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A phage RNA-binding protein binds to a non-cognate structured RNA and stabilizes its core structure

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ABSTRACT

Recent studies suggest that some RNA-binding proteins facilitate the folding of non-cognate RNAs. Here, we report that bacteriophage MS2 coat protein (MS2 CP) bound and promoted the catalytic activity of *Candida* group I ribozyme. Cloning of the MS2-bound RNA segments showed that this protein primarily interacts with the P5ab–P5 structure. Ultraviolet cross-linking and the T1 footprinting assay further showed that MS2 binding stabilized tertiary interactions, including the conserved L9–P5 interaction, and led to a more compact core structure. This mechanism is similar to that of the yeast mitochondrial tyrosyl-tRNA synthetase on other group I introns, suggesting that different RNA-binding proteins may use common mechanisms to support RNA structures.

RNA-binding proteins (RBPs) constitute a major class of proteins exerting critical functions in all living organisms [1,2]. It is believed that functional RNA molecules always associate with proteins in living cells, because there is no evidence that unbound RNA exists *in vivo* [3]. The interaction of several RBPs with structural RNAs has been shown to modulate RNA folding properties [4–6].

Group I introns are large ribozymes among these structural RNAs, and fold into defined tertiary structures required for their own splicing [7]. Assembly of the conserved P4–P6 and P3–P9 domains forms the ribozyme core, while highly variable peripheral elements are present to stabilize the core structure via long-range interactions [8,9]. Because their ribozyme activity provides a direct read-out for the native folds, group I introns have been widely used as a model for studying RNA folding mechanisms.

Self-splicing of group I introns *in vitro* in the absence of protein factors is much slower and less efficient than *in vivo* splicing, due to the thermodynamic and kinetic challenges that they encounter [10]. Early studies were limited to yeast mitochondrial proteins that, when mutated, resulted in splicing deficiency phenotypes. These studies led to the discovery of Cbp2 protein which captures the native-like structure of the group I intron bI5, and of Cyt-18 protein, which stabilizes the tertiary structure of the ribozyme by binding to the P4–P6 domain [4,11]. These two proteins are RNA-binding proteins with a general feature of binding directly to RNA and therefore stabilizing the RNA tertiary folds. Interestingly, CYT 18 also stimulates the activity of several non-cognate group I introns [12], questioning the specificity of the action of RNA-binding proteins.

MS2 CP tightly binds to a small RNA hairpin in its genomic RNA with a dissociation constant of 39 nM [13]. The binding represses translation of the early replicase gene and identifies the RNA for future packaging into virus particles [14]. In addition, MS2 CP binds to a number of non-native RNA hairpins produced by *in vitro* SELEX [15]. *Candida* group I ribozyme is a self-splicing intron located in the 26S rRNA gene of the pathogenic yeast *Candida albicans*. The folding and structure of this ribozyme have been extensively studied recently [16–18]. This intron contains 13 base-paired structures, this study sought to assess whether MS2 CP non-specifically interacts with this ribozyme.

Materials and methods

RNA and protein preparation. Preparation and radioactive labeling of the *Candida* group I intron ribozyme RNAs were performed as described [17]. MBP and MS2–MBP fusion protein were expressed and purified [19].

Analysis of the ribozyme activity. The purified RNAs randomly labeled by incorporation of [α-^{32}P]GTP during *in vitro* transcription was denatured and equilibrated as previously described [17]. The equilibrated RNA was incubated in the folding buffer containing 2 mM or 6 mM $MgCl_2$ and 1 μM of the indicated proteins at 4 °C for 15 min. Hydrolysis reactions were initiated by incubation at 37 °C for 90 min in the presence or in the absence of 2 μg/μl protease K, then stopped and analyzed as described previously [17]. To determine the 3′ hydrolysis kinetics, time-dependent accumulation

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of the hydrolysis products was determined by removal and analysis of reaction aliquots at the indicated rime points.

