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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 UUUAAC and UUUAUUU (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 UUUAAGA (15%) and UUUAAGG (40%) (underlined codon decoded by tRNA^Lys^, anticodon 5’ mmn5s2UUU 3’) was more complex, since the wobble base of tRNA^Lys^ is modified at two positions. Of four available mutants, only trmE (s2UUU) had a marked influence on frameshifting, increasing the efficiency of the process at the slippery sequence UUUAAG. No effect on frameshifting was seen in trmC1 (cmnm5s2UUU) or trmC2 (nm5s2UUU) strains and only a very small reduction (at UUUAAGG) was observed in an asuE (mnm5UUU) 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 mnm5 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 (UUUAAGG) 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.

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, wybutosine; HIV, human immunodeficiency virus; HTLV, human T-cell leukaemia virus; BLV, bovine leukaemia virus; IBV, infectious bronchitis virus; RRL, rabbit reticulocyte lysate; IPTG, isopropyl-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 a short 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 YY: 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 (XXYYYN). 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 (XXXYYYN), 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<sub>Asn</sub> (AAC, AAU), the wobble base is queuosine (Q), in tRNA<sub>Pro</sub> (UUC, UUU), wybutoxetine (Y) is present just 3' of the anticodon, in tRNA<sub>1<sup>5</sup>s</sub> (AAA, AAG), the wobble base is 5-methylaminomethyl-2-thiouridine (mnm<sub>2</sub>S<sub>2</sub>) (prokaryotes) and in tRNA<sub>Leu</sub> (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<sub>Pro</sub> lacked Y and in HTLV-1 and BLV infected cells, most of the tRNA<sub>Asn</sub> 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 (UUUAAX, 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_3^- mutants tested, only trnE_ (anticodon s'UUU) had a marked influence, increasing the efficiency of the process at the slippery sequence UUUAAAX.

**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 *BamHI* 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 that frameshifted prior to encountering the stop codon and continued to translate the 1b ORF in the −1 frame.

**Figure 1.** The basic plasmids used in this study, the pMM series, were prepared by subcloning 585 bp *NheI*-EcoRI fragments containing the IBV frameshift region from plasmids pFS7, pFS8 or mutant derivatives (Brierley et al., 1989, 1991) into *BamHI*-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 (S) and 1167 to 1500 (S'); 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.
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).
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, UUUAAC (pMM1), decoded by tRNAAsn (anticodon 5′ UQUU 3′) stimulated only low-levels of frameshifting (2%), as did UUUAAG (pMM2), UUUAAC (pMM8, 3%). In *E. coli*, therefore, tRNAAsn is considerably less slippery than it is in eukaryotic cells or the RRL (Brierley et al., 1989). In contrast, tRNALys (anticodon 5′ U8UUU 3′; where U8 is mnm5s2U), which reads UUUA (pMM9) and UUUAAG (pMM2), was highly slippery; frameshifting at these sites occurring with great efficiency (15% and 40%, respectively). The slipperiness of *E. coli* tRNALys 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 (UUUAAN) was N = G > A > U = C. This is almost the reverse of the situation seen in RRL, where the hierarchy for N is C > A = U > 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 +1 phase and a second immediately upstream (UAA) in the −1 phase, we were able to confirm that frameshifting takes place within the UUUAAG 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 UUUAAC (pMM30) was non-functional, since his mutation would prevent slippage of the A-site decoding tRNA. However a P-site mutation, UCUAAAG (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 tRNALys (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 greater than that seen previously in RRL, where frameshifting in *E. coli* meshifting 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 (UUUAAAN) 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 I and P l 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

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**Table 1. Bacterial strains used in this work**

| Strain | Genotype | Reference or source |
|--------|----------|---------------------|
| XA105  | ara Δ (lac-proB) nala rif thi metB argEsupG | Miller et al. (1977) |
| XA10B  | ara Δ (lac-proB) nala argE supG rif thi metB supB | Miller et al. (1977) |
| TH48   | As XA105 but fadL::Tn10 | Hagervall & Björk (1984) |
| TH49   | AsTH48 but trnC2 | Hagervall & Björk (1984) |
| TH69   | As TH48 but trnC1 | Hagervall & Björk (1984) |
| DEV1   | thi1 relI spoT1 lacZ(UAG) | Elseviers et al. (1984) |
| DEV16  | As DEV 1 but taut, trnE | Elseviers et al. (1984) |
| TH78   | As TH79 but trnE | This work |
| TH79   | As Δ (lac-proB) nala rif thi metB argE supG val8 supB | This work |
| TH15   | As TH160 but uaa107 | Hagervall & McCloskeya |
| TH160  | ara Δ (lac-proB) nala rif thi metB argE supG fadL::Tn10 | Hagervall & McCloskeya |
| SJ1502 | arad139 Δ (argF-lacU139) thi1 decO relA1 rpsL150 | Reuter et al. (1991) |
| SJ1505 | As SJ1502 but tgt1 | Reuter et al. (1991) |
| K12Agt | Δ (tgt) | Kenstenb |
| TG1    | supE, thi Δ (lac-proAB) F' (traT36 proAB+ lacI lacZ Δ M15) hsdA Δ 5 | Gibson et al. (1984) |
| BL21(DE3) | hsdS gal(lacI857 ind1 Sam7 nin5 lacIV5-17 gene 1) | Studier & Moffatt (1986) |

