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Three Modified Nucleosides Present in the Anticodon Stem and Loop Influence the *in vivo* aa-tRNA Selection in a tRNA-dependent Manner

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In *Salmonella typhimurium* seven tRNA species specific for leucine, proline and arginine have 1-methylguanosine (m^1G) next to and 3′ of the anticodon (position 37 of tRNA), five tRNA species specific for phenylalanine, serine, tyrosine, cysteine and tryptophan have 2-methylthio-*N*-6-(*cis*-hydroxy)isopentenyladenosine (ms^2io^6A) in the same position of the tRNA, and four tRNA species, specific for leucine and proline, have pseudouridine (Ψ) as the last 3′ nucleotide in the anticodon loop (position 38) or in the anticodon stem (positions 39 and 40). Mutants deficient in the synthesis of these modified nucleosides have been used to study their role in the first step of translation elongation, i.e. the aa-tRNA selection step in which the ternary complex (EF-Tu-GTP-aa-tRNA) binds at the cognate codon in the A-site on the mRNA programmed ribosome. We have found that the Ψ present in the anticodon loop (position 38) stimulates the selection of tRNA specific for leucine whereas Ψ in the anticodon stem did not affect the selection of tRNA specific for proline. The m^1G37 strongly stimulates the rate of selection of the three tRNA species specific for proline and one tRNA species specific for arginine but has only minor or no effect on the selection of the three tRNA species specific for leucine. Likewise, the ms^2io^6A, present in the same position as m^1G37 but in another subset of tRNA species, stimulates the selection of tRNA specific for tyrosine, stimulates to some extent also tRNA species specific for cysteine and tryptophan, but has no influence on the rate of selection of tRNA specific for phenylalanine. We conclude that function of m^1G and ms^2io^6A present next to and 3′ of the anticodon influences the *in vivo* aa-tRNA selection in a tRNA-dependent manner.

Keywords: modified nucleosides; tRNA selection; anticodon-codon; translation

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Introduction

Transfer RNA from all organisms contains modified nucleosides which are derivatives of the normal nucleosides adenosine, guanosine, uridine and cytosine. At present more than 80 different modified nucleosides have been characterised (Limbach *et al.*, 1994). Although the functions of these modified nucleosides were, for some time, obscure, they clearly have an important role in the translation process (Björk, 1995a,b, 1996). *In vitro* some modified nucleosides modulate the anticodon-codon interaction and others influence the rate of polypeptide chain elongation *in vivo*. However, it would be advantageous to study the action of the tRNA *in vivo* and the function of the modified nucleosides of specific tRNA species in each of the various steps of the translation elongation process. The first step is the tRNA selection step in which the ternary complex consisting of EF-Tu, GTP, and aa-tRNA binds to the mRNA in the A-site. Curran & Yarus (1989) devised a method that measures the relative rate of the selection of specific aa-tRNA species at 29 sense codons *in vivo*. We have used their method to study how three modified nucleosides, pseudouridine (Ψ), 1-methylguanosine (m^1G)

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Abbreviations used: Ψ, pseudouridine; m^1G, 1-methylguanosine; ms^2io^6A, 2-methylthio-N^6-(4-hydroxyisopentenyl)adenosine; EF-Tu, elongation factor Tu; aa, amino acid.

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and 2-methylthio-N^6-(4-hydroxyisopentenyl) adenosine (ms^2io^6A) influence the rate of the aa-tRNA selection *in vivo* at various codons.

The 46 tRNA species present in *Escherichia coli* and *Salmonella typhimurium* contain 29 characterised modified nucleosides and two that have not been identified (Björk, 1996). Some of them are present in all tRNA species, like 5-methyluridine (m^5U54), which is present in position 54, and Ψ55, while others, like Ψ38,39,40, m^1G37, and ms^2io^6A37 are present in a subset of tRNA species. The *truA* (*hisT*) product catalyses the synthesis of Ψ at three positions, 38 (last 3′ nucleoside in the anticodon loop), 39 and 40 (both in the anticodon stem), in 20 different tRNA species. A *truA* mutant will therefore contain tRNAs having U instead of Ψ at these positions in these tRNAs. Such Ψ-deficient tRNA reduces the rate of translation by 20%, increases translational fidelity, and reduces the growth rate of the cell (Palmer *et al.*, 1983) but does not influence the aminoacylation of tRNA (Brenner & Ames, 1972; Brenner *et al.*, 1972).

The methylated nucleoside m^1G is present in position 37, which is the position next to and 3′ of the anticodon, in tRNA species reading codon CUN (Leu), CCN (Pro) and CGG (Arg) from each of the three domains Archaea, Bacteria and Eucarya. Unless a convergent evolution has occurred, this modified nucleoside was present in the tRNA of the progenitor (Björk, 1986). If so, the m^1G37 may have a similar function in tRNA irrespective of the organism. Mutations in the structural gene for the tRNA(m^1G37)methyltransferase (*trmD*) result in a deficiency of m^1G37 and cause the cell to grow 20% slower than wild-type. The translation elongation rate is also reduced by 20%, indicating an inefficient anticodon-codon interaction due to the lack of m^1G37 (Li & Björk, 1995). Furthermore, lack of m^1G37 also induces +1 frameshifting (Björk *et al.*, 1989; Hagervall *et al.*, 1993). However, lack of m^1G37 does not influence the aminoacylation reaction of $tRNA^{Pro}$ *in vivo* (Qian & Björk, 1997; J.-N. L. & G. R. B., unpublished results).

The synthesis of ms^2io^6A37, which in *S. typhimurium* is present in nine tRNA species reading codons starting with U, proceeds in several steps and three genes have been identified that are involved in its synthesis (Figure 1). The first step, which is catalysed by the *miaA* gene product, is a transfer of an isopentenyl group from isopentenylpyrophosphate to A37 in the tRNA resulting in i^6A37. Since the following steps require the presence of i^6A37 (Esberg & Björk, 1995), the *miaA* mutant has A37 instead of the ms^2io^6A37 in its tRNA. The second step, thiolation and methylation at position 2 of A, requires the *miaB* and *miaC* gene products and produces ms^2i^6A37. A *miaB* mutant contains i^6A37 instead of ms^2io^6A37 (Esberg & Björk, 1995). Though the *miaA* mutation causes a 20 to 30% reduction in the polypeptide elongation rate (Diaz *et al.*, 1987; Ericson & Björk, 1986), the *miaB* mutation has no such effect (Esberg & Björk, 1995). Moreover, the efficiency of a suppressor tRNA is reduced two to threefold in a *miaB* mutant, whereas a 10 to 100-fold reduction, depending on the codon context, is observed in a *miaA* mutant (Esberg & Björk, 1995). Thus, both the i^6- and the ms^2-groups contribute to the efficiency of the tRNA, although to different degree. The aminoacylation reaction is, on the other hand, not affected by ms^2io^6A37 deficiency (Buck & Griffiths, 1982; Gefter & Russell, 1969; Wilson & Roe, 1989).

