Purification and Properties of an Endodeoxyribonuclease from Nuclei of Bovine Small Intestinal Mucosa*

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An endodeoxyribonuclease has been purified from nuclei of bovine small intestinal mucosa to a homogeneous state by a procedure involving affinity chromatography on heparin-agarose. The endonuclease, which was found to be bound to chromatin, has a pH optimum of 5.4. It requires Mn²⁺ or Co²⁺ for activity and its maximum activity with Mg²⁺ is about 80% of that with Mn²⁺. Its activity is strongly inhibited by sulfhydryl-blocking agents, and by ethidium bromide. The enzyme does not attack RNA and is inhibited by it. Its isoelectric point is 8.5 ± 0.1, and its molecular weight is 49,000 ± 3,000, determined by sucrose gradient sedimentation and gel filtration on Sephadex G-100. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate indicated that the enzyme is composed of two nonidentical subunits with molecular weights of 39,000 and 23,000. The enzyme catalyzes the endonucleolytic cleavage of circular duplex ColE1 DNA via single strand scissions from the initial stage of degradation. The average size of the limit products of native phage T7 or ColE1 DNA is about 2,000 to 1,500 base pairs, estimated by neutral sucrose gradient sedimentation or agarose gel electrophoresis. The enzyme degrades denatured DNA about 20 times faster than native DNA. The products contain 5'-phosphoryl and 3'-hydroxyl termini, and all four deoxynucleotides are present in almost equal amounts at the 5'-termini.

Nuclei disappear at the level of the granular layer during normal keratinization of epidermal tissue (1), and nuclear DNA is progressively lost from nuclei during terminal differentiation of fetal lens fiber cells (2). In both cases, the DNA degradation is supposed to be catalyzed by enzymes (1, 3). We are interested in this nuclear DNA degradation in situ observed at the final stage of normal cell differentiation, but unfortunately, these tissues cannot be obtained in sufficient quantity for enzymological studies. In this respect, a better system for studying DNA degradation during the course of normal cell differentiation in situ is rat small intestinal mucosa, in which rapid degradation of nuclear DNA by its own enzymes in situ can be observed on incubation in balanced salt solution (Krebs-Ringer phosphate buffer, pH 7.4) (4).

In previous work on the early events of nuclear DNA degradation in rat small intestinal mucosa incubated under these conditions, we separated many discrete bands of DNA by polyacrylamide gel electrophoresis and calculated that they had multiples of a molecular weight of 200 base pairs. Similar degradation products of DNA have been found in terminally differentiating fetal lens fiber cells (3). As a next step, we examined the endonuclease activity in the nuclei of the small intestinal mucosa responsible for breaking the nucleosome linker of chromatin in the initial stage of degradation of chromatin DNA.

This paper describes the purification and some properties of an endonuclease activity from the nuclei of bovine small intestinal mucosa. Bovine mucosa was used instead of rat tissue, because large quantities of mucosal scrapings were required to obtain sufficient enzyme from the nuclei for study. The endonuclease seems to bind to chromatin and appears to be a nicking enzyme.

The possible function of this endonuclease in vivo, and its participation in DNA synthesis in bovine small intestinal mucosa are discussed.

**EXPERIMENTAL PROCEDURES**

**Substrates**

32P-labeled Escherichia coli DNA was prepared as described previously (5). 32P- and 3H-labeled T7 DNAs were prepared by the methods of Friedman and Smith (6), and Richardson (7), respectively. 32P-labeled E. coli ribosomal RNA was prepared according to the method of Littauer et al. (8). ColE1 DNA (Form I) and phage λ DNA digested with restriction endonuclease HindIII were gifts of Dr. Y. Sakaki, and phage fd DNA was a gift of Dr. T. Takeya. DNA concentrations were expressed as nucleotide residues. γ-32P-ATP was prepared by the method of Glynn and Chappell (9) as modified by Weiss et al. (10).

