Nucleotides in precursor tRNAs that are required intact for catalysis by RNase P RNAs

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Received October 9, 1990; Revised and Accepted January 16, 1991

ABSTRACT

Precursor tRNA^{Asp} molecules, containing a 26-base 5' leader, were treated with diethylpyrocarbonate, 50% hydrazine or anhydrous hydrazine/3M NaCl and then subjected to processing by RNase P RNAs from *Escherichia coli* or *Bacillus subtilis*. Fully processed tRNAs and material not successfully cleaved by the catalytic RNAs were analyzed for their content of chemically altered nucleotides. Several bases were identified as being required intact for optimal activity as substrate as judged by exclusion of chemically modified residues from processed molecules, and simultaneous enhancement in material that was not recognized as substrate. Such nucleotides cluster near the site of cleavage at the mature 5' end and in the T stem and loop. Purines at residues 1 and 2 adjacent to the site of cleavage, position 57 in the T loop, and site 64 in the T stem exhibited the most pronounced effects. These results suggest a model of recognition of substrate by RNase P RNAs in which the ribozyme interacts with the corner of the precursor tRNA's three dimensional structure, where the T- and D-loops are juxtaposed, and extends along the top of the molecule back towards the site of catalysis.

INTRODUCTION

RNase P is a ribonucleoprotein particle that makes a single precise endonucleolytic cut in precursor tRNAs forming the mature 5' terminus of the tRNA molecule (1,2). The well-characterized activities from *Escherichia coli* and *Bacillus subtilis* contain a small basic protein with a molecular mass of approximately 14,000 daltons (3) and an RNA moiety approximately 400 nucleotides long (4,5). Under the appropriate ionic conditions, RNase P RNA in the absence of protein is catalytic (6). Significantly higher concentrations of mono- and divalent cations are required for optimal *in vitro* activity of RNase P RNA than other RNA-catalyzed reactions (7-9).

The sequences of several RNase P RNAs have been reported and credible structures based on comparative sequence analysis have been proposed (10). The nature of the interaction between the catalytic RNA and the substrate, as well as the mechanism

of catalysis are being actively studied using a variety of experimental approaches. Crosslinking studies have identified RNase P RNA nucleotides that are in close proximity to bound precursor tRNA (11,12). Studies focusing on the chemical and structural features of precursor tRNAs that are required in an active substrate have demonstrated that a 3' hydroxyl is not required (13); that the 2' hydroxyl of the nucleotide immediately adjacent to the mature 5' terminus enhances cleavage, but is not essential (14); that nucleotides near the site of cleavage and in the T-loop are required chemically intact (15,16); and that a minimal structure of the amino accepting stem and the T stem and loop are sufficient for accurate cleavage (17).

In this paper we identify nucleotides in precursor tRNA^{Asp} (pAsp) from *E. coli* that are required chemically intact for cleavage by RNase P RNAs from both *E. coli* and *B. subtilis*. Using precursor tRNAs that were chemically modified prior to processing by RNase P RNAs, we characterize the content of chemically altered bases in fully processed tRNAs and in precursor molecules that were not cleaved. Our results confirm the importance of the corner and top of the 'L-shaped' three dimensional structure of a tRNA (18), and help to define a minimal set of structural domains of the substrate required for catalysis by RNase P RNA.

MATERIALS AND METHODS

Buffers were prepared using deionized distilled water (Barnstead NANOpure II) and reagent grade chemicals. [α -³²P]ATP (>3000 Ci/mmol) was purchased from ICN Radiochemicals (Irvine, CA). Snake venom phosphodiesterase (46U/mg) was obtained from Worthington Biochemical (Freehold, NJ). ATP/CTP:tRNA nucleotidyltransferase from *Saccharomyces cerevisiae* was kindly provided by Dr. Jacek Wower (University of Massachusetts, Amherst, MA). T4 RNA ligase and T4 polynucleotide kinase were purchased from United States Biochemical (Cleveland, OH). T7 RNA polymerase was obtained from BRL (Gaithersburg, MD) or generously provided by Dr. Bernadette Pace (Indiana University, Bloomington, IN).

