In vitro transactivation of *Bacillus subtilis* RNase P RNA

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Received 26 June 2001; revised 12 September 2001; accepted 12 September 2001

First published online 25 September 2001

Edited by Takashi Gojobori

Abstract Deletion of the 'signature' PL5.1 stem-loop structure of a Type II RNase P RNA diminished its catalytic activity. Addition of PL5.1 in *trans* increased catalytic efficiency (k_{cat}/K_M) rather than k_{cat}. Transactivation was due to the binding of a single PL5.1 species per ribozyme with an apparent K_d near 600 nM. The results are consistent with the role of PL5.1 being to position the substrate near the active site of the ribozyme, and with the hypothesis that ribozymes can evolve by accretion of preformed smaller structures.

Key words: Catalytic RNA; RNA–RNA interaction; Kinetics; Phylogenetic variation; RNA world

1. Introduction

RNase P RNAs and other large catalytic RNAs typically contain phylogenetically variable structural elements which are not required for catalytic activity, although they may contribute to its efficiency [1]. The dispensable and variable nature of these elements suggests that their incorporation into catalytic RNA species could occur by accretion into a preexisting catalyst, as opposed to monophyletic descent from a single ancestor [2]. Thus, an RNA catalyst need not be derived solely from a pool of single-chain species. Instead, a large ribozyme could begin its existence as a multi-subunit array. Subsequently, these subunits could be joined into a single polynucleotide chain.

Several experiments are relevant to this scenario. Burke and Willis [3] combined two pools of preselected aptamers to generate two-domain chimeric molecules which retained the binding activities of both parents. An alternative approach is to examine the interactions between domains of contemporary large ribosomes. Large-order reconstructions of a Group I intron [4,5] and of RNase P RNA [6,7] have been carried out by associating domains through tertiary interaction. Similarly, the P5a and P2.1–P2.2 domains of the *Tetrahymena* Group Ia intron have been deleted and the remaining activity enhanced by adding the domains in *trans* [8–10].

The contributions of the various structural elements to the overall catalytic cycle of RNase P RNA have been more obscure. Watson–Crick and other interactions contribute to substrate recognition by RNase P [11–15]. Resolution of the contribution of these interactions may be difficult, since so many of the naturally-occurring RNA structures are poorly conserved and the catalytic core is relatively small [16,17].

We report here a combined mutational and kinetic study to analyze the contribution of one such non-conserved structural element to the action of the Type II RNase P RNA from *Bacillus subtilis*. The PL5.1 stem-loop element originally found in *B. subtilis* RNase P RNA is a distinguishing feature of Type II RNase P RNAs. PL5.1 is analogous, perhaps functionally so, to a well-conserved pseudoknot element (P6 in *Escherichia coli*) in Type I RNase P RNAs, including those from other Gram-positive organisms [17,18]. We deleted the PL5.1 stem-loop from Bsu RNase P RNA. This deletion mutant was deficient in catalytic activity. The PL5.1 element, when added in *trans*, stoichiometrically activated the mutant RNA, largely by affecting catalytic efficiency rather than the rate-determining step (k_{cat}) of the reaction.

2. Materials and methods

2.1. Preparation of RNAs

PL5.1 RNA, GCACGGUGCUGAGAUGCCCGUA, was synthesized by Cruachem, Ltd., Glasgow, UK, using F-moc chemistry, and purified by high pressure liquid chromatography. Unlabeled *B. subtilis* RNase P RNA and Δ5.1 RNA were prepared by in vitro transcription from plasmids pDW66 [19] and pΔ5.1 [20] linearized with *Dra*I. Transcription reactions (200 μl) were performed according to the manufacturer's directions using AmpliScribe T7 enzyme solution (Epicentre) and 60 μg linearized plasmid template. Internally labeled long pre-tRNA^{Asp} (with the 3′-CCA of pre-tRNA^{Asp}) and short pre-tRNA^{Asp} (without the 3′-CCA of pre-tRNA^{Asp}) were transcribed from plasmids pDW152 and pT7-1LCCA [12] linearized with *Bst*NI and *Nci*I respectively. RNAs were purified by denaturing polyacrylamide gel electrophoresis.

