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Ribosomal Frameshifting in the Yeast Retrotransposon Ty: tRNAs Induce Slippage on a 7 Nucleotide Minimal Site

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Summary

Ribosomal frameshifting regulates expression of the *TYB* gene of yeast Ty retrotransposons. We previously demonstrated that a 14 nucleotide sequence conserved between two families of Ty elements was necessary and sufficient to support ribosomal frameshifting. This work demonstrates that only 7 of these 14 nucleotides are needed for normal levels of frameshifting. Any change to the sequence CUU-AGG-C drastically reduces frameshifting; this suggests that two specific tRNAs, $tRNA^{Leu}_{UAG}$ and $tRNA^{Arg}_{CCU}$, are involved in the event. Our tRNA overproduction data suggest that a leucyl-tRNA, probably $tRNA^{Leu}_{UAG}$, an unusual leucine isoacceptor that recognizes all six leucine codons, slips from CUU-Leu onto UUA-Leu (in the +1 reading frame) during a translational pause at the AGG-Arg codon induced by the low availability of $tRNA^{Arg}_{CCU}$, encoded by a single-copy essential gene. Frameshifting is also directional and reading frame specific. Interestingly, frameshifting is inhibited when the "slip" CUU codon is located three codons downstream, but not four or more codons downstream, of the translational initiation codon.

Introduction

Establishment of the translational reading frame in eukaryotes occurs during initiation of protein synthesis when the first AUG codon in the mRNA is located by a component of the scanning 40S initiation complex, $tRNA_i^{Met}$ (Cigan et al., 1988). Following 80S ribosome assembly, translation continues in 3 nucleotide steps with a very high degree of accuracy, reflecting the fact that maintenance of the translational reading frame is essential for useful gene expression. Expression of the *TYB* gene of yeast Ty retrotransposons occurs by disruption of this highly accurate translocation mechanism at levels approaching 50% (Clare et al., 1988; Wilson et al., 1986). How is this level of "inaccuracy" achieved?

The Ty1 and Ty2 elements are members of a family of retrotransposons found dispersed throughout the genome of the yeast Saccharomyces cerevisiae (Cameron et al., 1979). They consist of 0.33 kb terminal direct repeats called "delta" (δ) flanking a 5.3 kb internal region termed "epsilon" (ε). Along with retroviruses of higher eukaryotes (reviewed in Varmus, 1983), the *copia*-like elements of Drosophila species (Emori et al., 1985; Mount and Rubin, 1985), and L1Md of mice (Loeb et al., 1986), Ty elements are members of a family of elements that replicate via an RNA intermediate.

Encoded by Ty elements are two genes, *TYA* and *TYB*. Ty elements replicate by a retroviral-like mechanism (Boeke et al., 1985) within a virus-like particle encoded by the products of the *TYA* gene, the analog of the retroviral *gag* gene (Adams et al., 1987; Garfinkel et al., 1985; Mellor et al., 1985b). The *TYB* gene includes sequence homologies to retroviral *pol* genes, which encode the reverse transcriptase, integrase, and protease proteins. As with many avian and mammalian retroviral *pol* genes, expression of *TYB* requires ribosomal frameshifting (Clare et al., 1988). *TYB*, like *pol*, is expressed as a protein fusion to the product of the upstream gene, *TYA* (Clare and Farabaugh, 1985; Mellor et al., 1985a).

While retroviral frameshift events occur in the −1 direction in a region of overlap between the *gag*, *pol* and sometimes *pro* genes (Jacks et al., 1987, 1988b; Jacks and Varmus, 1985; Moore et al., 1987; Wilson et al., 1988), Ty frameshift events occur in the +1 direction in the 38–44 bp overlap between *TYA* and *TYB*. In fact, we recently showed that a 14 bp region of this overlap is necessary and sufficient to promote normal levels of ribosomal frameshifting (Clare et al., 1988). In Rous sarcoma virus (RSV), mouse mammary tumor virus (MMTV), and the avian coronavirus infectious bronchitis virus (IBV), RNA secondary structure in the form of a pseudoknot plays a critical role in the ribosomal frameshift event, presumably by causing the ribosome to stall at the site of frameshifting (Brierley et al., 1989; Jacks et al., 1987, 1988a; Moore et al., 1987). The region of frameshifting in Ty elements contains no obvious secondary structure. In addition, Ty elements lack the characteristic homopolymeric run of nucleotides ("slippery" sequences) at the site of frameshifting that characterizes frameshift sites of retroviruses and coronaviruses (Brierley et al., 1989; Jacks et al., 1988a). In these systems, a simultaneous slippage of tRNAs in the A and P sites of the translating ribosome on the homopolymeric sequence results in a frameshift to the *pro* or *pol* reading frame and suppression of the *gag* frame termination codon. This appears not to be the case in Ty elements.

Results

An In Vivo Assay for Frameshifting

All constructions involve the use of a *HIS4A::lacZ* fusion gene contained on a 2μm DNA–based plasmid whose construction is described in Experimental Procedures (Figure 1). This plasmid is present at four copies per cell (Farabaugh et al., 1989). Oligonucleotides containing various versions of the frameshift sequence were introduced

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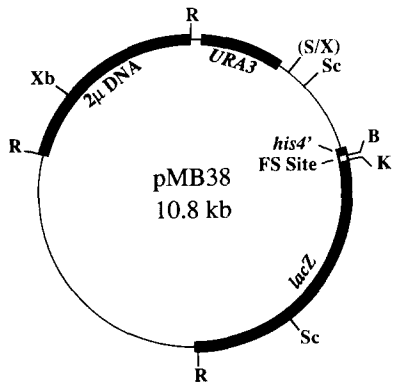


Figure 1. Plasmid pMB38

pMB38 is the base vector for all oligonucleotide constructions described in the text. B, BamHI; K, KpnI; R, EcoRI; Sc, SacI; (S/X), site formed by ligation of SalI to XhoI; FS Site, site of ribosomal frameshifting; *his4'*, 3' truncated *HIS4A* gene.

at the BamHI site of pMB25 and between the BamHI and KpnI sites of pMB38. Transcription from the *HIS4* promoter produces an mRNA with two overlapping genes: the 5' proximal gene derived from the first 100 nucleotides of the *HIS4A* gene and, in the +1 reading frame, the 3' proximal gene derived from the *lacZ* gene of Escherichia coli. Production of the *lacZ* gene product, β-galactosidase, depends upon a ribosomal frameshift event in the +1 direction within the sequences introduced on the oligonucleotides. The rate of frameshifting is measured by determining the ratio of β-galactosidase activity produced from a construct requiring a +1 frameshift to express *lacZ* to that of a construct in which the upstream and downstream genes are fused in frame. As shown below, the rate of frameshifting measured in this way is high, usually in the range of 40%; however, experimental variation in this rate occurs, both upward to the range of 60% and downward in the range of 20%. This variation results from unknown effects of the sequence context of individual constructions and slight variations in the physiology of yeast transformants. We will describe as abnormal only those transformants showing rates of frameshifting substantially lower than 20% (i.e., in the range of 2% or less).

The 14 Nucleotide Sequence Is the Site of Frameshifting

We previously demonstrated that a 14 nucleotide sequence conserved between the Ty1 and Ty2 families of Ty elements supports high levels of frameshifting (Clare et al., 1988). It is possible that the frameshift event occurs shortly before or after the 14 nucleotide sequence since neither the *TYA* nor the *TYB* reading frame is limited by translational termination codons in the region of overlap. In the construction used, the first *TYB* frame termination codon is 72 bp upstream of the overlap in *HIS4A* while the first *TYA* frame termination codon is 25 bp downstream in *lacZ*. An oligonucleotide was synthesized that contained the 14 nucleotide frameshift sequence flanked by termination codons, upstream in the *TYB* reading frame and downstream in the *TYA* reading frame (Table 7). The oligonucleotide was cloned at the BamHI site of pMB25 as described in Experimental Procedures and analyzed for its ability to promote frameshifting by assaying the expression of the downstream gene, *lacZ*. Expression of *lacZ* requires a +1 ribosomal frameshift within the sequence between the termination codons. Frameshifting is unaffected by the termination codons, occurring at levels of approximately 25% (data not shown). This result demonstrates that the 14 nucleotide sequence is the actual site of frameshifting.

