tmRNAs that encode proteolysis-inducing tags are found in all known bacterial genomes: A two-piece tmRNA functions in *Caulobacter*

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A general mechanism in bacteria to rescue stalled ribosomes and to clear the cell of incomplete polypeptides involves an RNA species, tmRNA (SsrA), which functions as both a tRNA and an mRNA. This RNA encodes a peptide tag that is incorporated at the end of the aberrant polypeptide and targets it for proteolysis. We have identified a circularly permuted version of the tmRNA gene in α-proteobacteria as well as in a lineage of cyanobacteria. The genes in these two groups seem to have arisen from two independent permutation events. As a result of the altered genetic structure, these tmRNAs are composed of two distinct RNA molecules. The mature two-piece tmRNAs are predicted to have a tRNA-like domain and an mRNA-like domain similar to those of standard one-piece tmRNAs, with a break located in the loop containing the tag reading frame. A related sequence was found in the mitochondrial genome of *Reclinomonas americana*, but only the tRNA-like portion is retained. Although several sequence and structural motifs that are conserved among one-piece tmRNAs have been lost, the α-proteobacterium *Caulobacter crescentus* produces a functional two-piece tmRNA.

Ribosomes stall on an mRNA if the message has no stop codon or if there is no cognate tRNA available for a particular codon. To rescue stalled ribosomes and eliminate partially completed polypeptides from the cell, bacteria maintain a quality-control system mediated by an RNA known as tmRNA (alternatively SsrA or 10Sa RNA) and at least one associated protein, SmpB (1–3). tmRNA has the unique capacity to act as both a tRNA and an mRNA (Fig. 1*A*). Its termini fold into a structure that mimics the acceptor stem and T arm of alanine-tRNA and can be charged with alanine by alanyl-tRNA synthetase (refs. 4 and 5; Fig. 1). Stalled ribosomes are recognized by tmRNA, and the tRNA-like domain is used as a substrate for transpeptidation, incorporating the alanine into the incomplete protein (1, 6). The ribosome then switches from the problematic mRNA to a specialized reading frame in tmRNA without releasing the nascent polypeptide. Translation resumes at the first codon of this reading frame (the resume codon), which is specified by a unique set of determinants involving neither an initiation codon nor a Shine–Dalgarno interaction (7). The tmRNA reading frame encodes a peptide tag with hydrophobic residues at the C terminus, which targets the tagged protein for degradation. The ribosome is released at the stop codon in the tmRNA tag reading frame.

The tmRNA quality-control system is nearly ubiquitous among eubacteria; tmRNA genes have been identified in all strains tested, except for those in the α-subdivision of the proteobacteria. tmRNA was believed to be absent from this group (8), because it could not be identified in the complete genome sequence of *Rickettsia prowazekii*. In fact, we have found that tmRNA does exist in α-proteobacteria but in a circularly permuted configuration in which the sequences normally found at the 3′ end are located upstream of the 5′ sequences. We provide evidence that this gene is transcribed as a single precursor RNA, but the intervening segment between the standard 3′ and 5′ ends is quickly excised, resulting in a two-piece tmRNA. We show that the two-piece tmRNA of the α-proteobacterium *C. crescentus* is capable of tagging proteins made from damaged mRNA. We have also identified tmRNA genes with the same permuted configuration in a lineage of cyanobacteria and in a mitochondrial genome.

Materials and Methods

Northern Blots and Primer Extension. For Northern blots, total cellular RNA was purified from log-phase cultures of *C. crescentus* strain CB15N by using the hot phenol method (9). RNA was separated by electrophoresis on 2.5% agarose/20% formaldehyde gels and transferred to Nytran N+ membrane (Schleicher & Schuell) by capillary action in 20× SSC. Blots were probed as described (9) with the ^{32}P-labeled oligonucleotides: acceptor 5′-TGG TGG AGC CGC CGG GAA TCG C-3′; tag sequence 5′-CGG CGA ACT CTT CAG CGA AGT TAT CGT TCG C-3′; intervening sequence 5′-ATG CAG TCC CGT GAT AAC GC-3′; 16S RNA 5′-TGG GCG TAA AGG GAG CGT AGG CGG ACT-3′. Primer extension assays were performed according to the manufacturer's protocol by using Superscript II (GIBCO/BRL) and the primers listed above.

Ribosome Association. Crude ribosome isolation and sucrose gradient fractionation were performed by the method of Komine *et al.* (10). RNA was purified from the crude ribosomal fractions by successive extractions with phenol and chloroform followed by ethanol precipitation, and the presence of RNA species was determined by primer extension assays. RNA was precipitated from sucrose gradient fractions by precipitation in the presence of one volume of saturated sodium chloride and two volumes of ethanol, and the presence of RNA species was determined by Northern blotting.

