Specific Limited Cleavage of Bihelical Deoxyribonucleic Acid by Wheat Seedling Nuclease*

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SUMMARY

Wheat seedling nuclease catalyzes the hydrolysis of intact, bihelical viral DNA or high molecular weight, native Escherichia coli DNA to produce limit polymers which are resistant to further hydrolysis by additional enzyme. These limit products are double-stranded polymers free of single strand interruptions and are terminated at their 5' ends with equal products. The average size of the duplex limit products, as determined by (a) alkaline and neutral sucrose gradient sedimentation, (b) viscometric determination of molecular weight, and (c) 5'-end labeling, varies from 2 to 4 \times 10^6 depending on the source of the DNA.

The involvement of regions rich in adenine-thymine base pairs at the sites of cleavage of the DNA molecule is suggested by the following experimental results: (a) the co-polymargenic duplex, poly(dA-dT) is hydrolyzed at a rate comparable to that found for denatured calf thymus DNA, a rate which is several orders of magnitude faster than that at which native calf thymus DNA is hydrolyzed; (b) \( \lambda \) DNA, which contains an adenine-thymine-rich region near its center, is rapidly cleaved to yield two fragments of similar size; (c) the rate of hydrolysis of native DNA is increased approximately 14-fold by increasing the reaction temperature from 20° to 30°.

Bacterial and bacteriophage genetics have progressed to the point where it is now feasible to examine a number of genetic problems with a biochemical approach utilizing enzymatic methods. In experiments of this type, nucleases with a high degree of specificity can be useful tools.

The ability to produce specific fragments of high molecular weight from intact viral DNA molecules has been demonstrated for several of the bacterial restriction endonucleases (1-3). Possible uses in genetic mapping (4), nucleotide sequencing (2), and in vitro recombination (5) have been suggested for these enzymes.

Another class of nucleases which promises to have utility in probing native DNA structure or function or both are a number of nucleases originally characterized as being specific for single-stranded DNA. Such enzymes have been isolated from lamb brain (6), sheep kidney (7), yeast cells (8), Neurospora crassa conidia (9), Staphylococcus aureus (10), Aspergillus oryzae (11), mung beans (12) and wheat seedlings (13, 14).

Two of these, the \( S. \) \( aureus \) and the mung bean enzymes, have been shown to have limited effects on native DNA. These effects have been interpreted (10, 15) as the preferential hydrolysis of areas rich in deoxyadenylic and thymidylic acid units, i.e. A-T-rich areas. In addition, the \( S. \) \( aureus \) nuclease from Aspergillus oryzae has recently been shown to attack native DNA to a very limited extent (16) and may be similar in its specificity to the mung bean nuclease (17). Finally, the single strand nuclease from Neurospora crassa has been used to cleave partially denatured regions of native DNA to yield unique DNA fragments (18).

This paper presents evidence demonstrating that the wheat seedling nuclease catalyzes the hydrolysis of a variety of intact, bihelical viral DNA molecules at a limited number of specific sites. The degree of specificity is of the same order as that observed with the restriction endonucleases. Evidence is also presented which suggests that the sites susceptible to hydrolytic cleavage are A-T-rich areas.

MATERIALS AND METHODS

Enzymes—Wheat seedling nuclease Type II (14) with a specific activity of about 4000 units per mg of protein was used in all of the experiments described in this paper. A unit of the nuclease is defined as that amount which catalyzes in 10 min the formation of lanthanum acid-soluble material with an absorbance of 1.0 per ml of incubation mixture. Polynucleotide kinase was purified through the phosphocellulose step as described by Richardson (19). The specific activity of this preparation was 51,000 units per mg. Alkaline phosphatase, Type III (25 units per mg) was purchased from Sigma and was carried through an additional purification by chromatography on a DEAE-cellulose column (20). DNase I (2500 units per mg, code DPF7) and venom phosphodiesterase (Code VPII, potency of 0.3) were obtained from Worthington. Trace amounts of 5-nucleotidase were removed from the phosphodiesterase by the method of Sukowski and Laskowski (21).

Nucleic Acid—Calf thymus DNA, Type I was purchased from Sigma. Poly(dA-dT) and poly(dG)-poly(dC) were products of Miles Laboratories. High molecular weight Escherichia coli DNA was prepared by Dr. D. M. Hanson by a modification (22) of the method of Bres and Thomas (23).

