Properties of a Novel Oligonucleotide-Releasing Bidirectional DNA Exonuclease from Mouse Myeloma†

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ABSTRACT: Highly purified, but not homogeneous, samples of helix-destabilizing protein 1 from mouse myeloma contain a novel oligonucleotide-releasing DNA exonuclease. This enzyme was separated from helix-destabilizing protein 1 and obtained in highly purified form. A polypeptide of M_r 41 000 is a main constituent of the purified enzyme, and this polypeptide comigrated with the exonuclease activity during the final step of the purification, Sephacryl S-200 gel filtration where the enzyme had a native M_r of 40 000. Overall purification of enzyme activity was >20 000-fold. This exonuclease releases 5'-oligonucleotides in a limited processive manner in both the 5' → 3' and 3' → 5' directions. Activity of the enzyme is resistant to 1 mM *N*-ethylmaleimide, requires a divalent cation, has an alkaline pH optimum, and degrades single-stranded DNA much faster than double-stranded DNA or RNA. The predominant oligonucleotide product with uniformly labeled substrates is $(pdN)_2$. With 3' end labeled substrates, >95% of the labeled products are $(pdN)_4$ and $(pdN)_5$; with 5' end labeled substrates, the main labeled product is $(pdA)_2$. The rate of product release from 3' and 5' end labeled substrates is nearly identical at 37 °C. A model of the action of this enzyme and a comparison with a human placenta exonuclease [Doniger, J., & Grossman, L. (1976) *J. Biol. Chem. 251*, 4579-4587] are discussed.

Mammalian cells can remove pyrimidine dimers and other bulky lesions from DNA by a pathway known as nucleotide excision repair (Hanawalt et al., 1978, 1979; Brown et al., 1979; Friedberg et al., 1979). This appears to be a complex series of reactions by which an oligonucleotide segment of DNA containing a lesion is excised and then new damage-free DNA is formed in its place. It now seems clear that this pathway plays an important role in protecting cells against UV light and a variety of chemicals that damage DNA. A model for the nucleotide excision-repair pathway involves four steps of distinct DNA enzymology: (1) incision of the DNA strand containing the damaged nucleotide, (2) excision of the damaged nucleotide plus some flanking DNA, (3) resynthesis of damage-free DNA using the complementary strand as template, and (4) ligation to reestablish continuity of the now damage-free strand.

To better understand the nucleotide excision-repair pathway in mammalian cells, detailed biochemical studies of critical enzymes and accessory proteins are needed. Information on the properties of the mammalian DNA polymerases, for example, is far more advanced than our understanding of the other enzymes and factors required for excision repair, and therefore, the resynthesis step has been the focus of much more experimental attention than the other steps. Concerning the enzymology of the excision step, multiple forms of thymidine dimer excising exonuclease were observed in KB cell extracts by Cook & Friedberg (1978), and Doniger & Grossman (1976) reported detailed studies of a putative excision-repair exonuclease from human placenta. In the present study, we found an oligonucleotide-releasing DNA exonuclease in preparations of a mouse single-stranded DNA binding protein. This exonuclease was obtained in highly purified form and characterized in detail.[1] The mouse enzyme shares many properties with the exonuclease from human placenta (Doniger & Grossman, 1976) and appears to be of the same general class.

Materials and Methods

Materials

Mouse myeloma (MOPC-104E) was grown subcutaneously and stored in liquid N_2 until use (Matsukage et al., 1976). Acrylamide, bis(acrylamide), ammonium persulfate, *N,N,N',N'*-tetramethylethylenediamine, and bromphenol blue (BPB)[2] were from Bio-Rad Laboratories. Formamide was from Fluka A.G.; urea (ultrapure) was from Schwarz/Mann; agarose was from Sea Kem; xylene cyanol FF was from Kodak; boric acid was from Fischer Scientific Inc.; tris(hydroxymethyl)aminomethane (Tris) was from Bethesda Research Laboratories, Inc. Cellulose powder CF11 was from Whatman, Ltd. Sephacryl S-200 and sodium dextran sulfate 500 were from Pharmacia Fine Chemicals. *N*-Ethylmaleimide and *p*-(hydroxymercuri)benzoate, sodium salt, were from Sigma Chemical, Inc. These compounds were dissolved in H_2O and used immediately in experiments. Dithiothreitol was from Calbiochem; 2-amino-2-methyl-1-propanol and 3-(*N*-morpholino)propanesulfonic acid were from P-L Biochemicals, Inc. Polynucleotide kinase and RNA ligase, RNase and DNase free, from T4-infected *Escherichia coli* were obtained from P-L Biochemicals, Inc. Polynucleotide kinase and RNA ligase, RNase and DNase free, from T4-infected *Escherichia coli* were obtained from P-L Biochemicals, Inc. Calf thymus terminal deoxynucleotidyltransferase was from Bethesda Research Laboratories, Inc. Calf alkaline phosphatase was from Boehringer-Mannheim, Inc. Nucleoside 5'-triphosphates were from Calbiochem, Inc. ^{32}P-Labeled nucleoside 5'-triphosphates in aqueous solution were from Amersham, Inc.

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[1] A brief report of these findings has been presented (Becerra & Wilson, 1982).

[2] Abbreviations: SDS, sodium dodecyl sulfate; AMP buffer, 2-amino-2-methyl-1-propanol; MOPS, 3-(*N*-morpholino)propanesulfonic acid; NEM, *N*-ethylmaleimide; pHMB, *p*-(hydroxymercuri)benzoate; TDT, terminal deoxynucleotidyltransferase; CAP, calf alkaline phosphatase; ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; BSA, bovine serum albumin; BPB, bromphenol blue; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; polynucleotide subscripts indicate the precise chain length, and subscripts with a bar indicate the average chain length.