*a Unpublished results.  
*b This strain was prepared in the laboratory of Professor Helga Kersten, Institut für Biochemie, Universität Erlangen-Nürnberg, Fahrstrasse 17, D-8520 Erlangen, Germany.
high that estimates of frameshifting efficiency were reliable and reproducible. We studied two anticodon modifications, the Q base of tRNA_Gln and the mmn5s2U base of tRNA_Arg (see Figure 4).

The role of Q

Plasmids pM11 (UUUAAC) 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 tgt). In these strains, Q is not incorporated into tRNA and tRNA_Gln 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_Gln does not lead to a dramatic increase or decrease in frameshifting.

The role of mmn5s2U

Unlike tRNA_Gln, the anticodon wobble base of tRNA_Arg 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 mmn5s2U, a fully unmodified strain is not available. Plasmids pMM9 (UUUAAA) 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 (mmn5s2U; TH69); trmC2 (Hagervall & Bjork, 1984), containing the undermodified 5-aminomethyl-2-thiouridine (nm5s2U; TH49); trmE (Elseviers et al., 1984), which possesses only the 2-thiouridine substitution (s2U; TH78, DEV 16) and asuE107, which contains only the 5-methylaminomethyl substitution (mm5U; 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 UUAUUAAAGUGA | 40 |
| | pMM27 UUAUUAAAGUGA | 32 |
| | pMM24 UUAUUAAAGUGA | 29 |
| | pMM25 UUAUUAAAGUGA | 41 |
| | pMM26 UAAUUAAAGUGA | 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 1 bp 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/tglI Q− | 1 |
| | K12Δgl Q− | 1 |
| UUUAAAU | SJ1512/wt Q+ | 3 |
| | SJ1515/tglI Q− | 6 |
| | K12Δgl Q− | 5 |
| UUUAAA | 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/asaE | 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/asaE | 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 mmn\textsuperscript{57}Se\textsuperscript{2}U has been
measured in an asuE strain and was not detected
(Kramer & Ames, 1988). Previous studies, how-
ever, 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 some-
what 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 frameshifting efficiencies
measured for the various mutants were not influ-
enced by the growth medium. No effect on frame-
shifting was seen in the trmc1 or trmc2 strains
when either the UUUA\textsuperscript{AAA} or UUUA\textsuperscript{AAG}-con-
taining test plasmids were expressed. The most no-
ticeable influence was in the trm\textsuperscript{E} strain, where
frameshifting was stimulated over twofold (from
15% to 32%) at the UUUA\textsuperscript{AAA} site and also in-
creased at the UUUA\textsuperscript{AAG} site, although to a lesser
extent (from 40% to 48%). These increases were
seen in two independent trm\textsuperscript{E} strains (TH78, DEV
16). Frameshifting in the asuE background was un-
altered at the UUUA\textsuperscript{AAA} site but was reduced a
little at the UUUA\textsuperscript{AAG} site (from 40% to 33%).

Discussion

Signals for frameshifting in \textit{E. coli}

The generality of the simultaneous slippage
model of frameshifting for sites expressed in \textit{E. coli}
is not fully established. Weiss \textit{et al} (1989) have
expressed a variant of the MMTV frameshifting signal
in \textit{E. coli} and have shown that ribosomes respond to
both of the tandem slippery codons of the MMTV
frameshifting signal as predicted by the simultaneous
slippage model. At the frameshift signal of the
\textit{E. coli} \textit{dnuX} gene, mutagenesis of the slippery se-
quence (A-AAA-AAG) has confirmed that both ly-
sine codons are required for efficient frameshifting
(Tsuchihashi & Brown, 1992). Similarly, frameshif-
ting at the G-T ORF overlap required to produce a
bacteriophage \textit{\lambda} tail assembly protein occurs by a
two tRNA slipp (Levin \textit{et al}, 1993). However, in the
\textit{E. coli} insertion element IS1, frameshif-
ting is known to occur by –1 slippage of a single lysyl
tRNA at the sequence A-AAA (from the under-
lined codon onto the overlapping AAA codon), de-
spite the fact that the A\textsubscript{6} stretch is embedded
within two potential and conventional slippery se-
quences (U-UUA-AAA-AAC; Sekine & Õhtsubo,
1992). An unexpected finding from our analysis of

