Specific Initiation Site for Simian Virus 40 Deoxyribonucleic Acid Replication

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Replicating simian virus 40 (SV40) deoxyribonucleic acid (DNA) molecules have been isolated under conditions in which the newly synthesized DNA is uniformly labeled with ^3H -thymidine. These newly synthesized strands are released from the replicative intermediate molecules by alkaline treatment, and it has been possible to isolate single-stranded SV40 DNA which varies in size from 157,000 daltons (from molecules that are 10% replicated) to 1,360,000 daltons (85% replicated). The rates of duplex formation of newly synthesized DNA have been used to relate their genetic complexity to the extent of DNA replication. As DNA replication proceeds, the time required to effect 50% renaturation of the newly synthesized DNA increases at a proportional rate. The data establish that DNA replication is not initiated at random, but rather that there is a single specific initiation site for DNA replication.

We previously examined simian virus 40 (SV40) deoxyribonucleic acid (DNA) replication (16) in lytically infected African green monkey kidney cells. A unique property of replicating molecules is the preservation of a covalently closed circular duplex structure of the parental DNA strands in the replicative intermediate (RI) molecules. As a consequence of the covalently closed structure, replicating molecules can be fractionated by isopycnic centrifugation in the presence of ethidium bromide based on the extent of DNA replication. When these replicating molecules are then sedimented in alkaline sucrose gradients, newly replicated DNA is dissociated from the parental DNA template. It has therefore been possible to isolate newly synthesized single-stranded SV40 DNA which varies in size from 157,000 daltons (from molecules that had completed 10% of a replication cycle) to 1,360,000 daltons (85% replication).

Determination of the rates at which newly synthesized SV40 DNA fragments of different sizes reassociate to form duplex DNA molecules provides a mechanism to determine the genetic complexity (1) of the fragments. One can predict that if DNA replication starts at a specific site and there is one growing point which moves in a single direction or if there are two growing points which move in opposite directions, then a newly synthesized DNA fragment which is one-tenth the length the viral genome will contain only 10% of the genetic information contained within the viral

genome. In contrast, if the initiation site is random, newly synthesized DNA obtained from RI molecules that are 10% replicated will be one-tenth the length of the viral genome, but will contain sequences homologous with the entire viral genome.

This paper reports the methods for isolating SV40 DNA fragments and the kinetics of duplex formation with these DNA fragments. The results establish that, as DNA replication proceeds, the genetic complexity of the DNA also becomes greater. The data indicate that there is a single initiation site for DNA replication.

MATERIALS AND METHODS

Cells. Primary African green monkey kidney (AGMK) cell monolayers were prepared from minced kidneys dispersed by trypsinization. Cells were re-fed 3 days after plating and became confluent by 6 days. Growth medium was Eagle medium supplemented with 10% fetal calf serum (Industrial Biological Laboratories) and 2 mM glutamine.

Infection of AGMK cells. Cells growing in 150-mm plastic petri dishes (Falcon Plastics) were infected at 6 to 8 days after preparation with a 3-ml inoculum at an input multiplicity of 50 to 100 plaque-forming units per cell. At the end of a 2-hr adsorption period, 40 ml of Eagle medium containing 2% fetal calf serum and 2 mM glutamine was added to each plate.

Preparation of the ^3H -thymidine-labeled low-molecular-weight (LMW) DNA fraction. At 28 or 48 hr post-infection, the medium was removed from the cells by aspiration, and 8 ml of prewarmed, serum-free me-

