Studies on Deoxyribonucleases from Saccharomyces cerevisiae. Characterization of Two Endonuclease Activities with a Preference for Double-Stranded DNA

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Summary

Two new endonuclease activities, endonuclease B and endonuclease C, obtained from yeast nuclear preparations have been separated and partially characterized. Endonuclease B has a primary requirement for Mn$^{2+}$ which cannot be replaced by Mg$^{2+}$ or Ca$^{2+}$, and makes single-strand scissions in double-stranded DNA. Endonuclease C is activated by either Mn$^{2+}$ or Mg$^{2+}$, and makes single-strand scissions with Mg$^{2+}$, while with Mn$^{2+}$, scissions are made which result in double-strand breaks. Neither enzyme is active on denatured DNA, and both are inhibited by yeast RNA. Both enzymes exhibit pH optima at pH 5.0 and pH 7.2, and leave 5'-phosphoryl termini.

Deoxyribonucleases have been shown to be essential components of the recombination, repair, and replication systems in prokaryotes [see review articles, refs. 1 to 3]. Since it is expected that this class of DNA enzymes will also have similar functions in eukaryotes, a study of these enzymes in the yeast, Saccharomyces cerevisiae, was begun. The existence of extensive genetic data on various aspects of recombination, as well as the availability of mutants affecting radiation repair (4), meiosis (5), meiotic (6), and mitotic recombination (7) make S.cerevisiae well suited to a molecular investigation of these processes. Moreover, recent work (8-9) which has defined major landmark events during the meiotic cycle and led to a significant improvement in the synchrony of cells
undergoing meiosis has encouraged us to think that a systematic investigation of DNases and other DNA enzymes would be particularly worthwhile at this time. Our previous efforts in this direction led to the characterization of an endonuclease (Endonuclease A) from whole cell extracts of *S. cerevisiae* (10). It soon became apparent that nuclei would probably be a better source of material for the study of enzymes involved in the replication, repair, or recombination of DNA, since it seemed reasonable to expect a substantial enrichment of such enzymes within nuclei. Moreover, the possibility of difficulties due to extraneous cytoplasmic enzymes such as proteases would be lessened.

The purpose of this report is to describe some of the properties of two new DNase activities, partially purified from yeast nuclear preparations which we propose to call endonuclease B and endonuclease C. Both enzymes have a decided preference for double-stranded DNA. Endonuclease B requires Mn$^{2+}$ for activity, a requirement not replaceable by either Mg$^{2+}$ or Ca$^{2+}$, and makes single-strand scissions in duplex DNA. Endonuclease C is activated by either Mn$^{2+}$ or Mg$^{2+}$ and make single-strand scission with Mg$^{2+}$. Activation by Mn$^{2+}$, on the other hand, leads to scissions resulting in double-strand breaks.

**Materials and Methods**

**Strains.** Diploid strain Z186, obtained from Drs. M. a R. Esposito was employed in this study.

**Media.** Z186 was grown in yeast extract peptone (YEP) (10) supplemented with 30 μg/ml of adenine, lysine, tryptophan, uracil, leucine, and histidine.

**DNA Substrates.** T5 and T4 DNA. Radioactively labelled T5 and T4 phage DNA were prepared as outlined by Thomas and Abelson (11). The specific absorbance at 260 nm was taken as 20 cm$^2$/mg. The optical density, taking into account the hyperchromic shift, was converted to nucleotide equivalents assuming a molar extinction coefficient of 10,000. Unless otherwise mentioned...
DNA concentration will be expressed as moles-nucleotide per milliliter. Dilutions for use in the various assays were made in 0.01M Tris, pH 7.5, 0.02M NaCl.

PM2-[3H] DNA. A stock of PM2 phage and its host, Pseudomonas Bal 31 were obtained from Dr. J. Richardson. Phage lysates and extracts of PM2 DNA were prepared according to the procedures of Richardson (12). Pseudomonas Bal 31 was grown at 27°C in Bal synthetic medium (13) supplemented with 0.2% casamino acids. 3H-labelled PM2 DNA was prepared by adding 3H-uracil (to a 250 ml culture volume) to a concentration of 0.2 uc/ml at 5 and 15 min after infection. Shaking was continued for 3-4 hrs until lysis occurred. The phage were purified and the DNA extracted. The 3H-PM2 DNA used in this report was used at a concentration of 21.5 ug/ml (0.06 umole-nucleotide/ml and a specific activity of 1.43 x 10^6 cpm/umole-nucleotide).