Tagging Assay. The λ-repressor (1–93)-M2-H6-trpAt construct described by Keiler *et al.* (1) was subcloned into a broad host range vector (pMR10 from Richard Roberts, Stanford University, Stanford, CA) and transformed into *Caulobacter*, and expression of the reporter protein was induced by addition of isopropyl β-D-thiogalactoside. The SsrA-DD variant was made by site-directed mutagenesis of the last two codons of the tag reading frame of *C. crescentus ssrA* from GCTGCG to GATGAC and was supplied on a compatible plasmid (JS14 from Jeffrey Skerker, Harvard University, Cambridge, MA). Soluble protein was extracted with detergent (BPER, Pierce), and reporter protein was purified by affinity chromatography with Ni-NTA

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. AF255738 for the *C. crescentus ssrA* gene and AF251551 for the *Cyanobium gracile* PCC 6307 *ssrA* gene).

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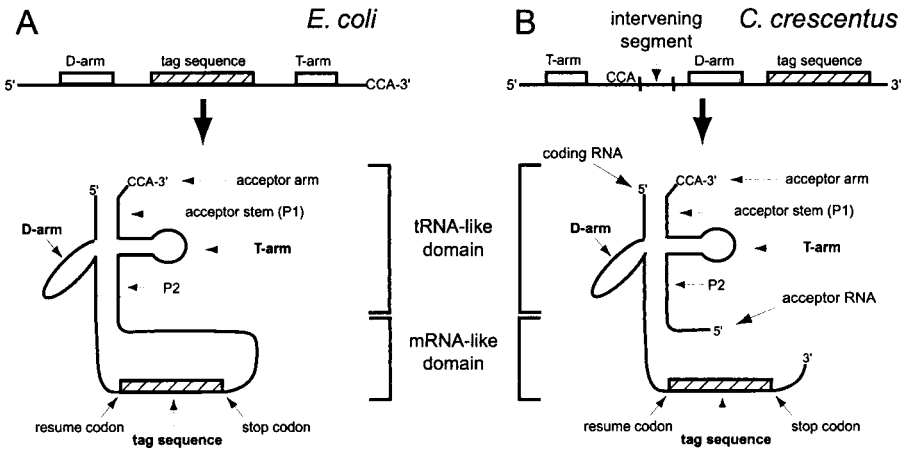

Fig. 1. Genetic and structural relationship between one-piece (*Escherichia coli*; *A*) and two-piece (*Caulobacter crescentus*; *B*) tmRNAs. Both tmRNAs contain the same functional elements, but a circular permutation in the gene of the two-piece tmRNA alters the linear order of these elements. The folded structures are both predicted to contain a tRNA-like domain and an mRNA-like domain, but in the two-piece tmRNA, this structure is formed by two distinct RNA molecules.

agarose (Qiagen, Chatsworth, CA). Western blots were probed with polyclonal anti-λ repressor antibody (a gift from Jennifer Leeds, Harvard University). Matrix-assisted laser desorption ionization time-of-flight mass spectrometry was performed on the affinity-purified proteins by the Stanford Protein and Nucleic Acid Facility.

Sequence Searches. Preliminary sequence data for *C. crescentus* strain CB15 were from the Institute for Genomic Research (Rockville, MD); we then sequenced the tmRNA gene from strain CB15N. Genomic databases were searched with the program PATSCAN by R. Overbeek (Argonne National Laboratory, Argonne, IL). Sequence data for *Sinorhizobium meliloti* strain 1021 were from S. Long and colleagues at Stanford University (http://cmgm.stanford.edu/~mbarnett/1xgenome.htm). Partial sequence data for *Methylobacterium extorquens* strain AM1 were from University of Washington (http://kandinsky.genome.washington.edu). Preliminary sequence data for *Prochlorococcus marinus* MED4 were from Department of Energy Joint Genome Institute (http://spider.jgi-psf.org/JGI_microbial/html). Genomic DNA was prepared from *Cyanobium gracile* PCC 6307 cells donated by S. Turner of Indiana University (Bloomington, IN). To test for the permuted tmRNA gene configuration, a pair of PCR primers complementary to conserved regions in the tRNA-like domain, 5′-GTCGAAACCATTACAACCCC and 5′-GTTCGATTCCGCTCAACTCCA, was designed to yield a small PCR product containing the intervening segment. A second pair of primers complementary to the above two was designed to test for the standard gene configuration. This test confirmed the permuted configuration for the *Cyanobium* gene. To generate sequence data for the DNA flanking the tRNA-like domain, the above four primers were used in two arbitrarily primed PCRs followed by nested PCR and sequencing (11). These data were used to design two new primers for a PCR from genomic DNA that yielded the final sequence data.

