Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 2527–2531, March 1994
Biochemistry

Further perspective on the catalytic core and secondary structure of ribonuclease P RNA

(rnpB/comparative analysis)

ELIZABETH S. HAAS*, JAMES W. BROWN*, CHRISTIAN PITULLE†, AND NORMAN R. PACE‡

Department of Biology and Institute for Molecular and Cellular Biology, Indiana University, Bloomington, IN 47405

Contributed by Norman R. Pace, November 29, 1993

ABSTRACT **Phylogenetic comparative analyses of RNase P RNA-encoding gene sequences from *Chlorobium limicola*, *Chlorobium tepidum*, *Bacteroides thetaiotaomicron*, and *Flavobacterium yabuuchiae* refine the secondary structure model of the general (eu)bacterial RNase P RNA and show that a highly conserved feature of that RNA is not essential. Two helices, comprised of 2 base pairs each, are added to the secondary structure model and form part of a cruciform in the RNA. Novel sequence variations in the *B. thetaiotaomicron* and *F. yabuuchiae* RNA indicate the likelihood that all secondary structure resulting from canonical base-pairing has been detected: there are no remaining unpaired, contiguous, canonical complementarities in the structure model common to all bacterial RNase P RNAs. A nomenclature for the elements of the completed secondary structure model is proposed. The *Chlorobium* RNase P RNAs lack a stem–loop structure that is otherwise universally present and highly conserved in structure in other (eu)bacterial RNase P RNAs. The *Chlorobium* RNAs are nevertheless catalytic, with kinetic properties similar to those of RNase P RNAs of *Escherichia coli* and other Bacteria. Removal of this stem–loop structure from the *E. coli* RNA affects neither its affinity for nor its catalytic rate for cleavage of a precursor transfer RNA substrate. These results show that this structural element does not play a direct role in substrate binding or catalysis.**

RNase P is the site-specific endoribonuclease that removes 5′ flanking sequences from precursors of transfer RNA (for reviews, see refs. 1–3). RNase P is a ribonucleoprotein; in Bacteria (formerly eubacteria), in which it is best studied, RNase P is composed of a single *ca.* 130-kDa RNA and a single *ca.* 14-kDa protein (see ref. 4 for review). The RNA component of bacterial RNase P is the catalytic moiety; at high ionic strength *in vitro* it is capable of efficient catalysis in the absence of protein (5). Understanding the mechanisms of substrate recognition and catalysis by RNase P RNA requires knowledge of the structure required for those functions. A model for the secondary structure§ of bacterial RNase P RNA has been derived from phylogenetic comparisons (4, 6), which included sequences from representatives of 5 of the approximately 12 "Kingdoms" [*sensu* Woese (7)] of Bacteria. This secondary structure model was expected to be incomplete; additional sequence variation was required to provide evidence for either the presence or absence of additional structure. Sequences from organisms that are phylogenetically distant from previously examined ones are especially useful for inferring structure because they generally vary greatly from those known. Moreover, phylogenetically disparate sequences may allow the identification of elements in the RNA that are not universally present and, therefore, are presumably not essential for the function of the RNA. Naturally occurring gene sequences that lack specific structural elements also provide information useful in designing meaningful deletions or substitutions that specifically test the biological function of the structural elements.

In this paper, we report the sequences encoding RNase P RNA from representatives of two additional bacterial kingdoms: *Chlorobium limicola* and *Chlorobium tepidum* (members of the green sulfur Bacteria); and *Bacteroides thetaiotaomicron* and *Flavobacterium yabuuchiae* (members of the *Bacteroides* and *Flavobacterium* group).¶ Variation from known RNA sequences identifies previously unknown secondary structure and, together with a mutational analysis of the *Escherichia coli* RNA, sheds light on the minimum RNase P RNA structure required for catalytic activity.