RNase P RNAs (*B. subtilis* and *E. coli*) were prepared by *in vitro* transcription of T7 expression vectors containing the appropriate genes (generously provided by Dr. David Waugh

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and Dr. Norman Pace, Indiana University). pDW25 (*B. subtilis* RNase P RNA) and pDW27 (*E. coli* RNase P RNA) were cut with Dra I (Promega, Madison, WI) and SnaBI (New England Biolabs, Beverly, MA) respectively, as recommended by the suppliers. 5 μ g of restricted plasmid was incubated at 37°C for 3 h with ATP, CTP, UTP and GTP (2 mM each) and T7 RNA polymerase (4U) in 50 μ L of 20 mM sodium phosphate buffer (pH 7.8), 10 mM dithiothreitol, 8 mM MgCl₂, 4 mM spermidine. After incubation, the transcripts were precipitated with ethanol, resuspended in 50 μ L of TBE buffer (90 mM Tris-borate, pH 8.3, 1 mM EDTA) containing 7 M urea, and loaded onto a 6% polyacrylamide gel (16 $\times$ 16 $\times$ 1.5 cm) in TBE/7 M urea. The gel was run at 250 V until the bromophenol blue tracking dye had migrated to the bottom. The transcripts were detected by UV shadow, cut from the gel and passively eluted overnight at 4°C in 10 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM EDTA, 0.1% SDS. The enzymatically active transcription products have two extra bases at the 5' terminus and several at the 3' terminus.

Precursor tRNA^{Asp}, derived from *E. coli*, was prepared by *in vitro* transcription of the T7 expression vector pDW128 (Dr. David Waugh and Dr. Norman Pace, Indiana University). Following cleavage of the plasmid with BstNI (New England Biolabs, Beverly, MA) according to recommendations of the supplier, the linearized plasmid was transcribed *in vitro* with T7 RNA polymerase as described above. The transcription product, having a 26 base 5' leader sequence and a 3'-terminal CCA, was isolated on a gel and purified as described for the RNase P RNAs.

pAsp (4 μ g) was labeled at its 3' end in one of two ways: following removal of single-stranded residues with snake venom phosphodiesterase, ATP/CTP:tRNA nucleotidyltransferase from yeast was used with [α -³²P]ATP as cosubstrate (19); or T4 RNA ligase was used to ligate [5'-³²P]pCp (20). Labeling of pAsp at its 5' end was carried out using [γ -³²P]ATP and polynucleotide kinase (21). Radioactively labeled pAsp was purified on a 0.4 mm $\times$ 18 cm $\times$ 35 cm 10% polyacrylamide sequencing gel (acrylamide:N-N'-methylene bisacrylamide, 19:1) in 8 M urea, 100 mM Tris-borate (pH 8.3), 2.5 mM Na₂EDTA. Material labeled using RNA ligase was repurified on a second such gel. The second repurification gel was necessary to separate the transcription product terminating at the expected nucleotide (A76) from transcripts containing three additional residues at the 3' end. Elution from gel slices was carried out as described previously (19).

Aliquots of the labeled pAsp were treated with diethylpyrocarbonate (DEP), 50% aqueous hydrazine or anhydrous redistilled hydrazine/3 M NaCl as described by Peattie (22). The times and temperatures of the treatments were as follows: DEP—90 s, 90°C; 50% hydrazine—5 min, 4°C; and hydrazine/3 M NaCl—10 min, 4°C. The final pellets were resuspended in water and aliquots of 10 μ L were added to an equal volume of concentrated buffer to final concentrations of 50 mM Tris-Cl (pH 7.6), 0.8 M NH₄Cl, 20 mM MgCl₂, and 1 μ M RNA substrate. 0.3–0.4 μ g of RNase P RNA from *E. coli* or *B. subtilis* was added (final concentration = 0.1 μ M) and incubation at 37°C was carried out from 30 min to 2 h (see figure legends). Unmodified controls were processed in exactly the same manner as the chemically modified samples. The processed tRNA and unprocessed precursor were separated on a 10% sequencing gel, recovered from the gel by elution and treated with aniline according to Peattie (22), except that the time of incubation at 65°C was shortened to 5 min.