Results

***C. crescentus* Has a Two-Piece tmRNA.** The *C. crescentus* tmRNA gene was identified by searching genomic sequence data (provided by the Institute for Genomic Research) for a tag reading frame. A region was found that has the expected features of a tmRNA reading frame (Fig. 1*B* and see also Fig. 4*A*), including an encoded peptide (ANDNFAEEFAVAA) that is hydrophobic and resembles previously identified tag sequences (12), a resume codon consensus sequence (WAUARNYGCNAANNANNA; ref. 7), and a normal stop codon. A sequence that could form the 5′ end of the acceptor stem is upstream of the tag reading frame, but there is no T arm or 3′ acceptor arm sequence downstream. Instead, a possible T arm and acceptor arm sequence was found upstream of the putative 5′ end (Fig. 1*B*).

Northern blots of total RNA from *Caulobacter* show that both the T arm/acceptor arm (acceptor RNA) and tag reading frame (coding RNA) are expressed at high levels *in vivo* but in distinct RNA molecules (Fig. 2*A*). Primer extension and nuclease protection assays were used to define more precisely the termini of these RNAs. A primer complementary to the acceptor arm and T arm sequences extended to a single site 7 nt from a putative σ^{70} promoter (Fig. 2 *B* and *C*, "acceptor"). S1 protection and RNase protection assays confirmed the 5′ end of the acceptor RNA and precisely mapped the 3′ end to the CCA tail, 83 nt downstream (data not shown). A primer complementary to the tag sequence extended to two sites: the majority extended to the expected 5′ end of the coding RNA (Fig. 2 *B* and *C*, "tag-first stop"), and a small fraction extended farther upstream to the 5′ end of the acceptor RNA (Fig. 2 *B* and *C*, "tag-second stop"). S1 protection and RNase protection assays confirmed the 5′ end of the coding RNA and mapped the 3′ end to a position 214 nt downstream (data not shown). The rare RNA observed with the tag primer (tag-second stop) was also detected by using a primer complementary to the intervening segment (Fig. 2 *B* and *C*, "intervening"). Together, these data define at least three RNAs: the abundant coding RNA (214 nt), the acceptor RNA (83 nt), and a longer rare RNA that is likely to be the precursor of the mature two-piece tmRNA (Fig. 2*C*).

The Two-Piece tmRNA of *Caulobacter* Is Functional. We tested the ability of the two-piece *Caulobacter* tmRNA to perform functions known for the single-chain *E. coli* tmRNA: association with 70S ribosomal particles and tagging of a nascent polypeptide translated from an mRNA that lacks an in-frame stop codon (1, 10, 13). A crude ribosome preparation sedimented from a *Caulobacter* lysate contained a substantial fraction of the acceptor and coding RNAs, but the rare precursor RNA remained in the supernatant (Fig. 3*A*). Further fractionation of the ribosomes on a sucrose gradient revealed that both the acceptor and coding RNAs sediment primarily with the 70S ribosomal particles (Fig. 3*B*). To ensure that cosedimentation is due to physical association between the 70S particles and tmRNA, the 70S particles were disrupted by incubation in buffer with low magnesium concentration before fractionation (10). Under these conditions, few 70S particles were observed, and both the acceptor and

