Programmed translational –1 frameshifting on hexanucleotide motifs and the wobble properties of tRNAs

Patricia Licznar1, Nina Mejlhede1,2,3, Marie-Françoise Prêre1, Norma Wills2, Raymond F.Gesteland2, John F.Atkins2 and Olivier Fayet1,4

1Microbiologie et Génétique Moléculaire, CNRS, 118 route de Narbonne, 31062 Toulouse Cedex, France and 2Department of Human Genetics, University of Utah, 15N 2030E Room 7410, Salt Lake City, UT 84112-5330, USA
3Present address: Department of Infectious Disease and Immunology, State Serum Institute, Artillerivej 5, 2300 Copenhagen S, Denmark
4Corresponding author
e-mail: olivier@ibcg.biotoul.fr

P.Licznar and N.Mejlhede contributed equally to this work

Programmed –1 ribosomal frameshifting, involving tRNA re-pairing from an AAG codon to an AAA codon, has been reported to occur at the sequences CGA AAG and CAA AAG. In this study, using the encoding region of insertion sequence IS3, we have investigated the influence on frameshifting in Escherichia coli of the first codon of this type of motif by changing it to all other NNA codons. Two classes of NNA codons were distinguished, depending on whether they favor or limit frameshifting. Their degree of shifting is correlated with wobble propensity, and base 34 modification, of their decoding tRNAs. A more flexible anticodon loop very likely makes the tRNAs with extended wobble more prone to liberate the third codon base, A, for re-pairing of tRNA15s in the –1 frame.

Keywords: frameshifting/hexanucleotide/insertion sequences/tRNA modification/wobble

Introduction

The expression of a minority of genes in probably all organisms involves a proportion of ribosomes shifting reading frame at specific sites. In some cases the function of this programmed event is regulatory and in others the synthesis of two products, with different C-termini, is the important consequence (Farabaugh, 1997; Atkins et al., 2001). Examples of the former often implicate +1 frameshifting, whereas many of the latter involve –1 frameshifting. Beside its role in gene expression, frameshifting elicits interest because of what it reveals about the functioning of the translational molecular machine, especially in view of the recent advances in our understanding of the ribosome at the atomic level (Ogle et al., 2001, 2002, 2003; Yusupova et al., 2001; Noller et al., 2002; Valle et al., 2002; Gao et al., 2003). In particular, frameshifting brings into light the intricacies of the relation between a tRNA, its codon and the ribosome, as illustrated by the present work, and also raises the question of the maintenance of the translational reading frame (Farabaugh and Björk, 1999; Atkins et al., 2000). All known cases of –1 ribosomal frameshifting involve dissociation of codon: anticodon pairing followed by anticodon re-pairing to mRNA at an overlapping –1 frame codon. Early work with frameshift mutant leakiness and synthetic constructs focused on low frequency dissociation and re-pairing events involving a single tRNA anticodon (Weiss et al., 1987; Gallant and Lindsley, 1992). The high frequency programmed frameshifting events involved in decoding potato virus M (Gramstad et al., 1994), bacterial insertion sequences IS3 (Sekine et al., 1994) and IS1222 (Mejlhede, P.Licznar, M.F.Prêre, N.Wills, R.F.Gesteland, J.Atkins and O.Fayet, in preparation) and that associated with decoding the Bacillus subtilis cytidine deaminase gene (cdd) (Mejlhede et al., 1999) have been considered in these terms. However, the great majority of known programmed –1 frameshifting involves re-pairing by tandem tRNAs at heptanucleotide sequences. Tandem slippage was discovered by Jacks and Varumus (1988) in their studies on the frameshifting required for retroviral gene expression and has since been found mostly in the decoding of viruses from diverse sources and in bacterial programmed frameshifting. Searches for additional cases of frameshifting were therefore concentrated on the characteristic heptanucleotide motifs for tandem re-pairing with little attention to single re-pairing possibilities.

The frameshifting that occurs in decoding B.subtilis cdd is 16% efficient. The intrinsic level of frameshifting at its A AAG shift site is 1.5%; as originally shown in Escherichia coli, tRNA15s (anticodon 3′-UUmm5’S5′-U5′, where mm5′S5′U5′ is 5-methylaminomethyl-2-thiouridine) is prone to shift –1 from AAG to AAA (Weiss et al., 1989; Tsuchihashi and Brown, 1992). A Shine–Dalgarno-like sequence within the coding sequence nine bases 5′ of the shift site acts to stimulate –1 frameshifting 10.6-fold (Mejlhede et al., 1999). Analogous stimulatory effects of nearby 5′ internal Shine–Dalgarno sequences are known for tandem –1 frameshifting (Larsen et al., 1994; Retberg et al., 1999). The identity of the codon, CGA, upstream of the AAG is crucial for high efficiency frameshifting, but not the base 5′ of it, leading to the hypothesis of a hexameric shift site (Mejlhede et al., 1999). The anticodon of the CGA-decoding tRNA15s (3′-GCI-5′) contains inosine, I. Previous studies have shown very inefficient A/I pairing in vivo (Curran, 1995; Carter et al., 1997). It was therefore suggested that apposition of the purine inosine in the anticodon with the purine A of the cdd CGA codon does not permit strong pairing and would frequently result in the liberation of the third codon base, thereby allowing re-pairing of tRNA15s from AAG to AAA.

