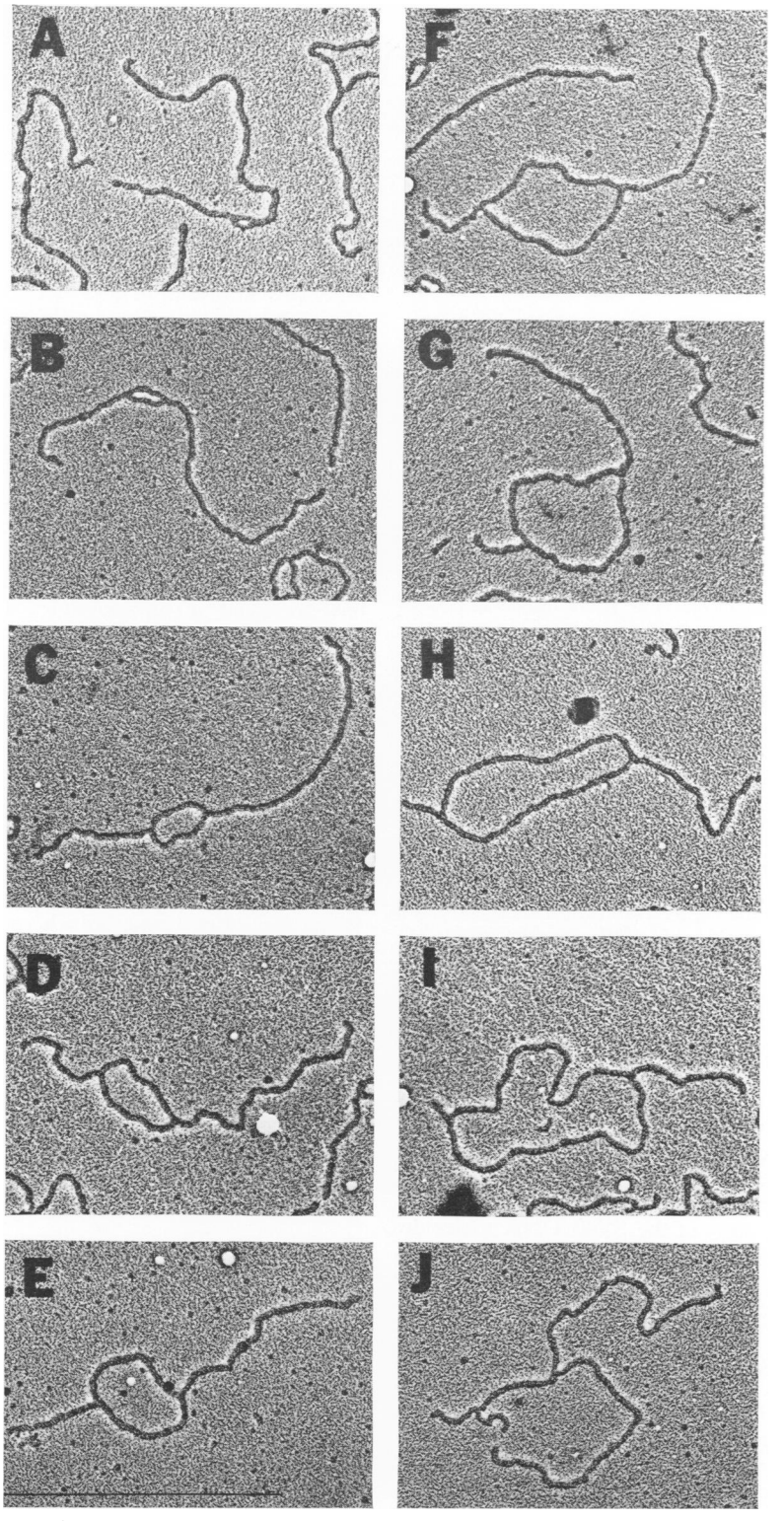

FIG. 4. *Replicating SV40 DNA molecules treated with R_I nuclease. DNA was mounted for electron microscopy by the aqueous technique of Davis et al. (5) and rotary-shadowed with platinum-palladium. Micrographs of selected molecules have been arranged in order of increasing degree of replication (A through J) and oriented with the short branch (L1) at the left. Panel J shows a molecule where replication has proceeded past the R_I nuclease cleavage site producing the terminally branched structure. Bar represents 1 μm.*