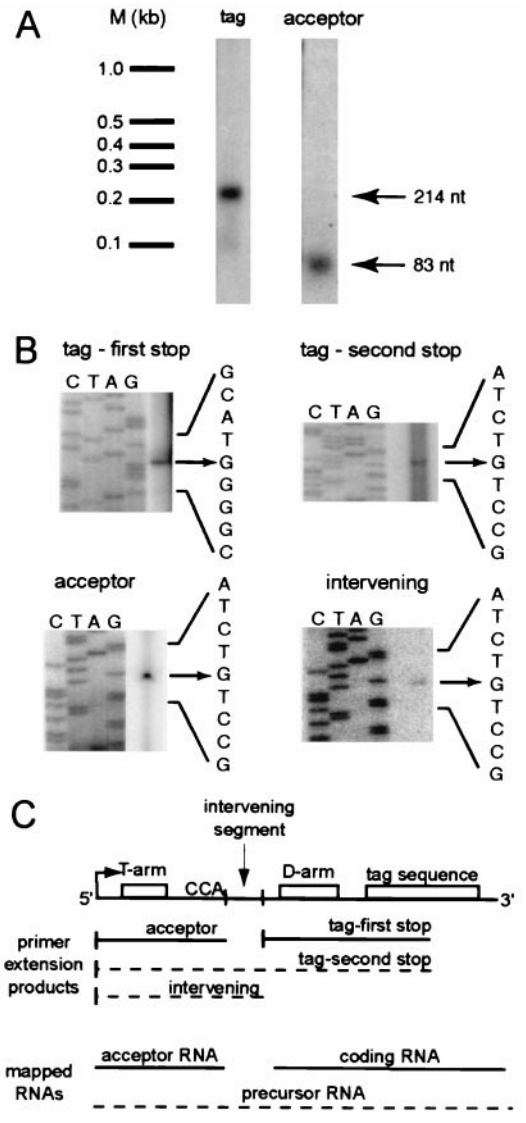

Fig. 2. Expression of *Caulobacter* tmRNA. (*A*) Northern blots of total RNA from *Caulobacter* were probed with oligonucleotides complementary to the tag reading frame (tag) or the acceptor arm (acceptor). The sizes indicated were determined by nuclease protection assays. M, size markers; kb, kilobase. (*B*) Primer extension assays of total *Caulobacter* RNA with primers complementary to the tag reading frame (tag), the acceptor arm (acceptor), and the intervening segment (intervening). Each primer extension is electrophoresed next to sequencing reactions performed with the cognate primer, and the local sequence is shown. The two panels for the tag primer are different exposures of the same extension reaction. (*C*) Schematic summary of primer extension results. The observed abundant (solid lines) and rare (dashed lines) extension products and deduced RNA species are shown relative to the tmRNA gene.

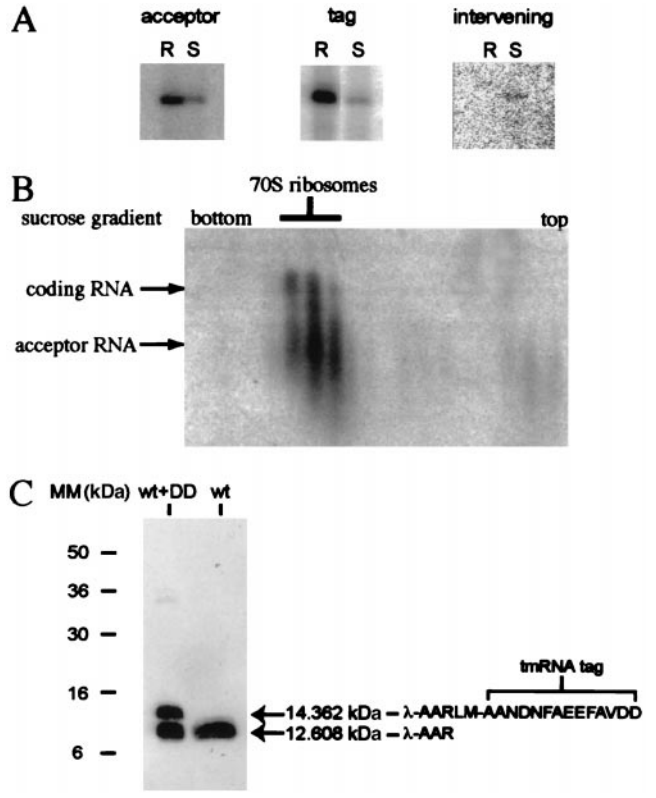

Fig. 3. The two-piece *Caulobacter* tmRNA is functional. (*A*) Ribosomal association of acceptor, coding, and precursor RNAs. The presence of each species of tmRNA in a crude ribosome preparation was assayed by primer extension on the ribosomal fraction (R) or the nonribosomal supernatant (S). (*B*) The ribosomal fraction was separated on a sucrose gradient, and the presence of the acceptor RNA and coding RNA in each fraction was assayed by Northern blotting. The fractions containing 70S ribosomes were determined by hybridization with a 16S RNA probe and are marked by the bar. The smearing of RNA bands is likely to be the result of nuclease activity in the ribosomal fraction. (*C*) Tagging activity of *Caulobacter* tmRNA. Western blot of a reporter protein expressed from a gene lacking an in-frame stop codon in wild-type *Caulobacter* (wt) or in *Caulobacter* with a plasmid-borne copy of SsrA-DD (wt + DD). The molecular masses (MM) indicated at the right were obtained from matrix-assisted laser desorption ionization time-of-flight mass spectrometry, and the corresponding proteins are shown schematically.

coding RNAs migrated at the top of the gradient (data not shown), consistent with release of tmRNA from the disrupted ribosomes. Thus, the mature two-piece tmRNA, but not its precursor, associates with whole ribosomes.

