Intragenic suppression in tRNA: evidence for crosstalk between the D and the T stems

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RNA 1997 3: 1220-1232

RNA (1997), 3:1220–1232. Cambridge University Press. Printed in the USA.

Intragenic suppression in tRNA: Evidence for crosstalk between the D and the T stems

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ABSTRACT

We showed previously that introduction of two of the three unique features of *Escherichia coli* initiator tRNA onto an elongator methionine tRNA conferred significant activity in initiation. Surprisingly, introduction also of the third unique feature, the A11:U24 base pair in the D stem, resulted in total lack of accumulation of the mutant Mi:3 tRNA. We show here that the Mi:3 tRNA gene is transcribed efficiently in vitro. Processing of the Mi:3 precursor transcript shows, however, that both the precursor and the mature Mi:3 tRNA are unstable in *E. coli* extracts. To understand the basis of instability caused by the A11:U24 base pair in the elongator methionine tRNA background, we have isolated and characterized intragenic suppressor mutations in the tRNA that restore its function in translation initiation. Sequence changes in the T stem that convert the existing A51 × C63 mismatch to a base pair in the Mi:3 tRNA result in accumulation of the tRNAs in vivo. The initiation activity and in vivo levels of accumulation of these suppressors are in the order Mi:3/G51:C63 > Mi:3/A51:U63 ≫ Mi:3/G51.U63. These results show that the in vivo accumulation of a tRNA with A11:U24 base pair in the D stem depends upon a base pair between positions 51 and 63 in the T stem. Structural analysis in vitro of the Mi:3 and Mi:3/G51:C63 transcripts suggests that the Mi:3 tRNA is unable to adopt a stable tRNA-like conformation. Various considerations suggest that this is most likely due to a high entropic barrier to tertiary interactions, between the D and the T loops necessary for the formation of a stable tRNA structure.

Keywords: tRNA structure; tRNA stability; tRNA-protein interactions; translation initiation; tRNA methionyl-tRNA formyltransferase interaction

INTRODUCTION

Two functionally distinct methionine tRNAs are present in all organisms studied to date (Kozak, 1983; Gold, 1988; Gualerzi & Pon, 1990; RajBhandary, 1994). In eubacteria, the initiator methionine tRNA ($tRNA^{fMet}$) is used in translation initiation, whereas the elongator methionine tRNA ($tRNA^{Met}$) is used to insert methionine into internal positions. Both these tRNAs are aminoacylated by methionyl-tRNA synthetase (MetRS) to form methionyl-tRNA (Met-tRNA). Subsequently, the initiator Met-$tRNA^{fMet}$ species is formylated and then used for the initiation of translation. Besides having a number of distinctive properties, the initiator $tRNA^{fMet}$ has three unique sequence elements not found in other tRNAs (RajBhandary, 1994). These are: (1) the absence of a base pair between positions 1 and 72 in the acceptor stem; (2) the presence of three consecutive G:C base pairs in the anticodon stem (G29G30G31:C39C40C41); and (3) the presence of an A11:U24 base pair in the dihydrouridine (D) stem. Structure–function relationship studies have elucidated the role of two of the three features. The mismatch at positions 1 and 72 is involved in: (1) formylation; (2) preventing the tRNA from acting in the elongation step of protein synthesis; and (3) preventing the tRNA from being a substrate for peptidyl-tRNA hydrolase (Schulman & Pelka, 1975; Seong & RajBhandary, 1987a; Lee et al., 1991, 1992; Guillon et al., 1992; Dutka et al., 1993). The three G:C base pairs in the anticodon stem are important for binding of the tRNA to the ribosomal P site (Seong & RajBhandary, 1987b; Mandal et al., 1996), possibly for its selection by the initiation factor IF3 (Hartz et al., 1990). The role of the third conserved sequence feature, the A11:U24 base pair in the D stem, in contrast to a pyrimidine 11:purine 24 base pair in other tRNAs, is not established. Mutation of A11:U24 to C11:G24 in the *Escherichia coli* initiator tRNA resulted in a sevenfold increase in K_m in the formylation reaction (Lee et al., 1991), suggesting a role for the A11:U24 base pair in formylation.

In attempts to convert an elongator tRNA to an initiator tRNA, we previously transplanted the three

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unique sequence elements of $tRNA^{fMet}$ into elongator $tRNA^{Met}$ to produce three mutant elongator tRNAs Mi:1, Mi:2, and Mi:3 (Varshney et al., 1993, Fig. 1). These tRNAs, which have one, two, or all three features unique to initiator tRNA, were assayed for their ability to initiate translation in vivo. The mutations were coupled to the anticodon sequence change from CAU → CUA, which allowed the mutants to be assayed for initiation activity using a reporter chloramphenicol acetyl transferase gene, CATam1.2.5, carrying UAG as the initiation codon (Varshney & RajBhandary, 1990).

Mutant tRNA, Mi:1, which has the C1 × A72 mismatch in the elongator $tRNA^{Met}$ background, showed very low, but detectable, activity in initiation in cells overproducing MetRS (Varshney et al., 1993; Li et al., 1996). The anticodon change from CAU → CUA makes the tRNA a very poor substrate for MetRS, which can be partially compensated for by overexpression of the synthetase. The mutant tRNA, Mi:2, with three G:C base pairs in the anticodon stem, in addition to the acceptor stem mismatch, was much more active in initiation under similar conditions. Surprisingly, however, introduction of the third characteristic feature, an A11:U24 base pair in the D stem, abolished the initiation activity of the tRNA. Further analysis revealed that the Mi:3 tRNA was not detectable in cells. These results suggest that the A11:U24 base pair can exist only in certain sequence contexts that are absent in the Mi:3 tRNA.

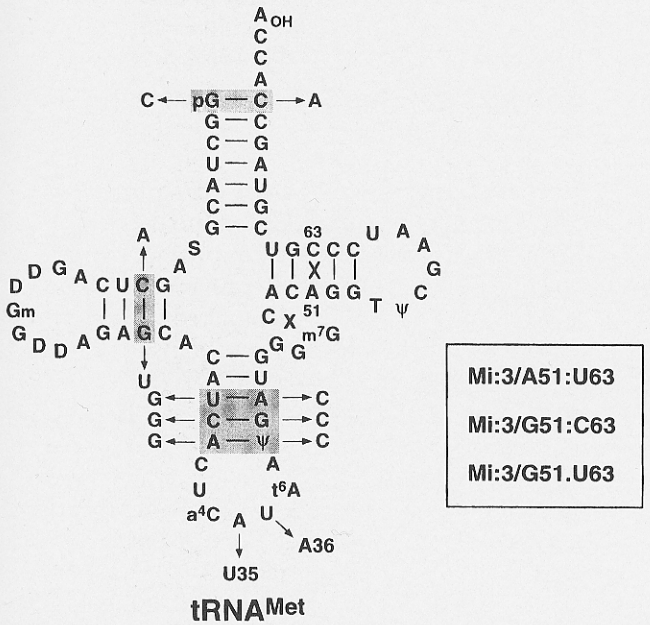


FIGURE 1. Sequence of elongator methionine $tRNA^{Met}$ of *E. coli* is shown in the backbone. The U35A36 mutation is common to all the tRNAs used here. Sites of mutation used to generate the three unique sequence elements of the initiator tRNA are shaded. These changes generate (1) a mismatch between nt 1 and 72, (2) three G:C base pairs in the anticodon stem, and (3) A11:U24 base pair in the D-stem. The mutant having change (1) alone is called Mi:1 and changes (1) and (2) is called Mi:2. The mutant with all the changes (1), (2), and (3) is called Mi:3. This mutant fails to accumulate in vivo. Changes that create a base pair at position 51:63 in the T stem and lead to accumulation of the tRNA in vivo are boxed.

To understand the structural basis of the instability and the lack of accumulation of the Mi:3 tRNA in vivo, we used random mutagenesis and screened for intragenic suppressor mutations in the tRNA gene (Anderson & Smith, 1972; McClain et al., 1988). We report here that a single mutation in the T stem, which converts an A51 × C63 mismatch to a Watson–Crick base pair, results in a tRNA that accumulates in vivo and is active in initiation.

Because the Mi:3 mutant tRNA failed to accumulate in vivo, we were previously unable to identify the role of the A11:U24 base pair in initiator tRNA in vivo. Isolation of the suppressor mutant(s) has allowed us to compare the effect of having either a C11:G24 base pair or A11:U24 base pair on the formylation levels of several mutant tRNAs. These studies have also shown that the A11:U24 base pair contributes toward formylation of the tRNA in vivo.

