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Synchronous Digestion of SV40 DNA by Exonuclease III†

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ABSTRACT: We have established an optimal condition for the synchronous digestion of SV40 DNA with *Escherichia coli* exonuclease III. Electron microscopy and polyacrylamide gel electrophoresis were used to obtain accurate measurements on the lengths of DNA before and after exonuclease III digestion. Based on this finding, a new method for determining the sequence of long duplex DNA can be realized. It involves (a) the synchronous digestion of the DNA from the 3′ ends with exonuclease III, followed by (b) repair synthesis with labeled nucleotides and DNA polymerase, and (c) sequence analysis of the repaired DNA.

Escherichia coli exonuclease III catalyzes the sequential hydrolysis of mononucleotides from the 3′ termini of duplex DNA (Richardson et al., 1964). At 5 °C, and using a high concentration of salt, exonuclease III catalyzes the synchronous hydrolysis of approximately six nucleotides from each 3′ terminus of the duplex DNA (Donelson and Wu, 1972). The use of this enzyme to produce single-stranded 5′ ends, followed by repair synthesis to label the ends, constitutes a method for the analysis of short sequences from the ends of a duplex DNA (Wu et al., 1972; Ghangas et al., 1973; Bambara and Wu, 1975).

Based on the above principles, a method is being developed for determining long sequences from the ends of a duplex DNA. In this method, exonuclease III is used to remove a large number of nucleotides (e.g., from 40 to 400 nucleotides) to produce long single-stranded ends (Figure 1, step 1). This DNA molecule can then be labeled with radioactive nucleotides (step 2), digested with a restriction enzyme so that the two labeled ends can be separated (step 3), and sequenced with one of the current methods, such as ribo substitution, for DNA sequence analysis (see review articles by Murray and Old, 1974; Salser, 1974; Wu et al., 1974), or with a new method to be perfected. A crucial factor in the success of this method is the degree to which the exonuclease III digestion proceeds synchronously under the optimal condition of incubation (step 1). In other words, for ease of sequence analysis, all DNA molecules should be digested to approximately the same extent.

In this communication, we describe our establishment of an optimal condition for the synchronous digestion of SV40 DNA with exonuclease III so that the method depicted in Figure 1 can now serve to determine long DNA sequences from each 3′ terminus of any duplex DNA.

Experimental Section

Materials

Cell Lines and Virus. Two lines of African green monkey kidney cells, CV-1 and TC-7, were kindly supplied by J.

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Sambrook and J. A. Robb, respectively. Plaque purified small plaque SV40 was kindly given to us by D. Nathans. SV40 was grown on either cell line by the procedure of Danna and Nathans (1971).

SV40 DNA. Confluent CV-1 or TC-7 monolayers in 100-mm plastic tissue culture dishes were infected with SV40 at a multiplicity of about 10 PFU/cell. For ^{32}P labeling, 0.5 mCi of carrier free [^{32}P]orthophosphate was added to each dish at 16 h after infection in 5 ml of phosphate-free MEM medium containing 2% fetal calf serum. At about 64–78 h, the cells were lysed according to the Hirt procedure (1967) and the DNA was extracted and purified by equilibrium centrifugation in CsCl-ethidium bromide (Danna and Nathans, 1971; Hirt, 1967; Wu et al., 1976). Form I DNA was used for all the experiments.

Enzymes. Exonuclease III from *E. coli* and restriction endonuclease *Eco* RI (Greene et al., 1974) were both purified by a modification of the published procedures from *E. coli* strain RY 13 which is deficient for endonuclease I. Our modifications are as follows: *Eco* RI was purified according to Greene et al. (1974) except that an agarose column (0.5 M, 100–200 mesh) step was introduced between ammonium sulfate fractionation and phosphocellulose column chromatography. During the hydroxylapatite chromatography, a linear gradient instead of a stepwise elution was used. This step partially removes a contaminating 3′–5′ single-stranded DNA-specific exonuclease activity. Our stock *Eco* RI has the activity of digesting completely 3.6 μg of λDNA in 2 h per μl of enzyme at 37 °C in a 50-μl volume.

Exonuclease III was purified by bringing the 50% saturated ammonium sulfate supernatant fluid of the extract to 70% saturation followed by agarose column fractionation (0.5 M, 100–200 mesh), DE52 and P11 phosphocellulose column chromatography using conditions similar to those described by Richardson and Kornberg (1964). The concentrated fraction has a specific activity of approximately 60 000 units per mg of protein (1 unit is defined as 1 nmol of nucleotides rendered acid soluble at 37 °C in 30 min under assay conditions). The *Hpa* II enzyme from *Haemophilus parainfluenzae* was purified by the method of Sharp et al. (1973). *H. influenzae* restriction enzyme (*Hind*) was purified according to Smith and Wilcox (1970). *Aspergillus* nuclease S_1 was purified according to Vogt (1973).

Methods

Restriction Enzyme Digestions. The superhelical, form I, SV40 DNA was digested by the *Eco* RI restriction enzyme to the linear form in 100 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, and 50 mM NaCl. Incubation was at 37 °C for 60 min with an amount of *Eco* RI enzyme previously determined to convert 1.5 times this amount of form I SV40 DNA to the linear molecule. The reaction was stopped by adding 20 mM EDTA and the DNA was extracted with phenol to denature the enzyme protein. The linear DNA was then dialyzed or precipitated with ethanol (Wu et al., 1976).

