ABSTRACT Using precursor tRNA molecules to study RNA-protein interactions, we have identified an RNA motif recognized by eukaryotic RNase P (EC 3.1.26.5). Analysis of circularly permuted precursors indicates that interruptions in the sugar-phosphate backbone are not tolerated in the acceptor stem, in the T stem-loop, or between residues A-9 and G-10. Prokaryotic RNase P will function with a minihelix consisting of the acceptor stem connected directly to the T stem-loop. Eukaryotic RNase P cannot use such a minimal substrate unless a linker sequence is added in the gap where the D stem and anticodon stem-loop were deleted.

Molecules of tRNA are synthesized as precursors, which, in turn, are converted to mature tRNA by a series of enzymatic reactions (1). The endonuclease RNase P (EC 3.1.26.5) is responsible for the generation of the 5' termini of mature tRNA molecules from their precursors (2). A variety of evidence indicates that, in both prokaryotes and eukaryotes, a single enzyme can cleave several precursors. Therefore, the enzyme must be cleaving directly or indirectly with features that are shared by all the precursors. The precursors probably present a tRNA-like tertiary structure in the mature domain of the molecule, even when an intervening sequence is present. Calculations of free-energy minima (3) and the use of chemical and enzymatic structure-specific probes (4) suggest that all of the precursor tRNAs (pre-tRNAs) (pre-tRNAs) have a common tertiary structure (5, 6). In this structure, the tRNA portion of the precursor maintains the L-shaped conformation that is stabilized by the interactions between the D and TWC loops.

We previously described a highly purified preparation of RNase P from Xenopus laevis oocyte nuclei (germinal vesicles) (7). The enzyme requires magnesium ions, and the end groups produced during the cleavage reaction are 5'P and 3'OH (8).

How is cleavage site specificity determined? Because the lengths of the leaders and their sequences are not, in general, conserved among different pre-tRNAs (9, 10), the main cleavage site determinants must be in the mature domain (11, 12).

To obtain information on the features of the pre-tRNA precursors recognized by Escherichia coli RNase P, McClain et al. (13) produced mutant precursors that lack specific domains of the normal tRNA sequence. The smallest tRNA precursor that was cleaved efficiently retained only the helical segment of the amino acid acceptor stem and the T stem-loop. The implication of these results is that the reduced substrate contains determinants for precursor recognition and cleavage.

Can a smaller substrate be defined for the eukaryotic enzyme?

MATERIALS AND METHODS

pre-tRNA Circularization. The yeast pre-tRNA(Pho) gene and the variant with an A-U base pair insertion in the pre-tRNA acceptor stem (AUV acc.s. pre-tRNA(Pho)), both of which were placed under the control of the bacteriophage T7 promoter, were assembled from a set of 10 synthetic oligodeoxynucleotides (14). The T7 promoter/pre-tRNA(Pho) gene constructs were inserted into the Pst I/BamHI site of the vector pUC13. Templates were prepared from the cloned wild-type and AUV acc.s. pre-tRNA genes by using the restriction enzyme BstNI so that the transcripts ended with 5'-CCA-3'. Transcription by T7 RNA polymerase was carried out in a volume of 40 mL containing 40 mM Tris-HCl (pH 8.0), 6 mM MgCl2, 10 mM dithiothreitol, 2 mM spermidine, each rNTP at 500 mL, 25 mM 5'-GMP, 0.1 mL [a-32P]UTP (800 Ci/mmol; 1 Ci = 37 GBq), 180 units of T7 RNA polymerase (Pharmacia), and 40 units of RNAsguard (Pharmacia) at 37°C for 60 min. The 5-fold excess of 5'-GMP over GTP was included in the reaction to generate RNA with 5'-monophosphate. The reaction products, after extraction with phenol and precipitation with ethanol, was purified on a denaturing 10% polyacrylamide gel, eluted, and precipitated with ethanol. Circularization of linear RNA molecules (15) was carried out in 50 mM Tris-HCl, pH 7.5/10 mM dithiothreitol/15% (vol/vol) dimethyl sulfoxide/200 mM ATP/1 unit of T4 RNA ligase per mL (Pharmacia) at 37°C for 120 min at an RNA concentration of 0.5–1 mL. The reaction mixture was extracted with phenol and precipitated with ethanol, and circular RNA was purified from unreacted linear RNA on a denaturing 10% polyacrylamide gel. Circular pre-tRNA (cir pre-tRNA) molecules were eluted and ethanol precipitated. The RNase P cleavage assay was performed as previously described (7).

