Mol. Cells, Vol. 10, No. 1, pp. 113–118

Molecules and Cells

Communication

Yeast Asparagine (Asn) tRNA without Q Base Promotes Eukaryotic Frameshifting More Efficiently than Mammalian Asn tRNAs with or without Q Base

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(Received on December 7, 1999)

In this study, we compare the efficiency of Asn tRNA from mammalian sources with and without the highly modified queuosine (Q) base in the wobble position of its anticodon and Asn tRNA from yeast, which naturally lacks Q base, to promote frameshifting. Interestingly, no differences in the ability of the two mammalian Asn tRNAs to promote frameshifting were observed, while yeast $tRNA^{Asn}_{-Q}$ promoted frameshifting more efficiently than its mammalian counterparts in both rabbit reticulocyte lysates and wheat germ extracts. The shiftability of yeast Asn tRNA is therefore not due, or at least not completely, to the lack of Q base and most likely the shiftiness resides in structural differences elsewhere in the molecule. However, we cannot absolutely rule out a role of Q base in frameshifting as wheat germ extracts and a lysate depleted of most of its tRNA and supplemented with calf liver tRNA contain both Asn tRNA with or without Q base.

Keywords: Asparagine tRNA; Queuosine Base; Ribosomal Frameshifting; Wheat Germ Extracts.

Introduction

An interesting feature of ribosomal frameshifting involving retroviruses of higher eukaryotes is that, when AAU or AAC, or for that matter, UUU or UUC, occur at the site of the frameshift (Farabaugh, 1996), there is a strong preference for only one of the pyrimidine bases in the 3′ position to promote efficient frameshifting (Carlson *et al.*, 1999; Chamorro *et al.*, 1992; Dinman *et al.*, 1991; Jacks *et al.*, 1988; Nam *et al.*, 1992; Wilson *et al.*, 1988). For example, AAC enhances frameshifting far more efficiently than AAU, while UUU enhances frameshifting far more efficiently than UUC. The fascinating feature about this phenomenon is that AAU/C are decoded by the same Asn tRNA and UUU/C are decoded by the same Phe tRNA. Thus, the same tRNA reads one of its codons far more efficiently than the other in promoting the frameshift process (reviewed in Hatfield *et al.*, 1992).

Asn and Phe RNAs are characterized by the fact that they contain a highly modified base in their anticodon loop (Sprinzl *et al.*, 1989). Asn tRNA contains queuosine (Q) base in the wobble position of its anticodon (position 34) and Phe tRNA contains wybutoxosine (Y) base in the position immediately 3′ to its anticodon (position 37). The presence of these highly modified bases are known to influence the coding properties of tRNAs containing them. For example, tyrosine tRNA from *Drosophila* (Bienz and Kubli, 1981) and tobacco plants (Beier *et al.*, 1984a; 1984b) lacking a modified Q base at position 34 suppresses the stop codon, UAG, while the fully modified isoacceptors do not. Histidine tRNA from *Drosophila* lacking Q base decodes CAC much more effectively than CAU, while the fully modified isoacceptor has a slight preference for CAU (Meier *et al.*, 1985). Lack of Y base in *E. coli* Phe tRNA results in the misreading of the leucine codon, CUU (Wilson and Roe, 1989), while the lack of the Y base in mammalian Phe tRNA promotes frameshifting

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Abbreviations: Asn, asparagine; GST, glutathione transferase; $GSTA_6U$ and $GSTA_6C$, GST constructs encoding a ribosomal frameshift signal, AAAAAAU(C), that is cloned downstream of the GST gene; PCR, polymerase chain reaction; Q base, queuosine base; Y base, wybutoxosine base.

(Carlson *et al.*, 1999). In addition, once mammalian Phe tRNA without Y translates its cognate codon, and is presumably being transferred to the ribosomal P site, it influences the selection of the incoming aminoacyl-tRNA to be decoded at the ribosomal A site (Carlson *et al.*, 1999; Smith and Hatfield, 1986).

Excellent reviews on the effect of modified bases in the anticodon loop of tRNAs on ribosomal frameshifting events that occur primarily in yeast and bacteria have been published recently (Bjork *et al.*, 1999; Farabaugh and Bjork, 1999).

