An RNA fragment consisting of the P7 and P9.0 stems and the 3′-terminal guanosine of the Tetrahymena group I intron

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ABSTRACT

On the basis of the nucleotide sequence of Tetrahymena group I intron, we constructed a 31 residue RNA that has the P7 stem and the 3′-terminal guanosine residue (3′-G) with a putative stem–loop structure (P9.0) intervening between them. For this model RNA (P7/P9.0/G), four residues around the guanosine binding site (GBS) in the P7 stem were found to exhibit much lower sensitivities to ribonuclease V1 than those of a variant having adenosine in place of the 3′-G, suggesting that the 3′-G contacts around the GBS. NMR analyses of the imino proton resonances of the P7/P9.0/G RNA indicated that the base pairing in the GBS is retained on the interaction with the 3′-G, and that the two base pairs of the putative P9.0 stem–loop are definitely formed. Comparison of the RNA with its variants with either A (3′-A) or a deletion in place of the 3′-G suggested that the stability of the P9.0 stem–loop is affected by the GBS–3′-G interaction. The melting temperatures of the P9.0 stem–loop were determined from the UV absorbances of these RNAs, which quantitatively indicated that the P9.0 stem–loop is significantly stabilized by the interaction of the GBS with the 3′-G, rather than the 3′-A, and also by direct interaction with divalent cations (Mg2+, Ca2+ or Mn2+). Upon replacement of the G-C base pair by C-G in the GBS of the P7/P9.0/G RNA, the specificity was switched from 3′-G to 3′-A, as in the case of the intact intron.

INTRODUCTION

The self-splicing of group I introns is a two-step transesterification reaction (1). In the first step, a guanosine molecule binds non-covalently to a ‘guanosine binding site’ (2–4) (abbreviated hereafter as GBS; Fig. 1), and then covalently to the 5′-end of the intron, resulting in a free 3′-terminus of the 5′ exon. The second step consists of ligation of the 5′ and 3′ exons and excision of the intron; the 3′ terminal G (3′-G’ or G414; Fig. 1A) of the intron binds to the GBS, and its 3′ phosphodiester bond is attacked. As for the tertiary interaction of the substrate G (free guanosine or 3′-G) with the GBS, which involves the G-C base pair next to the bulged residue (Fig. 1), several models have been proposed, based on phylogenetic and mutagenesis studies, and/or studies using guanosine analogs as alternative substrates (5–9). By replacement of the G-C base pair by C-G at the GBS, the substrate specificity for the 3′-terminal residue of the intron has been switched from G to A (5). Now, the mechanism of guanosine recognition by the GBS should be analyzed directly, for example, by NMR spectroscopy and/or X-ray crystallography. In such structural studies, short model RNAs may be useful, as in the case of NMR studies of oligonucleotides derived from the P4/P6 region (10,11) and the P1 helix (12) of group I introns.

The Tetrahymena group I intron requires divalent metal ions (Mg2+ or Mn2+) for the splicing reaction (1). Recently, it was found that divalent metal ions are required not only for catalysis, but also for the formation of the tertiary structure of the group I intron (13,14). In contrast, other catalytic RNAs, such as RNase P (15,16), hammerhead ribozymes (17), and hairpin ribozymes (18) have been reported to require divalent metal ions primarily for catalysis. For the Tetrahymena group I intron, it has been shown that Mg2+ stabilizes the tertiary interaction of the P1 stem with the catalytic core involving the P3, P4, P5, P6 and P7 stems (19). So far, Mg2+-binding sites in the P1 stem and the junction between the P7 and P8 stems have been identified (20,21). However, it is not clear how divalent metal ions are involved in the binding of the GBS in the P7 stem with the guanosine3′-G.

