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Cleavage of Simian Virus 40 DNA at a Unique Site by a Bacterial Restriction Enzyme

(DNA mapping/adenovirus–SV40 hybrid/T4 gene 32 protein/electron microscopy/double-strand cleavage)

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ABSTRACT **The R_I restriction endonuclease of *Escherichia coli* converts covalently-closed circular Simian Virus 40 (SV40) DNA to unit-length linear duplex molecules. Cleavage occurs at a unique site, since denaturation and renaturation of these linear molecules yield linear but no circular molecules. The distance from the cleavage site to the SV40 DNA sequence contained in the adenovirus–SV40 hybrid, Ad2$^+$ND$_1$, is 0.11 of the length of SV40 DNA. T4 gene 32 protein binds to SV40 DNA in a region 0.45 of the length of SV40 DNA from the R_I cleavage site. *E. coli* B restriction endonuclease can cleave SV40 DNA at several sites.**

SV40 DNA is a covalently-closed circular duplex DNA molecule [SV40(I)] (1). The observations that SV40(I) DNA is opened by treatment with *Escherichia coli* B or P1 phage restriction endonuclease (2), and particularly that *Hemophilus influenzae* restriction endonuclease (3) cleaves SV40 DNA into 11 specific, separable fragments, provide several new opportunities: analysis of nucleotide sequences of SV40 DNA, localization of segments that serve as template for the different RNA transcripts, and mapping of genetic and physical features of the SV40 genome. We wished to cleave both strands of SV40 DNA at only one site so as to create a linear molecule with defined ends. This was accomplished with the R_I restriction endonuclease isolated from *E. coli* carrying the drug-resistance transfer factor, RTF-1 (4). We characterized the product of this cleavage as a nonpermuted, full-length linear SV40 DNA molecule [SV40(L_{RI})]. With this product, we determined where the *E. coli* B restriction endonuclease (5, 6) cleaves SV40 DNA, where the T4 gene 32 protein binds to SV40 DNA (7), and what portion of the SV40 DNA sequence is in the adenovirus–SV40 hybrid, Ad2$^+$ND$_1$ (8). An earlier paper (9) showed how SV40(L_{RI}) was used to construct a circular SV40 DNA molecule containing several λ phage genes and the galactose operon of *E. coli*. Mertz and Davis (10) characterize the unusual termini produced by the endonuclease cleavage.

MATERIALS AND METHODS

Cell Lines. CV-1P cells obtained from S. Kit and MA-134 cells from J. S. Pagano are established lines of African green monkey kidney cells. KB-3, a human cell line, was obtained from M. Green. All were grown on plastic plates (Nunclon) in Eagle's medium as modified by Dulbecco (Gibco) with 10% calf serum (Microbiological Associates) and 100 μg/ml streptomycin sulfate (Pfizer) in a CO_2 incubator.

Abbreviations: SV40, Simian Virus 40; Ad2, adenovirus 2; PFU, plaque-forming units.

Viruses. Adenovirus 2 (Ad2) obtained from F. Rapp and Ad2$^+$ND$_1$ from A. M. Lewis, Jr. were grown on KB-3 cells at high multiplicity. SV40 strain Rh911, from J. Vinograd, was serially plaque-purified four times on CV-1P cells and used for two serial infections of 72 hr each at 0.05 plaque-forming units (PFU) per cell. Infected cells were sonicated, extracted at 0° with 0.1 volume chloroform, and centrifuged. Chloroform does not inactivate SV40 and prevents subsequent inactivation in such a suspension. After aeration of the surface to remove chloroform, the 12,000× *g* supernatant was stored at −20° and used for subsequent infections.

Viral DNAs. Ad2 and Ad2$^+$ND$_1$ were purified (11), and their DNAs were extracted (12).

