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Gene-Sized Pieces Produced by Digestion of Linear Duplex DNA with Mung Bean Nuclease†

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ABSTRACT: The single-strand specific endonuclease from mung bean sprouts catalyzes a limited number of double-strand cleavages in linear duplex DNA from bacteriophages T_7, gh-1 and PM2. A molecular weight range of ~1.4–0.2 × 10^6 for the limit products derived from each DNA was estimated by agarose gel electrophoresis. The number of cleavages per T_7 DNA molecule at the digestion limit is 37 as determined by ^{32}P end labeling using polynucleotide kinase and [γ-^{32}P]-ATP. These cleavages are probably at unique sites, since discrete bands are seen after agarose gel electrophoresis of the products in the early stages of digestion of each DNA. Observation of discrete bands at the endonucleolytic limit is complicated by the terminally directed (exonuclease-like) activity of the enzyme and by a small number of random endonucleolytic cleavages. The unique sites are thermolabile. A 10 °C increase in temperature results in a ca. tenfold increase in the initial hydrolysis rate of T_7 DNA while the size distribution of the limit products is unchanged. Also, a change from 0.10 to 0.14 M salt concentration produces a ca. fourfold rate decrease. Double-strand cleavages in T_7 DNA arise from the preferential cleavage opposite an enzyme-inflicted nick rather than by the accumulation of nicks at potential double-strand cleavage sites. The naturally occurring nicks in T_5 DNA, which are in G + C rich sequences, are not preferred cleavage sites for mung bean nuclease unless they are first enlarged to five residue (average) gaps by *Escherichia coli* exonuclease III. The specificity of mung bean nuclease on T_7, gh-1 and PM2 DNAs is attributed to thermolabile or structural "breathing" sites which occur in the range of 1 site ~300–2100 base pairs in each of the linear duplexes, independent of the overall percent A + T. This range encompasses that of most of the known structural genes in T_7 DNA.

The so-called "single-strand specific" nucleases are a class of endonucleases which show a pronounced specificity for denatured as opposed to native DNA. Such enzymes have been isolated from many sources (for a review see Kowalski and Laskowski, 1976), but the enzymes which have been purified

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to physical homogeneity from *Aspergillus oryzae* (S_1) (Vogt, 1973) and from mung bean sprouts (Ardelt and Laskowski, 1971; Kowalski et al., 1976) are the most widely used as probes of DNA structure. Using S_1 nuclease, it was found that some superhelical DNAs contain nonpaired or weakly hydrogen-bonded regions at specific loci (Beard et al., 1973). Mung bean nuclease has been shown to cleave λ DNA most rapidly in the A + T rich center of the molecule (Johnson and Laskowski, 1970) and preferentially at a total of six loci, all of which fall within the three A + T rich blocks revealed by electron microscopy partial-denaturation mapping (Chan et al., 1977).

In addition to these sites, there are sites in linear duplex DNA that are hydrolyzed more slowly. Under proper conditions, the single-strand specific nuclease from wheat seedlings hydrolyzes λ DNA to limit products (Kroeker and Fairley, 1975), the distribution of which shows that cleavages are made in the left half of the molecule as well as in the right half which contains the A + T rich blocks.

Similar experiments using mung bean nuclease and linear duplex DNA were initiated in this laboratory with the goal of determining the nature and specificity of the cleavage sites at the endonucleolytic limit. Conditions under which the enzyme is ~100% stable over the long reaction times required were found (Kowalski et al., 1976) and were slightly modified (see Methods). With the high enzyme concentrations required, DNA is degraded not only at a limited number of internal (endonucleolytic) sites, but also at the DNA termini in an exonucleolytic fashion (Kroeker et al., 1976). This terminally directed activity is the result of the relative thermodynamic instability of base pairing at the ends of the duplex. These ends are continuously degraded by the single-strand specific nuclease with the production of mono- and dinucleotides (Kroeker et al., 1976).

In this paper, we show that mung bean nuclease introduces a limited number of endonucleolytic cleavages in the linear duplex DNAs from bacteriophages T_7, gh-1, and PM2. For each DNA, the products are gene sized (300–2100 base pairs) and are the result of double-strand cleavages, most likely at specific sites. The mechanism of cleavage and the effects of conditions on the rate of hydrolysis of T_7 DNA are presented. Finally, studies on the specificity and relative rates of the enzyme on nicked and gapped linear duplex DNA are presented.

Experimental Procedures

Materials

Enzymes. Mung bean nuclease was isolated and purified to homogeneity as described (Kowalski et al., 1976). Polynucleotide kinase was isolated in a form which was devoid of any interfering activities (Kroeker and Laskowski, 1977). Alkaline phosphatase (Sigma) was further purified by the method of Weiss et al. (1968). Micrococcal nuclease was purified by Dr. E. Sulkowski (Sulkowski and Laskowski, 1966). *Eco*RI and *Hpa*II restriction endonucleases and *E. coli* deoxyribonuclease III were purchased from Miles.

DNA. T_7, T_5st(o), T_5(+), gh-1 and PM2 bacteriophage stocks and the appropriate bacterial hosts were supplied by Drs. R. L. Herrmann, D. J. McCorquadale, S. Weiss, J. Boezi, and J. C. Wang, respectively, except that the host for gh-1 phage, *Pseudomonas putida,* was obtained from the American Type Culture Collection. Both unlabeled and ^{33}P uniformly labeled T_7 and T_5st(o) DNA were made as described earlier (Kroeker et al., 1976) as was gh-1 DNA (Lee and Boezi, 1966). PM2 phage were grown and purified (Espejo and Canelo, 1968; Espejo et al., 1969), and form I DNA was isolated (Radloff et al., 1967) by Dr. A. Pritchard. PM2 form I DNA (400 μg/mL) was converted to form III by incubation with the restriction endonuclease *Hpa*II (80 units/mL for 3 h followed by an additional 40 units/mL for 1 h) at 37 °C in 0.01 M Tris-HCl[1] (pH 7.2), 0.01 M NaCl, 0.01 M magnesium acetate.