Clearly, the presence of Ψ, m^1G37 and ms^2io^6A37 in the anticodon region is important for the efficiency of the tRNA. It was early recognised that tRNA species reading codons starting with U or A have a hypermodified nucleoside at position 37. It was therefore suggested that the function of these hypermodified nucleosides, such as ms^2io^6A37, should stabilise the interaction between the first base in the codon and the last base in the anticodon (Nishimura, 1972). If so, the impact of these hypermodified nucleosides should be tRNA-independent, i.e. they would exert the same impact on all the tRNA species they are part of. This may be observed if individual steps of the translation process are studied. However, the effect may be obscured if the rate of the overall translation process is monitored, since the impact of a modified nucleoside may in one step improve the rate whereas in another step the modified nucleoside may reduce the rate. How these modified nucleo-

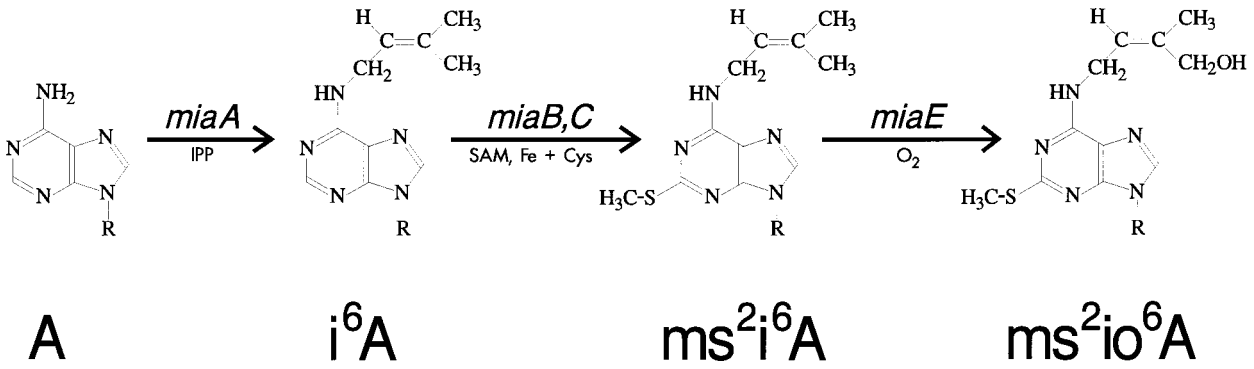

Figure 1. Biosynthesis of ms^2io^6A37 in *S. typhimurium*.

sides influence *in vivo* an individual step in the translation elongation process has not been analysed before. As a first step to elucidate the function of a particular modified nucleoside of an individual tRNA at one particular step of the translation elongation process, this paper focuses on the function of modified nucleosides at the first step of the translation process, the aa-tRNA selection step. Using the aforementioned assay devised by Curran & Yarus (1989), we have monitored how the presence or absence of Ψ in positions 38, 39, or 40, m^1G37 and the ms^2- and the i^6-groups of ms^2io^6A37 in various tRNA species influence the rate of aa-tRNA selection. Although all these modified nucleosides improve the efficiency of the translation elongation process, we find that they each influence the rate of tRNA selection differently and in a tRNA-dependent manner.

Results

We have used a competition assay that monitors the selection of aa-tRNA to specific codons at the A-site (Curran & Yarus, 1989). The assay system makes use of the fact that the synthesis of the release factor 2 (RF2) requires a +1 frameshift during the translation of its mRNA (Craigen *et al.*, 1985). In this system, the *lacZ* gene is fused into the +1 frame downstream of the frameshift site in *prfB* mRNA and thus β-galactosidase activity is a measurement of the efficiency of frameshifting (Figure 2). There are several experimental results that support the suggestion that this assay monitors the selection of the ternary complex at the A site (Curran & Yarus, 1989). First, since frameshifting at the P-site and translation of the test codon in the A-site are competing reactions, the frequency of frameshifting is inversely correlated to the rate of aa-tRNA selection at the A-site. Therefore, an increase of the aa-tRNA species reading the test codon should decrease the efficiency of frameshifting. Indeed, an overexpression of $tRNA^{Trp}$ reading the test codon UGG reduces the frameshifting ability (Curran & Yarus, 1989; Gao *et al.*, 1995). Furthermore, the efficiency of frameshifting is inversely correlated to the efficiency of suppressor tRNAs reading a nonsense codon (Curran & Yarus, 1988). Second, the rate constants *in vivo* obtained by this competition assay for five different codons are correlated (Curran & Yarus, 1989) to the rates obtained from *in vitro* programmed ribosomes (Thomas *et al.*, 1988). All these results and results presented here, support the conclusion that the competition assay used here measures the aa-tRNA selection step.

The efficiency of frameshifting is dependent on both a Shine-Dalgarno sequence upstream of the frameshifting site (Curran & Yarus, 1988; Weiss *et al.*, 1988) and on which tRNA decodes the slippery codon at the frameshifting point (Curran, 1993; Weiss *et al.*, 1987). In the *prfB* mRNA, which encodes the RF2, the shifty tRNA is $tRNA^{Leu}_{GAG}$ and the shifty codon is CUU. However, other tRNA species may also work, albeit at lower efficiencies (Curran, 1993; Weiss *et al.*, 1987). Since the shifty $tRNA^{Leu}_{GAG}$ in wild-type cells contains both Ψ in the anticodon stem and loop and m^1G37, we have used as the shifty tRNA the major $tRNA^{Val}_{cmo5UAC}$, which normally lacks Ψ38,39,40 and m^1G37, in our analysis of how Ψ and m^1G37 influence the aa-tRNA selection. In the analysis of the function of ms^2io^6A37 in the aa-tRNA selection, the $tRNA^{Leu}_{GAG}$

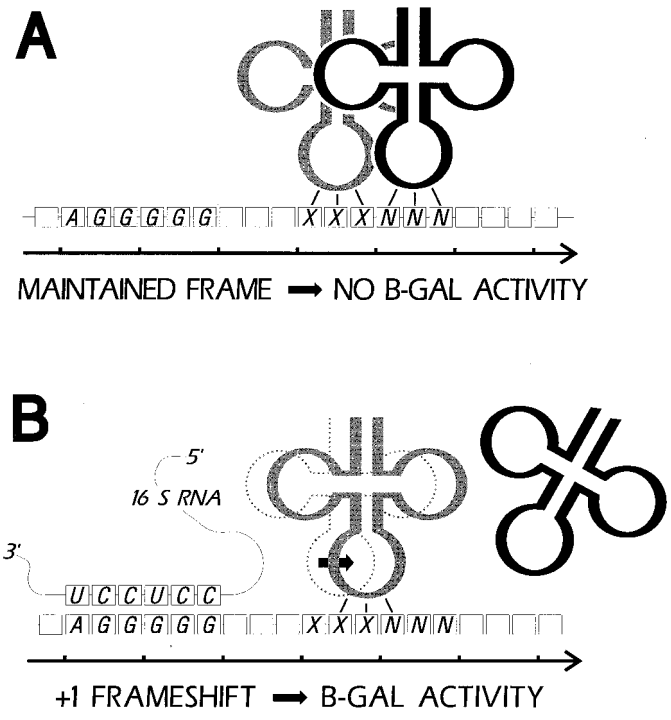

Figure 2. The "speedometer" assay system to determine the relative rate of the first step in the translation elongation cycle, the aa-tRNA selection. A, Successful binding of the aa-tRNA to the NNN codon in the A-site. The shifty tRNA cannot slip into +1 frame and no β-galactosidase is synthesised. B, Unsuccessful binding of aa-tRNA to the test codon and the shifty tRNA in the P-site slips into the +1 frame and active β-galactosidase is synthesised.

was used as the shifty tRNA, since it does not contain ms^2io^6A37. The rate of selection at the sense codon just downstream of the shifty codon (CUU or GUU) influences the efficiency of frameshifting in an inversely proportional manner. By placing different codons (the test codons) just downstream of the shifty codon, the rate of selection of the tRNA species that read these test codons can be determined. A high selection rate for the aa-tRNA reading the test codon will decrease the degree of frameshifting, whereas a low rate of aa-tRNA selection will increase the frameshifting. Thus, by comparing the ratio of the β-galactosidase activity expressed from the plasmid containing the test codon to the β-galactosidase activity from the in-frame *lacZ* gene present in the control plasmid pJC27 in the various tRNA modification deficient mutants to the ratio in the wild-type, the action of the modified nucleoside in question in the aa-tRNA selection step was monitored.