For the production of SV40 *Hind* DNA fragments, the form I SV40 DNA was digested by *Hind* enzyme according to Danna and Nathans and the DNA fragments were separated by polyacrylamide gel electrophoresis (Danna and Nathans, 1971).

Exonuclease III Digestion. The digestion was carried out in 10 × 75 mm siliconized glass test tubes in a volume of 20–60 μl which contained 67 mM Tris-HCl (pH 7.8 at room temperature), 90 mM NaCl, 4 mM $MgCl_2$, 4 mM dithiothreitol, 1–3 μg of [^{32}P]SV40 DNA (or unlabeled DNA), and 2–24 units of exonuclease III. Incubation was at 23, 28, or 37 °C for 5–60 min. The extent of digestion was followed by the release of acid-soluble ^{32}P counts. Samples (3 μl) were taken at time intervals and pipetted into 0.1 ml of chilled quenching mixture (50 μg of salmon sperm DNA, 100 μg of bovine serum albumin, and 10 mM EDTA); 0.5 ml of 10% trichloroacetic acid was then added to the tube. After chilling for 10 min and centrifuging at 8000*g* for 10 min, the supernatant solution was poured into a scintillation vial for counting (Wu et al., 1976).

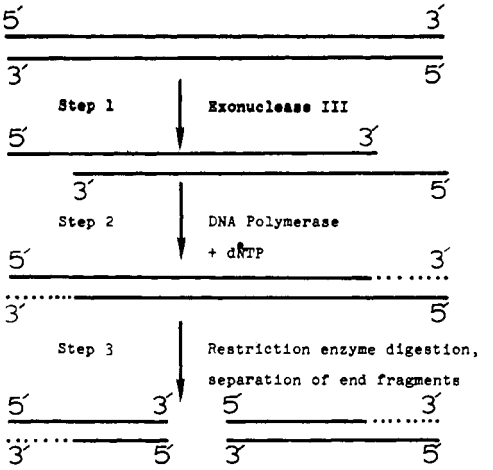

FIGURE 1: An illustration of a method for determining long sequences from the 3′ termini of a duplex DNA.

Exonuclease III Followed by Nuclease S_1 Digestion. After partial digestion of SV40 DNA to produce single-stranded tails, the incubation mixture is adjusted to pH 4.6 by the addition of 1/10 volume of 10 times concentrated S_1 buffer (0.5 M NaOAc-HOAc, 0.5 M NaCl, and 60 mM $ZnSO_4$ (pH 4.0)). Final concentration of $ZnSO_4$ is 6 mM, and NaCl from 140 to 300 mM. One unit of nuclease S_1 is added per 1 μg of SV40 DNA. The samples are incubated at 23 or 28 °C for 60–120 min to completely hydrolyze the single-stranded tails. The sample is chilled to 0 °C and (a) used for gel electrophoresis after the addition of 1/20 volume of 0.5 M EDTA, or (b) used for electron microscopy after the addition of 1/4 volume of 4 M NH_4OAc (pH 7).

Hpa II Restriction Enzyme Digestion. DNA sample (1–2 μg) was digested in a volume of 30 μl containing 10 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, 6 mM KCl, 1 mM dithiothreitol and 5 μl of *Hpa* II restriction enzyme (Sharp et al., 1973). Incubation was at 37 °C for 2 h and the reaction terminated by the addition of EDTA or NH_4OAc as given above.

Agarose Gel Electrophoresis. The method of Sharp et al. (1973) was used except that 20 cm × 20 cm slab gel and 1% agarose were used (Roberts et al., 1975).

Electron Microscopy. DNA molecules were picked up on 20–30 Å thick carbon-aluminum film (Ruben et al., 1975; Ruben and Siegel, 1975), and spread by the aqueous or formamide technique of Davis et al. (1971). A 7.5×10^{-5} M uranyl acetate staining solution in 90% ethanol was applied for 3 min. The DNA samples were imaged by tilted beam dark field EM at a magnification of 24 000× and enlarged to a magnification of 330 000× for measurement. The length of the DNA molecule is measured by a map reader (with reproducibility better than 2%) or on a "digitizer table" (with reproducibility better then 0.5%) where the DNA is traced by the cursor and the analog data are

