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Structural Requirements for the Formation of 1-Methylguanosine *in vivo* in $tRNA^{Pro}_{GGG}$ of *Salmonella typhimurium*

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Maturation of tRNA and rRNA and the assembly of the ribosome in all organisms occurs *in vivo* in a complex pathway in which various proteins such as endo- and exonucleases, tRNA and rRNA modifying enzymes and ribosomal proteins, act concomitantly and temporarily during the maturation process. One class of RNA binding proteins are the tRNA modifying enzymes, which catalyse the formation of various modified nucleosides present in tRNA. Here we analyse the consequences of various alterations in a tRNA on the formation of modified nucleosides in the tRNA and the aminoacylation of it under true *in vivo* conditions, i.e. in a cell with normal amounts of the tRNA substrate and the tRNA binding protein. We have devised a selection method to obtain mutants of $tRNA^{Pro}_{GGG}$ in *Salmonella typhimurium* that may no longer be a substrate *in vivo* for the tRNA(m^1G37)methyltransferase. These mutant tRNAs were purified from cells in balanced growth by a solid phase hybridisation technique and the presence of 1-methylguanosine (m^1G) in position 37 next to the anticodon was monitored. Of 13 different mutant $tRNA^{Pro}_{GGG}$ species analysed, eight of them had a drastically reduced level of m^1G. Some of these mutant tRNA species had alterations far from the nucleotide G37 modified by the enzyme; e.g. base-pair disruptions in the first, fourth and eighth (last) base-pair of the acceptor stem, in the D-stem, and in the top of the anticodon stem. The structure of all the mutant $tRNA^{Pro}_{GGG}$ species must deviate from the wild-type form, since they all induced +1 frameshifting. Still, $tRNA^{Pro}_{GGG}$ from five of the mutants had normal levels of m^1G. Thus, only a subset of mutations, all inducing an altered tRNA structure, resulted in m^1G deficiency. However, those alterations in $tRNA^{Pro}_{GGG}$, which influenced the tRNA(m^1G37)-methyltransferase activity, did not affect *in vivo* the formation of four other modified nucleosides and the aminoacylation of $tRNA^{Pro}_{GGG}$, demonstrating the extreme dependence of the tRNA(m^1G37)methyltransferase on an almost perfect three-dimensional structure of the tRNA. We discuss that the conformation of the anticodon loop may be a major determining element for the formation of m^1G37 *in vivo*.

Keywords: RNA-protein interactions; tRNA modifying enzyme; 1-methylguanosine; tRNA(m^1G37)methyltransferase; proline tRNA synthetase

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Abbreviations used: m^1G37, 1-methylguanosine present in position 37 (next to and 3′ of the anticodon) of the tRNA; Ψ, pseudouridine;a base substitution of G to A in position 72 of tRNA is denoted G72A and likewise for other alterations in the tRNA; tRNA(m^1G37)methyltransferase denotes the enzyme catalysing the formation of m^1G37; $tRNA^{Pro}_{GGG}$ denotes the proline specific tRNA with the anticodon 5′-GGG-3′; *trmD* is the structural gene for the tRNA(m^1G37)methyltransferase (TrmD peptide); ProRS and AaRS denote the proline-tRNA synthetase and any aminoacyl-tRNA synthetase, respectively.

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Introduction

During recent years much attention has been directed towards understanding how an RNA binding protein recognises its substrate (Draper, 1995). The intriguing aspect of RNA-protein interaction arose first in relation to how various aminoacyl-tRNA synthetases (AaRS) recognise their substrates, the tRNAs. These latter systems are presently the richest available sources of information and have been the major experimental systems to unravel some determinants for RNA-protein interactions. The selection of the appropriate tRNA for the attachment of the corresponding amino acid requires specific contacts between the AaRS and the tRNA. The sites have been identified by various techniques and several reviews on this topic have been published (see recent reviews: Giegé *et al*., 1993; McClain, 1993; Schulman, 1991). One important reason for the advancement in this area during recent years is the possibility to construct *in vitro* tRNA and other RNA species with specific alterations in the primary sequence of the RNA of interest (Sampson & Uhlenbeck, 1988). Such altered tRNAs have been used in various *in vitro* assays and important knowledge has been obtained regarding the determinants of the RNA-protein recognition process. However, *in vivo* all RNA species, including tRNA, are substrates for several RNA binding proteins. Transfer RNA and rRNA undergo complex maturation reactions in which several endo- and exonucleases, besides the tRNA modifying enzymes, act simultaneously on the primary transcript resulting in a mature tRNA, which in turn is a substrate for the aminoacyl-tRNA synthetases as well as other various proteins such as the elongation factors. Some of the modification reactions may even occur after the amino acid has been added to the tRNA. Furthermore, the same tRNA may interact with cognate as well as some non-cognate synthetases together with some late-acting tRNA modifying enzymes. Some of the tRNA modifying enzymes may even be unable to act upon a tRNA that has already accepted an amino acid or that has already been modified at another position provided that there is temporal synthesis of modified nucleosides (Björk, 1995a,b). If so, one interaction between a protein and the tRNA may inhibit the reaction of another tRNA-protein interaction. Clearly, the *in vivo* conditions are quite distinct from the conditions employed in an *in vitro* experiment, which is usually performed using a purified enzyme and a pure RNA species. Thus, the *in vitro* experiments are not performed under conditions reflecting the *in vivo* situation in which the enzyme of interest competes with other proteins having affinity for the same RNA molecule. Furthermore, many of the *in vitro* experiments have been performed using a completely unmodified RNA. Therefore, it would be highly advantageous to have a system in which one can study various mutations in the tRNA and their influence on a specific enzymatic reaction during true *in vivo* conditions; i.e. in a cell in balanced growth with normal gene copies for both the substrate and the enzyme of interest. Although a few *in vivo* assay methods have been developed to analyse the influence on various mutations in the tRNA on the aminoacylation or the modification-reaction (Grosjean *et al*., 1987; Schulman, 1991), they all have various limitations (see Discussion) and none measures the formation of the enzymatic product under true *in vivo* conditions as defined above. This paper describes such a true *in vivo* system in which the formation of 1-methylguanosine (m^1G), which is present in the anticodon of seven bacterial tRNAs, is monitored in various mutants of one specific tRNA species, the $tRNA^{Pro}_{GGG}$.

Transfer RNA of *Escherichia coli* or *Salmonella typhimurium* contains 31 different modified nucleosides of which 29 have been identified (Limbach *et al*., 1994). The synthesis of these modified nucleosides requires about 45 different tRNA modifying enzymes. The modified nucleosides in tRNA are important for accurate protein synthesis but also for some regulatory functions that tRNA has (Björk, 1995a). A few modified nucleosides, like the m^1G, are present in the same subset of tRNA from all organisms and may have been present in the tRNA of the progenitor (Björk, 1986). Indeed, the tRNA(m^1G37)methyltransferase from several organisms evolutionary far apart, show stretches of amino acid sequences of high similarities (J.–N. Li, & G.R.B., unpublished observation). The function and the mechanism by which the tRNA(m^1G37)-methyltransferase recognises the tRNA may therefore be similar in bacteria and in all other organisms.

