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Mutations affecting two distinct functions of the RNA component of RNase P

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The effect of structural changes on the functions of the RNA component (M1 RNA) of ribonuclease P (RNase P) of *Escherichia coli* has been studied using the thermosensitive mutants of the *rnpB* gene. One of the mutants, ts709, has two G-A substitutions at positions 89 and 365 from the 5′ end of M1 RNA. Of these substitutions, the one at position 89 from the 5′ end is responsible for the phenotype of this mutant. Although the RNase P activity of ts709 is thermosensitive, the mutant M1 RNA has the same catalytic activity as the wild-type RNA. M1 RNA of another mutant, ts2418, has a G-A substitution at position 329. This mutant RNA has extremely low catalytic activity. The upstream mutational site of ts709 appears to play a role in the association with the protein subunit, whereas the mutational site of ts2418 is related to the catalytic function of M1 RNA.

Key words: catalytic activity/M1 RNA/RNA processing/RNP formation

Introduction

RNase P is an endonuclease that cleaves tRNA precursors to generate the 5′ termini of mature tRNA molecules (Altman and Smith, 1971; Robertson *et al.*, 1972). The enzyme contains an essential RNA subunit which can be separated from the protein subunit and subseqently reassembled to reconstitute the enzyme activity (Stark *et al.*, 1978; Kole and Altman, 1979). The RNA component of *Escherichia coli* RNase P (M1 RNA) is 377 nucleotides in length and coded for by the *rnpB* gene located at 69 min on the *E. coli* chromosome (Sakamoto *et al.*, 1983a). The nucleotide sequence of the gene and its flanking regions have been determined (Reed *et al.*, 1982; Sakamoto *et al.*, 1983a).

Recently, the RNA subunit of RNase P from *E. coli* and *Bacillus subtilis* (P-RNA) has been shown to be able to cleave, by itself, tRNA precursors in buffers containing either 60 mM Mg^{2+} or 10 mM Mg^{2+} with 1 mM spermidine, and to represent the catalytic subunit of the enzyme (Guerrier-Takada *et al.*, 1983). The transcription product of the *rnpB* gene also has the same catalytic activity (Guerrier-Takada and Altman, 1984a), although the transcript carries an extra stretch of 36 or 37 nucleotides at the 3′ end of M1 RNA (Sakamoto *et al.*, 1983b; Reed and Altman, 1983; Gurevitz *et al.*, 1983). However, the protein and RNA subunits are required for cleavage of tRNA precursors in standard buffers containing 5-10 mM Mg^{2+} (Guerrier-Takada *et al.*, 1983). Also, M1 RNA alone is not active on the precursor of *E. coli* 4.5S RNA (Guerrier-Takada *et al.*, 1983), which has been shown to accumulate in the thermosensitive RNase P mutants at the restrictive temperature and to be processed by RNase P (Ikemura *et al.*, 1975; Bothwell *et al.*, 1976).

Thus, M1 RNA should contain at least two regions, or domains, which are responsible for the functions of the RNA, the catalytic activity and the association with the protein subunit. However, little is known about such functional regions of the RNA. In this paper, we describe the results of the analyses on the structures and functional defects of M1 RNAs from the thermosensitive *rnpB* mutants we previously isolated (Sakano *et al.*, 1974).

Results

Nucleotide sequence of the rnpB gene from ts2418

Among our temperature-sensitive mutants defective in RNase P activity and mapped at the *rnpB* locus of the *E. coli* chromosome, ts709 contains two G-A substitutions at positions 89 and 365 from the 5′ end of M1 RNA (Sakamoto *et al.*, 1983a). Another thermosensitive mutant, ts2418, has also been mapped at the *rnpB* locus (Sakano *et al.*, 1974; Shimura *et al.*, 1980). The phenotype of this mutant is indistinguishable from that of ts709. The crude extract (S30) prepared from this mutant showed very low RNase P activity at 30°C and its activity was almost undetectable at 42°C (data not shown). The *rnpB* gene of ts2418 was cloned and its nucleotide sequence was determined. The *rnpB* gene of ts2418 has a single G-A substitution at position 329 from the 5′ end of M1 RNA. In Figure 1, the base substitutions of ts2418 and ts709 are indicated in the secondary structure of M1 RNA proposed by Guerrier-Takada and Altman (1984b). As noted in the figure, the base substitutions of both mutants are in base-paired regions of the structure.

Construction of single mutants from ts709

We have shown that M1 RNA of ts709 is extremely unstable and rapidly degraded *in vivo* and *in vitro* (Sakamoto *et al.*, 1983a). To determine whether both substitutions are responsible for this and other mutational defects of ts709, we constructed single mutants from the *rnpB* gene of ts709 by replacing each of the mutant sequences with the wild-type counterparts. The 2.8 kb *Hin*cII fragment which includes the entire *rnpB* gene was recloned into pBR322 from λgrnpR-W and λgrnpR-709, the phage clones that contain the *rnpB* gene from strains 4273 (wild-type) and ts709, respectively (Sakamoto *et al.*, 1983a). As shown in Figure 2, the resulting two plasmids, pPR4273 containing the wild-type fragment and pPR709 containing the mutant fragment, were individually cut with *Bam*HI and *Sma*I, and two chimeric plasmids, pPR1 and pPR2, were constructed. Two single-mutant genes in which the upstream mutation (A89) and the downstream mutation (A365) were individually replaced by the corresponding wild-type sequences were thus obtained and designated the A365 and A89 genes, respectively. As seen in the figure, pPR1 and pPR2 contain the A89 and A365 genes, respectively.

