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The Q-base of Asparaginyl-tRNA is Dispensable for Efficient −1 Ribosomal Frameshifting in Eukaryotes

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The frameshift signal of the avian coronavirus infectious bronchitis virus (IBV) contains two *cis*-acting signals essential for efficient frameshifting, a heptameric slippery sequence (UUUAAAC) and an RNA pseudoknot structure located downstream. The frameshift takes place at the slippery sequence with the two ribosome-bound tRNAs slipping back simultaneously by one nucleotide from the zero phase (U UUA AAC) to the −1 phase (UUU AAA). Asparaginyl-tRNA, which decodes the A-site codon AAC, has the modified base Q at the wobble position of the anticodon (5′ QUU 3′) and it has been speculated that Q may be required for frameshifting. To test this, we measured frameshifting in cos cells that had been passaged in growth medium containing calf serum or horse serum. Growth in horse serum, which contains no free queuine, eliminates Q from the cellular tRNA population upon repeated passage. Over ten cell passages, however, we found no significant difference in frameshift efficiency between the cell types, arguing against a role for Q in frameshifting. We confirmed that the cells cultured in horse serum were devoid of Q by purifying tRNAs and assessing their Q-content by tRNA transglycosylase assays and coupled HPLC-mass spectroscopy. Supplementation of the growth medium of cells grown either on horse serum or calf serum with free queuine had no effect on frameshifting either. These findings were recapitulated in an *in vitro* system using rabbit reticulocyte lysates that had been largely depleted of endogenous tRNAs and resupplemented with Q-free or Q-containing tRNA populations. Thus Q-base is not required for frameshifting at the IBV signal and some other explanation is required to account for the slipperiness of eukaryotic asparaginyl-tRNA.

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

Introduction

Transfer RNAs have the highest density of modified nucleosides among cellular RNAs and more than 80 different modifications have been characterised (Limbach *et al*., 1994, 1995). The modifications are found at many sites in the tRNA, but they occur most commonly in the anticodon loop, especially at the wobble base (position 34) and the base immediately 3′ of the anticodon (position 37; Grosjean *et al*., 1995). Several of these modifications have been shown to play a role in extending or restricting the decoding capacity of the tRNA and can influence translational efficiency, fidelity, frame maintenance and codon choice (reviewed by Björk, 1992, 1995). Here, we investigate the potential involvement of one such modification, the hypermodified nucleoside queuosine (Q) at the wobble base of asparaginyl-tRNA ($tRNA^{Asn}$), in the process of −1 ribosomal frameshifting.

Programmed −1 ribosomal frameshifting is a translational event that allows the production of two (or more) proteins from a single mRNA and was first described as the mechanism by which the Gag-Pol polyprotein of the retrovirus Rous sarcoma virus (RSV) is produced from the overlapping

Abbreviations used: IBV, infectious bronchitis virus; RSV, Rous sarcoma virus; HIV, human immunodeficiency virus; BLV, bovine leukaemia virus; HTLV-1, human T-cell leukaemia virus type 1; TGT, tRNA transglycosylase; RRL, rabbit reticulocyte lysate; HS, horse serum; FCS, fetal calf serum; BHK, baby hamster kidney.

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gag and *pol* genes (Jacks & Varmus, 1985). The mRNA signals that specify frameshifting are comprised of two essential elements; a heptanucleotide "slippery" sequence, where the ribosome changes reading frame, and a stimulatory region of RNA secondary structure, often in the form of an RNA pseudoknot, located a few nucleotides downstream (Jacks *et al*., 1988; Brierley *et al*., 1989; Ten Dam *et al*., 1990). 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*., 1988).

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, 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 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 and colleagues (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. Experimentally, however, no consensus has emerged regarding a role for hypomodified tRNAs in frameshifting. Indirect support for the hypothesis has come 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-base and in HTLV-1 and BLV-infected cells, most of the $tRNA^{Asn}$ lacked Q-base. In contrast, it has been shown that the frameshift efficiency of the HIV-1 signal in T-lymphoid cell lines remains unchanged in cells uninfected or chronically infected with HIV-1 (Cassan *et al*., 1994) and also during a time-course of HIV-1 infection of CD4-expressing 293 cells (Reil *et al*., 1994). A comparison of the two sets of experiments is difficult, however, since in each case, only one parameter, either frameshift efficiency or modification status of the cellular tRNA population, was assessed.

In this study, we have investigated the role of Q in frameshifting by expressing a frameshift site derived from that of the coronavirus infectious bronchitis virus (IBV; Brierley *et al*., 1987, 1989) in tissue culture cells unable to synthesise Q-base. The IBV signal, which is present at the overlap of the 1a and 1b ORFs of the virus genomic RNA, is well characterised (Brierley *et al*., 1991, 1992) and comprises the slippery sequence UUUAAAC and a downstream RNA pseudoknot. We measured frameshifting in cos cells that had been passaged in growth medium containing calf serum or horse serum. Growth in horse serum, which contains no free queuine (q), eliminates Q from the cellular tRNA population (Langgut, 1993, 1995; Langgut *et al*., 1993; Reisser *et al*., 1993). Over ten cell passages, however, we found no significant difference in frameshift efficiency between the cell types. We confirmed that the cells cultured in horse serum were devoid of Q by purifying tRNAs and assessing their Q-content by tRNA transglycosylase (TGT) assays and coupled HPLC-mass spectroscopy. Supplementation of the growth medium of cells grown either on horse serum or calf serum with free queuine, the precursor of Q, had no affect on frameshifting either. These findings were recapitulated in an *in vitro* system using rabbit reticulocyte lysates (RRL) that had been depleted of endogenous tRNAs and resupplemented with Q-free or Q-containing tRNA populations. Taken together, the results argue against a specific role for the Q modification in frameshifting.

Results

IBV frameshifting *in vivo* is pseudoknot-dependent

Our analysis of the role of Q in frameshifting required that the process be measured in tissue culture cells. Hitherto, studies of the IBV frameshift signal had been restricted to *in vitro* translation systems and expression in *Escherichia coli* (Brierley *et al*., 1997). It was thus necessary to confirm that the key requirements for frameshifting, namely the slippery sequence and RNA pseudoknot (Brierley *et al*., 1991, 1992), were retained *in vivo*. The starting point for this analysis was the frameshift reporter construct pAC74Z (Stahl *et al*., 1995). This plasmid contains, under the control of the SV40 early promoter, β-galactosidase (β-gal) and luciferase (luc) reporter genes separated by a short linker into which candidate frameshift signals can be inserted. The minimal IBV frameshift signal (Brierley *et al*., 1992) was inserted into this intergenic region as a set of complementary oligonucleotides (see Materials and Methods) in such a way that the expression of luc, as a carboxy-terminal extension of the upstream β-gal, required a −1 frameshift at the IBV slippery sequence (pACFS1). In order to quantify the frameshift efficiency, an "in-frame" version of pACFS1 was prepared in which the two reporter genes were in the same

frame and luc expression was independent of a frameshift event (pACFS2). Indeed, every frameshift site variant tested was matched with an individual in-frame construct (see Table 1). Although this increased the number of constructs generated, it ensured that any amino acid changes introduced into the linker region, potentially changing the stability of the β-gal-luc fusion protein, were also present in the corresponding control construct, allowing a valid comparison to be made. The in-frame controls contained two changes; the spacing distance was increased from six to seven nucleotides to align β-gal and luc in the same frame (by insertion of an appropriate nucleotide immediately downstream of the slippery sequence) and the slippery sequence was changed to UUUAAAG, a change known to greatly reduce frameshifting in RRL (Brierley *et al*., 1992). Inactivation of the slippery sequence was important, since if this was not done, any ribosomes that frameshifted on the mRNA would terminate shortly after, decreasing β-gal-luc levels and introducing errors.

