Purification and Properties of the recBC DNase of Escherichia coli K-12*

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SUMMARY

The deoxyribonuclease controlled by the recB and recC loci of Escherichia coli K-12 has been purified to near homogeneity. It is composed of two nonidentical polypeptide chains with a combined molecular weight of approximately 270,000. In the presence of ATP the enzyme hydrolyzes linear duplex or single stranded DNA in an exonucleolytic manner to 5'-phosphoryl-terminated oligonucleotides of an average length of 4.5. The enzyme can also cleave single stranded DNA endonucleolytically, being stimulated 7-fold by ATP in this reaction. However, no nuclease activity is observed on closed circular duplex DNA. In the course of exonuclease digestion more than 20 ATP molecules are hydrolyzed to ADP and inorganic phosphate for each DNA phosphodiester bond broken. There is no ATP hydrolysis in the absence of degradable DNA.

Many bacteria have been found to contain ATP-dependent deoxyribonuclease activities (1-7). In Escherichia coli K-12 an ATP-dependent enzyme has been linked with genetic recombination and dark repair by the discovery that mutants of either the recB or recC loci lack the in vitro activity as it is assayed in crude extracts (1, 2, 4). This nuclease (the recBC DNase) was first reported to degrade native DNA in an exonucleolytic manner in the presence of ribo- or deoxyribotriphosphates. A subsequent report from this laboratory (7) showed that E. coli K-12 also contains a nucleoside triphosphate-stimulated endonuclease which acts on single stranded DNA. This activity is also lacking in mutants of either the recB or recC loci.

The present report describes the extensive purification and some physical properties of the recBC DNase. The enzyme has both endo- and exonucleolytic DNase activities as well as ATPase activity. The reaction requirements and products of the DNase digestion of several DNA substrates are presented.

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EXPERIMENTAL PROCEDURE

Materials

Enzymes—Snake venom phosphodiesterase from Crotalus adamanteus, pancreatic DNase, and bacterial alkaline phosphatase were purchased from Worthington. The alkaline phosphatase was further purified on DEAE-cellulose (8). A unit of phosphatase releases 1 μmole of PO4 from 5'-AMP in 1 hour at 37°. Polynucleotide kinase was purified through the hydroxyapatite step and assayed according to the procedure of Richardson (9) and Jacquemin-Sablon and Richardson (10); E. coli exonuclease I was the hydroxyapatite fraction of Lehman and Nussbaum (11); endonuclease R-B (12), a sucrose gradient fraction, was the gift of B. Eskin; homogeneous E. coli DNA polymerase was kindly provided by D. Brutlag. RNA polymerase was the gift of M. Chamberlin. Marker proteins used in SDS1 gel electrophoresis were provided by D. Tienberger.

DNA—Labeled fd phage DNA was prepared as previously described (7); RFI (labeled and unlabeled) from fd 8B-1° (13) was prepared by lysis of spheroplasts according to the procedure of Komano and Sinsheimer (14) and subsequent purification on Sephadex G-150 and nitrocellulose as described by Rush and Warner (15). E. coli DNA labeled with 32P either was provided by A. J. Clark, or was prepared by the method of Lehman (16). DNA from ϕX-174 was the gift of Dan Ray; PM2 DNA was prepared as described by Espejo, Comelo, and Sinsheimer (17). Linear DNA from phage fd was prepared as previously described (7). X DNA was prepared by heat induction of E. coli B806 λ c1857, banding of the phage on a CsCl density gradient, and phenol extraction of the purified phage. The oligonucleotide markers (pT)2-pT)G were obtained from I. R. Lehman. Denatured DNA was prepared by heating at 100° for 10 min in 0.02 M NaCl at a concentration of 1 mM, then cooling by immersion in ice. DNA concentrations are expressed as nucleotide residues.

Other Materials—ATP was purchased from Sigma Chemical

1 The abbreviations used are: SDS, sodium dodecyl sulfate; fd RF, the double stranded, circular replicative form of fd DNA (RFI, covalently closed circles; RFII, circles with single strand breaks); dAT, the synthetic alternating copolymer of deoxyadenylate and deoxythymidylate; d(pT)x is an oligonucleotide composed of X number of residues of deoxyribothymidine 5'-phosphate; [γ-32P]ATP, ATP labeled with 32P in the γ-phosphoryl group; DTT, dithiothreitol (Cleland's reagent); BSA, bovine serum albumin.
Corporation; all other unlabeled nucleotides were purchased from P-L Biochemicals, Inc. [γ-32P]ATP was prepared by the method of Glynn and Chapell (18), except that the product was purified on DEAE-cellulose as described by Wehrli et al. (19). ATP labeled with 35S was purchased from Schwarz. Bovine serum albumin was purchased from Pentex, and acetylated by the method of Dowhan (20) to reduce the amount of contaminating nuclease. Catalase was from Calbiochem. Poly(ethyleneimine) cellulose thin layer plates were purchased from Brinkmann Instruments.

Enzyme grade ammonium sulfate and density gradient grade sucrose were purchased from Mann Research Laboratories. DEAE-cellulose, type 40, was obtained from The Brown Co.; DNA-cellulose was prepared by the method of Alberts et al. (21) and then irradiated in powder form with an ultraviolet lamp ("Mineralight") for 10 min at a distance of 10 cm with constant mixing. The cellulose was then washed twice with buffer by centrifugation. Hydroxylapatite was prepared by the method of Siegelman et al. (22).

Methods

DNase Assays

Endonuclease Assay—The endonuclease assay measures the transformation of the circular fd phage DNA to a form susceptible to exonuclease I. The reaction (0.15 ml) was carried out under "standard assay conditions" (0.05 mM glycylglycine-NaOH, pH 7.0, 0.01 M MgCl2, 6.7 × 10−4 M DTT, and 1 mg per ml of acetylated BSA) and included 4 nmoles of •H-labeled fd DNA (6 to 9 × 106 cpm per n mole), 0.33 mM ATP, and 2 units (11) of exonuclease I. After 30 min at 37°C, the acid-soluble products were quantitated as previously described (7). One unit of endonuclease converts 1 n mole of fd DNA nucleotide to a form susceptible to exonuclease I in 30 min. The assay is linear in the range of 0.06 to 0.75 unit. The assay for endonuclease activity in the absence of ATP is exactly as above, except that ATP is omitted.