We previously proposed that the modification status of Asn and Phe tRNAs may affect their shiftability wherein the undermodified forms of each tRNA may promote frameshifting more efficiently (Carlson *et al.*, 1999; Hatfield *et al.*, 1989; 1992). To investigate this possibility, we examined the ability of Asn tRNA from yeast, which lacks the Q base, and hypomodified Phe tRNA from mammalian systems, which lacks the Y base, to stimulate frameshifting in rabbit reticulocyte lysates programmed with the mRNA encoding the appropriate frameshift signal (Carlson *et al.*, 1999). Both hypomodified tRNAs stimulated frameshifting demonstrating that they are indeed shifty tRNAs. However, with respect to Asn tRNA from yeast (designated herein as $\text{tRNA}^{\text{Asn}}{}_{-\text{Q(Y)}}$), there are several base differences between it and the corresponding mammalian, Q-containing or Q-deficient Asn tRNAs. This prompted us to examine the relative efficiencies of $\text{tRNA}^{\text{Asn}}{}_{-\text{Q(Y)}}$ and the Q-containing and Q-deficient mammalian Asn tRNAs (designated herein as $\text{tRNA}^{\text{Asn}}{}_{+\text{Q(M)}}$ and $\text{tRNA}^{\text{Asn}}{}_{-\text{Q(M)}}$, respectively) in promoting frameshifting. Interestingly, the level of frameshifting was indistinguishable with the two modified forms of mammalian Asn tRNA, but the level was enhanced with $\text{tRNA}^{\text{Asn}}{}_{-\text{Q(Y)}}$ in both rabbit reticulocyte lysates and wheat germ extracts. Hence, the enhanced shiftability of $\text{tRNA}^{\text{Asn}}{}_{-\text{Q(Y)}}$ is not due, at least completely, to the absence of Q base and is likely due to some other base difference or differences. Even though rabbit reticulocyte lysates were depleted of most of their endogenous tRNA, the lysates contained residual amounts of $\text{tRNA}^{\text{Asn}}{}_{+\text{Q(M)}}$ and $\text{tRNA}^{\text{Asn}}{}_{-\text{Q(M)}}$, and the tRNA added to supplement tRNA dependent lysates contained both isoacceptor forms, making it difficult to evaluate the role of Q base in frameshifting.

Materials and Methods

Materials [^{35}S]Methionine (Spec. Act. 1200 Ci/mMol), and [^{3}H]asparagine (Spec. Act. 20 Ci/mmol) were purchased from NEN and glutathione transferase gene (GST) backbone vector, pGEX4T-1, from Pharmacia. Rabbit reticulocyte lysates (nuclease treated and untreated) were purchased from Promega or were prepared as described (Hatfield *et al.*, 1979; Smith and Hatfield, 1986). Wheat germ extracts and Ribomax Large Scale RNA Transcription Kit were purchased from Promega, DH5a competent cells from Gibco, Qiafilter Maxi Kit from Qiagen, 12% polyacrylamide gels from Novex and calf liver tRNA from Sigma. Raw wheat germ was obtained from the local health food store.

Purification and chromatography of tRNAs Large scale amounts of rabbit reticulocyte lysates were prepared from phenylhydrazine treated rabbits and used as a source for preparing tRNA as described (Hatfield *et al.*, 1979; Smith and Hatfield, 1986). Total tRNA was isolated from nuclease treated and untreated rabbit reticulocyte lysates, from yeast [strain 3950-1B'2, obtained from D.C. Hawthorne (Hawthorne, 1976)] and from raw wheat germ as described (Diamond *et al.*, 1981; Hatfield *et al.*, 1979; Smith and Hatfield, 1986). Transfer $\text{RNA}^{\text{Asn}}{}_{+\text{Q(M)}}$ was purified from rabbit reticulocytes and $\text{tRNA}^{\text{Asn}}{}_{-\text{Q(Y)}}$ to homogeneity from yeast as given (Carlson *et al.*, 1999) and $\text{tRNA}^{\text{Asn}}{}_{-\text{Y(M)}}$ from reticulocytes by the same procedures. Amounts of specific tRNAs were determined by aminoacylating tRNAs in the presence of [^{14}C]amino acid and rabbit reticulocyte synthetases under limiting levels of tRNA as described (Hatfield *et al.*, 1979). Chromatography of tRNAs was carried out on RPC-5 columns (Kelmers and Heatherly, 1971) as described (Hatfield *et al.*, 1979).

