Articles

Contributions of Phylogenetically Variable Structural Elements to the Function of the Ribozyme Ribonuclease P†

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ABSTRACT: Ribonuclease P (RNase P) is a ribonucleoprotein enzyme which participates in processing precursor tRNAs. The RNA subunit contains the catalytic site and is capable of catalysis in the absence of the protein subunit. RNase P RNAs from various eubacteria consist of a core of conserved sequence and secondary structure which is evolutionarily modified in different organisms by the presence of discrete helical elements at various sites in the RNAs. The variable occurrence of these helical elements suggests that they have no important functional role in the enzyme. The *Escherichia coli* RNase P RNA contains four such elements. It has been shown that simultaneous deletion of all four of them produces an RNA that is functional but has several significant defects which could arise from general disruption of the RNA or from the loss of element-specific functions. This paper describes a more detailed analysis of the role of the variable elements in *E. coli* RNase P RNA. Removal of one of the elements had no apparent effect on RNase P activity in vitro. Two other elements are required for correct folding of the RNA: their absence confers a requirement for extremely high monovalent salt concentrations, apparently to reduce intramolecular electrostatic repulsion. The fourth element that was tested participates in a long-range structural interaction (pseudoknot) which contributes to the structural stability of the enzyme and affects substrate binding affinity. In the absence of this helix, the RNA becomes temperature-sensitive, and the K_M increases 100-fold. Thus, in RNase P RNA, and probably in other catalytic RNAs, phylogenetically variable structures may have important functions which are not predicted by simple phylogenetic secondary structure comparisons.

Ribonuclease P (RNase P) is a ribozyme composed of both RNA and protein subunits (Stark et al., 1978; Gardiner & Pace, 1980). It functions in tRNA synthesis by cleaving the 5′ leader of precursor tRNAs. The RNA subunit is catalytic: it binds substrate and catalyzes the enzyme reaction in vitro in the absence of the protein subunit (Guerrier-Takada et al., 1983). In contrast to the other major classes of ribozymes (i.e., group I introns, self-cleaving virusoids, etc.), RNase P is a true enzyme: it turns over catalytically in vivo, rather than promoting only one round of self-cleavage (Cech & Bass, 1986). In addition, unlike most ribozymes, RNase P does not appear to utilize base-pairing to bind its substrate. Further insight into the mechanisms of substrate recognition and catalysis will require knowledge of the structure of this unique enzyme.

A secondary structure model for RNase P RNA was proposed on the basis of phylogenetic covariation of base-paired nucleotides (James et al., 1988). This model predicted that RNase P is composed of a central core of conserved primary and secondary structure, which differs in various eubacteria by the presence or absence of discrete helical elements (compare the *Escherichia coli* and *Bacillus megaterium* structures in Figure 1). In order to test this model, a simplified RNase P RNA (Min 1) was constructed (Waugh et al., 1989). Four phylogenetically variable hairpin elements comprising 30% of the *E. coli* RNase P RNA were deleted or replaced with homologous sequences from *B. megaterium* RNase P RNA (Figure 1). Min 1 RNA was active (Waugh et al., 1989), demonstrating that the structure model had successfully predicted the central core of the molecule and that the phylogenetically variable structures were not absolutely essential for activity. However, Min 1 RNA differs from the native RNA in several ways: (1) Min 1 requires extremely high concentrations of monovalent salt for full activity, possibly to quell destabilizing internal electrostatic repulsions. (2) The K_M of Min 1 RNA for precursor $tRNA^{Asp}$ is 100-fold higher than that of the native RNA. (3) Min 1 RNA is more sensitive to temperature than native RNase P RNA. (4) Min 1 RNA does not respond to the RNase P protein subunit.

The differences in the properties of Min 1 and native RNase P RNAs are intriguing: it is expected that phylogenetically variable features would not contribute substantially to the function of otherwise structurally homologous enzymes from different organisms. Furthermore, structures which are totally absent in some examples of the enzyme (see *E. coli* regions 1, 3, and 4 in Figure 1) would be expected to have no general function. The observation that deletion of the four structures affects enzyme function seems most consistent with two possibilities: (1) Loss of each of the four helical elements results in an incremental, nonspecific destabilization of the molecule; or (2) the four variable regions have specific functions which are not predicted by the phylogenetic comparisons.

