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Trans-Activation of the *Tetrahymena* Ribozyme by Its P2-2.1 Domains[1]

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The *Tetrahymena* group I self-splicing intron contains peripheral domains P2-2.1. Mutant introns lacking these domains are hardly active. We found that if an independently prepared P2-2.1 RNA is added *in trans*, it efficiently enhances the catalytic activity of an intron lacking the domains. P2-2.1 RNA together with the previously identified activator, P5abc RNA, of the *Tetrahymena* intron can activate the intron lacking both of them. The *trans*-activation depends on the long-range interaction between P2.1 and P9.1 domains.

Key words: catalytic RNA, intron, ribozyme, self-splicing, *trans*-activation.

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The group I self-splicing intron is a member of the large ribozymes which catalyze the hydrolysis and/or *trans*-esterification at the phosphodiester bonds of RNA (*1*). The intron consists of a conserved core region and several non-conserved or weakly conserved peripheral domains that are characteristic of its subgroup, implying that the non-conserved domains are not essential for the catalytic activity (*2*). This is consistent with the experimental data obtained with mutant introns lacking the domains (*3-5*).

The subclass IC1 intron from *Tetrahymena thermophila* contains several peripheral elements including P5abc (Fig. 1A). P5abc, a large extension of the P5 domain, is highly conserved among subclass IC1 as well as subclass IC2 (*6*). Mutant *Tetrahymena* introns lacking P5abc, which are inactive under standard self-splicing conditions, can be activated *in trans* by adding a separately prepared P5abc domain (*7*). The formation of a stable RNA-RNA complex between P5abc RNA and the mutant intron is important for this *trans*-activation (*7*).

Compared with the P5abc domain, less is known about the functions of other peripheral elements in the *Tetrahymena* intron. For example, it still remains uncertain whether other peripheral elements can also function as *trans*-activators like P5abc does. In this study, we focused our attention on the peripheral domains termed P2-2.1. Mutant introns lacking these domains exhibited severely decreased activity, being hardly active under standard conditions (*3, 8*). We found that a separately prepared RNA consisting of the P2-2.1 domains efficiently activated a mutant intron lacking the corresponding domains *in trans* by forming a stable RNA-RNA complex with the intron RNA. The results indicate that the *Tetrahymena* intron contains at least two activator domains, *i.e.* P5abc and P2-2.1, that can function *in trans*.

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MATERIALS AND METHODS

Preparation of RNAs—All RNAs employed in this study were prepared by transcription *in vitro* with T7 RNA polymerase and purified by electrophoresis on polyacrylamide denaturing gels. Variant *Tetrahymena* introns were prepared using linearized plasmids bearing the T7 RNA polymerase promoter sequence followed by the respective introns. Plasmids were linearized with either *Hin*dIII for the hydrolysis reactions, or *Sca*I for the oligonucleotide-ligation reactions and the gel mobility-shift assays. Activator RNAs were prepared using synthetic oligonucleotide templates (*9*) with the following modification. To prevent the template oligonucleotides from forming secondary structures, we converted them into fully double stranded forms by means of primer extension using *Taq* polymerase.

Mutant Ribozyme Constructs—L-21 and L-30, lacking the first 21 and 30 nucleotides of the *Tetrahymena* intron, respectively, contain the P2-2.1 domains *in cis* (*10*). L-56 lacking the first 56 nucleotides contains P2.1 but not the P2 domain *in cis*. L-95 lacks the first 95 nucleotides including P2.1 as well as the P2 domain. L-30, L-56, and L-95 contain base-substitutions A31G, A57G, and A97G, respectively, as described previously (*8, 10, 11*). L-95ΔP5abc and L-95ΔP9.1T are variants of L-95 lacking the P5abc domain and the terminal region of the P9.1 domain, respectively (*3, 11*). The precursors of L-21, L-95, and L-95ΔP5abc contain the first 29 nucleotides of the 3′ exon of the *Tetrahymena* intron ribozyme. L-30 *Sca*I, L-56 *Sca*I, and L-95 *Sca*I lack the last five nucleotides of the *Tetrahymena* intron and the 3′ exon.