Complementation of ts709 with the single mutant genes

The 2.8 kb *Eco*RI fragments from pPR4273, pPR709, pPR1, and pPR2 were individually subcloned into the *Eco*RI site of pCP5, a miniF-pBR322 composite plasmid, and the resulting plasmids were designated pFR4273, pFR709, pFR1, and pFR2,

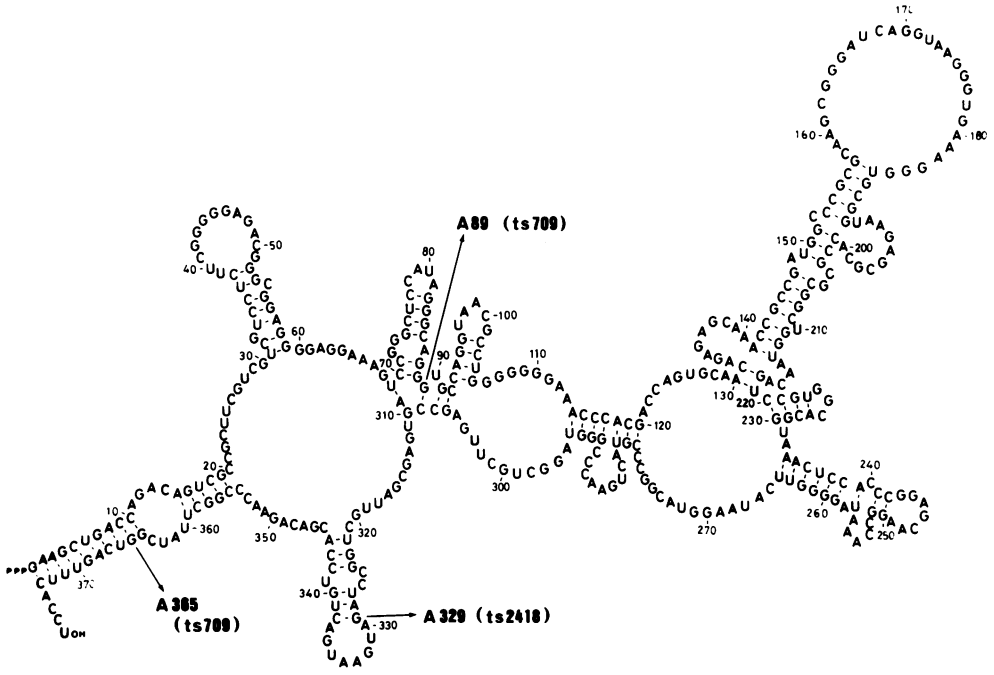

Fig. 1. Mutations of ts709 and ts2418. The base substitutions of ts709 and ts2418 are indicated in the secondary structure of M1 RNA proposed by Guerrier-Takada and Altman (1984b). Nucleotide residues are numbered in the 5′−3′ direction.

respectively. Each of these plasmids was introduced into ts709*polA* and the transformants were selected by ampicillin resistance. In these transformants, the copy number of the plasmids was under the control of the F replicon, because the pBR322 replicon could not function in the presence of the *polA* mutation. Thus, the dosage effect of the *rnpB* gene, if any, could be minimized in the marker rescue experiments.

The mutant cells transformed with pFR709 and pFR1 were still thermosensitive in their growth, while those transformed with pFR4273 and pFR2 grew at 42°C as the wild-type strain. The transformants were individually labelled with [^{32}P]orthophosphate at 42°C for 30 min and RNA extracted from the cells was electrophoresed on a 10% polyacrylamide gel. As shown in Figure 3, ts709*polA* showed a gel pattern which was characteristic of mutants defective in RNase P activity. Most of the RNA bands in the figure except for the 5S and 6S RNAs represent tRNA precursors as described previously (Ikemura *et al.*, 1975; Sakano and Shimura, 1978). These precursors do not accumulate in the wild-type cells at 42°C. When pFR1 was introduced into the mutant, the profile remained essentially the same as that obtained with ts709. However, the mutant cells harboring pFR2 showed a pattern indistinguishable from that of the wild-type cells. Thus, it is clear that pFR2 complements the mutant but pFR1 does not.

Since pFR2 contains the single base substitution at the downstream position, these results led us to conclude that the upstream mutation (at position 89) is responsible for the mutant phenotype of ts709, whereas the substitution at the downstream position (position 365) is little related to the defect of the mutant. It is worth noting that the *rnpB* gene from ts2418 does not complement ts709 (Figure 3).

Stability of mutant M1 RNAs

The amount of M1 RNA is extremely reduced in ts709 cells grown at the permissive temperature (Sakamoto *et al.*, 1983a; Reed *et al.*, 1982; Motamedi *et al.*, 1982). When the *rnpB* gene from ts709 was transcribed *in vitro* with *E. coli* RNA polymerase and the transcripts were subsequently incubated with crude extract from wild-type cells, the transcripts were processed to form mature M1 RNA, which was unstable in the extract and rapidly degraded, compared to M1 RNA prepared similarly from the *rnpB* gene from wild-type cells (Sakamoto *et al.*, 1983a). The instability of the mutant RNA was more pronounced at 44°C than at 30°C.