From comparison of primary sequences of different tRNAs, it was suggested that the modifying enzymes may recognise specific nucleotide motifs (Tsang *et al*., 1983); e.g. the formation of N^6-isopentenyladenosine (i^6A) in position 37 requires a specific A36-A37-A38 sequence and a five-base-paired anticodon stem that has an A,U(30)·Ψ,A(39) base-pair and G·C base-pairs at positions 27, 28·42, 43 (Grosjean *et al*., 1985). Another modifying enzyme, the tRNA-guanine transglycosylase, requires a specific sequence motif: the U33-G34-U35 sequence in the anticodon loop (Curnow *et al*., 1993; Nakanishi *et al*., 1994). However, tRNA modifying enzymes catalysing the formation of some other modified nucleosides present in the anticodon region (including the enzymes for the formation of m^1G37, wybutosine(yW37) and queuosine(Q34)) recognise not only a specific nucleotide sequence within the anticodon loop, close to the target nucleotide, but also more general features of the tRNA molecule (Grosjean *et al*., 1987, 1990). The major determinant for the tRNA (m^5U54)methyltransferase, which catalyses the formation of 5-methyluridine (m^5U) in position 54 of all tRNA species in *E. coli* and *S. typhimurium*, requires sequences in both the T-stem and in the T-loop (Gu & Santi, 1991). Systematic studies of yeast $tRNA^{Asp}$ *in vitro* revealed that the important struc-

tural requirements for the tRNA(m_2^2G26)methyltransferase (N^2,N^2-dimethylguanosine, m_2^2G), are not only the nearby nucleotides but also the presence of the two D stem base-pairs (C11·G24, G1·C25) as well as the three-dimensional structure (Edqvist *et al*., 1992, 1993, 1994). By injecting various unmodified yeast $tRNA^{Asp}$ into *Xenopus* oocytes and following the formation of different modified nucleosides, Grosjean and co-workers (Grosjean *et al*., 1996) suggested that there are two classes of oocyte tRNA modifying enzymes. One class, which catalyses the formation of modified nucleosides (m^1G37, m_2^2G26, Q34, Ψ40) in the anticodon region, in the D-stem and in the hinge between these two sub-domains, is sensitive to the overall structure of the tRNA. The other class of oocyte enzymes seems to require primarily local structural motifs such as the T-loop (m^5U54, Ψ40), the acceptor stem (m^2G6) or the anticodon loop and stem (Ψ32). The *E. coli* tRNA(m^1G37)methyltransferase may show similarity to the enzyme responsible for the formation of m^1G37 in yeast $tRNA^{Asp}$, since the secondary or tertiary structure of the tRNA molecule is important for recognition (Holmes *et al*., 1992). Since the activity of the tRNA(m^1G37)methyltransferase is inhibited by the dinucleotide GpG, the enzyme may accommodate the G36-G37 dinucleotide in the anticodon loop suggesting that this enzyme primarily recognises these two bases in the anticodon (Holmes *et al*., 1995). Although the structural requirements for several tRNA modifying enzymes mentioned above have been identified *in vitro*, it is not clear whether these requirements are valid for the conditions present *in vivo* (see above).

In this study we have devised a genetic selection method based on the fact that lack of m^1G of $tRNA^{Pro}_{GGG}$ mediates suppression of a specific set of frameshift mutations (Björk *et al*., 1989; Hagervall *et al*., 1993). Therefore, one subclass of +1 frameshift suppressor derivatives of $tRNA^{Pro}_{GGG}$ in *S. typhimurium* should be altered in the primary sequence of the tRNA in such a way that it no longer is a substrate *in vivo* for the tRNA(m^1G37)methyltransferase (TrmD protein). Such mutant tRNAs were purified by a solid phase hybridisation technique that overcomes the problem of labour-intensive tRNA purification. From the modification patterns of the mutant tRNAs, we were able to identify, for the first time, how structural changes in the primary sequence far from the target nucleotide influenced the catalytic properties of an RNA maturation enzyme under true *in vivo* conditions.

Results

Isolation of mutants that are not substrates for the tRNA(m^1G37)methyltransferase

Seven tRNA species ($tRNA^{Leu}_{1,2,3}$; anticodons CAG, GAG and UAG; $tRNA^{Pro}_{1,2,3}$, anticodons CGG, GGG, cmo^5UGG; and $tRNA^{Arg}_3$, anticodon CCG) in *E. coli* and *S. typhimurium* have m^1G at position 37, next to the 3′ end of the anticodon. In a standard Watson–Crick base-pair between G and C, the hydrogen of the nitrogen atom in position 1 of G is involved in a hydrogen bond to C. A methyl group at position 1 of G, which results in m^1G, blocks such a Watson–Crick base-pairing. Thus, an unmodified G at position 37 of a tRNA has therefore an ability to pair with C in the mRNA. In fact, a mutation in the structural gene (*trmD*) for the tRNA(m^1G37)methyltransferase, which results in lack of m^1G37, also induces suppression of certain frameshift mutations in the *his*-operon (Björk *et al*., 1989). It was further shown that $tRNA^{Pro}_{GGG}$ is one possible suppressor tRNA (Hagervall *et al*., 1993) and it was suggested that lack of m^1G allowed a quadruplet translocation, thereby inducing a shift in the reading frame. Thus, one way to change the $tRNA^{Pro}_{GGG}$ to frameshift is by m^1G37 deficiency caused by a defective tRNA(m^1G37)methyltransferase. However, other structural changes in the tRNA, such as an insertion of an extra G in the anticodon loop as in the *sufB2* frameshift suppressor derivative of $tRNA^{Pro}_{GGG}$ (Riddle & Roth, 1972; Sroga *et al*., 1992), may also promote frameshifts. Another way to create m^1G37 deficiency in a tRNA is to mutate the tRNA in such a way that it is no longer a substrate for the tRNA(m^1G37)methyltransferase. Since such mutants induce the ability to suppress certain frameshift mutations, they can be selected and an analysis of them should give information of the recognition elements for the tRNA(m^1G37)-methyltransferase. Moreover, if selected *in vivo*, the chromosomal mutations are derived under physiological conditions in which the formation of m^1G37 competes with the rest of the tRNA maturation machinery. Thus, analysis of such mutants should give information about the substrate requirement for the tRNA(m^1G37)methyltransferase under *in vivo* conditions in which the level of both the substrate tRNA and the enzymes of interest are the same as in the wild-type cell. The *trmD* gene and the structural gene for $tRNA^{Pro}_{GGG}$ (*proL*$^+$) are well separated on the *Salmonella* chromosome, which make it possible to mutate specifically the *proL* gene without changing the allelic state of the *trmD* gene.

To specifically introduce base substitutions in the $tRNA^{Pro}_{GGG}$, encoded by the *proL* gene, a P22 phage stock grown on strain GT1381(*proL*$^+$, *zef-2502*::Tn*10*; 95% co-transduced to *proL*) was mutagenized by hydroxylamine, which causes C to U and A to G transitions (Freese *et al*., 1961). Strain GT1405 (*hisO1242, hisD3749*) was transduced with the mutagenized phage stock and Tet^R transductants were selected. The *hisD3749* mutation is a frameshift mutation suppressed by a $tRNA^{Pro}_{GGG}$ lacking m^1G37 (Hagervall *et al*., 1993). In this way, we introduced mutations in the area of the chromosome where the *proL*$^+$ gene is located. The His^+ phenotype, which results from the suppression of the *hisD3749* mutation, was scored within 48 hours. His^+ transductants were obtained at a frequency of about 10^{-3} among the Tet^R clones. All