Assay of the 3′ Splice Site Hydrolysis Reaction—Uniformly ^{32}P-labeled precursor ribozymes were dissolved in distilled water with or without unlabeled activator RNA and then heated at 80°C for 3 min. After cooling and incubation at the reaction temperature for 10 min, the reaction was initiated by the addition of 5× concentrated reaction buffer with an appropriate concentration of $MgCl_2$ (as indicated in the figure legends). The resulting mixture was incubated at the indicated temperature. Aliquots were

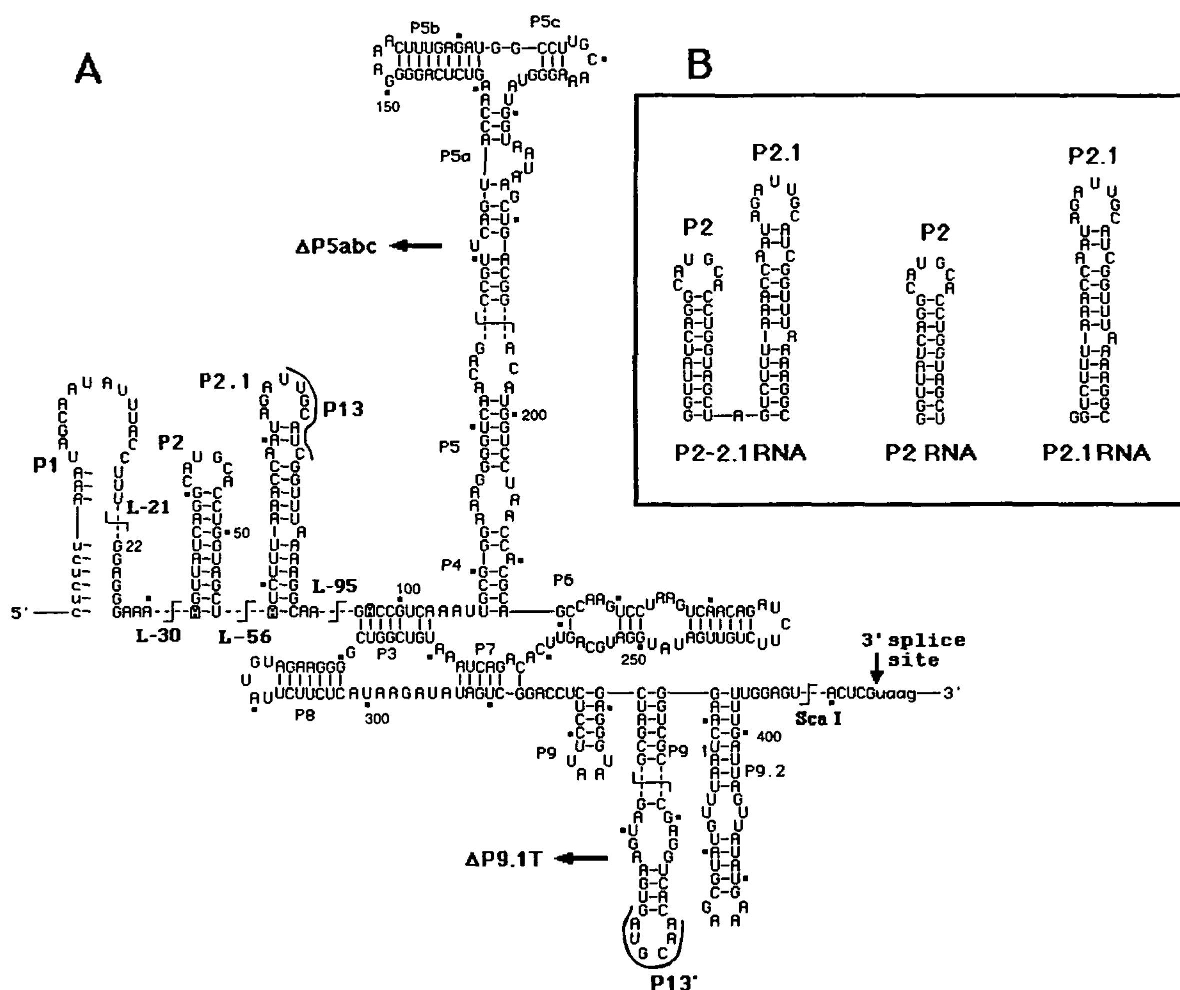

Fig. 1. **A: The secondary structure of the *Tetrahymena* group I intron ribozyme labeled according to the standard nomenclature.** The sites of deletion in this study are indicated by dotted lines and labels. **B: The secondary structures of separately prepared P2, P2.1, and P2-2.1 RNAs.**