MATERIALS AND METHODS

DNA Sources (Bacterial Strains). *C. limicola* forma sp. *thiosulfatophilum* (NCIB 8327) and *C. tepidum* (ATCC 49652) cell paste were gifts from M. Madigan (University of Southern Illinois, Carbondale). *B. thetaiotaomicron* strain 5482 (ATCC 29148) cell paste was a gift from A. Salyers (University of Illinois, Urbana). *F. yabuuchiae* (ATCC 49272) cell paste was a gift from C. Woese and L. Mandelco (University of Illinois, Urbana). Genomic DNAs were purified from these cells as described (8).

PCR Amplification. PCRs (9) were performed and product DNAs were cloned essentially as described (10). A fragment of the *C. limicola* RNase P RNA-encoding gene, for use as hybridization probe to screen gene banks, was obtained by amplification from genomic DNA using oligonucleotide primers 174F (5′-AGGGTGAAANGGTGSGGTAAGAG-3′) and 347R (5′-RTAAGCCGGRTTCTGT-3′). Plasmids from which functional RNase P RNAs from *C. limicola* (clone 53a) and *C. tepidum* (clone 55a) could be synthesized *in vitro* were constructed by cloning DNA amplified from genomic DNA using primers CLIM PCR 5 (5′-TAATACGACTCACTATAGGAAACCGCAAGTGTGCAG-3′) and CLIM PCR 3 (5′-CGGATCCAAACCGAAGCTGTAAG-3′). Because these primer sequences are based on the *C. limicola* gene, full-length amplification products and transcripts from the *C. tepidum* gene contain nonnative sequences, derived from the *C. limicola* gene, at both the 5′ and 3′ ends.

Genomic Southern Analysis. Southern analysis of genomic DNA was performed as described (8). The probe for the *C.*

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*Present address: Department of Microbiology, North Carolina State University, Raleigh, NC 27695.
†Present address: Department of Biochemical and Biophysical Sciences, University of Houston, Houston, TX 77204.
‡To whom reprint requests should be addressed.
§We define secondary structure as two or more contiguous, antiparallel, canonical (G·C or A·U) or G·U base pairs.
¶The sequences reported in this paper have been deposited in the GenBank data base [accession nos. L25702 (*B. thetaiotaomicron*), L25703 (*C. limicola*), L25704 (*C. tepidum*), and L25705 (*F. yabuuchiae*)].

limicola RNase P RNA-encoding gene was a uniformly ^{32}P-labeled run-off transcript of the cloned 174F/347R RNase P RNA amplification product synthesized using T7 RNA polymerase. The probe for the *B. thetaiotaomicron* genomic Southern analysis was an oligonucleotide (59F, 5′-GIIGAGGAAAGTCCIIGCT-3′) (8) that contains the most highly conserved sequence from known bacterial RNase P RNAs, which was 5′ end-labeled using [γ-^{32}P]ATP and T4 polynucleotide kinase (11).

Cloning of RNase P RNA-Encoding Genes. The cloning and screening methods used were essentially as described (8). The RNase P RNA gene from *B. thetaiotaomicron* was contained on a 4.4-kb *Eco*RI/*Hin*dIII DNA fragment cloned into pBluescript KS$^-$, and the *C. limicola* gene was contained on a 5.8-kb *Kpn* I/*Sal* I DNA fragment cloned into pBluescript KS$^+$. The *C. tepidum* gene was cloned by amplification directly from genomic DNA using oligonucleotides CLIM PCR 5 and CLIM PCR 3 as described above. A fragment comprising the majority of the *F. yabuuchiae* RNase P RNA-encoding gene was amplified using oligonucleotide primers 60FBam (5′-CGGGATCCGAGGAAAGTCCGGRC-3′) and 347REco (5′-CGGAATTCRTAAGCCGGRTTC-TGT-3′) and cloned into pBluescript KS$^+$ following digestion with restriction endonucleases *Eco*RI and *Bam*HI.