To determine whether *Caulobacter* tmRNA is capable of tagging proteins synthesized from damaged mRNA, we tested the ability of tmRNA to tag a reporter protein (a variant of phage λ-repressor) expressed from a gene with no stop codon (1). In a *Caulobacter* strain bearing only the wild-type tmRNA gene (*ssrA*), only a fragment of the reporter protein could be detected by Western blotting (Fig. 3*C*), consistent with efficient tagging leading to rapid degradation. To detect a tagged reporter protein, mutations were engineered in the predicted tag reading frame of *ssrA* that changed the two C-terminal tag residues from Ala-Ala to Asp-Asp (*ssrA-DD*). A similar change in *E. coli* ssrA inhibits proteolysis of tagged protein (14). In *Caulobacter* expressing *ssrA-DD* in addition to the wild-type gene, a band of the appropriate size for the full-length reporter protein was detected, suggesting that it was stabilized by tagging with the Asp-Asp peptide (Fig. 3*C*). The mass of the stabilized full-length protein determined by using mass spectrometry was within 1 Da of the predicted value for the reporter protein plus the peptide tag AANDNFAEEFAVDD (Fig. 3*C*). The mass of the smaller band observed in both *Caulobacter* strains is consistent with a fragment of the reporter protein and may be the result of translational termination without tagging or of tagging by the wild-type *ssrA* followed by proteolytic cleavage. These data indicate that *Caulobacter* does indeed have a tmRNA-based system for tagging proteins synthesized from damaged mRNA. Because the two-piece tmRNA can associate with 70S ribosomes, it is likely that this form is the active one *in vivo*.

Identification of Two-Piece tmRNAs in Other α-Proteobacteria, a Mitochondrion, and Cyanobacteria. Based on the permuted sequence of the *Caulobacter* tmRNA gene, we were able to identify

similar genes in other α-proteobacteria (Fig. 4*A*). Each sequence exhibits key features of tmRNA: an alanyl-tRNA-like domain and a specialized reading frame encoding a peptide of the size and sequence expected to induce proteolysis (Fig. 4 *B* and *C*). These genes were named *ssrA* in accord with the *E. coli* terminology.

The identification of a circularly permuted tmRNA gene in *Rickettsia*, which is closely related to the ancestral mitochondrion (15), prompted us to search for a similar tmRNA in the primitive mitochondrial genome of the protist *R. americana* (16). We found a tmRNA homologue that consists of the tRNA-like domain in a circularly permuted configuration. A tag reading frame was not found in the coding RNA, and homology to other tmRNAs was not observed outside of the tRNA-like domain. A normal ORF (*orf64*), with an AUG start codon and an excellent Shine–Dalgarno sequence, is located immediately downstream of the tRNA-like domain. Thus, it seems unlikely that *Reclinomonas* mitochondrial tmRNA has a peptide-tagging activity.

A permuted tmRNA sequence was identified in data from the genome project for the cyanobacterium *P. marinus* (Fig. 4*A*). Interestingly, the standard gene configuration had been found previously in a fairly close relative, *Synechococcus* PCC6301 (17). In a search for an additional example of the permuted cyanobacterial gene, a PCR test for tmRNA gene configuration was applied to an even closer relative of *Prochlorococcus*, *Cyanobium* PCC6307. The permuted configuration was observed for *Cyanobium*, and the region was sequenced (Fig. 4*A*).

Predicted Structures in α-Proteobacterial Two-Piece tmRNAs. The α-proteobacterial tmRNA sequences could be aligned with relatively few gaps (Fig. 4*A*). Based on this alignment, several pairings within and between the two mature tmRNA pieces were predicted, and some of these pairings are supported by base pair covariation (Fig. 4 *A* and *C*). The ends of the intervening segment seem to form an additional pairing that would be exclusive to the precursor. The acceptor and coding RNAs are held together by pairings that correspond to the P1 acceptor stem and the long interrupted P2 stem of standard tmRNAs (18). In standard tmRNAs, a large loop extends from the distal end of P2, containing the tag reading frame and four or more pseudoknots. The gene permutation results in an altered topology of the mature two-piece tmRNA by creating a break in this loop, downstream of the reading frame. There are no predicted pairings that would reconnect the break in the loop; little single-stranded RNA would be available in the acceptor RNA for such closure (Fig. 4*C*). The two new termini resulting from permutation are protected in stable secondary structures: the 5′ end of the acceptor RNA forms a stem with a loop from one of the tetraloop or triloop classes known to promote stability (19, 20), and the 3′ end of the coding RNA is folded into a pseudoknot (Ψ3).

