Saturation mutagenesis of a +1 programmed frameshift-inducing mRNA sequence derived from a yeast retrotransposon

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ABSTRACT

Errors during the process of translating mRNA information into protein products occur infrequently. Frameshift errors occur less frequently than other types of errors, suggesting that the translational machinery has more robust mechanisms for precluding that kind of error. Despite these mechanisms, mRNA sequences have evolved that increase the frequency up to 10,000-fold. These sequences, termed programmed frameshift sites, usually consist of a heptameric nucleotide sequence, at which the change in frames occurs along with additional sequences that stimulate the efficiency of frameshifting. One such stimulatory site derived from the Ty3 retrotransposon of the yeast *Saccharomyces cerevisiae* (the Ty3 stimulator) comprises a 14 nucleotide sequence with partial complementarity to a Helix 18 of the 18S rRNA, a component of the ribosome's accuracy center. A model for the function of the Ty3 stimulator predicts that it base pairs with Helix 18, reducing the efficiency with which the ribosome rejects erroneous out of frame decoding. We have tested this model by making a saturating set of single-base mutations of the Ty3 stimulator. The phenotypes of these mutations are inconsistent with the Helix 18 base-pairing model. We discuss the phenotypes of these mutations in light of structural data on the path of the mRNA on the ribosome, suggesting that the true target of the Ty3 stimulator may be rRNA and ribosomal protein elements of the ribosomal entry tunnel, as well as unknown constituents of the solvent face of the 40S subunit.

Keywords: *Saccharomyces cerevisiae*; programmed frameshifting; ribosome structure

INTRODUCTION

The ribosome is a large ribonucleoprotein complex that orchestrates the process of protein synthesis from an mRNA template (Green and Noller 1997; Ogle et al. 2003; Wintermeyer et al. 2004; Nilsson and Nissen 2005). The extension of a growing polypeptide chain proceeds by sequential recruitment of aminoacyl-tRNAs (aa-tRNAs) as a ternary complex with elongation factor 1A and GTP (aa-tRNA•EF-1A•GTP) to a binding site on the ribosome, the A site. The ribosome then transfers the growing peptide chain to the aminoacyl-tRNA from a peptidyl-tRNA bound to

the P site. The translational machinery employs multiple mechanisms to reduce the frequency of errors during protein synthesis, discriminating between correct (cognate) and incorrect (near or noncognate) aa-tRNAs by a process involving both kinetic proofreading (Hopfield 1974; Ninio 1975) and an induced fit mechanism (for review, see Rodnina et al. 2005). Kinetic proofreading can select against incorrect aa-tRNAs because of their rapid dissociation during two successive selection steps before and after GTP hydrolysis by EF-1A (for review, see Thompson 1988). The greatest effect on accuracy, however, appears not to result from these kinetic effects but rather from the ability of cognate aa-tRNAs to induce a change in ribosome structure increasing the rate of EF-1A GTPase activation and thus accelerating their acceptance relative to incorrect aa-tRNAs (for review, see Rodnina et al. 2005).

Despite these mechanisms, translation is not completely accurate partly because of the need to maximize translational output. For example, to achieve an elongation rate of 10 amino acids per second while still allowing correct tRNAs to outcompete the vast excess of incorrect tRNAs,

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the intrinsic rate of GTP hydrolysis must be extremely fast (Gromadski and Rodnina 2004). The reaction is kept far enough from equilibrium that differences in intrinsic stability cannot increase accuracy. Thus, maximizing protein output through GTP activation fails to maximize accuracy (Gromadski and Rodnina 2004).

The error rate is far from constant; it varies significantly with changes in mRNA sequence context (for review, see Parker 1989). The origin of context effects remains largely unexplained. Some variation could result from differences in the interaction between the mRNA and the ribosome's accuracy center. An extreme form of context effect occurs at mRNA sequences, termed "recoding sites" (Gesteland et al. 1992), which induce translational errors at a defined site. Recoding encompasses a variety of phenomena including programmed readthrough of nonsense codons, frameshifting, and bypassing of several codons (Farabaugh 1996; Namy et al. 2004). Errors at recoding sites occur at rates approaching 50% per codon (Farabaugh 1996; Namy et al. 2004), three orders of magnitude greater than the background rate of $\sim 5 \times 10^{-4}$ per codon (Parker 1989).