Nucleic Acid Sequencing. Double-stranded plasmid DNAs were sequenced by the dideoxy chain-termination method (12) using Sequenase version 2.0 (United States Biochemical).

Construction of *E. coli* ΔP18 RNase P RNA. The replacement of helix P18 (see the legend to Fig. 1 for a description of helix nomenclature) from the *E. coli* RNase P RNA-encoding gene in plasmid DW98 (13) was performed by the procedure of Kunkle (14), as described (15). A mutagenic oligonucleotide primer with the sequence 5′-GGTAGGCT-GCTTCAGATGAATGACTG-3′ was used to replace nucleotides 304–327 of the encoded *E. coli* RNase P RNA with a single C residue in plasmid *E. coli* ΔP18.

Determination of Kinetic Constants. Enzyme activity, analyzed by gel electrophoresis as described (16), was measured at 37°C in the presence of 25 mM $MgCl_2$, 50 mM Tris·HCl (pH 8), 0.1% SDS, 0.05% Nonidet P-40, 1–3 M NH_4Cl (as indicated), 0.9 nM RNase P RNA, and 10–1350 nM precursor tRNA (17, 18). Uniformly ^{32}P-labeled precursor tRNA^{Asp} from *Bacillus subtilis* was transcribed from plasmid DW128 (13). Unlabeled wild-type *E. coli*, *E. coli* ΔP18, *C. limicola*, and *C. tepidum* RNase P RNAs were transcribed from plasmids DW98 (13), *E. coli* ΔP18, *C. limicola* 53a, and *C. tepidum* 55a, respectively. Holoenzyme reactions using purified RNase P protein from *E. coli* were performed as described (19).

RESULTS

RNase P RNA-Encoding Genes. The RNase P RNA-encoding genes from *C. limicola* and *B. thetaiotaomicron*, and the majority of the genes from *C. tepidum* and *F. yabuuchiae*, were cloned and the nucleotide sequences were determined. The PCR-derived probes used to identify *C. limicola* and *B. thetaiotaomicron* RNase P RNA gene sequences in Southern analyses hybridized in each case to single DNA fragments (data not shown); these RNase P RNAs are therefore encoded by single-copy genes, as in previously examined Bacteria (6, 8, 19–22). Hybridization of the probes to RNAs of the predicted sizes in Northern analyses (data not shown) shows that the cloned genes are expressed *in vivo*. Transcripts of the sense strand of the complete genes, synthesized *in vitro* using T7 RNA polymerase from covalently closed circular plasmid DNA or PCR-generated templates, contain RNase P enzymatic activity (data not shown), demonstrating that the cloned genes encode functional RNase P RNAs. The secondary structures of these novel RNAs, inferred on the basis of comparative analysis, are shown in Fig. 1 and discussed below (see *Discussion*). The RNase P RNA secondary structure model is now sufficiently developed that it is useful to adopt a standard nomenclature. We use the nomenclature developed for group I introns (23), explained in the legend to Fig. 1.

Kinetic Analysis of RNase P RNAs Lacking P18. The *Chlorobium* RNase P RNAs substitute a single C residue for helix P18, which in other bacterial RNase P RNAs is universally present and highly conserved in structure. This finding indicates that, despite its conservative nature, P18 is not required for the catalytic activity of RNase P RNA. To verify this in the context of an RNase P RNA that normally contains P18, the kinetic properties of the *Chlorobium* RNAs were compared to those of a mutant *E. coli* RNA in which this element was likewise replaced by a single C residue (Fig. 1). Since these RNAs are homologs, their structures required for catalysis are expected to be similar. Consequently, the replacement of P18 in the *E. coli* RNA with a C, as occurs in the *Chlorobium* RNAs, should minimally perturb the remaining structure.