Polynucleotide concentrations are expressed on the basis of polymer molecules, not nucleotide residues. Oligonucleotide and polynucleotide preparations were from P-L Biochemicals, Inc.; ϕX174 viral DNA (+ strand) and ϕX174 RF DNA were from Bethesda Research Laboratories, Inc. Calf thymus DNA was from Worthington Biochemicals, Inc. [*adenyl*-8-^{14}C]-Poly(dA) (specific activity 2.5 μCi/μmol of adenine) was from P-L Biochemicals. Both ssDNA–cellulose and dsDNA–cellulose were prepared (Planck & Wilson, 1980) according to the method described by Litman (1968).

Methods

23% Polyacrylamide–7 M Urea Gel Electrophoresis. The sample to be analyzed was subjected to a denaturing treatment by heating at 80 °C for 3 min in the presence of 60% formamide and then cooling on ice. The sample then was mixed with a solution of bromphenol blue and xylene cyanol FF, and the resulting solution (usually 22 μL) was loaded directly on a 23% polyacrylamide slab gel (individual lanes of 36 × 1.2 × 0.08 cm) containing 7 M urea, 50 mM Tris–borate, pH 8.3, and 1 mM EDTA (Maxam & Gilbert, 1977). The gel had been preelectrophoresed at 900 V for at least 3 h. Electrophoresis was performed without cooling at 900 V until BPB had migrated to half the length of the gel. The electrophoresis running buffer was 50 mM Tris–borate, pH 8.3. The wet gel was transferred to a used X-ray film and wrapped in polyethylene. Autoradiography was performed at −80 °C, typically for 1–2 h, by using Kodak XR film and a Kodak lightning-plus intensifying screen (Detera et al., 1981; Detera & Wilson, 1982; Becerra et al., 1983).

Preparation of Circular $(pdA)_{16}$. The circular $(pdA)_{16}$ was obtained from linear $(pdA)_{16}$ after ligation with T4 RNA ligase (Sugino et al., 1977; Silber et al., 1972; Walker et al., 1975). The mixture (100 μL) contained the following: 66 mM Tris-HCl, pH 7.4, 6.6 mM $MgCl_2$, 10 mM dithiothreitol, 0.6 mM ATP, 6 μM [5′-^{32}P]$(pdA)_{16}$, 12 μM 5′-OH $(dA)_{15}$, d-$(pdA)_{15}$, and 27 units/mL RNA ligase. After an overnight incubation at 15 °C, the reaction mixture was placed in a 75 °C water bath for 5 min and then cooled immediately in an ice–water bath. Portions of this mixture were used directly in the nuclease reactions described. To assay the formation of circularized $(dA)_{16}$, an aliquot of the mixture was treated with CAP, and another aliquot was analyzed by 23% polyacrylamide–7 M urea gel electrophoresis. This CAP treatment did not remove the ^{32}P label from the oligonucleotide; the labeled oligonucleotide migrated in the gel as a sharp band located between $(pdA)_{13}$ and $(pdA)_{14}$ and, hence, was clearly distinguished from either $(pdA)_{16}$ or $(pdA)_{32}$.

Preparation of Substrates with [^{32}P]*dAMP at the 3′ End.*[3] We used a modification of the procedure described by Bollum (1962). Reaction mixtures (50 μL) contained the following: 60 mM Tris-HCl, pH 7.8, 7.5 mM magnesium acetate, 6% glycerol, 15 mM KCl, 0.24 mg/mL BSA, 5 μM [α-^{32}P]dATP (45 × 10^4 cpm/pmol), 40 μM oligo(pdA) and 850 units/mL TDT. After a 60-min incubation at 37 °C, reactions were terminated by placing the tubes in a 75 °C water bath. After 5 min, the reaction mixtures were cooled quickly in an ice–water bath, and portions were then used directly in the nuclease reactions. [^{32}P]dAMP incorporation was determined by chromatography on DE-81 paper. The migration on a 23% polyacrylamide–7 M urea gel of the labeled oligonucleotide was identical with that of the authentic oligo(pdA) molecules (Becerra et al., 1983) containing one or two dAMP residues more than the substrate.

Preparation of Substrates with [^{32}P]PO_4 *at the 5′ End.*[3] Various 5′-OH oligo(dA) molecules were labeled during a polynucleotide kinase reaction (50 μL) containing the following: 70 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 0.4 mg/mL BSA, 1 mM dithiothreitol, 40 μM oligo(dA), 100 μM [γ-^{32}P]ATP (22 × 10^3 cpm/pmol), and 100 units/mL polynucleotide kinase. After a 60-min incubation at 37 °C, the mixtures were placed in a 75 °C water bath for 5 min and then cooled immediately in an ice–water bath. Portions of these mixtures were used directly in the nuclease reactions. [^{32}P]PO_4 incorporation was measured by chromatography on DE-81 paper. The migration of the labeled oligonucleotide on a 23% polyacrylamide–7 M urea gel was identical with that of authentic oligo(pdA) molecules. Other polynucleotides were labeled similarly after treatment with CAP.

Preparation of Uniformly ^{32}*P-Labeled Oligo(dA).* We followed the procedure used for the preparation of substrates with [^{32}P]dAMP at the 3′ end. The reaction mixture (100 μL) contained the same reagents, except for 5 mM [α-^{32}P]dATP (80 cpm/pmol), 51 μM $(dA)_4$, and 400 units/mL TDT. After a 3-h incubation at 37 °C, 24 units of TDT were added; this was repeated 3 times. The reaction was terminated by placing the tube in a boiling water bath. After 5 min, the reaction mixture was cooled quickly in an ice–water bath, and portions were then used directly in nuclease reactions. [^{32}P]dAMP incorporation was determined by chromatography on DE-81 paper, indicating that an average of 50 dAMP residues had been incorporated per molecule of substrate. The migration on a 23% polyacrylamide–7 M urea gel of the labeled material corresponded to the migration of oligo(dA) molecules 45–55 residues in length.

