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Bidirectional Replication of Simian Virus 40 DNA*

(tumor virus/pulse-label/DNA cleavage/restriction endonuclease)

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ABSTRACT **SV40 (Simian Virus 40) DNA was pulse-labeled with [^{3}H]thymidine in infected monkey cells, and the distribution of label within newly completed molecules was determined by analysis of specific fragments produced by restriction endonuclease from *Hemophilus influenzae*. From these data, an order of synthesis or temporal order of the fragments was deduced. Comparison of the temporal order with the physical order of the fragments in the SV40 DNA molecule indicates a correspondence in these orders for two separate groups of fragments. From an analysis of the results, we conclude that SV40 DNA replication begins at a specific site and proceeds bidirectionally, terminating about halfway around the circular molecule from the initiation point.**

The genome of the oncogenic virus Simian Virus 40 (SV40) is a covalently-closed circular molecule of about 3 million daltons that replicates in infected monkey cells by means of a Cairns-type intermediate (1, 2). We have been analyzing the replication process and other functions of the genome by specific cleavage of the viral DNA with a bacterial restriction endonuclease (3, 4). As previously reported, the enzyme from *Hemophilus influenzae* (5) produces 11 DNA fragments separable by polyacrylamide gel electrophoresis (4). By analyzing fragments of pulse-labeled SV40 DNA isolated from infected cells, we have shown that there is a preferred or unique origin for SV40 DNA replication and a definite order of synthesis of different parts of the molecule (3). In the present paper we compare this temporal order with the physical order of the DNA fragments and conclude that the replication of SV40 DNA is bidirectional.

MATERIALS AND METHODS

Cell Lines and Virus. Small-plaque SV40 (from strain 776) was grown in the BSC-1 line of African green monkey kidney cells as described (4).

Preparation of Uniformly-Labeled SV40 [^{32}P]DNA I. Infection, labeling, and purification of covalently-closed SV40 DNA (DNA I) have been described (4). Briefly, infected BSC-1 cells were labeled with [^{32}P]orthophosphate; viral DNA was extracted by the method of Hirt (6) and purified by treatment with ribonuclease A, followed by phenol extraction, ethanol precipitation, equilibrium centrifugation in CsCl–ethidium bromide, and sedimentation in a neutral sucrose gradient.

Preparation of Pulse-Labeled SV40 [^{3}H]DNA I. As described previously (3), BSC-1 cells infected with SV40 were pulse-labeled with [^{3}H]thymidine 51 hr after infection for 5, 10, or 15 min. Cells were lysed by the Hirt procedure (6), and the labeled covalently-closed SV40 DNA I was purified by phenol extraction and ethanol precipitation, followed by sucrose gradient sedimentation. The 21S peak fractions were pooled and dialyzed against 15 mM NaCl–1.5 mM Na citrate (0.1 × SSC).

Digestion of SV40 DNA with H. influenzae Restriction Endonuclease. As described earlier (3), DNA samples of uniformly-labeled SV40 [^{32}P]DNA I mixed with pulse-labeled SV40 [^{3}H]DNA I (25–70 μg total DNA) were digested with 0.026 unit of *H. influenzae* restriction endonuclease in a volume of 0.05–0.09 ml of 50 mM NaCl–6.6 mM Tris·HCl (pH 7.5)–6.6 mM $MgCl_2$ at 37° for 2 hr. Under these conditions digestion was complete in less than 2 hr.

Slab Gel Electrophoresis. In preparation for electrophoretic separation of DNA fragments, samples were evaporated to dryness, dissolved in 20 μl of 1% sodium dodecyl sulfate in electrophoresis buffer, and heated at 37° for 30 min; 10 μl of 75% sucrose–1% bromphenol blue was added. The digests were then subjected to electrophoresis for 16–20 hr at a constant voltage of 160 V in 4% polyacrylamide vertical slab gels (15 × 40 × 0.16 cm) in a buffer consisting of 0.04 M Tris–0.02 M sodium acetate–2 mM sodium EDTA (pH 7.8). The gel chamber used was similar to those described by deWachter and Fiers (7) and by Reid and Bieleski (8). For quantitation of radioactive label in each fragment, [^{32}P]DNA bands were located by radioautography of the wet gel (7); segments corresponding to the bands were excised. Each was dissolved in 0.4 ml of 30% H_2O_2 at 65° (9) and counted in Triton X-100–toluene scintillation fluid. The ^{3}H/^{32}P ratio for each fragment was determined for mixtures of ^{3}H-pulse-labeled DNA I and reference uniformly-labeled [^{32}P]DNA I that had been digested with *H. influenzae* restriction endonuclease.

