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Expression of a Coronavirus Ribosomal Frameshift Signal in *Escherichia coli*: Influence of tRNA Anticodon Modification on Frameshifting

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Eukaryotic ribosomal frameshift signals generally contain two elements, a heptanucleotide slippery sequence (XXXYYYN) and an RNA secondary structure, often an RNA pseudoknot, located downstream. Frameshifting takes place at the slippery sequence by simultaneous slippage of two ribosome-bound tRNAs. All of the tRNAs that are predicted to decode frameshift sites in the ribosomal A-site (XXXYYYN) possess a hypermodified base in the anticodon-loop and it is conceivable that these modifications play a role in the frameshift process. To test this, we expressed slippery sequence variants of the coronavirus IBV frameshift signal in strains of *Escherichia coli* unable to modify fully either $tRNA^{Lys}$ or $tRNA^{Asn}$. At the slippery sequences UUUAAAC and UUUAAAU (underlined codon decoded by $tRNA^{Asn}$, anticodon 5′ QUU 3′), frameshifting was very inefficient (2 to 3%) and in strains deficient in the biosynthesis of Q base, was increased (AAU) or decreased (AAC) only two-fold. In *E. coli*, therefore, hypomodification of $tRNA^{Asn}$ had little effect on frameshifting. The situation with the efficient slippery sequences UUUAAAA (15%) and UUUAAAG (40%) (underlined codon decoded by $tRNA^{Lys}$, anticodon 5′ mnm^5s^2UUU 3′) was more complex, since the wobble base of $tRNA^{Lys}$ is modified at two positions. Of four available mutants, only *trmE* (s^2UUU) had a marked influence on frameshifting, increasing the efficiency of the process at the slippery sequence UUUAAAA. No effect on frameshifting was seen in *trmC1* ($cmnm^5s^2UUU$) or *trmC2* (nm^5s^2UUU) strains and only a very small reduction (at UUUAAAG) was observed in an *asuE* (mnm^5UUU) strain. The slipperiness of $tRNA^{Lys}$, therefore, cannot be ascribed to a single modification site on the base. However, the data support a role for the amino group of the mnm^5 substitution in shaping the anticodon structure. Whether these conclusions can be extended to eukaryotic translation systems is uncertain. Although *E. coli* ribosomes changed frame at the IBV signal (UUUAAAG) with an efficiency similar to that measured in reticulocyte lysates (40%), there were important qualitative differences. Frameshifting of prokaryotic ribosomes was pseudoknot-independent (although secondary structure dependent) and appeared to require slippage of only a single tRNA.

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Keywords: ribosomal frameshifting; tRNA anticodon modification; RNA pseudoknot; lysyl-tRNA; Q base

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Abbreviations used: RSV, Rous sarcoma virus; ORF, open reading frame; Q, queuosine; Y, wyebutoxine; HIV, human immunodeficiency virus; HTLV, human T-cell leukaemia virus; BLV, bovine leukaemia virus; IBV, infectious bronchitis virus; RRL, rabbit reticulocyte lysate; IPTG, isopropyl-β,D-thiogalactopyranoside; TGT, tRNA guanine transglycosylase; MMTV, mouse mammary tumour virus; pfu, plaque-forming units.

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Introduction

Several viruses use an efficient −1 ribosomal frameshifting mechanism to control expression of their replicases. Frameshifts of this class were first described as the process by which the gag-pol polyprotein of the retrovirus Rous sarcoma virus (RSV) is expressed from the overlapping *gag* and *pol* open reading frames (ORFs: Jacks & Varmus, 1985). Related frameshift signals have since been documented in an increasing number of systems, including several other retroviruses, a number of eukaryotic positive-strand RNA viruses, a double-stranded RNA virus of yeast, some plant RNA viruses and certain bacteriophage (reviewed by Brierley, 1995). The phenomenon is not restricted to viruses; frameshift signals of the "retrovirus type" have been described in a number of *Escherichia coli* insertion elements (reviewed by Chandler & Fayet, 1993; Farabaugh, 1996) and in a conventional cellular gene, the *dna X* gene of *E. coli* (Blinkowa & Walker, 1990; Flower & McHenry, 1990; Tsuchihashi & Kornberg, 1990; Tsuchihashi, 1991). The mRNA signals that specify frameshifting appear to be composed of two essential elements; a heptanucleotide "slippery" sequence, where the ribosome changes reading frame, and a region of RNA secondary structure, often in the form of an RNA pseudoknot, located a few nucleotides downstream (Jacks *et al.*, 1988a; Brierley *et al.*, 1989; ten Dam *et al.*, 1990). The molecular mechanism of the frameshift process is only poorly understood, but work from several groups supports a model (Jacks *et al.*, 1988a) in which the elongating ribosome pauses upon encountering the region of mRNA secondary structure, facilitating realignment of the slippery sequence-decoding tRNAs in the −1 frame. The heptanucleotide stretch that forms the slippery sequence contains two homopolymeric triplets and conforms, in the vast majority of cases, to the motif XXXYYYN. Frameshifting at this sequence is thought to occur by simultaneous slippage of two ribosome-bound tRNAs, presumably peptidyl and aminoacyl tRNAs, which are translocated from the zero (X XXY YYN) to the −1 phase (XXX YYY: Jacks *et al.*, 1988a). Following the slip, the tRNAs remain base-paired to the mRNA in at least two out of three anticodon positions. There is considerable experimental support for this model, particularly from site-directed mutagenesis studies (Jacks *et al.*, 1988a; Dinman *et al.*, 1991; Dinman & Wickner, 1992; Brierley *et al.*, 1992), sequencing of *trans*-frame proteins (Hizi *et al.*, 1987; Jacks *et al.*, 1988a,b; Weiss *et al.*, 1989; Nam *et al.*, 1993) and nucleotide sequence comparisons (Jacks *et al.*, 1988a; ten Dam *et al.*, 1990). The protein sequencing studies indicate that the frameshift occurs at the second codon of the tandem slippery pair, i.e. at that codon decoded in the ribosomal aminoacyl (A) site (XXXY$\underline{\text{YYN}}$). The importance of the A-site tRNA in frameshifting was also apparent from the mutagenesis studies; point mutations affecting the A-site tRNA were generally more inhibitory than those affecting the P-site tRNA.

A key question that has remained unanswered is whether frameshifting at the slippery sequence is mediated by canonical tRNAs, or requires the participation of special "shifty" tRNAs, more prone to frameshift than their "normal" counterparts (Jacks *et al.*, 1988a). At naturally occurring frameshift sites, of the codons that are decoded in the ribosomal A-site prior to tRNA slippage (XXXY$\underline{\text{YYN}}$), only five are represented in eukaryotes, AAC, AAU, UUA, UUC and UUU, and two in prokaryotes, AAA and AAG (Farabaugh, 1996). These codons are decoded by tRNAs with a highly modified base in the anticodon loop (see Hatfield *et al.*, 1992 and references therein). In $tRNA^{Asn}$ (AAC, AAU), the wobble base is queuosine (Q), in $tRNA^{Phe}$ (UUC, UUU), wyebutoxine (Y) is present just 3′ of the anticodon, in $tRNA^{Lys}$ (AAA, AAG), the wobble base is 5-methylaminomethyl-2-thiouridine (mnm^5s^2U) (prokaryotes) and in $tRNA^{Leu}$ (UUA), 2-methyl-5-formylcytidine is present at the wobble position (Debarros *et al.*, 1996). Hatfield *et al.* (1992) have suggested that hypomodified variants of these tRNAs may exist that function as specific "shifty" tRNAs, since such variants will have a considerably less bulky anticodon and be more free to move around at the decoding site. Indirect support for this hypothesis comes from an examination of the modification status of the anticodons of the aminoacyl-tRNAs that are required for translation at and around the frameshift sites of human immunodeficiency virus type 1 (HIV-1), human T-cell leukaemia virus type I (HTLV-1) and bovine leukaemia virus (BLV: Hatfield *et al.*, 1989). It was found that in HIV-1 infected cells, most of the $tRNA^{Phe}$ lacked Y and in HTLV-1 and BLV infected cells, most of the $tRNA^{Asn}$ lacked Q. However, research from other groups has suggested an alternative hypothesis, in which frameshifting is mediated by standard cellular tRNAs (Tsuchihashi, 1991; Tsuchihashi & Brown, 1992; Brierley *et al.*, 1992). These authors propose that frameshifting at a particular site depends, amongst other parameters, upon the strength of the interaction between the slippery sequence codons and the tRNAs decoding it and that if this interaction is relatively weak, then slippage is more likely to occur. The strength of the interaction between mRNA and tRNA is likely to be influenced considerably by the kind of base-pair that forms between the 3′ base of the codon and the 5′ base of the anticodon (position 34) at the wobble position. Modification of the anticodon wobble base of frameshift site-decoding tRNAs may well influence this interaction and hence the level of frameshifting observed. A prediction of the hypothesis is that the anticodon modifications present in tRNAs that decode highly efficient slippery sequences reduce recognition of the corresponding codons.