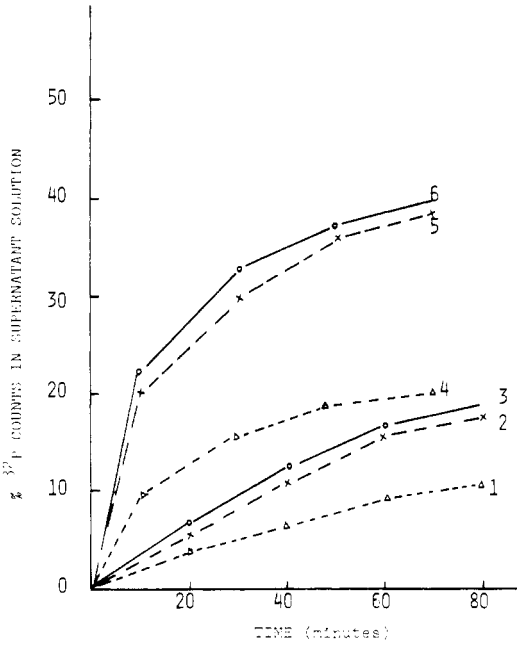

FIGURE 2: Kinetics of exonuclease III digestion of SV40 DNA at 23 and 37 °C. Each experiment contains 1 μg of [^{32}P]SV40 DNA (*Eco* RI linear molecules) in 20 μl of exonuclease III buffer. Two units of exonuclease III was used for experiments 1 and 4, 4 units for experiments 2 and 5, and 8 units for experiments 3 and 6. Experiments 1, 2, and 3 were incubated at 23 °C, and experiments 4, 5, and 6 were at 37 °C. Samples (3 μl) were taken at time intervals and the percent digestion was measured as acid-soluble ^{32}P counts which appeared in the supernatant solution. The percent counts were corrected for 2% acid-soluble counts which were present in the control experiment in the absence of exonuclease III. At 4 units of exonuclease III, the ratio of enzyme per 3′ end of DNA is considered 1:1.

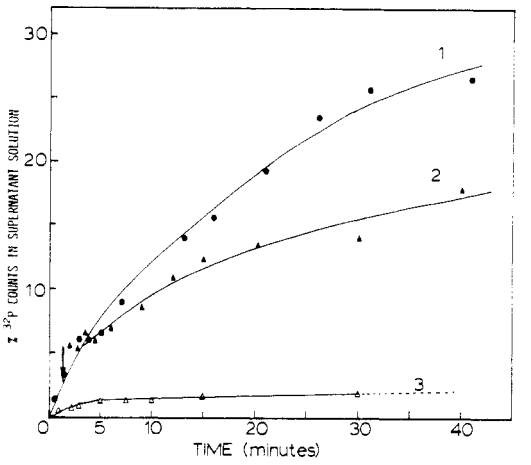

FIGURE 3: Study of processiveness of exonuclease III. Initial incubation mixture contains 0.28 μg of [^{32}P]SV40 DNA digested with *Hind* endonuclease in a final volume of 28 μl of buffer A (70 mM Tris-Cl (pH 7.5 at room temperature), 90 mM KCl, 5 mM $MgCl_2$, and 4 mM dithiothreitol) at 23 °C. Three units of exonuclease III was used to give a ratio of enzyme to 3′ end of DNA of approximately 0.4:1. At 30 s 2-μl aliquot was taken into 0.2 ml of quenching mixture (see Methods) at 4 °C. At 70 s, 13 μl of the incubation mixture was pipetted into 13 μl of buffer A containing 1.5 μg of cold SV40 DNA *Hind* digest (curve 2). At the same time, 13 μl of buffer A was added to the original reaction tube (curve 1); 2-μl samples were taken into the quenching mixture at time intervals indicated in the curves. Trichloroacetic acid soluble counts were determined as described in Methods. All cpm values are corrected for a dilution factor of 2 except for the 30-s time point in curve 1. 10% acid-soluble counts are equal to 500 cpm. (●) Original incubation mixture diluted with buffer A at 70 s; (▲) original incubation mixture diluted with 11-fold excess cold SV40 DNA *Hind* digest at 70 s; (△) same concentration of DNA, exonuclease III, and buffer A as curve 2 except that dilution was made at zero time.

digitized and entered into a computer. DNA molecules with length ±2 S.D. from the mean length ($\bar{L}$) are included in the histograms. In both the undigested and digested DNA preparations, approximately 3% of the DNA molecules are much longer and 10% of the DNA molecules are much shorter than the mean length.

Results

Establishing the Level of Exonuclease III for the Digestion of [^{32}P]SV40 DNA. To increase the probability of synchronous digestion, we have used an excess of exonuclease III so that every 3′ end of the duplex DNA may be associated with a molecule of enzyme. As shown in Figure 2, doubling the amounts of exonuclease III (from 4 to 8 units from experiments 2 to 3 or from experiment 5 to 6) did not appreciably increase the rate or extent of the digestion of [^{32}P]SV40 at either 23 or 37 °C. We interpret this to mean that at 4 units of exonuclease III per μg of SV40 DNA, the 3′ ends of the duplex DNA are saturated by the exonuclease molecules. Thus, this level of exonuclease III was used for all other experiments except in the experiments on the testing for the processiveness of this enzyme.