The minimal IBV frameshift signal (see the legend to Figure 1) and mutant variants were transfected into cos cells and subsequently, β-gal and luc levels measured 48 hours post-transfection (Figure 1 and Table 1). In contrast to the very high levels of frameshifting seen with the minimal IBV pseudoknot in RRL (45 %; Napthine *et al*., 1999), only about 9 % of ribosomes changed frame *in vivo*, a fivefold reduction. However, the response to frameshift signal variants was broadly similar to that seen in earlier *in vitro* studies. Firstly, frameshifting was sensitive to changes in the last nucleotide of the slippery sequence. The hierarchy of frameshifting for UUUAAAN variants closely paralleled that seen in RRL (Brierley *et al*., 1992) with C the most efficient (9 %/45 % [cos/RRL]) then A (8 %/25 %), U (5.8 %/22 %) and finally G (0.5 %/1 %). Secondly, frameshifting was RNA pseudoknot specific. Complete removal of the pseudoknot-dramatically reduced frameshifting (pACFS41; 0.05 %) and high levels of frameshifting were not supported by a large stem-loop structure of the same predicted size and nucleotide composition as the stacked stems of the pseudoknot (pACFS5, 1.6 %). The pattern of frameshifting observed for mutations within the pseudoknot closely paralleled that seen in the RRL, in that destabilisation of either stem of the pseudoknot reduced frameshifting efficiency dramatically (pACFS9, 13 in stem 1; pACFS 21 and 25 in stem 2) and compensatory mutations predicted to restore the structure increased frameshifting (pACFS17 in stem 1, 4 %; pACFS29 in stem 2, 6.5 %). A failure to restore fully the efficiency of frameshifting with compensatory changes in stem 1 has been documented in a number of studies of eukaryotic frameshifting (e.g. see Ten Dam *et al*., 1995) and may reflect a functional requirement for a particular pseudoknot

Table 1. Plasmid constructs used to study frameshifting *in vivo*

Construct	Description	Slippery sequence	Spacer	Stimulatory RNA	Frameshift efficiency (%)
pACFS1	Minimal IBV frameshift signal	UUUAA	UGAUACAC	Mimimal PK	9.1 ± 1.1
pACFS2	In-frame variant of pACFS1	UUUAAA**G**	**C**UGAUAC	Minimal PK	-
pACFS3	Slippery sequence mutant	UUUAAAG	UGAUAC	Minimal PK	0.5 ± 0.04
pACFS5	Structure mutant; PK replaced by SL	UUUAAAC	UGAUAC	Stem-loop	1.6 ± 0.18
pACFS6	In-frame variant of pACFS5	UUUAAA**G**	**C**UGAUAC	Stem-loop	-
pACFS9	Structure mutant, S1 arm 1 destabilised	UUUAAAC	UGAUAC	Disrupted PK	0.15 ± 0.02
pACFS10	In-frame variant of pACFS9	UUUAAA**G**	**C**UGAUAC	Disrupted PK	-
pACFS13	Structure mutant, S1 arm 2 destabilised	UUUAAAC	UGAUAC	Disrupted PK	0.4 ± 0.06
pACFS14	In-frame variant of pACFS13	UUUAAA**G**	**C**UGAUAC	Disrupted PK	-
pACFS17	Compensatory mutation, S1 reformed	UUUAAAC	UGAUAC	Reformed PK	4.0 ± 0.9
pACFS18	In-frame variant of pACFS17	UUUAAA**G**	**C**UGAUAC	Reformed PK	-
pACFS21	Structure mutant, S2 arm 2 destabilised	UUUAAAC	UGAUAC	Disrupted PK	1.0 ± 0.01
pACFS22	In-frame variant of pACFS21	UUUAAA**G**	**C**UGAUAC	Disrupted PK	-
pACFS25	Structure mutant, S2 arm 1 destabilised	UUUAAAC	UGAUAC	Disrupted PK	0.6 ± 0.05
pACFS26	In-frame variant of pACFS25	UUUAAA**G**	**C**UGAUAC	Disrupted PK	-
pACFS29	Compensatory mutation, S2 reformed	UUUAAAC	UGAUAC	Reformed PK	6.5 ± 0.7
pACFS30	In-frame variant of pACFS29	UUUAAA**G**	**C**UGAUAC	Reformed PK	-
pACFS33	Slippery sequence mutant	UUUAAA**U**	UGAUAC	Minimal PK	5.8 ± 1.2
pACFS34	In-frame variant of pACFS33	UUUAAA**G**	**U**UGAUAC	Minimal PK	-
pACFS36	Slippery sequence mutant	UUUAAA**A**	UGAUAC	Minimal PK	8.0 ± 0.8
pACFS37	In-frame variant of pACFS36	UUUAAA**G**	**A**UGAUAC	Minimal PK	-
pACFS41	Structure mutant, PK deleted	UUUAAAC	UGAUAC	No structure	0.05 ± 0.01
pACFS42	In-frame variant of pACFS41	UUUAAA**G**	**C**UGAUAC	No structure	-

The minimal IBV frameshift site in pACFS1 contains a slippery sequence (UUUAAAC) that is separated by a 6 nt spacer (UGAUAC) from the IBV minimal pseudoknot (PK) structure. Derivatives of the frameshift site (pACFS2-42) carried changes in the seventh nucleotide of the slippery sequence (bold), single nucleotide insertions into the spacer region (bold), or the pseudoknot was disrupted, deleted (no structure) or replaced by a stem-loop (SL) structure of the same predicted length and base-pair composition as the pseudoknot.

