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Nucleic Acids Research, 2001, Vol. 29, No. 12 2661–2665

The first phytoplasma RNase P RNA provides new insights into the sequence requirements of this ribozyme

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Received December 8, 2001; Revised March 21, 2001; Accepted April 29, 2001 DDBJ/EMBL/GenBank accession no. Y18869

ABSTRACT

A high variability of RNase P RNA structures is seen among members of the *Mycoplasma* group. To gain further insight into the structure–function relations of this ribozyme, we have searched for the RNase P RNA gene from more distant relatives, the phytoplasmas. These mycoplasma-like organisms are the aetiological agents of many severe plant diseases. We report the sequence and catalytic properties of RNase P RNA from the phytoplasma causing apple proliferation disease. The primary and postulated secondary structure of this 443 nt long RNA are most similar to those of *Acholeplasma*, supporting the phylogenetic position of this pathogen. Remarkably, the extremely AT-rich (73.6%) phytoplasma RNA differs from the known bacterial consensus sequence by a single base pair, which is positioned close to the substrate cleavage site in current three-dimensional models. Phytoplasma RNase P RNA functions as an efficient ribozyme *in vitro*. Conversion of its sequence to the full consensus and kinetic analysis of the resulting mutant RNAs suggests that neither the sequence alone, nor the type of pairing at this position is crucial for substrate binding or catalysis by the RNase P ribozyme. These results refine the bacterial consensus structure close to the catalytic core and thus improve our understanding of RNase P RNA function.

INTRODUCTION

RNase P is a ubiquitous enzyme essential for tRNA maturation. In bacteria and some archaea, the catalytic site resides in the RNA subunit, which is able to perform precise endonucleolytic cleavage of tRNA precursors to yield the mature 5′ end (1–4). Although RNase P RNAs from different bacterial groups vary considerably in sequence and length, covariation analysis of sequences covering the whole range of phylogenetic groups has made possible the definition of a universally conserved minimal consensus structure for these catalytically active RNAs (5). According to this model, two universally present regions of high sequence conservation constitute a long-range base pairing (P4), which is close to the catalytic center and involved in maintaining the three-dimensional structure of the ribozyme (6–10). The known bacterial RNase P RNA sequences fall into two well-defined structural classes (11): whereas class A comprises the Gram-negative and high GC Gram-positive bacteria, class B structures are mostly restricted to the low GC Gram-positives, including *Bacillus* and *Mycoplasma* species.

Phytoplasmas (formerly called mycoplasma-like organisms or MLOs) are the smallest known plant-pathogenic bacteria and are associated with diseases of several hundred species including many economically important fruit trees and ornamental plants (12); because of the failure of *in vitro* cultivation they are not well characterized. Their genomes consist of a circular, 640–1200 kb chromosome of low G+C content (13,14). For the phytoplasma causing apple proliferation disease (AP phytoplasma), a few genes could be identified and a physical map of the 645 kb chromosome was recently constructed from several gene sequences (15). Classification and determination of the phylogenetic position of phytoplasmas is mainly based on 16S rRNA and ribosomal protein sequences, leading to the establishment of one monophyletic clade consisting of 14 major groups (reviewed in 16). Comparisons of the few available sequences revealed that phytoplasmas are more related to the *Acholeplasma* species than to any animal-associated *Mycoplasma*. The knowledge of phytoplasma RNase P RNA sequences should not only help to confirm and further resolve this phylogenetic position, but also aid in developing tools for crop disease control.

Because an adaptation of RNase P RNA sequence and structure to the size and base content of genomes can often be observed (17–19), we have chosen the unusual phytoplasmas in order to search for new RNase P RNA structure variants and to gain insight into the way an organism adapts such a functional RNA to a highly biased genomic base composition.

Here we report the first sequence and analysis of catalytic properties of an RNase P RNA from a phytoplasma, the AP phytoplasma strain AT.

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MATERIALS AND METHODS

Isolation of nucleic acids

CsCl or PFGE purified phytoplasma DNA from *Nicotiana* plants infected with strain AT were a gift of Dr E.Seemüller. For RNA preparation, frozen stems of infected periwinkle plants (*Catharanthus roseus*) were ground to powder. Total RNA was prepared by phenol extraction and differential precipitation as described (20).