Decoding of a bacterial transposable element, insertion sequence IS1222 (Steibl and Lewecke, 1995), also uses –1
frameshifting at a CGA AAG hexamer. Frameshifting is required for synthesis of the transposase, and so for transposition, of IS1222 (N.Mejlhede, P.Licznan, M.F.Pré, N.Wills, R.F.Gesteland, J.Atkins and O.Fayet, in preparation). This recoding event, occurring at a frequency of ~7%, is stimulated by a weaker 5’ Shine-Dalgarno sequence than in cdd, but has a 3’ stimulatory stem-loop sequence. A stimulatory 3’ stem-loop is also not unique to this type of shift site, as it is known for several cases of bacterial dual-slipage frameshift regions including that for synthesis of a DNA polymerase component encoded by the *E.coli dnaX* gene (Larsen *et al.*, 1997). In another insertion sequence, IS3, an A AAG frameshift site is associated with a pseudoknot as 3’ stimulator (there is no 5’ stimulatory SD sequence) and the frequency of frameshifting was reported to be 6% (Sekine *et al.*, 1994). In this example the two upstream nucleotides are CA, which gives a CAA AAG hexamer. However, the role in frameshifting modulation of the CAA codon was not determined in the IS3 context, nor had it been tested within the IS1222 recoding signal. Possible different mechanistic consequences of the two types of sequences were examined in the current study.

The present work also determines whether NNA codons other than CGA, in the sequence NNA AAG, are decoded by tRNAs that liberate the third codon base, A, permitting realignment of tRNA<sup>5</sup> in the –1 frame. The incidence of the nucleotide 5’ of the NNA codon and the effect of the modification status of anticodon base 34 of the NNA-decoding tRNA were also analyzed. Two versions of a model for single re-pairing frameshifting are presented.

**Results**

Members of the IS3 family of insertion sequences have two partially overlapping open reading frames, *orfA* and *orfB*, with –1 ribosomal frameshifting at a specific site in the overlap region yielding an OrfAB transframe protein with transposase function (Mahillon and Chandler, 1998). IS3 itself, the archetype of the family, and IS1222 have this gene organization (Figure 1A). Their *orfA* gene encodes a protein containing a predicted α-helix–turn–α-helix motif, as well as a leucine-zipper motif, and their *orfB* gene encodes a protein with a domain characteristic of retroviral integrases and IS3 family transposases (Mahillon and Chandler, 1998). So far in IS elements, the OrfB polypeptide has only been found to be important for transposition activity when fused to the OrfA protein (Polard *et al.*, 1992). In the *orfA–orfB* overlap region of both IS3 and IS1222, frameshifting presumably occurs by re-alignment of one tRNA<sup>5</sup> on the A AAG sequence. To elucidate the exact role of the upstream codon in each IS, we cloned both frameshift regions into a reporter plasmid and changed the upstream codon of their respective hexamer to all 13 other N<sub>1</sub>N<sub>2</sub>A<sub>3</sub> sense codons (diagrammed in Figure 1A). In addition, we investigated the incidence of the nucleotide on the 5’ side of the hexamer (nucleotide N<sub>0</sub>). In one set, N<sub>0</sub> was different from N<sub>1</sub> to prevent re-pairing of the N<sub>1</sub>N<sub>2</sub>A<sub>3</sub>-decoding tRNA, and in the other set it was identical to N<sub>1</sub> in order to allow re-pairing of at least the third anticodon base (tRNA nucleotide 36). Since identical results were found with IS3 and IS1222, only the IS3 results are presented below.

![Fig. 1. The IS3 frameshift region, its various derivatives and the plasmid reporter system.](image_url)
For reasons discussed in the next section, we also analyzed in the case of IS3 the effect on frameshifting of the codon 3′ to the A AAG shift site (nucleotides N$_7$N$_6$N$_9$ in Figure 1A).

To study IS3 frameshifting, the 81-nucleotide segment shown in Figure 1A was inserted between, and fused to, two genes. The end of orfA is in-frame with gene 10 of phage T7 and the beginning of orfB is in-frame with the lacZ coding sequence on a plasmid-borne construct (Figure 1B; Retberg et al., 1999). Quantitation of the G10-OrfA'-OrfB'-LacZ transframe product (FS in Figure 1B) and G10-OrfA' (G10 in Figure 1B) products was performed by in vivo protein pulse labeling followed by PAGE separation or by β-galactosidase assay.