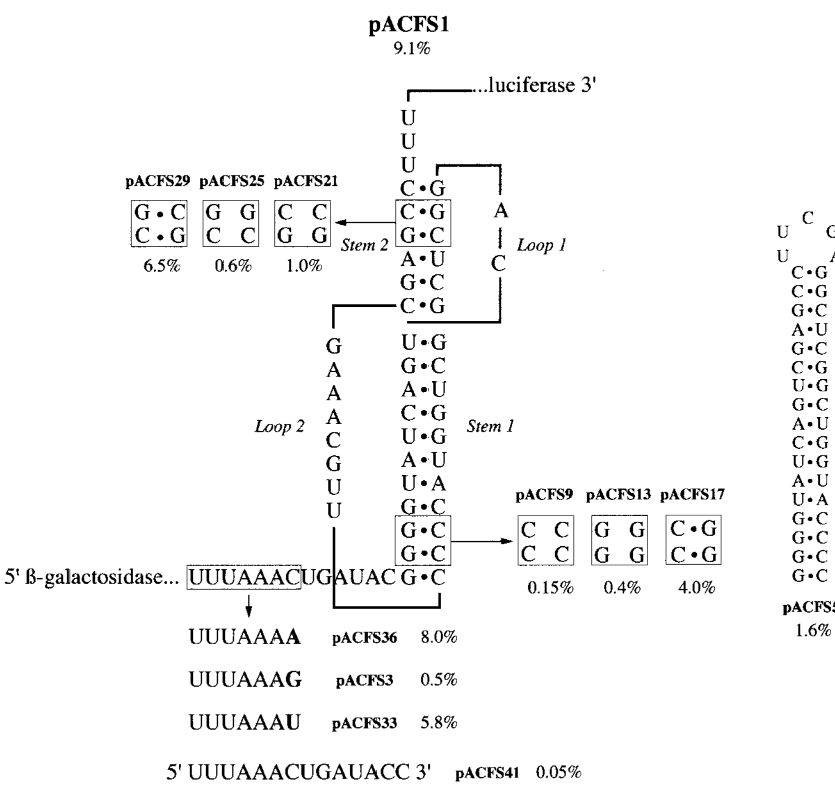


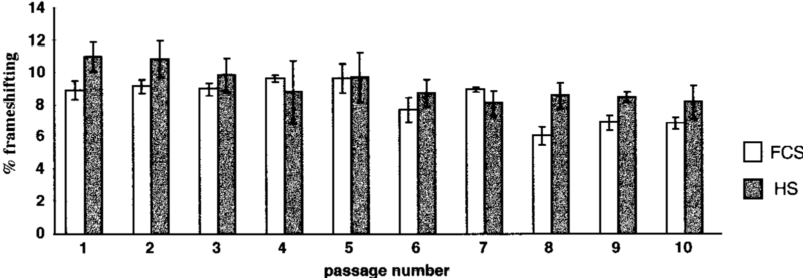
Figure 1. Ribosomal frameshifting at the IBV signal *in vivo*. The Figure shows the sequences present at the frameshift window of pACFS1 and the two main elements, the slippery sequence (UUUAAAC, boxed) and downstream pseudoknot structure. A variety of mutations were created within the frameshift window. (1) Slippery sequence variants were made in which the last nucleotide was changed to A, G or U (bold). (2) Complementary and compensatory changes were created within the pseudoknot region. In this representation of the pseudoknot, the stems are arranged vertically. For each stem, the two complementary changes (no base-pairing) and the compensatory change (base-pairing restored) are boxed and labelled with a mutant number. (3) A deletion mutant was tested (pACFS41) in which the entire pseudoknot was removed. (4) Finally, a construct was tested that formed a stem-loop structure rather than a pseudoknot. In plasmid pACFS5, the stem nucleotides are of the same length and nucleotide composition as the stacked stems of the pseudoknot in pACFS1. The frameshift efficiency (in cos cells) specified by each construct is also shown. A brief description of each construct and its relevant in-frame partner (see the text) is shown in Table 1.

conformation that is imprecisely reproduced in some of the compensatory mutants. Indeed, in pACFS17, the substitution of two G residues at the 5′-arm of stem 1 by C is predicted to have a detrimental effect on frameshifting (despite being paired), since in RRL, the IBV frameshift signal shows a preference for a G-rich stretch at the start of the 5′-arm (Napthine *et al*., 1999). The stem 2 compensatory mutant (pACFS29) reached 70 % of the wild-type level, consistent with earlier studies in RRL (Brierley *et al*., 1991). These experiments confirm for the first time a requirement for the IBV pseudoknot for efficient −1 frameshifting in *vivo*.

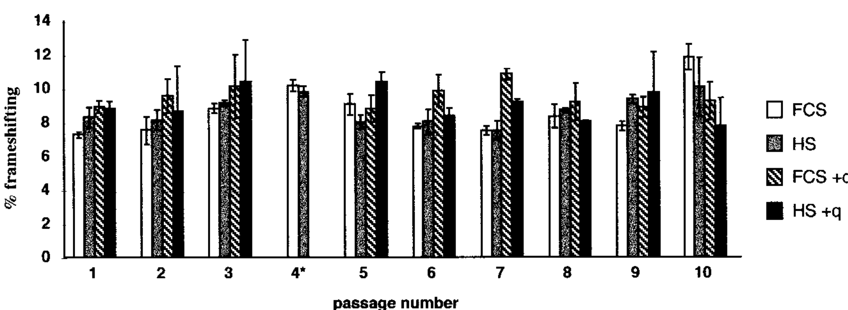
Q-base is not required for efficient −1 ribosomal frameshifting *in vivo*

Most animal sera contain q in concentrations varying between 50 nM and 0.3 μM, with the exception of horse serum (HS), which does not contain detectable amounts of the q-base (Kersten & Kersten, 1990). It has been shown that growth of HeLa cells in HS can induce Q-deficiency in the tRNA population (Langgut, 1993, 1995; Langgut *et al*., 1993; Reisser *et al*., 1993). We reasoned that a similar approach would allow us to examine the effect of Q-depletion of mammalian tRNAs on ribosomal frameshifting. cos cells were grown and passaged in fetal calf serum (FCS) or HS-containing medium and the frameshift efficiency of the IBV signal measured by transfection of the pACFS1 and two reporter plasmids over a series of ten passages. Each time the cells were split, 6 cm culture dishes were seeded with 3×10^5 cells from each population and on the following day transfected in triplicate with pACFS1 or pACFS2 using the DEAE-dextran method (see Materials and Methods). During the transfection procedure, cells were maintained in the appropriate serum supplement. At 48 hours post-transfection, the cells were lysed for analysis of β-galactosidase and luciferase expression and the assays performed in duplicate for each dish. Mean values of the frameshift efficiency calculated at each cell passage are shown in Figure 2(a). The frameshift efficiencies measured were found to be very similar in both cell populations, varying between 8 and 11 %. Continued passage in either q-containing or q-deficient serum led to a slight reduction in frameshift efficiency, but no significant difference was apparent between the two cell populations. This indicates that the modification status of the wobble base of $tRNA^{Asn}$, Q-containing or hypomodified, does not notably