Alkaline 1.5% Agarose Slab Gel Electrophoresis. We used the method described by McDonnell et al. (1977). A horizontal slab gel containing 1.5% agarose, 30 mM NaOH, and 2 mM EDTA was used. The electrophoresis running buffer was 30 mM NaOH–2 mM EDTA. DNA samples were adjusted to 10% glycerol, 0.025% BPB, 30 mM NaOH, and 2 mM EDTA and electrophoresed at 80 V for 3 h at 25 °C. The gel was removed from the electrophoresis chamber, neutralized, treated with ethidium bromide, and exposed to UV light.

Assay of Mouse Exonuclease. Assays used to monitor purification contained in a final volume of 10 μL the following: 50 mM Tris-HCl, pH 7.4, 1 mM dithiothreitol, 0.4 mg/mL BSA, 40 mM KCl, 6 mM magnesium acetate, 1 μM $(dA)_9$ as substrate (4 × 10^3 cpm/pmol), and sample (usually 1 μL) to be assayed. Assays used for characterization of fraction V enzyme contained in a final volume of 10 μL the following: 50 mM Tris-HCl, pH 8.4, 0.4 mg/mL BSA, 40 mM KCl, 6 mM magnesium acetate, 1 μM oligo(pdA) 5′-end or 3′-end ^{32}P labeled, and 2 ng of enzyme protein. Unless specified otherwise, incubations were for 20 min at 37 °C. The reactions were terminated by addition of formamide to a final concentration of 60% v/v and freezing in dry ice. Each reaction mixture then was analyzed by 23% polyacrylamide–7 M urea gel electrophoresis, as described above. Labeled reaction products were localized in the gel by autoradiography, and the portion of gel containing each product was cut out. Radioactivity was measured in a scintillation counter. One unit of enzyme activity is 1 pmol of product formed per h at 37 °C. The study of enzyme activity as a function of pH (6.0–9.5) was conducted with the following buffers: AMP buffer, MOPS, Tris-HCl, and NaP_i.

[3] The 5′-end ^{32}P atom was attached to the substrate through a phosphoester linkage at the 5′-OH group of the terminal deoxyribosyl group. The 3′-end ^{32}P atom was in the phosphodiester linkage between the penultimate and ultimate deoxyribonucleoside groups.

Table I: Partial Purification of Nuclease

fraction[a]	description	volume (mL)	protein (mg)	activity (units)[b]	sp act. [10^3(units/mg of protein)]
I	extraction and poly(ethylene glycol) treatment	967	5000	30000	0.006
II	dsDNA-cellulose/ssDNA-cellulose	52	18	15600	0.87
III	ssDNA-cellulose[c]	4.2	7	7700	1.1
IV	ssDNA-cellulose	6.2	0.6	1600	2.7
V	Sephacryl S-200, fractions 53 and 54	4.2	0.004	500	125

[a] From 150 g of frozen mouse myeloma, MOPC-104E. [b] 1 unit = 1 pmol of $(dpA)_4$ plus $(pdA)_5$ formed per 60 min at 37 °C. The substrate was 3'-end ^{32}P-labeled $(pdA)_9$. [c] Equivalent to fraction III helix-destabilizing protein 1 (Planck & Wilson, 1980).

Purification of Mouse Exonuclease. The procedure involved preparation of fraction III HD protein 1 exactly as described by Planck & Wilson (1980). In a series of preliminary experiments, we found that the exonuclease activities in fraction III could be separated from most of the HD protein 1 by chromatography on a small ssDNA–cellulose column. Thus, fraction III was applied to a 3-mL ssDNA–cellulose column (0.9 × 5 cm) at a flow rate of 1 mL/min, and the column was washed with 15 mL of buffer C. A 30-mL gradient of NaCl, from 25 to 500 mM in buffer C, was applied for elution (Figure 1). Fractions containing nuclease activity eluting in the flow through or just after the start of the gradient (Figure 1) were pooled and dialyzed against buffer D. The dialyzed solution (fraction IV) was pumped onto a Sephacryl S-200 column (0.9 × 90 cm) that was coupled in tandem to an identical column. Elution was with buffer D at 10 mL/h, and each tube contained 1 mL. All of the nuclease activity, measured with 5'- and 3'-end [^{32}P](pdA)$_9$, emerged from the column in a single symetrical peak centered in tube 54, corresponding to a molecular weight of about 39 000. The void volume of the column was in tube 38, and the marker proteins BSA, carbonic anhydrase, and lysozyme emerged in tubes 47, 51, and 59, respectively. The solution obtained by pooling tubes 53 and 54 was fraction V.

Buffers. Unless otherwise indicated, all pH values refer to the final solution at 0 °C. Buffer C is 10 mM Tris-HCl, pH 8.5, 25 mM NaCl, 0.5 mM EDTA, 50 μM dithiothreitol, and 5% glycerol. Buffer D is 20 mM Tris-HCl, pH 8.0, 500 mM NaCl, 0.1 mM EDTA, 1 mM dithiothreitol, and 10% glycerol.