RESULTS

Transcription of the tRNA genes in vitro and processing of the transcripts

Transcription and processing experiments were performed to test the synthesis and/or stability of the Mi:3 tRNA in vitro. The wild-type $tRNA_2^{fMet}$ (trnfM) gene cloned in a plasmid and restriction fragments containing the sequence coding for the Mi:3 tRNA or the anticodon sequence mutant (trnfMU35A36) of the initiator $tRNA_2^{fMet}$ were used as templates. The results show that, at least in vitro, the Mi:3 tRNA gene is transcribed by *E. coli* RNA polymerase as well as the initiator $tRNA_2^{fMet}$ gene or the U35A36 mutant tRNA gene to yield a predominant primary transcript of the same size (0.22 kb) (Fig. 2A, lanes 1, 3, and 4). The origin of an approximately 0.2-kb long doublet RNA is unknown. Incubation of the primary transcript with *E. coli* S100 extracts suggested that it probably goes through similar intermediate steps in processing (Fig. 2B, compare lanes 2, 3, 4, and 5 with lanes 7, 8, 9, and 10, respectively). However, compared to the stable accumulation of the mature U35A36 mutant initiator tRNA (Fig. 2B, lane 5), the Mi:3 tRNA was significantly less stable. After a period of 60 min, neither the precursor nor the product of the Mi:3 transcript is detectable (Fig. 2B, lane 10), whereas the U35A356 mutant initiator tRNA accumulates to a product of size corresponding to the mature $tRNA_2^{fMet}$ (Fig. 2B, lanes 5 and 14). The instability of the Mi:3 precursor tRNA and the mature Mi:3 tRNA in *E. coli* extracts in vitro most probably accounts for the lack of accumulation of the tRNA in vivo and suggests that the A11:U24 base pair cannot exist in the sequence context of the *E. coli* elongator methionine tRNA.

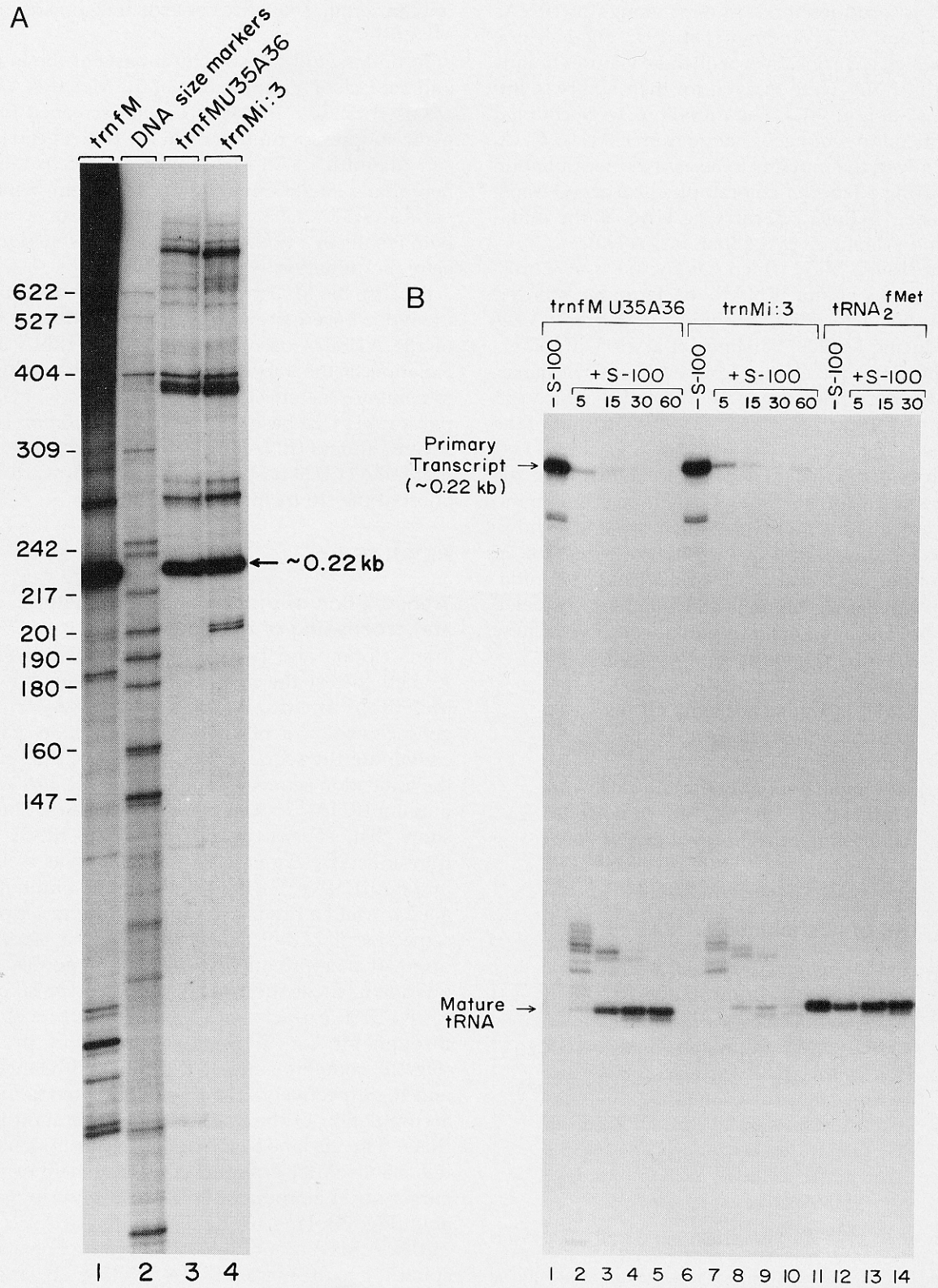


FIGURE 2. Synthesis and processing of transcripts in vitro. **A:** In vitro transcription. Supercoiled plasmid DNA (lane 1) or restriction fragments (lanes 3 and 4) containing the various tRNA genes were incubated with *E. coli* RNA polymerase.trnfM, wild-type initiator $tRNA_2^{fMet}$ gene, trnfM U35A36, the U35A36 mutant of the initiator tRNA gene, Mi:3, the Mi:3 mutant of *E. coli* elongator methionine tRNA gene. **B:** In vitro processing/stability. The trnfMU35A36 (lanes 1–5) and Mi:3 (lanes 6–10) transcripts and 5′-end-labeled $tRNA_2^{fMet}$ (lanes 11–14) were incubated with *E. coli* S100 extracts. Details in Materials and Methods.

Isolation of suppressor mutations in the Mi:3 tRNA gene

To identify the sequence context(s) necessary for accumulation of the Mi:3 tRNA in vivo, we have isolated intragenic suppressors in the Mi:3 tRNA gene. *E. coli* CA274 carrying the Mi:3 tRNA gene and the reporter CATam1.2.5 gene on pRSVCATam1.2.5 and the MetRS gene on pACMS3 was treated with the mutagen MNNG to generate a random pool of mutants (Fig. 3). Intragenic mutations in the tRNA gene that lead to its accumulation and participation in translation initiation should lead to the synthesis of CAT protein. Therefore, the ability of the mutants to grow in the presence of chloramphenicol was used to select for the suppressor mutations in the tRNA gene. The tRNA gene on the plasmid from 46 chloramphenicol-resistant mutants was sequenced and shown to have a C63 to U63 change in 18 of them; these could be derived from the same parent. The other chloramphenicol-resistant mutants could have changes in the chromosomal DNA or in the CAT gene on the pRSV *CATam1.2.5*; these were not pursued further. Subcloning of 1 of the 18 suppressor tRNA genes onto a fresh pRSVCATam1.2.5 vector followed by transformation of *E. coli* CA274 carrying the pACMS3 plasmid yielded transformants that were chloramphenicol resistant, confirming that the chloramphenicol-resistance phenotype was due to the mutation in the tRNA gene. The C63 to U63 mutation now generates a A51:U63 base pair in the T stem compared to a A51 × C63 mismatch in the Mi:3 tRNA (Fig. 1). This mutant is designated the Mi:3/A51:U63 mutant. Further mutations were introduced at this position using site-specific mutagenesis to yield Mi:3 tRNA mutants with G51:C63 (Watson–Crick) and G51.U63 (wobble) base pairs. These changes also conferred resistance to chloramphenicol in cells expressing these tRNAs.

