Determinants of *Escherichia coli* RNase P cleavage site selection: a detailed *in vitro* and *in vivo* analysis

Staffan G. Svärd and Leif A. Kirsebom*
Department of Microbiology, Box 581, Biomedical Center, S-751 23 Uppsala, Sweden

Received November 16, 1992; Revised and Accepted January 11, 1993

ABSTRACT

The location of the *Escherichia coli* RNase P cleavage site was studied both *in vitro* and *in vivo*. We show that selection of the cleavage site is dependent on the nucleotide at the cleavage site and the length of the acceptor-stem. Within the acceptor-stem the number of nucleotides on the 5'-half of the acceptor-stem appears to be the important determinant, rather than the number of base pairs in the acceptor-stem. We also demonstrate that the length of the T-stem and a G to C substitution at position 57 in the tRNA<sup>Sup3</sup> precursor influence the location of the cleavage site under certain conditions. With respect to the function of the subunits of RNase P our data suggest that the nucleotide at position 333 in M1 RNA, and the C5 protein, are important for the identification of the cleavage site.

INTRODUCTION

RNase P is an endonuclease which cleaves precursor tRNAs and generates mature 5'-termini for all known tRNAs in prokaryotes. The catalytic subunit of RNase P is an RNA, in *Escherichia coli* M1 RNA. The other subunit in *Escherichia coli*, the C5 protein, is not required for cleavage *in vitro* but is necessary for cleavage *in vivo* (1 and references therein). It is unknown how the enzyme targets the cleavage site on the large number of tRNA precursors it encounters *in vivo*. Previous reports have demonstrated that the length and primary structure of the amino acid acceptor-stem of the tRNA<sup>Sup3</sup> precursor is an important determinant in this process (2, 3). Furthermore, it has been proposed that the location of the site of cleavage is the result of a measuring of the length of the acceptor-stem and T-stem of a tRNA precursor (4–6). However, we showed recently that the location of the *E. coli* RNase P cleavage site on the tRNA<sup>Sup3</sup> precursor (pSu3) does not merely depend on the lengths of the acceptor-stem and T-stem *in vitro*. We demonstrated the importance of the identity of the nucleotides at and near the cleavage site and we proposed that the nucleotide at the site of cleavage functions as a guiding nucleotide. Furthermore, our results suggested that the length of the acceptor-stem has some role in identifying the cleavage site (7). Here we extend our analysis and show both *in vivo* and *in vitro* the importance of the identity of the nucleotide at the cleavage site and the length of the amino acid acceptor-stem of a tRNA precursor. Within the acceptor-stem it appears that the number of nucleotides in the 5'-half is the important determinant, rather than the number of base pairs in the acceptor-stem. In addition, our data suggest that the length of the T-stem and the nucleotide at position 57 in the T-loop influence the location of the cleavage site on a tRNA precursor. From our results it also appears that the C5 protein and the nucleotide at position 333 in M1 RNA are important for selection of the cleavage site. These results will be discussed with respect to the identification of the site of cleavage.

MATERIALS AND METHODS

Preparation of substrates and enzyme

The construction of tRNA<sup>Sup3</sup> gene behind the phage T7 promoter has previously been described (3). The different mutant derivatives of the tRNA<sup>Sup3</sup> gene were constructed using the polymerase chain reaction [PCR (8)] where one of the oligonucleotides carried a base-substitution(s). The resulting PCR-amplified gene(s) was cut with EcoRI and HindIII, and ligated into pUC19 which had been digested with the same enzymes. Plasmids were transformed according to standard procedures. Verification of the mutation(s) was done by DNA sequencing according to the method of Sanger (9). The various tRNA precursors of the sizes indicated in Figure 1 were prepared *in vitro* as run-off transcripts using T7 DNA-dependent RNA polymerase (7, 10). Preparation of wild-type and mutant M1 RNA molecules, and C5 protein were performed as described elsewhere (11, 12). The plasmid carrying the MIC333 RNA behind the T7 promoter was kindly provided by Dr C. Guerrier-Takada.