Constructs encoding frameshift signals The constructs encoding GST, designated GST-constructs, consist of the GST region and the *gag-pro* frameshift region of MMTV as described (Carlson *et al.*, 1999). The *gag-pro* region was prepared by amplifying a 214 bp fragment which begins 39 bases upstream of the frameshift site and is attached in-frame with the C-terminus of GST. A mutation was introduced 8 bp downstream of the hairpin structure (Carlson *et al.*, 1999) converting an A to a T to introduce a methionine codon in the –1 reading frame and mutation of the wild type construct (GST-A_6C) to the mutant construct (GST-A_6U) was then introduced within the heptanucleotide frameshift signal sequence by PCR. Constructs were amplified by transforming DH5a competent cells with the appropriate plasmid, growing transformed cells in 1 L batches, extracting and then purifying plasmids using a Qiafilter Kit by standard molecular cloning techniques (Park *et al.*, 1997).

Preparation of mRNA and protein synthesis GST-constructs were cut with *Eco*RI and mRNA generated from each construct (Carlson *et al.*, 1999) using a Ribomax large scale transcription kit as described by the vendor. Large scale preparation of rabbit reticulocyte lysates for making tRNA dependent and nuclease treated lysates were carried out as given (Hatfield *et al.*, 1979; Smith and Hatfield, 1986). Rabbit reticulocyte lysates were programmed with 5 µg of mRNA and ^{35}S-methionine, 19 unlabeled amino acids (minus methionine) added and reactions (25 µl total volume) carried out following the instructions of the vendor. For most studies, 5 pmol of $\text{tRNA}^{\text{Asn}}{}_{+\text{Q(M)}}$ (6 µg of tRNA), $\text{tRNA}^{\text{Asn}}{}_{-\text{Q(M)}}$ (4 µg of tRNA) and of $\text{tRNA}^{\text{Asn}}{}_{-\text{Q(Y)}}$ (4.6 µg of tRNA) were added to reactions where indicated in the figures. Rabbit reticulocyte lysates were depleted of endogenous tRNAs by Sepharose 6B (see Feng *et al.*, 1989, and references therein and Acknowledgements) and supplemented with total tRNA from calf liver (Sigma) or from rabbit reticulocytes. Following protein synthesis using rabbit reticulocyte lysates or wheat germ extracts, reactions were treated, electrophoresed and the level of frameshifting determined by analyzing developed gels using a Molecular Dynamics PhosphorImager as described (Carlson *et al.*, 1999) The number of methionine residues in the 0 frame and the –1 frame were compensated for in determining the level of frameshifting.

Results and Discussion

The level of frameshifting using frameshift signals encoding AAC and AAU at the frameshift site in response to the two mammalian Asn isoacceptors, $\mathrm{tRNA^{Asn}}_{+\mathrm{Q(M)}}$ and $\mathrm{tRNA^{Asn}}_{-\mathrm{Q(M)}}$, and to yeast Asn tRNA, $\mathrm{tRNA^{Asn}}_{-\mathrm{Q(Y)}}$, were examined. Rabbit reticulocyte lysates were programmed with either GST-A_6C or GST-A_6U mRNA and the appropriate Asn tRNA added as shown in Fig. 1. Neither mammalian isoacceptor (lanes 2 and 3) stimulated the level of frameshifting above the control level without added tRNA (lane 1). However, $\mathrm{tRNA^{Asn}}_{-\mathrm{Q(Y)}}$ stimulated the level of frameshifting dramatically with both frameshift signals (lane 4). The fact that frameshifting is reduced when AAC is mutated to AAU at the frameshift site has been known for many years (Jacks, 1990). The important observation in this study is that yeast $\mathrm{tRNA^{Asn}}_{-\mathrm{Q(Y)}}$ is indeed a shifter tRNA compared to either $\mathrm{tRNA^{Asn}}_{+\mathrm{Q(M)}}$ or $\mathrm{tRNA^{Asn}}_{-\mathrm{Q(M)}}$ under these assay conditions.

