RNA (2001), *7*:275–284. Cambridge University Press. Printed in the USA.

Programmed +1 frameshifting stimulated by complementarity between a downstream mRNA sequence and an error-correcting region of rRNA

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ABSTRACT

Like most retroviruses and retrotransposons, the retrotransposon Ty3 expresses its *pol* gene analog (*POL3*) as a translational fusion to the upstream *gag* analog (*GAG3*). The Gag3-Pol3 fusion occurs by frameshifting during translation of the mRNA that encodes the two separate but overlapping ORFs. We showed previously that the shift occurs by out-of-frame binding of a normal aminoacyl-tRNA in the ribosomal A site caused by an aberrant codon•anticodon interaction in the P site. This event is unlike all previously described programmed translational frameshifts because it does not require tRNA slippage between cognate or near-cognate codons in the mRNA. A sequence of 15 nt distal to the frameshift site stimulates frameshifting 7.5-fold. Here we show that the Ty3 stimulator acts as an unstructured region to stimulate frameshifting. Its function depends on strict spacing from the site of frameshifting. Finally, the stimulator increases frameshifting dependent on sense codon-induced pausing, but has no effect on frameshifting dependent on pauses induced by nonsense codons. Complementarity between the stimulator and a portion of the accuracy center of the ribosome, Helix 18, implies that the stimulator may directly disrupt error correction by the ribosome.

Keywords: mRNA-rRNA pairing; recoding; stimulator; translational accuracy

INTRODUCTION

During translational elongation, sequences in mRNAs can induce ribosomes to undergo unusual events termed recoding (reviewed by Gesteland & Atkins, 1996). These events include readthrough of termination codons, frameshifting, in which the reading frame changes by one or a few nucleotides, or translational hopping, in which the ribosome bypasses many nucleotides (reviewed by Gesteland & Atkins, 1996; Farabaugh et al., 2000). All of these events depend on special RNA sequences called recoding sites, which consist of the actual site of the noncanonical decoding event, as well as sequences near it that greatly increase its efficiency (stimulators). Stimulators either reduce the efficiency of continued normal decoding or increase the efficiency of a competing noncanonical event. The simplest form of stimulator is a pause-inducing codon. A ribosome cannot continue normal translational elongation if it cannot efficiently recruit the next aminoacyl-tRNA. In the frameshifts derived from Ty retrotransposons, poorly recognized codons cause the ribosome to pause with a frameshift-inducing codon in the P site (Belcourt & Farabaugh, 1990; Farabaugh et al., 1993). The length of that pause determines +1 frameshift efficiency, poorer recognition causing higher frameshifting (Kawakami et al., 1993; Vimaladithan & Farabaugh, 1994). Similarly, in the *Escherichia coli prfB* gene, encoding peptide release factor 2 (RF2), poorly recognized termination codons provide the pause that stimulates frameshifting (Craigen & Caskey, 1986; Donly et al., 1990). Slow recognition of an in-frame UGA termination codon, a codon recognized by RF2, during low availability of RF2 leads to increased frameshifting and therefore increased expression of the protein, completing an autogenous regulatory loop (Craigen et al., 1985; Craigen & Caskey, 1986; Donly et al., 1990). In general, pause codons reduce the kinetic advantage of continued normal decoding, allowing a more kinetically unfavorable alternative event.

Other stimulators appear to do more than simply cause the ribosome to pause. Downstream stimulators are more common. In the most common form of programmed frameshifting, −1 simultaneous slippage, a downstream secondary structure stimulates efficiency

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(Brierley et al., 1989). In eukaryotes, the sequence is nearly always a pseudoknot, whereas in prokaryotes, it is a stem-loop. The pseudoknots transiently pause the ribosome, though pausing is not sufficient to stimulate frameshifting (Tu et al., 1992; Somogyi et al., 1993). Presumably, frameshift-stimulating pseudoknots have some other function, which might explain why they have quite specific secondary structures (Shen & Tinoco, 1995; Chen et al., 1996; Liphardt et al., 1999; Napthine et al., 1999; Su et al., 1999). The nature of that function remains elusive.

The function of a third type of stimulator is clearer. High efficiency frameshifting in the *prfB* gene depends on the presence of a Shine–Dalgarno (SD) interaction site (Shine & Dalgarno, 1974) upstream of the frameshift site. With the ribosome paused at the frameshift site, an interaction between this site and the 16S rRNA appears to pull the ribosome into the shifted frame, causing the peptidyl-tRNA to slip +1 on the mRNA (Atkins et al., 1990; Weiss et al., 1990a). An SD interaction can also stimulate −1 frameshifting, for example in the *dnaX* gene of *E. coli*, with directionality of shifting depending on the distance between the SD and frameshift (Larsen et al., 1995). The programmed hop site of bacteriophage T4 gene *60* has a different type of upstream stimulatory site (Weiss et al., 1990b). A 48-nt upstream sequence encodes a 16 amino acid nascent peptide, which, by an unknown mechanism, stimulates hopping at the downstream site.