MA-134 cells were infected with SV40 (0.05 PFU per cell) and labeled with [^{3}H]thymidine (20 Ci/mmol, 25 μCi/100-mm plate) at 6 days and again at 8 days after infection. After 9 days cells were lysed (13), and the DNA in the supernatant fluid was extracted with phenol and centrifuged to equilibrium in CsCl–ethidium bromide (14). After removal of ethidium bromide (14) and incubation at pH 11 for 48 hr at 25° to degrade remaining RNA, SV40(I) DNA was purified by neutral sucrose gradient sedimentation.

Enzymes. R_I restriction endonuclease, purified 50-fold by DEAE-cellulose, phosphocellulose, and Sephadex chromatography, was the generous gift of H. W. Boyer and R. Yoshimori. *E. coli* B restriction endonuclease, DEAE fraction (15), was kindly provided by S. H. Linn, and T4 gene 32 protein (DEAE and hydroxylapatite fractions) was from B. Alberts by way of W. M. Huang. Electrophoretically purified DNase I was purchased from Worthington Biochemical Corp.

Enzyme Incubations. SV40(I) DNA (30 μg/ml) was incubated with R_I endonuclease (6000 units/ml) in 0.1 M Tris·HCl (pH 7.5)–10 mM $MgCl_2$ at 37° for 30 min. One unit of R_I endonuclease degrades half the λ DNA (0.2 μg/0.1 ml) to a form sedimenting at a slower rate in a neutral sucrose gradient (4). SV40 DNA and λ DNA are cleaved at about equal rates. DNase I (67 pg/ml) was incubated with SV40(I) DNA (1.1 μg/ml) at 28° for 5 min in 10 mM Tris·HCl (pH 7.4), 1 mM $MnCl_2$, and 40 μg/ml of bovine-serum albumin. This treatment causes double-strand scissions of SV40(I) DNA (D. Jackson and P. Berg, to be published). B restriction endonuclease was incubated in 75 mM Tris·HCl (pH 8.1), 7.8 mM $MgCl_2$, 1 mM 2-mercaptoethanol, 1.5 mM ATP, and 11 μM *S*-adenosylmethionine for 15 min at 37°. All endonuclease reactions were stopped by addition of EDTA to 20 mM and cooling to 0°. Sarkosyl was also added to B endonuclease

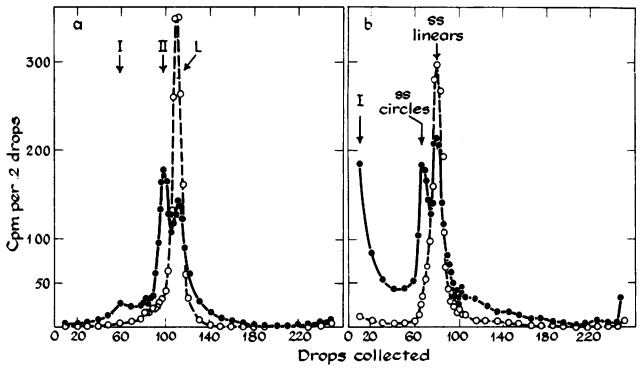

FIG. 1. Sedimentation analysis of R_I endonuclease cleavage of [^{3}H]SV40(I)DNA. (*a*) Neutral sucrose gradient, 4 hr: a mixture of ^{32}P-labeled SV40(I), SV40(II), and SV40(L) produced by DNase I served as markers (*I*, *II*, and *L*, respectively). (*b*) Alkaline sucrose gradient, 6.5 hr: ^{32}P-labeled SV40(I) (at bottom of gradient) and SV40(II) are markers. Alkaline denaturation of SV40(II) produces single-stranded circular DNA and single-stranded linear DNA (indicated as *ss circles* and *ss linears*). ●, ^{32}P; ○, ^{3}H.

incubation mixtures to 1% final concentration before sedimentation. Complexes of SV40(I) DNA with T4 gene 32 protein were prepared (7).

