Effects of 5′ Leader and 3′ Trailer Structures on Pre-tRNA Processing by Nuclear RNase P†

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ABSTRACT: Eukaryotic transfer RNA precursors (pre-tRNAs) contain a 5′ leader preceding the aminoacyl acceptor stem and a 3′ trailer extending beyond this stem. An early step in pre-tRNA maturation is removal of the 5′ leader by the endoribonuclease, RNase P. Extensive pairing between leader and trailer sequences has previously been demonstrated to block RNase P cleavage, suggesting that the 5′ leader and 3′ trailer sequences might need to be separated for the substrate to be recognized by the eukaryotic holoenzyme. To address whether the nuclear RNase P holoenzyme recognizes the 5′ leader and 3′ trailer sequences independently, interactions of RNase P with pre-tRNA^{Tyr} containing either the 5′ leader, the 3′ trailer, or both were examined. Kinetic analysis revealed little effect of the 3′ trailer or a long 5′ leader on the catalytic rate (k_{cat}) for cleavage using the various pre-tRNA derivatives. However, the presence of a 3′ trailer that pairs with the 5′ leader increases the K_m of pre-tRNA slightly, in agreement with previous results. Similarly, competition studies demonstrate that removal of a complementary 3′ trailer lowers the apparent K_I, consistent with the structure between these two sequences interfering with their interaction with the enzyme. Deletion of both the 5′ and 3′ extensions to give mature termini resulted in the least effective competitor. Further studies showed that the nuclear holoenzyme, but not the *B. subtilis* holoenzyme, had a high affinity for single-stranded RNA in the absence of attached tRNA structure. The data suggest that yeast nuclear RNase P contains a minimum of two binding sites involved in substrate recognition, one that interacts with tRNA and one that interacts with the 3′ trailer. Furthermore, base pairing between the 5′ leader and 3′ trailer hinders recognition.

Eukaryotic tRNA primary transcripts (pre-tRNAs) contain 5′ leader and 3′ trailer sequences, which must be removed to form mature tRNA molecules competent for aminoacylation. Synthesis of pre-tRNAs by RNA polymerase III is terminated by a polyuridine stretch at the end of the transcript, which becomes part of the 3′ trailer structure. Removal of the purine-rich 5′ leader of pre-tRNAs is accomplished by RNase P, a ribonucleoprotein metalloenzyme (*1*−*5*). In yeast, the steady-state pool of nuclear precursors suggests that RNase P cleavage precedes removal of the 3′ trailer by exo- or endonuclease activities (*6, 7*). Previous studies have demonstrated Watson−Crick base-pairing interactions between the purine-rich leader sequences and the polyuridine sequence of the 3′ trailer (*8*). The more stable stems between the leader and trailer sequences in naturally occurring yeast pre-tRNAs typically have mismatched bulges that prevent the formation of continuous extensions of the aminoacyl stem past the −1/+1 position (Figure 1). The −1 mismatch is important for RNase P activity; minor alterations that cause uninterrupted extension of the aminoacyl acceptor stem severely inhibit processing of the pre-tRNA in vitro and in vivo (*8*).

There are likely to be mechanisms that antagonize the stem structure in vivo, thereby making the separated 5′ and/or 3′ strands available for interaction with RNase P. One possible mechanism could be the binding of La protein to the 3′ poly-U[1] trailer (*9, 10*). In bacteria, both the 5′ leader and 3′ trailing sequences are thought to interact with the RNase P holoenzyme. The 3′ -CCA that is encoded in many bacterial pre-tRNAs immediately downstream of the aminoacyl stem interacts with an internal loop of the RNase P RNA subunit, helping to stabilize binding in the ribozyme reaction (*11, 12*). In addition, the protein subunit of bacterial RNase P has recently been shown to possess a single-stranded RNA binding cleft that recognizes the unpaired 5′ leader of bacterial pre-tRNAs (*13*−*15*). In eukaryotic nuclear pre-tRNAs, the 3′ terminal -CCA is not normally contained in the primary transcript, and the complementary sequence in the RNase P RNA P15 loop is also missing (*16*). If there is pairing between any 3′ trailing sequences in nuclear pre-tRNAs and the nuclear RNase P RNA subunit, it is not obvious and has not previously been shown to have a detectable positive effect on substrate recognition. Even in

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[1] Abbreviations: poly-A, poly-adenylic acid; poly-C, poly-cytidylic acid; poly-G, poly-guanylic acid; poly-U, poly-uridylic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EDTA, disodium ethylenediaminetetraacetate; Tris, tris(hydroxymethyl)aminomethane hydrochloride; SDS, sodium dodecyl sulfate; PEI, polyethylenimine.

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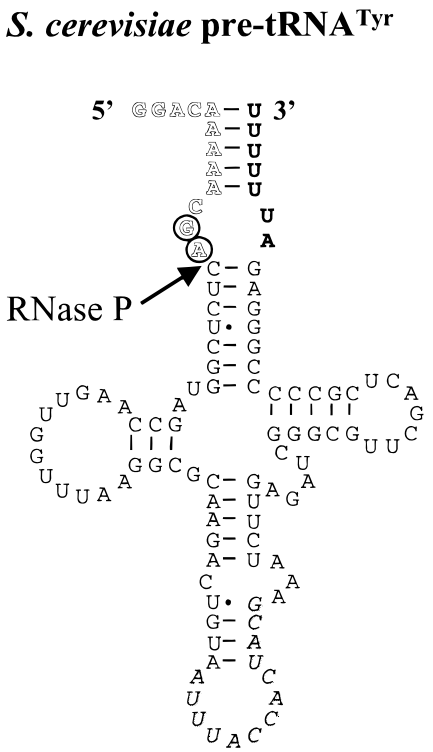


