Structure and Function of Eukaryotic Ribonuclease P RNA

Steven M. Marquez,1 Julian L. Chen,2 Donald Evans,1 and Norman R. Pace1,*
1Department of Molecular, Cellular and Developmental Biology
University of Colorado at Boulder Boulder, Colorado 80309
2Department of Chemistry and Biochemistry and School of Life Sciences
Arizona State University Tempe, Arizona 85287

Summary

Ribonuclease P (RNase P) is the ribonucleoprotein endonuclease that processes the 5′ ends of precursor tRNAs. Bacterial and eukaryal RNase P RNAs had the same primordial ancestor; however, they were molded differently by evolution. RNase P RNAs of eukaryotes, in contrast to bacterial RNAs, are not catalytically active in vitro without proteins. By comparing the bacterial and eukaryal RNAs, we can begin to understand the transitions made between the RNA and protein-dominated worlds. We report, based on crosslinking studies, that eukaryal RNAs, although catalytically inactive alone, fold into functional forms and specifically bind tRNA even in the absence of proteins. Based on the crosslinking results and crystal structures of bacterial RNAs, we develop a tertiary structure model of the eukaryal RNase P RNA. The eukaryal RNA contains a core structure similar to the bacterial RNA but lacks specific features that in bacterial RNAs contribute to catalysis and global stability of tertiary structure.

Introduction

Ribonuclease P (RNase P) removes 5′-leader sequences from precursor tRNAs and occurs in all three phylogenetic domains: Bacteria, Archaea, and Eukarya. The enzyme is universally composed of both protein and RNA moieties. The RNase P RNAs are all homologs, of common ancestry, but they have undergone substantial evolutionary diversification. The RNA moiety from bacterial and some archaeal holozymes can process precursor tRNA in vitro in the absence of proteins. In contrast, catalysis by the eukaryal RNase P requires multiple protein component(s) in addition to the RNA component. Insights into how the evolutionary process transformed a primordial ribozyme into a ribonucleoprotein can be gained by comparing the bacterial and eukaryal RNA components of RNase P.

The eukaryal RNase P holozyme differs from the bacterial and archaeal versions in both protein composition and RNA structure. In vivo, the bacterial RNase P RNA is associated with a single protein. The archaeal and eukaryal holozymes are more complex and contain at least four and nine proteins, respectively (Chamberlain et al., 1998; Hall and Brown, 2002; Jarrous, 2002; Kouzuma et al., 2003). In the case of human RNase P, two proteins in addition to the RNA are sufficient for activity in vitro (Mann et al., 2003). It is not known how these proteins contribute to RNase P activity.

Secondary structure models for the eukaryal RNase P RNA have been increasingly well developed through phylogenetic comparisons of diverse eukaryotic RNAs, and a core structure shared with bacterial RNAs is evident (Chen and Pace, 1997; Frank et al., 2000; Marquez et al., 2005; Pitulle et al., 1998; Tranguch and Engelke, 1993). The eukaryal RNA seems to be a more rudimentary version of the bacterial RNase P RNA. The typical eukaryotic RNase P RNA is roughly two-thirds the sequence length of the typical bacterial RNA. Although sequence conservation among the eukaryal RNAs is low, it includes distinct sequence elements, conserved regions (CRs) I–V, found in all archaeal and bacterial RNAs.

Eukaryal RNase P RNAs clearly are homologs of the bacterial RNAs and consequently are expected to have function and structure in common with those other RNase P RNAs. However, investigation of eukaryal RNase P RNA function in vitro has been limited by the lack of catalytic activity of the RNA alone. In this study, we used photoaffinity crosslinking to investigate the function of the eukaryal RNase P RNA and its global structure. Photoaffinity crosslinking has previously been used to map the active site of bacterial RNase P RNA and to generate distance constraints for inference of the RNA tertiary structure (Burgin and Pace, 1990; Chen et al., 1998; Harris et al., 1994; Nolan and Pace, 1996). The results reported here indicate that several eukaryal RNase P RNAs are capable of binding tRNA specifically and independently of protein, albeit with low affinity. This suggests that the eukaryal RNAs intrinsically possess information sufficient to fold into a structure similar to that of the bacterial RNAs independently of protein. We verify this with intramolecular crosslinking experiments. Data from crosslinking results and the recent bacterial RNA crystal structure were used to infer a specific tertiary structure model for the eukaryotic RNase P RNA. The model indicates that the eukaryal RNase P RNAs lack key elements in the active site and lack stabilizing helices important for proper structure.