Although there are many examples of recoding site stimulators, the evidence to date suggests that all of them work by one of two general mechanisms. They either passively allow more of the alternative decoding event by reducing the rate of in-frame decoding, or they actively and directly promote the alternative event, for example an mRNA•rRNA interaction that forces a ribosome to slip on the mRNA (reviewed in Farabaugh, 1996; Gesteland & Atkins, 1996). In studying a stimulatory sequence derived from the retrotransposon Ty3 in the yeast *Saccharomyces cerevisiae*, we have found evidence for a third mechanism in which the stimulator modulates the accuracy of decoding in the A site.

In *S. cerevisiae*, we have identified a sequence downstream of a programmed +1 frameshift site that stimulates frameshifting about 7.5-fold (Farabaugh et al., 1993). This sequence occurs in the retrotransposon Ty3, a transposable genetic element distantly related to retroviruses. The frameshift event occurs at a site between the *GAG3* and *POL3* genes of the element. The protein produced by this frameshift is the Gag3-Pol3 fusion protein, analogous to the gag-pol fusion expressed by retroviruses. We had previously identified a programmed frameshift site in the related retrotransposon Tyl, which occurs between the *gag-pol* homolog *TYA* and *TYB* genes (Belcourt & Farabaugh, 1990). The two Ty elements employ two quite distinct frameshifting mechanisms. In Tyl, frameshifting occurs when a peptidyl-tRNA$^{Leu}_{UAG}$ slips +1 between CUU and UUA Leu codons (Fig. 1A). Slippage occurs during a translational pause induced by the slow recognition of the next in-frame codon, AGG encoding arginine (Belcourt & Farabaugh, 1990; Kawakami et al., 1993). The sequence CUU-AGG-C is necessary and sufficient to allow 40% frameshifting.

Frameshifting in Ty3 occurs while a peptidyl-tRNA^{Ala} decodes a GCG codon during slow recognition of the next codon, AGU, as shown in Figure 1B (Farabaugh et al., 1993). What is different about this event compared with the Tyl frameshift is that peptidyl-tRNA^{Ala} cannot slip +1 at the site. Were the tRNA to slip, it would not be able to make the 2-bp interaction with the mRNA that the preponderance of the data shows is necessary for slippage (reviewed by Farabaugh, 1996). This fact implies that frameshifting occurs by out-of-frame binding of aminoacyl-tRNA to a +1 shifted codon in the ribosomal A site. Frameshifting on the heptameric recoding site GCG-AGU-U is only 2% efficient, but a 14-nt downstream sequence stimulates the event up to 17%. In a previous article (Farabaugh et al., 1993) this region was termed the Ty3 "context," but to adopt the standard nomenclature proposed by Gesteland, Weiss, and Atkins (1992), we will refer to the site as the Ty3 stimulator. Here we address the role played by the stimulator in Ty3 frameshifting.

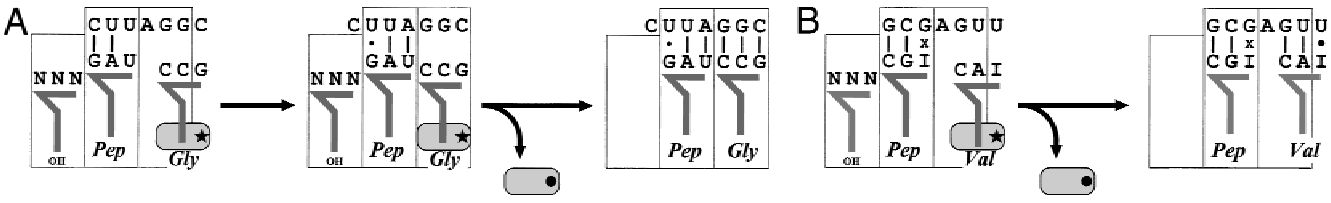

FIGURE 1. Two mechanisms of programmed +1 frameshifting in *S. cerevisiae*. **A**: Slippage frameshifting: At the frameshift site, CUU-AGG-C, peptidyl-tRNA$^{Leu}_{UAG}$ forms two base pairs with CUU, and slips +1 onto UUA probably during transient occupation of the A site by the +1 frame tRNA$^{Gly}_{GCC}$. The oval represents eIF-1A, and the black star and circle GTP and GDP, respectively. **B:** Nonslippage frameshifting: In this case, slippage by the peptidyl-tRNA$^{Ala}_{IGC}$ is impossible because it cannot form base pairs in the shifted frame. Instead, it appears to allow out-of-frame binding of tRNA$^{Val}_{IAC}$, apparently because the I•G wobble clash reduces the efficiency of in-frame decoding. The open boxes in each panel represent the three decoding sites of the ribosome, from right to left the A (aminoacyl), P (peptidyl), and E (exit) sites.

RESULTS

The influence of a 14-nt downstream sequence on programmed +1 frameshifting

Our analysis of the sequence requirements for +1 frameshifting in the retrotransposon Ty3 began with our mutagenesis of the 38-bp overlap between the *GAG3* and *POL3* genes (Farabaugh et al., 1993). This analysis established that the last 21 nt of the overlap were necessary for highly efficient frameshifting. Deletions successively removing 3-nt codons from the 3′ end of this region progressively reduced frameshifting to a level about sixfold lower when only a 7-nt sequence remained, GCG-AGU-U (Farabaugh et al., 1993). Intensive mutagenesis and sequencing of the protein across the frameshift site identified this 7-nt sequence as both essential and the site of frameshift event. The downstream region, which is important but not essential, is the Ty3 stimulator. At the time, we did not perform extensive functional analysis of the stimulator. Here we return to that issue to consider the structure and function of the Ty3 stimulator.