FIGURE 1: Structure of pre-tRNA^{Tyr}. The four experimental tRNA derivatives, pre-tRNA with leader and trailer (5′+, 3′+), product tRNA^{Tyr} with trailer (5′−, 3′+), pre-tRNA^{Tyr} without trailer (5′+, 3′−), and product tRNA^{Tyr} without trailer (5′−, 3′−), are based on the *S. cerevisiae* precursor tRNA^{Tyr} transcript. The 5′ leader structure is shown in outlined white letters, and the site of RNase P cleavage is indicated by an arrow. The truncated 5′ leader (2-Tyr) pre-tRNA^{Tyr} used in the k_{cat} and K_M determinations contained only the two circled nucleotides as a leader. The 3′ trailer is shown in boldface letters. The transcripts also contain an intron, denoted by italicized nucleotides. The 5′ leader and 3′ trailer interaction is shown as five A-U base pairs (*8*).

the bacterial enzyme, the effects of the -CCA/RNA subunit pairing are more pronounced in the ribozyme reaction (RNA-only) than when the holoenzyme is used (*12*).

In the absence of enzyme−substrate base pairing, it seems likely that the nuclear holoenzyme has the capacity to interact with multiple regions of the substrate. The yeast enzyme has 18 times more protein associated with the RNA subunit than is found in the eubacterial enzymes, and 7 out of the 9 protein subunits are quite positively charged (*17*). Thus, the nuclear holoenzyme could potentially interact with the 5′ leader and 3′ trailer separately. Sites of contact between the pre-tRNA substrate and the nuclear RNase P holoenzyme are currently unclear. As with the bacterial RNase P, multiple studies have shown that the tertiary structure of substrates, rather than specific sequences, is important for recognition (*18*−*23*). Unlike the bacterial ribozyme, however, the yeast nuclear holoenzyme does not easily recognize simple substrates that mimic only the coaxial aminoacyl/TΨC stems (*18, 24*; J. Y. Lee and D. R. Engelke, unpublished results). This suggests additional contacts in the D or anticodon arms might be required for efficient recognition.

To test the contribution of eukaryotic 5′ leader and 3′ trailer structures, their effects on recognition of a native substrate by *Saccharomyces cerevisiae* nuclear RNase P are investigated. In addition, the competitive binding of non-specific, single-stranded, and double-stranded RNA to the holoenzyme is tested. The results show that the single-stranded 3′ terminus plays a substantial role in substrate binding. Furthermore, it is shown that single-stranded RNAs can also efficiently inhibit the nuclear RNase P, but not the bacterial RNase P holoenzyme, in a partially sequence-specific fashion.

EXPERIMENTAL PROCEDURES

RNA Synthesis. The RNase P substrates and tRNA^{Tyr} derivatives used in the inhibition studies are transcribed from polymerase chain reaction products of the *S. cerevisiae* tRNA^{Tyr} gene (GenBank sequence accession number J01380). Wild-type pre-tRNA^{Tyr} with both leader and trailer is abbreviated by (5′+, 3′+), and pre-tRNA^{Tyr} minus the trailer is abbreviated by (5′+, 3′−). *S. cerevisiae* genomic DNA was used as the initial PCR template. Primers used to amplify the (5′+, 3′+) include forward primer A: 5′-GTAATACGACTCACTATAGGACAAAAACGACTCTCGGTAG-3′, and reverse primer B: 5′-AAAAATCTCCCGGGGGCGA-3′. Primers used to amplify the (5′+, 3′−) include forward primer A, and reverse primer C: 5′-CTCCCGGGGGCGAGTCG-3′. The amplification products of each PCR generate a DNA template containing the T7 RNA polymerase promoter. The templates were transcribed using the following conditions: 40 mM Tris, pH 8.1; 20 mM $MgCl_2$; 1 mM spermidine hydrochloride; 0.1% Triton X-100; 4 mM each of ATP, CTP, GTP, and UTP; and 20 mM dithiothreitol. The template DNA and T7 RNA polymerase were titrated to maximize transcript yield.

The tRNA^{Tyr} derivatives lacking a 5′ leader were generated by M1 ribozyme (*E. coli* RNase P RNA) catalyzed cleavage of the (5′+, 3′+) and (5′+, 3′−) pre-tRNA^{Tyr} transcripts. RNase P product tRNA with the trailer is abbreviated as (5′−, 3′+), and RNase P product tRNA minus trailer is abbreviated (5′−, 3′−). All four tRNA^{Tyr} derivatives were isolated from gel slices after denaturing gel electrophoresis, and further purified using DE52 ion exchange chromatography (Whatman). Figure 1 illustrates the mature domain, the 5′ leader (white letters), and the 3′ trailer (boldface letters) of the tRNA substrates and competitors.

A pre-tRNA^{Tyr} substrate (5′+, 3′+) with a reduced adenosine content in the 5′ leader was transcribed from a PCR product generated using forward primer D: 5′-GTAATACGACTCACTATAGGACtcActCGACTCTCGGTAGCCAAGTTGG-3′ and reverse primer B (described above) to give a pre-tRNA^{Tyr} template with a 5′ leader sequence marked by the underlined nucleotides. The lowercase letters in primer D indicate nucleotides that were formerly adenosines (Figure 1).

Five different RNAs were used as controls for nonspecific RNase P inhibition. *S. cerevisiae* 5S ribosomal RNA (120 nucleotide transcript) was prepared from T7 RNA polymerase transcription of a PCR-amplified DNA template that fused the T7 promoter to the 5S rRNA gene; transcription reaction conditions are described above. Poly-adenylic (poly-A), poly-cytidylic (poly-C), poly-guanylic (poly-G), and poly-uridylic (poly-U) homopolymers (Pharmacia-Upjohn) had heterogeneous lengths with the average being approximately 200 nucleotides. All RNAs were purified by DE52 ion exchange chromatography.

