Location of S1 Nuclease-Cleavage Sites on Circular, Superhelical DNAs between Polyoma Virus and Simian Virus 40

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Physical maps of the S1 nuclease-cleavage sites on double-stranded covalently closed, circular, superhelical DNAs of polyoma virus and simian virus 40 (SV 40) were constructed by the following technique: Polyoma and SV 40 DNAs were digested under defined conditions (30 mM sodium acetate buffer (pH 5.0), 60 mM NaCl at 37°C) with S1 nuclease in the presence of Mn^{2+} to generate unit length linear duplex molecules. Then, the linear DNAs were digested with *Eco* RI and *Bam* NI restriction endonucleases and size of the resulting DNA fragments were analyzed by agarose gel electrophoresis. Cleavage of those DNAs with S1 is more specific in the presence of Mn^{2+} than Zn^{2+} since discrete DNA bands can be obtained after electrophoresis in agarose gels. With Zn^{2+} a well known activator of the enzyme, S1 cleaves these DNAs within certain regions rather than at specific sites. With Mn^{2+} as activator S1 cleaves polyoma DNA at seven sites: map coordinate 0.11, 0.21, 0.30, 0.44, 0.56, 0.82 and 0.92 (fractional DNA lengths clockwise from the single *Eco* RI cleavage site (zero position)). SV 40 DNA is cleaved by S1 under these conditions at map coordinate 0.15, 0.28, 0.38, 0.39, 0.44, 0.56, 0.89 and 0.98 (also defined as fractional DNA lengths clockwise from SV 40's single *Eco* RI cleavage site (zero position)).

Map position 0.21 on polyoma DNA and 0.15 on SV 40 DNA are at or close to the termination site for DNA replication and the 3' ends of early and late mRNAs. Map positions 0.82, 0.92 and 0.11 on polyoma DNA and 0.56, 0.44 and 0.28 on SV 40 DNA occur at about 0.2, 0.4 and 0.8 respectively from the origin of DNA replication in the early region. The DNA segments between map positions 0.44 and 0.56 on polyoma and 0.89 and 0.98 on SV 40 contain the sequence coding for the COOH-terminus of VP2~VP3 and the NH₄-terminus of VP1.

Polyoma virus and SV 40 have almost identical molecular biologies and genome organization,^{3~6)} but have only limited sequence homology.^{7,8)} The genomes of both viruses are double-stranded covalently closed, circular DNAs with molecular weights of about 3.4×10^6 daltons (about 5300 nucleotide pairs).^{9,10)} A deficiency of helical turns causes the whole molecule to twist into a compact supercoil. These twisted DNAs contain approximately 24~26 negative superhelical turns,^{11,12)} thereby the introducing localized unwinding of helical base pairs into the molecule.

S1, a single-strand-specific endonuclease from *Aspergillus oryzae*,^{1,13)} can cleave, presumably at unpaired or weakly hydrogen-bonded regions,^{2,14,15)} both strands of superhelical DNA to generate unit length linear

duplex molecules with intact single strands. This reaction proceeds by two steps: first, cleavage occurs in either one of the two strands and then, the nicked, circular DNA is cleaved on the opposite strand at or near the nicks to yield a linear molecule.

To compare tertiary DNA structure between both viruses, an attempt has been made to map the S1-cleavage sites on the genomes. Beard *et al.* found using electronmicroscopic analysis that SV 40 DNA is cleaved once, most often within either one of the two regions, 0.15 to 0.25 and 0.45 to 0.55 fractional lengths clockwise from the *Eco*RI endonuclease-cleavage site.²⁾ Germond *et al.*, reported that polyoma DNA was also cleaved to linear duplexes by S1 nuclease, but the exact cleavage sites are still unknown.¹⁴⁾ In these experiments

the S1 reaction was performed in the presence of Zn^{2+} the well known activator of the enzyme.¹⁾ I have repeated these experiments using Mn^{2+} in place of Zn^{2+} and found that the enzyme is more restrictive under these conditions; S1 in Mn^{2+} cleaves DNA at defined sites yielding discrete DNA bands in agarose gel electrophoresis. Therefore I have reconstructed the map of the S1-cleavage sites of SV 40 and polyoma superhelical DNAs under these conditions. The results show that SV 40 and polyoma DNAs contain analogous S1-cleavage sites suggesting possible common functional requirements.

MATERIALS AND METHODS

Substrate DNAs. Polyoma DNA (from large plaque strain¹⁸⁾) was obtained from virus infected mouse 3T6 cells as described previously.¹⁷⁾ SV 40 DNA was prepared from a plaque purified isolate of strain 777 and was a generous gift from Dr. K. Oda, The Institute of Medical Science, The University of Tokyo. Tritium-labeled coliphage T7 DNA (specific activity 5×10^4 cpm/ μg) was prepared according to the procedure reported previously.¹⁹⁾