Our initial work concerned only the 14 nt downstream of the essential heptameric frameshift site. Larger regions of downstream sequence cause no appreciable increase in frameshift efficiency when they are included in our reporter construct (data not shown). Because distal sequences are dispensable, we focused our analysis on the 14-nt sequence.

The stimulator effect is not expressed through the nascent protein

Bacteriophage T4 gene *60* provides a precedent for a flanking sequence effect involving nascent peptide sequences (Weiss et al., 1990b). The ribosome clearly translates the Ty3 stimulator into a protein product, so we cannot a priori eliminate the possibility of a primary peptide sequence effect. The model may seem unlikely because the ribosome translates the peptide only after it commits itself to frameshifting. Frameshifting ribosomes pause at the site of frameshifting, and pausing is known to cause ribosomes to queue up on the mRNA (Wolin & Walter, 1988). Therefore, the nascent peptide expressed on ribosome might influence the frameshift efficiency of an immediately trailing ribosome.

To test this hypothesis we constructed a series of reporter plasmids replacing codons in the stimulator without changing the protein sequence encoded (see Table 1). The analysis is complicated by the fact that the ribosome can encode two different sequences depending on whether or not it frameshifts. To exclude an effect by either of these two forms, we constructed two mutated versions of the stimulator retaining either the zero frame peptide (Ser-Asn-Arg-Ser, or SNRS) or the +1 frame peptide (Leu-Thr-Asp-Leu, or LTDL). The normal construct for each of these mutants was a minimal frameshift site derived from the plasmid pMB38-Ty3, which retains the last 24 nt of the Ty3 GAG3/POL3 overlap (Farabaugh et al., 1993). Neither the construct retaining the zero frame nascent peptide (pMB38-Ty3Δ2-SNRS; Table 1, line 3) or the +1 frame nascent peptide (pMB38-Ty3Δ2–LTDL; Table 1, line 2) stimulated more frameshifting than the construct lacking the Ty3 stimulator (pMB38-Ty3Δ5; Table 1, line 4).

These data eliminate the hypothesis that frameshift stimulation requires the primary peptide sequence encoded in either frame. The stimulator must therefore function as a nucleic acid structure. Given its small size, it is likely that the stimulator functions as a primary sequence rather than a secondary structure. In

TABLE 1. The context is not a nascent peptide sequence.

Plasmid	Sequence[a]	Frameshifting (%)[b]
pMB38–Ty3Δ2	GUGAAGGCGAGUUCUAACCGAUCUUGAG V K A S S N R S * * R R\V L T D L E	14.8
pMB38–Ty3Δ2–LTDL	GUGAAGGCGAGUUCUcACgGAcCUgacUGAG V K A S S h g p d * * R R\V L T D L t E	3.3
pMB38–Ty3Δ2–SNRS	GUGAAGGCGAGUUCgAAuaGgagcUagG V K A S S N R S * * R R\V r i g a r	1.0
pMB38–Ty3Δ5	GUGAAGGCGAGU----------UGAG V K A S - - - - * * R R\V - - - - E	2.4[c]

[a]The sequence derived from the overlap is shown. Below the sequence are the predicted translation products in the 0 and +1 frame; the frameshift product is underlined. Changes from the wild-type RNA or protein sequence are shown in lowercase.

[b]All assays presented in this article had standard errors of the mean below 10%.

[c]Farabaugh et al. (1993).

fact, computer analysis predicts no stable structure for the stimulator (data not shown). It is formally possible that the stimulator forms a inducing long-range structure, though in these experiments, that would have to involve a fortuitous sequence downstream within the *lacZ* reporter; we have no evidence for such a structure. An argument against that explanation derives from our replacing the *lacZ* reporter with the *CUP1* gene of yeast, which encodes copper metallothionein, and quantifying frameshifting by *CUP1*-dependent resistance to the lethal effects of Cu^{2+}. We found no difference in the behavior of the stimulator using this unrelated reporter (H. Zhao & P.J. Farabaugh, unpubl. data). It is difficult to unequivocally eliminate a model dependent on long-range secondary structures, but these data strongly argue against it.

The stimulator's effect requires a specific distance to the recoding site

Cis-acting sites that stimulate frameshifting invariably are strictly spaced from the frameshift site. In −1 simultaneous slippage frameshifting, inserting as few as 2 nt between the frameshift site and the downstream secondary structure eliminates activation (Brierley et al., 1992). Spacing changes between the frameshift site and the upstream SD site in the *prfB* gene can have similar effects (Weiss et al., 1988). Does the Ty3 stimulator exhibit a similar requirement?

We tested this by introducing a single UGA codon between the recoding site and the stimulator. The construct mimics the structure of a construct in which the stimulator was completely deleted, pMB38-Ty3Δ5, used as the control in the previous experiment. That mutation in effect replaces the 14-nt stimulator with a single UGA codon eliminating the stimulators effect on frameshifting (Farabaugh et al., 1993 and data not shown). Reintroduction of the stimulator immediately downstream of the UGA codon in pMB38-Ty3Δ2 (+UGA) (Table 2, line 2) caused no further increase in frameshifting (compare lines 2 and 5 in Table 2). This result shows that the stimulator must maintain a strict spacing from the recoding site to stimulate frameshifting.