RNA electrophoretic mobility shift assay (EMSA). The internally labeled Ca.L-11 ribozyme RNA [17] was denatured and then incubated with the indicated concentrations of purified proteins in 10 μl folding buffer (6 mM Mg^{2+}) at 4 °C for 15 min as described above, except that 0.2 μg/μl tRNA and 5 μg/μl heparin were included. The samples were then fractionated on 5% native polyacrylamide gels [18].

In this study, all denature and native PAGE gels were analyzed using the variable scanner Typhoon 9200 (Amersham-Pharmacia Biotech). All data were plotted using the GraphPad Prism 4.0 program (www.graphpad.com).

Protein-protected RNA cloning method (PRC) to identify the MS2-binding site on Ca.L-11 ribozyme RNA. The method is detailed in Supplementary Data. In brief, the ribonucleoprotein (RNP) complex containing Ca.L-11 RNA and the MS2 fusion protein was formed; the unprotected RNA segments were removed by RNaseA to produce miniRNP. The miniRNP band was recovered from 5% native polyacrylamide gels, and the small RNAs in miniRNP were then cloned.

Results

MS2 CP binds to the Candida group I ribozyme

The binding capacity of MS2 CP with the *Candida* ribozyme was investigated by gel retardation assay, showing that the level of the MS2–ribozyme complex increased with the concentrations of MS2 protein (Fig. 1A). Quantification of three independent sets of experiments resulted in a K_d value of 610 ± 130 nM (Fig. 1B). Consistently, a K_d value of 226 ± 30 nM was obtained using fluorescence spectrum assay (Supplementary Figure S1). These data suggested that MS2 CP binds to ribozyme RNA at an affinity much lower than that of the native RNA substrate, while is comparable to some aptamer RNA of this protein [13,14]. The fluorescence data also suggest that one *Candida* ribozyme molecule was bound by one MS2 protein molecule (Supplementary Figure S1).

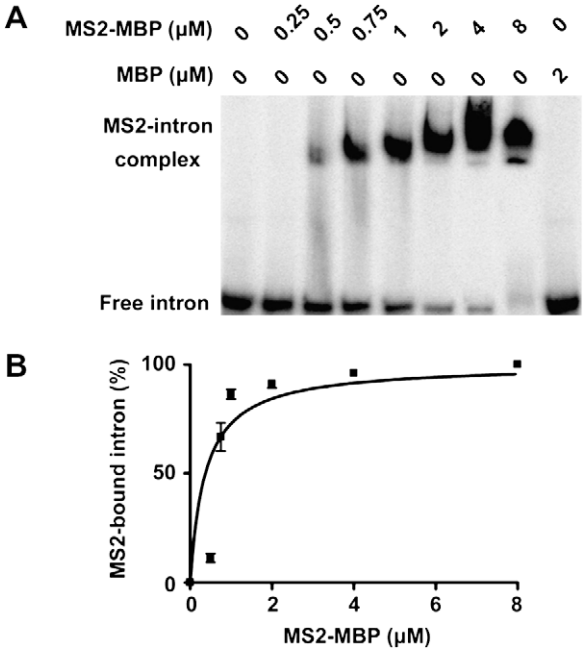


Fig. 1. Gel-shift analysis of MS2 binding to the ribozyme. (A) Varying concentrations of the purified MS2 CP and Ca.L-11 ribozyme RNA were incubated in 40 mM Tris–HCl, pH 7.9, 6 mM $MgCl_2$, 10 mM DTT, 10 mM NaCl, 2 mM spermidine, 0.2 μg/μl tRNA, and 5 μg/μl heparin for 15 min at 4 °C. The reactions were then analyzed by native gel electrophoresis. (B) The fraction of the labeled ribozyme bound to MS2 (Y) at each protein concentration was obtained from all three independent gel-shift experiments, which was then plotted against the MS2 concentrations (X) by fitting to the standard one-site binding equation $Y = 100 * X/(K_d + X)$. The bound fraction (Y) was the ratio of the radioactivity associated with the shifted band to total radioactivity in each lane. A K_d of 0.611 ± 0.138 μM was obtained.

MBP alone did not interact with the ribozyme RNA (Fig. 1A), supporting the conclusion that MS2–MBP fusion protein binds to the ribozyme using the RNA-binding function of the MS2 CP.

