Hybrid *E. coli*—Mitochondrial ribonuclease P RNAs are catalytically active

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ABSTRACT

RNase P is a ribonucleoprotein that cleaves tRNA precursors at their 5'-end. Mitochondrion-encoded RNA subunits of mitochondrial RNase P (mtP-RNA) have been identified in jakobid flagellates such as *Reclinomonas americana*, in the prasinophyte alga *Nephroselmis olivacea*, and in several ascomycete and zygomycete fungi. While the structures of ascomycete mtP-RNAs are highly reduced, those of jakobids, prasinophytes, and zygomycetes retain most conserved features of their bacterial counterparts. Therefore, these mtP-RNAs might be active in vitro in the absence of a protein subunit, as are bacterial P-RNAs. Here we present a comparative structural analysis including seven newly characterized jakobid mtP-RNAs. We investigate ribozyme activities of mtP-RNAs and find that even the most bacteria-like molecules of jakobids are inactive in vitro. However, when certain domains of jakobid and *N. olivacea* mtP-RNAs are replaced with those from *Escherichia coli*, these hybrid RNAs show catalytic activity. In vitro mutagenesis of these hybrid mtP-RNAs shows that various structural elements play a critical role in ribozyme catalysis and provide further support for the presence of these elements in mtP-RNAs. These include GNRA tetraloops in helix P14 and P18 of *Jakoba libera*, and a remnant P3 pairing in *Seculamonas ecuadoriensis*. Finally, we will discuss reasons for the failure of mtP-RNAs to show catalytic activity in the absence of P-proteins based on our mutagenesis analysis.

Keywords: jakobids; mitochondria; RNase P; RNA secondary structure; tetraloop motifs

INTRODUCTION

RNase P is an endonuclease that cleaves 5' leader sequence from tRNA precursors. It is usually composed of an RNA component (P-RNA) and one or several protein subunits (P-proteins) (Altman et al. 1995; Frank and Pace 1998; Hartmann and Hartmann 2003).

P-RNA genes (*rnpB*) have been identified in numerous species from all domains of life, also including plastids (Brown 1999) and mitochondria (Seif et al. 2003, 2005). When present, the P-RNA subunit seems indispensable for the endonucleolytic activity of RNase P (e.g., Stark et al. 1978; Gardiner and Pace 1980; Kline et al. 1981; Hollingsworth and Martin 1986; Jayanthi and Van Tuyle 1992; Baum et al. 1996; Lee et al. 1996a; Han et al. 1998;

Vainauskas et al. 1998; Thomas et al. 2000; Stathopoulos et al. 2001). In all tested bacteria and some archaeobacteria (Methanobacteria, Thermococci, and Halobacteria), even P-RNA alone is able to catalyze 5' tRNA processing in vitro, in the absence of a P-protein (Guerrier-Takada et al. 1983; Gardiner et al. 1985; Pannucci et al. 1999; Wagner et al. 2001).

Comparative studies suggest that P-RNAs from all domains of life are homologs, sharing up to five sequence motifs (CRI-V; Fig. 1; Chen and Pace 1997), a cage-shaped pseudoknot (pairings P1–P4) (Forster and Altman 1990), a four-way junction region including pairings P7–P10, and a large internal loop including P11–P14 (Chen and Pace 1997). *Escherichia coli* P-RNA (M1 RNA) has been studied in much detail. Secondary and tertiary RNA structure models have been established based on in vitro mutagenesis, enzymatic probing, and cross-linking experiments (Brown et al. 1996; Westhof et al. 1996; Haas and Brown 1998; Massire et al. 1998). Fe(II)-EDTA protection experiments demonstrate that *E. coli* (and *Bacillus subtilis*) P-RNAs can be dissected into two modules with independent tertiary architectures (Loria and Pan 1996). The

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FIGURE 1. Secondary structure models of bacteria-like mtP-RNAs. (A) Minimum bacterial consensus (Brown et al. 1994) and mtP-RNA secondary structure models for *R. americana* #50394, *N. olivacea* (Turmel et al. 1999) and *Rhizopus oryzae* (Seif et al. 2005). The S-domain is shown in red, and the C-domain is in black. Universally conserved motifs are presented as described by (Chen and Pace 1997). The arrows pinpoint experimentally determined RNA termini; the arrow length is proportional to the percentage of molecules ending at a given position. (B) Secondary structure models for mtP-RNAs of the jakobids *Histon aroides* #50634, *R. americana* #50283, *R. americana* #50284, *J. eucadoriensis*, *J. libera*, and *J. bahamiensis*. In *R. americana* #50284, nucleotides in green denote the few sequence differences compared to *Reclinomonas* sp. #50633. Positions in red are invariant or highly conserved in the minimum bacterial consensus. Uppercase letters indicate 100%; lowercase, >80% conservation of the minimum bacterial consensus sequence. Loops in green (L14 and L18) are GNRA motifs tested by mutational analysis (see Fig. 4). Potential L18/P8 interactions are shown by broken lines.