the slippery sequence requirements for IBV frame-
shifting in \textit{E. coli} was the high efficiency of the
UCUAAA\textsuperscript{G} mutant (pMM29), since such a mutant
has only low activity in RRL (Brierley \textit{et al}, 1989).
The simplest explanation for this observation is
that frameshifting at the IBV site in \textit{E. coli} occurs
by slippage of a single tRNA at the second homo-
polymeric triplet of the slippery sequence (UUUA\textsuperscript{AAX}) rather than by simultaneous slippage
of two tRNAs. The P-site codon in the mutant,
UCUAAA\textsuperscript{G}, is probably decoded by a minor
tRNA\textsubscript{Lys} 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\textsubscript{Lys} \textsubscript{1,3} slips when in
the P or A-site of the ribosome is not known. Recent
evidence supports the idea that frameshifting at the
HIV-1 slippery sequence (U-UUU-UUA) in
\textit{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 \textit{et al},
1995). This hypothesis was proposed following the
discovery that the presence of a termination codon
immediately downstream of the U\textsubscript{6}A stretch re-
duced frameshifting some five- to tenfold in a
manner that was independent of sequence context
and could be modulated by prokaryotic release fac-
tor 2. These observations were consistent with the
last six nucleotides of the slippery sequence occu-
pying 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 frame-
shift efficiency when the downstream terminators
UAG and UGA were employed, raising the possi-
bility that a fraction of the frameshift events moni-
tored involve the E-site; but the data are most
consistent with the slippery sequence occupying
the standard P and A-sites. This conclusion is sup-
ported by the fact that, as is also seen in RRL
(Brierley \textit{et al}, 1989, 1992), the spacing distance be-
 tween the slippery sequence and pseudoknot had to
be maintained at six nucleotides for efficient fra-
meshifting to occur. Protein sequencing studies
and further mutagenesis experiments are in pro-
gress in an attempt to improve our understanding of
the precise mechanism of tRNA slippage at the
IBV site in \textit{E. coli}.

The requirements for downstream RNA second-
ary structure were tested in constructs with the
most efficient (UUUA\textsuperscript{AAG}) 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 \textit{in vitro} translation system (Brierley \textit{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 \textit{E. coli} cells, in con-
contrast to the situation in RRL, where such a structure promotes only low levels of frameshifting. In naturally occurring frameshift sites in \textit{E. coli}, the requirements for stimulatory RNA structures appear to be variable. At the G-T ORF overlap of bacteriophage \(\lambda\), no stimulatory secondary structure is apparent (Levin \textit{et al.}, 1993). In contrast, the \textit{dnaX} frameshift signal includes both a downstream stem–loop (Tsuchihashi & Kornberg, 1990) and an upstream stimulatory element formed between a Shine-Dalgarno-like sequence (\(5'\ AGGGaG 3'\)) located 10 nt upstream of the slippery sequence and the 3'-end of 16 S rRNA (\(3'\ UCUCuC 5'\); Larsen \textit{et al.}, 1994). Variation is also evident at the frameshift sites of bacterial insertion sequences (Chandler & Fayet, 1993). In IS3, a pseudoknot is required for efficient frameshifting (Sekine \textit{et al.}, 1994) and in IS150, stimulation is via a downstream stem–loop that may form a pseudoknot (Vögele \textit{et al.}, 1991). In contrast, although a number of potential RNA structures are predicted to form downstream of the slippery sequence of IS1 (Sekine & Ohitsubo, 1989; Escoubas \textit{et al.}, 1991), they do not appear to play a role in frameshifting (Sekine & Ohitsubo, 1992). Whatever the case, a stimulatory structure is absolutely required for IBV frameshifting in \textit{E. coli}; the UUUAAAA sequence alone was unable to stimulate detectable frameshifting. This is perhaps unsurprising, since in the expression constructs employed, no obvious Shine-Dalgarno-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 \textit{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 frameshifter reporter constructs in \textit{E. coli} lgt mutants unable to biosynthesize Q. In these cells, however, we detected only a modest influence of hypomodified tRNA\textsubscript{Asn}, with frameshifting reduced by about twofold on the slippery sequence UUUAAA and increased by a similar magnitude at the UUUAAAU site. So for tRNA\textsubscript{Asn}, at least in \textit{E. coli}, anticodon hypomodification \textit{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\textsubscript{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 \textit{E. coli} at slippery sequences decoded by tRNA\textsubscript{Asn}, with or without Q, is in contrast to the situation in higher eukaryotic cells, where tRNA\textsubscript{Asn} is highly slippery (Brierley \textit{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\textsubscript{Lys}, is more complex, since the wobble base of this tRNA (\(mm\text{m}5\text{s}2U\)) is modified at two positions (see Figure 4) and an \textit{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\textsubscript{Lys}, only \textit{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 \(mm\text{m}2U\)) showed a small reduction in frameshifting at UUUAAAG. The increased efficiency seen in the \textit{trmE} strains is consistent with the idea that hypomodification of the anticodon stimulates frameshifting (Hatfield \textit{et al.}, 1992). However, this hypothesis cannot explain the intrinsic slipperness of the hypermodified “wild-type” tRNA\textsubscript{Lys}: The biological role (or at least one of the roles) of the tRNA\textsubscript{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 \textit{et al.}, 1985) indicate that the \(mm\text{m}5\text{s}2\) 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\textsubscript{Lys} has been shown to decode preferentially the AAA codon and has little affinity for AAG (Lustig \textit{et al.}, 1981). Tsuchihashi (1991) has rationalised the high-efficiency frameshift signal of the \textit{dnaX} gene on the basis that the slippery sequence, AAAAAG, is poorly recognised by tRNA\textsubscript{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 \textit{dnaX} site by expressing an additional tRNA\textsubscript{Lys} with anticodon \(5'\ CUU 3'\). In these cells, frameshifting at the AAAAAG slippery sequence is thought to have been prevented by the more stable recognition of the AAG codon by tRNA\textsubscript{Lys} CUU. We believe that the efficient frameshift observed at the IBV signal in \textit{E. coli} (at UUUAAAG) is also a result of a restricted capacity of tRNA\textsubscript{Lys} to pair with AAG. The fact that the UUUAAAA slippery sequence is also highly permissive suggests that recognition of AAA by tRNA\textsubscript{Lys} is also unusual when compared, for example, with the decoding of AAC or AAU by tRNA\textsubscript{Asn}.