Results

During studies of the influence of DNA binding proteins on the processivity of mouse α-polymerase (Detera et al., 1981), samples of partially purified mouse HD protein 1 were found to contain DNA exonuclease activities. The fact that homogeneous HD protein 1 was nuclease free (Planck & Wilson, 1980) suggested that the enzyme(s) responsible had been removed during subsequent purification steps. Several additional points were evident from these preliminary studies. First, $(pdN)_2$ was the predominant product with incubations containing 5' end labeled ssDNA as substrate, and dNMP, $(pdN)_4$, and $(pdN)_5$ were the predominant products with 3' end labeled ssDNA as substrate. The migration behavior of these oligonucleotide product molecules permited unequivocal assignment of the presence of a 5'-phosphate group (Becerra et al., 1983). Second, 1 mM NEM inhibited the formation of [^{32}P]dNMP but had no effect on ^{32}P-labeled oligonucleotide formation; changing the pH from 7.2 to 8.4 reduced the formation of [^{32}P]dNMP, whereas the formation of ^{32}P-labeled oligonucleotide was increased severalfold. These results suggested that dNMP formation was due to a different catalytic activity than oligonucleotide formation. The finding of different oligonucleotide products depending on whether the substrate was 5' or 3' end labeled raised the possibility of the

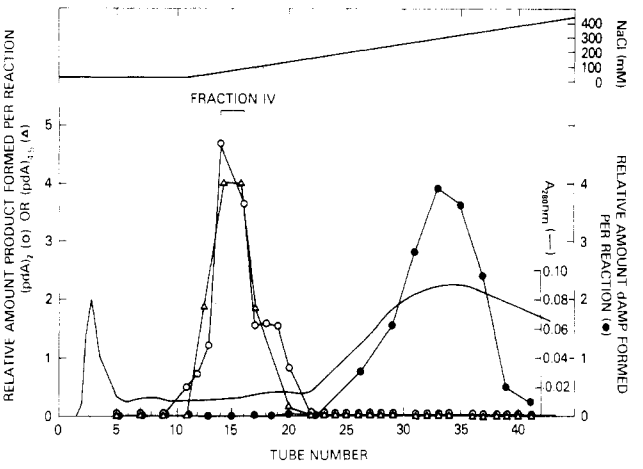


FIGURE 1: Purification of exonuclease by column chromatography on ssDNA–cellulose. Bands representing the products indicated were sliced from the gel and counted. Enzyme activity expressed as $(pdA)_{4,5}$ represents the sum of cpm in $(pdA)_4$ and $(pdA)_5$. Three milliliters of fraction III was applied to a column of 3-mL bed volume. The tubes indicated were pooled and dialyzed against buffer D to give fraction IV.

presence of two oligonucleotide-releasing enzymes, since there were no reports of a mammalian DNA exonuclease that releases products of different sizes depending upon which end of the substrate is labeled; this is well documented, however, with prokaryotic exonucleases (Kaplan et al., 1969; Chase & Richardson, 1974a,b). Hence, during subsequent purification of the exonuclease activities, starting with partially purified HD protein 1, it was of interest to follow the fractionation of three nuclease activities: [^{32}P](pdN)$_2$ formation with 5' end labeled ssDNA, [^{32}P]dNMP formation with 3' end labeled ssDNA; and [^{32}P](pdN)$_4$ plus [^{32}P](pdN)$_5$ formation with 3' end labeled ssDNA.[3]

Partial Purification of Oligonucleotide-Releasing Exonuclease. The overall purification from the crude extract is summarized in Table I. All enzyme activity measurements were at pH 7.2 with end-labeled oligo(dA) as substrate. In the ssDNA–cellulose column chromatography of fraction III (Figure 1), [^{32}P]dAMP-forming activity separated from the two oligonucleotide-forming activities, which cochromatographed with each other. The oligonucleotide-forming activities also comigrated through the Sephacryl S-200 column (data not shown). This final step resulted in an ~50-fold increase in enzyme specific activity (Table I) and removal of most of the Coomassie blue stained protein bands detected in SDS–polyacrylamide gel electrophoresis (Figure 2).

Migration of the oligonucleotide-forming activities in the Sephacryl S-200 gel filtration column was consistent with an average globular protein of native M_r 39 000, and a M_r 41 000 polypeptide in fraction V represented a large proportion of the stained protein (Figure 2). Analysis of portions from selected fractions of the S-200 column revealed that a M_r ~40 000 polypeptide comigrated with the enzyme activity. On the basis

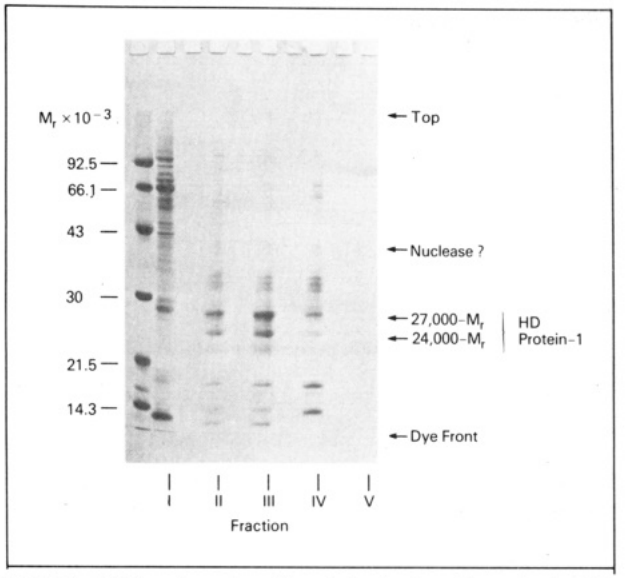


FIGURE 2: SDS–polyacrylamide gel electrophoretic analysis of the mouse exonuclease at various stages of purification. Results are with the purification described in Table I. For each sample, proteins in solutions of 20–100 μL were concentrated by acetone precipitation, denatured, and then subjected to electrophoresis in a 12% polyacrylamide gel (Planck & Wilson, 1980). The gels were stained with Coomassie blue. The amount of protein analyzed in each slot was 15.6, 4.6, 5.6, 5.3, and 0.4 μg for fractions I, II, III, IV, and V, respectively.

of these results, we conclude that the final fraction of exonuclease (fraction V) was highly purified but not homogeneous; the enzyme in a high ionic strength solution behaves as a single polypeptide of M_r ~40000, and both oligonucleotide-releasing activities appear to be tightly associated and could reside in this one polypeptide. The principal contaminants were M_r ~65000 and ~35000 polypeptides; the M_r 65000 polypeptide clearly did not comigrate with the enzyme activity. In an experiment not shown, where fraction III was applied directly to the gel filtration column, an enzyme activity profile identical with that observed by the usual procedure was obtained. Yet, the ssDNA–cellulose column was included as the penultimate step of the purification because this markedly reduced the amount of HD protein 1 and removed the dAMP forming 3′→5′-exonuclease activity (see Figure 1).