In the present study, we focused on the intramolecular interaction of the GBS with the 3′-G, rather than the intermolecular interaction with free guanosine. In addition to the 3′-G and the P7 stem bearing the GBS, structural elements, such as P9.0, P9-9.1-9.2 and P10, are considered to be important to locate the 3′ splice site at the GBS (22–24). Actually, van der Horst and Inoue (22) have prepared a shortened group I intron lacking P9-9.1-9.2 and P10, in which a sequence of 5′-GAGUACUC-3′ intervenes between the P7 stem and the 3′-G, and found it still capable of specific hydrolysis of the phosphodiester bond between the 3′-G residue and the 3′ exon. As shown in Figure 1B, the
The P7 RNA (Fig. 1 B) was synthesized with a MilliGen Studier (Stonybrook, NY), according to the described method was purified from an overproducing strain provided by Dr W. from chemically synthesized DNA templates. T7 RNA polymerase RNAs] (Fig. 1) were transcribed with T7 RNA polymerase (27) the P7/P9.0/A, P7/P9.0, P7(2CG)/P9.0/G and P7(2CG)/P9.0/A variants were also used to identify the small NOEs. Thus, we could demonstrate that the P9.0 base pairs are certainly formed to locate the 3′-G around the G6-C21 base pair centering the GBS (Fig. 1B), and that divalent cations stabilize the P9.0 stem–loop but have no effect on the specificity of the GBS–3′-G interaction.

**MATERIALS AND METHODS**

**Preparation of RNAs**

The 31 residue model RNA (P7/P9.0/G RNA) and variant RNAs [the P7/P9.0/A, P7/P9.0, P7(2CG)/P9.0/G and P7(2CG)/P9.0/A RNAs] (Fig. 1) were transcribed with T7 RNA polymerase (27) from chemically synthesized DNA templates. T7 RNA polymerase was purified from an overproducing strain provided by Dr W. Studier (Stonybrook, NY), according to the described method (28). The P7 RNA (Fig. 1B) was synthesized with a MilliGen Cyclone plus DNA/RNA synthesizer. The RNAs were purified by 8 M urea–20% polyacrylamide gel electrophoresis. The buffer was exchanged to 10 mM Tris–HCl (pH 7.5) by several rounds of ultrafiltration with a Centricon 10 unit (Amicon).

**Ribonuclease mapping experiment**

The P7/P9.0/G RNA and the P7/P9.0/A variant were transcribed with T7 RNA polymerase and [γ-32P]GTP (NEN), and were purified by 8 M urea–20% polyacrylamide gel electrophoresis. The 5′ end-labeled RNAs, in 10 mM Tris–HCl buffer (pH 7.5) containing 10 mM MgCl2, were partially digested at room temperature for 10 min with either ribonuclease T1 or ribonuclease V1 (Pharmacia Biotech) (29). The reaction (10 µl) was stopped by the addition of an equal volume of dye solution [30 mM sodium citrate buffer (pH 4.5) containing 1 mM EDTA, 0.03% bromophenol blue, 0.03% xylene cyanol and 9 M urea]. Samples were analyzed by electrophoresis on 20% polyacrylamide gels with 8 M urea and 1× TBE.

**NMR analysis**

The sample RNAs were each dissolved in 10 mM [2H]Tris–HCl buffer (pH 7.5) in 95% ¹H2O/5% ²H2O for exchangeable proton resonance measurements. The samples used to measure the non-exchangeable proton resonances were lyopholized several times from the ²H2O solution, and were then dissolved in 180 µl 99.95% ²H2O. The RNA concentrations were ~1 mM for the P7/P9.0/G RNA, the P7/P9.0/A variant, and the P7/P9.0 variant, and 0.15 mM for the P7 RNA. A more concentrated sample (8 mM) of the P7/P9.0 variant was also used to identify the small NOEs.

NMR spectra were recorded with Bruker AMX-600 and AMX-500 spectrometers. The ¹H chemical shifts were determined relative to internal DSS. The water signal was suppressed by the jump-and-return pulse sequence (30). Temperature dependencies of the exchangeable proton resonances of the P7/P9.0/G RNA and the two variants were observed between 20 and 50°C. One-dimensional NOE difference spectra were measured at 10°C at different decoupler powers and pre-irradiation times (200 ms or 1 s) to distinguish direct NOEs from spin-diffusion and spillover. NOESY spectra were measured for the ¹H2O solution at 10°C with a jump-and-return pulse to replace the last pulse of the NOESY sequence. The delay between the jump and return pulses was set to optimize the resonances at 13 p.p.m. Typically, 512 FIDs of 2 K points were collected, with a sweep width of 30 p.p.m. The relaxation delay was 1 s, and 128 scans were accumulated for each FID for the P7/P9.0 variant. NOESY spectra for the ²H2O solution were measured at 25°C. Typically, 512 FIDs of 4 K points were taken, with a sweep width of 10 p.p.m. and a relaxation delay of 1 s, whereas 64 scans were accumulated for each FID for the P7/P9.0 variant, and more scans were obtained for the other RNAs.