In this paper, we have analyzed the effects of each of the four deletions on the *E. coli* RNase P RNA in order to determine if the variable helical elements have a general or a specific role in the function of RNase P RNA. We found that the deleted structures have specific functions and that the

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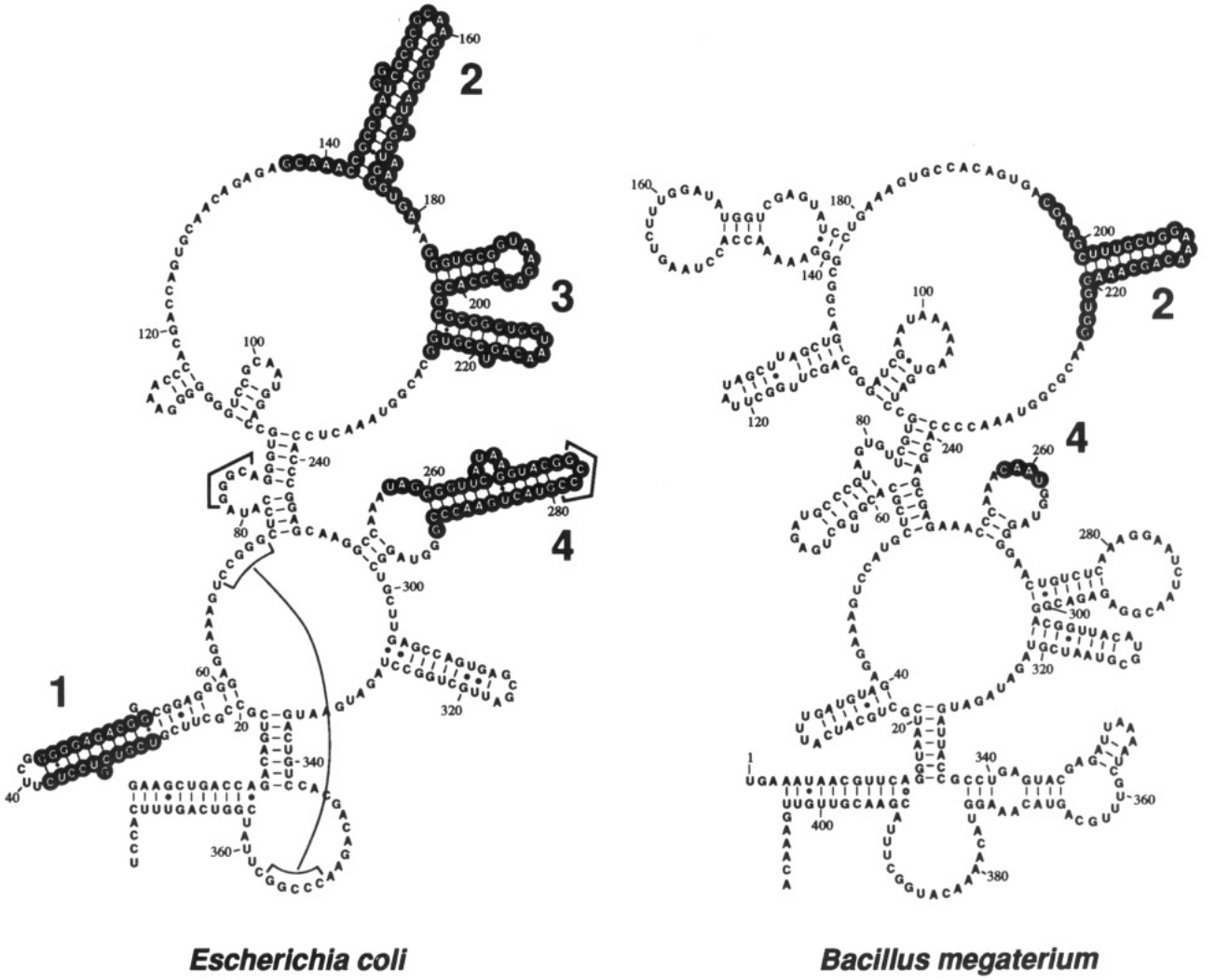

FIGURE 1: RNase P RNAs from *E. coli* and *B. megaterium*. The regions that were deleted from the *E. coli* RNase P RNA are highlighted and numbered. Their presence in RNase P RNAs is phylogenetically variable. In the case of regions 2 and 4, the highlighted nucleotides from *B. megaterium* RNase P RNA replace the highlighted regions in *E. coli*.

characteristics of Min 1 described above result from the sum of the effects of each deletion. These results have important implications for the use of phylogenetic comparative analysis to predict the function of structural elements in catalytic RNAs.

Materials and Methods

Construction of Deletion Mutants. The deletions that were individually made in the *E. coli* RNase P RNA gene are indicated and numbered in Figure 1. In two cases, Δ2 and Δ4, sequence from the RNase P of the Gram-positive bacterium *B. megaterium* was substituted into the *E. coli* RNA to create the deletion. In addition to the indicated deletion for Δ1, U25 was changed to a C in order to increase the theoretical stability of the shortened helix.