Here, we have attempted to test these hypotheses by measuring directly the influence of tRNA anticodon modification on frameshifting. Our approach was to determine the efficiency of the frameshift signal of the coronavirus infectious bronchitis virus (IBV; Brierley *et al.*, 1987, 1989) in mutant strains of *Escherichia coli* unable to modify fully either $tRNA^{Lys}$ or $tRNA^{Asn}$. The IBV frameshift signal, which is present at the overlap of the 1a and 1b ORFs of the virus genomic RNA, is a well-characterised eukaryotic system (Brierley *et al.*, 1991, 1992) that comprises the slippery sequence UUUAAAC and a downstream RNA pseudoknot. We began by determining the components of the IBV signal required for efficient frameshifting in *E. coli* and then proceeded with the investigation of the role of tRNA anticodon modification in frameshifting. Four slippery sequence variants of the IBV site (UUUAAAX, where X was A, C, G or U) were tested, focusing specifically on the A-site decoding tRNAs. The results obtained indicate that in *E. coli* there is little influence of the tRNA modification status on frameshifting. Hypomodification of $tRNA^{Asn}$ had only a slight effect on frameshifting and of the $tRNA^{Lys}$ mutants tested, only *trmE* (anticodon s^2UUU) had a marked influence, increasing the efficiency of the process at the slippery sequence UUUAAAA.

Results

Sequence requirements for IBV frameshifting in *E. coli*

The efficiency of frameshift signals of the IBV type in the eukaryotic rabbit reticulocyte lysate (RRL) *in vitro* translation system has been shown to be influenced by the nature of the slippery sequence, the integrity of the downstream RNA structure and the precise spacing between the two elements (Brierley *et al.*, 1991, 1992). We began by confirming that these requirements were maintained in *E. coli*. Complementary DNAs (cDNAs) containing variants of the IBV signal were subcloned (see Materials and Methods) into the *E. coli* expression vector pET3xc (Studier *et al.*, 1990) to create the pMM series of plasmids (see Figure 1). pET3xc contains the first 783 bp of coding sequence of the bacteriophage T7 gene 10, flanked by a T7 promoter and transcription termination signal. In *E. coli* BL21 cells, which contain an IPTG-inducible T7 RNA polymerase, a 261 amino acid residue portion of gene 10 is expressed from pET3xc as an abundant, Triton-insoluble product that is relatively easy to purify (see Materials and Methods). In the pMM plasmids, the IBV cDNAs were cloned in frame with and downstream of the gene 10 sequence of pET3xc at a unique *Bam*HI site. In BL21 cells, these constructs were predicted to express a 33 kDa non-frameshifted product corresponding to ribosomes that terminated at the IBV 1a stop codon and a 50 kDa frameshift product from ribosomes which frameshifted prior to encountering the stop codon and continued to translate the 1b ORF in the −1 frame. The constructs tested are shown in Figure 2, the expressions in Figure 3 and the results are summarised in Table 2. For the pET3xc plasmid, which does not contain an IBV insertion, only two major species were present, the N-terminal portion of gene 10, with an apparent molecular mass of about 34 kDa and lysozyme, carried over from the purification procedure (see Materials and Methods). For the pMM expressions, three species were seen; lysozyme, a 36 kDa species corresponding to the non-frameshifted product and for most constructs, a 50 kDa frameshift product. The identity of the 36 kDa and 50 kDa proteins was confirmed by demonstrating that both proteins reacted in Western blots with a monoclonal antibody directed against the N terminus of gene 10 (AMS Biotechnology Ltd, Europe; data not shown). We also confirmed that the 36 kDa and 50 kDa proteins were restricted to the insoluble fraction of *E. coli* cell extracts; no soluble non-frameshifted or frameshifted protein was detected (data not shown).

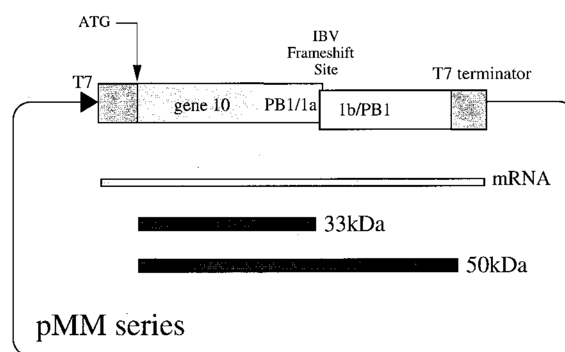
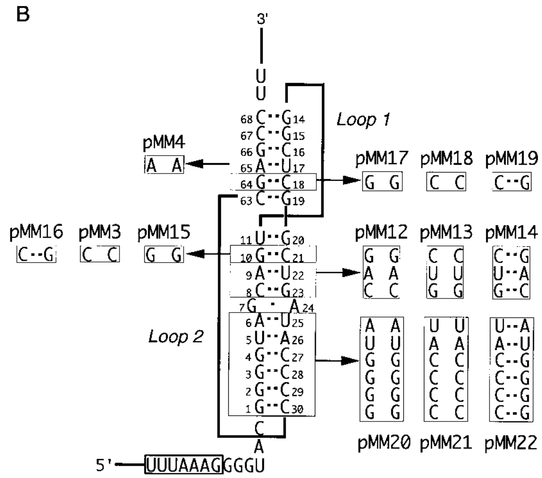


Figure 1. The basic plasmids used in this study, the pMM series, were prepared by subcloning 585 bp *Nhe*I-*Eco*RI fragments containing the IBV frameshift region from plasmids pFS7, pFS8 or mutant derivatives (Brierley *et al.*, 1989, 1991) into *Bam*HI-cleaved, plasmid pET3xc (Studier *et al.*, 1990). Both fragment(s) and vector were end-filled using the Klenow fragment of DNA polymerase I prior to ligation with T4 DNA ligase. The resulting plasmids contain the IBV ORF 1a/1b frameshift signal (sequence information from base-pairs 12,286 to 12,511; Boursnell *et al.*, 1987) flanked by portions of the influenza A/PR8/34 PB1 gene (sequence information from base-pairs 1140 to 1167 (5′) and 1167 to 1500 (3′); Young *et al.*, 1983) located downstream of, and in frame with, the first 783 bp of coding sequence of the bacteriophage T7 gene 10. The ensemble is under the control of the bacteriophage T7 promoter and a T7 transcription termination signal is present at the end of the coding sequences. In the relevant T7-expressing bacteria (see Materials and Methods), the constructs are predicted to express a 33 kDa non-frameshifted product, corresponding to ribosomes that terminate at the IBV 1a stop codon and a 50 kDa frameshift product from ribosomes that frameshift prior to encountering the stop codon and continue to translate the 1b ORF in the −1 frame.