The Frameshift Event Is Directional and Reading Frame Specific

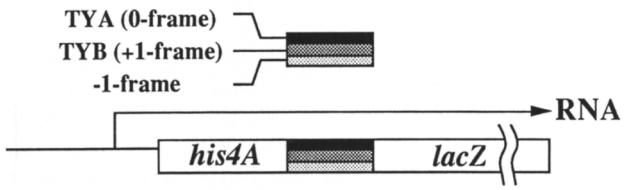
An open question in many of the site-specific frameshifting systems described in prokaryotes and eukaryotes is the degree to which the event is directional and reading frame specific. Is frameshifting constrained to occur in only one direction, or can it occur in either direction? Frameshifting in the human immunodeficiency virus (HIV-1) occurs only in the −1 direction (Wilson et al., 1988). Conversely, Weiss et al. (1987) demonstrated that ribosomes in E. coli may be made to frameshift both forward and backward within a sequence that incorporates a string of identical nucleotides. Shifts of −2, −1, +1, +2, +5, and +6 were identified. Will frameshifting occur only when the *TYA* reading frame is being translated by the ribosome? Changing the reading frame of E. coli frameshift sites decreases the frequency of frameshifting by about 100-fold (Weiss et al., 1987). Changing the frame disrupts both a required frame-specific pause induced by a nonsense codon, and a frame-specific tRNA slippage site. Removing the nonsense codon alone causes about a 10-fold decrease in frameshifting. This suggests that reading frame is important in the E. coli case, but not essential.

To test explicitly if frameshifting is reading frame specific or directional, we constructed a set of plasmids all of which had a single insert of the minimal region but designed such that translation comes into the region in each of the three reading frames and exits in each of the three reading frames (Table 1). To achieve all combinations required constructing nine plasmids. Each minimal region was flanked by termination codons to ensure that any frameshifting that occurred was taking place within the minimal sequence. These constructions were introduced into yeast and assayed for in vivo expression of β-galactosidase.

In three of the plasmids (pMB38-(0)fus, pMB38-(+1)fus, and pMB38-(−1)fus), the *HIS4A* and *lacZ* translational frames are fused. Two of these constructions give high levels of β-galactosidase activity (~3000 U; Table 1), but the construction in which translation occurs in the −1 frame of the minimal region expresses about 50-fold lower amounts. The latter construction has an in-frame UAG termination codon partway through the frameshift region, and thus translation terminates prematurely. Two −1 frameshift reporter constructs (pMB38-(0)−1, and pMB38-(+1)0) do not express significant amounts of β-galactosidase. We conclude that −1 frameshifts do not occur from either the 0 (defined as the wild-type or *TYA* reading

Table 1. Ty Frameshifting Occurs Only in the +1 Direction from the *TYA* Reading Frame

Construct		387-1D (sup+)		LO861 (*SUP4-3*, amber suppressor)	
Name	Diagram	β-galactosidase	% FS	β-galactosidase	% FS
pMB38-(0)fus		2800	n.a.	7200	n.a.
pMB38-(0)+1		1100	38	3500	48
pMB38-(0)-1		6	0.2	9	0.1
pMB38-(+1)fus		2900	n.a.	7200	n.a.
pMB38-(+1)-1		7	0.2	21	0.3
pMB38-(+1) 0		8	0.3	23	0.3
pMB38-(-1)fus		61	n.a.	2400	n.a.
pMB38-(-1) 0		11	n.a.	6	0.2
pMB38-(-1)+1		8	n.a.	30	1.3



frame) or +1 reading frame. By contrast, construct pMB-38-(0)+1, in which translation enters the minimal region in the 0 frame (*TYA*) and exits in the +1 frame (*TYB*), expresses high levels of β-galactosidase activity, indicating about 38% frameshifting as expected. Frameshifting from the +1 reading frame to the −1 reading frame (pMB38-(+1)−1) shows insignificant enzyme activity, indicating that +1 frameshifting does not occur from the +1 reading frame of the minimal sequence.

To test if frameshifting occurs in the +1 or −1 direction from the −1 reading frame, we transformed an amber suppressor strain, LO861, to allow readthrough past the in-frame UAG. In this background, the *TYA–TYB* +1 frameshift reporter plasmid, pMB38-(0)+1, promotes about 48% frameshifting (Table 1). Frameshifting in the +1 direction from the other two reading frames is not seen. Likewise, frameshifting in the −1 direction does not occur from any of the reading frames. We conclude from these results that frameshifting in the 14 nucleotide minimal region occurs only in the +1 direction and only from the 0, or *TYA*, reading frame.

Deletion Analysis Defines a Three Codon Minimal Frameshift Sequence

To determine whether all nucleotides of the 14 nucleotide frameshift site are required for frameshifting, a series of frameshift reporter constructs retaining increasingly smaller portions of the minimal sequence were made by synthesizing oligonucleotides that differ in length by single codons of the *TYA* reading frame. The remaining sequences in each oligonucleotide were flanked by termination codons to ensure that frameshifting occurs only within the sequence. The oligonucleotides were inserted into the *HIS4A::lacZ* frameshift reporter plasmid pMB25 at the BamHI site as described in Experimental Procedures. Frameshifting in the +1 direction will result in β-galactosidase production from the *lacZ* gene.

Table 2 shows the sequence of the deletion constructs as well as the results from each construct. Deleting the first two codons from the 3′ end of the 14 nucleotide sequence (including 5 nucleotides of the 14 nucleotide sequence) has no effect on frameshifting, demonstrating that only three codons from the 14 nucleotide sequence are necessary to direct frameshifting. No other Ty-derived sequences are present, yet frameshifting is occurring at wild-type levels. Deletion of an additional codon from the 3′ end or deletion of one or two codons from the 5′ end results in a much lower frequency of frameshifting (Table 2). All three constructs are at least 40-fold lower in expression than the plasmid retaining the first three codons of the 14 nucleotide sequence. Since no construction lacking any of the first three codons expresses significant levels of β-galactosidase, we conclude that the first three codons of the 14 nucleotide sequence, CUU-AGG-CCA, are necessary to direct frameshifting. This does not mean that each nucleotide is necessary, since the deletions do not allow us to map effects at a resolution of less than one codon.

Table 2. Deletion Analysis of the 14 Nucleotide Frameshift Site

Frameshift Site Sequence	Percent Frameshifting
GGAUCCGCUAGCACUUAGGCCAGGAACUUGAUCC	40
GGAUCCGCUAGCACUUAGGCCA——Δ——UGAUCC	40
GGAUCCGCUAGCACUUAGG——— Δ ———UGAUCC	0.8
GGAUCCGCUAGCA—Δ—AGGCCAGGAACUUGAUCC	0.7
GGAUCCGCUAGCA—— Δ —CCAGGAACUUGAUCC	≤0.1
AspProLeuAlaLeuArgProGlyThr***	
***HisLeuGlyGlnGluLeuAspPro	
GGAUCCGCUAGCACUUAGGCCAGGAACUUGAUCC	

Missense Mutagenesis Defines 7 Essential Nucleotides

The deletion analysis described above crudely defines the sequence that promotes frameshifting. To define which nucleotides are critical for frameshifting, we synthesized a series of nine oligonucleotides. Mixed synthesis was done with the nucleotides corresponding to the three desired mutations at each position of the 9 nucleotide minimal sequence defined above. The three mutations at each position were tested for their effect on frameshifting after cloning into the *HIS4A::lacZ* frameshift reporter plasmid pMB38 between the BamHI and KpnI sites as described in Experimental Procedures. The results are depicted graphically in Figure 2.

The nucleotide immediately 5′ of the 9 nucleotide minimal sequence is unimportant for frameshifting, as it can be any of the four nucleotides. The eighth and ninth nucleotides can also be changed to any base without affecting frameshifting. However, any change to the remaining 7 nucleotides drastically reduces frameshifting. The essential bases, CUU-AGG-C, include two codons of the *TYA* reading frame plus a seventh base. As can be seen from closer inspection of the data, some changes allow lower levels of frameshifting that are above background. C or G substitutions for U at the wobble position of the first codon allows approximately 2% frameshifting. Substitution of C or U for A at the first position of the second codon and replacement of G with U at the wobble position of the second codon also allow lower levels of frameshifting. The reason for the necessity of the seventh base is unknown but can probably be attributed to a context effect on decoding of the preceding codon. Context effects are known to involve the nucleotide immediately 3′ of a codon (Bossi, 1983; Bossi and Roth, 1980; Carrier and Buckingham, 1984; Fluck et al., 1977; Miller and Albertini, 1983; Murgola et al., 1984; Weiss, 1984; Weiss and Gallant, 1986).