Deletions and substitutions were constructed using the site-directed mutagenesis procedure of Kunkel (1985) as described (Sambrook et al., 1989). The Klenow fragment of DNA polymerase I was used for elongation of the mutagenic primer. The plasmid used for mutagenesis, pECP2, contains the *E. coli* RNase P gene downstream of a phage T_7 RNA polymerase promoter. To accomodate the T_7 polymerase promoter sequence, two G's are added preceding nucleotide 1 of the RNase P RNA sequence, and nucleotides 3 and 4 are changed from AG to UC. One of our deletion mutants, Δ3, was constructed in a different plasmid, pAB31, which does not change nucleotides 3 and 4, but adds seven nucleotides (GCGCGCC) to the 5′ end of the RNA. Transcripts of all the RNase P genes contain an additional seven nucleotides (GAUUUAC) at the 3′ end of the RNA. These nucleotide changes have no apparent effect on the activity of the RNAs. The insertion mutagenesis of Min 1 RNA utilized plasmid pDW133 (Waugh et al., 1989). RNase P RNA genes were transcribed in vitro using T_7 RNA polymerase as described (Reich et al., 1986; Burgin & Pace, 1990) and purified by polyacrylamide gel electrophoresis in the presence of 8 M urea.

Measurement of Enzyme Activity. Enzyme activity was measured at 37 °C in the presence of 50 mM *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid-NH_4OH (Hepes–NH_4OH), pH 8.0, 25 mM magnesium acetate, 0.05% Nonidet P-40, and the indicated concentration of ammonium acetate. Sodium dodecyl sulfate (0.1%) was included in most assays. ^{32}P-Labeled precursor $tRNA^{Asp}$ from *Bacillus subtilis* was transcribed in vitro from plasmid DW153 using T_7 RNA polymerase as described above. In order to assess the effects of salt, temperature, and the presence of the protein subunit on both substrate binding and catalytic aspects of the enzymes, all assays were carried out at $\leq 0.1K_M$ concentration of substrate. In all cases, enzyme concentrations and reaction incubation times were chosen so that steady-state rates were measured. To prevent substantial product inhibition, reaction conditions were adjusted so that less than approximately 30%

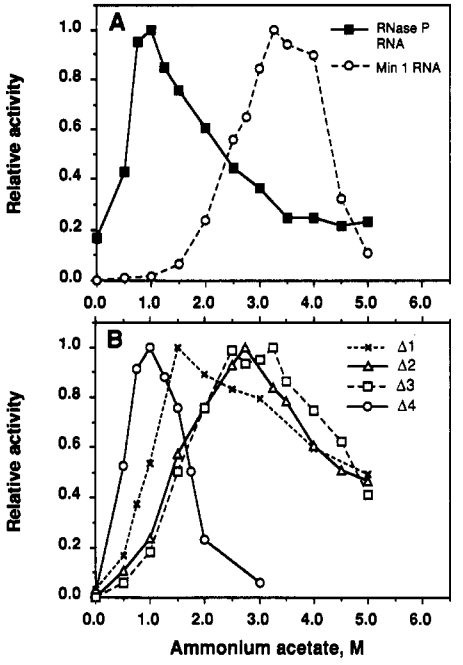

FIGURE 2: Effect of monovalent salt concentration on enzyme activity. (A) (■) Native RNase P RNA; (○) Min 1 RNA. (B) (×) Δ1 RNA; (△) Δ2 RNA; (□) Δ3 RNA; (○) Δ4 RNA. Enzyme activity in the presence of the indicated concentrations of ammonium acetate was measured at 37 °C. Results were normalized to the maximal value for each enzyme. The actual maximal rates of cleavage and the concentration of substrate and enzyme in each assay were the following: RNase P RNA, 18 pM/min, 5 nM substrate, 0.25 nM enzyme; Min 1 RNA, 4.9 nM/min, 0.5 μM substrate, 5.7 nM enzyme; Δ1 RNA, 5 pM/min, 2 nM substrate, 0.1 nM enzyme; Δ2 RNA, 97 pM/min, 17 nM substrate, 0.8 nM enzyme; Δ3 RNA, 97 pM/min, 25 nM substrate, 1 nM enzyme; Δ4 RNA, 2.2 nM/min, 50 nM substrate, 1 nM enzyme.

