Purification of potential 3' processing nucleases using synthetic tRNA precursors*

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Received 27 July 1978

ABSTRACT

The synthetic tRNA precursors, tRNA-C-[^{14}C]U and tRNA-C-C-A-[^{14}C]C-C, as well as poly(A) and diesterase-treated tRNA, have been used to identify and purify potential 3'processing nucleases. Four activities have been separated by this analysis; and three of them have been characterized. Two of the enzymes, which are well-separated on hydroxylapatite columns, act on poly(A), require K^+ and Mg^{2+} for activity, and have molecular weights of about 90,000. These activities have properties previously ascribed to RNase II. The third enzyme does not act on poly(A), requires Mg^{2+} for activity, and has a molecular weight of about 60,000. It is identical to RNase D, previously characterized as an exonuclease acting on tRNAs with altered structure. Each of the enzymes can remove nucleotides from the tRNA precursor containing extra nucleotides beyond the 3'terminus, whereas they are relatively inactive with intact tRNA or tRNA-C-U. The greatest specificity was displayed by RNase D. The possibility that RNase D is a 3'processing nuclease is discussed.

INTRODUCTION

Processing at the 3'terminus of tRNA precursors appears to differ among different precursor molecules. The $tRNA_1^{tyr}$ precursor found in the RNase P-deficient strain, A49 (1), contains 3 extra nucleotides at the 3'end in addition to the 41 nucleotide sequence at the 5'end (2), indicating that completion of 3'processing requires prior action of RNase P. However, some 3' processing of this precursor must have already occurred since *in vitro* transcripts (3-5) and some *in vivo* transcripts (6) contain many more extra 3' residues. A similar pathway is followed for processing of other *Escherichia coli* precursors (2). On the other hand, complete 3'processing of the bacteriophage T4 proline-serine dimeric precursor (6) or the *E. coli* $tRNA_3^{gly}$ precursor (2) proceeds *in vivo* without RNase P cleavage, suggesting that prior cleavage is not an obligatory requirement.

Attempts to identify the enzyme(s) responsible for 3' trimming of tRNA precursors have led to some confusion. Some mutants unable to carry out 3' processing appear to be defective in the 3' to 5' exonuclease, RNase II (1), and this enzyme is present in partially purified preparations of 3' processing

activity (2). In addition, partially purified preparations of RNase II can remove extra 3' nucleotides from tRNA precursors (3,5). Another mutant E. coli strain, termed BN (7), was also shown to be defective in processing some tRNA precursors, although others were processed normally (8). This has raised the possibility that more than one enzyme may be involved in 3' processing. In support of this possibility, an additional exonuclease, termed RNase P III, has been described (3) which appears to remove extra 3' nucleotides from the $tRNA^{tyr}$ precursor more efficiently than RNase II. Furthermore, Shimura et al. (9) have recently reported the identification of two exonucleases, termed RNase Q and RNase Y, which can remove nucleotides from the 3' end of tRNA precursors. Since these various possible processing nucleases have not been purified extensively or studied in any detail, it is not clear how these various activities are related, and whether they actually function in processing.

As discussed in the previous paper (10), the small amounts of substrate available and the cumbersome assay procedure have made purification and study of processing nucleases extremely difficult. However, using the specifically-labeled tRNA precursors (10) assay of processing nucleases could be made relatively simple. In this paper we describe the identification and purification of several enzymes which can act at the 3' end of tRNA precursors. The properties of these enzymes on a variety of substrates are compared, and the relation of these activities to previously described nucleases is discussed.

MATERIALS AND METHODS

tRNAs and tRNA nucleotidyltransferase were prepared as in the accompanying paper (10). Low molecular-weight contaminants were removed by chromatography on Sephadex G-100. [^{32}P]tRNA and [^{32}P] diesterase-treated tRNA (dt tRNA) were prepared as described previously (11). [^{3}H]poly(A) was purchased from Miles Laboratories. Whatman DEAE-cellulose (DE52) was purchased from Reeve-Angel, hydroxylapatite (Bio-Gel HT) was from Bio-Rad Laboratories, and Sephadex G-25 and G-100 were from Pharmacia. All salts were reagent grade except ultrapure ammonium sulfate (Schwarz/Mann). Phenylmethylsulfonylfluoride (PMSF) was obtained from Sigma Chemical Co.