Results

5′- and 3′-Arylazido Mature tRNA Crosslinks to Eukaryal RNase P RNA

Photoaffinity crosslinking was used to assay eukaryal RNase P RNAs for the capacity to bind tRNA. The photoagent was attached to the 5′-phosphate of mature tRNA because that is the phosphate acted upon by RNase P and so it is expected to be delivered to the active site of the RNase P RNA. The bacterial RNase P RNAs bind mature tRNA almost as tightly as precursor tRNAs (Reich et al., 1988), so we reasoned by homology that the eukaryal RNA would do so as well, if capable of specific binding. 32P-labeled mature Bacillus subtilis tRNAAsp was synthesized by transcription in the presence of guanosine 5′-monophosphorothioate (GMPS),
Figure 1. Intermolecular Crosslinking Analysis of Eukaryal RNase P RNA-tRNA Conjugates
(A) 5'-aryladzo-B. subtilis mature tRNA<sup>thio</sup> crosslinks to eukaryal RNase P RNA. All reactions are identical, with the following exceptions, given for each lane: (1) the reaction contained <i>E. coli</i> RNase P RNA but was not exposed to UV, (2) the thio-containing tRNA was not coupled to the azidophenacyl bromide, (3) no RNase P RNA was added, (4) RNA complementary to <i>E. coli</i> RNase P RNA was included instead of <i>E. coli</i> RNase P RNA, (5) the reaction contained <i>H. sapiens</i> RNase P RNA, (6) the reaction contained <i>C. elegans</i> RNase P RNA, (7) the reaction contained <i>D. melanogaster</i> RNase P RNA, (8) the reaction contained <i>S. cerevisiae</i> RNase P RNA, (9) the reaction contained 5'-aryladzo-B. subtilis mature tRNA<sup>thio</sup> crosslinks to eukaryal RNase P RNA.
which is incorporated only in the first nucleotide position of the tRNA. An arylazide photoaffinity agent (azidophenacyl bromide) then was coupled to the unique 5’-phosphorothioate of the tRNA. The modified tRNA was incubated with RNase P RNAs from a broad diversity of eukaryotic species and UV irradiated to activate crosslinking. RNase P RNAs were in vitro transcripts from the corresponding genes from Homo sapiens, Caenorhabditis elegans, Drosophila melanogaster, Saccharomyces cerevisiae, Schizosaccharomyces pombe, and Acanthamoeba castellanii. Crosslinked products were separated from unreacted substrate by polyacrylamide gel electrophoresis as shown in Figure 1A.

In general, more slowly migrating bands correspond to RNase P RNA-tRNA conjugates. Among those RNAs tested for binding tRNA, crosslinked products were observed only with the S. pombe RNA and the Escherichia coli bacterial positive control. The extent of crosslinking in the case of the E. coli RNA was more than 10-fold greater than with the S. pombe RNA. It is possible that the other eukaryal RNAs do bind tRNA, but irradiation does not give rise to crosslinked products (see below). To verify that crosslinking occurred through the photoaffinity agent and not between random parts of the RNAs due to UV exposure alone, we used phenylmercuric acetate to dissociate the E. coli and S. pombe RNase P RNA-tRNA conjugates (Burgin and Pace, 1990); organomercurials specifically cleave sulfur linkages (Neumann et al., 1967). Upon incubation with phenylmercuric acetate, all conjugates dissociated into RNase P RNA and tRNA (data not shown). Thus, the crosslinked products were due to the arylazide group linked to the 5’ end of tRNA via sulfur.

We used the crosslinking assay to test some additional aspects of the binding reaction. We determined that precursor tRNA crosslinks to S. pombe RNase P RNA with approximately the same efficiency as mature tRNA (see Figure S1A in the Supplemental Data available with this article online), indicating that the 5’-leader sequence does not contribute significantly to the binding of tRNA. In order to determine whether S. pombe RNase P RNA discriminates between eukaryal and bacterial tRNAs, a photoaffinity agent was placed at the 5’ end of S. cerevisiae 32P-labeled mature tRNA\textsuperscript{Amp} and then crosslinked to S. pombe RNase P RNA. S. cerevisiae tRNA\textsuperscript{Amp} crosslinked to S. pombe RNase P RNA with the same efficiency as B. subtilis mature tRNA\textsuperscript{Amp} (Figure S1B). These data indicate that eukaryotic RNase P does not discriminate between bacterial and eukaryal tRNAs.

The eukaryotic RNase P RNAs require cations to bind tRNA. When monovalent and divalent salts were omitted from the crosslinking reactions, no crosslink products were detected (data not shown). A titration of increasing monovalent salt (NH\textsubscript{4}\textsuperscript{+}), 0–5.0 M, with the divalent salt (Mg\textsuperscript{2+}) concentration constant at 25 mM, gave identical crosslink products and yielded for each reaction. A titration of increasing divalent salt (Mg\textsuperscript{2+}), 0–1.0 M, with the monovalent salt (NH\textsubscript{4}\textsuperscript{+}) concentration constant at 3.0 M, also gave identical crosslink products and yield for each reaction (data not shown). Thus, in the presence of minimum cation concentrations, binding is fairly insensitive to ionic strength. Binding of the tRNA by S. pombe RNase P RNA does not depend on Mg\textsuperscript{2+}, as is also the case with bacterial RNase P RNA (Beebe et al., 1996; Smith et al., 1992).