**Enzymes**

Bovine pancreatic DNase I, hog spleen DNase II, and alkaline phosphatase from E. coli (BAPF) were purchased from Worthington. Snake venom and calf spleen phosphodiesterases and micrococcal nuclease were products of Sigma. Polynucleotide kinase was purchased from P-L Biochemicals. Eco RI restriction endonuclease was purchased from Takara Biochemicals, Kyoto.

**Chromatographic Media**

CNBr-activated Sepharose 4B and heparin were obtained from Pharmacia and Nakarai Chemicals, Kyoto, respectively, and heparin-agarose was prepared according to the instructions in the manual attached to the CNBr-activated Sepharose 4B. DEAE-cellulose (DE32) was purchased from Whatman and Sephadex G-100, G-75, and DEAE-Sephadex A-25 were from Pharmacia.

**Other Reagents**

Bovine serum albumin Fraction V, and egg albumin (twice crystallized) were obtained from Armour, and ICN Pharmaceuticals, respectively. Cytochrome c, trypsin, and myoglobin were products of Sigma. G-Actin was prepared from an acetone powder of rabbit muscle by

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1. J. Nakayama, T. Fujiyoshi, and M. Anai (1978) Sekigaku 50, 634 (abstr.).
the method of Spudich and Watt (11). E. coli RNA was obtained from Mann. Dextran sulfate (Mr = 1,000) was from Pharmacia and calcium phosphate was from LKB. All other chemicals were of the highest grade available commercially and were used without further purification.

**Methods**

**Preparation of Nuclei from Bovine Intestinal Mucosa**—All operations were performed at between 0 and 4°C. Bovine small intestine was obtained at a local slaughterhouse and brought to the laboratory and remaining food particles. The mucosa was then obtained by scraping the intestine with the edge of a microscope slide glass by the method of Clark and Porteous (12). Tissue (wet volume 400 ml) was suspended in 3 volumes of 0.3 M sucrose containing 10 mM Tris/HCl, pH 8.0, 0.5 mM magnesium acetate, 3 mM CaCl2, 0.5 mM diethiothreitol, and 0.1% Triton X-100, and homogenized with five strokes of a Teflon pestle in a Potter homogenizer. The homogenate was centrifuged at 1,000 × g for 10 min. The pellet was washed twice by suspending it in 3 volumes of 0.3 M sucrose buffer, homogenizing it in a Waring blender at low speed for 2 min, and centrifuging the homogenate as described above. The final pellet was suspended in 5 volumes of 2.25 M sucrose containing 10 mM Tris/HCl, pH 8.0, 0.5 mM magnesium acetate, and 0.5 mM diethiothreitol. The suspension was layered over an equal volume of 2.25 M sucrose buffer and sedimented at 45,000 × g for 45 min. The resultant pellet was washed three times by suspension in 0.3 M sucrose containing 10 mM Tris/HCl, pH 8.0, and 2 mM MgCl2 and then stored at −30°C as a pellet.

**Preparation of DNA-Polyacrylamide Gel—** 32P-labeled E. coli DNA incorporated into polyacrylamide gel was used as a substrate for assay of the endonuclease activity. The DNA-gel suspension was prepared by the method of Melgar and Goldthwait (14) with a slight modification as follows. Air was removed under vacuum from a mixture of 2 ml of acrylamide stock solution (10% acrylamide, 2.5% bisacrylamide), 1 ml of 32P-labeled E. coli DNA (30 nmol/ml of DNA in 0.02 M KCl), and 0.25 ml of riboflavin solution (40 mg/ml). When polymerization under fluorescent lighting was complete, the gel was homogenized in a Waring blender at low speed for 1 min with 10 volumes of 0.05 M Tris/HCl, pH 7.2, 0.05 M KCl, and 0.2 mM EDTA. The homogenate was then passed through a 20-ml syringe twice. DNA-gel suspension was washed five times each by suspension and centrifugation at 15,000 × g for 5 min, first with 10 volumes of the same buffer, then with 10 volumes of 0.05 M Tris/HCl, pH 7.2, containing 0.02 M KCl and 0.2 mM EDTA, and finally with 10 volumes of 0.01 M potassium phosphate buffer, pH 7.0, containing 0.01 M KCl. The DNA concentration of DNA-gel suspension was adjusted to 15 nmol/ml. The yield of radioactivity in the DNA-gel was 40 to 60% of that of the input DNA. When heat-denatured DNA was incorporated in the polyacrylamide gel, DNA was released slowly during storage, so the gels were washed with the final buffer at least once a week.