RNase P assay

The RNase P and M1 RNA activities were monitored as previously described (7, 12–14). Cleavage reactions for M1 RNA or for the holoenzyme were also performed under identical buffer conditions: 50 mM Tris–HCl (pH 7.5), 5% (w/v) polyethylene glycol 6000, 100 mM NH₄Cl and 20 mM MgCl₂ (15).
Verification of the cleavage site and quantitation of the frequency of cleavage at different positions

The precursors were labelled with \([\alpha-32P]GTP\) or \([\alpha-32P]UTP\). The choice of which \([\alpha-32P]P\)NTP was used to label a precursor depended on which cleavage site we wanted to verify. After cleavage with M1 RNA or reconstituted holoenzyme the reaction products were separated on an 8% polyacrylamide gel in 7 M-urea and TEB-buffer (the reaction was stopped before the tRNA precursors were completely processed). The 5' cleaved tRNAs were excised and eluted from the gel in TE-buffer (16). The purified processed tRNA molecules were digested to completion with RNase T1, RNase T2 and pancreatic RNase A, and the products were analysed by 2-dimensional thin-layer chromatography according to Nishimura (17) in order to detect the 5'-phosphate-carrying nucleotide.

Quantitation of the frequency of cleavage at different positions was performed as described in detail elsewhere (7).

Genetic constructions and determination of the efficiency of suppression

The *E. coli* strain BL21(DE3) harbouring \(\Delta(proBlac)zah::Tn10\), which was used to determine the efficiency of suppression, has been described in a previous report (3).

To determine the efficiency of suppression, we used the nonsense codon, UAG, at position 189 in the *lacZ* system (18). Growth and assay conditions were as described elsewhere (19).

Analysis of RNase P cleavage in vivo

Northern blot and primer-extension analysis were performed as previously reported (3) using the following DNA oligonucleotide: 5'-TTCGAAGTCGATGACGGACAGATTTAG-3' [complementary to positions +34 through +51 in tRNA\(^{\text{Tyr}}\)Su3 (for numbering see Fig. 1)]. The primer-extension analysis was performed using the same amount of RNA extracted from cells carrying the respective mutant tRNA\(^{\text{Tyr}}\)Su3 gene behind the T7 promoter, as described elsewhere (3).

RESULTS

The length of the amino acid acceptor-stem together with a guanosine at the site of cleavage are important determinants for *E. coli* RNase P cleavage site selection

Previously it has been proposed that identification of the *E. coli* RNase P cleavage site is determined by the length of the amino acid acceptor-stem and T-stem (5, 6). This predicts that increasing the length of the acceptor-stem would shift the cleavage site accordingly. However, extension of the acceptor-stem of the tRNA\(^{\text{Tyr}}\)Su3 precursor (pSu3) with 3 base pairs results in a precursor which, upon cleavage by M1 RNA or RNase P, yields mainly a 5' cleaved tRNA with a 10 base pair (bp) long acceptor-stem rather than the expected 7 bp. Minor cleavage at a second position (+4) generating a tRNA with a 7 bp long acceptor-stem was also observed (7; Fig. 2). This pSu3 derivative (pSu3-3XUA) carries a G at the main cleavage site (position +1) and a U at the minor site (position +4, see Fig. 1). To test whether a guanosine at the cleavage site together with the length of the acceptor-stem are important for the location of the cleavage site we substituted the +4U with a G, A or C (see Fig. 1). These mutant precursors were cleaved by M1 RNA in the absence and presence of C5, the protein subunit of RNase P. The sites of cleavage were inferred from the mobilities of the 5'-cleavage products (which, if cleavage occurred at the +1 position, would migrate at the same position as the 5'-cleavage product generated in the cleavage reaction of the wild-type tRNA\(^{\text{Tyr}}\)Su3 precursor (7; Fig. 2)). Verification of the cleavage sites and quantitation of the frequency of cleavage at different positions were done as outlined in Materials and Methods. The results are shown in Figure 2 and are summarized in Figure 5.