MS2 CP binding significantly promotes the ribozyme activity

To examine whether MS2 binding affects the ribozyme activity, the 3′ hydrolysis reaction of the *Candida* ribozyme was performed. It was shown that 1 μM of MS2 CP enhanced the ribozyme hydrolysis, while MBP protein alone showed no appreciable effect (Fig. 2A and B). A greater stimulation by MS2 CP was observed at 2 mM than at 6 mM Mg^{2+}, suggesting that it stabilizes a functionally important structure similar as magnesium does. Protease K treatment almost completely abolished MS2-stimulated ribozyme activity (Fig. 2A and B), supporting a conclusion that the physical association of MS2 CP with the ribozyme is required for its promotion of the ribozyme activity.

Interestingly, MS2 CP did not alter the observed rate constant of the ribozyme catalysis, but significantly increased the maximal fraction of the catalytic active ribozyme (Fig. 2C–E), suggesting that binding of MS2 CP converts a larger fraction of the ribozyme to the active structure.

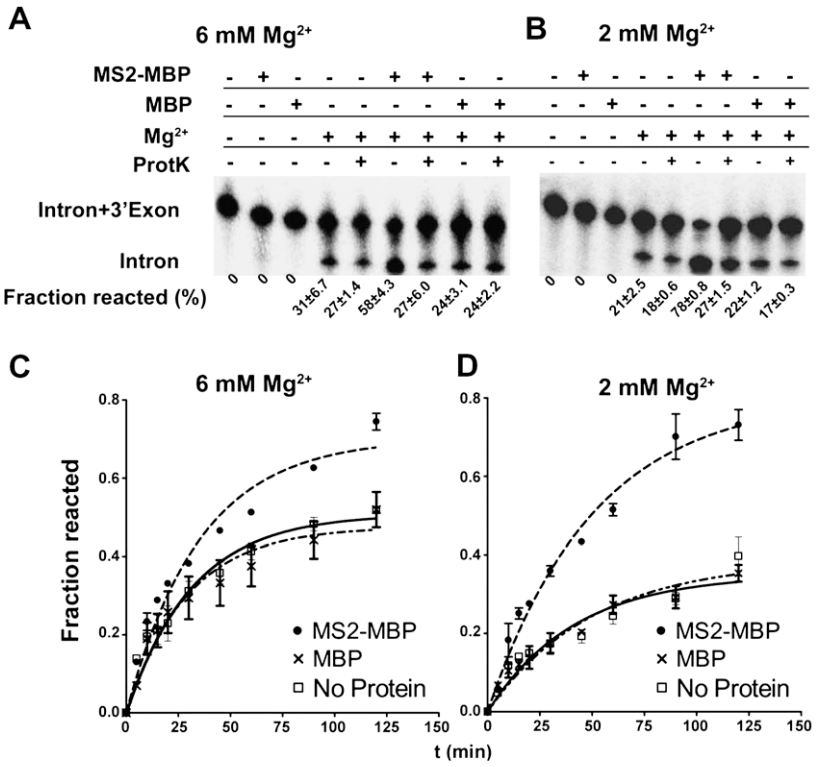
The similar effect of MS2 CP on the catalytic activity of two other eukaryotic group I introns from *Tetrahymena thermophila* (Tth.L1925) and *Pneumocystis carinii* (Pca.L1921) was also evident (Supplementary Figure S2), suggesting a general effect of MS2 CP on the group I ribozyme function, presumably reflecting a similar alteration of the ribozyme structure.

MS2 CP primarily interacts with the P5ab–P5 structure of the Candida ribozyme

Because *Candida* group I ribozyme contains no typical RNA-binding motif of MS2 CP, we were very interested in addressing where this protein binds on the group I intron. A new method called PRC was developed to identify the direct-binding site of MS2 CP on the ribozyme RNA. Interestingly, all the RNA sequences bound by MS2 CP were located in the P5ab–P5 structure (Fig. 3A). The shortest sequence was mapped to 135A to 151C, while the longest one was from sites 131A to 200C of the ribozyme RNA. It is particularly interesting to notice that the unpaired A139–A140 in J5/5a was protected from RNase A cleavage in all six clones. Because RNase A specifically cleaves the single-stranded RNA, the exclusive protection of J5/5a and P5ab harboring a number of unpaired nucleotides readily suggests that MS2 primarily binds to J5/5a–P5ab structure and protects it from RNase A digestion.