To determine how critical the spacing is, we created a second mutation by inserting a single nucleotide between the recoding site and the stimulator (Ty3Δ2G(+C); Table 2, line 4). This insertion shifts the stimulator 1 nt away from the recoding site, but also changes the reading frames read by frameshifting or nonframeshifting ribosomes as they pass through the stimulator. In particular, the insertion shifts the termination codon of the *GAG3* gene into the +1 reading frame so that ribosomes that frameshift at the recoding site terminate soon thereafter. To use the *lacZ* reporter to measure frameshift efficiency, we removed the terminator (UGA → UGG). To control for any effect this mutation might have on stimulation, we introduced the same mutation into a construct in which the spacing between stimulator and recoding site was normal (Ty3Δ2G; Table 2, line 3). Changing the UGA to UGG slightly reduced stimulation (compare lines 1 and 3 of Table 2). However, shifting the stimulator by 1 nt eliminated this stimulation (compare lines 3 and 4 of Table 2), reducing frameshifting to slightly lower than from a construct lacking the stimulator (compare lines 4 and 5 of Table 2). This result demonstrates that the spacing between the stimulator and recoding site is very critical, because insertion of even a single nucleotide eliminates frameshift stimulation.

TABLE 2. Altering spacing from the frameshift site eliminates context stimulation.

Plasmid	Sequence	Frameshifting (%)
pMB38–Ty3Δ2	GUGAAGGCGAGUUCUAACCGAUCUUGAG V K A S S N R S * * R R\V L T D L E	14.8
pMB38–Ty3Δ2(+UGA)	GUGAAGGCGAGUugaUCUAACCGAUCUUGAG V K A S * * R R\V d L T D L E	1.8
pMB38–Ty3Δ2G	GUGAAGGCGAGUUCUAACCGAUCUUGgc V K A S S N R S W * R R\V L T D L g	8.9
pMB38–Ty3Δ2G(+C)	GUGAAGGCGAGUcUCUAACCGAUCUUGg V K A S l * * R R\V s n r s w	1.5
pMB38–Ty3Δ5	GUGAAGGCGAGU----------UGAG V K A S - - - - * * R R\V - - - - E	2.4

The proximal two-thirds of the stimulator is more important

Progressive deletion of the stimulator from the 3′ side caused progressive loss of frameshift stimulation (Farabaugh et al., 1993). These data imply that all portions of the 14-nt region contribute to frameshift stimulation, but the analysis is too crude to allow us to draw a firm conclusion. We therefore created a series of missense mutations altering nucleotides across the entire stimulator, substituting single or several adjacent nucleotides. We constructed the mutations using a polymerase chain reaction strategy, as described in Materials and Methods. Table 3 shows that most of the mutations reduce frameshifting from two- to eightfold. Mutations in the first half of the stimulator reduced frameshifting in all but two cases. Mutations affecting the 6 nt at the 3′ end of the stimulator generally had no effect on frameshifting, though one (18G) increased frameshifting almost twofold, and two others (16A2 and 20C21U) reduced it about twofold.

These data show that sequences across the entire stimulator are important or essential to frameshift stimulation. However, the clear implication of the data is that the stimulator's 5′ half is more important than its 3′ half. Because single nucleotide changes in the 5′ half of the stimulator can eliminate stimulation (reducing it to the level of a completely deleted construct), the primary sequence in this region must be extremely important to stimulation. It would appear that the primary sequence in the 3′ half is not as important, though the fact that multiple substitutions in this region reduce frameshift stimulation implies that it is required for maximal stimulation. These data are consistent with a model in which stimulation depends on the ability of the 5′ half of the stimulator to base pair with an unknown target. It would be premature to conclude that the 3′ half does not need to base pair, but the data would be consistent with a less sequence-specific effect of the 3′ half of the stimulator.

TABLE 3. Missense mutagenesis of the Ty3 context.

Mutation	Sequence[a]	Frameshifting (%)
Wild type	GCGAGUUCUAACCGAUCUUGA	16
8U	gcgaguuUuaaccgaucuuga	5.3
9A	gcgaguucAaaccgaucuuga	6.5
9C	gcgaguucCaaccgaucuuga	1.9
10C	gcgaguucuCaccgaucuuga	9.7
10U	gcgaguucuUaccgaucuuga	16
11C	gcgaguucuaCccgaucuuga	17
13G	gcgaguucuaacGgaucuuga	7.5
13G14C	gcgaguucuaacGCaucuuga	4.4
14C	gcgaguucuaacCaucuuga	7.9
14C15U	gcgaguucuaaccCUucuuga	2.9
16A17G	gcgaguucuaaccgaAGuuga	17
16A2	gcgaguucuaaccgaAGAAga	10
18A19A	gcgaguucuaaccgaucAAga	16
18G	gcgaguucuaaccgaucGuga	27
18G2	gcgaguucuaaccgaucGACa	17
20C21U	gcgaguucuaaccgaucuuCU	7.8

[a]The sequence of the stimulator is underlined in line 1, wild type, and introduced mutations are capitalized and underlined in all other lines.