In addition to the arylazide group, which is 9 Å in length, we employed in crosslinking experiments another, shorter photoaffinity agent, 6-thioguanosine (s\textsuperscript{6}G) (1.5 Å) (Favre et al., 1998). This photoagent was incorporated during transcription, site specifically at the 5’ end of the tRNA. The resulting tRNA was submitted to crosslinking analysis with the same RNase P RNAs as above. As with the 5’-azido-labeled tRNA, crosslinked products were observed only with the E. coli and S. pombe RNAs (Figure S2).

We also analyzed the ability of the various eukaryotic RNase P RNAs to bind tRNA modified to contain an arylazido photoaffinity agent (4-azidobenzoyl) at the 3’ terminus (Oh and Pace, 1994). As a control, parallel crosslinking experiments were carried out with E. coli RNase P RNA, which resulted in products identical to those previously reported (Oh and Pace, 1994). No conjugates occurred in the absence of RNase P RNA or photoaffinity agent. Although the extent of crosslinking to the eukaryotic RNAs was very low, apparent conjugate bands nonetheless occurred with the H. sapiens, C. elegans, D. melanogaster, and S. pombe RNAs, but not with the S. cerevisiae RNA (Figure 1B). The reaction with the A. castellanii RNA did not yield defined bands, for unknown reasons. The observation that 3’ photoagent-labeled tRNA formed crosslinks with most eukaryal RNase P RNAs tested, whereas the 5’ photoagent tRNA does not, suggests that the positioning of the 5’ end of tRNA in these cases was unstable or too distant from the RNase P RNAs to crosslink. The only 3’ conjugate with sufficient material for use in mapping crosslinked sites was that of S. pombe.

To identify the tRNA crosslink sites in the S. pombe RNase P RNA, primer extension analyses were contained S. pombe RNase P RNA, and (11) A. castellanii RNase P RNA was included. The S. pombe RNase P RNA-5’-arylazido tRNA conjugates were analyzed by primer extension. Unlabeled S. pombe RNase P RNA-tRNA conjugates were prepared, purified, and quantified as described in Experimental Procedures. Lanes 1 and 2 contain primer extension products using oligonucleotides 150R and 250R, respectively. Lanes C, U, A, were analyzed by primer extension. Unlabeled (B) 32P-labeled mature tRNA was more than 10-fold greater than with the S. pombe RNA. It is possible that the other eukaryal RNAs do bind tRNA, but irradiation does not give rise to crosslinked products (see below). To verify that crosslinking occurred through the photoaffinity agent and not between random parts of the RNAs due to UV exposure alone, we used phenylmercuric acetate to dissociate the E. coli and S. pombe RNase P RNA-tRNA conjugates (Burgin and Pace, 1990); organomercurials specifically cleave sulfur linkages (Neumann et al., 1967). Upon incubation with phenylmercuric acetate, all conjugates dissociated into RNase P RNA and tRNA (data not shown). Thus, the crosslinked products were due to the arylazide group linked to the 5’ end of tRNA via sulfur.

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To identify the tRNA crosslink sites in the S. pombe RNase P RNA, primer extension analyses were performed in the presence of increasing amounts of S. pombe RNase P RNA (0–2.0 × 10\textsuperscript{-5} M) incubated with 32P-labeled 5’-arylazido mature tRNA (228 nM). • represents the fraction of tRNA bound to S. pombe RNase P RNA as assayed by radioactivity in the crosslinked band. The data were fit to the binding isotherm equation in Experimental Procedures.
S. pombe RNA revealed nucleotides where reverse transcriptase paused or terminated spontaneously, presumably due to structure in the RNA template (Lane et al., 1985). Pauses not seen in the unmodified S. pombe RNase P RNA are true crosslinks. Because reverse transcriptase cannot traverse the crosslink site, the last nucleotide incorporated is interpreted as one nucleotide prior to the actual site of the crosslink (Ehresmann et al., 1987).

All the crosslinking data for S. pombe RNase P RNA are summarized in Figure 1C. Crosslinks occurred in CRs II and IV and in regions of the eukaryotic RNA that are not conserved in the bacterial RNAs. The crosslinking into these CRs suggests the functional importance of the interaction (Burgin and Pace, 1990; Harris and Pace, 1995; Kazantsev et al., 2005; Pagan-Ramos et al., 1996). Primer extension analysis of the only resolvable conjugate formed between the S. pombe RNase P RNA and 5'-s6G tRNA indicated that three of the four premature terminations (A202, A207, and C212) were identical to those found when 5'-azidophenacyl tRNA was used for crosslinking. The unique termination site was at A139. The reason the 5' and 3' crosslink sites are in close proximity to each other likely is because the 5' and 3' ends of tRNA are adjacent to one another in the tRNA acceptor stem. These results indicate that eukaryal RNase P RNAs, although catalytically inactive, specifically bind tRNA in the absence of proteins. The crosslink sites in the eukaryal RNA are similar to those seen with the bacterial RNA, near the eukaryotic homolog of the bacterial active site (see below).