Kinetic analyses used three pre-tRNAs: (5′+, 3′+), (5′+, 3′−), and 2-Tyr. The 2-Tyr retains the 3′ trailer, but the 5′

Table 1: Kinetic Parameters

	(5′+, 3′+)	(5′+, 3′−)	2-Tyr[a]
k_{cat} (s^{-1})	1.3 ± 0.1	1.20 ± 0.07	1.14 ± 0.06
K_M (nM)	55 ± 10	9 ± 3	12 ± 4

[a] 2-Tyr = (5′+, 3′+) but with only a two nucleotide 5′ leader (Figure 1), and no pairing likely between leader and trailer sequences.

leader contains only two nucleotides (GA) (Figure 1). This substrate was designed to test whether, as with the bacterial holoenzyme, a longer 5′ leader enhances substrate affinity. The 2-Tyr pre-tRNA also eliminates interactions between the 3′ trailer and the full-length 5′ leader. For kinetic analyses (Table 1), these three pre-tRNAs were prepared by T7 RNA polymerase transcription as described above, but the GTP concentration was reduced to 0.4 mM, and 4 mM guanosine was added. The resulting 5′-hydroxyl of the guanosine in most transcripts was radiolabeled using [γ-^{32}P]ATP and T4 polynucleotide kinase (NEB) (*25*).

Internally labeled wild-type pre-tRNA^{Tyr}, used as substrate in the inhibition studies, was made by transcribing the (5′+, 3′+) DNA template as described above, but with only 0.1 mM UTP and including 100 μCi of [γ-^{32}P]UTP (NEN) at 3000 Ci/mmol. A 5′ end-labeled pre-tRNA without the 3′ trailer (5′+, 3′−), also used as a substrate for inhibition studies, was prepared by transcription in excess guanosine and then labeled using [γ-^{32}P]ATP and T4 polynucleotide kinase, as described above. End-labeled and internally labeled substrates gave identical results, within experimental error. Folding of radiolabeled and unlabeled substrate and inhibitor RNAs into predominantly single forms was monitored by electrophoresis through polyacrylamide gels under nondenaturing conditions.

Yeast RNase P Enzyme Preparation. Nuclear RNase P was purified from *S. cerevisiae* as previously described (*17*). Enzyme quantitation is based on RNA blot hybridization of a radiolabeled DNA oligomer complementary to *RPR1* RNA (yeast RNase P RNA), normalized to the signal from a known quantity of *RPR1* RNA synthesized in vitro.

Inhibition Reactions. RNase P activity was monitored using wild-type pre-tRNA^{Tyr} with (5′+, 3′+) or without (5′+, 3′−) the trailer sequence in the presence of variable concentrations of unlabeled competitor RNA, as indicated. Reactions were done in triplicate with conditions set to allow less than 20% cleavage. Substrate concentration was lower than K_M. Each reaction contained 10 mM HEPES, pH 7.8, 100 mM KCl, 10 mM $MgCl_2$, 15−20 nM internally or terminally radiolabeled pre-tRNA^{Tyr}, and 7−18 pM RNase P enzyme. Components were mixed, minus RNase P enzyme, and preincubated for 10−15 min. Enzyme was added; the reaction was mixed and then incubated for 0−10 min at 37 °C. Reactions were terminated by adding either 1.5 volumes of 95% formamide, 10 mM EDTA, 0.1% SDS, and 0.05% xylene cyanol and bromophenol blue, or 1 volume of 8 M urea, 200 mM EDTA, and 0.05% xylene cyanol and bromophenol blue. Reaction products were resolved by denaturing polyacrylamide gel electrophoresis. The percent product was determined by quantitation using a PhosphorImager (Molecular Dynamics), dividing the product quantity by the precursor plus product quantities. Dividing by the rate of product formation in the absence of inhibitor normalized each reaction rate.

Apparent inhibition constants (K_I's) were determined from curves fit to the titration data. Kaleidagraph software (Synergy) was used to fit the data to the following equation: normalized rate of product formation = $m1/(1 + [I]/[K_I]) + m3$, where $m1$ is the normalized activity in the absence of inhibitor (∼1), $m3$ is the activity extrapolated to saturating inhibitor, and $[K_I]$ is the concentration of inhibitor at 50% inhibition (*26*). In cases where the above equation does not fit the data, the data points are connected directly in the plots (Figures 3−5).

Kinetic Analysis. Steady-state turnover experiments were done in the presence of excess substrate ([S]/[E] > 5) in a reaction buffer containing 10 mM HEPES, pH 7.9, 100 mM KCl, and 10 mM $MgCl_2$. The pre-tRNA^{Tyr} derivative [either (5′+, 3′+), (5′+, 3′−), or 2-Tyr] was incubated in TE (10 mM Tris-HCl, pH 7.5, 0.1 mM EDTA) at 95 °C for 3 min prior to the addition of 2× reaction buffer and subsequent incubation at 37 °C for 15 min. Addition of RNase P initiated the cleavage reaction. At various times (10−30 s intervals from 0 to 6 min), an aliquot of the reaction mixture was diluted into an equal volume of stop solution, either 200 mM EDTA, pH 8, for the 2-Tyr substrate, or 200 mM EDTA, pH 8.0, 8 M urea, 0.05% bromophenol blue, and 0.05% xylene cyanol for (5′+, 3′+) and (5′+, 3′−). Reaction times were chosen so that ≤10% of substrate was cleaved (initial velocity conditions). Due to the 5′ γ-^{32}P on these tRNAs, only the leader and uncleaved substrate are detectable. For the 2-Tyr reactions, the 5′ leader product was resolved on polyethylenimine (PEI) cellulose thin-layer chromatography plates (Merck). After drying, the plates are soaked in methanol for 5 min to remove salts. The plates are then developed in 1 M LiCl (*27*). For (5′+, 3′+) and (5′+, 3′−), the substrate and 5′ leader product were separated on a 6% polyacrylamide/7 M urea gel. Quantitation was performed using a PhosphorImager with ImageQuant software (Molecular Dynamics).