DNA from each of the bacteriophages, T4, T7, \( \lambda \), and \( gh-1 \) was prepared by the following procedure. Na dodecyl-SO4 was added to a phage suspension (the concentration of phage was adjusted to give 10 to 25 \( A \) units at 260 nm per ml) to give a final concentration of 0.25%. The suspension was then heated to 60° for 3 min. The DNA was extracted three times with freshly distilled,
Bacteria and Bacteriophages—Bacteriophages T4 and T7 were produced by infecting the appropriate strains of E. coli. Following cell lysis, the phages were purified by differential centrifugation and banding in CsCl gradients. Bacteriophage λ was obtained by thermal induction of a temperature sensitive mutant of E. coli, C58 Cl8/875. The phages were purified in a manner analogous to that used for the T4 and T7 phages.

The bacteriophage, gh-1, was grown and purified from Pseudomonas putida by the method of Lee and Boezi (24).

Spectrophotometric Assays—In certain instances the increase in absorbance at 280 nm was used to measure the rate of hydrolysis of nucleic acid polymers by wheat seedling nuclease. These measurements were made in a Beckman DB spectrophotometer with the aid of a Sargent model SRL recorder. Rates were observed in the 0.5 to 1.5 optical density range and, except when noted, were linear for at least the first minute. Initial rates are reported as the increase in Α280 units per min.

Viscosity Assay—The reduction in molecular weight of E. coli DNA during hydrolysis by wheat seedling nuclease was examined by viscometry. Viscosities were determined with a Contraves Low-Shear Rheometer type LS100 (Contraves AG, Zurich) with a minimum shear rate of 0.07 per s. At the times specified, 0.35 ml of reaction mixture was placed in the viscometer. Viscometer values at various shear rates were recorded and essentially no shear dependence was noted at the lower shear rates. Molecular weight determinations were facilitated from the empirical equation of Eiger (25) where k, the concentration dependency constant, was 0.6.

Sedimentation of DNA in Neutral Sucrose Gradients—Alkaline sucrose gradients were prepared by forming a 4.5-ml linear gradient of 5 to 20% sucrose which was 0.9 M in NaCl and 0.1 M in NaOH. DNA samples (0.1 ml) were allowed to remain 30 min in 0.9 M NaCl, 0.1 M NaOH and then were layered on top of the gradient with polyethylene tubing (1.0 mm inside diameter) and a syringe microviscometer. The tubes were placed in a SW-39 rotor and spun in a Beckman Model L-2 centrifuge for 3 hours at 35,000 rpm at 20°C. In the case of 32P-labeled DNA, 1 to 5 µg of DNA was applied to the gradient and 30 fractions were collected. The 32P content of each fraction was determined either directly as Cerenkov radiation in a liquid scintillation counter. The polymers were then reduced to nucleotides, the mixture was separated on a Dowex 1 column and the percentage of the radioactivity in each nucleotide peak was determined.

RESULTS

Hydrolysis of Synthetic Polymers—The rates of hydrolysis of various nucleic acid polymers were measured by monitoring the increase in absorbance at 260 nm as described under “Materials and Methods.” The reactions were performed at 30°C in 0.04 M cacodylate buffer, pH 5, with 2.4 units of wheat seedling nuclease. Under these conditions the synthetic duplex, poly(dA-dT), was rapidly hydrolyzed by wheat seedling nuclease at a rate comparable to denatured calf thymus DNA. On the other hand, neither the homopolymeric duplex, poly(dG-dC), poly(dC), nor native calf thymus DNA produced any detectable hyperchromicity under the same incubation conditions.

Fig. 1 shows the effects of pH and ionic strength on the rate of hydrolysis of poly(dA-dT) and denatured DNA. An increase in ionic strength is seen to inhibit the hydrolysis of both polymeric substrates. Although the hydrolysis of denatured DNA has a distinct pH optimum at 5.3, the rate for poly(dA-dT) hydrolysis continues to increase with a decrease in pH, probably reflecting the localized denaturation of the substrate to form small single strand areas. The Tm for poly(dA-dT) under these conditions was determined to be at least 20°C above the incubation temperature of 30°C. Therefore, the synthetic polymer existed predominantly in the duplex form in all of these experiments.