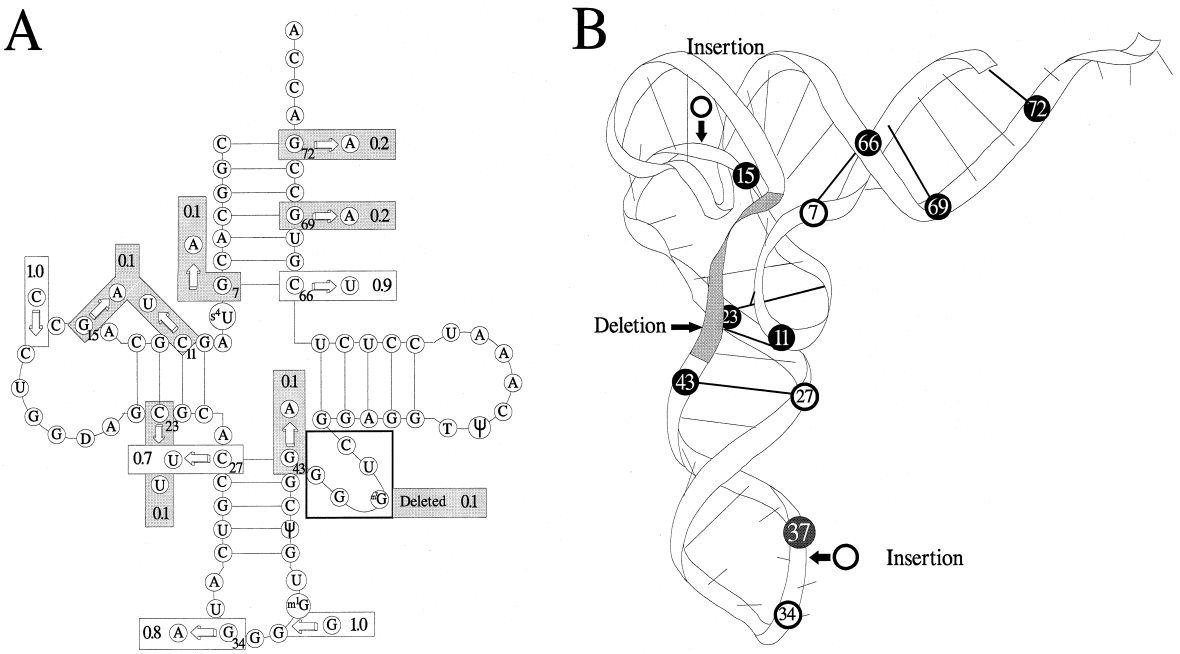

Figure 1. A, Diagram of $tRNA^{Pro}_{GGG}$ showing the various mutations in the *proL* gene and the corresponding m^1G level in $tRNA^{Pro}_{GGG}$. Shaded bars indicate m^1G level significantly different from the level in the wild-type; open bars indicate the same level of m^1G as in wild-type. Arrows pointing between nucleotides (between C16 and C17 or between G36 and m^1G37) indicate insertions. The box including the variable loop (nucleotides 44 to 48) indicates deletion of this loop. B, The various *proL* mutations with indicated level of m^1G in the corresponding mutant tRNA in a tertiary structure deduced from yeast $tRNA^{Phe}$. Filled circles indicate m^1G37 level significantly different from the wild-type level whereas open circles indicate the same level of m^1G37 as in the wild-type.

mutations, which resulted in a His^+ phenotype, were mapped by three-factor-crosses using a closely linked *zef-2516::*Tn*10d*Cm located on the opposite side of *proL* as compared to *zef-2502::*Tn*10*. Such genetic analysis suggested that all the mutations inducing the ability to suppress the *hisD3749* mutation were in the $proL^+$ gene region (data not shown).

DNA alterations in the $tRNA^{Pro}_{GGG}$ gene of the various mutants

The wild-type gene ($proL^+$) for $tRNA^{Pro}_{GGG}$ is located at minute 45 of the *S. typhimurium* chromosome and the gene has been cloned and sequenced (Kuchino *et al*., 1984; Sroga *et al*., 1992). The tRNA sequences deduced from the DNA sequences are shown in Figure 1 and Table 1. All mutations, except *proL214*, which is a deletion of four bases, were either G to A or C to T transitions, which are expected from the known mutagenic specificity of hydroxylamine. Mutants *proL202* and *proL203*, which had an insertion of a C in the dihydrouridine (D) loop, were obtained earlier after mutagenesis by nitrosoguanosine (Riddle & Roth, 1972; Sroga *et al*., 1992). Four of the mutants contained base substitutions in the acceptor stem (*proL208, proL210, proL211 and proL217*). Two mutants had insertions and three mutants had base substitutions in the D-stem and loop (*proL202, proL203, proL212, proL213, proL215*). Three mutants had base substitutions and one mutant had an insertion in the anticodon stem and loop including one substitution of the wobble base (*proL201, proL207, proL209*), and one mutant contained a deletion of the extra arm (*proL214*). Furthermore, one mutant (*proL209*) had three base substitutions (G to A transition in positions 37, 39 and 43) in the anticodon stem and loop. The striking feature of these mutants is that the base substitutions were scattered all over the tRNA molecule, except in the T-stem and loop.

Modification pattern of the wild-type tRNA

We wanted to determine the level of m^1G and other modified nucleosides of $tRNA^{Pro}_{GGG}$ and its aminoacylation during balanced growth of cells having wild-type gene copy number of the tRNA gene, of genes encoding the modifying enzymes and of the gene encoding the proline tRNA synthetase (ProRS). We therefore labelled tRNA of wild-type cells and of the various mutants with ^{32}P for two to three generations at 37°C in Mops-glucose minimal medium. Transfer RNA was purified by 12% PAGE and hybridised to the complementary strand of the wild-type or the corresponding mutant gene of $tRNA^{Pro}_{GGG}$. After hybridisation under stringent conditions the product migrated as a single band on 12% PAGE (Figure 1 A). Such a purified tRNA was subjected to nuclease P_1 diges-

Table 1. DNA sequence of various *proL* mutants of *S. typhimurium*

Mutants	Position in tRNA	Mutagen	Mutations	Reference
Acceptor stem:				
1 *proL208*	66	Hydroxylamine	C66T	This paper
2 *proL210*	69	Hydroxylamine	G69A	This paper
3 *proL211*	7	Hydroxylamine	G7A	This paper
4 *proL217*	72	Hydroxylamine	G72A2	This paper
D-loop and stem:				
5 *proL202*	16–17	Nitrosoguanosine	C insertion between C16 and C17	(Riddle &Roth, 1972)
6 *proL203*	16–17	Nitrosoguanosine	C insertion between C16 and C17	(Riddle & Roth, 1972)
7 *proL212*	23	Hydroxylamine	C23T	This paper
8 *proL213*	11, 15	Hydroxylamine	C11T, G15A	This paper
9 *proL215*	11, 15	Hydroxylamine	C11T, G15A	This paper
Anticodon stem:				
10 *proL201*	43	Nitrosoguanosine	G43A	(Riddle & Roth, 1972; Sroga *et al.*, 1992)
11 *proL216*	27	Hydroxylamine	C27T	This paper
12 *proL218*	27	Hydroxylamine	C27T	This paper
Anticodon loop:				
13 *sufB2*	34–37	ICR	G insertion between 34 and 37	(Riddle & Roth 1972)
14 *proL207*	34	Hydroxylamine	G34A G39A, G43A	This paper
Extra loop:				
16 *proL214*	44–48	Hydroxylamine	Deletions from 44 to 48	This paper

tion and the modification pattern was determined by two-dimensional thin-layer chromatography (Figure 1 B). The $tRNA^{Pro}_{GGG}$ is about 68% similar to the $tRNA^{Pro}_{GGG}$ and 78% to $tRNA^{Pro}_{cmo5UGG}$, and much less similar to the other m^1G-containing and non-m^1G containing tRNA species. We have three experimental evidences that the tRNA preparation consisted only of $tRNA^{Pro}_{GGG}$ and was not contaminated with the other tRNA species. First , all tRNA preparations showed the expected ratios of the major nucleotides, which makes it unlikely that our preparations were contaminated with $tRNA^{Pro}_{CGG}$ or $tRNA^{Pro}_{cmo5UGG}$, since the ratio for $tRNA^{Pro}_{GGG}$ is distinct from that of these two proline tRNA species. Second, $tRNA^{Pro}_{cmo5UGG}$, but not $tRNA^{Pro}_{GGG}$, contains cmo^5U. This nucleotide, which is well separated from pΨ and pU (it migrates below pU in the chromatographic systems used; Nishimura, 1979), was never observed in any of our tRNA preparations. Third, whereas both $tRNA^{Pro}_{CGG}$ and $tRNA^{Pro}_{cmo5UGG}$ contain pUm32, $tRNA^{Pro}_{GGG}$ does not. This nucleotide, which is also well separated in the chromatographic system used and migrates "north east" of pm^5U (Keith, 1995), was never observed in any of our tRNA preparations. Therefore, we conclude that the tRNA isolated was a pure $tRNA^{Pro}_{GGG}$ and the values shown in Table 2 represent the composition of $tRNA^{Pro}_{GGG}$ in the various mutants.