**Assay for Endonuclease Activity**—The reaction mixture (0.3 ml) contained 50 mM acetate buffer, pH 5.4, 3 mM MnCl2, 30 μg of bovine serum albumin, 0.1 ml of DNA-gel suspension (about 25,000 cpm) containing 3.3 mM potassium phosphate and 3.3 mM KCl, and enzyme preparation. After incubation for 30 min at 37°C, the reaction was terminated by adding 0.7 ml of 0.02 M EDTA, pH 8.0, at 0°C. The tube was centrifuged at 2,000 × g for 10 min, and 0.5 ml of the supernatant was removed, dried in a planchet, and counted in a gas flow counter. Reactions were always run in duplicate. In control tubes without enzyme, 1 to 2% of the input radioactivity was released during incubation. One unit of the enzyme was defined as the amount that released 1 nmol of DNA in 30 min at 37°C under these conditions. The release of 32P-labeled DNA from DNA-gel up to 40% of the input DNA was proportional both to the amount of enzyme added and to the time of incubation.

When the amount of acid-soluble DNA was measured, the reaction was terminated by adding 20 μl of 0.2 M NaOH and 0.25 ml of 2 N perchloric acid. The mixture was stood for 10 min at 0°C and then centrifuged, and the total supernatant was transferred quantitatively to a planchet, neutralized by adding 1 drop of 2 N KOH, dried in an oven, and counted as above. Unless otherwise noted, single-stranded DNA-gel was used as a substrate.

**Isoelectric Focusing**—Isoelectric focusing was performed according to the KLB instruction manual (15) with a 110-ml column using pH 7 to 10 carrier ampholite and a 0 to 50% sucrose gradient containing 10% glycerol. Enzyme solution previously dialyzed against 1 liter of 0.1% glycine containing 10% glycerol was mixed with the dense gradient solution. Electrophoresis was carried out at 450 V for 48 h at 4°C.

**Gel Electrophoresis—** Polyacrylamide gel electrophoresis was carried out at 4°C on a 7.5% polyacrylamide gel (0.6 × 7 cm) at 2 mA/gel with 0.45% β-aminolevulinic acid buffer, pH 4.5, for 1½ h, as described by Reisfeld et al. (16). After electrophoresis, the gel was stained with 0.1% ampicillin brilliant blue R 0.1:10 in 7% acetic acid and destained with 30% ethanol in 10% acetic acid and then with 5% methanol in 7.5% acetic acid. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed using a 10% gel column (0.6 × 7 cm) containing 0.1% sodium dodecyl sulfate by the method of Laemmli and Favre (17). The reaction was terminated by cooling the mixture to 0°C. The gel was stained as above.

ColEl DNA was applied to a 0.9% agarose disc or slab gel column containing 0.5 μg/ml of ethidium bromide and electrophoresis was carried out at 150 V for 2 h or at 50 V for 18 h at room temperature as described by Peacock and Dingman (18). DNA in the gel column was stained under fluorescent lighting and then photographed. Protein concentrations were determined by the method of Lowry et al. (19). Neutral and alkaline sucrose density gradient centrifugations of 32P-labeled Phage T7 DNA—Neutral and alkaline sucrose gradient sedimentations of DNA were performed in a 5 to 20% sucrose gradient containing 20 nm Tris/HCl, pH 8.5, 0.25 mM NaCl, and 1 mM EDTA, and in a 5 to 20% sucrose gradient containing 10 mM NaOH and 1 mM EDTA, respectively. The gradients were centrifuged for 2 h or 4½ h at 45,000 rpm at 4°C in a Hitachi RPS 50 rotor.