Deoxyribonuclease Assays.

Release of acid-soluble products. Assays were run at pH 5.0 using an acetate buffer, and at pH 7.2 using a TES (N-tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid) buffer. The incubation mixture (0.3 ml) contained 36 umoles of appropriate buffer, 2.0 umoles MnCl2, 6.0 umoles of 3H-T7 DNA, and various amounts (0.005-0.200 ml) of enzyme solution. After incubation at 37°C for 30 min, 0.2 ml of a solution of DNA (2.5 mg/ml) was added as "carrier", followed by 0.5 ml of cold 3.5% perchloric acid. After 5 min in an ice bath, the mixture was centrifuged for 15 min at 500 rpm in a Sorvall centrifuge; 0.2 ml was removed and added to 10 ml of Bray's (14) solution, and the radioactivity counted in a Beckman Scintillation counter. The supernatant obtained from control incubations (enzyme omitted) contained approximately 0.5% of the added radioactivity. A unit of activity was defined as that amount causing the production of 10^6 cpm of acid soluble 3H in 30 min. For the studies reported here, it was more convenient to express the activity as the percent of input counts rendered acid-soluble after incu-
bation at 37°C for 30 min.

Endonuclease Activity - Filter Assay. Endonucleolytic cleavage of DNA was monitored by a modification of the filter assay of Geiduschek and Daniels (15). A standard reaction mixture as described above was prepared and incubated for 30 min at 37°C. The reaction was stopped by adding 50 μl of 0.4M EDTA. To denature the DNA, 0.2 ml of 1N NaOH was added. After 10 min at room temperature, the contents of the reaction tube was mixed with 5 ml of 6X SSC, (16) (SSC, 0.015M Sodium citrate, 0.15M Sodium chloride, pH 7.0) and filtered slowly through nitrocellulose membrane filters (Schleicher and Schuell, type B-6). The filter was washed two times with 5 ml of 6X SSC, dried and counted in a toluene based scintillation fluid. Activity was expressed as the percent counts lost from the filter compared with a no-enzyme control.

Endonuclease Activity - Sedimentation Analysis. This endonuclease assay measures, by sedimentation in neutral and alkaline sucrose, the conversion of covalently-closed circular molecules of PM2 DNA to nicked ones. The sedimentation rate of PM2 DNA (molecular weight 6 x 10^6) in neutral and alkaline solution is 26.3 S and 50S, respectively (17). Nicked circles have a sedimentation coefficient of 21.2S in neutral sucrose; in alkaline solutions, the single-stranded circles and linear molecules are generally not resolved well and sediment with an S value of about 15 (18). From the Studier relations (19), linear, duplex molecules of PM2 DNA would be expected to have a sedimentation coefficient of 19.5S. Standard reaction mixtures, containing 1.2 nmoles ^3H-PM2 DNA were incubated at 37°C. The reaction was stopped at appropriate times by adding 25 μl 0.5M EDTA and heating for 10 min at 60°C. Then 0.1 ml of reaction mixture was layered on a 5 ml 5-20% sucrose gradient. Neutral sucrose gradients contained 0.9M NaCl, 0.01M EDTA, 0.3N NaOH. Centrifugation was carried out in a SW50.1 rotor at 4°C. Alkaline and neutral gradients were centrifuged at 40,000 rpm for 2.75 hr and 50,000 rpm for 3.5 hr, respectively. Under the assumption that the
number of breaks introduced by an endonuclease follows a Poisson distribution, the average number of nicks per molecule could be calculated from the expression,

$$P(0) = e^{-r},$$

where $$r$$ = average number of single-strand breaks per molecule, and $$P(0)$$ = relative amount of closed circular DNA remaining.

Identification of End-Groups Produced after Endonuclease Action.

Two methods were used to determine the nature of the end-groups produced after yeast endonuclease action. The first relies on the specificity of polynucleotide kinase to transfer a $^{32}$P-phosphoryl group from $\gamma$-$^{32}$P-ATP to each 5'-hydroxyl terminus in a DNA preparation (20). The second uses the difference in specificity between the two exonucleases, snake venom phosphodiesterase and bovine spleen phosphodiesterase, which require 3'-hydroxyl and 5'-hydroxyl end groups, respectively (21).