The stimulator increases +1 frameshifting dependent on "hungry" sense but not nonsense pause codons

Our study of programmed +1 frameshifting in *S. cerevisiae* provides many tools to study the mechanism of the stimulation. There are two forms of +1 programmed frameshift sites, one derived from the Tyl retrotransposon (Belcourt & Farabaugh, 1990) and one derived from Ty3 (Farabaugh et al., 1993). The Tyl frameshift occurs by slippage of peptidyl-tRNA during slow recognition of the next in-frame codon in the A site (Belcourt & Farabaugh, 1990). The Ty3 event appears to occur without slippage by out-of-frame recruitment of a cognate aminoacyl-tRNA in the A site (Farabaugh et al., 1993). Because the maximum efficiency frameshifting at the Ty3 site requires the downstream 14-nt stimulator, we hypothesized that the stimulator would be specific for frameshifting by the unusual out-of-frame binding mechanism. To test this, we compared stimulation of reporter constructs using the first codon of the Ty3 frameshift site, GCG, which directs out-of-frame recruitment (Farabaugh et al., 1993) or the codon from Tyl, CUU, which can allow peptidyl-tRNA slippage (Belcourt & Farabaugh, 1990). In addition, we made reporter constructs using the strong pause-inducing sequence of either the Tyl site (AGG-C) or the weaker sequence derived from Ty3 (AGU-U). Surprisingly, we found that the stimulator increased both types of frameshifting, as shown in Table 4 (lines 1 to 4). The absolute levels of frameshifting in the absence of the stimulator varied among the four sites used from a low of 2.4% to a high of 32%. The difference in frameshifting results

TABLE 4. The context only stimulates frameshifting dependent on pausing at sense codons.

	Frameshifting (%)	
Frameshift site	Without context	With context
GCG–AGU–U	2.4	15
GCG–AGG–C	5.9	31
CUU–AGU–U	4.1	23
CUU–AGG–C	32	71
GCG–AGU–U	2.4	15
GCG–AGG–C	5.9	31
GCG–UGG–C	1.5	19
GCG–UAG–C	12	12
GCG–UAA–C	30	48
GCG–UGA–C	37	31

from the stronger frameshift induction by CUU as compared to GCG (Farabaugh et al., 1993) and the stronger stimulatory effect of AGG-C than that of AGU-U (Pande et al., 1995). The effect of the stimulator in each case was to increase frameshifting drastically. The fold induction for the three weaker frameshift sites was about sixfold. Frameshifting on the stronger site, CUU-AGG-C increased a more modest 2.2-fold. For this construct, the smaller stimulation results both from its strength in the absence of the stimulator and the fact that a maximum mathematical effect of only threefold is possible. We have also noted a lack of linearity in our assay at high frameshift efficiencies (Vimaladithan & Farabaugh, 1994 and data not shown), which would cause an underestimation of stimulation. The clear implication of these data is that the stimulator is a general stimulator of programmed +1 frameshifting.

Programmed +1 frameshifting depends on a translational pause provided either by a slowly decoded sense codon (Belcourt & Farabaugh, 1990; Farabaugh et al., 1993) or a poorly recognized termination codon (Donly et al., 1990; Curran, 1993). The AGU codon that stimulates the pause at the Ty3 site can be replaced by five other codons: AGG, UGG, UAG, UAA, and UGA (Pande et al., 1995). Therefore, three sense codons (AGU, AGG, and UGG) and three termination codons (UAG, UAA, and UGA) can provide the pause. The low availability of the tRNAs recognizing the sense codons appears to stimulate frameshifting. This has been shown explicitly by showing that overexpressing the tRNA reduces frameshifting for AGG (Belcourt & Farabaugh, 1990), AGU (Farabaugh et al., 1993), and UGG (H. Zhao & P.J. Farabaugh, unpubl. data). Similarly, deleting the gene encoding the tRNA recognizing the AGG codon, forcing it to be read more inefficiently by a near-cognate isoacceptor, stimulates frameshifting (Kawakami et al., 1993; Vimaladithan & Farabaugh, 1994). Frameshift-inducing termination codons are also poorly recognized by peptide release factor; enhancing or restricting their recognition by peptide release factor (RF) also similarly affects efficiency (Donly et al., 1990; Tate & Brown, 1992).

To characterize further the effect of the Ty3 stimulator, we tested its effect on sites using each of these six pause codons. To do this, we constructed a set of matched *lacZ* fusions using each of the pause codons with and without the downstream stimulator. In accord with our previous results (Pande et al., 1995), all of these frameshift sites stimulate measurable levels of frameshifting in the absence of the stimulator (see column 1 of Table 4). The efficiency of frameshifting varied among the constructs, the three sense codons stimulating less frameshifting than the three termination codons. Combining the Ty3 stimulator with the frameshift sites including sense pause codons resulted in substantially increased frameshifting, increasing from 5.3- to 13-fold (lines 5 to 7 of Table 4). Surprisingly, the stimulator caused no significant or consistent increase in frameshifting with the constructs employing nonsense pause codons (lines 8 to 10 of Table 4). For UAG, there was no effect, whereas for UAA there was a slight 1.6-fold increase in frameshifting and for UGA an actual 1.2-fold decrease.

