Unwinding of Parental Strands During Simian Virus 40 DNA Replication

N. P. SALZMAN, E. D. SEBRING, AND M. RADONOVICH

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

Received for publication 25 April 1973

Pools of young (less than 60% replicated) and mature (60–90% replicated) replicating molecules of simian virus 40 (SV40) DNA have been treated at pH 12.2 in order to dissociate growing chains from the parental strands. The molecules are neutralized so that the parental strands can reassociate and they have then been isolated. They are covalently closed structures which sediment rapidly in alkaline sucrose gradients; however, the sedimentation rates are less than the sedimentation rate of SV40 DNA I. Isopycnic banding in CsCl-ethidium bromide and sedimentation velocity studies in the presence of various amounts of ethidium bromide indicate that these structures contain negative superhelical turns and several-fold-higher superhelix densities than SV40 DNA I (the covalently closed DNA molecule). These structures are those that would be predicted if nicking, unwinding, and sealing of the parental strands occurred as replication proceeded. These experiments provide a direct demonstration that there is a progressive decrease in the topological winding number which accompanies SV40 DNA replication.

For any covalently closed duplex DNA molecule, the topological winding number is a fixed value which can only be changed by introducing a break into one of the strands, allowing unwinding to occur, and then sealing the break. To effect semiconservative replication of simian virus 40 (SV40) DNA (8) the topological winding number of the molecule must decrease from a value of about 470 to a final value of zero. Since replicating molecules of SV40 do not contain swivels which allow the parental strands to unwind continuously as DNA replication proceeds (10, 16), we have postulated that nicking, unwinding, and then sealing of the parental strands occurs intermittently during the replication cycle (16). (In this work, the terms parental strands or parental molecule will refer to the SV40 DNA I which is used as the template for DNA replication. The parental strands can either be contained in a replicating SV40 DNA molecule, or will be that parental molecule which is generated when a replicating molecule is treated so as to dissociate the parental molecule from newly synthesized DNA strands.) A consequence of this postulate is that the topological winding number of the parental strands progressively decreases as the replication cycle proceeds. Experimental data in sup-

port of this idea were obtained when the sedimentation rates of the parental strands derived from molecules which have undergone different extents of replication were determined. The parental strands obtained from replicating molecules sedimented rapidly in alkaline sucrose gradients as would be expected for covalently closed duplex molecules. However, in each case they sedimented more slowly than DNA I (SV40 component I, the superhelical form of the molecule) (16).

A more direct measure of the winding number can be obtained by determining the number of superhelical turns in SV40, since this is related to the topological winding number by the relationship $\tau = \alpha - \beta$, where "the topological winding number, α , is the number of revolutions made by one strand about the duplex axis when the axis is constrained to lie in a plane; the duplex winding number, β , is the number of revolutions made by one strand about the duplex axis in the unconstrained molecule and the superhelix winding number, τ , is the number of revolutions made by the duplex about the superhelix axis" (19). As replication proceeds and unwinding of the parental strands occurs, there will be a decrease in α , the topological winding number. After displacement of the

newly synthesized strands from these replicative intermediate structures, the parental molecule will have the potential to form a Watson Crick base-paired structure in which the value β is the same as in DNA I. Since α has decreased as a result of replication, it is clear that these structures will have an increased number of superhelical turns, which can be quantitated experimentally. This paper describes the results of experiments which show that, after dissociation of newly synthesized strands from replicating molecules, covalently closed duplexes with a decreased winding number and a greater negative superhelical content than DNA I are generated, and it describes some properties of these molecules.

MATERIALS AND METHODS

Infection of cells. Confluent monolayers of African green monkey kidney (AGMK) cells growing in 150-mm plastic petri dishes were infected with 50 to 100 PFU per cell. At the end of a 2-h adsorption period, 40 ml of Eagle medium containing 2% fetal calf serum and 2 mM glutamine was added to each plate.

Preparation of ^3H -thymidine-labeled, low-molecular-weight DNA fraction. At 28 h postinfection, the medium was removed, and 8 ml of prewarmed, serum-free medium containing 50 μCi of ^3H -thymidine per ml was added to each plate. After incubation at 37 C for 3 h, the medium was removed, the cell monolayer was washed twice with cold phosphate saline, and the low-molecular-weight (LMW) viral DNA was extracted by the Hirt procedure (9). The Hirt supernatant fluid was dialyzed and concentrated by vacuum dialysis (16).