(a) Polynucleotide Kinase Activity. In this assay the conversion of $\gamma$-$^{32}$P of ATP into an acid-insoluble form after incubation with the yeast endonucleases was measured. A standard reaction mixture (0.3 ml) was prepared with yeast endonuclease B and C and incubated at 37°C. At different times the reaction was stopped by the addition of 10 μl of 0.2M EDTA, and heating the mixture for 10 min at 60°C. End group labelling with polynucleotide kinase was then carried out by the procedure of Weiss et al. (22). As controls we used DNase I (3.1.4.5) and DNase II (3.1.4.6) (Worthington) to produce nicks containing 5' phosphoryl-3' hydroxyl and 3' phosphoryl-5' hydroxyl end groups, respectively. The reaction mixture for DNase I was identical to our standard yeast reaction mix, except that it contained 0.4 μg DNase I. The reaction mixture for DNase II (0.3 ml) contained 28 μmoles acetate buffer, pH 4.8, 6 μmoles $^{3}H$-T5 DNA, and 0.2 μg DNase II (23). After incubation at 37°C for 20 min, the DNase I and DNase II reactions were stopped by heating 10 min at 65°C, and processed as in
the polynucleotide kinase assay.

(b) Snake Venom and Bovine Spleen Phosphodiesterase Assays. Digestion of yeast endonuclease treated $^3$H-T5 DNA with spleen and venom phosphodiesterase (Worthington) was carried out as follows: Replicate standard reaction mixtures were prepared and incubated at 37°C for 30 min. The reactions were stopped by heating for 10 min at 65°C. At this point 100 μg of snake venom phosphodiesterase (1 mg/ml in 0.12M Tris, pH 8.8) and 0.2 unit bovine spleen phosphodiesterase (2 units/ml in 0.2M succinate, pH 6.5) were added to replicate tubes. Incubation was continued for an additional 30 min at 37°C, and acid-soluble counts determined as described above. Appropriate controls were carried in parallel, and the data were expressed as the percent increase in acid soluble counts due to the action of the spleen or venom phosphodiesterase. In this case also DNase I and DNase II were used as controls to verify the specificity of the spleen and venom phosphodiesterases.

Buffers. SED buffer contained 0.9M sorbitol, 1 mM EDTA, 1 mM dithiotretilol (DTT), adjusted to pH 7.0. TEGD buffer contained 0.05M Tris, pH 7.9, 5% glycerol, 0.1 mM DTT, 0.1 mM EDTA, and 2 mM phenylmethylsulfonyl fluoride (PMSF). PMSF was added just prior to buffer use from a 0.04M solution in 95% ethanol (24). TAEGD buffer was like TEGD except that Tris was replaced by 0.05M Tris-acetate, pH 6.5.

Other Methods. Heat denatured DNA was obtained by heating a DNA solution (less than 100 μg/ml) for 10 min at 100°C, followed by immediate cooling in an ice bath. Protein was determined by the method of Lowry et al. (25) with bovine serum albumin as standard.

Growth of Cells and Preparation of Cell-Free Extracts. Cells were grown at 30°C in 3 liter Fernback flasks containing 1 liter of supplemented YEP medium. Cells were harvested in early stationary phase ($1 \times 10^8$ cells/ml) by centrifugation. The yield was 14-18 gm (wet weight) per liter of medium. All
subsequent steps were carried out at 0-4°C unless otherwise specified.

**Nuclear Preparations.** Spheroplasts were prepared as described previously (10). After spheroplast formation was judged optimal (greater than 90% in about 60 min), the spheroplasts were washed two times in 1M sorbitol by centrifuging at 1100 x g for 5 min. The spheroplast pellet (weighing normally 50-60% of the cell pellet) was resuspended in SED buffer at a concentration of less than 0.2 gm (wet weight) spheroplasts/ml. The spheroplast suspension was then broken in a Sorvall Omnimixer with care being taken to maintain low temperatures. Normally, 15 sec mixing at half-maximum speed resulted in greater than 90% spheroplast breakage.

The broken spheroplast suspension was centrifuged for 5 min at 650 g, and the supernatant was recentrifuged at 1000 5 for 5 min. The discarded pellet consists of debris and unlysed spheroplasts. The supernatant containing the nuclei and nuclear fragments was centrifuged for 10 min at 4000 g. The supernatant (cytoplasmic supernatant) was saved when protein, DNA and DNase recovery were being monitored.

