Characterization of the newly constructed domains that replace P5abc within the *Tetrahymena* ribozyme

Yoshiya Ikawa, Hideaki Shiraishi, Tan Inoue*

Department of Chemistry, Faculty of Science, Kyoto University, Kyoto 606-01, Japan

Received 1 July 1996; revised version received 5 August 1996

Abstract The P5abc domain of the *Tetrahymena* ribozyme has been shown to function as an activator that enhances core catalytic activity of the ribozyme. We reported previously that several new domains in that their primary sequences are different from that of P5abc are also capable of activating the ribozyme. It was unclear whether the mechanism of activation by the new domains is identical to that by P5abc. We have investigated structural and functional properties of the new domains and obtained evidence that strongly indicates that a particular domain activates the ribozyme in a different manner from that by P5abc.

Key words: *Tetrahymena*; Ribozyme; P5abc; Gel mobility shift

1. Introduction

P5abc within the group I intron, which is a large extension of the P5 domain (Fig. 1A), is conserved in the subclasses IC1 and IC2 [1]. The P5abc region plays an important role in the mechanism of activation of the group I intron ribozyme [2–4]. It has been demonstrated that an inactive mutant *Tetrahymena* intron lacking the P5abc element can be activated when separately prepared P5abc is added *in trans* [4]. This activation *in trans* has been shown to depend on the tertiary interactions between the two RNAs [4].

Using an in vitro selection technique, we recently performed the artificial construction of new domains that functionally replace P5abc and enhance the catalytic activity of the *Tetrahymena* intron. From the variant introns in which the P5abc domain was replaced by randomized sequences, several variant introns exhibiting 10–60% of the activity of the wild type were isolated [5,6]. The primary sequences of the domains have no apparent similarity to that of P5abc. This prompted us to investigate whether the new domains we isolated are structurally and functionally related to P5abc. In this paper, we report the characterization of the structural and functional properties of the variant introns with the new domains.

2. Materials and methods

2.1. Numbering of the nucleotides within the novel sequences

As shown in Fig. 1A,B, variant introns (20Ns, 40N-1s and 40N-2) contain novel sequences of 20 or 40 nucleotides in place of the original P5abc domain (69 nucleotides: C127–G195) [5]. We numbered the nucleotides within the novel sequence as follows. The type of the variants was referred to as 20 (for 20N), 41 (for 40N-1), or 42 (for 40N-2). The first and last nucleotide in the sequences are numbered as 1, and as 20 for 20N (or 40 for 40N), respectively. For example, 42-A3 refers to the third nucleotide (which is adenine) in the sequence of a 40N-2 variant.

2.2. Preparation of nucleic acids

The DNA templates used for transcription in this paper were prepared through PCR amplification as reported previously [6]. The RNAs were prepared by in vitro transcription with T7 RNA polymerase and purified by electrophoresis on 5% denaturing polyacrylamide gels.

2.3. Ribozyme activity assays

Bimolecular ligation reactions [5] and unimolecular circularization reactions [7] were performed in the reaction buffer containing 40 mM EPPS (pH 6.5), 200 mM NH_4OAc, and 7.5 mM of $MgCl_2$ (except for Fig. 2B) at 30°C for 30 min. The bimolecular ligation reactions were performed as described [5]. In all assays, the reactions were terminated by addition of 0.5 M EDTA (pH 8.0) to a final concentration of 50 mM. The reaction mixtures were electrophoresed on 5% denaturing polyacrylamide gels and the products were quantitated by using a Bio-Image Analyzer BAS-100 (Fuji Film).

2.4. Chemical modification by DMS

Chemical modifications of RNA with DMS were performed as described [8] with modification buffer containing 50 mM Tris-Cl (pH 7.5), 200 mM NaCl and 15 mM $MgCl_2$. Reverse transcriptions were also performed as described [8] using the primers complementary to A263–U289, or C216–A233.