Finally, attempts to further purify the fraction V enzyme by chromatography on ssDNA–cellulose or slab gel isoelectric focusing were not successful due to failure to bind or loss of activity, respectively. The oligonucleotide-forming activity of fraction V was lost upon freezing and thawing; activity was stable at −20 °C in the presence of 50% glycerol. Fraction V was free of DNA nicking or endonuclease activity against φX174 DNA and homopolymer DNA. Fraction V also was free of dNMP forming 3′→5′-exonuclease, DNA polymerase, and DNA-dependent ATPase activities.

Catalytic Properties. Catalytic properties of the fraction V enzyme were examined by using optimal reaction conditions determined in preliminary experiments, i.e., 50 mM Tris-HCl at pH 8.4, 6 mM Mg^{2+}, and 1 μM end-labeled oligo(dA) as substrate. The rate of ^{32}P-labeled oligonucleotide formation was about equal with the 5′ or the 3′ end labeled $(pdA)_9$ as substrate (Figure 3), and activity was proportional to the amount of enzyme in the reaction mixture in the range of 0–3 ng. Some requirements for ^{32}P-labeled oligonucleotide formation with the two end-labeled substrates are shown in Table II. Activity with both substrates was resistant to 1 mM NEM but was blocked by 0.1 mM pHMB. This inhibition picture of NEM resistance and pHMB sensitivity is reminiscent of

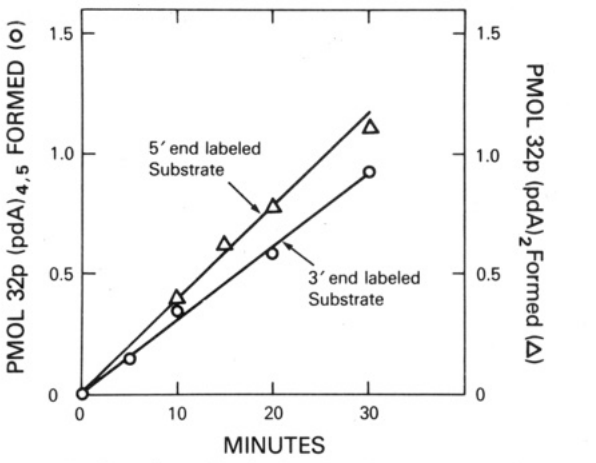


FIGURE 3: Product formation by fraction V mouse exonuclease as a function of time of incubation. The substrates were $(pdA)_9$ 5′ or 3′ end labeled. Incubations were as described under Methods and contained 2 ng of fraction V proteins.

Table II: Some Properties of Nuclease Activities[a]

modification[a]	amount of ^{32}P product formed (%)	
	$(pdA)_{4,5}$	$(pdA)_2$
none	100 (0.6)	100 (0.8)
+1 mM DTT	100	100
+1 mM NEM	115	100
+4 mM NEM	65	60
+1 mM ATP	100	90
+0.1 mM pHMB	5	5
+0.5 mM pHMB	0	0
+40 mM EDTA	0	0
+200 mM NaCl	50	60
enzyme preincubation 10 min at 70 °C	<1	<1
enzyme preincubation 3 min at 70 °C	40	50
$+(pdA)_{300}$	100	100
$+(pdA)_{16}$	50	50
$+(pdA)_6$	45	50

[a] Each reaction mixture (10 μL) contained 50 mM Tris-HCl, pH 8.4, 0.4 mg/mL BSA, 40 mM KCl, 6 mM magnesium acetate, and 1 μM $(pdA)_9$ 5′ or 3′ end ^{32}P labeled. Values representing 100% shown in parentheses are picomoles per 20 min.

observations with mammalian DNA polymerase β (Matsukage et al., 1975). Activities were blocked by an excess of EDTA and by a 10-min preincubation at 70 °C. Activities were reduced about equally by a shorter preincubation at 70 °C. Addition of 200 mM NaCl resulted in only 50% inhibition, indicating that the activities were relatively salt resistant. Addition of $(dA)_{\overline{300}}$, in a microgram amount equal to the amount of $(pdA)_9$ substrate, had no effect on the activities, whereas addition of an equal amount of $(pdA)_{16}$ or $(pdA)_6$ reduced labeled product formation by about 50%. This suggested that the enzyme recognizes termini and that the affinity for the substrate was about equal to that for the other two oligonucleotides. In a series of experiments not shown, the pH optimum was found to be alkaline and broad (pH 7.6–9); sodium phosphate buffer was inhibitory. Activity was not observed in the absence of a divalent cation, and Mn^{2+} and Mg^{2+} were equally effective at saturating levels, which were 0.5 and 5 mM, respectively. The apparent Michaelis constant for the substrate was 0.3 μM. With the 3′ end labeled substrate, the ratio of $(pdA)_5$ to $(pdA)_4$ formed was not constant under all of the conditions evaluated. Under the optimal reaction conditions, the ratio was about 1:1. At 0.5 and 1 mM