Replacement of the uridine at the +4 position with a guanosine (pSu3-3XGA) resulted in cleavage at two positions, +1 and +4. The frequencies of cleavage at positions +1 and +4 were approximately 60% and 40%, respectively, and were independent of C5. In contrast, cleavage of precursors harbouring a A or C at this position occurred at the +1 position as determined from the mobilities of the 5'-cleavage fragments. However, cleavage of these precursors generated two species of 5'-cleaved tRNAs. Analysis of the cleavage sites demonstrated that cleavage also occurred at position +4 (Fig. 5). These results indicate that these tRNA precursors were first cleaved at the +1 position generating products which could function again as substrates. Cleavage of pSu3-3XGA at position +1 also generated a product which was subsequently cleaved at position +4. In addition, we restored base-pairing at position +4 in pSu3-3XGA by changing the A

![Figure 1. Predicted secondary structure of the precursor to tRNA\(^{\text{Tyr}}\)Su3-3XUA (pSu3-3XUA) which carries three extra base-pairs: two U:A base pairs and one G:C base pair in the acceptor-stem. The changed nucleotides are indicated, where $\Delta =$ deletion and $\triangledown =$ insertion. The wild-type tRNA\(^{\text{Tyr}}\)Su3 precursor is designated pSu3, whereas the different mutant precursors are designated as shown in the text. Arrows indicate the RNase P cleavage sites. The numbering of the tRNA precursor is according to Sprindl et al. (22).](image-url)
In the 3'-half of the acceptor-stem to a C, as depicted in Figure 1. The resulting precursor, pSu3-3XGC, was cleaved mainly at position +4 with some residual cleavage at position +1 in the absence of C5, whereas addition of C5 resulted in cleavage at both position +1 and +4 with approximately equal frequency. Taken together, these results suggest that a G at the cleavage site together with the length of the acceptor-stem of a tRNA precursor are important determinants for selection of the site of cleavage by E.coli RNase P. Our results also suggest that a G:C bp at the site of cleavage affects the location of the cleavage site. However, it is not the presence of a C at the position opposite +4 since pSu3-3XUC (Fig. 1) was cleaved only at position +1 (Fig. 2 lanes 12 and 19). Note that disruption of base-pairing in the acceptor-stem resulted in an increase in the rate of cleavage. The slower migration of pSu3-3XGC and 5'-matured tRNA™5'Su3-3XUA is most likely due to residual RNA secondary structure which has previously been observed in denaturing polyacrylamide gels (20).

If a guanosine at the cleavage site together with the length of the acceptor-stem is important for the location of the cleavage site then a deletion of one of the G:C base-pairs of the acceptor-stem of pSu3-3XGA downstream of position +4 (Fig. 1) would abolish cleavage at this position. Indeed, the resulting mutant precursor (pSu3-3XGAGC) was cleaved only at position +1 by both forms of the enzyme (Fig. 3a, lane 8 and Fig. 5). Thus, the length of the acceptor-stem is important for the selection of the cleavage site. Cleavage at the +4 position was also abolished when we deleted only the guanosine on the 5'-half of the acceptor-stem downstream position +4 as indicated in Figure 1 (Fig. 3a, lane 9 and Fig. 5). Deletion of the cytosine on the 3'-half of the acceptor-stem resulted in cleavage at position +1. However, in

---

**Figure 2.** Processing in vitro of different precursors by wild-type M1 RNA and reconstituted holoenzyme at 37°C. The cleavage activities were monitored as outlined in Materials and Methods. The final concentrations of the reactants were respectively, no enzyme added, time of incubation (toi) = 120 min.