2.5. Gel mobility shift assay

The gel mobility shift assay was performed as described [4] using uniformly ^{32}P-labeled P5abc RNA (≤10 nM) and unlabeled RNA of the intron variant (1.0 μM).

3. Results and discussion

3.1. Catalytic activities of the variant introns containing new domains

As reported previously, we isolated six representative variants of the *Tetrahymena* ribozyme containing new domains in place of the P5abc by in vitro selection [5]. Among the new domains of the six variants, three consist of 20 nucleotides and the rest consist of 40 nucleotides. The domains can be classified into three distinctive families of 20N, 40N-1 and 40N-2 on the basis of their primary sequences and secondary structures predicted by minimum energy RNA folding calculations (Fig. 1B) [9]. 20N and 40N-1 families can be further categorized into subvariants 20N-a, b, c and subvariants 40N-1a and 1b, respectively.

To examine the catalytic activity of each variant intron, bimolecular ligation reactions that were employed for selecting the variants were performed under the conditions where the intron lacking P5abc is inactive [5]. 40N-1b exhibited the highest activity of all, and 40N-1a was second to that. The 20N family and 40N-2 were less active than the 40N-1 family (Fig. 2A).

To further characterize the nature of the three families,

*Corresponding author. Fax: (81) (75) 753 3996.

