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A Novel Programed Frameshift Expresses the *POL3* Gene of Retrotransposon Ty3 of Yeast: Frameshifting without tRNA Slippage

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Summary

Most retroviruses and retrotransposons express their *pol* gene as a translational fusion to the upstream *gag* gene, often involving translational frameshifting. We describe here an unusual translational frameshift event occurring between the *GAG3* and *POL3* genes of the retrotransposon Ty3 of yeast. A +1 frameshift occurs within the sequence GCG AGU U (shown as codons of *GAG3*), encoding alanine-valine (GCG A GUU). Unlike other programed translational frameshifts described, this event does not require tRNA slippage between cognate or near-cognate codons in the mRNA. Two features distal to the GCG codon stimulate frameshifting. The low availability of the tRNA specific for the "hungry" serine codon, AGU, induces a translational pause required for frameshifting. A sequence of 12 nt distal to the AGU codon (termed the Ty3 "context") also stimulates the event.

Introduction

The accuracy of decoding of any informational macromolecule is critical to the expression of genetic information. In each of the three steps of information transfer—DNA replication, transcription, and translation—biochemical mechanisms have evolved that ensure production of a faithful copy of the template. Errors in translation are of two kinds: processivity failures (the failure to complete a chain) and misreading (incorporating an incorrect amino acid) (Kurland, 1992). Each type of error occurs rarely, and the aggregate error rate during translation probably does not exceed 5×10^{-4}. Among processivity errors, frameshift errors probably occur most infrequently, at rates estimated as less than 5×10^{-5} per codon (Kurland, 1992). This low frequency of random error reflects the fact that frame maintenance occupies a central position in the mechanism of elongation. The interaction between successive tRNAs bound to the mRNA in the ribosomal decoding sites, the A and P sites, defines the 3 nt translational step size, the codon (Atkins et al., 1991; Curran and Yarus, 1987; Smith and Yarus, 1989). The translational machinery has evolved to ensure that the incoming aminoacyl-tRNA inserts into the ribosomal A site so that the anticodon of the tRNA exactly adjoins the codon–anticodon helix formed by the peptidyl-tRNA in the ribosomal P site.

The details of how translational reading frame can be so strictly maintained are still not clear, despite several decades of work on the subject. What we know about frame maintenance derives from studies of the variety of ways that translational reading frame can be disrupted. Mutant tRNAs can reestablish the reading frame of frameshift mutants, emphasizing the importance of tRNA structure in defining translational step size (Atkins et al., 1991). Mutations in elongation factor Tu (EF-Tu) or its eukaryotic analog EF-1α can increase the frequency of random frameshifting (Hughes et al., 1987; Sandbaken and Culbertson, 1988; Tuohy et al., 1990; Vijgenboom and Bosch, 1989), as can limiting availability of certain aminoacyl-tRNAs (Gallant and Foley, 1980; Kurland and Gallant, 1986; Weiss and Gallant, 1983, 1986). These effects imply that, at least in some cases, shifting of reading frames is excluded through the action of EF-Tu.

Sequences in the mRNA can program shifts in translational reading frame at rates from a few percent to nearly 100% (reviewed by Atkins et al., 1990; Gesteland et al., 1992; Jacks, 1990). All programed frameshifts consist of two elements, a recoding site (Gesteland et al., 1992), which allows nontriplet decoding of the mRNA, and a stimulator (Atkins et al., 1990), which increases the efficiency of recoding. Recoding sites exist that shift the reading frame in the upstream, or minus, and downstream, or plus, direction. The most common types of frameshifts shift either −1 or +1, but other events result in shifts of multiple nucleotides in either direction (translational hops). In previously described +1 and −1 frameshifts and translational hops, the recoding site consists of alternative cognate or near-cognate codons. A ribosome-bound tRNA slips from the codon in the upstream frame onto an out-of-frame codon. Translation then continues in the new translational reading frame with the production of a translational fusion of two reading frames. In the best known example of frameshifting, retroviral −1 frameshifting, the slip site consists of a heptameric site on which two tRNAs bound to nucleotides 2 to 7 simultaneously slip onto nucleotides 1 to 6. A secondary structure, commonly a pseudoknot, stimulates the event at least partially by causing a translational pause analogous to that produced by hungry codons, as has recently been demonstrated biochemically (Tu et al., 1992). Such frameshift sites have been found in retroviruses, coronaviruses, retrotransposons, bacterial transposons, and a prokaryotic gene (for a review see ten Dam et al., 1990). A second common form of frameshift site found in prokaryotes consists of a slippery sequence followed by an in-frame termination codon. The canonical example of such a site is from the Escherichia coli release factor 2 gene (Craigen and Caskey, 1986). A +1 frameshift occurs at a slippery codon stimulated by a combination of an adjacent in-frame termination codon and a Shine–Dalgarno interaction between 16S rRNA and a sequence upstream of the shift site (Craigen and Caskey, 1986; Curran and Yarus, 1988; Weiss et al., 1988a; Weiss et al., 1987; Williams et al., 1989).

We previously described a +1 frameshift in the overlap between the *TYA* and *TYB* genes of Ty1 retrotransposons in the yeast Saccharomyces cerevisiae (Belcourt and Farabaugh, 1990). The event requires only a 7 nt sequence

CUU AGG C, shown as codons of the upstream gene, *TYA*. Frameshifting occurs by slippage of the CUU-decoding tRNA, $tRNA^{Leu}_{UAG}$, from CUU to UUA during a translational pause induced by the low availability of the AGG-decoding tRNA, $tRNA^{Arg}_{CCU}$. Frameshifting requires no other factors, such as messenger secondary structures, and no additional sequences either upstream or downstream. The low availability of tRNAs induces frameshifting in a variety of other systems. Interestingly, in E. coli, tandem AGG codons or tandem AGA codons induce translational frameshifting (Spanjaard et al., 1990; Spanjaard and van Duin, 1988). As with the yeast AGG codon, rare tRNAs decode both AGG and AGA in E. coli. A tandem pair of either codon severely restricts elongation, probably since the first codon sequesters the only cognate tRNA readily available to a particular ribosome.

The one similarity among all programed frameshift and translational hop sites is that they require alternate coding sites for tRNAs on the mRNA. We describe here an unusual frameshift system from the retrotransposon Ty3 of yeast, a distant relative of Ty1. Ty3 encodes two genes, termed *GAG3* and *POL3* by analogy to the retroviral genes to which they are functionally related. *POL3* is expressed as a fusion to the upstream *GAG3* gene (Kirchner et al., 1992). We show here that translational frameshifting within the 38 nt overlap between the genes produces the GAG3–POL3 fusion protein. The last 18 nt of the overlap promotes frameshifting with wild-type efficiency, about 10%. Sequencing of the protein product across this region and targeted mutagenesis indicate that this programed frameshift is unlike others previously characterized. The event does not require slippage of a tRNA at the site of frameshifting; a similar mechanism is proposed for a fortuitous, very low level frameshift that can be stimulated artificially by amino acid starvation (Peter et al., 1992; Weiss et al., 1988b). In addition, the Ty3 frameshift is unlike a translational hop. In both characterized translational hops recoding occurs by the repositioning of peptidyl-tRNA from a conventionally decoded codon to the start of the shifted reading frame (Benhar and Engelberg, 1993; Weiss et al., 1990). By contrast, Ty3 frameshifting appears to occur by noncanonical positioning of the incoming aminoacyl-tRNA. This result increases the phenomenology of programed frameshifting to include a mechanism previously seen in fortuitous sites.