**Binding Affinity of S. pombe RNase P RNA for Mature tRNA**

We used the crosslinking assay to evaluate the dissociation constant for the complex between the S. pombe RNase P RNA and 5'-arylazido mature tRNA. A constant concentration of [32P]-labeled 5'-arylazido tRNA was incubated with different concentrations of unlabeled S. pombe RNase P RNA and then irradiated with UV. The resulting complexes were resolved by denaturing polyacrylamide gel electrophoresis as above. We assume that crosslinking occurs at equilibrium, so the crosslinked conjugate is expected to be proportional to the concentration of the RNase P RNA-tRNA complex at the time of UV exposure. Crosslinking reactions were conducted in triplicate at each RNA concentration, and the data were in good agreement with the binding isotherm equation shown in Figure 1D. We extract from the data a dissociation constant (K_d) of \( \sim 2.2 \mu M \). The binding is specific: nonlabeled mature tRNA competes with 5'-arylazido tRNA, while unlabeled antisense E. coli RNase P RNA is unable to compete (data not shown). This means that the eukaryal RNase P RNA is capable of part of the catalytic cycle, binding of substrate.

**Eukaryotic RNase P RNAs Are Structurally Less Stable than Bacterial RNAs**

The eukaryotic RNase P RNAs typically lack hairpin elements that in bacteria are known to contribute to the stability of the tertiary structural folding of the RNA. This suggests that the global packing of the...
Figure 3. Intramolecular Crosslinking Analysis of Circularly Permuted S. pombe RNase P RNAs cp69, cp118, cp140, and cp242
(A) Uniformly $^{32}$P-labeled photoagent-containing cpRNAs without (UV−) or with (UV+) 302 nm UV irradiation were analyzed by gel electrophoresis and autoradiography. The crosslinking reactions contained 1.0 μM photoagent-containing RNAs. The cpRNA crosslink reactions resulted in crosslinked bands, designated x1, x2, x3, etc.

(B) Primer extension mapping of crosslinked RNAs was carried out with 5'-32P-labeled oligonucleotides complementary to S. pombe RNase P RNA (Supplemental Experimental Procedures). Primer extension mapping of some crosslinked RNAs indicated that they were circular molecules and uninformative (data not shown). Lanes C, U, A, and G correspond to sequencing reactions with noncrosslinked RNA template, and lane N is a control primer extension without dideoxynucleotides of unmodified S. pombe RNase P RNA. The primer extension reactions using the crosslinked species are indicated above each lane. The termination sites of primer extension are indicated to the right of the gel. The RNA sequence of the termination sites is shown on the left. Filled circles denote the actual crosslink sites which are one nucleotide 5' to the primer extension termination sites.

(C) Crosslinking sites in the RNA secondary structures are shown. The boxed G indicates the photoagent attachment site located at the 5' end of the cpRNA. The circled bases in the secondary structure represent the corresponding crosslinking sites. Arrowheads indicate the direction of the crosslinks.
Figure 4. Commonalities in Structure and Function between the Crystal Structure of Bacterial RNase P RNA and the Modeled Eukaryal RNase P RNA

(A) Coaxial stack representation of the secondary structures of \textit{B. stearothermophilus} and \textit{S. pombe} RNase P RNAs. The RNA that is homologous between \textit{B. stearothermophilus} and \textit{S. pombe} RNase P RNAs is represented as blue; nonhomologous RNA is colored gray; and RNA not represented in the \textit{B. stearothermophilus} crystal structure is colored black in both molecules. Arrows indicate the 5' to 3' direction. Sites of 5' tRNA crosslinking are represented as red spheres. \textit{B. stearothermophilus} long-range tertiary interactions between helices are indicated by dashed lines, while homologous interactions are lacking in \textit{S. pombe} RNA. The main site of 3' tRNA crosslinking (P15) in \textit{B. stearothermophilus} RNA is colored gold. A main site of 3' tRNA crosslinking in \textit{S. pombe} RNA is the P3 bulge loop, which, while not homologous to bacterial P15, is also colored gold.