**Frameshifting occurs while the AAG codon is in the ribosomal A-site**

To gain evidence concerning the ribosomal site at which the frameshift occurs with the IS3 motif, the GCC codon 3′ to the C CAA AAG wild-type sequence was changed to all 16 possible N$_7$G$_6$N$_9$ codons (Figure 1A); in another set of constructs, a different shift site was used, C GCA AAG, and the 3′ codon was changed to the 32 possible N$_7$(A/G)$_6$N$_9$ codons. Slow-to-decode codons, especially stop codons, in the ribosomal A-site can stimulate non-programmed (i.e. low level) −1 frameshifting of peptidyl-tRNA in the P-site, if upstream re-pairing is possible (Weiss et al., 1987; Gallant and Lindsley, 1992). Consequently, if a 3′ stop or rare (e.g. AGG or AGA in E.coli) codon has a stimulatory effect, this indicates P-site slippage, whereas absence of an effect suggests that frameshifting occurred while the shifty motif was in the A-site.

Figure 2A and B shows the results of the analysis carried out on the wild-type and mutant IS3 signals, respectively. Interestingly, in this set of 48 N$_7$(A/G)$_6$N$_9$ constructs the absolute level of frameshifting varies with the 3′ context. In Figure 2A, for example, there is a 2-fold difference between GGA and GCC or a 3-fold factor between GGC and CGG. In Figure 2B, β-galactosidase assay was used to measure frameshifting. Even if levels of frameshifting thus measured are lower than with the pulse-labeling method (see Materials and methods), significant differences also exist between constructs (e.g. 2.5-fold between GAA and CAG). In a recent study we observed a similar effect of the 3′ context with the four heptameric X XXA AAG dual slippage motifs (Bertrand et al., 2002). The statistical analysis of nearly 200 mutants showed that the first nucleotide after the motif has the primary effect on frameshifting, with, in order of decreasing efficiency, U > C > A > G. Our interpretation was that when the AAG slippery codon enter the A-site, there is a competition between standard decoding and −1 frameshifting the outcome of which could be in part determined by the stacking of the next nucleotide of the message on the AAG codon–anticodon helix. Purines, having a higher stacking potential than pyrimidines, would therefore tend to limit frameshifting (see discussion in Bertrand et al., 2002). Comparison of the mean value after grouping of the constructs according to the identity of the first nucleotide of the 3′ codon (Figure 2) demonstrates that, for the IS3 shift site also, pyrimidines in this position generally results in a higher level of frameshifting than when it is a purine.

![Fig. 2. Effect on frameshifting of variants of the codon 3′ of the IS3 shift site. The mutations indicated in Figure 1A were introduced on the 3′side (nucleotides N$_7$N$_6$N$_9$) of the A AAG shift site and the modified frameshift regions were cloned into the pOFX302 reporter plasmid. (A) Summary of the results obtained by pulse-labeling of C CAA AAG N$_7$G$_6$N$_9$ constructs. (B) Results obtained by performing β-galactosidase assays with C GCA AAG N$_7$(A/G)$_6$N$_9$ constructs. The value for both motifs with GCC as 3′ codon is also given (wt stands for C CAA AAG).](image)
between the eight constructs with a 3' stop, or rare, codon (marked with an asterisk in Figure 2A and B) and most of their related sense codon constructs. There is no increase in frameshifting caused by the presence of a 3' stop or rare codon. Therefore, in the context of the IS3 recoding region, frameshifting of tRNA<sup>39</sup> from AAG to AAA is most likely initiated while AAG is in the A-site.

**First codon of the hexanucleotide shift site**
tRNA<sup>39</sup> re-pairing to mRNA at a cognate codon requires that the last base of the previous codon be A. Previous partial mutational analysis of the cdd signal suggested a strong influence on recoding of the identity of the NNA codon, with CGA apparently being the most shift prone (Mejlihed <i>et al.</i>, 1999). To investigate this question more systematically, CAA was substituted in the IS3 recoding region by all other NNA codons except for UAA and UGA that would be in-frame stop codons. The 5' nucleotide, N<sub>0</sub>, was also changed as indicated to preclude its involvement in Watson–Crick pairing with a tRNA attempting to repair to mRNA at the overlapping −1 frame codon. The results presented in Figure 3A show clearly that the level of frameshifting is strongly influenced by the identity of the first two nucleotides of the NNA AAG hexamer and that several codons are equal to, or better than, CGA. The 14 NNA codons can be separated into two classes, the ones that lead to ‘low’ frameshifting and those that give ‘high’ frameshifting. Within each class, there is a notable amount of variation indicative of an extra layer of idiosyncratic behavior. For example, GUA and AGA are respectively remarkably higher and lower than the others. Codons UCA and ACA first appeared as intermediate, but not overlapping with any in the low category (>95% confidence level).