At their optimal ionic strengths, the *Chlorobium* RNAs and the P18 deletion mutant of the *E. coli* RNA are comparable to the native *E. coli* RNA in affinity for substrate (K_m), rate of catalysis and product release (k_{cat}), and overall catalytic efficiency (k_{cat}/K_m) (Table 1). Clearly, helix P18 does not contribute critically to the function of RNase P RNA. However, the deletion of P18 from the *E. coli* RNA results in an increase in the optimal concentration of monovalent salt (NH_4Cl) from 1 M to *ca.* 3 M, indicating a structural defect in the deletion mutant. At the lower ionic strength, the affinity ($1/K_m$) of the mutant RNA for substrate is decreased 60-fold. The higher k_{cat} for the mutant than the native RNA at low ionic strength is a consequence of the fact that the turnover rate of the RNase P RNA reaction is limited by the rate of product release. Poorer binding of substrate (and product) therefore can result in higher k_{cat} (24). Increased ionic strength affects to a lesser extent the kinetic properties of the two *Chlorobium* RNAs; K_m values at high ionic strength are 5- to 10-fold lower than at low ionic strength, resulting as well in reductions in k_{cat}. Catalytic activity of the *Chlorobium* RNAs, and to a lesser extent that of the *E. coli* ΔP18 RNA, is conferred at physiological ionic strength (100 mM NH_4Cl) by the presence of the *E. coli* RNase P protein (data not shown). Helix P18, therefore, is also not required for binding of the protein subunit.

DISCUSSION

The *B. thetaiotaomicron* and *F. yabuuchiae* RNase P RNAs are unusual in several respects. Most importantly, these RNAs are richer in A+U (48.5% and 54.7%, respectively) than previously examined RNase P RNAs (e.g., 38.2% for the *E. coli* RNA), so they provide a large number of novel sequence variations. New covariations provide evidence for the occurrence of two previously unidentified helices (P10 and P11); each of the pairings in these helices is supported by the occurrence of compensatory base substitutions that nevertheless maintain complementarity. One of these pairings (nucleotides A121 and U236 in *E. coli*) is also confirmed by mutation and second-site reversion (25). Other covariations in the *B. thetaiotaomicron* and *F. yabuuchiae* RNAs verify base pairs that were presumed in the previous structure model but had not been proven because of their constancy among available RNase P RNA sequences. Only seven predicted base pairs in the bacterial consensus structure (Fig. 2) remain to be specifically proven by the encounter of sequence covariation. The *B. thetaiotaomicron* and *F. yabuuchiae* RNase P RNA gene sequences also contribute to