Enzymes and reactions. S1 nuclease provided by Kaken Chem. Co. was further purified with SP-Sephadex according to the procedure of Vogt.¹³⁾ This preparation showed a single band in sodium dodecyl sulfate-polyacrylamide gel electrophoresis. *Eco* RI endonuclease prepared according to Green *et al.*¹⁰⁾ was a gift from Dr. T. Tanaka, Mitsubishi Kasei Institute for Life Science. *Bam* NI endonuclease²⁰⁾ (isoschizomer to *Bam* HI) was provided by Dr. T. Shibata in our laboratory. *Bce* 170 endonuclease²¹⁾ (isoschizomer to *Pst* I) was provided by Dr. E. Hayase in our laboratory. *Hae* II, *Hind* III, *Hpa* II, *Bgl* I and *Taq* I endonucleases were purchased from Bethesda Research Laboratories, Inc. *Hga* I endonuclease was prepared according to the Takanami's procedure.²²⁾

The digestions were as follows: (1) S1 nuclease-DNA (0.1 μg) was incubated for 30 min at 37°C with indicated amounts of the enzyme (dialyzed against 50% glycerol) in 10 μl buffer (30 mM sodium acetate buffer (pH 5.0), 2 mM divalent metal, 60 mM NaCl and 5% glycerol), unless indicated otherwise. One unit of S1 activity converts 50% of heat-denatured (^3H)-labeled T7 DNA to acid-soluble form in this condition except no addition of the metals. For sequential digestion with restriction endonuclease, S1-digested DNA was extracted with an equal volume of phenol saturated with TEN buffer (10 mM Tris-HCl (pH 7.5), 1 mM

EDTA and 10 mM NaCl), then 5 M NaCl was added to the aqueous layer to give a final concentration of 1 M. After treatment with ether, the DNA was precipitated with two volume of ethanol, centrifuged, and then dissolved in TEN buffer and digested further with restriction endonuclease. (2) *Eco* RI-0.5 μg DNA was digested for 60 min at 37°C in 25 μl of buffer (100 mM Tris-HCl (pH 7.5) and 10 mM MgCl_2). (3) *Bam* NI and/or *Bce* 170-0.5 μg DNA was digested for 2 hr at 37°C with each enzyme in 25 μl of buffer (50 mM Tris-HCl (pH 7.5), 10 mM MgCl_2 and 10 mM β -mercaptoethanol). (4) *Hae* II, *Hpa* II, *Bgl* I and *Hga* I-0.5 μg DNA was digested for 2 hr at 37°C with each enzyme in 25 μl of buffer (20 mM Tris-HCl (pH 7.5), 10 mM MgCl_2 and 7 mM β -mercaptoethanol). (5) *Hind* III-0.5 μg DNA was digested for 60 min at 37°C in 25 μl of buffer (20 mM Tris-HCl (pH 7.5), 60 mM NaCl, 6 mM MgCl_2 and 1 mM dithiothreitol). (6) *Taq* I-0.5 μg DNA was digested for 60 min at 50°C in 25 μl of buffer (10 mM Tris-HCl (pH 8.4), 6 mM MgCl_2 and 6 mM β -mercaptoethanol). (7) For sequential digestion with *Eco* RI, the first reaction buffer was adjusted to *Eco* RI buffer and further digestion was performed. (8) For incomplete digestion with *Hind* III and *Hga* I, 1/5 the amount of each enzyme was used under the same conditions.

Agarose gel electrophoresis. Agarose tube gels (1.2%, 6 mm in diameter, 180 mm long) were prepared and electrophoresed in Tris-borate buffer (89 mM Tris-OH, 89 mM boric acid and 2.5 mM EDTA, pH 8.2²³⁾) at 150 volts for 3~4 hr. Agarose horizontal slab gels (1.0%, 15 $\times$ 20 $\times$ 0.3 cm) were formed and electrophoresed in Tris-acetate buffer (50 mM Tris-HCl, 20 mM sodium acetate, 18 mM NaCl and 2 mM EDTA, pH 8.2) at 1 V/cm for 15 hr. The DNA bands were stained with ethidium bromide (EtBr), visualized using a short wavelength UV light, and then photographed using a orange filter (Kenko MC YA3) and Polaroid black and white Land Pack film type 665.

RESULTS

Manganese can activate S1 nuclease activity to almost same extent as zinc

Figure 1 shows S1 nuclease activity on heat-denatured T7 DNA as a function of various divalent metals and enzyme concentrations. Mn^{2+} stimulates S1 nuclease action to almost the same extent as does Zn^{2+} the well established activator.¹⁾ Activation of the enzyme by these metals was more notable at low enzyme concentrations. As already reported by Ando,¹⁾ Ca^{2+} and Mg^{2+} did not stimulate S1 nuclease activity.

FIG. 1. S1 Nuclease Activity on Single-stranded DNA Under the Presence of Various Divalent Metals. Tritium-labeled T7 DNA (0.1 μ g, 5,000 cpm) was digested with indicated amounts of S1 using conditions described in MATERIALS AND METHODS. After the reaction, 30 μ l of buffer (0.05 M Tris-HCl (pH 7.5), 0.1 M NaCl, 0.04 M EDTA), 10 μ l of salmon sperm DNA (2.5 mg/ml) and 50 μ l of 15% trichloroacetic acid were added and the radioactivity retained on the filters was determined.