The modification pattern of the mutant tRNAs

Base substitutions at position 27 (*proL216* and *proL218*) in the anticodon stem and at position 66 (*proL208*) in the acceptor stem, which changed a G·C base-pair to a G·U base-pair, or a base substitution at the wobble position (*proL207*, G34A), a G insertion in the anticodon (*sufB2*), and a C insertion at the dihydrouridine (D) loop (*proL202* and *proL203*), did not influence the level of m^1G. However, a single base substitution at position 43 (*proL201*, G43A) in the anticodon stem (Figure 2 C), at positions 72 (*proL217*, G72A), 69 (*proL210*, G68A), and 7 (*proL211*; G7A) all in the acceptor stem reduced the level of m^1G by more than 80%. Base substitutions at both positions 11 and 15 (*proL213* and *proL215*) in the D-stem, and a deletion of the extra arm (*proL214*) also severely reduced the level of m^1G. We suggest that these mutations (G to A changes, resulting in disruptions of a G·C base-pair in most of the cases) may open some of the tertiary structures in the tRNA so that these tRNA species were no longer recognised by TrmD. Thus, several alterations even far from the target nucleotide resulted in a lack of m^1G. Note, that all mutations must change the structure of the tRNA in some way, since all of them induce a +1 frameshift. Still, only a subset of these mutations resulted in a m^1G37-deficiency. Therefore, only certain specific changes in the tRNA structure were sensed by the tRNA(m^1G37)methyltransferase.

Table 2. The molar yield of modification of mutant tRNAs

Strain	Mutations	m^1G37	Ψ	m^5U54	D20	No. of exp.
proL$^+$		0.9 ± 0.1	2.1 ± 0.1	0.9 ± 0.1	Yes	3
Acceptor stem:						
proL208	C66T	0.9 ± 0.1	1.9 ± 0.1	1.0 ± 0.1	Yes	2
proL210	G69A	0.2 ± 0.1	1.9 ± 0.1	0.9 ± 0.1	Yes	4
proL211	G7A	0.1 ± 0.1	1.8 ± 0.2	1.0 ± 0.1	Yes	4
proL217	G72A	0.2 ± 0	1.7 ± 0.2	1.0 ± 0.1	Yes	3
D-loop:						
proL202	C insertion between C16 and C17	1.0 ± 0.2	1.8 ± 0.2	0.9 ± 0.1	Yes	3
proL212	C23T	0.1 ± 0.1	1.7 ± 0.1	0.8 ± 0.2	Yes	3
proL213	C11T, G15A	0.1 ± 0.1	1.7 ± 0.2	0.7 ± 0	Yes	2
proL215	C11T, G15A	0.2 ± 0.1	1.7 ± 0.1	1.0 ± 0.3	Yes	3
Anticodem stem:						
proL201	G43A	0.1 ± 0.1	1.5 ± 0.3	0.9 ± 0.1	Yes	4
proL216	C27T	0.9 ± 0.1	2.0 ± 0.2	0.8 ± 0	Yes	2
proL218	C27T	0.7 ± 0.1	2.0 ± 0.2	1.0 ± 0	Yes	3
Anticodon loop:						
sufB	G insertion between 34 and 37	1.0 ± 0.2	2.0 ± 0.0	0.9 ± 0.1	Yes	3
proL207	G34A	0.8 ± 0.1	1.9 ± 0.1	0.8 ± 0.3	Yes	3
proL209	G37A, G39A, G43A	0.1 ± 0.1	1.9 ± 0.3	0.8 ± 0.1	Yes	3
Extra loop:						
proL214	Deletion from 44 to 48	0.1 ± 0.1	1.2 ± 0.1	0.7 ± 0.1	No	2

D (dihydrouridine) migrates at the tail of pU on the TLC and is therefore difficult to quantify. Therefore, only the presence or absence of this modified nuclecoside is indicated. 7-Methylguanosine migrates between pA and pC and was not well separated and is therefore excluded from the measurements. Furthermore, 4-thiouridylic acid was not recorded.

The two-dimensional thin-layer chromatography analysis also gave information of the level of Ψ (positions 40 and 55), m^5U54 and dihydrouridine (D20). In most mutant tRNA species, the level of these modified nucleotides was not altered. Thus, the corresponding enzymes do not seem to be sensitive to the same structural alterations as the tRNA(m^1G37)methyltransferase. However, in the *proL214* mutant (deletion of the the extra arm) and perhaps in the *proL201* mutant (G43A at the anticodon stem and close to the extra arm) the level of Ψ in the tRNA was reduced. In the *proL201* mutant, the level of m^5U54 was normal whereas the levels of Ψ40 or Ψ55 were slightly reduced. The level of m^1G was also dramatically reduced in these two mutants, suggesting that the anticodon loop may not be in its optimal conformation. If so, the observed reduction of the level of Ψ may be due to a reduction at position 40 and not at position 55. This is consistent with the suggestion that the formation of Ψ55 may only require an intact T-loop and stem (Nurse *et al*., 1995). These results suggest that the tRNA(Ψ40)synthetase, which is encoded by the *hisT* gene, is also sensitive, as the tRNA(m^1G37)methyltransferase, to the overall conformation of the tRNA and especially to the conformation of the anticodon stem and loop. However, most of the mutations that affect the level of m^1G37 did not reduce the level of Ψ40 (and Ψ55). Thus, the tRNA(Ψ40)synthetase is not sensitive to the same alterations of $tRNA^{Pro}_{GGG}$ as the tRNA(m^1G37)methyltransferase.

Mutant tRNAs do not show reduced level of Pro-$tRNA^{Pro}_{GGG}$

Transfer RNA from both the wild-type and mutants were prepared and the *in vivo* level of Pro-$tRNA^{Pro}_{GGG}$ was determined (Varshney *et al*., 1991). Figure 3 shows that $tRNA^{Pro}_{GGG}$ is fully aminoacylated in all the mutants examined. Thus, all structural alterations in the isolated mutant $tRNA^{Pro}_{GGG}$ species, including those mutations that effect the modification of m^1G at position 37, did not influence the level of Pro-$tRNA^{Pro}_{GGG}$ *in vivo*.

Discussion

Some of the earliest information of the recognition process for the aminoacyl-tRNA synthetases were obtained *in vivo* by the identification of mutations in the tRNAs that alter the amino acid specificity

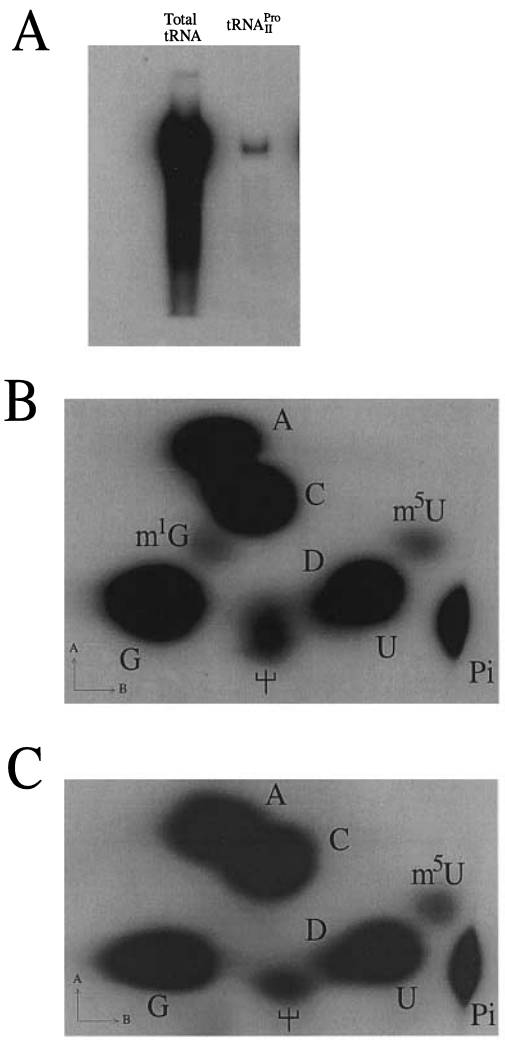

Figure 2. A, PAGE of total tRNA (left lane) and the purified $tRNA^{Pro}_{GGG}$ (right lane) eluted from the Dynabeads after hybridisation. B, Two-dimensional thin-layer chromatogram of wild-type $tRNA^{Pro}_{GGG}$. Position of m^1G is indicated. C, Two-dimensional thin-layer chromatogram of $tRNA^{Pro}_{GGG}$ isolated from mutant *proL201*. No m^1G was detected.