Activity of Mi:3 suppressor tRNAs in translation initiation in vivo

Extracts of *E. coli* CA274 transformants carrying the genes for mutant tRNAs, the CATam1.2.5 reporter gene, and the MetRS gene on plasmids were used for measuring the CAT activity. Extracts from cells carrying any of the three suppressors showed significantly more CAT activity compared to cells carrying the Mi:3 tRNA (Table 1). Although the activity of the Mi:3/A51:U63 mutant tRNA was comparable to that of the Mi:2 tRNA, the Mi:3/G51:C63 mutant tRNA showed a twofold increase in CAT activity. The Mi:3/G51.U63 mutant tRNA having the wobble base pair was much less active among the three suppressors, although this level of CAT activity is sufficient for growth on plates containing 50 μg/mL chloramphenicol. Immunoblot analysis of cell extracts for CAT protein confirmed the results of CAT activity assays, with no detectable protein in transformants carrying the Mi:3 tRNA and only small amounts in cells expressing the Mi:3/G51.U63 tRNA (data not shown). The results with the Mi:3 suppressor tRNAs suggest a very good correlation between the strength of the base pair at positions 51 and 63 and the overall amounts of CAT protein synthesized.

Accumulation of the Mi:3 suppressor tRNAs in vivo

To assess the steady-state levels of the Mi:3 suppressor tRNAs in vivo, total tRNA was isolated from cells expressing the different mutant tRNAs. Northern blot

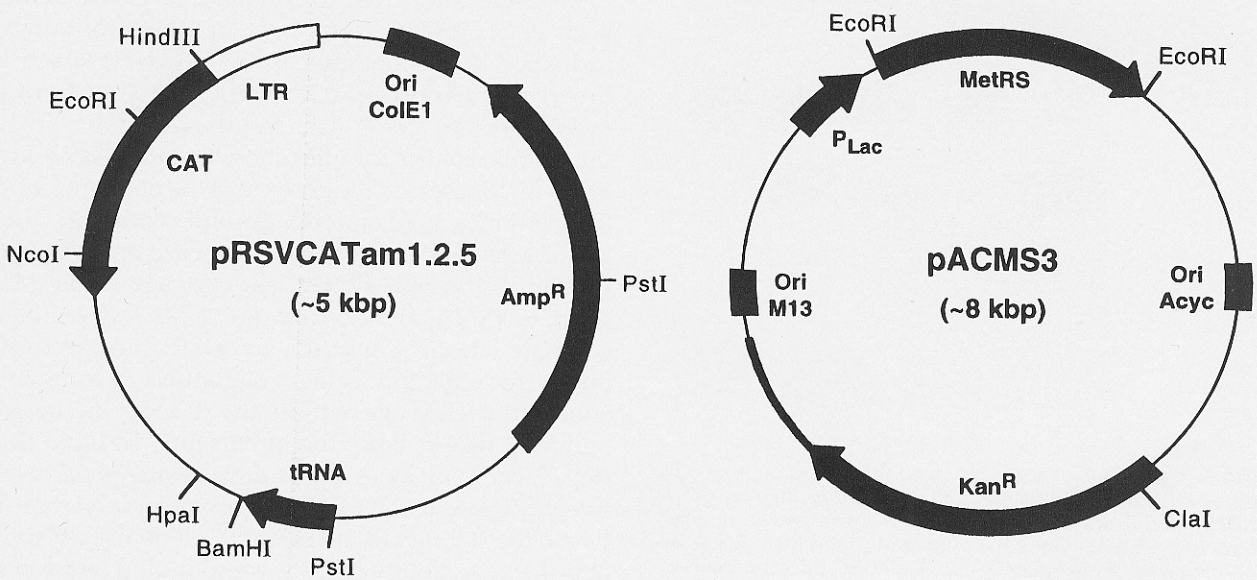


FIGURE 3. Plasmids used in this study. The pRSVCATam1.2.5 (left) has the genes for the mutant tRNAs and the reporter CAT and the pACMS3 plasmid has the gene for *E. coli* MetRS (right).

TABLE 1. Relative CAT activities in extracts of *E. coli* CA274 transformed with pRSVCATam1.2.5 carrying the various mutant tRNA genes and pACMS3.

tRNA	% CAT activity
Mi:2	100[a]
Mi:3	0.3
Mi:3/A51:U63	106
Mi:3/G51:C63	198
Mi:3/G51:U63	13

[a]100% corresponds to 4.35 units of CAT activity per μg of crude extract; 1 unit is defined as nanomoles of chloramphenicol converted to acetyl-chloramphenicol in 15 min at 37 °C.

analysis revealed that the suppressor mutant tRNAs accumulate to different levels (Fig. 4). As shown before, there is no accumulation of the Mi:3 tRNA (Varshney et al., 1993). Quantitation of the blots using $tRNA^{Tyr}$ as an internal control showed that the accumulation of the tRNAs was 6-and 12-fold more, respectively, for the Mi:3/A51:U63 and the Mi:3/G51:C63 tRNAs compared to the Mi:3/G51.U63 tRNA (Fig. 4, lanes 3–5).

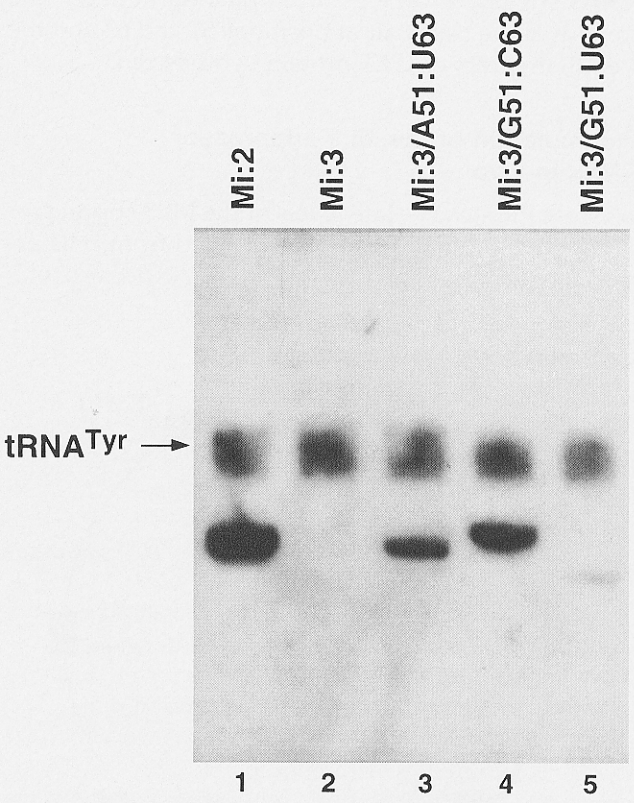


FIGURE 4. Northern blot analysis of total tRNA isolated from cells expressing the indicated mutant *E. coli* elongator methionine tRNA genes. The hybridization signal from $tRNA^{Tyr}$ was used as an internal control for quantitation of the mutant Mi:2 and Mi:3 tRNAs by phosphorimaging. A mixture of 5′-^{32}P-oligonucleotides complementary to the Mi:2 tRNA and *E. coli* $tRNA^{Tyr}$ were used simultaneously as probes to detect the corresponding tRNAs (details in Materials and Methods).

These results show that a base pair in the T stem between nt 51 and 63 is important for accumulation of the tRNAs carrying an A11:U24 base pair in the D stem. Also, there is a direct correlation between the strength of the base pair between nt 51 and 63 on the overall accumulation of the mutant tRNAs in vivo. The levels of accumulation of the mutant tRNAs are in the same order (Mi:3/G51:C63 > Mi:3/A51:U63 ≫ Mi:3/G51.U63) as amounts of the CAT protein in cell free extracts. The different mobilities of the Mi:3 series of mutant tRNAs in lanes 3–5 of Figure 4 are most likely due to conformation variations.