Formation of Limit Polymers—Fig. 2 shows the time course for the hydrolysis of native Escherichia coli DNA by wheat seedling nuclease. The progress of the reaction was followed by calculat-
Fig. 2. Time course for the decrease in molecular weight during the hydrolysis of native *Escherichia coli* DNA by wheat seedling nuclease. The reaction mixture contained 0.08 ml of 0.5 m sodium acetate buffer (pH 5.0), 0.2 ml of 0.1 m 2-mercaptoethanol, and 1.92 ml of *E. coli* DNA (20.5 pg/ml in 0.2 m NaCl) plus 10.5 DNase units (2.6 ng) of enzyme. The reaction was carried out at 22°C and an additional 10.5 units of enzyme was added at 4 hours. At the time indicated, 0.35-ml aliquots were removed and placed in the viscometer. Viscosities and molecular weights were determined as described under "Materials and Methods." The inset in the figure is a replot of the same data after subtracting the "limit" molecular weight of the reaction products and taking the logarithm at the viscometer. Viscosimetric analysis used for determination.

Fig. 3. Neutral sucrose gradient sedimentation of gh-1 DNA and its reaction products. The reaction mixture contained 0.05 ml of 32P-labeled gh-1 DNA (445 pg/ml, 117 cpn/pg), 0.015 ml of 10 mM 2-mercaptoethanol, 0.045 ml of H2O, and 0.15 ng of enzyme (0.5 DNase units). The reaction was conducted at 22°C and 0.1-ml aliquots were removed at the times indicated and combined with 0.025 ml of 1.5 mM Tris base. Neutral sucrose gradient sedimentation was accomplished as described under "Materials and Methods." The S value and molecular weight calibration shown on the figure is based on the sedimentation of gh-1 DNA as a marker (31 S, M, = 23 X 10^6).

TABLE I

| Source | Original DNA | Limit polymer |
|--------|--------------|---------------|
|        | Single strand | Double strand |
| *Escherichia coli* | 75 X 10^6 | 2.6 X 10^6 |
| T7     | 75 X 10^6 | 1.2 X 10^6 |
| T4     | 75 X 10^6 | 0.9 X 10^6 |
| λ      | 75 X 10^6 | 1.05 X 10^6 |
| gh-1   | 75 X 10^6 | 1.7 X 10^6 |
| gh-1   | 75 X 10^6 | 1.5 X 10^6 |

Viscosimetric analysis used for determination.

Alkaline sucrose gradient centrifugation used for determination.

Neutral sucrose gradient sedimentation used for determination.

32P end labeling used for determination.

Agarose gel electrophoresis used for determination.

When gh-1 DNA, a linear duplex molecule of molecular weight 23 X 10^6 with 57% G + C (29), was incubated with the wheat seedling nuclease, a very limited amount of hydrolysis was again observed. Fig. 3 shows the results of sedimenting the reaction products from such an incubation in neutral sucrose gradients. In a 12-hour incubation period, the gh-1 DNA was reduced to fragments with an average molecular weight of 3 X 10^6. No further reduction in size occurred over a 24-hour period in the presence of additional enzyme.

The formation of limit polymers was observed for all of the native, intact viral DNAs tested as well as for high molecular weight *E. coli* DNA. Table I lists the average molecular weights obtained for the various limit polymers. Both alkaline and neutral sucrose gradient sedimentations were performed on the limit polymers from gh-1 and λ DNA. The molecular weights obtained from the alkaline sucrose gradient data were very close to half the values obtained from the neutral sucrose gradients. Since the polymers are sedimented as single-stranded molecules in the alkaline gradients, these molecular weight values indicate that the limit polymers are intact double-stranded molecules free of any single strand breaks. Therefore it is apparent that wheat seedling nuclease produces only double strand scissions in the native DNA molecules.

As can be noted from the table, the number-average molecular weight of the gh-1 DNA limit polymers as determined by the end-labeling experiment agrees rather closely with the weight average molecular weight determined by sucrose sedimentation experiments. These results would be expected if the limit polymer molecules were all essentially the same size. In order to further explore this possibility, the limit polymers from gh-1 DNA were subjected to electrophoresis through agarose gels according to the method of Sharp et al. (27). More than 80% of the DNA was judged to migrate within ±0.5 X 10^6 daltons of the Φ-X174 marker DNA. These results indicate a roughly uniform size for the limit polymers; however, a more precise evaluation of homogeneity awaits further experimentation.