Yokoyama \textit{et al.} (1985) have suggested that both the 2 and 5-substituents contribute to the confor-
mational rigidity of tRNA^{15s} 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'-substituents containing an aminomethyl moiety (mnm5s and cmmms) 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 N3 threonylcarbamoyl (t6) modification of base A37 across the loop (Watanabe et al., 1993). Support for this idea comes from studies of a chemically synthesized short oligoribonucleotide comprising U33-mmm5s2U-U-U-t6A37 (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 mnm5s and the amino acid residue of t6A, by hydrogen or ionic bonding. Furthermore, model-building studies predict that the anticodon domain U-turn in tRNA^{15s} is at the mnm5s2U33 base rather than at the usual invariant residue U33.

The structural studies described above suggest that the intrinsic shiftyness of tRNA^{15s} arises from its inability to recognize 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^{15s}, most likely in inter-loop contact with t6A37. 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 aminoacylated; efficient aminoacylation appears to be correlated with the presence of the s2 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^{15s} in these strains is not sufficiently different to influence codon recognition. In the case of trmC1 (cmmm5s2U; TH69) and trmC2 (nm5s2U; 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^{15s} with anticodon 5'-mm5s2UUA 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^{15s} 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^{15s} 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^{15s} 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^{15s} argues strongly against this hypothesis. Nevertheless, the data obtained for the other tRNA^{15s} 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^{15s} 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^{15s} 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 *NheI-EcoRI* fragments encompassing the frameshifting region were subcloned from the mutated plasmids into BamHI-cleaved pET3x, 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 PBl1a:1b:PBl1 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 XA108 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<sup>+</sup> phenotype. In this screen, the presence of *trmE* was observed as an antisuppressors activity of *supE*, which suppresses poorly the amber mutation in *argE*. A lack of mmm<sup>U</sup>U and the presence of sU in tRNA from TH78 was verified by HPLC analysis according to Gehre & Kuo (1990). TH79 is an isogenic trmL<sup>+</sup> strain. The isolation of the *asuE*<sup>107</sup> mutation used in this study (TH159) will be described elsewhere. TH160 is an isogenic asuE<sup>+</sup> strain. Transfer RNA purified from TH159 was shown to contain mmm<sup>U</sup>U instead of mmm<sup>U</sup>U 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 lacUV5 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 (2 mg/ml) for a further 30 minutes at 37°C in the presence of 8 mM MgCl₂ and 1 mM MnCl₂. 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 frameshifting 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<sub>I</sub> and P<sub>L</sub> 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<sup>10</sup> 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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