The Sequence of the Frameshift Peptide Indicates That Frameshifting Occurs When Peptidyl-tRNA^{Leu} Is Bound to the CUU Codon and Does Not Involve Simultaneous Slippage

The 7 nucleotide minimal sequence defined above includes two unusual features. First, there are overlapping leucine codons (CUU and UUA) in the 0 and +1 reading frames. In nearly all organisms these two codons are decoded by distinct isoaccepting species. Yeast is unusual in that it encodes a tRNA, $tRNA^{Leu}_{UAG}$ (previously known as $tRNA^{Leu}_{1}$) that decodes all six leucine codons (Weissenbach et al., 1977). The expanded recognition requires an unmodified uracil at the wobble position of the anticodon (Randerath et al., 1979). Frameshifting could involve slippage of this tRNA between the two leucine codons. Second, the next codon, AGG (Arg), is recognized by a low-abundance tRNA encoded by a single nuclear gene, $tRNA^{Arg}_{CCU}$. Deletion of this gene is lethal to yeast (Gafner et al., 1983).

Two models can account for the necessity of the CUU

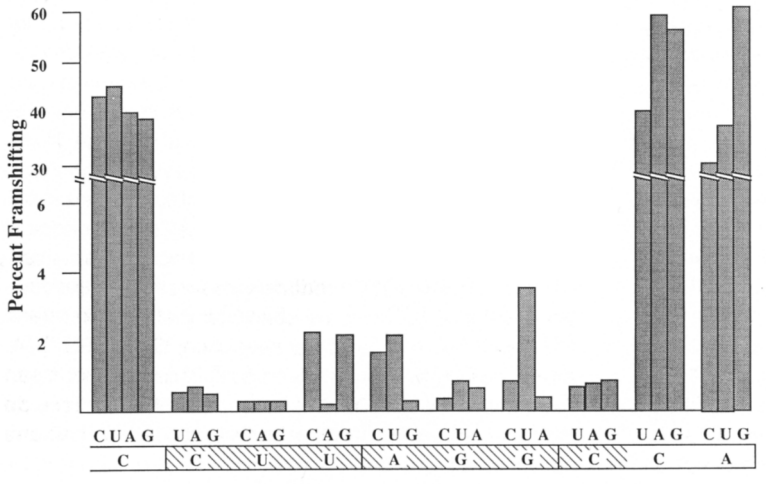


Figure 2. Results of Missense Mutagenesis Analysis of the Three Codon Minimal Frameshift Sequence

Shown boxed on the x-axis is the wild-type sequence of the frameshift site. The hatched portion indicates the essential nucleotides for frameshifting. Above each boxed nucleotide are the missense substitutions generated for each position of the sequence. The percent frameshifting for each substitution is shown graphically.

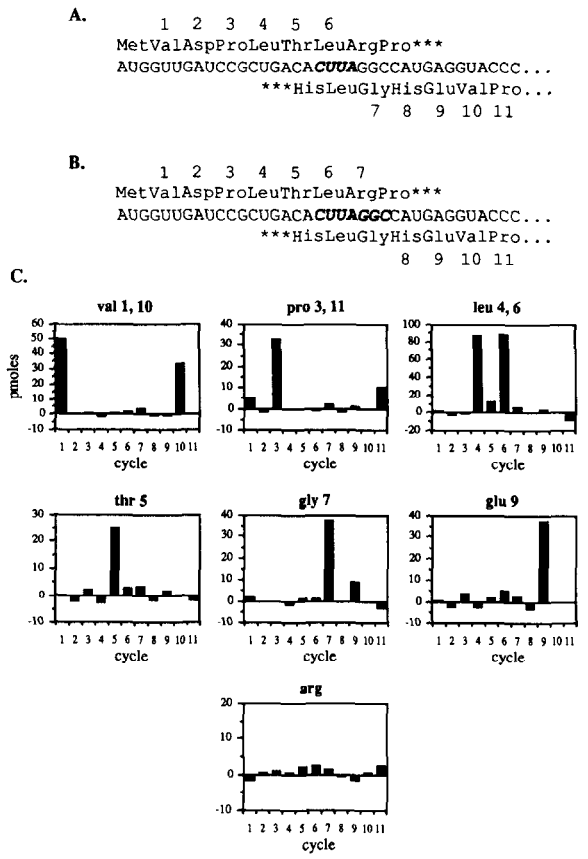


Figure 3. Predicted and Actual Peptide Sequences through the Frameshift Site

(A) Predicted amino acid sequence through the 7 nucleotide frameshift site (amino acids shown numbered) if Ty elements employ a peptidyl-tRNA slippage method of frameshifting as described in the text. A partial RNA sequence from the AUG codon is provided for reference.

(B) Predicted amino acid sequence through the 7 nucleotide frameshift site (amino acids shown numbered) if Ty elements employ the simultaneous-slippage method of frameshifting seen in retroviruses of higher cells. A partial RNA sequence from the AUG codon is provided for reference.

(C) Amino acid sequence data through the 7 nucleotide frameshift site. Histograms of relevant PTH-amino acids through 11 cycles of Edman degradation are shown with the major amino acid present in each cycle indicated above the histograms and above the mRNA sequence in (B). Cycle 8, which is predicted to yield His according to the RNA sequence, gave a weak signal, and no assignment was made. No other amino acid can be detected. The 14 nucleotide frameshift site has a Gln residue (rather than His) following Gly. The amino acid sequence through the 14 nucleotide site confirms the Gln residue after Gly (data not shown), supporting the notion that translation shifts to the +1 reading frame by peptidyl-tRNA slippage on the CUU-Leu codon.

and AGG codons. In RSV, HIV-1, MMTV, IBV, bovine leukemia virus (BLV), and the simian retrovirus type 1 (SRV-1), a simultaneous slippage of two tRNAs causes a −1 frameshift (Jacks et al., 1988a, 1988b; Wilson et al., 1988). The model requires that both the A and P sites be occupied simultaneously to accomplish the frameshift. This could be the case for Ty, yet only the simultaneous slip would be in the +1 direction from CUU-AGG to UUA-GGC. This would predict a protein sequence of Leu-Arg-His through the frameshift site (Figure 3B). Another model predicts that the ribosome encountering the AGG codon pauses because of the low availability of $tRNA^{Arg}_{CCU}$. In this state, with peptidyl-$tRNA^{Leu}_{UAG}$ in the P site and a vacant A site, the peptidyl-$tRNA^{Leu}_{UAG}$ slips from the CUU reading frame to the UUA (+1) reading frame. This places the GGC (Gly) codon in the A site. $tRNA^{Gly}_{GCC}$ enters the A site and translation continues in the +1 reading frame. This model predicts a protein sequence of Leu-Gly-His (Figure 3A).

To distinguish between the two above scenarios as well as other models, we determined the peptide sequence through the frameshift site. The *HIS4A::lacZ* fusion plasmid p3p has a BamHI site incorporated after the first two codons of the *HIS4A* gene. The construction is described in Experimental Procedures and depicted in Figure 3. The 9 nucleotide frameshift site, flanked by termination codons, was cloned at the BamHI site such that *lacZ* expression requires a +1 frameshift within the minimal sequence. After purification of the *HIS4A::lacZ* fusion product, the protein was subjected to 14 cycles of Edman degradation. The relevant PTH-amino acids through the first 11 cycles are shown in Figure 3C. The data clearly support the $tRNA^{Leu}_{UAG}$ slippage model. Glycine rather than arginine is incorporated after leucine, indicating that the frameshift event occurs on the CUU leucine codon. No significant amount of arginine is detected at cycle 7, ruling out a CUU-AGG simultaneous-slippage model of frameshifting. These data do not rule out simultaneous slippage on CUU and the codon immediately upstream of it; this is very unlikely since that codon is not from the Ty overlap, and can be changed at any position with no effect on frameshifting. Therefore, the most likely model is one involving slippage of a single tRNA bound to the CUU codon.