Inhibition of Bacillus subtilis RNase P. The inhibition of either *B. subtilis* RNase P RNA or holoenzyme by the ribonucleic acid homopolymers (poly-A, poly-C, poly-G, or poly-U) was investigated by measuring pre-tRNA^{Tyr} cleavage at varying homopolymer concentrations (0−4 μM). The assays were determined at optimal reaction conditions with substrate concentrations lower than K_M (*5*, *25*, *28*). Ribozyme reactions contained 100 mM $MgCl_2$, 800 mM NH_4Cl, 50 mM Tris-HCl, pH 8, 0.1% SDS, 50 nM 5′ terminally radiolabeled pre-tRNA^{Tyr}, and 1.25 nM P RNA. *B. subtilis* holoenzyme reactions contained 10 mM $MgCl_2$, 100 mM NH_4Cl, 50 mM Tris-HCl, pH 8, 100 nM 5′ terminally radiolabeled pre-tRNA^{Tyr}, and 2.5 nM RNase P. The RNA components were incubated in TE (10 mM Tris-HCl, pH 7.5, 0.1 mM EDTA) at 95 °C for 3 min prior to the addition of 2× reaction buffer and subsequent incubation at 37 °C for 15 min to allow folding. Reactions were initiated by addition of enzyme (P RNA or RNase P holoenzyme). At time intervals (t = 0−90 s) before 10% of the substrate was cleaved, an aliquot of the reaction mixture was diluted into an equal volume of stop solution (200 mM EDTA, pH 8.0, 8 M urea, 0.05% bromophenol blue, and 0.05% xylene cyanol). The substrate and 5′ leader product were separated on a 6% polyacrylamide/7 M urea gel. Quantitation was performed using a PhosphorImager with ImageQuant software (Molecular Dynamics).

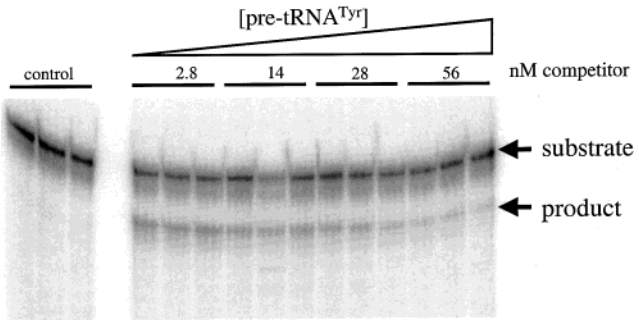


FIGURE 2: Denaturing polyacrylamide gel electrophoresis of radiolabeled pre-tRNA^{Tyr} reaction products. RNase P processing of radiolabeled pre-tRNA^{Tyr} was monitored in the presence of increasing concentrations of pre-tRNA^{Tyr} derivatives. An individual reaction consisted of 7 pM RNase P enzyme, 15 nM radiolabeled pre-tRNA, and increasing concentrations of the pre-tRNA derivative from 0 to 1 μM. Each derivative titration was done 3−4 times, with points taken in triplicate each time. The reaction was run for 10 min at 37 °C. Only 10−20% of the radiolabeled pre-tRNA, in the absence of competitor pre-tRNA derivative, was processed during the course of the reaction.

RESULTS

The Pairing between 5′ Leader and 3′ Trailer Hinders Recognition by RNase P. Kinetic analysis was used to determine effects of the 5′ leader and 3′ trailer structures on the Michaelis constant (K_M) and catalytic rate constant (k_{cat}) of yeast nuclear RNase P. To monitor turnover, three substrate pre-tRNA^{Tyr} derivatives were used. These included radiolabeled (5′+, 3′+), (5′+, 3′−), and 2-Tyr. The (5′+, 3′−) substrate eliminated the leader−trailer interaction by simply removing the trailer. This substantially reduced the K_M without affecting k_{cat} (Table 1). The 2-Tyr substrate contains the 3′ trailer structure, but only a two nucleotide leader that also eliminates the leader−trailer interaction. The 2-Tyr substrate also has a lower K_M. Therefore, the presence of paired 5′ leader and 3′ trailer sequences hinders the apparent binding of substrate, as evidenced by the larger K_M of (5′+, 3′+), and decreases the RNase P catalytic efficiency (k_{cat}/K_M) approximately 5-fold.

The Interaction of RNase P with tRNA Derivatives Is Enhanced by the 3′ Trailer. Four unlabeled tRNA^{Tyr} derivatives were prepared for use as competitors. These tRNA^{Tyr} derivatives either contained the native 5′ leader and 3′ trailer [i.e., (5′+, 3′+) substrate], were missing one of these [i.e., (5′+, 3′−) substrate and (5′−, 3′+) product], or lacked both [i.e., (5′−, 3′−) product], shown in Figure 1. The concentrations of these RNAs in a yeast nuclear RNase P reaction were titrated to compare their relative efficiencies at inhibiting cleavage of radiolabeled wild-type pre-tRNA^{Tyr} substrate. Quantitation and normalization using the rate of product formation in the absence of inhibitor followed electrophoretic separation of reaction substrates and products. An example of these data is shown in Figure 2. Inhibition by the (5′−, 3′+) and (5′−, 3′−) products and the 5S rRNA nonsubstrate control is shown in Figure 3as examples of plotted data. Apparent K_I values for all the tRNA^{Tyr} derivatives are listed in Table 2.