The decoding properties of the wobble base (Crick, 1966; Yokoyama and Nishimura, 1995) of the cognate tRNAs for NNA codons are given in Figure 3B. With the exception of GGA, the NNA codons which give a low level of frameshifting belong to split codon boxes. These codons are decoded by tRNAs with a ‘restricted wobble’ capacity, i.e. they have a 3'-N<sub>36</sub>N<sub>15</sub>U<sub>45</sub>-5' anticodon (except tRNA<sup>Glu</sup>, which has a 3'-UAC-5' anticodon) that read NNA and NNG codons only (or UUA only for tRNA<sup>Glu</sup>). In contrast, the NNA codons associated with high frameshifting come from four-codon family boxes. Their respective tRNAs also have a 3'-N<sub>56</sub>N<sub>65</sub>U<sub>34</sub>-5' anticodon, except tRNA<sup>Arg</sup> 3'-GCI-5', but can read three codons, those ending with A, G and U (or C, U and A for tRNA<sup>Arg</sup> 3'-GCI-5'). All the NNA-specific tRNAs have a modified anticodon base U<sub>34</sub> (or C<sub>34</sub> to 1<sup>12</sup>C in tRNA<sup>Glu</sup>; or A<sub>34</sub> to I in tRNA<sup>Arg</sup>) and the type of U<sub>34</sub> modification is clearly correlated with ‘low’ or ‘high’ shiftiness (Figure 3B). The tRNAs which have an x<sup>5</sup> type modification (i.e. mm<sub>5</sub>, cmn<sub>5</sub> and mm<sub>5</sub> for each tRNA<sup>NNA</sup>) restrict frameshifting, whereas those having a modification of the x<sup>5</sup> type allow more frameshifting to occur.

**Possible upstream re-pairing for tRNA<sup>NNA</sup>**
A second set of 14 constructs was generated by changing the 5' N<sub>0</sub> nucleotide to one identical to the first of the NNA codon. The consequences in terms of re-pairing in the −1 frame vary according to each tRNA/N<sub>0</sub>N<sub>1</sub>N<sub>2</sub>A<sub>3</sub> pair (Figure 3C). In four cases a consensus heptameric X

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**Fig. 3.** Effect on frameshifting of N<sub>0</sub>N<sub>1</sub>N<sub>2</sub> variants: correlation with wobble properties and modification of base 34 of the N<sub>1</sub>N<sub>2</sub>-decoding tRNA (A and B), and variants where N<sub>0</sub> is identical to N<sub>1</sub> (C). In (A) and (C), frameshift efficiencies were measured by quantitation of [35]<sup>3</sup>S- methionine labeled products. The error bars indicate the 95% confidence interval. In (B), the sequences of the anticodons of the E.coli NNA-decoding tRNAs including modifications of base 34 are shown. The modifications are abbreviated as follows: 5-methylaminomethyluridine (mm<sub>5</sub>U), 5-methylaminomethyl-2-thioridine (mm<sub>5</sub>SU), 5-caboxymethylaminomethyluridine (cmnSU), 5-methoxyuridine (mo<sub>5</sub>U), uridine-5-oxoacetic acid (emo<sub>5</sub>U), inosine (I) and lysidine (k<sub>5</sub>C). Three anticodon sequences are not from E.coli but from Bacillus subtilis (B.s.) or Mycoplasma capricolum (M.c.) as indicated.

XXX YYZ site for tandem slippage is generated, allowing cognate (A AAA AAG, G GGA AAG) or near cognate (C CCA AAG and U UUA AAG) interaction of P-site tRNA<sup>NNA</sup> in the −1 phase. Accordingly, frameshifting is greatly stimulated from 6- to nearly 50-fold. For the 10 other NNA codons, the outcome is variable. In two cases, G GAA and C CAA, there is a 4-fold stimulation perhaps related to Watson–Crick pairing of tRNA bases 34 and 36 with the −1 frame codon (middle base 35 would form either an acceptable U-G pair or a less favorable U-C pair). Two others have a 2-fold increase (A ACA and C CGA) and the six remaining cases are not affected; for all eight, Watson–Crick pairing is limited to the interaction between N<sub>0</sub> and anticodon base 36. From this we conclude that providing tRNA<sup>NNA</sup> with an opportunity to re-pair in a
cognate or near-cognate manner in the −1 phase increases frameshifting efficiency. Restricting pairing to the first position, N0, of the codon in the new frame has no, or only a marginal, positive effect on frameshifting which must then proceed via re-pairing of the AAG-decoding tRNA only.