Molecular weights for SV40 DNA fragments produced by cleavage with *H. influenzae* restriction endonuclease were estimated from the distribution of radioactive label in each fragment from a complete digest of SV40 [^{32}P]DNA (4).

Base Analysis of SV40 [^{32}P]DNA and of Purified ^{32}P-Labeled Fragments. Base composition of SV40 [^{32}P]DNA and of purified fragments (after electrophoresis and elution from the gel) was determined by hydrolysis of the DNA to 5′-deoxyribonucleotides with pancreatic deoxyribonuclease and snake venom phosphodiesterase (Worthington Biochemicals)

Abbreviations: SV40, Simian Virus 40; SV40 DNA I, covalently-closed SV40 DNA.

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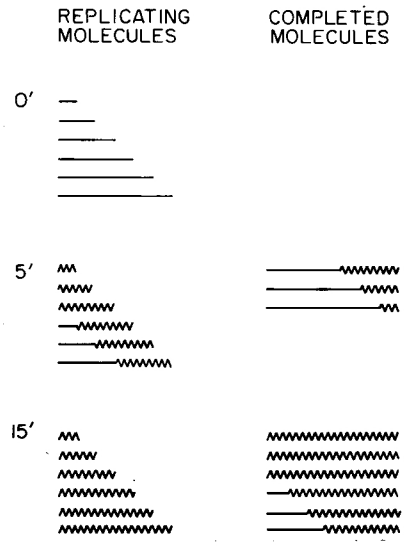

FIG. 1. A diagram of the distribution of radioactivity in pulse-labeled daughter strands at different times after addition of [^{3}H]dT, assuming that DNA replication begins at a specific point in the molecule (the left of each line) and that the time needed for one round of replication is about 10 min. Labeled regions are drawn in *wavy lines*.

(10), followed by high-voltage paper electrophoresis (11) and quantitation of the amount of radioactive label in each deoxyribonucleotide.

RESULTS

Relative specific activity of each fragment from pulse-labeled, newly-synthesized SV40 DNA

Exposure of SV40-infected cells, which are synthesizing viral DNA, to a brief pulse of [^{3}H]thymidine results in incorporation of label at the growing points of replicating DNA (3) (Fig. 1). Molecules completed during a pulse time shorter than the time needed for complete replication will be labeled in those regions synthesized last (i.e., near the terminus of replication), but not in those regions synthesized first. For a pulse time longer than the replication time, all regions of newly-completed molecules will contain label, but there will still be a gradient of labeling of various regions of the DNA (with the origin region containing the least amount of label), from which a temporal order of synthesis of different parts of the molecule can be deduced. In this way, we have determined the order of synthesis of those parts of the SV40 DNA corresponding to each of the fragments produced by cleavage with restriction endonuclease from *H. influenzae*.

For determination of the relative specific activity of each fragment from newly synthesized, completed molecules of SV40 DNA, infected cells were pulse-labeled with [^{3}H]-thymidine, and covalently-closed circular DNA (DNA I) was isolated virtually free of replicative molecules (see *Methods* and ref. 3). Each [^{3}H]DNA I sample was then mixed with uniformly-labeled SV40 [^{32}P]DNA I, and the mixture was completely digested with *H. influenzae* restriction endonuclease. After separation of the products by electrophoresis in slabs of polyacrylamide gel, each fragment was analyzed for its ^{3}H/^{32}P ratio. A typical separation of fragments is shown in Fig. 2, and the relative specific activity of each fragment (^{3}H/^{32}P ratio relative to that of fragment *A* and corrected for the slightly different thymidine contents of the fragments) is presented in Table 1. The results are essentially the same as those reported earlier (3), but, because of the better separation, they now include all the fragments produced by the *H. influenzae* restriction enzyme. As shown in the table (and in Fig. 4), there is a consistent gradient of labeling, indicating a specific order of synthesis of different parts of the SV40 DNA molecule. Since newly-completed molecules were analyzed, fragments with the lowest amount of pulse label (*C* and *D*) are from that part of the DNA synthesized first. Fragments with the highest amount (*G* and *J*) are from that part of the DNA synthesized last.