MS2 binding stabilizes the L9/P5 tertiary interaction and the core structure

We then addressed how MS2 binding globally affects the ribozyme structure. The T1 ribonuclease footprinting experiment was performed as described in Supplementary Data. Consistent with the view that MS2 primarily interacts with P5a, T1 protection was clearly evident at G183–186, a G-rich region in the P5a helix, in the presence of MS2 CP but not in the presence of the control protein (Fig. 4A). Strikingly, G286/287 in the J8/7 junction, G256/G257 in the P3 helix, and G245–247 in the P7 helix became strongly resistant to T1 cleavage in the presence of MS2 CP (Fig. 4A). A weak protective effect was also observed at G308 in



E

	6 mM Mg^{2+}		2 mM Mg^{2+}	
	Maximal hydrolyzed fraction (%)	kobs (min-1)	Maximal hydrolyzed fraction (%)	kobs (min-1)
● MS2-MBP	70±3	0.028±0.003	80±4	0.02±0.002
× MBP	48±3	0.034±0.006	35±2	0.024±0.003
□ NO protein	51±2	0.032±0.004	37±4	0.019±0.004

Fig. 2. MS2 CP-binding promotes the 3′ hydrolysis of the wild-type ribozyme. The gel-purified I-3′E RNA was hydrolyzed at the 3′ splice site in the presence of 6 mM Mg^{2+} (A) or 2 mM Mg^{2+} (B). Two independent experiments were performed and one representative gel is shown here. Hydrolysis of the *Candida* group I ribozyme was performed at 6 mM (C) and 2 mM (D) $MgCl_2$, and then reaction aliquots were taken for gel fractionation after 5, 10, 15, 20, 30, 45, 60, 90, and 120 min of hydrolysis at 37 °C. (E) The reacted fraction was plotted against the reaction time by fitting curves to a single exponential, and the observed rate constant and the maximal reacted fraction deduced from the curve are shown.

L9 (Fig. 4B), hinting that MS2 binding may stabilize the tertiary interaction between P5 and L9 [18].

To validate this observation, UV cross-linking coupled with primer extension experiment (CLiPE) was performed as described in Supplementary Data. Two different primers pairing 356–379, 179–202 of *Candida* ribozyme were used to determine the ribozyme structure (Fig. 4E), with the results being shown in Fig. 4B and C, respectively. The stop signals in the presence of MBP protein show similar profile and intensity as those of the no-protein control, indicating that neither does MBP protein bind to the ribozyme RNA nor alter the ribozyme structure. Strikingly, the presence of MS2 CP induced a number of unique stop signals (lane 2), including the newly appearing bands and those with increased band intensity. These induced stop signals are readily interpreted as the sites of MS2 CP-dependent cross-linking, which could be resulted from either RNA–protein cross-linking or protein-dependent tertiary RNA–RNA interactions [20,21].

Among the detectable region between C135 and A172 in P5-P5ab structure, most of the nucleotides in P5-J5/5a-P5a-J5b/5a were cross-linked, which strongly supports our conclusion that MS2 CP binds to this structure (Fig. 4B and C). The cross-link signals at C135–G138 of the base-paired region of P5 might not be attributable to a direct binding by MS2 CP; they may instead reflect an increased tertiary interaction with L9, because the GAAAA sequence in L9 also displayed strong cross-link signals (Fig. 4B and C). The L9 GNRA tetraloop are expected to interact with the minor groove of P5 [8,22] and this tertiary interaction is subjected to cross-linking by UV irradiation [20,21]. The increase of L9/P5 tertiary interaction by MS2 CP binding is further supported by T1 footprinting data showing a moderate protection at region around G308 (Fig. 4A and B).