specificity, or S-, domain binds the T-stem-loop region of a pre-tRNA substrate, whereas the catalytic, or C-, domain carries the active site that consists of the pairing P4 and the junction J3/4 between P3 and P4 (Fig. 1; Guerrier-Takada and Altman 1992; Christian et al. 2000; Kaye et al. 2002; Tanaka et al. 2004a). In most bacteria including *E. coli*, the C-domain further interacts with the 3' terminal RCCA sequence of the tRNA via a GGU motif in the internal loop between P15 and P16 (Kirsebom and Svard 1994; Kufel and Kirsebom 1998; Brannvall et al. 2004). Phylogenetic comparative studies and cross-linking experiments in *E. coli* suggest that C- and S-domains interact through tertiary interactions of GNRA tetraloops (L18/P8 and L9/P1) (Brown et al. 1996; Massire et al. 1997), and that similar interactions occur within the S-domain (L14/P8 and L13/P12). These interactions are confirmed in a recent study of the crystal structure of P-RNA from the bacterium *Thermotoga maritima* (Torres-Larios et al. 2005). Tetraloops of the GNRA-type confer thermodynamic stability on short

RNA double helices (Heus and Pardi 1991), and stabilize tertiary structures of large RNAs by intramolecular interactions (Jaeger et al. 1994; Murphy and Cech 1994; Cate et al. 1996). GNRA motifs also contribute to the catalytic activity of P-RNA, as shown by in vitro mutagenesis of the *E. coli* molecule: Substitution of certain GNRA by UNGC tetraloops significantly reduces P-RNA activity (Pomeranz-Krummel and Altman 1999).

Across eukaryotes, secondary structure models of mitochondrial P-RNAs (mtP-RNAs) deviate to various degrees from the established minimum bacterial consensus (Fig. 1A; Lang et al. 1997), reflecting consecutive losses of structural elements from an ancestral α -proteobacterium-like P-RNA (Seif et al. 2003, 2005). In budding yeasts and in the zygomycete *Smittium culisetiae*, secondary structures of mtP-RNAs are reduced to two elements only: P1 and P4 (Seif et al. 2003, 2005; Talla et al. 2005). In contrast, a bacteria-like mtP-RNA structure is found in the bacterivorous protist *Reclinomonas americana* (member of the

jakobid flagellates), which also possesses the most bacteria-like mitochondrial genome (Lang et al. 1997; Jacob et al. 2004). *Reclinomonas* mtP-RNA not only contains all structural elements of the minimum bacterial consensus but also a P19 helix otherwise typical for α -proteobacterial P-RNAs (Fig. 1A,B). P19 is otherwise only present in the mtP-RNA of the (green algal) prasinophyte *Nephroselmis olivacea* (Turmel et al. 1999).

Whereas mtP-RNAs have been well studied in various fungi (Shu and Martin 1991; Wise and Martin 1991; Lee et al. 1996b; Seif et al. 2003, 2005), little is known about the bacteria-like mtP-RNAs in jakobids or *N. olivacea*. Jakobids contain the most bacteria-like mitochondrial genomes in eukaryotes. Studying this lineage provides an insight into the early stages of mitochondrial evolution and, in particular, that of mtRNase P. It is unknown whether these mtP-RNAs, which have been assigned based on in silico analysis, are transcribed in vivo, and if so, whether they are catalytically active in vitro, in the absence of a protein subunit. In this study, we conducted comparative structural analyses of jakobid and *N. olivacea* mtP-RNAs, mapped 5' and 3' termini for *R. americana* and *N. olivacea* mtP-RNAs, and tested the in vitro activity of the wild-type and various mutagenized hybrid molecules.

RESULTS

Structural variation among jakobid mtP-RNAs

Bacteria-like mtP-RNA have been previously identified in the jakobid *R. americana* #50394 (Lang et al. 1997), in *N. olivacea* (Turmel et al. 1999), and in zygomycetes (Seif et al. 2005). To obtain additional data for the comparative analysis of this RNA molecule, we sequenced mitochondrial DNAs (mtDNAs) of seven other jakobids: *R. americana* #50283, *R. americana* #50284, *Reclinomonas* sp. #50633, *Histiona aroides*, *Seculamonas ecuadoriensis*, *Jakoba libera*, and *Jakoba bahamiensis* (G. Burger and B.F. Lang, unpubl.). We located *rnpB* genes in all jakobid mtDNAs, initially by searching for the conserved motifs CRI, CRIV, and CRV, the pseudo-knot P4, and the P1 pairing (Forster and Altman 1990; Chen and Pace 1997; Seif et al. 2003). (The multiple alignment of these sequences is available at <http://megasun.bch.umontreal.ca/People/lang/rnpB/secondarystructuremodels.html>).