Solution structure of the Mi:2, Mi:3, and Mi:3/G51:C63 tRNAs

Accessibility of the D and T loops to oligonucleotide hybridization

tRNAs were made by in vitro transcription with T7 RNA polymerase and then labeled at the 5′ end with ^{32}P. For purposes of T7 RNA polymerase transcription, the tRNA transcripts contained a G as the first nucleotide instead of a C. These tRNA transcripts differ from each other in two respects. (1) Mi:3 and Mi:3/G51:C63 tRNAs have A11:U24 base pair in the D stem instead of a C11:G24 base pair in the Mi:2 tRNA. This could make the D stem and loop structure of the Mi:3 and Mi:3/G51:C63 tRNA less stable than that of the Mi:2 tRNA. (2) Mi:2 and Mi:3 tRNAs have an A51 × C63 mismatch in the T stem instead of a G51:C63 base pair in the Mi:3/G51:C63 tRNA. This could make the T stem and loop structure of the Mi:2 and Mi:3 tRNAs less stable than that of the Mi:3/G51:C63 tRNA. Oligonucleotides complementary to the D loop and the T loop were used to probe the accessibility of these loops to oligonucleotide hybridization in the tRNAs (Uhlenbeck et al., 1970). The extent of RNA:DNA hybrid formation was monitored using RNaseH, which hydrolyzes RNA only in an RNA:DNA duplex (Zarrinkar & Williamson, 1994). The results are shown in Figure 5. First, control samples show that there is no cleavage with RNaseH in the absence of the oligonucleotides (Fig. 5, lanes 1, 6, and 11). Second, with the D loop oligonucleotide, there is extensive cleavage within the D loop in the Mi:3 tRNA, less cleavage in the Mi:3/G51:C63 tRNA, and no cleavage in Mi:2 tRNA (Fig. 5, compare lanes 7, 8 with 12, 13 and 2, 3, respectively). Similarly, with the T loop oligonucleotide, there is substantial cleavage within the T loop in the Mi:2 and Mi:3 tRNAs but none in the Mi:3/G51:C63 tRNA (Fig. 5, compare lanes 4, 5 with 9, 10 and 14, 15 respectively). These results indicate a good correlation between the stability of the D and the T stems (see above) and the accessibility of the corresponding loops to hybridization with oligonucleotides. Preincubation of the tRNA transcripts with folding buffer containing Mg^{+2}

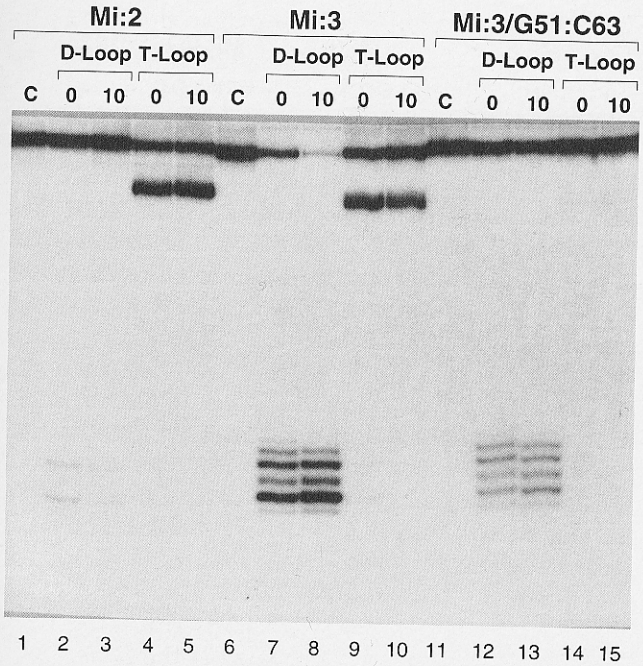


FIGURE 5. Oligonucleotide binding and cleavage with RNaseH. The hybridization reaction was performed with a large excess of the oligonucleotides and hybridization and cleavage with RNaseH was performed at 37 °C for 30 s. C, transcripts incubated in the absence of oligonucleotide. Time points 0 and 10 refer to the periods the tRNA are incubated in presence of folding buffer prior to the addition of D and T loop probes and RNaseH. Positions of cleavage were identified using oligonucleotide fragments present in RNaseT1 digests of the corresponding tRNAs as size markers.

for 10 min prior to addition of the oligonucleotide probes and RNaseH has essentially no effect on the extent of cleavage (Fig. 5, compare lanes 4 to 5, 7 to 8, 9 to 10, and 12 to 13). Therefore, if the in vitro transcripts fold into a "tRNA like tertiary structure" involving interaction between the D and the T loop nucleotides during the preincubation time, the oligonucleotide probes compete against the tertiary structure under the conditions used.

Fe(II)–EDTA cleavage

The Fe(II)–EDTA complex has been used for the generation of hydroxyl radicals to cleave the nucleic acid backbone of DNA and to footprint the DNA following its binding to proteins (Hertzberg & Dervan, 1984; Tullius & Dombroski, 1986). Latham and Cech (1989) have used this reagent to define the "inside and outside" of a folded RNA molecule. Because of the solvent-based nature of the metal complex, the cleaved positions on native tRNA define the exterior solvent-accessible surface. The regions of decreased cleavage are due to the exclusion of the reagent from the interior solvent-inaccessible regions of the RNA. The Mg^{+2}-dependent differences in the cleavage pattern have been interpreted to indicate the formation of divalent ion-dependent tertiary structure. An in vitro transcript of yeast $tRNA^{Phe}$ showed uniform cleavage in the absence of Mg^{+2}. Addition of Mg^{+2} significantly suppressed cleavage at phosphates 58, 59, 60, and, to a lesser extent, at phosphates 48, 49 and 18, 19, 20 (Latham & Cech, 1989). The cleavage of native yeast $tRNA^{Phe}$ was also similar, except that it was not dependent on the presence of Mg^{+2} ions (Huttenhofer & Noller, 1992).

Here, we have used this reagent to probe structural differences between the in vitro transcripts corresponding to the Mi:2, Mi:3, and Mi:3/G51:C63 tRNAs. The transcripts, labeled at the 5′ end, were treated with the reagent in the absence and presence of 10 mM Mg^{+2} and the products of cleavage were analyzed on denaturing gels (Fig. 6A). The sequence or secondary structure-independent nature of cleavage is consistent with the observed pattern of cleavage of the RNAs in the absence of Mg^{+2} (Fig. 6A, lanes 3, 7, 11). In the presence of 10 mM Mg^{+2}, the overall cleavage is significantly enhanced (Fig. 6A, lanes 4, 8, 12). A clear protection in cleavage is seen for the Mi:2 and Mi:3/G51:C63 tRNA transcripts in the presence of Mg^{+2} at phosphates 58, 59, 60 in the T loop (Fig. 6A, lane 4, 12). In contrast, this region in Mi:3 tRNA is not protected in the presence of Mg^{+2} (Fig. 6A, lane 8). The cleavage at different concentrations of Mg^{+2} ranging from 0.625 mM to 20 mM indicated maximal protection at 5 mM Mg^{+2} in both Mi:2 and Mi:3/G51:C63 tRNAs (data not shown).

Quantitative analysis of the products of Fe–EDTA cleavage

The dried gels were exposed to phosphorimager screens and the images were scanned. The scans for Mi:3 and Mi:3/G51:C63 tRNAs, plotted as line graphs, are shown in Figure 6B (I) and (II), respectively. The lane containing the products of partial cleavage with RNaseT1 served as markers and reference points to facilitate the alignment of the peaks in the scanned line graphs. The top and bottom panels are scans of Fe-EDTA reaction in the absence and presence of Mg^{2+}, respectively. Comparison of the top and bottom panels in Figure 6B (I) shows that, for the Mi:3 tRNA, there are no significant changes in the pattern of peaks except that the area of the peaks in the bottom panel is more than the corresponding peaks in the top panel. This is expected because we see an overall enhanced rate of cleavage in the presence of Mg^{2+}. A similar comparison (Fig. 6B, II) for the Mi:3/G51:C63 tRNA clearly indicates a decrease, at positions corresponding to phosphates 58, 59, and 60 in the bottom panel, when the Mi:3/G51:C63 tRNA is incubated in the presence of Mg^{2+} and then subjected to the Fe-EDTA-induced cleavage. These results suggest that the Mi:3/G51:C63 tRNA can form a stable tRNA-like tertiary structure, whereas the Mi:3 tRNA cannot. The sites of protection against Fe-EDTA cleavage seen in the Mi:3/G51:C63 tRNA are indicated

