Three Modified Nucleosides Present in the Anticodon Stem and Loop Influence the \textit{in vivo} aa-tRNA Selection in a tRNA-dependent Manner

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In \textit{Salmonella typhimurium} seven tRNA species specific for leucine, proline and arginine have 1-methylguanosine (m$^1$G) 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-hydroxyisopentenyl)adenosine (ms$^2$io$^6$A) 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$^1$G37 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$^2$io$^6$A, present in the same position as m$^1$G37 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$^1$G and ms$^2$io$^6$A present next to and 3’ of the anticodon influences the \textit{in vivo} aa-tRNA selection in a tRNA-dependent manner.

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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 \textit{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). \textit{In vitro} some modified nucleosides modulate the anticodon-codon interaction and others influence the rate of polypeptide chain elongation \textit{in vivo}. However, it would be advantageous to study the action of the tRNA \textit{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 \textit{in vivo}. We have used their method to study how three modified nucleosides, pseudouridine (Ψ), 1-methylguanosine (m$^1$G)
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-methyluridylate (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. A trueA (hisT) product catalyses the synthesis of the modified nucleoside m^1G 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 trueA mutant will therefore contain tRNAs having U instead of Ψ at these positions in these tRNAs. Such Ψ- and mG37-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.

The lack of m1G37 also induces frameshifting (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 Ψ- and the ms-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; Getter & Russell, 1969; Wilson & Roe, 1989).

Clearly, the presence of Ψ, mG37 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](image-url)
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, m1G37 and the ms2- and the i6-groups of ms2io6A37 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<sup>Trp</sup> 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, 1988) 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<sub>Leu<sup>GAG</sup></sub> 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<sub>Leu<sup>GAG</sup></sub> in wild-type cells contains both Ψ in the anticodon stem and loop and m1G37, we have used as the shifty tRNA the major tRNA<sub>Val<sup>cmo5UAC</sup></sub>, which normally lacks Ψ38,39,40 and m1G37, in our analysis of how Ψ and m1G37 influence the aa-tRNA selection. In the analysis of the function of ms2io6A37 in the aa-tRNA selection, the tRNA<sub>Leu<sup>GAG</sup></sub>
was used as the shifty tRNA, since it does not contain ms^ioA37. 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 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^G37-, and ms^ioA37-deficient strains compared to the wild-type strain.

Lack of ψ in three Leu-tRNA^{1 eu} species, but not in tRNA^{pro}_{CGG}, reduced the rate of tRNA selection to the cognate codons

The three tRNA^{1 eu} species, which read codons of the CUN type, all contain ψ in the anticodon loop and stem (positions 38 and 40 for tRNA^{1 eu}_{CGG}, position 38 for tRNA^{1 eu}_{GGG}, and positions 38 and 39 for tRNA^{1 eu}_{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 congenic wild-type strain GT2529 (hisT^{+}). The lack of ψ in the major tRNA^{1 eu} and the minor tRNA^{1 eu} increased the frameshifting about threefold (Table 2). According to Curran & Yarus (1989), this value can be calculated as the relative rate of selection (R_s/R; R_s denotes the rate of aa-tRNA selection and R 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

<table>
<thead>
<tr>
<th>Codon</th>
<th>tRNA anticodon</th>
<th>lacZ/rpo8^b (mRNA)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Wild-type^b</td>
</tr>
<tr>
<td>CUU (Leu)</td>
<td>GAG</td>
<td>3.5/2.1</td>
</tr>
<tr>
<td>CUC (Leu)</td>
<td>GAG</td>
<td>0.70/1.0</td>
</tr>
<tr>
<td>CUA (Leu)</td>
<td>UAG</td>
<td>1.1/0.95</td>
</tr>
<tr>
<td>CUG (Leu)</td>
<td>CAG, UAG</td>
<td>0.84/0.86</td>
</tr>
<tr>
<td>CCA (Pro)</td>
<td>GGG; cmo^5U34GG</td>
<td>0.54/0.51</td>
</tr>
<tr>
<td>CCC (Pro)</td>
<td>GGG</td>
<td>1.0/1.0</td>
</tr>
<tr>
<td>CCA (Pro)</td>
<td>cmo^5U34GG</td>
<td>1.08/1.25</td>
</tr>
<tr>
<td>CCG (Pro)</td>
<td>CGG; cmo^5U34GG</td>
<td>1.0/1.25</td>
</tr>
<tr>
<td>CGG (Arg)</td>
<td>CGG</td>
<td>0.99/n.d.</td>
</tr>
<tr>
<td>pJC27</td>
<td>n. r.</td>
<td>1.61/1.61</td>
</tr>
</tbody>
</table>

^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:::rpoB, hisT^{+}) and the second values to strain GT874 (trmD^{+}).
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<sup>Leu</sup> species. The rate of selection of tRNA<sup>Leu</sup><sub>GAG</sub> at the CUU codon may also be reduced but not at the CUC codon.