dium containing 50 or 100 μCi of ^3H -thymidine (New England Nuclear Corp.) per ml was added to each plate. After incubation at 37 C for 60 min, the medium was removed and the cell monolayer was washed twice with cold phosphate-buffered saline. Viral DNA was separated from cellular DNA as described by Hirt (6). Monolayer cultures were lysed by the addition of 3 ml of a solution containing 0.01 M tris(hydroxymethyl)-aminomethane (Tris), 0.01 M ethylenediaminetetraacetic acid (EDTA), 0.05 M NaCl, and 0.6% sodium dodecyl sulfate (SDS), pH 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, the tube was gently inverted 10 times to effect mixing, and the mixture was stored overnight at 4 C. The insoluble material was pelleted by centrifugation at 4 C for 60 min at 10,000 rev/min in a Sorvall RC2-B centrifuge. The supernatant fluid, containing the LMW DNA, was dialyzed against a solution containing 0.01 M Tris, 0.01 M EDTA, and 0.05 M NaCl, and was concentrated by vacuum dialysis using a colodion bag filter apparatus (Schleicher & Schuell, Inc., Keene, N.H.).

Velocity gradient fractionation of DNA. Analytical and preparative alkaline sucrose gradients were carried out by layering 0.01- to 0.3-ml portions of the sample onto 11.8-ml 10 to 30% sucrose gradients containing 0.7 M NaCl, 0.3 M NaOH, 0.01 M Tris, 0.001 M EDTA, and 0.015% Sarkosyl. Approximately 1,500 counts/min of ^{14}C -thymidine-labeled SV40 DNA II was added to the analytical gradients as a sedimentation marker. Centrifugation was for 16.5 hr at 40,000 rev/min in an SW41 rotor at 10 C.

Analytical and preparative neutral sucrose gradients were carried out as described for alkaline gradients except that samples were layered onto 11.8-ml 5 to 30% sucrose gradients in 0.05 M Tris, 0.1 M NaCl, 0.0025 M EDTA, pH 7.5. ^{14}C -thymidine-labeled SV40 components I and II (Comp. I and Comp. II) were added to analytical gradients as sedimentation markers. Samples were centrifuged for 7 hr at 40,000 rev/min in an SW41 rotor at 10 C.

Collection of fractions and counting conditions were the same as those previously described (16).

Isopycnic banding in ethidium bromide-cesium chloride. Ethidium bromide and cesium chloride were added to the dialyzed LMW DNA fraction to give a final density of 1.564 and an ethidium bromide concentration of 200 $\mu\text{g}/\text{ml}$. Gradients (6 to 9 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, 0.25-ml fractions were collected, and 5 μliters of each fraction was counted to determine the distribution of the ^3H -labeled LMW DNA.

Reannealing of DNA fragments. Newly synthesized ^3H -thymidine-labeled SV40 DNA fragments were obtained from alkaline sucrose gradients. The fractions were dialyzed against 0.01 M Tris, 0.01 M EDTA, pH 7.2, and stored at 4 C. Each reaction mixture contained 2,000 to 5,000 counts per min of sized DNA fragments per ml plus 100 μg of sheared, denatured salmon sperm DNA per ml. (Appropriate normalizations for the varying concentrations of DNA fragments were made

as described below in the section on Calculations.) Immediately before the reannealing experiment, each reaction mixture was adjusted to 0.1 N NaOH and incubated at room temperature for 30 min. The phosphate concentration of the sample was then adjusted to 0.4 M and the final pH to 6.8 by the addition of one-tenth volume of 4.4 M phosphate buffer (1.2 parts Na_2HPO_4 and 3.2 parts NaH_2PO_4). The reaction mixture was incubated at 68 C. Samples (0.1 ml) were removed at the beginning of the incubation period and at various times during the next 26 hr and diluted to 1.0 ml with 0.11 M PO_4 buffer, pH 6.8, to adjust the final PO_4 concentration to 0.14 M. Samples were stored at 4 C. The end-point value (100% reannealing) was obtained by removing a 0.1-ml sample from the reaction mixture and incubating it for 1 hr at 68 C in the presence of an excess (20 $\mu\text{g}/\text{ml}$) of sheared, denatured, unlabeled SV40 DNA. At the end of the incubation period, the sample was diluted and stored as described above.