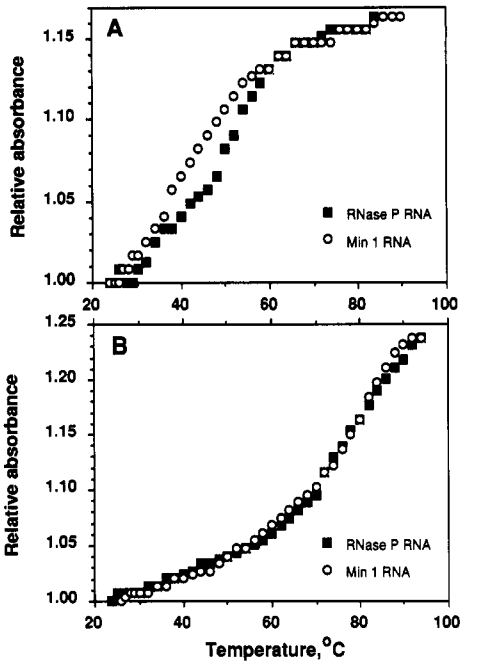

FIGURE 3: Thermal denaturation of native RNase P RNA and Min 1 monitored by the absorbance at 260 nm. (A) 5.3 mM sodium phosphate buffer; (B) 5.3 mM sodium phosphate/1 M NaCl. (■) Native RNase P RNA; (○) Min 1 RNA.

of the substrate was cleaved. Enzyme reactions were stopped by ethanol precipitation of the RNAs, and the products were resolved by polyacrylamide gel electrophoresis in the presence of 8 M urea. The gels were fixed, dried, and exposed to film. Radioactive products were excised from the gels and quantified using Cerenkov scintillation counting.

Reconstitution of RNA and Protein Subunits. RNase P protein was isolated (by B. Pace in our laboratory) from an overexpressing strain of *E. coli*, BL21 (DE3), carrying plasmid pARN1 (gift of C. Guerrier-Takada and S. Altman) using the method of Vioque et al. (1988). Aliquots of the protein subunit (1 μM) were stored at −80 °C in a solution containing 50 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), pH 7.8, 100 mM NH_4Cl, 10 mM $MgCl_2$, 0.1% (w/v) Nonidet P-40, and 50% (w/v) glycerol. Protein was used immediately upon thawing.

The protein and RNA subunits were reconstituted by incubating them at the indicated ratios at 37 °C for 20 min in the presence of 50 mM Hepes–NH_4OH, pH 8.0, 10 mM magnesium acetate, 100 mM ammonium acetate, and 0.05% (w/v) Nonidet P-40. Reconstituted enzyme activity was measured by adding ^{32}P-labeled precursor tRNA to the mixture and assaying enzyme activity as described above.

Thermal Melting Curves. Thermal melting curves were measured using a water-jacketed cuvette and a Gilford Model 250 spectrophotometer. RNAs were resuspended in 5.3 mM sodium phosphate buffer (final Na^+ concentration, 10 mM). for high-salt measurements, 1 M NaCl was included. Absorbance was meausred after heating the RNAs once to 80 °C and slowly cooling to room temperature. The rate of heating was approximately 3 °C/min.

RESULTS

Min 1 RNA, the simplified RNase P RNA, requires unusually high concentrations of monovalent salt for optimal activity as shown in Figure 2A. The optimal monovalent salt concentration (ammonium acetate) is 3.25 M for Min 1 RNA, while native RNase P RNA requires 1 M. This result is consistent with the hypothesis that Min 1 RNA is destabilized and requires more electrostatic screening than the native RNA in order to fold into a functional structure. The monovalent salt requirements of the RNAs containing the individual deletions are shown in Figure 2B. Two of the deletions (Δ1 and Δ4) do not significantly change the salt optimum of the RNA, while two others (Δ2 and Δ3) each require 2.75 M or greater monovalent salt for maximum activity. Thus, the salt requirement of Min 1 RNA appears to result primarily from the deletion of regions 2 and 3 and is not the result of incremental requirements for salt conferred by each of the four deletions. We tested whether regions 2 and 3 are independent of each other by constructing a RNA which contained both deletions. This RNA, Δ23, also requires 2.75 M monovalent salt for optimal activity (result not shown), suggesting that deletion of either region 2 or region 3 has the same effect on the RNA.

The stabilizing effect of salt on RNA can also be observed in thermal denaturation. At low salt (Figure 3A), Min 1 RNA has a T_m of 43.0 ± 1.4 °C, while the T_m of native RNase P RNA is 48.4 ± 1.9 °C. The shape of the melting curves is also different: Min 1 RNA melts continuously while native RNase P RNA displays a discontinuous melting curve. This result suggests that native RNase P RNA contains structural domains with differing stabilities to thermal denaturation which apparently are destabilizied in Min 1 RNA. Addition of monovalent salt stabilizes the structure of both RNAs: at 1 M NaCl (Figure 3B), the T_m of both RNAs shifts to greater than 73 °C, and the shapes of the melting curves are nearly identical.