PII S0014-5793(96)00918-0

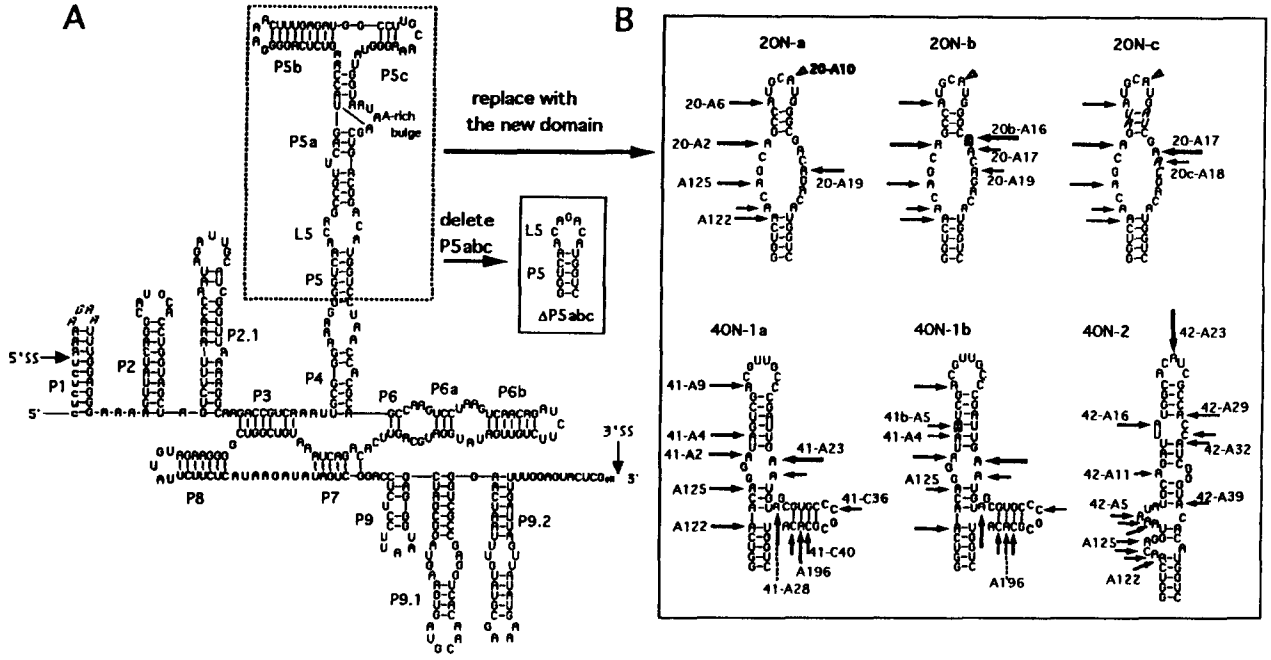

Fig. 1. A: Secondary structure of the wild type and ΔP5abc mutant of the *Tetrahymena* ribozyme. The wild type L1 loop has been replaced by the AGAA tetraloop (shown in italic type) to exclude unfavorable reaction sites [5]. A box with broken lines indicates the P5abc region, which is truncated in the ΔP5abc mutant (indicated in box with solid lines), or replaced by new sequences as shown in B. B: Predicted secondary structures and summary of DMS modifications of new domains that replace the wild type P5abc. 20N-b and 40N-b differ from 20N-a and 40N-a by single base changes (shown in outline type). 20N-c differs from 20N-a by six base changes (shown in italic type). Positions modified by DMS are indicated as black arrows (see also Fig. 3). The degree of modification is classified as strong (large arrow), moderate (middle arrow), or weak (small arrow). 20A-10 of 20N variants are also indicated as white arrow heads.

bimolecular ligation reactions were carried out by varying concentrations of $MgCl_2$ for 40N-1b, 40N-2, and 20N-a (Fig. 2B). The catalytic activities of 40N-1b and 40N-2 increased gradually relative to the increase in $MgCl_2$ concentration. In contrast, that of 20N-a dropped sharply in the presence of more than 12.5 mM of $MgCl_2$. 20N-b and 20N-c also exhibited a similar tendency (not shown).

3.2. Structural mapping of the new domains

To analyze the secondary structure of the new domains, chemical modifications were performed using DMS, which specifically modifies N1 of adenines (or N3 of cytosines with less efficiency) that are free from secondary or tertiary interactions [10]. The experiments were done under native conditions where the RNAs are active (Figs. 1B and 3).

In the 20N family, the experiments supported the predicted secondary structures of the subvariants 20N-a,b,c as summarized in Fig. 1B. However, in the region between the nucleotides 20-A16 and A19, the accessibility of DMS varied for each variant. The adenine at 20-A10 of the 20N family, which is predicted to be in a loop region, was unmodified under native conditions.

The results in the 40N-1 and 40N-2 families also support the predicted secondary structure. As in the cases of 20Na, b and c, minor differences were observed for 40N-1a and 1b. Three adenines (41-A4, A125, and A196 in 40N-1a) were dis-

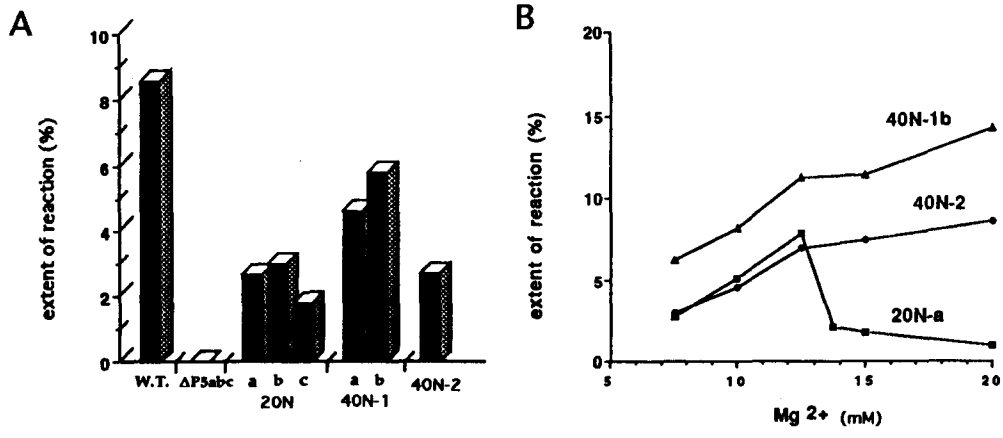

Fig. 2. A: Bimolecular ligation activities of wild type, ΔP5abc and variant introns. The uniformly ^{32}P-labeled 5′-half RNA of each variant (1.0 μM) and the unlabeled 3′-half RNA (2.0 μM) were incubated. B: Effect of $MgCl_2$ concentration on the activity of variant introns. Each RNA was incubated as described in Section 2 except that the reaction buffer contained 7.5, 10, 12.5, 15 or 20 mM of $MgCl_2$.