Methods

Reaction of Duplex DNA with Mung Bean Nuclease. The reaction buffer used throughout this work was 0.05 M sodium acetate adjusted to pH 5.0 with acetic acid. The total salt concentration was varied by the addition of a NaCl solution. In all cases, the total salt concentration refers to the sum of all ionic species at pH 5.0, i.e., sodium acetate, NaCl, and Tris-HCl. Tris-HCl is a contribution of the DNA storage buffer (0.01 M Tris-HCl, pH 7.3; 0.01 M NaCl). In addition to the above, reaction mixtures contained 0.01 mM zinc acetate, 0.005% Triton X-100 (in some cases 0.001%), and 1.0 mM L-serine. L-Serine was substituted for the sulfhydryl compounds previously employed (Kowalski et al., 1976), since it is equally effective in stabilizing the enzyme activity and does not cause nicking of the DNA (unpublished experiments from this laboratory). The enzyme was stable (>90%) with respect to its activity toward denatured DNA under all reaction conditions and all incubation times used in this study. The enzymatic reaction was terminated by cooling the reaction mixture on ice followed by the addition of 1/20 vol of 1 M Tris (free base) which increased the pH from 5.0 to 8.0. Gel electrophoretic analysis (see below) of reactions carried out in the absence of enzyme showed that the DNA was free of single- and double-strand breaks.

Quantitation of the DNA Termini Produced by Mung Bean Nuclease. The method of Weiss et al. (1968) for labeling the 5′-phosphorylated termini of DNA with polynucleotide kinase after treatment with alkaline phosphatase at either 25 (to determine double-strand cleavages) or 65 °C (to determine nicks plus double-strand cleavages) was modified to provide an accurate and convenient procedure for measuring the limited endonucleolytic cleavages of native DNA. The procedure (Kroeker and Laskowski, 1977) takes advantage of the rapid diffusion of unreacted [^{32}P]ATP from agarose pellets which retain the large, terminally labeled DNA products. Acid-soluble products, which were <2% of the total DNA, did not interfere with the end-labeling reaction.

Viscometric Analysis. Molecular weight values for DNA and its nuclease digestion products were obtained from an ultra low sheer rheometer, Contraves LS-100. Viscosity was essentially independent of the shear setting. Using the empirical formula of Eigner (1968), the molecular weight was independent of DNA concentration from 20 to 60 μg/mL when the total salt concentration was 0.1 to 0.2 M. The average molecular weight (M) was correlated to the average number of cleavages per T_7 DNA (N) by the formula $N = [(2.6 \times 10^7)/M] - 1$. N does not represent the true number of cleavages, since M is not a number average but is closer to a weight average. Molecular weights determined for T_5 DNA (76×10^6), T_7 DNA (26×10^6), and PM2 form III DNA (6.3×10^6) were all within ±5% of their known values. The cup and bob of the viscometer were wetted by rinsing with 1% Triton X-100. They were then wiped dry with tissue and equilibrated in the viscometer to the desired temperature. A reaction mixture of 0.5 mL was used and evaporation was minimized by the use of a small humidifying chamber surrounding the cup and bob when following reactions for longer than 1 h.

[1] Abbreviations used are: Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; EDTA, (ethylenedinitrilo)tetraacetic acid.

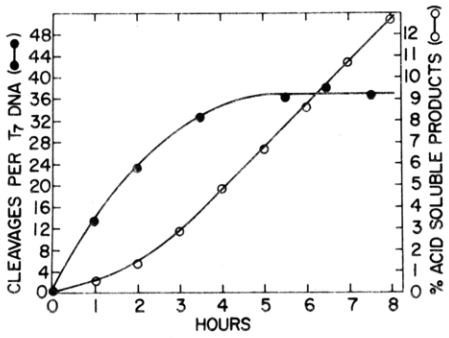

FIGURE 1: Time course for the hydrolysis of T_7 DNA by mung bean nuclease. The reaction (0.50 mL) contained 262 μg/mL T_7 DNA, 50 mM sodium acetate (pH 5.0), 0.01 mM zinc acetate, 0.005% Triton X-100, 1.0 mM L-serine, 32 mM NaCl, and 6 units/mL mung bean nuclease. The reaction was carried out at 37 °C and 50-μL aliquots were removed at the times indicated, added to 0.3 mL of 0.1 M Tris-HCl (pH 8.2), and dialyzed at 4 °C vs. 0.5 M NaCl, 0.01 M Tris-HCl (pH 8.0). After a final dialysis vs. 0.01 M NaCl, 0.01 M Tris-HCl (pH 8.0), the DNA concentration was checked by measuring the absorbance at 260 nm. Triplicate samples (0.25 mL) were used for the quantitation of termini (see Methods) and double-strand cleavages per T_7 DNA (●) were calculated. A parallel reaction was run with T_7 [^{33}P]DNA (840 cpm/μg) and the percent acid-soluble products (○) was determined (see Methods) at the times indicated.