of an amber suppressor tRNA (Hooper *et al*., 1972; Shimura *et al*., 1972). Other attempts to analyse a nonsense suppressor with mutations in the anticodon *in vivo* also gave information of the requirements for different aminoacyl-tRNA synthetases (Schulman, 1991). Since the anticodon is an important recognition element for several aminoacyl-tRNA synthetases, such analysis of nonsense suppressors has its limitations. Therefore, an assay was developed that measures aminoacylation of a non-initiator tRNA, which initiates protein synthesis (Chattapadhyay *et al*., 1990). However, both the suppression assay and the initiation assay are indirect measurements of the aminoacylation reaction and neither the suppressor efficiency nor the efficiency of initiation can be directly correlated to the efficiency of aminoacylation. Recently, one of the two $tRNA^{Ala}$ genes has been deleted (Gabriel *et al*., 1996). This strain has been used to introduce various mutant forms of $tRNA^{Ala}_{GGC}$ and the acceptor activity of the various tRNAs were monitored. However, in this case as well as in the suppression and initiation assay mentioned above, the tRNA of interest is encoded by a plasmid and therefore the ratio between the enzyme and its substrate is not the same as that in a cell in which both these components are encoded by the chromosome.

Analyses of the requirement of tRNA modifying enzymes have also utilised *Xenopus* oocytes as an *in vivo* system. Radioactively labelled unmodified tRNA can be injected into the cytoplasm or into the nucleus and the modification process can be monitored as a function of time (Grosjean *et al*., 1987). Although several important conclusions about the structural requirements for tRNA modifying enzymes were obtained by such studies, it is an artificial system since the tRNA analysed is heterologous to the modifying enzymes. Furthermore, the reactions are very slow and an incubation time of up to 20 hours is required for the formation of appreciable amounts of the modified nucleosides. Moreover, the maturation process was not followed during concomitant synthesis of the primary transcript. Note also that although most of the

Table 3. *S. typhimurium* strains

Strains	Geontype	Source
TR1034	*hisO1242, hisD3749*	J. Roth
GT1381	*hisO1242, hisC3737, zef-2502::*Tn*10*	This lab
GT2439	*hisO1242, hisC3737, zef-2516::*Tn*10d*Cm	This lab
GT857	*hisO1242, hisD3018, sufB2, zef-2502::*Tn*10*	This lab
GT1380	*hisO1242, hisC3737, proL201 (sufX201), zef-2502::*Tn*10*	This lab
GT1480	*hisO1242, hisD3737, proL202, zef-2502::* Tn*10*	This study
GT1482	*hisO1242, hisD3737, proL203, zef-2502::*Tn*10*	This study
GT2919	*hisO1242, hisD3749, proL207, zef-2502::*Tn*10*	This study
GT2920	*hisO1242, hisD3749, proL208, zef-2502::*Tn*10*	This study
GT2921	*hisO1242, hisD3749, proL209, zef-2502::*Tn*10*	This study
GT2922	*hisO1242, hisD3749, proL210, zef-2502::*Tn*10*	This study
GT3022	*hisO1242, hisD3749, proL211, zef-2502::*Tn*10*	This study
GT3023	*hisO1242, hisD3749, proL212, zef-2502::*Tn*10*	This study
GT3024	*hisO1242, hisD3749, proL213, zef-2502::*Tn*10*	This study
GT3025	*hisO1242, hisD3749, proL214, zef-2502::*Tn*10*	This study
GT3026	*hisO1242, hisD3749, proL215, zef-2502::*Tn*10*	This study
GT3027	*hisO1242, hisD3749, proL216, zef-2502::*Tn*10*	This study
GT3028	*hisO1242, hisD3749, proL217, zef-2502::*Tn*10*	This study
GT3029	*hisO1242, hisD3749, proL218, zef-2502::*Tn*10*	This study

tRNA modifications occur in the nucleus, the tRNA is usually injected into the cytoplasm, where only the synthesis of a few modified nucleotides (usually those present in the anticodon) normally occurs. The fact that modification can occur in the cytoplasm is due to the presence of a subpopulation of the enzyme molecules that just have been synthesised on the ribosome and not yet translocated into the nucleus. Thus, the conditions in which these studies were performed are, most likely, far from those in the nucleus.

To circumvent all these obstacles we devised a method to study the requirements primarily for tRNA(m^{1}G37)methyltransferase but also for other modifying enzymes and the ProRS during true *in vivo* conditions in which the ratio between the tRNA and the various enzymes are the same as in the wild-type cell. Moreover, the analysis of the modification reactions was performed under normal logarithmic growth. Note that the selection was performed in strain containing a normal gene copy of both the tRNA and the tRNA(m^{1}G37)-methyltransferase. We analysed the level of m^{1}G37 in this particular tRNA from several mutants with specific alterations in the primary sequence of the tRNA. Indeed, many but not all mutant tRNAs were lacking m^{1}G37 although a functional tRNA(m^{1}G37)methyltransferase was present in the cell.

Of all the 497 tRNA sequenced (Steinberg *et al*., 1993) from cytoplasm of the three domains and mitochondria and chloroplasts, only two ($tRNA^{His}_{GUG}$ from human HeLa cells (Rosa *et al*., 1983) and $tRNA^{Leu}_{AAG}$ from *Caenorhabditis elegans* (Tranquilla *et al*., 1982)) have an unmodified G in position 37. Instead the G37 is modified to m^{1}G37 or wybutosine demonstrating that an unmodified G37 may be deleterious to the cell. Indeed, m^{1}G37 deficiency induces frameshift errors in *S. typhimurium* (Björk *et al*., 1989; Hagervall *et al*., 1993) and also inefficient cognate codon interactions (Li & Björk, 1995). Furthermore, a deletion of the *trmD* gene in *E. coli* mediates extremely slow growth (Persson *et al*., 1995). As pointed out in the Introduction, it seems likely that the recognition requirements of the tRNA(m^{1}G37)methyltransferase are similar in all organisms. Therefore the results presented here are expected to be of general significance.