The complete predicted mtP-RNA secondary structures (Fig. 1B) are similar to those of *R. americana* #50394 (Lang et al. 1997), containing all structural elements of the minimum bacterial consensus. The only minor deviation is a highly reduced P3 in *S. ecuadoriensis* (Fig. 1B). Following a comparative analysis of jakobid mtP-RNAs, we updated the previously published secondary structure model for *R. americana* #50394 (Lang et al. 1997) by extending the P18 helix and by assigning a GNRA tetraloop in L18 that is conserved in jakobids (except in *S. ecuador-*

iensis and *H. aroides*) (Fig. 1B). Note that P19 is present in jakobids (except *J. bahamiensis*) and in the prasinophyte mtP-RNAs.

To verify that the in silico predicted *rnpB* genes of a jakobid representative (*R. americana* #50394) and *N. olivacea* are expressed in vivo, we mapped their precise termini by ligation of 5' and 3' ends, RT-PCR amplification, and sequencing of the amplification products. In both cases, the 5' and 3' extremities are moderately heterogeneous and match the ends inferred from the predicted secondary structure (Fig. 1A; Lang et al. 1999; Turmel et al. 1999).

Native mtP-RNAs are catalytically inactive in vitro

We tested the catalytic in vitro activity of seven mtP-RNAs, which contain all or most of the structural elements as defined in the minimum bacterial P-RNA consensus (Fig. 1A), i.e., the molecules of the jakobids *R. americana* #50394, *S. ecuadoriensis*, *J. libera*, and *J. bahamiensis*, the prasinophyte *N. olivacea*, and the zygomycetes *Rhizopus oryzae* and *Rhizopus oligosporus* (Seif et al. 2005). The catalytic activity of the in vitro synthesized mtP-RNAs was tested using RNA precursors of mitochondrial tRNA^{Pro} from *R. americana* and tRNA^{Lys} from *R. oryzae* as substrates. None of the listed mtP-RNAs exhibit a catalytic activity under any assay condition in which the similarly "reduced" archaeal or synthetic "micro" RNase P RNAs are active (Siegel et al. 1996; Pannucci et al. 1999), including a wide range of ribozyme concentrations, extreme salt concentrations, and addition of PEG. The activity of the *R. americana* #50394 mtP-RNA was also tested in the presence of the *E. coli* protein subunit and in an in vivo complementation assay using an *E. coli* mutant with a defective (thermosensitive) P-protein (Baer et al. 1989). These assays also turned out negative (results of both experiments not shown).

Fusions of mtP-RNAs with *E. coli* P-RNA are catalytically active

To locate specific structural elements that are responsible for the inactivity of mtP-RNAs, we constructed chimeric RNA molecules by combining half molecules of *E. coli* and mtP-RNAs of the jakobids *R. americana* (ATCC 50394), *J. libera*, and *S. ecuadoriensis*, the prasinophyte *N. olivacea*, and the zygomycete *R. oryzae*, which has the least derived structure among Fungi (Seif et al. 2005).

C-type chimeras contain the most conserved structural elements of mtP-RNAs and their equivalent of the catalytic C-domain (Fig. 1A), including P1–P4, P18, and P19; the remaining elements are from *E. coli* (Fig. 2A). C-type chimeras possess various degrees of in vitro activity, highest in *J. libera*, and followed in order by *R. americana*, *S. ecuadoriensis*, and *N. olivacea* (Table 1; Fig. 2B,C). The