Measurement of the K_M of the individual deletion mutants (Table I) shows that the high K_M of Min 1 RNA is caused

Table I: Kinetic Characteristics of *E. coli* RNase P RNA and Deletion Mutants[a]

RNA	K_M (μM)	k_{cat} (min^{-1})	k_{cat}/K_M (min^{-1} μM^{-1})
RNase P	0.05	0.7	14
Min 11[b]	5.0	10	2
Δ1	0.02	0.4	20
Δ2	0.12	0.3	2.5
Δ3	0.25	0.6	2.4
Δ4	3.0	43	14
Δ23	0.13	0.6	4.6
Δ4Ec	2.3	28	12

[a] All measurements were made at the optimal concentration of ammonium acetate for each RNA: native RNase P RNA, 1 M; Δ1, 1.5 M; Δ2, 2.75 M; Δ3, 3.0 M; Δ4, 1 M; Δ23, 3.0 M; Δ4Ec, 1.0 M. [b] Min 1 kinetic data from Waugh et al. (1989).

by deletion of helix 4. The K_Ms of the other three mutants are all within 5-fold of that of the native RNA. Previous work has suggested that the K_M approximates the K_D in the in vitro RNase P RNA reaction; the activity of RNase P RNA is rate-limited by release of product, not by binding of substrate (Reich et al., 1988; Smith et al., 1992). Therefore, the 100-fold increase in K_M caused by deletion of helix 4 is equivalent to about 2.7 kcal of binding energy, approximately that expected from one to five hydrogen bonds (Fersht, 1987). Thus, the deletion of helix 4 may have disrupted at least one contact point between RNase P and its substrate tRNA.

In constructing Δ4, the phylogenetically variable helix (nucleotides 260–290, Figure 1) was deleted, and four nucleotides flanking the deleted helix were changed to the *Bacillus megaterium* sequence (UAG-G to CAAU). We tested whether these nucleotide changes in loop 4 cause the loss of the tRNA contact by creating another version of Δ4 that contains exactly the *E. coli* sequence in that region. The K_M of that RNA, Δ4Ec (Table I), was not significantly different from that of Δ4. This suggests that it is the deletion of helix 260–290, not the sequence changes in the loop, that causes the loss of tRNA contact(s). This result was unexpected: since helix 260–290 is not conserved phylogenetically (Figure 1), it was not expected to participate in substrate binding. However, recent structural information from phylogenetic covariation and random mutagenesis (Haas et al., 1991) rationalizes these observations (see Discussion).

The k_{cat} values of the Δ4 and Min 1 RNAs are more than 10-fold greater than that of native RNase P RNA (Table I). This result is predicted, since the RNase P reaction in vitro is rate-limited by product release (Reich et al., 1988). Assuming that product binds with the same affinity as substrate (Reich et al., 1988; Smith et al., 1992) k_{cat} is expected to increase in the cases of enzymes with reduced affinity for substrate (i.e., increased K_M), up to the limit of the next rate-limiting step. The observation that Δ4 and Δ4Ec both display k_{cat} values that are greater than that of Min 1 RNA, even though their K_M values are slightly less than that of Min 1, suggests that the catalytic competence of Min 1 RNA is reduced. Perhaps deletion of regions 1–3 alters a structure affecting the catalytic step, thereby reducing the k_{cat} of Min 1. Alternatively, the rate of cleavage may be reduced in the high ionic strength conditions used for assaying Min 1 RNA activity. These effects may not be manifest in Δ1–Δ3 RNAs because those enzymes have relatively low K_Ms and are rate-limited by product release.

Min 1 RNA activity is also more temperature-sensitive than that of the native RNase P RNA (Figure 4A). At low substrate concentrations, where both K_M and k_{cat} effects can be observed, the temperature optimum of Min 1 RNA is 35 °C, whereas that of native RNase P RNA is 45 °C. Similar analysis of the four deletion mutants (Figure 4B) showed that temperature sensitivity is caused by the deletion of region 4. At their respective optimal ionic strength, the other RNAs have temperature optima equal to or slightly greater than that of the native RNA.