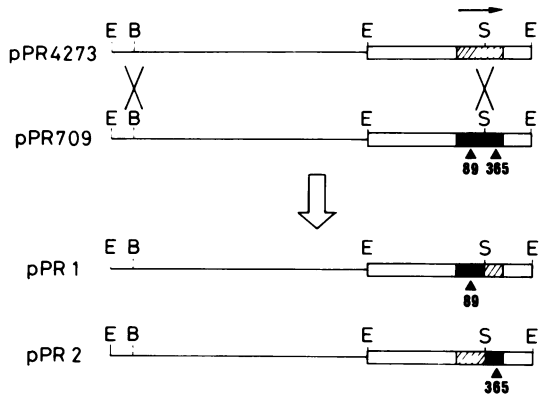

Fig. 2. Construction of single mutants from ts709. pPR4273 and pPR709 were individually digested with *Bam*HI and *Sma*I, and two chimeric plasmids were constructed as described in Materials and methods. As both plasmids contain a single *Sma*I site in the *rnpB* sequences between the two mutational sites (position 290) of ts709, the chimeric plasmids contain single-mutant genes, A89 and A365. Each plasmid is presented in a linearized form placing the 2.8 kb insert to the right in the figure. The 2.8 kb *Eco*RI fragments carrying the *rnpB* genes are indicated by boxes and the pBR322 sequences by thin lines. The hatched boxes represent the *rnpB* gene sequences derived from the wild-type strain (4273) and the filled boxes those derived from ts709. The apexes of the solid triangles indicate the sites of the G−A substitutions. Numbers below the triangles indicate the nucleotide positions from the 5′ end of M1 RNA. The direction of transcription of the *rnpB* gene is shown by an arrow above pPR4273. The following restriction sites are indicated: **E**, *Eco*RI; **B**, *Bam*HI; **S**, *Sma*I.

The *rnpB* gene of the two single mutants, pPR1 (A89) and pPR2 (A365), was transcribed *in vitro* and the transcripts were

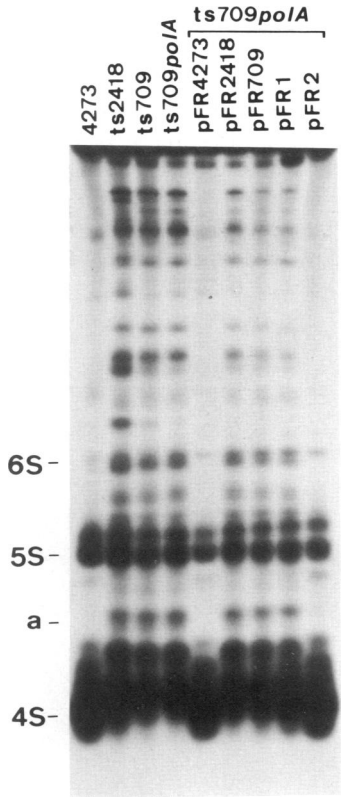

Fig. 3. Complementation of ts709 with the plasmids carrying the mutant *rnpB* genes. ts709*polA* was transformed individually with pFR4273, pFR2418, pFR709, pFR1, and pFR2 which carry the *rnpB* genes from 4273 (wild-type), ts2418, ts709, pPR1 (A89), and pPR2 (A365), respectively, and the ampicillin resistant colonies were selected at 30°C. The transformants were labeled with [^{32}P]orthophosphate at 42°C for 30 min and RNA was extracted with phenol. The RNAs were electrophoresed on a 10% polyacrylamide gel and autoradiographed as described (Sakano and Shimura, 1978). The wild-type strain (4273) and the *rnpB* mutants (ts2418, ts709, and ts709*polA*) were similarly labeled and RNAs were fractionated on the same gel. The positions of 6S RNA, 5S RNA, and 4S RNAs are indicated. Band a represents a monomeric precursor to $tRNA^{Asp}_1$.

incubated with crude extract at 37°C. As shown in Figure 4, the processed RNA from the A89 gene was extremely labile and rapidly degraded, as was the case with the RNA from the ts709 gene. In contrast, the RNA from the A365 gene was as stable as wild-type RNA. Thus, it is clear that the instability of ts709 M1 RNA is primarily due to the base substitution at position 89 from the 5′ end of the RNA. These results support the view that the mutation at position 89 is responsible for the mutant phenotype of ts709 but the mutation at position 365 is not directly related to it.

Also noted in this figure is that the RNA derived from the transcripts of the *rnpB* gene from ts2418 is as stable as the wild-type RNA. This was also the case *in vivo* at both 30° and 42°C (data not shown). Thus, mutant M1 RNA appears to be stable in ts2418.

Temperature-sensitive property of RNase P from the mutants

To examine the thermosensitive nature of RNase P from ts2418 and ts709, the enzyme was partially purified from the mutant cells and also from wild-type cells. The enzyme activity was assayed using a monomeric precursor of $tRNA^{Asp}_1$ (band a in Figure 3) as substrate and quantitated by measuring the amount of RNA product containing the mature tRNA sequence and the 3′ extra sequence. The mutant enzymes showed lower activity than the wild-type enzyme even at 30°C (data not shown). The activity of the mutant enzymes was hardly detectable when assayed at 40°C, whereas the activity of the wild-type enzyme at 40°C was almost three times higher than that at 30°C. The activity of the ts2418 enzyme was lower than that of the ts709 enzyme at 30°C.