Separation of single-stranded and double-stranded DNA. Hydroxyapatite (Biogel HTP, Bio-Rad Laboratories, Richmond, Calif.) used for the strand separation was mixed 1:4 with 0.14 M PO_4 buffer containing equimolar parts Na_2HPO_4 and NaH_2PO_4 , pH 6.8.

The 1.0-ml samples from the reaction mixtures were preincubated at 60 C for 5 min. A 0.4-ml volume of hydroxyapatite was then added to each sample, and the mixture was incubated at 60 C for 7 min. At the end of the incubation period, samples were centrifuged for 1 min at 1,000 rev/min, and the supernatant fluid containing the single-stranded DNA was transferred to a liquid scintillation vial. A 1-ml volume of the 0.14 M PO_4 buffer was added to the hydroxyapatite as a wash. This was incubated and centrifuged in the same manner, and the supernatant fluid was pooled with the first eluate.

Double-stranded DNA was eluted from the hydroxyapatite by adding 1.2 ml of 0.14 M PO_4 buffer, heating the sample to 100 C for 5 min, centrifuging, and transferring the supernatant fluid to a scintillation vial. The hydroxyapatite was eluted a second time in the same way with 1.0 ml of 0.14 M PO_4 buffer which was then combined with the first eluate.

A 10-ml amount of Aquasol (New England Nuclear Corp.) was added to the eluates, and the vials were counted in a Beckman LS250 liquid scintillation spectrometer.

Calculation of the rates of reassociation. In the present study the concentrations of the DNA fragments have been determined based on the amounts of radioactivity that they contain. Therefore, only relative rate constants can be calculated. Relative " $C_0t_{1/2}$ " values (reciprocal of the rate constant) were determined from the midpoint of a plot of C/C_0 versus $\log C_0t$, according to the second-order equation derived by Britten and Kohne (1) (equation i): $C/C_0 = 1/(1 + kC_0t)$, where C_0 is the initial concentration of single-stranded DNA and C is the concentration of single-stranded DNA at time, t . The counts per minute contained in single- and double-stranded DNA were determined by differential elution from hydroxyapatite. Data were normalized to give 100% reassociation at infinite time (end point), 0% reassociation at zero time, and an ini-

tial concentration of single-stranded DNA (C_0) of 5,000 counts per min per ml. The fraction of unreacted DNA (C/C_0) was determined by dividing the single-stranded DNA counts per minute by the sum of the single-stranded and double-stranded DNA counts per minute. For the samples examined, 3 to 8% of the DNA was double-stranded at zero time, and at the end point more than 90% of the DNA had reannealed.

The rate of reannealing varies as a function of the square root of the length of the reannealing fragments (18). In the present study, it was felt that shearing the fragments to the same size was impractical since it would lead to a more heterogeneous size distribution. Therefore, the various fractions of newly synthesized fragments were reannealed as isolated. The data for each time point were then corrected to the time that would have been required to effect the same amount of reannealing had the fragments been the length of a complete SV40 DNA molecule, using the following relationship (equation ii): $C_{0t(16S)} = C_{0t(F)} \times (S_{(F)}/S_{(16S)})^{1.28}$, which was derived from the empirical relationship (equation iii): $\log S = 0.78 \log k_2 - \text{constant}$, determined by Wetmur and Davidson (18) for T2 and T7 DNA.

In equation ii, $S_{(F)}$ and $S_{(16S)}$ are the S values in alkali for the fragment and for a complete linear SV40 DNA strand, respectively. $C_{0t(F)}$ is the C_{0t} for the fragment and $C_{0t(16S)}$ is the corrected C_{0t} that would have been obtained if the fragment were 16S. The value of $C_{0t_{1/2}}$ was determined from a plot of C/C_0 versus $\log C_{0t(16S)}$ (1).