Synthesis of tRNA-C-C-[^{14}C]A was carried out by exchange of AMP rather than repair of tRNA-C-C since the latter substrate has had its 5' phosphate group removed by alkaline phosphatase treatment, and we have previously shown that the 5' phosphate of tRNA affects the action of RNase D (11). Reaction mixtures contained in 10 ml: 50 mM Tris-Cl, pH 7.0; 10 mM $MgCl_2$;

0.5 mM ATP (∿ 4,000 cpm per nmole); 2 mM sodium pyrophosphate; 25 mg tRNA and 12 units of tRNA nucleotidyltransferase. After incubation for 2 hours at 37°, tRNA was precipitated with ethanol, redissolved, and purified on Sephadex G-25 containing silicic acid on top. Recovery of tRNA was 15 mg containing approximately 2500 cpm per nmole. Alkaline hydrolysis of the radioactive product indicated that > 90% of the added [^{14}C]AMP was in the terminal position.

Assays for measurement of acid-soluble radioactivity were carried out in 0.1 ml reaction mixtures containing: 10 mM Tris-Cl, pH 7.5; 5 mM $MgCl_2$ and 0.1 M KCl. Details of individual experiments with regard to substrate and enzyme are in the legends. After incubation, 0.3 ml of carrier yeast RNA (5 mg per ml) and 0.4 ml of cold 20% trichloroacetic acid were added. The samples were left in ice for 10 min and then centrifuged for 10 min at 10,000 xg. An aliquot of the supernatant fluid (0.4 ml) was withdrawn and the radioactivity determined after addition of 0.6 ml H_2O and 10 ml of Triton-toluene scintillation fluid. One unit of activity for each substrate is the amount of enzyme that will solubilize 1 μmole of radioactive material in 1 hour.

RESULTS

1. Purification of potential processing nucleases

Purification of the various nucleases was followed by examining the ability of fractions to release acid-soluble radioactivity from several different labeled substrates. Trial purification experiments revealed that all fractions active with the synthetic tRNA precursors also could act on [^{32}P] diesterase-treated tRNA, so that in the early stages of the large-scale purification described here, the latter substrate was used to conserve synthetic precursors. In addition, [^{3}H]poly(A) was used to assess RNase II-like activity throughout purification. In the later purification steps tRNA-C-C-[^{14}C]U and tRNA-C-C-A-[^{14}C]C-C were also used to follow nuclease activities.

All procedures were carried out at 4°. *E. coli* A19 cells (100 g), grown to late log phase and kept frozen, were suspended in 5 volumes of 20 mM Tris-Cl, pH 7.5; 5 mM $MgCl_2$; 1 mM dithiothreitol; 0.1 mM EDTA; 0.1 mM PMSF (buffer A). The suspended cells were ruptured in an Aminco French Press at 11,000 p.s.i., and the extract was centrifuged at 30,000 xg for 30 min. The supernatant (S_{30}) fraction was collected (500 ml), and fractionated with ammonium sulfate, maintaining the pH at 7.5 with 1 N NaOH. The fraction precipitating between 30 and 75% saturation was collected by

centrifugation, dissolved in 300 ml of buffer A and dialyzed against 4 l of buffer A containing 0.1 M KCl and 10% glycerol for 24 hours with two changes of buffer. Non-dissolved precipitate was removed by centrifugation. In addition to purification, the ammonium sulfate step served to extract nucleases bound to ribosomes.

The dialyzed material was applied to a DEAE cellulose column (8 x 29 cm) equilibrated with the dialysis buffer, and was washed with this buffer until the A_{280} was about 0.3. At this point the column was eluted with the same buffer containing 0.2 M KCl. Fractions active against poly(A) and diesterase-treated tRNA were eluted, pooled and dialyzed as above. The DEAE cellulose step was effective in removing most of the nucleic acid in the ammonium sulfate fraction. Half of the dialyzed sample was then applied to a second DEAE cellulose column as above, and after washing, the column was eluted with a gradient from 0.1 to 0.2 M KCl in the same buffer (Fig. 1). With this gradient activity degrading diesterase-treated tRNA and synthetic tRNA precursors (pool I) could be separated from another activity which acted on poly(A) in addition to the other substrates (pool II). Each of these pooled fractions was then further purified by chromatography on hydroxylapatite.