Figure 2 indicates the cleavage patterns of covalently closed circular polyoma DNA by S1 as a function of enzyme concentration in the presence of Zn^{2+} and/or Mn^{2+} ions. The efficiency of conversion of a superhelical DNA to a unit length linear form in the presence of Mn^{2+} was almost the same as Zn^{2+} and was proportional to the concentration of enzyme used. With 5 units of S1, about 80% of the polyoma DNA (0.1 μ g) was converted

to the linear form in the presence of both metals with only slight production ($\sim 5\%$) of fragments smaller than the unit length linear DNA. Without the metal ions or with Mg^{2+} and/or Ca^{2+} the conversion of superhelical polyoma DNA to a linear form was poor (the conversion to a open circular form was complete).

S1 nuclease in the presence of manganese cleaves Polyoma DNA at specific locations

Beard *et al.* have already reported that S1 nuclease cleaves SV 40 DNA once within two localized regions.²¹ These regions were mapped using electronmicroscopy to size the DNA fragments produced by *EcoRI* and *HpaII* endonuclease-digestions of the S1-generated unit length linear DNA. I have characterized the DNA fragments produced by sequential digestions with S1 nuclease (Mn^{2+}) and restriction endonucleases using agarose gel electrophoresis. Figure 3 shows two electrophoretic patterns of the polyoma DNA fragments produced by digestion of S1-generated unit length linear DNA (also a small amount of open circular DNA) with *EcoRI* endonuclease. With Mn^{2+} as the cation in the S1 reaction, discrete DNA bands were evident in the gel, but with Zn^{2+} , these bands were fainter, broader and more numerous. This means that

FIG. 2. Conversion of Superhelical Polyoma DNA to Unit Length Linear Form by S1 Nuclease Under the Presence of Various Divalent Metals.

Non labeled 0.1 μ g polyoma DNA was digested in 10 μ l of reaction buffer with the indicated amounts of S1 under conditions described in MATERIALS AND METHODS. The digests were electrophoresed in horizontal agarose slab gels as described in MATERIALS AND METHODS. Form I, circular superhelical; II, open circular; III, unit length linear.

FIG. 3. Agarose Gel Electrophoretic Patterns of DNA Fragments Produced by Sequential Digestion of S1 Nuclease (Mn^{2+} or Zn^{2+})-Generated Unit Length Linear DNA with *Eco* RI Restriction Endonuclease.

5 μ g polyoma DNA was digested in 500 μ l of reaction buffer with 250 units S1 as described in MATERIALS and METHODS. S1 digestion was performed with Mn^{2+} (track 1) and/or Zn^{2+} (track 2). After recovery of the DNA as described in MATERIALS AND METHODS, 0.5 μ g aliquot of the S1-generated unit length linear DNAs containing slight amount of open circular DNA was digested with *Eco* RI endonuclease. The *Eco* RI endonuclease-digest was electrophoresed in agarose tube gels as described in MATERIALS AND METHODS. The outer tracks are the S1 nuclease-digests without subsequent *Eco* RI digestion.

the length distribution of the fragments by the *Eco*RI nuclease-digestion is large. Since S1 nuclease is almost equally active on single-stranded DNA with Mn^{2+} and Zn^{2+} , it is likely that the cleavage of polyoma DNA within the unpaired regions occurs more specifically with Mn^{2+} than Zn^{2+} .

S1 nuclease cleaves Polyoma DNA at seven specific sites

Mapping of the polyoma DNA sites cleaved by S1 nuclease in the presence of Mn^{2+} was performed using *Eco*RI and *Bam*NI endonucleases. The results are summarized in Figure 8-A. The *Eco*RI endonuclease-digest of S1-generated unit length linear polyoma DNA

FIG. 4. Fragments of Polyoma DNA Produced after Digestion of S1 Nuclease (Mn^{2+})-Generated Unit Length Linear DNA with *Eco* RI or *Bam* NI Restriction Endonuclease.

0.5 μ g each S1 nuclease-generated linear DNA (prepared as described in Fig. 3) was digested with *Eco* RI or *Bam* NI endonuclease. Each digest was electrophoresed at same time in agarose gel as described in MATERIALS AND METHODS.

(containing a slight amount of partial product, open circular DNA) yielded eleven discrete DNA bands on gel electrophoresis (*Eco*-A to *Eco*-K) as shown in Fig. 4-1. There is also a background smear of DNA because the polyoma DNA preparation contained some randomly nicked, open circular DNA (see Fig. 2) which generate a circularly permuted set of linear DNAs that are then cleaved by the *Eco*RI endonuclease. As a consequence, the background in the gel is relatively high compared with that of typical restriction endonuclease-digest. Table I summarizes the sizes of these fragments, expressed in polyoma DNA fractional lengths, which were deduced from their electrophoretic mobilities using molecular length standards (see Fig. 5).

Grouped in pairs (*Eco*-D (0.82)+*Eco*-K (0.19); *Eco*-E (0.79)+*Eco*-J (0.21); *Eco*-F (0.70)+*Eco*-I (0.30); *Eco*-G (0.56)+*Eco*-H (0.44)) the fragments add up to polyoma unit length. The pairs to *Eco*-B (0.91~0.92) and

FIG. 5. A Plot of the Electrophoretic Mobility as a Function of the Logarithm of the Molecular Lengths of Polyoma DNA Fragments.