Circular Permutation Analysis (CPA). DNA templates were transcribed by T7 RNA polymerase, and the pre-tRNAs were purified. To introduce a unique 32P label at the RNase P cleavage site, the transcript was dephosphorylated and labeled with 32P by using T4 polynucleotide kinase and [γ-32P]ATP (3000 Ci/mmol; Amersham). After labeling, the precursor was precipitated with ethanol and repurified on a 10% polyacrylamide/4 mL urea gel. The RNAs were circularized by incubating them at a concentration of 0.5–1 mL with T4 RNA ligase (15). Alkaline hydrolysis was carried out with 0.7 mL cir pre-tRNA(Pho) in 50 mM NaHCO3, pH 9.0, at 90°C for 1 min, the products of the reaction were precipitated with ethanol in the presence of 20 μL of glycerol per mL, and the precipitate was washed twice with 70% ethanol. The RNase P cleavage reaction was performed as described (7). For the wild-type pre-tRNA(Pho) the size of the products can be determined by comparing them to alkaline hydrolysis products and endoribonuclease T1 digests of linear pre-tRNA(Pho) labeled at the 5' end at the RNase P cleavage site. On the other hand, products from the cir pre-tRNA(Pho) AUV acc.s. variant were electrophoresed alongside the alkaline hydrolysis products of the 3'
end-labeled control. The combined analysis of the two different CPAs allows for the precise identification of the bases of the tRNA molecule affected by RNase P cleavage.

**RNA Structure Analysis.** Structure-probing reactions of 32P 5' end-labeled precursors (5000 cpm) under non-denaturing conditions were performed at 37°C in 10 μl of JB buffer [10 mM Heps, pH 7.4/7 mM MgCl2/0.1 mM EDTA/2.5 mM dithiothreitol/10% (vol/vol) glycerol] containing 70 mM NH4Cl (JB70) (7) by using 0.025 unit of endoribonuclease T1 (guanosine specific) (BRL), 0.5 unit of *Bacillus cereus* endoribo-
 nuclease (uridine and cytosine specific) (Pharmacia), and 20-40 units of endonuclease S1 (no known sequence specificity) (Pharmacia). Reaction mixtures with endonuclease S1 contained 1 mM Zn2+ in JB70. In all reaction mixtures 4 μg of carrier tRNA was added. Times of incubation were 1 min for endoribonuclease T1, 3 min for *B. cereus* endoribo-
 nuclease, and 3 min for endonuclease S1. Reactions were extracted with phenol and precipitated with ethanol in the presence of 20 μg of glycin per ml. The samples were loaded on an 8 M urea/16% polyacrylamide gel. The RNA sequences were determined by partial digestion with alkali and, under fully denatured conditions, with endoribonuclease T1 and *B. cereus* endoribo-
 nuclease (16). The specific cleavage sites in the structure-probing reactions were assigned by comparing the mobilities of the nucleotide fragments with those produced by base-specific sequencing reactions and alkaline hydrolysis.

**Small Model Substrates.** The pre-tRNAs were generated by in vitro T7 RNA polymerase transcription of 2.5 pmol of double-stranded DNA templates made by annealing and ex-
tending complementary pairs of synthetic oligodeoxynucleoti-
des. The oligonucleotides (50 pmol) of each were annealed for 5 min at 80°C and extended with 500 units of Moloney murine leukemia virus reverse transcriptase (GIBCO/BRL) in 50 μl containing 50 mM TrisHCl (pH 8.3), 75 mM KCl, 10 mM dithiothreitol, 3 mM MgCl2, and each dNTP at 300 μM at 37°C for 60 min. The reaction mixture was phenol extracted and ethanol precipitated.

**RESULTS**

**The Minihelix Consisting of Acceptor Stem Plus T Stem–Loop Is Not a Substrate.** We produced the mutant AT derived from yeast pre-tRNA Phe (Table 1) lacking the extended anti-

codon stem and the D stem and loop (Fig. 1). In the AT minihelix, nucleotide residue U-7 is joined directly to C-68. AT is practically unreactive with *X. laevis* RNase P (Fig. 2, lanes 1 and 2). We also tested the equivalent deletion mutant (termed AT-1) originally shown by McClain *et al.* (13) to be a substrate for the prokaryotic enzyme; this RNA was not cleaved by the eukaryotic enzyme (data not shown).