FIGURE 2. Activity assays of C-type chimeras. (A) Secondary structure model for C-type chimeras. The *E. coli* domain is shown in blue, and the mitochondrial domain is in black. Green nucleotides followed by upward arrows denote mutations that increase the catalytic activity of the chimera (see also Table 2). Red nucleotides followed by downward arrows indicate mutations that decrease the catalytic activity of the chimera (see also Table 2). Δ P19, deletion of the P19 helix. (B) Catalytic assays of C-type chimeras with pre-tRNA^{Pro}, under conditions optimal for archaeobacterial P-RNAs. The substrate (1000 dpm) was incubated at 37°C for 18 h in the presence of 100 nM ribozyme in 300 mM MgCl₂, 2.5 M ammonium acetate (NH₄OAc), 50 mM Tris (pH 8), 0.1% SDS, and 0.05% Nonidet P-40. All tested constructs except the *Rhizopus oryzae* chimera are active. The positive control is with *E. coli* P-RNA (10 nM); the negative control contains substrate tRNA precursor only. (C) Catalytic assays of C-type chimeras with pre-tRNA^{Pro}, under conditions optimal for bacterial P-RNAs (50 mM Tris at pH 7.5, 100 mM NH₄Cl, 110 mM MgCl₂). The ribozyme concentrations were as in Figure 2B. Under these conditions, only the *J. libera* and *R. americana* #50394 chimeras are catalytically active.

chimera containing *R. oryzae* mtP-RNA is inactive. In S-type chimeras, the catalytic C-domain is from *E. coli*, and the residual elements are from mitochondrial molecules (Fig. 3A). In this configuration, the *R. americana* construct has the highest catalytic activity, followed by *J. libera* and *S. ecuadoriensis*, while that of *N. olivacea* is inactive (Fig. 2B,C). S-type chimeras are only active at high salt concentrations and require ~ 10 times higher ribozyme concentrations to achieve levels of activity comparable to C-type constructs (Fig. 2B,C).

In the presence of 1 nM of *E. coli* P-protein and 0.1 nM of chimera P-RNA, a slight activity increase was only detected in S-type chimera of *R. americana*. When the P-protein and P-RNA concentrations were raised to 5 nM and 0.5 nM, respectively, activities of S-type chimeras increased strongly for the *R. americana* and moderately for *J. libera* (Fig. 3C).

Both C- and S-type chimeras of *R. americana* are able to complement an *E. coli* mutant with a defective (thermo-

sensitive) P-protein at the nonpermissive temperature (Baer et al. 1989), demonstrating that the chimeric RNAs are not only active under the nonphysiological in vitro conditions but also in vivo (Fig. 4).

Mutations in GNRA tetraloops reduce catalytic activity

GNRA tetraloops, which are highly conserved and functionally important in bacterial P-RNAs (Brown 1999; Pomeranz-Krummel and Altman 1999), are only present in some jakobid mtP-RNAs (L18 in *Reclinomonas* and *Jakoba* species, L14 in *Jakoba* species, and L9 in *Histiona aroides*) (Fig. 1). In *E. coli*, mutations in the L14 and L18 GNRA motifs lead to the loss of catalytic activity at low (<20 mM) MgCl₂ concentration (Pomeranz-Krummel and Altman 1999). A corresponding mutation in *J. libera* C-type chimera has a similar effect: A GUAA to AUAA mutation in the L18 tetraloop has no significant effect on activities measured at 100 mM MgCl₂ or higher (data not shown), but catalysis drastically decreases at low salt concentration (~ 20 mM MgCl₂) (Fig. 5A). A mutation of the L14 tetraloop in *J. libera* from GAGA to GAGG or AAGA (applied to the S-type chimera) significantly lowers the activity even at 300 mM MgCl₂ (Fig. 5B). In turn, introduction of a GNRA motif in L9 of the

R. americana S-type chimera increases its activity by more than twofold (Fig. 5C).

Functionally important P3 remnant in mtP-RNA of *S. ecuadoriensis*

P3 is found in all bacterial P-RNAs (Tanaka et al. 2004b) and in jakobid mtP-RNAs, varying in length between 5 and 8 bp, except in *S. ecuadoriensis*, where only a single A–U pair is predicted (Fig. 1B). To verify the functional significance of the postulated remaining A–U pair, it was mutated to either an AxA mismatch or a C–G base pair (Fig. 6B). At 300 mM MgCl₂, the catalytic activity was high in all three cases. However, at 100 mM MgCl₂, the activity decreases more than twofold in the AxA mutant but remains unaffected in the C–G double mutation (Fig. 6). These results show that the remaining, single P3 Watson–Crick base pair in the *S. ecuadoriensis* mtP-RNA plays a critical role in the molecule's function.

TABLE 1. Summary of species-specific catalytic activity for C- and S-type chimeras

Species	Catalytic activity		
	C-type chimera high salt conditions 300 mM MgCl ₂ 2.5 M CH ₃ COONH ₄ (50 mM Tris at pH 8, 0.1% SDS, and 0.05% NP-40)	C-type chimera intermediate salt conditions 100 mM MgCl ₂ 100 mM NH ₄ Cl (50 mM Tris at pH 7.5)	S-type chimera high salt conditions 2.5 M CH ₃ COONH ₄ (50 mM Tris at pH 8, 0.1% SDS, and 0.05% NP-40)
<i>R. americana</i>	+	+	+
<i>J. libera</i>	+	+	+
<i>S. ecuadoriensis</i>	+	—	+
<i>N. olivacea</i>	+	—	—
<i>R. oryzae</i>	—	—	N.C.