---

**Figure 3.** Processing in vitro of different precursors by wild-type M1 RNA and reconstituted holoenzyme at 37°C. The experimental conditions were as outlined in Materials and Methods and in Figure 2 legend. A. Lanes 1 to 6; pSu3-3XGA, pSu3-3XGAGC, pSu3-3XGAAAG, pSu3-3XGAAAGC, pSu3-3XGAGC+[T-GC]-10, respectively, no enzyme added, time of incubation (toi) = 120 min. Lanes 7 to 12, M1 RNA added; lane 7 = pSu3-3XGA, toi = 15 min, lane 8 = pSu3-3XGAGC, toi = 15 min, lane 9 = pSu3-3XGAAAG, toi = 15 min, lane 10 = pSu3-3XGAAAGC, toi = 120 min, lane 11 = pSu3-3XGAGC+[T-GC]-10, toi = 120 min and lane 12 = pSu3-3XGAAAGC+[T-GC]-10, toi = 120 min. Lane 13 (reconstituted holoenzyme added); lane 14 = pSu3-3XGA, toi = 40 min, lane 15 = pSu3-3XGAGC, toi = 40 min, lane 16 = pSu3-3XGAAAGC, toi = 40 min, lane 17 = pSu3-3XGAAAGC+[T-GC]-10, toi = 40 min, lane 18 = pSu3-3XGAAAGC, toi = 120 min and lane 19 = pSu3-3XGAAAGC+[T-GC]-10, toi = 120 min. B. Lanes 1 and 2; pSu3-3XGAAAC and pSu3-3XGAAAGC+[T-GC], no enzyme added, time of incubation (toi) = 93 min. Lanes 3 to 7, M1 RNA added; lane 3 = pSu3-3XGAAAC, toi = 15 min, lane 4 = pSu3-3XGAAAGC, toi = 90 min, lane 5 = pSu3-3XGAAAGC+[T-GC], toi = 15 min, lane 6 = pSu3-3XGAAAGC+[T-GC], toi = 90 min, lane 7 = pSu3-3XGAAAC, toi = 15 min. C. Lanes 1 to 4; pSu3-3XGA-C57, pSu3-3XGAAAGC, pSu3-3XGAAAGC+[T-GC], respectively, no enzyme added, time of incubation (toi) = 128 min. Lanes 5 to 9, M1 RNA added; lane 5 = pSu3-3XGA-C57, toi = 180 min, lane 6 = pSu3-3XGAAAGC, toi = 120 min, lane 7 = pSu3-3XGAAAGC, toi = 120 min, lane 8 = pSu3-3XGAAAGC, toi = 30 min and lane 9 = pSu3-3XGAAAGC, toi = 40 min.
Figure 4. Processing *in vitro* of pSu3 and pSu3-C57 by various M1 RNAs in the absence and presence of C5 at 37°C. Also shown is the predicted secondary structure of M1 RNA according to Brown and Pace (34). The substitutions are indicated by boxes, and shaded nucleotides are those involved in pseudoknot formation. The cleavage activities were monitored as outlined in Materials and Methods. The final concentrations of the reactants were as follows: precursors, ∼0.11 pmol/μl; M1 RNA in the absence of C5 0.082 pmol/μl and 0.0082 pmol/μl in the presence of C5. Cleavage by M1 RNA and by the holoenzyme was performed under identical buffer conditions, see Materials and Methods. The cleavage products and the precursors were separated on an 10.5% denaturing polyacrylamide gel. Lanes 1 and 2; pSu3-C57 and pSu3, respectively, no enzyme added, time of incubation (toi) = 130 min. Lanes 3 to 6, in the absence of C5; lane 3 = pSu3, wild-type M1 RNA, toi = 60 min; lane 4 = pSu3-C57, wild-type M1 RNA, toi = 125 min; lane 5 = pSu3-C57, M1G294 RNA, toi = 125 min; lane 6 = pSu3-C57, M1C333 RNA, toi = 125 min. Lanes 7 to 10, in the presence of C5; lane 7 = pSu3, wild-type M1 RNA, toi = 15 min; lane 8 = pSu3-C57, wild-type M1 RNA, toi = 30 min; lane 9 = pSu3-C57, M1G294 RNA, toi = 30 min; lane 10 = pSu3-C57, M1C333 RNA, toi = 50 min.