Determination of phytoplasma RNase P RNA sequence

DNA of AT phytoplasma was first amplified with the primer pair MYC5 (GAGGAAAGTCCRYGCT; all primer sequences are given in 5′→3′ direction) and MYC3 (ATAAGCCRCGT-TYTGT) derived from known mycoplasma sequences (17,18), using *Tth* DNA polymerase (Biozym) in a Hybaid OmniGene PCR Incubator (1 µM each primer, 200 µM each dNTP, 0.1 U *Tth* DNA polymerase/µl). After denaturation for 2 min at 94°C, 35 cycles of PCR were performed (30 s at 94°C, 30 s of annealing at 40°C, 1 min at 72°C). The PCR product was purified by agarose gel electrophoresis and eluted using a JETquick kit (Genomed), sequenced (Sequenase 2.0 kit; United States Biochemicals), and the primer sequences were refined. A second genomic PCR was performed using the proofreading *Pfu* DNA polymerase (Stratagene) and the refined primer pair PHY5-1 (GAGGAAAGTCCATGYTAGCAC) and PHY3-1 (ATAAGCCGCGTTTTGTTCTTG) derived from the previous sequence (0.5 µM each primer, 200 µM each dNTP, 0.05 U *Pfu* DNA polymerase/µl). The cycle conditions were the same, except that the annealing temperature was raised to 56°C. The resulting PCR product was purified and sequenced as above.

5′ and 3′ ends of the RNA were determined by rapid amplification of cDNA ends (RACE), using a 5′/3′ RACE kit (Roche Diagnostics). 5′ RACE was performed with 1 µg total RNA and the nested primer set PHY3-1 and PHY3-2 (TTATCTCGTCTCTGTGGCAC). As prerequisite of the 3′ RACE, 1 µg total RNA was used in a polyadenylation reaction for 20 min at 30°C using 50 U yeast poly(A) polymerase (United States Biochemicals) and 0.5 mM ATP. Subsequently, the 3′ RACE was performed according to the manufacturer's protocol, using the nested primer set PHY5-1 and PHY5-2 (CCCCTCAAGCTAACAACCC). The respective PCR products were purified and sequenced as above.

For sequence data analysis the GCG program package v9.0 (University of Wisconsin, Madison, WI) was used. Homology searches were performed with the programs FASTA or BESTFIT.

Construction of transcription clones for phytoplasma RNase P RNA and its mutants

For first strand cDNA synthesis, 20 pmol of primer Phyto3′ (GCGCGGATGAATTCGAATAAAAGTACCAAATAATA-TGCATAAGCC) were annealed to 1 µg total RNA and extended using AMV Reverse Transcriptase (Promega) for 1 h at 42°C. One-tenth of this cDNA was amplified in a PCR with the primer pair T7PhyRP (GCGCTAATACGACTCACTAT-AGGGAGTTACCAAATTAATAAAGGCG) and Phyto3′ using *Pfu* DNA polymerase (Stratagene) under the same conditions as above; the annealing temperature was 48°C for three cycles and 64°C for the subsequent 35 cycles. The amplification products containing a T7 promoter and a *Fok*I restriction site to allow runoff transcription were digested with *Eco*R1 and *Pst*1, purified on a QIAquick spin column (Qiagen) and ligated into an appropriately digested pUC19 vector resulting in the plasmid pT7ATRP. Mutant RNase P RNA clones were obtained by megaprimer mutagenesis essentially as described (21), using pT7ATRP as template, *Pfu* DNA polymerase, and an annealing temperature of 48°C for the mutant primer. For the C77 mutant, the megaprimer was generated with PhytoC77 (CCTTTAAGTATGTGCTAG-CATGGACTTTCCTAAAATT) and T7PhyRP, and the full length clone amplified with T7PhyRP and Phyto3′, as in all other cases; for G283, the megaprimer was synthesized with Phyto3′ and PhytoG283 (CTCAAGCTAGCAACCCAAAATATG). For the double mutant C77-G283, the megaprimer was generated from the template pT7ATRP-G283, using T7PhyRP and the mutant primer PhytoC77. Preparative runoff transcription from *Fok*I-restricted templates was performed as described (22).

Analysis of processing activity

Due to the lack of any homologous tRNA sequence from phytoplasmas, *Escherichia coli* pre-$tRNA^{Tyr}$ (23) was used as substrate for all processing reactions. Runoff transcription from *Fok*I-cut template was performed as described (22). All RNase P RNA reactions were performed at 37°C; optimum reaction conditions were determined at 0.3–1 nM substrate. Monovalent cation concentration was optimized at 50 mM $MgCl_2$; the Mg^{2+}-dependence of the RNase P RNA reaction was then determined in 2 M NH_4Cl. For the determination of kinetic parameters, reactions contained 20–400 nM of substrate (1000–2000 c.p.m.) and 5–15 nM RNase P RNA in 50 mM Tris–Cl pH 7.5, 2 M NH_4Cl and 37.5 (wt or C-G mutant) or 50 mM $MgCl_2$ (U·G or C·A mutants). The identity of the reaction products was confirmed by size determination on denaturing polyacrylamide gels, and comparison to reaction products obtained by cleavage with *E.coli* M1 RNA (not shown). Time points during the initial phase of the reaction (<10 min) were taken at appropriate intervals so that substrate conversion was <20%. Reaction products were quantitated with a PhosphorImager system (Molecular Dynamics). Data were evaluated by Lineweaver–Burke plots; at least three independent experiments were analyzed for each RNase P RNA variant.