removed at specific times and quenched on ice by the addition of an equal volume of a stop solution (150 mM EDTA, 70% formamide, and 0.25% of xylene cyanol). The products were electrophoresed on 5% polyacrylamide denaturing gels. Derivatives of the *Tetrahymena* ribozyme employed in this study all generated a 3′ exon fragment (29 nucleotides) exhibiting the same mobility on 12% polyacrylamide denaturing gels, indicating that they were cleaved at the accurate 3′ splice site (data not shown).

Assay of the Oligonucleotide-Ligation Reaction—Uniformly ^{32}P-labeled P1 RNA (<10 nM), unlabeled 5′ exon RNA (1.0 μM), and ribozymes (1.5 μM) were dissolved in distilled water with or without P2-2.1 RNA (5 μM), and then heated at 80°C for 3 min. After cooling and incubation at 37°C for 10 min, the reactions were initiated by the addition of 5× concentrated reaction buffer, followed by incubation at 37°C for 1 h. The reactions were stopped by the addition of an equal volume of the stop solution (150 mM EDTA, 70% formamide, and 0.25% of xylene cyanol), and then the products were electrophoresed on 10% polyacrylamide denaturing gels.

Gel Mobility-Shift Assay—The gel mobility-shift assays were performed as described (*12*) on 5% polyacrylamide native gels comprising 50 mM Tris-Cl (pH 7.5) and 25 mM $MgCl_2$ with uniformly ^{32}P-labeled activator RNA (<10 nM) and the indicated amount of unlabeled introns.

RESULTS AND DISCUSSION

Assay Systems for Trans-Activation by the P2-2.1 Domains—Previously, studies on the P2-2.1 domains were performed employing mutant introns having base-substitutions and/or deletions in the domains by employing *trans*-esterification reactions at the 5′ splice site for the investigation of their catalytic activity (*3, 13-15*). However, the assay system has a potential problem, as follows. For example, if the activity of a mutant is less than that of the wild type, one has to consider two dissimilar interpretations: (i) the deletion of the P2-2.1 domains is directly responsible for the decrease in the catalytic activity, as in the case of the P5abc domain (*7*), or (ii) the deletion does not influence the activity but disturbs the correct positioning of the P1 domain containing the 5′ splice site to the catalytic center, as in the case of the P2 domain of the T4 *td* intron (*2, 16*).

To avoid the positioning effect described above, we

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employed two methods for assaying of the mutant introns in this study. One involves comparison of the specific hydrolysis reaction at the 3′ splice site that is not influenced by deletion of the P1 domain (*10*) which is required for both the first and second steps of the splicing reaction (*17*). The other involves comparison of the reaction at the artificially created 5′ splice site employing the previously reported oligonucleotide-ligation system consisting of the 5′ exon and the P1 RNA, that were prepared separately from the rest of the intron (*18*). This reaction is equivalent to the reverse of the first step of the splicing (Fig. 2B).