None of the mutations affecting tRNA modification influenced the level of *lacZ* mRNA

If the competing tRNA is less efficient, more frameshifting occurs and more ribosomes enter the *lacZ* mRNA and counteract a possible degradation of the mRNA. A correction of the β-galactosidase activity with the mRNA level compresses therefore the range of tRNA selection but does not change the direction of the effect induced by the changed frequency of frameshifting. In contrast, it would rather make the difference less pronounced. However, the mRNA correction induces additional experimental errors in the comparison of frameshifting in the various mutants compared to the wild-type. Although there is no indication that deficiency in tRNA modification would influence the copy number of the plasmid, a measurement of the *lacZ* mRNA level will also give information of the copy number of the plasmid. Table 1 shows that the level of *lacZ* mRNA was similar for each plasmid in the mutant and in the wild-type strains (for *hisT1504* and *hisT*$^+$ these values were within +/− 20% except for CUA/G (+/− 100%) and CCA (+/ − 30%); for the *trmD3* and *trmD*$^+$ these values were within +/− 20% except for CUA (+/ − 36%) and CCA (+/ − 153%)). Furthermore, there is not always a correlation between the mRNA level and the level of β-galactosidase (cf. Tables 1 and 2). Likewise, no difference in the *lacZ* mRNA level was observed for the experiments reported in Table 4 (see the legend to Table 4). Thus, the various efficiency of frameshifting did not influence the *lacZ* mRNA level. As expected, the similar mRNA level also suggested that the plasmid copy number did not vary much between the mutant and the wild-type. Therefore, Figures 3 and 4 show the relative activities not corrected for the mRNA level of the various tRNAs in Ψ-, m^1G37-, and ms^2io^6A37-deficient strains compared to the wild-type strain.

Lack of Ψ in three Leu-tRNA^{Leu} species, but not in $tRNA^{Pro}_{GGG}$, reduced the rate of tRNA selection to the cognate codons

The three $tRNA^{Leu}$ species, which read codons of the CUN type, all contain Ψ in the anticodon loop and stem (positions 38 and 40 for $tRNA^{Leu}_{CAG}$, position 38 for $tRNA^{Leu}_{GAG}$ and positions 38 and 39 for $tRNA^{Leu}_{UAG}$; Figure 3). In the *hisT1504* mutant, these tRNAs have an unmodified U instead of Ψ in these positions. Plasmids having the four different leucine codons of the type CUN as the test codons, were introduced into the strain GT2530 (*hisT1504*), which lacks Ψ in the anticodon region, and to its congenetic wild-type strain GT2529 (*hisT*$^+$). The lack of Ψ in the major $tRNA^{Leu}_{CAG}$ and the minor $tRNA^{Leu}_{UAG}$ increased the frameshifting about threefold (Table 2). According to Curran & Yarus (1989), this value can be calculated as the relative rate of selection (R_t/R_s; R_t denotes the rate of aa-tRNA selection and R_s denotes the rate of frameshift) of the ternary complex as explained in the legend to Table 2. Figure 3 shows that lack of Ψ in these two tRNA species specific for leucine, the relative rate of tRNA selection was reduced at the

Table 1. *lacZ* mRNA level in wild-type strain and *hisT1504* and in *trmD3* mutants

Codon	tRNA anticodon	*lacZ*/*rpoB*[a] (mRNA)		
		Wild-type[b]	*hisT1504* (no Ψ at 38-40)	*trmD3* (no m^1G37)
CUU (Leu)	GAG	3.5/2.1	3.1	2.5
CUC (Leu)	GAG	0.70/1.0	0.82	0.97
CUA (Leu)	UAG	1.1/0.95	2.3	1.3
CUG (Leu)	CAG; UAG	0.84/0.86	1.69	0.89
CCU (Pro)	GGG; cmo^5U34GG	0.54/0.51	0.62	0.68
CCC (Pro)	GGG	1.0/1.0	1.0	0.87
CCA (Pro)	cmo^5UGG	1.08/1.25	1.43	1.91
CCG (Pro)	CGG; cmo^5U34GG	1.0/1.23	0.86	1.3
CGG (Arg)	CCG	0.99/n.d.	1.4	n.d.
pJC27	n. r.	1.61/1.61	2.06	1.45

[a] The mRNA levels are the ratio of the *lacZ* to *rpoB* (internal control) mRNA levels.
[b] The first number relates to the level obtained with strain GT2529 (*zej-635* :: Tn*5*, *hisT*$^+$) and the second values to strain GT874 (*trmD*$^+$).
n.r., not relevant; n.d., not determined.

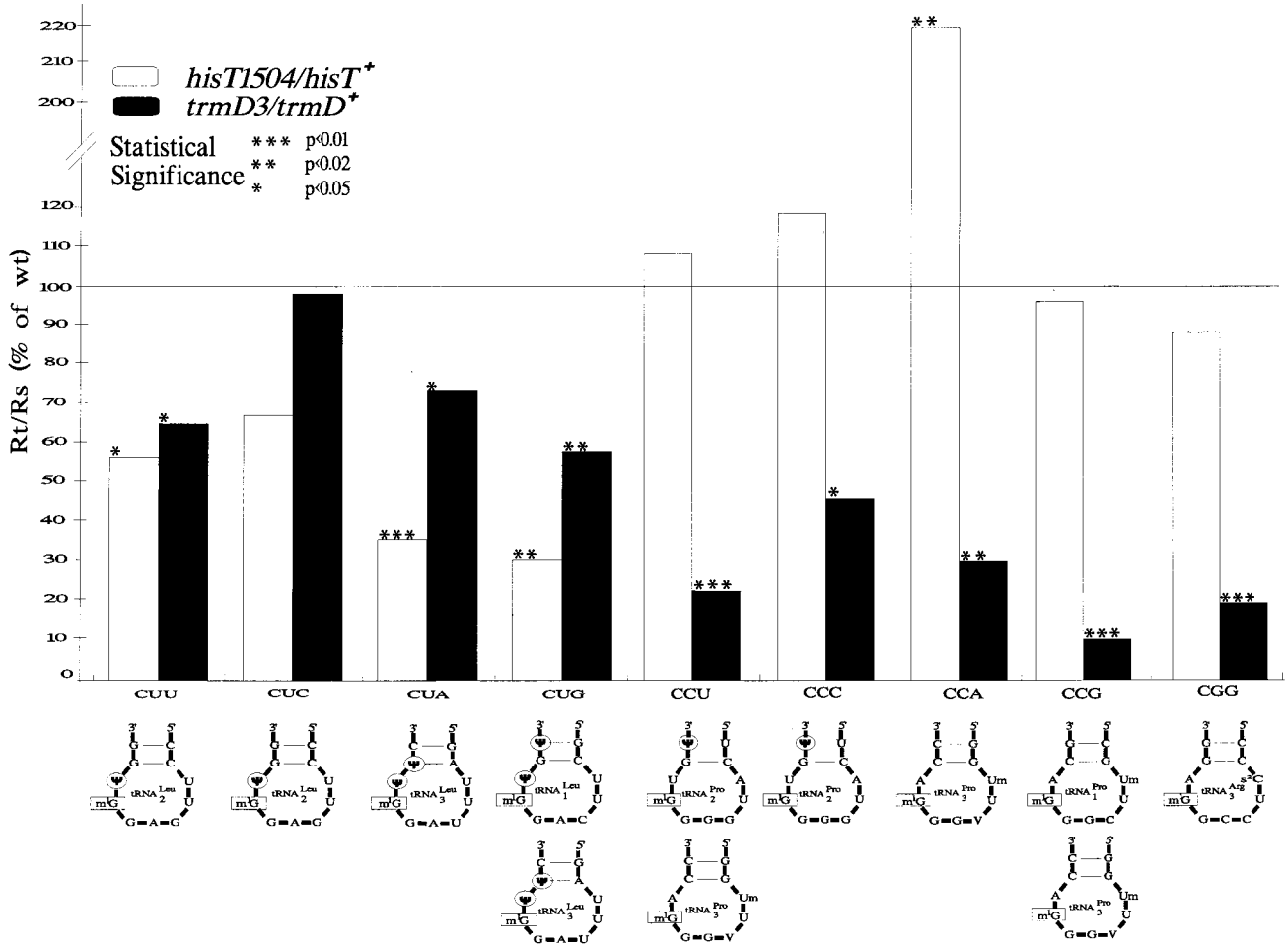

Figure 3. Intrinsic rate (R_t/R_s) of aa-tRNA selection based on relative β-galactosidase activity in a *hisT1504* (open bars) mutant and in a *trmD3* mutant (shaded bars) relative to the wild-type (%). The statistical variations were calculated with the *t*-test. A difference between wild-type and the mutant is considered significant when $p < 0.05$ (*), 0.02 (**), or 0.01 (***).