Aniline cleavage products were adjusted such that their content of radioactivity, determined using a Bioscan 2000 detector (Bioscan Inc., Washington, DC) were approximately equal ($\pm$ 15%), and were analyzed on 20% sequencing gels. Autoradiograms were made using Kodak X-Omat AR film and were exposed at –70°C for 1–15 days.

RESULTS

³²P-labeled pAsp was chemically modified with DEP, 50% hydrazine or hydrazine/3M NaCl and processed using RNase P RNA from *E. coli* or *B. subtilis*. Mature tRNA was separated from unprocessed material on a 10% sequencing gel. Unprocessed, modified controls and material that had been processed prior to modification were purified on the same gel. As judged by visual comparisons of bands on autoradiograms corresponding to processed and unprocessed material, cleavage of DEP-modified RNA was consistently inhibited more than that of 50% hydrazine- or hydrazine/3M NaCl-treated samples. The largest inhibition observed with DEP-modified samples resulted

Figure 1. Autoradiogram identifying carbethoxylated guanines in pAsp that interfered with processing by RNase P RNA from *B. subtilis*. 3'³²P-labeled samples were modified with DEP but not subjected to processing (lane 1); modified before processing and recovered as unprocessed pAsp (lane 2); modified before processing and recovered as mature tRNA^{Asp} (lane 3); or processed before modifying (lane 4). Incubation with RNase P RNA was at 37°C for 2 h. All samples were purified on a 10% sequencing gel (see Fig. 1) and then treated with aniline. The resulting fragments were resolved on a 20% sequencing gel run at 30 mA until the xylene cyanole had migrated 25 cm. Numbers refer to nucleotide positions numbered according to Sprinzl *et al.* (23). Arrows indicate sites where modified bases interfered with processing.

in approximately 40% of the pAsp remaining unprocessed; in control and hydrazine- or hydrazine/3M NaCl-treated samples, greater than 90% of the sample was converted to mature tRNA.

Chemically modified nucleotides that interfered with processing were identified by characterizing the content of modified bases in fully processed tRNA and in material that was not successfully processed. As a specific example, a carbethoxylated guanine at position 57, numbered according to Sprinzl *et al.* (23), was essentially absent from the processed tRNA, following cleavage by RNase P RNA from *B. subtilis* (Fig. 1, lane 3), and was simultaneously enhanced in the unsuccessfully processed substrate (Fig. 1, lane 2). Bands corresponding to guanines 1, 2, 64, and, to a lesser extent, guanines 49, 51, 52, and 53, were also reduced

in intensity in the mature tRNA and enhanced in unprocessed material. A similar pattern of reductions and enhancements were observed when the catalytic RNase P RNA from *E. coli* was used (Fig. 2).

These reduced and enhanced intensities were not artifacts of purifying modified material on gels (see ref. 24). Such artifacts would have been detected in the control samples. For example, in Figure 1, the sample in lane 1 had been modified but not processed with RNase P RNA, and then gel purified; that in lane 4 was processed prior to modification and then gel purified. In neither case was the intensity of the band corresponding to the

Figure 2. Autoradiogram identifying carbethoxylated guanines in pAsp that interfered with processing by RNase P RNA from *B. subtilis* or *E. coli*. 3^{32}P -labeled samples were modified with DEP before processing with RNase P RNA from *E. coli* and recovered as unprocessed pAsp (lane 1); modified before processing with RNase P RNA from *E. coli* and recovered as mature tRNA^{Asp} (lane 2); modified with DEP but not subjected to processing (lane 3); modified before processing with RNase P RNA from *B. subtilis* and recovered as mature tRNA^{Asp} (lane 4); or modified before processing with RNase P RNA from *B. subtilis* and recovered as unprocessed pAsp (lane 5). Incubation with the catalytic RNA was at 37°C for 30 min. All samples were purified on a 10% sequencing gel (see Fig. 1) and then treated with aniline. The resulting fragments were resolved on a 20% sequencing gel run at 30 mA until the xylene cyanole had migrated 10 cm (a), 25 cm (b), or 42 cm (c). Numbers refer to nucleotide positions numbered according to Sprinzl *et al.* (23). Arrows indicate sites where modified bases interfered with processing.

