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Detection of a key tertiary interaction in the highly conserved GTPase center of large subunit ribosomal RNA

(L11/thiostrepton/RNA–protein recognition/phylogenetic comparisons)

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ABSTRACT **Searches of ribosomal RNA sequences for compensatory base changes preserving Watson–Crick base pairing have led to detailed models of the conserved secondary structures of these RNAs. In principle, tertiary interactions can also be detected by searches for phylogenetically covariant bases. Within a highly conserved region of the large subunit ribosomal RNA termed the "GTPase center," the bases G-1056·U-1082·A-1086 are found in all eubacteria (*Escherichia coli* numbering), while A-1056·C-1082·G-1086 are found at the homologous positions in eukaryotes; archaebacteria fall into either category with some exceptions. Either sequence can potentially form a similar set of hydrogen bonds connecting the 3 bases. To determine the contribution of these 3 bases to RNA tertiary structure, sequence variants were made in RNA fragments covering the GTPase center. Correct folding of the RNA fragments was assayed by measuring the binding affinities of two different ligands that recognize the RNA tertiary structure: the highly conserved ribosomal protein L11, which is normally associated with the GTPase center RNA, and the peptide antibiotic thiostrepton, which inhibits the GTPase activity of eubacterial and some archaebacterial ribosomes. The results strongly support the existence of a base pair between positions 1082 and 1086: single mutations at either position weaken both L11 and thiostrepton binding by ≈10-fold or more, while compensatory double mutations bind the ligands nearly as well as the wild-type *E. coli* sequence. Variants at position 1056 have little effect on either L11 or thiostrepton binding; a 3-base interaction is therefore not supported by these experiments. A base pair between positions 1082 and 1086 strongly constrains the geometry with which three helical segments join in the middle of the GTPase center.**

Ribosomes are large protein–RNA complexes that catalyze the translation of the genetic code into proteins. Both the size and complexity of ribosomes have made a description of their structure difficult, although in the past decade phylogenetic studies of rRNA sequences have been the basis for a major advance in describing RNA folding within the ribosome. It has become clear that rRNAs from all organisms are related, and comparisons of sequences from distantly related organisms show that rRNA secondary structures are conserved despite wide divergences in primary structure (1). Physical and chemical data have been used to construct rough models of the packing of the rRNA secondary structure within the large and small subunits (2, 3). Some regions of the rRNA are very highly conserved in primary as well as secondary structure. These regions presumably contribute crucial functions, which have been present in ribosomes since the earliest times and, in some cases, have been identified with specific ribosomal functions such as the sites of codon–anticodon interaction or peptidyltransferase activity (4).

A next step in the analysis of rRNA structure must be the identification of tertiary structures that precisely define the three-dimensional foldings of domains within the ribosome subunits. Large data bases of rRNA sequences have been searched for covariant base changes that might reflect tertiary interactions. These searches have not been confined to canonical pairing rules or even pairwise interactions: any sequence change that is always accompanied by specific changes in one or more other bases will be detected (5). A number of potential interactions have been detected in this way, and some suggest unusual structures containing pseudoknots or noncanonical pairings (6–8).

A current limitation in the prediction of rRNA tertiary structures from phylogenetic comparisons is that the functionally most interesting regions of the rRNAs tend to show little sequence divergence and few examples of covariance. Conclusions about structure then become difficult to make: a covariant change that has arisen only a few times during evolution might represent a real interaction or might simply be an evolutionary coincidence. Experimental confirmation of some of the tertiary interactions and structures predicted from phylogenetic analysis would be helpful at this time and would help establish the reliability of predictions made on the basis of rare covariances.