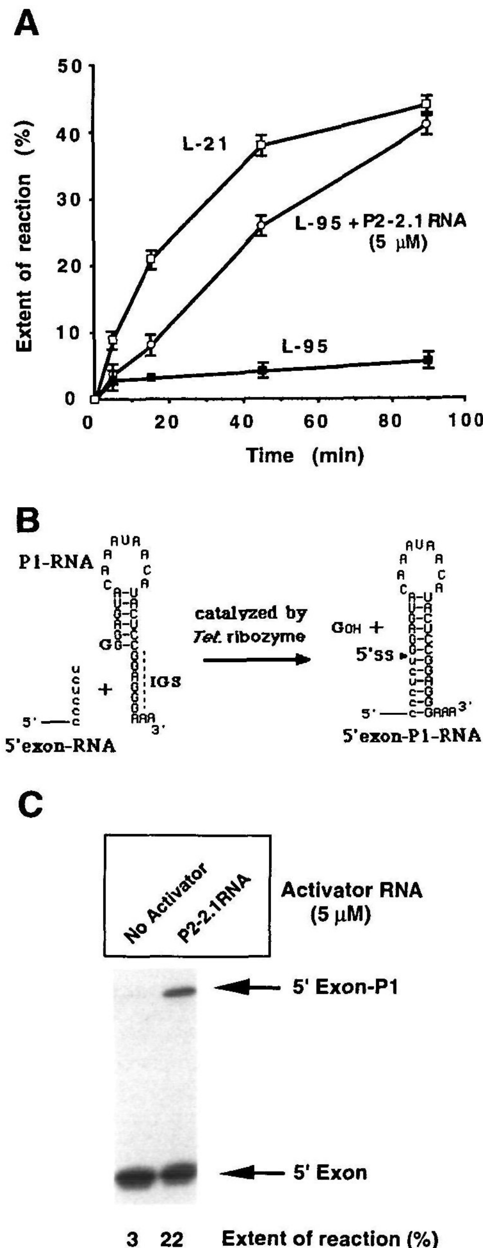

Fig. 2. **A: Effect of P2-2.1 RNA on the 3′ splice site hydrolysis reactions.** Time courses for L-95 with P2-2.1 RNA (5 μM), L-95, and L-21. ^{32}P-labeled precursor RNAs having the 3′ exon (29 nt) were incubated at 30°C in a hydrolysis buffer comprising 50 mM Tris-Cl (pH 8.3) and 12 mM $MgCl_2$. **B: Schematic representation of the oligonucleotide-ligation reaction catalyzed by the *Tetrahymena* intron. C: Effect of P2-2.1 RNA on oligonucleotide-ligation reactions.** ^{32}P-labeled 5′ exon RNA (<10 nM), unlabeled P1 RNA (1.0 μM), and L-95 *Sca*I (1.5 μM) were incubated with/without P2-2.1 RNA (5 μM) at 37°C for 1 h in a buffer comprising 50 mM Tris-Cl (pH 7.5) and 25 mM $MgCl_2$.

P2-2.1 RNA Activates the Intron Ribozyme In Trans—To determine whether the P2-2.1 domains could activate ribozymes lacking the corresponding domain(s) *in trans*, we first attempted the hydrolysis reaction at the 3′ splice site. A mutant intron that lacks both the P2 and P2.1 domains (termed L-95, Fig. 1A) was incubated with a separately prepared RNA consisting of the sequence of the P2-2.1 domains (Fig. 1B) under our standard conditions, where L-95 by itself is hardly active. P2-2.1 RNA efficiently activated L-95 (Fig. 2A, see also Fig. 5A). We also compared the hydrolysis reaction activated by P2-2.1 RNA *in trans* with that of the L-21 possessing P2-2.1 *in cis*. The activity of L-95 with P2-2.1 RNA was less than that of the L-21 (Fig. 2A).

To determine whether the activation by P2-2.1 RNA is applicable to other reactions catalyzed by the *Tetrahymena* ribozyme, we attempted the oligonucleotide-ligation reaction, that is equivalent to the reverse of the first step of the splicing (Fig. 2B) (*18*). Under the conditions where L-95 *Sca*I is barely active, P2-2.1 RNA enhanced the activity of L-95 *Sca*I approximately 7-fold (Fig. 2C), demonstrating that P2-2.1 RNA is responsible for the enhancement of the core catalytic activity.

To examine the physical affinity of P2-2.1 RNA to L-95, we attempted RNA-RNA gel mobility-shift assays. ^{32}P-labeled P2-2.1 RNA was incubated with L-95 *Sca*I and then electrophoresed on a non-denaturing polyacrylamide gel. A

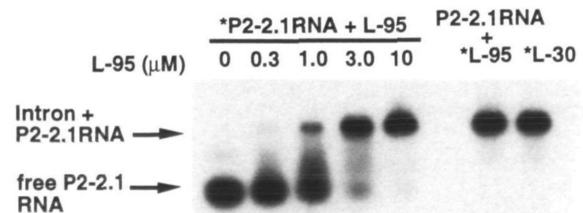

Fig. 3. **Physical affinity of the activator RNA for L-95.** Gel mobility-shift assay of L-95 *Sca*I with P2-2.1 RNA. Asterisks denote to the RNAs labeled with ^{32}P.