**Melting profile analysis**

Melting profiles of the RNAs were measured with a Gilford Response II UV spectrometer at a heating rate of 1°C/min (31).
The UV absorbance at 260 nm of the sample, in 50 mM sodium cacodylate buffer (pH 7.0) containing 100 mM NaCl, was measured at 0.5°C increments from 25 to 100°C. When the metal ion dependencies were measured, a small aliquot of a concentrated metal ion solution (MgCl₂, CaCl₂ or MnCl₂) was added to the RNA sample in the buffer described above. The final concentration of the divalent cation was 10 mM. The RNA concentration was adjusted to 1.2 A₂₆₀ units at 25°C.

RESULTS AND DISCUSSION

Ribonuclease mapping experiment

Ribonucleases (RNases) T₁ and V₁ were used to probe the folding of the P7/P9.0/G and P7/P9.0/A RNAs (Fig. 2). The strongest cleavage by RNase T₁ (specific for single-stranded G) was observed after G1 and G2, which must be in the unstructured, single-stranded region. In addition, much weaker cleavage was observed after G23 and G25, suggesting that the P7 stem–loop is more stable than the P9.0 stem–loop. As the cleavage patterns are the same between the two RNAs, the overall secondary structure is preserved.

On the other hand, RNase V₁, which cleaves specifically in helical or stacked regions of RNA structure (32), cleaved the RNAs at the P7 and P9.0 stems and the GUAC loop region. Although the cleavage sites were the same between the two RNAs, the efficiencies of cleavage after A5, A7, C21 and G22 were much weaker in the P7/P9.0/G RNA than in the P7/P9.0/A variant. These sites exhibiting the 3'G-dependent protection from RNase V₁ are all located around the G6·C21 base pair in the P7 stem. This clearly indicates that the 3'-G and the P7 stem interact with each other around the GBS, which is separate, in the primary structure, from the 3'-G or A. The decrease in the cleavage efficiency in the P7 stem may be due to some local conformational change induced upon 3'-G binding and/or steric inhibition of nuclease access by the bound 3'-G. We tried, unsuccessfully, another chemical modification protection experiment for N7 of guanine base with dimethyl sulfate (data not shown); gel electrophoresis patterns of fragments were much less clean by 5’ end-labeling than by 3’ end-labeling, as has already been reported (33).

Assignments of the imino proton resonances

The above-mentioned nuclease mapping analysis clearly demonstrated that the P7/P9.0/G RNA forms the P7 stem structure, and that the 3'-G specific interaction occurs around the G6·C21 base pair in the P7 stem. However, the formation of the P9.0 stem–loop, composed of only two putative base pairs, was not confirmed from the ribonuclease mapping. In the next step, therefore, we performed NMR measurements. Base-paired imino proton resonances of the P7/P9.0/G, P7/P9.0/A and P7/P9.0 RNAs were assigned by the conventional method (34) using sequential nuclear Overhauser effects (NOEs) observed in the 1D NOE difference spectra and the 2D NOESY spectra of H₂O solutions, as follows. We started the NH resonance assignment with the P7/P9.0 variant (Fig. 3). First, on the basis of the characteristic high-field chemical shift (≈ 10 p.p.m.) of the imino proton resonance of the G in the UUCG tetra-loop (25,26), peak j was assigned to the N1H of G15. Peaks a, b, d and g were classified as the N3H proton resonances of U base-paired with A, from their sharp NOEs on the adenine C2H proton resonances. Then, the NOE connectivities of j-f and d-g-e-a-h were assigned to G15–G16(C11) and U17(A10)–U19(A18)–G19(C8)–U20(A7)–G6(C21), respectively (the base pairing partners are shown in parentheses). The NOE between G15 and G16 was weak in the 1D different spectrum (Fig. 3), but was confirmed in the NOESY spectrum with a mixing time of 200 ms. The overlapping of the imino proton resonances of G16 and U17 prevented detection.
Figure 3. The regions of imino proton and aromatic CH proton resonances of a 1D proton NMR spectrum (A) and the NOE difference spectra (B–F) of the P7/P9.0 variant. ▼, the irradiated proton resonance with its assignment underlined; ◀, the observed sequential NOE; ▲, the NOE to the adenine C2H proton; ◖ and ◗, imino and CH proton peaks, respectively, due either to spin-diffusion or to spillover.