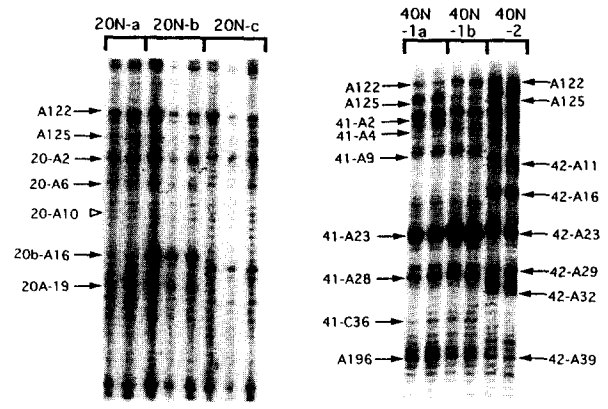

Fig. 3. DMS modifications of the variant introns. Positions of methylation were detected by reverse transcription with 5′-end labeled primer (see also Fig. 1B). Under the conditions employed, adenines were methylated preferably to cytosines by DMS [8,10].

tinctly more accessible to DMS than those in 40N-1b. Similar differences were also observed for the modification around 41a-G5 (or 41b-A5) which is characteristic for the two 40N-1s. The differences indicate that the domain in 40N-1a is less stable than that of 40N-1b, implying that it might be reflected to the activity of 40N-1a and 40N-1b. The modification experiments demonstrate that the secondary structures of the new domains are unrelated to that of P5abc.

3.3. *Analysis of new domains by gel mobility shift assay and trans-activation assay*

We next investigated whether the new domains enhance catalytic activity of the *Tetrahymena* ribozyme in a manner distinct from P5abc. As it was previously demonstrated that ΔP5abc is capable of forming a stable complex with the separately prepared P5abc RNA [4,11] (see also Fig. 4A), we first investigated whether the variants are also capable of forming a stable complex with P5abc RNA using RNA-RNA gel mobility shift assay. The assay revealed that the variant 20N-a and 40N-2 were unable to form a stable complex (Fig. 4A). More than one half of P5abc RNA molecules disassociated with either the intron variant 20N-a or 40N-1. This shows that the dissociation constants (K_d) of their complexes are apparently more than 1.0 μM [11]. Because the K_d of the complex of ΔP5abc intron with P5abc RNA is approximately 20 nM (Naito and Inoue, unpublished results), the K_d of the complex of either 20N-a or 40N-1 variant with P5abc is 50 times higher than that of the complex of ΔP5abc intron with P5abc. In contrast, the 40N-1b variant can form a complex that is as stable as that of ΔP5abc intron with P5abc (Fig. 4A).

The difference in the affinities of the variant introns to P5abc RNA can be explained as follows. In the case of 20N and 40N-2 variants, the new domain blocked the binding of P5abc RNA (like B1 in Fig. 4B for example). In contrast, that the 40N-1b variant efficiently associates with P5abc RNA in-

