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RNase PH: An *Escherichia coli* phosphate-dependent nuclease distinct from polynucleotide phosphorylase

(tRNA processing/phosphorolysis)

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ABSTRACT **Final trimming of the 3′ terminus of tRNA precursors in *Escherichia coli* is thought to proceed by an exonucleolytic mechanism. However, mutant strains lacking as many as four exoribonucleases known to act on tRNA still grow normally and process tRNA normally. Extracts from such a multiple-RNase-deficient strain accurately mature tRNA precursors exonucleolytically *in vitro* in a reaction that requires inorganic phosphate. Here we show that this reaction is not due to polynucleotide phosphorylase (PNPase) but, rather, that it is mediated by a phosphate-requiring exonuclease that we have named RNase PH. Purified PNPase is incapable of completely processing tRNA precursors, and extracts from a PNPase$^-$ strain retain full activity for phosphorolytic processing. Although both PNPase and RNase PH act in a phosphorolytic manner, they differ substantially in size and substrate specificity. RNase PH has a molecular mass of 45–50 kDa and favors tRNA precursors as substrates. The possible physiological role of RNase PH and the advantages of phosphorolytic processing are discussed.**

In contrast to the 5′ processing of tRNA precursors by RNase P, our understanding of processing reactions at the 3′ terminus is extremely limited. It is thought that 3′ processing in *Escherichia coli* is initiated by an endonucleolytic cleavage downstream from the mature 3′ end followed by one or two exonucleolytic trimming reactions that stop at the encoded -C-C-A sequence (1). One exoribonuclease isolated from *E. coli*, RNase D, can accurately carry out the final trimming reaction *in vitro* (2). However, mutant strains devoid of this enzyme grow normally and are not impaired in tRNA biosynthesis (3). Thus, even though RNase D might be the normal 3′ processing enzyme *in vivo*, another RNase must be able to substitute in its absence.

In the course of trying to identify an alternative RNase, it was shown that *E. coli* contains several additional exoribonucleases able to remove nucleotides from the 3′ terminus of tRNA precursors, albeit with varying efficiencies, namely, RNase II (2), RNase BN (4), and RNase T (5). However, a multiple-mutant strain deficient in these enzymes, as well as RNase D, appears unaffected (6). To resolve this paradox, we recently developed an *in vitro* tRNA-processing system that uses as substrates SP6 RNA polymerase-generated transcripts of the gene for $tRNA^{Tyr}\ su_3^+$ (7). With this system and extracts from the multiple-RNase-deficient strain, we identified another activity that accurately processes the 3′ terminus of tRNA precursors in an exonucleolytic manner (7). Surprisingly, this activity requires inorganic phosphate (P_i) as a substrate. The reaction is phosphorolytic, generating a mature 3′ terminus on tRNA and nucleoside diphosphates (7).

These properties of the processing activity suggested initially that polynucleotide phosphorylase (PNPase), the only known P_i-dependent nuclease (8), might be involved in the reaction. In this report, however, we show that purified PNPase is not able to catalyze the 3′ processing reaction and that extracts from a strain devoid of PNPase are still active. In addition, we have identified and partially characterized a phosphorolytic nuclease, which we have termed RNase PH, with structural and catalytic properties distinct from PNPase, that can carry out the 3′ processing reaction *in vitro*.

MATERIALS AND METHODS

Bacterial Strains, Construction of Mutants, and Growth Conditions. The multiple-RNase-deficient *E. coli* strain 20-12E/18-11 (RNase I$^-$, II$^-$, D$^-$, BN$^-$, T$^-$), herein called 18-11, has been described (9). Strain NK5151 (*trp*::Tn*10*, *rnb*$^+$) was obtained from Nancy Kleckner (Harvard University); strain LM160 (*pnp*::Tn*5*) (10), from Stuart Levy (Tufts University); and strain SK5005 (*rnb-500*, Cm^r) (11), from Sidney Kushner (University of Georgia). Since RNase II$^-$, PNPase$^-$ strains are inviable (11), it was necessary to reintroduce the *rnb*$^+$ gene into 18-11 prior to making it *pnp*. A tetracycline-sensitive derivative of strain 18-11 was converted to *rnb*$^+$ by phage P1-mediated transduction using a P1 lysate grown on strain NK5151. Tetracycline-resistant transductants were selected and assayed for RNase II. One *rnb*$^+$ derivative, 18-11A, was made *pnp* by transduction with P1 grown on LM160 and selection for kanamycin-resistant transductants. The transduction to *pnp* proceeded with poor efficiency and the transductants grew slowly, although faster-growing revertants could be isolated. Presumably, the PNPase$^-$ phenotype is also incompatible with one of the other RNase$^-$ mutations in this genetic background. One *rnb*$^+$, *pnp* derivative, 18-11B, was retained for further study.