PL5.1 RNA for gel retardation assay was labeled at the 3′ end with [5′-^{32}P]pCp (3000 Ci/mmol, New England Nuclear). 1.6 mmol of RNA was incubated with 5 U T4 RNA ligase (Epicentre) and 150 μCi of [5′-^{32}P]pCp in 50 mM HEPES, pH 7.5, 20 mM $MgCl_2$, 3.3 mM DTT, 10 μg/ml BSA, 6 μM ATP, and 10% DMSO at 4°C for 16 h. The transcription products and ligation mixtures were purified by electrophoresis on 10% polyacrylamide/7.6 M urea gels.

2.2. Kinetic assays

RNase P RNA and substrate pre-RNAs were renatured separately in 50 mM Tris–HCl, pH 8.0, 0.05% Nonidet P-40, 100 mM $MgCl_2$, 800 mM NH_4OAc for 10 min at 55°C. Reaction was initiated by addition of ribozyme to substrate in the same buffer; final concentrations were 1 nM P RNA or 10 nM Δ5.1 RNA+2 μM PL5.1 RNA. Reactions were stopped by the addition of 2.5 volumes of cold ethanol. The reaction products were resolved by electrophoresis in 6% polyacrylamide/7.6 M urea gels. The intensities of RNA bands were measured with a phosphorimager (Molecular Dynamics, Sunnyvale, CA, USA) and the resulting data fitted using Kaleidagraph (Synergy Software).

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0014-5793/01/$20.00
PII: S0014-5793(01)02929-5

2.3. Electrophoretic mobility shift assays

Mixtures (5 μl) containing 100 nM of 3′ end-labeled PL5.1 RNA and 100 nM to 3.5 μM of Δ5.1 RNA were prepared in 50 mM Tris–HCl, pH 8.0, 0.05% Nonidet P-40, 100 mM $MgCl_2$, 800 mM NH_4OAc, heated at 80°C for 2 min and slowly cooled to 37°C. Reaction mixtures were loaded on a 6% polyacrylamide gel containing 50 mM Tris–acetate, pH 8.0, 0.1 mM EDTA, 50 mM Mg–acetate, 800 mM NH_4Cl. Gels were run at a constant current of 35 mA for 3–4 h at 4°C. The running buffer was 50 mM Tris–acetate, pH 8.0, 0.1 mM EDTA, 50 mM Mg–acetate, 800 mM NH_4Cl.

In further experiments, mixtures were prepared as above, but with a constant ratio of Δ5.1 RNA to labeled PL5.1 RNA, and increasing amounts of total RNA. The reaction mixtures were applied to a 6% polyacrylamide gel and run as above. The intensities of the bands were measured by phosphorimaging. The K_d was determined from the slope of a plot of $(1-r)(\alpha-r)/r$ vs. $[PL5.1]_t$, where r is the fraction of radioactivity in the complex band, $[PL5.1]_t$ is the total concentration of PL5.1 RNA added, and α is an empirically derived constant adjusted so that the y-intercept of the plot is zero [21].

3. Results and discussion

3.1. Enhancement of the catalytic activity of a deletion mutant of Bsu RNase P RNA

We previously reported the construction of a mutant form of Bsu RNase P RNA (Fig. 1), in which the sequence specifying the PL5.1 element was replaced by a *Sna*BI recognition sequence [20]. In the present work, the corresponding RNA, termed Δ5.1 RNA, was synthesized by in vitro transcription and used to catalyze the hydrolysis of a pre-$tRNA^{Asp}$ substrate [22].

The Δ5.1 RNA was deficient in cleaving its pre-tRNA substrate (Fig. 2, lanes 1–3, and Table 1) relative to native RNase P RNA. Enzymatic activity was enhanced by the inclusion of synthetic PL5.1 RNA in the reaction mixture (Fig. 2, lanes 4–12, and Table 1). RNase P activity was not a function of PL5.1 RNA alone (Fig. 2, lanes 13–15).