Pool I was concentrated with an Amicon PM-10 membrane and applied to a hydroxylapatite column equilibrated with 50 mM KPO_4, pH 7.5; 1 mM dithiothreitol; 0.1 mM PMSF. The column was washed with the same buffer until no more activity was eluted and the A_{280} was less than 0.05. The column was then eluted with a gradient from 50 mM to 125 mM KPO_4, pH 7.5, which removed a second activity (Fig. 2). Although the material applied to the column was inactive against poly(A), the activity which did not stick to hydroxylapatite, termed fraction A, was capable of hydrolyzing poly(A), as well as diesterase-treated tRNA and the synthetic tRNA precursors. Presumably, an inhibitor of poly(A) hydrolysis was removed during chromatography. The second activity, which bound to hydroxylapatite, was unable to hydrolyze poly(A), but it was active against diesterase-treated tRNA, tRNA-C-U and tRNA-C-C-A-C-C. This activity was identical to RNase D which we described previously as a nuclease which acted on tRNA molecules with altered structures (11).

Pool II from DEAE cellulose was concentrated as above and applied to a hydroxylapatite column equilibrated with 10 mM KPO_4, pH 7.5; 1 mM dithiothreitol; 0.1 mM PMSF. After washing with the same buffer until the A_{280} was less than 0.05, the column was eluted with a gradient from 10 mM to 100

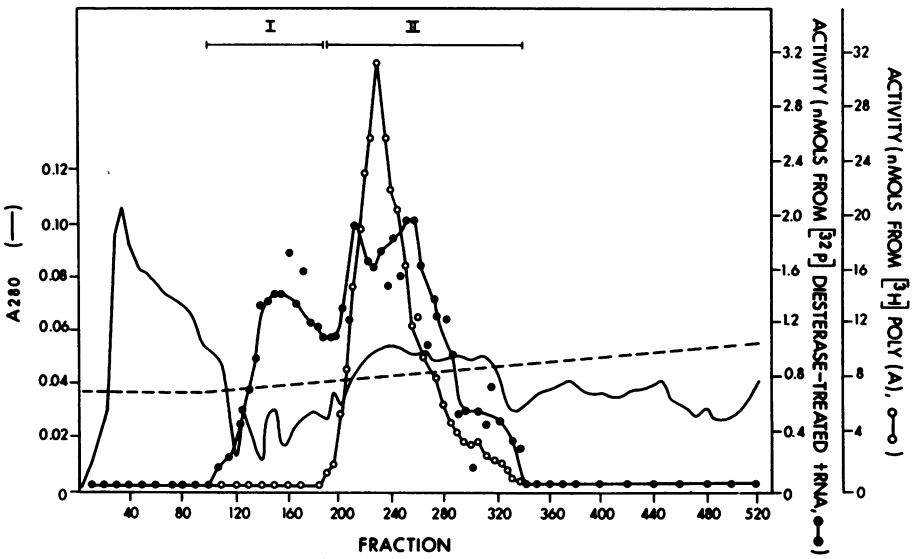

Fig. 1. DEAE cellulose chromatography. Half of the dialyzed sample from the first DEAE cellulose column was applied to a second DEAE cellulose column (5.5 x 28 cm) and eluted with a 12liter gradient from 0.1 to 0.2 M KCl in buffer A containing 10% glycerol. The column was run at a flow rate of 250 ml/hour and fractions of 21 ml were collected. Aliquots (25 µl) were assayed for activity on [^{32}P] diesterase-treated tRNA (89 µg) or [^{3}H] poly(A) (98 nmoles nucleotide) for 30 min or 10 min, respectively, as described in Methods.

mM KPO_4, pH 7.5. Two distinct peaks of activity, termed fractions B and fraction C, were resolved, each of which was active against poly(A), diesterase-treated tRNA, tRNA-C-U and tRNA-C-C-A-C-C (Fig. 3).