Since the presence of RNase II complicated assays of extracts from strain 18-11B, an additional derivative containing a temperature-sensitive *rnb* (*rnb*^{ts}) allele was constructed by transduction with P1 grown on strain SK5005 and selection for chloramphenicol-resistant transductants. One *rnb*^{ts}, *pnp* derivative, termed 18-11BP, was used for the studies described. Extracts of this strain were devoid of PNPase and contained temperature-sensitive RNase II activity (see *Results*).

Cells were routinely grown in YT medium (12). When required, antibiotics were present at the following concentrations: tetracycline, 12.5 μg/ml; kanamycin, 50 μg/ml; and chloramphenicol, 20 μg/ml.

Substrates. [^{3}H]Poly(A) was obtained from Amersham and poly(A) from Miles. tRNA-C-C-A-[^{14}C]C_n and tRNA-C-C-[^{14}C]A were prepared from *E. coli* tRNA and [^{14}C]CTP or

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Abbreviations: PNPase, polynucleotide phosphorylase; superscript ts, temperature-sensitive.

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[^{14}C]ATP by using rabbit liver tRNA nucleotidyltransferase as described (13). The preparation used in these studies contained an average of 3.3 CMP residues beyond the -C-C-A terminus. The [^{32}P]tRNA^{Tyr} precursors, HB3 and 1HR, containing 5 extra and 25 extra 3′ residues, respectively, and 49 extra 5′ residues were synthesized by using bacteriophage SP6 RNA polymerase (7). [^{14}C]CDP was purchased from Schwarz/Mann.

Other Materials. *E. coli* tRNA was prepared as described (13). *E. coli* PNPase was purchased from Amersham and RNase T1 was purchased from Boehringer Mannheim. Ultrogel AcA 44 was obtained from LKB. All other chemicals were reagent grade.

Preparation of Extracts. Routine extracts for determination of RNase activities were prepared from cells grown to an OD_{550} of 1, concentrated 10-fold in 20 mM glycine·NaOH, pH 8.5/0.1 mM EDTA/0.1 mM dithiothreitol/0.1 mM phenylmethylsulfonyl fluoride, and treated with two 15-sec pulses from a sonicator (Heat System/Ultrasonics, Plainview, NY). Extracts were centrifuged at 10,000 × *g* for 10 min and the supernatant fractions were used for assays.

Large-scale preparation of 100,000 × *g* supernatant (S100) fractions were made from cells passed through an Aminco French pressure cell as described (7). In some cases S100 fractions were concentrated about 3-fold with an Amicon YM10 membrane prior to chromatography.

Assays. Processing of the SP6 RNA polymerase-generated HB3 and 1HR transcripts was carried out as described (7), with electrophoresis in 20% polyacrylamide gels to separate the oligonucleotides generated by RNase T1.

PNPase was routinely assayed by polymerization of CDP in a reaction mixture (100 μl) containing 100 mM glycine·NaOH (pH 8.9), 200 mM NaCl, 0.5 mM $MgCl_2$, 0.5 mM [^{14}C]CDP (930 cpm/nmol), and 50 μl of cell sonicate. After incubation for 10 min at 37°C the reaction was stopped by the addition of 10% (wt/vol) trichloroacetic acid, and acid-precipitable radioactivity was determined.