We have been interested in the structure of a limited domain of large subunit rRNA termed the "GTPase center," which covers approximately bases 1030–1124 in the numbering of the *Escherichia coli* rRNA. Elongation factor G, which catalyzes mRNA translocation accompanied by GTP hydrolysis, protects bases A-1067 and A-1069 within this domain from chemical modification when bound to the ribosome (9). The thiostrepton family of related peptide antibiotics binds directly to this RNA (10) and affects the elongation factor G-dependent GTPase activity: thiostrepton inhibits the activity, while micrococcin stimulates it (11). An *E. coli* ribosomal protein, L11, binds to this RNA domain from *E. coli* (12, 13) as well as to the homologous RNA from eukaryotic or archaebacterial sources (12–15). Homologues of L11 have been sequenced from yeast (16) and archaebacteria (17); the protein–RNA interaction thus appears highly conserved. Ribosomes deficient in protein L11 are still active but are stimulated in both protein synthesis and the elongation factor-dependent GTPase by addition of purified L11 (18). Elongation factor G, L11, and thiostrepton have all been located at the base of the L7/L12 stalk of the 50S subunit by immunoelectron microscopy (19). The associated RNA domain is therefore thought to help mediate mRNA translocation by elongation factor binding and GTP hydrolysis during the ribosome elongation cycle.

A 3-base tertiary interaction within this RNA domain has been proposed on the basis of phylogenetic comparisons (20). In this paper, we use the recognition of this domain by two

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ligands, L11 and thiostrepton, to assay for the correct folding of RNA fragments containing the GTPase center. By measuring the binding affinities of these ligands for sequence variants of the fragments, we show that a 2-base tertiary interaction does take place at the predicted positions, but that the 3rd base is irrelevant. This pairing greatly constrains the overall folding of the RNA domain.

MATERIALS AND METHODS

RNA fragments containing nucleotides 1029–1122 of the *E. coli* 23S rRNA sequence (referred to as 1029–1122 RNA in the text; see Fig. 1) were prepared by transcription of the plasmid pLL1 with T7 RNA polymerase as described (13). The RNA fragment contained a GG sequence at the 5′ end that is not present in the rRNA sequence. Sequence variants of this RNA were prepared by oligonucleotide site-directed mutagenesis of an M13 phage derivative of this clone; the DNA sequence was then cloned into pUC18 for transcription.

Nitrocellulose filter binding assays using purified *E. coli* ribosomal protein L11 have been described (13). Titrations of ^{35}S-labeled RNA fragments with L11 were performed under standard conditions of 30 mM Tris·HCl/175 mM KCl/2 mM $MgSO_4$, pH 7.6, at 0°C. Since protein is stored in 6 M urea and diluted and renatured just before use, the assay also contains 0.12 M urea. Filter binding assays with thiostrepton were performed under slightly different conditions (30 mM Tris·HCl/175 mM KCl/11 mM $MgSO_4$, pH 7.6, at 0°C). Because of low thiostrepton solubility in water, 2% dimethyl sulfoxide was also included in these assays. A more detailed account of thiostrepton binding to RNA fragments will be published elsewhere (P.C.R., M. Lu, and D.E.D., unpublished data). Filter retention data were fit to hyperbolic binding isotherms by a nonlinear least-squares method; both the filter retention efficiency, r_m (i.e., the extrapolated retention at infinite ligand concentration), and the binding constant, K, are variables (21).

RESULTS

Phylogenetic Evidence for a Tertiary Structure Within the GTPase Domain. Fig. 1 shows the phylogenetically conserved secondary structure of the GTPase domain from *E. coli*. Within the most highly conserved section, nucleotides 1051–1108, a number of positions have the same base in all three kingdoms. Several potential base pairs are universally conserved; they are indicated as pairs in the structure since they are adjacent to phylogenetically supported base pairs. Two pairs of bases that would appear to continue Watson–Crick base pairing in the *E. coli* sequence, U-1061/A-1077 and U-1065/A-1073, are not conserved and therefore are not marked as base pairs. A potential wobble pair between G-1056 and U-1082, which would create a continuous 9-base-pair helix with a bulge in the middle, is present in all eubacteria but becomes an A·C mismatch in eukaryotes (Table 1). Instead, U-1082 shows a consistent covariance with A-1086. The covariance is strongly correlated with kingdom (Table 1): all eubacteria have U-1082/A-1086, all eukaryotes have C-1082/G-1086, and some archaebacteria (Halobacteria and the extreme thermophile *Sulfolobus solfataricus*) have C-1082/G-1086 while the remainder have U-1082/A-1086 (see examples in Table 1). It may be that substitutions at these two positions have taken place only one or two times in this very slowly evolving region; therefore, the significance of the covariance is dubious. A recent compilation of higher-order structures detected by covariances in the rRNAs conservatively omits a 1082–1086 interaction (8).

