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# Different cleavage sites are aligned differently in the active site of M1 RNA, the catalytic subunit of *Escherichia coli* RNase P

**(ribozyme/tRNA precursors/tRNA processing)**

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**ABSTRACT** **We have studied RNase P RNA (M1 RNA) cleavage of model tRNA precursors that are cleaved at two independent positions. Here we present data demonstrating that cleavage at both sites depends on the 2′-OH immediately 5′ of the respective cleavage site. However, we show that the 2-amino group of a guanosine at the cleavage site plays a significant role in cleavage at one of these sites but not at the other. These data suggest that these two cleavage sites are handled differently by the ribozyme. This theory is supported by our finding that the cross-linking pattern between M1 RNA and tRNA precursors carrying 4-thioU showed distinct differences, depending on the location of the 4-thioU relative to the respective cleavage site. These findings lead us to suggest that different cleavage sites are aligned differently in the active site, possibly as a result of different binding modes of a substrate to M1 RNA. We discuss a model in which the interaction between the 3′-terminal "RCCA" motif (interacting residues are underlined) of a tRNA precursor and M1 RNA plays a significant role in this process.**

RNase P is an endoribonuclease responsible for the maturation of the 5′ termini of the majority of all known tRNAs in prokaryotes as well as in eukaryotes (see refs. 1 and 2, and references therein). In *Escherichia coli*, RNase P consists of an RNA subunit and a small basic protein subunit, M1 RNA and C5, respectively. The catalytic activity is associated with the RNA, and cleavage of tRNA precursors *in vitro* does not require the presence of C5 (3), but the protein subunit is essential for activity *in vivo*. Considering the large number of different tRNA precursors in a cell, it is important to understand how this enzyme selects its cleavage site because cleavage at the correct position on tRNA precursors is crucial to generate functionally matured tRNA molecules.

RNase P recognizes the folding of the tRNA domain of a tRNA precursor, and studies (for reviews, see refs. 1 and 2) using different tRNA precursors suggest that there are several determinants on a tRNA precursor that dictate the location of the cleavage site. Among these is the 3′-terminal CCA, encoded in all *E. coli* tRNA genes (see ref. 4 and references therein), which base pairs with RNase P RNA (5, 6, 36). It has also been suggested that a guanosine at the cleavage site functions as a guiding nucleotide for RNase P (7–9). Recent studies indicate the existence of more than one binding site of precursor tRNA and tRNA on M1 RNA (refs. 10 and 11; S. Altman, personal communication). It has also been discussed that the active conformation of M1 RNA in the enzyme-substrate complex depends on the identity of the tRNA precursor (12, 13). To identify factors influencing cleavage site selection, we previously used model tRNA precursors that are cleaved at two positions (8, 9, 14). Here we have investigated the importance of specific chemical groups at and near these two independent RNase P cleavage sites and their influence on cleavage site selection. Our data demonstrate that the 2′-hydroxyl immediately 5′ of the cleavage site plays a significant role for cleavage irrespective of context near the site of cleavage. By contrast, the presence of the exocyclic amine of the guanosine immediately 3′ of the scissile bond influences cleavage significantly at one site but not at the other. These results and cross-linking data, with which we identified close contact points between the nucleotide 5′ of two different cleavage sites and M1 RNA, lead us to suggest that different cleavage sites are aligned differently in the active site. The importance of the base pairing between the 3′-terminal "RCCA" motif (interacting nucleotides are underlined) and M1 RNA in relation to these findings will be discussed.

## MATERIALS AND METHODS

**Preparation of Substrates and Enzyme.** Plasmids carrying the genes encoding pSu3–3XGC and pSu3–3XGA starting at the G at position +15 were constructed by PCR according to standard procedures using a primer complementary to residues +15 through +35 [numbering refers to pSu3 (15)] in pSu3–3XGC and pSu3–3XGA (9). This primer also carried the T7 RNA polymerase promoter sequence. The 3′ primer was complementary to a sequence downstream of the *Hin*dIII site in pUC19. The PCR products were blunt-end ligated into pUC19 digested with *Sma*I. Plasmid DNA was transformed and prepared according to standard protocols. The constructs were verified by DNA sequencing according to the method of Sanger and coworkers (16).

The RNA molecules were generated as run-off transcripts using T7 DNA-dependent RNA polymerase as described (15, 17). Transcription of the genes starting at G15 was performed in the presence of 5 mM GMP and 1 mM GTP to ensure incorporation of monophosphate at the 5′ end of the substrate (18). These RNA molecules were designated +15pSu3–3XGC and +15pSu3–3XGA.

RNA oligonucleotides harboring modified nucleotides at specific positions were purchased from Scandinavian Gene Synthesis (Köping, Sweden). For the ligation reaction (see below), ≈20 pmol was 5′-end labeled with [$\gamma$-$^{32}$P]ATP using T4 polynucleotide kinase (T4 PNK; United States Biochemical) according to standard procedures. Construction and generation of M1 RNA has been described elsewhere (19).