From previous *in vitro* studies it is known that removing any of the variable loop, the D-loop or the T-loop of $tRNA^{Leu}_{CAG}$ reduces the V_{max} by one order of magnitude, whereas elimination of all three loops simultaneously reduces the V_{max} by two orders of magnitude. However, the K_m is moderately affected when one loop is eliminated and not affected when the V-, D, and T-loops and stems are eliminated simultaneously (Holmes *et al*., 1992). These results show that the tRNA(m^{1}G37)methyltransferase does not catalyse the formation of m^{1}G37 efficiently when only the anticodon-stem and loop is presented to the enzyme suggesting that the enzyme requires more than a simple mini-helix containing the target nucleotide. Base substitutions in the AC-mini-helix revealed that base-stacking and not the sequence *per se* is important for recognition. However, the G residue at position 36, which is next to the target nucleotide G37, is important for activity (Holmes *et al*., 1992). Thus, the dinucleotide G36-G37 may be crucial in the recognition process of the tRNA(m^{1}G37)methyltransferase and indeed the dinucleotide GpG is a potent inhibitor *in vitro* of the enzyme (Holmes *et al*., 1995). Furthermore, introducing a G instead of the normal A in position 37 of $tRNA^{His}$, which results in the sequence G36-G37, makes this tRNA a good substrate *in vitro* for the tRNA(m^{1}G37)methyltransferase although there is little homology between the $tRNA^{Leu}_{CAG}$ and the $tRNA^{His}$. These results suggest that the G36-G37 dinucleotide may be accommodated in the active site of the enzyme. Our selection procedure required that the tRNA frameshifted at the CCC-U sequence, which most likely requires the presence of G36-G37 sequence (Hagervall *et al*., 1993), and this may explain why no base alterations were obtained at these two positions. However, our results show that changes in other parts of the molecule eliminate m^{1}G, demonstrating that even if this G36-G37 dinucleotide is present, this is not sufficient to allow the methylation of G37 *in vivo*.

Disruption of a base-pair in the acceptor stem, in the D-stem or in the anticodon stem reduced the level of m^{1}G37

The tRNA(m^{1}G37)methyltransferase, whose target nucleotide is in the anticodon loop, is unexpectedly dependent on an intact acceptor stem, since the disruption of the base-pairs C1·G72 (*proL217*), C4·G69 (*proL210*) or G7·C66 (*proL211*) in this stem all reduced the level of m^{1}G37. However, changes in the last base-pair of the acceptor stem from a Watson–Crick base pair (G7·C66) to a wobble base-pair (G7-U66) did not influence the formation of m^{1}G37. Thus, such a reduction in the stability of the acceptor stem does not induce changes in the anticodon around the target nucleotide (G37) that are sensed by the tRNA(m^{1}G37)methyltransferase. One explanation for the inability for the tRNA(m^{1}G37)methyltransferase to methylate the mutants *proL210* (G69A), *proL211* (C66U) and *proL217* (G72A), may be that the enzyme makes specific contacts with the acceptor stem. Alternatively, these alterations induce long range changes in the anticodon region resulting in a low catalytic activity. The tRNA(m^{1}G37)methyltransferase is most likely active as an monomer (Hjalmarsson *et al*., 1983). The molecular mass is 28.4 kDa and therefore large enough to interact with various loops and stems. However, if the enzyme is a globular protein it is unlikely that it will simultaneously interact with both the acceptor stem and the anticodon loop, similarly to the way the GlnRS binds to $tRNA^{Gln}$ ((Rould *et al*., 1989; *c.f.* GlnRS is 63.3 kDa and is also active as a monomer). If so, the changed structure of the acceptor stem may

rather influence the structure of the anticodon region in such a way that the TrmD peptide is unable to bind or that the structure around the target nucleotide is distorted resulting in no catalytic reaction.

Base substitutions in both the D-stem (C11U) and the D-loop (G15A) (mutants *proL213* and *proL215*) resulted in complete m^1G37 deficiency as did a single base substitution in the D-stem (C23U, *proL212*). Whereas the base substitution in position 11 in the D-stem (C11U, *proL213* and *215*) resulted in a disruption of a Watson–Crick base-pair, the alteration in position 23 resulted in the formation of a wobble base-pair (G12-U23 instead of G12·C23) in the D-stem. Although the *proL213* and *proL215* (C11U) mutants also had an altered D-loop (G15 to A15), the change at position 23 in the D-stem in mutant *proL212* may also change the tertiary interaction with the base at position 9, if the $tRNA^{Pro}_{GGG}$ has a similar tertiary interaction to that of yeast $tRNA^{Phe}$. In the 3D structure of yeast $tRNA^{Phe}$, the nucleotide at position 23 is not only making a Watson–Crick base-pair with the nucleotide at position 12 in the D-stem but it also interacts with the nucleotide at position 9 (Kim, 1980). In fact, all class I tRNAs, to which $tRNA^{Pro}_{GGG}$ with its short extra arm belongs, may have such a triplet interaction between nucleotide 9 and the base-pair 12·23 . Thus, changes from Watson–Crick base-pair to a wobble base-pair may effect the triplet interaction resulting in a changed structure of the tRNA. Since the base-pairs 13·22 and 10·25 also make a triplet interaction with the nucleotides 46 and 45 of the extra loop, we note that also a deletion of the variable loop (G44 to G48 in mutant *proL214*) resulted in a reduction of the formation of m^1G37. It is reasonable that mutations changing the tertiary interactions between the D-stem, the variable loop and the acceptor stem result in a changed structure of the anticodon region. This may decrease the binding of the enzyme to this part of the tRNA resulting in the lack of m^1G37. It is also known that there is a tertiary interaction between the D-loop and the TΨC-loop. Therefore, the insertion of a C between G15-U18 (mutants *proL202* and *proL203*) may also result in an altered structure. However, this structural change did not affect the ability of the tRNA(m^1G37)methyltransferase to recognise its substrate since the tRNA from this mutant is fully methylated. Still, all these alterations induce a structural change of $tRNA^{Pro}_{GGG}$, since they all induce +1 frameshifting.

Complete disruption of the first base-pair in the anticodon stem, as in mutant *proL201* (C27·G43 base pair to C27-A43 non-base-pair), severely reduced the level of m^1G37 indicating a strong structural alteration of the anticodon region. However, changes at the same position but to a wobble base-pair (C27·G43 to U27-G43 in mutants *proL216* and *proL218*) did not at all or only slightly affect the level of m^1G37. These results agree with earlier data (Hüttenhofer *et al*., 1990) showing that a single base change at position 42, which exchanges a Watson–Crick base pair (G28·C42) with a wobble base-pair (G28-U42), in a yeast mitochondrial Ser-tRNA induces also a +1 frameshift activity and does not affect the formation of Ψ32 and m^1G37 in the anticodon. However, the formation of Ψ27, which is part of the base-pair next to the one affected by the mutation, is abolished. Thus, changes in the anticodon stem may influence the anticodon modification by a drastic change, such as a disruption of a base-pair, whereas introduction of a wobble base pair in the anticodon stem does not influence the modification in the anticodon loop but affects only the modification in the close neighbourhood of the base substitution.

Base substitutions or insertions close to the target nucleotide in the anticodon loop did not influence the level of m^1G37

Insertion of a G as in *sufB2* or a base substitution of G34A as in *proL207* in the anticodon loop did not affect the level of m^1G37 suggesting that the size of the anticodon loop or that the wobble nucleotide are not recognition determinants for the tRNA(m^1G37)methyltransferase. However, insertions in the anticodon loop of yeast $tRNA^{Asp}$ drastically reduce the formation of m^1G37 as monitored in the *Xenopus* oocyte system (Grosjean *et al*., 1996). This may either be explained by the difference between the systems used in the analysis or that the insertion in the $tRNA^{Asp}$ was on the 5′side of the anticodon which may induce other structural changes than an insertion on the 3′side of the anticodon as in the *sufB2* mutant. Although the oocyte and the bacterial enzymes catalyse the formation of the same nucleoside at the same position in the tRNA, they may be quite different. The enzyme using yeast $tRNA^{Asp}$ as substrate recognises a tRNA having a C36 whereas the bacterial enzyme has one of the major recognition requirements, a G36 (Holmes *et al*., 1995). Thus in yeast and in other eukaryotes there may be more than one enzyme catalysing the formation of m^1G37, one related to the bacterial enzyme, which methylates the subpopulation of tRNA from all organisms having m^1G37 and reading codons of the type CUN, CCN and CGG and another enzyme that methylates other tRNAs having m^1G37, e.g. $tRNA^{Asp}$.