CUG and CUA codons by about 70%. These results suggest that modification of U to Ψ in the anticodon region improved the efficiency of selection for these Leu-tRNA^{Leu} species. The rate of selection of tRNA$^{Leu}_{GAG}$ at the CUU codon may also be reduced but not at the CUC codon.

Of the three tRNA^{Pro} species only the tRNA$^{Pro}_{GGG}$ contains Ψ in the anticodon stem and loop (position 40). As expected, the selection rates of the other two Pro-tRNA^{Pro} species were the same in the *hisT1504* mutant as in the wild-type (Table 2). However, the relative β-galactosidase activity was also the same for CCU/C codons, which are read by Ψ40 containing tRNA$^{Pro}_{GGG}$. The CCU codon is also read by tRNA$^{Pro}_{cmo5UGG}$ and the concentration of this tRNA is much higher than that of the minor tRNA$^{Pro}_{GGG}$ (Ikemura, 1981). Thus, the possible effect of lack of Ψ40 in tRNA$^{Pro}_{GGG}$ in the *hisT1504* mutant might be obscured by the reading of tRNA$^{Pro}_{cmo5UGG}$, whose structure is not influenced by the *hisT1504* mutation, since it normally does not contain Ψ in the anticodon loop or stem. However, the CCC codon is only read by tRNA$^{Pro}_{GGG}$, so the observed lack of effect due to Ψ40 deficiency at this codon cannot be obscured by any dominant effect of the other isoacceptors. Thus, whereas Ψ in the anticodon loop (position 38) and in the stem (position 39) influenced the rate of selection of tRNA^{Leu}, it does not seem to influence the rate of selection of Pro-tRNA$^{Pro}_{GGG}$, which has the Ψ only in the anticodon stem (position 40).

Absence of m^{1}G in all three tRNA^{Pro} species and in tRNA$^{Arg}_{CCG}$ (Arg3) reduced the aa-tRNA selection rate whereas m^{1}G37 deficiency in the three tRNA^{Leu} only affected the selection to the CUG codon

All three Pro-tRNAs contain m^{1}G37. Two of the four proline codons, CCC and CCA, are each read by only one of the three tRNA^{Pro} isoacceptors, tRNA$^{Pro}_{GGG}$ and tRNA$^{Pro}_{cmo5UGG}$, respectively. Lack of m^{1}G37 increased the β-galactosidase activity 2.2 and 3.3-fold (Table 2), implying a reduction of the relative rate of aa-tRNA selection by 50 and 70% (Figure 3), respectively, to these two codons. The CCU codon is read by both tRNA$^{Pro}_{GGG}$ and tRNA$^{Pro}_{cmo5UGG}$ and lack of m^{1}G37 reduced the

Table 2. Aminoacyl-tRNA selection at various test codons in wild-type strain and in *hisT1504* and *trmD3* mutants

Codon	tRNA anticodon	% pJC27 (β-Gal[a]) Wild-type[b]	*hisT1504* (no Ψ at 38-40)	*trmD3* (no m^1G37)	R_t/R_s[c] wt	$(R_t/R_s)/t$[c] wt
CUU (Leu)	GAG	0.43/0.51	0.77	0.78	232/195	773/650
CUC (Leu)	GAG	0.30/0.43	0.45	0.44	332/232	1106/773
CUA (Leu)	UAG	0.49/0.48	1.35	0.66	203/207	2030/2070
CUG (Leu)	CAG; UAG	0.14/0.14	0.45	0.24	712/713	712/713
CCU (Pro)	GGG; cmo^5UGG	0.29/0.34	0.27	1.46	344/293	−/−[d]
CCC (Pro)	GGG	0.31/0.38	0.26	0.83	322/262	3222/2620
CCA (Pro)	cmo^5UGG	0.39/0.42	0.18	1.4	255/237	319/296
CCG (Pro)	CGG; cmo^5UGG	0.51/0.47	0.53	4.0	195/212	195/212
CGG (Arg)	CCG	3.4/4.3	3.9	18.7	28.4/22.3	284/223
pJC27	n. r.	100/100	100	100	n. r.	n. r.

[a] The values given are the β-galactosidase activities relative to the values obtained in the control pseudo-wild-type plasmid pJC27 that does not require a +1 frameshift for the production of β-galactosidase activity. The experimental errors were in the range +/− 16% except for CCG in the *hisT1504* mutant, which was +/−20%.

[b] The first number relates to the level obtained with strain GT2529 (*zej-635 ::* Tn*5*, *hisT*$^+$) and the second value to strain GT874 (*trmD*$^+$).

[c] The relative rates (R_t/R_s) of the rate of the aa-tRNA selection (R_t) to that of the frameshift (R_s) was calculated from the equation $R_t/R_s = 1/F - 1$ (derived by Curran & Yarus, 1989) in which F is the frequency of frameshift (β-galactosidase activity of the test-plasmid relative to the activity of the control plasmid). The overall rate of aa-tRNA selection $(R_t/R_s)/t$ is further dependent on the concentration (t) of the tRNA species reading the test codon. The two numbers are related to the wild-type strains as specified under [b].

[d] $tRNA^{Pro}_{GGG}$and $tRNA^{Pro}_{cmo5UGG}$ are a minor and major tRNA, respectively. Since it is not known which of these two tRNAs is the main reader of CCU, no calculation of the overall rate of Pro-tRNA selection was made.

n.d., not determined; n.r., not relevant.

aa-tRNA selection about 75% at this codon (Figure 3). The CCG codon is read by both the major $tRNA^{Pro}$ isoacceptors, $tRNA^{Pro}_{CGG}$ and $tRNA^{Pro}_{cmo5UGG}$. The reduction of aa-tRNA selection at this codon is drastically reduced by lack of m^1G37 (R_t/R_s is only 11% of wild-type). Thus, the selection of the four different proline codons was reduced, but to quantitatively different degrees (50 to 90%) indicating a tRNA-dependent influence by m^1G37 in the tRNA selection step.

There are four $tRNA^{Arg}$ species in *S. typhimurium*, of which only the CGG reading $tRNA^{Arg}_{CCG}$(Arg3) contains m^1G37. However, this tRNA does not contain any Ψ in the anticodon region. Therefore, we expected that the CGG codon should be selected at the same rate in the *hisT1504* mutant as in the wild-type, which was the case (Table 2). However, the Arg-$tRNA^{Arg}_{CCG}$ selection rate was decreased by 80% in the *trmD3* strain (Figure 3). Thus, the presence of m^1G37 improves the rate of $tRNA^{Arg}_{CCG}$ selection fivefold.

The three $tRNA^{Leu}$ species, which read CUN codons, contain m^1G37, next to and 3′ of the anticodon (Figure 3). Compared with the wild-type control, the relative β-galactosidase activity was significantly different in the *trmD3* mutant only in the selection at the CUG codon (Table 2; Figure 3), suggesting that m^1G37 has no (at CUC) or only minor (at CUU and CUA) influence of the selection of any other leucine codons than CUG.