These data show that the stimulator can distinguish between sense and nonsense codons in the pause position of the frameshift site. Frameshifting occurs at a time when the ribosomal A site is empty and about to be filled with aminoacyl-tRNA. These results suggest that the stimulator distinguishes between inefficient binding of aminoacyl-tRNA to the A site (perhaps by affecting the efficiency of decoding) and inefficient binding of RF to the same site.

DISCUSSION

The results of the experiments described here significantly limit the possible models to explain the frameshift-inducing effect of the Ty3 stimulator. The stimulator appears unable to form a stable secondary structure, arguing against stimulation by a structure that blocks ribosome progress. We have specifically tested the alternative hypothesis that stimulation requires the primary protein product expressed from the stimulator in either the zero or +1 reading frames. This model is clearly incorrect, leaving the hypothesis that the primary sequence of the stimulator increases frameshifting.

The fact that the stimulator must be spaced a precise distance downstream from the ribosomal decoding sites argues that the geometry of its placement on the ribosome must be important. This effect is reminiscent of the effect of altering spacing between SD interaction sites and frameshift signals in bacteria (Weiss et al., 1987; Larsen et al., 1994), and of pseudoknots and frameshift signals in eukaryotes (Brierley et al., 1992). Quite small changes can have drastic effects on frameshift stimulation by these structures. Changing the spacing between the SD site and the frameshift site in the *prfB* +1 frameshift site in *E. coli* reduced frameshifting as much as 17-fold, and spacing mutants reduced −1 frameshifting on the *dnaX* site up to 25-fold (Weiss et al., 1987; Larsen et al., 1994). The effect of altering spacing between the slip site and pseudoknot of simultaneous slippage sites is rather smaller but still a quite significant effect (e.g., see Brierley et al., 1992). The mechanism by which pseudoknots stimulate frameshifting remains a mystery, despite recent progress in defining structures of frameshift-stimulating pseudoknots (Shen & Tinoco, 1995; Chen et al., 1996; Liphardt et al., 1999; Napthine et al., 1999; Su et al., 1999), but we do have a model for the action of SD sites (Weiss et al., 1987; Curran & Yarus, 1988). Base pairing between the SD site and the 3′ end of 16S rRNA appears to occur during frameshifting much as it does during initiation site selection. The spacing between the SD site and

the frameshift signal is different than the optimum used in initiation, suggesting that mRNA•rRNA base pairing strains the ribosome, with tRNA slippage allowing the mRNA to move so as to reduce the strain. Clearly, changing the spacing between the SD site and the frameshift signal could reduce this strain, reducing the stimulatory effect of the SD site.

A strict spacing requirement for the Ty3 stimulator is consistent with the hypothesis that it interacts with the ribosome to stimulate frameshifting. Because the effect of the stimulator appears to depend on its primary sequence, the simplest model is that it base pairs with a segment of the rRNA. In vitro crosslinking studies confirmed that the region of the SD site is in close contact with the 3′ end of 16S rRNA during initiation (Stade et al., 1989; Dontsova et al., 1991), as predicted by Shine and Dalgarno (1974). Similar studies (Rinke-Appel et al., 1993) have identified an in vitro crosslink between a position 4–6 nt downstream of the A site and nucleotide 532 of the 16S rRNA (corresponding to 579 in yeast 18S rRNA). This crosslink is interesting because the structure including this base, termed the 530 loop or Helix 18, is implicated in regulating the accuracy of decoding in the A site (Powers & Noller, 1994). Importantly, the sequence of the loop is identical between bacteria, yeast, and humans and closely related in the Archea; it is one of the most highly conserved structures in rRNA. Given this conservation and the critical importance of the loop to ribosomal accuracy, it is very likely that position of the structure in eukaryotic ribosomes is identical. A comparison of the Ty3 stimulator and the yeast Helix 18 shows that they are partially complementary (Fig. 2). More importantly, this complementarity is virtually in register with the bacterial crosslinking data. Though the pairing appears weak, the crosslink data suggests that translation elongation juxtaposes the two sequences extremely closely.

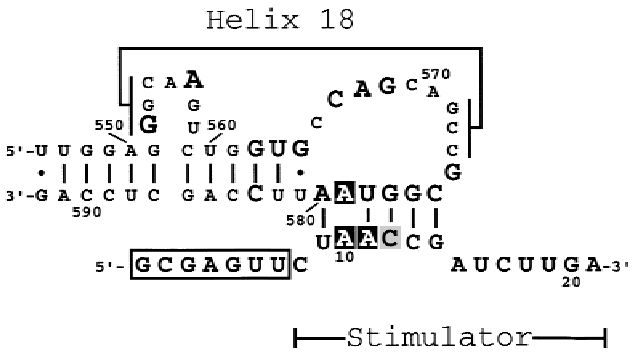

FIGURE 2. A possible mRNA•rRNA pairing scheme. The context is complementary to a part of the 530 loop, or Helix 18, of 18S rRNA. Nucleotides of rRNA that are conserved in all three domains (Eukarya, Prokarya, and Archaea) are shown in larger letters. White letters on black background represent the nucleotides in the rRNA and mRNA that can be efficiently crosslinked and black letters on grey background represent the nucleotides that are crosslinked much less efficiently (Dontsova et al., 1992).