The apparent K_I values of the two substrates, (5′+, 3′+) and (5′+, 3′−), are larger than or equal to the K_M for each substrate (Table 1), as expected for a simple competitive binding mechanism. For a simple rapid equilibrium binding model, the decreased cleavage rate caused by competition with a second substrate is described by: rate = $k_{cat}/[1 + (K_{M1}/S_1)(1 + (S_2/K_{M2})]$. Furthermore, cleavage of the radiolabeled substrate is completely inhibited by the unlabeled substrate at high concentrations. Surprisingly, the (5′+, 3′−) pre-tRNA^{Tyr} substrate does not completely inhibit cleavage of the radiolabeled (5′+, 3′+) pre-tRNA^{Tyr}; at saturating (5′+, 3′−), the apparent inhibition curve extrapolates to a rate of cleavage of (5′+, 3′+) that is 5% of the uninhibited rate. To further illustrate this point, at 1 μM (5′+, 3′−), a concentration that is 45-fold higher than the K_I, 5% of the cleavage rate of radiolabeled (5′+, 3+) is still observed. These data demonstrate that the (5′+, 3′−) substrate cannot completely compete with (5′+, 3′+) binding to eukaryotic RNase P, suggesting the existence of a binding site for the 3′ trailer.

The two tRNA^{Tyr} product derivatives, (5′−, 3′+) and (5′−, 3′−), are also effective inhibitors with K_I's ∼ 30 nM. In this case, the apparent inhibition constant is unaffected by the presence of the 3′ trailer sequence (Table 2). Despite this similarity at low inhibitor concentrations, these RNase P product derivatives, (5′−, 3′+) and (5′−, 3′−), display strikingly different characteristics at high concentrations. The

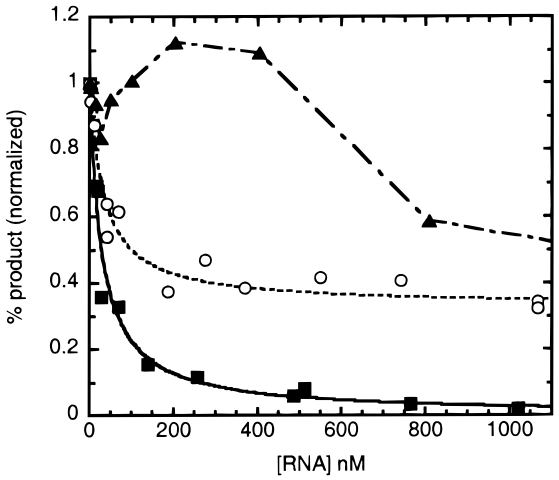


FIGURE 3: Inhibition of RNase P activity by tRNA derivatives and 5S rRNA. The ability of each tRNA derivative and 5S rRNA to inhibit cleavage of radiolabeled pre-tRNA^{Tyr} (5′+, 3′+) was determined by plotting the normalized percent of product formed versus the tRNA derivative concentration; (5′−, 3′+) is indicated by filled squares, (5′−, 3′−) by open circles, and 5S rRNA by filled triangles. Both (5′−, 3′+) and (5′−, 3′−) data are fit to the equation: $\nu = [k_{obs}/(1 + [I]/K_I)] +$ endpt. The 5S rRNA data are connected by stippled lines, with no fit implied.

Table 2: Competition

competitor	$K_{I,app}$(nM)	endpoint[a]
substrate		
(5′+, 3′+)[b]	53 ± 8	0
(5′+, 3′−)[b]	23 ± 3	5.3 ± 0.8
inhibitor		
(5′−, 3′+)[b]	29.4 ± 0.8	0
(5′−, 3′−)[b]	28 ± 7	31 ± 4
(5′−, 3′−)[c]	≤1.7	0
poly-G[b]	≤0.6	0
poly-U[b]	4.3 ± 0.8	0

[a] Endpoint = percent residual activity at high concentration of inhibitor as determined by the equation: $\nu = [k_{obs}/(1 + [I]/K_I)] +$ endpt. [b] Inhibition using (5′+, 3′+) as substrate. [c] Inhibition using (5′+, 3′−) as substrate.

product containing the 3′ trailer completely inhibits cleavage of radiolabeled (5′+, 3′+) pre-tRNA^{Tyr}. However, the tRNA product without the trailer, (5′−, 3′−), was unable to inhibit more than 70% of the RNase P-catalyzed cleavage of a radiolabeled substrate containing both leader and trailer sequences (5′+, 3′+), even at 1 μM, which is 36-fold higher than the apparent K_I (Table 2, Figure 3). Again, the 3′ trailer sequence is essential to completely compete with a pre-tRNA^{Tyr} substrate containing this sequence.

These data indicate that the trailer sequence enhances the binding of substrates and inhibitors. To further investigate this effect, the (5′−, 3′−) tRNA was titrated to determine if this product could block RNase P-catalyzed cleavage of a radiolabeled (5′+, 3′−) substrate. In fact, the (5′−, 3′−) tRNA completely inhibited processing of radiolabeled pre-tRNA^{Tyr} lacking a 3′ trailer (5′+, 3′−) with a much smaller inhibition constant ($K_I \leq 1.7$ nM, Table 2). This decrease in K_I for (5′−, 3′−) tRNA, which is dependent on the substrate structure, indicates that the observed K_I is not measuring simple inhibition due to the formation of an E·I complex. Further, these data demonstrate that an interaction between RNase P and the 3′ trailer sequence is responsible for the observed noncompetitive behavior. Models for such interactions are considered under Discussion. While we cannot formally exclude the possibility that the leader and trailer sequences are having minor, indirect structural effects, the more straightforward interpretation is direct interactions between the holoenzyme and these extensions.

