

Cleavage of Circular, Superhelical Simian Virus 40 DNA to a Linear Duplex by S_1 Nuclease

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S_1 nuclease, the single-strand specific nuclease from *Aspergillus oryzae* can cleave both strands of circular covalently closed, superhelical simian virus 40 (SV40) DNA to generate unit length linear duplex molecules with intact single strands. But circular, covalently closed, nonsuperhelical DNA, as well as linear duplex molecules, are relatively resistant to attack by the enzyme. These findings indicate that unpaired or weakly hydrogen-bonded regions, sensitive to the single strand-specific nuclease, occur or can be induced in superhelical DNA. Nicked, circular SV40 DNA can be cleaved on the opposite strand at or near the nick to yield linear molecules. S_1 nuclease may be a useful reagent for cleaving DNAs at regions containing single-strand nicks. Unlike the restriction endonucleases, S_1 nuclease probably does not cleave SV40 DNA at a specific nucleotide sequence. Rather, the sites of cleavage occur within regions that are readily denaturable in a topologically constrained superhelical molecule. At moderate salt concentrations (75 mM) SV40 DNA is cleaved once, most often within either one of the two following regions: the segments defined as 0.15 to 0.25 and 0.45 to 0.55 SV40 fractional length, clockwise, from the $EcoR_1$ restriction endonuclease cleavage site (defined as the zero position on the SV40 DNA map). In higher salt (250 mM) cleavage occurs preferentially within the 0.45 to 0.55 segment of the map.

The DNA of simian virus 40 (SV40) is a double-stranded covalently closed, circular, superhelical molecule (5, 33). The development of ways to cleave SV40 DNA at specific locations has been useful both for introducing DNA segments at defined locations in the viral chromosome (17) and for providing unique linear molecules for mapping genetic and physical loci (26). Specific breakage of SV40 DNA has been achieved with restriction endonucleases from *Haemophilus influenzae* (6) from *Escherichia coli* carrying the drug resistance transfer factor RTF-1 (R.N. Yoshimori, Ph.D. dissertation, University of California, San Francisco Medical Center) (26, 28), from *Haemophilus aegyptius* (16a) and from *Haemophilus parainfluenzae* (8, P. A. Sharp, B. Sugden, and J. Sambrook, Biochemistry, in press). We now report that a single strand-specific nuclease, the S_1 endonuclease of *Aspergillus oryzae* (2), cleaves plaque-purified superhelical SV40 DNA preferentially within either of two alternative regions, or predominantly within one of these regions, depending upon the reaction conditions. These regions have been mapped relative to the segment of SV40 DNA contained in the adenovi-

rus-SV40 hybrid, Ad2⁺ND₁ (22) and to the locations at which the R_1 restriction endonuclease, $EcoR_1$, and a restriction enzyme from *H. parainfluenzae* (HpaII) (P. A. Sharp, B. Sugden, and J. Sambrook, Biochemistry, in press), cleave SV40 DNA. The cleavage of SV40 DNA by S_1 nuclease most likely occurs within regions that contain localized interruptions in base pairing resulting from the superhelicity of the natural SV40 DNA molecule. S_1 nuclease also attacks nicked circular SV40 DNA so as to cleave the duplex on the opposite strand at or near the nick; consequently a single-strand nick is converted to a double-strand break.

MATERIALS AND METHODS

Cells and virus. The methods of cell culture and virus growth have been described previously (26). Plaque-purified SV40 strain Rh911 was grown on CV-1P, a line of African green monkey kidney cells. The adeno-SV40 hybrid virus, Ad2⁺ND₁, was grown on the human cell line KB-3.

Isolation of viral DNA. Ad2⁺ND₁ was purified and the DNA isolated as described earlier (12, 20). SV40 DNA labeled with ³H-thymidine was isolated by the method of Hirt (15) from SV40-infected CV-1P cells (20 plaque-forming units/cell) after incubation

with ³H-thymidine (5 μCi/ml, 20 Ci/mmol) between 24 and 48 h after infection. The DNA solution was extracted with chloroform-isoamyl alcohol (24:1) to remove protein, then treated with ribonuclease (60 μg/ml, heated for 5 min at 100 C to inactivate deoxyribonuclease). Supercoiled SV40 DNA was purified by sedimentation in a neutral sucrose gradient followed by equilibrium centrifugation in CsCl-ethidium bromide (29). SV40 DNA labeled with ³²P was prepared similarly except that 24 h after infection the medium was replaced with phosphate-free medium containing 10% dialyzed calf serum and 50 μCi of ³²P-phosphate per ml. The superhelical, nicked circular, and linear forms of SV40 DNA are referred to as SV40(I) DNA, SV40(II) DNA, and SV40(L) DNA, respectively. [Linear molecules generated by S₁ nuclease, EcoR₁, or HpaII endonucleases are designated SV40(L_{S1}), SV40(L_{R1}), and SV40(L_{HpaII}) DNA, respectively.]

Closed circular duplex λdv-120 (λdvgal) DNA labeled with ³H-thymidine was isolated from an *E. coli* strain containing this DNA as a plasmid by the methods described earlier (17).

Enzymes. S₁ nuclease from *A. oryzae* (Sephadex G-75 fraction, purified 1000-fold) (2) was generously provided by T. Ando. In some experiments a preparation made by H. Marriott and C. Rhodes according to the procedure of Vogt (35) was used; this preparation was approximately 100-fold more pure than Ando's, as judged by their activities per A₂₈₀. The results with the two enzyme preparations, however, were virtually identical. EcoR₁ restriction endonuclease and T4 gene 32 protein (1) were the preparations described previously (26). A restriction endonuclease of *H. parainfluenzae* (HpaII) was prepared by a modification of a procedure kindly sent to us prior to publication by Sharp, Sugden, and Sambrook (P. A. Sharp, B. Sugden, and J. Sambrook, *Biochemistry*, in press). Mung bean nuclease (18) was very generously provided by M. Laskowski, Sr. A preparation of polynucleotide ligase from *E. coli* (25) was given to us by P. Modrich.