Results

The retrotransposon Ty3 includes two genes, *GAG3* and *POL3*, which correspond to the *TYA* and *TYB* genes of the Ty1 yeast retrotransposons. The *GAG3* gene encodes the protein constituents of the Ty3 virus-like particle (Hansen et al., 1992). The *POL3* gene encodes the enzymatic activities that catalyze the conversion of the Ty3 mRNA into a double-stranded DNA and the insertion of this DNA into a target DNA molecule. These activities are a protease, reverse transcriptase, RNAase H, and integrase (Chalker and Sandmeyer, 1990; Hansen et al., 1992; Hansen and Sandmeyer, 1990). The *GAG3* gene appears to be a normal cellular gene in that the initiation codon for *GAG3* is the first AUG in the Ty3 mRNA (Hansen et al., 1988; Hansen and Sandmeyer, 1990). *POL3* lies about 1.5 kb into the Ty3 transcript, overlapping the last 38 bp of *GAG3* in a reading frame shifted +1 from *GAG3*. As in retroviruses, the Ty3 *POL3* gene is expressed as a GAG3–POL3 translational fusion (Kirchner et al., 1992). By analogy to retroviruses and other retrotransposons, we presumed that expression of the GAG3–POL3 fusion required +1 translational frameshifting.

As a first step in analyzing the mechanism of this expression, we used a convenient reporter system previously used in analyzing frameshifting in Ty1 retrotransposons (Belcourt and Farabaugh, 1990). This system, carried on plasmid pMB38, consists of a translational fusion between the *HIS4* gene of yeast and the E. coli *lacZ* gene. Transcription and translational initiation of the hybrid gene in yeast depend on signals provided by the *HIS4* gene. Between convenient BamHI and KpnI restriction sites at the *HIS4::lacZ* fusion junction we can insert various fragments encompassing putative translational frameshifting sites. The *HIS4* reading frame is fused to the upstream or 0 frame of each site, while *lacZ* is fused to the +1 frame. Ribosomes must shift reading frames +1 within the inserted region to read the sense frame of *lacZ*. To constrain frameshifting to this interval, termination codons flank the region both downstream (in the 0 frame) and upstream (in the +1 frame).

As a first attempt we inserted a copy of the entire Ty3 overlap into this vector to create pMB38–Ty3. If the overlap region is sufficient to promote efficient translational frameshifting, we would see appreciable expression of *lacZ* from this vector. The amount of enzyme expressed by pMB38–Ty3 was compared with the amount expressed by a construct in which the *HIS4* and *lacZ* genes are in the same translational reading frame, pMB38–Ty3FF (a single base pair within the overlap region was deleted to eliminate the need for frameshifting in expressing *lacZ*; see Experimental Procedures). Translating through the Ty3 overlap, about 11% of the ribosomes shift reading frames +1 within the overlap (data not shown). Thus, the 38 bp Ty3 overlap is sufficient to promote efficient translational frameshifting.

5′ and 3′ Deletions Identify a 21 nt Region Required for Maximal Efficiency of Frameshifting

To identify the minimal region capable of stimulating efficient translational frameshifting, we constructed deletions that truncated the Ty3 overlap region from either the upstream (toward the Ty3 promoter) or downstream end. These deletions were made by the polymerase chain reaction (PCR), using pMB38–Ty3 as template. All of the deletions were sequenced prior to use to preclude the introduction of extraneous mutations by PCR within the overlap region. The synthesized primers (shown in Table 1) created a series of nested deletions from either the 5′ or the 3′ end of the overlap. The construction of these deletions is described in Experimental Procedures.

Removing either the first 9, 18, or 21 bp of the overlap (Ty3Δ1, Ty3Δ2, and Ty3Δ7G) actually increased expression of *lacZ* less than 2-fold (Table 1). Further deletion of

Table 1. Sequences of 5′ and 3′ Deletions of the Ty3 *GAG3–POL3* Overlap

Plasmid	Sequence[a]	Frameshifting (%)
pMB38–Ty3	ggauccagUGAACGAAUGUAGAGCACGUAAGGCGAGUUCUAACCGAUCUUGAgguacc	11.4
pMB38–Ty3Δ1	ggauccagUG——AGAGCACGUAAGGCGAGUUCUAACCGAUCUUGAgguacc	16.5
pMB38–Ty3Δ2	ggauccagUG——AAGGCGAGUUCUAACCGAUCUUGAgguacc	14.8
pMB38–Ty3Δ7G	ggauccagUA——GCGAGUUCUAACCGAUCUUGAgguacc	16.9
pMB38–Ty3Δ7	ggauccagUGA——CGAGUUCUAACCGAUCUUGAgguacc	0.6
pMB38–Ty3Δ8	ggauccagUG——AGUUCUAACCGAUCUUGAgguacc	0.8
pMB38–Ty3Δ3	ggauccagUGA——CUAACCGAUCUUGAgguacc	1.0
pMB38–Ty3Δ9	ggauccagUG——AAGGCGAGUUCUAACCGAU——GAgguacc	8.4
pMB38–Ty3Δ4	ggauccagUG——AAGGCGAGUUCUAAC——UGAgguacc	5.2
pMB38–Ty3Δ10	ggauccagUG——AAGGCGAGUUCU——UGAgguacc	1.8
pMB38–Ty3Δ5	ggauccagUG——AAGGCGAGUU——GAgguacc	2.4
pMB38–Ty3Δ6	ggauccagUG——AAGGCG——UGAgguacc	2.5

[a] The sequence derived from the overlap is shown in capitals, and adjoining linkers are shown in lowercase. Nucleotides removed by deletion are denoted by lines. The essential GCG AGT codons are underlined.

1 bp (Ty3Δ7), removing the first G of the sequence GCG AGU (shown in the *GAG3* reading frame), virtually eliminated expression of β-galactosidase, a decrease of about 27-fold. Deletion of the next 2 or 5 bp (Ty3Δ8 and Ty3Δ3) had little further effect on expression. The abrupt loss of expression caused by removing the 1 bp between Ty3Δ7G and Ty3Δ7 establishes an upstream border of the minimal frameshift region as a GCG alanine codon of *GAG3*, the eighth *GAG3* codon in the overlap region. To determine the downstream boundary of the minimal site, 3′ deletions of the Ty3Δ2 fusion were constructed by PCR. The effect of progressive 3′ deletions was qualitatively different from the 5′ deletions. As 2, 6, or 9 bp was deleted from the end of the overlap (Ty3Δ9, Ty3Δ4, and Ty3Δ10, respectively), expression of *lacZ* declined gradually, each deletion causing an approximately 2-fold decrease. The expression of Ty3Δ10 corresponded to a frameshift efficiency of 1.8%, 8-fold below the Ty3Δ2 control. Further 2 or 6 bp deletions (Ty3Δ5 and Ty3Δ6) had no further effect on expression. We conclude that maximal frameshifting requires the last 18 bp of the Ty3 overlap. The extreme 5′ end of this region is essential, since 5′ deletions of the region showed little expression and inclusion of only the first one or two codons from this region, GCG AGU, stimulated low but measurable expression. The 3′ end of the region, including the last 12 bp, though not essential, increased the efficiency of frameshifting when present. This region, which we term the Ty3 context, either may directly stimulate the shift of reading frames or may prolong a translational pause on the recoding site. Experiments are in progress to determine how the context stimulates frameshifting.