particular in the absence of C5 the resulting 5'-matured tRNA was cleaved a second time at the +4 position (Fig. 3b, lanes 3 and 4; Fig. 5). We also prepared and tested truncated derivatives of pSu3-3XGA and pSu3-3XGAΔGC. These precursors, pSu3-3XGA-10 and pSu3-3XGAΔGC-10, lacked 12 nucleotides at their 3'-ends (Fig. 1). Recently we showed by structural probing that a similar 3'-truncated tRNA*Su*3 derivative was single stranded on both sides the cleavage site and no significant structural changes in other regions of this precursor was observed when compared to the wild-type precursor (7). In the absence of C5, pSu3-3XGA-10 was cleaved mainly at position +4 with residual cleavage at positions +1 and +6 as well as at a position in the 5'-leader (Fig. 3c, lane 6 and Fig. 5). The other truncated precursor, shortened by a G:C bp downstream position +4 (pSu3-3XGAΔGC-10), was not cleaved at position +4. Instead, significant cleavage at positions +1, +2 and +3 was observed in the absence of C5 (Fig. 3a, lane 10 and Fig. 5). This precursor was also cleaved in the 5'-leader. In conclusion, the combined data suggest that a guanosine at the cleavage site together with the number of nucleotides in the 5'-half of the amino acid acceptor-stem are important in identifying the *E. coli* RNase P cleavage site.

The length of the T-stem and the location of the cleavage site

The selection of the *E. coli* RNase P cleavage site was proposed to be dependent on the length of the acceptor-stem and T-stem (5, 6). This predicts that the cleavage sites on pSu3-3XGAΔGC and pSu3-3XGAΔGC-10 should be shifted to the +4 position by an extension of the T-stem with one base-pair. To test this we extended the T-stem of pSu3-3XGAΔGC and pSu3-3XGAΔGC-10 with one G:C base-pair (Fig. 1). Cleavage of the resulting precursors, pSu3-3XGAΔGC+[T-GC] and pSu3-3XGAΔGC-10+[T-GC], are shown in Figures 3a and 3b, and are summarized in Figure 5. The former precursor was cleaved first at the +1 position generating a product which was subsequently cleaved at the +4 position in the absence of C5 (Fig. 3b, lanes 5 and 6). Addition of C5 resulted in cleavage only at the +1 position (Fig. 5). The main site of cleavage (at
position +4) in the absence of C5 on the other precursor (pSU3-3XGAAGC + [T-GC]-10) was the same as observed for cleavage of a truncated version of pSU3-3XGA (pSU3-3XGA + [T-GC]-10) which also carried an insertion of an extra G:C base-pair in the T-stem (Fig. 3a, compare lanes 11 and 12). These truncated precursors were also cleaved at position +6 by M1 RNA alone. Taken together, these results suggest that the length of the T-stem influences the identification of the E.coli RNase P cleavage site.

The identity of the nucleotide at position 57 in the T-loop influences the location of the cleavage site

Earlier reports suggested that nucleotides in the T-loop are in contact with RNase P during the cleavage reaction (5, 6). To investigate whether the primary structure of the T-loop is important for the selection of the E.coli RNase P cleavage site we replaced G57 in pSU3 and in some of the mutant derivatives (pSU3-3XGA and pSU3-3XGA-10) with a C (Fig. 1). These precursors were designated pSU3-C57, pSU3-3XGA[C57] and pSU3-3XGA[C57]-10. We chose G57 since the nucleotide at this position in yeast tRNA* is not involved in any Watson–Crick base-pairing (21). The results of RNase P cleavage analysis of these mutant precursors are shown in Figure 3c and are summarized in Figure 5.

![Table 1. The efficiency of suppression of the different tRNA nonsense suppressors used in this study. The averages of uncorrected numbers of several independent measurements are shown. *The values given for Su3+ and pUC19 were taken from Kirsebom and SvHrd (3); standard deviation is given only for pUC19.](https://ncbi.nlm.nih.gov/pubmed/8222586)
or by the holoenzyme was performed under identical buffer conditions (Fig. 4, lanes 4 and 8). Thus, C5 influences the location of the cleavage site. When the G to C substitution at position 57 was introduced into pSu3-3XGA we observed cleavage mainly at position +1. Minor cleavage was observed at positions +4 and +6, and at a position upstream of +1 (Fig. 3c, lane 5 and Fig. 5). The latter cleavage site was observed only in the absence of C5. We also note that pSu3-3XGA[C57] was cleaved at a reduced rate relative to cleavage of pSu3-3XGA. Cleavage of pSu3-3XGA[C57]-10 by M1 RNA alone resulted in a 5'-matured tRNA which carried a pGp at its 5'-end. This, together with the observed mobility of the 5'-cleavage product, suggested that cleavage occurred at position +6. Significant cleavage of pSu3-3XGA[C57]-10 at positions within the 5'-leader region was also observed (Fig. 3c, lane 7). These cleavage sites were not further characterized. Taken together, these results show that a substitution of G57 in the T-loop can alter the location of the cleavage site on the tRNA^Su3 precursor.

