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Functional domains of the RNA component of ribonuclease P revealed by chemical probing of mutant RNAs

Hideaki Shiraishi and Yoshiro Shimura

Department of Biophysics, Faculty of Science, Kyoto University, Kyoto 606, Japan

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The higher-order structure of the RNA component of ribonuclease P from *Escherichia coli* was analyzed using chemical probes. The secondary structure model which had been constructed from the comparative sequence analysis of the RNA was refined using the experimental data. In a mutant RNA (A89 RNA), which contains a G→A substitution at nucleotide 89, we detected a number of conformational alterations clustered between nucleotides 90 and 239. In view of the fact that A89 RNA is as catalytically active as wild-type RNA, but defective in association with the protein component, it is clear that the catalytic function of the RNA component resides on the structure which is not disrupted by the A89 mutation and that the structures altered by the mutation represent the region(s) interacting with the protein component. Another mutant (A329 RNA), which has a G→A substitution at nucleotide 329 and is defective in catalytic function, showed no detectable change in higher-order structure.

Key words: catalytic RNA/chemical modification/reverse transcription/secondary structure

Introduction

Ribonuclease P (RNase P) is an endonuclease which cleaves tRNA precursors to generate the 5′ termini of mature tRNAs (Altman and Smith, 1971; Robertson *et al.*, 1972). The enzyme contains an essential RNA molecule which exhibits catalytic activity in the presence of high concentrations of magnesium ion, although the protein component is essential for its activity under physiological conditions (Guerrier-Takada *et al.*, 1983). The RNA component of RNase P from *Escherichia coli* is 377 nucleotides in length and is coded for by the *rnpB* gene (Sakamoto *et al.*, 1983a). The nucleotide sequence of the gene has been determined (Reed *et al.*, 1982; Sakamoto *et al.*, 1983a).

We have previously investigated the effect of structural changes on the functions of the RNA component using thermosensitive *rnpB* mutants of *E.coli* (Shiraishi and Shimura, 1986). One of the mutants, ts709 (Sakano *et al.*, 1974), has two G→A substitutions at positions 89 and 365 from the 5′ end of the RNA (Sakamoto *et al.*, 1983a). Of these substitutions, the one at position 89 is responsible for the mutant phenotype, i.e. the defect of the mutant RNA in association with the protein component. However, the catalytic activity of the mutant RNA is indistinguishable from that of wild-type RNA when assayed *in vitro* in the absence of the protein component. These results suggest that the two functions of the RNA component—the catalytic activity and the interaction with the protein component—are carried out by distinct regions of the RNA, and that the upstream mutational site of ts709, nucleotide position 89, is within or close to the region where the protein component of RNase P binds. The RNA component of another mutant, ts2418 (Shimura *et al.*, 1980), has a G→A substitution at nucleotide 329 from the 5′ end of the RNA. This mutant RNA (A329 RNA) is defective in catalytic function; V_{max} of the reaction catalyzed by A329 RNA is reduced 400-fold compared to that catalyzed by wild-type RNA. Thus, the mutational site is within or close to the region which is responsible for catalytic function.

Although it has been shown that the two single-base substitutions affect the two distinct functions of the RNA, it is not known whether the bases at the substituted positions affect the functions directly or whether the mutational effect is exerted by alterations in higher-order structures of the RNA. In this respect, it is worth noting that the RNA component containing the upstream mutation of ts709 (A89 RNA) is extremely sensitive to cellular nucleases compared to wild-type RNA, and is rapidly degraded both *in vivo* and *in vitro* (Shiraishi and Shimura, 1986; Sakamoto *et al.*, 1983a). This should imply that there are some conformational differences between A89 RNA and wild-type RNA. To clarify the structural changes of the mutant RNAs defective in ribonucleoprotein formation and in catalytic activity, we analyzed higher-order structures of the mutant RNAs using structure-specific chemical probes. Functional domains of the RNA component were localized by comparing the functional defects and structural changes.