Inhibition of RNase P Occurs with Single-Stranded, but Not Double-Stranded, Nonsubstrate RNA. The lack of specific sequence requirements in naturally occurring 5′ leader and 3′ trailer sequences suggests that RNase P might have nonspecific binding sites for single-stranded or double-stranded RNA. To test this for double-stranded RNA, we attempted to block RNase P with 5S ribosomal RNA, which is not a substrate for RNase P, but has a sufficiently tight tertiary structure so that few nucleotides are left unpaired. Synthetic 5S rRNA was generated from in vitro transcription and was used to determine nonspecific effects of double-stranded RNA on RNase P activity. Titration of unlabeled 5S exhibited only mild perturbations of RNase P activity (Figure 3). Initially, there is less than 20% inhibition at low 5S concentrations (2.5−100 nM) followed by stimulation of RNase P activity (200−400 nM). Above 400 nM 5S rRNA, the maximum inhibitory effect is less than 50%. The stimulation of RNase P activity, in the 200−400 nM range, was observed in several independent experiments, although the range of stimulation varied from 1.2-fold (Figure 3) to nearly 4-fold for unknown reasons. Comparing these results to those obtained with the tRNA product missing 5′ leader and 3′ trailer (5′−, 3′−), it is clear that the highly structured tRNA product is a better inhibitor. This is consistent with the likelihood that nuclear RNase P contains a binding site for specific recognition of the tRNA product domain tertiary structure.

The four poly-ribonucleic acid homopolymers were used as controls for the general effect of single-stranded RNA on RNase P activity. All four homopolymers inhibited RNase P cleavage to some extent (Figure 4). Unexpectedly, poly-G and poly-U were even more effective at blocking pre-tRNA cleavage than the RNase P substrates (5′+, 3′+) and (5′+, 3′−). Poly-A was a considerably less effective inhibitor than

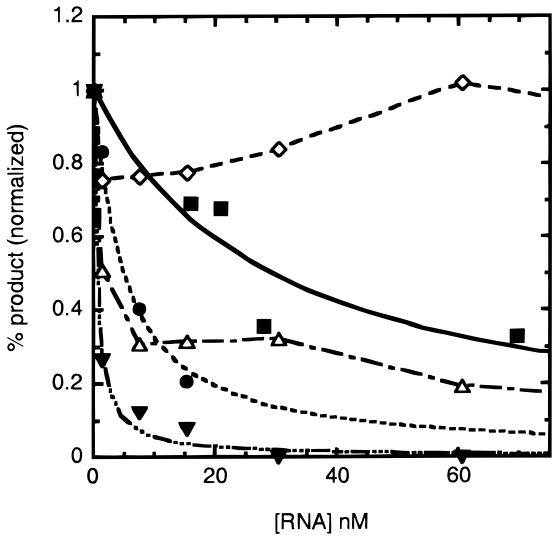


FIGURE 4: Ribonucleic acid homopolymer inhibition of RNase P. Cleavage of radiolabeled pre-tRNA^{Tyr} (5′+, 3′+) was assayed in the presence of increasing concentrations of homopolymers. Poly-A is indicated by open triangles, poly-C by open diamonds, poly-G by filled inverted triangles, and poly-U by filled circles. Inhibition by the tRNA derivative (5′−, 3′+) is shown in filled squares for comparison. Poly-G, poly-U, and (5′−, 3′+) data are fit to the equation: $\nu = [k_{obs}/(1 + [I]/K_I)]$ + endpt. Lines connect poly-A and poly-C data points, with no fit implied.

poly-G and poly-U, roughly equivalent to the (5′+, 3′+) pre-tRNA in successive experiments. Poly-C is much less inhibitory than the other homopolymers. There is a reproducible inhibitory effect of poly-C in the low nanomolar range, which is alleviated between 40 and 80 nM. Above 100 nM, poly-C gradually becomes inhibitory once again.

Poly-G and Poly-U Do Not Inhibit through Watson−Crick Interactions with the Radiolabeled Pre-tRNA Substrate. The potent inhibition of RNase P by poly-G and poly-U was unexpected, and explanations other than competitive inhibition were explored. Both poly-G and poly-U could hypothetically interact with the radiolabeled pre-tRNA substrate via Watson−Crick base pairing. Poly-U has the potential to base pair with the purine-rich leader, and poly-G could pair with the U-rich trailer. The homopolymers might act to sequester substrate molecules by annealing to the 5′ leader or 3′ trailer to form a complex that cannot be recognized by RNase P. To eliminate this possibility, poly-G was assayed for its ability to inhibit cleavage of radiolabeled (5′+, 3′−) substrate. The result, shown in Figure 5A, shows that poly-G inhibition is not dependent on the presence of the 3′ U-rich trailer of the RNase P substrate. Similarly, a radiolabeled pre-tRNA^{Tyr} substrate with a reduced adenosine content in the 5′ leader sequence was assayed for poly-U competition, to eliminate potential substrate−homopolymer pairing. Substitution of pyrimidines for most adenosines in the 5′ leader did not alleviate RNase P inhibition by poly-U (Figure 5B).

Homopolymers Do Not Inhibit the B. subtilis Holoenzyme. The *B. subtilis* ribozyme and holoenzyme were used to test if the inhibition by homopolymers is an intrinsic property of RNase P. Poly-A, poly-C, poly-G, and poly-U were examined as possible inhibitors of cleavage of the wild-type substrate (5′+, 3′+) catalyzed by the P RNA alone in high salt (100 mM $MgCl_2$, 800 mM NH_4Cl) under k_{cat}/K_M conditions. In the RNA-only ribozyme reaction, poly-U, poly-C, and poly-A at 4 μM inhibit the reaction <10%,