Role of tRNA base U34 modification

The correlation between base U34 modification and frameshifting propensity, as well as many data suggesting that U34 modification may contribute to the wobble property of the tRNAs, prompted us to investigate the effect of mutations affecting specifically the xo3 or the x3 modification. Inactivation of the aroD gene prevents the formation of cmo3U (Björk, 1995). Inactivation of mmmA and mmmE, respectively, precludes replacement of xo3 by x3 and insertion of the mmm3 group (Björk, 1995). Two subsets of the NNA AAG constructs, three ‘low’ and three ‘high’ frameshifters, were tested in the three modification-deficient mutants (Figure 4); in the chosen constructs, the N0 nucleotide does not allow upstream re-pairing. The aroD mutation did not appear to have any significant effect on frameshifting modulation by the two classes of NNA codons. This indicates that the cmo3 modification is not what makes the tRNAs with extended wobbling more shift prone. In contrast, the mmmE and, more clearly, the mmmA mutations led to reduced frameshifting frequency, especially in the case of the ‘high’ frameshifting NNA codons, for which there is a 2- to 4-fold reduction. With these two mutants, the modification deficiencies affect not only the ‘low’ frameshifting NNA-decoding tRNAs, but also the downstream tRNA159, the one that shifts from the 0 to the −1 frame. So in the case of ‘high’ frameshifting NNA codons, the assay in the mmm mutants reveals the importance of modification for the frameshifting capacity of tRNA159. Obviously, both mmmA and mmmE alter this capacity, the former more than the latter.

Discussion

P-site pairing maintained or irreversibly disrupted in hexanucleotide shifting

Influence of a stop codon (or rare codon) on −1 frameshifting is evidence that disruption of codon-anticodon base-pairing and re-pairing in a new frame occurs in the P-site. The observed lack of influence of a stop or rare codon placed immediately 3’ of the CAA AAG or GCA AAG hexanucleotide shift site is interpreted to mean that CAA or GCA, and by extension any other NNA codon, is in the ribosomal P-site and AAG is in the A-site when frameshifting occurs. This is comparable to classical heptanucleotide frameshifting on XXY YYZ sequences, where the XXY and YYZ 0 frame codons are in the P- and A-site, respectively (Jacks et al., 1988; Weiss et al., 1989; Harger et al., 2002).

An interesting feature of the results is the involvement of hexanucleotide, rather than heptanucleotide, shift sites for the lesser, but still significantly efficient, −1 frameshifting studied. This hexanucleotide frameshifting likely involves the same mRNA movement as in tandem slippage in −1 heptanucleotide frameshifting (Jacks et al., 1988; Weiss et al., 1989; Harger et al., 2002). The difference is that in hexanucleotide frameshifting there is no re-pairing of the P-site tRNA to mRNA. In one model of hexanucleotide frameshifting, outlined in Figure 5A, there is dissociation of pairing in the P-site without re-pairing to mRNA. Lack of involvement of P-site re-pairing is quite plausible, since peptidyl-transfer can sometimes be carried out, in vitro and in vivo, in the absence of codon–anticodon interaction (Yusupova et al., 1986; Atkins et al., 2001; A.J.Herr, N.M.Wills, C.Nelson, R.F.Gesteland and J.F.Atkins, in preparation). Our data show that −1 frameshifting is more efficient when there is limited P-site re-pairing potential on non-standard heptamers (e.g. CCAA AAG or GAA AAG; see Figure 3), provided that two conditions are met: N0 and N1 have to be identical and at least one other Watson–Crick pair exists between the tRNA and the −1 frame N0N1N2 codon.

An alternative model for hexanucleotide −1 frameshifting is that P-site pairing is partially maintained, detachment of only anticodon base 34 from the third codon base is involved allowing tRNA159 to re-pair in the −1 frame (Figure 5B). Retention of codon pairing by P-site anticodon bases 36 and 35 would require not only a change of the relative positions of anticodon bases 35 and 34, but also a large change in position within the P-site of the whole anticodon to permit pairing between anticodon base 36 of A-site tRNA159 and what was the third codon base of the P-site. It is unlikely that tRNA159 initially pairs in the −1 frame with AAA in the sequence A AAG since the AAG lysine codon is required for efficient frameshifting.