A

pMM1	UUA[UUUAAA**C**]GGGUAC....pseudoknot
pMM2	UUA[UUUAAAG]GGGUAC....pseudoknot
pMM8	UUA[UUUAAA**U**]GGGUAC....pseudoknot
pMM9	UUA[UUUAAA**A**]GGGUAC....pseudoknot
pMM10	UUA[UUUAAAG]UAC....pseudoknot
pMM11	UUA[UUUAAAG]GGGU**AC**UAC....pseudoknot
pMM6	UUA[UUUAAA**C**]GGGUAC
pMM5	UUA[UUUAAAG]GGGUAC
pMM28	UUA[UUUAAAG]**UAA**UAC....pseudoknot
pMM27	UUA[UUUAAAG]**UAG**UAC....pseudoknot
pMM24	UUA[UUUAAAG]**UGA**UAC....pseudoknot
pMM25	UUA[UUUAAAG]**UGG**UAC....pseudoknot
pMM26	**UAA**[UUUAAAG]**UAG**UAC....pseudoknot
pMM29	UUA[UCUAAAG]GGGUAC....pseudoknot
pMM30	UUA[UUUACAG]GGGUAC....pseudoknot



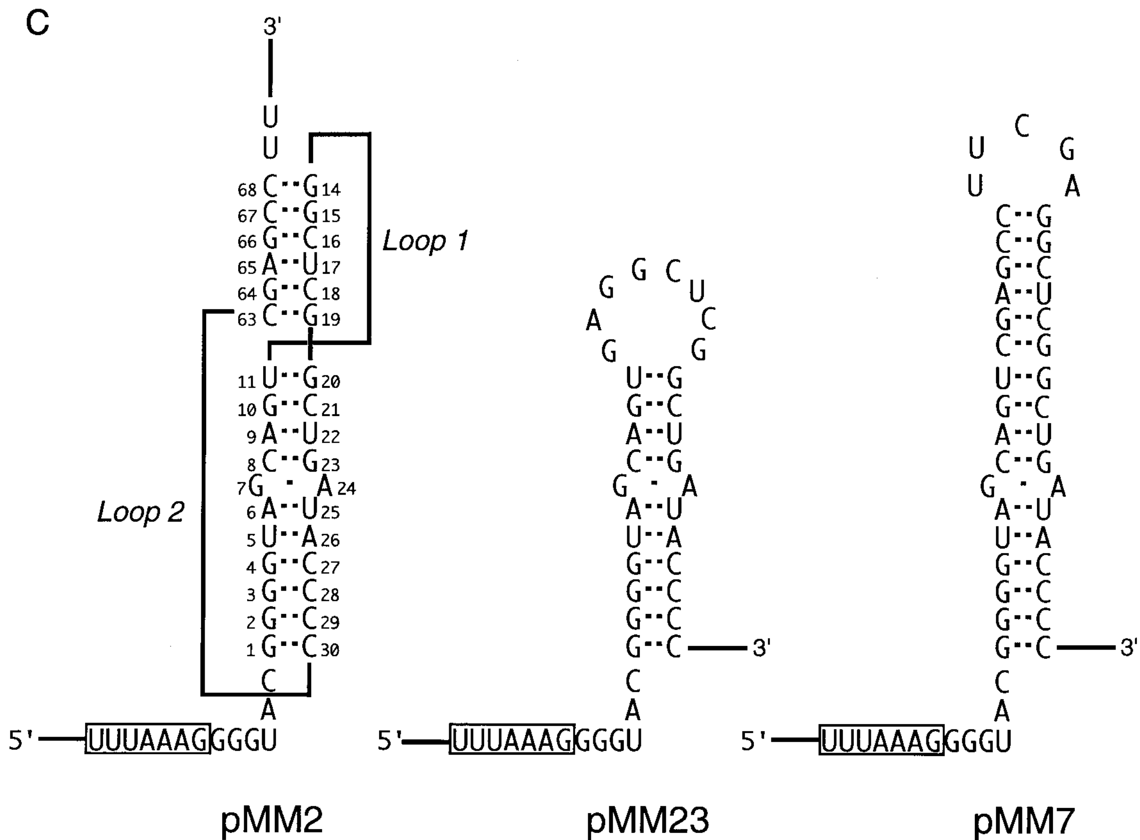


Figure 2. Frameshift constructs tested in *E. coli*. A, Slippery sequence variants. In each construct, the slippery sequence is boxed. Nucleotides that differ from the wild-type sequence (pMM2) are indicated in bold. In pMM10 and pMM11, the distance between the slippery sequence and RNA pseudoknot was decreased (GGG deleted) or increased (UAC inserted) by 3 nt, respectively. In pMM6 and pMM5, the pseudoknot was deleted (as in construct pFS7.6; Brierley *et al*., 1989). B, Pseudoknot variants. Complementary and compensatory changes were created within the pseudoknot region. In this representation of the pseudoknot, the stems are arranged vertically and the loops are shown as thick lines. For each base-pair(s) studied, the two complementary changes (no base-pairing) and the compensatory change (base-pairing restored) are boxed and labelled with a mutant number. C. Stem–loop constructs tested. Two constructs were tested that formed a stem–loop structure rather than a pseudoknot. Plasmid pMM23 forms only stem 1 due to a deletion that removed the downstream pseudoknot-forming region (as in construct pFS7.6; Brierley *et al*., 1989). Plasmid pMM7 is a stem–loop construct in which the stem nucleotides are of the same length and nucleotide composition as the stacked stems of the pseudoknot in pMM2 (as in construct pFS8.26; Brierley *et al*., 1991).

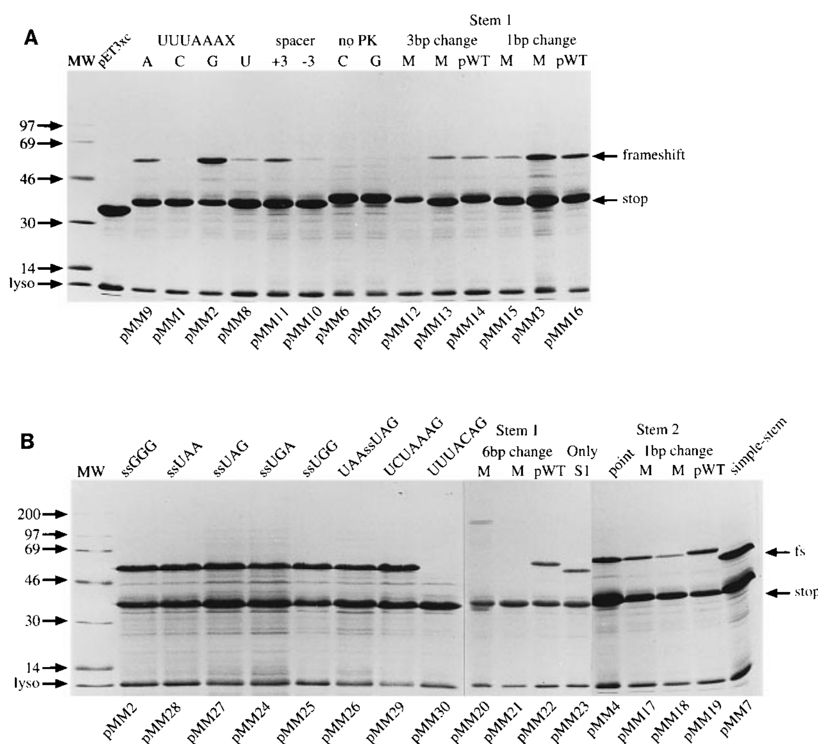


Figure 3. A and B, Frameshift assays in *E. coli*. Purified proteins expressed from the relevant frameshift plasmids were separated on SDS 15% polyacrylamide gels and detected by staining with Coomassie brilliant blue R as described in Materials and Methods. The non-frameshifted (stop) and frameshifted (frameshift or fs) products are indicated by arrows. Lysozyme (lyso) carried over from the purification procedure is indicated by an arrow. The relevant mutant number is indicated at the bottom of each track. A number of abbreviations are employed: MW, high molecular mass protein size standards (Amersham); UUUAAAX, variant slippery sequence where X is A, C, G or U as indicated above the relevant track; spacer, the slippery sequence-pseudoknot spacing distance was increased (+3 nt) or decreased (−3 nt) as indicated; no PK, pseudoknot deletion mutants with slippery sequence UUUAAAC (C) or UUUAAAG (G); stem 1 or stem 2 complementary (M) and compensatory (pWT) mutations were analysed in blocks of six (6 bp change), three (3 bp change) or single base-pairs (1 bp change); ssGGG, slippery sequence (UUUAAAG) and downstream codon are indicated; UAAssUAG, slippery sequence flanked by termination codons; point, single point mutation in stem 2 of construct pMM4; only S1, construct can form only stem 1; simple-stem, construct forming long stem–loop structure as detailed in Figure 2C.