Interestingly, CYT 18 also directly contacts the L9/P5 interaction of its non-cognate RNA substrate Twort group I intron [23]. Our results could be explained by a direct contact of MS2 CP with the L9/P5 structure. However, it is also possible that the MS2 CP binding of J5/5a–P5ab stabilizes the ribozyme structure and thus contributes to the increased L9/P5 interaction, because P5ab helix is packed along with P5-P4-P6 helices and this packing stabilizes the compact structure of the ribozyme [16] (M. Xiao and Y. Zhang, unpublished data).

Consistent with the requirement of L9/P5 structure for the action of MS2 on the ribozyme, we showed that the MS2-promoted

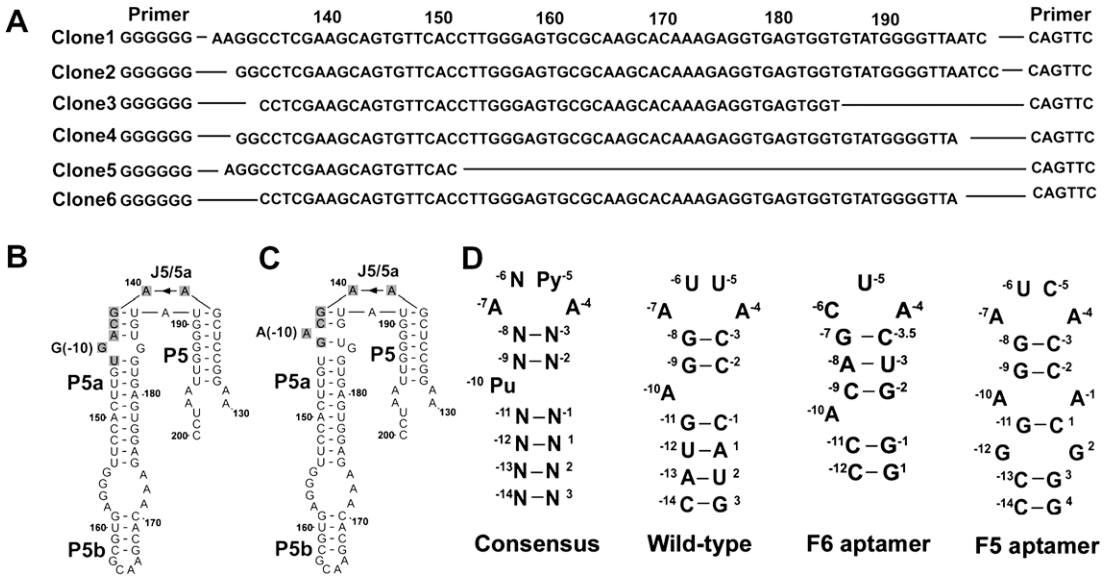


Fig. 3. MS2 CP binds to the P5ab substructure. The PRC method described under Materials and methods was used to identify the MS2-binding site on the *Candida* group I ribozyme. (A) The MS2-bound sequences identified from six positive clones. Their corresponding positions on the ribozyme are indicated. (B) The MS2-bound sequences are located inside of the P5ab–P5 structure of the *Candida* ribozyme. The nucleotides resemble the conserved binding sites of MS2 CP were shaded. (C) An alternative structure of P5ab–P5 with different base pairing pattern in P5a, and the potential binding sites of MS2 CP were shaded. (D) Secondary structures of some previously reported typical RNA stem-loops bound by the MS2 coat protein [26].

ribozyme activity was dependent on the presence of both P5ab and P9, as reflected by the diminishment of such effect when either of this structure element was deleted (Supplementary Figure S3).

Discussion

We have demonstrated that MS2 CP binds to a eukaryotic group I ribozyme, which alters the ribozyme 3′ hydrolysis activity. A new method developed in this report showed that MS2 primarily interacts with the P5ab peripheral structure of the ribozyme. T1 footprinting and CLiPE (UV cross-linking coupled with primer extension) assays showed that MS2 binding stabilizes the L9–P5 tertiary interaction and leads to a more compact ribozyme core important for the 3′ hydrolysis activity of the ribozyme.