The nuclear pellet was resuspended in 10-15 ml of TEGD buffer containing 1M ammonium sulfate. After mixing well the suspension was allowed to stand for 10 min in an ice bath, and then passed through a pre-cooled French cell at 14,000 lb/in. The broken nuclear suspension was resuspended in 3 ml TEGD + 1M ammonium sulfate and recentrifuged. The supernatants from the two spins were combined (nuclear supernatant), and after dialysis against 100 volumes TEGD buffer, served as the starting material for DNase assays and further purification.

**DEAE-Sephadex Chromatography.** The dialyzed nuclear supernatant was applied to a 50 ml bed volume of DEAE-sephadex equilibrated with TEGD buffer. The column was then washed with one column volume of TEGD buffer, and eluted either with a linear gradient (400 ml) with 0 and 0.3M ammonium sulfate as limiting concentrations, or by step gradients consisting of 60 ml each of
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0.05M, 0.10M, 0.20M, 0.30M ammonium sulfate in TEGD buffer. The flow rate was maintained at 30 ml/hr, and 4 ml fractions were collected.

Phosphocellulose Chromatography. Phosphocellulose powder (Whatman, P-11) was washed as described by Burgess (26). A 20 ml bed volume of phosphocellulose was equilibrated with TAEgd buffer. The "flow-through" fractions from the DEAE-sephadex column, hereafter called the "AI" fraction were pooled, dialyzed against TAEgd and applied to the phosphocellulose column. Elution was performed with a linear gradient with 0 and 0.3M ammonium sulfate in TAEgd buffer as limiting concentrations. Flow rate and fraction volume were 30 ml/hr and 2ml, respectively.

RESULTS

The nuclear supernatant as defined above contained 16-25% of the spheroplast protein and 33-50% of the total DNA. Since under the conditions of growth, mitochondrial DNA amounts to 10-12% of the total cellular DNA, in the best preparations 35-40% of the nuclei were being lost. DNase activity as monitored by release of acid soluble products from both single and double-stranded DNA was present in the dialyzed nuclear supernatant from pH 4.6 to pH 9.0. These activities were Mg2+ or Mn2+ dependent and were inhibited by EDTA. Maximum extraction of DNase activity into a soluble form required both high salt and vigorous disruption of the nuclear preparation.

Summary of Column Chromatography. The elution profile of the nuclear supernatant on DEAE-sephadex is shown in Fig. 1. Endonuclease activity monitored by the filter assay is shown in Fig. 1a, while acid-soluble activity on native and denatured 3H-T5 DNA is shown in Fig. 1b.

The elution profile of the AI fraction from a phosphocellulose column is shown in Fig. 2. The characterization of the "flow-through" activity from the phosphocellulose column (designated as PI) and the activity eluting at about 0.1M ammonium sulfate (designated as PII) form the subject of this
Sephadex chromatography. In this experiment, 14.5 ml of a nuclear supernatant (140 mg) were loaded onto a 45 ml column of DEAE-Sephadex equilibrated with TEGD buffer. The column was then washed successively with 60 ml TEGD, 60 ml TEGD + 0.1M (NH₄)₂SO₄, 60 ml TEGD + 0.2M (NH₄)₂SO₄, and 60 ml TEGD + 0.5M (NH₄)₂SO₄. Fractions of 5 ml were collected, and 100 μl were used to assay for DNase activity. Reactions were carried out at pH 7.2 using the standard conditions (using 6 mM MnCl₂). (a) Endonuclease activity was monitored by the Filter assay. Control incubations retained 3500 ± 200 cpm on the filter. (b) Release of acid-soluble fragments from native (●●●) and denatured (○○○) DNA was expressed as percent of input DNA.

Fig. 1. Elution profile of DNase activity after DEAE-Sephadex.

Rough estimates of the extent of purification of the PI and PII fractions is given in Table I. It should be recognized that this estimate is only approximate. Assay of endonuclease activity in the cytoplasmic supernatant is unreliable. Reliable estimates from the nuclear supernatant are also difficult to obtain not only because of contaminating enzymes, but because of inhibitory activities present. Thus, for example, the A1 fraction normally shows a substantial increase in activity over that in the nuclear supernatant, indicating that the activity in the nuclear supernatant is being masked. Hence, it is difficult to estimate reliably the recovery of the PI
Fig. 2. Separation of DNase activities on a phosphocellulose column.