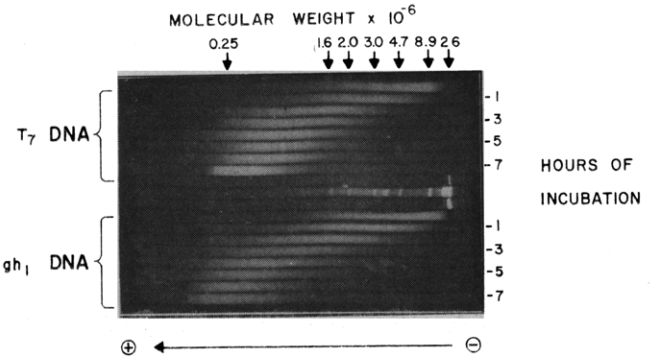

FIGURE 2: Agarose gel electrophoresis of the digestion products of T_7 and gh-1 DNAs at various incubation times with mung bean nuclease. Mung bean nuclease (5 units/mL) was incubated at 37 °C with either T_7 DNA (200 μg/mL) or gh-1 DNA (100 μg/mL) in 50 mM sodium acetate (pH 5.0), 30 mM NaCl, 1.0 mM L-serine, 10 μM zinc acetate, and 0.005% Triton X-100. At 0.5 h, 1.0 h, and every hour thereafter up to 7.0 h, aliquots containing 2 μg of DNA were removed from the reaction, cooled on ice, and combined with an equal volume of 0.1 M Tris (free base), 5 mM EDTA, and stored at −20 °C. After adding 0.5 volume of 15% glycerol saturated with bromophenol blue, aliquots containing 1 μg of DNA were electrophoresed through 0.7% agarose gels for 16 h at 22 V (constant voltage). The gels were stained and photographed as described under Methods. Migration is from right to left. The gels on which the products of the T_7 DNA and gh-1 DNA incubations were electrophoresed are enclosed in brackets and correspond to incubation times, from top to bottom, of 0.5, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, and 7.0 h. The three gels in the center of the photograph correspond, from top to bottom, to T_7 DNA, a mixture of the *Eco*RI digests of both λ and T_5(+) DNAs, and gh-1 DNA.

Exonuclease III Reaction. E. coli exodeoxyribonuclease III (exonuclease III) removes nucleotides from the 3′ termini of nicks and duplex ends at approximately equal rates (Masamune et al., 1971). In order to estimate the average number of nucleotides removed at the nicked sites of T_5st(o) DNA as a function of exonuclease III treatment, a time course of the production of acid-soluble radioactivity from T_5st(o) [^{33}P]-DNA was determined. The reactions (0.1 mL) were performed at 30 °C, pH 8.0, and contained 16.5 μg of T_5st(o)[^{33}P]DNA (806 cpm/μg), 33 μmol of Tris, 0.33 μmol of $MgCl_2$, 0.5 μmol of 2-mercaptoethanol, and 0.001 mL of enzyme solution (500 units/mL; prepared by a tenfold dilution of the commercial solution with 0.05 M Tris-HCl, pH 7.4, and 1 mM 2-mercaptoethanol). Reactions were stopped after fixed time intervals with the addition of 0.01 mL of calf thymus DNA (2 mg/mL) and 0.06 mL of cold $La(NO_3)_3$–HCl reagent (0.02 M–0.2 N). The reaction was linear for 60 min, at which time 0.13% of the DNA (312 nucleotides/T_5 DNA) was acid soluble.

Agarose Gel Electrophoresis. Gel electrophoresis was performed in 0.5 cm i.d. × 16 cm constricted glass tubes. The method of Helling et al. (1974) was used for 0.7 and 1.0% agarose gels. The method of Sharp et al. (1973) was used for 1.4% gels, except that the samples were electrophoresed for 1.5 h at 2.5 mA/gel. Gels were stained in a solution of ethidium bromide (1 μg/mL) and the fluorescence produced by excitation from below with a long-wavelength UV light source was photographed on Polaroid Type 52 or 55 P/N film through a Wratten 23A filter.

Direct scanning of a 5-cm section of 1.4% agarose gels was performed in an Aminco fluorocolorimeter equipped with a gel scanning attachment and a high-intensity Hg vapor lamp (excitation filter, Corning 7-51; emission filter, Wratten 23A; slits, 0.1 mm). Alternatively, the negatives obtained with Type 55 P/N film were cut into ~1 × 10 cm sections, taped onto a 10-cm quartz cuvette, and scanned at 550 nm in a Gilford recording spectrophotometer equipped with a linear transport.

Agarose gels were calibrated using linear duplex DNA markers produced by restriction endonuclease *Eco*RI digestion of λ DNA (13.7, 4.7, 3.7, 3.5, 3.0, and 2.1 × 10^6 daltons, Helling et al., 1974) and T_5(+) DNA (28, 19, 16, 8.9, 2.0, 1.6, and 0.25 × 10^6 daltons, Rhoades, 1975). An *Eco*RI digest of λ DNA was also kindly provided by Drs. R. Wu and D. Tu at the outset of this work. The logarithm of the molecular weight was a linear function of mobility relative to bromophenol blue from 5 to 0.25 × 10^6 for the 0.7% gels and from 2 to 0.25 × 10^6 for the 1.4% gels.

Homogeneity with respect to the molecular weight of the purified bacteriophage DNAs used in this work was routinely checked after agarose gel electrophoresis. The 0.7% agarose gel system described above was used for native DNA and the 0.6% agarose gel system described by Hayward and Smith (1972) was used for alkali-denatured DNA. In the latter case, gels were also stained with ethidium bromide and molecular weight markers were obtained by denaturation of T_5st(o) DNA. The logarithm of the molecular weight was a linear function of mobility relative to bromophenol blue from 35 to 3.8 × 10^6.

General Procedures. The T_7 [^{33}P]DNA acid-soluble assay was previously described (Kroeker et al., 1976). Mung bean nuclease units refer to the standard assay procedure using denatured calf thymus DNA (Kowalski et al., 1976).