Testing for the Processiveness of Exonuclease III. Once a molecule of exonuclease III binds to the 3′ end of a duplex DNA and proceeds to hydrolyze the DNA by removing a mononucleotide, the enzyme may dissociate and bind to a second DNA molecule, etc., and hydrolyze the DNA in a nonprocessive manner. Alternatively, the exonuclease III may function in a processive manner by staying with the same DNA molecule and removing many mononucleotides before leaving this DNA molecule. To differentiate these two possibilities, the following experiment was carried out. Limiting amounts of exonuclease III were used so that practically all exonuclease III molecules were bound to the 3′ ends of DNA molecules, and there were excess DNA molecules in solution without bound enzyme. Shortly after the digestion of [^{32}P]SV40 DNA with limiting amounts of exonuclease III has started, a tenfold excess of unlabeled SV40 DNA was added to the sample. If exonuclease III is processive at 23 °C, the rate or extent of hydrolysis of the [^{32}P]DNA will not be affected. However, if exonuclease III is nonprocessive, the rate or extent of hydrolysis of the [^{32}P]DNA should decrease approximately tenfold. Results in Figure 3 (curve 2) show that after the addition of a tenfold excess of unlabeled DNA at 70 s (see arrow), the rate or extent of hydrolysis of the [^{32}P]DNA remained essentially unchanged for the next 10 min. As a control, in curve 3, when the unlabeled DNA was added to the labeled DNA before the addition of exonuclease III, the hydrolysis of [^{32}P]DNA was strongly inhibited. Therefore, from the way exonuclease III hydrolyzes the SV40 DNA it would be classified as processive. From data given in Figures 2 and 3, it can be estimated that in 10 min at 23 °C, approximately 100 nucleotides were hydrolyzed by each molecule of exonuclease III. Thus, the majority of the enzyme molecules digest at least 100 nucleotides from the same DNA terminus before leaving the DNA molecule.

Digestion of SV40 DNA with Exonuclease III and Nuclease S_1 at Different Temperatures. As shown in Table I, at all three temperatures of exonuclease III digestion, the more extensive the digestion (as measured by the acid-soluble counts) the lower the percent of [^{32}P]DNA that remained with the main agarose gel band. At a given extent

Table I: Measurement of Acid-Soluble ^{32}P and Electrophoretic Mobility of SV40 DNA after Digestion with Exonuclease III and Nuclease S_1.[a]

	Exo III Digestion		% Acid-Soluble Counts		Gel Electrophoresis	
Expt	Temp (°C)	Time (min)	Exo III	Exo III + S_1	Migration (mm)	% Counts in Main Band
1	23	25	14.7	28.3	38.0	86
2	23	34	17.2	34.0	39.5	79
3	23	52	24.5	45.5	45.0	70
4	28	15	15.4	29.1	38.2	80
5	28	25	23.7	42.2	44.5	74
6	28	37	29.6	56.7	54.0	52
7	37	4	7.8	13.5	33.3	84
8	37	4.5	8.6	16.4	34.3	80
9	37	8	16.9	31.7	38.9	76
10	28	35		3.3	32.1	91
11	28	35			32.4	96

[a] Each experiment contains 1.0 μg of SV40 DNA (*Eco* RI linear, form III) in 20 μl of buffer. After exonuclease III (exo III) digestion, the buffer was adjusted to pH 4.5, and $ZnSO_4$ (6 mM), NaCl (300 mM), and nuclease S_1 (1 unit) were added. Incubation was at 28 °C for 60 min. The reaction was terminated by the addition of 1 μl of 0.5 M EDTA and 6 μl of loading dye, and the samples were loaded on 1% agarose gel for electrophoresis. No exonuclease III was added to experiments 10 and 11. No nuclease S_1 was added to experiment 11.

of digestion, as shown in experiments 1, 4, and 9, a higher degree of synchrony of exonuclease III digestion was obtained at the lower temperature (23 °C). The pattern of agarose gel electrophoresis (data not presented here) showed that the DNA bands are quite sharp in experiments where less than 30% of the DNA was digested (750 nucleotide pairs removed per end) with exonuclease III and nuclease S_1. However, to ensure a high degree of synchrony of exonuclease III digestion, it is desirable to digest the DNA less than 20%, because an endonuclease activity in the exonuclease III preparation can produce some single- and double-stranded breaks with longer incubation.

Electron Microscopy as a Quantitative Method for Measuring the Length and Distribution of DNA Molecules. Dark field electron microscopy is used since it is easier to define the ends of DNA molecules and see small DNA pieces as short as 80 Å in length (Ruben et al., 1975). Thus, the length of DNA molecules can be measured with greater accuracy and ease. A composite electron micrograph of some representative DNA molecules is shown in Plate 1. The molecule on top is the linear form of SV40 DNA which was produced by digesting the superhelical SV40 DNA with *Eco* RI restriction enzyme. The next two molecules below are those digested with exonuclease III only and spread with the formamide and the aqueous Kleinschmidt procedure (Davis et al., 1971), respectively. The extended and just below it the collapsed single-stranded tails can be seen. The molecule at the bottom represents one that has been digested by exonuclease III followed by nuclease S_1 to remove the single-stranded tails. For most experiments, the molecules like the bottom one with the single-stranded ends removed are measured for length.