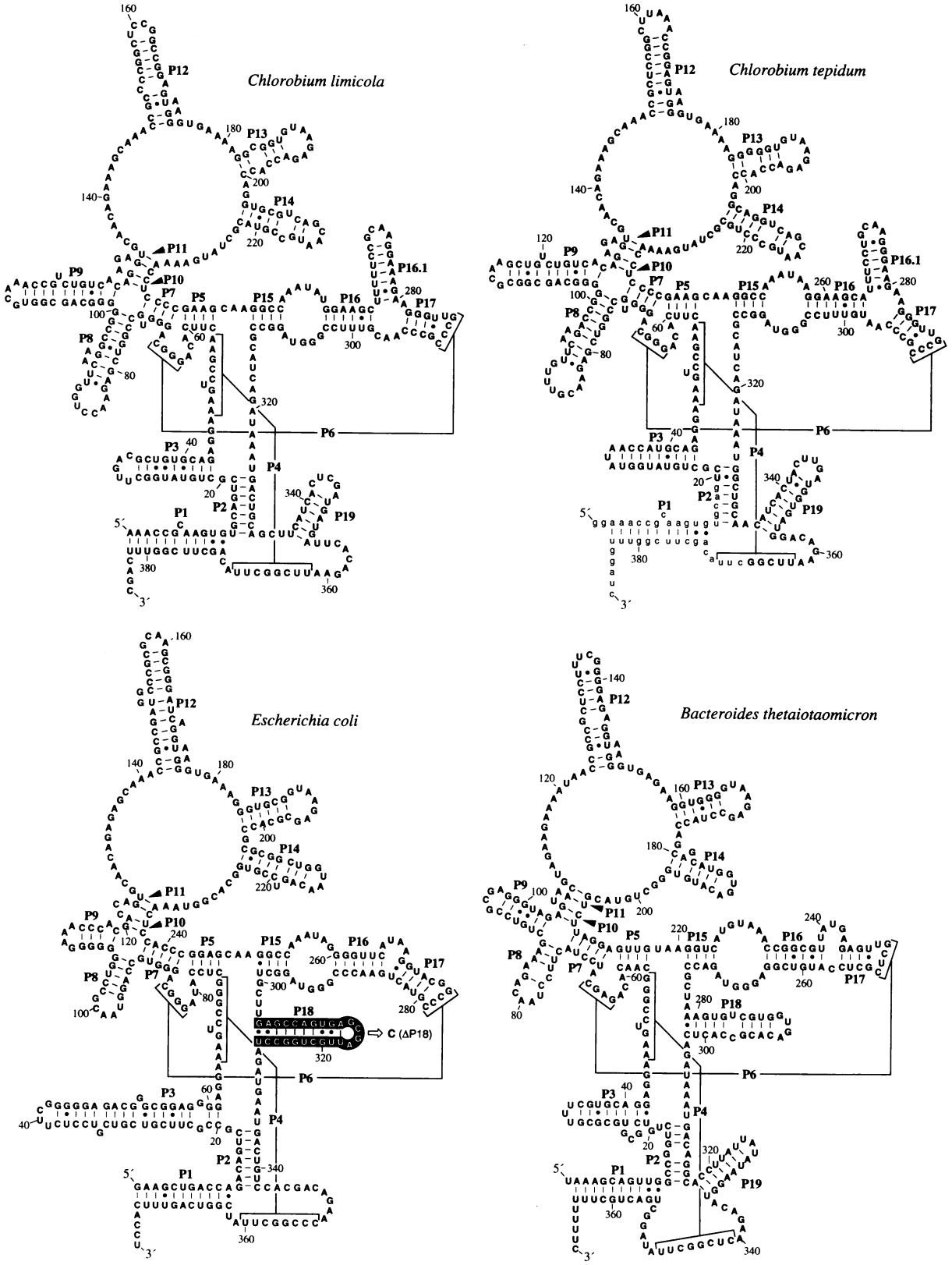

FIG. 1. Secondary structures of the *Chlorobium* and *B. thetaiotaomicron* RNase P RNAs. Base-pairings in P4 and P6 are shown as brackets and lines. The naturally occurring 5′ and 3′ termini of the *Chlorobium* and *B. thetaiotaomicron* RNAs were not determined experimentally but are predicted based on homology with those of *E. coli*; the ends shown for the *Chlorobium* RNAs are those of the transcripts synthesized *in vitro* that were used in kinetic analyses (see text). Nucleotides in lowercase in the *C. tepidum* RNA are derived from the *C. limicola* sequence and represent portions of the amplification primers used to clone the *C. tepidum* gene. Nucleotides replaced by a single C residue in the *E. coli* ΔP18 RNA are highlighted. The helix nomenclature used is that developed for group I introns (23). Helices are numbered as they occur 5′ to 3′ preceded by P (paired; e.g., P3 is the third helix in the structure). The helix numbers are based on the ancestral form of the secondary structure (19)—for instance, that of *E. coli*. The representation of the secondary structure has been changed somewhat from the previous one (4) for clarity and accommodation of the newly discovered P10 and P11.