Sedimentation. DNA samples (0.1 ml) were layered on linear 5–20% sucrose gradients in 4.2-ml polyallomer tubes and sedimented at 4° in a Beckman SW56 rotor at 55,000 rpm for the indicated times. Neutral gradients contained 10 mM NaCl–1 mM EDTA–10 mM Tris·HCl (pH 7.4). Alkaline gradients contained 5 mM EDTA, 0.2–0.8 N NaOH (proportional to sucrose concentration), 0.8–0.2 M NaCl (to make the Na^+ concentration up to 1 M). Drops from the bottom of the tube were collected on Whatman 3 mm paper discs, dried and counted in toluene-based scintillation fluid in a scintillation spectrometer.

DNA Denaturation and Renaturation. Alkaline denaturation and renaturation in 48% formamide were done (16). A mixture of 1.5 μg/ml Ad2$^+$ND$_1$, 1.5 μg/ml Ad2, and 0.03 μg/ml SV40(L_{RI}) DNAs was annealed for 24 hr to permit only 50% renaturation of SV40(L_{RI}) DNA and to minimize renaturation of free SV40 single strands with SV40(L_{RI}) strands already annealed to Ad2$^+$ND$_1$ DNA.

Electron Microscopy. Samples were mounted for electron microscopy by the formamide technique (16). Shadowed grids were examined and photographed with a Philips EM 300. Measurements were made with a Keuffel and Esser map measure or, in the adenovirus experiment, with a Hewlett–Packard 9864A Digitizer and 9810A Calculator with a fully smoothed length calculation program giving an accuracy of ±0.5% and a greater degree of reproducibility on sample figures of known length. Absolute length calibrations were made with a diffraction grating replica.

RESULTS

R_I Restriction Endonuclease Cleaves SV40(I) DNA to Unit Length Linear Molecules. Covalently-closed SV40(I) DNA, after incubation with R_I restriction endonuclease, sediments more slowly than does the circular nicked DNA, SV40(II), but cosediments with linear SV40 DNA molecules, SV40(L), in a neutral sucrose gradient (Fig. 1*a*). In an alkaline sucrose gradient, the R_I endonuclease product sediments as a quite homogeneous component (very little trailing material) somewhat slower than single-stranded circles and in the position expected for linear single-stranded DNA the same length as SV40 DNA (Fig. 1*b*). Prolonged incubation or addition of more R_I endonuclease produces no substantial change in the sedimentation profiles. These two findings suggested that R_I endonuclease cleaves each strand of SV40 DNA once, causing the molecule to become linear.

DNA molecules produced by R_I endonuclease action are almost exclusively linear when spread by the formamide technique (16) and viewed in an electron microscope (Fig. 2*A*) (only 2–3% of the molecules remained supercoiled circles*). Comparative length measurements of these molecules with SV40(II) DNA on the same grid established that the linear products have the same contour length as the circular viral DNA (Fig. 3).

R_I Cleavage Occurs at Only One Site on the SV40 DNA Molecule. If the circular SV40 DNA molecules were being cleaved by double-strand scissions at either of two or more separated sites, the linear products would have circularly permuted nucleotide sequences. Such circularly permuted linear DNAs, after denaturation and renaturation, should yield circular molecules by virtue of complementary ends generated by random reassociation of the permuted strands (18). On the other hand, a unique cleavage point yields linear molecules with identical sequences that should not produce circular molecules after strand separation and reassociation. By electron microscopic examination of 500 double-stranded molecules recovered after denaturation and renaturation of SV40(L_{RI}), only full-length linear molecules were seen. As predicted, SV40 linear DNA molecules, produced by exposure to DNase I with Mn^{2+} (D. A. Jackson and P. Berg), yielded circular molecules (40% of the total) after denaturation and renaturation. We conclude from these, as well as from the following experiments, that R_I endonuclease cleavage occurs at only one site on the SV40 DNA molecule.