Frameshifting at CCN codons mediated by m^1G37 deficiency contributed less than 5% to the observed programmed +1 frameshifting

We have earlier shown that lack of m^1G37 induces +1 frameshifting at sites, such as CCC-N, due to a quadruplet translocation (Björk *et al.*, 1989; Hagervall *et al.*, 1993). Since the β-galactosidase activity is a result of a +1 frameshifting event, the observed increase of β-galactosidase activity in the *trmD3* mutant may be due to an induction of +1 frameshifting as earlier observed. However, the frameshifting activity in the *prfB* system used by us is dependent on a Shine-Dalgarno sequence at a critical distance from the shifty codon (Curran & Yarus, 1988; Weiss *et al.*, 1988). Although the distance between the potential +1 frameshifting site mediated by lack of m^1G37 is too far away from the Shine-Dalgarno sequence (the potential frameshifting site is the test codon in the A-site), we still wanted to estimate how much of the observed β-galactosidase activity was independent of the Shine-Dalgarno sequence. Therefore, we replaced the Shine-Dalgarno sequence AGGGGG by the non-Shine-Dalgarno sequence CGTGGC. In such constructs, the expression of *lacZ*, if there is any, will only be due to the frameshifting event caused by a m^1G37 deficient tRNA at a site upstream of the fusion point in the *prfB-lacZ* hybrid and thus including the A-site test tRNA. As shown in Table 3, no more than 5% of the *lacZ* expression was retained in all non-Shine-Dalgarno constructs though the β-galactosidase activity is about threefold higher in the *trmD3* mutant than that in the *trmD*$^+$ cells in all cases. Therefore, the major part (at least 95%) of the frameshifting activity observed in the *trmD3* mutant strain was caused by a frameshifting of $tRNA^{Val}$ and not by any m^1G37 containing tRNAs reading codons within the frameshifting window.

Table 3. Frameshifting mediated by m^1G deficient tRNA species independent of the Shine-Dalgarno sequence

Plasmid[a]	β-Gal activity[b] (% of the original construct)[d]			β-Gal activity/mRNA level[c] (% of the original construct)[d]		
	trmD$^+$	*trmD3* (no m^1G37)	*trmD3*/*trmD*$^+$	*trmD*$^+$	*trmD3* (no m^1G37)	*trmD3*/*trmD*$^+$
non-SD	0.018	0.052	2.9	0.015	0.037	2.5
CCA	(4.3%)	(3.7%)		(4.3%)	(5.0%)	
non-SD	0.0082	0.027	3.3	0.018	0.059	3.3
CCG	(1.7%)	(2.3%)		(4.6%)	(1.9%)	
non-SD	0.012	0.033	2.8	0.011	0.022	2.0
CCU	(3.5%)	(2.3%)		(3.7%)	(2.2%)	

[a] In these plasmids, the Shine-Dalgarno-like sequence AGGGGG in the original constructs was replaced by sequence CGTGGC, which will eliminate the frameshifting event by the P-site shifty tRNA.
[b] β-Galactosidase activity, relative to that of the pJC27 control (does not require a frameshift for the production of β-galactosidase).
[c] *lacZ* message level, relative to that of the pJC27 control.
[d] Data in parenthesis represent percentage of A-site frameshifting (without Shine-Dalgarno effects) to the observed total frameshifting (with Shine-Dalgarno effects).

Presence of ms^2io^6A37 improves the selection of Tyr-, Trp-, Cys- and Ser-tRNA species but not the selection of Phe-tRNA

Nearly all tRNAs that read codons starting with U contain ms^2io^6A37. Thus, these tRNAs have an unmodified A37 in the *miaA1* mutant and i^6A37 in the *miaB2508* :: Tn*10d*Cm mutant. This modified nucleoside is not present in $tRNA^{Leu}_{GAG}$, which reads CUU and is the slippery tRNA in the wild-type form of the *prfB* gene. Since this tRNA is more slippery than the $tRNA^{Val}$ used in the earlier experiments, we used a set of plasmids having this CUU as the slippery codon and various test codons 3′ of it. Plasmids with various test codons were introduced into these two modification-deficient mutants and the wild-type strain GT522. As expected, test codons read by tRNAs that normally do not have ms^2io^6A37, like CUU/C/G, were unaffected by the *miaA1* or the *miaB2508* :: Tn*10d*Cm mutations (Table 4 and Figure 4). The observed level of frameshifting was similar to that found by Curran & Yarus (1989) which demonstrates that the system works similarly in *S. typhimurium* as in *E. coli*. Table 4 shows that the decoding by the single $tRNA^{Phe}$ species present in *S. typhimurium*, which read both UUU and UUC, was unaffected by the lack of ms^2io^6A37 or lack of only the ms^2-group as in the *miaB2508* :: Tn*10d*Cm mutant. There are two $tRNA^{Tyr}_{QUA}$ species with the same anticodon reading UAU/C and both contain ms^2io^6A37. Lack of the ms^2io^6 or the ms^2-group reduced the rate of selection of these two $tRNA^{Tyr}_{QUA}$ species (Table 4). The reduction was most pronounced in the *miaA1* mutant (a reduction of R_t/R_s to 10% of wild-type; Figure 4) although the reduction in the relative rate of Tyr-tRNA selection in the *miaB* mutant was also significant (R_t/R_s reduced to 60% of wild-type; Figure 4). Also the unique $tRNA^{Trp}$ present in *S. typhimurium* had a reduced rate of selection (reduced to 30% of wild-type) at the UGG codon when lacking the ms^2io^6-

Table 4. Aminoacyl-tRNA selection in wild-type and in the mutants *miaA1* and *miaB2508* :: Tn*10d*Cm

Test codon	tRNA anticodon	% pJC27			R_t/R_s wt	$(R_t/R_s)/t$ wt
		wt	*miaA1*	*miaB2508*		
CUU (Leu)	GAG	7.3	7.0	6.3		
CUC (Leu)	GAG	4.0	3.8	3.4		
CUG (Leu)	CAG; UAG	2.0	2.1	2.5		
UUU (Phe)	GAA	8.1	8.8	6.7	11	32
UUC (Phe)	GAA	2.9	2.8	2.4	33	100
UAC (Tyr)	QUA	12	59	19	7.3	15
UAU (Tyr)	QUA	14	62	19	6.1	12
UGG (Trp)	CCA	23	53	26	3.3	11
UGU (Cys)	GCA	17	58	22	4.9	49
UGC (Cys)	GCA	8.8	13	8.5	10	100
UCG (Ser)	CGA	11	16	14	8.1	32
UAGA	Tyr-amber (Su3)	24	52	32	3.2	
UAGC	Tyr-amber (Su3)	34	70	71	2.0	
UAGG	Tyr-amber (Su3)	17	26	21	5.0	
UAGU	Tyr-amber (Su3)	21	46	27	3.7	

Values (%) given are the β-galactosidase activities obtained by the various plasmids relative to the β-galactosidase activity obtained by the pseudo-wild-type plasmid, pJC27. The ratio of *lacZ*/*rpoC* mRNA was determined in each experiment from three samples of two independent cultures. The ratios were the same in all cultures (variation +/− 26 % except for pJCTet27 in *miaB2508*::Tn*10d*Cm background which was +/ − 47%) and no systematic variation was observed (data not shown). Therefore, the (β-galactosidase levels have not been corrected for the mRNA levels. The relative rates (R_t/R_s) are calculated as described in the legend to Table 2.