Results

Chemical probing of the higher-order structure of RNA

For the analysis of the higher-order structure of RNA, it is necessary to prepare the RNA in sufficient quantities. However, one of the mutant RNAs, A89 RNA, is extremely labile in *E.coli* cells (Sakamoto *et al.*, 1983a; Shiraishi and Shimura, 1986) and difficult to recover from the bacteria. To circumvent this problem, we prepared the RNA and other RNAs by *in vitro* transcription of the *rnpB* genes according to the procedures described previously (Sakamoto *et al.*, 1983b). In the course of these experiments, special care was taken to minimize denaturation of the RNA before chemical modification, since this could cause the RNA to form artificial structures upon renaturation. RNAs recovered from the transcription mixtures were subjected to chemical modification by dimethyl sulfate (DMS) or water-soluble carbodiimide, 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-*p*-toluenesulfonate (CMCT). DMS reacts with N7-G, N1-A and N3-C, whereas CMCT reacts with N3-U and N1-G. The modification reactions were carried out in the presence of 10 mM Mg^{2+} under appropriately controlled conditions to modify about one base per 150 nt. In

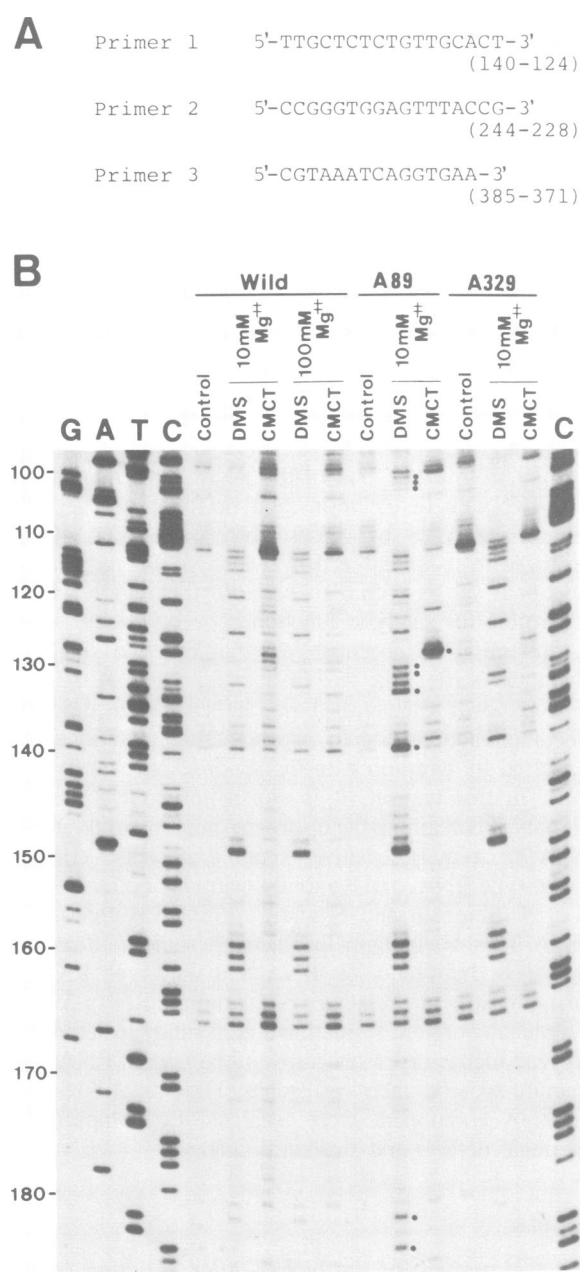

Fig. 1. Determination of modified sites by reverse transcription. (**A**) Nucleotide sequences of synthetic primers. The sites of hybridization on the transcription product of the *rnp*B gene are shown in parentheses. (**B**) An example of reverse transcription using modified RNA as substrate. Primer 2 was hybridized to each RNA and extended by reverse transcriptase. Unmodified RNAs were used as templates in the control reactions. Products of reverse transcription were fractionated on a 6% polyacrylamide gel containing 8 M urea. Nucleotide positions from the 5′ end of the RNA are shown on the left side of the panel. Termination sites enhanced in A89 RNA are indicated by dots on the right sides of lanes.

the case of wild-type RNA, however, the modification reactions were also carried out in a buffer containing 100 mM Mg^{2+}. Under these conditions, the RNA exhibits catalytic activity. After the modification reactions, synthetic primers (Figure 1A) were hybridized to the modified RNAs and elongated by reverse transcriptase. The sites of single-strand specific modification could be determined from termination of the cDNA synthesis directed by the modified RNA (Inoue and Cech, 1985; Moazed *et al.*, 1986;