All RNA molecules (RNA oligonucleotides and RNA transcripts) were purified on denaturing polyacrylamide gels, and the RNAs of the correct sizes were excised and eluted according to standard protocols (20). The RNA molecules were renatured by incubation in 55°C for 5 min before further use.

**Preparation of RNA Precursors Harboring Modified Nucleotides at Specific Positions.** Precursors harboring modified nucleotides were prepared by ligation of two RNA fragments according to Moore and Sharp (ref. 21; Fig. 1*A*). A 5′-end labeled RNA oligonucleotide (a 27-mer corresponding to residues −10 in the 5′ leader through A14 in the D-loop)

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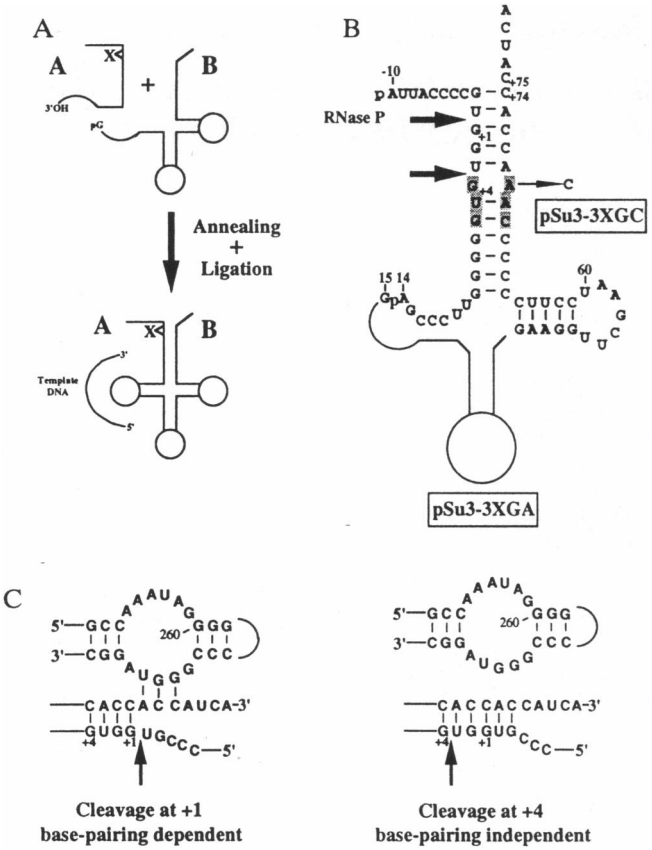


FIG. 1. The secondary structure of the different tRNA precursors used in this study. (*A*) Preparation of tRNA precursors harboring modified nucleotides at specific positions. The procedures were performed as outlined in the text. The 5′-end labeled modified RNA oligonucleotide corresponding to the 5′ part of the tRNA precursor (*A*) was annealed to the rest of the tRNA molecule (*B*) using a complementary DNA oligonucleotide template. The RNAs were ligated using T4 DNA ligase. X indicates the modified position. (*B*) Predicted secondary structure of the precursor derivatives to tRNA$^{Tyr}$Su3–3XGA and tRNA$^{Tyr}$Su3–3XGC (pSu3–3XGA and pSu3–3XGC, respectively) obtained using the approach described in *A*. These precursors all carry a 10-nt-long 5′ leader. Thick arrows indicate RNase P cleavage sites. Thin arrow indicates the difference between pSu3–3XGA and pSu3–3XGC. The numbering of the tRNA precursors refers to pSu3 (15). The shaded nucleotides correspond to the extra base pairs that were inserted in the original construction (8). (*C*) Illustration of the dependence of the base pairing between M1 RNA and its substrate for the two cleavage sites studied in this report.

carrying the modification was ligated to the second RNA fragment covering the rest of the tRNA molecule starting at position G15 (+15pSu3–3XGC or +15pSu3–3XGA; see above). These RNA molecules were annealed to a DNA oligonucleotide (Su3:2; ref. 15), complementary to the junction region in the D-loop, in 50 mM KCl/50 mM Tris·HCl (pH 7.9) by heating at 95°C for 5 min and then by cooling on ice. Ligation was performed in ligation buffer (21) supplemented with polyethylene glycol (PEG) 6000 (12–14% wt/vol), RNA guard (0.4 units/μl; Pharmacia) using T4 DNA ligase (1 unit/μl; Pharmacia). After incubation overnight at 16°C, the products were separated on a denaturing polyacrylamide gel and purified as described above. The resulting precursors all carry a 10-nt-long 5′ leader.

**M1 RNA Assay.** The M1 RNA activity was monitored as described elsewhere (3, 15) in our standard reaction buffer (50 mM Tris·HCl, pH 7.5/5% (wt/vol) PEG 6000/100 mM $NH_4Cl$/100 mM $MgCl_2$ or $CaCl_2$ as indicated). The rate of cleavage under single-turnover conditions was determined using 0.02 pmol/μl of substrate and 0.625 pmol/μl of M1 RNA. Measurements were carried out in the linear range of the reaction, and the single-turnover rate constant, $k_{obs}$, is given as picomole of substrate cleaved per picomole of enzyme per minute.