Of the three tRNA<sup>Pro</sup> species only the tRNA<sup>Pro</sup><sub>GGG</sub> contains Ψ in the anticodon stem and loop (position 40). As expected, the selection rates of the other two Pro-tRNA<sup>Pro</sup> species were the same in the wild-type (Table 2). However, the relative β-galactosidase activity was also the same for CCU/C codons, which are read by Ψ in the anticodon loop. Thus, the possible effect of lack of Ψ in tRNA<sup>Pro</sup><sub>GGG</sub> 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<sup>Leu</sup><sub>GAG</sub>, which has the Ψ only in the anticodon stem (position 40),

Absence of m<sup>1</sup>G in all three tRNA<sup>Pro</sup> species and in tRNA<sup>Arg</sup><sub>GGG</sub> (Arg3) reduced the aa-tRNA selection rate whereas m<sup>1</sup>G37 deficiency in the three tRNA<sup>Leu</sup> only affected the selection to the CUG codon. All three Pro-tRNAs contain m<sup>1</sup>G37. Two of the four proline codons, CCC and CCA, are each read by only one of the three tRNA<sup>Pro</sup> isoacceptors, tRNA<sup>Pro</sup><sub>GGG</sub> and tRNA<sup>Pro</sup><sub>cmo5UGG</sub>, respectively. Lack of m<sup>1</sup>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 CCC codon is read by both tRNA<sup>Pro</sup><sub>GGG</sub> and tRNA<sup>Pro</sup><sub>cmo5UGG</sub> and lack of m<sup>1</sup>G37 reduced the
aa-tRNA selection about 75% at this codon (Figure 3). The CCG codon is read by both the major tRNA Pro isocaccptors, tRNA Pro(Pro) and tRNA Pro(cmo5UGG). The reduction of aa-tRNA selection at this codon is drastically reduced by lack of m1G37 (Rf/Rs 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 m1G37 in the tRNA selection step. There are four tRNA Arg species in S. typhimurium, of which only the CCG reading tRNA Arg(CCG) contains m1G37. However, this tRNA does not contain anyΨ in the anticodon region. Therefore, we expected that the CCG 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 m1G37 improves the rate of the wild-type -galactosidase activity. The experimental errors were in the range +/−16% except for CCG in the hisT1504 mutant, which was +/−20%.

The first number relates to the level obtained with strain GT2529 (CJ-635 ∶ TrnS, hist+1) and the second value to strain GT874 (trmD3). The relative rates (Rf/Rs) of the rate of the aa-tRNA selection (Rf) to that of the frameshift (Rs) was calculated from the equation Rf/Rs = 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 (Rf/Rs)/f is further dependent on the concentration (f) of the tRNA species reading the test codon. The two numbers are related to the wild-type strains as specified under b.

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

<table>
<thead>
<tr>
<th>Codon</th>
<th>tRNA anticodon</th>
<th>Wild-type</th>
<th>hisT1504 (no Ψ at 38-40)</th>
<th>trmD3 (no m1G37)</th>
<th>Rf/Rs wt</th>
<th>(Rf/Rs)/f wt</th>
</tr>
</thead>
<tbody>
<tr>
<td>CUU (Leu)</td>
<td>GAG</td>
<td>0.43/0.51</td>
<td>0.77</td>
<td>0.78</td>
<td>232/195</td>
<td>773/650</td>
</tr>
<tr>
<td>CUC (Leu)</td>
<td>GAG</td>
<td>0.30/0.43</td>
<td>0.45</td>
<td>0.44</td>
<td>332/232</td>
<td>1106/773</td>
</tr>
<tr>
<td>CUA (Leu)</td>
<td>UAG</td>
<td>0.49/0.48</td>
<td>1.35</td>
<td>0.66</td>
<td>203/207</td>
<td>2030/2070</td>
</tr>
<tr>
<td>CUG (Leu)</td>
<td>CAG; UAG</td>
<td>0.14/0.14</td>
<td>0.45</td>
<td>0.24</td>
<td>712/713</td>
<td>712/713</td>
</tr>
<tr>
<td>CCC (Pro)</td>
<td>GGC; cmo5UGG</td>
<td>0.29/0.34</td>
<td>0.27</td>
<td>1.46</td>
<td>344/293</td>
<td>322/2620</td>
</tr>
<tr>
<td>CCA (Pro)</td>
<td>GGG</td>
<td>0.31/0.38</td>
<td>0.26</td>
<td>0.83</td>
<td>322/262</td>
<td>322/2620</td>
</tr>
<tr>
<td>CCG (Pro)</td>
<td>cmo5UGG</td>
<td>0.39/0.42</td>
<td>0.18</td>
<td>1.4</td>
<td>255/237</td>
<td>319/296</td>
</tr>
<tr>
<td>CCCG</td>
<td>CCG</td>
<td>0.51/0.47</td>
<td>0.53</td>
<td>4.0</td>
<td>195/212</td>
<td>195/212</td>
</tr>
<tr>
<td>pJC27</td>
<td>n. r.</td>
<td>100/100</td>
<td>100</td>
<td>100</td>
<td>n. r.</td>
<td>n. r.</td>
</tr>
</tbody>
</table>