Missense Mutagenesis Identifies 6 nt, the *GAG3* Codons GCG AGU, as Essential to Ty3 Frameshifting

Deletion analysis can identify the region involved in Ty3 frameshifting, but only to a resolution of 3 bp, since all of the deletions must maintain the translational reading frame. In addition, it cannot determine which nucleotides are essential or inessential within that region. The 18 bp minimal region could be characterized in detail by saturation missense mutagenesis. Initially, we concentrated on the region toward the 5′ end of the 18 bp region, which seems to be essential for frameshifting. Using appropriate mutagenic PCR primers, we introduced a nearly saturating set of single base mutants in each of the first 13 bp of the minimal region. The context for these mutations was the Ty3Δ2 fusion construct. As a control we changed the nucleotide immediately upstream of the region to all four nucleotides. Each mutant form was introduced into yeast, and the level of β-galactosidase expressed by each was determined.

As shown in Figure 1, the mutants clearly identify a sequence of 5 nt that could not be changed without either greatly reducing or eliminating frameshifting. This sequence CG AGU corresponds to the second and third nucleotides of the second codon of the region, GCG (Ala), and the next codon, AGU (Ser). In fact, in these two codons

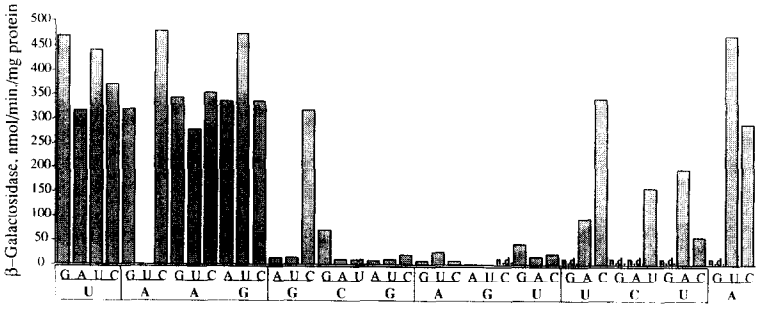

Figure 1. β-Galactosidase Activity of Missense Mutants of the pMB38–Ty3Δ2.

The sequence of Ty3Δ2 is shown boxed as codons of *GAG3* below the X axis, starting with the nucleotide, U, immediately 5′ of the overlap derived sequence (the −1 position). Above each boxed nucleotide are the missense mutants generated for each position. The β-galactosidase activity of each appears graphically.

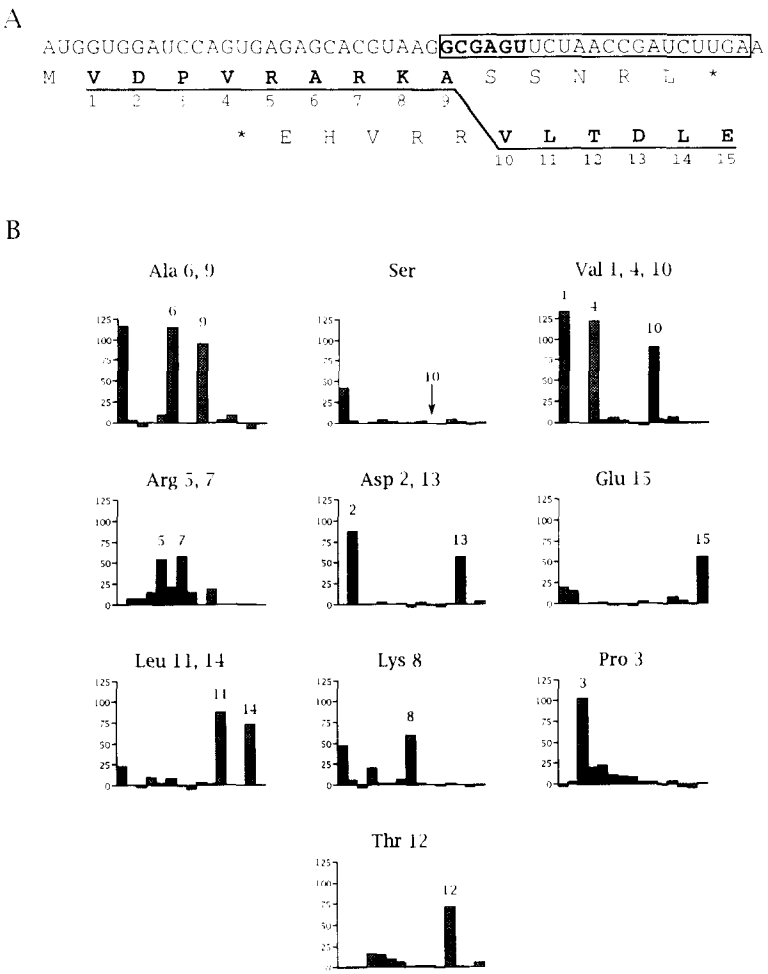

Figure 2. Predicted and Actual Peptide Sequence through the Frameshift Site

(A) The predicted amino acid sequences of the 0 and +1 reading frames appear below the sequence of p3p-Ty3Δ1. The region of the sequence essential to frameshifting is boxed, and the essential GCG AGU codons are in bold. The sequence of amino acids deduced from the data in (B) is shown in bold with a line to indicate the postion of the frameshift.

(B) Amino acid sequence data through residue 15. Histograms show the amount of relevant PTH-amino acids through 15 cycles of Edman degradation. The values have been corrected for background. The first cycle included significant amounts of several PTH-amino acids in addition to the predicted Val (note for example Ala and Ser); at all other steps little unpredicted product was observed. The tenth residue is critical to defining the frameshift. Note that the tenth cycle includes significant amounts of Val but no Ser (the tenth 0 frame amino acid after the initial Met, which is cleaved off in vivo).

only one mutation had no phenotypic effect on expression, GCG to CCG in codon 2. Clearly these two codons must play a central role in the frameshift. At each of the other mutated positions, there was at least one change that had no effect or reduced expression about 2-fold. Changes to the nucleotide immediately upstream of the region had no effect. Thus, the sequence outside of codons 2 and 3 is less constrained, though still important to frameshifting.

These data for the most part correlate well with the evidence from 5′ and 3′ deletions. The first deletion that impinged on the sequence GCG AGU (Ty3Δ7), removing the AAG codon and changing GCG AGU to ACG AGU, greatly reduced expression. The deletions from the 3′ side gradually reduced expression, reaching their lowest level when nearly the entire sequence downstream of GCG AGU was removed. That expression was not eliminated when this crucial sequence was retained is consistent with its playing a central role in frameshifting. The distal sequence must simply increase the efficiency of an event dependent on the sequence upstream. Suprisingly, further deletion into this region by one codon, replacing the AGU codon with UGA (Stop), had no further effect on expression. Since the AGU codon is entirely essential for frameshifting, we must conclude that the UGA codon can perform that same function. In several bacterial systems a termination codon causes a translational pause that increases the efficiency of frameshifting or translational hopping (reviewed by Atkins et al., 1990). This result suggests that the AGU codon functions to induce a translational pause, which would also suggest that frameshifting must occur after the ribosome decodes the immediately upstream GCG or CCG codon.