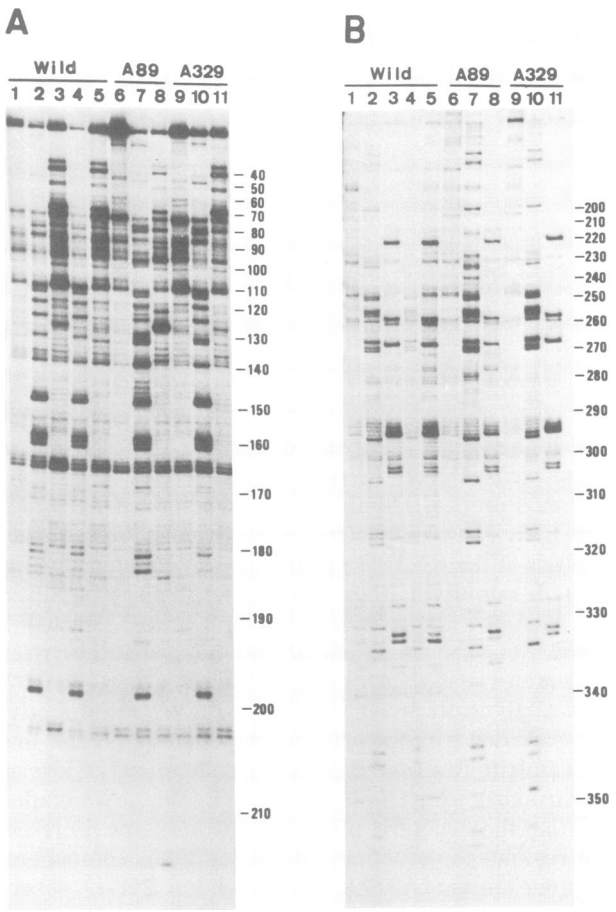

Fig. 2. Modification sites in the RNA component. Primer extension was performed using primer 2 (**panel A**) and primer 3 (**panel B**). Most of the modification sites in the RNA are visible in these two autoradiograms. Template RNAs used were as follows: unmodified RNA (**lanes 1, 6** and **9**), DMS-modified RNA (**lanes 2, 4, 7** and **10**), CMCT-modified RNA (**lanes 3, 5, 8** and **11**). Modification of the RNA was carried out in buffers containing 10 mM Mg^{2+} (**lanes 2, 3, 7, 8, 10** and **11**) or 100 mM Mg^{2+} (**lanes 4** and **5**).

Ehresmann *et al.*, 1987). When the chemical probes are covalently bound to specific positions on bases forming hydrogen bonds with complementary bases, elongation of the cDNA chain along the RNA molecule terminates one nucleotide before the modified residues. As shown in Figure 1B, there were many additional termination sites which were undetectable or faint, if present at all, in the control reactions with unmodified RNA when modified RNAs were used as templates for reverse transcription. Modification sites between nt 1 and 365 were thus determined (Figure 2) using the three kinds of primers indicated in Figure 1A. The probing experiments were carried out three times using template RNAs which were prepared independently. Reproducible modification sites determined by these experiments are shown in Figure 3.

Structure of the wild-type RNA

In Figure 3A, modification sites of the wild-type RNA are superimposed on a refined model of the secondary structure of the RNA component of RNase P. Most of the experimental data were consistent with the secondary structure model of the RNA which had been constructed theoretically from a phylogenetic comparative analysis (James *et al.*, 1988). However, the strong reactivity of nt 148 and 266 with