RESULTS AND DISCUSSION

Phytoplasma RNase P RNA deviates from the bacterial consensus and reveals novel structural elements

The primary sequence of RNase P RNA from the aetiological agent of apple proliferation disease (AP phytoplasma, strain AT) is most similar to those from the *Mycoplasma* cluster, including *Acholeplasma laidlawii;* the extremely low G-C content of 26.4% is consistent with the biased base composition typical for phytoplasma genomes (13). The postulated secondary structure corresponds to the type B RNase P RNAs (Fig. 1) (11). One major difference between the ancestral (A) and *Bacillus*-type (B) classes is the lack of the distal helices P16 and P17 in the B-type structures and, as a consequence, of the long-distance pairing P6 between L17 and J5/7. A comparable interaction between L5.1 and L15.1 has been implied

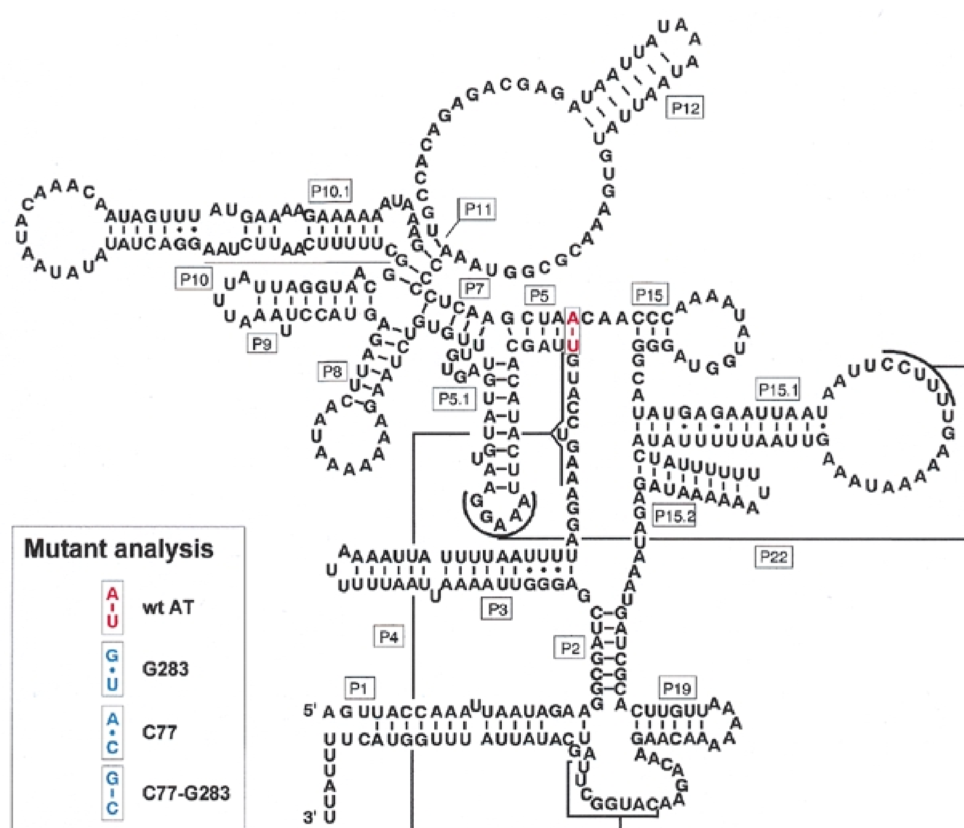

Figure 1. Secondary structure of phytoplasma RNase P RNA. Domain nomenclature is according to Haas *et al.* (5); the postulated long-range interaction between L5.1 and L15.1 is named P22. The two deviations from the bacterial consensus are highlighted in the structure (red); changes in the mutant analysis are given in the inset (blue). The sequence will be accessible under the GenBank number Y18869.

from covariation analysis of type B sequences (11); this helix (P22) does not exceed two consecutive base pairs in any of the known RNase P RNAs (24). In striking contrast, the postulated P22 in the phytoplasma RNA consists of 5 bp (or up to 8, if P5.1 opens at the distal end), and thus strongly supports the existence of this interaction in all type B RNase P RNAs. The unusual length of P22 may be required to stabilize the tertiary structure of this extremely A-U rich ribozyme. The unambiguous confirmation of existence and function of this interaction, however, will require additional sequence data and corresponding functional studies.