Enzyme reactions. Assay mixtures for S₁ nuclease contained the following in a volume of 0.1 ml: 30 mM sodium acetate buffer, pH 4.6, 0.5 mM ZnCl₂, 75 mM NaCl, 1 μg of heat-denatured ³H-labeled SV40(II) DNA, and S₁ nuclease. After incubation for 10 min at 37 C, 50 μg of carrier salmon sperm DNA and 3 ml of cold 1 N HCl were added. The precipitate was collected on glass filters, washed, and its radioactivity was measured. One unit of S₁ nuclease activity converts 50% of heat-denatured SV40 DNA to an acid soluble form in this assay. Cleavage of SV40(I) DNA (10 μg/ml) by S₁ nuclease (200 U/ml, approximately 9 μg of protein/ml) was performed under the conditions described above, except that different concentrations of NaCl were used. After the reaction samples were cooled in ice and neutralized by the addition of Tris-hydrochloride buffer, pH 8, to a final concentration of 0.1 M and EDTA to 10 mM.

SV40(L_{S1}) DNA and SV40(I) DNA were treated with EcoR₁ endonuclease under the conditions described previously (26). SV40(L_{S1}) DNA, 3 μg/ml, was incubated with HpaII restriction endonuclease for 2 h at 37 C in 10 mM Tris-hydrochloride buffer, pH 7.4, 5

mM MgCl₂, 0.4 mM dithiothreitol, and 50 μg of autoclaved gelatin (Difco) per ml. Polynucleotide ligase reactions were performed according to Mertz and Davis (24); 1 μg of SV40(L_{R1}) DNA per ml was incubated with excess ligase for 48 h at 15 C. These conditions favor intramolecular joining to produce circular molecules. The complex of SV40(I) DNA and T4 gene 32 protein was made by the method of Delius et al. (11). After glutaraldehyde fixation the complexes were dialyzed against 10 mM Tris-hydrochloride, pH 7.4, 1 mM EDTA before incubation with enzymes.

Centrifugation. Solutions for equilibrium centrifugation contained SV40 DNA, 10 mM Tris-hydrochloride buffer, pH 7.5, 1 mM EDTA, 370 μg of ethidium bromide and CsCl per ml to bring the final density to 1.566 g/ml. Centrifugation was for 36 h at 40,000 rpm at 20 C in a Beckman SW50.1 rotor. Gradients were fractionated by collecting drops from the bottom of the tube. Samples of each fraction were spotted and dried on Whatman 3 MM paper disks and the radioactivity was determined by using toluene-based scintillation fluid and a scintillation spectrometer. Velocity sedimentation on linear 5 to 20% sucrose gradients occurred at 55,000 rpm at 4 C in a Beckman SW 56 rotor. Neutral gradients containing 10 mM Tris-hydrochloride buffer, pH 7.5, 1 mM EDTA, 1 M NaCl, were centrifuged for 4 h. Alkaline sucrose gradients containing 2 mM EDTA, 0.2 to 0.8 N NaOH (in proportion to sucrose concentration), 0.8 to 0.2 M NaCl (final Na⁺ concentration 1 M) were centrifuged at 55,000 rpm for 4 h. Gradient fractions were collected as above and the radioactivity was determined without correcting for an overlap of 0.4% ³²P in the ³H channel.

Electron microscopy. The methods used to prepare heteroduplex DNA molecules containing Ad2, Ad2⁺ND₁ and SV40 DNA have been described (26). Heteroduplex DNA and the cleavage products of SV40 DNA by S₁ nuclease, EcoR₁, or HpaII nucleases were mounted by the formamide technique, shadowed, and examined by electron microscopy (9). Lengths were measured using a Hewlett-Packard 9864A digitizer and 9810A calculator with a length calculation program which gives an accuracy of ±0.5%.

RESULTS

S₁ nuclease cleaves double-stranded SV40 DNA. S₁ nuclease, which normally degrades single-stranded but not double-stranded DNA to 5'-deoxynucleotides (2), cleaves superhelical SV40 DNA; the product of the cleavage depends on the concentration of NaCl in the reaction. The product of cleavage in 10 mM NaCl sediments in neutral sucrose gradients more slowly than unit length linear SV40 DNA, in the region expected for shorter length DNA (Fig. 1). The major product formed in 75 or 250 mM NaCl sediments as unit length linear SV40 DNA, with some nicked circular DNA but little material sedimenting more slowly than complete length

molecules (Fig. 1). The amount of S_1 nuclease in these reactions is five times the amount required to completely degrade an equivalent amount of single-stranded DNA. Additional S_1 nuclease does not appreciably degrade the unit length linear products of reaction; therefore, the number of cleavages per SV40 DNA molecule is not limited by the amount of enzyme present. A purer preparation of S_1 nuclease, made by the procedure of Vogt (35), cleaved SV40 DNA to identical products (data not shown); therefore, the cleavage is most likely by S_1 nuclease and not by a contaminating activity.

Conversion of supercoiled DNA to nicked circles and linear DNA is more than five times slower in 250 mM NaCl than in 75 mM NaCl although the degradation of denatured DNA by S_1 nuclease is only 30% slower at the higher salt concentration (32, 35). In an S_1 nuclease reaction with 250 mM NaCl, the conversion of SV40(I) DNA to SV40(L_{S1}) DNA continues for 60 min, whereas the proportion of nicked circular DNA (SV40(II)) rises rapidly, initially, and then remains nearly constant (Fig. 2). We have observed some variability in the relative propor-

tions of nicked circular and linear molecules produced in 250 mM NaCl (compare Fig. 1 and 2); generally, the amount of SV40(I) DNA which disappears is reproducible (within 25%) although the ratio of SV40(L) to SV40(II) DNA has varied from 2 to about 0.75.