RESULTS

DNA species contained in the LMW DNA fraction. The ultimate experimental bases for the determination of the specificity of the site of DNA initiation and the direction of replication are the rates of duplex formation of newly synthesized DNA strands. However, a number of conditions must be met before this experiment can be carried out. Newly replicated DNA contained in RI molecules cannot readily be obtained in amounts sufficient to determine their concentrations by chemical means. Because of this, the experimental approach that has been followed in this study is to expose infected cells to ^3H -thymidine for sufficient time so that newly replicated DNA strands in the RI molecules are uniformly labeled.

In Fig. 1, the sedimentation properties of the various DNA species contained in the LMW DNA fraction have been examined after exposing cells to ^3H -thymidine for 60 min, when the labeled precursor is added at 28 hr postinfection. A comparison of the isotope incorporated into Comp. I and II and into replicating SV40 DNA (22 to 28S) (10, 16) indicates that the replicating pool must have turned over at least three times during this 1-hr period. Thus, 60 min is ample time to assure uniform specific activity within the newly synthesized DNA. Comp. I and Comp. II will accumulate within infected cells, and quantities of these two species will be present in infected cells in unlabeled as well as in labeled forms. To a lesser

FIG. 1. Velocity sedimentation in neutral sucrose of low-molecular-weight (LMW) DNA. An SV40-infected AGMK monolayer was pulsed, at 28 hr postinfection, for 1 hr with 10 μCi of ^3H -thymidine per ml. The LMW DNA was selectively extracted with 0.6% SDS as described in Materials and Methods. After dialysis and concentration, the sample was layered onto an 11.8-ml 5 to 30% neutral sucrose gradient and sedimented at 10 C for 16 hr at 26,500 rev/min in an SW41 rotor. Sedimentation is from right to left. ^{14}C -thymidine SV40 Comp. I and Comp. II were added to the gradient as a sedimentation marker. Fractions were collected in vials containing 0.25 mg of yeast ribonucleic acid and precipitated with 5% trichloroacetic acid. The precipitate was put on glass filter papers, washed with 5% trichloroacetic acid and with ethanol. Filters were dried and counted in Liquofluor.

extent, the same is true of cellular DNA. Under neutral conditions, it is seen (Fig. 1) that the LMW DNA fraction does not contain any species of DNA that sediments more slowly than 16S, the sedimentation constant of SV40 DNA Comp. II. After alkaline treatment, newly synthesized DNA fragments are released from RI molecules and sediment more slowly than the 16S single-strand component in Comp. II (16). Comp. II is the only extraneous DNA that is likely to contaminate newly replicated DNA strands which are isolated after sedimentation in alkali since it will give rise to 18S and 16S components. In alkaline gradients Comp. I will sediment rapidly with an S of 53, and cellular DNA will have an S value somewhat greater than the S value observed in neutral gradients (greater than 21S) (17).

Isolation of newly synthesized SV40 DNA. Ten 150-mm dishes containing confluent AGMK cell monolayers were infected with SV40 at an added input multiplicity of 50 plaque-forming units per cell. At 48 hr postinfection, ^3H -thymidine (50 $\mu\text{Ci}/\text{ml}$) was added, and after 1 hr the LMW DNA fraction was prepared. The material was layered onto 10 to 30% alkaline sucrose gradients and centrifuged for 16.5 hr at 40,000 rev/min. The

results obtained from an analytical gradient are shown in Fig. 2. Fractions, as indicated in Fig. 2, were taken from parallel preparative gradients, and each fraction was centrifuged a second time in an alkaline sucrose gradient. Selected regions were taken from each of the alkaline gradients, and the size of the SV40 DNA fractions was determined by using a portion of the material. The remainder of each fraction was used in reassociation experiments (*see below*). The results (Fig. 3 and Table 1) show that single-stranded SV40 DNA fragments which have been isolated range in S values from 6.3 to 12.7, corresponding to single-stranded DNA of 157,000 to 901,000 daltons (17). It can be seen (Fig. 3) that a narrow size distribution is obtained in each of the DNA pools.