DNA fragments obtained by sequential digestion with S1 nuclease and *Eco* RI or *Bam* NI endonuclease were at same time electrophoresed in parallel with the molecular length standards produced by cleavage with several restriction endonucleases; full length linear polyoma DNA produced by *Eco* RI cleavage,²⁴ six fragments (0.89, 0.75, 0.66, 0.54, 0.21 and 0.13) produced by *Hga* I-limit and/or incomplete cleavage,¹⁷ two fragments (0.58 and 0.42) generated by sequential digestion with *Bam* NI⁴ and *Eco* RI,²⁴ and four fragments (0.345, 0.29, 0.185, and 0.165) produced by *Bce* 170.^{4,21} The analysis of molecular lengths of the fragments (*Eco*-A to *Eco*-G) was done separately.

Eco-C (0.89) fragments are not detectable in this gel. The production of unit length linear DNA (*Eco*-A) is due to digestion of open circular DNA. It is unlikely that one of the S1 nuclease-cleavage sites is located at or close to the *Eco*RI site²⁴ because no fragments of about 0.58 and 0.42 fractional lengths were produced by digestion of S1-generated unit length linear DNA with *Bam*NI whose single cleavage site is located at 0.58 map unit on DNA⁴ (see Fig. 4-2 and Table I).

To map the cleavage sites, the linear DNA was digested with *Bam*NI endonuclease (see Fig. 4-2). Table I represents the "expected" fragment lengths assuming the S1-cleavage occurred at seven sites concluded in Fig. 8-A. The 0.79 and 0.21 fractional length fragments produced by *Eco*RI digestion indi-

cated that S1 nuclease cleaved polyoma DNA at either 0.21 or 0.79 map position or at both. If S1 cleaved the DNA at map position 0.21, *Bam*NI would generate two fragments of 0.37 and 0.63 fractional length; on the other hand, if S1 cleavage occurred at map position 0.79, *Bam*NI should produce two fragments of 0.21 and 0.79 fractional length. Experimentally only the 0.37 and 0.62 fragments were produced (Fig. 4-2 and Table I) indicating that one of S1 nuclease-cleavage sites is located at map position 0.21 on the DNA. The other cleavage sites at map position 0.11, 0.30, 0.44, 0.82 and 0.92 were determined in the same way. The cleavage site at 0.56 map unit was determined as follows: The production of corresponding 0.98 and 0.02 fractional length fragments (*Bam* NI single cleavage site occurs at 0.58 map unit)

TABLE I. MOLECULAR LENGTHS OF FRAGMENTS PRODUCED IN SEQUENTIAL DIGESTS OF POLYOMA DNA WITH S1 NUCLEASE AND *Eco* RI OR *Bam* NI ENDONUCLEASE

Molecular lengths of fragments were deduced from the plot shown in Fig. 5.

<i>Eco</i> RI digest		<i>Bam</i> NI digest		
Fragment	Polyoma fractional length (observed)	Fragment	Polyoma fractional length Expected ^a	Observed
<i>Eco</i> -A	1.00	<i>Bam</i> -A	1.00+0.98	1.00 ^b
<i>Eco</i> -B	0.91~0.92	<i>Bam</i> -B	0.87	0.865
<i>Eco</i> -C	0.89	<i>Bam</i> -C	0.76	0.765
<i>Eco</i> -D	0.82	<i>Bam</i> -D	0.72	0.73
<i>Eco</i> -E	0.79	<i>Bam</i> -E	0.67	0.66
<i>Eco</i> -F	0.70	<i>Bam</i> -F	0.63	0.62
<i>Eco</i> -G	0.56	<i>Bam</i> -G	0.53	0.53
<i>Eco</i> -H	0.44	<i>Bam</i> -H	0.47	0.47
<i>Eco</i> -I	0.30	<i>Bam</i> -I	0.37	0.37
<i>Eco</i> -J	0.21	<i>Bam</i> -J	0.34	0.33
<i>Eco</i> -K	0.19	<i>Bam</i> -K	0.28	0.28
		<i>Bam</i> -L	0.24	0.235
		<i>Bam</i> -M	0.13	0.135
		<i>Bam</i> -N	0.02	— ^c

^a "Expected" in *Bam* NI endonuclease-digest are calculated fractional lengths assuming the S1 nuclease-cleavage occurred at seven sites shown in Fig. 8-A.

^b *Bam*-A band contains both unit length linear DNA generated by digestion of open circular DNA (partial product in S1 nuclease reaction) with *Bam* NI endonuclease and the fragment of 0.98 fractional length generated by digestion of unit length linear DNA which was produced by cutting of the DNA with S1 nuclease at 0.56 map unit, with *Bam* NI endonuclease (it's single cleavage site is located at 0.58 map unit.⁴⁾

^c *Bam*-N band run off from the gel (see Fig. 4-2).

could not be detected in the gel of Fig. 4-2. So, the S1 nuclease-generated linear DNA was digested with *Hae*II endonuclease (it's cleavage site occurs at 0.73 map unit⁴⁾; in this way, two fragments 0.83 and 0.17 fractional lengths were produced. In Fig. 4-2, the 0.53 and 0.47 *Bam*NI fragments were relatively faint indicating that S1 nuclease-cleavage at map position 0.11 is infrequent.