These results are a summary of experiments shown in Figures 2, B and C, and 3B. (+) indicates detection of a positive result; (—), of a negative result. N.C. (not constructed), no S-type chimera was designed for *R. oryzae* due to the high deviation in the secondary structure of the S-domain of its mtP-RNA.

Other structural elements with impact on the catalytic activity of mtP-RNAs

We further tested the impact of other features of jakobid mtP-RNAs, such as the presence of P19, a structural remnant from the α -proteobacterial ancestor (except in *J. bahamiensis*). The deletion of P19 from the *R. americana* C-type chimera causes only an insignificant decrease of the initial catalytic activity (Table 2). This observation agrees with the evolutionary volatility of this variably present helix.

To further elucidate the failure of bacteria-like mtP-RNAs to show catalytic activity in vitro in the absence of the protein subunit, we tested three different features encountered frequently in mtP-RNAs: first, the impact of deviations from the bacterial consensus sequence that are often noticed in mtP-RNAs (Seif et al. 2003); second, the impact of absence of the GGU motif in L15 that is responsible for binding of the 3' terminal RCCA in most bacterial tRNAs; and finally, the impact of the high A + U

content, which in helical regions diminishes mtP-RNA stability.

The replacement of A292 in CRV of C-type chimera of *R. americana* with a G (preferred in 90% of bacterial mtP-RNAs) increases the catalytic activity more than twofold at low MgCl₂ concentrations (Table 2). Replacing the GGU motif by CCA (Fig. 2A) causes a twofold decrease in C-type chimera activity (Table 2), inferring that the absence of this motif is a handicap for the ribozyme activity of mtP-RNAs. Finally, augmenting the G + C content in P4 of a C-type chimera by replacing two A–U with G–C pairs (Fig. 2A), results in a twofold increase of the catalytic activity at an intermediate salt concentration (Table 2).

DISCUSSION

We have shown that even the most bacteria-like mtP-RNAs are catalytically inactive in vitro, which is likely due to the cumulative effect of (1) their relatively high A + U content

TABLE 2. Effect of mutations in *R. americana* C-type chimera

Mutations	Percentage of cleaved substrate		
	High salt conditions 300 mM MgCl ₂ (50 mM Tris at pH 8, 0.1% SDS, and 0.05% NP-40)	Intermediate salt conditions 100 mM MgCl ₂ 100 mM NH ₄ Cl (50 mM Tris at pH 7.5)	Low salt conditions 20 mM MgCl ₂ 100 mM NH ₄ Cl (50 mM Tris at pH 7.5)
C-type chimera	72%	15%	5%
L15/16-CCA	37%	—	—
Δ P19	57%	—	—
P4 G + C rich	73%	30%	7%
A-G 292	82%	13%	13%

L15/16-CCA, mutation of GGU motif to CCA; Δ P19, deletion of P19; P4 G + C rich, exchange of A–U base pairs by G–C pairs in P4; A–G 292, mutation of A292 to G in CRV. Positions of these mutations are shown in Figure 2A. Assays were performed as described in the Materials and Methods section, and the reaction buffers are described in the table. The values shown in the table correspond to averages of four measures for C-type chimeras, three measures for mutants at low salt conditions, and two measures for the other mutants. Errors were calculated as the standard deviation of four measures of C-type chimera and three measures for mutants at low salt concentration (3% in all instances).

FIGURE 3. Activity assays of S-type chimeras. (A) Secondary structure model for S-type chimeras. The *E. coli* domain is shown in blue, and the mitochondrial domain is in black. Green nucleotides followed by upward arrows represent mutations that cause an increase in the catalytic activity of the chimera. (B) Catalytic assays of S-type chimeras with pre-tRNA^{Pro} and pre-tRNA^{Lys}, at salt conditions optimal for archaeobacterial RNase P. Substrate (1000 dpm) was incubated at 37°C for 18 h in the presence of 1 μM of ribozyme, in 300 mM MgCl₂, 2.5 M NH₄OAc, 50 mM Tris (pH 8), 0.1% SDS and 0.05% Nonidet P-40. *J. libera* and *R. americana* #50394 chimeras have the highest catalytic activity, followed by *J. libera* and *S. ecuadoriensis*; the *N. olivacea* construct is inactive. (C) Catalytic assays of S-type chimeras in the presence of *E. coli* P-protein. The assays were performed as described in Materials and Methods except that the concentrations of P-RNAs and P-protein were fivefold higher.