(B) (Crystal Structure)

(C) (Model)
eukaryotic RNAs might be less stable than that of bacterial RNase P RNAs, perhaps the cause of the inactivity of the eukaryotic RNAs. To examine the conformations of the eukaryotic RNase P RNA compared to the bacterial RNAs, we used native polyacrylamide gel electrophoresis. In the conditions of 100 mM NH₄⁺ and 0, 0.5, 1.0, and 5.0 mM Mg²⁺, all RNAs migrated mainly as single bands (Figure 2A), indicating hydrodynamic and, thereby, conformational homogeneity under the particular conditions. However, the eukaryotic and bacterial RNAs respond differently to elevated Mg²⁺, as summarized in Figure 2A. The bacterial RNAs display an increase in relative mobility, an apparent compaction, between 0 and 5.0 mM Mg²⁺. This would be consistent with a tightening of tertiary structure as Mg²⁺ ions counteract electrostatic repulsion due to the phosphodiester chain. In contrast, none of the eukaryal RNAs undergo appreciable change in electrophoretic mobility at higher Mg²⁺ concentration (Figure 2A). This would be consistent with the notion that the eukaryal RNAs cannot adopt the stable tertiary structure equivalent to their bacterial homologs and so are not amenable to further compaction. Thus, the Mg²⁺-independent electrophoretic properties of the eukaryal RNAs may reflect lack of assembly of the core helices to form the tertiary structure that in the bacterial RNAs is stabilized by hairpin elements not present in the eukaryal RNAs.

We additionally tested the relative thermal stabilities of the eukaryotic RNase P RNAs compared to their bacterial homologs by using temperature gradient gel electrophoresis (TGGE). With this method, RNAs electrophoretically migrate perpendicular to a linear temperature gradient. When RNA structure changes from the folded structures (Kazantsev et al., 2005; Pan et al., 1997). The azidophenacyl crosslinking agent was attached to the 5′ end of the cpRNA. Six circularly permuted S. pombe RNase P RNAs, cp69, cp118, cp140, cp210, and cp242, (e.g., cp60 signifies that the 5′ end of cp60 begins at native nucleotide 60) were made for site-specific crosslinking analysis. These photoagent-attachment sites in the S. pombe cpRNAs are homologous to those previously subjected to similar analysis with the bacterial RNAs. The introduction of internal nicks in RNAs usually has no impact on the folded structures (Kazantsev et al., 2005; Pan et al., 1991). In our study, the cpRNAs migrated as single bands in native gels (100 mM NH₄⁺ and 1.0 mM Mg²⁺) and retained the capacity to bind tRNA in the crosslinking assay (Figure S4). cpRNAs therefore adopt the functional structure.

Intramolecular crosslinking experiments were conducted under conditions determined to be optimal for intermolecular crosslinking reactions (see above). Two classes of crosslinks form in the intramolecular crosslinking reaction: circular and lariat molecules (Harris et al., 1997). Circular molecules, usually the most slowly migrating bands in gels, result from crosslinking from the photocrosslinking to the nearby 3′ end of the same RNA molecule. Mapping of these crosslink sites provides distance constraints with which to orient the structural elements of the S. pombe RNA. As in the S. pombe RNA, we used circularly permuted RNAs (cpRNAs) specific sites within the sequence of the S. pombe RNase P RNA.
The eukaryal RNase P RNA forms a self-folded core structure similar to that of the bacterial ribozyme.

A few results were not useful or differed between the bacterial and eukaryal RNAs. For instance, crosslinking of cp60 resulted only in local crosslinks within the P3 bulge loop, providing no useful structural information. Unexpectedly based on the bacterial structure, when the photoagent is located in CR II (cp140) it forms crosslinks to nucleotides in CR I and to nucleotides in the internal loop of P3 (Figure 3C). These results would indicate that CR II and the internal loop of P3 are in close proximity to CR I. However, compared to the crystal structures of Thermotoga maritima and B. stearothermophilus RNase P RNAs, CR II and CR I are not within...
crosslinking distance of each other (see Discussion) (Kazantsev et al., 2005; Torres-Larios et al., 2005). Nonetheless, the similarities of most of the bacterial and eukaryal crosslinking results, and the clear homology of the RNAs, justify specification of a working model for the tertiary structure of the S. pombe RNase P RNA based on the crystal structure of the bacterial RNA from B. steatornerophilus (Kazantsev et al., 2005).