The level of frameshifting was also examined in wheat germ extracts (Fig. 2). Wheat germ extracts were programmed with GST-A_6C and GST-A_6U and the appropriate Asn tRNA added. Although the level of frameshifting was much lower in wheat germ extracts (Fig. 2) compared to that found in rabbit reticulocyte lysates (Fig. 1), similar patterns of frameshifting were observed in both sources of *in vitro* protein synthesis systems. That is, GST-A_6C was a more slippery, frameshifting template than GST-A_6U and $\mathrm{tRNA^{Asn}}_{-\mathrm{Q(Y)}}$ enhanced frameshifting, whereas $\mathrm{tRNA^{Asn}}_{+\mathrm{Q(M)}}$ and $\mathrm{tRNA^{Asn}}_{-\mathrm{Q(M)}}$ had little or no effect on the level of frameshifting. It should be noted that repeated experiments using wheat germ extracts and each Asn tRNA showed a slight stimulation with either $\mathrm{tRNA^{Asn}}_{+\mathrm{Q(M)}}$ and $\mathrm{tRNA^{Asn}}_{-\mathrm{Q(M)}}$ and GST-A_6C mRNA (ranging between 1.25 and 1.5 fold) and a more pronounced stimulation with $\mathrm{tRNA^{Asn}}_{-\mathrm{Q(Y)}}$ (ranging between 2 and 2.5 fold). Similarly, very little or no stimulation in frameshifting was observed with either mammalian Asn tRNA and GST-A_6U mRNA in repeated experiments, whereas $\mathrm{tRNA^{Asn}}_{-\mathrm{Q(Y)}}$ always enhanced frameshifting by about 2 fold.

The above data demonstrate that yeast $\mathrm{tRNA^{Asn}}_{-\mathrm{Q(Y)}}$ enhances frameshifting, while the corresponding mammalian isoacceptors with and without Q base have little or no effect on frameshifting when added exogenously. Since the only difference between the two mammalian isoacceptors is the presence or absence of Q, does this mean that Q has no influence on frameshifting? To attempt to resolve this question, we prepared tRNA dependent reticulocyte lysates as described in Materials and Methods. Most of the tRNA was removed from lysates as shown in Fig. 3, lane 4. For comparison, untreated lysates (lane 2), nuclease treated lysates supplemented with calf liver tRNA (lane 3) and tRNA dependent lysates supplemented with calf liver tRNA (lane 5) are shown. In addition, tRNA preparations from calf liver (Sigma) and from rabbit reticulocytes and yeast (prepared as given in Materials and Methods) are shown in lanes 6–8, respectively.

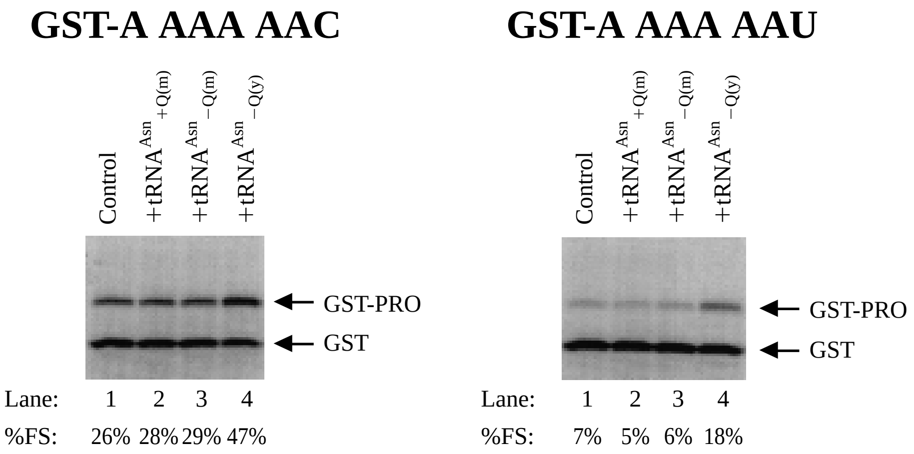

Fig. 1. Ribosomal frameshifting in rabbit reticulocyte lysates supplemented with Q-containing and Q-deficient Asn tRNA. Nuclease treated rabbit reticulocyte lysates were programmed with mRNA generated from constructs encoding GST-A_6C or GST-A_6U as shown and supplemented with $\mathrm{tRNA^{Asn}}_{+\mathrm{Q(M)}}$ (lane 2), $\mathrm{tRNA^{Asn}}_{-\mathrm{Q(M)}}$ (lane 3) or $\mathrm{tRNA^{Asn}}_{-\mathrm{Q(Y)}}$ (lane 4) or no additional tRNA (lane 1). Following incubation, reactions were electrophoresed and the level of frameshifting determined as given (see Materials and Methods and Carlson *et al.*, 1999).