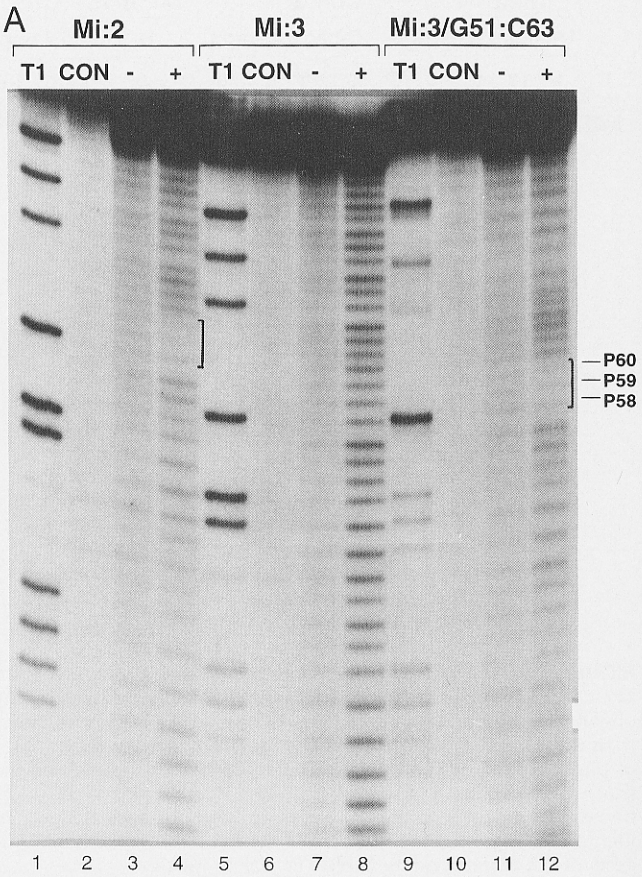
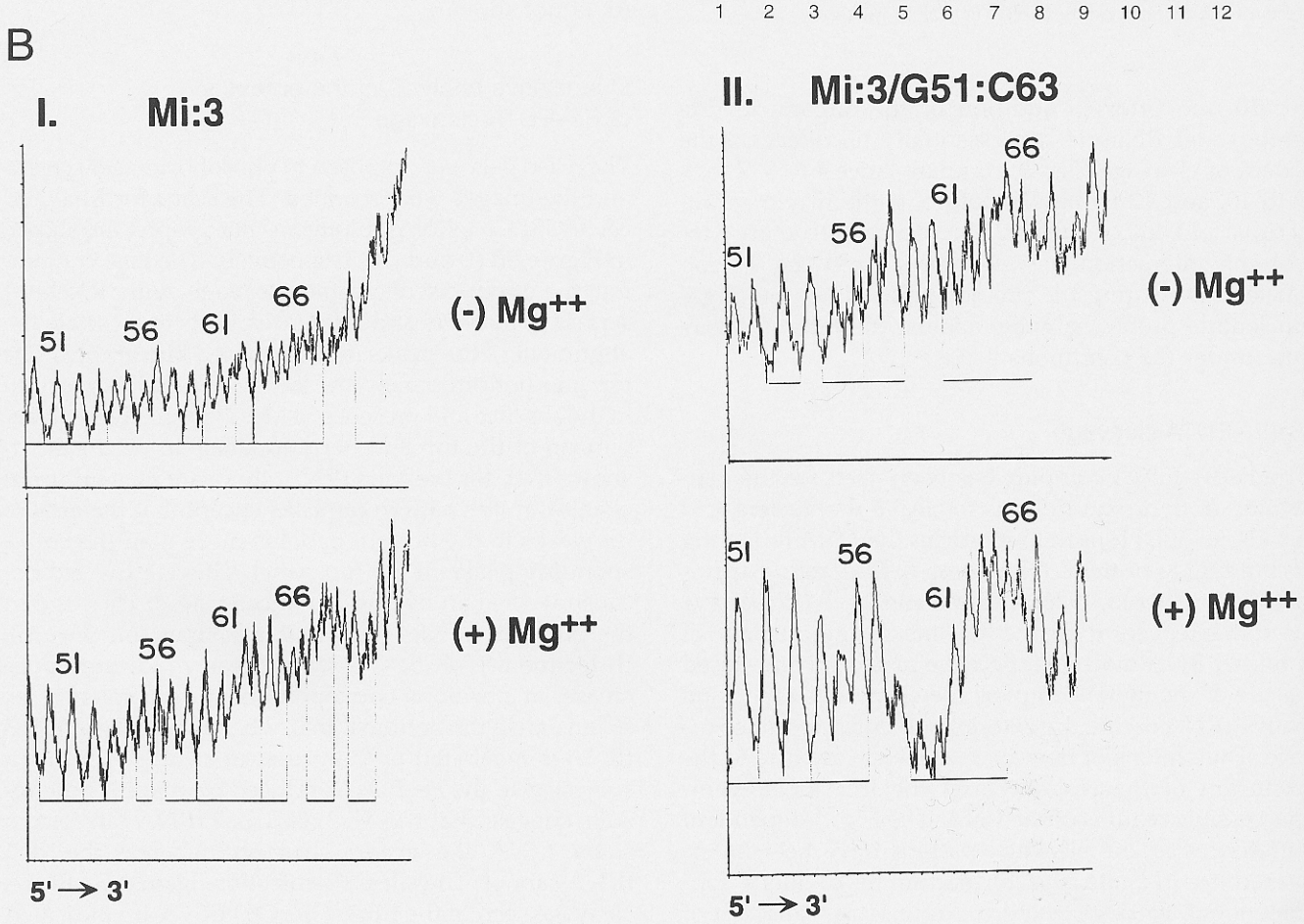


FIGURE 6. **A:** Cleavage of the 5′-end-labeled transcripts corresponding to Mi:2, Mi:3, and Mi:3/G51:C63 tRNAs by Fe(II)-EDTA. CON, transcript incubated in the absence of cleavage reagents; T1, partial RNaseT1 digest. (−) and (+) indicate the cleavage in the absence and presence of 10 mM Mg^{+2}. Phosphates that show differences in reactivity are indicated at the right and numbered from the 5′-terminal residue. **B:** Phosphorimager analysis and scanning of Fe(II)-EDTA cleavage reactions of Mi:3 (I) and Mi:3/G51:C63 (II) tRNA gene transcripts. Cleavage sites were identified using the fragments present in RNaseTI digests as size markers.



on the cloverleaf structure of the *E. coli* elongator $tRNA^{Met}$ in Figure 7.

Comparison of in vivo formylation levels of mutant tRNAs carrying A11:U24 vs. C11:G24 base pairs

The Mi:2 and Mi:3 mutant tRNAs differ only in that the former has a C11:G24 base pair, whereas the latter has a A11:U24 base pair. As described above, the Mi:3 tRNA does not accumulate in vivo, however, suppressors derived from the Mi:3 tRNA (Mi:3/A51:U63, Mi:3/G51:C63, and Mi:3/G51.U63) do. To study the role of the A11:U24 base pair conserved in eubacterial initiator tRNAs in formylation, we have compared the formylation levels of the Mi:3/A51:U63, Mi:3/G51:C63, and Mi:3/G51.U63 tRNAs with those of the Mi:2/A51:U63, Mi:2/G51:C63, and Mi:2/G51.U63 tRNAs, respectively. For this purpose, the mutant tRNAs were isolated from *E. coli* CA274 overproducing MetRS under acidic conditions, fractionated on acid-urea gels, and analyzed by northern blotting. The pattern of hybridization is shown in Figure 8. The assignment of the bands is shown on the sides (Li et al., 1996). The four bands of hybridization detected in the case of the

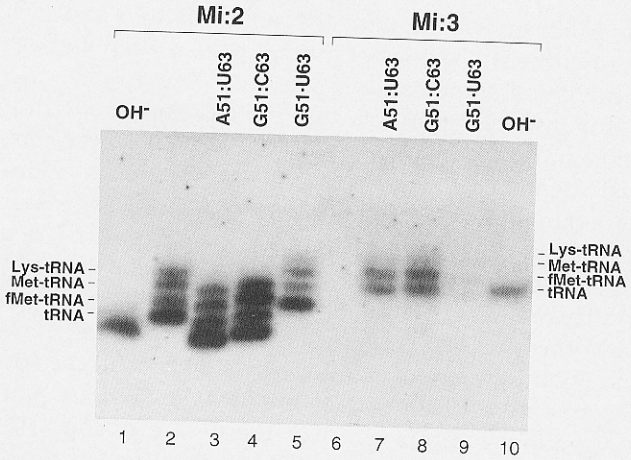


FIGURE 8. Acid-urea gel analysis of mutant tRNAs isolated from *E. coli* CA274 transformants carrying the mutant tRNA genes and overproducing MetRS. tRNAs were separated on 6.5% polyacrylamide gels and detected by northern blot analyses. The mutant tRNAs are indicated on the top with Mi:2 in lane 2 and Mi:3 in lane 6. Identification of tRNA bands for two of the lanes are indicated on the sides (lane 2, left side; lane 8, right side). Lanes 1 and 10 marked OH contain the deacylated tRNA samples of the Mi:2/G51:C63 and the Mi:3/G51:C63 tRNAs, respectively.