Computer Modeling of S. pombe RNase P RNA

A model of the tertiary structure of the S. pombe RNA was inferred by homologous replacement and is shown in comparison to the bacterial structure in Figure 4. Seventy-two percent of the nucleotides in the S. pombe RNA could be reliably modeled from the alignment of the B. steatornerophilus and S. pombe RNase P RNA sequences. The accuracy of the homologous modeling approach has been confirmed by the correspondence of comparative and crystal structures for 16S and 23S rRNAs (Gutell et al., 2002). Comparison of the bacterial and eukaryal RNase P RNA structures shows that both share a similar core structure, but the eukaryal RNA lacks peripheral helices known to stabilize the bacterial RNA structure (e.g., interactions between P5.1 and P15.1 in the B. steatornerophilus RNA, and P14 and P8 in the E. coli RNA), as well as nucleotides known to be important for binding and catalysis (e.g., B. steatornerophilus and E. coli, L15) (Brown et al., 1996; Darr et al., 1992; Kirshebom and Svard, 1994). In addition, the internal loop of P3, missing in bacterial RNAs, lies near the bacterial P15, which does not occur in the eukaryotic RNAs. Of the 45 nucleotides that comprise the B. steatornerophilus RNase P RNA catalytic core, shown in Figure 5, at least 37 homologous nucleotides are found in the S. pombe RNA. Of the 13 base pairs identified in the B. steatornerophilus core, all are present in the S. pombe RNA as well. Although the sequences in these base pairs vary, compensatory base substitutions found in the S. pombe RNA should not perturb the tertiary structure in the bacterial model because they are isosteric with the bacterial base pairs (Leontis et al., 2002). The only possible variation to isostericity is S. pombe A249G:74, which is a C:G pair in B. steatornerophilus (Figure 5). However, this P4 base pair also is likely isosteric, because P4 is a highly conserved helix and the homologous base pairs in other eukaryal indeed are canonical Watson-Crick base pairs. The structural relationships of three nucleotides in J4/1 of the S. pombe RNA (A253, U254, and C255) are uncertain and therefore are not paired in the model. However, because of positional homology with the comparable bacterial nucleotides, they likely form the same structure seen in the bacterial RNA.

Discussion

In this study, we used photoaffinity crosslinking to investigate the structure and function of eukaryotic RNase P RNA. We show that the eukaryal RNAs, although catalytically inactive under conditions in which bacterial RNase P RNAs are active, are capable of specifically binding tRNA. We additionally show that the S. pombe RNase P RNA adopts a near-native conformation in the absence of proteins. Utilizing this information as well as the recently solved bacterial RNase P RNA structure, we have modeled the structure for a eukaryal RNase P RNA. The model allowed us to see, in detail not previously possible, some likely reasons for the inactivity of the eukaryal RNA alone and suggests possible roles for the required protein subunits.

The S. pombe RNase P RNA structure model (Figure 4) can be interpreted functionally by homology with the bacterial structure. In bacterial as well as eukaryal RNase P RNAs, structural elements thought to be involved in catalysis include P4, CR I, CR IV, and CR V. These elements constitute the phylogenetically conserved structural core and face the substrate. In bacterial and archaeal RNase P RNAs, hairpin structures are attached to the phylogenetically conserved core and dock elsewhere to form struts that stabilize the global conformation of the RNA (Pace and Brown, 1995). An example of such an interaction is between P5.1 and P15.1 in Bacillus-type RNAs, shown in Figure 4. Comparable stabilizing structures are absent from the eukaryal RNAs. Some nonconserved hairpin elements occur in some eukaryal RNase P RNAs; however, comparative studies indicate that these are not homologs of the stabilizing helices found in bacterial RNAs (Marquez et al., 2005). It is evident from the crystal structure of the bacterial RNA, and the eukaryal model derived from it, that structural elements attached to the core are positioned to expand in length without distortion of the active core elements. Presumably, in eukaryal holoenzymes, one role of the multiple protein components is to assume structural functions that in bacterial holoenzymes are performed by RNA structural elements, for instance to stabilize the global structure.

We used native gel electrophoresis to investigate the effects of Mg²⁺ and temperature on the folding and stability of the eukaryotic RNase P RNA structures. As Mg²⁺ concentration is increased, eukaryotic RNAs undergo little change in electrophoretic mobility, whereas the bacterial RNAs undergo an increase in mobility consistent with further compaction of the global structures. Since Mg²⁺ generally stabilizes RNA structure, we interpret the influence of Mg²⁺ on the mobility of the bacterial RNAs compared to the eukaryal RNAs as a manifestation of structural ordering that the bacterial but not the eukaryal RNA can undergo independently of protein. One explanation for this difference in electrophoretic behavior would be the stabilizing influence of long-range interactions on the bacterial RNA tertiary structure and their absence in the eukaryotic version of the RNA. The thermal gradient gel results are consistent with the relative instability of the global tertiary structures of the eukaryal RNAs compared to the bacterial RNAs: the eukaryal RNase P RNA structures denature at lower temperatures than do the bacterial RNAs.

The less stable structures of the eukaryotic RNase P RNAs compared to the bacterial versions may result in increased potential for misfolding of the eukaryotic RNAs, which may explain a few minor disagreements in the crosslink data obtained with the eukaryal and bacterial RNAs. Inter- and intramolecular crosslinking results both place CR II near the 5’ end of tRNA and near CRs I, IV, and V. A photoagent in the homologous position in the bacterial RNA, near CR II, crosslinked only locally (N.R.P., unpublished data). Furthermore, in the
crystal structures of the bacterial RNase P RNAs CR II is not in proximity to CR I (Kazantsev et al., 2005; Torres-Larios et al., 2005). Additionally, the 5' end of tRNA crosslinks to S. pombe RNase P RNA in locations that are not supported by comparable bacterial crosslink sites (Figure 4A). It is possible that, without structurally stabilizing hairpins comparable to those in the bacterial RNAs, eukaryal RNAs are to some extent conformationally heterogeneous in the absence of proteins. This again would be consistent with a role for proteins in stabilization of parts of the molecule that in the case of the bacterial RNase P are stabilized by RNA structure.