A-site tRNA

Both models require re-pairing of the A-site tRNA in the −1 frame. The frame change could then happen before and/or after GTP hydrolysis and EF-Tu release, after codon recognition, during the second-half of the ‘initial selection’ steps or at the onset of the following ‘accommodation’ step, as defined by Rodnina and Wintermeyer (2001). It possibly occurs after the correct codon–anticodon interaction in the A-site triggers adoption of a ‘closed’ conformation by the 3OS subunit (Ogle et al., 2002, 2003). Once accepted-in, the A-site tRNA, still in the A/T hybrid state, with or without EF-Tu attached, must have more
adjustment by one nucleotide of the mRNA position, brought along by the 3’ pseudoknot (Plant et al., 2003), probably occurs at that time (stage 2 in Figure 5). The A-site tRNA eventually moves to reach the A/A state; this requires a large movement (56 Å) of its CCA end and the repositioning by 9 Å of anticodon base 34, accompanied by a rotation around the phosphodiester bond between the P and A codons (Noller et al., 2002). Locking in the P/P and A/A states of the two tRNAs and their anticodons probably makes the change in frame irreversible. There is, then, a kink in the message between the P and A codons (stage 3 in Figure 5) and the phosphate group of base 1401 of 16S RNA is wedged between the last and the first bases of each codon (Ogle et al., 2001; Yusupova et al., 2001). What is unusual here is that the P-site codon should contain at most two paired bases. Re-pairing of the A-site tRNA in the –1 frame and mRNA movement resets the reading frame. Peptidyl transfer can now take place and be followed by a normal three-base translocation (stage 4 in Figure 5).

This model of re-alignment of aminoacyl tRNA^_Arg occurring prior to translocation derives from the one originally proposed for tandem –1 slippage (Jacks et al., 1988) and very recently refined and justified by Harger et al. (2002). However, for both tandem –1 slippage (Weiss et al., 1989; Atkins and Gesteland, 1995) and the P-site pairing model presented here, another proposal invokes slippage after transpeptidation, and perhaps during translocation resulting effectively in a two-base translocation. Arguments against this version were presented in detail by Harger et al. (2002). What we would like to add, in view of the three-dimensional model for tRNAs movements outlined by Noller et al. (2002), is that directly after transpeptidation the tRNAs are still in the same ‘locked’ configuration, and are therefore unlikely to change frame. Translocation per se is, together with peptidyl-transfer, the fundamental function of the ribosome, and as such it probably is the most constrained one. Again it is difficult to envision the tRNAs and the mRNA being loose at this critical stage. The advantage of frameshifting occurring at the accommodation step is that it does not tamper with the strict three-base translocation mechanism.

**P-site tRNA**

In both versions of the model, anticodon base 34 of the P-site tRNA has the ability (to different extents depending on the tRNA) of un-pairing from the third codon base. In the version where P-site pairing is irreversibly disrupted, there is no necessary tRNA distortion. However, in the version where anticodon bases 35 and 36 maintain pairing, disruption of the anticodon base 34 interaction is most likely due to its flipping out of the anticodon stack. tRNAs are known to possess a large degree of structural flexibility. Recent studies by cryo-electron microscopy suggest that the aminoacyl-tRNA could participate actively in the accommodation step via conformational changes in its anticodon arm (Stark et al., 2002; Valle et al., 2002). At a more refined level, molecular dynamics studies indicates that the anticodon loop (as well as the acceptor arm) is potentially a region with a large amplitude mobility (Matsumoto et al., 1999). NMR analyses of synthetic anticodon regions derived from a few tRNAs

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Fig. 5. Models for frameshifting on hexameric motifs. The N1N2A3 AAG hexamer is normally read as N1N2A3 and A3A4G5 in frame 0 (top). Base 34 of the N1N2A3-decoding tRNA disengages from pairing with A3 (stage 1). A-site tRNA^_Arg^ re-pairs on the –1 frame A3A4A5 codon with re-positioning of the mRNA (stage 2). In (A), the P-site anticodon irreversibly dissociates from the mRNA whereas in (B), anticodon bases 36 and 35 maintain pairing. Accommodation is then completed (stage 3), bringing the two tRNAs and the mRNA in the configuration seen in crystallized complexes (Yusupova et al., 2001) allowing peptidyl-transfer and standard three-base translocation establishing the change in frame (stage 4).