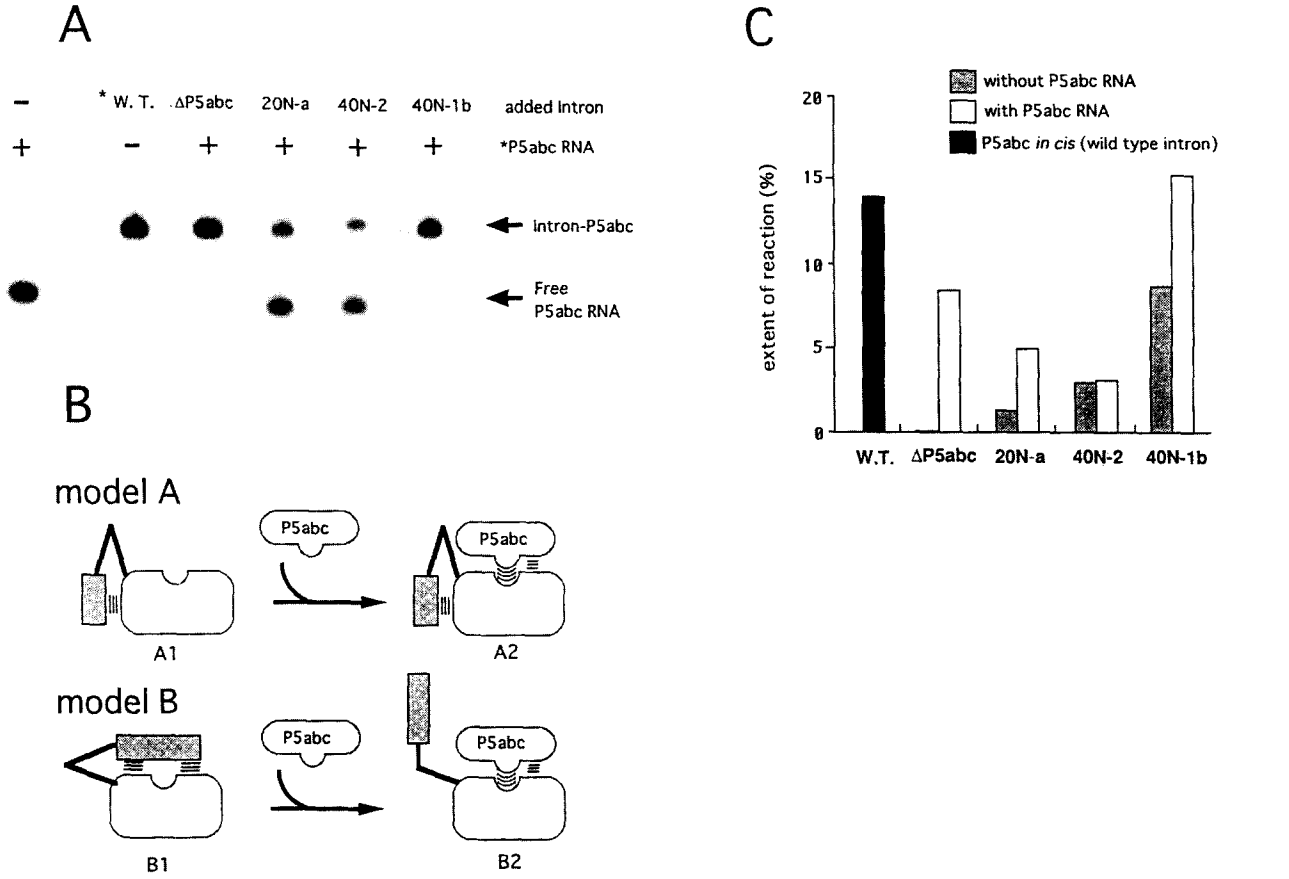

Fig. 4. A: Gel mobility shift assay of variant introns with P5abc RNA. Asterisks indicate that the corresponding RNAs are labeled with ^{32}P. B: Models A and B for the *trans*-activation of the variant introns by P5abc RNA. The new domain is denoted as a gray box. C: Effect of P5abc RNA on activities of selected variants. Uniformly ^{32}P-labeled variants were incubated with or without unlabeled P5abc RNA (5.0 μM) under the conditions described in Section 2. The wild type intron was also incubated under the same conditions without P5abc RNA.

dicates that the new domain in this variant does not interfere with the binding of P5abc RNA as in model A (Fig. 4B), or that the domain can be displaced easily by P5abc RNA as in model B.