Figure 3. Autoradiogram identifying DEP-, 50% hydrazine-, or hydrazine/3M NaCl-modified nucleotides in pAsp that interfered with processing by RNase P RNA from *B. subtilis* or *E. coli*. 3^{32}P -labeled samples were modified with DEP (a), 50% hydrazine (b) or hydrazine/3 M NaCl (c). Aliquots of the modified samples were processed with RNase P RNA from *E. coli* and recovered as unprocessed pAsp (lane 1) or as mature tRNA^{Asp} (lane 2); other aliquots were modified before processing with RNase P RNA from *B. subtilis* and recovered as unprocessed pAsp (lane 3) or mature tRNA^{Asp} (lane 4). Control material was modified, but not subjected to processing (lane 5). Incubation with the catalytic RNA was at 37°C for 2 h. All samples were purified on a 10% sequencing gel (see Fig. 1) and then treated with aniline. The resulting fragments were resolved on a 20% sequencing gel run at 30 mA until the xylene cyanole had migrated 11 cm. Numbers refer to nucleotide positions numbered according to Sprinzl *et al.* (23). Arrows indicate sites where modified bases interfered with processing.

Figure 4. Autoradiogram showing enhancement of chemically modified nucleotides in 5'-³²P-labeled pAsp that was not successfully processed by RNase P RNA from *B. subtilis* or *E. coli*. pAsp was labeled with ³²P at its 5' end, modified with DEP (a), 50% hydrazine (b), or hydrazine/3 M NaCl (c), and subjected to processing by RNase P RNA from *E. coli* (lanes 1) or *B. subtilis* (lanes 3). Material remaining intact following incubation with the catalytic RNA for 1 h (lanes 1 and 3), and control material that was modified but not subjected to processing (lanes 2), were purified on a 10% sequencing gel and treated with aniline. The resulting fragments were resolved on a 20% sequencing gel run at 30 mA until the xylene cyanole had migrated 10 cm. Numbers refer to nucleotide positions numbered according to Sprinzl *et al.* (23). Arrows indicate sites where modified bases interfered with processing.

guanine at position 57 significantly reduced or enhanced, relative to other guanine residues. In contrast, the bands in the experimental samples (lanes 2 and 3), which had been modified prior to processing, were significantly reduced or enhanced.

Modification of cytidines by hydrazine/3 M NaCl also affected processing by both RNase P RNAs (Fig. 3), especially those located at positions 56 and 61–63 (Fig. 3c). Similarly, alteration of uridines 54 and 55 using 50% hydrazine inhibited the reaction (Fig. 3b). In these instances, the enhanced intensities in material that was not successfully processed was readily apparent, whereas intensities in the mature tRNA were only slightly reduced.

When 5'-labeled material was treated in a similar manner (Fig.

Figure 5. The location of residues in the secondary and tertiary structure of pAsp identified as being important for interaction with RNase P RNAs from *E. coli* and *B. subtilis*. Bases that influence the reaction when chemically modified are indicated by stippling on the model of secondary structure (A) and in black on a model of tertiary structure (B) of pAsp. In (A) only the five 3' residues of the 5' leader are shown. The sequence of the entire 5' leader is GGGAAUUCG-AGCUCGGUACCCAAAUAU. In (B) the general regions implicated as being important for interaction with the ribozyme, along the top and back and at the corner of the tRNA precursor, are shaded. The bases are numbered according to Sprinzl *et al.* (23).

4), enhancements in the unprocessed precursor could only be compared to material that was not subjected to processing; the mature tRNA released from the precursor did not contain a radioactive label. From such experiments, it was clear that modified uridines at sites -1 (just 5' of the site of cleavage) and +3, as well as chemically altered cytidines at positions 4 and 5 in the acceptor stem, also interfered with processing.

A summary of bases that reproducibly interfered with processing when they had been chemically altered is presented in Figure 5a. The location of such residues in the tertiary structure of a mature tRNA is depicted in Figure 5b.

It was also interesting to note that bands corresponding to purines on the 5' side of the site of cleavage, were reduced in intensity in the track containing material that had not been successfully processed (Fig. 1, lane 2 and Fig. 2, lanes 1 and 5). We do not know the basis for these reduced intensities, but note that similar results were evident in the data presented by Kahle *et al.* (16). They probably do not reflect instances where modified bases facilitated processing, because the 5' fragment released following processing was not enhanced in its content of modified bases (data not shown).