Results

Limited Endonucleolytic Cleavage of Duplex DNA. In order to provide a direct measurement of the limited hydrolysis of duplex DNA catalyzed by mung bean nuclease, polynucleotide kinase was used to label the newly generated termini with ^{32}P as described under Experimental Procedures. A time course for the double-strand cleavage of T_7 DNA is presented in Figure 1. Under the conditions of the reaction, the number of endonucleolytic cleavages reaches a limit of 37 per T_7 DNA molecule after 5.5 h of incubation. Also shown in Figure 1 is the time course for the liberation of acid-soluble products. These products [mainly mono- and dinucleotides (Kroeker et al., 1976)] were removed by dialysis before the end-labeling procedure was used. As the number of endonucleolytic cleavages approaches a limit, the rate of liberation of acid-soluble

TABLE I: Comparison of the Molecular Weight and Number of Cleavages at the Digestion Limit with the Original Molecular Weight and A + T Content of the Bacteriophage DNA.

	bacteriophage DNA		
	T_7	gh-1	PM2, III
orig mol wt[a] $\times 10^{-6}$	26	23	6.3
% A + T	52	43	57
limit mol wt[b] $\times 10^{-6}$	0.62	0.65	0.65
cleavages/molecule[c]	41	35	10

[a] Molecular weight values: T_7, Richardson (1966); gh-1, MacHattie and Thomas (1970); PM2, Parker et al. (1977). [b] Limit molecular weight is the value of the molecular weight at the center of the symmetrical peak (molecular weight range ~ 1.4–0.2×10^6) obtained in fluorometric scans of 1.4% agarose gels (see Experimental Procedures) and corrected for the percent acid-soluble DNA. Molecular weight values represent an average of determinations at several postlimit incubation times. [c] Cleavages/molecule = (original mol wt/limit mol wt) − 1.

products becomes constant (Figure 1). These data are consistent with previously described experiments demonstrating two modes of hydrolysis of duplex DNA by mung bean nuclease: endonucleolytic and terminally directed (Kroeker et al., 1976).

The limited nature of the endonucleolytic hydrolysis of native T_7 and gh-1 DNAs by mung bean nuclease can be seen in a qualitative sense from the size distribution evidenced after electrophoresing the products at various stages of digestion through agarose gels. Such an analysis is shown in Figure 2. The heterogeneous distribution of products formed in the early stages of the reaction gradually gives way to a relatively narrow molecular weight distribution (~ 1.4–0.2×10^6) which remains essentially constant from 5–7 h, in spite of the presence of 100% of the original enzyme activity. The product distribution continues to slowly shift downward in molecular weight during this period as a result of the terminally directed activity. When the reaction is carried out at 30 °C instead of 37 °C, the rates of endonucleolytic cleavage and acid-soluble production are reduced, but the same size distribution is obtained at the digestion limit.

Agarose gel electrophoresis was also used to follow the reaction of mung bean nuclease with linear duplex PM2 DNA (form III) which was generated by a single, site-specific cleavage of superhelical PM2 DNA (form I) with the restriction endonuclease *Hpa*II (Wang, 1974). A stage in the reaction with PM2 form III DNA was also seen in which the product size distribution was constant with time. The molecular weight range of this distribution was the same as that observed with T_7 and gh-1 DNAs.

The values of the average molecular weight at the limit of digestion of the three bacteriophage DNAs obtained from analysis of direct fluorometric scans of ethidium-stained agarose gels are shown in Table I along with other data. The number of cleavages/molecule correlate with the original molecular size of the DNA and are independent of the overall A + T content (Table I).

Uniqueness of the Endonucleolytic Cleavages. Given the limited number of sites in native DNA susceptible to the endonucleolytic action of mung bean nuclease, the question naturally arises as to whether they are unique (i.e., at fixed intervals from the ends of every molecule) or randomized among all the molecules. If they were truly unique as in the case of the sites recognized by the restriction endonucleases, then one would expect to see discrete bands when limit products were electrophoresed on agarose gels instead of the single broad band observed. However, the observation of discrete bands is complicated by (1) the continuous, exonuclease-like activity of the enzyme on all termini (Kroeker et al., 1976), (2) the relatively narrow molecular weight range (~ 1.4–0.2×10^6) of the limit digestion products, and (3) the possible existence of a slow, random endonuclease activity. Each of these complications can be circumvented if the reaction products are examined at the early stages of hydrolysis (one to two cleavages/molecule) where the fraction of DNA rendered acid soluble is very small (<0.3%), the products range in size from that of the intact DNA to the lower limit of ca. 200 000 daltons, and the fraction of the total cleavages produced by a slow reaction at random sites is minimal. Under these conditions, discrete bands are observed on agarose gels with all three bacteriophage DNAs tested.