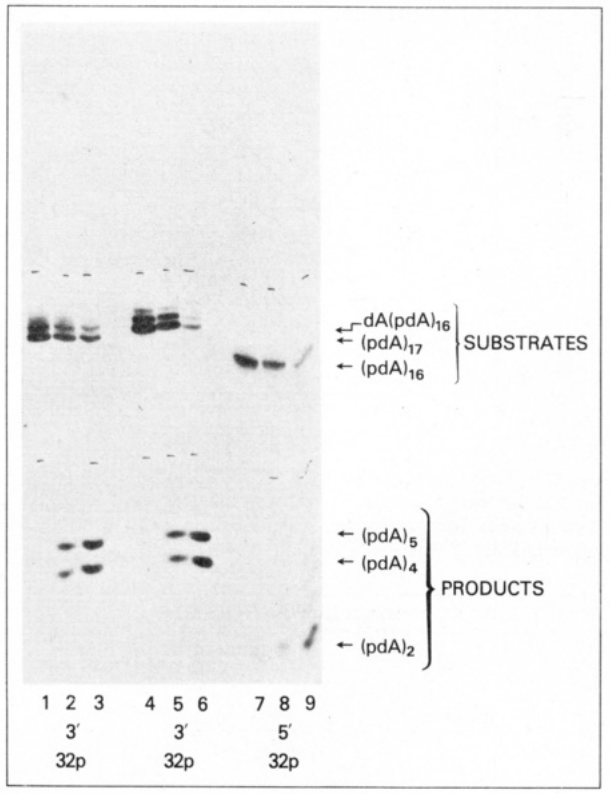


FIGURE 4: Autoradiograms showing analysis of mouse exonuclease reaction products formed with end-labeled $(pdA)_{17}$ (lanes 1–3), dA-$(pdA)_{16}$ (lanes 4–6), or $(pdA)_{16}$ (lanes 7–9) as substrate. For each substrate, results with three different incubation times are shown: lanes 1, 4, and 7, 0 min; lanes 2, 5, and 8, 15 min; lanes 3, 6, and 9, 60 min.

Mg^{2+}, the ratio of $(pdA)_5$:$(pdA)_4$ was 3:1, and at all levels of Mn^{2+}, the ratio of $(pdA)_5$:$(pdA)_4$ was 1:3. At pH values lower than 7, the ratio of $(pdA)_5$:$(pdA)_4$ was 2:1, and $(pdA)_7$ and $(pdA)_6$ also were formed in amounts about equal to $(pdA)_4$.

Substrate specificity was examined by using a variety of homopolymer and heteropolymer polynucleotides. Circular $(pdA)_{16}$ was not degraded, nor was form 1 ϕX174 DNA. Oligoribonucleotides were not degraded. $(prN)_2$ was released from [5′-^{32}P]tRNA at about $^1/_{30}$th the rate observed with [5′-^{32}P]$(pdA)_9$. Oligo- and polydeoxynucleotides were degraded without specificity for base composition. As will be illustrated below, we found that ssDNA substrates generally were degraded faster than dsDNA substrates. The presence or absence of a 5′-terminal phosphate did not influence the formation of $(pdA)_4$ and $(pdA)_5$ from 3′ end labeled oligonucleotide substrate.

Mode of Action: Processive Bidirectional Activity. We did not observe product molecules intermediate in chain length between the usual products and the substrate $(pdA)_9$. This also was true with reaction mixtures where <10% of the substrate was consumed during the incubation. In other incubations where 3′ end labeled $(pdA)_{17}$ or $dA(pdA)_{16}$ was the substrate, no formation of $(pdA)_{15}$ was observed (Figure 4). This absence of intermediate products indicated that the mode of $(pdA)_2$ release was not by distributive action in the 5′ → 3′ direction. Similarly, with 5′ end labeled $(pdA)_{16}$ as substrate, no formation of $(pdA)_{11}$ and $(pdA)_{12}$ was detected (Figure 4), thus ruling out distributive release of $(pdA)_4$ and $(pdA)_5$ in the 3′ → 5′ direction. The results, therefore, taken together, are consistent with two possible modes of action: processive degradation in one direction or processive degradation in either direction. To distinguish between these possibilities, we compared the rates of product formation using

Table III: Molecular Ratio of Products Formed with $(dA)_{\overline{50}}$ as Substrate

product	position of ^{32}P label[a] 3′ end	5′ end	uniform	difference (pmol) between end and uniformly labeled
$(pdA)_2$	0	0.8	2.3	1.5
$(pdA)_3$	0	0.3	0.9	0.6
$(pdA)_4$	0.3	0.3	1.0	0.4
$(pdA)_5$	0.4	0.2	0.7	0.1

[a] Values in these three columns are the picomoles of product formed.

double-labeled substrates: poly$(dA)_{\overline{800}}$ uniformly labeled with ^{14}C and ^{32}P end labeled at either the 5′ or the 3′ end. The rates of release of [^{32}P]$(pdA)_2$ and [^{32}P]$(pdA)_{4,5}$ were approximately equal. With an incubation where 60% of the 5′-^{32}P had been released and 50% of the 3′-^{32}P had been released, only 20% of the ^{14}C label was degraded. Similar results were obtained with three preparations of $(pdA)_{50}$ that were either ^{32}P uniformly labeled or ^{32}P end labeled. With a set of incubations containing 0.1 μM substrate, 80% of the 5′ end labeled substrate was released, and 60% of the 3′ end label was released. Yet, only about 10% of the uniformly labeled substrate was degraded to the usual oligonucleotide products. With this uniformly labeled substrate, faint bands due to intermediate-size products were detected, and a mixture of partially degraded molecules formed a prominent smear just below $(pdA)_{50}$. These results indicate that the enzyme degrades poly(dA) by partial or "limited" processive action in both directions; the results are inconsistent with fully processive action in one or either direction.

Stoichiometry of Products. Results on the stoichiometry of products that accumulated in reactions with end-labeled and uniformly labeled $(pdA)_{50}$ as substrate are summarized in Table III. Products with the two end-labeled substrates were similar to those observed when $(pdA)_9$ was the substrate. The predominant product with the uniformly labeled substrate was $(pdA)_2$. Thus, as shown in the last column of Table III, most of the internal portion of this substrate was degraded to $(pdA)_2$, not $(pdA)_4$ and $(pdA)_5$.