Fractions A, B and C from hydroxylapatite were each concentrated to 2-3 ml by filtration through Amicon PM-10 membranes. An aliquot of each of these fractions (1 ml) was further purified by chromatography on Sephadex G-100. The peak tubes from Sephadex chromatography were used for the studies described here. RNase D from hydroxylapatite was concentrated by adsorption and elution from a small hydroxylapatite column. Chromatography of this material on Sephadex G-100 did not lead to any further purification. The RNase D used in the experiments described here was either the concentrated hydroxylapatite fraction or the Sephadex G-100 fraction.

A summary of the purification of the various activities, based on their action on poly(A) or diesterase-treated tRNA is shown in Table I. Due to the multiplicity of activities acting on each substrate in the cruder fractions, and the fact that the different activities could act synergistically (11), it was not possible to get an accurate measure of the overall

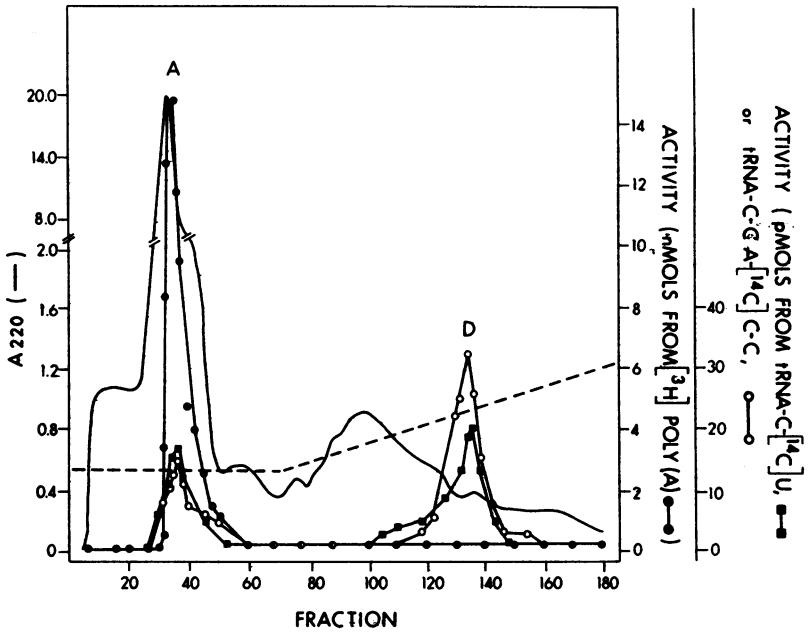

Fig. 2. Hydroxylapatite chromatography of pool I. DEAE cellulose pool I was concentrated with an Amicon PM-10 membrane to 112 ml and applied to a hydroxylapatite column (2.6 x 8 cm). After washing with the equilibration buffer (290 ml), the column was eluted with a 750 ml gradient from 50 to 125 mM KPO_4, pH 7.5. The column was run at a flow rate of 24 ml/hour and fractions of 5.3 ml were collected. Aliquots (25 µl) were assayed for activity on [^{3}H] poly(A) and [^{32}P] diesterase-treated tRNA as described in Fig. 1, and for activity on tRNA-C-[^{14}C]U (29 µg) and tRNA-C-C-A-[^{14}C]C-C (9 µg). For clarity, the activity against diesterase-treated tRNA is not shown.

purification or the yield for each activity. However, at a minimum, RNase D was purified about 250-fold using diesterase-treated tRNA as substrate, and the peaks of fractions B and C about 200- and 600-fold, respectively, using poly(A) as substrate. Although peak A differed in substrate specificity somewhat compared to the other enzymes, it has not been purified sufficiently to determine whether it is a distinct enzyme or a mixture of several activities, and was not studied in any detail. On the other hand, the properties of peak B and peak C are identical to those previously described for E. coli RNase II (12,13). Furthermore, when assayed according to the conditions of Singer and Tolbert (12), the peak tube from Sephadex G-100 of fraction C had a specific activity identical to pure RNase II (12,13), and the peak tube from fraction B had about one-third of this specific activity.

