Probing of tertiary interactions in RNA: 2'-Hydroxyl–base contacts between the RNase P RNA and pre-tRNA

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ABSTRACT A general method has been developed to analyze all 2' hydroxyl groups involved in tertiary interactions in RNA in a single experiment. This method involves comparing the activity of populations of circularly permuted RNAs that contain or lack potential hydrogen-bond donors at each position. The 2' hydroxyls of the pre-tRNA substrate identified as potential hydrogen bond donors in intermolecular interactions with the ribozyme from eubacterial RNase P (P RNA) are located in the T stem and T loop, acceptor stem, and 3' CCA regions. To locate the hydrogen-bond acceptors for one of these 2' hydroxyls in the P RNA, a phylogenetically conserved adenosine was mutated to a guanosine. When this mutant P RNA was used, increased cleavage activity of a single circularly permuted substrate within the population was observed. The cleavage efficiency (kcat/Km) of a singly 2'-deoxy-substituted substrate at this position in the T stem was also determined. For the wild-type P RNA, the catalytic efficiency was significantly decreased compared with that of the all-ribo substrate, consistent with the notion that this 2' hydroxyl plays an important role. For the P RNA mutant, no additional effect was found upon 2'-deoxy substitution. We propose that this particular 2' hydroxyl in the pre-tRNA interacts specifically with this adenosine in the P RNA. This method should be useful in examining the role of 2' hydroxyl groups in other RNA–RNA and RNA–protein complexes.

The 2' hydroxyl (2' OH) groups in RNA play an important role in the folding and in the biological functions of RNA. In particular, 2' OH groups as hydrogen-bond (H-bond) donors or acceptors can participate in tertiary interactions in specific recognition between two RNA molecules. For example, interactions involving four 2' OH groups in the P1 helix of the Tetrahymena group I ribozyme are crucial for increasing the affinity of substrate binding and for correct docking of the substrate into the active site (1–4). The ribozyme (catalytic RNA) from eubacterial RNase P (P RNA) recognizes its highly structured pre-tRNA substrate primarily through tertiary interactions (5, 6). In this case, functional groups in the ribose-phosphate moiety, especially 2' OH groups, within the structural context of tRNA can serve as H-bond donors to or acceptors from specific residues in the P RNA.

Studies of the role of 2' OH groups in RNA–RNA interaction require modification of 2' OH groups, in most cases to 2' H, followed by analysis of these “atomic” mutants (1, 4, 7). In general, such 2' OH → 2' H modifications are made individually and the observed effects show decreases in the activity of the RNA of interest. Here we report an experimental technique that allows analysis of the role of 2' OH groups in a mixture of RNA molecules and its application to P RNA–substrate recognition. In this method, the RNA is circularly permuted to convert a 2' OH and its adjacent phosphodiester bond into a 2',3' cyclic phosphate and a 5' OH, followed by removal of the cyclic phosphate to generate 2' and 3' OH groups. In this process, a 2' H-bond donor or acceptor (2' OH) is first modified into an H-bond acceptor (2',3' cyclic phosphate) and then modified again to an H-bond donor or acceptor in an altered form (2' or 3' OH). If the original 2' OH were acting as an H-bond donor, the activity of the RNA would initially decrease (2' donor to acceptor) and then increase (2' acceptor to donor). Thus, this method can detect 2' OH groups involved as potential H-bond donors through an observed increase in the activity of the RNA upon removal of the cyclic phosphate. Several 2' OH groups in the pre-tRNA substrate were found to be candidates for direct contacts with the P RNA, and one of these was implicated in forming a H-bond with a specific adenosine residue in the P RNA.

MATERIALS AND METHODS

Preparation of the Circular pre-tRNA Substrate. The pre-tRNA\(^{\text{pre}}\) substrate was obtained by in vitro transcription using bacteriophage T7 RNA polymerase and purified as described (8, 9). To form an intramolecular circle, \(^{32}\text{P}\)-labeled pre-tRNA\(^{\text{pre}}\) was incubated in 50 mM Tris, pH 7.6/10 mM MgCl\(_2\)/10 mM 2-mercaptoethanol/8 μM ATP/15% dimethyl sulfoxide with bacteriophage T4 RNA ligase at 1 unit/μl for 1.5 hr at 37°C. Purification by electrophoresis in 8% polyacrylamide gels containing 7M urea allowed complete separation of circular pre-tRNAs that differed by a single nucleotide.