DISCUSSION

Experimental approach

Chemical modification of precursor tRNAs, especially that by DEP, interfered with the extent of cleavage of precursor tRNAs by the catalytic RNA component of RNase P from *B. subtilis* and *E. coli*. We have utilized this inhibition to identify individual nucleotides in a precursor of tRNA^{Asp} that, when chemically modified, interfered with processing by RNase P RNAs from both *E. coli* and *B. subtilis* (Figs. 1–4). The experimental approach of chemically modifying a tRNA substrate prior to interaction with processing enzymes has been used previously to identify nucleotides essential for interaction with nucleotidyltransferase (19), and with RNase P RNA from *E. coli* and RNase P (holoenzyme) from *Schizosaccharomyces pombe* (16).

The rationale for such experiments is based on the assumption that both the extent of modification of each molecule and the susceptibility to modification of each nucleotide are controlled by experimental conditions according to the following criteria. Prior to interaction with the enzyme of interest, each molecule should, on average, be modified only once. The amount of chemical modifier and length of incubation are controlled such that most of the material following cleavage at modified bases with aniline remains intact (indicating that most of the material was not modified) and such that small fragments do not predominate (indicating that there were not multiple hits). The RNA must also be fully denatured by high temperatures (90°C for treatment with DEP) or high pH (>12 for treatment with hydrazine), ensuring that each nucleotide is equally susceptible to modification.

Care must also be taken when chemically treated RNA molecules are purified on polyacrylamide gels, because modification-induced electrophoretic conformers may arise (24). We utilized relatively low percentage gels and short migration times to minimize the likelihood of resolving such conformers. We also made all comparisons of band intensities to those in control material, which had been isolated on the same gel as the experimental samples. Given the above caveats, the damage-selection experimental approach can be a powerful means of identifying individual nucleotides that play vital roles in interactions with enzymes. The results cannot in all instances distinguish points of direct contact from residues required intact for higher order structure, nor can they discriminate bases that affect binding to the enzyme from those that may affect the rate of catalysis. However, the identity of individual residues that play important roles can be quickly and precisely determined, thereby

targeting sites for future mutagenesis experiments in which the precise function of a given base can be established.

Relative effects of chemical modifiers

Each chemical modifier used in the present study resulted in exclusion of at least some molecules that contained chemically altered residues from the population of fully processed tRNAs. Carboxymethylated purines (DEP) were most frequently responsible for such exclusions, followed by excised cytidines (hydrazine/3 M NaCl) and excised uridines (50% hydrazine). This is consistent with the observation that treatment with DEP noticeably increased the fraction of uncleaved precursor following processing, whereas 50% hydrazine or hydrazine/3 M NaCl did not, at least at the enzyme:substrate ratios used in our experiments (data not shown). It may be that addition of bulky substituents, such as a carboxymethyl group, which would interfere with close approach by the ribozyme, is more inhibitory than removal of any single base.

General features of recognition by RNase P RNAs

Six residues in pAsp that we have identified as being involved in the interaction with RNase P RNAs are located in the acceptor stem near the site of cleavage (U-1, G1, G2, U3, C4 and C5; see Fig. 5). The ability of such chemically altered residues to inhibit the cleavage reaction is not surprising. One can easily imagine that the excision of nearby uridines or cytidines with hydrazine or the carboxymethylation of adjacent purines by DEP would interfere with such a precise enzymatic reaction. Either points of direct contact with the catalytic RNA or vital local helical structure could easily be disrupted by such modifications.

In addition, 12 bases in the T-stem/loop region of the precursor molecule were able to interfere with the interaction when modified. Residues 61–64 are located near the top of the tRNA's L-shaped three dimensional structure, on the same face as bases 1–6 in the acceptor stem (on the backside as depicted in Fig. 5b). Nucleotides 49 and 51–57 appear near the corner of the tRNA, on the opposite face (in front as depicted in Fig. 5b). Our results are consistent with the ability of structural analogs to the stacked array of bases in the acceptor and T-stems, found along the top of a tRNA precursor, to be successfully processed by M1 RNA (17). They suggest that the ribozyme extends from the corner of the tRNA precursor, across the top of the molecule, to the site of cleavage. This model of recognition of substrate by RNase P RNAs is consistent with the ability of these catalytic RNAs to recognize and process any tRNA, or tRNA-like, molecule (25), because the tertiary structural arrangement involving the juxtaposition of the T- and D-loops at the corner of the molecule appears to be highly conserved (18). In a similar manner, nucleotidyltransferase, which also does not discriminate among tRNAs, and tRNA-like molecules, interacts primarily with the top and corner of a tRNA (19,26).