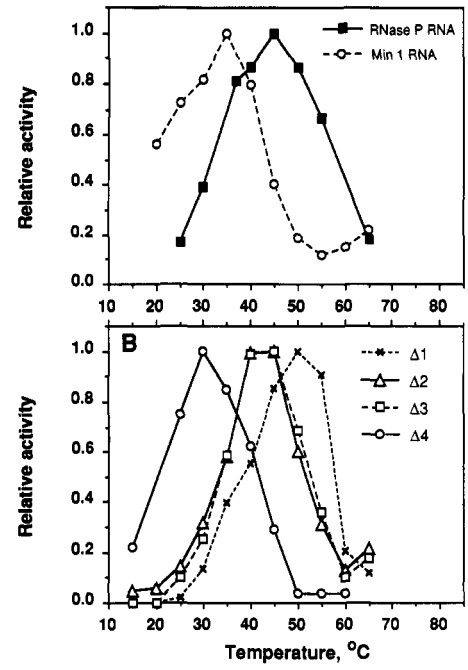

FIGURE 4: Temperature sensitivity of RNase P activity. (A) (■) Native RNase P RNA, (○) Min 1 RNA. (B) (×) Δ1 RNA; (△) Δ2 RNA; (□) Δ3 RNA; (○) Δ4 RNA. Enzyme activity was measured at the indicated temperatures in the presence of optimal monovalent salt concentrations for each RNA. The ammonium acetate concentrations used were as follows: native RNase P RNA, 1 M; Min 1 RNA, 3.25 M; Δ1, 1.5 M; Δ2, 2.75 M; Δ3, 2.75 M; Δ4, 1.0 M. Activity was normalized to the maximum value in each case. Actual maximal rates of cleavage and the concentrations of substrate and enzyme in each assay were the following: RNase P RNA, 16 pM/min, 5 nM substrate, 0.2 nM enzyme; Min 1 RNA, 272 pM/min, 0.1 μM substrate, 1.3 nM enzyme; Δ1 RNA, 16 pM/min, 3 nM substrate, 0.1 nM enzyme; Δ2 RNA, 29 pM/min, 12 nM substrate, 0.6 nM enzyme; Δ3 RNA, 87 pM/min, 25 nM substrate, 0.9 nM enzyme; Δ4 RNA, 330 pM/min, 50 nM substrate, 0.5 nM enzyme.

The final characteristic of the deletion mutants that we have examined is their response to the protein subunit. The protein subunit is essential for RNase P enzyme activity in vivo (Kole et al., 1980). At 100 mM ammonium acetate, the RNA is nearly inactive in the absence of the protein subunit. Figure 5A compares the response of native RNase P RNA and Min 1 RNA to added protein subunit. Min 1 RNA does not respond to the presence of the protein subunit, whereas the activity of the native RNA is greatly stimulated. The results with the four deletion mutants are shown in Figure 5B. Only the Δ1 RNA displays the normal response to the protein. The rest of the mutant RNAs, Δ2–Δ4, all respond poorly. From these data, it is not possible to distinguish whether Min 1 RNA or the Δ2–Δ4 mutants are incapable of binding the protein subunit or whether they are not stimulated by bound protein. It is clear, however, that Δ2–Δ4 are capable of responding at a reduced level and that the response qualitatively appears to saturate at protein:RNA ratios similar to that of the native RNase P RNA. This suggests that the deletion mutants can bind the protein subunit but fail to fully respond to it.

Thus, deletion of regions 2, 3, or 4 reduces but does not eliminate the response to the protein subunit. In contrast, Min 1 RNA does not respond to the protein. This is the only aspect of Min 1 RNA that cannot be attributed to the loss of a single