A. Frameshift efficiency in cos-7 cells.



B. Frameshift efficiency in cos-7 cells grown with exogenous q-base.



C. [^{14}C]-guanine incorporation by purified tRNAs

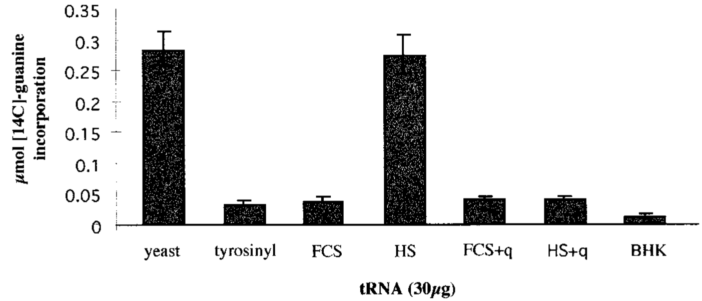


Figure 2. Influence of Q-base on −1 ribosomal frameshifting at the IBV signal *in vivo*. (a) cos cells were cultured over ten passages in medium containing either 10 % FCS or 10 % HS. The frameshift efficency of the minimal IBV frameshift signal was examined in the two cell populations using the frameshift reporter plasmids pACFS1 and 2 (see the text). Frameshifting was measured at each cell passage in three transient transfection experiments and the average values and range are shown. (b) cos cells were cultured over ten passages in medium containing either 10 % FCS or 10 % HS. After this time, a further ten passages were performed in the same medium, or in medium containing exogenous q-base (at 300 nM). Frameshifting was measured at each cell passage in three transient transfection experiments and the average values and range are shown. Two of the samples from passage 4 (*) were lost. (c) The queuosine-content of tRNAs purified from passage 10 cos cells cultured in FCS or HS with (+q) and without (−q) added q was measured by the guanine exchange assay (see Materials and Methods). The assay was performed in triplicate using 30μg aliquots of tRNA and the average values and range are shown. Controls included commercial yeast and tyrosinyl tRNAs, and tRNAs extracted from baby hamster kidney (BHK) cells.

influence frameshifting at the IBV signal. However, it was conceivable that the FCS-fed cos cell tRNAs had low levels of Q to begin with, and that switching to HS would not lead to any significant further reduction in Q-content. We tested this by adding back free queuine to the growth medium. Previous studies have shown that this can fully revert the Q-deficiency of HeLa cell

tRNAs within 72 hours (Langgut & Reisser, 1995). To this end, cells that had been passaged ten times in medium containing either HS or FCS were supplemented with free q-base at 300 nM and passaged a further ten times. To control for the effects of the q-supplementation, cos cell passage was also continued in unsupplemented FCS or HS-containing medium. Each time the cells were split, transfections with the frameshift reporter plasmids pACFS1 and pACFS2 were carried out as above and frameshift efficiency measured. As can be seen in Figure 2(b) the frameshifting efficiencies in the four cos cell populations (FCS, FCS + q, HS and HS + q) were found to be very similar to each other and to those measured in the previous analysis (Figure 2(a)), between 8 % and 12 %, with no noticeable effect of q-supplementation. Nevertheless, it was important to determine the Q-content of the various tRNA populations. To this end, cellular tRNAs were purified and assayed for Q-content enzymically, using the *E. coli* tRNA guanine transglycosylase (TGT) assay and biophysically, by HPLC-combined mass spectrometry. The TGT assay exploits the inability of bacterial TGT to recognise q or Q as a substrate; the enzyme can use only a q precursor or guanine in an exchange reaction in the anticodon. When offered hypomodified tRNAs of the Q-family ($tRNA^{Asn}$, $tRNA^{Asp}$, $tRNA^{His}$, $tRNA^{Tyr}$) and a radioactive guanine isotope, the bacterial TGT introduces the radioactive isotope into the anticodon loop in a base exchange reaction (Okada *et al*., 1978). Thus the incorporation of the radioactive isotope is a direct measure of the Q-content of tRNA.

The guanine exchange assay was set up with 30 μg of tRNA purified from passage 10 cells from both the initial comparison of frameshifting in FCS or HS-fed cells (FCS, HS; Figure 2(a) and the q-supplementation experiment (FCS + q, HS + q; Figure 2(b)). As controls, the assay was performed with Q-deficient yeast tRNAs, Q-containing *E. coli* $tRNA^{Tyr}$ and baby hamster kidney (BHK) cell tRNAs. All reactions were carried out in duplicate and the results are summarised in Figure 2(c). The results obtained were entirely consistent with the predicted Q-content of the various tRNA species. Those tRNAs that were expected to be hypomodified (yeast, HS) were good substrates for TGT. In contrast, those tRNAs expected to contain Q ($tRNA^{Tyr}$, FCS, FCS + q, HS + q, BHK) showed very low levels of [^{14}C]guanine incorporation.

From this experiment, we concluded that growth in HS did eliminate Q from the cos cell tRNAs and that supplementation with free q restored the level of Q. Furthermore, the addition of q to cells grown in FCS did not alter the guanine exchange activity of the tRNAs, suggesting that the tRNAs already contained high levels of Q. Consistent with this is the observation that the level of guanine