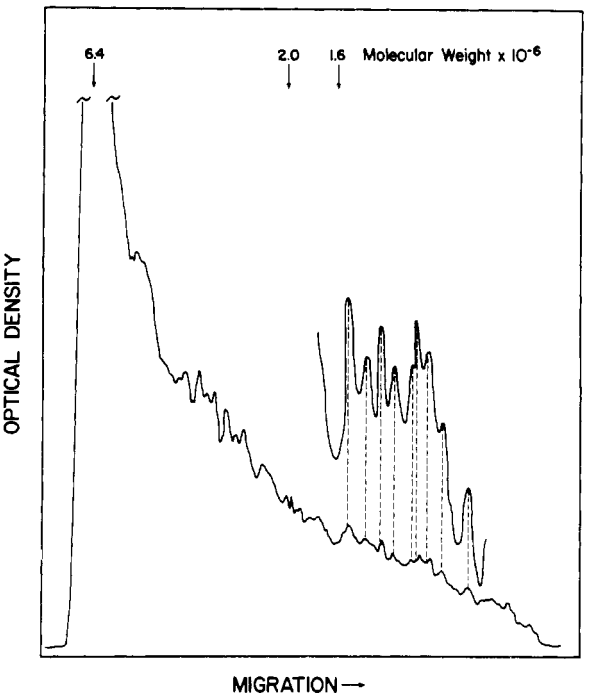

FIGURE 3: Agarose gel electrophoresis of the early digestion products of PM2 form III DNA. Mung bean nuclease (5 units/mL) was incubated with PM2 form III DNA (125 μg/mL) for 10 min as described in Figure 2. The reaction was stopped, the solution was prepared for electrophoresis, and aliquots containing either 1 or 2 μg of DNA were electrophoresed on 1.0% agarose gels as described in Figure 2. The gels were stained with ethidium, two photographs were taken using Polaroid Type 55 P/N film, and the negatives were scanned at 550 nm as described under Methods. The scans were refined to eliminate noise produced by imperfections in the negatives by superimposing, in turn, the two scans of the gel containing the 1-μg sample, the 2-μg sample, and, finally, the refined scans of the 1- and 2-μg samples. In each superposition, the fingerprint of the digestion products was readily apparent while small "peaks" due to film imperfections occurred at random positions and at low frequency. Shown in the figure is the refined scan of the 2-μg DNA sample. The inset is a 5× scale expansion. Molecular weight markers (indicated by arrows) are PM2 form III DNA (6.4×10^6) and two DNA fragments from an *Eco*RI digest of $T_5(+)$ DNA.

The products obtained at various times during the early stages of digestion of linear PM2 DNA were electrophoresed through 1% agarose gels. The gels were stained and photographed, and the negatives were scanned as described under Methods. A scan of the electrophoretic pattern seen after 10-min digestion is shown in Figure 3. The pattern shows a large number of discrete peaks. The distance of any given peak from the origin was identical for 5, 10, and 20 min of digestion. The characteristic banding pattern was also observed in the early stages of digestion at various temperatures (10–37 °C),

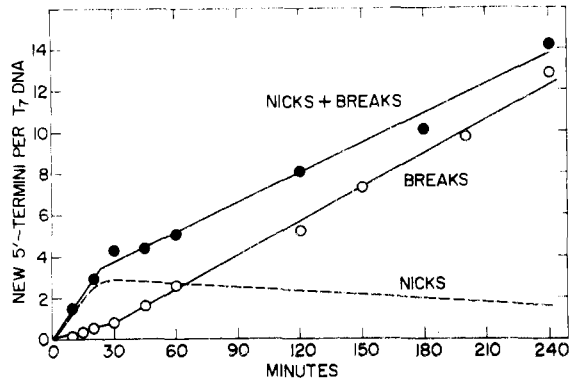

FIGURE 4: Early time course for the production of new 5′ termini per T_7 DNA resulting from double-strand cleavages (○, breaks) and nicks plus breaks (●) catalyzed by mung bean nuclease. The difference between the two lines (---) represents new 5′ termini/T_7 DNA resulting from nicks alone. The reaction (0.40 mL) was conducted at 37 °C and consisted of 245 μg/mL T_7 DNA, 3 units/mL mung bean nuclease, 50 mM sodium acetate (pH 5.0), 30 mM NaCl, 1.0 mM L-serine, 10 μM zinc acetate, and 0.001% Triton X-100. The number of nicks plus breaks (●) and breaks (○) were determined (see Methods) at the indicated times. A parallel reaction was run with T_7 [^{33}P]DNA (840 cpm/μg) and the percent acid-soluble products determined (see Methods) at 21, 87, 150, and 240 min were 0.10, 0.25, 0.50 and 1.85%, respectively.

TABLE II: Effects of Various Conditions on the Initial Rates for the Endonucleolytic Hydrolysis of T_7 DNA as determined by Viscometric Analysis.

conditions[a]	rate[b] (*p* molar cleavages/h) 1st phase	2nd phase
[DNA], μg/mL		
25	0.48	0.76
50	0.88	1.44
100	2.08	2.68
temp, °C		
20	0.28	0.41
30	0.88	1.44
40	8.40	10.68
[salt], M		
0.10	0.88	1.44
0.12	0.76	0.76
0.14	0.60	0.32

[a] Except for the parameter varied, all other conditions were: T_7 DNA, 50 μg/mL; mung bean nuclease, 5 units/mL; temperature, 30 °C; pH 5.0; salt concentration, 0.05 M sodium acetate, 0.05 M NaCl ([salt] was varied by changing [NaCl]). [b] The average number of cleavages per T_7 DNA molecule was calculated from average molecular weight data (see Experimental Procedures) and multiplied by the T_7 DNA concentration to derive *p* molar cleavages/h.

salt concentrations (0.08–0.14 M), and molar ratios of enzyme to DNA (1:1–5:1). The observation of unique fragments in the early stages of the reaction of mung bean nuclease with PM2 form III DNA coupled with the limited nature of the reaction suggests that the estimated ten cleavages/molecule at the limit of digestion (Table I) occur at specific sites.

Under conditions in which the formation of the discrete bands of DNA observed on agarose gels is slow (e.g., 30 °C, 0.14 M salt, pH 5.0, 5 units/mL, 200 μg of DNA/mL), a more rapid hydrolytic event could be detected. In the case of T_7 DNA, agarose gel electrophoresis shows a smear of DNA ranging between intact DNA and ca. 0.6×10^6 daltons. The number of sites per molecule which are responsible for this early hydrolytic event is small relative to the number of unique sites, since 25–50% of the T_7 DNA remains intact when the early hydrolytic event ceases (1 h under the above conditions). With PM2 DNA, the smear of DNA seen on agarose gels is less apparent then that seen with T_7 DNA and ca. 75% of the DNA molecules remain intact. This early, apparently random, cleavage of some of the DNA molecules provides a background of fluorescence on the ethidium-stained agarose gels which tends to obscure the discrete banding pattern.