of the NOE between them. Instead, in the 300 ms NOESY spectrum for the 3H2O solution (data not shown), the C2H proton of A10(U17) exhibited, in addition to a strong NOE with the C2H proton of A18(U9), weak NOEs with the C6H and C1′H protons of C11(G16). These two CH protons of C11 were assigned from the NOE connectivities to the C5H proton of U12, which showed a NOE in turn with the N1H proton of G15, in accordance with the previous results for the UUCG tetra-loop (25,26).

Similarly, for the P7/P9.0/G and P7/P9.0/A RNAs, the imino proton resonances from the UUCG tetra loop and the six consecutive base pairs of the P7 stem (G6 to C21) were assigned. The resonance assignments of the P7 RNA could be made through simple comparison with the other RNAs, but not by direct NOE measurements. Figure 4 summarizes the assignments of the P7 RNA. The two imino proton resonances of the P9.0 stem, which were assigned for the P7/P9.0/G and P7/P9.0 RNAs, are indicated with their assignments in parentheses (A and C, respectively).

Peak b was found to be due to the imino proton resonance of an A·U base pair, and to have an NOE connectivity with a non-A·U imino proton, peak i, in the cases of the P7/P9.0/G and P7/P9.0 RNAs. Therefore, peaks b and i were assigned to U29(A24) and G23(C30), respectively, in the P9.0 stem. These two resonances were certainly observed for the P7/P9.0 variant, but were much broader or missing in the P7/P9.0/A variant spectrum (Fig. 4).

Another peak (13.35 p.p.m.) was observed for the P7/P9.0/G RNA, but not for the other RNAs (Fig. 4). This peak was tentatively assigned to the imino proton resonance of the 3′-G residue, although signal overlapping prevented us from identifying the NOE between this resonance and others, such as that of G6(C21).

The present NMR analysis of the P7/P9.0/G RNA showed that the P9.0 stem is certainly base paired as well as the P7 stem. The P7 stem is formed in the same manner in all the four RNAs (Fig. 4). Therefore, the site-specific decrease in the ribonuclease V1 cleavage efficiency in the P7 stem of the P7/P9.0/G RNA (Fig. 2) is ascribed to steric inhibition of ribonuclease access by the bound 3′-G, rather than some 3′-G-induced local unwinding of the P7 stem. On the other hand, the ribonuclease-mapping patterns are
much the same for the P9.0 stem–loop region between the P7/P9.0/G and P7/P9.0/A RNAs (Fig. 2). By contrast, the P9.0 stem appears to be more rigid in the P7/P9.0/G RNA than in the P7/P9.0/A RNA (Fig. 4). These results prompted us to compare the stabilities of the P9.0 stem among the RNAs.

Temperature dependencies of the imino proton resonances

For each of the P7/P9.0/G, P7/P9.0/A and P7/P9.0 RNAs, the temperature dependencies of the imino proton resonances were measured from 20 to 50°C, as shown in Figure 5. In this range, the imino-proton resonances underwent temperature-dependent broadening without any appreciable change in the chemical shifts, probably because of accelerated chemical exchange with the solvent water. In general, such phenomena occur below the melting temperature, \( T_m \), but certainly reflect the stabilities of the base pairs. For the P7/P9.0/G RNA, the base pairs in the P7 stem (G6·C21, A7·U20, C8·G19, U9·A18, A10·U17 and C11·G16) were found to be more stable than others; the peak heights of their imino proton resonances gradually decreased with the temperature increase from 30 to 50°C (Fig. 5). Actually, the imino proton resonance of G15 in the UUCG tetra-loop at the end of the P7 region showed a decrease in its peak height at lower temperatures than the imino proton resonances due to the P7-stem base pairs (Fig. 5). This is probably because the imino proton of the G in the UUCG tetra-loop is less shielded from the solvent water than those of the ordinary base pairs inside the double-stranded stem. The P7-stem base pairs of the P7/P9.0/A and P7/P9.0 RNAs were shown to be as stable as those of the P7/P9.0/G RNA; their temperature dependencies were essentially the same (Fig. 6).