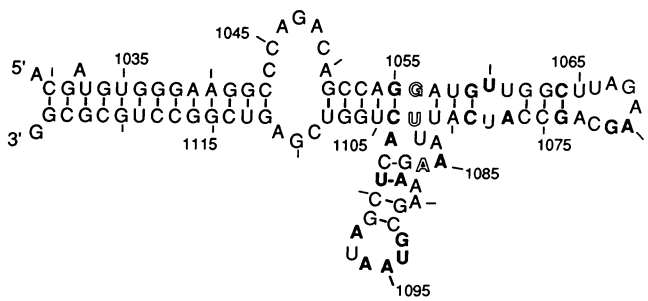

FIG. 1. Sequence of the GTPase center from *E. coli* large subunit ribosomal RNA. Tic marks are located every 5 bases; numbering is from the 5′ terminus of the mature *E. coli* rRNA. The base pairing indicated is according to the most recent phylogenetic evidence (8). L11 and thiostrepton recognize the 1051–1108 sequence within this domain, although binding studies were done with a larger RNA fragment (bases 1029–1122). Bases shown in boldface type within the 1051–1108 region are universally conserved in eubacteria, archaebacteria, and eukaryotes (22).

Egebjerg *et al.* (20) have made the intriguing suggestion that bases 1056, 1082, and 1086 form a triple-base interaction. As shown in Table 1, the base at position 1056 also differs between kingdoms: it is a G in eubacteria, an A in eukaryotes, and either A or G in archaebacteria. Egebjerg *et al.* propose that 1082 and 1086 form a Watson–Crick pair, with G-1056 in eubacteria hydrogen bonding in the major groove of the pair via G·U (HN-1···O-4) and G·A (O-6···HN-6). Eukaryotic sequences can form the isosteric hydrogen bonds A·C (N-1···HN-4) and A·G (HN-6···O-6). A weak point of the proposal is that there are exceptions to this scheme among the archaebacteria; several examples of A-1056 with U-1082·A-1086 and G-1056 with C-1082·G-1086 are known (Table 1). Egebjerg *et al.* point out that these exceptions can still form one hydrogen bond between the base at 1056 and the base pair at positions 1082 and 1086.

L11 and Thiostrepton Binding to Sequence Variants Within the GTPase Domain. We have used recognition of the GTPase domain RNA by two different ligands, ribosomal protein L11 and the antibiotic thiostrepton, as assays for folding the RNA into its native tertiary structure. Neither ligand will recognize RNA fragments smaller than bases 1051–1108, and both have a very strong requirement for Mg^{2+} or other multivalent ions for recognition to take place (12, 13, 23; P.C.R., M. Lu, and D.E.D., unpublished observations). Since multivalent ions are known to stabilize the tertiary structure of tRNA (24, 25), we have interpreted the Mg^{2+} dependence of ligand binding as a requirement that the RNA be folded correctly before ligands are able to recognize their binding sites. The melting temperature of an RNA fragment covering bases 1022–1129

Table 1. Phylogenetic variation of bases within the GTPase center RNA

Kingdom	Base 1056		Base 1082				Base 1086
Eubacteria	G	...	U	N	R	A	A
Eukaryotes	A	...	C	U	A	A	G
Archaebacteria							
Sulfolobus solfataricus	A	...	C	U	A	A	G
Methanococcus vanielii	G	...	U	U	A	A	A
Methanobacterium thermoautotrophicum	A	...	U	U	A	A	A
Halococcus morrhuae	G	...	C	U	A	A	G

Sequence conservations in 13 eubacterial or 16 eukaryotic large subunit RNA sequences compiled by Gutell and Fox (22) are summarized in the first two lines (R = G or A; N = U, G, or A). Numbering is according to the *E. coli* sequence. Individual archaebacterial sequences are shown; all other archaebacteria sequences compiled by Gutell and Fox (9 total) are identical with one of the four sequences shown.

of the *E. coli* rRNA sequence is increased by ≈20°C in the presence of Mg^{2+}, reminiscent of similar experiments with tRNA (25, 26) and supporting the idea that a Mg^{2+}-stabilized RNA structure is needed for protein binding (L. Laing and D.E.D., unpublished observations).