Early Velocity Measurements for the Endonucleolytic Hydrolysis of T_7 DNA. Initial rates for the hydrolysis of T_7 DNA were determined by viscometric analysis as described under Experimental Procedures. This type of analysis led to linear initial rates when the hydrolysis of T_7 DNA was catalyzed by micrococcal nuclease or *Hpa*II restriction endonuclease. With mung bean nuclease, however, the time course consisted of two linear phases, the second of which began between 0.5 and 0.8 cleavages per T_7 DNA molecule. The time course closely resembles that observed for double-strand cleavages determined by the ^{32}P end-labeling procedure (described below, see Figure 4). Both the first and second phase rates were proportional to enzyme concentration over the fivefold range examined.

Table II shows the effect of DNA and salt concentration as well as temperature on the early rates of cleavage of T_7 DNA. The fourfold increase in substrate produces a proportional increase in both the first and second phase rates. Thus, these substrate concentrations are well below saturation. A temperature increase from 20 to 30 °C shows a threefold rate increase in both phases of the reaction, approximately that expected from the thermal activation of the enzyme-catalyzed reaction. However, an additional 10 °C increase in temperature results in a nearly tenfold rate increase in both phases of the reaction, suggesting a major change in the substrate structure. Unlike the other parameters investigated, the salt concentration had a pronounced differential effect on the rates of the two phases. The first phase is only slightly inhibited by increased salt, while the second phase is quite sensitive to the concentration of salt present. The salt sensitivity of the second phase is not the result of inactivation of the enzyme, since it remains 100% active toward denatured DNA as assayed in situ during the second phase of the reaction with native DNA. The different effect of salt on the two rates suggests the possibility of separate classes of sites in T_7 DNA. The endonucleolytic hydrolysis of almost all of these sites is salt sensitive.

Mechanism of the Double-Strand Cleavage. The double-strand cleavage of bihelical DNA by a single-strand-specific enzyme could be produced through two possible mechanistic extremes, namely: (1) the accumulation of nicks at potential double-strand cleavage sites until two nicks on opposite strands are either coincident or proximate or (2) the rapid and preferred cleavage of the strand opposite a preexisting nick. Using the ^{32}P end-labeling procedure (see Experimental Procedures), the number of double-strand cleavages (breaks) and the number of nicks plus breaks were measured as a function of reaction time of T_7 DNA and mung bean nuclease. The data are presented in Figure 4 as the number of new 5′ termini per T_7 DNA resulting from breaks (⊙) and those resulting from nicks plus breaks (●). The difference between the two lines (dashed line) represents new 5′ termini/T_7 DNA resulting from nicks alone. Nicks do not accumulate at the 37 potential cleavage sites but reach a maximum of only three nicks/T_7 DNA at 30 min and slowly decrease in number thereafter. The introduction of breaks initially lags behind that of nicks but then increases at a constant rate during the steady-state nicking period. Thus, the enzyme catalyzes double-strand cleavages at a limited number of sites in T_7 DNA by the preferred cleavage of the strand opposite a preexisting nick.

Cleavage at Nicks and Gaps in Duplex DNA. To determine if nicks that were not produced by the enzyme would also be preferred cleavage sites, T_5st(o) DNA, which possesses nicks in one of its strands, was used as a substrate. Using the positions of the three primary nicks (Hayward, 1974), the weight average molecular weight of the duplex fragments obtained by the hypothetical cleavage at these nicks is 28×10^6. Using this value as an "end point", the molecular weight as a function of incubation time was determined by viscometric analysis. As shown in Figure 5, with 2 units/mL mung bean nuclease (closed triangles), there is no rapid drop in molecular weight to a limit value around 28×10^6 but rather a slow and continuous reduction to 11.6×10^6 after 3 h. This result indicates the lack of a high preference of mung bean nuclease for the phosphodiester bonds in the strand opposite the T_5st(o) nicks.

In order to determine approximately how many nucleotides must be deleted from the T_5 DNA nicks to create a highly susceptible site for cleavage by mung bean nuclease, we have used *E. coli* exonuclease III to elongate the nicks into gaps of defined size (see Experimental Procedures). T_5 DNA possessing gaps of approximately 40 nucleotides in length was incubated with mung bean nuclease, and the size of the DNA as a function of time is plotted in Figure 5. With 2 units/mL of enzyme (closed circles), the DNA is very rapidly degraded to an average size of $\sim 30 \times 10^6$ daltons followed by a much slower size reduction. This second slow reaction is eliminated if the level of mung bean nuclease is 0.2 unit/mL (open triangles). Using still lower levels of mung bean nuclease (0.03 unit/mL), the time required to reach the limit molecular weight was sufficiently long to permit a measurement of the initial rate. These rates were determined as a function of the number of nucleotides deleted from the T_5 DNA nicked sites. When the average number of deleted nucleotides per nick was 5, 10, 20, and 40, the rate was 84, 116, 183, and 577 times as fast, respectively, as the rate of cleavage of T_5st(o) DNA without prior exonuclease treatment. Therefore, the removal of only a few nucleotides at the nicked sites of T_5 DNA creates a highly favorable region for the endonucleolytic cleavage catalyzed by mung bean nuclease.