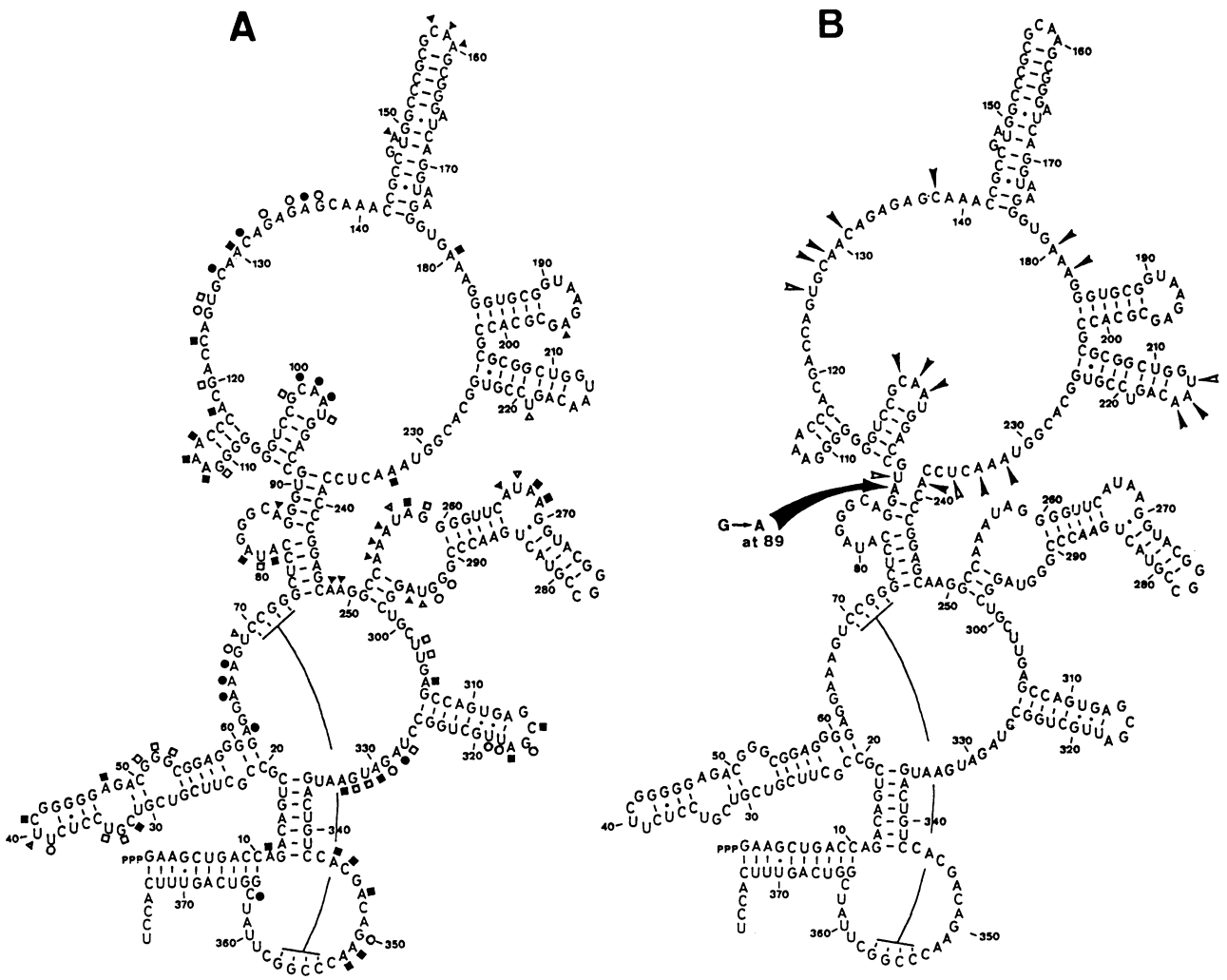

Fig. 3. Secondary structures of wild-type and A89 RNAs. (**A**) Modification sites and secondary structure of wild-type RNA. Modification sites are shown as follows: ▼ strongly attacked by DMS, ■ moderately attacked by DMS, ● marginally attacked by DMS, ▽ strongly attacked by CMCT; □ moderately attacked by CMCT, ○ marginally attacked by CMCT. (**B**) Conformational alterations of A89 RNA. Enhanced modification sites of A89 RNA are shown by arrowheads. Filled arrowheads: enhanced attack by DMS; open arrowheads: enhanced attack by CMCT.

DMS was incompatible with the previous model in which these nucleotides were within stem structures. These inconsistencies were resolved by placing these two nucleotides in bulges and by changing the structures of the neighboring stems. In addition, the stem-and-loop structure constructed between nt 20 and 61 in the previous model was slightly modified by placing two small bulges in the middle of the stem. This new secondary structure fits the experimental results better than the previous model.

Even in the presence of 100 mM Mg^{2+}, the modification patterns obtained with the two reagents were indistinguishable from those obtained in the presence of 10 mM Mg^{2+} except that the reactivity of most bases with the chemical reagents was reduced. Therefore, no conformational change of the RNA was detectable in the presence of the low (10 mM) and high (100 mM) concentrations of Mg^{2+}.