Exonuclease Assay—The exonuclease assay measures the release of acid-soluble fragments from linear E. coli DNA, and was carried out under standard assay conditions in a volume of 0.15 ml with 4 nmoles of 32P-labeled E. coli DNA (1 to 10 × 106 cpm per n mole). Unless otherwise indicated, assays with duplex or single stranded DNA contained 60 to 200 n moles of ATP or DNA, respectively. After 30 min at 37°C, the acid-soluble products were quantitated as previously described (7). One unit of exonuclease activity releases 1 n mole of acid-soluble fragments from E. coli DNA in 30 min. The assay is linear in the range of 0.05 to 0.80 unit. The exonuclease activity is optimal at pH 7.0; however, for consistency of conditions, all exonuclease reactions have been carried out at pH 7.0.

The enzyme was diluted in a solution containing 0.01 M KPO4, pH 6.7, 1 × 10−4 M DTT, 1 × 10−4 M EDTA, 1 mg per ml of acetylated BSA, and 10% glycerol.

Polynucleotide Kinase Treatment

Reactions including polynucleotide kinase were adjusted to the following conditions: 0.05 M Tris-HCl, pH 8.2, 0.01 M MgCl2, 0.002 M KPO4, pH 6.7 (to inhibit any residual alkaline phosphatase present), and the indicated amount of ATP and polynucleotide kinase.

Determination of 32PO4 Released from ATP or DNA

The assay measures the amount of 32P which does not adsorb to Norit. The sample was diluted to 0.3 ml with water, and 0.1 ml of each of the following was added in order: 1 x HCl, 1 x KPO4, pH 6.7, 20% acid-washed Norit, and 1 mg per ml of BSA. After 5 min at 56°C, the suspension was centrifuged for 10 min at 18,000 × g and the supernatant fluid was removed for counting. A second wash of the Norit was accomplished as above, and both supernatants were counted on a gas flow counter.

Thin Layer Chromatography

Poly(ethylenimine) cellulose plates were spotted with the sample (0.002 to 0.005 ml) and unlabeled nucleotide (10 nmoles each) as marker, dried in a stream of air, and developed by ascending elution with 1 x acetic acid 4 x LiCl (8:2) for 2 hours. After air drying, the areas of the marker nucleotides were identified with an ultraviolet lamp (2537 A), then cut out and counted in a low background gas flow counter. In this system the RF values of ATP, ADP, AMP, adenosine, pyrophosphate, and orthophosphate were 0.16, 0.55, 0.72, 0.76, 0.10, and 0.80, respectively.

Sucrose Gradient Sedimentation

All DNA sedimentation analysis was carried out in the Spinco SW 50.1 rotor run at 50,000 rpm and 4°C. Previously formed to 20%, sucrose gradients contained 0.25 M NaOH, 1 × 10−4 M EDTA for alkaline sedimentation, or 0.25 M NaCl, 0.02 M Tris-HCl, pH 8.2, 1 × 10−4 M EDTA for neutral pH sedimentation. Fractions of 8 drops were collected from a hole in the bottom of the tube, the fractions neutralized if run under alkaline conditions, and the radioactivity determined by liquid scintillation.

Other Methods

Liquid scintillation counting was accomplished by adding 10 ml of scintillator (9.1 g of 2,5-diphenyloxazole (POPOP), 0.61 g of 1,4-bis[2-(5-phenyloxazolyl)]benzene (POPOP), 1250 ml of Triton X-100, 2140 ml of toluene) to 0.9 ml of aqueous phase sample, or by placing a nonaqueous sample (GF/C glass filter, or chromatography paper) in 10 ml of nonaqueous scintillator (12 g of 2,5-diphenyloxazole, 0.3 g of 1,4-bis[2-(5-phenyloxazolyl)]benzene, 3 liters of toluene). Protein was determined by the method of Lowry et al. (23) with BSA as a standard. Protein concentrations below 0.04 mg per ml were measured by ultraviolet absorbance at 280 nm.

Results

Purification of Enzyme

All operations were carried out at 0–4°C and, unless otherwise noted, centrifugations were for 10 min at 16,000 × g.

Preparation of Extract—In a typical preparation (Table I) 12 liters of E. coli K-12 (JC 4583) were grown and disrupted by sonic irradiation as previously described (7), except that the cells were suspended in 0.05 M glycylglycine-NaOH, pH 7.0, 5 × 10−4 M DTT, and 5 × 10−4 M EDTA (Buffer A). Cell debris were removed by centrifugation, and the absorbance of the extract at 260 nm was adjusted to 200 with Buffer A (Fraction I).
Streptomycin Precipitation—To Fraction I was slowly added with stirring 0.08 volume of freshly prepared 5% streptomycin sulfate. After 30 min the suspension was centrifuged and the pellet discarded (Fraction II).

Ammonium Sulfate Precipitation—Solid ammonium sulfate (37.9 g/100 ml) was slowly added to Fraction II with stirring and then stirring was continued for 30 min. The precipitate was collected by centrifugation at 27,000 × g for 15 min, and then dissolved in 50 ml of Buffer A (Fraction III).

Potassium Phosphate Fractionation—Fraction III was treated with dibasic potassium phosphate by slowly adding 0.14 part (v/v) of the 4 M salt, stirring for 10 min, and then removing the precipitate by centrifugation at 27,000 × g for 10 min. To the supernatant fraction was added 0.41 part (v/v) of 1 M dibasic potassium phosphate. After 30 min of stirring, the precipitate was collected by centrifugation at 27,000 × g for 30 min and resuspended in 15 ml of Buffer B (0.02 M KPO₄, pH 6.7, 10% glycerol, 1 × 10⁻³ M EDTA, 1 × 10⁻⁴ M DTT) (Fraction IV).