The length distribution of the control and nuclease digested DNA molecules are shown in Figure 4. The no-enzyme control gave a length of 1.50 μ and a narrow distribution (spread of 2.7%). Addition of nuclease S_1 alone (panel B) broadens the peak somewhat (spread of 5.0%) indicating that this single-strand specific nuclease preparation is not entirely free of contaminating nucleases. Addition of exonuclease III alone (panel C) reduced the length of 1.18 μ and the distribution became broader (6.7%) due to the difficulty of precisely locating the junction between double- and single-stranded DNA. Digestion of the DNA with exonuclease III followed by nuclease S_1 to remove the single-stranded tails gave completely double-stranded DNA molecules which are easier to measure. DNA molecules of this type were measured for all other experiments presented in this figure and in Figures 6 and 7. In experiments D and E, after digestion of SV40 DNA with exonuclease III followed by nuclease S_1, the length distributions of the DNA molecules are quite narrow (5.1 and 4.5% spread which is similar to the control value in experiment B). This indicates that the exonuclease III digestion is relatively synchronous at 23 or 28 °C when 10–13% of the DNA molecules are hydrolyzed. Since SV40 DNA contains approximately 5 000 base pairs, a 10% digestion corresponds to the removal of 500 base pairs from the two ends of a DNA molecule, or 250 base pairs per end.

At 37 °C, the exonuclease III digestion was almost as synchronous. For example, when 27% of each DNA molecule was hydrolyzed the spread is only 5.7%. However, results at 37 °C are not as reproducible as those at lower temperatures. It appears that the trace contaminants in the exonuclease III preparation are more active at 37 °C and produce more single- and double-stranded breaks in the DNA.

The Synchrony of Exonuclease III Digestion of SV40 DNA. To precisely measure the distribution of the decrease in length of a single 3′ end after exonuclease III digestion of SV40 DNA, the DNA was further cleaved by *Hpa* II restriction enzyme to give two fragments. As shown in Figure 5, the small fragment amounts to 26% of the length of the original SV40 DNA in agreement with the value of Sharp et al. (1973). Therefore, if the digestion removes the same number of nucleotides from each end, a 10% digestion (5% per end) of the original SV40 DNA should produce a 20% decrease in the length of the *Hpa* II small fragment (from 26% down to 21%). As shown in Table II, in experiment 3, when 9.6 ± 0.7% of the full length SV40 DNA was made acid-soluble, a 17% decrease in the length of the *Hpa* II small fragment was observed by using either gel electrophoresis or electron microscopy. As expected, the smaller percent decrease in the length of the *Hpa* II large fragment showed greater variations in comparing the two methods of measurement. The data on the mobility on agarose gel elec-

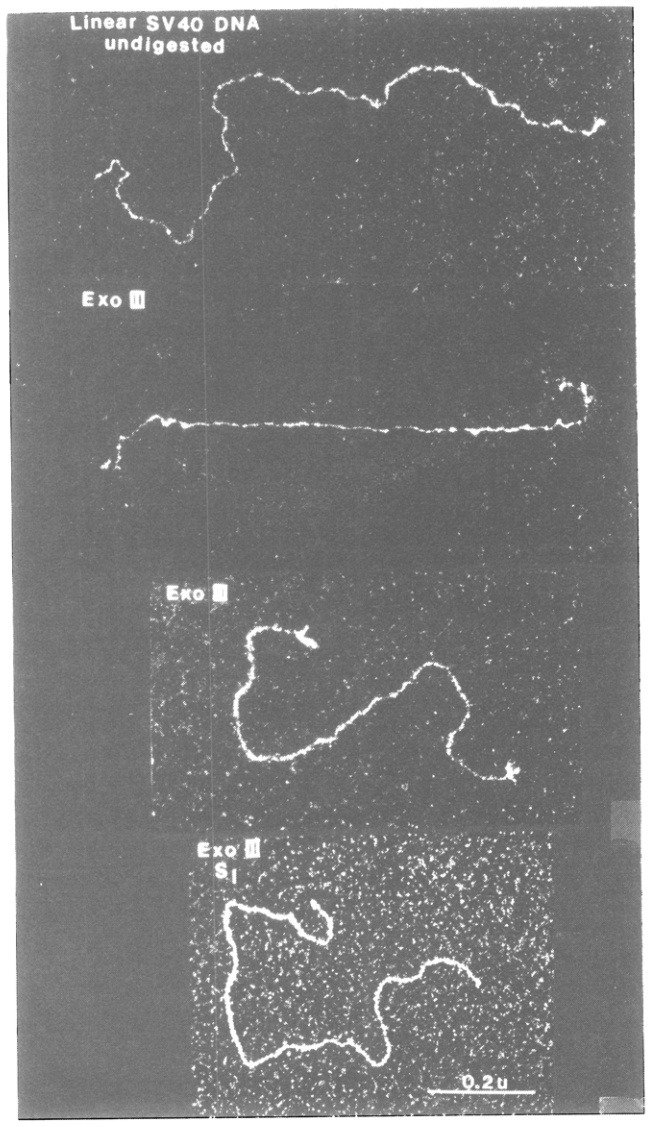

PLATE 1: Electron micrograph of SV40 DNA molecules. (Top panel) Undigested SV40 DNA (*Eco* RI linear molecules) spread by aqueous Kleinschmidt procedure. (Middle panels) SV40 DNA digested with exonuclease III; (second panel) DNA spread with 35%/5% formamide Kleinschmidt procedure; (third panel) DNA spread with aqueous Kleinschmidt procedure. (Lower panel) SV40 DNA digested by exonuclease III followed by nuclease S_1 (aqueous Kleinschmidt procedure).