These observations suggested that the PL5.1 RNA associated with the Δ5.1 form of RNase P RNA to make a structure which resembled the native ribozyme. A simplified steady-state kinetic model for such a process is given by:

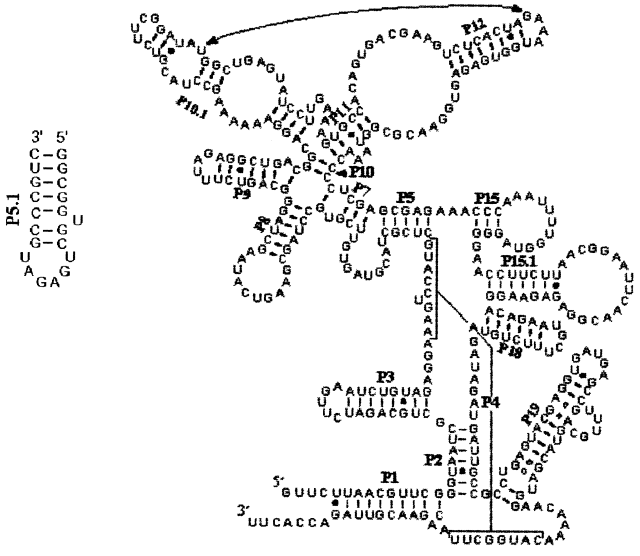

Fig. 1. Secondary structure of Δ5.1 mutant of RNase P and of the PL5.1 structural element, adapted from the RNase P database [36]. The deletion mutant was constructed by inverse PCR so that the PL5.1 element was replaced by a *Sna*BI recognition sequence [21].

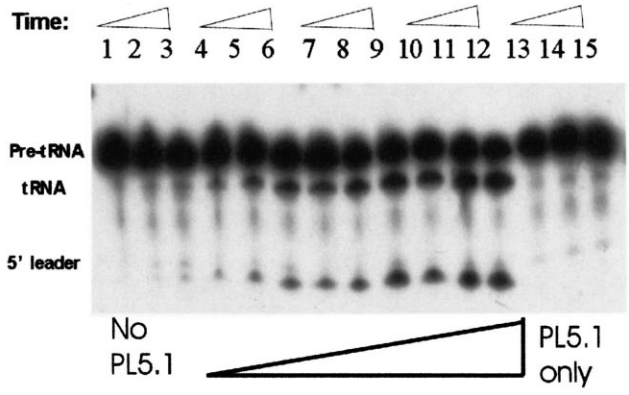

Fig. 2. Transactivation of the catalytic activity of Δ5.1 mutant RNase P RNA by PL5.1. The reaction mixture contained either no PL5.1 (lanes 1–3), 0.2 μM PL5.1 (lanes 4–6), 0.5 μM PL5.1 (lanes 7–9), or 1.0 μM PL5.1 (lanes 10–12), together with 25 nM Δ5.1 RNA and 11 nM pre-tRNA in 12.5 mM Tris–HCl, pH 8.0, 50 mM NH_4–acetate and 50 mM $MgCl_2$. Lanes 13, 14 and 15 contained no Δ5.1 RNA, with 0.2, 0.5 and 1 μM PL5.1, respectively. Aliquots were removed after 5 (lanes 1, 4, 7 and 10), 10 (lanes 2, 5, 8 and 11) and 20 min (lanes 3, 6, 8, 12–15) incubation.

$$\text{pre-tRNA} + \Delta 5.1 + \text{PL5.1} \overset{K_d}{\rightleftarrows}$$

$$[E^*] \underset{k_2}{\overset{k_1}{\rightleftarrows}} [E^* \cdot \text{pre-tRNA}] \xrightarrow{k_3} E^* + L + \text{tRNA}$$

where E* is the complex of PL5.1 and Δ5.1 RNA, K_d refers to the dissociation constant for formation of E*, k_1, k_2, and k_3 are the rate constants for the reaction, and L is the precursor-specific leader sequence of the pre-tRNA substrate. The model has several consequences: First, PL5.1 and Δ5.1 RNA should form a saturable 1:1 complex. Secondly, under initial velocity conditions (i.e. when [tRNA] is near zero), the reconstituted RNA species E* should form a saturable (Michaelis) complex with substrate. These predictions were examined experimentally.