A333 in M1 RNA appears to be important for the location of the cleavage site on pSu3-C57

The results described in the previous section showed that a mutation in G57 in the tRNA^Su3 precursor can influence the selection of the cleavage site in a C5 dependent manner. The nucleotide at this position is part of the well-conserved -GUUCR-sequence in tRNAs (22). There are two regions in M1 RNA which are complementary to this region, the nucleotides at positions 284 to 288 and 331 to 335 (23). Therefore to investigate whether one of these regions (or both) in M1 RNA are important for the selection of the cleavage site we cleaved pSu3-C57 with two M1 RNA variants (Fig. 4). One carried a G at position 284 which could form a potential Watson–Crick base pair with C57 in the mutant precursor. The other carried an A to C substitution at position 333. The cleavage analysis was performed in the absence or presence of C5 under various buffer conditions (see Materials and Methods). The results are shown in Figure 4 and are summarized in Figure 5.

Irrespective of buffer conditions both the wild-type and the mutant M1 RNAs cleaved pSu3-C57 at the same position, +1, in the absence of C5. In the presence of C5, under various buffer conditions (see Materials and Methods), M1C333 RNA cleaved pSu3-C57 preferentially at position +1 with some residual cleavage at position −2 whereas significant cleavage was observed at both these positions for wild-type M1 RNA and M1G284 RNA. Thus, a change of A333 to a C in M1 RNA affects the location of the cleavage site on pSu3-C57 in a C5 dependent manner. Cleavage of pSu3 in the absence and presence of C5, under the various buffer conditions tested, occurred at the +1 position independent of M1 RNA (data not shown).

In vivo processing

We have earlier reported that when the tRNA^Su3 gene behind the T7 promoter is expressed in vivo, the resulting tRNA functions as a nonsense suppressor (3). In order to understand the importance of the length of the acceptor-stem of a tRNA precursor and the identity of the nucleotide at the cleavage site in vivo we determined the efficiency of suppression for some of the tRNA^Su3 variants tested above. The results are shown in Table 1.

Expression of the three tRNA^Su3 mutant derivatives tRNA^Su3-3XGA, tRNA^Su3-3XAA and tRNA^Su3-3XGC resulted in functional tRNAs as determined by suppression of UAG. This result suggested that the corresponding tRNA precursors were correctly processed. By contrast, very little suppression if any was observed for the other tRNA^Su3 derivatives. To further evaluate the processing of these tRNA precursors in vivo total RNA was extracted from cells harbouring various tRNA mutant gene constructs. The RNA was subjected to Northern blot-hybridization analysis and showed that some of the tRNA mutants were longer than the ‘wild-type’ tRNA^Su3 (data not shown). The RNase P cleavage site on these mutant precursors was determined by primer-extension analysis. The results demonstrated clearly that all precursors tested were cleaved at position +1 (Fig. 5 and Fig. 6). Furthermore, the presence of pSu3-3XGA, pSu3-3XGC and pSu3-3XAA yielded functional tRNAs. We therefore concluded that these precursors...
were also cleaved at position +4 generating tRNAs with 7 bp long acceptor-stems. The tRNA^{Ty}Su3-3XGAAGC precursor was cleaved mainly at position +1 but not at position +4, in keeping with the finding that the corresponding mutated tRNA did not function as a tRNA nonsense suppressor (Fig. 6 and Table 1). Very little matured tRNA^{Ty}Su3-3XGC and tRNA^{Ty}Su3-3XUA were produced (data not shown) suggesting that the rate of processing of their tRNA precursors was significantly reduced in comparison to processing of the tRNA^{Ty}Su3-3XGA precursor. These findings were consistent with our in vitro results (Fig. 2 and 5). We conclude that the length of the acceptor-stem of a tRNA precursor together with the identity of the nucleotide at the cleavage site are important for the location of the E. coli RNase P cleavage site in vivo.