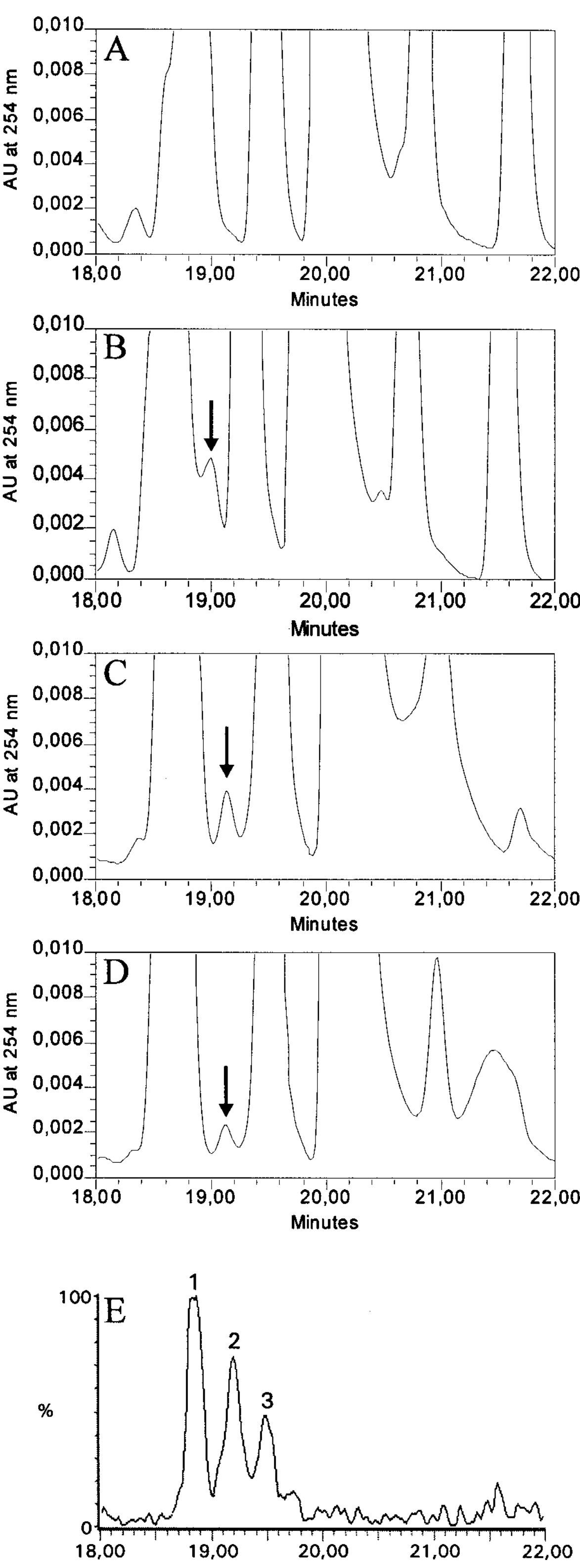


Figure 3. HPLC and mass spectrometric analysis of tRNA. (a) to (d) HPLC analysis of nucleosides from tRNA derived from cos cells grown in (a) HS, (b) FCS, (c) HS with added q, and (d) FCS with added q. Arrows indicate the position of Q. (e) Identification of Q and manQ/galQ in tRNA from cos cells grown in FCS medium with added q using selected ion profiles for the protonated nucleosides, *m/z* 572 (manQ/galQ) and *m/z* 410 (Q). 1, manQ or galQ; 2, Q; 3, manQ or galQ.

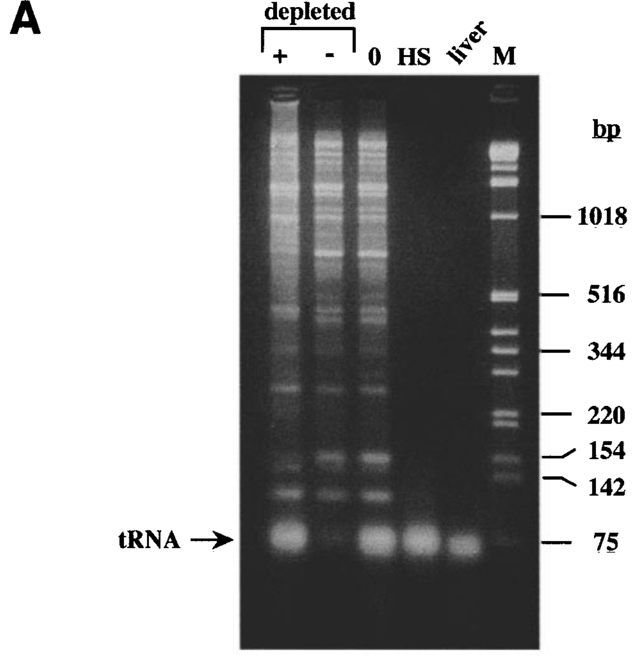
exchange seen with the tRNAs was similar to that seen with the hypermodified $tRNA^{Tyr}$. To confirm these data biophysically, the purified tRNAs were subjected to combined HPLC-mass spectrometry. As judged by HPLC analysis, the HS-derived tRNA was free of Q, and the FCS-derived tRNA was Q-containing (Figure 3(a) and (b)). As expected, addition of q to HS medium restored the Q-modification in tRNA (Figure 3(c)), whereas addition of q to FCS medium did not significantly increase the Q-content of the tRNA (Figure 3(d)), indicating that tRNA from cells grown in FCS medium is fully modified. The separation of manQ and galQ (glycosylated derivatives (mannose and galactose) of Q present in mammalian cells) from other compounds was not optimal using the gradient adapted for mass spectrometry. To detect the presence of manQ and galQ in tRNA, mass spectrometric analysis in the form of selected-ion recordings was carried out. The ions used for detection of Q was *m/z* 410 and for manQ/galQ *m/z* 572. The analyses revealed that tRNA from cells grown in FCS medium with or without added q and in HS medium with added q, contained Q, manQ and galQ (Figure 3(e) and data not shown). No ion corresponding to Q or manQ/galQ could be detected in tRNA from cells grown in HS medium (data not shown). In conclusion, tRNA from cells grown in HS medium lack Q-derived nucleosides, while tRNA from cells grown in FCS medium is fully modified with respect to these nucleosides.

Q-base is not required for efficient −1 ribosomal frameshifting *in vitro*

We went on to test the influence of Q on *in vitro* frameshifting, using a tRNA-dependent *in vitro* translation system (unpublished results). RRL was depleted of tRNAs by gel filtration through an ethanolamine DEAE-Sepharose column and fractions judged to be greater than 90 % depleted of tRNA, yet retaining all other nucleic acid species, were used in translation assays. The nucleic acid content of such a depleted fraction is shown in Figure 4(a), lane [−]. Supplementation of this fraction with HS tRNA (to 50 μg/ml, lane [+]) restored the level of tRNA to that of the undepleted lysate (lane [0]). As a frameshift reporter plasmid, we chose pFScass 6 (Brierley *et al*., 1992), which contains the same minimal IBV frameshift signal as used in pACFS1 cloned into the influenza A/PR/8/34 PB2 gene (Brierley *et al*., 1992) at a unique *Bgl*II site. Transcription of *Bam*HI-digested pFScass 6 *in vitro* using SP6 RNA polymerase generates a 2.4 kb mRNA that when translated in RRL yields a 19 kDa non-frameshifted species and a 28 kDa −1 frameshift product. Transfer RNA-depleted RRL was programmed with capped pFScass 6/*Bam*HI mRNA and supplemented with either Q-containing (FCS-grown cos cells, BHK cells, q-supplemented cos-FCS and q-supplemented cos-HS cells) or Q-free tRNAs (HS-grown cos cells, yeast tRNAs). The translation products, along with the calculated frameshift efficiencies, are shown in Figure 4(b). As controls, translations were also carried out with undepleted RRL, undepleted RRL that had been diluted 30 % to account for the dilution that occurs during chromatographic depletion of tRNAs, and depleted RRL that was supplemented with calf-liver tRNA (the tRNA used to supplement standard RRL translations (Jackson & Hunt 1983)). The translation of the pFScass 6 mRNA in the undepleted lysates (Figure 4, lanes 2 and 4) yielded the expected 19 kDa non-frameshifted and 28 kDa −1 frameshifted products and the frameshift efficiency was about 40 %, the expected value for this particular signal (Brierley *et al*., 1992; Napthine *et al*., 1999). Indeed, both products were observed in the translation reactions that contained mRNA, except for the translation reaction without tRNA supplement (lane 5). Thus all purified tRNAs were able to restore translation and frameshift activity. The relative abundance of non-frameshifted and frameshifted products on the gels was estimated by scanning densitometry and adjusted to take into account the differential methionine content of the 19 kDa and 28 kDa products (11 and 12, respectively). Although the overall frameshift efficiency seen in translations with depleted RRL was slightly reduced (to 30-35 %), the type of tRNA employed as a supplement, whether Q-modified or Q-deficient, did not appear to influence the degree of frameshifting. Hence the *in vitro* translations paralleled the *in vivo* studies and reinforce the conclusion that the Q-modification of $tRNA^{Asn}$ is not required for −1 ribosomal frameshifting at the IBV signal.