If the putative 1056–1082–1086 interaction is important for stabilizing the GTPase center tertiary structure, then disruption of the interaction by mutagenesis should have a major effect on both L11 and thiostrepton binding. Accordingly, a number of sequence variants in an RNA fragment encompassing bases 1029–1122 of the *E. coli* sequence were prepared and tested for ligand recognition. Representative filter binding assays are shown in Fig. 2, and relative binding constants are compiled in Table 2. A pairing interaction between bases 1082 and 1086 is supported by the data. Mutations in either base alone (U-1082 → C or A-1086 → G) drastically weaken binding of both L11 and thiostrepton, while the compensatory double mutant, which substitutes the eukaryotic C·G pair at these positions, binds both ligands with about wild-type affinity. The substitution of A-1082·U-1086 at these positions, a combination of bases that is not known to exist in nature, also showed normal binding affinities and provides strong evidence that bases 1082 and 1086 interact.

Whether G-1056 contributes to a 3-base interaction was asked by substituting either A or C at this position. C has no effect on either L11 or thiostrepton binding (within the error of the measurements), while A has a small, 2- to 3-fold effect. These results seem to argue against any contribution of base 1056 to the RNA tertiary structure, since neither C nor A can form a structure isosteric with the 3-base interaction proposed by Egebjerg *et al.* (20).

Table 2. Relative equilibrium constants for L11 and thiostrepton binding variants of the 1029–1122 RNA

RNA sequence			Relative K	
1056	1082	1086	L11	Thiostrepton
G	U	A	1.00	1.00
G	U	G	0.15	<0.1
G	C	A	<0.1	<0.1
G	C	G	0.62	1.27
G	A	U	0.70	0.84
A	U	A	0.44	0.33
C	U	A	1.11	0.77

Equilibrium constants were determined by filter binding assays. Equilibrium constants have been divided by the value for wild-type RNA (top line), 11.0 μM^{-1} for L11 and 0.79 μM^{-1} for thiostrepton. The values are the averages of at least two independent titrations. Standard errors are about ±30% for L11 titrations and ±40% for thiostrepton.

DISCUSSION

Interpretation of Covariance Data. Even though the compensatory change at bases 1082 and 1086 may have arisen only a few times during evolution, the covariance is completely consistent with our binding data and a tertiary interaction between these 2 bases is very likely. While a triple base interaction with base 1056 cannot be ruled out by our experiments, the archaebacterial exceptions to a consistent set of hydrogen bonds between bases 1082 and 1086 and base 1056 (Table 1), together with the absence of any strong effects of 1056 mutations on ligand binding (Table 2), argue against participation of base 1056 in the RNA tertiary structure. It is worth noting that a somewhat smaller data base of rRNA sequences excluding only two groups of archaebacteria (methanobacteria and halobacteria; see ref. 27) would not have shown any exceptions to the covariance of base 1056 with the base pair at positions 1082 and 1086. A thorough search of the phylogenetic tree for exceptions to a covariance seems a necessary prerequisite before the covariance is seriously considered.