The Frameshift Occurs at the Sequence GCG A GUU Encoding Ala-Val

To show that expression of the Ty3 GAG3–POL3 fusion occurs by translational frameshifting requires that we determine the sequence of the primary translation product. We have previously used immunoaffinity chromatography to purify β-galactosidase fusion proteins and automated Edman degradation to determine the amino acid sequence near the N-terminus of the protein (Belcourt and Farabaugh, 1990). This approach requires that the site at which the putative translational frameshift occurs be within the first 20 amino acids of the protein, the limit of our ability to perform automated Edman sequencing of β-galactosidase. We created a plasmid carrying a Ty3Δ1::*lacZ*

translational fusion in which the sixth codon is the first derived from Ty3 and the GCG AGU putative frameshift site is codons 9 and 10. The sequence of the first 16 codons of the Ty3Δ1::*lacZ* fusion gene, including all codons from the Ty3 overlap region (codons 6 to 16), is shown in Figure 2A, along with the predicted translational product in both the 0 frame (in-frame with the initiation codon) and +1 frame. About 100 μg of the fusion protein was analyzed, and a clear sequence was derived for the first 19 residues. The amounts of the relevant phenylthiohydantoin (PTH)-amino acids through the first 15 cycles of Edman degradation are shown in Figure 2B. The sequence matched the predicted 0 frame sequence starting from codon 2 (the N-terminal methionine appeared to be cleaved off) to codon 9, the GCG alanine codon essential for frameshifting. The next residue is valine, which could be encoded by GUU, the first +1 frame codon past the GCG codon. The residues match the +1 frame predicted sequence from the valine codon through the next nine +1 frame codons (Figure 2B; data not shown). There is no ambiguity in the determined sequence; at each cycle one PTH-amino acid is clearly predominant, and in each case it corresponds to a Ty3Δ1 codon. These data clearly show, as diagramed in Figure 2A, that expression of the GAG3–POL3 fusion occurs by change of reading frames between the GCG and GUU codons of the sequence GCG AGU U (shown as *GAG3* codons).

The predicted Ty3 frameshift site is unusual. The last 0 frame codon, GCG, is not a "slippery codon." A tRNA decoding this codon would not be able to slip +1 on the message to allow realignment of the ribosome in the new frame. Other programed frameshifts employ codons that have alternate near-cognate codons in the shifted frame that allow tRNAs to "recode," or slip from a codon in one frame to a codon in a second frame (Gesteland et al., 1992). It is provocative that the Ty3 frameshift does not comply with this mechanism of frameshifting.

Overproduction of $tRNA^{Ser}_{GCU}$, Which Decodes AGU, Abolishes Ty3 Frameshifting

The position of the AGU codon relative to the frameshift site, the codon immediately downstream of the last decoded 0 frame codon, combined with the fact that the codon is essential, suggests that the AGU codon may function by inducing a translational pause. In several systems the first 0 frame codon past the shift site is either a slowly decoded sense codon or a nonsense codon, presumably also slowly recognized by peptide release factor. For example, in the case of Ty1 retrotransposons in yeast, the AGG (Arg) codon downstream from the CUU (Leu) slip codon is a low abundance codon that is presumably inefficiently decoded. Overproducing the tRNA specific for this codon, $tRNA^{Arg}_{CCU}$, reduced frameshifting 43-fold to nearly background levels (Belcourt and Farabaugh, 1990).

We hypothesized that the AGU codon also limits translation, inducing a translational pause that stimulates Ty3 frameshifting. To test this hypothesis we obtained the clone for tRNA that decodes AGU, $tRNA^{Ser}_{GCU}$, by PCR from genomic DNA, using the sequence of the gene available from the GenBank data base (accession number X06992). The primers used in PCR introduced SalI restriction sites flanking the clone. These were used to insert the gene into the unique SalI site upstream of the *HIS4* promoter. By introducing the tRNA gene onto the multicopy plasmid carrying the *lacZ* reporter gene, we ensured that, when introduced into yeast, each cell expressing *lacZ* would simultaneously express multiple extra copies of the tRNA gene. Since the plasmid is present in four copies per haploid genome (Belcourt and Farabaugh, 1990), when introduced into yeast it would increase the copy number of the tRNA gene by four.

To assess the effect of the tRNA on Ty3 frameshifting, we introduced into yeast matched plasmids with and without the $tRNA^{Ser}_{GCU}$ gene. As shown in Figure 3, introducing the tRNA gene into pMB38–Ty3FF reduced expression only slightly; however, overexpression of the tRNA reduced +1 frameshift expression of pMB38–Ty3 15-fold. Comparing the +1 frameshift and frame fusion constructs shows that overproducing $tRNA^{Ser}_{GCU}$ reduces apparent frameshift efficiency 10-fold. Although we do not know the copy number of the $tRNA^{Ser}_{GCU}$ structural gene and thus cannot estimate the degree of overproduction of the tRNA, frameshift expression is clearly sensitive to the concentration of this tRNA.

That overproducing $tRNA^{Ser}_{GCU}$ reduced Ty3 frameshifting demonstrates two things. First, the process leading to expression of the Ty3 GAG3-POL3 fusion protein occurs during translation. Had the event occurred either during transcription or posttranscriptionally (e.g., by inaccurate transcription or RNA editing leading to creation of a fused *GAG3–POL3* reading frame in the mRNA), then the concentration of any particular tRNA would have been irrelevant. Therefore, the event must be translational. Second, since overproducing the tRNA eliminated expression, frameshifting must depend on the slow decoding of AGU by $tRNA^{Ser}_{GCU}$, and therefore the presence of the codon must

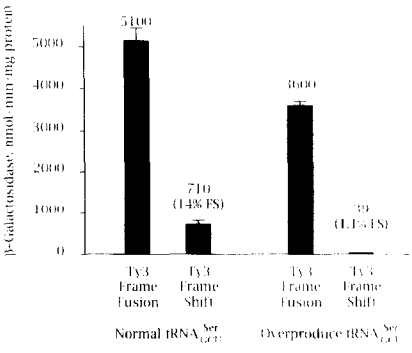

Figure 3. Overproduction of $tRNA^{Ser}_{GCU}$ Virtually Eliminates Ty3 Frameshifting

The histograms present the β-galactosidase activity of transformants of plasmids with or without $tRNA^{Ser}_{GCU}$. The units expressed and, for frameshift constructs, the frameshift efficiency appear above each bar. Error bars represent the SEM.

cause a translational pause. The AGU codon of the Ty3 frameshift site is analogous to the AGG (arginine) codon of the Ty1 frameshift site. Thus, the two related retrotransposons share a similar mechanism of frameshifting, at least to the extent of the nature of the translational pause induced. It is interesting that the codons involved in these two mechanisms are so closely related, differing only in the wobble position, though encoding different amino acids. It is tempting to speculate that, though the retrotransposons are only distantly related (Hansen et al., 1988), the two frameshift sites may have diverged from a common progenitor that used one or the other of these codons. We note that, in our previous analysis of Ty1 frameshifting, no codon other than AGG could subsitute to allow high level (~20%) frameshifting (Belcourt and Farabaugh, 1990); however, several codons did allow significant though low level frameshifting (1%–2%). Among these was the AGU serine codon. The low level of frameshifting indicated that AGU was a poor pause codon. How then is it possible that the Ty3 site, employing this site, is nearly as efficient as the wild-type Ty1 site? Clearly, if the two sites have diverged from each other, they have achieved this efficiency by distinct methods.