Fig. 3A shows the effect of increasing [PL5.1] on the initial velocity at which a pre-$tRNA^{Asp}$ substrate was processed by the Δ5.1 RNA. The data fit well to a single hyperbola whose half-maximum (equivalent to the apparent K_d for the dissociation of the PL5.1–Δ5.1 complex) was 600 ± 100 nM. Consistent with the good fit to a single-binding model ($R > 0.98$), a Hill plot (Fig. 3B) of these data had a slope of 1.1, consistent with the catalytically active species being a 1:1 complex of the two RNAs.

When examined by electrophoretic mobility shift analysis (Fig. 4) under conditions of 10-fold higher ionic strength ($\mu = 1.1$ vs. 0.1), the binding of the PL5.1 structural element to Δ5.1 RNA again fitted to a single exponential with apparent $K_d = 600 \pm 200$ nM [20,23,24]. The experiments in Fig. 4 were carried out using constant amounts of [^{32}P]-labeled PL5.1 and increasing amounts of Δ5.1 RNA. Complementary experiments (not shown) in which the concentrations of Δ5.1 RNA and PL5.1 RNA were varied at a constant ratio [21] yielded apparent K_d values within experimental error of that determined by the experiment shown in Fig. 4. Thus, three different methods yielded similar apparent K_ds for the association of PL5.1 and Δ5.1 RNAs. While K_d values determined by kinetic and mobility shift analysis may not be directly

Table 1
Steady-state kinetic properties of RNase P RNA species

Catalytic RNA	Substrate RNA	k_{cat} (s^{-1})	K_M (nM)	k_{cat}/K_M (M^{-1} s^{-1})
P RNA	long (3′-CCA)	0.04 ± 0.01	190 ± 30	$2.1 \pm 0.6 \times 10^5$
Δ5.1	long (3′-CCA)	0.009 ± 0.002	400 ± 80	$1.9 \pm 0.5 \times 10^4$
Δ5.1+PL5.1	long (3′-CCA)	0.010 ± 0.001	200 ± 20	$4.1 \pm 0.3 \times 10^4$
P RNA	short (no CCA)	0.79 ± 0.09	1200 ± 200	$6 \pm 1 \times 10^5$
Δ5.1	short (no CCA)	0.03 ± 0.02	6000 ± 1000	$5 \pm 3 \times 10^3$
Δ5.1+PL5.1	short (no CCA)	0.028 ± 0.005	1900 ± 500	$1.5 \pm 0.4 \times 10^4$

comparable, the fact that they are similar under different ionic conditions is consistent with the binding of PL5.1 to Δ5.1 RNA being by non-Watson–Crick interaction, rather than by helix formation [25].

3.2. Kinetics and substrate specificity of transactivated RNase P RNA

The enhancement of the catalytic activity of Δ5.1 RNA by PL5.1 RNA was examined by steady-state kinetic analysis (Table 1). Deletion of the PL5.1 element decreased the catalytic efficiency, k_{cat}/K_M, of Bsu RNase P RNA about 10-fold when the substrate contained the 3′-CCA terminus common to bacterial RNase P substrates. The maximal velocity, k_{cat}, of the ribozyme was decreased about 4-fold by the deletion. Addition of the PL5.1 element in *trans* restored the efficiency of the ribozyme without increasing k_{cat}. The ability of PL5.1 to affect k_{cat}/K_M without affecting k_{cat} indicates that there is a slow step in the mechanism which occurs in k_{cat} but not in K_M. This slow step must occur after the first irreversible step (else it would appear in K_M) and could therefore be product release. In the steady-state, k_{cat} of full-length RNase P RNA is determined by the rate of product release [26].