leeway: it can disengage, re-pair in the –1 frame and stay there, especially in the case of *E. coli* tRNA^_Lys^, which has a stronger interaction with AAA than with AAG (Lustig et al., 1981; Yokoyama and Nishimura, 1995). Re-
give an even more precise idea of the degree of mobility of individual nucleotides in the anticodon loop and of the effect of modification of bases 34 and 37 in particular (Clore et al., 1984; Schweiguth and Moore, 1997; Sundaram et al., 2000; Cabello-Villegas et al., 2002). Even if most, in their fully modified form, adopt, in solution, a classical 3’-stacked loop configuration, with a U-turn between nucleotide 33 and 34, anticodon bases 34, 35 and 36 are still fairly mobile. In one case, E. coli tRNA<sup>Pro</sup>; the loop is reduced to bases 34 to 36 even when base 37 is modified as it is in vivo, suggesting that some anticodon loops may adopt, in solution, a conformation differing from the classical one (Cabello-Villegas et al., 2002). However, two missing pseudouridine modifications (U32 and U39) in the analyzed anticodon stem–loop may be in part responsible for this unorthodox configuration. In the case of tRNA<sup>Val</sup>, absence of modification also lead to a pseudo tri-loop anticodon, and addition of the modifications (t6A<sub>37</sub>, mm<sup>5</sup>S<sub>2</sub>U<sub>34</sub>, ψ(U<sub>39</sub>)) bring the structure to the standard 7-nucleotide loop, which, however, remains flexible (Durant and Davis, 1999; Sundaram et al., 2000). It therefore appears that the S<sup>2</sup> and mm<sup>5</sup> modifications increase the rigidity of the anticodon loop, in particular by strongly shifting the ribose conformation toward the C3’-end form, and thus allowing reading of A- and G-ending codons only (restricted wobble). In contrast the cmo<sup>5</sup>, and related modification of U34, have been proposed to tilt the balance in a more moderate manner. The C2’-end form predominates but the C3’-end form is also present and the end result is more flexibility. This allows interaction of U34 with G- or U-ending codons (extended wobble), when in the C2’-end configuration, and also with A-ending codons, when in the C3’-end form (Yokoyama et al., 1985; Yokoyama and Nishimura, 1995). Thus these data, linking anticodon base flexibility and wobble capacity, are in agreement with our finding that there are two types tRNAs as judged by their effect on frameshifting on NNA AAG hexamers. We can now re-formulate our conclusion and say that tRNAs with a more flexible base 34 (xmo<sup>5</sup>U in six cases and I in one; see Figure 3) are more frameshift-promoting than those with less flexibility at that position (xm<sup>3</sup>U in six cases and k<sup>3</sup>C in one). At the molecular level, the C2’/C3’-end interconversion may be what temporarily brings base 34 out of pairing with the third base of NNA codons (or what causes all three bases of tRNA<sup>NNA</sup> anticodon to disengage, according to the alternate scenario). To conclude, it appears that tRNAs anticodons are not extremely rigid and that there are probably large differences among them from that point of view. Such flexibility may well allow transitory un-pairing of base 34 (and perhaps of bases 35 and 36 also), especially since there is not a close monitoring of the codon–anticodon interaction in the P-site (Ogle et al., 2001).

**Modification of tRNA base 34**

The apparent correlation between frameshifting, wobbling and the modification pattern of base U<sub>34</sub> (Figure 3) led us to examine frameshifting in modification-deficient mutants, with the hope it would provide a new window for assessing the function of modification at that position in the anticodon (Figure 4). This hope was only partly fulfilled. Absence of the cmo<sup>5</sup> modification did not change the incidence on frameshifting of the relevant NNA-tRNAs. This means that the flexibility of base U<sub>34</sub> is still the same without the cmo<sup>5</sup> group. The result was not entirely unexpected in view of the higher mobility displayed by that nucleotide when it is non-modified. This higher mobility expands wobbling further, since unmodified U<sub>34</sub> recognizes codons ending with any nucleotide, at least in vitro (Yokoyama and Nishimura, 1995). Thus, the cmo<sup>5</sup> modification is not the cause of U<sub>34</sub> mobility, it probably limits it to prevent pairing with C; it rather is, like frameshifting stimulation, a consequence of structural properties of the anticodon region shared by one class of NNA-decoding tRNAs (Grosjean et al., 1996). In contrast, absence of either the S<sup>2</sup> or mm<sup>5</sup> modifications had a negative effect on frameshifting on the most efficient NNA AAG motifs. There, it was the A-site tRNA<sup>Val</sup> that was affected by the mutation. A known effect of S<sup>2</sup> and of mm<sup>5</sup> to a lesser extent, is to favor pairing of tRNA<sup>Val</sup> on AAA over AAG by increasing the rigidity of the anticodon (Yokoyama et al., 1985; Yokoyama and Nishimura, 1995). In the absence of one or other modification, anticodon base 34 is more flexible, allowing easier adjustment for proper pairing with G. This makes re-pairing from AAG to AAA energetically less advantageous and therefore frameshifting becomes less frequent.

The finding of significant levels of frameshifting at multiple hexanucleotide sequences has substantial relevance for ongoing searches to discover where programmed frameshifting is utilized for gene expression. While utilization by the IS elements mentioned above provide some initial examples, the generality of this form of recoding remains to be determined.

**Materials and methods**

**Bacterial strains and growth conditions**

The E. coli K12 strain JS238 [MC1061, araD30 Δ(ara leu) galU galK hsdS rpsL ΔlacOPZYM47.6 lacFΔ (lacI Q16 lacZ Δ(Tn10 recA1))] was used for all cloning experiments.