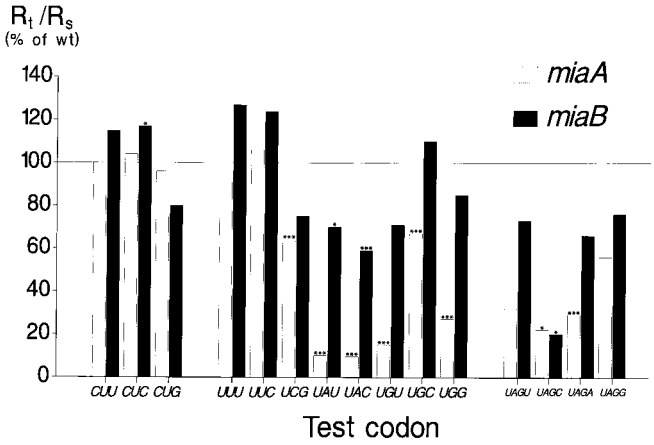

Figure 4. Intrinsic rate (R_t/R_s) of aa-tRNA selection based on relative β-galactosidase activity in a *miaA1* (open bars) mutant and in a *miaB2508 ::* Tn*10d*Cm mutant (shaded bars) relative to the wild-type (%). The statistical variations were calculated with the *t*-test. A difference between wild-type and the mutant is considered significant when $p < 0.05$ (*), 0.02 (**), or 0.01 (***).

group as in the *miaA1* mutant, whereas no significant effect was observed when the $tRNA^{Trp}$ only lacked the ms^2-group. The selection of Cys-$tRNA^{Cys}$ at the two cognate codons UGU and UGC was affected differently by ms^2io^6A37 deficiency. Whereas the selection to the UGU codon was reduced by 85%, the selection to UGC was only reduced by 30%. The Ser-tRNA selection to UCG codon was only slightly affected (a 35% reduction) by the lack of ms^2io^6A37 (Table 4; Figure 4). Thus, the ms^2io^6A37 modification reduced the rate of selection of especially Tyr-tRNA, to a less extent of Trp-, Cys-, and Ser-tRNAs, but not of Phe-tRNA.

The amber suppressor efficiency is thought to depend on the rate of selection of the suppressor tRNA, relative to the rate of action of the RF1. We (Bouadloun *et al.*, 1986; Ericson & Björk, 1991) and others (Björnsson & Isaksson, 1993; Petrullo *et al.*, 1983) have shown that, depending on the codon context, the efficiency of amber suppression is reduced by up to 99% in a *miaA1* strain. Moreover, lack of the ms^2-group reduces the efficiency of amber suppressor two to threefold compared to more than tenfold when the tRNA lacks the ms^2io^6 modification (Esberg & Björk, 1995). To relate the extensive data set to results obtained with the frameshift assay, we thought that it might be useful to study the effects of ms^2io^6A37 at RF2 alleles that have an amber test codon.

Strains were constructed that contain the *supF30* mutation, which encodes the amber suppressor $tRNA^{Tyr}_{CUA}$, and the *miaA1* mutation, the *miaB2508 ::* Tn*10d*Cm or their wild-type alleles. We then introduced plasmids that have UAG as the test codon into these strains. To monitor context effects on translation of the UAG, we made four constructs with each major base 3′ to the amber codon (Figure 4). As in the case of the wild-type $tRNA^{Tyr}_{QUA}$, the lack of ms^2io^6A37 strongly reduces the rate of the suppressor, particularly at the allele with the 3′C context. Furthermore, the 3′C context is especially poor for a suppressor lacking only the ms^2 group. However, the *miaB* mutation did not strongly affect the rate of the wild-type $tRNA^{Tyr}_{QUA}$ (see UAU and UAC in Figure 4) although the 3′ nucleotide was a C (see Materials and Methods). Therefore, it seems as if the *miaB* effect at the 3′C context is specific to the amber suppressor $tRNA^{Tyr}$. We note that the only difference between the amber suppressor $tRNA^{Tyr}$ and the wild-type form of it is the wobble nucleoside (C34 in the amber suppressor and Q34 in the wild-type $tRNA^{Tyr}_{QUA}$). In any case, the fact that these modification-deficiency mutations decrease amber suppression and increase frameshifting of RF2-UAG sites strengthens the conclusion that the ms^2io^6-modification increases the rate of suppressor tRNA selection at amber codons.

Discussion

Here we have compared the rate of aa-tRNA selection of several tRNA species with and without the modified nucleosides m^1G37, Ψ38,39,40 and ms^2io^6A37 (tRNA species lacking only the ms^2-group of ms^2io^6A as in the *miaB* mutant were also studied). These three modified nucleosides are all present in the anticodon region of the tRNA. Whereas the presence of Ψ38,39,40 improved the selection rate of Leu-RNA to CUA and CUG codons (Table 2; Figure 3), the m^1G37 in the same tRNAs only improved the tRNA selection to the CUG codon. On the other hand, the m^1G37 strongly influenced the Pro-tRNA selection to all proline codons (a possible exception may be CCC, see Figure 3) whereas the Ψ40 of Pro-$tRNA^{Pro}_2$ did not. Although the ms^2io^6A37 strongly influences the overall activity of the translation cycle of all tRNAs that have ms^2io^6A37 (Björk, 1995a,b), the rate of selection of the Phe-tRNA was not affected by the absence of this modified nucleoside. However, the same modification reduced the rate of selection of Tyr-, Trp-, Cys- and Ser-tRNAs. Thus, the various modified nucleosides tested, which all are known to reduce the overall activity of the tRNA in the translation cycle, influenced the aa-tRNA selection step differently and in a tRNA-dependent manner.

Effects of the Ψ modification at positions 38 to 40

The *hisT1504* mutant was isolated as able to derepress the *his*-operon (Roth *et al.*, 1966). This operon is preceded by a leader sequence containing seven histidine codons in a row (Barnes, 1978).

The *hisT*-mediated derepression is caused by a slower decoding at these histidine codons by a Ψ38,39-deficient $tRNA^{His}$ (Johnston *et al.*, 1980). This is consistent with the observed reduced polypeptide chain elongation rate in the *hisT* mutant (Palmer *et al.*, 1983). The effect of the *hisT1504* mutation on other amino acid biosynthetic operons can be explained in a similar way. The leader region of the leucine operon includes a 28 codon leader that contains four consecutive leucine codons (CUA-CUA-CUA-CUC; Carter *et al.*, 1985). The $tRNA^{Leu}_{GAG}$ (reads CUC) and $tRNA^{Leu}_{UAG}$ (reads CUA) normally contain Ψ in the anticodon region (Figure 3). In a *hisT* mutant, these tRNAs contain an unmodified U instead of Ψ. A mutation in the *hisT* gene leads to derepression of the *leu*-operon, which can be reconciled with an increased step time at these leucine regulator codons. Apparently, the increased step time of these two $tRNA^{Leu}$ species lacking Ψ in the anticodon is enough to derepress the *leu*-operon. Therefore, part of this increased step time seems to be caused by less efficient selection of Leu-$tRNA^{Leu}_{UAG}$ (Table 2; Figure 3).