Frameshifting Occurs When the AGG Codon Is Unoccupied by Its Cognate tRNA

A correlation exists between the abundance of yeast tRNAs and the occurrence of their respective codons (Ikemura, 1982). The second codon (AGG) of the frameshift site is recognized by a low-abundance tRNA encoded by a single gene. As expected, the codon AGG is rare in yeast genes (Aota et al., 1988). The fact that the rate of aminoacyl-tRNA binding to the ribosomal A site is proportional to its concentration (Thompson et al., 1980) has been taken to mean that codons recognized by abundant tRNAs are decoded quickly, while codons recognized by nonabundant tRNAs are decoded slowly. Recent evidence suggests that translation rate is not strictly correlated with tRNA concentration, since some nonabundant tRNAs are decoded more quickly than some much more abundant tRNAs (Bonekamp et al., 1989). Though the rule that all low-abundance tRNAs decode slowly may not be universal, it is true that some codons are very slowly decoded. Interestingly, the $tRNA^{Arg}_{CCU}$ of E. coli, which is also a rare tRNA, has a low intrinsic decoding rate (Bonekamp and Jensen, 1988). It is possible that the very low abundance of $tRNA^{Arg}_{CCU}$ is necessary to promote frameshifting by causing a translational pause analogous to the function of the nonsense codon and "hungry" codons in E. coli frame-

Table 3. Overproduction of Specific tRNAs Abolishes Frameshifting

Plasmid	tRNA Gene	Copies	β-Galactosidase Activity (U)		Percent Frameshifting
			In Frame	+1 Frameshift	
pMB38-9merWT	none	NA	2800	1200	43
pMB38-9merWT-CCU	$tRNA^{Arg}_{CCU}$	5	2500	25	1.0
pMB38-9merWT-AAG	$tRNA^{Leu}_{AAG}$	4	4200	40	1.0
pMB38-9merWT-UAG	$tRNA^{Leu}_{UAG2}$	>4	6800	1400	21
pMB38-9merWT-UUA	$tRNA^{Leu}_{UUA}$	1	1200	270	23

shifts (Weiss et al., 1987, 1988). If this is true, then it may be possible to modulate frameshifting by modulating the amount of the rare tRNA. In particular, increasing the in vivo concentration of the tRNA should decrease the putative pause, and thus decrease the rate of frameshifting.

The gene for $tRNA^{Arg}_{CCU}$ was cloned onto a frameshift reporter plasmid, pMB38-9merWT, as described in Experimental Procedures. The copy number of this plasmid is approximately four per cell, increasing the total number of copies of the $tRNA^{Arg}_{CCU}$ gene to five per cell (including the one endogenous copy). The plasmid contains the *HIS4A*–9 nucleotide frameshift site–*lacZ* reporter gene, which normally shows 40% frameshifting (Table 3). The 5-fold increase in copy number of the $tRNA^{Arg}_{CCU}$ severely inhibited frameshifting (Table 3). The fact that increasing the concentration of $tRNA^{Arg}_{CCU}$ causes frameshifting to decrease is incompatible with any model in which the frameshift event is stimulated in part by binding of $tRNA^{Arg}_{CCU}$ to the ribosomal A site. Rather, $tRNA^{Arg}_{CCU}$ must "act" by being absent from the ribosome since increasing its ability to occupy the A site has a negative effect on frameshifting. We conclude that competition for the rare $tRNA^{Arg}_{CCU}$ induces a translational pause that is essential for the frameshift event and that frameshifting occurs when the ribosomal A site is unoccupied.

Frameshifting Depends upon tRNA Slippage between the CUU and UUA Leucine Codons

The missense mutagenesis data demonstrate that the CUU codon is essential for frameshifting (Figure 2). We wanted to test directly if frameshifting depended upon frame slippage by a peptidyl-$tRNA^{Leu}$. If $tRNA^{Leu}_{UAG}$ promotes frameshifting by frame slippage, then the rate of frameshifting should be directly related to the frequency that that tRNA is used to decode the CUU codon. If another isoaccepting species, incapable of frame slippage, were to compete with $tRNA^{Leu}_{UAG}$ for binding to the CUU codon, frameshifting would be reduced. Weiss et al. (1988) have proposed a different model for ribosomal frameshifting at "hungry" codons in E. coli. The model predicts that in a situation where the A site is unoccupied during a very long pause, a tRNA can bind out of frame, either +1 or −1, in violation of the normal frame maintenance mechanism. If this model were to operate in the Ty case, one would see the same peptide sequence at the frameshift site that we determined, Leu-Gly-His. However, the ability of the peptidyl-$tRNA^{Leu}$ to slip would be irrelevant.

To test the model of peptidyl-$tRNA^{Leu}$ slippage, we constructed three novel tRNAs bearing modified anticodons by modifying the anticodon of a gene for the most abundant isoacceptor, $tRNA^{Leu}_{CAA}$, also known as $tRNA^{Leu}_3$ (see Experimental Procedures). The first, with the anticodon AAG ($tRNA^{Leu}_{UAG}$), can decode CUU but is incapable of recognizing the overlapping UUA codon. The second has the anticodon UAG (termed $tRNA^{Leu}_{UAG2}$ to distinguish it from the wild-type $tRNA^{Leu}_{UAG}$), and the third is an ochre suppressor with the anticodon UUA ($tRNA^{Leu}_{UUA}$). The latter two serve as controls since neither is expected to interfere with frameshifting. Changing the anticodon should have no effect on charging of the tRNA by leucine aminoacyl-tRNA synthetase for two reasons. First, leucine is encoded by six codons, the only common feature of which is the central U residue. Second, functional amber and ochre suppressor forms of $tRNA^{Leu}$ exist (Sherman, 1982).

The tRNA genes were cloned into the plasmid pMB38-9merWT (see previous section) and introduced into yeast. The rate of frameshifting was drastically lower in the presence of $tRNA^{Leu}_{AAG}$, but not with $tRNA^{Leu}_{UAG2}$, while $tRNA^{Leu}_{UUA}$ had no effect (Table 3). The 43-fold decrease in frameshifting in the presence of $tRNA^{Leu}_{AAG}$ suggests that it has competed away 98% of the binding of a tRNA responsible for frameshifting. Although the data do not directly identify $tRNA^{Leu}_{UAG}$ as the "shifty" tRNA, they do demonstrate that the ability of the tRNA decoding the CUU codon to slip to UUA is essential for Ty ribosomal frameshifting.

Other Codons Decoded by Rare tRNAs Cannot Substitute for AGG

If the AGG codon of the frameshift site induces a translational pause as a result of the low availability of its cognate tRNA, is it possible to induce a translational pause by substituting a codon recognized by other low-abundance tRNAs? We replaced the AGG codon with two other codons known to be recognized by tRNAs present in low concentration: CGG, decoded by $tRNA^{Arg}_{CCG}$ (M. Culbertson and I. Edelman, personal communication); and UCG, decoded by $tRNA^{Ser}_{CGA}$ (Etcheverry et al., 1982; Olson et al., 1981). Like the $tRNA^{Arg}_{CCU}$ gene, the $tRNA^{Arg}_{CCG}$ and $tRNA^{Ser}_{CGA}$ genes are present in single copies in the haploid yeast genome and are lethal to the cell if deleted, reflecting the fact that no other tRNAs are capable of decoding the CGG or UCG codons.

Table 4. Other Codons Decoded by Rare tRNAs Cannot Substitute for the AGG "Pause" Codon

Sequence of Frameshift Site	β-Galactosidase Activity (U)	Percent Frameshifting
UGACACUUAGGCCAUGA	1200	43
UGACACUUCGGCCAUGA	50	1.8
UGACAUUUCGGCCAUGA	33	1.6
UGACACUUUCGCCAUGA	9	0.3
UGACAUUUUCGCCAUGA	2	<0.1

Oligonucleotides containing CGG or UCG at the AGG position of the frameshift site were cloned into the frameshift reporter plasmid pMB38 as described in Experimental Procedures. After introduction into yeast, the constructs were analyzed for β-galactosidase production. Both produced very low levels of enzyme, corresponding to 1.8% frameshifting for CGG and 0.3% frameshifting for UCG (Table 4). The CGG construct does support a significant level of frameshifting but still is about 25-fold below that of the wild-type construct with identical flanking sequences. A possible problem with this experiment is the elimination of the UUA leucine codon in the +1 reading frame. Perhaps $tRNA^{Leu}_{UAG}$ cannot slip from CUU to UUC or UUU, both phenylalanine codons recognized by a single isoacceptor, $tRNA^{Phe}_{GAA}$, also known as $tRNA^{Phe}$ (RajBhandary et al., 1967; Valenzuela et al., 1978). To control for this, oligonucleotides were constructed containing the same substitutions for AGG as above, but with only the first position C of the CUU codon changed to U. We reasoned that $tRNA^{Phe}_{GAA}$ should be able to shift from UUU to UUU or UUC of the +1 reading frame of each construct (Table 4) because the HIV-1 frameshift event can occur in the −1 direction on a string of 6 uracil residues in yeast (Wilson et al., 1988). As can be seen from the data, this change did not improve the ability of the sequences to support frameshifting (Table 4).