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 (CJ-635 ∶ TrnS, hist+1) and the second value to strain GT874 (trmD3).

c The relative rates (Rf/Rs) of the rate of the aa-tRNA selection (Rf) to that of the frameshift (Rs) was calculated from the equation Rf/Rs = 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 (Rf/Rs)/f is further dependent on the concentration (f) of the tRNA species reading the test codon. The two numbers are related to the wild-type strains as specified under b.

d RNA Pro(cmo5UGG) 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.

Frameshifting at CCN codons mediated by m1G37 deficiency contributed less than 5% to the observed programmed +1 frameshifting

We have earlier shown that lack of m1G37 induces +1 frameshifting at sites such as CCG-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 coding (Curran & Yarus, 1988; Weiss et al., 1988). Although the distance between the potential +1 frameshifting site mediated by lack of m1G37 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 m1G37 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 m1G37 containing tRNAs reading codons within the frameshifting window.
Table 3. Frameshifting mediated by m^1G deficient tRNA species independent of the Shine-Dalgarno sequence

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>trmD^+</th>
<th>trmD^+ (no m^1G37)</th>
<th>trmD^+/trmD^+</th>
<th>trmD^+</th>
<th>trmD^+ (no m^1G37)</th>
<th>trmD^+/trmD^+</th>
</tr>
</thead>
<tbody>
<tr>
<td>non-SD</td>
<td>0.018</td>
<td>0.052</td>
<td>2.9</td>
<td>0.015</td>
<td>0.037</td>
<td>2.5</td>
</tr>
<tr>
<td>CCA</td>
<td>(4.3%)</td>
<td>(3.7%)</td>
<td>3.3</td>
<td>(4.6%)</td>
<td>(1.9%)</td>
<td>3.3</td>
</tr>
<tr>
<td>CCG</td>
<td>(1.7%)</td>
<td>(2.3%)</td>
<td>2.8</td>
<td>0.011</td>
<td>0.022</td>
<td>2.0</td>
</tr>
<tr>
<td>CCU</td>
<td>(3.5%)</td>
<td>(2.5%)</td>
<td>3.7</td>
<td>(3.7%)</td>
<td>(2.2%)</td>
<td></td>
</tr>
</tbody>
</table>

*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 :: Tn10delCm mutant. This modified nucleoside is not present in tRNA^Leu^, 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 :: Tn10delCm 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 :: Tn10delCm mutant. There are two tRNA^Tyr^ 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^ species (Table 4). The reduction was most pronounced in the miaA1 mutant (a reduction of R_e/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_e/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 :: Tn10delCm

<table>
<thead>
<tr>
<th>Test codon</th>
<th>tRNA anticodon</th>
<th>% pJC27</th>
<th>miaA1</th>
<th>miaB2508</th>
<th>R_e/R_s wt</th>
<th>(R_e/R_s)/t wt</th>
</tr>
</thead>
<tbody>
<tr>
<td>CUU (Leu)</td>
<td>GAG</td>
<td>7.3</td>
<td>7.0</td>
<td>6.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CUC (Leu)</td>
<td>GAG</td>
<td>4.0</td>
<td>3.8</td>
<td>3.4</td>
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<td>2.5</td>
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<tr>
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<td>46</td>
<td>27</td>
<td>3.7</td>
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*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 message level, relative to that of the pJC27 control (does not require a frameshift for the production of β-galactosidase). The ratios were the same in all cultures (variation +/− 26% except for pJC27 in miaB2508 :: Tn10delCm 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_s/R_e) are calculated as described in the legend to Table 2.
A difference between wild-type and the mutant is to a less extent of Trp-, Cys-, and Ser-tRNAs, but tRNA lacks the ms2io6 modification (Esberg & BjoÈrk, 1995). To relate the extensive data set to effects of ms 2io6A37 at RF2 alleles that have an amber test codon (Figure 4). Thus, the ms2io6A37 modification re-
p
p
sidered significant when 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^{Try} only lacked the ms2-group. The selection of Cys-tRNA^{Cys} at the two cognate codons UGU and UGC was affected differently by ms2io6A37 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 ms2io6A37 (Table 4; Figure 4). Thus, the ms2io6A37 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 cognate codons 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 ms2-group reduces the efficiency of amber suppressor two to three-fold compared to more than tenfold when the tRNA lacks the ms2io6 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 ms2io6A37 at RF2 alleles that have an amber test codon.