Discussion

Here we describe the analysis of a ribosomal frameshift occurring between *GAG3* and *POL3* of the retrotransposon Ty3. Since the genes overlap in an arrangement identical to that of the Ty1 genes *TYA* and *TYB*, we suspected that expression of Ty3 *POL3* required, like Ty1, a +1 frameshift within the overlap region. Since the Ty3 overlap does not include the 7 nt Ty1 frameshift site, CUU AGG C (Belcourt and Farabaugh, 1990), the mechanism must necessarily be different. We show here that the Ty3 mechanism differs in several respects. That maximal Ty3 frameshifting required a longer 21 nt region indicated that the mechanism was probably more complex than that operating on the shorter Ty1 site. Peptide sequencing identified the site of frameshifting at the beginning of the 21 nt required region, the sequence GCG AGU U, which is decoded alanine (GCG)-valine (GUU). Unexpectedly, the shift in frames occurs at a codon GCG, decoded by a tRNA, $tRNA^{Ala}_{CGC}$, that cannot slip onto the overlapping +1 frame codon, CGA. In other efficient programed frameshifts and translational hops, the site of the shift includes a site where a tRNA recognizes a cognate codon in the upstream frame but can also recognize a cognate or near cognate in the shifted frame (Gesteland et al., 1992). The idea that the Ty3 event does not involve tRNA slippage is underscored by the observation that mutating GCG to GGG, which creates a potential site for slippage of $tRNA^{Gly}_{CCC}$ from GGG to GGA, actually reduces frameshifting about 4-fold (see Figure 1). A similar event occurs at the sequence GCC AAG C in E. coli. Lysine starvation causes +1 frameshifting on this site, decoding it as Ala-Ser (GCC A AGC) (Peter et al., 1992; Weiss et al., 1988b). This is a fortuitous, rather than programed, frameshift. The site induces very low levels of frameshift expression in the absence of starvation (~0.01%), rising to about 1% frameshifting under severe starvation (Weiss et al., 1988b). However, as with the programed Ty3 event, slipping of peptidyl-$tRNA^{Ala}$ +1 from GCC to CCA is unlikely, and therefore frameshifting must occur by another mechanism.

The tRNA decoding GCG, though it does not slip, does have a role in promoting frameshifting. Single base substitutions of GCG create nine other codons: three other alanine codons (decoded by two other isoacceptors) and codons for glutamic acid, glycine, proline, threonine, serine, and valine. Replacing GCG with seven of these codons eliminated frameshifting, and substituting GGG greatly reduced it, demonstrating that the corresponding tRNAs cannot substitute for $tRNA^{Ala}_{CGC}$. Only one mutation, GCG (Ala)→CCG (Pro), had no phenotypic effect, suggesting that $tRNA^{Pro}_{CGG}$ can function interchangeably with $tRNA^{Ser}_{GCU}$. If these mutations are a fair sample of the estimated 44 tRNA species in yeast other than GCG, one would predict that around 10 tRNAs might substitute for $tRNA^{Ala}_{GCU}$. This number seems unacceptably high; we are attempting now to identify all substitutes for GCG by random mutagenesis.

If the Ty3 shift occurs without slippage of a peptidyl-tRNA, then how is the change of reading frames accomplished? Four general mechanisms are possible: out-of-frame binding of an aminoacyl-tRNA in the ribosomal A site, use of a 4 nt anticodon by either the P site or A site tRNA, bulging out of the A in the message between GCG and GUU, or RNA editing to remove one nucleotide and put the *GAG3* and *POL3* genes in the same reading frame. The fact that overproduction of $tRNA^{Ser}_{GCU}$ eliminates frameshifting excludes RNA editing.

The first model requires out-of-frame binding of valyl-$tRNA^{Val}_{IAC}$ to the +1 frame codon GUU (cartooned in Figure 4A). In elongation the incoming aminoacyl-tRNA must bind immediately adjacent to the peptidyl-tRNA, ensuring a 3 nt translational step size. This binding is probably stabilized by interactions between the two tRNAs (Curran and Yarus, 1987; Smith and Yarus, 1989). However, this model requires the incoming $tRNA^{Val}_{IAC}$–EF-1α–GTP complex to enter the A site and bind one nucleotide away from peptidyl-$tRNA^{Ala}_{GCU}$ in the ribosomal P site. It is not clear why this normally very rare event would occur at such a high rate at the Ty3 frameshift site, though presumably it depends on some feature of the structure of $tRNA^{Ala}_{GCU}$ and/or $tRNA^{Val}_{IAC}$. We know that frameshifting is eliminated by replacing GCG with 7 out of 9 other codons or by replacing GCG AGU U with GCG AGC U (replacing the GUU (Val) codon with GCU (Ala)), and preliminary results suggest that several other codons also fail to replace GUU (S. Pande and P. J. F., unpublished data). In addition, the downstream context would stimulate this event either directly by aiding out-of-frame binding or indirectly by increasing the required translational pause.

The second model, use of a 4 nt anticodon, also presumes the involvement of specific tRNAs. The normal three base codon is defined by the structure of the 7 nt tRNA anticodon loop. The three-dimensional structure of stacked bases within the anticodon loop causes the central three base pairs of the loop to function as the anticodon in binding the codon. Expanding the anticodon loop to 8 nt allows a tRNA to efficiently decode 4 nt codons and