Gradient fractionation of the LMW DNA. The conditions for the fractionation of the LMW DNA by neutral and alkaline sucrose velocity sedimentation and by isopycnic banding in ethidium bromide-cesium chloride have already been described (16). Detailed conditions for each experiment are given in the legends to the figures.

RESULTS

Preparation of replicating SV40 DNA molecules labeled in both parental growing strands. In order to incorporate isotope into the template strands of replicating molecules, as well as into growing SV40 DNA chains, it is necessary to use extended labeling periods. Under these conditions, labeled progeny molecules (DNA I) are formed, re-enter the replicating pool, and are used as templates for the synthesis of additional progeny molecules (16). At 28 h postinfection, cells were labeled for 3 h with tritiated thymidine, and the LMW fraction was prepared and banded isopycnicly in CsCl-ethidium bromide (Fig. 1). As expected under

these conditions, the bulk of the isotope is incorporated into DNA I and the remainder bands at lower densities in the region that contains replicating molecules and DNA II (SV40 component II, the nicked form of the molecule). This latter region was divided to give the young replicating molecules (pool 1, less than 60% replicated) and the more mature replicating molecules (pool 2, 60 to 90% replicated). Each of these pools was freed of ethidium bromide, concentrated, and sedimented in neutral sucrose gradients. This gradient procedure separates the replicating molecules from contaminating DNA I and II, since in neutral sucrose DNA I and II both sediment more slowly than the replicating molecules (12, 16). The pools of young (pool 1) and mature replicating molecules (pool 2) that were obtained after neutral sedimentation are shown in Fig. 2.

Dissociation of replicating molecules into parental and newly synthesized strands. In studies with SV40 DNA I, we observed that molecules held at pH 13 for 3 min and then neutralized were irreversibly denatured and sedimented in neutral gradients at 29S as compared with a sedimentation rate of 21S for untreated DNA I. However, after treatment at

FIG. 1. Isopycnic banding of SV40 replicating molecules in ethidium bromide CsCl gradients. Infected AGMK monolayers were pulsed for 3 h with ^3H -thymidine (50 $\mu\text{Ci}/\text{ml}$) at 28 h after infection. The Hirt supernatant fluid (9) was prepared and dialyzed and then was centrifuged at 42,000 rpm for 40 h in an ethidium bromide-CsCl gradient (9 ml; ethidium bromide, 200 $\mu\text{g}/\text{ml}$; CsCl density, 1.56). Fractions were collected, and 5 μl of each fraction were counted (16).

pH values below 12.6, the neutralized DNA I still sedimented at 21S. Similar results have been reported previously for SV40 (22) and for the replicating form of ϕ X DNA (15). The neutral sedimentation profiles of the two pools of replicating molecules (pools 1 and 2 of Fig. 2) that had been treated at pH 12.2 for 3 min and then neutralized are shown in Fig. 3. The rapidly sedimenting material is the parental DNA, and the more slowly sedimenting material is the newly synthesized DNA. After treating young replicating molecules (pool 1 of Fig. 2) under these conditions, 59.1% of the labeled DNA sediments as parental strands and 40.9% sediments as dissociated newly synthesized strands. On sedimentation of the same replicating molecules at pH 13, conditions which completely dissociate parental and newly syn-

thesized strands (16), we obtained values of 57.6 and 42.4%, respectively, indicating that a quantitative dissociation had been effected at pH 12.2. For mature molecules after treatment at pH 12.2 or pH 13 the distribution of the labeled DNA was 45.7 and 41.6% in parental strands and 54.3 and 58.4% in daughter strands, again indicating that a complete dissociation had been effected.

In pools 1 and 2 (see Fig. 3) the parental strands sediment with *S* values of 22.2 and 23.6, respectively. This is somewhat more rapid than the sedimentation rate of SV40 DNA I (21S), which is also covalently closed and has the same mass. It is possible that the parental strands sediment more rapidly as a consequence of an alteration in structure which results from an increased number of superhelical turns in the molecules. In 1 M NaCl, the parental strands from young molecules (pool 1 of Fig. 3) sediment at 29S (see Fig. 5, zero ethidium bromide concentration).