S1 nuclease cleaves SV 40 DNA at eight specific sites

Beard *et al.*, have already reported that S1 nuclease (Zn²⁺) cleaves SV 40 DNA once with-

TABLE II. MOLECULAR LENGTHS OF FRAGMENTS PRODUCED IN SEQUENTIAL DIGESTS OF SV 40 DNA WITH S1 NUCLEASE AND *Eco* RI OR *Bam* NI ENDONUCLEASE

Molecular lengths of fragments were deduced from the plots shown in Fig. 7.

<i>Eco</i> RI digest		<i>Bam</i> NI digest		
Fragment	SV 40 fractional length (observed)	Fragment	SV 40 fractional length Expected ^e	Observed
<i>Eco</i> -A	1.00 ^a	<i>Bam</i> -A	1.00	1.00 ^d
<i>Eco</i> -B	0.89~0.90	<i>Bam</i> -B	0.87	0.88
<i>Eco</i> -C	0.85	<i>Bam</i> -C	0.83	0.84
<i>Eco</i> -D	0.73	<i>Bam</i> -D	0.77+0.76	0.76 ^e
<i>Eco</i> -E	0.61 ^b	<i>Bam</i> -E	0.75	0.75
<i>Eco</i> -F	0.56	<i>Bam</i> -F	0.71	0.70
<i>Eco</i> -G	0.44	<i>Bam</i> -G	0.59	0.57
<i>Eco</i> -H	0.39	<i>Bam</i> -H	0.41	0.43
<i>Eco</i> -I	0.38	<i>Bam</i> -I	0.29	0.293
<i>Eco</i> -J	0.28	<i>Bam</i> -J	0.26	0.255
<i>Eco</i> -K	0.15	<i>Bam</i> -K	0.23+0.24	0.24 ^e
<i>Eco</i> -L	0.12	<i>Bam</i> -L	0.17	0.17
		<i>Bam</i> -M	0.13	— ^f

^a *Eco*-A band contains both unit length linear DNA generated by digestion of open circular DNA (partial product in S1 nuclease reaction) with *Eco* RI endonuclease and the fragment of 0.98 fractional length generated by digestion of unit length linear DNA which was produced by cutting of the DNA with S1 nuclease at 0.98 map unit with *Eco* RI endonuclease (it's cleavage site occurs at 0/1.0 map unit.^{4b)}

^b *Eco*-E band is doublet containing two fragments of 0.62 and 0.61 fractional lengths which are pairs to *Eco*-I (0.38 fractional length) and *Eco*-H (0.39 fractional length) respectively. These fragments are not separable in the gel (see Fig. 6-1).

^c "Expected" in *Bam* NI endonuclease-digest are calculated fractional lengths assuming the S1 nuclease-cleavage occurred at eight sites shown in Fig. 8-B.

^d *Bam*-A band contains the two sorts of unit length linear DNA generated by *Bam* NI endonuclease; one is the digest of open circular DNA and another is the digest of unit length linear DNA produced by cutting of the DNA with S1 nuclease at 0.15 map unit (just *Bam* NI-cleavage site).^{2b)}

^e *Bam*-D and *Bam*-K bands are doublets containing the fragments of 0.77 and 0.76 fractional lengths and 0.23 and 0.24 fractional lengths, respectively. Two sets of the fragments are not separable in the gel (see Fig. 6-2). These four fragments were generated after digestion of unit length linear DNA which was produced by cutting of SV 40 DNA with S1 nuclease at 0.38 or 0.39 map unit, with *Bam* NI endonuclease.

^f *Bam*-M band is not clear in the gel shown in Fig. 6-2.

FIG. 6. Fragments of SV 40 DNA Produced after Digestion of S1 Nuclease (Mn^{2+})-Generated Linear DNA with *Eco* RI or *Bam* NI Restriction Endonuclease.

0.5 μ g each S1 nuclease-generated unit length linear DNA containing slight amount of open circular form prepared as described with polyoma DNA was digested with *Eco* RI (track 1) or *Bam* NI (track 2) endonuclease. Each digest was electrophoresed independently in agarose gels.

in the region 0.15~0.25 or 0.45~0.55 map unit.²⁾ Here, I have mapped the cleavage sites on SV 40 DNA produced by S1 nuclease in the presence of Mn^{2+} . The results are summarized in Fig. 8-B. Figure 6-1 shows the electrophoretic pattern of the digest of the S1 nuclease generated linear SV 40 DNA with *Eco* RI endonuclease. The sizes of the twelve DNA bands (*Eco*-A to *Eco*-L), expressed in SV 40 fractional lengths (see Fig. 7), are summarized in Table II. The map locations of the S1-cleavage sites were determined as described for polyoma. Grouped in pairs (*Eco*-B (0.89~0.90)+*Eco*-L (0.12); *Eco*-C (0.85)+*Eco*-K (0.15); *Eco*-D (0.73)+*Eco*-J (0.28); *Eco*-E (0.61)+*Eco*-H, I (0.39, 0.38); *Eco*-F (0.56)+*Eco*-G (0.44)) the fragments add up to SV 40 unit length. The band at the position of unit length linear (*Eco*-A) is produced from the open circular DNA. The fragment of 0.98 fractional length results from S1 nuclease-cleavage at 0.98 map unit based on the following data: (1) As shown in Fig. 6-2 and