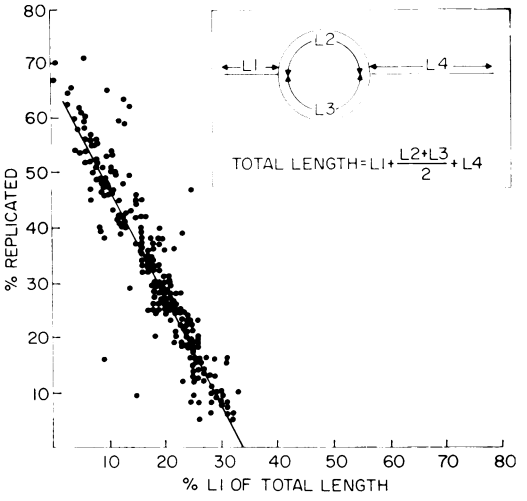

FIG. 5. *Length of the short, linear branch in cleaved replicating molecules as a function of the extent of replication. Length measurements were made on replicating SV40 DNA molecules cleaved by the R_I restriction enzyme (see Materials and Methods). The same population of molecules as illustrated in Fig. 4 was used for this analysis. Inset shows the contour lengths which were measured (14) as well as the computation for the total length of an individual molecule. The length of short, linear branch or tail segment (L1) was divided by the total length, and the quotient was converted to a percent of the total length by multiplying by 100. The percent replicated was determined by dividing the average of the two replicated branches (L2 and L3) by the total length and then multiplying by 100.*

molecules in Fig. 4J. These configurations arise in molecules in which the cleavage site has been replicated. The population of molecules examined in this study excluded most of the "mature" or almost fully replicated molecules as well as the bulk of contaminating cellular DNA. When the pool of molecules containing the "mature" molecules was studied after cleavage, a larger number of the terminally branched molecules was seen.

About 9 to 10% of the SV40 DNA molecules exhibited single branch points in a Y configuration. These were interpreted as representing cleaved molecules which had been broken at one of the forks in addition to the R_I cleavage site either during handling or possibly in vivo. A small number of the single branch molecules contained an intact circular region which was the same length as SV40 DNA (1.5 μm). These may have arisen by breakage at one of the forks in an uncleaved molecule.

DISCUSSION

Replicating SV40 DNA molecules, which are circular and have two branch points and no free ends, were cleaved by the R_I restriction endonuclease from *E. coli*. The R_I enzyme produces one double-strand break in SV40 DNA, presumably at a specific nucleotide sequence.

Sedimentation studies on cleaved, pulse-labeled replicating molecules located the R_I cleavage site near to the termination point for replication. With this information and electron microscopy data, it has been possible to differentiate between the unidirectional and bidirectional models for the orientation of replication. In unidirectional replication, the termination point is adjacent to the initiation site on the circular map for SV40 DNA. This implies that the branch point nearest the cleavage site is fixed while the other branch point moves around the genome, eventually (after 83% replication) passing the cleavage site. Electron microscopic examination of cleaved molecules which were less than 83% replicated should have revealed in all molecules a short, linear branch or tail measuring about 17% of the genome length. The bidirectional model assumes that both branch points are growing points moving in opposite directions, and, therefore, the linear branches would be expected to decrease in length as replication proceeds. Furthermore, in this model the cleavage site would be predicted to be about 33% of the genome length from the origin, based on the sedimentation data available and assuming that the termination point is 50% of the genome length or 180° from the origin. When the short, linear branch was measured, it was found to decrease in length as the percent replication increased. By plotting the length of the short tail segment versus the extent of replication, a linear relationship between these parameters was detected. Extrapolation of this line to the point on the graph corresponding to 0% replication intercepted the abscissa at 33% of the genome length or the distance from the origin to the cleavage site. Thus, two separate methods of analysis, sedimentation through linear sucrose gradients and electron microscopy, yield complementary data. These results demonstrate that the branch points are, in fact, two growing points which move in opposite directions from a specific origin.

A bidirectional orientation for DNA synthesis has been found in a number of systems studied including *E. coli* (10), phage lambda (12, 15), phage T7 (6), and developing chick erythroblasts (18). Phages T4 and P2 and the replicative form I of phage φX174 (2, 9, 13) appear to undergo unidirectional replication. We have previously demonstrated that SV40 DNA chain growth occurs by a discontinuous mechanism (Fareed and Salzman, Nature [London], *in press*), per-

haps similar to the mechanism involved in chain elongation in larger chromosomal DNA species. Two other fundamental features of replication are initiation and orientation. The evidence presented in this paper points out that, in SV40 DNA replication, the origin is specific and the orientation is bidirectional. These, then, may be two additional features which SV40 DNA replication has in common with the replication of large chromosomes.

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