It may turn out that most tRNA processing enzymes recognize the highly conserved tertiary structural arrangement at the corner of the molecule, involving the T- and D-loops, and then measure along the molecule to their site of interaction. Nucleotidyltransferase (19) and RNase P measure across the top of the tRNA to the 3' end; yeast tRNA splicing enzymes measure along the anticodon stem to the intron within the anticodon loop (27). Aspects of tRNA structure required for recognition by a host of other 3'-processing enzymes have yet to be fully characterized (28). In contrast, aminoacyl-tRNA synthetases, which discriminate among tRNAs, seem to interact with more variable regions of a tRNA, including the variable and anticodon loops (29).

Role of the T-loop

Based on the highly reproducible, nearly total exclusion of chemically modified guanosines at position 57 from fully processed tRNAs, we propose that this universally conserved purine plays an especially important role in mediating recognition by RNase P RNA. This is not totally surprising in view of the critical location of site 57, near a tight binding site for Mg^{2+} , which is well known to stabilize tRNA structure. Purine-57 is also required intact for interaction with nucleotidyltransferases from *E. coli*, yeast, and rabbit liver (19,30). We cannot conclude that this residue necessarily forms a base pair with a nucleotide in the ribozyme, because even slight disruption of local structure in this region of the molecule may interfere with the recognition process. However, it is clear that the corner of the precursor tRNA is required intact by RNase P RNAs and that this may constitute the point from which an appropriate distance to the site of cleavage is 'measured' by the ribozyme.

Comparison between RNase P RNAs from *E. coli* and *B. subtilis*

In general the two RNase P RNAs were remarkably similar in their requirements for unmodified bases in the precursor tRNAs. However, the RNase P RNA from *B. subtilis* displayed a slight, but consistent, enhanced sensitivity to a chemically altered guanine at position 57. We have not tested either holoenzyme, but based on the results of Kahle *et al.* (16), anticipate that exclusions would be more pronounced and widespread.

Comparison of results reported here to those of similar experiments

During the course of our experiments, results from another study using a similar approach were reported in which precursors to tRNA^{Ser} and tRNA^{Met} from *S. pombe* were chemically modified prior to processing by M1 RNA from *E. coli* and by RNase P holoenzyme from *S. pombe* (16). A few instances of inhibition by modified bases in the anticodon stem and other regions of the precursor tRNAs were reported by Kahle *et al.* (16), but were not observed by us. Such effects were very minor, and we cannot exclude the possibility that we could not detect comparable reductions in intensity by simple visual inspection. We did not attempt to quantify extents of exclusion, but, rather, focused on identifying instances where exclusions were reproducibly apparent from visual inspection of the autoradiograms. We thereby present our results as a minimum set of bases that have substantial effects on the interaction. We cannot exclude the possibility that other nucleotides may be identified as being important under different experimental conditions.

We cannot directly compare our results to those of Kahle *et al.* (16), because the concentration of enzyme, the ratio of enzyme:substrate, the ionic conditions, and the nature of the chemical modifiers were substantially different in the two sets of experiments. Nevertheless, we consider it significant that both our data and those of Kahle *et al.* (16) support the notion that nucleotides near the site of cleavage, as well as those along the top and at the corner of precursor tRNAs' three-dimensional structure, are required chemically intact in active substrates for RNase P RNAs.

ACKNOWLEDGEMENTS

This work was supported in part by NSF grant DMB-8804218 to TLM. DS gratefully acknowledges support from The James S. and Ada Bickman Summer Science Research Internship for

Undergraduates at Clark University. DLT sincerely acknowledges continued support from the department of Chemistry at Clark University.

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