**5'-Terminal Nucleotide Analysis—** Phage fd DNA (12 nmol) or ColEl DNA (5 nmol) was incubated with 1 unit of endonuclease at 37°C for 60 min in 0.1 ml of reaction mixture containing 50 mM acetate buffer, pH 5.4, 3 mM MgCl2, and 10 μg of bovine serum albumin. A control without endonuclease was run in parallel. The reaction was terminated by heating the mixture for 5 min at 100°C and then rapidly placing it in an ice bath. The solution was dialyzed against 10 mM Tris/HCl buffer, pH 7.5, containing 0.1 mM EDTA for 5 h and then incubated for 90 min at 45°C with 20 μg of alkaline phosphatase. The reaction was terminated by cooling the mixture to 0°C. The mixture was treated three times with an equal volume of saturated phenol, and twice with an equal volume of ether. The DNA solution was dialyzed against 1 mM Tris/HCl buffer, pH 7.5, containing 0.01 mM EDTA overnight. Then it was phosphorylated with 4 units of polynucleotide kinase and 20 nmol of [γ-32P]ATP (5 × 106 cpm/nmol) in 0.2 ml of reaction mixture containing 20 mM Tris/HCl buffer, pH 8.0, 10 mM dithiothreitol, 10 mM MgCl2, and 1 mM spermidine for 40 min at 37°C as described by Takeya et al. (19). Additional polynucleotide kinase (4 units) was added and the incubation was continued for 20 min. The mixture was treated first with phenol and then with ether twice as described above. The solution was applied to a Sephadex G-75 column (1.2 × 32 cm) previously equilibrated with 10 mM Tris/HCl buffer, pH 7.5, containing 0.1 mM EDTA. The 5'-32P-labeled polynucleotides were detected in the exclusion volume and were lyophilized. 5'-32P-labeled polynucleotides were dissolved in 20 μl of distilled water and incubated with 5 μg of pancreatic DNase I in 0.1 ml of reaction mixture containing 20 mM Tris/HCl buffer, pH 7.6, and 10 mM MgCl2 for 90 min at 37°C. Then a final concentration of 70 mM glycine/NaOH buffer, pH 9.2, was added and the solution was treated with 4 μg of snake venom phosphodiesterase for 60 min, and 100 μg of RNase A and 100 μg of mononucleotide solution was applied to Whatman No. 3MM paper together with authentic samples of the four mononucleotides. Ascending paper chromatography was performed in a solvent system of 0.1 M sodium phosphate buffer, pH 6.8/ammonium sulfate/n-propyl alcohol 100/0/2. Spots of nucleotides were located under ultraviolet light, and cut out for counting in a liquid scintillation counter.

**Others—** RNase activity was measured using 32P-labeled E. coli RNA as a substrate by counting acid-soluble radioactivity. Micrococal nuclease digestion of E. coli DNA was carried out as described by Webb and Felgner (20). Molecular weights of proteins were measured by centrifugation on a 5 to 20% sucrose density gradient at 45,000 rpm for 18 h at 4°C in a Hitachi RPS 50 rotor. Fractions collected from the bottom of the gradient were assayed for endonuclease and hog spleen DNase II activities, respectively. Protein concentration was determined by measuring the absorbance at 280 nm, assuming A280 = 1.00 for a solution of 1 mg of protein/ml.
Detection of Endonuclease Activity in Crude Extracts