To see whether the stability of the intron-P5abc complexes is directly related to the efficiency of activation by P5abc *in trans*, we compared the activity of the complexes by attempting the circularization reaction [7]. ΔP5abc mutant and the three variants were incubated in the presence or absence of P5abc RNA (Fig. 4C) [4]. The ΔP5abc mutant that forms a stable complex with P5abc RNA was activated up to 60% of that of the wild type intron by P5abc RNA *in trans* [4]. Compared with ΔP5abc, 20N-a and 40N-2, which form rather less stable complexes, were less efficiently activated by P5abc. The data suggested that weak *trans*-activation of the variants is due to the instability of the intron-P5abc complexes.

In contrast to 20N-a and 40N-2, the 40N-1b variant, which forms a stable complex with P5abc, was *trans*-activated distinctly compared with others (Fig. 4C). On the basis of this result, we would like to discuss a possible mechanism of activation in 40N-1b employing two models as shown in Fig. 4B. In model A, the 40N-1b variant that is capable of binding P5abc without altering the structure of the new activator domain present *in cis*, is further activated by P5abc *in trans* in addition to the activation by the domain existing *in cis*. On the basis of this model, it is possible that the intron-P5abc complex A2 becomes more active than A1 or the complex of ΔP5abc with P5abc RNA. This model does not contradict the results of both gel mobility shift assay and *trans*-activation assay. Contrary to model A, if the effective binding between P5abc and the 40N-1b variant is due to displacement as shown in model B, the activity of complex B2 is unlikely to exceed that of ΔP5abc with P5abc because the effect of the *cis*-existing domain is abolished by the displacement. Although a possibility that the displacement in model B happens to cause unusually efficient *trans*-activation of 40N-1b is not denied, we think that model B is unlikely to be the case. Given that the *trans*-activation of the variant 40N-1b is performed in a manner shown in model A, the result implies that the mechanism of activation of variant 40N-1b is different from that of P5abc and the catalytic core of the *Tetrahymena* ribozyme has potential to acquire various forms of activator domains independent of those in natural introns.

Acknowledgements: We thank Y. Naito for useful advice on the gel mobility shift assay and valuable discussions. This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture, Japan and Tokuyama Foundation.

References

[1] Michel, F. and Westhof, E. (1990) J. Mol. Biol. 216, 585–610.
[2] Joyce, G.F., van der Horst, G. and Inoue, T. (1989) Nucleic Acids Res. 17, 7879–7889.
[3] Pace, U. and Szostak, J.W. (1991) FEBS Lett. 280, 171–174.
[4] van der Horst, G., Christian, A. and Inoue, T. (1991) Proc. Natl. Acad. Sci. USA 88, 184–188.
[5] Williams, K.P., Imahori, H., Fujimoto, D.N. and Inoue, T. (1994) Nucleic Acids Res. 22, 2003–2009
[6] Ikawa, Y., Shiraishi, H. and Inoue, T. (1995) in: Tracing Biological Evolution in Protein and Gene Structures (M. Go and P. Schimmel, Eds.), pp. 115–123, Elsevier, Amsterdam.
[7] Inoue, T., Sullivan, F.X. and Cech, T.R. (1986) J. Mol. Biol. 189, 143–165.
[8] Murphy, F.L. and Cech, T.R. (1993) Biochemistry 32, 5291–5300.
[9] Jaeger, J.A., Turner, D.H. and Zuker, M. (1989) Methods Enzymol. 183, 281–306.
[10] Inoue, T. and Cech, T.R. (1985) Proc. Natl. Acad. Sci. USA 82, 648–652.
[11] Pyle, A.N., McSwiggen, J.A. and Cech, T.R. (1990) Proc. Natl. Acad. Sci. USA 87, 8187–8191.