S_1 nuclease probably converts supercoiled SV40 DNA to a linear structure, not by making simultaneous cleavages on opposite strands of the duplex, but rather, by making sequential, relatively closely spaced scissions on opposite strands. This is indicated by the observation that nicked, circular SV40 DNA can be converted to linear molecules by S_1 nuclease. ^{32}P -SV40(II) DNA, isolated after spontaneous decay of ^{32}P -SV40(I) DNA, and containing an average of one to two single-strand nicks per circular molecule (after decay of about 75% of the ^{32}P -SV40(I) DNA and assuming random nicks during ^{32}P decay, we estimate that the isolated ^{32}P -SV40(II) DNA contains, on the average, between one to two single-strand nicks per circular molecule) was converted by S_1 nuclease in 75 mM NaCl to slower sedimenting material; of the approximately 90% of the

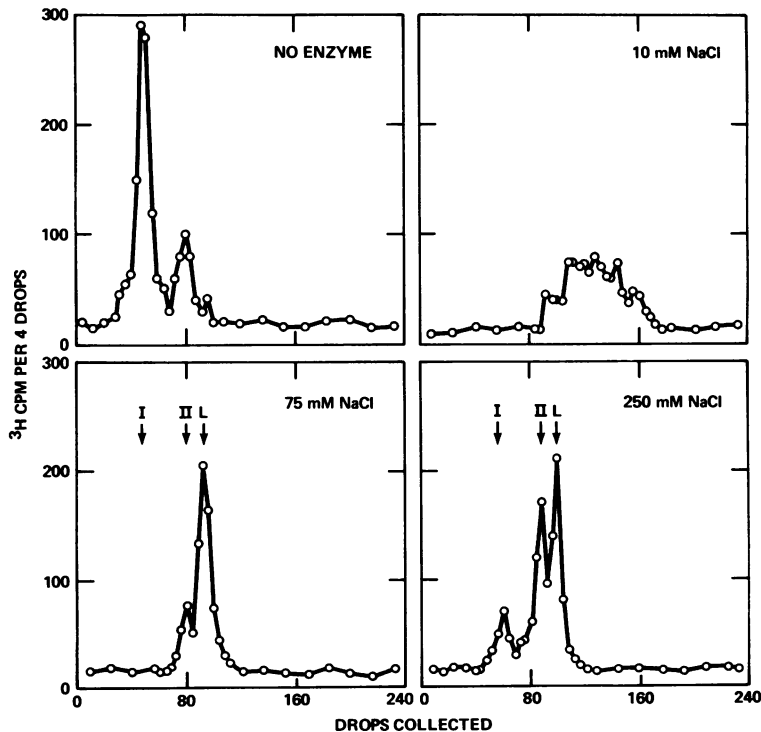


FIG. 1. Neutral sucrose gradient sedimentation analysis of the products of S_1 nuclease digestion of ^3H -SV40(I) DNA. SV40(I) DNA was incubated in a reaction mixture without S_1 nuclease or with S_1 nuclease in 10 mM NaCl, in 75 mM NaCl, or in 250 mM NaCl. Incubation in 250 mM NaCl was for 60 min; the other incubations were for 10 min. The positions of marker ^{32}P -labeled SV40(I), (II), and (L) DNA are indicated. In this and subsequent sedimentation profiles the direction of sedimentation is from right to left.

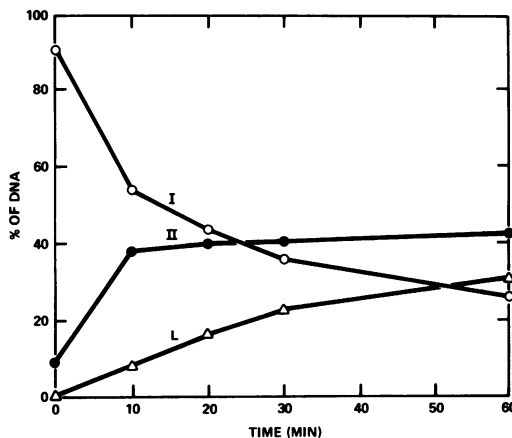


FIG. 2. Time course of cleavage of ^3H -labeled SV40(I) DNA by S_1 nuclease in 250 mM NaCl. Samples of the reaction mixture were removed at intervals and analyzed by sedimentation in neutral sucrose gradients to determine the proportions of SV40(I), (II), and (L) DNA.

^{32}P -SV40(II) DNA which disappeared, about 70% sedimented as unit length linear molecules and the remainder formed a heterogeneous population sedimenting at less than 12S. This is the expected result if a population of singly and multiply nicked circular forms are cleaved at the nicks to linear forms by the S_1 nuclease.

When SV40 DNA, cleaved by S_1 nuclease in 75 or 250 mM NaCl, and isolated from a neutral sucrose gradient (as in Fig. 1), is mounted by the formamide technique and examined by electron microscopy, almost all the molecules are linear. The length of these linear molecules is 0.99 (standard error 0.01) SV40 fractional length relative to nicked circular SV40 DNA measured on the same grid. We conclude that under these conditions S_1 nuclease can cleave both strands of superhelical SV40 DNA to produce linear molecules (SV40(L_{S_1})) whose length is virtually unchanged. Most of the SV40(L_{S_1}) DNA molecules contain no internal single strand interruption since very little material sediments more slowly than single-stranded linear SV40 DNA in an alkaline sucrose gradient (Fig. 3).

S_1 nuclease cleaves SV40 DNA at specific locations. SV40(L_{S_1}) DNA might arise by cleavage of circular SV40 DNA at a unique site, at a small number of alternative sites or at any one of many sites distributed at random. Since EcoR_I endonuclease introduces a double-stranded break at a unique site in SV40 DNA (26, 28), these possibilities can be distinguished by measuring the lengths of the DNA fragments obtained by treating SV40(L_{S_1}) DNA with

EcoR_I. (i) If there is a unique S_1 nuclease cleavage site, digestion of SV40(L_{S_1}) DNA with EcoR_I would yield two segments of DNA; the lengths of these segments would correspond to the distances between the S_1 site and the EcoR_I site in each direction around the circular DNA. (ii) If there were two alternative S_1 cleavage sites, the products of EcoR_I digestion would contain two lengths of DNA for each S_1 site (four different lengths at most). (iii) If the site of cleavage by S_1 nuclease were located randomly relative to the EcoR_I cleavage site, the DNA fragments produced by sequential digestion with both enzymes would form a random distribution of lengths, up to the length of complete SV40 DNA.

With SV40(L_{S_1}) DNA formed by cleavage in 250 mM NaCl the lengths of DNA fragments observed after treatment with EcoR_I were largely between 0.45 and 0.55 SV40 fractional length (Fig. 4A). This result indicates that, in the presence of 250 mM NaCl, S_1 nuclease cleaves SV40 DNA near 0.5 SV40 fractional length from the EcoR_I cleavage site more frequently than at any other single region. Our result does not distinguish between one cleavage site near 0.5 and two or more alternative sites between 0.45 and 0.55 SV40 fractional length from the EcoR_I site.