DEAE-cellulose Chromatography—A column (4 × 23 cm) of DEAE-cellulose was equilibrated with 2 liters of Buffer B. Fraction IV was diluted with Buffer B to a conductivity corresponding to 0.15 M KCl in Buffer B, and then loaded onto the column at 2 ml per min. The column was washed with 200 ml of Buffer B containing 0.15 M KCl, and then a linear gradient (1500 ml total volume) from 0.15 to 0.45 M KCl in Buffer B was applied. The active fractions (0.30 to 0.34 M KCl) were pooled, and solid ammonium sulfate was added (39 g/100 ml) with stirring. The pH was held constant by the addition of 0.5 ml of 1 N NaOH per 100 g of ammonium sulfate. After stirring for 30 min, the precipitate was collected by centrifugation at 13,000 × g for 30 min, and then dissolved in a minimal volume of Buffer C (0.01 M KPO₄, pH 6.7, 10% glycerol, 1 × 10⁻³ M EDTA, 1 × 10⁻⁴ M DTT) (Fraction V).

Sephadex G-150 Filtration—A column, 2.5 x 95 cm, of Sephadex G-150 was equilibrated with 3 liters of Buffer C. Fraction V was layered onto the column, and the column was eluted with Buffer C at 15 ml per hour. Fractions (4.4 ml) were collected, and 92% of the applied activity appeared in a single peak just after the void volume of the column. Fractions with a specific activity greater than 1400 were pooled (Fraction VI).

Hydroxylapatite Chromatography—Fraction VI was eluted to a column of hydroxylapatite (0.9 x 3.5 cm) previously equilibrated with 70 ml of Buffer C. The column was washed with 2 ml of Buffer C, and then with 4-ml aliquots each of 0.05 and 0.10 M KPO₄, pH 6.7, in Buffer C at 11 ml per hour. Fractions (2 ml) with a specific activity greater than 7200 were pooled and dialyzed for 8 hours versus 1 liter of Buffer D (0.02 M KPO₄, pH 8.1, 10% glycerol, 1 × 10⁻³ M EDTA, 1 × 10⁻⁴ M DTT) (Fraction VII).

DNA-cellulose Chromatography—A column of DNA-cellulose (0.6 x 3.5 cm) was equilibrated with 100 ml of Buffer D. Fraction VII was made 0.05 M in KCl and put on the column at 1.9 ml per hour with a pressure bulb. The column was then washed with successive 1.5-ml portions of Buffer D containing the following KCl concentrations: 0.05, 0.15, 0.25, 0.45, and 0.70 M. Of the activity applied to the column, 88% appeared in the 0.45 M KCl eluate. This fraction (Fraction VIII) was dialyzed for 4 hours versus 500 ml of Buffer E (Buffer C plus 1 × 10⁻⁴ M EDTA).

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume</th>
<th>Protein</th>
<th>Endonuclease *</th>
<th>Exonuclease *</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Crude extract</td>
<td>283</td>
<td>21.7</td>
<td>(3.8)</td>
<td>(23.6)</td>
</tr>
<tr>
<td>II. Streptomycin</td>
<td>300</td>
<td>20.0</td>
<td>(4.0)</td>
<td>(26.0)</td>
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<tr>
<td>III. Ammonium sulfate</td>
<td>118</td>
<td>34.4</td>
<td>(20.3)</td>
<td>(59.3)</td>
</tr>
<tr>
<td>IV. Potassium phosphate</td>
<td>27</td>
<td>76</td>
<td>(10)</td>
<td>(63)</td>
</tr>
<tr>
<td>V. DEAE concentrate</td>
<td>1.9</td>
<td>64</td>
<td>19.0</td>
<td>128</td>
</tr>
<tr>
<td>VI. Sephadex G-150 pool</td>
<td>29</td>
<td>0.23</td>
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</tr>
<tr>
<td>VII. Hydroxylapatite pool</td>
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<td>0.28</td>
<td>1,505</td>
<td>10,200</td>
</tr>
<tr>
<td>VIII. DNA cellulose</td>
<td>1.9</td>
<td>0.04</td>
<td>14,000</td>
<td>121,000</td>
</tr>
<tr>
<td>IX. Glycercid gradient</td>
<td>14.3</td>
<td>-0.002</td>
<td>42,000</td>
<td>572,000</td>
</tr>
</tbody>
</table>

* As reported previously (7) nuclease assays of the early fractions of the purification were not dependable due to the presence of inhibitors. For this reason with Fractions I to IV, assays were done with less than 0.25 unit of enzyme, a range in which the deviation of the assay from a linear response to added enzyme is no greater than 50%. For this reason activity values for Fractions I to IV should be considered to be semiquantitative.

Corrected for the fact that only a portion of Fraction VIII was taken for this step.

Glycerol Gradient Sedimentation—A portion of Fraction VIII (0.25 ml) was layered onto a 5-ml gradient of glycerol (20 to 40%) made in Buffer E. After centrifugation for 14 hours in an SW 50.1 rotor at 50,000 rpm and 4 °C, a single symmetrical peak of activity appeared about 60% of the distance down the tube (Fraction IX). (See Fig. 3 below.) After collection, the active fractions were pooled and stored either at −20 °C, or over liquid nitrogen.

The purification procedure has been successfully scaled up to process 1 kg of cell paste. Except for the unconcentrated DEAE-cellulose eluates, the DNase is stable during purification. Fraction IX represents approximately a 17,000-fold purification of the activity, with a 7% yield, and is approximately 90% homogeneous (see Fig. 5). It has been stored over liquid nitrogen.

Properties of Purified DNase

Requirements of Nuclease Activities—As previously reported, the recBC DNase can endonucleolytically cleave the single stranded circular DNA from phage fd (7) and can exonucleolytically...
4.4 units were used. Assays were as described under "Methods," except that ATP was as indicated, and, in the case of the circular fd DNA, samples were incubated for 30 min in the absence of exonuclease I. They were then heated to 90° for 5 min, and cooled on ice. Exonuclease I was added and the incubation was continued for an additional 30 min at 37°. If heat-denatured E. coli DNA was used as substrate, the results were the same as found for linear fd DNA.