Table 1. Kinetic analysis of RNase P RNAs

RNA	1 M NH_4Cl			3 M NH_4Cl		
	K_m, nM	k_{cat}, min^{-1}	k_{cat}/K_m	K_m, nM	k_{cat}, min^{-1}	k_{cat}/K_m
E. coli	18 ± 4.9	0.21 ± 0.11	0.012 ± 0.0051	19.0 ± 9.2	0.18 ± 0.050	0.010 ± 0.0025
E. coli ΔP18	1200 ± 260	2.76 ± 1.5	0.0023 ± 0.0012	26.0 ± 6.8	0.31 ± 0.064	0.012 ± 0.0018
C. tepidum	23 ± 6.6	0.42 ± 0.072	0.018 ± 0.0028	2.3 ± 0.58	0.13 ± 0.020	0.056 ± 0.0058
C. limicola	71 ± 36	0.18 ± 0.074	0.0028 ± 0.00076	11.0 ± 4.4	0.062 ± 0.015	0.0060 ± 0.0017

evidence *against* the occurrence of any remaining unidentified helices composed of canonical base pairs. There are no remaining unpaired, phylogenetically consistent, dinucleotide complements in the conserved core of the RNase P RNA structure model. Of course, additional noncanonical associations are expected to exist. The fraction of paired nucleotides in the current RNase P RNA secondary structure model is similar to those of other well-defined secondary structure models of RNA. For instance, 64% of the nucleotides in the *E. coli* RNase P RNA are paired, whereas 60% and 55% of the small subunit ribosomal RNA and transfer RNA nucleotides, respectively, are paired (26).

The juxtaposition of helices P7–P10 without intervening unpaired residues creates a cruciform in the RNase P RNA

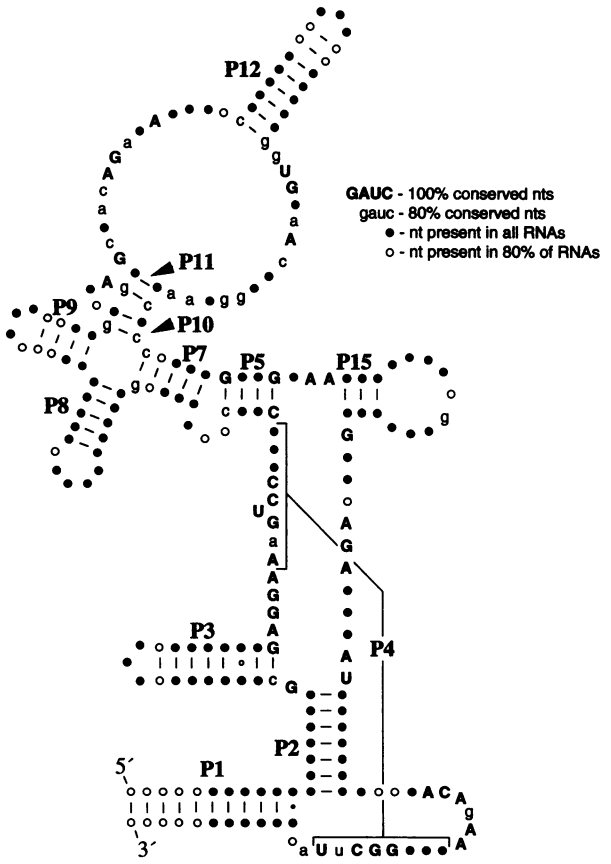

FIG. 2. Phylogenetic minimum bacterial consensus RNase P RNA. Helices are labeled as described in the legend to Fig. 1. Base-pairings in P4 are shown as brackets and lines. As described in the text, only the structural elements that are conserved in all known bacterial RNase P RNAs are included in the consensus. Universally conserved nucleotides are in uppercase letters; those that are at least 80% conserved, but are not invariant, are in lowercase letters. Nucleotides that are not conserved in identity but are present in all sequences are indicated by filled circles; those that are present in at least 80% of sequences, but absent in at least one, are indicated by open circles. The base-pairings indicated by closed and open dots are, respectively, a conserved noncanonical (G·A or A·C) interaction and a pairing that is frequently G·G (e.g., in *E. coli*) rather than canonical.

secondary structure. The four helices are expected to stack in some fashion. Analysis of Holliday junction-like DNA cruciforms suggests that the helices form two pairs of coaxial stacks (27, 28). If the cruciform in RNase P RNA is similarly arranged, there are two possible conformations of the resulting structure: either P7 stacked with P8 and P9 with P10 or P7 with P10 and P8 with P9. Available comparative and experimental data provide no convincing evidence for one conformation over the other, and it is possible that both exist during different phases of the catalytic cycle.