With SV40(L_{S_1}) DNA formed by cleavage in 75 mM NaCl a different result was obtained. In

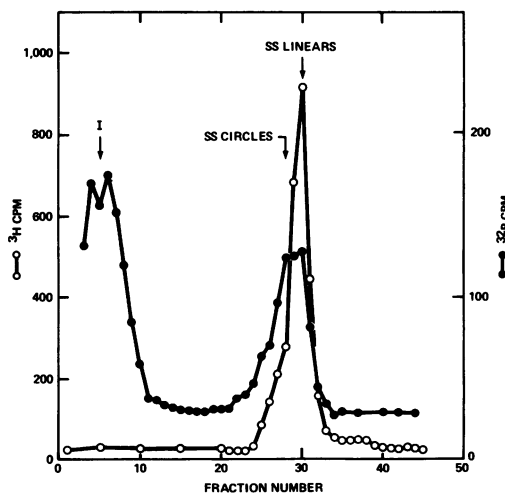


FIG. 3. Alkaline sucrose gradient sedimentation analysis of ^3H -labeled SV40(L_{S_1}) DNA formed by cleavage in 75 mM NaCl and isolated from a neutral sucrose gradient as in Fig. 1. Centrifugation was for 4 h. A mixture of ^{32}P -labeled SV40(I) and (II) DNA is present as marker; SV40(II) DNA denatures to single-stranded linear and single-stranded circular DNA (ss linears and ss circles). Under these conditions SV40(I) DNA sediments to the bottom of the gradient.

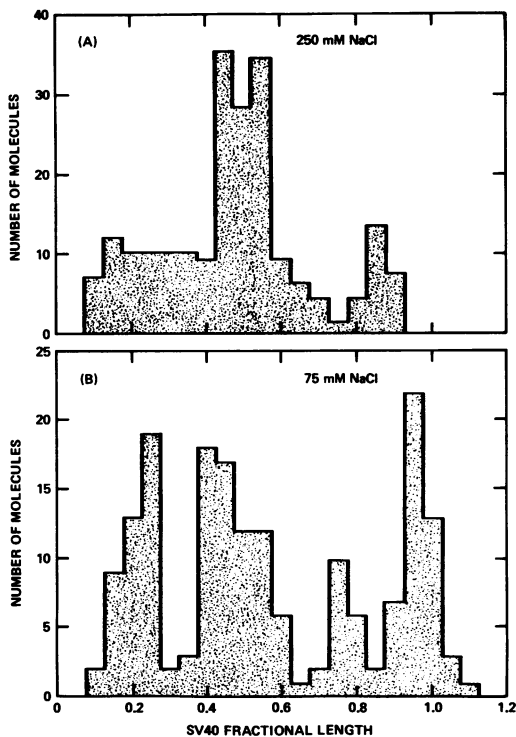


FIG. 4. Histograms of length measurements of SV40(L_{S1}) digested with R_1 endonuclease. A, SV40(L_{S1}) DNA formed by cleavage in 250 mM NaCl was incubated with EcoRI endonuclease and examined by electron microscopy as described in Materials and Methods. Lengths are presented relative to mean length of 56 SV40(II) DNA molecules (standard error of 0.0039 fractional length) in the same photographs. Molecules not cleaved by EcoRI (15% of the total) had a mean length of 1.01 SV40 fractional length (standard error 0.01) and are not shown for the purpose of clarity. B, SV40(L_{S1}) DNA formed by cleavage in 75 mM NaCl was treated as in A. Lengths are presented relative to mean length of 36 SV40(II) molecules (standard error 0.0078 fractional length) in the same photographs. All linear molecules are shown.

this case cleavage of SV40(L_{S1}) by EcoRI endonuclease generated DNA fragments of four broad length classes with mean lengths of 0.22, 0.44, 0.56, and 0.78 SV40 fractional length (Fig. 4B). Grouped in pairs (0.22 + 0.78; 0.44 + 0.56) the fragments add up to SV40 unit length. This result indicates that in 75 mM NaCl there are two alternative regions for cleavage of SV40 DNA by S_1 nuclease. These are located approximately 0.2 and 0.45 fractional length from the EcoRI endonuclease cleavage site. These two regions are attacked at about equal frequency.

Since the major product of such digestion is unit length linear DNA, cleavage of a given molecule at one site very likely prevents cleavage at the other site.

Mapping of the S_1 nuclease cleavage sites in SV40 DNA. The SV40 DNA sequence contained in the adeno-SV40 hybrid, Ad2⁺ND₁ (Fig. 5), occupies the region 0.11 to 0.28 SV40 fractional length from the EcoRI cleavage site (26). We have attempted to map the S_1 nuclease cleavage sites relative to the DNA segment in Ad2⁺ND₁.

Heteroduplex molecules made from the DNA of Ad2 and Ad2⁺ND₁, as seen in the electron microscope, contain double-stranded segments corresponding to the shared sequences and single-stranded segments corresponding to sequences not shared; the SV40 sequence contained in Ad2⁺ND₁ DNA appears as the shorter of the two single strand segments in the substitution loop (19a, 26). Two additional types of heteroduplex molecules are seen when single strands of SV40(L_{S1}) DNA formed in 75 mM salt are annealed to the Ad2-Ad2⁺ND₁ heteroduplexes (Fig. 6). In the first (Fig. 6A) the shorter substitution loop (segment t) appears double stranded and the two ends of this duplex region are joined by an additional single strand (segment s). In the second type (Fig. 6B), the shorter segment is also double stranded but single-stranded DNA "tails" (segments o and m) originate from each end of the duplex segment. We interpret these structures as Ad2-Ad2⁺ND₁ heteroduplexes with one of two classes of SV40(L_{S1}) DNA single strands annealed to the complementary SV40 sequence in Ad2⁺ND₁. Heteroduplexes of the first type are formed with an SV40(L_{S1}) DNA strand broken at a point within the SV40 segment of Ad2⁺ND₁. Those of the second type are produced when SV40(L_{S1}) strands generated by a cleavage outside of the segment in Ad2⁺ND₁ annealed to the SV40 sequence of Ad2⁺ND₁.