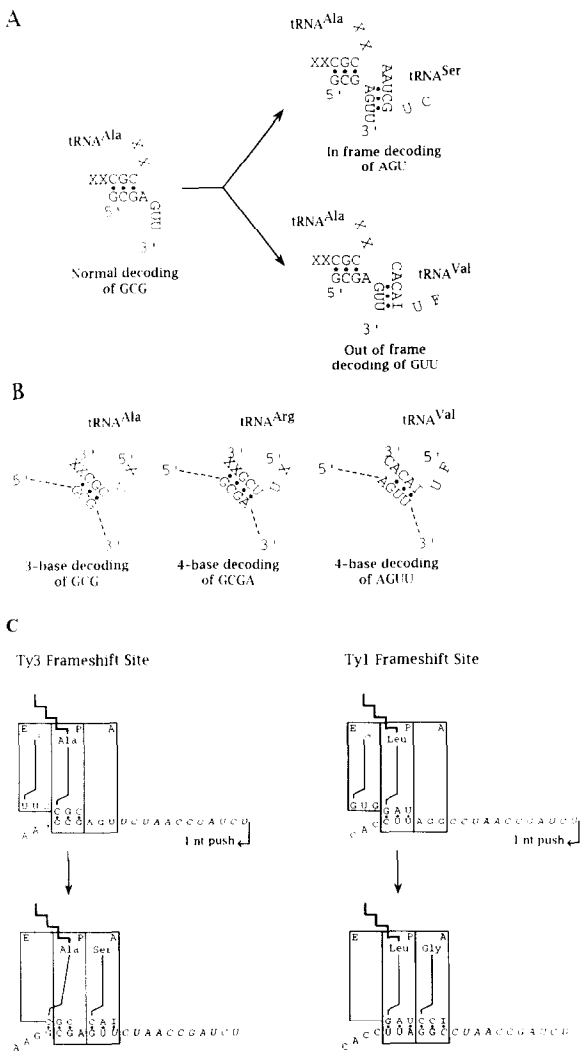

Figure 4. Models of Ty3 Frameshifting

(A) Out-of-frame decoding of GUU by $tRNA^{Val}_{IAC}$. The sequence of the mRNA at the recoding site, GCG AGU U, is shown. The kink in the mRNA that occurs between the A and P sites is exaggerated here for clarity. The sequences of the anticodon loops of each tRNA are shown to indicate stacking relationships. Those bases that are stacked are represented by in-line letters (after Tuohy et al., 1992); for example, the bases of the sequence GCUAA of $tRNA^{Ser}_{GCU}$ are stacked, as are those of the dinucleotide CU. The sequence of $tRNA^{Ala}_{CGC}$ is not known; the predicted anticodon is shown, and other anticodon loop bases are indicated by X. Base pairing interactions are indicated by bullets. Normal in-frame decoding of AGU by $tRNA^{Ser}_{GCU}$ occurs when the mRNA is in the normal conformation; decoding of GUU is proposed to occur by a rearrangement of the mRNA moving the GUU codon into the A site.

(B) Four base decoding of GCGA or AGUU. Normal decoding of GCG by $tRNA^{Ala}_{CGC}$ is shown, as well as four base decoding of GCGA by $tRNA^{Arg}_{UCG}$ (whose sequence is not known) and of AGUU by $tRNA^{Val}_{IAC}$. The stacking pattern within the anticodon loop, as cartooned, requires that GCGA be decoded by $tRNA^{Arg}_{UCG}$ rather than $tRNA^{Ala}_{CGC}$.

(C) A model for the effect of the Ty3 context: pushing the mRNA. The interaction of tRNAs in the E ("exit"), P, and A sites as influenced by the Ty3 context is cartooned, adapting the convention of Moazed and Noller (1989). The peptidyl-tRNA is indicated, with the nascent peptide indicated by a zigzag line. The Ty3 context is shown in italics. The model proposes that for the Ty3 site an intermolecular interaction with the context (and rRNA?) pushes the mRNA 1 nt in the 5′ direction. $tRNA^{Ala}_{CGC}$, which cannot slip, is forced 1 nt into the E site; the GUU codon in the A site is recognized by its cognate tRNA, causing the frameshift. For the Ty1 site, $tRNA^{Leu}_{UAG}$ can slip onto the adjacent UUA codon in response to the mRNA movement; the GGC codon in the A site is then decoded in the +1 frame.

therefore function as a +1 frameshift suppressor, by increasing the number of adjacent nucleotides accessible to the mRNA for pairing (Atkins et al., 1991; Bossi and Smith, 1984; Curran and Yarus, 1987). Since it must use normal tRNAs to achieve four base decoding, the Ty3 frameshift cannot depend on expansion of the anticodon loop in either $tRNA^{Ala}_{CGC}$ or $tRNA^{Val}_{IAC}$. However, some +1 frameshift suppressor tRNAs have 7 nt anticodon loops and can bind the mRNA with either a three or four base anticodon (Atkins et al., 1991). Because of the three-dimensional structure of the anticodon loop, when four bases pair with the mRNA they consist of the normal anticodon and its 3′ neighbor (as shown in Figure 4B). It is not possible to use an alternative expanded anticodon consisting of the normal anticodon and its 5′ neighbor, the universal U_{33} base. U_{33} interacts by a tertiary hydrogen bond with phosphate-36, which stabilizes the structure of the anticodon loop (Quigley and Rich, 1976), so it cannot stack beneath nucleotide 34 of the anticodon to pair with the mRNA (Ayer and Yarus, 1986; Bossi and Smith, 1984; Curran and Yarus, 1987). The same constraint probably operates on the tRNA responsible for Ty3 frameshifting; since the four base decoding of GCGA by $tRNA^{Ala}_{CGC}$ would require that U_{33} participate in the anticodon, this model is probably wrong. In fact, assuming this to be correct, four base decoding of GCGA by a 7 nt loop tRNA should result in insertion of Arg (CGA) rather than the observed Ala (GCG) (Figure 4B). By contrast, four base decoding of AGUU would insert Val (GUU), as observed, not Ser (AGU) (see Figure 4B). Therefore, if the Ty3 frameshift occurs by four base decoding, it must occur in the ribosomal A site by noncanonical binding of $tRNA^{Val}_{IAC}$. As shown in the Figure, the base 5′ of the codon of $tRNA^{Val}_{IAC}$, an A, cannot base pair with the A of AGUU. Some frameshift suppressor tRNAs recognize only 3 of the 4 base pairs of the expanded codon, presumably because base pairing at the fourth position is not required for codon recognition (Atkins et al., 1991). However, the nonpairing base is invariably in the wobble position, the 5′ base of the anticodon. The unusual pairing scheme proposed for $tRNA^{Val}_{IAC}$ weakens this model of Ty3 frameshifting.

The third model proposes that the A of GCG A GUU bulges out so that it is ignored in base pairing in the ribosomal A site. The structure of the pseudohelix formed by the two tRNAs binding to such a message would resemble an RNA helix in which an extra nucleotide on one side of the helix is excluded from base pairing. However, a single A inserted into a helix does not bulge out, as proposed by the model, but rather stacks between the paired nucleotides on either side (Kalnik et al., 1989). For this reason the model seems unlikely, though it cannot be definitely excluded.

If the mechanism by which Ty3 shifts frames is fundamentally different from other programed frameshifts and translational hops, is the mechanism used to stimulate

this event more conventional? All programed frameshifts appear to occur during a translational pause during elongation. That pause can be induced by the slow recognition of a termination codon by peptide release factor, by a stable secondary structure distal to the site, or by the slow decoding of a sense codon decoded by a low abundance tRNA (Gesteland et al., 1992). As stated above, the Ty1 frameshift site stimulates frameshifting by the last mechanism (Belcourt and Farabaugh, 1990). Ty3 uses a similar stimulator. The codon following the site of frameshifting in Ty3 is the serine codon AGU, which is decoded by $tRNA^{Ser}_{GCU}$. Though neither the copy number of this gene nor the abundance of its product has yet been determined, we have shown that overproducing $tRNA^{Ser}_{GCU}$ virtually eliminated Ty3 frameshifting, reducing its efficiency about 14-fold. Thus, the shift must occur when translation pauses owing to the low availability of $tRNA^{Ser}_{GCU}$. This implies that the event occurs when the AGU codon occupies an empty ribosomal A site and peptidyl-$tRNA^{Ala}_{CGC}$ occupies the P site. The pause allows sufficient time for the frameshift to occur on up to 10% of the paused ribosomes.