Unless otherwise noted, centrifugation was carried out at 20,000 × g for 10 min, and all operations were performed at 0–4°C. Crude extracts were eluted from nuclei stepwise with increasing NaCl concentrations as follows. The purified nuclear pellet was thawed in 3 volumes of Buffer A (20 mM Tris/HCl, pH 7.5, 1 mM EDTA, and 10 mM 2-mercaptoethanol) and disrupted by sonication in a Branson sonifier (model B-12) operated at maximum output three times for 1 min each. The disrupted nuclear suspension was stirred for 10 min, centrifuged, and the supernatant was saved. The pellet was resuspended in 6 volumes of Buffer A containing 0.15 M NaCl, homogenized, stirred for 30 min, and centrifuged as above. These processes were performed successively in 6 volumes of Buffer A containing 0.3 M, 0.6 M, and 1.0 M NaCl. The resultant 105,000 × g supernatants were dialyzed extensively against Buffer B (20 mM potassium phosphate buffer, pH 7.0, containing 5 mM 2-mercaptoethanol, 0.1 M EDTA, 10% glycerol), and the slight precipitates formed were removed by centrifugation.

The endonuclease activity for degrading denatured DNA-gel and the protein concentration of each fraction were measured (Fig. 1). The specific activity and yield of endonuclease activity were highest in the fractions extracted with increasing NaCl concentrations. The specific activity, as well as the yield, of the starting material was extracted after washing with 0.1 M and 0.25 M NaCl were pooled (Fraction I1, 18 ml) that had been equilibrated with the same buffer. It was then eluted with the same buffer at a flow rate of 20 ml/h. The active fractions were collected (Fraction IV, 60 ml), concentrated by dialysis against polyethylene glycol solution, and then dialyzed against 50 mM potassium phosphate buffer, pH 7.0, containing 5 mM 2-mercaptoethanol, 0.1 M EDTA, 50 mM NaCl, and 30% glycerol for 16 h. The results of a typical purification are summarized in Table I.

Although only 30-fold increase of specific activity was achieved, the starting material was extracted after washing the nuclei twice with Buffer A, which removed large quantities of protein. Thus the increase of specific activity is several hundredfold if calculated on the basis of the total protein. The total activity of the DEAE-cellulose fraction was always 1.5- to 2-fold greater than that of the crude extract. This may be due to the existence of an inhibitor(s) for the endonuclease. The final preparation of enzyme lost 20% of its activity on storage for 1 month at ~20°C. When NaCl was omitted from Buffer B, the enzyme activity was completely lost during the purification procedures. Unless otherwise noted, Fraction IV was used in all subsequent experiments.

Purity of the Enzyme—When Fraction IV was subjected to 7.5% polyacrylamide gel electrophoresis (pH 4.5), only one major band was evident (Fig. 3A), but the enzyme activity eluted from the gel was low. When an excess amount of

**TABLE I**

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| Enzyme activity was measured under standard assay conditions as described under “Methods.” |

| Fraction | Volume | Total activity | Total protein | Specific activity | Yield |
|----------|--------|----------------|---------------|-----------------|-------|
| Crude extract | 25 | 314 | 37.0 | 9.5 | 100 |
| DEAE-cellulose | 33 | 631 | 7.1 | 89 | 201 |
| Heparin-agarose | 18 | 404 | 1.7 | 238 | 129 |
| Sephadex G-100 | 60 | 242 | 1.0 | 242 | 77 |
Fraction III was subjected to gel electrophoresis, the position of the enzyme activity corresponded with that of the major band of Fraction IV.

Fraction IV had no detectable ribonuclease activity; when 5 nmol of 32P-labeled E. coli rRNA (2,000 cpm/nmol) was incubated with 1/2 units of the enzyme for 60 min at 37°C, the formation of acid-soluble material was less than 5%.

Properties of the Enzyme

Molecular Weight—The molecular weight of the purified enzyme was estimated by Sephadex G-100 gel filtration with marker proteins and by zone sedimentation in a 5 to 20% sucrose density gradient as described under "Methods." Values of 49,000 ± 3,000 were obtained by these procedures assuming that the enzyme is a globular protein. Electrophoresis of the purified enzyme in the presence of 0.1% sodium dodecyl sulfate on 10% polyacrylamide gel showed that it was composed of two nonidentical polypeptide chains with molecular weights of about 30,000 and 23,000 (Fig. 3, A, B, and C).