Discussion

The potential influence of tRNA anticodon nucleoside modifications on the process of −1 ribosomal frameshifting was first considered by Hatfield and colleagues (Hatfield *et al*., 1989). These authors reasoned that as most slippery sequence-decoding tRNAs have a hypermodified nucleoside in the anticodon, hypomodification would result in a tRNA with a less bulky anticodon and be more free to move around during decoding thus increasing frameshifting. An alternative hypothesis proposed a different role for the hypermodified base (Tsuchihashi, 1991; Tsuchihashi & Brown, 1992). In this scenario, the modification influences frameshifting by modulating the strength of the codon-anticodon interaction. From studies of ribosomal frameshifting in *E. coli*, the latter hypothesis seems the likelier. Firstly, it has been shown that hypomodification *per se*, does not necessarily lead to a stimulation of frameshifting. In *E. coli*, the IBV signal, or a



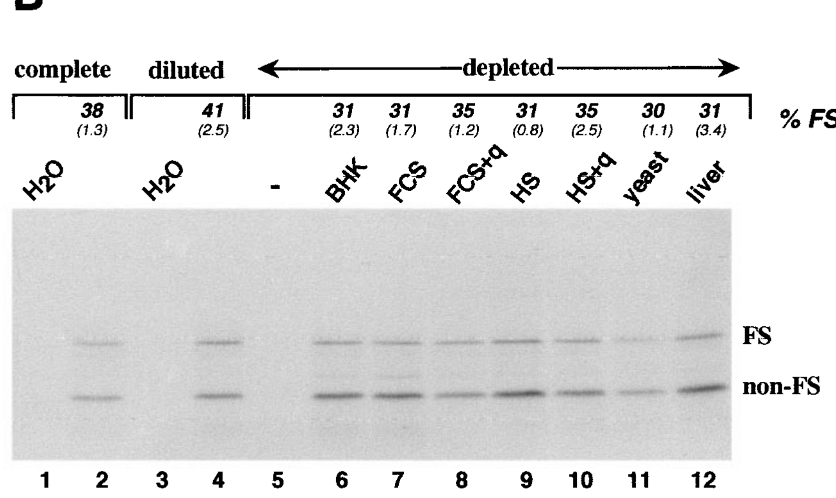


Figure 4. Influence of Q-base on −1 ribosomal frameshifting at the IBV signal *in vitro*. (a) Transfer-RNA-depleted RRL (depleted; 30 μl), which had been left unsupplemented (−) or had been supplemented with HS tRNA to 50 μg/ml (+) was analysed for nucleic acid content following extraction with phenol/chloroform and precipitation in ethanol. Aliquots (5 μl) of the final nucleic acid preparation (which was dissolved in 10 μl of water) were analysed on a 3 % agarose gel and visualised by staining with ethidium bromide. Also analysed were 5 μl of the nucleic acid preparation from undepleted, RRL (0), 0.75 μg of purified HS tRNA (HS) and 0.75 μg of commercial calf liver tRNA (liver). Molecular mass DNA markers (M; Life Technologies) were included. (b) *In vitro* translation of pFScass 6/*Bam*HI mRNA (Brierley *et al.*, 1992) in normal RRL (complete), normal RRL diluted to 70 % with water (diluted) or RRL depleted of endogenous tRNAs (depleted). The depleted RRL was either unsupplemented (−) or supplemented with tRNAs (at 50 μg/ml) derived from either BHK cells (BHK), FCS-grown cos cells (FCS), FCS-grown cos cells supplemented with endogenous q (FCS +q), HS-grown cos cells (HS), HS-grown cos cells supplemented with endogenous q (HS + q), yeast cells (yeast) or calf liver cells (liver). Tracks labelled H_2O represent water-programmed translations. The pFScass 6/*Bam*HI template directs the synthesis of a 19 kDa non-frameshift (non-FS) and 28 kDa frameshift product (FS) (Brierley *et al.*, 1992). Products were labelled with [^{35}S]methionine, separated on an SDS/15 % polyacrylamide gel and detected by autoradiography. The frameshift efficiencies quoted above the relevant lanes are the average of two independent measurements and the range is shown in parentheses.

variant with slippery sequence UUUAAAU, functions inefficiently (1-2 %), whether tRNA^{Asn} possesses the anticodon QUU or GUU (Brierley *et al.*, 1997). Secondly, there is evidence to support a role for the strength of the codon-anticodon interaction in frameshifting. Tsuchihashi & Brown (1992) were able to diminish frameshifting at the *dnaX* signal (slippery sequence AAAAAAG) by co-expressing a variant tRNA^{Lys} in which the anticodon had been changed from UUmnm^{5}s^{2}U to UUC. Expression of this tRNA, an isoacceptor not present naturally in *E. coli* cells, was considered to have reduced frameshifting by recognising more strongly the AAG codon. It was subsequently shown that the natural anticodon of *E. coli* tRNA^{Lys} has an unusual conformation; it can probably form only a weak wobble pair with the anticodon, perhaps accounting for the high frameshift efficiency in *E. coli* at slippery sequences ending in AAG (Watanabe *et al.*, 1993; Agris, 1996; Agris *et al.*, 1997).

The relevance of these experiments to the eukaryotic frameshifting, however, is uncertain. All efficient −1 frameshift sites in *E. coli* employ tRNA^{Lys} as the A-site decoding tRNA and, given

that this tRNA has a very unusual anticodon structure, it may be the case that frameshifting in prokaryotic systems is distinct from that in eukaryotes and that $tRNA^{Lys}$ is a special case. In this study, we have performed a detailed examination of the influence of the hypermodified base Q on ribosomal frameshifting in eukaryotes, using both *in vivo* and *in vitro* systems. During all experiments, the predicted modification status of purified cellular tRNAs with respect to queuosine was confirmed with an enzymatic guanine exchange assay and combined HPLC-mass spectrometry. Frameshifting was measured in cos cells that had been passaged in growth medium containing either FCS or HS, and in cos cells grown in FCS or HS supplemented with free queuine base. Addition of free queuine converts hypomodified tRNA (in HS) back to the hypermodified state (in FCS). The purified cellular tRNAs of defined modification status were also used in a tRNA-dependent *in vitro* translation system to study the effect of $tRNA^{Asn}$ hypomodification on *in vitro* frameshifting at the IBV signal. The efficiency of the IBV frameshift signal was not influenced by the nature of the tRNA population present in either system. The simplest interpretation of these observations is that both hypo- and hypermodified $tRNA^{Asn}$ can support efficient ribosomal frameshifting. However, as we cannot rule out the possibility that the tRNAs purified from FCS-grown cos cells (or FCS-grown cos cells supplemented with q) still retain low levels of hypomodified tRNAs, it is not possible to conclude categorically that frameshifting can be mediated by hypermodified tRNAs alone. However, it is clear that hypomodified tRNAs are fully functional.