Acid-Soluble Products—Assays based on the formation of acid-
soluble material were performed on the reaction products from the limit digestion of gh-1 DNA. The results from these assays are shown in Table II. At incubation temperatures of 22° and 37° only a negligible amount of acid-soluble material was detected. This amount of material, 0.04% and 0.07%, respectively, of the total nucleic acid, represents the lower limit of detectability under these experimental conditions, corresponding to fewer than eight nucleotide equivalents released per limit polymer formed. The molecular weight values indicate that at both temperatures the digestion had proceeded to the limit digest stage.

5'-End Labeling of Limit Polymers—The 5'-terminal nucleotides of the gh-1 DNA limit polymers, as well as intact gh-1 DNA, were labeled with 32P by the action of polynucleotide kinase as described under “Materials and Methods.” In an experiment in which an amount of gh-1 DNA equivalent to 86 nmol of nucleotide was labeled, assuming an average molecular weight of 350 for the nucleotide components, 600 cpm were incorporated. From these data and the specific activity of the [γ-32P]ATP (2.38 x 10⁶ cpm/μmol), it was calculated that only one of 3.4 x 10⁶ nucleotides was phosphorylated. If indeed one nucleotide was labeled per DNA chain, the molecular weight of the chain would be about 12 x 10⁶. This value is in very good agreement with the known molecular weight of 23 x 10⁶ for duplex gh-1 DNA.

In a similar experiment in which the limit polymers formed by the nuclease from an amount of gh-1 DNA corresponding to 80 nmol of nucleotide were labeled, the extent of 32P incorporation was 4450 cpm. These data yield an average chain length of 4.3 x 10⁶ nucleotides and an average molecular weight of 1.5 x 10⁶, a value essentially the same as determined by other methods indicated in Table I.

Control reactions run in the absence of alkaline phosphatase indicated that the activity of this enzyme was required for complete labeling during the subsequent kinase reaction. This observation is consistent with the likelihood that wheat seedling nuclease produces reaction products from native gh-1 DNA bearing 5'-terminal phosphate groups. Furthermore, the data above indicate strongly that essentially complete, specific labeling of the 5' ends of the polynucleotide chains was accomplished.

The nucleotides labeled in experiments with the gh-1 polymers were found to be predominantly the G and C components while the experiments with intact gh-1 DNA demonstrated that all four of the usual nucleotides were labeled to a major extent. The data is presented in Table III. These data indicate that the products of limited digestion bear roughly equal amounts of C and G residues at the termini.

Effect of Temperature on Hydrolysis of Native DNA—A large increase in the rate of hydrolysis of native DNA with moderate increases in temperature had been observed in earlier studies. In order to quantitate this effect, the extent of hydrolysis of λ DNA by wheat seedling nuclease at 20° and 30° was investigated. Reactions containing 30 μg of λ DNA in 0.05 ml, 0.01 ml of 0.5 m sodium acetate, pH 5.0, and 0.085 ml of H₂O, and 1 unit of wheat seedling nuclease were incubated at the two temperatures for 1 hour. Reactions were terminated by adding 0.013 ml of 1 N NaOH and 0.08 ml of 4.5 M NaCl. Samples were then sedimented in alkaline sucrose gradients as described under “Materials and Methods.” The average duplex-equivalent molecular weight of the products from the 20° incubation was 15 x 10⁶. Comparison of the molecular weights of the products with the 30 x 10⁶ molecular weight of intact λ DNA indicates that the average number of double strand scissions produced by the nuclease at 20° was 1 per DNA molecule and at 30° was 14. Since a 14-fold increase in activity is much larger than that expected from the thermal activation of the enzymatic catalysis, the increase is attributed to changes in substrate structure at a limited number of sites, presumably those rich in A-T base pairs.

### DISCUSSION

The production of large limit fragments from high molecular weight, intact DNA molecules suggests that wheat seedling nuclease recognizes and cleaves only a few, specific sites. The average size of the limit polymers indicates that fewer than 0.1% of the potentially available phosphodiester bonds are cleaved. Furthermore, these sites may be distributed with some degree of periodicity along the DNA duplex since other preliminary evidence indicates that the limit polymers formed appear to be relatively homogeneous in size.

As was pointed out in the preceding paper (14), wheat seedling
nuclease hydrolyzes denatured DNA at a much faster rate than native DNA. This observation was made on the basis of the amount of acid-soluble material released from commercial calf thymus DNA. The small amount of material released from native calf thymus DNA could have arisen from contamination with single-stranded DNA, RNA, or traces of other nucleolytic enzymes. In the main, this does appear to be the case, because the experiment with carefully prepared, native gh-1 DNA yielded only a trace of acid-soluble material. In this experiment, less than 0.07 % of the gh-1 DNA was converted to acid-soluble form during extensive incubation. This corresponds to less than eight nucleotide equivalents released per limit polymer formed.