Another B-type peculiarity is the extended P10.1 close to the cruciform region. A postulated tertiary interaction between a receptor motif in P10.1 and the L12 tetraloop may result in overall stabilization of the ribozyme structure (11,25,26); this view is supported by the observation that RNase P RNAs lacking P12 are catalytically active under conditions of increased ionic strength (18). L12 is GAAA in all B-type RNase P RNAs containing the P10.1 extension, but UAAA in the phytoplasma RNA. The existence of a similar tertiary interaction despite the sequence divergence is supported by covariation of the length of P12 and P10.1 (26).

The phytoplasma RNase P RNA deviates at two additional positions from the established bacterial ribozyme consensus: the fully conserved proximal C-G pair of P5 is replaced by a U-A pair in this organism. The colinearity of genomic and RNA-derived sequence (obtained by RT–PCR) excludes any post-transcriptional sequence changes, such as RNA editing, during expression of this RNA. As for the L12 sequence, these changes might thus be explained by base transitions that have occurred to accommodate the sequence to the low G-C content in the phytoplasma genome.

A consensus base pair close to the catalytic center modulates, but is not essential for ribozyme activity of phytoplasma RNase P RNA

Sequence conservation and functional data have led to the conclusion that the catalytic core of bacterial RNase P RNAs is constituted at least in part by the conserved helical element P4 (27,28). The absolute conservation of the adjacent C-G pair suggests a significant role in the function of bacterial RNase P RNAs. The unexpected discovery of a U-A pair at the corresponding position in the phytoplasma RNA implies, however, that the conserved sequence may not be important for RNase P function *in vivo*. We have thus investigated the influence of nucleotide identity and base-pairing properties at this position on ribozyme function by converting the U-A back to the consensus C-G, and by introducing a 'wobble' U·G or a non-canonical C·A pair, respectively.

The steady-state parameters, K_m^{app} and k_{cat}^{app} of the wild-type phytoplasma ribozyme are 116 nM and 0.75 min^{-1}, respectively, and are well within the range described for other bacterial RNase P RNAs (Table 1) (29–32). Remarkably, although these parameters are significantly different for the (A+U)-rich RNase P

Table 1. Kinetic parameters of phytoplasma RNase P RNA mutants

Mutant name	Base pair sequence	$MgCl_2$ (mM)	K_M^{app} (nM)	k_{cat}^{app} (min^{-1})	k_{cat}/K_M ($nM^{-1}min^{-1}$) × 10^{-3}
wt AT	U-A	37.5	116 ± 6	0.8 ± 0.1	6.5 ± 1.3
G283	U·G	37.5	301 ± 48	1.7 ± 0.5	5.6 ± 1.5
C77	C·A	37.5	327 ± 26	1.1 ± 0.2	3.3 ± 0.6
C-G	C-G	37.5	191 ± 13	0.8 ± 0.1	3.9 ± 0.9
G283	U·G	50	126 ± 50	0.4 ± 0.1	3.4 ± 0.8
C77	C·A	50	238 ± 23	0.9 ± 0.1	3.9 ± 0.6
M.hyopneumoniae	C-G	100	1200	6.3	5.3

Kinetic analysis of each RNase P RNA variant was performed as described in the Materials and Methods; standard deviations are given for each experimental value. Parameters were obtained at 37.5 mM $MgCl_2$ ('wt buffer'; rows 1–4), and in addition at 50 mM $MgCl_2$ ('mutant buffer'; rows 5 and 6) for the non-Watson–Crick mutants. For comparison, the corresponding values for *M.hyopneumoniae* RNase P RNA, determined in 800 mM NH_4Cl (17), are included.

RNA from *Mycoplasma hyopneumoniae*, the specificity constant k_{cat}^{app}/K_m^{app} of that ribozyme is close to that observed in the phytoplasma RNA (17) (Table 1). The disruption of the Watson–Crick U-A pair, or the conversion to the consensus C-G, results in moderate to significant changes in the catalytic properties of the phytoplasma ribozyme if tested under the conditions established for the wild-type RNA (we consider a factor of 2 as significant): for the mutants lacking the Watson–Crick pair, K_m^{app} increases to >2-fold, indicating an impairment in substrate binding. Concomitantly, k_{cat}^{app} increases in the U·G and C·A variants. This might be explained by the observation that moderately reduced affinities can result in higher substrate turnover numbers (33). In contrast, k_{cat}^{app} remains essentially unchanged for the C·G mutant. Consequently, all three mutants show a reduction in catalytic efficiency compared to the wild-type due to their increase in K_m^{app}.