Slippery sequence requirements

The generality of the simultaneous slippage model of frameshifting for sites expressed in *E. coli* is not fully established. It was important, therefore, to determine whether frameshifting at the IBV slippery sequence in *E. coli* deviated from the conventional simultaneous slippage mechanism ascertained in RRL. We created a series of mutations at or around the IBV slippery sequence and tested for frameshifting by expression in *E. coli* BL21 (see Figure 3A). The wild-type IBV slippery sequence, UUUA<u>AAC</u> (pMM1), decoded by $tRNA^{Asn}$ (anticodon 5′ QUU 3′) stimulated only low-levels of frameshifting (2%), as did UUUA<u>AAU</u> (pMM8, 3%). In *E. coli*, therefore, $tRNA^{Asn}$ is considerably less slippery than it is in eukaryotic cells or the RRL (Brierley *et al.*, 1989). In contrast, $tRNA^{Lys}$ (anticodon 5′ U^8UU 3′; where U^8 is mnm^5s^2U), which reads UUUA<u>AAA</u> (pMM9) and UUUA<u>AAG</u> (pMM2), was highly slippery; frameshifting at these sites occurring with great efficiency (15% and 40%, respectively). The slipperiness of *E. coli* $tRNA^{Lys}$ has been documented and will be discussed in detail later. In *E. coli* the hierarchy of frameshifting for the seventh nucleotide of the slippery sequence (UUUAAAN) was $N = G > A \gg U = C$. This is almost the reverse of the situation seen in RRL, where the hierarchy for N is $C > A = U \gg G$ (Brierley *et al.*, 1992), and is consistent with earlier studies of frameshifting in *E. coli* (Weiss *et al.*, 1989; Tsuchihashi & Brown, 1992). The introduction of different termination codons (UGA, pMM24; UAG, pMM27; UAA, pMM28) or an alternative sense codon (UGG, pMM25) immediately downstream of the slippery sequence had little effect on frameshifting, although a discernible reduction was seen with the UAG (32%) and UGA (29%) terminators. By flanking the slippery sequence with termination codons (pMM26), one immediately downstream (UGA) in the zero phase and a second immediately upstream (UAA) in the −1 phase, we were able to confirm that frameshifting takes place within the UUUAAAG stretch (Figure 3B). As with pMM27, we observed a slight reduction in frameshift efficiency with this construct, which had UAG as the downstream termination codon (see Discussion). We also introduced mutations within the slippery sequence. Unsurprisingly, the sequence UUUA<u>C</u>AG (pMM30) was non-functional, since his mutation would prevent slippage of the A-site decoding tRNA. However a P-site mutation, U<u>C</u>UAAAG (pMM29) was fully competent in frameshifting, suggesting that in the case of the IBV signal in *E. coli*, the process does not involve simultaneous slippage of two tRNAs, but rather −1 slippage of a single $tRNA^{Lys}$ (from AAG to AAA).

RNA secondary structure requirements

Efficient frameshifting at the IBV signal, at least in the RRL, depends upon the RNA pseudoknot structure, which cannot be replaced functionally by a stable stem–loop structure of the same predicted size and nucleotide composition as the stacked stems of the pseudoknot (Brierley *et al*., 1991). We investigated whether the RNA secondary structure requirements for frameshifting in *E. coli* were conserved by measuring the frameshift efficiency of a series of pseudoknot mutants (see Figures 2 and 3). Complete removal of the pseudoknot dramatically reduced frameshifting, irrespective of whether the slippery sequence was UUUAAAC (pMM6) or UUUAAAG (pMM5). The pattern of frameshifting observed for mutations within the pseudoknot closely paralleled that seen in the RRL, in that destabilization of either stem of the pseudoknot reduced frameshifting efficiency (pMM3, 12, 13, 15, 20, 21 in stem 1; pMM4, 17, 18 in stem 2) and compensatory mutations predicted to restore the structure in general increased frameshifting (pMM16, 22 in stem 1; pMM19 in stem 2). Of the compensatory mutants in stem 1, two of the three analysed had frameshift efficiencies considerably below that seen for the wild-type structure (pMM14, 12% and pMM16, 20%). This is a phenomenon that has been observed in a number of studies of eukaryotic frameshifting (see ten Dam *et al*., 1995) and may reflect a functional requirement for a particular pseudoknot conformation that is imprecisely reproduced in some of the compensatory mutants. The efficiencies of frameshifting measured for the stem 2 point mutations (pMM4, 15%; pMM17, 15% and pMM18, 9%) were considerably greater than that seen previously in RRL, where frameshifting in these mutants is reduced to about 5% (Brierley *et al*., 1991). This supports the idea that efficient frameshifting in *E. coli* can be mediated by simple hairpin loop stimulators. Indeed, in construct pMM23, which contains the potential for formation of only stem 1, frameshifting occurred efficiently (22%). Moreover, when the pseudoknot of pMM2 was replaced by a large stem–loop structure of the same predicted size and nucleotide composition as the stacked stems of the pseudoknot (pMM7), frameshifting occurred at a high level in *E. coli* cells (32%), in contrast to the situation in RRL, where such a structure promotes only low levels (1 to 2%) of frameshifting. This observation highlights an important difference between prokaryotic and eukaryotic ribosomes in terms of their response to RNA stimulators associated with frameshift sites. A similarity that is maintained, however, is the necessity for precise spacing between the stimulatory structure and the slippery sequence. As in the RRL (Brierley *et al*., 1989), increasing (pMM11) or decreasing (pMM10) the spacing distance by three nucleotides greatly reduced frameshifting at the IBV site in *E. coli*.

The role of tRNA anticodon modification in frameshifting

To investigate the role of tRNA anticodon modification in the frameshift process, pairs of plasmids with variations in the last nucleotide of the slippery sequence (UUUAAA$\underline{N}$) were expressed in *E. coli* strains deficient in tRNA modification (see Table 1). In these experiments, T7 RNA polymerase was provided by infecting cells with bacteriophage λ CE6 (see Materials and Methods), which contains the gene for T7 RNA polymerase under the control of the P_L and P_I promoters. Under these circumstances, we found that expression levels were generally lower than those seen upon IPTG-induction of BL21 cells and were influenced by the growth-rate of the cells (reflecting the efficiency of the λ CE6 infection). However, the signal to noise ratios (in terms of expressed proteins to cellular background) were sufficiently

Table 1. Bacterial strains used in this work

Strain	Genotype	Reference or source
XA105	*ara* Δ *(lac-proB) nalA rif thi metB* $argE_{am}$ *supG*	Miller *et al*. (1977)
XA10B	*ara* Δ *(lac-proB) nalA* $argE_{am}$ *rif thi metB supB*	Miller *et al*. (1977)
TH48	As XA105 but *fadL*::Tn*10*	Hagervall & Björk (1984)
TH49	AsTH48 but *trmC2*	Hagervall & Björk (1984)
TH69	As TH48 but *trmC1*	Hagervall & Björk (1984)
DEV1	*thi1 rel1 spoT1 lacZ(UAG)*	Elseviers *et al*. (1984)
DEV16	As DEV 1 but val^R, *trmE*	Elseviers *et al*. (1984)
TH78	As TH79 but *trmE*	This work
TH79	*ara* Δ *(lac-proB) nalA rif thi metB* $argE_{am}$ val^R *supB*	This work
TH159	As TH160 but *asuE107*	Hagervall & McCloskey[a]
TH160	*ara* Δ *(lac-proB) nalA rif thi metB* $argE_{am}$ *supB fadR*::Tn*10*	Hagervall & McCloskey[a]
SJ1502	*araD139* Δ *(argF-lacU139) thi1 deoC relA1 rpsL150*	Reuter *et al*. (1991)
SJ1505	As SJ1502 but *tgt1*	Reuter *et al*. (1991)
K12Δtgt	Δ *(tgt)*	Kersten[b]
TG1	*supE, thi* Δ *(lac-proAB) F′ (traD36* proAB+ $lacI^q$ *lacZ*Δ *M15) hsh*Δ *5*	Gibson *et al*. (1984)
BL21(DE3)	*hsdS gal (λcIts857 ind1 Sam7 nin5 lacUV5-T7 gene 1)*	Studier & Moffatt (1986)

[a] Unpublished results.
[b] This strain was prepared in the laboratory of Professor Helga Kersten, Institüt für Biochemie, Universität Erlangen-Nürnberg, Fahrstrasse 17, D-8520 Erlangen, Germany.