The largest difference between the wild-type and the *hisT1504* mutant was observed for the selection of CUG, which is read by $tRNA^{Leu}_{CAG}$ and $tRNA^{Leu}_{UAG}$ species. The overall rate of tRNA selection, which is a function of the R_t/R_s and the concentration (t) of the tRNA species (Tables 2 and 4), determines the efficiency of frameshifting. Whereas the CUG codon has a much lower overall rate of tRNA selection than the CUA codon (712 *versus* 2030, Table 2), the reduction of the R_t/R_s due to lack of Ψ was about the same (31 and 36 %, respectively; see Figure 3; the synthesis of various tRNA species is likely not to be affected by the modification pattern; experimentally this suggestion has been verified for $tRNA^{Pro}$ species 1 and 2 in the *trmD3* mutant, unpublished results). An even smaller effect induced by the Ψ-deficiency was observed for the CUU/C codons (Figure 3), which also have a lower overall rate of tRNA selection than the CUA codon (773 and 1106 *versus* 2030, Table 2). Thus, for the Leu-$tRNA^{Leu}$ and Ψ-deficiency, we did not observe a simple correlation of the magnitude of the reduction and the overall rate of tRNA selection. Since $tRNA^{Pro}_{GGG}$ has the highest overall rate (about 3000 in our system and it was also the highest reported by Curran & Yarus (1989)), a possible weak effect induced by Ψ-deficiency in this tRNA may not have been detectable with our system. However, whereas Ψ in all three $tRNA^{Leu}$ are in position 38 in the anticodon loop ($tRNA^{Leu}_{UAG}$ also contains Ψ39), $tRNA^{Pro}_{GGG}$has Ψ at position 40 in the anticodon stem. The Ψ in position 38 is part of the anticodon loop, so it may impose a stronger effect on the conformation of the anticodon than Ψ40. The Ψ modification creates an additional NH moiety when the sugar is shifted from N1 in U to C5 in Ψ and this proton presents a site for formation of a hydrogen bond. Indeed, Ψ32 of $tRNA^{Phe}$ forms an internal hydrogen bond to a bridging water molecule or to a 2′-hydroxyl of a nearby nucleoside (Griffey *et al.*, 1985). Although Ψ40 may stabilise the anticodon stem by improved stacking (Davis, 1995), the effect exerted by Ψ38 may be much more pronounced. Some experimental results support this suggestion. The efficiency of the amber suppressor derivative of $tRNA^{Tyr}$, which has Ψ39, is only decreased by 40% when lacking Ψ (Hagervall *et al.*, 1990), whereas the activity of the *supE* amber suppressor, which is a derivative of Ψ38 containing $tRNA^{Gln}$, is reduced by more than 95% (Bossi & Roth, 1980). Moreover, a reduced misincorporation is correlated to a hypomodification of Ψ38 (Parker, 1982). One explanation is that the hypomodification of Ψ38 reduces the activity of the misreading tRNA much more than if the misreading tRNA is hypomodified in position 39. Thus, the impact of Ψ modification may be related to its presence in the anticodon loop or in the anticodon stem, which we also observed.

Effects of the m^1G37 modification

In Mops-glucose minimal medium lack of m^1G37 increases the average step time 3.3-fold (Li & Björk, 1995). As stated above, the *leuABCD* operon is regulated by the speed with which the ribosome traverses the four regulatory leucine codons (CUA-CUA-CUA-CUC) present in the leader mRNA. These leucine codons are read by m^1G37 containing $tRNA^{Leu}_{UAG}$ (reads CUA) and $tRNA^{Leu}_{GAG}$ (reads CUC). However, unlike the *hisT1504* mutation, which derepresses the *leu*-operon several-fold, the *trmD3* mutation does not (Li & Björk, 1995). These results indicate that although the *trmD3* mutation increases the average step time 3.3-fold, it does not influence the step time of these two leucine tRNAs. If so, the average step time for the $tRNA^{Leu}_{CAG}$, the three $tRNA^{Pro}$ isoacceptors and $tRNA^{Arg}_{CCG}$ increases to 3.8-fold. Consistent with these results, we observed no effect on the selection of $tRNA^{Leu}_{GAG}$ at CUC codons (0.43 *versus* 0.44, Table 2) and only a small effect at CUA ($tRNA^{Leu}_{UAG}$) codon (Table 2).

All three $tRNA^{Pro}$ isoacceptors as well as the $tRNA^{Arg}_{CCG}$ were strongly affected in the tRNA selection by m^1G37 deficiency. Among the proline tRNAs the largest effect (reduction by 90%) was observed for CCG, which is read by $tRNA^{Pro}_{CGG}$ and $tRNA^{Pro}_{cmo5UGG}$. The overall rate was also the lowest among the proline tRNAs (about 200, Table 2) and similar to CCA (about 300, Table 2) for which we observed the next largest reduction (by 70%, Figure 3). However, m^1G37 deficiency reduced the rate of tRNA selection by 50% to CCC (Pro) and by 80% to CGG (Arg) although the overall rate is much higher (3000) for CCC than that for CGG (less than 300, Table 2). Thus, as for the Ψ-deficiency, we did not observe a strict correlation between the magnitude of reduction and the overall rate of selection for m^1G37-deficient tRNA species.

Effects of the ms^{2}io^{6}A37 modification

It has been suggested that the presence of ms^{2}i^{6}A37 stabilises the intrinsically weak interaction of the A36-U base-pair (Jukes, 1973; Nishimura, 1972). Indeed, the presence of the ms^{2}i^{6}A37 modification stabilises tRNA-tRNA dimers with complementary anticodons mainly due to an improved stacking of the hypermodified nucleoside (Houssier & Grosjean, 1985; Vacher *et al.*, 1984). Thus, the results from such model experiments suggest that the modification of A37 to ms^{2}io^{6}A37 stabilises the 3′ stacking features of the anticodon and thereby improves its interaction with the codon. Indeed, the average step time *in vivo* of an ms^{2}io^6-deficient tRNA increases four times (Ericson & Björk, 1986) and the efficiencies of nonsense suppressors are reduced up to 99% depending on the codon context (Björnsson & Isaksson, 1993; Bouadloun *et al.*, 1986; Ericson & Björk, 1991; Petrullo *et al.*, 1983). Also *in vitro* ms^{2}i^{6}A37 deficiency decreases the activity of the tRNA (Buck & Griffiths, 1982; Diaz & Ehrenberg, 1991; Gefter & Russell, 1969; Vacher *et al.*, 1984). Based on the model experiments with tRNA dimers in conjunction with the strong effect on the efficiency of the tRNA both *in vivo* and *in vitro*, one would expect that the ms^{2}io^{6}A37 modification should have a great influence on the aa-tRNA selection. This was not the case for tRNA^{Phe} *in vivo* (Table 4), as well as *in vitro* (Diaz & Ehrenberg, 1991). This unexpected result may be explained by the fact that when the stability of the tRNA-tRNA dimers was measured, the EF-Tu was not present. Since it has been shown that binding of EF-Tu alters the conformation of the anticodon (Wikman *et al.*, 1982), it is possible that the modification does not improve the stacking interaction when the tRNA has assumed such a conformation. If so, the strong effect by the presence of ms^{2}io^{6}A37 on the efficiency of tRNA must be in a step of the translation cycle after the aa-tRNA selection step when the EF-Tu has left the ribosome.

Of the five tested tRNAs normally having ms^{2}io^{6}A37, tRNA^{Tyr} and tRNA^{Trp} have the lowest overall rate of aa-tRNA selection (11 and 12, respectively, Table 4). We observed a 90% reduction in the tRNA selection for tRNA^{Tyr} lacking the ms^{2}io^6-group but also if it was deficient only in the ms^2-group (UAU, Figure 4). Although the tRNA^{Trp} has an even lower overall rate, the selection of ms^{2}io^{6}A37 deficient tRNA^{Trp} to the cognate codon UGG was not reduced as much as that observed for tRNA^{Tyr}. The tRNA^{Cys} has a higher overall rate of selection than tRNA^{Trp}. Still, ms^{2}io^{6}A37 deficiency caused almost as large a reduction (85%; UGU Figure 4) as that observed for tRNA^{Trp} (UGG, Figure 4). Furthermore, the selection to UGC(Cys) and UCG(Ser) was reduced (Figure 4) although both have as high overall rate of tRNA selection as that of tRNA^{Phe} (Table 4). Thus, we did not observe a correlation between a low overall rate of selection and a reduction due to ms^{2}io^{6}A37-deficiency.

The *supF30* encodes the amber suppressor tRNA$^{Tyr}_{CUA}$, and its ability to influence the frameshifting at the test codon UAG (amber) with various 3′-nucleotides was monitored (Table 4). The ms^{2}io^{6}A37 deficient amber suppressor tRNA had a much reduced activity to compete with the frameshifting tRNA, and its activity was dependent on the nature of the 3′-nucleotide. The largest reduction was observed at UAG-C context (37 *versus* 71, Table 4; Figure 4), which is also the context that the efficiency of the amber suppressor tRNA species are reduced the most by ms^{2}io^{6}A37 deficiency (Bouadloun *et al.*, 1986; Ericson & Björk, 1991). Note also that in this case the same reduction of activity was observed for the ms^{2}io^6- and ms^2-deficient tRNA. Although the largest reduction was observed at this UAG-C context, it has the second highest relative rate of selection of the four codon contexts tested. Therefore, in the case of amber suppressors, the observed effect caused by modification deficiency is not correlated to a low overall rate of tRNA selection.