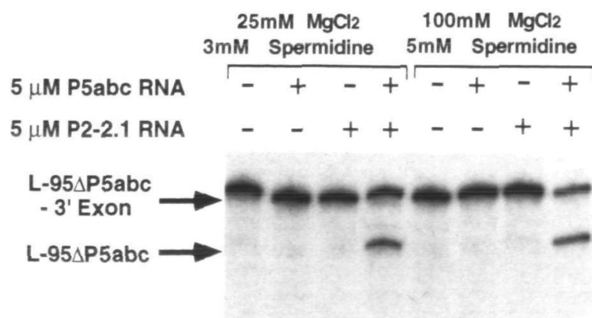

Fig. 4. **Reconstitution of an active ribozyme from the core RNA and two distinct activator RNAs.** The precursor of the mutant intron (L-95ΔP5abc) lacking P2-2.1 as well as P5abc was incubated at 37°C for 30 min in a hydrolysis buffer comprising 50 mM Tris-Cl (pH 8.3) and other additives, as indicated in the figure.

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slowly migrating band was observed relative to the increase in the amount of L-95 *ScaI* (Fig. 3). Its mobility was similar to that of ^{32}P-labeled L-30 *ScaI* possessing P2-2.1 *in cis*, indicating that the band corresponds to the complex of P2-2.1 RNA and L-95 *ScaI*. The dissociation constant of the complex was determined to be 1 to 3 μM. The constant for P5abc RNA and the mutant intron lacking P5abc that forms active complex with P5abc RNA was previously determined to be 20 nM (*12*), so the binding between the P2-2.1 RNA and L-95 is much weaker.

Trans-Activation by Both P2-2.1 and P5abc RNA—Our finding that P2-2.1 RNA functions as a *trans*-activator provides an additional evidence supporting the view that a group I intron is composed of independent structural domains that are physically separable (*7, 19*). To further explore this line of thought, we attempted reconstitution of the ribozyme by assembling three RNA molecules; the core region of the ribozyme and two activator RNAs (Fig. 4). A mutant intron lacking both P2-2.1 and P5abc (L-95ΔP5abc), that is inactive even in the presence of a high concentration of $MgCl_2$ (100 mM) together with spermidine, exhibited the hydrolysis activity only if both P2-2.1 and P5abc RNA were present, demonstrating the formation of a tri-molecular ribozyme.

The P2.1 Domain Is Important for Trans-Activation—To determine the region important for the *trans*-activation by P2-2.1 RNA, we prepared two RNA fragments, P2 and P2.1 RNA, that consist of the sequences of the P2 domain and the P2.1 domain, respectively. P2 RNA cannot activate L-95 whereas P2.1 RNA functions as an activator RNA that is as effective as P2-2.1 RNA (Fig. 5A). The result shows that the P2.1 domain plays a critical role for the *trans*-activation.

To further analyze the activation by P2-2.1 or P2.1 RNA, we attempted the hydrolysis reaction by varying the amounts of the two activator RNAs (Fig. 5B). At 1 μM or less, P2-2.1 RNA activated L-95 more efficiently than P2.1 RNA. However, at 3 μM, P2.1 RNA was more efficient than P2-2.1 RNA. At more than 5 μM, the reaction efficiency was further enhanced relative to the increase in the amount of the RNA at least until it exceeded 10 μM for P2.1 RNA, whereas it remained unchanged for P2-2.1 RNA. Interestingly, no stable complex of P2.1 RNA and L-95 *ScaI* was detected by gel-mobility shift assay (Fig. 5C). So P2.1 RNA exhibited the characteristic feature that it can act more efficiently than P2-2.1 RNA, although it interacts with L-95 less stably than P2-2.1 RNA.

Two distinctive aspects of the P2.1 domains of subclass IC1 introns were observed on phylogenetic comparisons. One is that the base-pairings in P2.1 that correspond to 58G-93C, 59U-92G, and 60C-91G of the *Tetrahymena* intron are highly conserved (*15*). The other is that the introns can form Watson-Crick base pairs termed P13 between the L2.1 loop and the L9.1a loop (Fig. 1A) (*11, 15*).

To examine the possible role of the conserved base-pairs of the stem of P2.1 in the activation, we prepared two variant P2-2.1 RNAs (Y1 and Y2) with base-substitutions in the corresponding region, and examined their activity (Fig. 6). It was found that they are still capable of activating L-95, demonstrating that this conserved stem is not essential for the *trans*-activation.