How do these observations fit with the view that anticodon loop modifications may influence frameshifting by influencing the strength of the codon-anticodon interaction during decoding of the slippery sequence? The three-dimensional structure of queuosine 5′-monophosphate, determined by X-ray crystallography, and incorporated into $tRNA^{Tyr}$ on the basis of the coordinates of yeast $tRNA^{Phe}$, suggests that Q in the anticodon loop does not interfere with codon-anticodon interactions (Yokoyama *et al*., 1979). The bulky 7-substituent cyclopentenediol group of Q is fully extended outwards away from the anticodon, and the bond length and bond angles of the pyrimidine moiety of the 7-deazaguanine ring are almost equal to those of the unmodified guanosine base. Thus the conformational characteristics imply very similar decoding properties of Q and G-containing $tRNA^{Asn}$, consistent with our observation that frameshifting efficiencies are similar with Q or G-containing $tRNA^{Asn}$. However, measurements of the relative stability of anticodon-anticodon complexes suggest a slight reduction in stability when a G-C pair is replaced by Q-C pair at the wobble position in the same anticodon sequence; the G-C-containing complex was stable for 840 ms compared with 620 ms for Q-C (Grosjean *et al*., 1978). So we might have expected a decrease in frameshift efficiency in Q-free cells if the stability hypothesis is correct.

Our observation that supplementation of the tRNA-dependent RRL with Q-free tRNAs (HS-derived tRNAs or yeast tRNAs) did not stimulate frameshifting at the IBV site is inconsistent with recent work by Carlson *et al*. (1999), who studied frameshifting using *in vitro* translation reactions supplemented with hypomodified variants of $tRNA^{Asn}$ or $tRNA^{Phe}$. In these experiments, slippery sequence variants of the mouse mammary tumour virus *gag/pol* frameshift signal were translated in RRL in the presence or absence of the exogenous hypomodified tRNAs. It was found that frameshifting at AAAUUUU was stimulated by addition of Y^- $tRNA^{Phe}$ (purified from rabbit reticulocytes) and at AAAAAAC or AAAAAAU by addition of Q^- $tRNA^{Asn}$ (purified from yeast strain 3950-1B′2). Currently, it is not known why these data contradict those of the present study. In the analysis by Carlson *et al*. (1999), standard, non-depleted RRL was employed. Although it is possible that the tRNA-depleted lysate employed in our study is also depleted of an additional component(s) that is required to mediate the stimulation afforded by hypomodified, but not hypermodified tRNAs, this seems unlikely. Although the frameshift efficiencies measured in the tRNA-depleted RRL are slightly reduced from those measured in standard RRL (38 to about 32 %), this is probably a consequence of a reduced processivity of translation in the re-supplemented reactions. This leads to an underestimation of the amount of frameshift product and, consequently, an apparent reduction in frameshifting. A more likely explanation is that the influence of hypomodified tRNAs is seen only at certain slippery sequences; i.e. at certain P and A-site tRNA combinations. Alternatively, the IBV site may represent an ''upper limit'' of frameshift efficiency that cannot be stimulated further by hypomodified tRNAs. We are currently testing these possibilities. Whatever the case, Q is not required for frameshifting at the IBV site *in vivo*.

The study of IBV frameshifting in eukaryotic cells has confirmed for the first time the requirement for a pseudoknot *in vivo*. Indeed, the pseudoknot was about six times more effective at promoting frameshifting than a related hairpin-loop. However, the level of frameshifting observed was reduced some four to fivefold in comparison to *in vitro* translation systems (RRL; wheat-germ extract, data not shown) and the reason for this is not known. In RRL, the translation process is physiologically detached from other post-transcriptional events such as splicing, polyadenylation and nuclear export of mRNA, all of which might influence the kind of proteins associated with the mRNA, and hence potentially frameshifting. Alternatively, differences with respect to the ribosome load on mRNAs between the two systems cannot be ruled out. The molecular basis for this difference

may be informative in terms of the mechanism of the frameshift process.

Materials and Methods

Plasmid construction

The pACFS series of plasmids were prepared in a two-step cloning strategy. Plasmid pAC74Z (Stahl *et al.*, 1995) was digested sequentially with *Bcl*I and *Nhe*I (which removes the β-galactosidase gene) and wild-type or mutant IBV frameshift sequences inserted as three pairs of complementary, phosphorylated oligonucleotides. The design of the oligonucleotides was such that the zero reading frame of the inserted frameshift site was in the same frame as the deleted β-galactosidase gene, and the −1 reading frame was that of luciferase. The ends of the oligonucleotides restored the *Nhe*I site at the 5′-site of the frameshift window but the *Bcl*I site and the termination codon at the 3′-site were not reformed. Ligation reactions were transformed into *E. coli* DH5α and correct clones (pACIBV* series) identified by DNA sequencing (Sanger *et al.*, 1977). Plasmids of the pACIBV series were digested with *Nhe*I and ligated with the β-galactosidase gene fragment isolated from pAC74Z by *Nhe*I restriction digestion. The resulting plasmids (pACFS series, see Table 1) were transformed into *E. coli* JM101 cells and plasmid DNA isolated by CsCl-EtBr density-gradient centrifugation.

Tissue culture transfection protocol

cos cells were maintained in Dulbecco's modification of Eagle's medium (DMEM) containing 10 % (v/v) fetal calf serum (FCS) or 10 % (v/v) horse serum (HS) (all from Sigma). Cells were passaged when they reached 70 % confluence. Where required, queuine (a generous gift from Dr Susumu Nishimura, Banyu Tsukuba Research Institute, Tsukuba, Japan) was added to 300 nM. Plasmid transfections were carried out using the DEAE-dextran method. Cells (3×10^5) were seeded in 60 mm dishes and grown for 18-24 hours. The cells were washed consecutively with phosphate-buffered saline (PBS) and Optimem (Gibco), before overlaying with 0.5 ml of a transfection mix containing 3 μg of plasmid DNA in 0.25 ml of Optimem and 0.25 ml of 1 mg/ml DEAE-dextran in TBS (25 mM Tris-HCl (pH 7.4), 140 mM NaCl, 3 mM KCl). After 30 minutes, the mix was removed by aspiration and replaced with 5 ml of the relevant growth medium containing 60 μg/ml chloroquine. Incubation was continued for a further one to four hours before the medium was replaced with fresh growth medium without chloroquine. The cells were harvested 48 hours post-transfection and reporter gene expression determined as below.

β-Galactosidase and luciferase assays

Transfected cells were washed with PBS, drained and 450 μl of lysis buffer (25 mM glycyl-glycine (pH 7.8), 15 mM $MgSO_4$, 4 mM EGTA, 0.1 % (v/v) Triton-X-100, 1 mM DTT) added. Following ten minutes agitation at room temperature, cell debris was removed by centrifugation (10,000 *g*, one minute), the supernatants transferred to fresh tubes on ice and 100 μl aliquots tested immediately for β-galactosidase and luciferase activity according to Sambrook *et al.* (1989) and de Wet *et al.* (1987), respectively. Enzyme assays were performed in duplicate. Each plasmid was tested in at least three independent transfections.