Programmed frameshifting occurs typically by shifts of 1 nucleotide (nt) in either the 3' direction with respect to the mRNA (e.g., programmed -1 frameshifting) or the 5' direction (e.g., programmed $+1$ frameshifting). Programmed $+1$ frameshifting in *Saccharomyces cerevisiae* occurs at special heptameric sites composed of codons that occupy the ribosomal P and A sites during the frameshift. Frameshifting requires that during the shift a near-cognate peptidyl-tRNA occupy the P site codon that forms a weak pyrimidine•pyrimidine or a purine•purine third position ("wobble") pair (Sundararajan et al. 1999). We have hypothesized that the unusual pairing in the P site disturbs the suite of interactions between the codon•anticodon complex and the ribosome and interferes with acceptance of even perfect cognate tRNAs in the A site (Stahl et al. 2002). This effect would eliminate the nearly absolute preference for in-frame cognate tRNAs and increase the probability of frameshifting by recruitment of a cognate tRNA in the $+1$ frame. The ability of the P site tRNA to slip $+1$ on the mRNA further increases frameshift efficiency (Belcourt and Farabaugh 1990; Sundararajan et al. 1999). The requirement for slippage is not absolute; frameshifting can also occur by out-of-frame recruitment of aa-tRNA without slippage (Farabaugh et al. 1993; Sundararajan et al. 1999).

Sequences surrounding a recoding site can stimulate translational errors (Larsen et al. 1995; Farabaugh 1996; Namy et al. 2004). Some sequences locally reduce the rate of canonical translation, inducing a translational pause. Ribosome pausing may provide sufficient time for a slow noncanonical event (frameshift, readthrough, or bypass). For example, the efficiency of $+1$ frameshifting in yeast is determined by competition between tRNAs that recognize the zero and $+1$ frame codons in the A site. Slow recog-

nition of an in-frame codon tends to increase frameshifting efficiency (Belcourt and Farabaugh 1990; Kawakami et al. 1993), as does rapid recognition of the overlapping $+1$ frame codon (Pande et al. 1995). Alternatively, context sequences can directly stimulate the frequency of the noncanonical decoding event. For example, programmed frameshift sites in bacteria include a Shine-Dalgarno (SD) site immediately upstream of the shift site (Larsen et al. 1995). The sequence appears to base pair with the anti-SD site near the 3' end of the 16S rRNA. The spacing between the SD and shift sites is not optimal. The mRNA•rRNA pairing may introduce strain that is lessened by the ribosome moving into the shifted frame. Thus, the SD interaction would effectively pull the ribosome into the shifted frame (Curran and Yarus 1988; Weiss et al. 1988).

The mechanism of other context effects remain unexplained. For example, a 14-nt sequence downstream from the retrotransposon Ty3 frameshift site in the yeast *S. cerevisiae*, termed the Ty3 stimulator, increases programmed $+1$ frameshifting by as much as 7.5-fold (Farabaugh et al. 1993). It functions as a primary mRNA sequence has, and has limited complementarity with a region of the rRNA within the loop of Helix 18, an element of the ribosome's accuracy center (Li et al. 2001). This target region includes a highly conserved nucleotide with a major role in aa-tRNA recognition in the A site, guanosine 577 (G577), which corresponds to G530 of bacterial ribosomes. In bacterial ribosomes, G530 contacts both the second and third base pair of the cognate codon•anticodon complex, facilitating its recognition by the ribosome (for review, see Ogle et al. 2003). DNA cross-linking assays showed that only when the A site is empty, G530 lies very close to the mRNA region corresponding to the Ty3 stimulator (Dontsova et al. 1992; Rinke-Appel et al. 1993). Based on the complementarity and proximity of the mRNA and rRNA, Li et al. (2001) predicted that the Ty3 stimulator might pair with this region (Fig. 1) and interfere with its rapid recognition of