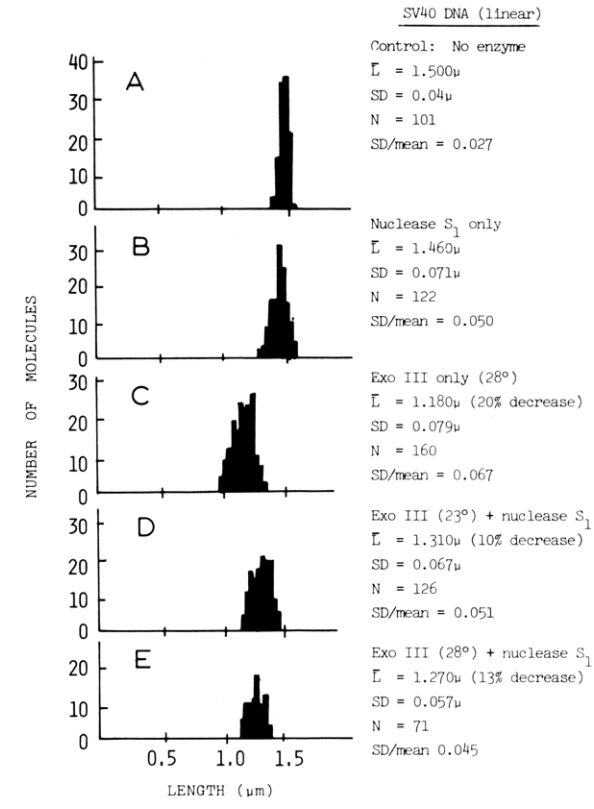

FIGURE 4: The length distribution of the undigested *Eco* RI linear molecules and the nuclease-digested SV40 DNA at 23 and 28 °C as determined by electron microscopy. The conditions for the S_1 nuclease and exonuclease III (Exo III) digestions are given in Methods.

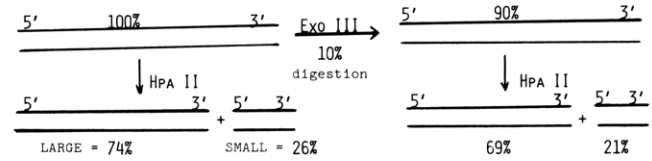

FIGURE 5: A schematic representation of the cleavage of SV40 DNA (*Eco* RI linear molecules) by *Hpa* II restriction enzyme. Although S_1 digestion is not shown, it is used to remove the single-stranded ends after exonuclease III digestion.

trophoresis of the *Hpa* II large and small fragments are shown in Plate 2. The length distributions as measured by the electron microscopy are shown in Figure 6. Quantitative results on the DNA length measurements are summarized in Table II. In experiment 2, approximately 150 base pairs were digested from each end of the SV40 DNA—as accurately reflected by the decrease in length of the *Hpa* II small fragment. Since the distribution of the DNA population in this experiment is as narrow as the control (experiment 1, in Figure 6) the synchrony is maintained within the sensitivity of the measurements. In experiment 3, approximately 230 base pairs have been digested. The 2% greater standard deviation indicates that the uncertainty of length is ± 25 base pairs.

Digestion of Hind-B DNA with Exonuclease III. The synchrony of exonuclease III digestion was also investigated with a shorter DNA fragment. SV40 DNA was digested with *Hind* restriction enzyme and the fragments were separated by polyacrylamide gel electrophoresis. The fragment B (*Hind*-B) was chosen for this study since it is well separated from other fragments. The size of this fragment is approximately 750 base pairs. This DNA was extensively digested at 23 °C by exonuclease III and nuclease S_1 to remove approximately 40% of the nucleotides. The histogram for the length measurements by electron microscopy is given in Figure 7. This shows that the difference in the spread of DNA length of exonuclease III-digested DNA (expt 2) is only 5% above that of the control (expt 1). This 5% value is somewhat inflated because the control DNA was not treated with nuclease S_1. Nonetheless, it seems that even by digesting away 40% of the nucleotides, the synchrony of digestion remains good. In other words, when 320 base pairs were removed from this DNA fragment, the length of the remaining DNA is 430 ± 23 base pairs. This extent of synchrony is more than adequate for making the method shown in Figure 1 a useful one for sequence analysis.

Discussion

Length variations of less than 5% were found after exo-

Table II: Length Measurements of SV40 DNA after Digestion with Exonuclease III, Nuclease S_1, and *Hpa* II.[a]

Expt	Enzyme Addition	Exo III Time (min)	% Acid-Soluble Counts[b]	Gel Electrophoresis: *Hpa* II fragment	Migration (mm)	Calcd Base Pairs[c]	% Decrease	Electron Microscopy: Length (μ)	Calcd Base Pairs[c]	% Decrease
1	S_1	0	0	Small	143.0	1350		0.400	1380	
				Large	82.7	3650		1.050	3620	
2	Exo III, S_1	15	6.0	Small	149.5	1200	*11.1*	0.358	1220	*11.5*
				Large	83.2	3600	1.4	1.030	3530	2.5
3	Exo III, S_1	25	9.6	Small	154.6	1120	*17.0*	0.336	1150	*16.6*
				Large	84.0	3540	3.0	0.989	3380	6.6