Measurements of the lengths of the single-stranded rings and tails originating from the double-stranded SV40 segment of the heteroduplexes are consistent with this conclusion (Table 1). For example, the length of the duplex SV40 segment (segment t in Fig. 6A) plus the length of the single-stranded ring joined to it (segment s) is, within the error of the measurements, equal to that of the SV40(L_{S1}) strands. Therefore, the cleavage by S_1 nuclease in 75 mM NaCl at about 0.2 SV40 fractional length from the EcoRI site is on the same side as the

segment contained in $Ad2^+ND_1$. In heteroduplexes of the type shown in Fig. 6B, the sum of the lengths of the single-stranded tails (segments o and m) plus the length of the double-stranded SV40 portion (segment n) is also equal to one SV40(L_{S_1}) strand; one of the single-stranded tails is 0.185 and the other is 0.615 SV40 fractional length (Table 1). From these results we conclude that the alternative cleavage site is about 0.48 SV40 fractional length from the $EcoR_1$ site, very near the midpoint of the map.

The major site of S_1 nuclease cleavage of

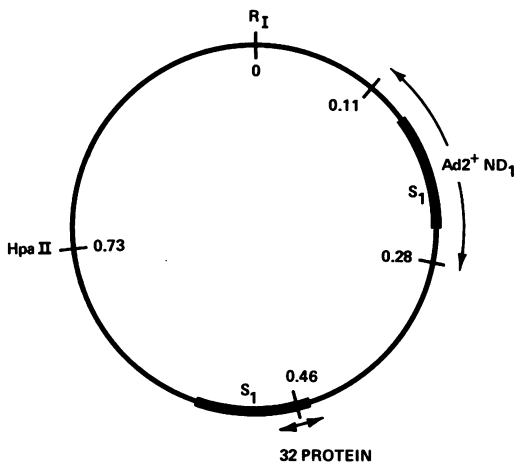


FIG. 5. Physical map of SV40 DNA. Map positions are presented in SV40 fractional length.

SV40 DNA in 250 mM NaCl (probably the same as the one made near the midpoint in 75 mM NaCl), was determined in another way. One of the restriction endonucleases from *H. parainfluenzae*, HpaII, cleaves SV40 DNA at 0.73 map unit (our measurement) from the $EcoR_1$ endonuclease site (P. A. Sharp) (see Fig. 5). Digestion of SV40(L_{S_1}) with HpaII generates a population of fragments with a non-random distribution of lengths (Fig. 7). One of the major classes (75 molecules or 34% of the total) has a mean between 0.15 and 0.25 SV40 fractional length, suggesting that the majority of the SV40(L_{S_1}) molecules had been cleaved by S_1 nuclease between 0.48 and 0.58 SV40 fractional length from the $EcoR_1$ endonuclease cleavage site; the HpaII endonuclease cleavage of such an SV40(L_{S_1}) DNA molecule should also generate an equal number of fragments with a mean between 0.75 and 0.85 SV40 fractional length. Such a class, containing 74 molecules or 33% of the total, is also discernible. There are also one or two classes of fragments produced by the HpaII cleavage which are between 0.40 and 0.60 SV40 fractional length (64 molecules or 29% of the total). These would be expected to arise from SV40(L_{S_1}) molecules generated by S_1 nuclease cleavage in the region between 0.15 and 0.25 SV40 fractional length from the $EcoR_1$ site, that is, the one within the segment present in $Ad2^+ND_1$. It is important to note that a linear molecule cleaved within the region 0.15 to 0.25 by S_1 yields two fragments in the length class

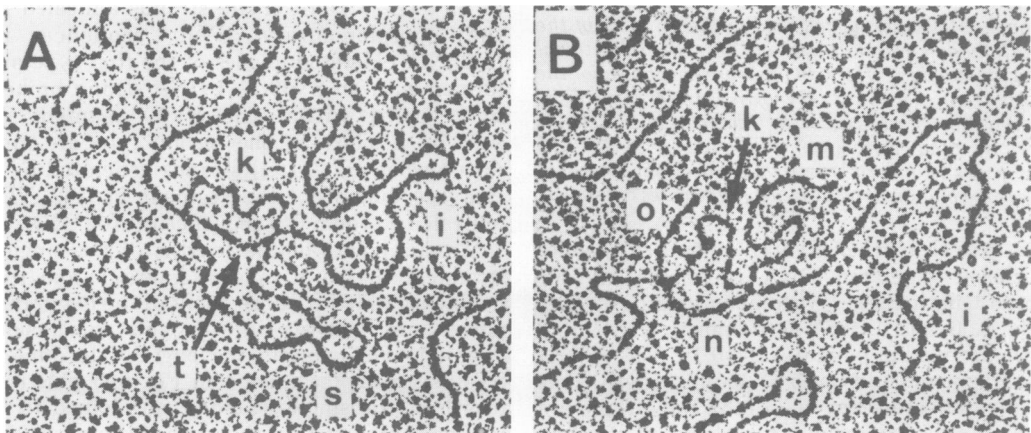


FIG. 6. Electron micrographs of SV40(L_{S_1})- $Ad2^+ND_1$ - $Ad2$ heteroduplex DNA molecules. A, Heteroduplex of $Ad2^+ND_1$ and $Ad2$ DNA with SV40(L_{S_1}) cleaved in the 0.15 to 0.25 map region. Segment i: duplex formed from $Ad2^+ND_1$ and $Ad2$ DNA. Segment t: duplex formed from SV40(L_{S_1}) and $Ad2^+ND_1$ DNA. Segment s: single strand from SV40(L_{S_1}) DNA. Segment k: single strand from $Ad2$ DNA. B, Heteroduplex of $Ad2^+ND_1$ and $Ad2$ DNA with SV40(L_{S_1}) cleaved in the 0.45 to 0.55 map region. Segment n: duplex formed from SV40(L_{S_1}) and $Ad2^+ND_1$ DNA. Segments m and o: single strands from SV40(L_{S_1}) DNA. Segments i and k as in A. Magnification in A and B is $\times 50,000$.