A more controlled experiment utilizes a well-characterized set of $tRNA^{Tyr}_{GUA}$ gene deletion constructs. The UAY codon is decoded by an abundant tRNA ($tRNA^{Tyr}_{GUA}$; also called $tRNA^{Tyr}$) encoded by eight unlinked genes in the haploid yeast genome (Olson et al., 1977). Burke (1988) demonstrated that the sequential deletion of each of the $tRNA^{Tyr}_{GUA}$ genetic loci results in a linear decrease in the pool of aminoacyl-$tRNA^{Tyr}$ within a cell. Deletion of up to four of the genes has no detectable phenotype; however, deleting five results in a 50% increase in doubling time, deleting six increases doubling time by 80%, and deleting seven is lethal. The increase in doubling time unambiguously indicates that cell growth is limited by the availability of the tRNA, suggesting that competition for the tRNA would be severe in at least those strains.

We introduced a frameshift reporter plasmid into strains containing two to eight copies of the $tRNA^{Tyr}_{GUA}$ genetic loci. This plasmid contained one of three versions of the 9merWT frameshift site: one with the wild-type sequence (CUU-AGG-CCA), one substituting a UAU tyrosine codon for AGG, or one substituting the codons UUU-UAU for CUU-AGG (Table 5). The second construct would require $tRNA^{Leu}_{UAG}$ to slip from CUU (Leu) to UUU (Phe). To make a slip more likely, the last construct requires $tRNA^{Phe}_{GAA}$ to slip from the 0 frame UUU codon to the +1 frame UUU codon to express *lacZ*. We reasoned that as the aminoacyl-$tRNA^{Tyr}_{GUA}$ pool drops with decreasing copies of the $tRNA^{Tyr}_{GUA}$ gene, the possibility of a translational pause at the UAU codon would increase because of the apparent severe competition for the tRNA. This pause may allow a frameshift event to occur at the upstream codon. The results are displayed in Table 5. Neither construct incorporating a UAU "pause" codon allows appreciable levels of frameshifting in any of the deletion strains, while the wild-type construct shows normal amounts of frameshifting. We conclude from these data that the fact that a codon is decoded by a limiting tRNA in itself is not sufficient to cause a translational pause capable of inducing detectable translational frameshifting.

Frameshifting Is Inhibited by Proximity to the Initiation Codon

We previously demonstrated that placing the 14 nucleotide frameshift site in immediate proximity to the translational initiation codon inhibits frameshifting (Clare et al., 1988). Placing the CUU codon of the frameshift site three codons downstream of the Ty2-917 AUG codon abolished frameshifting. When this codon was positioned 31 codons downstream, frameshifting was restored. This suppression could be a context effect around the Ty2-917 initiation codon (in which case the sequence around the initiation codon would suppress frameshifting even if positioned far downstream within a gene), or it could be an effect of initiation itself. To test the former possibility, we synthesized an oligonucleotide encompassing a region from 15 bp upstream of the Ty2-917 AUG, through the 14 nucleotide frameshift site (which was fused at the Ty2-917 AUG), and 15 bp downstream. The oligonucleotide, when inserted between a *TYA917* deletion 929 bp downstream of the initiation codon and the *lacZ* gene (in the +1 reading frame), supported normal levels of frameshifting (data not shown).

Table 5. Limiting Availability of $tRNA^{Tyr}$ Does Not Induce Frameshifting

Sequence at Frameshift Site	$tRNA^{Tyr}$ Gene Copy Number					
	7	6	5	4	3	2
UGACACUUUAUCCAUGA	0.1%	0.1%	0.1%	0.1%	0.2%	0.6%
UGACAUUUUAUCCAUGA	0.2%	0.1%	0.1%	0.1%	0.1%	<0.1%
UGACACUUAGGCCAUGA	33%	19%	20%	30%	34%	60%

Table 6. Frameshifting Is Inhibited When Too Near the Initiator AUG

Plasmid	Sequence of 5′ End of the Frameshift Reporter Gene	Percent Frameshifting
p2p	AUG · GAU · CCC · CUU · AGG · C	0
p3p	AUG · GUG · GAU · CCC · CUU · AGG · C	7
p4p	AUG · GUU · UUG · GAU · CCC · CUU · AGG · C	12
p5p	AUG · GUU · UUG · CCG · GAU · CCC · CUU · AGG · C	13
p6p	AUG · GUU · UUG · CCG · AUG · GAU · CCC · CUU · AGG · C	17
p7p	AUG · GUU · UUG · CCG · AUU · CUG · GAU · CCC · CUU · AGG · C	12

This suggests that proximity alone causes the suppression of frameshifting.

To determine the minimal distance between the AUG initiation codon and the 14 nucleotide frameshift site, we placed the frameshift site at increasingly farther codon intervals from the *HIS4A* initiation codon. BamHI sites were introduced in the *HIS4A* gene at codon intervals beginning at the second codon and ending at the seventh codon as described in Experimental Procedures. Introduction of the 14 nucleotide frameshift sequence into each of the six "proximity constructs" allowed a measure of frameshifting into the downstream *lacZ* gene. As we saw with the Ty2-917 construct, placement of the CUU codon of the frameshift site three codons downstream of the *HIS4* AUG in construction p2p resulted in no frameshifting (Table 6). However, construction p3p, which places the frameshift site at a position four codons downstream, shows appreciable levels of frameshifting, with frameshifting occurring at about 7%. Placement of the site five, six, seven, or eight codons downstream of the AUG codon showed frameshift activity at near wild-type levels. We hypothesize that the ribosome becomes competent to frameshift during the initial stages of elongation following initiation, apparently with an abrupt transition after the fourth codon of the gene.

Discussion

Translation initiation in eukaryotes occurs by a mechanism fundamentally different from that in prokaryotes. The ribosome binds to the 5′ end of the mRNA and "scans" to the initiation codon, usually the first AUG codon in the message. The AUG codon acts as a starting point for translation and establishes the reading frame of the message. Disruption of the reading frame occurs only rarely during elongation, but several instances of reading frame shifts have been observed. The phages f2 and MS2 (Beremand and Blumenthal, 1979; Kastelein et al., 1982) express their lysis genes through a mechanism that involves frameshifting within the upstream coat cistron. Spontaneous readthrough of leaky frameshift mutations occurs within the *oxi1* gene of yeast mitochondria (Fox and Weiss-Brummer, 1980). The addition of a carboxy-terminal extension onto the phage T7 major coat protein also occurs by a frameshift during translation (Dunn and Studier, 1983). Synthesis of the E. coli peptide release factor 2 (RF2) requires a +1 frameshift at an in-frame UGA codon (Craigen and Caskey, 1986; Craigen et al., 1985), while some retroviruses and coronaviruses of higher eukaryotes employ a −1 frameshift to express the products of their *pol* (or *pro*) and F2 genes, respectively (Brierley et al., 1987, 1989; Jacks et al., 1987, 1988a, 1988b; Jacks and Varmus, 1985; Wilson et al., 1988).

Frameshifting in the two best-studied systems, the RF2 gene of E. coli and retroviruses of higher eukaryotes, have several unifying features. Codon 26 of the RF2 gene of E. coli is an in-frame UGA termination codon, one of two codons recognized by RF2 (Scolnick and Caskey, 1969). The UGA codon is probably the site of an autogenous control system. Instead of prematurely terminating at the UGA codon, 50% of the ribosomes shift into the +1 frame at the position of the CUU (Leu) codon immediately preceding the UGA (Craigen and Caskey, 1986; Craigen et al., 1985). In an exhaustive study, Weiss et al. (1987, 1988) have defined the minimal requirements of frameshifting in E. coli. They find that the three things necessary for frameshifting to occur are a "slippery run," or repeat of several identical nucleotides in the RNA, followed immediately by a termination codon and preceded by a Shine–Dalgarno sequence (Shine and Dalgarno, 1974) a precise distance away. Constructs were made that frameshifted −2, −1, +1, +2, +5, and +6 with varying efficiency. The proposed mechanism involves binding of a tRNA in the "slippery run." A translational pause, induced by the slow process of termination at the nonsense codon, allows an interaction between the Shine–Dalgarno-like sequence upstream of the frameshift site and the 16S RNA. The interaction "pulls" or "pushes" the ribosome, causing the tRNA to slip along the slippery run. Translation then continues in the new frame.