The peak fractions of the 'flow-through' from the DEAE-Sephadex column (Fig. 1), the \( \Delta I \) fraction, were pooled and dialyzed against TAEGD buffer. In this experiment, 6-0 mg of the \( \Delta I \) fraction were applied to a 20 ml phosphocellulose column equilibrated with TAEGD buffer. The column was washed with 30 ml TAEGD buffer, followed by a gradient (100 ml) of 0 to 0-3M \((NH_4)_2SO_4\) in TAEGD. Fractions of 2 ml were collected and assayed as described in Fig. 1. (a) Endonuclease filter activity; (b) Release of acid-soluble fragments from native (•—•) and denatured (o—o) DNA.

and PII fractions. The units of activity were determined by the filter assay described in Methods.

Properties of Endonuclease B and Endonuclease C.

pH Optima and Cation Requirements. The endonucleolytic activities of the P1 and PII fractions from the phosphocellulose column, hereafter called endonuclease B and endonuclease C, respectively, exhibit activity at neutral as well as acid pH. The optimum at neutral pH is about pH 7.2 when assayed in TES buffer; the optimum at acid pH is pH 5.0 when assayed in acetate buffer. The level of activity at pH 5.0 is 50-100% higher than at pH 7.2 for endonuclease B; endonuclease C is some preparations exhibited as much as 3-fold higher activity at pH 5.0 than at pH 7.2. No differences in substrate specifi-
Table I

Summary of purification procedure of exonuclease B and endonuclease C.

| Fraction          | Volume (ml) | Protein mg/ml | Specific Activity units/mg protein | Total units | Purification |
|-------------------|-------------|---------------|-----------------------------------|-------------|--------------|
| Cytoplasmic supernatant | 47          | 12.0          | ----a                             | ----        | ----         |
| Nuclear supernatant | 14.5        | 9.5           | 200                               | 27980       | 1.0          |
| AI                | 20          | 1.28          | 5500                              | 140800      | 27.5         |
| PI                | 8           | 0.08          | 77000                             | 49300       | 385          |
| PII               | 15          | 0.30          | 39500                             | 177800      | 198          |

aMeasurement of DNase activity was unreliable at this step, and hence was not presented. DNase activity refers to exonuclease activity expressed in the units defined by the filter assay described in Methods.

City or cation requirements between the activities at pH 5.0 and pH 7.2 for either endonuclease B and endonuclease C were found. In the detailed description of these two activities which follows, assays were carried out at both pHs and is indicated in figure legend. It should be noted that the activity which releases acid-soluble fragments from denatured DNA and which contaminates endonuclease C (see Fig. 2) has a pH optimum between pH 7.0 and pH 7.4, and has no activity at pH 5.0. Endonuclease B, as will be shown below, has a primary requirement for Mn²⁺, which cannot be replaced by either Mg²⁺ or Ca²⁺; endonuclease C can be activated by Mn²⁺ or Mg²⁺, but not by Ca²⁺. Both activities were also completely inhibited by low levels of EDTA.

Substrate Specificity. In Fig. 3 the activity of endonuclease B and endonuclease C on double-stranded DNA measured by the filter assay and the
Fig. 3 Activity of endonuclease B and endonuclease C on native $^3$H-T5 DNA.

Incubation mixtures were prepared at pH 5.0 as described in Methods using 30 units of endonuclease B and 30 units of endonuclease C, and incubated at 37°C for the indicated times. Activity was monitored by the acid-soluble and filter assays.

release of acid-soluble fragments is compared. After a time when almost 100% of the counts have been lost from the filter, less than 3% have been detected as acid-soluble fragments.

Endonuclease B was further characterized by its activity on $^3$H-PM2 DNA as described in Methods. The sedimentation analysis of $^3$H-PM2 DNA incubated with endonuclease B indicates that the enzyme has a primary requirement for Mn$^{2+}$, which cannot be replaced by Mg$^{2+}$. Hence, as can be seen in Fig. 4, whereas a 15 min incubation with endonuclease B and Mn$^{2+}$ resulted in almost complete conversion of covalently closed circles to nicked ones, a 30 min incubation with Mg$^{2+}$ indicated no nicking activity. More precisely, 15 min with Mn$^{2+}$ gave $P(0) = 0.214$ and $r = 1.6$ nicks per molecule. In contrast, 30 min with Mg$^{2+}$ gave $P(0) = 0.956$ and $r = 0.04$. Moreover, from the sedimentation profiles in neutral sucrose, (Fig. 4) it is possible to say that endonuclease B is making primarily single-strand scissions. If we assume a sedimentation coefficient of 26.3 for the faster sedimenting component, then we calculate that the $S$ value for the slower component is 21.4, which is what

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Fig. 4. Neutral and alkaline sedimentation analysis of $^{3}$H-PM2 DNA treated with endonuclease B.

