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The unusually long amino acid acceptor stem of *Escherichia coli* selenocysteine tRNA results from abnormal cleavage by RNase P

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ABSTRACT

The nucleotide sequence of the gene encoding the *Escherichia coli* selenocysteine tRNA ($tRNA^{SeCys}$) predicts an unusually long acceptor stem of 8 base pairs (one more than other tRNAs). Here we show by *in vivo* experiments (Northern blots, primer extension analysis) and by *in vitro* RNA processing studies that *E. coli* $tRNA^{SeCys}$ does contain this additional basepair, and that its formation results from abnormal cleavage by RNase P.

INTRODUCTION

Several prokaryotic and eukaryotic enzymes are known to contain selenium incorporated as selenocysteine (1). For *E. coli* formate dehydrogenase H it was shown that selenocysteine incorporation occurs cotranslationally in response to an in-frame UGA nonsense codon (2). The gene *selC* encoding the tRNA inserting selenocysteine at the termination site of this UGA codon, has recently been cloned and characterized (3). The RNA sequence deduced from the gene predicts an unusual structure for the amino acid acceptor stem and poses some questions about its biosynthesis (4). According to the deduced RNA sequence and conserved tRNA secondary structure, it may consist of either eight basepairs, and an unpaired nucleotide followed by the CCA-end, or of the usual seven basepairs followed by two unpaired nucleotides and 3'-terminal CCA. This is somewhat reminiscent of histidine tRNA which carries an additional nucleotide at its 5'-terminus. RNase P is the enzyme responsible for 5'-end maturation of tRNA. In the case of $tRNA^{His}$ the unusually long amino acid acceptor stem is generated by an abnormal cleavage by RNase P at position -1 to generate the tRNA in *E. coli* (5) and in *Bacillus subtilis* (6). It appears that the G_{-1}/C_{73} sequence is important for the cleavage of tRNA precursors at position -1 (7). Therefore we determined whether *E. coli* $tRNA^{SeCys}$ also has an unusually long amino acid acceptor stem of eight base pairs and whether this results from abnormal cleavage by RNase P analogous to *E. coli* $tRNA^{His}$.

MATERIALS AND METHODS

Plasmid constructions. Plasmid pSeCAlu was constructed by recloning the $tRNA^{SeCys}$ gene from pMN81 (3) as an *AluI*/*Hin*dIII fragment into pSP72 (Promega Biotec) which had been cut

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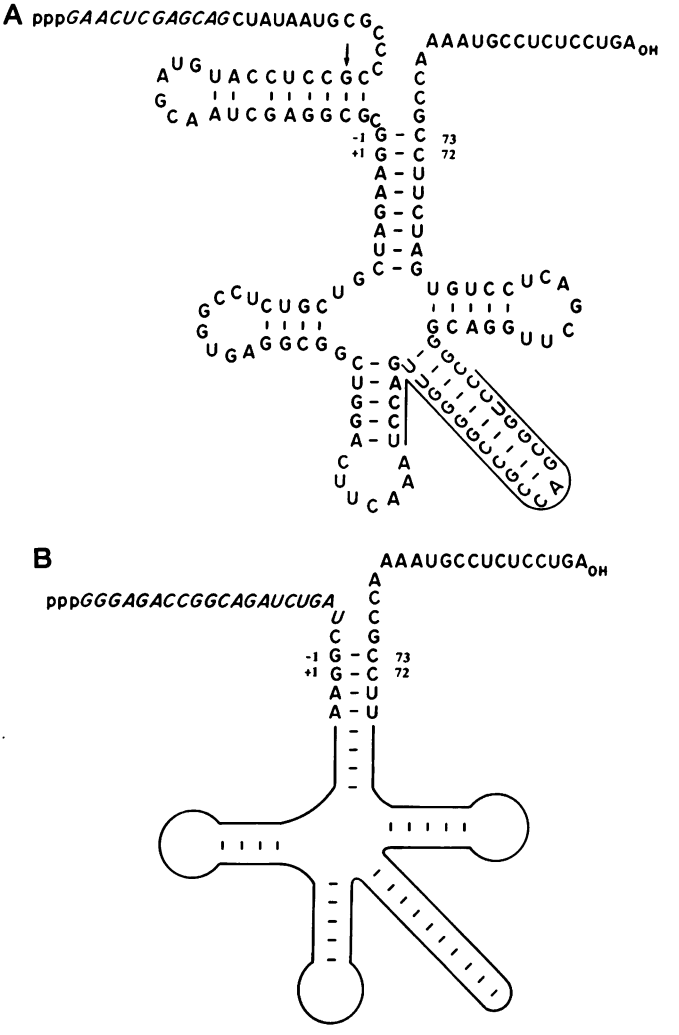