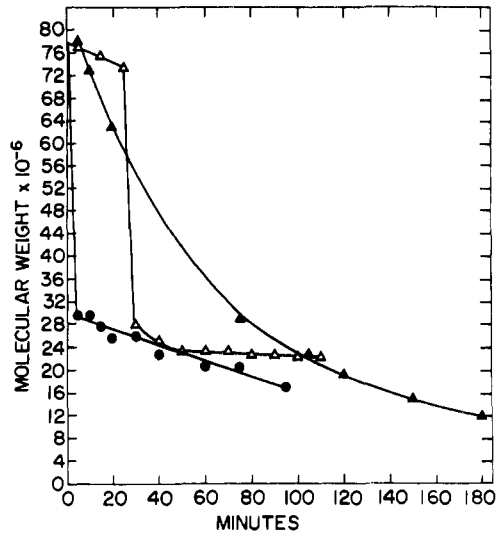

FIGURE 5: Effects of incubation with mung bean nuclease on the molecular weight of T_5st(o) DNA, with and without prior *E. coli* exonuclease III treatment: (▲) no exonuclease III, 2 units/mL mung bean nuclease; (●) 10 units/mL exonuclease III, 2 units/mL mung bean nuclease; (△) 10 units/mL exonuclease III, 0.2 unit/mL mung bean nuclease (added at 26 min). Exonuclease III reactions (50 μL) were carried out at 30 °C in the presence of 300 μg/mL T_5st(o) DNA, 66 mM Tris-HCl (pH 8.0), 0.66 mM $MgCl_2$, 1.0 mM 2-mercaptoethanol. The reaction was stopped after 45 min by the addition of 0.45 mL of a solution of 55 mM sodium acetate (pH 5.0), 55 mM NaCl, 1.1 mM L-serine, 11 μM zinc acetate, and 0.001% Triton X-100. The reaction with mung bean nuclease was followed in the viscometer at 30 °C and the viscosity readings at the indicated times were converted to molecular weight (see Methods). Control reactions in the absence of mung bean nuclease resulted in less than 5% of the rate observed at enzyme concentrations of 2 units/mL.

Discussion

Hydrolysis of Linear Duplex DNA into Gene-Sized Pieces. The linear duplex DNAs from the bacteriophages T_7, gh-1, and PM2 each contain a limited number of sites which have sufficient "single-strand character" to be cleaved by mung bean nuclease. The size range of the limit products from each of the DNAs is identical (~300–2100 base pairs) even though the DNA substrates differ widely in molecular size and percent A + T (Table I). This size range encompasses that of almost all of the known structural genes in T_7 DNA (see below). The available evidence suggests that the cleavage sites are at specific loci. It is unlikely that a single, specific nucleotide sequence is cleaved at one position. Unfortunately, the terminally directed activity of the enzyme prohibits the determination of the base sequence of the sites.

Several models for sites in duplex DNA which are cleaved by a single-strand-specific nuclease are (for a review see Wells et al., 1977): (1) thermolabile or structural "breathing" regions, i.e., regions rich in A + T (von Hippel and Felsenfeld, 1964; Wingert and von Hippel, 1968) or regions which contain modified nucleotides (Chan and Wells, 1974); (2) unpaired bases, as in cruciforms (Gierer, 1966), kinks (Crick and Klug, 1975), and base mismatches (Shenk et al., 1975); and (3) duplex but non-DNA B-type helix, i.e., regions with altered helical pitch or altered tilt of base pairs (Chan and Wells, 1974).

The thermolabile region model is consistent with all of the data presented here. This model accounts for the sensitivity of the rate of hydrolysis to both salt concentration and temperature (Table II). Also, it accounts for the preferred cleavage across from enzyme-inflicted nicks in T_7 DNA, since introducing a nick into a duplex region would increase its thermolability. Alternatively, binding of the enzyme to the strand to be nicked could also facilitate cleavage in the opposite strand by the same or by an additional enzyme molecule. Finally, indirect evidence supporting the thermolabile region model is the lack of preference of the enzyme for nicks in regions which are *not* thermolabile (see below).

Given the known preference of the enzyme for A↓pN and T↓pN linkages (Sung and Laskowski, 1962), the thermolability of the cleavage sites is probably due to enriched A + T sequences rather than the presence of modified nucleotides. Comparison of the number of enzyme susceptible sites with the number of A + T rich blocks determined by electron microscopy partial-denaturation mapping (Inman and Schnös, 1970) shows that there are more susceptible sites than there are A + T rich blocks. T_7 DNA has 37 susceptible sites and only 13 A + T rich blocks (Gomez and Lang, 1972). PM2 form III DNA has ca. ten susceptible sites and eight A + T rich blocks (Brack et al., 1975). Of these two estimates of susceptible sites, the number for T_7 DNA obtained by ^{32}P end-labeling is more reliable. Thus, the enzyme probably recognizes additional A + T rich sites which are too small to be mapped in the electron microscope and/or additional sites of a different nature.

The nonrandom distribution and low frequency of occurrence of the sites recognized by mung bean nuclease suggest

that they may serve some biological function(s). Furthermore, the identical size distribution of limit products derived from T_7, gh-1, and PM2 form III DNAs suggests that this function(s) may be common to the three bacteriophage DNAs. For T_7 DNA, the number of sites (37) and the size range (~300–2100 base pairs) of the limit products are similar to the number of genes (~30; Studier, 1972) and their size range (21 genes of 230–2200 base pairs; 2 genes of 2600 and 3900 base pairs; calculated from the known molecular weights of proteins specified by T_7 genes; Studier, 1972). The known occurrence of sizeable A + T rich regions between some of the T_7 genes suggests the possibility that some of the gene-sized pieces produced by mung bean nuclease digestion may in fact be individual genes. Base sequences 8–11 residues in length and ~90% A + U have been found in the intercistronic regions of the polycistronic mRNA transcribed from the early region of the T_7 genome (Rosenburg et al., 1974; Rosenburg and Kramer, 1977; Robertson et al., 1977). In each intercistronic region, one A + U rich sequence corresponds to the *E. coli* ribonuclease III cleavage site. Two additional A + U rich regions (eight to nine residues, ~90% A + U) are seen in the extensively sequenced mRNA coded by the gene 0.3–0.7 junction (Rosenburg and Kramer, 1977).