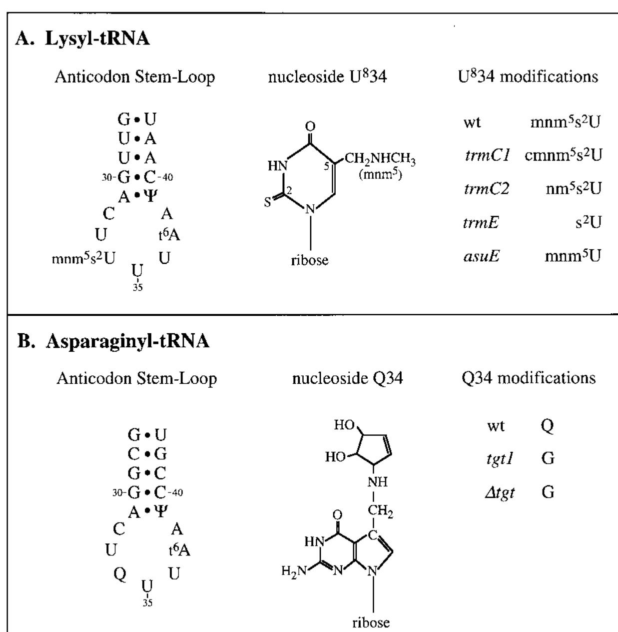


Figure 4. Anticodon modifications studied. The Figure shows the anticodon stem–loop nucleotides, the nucleoside modifications and the mutants tested for both lysyl and asparaginyl-tRNAs.

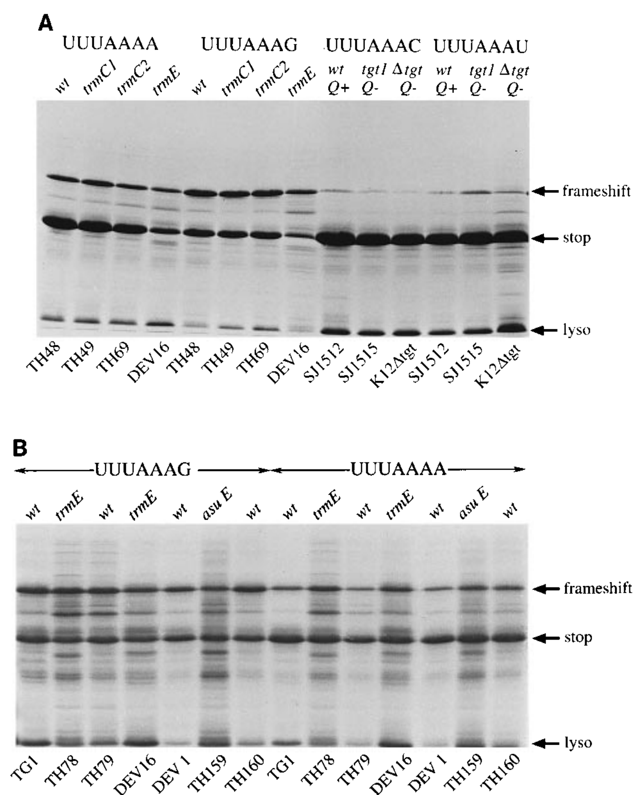


Figure 5. Frameshift assays in modification-deficient *E. coli* hosts. Frameshift plasmids were expressed in a variety of *E. coli* strains (see Table 1), the purified expression products separated on SDS 15% polyacrylamide gels and detected by staining with Coomassie brilliant blue R as described in Materials and Methods. The non-frameshifted (stop) and frameshifted (frameshift) products are indicated by arrows. Lysozyme carried over from the purification procedure is indicated by an arrow (lyso). The *E. coli* strain employed is indicated at the bottom of each track. The plasmids expressed were pMM1 (UUUAAAC), pMM2 (UUUAAAG), pMM8 (UUUAAAU) and pMM9 (UUUAAAA). The relevant genotype is indicated above each track (wt, wild-type with respect to the modification genotype); Q; Q status, either absent (−) or present (+). A, Bacteria cultured in LB medium; B, bacteria cultured in a defined minimal medium (see Materials and Methods), except *asuE* (LB medium).

high that estimates of frameshifting efficiency were reliable and reproducible. We studied two anticodon modifications, the Q base of $tRNA^{Asn}$ and the mnm^5s^2U base of $tRNA^{Lys}$ (see Figure 4).

The role of Q

Plasmids pMM1 (UUUAAAC) and pMM8 (UUUAAAU) were expressed in a wild-type host (SJ1512) and in two strains deficient in the biosynthesis of Q due to a lack of functional tRNA guanine transglycosylase (TGT: SJ1515, K12 Δtgt). In these strains, Q is not incorporated into tRNA and $tRNA^{Asn}$ has the anticodon 5′ GUU 3′. As can be seen in Figure 5A, the absence of Q had only a modest effect on frameshifting, resulting in a twofold increase (UUUAAAU) or decrease (UUUAAAC) in frameshift efficiency (see Table 2). It is clear therefore that in *E. coli*, hypomodification of the anticodon of $tRNA^{Asn}$ does not lead to a dramatic increase or decrease in frameshifting.

The role of mnm^5s^2U

Unlike $tRNA^{Asn}$, the anticodon wobble base of $tRNA^{Lys}$ is modified at two positions, at position 5, where hydrogen is replaced by a methylaminomethyl group and at position 2, where oxygen is replaced by sulphur. Although a number of *E. coli* mutants exist that are defective in the synthesis of mnm^5s^2U, a fully unmodified strain is not available. Plasmids pMM9 (UUUAAAA) and pMM2 (UUUAAAG) were expressed in wild-type hosts (TG1, TH48, DEV 1, TH79, TH160) and in the relevant defective strains listed in Table 1. Essentially, four mutants were examined; *trmC1* (Hagervall & Bjork, 1984), which has the hypermodification 5-carboxymethylaminomethyl-2-thiouridine ($cmnm^5s^2U$; TH69); *trmC2* (Hagervall & Bjork, 1984), containing the undermodified 5-aminomethyl-2-thiouridine (nm^5s^2U; TH49); *trmE* (Elseviers *et al.*, 1984), which possesses only the 2-thiouridine substitution (s^2U; TH78, DEV 16) and *asuE107*, which contains only the 5-methylaminomethyl substitution (mnm^5U; TH159). In the *trmC1*, *trmC2* and *trmE* strains, the presence of the sulphur atom was potentially problematic, since it can be replaced by selenium, which is thought to confer altered decoding properties (Wittwer &

