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Oligoribonuclease Is Distinct from the Other Known Exoribonucleases of *Escherichia coli*

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Oligoribonuclease, an exoribonuclease specific for small oligoribonucleotides, was initially characterized 20 years ago (S. K. Niyogi and A. K. Datta, J. Biol. Chem. 250:7307–7312, 1975) and shown to be different from RNase II and polynucleotide phosphorylase. Here we demonstrate, using mutant strains and purified enzymes, that oligoribonuclease is not a manifestation of RNases D, BN, T, PH, and R, exoribonucleases discovered subsequently. Thus, oligoribonuclease is the eighth distinct exoribonuclease discovered in *Escherichia coli*. We also show that oligoribonuclease copurifies with polynucleotide phosphorylase.

RNases play a central role in RNA metabolism (6). Close to 20 RNases have already been identified in *Escherichia coli*, 8 of which are exoribonucleases, all acting in the 3′-to-5′ direction. These enzymes include polynucleotide phosphorylase (PNPase), RNases II, D, BN, T, PH, and R, and oligoribonuclease (5). Of these enzymes, the first seven are clearly distinct on the basis of their biochemical and structural characterization and genetic properties. In contrast, the uniqueness of oligoribonuclease is much less certain (4, 13). When oligoribonuclease was initially identified and characterized as an enzyme active only on short oligoribonucleotides, it was shown to be different from PNPase and RNase II, the only other *E. coli* exoribonucleases known at that time, on the basis of its purification properties, substrate specificity, ion requirements, and thermostability (4, 13). However, the subsequent discovery of five additional exoribonucleases raised the possibility that oligoribonuclease activity is simply a manifestation of one of these other enzymes.

In this report, we present evidence that oligoribonuclease is, in fact, a distinct enzyme. Using a modified assay for oligoribonuclease, we demonstrate that this activity is unaffected in extracts from mutant cells lacking each of the newly discovered exoribonucleases. Furthermore, we show that several of these RNases, in purified form, lack oligoribonuclease activity. These data support the conclusion that oligoribonuclease is the eighth distinct 3′→5′ exoribonuclease present in *E. coli*. As a sidelight of these studies, we have also found that oligoribonuclease copurifies with PNPase.

These studies were carried out primarily with *E. coli* K-12 strains derived from strain CA244 (*lacZ trp relA spoT*) and have been described earlier (11, 12, 15). Strain S296-6808, lacking RNase R, has also been described (8). The RNase D^-, T^-, PH^-, and $PNPase^-$ strains contain null mutations, either deletions or interruptions, and are devoid of the relevant activity, while the other strains, although not completely characterized, exhibit very low levels of the relevant activities.

Preparation of [^{3}H]oligo(U). The oligoribonuclease substrate, [^{3}H]oligo(U), was prepared by controlled alkaline hydrolysis of [^{3}H]poly(U) (Dupont-NEN), made up to ~500 cpm/nmol of UMP residue by mixing with unlabeled poly(U). A sample of poly(U) containing 0.7 to 2.5 μmol of UMP residues was incubated in 0.3 N KOH at 37°C for 18 min (determined experimentally for the average chain length desired). After incubation, the pH was adjusted to 8.0 by the addition of perchloric acid, and the sample was left on ice for 10 min. The potassium perchlorate precipitate was removed by centrifugation, and the resulting solution was made 0.1 M in Tris-Cl, pH 8.0. The [^{3}H]oligo(U) preparation was stored at −20°C until use.

The average chain length of the [^{3}H]oligo(U) was determined by measuring the ratio of nucleotide to nucleoside after alkaline phosphatase treatment and complete hydrolysis by alkali. A small portion of the [^{3}H]oligo(U) was treated with alkaline phosphatase (Worthington) which converted the oligonucleotides from the form (Up)nUp to (Up)nU. This material was then subjected to alkaline hydrolysis at 80°C in 0.1 N KOH for 2 h. This procedure converted the 3′-terminal U residue to uridine, whereas the others were converted to a mixture of 2′ and 3′ UMP. The hydrolysis mixture was neutralized with perchloric acid and cleared of precipitate by centrifugation, and uridine and UMP were separated by chromatography on columns (0.5 ml) of AG-1-X2 (Bio-Rad). [^{3}H]uridine was eluted with H_2O, while the nucleotides were eluted with 0.5 N HCl. The average chain length of the original [^{3}H]oligo(U) is the ratio of total radioactivity to that eluted by H_2O. Under these conditions, average chain lengths of 3.0 to 3.2 were obtained.