Strains with mutations in tRNA<sup>Val</sup> modification genes were provided by Professor G.Björk: TH194 (aroD<sup>+</sup>, mmnA<sup>+</sup>, mmnE<sup>+</sup>, GRB2162 (aroD<sup>-</sup>), TH193 (mmnA<sup>-</sup>) and TH99 (mmnE<sup>-</sup>) (Urbanovics et al., 2001). These strains were transformed with plasmid pPA2-lacP<sup>+</sup> (P.Polard, unpublished) before introduction of the various pOFX302-based frameshift constructions. This plasmid, being based on a p15A replicon, is compatible with pBR322 derivatives and carries a kanamycin resistance gene as well as the lacP<sup>+</sup> gene, which ensure a tight control of the Tac promoter carried by pOFX302. Bacteria were grown in LB medium (Sambrook et al., 1989) or, for protein labeling, in MOPS medium (Neidhardt et al., 1974) supplemented with glucose (0.5%), thiamine (2 mg/l) and all amino acids at 50 μg/ml each (except methionine, tryptophan and tyrosine). Rhabdophaga agar plates (Merck) were used to identify clones expressing β-galactosidase. Ampicillin (40 μg/ml) plus oxacillin (200 μg/ml), and kanamycin (25 μg/ml) were added when necessary.

**DNA techniques and quantitation of radioactive macromolecules**

Plasmid DNA was prepared using the Qiaprep or Qiagen-tip100 systems as indicated by the supplier (Qiagen). Restriction enzymes, T4 poly nucleotide kinase and T4 DNA ligase were from New England Biolabs. AmpliTaq DNA polymerase and the Amplicycle sequencing kit were from Applera. Cloning, transformation, agarose gel electrophoresis, and sequencing gels were carried out according to standard procedures (Sambrook et al., 1989). Radioactive products ([γ<sup>32</sup>P]ATP for DNA sequencing and [<sup>35</sup>S]methylmethionine for in vivo protein labeling) were obtained from Amersham. The Fuji X BAS1000 phosphorimager and the
PCBAs software were used for the quantitative analysis of electrophoresis gels in which 35S-labeled proteins were separated.

**Plasmids constructions**

Mutants of the IS3 (or IS1222) frameshift region were cloned into the reporter plasmid pFX302 described by Rettenberg et al. (1999). In one set the second and third nucleotides of the C CAA AAG sequence containing the IS3 shift site was changed to all possible sequences (except TA and TG, to avoid in-frame stop codon); the first base was changed to G when the second was C. In a second set of 14 constructions, the first base was made identical to the second. Two control plasmids were also generated, in one the C CAA AAG was changed to C CAG AAA, to prevent frameshifting (0% frameshifting construct) and in the other a base was added to C CAA AAA G in order to set g10 and lacZ in the same phase (100% frameshifting construct).

**Measurement of frameshifting frequency**

Frameshifting frequency was determined by in vivo protein pulse labeling with [35]S-methionine on four independent clones for each construct, following a previously described protocol (Rettenberg et al., 1999; Bertrand et al., 2002). To calculate the frequency of frameshifting, the fraction of the total radioactivity present in the relevant band was divided by the corresponding value obtained for the in-phase control. Precision was assessed by calculation of the 95% confidence interval.

Experiments were conducted by measuring β-galactosidase activity. For each strain, four to eight tubes containing 0.5 ml of LB (supplemented with kanamycin, ampicillin and oxacillin) were inoculated with independent clones and incubated overnight at 37°C. After 1/5 dilution in LB, the absorbance at 600 nm of each culture was measured on 125 µl in a 96 flat-bottomed wells microplate (optical path of 0.38 cm) with a spectramax 340PC spectrophotometer (Molecular Devices). The diluted cultures (0.5 ml) were adjusted to 1X Z+ buffer [Z buffer from Miller (1992), supplemented with 0.005% SDS, 1 mg/ml BSA and 10 mM DTT instead of β-mercaptoethanol] and treated for 10 min at 0°C with 10 µl of CHCL3. Assays were prepared in 96-well microplates. A volume of extract depending on the activity was completed to 200 µl with Z+ buffer and 50 µl of 4 mg/ml ONPG was added. Absorbance was read at 420 nm each minute over a 30 min period with a Spectramax 340PC spectrophotometer. In order to be directly comparable to those obtained with the classical protocol of Miller (1992), our specific activities were calculated for a volume of extract of 125 µl and for an OD600 of 1. As in the pulse-labeling experiments, the in-phase control served as 100% reference and precision was assessed by determining the 95% confidence interval.

Note that absolute levels of frameshifting measured that way are about nine times lower than those obtained by the same constructs. This is likely due to underestimation of the 100% value in the case of pulse-labeling (induction of the strong pTac promoter probably saturates the protein synthesis capacity). However, both methods gave identical results in terms of relative activities of the various constructs.

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**References**


during decoding of Bacillus subtilis cdd occurs at the sequence CGA AAG. J. Bacteriol., 181, 2930–2937.

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Note added in proof