Table 2. The −1 frameshifting efficiencies from the analyses of Figures 3 and 5

Construct		Feature	Efficiency (%)
A. *Slip-site variants*	pMM9	UUUAAAA	15
	pMM1	UUUAAAC	2
	pMM2	UUUAAAG	40
	pMM8	UUUAAAU	3
	pMM29	UCUAAAG	40
	pMM30	UUUACAG	1
B. *Flanking codons*	pMM28	UUAUUUAAAGUAA	40
	pMM27	UUAUUUAAAGUAG	32
	pMM24	UUAUUUAAAGUGA	29
	pMM25	UUAUUUAAAGUGG	41
	pMM26	UAAUUUAAAGUAG	33
C. *Spacing variants*	pMM11	Spacer +3 nucleotides	8
	pMM10	Spacer −3 nucleotides	2
D. *PK mutations*	pMM6	Pseudoknot deletion	1
	pMM5	Pseudoknot deletion	1
	pMM23	Stem 2 deleted	22
	pMM7	Long stem-loop	32
	pMM12	Stem 1 3 bp change	8
	pMM13	Stem 1 3 bp change	10
	pMM14	Stem I pseudo-wt	12
	pMM15	Stem 1 1 bp change	10
	pMM3	Stem 1 1bp change	19
	pMM16	Stem 1 pseudo-wt	20
	pMM20	Stem 1 6 bp change	2
	pMM21	Stem 1 6 bp change	2
	pMM22	Stem 1 pseudo-wt	38
	pMM4	Stem 2 point mutation	15
	pMM17	Stem 2 1 bp change	15
	pMM18	Stem 2 1 bp change	9
	pMM19	Stem 2 pseudo-wt	37
E. *Role of tRNA anticodon modification*			
	Slip sequence tested	Host/modification	
	UUUAAAC	SJ1512/wt Q+	2
		SJ1515/*tgt1* Q−	1
		K12Δ*tgt* Q−	1
	UUUAAAU	SJ1512/wt Q+	3
		SJ1515/*tgt1* Q−	6
		K12Δ*tgt* Q−	5
	UUUAAAA	TG1/wt	15
		TH48/wt	15
		TH49/*trmC1*	16
		TH69/*trmC2*	15
		DEV1/wt	15
		DEV16/*trmE*	32
		TH79/wt	15
		TH78/*trmE*	32
		TH160/wt	15
		TH159/*asuE*	15
	UUUAAAG	TG1/wt	40
		TH48/wt	41
		TH49/*trmC1*	40
		TH69/*trmC2*	40
		DEV1/wt	40
		DEV16/*trmE*	48
		TH79/wt	40
		TH78/*trmE*	49
		TH160/wt	40
		TH159/*asuE*	33

Each value quoted represents the average of three to five independent measurements, which varied by less than 5%; i.e. a measurement of 40% frameshift efficiency was between 38% and 42%. The abbreviations used are: PK, pseudoknot; wt, wild-type.

Ching, 1989). This was not a problem with the *asuE* strain; the level of mnm^5Se^2U has been measured in an *asuE* strain and was not detected (Kramer & Ames, 1988). Previous studies, however, have indicated that selenium is not detectably incorporated into tRNA when bacteria are cultured in minimal medium (Wittwer & Stadtman, 1986). Although these authors have suggested that this is also the case for LB medium, we decided to measure frameshifting in the *trmC1*, *trmC2* and *trmE* strains during culture in a defined minimal medium prepared from chemicals of high purity in addition to assays in standard LB medium. The results of the analysis are shown in Figure 5 and are summarised in Table 2. The signal to noise ratio in the minimal medium assays was increased somewhat as a consequence of the slower growth-rate of the strains. The *asuE* strain (Figure 5B) grew very slowly in minimal medium and was only tested in LB medium. The frameshift efficiencies measured for the various mutants were not influenced by the growth medium. No effect on frameshifting was seen in the *trmC1* or *trmC2* strains when either the UUUAAAA or UUUAAAG-containing test plasmids were expressed. The most noticeable influence was in the *trmE* strain, where frameshifting was stimulated over twofold (from 15% to 32%) at the UUUAAAA site and also increased at the UUUAAAG site, although to a lesser extent (from 40% to 48%). These increases were seen in two independent *trmE* strains (TH78, DEV 16). Frameshifting in the *asuE* background was unaltered at the UUUAAAA site but was reduced a little at the UUUAAAG site (from 40% to 33%).

Discussion

Signals for frameshifting in *E. coli*

The generality of the simultaneous slippage model of frameshifting for sites expressed in *E. coli* is not fully established. Weiss *et al.* (1989) have expressed a variant of the MMTV frameshift signal in *E. coli* and have shown that ribosomes respond to both of the tandem slippery codons of the MMTV frameshift signal as predicted by the simultaneous slippage model. At the frameshift signal of the *E. coli dnaX* gene, mutagenesis of the slippery sequence (A-AAA-AAG) has confirmed that both lysine codons are required for efficient frameshifting (Tsuchihashi & Brown, 1992). Similarly, frameshifting at the G-T ORF overlap required to produce a bacteriophage λ tail assembly protein occurs by a two tRNA slip (Levin *et al.*, 1993). However, in the *E. coli* insertion element IS*1*, frameshifting is known to occur by −1 slippage of a single lysyl tRNA at the sequence A-AAA (from the underlined codon onto the overlapping AAA codon), despite the fact that the A_4 stretch is embedded within two potential and conventional slippery sequences (U-UUA-AAA-AAC; Sekine & Ohtsubo, 1992). An unexpected finding from our analysis of the slippery sequence requirements for IBV frameshifting in *E. coli* was the high efficiency of the UCUAAAG mutant (pMM29), since such a mutant has only low activity in RRL (Brierley *et al.*, 1989). The simplest explanation for this observation is that frameshifting at the IBV site in *E. coli* occurs by slippage of a single tRNA at the second homopolymeric triplet of the slippery sequence (UUUAAAX) rather than by simultaneous slippage of two tRNAs. The P-site codon in the mutant, UCUAAAG, is probably decoded by a minor $tRNA^{Leu}$ isoacceptor with anticodon 5′ UAG 3′ (Inokuchi & Yamao, 1995). If this tRNA were to slip into the −1 reading frame (in accordance with the simultaneous slippage model) it could form only a single G-U base-pair with the −1 frame codon. At present, therefore, we favour single-tRNA slippage. Whether $tRNA^{Lys}$ slips when in the P or A-site of the ribosome is not know. Recent evidence supports the idea that frameshifting at the HIV-1 slippery sequence (U-UUU-UUA) in *E. coli* occurs not by simultaneous slippage of P and A-site-bound tRNAs, but when these tRNAs are in the ribosomal E and P-sites (Horsfield *et al.*, 1995). This hypothesis was proposed following the discovery that the presence of a termination codon immediately downstream of the U_6A stretch reduced frameshifting some five- to tenfold in a manner that was independent of sequence context and could be modulated by prokaryotic release factor 2. These observations were consistent with the last six nucleotides of the slippery sequence occupying the E and P-sites and the termination codon the A-site prior to tRNA slippage. In the present study, we did detect a slight reduction in frameshift efficiency when the downstream terminators UAG and UGA were employed, raising the possibility that a fraction of the frameshift events monitored involve the E-site; but the data are most consistent with the slippery sequence occupying the standard P and A-sites. This conclusion is supported by the fact that, as is also seen in RRL (Brierley *et al.*, 1989, 1992), the spacing distance between the slippery sequence and pseudoknot had to be maintained at six nucleotides for efficient frameshifting to occur. Protein sequencing studies and further mutagenesis experiments are in progress in an attempt to improve our understanding of the precise mechanism of tRNA slippage at the IBV site in *E. coli*.

The requirements for downstream RNA secondary structure were tested in constructs with the most efficient (UUUAAAG) slippery sequence, using a series of complementary and compensatory pseudoknot mutants. The response of prokaryotic ribosomes to these mutants was generally similar to that seen with the eukaryotic ribosomes of the RRL *in vitro* translation system (Brierley *et al.*, 1991). However, an important difference was noted; when the IBV pseudoknot was replaced by a stable stem–loop structure of the same predicted size and nucleotide composition, frameshifting was maintained at a high level in *E. coli* cells, in con-

trast to the situation in RRL, where such a structure promotes only low levels of frameshifting. In naturally occurring frameshift sites in *E. coli*, the requirements for stimulatory RNA structures appear to be variable. At the G-T ORF overlap of bacteriophage λ, no stimulatory secondary structure is apparent (Levin *et al*., 1993). In contrast, the *dnaX* frameshift signal includes both a downstream stem–loop (Tsuchihashi & Kornberg, 1990) and an upstream stimulatory element formed between a Shine-Delgarno-like sequence (5′ AGGGaG 3′) located 10 nt upstream of the slippery sequence and the 3′-end of 16 S rRNA (3′ UCCUcC 5′: Larsen *et al*., 1994). Variation is also evident at the frameshift sites of bacterial insertion sequences (Chandler & Fayet, 1993). In IS*3*, a pseudoknot is required for efficient frameshifting (Sekine *et al*., 1994) and in IS*150*, stimulation is *via* a downstream stem–loop that may form a pseudoknot (Vögele *et al*., 1991). In contrast, although a number of potential RNA structures are predicted to form downstream of the slippery sequence of IS*1* (Sekine & Ohtsubo, 1989; Escoubas *et al*., 1991), they do not appear to play a role in frameshifting (Sekine & Ohtsubo, 1992). Whatever the case, a stimulatory structure is absolutely required for IBV frameshifting in *E. coli*; the UUUAAAG sequence alone was unable to stimulate detectable frameshifting. This is perhaps unsurprising, since in the expression constructs employed, no obvious Shine-Delgarno-like sequences are present upstream of the slippery sequence.