The high degree of single strand specificity exhibited by wheat seedling nuclease raises the possibility that the enzyme might be able to recognize areas in native DNA molecules which possess a certain amount of single strand character. Von Hippel and Felsenfeld (30) have postulated a "structural breathing" phenomenon for areas in DNA which are A-T rich. They suggest that areas certain amount of single strand character. Von Hippel and Felsenfeld (30) have postulated a "structural breathing" phenomenon for areas in DNA which are A-T rich. They suggest that areas rich in A-T base pairs undergo local strand separation at temperatures under the Tm to a greater extent than areas rich in G-C. In other words, the dynamic equilibrium (single strand ⇒ double strand) lies further to the left for A-T-rich areas as compared with other areas of DNA.

The fact that wheat seedling nuclease readily degrades the co-polymeric duplex, poly(dA-dT), probably reflects this phenomenon of structural breathing. This is particularly apparent from the sharp increase in the rate of hydrolysis of this compound under conditions such as low ionic strength and low pH which would be expected to favor partial denaturation.

Other evidence which suggests that wheat seedling nuclease may recognize and cleave A-T-rich areas in native DNA is provided by experiments with λ DNA. After λ DNA is incubated with wheat seedling nuclease, sucrose gradient sedimentation of the reaction products reveals an early and rapid production of molecules which are one-half the size of λ DNA. This observation is possibly significant because λ DNA is known to contain an A-T-rich region near its center. In addition, the fact that the rate of λ DNA hydrolysis is increased 15-fold with an increase in temperature from 20° to 30°, suggests a substantial change in the base component of the nucleotide moieties in denatured DNA, RNA, and 3'-nucleoside monophosphates. Thus, the phosphate ester bonds adjacent primarily to λ and secondarily to T or U units are much more susceptible to hydrolysis than bonds adjacent to C and G moieties. Therefore, it is possible that the base preference is acting in concert with the enzyme's ability to recognize localized, temporary disruptions of the ordered structure of native DNA at AT-rich regions to result in cleavage only at specific sites.

As was pointed out in the preceding paper, wheat seedling nuclease exhibits a rather pronounced specificity with respect to the base component of the nucleotide moieties in denatured DNA, RNA, and 3'-nucleoside monophosphates. Thus, the phosphate ester bonds adjacent primarily to λ and secondarily to T or U units are much more susceptible to hydrolysis than bonds adjacent to C and G moieties. Therefore, it is possible that the base preference is acting in concert with the enzyme's ability to recognize localized, temporary disruptions of the ordered structure of native DNA at AT-rich regions to result in cleavage only at specific sites.

However, other factors may also contribute to the specificity observed in the hydrolysis of native DNA by the wheat seedling nuclease. For example, symmetry elements in the DNA or unique nucleotide sequences may define the recognition site for the enzyme. The binding of the enzyme to the DNA at AT-rich areas for example, may also contribute to the specificity of the catalytic event by enhancing the local separation of the duplex.

The following "mechanistic model" for the specific cleavage of native DNA by wheat seedling nuclease is consistent with the data obtained thus far. An A-T-rich site in the DNA molecule is recognized by the enzyme and a small number of adenylie and thymidylic acid residues (probably less than 8) are removed. Hydrolysis ceases when a G-C pair is encountered. This would account for the C and G residues found at the 3'-termini of the limit polymers.

As mentioned previously, several other single strand specific nucleases have been reported which are capable of degrading native DNA. Evidence implicating attack at A-T-rich, or partially denatured areas in native DNA has been presented for the nucleases from mung bean (12), Staphylococcus aureus (10) and Neuspora crassa (15). It remains a possibility that many of the single strand specific nucleases may behave similarly to the wheat seedling nuclease in their ability to produce "limit polymers" from high molecular weight, native DNA molecules.

Wheat seedling nuclease, readily prepared in a highly purified form, offers a potentially useful tool for probing structural or structure-function relationships in native DNA molecules. For example, prophage insertion sites (31) and promoter sites in T1 and T2 DNA (32) have been shown to possess unusually high A-T content. In addition, wheat seedling nuclease provides a tool auxiliary to the restriction endonucleases to produce large fragments from intact DNA molecules which may be useful in studies of viral genetics.

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