Phosphorolysis of poly(A) by PNPase or RNase PH was determined as P_i-dependent radioactivity released into an acid-soluble form from [^{3}H]poly(A). Reaction mixtures (100 μl) contained 50 mM Tris Cl (pH 8.0), 5 mM $MgCl_2$, 1 mM [^{3}H]poly(A) (≈60 cpm/nmol), 60 mM NaCl, 10 mM potassium phosphate (pH 7.5), and enzyme fraction. After incubation for the indicated time at 37°C reactions were stopped by the addition of carrier RNA and trichloroacetic acid, and acid-soluble radioactivity was determined. RNase PH was determined exactly as for PNPase phosphorolysis except that tRNA-C-C-A-[^{14}C]C_n (12 μg, 8200 cpm/nmol) was substituted for [^{3}H]poly(A).

RNase II was assayed by the release of acid-soluble radioactivity from [^{3}H]poly(A) as described (5).

RESULTS

The Phosphate-Requiring Exoribonuclease May Not Be PNPase. Our earlier studies (7) of 3′-end maturation in an *in vitro* tRNA-processing system revealed that nucleotides were removed exonucleolytically in a P_i-dependent reaction that generated nucleoside diphosphates. These properties immediately suggested that PNPase, the only known P_i-requiring nuclease, might be the enzyme carrying out the 3′ processing. One piece of evidence arguing against this possibility, however, was the finding that nucleotide removal from the precursor proceeded randomly (7), whereas PNPase is known to phosphorolyze RNA molecules in a processive fashion (8).

To directly examine the possible role of PNPase, we tested whether commercial, purified PNPase or PNPase partially purified from the active processing system could substitute for the processing activity in the complete extract. Neither commercial PNPase nor fraction A (containing PNPase from the S100 fraction) was able to generate the mature 3′ terminus from either tRNA^{Tyr} precursor (Fig. 1). On the other hand, both an S100 fraction and a dialyzed S100 fraction supplemented with P_i were able to catalyze complete conversion of either the HB3 precursor (5 extra 3′ nucleotides) or the 1HR precursor (25 extra 3′ nucleotides) to mature tRNA. The same amount of PNPase activity [based on phosphorolysis of poly(A)] in fraction A or the commercial preparation did not remove the last 3 or 4 extra nucleotides from the 3′ end of the precursor. PNPase did remove extra nucleotides from the longer 1HR precursor, converting it to the size of the smaller one (Fig. 1), but trimming did not proceed past this point.

A similar result was obtained when the artificial tRNA precursor tRNA-C-C-A-[^{14}C]C_n was substituted for the SP6 transcripts (Table 1). The rate of phosphorolysis of [^{3}H]poly(A) by purified PNPase was >2000 times as rapid as phosphorolysis of the terminal residues of tRNA-C-C-A-[^{14}C]C_n. When the same assays were carried out with an S100