Influence of tRNA anticodon modification on frameshifting

Our investigation of the role of tRNA anticodon modification in frameshifting was prompted by the experiments reported by Hatfield *et al*. (1989, 1992), who proposed that hypomodification of the anticodons of those tRNAs implicated in decoding frameshift sites may promote efficient frameshifting. We began by expressing our frameshift reporter constructs in *E. coli tgt* mutants unable to biosynthesize Q. In these cells, however, we detected only a modest influence of hypomodified $tRNA^{Asn}$, with frameshifting reduced by about twofold on the slippery sequence UUUAAAC and increased by a similar magnitude at the UUUAAAU site. So for $tRNA^{Asn}$, at least in *E. coli*, anticodon hypomodification *per se* is insufficient to promote highly efficient frameshifting. The modest effects noted, however, are consistent with the view that the strength of the pre-slippage codon-anticodon interaction is important in frameshifting. The hypomodified variant of $tRNA^{Asn}$ with anticodon 5′ GUU 3′ would be expected to pair more strongly with the AAC codon than with AAU, and hence frameshifting should decrease at the AAC codon and increase at AAU, as was observed. The low levels of frameshifting seen in *E. coli* at slippery sequences decoded by $tRNA^{Asn}$, with or without Q, is in contrast to the situation in higher eukaryotic cells, where $tRNA^{Asn}$ is highly slippery (Brierley *et al*., 1992), despite possessing an identical anticodon loop (Chen & Roe, 1978). The altered frameshift capacity may simply be a reflection of the eukaryotic translational environment, but a role for Q in eukaryotic frameshifting cannot be ruled out.

The situation with UUUAAAA and UUUAAAG, decoded by $tRNA^{Lys}$, is more complex, since the wobble base of this tRNA (mnm^5s^2U) is modified at two positions (see Figure 4) and an *E. coli* strain expressing a fully unmodified tRNA is unavailable. In the present study, of the four available mutants with altered modification of the wobble base of $tRNA^{Lys}$, only *trmE* (wobble base is 2-thiouridine) had a marked influence on frameshifting, increasing the efficiency of the process over twofold at UUUAAAA and to a lesser extent at UUUAAAG. The *asuE* mutant (wobble base mnm^5U) showed a small reduction in frameshifting at UUUAAAG. The increased efficiency seen in the *trmE* strains is consistent with the idea that hypomodification of the anticodon stimulates frameshifting (Hatfield *et al*., 1992). However, this hypothesis cannot explain the intrinsic slipperiness of the hypermodified "wild-type" $tRNA^{Lys}$. The biological role (or at least one of the roles) of the $tRNA^{Lys}$ modifications is thought to be in the regulation of the conformational flexibility or rigidity of the base to ensure the correct translation of codons during protein synthesis, particularly to prevent decoding of AAC and AAU. Proton NMR studies of the conformational characteristics of modified uridine bases (Yokoyama *et al*., 1985) indicate that the mnm^5s^2 modification introduces conformational rigidity such that the ribose exclusively adopts a C3′-*endo* form, which favours recognition of adenosine, allows weak recognition of guanosine and precludes binding to cytosine and uridine. In support of this, $tRNA^{Lys}$ has been shown to decode preferentially the AAA codon and has little affinity for AAG (Lustig *et al*., 1981). Tsuchihashi (1991) has rationalised the high-efficiency frameshift signal of the *dnaX* gene on the basis that the slippery sequence, AAAAAAG, is poorly recognised by $tRNA^{Lys}$, which slips efficiently at the AAG codon due to a weak wobble base-pair. This concept is supported by the experiments reported by Tsuchihashi & Brown (1992), who were able to inhibit frameshifting at the *dnaX* site by expressing an additional $tRNA^{Lys}$ with anticodon 5′ CUU 3′. In these cells, frameshifting at the AAAAAAG slippery sequence is thought to have been prevented by the more stable recognition of the AAG codon by $tRNA^{Lys}$ CUU. We believe that the efficient frameshift observed at the IBV signal in *E. coli* (at UUUAAAG) is also a result of a restricted capacity of $tRNA^{Lys}$ to pair with AAG. The fact that the UUUAAAA slippery sequence is also highly permissive suggests that recognition of AAA by $tRNA^{Lys}$ is also unusual when compared, for example, with the decoding of AAC or AAU by $tRNA^{Asn}$.

Yokoyama *et al*. (1985) have suggested that both the 2 and 5-substituents contribute to the confor-

mational rigidity of $tRNA^{Lys}$, with the major input from the 2-thiocarbonyl group. The NMR studies by Agris and colleagues (Sierzputowska-Gracz *et al.*, 1987; Agris *et al.*, 1992) indicate a role for only the 2-position thiolation. A second interaction, a hydrogen bond, has been proposed, which forms between the amino group of 5′-substitutents containing an aminomethyl moiety (mnm^5 and $cmnm^5$) and the 2′-OH residue of the adjacent, unmodified U33 base in the U-turn structure of the anticodon loop (Hillen *et al.*, 1978; Yokoyama & Nishimura, 1995; and see Figure 4). Circular dichroism analysis of the tRNA suggests that the anticodon has an unusual conformation in which the wobble base is buried in the anticodon loop, possibly interacting *via* its 5′-substituent with the N^6 threonylcarbamoyl (t^6) modification of base A37 across the loop (Watanabe *et al.*, 1993). Support for this idea comes from studies of a chemically synthesised short oligoribonucleotide comprising U33-mnm^5s^2U-U-U-t^6A37 (cited by Agris, 1996). In this doubly modified pentamer, a unique interaction occurs between the two modifications that is thought to be between the amine group of mnm^5 and the amino acid residue of t^6A, by hydrogen or ionic bonding. Furthermore, model-building studies predict that the anticodon domain U-turn in $tRNA^{Lys}$ is at the mnm^5s^2U34 base rather than at the usual invariant residue U33.

The structural studies described above suggest that the intrinsic shiftyness of $tRNA^{Lys}$ arises from its inability to recognise efficiently lysine codons as a consequence of the anticodon modifications, which are required to prevent erroneous decoding of asparagine codons. In this light, we expected that gross alterations in the modification status (absence of thiolation in *asuE*, absence of the 5′-substituent in *trmE*) would result in a tRNA that would be less restricted and perhaps pair more readily with lysine codons. Following this logic, frameshifting would be reduced in these mutants, since the codon-anticodon interaction would be more stable. Clearly this was not the case, and at present we are unable to explain these observations satisfactorily. The most likely explanation is that possession of either of the two modifications is sufficient to reduce recognition of lysine codons and allow efficient frameshifting. The structural studies imply a role for the amino group of the 5′-substituent of $tRNA^{Lys}$, most likely in inter-loop contact with t^6A37. The increase in frameshifting seen with *trmE*, which does not possess the amino group, may well reflect the loss of such an interaction, resulting in altered recognition of the AAA codon (and to a lesser extent the AAG codon) by the mutant tRNA. It seems unlikely that the effect seen with *trmE* is related to how well the mutant tRNA is aminoacetylated; efficient aminoacetylation appears to be correlated with the presence of the s^2 moiety in tRNAs of this class (Rogers *et al.*, 1995). The lack of effect of the *trmC1* and *trmC2* mutations, which retain the amino group, may suggest that the conformation of $tRNA^{Lys}$ in these strains is not sufficiently different to influence codon recognition. In the case of *trmC1* ($cmnm^5s^2$U; TH69) and *trmC2* (nm^5s^2U; TH49), model-building studies (Lim, 1994) support the idea that the modified tRNAs would be expected to have similar decoding properties as the wild-type tRNA. Mutants defective in *asuE*, *trmE* and *trmC* all decrease the efficiency of the ochre suppressor tRNA *supG*, which is a derivative of $tRNA^{Lys}$ with anticodon 5′ mnm^5s^2UUA 3′ (reviewed by Björk, 1992). Whether this reflects a reduced affinity for the nonsense codon or another step in the suppression pathway is not clear, although the *trmC1* mutation is known not to affect the binding properties of $tRNA^{Lys}$ to AAA or AAG programmed ribosomes (Elseviers *et al.*, 1984).