Next we examined whether the P13 base-pairs are required for the *trans*-activation. In the case of the *Tetrahymena* intron, P13 is predicted to consist of seven base-pairs, although they partially overlap with the predicted stem regions of the P2.1 and P9.1 domains, as shown in Fig. 1A. Considering this, we prepared and examined the activating ability of a series of variant P2-2.1 RNAs (Y3-Y11), as shown in Fig. 6. The activating activities of the RNAs having the mutations that were designed not to affect the formation of P13 (Y4, Y6, and Y11) were all more than 60% of that of the wild type P2-2.1. In contrast, mutants designed to disrupt the predicted seven base-paired P13 interaction (Y3 and Y5) exhibited only weak activity. Y10, in which only one terminal base-pair of P13 was disrupted,

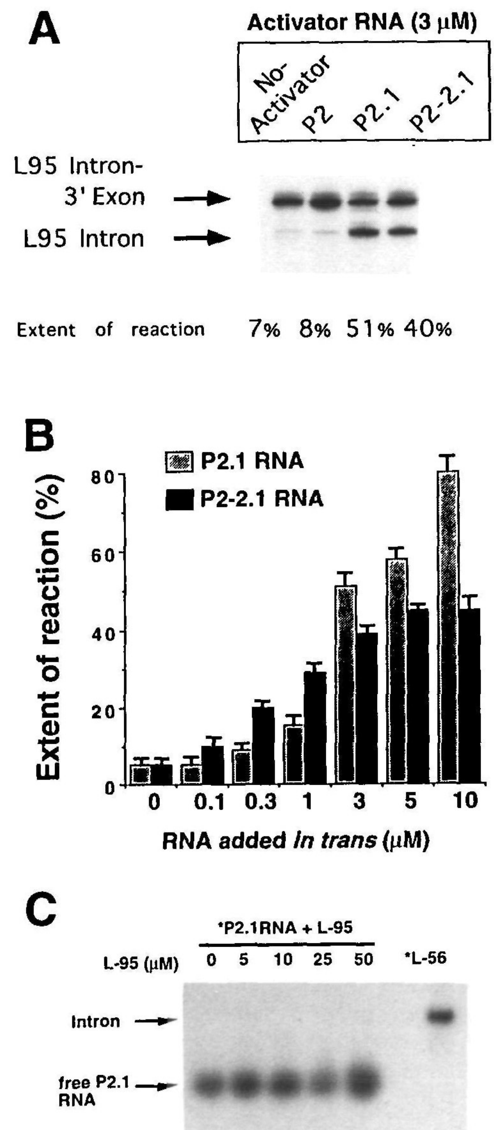

Fig. 5. **A: Effect of P2, P2.1, or P2-2.1 RNA on the hydrolysis reaction of L-95.** L-95 precursors were incubated at 37°C for 30 min in a hydrolysis buffer comprising 50 mM Tris-Cl (pH 8.3) and 12 mM $MgCl_2$ with 3 μM P2, P2.1 or P2-2.1 RNA. **B: Effect of the amount of P2-2.1 or P2.1 RNA.** L-95 precursors were incubated at 37°C for 30 min in the hydrolysis buffer containing 50 mM Tris-Cl (pH 8.3) and 12 mM $MgCl_2$ with the indicated amounts of P2-2.1 (black bars) RNA or P2.1 (gray bars). **C: Gel mobility-shift assay of L-95 *ScaI* with P2.1 RNA.** Asterisks denote the RNAs labeled with ^{32}P.