Specific Sites or Segments in SV40 DNA Can Be Located Relative to the R_I Endonuclease Cleavage Site. Delius *et al.* (7) showed that incubation of SV40(I) DNA with T4 gene 32 protein (19) under defined conditions followed by fixation with glutaraldehyde yields circular structures with one small "denaturation loop" per molecule (Fig. 2*B* and *C*); molecules with two "denaturation loops" are seen at a low frequency (2%). The basis for the preferential denaturation at one location is not yet established. Is this "denaturation loop" at a unique location on the SV40 DNA? Cleavage of the SV40 DNA–gene 32 protein complex with R_I endonuclease produces linear molecules containing the denaturation loop (Fig. 2*D* and *E*); measurement of the DNA lengths from the midpoint of the denaturation loop to the ends of each molecule shows that in 90% of 106 molecules measured the midpoint is

* This result was observed with DNA obtained after infection with plaque-purified virus. The R_I-resistant supercoiled DNA molecules are at least 10-fold less infectious than SV40(I) and have a shorter mean length (1.54 ± 0.18 μm SD) than SV40(L_{RI}) (1.82 ± 0.05 μm SD). R_I-resistant molecules with similar properties are 25% of the SV40(I) DNA recovered after infection with SV40 virus propagated through repeated cycles at high multiplicity (J. Mertz and P. Berg, unpublished observations).

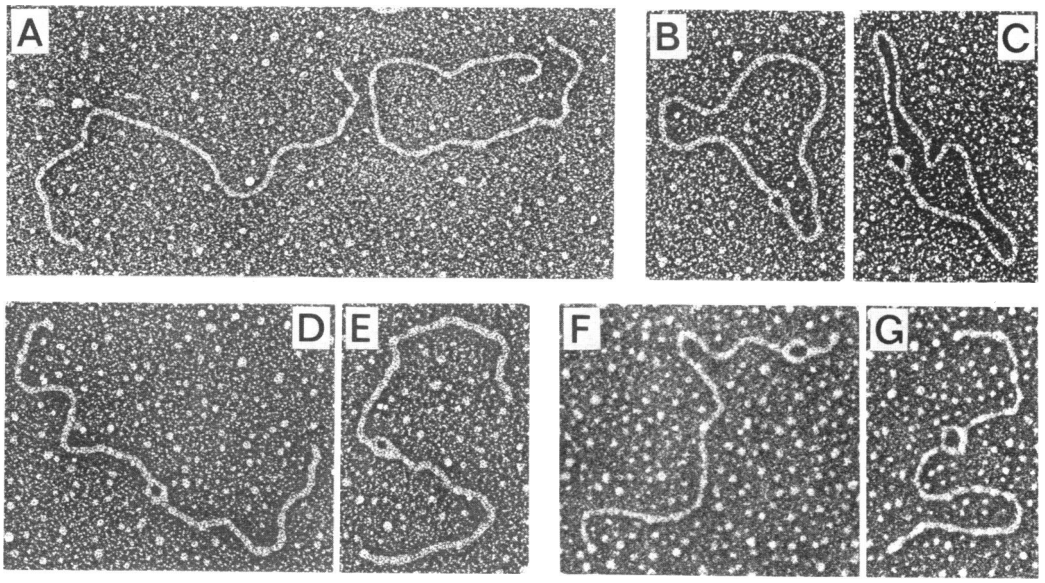

FIG. 2. Electron micrographs of SV40 DNA treated in various ways. (*A*) SV40(L_{RI}); (*B, C*) SV40(I) complexed to T4 gene 32 protein; (*D, E*) SV40(I)–T4 gene 32 protein complexes treated with R_I endonuclease; (*F, G*) SV40(I)–T4 gene 32 protein complexes treated with DNase I. Magnification 45,000 ×.

0.45 fractional SV40 length from one end (Fig. 4*a*). The denaturation loop occupies 5% of the total length. When the SV40 DNA–gene 32 protein complex is cleaved with DNase I (in the presence of Mn^{2+}), the denaturation loop does not appear at a preferred location (Fig. 4*b*) relative to the ends of the molecules of unit length that were examined (Fig. 2*F* and *G*).