The triple mutant *proL209* has a disrupted anticodon stem and a base substitution at position 37 (G37A) in the anticodon loop. Of course, this change ruled out any formation of m^1G in position 37, but also in any other positions in the anticodon loop in which G were present (G34, G35 and G36).

None of the structural alterations in the various mutant $tRNA^{Pro}_{GGG}$ influenced the level of three other modified nucleosides

Table 2 shows that all mutations that changed the structure of $tRNA^{Pro}_{GGG}$ in such a way that the

tRNA(m¹G37)methyltransferase was unable to methylate it, did not affect the formation of Ψ40, m⁵U54, Ψ55. Note, the triple mutant (*proL209*), which has two disrupted base-pairs in the anticodon stem and a base substitution G37A and the two double mutants *proL213*, *proL215*, which affect the D-loop, still had a normal level of other modified nucleosides although they all lacked m¹G. Thus, the corresponding enzymes that catalyse the formation of these modified nucleosides are not sensitive to the same structural perturbation as the tRNA(m¹G37)methyltransferase. Only mutant *proL214*, which has the entire extra arm (G44 to G48) deleted, showed a significant reduction in the formation of Ψ and to a lesser degree of m⁵U54 as well as possibly a lack of D20. Although our analysis method does not distinguish between Ψ40 and Ψ55, we suggest that this reduction of Ψ is primarily due to a reduction at position 40, since this mutant lacks the nearby m¹G37 but has only a slight reduction of m⁵U54, which is next to Ψ55. The consensus recognition sequence for the tRNA(m⁵U54)methyltransferase consists of only 11 nucleotides of the T-arm (Gu & Santi, 1991). Although this consensus sequence is present in the mutant *proL214*, we observed a reduction in the formation of m⁵U54 suggesting that even this enzyme is sensitive to some specific perturbations of its substrate for optimal function *in vivo*. However, the tRNA(m¹G37)methyltransferase seems to be unique among the tRNA modifying enzymes monitored, since many of the structural perturbations which this enzyme was sensitive to, did not affect the other tRNA modifying enzymes.

None of the structural alterations in the various mutants influenced acceptor activity of the $tRNA^{Pro}_{GGG}$

A computer analysis of several tRNA sequences suggested that the C1·G72 base-pair in the acceptor stem, U17A in the D-loop and G35, G36 and G37 in the anticodon loop are important for the $tRNA^{Pro}$ identity (McClain *et al*., 1994). The nucleoside G72 as an identity element was confirmed *in vitro* by various base substitutions, including G72A (Liu *et al*., 1995). The G72A alterations reduced the specificity constant (k_{cat}/K_m) almost 200-fold. Moreover, changes in the core region, as disruption of the C48·G15 interaction, also reduced the acceptor activity. However, the acceptor activity of the $tRNA^{Pro}_{GGG}$ of the *proL217* (G72A) mutant was not affected *in vivo* (Figure 3) as well as $tRNA^{Pro}_{GGG}$ from several mutants (e. g. from the mutants *proL212*(C23U) and *proL214* (deletion of the extra loop)) with alterations that probably influence the stability of the core region. Reversing the C1·G72 base-pair to G1·C72, abolishes the acceptor activity both *in vitro* (Liu *et al*., 1995) and *in vivo* (McClain *et al*., 1994). Since a G·C base-pair in the end of a helix is more stable than a C·G base-pair (Limmer *et al*., 1993; Sugimoto *et al*., 1987), these result suggests that a too strong base-pair in the end of the acceptor stem is detrimental for the acceptor activity. Indeed, a disruption of the second base-pair C2·G71 by a mutation G71A increased *in vivo* the acceptor activity (McClain *et al*., 1994). Our results suggest that destabilisation of the first base-pair, as in our *proL217*(G72A) mutant, as well as at base-pair C4·G69 and G7·C66 were tolerated by the ProRS. These results and others (Liu *et al*., 1995; McClain *et al*., 1994) support the suggestion that the ProRS requires a distinct conformation of the acceptor stem. Our results extend this suggestion by the observation that the ProRS can tolerate *in vivo* even a more destabilised acceptor stem than that present in the wild-type.

Taken together our results suggest that whereas the tRNA(m¹G37)methyltransferase is extremely sensitive to small but specific perturbations of the structure of $tRNA^{Pro}_{GGG}$ *in vivo*, other tRNA modifying enzymes, as those involved in the formation of D20, Ψ40, m⁵U54, Ψ55 and the ProRS are not. We conclude that contrary to other enzymes using $tRNA^{Pro}_{GGG}$ as substrate, the tRNA(m¹G37)methyltransferase has evolved a sophisticated recognition process that carefully monitors the structure of the tRNA, especially the structure of the anticodon loop in much more detail than the other modifying enzymes and the ProRS. To obtain a more detailed molecular understanding of the way with which tRNA(m¹G37)methyltransferase recognises the $tRNA^{Pro}_{GGG}$ and its anticodon loop architecture, it is necessary to perform careful *in vitro* studies of the various mutants analysed *in vivo* in this report. Such analyses are in progress (Holmes, M., personal communication) and they will reveal whether or not these reported *in vivo* effects can also be observed *in vitro* under conditions in which the formation of m¹G37 does not compete with other reactions occurring concomitantly with the methylation reaction or in which the stoichometric conditions between the enzymes and tRNA are not as found *in vivo*. Such comparisons are important since the outcome will reveal how valid the detailed molecular information obtained *in vitro* is *in vivo*.

Material and methods

Strains and materials

The strains used are listed in Table 3. The Dynabeads M-280 were purchased from Dynal Company, Oslo, Norway. The biotinylated primer was purchased from Symbicom AB, Umeå, Sweden. The second primer was synthesised by a Cyclone Plus DNA synthesiser, Millipore, England. RNase P_1 was from Sigma, USA. [^{32}P] Orthophosphate was from Amersham, England. For genetic and suppression experiments, medium E was used (Vogel & Bonner, 1956). For the labelling experiments,

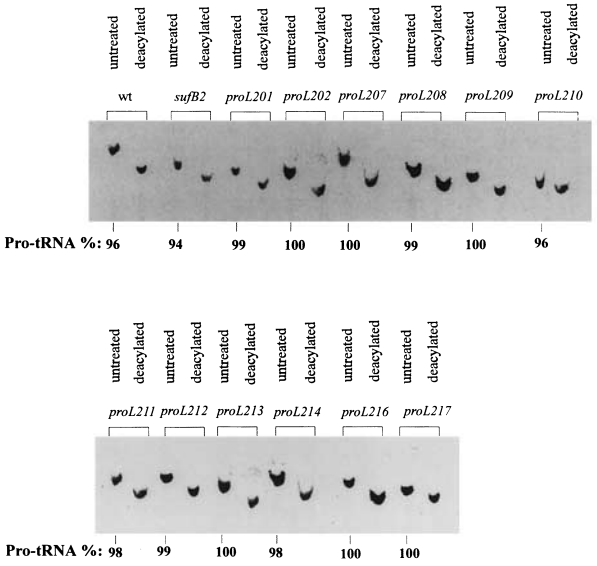

Figure 3. The *in vivo* amounts of Pro-tRNA$^{Pro}_{GGG}$ and uncharged tRNA$^{Pro}_{GGG}$ in various *proL* mutants. Transfer RNA from each mutant was prepared under acid conditions (pH 5.2). Half of the tRNA sample was stripped of amino acids by alkali treatment (pH 9.0). This part (denoted deacylated) and the untreated other half (denoted untreated) were fractionated by acid-gel electrophoresis, transferred to a membrane and hybridised with a probe specific for tRNA$^{Pro}_{GGG}$. Samples wt to *proL208*, samples *proL209* to *proL210*, and samples *pro211* to *proL217* were run on separate gels. The percentages of Pro-tRNA$^{Pro}_{GGG}$ in the untreated sample was calculated as: $100 \times$ (Pro-tRNA$^{Pro}_{GGG}$)/(Pro-tRNA$^{Pro}_{GGG}$ + tRNA$^{Pro}_{GGG}$) and for the various mutants are shown below the gel.