Parallel preparative gradients were carried out after pH 12.2 treatment of pools 1 and 2 of Fig. 2 in order to obtain the parental strands from the two pools of replicating molecules. The pool of "young" parental molecules obtained after treatment at pH 12.2 and neutral sedimentation was further characterized by alkaline sedimentation analysis. The parental strands sediment in alkali as a relatively homogeneous material at 49.5S (not shown). This analysis confirmed that all of the radioactivity contained in the newly synthesized strands was removed by the prior treatment at pH 12.2 and the subsequent neutral sucrose gradient. The parental strands sediment somewhat slower than SV40 DNA I which is in agreement with our

FIG. 2. Preparative neutral sucrose sedimentation of SV40 replicating DNA molecules. Pools 1 and 2 from Fig. 1 were freed of ethidium bromide and then dialyzed and concentrated. Samples were then layered onto 12-ml 5 to 30% sucrose gradients containing 0.1 M NaCl, 0.01 M Tris, 0.01 M EDTA, pH 7.5. Gradients were centrifuged for 16 h at 26,000 rpm in a SW41 Ti rotor at 10 C. Fractions were collected, and 5 μ l of each fraction were counted. Sedimentation is from right to left.

FIG. 3. Separation of parental strands of replicating molecules from newly replicated strands. The two pools of ^{3} H-replicating molecules (from Fig. 2A and B) were adjusted to pH 12.2 and then neutralized after 3 min. A portion of each pool and ^{14} C-SV40 DNA I and DNA II was layered onto a 12-ml 5 to 30% sucrose gradient containing 0.01 M NaCl, 0.01 M Tris; and 0.001 M EDTA, pH 7.5. Gradients were centrifuged for 16 h at 26,000 rpm at 10 C in a SW41 Ti rotor. Sedimentation is from right to left.

previous findings, in which parental strands from replicating molecules that had undergone different degrees of replication were examined (16), and is related to the alteration in the winding number of these parental strands (see Discussion). When the young replicating molecules which had not been treated at pH 12.2 (pool 1 of Fig. 2) are centrifuged in alkaline gradients, the parental strands sediment in the same manner, and the newly synthesized strands which are dissociated from them are observed near the top of the gradient, sedimenting less rapidly than 16S. The increase in sedimentation rate from 29S in 1 M neutral sucrose gradients (see Fig. 5, where zero ethidium bromide is present) to 49.5S in alkaline gradients is expected for parental strands that are in a covalently closed superhelical configuration.

Isopycnic centrifugation of young and mature parental molecules in the presence of ethidium bromide. Gray, Upholt, and Vinograd (7) have published a procedure by which the superhelix density of a covalently closed molecule can be calculated based on the density at which it bands in CsCl-ethidium bromide. When the parental strands derived from the replicating molecules by alkaline treatment were banded in this manner, the parental strands from young replicating molecules banded at about the same position as DNA II although there is clear evidence of heterogeneity. Those from the mature replicating molecules banded at a lower density than DNA II (Fig. 4).

Effect of ethidium bromide on the sedimentation rate of parental strands. The tritium-labeled parental strands were obtained

from the young replicating molecules after treatment at pH 12.2. Their sedimentation rate in neutral sucrose gradients containing 1 M NaCl and either 0, 1.5, 7.5, 10, or 20 μg of ethidium bromide per ml are seen in Fig. 5 and Table 1. DNA II labeled with ^{32}P and DNA I containing ^{14}C are also included as sedimentation references. The sedimentation values of parental strands and DNA I are expressed relative to DNA II which is assigned a sedimentation rate of 16S at each ethidium bromide concentration.

The sedimentation rate of DNA I decreases in gradients containing 1.5 μg of ethidium bromide per ml. With further increases in the concentrations of ethidium bromide its sedimentation rate increases to a maximum *S* value of 20.5. The parental strands can be seen to be heterogeneous in their sedimentation at 1.5 $\mu\text{g}/\text{ml}$. At this concentration a fraction of the molecules sediment at 27S while the remainder sediment at 20.3S. The presence of two peaks may result from differential binding of ethidium bromide by molecules with differing numbers of superhelical turns. At a higher ethidium bromide concentration of 7.5 $\mu\text{g}/\text{ml}$, the *S* value decreases to 19 and there is only a slight further decrease in the sedimentation rate when the ethidium bromide concentration is increased to 10 or 20 μg of ethidium bromide per ml. The free energy of superhelix formation for such a highly underwound molecule may be large and so there is an enhanced affinity for drug in order to remove the large number of supercoils. As a consequence, all binding sites are saturated at relatively low concentrations of ethidium bromide. This would explain the small change in *S*

FIG. 4. Isopycnic banding of parental strands of SV40 replicating DNA molecules in ethidium bromide-CsCl gradients. A portion of the two pools of parental strands (fractions 15 to 20 of Fig. 3A and B) were mixed with ^{14}C -SV40 DNA I and DNA II and adjusted to 330 μg of ethidium bromide per ml, and CsCl to a density of 1.58. Gradients were centrifuged for 40 h at 40,000 rpm in a SW50.1 rotor (7). A, Parental strands from "young" replicating molecules (pool 1); B, parental strands from "mature" replicating molecules (pool 2).