Mi:2 series of mutant tRNAs (Fig. 8, lanes 2–5) correspond, in order of increasing mobility, to Lys-tRNA, Met-tRNA, fMet-tRNA, and tRNA. The mutant tRNAs are normally aminoacylated with lysine in vivo, but the tRNA aminoacylated with lysine is an extremely poor substrate for formylation, hence there is no accumulation of fLys-tRNA (Li et al., 1996). In cells overproducing MetRS, part of the tRNA is now aminoacylated with methionine and the tRNA carrying methionine is now a substrate for formylation. Quantitation of radioactivity in Met-tRNA and fMet-tRNA bands shows 39.5–52.5% formylation of the Mi:2 series of mutant tRNAs (Table 2), suggesting that the changes in the T-stem at positions 51 and 63 do not have any significant effect on formylation. Interestingly, all of the Mi:3 series of mutant tRNAs (Fig. 8, lanes 7–9) show much higher levels of formylation, ranging from 75.8 to 82.6% (Table 2). Thus, the presence of A11:U24 base pair in the D stem instead of a C11:G24 base pair leads to an increase in formylation of the tRNA. As for

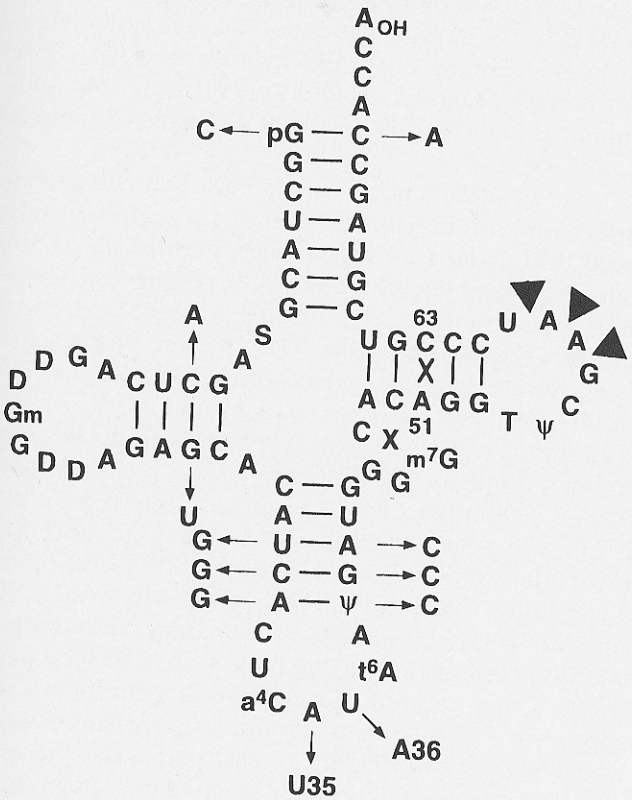


FIGURE 7. Localization of the phosphates on the cloverleaf structure of tRNA protected against cleavage by Fe–EDTA. The backbone sequence is that of *E. coli* elongator $tRNA^{Met}$. Arrows indicate the other changes explained in Figure 1. Darkened triangles represent phosphates 58, 59, 60 protected in the Mi:2 and Mi:3/G51:C63 tRNAs, but not in the Mi:3 tRNA.

TABLE 2. Extent of formylation of mutant tRNAs in vivo.

tRNA	% Formylation[a]	tRNA	% Formylation[a]
Mi:2	52.2	Mi:3	—[b]
Mi:2/A51:U63	51.6	Mi:3/A51:U63	82.6
Mi:2/G51:C63	39.5	Mi:3/G51:C63	75.8
Mi:2/G51:U63	52.5	Mi:3/G51:U63	80.2

[a]Derived from phosphorimager analysis of radioactivity in bands corresponding to fMet-tRNA and Met-tRNA (Fig. 8). % Formylation = [fMet-tRNA/fMet-tRNA + Met-tRNA] × 100.
[b]tRNA not produced in cells.

the Mi:2 series of mutant tRNAs, mutations in the T stem of the Mi:3 series of mutant tRNAs do not affect formylation significantly.

It is interesting to note that the electrophoretic mobility of the Mi:2 series of mutant tRNAs on the partially denaturing acid–urea gels (Varshney et al., 1991a) depends upon the presence or absence of a base pair and upon the nature of the base pair, Watson–Crick versus wobble, at positions 51 and 63 (Fig. 8, lanes 2–5). This suggests an effect on tRNA structure. Similarly, the Mi:3 suppressor tRNAs carrying the A11:U24 base pair all migrate significantly slower than the corresponding Mi:2 series carrying the C11:G24 base pair, suggesting a strong effect of the nature of the 11:24 base pair in the D stem on tRNA structure.

DISCUSSION

The surprising result of this work is that the detrimental effect of a C11:G24 to A11:U24 mutation in the D stem of Mi:3 tRNA (a mutant derived from *E. coli* elongator methionine tRNA) can be suppressed by single base mutations in the T stem that convert a A51 × C63 mismatch to A51:U63 or G51:C63 base pairs. In contrast to the Mi:3 tRNA, which does not accumulate in vivo, the Mi:3/G51:C63 and Mi:3/A51:U63 tRNAs accumulate in vivo and are active in initiation of protein synthesis. In vitro studies on processing of the Mi:3 transcript suggest that the most likely reason for the lack of accumulation of the Mi:3 tRNA in vivo is due to instability of the mature Mi:3 tRNA and its precursor (Fig. 2). Structural studies on the tRNAs using Fe-EDTA as a reagent suggest that the Mi:3 tRNA does not fold into a stable tRNA-like tertiary structure, whereas the Mi:3/G51:C63 tRNA does (Fig. 6A,B).

Why does mutation of the C11:G24 base pair to A11:U24 base pair have such a striking effect on accumulation of the tRNA, and how does the generation of a 51:63 base pair in the T stem compensate for the effect of the D stem mutation? There are no known interactions between bases in the D stem and those in the T stem. The most likely explanation is that the C11:G24 to A11:U24 mutation destabilizes the D stem and loop structure in such a way that bases in the D loop become more mobile. Similarly, the presence of a A51 × C63 mismatch in the T stem of the Mi:3 tRNA would destabilize the T stem and loop structure and make bases in the T loop more mobile. Although the mobility of the D loop or the T loop alone may not greatly affect tRNA structure and stability, the mobility of both the D loop and T loop in a tRNA would impose a high entropic barrier (de Pouplana et al., 1996) to tertiary interactions, within the T loop (the T54–A58 reversed Hoogsten base pair), and between the D and the T loops, which are critical for stabilization of the three-dimensional structure of the tRNA (reviewed in Dirheimer et al., 1995). Mutations that generate a base pair between bases 51 and 63 would stabilize the T stem and loop structure and thereby lower the entropic cost of the intra T loop and the D loop–T loop tertiary interactions. A prediction based on this interpretation is that the A11:U24 base pair can exist only in tRNAs that have a base pair between positions 51 and 63. Therefore, the effect of a C11:G24 to A11:U24 mutation on tRNA structure should depend upon the nature of bases of 51 and 63 in other tRNAs also. In other words, there is crosstalk between sequences in the D stem and in the T stem that is not obvious from the three-dimensional structure of tRNAs. Similarly, the effect of a A51 × C63 mismatch on tRNA structure should also be dependent on the tRNA context. Several lines of evidence summarized below support the above interpretations.

1. We have shown that the D loop is accessible to oligonucleotide hybridization and, therefore, probably "unstructured" in those tRNAs that contain an A11:U24 base pair (Fig. 5, lanes 7, 8, 12, and 13). Similarly, the T loop is accessible and, therefore, probably "unstructured" in those tRNAs that contain an A51 × C63 mismatch (Fig. 5, lanes 4, 5, 9, and 10). Although we have referred to A51 and C63 as being mismatches within the T stem, it is possible that A51 and C63 form a "wobble" base pair in which the A51 is protonated, as seen in the crystal structure of a deoxyribooligonucleotide (Hunter et al., 1987). However, such a base pair between A and C residues would still cause substantial destabilization of the RNA helix (Aboul-ela et al., 1985).

2. The A11:U24 base pair is found in all eubacterial and chloroplast initiator tRNAs. Out of approximately 3,000 tRNA and tRNA gene sequences in the tRNA database (Steinberg et al., 1993; R. Cedergren & S. Steinberg, pers. comm.), 56, including 33 initiator tRNAs and tRNA genes, have the A11:U24 base pair. Of the 56, 53 have a Watson–Crick base pair and 3 have a G.U wobble base pair between bases 51 and 63.