The crosslinks between the mRNA and rRNA are of "zero length," so that the nucleotides are in direct contact with each other (Rinke-Appel et al., 1994). With direct contact, and complementarity nearly in register, the forward rate of binding should be virtually instantaneous, giving an apparently extremely high K_d even if the $t_{1/2}$ of binding were short. Recently, structures of the 30S ribosome of *Thermus thermophilus* were solved (Schluenzen et al., 2000; Wimberly et al., 2000). This structure, combined with the data from the 7.8 Å structure of 70S functional ribosome•mRNA•tRNA structures (Cate et al., 1999), identify a region termed the latch, a deep notch in the 30S structure between its head and shoulder. The mRNA enters the ribosome through the latch as it passes toward the decoding center. Helix 18 projects from the shoulder to form part of the surface of the latch, putting it in close contact with the mRNA downstream of the decoding center, as predicted by the crosslinking data (Schluenzen et al., 2000). The nucleotides of Helix 18 predicted to base pair with the mRNA are at the surface of the latch close to the mRNA (Fig. 3). This structural information makes the model of mRNA•Helix 18 base pairing very plausible.

The missense mutant data provides a measure of the plausibility of this model. If this model were correct, then mutations that affect putative base-paired nucleotides should have predicted effects on frameshift stimulation. Table 5 shows a correlation between phenotypes and predicted effects on base pairing. Five of the eight relevant mutations are completely consistent with the model, that is, mutations predicted to decrease stability of the interaction reduce the stimulator's effect (mutants 9A, 9C, 13G, 13G14C, and 14C). Two mutations affecting the A at position 10 of the stimulator have different effects. Mutation 10C slightly reduces stimulation, whereas 10U has no effect. The mutations would replace an A•A pair with either a U-A or a C-A pair. In Figure 3, position 10 is diagrammed as not pairing with the rRNA, but since non-Watson–Crick pairing occurs frequently in RNA structures (Westhof & Fritsch, 2000) a non-Watson–Crick A•A pair may actually form. The lack of effect of mutation 10U may mean that replacing this pair with a Watson–Crick A-U pair does not affect function, whereas mutation 10C slightly reducing stimulation may mean that a non-Watson–Crick C•A pair is less able to replace an A•A pair. The final mutation, 11C, replaces an A-U pair with C U, which are not expected to base pair. Despite the prediction that this mutation would reduce overall stability, it has no effect on stimulation, which is inconsistent with the model. It is conceivable that the lack of pairing at this central position does not significantly effect function. Though more mutations are necessary to be completely certain, the overall impression is that the mutagenesis is consistent with the pairing model. Experiments in progress should provide a more definitive genetic test of the model.

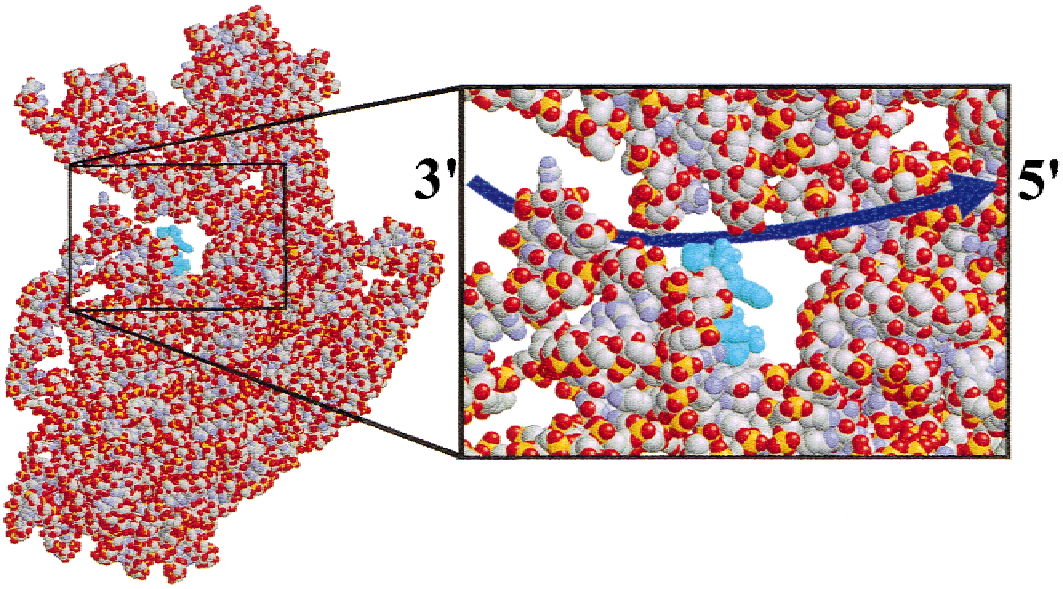

FIGURE 3. The mRNA passes near Helix 18 as it enters the ribosome. The blue arrow represents the approximate pathway of mRNA on the 30S ribosomal subunit of *T. thermophilus* based on published X-ray crystallographic data (Cate et al., 1999; Schluenzen et al., 2000; Wimberly et al., 2000). The residues corresponding to nt 576–581 of yeast 18S rRNA are colored light blue in the figure. The figure was prepared using RasMac using the X-ray crystallography coordinate file 1FJF (Wimberly et al., 2000) downloaded from the Protein Data Bank (PDB) maintained by the Research Collaboratory for Structural Bioinformatics (RCSB).