A new method for identification of the interaction sites between proteins and folded RNAs

For the structurally complex RNA molecules, binding of a protein at one site may alter the structure of another site; this feature precludes the use of regular footprinting method to accurately map the direct protein-binding sites on RNA. To circumvent this problem, direct hydroxyl-radical cleavage assay has been used to map the protein-binding sites on some group I introns [24], in which series of protein mutants containing a single cysteine at different positions needed to be constructed, expressed, and purified. In this study, the protein-protected RNA cloning (PRC) method has been developed to successfully identify the MS2-binding site on the *Candida* group I ribozyme RNA. In principle, the RNA bound by MS2 CP is protected from nuclease digestion and can be successfully cloned after RT-PCR amplification. This method is cost-effective and easy to be performed, which provides an attractive alternative to identify RNA sequences bound by RNA-binding proteins *in vitro*.

An atypical MS2 CP-binding motif on the Candida ribozyme

The P5ab structure does not closely resemble the wild-type target of the MS2 protein or the typical MS2 CP aptamers [15,25], and no typical MS2 CP-binding hairpin structure is present in the *Candida* ribozyme (comparison of Fig. 3C and D). MS2 interacts with the cognate RNA hairpin asymmetrically. The adenine at the −4 position in the terminal loop and the bulged purine at −10 in the paired region establish four and three hydrogen bonds with some threonine and serine residues in the MS2 protein (Fig. 3D) [26]. In the *Candida* P5ab structure, two symmetrically bulged Gs are present in P5a, and one recovered sequence contains only G141–C152 in P5a, indicating that this side of P5a may directly contact MS2 protein and that G144 might represent the −10 purine, resembling the base-paired structure in the F6 aptamer (Fig. 3B). We also noted that P5a could form an alternative structure, with one bulged G144 pairing with U185 and the other bulged G184 pairing with U145. This alternative pairing ending up with a single bulged A143 after the first two base pairs in P5a represents a conserved paired structure for the MS2 protein targets (Fig. 4C). Interestingly, previous research has shown that A-4 is essential for MS2 recognition, and base substitution at this position reduces the dissociation constant of the protein binding to 670 nM [13], consistent with the lack of a conserved A(-4) in the P5ab–P5 structure and a similar low binding affinity.

In summary, the finding of MS2 CP binding of the P5ab–P5 structure of the *Candida* group I ribozyme suggests that this phage protein can interact with a bulged RNA paired region connected to an asymmetric internal loop containing three adenines.

The implication of the MS2 CP in supporting the active structure of a fungal group I ribozyme

In addition to the early mentioned mitochondrial proteins CBP2 and Cyt-18, several general RNA-binding proteins have been demonstrated to help RNA folding. The hnRNP A1 protein, HIV-1 nucleocapsid protein (NCp7), coronavirus nucleocapsid protein, and the GAPDH protein have been shown to stimulate hammerhead ribozyme catalysis. And La, hnRNP K, and hnRNP I are reported to possess RNA chaperone activity [4]. This study shows that MS2 CP, a bacteriophage protein, can bind to a peripheral structure of a group I ribozyme, which in turn stabilizes the core