The mechanism of −1 frameshifting in retroviruses has been shown to involve both a specific "slippery" sequence and, in some cases, a conserved secondary structure. Jacks et al. (1988a) showed that three subsets of repetitive nucleotides conserved among retroviruses constitute the sites of frameshifting. Protein sequence analysis and site-directed mutagenesis of the *gag–pro* fusion junction from MMTV (Jacks et al., 1987), HIV-1 (Jacks et al., 1988b; Wilson et al., 1988), and RSV (Jacks et al., 1988a) demonstrated that the event occurs at these repetitive sequences: in MMTV at the sequence A-AAA-AAC (shown as *gag* frame codons), in HIV-1 at the sequence U-UUU-UUA, and in RSV at the sequence A-AAU-UUA. Similar sequences from IBV, BLV, and SRV-1 can also support frameshifting (Jacks et al., 1988a). Most retrovirus frameshift events also require a conserved pseudoknot

structure located immediately downstream of all retroviral frameshift sites (Jacks et al., 1988a), a requirement also seen in IBV frameshifting (Brierley et al., 1989). Notably, HIV-1 does not require its pseudoknot structure for frameshifting (Jacks et al., 1988a; Wilson et al., 1988). This pseudoknot structure may be required to induce a translational pause (Jacks et al., 1988a). Like the translational pause in the E. coli case, the pause induced by the pseudoknot allows frameshifting to occur on the slippery sequence. Unlike E. coli, frameshifting appears to involve two tRNAs that recognize two codons of the slippery sequence in the *gag* reading frame. Jacks et al. (1988a) showed that the frameshift event involves a simultaneous slippage of these tRNAs in the ribosomal A and P sites. Because certain changes are allowed in the A and P site codons and because several different sites exist (see above), more than one tRNA (termed "shifty" tRNAs) is competent to frameshift.

Frameshifting in Ty elements initially appeared to lack all of the features identified in other frameshift sites. No homopolymeric string of nucleotides is necessary in the region of the frameshift event. Frameshifting requires neither a nearby termination codon nor a downstream RNA secondary structure to induce a translational pause. Previously, we showed that a 14 nucleotide sequence is necessary and sufficient to induce ribosomal frameshifting even when placed within a completely heterologous context, the *HIS4* gene of yeast (Clare et al., 1988). RNA sequencing of an mRNA containing this frameshift site demonstrated that the DNA and RNA sequences are exactly colinear, ruling out any pretranslational mechanism.

In this report we show that frameshifting occurs within the 14 nucleotide sequence. Since the sequence is flanked with termination codons, upstream in the *TYB* reading frame and downstream in the *TYA* reading frame, frameshifting is confined to occur within the window between the termination codons. Expression of the downstream *lacZ* gene, in the +1 reading frame, is unaffected by the termination codons. We show that the frameshift event is directional and reading frame specific. Three different constructs allow translation to occur into each of the three reading frames of the frameshift site. For each construct, the *lacZ* gene is fused downstream in all three reading frames. This allows an assay for frameshifting from each of the reading frames in either the +1 or −1 direction. Translation into the frameshift sequence in the 0, or *TYA*, reading frame (the normal reading frame of the frameshift site) allows wild-type levels of frameshifting in the +1 direction. However, translating the +1 or −1 reading frames allows no frameshifting in the +1 direction, demonstrating the reading frame specificity of frameshifting. Frameshifting does not occur in the −1 direction from any of the three reading frames, indicating that frameshifting is directional.

A Model for Ty Frameshifting

Deletion analysis and missense mutagenesis define 7 nucleotides essential for frameshifting. This sequence, CUU-AGG-C, has some interesting characteristics. Overlapping leucine codons, CUU in the 0 frame and UUA in the +1 frame, are recognized by a single isoacceptor in yeast, $tRNA^{Leu}_{UAG}$. This tRNA can recognize all six of the leucine codons by virtue of an unmodified uracil in its wobble position (Randerath et al., 1979; Weissenbach et al., 1977). Modified uracil residues recognize only purines, whereas an unmodified uracil can recognize all four bases (Heckman et al., 1980; Sibler et al., 1986); unmodified uracil residues (and consequently, recognition of four nucleotides) is common in mitochondrial tRNAs (Barrell et al., 1980; Bonitz et al., 1980; Heckman et al., 1980; Sibler et al., 1986), where a much smaller number of tRNAs are sufficient to translate all the codons of the mitochondrial genome. This characteristic makes this tRNA an ideal candidate for a shifty tRNA, as it is a major isoacceptor for leucine (present at 48% the level of the most abundant isoacceptor, $tRNA^{Leu}_{CAA}$; Ikemura, 1982) and satisfies wobble rules in both reading frames. The second unique characteristic of this sequence is the AGG codon. This codon is recognized by a low-abundance tRNA, $tRNA^{Arg}_{CCU}$, a single-copy essential gene.

These two characteristics suggest a model for frameshifting that retains features of frameshift sites in other systems. A slippery sequence, CUU in the 0 frame to UUA in the +1 frame, is possible because of the unique $tRNA^{Leu}_{UAG}$. A translational pause could be induced by the low-abundance $tRNA^{Arg}_{CCU}$. Pausing and frameshifting at "hungry" codons, codons whose cognate tRNA is in short supply, occurs in other systems (Spanjaard and van Duin, 1988; Weiss et al., 1988) and, in Ty elements, may satisfy the apparent requirement for a translational pause to induce frameshifting. The model, diagrammed in Figure 4, states that ribosomes encountering the AGG codon pause because of the low availability of $tRNA^{Arg}_{CCU}$. Peptidyl-$tRNA^{Leu}_{UAG}$, in the P site of the ribosome (on the CUU codon), slips forward +1 during the pause onto the UUA codon. Translation continues in the +1 reading frame.

We have provided experimental evidence to support this hypothesis. Peptide sequencing through the frameshift site indicates frameshifting at the CUU codon, eliminating the simultaneous-slippage model of frameshifting seen in retroviruses. Overproduction of $tRNA^{Arg}_{CCU}$ decreases frameshifting approximately 43-fold, presumably by eliminating the translational pause at the AGG codon. Since $tRNA^{Arg}_{CCU}$ is now abundant in this background, translation continues in the 0 frame and terminates immediately downstream. This suggests that a translational pause is an essential component of the frameshift event in Ty as it seems to be in other systems. Significantly, a high copy number suppressor of Ty transposition maps to the $tRNA^{Arg}_{CCU}$ gene, a result that directly supports the importance of frameshifting in the expression of the *TYB* gene and hence in transposition (H. Xu and J. D. Boeke, personal communication). A second result implicates $tRNA^{Leu}_{UAG}$ as the slippery tRNA. Since this tRNA may be the only tRNA that can decode CUU, we reasoned that overproduction of a second tRNA that decodes CUU exclusively would compete with $tRNA^{Leu}_{UAG}$ and reduce frameshifting. This second tRNA would not be expected

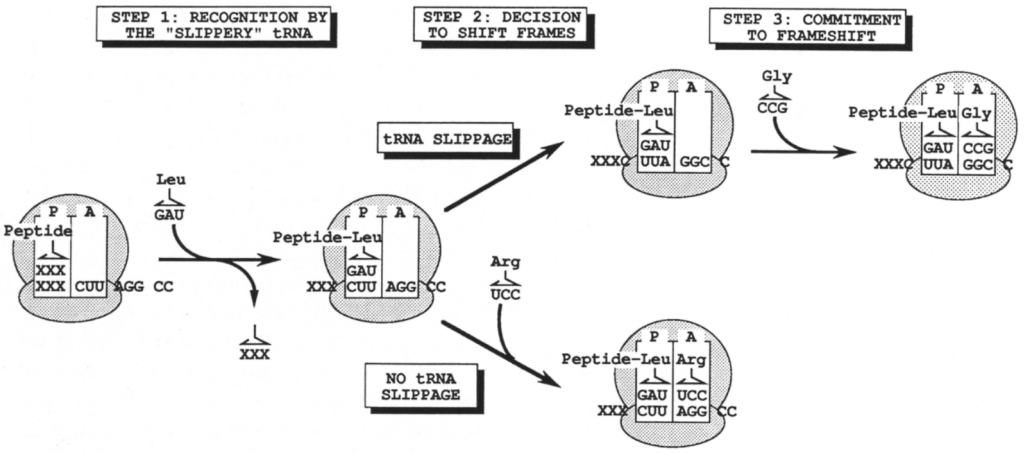


Figure 4. Frameshifting in Ty Elements Occurs in Three Steps

The first step necessitates recognition of the CUU codon by $tRNA^{Leu}_{UAG}$. Owing to the low availability of $tRNA^{Arg}_{CCU}$, a translational pause at the AGG codon allows time for a frameshift to occur at step 2. The commitment to frameshifting occurs at step 3 when the next +1 frame codon is recognized by its cognate tRNA.

to be able to slip onto UUA. Changing the anticodon of $tRNA^{Leu}_{CAA}$ to create $tRNA^{Leu}_{AAG}$ and overproducing this tRNA results in a 43-fold inhibition of frameshifting. Clearly, this result demonstrates the importance of $tRNA^{Leu}_{UAG}$ in the frameshift event.