2. Properties of the purified enzyme

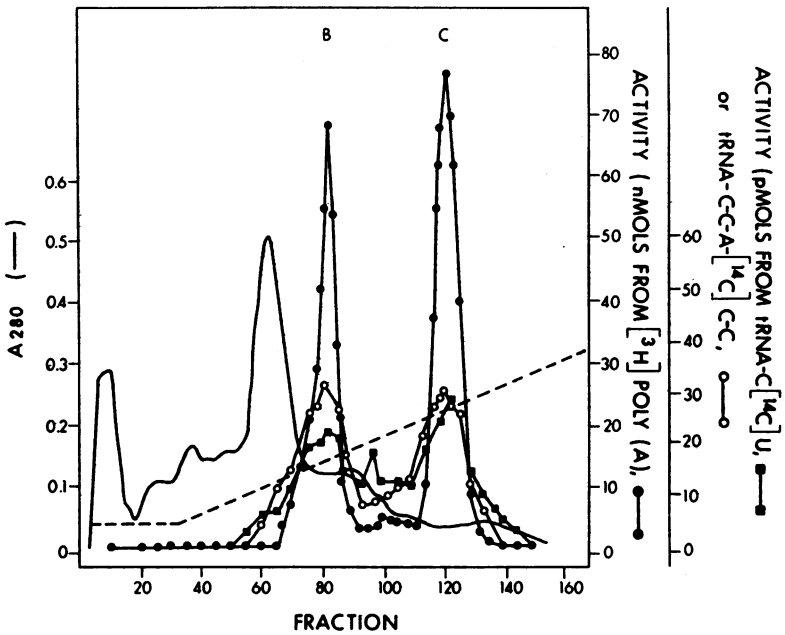

Fig. 3. Hydroxylapatite chromatography of pool II. DEAE cellulose pool II was concentrated as in Fig. 2 to 80 ml and applied to a hydroxylapatite column (2.6 x 8 cm). After washing with the equilibration buffer (240 ml), the column was eluted with a 1400 ml gradient from 10 to 100 mM KPO_4, pH 7.5. The column was run at a flow rate of 24 ml/hour and fractions of 10 ml were collected. Assays were performed exactly as described in Fig. 2. For clarity, the activity against diesterase-treated tRNA is not shown.

One measure of the specificity of each of the purified enzymes for the different substrates could be obtained from their relative enrichment with respect to the crude extract using each of the substrates. The data in Table II show that fraction B and fraction C have been most highly enriched with respect to hydrolysis of poly(A), whereas the RNase D fraction has lost all activity against poly(A), but has been highly enriched for activity against the synthetic precursor tRNA-C-C-A-C-C. In contrast, none of the enzymes has been as well purified for activity against tRNA-C-U as for the other substrates. Although none of the enzymes show absolute specificity for any substrate, these data suggest that RNase D may be the enzyme acting on tRNA precursors with extra residues after the -C-C-A terminus. Furthermore, since the relative activity against tRNA-C-U compared to other substrates decreased for each of the enzymes compared to the crude extract, it is possible that a nuclease more specific for tRNA-C-U has been removed during

TABLE I
Summary of Nuclease Purification

Fraction	Protein	Total Activity (dt tRNA)	Sp. Act.	Total Activity (poly(A))	Sp. Act.
	mg	units	units/mg	units	units/mg
S_{30}	17,900	2,800	0.16	64,800	3.6
Dialyzed 30-75% $(NH_4)_2SO_4$	3,200	3,400	1.1	51,600	16
1st DEAE cellulose	750	1,860	2.5	27,600	37
2nd DEAE cellulose					
pool I	174	600	3.4	0	-
pool II	384	1,120	2.9	18,000	47
Hydroxylapatite					
A	44			156	3.5
B	5.6			1,440	260
C	5.1			2,280	450
D	3.8	157	41	0	
Sephadex G-100					
A	34			95	2.8
B[1]	3.8(0.08)			1,730(55)	460(690)
C[1]	1.7(0.04)			2,450(86)	1,400(2100)

[1] The numbers in parenthesis represent the values for the peak tube.

purification (8).

Using poly(A) as substrate, fraction B and fraction C had a broad pH optimum between pH 7 and 10. Both enzymes required a divalent cation for activity. Optimal activity was obtained at 1 mM Mg^{2+}; Mn^{2+} was 50% as effective, but Ca^{2+} was inactive. The two enzymes also required a monovalent cation for activity. Optimal activity was obtained at 0.1 M K^+ or NH_4^+ for fraction B, and at 0.15 M cation for fraction C. Na^+ could not satisfy the monovalent cation requirement in either case. The molecular weight of each fraction, determined by chromatography on Sephadex G-100 was about 90,000. These properties of fractions B and C are identical to those previously ascribed to ribonuclease II (12,13).