The reaction products were separated on 18% (wt/vol) denaturing polyacrylamide gels. The positions of the cleavage sites were inferred from the mobility of the 5′ cleavage products, and the frequency of cleavage at different positions was quantified from the relative amount of 5′ cleavage products generated from cleavage at the different positions using a PhosphorImager (Molecular Dynamics).

**Specific UV-Induced Cross-Linking Between M1 RNA and Precursor tRNA.** Precursor tRNAs (−10pSu3–3XGC) harboring 4-thioU at position −1 or +3 (Fig. 1) were generated using the ligation protocol described above. Approximately 20 μg of tRNA precursor was incubated with 2.5-fold molar excess of wild-type M1 RNA at 37°C for 10 min in 50 mM Tris·HCl, pH 7.0/100 mM $NH_4Cl$/100 mM $MgCl_2$ to allow enzyme-substrate complex formation. The mixture was UV irradiated using a long-wave UV lamp (366 nm) on ice for 30 min. After precipitation, the products were separated on 5% (wt/vol) denaturing polyacrylamide gels. Cross-linked species were excised from the gel and eluted as described above. This material was further analyzed by primer-extension using four primers complementary to residues 358–377, 280–306, 240–255, and 166–190 in M1 RNA (AV22, MLK37, SS4, and SS12, respectively (14, 19). For the analytical cross-linking experiments, we used 0.4 pmol of [γ-$^{32}$P]ATP 5′-labeled tRNA precursor and 1 pmol of M1 RNA.

## RESULTS

**Cleavage at Different Positions Depends on the 2′-Hydroxyl Immediately 5′ of the Scissile Bond.** We (8, 9, 14) previously used precursor derivatives of the tRNA$^{Tyr}$Su3 carrying three base pairs inserted in the acceptor-stem, pSu3–3XGA and pSu3–3XGC (Fig. 1), as model substrates to study cleavage site selection. These precursors are particularly suitable to investigate how M1 RNA selects its cleavage site, because they are initially cleaved at two positions, either at +1 or at +4. A major determinant for cleavage at the former position is the base pairing between the 3′-terminal RCCA motif and M1 RNA (Fig. 1*C*). For the latter position, in contrast, it is the length of the amino acid acceptor-stem that plays a major role (9, 14). Furthermore, although an increase in pH stimulates cleavage at +1 significantly, cleavage at +4 is essentially unaffected (14). These data indicate that these two cleavage sites are handled differently by M1 RNA. Several reports have demonstrated that the 2′-OH at the −1 position is important for cleavage (7, 22–25). To investigate whether cleavage at +1 and at +4 also depends on the 2′-OH immediately 5′ of the respective cleavage site, we introduced a deoxyU at either −1 or at +3 as outlined in Fig. 1*A*. These precursors carrying a 10-nt 5′ leader were cleaved by M1 RNA in the absence of C5. The cleavage site was identified from the mobility of the 5′-cleavage fragment. The results are summarized in Table 1. A "preferred" cleavage site is defined as the site where initial cleavage occurs (14). Accordingly, when initial cleavage is changed from one site to another, this change is referred to as a shift of cleavage site selection.

The "normal" precursors lacking any modified nucleotides were cleaved at two positions, +1 or +4, in keeping with our previous results. Precursors with a deoxyU either at −1 or at +3 were not cleaved at +1 and at +4, respectively, whereas cleavage at the other position was essentially unaffected. Cleavage was also observed at other positions as a result of the introduction of a deoxyU at either position. Previous data show that an increase in [$Mg^{2+}$] shifts the preferred cleavage site from +4 to +1 (14). However, an increase of [$Mg^{2+}$] to 1 M did not influence cleavage site selection on the deoxyU-substituted precursors (here we only used pSu3–3XGA; data

Table 1. Summary of the cleavage sites using different derivatives of pSu3-3XGC and pSu3-3XGA harboring modified nucleotides at the cleavage sites

| | Cleavage site [$Mg^{2+}$] = 100 mM | | | | | Cleavage site [$Ca^{2+}$] = 100 mM | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Precursor | −2 | +1 | +2 | +3 | +4 | −2 | +1 | +2 | +3 | +4 |
| pSu3-3XGA | | | | | | | | | | |
| Normal | | 84 | | | 16 | | 72 | 15 | | 13 |
| −1 deoxyU | | | 5 | | 95 | | | 43 | | 57 |
| +3 deoxyU | 5 | 93 | 2 | | | 12 | 73 | 15 | | |
| +1 Inosine | | 4 | 15 | 6 | 75 | | 6 | 75 | 4 | 14 |
| +4 Inosine | | 88 | 2 | | 10 | | 75 | 20 | | 5 |
| pSu3-3XGC | | | | | | | | | | |
| Normal | | 65 | | | 35 | | 45 | 4 | | 51 |
| −1 deoxyU | | | 4 | | 96 | | | 7 | | 93 |
| +3 deoxyU | 11 | 84 | 5 | | | 11 | 86 | 3 | | |
| +1 Inosine | | 5 | 12 | 10 | 73 | | 5 | 12 | 11 | 72 |
| +4 Inosine | | 38 | | | 62 | | 78 | 6 | 7 | 9 |

The frequency of cleavage at the different positions is given as percentage values and was quantitated as described in *Materials and Methods*. Each value is an average of several independent experiments.

not shown). These data suggest that cleavage at both +1 and +4 requires a 2′-OH immediately 5′ of these cleavage sites.