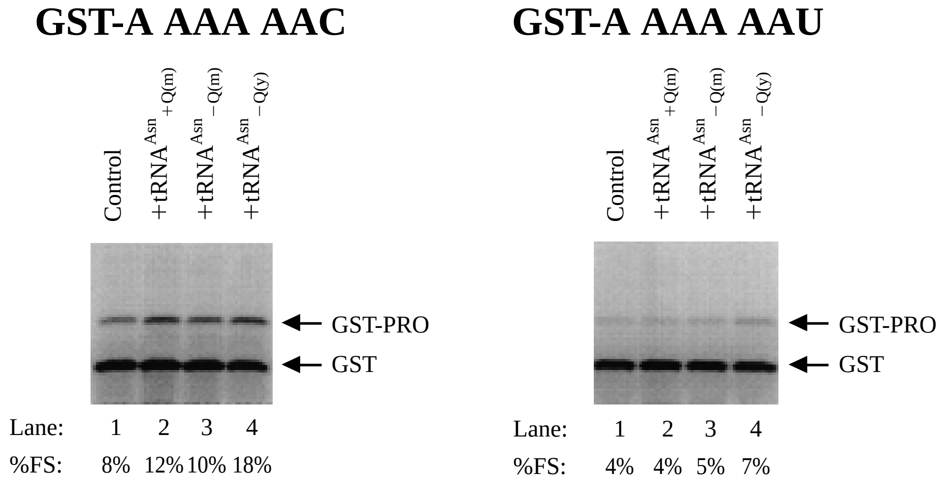

Fig. 2. Ribosomal frameshifting in wheat germ extracts supplemented with Q-containing and Q-deficient Asn tRNA. Wheat germ extracts were programmed with mRNA generated from constructs encoding GST-A_6C or GST-A_6U as shown and supplemented with $tRNA^{Asn}_{+Q(M)}$ (lane 2), $tRNA^{Asn}_{-Q(M)}$ (lane 3) or $tRNA^{Asn}_{-Q(Y)}$ (lane 4) or no additional tRNA (lane 1). Following incubation, reactions were electrophoresed and the level of frameshifting determined as given (see Materials and Methods and Carlson *et al.*, 1999).

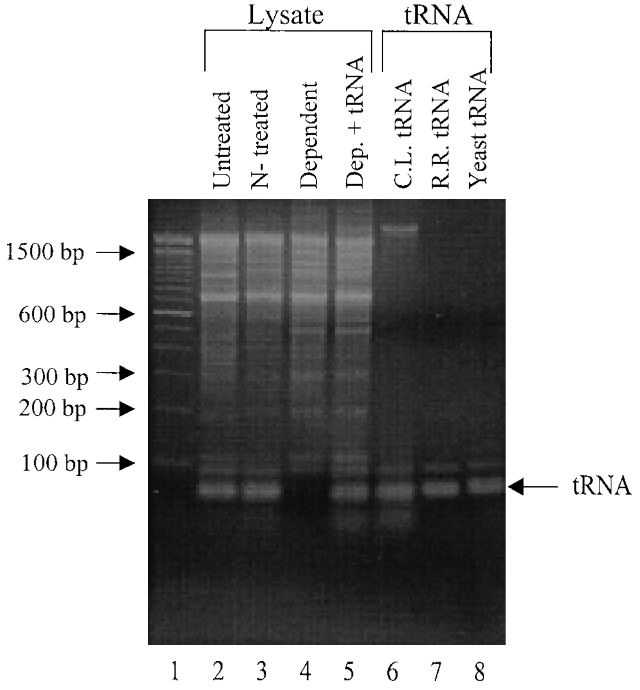

Fig. 3. Transfer RNA in rabbit reticulocyte lysates. RNA was extracted from untreated (lane 2) and nuclease treated lysates (lane 3), tRNA dependent lysates (lane 4) and tRNA dependent lysates supplemented with calf liver tRNA (lane 5), run on a 3% agarose gel and stained with EtBr. In addition, total calf liver tRNA from Sigma, total tRNA prepared from rabbit reticulocytes and total tRNA prepared from yeast (see Materials and Methods) are shown in lanes 6–8, respectively. Standard DNA MW markers (at 100 bp increments) are shown in lane 1.