Circular Permutation Analysis (CPA). Alkaline hydrolysis of ~50 fmol of circular pre-tRNA\(^{\text{pre}}\) was carried out in 1 mM glycine/0.4 mM MgSO\(_4\), pH 9.5, by boiling for 30 s to generate circularly permuted (CP) substrates containing 2',3' cyclic phosphates. These CP substrates were incubated in 50 mM

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Tris, pH 8.1/10 mM MgCl₂ with T4 polynucleotide kinase at 1 unit/µl to produce CP substrates containing 2' and 3' OH groups (10). Kinase and Mg²⁺ were then removed through extraction with phenol/CHCl₃ and ethanol precipitation. The CP substrates were renatured in Tris (pH 8.1) by heating for 2 min at 85°C, followed by addition of MgCl₂ and KCl and incubation at 37°C for 5 min. Bacillus subtilis P RNA was prepared and renatured as described (9, 11). The cleavage reaction was initiated by mixing the ribozyme (30 nM wild-type P RNA), with <1 nM circular substrate, 50 mM Tris (pH 8.1), 25 mM MgCl₂, and 1 M KCl. The cleavage reaction was stopped by addition of 2 volumes of 9 M urea/25 mM EDTA, and the reaction mixture was then directly loaded onto a polyacrylamide gel containing 7 M urea. The gel was dried and the profile of the reaction was obtained with a phosphor imager (Fuji). Reactions with the A230G and A229G mutants were performed as described above, except that the concentrations of the mutant ribozymes were 200 nM and 150 nM, respectively.

**Cleavage Activities of 2'-Deoxy-Substituted Substrates.** An RNA containing nt 1–57 of the yeast tRNAпле plus 14 nt 5' to the cleavage site was obtained by *in vitro* transcription using PCR-generated DNA templates. RNAs containing nt 58–76 of the yeast tRNAпле with a single 2' H at nt 61, 62, and 64 were synthesized by standard phosphoramidite chemistry. The two RNA molecules were then ligated by use of T4 DNA ligase (12) and a bridging oligodeoxynucleotide complementary to nt 47–76 of yeast tRNAпле. Single-turnover kinetics of the 2' H-modified substrates was carried out under *k_cat/K_m* conditions with 30 nM wild-type ribozyme, 80 nM A229G mutant, or 150 nM A230G mutant.
RESULTS

The experimental strategy (Fig. 1) represents a modified protocol of CPA (9, 13). Partial alkaline hydrolysis of a circular pre-tRNA substrate under denaturing conditions generates, on average, one break per circular molecule at approximately equal frequency at each ribose-phosphate position. At this point, all CP RNAs in this mixture contain 2',3' cyclic phosphates at their 3' termini, corresponding to the first modification of the 2' OH groups (potential donor/acceptor to acceptor only). A fraction of the CP RNAs is then treated with a phosphatase to generate CP substrates containing 2' and 3' OH groups, corresponding to the second modification (acceptor to potential donor). These two mixtures of CP substrates are then cleaved by the B. subtilis P RNA. Cleavage of an active CP pre-tRNA generates a 32P-labeled shorter product with the 5' terminus at the site originated from alkaline hydrolysis and the 3' terminus at the P RNA cleavage site. For inactive CP substrates, no such shorter products are formed. The most interesting CP substrates are those whose cleavage activity is decreased in the 2',3'-cyclic-phosphate form and subsequently increased in the 2',3'-OH form. Since the removal of the cyclic phosphate is the crucial step, the method is termed CPA–DP.