and low thermodynamic stability; (2) the absence of certain GNRA motifs, reducing the overall strength of tertiary interactions; (3) the absence of the GGU motif in L15/L16, which is involved in substrate binding of the *E. coli* molecule; and (4) deviations from the catalytically important bacterial consensus sequences (as demonstrated here with CRV). Any of these factors alone is not responsible for the lack of activity of mtP-RNAs, since also a “micro P-RNA” of *E. coli* lacking several GNRA motifs and P6 (Siegel et al. 1996) is still catalytically active and because minor deviations from the consensus sequences are also tolerated in bacterial P-RNAs (Rasmussen and Nolan 2002).

MtP-RNAs are catalytically active in vitro, in *E. coli*/mtP-RNA chimeras

The jakobid and *N. olivacea* mtP-RNAs retain sufficient structural features that allow restoration of their activity following partial replacements with *E. coli* sequences. The activities of these chimeras essentially correlate with (1) the overall degree of structural conservation of mtP-RNAs (with respect to their bacterial counterparts) and (2) the presence of features that

stabilize RNA structure, such as high G + C content in helices, and tetraloops.

Both types of chimeras are catalytically active in jakobids. Yet, C-type chimeras are an order of magnitude more active than S-type chimeras, likely because their mitochondrial C-domain is most conserved. In fact, the S-domain of mtP-RNAs is highly variable in all species, especially in fungi, where some structural elements have been lost several times independently in the course of mitochondrial evolution (Seif et al. 2003). It is therefore not surprising that the *N. olivacea* C-type chimera is active, but that the S-type chimera lacks catalytic activity because its S-domain lacks P13, has a very short P14, and contains a 30-nucleotide (nt) insertion between P5 and P7.

Potential tertiary interactions and stability of secondary structure in mtP-RNAs

We note that the *J. libera* C-type chimera has the highest activity among all chimeras, which we attribute to the relatively high G + C content in the helical regions of its mtP-RNA. This view is supported by an experiment with the *R. americana* C-type chimera in which two A–U pairs in P4 are

exchanged by G–C pairs, resulting in an increase of catalytic activity (Table 2).

In addition, tertiary interactions mediated by GNRA motifs are crucial for the correct folding of P-RNAs into functional molecules (Pomeranz-Krummel and Altman 1999). Results from mutational experiments conducted on mitochondrial GNRA tetraloops are in line with this observation, suggesting possible long-range interactions with P8, as in *E. coli* and *Thermotoga maritima* (Brown et al. 1996; Torres-Larios et al. 2005): Their elimination in L14 and L18 of S-type and C-type chimeras of *J. libera* diminishes catalytic activity, whereas mutation of the L9 tetraloop sequence AAAA to GAGA in S-type chimera of *R. americana* results in an increase.

Importance of P-proteins for mitochondrial RNase P activity

All tested mtP-RNAs are catalytically inactive in vitro and, in this respect, resemble those from eukaryotes (i.e., nucleus-encoded), chloroplasts, and most archaeobacteria that also require protein subunits for in vitro activity. We

FIGURE 4. In vivo complementation assays of C- and S-type chimeras. *E. coli* P-RNA (positive control) and the *R. americana* C- and S-type chimeras were cloned downstream of a T7 promoter in an *E. coli* plasmid vector (pFBS) carrying the ampicillin resistance gene. Transformants of the three constructs and a negative control (plasmid without insert) were deposited three times independently on LB agar plates supplemented with antibiotics (for more details, see Materials and Methods). The picture was taken after overnight incubation at 43°C.

have therefore conducted complementation experiments of mtP-RNAs with the purified *E. coli* P-protein.

Whereas the *E. coli* P-protein does not reconstitute the activity of native mtP-RNAs, our results show that the in vitro catalytic activities of *R. americana* and *J. libera* S-type (but not C-type) P-RNA chimeras significantly increase in the presence of the *E. coli* P-protein. We think that this activation is favored by binding of the P-protein to the bacterial C-domain of the chimera that is also known to bind the P-protein in vitro (Loria and Pan 2001).

Overexpression of both *R. americana* chimeras complements a thermosensitive *E. coli* mutant that carries a point mutation in the protein subunit, implying that the mtP-RNA chimeras interact with the *E. coli* P-protein in vivo. We interpret the activity of C-type chimera in the in vivo assay (and lack of activity in vitro) by differences in RNA and in protein folding and binding conditions.