It does not seem that all of the four or more pseudoknots found in other bacterial tmRNAs are retained in α-proteobacteria. As many as three pseudoknots can be predicted in some of the sequences, but strict reliance on base pair covariation in the sequences identified thus far supports only the terminal pseudoknot (Ψ3) in the coding RNA. The first pseudoknot (Ψ1) is plausible but is not supported by base pair covariation, and the least likely of these potential pseudoknots (Ψ2) would be degenerated in *Sinorhizobium* and precisely deleted in *Rickettsia*. Full elucidation of secondary structure awaits further phylogenetic analysis and chemical probing.

Discussion

Searches for tmRNAs by PCR and in genomic databases, taking advantage of the highly conserved 5′ and 3′ termini, led to the conclusion that tmRNA was not present in the α-proteobacteria. We report herein that the tmRNAs are indeed present and functional in these bacteria but in a permuted conformation with the normal 3′ sequences lying upstream of the normal 5′ sequences. In light of this unexpected plasticity in the genetic structure of the tmRNA gene, great care should be taken before a tmRNA or other RNA gene is said to be absent in genomic sequence data. With the addition of the sequences described herein, tmRNA has been found in every bacterial species that has been examined.

Each of the permuted tmRNA genes presented herein is previously undescribed, except for that of *B. japonicum.* A 213-nt RNA (named sra) was identified in *Bradyrhizobium* in a screen for mutations that prevent growth in root nodules (21). *sra* is actually the coding RNA of a circularly permuted tmRNA gene, and it is likely that the failure of *sra* mutants to colonize root nodules is caused by loss of tmRNA function. Although tmRNA is not essential for growth of *E. coli* in culture, *Bradyrhizobium* provides another example of bacteria for which tmRNA is required for all or part of the life cycle: tmRNA is required for virulent growth of *Salmonella typhimurium* (22, 23) and is essential in *Neisseria gonorrheae* (24), and *C. crescentus* (K.C.K. and L.S., unpublished observation), and possibly *Mycoplasma genitalium* (25), *Mycoplasma pneumoniae* (25), and *Synechococcus* PCC 6301 (17).

The circular permutation in the tmRNA gene presents a functional problem: although the structure should not be disturbed by the addition of a loop connecting the normal 5′ and 3′ ends, a free 3′ CCA is required for charging with alanine. We have shown that, in *Caulobacter*, this problem is solved by expressing tmRNA as two distinct RNA molecules. The primer extension and Northern blot data can be explained by the RNAs shown in Fig. 2*C*. In this model, a precursor tmRNA is transcribed, most likely by using the conserved σ^{70}-like promoter and initiating transcription at the site of the mature 5′ end of the acceptor RNA, and is processed rapidly to produce the mature acceptor RNA and coding RNA. In principle, removal of the intervening segment from the permuted precursor could be similar to processing of a standard tmRNA precursor. The endonuclease RNase P removes a leader sequence to generate the mature 5′ end in tRNAs and single-molecule tmRNAs (5). RNase P can process tRNAs that have been engineered with circular permutations (26); thus, it should be unimpeded by the internal loop in the permuted precursor tmRNA. Once RNase P or other endoribonucleases have cut the intervening segment, the exonucleases and other enzymes that normally produce the CCA tail (27) could proceed. The data do not exclude an alternative model in which the acceptor RNA is transcribed from the σ^{70}-like promoter and the coding RNA is transcribed from a distinct promoter in the intervening segment.

To maintain the tmRNA structure after processing of the precursor, the two-piece tmRNAs might be expected to contain extensive intermolecular interactions. In fact, although the P1 and P2 pairings create an interface along most of the acceptor RNA, the P2 interactions are less extensive than those observed in one-piece tmRNAs. The P1 and P2 interactions may be sufficient to stabilize the two-piece structure, or other factors such as proteins may be required.

Curiously, the α-proteobacterial tmRNAs lack some of the sequences that are found in all other known tmRNAs and most tRNAs. The primary transcript of one-piece tmRNAs and most tRNAs, including α-proteobacterial tRNAs, contains the consensus sequence UUCRANY in the T loop, and this sequence is modified to TΨCRANY (28). In tRNAs, the T loop makes tertiary interactions within itself and with a conserved sequence in the D loop. In the α-proteobacterial tmRNAs, the T loop and D loop motifs are wholly or partially degenerated. It is unlikely that the modified bases T and Ψ occur in all α-proteobacteria, because guanines usually replace both modified uracils. It is possible that the α-proteobacterial tmRNAs have evolved compensating interactions, but it is clear that neither the conserved sequence in the T loop nor the associated base-modifications are universally required for tmRNA function. Likewise, the changes observed in the α-proteobacterial T loop sequence are not generally required for the two-piece tmRNA composition, be-