As previously reported, in all cases the ATP can be replaced by another nucleoside triphosphate, or by mixtures of the four ribo- or deoxyribonucleoside triphosphates (1, 2, 7).

The highly purified DNase requires the presence of a protein such as BSA for maximal endonuclease activity (Fig. 2). In the absence of BSA, the endonuclease is active for only a short time (about 1 min), whereas in the presence of 1 mg per ml of BSA the endonuclease continues to act over a 30-min incubation. Other proteins including egg albumin and carbonic anhydrase have similar stimulatory effects. The exonuclease activities do not show any similar dependence on added protein, but are stimulated 2- to 3-fold by its presence.

Cosegregation and Coelectrophoresis of Nuclease Activities—Although all of the nuclease activities appear to be under the control of the recB and recC loci of E. coli K-12 (1, 2, 4, 7), the varying response to ATP concentration, BSA, and pH might suggest that the activities are not all associated with the same molecular species. However, this does not appear to be the case. If the highly purified DNase is sedimented through a 20 to 40% glycerol gradient (Fig. 3), the endonuclease assayed in the presence or absence of ATP and the exonuclease activities sediment together.

As another test, the purified DNase was subjected to electrophoresis on a 5% polyacrylamide gel at pH 9.0. The gel was then sliced, and the slices were assayed for nuclease activity after elution with buffer (Fig. 4). Both the endonuclease assayed in the presence or absence of ATP, and the exonucleases migrated to the same position in the gel. A parallel gel was run at the same time and stained for protein with Coomassie blue (Fig. 5). This is a dark band of stain at the position corresponding to the eluted activities, and a very faint contaminant (less than 10% of the dye intensity) that migrated farther down the gel. These data show that the enzyme preparation is approximately 90% homogeneous and that the nuclease activities are inseparable by electrophoresis at pH 9.0. The electrophoresis results taken together with the sedimentation data and copurification of the nuclease activities during the latter stages of purification (Table I), strongly support the conclusion that all of the DNase activities are embodied in one molecular species.

Physical Structure of DNase—The highly purified enzyme sedimented together with catalase through a glycerol gradient (Fig. 3) and hence has an e280 of approximately 12. (This value corresponds to a molecular weight of about 250,000.)

**Fig. 1.** Effect of ATP concentration on DNA breakdown. For the circular fd DNA, 0.8 unit of enzyme was added; for the others, 4.4 units were used. Assays were as described under "Methods," except that ATP was as indicated, and, in the case of the circular fd DNA, samples were incubated for 30 min in the absence of exonuclease I. They were then heated to 90° for 5 min, and cooled on ice. Exonuclease I was added and the incubation was continued for an additional 30 min at 37°. If heat-denatured E. coli DNA was used as substrate, the results were the same as found for linear fd DNA.

**Fig. 2.** Effect of BSA on the endonuclease activity. The reaction mixtures (1.35 ml) contained 0.05 M glycylglycine-NaOH, pH 7.0, 0.01 M MgCl₂, 6.7 × 10⁻⁴ M DTT, 75 nmoles of fd phage [α²H]-DNA (9 × 10⁵ cpm per nmole), and 1 mg per ml of acetylated BSA where indicated. After warming the mixtures to 37°, DNase (24 units) was added, and then aliquots (0.15 ml) were removed at the indicated times, immediately heated to 90° for 5 min, and cooled on ice. Exonuclease I (2 units) was next added and the incubation was continued for another 30 min at 37°. Acid-soluble products were quantitated as described under "Methods." Control experiments have shown that exonuclease I is unaffected by the presence of acetylated BSA.
FIG. 3. Glycerol gradient sedimentation of the DNase. Sedimentation of the DNase was accomplished as described in the purification procedure, and the nuclease activities were assayed as described under "Methods." A parallel gradient showed that the nuclease sedimented at the same rate as catalase.

FIG. 4. Polyacrylamide gel electrophoresis of the DNase. A 5% polyacrylamide gel was prepared according to the procedure of Davis (24), except that 0.008% thioacetic acid was included in the electrophoresis buffer. The gel was subjected to preliminary electrophoresis at 4° for 3½ hours at 1.6 ma per gel, then the enzyme (0.1 ml of Fraction IX in 30% glycerol) was added. Electrophoresis was resumed for 2 hours at 2 ma per gel at 4°; then the gel was sliced into 30 slices (2.5 mm each), macerated in a tube, and eluted with 0.2 ml of enzyme diluent (see "Methods") for 5 hours. Each fraction was assayed for the nuclease activities as described under "Methods."

When enzyme was dissociated by boiling for 2 min in 2% SDS, 5% 2-mercaptoethanol, and then subjected to electrophoresis on 5% polyacrylamide gels containing 0.1% SDS (25), only two protein bands were seen after staining with Coomassie blue. By comparison of the mobilities of these bands to those of proteins of known molecular weight, the two polypeptides were determined to have molecular weights of approximately 140,000 and 128,000 (Fig. 6). Also, from a comparison of intensities of staining of the bands, it appeared that the two polypeptides were present in approximately equal amounts. When combined with the size estimate of the active enzyme obtained by glycerol gradient sedimentation, these results show that the recBC DNase is composed of two large, nonidentical polypeptide chains with a combined molecular weight of approximately 270,000.