Isoelectric Point—The heparin-agarose fraction (Fraction III) was used for determination of the isoelectric point. The sample (19 ml, 200 units) was dialyzed and subjected to electrofocusing as described under "Methods." After electrofocusing, fractions (2.0 ml) were collected from the bottom of the column, and the endonuclease activity and pH of each were measured (Fig. 4). The isoelectric point of the enzyme was 8.5 ± 0.1.

Requirements for Activity—The optimal pH was 5.4 in 50 mM sodium acetate buffer. In 50 mM sodium cacodylate buffer, pH 5.8, and 50 mM potassium phosphate buffer, pH 6.0, the activities were 90% and 30%, respectively, of the maximum activity in acetate buffer. The enzyme required divalent cations for activity. Addition of 1 mM EDTA to the reaction mixture instead of metal ions resulted in complete loss of endonuclease activity. Maximum activity was obtained with either Mn2+ or Co2+ at a concentration of 3 mM. The maximum activity with 3 mM Mg2+ was about 80% of that with 3 mM Mn2+. Addition of 5 mM CaCl2 with Mn2+ or Mg2+ inhibited the activity about 70%.

Sulfhydryl-blocking agents, such as N-ethylmaleimide (5 mM) and p-chloromercuribenzoate (0.2 mM), completely inhibited the reaction, while 2-mercaptoethanol and dithiothreitol stimulated the reaction about 26 to 40%. NaCl or potassium phosphate at 50 mM inhibited the enzyme activity about 80%. Ethidium bromide (1 µg/0.3 ml) completely inhibited the reaction, and E. coli RNA (10 µg/0.3 ml) also inhibited the activity 50%.

Rabbit muscle G-actin, which is a specific inhibitor of pancreatic DNase I, had no effect. Bovine serum albumin (30 µg/0.3 ml) increased the activity about 2-fold.

Comparison of the Activities on Native and Denatured DNA-Gels—The rates of hydrolysis of native and denatured DNA-gels were linearly proportional to the time of incubation with the enzyme (Fig. 5). The rate of degradation of denatured DNA-gel was about 20 times that of degradation of native DNA-gel. On incubation for 2 h, formation of acid-soluble nucleotides from denatured DNA-gel amounted to about 5% of the total, while none were released from native DNA-gel. When heat-denatured E. coli DNA (1.5 nmol) was incubated with excess endonuclease (3 units) for 12 h, about 30% of the input DNA became acid-soluble.

Mode of Action of the Enzyme—ColE1 DNA (Form I) was digested with the enzyme and subjected to agarose slab gel electrophoresis as described under "Methods." The purified

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**Fig. 3.** Polyacrylamide gel electrophoresis of endonuclease in the absence (A) and presence of sodium dodecyl sulfate (B), and the molecular weight of endonuclease (C). A, Endonuclease (20 µg) in 100 µl of 0.02 M potassium phosphate buffer, pH 7.0, containing 0.1 mM EDTA, 5 mM 2-mercaptoethanol, and 10% glycerol was layered on a gel column. Electrophoresis, staining, and destaining were performed as described under "Methods." B, Endonuclease (20 µg) and marker proteins (15 µg each) in 200 µl of 0.125 M Tris/HCl, pH 6.8, 2% sodium dodecyl sulfate, 0.001% bromophenol blue (BPP), 5% 2-mercaptoethanol, and 10% glycerol were applied to gel columns containing 0.1% sodium dodecyl sulfate. Electrophoresis was carried out as described under "Methods." C, The migration of each protein relative to that of the tracking dye is plotted against the logarithm of the molecular weight on a semilogarithmic scale. The locations of the subunits of endonuclease are indicated by arrows.