Catalytic properties of the mutant M1 RNAs

The catalytic activity of M1 RNAs from the mutant and wild-type strains was examined. The *rnpB* genes from the wild-type strain (4273) and the four mutants including the two single mutants derived from ts709 were individually transcribed *in vitro* with purified *E. coli* RNA polymerase, and the transcription products purified by electrophoresis on an 8 M urea/4% polyacrylamide gel. The transcripts were recovered from the gel and their catalytic activity was assayed in the presence of 60 mM Mg^{2+} using the monomeric precursor of $tRNA^{Asp}_1$ as substrate.

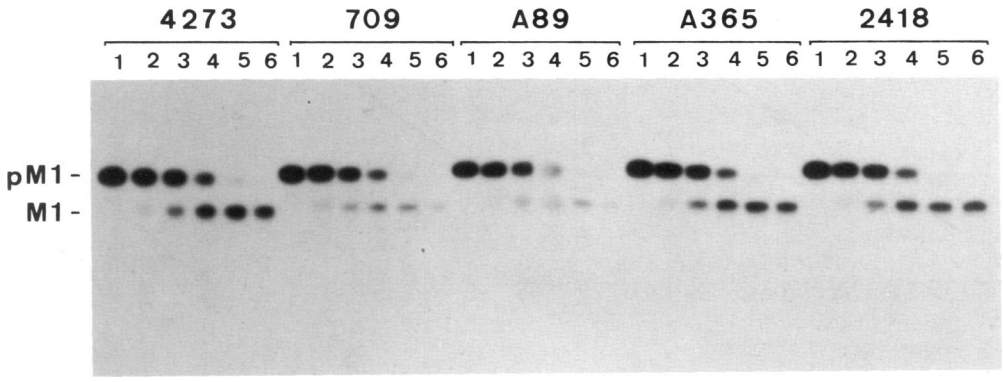

Fig. 4. Stability of mutant M1 RNAs *in vitro*. The *rnpB* genes from wild-type (4273) and mutants (ts709, A89, A365, and ts2418) were individually transcribed *in vitro* in the presence of [α-^{32}P]GTP as the radioactive substrate, and the major transcription products (pM1; ~15 fmol, 3 × 10^4 c.p.m. for each) were incubated at 37°C with the S30 extract of *E. coli* Q13. Incubation times (minutes) were as follows: 0 **(lane 1)**, 10 **(lane 2)**, 30 **(lane 3)**, 60 **(lane 4)**, 120 **(lane 5)**, 180 **(lane 6)**. After incubation, RNA was extracted with phenol, electrophoresed on an 8 M urea/4% polyacrylamide gel, and autoradiographed.

As shown in Figure 5A, the transcription products from the ts709 gene and the two single-mutant (A89 and A365) genes were as active as the products from the wild-type gene both at 30°C and at 44°C. The transcripts of these mutant and wild-type genes were more active at 44°C than at 30°C. However, the catalytic activity of the transcript of the ts2418 gene was not detectable under the same conditions. When the amount of ts2418 RNA was increased eight times and the reaction mixture was incubated for prolonged time (5 h), the activity of the mutant RNA became barely detectable (Figure 5B). Unlike the cases with the other mutant RNAs, the catalytic activity of this mutant RNA at 44°C was not higher than that at 30°C. The kinetics of the reactions catalyzed by the wild-type and mutant RNAs were examined. Figure 6 shows the Lineweaver–Burk plots of the reactions. The V_{max} and K_m values for each reaction determined from the plots are listed in Table I. As noted in the table, the reactions catalyzed by ts709 and ts2418 RNAs showed the same K_m values as that catalyzed by wild-type RNA. On the other hand, the V_{max}