Analysis of Digestion Products from Various DNA Substrates

Closed Circular Duplex DNA—In an earlier report (7) we suggested that the recBC DNase had very little, if any, activity on closed duplex DNA. To rigorously test the extent of activity on double stranded DNA where no termini are present, RF1 from phage fd was incubated with 10 times the amount of enzyme which would nick an equivalent number of single stranded DNA circles. Sedimentation analysis on sucrose gradients showed no change in the DNA after enzyme treatment with either 300 μM, 30 μM, or no ATP present (Fig. 7). To show that the DNase is not inhibited by the RFI, single stranded, circular 14C-labeled DNA from phage fd was included in a digestion, and the products were analyzed on a sucrose gradient (Fig. 8). The phage DNA was completely degraded to low molecular weight fragments; whereas the RF remained intact. Hence, the RF does not inhibit the endonuclease. Since these...
FIG. 6. Molecular weight estimate of the DNase polypeptides by SDS gel electrophoresis. Fraction IX (0.10 ml) was subjected to electrophoresis on a 5% polyacrylamide gel at pH 9.0 as described in the legend to Fig. 4. The gel was sliced into 2.5-mm slices and each slice eluted overnight at 4°C in 0.15 ml of “final sample buffer” as described by Laemmli (25). The eluates containing the DNase were then boiled for 2 min in 2% SDS, 5% 2-mercaptoethanol to dissociate subunits (25). They were then subjected to electrophoresis on 5% polyacrylamide gels containing 0.1% SDS (25), after which the gels were stained with Coomassie blue and destained electrophoretically. The mobilities of the DNase polypeptides were compared to the mobilities of protein species of known molecular weight as suggested by Weber and Osborn (26). Marker proteins and their molecular weights were: bovine serum albumin, 67,500; glutamic dehydrogenase, 53,000; β-galactosidase, 130,000; RNA polymerase: α′, 160,000; β, 150,000; α, 39,000; and bovine thyroglobulin, 335,000 (27); and DNA polymerase, 109,000 (28). The mobilities of the DNase polypeptides are indicated by arrows. Identical results were obtained with Fraction IX enzyme which was put directly onto the SDS-containing gels.

Gradients can detect the conversion of RFI to RFII (closed to open form), and since this did not occur under all conditions examined, it appears as if the recBC DNase has no effect on closed native DNA.

In a related experiment (Table II) the 3H-labeled RFI was mixed with 32P-labeled E. coli DNA and exposed first to E. coli endonuclease R-B (12), and then to the recBC nuclease. (Endonuclease R-B, the “B restriction enzyme,” makes one double stranded cleavage in the RFI.) Only the linear E. coli DNA was digested by the recBC DNase without preliminary treatment by endonuclease R-B; however, after treatment with endonuclease R-B to convert the RFI to a linear form, both of the DNAs were converted to small fragments by the DNase. Hence, it is concluded that the RFI DNA is resistant to digestion by the recBC enzyme only because it lacks ends.

Circular Single Stranded DNA—The recBC DNase can degrade the single stranded circular DNA from phage fd (7). Likewise, it can endonucleolytically degrade DNA from phage φX-174 or denatured DNA from phage λ. This action does not affect

P. J. Goldmark and S. Linn, unpublished.
...and then treated with polynucleotide kinase and [γ-32P]ATP as described by Weiss, Live, and Richardson (8). DNA was digested with the DNase to produce 5.3 breaks per fd DNA molecule, treated with phosphomonoesterase to remove previously existing 5′-phosphomonoester groups, and then treated with polynucleotide kinase. The phosphorylated DNA was then treated with mononucleotide 5′-phosphates and, after separation of these monomers by chromatography, the amount of label present in each nucleoside 5′-phosphates and, after separation of these monomers was determined. When comparing the bases found at the 5′ end of the oligonucleotides to the overall base composition of the DNA, it is seen (Table III) that, whereas the enzyme has no apparent absolute base specificity, it does appear to have some discretion for breaks next to adenine, and against breaks next to thymine.

In order to determine whether the recBC DNase makes 5′-hydroxyl or 5′-phosphomonoester groups during digestion of DNA in the absence of ATP, the susceptibility of a digest to phosphorylation by polynucleotide kinase without prior treatment of the digest with phosphomonoesterase was explored. (DNA with previously existing 5′-phosphomonoester groups is not phosphorylated by polynucleotide kinase.) The omission of phosphatase treatment reduced the incorporation of phosphate by 78% (Table IV). Hence, while the enzyme produces predominantly 5′-phosphomonoester termini on fd DNA in the absence of ATP, it cannot be ruled out that the enzyme makes some 5′-hydroxyl groups as well under these conditions.3

1 The reason for the apparent production of 5′-hydroxyl termini is unknown. The same ratio of 5′-hydroxyl groups is repeatedly observed, even when Fraction VIII enzyme is used. No DNA phosphomonoesterase activity can be detected either in the recBC enzyme preparation, or in the various reagents used in the experiment. When the base distribution at the 5′ termini which were phosphorylated without prior phosphatase treatment was examined, it was found to be 35% adenine, 28% thymine, 16% guanine, and 21% cytidine. This distribution is essentially identical with that found for the 5′ termini of the total digest, and does not reflect random breakage of the DNA (Table III). Hence, there does not appear to be a contaminating nuclease present which produces the 5′-hydroxyl ends.

Linear Duplex DNA—To examine the products of the reaction of the DNase with linear native DNA, the enzyme was incubated with E. coli DNA labeled with 32P until no further digestion was brought about by additional enzyme, ATP, or incubation time. These conditions rendered the DNA 88% soluble in acid, and reduced it to an average oligonucleotide chain length of 4.5 as determined by susceptibility of the 32P to alkaline phosphatase. Among the products, no detectable mono- or dinucleotides were found. The majority of oligonucleotides had chain lengths of 3 to 5, and greater than 95% had lengths from 3 to 7 (Table V).