[a] Each experiment contains 1.5 μg of SV40 DNA (*Eco* RI linear) in 30 μl of buffer. Exonuclease III digestion was carried out at 23 °C for experiments 2 and 3. It was followed by nuclease S_1 (1.5 units) digestion at 23 °C for 2 h. The samples were deproteinized by phenol and ether extraction, and the DNA was precipitated with 66% ethanol. The DNA was resuspended in 30 μl of *Hpa* II buffer containing 5 μl of *Hpa* II restriction enzyme. Incubation was at 37 °C for 2 h and the reaction terminated by addition of 1 μl of 0.5 M EDTA. Each sample was divided into equal parts for gel electrophoresis (1% agarose) and electron microscopy analyses. [b] % acid-soluble ^{32}P counts released in experiments 2 and 3 have been corrected for 3.1% counts found in control (expt 1). [c] SV40 DNA is assumed to contain 5000 base pairs.

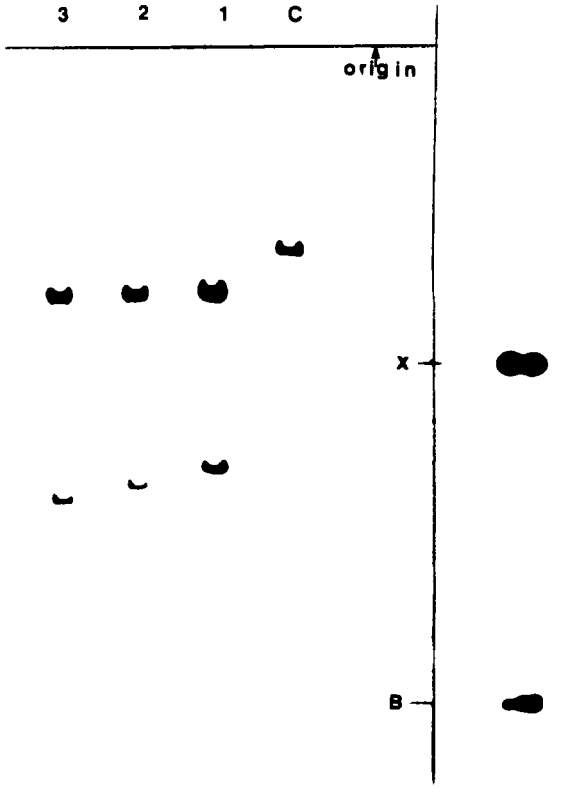

PLATE 2: Agarose gel electrophoresis pattern of SV40 DNA after digestion by exonuclease III and nuclease S_1 followed by *Hpa* II restriction enzyme. Radioautograph of the gel is shown. (Lane C) Control SV40 DNA (*Eco* RI linear molecule) without any enzyme digestion. Lanes 1, 2, and 3 correspond to those experiments shown in Table II. The DNA samples were digested with nuclease followed by *Hpa* II enzyme. X and B correspond to the positions where tracking dyes xylene cyanol FF and bromophenol blue migrated.

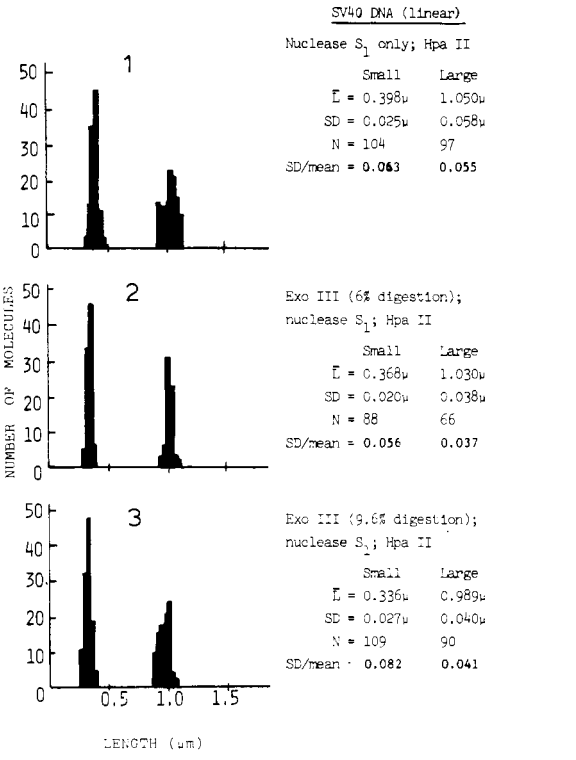

FIGURE 6: The length distribution of the control (nuclease S_1 digested) and the exonuclease III and nuclease S_1-digested SV40 DNA after cleavage by *Hpa* II restriction enzyme.