Preparation of tRNA-guanine transglycosylase (TGT)

E. coli TGT was prepared from the prokaryotic expression plasmid pTGT5 (provided by Dr George Garcia, University of Michigan, Ann Arbor, USA) essentially as described (Garcia *et al.*, 1993). Briefly, TGT was overexpressed in *E. coli* BL21 cells and purified by selective ammonium sulphate precipitation followed by anion-exchange chromatography. The product was judged to have a purity greater than 95 % and was free of contaminating nucleic acids. The specific activity of the preparation was determined by the guanine exchange assay (see below) and was 817 units/mg. This value was derived according to the definition (Garcia *et al.*, 1993), that one unit of TGT catalyses the incorporation of 1 μmol of [^{14}C]guanine into tRNA per minute at 100 μM yeast tRNA at 37 °C per mg of TGT $\times 10^5$. The specific activity of the TGT was almost identical with that prepared by others (804 units/mg ; Garcia *et al.*, 1993). TGT was stored at −70 °C at 2 mg/ml in 100 mM Hepes (pH 7.7), 5 mM DTT, 0.3 M KCl.

Guanine exchange assay

The presence or absence of Q-base in tRNAs was assessed by the guanine exchange assay, which exploits the ability of the *E. coli* TGT enzyme to catalyse the exchange of a guanine base at the first position of the anticodon of queuosine-unmodified tRNAs with radiolabelled [^{14}C]guanine (46 mCi/mmol; Amersham, UK). The enzyme cannot use queuosine-modified tRNAs as a substrate, thus the guanine acceptance is maximal when the tRNAs are Q-deficient. Radiolabelled guanine incorporation is therefore a measure of queuosine content (Okada *et al.*, 1978). The assay was performed for one hour at 37 °C in a total volume of 50 μl and contained 0.1 M Hepes KOH (pH 7.5), 20 mM $MgCl_2$, 1.6 units of TGT, 0.2 μCi of [^{14}C]guanine (46 mCi/mmol) and 0-1 mg of tRNA. Aliquots were spotted onto GF/C filter disks, washed once with 3 ml of 10 % trichloroacetic acid (TCA), twice with 3 ml of 5 % TCA, once with 3 ml of 70 % ethanol, dried and counted in a PackardTri-Carb® 1500 scintillation counter. Assays were performed in triplicate. Commercial yeast and tyrosinyl-tRNAs used in the assay were from Sigma.

Purification of tRNAs from tissue culture cells

Pellets of 2.5×10^8 cells, previously washed in PBS, were resuspended in 4 ml of NTE lysis buffer (0.1 M NaCl, 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.5 % (v/v) NP40) and incubated on ice for two minutes. The lysates were cleared by centrifugation (6000 *g*, five minutes), the supernatants made 1 % (w/v) in SDS and subjected to two rounds of extraction with phenol/chloroform and one round with chloroform. Following precipitation in ethanol, the crude nucleic acid pellet was resuspended in 0.5 ml of water. Aliquots (100 μl) were mixed with an equal volume of formamide, boiled for two minutes and loaded onto a denaturing 15 % polyacrylamide gel. The tRNA-containing bands were identified by UV shadowing, sliced out, chopped up and eluted in 0.5 M ammonium acetate, 10 mM magnesium acetate, 1 mM EDTA, 0.1 % (w/v) SDS, at 4 °C overnight. The eluted tRNA was concentrated by precipitation in

ethanol. Typically 0.6 to 0.8 mg of tRNA was obtained from 10^9 cells and was dissolved in water at a concentration of 2 mg/ml.

Analysis of the Q-content by HPLC-coupled mass spectroscopy (MS)

Transfer RNA was degraded to nucleosides using nuclease P_1 and alkaline phosphatase (Gehrke & Kuo, 1989). The nucleoside mixture was analysed on a Waters™ System liquid chromatograph with a Waters™ 996 diode array UV detector (Waters™ Corporation, Milford, MA, USA), directly interfaced to a VG platform mass spectrometer equipped with an electrospray ionisation source (Fisons Instruments, Altrincham, UK). Separation of nucleosides by HPLC was achieved using a Supelcosil LC-18-S reverse-phase column (2.1 mm by 250 mm, particle diameter 5 μm) and a Supelguard LC-18-S, 2.1 mm by 20 mm guard column (Supelco, Bellefonte, PA, USA) held at 26 °C, at a flow-rate of 2 ml/minute. Nucleosides were eluted using the gradient described by Buck *et al*. (1983) but altered to accommodate a lower ammonium acetate concentration (5 mM), which is more compatible with electrospray ionisation. UV data were recorded continuously, and mass spectra were taken every 1.0 second during the 60 minutes of chromatography. The procedures and interpretation of data for qualitative LC-MS analysis of nucleosides in RNA hydrolysates have been described (Pomerantz & McCloskey, 1990).

In vitro transcription, translation and preparation of tRNA-dependent RRL

In vitro transcription of plasmid pFScass 6 (Brierley *et al*., 1992) employing the bacteriophage SP6 RNA polymerase, was carried out essentially as described by Melton *et al*. (1984) and included the synthetic cap structure 7meGpppG (New England Biolabs) to generate capped mRNA. Product RNA was recovered by a single extraction with phenol/chloroform/isoamyl alcohol (49:49:2, by vol.) followed by precipitation in ethanol in the presence of 2 M ammonium acetate. The RNA pellet was dissolved in water, and remaining unincorporated nucleotide triphosphates removed by Sephadex G-50 chromatography. RNA was recovered by precipitation in ethanol, dissolved in water and checked for integrity by electrophoresis on 1.5 % agarose gels containing 0.1 % SDS. In ribosomal frameshift assays, serial dilutions of purified mRNAs were translated in RRL as described (Brierley *et al*., 1989). Translation products were analysed on SDS/15 % (w/v) polyacrylamide gels according to standard procedures (Hames, 1991). The relative abundance of non-frameshifted or frameshifted products on the gels was determined by direct measurement of [^{35}S]-methionine incorporation using a Packard Instant Imager 2024. Frameshift efficiencies were calculated from those dilutions of RNA where translation was highly processive (RNA concentrations of 10 μg to 25 μg RNA/ml of reticulocyte lysate). The frameshift efficiencies quoted are the average of at least three independent measurements, which varied by less than 10 %, i.e. a measurement of 40 % frameshift efficiency was between 36 % and 44 %. The calculations of frameshift efficiency take into account the differential methionine content of the various products (19 kDa, 11; 28 kDa, 35). Transfer RNA-depleted RRL was prepared by ethanolamine-Sepharose column chromatography (unpublished results).

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