Preparation of cell extracts. The desired strains were grown in YT medium to an A_{550} of ≈1. Portions (25 ml) of culture were centrifuged, and cells were resuspended in 1-ml amounts of sonication medium containing 10 mM Tris-Cl (pH 8.0), 10 mM $MgCl_2$, 20 mM NH_4Cl, and 10% glycerol. This medium was slightly modified from that of Niyogi and Datta (13) by the addition of glycerol. The cell suspension was subjected to sonication by using four 15-s pulses with 30-s cooling periods. Cell debris was removed by centrifugation. Cell extracts were used fresh or after storage at −20°C. No change in oligoribonuclease activity was observed upon freezing.

Assay of oligoribonuclease activity. Oligoribonuclease activity was determined by monitoring the release of [^{3}H]UMP from [^{3}H]oligo(U) after its conversion to uridine upon treatment with alkaline phosphatase. The reaction mixture of 100 μl contained 100 mM Tris-Cl (pH 8.0), 5 mM $MnCl_2$, 0.06 mM [^{3}H]oligo(U) (average chain length, ≈3), and 1 μg of bacterial alkaline phosphatase. Samples were preincubated to remove the 3′-terminal phosphate residue from the substrate which is inhibitory to oligoribonuclease activity (4, 13). Cell extract was added, and the sample was incubated at 37°C for 5 min. Two

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TABLE 1. Oligoribonuclease activities in exoribonuclease mutant strains[a]

Relevant phenotype	Sp act[b]	Relative activity[c]
Wild type	117	100
RNase I$^-$	116	99
PNPase$^-$	112	96
RNase D$^-$	119	103
RNase T$^-$	112	96
RNase PH$^-$	118	101
RNase BN$^-$	115	98
RNase R$^-$	120	103
RNase II$^-$	50	43
RNase II$^-$, D$^-$, BN$^-$, T$^-$, I$^-$	48	41

[a] Extracts were prepared and assayed as described in the text.
[b] Units per milligram of protein.
[c] Wild-type activity set at 100.

TABLE 2. Oligoribonuclease activity of purified exoribonucleases[a]

Enzyme[b]	Normalized oligoribonuclease activity[c]
RNase D	<5
RNase T	<5
RNase PH	<5
RNase II	42
PNPase	95

[a] Enzyme activities were assayed as described in the text.
[b] All the enzymes were homogeneous except PNPase, which was estimated to be 50% pure (3, 7, 9, 16).
[c] Oligoribonuclease activity was normalized by setting the ratio of oligoribonuclease activity to RNase activity in the extract to 100 and determining the same relative ratio for a comparable amount of purified enzyme. For PNPase, normalization was carried out with RNase II$^-$ extracts.

micromoles of EDTA (pH 8.0) was added to stop the reaction, and the complete reaction mixture was then applied to a 0.5-ml column of AG-1-X2 in a Pasteur pipette. The total [^{3}H]uridine released by the combined action of oligoribonuclease and alkaline phosphatase was washed off the column by five 1-ml portions of H_2O, and radioactivity was determined in a liquid scintillation counter. One unit of oligoribonuclease activity is defined as that amount of enzyme which produces 1 nmol of uridine in 5 min. All assays were carried out in duplicate and with extracts prepared at least twice.

Other assays. Purified RNases II, D, T, PH, and PNPase were prepared and assayed as described elsewhere (10–13, 17) with either [^{3}H]poly(A) (Amersham), tRNA-CCA-[^{3}H]C_3 or tRNA-CC-[^{14}C]A as substrate (10). The protein level was determined by the method of Bradford (1).