Strains were constructed that contain the supF30 mutation, which encodes the amber suppressor tRNA^{Try}QUA, and the miaA1 mutation, the miaB2508::Tn10dCm 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^{Try}QUA, the lack of ms2io6A37 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 ms2 group. However, the miaB mutation did not strongly affect the rate of the wild-type tRNA^{QUA} (see UAU and UAC in Figure 4) although the 3’ context 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^{Try}. We note that the only difference between the amber suppressor tRNA^{Try} and the wild-type form of it is the wobble nucleoside (C34 in the amber suppressor and Q34 in the wild-type tRNA^{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 ms2io6-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'G37, 38,39,40 and ms2io6A37 (tRNA species lacking only the ms2-group of ms2io6A 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 38,39,40 improved the selection rate of Leu-tRNA to CUA and CUG codons (Table 2; Figure 3), the m'G37 in the same tRNAs only improved the tRNA selection to the CUG codon. On the other hand, the m'G37 strongly influenced the Pro-tRNA selection to all proline codons (a possible exception may be CCC, see Figure 3) whereas the 340 of Pro-tRNAPro did not. Although the ms2io6A37 strongly influences the overall activity of the translation cycle of all tRNAs that have ms2io6A37 (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 differentantly 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 tRNAHis (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\textsubscript{Leu}\textsubscript{CUA} (reads CUC) and tRNA\textsubscript{Leu}\textsubscript{CUG} (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\textsubscript{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\textsubscript{Leu}\textsubscript{CUA} (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\textsubscript{Leu}\textsubscript{CUG} and tRNA\textsubscript{Leu}\textsubscript{CUA} species. The overall rate of tRNA selection, which is a function of the \( R_s/R_c \) and the concentration (\( t_1 \)) 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 \( R_s/R_c \) 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\textsubscript{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\textsubscript{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\textsubscript{Pro}\textsubscript{CUC} 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\textsubscript{Leu} are in position 38 in the anticodon loop (tRNA\textsubscript{Leu}\textsubscript{LAG} also contains Ψ39), tRNA\textsubscript{Pro}\textsubscript{CUC} 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, Ψ52 of tRNA\textsubscript{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 stabilize 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\textsubscript{Pro} 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\textsubscript{Gln}, is reduced by more than 95% (Bössi & 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\textsuperscript{1}G37 modification

In Mops-glucose minimal medium lack of m\textsuperscript{1}G37 increases the average step time 3.3-fold (Li & Böjörk, 1995). As stated above, the leu\textsubscript{ABCD} 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\textsuperscript{1}G37 containing tRNA\textsubscript{Leu}\textsubscript{CUA} (reads CUA) and tRNA\textsubscript{Leu}\textsubscript{CUG} (reads CUC). However, unlike the hisT1504 mutation, which derepresses the leu-operon severalfold, the trmD3 mutation does not (Li & Böjörk, 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\textsubscript{Leu}\textsubscript{CUA} of the three tRNA\textsubscript{Pro} isoacceptors and tRNA\textsubscript{Arg}\textsubscript{CUC} increases to 3.8-fold. Consistent with these results, we observed no effect on the selection of tRNA\textsubscript{Leu}\textsubscript{CUG} at CUC codons (0.43 versus 0.44, Table 2) and only a small effect at CUA (tRNA\textsubscript{Leu}\textsubscript{CUA}) codon (Table 2).