It has been shown previously that the effect of mutations or deletions at conserved positions in RNase P RNA can frequently be overcome by increased ionic strength (reviewed in 34). We have thus determined the kinetic parameters under the ionic conditions needed for optimum activity of each RNA mutant. All phytoplasma RNase P RNA variants require 2 M NH_4Cl for maximum activity. In contrast, the optimal concentrations of $MgCl_2$ are higher for the mutants containing non-Watson–Crick base pairs, than for the wild-type and C-G mutants, respectively (Fig. 2; Table 1). The requirements for high mono- and divalent cation concentrations thus resemble those determined for *Bacillus subtilis* RNase P RNA (35) and are unlike those of *E.coli* M1 RNA, which belongs to the structurally different A-type (36). Such a high ionic strength environment may help to alleviate minor deviations from an optimal RNase P RNA structure by supporting the correct folding of the ribozyme core. This hypothesis is supported by the finding that, under increased Mg^{2+} concentrations, a decrease of K_m^{app} is observed for the two 'non-Watson–Crick' mutants, which approaches wild-type level and is paralleled by a significant drop in k_{cat}^{app} for the U·G mutant. Thus, our data support the hypothesis that, although the conserved C-G pair may modulate catalytic efficiency of bacterial RNase P RNAs, neither its presence as a Watson–Crick pair nor its sequence alone are crucial for efficient ribozyme function. Interestingly, two deviations from the bacterial consensus at positions

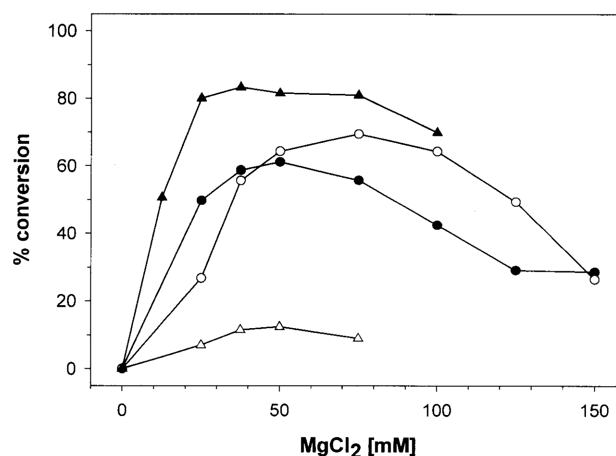

Figure 2. Mg^{2+}-dependence of the phytoplasma RNase P RNA reaction. Reactions were performed and analyzed as described in the Materials and Methods, using 2 M NH_4Cl as monovalent cation. Substrate turnover after 20 min is compared for wild-type (filled circles) and mutant RNAs (U·G, open circles; C-G, filled triangles; and C·A, open triangles).

different from those in phytoplasma are present in RNase P RNA from a primitive plastid, which is not a ribozyme *in vitro* (19). Restoration of the consensus does not confer ribozyme activity on this RNA; however, heterologous reconstitution with a cyanobacterial protein subunit yields a functional holoenzyme, irrespective of the RNA sequence (22 and unpublished results). Taken together, these results support the notion that even fully conserved nucleotides in the central region of *B.subtilis* RNase P RNA might not be essential for catalysis, but rather for stabilization of a favorable tertiary structure (28). This view is corroborated by the recent finding that the conserved helix P4 is a metal binding domain, possibly serving a structural role in the folding of the ribozyme core (33,37). The Mg^{2+}-binding properties of P4 may well be influenced by adjacent structural elements (38). Accordingly, the requirement for higher concentrations of Mg^{2+} in the phytoplasma RNase P RNA mutants containing non-Watson–Crick pairs suggests that the terminal base pair could play a role in stabilization of P5 or other structural elements adjacent to the

catalytic core, and—among other possible reasons—may influence the binding of functionally important divalent cations.

ACKNOWLEDGEMENTS

We thank Dr E.Seemüller (Dossenheim) for the kind gift of purified DNA from AP phytoplasma (AT strain) and infected plant material. This work was supported by grants from the Deutsche Forschungsgemeinschaft to A.S. (Scho 515/4-1) and H.J.G. (SFB 251).

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