3. The A11:U24 base pair has been introduced into amber suppressor tRNAs derived from *E. coli* alanine (Hou & Schimmel, 1988), tryptophan (Smith & Yarus, 1989), and phenylalanine tRNAs (McClain & Foss, 1988). The results obtained with these tRNAs support our findings with the Mi:3/G51:C63, Mi:3/A51:U63, and Mi:3/G51:U63 tRNAs. The alanine and tryptophan suppressor tRNAs that have a G51:C63 base pair in the T stem accumulate in vivo and are active in suppression. The phenylalanine suppressor tRNA, which has a "weaker" U51:G63 base pair, is also present in cells; however, the tRNA is inactive in suppression and it is not known how much of this tRNA accumulates in cells (McClain & Foss, 1988). It is quite possible that the levels of this mutant tRNA are reduced and comparable to those seen in this work with the Mi:3/G51:U63 mutant tRNA (Fig. 4).

4. There are other examples of tRNAs that do not accumulate in vivo because they contain an A51 × C63 mismatch in the T stem. A mutant of the phage T4 serine ochre suppressor tRNA, which has an A51 × C63 mismatch in the T stem, is inactive in suppression and does not accumulate in vivo. Revertants derived from this tRNA that accumulate in vivo and that are active in suppression have regenerated either an A51:U63 base pair or a G51:C63 base pair in the T stem (McClain et al., 1988).

5. Another revertant of phage T4 serine ochre suppressor tRNA mutant that accumulates in vivo and is active in suppression has a change in the T loop from A57 to G57. In yeast $tRNA^{Phe}$, G57 stabilizes the tertiary interactions between the highly conserved G18 and G19 in the D loop and the Ψ55 and C56 in the T loop by intercalating between the G18:Ψ55 and the G19:C56 tertiary base pairs, and by formation of two hydrogen bonds between the N2-amino group of G57 and the O2′-ribose of G18 (Quigley & Rich, 1976). In the serine ochre suppressor tRNA, A57 is also able to intercalate between the G18:Ψ55 and G19:C56 base pairs, but is not able to form the two hydrogen bonds to O2′ of G18. Therefore, it is most likely that, in the G57 revertant derived from the phage T4 serine ochre suppressor tRNA, G57 compensates for the destabilizing effect of the A51 × C63 mismatch on the T loop structure by augmenting further the tertiary interactions between the D and the T loops and thereby the three-dimensional structure of the tRNA (McClain et al., 1988).

The base pairs in the D stem of tRNAs are involved in complex tertiary interactions with bases in the variable loop and with the purine at position 9. Therefore, another possible explanation for the striking effect of the C11:G24 to A11:U24 base pair mutation is that this mutation affects such tertiary interactions. However, although the 10:25 and 12:23 base pairs are involved in such tertiary interactions, the 11:24 base pair is not.

Another possibility is that mutation of the 11:24 base pair results in an alternate tertiary interaction, via triple base pair formation, between purine 9 and the 11:24 base pair instead of purine 9 and the 12:23 base pair, and this results in destabilization of the Mi:3 tRNA. Such an alternate pairing scheme has been proposed to explain the effect of covariations of nucleotides at position 9 and the 11:24 base pair on the ribosomal property of a suppressor tRNA derived from *E. coli* tryptophan tRNA (Smith & Yarus, 1989). We have changed A9 to G9 in the Mi:3 tRNA such that bases 9, 11, and 24 in the Mi:3 tRNA are now the same as in *E. coli* initiator $tRNA^{fMet}$. This additional mutation does not have a significant effect on the accumulation of the Mi:3 tRNA in vivo (C.P. Lee & U.L. RajBhandary, unpubl.). Therefore, we consider this possibility unlikely, at least in the sequence context of the Mi:3 tRNA.

The isolation of suppressor mutants of the Mi:3 tRNA has allowed us to compare directly the in vivo formylation levels of tRNAs differing only in having either a C11:G24 or A11:U24 base pair in the D stem. Results of such studies have shown that the A11:U24 base pair contributes to formylation of the tRNA in vivo (Table 2). These results are in agreement with our previous in vitro studies showing that mutation of the A11:U24 base pair to C11:G24 base pair in *E. coli* initiator tRNA resulted in an approximately sevenfold increase in the K_m for tRNA in formylation (Lee et al., 1991).

Finally, use of intragenic suppressors has in the past provided much useful information on structure, biosynthesis, and function of tRNAs (Altman & Smith, 1971; Anderson & Smith, 1972; McClain et al., 1988). Use of this approach has, in the current work, uncovered an unsuspected connection between the structure and stability of the D stem and the T stem of tRNAs.

MATERIALS AND METHODS

Strains and plasmids

E. coli strain CA274 (*HfrH lacZam trpEam*) was used in this work. The mutant Mi:3 tRNA gene and the CAT reporter gene were cloned into the pRSV vector to yield pRSV-CATam1.2.5 (amp^r) (Varshney et al., 1993). The gene for *E. coli* MetRS was cloned into a compatible pACYC vector to yield pACMS3 (kan^r) (Varshney & RajBhandary, 1992). The plasmids were selected for in the presence of ampicillin (100 μg/mL) and kanamycin (25 μg/mL). Chloramphenicol was used at a concentration of 50 μg/mL to select for the cells in which the mutant tRNAs initiate translation from the CATam1.2.5 mRNA. When selected for growth at 37 °C in presence of all the three antibiotics, cells harboring Mi:2 tRNA gene grow, whereas the cells harboring the Mi:3 tRNA gene fail to grow.

Mutagenesis of *E. coli* and selection for chloramphenicol resistance

E. coli CA274 harboring the pRSVCATam1.2.5 plasmid with the Mi:3 tRNA gene and the pACMS3 plasmid (Fig. 3) was grown in 2× YT medium containing ampicillin and kanamycin. Random mutagenesis was performed with *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) as described (Miller, 1992). The cells were treated with the mutagen at a final concentration of 50 μg/mL, washed twice with 0.1 M KH_2PO_4, pH 7.0, and used to generate overnight cultures by growing in 2× YT in the presence of ampicillin and kanamycin at 37 °C with aeration. These cultures were then selected for growth on 2× YT plates containing ampicillin, kanamycin, and chloramphenicol [AKC plates]. Single chloramphenicol-resistant colonies isolated from such selections were grown in liquid cultures in presence of ampicillin and kanamycin and streaked for growth on AKC plates. Plasmid DNAs were prepared from chloramphenicol-resistant cultures and screened by sequencing the tRNA gene, leading to the identification of a C63 → U63 mutation in the Mi:3

tRNA gene. This mutant gene was named Mi:3/A51:U63 tRNA. An ~450-bp *Pst* I + *Bam*HI fragment bearing the mutant tRNA gene was cut from this plasmid and cloned at the corresponding sites of pRSVCATam1.2.5 (Fig. 2). The entire tRNA gene and the flanking region was sequenced again to confirm the C63 → U63 change; the resistance to chloramphenicol was also verified.

Site-specific mutagenesis of tRNA genes

The mutant tRNA genes, Mi:3/G51:C63 carrying a G51:C63 Watson-Crick base pair and Mi:3/G51.U63 carrying a G51.U63 wobble base pair, were constructed by site-specific mutagenesis. Similar mutations were introduced into the Mi:2 tRNA gene to yield Mi:2/G51:C63, Mi:2/A51:U63, and Mi:2/G51.U63 mutant tRNAs.

Isolation of RNAs for northern blot analysis

Total RNA was isolated from *E. coli* CA274 expressing the mutant tRNAs at pH 8.0 and at room temperature and fractionated on a 12% native polyacrylamide gel (Lee et al., 1991). For comparing the levels of formylation, RNA was isolated from *E. coli* CA274 expressing the mutant tRNAs and MetRS under acidic conditions (Varshney et al., 1991a) and subjected to PAGE under acidic conditions. Nucleic acids were electroblotted onto a Nytran plus membrane (Schleicher & Schuel), baked for 4 h at 70 °C, and washed sequentially at room temperature and at 42 °C for 30 min each with 4× SET + 1% SDS (1× SET = 0.15 M NaCl, 0.03 M Tris-HCl, 2 mM Na_2EDTA, pH 8.0). Prehybridization was at 42 °C for 4 h in 4× SET containing 250 μg/mL sheared denatured salmon sperm DNA, 0.1% SDS, and 10× Denhardt's solution (1× Denhardt's solution = 0.02% BSA, 0.02% polyvinylpyrrolidone 40, and 0.02% Ficoll). Hybridization was performed with a 5′-^{32}P-labeled oligodeoxyribonucleotide complementary to nt 25–45 of Mi:2 tRNA (2×10^6 cpm/mL) in the same solution for 12–16 h. For the experiment described in Figure 4, a 5′-^{32}P-labeled oligodeoxyribonucleotide complementary to nt 2–44 of *E. coli* $tRNA^{Tyr}$ was used simultaneously with the Mi:2 tRNA probe. Membranes were washed three times with 3× SET + 0.2% SDS at 42 °C for 30 min each and once at room temperature with 1.5× SET + 0.1% SDS for 30 min and subjected to autoradiography.