FIG. 5. Sedimentation of parental strands of young replicating molecules in the presence of ethidium bromide. Portions of the parental strands of "young" replicating molecules (pool I) were layered onto 12-ml 5 to 30% sucrose gradients containing 1 M NaCl, 0.01 M EDTA, 0.01 M Tris, pH 7.5 and 0, 1.5, 7.5, 10, or 20 µg of ethidium bromide per ml. ¹⁴C-SV40 DNA I and ³²P-SV40 DNA II were also added to gradients. Samples were centrifuged for 16 h at 26,000 rpm at 10 C in a SW41 Ti rotor. Sedimentation is from right to left.

in going from 7.5 to 20 µg of ethidium bromide per ml and the fact that a minimum is not observed in the sedimentation titration curve.

DISCUSSION

In previous studies it has been shown that replicating SV40 DNA molecules contained parental strands in a covalently closed structure (10, 16). It is clear, however, that DNA replication cannot proceed without the introduction of

TABLE 1. Rate of sedimentation of replicating molecules in the presence of ethidium bromide

Ethidium bromide concn (µg/ml)	Sedimentation rate of the parental strands
0	28.8
1.5	20.3, 27.0
7.5	19.5
10	19.4
20	19.1

a "swivel" in the parental strands. Our previous data on the mode of SV40 DNA replication seemed most consistent with a model for DNA replication in which there was an intermittent swivel which permitted unwinding of the parental strands to occur as DNA replication proceeded. A preliminary characterization of parental strands from replicating molecules of different ages was consistent with this model (16). The parental strands in alkaline sucrose gradients had sedimentation properties consistent with covalently closed circles, but sedimented somewhat more slowly than SV40 DNA I. We postulated that the decrease in the sedimentation rate was the consequence of a decrease in the topological winding number which would be expected to occur as replication proceeded. The sedimentation rate of parental strands derived from young replicating molecules (47S) was considerably more rapid than those from mature replicating molecules (36S) but less than the reference 53S value of DNA I, data which are in agreement with this idea.

If there is progressive unwinding and subsequent sealing of the parental strands as replication proceeds, one can predict the structures that would be generated if the parental strands contained in the replicating molecules were separated from the newly synthesized strands under conditions where the parental strands could then renature. The topological winding number of SV40 DNA I is 467, and this underwound structure contains approximately 19 negative superhelical turns and a superhelix density of -0.039 (7). We have observed that replicating molecules contain approximately the same superhelix density in the unreplicated portion of the molecule as is observed for DNA I (Sebring, Garon, and Salzman, unpublished results). Based on this observation we would predict that the parental strands of a molecule which was 50% replicated would have a topologic winding number of about 234. When the newly synthesized strands were dissociated and the parental strands were allowed to renature, if renaturing were complete they should generate structures with about 252 negative superhelical turns, which corresponds to a superhelix density of -0.5 . Upholt, Gray, and Vinograd (7, 18) have provided a precise characterization of molecules with superhelix densities as high as -0.08 . These covalently closed molecules were generated by the action of ligase on DNA II in the presence of ethidium bromide (7). However, it is not possible by this procedure to generate structures with superhelix densities in the same range as those which are formed from parental strands of replicating molecules.

We have observed that after treatment at pH 12.2, a pH value at which DNA I is reversibly denatured, there is a complete release of newly synthesized strands from replicating molecules. When the parental strands derived from replicating molecules are banded in CsCl-ethidium bromide, the covalently closed parental molecule derived from young replicating molecules banded heterogeneously at a density close to that of DNA II, while those parental strands derived from mature replicating molecules band at an even lower density than DNA II. Since single-stranded SV40 DNA bands at a slightly higher density than DNA II, the banding of the parental molecules at a lower density than DNA II can only be attributed to the presence of a very high negative superhelix density in these molecules. Bauer and Vinograd (2) have predicted that closed circular DNA molecules of sufficiently high negative superhelix density should bind more ethidium bromide than DNA II at a dye concentration of $330 \mu\text{g/ml}$. This is

FIG. 6. Binding isotherms for the interaction of ethidium bromide with closed circular SV40 DNA, calculated by the method of Bauer and Vinograd (2), as a function of the number of superhelical turns. The binding ratio is plotted as a function of $\log(kc)$ for various numbers of superhelical turns ranging from -19 (DNA I) to -200 . This corresponds to superhelical densities of from -0.039 (DNA I) to -0.5 . The upper abscissa shows the free dye concentration calculated with a binding constant $k = 7.3 \times 10^3 \text{ liter mol}^{-1}$. The dashed line represents a free ethidium bromide concentration of $330 \mu\text{g/ml}$, the concentration at which isopycnic banding of parental strands was carried out.