The *Chlorobium* RNase P RNAs also are unique in several respects, notably in their lack of helix P18. Its replacement in these RNAs with a single C residue indicates that P18 is a discrete structural unit and that it is not required intrinsically for function *in vivo*. The *Chlorobium* RNAs evidently do not contain a replacement for some catalytically important feature of P18, since the *E. coli* mutant RNA lacking P18 has native activity at its optimal ionic strength; it is unlikely that the native *E. coli* RNA contains both P18 as well as a replacement for its function. Previous analyses found that deletion mutants of *E. coli* (29) and *B. subtilis* (30) RNAs that lacked P18 had greatly diminished activities; however, those mutants lacked additional sequences, as well. The high level of activity of the ΔP18 mutant described here attests to the utility of phylogenetic comparisons in the design of deletion endpoints, in this case mimicking the natural deletion in the *Chlorobium* RNAs by replacement of P18 with a single nucleotide.

The ΔP18 mutant RNA requires higher ionic strength for maximal activity than required by the native RNA. This suggests that the structure of the mutant RNA is slightly destabilized, possibly due to some rearrangement in packing of other structural elements in order to accommodate the cavity left by removal of helix P18. The very high ionic strength required for activity by the mutant RNA presumably screens electrostatic repulsion within the RNA and thereby allows it to assume the active structure. In the *Chlorobium* RNAs, additional intramolecular interactions, perhaps only space-filling or stacking, could provide stability beyond that seen for the *E. coli* deletion mutant. There are several helical elements in the *Chlorobium* RNAs that are larger than their *E. coli* counterparts and might be involved in structural compensation for the lack of P18. We conclude that helix P18 has a modest structural role in bacterial RNase P RNA but does not participate directly in substrate binding or catalysis. The phylogenetically conserved nature of that helix perhaps points to some other important role *in vivo*.

Conservation of a particular structural element in *all* instances of a macromolecule indicates an important role for the element in structure or function. Conversely, the *absence* of a structural element from some instances of a macromolecule indicates that the element is not fundamentally essential. Our current view of the minimum core of the bacterial RNase P RNA, as indicated by evolutionary conservation, is summarized in Fig. 2. This minimum consensus structure includes only the secondary structural features present in all bacterial RNase P RNAs and nucleotide positions that are present in at least 80% of the RNAs. The helices and sequences identified in the consensus structure probably constitute the most important elements in the native RNAs

and so are particularly interesting targets for mutational and other analyses. At approximately 192 nucleotides, this phylogenetically defined core structure is far smaller than native RNase P RNAs, which usually are 350–400 nucleotides in length. Additional sequence lengths in the native RNAs are generally in helical domains. Some of the nonconserved sequences—for instance, P18 as analyzed in this study—contribute to structural stability; some may have other important but noncatalytic roles *in vivo*.

We thank Dr. Michael Madigan for the gift of *C. tepidum* and *C. limicola* cells, Dr. Abigail Salyers for the gift of *B. thetaiotaomicron* cells, Drs. Carl Woese and Linda Mandelco for the gift of *F. yabuuchiae* cells, and Dr. Bernadette Pace for the gift of *Thermus aquaticus* DNA polymerase and *E. coli* RNase P protein. This work was supported by National Institutes of Health Grant 34527-10 to N.R.P. and a Walther Cancer Institute Postdoctoral Fellowship to J.W.B.

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