Comparison of cation requirements of endonuclease B. Incubation mixtures at pH 7·2 containing 30 units of endonuclease B, 1·2 nmoles $^{3}$H-PM2 DNA, and 6 mM MnCl$_2$ and 6 mM MgCl$_2$, respectively, were prepared. The Mn$^{2+}$ containing mixture was incubated for 15 min at 37°C. The reaction was stopped by adding 25 μl 0·5M EDTA, and heating the mixture for 10 min at 60°C. Then 0·1 ml was layered on a neutral and alkaline sucrose gradient. Centrifugation conditions were as described in Methods. A control incubation mixture was treated in an identical manner.

would be expected if only single-strand scissions are made.

In contrast to endonuclease B, endonuclease C is activated by Mn$^{2+}$ or Mg$^{2+}$. This is shown in Fig. 5 where a comparison of the activities of endonuclease C with Mg$^{2+}$ and Mn$^{2+}$ on $^{3}$H-PM2 DNA is presented. The results also indicated a difference in the specificity of the enzyme when activated with Mg$^{2+}$ as compared with Mn$^{2+}$. Thus, for example, in neutral sucrose, the slower moving component after 30 min with Mg$^{2+}$ has an S value of 21·4. After 30 min with Mn$^{2+}$ the distribution is decidedly more heterogeneous with an average S value of less than 18. The appearance of molecules with sedimentation coefficients of less than 18 indicates that the PM2 DNA has sustained double-strand breaks. However, the fact that the formation of the linear molecules follows that of the nicked circular molecules suggests that
Fig. 5. Neutral and alkaline sedimentation analysis of $^3$H-PM2 DNA treated with endonuclease C.

Comparison of activity with MnCl$_2$ and MgCl$_2$. Incubation mixtures containing 30 units of endonuclease C were prepared as described in Fig. 4. Centrifugation conditions were as described in Methods.

More double-strand breaks are being produced than random endonuclease activity would predict. Hence, endonuclease C when activated by Mn$^{2+}$ may have some preference for attacking a DNA strand opposite a nick, as has been suggested for pancreatic DNase (27).

Results consistent with this interpretation were also obtained by comparing the number of single-strand scissions leading to double-strand breaks following Mn$^{2+}$ activated endonuclease C attack on linear duplex DNA molecules. In Fig. 6 the sedimentation profiles in neutral and alkaline sucrose of T4 $[^{14}$C]$DNA$ after incubation with 20 units of endonuclease C for 10 min are shown. The number of double-strand breaks and single-strand nicks have been calculated using the expression derived by Charlesby (28)

$$N_t = \frac{2(e^{P} + P - 1)}{P^2}$$
Fig. 6. Sedimentation analysis of $^{14}$C-T4 DNA treated with endonuclease C activated with Mn$^{2+}$.

A standard incubation mixture at pH 5-0 containing 6.5 nmoles $^{14}$C-T4 DNA, 6 mM MnCl$_2$, and 20 units endonuclease C were prepared. After 10 min at 37°C, the reaction was stopped by adding 50 μl 0.5M EDTA and heating at 60°C for 10 min. Half of the reaction mixture was layered on a neutral sucrose gradient, and the remainder was layered on an alkaline sucrose gradient. Centrifugation was carried out in a SW50.1 rotor at 4°C. Neutral gradients were centrifuged for 2 hr at 46,000 rpm, and alkaline ones for 3 hr at 46,000 rpm.

$M_c/M_o$ is the ratio of final to initial molecular weights, and $p$, for neutral gradients, is the number of breaks per molecule, and for alkaline gradients, is the number of nicks per strand. After 10 min with endonuclease C we calculated that 12-16 nicks per strand resulted in 10-12 breaks per molecule. Since every double-strand break required at least one nick per strand, we see that most of the single-strand scissions made by endonuclease C, when activated with Mn$^{2+}$, lead to double-strand breaks.