An enzymatic reaction can be considered in essence as two steps: one, the binding of substrate; and two, chemical action. Clearly the eukaryotic RNase P RNA is capable of binding substrate; however, cleavage of the scissile bond does not occur. The clear homology between the eukaryal and bacterial RNase P RNAs is strong evidence that the eukaryotic RNA, like the bacterial RNAs, performs an essential role in the catalytic function of the holoenzyme. The structural model can address the failure of the eukaryal RNA to carry out the catalytic step. The S. pombe RNase P RNA contains most of the catalytic core found in bacterial RNAs as seen in Figure 5. Importantly, the only bases missing from the S. pombe RNase P RNA core compared to the bacterial catalytic core are the 3' flanking sequences (three nucleotides) of the bacterial helix P15 and nucleotides homologous to A255 and A256 (B. stearothermophilus numbering). In the bacterial RNA, mutants of the homolog of A255 show a significant decrease in binding affinity and catalysis. Additionally, nucleotide A255 in the bacterial RNA has been shown to interact with the base immediately 5' of the cleavage site of tRNA (Zahler et al., 2003). Mutations of A256 show little effect on catalysis but show defects in RNA structure near the active site. The S. pombe RNase P RNA catalytic core, while containing most of the catalytic core found in bacteria, clearly lacks bases thought to be critical for binding and catalysis in bacterial RNAs.

In addition, the S. pombe and other known eukaryotic RNase P RNAs lack a homolog of the bacterial P15 and L15, the binding site for the 3'-CCA of tRNAs and potentially an important part of the active site in the bacterial RNA (Kirsebom, 2002; Oh and Pace, 1994; Oh et al., 1998). In eukaryal RNAs, the homologous position is empty or occupied by a region that is particularly variable in length and sequence and is not homologous with the bacterial P15/L15. We used the 3' photoagent-labeled tRNA as a probe to identify residues in the eukaryal RNase P RNA that are in the vicinity of the 3' end of tRNA. The 3' end of tRNA crosslinks to the S. pombe RNA near the location of P15 in the bacterial RNA and to an eukaryote-specific structure, the bulge loop of P3 (Figure 1C). Notably, the 5' end of tRNA also crosslinks to this bulge loop. These results suggest that this eukaryote-specific bulge loop is near the active site of the RNA. In bacterial RNase P RNA, the 5' photoagent in tRNA crosslinks to P15/L15 (Burgin and Pace, 1990). According to the eukaryal tertiary structure model (Figure 4), the bulge loop in P3 is located near the position of the bacterial helix P15/L15. The absence of a bacterial P15 from the eukaryotic version nominally provides an empty space that could be occupied by the bulge loop in P3 and possibly a protein component in vivo (Ziehler et al., 2001).

The absence of the bacterial P15/L15, 3'-CCA binding site from the eukaryotic RNase P RNAs may account for some of the lack of affinity for tRNA, and it is unknown what role proteins may have in binding the substrate (Jarrous, 2002). In S. pombe, in vivo, the 3'-CCA sequences of the tRNAs are deposited after RNase P RNA cleaves the 5' end of pre-tRNA (Van Horn et al., 1997). If this order of processing is true for all eukaryotes, then the eukaryotic RNase P RNA may not require a structural homolog of the bacterial loop, at least for binding CCA (Dubrovsky et al., 2004).

Eukaryal RNase P RNA homologs are expected to possess commonalities in function and structure with the bacterial RNase P RNAs. These common structures must have been established early in evolution and have persisted in all domains of life, throughout time. Our results show that the eukaryal core contains the requisite information, independently of protein, to determine the near-native fold for the RNase P RNA, and to bind substrate, the first step in catalysis. The eukaryal and bacterial RNase P RNAs had common primordial ancestry; however, they followed different evolutionary paths, during the course of which the eukaryotic RNAs became dependent on protein for catalytic function. Our eukaryal tertiary structure model in comparison with the bacterial structure clearly identifies nucleotides and helical elements that in evolution were lost from the eukaryal RNA or never acquired. Instead, evolution of the eukaryal RNase P incorporated multiple proteins to maintain catalytic activity.