**DISCUSSION**

To understand the function of RNase P it is important to elucidate how the enzyme localizes the cleavage site. It has been suggested that RNase P makes contacts in the T-loop and measures the combined lengths of the T-stem and acceptor-stem in order to localize the cleavage site (4–6). However, the selection of the E. coli RNase P cleavage site both in vivo and in vitro is not merely dependent on the length of these stem structures, as shown by our present work.

Different regions on a tRNA precursor determine the selection of the E. coli RNase P cleavage site dependent on the identity of the substrate

Previous reports have demonstrated that RNase P cleavage is dependent on the identity of the substrate. For example, a substitution of the nucleotide at the cleavage sites in the tRNA^{Ty}Su3 and tRNA^{Ser}Su1 precursors did not cause a shift in the position of cleavage (3, 11). By contrast, a guanosine at the cleavage site is important for the location of the cleavage site on the tRNA^{His} precursor and on a tRNA^{Ty}Su3-tRNA^{His} chimeric precursor (3, 24, 25). The location of the cleavage site on the tRNA^{His} precursor is also dependent on the primary structure and length of its acceptor-stem (2, 3). Furthermore, recent results suggest that the identity of the nucleotide at position —2 in the tRNA^{Ser}Su1 precursor is crucial for the location of the cleavage site whereas this is not the case for cleavage of the tRNA^{Ty}Su3 precursor (7, 11). The selection of the cleavage site on the tRNA^{Ser}Su1 precursor is also absolutely dependent on the presence of the CCA sequence (11). The positioning of CCA in a tRNA precursor was suggested to be important for the location of the cleavage site (26). A shift of the cleavage site relative to the position of CCA was observed for certain tRNA^{Ty}Su3 precursor variants (7). Thus, the relative position of the CCA sequence appears to be of significance for the selection of the E. coli RNase P cleavage site in a substrate-dependent manner. In conclusion, depending on the identity of the substrate, different regions on a tRNA precursor determine the selection of the E. coli RNase P cleavage site.

Features of the tRNA^{Ty}Su3 precursor important for cleavage-site selection

The length of the acceptor-stem of the tRNA^{Ty}Su3 precursor is of some importance for the location of the cleavage site (7; this report). Recently we showed that the identity of the nucleotides near the cleavage site is important for recognition of the cleavage site. We proposed that the nucleotide at the cleavage site functions as a guiding nucleotide (7). The results reported here demonstrate the importance of this nucleotide both in vivo and in vitro. From the combined data we conclude that the length of the acceptor-stem together with the nucleotide at the cleavage site are important determinants for selection of the cleavage site on the tRNA^{Ty}Su3 precursor. Recently we discussed the possibility that the identity of the nucleotides at positions —2, —1, +1, +72, +73 and +74 are important for Mg^{2+} binding (7). It is conceivable that this is the explanation to why we observed cleavage only at positions +1 and +4 using the pSu3-3X derivatives. A possibility is also that the positioning of CCA which is the same at both positions played a role in this process.

Deletion of a G in pSu3-3XGA in the 5'-half of the acceptor-stem downstream of the cleavage sites, as in pSu3-3XGAAG, resulted in cleavage exclusively at position +1, whereas deletion of the cytosine in the 3'-half of the acceptor-stem resulted in cleavage both at position +1 and at position +4. Therefore, it appears that the number of nucleotides in the 5'-half of the acceptor-stem plays a more significant role than the number of the nucleotides in the 3'-half of the acceptor-stem. Perhaps the number of nucleotides on the 5'-side of the acceptor-stem is important for alignment of the precursor on the enzyme. Consistent with this hypothesis is the observation that cleavage of truncated derivatives of pSu3 with only 4 or 5 base pairs in the acceptor-stem generated mainly 5'-matured 'tRNAs' with 7 bases in the 5'-half of the acceptor-stem (7; this report).