Since labeled $(pdN)_4$ and $(pdN)_5$ accumulate in reactions with 3′ end labeled substrates, we did not expect that these molecules would be used as substrate for $(pdA)_2$ formation. This was found to be the case with both $(pdA)_4$ and $(pdA)_5$. Interestingly, $(pdA)_6$ was cleaved to $(pdA)_2$ and $(pdA)_4$, and $(pdA)_7$ was cleaved to $(pdA)_2$ and $(pdA)_5$. Both 3′-^{32}P-labeled $(pdA)_7$ and $dA(pdA)_6$ were cleaved to [^{32}P]$(pdA)_5$.

Degradation of Mismatched Terminal Nucleotides. In preliminary experiments, we found that $(pdN)_2$ formation with 5′ end labeled poly(dC) or poly(dT) was >90% inhibited by annealing these substrates with a base pair complementary polynucleotide. It was of interest to determine if this was also true with other polynucleotides and with 3′ end labeled substrates, and whether a mismatched polynucleotide "tail" would be degraded. These questions were evaluated in the experiment shown in Table IV. In experiment I of the table, it is shown that activity with $(pdA)_{10}$ labeled at either end was reduced by ~50% by annealing with poly(dT). Inhibition was not observed when poly(dA) was substituted for poly(dT), suggesting that the inhibition was not due to simple polynucleotide competition for the enzyme. In experiment II of Table IV, it is shown that annealing with poly(dA) inhibited activity with 3′ end labeled poly(dT) less than with 5′ end labeled poly(dT).

Table IV: Effect of Base Pair Complementary Polynucleotides on Substrate Utilization by Mouse Exonuclease

expt	5′ and 3′ end labeled polymer[a]	annealed polymer[b]	$(pdN)_2$ formed (%)	$(pdN)_4$ and $(pdN)_5$ formed (%)
I	$(pdA)_{10}$		100 (1120)[c]	100 (5300)
	$(pdA)_{10}$	$(pdT)_n$	53	47
	$(pdA)_{10}$	$(pdA)_n$	90	116
II	$(pdT)_n$		100 (310)	100 (3700)
	$(pdT)_n$	$(pdA)_n$	10	44
	$(pdC\text{-}pdA)_5$	$(pdT)_n$	100 (6500)	100 (1300)
	$(pdC\text{-}pdA)_5$-$(pdT)_n$	$(pdA)_n$	103	52
III	$(pdT)_{10}$			100 (6400)
	$(pdT)_{10}$	$(pdA)_n$		33
	$(pdT)_{10}$-$(pdC)_{\overline{15}}$			100 (3570)
	$(pdT)_{10}$-$(pdC)_{\overline{15}}$	$(pdA)_n$		98
	$(pdC)_{\overline{15}}$			100 (7250)
	$(pdC)_{\overline{15}}$	$(pdI)_n$		74

[a] For each polymer, separate incubations were conducted for 5′ and 3′ end labeled molecules. [b] Polymers were present at a 1:1 weight ratio with the labeled base pair complementary substrate. Annealing was conducted by a preincubation in 10 mM Tris-HCl, pH 7.2, and 180 mM KCl; the solution was held at 65 °C for 1 min and then cooled to 5 °C over a period of 16 h. [c] Values shown in parentheses representing 100% are cpm per 20 min.

When poly(dT) contained a mismatched 5′-end tail, release from the 3′ end labeled substrate was inhibited by annealing with poly(dA). In contrast, release of $(pdN)_2$ from the mismatched tail was not inhibited by annealing the poly(dT) portion of the polymer with poly(dA).

In experiment III of Table IV, it is shown that $(pdT)_4$ and $(pdT)_5$ formation with 3′ end labeled $(pdT)_{10}$ was inhibited ~70% by annealing with poly(dA). However, when the $(pdT)_{10}$ contained a 3′-end $(pdC)_{15}$ tail, annealing of the $(pdT)_{10}$ portion of this mixed polymer did not inhibit release of $(pdC)_4$ and $(pdC)_5$ from the mismatched tail. Release of these products from $(pdC)_{15}$ itself was partially inhibited by annealing with poly(dI). These results with homopolymer substrates indicate that a single-stranded DNA tail can be recognized and preferentially degraded by this mouse exonuclease.

Discussion

Two oligonucleotide-releasing exonuclease activities were present in the final enzyme preparation described in this work. Since the preparation was not homogeneous, the assignment of both activities to one enzyme is not conclusive. Yet, this appears to be the most reasonable interpretation of the present data. Both activities have very similar general catalytic properties and mode of action, and both appear as an M_r ~40 000 native protein during gel filtration in high ionic strength solution. The activities are similarly inactivated by heating at 70 °C, by 200 mM NaCl in the assay, and by pHMB. Thus, we believe the mouse enzyme can be described as a limited processive bidirectional DNA exonuclease that releases 5′-phosphate oligonucleotides from ssDNA substrates. Several properties of this enzyme are shared with the bidirectional oligonucleotide-releasing exonuclease from human placenta described by Doniger & Grossman (1976). For example, both enzymes have a native molecular weight of about 40 000 in high ionic strength solution, alkaline pH optima, and require a divalent cation for activity. Therefore, these mouse and human enzymes appear to be of the same general class. However, there are differences in the reported sensitivities to high salt and in the chain-length distribution of products formed. For example, the human exonuclease produced a mixture of $(pdT)_5$–$(pdT)_{10}$ with either 5′ or 3′ end labeled poly(dT) as substrate (Doniger & Grossman, 1976), whereas the mouse enzyme produced $(pdT)_2$ with 5′ end labeled poly(dT) as substrate, and it produced $(pdT)_4$ and $(pdT)_5$ when 3′ end labeled poly(dT) was the substrate. We believe that these differences in the chain length of products formed are probably more apparent than real. This is because the conditions for purification and assay of the two enzymes were different, as were the methods for identification of the precise chain length of products.