Figure 1: Secondary structure models for *in vitro* transcribed *E. coli* $tRNA^{SeCys}$ precursors. (A) Precursor 1, derived by SP6 polymerase transcription from plasmid pSeCAlu. (**B**) Precursor 2, derived by T7 polymerase transcription from plasmid pSeCHha. Nucleotides derived from vector sequence are given in italics. The underlined nucleotides (Fig. 1A) are complementary to the synthetic oligonucleotide, which was used for Northern analysis and primer extension. The arrow indicates the start of transcription *in vivo*. The numbers of the nucleotides are according to Sprinzl *et al.* (12).

with *Pvu*II/*Hin*dIII. Plasmid pSeCHha was obtained by recloning the same tRNA gene as a *Hin*dIII/*Hha*I fragment, which had been blunt-ended at the site of *Hha*I cleavage, into *Eco*RV/*Hin*dIII-cleaved pSP72. The structures of the expected precursors produced by *in vitro* transcription are presented in Fig. 1.

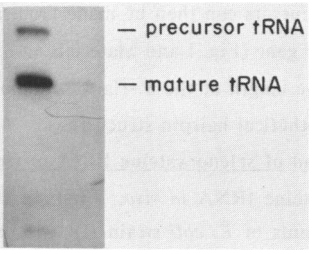

Figure 2: Autoradiogram of a Northern blot of *E. coli* tRNA probed with the synthetic oligonucleotide complementary to the extra arm of $tRNA^{SeCys}$ (see Fig. 1A). The tRNA was from cells transformed with pMN81, the plasmid carrying *selC* (lane 1) or with pUC18 (lane 2).

RNase P enzymes. M1-RNA, the catalytic subunit of *E. coli* RNase P (8) and P RNA, the catalytic subunit of *B. subtilis* RNase P (9) were synthesized by *in vitro* RNA transcription according to Reich *et al.* (9). *Schizosaccharomyces pombe* RNase P (10) was kindly provided by Denis Drainas in this laboratory.

Processing of tRNA precursors. Reactions catalyzed by M1-RNA, P RNA and *E. coli* RNase P were performed as described earlier (7). Processing by *S. pombe* RNase P was carried out at 37°C in buffer containing 30 mM Tris.HCl (pH 8.0), 100 mM NH_4Cl, 5 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM 2-mercaptoethanol. RNA 5'-end analysis was performed as described (7).

Primer extension and Northern analysis. *E. coli* tRNA was prepared according to Caillet *et al.* (11). Primer extension and Northern analysis were carried out with an oligodeoxynucleotide complementary to the extra arm of selenocysteine tRNA (Fig. 1A). Dideoxysequencing of the RNA transcripts was by primer extension, performed with reverse transcriptase (IBI) at 42°C after denaturing and annealing by heating at 95°C for 1 min. and at 65°C for 10 min. For Northern analysis equal amounts of tRNA from cells transformed with pMN81, the plasmid carrying the *selC* gene (3) or with vector pUC18 were run on an 8% polyacrylamide gel containing 8M urea and transferred to Zetaprobe membrane by electroblotting as described by the manufacturer (BioRad). Prehybridization and hybridization were carried out at 25°C in 50% formamide, 50 mM sodium phosphate (pH 6.5), 5 x SSC, 0.1% SDS, 1 mM EDTA, 200 μg/ml denatured salmon sperm DNA, 2.5 x Denhardt's solution. The membrane was washed at 25°C in 2 x SSC before autoradiography.

RESULTS

In order to determine the nature and origin of the 5'-end of selenocysteine tRNA we needed to determine *in vitro* the cleavage site in the $tRNA^{SeCys}$ precursor produced by *E. coli* RNase P and to compare this result with the structure of the 5'-end of the *in vivo* generated product. For the *in vitro* studies we used two tailored $tRNA^{SeCys}$ genes; one has additional 5' flanking sequence which potentially can form a hairpin structure upstream of the mature 5'-

end of the tRNA. The two precursors can then be made from pSeCAlu and pSeCHha, the two plasmids carrying the $tRNA^{SeCys}$ gene (Fig. 1 and Materials and Methods). The main distinction between the two precursors is the length of the 5'-flank; the longer RNA, precursor 1, (derived from pSeCAlu), contains a hypothetical hairpin structure.