**Fig. 4.** Isoelectric focusing of endonuclease. Fraction III (19 ml, 200 units) was used instead of Fraction IV, because the activity of the latter was labile during overnight dialysis against 1% glycin containing 10% glycerol at 4°C. Electrophoresis was carried out as described under "Methods." After focusing, fractions (2 ml) were collected from the bottom of the column. The absorbance at 280 nm and the pH were determined and aliquots (60 µl) were assayed as described under "Methods," except that the incubation time was 150 min. ○, pH; ●—●, enzyme activity.

**Fig. 5.** Rate and time course of hydrolysis of native and denatured DNA-gels and production of acid-soluble material by the endonuclease. Assays with 0.7 unit of enzyme were performed as described under "Methods," except that the native and denatured DNA-gels were hydrolyzed with endonuclease for the indicated periods. The acid-soluble fraction was determined as described under "Methods." ○—○, denatured DNA-gel; △—△, acid-soluble nucleotides from denatured DNA-gel; ■—■, acid-soluble nucleotides from native DNA-gel.
endonuclease converted double-stranded superhelical DNA (Form I) first to an open circular DNA (Form II), and then to a unit length of DNA (Form III) (Fig. 6). The above results indicate that the enzyme acts on double-stranded DNA endonucleolytically with single strand scissions. In order to indicate that the enzyme acts on double-stranded DNA, the digestion was first digested with the enzyme, and then the digest was treated with Eco RI endonuclease. No new band was observed on agarose gel electrophoresis, indicating that the endonuclease did not act on ColEl DNA at specific sites (data not shown).

Size of Products of Hydrolysis of Native T7 DNA—The size of the products in limit digests of native T7 DNA was analyzed by neutral sucrose density gradient centrifugation (Fig. 7). The average molecular weight of the products was estimated as 1.35 × 10^6, which corresponded to about 2,000 base pairs (22). No further shift of the peak toward the top of the gradient was detected on increasing the incubation time. When the products of limit digestion were analyzed by alkaline sucrose gradient centrifugation, however, the peak fraction was shifted further to the top, and this material was calculated to contain several hundred nucleotide residues. When ColEl DNA was digested with excess endonuclease, the final product was calculated to be about 1,500 base pairs on agarose gel electrophoresis with a marker of phage λ DNA digested with HindIII endonuclease (data not shown). The above results indicate that limited numbers of single strand scissions were introduced into native T7 and ColEl DNA even after extensive digestion with the enzyme.

Digestion of E. coli DNA of Various Sizes with Endonuclease—To examine how the endonuclease acts on DNA of smaller size (less than 2,000 base pairs), we first digested E. coli DNA with various amounts of micrococcal nuclease. The size of the products was estimated by agarose gel electrophoresis and neutral sucrose density gradient sedimentation. Then the digested E. coli DNA of various sizes was further digested with the endonuclease and the final products were analyzed by alkaline sucrose density gradient sedimentation. When the size of E. coli DNA was about 20,000 bases, 16 nicks were introduced into the DNA (Fig. 8, a and b). On the other hand, when it was about 2,000 bases only one nick was introduced (Fig. 8, c and d).