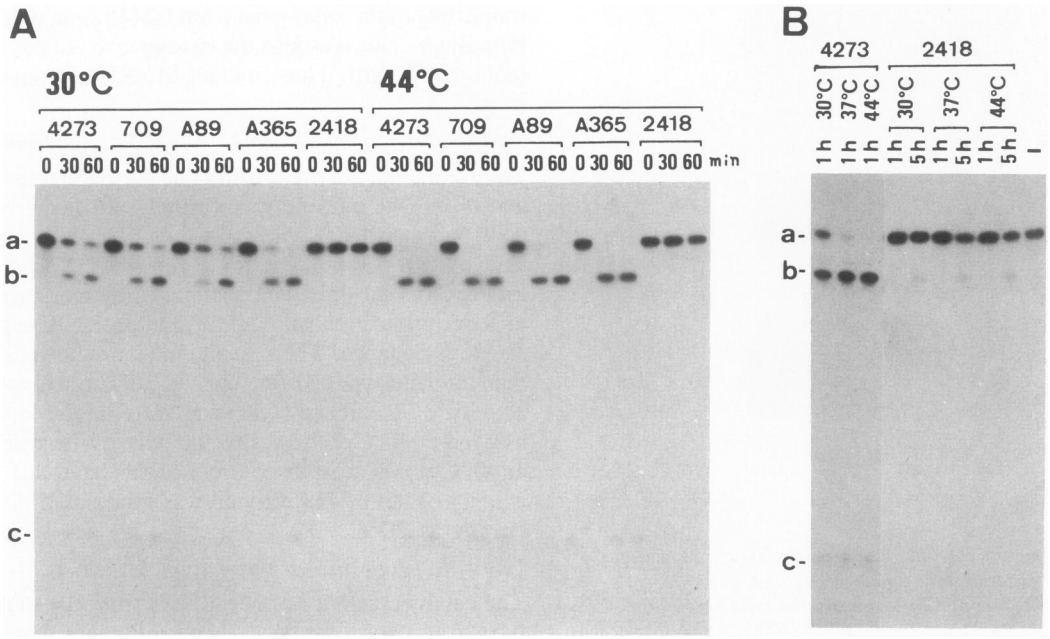

Fig. 5. Cleavage reactions catalyzed by M1 RNA precursors. **(A)** The *rnpB* genes from wild-type stain (4273) and mutants (ts709, A89, A365, and ts2418) were individually transcribed *in vitro* and the major transcription products were purified. The M1 RNA precursors thus prepared were individually incubated at 30°C or 44°C with the $tRNA^{Asp}_1$ precursor (1.5×10^4 c.p.m., ~6.6 ng) in 20 μl of the reaction mixture described in Materials and methods. The final concentration of the M1 RNA precursors was 10 nM for each reaction. At the times indicated, aliquots of 6 μl were withdrawn from the reaction mixtures. After the reaction was terminated, RNA was electrophoresed on an 8 M urea/8% polyacrylamide gel. **(a)**, Monomeric precursor of $tRNA^{Asp}_1$; **(b)**, the mature tRNA sequence with the 3′ extra sequence; **(c)**, the 5′ leader sequence. **(B)** The transcription products of the wild-type gene (4273) and the ts2418 gene were individually incubated with the $tRNA^{Asp}_1$ precursor for the times indicated at 30°C, 37°C, or 44°C in 20 μl of the reaction mixtures. The final concentration of the M1 RNA precursors was 10 nM for 4273 and 80 nM for ts2418. The samples were analyzed by electrophoresis on an 8 M urea/8% polyacrylamide gel. (−), No M1 RNA precursor was added.

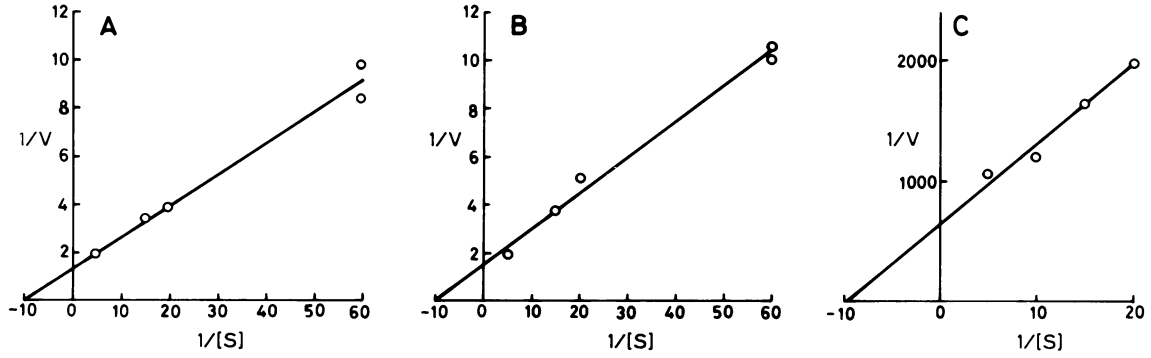

Fig. 6. Lineweaver–Burk plots of the reactions catalyzed by M1 RNA precursors. The $tRNA^{Asp}_1$ precursor and the transcription products of the *rnpB* gene from strains 4273 **(Panel A)**, ts709 **(Panel B)**, or ts2418 **(Panel C)** were individually preincubated in the reaction buffer at 37°C for 10 min and then mixed. The final concentration of the M1 RNA precursor was 10 nM in the reactions shown in **Panels A** and **B** and 80 nM in **Panel C**. The mixed samples (20 μl) were incubated further at 37°C and 4 μl-aliquots were withdrawn at 5 sec, 3.5 min, 7 min and 12 min in the reactions shown in **A** and **B**, and at 5 sec, 60 min, 120 min and 240 min in the reaction shown in **C**. The reaction products were fractionated by electrophoresis on an 8 M urea/8% polyacrylamide gel. After autoradiography, RNAs corresponding to bands a, b and c in Figure 5 were excised and the radioactivity of each RNA species was determined by liquid scintillation counting. The extent of the reaction was calculated as the sum of the radioactivity of bands b and c relative to that of bands a, b, and c, and the initial velocity of the reaction was determined. The substrate concentration [S] was calculated from the estimated mol. wt of 30 000 for the band-a RNA. Units: [S], μM; V, min^{-1}.

for the reaction obtained with ts2418 RNA was about 0.25% of the value with wild-type RNA, while the V_{max} obtained with ts709 RNA was the same as that with wild-type RNA. Thus, the catalytic activity of ts709 RNA is indistinguishable from that of wild-type RNA, whereas ts2418 RNA is defective in this activity. Essentially the same results were obtained when mature M1 RNAs, prepared *in vitro* from the transcription products of wild-type and mutant *rnpB* genes by the action of purified processing enzyme, were used instead of the transcript RNAs (data not shown). Therefore, it appears that the mutational site of ts2418 (position 329) is within, or close to, the region which is responsible for the catalytic function of M1 RNA.