**Determination of Domains That Are Not Important for Interaction with RNase P.** At this point we returned to a full-length substrate to determine if there are domains that are not important for interaction with the eukaryotic enzyme.

We performed CPA (15) on wild-type cir pre-tRNA and on cir pre-tRNA Phe AUU accs. (Fig. 1A), which is cleaved by RNase P one nucleotide downstream of the wild-type cleavage site (8). CPA permits one to determine which of the possible circularly permutated linear precursor molecules can be cut by RNase P. In CPA a unique position in the circular sequence is labeled with 32P; in our experiments this was the phosphate on the 5' side of nucleotide G-1. These circles were subjected to partial alkaline hydrolysis to create a collection of linear molecules with uniform representation of each permutation. The collection was treated with RNase P, and the permutations that formed poor substrates were visualized as “holes” in the oligonucleotide ladder after electrophoresis. RNase P cleavage of the wild-type precursor molecules left the 32P on the 5' end of the products, whereas cleavage of the AUU molecules left the 32P on the 3' end of the products, facilitating the CPA while providing confirmatory results. Fig. 1A presents a summary of CPA results: the nonpermissive sites are all located in the acceptor stem, in the T stem–loop, and between A-9 and G-10.

To verify the results of CPA we constructed two individual isomers, the first starting with G-18 (wild type, G-18 start) and the second with G-76 (wild type, G-76 start). As expected on the basis of CPA results, we observed that the isomer starting with G-18 is cleaved by RNase P with kinetics indistinguishable from those of the wild-type precursor; the isomer starting with G-76, on the contrary, is severely affected (Fig. 1B). It is remarkable that a large number of circularly permuted pre-

RNA Phe molecules are still cleavable by RNase P.

**Cleavage of a Precursor That Contains a Large Deletion.** The results of CPA encouraged attempts to define a smaller substrate. We therefore decided to prepare pre-tRNA with internal deletions of the domains that did not appear by CPA to be crucial for activity. Mutant ADT lacks nucleotides C-28 to G-61 (Fig. 1A and Table 1) but is a good substrate (Fig. 2, lanes 8 and 9). The $K_m$ and $k_{cat}$ relative to the wild-type substrate (wild-type: $K_m = 0.13 \times 10^{-8}$ M, $V_{max} = 3.5 \times 10^{-15}$ mol/min) are 1.5 and 2.0, respectively. This result indicates that the extended anticodon stem is not required for RNase P action. Yuan and Altman (17) have recently presented evidence indicating that the anticodon stem is not required for the action of human RNase P.

**Small Model Substrates for RNase P.** The results suggested that a set of nucleotides should be introduced to replace the part of the molecule that had been removed from ADT to form AT. We produced a series of variant substrates that represent the insertion of various linker sequences at the site of the internal deletion. Fig. 3A shows that AT-1a (Table 1) is a better substrate than AT (relative $K_m$ and $k_{cat}$ for AT-1a are 8.0 and 0.05, respectively). The same is true for AT-1b, AT-1c, and AT-1d (data not shown). An even more notable improvement takes place when two nucleotides are added (AT-2) (Fig. 3A). AT-3, AT-4, AT-5a, and AT-9a (Fig. 3A) represent increasingly efficient substrates (relative $K_m$ and $k_{cat}$ for AT-5a are 1.9 and 0.9, respectively). These results show that the addition of up to nine nucleotides increases substrate activity, as might have been expected given the size of the interphosphate gap. For optimal results the replacement sequence must be added

<table>
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<th>Mutant precursor</th>
<th>Linker</th>
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<td>AT-1a</td>
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<tr>
<td>ADT</td>
<td>UAGCCUGAGUGGAAGCGGCAGGCAGGUC</td>
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The inset shows the position (between U-7 and C-68) where the linkers were inserted.

Fig. 3B shows that not only the number of nucleotides but also their identities are important for substrate activity. For example, AT-5b is not as efficient a substrate as AT-5a.

The secondary structures of AT and selected derivatives were investigated by 5' end-labeling with [γ-32P]ATP and partial digestion with endonuclease T1, which cleaves preferentially after Gp in single-stranded regions, with endonuclease S1, which also cleaves in the single-stranded region, though apparently without any base specificity, and with B. cereus endonuclease, which cleaves in single-stranded regions preferentially after Up and Cp. Fig. 4A shows that there is only one sensitive region, in the T loop of AT. The derivatives are cleaved not only in the T loop but also in the region of the linker (Fig. 4 B–D). These results indicate that the linker region cannot be helical.