![Fig. 9. Sucrose gradient sedimentation of fd phage DNA treated with the DNase in the absence of ATP. The reaction mixtures (0.02 ml) contained 4.0 nmoles of 3H-labeled fd DNA (6.4 × 10^5 cpm per nmole), and 190 units of the DNase where indicated, and were incubated under "standard assay conditions" for 30 min at 37°C. Reaction was terminated by the addition of 0.03 ml of 0.2 M EDTA, 0.10 ml of distilled water, and 0.005 ml of 5 N NaOH. Marker 14C-labeled fd DNA was added, and the material was layered onto alkaline sucrose gradients. Sedimentation was for 315 min as described under "Methods." The marker 14C-labeled fd phage DNA is a mixture of circular (19 S) and linear (17 S) forms.](http://www.jbc.org/)
The reaction (0.10 ml) was run under standard assay conditions and contained 30 nmole of 3H-labeled fd phage DNA (6.4 × 10^6 cpm per nmole) and 1700 units of DNase. A blank reaction containing no DNase was also run. After 30 min at 37°, the mixtures were made 0.05 M in Tris-HCl, pH 8.2, and heated for 5 min at 90° to terminate the DNase reaction. Six units of alkaline phosphatase were added and incubation was continued for 30 min at 37°. The reactions were then adjusted to "poly nucleotide kinase conditions" (see "Methods"), and 21 units of polynucleotide kinase and 3.03 nmoles of [γ-32P]ATP (8.7 × 10^6 cpm per nmole) were added in a final volume of 0.148 ml. After 30 min at 37°, the kinase reaction was terminated by adding 0.5 μ mole of ATP, 0.3 μ mole of salmon sperm DNA, and 0.025 ml of 7 N perchloric acid. After 5 min at 0°, the precipitate was collected by centrifugation at 16,000 × g for 5 min. It was suspended in 0.1 ml of 0.1 N NaOH and reprecipitated by the addition of 0.02 ml of 7 N perchloric acid and 0.5 ml of distilled water. After collection and resuspension of the precipitate again, the mixtures were made 0.15 M in Tris-HCl, pH 7.5, and 0.5 ml of distilled water. After heating to 90° for 10 min, 1 unit of alkaline phosphatase was added to one digest and to one blank tube. After 30 min at 37°, the reactions were adjusted to polynucleotide kinase conditions as described under "Methods," and polynucleotide kinase (3 units) and [γ-32P]ATP (4.1 × 10^6 cpm per pmole, final concentration, 0.016 μM) were added to each reaction (0.055 ml final volume). Incubation was continued for 30 min at 37°, then 0.10 ml of 0.1 M ATP, 0.2 ml of 0.1 M sodium pyrophosphate, 0.1 ml of 2 mg per ml of salmon sperm DNA, and 0.5 ml of 10% triethanolamine-acetic acid were added. The 3P incorporated was determined by filtration through glass filters (8) and counted by liquid scintillation. Blank values were 0.74 and 0.40 pmole of 32PO4 retained, and have been subtracted from the enzyme digest values obtained with and without phosphatase, respectively.

**Table III**  
**Base composition of 5' termini produced by digestion of fd DNA in absence of ATP**

<table>
<thead>
<tr>
<th>Base</th>
<th>Frequency at 5' terminus of digest</th>
<th>Reported fd DNA base composition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenine</td>
<td>34</td>
<td>24</td>
</tr>
<tr>
<td>Thymine</td>
<td>25</td>
<td>34</td>
</tr>
<tr>
<td>Guanine</td>
<td>15</td>
<td>20</td>
</tr>
<tr>
<td>Cytidine</td>
<td>25</td>
<td>22</td>
</tr>
</tbody>
</table>

Since the DNase produces a rather narrow length distribution of oligonucleotides, it seemed probable that the DNase does not hydrolyze phosphodiester bonds at a specific nucleotide. To affirm this hypothesis, the oligonucleotides from a native E. coli DNA digest were labeled at the 5' terminus with polyadenylosine kinase, then digested to mononucleotides with snake venom phosphodiesterase. After separation of the mononucleotides by electrophoresis, the amount of label in each was determined (Table VI). Comparison of the frequency of labeling with the base composition of E. coli DNA shows that each base has nearly an equal probability of appearing at the 5' terminus of the oligonucleotide product. Finally, the sus-

**Table IV**  
**Phosphorylation of DNA digestion products by polynucleotide kinase**

DNA from phage fd was digested with the DNase in a reaction mixture (0.03 ml) under standard assay conditions in the absence of ATP. Four mixtures were run, each containing 4.6 nmole of fd 3H-labeled DNA (0.4 × 10^6 cpm per pmole); two contained 102 units of recBC DNase, the two remaining reactions served as blanks. After 30 min at 37°, the four mixtures were chilled and 0.01 ml of 0.05 M Tris-HCl, pH 8.2, 0.01 M MgCl2 was added to each. After heating to 90° for 10 min, 1 unit of alkaline phosphatase was added to one digest and to one blank tube. After 30 min at 37°, the reactions were adjusted to polynucleotide kinase conditions as described under "Methods," and polynucleotide kinase (3 units) and [γ-32P]ATP (4.1 × 10^6 cpm per pmole, final concentration, 0.016 μM) were added to each reaction (0.055 ml final volume). Incubation was continued for 30 min at 37°, then 0.10 ml of 0.1 M ATP, 0.2 ml of 0.1 M sodium pyrophosphate, 0.1 ml of 2 mg per ml of salmon sperm DNA, and 0.5 ml of 10% triethanolamine-acetic acid were added. The 3P incorporated was determined by filtration through glass filters (8) and counted by liquid scintillation. Blank values were 0.74 and 0.40 pmole of 32PO4 retained, and have been subtracted from the enzyme digest values obtained with and without phosphatase, respectively.

**Table V**  
**Base composition of 5' termini produced by digestion of fd DNA in absence of ATP**

<table>
<thead>
<tr>
<th>Base</th>
<th>Frequency at 5' terminus of digest</th>
<th>Reported fd DNA base composition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenine</td>
<td>34</td>
<td>24</td>
</tr>
<tr>
<td>Thymine</td>
<td>25</td>
<td>34</td>
</tr>
<tr>
<td>Guanine</td>
<td>15</td>
<td>20</td>
</tr>
<tr>
<td>Cytidine</td>
<td>25</td>
<td>22</td>
</tr>
</tbody>
</table>

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and there was no apparent base specificity of the DNase exphosphomonoesters were created by the DNase (Table IV), exhibited at the 5' terminus (Table VI). However, there are distribution was seen (Table V). As with duplex DNA, 5' groups.