Rate of reassociation of newly replicated SV40 DNA. Newly synthesized single-stranded SV40 DNA that was isolated by two cycles of alkaline sucrose sedimentation (*see above*) was reannealed in 0.4 M PO_4 buffer at 68 C (5). Samples were removed after various times of incubation, and the fraction of the DNA present as single-stranded and double-stranded DNA was determined by batchwise treatment with hydroxyapatite. The re-

FIG. 2. Velocity sedimentation in alkaline sucrose of low-molecular-weight (LMW) DNA. SV40-infected AGMK monolayers were pulsed, at 48 hr postinfection, for 1 hr with 50 μCi of ^3H -thymidine per ml. The LMW DNA was selectively extracted as described in Materials and Methods. After dialysis and concentration, the LMW DNA was layered onto an 11.8-ml 10 to 30% alkaline sucrose gradient and sedimented at 10 C for 16.5 hr at 40,000 rev/min in an SW41 rotor. Sedimentation is from right to left. Figure 2 represents an analytical gradient from which fractions were collected directly into scintillation vials, neutralized by the addition of two drops of glacial acetic acid, and counted. Parallel preparative alkaline sucrose gradients were run, and four pools were made from fractions in regions of the gradient indicated by the bars.

FIG. 3. Velocity sedimentation in alkaline sucrose of low-molecular-weight (LMW) DNA samples derived from two successive preparative alkaline gradients. Microliter amounts of a sample to be used in a reannealing experiment were layered onto an 11.8-ml 10 to 30% alkaline sucrose gradient. ^{14}C -thymidine SV40 DNA II was added to the gradient as a sedimentation marker. Samples 1 and 4 shown in Fig. 3 were actually run on separate analytical alkaline sucrose gradients, but here are shown superimposed on one graph to indicate the homogeneity of the samples used in the reannealing experiments. Sedimentation is from right to left. Gradients were punctured at the bottom, and fractions were collected directly into scintillation vials, neutralized by the addition of two drops of glacial acetic acid, and counted.

TABLE 1. Relationship between size of newly synthesized SV40 DNA and rate of reassociation

Fraction	Sedimentation coefficient	Mol wt	" $\text{C}_0\text{t}_{1/2}$ " ^a	Fold decrease	
				Size	$\text{C}_0\text{t}_{1/2}$
1	12.7	901,000	0.350	1.00	1.00
2	10.8	598,000	0.203	1.50	1.72
3	6.7	183,000	0.069	4.92	4.68
4	6.3	157,000	0.048	5.74	7.29

^a Radioactive DNA species that are used in reannealing experiments differ in their specific activities in the different experiments. For this reason, corrected " $\text{C}_0\text{t}_{1/2}$ " values can be compared within an experiment, but not between two separate experiments. The calculations involved in determining corrected " $\text{C}_0\text{t}_{1/2}$ " values are detailed in Materials and Methods.

sults are presented graphically in Fig. 4, and the " $\text{C}_0\text{t}_{1/2}$ " values for DNA species of various sizes are seen in Table 1. It can be seen that the time

FIG. 4. Kinetics of reassociation of newly replicated SV40 DNA fragments isolated by two cycles of alkaline sucrose sedimentation. Newly replicated SV40 DNA fragments of various sizes were isolated by two cycles of sedimentation in alkaline sucrose. Fragments were then reannealed in 0.4 M PO_4 buffer, pH 6.8, at 68 C. The amount of single- and double-stranded DNA at each time point was determined by elution from hydroxyapatite. Values were normalized for 0% reannealing at zero time and 100% reannealing at the end point. Corrections were also made for variation in the DNA concentrations at zero time and for difference in the size of the fragments. See Materials and Methods for details of the procedure. Symbols: ▼, DNA fragments of 901,000 daltons; ●, 598,000 daltons; ■, 183,000 daltons; ▲, 157,000 daltons.

required to effect 50% reassociation of the DNA ($C_{0t_{1/2}}$) increases at a rate that is proportional with the increasing size of the DNA. Since the " $C_{0t_{1/2}}$ " value has been determined based on equal zero time concentrations of DNA, this finding is that predicted if genetic complexity of the DNA strands increases as replication proceeds. This will be the case if there is a specific site for initiation of DNA replication. In this experiment, newly synthesized DNA strands larger than 900,000 daltons were not used because of possible contamination with unlabeled 16S DNA derived from Comp. II. With DNA of a molecular weight less than 900,000, contamination is not a problem.