TABLE 1. Molecular lengths of segments of heteroduplexes*

| DNA segment | Fractional length | |
|--|-------------------|---------------|
| | SV40 | Ad2 |
| SV40 sequences in Ad2 ⁺ ND ₁ (segment n) | 0.164 ± 0.006 | |
| Longer single strand from SV40(L _{S1}) (segment m) | 0.615 ± 0.025 | |
| Shorter single strand from SV40(L _{S1}) (segment o) | 0.185 ± 0.025 | |
| Total | 0.964 ± 0.036 | |
| SV40 sequences in Ad2 ⁺ ND ₁ (segment t) | 0.158 ± 0.010 | |
| Single-stranded loop from SV40(L _{S1}) (segment s) | 0.877 ± 0.050 | |
| Total | 1.035 ± 0.051 | |
| Shorter duplex of Ad2 and Ad2 ⁺ ND ₁ (segment i) | | 0.134 ± 0.003 |
| Ad2 DNA deleted from Ad2 ⁺ ND ₁ (segment k) | | 0.052 ± 0.002 |

* Measurements are presented as mean fractional length ± twice the standard error of the mean. Forty duplex and 20 single-strand SV40 circular DNA molecules in the same photographs were the length standards for conversion of duplex or single-strand SV40 segments, respectively, to SV40 fractional length. The duplex and the single-strand Ad2 segments were converted to Ad2 fractional length by use of the SV40/Ad2 length ratio (26) and the duplex or single-strand SV40 DNAs, respectively, in these photographs. Twenty-one heteroduplexes of the class shown in Fig. 6B and eight of the class shown in Fig. 6A were measured. Two SV40(L_{S1})-Ad2⁺ND₁-Ad2 heteroduplexes had SV40 segments whose measurements did not fit in either class and were not included in Table 1.

0.40 to 0.60 after HpaII digestion, whereas linear molecules cleaved near 0.5 by S₁ yield one short and one long fragment after HpaII. Since HpaII endonuclease cleavage of SV40(L_{S1}) yields more than twice as many fragments of 0.2 and 0.8 SV40 fractional length as those of the 0.40 to 0.60 length class, we conclude that cleavage of SV40(I) DNA by S₁ nuclease in high salt occurs more frequently within the region 0.45 to 0.55 than 0.15 to 0.25 on the SV40 DNA map.

Nature of the sites on SV40 DNA cleaved by S₁ nuclease. Why does S₁ nuclease, which normally degrades only single-stranded DNA, cleave double-stranded SV40 DNA? The simplest hypothesis is that there are specifically localized, perhaps transient, short interruptions of the DNA base pairing which permit endonuclease cleavage at these locations. A number of observations support this hypothesis.

First, cleavage by S₁ nuclease requires that the SV40 DNA be superhelical. (i) SV40(L_{R1}) DNA is not degraded further by S₁ nuclease although superhelical SV40 DNA, present in the same reaction mixture is converted to linear molecules (Fig. 8A, B). (ii) Covalently circular but nonsuperhelical SV40 DNA, prepared by

resealing SV40(L_{R1}) DNA with DNA ligase at the same ionic strength and temperature used for S₁ nuclease digestion, is also resistant to breakage by S₁ nuclease (Fig. 8C, D). Therefore, a superhelical structure is necessary for cleavage by S₁ nuclease.

Second, the rate of cleavage of SV40(I) DNA by S₁ nuclease decreases more than fivefold when the salt concentration is raised from 10 mM to 250 mM NaCl although there is only a slight decrease in the rate of degradation of single-stranded DNA over this range of ionic strength (32, 35). One would expect the fraction of nonhelical DNA to decrease with increasing ionic strength.

Third, the location of the S₁ nuclease cleavage site in high salt is within the region that was identified by Mulder and Delius (28) as the one most easily denatured at high pH. Moreover, the T4 gene 32 protein, which binds selectively to single-stranded DNA (1), also binds to superhelical and not nonsuperhelical SV40 DNA (11). It is interesting that the specific region to which the gene 32 protein binds is located at 0.46 SV40 fractional length clockwise from the EcoR_I endonuclease cleavage site (27), that is, at or very close to the S₁ nuclease cleavage site preferred in high salt. We find that S₁ nuclease cleaves less than 15% of SV40 DNA molecules to which gene 32 protein has been fixed, whereas more

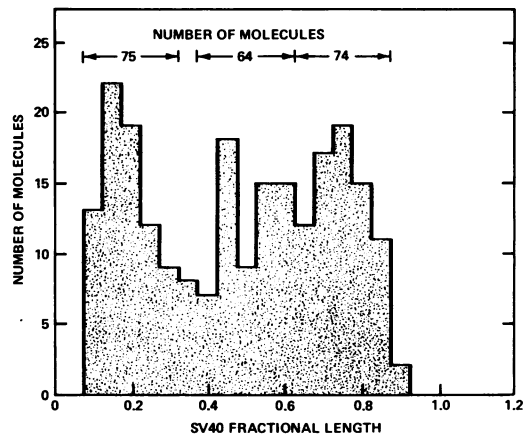


FIG. 7. Histogram of length measurements of SV40(L_{S1}) digested with HpaII endonuclease. SV40(L_{S1}) DNA formed by cleavage in 250 mM NaCl was incubated with HpaII endonuclease and examined by electron microscopy as described in Materials and Methods. Lengths are presented relative to mean length (standard error of the mean was 0.0052 fractional length) of 30 SV40(II) molecules in the same photographs. Molecules not cleaved by HpaII (37% of the total) had a mean length of 0.968 SV40 fractional length (standard error 0.004) and are not shown for the purpose of clarity.