The tRNA "depleted" lysates were dependent on exogenous tRNA to restore protein synthesis and were therefore supplemented with either calf liver tRNA or rabbit reticulocyte tRNA. The level of frameshifting was restored to that shown in Fig. 1 (data not shown). Although the level of frameshifting was not enhanced by further addition of either $tRNA^{Asn}_{+Q(M)}$ or $tRNA^{Asn}_{-Q(M)}$ (data not shown) the role of Q base in frameshifting cannot be assessed by these studies. About 10% of the Asn tRNA population in calf liver consists of $tRNA^{Asn}_{-Q(M)}$ (Hatfield *et al.*, 1979). Thus, a tRNA population fully Q-containing or fully Q-deficient is difficult to obtain from mammals. Although yeast Asn tRNA is totally devoid of Q, the shiftability of this tRNA in ribosomal frameshifting is not due, or at least not completely, to the absence of Q (see below).

We examined the distribution of Q-containing and Q-lacking Asn tRNAs in the protein synthesis systems used in this study. As shown in Fig. 4A, about 25% of the Asn tRNA population consists of a late eluting species in nuclease-treated lysates that were supplemented with calf liver tRNA. This late eluting species lacks Q base (Hatfield *et al.*, 1979), and presumably most of the Q-deficient Asn tRNA observed in lysates, comes from the endogenous reticulocyte tRNA population as this source of tRNA is known to be rich in Q deficient tRNAs (Hatfield *et al.*, 1979). Interestingly, Asn tRNA could be recovered from tRNA "depleted" lysates (Fig. 4A) even though virtually no tRNA was detected by gel electrophoresis (Fig. 3, lane 4). Thus, not all of the tRNA was removed from tRNA dependent lysates and the distribution of the two forms was similar to that observed in nuclease-treated-supplemented lysates. Furthermore, isolation of tRNA from tRNA

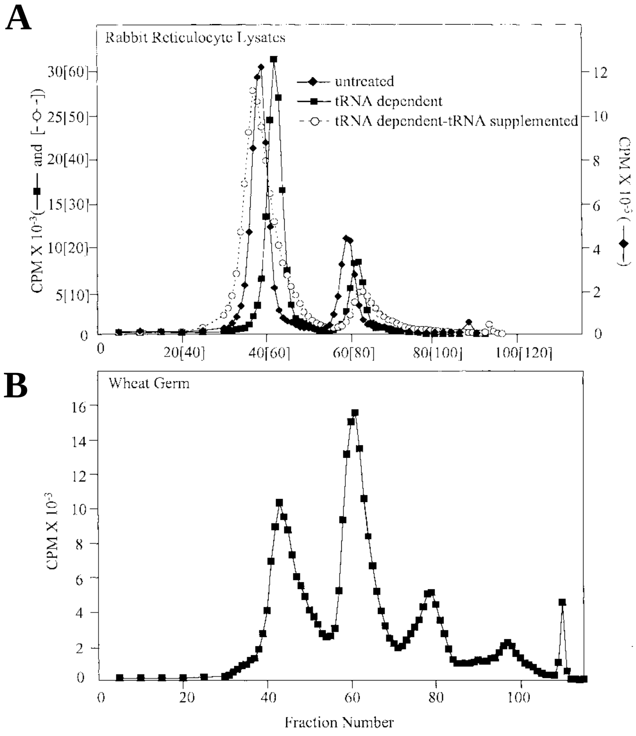

Fig. 4. Chromatography of Asn tRNAs from protein synthesis systems. Transfer RNA was isolated from 20 ml of untreated lysate, 20 ml of tRNA dependent lysate, 20 ml of tRNA dependent lysate that was supplemented with calf liver tRNA and 20 g of raw wheat germ and aminoacylated with ^{3}H-asparagine as given in Materials and Methods (see also Hatfield *et al.*, 1979; Smith and Hatfield, 1986). ^{3}H-Asparaginyl-tRNAs were chromatographed on a RPC-5 column in a 0.45 to 0.65 M NaCl gradient as described (Hatfield *et al.*, 1979), eluted fractions collected and counted in a Scintillation Counter and the resulting elution profiles from an untreated lysate (—♦—), from a tRNA dependent lysate (—■—), and from a tRNA dependent, calf liver supplemented lysate (—○—) are shown in A and from wheat germ extracts in B. Although the ^{3}H-asparaginyl-tRNAs from reticulocytes are plotted on the same graph, they were obtained from individual chromatographic runs on the column. The elution profile of ^{3}H-asparaginyl-tRNA from tRNA dependent, tRNA supplemented lysates was obtained on a separately packed RPC-5 column which retarded its elution as indicated by the fraction numbers in brackets.

dependent lysates that were supplemented with calf liver tRNA showed that this population contained both Q-containing and Q-deficient Asn isoacceptors (Fig. 4A). Wheat germ extracts also contained both Asn isoacceptors in high amounts (Fig. 4B).