Further interpretation of a covariance or compensatory change in terms of tertiary hydrogen bonding schemes presents some problems. The first is whether a covariance necessarily implies a hydrogen-bonded interaction between bases. In the case of canonical secondary structure, where changes preserving Watson–Crick complementarity may be observed at several neighboring base-pair positions, a helical segment seems the only plausible interpretation. In the cases of covariant bases that predict tertiary interactions, such as bases 1082 and 1086 or some of the predicted interactions between single bases in two different loops of the large subunit rRNA (8), might it be possible that the structural alteration caused by a mutation at one site could be compensated by a mutation elsewhere in the molecule, without a direct interaction between the two sites? An example of this phenomenon comes from studies of yeast suppressor tRNAs (28). Two mutations were found, each of which inactivates the suppressor phenotype; both are G → A mutations, which introduce an A·C mismatch in either the anticodon or the T stems. The suppressor phenotype can be restored in each case by a second mutation that changes a G·U wobble pair in

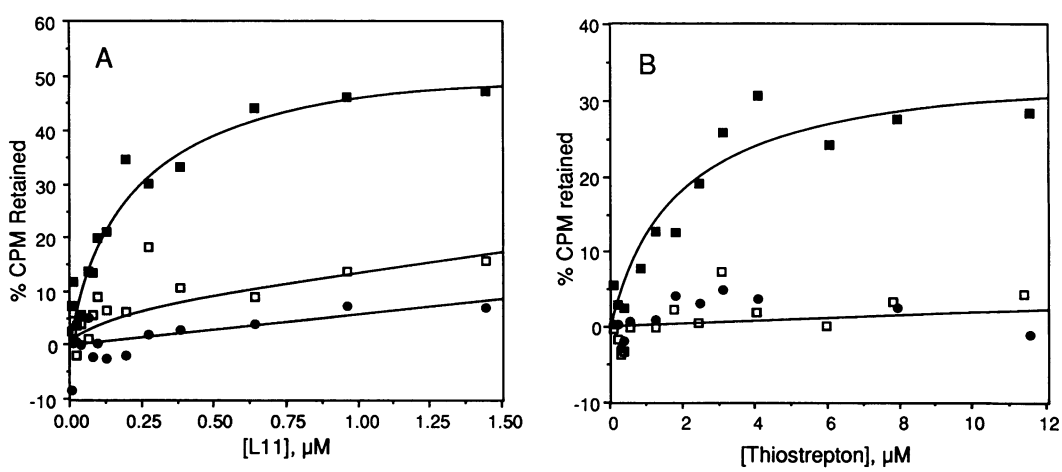

FIG. 2. Filter binding titrations of sequence variants in the 1029–1122 RNA. (*A*) L11 titration of U-1082 → C (●), A-1086 → G (□, curve calculated for K = 1.1 μM^{-1}, r_m = 0.60), and U-1082 → C + A-1086 → G (■, curve calculated for K = 5.57 μM^{-1}, r_m = 0.54) RNAs. (*B*) Thiostrepton titration of the same RNAs (symbols same as in *A*; upper line calculated for K = 0.55 μM^{-1}, r_m = 0.35). Background retentions of [^{35}S]RNA in the absence of ligands have been subtracted from the data.

the acceptor stem to an A·U pair, although this second site mutation by itself has no phenotype. It is clear from the known tRNA tertiary structure that there is no direct interaction between the bases involved. This observation certainly argues for some caution in assuming that covariant bases necessarily predict tertiary structures.

We think that the compensatory base changes at bases 1082 and 1086 reflect a direct interaction, and not some more subtle communication between different parts of the molecule, for several reasons. First, the compensatory change at bases 1082 and 1086 differs from the yeast suppressor case in that mutations of both 1082 and 1086 have nearly equal and severe effects on ligand binding, as expected if they bond together to stabilize a structure. Second, two different assays for functional folding of the RNA (L11 or thiostrepton binding) give the same result. L11 and thiostrepton bind cooperatively to the GTPase center as well as independently (23), which means that the two ligands must make completely different sets of contacts with the RNA. This argues that a fundamental feature of the RNA tertiary folding has been detected in the double mutants. Lastly, we find that pairing between bases 1082 and 1086 can be incorporated into models of the RNA tertiary structure that are reasonable in terms of the known stacking and hydrogen bonding properties of nucleotides (see below). We therefore argue that there is a direct interaction between bases 1082 and 1086, although only detection of hydrogen bonds between these bases by more direct experiments (e.g., NMR) can unequivocally demonstrate their existence.