**The 2-Amino Group of the Guanosine 3′ of the Scissile Bond Affects Cleavage at One Position but Not at the Other.** Previous reports suggest that for the tRNA precursors used here, a guanosine at the cleavage site is a major determinant for the identification of the cleavage site (9, 14). To investigate why a guanosine is preferred at the respective cleavage site, we introduced an inosine either at position +1 or at position +4 as outlined in Fig. 1*A*. Note that the difference between an inosine and a guanosine is that the former lacks the exocyclic amine. The results are shown in Fig. 2 and summarized in Table 1.

An introduction of an inosine at the +1 position resulted in a significant reduction of cleavage at this position, whereas the rate of cleavage at the +4 position was increased. This was observed irrespective of precursor (Table 2). By contrast, the pSu3–3XGA precursor carrying an inosine at +4 was cleaved to the same extent at this position as the precursor harboring a +4G, whereas a shift of the main cleavage site was observed using pSu3–3XGC with an inosine at the +4 position. However, this precursor was still cleaved significantly at +4. Interestingly, the substitution of the +1G with an inosine resulted in cleavage also at positions +2 and +3. This was observed using either pSu3–3XGA or pSu3–3XGC. Determinations of the rate constant under single-turnover conditions for cleavage of the various pSu3–3XGA derivatives were consistent with these findings (Table 2). We also studied cleavage site selection as a function of increasing [$Mg^{2+}$] due to reasons discussed above. However, no change in the cleavage pattern of these pSu3–3XGA derivatives was observed even if the [$Mg^{2+}$] was increased to 1 M (data not shown). From these results, we conclude that the 2-amino group of the guanosine at the cleavage site is not essential for the mechanism of cleavage, but it plays a significant role in cleavage site selection in a context-dependent manner. Our data presented here also suggest that a likely reason why most tRNAs (>80%) carry a guanosine at the +1 position (26) is to provide a 2-amino group in the correct position in the active site of M1 RNA. Several determinants dictate the localization of the cleavage site (2). Therefore, other determinants might compensate for the loss of the 2-amino group of the guanosine at +1 in those tRNA precursors that lack a G at this position.

**Cleavage Site Selection and the Nature of the Divalent Metal Ion.** Replacement of $Mg^{2+}$ with $Ca^{2+}$ can result in a shift of the preferred cleavage site, depending on the structure at

Table 2. Single-turnover rate constants for cleavage of pSu3-3XGA derivatives substituted with inosine at the different cleavage sites, +1 or +4

| Cleavage site | Precursor | | |
|---|---|---|---|
| | 3XGA | 3XGA + 1I | 3XGA+4I |
| +4 | 0.0067 | 0.0135 | 0.0062 |
| +3 | | 0.004 | |
| +2 | | 0.008 | |
| +1 | 0.023 | 0.0058 | 0.013 |

Cleavage was performed as described in *Materials and Methods*. Values for $k_{obs}$ are given as picomole of substrate cleaved per picomole of enzyme per minute.