Of the seven m^{1}G37 containing tRNAs, four species (the three tRNA^{Pro} isoacceptors and tRNA$^{Arg}_{CGG}$) have a short variable loop, whereas the three tRNA^{Leu} isoacceptors have a long variable loop. The four tRNAs with a short variable loop were strongly affected by m^{1}G37-deficiency in the aa-tRNA selection step, whereas those with a long variable loop were not (Figure 3). Thus, the impact of m^{1}G37 may be related to the length of this loop. Interestingly, a correlation also exists between the length of the variable loop and 3′-context sensitivity on aa-tRNA selection (Curran *et al.*, 1995). Although we observed a strict correlation between the impact of m^{1}G37 on aa-tRNA selection and a short variable loop, such a strict correlation was not observed for ms^{2}io^{6}A37, e.g. the largest effect observed by ms^{2}io^{6}A37-deficiency was for tRNA^{Tyr} although it has a long variable loop whereas tRNA^{Phe} with a short variable loop was not sensitive to the level of modification. Therefore, some modified nucleosides (like m^{1}G37) may compensate for a short variable loop, whereas another modified nucleoside in the same position in a tRNA, like ms^{2}io^{6}A37, may not.

We observed a tenfold reduction caused by modification deficiency in the selection of a few tRNAs (e.g. m^{1}G37 (Pro) and ms^{2}io^{6}A37 (Tyr)). In an extensive analysis of how various base substitutions in the anticodon loop and stem influence the efficiency of an amber suppressor, Yarus *et al.* (1986) also observed a tenfold reduction when some critical base substitutions were introduced into the anticodon loop; e.g. substitution of A37 to G37 or A38 to U38 of the amber suppressor tRNA. The effects observed by base substitutions are therefore in the same range as those observed by removing a modification. Thus, a simple methyl group or a hypermodification at position 37 of the

tRNA may be quantitatively as important as the identity of a base.

In conclusion, our results suggest that some modifications in the anticodon region like Ψ, m^1G37 and ms^2io^6A37 may have an effect on the tRNA selection at the A-site. This effect seems not to be correlated in a simple way to a low overall rate of the wild-type tRNA selection. The same modification may or may not have an effect on the tRNA selection suggesting that the function of these investigated modified nucleosides are different depending on which tRNA species they are part of.

Material and Methods

Bacterial strains and plasmids

The bacterial strains used were all derivatives of *S. typhimurium* LT2 (Table 5). *prfB-lacZ* fusion plasmids carrying different test codons were constructed as described earlier (Curran & Yarus, 1986). A pseudo-wild-type *lacZ* (pJC27) that does not require frameshifting for expression of the β-galactosidase was used as the control plasmid. By relating all β-galactosidase values to the β-galactosidase activity of the control plasmid pJCTet27, any general influence on translation, such as reduction of polypeptide chain elongation rate or reduced growth rate, is thus averaged out. The frameshifting tRNA in the original *prfB* mRNA is $tRNA^{Leu}_{GAG}$, which is the only $tRNA^{Leu}$ that reads CUU. However, this tRNA contains m^1G at position 37 and Ψ at position 38, which may influence its ability to frameshift. Therefore, to analyse the function of these two modified nucleosides, we used a plasmid that instead contained GUU (Val1) as the shifty codon. Although the GUU codon is less shifty than the CUU codon, it is still the second most shifty codon tested (Curran, 1993; Weiss *et al.*, 1987). These plasmids are denoted pJCCmGUU and the test codon; e.g. pJCCmGUUCCC. In the analysis of the function of ms^2io^6A37 the CUU (Leu2) was the shifty codon. These plasmids are denoted pJCTetCUU and the test codon. The sequence on the 3′ side of the test codon was the same in all constructs. The sequence around the test codon is: GUU-NNN-CUA-C or CUU-NNN-CUA-C (dashes indicate the zero frame).

Genetic techniques

The plasmids were transformed into strain GT907 (*galE503*, *hsdL66*, *hsdA29*). The *galE503* mutation makes the *S. typhimurium* strain competent for transformation (MacLachlan & Sanderson, 1985) and the *hsd*-mutation abolishes the restriction of *E. coli* grown plasmids. The various plasmids were transferred from this strain into various *Salmonella* strains by transduction using phage P22 HT105/I (*int-201*) (Davis *et al.*, 1980).

Determination of β-galactosidase activity

Medium E (Vogel & Bonner, 1956) supplemented with Casamino acids and 0.4% (w/v) glucose was used in all experiments. Chloramphenicol (17 μg/ml) or tetracycline (7.5 μg/ml) were added for overnight growth to select for the plasmids. Cultures were grown at 37°C for several generations to about 2×10^8 cells/ml at which cell density the cells were harvested. The β-galactosidase activity was measured as described by Miller (1972). The relative activity was calculated as the percentage of the value from the corresponding strain that harbours the pseudo-wild-type plasmid (pJC27). Each value is an average of at least three independent clones. From each culture four enzymatic determinations were made at two different cell concentrations.

Determination of the level of *lacZ* mRNA

At the time of harvest for the determination of the β-galactosidase activity, 2 ml of the culture was poured directly into a 10 ml tube containing crushed ice. The cells were pelleted in the cold and total RNA was extracted with hot acidic phenol (von Gabain *et al.*, 1983). An endonuclease S_1 protection assay was performed essentially as described by Maniatis *et al.* (1982). We used a DNA fragment containing both *lacZ* (about 200 bp) and *rpoB* (about 400 bp) sequence of a *rpoB-lacZ* fusion as probe. The probe was labelled by PCR with [α-^{32}P]dATP. Since the level of the *rpoB* message is known to be a constant fraction of total RNA over a wide range of growth rates (Dennis, 1977), we measured the *lacZ* mRNA level relative to that of the *rpoB* mRNA. About 40,000 cpm probe fragment was added to each hybridisation reaction. After S_1 digestion, the products were separated on a 6% (w/v) denaturing polyacrylamide gel. Two bands, one for *rpoB* (400 nucleotides long) and one for *lacZ* (200 nucleotides long) were quantified using a Phosphorimager. Each value is an average from three independent clones, and a triplicate measurement was performed for each clone. The relative mRNA level was calculated as the percentage of the value from the corresponding strain which harbours the pseudo-wild-type plasmid pJC27.

Table 5. *S. typhimurium* strains used

Strain	Genotype	Source
GT522	*wt*	Ericson & Björk (1986)
GT523	*miaA1*	Ericson & Björk (1986)
GT874	*trmD*$^+$	Björk *et al.* (1989)
GT875	*trmD3*	Björk *et al.* (1989)
SL1306 (GT907)	*metA22*, *metE551*, *ilv-452*, *trpB2*, *xyl-404*, *rpsL120*, *flaA66*, *hsdL66*, *hsdA29*, *galE503*	MacLachlan & Sanderson (1985)
GT956	*hisO1242*, *hisD6404*(Am), *leuA414*(Am), *supF30*	This work
GT1066	*hisO1242*, *hisD6404*(Am), *leuA414*(Am), *supF30*, *miaA1*	This work
GT2176	*miaB2508* :: Tn*10d*Cm, *phs*	Esberg & Björk (1995)
GT2529	*hisT*$^{+}$, *zej-635* :: Tn*5*	This work
GT2530	*hisT1504*, *zej-635* :: Tn*5*	This work
GT3555	*hisO1242*, *hisD6404*(Am), *leuA414*(Am), *supF30*, *miaB2508* :: Tn*10d*Cm	This work

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