A typical experiment with the wild-type B. subtilis P RNA is shown in Fig. 2 and the results are summarized in Fig. 3. Hydrolysis of the linear pre-tRNA product resulted in a ladder of nearly uniform intensity (Fig. 2, lane C → L OH−), suggesting that most CP substrates were present in similar proportions in the mixture prior to P RNA cleavage. A large number of CP pre-tRNAs were active substrates, regardless of the identity of their termini. One of the CP substrates in the population was the original linear RNA substrate (the fastest migrating product band in Fig. 2). By comparing the amount of product of any CP substrate to that of the linear substrate as a function of time, the cleavage efficiency could be estimated. By this measure, the cleavage efficiency of many CP substrates was reduced by a factor of 5–10 relative to the linear pre-tRNA. These values are close to the reduction in $k_{cat}/K_m$ of the circular tRNA substrate as well as those of several other CP tRNA substrates that have been characterized with Escherichia coli P RNA (5, 14). The 5' termini for these previously studied CP pre-tRNAs are located at nt 53, 64, and 69, all of which are active substrates as indicated by our CPA experiment. Folding studies of CP tRNA (13) show that backbone breaks at nt 7–15 and 54–57 result in CP tRNAs that are misfolded. Therefore, the low reactivity of the pre-tRNA with breaks at some of the nonpermissive positions—e.g., at nt 54–57—may also be due to misfolding of the tertiary structure of tRNA.

Comparison of the reactivity of CP substrates containing 2',3' cyclic phosphates with those containing both 2' and 3' OH groups revealed that the cleavage activity increased for several CP substrates through removal of the cyclic phosphates, as shown in Figs. 2 and 3. In these and subsequent figures, the nucleotide residues are labeled according to the standard phosphate numbers of tRNA. These numbers also correspond to the 5' terminal residues of CP substrates in the population. However, the 2' oxygen in the cyclic phosphate originates from the 2' OH of the preceding residue. For example, the band labeled 63 in Fig. 2 involves the phosphorus atom from nt 63 but the 2' OH from nt 62. Therefore, these CP substrates represent conversion of 2' OH groups at nt 74, 72, 62, 61, 57, and 54. Increases in activity were not due to cleavage at a different site in the CP substrates by the P RNA, as confirmed by analysis of isolated substrates with the termini at nt 62 and 63 (data not shown). A previous study using a minimal substrate with E. coli P RNA showed that 2' OH groups at positions 74 and 72 were important in the catalytic efficiency, whereas the 2' OH at position 54 was not (15). Unfortunately, effects of 2' OHs at nt 62, 61, and 57 were not tested in that study. One interpretation of our results is that at least some of these 2' OH groups are involved in direct tertiary contact with the P RNA. When these 2' OH groups are converted to cyclic phosphates, their ability to serve as a H-bond donor is eliminated. Removal of the cyclic phosphate restores its ability to form a H bond. However, H-bonding to the new 2' OH groups may not fully compensate for backbone breakage and phosphate removal at these positions, thus resulting in a varying extent of increased activity.

What are the possible H-bonding acceptors to these 2' OH groups in P RNA? The 2' OH groups can form H-bonds with N-1 of adenosine residues, as observed in the crystal structure of yeast tRNA (16) and in the group I intron ribozyme–substrate complex (2). An A → G mutation changes the N-1 of the purine from an H-bond acceptor to an H-bond donor at the equivalent position, if we assume an identical geometry for the purine base in the RNA. Thus, a specific A → G mutation of the P RNA may rescue the activity of a specific CP substrate containing 2',3' cyclic phosphate with the N-1 H of guanine as the H-bond donor. With CPA–DP, all 2' OH groups implicated as H-bond donors can be examined simultaneously in one experiment.

Several considerations were made in selecting specific adenosine residues to be mutated in the B. subtilis P RNA. Since a 2' OH is invariant in all pre-tRNA substrates, it is likely that an A residue involved in H-bonding would be phylogenetically conserved to preserve this interaction. One might also expect that such a base would be exposed to solvent in the absence of the substrate and therefore susceptible to chemical modification, whereas the modification would be suppressed in the ribozyme–substrate complex (2, 17, 18). If we consider residues outside of the 3' CCA binding site (19–21), two adenosines, A130 and A230, in the B. subtilis P RNA fulfill both criteria (19, 22). One of the two, A230, was mutated to guanosine. Another conserved adenosine, A229, was also mutated to guanosine for comparison. The A230G mutant had $k_{cat}/K_m$ reduced by a factor of ~150 with the linear pre-
tRNA\textsuperscript{Phe} substrate, indicating that the adenosine at this position is important for \textit{P} RNA function. This reduction in cleavage activity is not due to alteration in global folding, since this mutant showed an Fe(II)-EDTA protection pattern and Mg\textsuperscript{2+} dependence comparable to that of the wild-type \textit{P} RNA (data not shown; ref. 11).