TABLE 1. *Relative ^{3}H yield of each fragment from pulse-labeled SV40 DNA I (see Fig. 2)*

Fragment	Fractional length*	% A + T†	Relative amount of pulse label‡ 5 min	10 min	15 min
A	0.225	62	1.0	1.0	1.0
B	0.150	64	3.9	3.0	2.3
C	0.105	50	0	0.75	0.75
D	0.100	55	0.92	0.86	1.1
E	0.085	63	1.8	2.0	1.7
F	0.075	63	4.0	3.1	2.4
G	0.070	62	5.4	4.2	2.6
H	0.055	67	1.7	2.5	2.0
I	0.050	63	2.7	3.0	2.2
J	0.045	57	4.9	3.7	2.6
K	0.040	57	2.4	2.9	1.9

* Fractional length is based on the yield of each fragment in a complete digest of uniformly-labeled SV40 [^{32}P]DNA I.

† Most of the values for % *A* + *T* are based on duplicate determinations for each fragment (see *Methods*). The base composition of SV40 DNA determined at the same time was 59–60% *A* + *T*, in agreement with reported values (1).

‡ The relative amount of pulse label is the ^{3}H/^{32}P ratio of each fragment, corrected for thymidine content and normalized to 1 for fragment *A*. 5, 10, and 15 min refer to the labeling period.

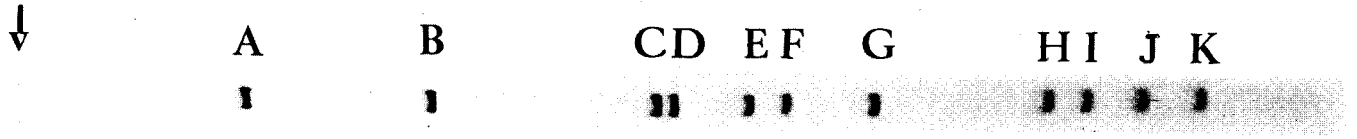

FIG. 2. Radioautogram of ^{32}P-labeled fragments of SV40 DNA. Conditions for digestion and electrophoresis are described in *Methods*. For the purpose of this figure, the gel was dried by the method of Maizel (12) before autoradiography. The actual distance of *A* from the origin is 7 cm. The *arrow* indicates the origin.

Comparison of the temporal and physical orders of DNA fragments

A comparison of the order of synthesis (or temporal order) with the physical order of the fragments should allow one to distinguish unidirectional from bidirectional modes of replication of a circular molecule. We have recently determined the order (shown in Fig. 3) of the SV40 DNA fragments produced by the *H. influenzae* restriction endonuclease by analyzing incompletely digested DNA fragments, as well as an overlapping set of fragments, produced by a restriction endonuclease from *H. parainfluenzae* (ref. 13; Sack and Nathans, manuscript in preparation); these experiments will be reported elsewhere (Danna, Sack, and Nathans, manuscript in preparation). In Fig. 4 we compare the physical order of the fragments, noted at the top of the figure, with the relative specific activity of each fragment from pulse-labeled, newly completed molecules. For the purpose of this presentation, the SV40 DNA map has been opened at fragment *G* (shown at both ends), which is the part of the molecule synthesized last. The restriction enzyme site between fragments *A* and *C* has been designated the zero point.

It is apparent from Fig. 4 that for two groups of fragments (*C,D . . . G* and *A,H . . . G*) the temporal and physical orders correspond for all three sets of pulse-label data. For each group, the relative specific activity of the fragments extrapolates to a minimal point within fragment *C* near the junction of fragments *C* and *A* and to a maximal value within fragment *G*, which is roughly halfway around the physical map from the *C–A* junction. Since a higher relative specific activity for a fragment indicates a later time of synthesis in the replication cycle, we conclude that SV40 DNA replication begins in fragment *C* near the *C–A* junction and proceeds bidirectionally, terminating in fragment *G*.