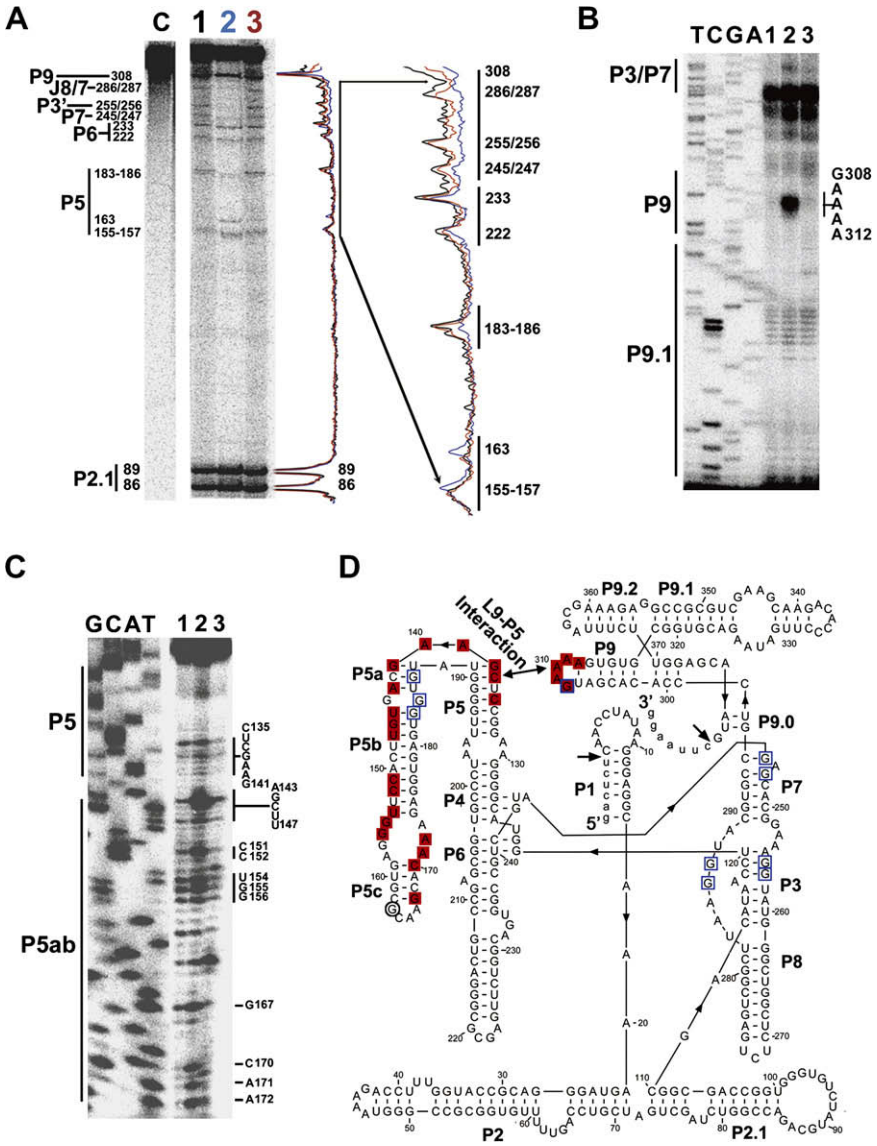


Fig. 4. MS2 CP binding stabilizes the L9/P5 tertiary interaction and the core structure of the ribozyme. **(A)** T1 ribonuclease footprinting analysis. The gel-purified Ca.L-11 RNA radiolabeled at its 5′ end was analyzed in the absence of protein (lane 1), or in the presence of MS2–MBP fusion protein (lane 2), or MBP alone (lane 3). Lane C indicates the labeled ribozyme was incubated in the folding buffer without adding T1 ribonuclease. The gel result was further analyzed by normalization of the intensity of each band to that of G89 in P2.1 in each lane, and shown on the right of the gel. Black, blue, and red lines represent the analyzing results from lanes 1, 2, and 3, respectively. (B–D) UV cross-linking coupled with primer extension (CLiPE) analysis. The unlabeled purified ribozyme was folded in the absence (lane 1) or in the presence of the MS2–MBP fusion protein (lane 2), or MBP alone (lane 3). The radiolabeled primers were 356–379 primer (B), 179–202 primer (C). (D) Mapping the MS2 CP-altered positions on the secondary structure of the *Candida* ribozyme. The MS2-protected nucleotides identified by UV cross-linking-coupled primer extension (CLiPE) are shaded in Red; those identified by T1 footprinting are boxed. The MS2-opened G163 identified by T1 footprinting is circled. The L9–P5 interaction is indicated.

structure and promotes the activity of the ribozyme. The action mechanism of this protein is strikingly similar to that of the CYT-18 protein on its cognate and non-cognate group I ribozymes. These findings support a model that different RNA-binding proteins may use common mechanisms to promote RNA structures.

This model is supported by the notion that splicing factors without host specificity are present to facilitate the group I intron splicing *in vivo*. For example, the *Tetrahymena* group I intron splices in *Escherichia coli* and human cells as efficiently as in its natural host [27,28].

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2008.10.160.

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