Isolation of SV40 DNA fragments by two cycles of centrifugation in alkaline sucrose gradients is not a suitable procedure for obtaining single-stranded DNA from RI molecules more than 60% replicated. These larger newly replicated fragments, however, can be obtained from the LMW DNA fraction by an alternative purification

scheme. Eighteen 150-mm AGMK monolayers were infected, and at 28 hr postinfection 3H -thymidine (100 μ Ci/ml) was added for 1 hr. The LMW DNA fraction was prepared and fractionated by isopycnic centrifugation in cesium chloride-ethidium bromide. The DNA banding at the lighter density contains cellular DNA, Comp. II, and RI molecules (9, 16). This fraction was concentrated, and after removal of ethidium bromide (2, 16) and cesium chloride, the material was layered onto a 5 to 30% neutral sucrose gradient. By this gradient procedure, the RI molecules which sediment with S values of 23 to 28S are effectively freed from contamination with Comp. II. Fractions corresponding to replicating molecules were then taken from the neutral gradients and centrifuged in alkaline gradients. Selected regions from the alkaline gradients were pooled to obtain SV40 DNA fragments of various sizes. A sample from each fraction was sized by alkaline velocity sedimentation before use in reannealing experiments. Results identical to those noted previously (see Fig. 3) demonstrate that there was little size heterogeneity in the fractions that were used. The size range of the fractions that were obtained is shown in Table 2. For each fraction, the kinetics of reassociation were determined, and the time that was required for 50% of the DNA to reassociate was calculated. Each of the samples reannealed with the expected second-order kinetics predicted for a genetically homogeneous material (1). The data for the DNA fragments shown in Table 2 indicate that the observed " $C_{0t_{1/2}}$ " value increased as the size of the newly synthesized DNA increased. This relationship is the same as that observed with the shorter, newly synthesized DNA fragments.

TABLE 2. Relationship between size and rate of reassociation of DNA fragments from molecules 42 to 85% replicated^a

Fraction	Sedimentation coefficient	Mol wt	" $C_{0t_{1/2}}$ "	Fold decrease	
				Size	" $C_{0t_{1/2}}$ "
1	15.0	1,360,000	2.35	0.80	0.91
2	14.7	1,293,000	2.8	0.84	0.76
3	13.7	1,084,000	2.13	1.00	1.00
4	13.1	969,600	1.8	1.12	1.18
5	12.6	879,700	1.6	1.23	1.33
6	12.5	862,300	1.7	1.26	1.25
7	12.2	811,500	1.6	1.34	1.33
8	11.5	700,100	1.6	1.55	1.33
9	11.3	670,000	1.1	1.62	1.94

^a The newly synthesized DNA fragments were isolated by fractionation in ethidium bromide-cesium chloride, followed by neutral and alkaline sucrose sedimentation. The fold decreases for size and " $C_{0t_{1/2}}$ " values are expressed in relation to fraction 3 since that value was the average of three determinations.