As shown in Fig. 4, for each pulse time the change in relative specific activity per unit length of DNA is about the same in the two replication arms of the molecule, although there is some scatter in the data, especially involving the small fragments *H* and *K*. (For simplicity of presentation, straight lines have been drawn through the points.) Since a significant disparity in the rate of extension of daughter strands in the two arms would have resulted in different gradients of labeling along the two replication arms, we conclude that the rates of replication in each direction are very similar. The finding that the terminus of replication is about halfway around the molecule from the origin substantiates this conclusion.

Finally, we can make a somewhat more precise estimate than reported previously (3) of the time between addition of [^{3}H]thymidine and complete replication of a molecule of SV40 DNA by noting the earliest time at which the intercept of the lines in Fig. 4 is above the baseline. This occurs between 10 and 15 min after addition of labeled thymidine.

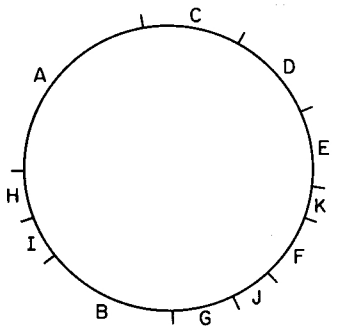

FIG. 3. Physical order of SV40 DNA fragments produced by cleavage with *H. influenzae* restriction endonuclease.

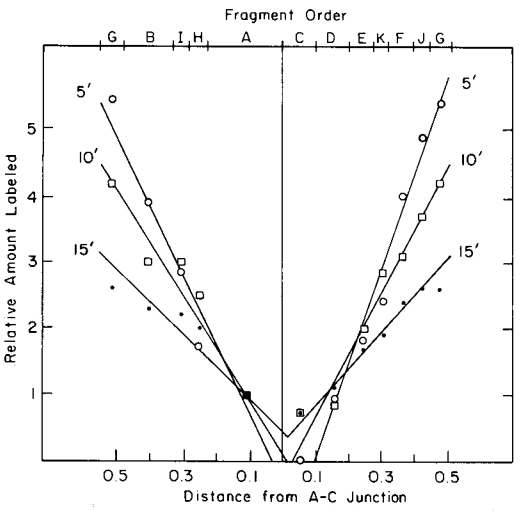

FIG. 4. Comparison of the position of each fragment in SV40 DNA and the relative amount of ^{3}H-labeled fragment obtained from pulse-labeled newly completed molecules. Pulse-labeled, newly completed molecules of SV40 DNA were isolated after infected cells were exposed to [^{3}H]thymidine for 5, 10, or 15 min. Each [^{3}H]DNA sample was then mixed with uniformly labeled SV40 [^{32}P]DNA and digested with *H. influenzae* enzyme. Individual DNA fragments were isolated by electrophoresis and counted. On the *ordinate* is plotted the relative amount of each fragment labeled (^{3}H/^{32}P ratio, corrected for thymidine content and normalized to the value for fragment *A;* see Table 1). On the *lower horizontal axis* is the distance of the midpoint of each fragment from the *A–C* junction. ○, 5-min pulse time; □, 10-min pulse time; ●, 15-min pulse time.

DISCUSSION

The major conclusion of the experiments reported in this paper is that SV40 DNA replication begins at a specific site and proceeds in both directions at about the same rate around the circular molecule. Earlier suggestions that SV40 DNA replication is bidirectional were based on the observation by electron microscopy of single-stranded regions at both forks of replicating molecules (14). Since the initiation site and the termination site have been localized, and DNA fragments containing these sites are available, it will be interesting to determine what special nucleotide sequences might represent the signals for initiation and termination.

When SV40 DNA replication is compared with that of other viral DNAs, SV40 DNA is similar to λ DNA in having a bidirectional mode of replication (15), in contrast to that of coliphage P2, which is predominantly unidirectional (16). What general significance uni- or bidirectional replication may have is not known.

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