Determination of the 5'-end of selenocysteine tRNA *in vivo*. Two methods were used to analyze the 5'-end of selenocysteine tRNA *in vivo*. First we carried out Northern analysis of tRNA prepared from transformants of *E. coli* strain HB101 containing plasmid pMN81 (3) or pUC18. We chose as a hybridization probe an oligonucleotide complementary to the extra arm of $tRNA^{SeCys}$ (Fig. 1A). The Northern blot (Fig. 2) reveals two major bands; mature tRNA and a precursor tRNA in tRNA from *E. coli* cells transformed with pMN81 (containing the $tRNA^{SeCys}$ gene). Control tRNA from *E. coli* transformed with vector pUC18 with no insert revealed that the same RNAs were present, but at a much lower concentration. Primer extension analysis was used to determine the exact position of the 5'-terminus of mature $tRNA^{SeCys}$. Fig. 3 shows the sequencing reactions for the nucleotides near the 5'-end of the tRNA. The 3'-end of the sequence reads ...TCTTCC, which corresponds to GGAAGA... as the sequence of the 5'-end of the tRNA. After a longer exposure of the sequencing gel, a precursor starting at position -25 (see arrow in Fig. 1A) was detected.

Processing of *in vitro* transcribed $tRNA^{SeCys}$ precursors. Our studies on 5'-end maturation of $tRNA^{SeCys}$ were carried out with *in vitro* transcribed precursors derived from plasmids pSeCAlu and pSeCHha using bacteriophage SP6 and T7 RNA polymerase, respectively. The construction of the clones is described in the Materials and Methods section. In order to determine the site of cleavage by RNase P we carried out *in vitro* processing and analyzed the

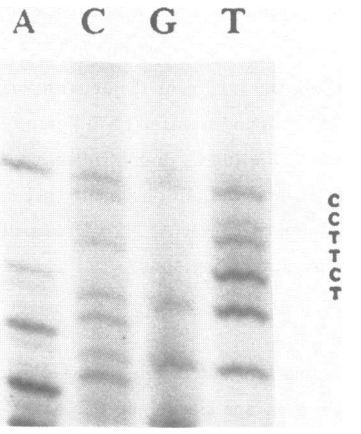

Figure 3: Autoradiogram of a sequencing gel showing the 3'-terminal sequence of primer extension reactions on tRNA from cells transformed with pMN81, as described in Materials and Methods.

5'-end of the resulting tRNA products as well as the length of the flanks generated in this reaction. We used several different RNase P activities of prokaryotic and eukaryotic origin; *E. coli* RNase P holoenzyme, *E. coli* M1-RNA, *B. subtilis* P RNA, and *S. pombe* RNase P. Fig. 4 shows examples of the gel electrophoretic separation of precursor RNAs, tRNA products and 5'-flanks after processing precursor tRNA 1, derived from pSeCAlu and precursor tRNA 2,

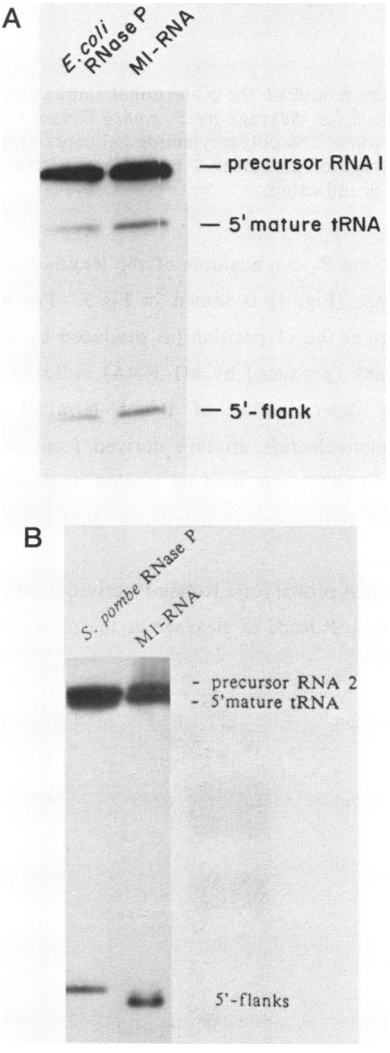

Figure 4: Autoradiogram of gel electrophoretic separation of RNA products derived from processing of $tRNA^{SeCys}$ precursors. (A) for precursor 1 (separation on an 8% polyacrylamide gel containing 8M urea) (B) for precursor 2 (separation on a 15% polyacrylamide gel containing 8M urea). The different RNase P activities that were used are indicated.