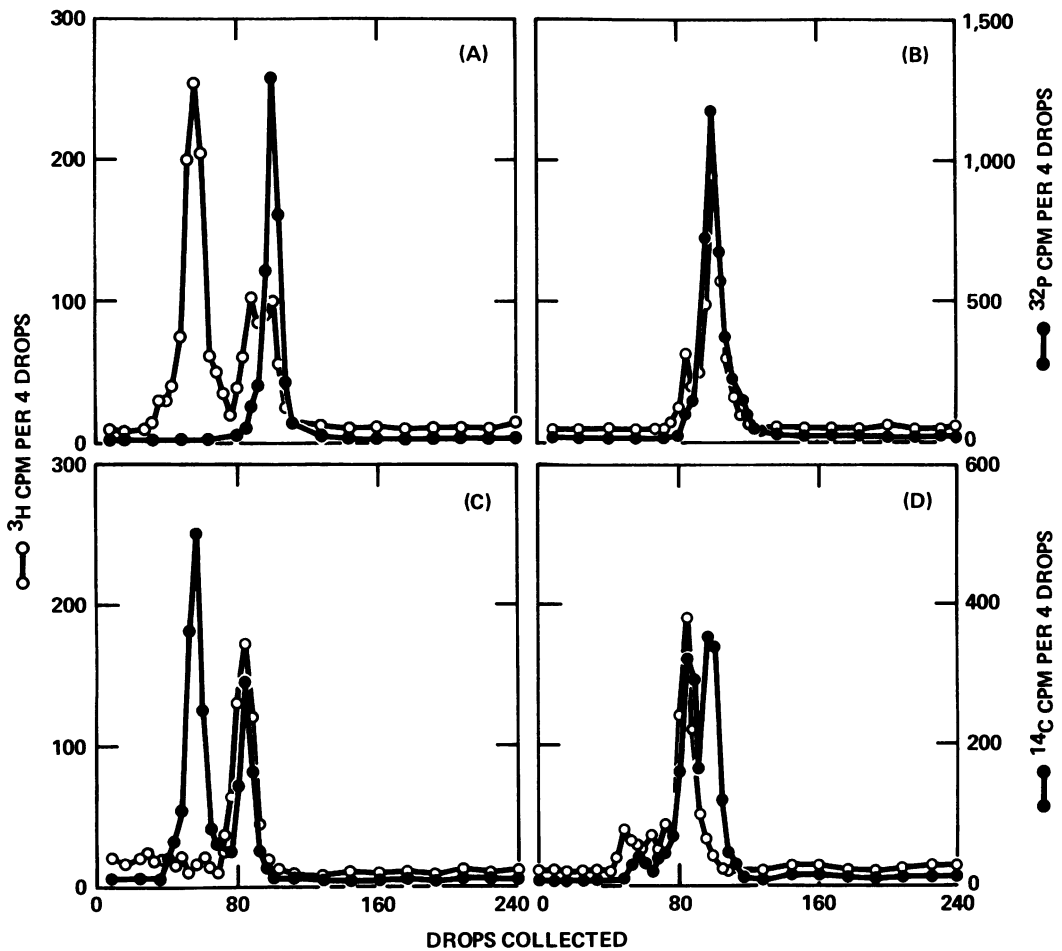


FIG. 8. Neutral sucrose gradient sedimentation analysis of products of treatment of various forms of SV40 DNA with S_1 nuclease. (A) and (B) incubation mixtures contained ^3H -SV40(I) DNA and ^{32}P -SV40(L_{R1}) DNA, (A) without S_1 nuclease, (B) with S_1 nuclease. (C) and (D) incubation mixtures contained ^{14}C -SV40(I) and (II) DNA and ^3H -labeled nonsuperhelical closed circular SV40 DNA, (C) without S_1 nuclease, (D) with S_1 nuclease.

than 80% of supercoiled SV40 DNA, incubated without gene 32 protein but treated with glutaraldehyde, is cleaved normally. The effect of gene 32 protein may be due either to actual blocking of the cleavage site or to the attendant relaxation of the supercoiled conformation when gene 32 protein is bound.

These three lines of evidence suggest that regions of localized denaturation, probably generated by the superhelicity, are the sites within which S_1 nuclease cleaves both strands of the SV40 DNA. When cleavage of both strands occurs at one site, superhelicity is lost and a molecule is generated which, in the absence of single strand nicks, is now immune to further cleavage. Therefore, though there may be more than one potential S_1 nuclease cleavage site,

each molecule is cleaved only once.

S_1 nuclease cleaves other superhelical DNA. S_1 nuclease also cleaves superhelical polyoma DNA to unit length linear molecules. SV40 and polyoma DNA containing deletions of the EcoR_I endonuclease site remain fully susceptible to S_1 nuclease cleavage. The location of the cleavage sites in the defective molecules has not been determined. The covalently circular, superhelical plasmid DNA, $\lambda\text{dv-120}$ (17), is cleaved once by S_1 nuclease to yield a linear molecule of the same length as the circular DNA substrate (Fig. 9). By contrast the EcoR_I restriction endonuclease cleaves $\lambda\text{dv-120}$ DNA into two equal lengths; the $\lambda\text{dv-120}$ molecule is very likely a head to tail dimer containing two EcoR_I sites 180° apart (17).

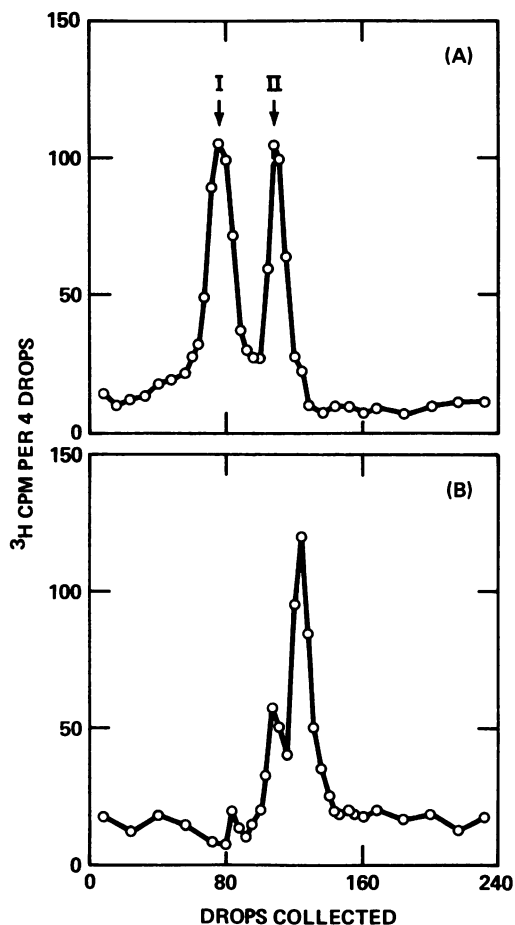


Fig. 9. Cleavage of λ dv120 DNA to complete length linear molecules by S_1 nuclease. A mixture of closed circular (I) and nicked (II) ^3H - λ dv120 dimer DNA was incubated either (A) without S_1 nuclease or (B) with S_1 nuclease under the conditions used for SV40 DNA (75 mM NaCl), then sedimented in a neutral sucrose gradient for 150 min at 55,000 rpm, 4 C in Beckman SW56 rotor.