Analysis of Acid-soluble Nucleotides of Denatured E. coli DNA—The size of the products of limit digestion of denatured E. coli DNA was analyzed by DEAE-Sephadex A-25 chromatography in the presence of 7 M urea (23). Only small amounts of mono- to pentanucleotides were produced and more than 90% of the acid-soluble nucleotides were bigger than 8 nucleotides (data not shown).
In this work we purified an endonuclease to homogeneity from nuclei of bovine small intestinal mucosa and studied its properties. The enzyme has several unique properties which distinguish it from other mammalian endonucleases reported previously. (a) On repeated washing, the enzyme activity remains associated with the nuclear pellet and is eluted best with 0.3 to 0.6 M NaCl. These results indicate that the enzyme is bound fairly tightly to chromatin, although not so tightly as non-histone proteins. Recently, two endonucleases associated with chromatin have been reported. One is Ca"⁺⁺-Mg"⁺⁺-dependent DNase (21), which is eluted with 0.6 M NaCl from rat liver nuclei (24). This enzyme has not been characterized in detail, but it is strongly activated by Ca"⁺⁺ in the presence of Mg"⁺⁺. In contrast, the bovine intestinal endonuclease described in this paper was inhibited by Ca"⁺⁺ in the presence of Mn"⁺⁺ or Mg"⁺⁺. The other endonuclease reported was purified from HeLa S3 cells (25). It has a molecular weight of 22,000, a pH optimum of 7.2 and isoelectric point of 5.1 ± 0.2. In contrast, the molecular weight, pH optimum, and isoelectric point of bovine intestinal endonuclease are 49,000 ± 3,000, 3.4 in 50 mM acetate buffer, and 8.5 ± 0.1, respectively. (b) The results obtained by agarose gel electrophoresis of ColEI DNA clearly indicate that the enzyme makes single-strand breaks without showing any base specificity at the initial stage of attack. The enzyme hydrolyzes denatured DNA about 20 times faster than native DNA. In this respect, the purified endonuclease has common characteristics with mammalian DNase V, which is located in nuclei and hydrolyzes denatured DNA about 5 times faster than native DNA (26). However, there are several differences in the properties of the purified endonuclease and DNase V. First, all the DNase V's so far examined (26-28) were co-purified with DNA polymerase α, which is extracted from the cytosol or nucleokarysm fraction of cells. DNA polymerase α in rat small intestinal mucosa, although not characterized in detail, has been reported to be extracted from nuclei in the absence of NaCl (29). On the contrary, our purified enzyme is extracted from nuclei in the presence of 0.3 to 0.6 M NaCl. Second, calf thymus DNase V has a molecular weight of 53,000 ± 2,500, and is composed of four identical subunits. Bovine intestinal endonuclease is, however, composed of two nonidentical subunits with molecular weights of 30,000 and 23,000. Third, the endonuclease co-purified with DNA polymerase α from rat regenerating liver is not adsorbed to DNA-cellulose (28), while the endonuclease reported here is eluted with 0.15 M NaCl from heparin-agarose, which has many properties in common with DNA-cellulose (39). Thus, bovine intestinal endonuclease appears to be essentially a nicking endonuclease, and has some unique properties different from those of other mammalian endonucleases so far reported.

The size of limit digestion products of native T7 and ColEI DNA is about 2,000 to 1,500 nucleotides. The enzyme reported here cannot act on 3²P-labeled E. coli DNA of smaller size (less than 1,000 bases). Why the endonuclease does not act on DNA of smaller size is still unknown, but there are several restriction endonucleases that recognize specific nucleotide sequences but are nonspecific in their cleavage (31,32), and in this respect, it should be further studied whether the purified endonuclease recognizes specific nucleotide sequences or not. Meanwhile, this property is interesting in connection with the physiological function of the endonuclease. The enzyme may well be associated with DNA synthesis, judging from its location in nuclei and its production of 3'-hydroxyl ends, which activate DNA polymerase α in vitro. It is reported that the size of Okazaki pieces in mammalian cells is about 100 nucleotides, while that of E. coli is 1,000 nucleotides (33). The distance between the two origins of replication in eukaryotic cells is about 10,000 bases (34). If an endonuclease is needed for relieving the torque in unwinding duplex DNA before...
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DNA polymerase initiates incorporation of nucleotides in mammalian cells, the enzyme should be an endonuclease such as that reported here which nicks native duplex DNA but does not act on DNA of smaller size (i.e. less than 100 nucleotides). The precise nature of the endonuclease, however, requires further characterization especially with respect to how the enzyme recognizes its action sites in the replicative form of chromatin.

Our purpose in isolating and characterizing this endonuclease from nuclei of bovine small intestinal mucosa was to identify the enzyme that breaks the nucleosome linker of chromatin during the initial stage of DNA degradation in situ. We are now attempting to determine whether the endonuclease activity reported here is also present in the nuclei of rat small intestinal mucosa. It would be interesting to study how chromatin DNA is digested by the purified endonuclease in a reconstituted system in vitro.

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