**TABLE V**

<table>
<thead>
<tr>
<th>Deoxyribonucleotide spot</th>
<th>Fraction of total nucleotide material</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Duplex DNA substrate</td>
</tr>
<tr>
<td>Mononucleotides</td>
<td>%</td>
</tr>
<tr>
<td>Dinucleotides</td>
<td>0</td>
</tr>
<tr>
<td>Trinucleotides</td>
<td>20</td>
</tr>
<tr>
<td>Tetranucleotides</td>
<td>38</td>
</tr>
<tr>
<td>Pentanucleotides</td>
<td>18</td>
</tr>
<tr>
<td>Hexanucleotides</td>
<td>14</td>
</tr>
<tr>
<td>&gt;Hexanucleotides</td>
<td>10</td>
</tr>
</tbody>
</table>

Reported E. coli DNA base composition (31)

<table>
<thead>
<tr>
<th>Base</th>
<th>%</th>
<th>%</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenine</td>
<td>27</td>
<td>27</td>
<td>25</td>
</tr>
<tr>
<td>Thymine</td>
<td>21</td>
<td>24</td>
<td>24</td>
</tr>
<tr>
<td>Guanine</td>
<td>24</td>
<td>29</td>
<td>26</td>
</tr>
<tr>
<td>Cytidine</td>
<td>28</td>
<td>26</td>
<td>26</td>
</tr>
</tbody>
</table>

**TABLE VI**

<table>
<thead>
<tr>
<th>Base composition of 5' termini produced by DNase digestion of E. coli DNA in presence of ATP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Base</td>
</tr>
<tr>
<td>------</td>
</tr>
<tr>
<td>Adenine</td>
</tr>
<tr>
<td>Thymine</td>
</tr>
<tr>
<td>Guanine</td>
</tr>
<tr>
<td>Cytidine</td>
</tr>
</tbody>
</table>

Two differences between digestion of native and denatured DNA by the recBC DNase. The maximal rate of appearance of acid-soluble fragments with denatured DNA is 50% of the maximal rate observed on native DNA, and the effect of ATP concentration on the rate of digestion of the two forms of substrate are drastically different (Fig 1). These data suggest that the mechanisms for degradation of duplex and denatured DNA are quite similar, but not identical.

**Breakdown of ATP**

All of the nuclease activities of the recBC DNase are affected by ATP: the exonuclease activities absolutely require ATP (or another nucleoside triphosphate (1, 2, 7)), whereas the endonuclease is stimulated 7- to 9-fold by its presence. To study the fate of the ATP during DNA digestion, native E. coli DNA was degraded in the presence of γ-32P- and 32P-labeled ATP, and the ATP products were analyzed. ADP and orthophosphate appeared in approximately equimolar amounts and no other adenosine or phosphate derivatives were detected (Table VII). When ATP was incubated with enzyme in the absence of DNA, however, no ATP hydrolysis occurred.

To determine the number of ATP molecules hydrolyzed per phosphodiester bond cleaved, 32P-labeled ATP was included during digestion of native or denatured 32P-labeled E. coli DNA. The number of phosphodiester bonds broken in the course of the reaction was determined by sensitivity of the DNA phosphorous to alkaline phosphatase, and the ATP consumed was measured by thin layer chromatography (Table VIII). In both cases 20 molecules of ATP were consumed per phospho-

*P. J. Goldmark and S. Linn* 1857
Table VII

Fate of ATP during digestion of E. coli DNA

The reactions (0.05 ml) were carried out under standard assay conditions with 0.12 nmol ATP labeled with both 32P and 14C (final specific activities: 32P, 5.5 × 10^6 cpm per nmole; 14C, 5.0 × 10^6 cpm per nmole), and 5 nmoles of duplex E. coli DNA, and 6.7 units of DNase where indicated. After 10 min at 37°, the mixtures were chilled and 0.01 ml aliquots were removed and treated with Norit to determine the amount of 32PO_4 released. Ten microliters of each mixture was spotted on Whatman No. 1 paper together with 0.1 nmole each of ATP, ADP, AMP, and adenine, as markers. The chromatogram was developed by descending elution with isobutyric acid-ammonia-water (66:1:33) for 16 hours. The spots were cut out and dried, and the amount of radioactivity determined by liquid scintillation. The 32P released was verified to be orthophosphate by thin layer chromatography on poly(ethyleneimine) cellulose plates as described under "Methods."1

<table>
<thead>
<tr>
<th>Additions</th>
<th>Compounds present after reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ATP</td>
</tr>
<tr>
<td>None</td>
<td>5.60</td>
</tr>
<tr>
<td>DNA</td>
<td>5.51</td>
</tr>
<tr>
<td>Enzyme, no DNA</td>
<td>5.49</td>
</tr>
<tr>
<td>Enzyme plus DNA</td>
<td>3.57</td>
</tr>
</tbody>
</table>

Table VIII

Stoichiometry of DNA-dependent ATPase

The reactions (0.05 ml) were carried out under standard assay conditions with 10 nmoles of 14C-labeled ATP (5 × 10^6 cpm per nmole), 7.3 units of DNase, and 4.8 nmoles of duplex or denatured 32P-labeled E. coli DNA (6 × 10^6 cpm per nmole), where indicated. After 30 min at 37°, the reactions were heated to 90° for 5 min, then an aliquot (0.002 ml) of each mixture was spotted onto a thin layer chromatogram (see "Methods") together with 0.1 nmole each of ATP, ADP, AMP, and adenine, as markers. After the chromatogram was developed, the spots were cut out and counted on a low background gas flow counter. The remainder of the reaction mixtures were diluted to 0.25 ml with water, mixed with 4 units of alkaline phosphatase, and incubated for 30 min at 37°. The fraction of the total 32PO_4 that was Norit-nonadsorbable was measured to determine the DNA termini produced. In all cases, the disappearance of ATP was accompanied by a concomitant appearance of ADP.