TABLE II

Enrichment of Purified Enzymes Against Various Substrates

Each of the purified enzymes and the S_{30} fraction were assayed as described in Methods using [^{3}H] poly(A) (98 nmoles nucleotide), [^{32}P] diesterase-treated tRNA (180 µg); tRNA-C-[^{14}C]U (37 µg) and tRNA-C-C-A-[^{14}C]C-C (44 µg) and the specific activities calculated. The data presented are the increases in specific activity with respect to each substrate from the S_{30} fraction to the purified enzyme. The initial specific activities in the S_{30} fraction in this experiment were poly(A), 2.7 units per mg; diesterase-treated (dt tRNA) 0.15; tRNA-C-U, 0.0015; tRNA-C-C-A-C-C, 0.003.

	Substrate			
Enzyme	Poly(A)	dt tRNA	tRNA-C-U	tRNA-C-C-A-C-C
		- fold purification		
Fraction B	250	29	33	150
Fraction C	780	250	66	360
RNase D	-	270	53	870

In contrast, using diesterase-treated tRNA as substrate, RNase D had a pH optimum between pH 9 and 10. The enzyme required 5 mM Mg^{2+} for optimal activity; Mn^{2+} was about 80% as effective, and Ca^{2+} about 10%. RNase D was stimulated slightly by K^+ or NH_4^+, but was inhibited by Na^+. Essentially identical requirements were found when tRNA-C-C-A-[^{14}C]C-C was used as substrate except that K^+ inhibited slightly. The molecular weight of RNase D, determined on Sephadex G-100, was about 60,000. The properties of RNase D clearly distinguish this enzyme from fractions B and C, and from other *E. coli* nucleases.

The action of fractions B and C and RNase D on the substrates tRNA-C-C-[^{14}C]A, tRNA-C-[^{14}C]U and tRNA-C-C-A-[^{14}C]C-C are shown in Fig. 4. Each enzyme's activity has been normalized to the same amount of hydrolysis using [^{32}P] diesterase-treated tRNA as substrate. Each of the enzymes is relatively inactive against intact tRNA and against the synthetic precursor tRNA-C-U, whereas they display substantial activity against the other synthetic precursor, tRNA-C-C-A-C-C. Thus, each of these enzymes is capable of removing extra nucleotides following the -C-C-A sequence, with the greatest specificity being shown by RNase D. In the case of the latter enzyme intact tRNA is digested at < 3% of the rate of tRNA-C-C-A-C-C, as might be expected for a 3' processing nuclease.