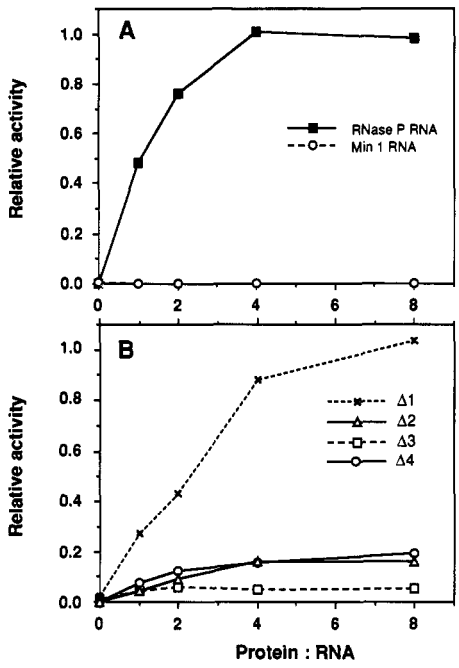

FIGURE 5: Response of RNase P and the deletion mutants to the protein subunit. The protein subunit was reconstituted at the indicated molar ratios with the RNA subunit, and the resulting holoenzyme was assayed at 37 °C in the presence of 100 mM ammonium acetate. (A) (■) Native RNase P RNA; (○) Min 1. (B) (×) Δ1; (△) Δ2; (□) Δ3; (○) Δ4. Data were normalized to the rate of cleavage of each RNA in the absence of protein at its optimal salt concentration (see Figure 4 legend). The actual rates for the holoenzyme at an 8:1 protein:RNA ratio and the concentrations of the substrate and the RNA subunit of the enzyme in each assay were as follows: RNase P RNA, 8.2 pM/min, 4 nM substrate, 0.2 nM RNA; Min 1 RNA, no detectable cleavage, 50 nM substrate, 4.2 nM RNA; Δ1, 10 pM/min, 4 nM substrate, 0.1 nM RNA; Δ2, 6.0 pM/min, 17 nM substrate, 0.84 nM RNA; Δ3, 6.9 pM/min, 25 nM substrate, 1.2 nM RNA; Δ4, 42 pM/min, 50 nM substrate, 0.5 nM RNA.

helical region. Apparently the combination of more than one deletion is required to produce an RNA with no response to the protein subunit. The deletion of regions 2 and 3 is not sufficient to eliminate the response, since Δ23 RNA responds to the protein subunit at approximately the same level as Δ2 and Δ3 RNAs (data not shown). Thus, the combined deletion of regions 2 and/or 3, and region 4 appears to be required to produce the phenotype of Min 1 RNA. These data are consistent with previous results demonstrating footprinting by the protein subunit spanning regions 2, 3, and 4 (Vioque et al., 1988).

Discussion

E. coli RNase P RNA contains four helical elements which are not present in the RNAs of other organisms and, therefore, are phylogenetically variable. Deletion of all four helical elements (to produce Min 1 RNA) leaves only the core of RNase P, which is enzymatically active, though different from the native RNase P RNA in several aspects (Waugh et al., 1989). We have deleted the four structures individually from *E. coli* RNase P RNA and can assign functions to three of the four helical elements. The sum of the functions of the individual helical elements accounts for the phenotype of Min 1 RNA described in the introduction.

Deletion of region 1 (Figure 1) shortens the hairpin between nucleotides and 20 and 61 by 21 residues. It is the only deletion which has no substantial effect on the RNA in any of the characteristics we measured. This hairpin varies widely in length within the proteobacteria, the eubacterial phylum which includes *E. coli* (Brown et al., 1991). Thus, we confirmed the predictions of the phylogenetic comparative analysis: the length of this helix is not important to enzyme function.

Deletion of regions 2 and/or 3 confers the requirement for high concentrations of monovalent salt. This suggests that the deletions cause structural disruptions, since high concentrations of salt increase the strength of base stacking and reduce electrostatic repulsion in nucleic acids. The two regions appear to interact, since no additive effect could be demonstrated between them; Δ2, Δ3, and Δ23 RNAs display essentially the same characteristics. Deletion of either region probably disrupts the same structure or domain in the enzyme.

There is no indication from the phylogenetic data that helix 2 plays an important structural role. Helix 2 varies somewhat in length and sequence among the proteobacteria. Of the 35 nucleotides in the helix, 7 are conserved in all 12 of the proteobacterial sequences known, and 7 more vary in only 1 known case (Brown et al., 1991). Substitution of the helix from the Gram-positive organism *B. megaterium* changes all of these nucleotides, eliminates the two conserved unpaired A residues at nucleotides 173 and 174, and alters four nucleotides adjacent to the helix. The length of the new helix, however, is only one base pair longer than the proteobacterial consensus length. Thus, the presence of any helix is not sufficient to substitute for helix 2 in *E. coli*. Apparently there are some sequence requirements in the base-paired or bulged nucleotides. It is difficult to imagine that changing the identity of a few nucleotides in a variable helix or adjacent to it would confer such a large requirement for ionic strength. Perhaps the conserved, unpaired A residues participate in a tertiary contact which is lost when the *B. megaterium* helix is substituted.

The role of region 3 also is not apparent from phylogenetic information. Although the two helices are present in most RNase P RNAs, they are totally absent in one example of the proteobacteria, *Alcaligenes eutrophus*, and also in some of the Gram-positive bacteria (James et al., 1988; Brown et al., 1991). The *A. eutrophus* RNase P RNA does not contain any obvious differences from the *E. coli* RNA which would compensate for the loss of region 3. The influence of ionic strength on the activity of the *A. eutrophus* RNase P RNA is not known. It is possible that this RNA also requires high ionic strength for optimal activity.