<table>
<thead>
<tr>
<th>DNA present</th>
<th>ATP consumed</th>
<th>DNA termini produced</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.02</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Duplex</td>
<td>3.51</td>
<td>0.151</td>
<td>23.3</td>
</tr>
<tr>
<td>Denatured</td>
<td>2.59</td>
<td>0.112</td>
<td>23.1</td>
</tr>
</tbody>
</table>

diester bond cleaved. This value is somewhat dependent upon the ATP concentration and pH used, however.4

To elucidate the degree of coupling of the ATPase to the DNase, various DNA species were used in the ATPase assay (Table IX). Native E. coli DNA served as the best cofactor for the DNA-dependent ATPase, and denatured E. coli DNA was about half as effective in stimulating the ATPase. Intact DNA from the phage fd also acted as an activator for the ATPase, but DNA substrates resistant to nuclease attack such as closed duplex DNA from the phage PM2, or RFI of phage fd were very poor cofactors for the DNA-dependent ATPase. The oligonucleotides (pT)_2 and (pT)_3 also failed to stimulate the ATPase. Thus, the ATPase appears to be coupled in some manner to the DNase activity, but the coupling is curious in that a large number of ATP molecules are consumed per phosphodiester bond broken, and this number is dependent upon reaction conditions.

Discussion

The deoxyribonuclease reported here is a nearly homogeneous preparation of an enzyme first detected by Buttin and Wright in 1968 (1). This, and subsequent studies (2–4), described an activity which degraded duplex DNA in the presence of a nucleoside triphosphate, and which was somehow controlled by both the recB and recC loci of E. coli K-12. Later, an endonuclease active on single stranded fd phage DNA, which was also ATP-dependent and controlled by the recB and recC loci, was described (7). The present report shows that both of these nuclease activities as well as an ATPase are various facets of the same molecular species. Also, in contrast to the less pure enzyme, the purified DNase can exonucleolytically degrade linear single stranded DNA only in the presence of ATP.

Studies of the nearly homogeneous DNAse reported here, show that the enzyme has a molecular weight of approximately 270,000 and is made of two nonidentical polypeptides which are very similar in size. It is plausible, then, that the recB and recC loci are the structural genes for the two polypeptides which constitute the DNase.

The endonuclease activity is remarkably specific for single stranded DNA. No endonuclease activity has been detected on duplex DNA (less than 0.01%). With less purified enzyme we noted earlier that the endonucleolytic cleavage of fd DNA was strictly dependent upon ATP (7). However, with the nearly homogeneous preparation reported here, the enzyme retains 10 to 15% of its endonuclease activity in the absence of ATP. The reason for this change is unknown.

It is noteworthy that the end group and size analysis of the products of the endonucleolytic reaction were carried out in the absence of ATP so that the product would not be degraded.
by the exonuclease function of the DNase. It is possible, however, that the endonucleolytic reaction is altered qualitatively as well as quantitatively by the presence of ATP. The endonucleolytic cleavage of single stranded DNA by the recBC DNase is very slow: approximately 30 substrate molecules are cleaved from closed to linear form per enzyme molecule in the course of a 30-min incubation with ATP present. This calculation assumes that all enzyme molecules are active, and that the active form of the DNase is $3 \times 10^5$.

In the presence of ATP the products of degradation of linear duplex and linear single stranded DNA are remarkably similar. Both substrates are degraded with no apparent base specificity to short fragments bearing 5'-phosphomonophosphates. However, single stranded DNA is degraded at about one-half the rate, and the rates of degradation of the two substrates differ dramatically in response to ATP concentration. Anai et al. (6) have described a DNase from Micrococcus lysodeikticus which attacks duplex DNA in the presence of ATP to produce very similar products; this DNase does not, however, exomunecolytically attack single stranded DNA.

With linear DNA, the rate of nuclease digestion at 37° is about 500 breaks per min introduced per enzyme molecule. This rate is similar to that for the digestion of native DNA by E. coli exonuclease III.2 In light of the large extent of ATP hydrolysis and ADP or Pi. It is not clear why the ATP is consumed, nor is the presence of degradable DNA substrate, so the DNase and enzyme-catalyzed exchange between ATP and ADP or Pi. Nevertheless, the hydrolysis of ATP is dependent upon the presence of degradable DNA substrate, so the DNase and ATPase are evidently coupled in some way. This coupling is not absolute, however, as there is no simple (or constant) correlation between the number of ATP molecules degraded and the number of DNA phosphodiester bonds cleaved. In solution, the requirement is not obviously related to the base content of the DNA substrate, since we have found that when the alternating copolymer dAT is used as substrate, the enzyme shows the same response to GTP, ATP, or CTP as when DNA is present.

It would be of interest to know from which terminus of the DNA exonuclease digestion is initiated. Accordingly, duplex and single stranded DNA have been labeled at the 5' terminus (with DNA polymerase), or at the 3' terminus (with polynucleotide kinase), then exposed to low, or saturating levels of the recBC DNase. In all cases the same results were observed: the termini of the molecules were degraded with a very slight, although consistent, bias over the bulk of the molecule. This anomalous result is probably due to a peculiar property of the enzyme: it appears to initiate hydrolysis of a DNA molecule very slowly, but once hydrolysis is commenced, the molecule is rapidly degraded. Thus, even with saturating enzyme concentrations, at least 20 min is required to degrade completely a DNA population. During this period only intact, or completely degraded fragments are observed (see, for example, Figs. 1 and 2, Reference 7). Attempts at synchronizing the initiation of digestion by preliminary incubations (e.g. with ATP absent) have failed. For this reason no definite conclusions can be drawn about the polarity of digestion by the enzyme, although it is perhaps indicated that the enzyme degrades the DNA from both termini.

The most intriguing question to be answered is the role of the DNase in DNA metabolism, specifically in recombination and repair. Several elegant models (32-34) have proposed intermediate steps in recombination and repair that could be mediated by the recBC DNase. Diligent in vitro experiments with the enzyme as a component may be able to test these, or other models, for the molecular mechanism of genetic recombination and repair.

Acknowledgments—We are indebted to Helen Mobach, Erin Hawkins, and Alexander Karu for their help with some of the experiments.

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Purification and Properties of the recBC DNase of Escherichia coli K-12
Peter J. Goldmark and Stuart Linn


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