DISCUSSION

In the present study, the rates of reassociation of newly synthesized SV40 DNA strands have been examined. It has been demonstrated that DNA renaturation is a second-order reaction and that the rate of renaturation is inversely proportional to the genetic complexity of the DNA (1). Britten and Kohne have shown that the DNA of every organism may be characterized by the value of $C_0t_{1/2}$, the product of the initial DNA concentration and the time at which the renaturation reaction is half completed. There are clear predictions as to the expected rates of renaturation of newly synthesized fragments of SV40 DNA depending on whether DNA replication is initiated at a specific site or is initiated at random. In Fig 5 we show molecules which are either 17% or 50% replicated, and have indicated the genetic com-

plexity of the newly synthesized DNA strands that are replicated when there is a specific initiation site (A, C) or when DNA initiation is at a random site (B, D). When there is a specific initiation site in the molecule that is 17% replicated, only gene a will have been replicated. Under these conditions, when replication has proceeded to 50%, genes a, b, and c will have been replicated. If the rates of reassociation of the newly synthesized DNA from RI molecules A and C are compared (when C_0 and the size of the DNA fragments are the same), the molecules which are 17% replicated will reanneal three times more rapidly, since molecule C releases DNA which is three times more complex. In molecules B and D, where DNA replication is initiated randomly, RI molecule D will release DNA strands which are three times the length of those obtained from RI molecule B, but RI molecules B and D will both release DNA that

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FIG. 5. Schematic representation of the genetic complexity of newly synthesized DNA fragments that would be released from RI molecules if the initiation site is specific (A and C) or is random (B and D). RI molecules which have completed 17% and 50% of a replication cycle are diagrammed to illustrate that as replication progresses the genetic complexity of newly synthesized DNA fragments will increase if the initiation site is specific. If the initiation site is random, the genetic complexity of a population of RI molecules remains the same as replication progresses.

is homologous with the entire viral genome. Both B and D, when compared when C_0 and the size of the DNA fragments are the same, will reanneal at the same rates. The data demonstrate that, as DNA replication progresses, the rate of reassociation of the newly synthesized DNA decreases. These findings are those expected when there is a specific initiation site for DNA replication. Since new genetic sequences are generated as replication proceeds, certain conclusions can be made about the direction of replication. The present results cannot differentiate between a unique site where there is unidirectional replication or bidirectional replication. However, the data do exclude a model in which replication proceeds unidirectionally and in which molecules are equally likely to replicate in either a clockwise or counterclockwise direction, since under these conditions molecules would not increase in genetic complexity when they became more than 50% replicated.

When examined by electron microscopy, replicating lambda DNA structures contain three branches, two branch points, and no free ends (12). Schnos and Inman (14) have examined the structure of these replicating lambda DNA molecules after partial denaturation and determined the positions of the branch points relative to the denatured sites which form at unique positions on the lambda DNA molecule (7, 8). Based on these measurements, they concluded that lambda DNA replication starts at a specific initiation site 18.3% from the right end of the mature lambda DNA molecule. A fraction of the replicating molecules contained a single growing point. For these molecules the direction of replication is either to the right or to the left of the initiation site. However, the majority of molecules have two growing points which move in opposite directions from the origin. In a similar study of P2 phage DNA replication (15), a unique DNA initiation site was also observed, but in contrast to lambda DNA, P2 DNA replication is unidirectional.

Evidence for a preferred initiation site in SV40 has been obtained by Nathans and Danna (11) who determined the pattern of distribution of isotope in fragments that were obtained from SV40 DNA Comp. I and replicative intermediates after treatment with the restriction endonuclease of *Haemophilus influenzae*. Our findings are in agreement with their results. Previously we examined (4) properties of replicating SV40 DNA molecules that were cleaved with the restriction endonuclease obtained from *Escherichia coli* B (13). While parental strands in replicating molecules were cleaved by this enzyme, newly synthesized strands contained in the replicative intermediates were not cleaved, even with molecules that were 90% replicated. These findings indicate that the cleavage site is not contained in the replicated regions and

may be located close to the site at which DNA termination occurs. The findings also suggest a specific initiation site for SV40 DNA replication.

Using an *fi*⁺ R restriction endonuclease of *E. coli* which cleaves SV40 at one specific site we have confirmed the presence of a specific site for initiation of DNA synthesis. Furthermore, we have been able to show that replication occurs at two branch points which move in opposite directions from the initiation site (3a).

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