Table II, a small fragment of 0.17 fractional length is formed after digestion of S1-generated linear DNA with *Bam* NI (cleavage site is located at 0.15 map unit.²⁵⁾ (2) Of the two possible S1 nuclease-cleavage sites, either map location 0.98 or 0.32, the latter can be ruled out because no band of 0.32 fractional length is formed after digestion with *Eco* RI (see Fig. 6-1 and Table II). (3) A new fragment of 0.25 fractional length is produced by digestion of S1-generated linear DNA with *Hpa* II endonuclease (cleavage site is located at 0.73 map unit.²⁸⁾

To map the S1 nuclease-cleavage sites the SV 40 linear DNA was digested with *Bam* NI (Fig. 6-2). Table II represents the "expected" fragments assuming the S1-cleavage occurred at the eight sites shown in Fig. 8-B. The cleavage sites at map unit 0.15 and 0.28, and 0.44 and 0.56 correspond to those reported by Beard *et al.*²⁾ The cleavage site at 0.15 map unit was confirmed by production of two fragments of 0.52 and 0.48 fractional lengths after digestion of S1-generated linear DNA with *Bgl* I endonuclease (it's single cleavage site occurs at 0.67 map unit (Roberts, personal communication). In my experiment, additional S1-sensitive sites were located at 0.38, 0.39 and 0.89 on the SV 40 DNA map. These were confirmed by digestion of the S1 nuclease-generated linear DNA with *Taq* I endonuclease²⁶⁾ (cleavage site is located at 0.56 map unit (Roberts, personal communication)). This leads to the production of fragments with fractional lengths of 0.18, 0.17 and 0.33.

In Figs. 6-1 and -2, there are also one or three faint DNA bands after. This indicates that there are one or two extra minor cleavage site(s) on SV 40 DNA.

DISCUSSION

In this paper, I have shown that covalently closed circular DNAs of polyoma virus and SV 40 are cleaved more specifically by S1 nuclease in the presence of Mn^{2+} than with Zn^{2+} although both divalent cations are almost equally effective activators of the enzyme with

FIG. 7. Plots of the Electrophoretic Mobility as a Function of the Logarithm of the Molecular Lengths of SV 40 DNA Fragments.

DNA fragments obtained by sequential digestion with S1 nuclease and *Eco* RI or *Bam* NI endonuclease were independently electrophoresed in parallel with the molecular length standards produced by cleavage with several restriction endonucleases; full length linear DNA produced by *Eco* RI cleavage,⁴⁰⁾ two fragments (0.85 and 0.15) generated by sequential digestion with *Bam* NI²⁵⁾ and *Eco* RI, two fragments (0.73 and 0.27) generated by sequential digestion with *Hpa* II²³⁾ and *Eco* RI, two fragments (0.56, and 0.44) obtained by sequential digestion with *Taq* I²⁶⁾ and *Eco* RI and six fragments (0.34, 0.225, 0.205, 0.125, 0.105 and 0.085) produced by limit and incomplete *Hind* III cleavage.^{47, 48)}

single-stranded DNA. How and why Mn^{2+} produces this effect is not clear. Mn^{2+} also alters the specificity of pancreatic DNase I²⁷⁾; with Mg^{2+} , DNase I introduces random single-strand breaks into double-stranded DNA, but with Mn^{2+} , the enzyme also produces double-strand scissions into the DNA.

The genomes of polyoma and SV 40 show striking similarities in the structure and genetic organization³⁻⁶⁾ even though their base sequences have only limited homology; the region between 0.93 and 0.98 map unit on SV 40 DNA is partially homologous with the region between 0.47 and 0.52 map unit on polyoma DNA⁷⁾, the nucleotide arrangement of their origins of genome replication are

similar,⁸⁾ and some sequences in early region coding tumor antigen are analogous (E. Soeda, personal communication). My experiments show that their sensitivities to S1 nuclease-cleavage are also strikingly similar: (1) Both are cleaved at or close to the site at which DNA replication terminates and the 3' ends of the early and late mRNAs (map unit 0.15 in SV 40 and 0.21 in polyoma); (2) the region in which the coding sequences for VP2/VP3 terminate and the coding sequence for VP1 begins is placed between two cleavage sites (map unit 0.89 and 0.98 on SV 40 and map unit 0.44 and 0.56 on polyoma); (3) three cleavage sites on each DNA (0.56, 0.44 and 0.28 on SV 40, 0.82, 0.92 and 0.11 on polyoma)

FIG. 8. Summary of S1 Nuclease-cleavage Sites on Polyoma DNA and SV 40 DNA from Point of View of Viral Genome Organization.