The results of CPA experiments with both mutants are shown in Fig. 4. For \textit{CP} substrates containing 2',3' cyclic phosphates, the A230G mutant was able to partially restore the cleavage activity of the substrate containing a cyclic phosphate involving the 2' OH group at nt 62. When the cyclic phosphates were removed, the activity was restored to a higher extent. These results are consistent with the hypothesis that A230 in the ribozyme forms direct contact with the 2' OH at nt 62 in the substrate. Further rescue in activity for the \textit{CP} substrate containing 2' and 3' OH groups may be explained by the terminal 2' OH being in a better position to accept the H-bond than the 2' oxygen in the 2',3' cyclic phosphate. Results obtained with the A229G mutant, on the other hand, are identical to those with the wild-type \textit{P} RNA, suggesting that A229 does not interact directly with a 2' OH in the substrate.

The role of 2' OH groups at nt 61 and 62 in substrate recognition by \textit{P} RNA was also analyzed by single 2' deoxy substitutions (Table 1): 2' OH → 2' H modifications were made at three positions, 61 (dc61), 62 (da62), and 64 (da64). The da64 control has \( k_{\text{cat}}/K_{\text{m}} \) reduced by a factor < 2 compared with the all-ribo substrate. This value presumably represents the general effect for a single 2' OH → 2' H substitution in the T stem. The dc61 variant has \( k_{\text{cat}}/K_{\text{m}} \) reduced consistently by a factor of 2–3, suggesting that the effect observed in the corresponding \textit{CP} substrate may be largely due to the phosphodiester bond breakage. On the other hand, da62 has \( k_{\text{cat}}/K_{\text{m}} \) decreased by a factor of 15–20 for the wild-type \textit{P} RNA and the A229G mutant, suggesting that the 2' OH at position 62 is crucial for efficient \textit{P} RNA function. For the A230G mutant all three 2-deoxy substrates give similar \( k_{\text{cat}}/K_{\text{m}} \) values (Table 1). This is consistent with the idea that no interaction is present initially between the 2' OH at residue

![Fig. 4](image)

**Fig. 4.** CPA–DP using the A230G (A) and A229G (B) mutants of \textit{B. subtilis} \textit{P} RNA. For symbols, see legend to Fig. 2. For the A230G mutant, the positions of the \textit{CP} substrates with 3' termini at nt 62 (5' at nt 63) are indicated by large arrowheads.

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**Table 1.** Relative \( k_{\text{cat}}/K_{\text{m}} \) values of singly 2' OH → 2' H substituted pre-tRNA\textsuperscript{Phe} substrates

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<th>P RNA</th>
<th>dc61</th>
<th>da62</th>
<th>da64</th>
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<tr>
<td>Wild type</td>
<td>0.32</td>
<td>0.053</td>
<td>0.51</td>
</tr>
<tr>
<td>A230G</td>
<td>0.31</td>
<td>0.35</td>
<td>0.67</td>
</tr>
<tr>
<td>A229G</td>
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<td>0.054</td>
<td>0.61</td>
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*Relative to the \( k_{\text{cat}}/K_{\text{m}} \) of the all-ribo substrate (value defined as 1.00) for the indicated conditions and P RNA.
†Conditions: 25 mM MgCl\(_2\)/1 M KCl/50 mM Tris, pH 8.1, 37°C. The absolute values are \( 4 \times 10^5 \), \( 0.23 \times 10^5 \), and \( 7.8 \times 10^6 \) M\(^{-1}\)min\(^{-1}\) for wild type, A230G, and A229G, respectively.
‡Conditions: 25 mM MgCl\(_2\)/50 mM Tris, pH 8.1, 37°C. The absolute values are \( 3.5 \times 10^6 \), \( 0.031 \times 10^6 \), and \( 0.77 \times 10^6 \) M\(^{-1}\)min\(^{-1}\) for wild type, A230G and A229G, respectively.
62 and the A230G mutant. 2'-Deoxy (2' OH → 2' H) substitution at this position should have no further effect on the cleavage activity.