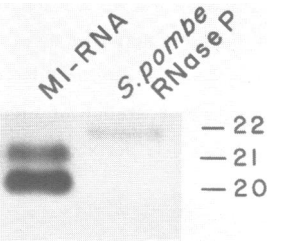

Figure 5: Determination of the length of the 5'-terminal flanks generated by RNase P. Line 1 for cleavage by M1-RNA, line 2 for cleavage by *S. pombe* RNase P. The autoradiogram shows gel electrophoretic separation (on a 20% polyacrylamide gel containing 8M urea) of the 5'-flanks obtained after enzymatic cleavage of precursor 2 by different RNase P activities. The length of the flanks (in nucleotides) is indicated.

derived from pSeCHha by RNase P. An example of the length determination of the 5'-flanks generated by RNase P cleavage (Fig. 4) is shown in Fig.5. For precursor 2 a length of 22 nucleotides represents cleavage at the +1 position (as produced by *S. pombe* RNase P), while a 21 and 20 nucleotide long flank (produced by M1-RNA) indicates cleavage at the -1 and -2 position, respectively. The determination of the 5'-terminal nucleotide by thin layer chromatography (7) of a mononucleotide mixture derived from the tRNA sized products is shown in Fig. 6. When [α-^{32}P]ATP was used as labeled nucleotide during synthesis of the tRNA precursors, the signal of radioactive pGp indicates that RNase P cleavage occurs at the position +1.

These experiments show that prokaryotic RNase P activities cleave at the -1 position, while processing with *S. pombe* RNase P leads to cleavage at the position +1. Precursor 1 with the

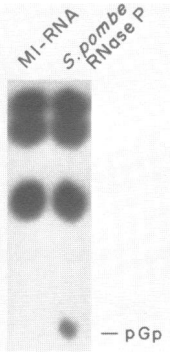

Figure 6: 5'-end analysis of the tRNA products labeled with [α-^{32}P]ATP derived from precursor 2 after cleavage by *E. coli* M1-RNA and *S.pombe* RNase P. Autoradiogram of a PEI-cellulose thin layer chromatogram separating the nucleotide mixtures resulting from digestion with RNases T_2 and A is shown.

additional putative hairpin in the 5'-flank (Fig. 1A) was cleaved only at position -1 by prokaryotic RNase P activities. However, the shorter precursor 2 (Fig. 1B) lacking this hairpin was also partially cleaved at C_{-2} (see Fig. 5). The cleavage at -2 was confirmed by the finding of pCp as 5'-terminal nucleotide in an analysis of the hydrolysate of a [α-^{32}P]CTP labeled precursor 2 (data not shown).

DISCUSSION

The gene for *E. coli* selenocysteine tRNA potentially encodes an eight basepair amino acid acceptor stem, analogous to *E. coli* $tRNA^{His}$; in addition it contains an unpaired nucleotide preceding the 3'-terminal CCA-sequence. Earlier we had shown that the base pair G_{-1}/C_{73} of an *E. coli* $tRNA^{His}$ precursor is important for directing cleavage by *E. coli* RNase P to the -1 position (7). Thus, we expected that the *E. coli* enzyme should cleave the $tRNA^{SeCys}$ precursor at the -1 position. The *in vivo* results of Northern blot and primer extension analysis (Fig. 2 and 3) support this conclusion. *In vitro* processing studies of two $tRNA^{SeCys}$ precursors confirm the *in vivo* results. Thus both precursors were cleaved at position -1 by prokaryotic RNase P. The amino acid aceptor stem of *E. coli* selenocysteine tRNA is indeed eight base pairs in length, and like $tRNA^{His}$, this tRNA is one base pair longer than all other tRNAs. Furthermore it shows that the additional 5'-terminal G of the tRNA, which is encoded in the gene, results from cleavage of RNase P at this unusual site (7). In addition to the -1 cleavage site, the shorter precursor was also cleaved at the -2 position. Possibly the putative upstream hairpin structure of precursor 1 may be important for the accurate recognition of the cleavage site. Formation of this additional stem may prevent hydrogen bonding between C_{-2} and G_{74}, which in contrast may be possible in the shorter tRNA precursor. An additional C/G base pair may then lead to partial miscleavage by RNase P, generating a nine base pair amino acid acceptor stem of the tRNA. As shown earlier (7), this kind of miscleavage can take place when a G_{-2}/C_{74} base pairing possibility was introduced in an *E. coli* $tRNA^{His}$ precursor. Thus, RNase P has some flexibility in substrate recognition to fit its role as the sole enzyme in prokaryotic cells generating the 5'-ends of mature tRNA (13).

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