(A) Polyoma DNA: ●, seven S1 nuclease-cleavage sites; ○, five preferential binding sites of T4 gene 32 protein;²⁸⁾ △, five preferential binding sites of *E. coli* RNA polymerase.²⁹⁾ Ori. and T. indicate the positions of origin and terminus of genome replication.³⁻⁶⁾ Restriction endonuclease (*Eco* RI, *Bam* NI and *Hae* III⁴²⁾-cleavage sites are shown. The maps of mRNA and gene products are also shown.^{3, 4, 8, 34, 40)}

(B) SV 40 DNA: ●, eight S1 nuclease-cleavage sites; ○, one strong binding site of T4 gene 32 protein;³⁰⁾ △, one strong binding site of *E. coli* RNA polymerase.³¹⁾ —→ (inner circle), the initiation sites for RNA synthesis by *E. coli* RNA polymerase.⁵⁰⁾ Ori. and T. indicate the positions of origin and terminus of genome replication.^{3-6, 48)} Restriction endonuclease (*Eco* RI, *Bam* NI, *Hpa* II, *Bgl* I and *Taq* I)-cleavage sites are shown. The maps of mRNA and gene products are also shown.^{3, 4, 8, 35, 39, 43)}

are located at around 0.2, 0.4 and 0.8 respectively the distance from the origin of DNA replication in the early regions of the two viruses.

It is known that T4 gene 32 protein (helix destabilizing protein) and *Escherichia coli* RNA polymerase bind preferentially to unpaired and/or weakly hydrogen-bonded regions on polyoma^{28, 29)} and SV 40 DNAs^{30, 31)}; these sites are also indicated in Fig. 8. Of interest is the fact that these sites occur at or near S1-cleavage sites in polyoma DNA (see Fig. 8-A) and that there is tendency in that direction in SV 40 DNA (see Fig. 8-B).

Recently, Shen and Hearst,³²⁾ (Hearst, personal communication) and Hsu and Jelinek³³⁾ have reported that SV 40 DNA contains inverted repeated regions (sequences with 2-fold symmetry). The positions of inverted repeats coincide with the positions at which 3'- and 5'-ends of early and late mRNAs have been mapped.³⁴⁻³⁸⁾ Moreover, recombination events either within the SV 40 genome^{39, 40)} or between SV 40 and the adenovi-

rus to form a family of Adeno-SV 40 hybrids⁴¹⁻⁴⁴⁾ have frequently occurred at the position of inverted repeated sequences.³³⁾ It is noteworthy that these inverted repeated regions exhibit nice agreement with S1 nuclease-cleavage sites.

What the structure-function correlates between S1 nuclease sensitivity and mRNA production (*e. g.*, processing, termination, *etc.*) or recombination is not clear. Whether it reflects the existence and functional requirement of cloverleaf-like folding with S1 nuclease-sensitive-single-strand portions in the supercoiled DNA⁴⁵⁾ remains to be determined.