Sedimentation analysis of denatured T5[$^3$H]DNA treated with endonuclease B and endonuclease C indicated that neither enzyme is active on denatured DNA. Incubation of heat denatured T5[$^3$H]DNA for 20 min with 20 units of endonuclease B and 20 units of endonuclease C (with either Mn$^{2+}$ or Mg$^{2+}$ resulted in no reduction in the average single-strand molecular weight of the enzyme treated DNA as compared with an untreated control. Hence, it...
appears that both endonuclease B and endonuclease C require a duplex structure for activity.

As will be recalled from Fig. 2, the phosphocellulose fraction, PII, also contains an activity which released acid-soluble fragments from denatured DNA. This activity was also activated by either Mg$^{2+}$ of Mn$^{2+}$. In order to determine whether this activity, the endonuclease C, and endonuclease B activities resided in the same protein, the thermal stability of all three activities was examined. The results shown in Fig. 7, indicate that all three activities differ significantly in their thermal stabilities. In particular, whereas only 10% endonuclease B activity is retained after 15 min at 50°, there has been no loss of endonuclease C activity. The acid-soluble activity of the phosphocellulose PII fraction is also clearly distinguishable from the endonuclease C activity. These results suggest strongly that these three activities reside in different proteins.

Fig. 7. Relative heat inactivation of endonuclease B, endonuclease C, and the PII acid-soluble activity.

Portions of the PI (endonuclease B), and the PII fractions were heated at 50°C. At the times indicated aliquots were removed and assayed for endonuclease and acid-soluble activity. Endonuclease activity was determined by the PM2 DNA assay described in Methods.
End-Group Specificity. The end-group specificity of endonuclease B and endonuclease C determined by the polynucleotide kinase assay described in Methods has indicated that both have the same specificity as DNase I. Thus, for example, under conditions where less than 2% acid-soluble $^3H$ counts were produced with DNase I and DNase II, 99 cpm and 665 cpm $^{32}P$, respectively, were recovered in acid precipitable form after incubation with polynucleotide kinase. A similar incubation with 20 units of endonuclease B for up to one hour resulted in only 120 cpm of acid-precipitable $^{32}P$. Similarly, incubation of endonuclease B-treated $^3H$-T5 DNA with snake venom phosphodiesterase resulted in a 4-fold increase in acid-soluble counts compared with only the venom phosphodiesterase control. In contrast, incubation with bovine spleen phosphodiesterase resulted in no increase in acid-soluble counts. The polynucleotide kinase and phosphodiesterase results are consistent with each other and indicate that the reaction products of endonuclease B and endonuclease C are 5'-phosphoryl terminated oligonucleotides.

RNA Inhibition. Both endonuclease B and endonuclease C are inhibited by yeast RNA. Addition of yeast RNA in the incubation mixture to a concentration of 40 nmoles/ml resulted in 70% inhibition of endonuclease C, and about 80% inhibition of endonuclease B. In contrast, the acid-soluble PII activity was inhibited to only about 10% at the highest concentrations of RNA used. This result also supports our conclusion that the endonuclease C activity and the acid-soluble PII activity reside in different proteins.

Stability. The phosphocellulose fractions have shown no significant loss of activity after repeated freezing (-20°C) and thawing for over a period of 16 months.

DISCUSSION

Two endonuclease activities, which we have called endonuclease B and endonuclease C, obtained from yeast nuclear preparations have been separated.
and partially characterized. Both enzymes exhibit pH optima at pH 5.0 and pH 7.2, and leave 5′-phosphoryl termini. Endonuclease B has a primary requirement for Mn$^{2+}$ that cannot be replaced by Mg$^{2+}$ or Ca$^{2+}$, and makes single-strand scissions in double-stranded DNA. Endonuclease C is activated by either Mn$^{2+}$ or Mg$^{2+}$ and appears to make single-strand scissions with Mg$^{2+}$, while with Mn$^{2+}$ scissions are made which result in double-strand breaks.

Neither enzyme is active on single-stranded DNA. An enzyme comparable to endonuclease B is the Mn$^{2+}$ activated T4-induced endonuclease (29), except that the specificity with Mn$^{2+}$ of endonuclease B appears to be more absolute.

We have found that the endonuclease C activity eluting at about 0.1M salt from a phosphocellulose column is contaminated with an activity which produces acid-soluble products from single-stranded DNA. Differences in thermal stability and substrate specificity indicate that this activity and the endonuclease C activity reside in different proteins. This (acid-soluble) activity is also distinguishable from endonuclease A described earlier (10), since it does not exhibit endonucleolytic activity on single-stranded DNA, which is the characteristic feature of endonuclease A.