DISCUSSION

Our experiments show that circular covalently closed, superhelical SV40 DNA can be converted to a linear duplex of equal length by the action of the single strand-specific nuclease, S_1 . We have attempted, in this work, to clarify two aspects of the reaction: the location on the SV40 DNA at which the double strand cleavage occurs and the unexpected susceptibility of the duplex DNA to a single strand-specific endonucleolytic attack.

Unlike the restriction endonucleases, S_1 nuclease probably does not cleave SV40 DNA at a specific nucleotide sequence. Most significant

in this regard is the finding that S_1 nuclease does not appreciably cleave covalently circular SV40 DNA lacking superhelical turns (because covalent closure of nicked SV40 DNA with ligase occurred at the ionic strength used for S_1 nuclease action) (38). Sensitivity of native SV40 DNA to S_1 nuclease appears, therefore, to be dependent upon a superhelical conformation. This explains why unit length linear molecules, generated by S_1 nuclease or by EcoR_I restriction endonuclease, which still contain the regions within which S_1 cleaves supercoils, are relatively resistant to further double-strand cleavages or even nicking of single strands. (Our finding that Ando's and our own purified S_1 nuclease preparations do not nick linear duplex DNA agrees with Vogt's observations on λ phage DNA [35] but disagrees with Godson's report of extensive nicking of T7 DNA [14].)

S_1 nuclease probably does not make simultaneous cleavages in opposite strands of the SV40 DNA in generating the linear product. More likely, a singly nicked, circular molecule, SV40(II) DNA, is a transitory intermediate which is subsequently cleaved on the opposite strand to produce the linear structure. If this model is correct superhelicity is necessary for S_1 nuclease to introduce the first nick; the second break, on the opposite strand, at or near the nick, is, therefore, not dependent on superhelicity. Other single strand-specific endonucleases are also capable of cleaving circular, superhelical DNA. Kato et al. (19) and Bartok (private communication) have found that *Neurospora* endonuclease (23) converts the supercoiled but not relaxed form of ϕ X-RF-I DNA to the nicked circular structure, ϕ X-RF-II. Mung bean nuclease (18) also converts SV40(I) DNA to the nicked circular but not linear structure (unpublished result). Kato et al. (19) reported that only after the denatured region(s) is fixed by reaction with formaldehyde can *Neurospora* endonuclease convert the circular molecule to its linear derivative. These findings indicate that S_1 nuclease may be unique amongst these single strand-specific nucleases in being able to convert the nicked form to the linear structure although the basis for this property is not clear.

S_1 nuclease can convert other supercoiled DNA to a unit length linear form. Defective SV40 DNA (supercoiled molecules lacking the EcoR_I cleavage site), polyoma DNA (both normal and defective molecules) and covalently circular, superhelical λ dv120 DNA are readily converted to linear duplexes of equivalent length. In the latter instance, the number of cleavages is limited to one per supercoiled molecule even though λ dv120 DNA is a head to

tail dimer; by contrast, the EcoR_I restriction endonuclease cleaves λdv120 DNA at each of the two paired restriction sites per supercoiled molecule (17).

In seeking an explanation for the sensitivity of supercoiled DNA molecules to S₁ nuclease we were aware, of course, that another single strand-specific reagent, the T4 gene 32 protein, binds to supercoiled but not nicked SV40 DNA (11). Significantly, the gene 32 protein binds to and can be fixed to a specific region located at 0.46 on the SV40 DNA map (27). Dean and Lebowitz (10) had already observed that a significant number of bases in supercoiled φX-RF and PM2 DNA, but not in their nicked forms, behave as if they are unpaired because they are readily hydroxymethylated by formaldehyde. Subsequently, using methyl mercury binding as a probe of unpaired bases, Beerman and Lebowitz (*J. Mol. Biol.*, in press) estimated that 3 to 4% of the bases in supercoiled PM2 DNA (about 300 to 400 base pairs) are unpaired as judged by their ability to bind methyl mercury. This conclusion probably explains the sensitivity of φX-RF I to the single strand-specific endonuclease of *N. crassa* (19). Thus, both chemical and enzymatic probes of single strandedness detect non-base-paired regions in native, circular, superhelical DNA.

Why do circular, superhelical DNA behave as if they contain unpaired bases? Possibly superhelicity alters the structure or dimensional parameters of the helix, thereby causing the bases to behave partially or completely unpaired (36). Alternatively, superhelicity promotes or stabilizes cloverleaf-like foldings with single-stranded loops of the type postulated by Sobell (31). Conceivably, certain regions may undergo localized denaturation as a result of the increased free energy of negative superhelicity. Bauer and Vinograd (3, 4) have estimated that SV40 DNA with 12 to 15 negative superhelical turns has a positive free energy of about 84 kcal/mol relative to the nicked form. If we assume that this estimate, made under conditions different from those used in the S₁ nuclease reaction, is appropriate under these conditions, and that 10 to 15 kcal are required to denature ten base pairs (21, 30), then superhelicity can supply enough free energy to denature several turns of DNA duplex. Whether part or all of the denaturation required for S₁ nuclease or gene 32 protein action preexist or is facilitated by binding of the proteins can presently only be conjectured.

Unwinding of helical base pairs due to superhelicity would be expected to occur in regions that are most readily denatured by other physi-

cal or chemical perturbations. Of interest, therefore, is the finding that in SV40 DNA the gene 32 protein binding site and one of the S₁ nuclease cleavage zones are virtually coincident; moreover, both lie within the region which is most readily denaturable at elevated pH (28). Whether this ready denaturability results from a high AT content or some other structural feature remains to be determined. The lack of binding of gene 32 protein and S₁ nuclease insusceptibility at high salt in the region of 0.15 to 0.25 on the SV40 map could be due to more limited denaturation in that region.

Finally, there is the question of relevance. Do these localized regions of denaturation have a specialized physiologic function? The most readily denatured region of SV40 DNA does not coincide with the replication origin at 0.67 on the map (7, 13) although termination of replication occurs near the alternate S₁ nuclease site. Until the promoter regions for transcription of SV40 DNA have been mapped, one can only speculate on the role of unpaired regions in this process. Of interest, however, is the identification of 0.17 map unit as the origin of transcription in vitro by *E. coli* RNA polymerase (39).

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