Oligoribonuclease activity in exoribonuclease mutants. In order to determine whether any of the other known *E. coli* exoribonucleases was responsible for oligoribonuclease activity, extracts were prepared from wild-type cells and from cells lacking each of the RNases. In addition, extracts were made from cells lacking RNase I or lacking RNase I plus four of the exoribonucleases. As shown in Table 1, cells devoid of RNase I, PNPase, RNase D, RNase T, RNase BN, RNase PH, or RNase R displayed essentially the same level of oligoribonuclease activity as the wild-type strain. These data indicate that oligoribonuclease activity is not a consequence of any of the aforementioned RNases.

Cells lacking RNase II, on the other hand, showed a reduction in oligoribonuclease activity (Table 1) despite the fact that earlier studies showed (13) that these two activities were distinct. The explanation for this apparently anomalous observation is that RNase II is active on oligoribonucleotides with a chain length of five or more (14), and the [^{3}H]oligo(U) preparation used as the substrate, although having an average chain length of 3.2, contains some longer chains which are substrates for the enzyme. Thus, RNase II contributes to the total oligoribonuclease activity in extracts under these conditions, although about half of the overall activity is independent of this enzyme and the other known RNases as well. In support of this conclusion, a multiple mutant strain lacking RNase II and three other exoribonucleases, RNases D, BN, and T, shows the same level of activity as a strain lacking RNase II alone. This result confirms that RNases D, BN, and T do not contribute to oligoribonuclease activity.

Oligoribonuclease activity of purified RNases. As a second test of the relationship between oligoribonuclease and other RNases, several purified exoribonucleases were assayed for oligoribonuclease activity. To normalize the different enzymes, the ratio of oligoribonuclease activity to the activity of the relevant RNase was measured in cell extracts, and the ratio of activity for an equivalent amount of purified enzyme was compared. In this way, it was possible to determine whether the two activities could be separated as the particular RNase was purified. The data presented in Table 2 demonstrate that purified RNases D, T, and PH were essentially devoid of oligoribonuclease activity. As expected, RNase II displayed a level of oligoribonuclease activity consistent with the data presented in Table 1. These data support the conclusions that oligoribonuclease is distinct from RNases D, T, and PH and that RNase II has some activity on the [^{3}H]oligo(U) substrate.

To our surprise, the partially purified PNPase preparation used for these experiments retained the full oligoribonuclease activity of a crude extract. This observation suggested either that PNPase is responsible for oligoribonuclease activity, in contradiction with the data in Table 1, and earlier results (13), or that the two enzymes had copurified. In support of the latter possibility, sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the PNPase preparation revealed several proteins in addition to PNPase itself, one of which had a mass of 38 kDa (17), the reported size of oligoribonuclease (13). Moreover, while PNPase requires P_i for degradative activity, oligoribonuclease does not (unpublished observation). Inasmuch as this PNPase preparation had been purified approximately 100-fold from an overexpressing strain and is estimated to be at least 50% pure, the continued presence of oligoribonuclease is of considerable interest. Oligoribonuclease would be the second RNase found to associate with PNPase, as RNase E has also recently been shown to copurify with that enzyme (2). The possibility of a RNA degradation complex needs to be considered in light of these observations.

The data presented here demonstrate that oligoribonuclease is distinct from RNases D, BN, T, PH, and R. This, coupled with earlier information (13) that oligoribonuclease is independent of RNase II and PNPase, indicates that it is the eighth 3′→5′ exoribonuclease of *E. coli*. What might be the function of such an enzyme? As previously suggested by Datta and Niyogi (4), oligoribonuclease may serve as the "finishing enzyme" to degrade the small oligoribonucleotides left after the action of RNase II and PNPase. The apparent association of PNPase and oligoribonuclease supports this contention. Clearly, the isolation of a mutant strain lacking oligoribonuclease is needed to sort out its function. Studies along these lines are under way.

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