Association of the transcript of the A89 gene with the protein subunit of RNase P

Although the catalytic activity of M1 RNA of ts709 is the same as that of wild-type M1 RNA, RNase P activity of this mutant is thermosensitive and the mutant RNA is extremely unstable in cells particularly at high temperature, it is most likely that the association of M1 RNA with the protein subunit of the enzyme is not normal in ts709 and that the base substitution at position 89 from the 5′ end of the RNA is responsible for the weak association with the protein. This view is supported by the following experiment in which we examined the formation of a ribonucleoprotein complex *in vitro* between the transcription product of the A89 gene and the protein subunit of RNase P from wild-type cells. The ^{32}P-labeled transcription products of wild-type and A89 *rnpB* genes were individually incubated with the protein subunit of RNase P at 0°C for 60 min and subsequently at 40°C for 10 min. The mixtures were then subjected to density gradient centrifugation in CsCl and radioactivity of each fraction was determined. As seen in Figure 7B, when the transcript of the wild-type gene was incubated with the protein subunit, a radioactive peak was observed at a density of about 1.70. The appearance of the radioactive peak is dependent on the presence of the protein subunit in the incubation mixture (Figure 7A). In the CsCl gradient, free RNA sedimented as a pellet at the bottom of tubes. In a separate experiment, we found that the peak fraction and a purified RNase P preparation banded at the same density in CsCl. Thus, it is almost certain that the peak fraction represents a ribonucleoprotein complex of RNase P activity. On the other hand, such a radioactive peak was not detectable when the transcript of the A89 gene was incubated with the protein subunit (Figure 7C). Although these results do not clarify whether the formation of the ribonucleoprotein complex is impaired with the mutant RNA or the complex is formed with the mutant RNA but rapidly dissociated under the experimental condition, it is almost certain that the association of the mutant RNA with the protein subunit to form the ribonucleoprotein of RNase P activity is affected by the point mutation at position 89 from the 5′ end of the RNA.

Table I. K_m and V_{max} for the cleavage reactions catalyzed by wild-type and mutant M1 RNAs

Strain	V_{max} (min^{-1})	K_m (M)
4273	0.8	1×10^{-7}
ts709	0.8	1×10^{-7}
ts2418	0.002	1×10^{-7}

The K_m and V_{max} values were determined from the plots shown in Figure 6.

Discussion

To obtain information on functional domains of the RNA subunit of RNase P, we undertook a close examination of the structure and functional defects of the RNA component from the two temperature-sensitive mutants, ts709 and ts2418 (Sakano *et al.*, 1974). These mutants were independently isolated and mapped at the *rnpB* locus on the *E. coli* chromosome (Shimura *et al.*, 1980).

We have shown that M1 RNA of ts709 is normal in its catalytic activity despite the fact that the RNase P activity of the mutant is thermosensitive and that the instability of this mutant RNA both *in vitro* and *in vivo* especially at the high temperature is due to the base substitution at position 89. In addition, we have demonstrated that this mutant RNA is defective in the association with the protein subunit to form a ribonucleoprotein complex *in vitro*, because of the base substitution. Presumably this could account for the thermosensitive phenotype of ts709. Thus, it is likely that the mutation site (position 89) is within or sufficiently close to the domain for the complex formation with the protein subunit.

Another *rnpB* mutant, ts2418, has a single G−A substitution at position 329. The transcript of this mutant gene is defective in cleavage of the monomeric precursor to tRNA$^{Asp}_1$. The V_{max}

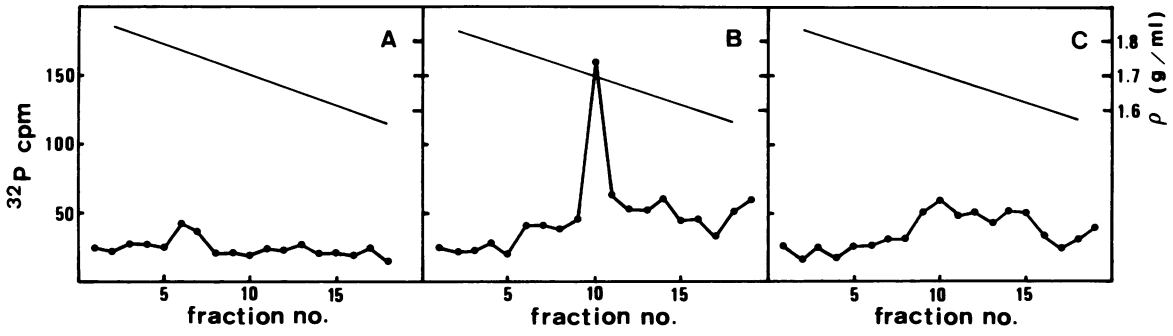

Fig. 7. Complex formation between the transcripts of the *rnpB* gene and the protein subunit of RNase P. The transcripts (2 × 10^5 c.p.m., ~14 ng) from wild-type **(Panels A** and **B)** and A89 **(Panel C)** *rnpB* genes were individually incubated at 0°C for 60 min in the absence **(Panel A)** or presence **(Panels B** and **C)** of the protein subunit (about 50 ng) of RNase P purified from *E. coli* Q13 in 100 μl of 20 mM Tris−HCl (pH 7.8), 1 mM $MgCl_2$, 10 mM KCl, and 7 mM 2-mercaptoethanol. The mixtures were subsequently incubated at 40°C for 10 min. To each mixture, CsCl was added to a final concentration of 52% (w/w) and its volume adjusted to 1 ml with the buffer. Each solution was overlaid onto two layers of CsCl solutions, 1 ml each of 58% and 55% solutions in the same buffer, and centrifuged at 40 000 r.p.m. in the Beckman Spinco SW50 rotor for 20 h. Twelve-drop fractions (about 160 μl) were collected from the bottom of centrifuge tube, omitting free RNA sedimented at the bottom of the tube. Shown in the figure is the analysis of CsCl fractions for radioactivity measured on 50 μl of each fraction by liquid scintillation counting.