Although it is tempting to speculate that Q base does not have a role in the frameshift event, we cannot conclude that this is true based on the protein synthesis data observed with the two forms of mammalian Asn tRNA. Clearly, the protein synthesis systems used are never completely devoid of the Q-containing or Q-deficient isoacceptor and one form may have a preference in decoding AAC. The work of Meier *et al.* (1985) clearly demonstrates that histidine tRNA lacking Q base from higher eukaryotes translates its cognate code word terminating in C (CAC) far more efficiently than its cognate code word terminating in U (CAU). Histidine tRNA containing Q base translated CAU/C with a slight preference for the U containing code word. These observations favor a model in which Q-deficient Asn tRNA promotes frameshifting more efficiently than the Q-containing form. Indeed, there must be a subtle translational mechanism operating at the frameshift site that accounts for the more slippery AAC than AAU codon. A logical explanation for a codon preference at the frameshift site is that the modified status of the decoding tRNA influences this process.

It can definitely be concluded from these studies that yeast Asn tRNA is a tRNA that stimulates frameshifting, and, thus, a tRNA lacking Q base promotes frameshifting. It is questionable, however, the extent to which Q base may play a role in this event. Since yeast and mammalian Asn isoacceptors differ in their primary structures, their primary structures are shown in Fig. 5. The anticodon loops are identical in $\text{tRNA}^{\text{Asn}}_{-\text{Q(Y)}}$ and $\text{tRNA}^{\text{Asn}}_{-\text{Q(M)}}$. Thus, the shiftiness of the yeast isoacceptor does not appear to be associated with the anticodon loop unless the anticodon stem or other regions of $\text{tRNA}^{\text{Asn}}_{-\text{Q(Y)}}$ render the anticodon more favorable to decoding AAU/C code words. Another important feature about yeast Asn tRNA is that it stimulates frameshifting with either AAU or AAC as the frameshift code word. This is unlike the natural decoding of the frameshift site in retroviruses that utilize Asn tRNA where AAC is strongly preferred. It is important to note that yeast Asn tRNA can stimulate ribosomal frameshifting in higher eukaryotes and the reason for the shiftability of this tRNA will likely elucidate some of the

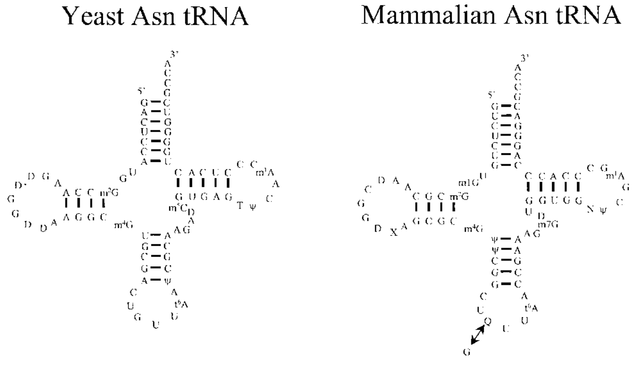

Fig. 5. Primary structures of yeast ($\text{tRNA}^{\text{Asn}}_{-\text{Q(Y)}}$) and mammalian Asn tRNAs ($\text{tRNA}^{\text{Asn}}_{+\ \&\ -\text{Q(M)}}$) shown in a cloverleaf model. Primary structures were taken from Sprinzl *et al.* (1989).

subtleties of translational regulation in higher eukaryotes. However, our focus of attention in future studies will be directed toward resolving the role of Q base in mammalian Asn tRNA and of Y base in mammalian Phe tRNA in retroviral ribosomal frameshifting. It should be emphasized that base modifications at position 37 in tRNA have a strong influence on retarding frameshifting and perhaps more so than modifications at position 34 (Bjork *et al.*, 1999; Farabaugh and Bjork, 1999). Hence, the Y base at position 37 in Phe tRNA may strongly retard ribosomal frameshifting, while Q base at position 34 in Asn tRNA may only have a moderate effect.

Acknowledgments We thank Dr. Y.-X. Feng for her help in obtaining the tRNA dependent rabbit reticulocyste lysates. This work was supported in part by the Molecular Medicine Research Group Program, Ministry of Science and Technology of Korea (B. J. L).

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