The AGG pause codon of Ty1 is the only codon decoded by its cognate $tRNA^{Arg}_{CCU}$. This is not the case with $tRNA^{Ser}_{GCU}$, which decodes both the AGC and AGU serine codons. If the tRNA's concentration were the only relevant factor in determining the length of the translational pause, then one would predict that either of these codons could serve as the Ty3 pause codon. However, mutating the pause codon from AGU to AGC eliminated Ty3 frameshifting. How can two codons decoded by the same isoacceptor have such different phenotypic effects? It is possible that the details of the codon–anticodon interaction would affect frameshift efficiency, that is, that its strength would affect the length of the translational pause. The binding energy of $tRNA^{Ser}_{GCU}$ to the AGC codon should be greater than to AGU because of the G–C rather than G–U wobble base pair. This difference in energy could affect the rate of selection of $tRNA^{Ser}_{GCU}$ by either increasing the rate at which seryl-$tRNA^{Ser}_{GCU}$–EF-1α–GTP ternary complex binds to the AGC codon in the A site or increasing the rate at which ternary complex or seryl-$tRNA^{Ser}_{GCU}$ dissociates from AGU during kinetic proofreading. In vitro biochemical analysis has demonstrated that synonymous codons can differ in rate of decoding and that that difference reflects the more rapid binding of a tRNA to one of the codons (Dix and Thompson, 1989; Thomas et al., 1988). Rates of dissociation of synonomous tRNAs from the ribosome have no effect on rates of decoding, since they are very much less than the rate of peptide bond formation; essentially all cognate tRNAs selected by the ribosome remain bound and yield peptidyl-tRNA (Dix and Thompson, 1989). However, the difference in decoding between synonymous cognate codons is very small; the differences between UUU and UUC and between CUU and CUC were no more than 2-fold (Thomas et al., 1988). Since translation is a kinetically driven process, changes of as little as 2-fold can have a much larger effect on overall expression, so it is possible that such a small difference in decoding of the AGU and AGC codons could result in a 13-fold difference in frameshifting.

If, however, an intrinsic difference in the kinetics of their decoding by $tRNA^{Ser}_{GCU}$ does not explain why AGC cannot replace AGU as the pause codon, then the difference may depend on the overlapping +1 frame codon. The identity of the tRNA that decodes the first +1 frame codon may affect frameshift frequency if the interaction of that tRNA with the mRNA drives the shifting of frames. Either of the proposed models of Ty3 frameshifting would require an unconventional 4 nt translocation at the position of the frameshift. Out-of-frame or four base decoding by $tRNA^{Val}_{IAC}$ may require an unusual interaction between tRNAs in the A and P sites of the ribosome. It may be that only particular tRNAs entering the A site can promote such a frameshift. Substituting the GUU valine codon of GCG AGU U with the GCU alanine codon of GCG AGC U might eliminate frameshifting by substituting $tRNA^{Ala}_{IGC}$ for $tRNA^{Val}_{IAC}$. However, $tRNA^{Val}_{IAC}$ cannot be unique, since frameshifting is unchanged when GCG AGU-U (Ty3Δ5) is replaced by GCG UGA G (Ty3Δ6) and increases when it is replaced by GCG AGG C (data not shown). These sites would require noncanonical decoding by $tRNA^{Glu}_{CUC}$ and $tRNA^{Gly}_{GCC}$. Thus, three tRNAs, under this model, efficiently stimulate Ty3 frameshifting, $tRNA^{Val}_{IAC}$, $tRNA^{Glu}_{CUC}$, and $tRNA^{Gly}_{GCC}$, and one, $tRNA^{Ala}_{IGC}$, does not. We are pursuing other sequences that can substitute for AGUU in hopes of determining the rules governing stimulation of the frameshift.

In summary, we have identified the site responsible for translational frameshifting at the junction of the *GAG3* and *POL3* genes of the retrotransposon Ty3 in yeast. The frameshift site has several notable features. The mechanism of frameshifting is unlike that of other programed frameshift sites or translational hops. While other sites require slippage of a tRNA between alternate cognate or near cognate codons, the Ty3 site does not. Frameshifting either occurs by out-of-frame binding of aminoacyl-tRNA or four base decoding by a normal aminoacyl-tRNA. Either event occurs efficiently during normal elongation. Frameshifting is stimulated by two elements, a slowly decoded ("hungry") codon and a short RNA sequence distal to the frameshift site, the Ty3 context. The frameshift defines a phenomenology unlike other programed frameshifts, identifying a distinct mechanism by which an mRNA can efficiently manipulate the mechanism of reading frame maintenance. It should provide additional tools to probe that mechanism, to determine how ribosomes so accurately monitor decoding of the messenger.

Experimental Procedures

Yeast Strains, Media, and General Methods

The S. cerevisiae strain used for this work is 387-1D (α *his4*Δ38 *ura3-52 trp1-289 HOL1-1*). All strains were grown in SD minimal media supplemented with the appropriate amino acids to allow selection for *URA3*$^+$-containing plasmids (Rose et al., 1990; SD, synthetic media plus dextrose). DNA transformations of yeast were performed by the lithium acetate method (Ito et al., 1983). The activity of β-galactosidase expressed by transformants was determined as described (Farabaugh et al., 1989). Oligonucleotides were synthesized on a Biosearch Cyclone DNA synthesizer (Milligen) and purified by chromatography on Oligo-Pak columns (Milligen) according to manufacturer's directions.