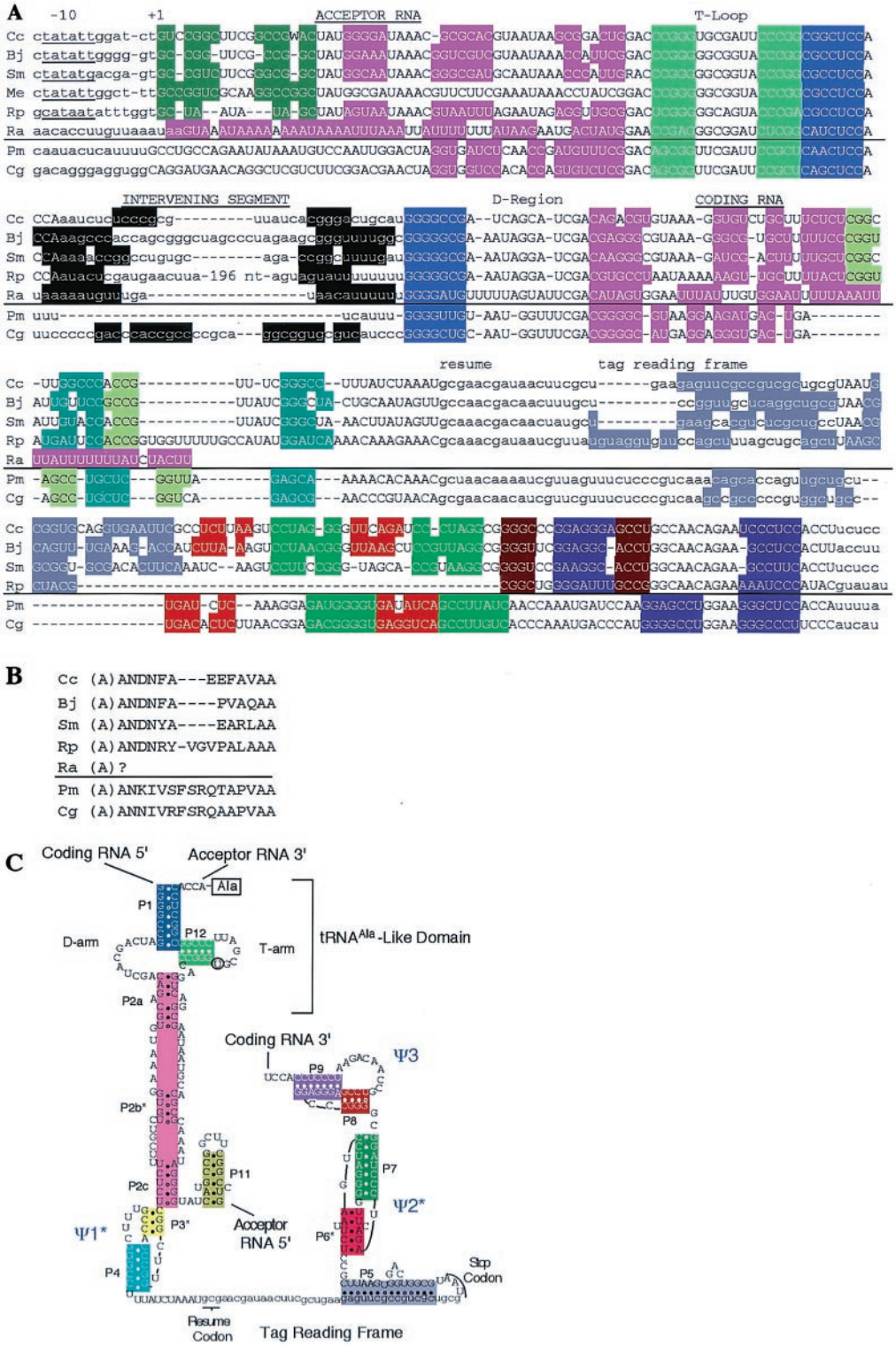

Fig. 4. Permuted tmRNA genes. (*A*) Alignment. Sequences from the α-proteobacterial lineage (including the mitochondrial sequence, Ra) are shown above the line in each section; those from the cyanobacterial lineage are shown below the line. Color coding marks potential base pairing; note that some marked pairings are not confirmed by base pair covariation. Lowercase is used to represent 5′ and 3′ segments that are predicted to be absent in mature tmRNAs, intervening segments, and tag reading frames. Likely −10 promoter sequences are underlined. Intrastrain sequence variation (ambiguously called bases) is suggested by conflict in equally reliable sequence data for *C. crescentus* and *S. meliloti*. Abbreviations for sequence data: Cc, *C. crescentus*; Bj, *Bradyrhizobium japonicum*; Sm, *S. meliloti*; Me, *M. extorquens*; Rp, *R. prowazekii*; Ra, *Reclinomonas americana* mitochondrion; Pm, *P. marinus*; Cg, *C. gracile* PCC 6307. An enhanced version of this alignment is displayed at www.indiana.edu/~tmrna. (*B*) Encoded peptide tags. Because no tag-like sequence is found in *Reclinomonas*, it is unclear whether it donates its charging alanine (in parenthesis). (*C*) Predicted secondary structure of *Caulobacter* tmRNA. Pairings are numbered by possible analogy with standard tmRNA; those not supported by base pair covariation are marked with asterisks, as are the corresponding pseudoknots (Ψ) that would contain them. The U, which may be modified to T in the T arm, is circled.

cause the cyanobacterial two-piece tmRNAs have the canonical T loop and D loop sequences.

Circular permutation has been recognized recently in the evolution of several protein-coding genes (29), but the only previous observation in an RNA gene is for the large subunit rRNA in the *Tetrahymena pyriformis* mitochondrion (30, 31). Strikingly, two independent yet similar circular permutation events seem to have produced the permuted tmRNA genes in α-proteobacteria and cyanobacteria. The cyanobacteria *P. marinus* and *Cyanobium* PCC 6307 are very closely related according to rRNA sequences (32), and both have permuted tmRNA genes. However, their near relative *Synechococcus* PCC 6301 has its tmRNA gene in the standard configuration, as do all other tested cyanobacteria (Fig. 4*D*). Despite the two different configurations, these three cyanobacterial tmRNA genes show remarkable conservation in the tRNA-like domain (differing at only 3 of its 47 positions) and in the encoded peptide tag. These conserved features contrast distinctly with the corresponding features in α-proteobacteria (Fig. 4*A*). In particular, the T loop sequence matches that of tRNAs and standard tmRNAs, not that of α-proteobacteria. Thus, it seems likely that there were two independent tmRNA gene permutation events and not lateral transfer between the groups.