All three tRNA\textsubscript{Pro} isoacceptors as well as the tRNA\textsubscript{Arg}\textsubscript{CUC} were strongly affected in the tRNA selection by m\textsuperscript{1}G37 deficiency. Among the proline tRNAs the largest effect (reduction by 90%) was observed for CCG, which is read by tRNA\textsubscript{Pro}\textsubscript{CUC} and tRNA\textsubscript{Pro}\textsubscript{CUGS}. 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\textsuperscript{1}G37 deficiency reduced the rate of tRNA selection by 50% to CCC (Pro) and by 80% to CCG (Arg) although the overall rate is much higher (3000) for CCA than that for CCG (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\textsuperscript{1}G37-deficient tRNA species.
Effects of the ms\textsuperscript{2}io\textsubscript{6}A\textsubscript{37} modification

It has been suggested that the presence of ms\textsuperscript{2}io\textsubscript{6}A\textsubscript{37} stabilises the intrinsically weak inter-
action of the A36-U base-pair (Jukes, 1973; Nishimura, 1972). Indeed, the presence of 
the ms\textsuperscript{2}io\textsubscript{6}A\textsubscript{37} modification stabilises tRNA-tRNA di-
mers with complementary anticodons mainly due 
to an improved stacking of the hypermodified nu-
cleoside (Houssier & Grosjean, 1985; Vacher et al., 1984). Thus, the results from such model exper-
iments suggest that the modification of A\textsubscript{37} to 
ms\textsuperscript{2}io\textsubscript{6}A\textsubscript{37} de\textsuperscript{ciency causes almost as large a 
reduction in the tRNA selection efficiency as that observed for tRNA Tyr. The tRNA Cys has a higher 
overall rate of selection than tRNA Trp. Still, the ms\textsuperscript{2}io\textsubscript{6}A\textsubscript{37}-deficient amber suppressor tRNA 
had a much reduced activity to compete with the frameshifting tRNA, and its activity was depen-
dent on the nature of the 3'-nucleotide. The largest 
reduction was observed at UAG-C context (37 ver-
sus 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\textsuperscript{2}io\textsubscript{6}A\textsubscript{37} 
deficiency (Bouadloun et al., 1986; Ericson & Björk, 1991). Note also that in this case the same 
reduction of activity was observed for the ms\textsuperscript{2}io\textsubscript{6}- 
and ms\textsuperscript{2}-deficient tRNA. Although the largest 
reduction was observed at this UAG-C context, it 
had 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\textsuperscript{1}G37 containing tRNAs, four 
(species the three tRNA\textsubscript{Leu}\textsuperscript{CUN}, tRNA\textsubscript{Leu}\textsuperscript{UGA}) have a short variable loop, whereas 
the three tRNA\textsubscript{Leu}\textsuperscript{CAG} isoacceptors have a long variable 
loop. The four tRNAs with a short variable loop 
were strongly affected by m\textsuperscript{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\textsuperscript{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 sensi-
tivity on aa-tRNA selection (Curran et al., 1995). 
Although we observed a strict correlation between 
the impact of m\textsuperscript{1}G37 on aa-tRNA selection and a 
short variable loop, such a strict correlation was 
ot observed for m\textsuperscript{2}io\textsubscript{6}A\textsubscript{37}, e.g. the largest effect 
observed by ms\textsuperscript{2}io\textsubscript{6}A\textsubscript{37}-deficiency was for tRNA\textsubscript{Arg} in 
even a long variable loop whereas tRNA\textsubscript{His} with a short variable loop was not sensi-
tive to the level of modification. Therefore, some 
modified nucleosides (like m\textsuperscript{1}G37) may config-
erate for a short variable loop, whereas another 
modified nucleoside in the same position in a 
tRNA, like ms\textsuperscript{2}io\textsubscript{6}A\textsubscript{37}, may not.

We observed a tenfold reduction caused by 
modification deficiency in the selection of a few 
tRNAs (e.g. m\textsuperscript{1}G37 (Pro) and ms\textsuperscript{2}io\textsubscript{6}A\textsubscript{37} (Tyr)). In 
an extensive analysis of how various base substi-
tutions 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 A\textsubscript{37} to 
G\textsubscript{37} or A\textsubscript{38} to U\textsubscript{38} 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 \( \Psi \), \( m^1G37 \) and \( m^2io^6A37 \) 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 \( \beta \)-galactosidase was used as the control plasmid. By relating all \( \beta \)-galactosidase values to the wild-type tRNA selection, the function of these plasmids was determined for each clone. The relative mRNA level 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 \( \beta \)-galactosidase activity**

Medium E (Vogel & Bonner, 1956) supplemented with Casamino acids and 0.4% (w/v) glucose was used in all experiments. Chloramphenicol (17 \( \mu \)g/ml) or tetracycline (7.5 \( \mu \)g/ml) were added for overnight growth to select for the plasmids. Cultures were grown at 37°C for several generations to about 2 \( \times \) \( 10^8 \) cells/ml at which cell density the cells were harvested. The \( \beta \)-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).

**Table 5. S. typhimurium strains used**

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Acknowledgements

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