Conclusions

In the course of evolution, mtP-RNAs have progressively lost structural elements that are conserved among their bacterial counterparts. We posit that this loss of RNA domains is compensated by proteins, a process that may ultimately lead to a protein-only RNase P. Currently, only one mtP-protein has been identified in *S. cerevisiae* that is 10 times larger than in bacteria (Morales et al. 1992). In other instances, mitochondrial RNase has been purified and shown to contain multiple protein subunits (e.g., Marchfelder and Brennicke 1994; Lee et al. 1996a), but these remain unidentified. Evidently, we require information on mitochondrial P-proteins from a wide range of species covering various degrees of mtP-RNA reduction to better understand functional aspects, as well as test the hypothesis of a functional shift from RNA to protein during evolution.

MATERIAL AND METHODS

Cell cultures and nucleic acid extractions

An axenic strain of *N. olivacea* (NIES-484, a prasinophyte green alga distributed by the National Institute for Environmental Forum, Japan) was kindly provided by Drs. M. Turmel and C. Lemieux (Université Laval). Cells from this species were grown as previously described (Turmel et al. 1999). The jakobids *Reclinomonas americana* (New Zealand strain; ATCC 50,394), *R. americana* (ATCC 50,283), *R. americana* (ATCC 50,284), *Reclinomonas* sp. (ATCC 50,633), *Histiona aroides* (ATCC 50,634), and *Seculamonas ecuadoriensis* (ATCC 50,688) were grown in WCL medium, whereas the two jakobids adapted to saline growth conditions, *Jakoba libera* (ATCC 50,422) and *Jakoba bahamiensis* (ATCC 50,695), were cultivated in F/2 medium. The recipes for these growth media are posted at <http://megasun.bch.umontreal.ca/People/lang/FMGP/methods.html>. For large-scale cultures, jakobids were incubated in 2.5-L culture flasks under gentle shaking at ~24°C and were daily fed with live *Enterobacter aerogenes* bacteria (ATCC 13,048). When cells reached the late logarithmic growth phase (after ~5 d), they were harvested by centrifugation, and nucleic acids were extracted as described (Seif et al. 2003).

MtP-RNA 5' and 3' end mapping

About 10 µg of mitochondrial RNA were used to determine the precise 5' or 3' ends of mtP-RNAs by RNA ligation, RT-PCR

FIGURE 5. Catalytic assays of GNRA tetraloop mutants. (A) Assays for mutants in L18 of *J. libera* C-type chimera. Experiments with the respective wild-type (GUAA) and mutant sequences (AUAA) are labeled in the figure. The negative control lacks ribozyme. The assays were performed with 100 nM of P-RNA, at 37°C for 2 h with 1000 dpm of pre-tRNA^{Pro} in a buffer containing 20 mM MgCl₂, 2.5 M NH₄Cl, 50 mM Tris (pH 8), 0.1% SDS and 0.05% Nonidet P-40. (B) Assays of an L14 mutant of the *J. libera* S-type chimera. Experiments with the wild-type (GAGA) and mutant sequence (AAGA and GAGG) are labeled in the figure. In the lane labeled *substrate*, no ribozyme was added. The assay conditions were 1 µM of ribozyme for 18 h at 37°C, with the conditions optimal for archaeobacterial P-RNAs (see Materials and Methods). (C) Assays of an L9 tetraloop mutant of the *R. americana* S-type chimera. Experiments with the wild-type tetraloop (AAAA) and the mutant sequence (GAGA) are labeled in the figure. In the lane labeled *substrate*, no ribozyme was added. Assay conditions are otherwise the same as described in B.

FIGURE 6. P3 mutagenesis in *S. ecuadoriensis*. (A) Assays for mutants of the postulated single P3 base pair. The postulated base pair is marked with an asterisk (*). Ribozymes (100 nM) were incubated with 1000 dpm of pre-tRNA^{Pro} in a buffer containing 100 mM MgCl₂, 2.5 M NH₄OAc, 50 mM Tris (pH 8), 0.1% SDS, and 0.05% Nonidet P-40. The *AXA mutant has a twofold decrease in catalytic activity compared to the *G-C mutant and the *U-A wild-type sequence. “Sub.” denotes substrate only (negative control). (B) Secondary structure model of P3 and surrounding nucleotides.

amplification, and cDNA sequencing, as previously described (Seif et al. 2003). Primer sequences for *R. americana* were 5'-TAAAT-GAGTCCAGACTTTCCT-3' and 5'-GAGCAAAACCATAGG-TTGCT-3'; and for *N. olivacea*, 5'-CTATTTCATACTAGC-TCT-3' and 5'-GTGCAAGAATTTAATTCGCT-3'.