Once a convincing case is made that a covariance reflects a direct interaction between bases, there is the further problem of whether a unique hydrogen bonding scheme can be deduced. In the case of bases 1082 and 1086, the phylogenetic covariance as well as our compensatory mutations would seem to predict either Hoogsteen or Watson–Crick pairing. However, a similar covariance in tRNA has been found that reflects a much more unusual pairing scheme. A base pair between positions 15 and 48 (yeast $tRNA^{Phe}$ numbering) was predicted by Levitt (29), since the bases are phylogenetically conserved as either G-15·C-48 or A-15·U-48. The prediction of an interaction is correct, but the crystal structure of yeast $tRNA^{Phe}$ revealed that the hydrogen bonding is actually G·C (HN-1···O-2) and G·C (HN-2···N-3) between parallel-stranded nucleotides, rather than antiparallel Watson–Crick pairing. U and A at the same positions can hydrogen bond at NH-6···O-2 (A·U) and N-1···NH-3 (A·U) to form a similar, but not precisely identical, structure. Since many more examples of unusual tertiary hydrogen bonding patterns probably remain to be discovered, it seems premature to make confident predictions of tertiary pairing schemes on the basis of compensatory base substitution patterns.

It is probably not possible for the pairing of bases 1082 and 1086, which is conserved in the same way as the tRNA pairing of bases 15 and 48, to be a parallel-stranded structure. Models can be built with bases 1082 and 1086 bonded in the same fashion as tRNA, but there is one example of a 1-base deletion of base 1083 (30). With only 2 intervening bases, it is very difficult to bring the nucleotides together with parallel strands.

A Model of the Helix Junction Within the GTPase Center RNA. If base pairing takes place between U-1082 and A-1086, then the RNA domain at bases 1051–1108 can be thought of as a junction of four helices, with one of the "helices" only 1-base-pair long, and with two unpaired nucleotides (G-1056 and A-1103) at the base of one of the other helices. A single base pair is not stable in the absence of other interactions, but U-1082·A-1086 could stack onto either A-1057·U-1081 or G-1087·C-1102. Diagrams of the two possible stacking patterns are shown in Fig. 3. We have built models of these structures and find that other hydrogen bonding interactions

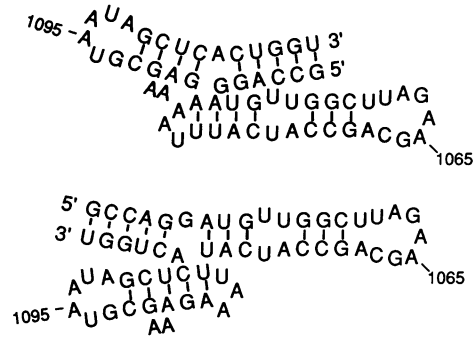

FIG. 3. Possible tertiary stacking of bases within the helix junction region of 1051–1108 RNA. The U-1082·A-1086 base pair is stacked either on A-1057·U-1081 (*Upper*) or G-1087·C-1102 (*Lower*).

are possible between universally conserved bases. The two different stacked helical segments are able to rotate with respect to each other in both models, suggestive of potential conformational transitions. At this time these models are quite speculative, but the relatively limited number of conformations possible when a base pair at positions 1082 and 1086 is included shows that this is a key interaction in determining the overall tertiary structure of this RNA domain. ^1H NMR experiments may be useful to identify a base pair at positions 1082 and 1086 and neighboring base pairs.

This proposed structure for the GTPase center RNA has some analogy to DNA four-helix junctions, which have been synthesized and studied as models for Holliday recombination junctions (31). It has been found that pairs of helices tend to stack coaxially, so that the overall structure looks like two longer helices joined to form an X (32, 33), as we propose for the RNA at bases 1051–1108. Formation of this structure requires Mg^{2+} (33), presumably because backbone phosphates come into closer contact than usual at the joint. A similar strong dependence on Mg^{2+} is seen in L11 and thiostrepton binding of the GTPase RNA (13).

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