As indicated by the missense mutagenesis data, some changes in the 7 nucleotide sequence allow frameshifting at low levels. These changes allow frameshifting at levels of approximately 20- to 30-fold below that of the wild-type sequence. It is difficult to ascertain why frameshifting can occur in some of these cases; however, the two cases showing the highest levels of frameshifting may share some features with the wild-type sequence. The A to C change at the first position of the second codon produces a CGG arginine codon in the 0 frame and a UUC phenylalanine codon in the +1 frame. CGG is a codon recognized by a rare tRNA, $tRNA^{Arg}_{CCG}$. A possible reason why this sequence does not support higher levels of frameshifting is seen in the codon usage pattern for CGG. CGG appears at a level 7-fold below that of AGG in yeast genes (Aota et al., 1988). The lower demand for $tRNA^{Arg}_{CCG}$ could reduce the length of the translational pause needed for frameshifting. This should result in a lower proportion of frameshift events occurring before translation continues by insertion of arginine at the in-frame CGG codon. The G to U change at the third position of the second codon retains the CUU to UUA slippery sequence but changes the AGG-Arg codon to AGU-Ser. AGU must also allow a shorter pause; the tRNA that decodes it has not been identified, so we do not know if it is a rare or abundant tRNA.

Thus far, attempts to create a frameshift site by replacing the AGG codon with other codons recognized by nonabundant tRNAs have failed. An experiment that definitively tests this hypothesis uses a family of strains retaining from eight to only two of the $tRNA^{Tyr}_{GUA}$ genes, the expectation being that when the cell is limited for tRNA decoding the two tyrosine codons, competition for the aminoacyl-tRNA will be severe and a substantial translational pause should result. The fact that this, too, failed forces us to conclude either that competition for the $tRNA^{Arg}_{CCU}$ is especially severe or that the translational pause induced by competition for the tRNA is not the only function of the AGG codon, the second role not being satisfied by the other codons tested. A more exhaustive search for possible substitute "pause" codons may determine which possibility is correct.

Remarkably, frameshifting in Ty elements is inhibited by the proximity of the frameshift site to the translational initiation codon. This phenomenon was first observed by placement of the 14 nucleotide frameshift site within four codons of the initiation codon of Ty2-917 (Clare et al., 1988). Frameshifting was completely inhibited. Similarly, placement of the site the same distance from the *HIS4A* initiation codon inhibits frameshifting. Addition of another codon between the initiation codon and the frameshift site restores frameshifting. This proximity effect has not been observed or examined in other systems and may be a general phenomenon. We hypothesize that translation immediately after initiation might be fundamentally different from that during the elongation phase. This difference could perturb the ribosome's frameshifting ability. Experiments are under way to understand what elements determine the ribosome's ability to frameshift early in elongation.

Experimental Procedures

Yeast Strains, Media, and General Methods

The S. cerevisiae strain used for this work unless otherwise indicated is 387-1D (α *his4Δ38 ura3-52 trp1-289 HOL1-1*). Analysis of directionality plasmids was also done in strain LO861 (**a** *his1-7,8 lys1-1-o met8-1-am sup4-3 ura3-52*), kindly provided by Gerald Fink. This strain carries a temperature-sensitive amber suppressor, suppressing at 30°C but not at 37°C. Analysis of tyrosine codon substitutions was done in strains kindly provided by Maynard Olson. The strains are deleted for one to six copies of the eight $tRNA^{Tyr}_{GUA}$ genetic loci (*sup2-8, 11*) and have the following genotype: *MAT***a** or α *ade2-1-o lys2-1-o trp5-2-o leu1-12 can1-100-o ura3-1 met4-1-o* (Burke, 1988). Strain AB1143 is deleted for *sup8*;

Table 7. Oligonucleotides

Oligonucleotide	Sequence
Flanking termination codons: top strand	GATCCGCTAGCCCTTAGGCCAGGAACTT BamHI
Flanking termination codons: bottom strand	GATCAAGTTCCTGGCCTAAGGGCTAGCG
MB38-(0)fus	GATCCGATGCTTAGGCCAGGAACTGAGGTAC BamHI KpnI
MB38-(0)+1	GATCCGATGAACCTTAGGCCAGGAACCTGAGGTAC
MB38-(0)-1	GATCCGATGCATGAACTTAGGCCAGGAACCTGACGGTAC
MB38-(+1)fus	GATCCGATGCACTTAGGCCAGGAACCTGAGGTAC
MB38-(+1)-1	GATCCGATGCTGAACTTAGGCCAGGAACACTGAGGTAC
MB38-(+1) 0	GATCCGATGCATGACTTAGGCCAGGAACACTGACGGTAC
MB38-(-1)fus	GATCCGATGACTTAGGCCAGGAACACTGAGGTAC
MB38-(-1) 0	GATCCGATGACTTAGGCCAGGAACTGAGGTAC
MB38-(-1)+1	GATCCGATGCATGAACCTTAGGCCAGGAACTGACGGTAC
Codon Δ1-3' top	GATCCGCTAGCACTTAGGCCAT
Codon Δ1-3' bottom	GATCATGGCCTAAGTGCTAGCG
Codon Δ2-3' top	GATCCGCTAGCACTTAGGT
Codon Δ2-3' bottom	GATCACCTAAGTGCTAGCG
Codon Δ1-5' top	GATCCGCTAGCAAGGCCAGGAACTT
Codon Δ1-5' bottom	GATCAAGTTCCTGGCCTTGCTAGCG
Codon Δ2-5' top	GATCCGCTAGCACCAGGAACTT
Codon Δ2-5' bottom	GATCAAGTTCCTGGTGCTAGCG
9mer-WT	GATCCGCTGACACTTAGGCCATGAGGTAC
9mer-Fusion	GATCCGCTGACACTTAGGCCACGTGAGGTAC
9mer-CGG	GATCCGCTGACACTTCGGCCATGAGGTAC
9mer-TTT-CGG	GATCCGCTGACATTTCGGCCATGAGGTAC
9mer-TCG	GATCCGCTGACACTTTCGCCATGAGGTAC
9mer-TTT-TCG	GATCCGCTGACATTTTCGCCATGAGGTAC
9mer-TAT	GATCCGCTGACACTTTATCCATGAGGTAC
9mer-TTT-TAT	GATCCGCTGACATTTTATCCATGAGGTAC
Sal-upstream	GGGGGTCGACCTTTCTCATAATCAACCCAC SalI
2p	CCCCGGATCCATTATTCAGAAAAAAAA BamHI
3p	CCCCGGATCCACCATTATTCAGAAAAA
4p	CCCCGGATCCAAAACCATTATTCAGAA
5p	CCCCGGATCCGGCAAAACCATTATTCA
6p	CCCCGGATCCATCGGCAAAACCATTAT
7p	CCCCGGATCCAGAATCGGCAAAACCAT
Sal-downstream	TGTTATGAACTTCAGCTGTTATAA SalI
ENX-upstream	GCGCGCGAATTCCATATGCTCGAGGAGAACTTCTA EcoRI NdeI XhoI
NarI-opposite strand	TTAATTGGCGCCTTAGACCGCTCGGC NarI
AAG	TATATAGGCGCCTGATTAAGGAAATATCTTGACCG
UAG	TATATAGGCGCCTGATTUAGGAAATATCTTGACCG
UUA	TATATAGGCGCCTGATTUUAGAAATATCTTGACCG

Relevant restriction sites are indicated by underlining and labeled by name. The position of the $tRNA^{Leu}$ anticodon is double underlined. All oligonucleotides are displayed 5' to 3'.