illustrated in Fig. 6 which shows theoretical binding isotherms for SV40 DNA molecules containing varying numbers of negative superhelical turns. It can be seen that molecules containing 75 superhelical turns will bind more ethidium bromide than DNA II at a concentration of ethidium bromide at 330 $\mu\text{g/ml}$. Based on the buoyant density of parental strands in ethidium bromide, we can conclude that they contain at least 75 superhelix turns. We had noted previously that structures with as many as 250 negative superhelix turns could arise from replicating molecules if strand renaturation was complete. When newly synthesized strands of replicating molecules are displaced from parental strands and the parental strands are allowed to renature, one superhelical turn should be introduced for each ten base pairs which renature. Since the free energy required to introduce each additional superhelical turn into a molecule increases as the number of superhelical turns in the molecule becomes greater (1), the process of renaturation should continue only until the free energy required to introduce an additional superhelical turn is greater than the free energy released by the renaturation of 10 base pairs. At this point strand renaturation would not continue, thus generating molecules with single-stranded regions.

The properties of renatured parental strands are consistent with the presence of extensive single-stranded regions in the molecules. One evidence of this is a marked dependence of the rate of sedimentation on ionic strength (17). Parental strands sediment at 22S in 0.01 M NaCl and at 29S in 1 M NaCl (see Fig. 3 and Fig. 5, zero ethidium bromide concentration). When parental strands obtained from replicating molecules are banded isopycnicly in CsCl they band between the position of single-stranded and double-stranded DNA (Salzman and Radonovich, unpublished results). This would also suggest the presence of extensive single-stranded regions in these molecules. Replicating mitochondrial DNA molecules have also been examined after they were denatured and then renatured. Loops of unrenatured single-stranded DNA were observed (14), findings which are similar to those which we have observed with SV40. The behavior of parental strands of SV40 during isopycnic banding in CsCl and their sedimentation properties suggest that their degree of single strandedness is greater than that of native superhelical DNA (5, 11).

DNA, in the presence of excess amounts of ethidium bromide, is not able to bind more than

0.25 mol of dye/mol of nucleotide (2). For a DNA molecule which contained 4860 nucleotide base pairs, 2,430 is the maximum number of molecules of ethidium bromide that can be bound. With an unwinding angle ϕ of 12° for each mole of drug bound (21), even when the maximum number of molecules of ethidium bromide are bound, no more than 81 superhelical turns would be lost from the parental structures. Since they may contain as many as 250 superhelical turns, a tight, negatively wound superhelical structure would still be present. Since all of the negative superhelical turns cannot be removed by the binding of ethidium bromide, it is not surprising that, when the sedimentation rate of parental strands is studied as a function of increasing ethidium bromide concentration, a decrease in the *S* value of parental strands is observed but no increase in sedimentation is observed at higher dye concentrations. Such an increase would only be expected when sufficient ethidium bromide had been bound to first remove all of the negative superhelical turns and then generate positive superhelical turns by binding additional dye.

These data provide direct evidence for the progressive unwinding of parental strands during DNA replication. Evidence for unwinding has also recently been reported for replicating molecules of polyoma virus (3). It does not provide information on the *in vivo* mechanism of unwinding. This may occur by the action of proteins which function as swivels and allow unwinding of superhelical molecules to occur (4, 20). As yet, however, these proteins, which function *in vitro*, have not been shown to have an essential role in *in vivo* replication.

Paoletti and LePecq (13) have reported that intercalation of ethidium bromide winds the DNA helix rather than unwinding it, as previously reported (6). If this were correct, binding ethidium to covalently closed DNA would result in the introduction of negative superhelical turns. It would follow that a DNA molecule containing positive superhelical turns would bind more ethidium bromide than one containing negative superhelical turns. The authors concluded that DNA I contains a positive superhelix. There is a clear prediction that the parental molecules formed by dissociating the newly replicated strands from replicating molecules will contain negative superhelical turns. Since these molecules bind more ethidium than either DNA I or DNA II, it must be concluded that ethidium does cause unwinding of the DNA helix, as Fuller and Waring (6) originally reported.

ACKNOWLEDGMENTS

We wish to acknowledge many thoughtful and valuable discussions with Jacob Lebowitz.

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