The effect of the deletion of regions 2 and 3 on RNase P RNA might best be explained not by the loss of stabilizing structures but by the creation of an unstable structure. Perhaps deletion of the hairpins in region 3 or substitution of a helix with no unpaired nucleotides in region 2 produces a RNA which cannot fold properly. This misfolding might cause increased electrostatic repuslion between phosphates in the RNA backbone, which in turn might destabilize a functionally important element. High concentrations of monovalent salt would reduce or eliminate the electrostatic repulsion, thereby minimizing the functional disruption. This interpretation is consistent with the temperature sensitivity of Δ2 and Δ3 RNAs. At high salt concentrations, the two mutants are not temperature-sensitive relative to native RNase P RNA. If the deletions had caused the loss of a stabilizing structure, it is expected that the RNAs might be more temperature-sensitive even in the presence of salt. However, if the deletions only created an unstable structure which can be ameliorated by counterion, then, in the presence of salt, the deletion mutants should still be as stable as the native RNA.

Deletion of region 4 from the *E. coli* RNase P RNA increases the K_M for precursor tRNA nearly 100-fold. The increased K_M is due directly to the loss of helix 4 and not to

the nucleotide changes made in the loop region adjacent to the deleted helix. K_M is equivalent to K_D for RNase P RNA in vitro (Reich et al., 1988; Smith et al., 1991). Thus, the increase in K_M may be the result of a reduction in binding affinity between enzyme and substrate. We were surprised by these data: since helix 4 is not phylogenetically conserved, it was not expected that it would play a significant role in an essential function such as substrate binding. However, recent revisions in the structure model of RNase P RNA shed light on our observations.

It has been discovered, using both new phylogenetic covariation data and site-directed mutations, that helix 4 participates in a pseudoknot with another region of the RNA (Haas et al., 1991). Specifically, nucleotides 276–279 at the end of helix 4 base-pair with nucleotides 82–85 (indicated by brackets in Figure 1). Thus, deletion of helix 4 disrupts a long-range interaction which probably has large effects on the structure of the molecule. The reduced binding affinity for substrate (increased K_M) in Δ4 RNA may, therefore, be either a direct effect of the loss of the helix or an indirect effect resulting from the structural disruption. We have observed that in nondenaturing gel electrophoresis (in the absence of Mg^{2+}) both Δ4 and Min 1 RNAs migrate somewhat more rapidly than their size would predict (data not shown). This suggests that the RNAs are collapsed or made more flexible by the loss of the pseudoknot.

Deletion of region 4 also confers temperature-sensitivity on the RNA (Figure 4B). Two explanations are consistent with this effect. The loss of activity of the RNA at moderate temperatures may result from reduced structural stability in the absence of the pseudoknot, causing reduced catalytic competence. Alternatively, the rate of substrate dissociation of the high-K_M Δ4 mutant may contribute significantly to k_{cat}/K_M at moderate temperatures, causing a rate decrease as the temperature increases. For the low-K_M native ribozyme, the rate of substrate dissociation may not be significant until much higher temperatures are reached. These alternatives are not mutually exclusive, and it is possible that both may contribute to temperature-sensitivity in Δ4 and Min 1 RNAs.

Helix 4 is absent in the RNase P RNA from four species of the Gram-positive genus *Bacillus* (James et al., 1988; and see the *B. megaterium* RNase P RNA in Figure 1). The K_M for precursor $tRNA^{Asp}$ of the *Bacillus subtilis* RNase P RNA is 0.2 μM (Reich et al., 1988), only 4-fold greater than that of the *E. coli* RNase P RNA. Thus, the *Bacillus* RNase P RNAs have lost helix 4 without great reduction of enzyme–substrate binding energy. Haas and colleagues (Haas et al., 1991) have proposed that the *Bacillus* RNase P RNAs have compensated for the loss of helix 4 with another helix inserted adjacent to the other side of the helix 4 pseudoknot (helix 59–77 in the *B. megaterium* RNA in Figure 1). This inserted helix might allow adjacent regions in the RNA to take on the same conformation as if the helix 4 pseudoknot was present (Haas et al., 1991).

In summary, our analysis has shown that three of four phylogenetically variable helices in the *E. coli* RNase P RNA contribute to the stability and structure of the RNA. Although these helices are not absolutely essential to the enzyme, they clearly are not dispensable. Deletion of region 4, in particular, produces a large, specific reduction in the stability of the enzyme–substrate complex. These results suggest that the use of phylogenetic comparisons to predict the dispensability of some structures in RNA enzymes must be regarded with caution. Structural elements which appear to vary widely among examples of an enzyme isolated from phylogenetically distant organisms may still have specific roles in the structure and function of that enzyme.

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