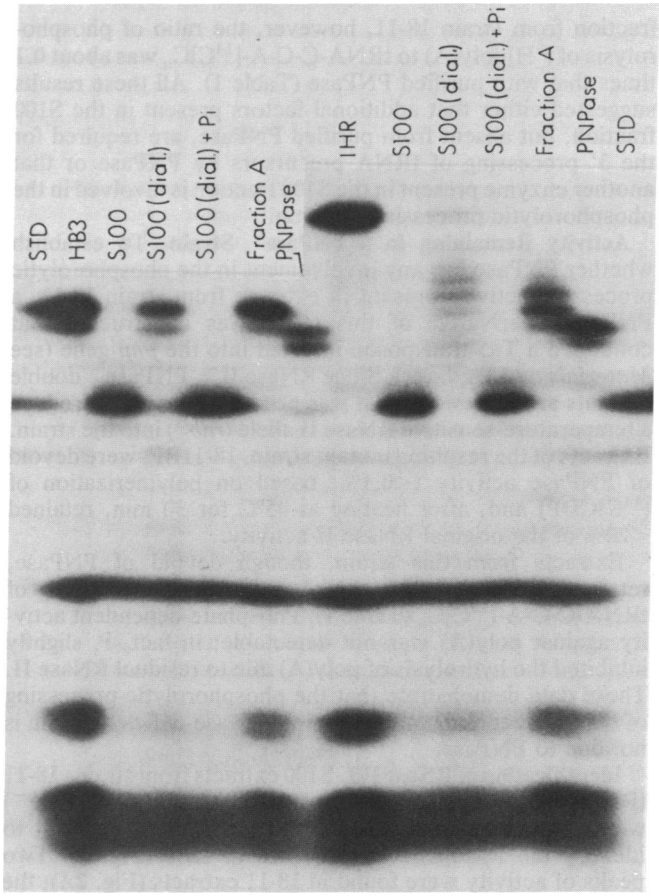

FIG. 1. Effect of fractionated S100 or purified PNPase on 3′ processing of tRNA precursors. An S100 fraction from strain 18-11 was partially purified by chromatography on Ultrogel AcA 44, and the fractions containing PNPase activity [based on phosphorolysis of poly(A)] were combined as fraction A. The tRNA precursors HB3 and 1HR were incubated for 15 min at 37°C in a reaction mixture (10 μl) containing 50 mM Tris Cl (pH 8.0), 5 mM $MgCl_2$, 40 mM KCl, 10,000–20,000 cpm of ^{32}P-labeled precursor, and the indicated enzyme fraction. All enzyme fractions were normalized to the same level of PNPase phosphorolysis activity corresponding to 16 μg of S100 fraction. For fraction A or PNPase, purified RNase P also was added. After incubation, samples were boiled, treated with RNase T1, and electrophoresed in a 20% polyacrylamide/8 M urea gel as described (7). The migration position of the 19-mer oligonucleotide derived from the 3′ terminus of mature tRNA$_1^{Tyr}$ is shown in the STD lanes. The 3′ T1 oligonucleotide derived from precursor HB3 is 24 nucleotides long, and that from precursor 1HR is 32 nucleotides long (7). Dial., dialyzed.

Table 1. Relative phosphorolysis activity of various enzyme fractions against poly(A) and tRNA-C-C-A-C_n

Enzyme	P_i-dependent activity, nmol		
	Poly(A)	tRNA-C-C-A-C_n	Ratio
Purified PNPase	14.3	0.006	2400
Strain 18-11 S100 (RNase II$^-$, PNPase$^+$)	16.6	0.061	270
Strain 18-11BP S100 (RNase II^{ts}, PNPase$^-$)	ND	0.064	—
PNPase peak from AcA 44	37.2	0.027	1400
RNase PH peak from AcA 44	2.4	0.172	14

Phosphorolysis activity against [^{3}H]poly(A) and tRNA-C-C-A-[^{14}C]C_n was determined with the indicated enzyme fraction. In each case the hydrolytic activity in the absence of P_i has been subtracted. Only with the 18-11BP S100, which has residual RNase II, was this significant, since with poly(A) as substrate there was less activity with P_i than in its absence. The PNPase and RNase PH peaks are the peak tubes from the experiment of Fig. 2*A*. ND, not detectable.

fraction from strain 18-11, however, the ratio of phosphorolysis of [^{3}H]poly(A) to tRNA-C-C-A-[^{14}C]C_n was about 0.1 times that with purified PNPase (Table 1). All these results suggested either that additional factors present in the S100 fraction, but absent from purified PNPase, are required for the 3′ processing of tRNA precursors by PNPase or that another enzyme present in the S100 fraction is involved in the phosphorolytic processing reaction.

Activity Remaining in a PNPase$^-$ Strain. To establish whether PNPase has any involvement in the phosphorolytic processing activity present in extracts from strain 18-11, a PNPase$^-$ derivative of this strain was constructed that contained a Tn*5* transposon inserted into the *pnp* gene (see *Materials and Methods*). Since RNase II$^-$, PNPase$^-$ double mutants are inviable (13), it was necessary first to introduce a temperature-sensitive RNase II allele (*rnb*^{ts}) into the strain. Extracts of the resulting mutant strain, 18-11BP, were devoid of PNPase activity (<0.1%, based on polymerization of [^{14}C]CDP) and, after heating at 45°C for 30 min, retained <20% of the original RNase II activity.