for the reaction catalyzed by this mutant RNA is 1/400 of that observed with wild-type and ts709 RNAs, although the K_m values for the reactions catalyzed by all three RNAs are the same. The mechanism underlying the decrease of catalytic activity of ts2418 RNA is not well understood. It is possible to assume that the initial binding of this mutant M1 RNA to the tRNA precursor may take place normally but some subsequent step(s) in the cleavage reaction is impaired. In any case, it is highly likely that the mutational site of ts2418 is within or close to the catalytic domain of M1 RNA. In this connection, it has recently been reported by Guerrier-Takada and Altman (1986) that fragments of M1 RNA missing as many as 122 nucleotides at the 3′ terminus retain catalytic activity, although at a much lower level than M1 RNA. They suggest that M1 RNA might dimerize to form the catalytic complex (Guerrier-Takada *et al.*, 1986) and that the fragments of M1 RNA form dimers at a much lower rate than wild-type M1 RNA (Guerrier-Takada and Altman, 1986). It is conceivable that ts2418 M1 RNA form a dimer which is not capable of generating the conformation of an active RNA species. On the other hand, in the case of the RNA component of the *B. subtilis* RNase P, which is 400−401 nucleotides long, an RNA molecule missing 121−122 nucleotides at the 3′ terminus is inactive in processing of a tRNA precursor *in vitro* (Reich *et al.*, 1986). However, as the *B. subtilis* RNA and M1 RNA are different in length and show less than 50% similarity in sequence (Reich *et al.*, 1986), it would not be simple to compare the results obtained with the two RNAs about the regions of catalytic activity. It has been pointed out by Reed *et al.* (1982) that there is a pentanucleotide sequence (5′-UGAAU) in M1 RNA, which is complementary to an invariant sequence (5′-GTΨCPu) of tRNA. The pentanucleotide sequence is located at positions 331−335, which are 2−6 nucleotides downstream from the mutational sites of ts2418. The importance of the GTΨCPu sequence of tRNA for processing of its precursor *in vivo* has been observed by Kudo *et al.* (1981). However, the possible interaction between the pentanucleotide sequence (UGAAU) of M1 RNA and the invariant sequence (GTΨCPu) of tRNA precursors still remains to be examined.

We have demonstrated that the two distinct sites in M1 RNA may play important roles in the functions of the RNA, association with the protein subunit and the catalytic activity of RNase P. Obviously these functions must be carried out by specific contact of functional domains of M1 RNA which possibly include interaction of those two sites with the protein moiety of RNase P and tRNA precursors. Such domains are yet to be clarified. Analysis of more mutants, particularly those created by site-specific mutagenesis near the two sites, would provide vital information on the domain structures.

Materials and methods

Bacterial strains, phage strains, and growth media

E. coli strains 4273, ts709, and ts2418 have been described previously (Sakano *et al.*, 1974; Shimura *et al.*, 1980). ts709*polA* was constructed by transduction with phage P1. *E. coli* strains Q13 was from our laboratory stock. λgrnpR-W and λgrnpR-709 were described previously (Sakamoto *et al.*, 1983a). A miniF-pBR322 composite plasmid, pKP1033 (Ogura and Hiraga, 1983), was obtained from Dr S.Hiraga. Plasmids were prepared as described by Maniatis *et al.* (1982). Growth media and low-phosphate medium used for preparation of ^{32}P-labeled cells were described previously (Sakano and Shimura, 1978; Nakajima *et al.*, 1981). Phages were purified as described previously (Nakajima *et al.*, 1981).

Enzymes

E. coli RNA polymerase was purified as described previously (Nakajima *et al.*, 1982). Restriction enzymes and T4 DNA ligase were purchased from Takara Shuzo Co. RNase P was purified as described by Shimura *et al.* (1978) with the following modifications. Cells were disrupted by sonication instead of grinding with alumina and the enzyme was purified up to the step of the first DEAE−cellulose chromatography. When the protein subunit of RNase P was prepared, the enzyme was purified further by gel filtration through cellurofine GCL-2000 (Seikagakukogyo Co.).