DISCUSSION

The results presented in this paper demonstrate the usefulness of the

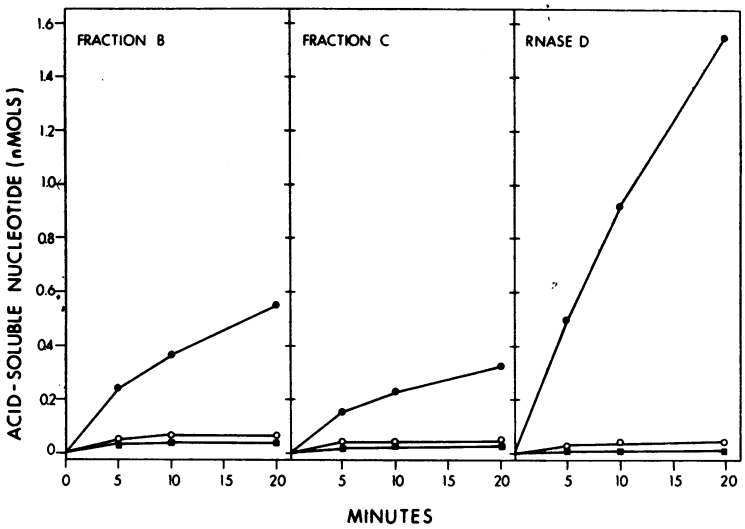

Fig. 4. Rate of hydrolysis of intact tRNA and synthetic tRNA precursors with fractions B and C and RNase D. Assays were performed as described in Methods except that no KCl was present in the RNase D assays. Approximately 70 µg of each tRNA substrate was present, and varying amounts of enzyme depending on the substrate. Samples were incubated at 37° for the times indicated, and radioactivity made acid-soluble was determined. All rates have been normalized to 7 milliunits of enzyme using 70 µg of [^{32}P] diesterase-treated tRNA as substrate. tRNA-C-C-A-[^{14}C]C-C, ●—●; tRNA-C-C-[^{14}C]A, o—o, tRNA-C-[^{14}C]U, ■—■.

synthetic tRNA precursors for identifying, purifying and characterizing potential 3' processing nucleases by simple assay procedures. This initial analysis has led to the separation of four activities which are capable of removing nucleotides from the 3' end of synthetic precursor tRNAs. One of these activities, fraction A, was not well-purified. Its requirements were similar to fractions B and C, but its substrate specificity differed (data not shown). Further work is necessary to determine whether this activity is a distinct enzyme.

Fractions B and C, on the other hand, had enzymatic properties and molecular weights identical to those previously ascribed to RNase II (12,13), and one of the activities, fraction C, was highly purified (12). However, it is not yet clear why we repeatedly observed two easily separable peaks of this activity on hydroxylapatite columns. The resolution of two peaks of activity was probably not due to proteolytic digestion of RNase II since all buffers contained the protease inhibitor, phenylmethylsulfonylfluoride. However, since the two fractions could be separated, they must differ in

structure. Nevertheless, the data presented here confirm earlier suggestions (1-5) that RNase II can act at the 3' end of tRNA precursors, although with less specificity than RNase D (see below).

RNase D has previously been shown to act on tRNAs with altered structures (11). The results presented here demonstrate that this enzyme also displays high specificity for a tRNA precursor containing extra residues following the -C-C-A sequence. Obviously, such a precursor is recognized as a tRNA with an altered structure. In contrast, RNase D acts relatively poorly on the other type of tRNA precursor, tRNA-C-U. These results support the suggestion (8) that two different nucleases would be required to process the two different types of tRNA precursors. The properties of RNase D during purification also suggest that it may be related to the RNase P III reported by Bikoff et al. (3).

The properties of RNase D make it the most likely candidate for the nuclease involved in processing the 3' terminus of E. coli tRNA precursors. Our previous results with E. coli mutants lacking tRNA nucleotidyltransferase (14) suggested that whatever was the active 3' processing nuclease in vivo, it would stop without removing residues from the -C-C-A sequence (15,16). The very low activity of RNase D on intact tRNA is consistent with this observation. Furthermore, preliminary results obtained in collaboration with Dr. Sidney Altman, have shown that RNase D removes mononucleotides from the $tRNA^{tyr}$ precursor, but only after prior cleavage with RNase P. This order of processing is the same as observed in vivo (2). RNase D also releases [^{14}C]CMP from tRNA-C-C-A-[^{14}C]C-C to regenerate tRNA-C-C-A with amino acid acceptor activity (Ghosh and Deutscher, unpublished result). In contrast, fractions B and C act on the $tRNA^{tyr}$ precursor without RNase P action, and lead to more extensive release of mononucleotides. Shimura et al. have recently reported the existence of two nucleases, termed RNase Q and Y (9). From their action on natural precursors, RNase Q may be identical to RNase D, and RNase Y to fractions B and C.

These results show that the use of synthetic tRNA precursors greatly simplifies the identification and purification of potential processing nucleases. Coupled with the use of other substrates, such as poly(A), these studies have been able to distinguish among the various activities and relate them to known enzymes. In addition, we have been able to attain a high degree of purification for several of these enzymes. Studies are now in progress to determine the purity of each of these enzymes, and to determine their mode of action on natural tRNA precursors.

ACKNOWLEDGEMENTS

This work was supported by grant GM16317 from the National Institutes of Health. The technical assistance of Mr. Rong-Chang Ni is greatly appreciated.

*This paper is number 25 in the series, "Reactions at the 3'Terminus of tRNA". The previous paper in the series is reference 10.

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