Extracts from this strain, though devoid of PNPase, retained full activity for the phosphorolytic processing of tRNA-C-C-A-[^{14}C]C_n (Table 1). Phosphate-dependent activity against poly(A) was not detectable; in fact, P_i slightly inhibited the hydrolysis of poly(A) due to residual RNase II. These data demonstrate that the phosphorolytic processing of tRNA precursors in the multiple-RNase-deficient strain is not due to PNPase.

Identification of RNase PH. S100 extracts from strains 18-11 (RNase II$^-$, PNPase$^+$) and 18-11BP (RNase II^{ts}, PNPase$^-$) were fractionated by gel filtration on Ultrogel AcA 44 to identify the P_i-dependent activities in each (Fig. 2). Two peaks of activity were found in 18-11 extracts (Fig. 2*A*); the first was eluted at the void volume of the column and was very active against poly(A), and the second was included in the column and was much more active with tRNA-C-C-A-[^{14}C]C_n as substrate than with poly(A). The properties and elution position of the first peak correspond to those of PNPase, and this was confirmed by the absence of this activity in strain 18-11BP (Fig. 2*B*). The second peak is an activity that we have named RNase PH (for *ph*osphate-dependent nuclease). RNase PH was also found at similar levels upon gel filtration of 18-11BP extracts, although in this case the residual RNase II activity overlapped to some degree. These findings indicate that *E. coli* contains a P_i-dependent nuclease, distinct from PNPase, that participates in the 3′ processing of tRNA precursors.

Properties of RNase PH. The gel-filtration data (Fig. 2) suggest that the native molecular weight of RNase PH is 45,000–50,000. This size for RNase PH, combined with its

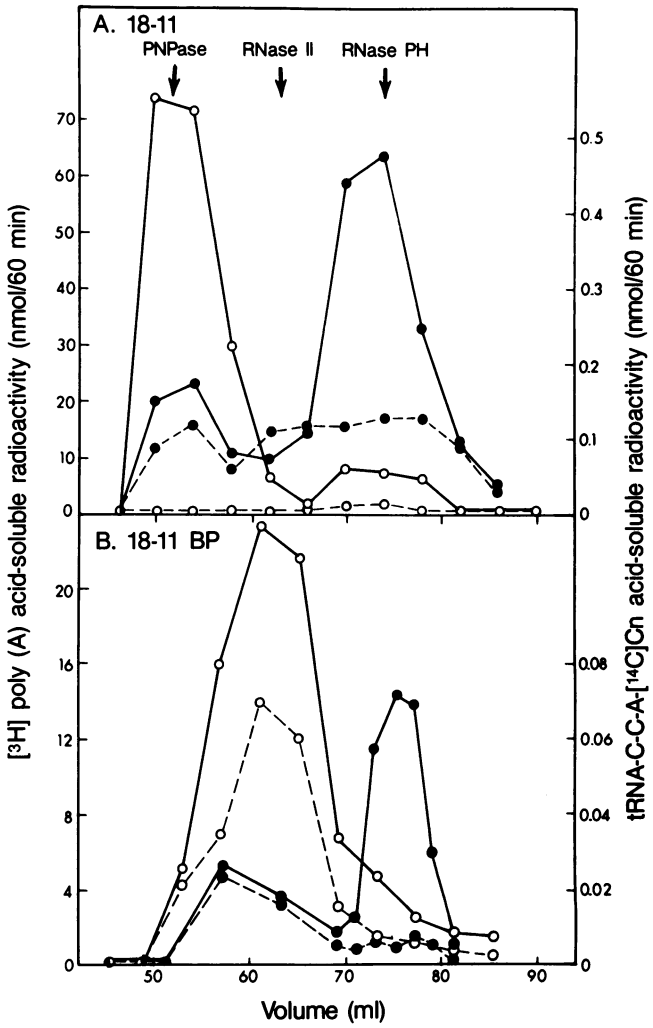

FIG. 2. Gel filtration of S100 fractions from strains 18-11 (*A*) and 18-11BP (*B*). S100 fractions were concentrated 2- to 3-fold, and 2 ml was applied to a column of Ultrogel AcA 44 (76 × 1.5 cm) equilibrated with 20 mM Tris Cl, pH 8.0/200 mM NaCl/0.1 mM EDTA/0.1 mM dithiothreitol/10% (vol/vol) glycerol. Columns were run at a flow rate of 5–6 ml/hr, and 2.0-ml fractions were collected. Portions were assayed against [^{3}H]poly(A) (○) and tRNA-C-C-A-[^{14}C]C_n (●) in the presence (solid lines) or absence (broken lines) of 10 mM potassium phosphate (pH 7.5). The low level of stimulation of RNase II by potassium phosphate is due to K^+.

requirement for P_i, distinguishes it from all the previously described ribonucleases of *E. coli*.