$Ad2^+ND_1$ is a nondefective adenovirus–SV40 hybrid that contains a small segment of SV40 DNA covalently inserted into the adenovirus 2 (Ad2) DNA (8). This portion of the SV40 genome is presumably responsible for the ability of $Ad2^+ND_1$ to multiply in African green monkey kidney cells (a nonpermissive host for Ad2) and to induce SV40 U antigen in infected cells (20). Electron microscopic examination of heteroduplexes formed from Ad2 and $Ad2^+ND_1$ DNAs indicated that 5.4% of the Ad2 DNA sequence is deleted in $Ad2^+ND_1$ and that DNA equivalent in length to 17.8% of the SV40 genome is inserted at the deletion site (T. J. Kelly, Jr. and A. M. Lewis, Jr., in preparation). We have tried to fix the location of the SV40 sequence contained in $Ad2^+ND_1$ along the length of SV40(L_{RI}) in order to map that segment relative to the R_I endonuclease cleavage point.

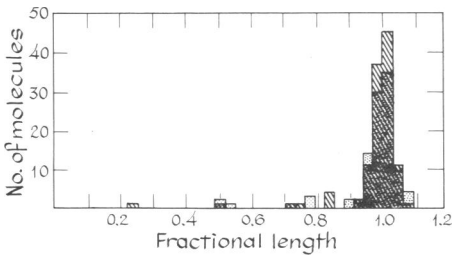

FIG. 3. Histograms of length measurements of SV40(II) (*dotted*, 103 molecules) and SV40(L_{RI})(*diagonal stripe*, 115 molecules) mounted on the same grid. SV40(II) lengths greater than 0.85 fractional length were (mean ±SD) 1.84 (defined as 1.00 fractional length) ±0.06 μm. SV40(L_{RI}) lengths greater than 0.85 fractional length were 1.85 ± 0.05 μm.

Heteroduplexes formed from Ad2 and $Ad2^+ND_1$ DNAs contain duplex regions corresponding to their common sequences and single-stranded segments corresponding to those sequences that are not shared (a typical view of an entire molecule is seen in Fig. 5*A*, and a higher magnification of the unpaired segments is in Fig. 5*B*). About half of renatured DNA molecules of adenovirus DNA length (13.0 μm) have a substitution loop 1.77 μm (0.136 fractional Ad2 DNA length) from one end. We consider these molecules to be the predicted Ad2–$Ad2^+ND_1$ heteroduplexes because they form a homogeneous population (shown by length measurements), occur at the expected frequency, and contain a single-stranded

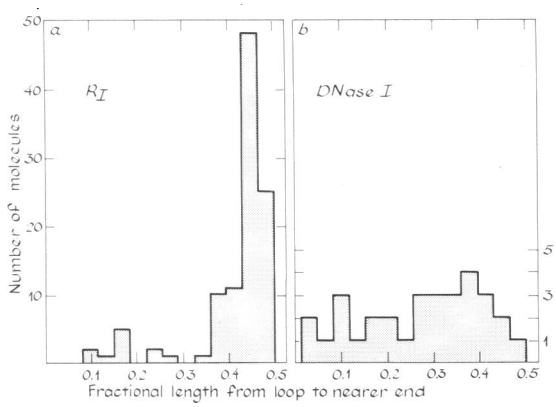

FIG. 4. Endonuclease cleavage of SV40(I) DNA–T4 gene-32 protein complexes. Distance from the midpoint of the "denaturation loop" to the nearer of the two ends is presented as a fraction of the total length of the complex. (*a*) R_I-cleaved complexes. Fractional lengths between 0.3 and 0.5 = 0.446 ± 0.028 (mean ± SD). Total lengths of 106 complexes = 1.85 ± 0.10 μm (mean ± SD). SV40(II) molecules on the same grid = 1.84 ± 0.11 μm. (*b*) DNase I-cleaved complexes. Fractional lengths presented (31 complexes) = 0.270 ± 0.131. Total lengths of complexes = 1.77 ± 0.07 μm. SV40(II) molecules on the same grid = 1.81 ± 0.08 μm.