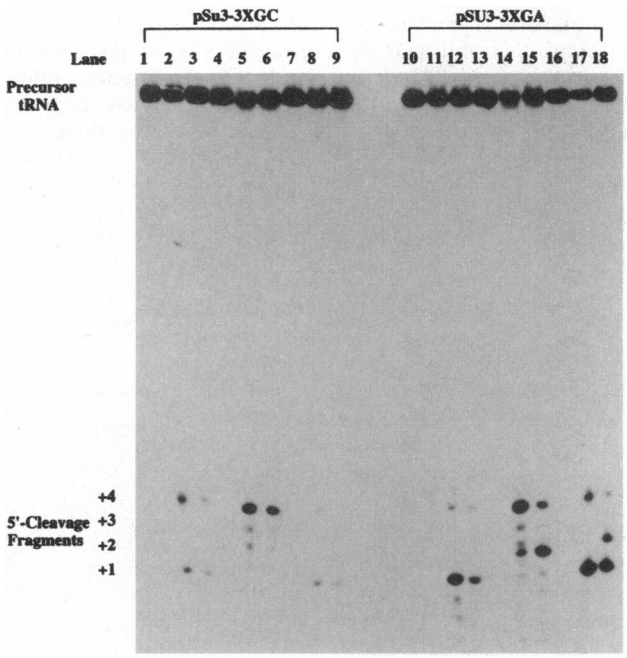


FIG. 2. Processing *in vitro* of different precursors carrying inosine at the respective cleavage site by wild-type M1 RNA. The cleavage was performed as outlined in *Materials and Methods*. The final concentrations of reactants were as follows: precursor ≈0.05 pmol/μl, M1 RNA ≈0.41 pmol/μl. The cleavage products were separated from the precursors on a 18% denaturing polyacrylamide gel. Lanes 1–9, derivatives of pSu3–3XGC. Lanes 10–18, derivatives of pSu3–3XGA. Lanes 1, 4, 7, 10, 13, and 16, no enzyme added. Lanes 2, 3, 5, 6, 8, 9, 11, 12, 14, 15, 17, and 18, M1 RNA added. Lanes 1–3 and 10–12, "normal" unmodified precursors. Lanes 4–6 and 13–15, precursors carrying an inosine at +1. Lanes 7–9 and 16–18, precursors carrying an inosine at +4. Lanes 1, 2, 4, 5, 7, and 8, [$Mg^{2+}$] = 100 mM, time of incubation (toi) = 90 min. Lanes 3, 6, and 9, [$Ca^{2+}$] = 100 mM, toi = 120 min. Lanes 10, 11, 13, 14, 16, and 17, [$Mg^{2+}$] = 100 mM, toi = 30 min. Lanes 12, 15, and 18, [$Ca^{2+}$] = 100 mM, toi = 60 min.

and near the cleavage site (14). To investigate this in more detail, we studied the influence of the 2-amino group and the 2′-OH at the cleavage site on cleavage site selection by performing the same experiment as described above in the presence of $Ca^{2+}$. The results are shown in Fig. 2 and Table 1.

No significant differences were observed in cleavage of the deoxyU-substituted precursors as a result of replacing $Mg^{2+}$ with $Ca^{2+}$ except that pSu3–3XGA was cleaved significantly at the +2 position in the presence of $Ca^{2+}$. Furthermore, cleavage of both pSu3–3XGA and pSu3–3XGC at +1 was clearly sensitive to the absence of the 2-amino group at this position. By contrast to the result observed in the presence of $Mg^{2+}$, cleavage at +4 was also significantly affected by the absence of the 2-amino group at this position. We also observed significant cleavage of the various inosine-substituted precursors at +2 and +3. In particular, the main cleavage site on pSu3–3XGA carrying an inosine at +1 was shifted from +4 to +2, compared with cleavage in the presence of $Mg^{2+}$. These data suggest that specific chemical groups at the cleavage site are responsible for the different structural requirements for cleavage in the presence of different divalent metal ions. This might reflect different binding of these two metal ions in the vicinity of the cleavage site (14). However, $Mg^{2+}$ and $Ca^{2+}$ can also affect the structure of M1 RNA or the enzyme-substrate complex differently. Nevertheless, this suggests that the M1 RNA-catalyzed reaction requires $Mg^{2+}$ not only to promote efficient cleavage rates (27, 28) but also to obtain cleavage at the correct position.

**Cross-Linking Indicates That Different Cleavage Sites Are Positioned Differently in the Enzyme-Substrate Complex.** The differential response with respect to cleavage at +1 and at +4 as a function of replacing G with inosine suggests structural differences at and near these cleavage sites in the enzyme-substrate complex. Therefore, to identify close contact points between these two cleavage sites and M1 RNA by cross-linking, a photoreactive group, 4-thioU, was introduced at either position −1 or position +3 in pSu3–3XGC (see above). This precursor was suitable to use because it is cleaved very slowly, which improved the efficiency of cross-linking (9, 14). Furthermore, introduction of 4-thioU did not change the cleavage patterns (data not shown). The modified precursors were cross-linked to M1 RNA, and the cross-linked residues in the ribozyme were mapped (*Materials and Methods*). The results are shown in Fig. 3 and summarized in Fig. 4.

Three cross-linked products were observed both when 4-thioU was located at −1 or at +3 (Fig. 3*A*). Primer-extension analysis revealed that all products represented cross-linked enzyme-substrate complexes. Irrespective of whether the 4-thioU was located at −1 or at +3, several cross-linked residues in M1 RNA were detected. These were mapped to two major regions, 248–256 and 331–337 (Fig. 4). In keeping with previous work, cross-links to these residues have also been observed using tRNA precursors carrying a photoreactive group in the vicinity of the cleavage site (30, 31). This suggest that we are looking at the cross-links in a complex that is the result of productive interactions between M1 RNA and its substrate. Comparison of the cross-linking patterns for the two cases revealed two striking differences. First, the cross-link between the 4-thioU at the +3 position and residues G332 and A333 in M1 RNA was significantly reduced compared with when the precursor harboring the 4-thioU at the −1 position was used (Fig. 3*B*). These cross-linked residues were only detected in the cross-linked product corresponding to band 1, whereas the other cross-linked residues in M1 RNA were observed in all three cross-linked products (Fig. 3*A*). These findings suggest that G332 and A333 are in close contact with the nucleotide immediately 5′ of the +1 cleavage site in the enzyme-substrate complex, whereas these residues in M1 RNA and the uridine at +3 are positioned in such way that cross-linking becomes significantly reduced. Second, the cross-link to G262 was only observed using the precursor with the 4-thioU located at the −1 position (Fig. 3*C*). The cross-linking pattern for the other residues in M1 RNA were approximately the same irrespective of the location of the 4-thioU. These data suggest that the structural organization in the vicinity of these two cleavage sites is different in the enzyme-substrate complex.