In contrast, for the A24·U29 and G23·C30 base pairs in the P9.0 stem of the P7/P9.0/G RNA, the imino proton peak heights decreased immediately from 20°C, and became nearly undetectable at 45°C (Figs 5 and 6A). Thus, the P7/P9.0/G RNA is now shown to be composed of two distinct subdomains with different thermal stabilities, a more stable one with the P7 stem, and a less stable one with the P9.0 stem–loop. Furthermore, the P7/P9.0/A and P7/P9.0 variants showed decreases in the peak heights of the P9.0-stem imino proton resonances at slightly lower temperatures than the P7/P9.0/G RNA (Fig. 6).

Two-step melting of the P7/P9.0/G RNA

In order to determine the melting temperatures \( T_m \) of the P7 stem and the P9.0 stem–loop separately and precisely, the UV melting profiles of the P7/P9.0/G, P7/P9.0/A, P7/P9.0 and P7 RNAs were measured, and their first derivatives were obtained, as shown in Figure 7. The P7/P9.0/G, P7/P9.0/A and P7/P9.0 RNAs were found to melt in two steps, which is consistent with the NMR observations that these RNAs are composed of two distinct subdomains with different thermal stabilities. In contrast, the P7 RNA exhibited a single-step melting profile (Fig. 7). The melting temperatures \( T_m \) are listed in Table 1. The higher \( T_m \) values, of 78 ± 0.5°C, are common to all four RNAs. In combination with the NMR assignment of the two subdomains, the subdomains corresponding to the higher and lower \( T_m \) are unambiguously assigned to the P7 and P9.0 stem–loops, respectively. Therefore, the higher and lower \( T_m \) will be denoted hereafter as the P7 and P9.0 \( T_m \) (Table 1). The greater stability of the P7 subdomain may be ascribed to the stability of the UUCG tetra-loop (24,25), in addition to the larger number of base pairs in this stem than in the other. On the other hand, the P9.0 \( T_m \) is much lower than the P7 \( T_m \), because the P9.0 subdomain has only two base pairs and an ordinary GUAC loop (Fig. 1).

The P9.0 \( T_m \) values of the P7/P9.0/G, P7/P9.0/A and P7/P9.0 RNAs, which vary in the 3′-terminal structures, are appreciably different from each other, as shown in Table 1. The P7/P9.0/G RNA showed the highest P9.0 \( T_m \) (46.0°C), which was reduced by 4.5 and 8 degrees upon replacement with A and deletion, respectively, of the 3′-G. This is consistent with the temperature dependence of the NMR signals (Fig. 6). As the ribonuclease mapping analysis showed that the 3′-G interacts with the GBS more strongly than the 3′-A (Fig. 2), the stability of the P9.0 stem–loop structure is correlated with the stability of the interaction between the GBS and the 3′-terminal.
Figure 6. The relative peak heights of the imino proton resonances of G6 (■), U20 (●), U17+G19 (●), U9 (○), G16 (▲), and G15 (▼) of the P7 region, and G23 (●) and U29 (▲) of the P9.0 stem for the P7/P9.0/G RNA (A), the P7/P9.0/A RNA (B), and the P7/P9.0 RNA (C) from 20 to 50°C.

Table 1. Melting temperatures ($T_m$) of the RNAs

<table>
<thead>
<tr>
<th>RNA</th>
<th>P9.0 $T_m$</th>
<th>P7 $T_m$</th>
</tr>
</thead>
<tbody>
<tr>
<td>P7/P9.0/G</td>
<td>46.0</td>
<td>77.5</td>
</tr>
<tr>
<td>P7/P9.0/A</td>
<td>41.5</td>
<td>78.0</td>
</tr>
<tr>
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<td>38.0</td>
<td>78.0</td>
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<tr>
<td>P7 RNA</td>
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<td>78.5</td>
</tr>
<tr>
<td>P7(2CG)/P9.0/G</td>
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</tr>
<tr>
<td>P7(2CG)/P9.0/A</td>
<td>41.0</td>
<td>74.5</td>
</tr>
</tbody>
</table>

n.d., not determined.