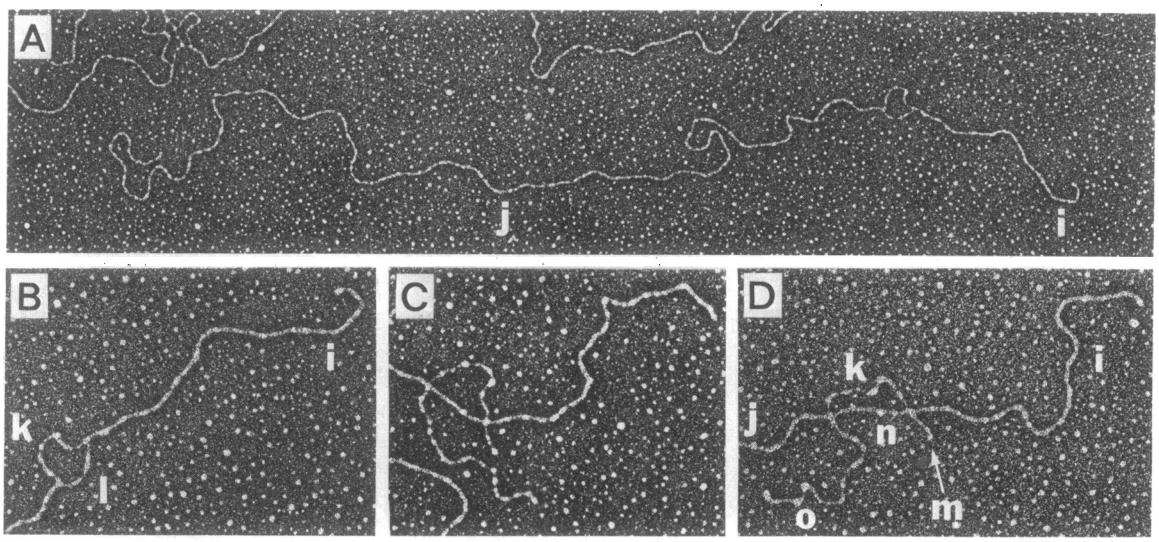

FIG. 5. Electron micrographs of Ad2$^+$ND$_1$ heteroduplexes. (*A*) Heteroduplex of Ad2$^+$ND$_1$ and Ad2 DNAs. *Segments i* and *j:* duplexes formed from Ad2$^+$ND$_1$ and Ad2 DNAs. Magnification 15,300×. (*B*) Enlarged view of the substitution loop in the same Ad2$^+$ND$_1$–Ad2 heteroduplex. *Segment k:* single strand from Ad2 DNA; segment *l:* single strand containing SV40 sequences in Ad2$^+$ND$_1$ DNA. (*C, D*) Heteroduplexes of Ad2, Ad2$^+$ND$_1$, and SV40(L$_{RI}$) DNAs. *Segments i* and *j:* duplexes formed from Ad2$^+$ND$_1$ and Ad2 DNAs; *k:* single strand from Ad2 DNA; *n:* duplex formed from Ad2$^+$ND$_1$ and SV40(L$_{RI}$) DNAs; *m* and *o:* single strands from SV40(L$_{RI}$) DNA. Magnification in *B, C,* and *D* is 26,600×.

segment about equal to the length expected of the SV40 sequence in Ad2$^+$ND$_1$ (8).

7% of Ad2$^+$ND$_1$–Ad2 heteroduplexes (68 of 915 molecules) were annealed to an SV40(L$_{RI}$) strand (Fig. 5*C* and *D;* Table 1). In these the smaller of the two previously single-stranded loops appears double-stranded (*segment n*) and emanating from the two branches are single-stranded tails (*segments m* and *o*). We believe these are Ad2–Ad2$^+$ND$_1$ heteroduplexes containing an SV40(L$_{RI}$) DNA strand paired with the SV40 sequence in Ad2$^+$ND$_1$ because: (*i*) the length of the two Ad2 duplex segments emanating from the two branch points (*segment i* = 0.138; *segment j* = 0.799 Ad2 DNA length) plus the length of the single-strand DNA connecting the two branch points (*segment k* = 0.055 Ad2 DNA length) equals one complete Ad2 DNA length as it does for the Ad2–Ad2$^+$ ND$_1$ heteroduplex (Fig. 5*D;* Table 1); (*ii*) the length measurements of single-strand *segments m* and *o* are 0.110 and 0.732