The data presented in Fig. 2 and Table 1 show that the substrate specificity of RNase PH is very different from that of PNPase. Whereas PNPase phosphorolyzes poly(A) almost 1500 times more rapidly than tRNA-C-C-A-C_n, this ratio is only about 15 with RNase PH. These findings make it unlikely that RNase PH is an altered form or fragment of PNPase present in the mutant strain.

RNase PH requires Mg^{2+}, with maximal activity in the range 3–10 mM. Its pH optimum is about 8, although activity is relatively constant from pH 6.8 to pH 9.5. RNase PH is stimulated dramatically by P_i or arsenate with optimum activity at about 10 mM for each. A low level of tRNA-C-C-A-C_n hydrolysis was observed in the absence of P_i (see Fig. 3) and appeared to be due to a contaminating endonuclease activity, but further purification is required to substantiate this point.

That RNase PH actually phosphorolyzes its RNA substrate is demonstrated by the results shown in Fig. 3.

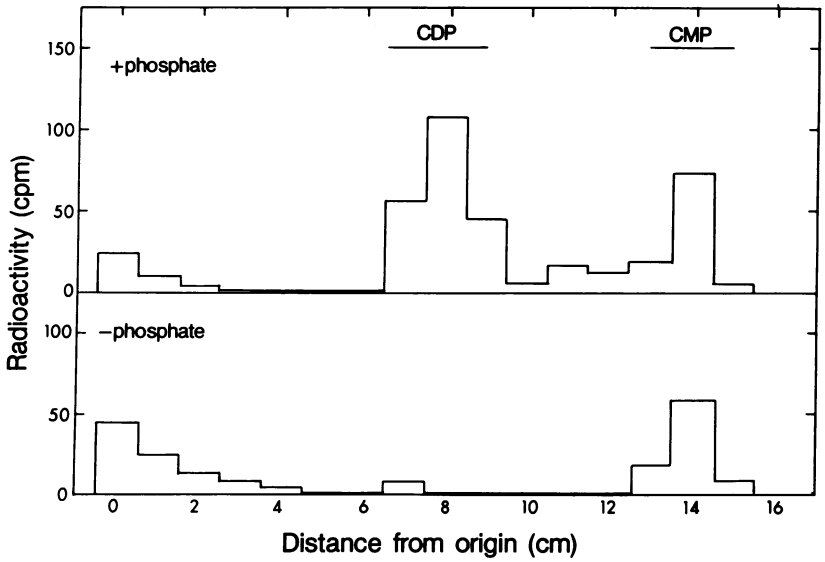

FIG. 3. Paper chromatography of low molecular weight products released from tRNA-C-C-A-[^{14}C]C$_n$ by RNase PH. tRNA-C-C-A-[^{14}C]C$_n$ was treated under standard conditions with RNase PH (peak fraction from Fig. 2*A*) in the presence or absence of 10 mM potassium phosphate (pH 7.5). The reactions were terminated with carrier RNA and trichloroacetic acid, as usual, and half of the acid-soluble fraction (0.4 ml) from each reaction mixture was taken for paper chromatography. The samples were extracted eight times with ether, lyophilized, and dissolved in 50 μl of water. The complete samples were spotted on DEAE-paper for chromatography in 0.4 M ammonium formate (pH 2.5). Strips of 1 cm were cut out and radioactivity was determined. A blank value of 50 cpm has been subtracted from each strip. The migration positions of CMP and CDP standards included with the samples, and of [^{14}C]CDP included in a mock reaction mixture, are shown.