**DISCUSSION**

We have described an experimental method capable of detecting changes in activity upon 2' OH modification, thereby identifying 2' OH groups potentially involved in tertiary hydrogen bonding. By substituting P RNA cleavage with another functional activity—e.g., protein binding—it should be possible to apply this method directly to other RNAs. In cases where specific cleavage at a second site is not available, run-off reverse transcription can be used to determine the 5' termini of the CP RNA molecules (9, 23).

The location of the 2' OH groups implicated in direct contacts with the P RNA shows once again that the P RNA recognizes the structure formed by the T stem–loop and the acceptor stem of tRNA. In the three-dimensional structure of tRNA^{Phe} (16), 2' OH groups of nt 72 and 74 are within 16 Å of the reactive phosphate. The 2' OH groups of nt 54, 61, and 62 are located in the same groove, suggesting that these 2' OH groups may function as a unit for recognition by P RNA.

In a CPA experiment using Xenopus laevis RNase P (24), backbone breakage in three regions in the pre-tRNA substrate resulted in significant reduction in the cleavage efficiency. One region is in the conserved T loop at nt 54–57. A second region is in the 5' half of the acceptor stem, at nt 2–3. A third region is at the junction of the acceptor stem and the D stem, at nt 10. Interestingly, none of the ribose phosphates in the T stem was found to be important in the cleavage efficiency. Thus, it seems that to some extent, eukaryotic RNase P may specifically recognize different ribose moieties of the tRNA substrate. Since the eukaryotic RNase P can be used only as a ribonucleoprotein complex, the presence of its proteins may alter the mode of specific recognition of tRNA substrates. Indeed, some minimal substrates for the eubacterial RNase P are inactive as substrates for eukaryotic RNase P (25, 26).

The six 2' OH groups identified here certainly represent only a subset of all the 2' OH groups involved in specific contacts with the P RNA. If a 2' OH serves as a H-bond acceptor it cannot be detected by our method, since no increase in activity would be apparent upon removal of the cyclic phosphate. In addition, nucleotides near and at the cleavage site have not been analyzed here, although they have been implicated in direct contacts with P RNA in previous work (15, 27). In principle, the 5' half of the tRNA acceptor stem can be examined by incorporating the unique 32P label at the cleavage site. In this case, the corresponding circular RNA can be made by using T4 DNA ligase and a bridging oligodeoxynucleotide (12).

The P RNA–tRNA tertiary interaction identified here provides another example of specific H-bonding between the adenine and the 2' OH of ribose. In the crystal structure of yeast tRNA^{Phe} (16), one tertiary interaction involves N-1 of A21 H-bonding to the 2' OH of the ribose of nt 8. Three other 2' OH–base interactions utilize the N-7 of purine bases. In the Tetrahymena group I ribozyme, one interaction also involves the N-1 of an adenosine, A302, to the 2' OH of ribose −3, and at least two other 2' OH–purine interactions have not been identified as precisely (1, 2, 4). With the crystal structure of yeast tRNA^{Phe} as a guide, some of the interactions in the group I ribozyme as well as in the P RNA–tRNA system may also involve N-7 of purine bases.

The finding of the tertiary interaction between the 2' OH at nt 62 in the pre-tRNA and A230 in the P RNA provides an important constraint in refining the structural models of the P RNA–substrate complex. In the current model of Pace and coworkers, the part of substrate T stem containing nt 62 is proximal to the helices of P10 and P11 in the P RNA (N. Pace, personal communication). The location of A230, immediately 5' to P11, suggests that direct contact between these two residues is plausible in the model.

The tertiary interaction between the 2' OH at residue 62 and A230 also provides some insights on the function of domains in eubacterial P RNAs (11). In the domain structure of the B. subtilis P RNA, A230 is located in the independently folded domain I. The other independently folded portion of P RNA includes domains II and III (A.L. and T.P., unpublished results), which contain the 3' CCA binding loop, L15, and the active site residues in J4/5, P4, and J18/2. The 2' OH groups identified here are clustered in two structural elements in tRNA: the T stem–loop and the 3' CCA with the residues at and near the cleavage site. It will be interesting to see whether the isolated domains from P RNA can specifically interact with these helical stem–loops of tRNA.

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