Experimental Procedures

Formation of RNase P RNA-Mature tRNA Conjugates
In the standard intermolecular crosslinking reaction, RNase P RNAs were folded prior to crosslinking: 100 ng of photoagent-containing mature tRNA (Supplemental Experimental Procedures) and 4 μg of RNase P RNA in 25 μl of 50 mM Tris-HCl (pH 8.0), 100 mM NH₄OAc, and 0.05% SDS were incubated for 10 min at 65°C, followed by adding MgOAc to 25 mM solution and incubating at 37°C for 15 min. Crosslinking reactions were performed essentially as described previously (Burgin and Pace, 1990). Crosslinking was done at 2 cm for 30 min. The crosslinking reaction products were ethanol precipitated and resolved on a 4% polyacrylamide-8 M urea gel. For analytical gels, the crosslinked products were detected by autoradiography and phosphorimager. For preparative gels, the crosslinked RNAs were excised from ethidium bromide-stained gels, eluted into elution buffer at 4°C overnight, and ethanol precipitated. RNase P RNA crosslinks to mature tRNA were cleaved with mercury essentially as described (Burgin and Pace, 1990).

Determination of Kd
To measure the dissociation constant, S. pombe RNase P RNA at various concentrations (5.0 × 10⁻⁷, 1.0 × 10⁻⁶, 2.0 × 10⁻⁶, 5.0 × 10⁻⁶, 1.0 × 10⁻⁵, and 2.0 × 10⁻⁵ M) and 32P-labeled aryldiazo mature tRNA (228 nm) were prepared as described, incubated together for 5 min at 37°C, and then placed on ice. The crosslinking reaction was performed as described above for the formation of RNase P RNA-mature tRNA conjugates. RNAs were resolved by electrophoresis through an 8% polyacrylamide-8 M urea gel. Following UV irradiation, each labeled crosslink band was quantified using a phosphorimager. Background was determined by measurement of radioactivity in a lane in the absence of RNase P RNA (cpm background). The fraction of tRNA bound ([P/C14 tRNA]/tRNA total) was calculated as (cpm crosslink band – cpm background) / (cpm tRNA total – cpm background), where cpm is radioactivity in counts per minute. We assume that crosslinking is much faster than the rate...
of equilibrium, so at equilibrium $[P_{\text{total}}] = [\text{crosslink band}]$, and the complex reacts to form a crosslink with efficiency $0 < \beta < 1$. Then, the data are fit to the hyperbolic binding isotherm weighted by the efficiency of crosslinking $(\beta)$: $[P_{\text{total}}] / [\text{RNA}]_{\text{total}} = \beta / (1 / [1 + K_d / [P_{\text{total}}])]$. The data were fit with the Kaleida Graph (Synergy Software, Reading, PA) curve-fitting program using the above equation.

Native and Temperature Gradient Gel Electrophoresis Assays

RNase P RNAs and cpRNAs were folded before native and TGGE experiments in 1× THE (66 mM HEPES, 33 mM Tris-HCl [pH 7.4], and 0.1 mM EDTA), 100 mM NH$_4$OAc, 10 mM MgOAc, and 0.05% NP40 for 10 min at 60°C followed by 20 min at 37°C. For native gel Mg$^{2+}$-titrations, samples were diluted 1:4 with loading buffer (1× THE, 13% sucrose, and 0.2% bromophenol blue) and ~200 nM RNA. The samples were loaded onto four 4.5% acrylamide 1× THE native gels containing 100 mM NH$_4$OAc and various concentrations of MgOAc (0.5, 1.0, and 5.0 mM). It was not necessary to dialyze the samples to the exact gel conditions because they equilibrated rapidly once in the gel. Electrophoresis running buffer contained 1× THE, 100 mM NH$_4$OAc, and the requisite MgOAc concentration. The running buffer was changed every hour. Each gel was run at constant voltage, 250V, and detected by autoradiography or phosphorimager. TGGE gel samples were treated the same as above, and the samples were loaded in 15 min intervals in the single well. TGGE gels, buffers, and running conditions were the same as above.

Computer Modeling

The crystal structure of B. stearothermophilus RNase P RNA, bacterial and eukaryal crosslinking data, and the constraints of secondary structure were used to develop the S. pombe RNA tertiary structure model. Nonhomologous helices and single-stranded regions of S. pombe RNA were constructed and minimized individually using the Discover modules of Insight II program (Accelrys) and Crystallography and NMR System (CNS). The S. pombe RNA model was then further refined with energy minimization using CNS. The S. pombe RNA model was visualized using the modeling software by MacPyMOL, W.L. DeLano, the PyMOL Molecular Graphics System (2002), on the World Wide Web at http://www.pymol.org.

Supplemental Data

Supplemental Data include four figures and Supplemental Experimental Procedures can be found with this article online at http://www.molecule.org/cgi/content/full/24/3/445/DC1/.

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References


Accession Numbers

The atomic coordinates have been deposited in the RNase P Database http://www.mbio.ncsu.edu/RNaseP (Brown, 1999) under the designation spo.pdb.