Hydrolysis of Linear Duplex DNA Containing Nicks or Gaps. The experiments performed with T_5 DNA show that a nicked site in itself is not sufficient for the recognition of duplex DNA by mung bean nuclease. The major nicks in T_5(+) DNA contain the sequence 5′-p-G·C·G·C– in common (Nichols and Donelson, 1977a). Where determined, the 3′-terminal sequence is –R·R·A-OH, in which R = G at two major nicks and R = A at one (Nichols and Donelson, 1977b; Brian Nichols, personal communication). The site where R = A is situated such that preferential cleavage there would produce only an 8% decrease in the molecular weight of T_5 DNA and thus might go undetected. The sites where R = G are not preferred cleavage sites, presumably due to their G + C rich nature which would tend to minimize structural breathing.

In contrast to mung bean nuclease, S_1 nuclease preferentially cleaves T_5 DNA at the major nicks (Shishido and Ando, 1975; Weigand et al., 1975) and converts nicked circular DNA to the linear duplex form (Beard et al., 1973). It has not been shown whether S_1 nuclease actually recognizes a nicked site itself or, alternatively, recognizes a gap which is the result of intrinsic and/or contaminating exonuclease action at the nicked site. As shown here, exonuclease III removal of only a few nucleotides from the 3′ terminus of the nicked sites in T_5 DNA creates a highly favorable cleavage site for mung bean nuclease. The lack of preferential cleavage at nicks in T_5 DNA coupled with the preferential cleavage at gaps shows that for mung bean nuclease the level of contaminating exonuclease activity is very low and the intrinsic, terminally directed activity of the enzyme is not very efficient in the removal of nucleotides from the nicked sites in T_5 DNA.

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Procollagen Complementary DNA, a Probe for Messenger RNA Purification and the Number of Type I Collagen Genes†

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ABSTRACT: Type I procollagen mRNAs were separated from contaminating low-abundance messenger and nuclear RNAs by chromatography over Sepharose 4B in 0.65 M NaCl at room temperature. All of 27S rRNA and four-fifths of procollagen mRNAs bind to Sepharose under these conditions, while 18S rRNA and about three-fourths of other poly(A)-containing RNAs do not bind. AMV reverse transcriptase was used to prepare complementary DNA to procollagen mRNA at each purification step. Hybridization studies, in RNA excess, were carried out to establish the enrichment at each step both with respect to total RNA and to poly(A)-containing RNA. While "purified" procollagen mRNA preparations still consist of about 50% 27S rRNA, over 80% of cDNA prepared from it back hybridizes to its template at a log $cr_0t_{1/2}$ of -1.9. This type I procollagen cDNA hybridizes in DNA excess to DNA isolated from chicken erythrocytes and from embryonic chick calvaria at a log $c_0t_{1/2}$ of 3.1, demonstrating that procollagen cDNA is complementary to unique gene sequences in both tissues and that procollagen genes are not reiterated.

At least four different types of collagen are expressed in different tissues of the same organism or in the same tissue at different developmental stages (Miller and Matukas, 1974; Gross, 1973; Miller, 1976). The expression of these collagen genes is closely coupled to major differentiational events starting in gastrula (Green et al., 1968; Golob et al., 1974) and thus offers an extremely interesting system for studying the regulation of gene expression and its role in development in eucaryotes.

The isolation and characterization of procollagen mRNAs constitute an essential prerequisite to the study of control of the expression of these genes. We report here the extension of the initial purification of the two mRNAs coding for type I pro-α1 and pro-α2 collagen (Boedtker et al., 1976) resulting in an mRNA preparation which is essentially free of contaminating poly(A)-containing RNA species. AMV reverse transcriptase was used to synthesize complementary DNA to both purified procollagen mRNAs and to partly purified preparations to provide a quantitative determination of both the yields and the purification at each step.

cDNA prepared from purified procollagen mRNA was used to investigate the possibility of collagen gene amplification in both collagen and noncollagen producing tissues.

Materials and Methods

Isolation of Procollagen mRNAs. Type I procollagen mRNAs were isolated as described previously (Boedtker et al., 1976), except the 0.1 M KCl wash was omitted in the oligo(dT)-cellulose chromatography of total calvaria RNA, and the poly(A)-containing RNA was chromatographed over Sepharose 4B as described below.

Isolation of Embryonic Chick Calvaria DNA. Total calvaria RNA was separated from calvaria DNA following phenol extraction by pelleting through a cushion of 6.1 M CsCl, a modification of the procedure described by Glisin et al. (1974). The CsCl containing the DNA was pooled, dialyzed against 3 mM Na_2EDTA (pH 7.0) at 4 °C for 3 days, and precipitated with 3 volumes of absolute ethanol.

Isolation of Chick Erythrocyte DNA. Reticulocytes from an adult anemic chicken were frozen in liquid nitrogen. DNA was isolated according to the procedure of Sullivan et al. (1973). After alcohol precipitation, the DNA was dissolved in 0.1 M NaCl, 0.05 M Tris-HCl,[1] 10 mM Na_2EDTA (pH 7.0) and incubated overnight at 37 °C in RNase (100 μg/mL). The RNase was then removed by phenol extraction followed by dialysis against 3 mM Na_2EDTA (pH 7.0).

Sepharose 4B Chromatography. Sepharose 4B (Pharmacia, Sigma) was washed with diethyl pyrocarbonate (100 ppm), using 50 mL for a 10-mL column. After thoroughly rinsing with sterile deionized water, the column was equilibrated with 0.65 M NaCl, 0.02 M Tris-HCl (pH 7.5), 2.5 mM Na_2EDTA (pH 7.5), and 0.1% recrystallized Sarkosyl (ICN, K & K Laboratories, Inc., Plainview, N.Y.) at room temperature.

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[1] Abbreviations used are: Tris-HCl, 2-amino-2-hydroxymethyl-1,3-propanediol hydrochloride; Na_2EDTA, disodium (ethylenedinitrilo)-tetraacetic acid; DTT, dithiothreitol; $NaDodSO_4$, sodium dodecyl sulfate.

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