The stabilization of the P9.0 stem–loop by the GBS–3'-G interaction is probably because the two ends of the GAGUACUC sequence are kept close to each other. It is possible, in turn, that the interaction of the 3'-G with the GBS depends on the P9.0 stem–loop formation. Note that the P7 $T_m$s are nearly the same among the wild-type and variant RNAs. At higher temperatures around the P7 $T_m$, the P9.0 stem–loop region is already melted, so that the GBS hardly interacts with the 3'-terminus.

Effects of a mutation at the GBS on the UV melting profiles

It has been reported that the specificity of the intron is switched from 3'-G to 3'-A by replacement of the G·C base pair at the GBS by C·G (5). Therefore, we prepared variants of the P7/P9.0/G and P7/P9.0/A RNAs by introduction of the G·C→C·G mutation into the GBS [the P7(2CG)/P9.0/G and P7(2CG)/P9.0/A RNAs, respectively] (Fig. 1C), and measured their UV melting profiles (Fig. 8). The P7 $T_m$ values of the variant RNAs [74.0 and 74.5°C, respectively] are lower by 3.5 degrees than those of their parent RNAs, P7/P9.0/G and P7/P9.0/A (Table 1). This suggests that the G·C base pair contributes to the conformational stability of the GBS. The P7(2CG)/P9.0/G RNA exhibited no clear peak for the P9.0 melting in the first derivative of its UV melting profile (Fig. 8), indicating that the interaction of the 3'-G with the C·G mutant of GBS is too weak to stabilize the P9.0 stem–loop structure, or even destabilizes it. In contrast, we clearly observed the peak of the P9.0 melting for the P7(2CG)/P9.0/A RNA (Fig. 8). It is indicated, therefore, that the mutant GBS can bind the 3'-A rather than the 3'-G, while the wild-type GBS is specific to the 3'-G. Thus, we successfully reproduced the specificity switching for the 3'-terminal residue of intron by the same GBS mutation as that reported for the intact group I intron (5). Actually, the P9.0 $T_m$ value of the P7(2CG)/P9.0/A (41°C) is not as high as that of the wild-type P7/P9.0/G RNA (46°C) (Table 1). This probably agrees with the previous proposal that the G·C–3'-G and C·G–3'-A interactions involve two hydrogen bonds and one hydrogen bond, respectively (5). All these results conclude that
Metal ions control the P9.0 observed, to a somewhat lesser extent, for calcium and manganese ions, which were not affected by magnesium ion. Similar effects were introduced UUCG tetraloop. On the other hand, magnesium ion might reflect some interaction of the ion with the artificially introduced GBS–3′-G interaction in the group I intron (6,7). Figure 2 shows that, on the two opposed strands of the P7 stem, the sequences protected from the ribonuclease attack are mutually 3′-shifted. This type of skewing has been observed for a minor groove-binding protein (36). Therefore, one possibility is that the 3′-G is located in the minor groove of the P7 stem. On the other hand, if the 3′-G binds to the major groove, it must be stretched along the groove. This situation is consistent with the axial binding model (7) but not with the coplanar model (6). In order to elucidate the detailed mechanisms of the 3′-G recognition by the GBS, isotope-aided NMR analyses as well as mutational analyses are in progress in our laboratories.

In contrast, no interaction was detected between free guanosine (substrate of the first step of the self-splicing) and the GBS for the present model RNAs. By UV (the P7/P9.0/G, P7/P9.0/A and P7/P9.0 RNAs) and NMR (the P7/P9.0/G and P7/P9.0 RNAs) the present model RNA, P7/P9.0/G, mimics well the recognition of the 3′-G by the GBS of the intact intron.