Analysis of formylation levels of mutant tRNAs

The amount of radioactivity in the bands corresponding to the fMet-tRNA and Met-tRNA (Fig. 8) was quantitated using Phosphorimager (Molecular Dynamics). % Formylation is defined as the ratio of the radioactivity of fMet-tRNA to (fMet-tRNA + Met-tRNA) × 100.

Assays for chloramphenicol acetyl transferase activity in extracts

E. coli CA274 carrying the plasmids pRSVCATam1.2.5 with the mutant tRNAs and pACMS3 were grown at 37 °C overnight in 2× YT medium containing ampicillin and kanamycin. This culture was inoculated into fresh media containing the antibiotics and grown for 4 h at 37 °C. Cell extracts were prepared and assayed for CAT and β-lactamase as described (Varshney et al., 1991b). The CAT activities were normalized to β-lactamase activity to account for any possible fluctuations in copy number of the pRSVCATam1.2.5 plasmid. The relative CAT activity in cells carrying the Mi:2 mutant tRNA was fixed at 100%.

In vitro transcription of the tRNA genes and processing of the transcripts

Either 0.3 μg of the supercoiled plasmid (pBR322trnfM carrying the $tRNA_2^{fMet}$ gene) or 0.1 μg of purified restriction fragments (*Pst* I + *Bam*H I, ~ 450 bp) carrying the promoter and termination sequences of the *E. coli* $tRNA_2^{fMet}$ gene and encoding the respective tRNAs (U35A36 mutant of $tRNA_2^{fMet}$ or Mi:3) was used as a template for transcription by *E. coli* RNA polymerase. The incubation mixture (25 μL) contained 40 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 0.1 mM Na_2EDTA, 0.1 mM DTT, 150 mM KCl, 500 μg/mL BSA, 250 μM each of all the four NTPs (Promega), 40 μCi of [α-^{32}P]GTP (3,000 Ci/mmol), and 1.5 units of *E. coli* RNA polymerase (Boehringer Mannheim). Incubation was at 37 °C for 30 min. Products were analyzed on 6% polyacrylamide gels containing 8 M urea. Transcripts were located by autoradiography of the wet gels and eluted from the gels by incubating the gel pieces at 37 °C overnight in 0.5 mL of 0.3 M sodium acetate, 1 mM Na_2EDTA, and 5% phenol. Labeled RNA was recovered by precipitation with three volumes of ethanol in the presence of total yeast RNA (20 μg/mL) and dissolved in 10 mM Tris-HCl, pH 8.0, 1.0 mM Na_2EDTA. Aliquots containing ~ 75,000 cpm of radioactivity were diluted to 50 μL with transcription buffer. Processing was started by addition of 6 μL of an *E. coli* S-100 extract (~30 μg) to each reaction and incubating at 37 °C. Aliquots (10 μL) were removed before (0 min) or at various times after addition of the S-100 extract (5, 15, 30, and 60 min) and extracted with phenol. The RNAs were precipitated with ethanol in the presence of carrier RNA and analyzed on 0.4-mm thick 7% polyacrylamide 8 M urea gels. As a control for nuclease activity in the S100 extracts and also for an exact size marker, 5′-^{32}P-labeled $tRNA_2^{fMet}$ was also treated under similar conditions.

T7 RNA polymerase transcription of the tRNA genes and 5′-^{32}P labeling of the tRNA transcript

PCR was used to amplify tRNA coding sequences for transcription. The forward primer for PCR has the promoter sequence for the bacteriophage T7 RNA polymerase (Milligan & Uhlenbeck, 1989). The underlined sequence in these primers corresponds to the 5′ end of tRNAs. The reverse primer (A72R), which begins with TGG, encodes the CCA 3′ end of the tRNA transcripts.

1. T7G1F Mi:3: 5′ TAATACGACTCACTATAGGCTACGTAG ATCAGTT 3′.
2. T7G1F Mi:2: 5′ TAATACGACTCACTATAGGCTACGTAG CTCAGTT 3′.
3. A72R Mi:3: 5′ TGGTTGCTACGACGGG 3′.

PCR was performed using the plasmids pRSVCATam1.2.5/Mi:2, pRSVCATam1.2.5/Mi:3, or pRSVCATam1.2.5/Mi:3/

G51:C63 as templates and *Taq* DNA polymerase (Boehringer Mannheim) for 20 cycles (94 °C for 1 min; 50 °C for 1 min; 72 °C for 20 s) followed by a final extension at 72 °C for 5 min. Products were extracted with phenol and chloroform and precipitated with ethanol. Approximately 100 ng of the PCR product was used as template for transcription with T7 RNA polymerase. The reaction (15 μL) contained 40 mM Tris-HCl, pH 8.0, 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl, 10 mM DTT, 0.5 mM each of all four NTPs, and 10 units of T7 RNA polymerase (Promega). Incubation was at 37 °C for 60 min. Products were analyzed on 15% polyacrylamide gels containing 8 M urea. tRNA transcripts were located by UV shadowing and eluted from the gel. tRNAs were dephosphorylated using calf intestinal alkaline phosphatase (Boehringer Mannheim), 5′-end labeled using [γ-^{32}P]ATP and polynucleotide kinase (Silberklang et al., 1979) and the labeled tRNAs were purified by electrophoresis as described above.

Oligonucleotide hybridization and RNaseH cleavage

A deoxy undecanucleotide (CTAACCAACTG) complementary to the D loop and the adjoining base pair in the D stem and a nonanucleotide (GATTCGAAC) complementary to the T loop and the adjoining base pair in the T stem of the *E. coli* elongator methionine tRNA were used as probes. The 5′-end labeled transcripts corresponding to Mi:2, Mi:3, and Mi:3/G51:C63 tRNAs were denatured by heating to 70 °C for 1 min in 10 mM Tris-HCl, pH 8.0 (5 μL; ~10,000 cpm), and left at 37 °C for 3 min. Zero time point refers to the time of addition of 5 μL of 2× folding buffer (20 mM Tris-HCl, pH 8.0, 20 mM KCl, 10 mM $MgCl_2$, 0.1 mM Na_2EDTA, 0.1 mM DTT) containing DNA oligonucleotide probe (final concentration ~10 μM) and RNaseH (Amersham, final concentration 0.1 U/μL), to the equilibrated RNA sample. The RNaseH cleavage (final volume 10 μL) was performed at 37 °C for 30 s and stopped by the addition of 10 μL stop buffer (gel loading dyes in 50 mM Na_2EDTA and 8 M urea) and freezing in dry ice. For the other time point (10 min), 2.5 μL of 3× folding buffer was added to the equilibrated tRNA and tRNA was allowed to fold at 37 °C for 10 min. Cleavage was initiated by the addition of 2.5 μL of 1× folding buffer containing the appropriate oligonucleotide and RNaseH, left for 30 s at 37 °C, and stopped as before. Products were resolved on 15% polyacrylamide gels containing 8 M urea.

FeII–EDTA cleavage reactions

The reaction (10 μL) contained ~10^5 cpm of 5′-^{32}P-end labeled tRNA in the absence or presence of 10 mM Mg^{2+} in 10 mM Tris-HCl, pH 8.0 (Latham & Cech, 1989; Celander & Cech, 1991). The tRNA solution (7 μL) was heated at 70 °C for 1 min and allowed to equilibrate at 37 °C for 20 min prior to the addition of reagents. One microliter each of 10 mM $FeSO_4(NH_4)_2SO_4 \cdot 6H_2O$, 20 mM EDTA, and 5 mM DTT was added and the incubation continued for 90 min at 37 °C. The reaction was quenched by the addition of 10 μL of a solution containing 8 M urea, 40 mM thiourea, and gel loading dyes, and analyzed on 15% polyacrylamide gels containing 8 M urea. The gels were dried and quantitated by phosphorimaging (Molecular Dynamics Image Quant Software) using area integration of the bands in individual lanes. Products of partial cleavage with RNase T1 served as markers to identify the bands.

ACKNOWLEDGMENTS

We thank Drs. Bob Cedergren and Sergey Steinberg for help with access to the tRNA sequence database and Drs. Paul Schimmel, Jamie Williamson, Christophe Bruel, and Anne Kowal for comments and suggestions on this manuscript. We thank the reviewers of this manuscript for a very prompt and thorough review. We also thank Annmarie McInnis for her patience and care in the preparation of this manuscript. This work was supported by grant GM17151 from the National Institutes of Health.

Received August 1, 1997; returned for revision August 14, 1997; revised manuscript received September 1, 1997

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