AB1144 for *sup8* and *11*; AB1145 for *sup2, 8,* and *11*; AB1146 for *sup2, 3, 8,* and *11*; AB1147 for *sup2, 3, 4, 8,* and *11*; and AB1148 for *sup2, 3, 4, 5, 8,* and *11*. All strains were grown in SD minimal medium supplemented with the appropriate amino acids to allow selection for *URA3*-containing plasmids (Sherman et al., 1986). DNA transformations of yeast were performed by the lithium acetate method (Ito et al., 1983). Oligonucleotides were synthesized on a Biosearch Cyclone DNA synthesizer (Millipore).

Plasmid Constructions and Oligonucleotides

The plasmid pMB38 is shown in Figure 1. This plasmid contains the *URA3* gene and the 2μm origin of replication from pLGSD5 (Guarente et al., 1982), and a 5' truncated *lacZ* gene from pMC1790 (Casadaban et al., 1983); the construction of this portion of the plasmid has been described (Liao et al., 1987). The *HIS4A* gene was introduced as a 1.2 kb SalI–BamHI fragment between the XhoI and BamHI sites of pNoUp (Liao et al., 1987). Replacement of the BamHI–SacI *lacZ* fragment with the complementary fragment containing the BamHI–KpnI linker at the 5' end of *lacZ* creates pMB38. The *lacZ* gene is fused via a BamHI–KpnI linker to the *HIS4A* gene 33 codons downstream of the *HIS4A* initiation codon. Missense, codon substitution, and directionality oligonucleotides containing the 9 bp and 14 bp frameshift sites were introduced between the BamHI and KpnI sites as described by Derbyshire et al. (1986). The sequences of directionality oligonucleotides are shown in Table 7. Missense and codon substitution oligonucleotides have the following general sequence: 5'-GATCCGCTGACACTTAGGCCA-TGAGGTAC-3'. This oligonucleotide contains 5' and 3' overhangs complementary to BamHI and KpnI half-sites, respectively. This allowed cloning into pMB38 as described above. The underlined region was subjected to missense mutagenesis as described in the text or changed to specific nucleotides as described in the section on codon substitutions (see Table 7). An oligonucleotide identical to this except for an addition of 2 nucleotides following the underlined sequence fuses the upstream and downstream reading frames in frame. This oligonucleotide (called 9mer-fusion; Table 7) allows for quantitation of the level of frameshifting. Top and bottom strands of flanking terminator and codon deletion oligonucleotides, depicted in Table 7, were annealed and cloned into the BamHI site of pMB25 as described in Clare et al. (1988). pMB25 is identical to pMB38 except that it lacks the KpnI site at the junction of *HIS4A* and *lacZ*.

Construction of "proximity" mutations in the *HIS4A* gene was done by utilizing the polymerase chain reaction (PCR). An oligonucleotide, Sal-upstream (see Table 7), of which the 3' 20 nucleotides are complementary to the noncoding strand of the *HIS4A* gene, primes synthesis of the coding strand beginning 708 nucleotides upstream of the protein coding region (Donahue et al., 1982). Downstream, six oligonucleotides, of which the 3' 20 nucleotides are complementary to the coding strand, prime synthesis of the noncoding strand at codon intervals immediately after the initiation codon. The sequences of these oligonucleotides (called 2p–7p) are listed in Table 7. PCR reactions were done as described by the manufacturer (Cetus Corporation) on plasmid pSal1, which contains the complete *HIS4* gene. The upstream oligonucleotide incorporates a SalI restriction site with the downstream oligonucleotides incorporating a BamHI site. The PCR-generated fragments were digested with SalI and BamHI and cloned into the XhoI and BamHI sites of pNoUp. Subsequently, the BamHI–SacI *lacZ* fragment of pNoUp was replaced by either the BamHI–SacI *lacZ* fragment of pMB38-9merWT or the BamHI–SacI *lacZ* fragment from pTy1-912Δ1225-CO (Clare et al., 1988) to incorporate the 9 bp and 14 bp frameshift sites, respectively.

The plasmid used to overexpress $tRNA^{Arg}_{CCU}$ was pMB38-9merWT. This plasmid contains the missense oligonucleotide depicted above with the wild-type 9 nucleotide frameshift site. The unique TtHI11I site was blunted with DNA polymerase I large fragment and joined to SalI 8-mer linkers (New England Biolabs). The plasmid HI3 (Gafner et al., 1983), the kind gift of Peter Philippsen, contains a 2.15 kb XhoI fragment carrying the gene for $tRNA^{Arg}_{CCU}$. This XhoI fragment was cloned into the SalI site of pMB38-9merWT to create the plasmid pMB38-9merWT-$tRNA^{Arg}_{CCU}$.

The tRNA genes encoding $tRNA^{Leu}_{AAG}$, $tRNA^{Leu}_{UAG2}$, and $tRNA^{Leu}_{UUA}$ were overexpressed on the same pMB38-9merWT plasmid. A gene for $tRNA^{Leu}_{CAA}$ is found immediately upstream of the *LEU2* gene. PCR was performed on a plasmid bearing the 2.2 kb XhoI–SalI fragment carrying *LEU2* using the Sal-downstream and ENX-upstream primers (see Table 7). The product was a 548 bp fragment with EcoRI, NdeI, and XhoI sites at the upstream end of the tRNA fragment and a SalI site at the downstream end. NdeI- and SalI-cleaved fragment was cloned into pUC13 between the unique NdeI and SalI sites. The resulting clone lacks the unique NarI site of pUC13, but introduces a unique NarI site in the insert starting 11 bp upstream of the anticodon of $tRNA^{Leu}_{CAA}$. To construct the three codon replacements of $tRNA^{Leu}_{CAA}$, we adapted a PCR procedure in which a whole plasmid is amplified using two adjacent, divergent primers (Hemsley et al., 1989). The NarI-opposite strand primer (Table 7) primes synthesis from the unique NarI site in

the direction away from the $tRNA^{Leu}_{CAA}$ anticodon. Three primers incorporating the novel anticodons, the AAG, UAG, and AAU primers (Table 3), prime synthesis from the NarI site in the opposite direction. The product is a linear form of the plasmid with NarI sites at either end carrying the novel anticodons. The product is digested with NarI and recircularized to recover the plasmids bearing the novel tRNA genes. The XhoI–SalI fragment encompassing each gene was transferred into the SalI site of pMB38-9merWT, and isolates of each in which the tRNA gene is oriented pointing toward the 3′ end of the *lacZ* gene of the plasmid were selected. These plasmids are pMB38-9merWT-$tRNA^{Leu}_{AAG}$, pMB38-9merWT-$tRNA^{Leu}_{UAG2}$, and pMB38-9merWT-$tRNA^{Leu}_{UUA}$.

β-Galactosidase Purification and Protein Sequencing

The protocol for β-galactosidase purification from yeast was provided by Robert Weiss and is essentially as described (Weiss et al., 1989). Yeast strain 387-1D, transformed with plasmid p3p-9merWT, was grown at 30°C in 18 liters of SD minimal medium supplemented with 20 mg/l of histidine and tryptophan and 2% glucose. At a culture density of $OD_{600} \approx 1$, the cells were pelleted, washed with one-fourth volume of buffer A (50 mM Tris–HCl [pH 7.4], 150 mM NaCl, 5 mM EDTA, 0.1% Tween 20, 10 mM β-mercaptoethanol, and 0.5 mM PMSF), and then resuspended in the same buffer (3 ml of buffer per gram of cells). Cells were broken using 10 g of 400 μm glass beads (Sigma) per 4 ml of suspension by vortexing for 4 min with 1 min incubations on ice between each minute of vortexing. Cell debris and beads were pelleted by centrifugation at 2000 × g for 15 min. The resulting supernatant was then centrifuged for 90 min at 100,000 × g in a Beckman TL100 centrifuge to pellet organelles. A clear supernatant was collected and passed over an anti-β-galactosidase immunoaffinity column (Protosorb, Promega Biotec) equilibrated with buffer A. The eluate was concentrated with a Centricon 30 centrifuge chamber (Amicon), washed four times with HPLC-grade water, and then placed in the cartridge of an Applied Biosystems 475A protein sequencer equipped with an on-line Applied Biosystems 120A high performance liquid chromatography analyzer.

β-Galactosidase Assay and DNA Sequencing

Three transformants of each plasmid were each assayed in triplicate for β-galactosidase activity as described (Farabaugh et al., 1989). All constructions described in this paper were sequenced by the chain termination technique (Sanger et al., 1977) from plasmids prepared as described by Mierendorf and Pfeffer (1987).

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