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REFERENCES

- 1) T. Ando, *Biochim. Biophys. Acta*, **114**, 158 (1966).
- 2) P. Beard, J. F. Morrow and P. Berg, *J. Virol.*, **12**, 1303 (1973).
- 3) G. C. Fareed and D. Davoli, "Ann. Rev. Biochem.," Vol. 46, Ann. Rev. Inc., Palo Alto, California, 1977, p. 471.
- 4) M. Fried and B. E. Griffin, "Adv. Cancer Res.," Vol. 24, ed. by G. E. Orge and S. Weinhouse, Academic Press Inc., New York, N. Y., 1977, p. 67.
- 5) K. Shishido, *Protein Nucleic Acid Enzyme*, **23**, 878 (1978).
- 6) In abstracts of the International Symposium on Papovaviruses and Their Role in Cell Transformation and Oncogenesis, National Institutes of Health, U.S.A., 1978.
- 7) J. Ferguson and R. W. Davis, *J. Mol. Biol.*, **94**, 135 (1975).
- 8) E. Soeda, K. Kimura and K. Miura, *Proc. Natl. Acad. Sci. U.S.A.*, **75**, 162 (1978).
- 9) L. V. Crawford and P. H. Black, *Virol.*, **24**, 388 (1964).
- 10) J. Vinograd, J. Lebowitz, R. Radloff, R. Watson and P. Laipis, *Proc. Natl. Acad. Sci. U.S.A.*, **53**, 1104 (1965).
- 11) W. Keller, *ibid.*, **72**, 4876 (1975).
- 12) M. Shure, D. E. Pulleyblank and J. Vinograd, *Nucleic Acid Res.*, **4**, 1183 (1977).
- 13) V. M. Vogt, *Eur. J. Biochem.*, **33**, 192 (1973).
- 14) J.-E. Germond, V. M. Vogt and B. Hirt, *Eur. J. Biochem.*, **43**, 591 (1974).
- 15) K. Shishido and T. Ando, *Agric. Biol. Chem.*, **39**, 673 (1975).
- 16) R. Dulbecco, L. H. Hartwell and M. Vogt, *Proc. Natl. Acad. Sci. U.S.A.*, **53**, 403 (1965).
- 17) K. Shishido and P. Berg, *J. Virol.*, **18**, 793 (1976).
- 18) K. Shishido, *Biochim. Biophys. Acta*, **312**, 708 (1973).
- 19) P. J. Greene, M. C. Betlach, H. M. Goodman and H. W. Boyer, "Methods Mol. Biol.," Vol. 7, ed. by R. B. Wickner, Marcel Dekker, New York, 1974, p. 87.
- 20) T. Shibata and T. Ando, *Biochim. Biophys. Acta*, **442**, 184 (1976).
- 21) T. Shibata, S. Ikawa, C. Kim. and T. Ando, *J. Bacteriol.*, **128**, 473 (1976).
- 22) M. Takanami, "Methods Mol. Biol.," Vol. 7, ed. by R. B. Wickner, Marcel Dekker, New York, 1974, p. 113.
- 23) P. A. Sharp, B. Sugden and J. Sambrook, *Biochemistry*, **12**, 3055 (1973).
- 24) B. E. Griffin, M. Fried and A. Cowie, *Proc. Natl. Acad. Sci. U.S.A.*, **71**, 2077 (1974).
- 25) C. N. Cole, T. Landers, S. P. Goff, S. Manteuil-Brudlag and P. Berg, *J. Virol.*, **24**, 277 (1977).
- 26) S. Sato, C. A. Hutchison and J. I. Harris, *Proc. Natl. Acad. Sci. U.S.A.*, **74**, 542 (1977).
- 27) E. Melgar and D. A. Goldthwait, *J. Biol. Chem.*, **243**, 4409 (1968).
- 28) J. Monjardino and A. W. James, *Nature*, **255**, 249 (1975).
- 29) B. Lescure, P. Oudet, P. Chambon and M. Yaniv, *J. Mol. Biol.*, **108**, 83 (1976).
- 30) J. F. Morrow and P. Berg, *J. Virol.*, **12**, 1631 (1973).
- 31) B. Allet, R. J. Roberts, R. F. Gesteland and R. Solem, *Nature*, **249**, 217 (1974).
- 32) C.-K. Shen and J. E. Hearst, *Proc. Natl. Acad. Sci. U.S.A.*, **74**, 1363 (1977).
- 33) M.-T. Hsu and W. R. Jelinek, *ibid.*, **74**, 1631 (1977).
- 34) N. H. Acheson, *Cell*, **8**, 1 (1976).
- 35) W. Fiers, R. Contreras, G. Haegeman, R. Rogiers, A. Van de Voorde, H. Van Heuverswyn, J. Van Herreweghe, G. Volckaert and M. Ysebaert, *Nature*, **273**, 113 (1978).
- 36) S. Lavi and Y. Groner, *Proc. Natl. Acad. Sci. U.S.A.*, **74**, 5323 (1977).
- 37) E. May, J. V. Maizel and N. P. Salzman, *ibid.*, **74**, 496 (1977).
- 38) V. B. Reddy, B. Thimmappaya, R. Dahr, K. N. Subramanian, B. S. Zain, J. Pan, P. K. Ghosh, M. L. Celma and S. M. Weissman, *Science*, **200**, 494 (1978).
- 39) G. Khoury, G. C. Fareed, K. Berry, M. A. Martin, T. N. H. Lee, and D. Nathans, *J. Mol. Biol.*, **87**, 289 (1974).
- 40) J. E. Mertz, J. Carbon, M. Hertzberg, R. W. Davis and P. Berg, Isolation and Characterization of Individual Clones of Simian Virus 40 Mutants containing Deletions, Duplications and Insertions in Their DNA, Cold Spring Harbor Symposium, *Quant. Biol.*, **39**, 69 (1974).
- 41) T. J. Kelly Jr., *J. Virol.*, **15**, 1267 (1975).
- 42) T. J. Kelly Jr., A. M. Lewis Jr., A. S. Levine and S. Siegal, *J. Mol. Biol.*, **89**, 113 (1974).
- 43) T. J. Kelly Jr. and D. Nathans, "Adv. Virus Res.," Vol. 21, ed. by M. A. Lauffer, F. B. Bang, K. Maramorosch and K. M. Smith, Academic Press Inc., New York, N.Y., 1977, p. 86.
- 44) P. Lebowitz, T. J. Kelly Jr., D. Nathans, T. N. H. Lee and A. M. Lewis Jr., *Proc. Natl. Acad. Sci. U.S.A.*, **71**, 441 (1974).
- 45) H. M. Sobell, *ibid.*, **69**, 2483 (1972).
- 46) J. F. Morrow and P. Berg, *ibid.*, **69**, 3365 (1972).
- 47) K. J. Danna, G. H. Sack and D. Nathans, *J. Mol. Biol.*, **78**, 363 (1973).
- 48) R. Yang, K. Danna, A. Van de Voorde and W. Fiers, *Virol.*, **68**, 260 (1975).
- 49) R. Kamen and H. Shure, *Cell*, **7**, 361 (1976).
- 50) P. Lebowitz, R. Stern, P. K. Ghosh and S. M. Weissman, *J. Virol.*, **22**, 430 (1977).