## DISCUSSION

Here we have investigated cleavage site selection by the catalytic subunit of *E. coli* RNase P, M1 RNA, as a function of different chemical groups at and near the cleavage site using

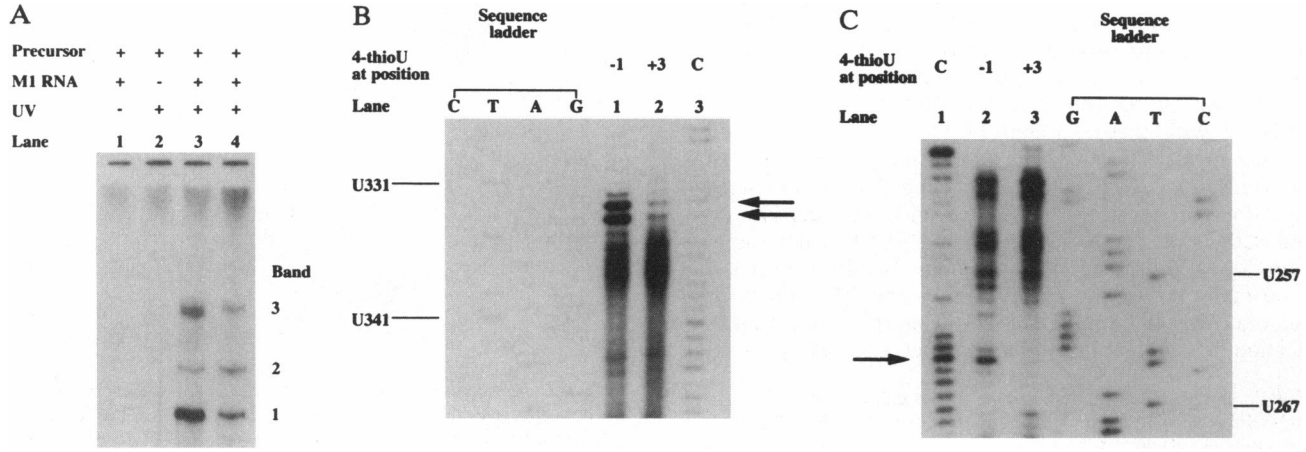


FIG. 3. Cross-linking of wild-type M1 RNA and pSu3–3XGC harboring a 4-thioU at position −1 or position +3. The experiments were performed as outlined in the text. (*A*) 5′-end labeled pSu3–3XGC carrying 4-thioU at a specific position was incubated with 2.5-fold molar excess of M1 RNA and cross-linked by UV irradiation (366 nm). The cross-linked products were separated on a 5% denaturing polyacrylamide gel. Bands 1–3 indicate the major cross-linked complexes between M1 RNA and pSu3–3XGC. M1 RNA and pSu3–3XGC were mixed and UV irradiated as indicated. Lanes 1–3, pSu3–3XGC carrying 4-thioU at −1; lane 4, pSu3–3XGC carrying 4-thioU at +3. (*B* and *C*) Mapping of the nucleotides in M1 RNA involved in cross-linking with the substrate by primer extension using AV22 as primer (complementary to residues 358–377 in M1 RNA) (*B*) and MLK37 as primer (complementary to residues 280–306 in M1 RNA) (*C*). The analysis was performed as outlined in the text. Primer-extension reactions were separated on an 8% denaturing polyacrylamide gel. The photoreactive agent 4-thioU was located at −1 or at +3 as indicated. C, control experiments [i.e., primer extension of M1 RNA that had not been cross-linked to the substrate (*B*, lane 3, and *C*, lane 1)]. The sequence ladder was applied as indicated, and U331, U341, U257, and U267 refer to the structure of M1 RNA (see Fig. 4). Differences in the cross-linking patterns are indicated with arrows.