DEAE-paper chromatography of the low molecular weight products from the action of RNase pH on tRNA-C-C-A-[^{14}C]C$_n$ showed that [^{14}C]CDP is generated in the presence of P_i but not in its absence. These data indicate that P_i participates directly in RNase PH action and is not simply an activator of hydrolysis by the enzyme. Small amounts of oligonucleotide and CMP were also found, but generation of these products was not P_i-dependent, and as noted above, was probably due to contaminating nuclease activity.

The catalytic properties of partially purified RNase PH with tRNA-C-C-A-C$_n$ as substrate reported here are essentially identical to those previously observed (7) with crude S100 fractions of strain 18-11 using SP6 RNA polymerase-generated precursors. Furthermore, RNase PH displayed the specificity expected for a processing enzyme, being about 100 times more active against the precursor substrate than against mature tRNA (Table 2). These findings suggest that the activity of the crude extract against tRNA precursors was largely, if not exclusively, due to RNase PH.

DISCUSSION

The studies presented here demonstrate the existence of a P_i-dependent exoribonuclease, distinct from PNPase and termed RNase PH, in extracts of *E. coli*. With the identification of RNase PH, seven exoribonucleases have now been observed in this bacterium (14). This large number of seemingly similar activities makes it imperative to ensure that each new one added to the list is really a distinct enzyme. In this case the P_i requirement for activity eliminated all the other ribonucleases except PNPase.

Table 2. Specificity of RNase PH against precursor and mature tRNA

Substrate	Activity, pmol		
	− P_i	+ P_i	P_i-dependent
tRNA-C-C-A-[^{14}C]C$_n$	93	582	489
tRNA-C-C-[^{14}C]A	12	17	5

Release of acid-soluble radioactivity from each of the two tRNA substrates was determined in the presence or absence of 10 mM potassium phosphate (pH 7.5). The RNase PH used was 30 μl of the peak tube from Fig. 2*A*. Incubation was for 30 min at 37°C. Blank values in the absence of RNase PH have been subtracted from the values presented.

Our first indication that the P_i-dependent activity was not due to PNPase was the observation that purified PNPase could not substitute for an S100 fraction in the 3′ processing of tRNA precursors. This was substantiated by the finding that a strain devoid of PNPase retained 3′ processing activity. The identification of RNase PH, and its presence at comparable levels in PNPase$^+$ and PNPase$^-$ strains, likewise, made it unnecessary to invoke some manifestation of PNPase as the cause of the P_i-dependent activity. Finally, the distinct molecular weight and substrate specificity of RNase PH clearly distinguished it from PNPase and indicated that *E. coli* has yet another exoribonuclease in its degradative repertoire.

The possible presence in *E. coli* of phosphorolytic nuclease activities distinguishable from normal PNPase has been observed occasionally during studies of PNPase mutants (15, 16). In one case (15) a phosphorolytic activity of M_r 100,000 was identified that was suggested to be a mutant form of PNPase. In a second instance (16) a strain with a *pnp* gene inactivated by a Tn*5* transposon was found to exhibit a low level of phosphorolytic activity, apparently due to unspecified lower molecular weight fractions, but these were not examined further. It is not clear from these earlier studies, which used RNA homopolymers as substrates, whether the activities have any relation to RNase PH. They do suggest, however, that the number of P_i-dependent nucleases may not be limited to just PNPase and RNase PH. Phosphorolytic nucleases would appear to be advantageous to cells because they recapture the energy present in the phosphodiester bond. It would not be surprising if a class of these enzymes participated in various RNA-processing and -degradative reactions and thereby lowered the energy requirements of RNA metabolism under certain conditions.

The identification of RNase PH raises the interesting question of whether its normal function *in vivo* is in tRNA processing or whether it only takes over this function when

other exoribonucleases are absent. Since *E. coli* contains a number of enzymes that can act at the 3′ terminus of tRNA precursors *in vitro*, and mutations eliminating these activities have not affected tRNA processing, it has been extremely difficult to pinpoint a primary enzyme, if one exists, for 3′ tRNA processing. Nevertheless, we suspect that we are very near or at the end of the catalogue of nucleases able to process tRNA precursors, and we anticipate that mutant strains deficient in RNase PH and the other nucleases will have an observable phenotype that will allow us to sort out the functions of this group of enzymes.

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