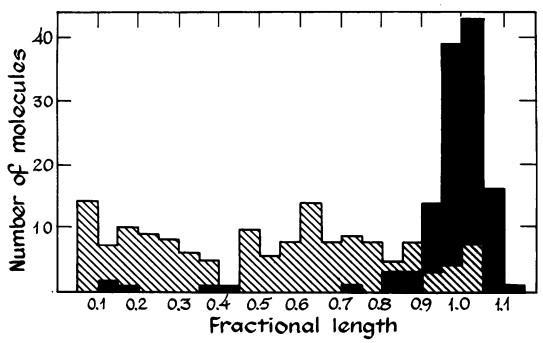

FIG. 6. Histogram of length measurements of SV40(L$_B$) and SV40(L$_B$) digested with R$_I$ endonuclease. SV40(L$_B$) DNA (*black histogram*) was prepared and mounted for electron microscopy as in *Methods*. This material was digested with R$_I$ endonuclease to yield fragments whose lengths are shown in the *diagonally striped histogram*. All lengths are fractional length relative to SV40(II) on the same grids.

SV40 DNA length, respectively, and with the 0.172 length of the paired *segment n* they comprise one SV40 DNA length. From this experiment we conclude that the SV40 sequence inserted into Ad2$^+$ ND$_1$ begins 0.11 SV40 DNA length from one of the ends produced by R$_I$ endonuclease cleavage and terminates 0.73 SV40 DNA length from the other end.

With 0.05 μg of SV40(I) and a 3-fold excess of B restriction endonuclease over the amount required to destroy 80% of the supercoiled molecules, the mean fractional length of 117 linear duplex molecules was 0.511 ± 0.340 (SD). Under these conditions, then, there are an average of two double-strand hits per SV40 DNA molecule. *S*-adenosylmethionine markedly stimulates endonuclease activity, since only one-fiftieth as much cleavage occurs in its absence.

With a lower amount of enzyme, just sufficient to eliminate 90% of SV40(I), we could recover, after sucrose gradient centrifugation, full length DNA (Fig. 6). Treatment of these SV40(L$_B$) molecules with R$_I$ endonuclease generated a polydisperse collection of molecular lengths (Fig. 6). If the B enzyme had cleaved preferentially at a single site, at most two-length classes should predominate in the products of digestion by both enzymes. Instead, representatives of six or more length classes are found in roughly equal proportions, suggesting that the B endonuclease can cleave at any of several sites.

DISCUSSION

Four lines of evidence show that R$_I$ restriction endonuclease cleaves SV40(I) DNA at a unique site: (*i*) an R$_I$ endonuclease limit digest of DNA from plaque-purified SV40 contains virtually only unit-length duplex molecules with no internal nicks; (*ii*) denaturation and renaturation of these linear molecules yield linear but no circular molecules; (*iii*) the distance from the R$_I$ cleavage site to the region of SV40 DNA denatured by T4 gene 32 protein is the same in 90% of the molecules examined; (*iv*) the distance from the R$_I$ cleavage

TABLE 1. *Molecular lengths of various segments of heteroduplexes of Ad2, Ad2+ND1, and SV40 DNAs*

DNA segment	Ad2–Ad2+ND1 heteroduplexes	Ad2–Ad2+ND1–SV40(L_{RI}) heteroduplexes
Longer duplex of Ad2 and Ad2+ND1 (*segment j*)*	0.809 ± 0.007	0.799 ± 0.009
Shorter duplex of Ad2 and Ad2+ND1 (*segment i*)*	0.136 ± 0.002	0.138 ± 0.001
Ad2 DNA deleted from Ad2+ND1 (*segment k*)*	0.055 ± 0.001†	0.056 ± 0.001†
Total*	1.000 ± 0.010	0.993 ± 0.011
SV40 sequences in Ad2+ND1 (*segment l, n*)‡	0.167 ± 0.006†	0.172 ± 0.002
Shorter single strand from SV40(L_{RI}) (segment *m*)‡	—	0.110 ± 0.003†
Longer single strand from SV40(L_{RI}) (*segment o*)‡	—	0.732 ± 0.014†
Total‡	—	1.014 ± 0.019