Mops-glucose minimal medium supplied with histidine was used (Neidhardt *et al.*, 1974). Cells were grown at 37°C.

Hydroxylamine mutagenesis

Transductions were performed with a derivative of phage P22 containing the mutations HT105/I, which leads to increased frequency of generalised transduction (Schmieger, 1972), and *int*-201, which prevents formation of stable lysogens (Scott *et al.*, 1975). Transductions were performed as described (Davis *et al.*, 1980). Phage free recombinants were isolated by streaking non-selectively on green indicator plates (Chan *et al.*, 1972). The phage (2×10^{11} to 2×10^{12} PFU/ml) grown on strain GT1381(*zef*-2502::Tn*10*) were treated with the transition mutagen hydroxylamine for 36 hours at 37°C to reach a phage survival of 10^{-3} as described (Davis *et al.*, 1980). Each transducing phage particle in such a phage stock contains about 40 kb of chromosomal DNA in which deamination of C has occurred randomly. By selecting Tc^R transductants chemically altered DNA located near the *zef*-2502:Tn*10* insertion was recombined into the bacterial chromosome. Since this Tn*10* is located only about 1 kb from the *proL* gene, it is likely that some of these Tc^R transductants also had a mutated *proL* gene. To isolate mutants with an altered *proL* gene the following procedure was used. The hydroxylamine treated phage stock was mixed with strain GT1405 which contains the *hisD3749* mutation resulting in a +1 frameshift site CCCU. The donor strain GT1381 contains a transposon Tn*10* (*zef*-2502:Tn*10*) which is 95% co-transducable with *proL*$^+$ gene and far away form the *hisD*$^+$ gene. Tetracycline resistant transductants were selected and replicated to different selective plates. Potential *proL* mutants, which synthesise a tRNA$^{Pro}_{GGG}$ that was able to correct the +1 frameshift mutation *hisD3749*, were those mutants that had a His$^+$ phenotype after 48 hours incubation at 37°C. Such potential mutants were confirmed by backcrossing to the parental stain.

DNA sequencing

Two primers were designed to amplify by PCR a 283 bp fragment that contains the tRNA$^{Pro}_{GGG}$ gene (*proL*$^+$) from the chromosome DNA(Primer 1: 5′-GGGAATTCT-GCTGCTGCAAGTGC-3′; Primer 2: 5′-BIOTIN-GGGT-CCCCCGTTGCTTTCCCG-3′) (Sroga *et al.*, 1992). The biotinylated primer binds to the sense strand and is therefore complementary to the tRNA. After a standard PCR reaction, the amplified products were incubated at room temperature for 12 hours with 1 mg pre-washed Dynabeads M-280 (washed by TE buffer (10 mM Tris-HCl(pH 8.0)) containing 1 M NaCl). The Dynabeads were then washed three times with deionized water, and incubated in 20 µl of 0.15 M sodium hydroxide at room temperature for one hour to separate the strands. The supernatant was removed by magnetic separation of the beads and applied to (0.8% (w/v) agarose to check the binding efficiency. The Dynabeads containing anti-sense strand of the tDNA strand were used either as template as described by the standard DNA sequencing method (Sanger *et al.*, 1977) or as probe to isolate tRNA$^{Pro}_{GGG}$ (see below). The *proL* gene from the various mutants was sequenced two or three times.

tRNA purification by hybridisation

The cells were pre-grown at 37°C in the low phosphate (0.5 mM) Mops-glucose medium for three generations before they were diluted to $A_{550} = 0.05$. Five ml of such culture was grown to $A_{550} = 0.3$ when 0.5 mCi [^{32}P] orthophosphate was added (Yarus *et al.*, 1986). After two hours of incubation, total RNA was extracted by adding an equal volume of phenol pre-equilibrated with chloroform. Bulk tRNA was purified by 12% (w/v) polyacrylamide gel and recovered by mixing the gel slices with hybridisation buffer ($6 \times$ SSC, $2 \times$ Denhart, 50% formamide and 0.1% SDS) at 37°C with vigorous shaking. Such bulk tRNA was then hybridised with Dynabeads containing the anti-sense strand of tDNA at 42°C overnight. The beads were then washed with 2xSSC (ssc is 0.15 m NaCl, 0.015 m sodium citrate, pH 7.0) containing 0.1% SDS for 15 minutes, $1 \times$ SSC containing 0.1% SDS for 30 minutes and finally with stringent washing with $0.1 \times$ SSC, 0.1% SDS for 15 minutes at 42°C. The hybridised tRNAs were eluted with 0.15 M NaOH at 65°C for 5 minutes and purified once more by 12% PAGE.

Analysis of modified nucleosides by two-dimensional thin-layer chromatography

10,000 to 20,000 cpm of purified tRNA was hydrolysed by nuclease P_1 overnight in the presence of $ZnSO_4$

(Gehrke *et al*., 1982). Such hydrolysed tRNA was applied onto cellulose-coated plastic plates. The chromatograms were developed for about four hours in isobutyric acid-50% NH_4OH (50/3, v./v) and in the second dimension for about seven hours in HCl-isopropanol-H_2O (15/70/10, by vol.) (Nishimura, 1979). The radioactivity of each nucleotide was measured in a Phosphor imager instrument from Molecular Dynamic. The molar yield of each nucleotide was determined.

Determination of the level of Pro-tRNA in the cell

The level of Pro-tRNA$^{Pro}_{GGG}$ was determined according to Varshney et al., 1991). Transfer RNA was prepared at pH 4.5 and half of the sample was stripped of its amino acids by incubation for 20 minutes at 37°C in 0.5 M Tris-HCl (pH 9.0). The tRNA samples was fractionated in an acid gel (pH 5.2) to preserve the aminoacylated tRNA species and transferred by electroblot to a Zeta probe (GT genomic) blotting membrane (Bio-Rad), pre-hybridised at 37°C for one hour in 0.9 M NaCl, 90 mM Tris-HCl (pH 7.6), 1 mM EDTA, 1% (w/v) SDS, 100 µg/ml calf thymus DNA. The filter was then incubated with the ^{32}P-labelled oligo 5′-GACCCCCGACACCC-CAUGACGGU-3′ at 70°C for five minutes and cooled slowly to room temperature (took usually about three hours) and then the filter was washed twice in 6 × SSC, 0.1% SDS for five minutes at 37°C. Since the mutant *proL214*, which has a four base deletion, did not migrate significantly different from the wild-type tRNA we tested the specificity of the probe. The probe was bound to Dynabeads and tRNA$^{Pro}_{GGG}$ was purified using the same hybridisation conditions as used for the Northern-blot analysis. The molar ratio of the major nucleotides was the same as for a pure tRNA$^{Pro}_{GGG}$. Moreover, none of the modified nucleosides specific for the other two tRNA^{Pro} species were present. We have isolated a mutant containing a mutated tRNA$^{Pro}_{CGG}$ with a nine base-pair insertion that migrates differently from that of the wild-type tRNA$^{Pro}_{CGG}$ (J.-N. Li and G. R.B., unpublished observation). The tRNA$^{Pro}_{GGG}$ specific probe used by us did not bind to this tRNA$^{Pro}_{CGG}$ derivative under the same hybridisation conditions as used to detect tRNA$^{Pro}_{GGG}$. Taken together these results demonstrate that the probe is specific for tRNA$^{Pro}_{GGG}$. We do not have an explanation why the tRNA with a four base deletion did not migrate distinctly from that of the wild-type tRNA$^{Pro}_{GGG}$. However, the aforementioned derivative of tRNA$^{Pro}_{CGG}$ with a nine base-pair insertion was only barely separated from the wild-type form. Thus, it seems as if the various tRNA^{Pro} species must retain some secondary structure in the acid gel that may interfere with their migration.

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