In conclusion, our data are not wholly consistent with either of the hypotheses advanced to explain the role of tRNA anticodon modification in frameshifting. The observed effects of hypomodified $tRNA^{Asn}$ on frameshifting at UUUAAAU/C are most consistent with the idea that the strength of the codon-anticodon interaction determines frameshift efficiency rather than being mediated primarily by hypomodified tRNAs. However, the data obtained with variantly modified $tRNAs^{Lys}$ are not readily explainable in terms of either model. Although the increased frameshifting seen in *trmE* strains is consistent with a role for hypomodified tRNAs, the inherent slipperiness of the wild-type hypermodified $tRNA^{Lys}$ argues strongly against this hypothesis. Nevertheless, the data obtained for the other $tRNA^{Lys}$ modification mutants are difficult to interpret solely in terms of the predicted strength of codon-anticodon recognition. All efficient −1 frameshift sites in *E. coli* employ $tRNA^{Lys}$ as the A-site decoding tRNA and in all likelihood, this tRNA has a very unusual anticodon structure. In attempting to compare the role of tRNA anticodon modification in frameshifting between prokaryotic and eukaryotic systems, we remain mindful that $tRNA^{Lys}$ may be a special case and that the role of modified bases in eukaryotic frameshifting needs to be tested directly. Such studies are underway.

Materials and Methods

Site-specific mutagenesis

Site-directed mutagenesis was carried out by a procedure based on that of Kunkel (1985) as described (Brierley *et al.*, 1989). Mutants were identified by dideoxy sequencing of single-stranded templates (Sanger *et al.*, 1977).

Construction of plasmids

The starting plasmids pFS7, pFS8 or mutant derivatives (Brierley *et al.*, 1989, 1991), which contain the IBV ORF 1a/1b frameshift signal flanked by portions of the influenza virus A/PR8/34 PB1 gene (Young *et al.*, 1983), were subjected to site-directed mutagenesis. In most cases, this was to change the IBV slippery sequence from

UUUAAAC to UUUAAAG. Following mutagenesis, 585 bp *Nhe*I-*Eco*RI fragments encompassing the frameshift region were subcloned from the mutated plasmids into *Bam*HI-cleaved pET3xc, an *E. coli* expression vector (Studier *et al*., 1990). Both fragments and vector were end-filled using the Klenow fragment of DNA polymerase I prior to ligation with phage T4 DNA ligase. The resulting plasmids, which comprise the pMM series, are shown in Figure 1. The PB1:1a/1b:PB1 fragment is located downstream of, and in frame with, the first 783 bp of coding sequence of the bacteriophage T7 gene 10 and the ensemble is under the control of the bacteriophage T7 promoter.

Bacterial strains and culture conditions

Bacteria were grown at 37°C in rich medium (LB; Maniatis *et al*., 1982) or in minimal salt medium (M9; Maniatis *et al*., 1982) containing thiamine (5 mg/l), glucose (0.2%, w/v) and the required amino acids (50 mg/l). The M9 medium was prepared using reagents of high purity (Aldrich Chemical Company). The genotypes and origins of the *E. coli* strains used are given in Table 1. TH78 was constructed by transferring the *trmE* allele from DEV 16 into XA10B by phage P1 transduction, essentially according to Miller (1972). Transductants were selected for valine resistance and co-transduction of *trmE* monitored by screening for an Arg^- phenotype. In this screen, the presence of *trmE* was observed as an antisuppresor activity of supB, which suppresses poorly the amber mutation in *argE*. A lack of mnm^5s^2U and the presence of s^2U in tRNA from TH78 was verified by HPLC analysis according to Gehrke & Kuo (1990). TH79 is an isogenic trmE + strain. The isolation of the *asuE107* mutation used in this study (TH159) will be described elsewhere. TH160 is an isogenic asuE + strain. Transfer RNA purified from TH159 was shown to contain mnm^5U instead of mnm^5s^2U by combined liquid chromatography-mass spectrometry (LC/MS; unpublished results).

Frameshifting in *E. coli* BL21

The sequence requirements for IBV frameshifting in *E. coli* were investigated by expressing the pMM plasmid series in *E. coli* BL21/DE3/pLysS cells (Figure 1, Table 1), largely as described by Studier *et al*. (1990). These cells contain, under the control of the *lac*UV5 promoter, the gene for T7 RNA polymerase inserted within the *int* gene of the prophage DE3, a λ derivative. Expression of T7 RNA polymerase in BL21 cells can be induced by addition of isopropyl-β,D-thiogalactopyranoside (IPTG). Freshly transformed BL21 cells prepared by the method of Hanahan (1983) and containing plasmids of the pMM series were inoculated into 1.5 ml LB cultures containing ampicillin (50 μg/ml) and chloramphenicol (30 μg/ml). After three hours at 37°C, IPTG was added to 0.4 mM and incubation continued for a further three hours. Cells were pelleted, resuspended in 150 μl of lysis buffer (25 mM Tris (pH 8), 10 mM EDTA, 50 mM sucrose, 2 mg/ml lysozyme), placed on ice for 30 minutes and treated with DNase I (30 μg/ml) for a further 30 minutes at 37°C in the presence of 8 mM $MgCl_2$ and 1 mM $MnCl_2$. Detergent solution (300 μl of 20 mM Tris (pH 7.5), 2 mM EDTA, 0.2 M NaCl, 1% (w/v) deoxycholic acid, 1% (v/v) Nonidet P-40) was added and the insoluble material harvested by centrifugation at 8000 *g* for two minutes. The pellet was washed three times with 0.5% (v/v) Triton X-100, 1 mM EDTA and the Triton-insoluble material, which contained predominantly the pMM expression products, dissolved in 100 μl of sample buffer (Laemmli, 1970). Aliquots were analysed on SDS/15% (w/v) polyacrylamide gels according to standard procedures (Hames, 1991). Proteins were stained with Coomassie brilliant blue R (0.05%, w/v) in 10% (v/v) acetic acid, 50% (v/v) methanol, and destained in 10% acetic acid, 20% methanol. The relative abundance of non-frameshifted or frameshifted products was estimated (Adobe Photoshop and NIH Image software) by scanning densitometry and adjusted to take into account the differential size of the products. Scans were performed on gels whose proteins were stained to an intensity at the centre of the dynamic range of the scanner (Microtek IIXE Scanmaker). The frameshift efficiencies quoted in the text and summarised in Table 2 are the average of three to five independent measurements that varied by less than 5%, i.e. a measurement of 40% frameshift efficiency was between 38% and 42%.

Frameshifting in modification-deficient *E. coli* strains

The influence of tRNA anticodon modification on IBV frameshifting was probed by expressing pMM plasmids in modification-deficient *E. coli* strains (Table 1). Since the strains were non-lysogenic for DE3, expression of T7 RNA polymerase was achieved by infecting plasmid-bearing cells with λ CE6 (AMS Biotechnology UK Ltd.)> This phage contains the gene for T7 RNA polymerase under the control of the P_L and P_I promoters. Stocks of CE6 were prepared in *E. coli* LE392 by plate lysis (Maniatis *et al*., 1982), collected by centrifugation (100,000 *g* for two hours) and resuspended at 10^{12} pfu/ml. Modification-deficient strains harbouring pMM plasmids were grown until the absorbance at 600 nm was between 0.6 and 0.8, and infected with CE6 at 10 to 20 pfu/cell. After three hours, the cells were harvested and pMM expression products purified and analysed as above.

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