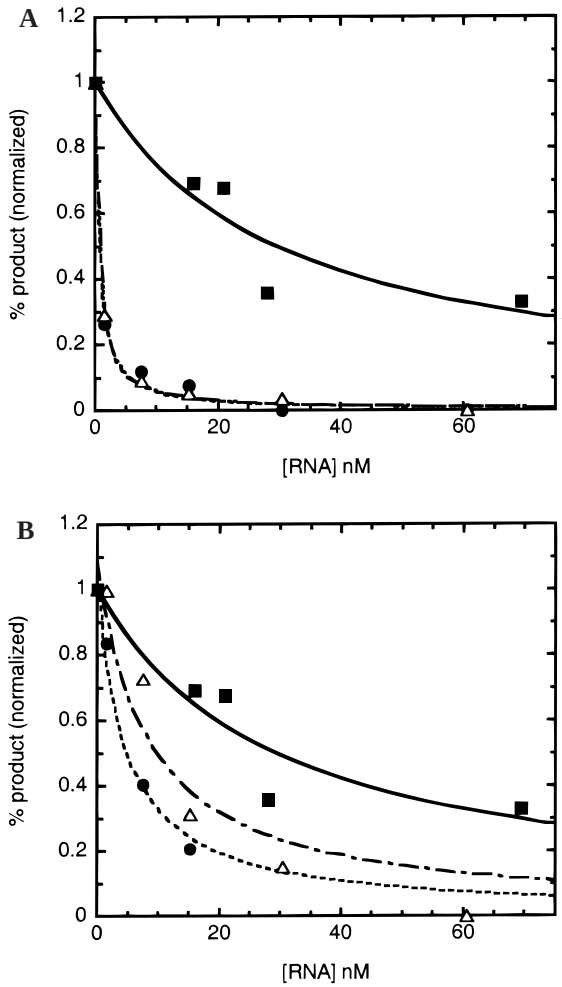


FIGURE 5: Poly-G and poly-U do not inhibit RNase P activity by interaction with the radiolabeled pre-tRNA^{Tyr} substrate. (A) Poly-G inhibition of RNase P cleavage. Poly-G with radiolabeled (5′+, 3′+) substrate is indicated by filled circles, with radiolabeled (5′+, 3′−) substrate indicated by open triangles. (B) Poly-U inhibition of RNase P cleavage. Poly-U inhibition using radiolabeled (5′+, 3′+) substrate is indicated by filled circles. Cleavage of radiolabeled (5′+, 3′+) containing a reduced adenosine leader is indicated by open triangles. Inhibition of RNase P by (5′−, 3′+) is shown on both plots as a reference. All data shown are fit to the equation: $v = [k_{obs}/(1 + [I]/K_I)] + \text{endpt}$.

<30%, <40%, respectively. Poly-G, on the other hand, completely inhibits the ribozyme-catalyzed cleavage with a K_I of 36 nM, only slightly higher than needed to inhibit the nuclear enzyme. This ribozyme inhibition is likely to be a specific interference of poly-G with folding of the ribozyme RNA, however, since the *B. subtilis* holoenzyme is only inhibited less than 40%, even at 4 μM poly-G. Thus, the nuclear holoenzyme is 1000-fold more sensitive to homopolymer inhibition than the bacterial holoenzyme. This specificity of inhibition for the nuclear RNase P suggests the homopolymers can be used as selective inhibitors of the eukaryotic enzyme.

DISCUSSION

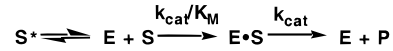
S. cerevisiae nuclear RNase P appears to have binding sites for both the tRNA structure and single-stranded RNAs, including the 3′ trailer structure and poly-ribonucleotide homopolymers. The inhibition studies suggest, not surprisingly, that tRNA^{Tyr} derivatives that are RNase P substrates compete well for RNase P cleavage. Inhibition of RNase P by the (5′-, 3′+) product further suggests that single-stranded RNA extending 3′ of the aminoacyl stem terminus enhances binding. Product tRNA lacking free single-stranded termini, (5′−, 3′−), is a relatively poor inhibitor (Figure 3), and 5S rRNA does not appear to block the tRNA structure recognition site of RNase P. The fact that tRNA is a significantly better inhibitor than the tightly structured 5S rRNA suggests that the tRNA product mature domain, lacking termini, does possess a measure of specific binding to nuclear RNase P.

Previous studies have shown that the formation of continuously base-paired stems between the 5′ leader and 3′ trailer sequences of yeast pre-tRNAs strongly inhibited recognition by nuclear RNase P (*8*). This was not surprising, given that tertiary structure models of the RNA subunit predict steric obstructions if the aminoacyl stem of the bound substrates is extended linearly without either bending the duplex extension or denaturing it into single-stranded 5′ leader and 3′ trailer (*29*, *30*). This second mode of binding is more consistent with the observed separate interactions of bacterial 5′ leaders and 3′ trailers with different parts of the bacterial holoenzyme. The present data show that the 3′ trailer sequence of the pre-tRNAs can interact with nuclear RNase P. The inhibitory properties of the tRNA product with a 3′ trailer are surprising, and this observation suggests that the eukaryotic enzyme is able to interact with the trailer structure.

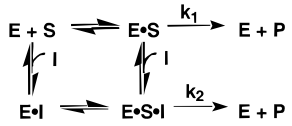
The steady-state kinetic data (Table 1) indicate that pre-tRNA^{Tyr} substrates containing 5′ leader and 3′ trailer sequences that hybridize have a 5-fold lower k_{cat}/K_M and K_M than the (5′+, 3′−) and 2-Tyr tRNA substrates. The second-order rate constant, k_{cat}/K_M, for (5′+, 3′−)-pre-tRNA cleavage catalyzed by nuclear RNase P is at or near the diffusion-controlled limit at 1.3×10^8 M^{-1} s^{-1} (*31*, *32*). This indicates that the association rate constant for substrate binding to enzyme is likely rate-limiting under these conditions, similar to *B. subtilis* RNase P (*25*, *33*; Day and Fierke, unpublished data). Therefore, the loss in k_{cat}/K_M caused by base-pairing of the leader/trailer sequences suggests that the substrate with denatured ends associates with RNase P more rapidly and, perhaps, k_{cat}/K_M correlates with the concentration of this form of the substrate (see Scheme 1). On the other hand, k_{cat}/K_M, is not affected by deletion of either the 5′ leader or the 3′ trailer sequences (Table 1). These data only demonstrate that neither of these sequences is required for the substrate binding step and do not indicate whether the substrate binding affinity is enhanced by the presence of these sequences. For example, favorable interactions between the 5′ leader and the protein component in *B. subtilis* RNase P are not observed in the steady-state kinetic parameters k_{cat}/K_M or K_M, since they do not reflect substrate affinity (*33*).