nuclease III digestion of SV40 DNA, at 23 or 28 °C, with an excess of enzyme molecules over DNA termini. This means that for a 200 base pair long DNA, if 40 bases are removed by exonuclease III from each end, the probable range of the length of the single-stranded tails is between 36 and 44 bases long. This degree of synchrony is more than adequate for using this partially digested DNA for sequence analysis. For example, as shown in Figure 8, the exonuclease III-digested DNA can be repaired by ribo substitution and the product digested with pancreatic RNase to cleave next to each rC to produce rC-terminated oligodeoxynucleotides. After fractionation of these oligonucleotides by two-dimensional homochromatography, spots 1 through 6 can be separated, and they can be sequenced after partial digestion followed by the mobility method (Wu et al., 1974). Since spots 1 through 4 are present in all DNA molecules, they are expected to be more abundant than spots 5 and 6. If the distribution of molecules of type A, B, and C is 25, 50, and 25%, respectively, then the relative amounts of spots 4, 5, and 6 are 100, 75, and 25%, respectively. Therefore, there is no question that spot 6 includes new sequences beyond those included in spots 5 and 4. The order of spots 1 through 5 can be determined after partial pancreatic RNase digestion of the labeled DNA followed by two-dimensional homochromatography and redigestion of each partial product (Sanger et al., 1973; Marians et al., 1975). Alternatively, the order of the rC-terminated oligodeoxynucleotides can be determined by the following method. The

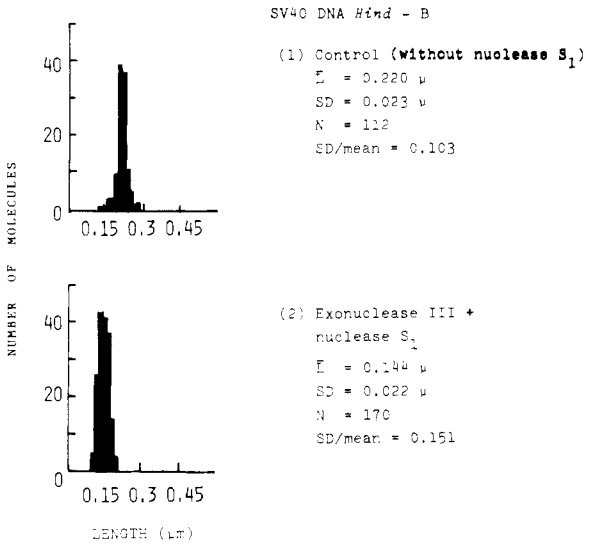

FIGURE 7: The length distribution of the control (without nuclease S_1 digestion) and exonuclease III and nuclease S_1-digested *Hind* fragment B of SV40 DNA.

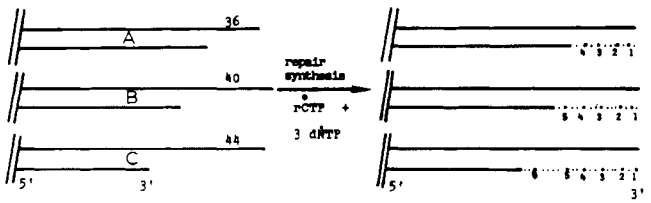

FIGURE 8: An illustration of a method for sequence analysis of exonuclease III-digested duplex DNA. Only one end of a double-stranded DNA is shown here. The length of the single-stranded tails produced by the action of exonuclease III is 36, 40, and 44 nucleotides long in molecules A, B, and C, respectively. Repair synthesis is carried out using DNA polymerase I with [^{32}P]rCTP (large dots) and three dNTPs (small dots). These labeled DNA molecules correspond to one family of the end-labeled fragments produced after step 3 in Figure 1.

original DNA can be digested by exonuclease III to different extents, such as removing approximately 10 (sample D), 20 (sample E), and 30 (sample F) nucleotides from each end by varying the time of enzyme digestion. After repair synthesis, pancreatic RNase digestion, and homochromatography, spot 1 and small amounts of spot 2 may be present in sample D, and spots 1, 2, and small amounts of spot 3 may be present in sample E, etc. Thus, the order of oligonucleotides can be established.

The exonuclease III digestion of DNA at 37 °C can be as synchronous as that obtained at 23 or 28 °C in the absence of any enzyme impurities. Unfortunately, the exonuclease III preparation seems to contain some endonuclease activity and incubation at 37 °C makes the effects of this activity more pronounced. Furthermore, if less than 10% digestion is desired, the short interval of incubation may be more difficult to control (see Table I). For the above reasons, we prefer to use 23 °C for the exonuclease III digestion of DNA, to give more reproducible results. In a previous study, λDNA specifically labeled with 12 nucleotides at each termini was used as the substrate for testing the exonuclease III digestion at 5 and 23 °C (Donelson and Wu, 1972). With this substrate, the synchrony of the exonuclease III digestion in removing the 12 nucleotides was rather poor at 23 °C. This was perhaps due to a lower ratio (less than 0.5) of exonuclease III/DNA used so that the exonuclease III had to move from one DNA to another before all the labeled DNA was digested. Golomb and Chamberlin (1974) reported that the digestion of T7 DNA by exonuclease III was relatively synchronous at 37 °C.

It is of interest to note that the synchrony of exonuclease III digestion is possible at the in vivo temperatures (37 °C), and thus the synchronous digestion of DNA by this processive enzyme may play some physiologically important role.

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