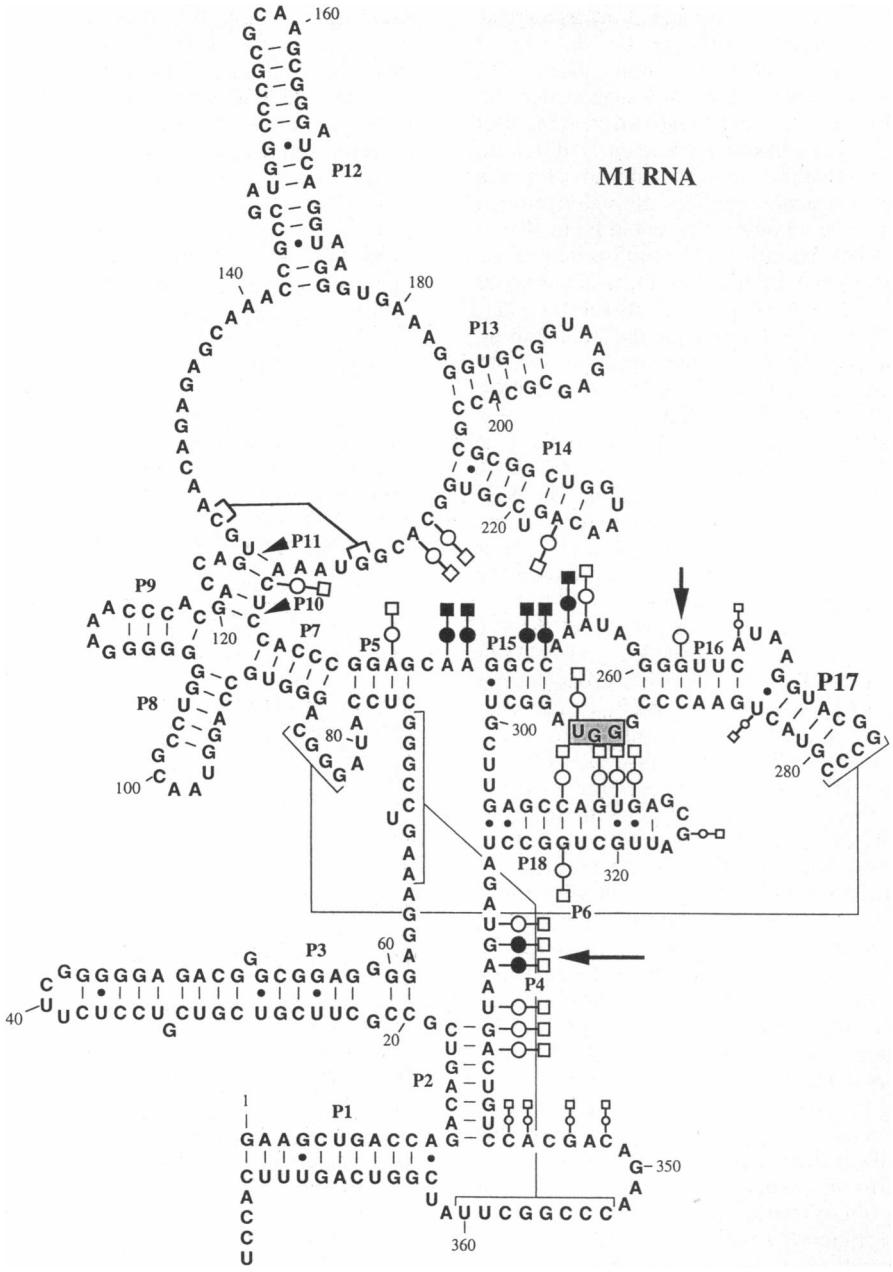


FIG. 4. Secondary structure of M1 RNA according to Brown and coworkers (29) and a summary of residues that were cross-linked to 4-thioU positioned either at −1 or at +3 in pSu3–3XGC. ●, residues in M1 RNA cross-linked to 4-thioU at −1 in the substrate; ■, residues in M1 RNA cross-linked to 4-thioU at +3 in the substrate; ■ and ●, major cross-links; □ and ○, less efficient cross-links; ▫ and ◦, minor cross-links. Arrows indicate the positions where we observed distinct differences in the cross-linking pattern, depending on the position of the 4-thioU in the substrate. This is a summary of several independent experiments, and only residues for which the results were reproducible are included.

precursors that are normally cleaved at two positions (+1 or +4; Fig. 1*B*). Cleavage at +1 was significantly reduced by removing the 2-amino group of the guanosine 3′ of this cleavage site. By contrast, removal of the same group from the guanosine at +4 did not affect cleavage at this position to any significant extent. Furthermore, the cross-linking pattern showed distinct differences using tRNA precursors carrying the photoreactive agent 4-thioU at −1 or at +3 (Fig. 4). In addition, cleavage at +1 increases significantly, whereas cleavage at +4 is essentially unaffected with increasing pH (14). Thus, these data suggest that these cleavage sites are handled differently by the enzyme. Cleavage at either of these positions results in a "5′-matured" tRNA carrying a 5′-phosphate and a 3′-hydroxyl, as is the case for cleavage of normal tRNA precursors (1). Furthermore, the 2′-OH immediately 5′ of the scissile bond has been suggested to function as a ligand for a functionally important $Mg^{2+}$, and the importance of this hydroxyl was demonstrated irrespective of cleavage site. This result is in keeping with previous observations, where other precursors were used (7, 22–25). We cannot exclude the possibility that substitution of a 2′-OH 5′ of the cleavage site leads to a change in the interaction with M1 RNA. Taken together, we consider it unlikely that the differential effects observed for cleavage at the two sites is due to differences in the chemistry of cleavage. Instead, we suggest that our results reflect differences in the structural arrangements at and near the respective cleavage site in the enzyme-substrate complex.