Bacterial RNase P contains a single, small protein subunit that enhances catalytic efficiency at low ionic strength. Crystallographic data of the *B. subtilis* protein subunit have identified the presence of a single-stranded RNA binding cleft (*13*). This has been recently confirmed by NMR analysis of the *S. aureus* P protein interacting with poly-U (*15*). Cross-linking between the pre-tRNA leader region and the P protein

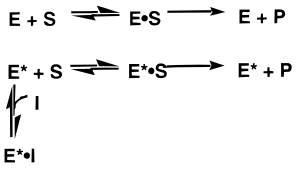
Scheme 1



Scheme 2



Scheme 3



in the bacterial holoenzyme has demonstrated that the single-stranded RNA binding cleft of the P protein is in close proximity to the pre-tRNA leader sequence (*14*). Furthermore, interactions between the pre-tRNA leader and the protein enhance the binding affinity of pre-tRNA (*27*). Therefore, binding of the leader sequence is a major function of the protein subunit in bacterial RNase P. Yeast nuclear RNase P holoenzyme has a larger and more diversified protein contingent. Nine protein subunits ranging from 15.5 to 100 kDa are essential components of the holoenzyme (*17*). Although none of these proteins share significant sequence homology with the bacterial P protein, one or more of the yeast proteins may similarly interact with the 5′ leader sequence.

As expected, unlabeled pre-tRNA substrates and tRNA product derivatives can compete with labeled wild-type pre-tRNA^{Tyr}. However, unexpectedly, in the absence of a 3′ trailer sequence, neither the (5′+, 3′−) substrate nor the (5′−, 3′−) product can completely inhibit the cleavage of the wild-type substrate. These data indicate that cleavage of the wild-type pre-tRNA substrate can occur even in the presence of saturating (5′+, 3′−) or (5′−, 3′−) tRNA derivatives. Additionally, the dependence of the value of the inhibition constant for (5′−,3′−) tRNA on the structure of the substrate (Table 2) indicates that inhibition of RNase P cannot be explained by the formation of an E·I complex where the product binds competitively to the substrate binding pocket. The simplest explanation for these data is the formation of a ternary complex where yeast RNase P can still catalyze wild-type pre-tRNA cleavage in the presence of bound (5′+, 3′−) or (5′−, 3′−) tRNA derivatives. One possible mechanism (see Scheme 2) consistent with the data is the formation of an E·S·I complex that can still catalyze pre-tRNA cleavage and dissociation, only with a decreased rate constant. The present data do not preclude the formation of an inactive E·I complex competitive with E·S. Similarly, the formation of any ternary complex (such as E·P·I) that is in equilibrium with the E·S complex would also fit the observed data. A second type of model (Scheme 3) consistent with the data is the existence of two forms of nonequilibrating enzyme where one species interacts with a 3′ trailer sequence and the other does not. Although there is no reason to believe that there are two forms of the enzyme in our preparations, we cannot formally exclude such a model.

All of these models require the presence of two types of binding sites in nuclear RNase P: one binding site that interacts with a 3′ trailer sequence and one binding site that is competitive with tRNA and pre-tRNA structures, regardless of the structure of the 3′ end. Therefore, these data suggest that yeast RNase P contains a minimum of two binding sites that interact with the substrate. These binding sites could reside either in the P RNA subunit, similar to the tRNA binding site in bacterial RNase P RNA (*34*), or in one or more of the protein subunits.

Double-stranded RNA molecules, such as 5S RNA, do not significantly inhibit RNase P, indicating specificity for tRNA in one or more of the yeast RNase P binding sites (Figure 3). However, single-stranded poly-ribonucleotide RNA homopolymers, except poly-C (Figure 4), have lower inhibition constants than the tRNA product derivatives and demonstrate some sequence specificity. It is not clear why poly-A, poly-G, and poly-U inhibit RNase P so effectively, but multiple contacts with the holoenzyme over significant lengths of RNA could be involved. Poly-A has a biphasic inhibition curve, reminiscent of inhibition by (5′−, 3′−) tRNA, suggesting that this poly-ribonucleotide might compete effectively for at most one of the two substrate binding sites. On the other hand, poly-U and poly-G completely inhibit RNase P with low K_I values. Therefore, these homopolymers must either compete for both RNase P binding sites or significantly decrease the activity of the ternary E·I·S complex compared to bound tRNA. This latter effect could be caused by interactions with RNase P outside of the substrate binding pockets. Yeast RNase P could potentially have numerous sites that bind RNA either specifically or nonspecifically so that long homopolymers can interact with several sites simultaneously to achieve stable binding. This does not appear to be a general property of RNase P; bacterial RNase P contains two main RNA binding domains: one on the P RNA subunit and one on the protein subunit (*13*, *14*, *34*). It is interesting to speculate that the increased catalytic efficiency of nuclear RNase P (1×10^8 M^{-1} s^{-1}) compared to bacterial RNase P (1×10^7 M^{-1} s^{-1}), despite the increased size and complexity, could be due to the presence of multiple sites on the 10 protein subunits that interact weakly with RNA substrates to facilitate diffusion of the substrate to the catalytic site of the enzyme.

An interesting corollary to these observations is that in vivo, the enzyme might need to be somehow protected from exposure to significant levels of free, unstructured RNAs. It is not known at this time whether this protection is achieved by subnuclear compartmentalization (*35*) or exclusion at a more molecular level.

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