The correlation between the in vitro specificity of these endonucleases and their function in vivo is far from clear. Experience with prokaryotic systems has shown that the generation of either single or double-strand scissions, whether as a result of specific recombination enzymes or as a consequence of replication or repair processes, are necessary features of a recombination event. The specific processes by which, in Bernardi's terms (30), haplotomic (single-break) and diplotomic (double-break) mechanisms lead to recombination events remain to be worked out. The diplotomic activity of endonuclease C is similar to that of pancreatic DNase, which can make either single or double-strand breaks depending upon the divalent cations present (31). The enzymological analysis of strains in S. cerevisiae with altered repair, recombination, and replication properties should begin to define the
function of these and other DNA enzymes. The procedural framework described in this report will, we hope, aid in such studies.

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REFERENCES

1. Richardson, C.C. (1969) Ann. Rev. Biochem. 38, 795.
2. Clark, A.J. (1971) Ann. Rev. Microbiol. 25, 437-464.
3. Radding, C.M. (1973) Ann. Rev. Genetics 7, 87-112.
4. Cox, B.S. and Parry, J.M. (1968) Mutation Res. 6, 37-55.
5. Esposito, M.S. and Esposito, R.E. (1969) Genetics 61, 79-89.
6. Roth, R. and Fogel, S. (1971) Molec. Gen. Genetics 112, 295-305.
7. Rodarte-Ramén, U.S. and Mortimer, R.K. (1972) Radiation Res. 49, 141-147.
8. Simchen, G., Piñon, R. and Salts, Y. (1972) Expt. Cell Res. 75, 205-218.
9. Esposito, M.S. and Esposito, R.E. Genetics, in press.
10. Piñon, R. (1970) Biochemistry 9, 2839-2865.
11. Thomas, C.A., Jr. and A'nelson, J. (1965) in Procedures in Nucleic Acid Research (Cantoni, G.L. and Davies, D.R., eds) p. 235, Harper and Row, New York.
12. Richardson, J. (1973) J. Mol. Biol. 78, 703-714.
13. Franklin, R.M., Salzett, M., and Silbert, J.A. (1969) Virology 38, 627-240.
14. Bray, G.A. (1960) Anal. Biochem. 1, 279-285.
15. Geiduschek, E.P. and Daniels, A. (1965) Anal. Biochem. 11, 133-137.
16. Center, M.S., Studier, F.W., and Richardson, C.C. (1970) Proc. Nat. Acad. Sci. U.S.A. 65, 242-248.
17. Espejo, R.T., Canelo, E.S., and Sinheimer, R.L. (1969) Proc. Nat. Acad. Sci. U.S.A. 65, 1164-1168.
18. Espejo, R.T. and Canelo, E.S. (1969) Virology 37, 495-498.
19. Studier, F.W. (1965) J. Mol. Biol. 11, 373-390.
20. Richardson, C.C. (1965). Proc. Nat. Acad. Sci. U.S.A. 54, 158-165.
21. Razzell, W.E. (1963). Methods in Enzymology VI, 236-258.
22. Weiss, B.W., Live, T.R. and Richardson, C.C. (1968). J. Biol. Chem. 243, 4530-4542.
23. Laskowskl, M., Sr. (1961). In The Enzymes 5, 2nd edit. (Boyer, P.D., Lardy, H. and Myrback, K., eds) p. 123, Academic Press, New York.
24. Schulze, I.T. and Colowick, S.P. (1969). J. Biol. Chem. 244, 2306-2315.
25. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1961) J. Biol. Chem. 193, 265-275.
26. Burgess, R.R. (1969). J. Biol. Chem. 244, 6160-6167.
27. Masamune, Y., Fleischman, R.A., and Richardson, C.C. (1971). J. Biol. Chem. 246, 2680-2691.
28. Charlesby, A. (1954). Proc. Roy. Soc. A. 224, 120-128.
29. Kemper, B. and Hurwitz, J. (1973). J. Biol. Chem. 248, 91-99.
30. Bernardi, G. (1968). Advan. Enzymol. 31, 1-49.
31. Melgar, E. and D.A. Goldthwait (1968). J. Biol. Chem. 243, 4409-4416.