Structures of mutant RNAs

In A89 RNA, many bases are more susceptible to the chemical modification reactions than in wild-type RNA (Figures 1B and 2). The hypersensitive sites are clustered at several positions between nt 90 and 239 (Figure 1B). The rest of the nucleotide residues of the mutant RNA showed essentially the same susceptibility as wild-type RNA (Figure 2). It is obvious that in mutant RNA a helix 87–91/238–242 is disrupted, at least partially, by the substitution of nt 89, since nt 90 and 239 became susceptible to the chemical modification. As shown in Figure 3B, all of the hypersensitive sites except nt 90 and 239 are located in single-stranded regions of the secondary structure model presented above. It is likely that these sites are partially protected from chemical modification by tertiary interactions in wild-type RNA, but, in A89 RNA, the tertiary interactions are distorted by the base substitution at nt 89.

In the case of A329 RNA, reactivity with the chemical reagents was essentially the same as that of wild-type RNA. Thus, the higher-order structure of this RNA seems to be the same as wild-type RNA. It appears that the A329 mutation reduces the catalytic activity without changing the higher-order structure of the RNA.

Discussion

Two models have been proposed for the secondary structure of the RNA component of RNase P. One was based mainly

on the cleavage experiments using structure-specific nucleases (Guerrier-Takada and Altman, 1984; Lawrence *et al.*, 1987), and the other was constructed theoretically by comparative sequence analyses of various bacterial RNA moieties of RNase P (James *et al.*, 1988). The present experimental results fit the latter model better. However, minor modifications of this model were required to make it more compatible with our experimental results. The refined model depicted in Figure 3A is consistent with both the experimental results and the phylogenetic analyses.

Our previous analyses concerning the functional defects of the RNA components from temperature-sensitive RNase P mutants of *E.coli* show that the RNA consists of two functional domains; one is responsible for the catalytic activity and the other for the association with the protein component (Shiraishi and Shimura, 1986). The present studies on the structure of mutant RNAs are consistent with the previous findings. In A89 RNA, we detected a number of conformational alterations clustered between nt 90 and 239. In view of the fact that this RNA is as active as wild-type RNA in the catalytic reaction but defective in the interaction with the protein component, it is clear that the catalytic function of the RNA resides in the regions which are not altered by the mutation (nt 1–88 and 240–377) and that the structure altered by the mutation (nt 89–239) contains the region(s) which is involved in the association with the protein component. The boundaries of the two domains should be clarified by further experiments.

Based on their secondary structure model, James *et al.* (1988) suggested that the RNA component of RNase P consists of two major structural domains in which several adjacent helical elements are stacked coaxially. Their proposal was based on the fact that coaxial stacking of helices is a common structural motif in the rRNAs and tRNAs. One of the coaxial stacks (coaxial stack I) consists of the helices 1–10/364–373, 12–18/336–342 and 20–38/43–61 in Figure 3A. Another (coaxial stack II) is formed by the helices 70–73/354–357, 74–78/243–247, 87–91/238–242 and 92–96/102–106. Thus, the RNA appears to have two structural domains which consist of the two coaxial stacks and the structures attached to them. The catalytic domain inferred from the analyses of A89 RNA contains coaxial stack I, a portion of coaxial stack II and the structures attached to them. The protein-binding domain consists of the remaining portion of the coaxial stack II and the structures attached to it.

Since the combination of the protein from the *E.coli* enzyme and the RNA from *Bacillus subtilis* can reconstitute an active enzyme complex *in vitro* (Guerrier-Takada *et al.*, 1983), the RNA component of the two species should share a common structural element where the protein component binds. Although the homology between the RNAs from the two bacterial species is very low (<50% similarity in the sequences), there are a few stretches of the sequences which are conserved between the two species (Reich *et al.*, 1986). The conserved sequence motifs in the protein-binding domain, which are longer than five consecutive nucleotides, are GUGCC at nt 89–93, AGUGCNACAG at 124–133 and GGUAAACPyCC at 229–238. It is possible that these sequences represent the binding sites for the protein component.