The 2-amino group of guanosine at the cleavage site was shown to be important for cleavage at one site but not at the other. There is a possibility that this exocyclic amine could function as a ligand for $Mg^{2+}$. However, the exocyclic amine of a guanosine is generally not used as a ligand for $Mg^{2+}$ (32).

Second, we observed no increase in cleavage at +1 as a result of increasing [$Mg^{2+}$]. Note that removal of a possible $Mg^{2+}$ ligand in the substrate results in an increase in the optimal [$Mg^{2+}$] (7). We consider this possibility unlikely. Our data suggest that the positioning of M1 RNA residues relative to the two cleavage sites (+1 and +4) in the enzyme-substrate complex is different. Therefore, it is conceivable that the exocyclic amine at +1 makes a functionally important interaction, perhaps through hydrogen bonding with M1 RNA. Such an interaction could be mediated through C247 in M1 RNA because it is positioned in close proximity to the cleavage site in the two three-dimensional structural models of M1 RNA in complex with its substrate (11, 30). Furthermore, cleavage at +1 relies on the base pairing between the 3′-terminal RCCA motif of the precursor and M1 RNA, whereas this is not the case for cleavage at +4 (see below; refs. 9 and 14; Fig. 1*C*). Since the 2-amino group at +1 is important for cleavage at this position, we suggest that an efficient interaction of a 2-amino group at the cleavage site and M1 RNA also depends on the RCCA–M1 RNA interaction. The RCCA–M1 RNA interaction has been suggested to unfold the precursor such that the cleavage site is exposed (5). As a consequence, this might also result in an interaction between M1 RNA and the 2-amino group at the +1 position.

Our cross-linking data suggest that the uridine at the −1 position is in close contact with residues G332 and A333 in M1 RNA. By contrast, a significant reduction in cross-linking to these residues was observed with the precursor carrying the 4-thioU at the +3 position. Cross-linking to these residues has also been observed using a matured $tRNA^{Phe}$ carrying an azidophenacyl group at the 5′ termini or tRNA precursors harboring this photoreactive agent at different positions in the vicinity of the cleavage site (30, 31, 33). In addition, chemical modification studies suggest that the −1 residue in a tRNA precursor is in close contact with these residues in M1 RNA (34). In fact, in the three-dimensional structural models of M1 RNA in complex with its substrate G332 and A333 are located on the other side of the cleavage site relative to the positioning of C247 (11, 30). As discussed above, cleavage at +1 relies on the presence of the 3′-terminal CCA sequence (Fig. 1*C*). Mutant M1 RNA molecules harboring changes in the region involved in base pairing with this motif cleave the pSu3–3XGA precursor mainly at the +4 position, whereas wild-type M1 RNA cleaves this precursor preferentially at the +1 position (Table 1; unpublished data). Cross-linking experiments similar to those presented here, using one of these mutant M1 RNAs, show similar cross-linking efficiency relative to G332 and A333 irrespective of the location of the 4-thioU (unpublished data). The combined data suggest a model for cleavage at +1 where the base pairing between the 3′-terminal RCCA motif of the tRNA precursor and M1 RNA is involved in correct positioning of G332 and A333 close to the nucleotides at the +1 cleavage site such that efficient cleavage is ensured. This model does not apply to cleavage at the other position, +4, because cleavage at this position depends more on the distance between the T-loop and the cleavage site (9, 14). The importance of this interaction is supported by the finding that a tRNA precursor lacking the 3′-terminal CCA motif is cross-linked to different residues in M1 RNA compared with when this sequence element is present (12). We note that A333 is not essential for cleavage because a mutant M1 RNA, harboring a C at this position, is still active (35). However, our preliminary results suggest that substitutions of G332 affects cleavage site selection. At present, we do not know whether there is a direct interaction between the −1U and this region of M1 RNA. A majority (≈67%) of the tRNA precursors in *E. coli* harbor a −1U, and only ≈15% carry a purine at this position (see ref. 4 and references therein). In addition, a majority of bacterial RNase P RNAs characterized so far carry purines at the positions corresponding to 332 and 333 in M1 RNA (see ref. 29 and references therein). Interaction between the uridine at −1 and G332 (or A333) is therefore not excluded. To gain further insight into the way different tRNA precursors interact with RNase P RNA requires detailed functional as well as structural analysis using different M1 RNA derivatives and various wild-type tRNA precursors.

In summary, the possibility of more than one binding mode of a tRNA domain to M1 RNA has recently been suggested (refs. 10 and 11; S. Altman, personal communication) as well as that the active conformation of M1 RNA depends on the identity of the substrate (12, 13). Thus, we suggest that different cleavage sites are aligned differently in the active site, possibly as a consequence of different binding modes of a substrate to M1 RNA.

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