Metal-ion dependence of the UV melting profiles

Then, effects of divalent metal ions on the melting temperatures were examined for the P7/P9.0/G, P7/P9.0/A and P7/P9.0 RNAs. In both the presence and absence of various metal ions (magnesium, calcium and manganese ions) at 10 mM, all three RNAs melted in two steps (data not shown). Table 2 lists the P7 and P9.0 Tm values. The P7.0 Tm's of the RNAs were slightly affected (±3.5 degrees) upon the addition of metal ions, which might reflect some interaction of the ion with the artificially introduced UUCG tetraloop. On the other hand, magnesium ion largely affected the P9.0 Tm of the P7/P9.0/G RNA (60.0°C in the presence, and 46.0°C in the absence of magnesium ion). Thus, it is shown that the P9.0 stem with only two base pairs is considerably stabilized by magnesium ion.

The P9.0 Tm's of the P7/P9.0/A and P7/P9.0 RNAs were also increased in the presence of magnesium ion to the same extent (by 14 degrees). The differences between those of the 3′-G and the 3′-A (4.5 degrees) or those of the 3′-G and the 3′-Δ (8 degrees) were not affected by magnesium ion. Similar effects were observed, to a somewhat lesser extent, for calcium and manganese ions, which increased the P9.0 Tm's of the P7/P9.0/G RNA by 11.5 and 9 degrees, respectively. Thus, the differences between the P9.0 Tm's of the 3′-G and the 3′-A or those of the 3′-G and the 3′-Δ were not greatly affected by the presence of any of the three divalent ions. Therefore, divalent metal ions do not contribute to the specificity of the tertiary interaction of the GBS with the 3′-G in this model RNA. Thus, it is indicated that the divalent metal ions bind to the P9.0 stem–loop (probably to the GUAC loop), and directly increase the stability of the P9.0 stem–loop.

Table 2. Effect of metal ions (10 mM) on the melting temperatures (Tm's, °C) of the P7/P9.0/G, P7/P9.0/A and P7/P9.0 RNAs

<table>
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<th>RNA</th>
<th>Metal ions</th>
<th>P9.0 Tm</th>
<th>P7 Tm</th>
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<tr>
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<td></td>
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<td>P7/P9.0</td>
<td>control</td>
<td>38.0</td>
<td>78.0</td>
</tr>
<tr>
<td></td>
<td>Mg2⁺</td>
<td>52.0</td>
<td>81.0</td>
</tr>
<tr>
<td></td>
<td>Ca2⁺</td>
<td>52.0</td>
<td>77.0</td>
</tr>
<tr>
<td></td>
<td>Mn2⁺</td>
<td>48.0</td>
<td>74.5</td>
</tr>
</tbody>
</table>

Relation between the structures of the model RNA and the shortened group I intron

In the present study, we showed, for the model RNA, that the P9.0 base pair is certainly formed and the 3′-G-specific interaction occurs at the GBS. Magnesium ion stabilizes the P9.0 stem–loop structure, but has no effect on the specificity of the GBS–3′-G interaction. These characteristic features of the model RNA give insight into the structure of the shortened intron (22). The local tertiary structure involving the P9, P9.1 and P9.2 stem–loops was proposed to bring the 3′ splice site into the catalytic core of the intron (35), while these stem–loops are dispensable in the presence of the GAGUACUC sequence between the P7 stem and the 3′-G (22). This GAGUACUC sequence may be folded into the P9.0 stem–loop structure, and therefore locate the 3′-G at the GBS, in the shortened group I intron, as we observed for the model RNA.

It is interesting to compare the properties of the present model RNA with those of the proposed model structures for the GBS–3′-G interaction in the group I intron (6,7). Figure 2 shows that, on the two opposed strands of the P7 stem, the sequences protected from the ribonuclease attack are mutually 3′-shifted. This type of skewing has been observed for a minor groove-binding protein (36). Therefore, one possibility is that the 3′-G is located in the minor groove of the P7 stem. On the other hand, if the 3′-G binds to the major groove, it must be stretched along the groove. This situation is consistent with the axial binding model (7) but not with the coplanar model (6). In order to elucidate the detailed mechanisms of the 3′-G recognition by the GBS, isotope-aided NMR analyses as well as mutational analyses are in progress in our laboratories.
measurements (data not shown). Accordingly, interaction of free guanosine with the GBS appears to require additional structural elements such as the P3, P4, P5 and P6 stems, which have been proposed to make, together with the P7 stem, a core structure of the ribozyme (37).

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