**DISCUSSION**

Folded RNA molecules, like proteins, have an interior and an exterior (18), despite striking differences between RNA and protein secondary structures. RNA secondary structure involves hydrogen bonding between the bases to form double-helical regions, leaving the repetitious backbone on the outside. The helical elements of the RNA must specifically pack together to form the tertiary structure by utilizing hydrogen bonding in base triples and in backbone interactions and divalent cations such as Mg^{2+}. In this scenario dominated by the individual secondary structural elements, it may often be possible to attribute discrete aspects of RNA function to a subset of the helices.

We report in this paper that the mutant AT, deleted of anticodon and D stem–loops, is not a good substrate for the eukaryotic RNase P enzyme and a set of nucleotides needs to be introduced into the gap to obtain an active precursor. The number of residues of the linker is important; the addition of up to nine nucleotides increases substrate activity. Although the sequence of the added linker is important, there is no absolute requirement for a specific sequence.

What is the role of the linker? The inserted sequences could provide functional groups for enzyme binding or permit an optimal structure of RNA, perhaps reproducing the slight
bend between acceptor- and T-helix axes that is seen in the x-ray structure of tRNA\(^{\text{Phe}}\).

The kinetic parameters suggest that the interactions of the wild-type precursor or a good reduced substrate like AT-5a with the enzyme are quite similar, but they may not be identical. It will be interesting to determine whether, for example, an insertion of a base pair in the acceptor stem is still tolerated in the small model substrate.

Recently an \textit{in vitro} selection strategy was used to isolate a substrate for elongation factor Tu (EF-Tu) from an RNA library where nine random nucleotides were inserted between A-7 and A-49 in the Ala-minihelix (19). After six rounds of enrichment, two groups of RNA were obtained that bound \textit{Thermus thermophilus} (EF-Tu) as well as does Ala-tRNA\(^{A_{\text{Ala}}}\).

One of these groups has the consensus sequence UNDUGACUY (where D = G, A, or T) in the randomized region. We have shown that the corresponding AT derivative with the linker sequence UGUUGACUC (AT-9b) is an excellent substrate for the \textit{Xenopus} RNase P. This suggests that the substrate recognition properties of the two very different enzymes, EF-Tu and RNase P, may be similar. A similar model substrate has been identified for human RNase P in which the identity of the nucleotide in a linker sequence of only one nucleotide plays an important role in the determination of cleavage efficiency (20). It is possible that the enzymes, in addition to recognizing specific bases, interact with particular structural features of the sugar-phosphate backbone, features that can be reproduced by the short linkers of our reduced substrates. Perhaps even smaller substrates can be discovered that provide these essential structural features.

pre-tRNA\(^{\text{Phe}}\) is also the substrate for another processing enzyme, the tRNA-splicing endonuclease. This latter enzyme, in contrast to RNase P, is unable to function efficiently on extremely reduced substrates (ref. 21; D. Gandini-Attardi, E. De Nicola, and G.P.T.-V., unpublished data). A minimal substrate for a presumably ancient RNA-based enzyme provides an interest-

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**Fig. 3.** Kinetic analysis of the RNase P cleavage of wild-type and various mutant precursors. Assays were performed and analyzed as described (8). (4) Wild type; \(\triangledown\), ADT; \(\blacktriangle\), AT-9b; \(\Delta\), AT-9a; \(\bullet\), AT-5a; \(\bigcirc\), AT-4; \(\triangle\), AT-3; \(\bigtriangleup\), AT-2; \(\bigstar\), AT-1a; \(\ast\), AT.

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**Fig. 4.** Structure-probing reactions of \(\text{\textsuperscript{32}}\)P 5' end-labeled mutant precursors. (A) AT; (B) AT-3; (C) AT-5a; and (D) AT-9a. C, untreated precursor; OH, alkaline hydrolysis; T1\(^{d}\), nuclease T1 digestion under denaturing conditions; T1, nuclease T1 digestion under native conditions; S1, nuclease S1 digestion under native conditions; Cer\(^{d}\), \textit{B. cereus} nuclease digestion under denaturing conditions; Cer, \textit{B. cereus} nuclease digestion under non-denaturing conditions; \(\bullet\), linker; \(\bigtriangleup\), T loop.
ing parallel and an interesting twist to the view emerging from diverse considerations (22–24) of an ancient, functional prototRNA corresponding to just the acceptor stem/T stem–loop half of modern tRNA.

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