If k_{cat} of Δ5.1 RNA is determined by product release, then a substrate with faster turnover should exhibit the same qualitative behavior as did the native pre-$tRNA^{Asp}$ substrate. Alternatively, if Δ5.1 RNA were to have a different rate-determining step than the native ribozyme, then k_{cat} should not be influenced by the choice of substrate. To test this, we synthesized a pre-$tRNA^{Asp}$ substrate lacking the 3′-terminal CCA sequence and determined its steady-state kinetic parameters. Consistent with previous work [11,12], the k_{cat} of all the RNase P RNA variants increased when the substrate lacked CCA. Product release is facilitated by deletion of the CCA sequence because it is no longer necessary to break the Watson–Crick base pairs between the CC sequence and the invariant GG at nt 258–259 of Bsu RNase P RNA (Fig. 1). On the other hand, k_{cat}/K_M should be affected by two factors: the strength of the substrate interaction with the ribozyme is diminished by deletion of the CCA sequence because important contacts are unavailable. Simultaneously, a catalytically active Mg^{2+} ion becomes unavailable for participation in the chemical step of catalysis [12,13].

Although the numerical values of the kinetic parameters were different, the effects of deleting the PL5.1 element and of adding it in *trans* were similar whether or not the pre-tRNA substrate contained the CCA sequence. The Δ5.1 RNase P RNA had a reduced k_{cat} and k_{cat}/K_M for the substrate lacking CCA. Addition of PL5.1 in *trans* to the Δ5.1 ribozyme partially restored k_{cat}/K_M, without altering k_{cat}. The data taken together indicate that transactivation by the PL5.1 element does not affect the rate-limiting step, most likely product release, but rather a preceding step in the kinetic mechanism.

3.3. Proposed role of PL5.1 in RNase P catalysis

The kinetic behavior of the Δ5.1 RNase P RNA and its transactivation by PL5.1 RNA are consistent with the involvement of the PL5.1 element in substrate binding. This effect is likely to be indirect. If PL5.1 were able to interact directly with substrate, then, at the highest concentrations used in Fig. 3, free PL5.1 RNA would likely have competed for substrate with the reconstituted ribozyme and the overall reaction rate would have decreased. This effect, analogous to substrate inhibition, was not observed; the data fit well to a single-binding model, and Lineweaver–Burk plots of the data in Fig. 3 were linear. Similarly, no evidence was found by

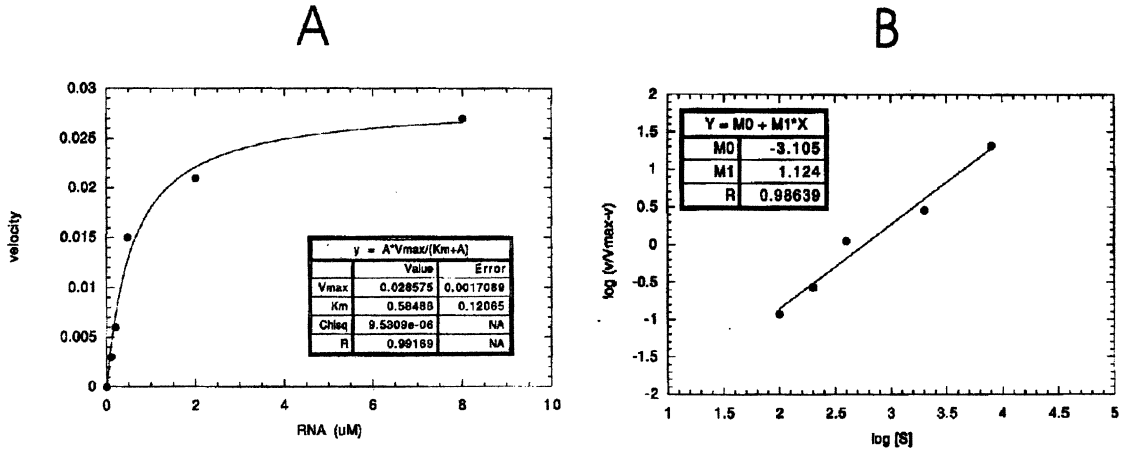

Fig. 3. Binding of Δ5.1 RNA to PL5.1 RNA. A: Dependence of the initial velocity increase over the velocity of Δ5.1 RNA alone (μmol substrate cleaved per min) on the concentration of PL5.1 RNA. B: Hill plot of the data in A.