Construction of plasmids

λgrnpR-W and λgrnpR-709 contain the *rnpB* gene from strains 4273 (wild-type) and ts709, respectively, in the 10 kb *Eco*RI fragment inserted into the phage vector (Sakamoto *et al.*, 1983a). Digestion of the *Eco*RI fragment with *Hin*cII generated a fragment of 2.8 kb in length (2.8 kb *Hin*cII fragment) which contained the *rnpB* gene. The 2.8 kb *Hin*cII fragment from λgrnpR-W and λgrnpR-709 were individually cloned, after addition of an *Eco*RI linker at both termini, into the *Eco*RI site of pBR322; the resulting plasmids were designated pPR4273 and pPR709, respectively. The 2.8 kb *Hin*cII fragment was similarly cloned from strain ts2481 and the plasmid thus obtained designated pPR2418. To make the chimeric *rnpB* genes of wild-type and ts709, pPR4273 and pPR709 were individually cut with *Bam*HI/*Sma*I and the resulting 1.2 kb and 6 kb fragments recovered. Each fragment from pPR4273 was replaced by the corresponding fragment from pPR709 and ligated as shown in Figure 2. The chimeric plasmids pPR1 and pPR2 were thus obtained. The gene sequences in the single mutants were confirmed by restriction with *Fok*I and *Sau*3A that cleave the upstream (A89) and downstream (A365) mutational sites of ts709 gene, respectively (Sakamoto *et al.*, 1983a), and by direct DNA sequencing of the regions in the two single mutant genes. When the wild-type and mutant *rnpB* genes were subcloned into a miniF-pBR322 composite plasmid, we employed pKP1033. The 0.8 kb *Sal*I fragment was deleted from pKP1033 and the resulting plasmid containing a single *Eco*RI site designated pCP5. The 2.8 kb *Eco*RI fragments from pPR4273, pPR709, pPR2418, pPR1 and pPR2 were individually cloned into the *Eco*RI site of pCP5, and those plasmids having the inserts of the same orientation were chosen and designated pFR4273, pFR709, pFR2418, pFR1, and pFR2, respectively.

Complementation of ts709 with plasmids

ts709*polA* was transformed individually with pFR4273, pFR709, pFR2418, pFR1, and pFR2, and the ampicillin resistant colonies were selected. Growth of the transformants at the restrictive temperature (42°C) was tested on a rich medium (λ-agar plate). The transformants were labeled with [^{32}P]orthophosphate at 42°C for 30 min, and RNA was extracted and electrophoresed as described previously (Sakano and Shimura, 1978).

Cloning and sequencing of the rnpB gene from ts2418

The *rnpB* gene was cloned from ts2418 as described previously (Sakamoto *et al.*, 1983a), except that EMBL3 (Frischauf *et al.*, 1983) was the phage vector. DNA sequence analysis was performed according to the dideoxy chain termination method (Sanger *et al.*, 1977) using M13mp18 and M13mp19.

Preparation of ^{32}P-labeled tRNA$^{Asp}_1$ precursor

The ^{32}P-labeled monomeric precursor of tRNA$^{Asp}_1$ was prepared as described previously (Sakano and Shimura, 1978). The specific activity of the RNA was $1.5-2 \times 10^6$ d.p.m./μg.

Preparation of the transcription products of the rnpB gene

The ^{32}P-labeled transcription products of the *rnpB* gene were prepared *in vitro* as described previously (Sakamoto *et al.*, 1983b), using [α-^{32}P]GTP (specific activity 20 Ci/mmol) as a radioactive substrate. The transcripts were purified by electrophoresis on an 8 M urea/4% polyacrylamide gel. The non-labeled transcripts of the *rnpB* gene were also prepared in the same *in vitro* system, except that the reaction volume was increased 10 times (0.5 ml) that of the radioactive reaction mixture described above and the concentration of four nucleoside triphosphates was increased to 0.25 mM. After incubation, the transcripts were purified by gel electrophoresis as above and detected, after staining with ethidium bromide (0.5 μg/ml), under u.v. irradiation.

Assays for the reactions catalyzed by RNase P and by M1 RNA precursor

Unless otherwise stated, RNase P was assayed in a reaction mixture (50 μl) containing 20 mM Tris−HCl (pH 7.8), 5 mM $MgCl_2$, 100 μM EDTA, 10 mM 2-mercaptoethanol, the monomeric precursor of tRNA$^{Asp}_1$ (5×10^4 d.p.m., 33 ng), and 0.1 vol of purified enzyme preparation. The mixture was incubated as indicated. The reaction was terminated by the addition of 10−20 vol of stop solution which contained 0.3 M Na-acetate (pH 5.1), 0.1% SDS, and yeast RNA (100 μg/ml), followed by phenol extraction. The enzyme activity was quantitated by measuring the radioactivity of the reaction product relative to that of the substrate RNA.

The reaction catalyzed by the M1 RNA precursor was performed in a reaction mixture (20 μl) containing 50 mM Tris−HCl (pH 7.6), 100 mM NH_4Cl, 60 mM $MgCl_2$, the tRNA$^{Asp}_1$ precursor (1.5×10^4 d.p.m., ~6.6 ng) and transcript RNA as indicated. The reaction was terminated as described above.

Assay for in vitro stability of M1 RNA precursor

The M1 RNA precursor was incubated with crude extract (S30) of *E. coli* Q13 and its stability was assayed as described by Sakamoto *et al.* (1983a).

Protein determination

Protein was determined with the Bio-Rad Protein Assay Kit, with BSA as a standard.

Preparation of the protein component of RNase P

The RNase P preparation (75 μg protein) purified from *E. coli* Q13 was dialyzed against 10 mM Tris−HCl (pH 7.8), 1 mM $MgCl_2$, 10 mM KCl, 7 mM 2-mercaptoethanol, 7 M urea and subsequently applied to a DEAE-cellulose column (0.8 × 7 cm) equilibrated with the same buffer. The protein component eluted in the flow-through fraction was dialyzed against 20 mM Tris−HCl (pH 7.8), 1 mM $MgCl_2$, 10 mM KCl, and 7 mM 2-mercaptoethanol.

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