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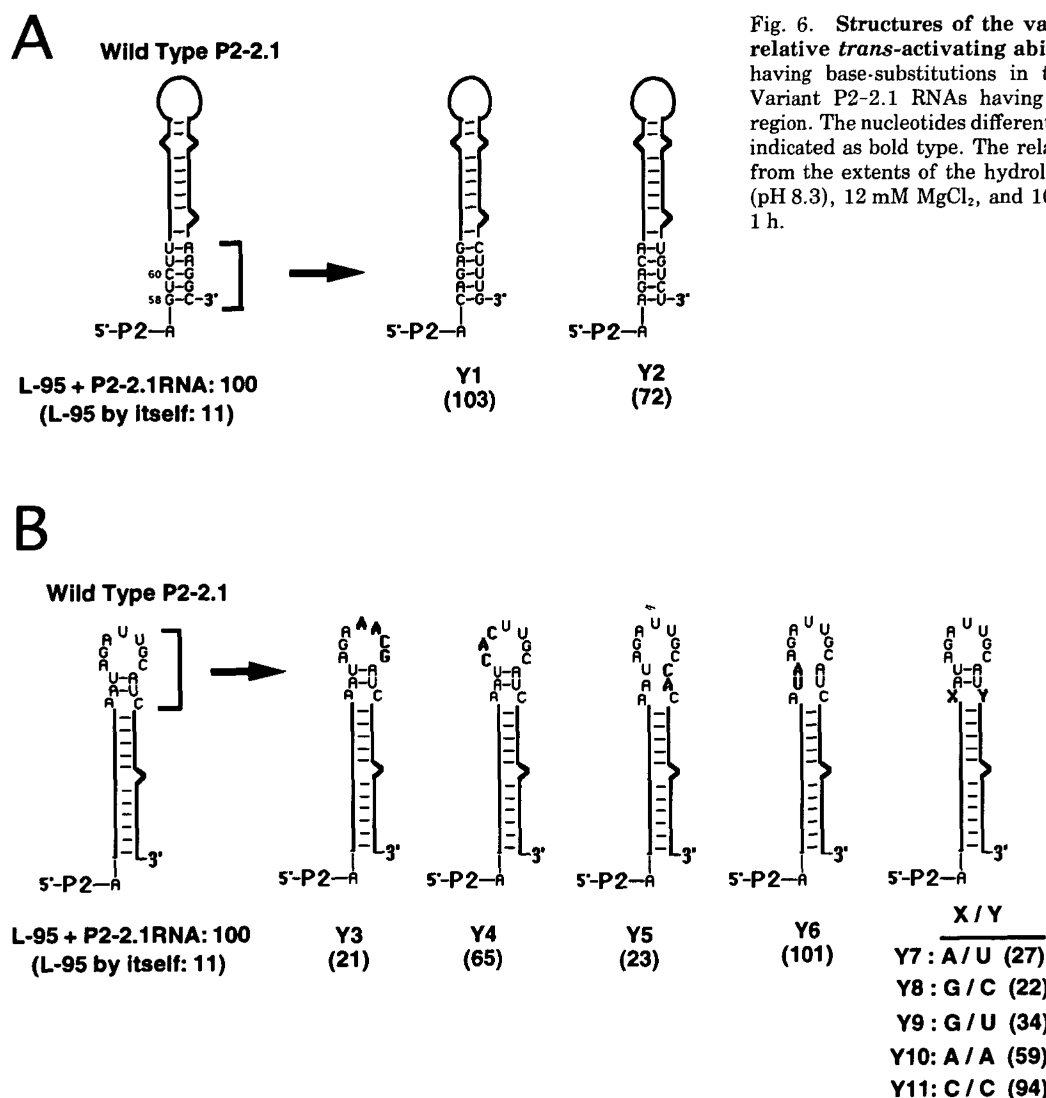

Fig. 6. **Structures of the variant P2-2.1 RNAs and their relative *trans*-activating abilities.** A: Variant P2-2.1 RNAs having base-substitutions in the conserved stem region. B: Variant P2-2.1 RNAs having base-substitutions in the loop region. The nucleotides different from the wild type sequence are indicated as bold type. The relative activities were determined from the extents of the hydrolysis reactions in 50 mM Tris-Cl (pH 8.3), 12 mM $MgCl_2$, and 10 μM activator RNA at 37°C for 1 h.

was more active than Y3 and Y5 but distinctly less active than the wild type P2-2.1. Mutant RNAs having base-substitutions that stabilize the stem structure of P2.1 (Y7, Y8, and Y9) also only exhibited weak activity. The results indicate that the P13 interaction is important for the mechanism underlying the activation. This is consistent with other evidence, *i.e.* that a variant L-95 lacking the terminal region of P9.1 (L-95ΔP9.1T) was not activated by P2-2.1 RNA *in trans* (data not shown). The results demonstrate that the predicted seven base-pairs between L2.1 and L9.1a are important for the *trans*-activation by P2-2.1 RNA.

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