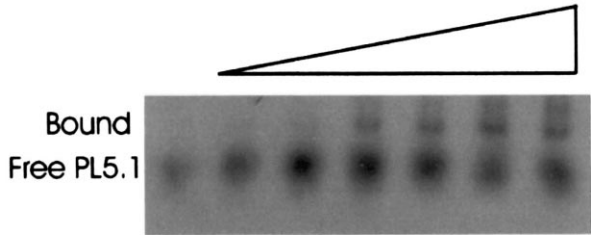

Fig. 4. Gel electrophoretic mobility shift assay of PL5.1 binding to Δ5.1 RNA. [PL5.1] were: Lane 1, none; lane 2, 25 nM; lane 3, 100 nM; lane 4, 500 nM; lane 5, 1 μM; lane 6, 2 μM; lane 7, 3.5 μM. The apparent lower level of complex formation in lane 7 than in lane 6 is likely due to differences in the total amounts of RNA that were loaded.

mobility shift assay for the direct binding of PL5.1 to the pre-tRNA substrate, either under the conditions used for enzyme assay or under those used previously to detect other RNA–RNA interactions [20]. Structural models place PL5.1 near where the CCA terminus of the substrate binds [27,28]. Available data therefore imply the involvement of PL5.1 in the overall positioning of the substrate binding region.

3.4. Modular mode of ribozyme evolution

Transactivation of large ribozymes by small structures derived from them suggests a mode of ribozyme evolution in vitro, and possibly in vivo [2]. Weakly catalytic RNAs would be activated by small RNAs in *trans*, followed by covalent association to form larger polynucleotide chains. Transactivating structural elements bind to their cognate ribozymes with K_d values in the micromolar or slightly lower, but clearly not in the nanomolar range (Fig. 2) [8–10]. These interactions are considerably weaker than those available from Watson–Crick base pairing, providing a selective force favoring the incorporation of transactivating domains into a single polynucleotide chain. Further selective pressure in favor of a larger RNA would arise from the enhanced k_{cat} provided by the presence of the small structure in the single chain (Table 1) [29].

The idea that ribozymes arose by association of preformed subunits might favor the prebiotic formation of large RNA catalysts (the RNA world model) relative to protein enzymes. Protein enzymes in a prebiotic situation would have to be assembled from a family of encoded units (domains) that are large enough to retain some secondary structure. In contrast, catalytic activity can reside in very small RNA structures [30–32]. Given that any prebiotic polymerizing machinery would be poorly processive, only relatively small catalysts could have been synthesized [2,33,34]. This consideration implies that the concentrations, and indeed the absolute number, of molecules necessary for selection to operate would be more easily attained with a library of small molecular species than with fewer large species. Association of two distinct RNA molecules could lead to an enhancement of catalytic activity followed by an RNA-catalyzed transesterification event [35]. Because small RNAs attain stable, compact secondary structure more easily than do peptides of similar length, this scheme might indeed be favored in an RNA world, even though contemporary genetic rearrangements occur at the genomic level.

The Type II RNase P RNAs diverged relatively recently from those of other bacteria [17], making the above scenario unlikely as an explanation for the incorporation of PL5.1 into the ribozyme. However, PL5.1 and its interaction with the rest of RNase P RNA provides a model to evaluate the structure, function and in vitro evolution of this element.

Acknowledgements: We thank Dr. Vaman Rao for synthesizing PL5.1 RNA, Drs. Peter Tipton, Steve Van Doren, and Dave Taylor for discussions and advice. This work was supported by the University of Missouri Research Council and by NASA grant NAG5-9555. T.C.L. was a fellow of the University of Missouri-Columbia Molecular Biology Program during this work.

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