Measurements are presented as mean fractional length ± standard error of the mean, relative to 86 SV40(L_{RI}) molecules or 79 Ad2 DNA molecules in the same photographs with the 40 Ad2–Ad2+ND1 heteroduplexes and 68 Ad2–Ad2+ND1–SV40 (L_{RI}) heteroduplexes measured. The length of SV40(L_{RI}) DNA is 0.137 ± 0.001 relative to Ad2 DNA. For DNA segments, see Fig. 5.

* These lengths are expressed as fractional length of Ad2 DNA.

† Contour lengths of single-stranded DNA segments in the heteroduplexes converted to double-stranded DNA equivalent length as follows: (*i*) the measured length of single-stranded SV40 DNA was divided by 0.759, the ratio found for the lengths of single- and double-stranded SV40 DNA on the same grid (SEM 0.001; 79 molecules of each); (*ii*) the measured length of single-stranded adenovirus DNA was divided by 0.724, the ratio of lengths found for circular single-stranded adenovirus DNA (17) to the length of an Ad2 or Ad2+ND1 homoduplex in the same frame (SEM 0.006; 43 determinations). The difference between these ratios is not accounted for by the short duplex segment in single-stranded circular adenovirus DNA (17).

‡ These lengths are expressed as fractional length of SV40 DNA.

site to the SV40 DNA sequence in Ad2+ND1 is constant in the heteroduplexes examined. The SV40(L_{RI}) has already proved to be valuable for synthesizing covalently-closed, circular molecular hybrids in which different DNA segments have bridged the ends of an SV40(L_{RI}) molecule (9).

The formation of triple heteroduplexes between SV40(L_{RI}), Ad2, and Ad2+ND1 DNAs has made it possible to fix uniquely the location of the SV40 DNA sequence carried in the Ad2+-ND1 hybrid relative to the R_I endonuclease cleavage site. That sequence, equivalent to 0.172 SV40 DNA length, which causes the induction of SV40 U antigen (20), begins 0.11 SV40 DNA length from one of the ends produced by R_I endonuclease.

The experiments with heteroduplexes of SV40(L_{RI}), Ad2+ND1, and Ad2 DNAs confirm that the shorter single-stranded segment of Ad2+ND1–Ad2 heteroduplexes is the SV40 DNA segment contained in Ad2+ND1 (T. J. Kelly, Jr. and A. M. Lewis, Jr., in preparation). Moreover, the electron micrographs show that this DNA segment contains only SV40 sequences with no internal deletions or other aberrations relative to the SV40 chromosome.

We have examined heteroduplexes of SV40(L_{RI}) with Ad2 and Ad2+ND4 DNAs. The SV40 DNA sequence contained in Ad2+ND4 includes that of Ad2+ND1 and appears to include all the sequences expressed early in SV40 lytic infection (A. S. Levine, M. J. Levin, M. N. Oxman, and A. M. Lewis, Jr., in preparation). Kelly and Lewis (in preparation) have found that, as in Ad2+ND1, the SV40 DNA sequence of Ad2+ND4 begins 0.138 adenovirus fractional length units from one end of the adenovirus DNA molecule, but the SV40 DNA sequence in Ad2+ND4 is 0.26 SV40 fractional length units longer than in Ad2+ND1. In experiments done collaboratively with Kelly and Lewis (to be published), we find that the R_I cleavage site is also outside the SV40 DNA sequence of Ad2+ND4. Taken together these observations suggest that the R_I cleavage site is in the late gene region of SV40 DNA.

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