The mechanism usually proposed for gene permutation is tandem duplication of all or part of the gene, followed by degeneration of duplicated outer segments (29). For the tmRNA gene, viruses may have been agents of these permutation events, because integrating bacteriophages use its 3′ end as an attachment site in several bacteria (22, 23, 33–36). Particularly for the more recent cyanobacterial rearrangement, further comparative analysis may allow a fairly precise reconstruction of the molecular events of gene permutation.

Because permuted tmRNA genes have been conserved in at least two independent lineages, it is likely that there are benefits from a two-piece construction that offset possible costs to stability. One possibility is that folding is faster in the permuted precursor. The P1 and P2 stems are formed by opposite ends of the standard tmRNA precursor, but in the permuted precursor, they are much closer together. The formation of the predicted stem within the intervening segment may provide additional folding rate enhancement. A second possibility is that the pieces have distinct activities independent of each other, with separation allowing for independent regulation. Another benefit from a two-piece construction may relate to the topology of tmRNA translation. The tag reading frame in standard tmRNA is part of a large loop, the topological equivalent of a circular mRNA if P2 and P1 do not unwind during translation. With the reading frame engaged in the mRNA track of the small subunit, the remainder of the RNA circle must either wrap around the outside of the ribosome or double back within the subunit interface. It is a possibility that pseudoknots provide structural plasticity that enables the standard tmRNA to be translated. If such plasticity exists, the two-piece tmRNAs may have found an alternative solution to the topological problem by breaking the loop and presenting the reading frame as a linear molecule.

The tmRNA homologue of *R. americana* is, to our knowledge, the first to be identified in mitochondria. Its descent from the tRNA-like domain of the α-proteobacterial tmRNA gene is evident (Fig. 4*A*); however, because there is no associated tag reading frame, it is very unlikely that message-switching activity is retained. On the other hand, some residual or previously unidentified activity is suggested for the *Reclinomonas* tmRNA by its intact tRNA-like domain, complete with determinants for charging with alanine and by the abrupt halt to tmRNA homology at the end of the this domain. Alternate mechanisms for ribosome release and/or nascent polypeptide degradation may have evolved that do not require mRNA-like function. For example, release factors or proteases could be recruited directly to the ribosome-tmRNA complex, or transfer of a single alanine residue from the tRNA-like domain of tmRNA to the protein may be sufficient for proteolysis. Identifying additional mitochondrial homologues and mapping RNA ends should help resolve the status of this unusual tmRNA variant. This work also renews interest in searching for tmRNA-related genes in archaeal and eukaryotic nuclear genomes.

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