How could the ability of the stimulator to pair with this loop of the rRNA explain frameshift stimulation? Powers and Noller (1994) have hypothesized that Helix 18 undergoes a structural transition between hairpin and pseudoknot form in response to the presence of cognate or noncognate tRNAs in the ribosomal A site. With the pseudoknot ("closed"), which forms when cognate tRNAs occupy the A site, the ribosome has high affinity for EF-1A•GTP and low affinity for EF-1A•GDP. The hairpin ("open"), formed when the tRNA in the A site is not cognate, gives the ribosome low affinity for EF-1A•GTP and high affinity for EF-1A•GDP. The closed form retains aminoacyl-tRNA in the A site by stabilizing aminoacyl-tRNA•EF-1A•GTP ternary complexes and, after GTP hydrolysis, by allowing rapid dissociation of EF-1A•GDP and retention of aminoacyl-tRNA. The open form would promote the opposite effect, allowing dissociation of the GTP ternary complex and retention of EF-1A•GDP, which would allow dissociation of aminoacyl-tRNA. If the Helix 18 were unable to undergo the open-to-closed transition, the ribosome could not preferentially select cognate aminoacyl-tRNA, leading to translational errors. We propose that base pairing between the Ty3 stimulator and the loop interferes with the transition between the two forms, causing increased frameshift errors at the programmed site.

TABLE 5. Correlation of missense mutations with the predicted mRNA•rRNA pairing scheme.

	Predicted base pairing[a]			
Mutation	WT	Mutant	Predicted effect	Observed effect
9A	U-A	A A	Down	↓ 2.5 ×
9C	U-A	A C	Down	↓ 8.0 ×
10C	A A	C A	None?[b]	↓ 1.6 ×
10U	A A	U-A	Up?[b]	None
11C	A-U	C U	Down	None
13G	C-G	G G	Down	↓ 2.1 ×
13G14C	C-G	G G	Down	↓ 3.6 ×
	G-C	C C		
14C	G-C	C C	Down	↓ 2.0 ×

[a]Base pairs shown with mRNA on the left, rRNA on the right.
[b]See text.

MATERIALS AND METHODS

Yeast strains, media, and general methods

The *S. cerevisiae* strain used for this work is 387-1D (*MAT***a** *his4 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). 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). Briefly, the assays are done of at least three independent transformants with three replicates of each. The standard error of the mean of all data presented is less than 10%. Oligonucleotides were synthesized on an Expedite 8909 DNA synthesizer (Applied Biosystems) according to the manufacturer's directions.

Plasmid constructions

All plasmids used in this study are derivatives of pMB38 (Belcourt & Farabaugh, 1990), a 2μ-*URA3*-based shuttle vector carrying a *lacZ* gene used to report expression dependent on +1 frameshifting. The plasmid carries a triple gene fusion. The yeast *HIS4* gene is fused to the *E. coli lacZ* gene through an intervening oligonucleotide, which includes a potential +1 translational frameshift site. Translation initiates at the normal *HIS4* start site and proceeds into the Ty3 frameshift site; ribosomes that shift +1 then continue into *lacZ*, producing β-galactosidase, whereas ribosomes which do not shift terminate at an in-frame UGA codon immediately downstream. β-galactosidase activity was determined as described (Farabaugh et al., 1989). To determine the efficiency of frameshifting, we compared expression of the frameshift constructs to that of a construct, pMB38-Ty3FF (Farabaugh et al., 1993), in which a single nucleotide within the frameshift region was deleted, putting lacZ in frame with *HIS4*. By definition, frameshift efficiency is the ratio of expression of the frameshift to the frame fusion construct.

Unique restriction sites, *Bam*HI site (upstream) and a *Kpn*I site (downstream), flank the frameshift region in pMB38, and unique sites exist upstream of the *HIS4* promoter (*Sal*I) and within *lacZ* (*Sac*I). These restriction sites allow us to alter the frameshift site by inserting appropriate PCR fragments as described (Farabaugh et al., 1993). Briefly, construction of each mutation requires a PCR primer extending from either the *Bam*HI or *Kpn*I site through the sequence to be altered to either the *Sal*I or *Sac*I site. PCR in combination with a oligonucleotide that primes synthesis far outside the recoding region produces a DNA fragment that can be inserted to replace a unique *Sal*I/*Kpn*I or *Bam*HI/*Sac*I fragment of the reporter plasmid. Insertion of the fragment introduces the novel sequence of the mutated recoding site.

ACKNOWLEDGMENTS

We thank Hong Zhao for her technical assistance. Grants from the National Institute of General Medical Sciences (GM 29480) and the Fogarty International Center (TW02211-01) of the National Institutes of Health, and from the Swedish Cancer Foundation (3717-B95A)1VAA) supported this work.

Received September 21, 2000; returned for revision October 17, 2000; revised manuscript received November 7, 2000

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