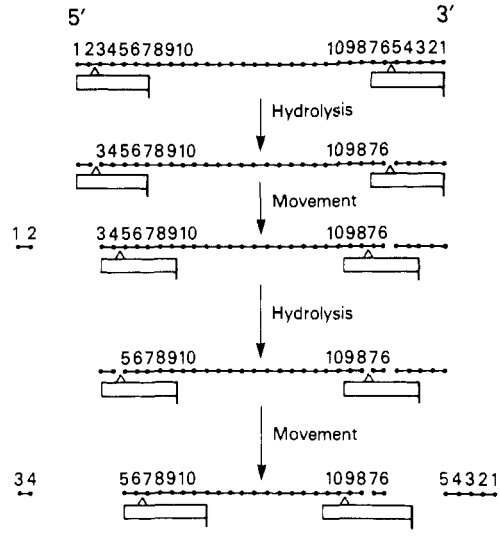
A distinguishing feature of the mouse exonuclease, *N*-ethylmaleimide resistance, was not reported for the human exonuclease. In order to compare the two enzymes with regard to this property, a sample of highly purified human placenta exonuclease was obtained[4] and studied under the optimum conditions described here for the mouse exonuclease. We found that the human exonuclease produced labeled $(pdA)_4$ and $(pdA)_5$ only, in incubations with 3′-end $[^{32}P](pdA)_9$ as substrate; this activity did not require dithiothreitol and was completely resistant to 1 mM NEM. With 5′-end $[^{32}P](pdA)_9$, the human exonuclease formed $(pdA)_2$ predominantly. It appears therefore that the two enzymes are analogous.

Because of the characteristic products with 3′ end labeled substrates, the alkaline pH optimum, and the resistance to 1 mM NEM and high salt, it should be possible to readily measure the mouse exonuclease in crude extracts and partially purified fractions. This is important as it will facilitate studies of levels of the enzyme in cells deficient in DNA repair and in cells responding to growth control and modulation of DNA synthesis. Another point worth mentioning is the relationship between HD protein 1 and the exonuclease. HD protein 1 is an abundant DNA binding protein in mouse myeloma extracts (Planck & Wilson, 1980). The binding affinity of this protein to single-stranded DNA is much higher than to double-stranded DNA, and it appears that there is enough HD protein 1 in the nuclei of mouse cells to cover most, if not all, of the single-stranded DNA present at any one time. Hence, enzymes tightly associated with HD protein 1 could be targeted specifically to regions of single-stranded DNA. The mouse exonuclease described here may be an example of such an enzyme. The mouse exonuclease in highly purified form, essentially free of HD protein 1, binds only weakly to single-stranded DNA–cellulose. Yet, in the presence of HD protein 1, the enzyme is tightly and specifically bound to single-stranded DNA, presumably through a protein–protein interaction with HD protein 1.

Characterization of the mode of action of the mouse exonuclease presents a challenging enzymological problem. Our evidence that the enzyme acts bidirectionally in a limited processive fashion with long substrate molecules appears conclusive, and results on the stoichiometry of products released from the internal portion of this substrate indicate that $(pdN)_2$ is the predominant product (Table III). When the enzyme initiates at the 3′ end, the terminal residue of the substrate is released as either $(pdN)_4$ or $(pdN)_5$. The possibility that the enzyme releases $(pdN)_2$ predominantly as it then proceeds to digest the substrate in the 3′ → 5′ direction was confirmed in an experiment with $(pdT)_{10}$-$(pdC)_{\overline{20}}$ as substrate.

[4] The enzyme, provided by J. Doniger, was fraction V human placenta correxonuclease further purified by isoelectric focusing, as described (Doniger & Grossman, 1976).

Scheme I: Speculative Model on the Mode of Action of Mouse Exonuclease[a]



[a] The data are consistent with this model in which all enzyme functions are oriented in a single active center. Specific binding sites on the enzyme direct initial binding to either the 5′ or the 3′ end of the substrate, as shown. Binding covers seven nucleotides, and orientation of the enzyme is the same for binding at each end; the active site is indicated by the groove. The oligonucleotide products remain bound to the enzyme after hydrolysis and eventually are displaced by enzyme movement along the substrate.

The $(pdC)_{\overline{20}}$ portion of this molecule was uniformly ^{32}P labeled. By annealing $(pdA)_n$ to the $(pdT)_{10}$ portion of this molecule, the 5′ → 3′ degrading activity was blocked (see Table IV). We found that the size distribution of labeled products was consistent with release of $(pdC)_4$ and $(pdC)_5$ from the 3′ end and release of $(pdC)_2$ from the internal portion of this substrate.

A speculative model on the mode of action of the mouse exonuclease is shown in Scheme I. The model illustrates that the enzyme binds at the 3′ end and/or the 5′ end of a substrate through interaction with separate binding domains. With binding at either end, the enzyme covers six or seven nucleotides, and the orientation of the enzyme with respect to the polynucleotide chain is the same. Therefore, the position of the active site relative to the end of the substrate depends upon whether the enzyme has bound to the 5′ or 3′ end. After initial binding and hydrolysis, the enzyme remains associated with the substrate through binding by one of the domains. Thus, in the case of initial binding at the 5′ end, the enzyme covers seven residues, cleaves as shown, and moves rightward along the substrate releasing $(pdN)_2$. In the case of initial binding at the 3′ end, the enzyme covers seven residues and cleaves between residues 5 and 6, as shown. The $(pdN)_5$ product remains in the active site as the enzyme moves leftward along the substrate, cleaving at every other phosphodiester bond. In this way, $(pdN)_5$ is displaced from the active site. A problem with this model is that it does not explain why the enzyme has high activity with $(pdN)_7$ as substrate. If the products remain tightly associated with the active site unless they are displaced through enzyme movement, the products of $(pdN)_7$ hydrolysis would be dead-end inhibitors, and this clearly was not observed.

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