Construction of chimeric P-RNAs

The *rnpB* genes of *E. coli*, *R. americana* #50394, *J. libera*, *S. ecuadoriensis*, *R. oryzae*, and *N. olivacea* were cloned into pFBS (a modified bluescript vector) and used as templates for the PCR amplifications described below.

The cloning vector was amplified together with one of the two (mitochondrial or *E. coli*) domains, which permits direct cloning of chimeric *rnpB* genes after DNA ligation with a corresponding section of P-RNA. For construction of C-type chimeras (see Fig. 2A for the precise extensions of the mitochondrial and bacterial sequences), mitochondrial P-RNAs were amplified to include P1–P4, P18, P19, and J15/18. The respective *E. coli* P-RNA domain was amplified to include P5–P17. For S-type chimeras (see Fig. 3A), the amplified mitochondrial domain contained P5 and P7–P15, whereas the *E. coli* domain comprised P1–P4, J15/18, and P18. All amplification products were end-repaired (unmodified T7 DNA polymerase from New England Biolabs, no. M02565S, plus the Klenow fragment from Roche, no. 1008404) at 14°C for 30 min. After denaturation of the polymerases at 65°C for 10 min, the DNA fragments were purified on low-melting agarose gel (FMC Bioproducts, no. 50102). The PCR product including the vector sequence was dephosphorylated with alkaline phosphatase (Boehringer Mannheim, no. 713023), followed by extraction with phenol/chloroform and ethanol precipitation, whereas the corresponding fragment containing the other half of the P-RNA sequence was phosphorylated with T4 polynucleotide kinase (USB, no. 27073602). The two fragments were ligated at 14°C overnight and transformed into *E. coli*. Finally, transformants were verified by DNA sequencing.

In vitro synthesis of RNA and RNase P activity assays

Internally radiolabeled precursor tRNA substrates and unlabeled P-RNAs were synthesized in vitro and purified as described previously (Jacob et al. 2004). DNA templates for in vitro RNA synthesis were prepared by PCR amplification of respective P-RNA sequences, by inclusion of a T7 RNA polymerase promoter in one of the two PCR primers. Purified RNAs were dissolved in 1× PA buffer (50 mM Tris at pH 7.5, 100 mM NH₄Cl, 10 mM MgCl₂), heated for 5 min at 65°C, and then allowed to slowly cool to 37°C.

The catalytic activity of P-RNAs was tested with the pre-tRNA substrate (~1000 dpm, 0.013 pmol) under two conditions: (1) activity assays adapted to bacterial P-RNAs at intermediate salt conditions (Guerrier-Takada et al. 1986), including 1× PA buffer, 100 mM MgCl₂, and 4% PEG (polyethylene glycol), or (2) archaeobacterial or high salt conditions (50 mM Tris at pH 8, 300 mM MgCl₂, 2.5 M NH₄OAc, 0.1% SDS, and 0.05% Nonidet P-40) (Pannucci et al. 1999). For assays including the *E. coli* P-protein, 5 nM of the protein was added to 0.5 nM of P-RNA in binding buffer (20 mM HEPES-KOH at pH 8, 400 mM NH₄OAc, 10 mM Mg(OAc)₂, 5% glycerol), the mixture was heated for 5 min at 37°C, and 1000 dpm of substrate was added and incubated at 37°C overnight.

Reaction products were loaded on 7% denaturing polyacrylamide gels as described (Jacob et al. 2004), gels were exposed for 2 h on a BioRad molecular imaging screen K (no. 1707841), and images were developed using the BioRad molecular imager FX and analyzed with the “Quantity One” software.

Genetic complementation assay

E. coli BL21 carrying a *rnpA* mutation in the chromosome (C5 R46H) (Guerrier-Takada et al. 1995) and containing λ prophages that encode T7 RNA polymerase were used for the genetic complementation assay. The permissive and nonpermissive temperatures for this *E. coli* strain are 30°C and 43°C, respectively. This *E. coli* strain was transformed with plasmids encoding various mtP-RNA constructs and grown overnight at 30°C on LB media with 5 μg/mL tetracycline and 100 μg/mL ampicillin. Several independent transformants were isolated and grown on liquid LB media to an optical density of 0.7 at the permissive temperature. From each culture, 7.5 μL was dropped on two (prewarmed) agar plates and incubated overnight, one at 30°C and the other at 43°C. Genetic complementation by various P-RNA constructs was assessed by comparison of growth at the two temperatures (Baer et al. 1989). Cells transformed with vector alone and a vector encoding *E. coli* P-RNA served as negative and positive controls.

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