An extension of the T-stem of the tRNA^{Ty}Su3 precursor by one base-pair did not change the position of cleavage (7). Neither did an extension of the T-stem of pSu3-3XGA-10 (this report). However, an insertion of a G:C base pair in the T-stem compensated for a deletion of one base pair in the acceptor-stem (this report). Thus, the importance of the length of the T-stem for the location of the cleavage site became evident when other landmarks in the precursor where changed or deleted.

A mutation of G57 to C resulted in a shift of the cleavage site. Surprisingly, cleavage by the holoenzyme of pSu3-C57 occurred at several positions (this report). Processing of this precursor yielded small amounts of matured tRNA in vivo (data not shown). Conceivably the base-substitution at position 57 influences the local folding of the T-loop and/or the folding of the tRNA precursor, resulting in aberrant cleavage. Furthermore, a substitution of the conserved U54 in the tRNA^{Ty}Su3 precursor resulted in a significant decrease in the rate of RNase P cleavage (27, 28). Taken together, these data are compatible with the suggestion that the T-loop are in contact with M1 RNA during the cleavage reaction (5, 6).

**How does E. coli RNase P localize the cleavage site?**

From the combined data we envision the following scenario for the location of the E. coli RNase P cleavage site on a tRNA precursor (Fig. 7). RNase P recognizes the conformation of the tRNA portion of a tRNA precursor, as suggested by Altman (29). This is supported by the finding that mutation of a nucleotide which is important for the folding of the tRNA results in an impairment in the interaction with the enzyme (30). The enzyme makes contact with nucleotides in the T-loop. From the results reported here it appears that the nucleotide at position 333 in M1 RNA influences this process, however only in the presence of C5. A previous report suggested that this region of M1 RNA is not involved in conventional base-pairing with the precursor (28). Further experiments are therefore required to understand...
how the nucleotide at position 333 in M1 RNA affect the identification of the cleavage site. The lengths of the T-stem and acceptor-stem are measured (indicated by the ruler) to localize the nucleotide at the cleavage site which functions as a guiding nucleotide. In most tRNA precursors it is a guanosine (22). Here, the number of nucleotides in the 5'-half of the acceptor-stem appears to be important, conceivably in such a way that correct alignment of the precursor on the enzyme is achieved. This could be accomplished through unconventional base-pairing since there is no sequence homology between different tRNA acceptor-stems (22). The local structure near the cleavage site plays a significant role in the location of the cleavage site, possibly through the coordination of Mg++ (7, 31). The EGS [external guide sequence (32)] influences the location of the cleavage site, albeit to a minor extent (7; this report). Perhaps the EGS together with the CCA sequence, when present, has a "tuning" function which ensures cleavage at the correct position. We emphasize that the significance of the different determinants is dependent on the identity of the tRNA precursor. In addition, changing one determinant does not necessarily result in a shift of the cleavage site (7; this report).

The C5 protein plays a role in the location of the cleavage site

The role of C5 is to facilitate product-release and to prevent re-binding of the 5'-matured tRNA to the enzyme (15, 33). Here we showed that pSu3-C57 was cleaved at position +1 in the absence of C5 whereas addition of C5 resulted in cleavage at several positions. Cleavage by M1 RNA alone and by holoenzyme was performed under identical buffer conditions. Thus, we conclude that C5 also plays a role in the location of the cleavage site.

Concluding remarks

The results reported here demonstrate which regions of the tRNA\textsuperscript{\textasciitilde}Su3 precursor are important for the location of the E.coli RNase P cleavage site. The different variants of the tRNA\textsuperscript{\textasciitilde}Su3 precursor described here will be used to generate mutant M1 RNA molecules which show substrate-specificity with respect to the selection of the cleavage site. This approach will give information about nucleotides in M1 RNA which are significant for the interaction between the enzyme and its substrate. Here, we present data which indicate that A333 in M1 RNA is one of these nucleotides.

ACKNOWLEDGEMENTS

This work was supported by a grant to L.A.K. from the Swedish Research Council. We wish to thank Drs S.Burnett, T.Russala and G.Wagner for critical reading of the manuscript. Miss U.Kagardt is acknowledged for skillful technical assistance and Miss A. Tallsjo for preparation of C5.

REFERENCES