Plasmid Construction

All of the plasmids used in this work are derived from the plasmid

Table 2. Sequences of Oligonucleotides Used

Number	Alias	Sequence[a]	
Wild-Type Ty3 *GAG3–POL3* Overlap (Frameshift Site)			
oli 140	Top	gatccagTGAACGAATGTAGAGCACGTAAGGCGAGTTCTAACCGATCTTGAggtac	
oli 141	Bottom	cTCAAGATCGGTTAGAACTCGCCTTACGTGCTCTACATTCGTTCActg	
5′ and 3′ Deletions of the Frameshift Site			
oli 144	Ty3Δ1	aattggatccagtgaGAGCACGTAAGGCGAGT	
oli 145	Ty3Δ2	aattggatccagtgaAGGCGAGTTCTAACCGA	
oli 146	Ty3Δ3	aattggatccagtgaCTAACCGATCTTGAGGT	
oli 147	Ty3Δ4	atcgggtacctcaGTTAGAACTCGCCTTCA	
oli 148	Ty3Δ5	atcgggtacctcAACTCGCCTTCACTGGAT	
oli 149	Ty3Δ6	atcgggtacctcaCGCCTTCACTGGATCCG	
oli 153	Ty3Δ7	aattggatccagtgaCGAGTTCTAACCGATCT	
oli 267	Ty3Δ7G	aattggatccagtaGCGAGTTCTAACCGATCT	
oli 154	Ty3Δ8	aattggatccagtgaGTTCTAACCGATCTTGA	
oli 155	Ty3Δ9	atcgggtacctcATCGGTTAGAACTCGCCT	
oli 232	Ty3Δ10	gaccggtacctcaAGAACTCGCCTTCACTGGATCC	
Site Mutations of the Frameshift Site			
oli 156	G–1X	aattagatctagTXAAGGCGAGTTCTAACCGA	(X = A, C, T)
oli 157	A1X	aattagatctagTGXAGGCGAGTTCTAACCGA	(X = C, G, T)
oli 158	A2X	aattagatctagTGAXGGCGAGTTCTAACCGA	(X = C, G, T)
oli 159	G3X	aattagatctagTGAAXGCGAGTTCTAACCGA	(X = A, C, T)
oli 160	G4X	aattagatctagTGAAGXCGAGTTCTAACCGA	(X = A, C, T)
oli 161	C5X	aattagatctagTGAAGGXGAGTTCTAACCGA	(X = A, G, T)
oli 162	G6X	aattagatctagTGAAGGCXAGTTCTAACCGA	(X = A, C, T)
oli 163	A7X	aattagatctagTGAAGGCGXGTTCTAACCGA	(X = C, G, T)
oli 182	G8X	aattagatctagTGAAGGCGAXTTCTAACCGAT	(X = A, C, T)
oli 183	T9X	aattagatctagTGAAGGCGAGXTCTAACCGAT	(X = A, C, G)
oli 188	T10X	aattagatctagTGAAGGCGAGTXCTAACCGATCTTG	(X = A, C, G)
oli 189	C11X	aattagatctagTGAAGGCGAGTTXTAACCGATCTTG	(X = A, G, T)
oli 190	T12X	aattagatctagTGAAGGCGAGTTCXAACCGATCTTG	(X = A, C, G)
oli 191	A13X	aattagatctagTGAAGGCGAGTTCTXACCGATCTTG	(X = C, G, T)
Frame Fusion Constructs			
oli 169	Ty3FF	aaccggtaccTCAAGACGGTTAGAA	

[a] Nucleotides derived from the Ty3 overlap are shown in capital letters.

pMB38 (Belcourt and Farabaugh, 1990). The plasmid carries the *URA3* gene and 2μm origin of replication to allow selection of autonomous replication in yeast. It also includes a translational fusion between the *HIS4* gene of yeast and the E. coli *lacZ* gene. The *lacZ* gene is fused via a BamHI–KpnI linker to the *HIS4A* gene 33 codons downstream of the *HIS4* initiation codon. The construction and structure of this plasmid have been described (Belcourt and Farabaugh, 1990).

To assay for frameshifting using this plasmid, a double-stranded oligonucleotide encompassing the entire Ty3 overlap flanked upstream by a BamHI site and downstream by a KpnI site (see Table 2) was inserted between the BamHI and KpnI sites of pMB38. The oligonucleotide was designed so that translation enters from the upstream *HIS4A* gene in the Ty3 *GAG3* reading frame and terminates in-frame at the *GAG3* termination codon, UGA. The +1 frame of the oligonucleotide begins at the TGA codon, which defines the upstream end of the Ty3 overlap, and continues through into *lacZ*. Expression of *lacZ* from the transcript of this chimeric gene would require the translational reading frame to shift within the region of the Ty3 overlap, between the two flanking nonsense codons. This plasmid was named pMB38–Ty3. A variant of this plasmid, pMB38–Ty3FF, was constructed in which nucleotide 35 of the overlap, the A of the sequence 5′-CCGATCTT-3′ (see Figure 1), is deleted, putting the upstream *HIS4A* and downstream *lacZ* genes in the same translational reading frame. We define the ratio of expression of transformants of pMB38–Ty3 and pMB38–Ty3FF as the frequency of translational frameshifting.

pMB38–Ty3FF and other variants of the original pMB38–Ty3 were constructed using PCR. The general approach used, which has been described (Farabaugh et al., 1993), is to prime synthesis with an oligonucleotide that includes near its 5′ end either a BamHI or KpnI, to allow subcloning back into a pMB38-derivative plasmid, and that modifies the overlap in the way desired. Oligonucleotides having a BamHI site prime DNA synthesis from the overlap region toward the downstream *lacZ* gene; the corresponding second primer used in the PCR, termed 3′ to Sac (Farabaugh et al., 1993), primes synthesis from downstream of the unique SacI site in *lacZ*. Oligonucleotides having a KpnI site prime toward the upstream *HIS4* gene; they were used in PCR with an oligonucleotide, Sal-upstream (Belcourt and Farabaugh, 1990), that primes from upstream of a unique SalI site at the 5′ border of the *HIS4* promoter. The sequence of each of the mutagenic primers is given in Table 2.

To test whether overproduction of the tRNA specific for the serine codon AGU would affect frameshift efficiency, we cloned the $tRNA^{Ser}_{GCU}$ structural gene by PCR. The sequence of the gene was contributed to EMBL/GenBank by H. Feldmann (accession number X06992). We designed PCR primers that amplify the entire sequenced region (720 bp) flanked by SalI restriction sites. The PCR product was digested with SalI and inserted into the unique SalI site of pMB38–Ty3, immediately upstream of the *HIS4* promoter. The tRNA gene insertion was transferred by standard cloning methods into pMB38–Ty3FF.

The sequence of the peptide expressed from the Ty3 frameshift site was determined from a construct in which the frameshift was inserted near the 5′ end of the reporter *lacZ* fusion construct. The plasmid was constructed starting with the plasmid p3p (Belcourt and Farabaugh, 1990), which introduces a BamHI site beginning at the sixth nucleotide of the gene (i.e., ATG GTG GAT CC, shown as codons with the BamHI site underlined). The Ty3Δ1 truncated frameshift site

was inserted downstream of this BamHI site, using the unique BamHI and SacI (in *lacZ*) sites, to create a fusion gene in which the essential GCG AGU codons of the frameshift site are codons 9 and 10. The sequence of the first 15 codons of this construct is shown in Figure 2A.

Each overlap variant construct was sequenced by primer-directed sequencing using Sequenase version 2.0 (US Biochemical) according to manufacturer's specifications.

β-Galactosidase Purification and Protein Sequencing

The protocol for β-galactosidase purification from yeast was essentially as described (Belcourt and Farabaugh, 1990) with the following modifications. Yeast strain 387-1D, transformed with plasmid pTy3Δ1(PsK)-3p, was grown at 30°C in 8 liters of SD minimal media supplemented with 30 mg/l of histidine and tryptophan and 2% glucose. Cells grown to saturation were pelleted, and a cleared lysate was prepared. To cells resuspended in 200 ml of buffer A was added an equal volume of glass beads. Cells were broken in a Bead-Beater (Biospec Products) by 6 cycles of 45 s of grinding and 1 min of cooling. The lysate was cleared by centrifugation at 39,000 rpm in a Ti 50 rotor (Beckman) for 90 min. The cleared supernatant was collected and passed over an anti-β-galactosidase immunoaffinity column (Protosorb, Promega Biotech) equilibrated with buffer A, and eluted with 3 times 1 ml of high pH elution buffer (0.1 M $NaHCO_3$–NaOH [pH 10.8]). The eluent was concentrated with a Centricon 30 centrifuge chamber (Amicon) and washed four times with high performance liquid chromatography grade water. Since the eluent was contaminated with small molecular weight proteins, it was diluted into 5 ml of buffer A and again bound and eluted from the affinity column. The second eluent was again concentrated into high performance liquid chromatography 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.

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