The catalytic center of the RNA component is not well understood. Although we assigned the catalytic domain to nt 1–88 and 240–377, the minimum structure required for catalysis could be a portion of the domain. Using the precursor of *E.coli* $tRNA^{Tyr}$ as a substrate, Guerrier-Takada and Altman (1986) have shown that the RNA component missing as many as 122 nucleotides at the 3′ terminus retains catalytic activity, although at a much lower level than the intact RNA. This result suggests that the catalytic center of the RNA is located at the 5′ proximal region of the RNA. On the other hand, A329 RNA, which contains a base substitution in the 3′ proximal region, shows greatly reduced catalytic activity (Shiraishi and Shimura, 1986). In addition, we failed to detect any activity for cleaving a precursor of *E.coli* $tRNA^{Asp}$ using the RNA missing 87 nucleotides at the 3′ terminus (unpublished result). The importance of the 3′ proximal sequences for the catalytic activity has also been shown with the RNA component of *B.subtilis* RNase P by Reich *et al.* (1986). In this case, a shortened version of the RNA component missing 120–121 nt at the 3′ terminus has no detectable catalytic activity. These results imply that the sequences at both the 5′ proximal portion (nt 1–88) and the 3′ proximal portion (nt 240–377) are required for maximal catalytic activity. In these regions, there are several sequence elements which are conserved between Gram-positive and purple bacteria (James *et al.*, 1988). The conserved sequences contain a sequence element, 5′-UAGA-3′, spanning nt 327–330 of the *E.coli* RNA (Figure 3A), which includes the mutational site of A329 RNA. Also conserved are the sequences spanning nt 60–70, 252–256, 292–297, 347–352 and 356–360 of the *E.coli* RNA, most of which are located in the single-stranded region of the RNA (see Figure 3A). It is worth noting that some of these sequences are conserved even in the RNA component of yeast mitochondrial RNase P (Miller and Martin, 1983), which has no homology to the bacterial RNAs in the putative protein-binding domain. Proper configuration of the conserved sequences in the catalytic domain might play a key role in substrate recognition and catalysis. Analysis of more mutants generated by site-directed mutagenesis (Shiraishi and Shimura, 1988) would provide vital information both on the domain structures and on the role of the conserved structures of the RNase P RNA.

Materials and methods

Enzymes and reagents

E.coli RNA polymerase was purified from *E.coli* A19 (RNase I^-) to near homogeneity. Reverse transcriptase from Rous-associated virus 2 was obtained from Takara Shuzo Co. DMS and CMCT were purchased from Eastman Kodak Co. and Aldrich Chemical Co. respectively.

In vitro transcription of the rnpB gene

The 2.8-kb *Eco*RI fragments from pPR4273, pPR1 and pPR2418, the plasmids carrying the wild-type, A89 and A329 *rnpB* genes respectively were transcribed in 100 μl of the reaction mixtures as described previously (Shiraishi and Shimura, 1986). After phenol extraction and ether extraction, an equal volume of 4 M ammonium acetate was added and nucleic acids were precipitated with 2.5 vol of ethanol. The precipitates were dissolved in 50 μl of 10 mM Tris–HCl (pH 7.5), 5 mM $MgCl_2$. Ten microliters of this solution containing ~5–10 pmol of the major transcription product of the *rnpB* gene were used for each modification reaction.

Chemical modification of RNA

For DMS modification, the transcription products of the *rnpB* gene were preincubated at 37°C in 100 μl of a buffer containing 50 mM sodium cacodylate (pH 7.5), 10 or 100 mM $MgCl_2$, 100 mM KCl. After 10 min, 2 μl of DMS diluted 5-fold with ethanol was added, and the incubation was

continued at 37°C for 10 min. The reaction was terminated by the addition of 50 μl of DMS stop solution [1.5 M sodium acetate (pH 7.0), 1 M β-mercaptoethanol], and nucleic acids were recovered by ethanol precipitation. CMCT modification was performed by the same procedures as DMS modification except that the reaction buffer was 50 mM potassium borate (pH 8.0), 10 or 100 mM $MgCl_2$, 100 mM KCl. The final concentration of CMCT was 21 mg/ml. After the reaction, 10 μl of 3 M sodium acetate (pH 7.0) was added, and nucleic acids were recovered by ethanol precipitation.

After the modification reactions, the major transcription products of the *rnpB* genes (Sakamoto *et al.*, 1983b) were purified by electrophoresis on a 5% polyacrylamide gel containing 8.3 M urea.

Primer extension

Primer extension was performed as described previously (Moazed and Noller, 1986). In some experiments, dGTP in the reaction mixtures was substituted with the same concentration of dITP to avoid band compressions (Mills and Kramer, 1979; Gough and Murry, 1983; Tabor and Richardson, 1987). The sequence ladder was generated by the method of Tabor and Richardson (1987) using the single-stranded *rnpB* gene as template. Electrophoresis was performed in 5%, 6% and 8% polyacrylamide gels containing 8.3 M urea.

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