FIGURE 1. The Helix 18 pairing model. The Ty3 stimulator was proposed to pair with the loop of Helix 18 at a short region of complementarity as shown (after Li et al. 2001). Black lines indicate Watson-Crick base pairing; dots indicate G•U non-Watson-Crick pairing. The dashed line indicates a pseudoknot tertiary interaction within Helix 18. Base pairing within Helix 18 is based on the most recent predicted secondary structure of Gutell (1996). Note that pairing of C575 to G14 of the stimulator would necessitate disruption of the G568–C575 pair within Helix 18.

cognate aa-tRNA. The data supporting this conclusion include a set of missense mutations across the Ty3 stimulator. Almost all of these mutations had phenotypes consistent with the model. We have revisited this issue to further test the Helix 18 pairing model by constructing a saturating set of single-base missense mutations. These data show that there is no correlation between the effect of these mutations on frameshift stimulation and on predicted pairing. We conclude that the Helix 18 pairing model is incorrect and suggest an alternative explanation for the Ty3 stimulator effect.

RESULTS AND DISCUSSION

A saturating set of single-base point mutations of the Ty3 stimulator

The genetic evidence that provided support for the Helix 18 pairing model (Fig. 1) consisted of a set of 16 single and double-mutations of the Ty3 stimulator, which together altered 13 out of 14 of the stimulator bases (Li et al. 2001). The stimulator corresponds to the nucleotides 8–21 downstream of the 5' nucleotide of the P site codon, GCG. Mutations of positions 8–15 tended to reduce frameshift stimulation about twofold, but some had no effect (A10U and A11C); only one mutation (U9C) much more strongly reduced stimulation (Li et al. 2001). Eight of the mutations altered positions predicted to base pair with Helix 18, and five of those had effects that were consistent with the model; that is, mutations predicted to weaken the predicted pairing also reduced stimulation. Of the three other relevant mutations, only one (A11C) was explicitly inconsistent with the model, since it failed to reduce stimulation even though it was predicted to replace an A–U pair with a pairing of C and U, which cannot form a Watson–Crick base pair. The combination of genetic results that generally support the model and cross-linking data suggesting that the mRNA and rRNA regions were in close apposition (Dontsova et al. 1992) increased the plausibility of the model, although it remained hypothetical.

A critical test of the model would have involved combining one or more defective mutants of the Ty3 stimulator with mutations altering rRNA nucleotides to which they are predicted to pair. We could conclude that the pairing model were correct if we found that pairing with the mutant stimulator restored frameshifting. This kind of analysis has been successfully used to test many RNA–RNA pairing schemes, for example, testing mRNA•snRNA pairing (Schaufele et al. 1986; Zhuang and Weiner 1986; Parker et al. 1987). Unfortunately, mutations altering the rRNA region predicted to pair with the Ty3 stimulator confer either a lethal or extremely error-prone phenotype (Powers and Noller 1990; Santer et al. 1993; Taliaferro and Farabaugh 2007b). We have therefore chosen to test the model in a different way. The fact that

the previous missense mutagenesis created only a small subset of all possible mutations weakens support for the pairing model, because, by chance, the subset tested may fail to invalidate the model, because untested mutations might exist that would have invalidated it. To be sure whether this is the case, we constructed and tested a saturating set of missense mutations of the Ty3 stimulator.

The mutations were constructed in the context of the frameshift reporter plasmid created by Li et al. (2001), in which the Ty3 frameshift site is fused to a downstream reporter, the bacterial *lacZ* gene encoding β -galactosidase. Expression of the enzyme requires the ribosome to enter the frameshift site in the zero frame and shift into the +1 frame and continue translating into *lacZ*. To calculate frameshifting efficiency, the activity of β -galactosidase from this construct is compared with the activity of a matched reporter, in which *lacZ* is in the zero frame and therefore does not require frameshifting for its expression. Each of the 42 possible single-base mutations of the 14-nt Ty3 stimulator were constructed, and the activity of β -galactosidase expressed from each mutant plasmid determined as described in Materials and Methods.

The values we obtained for frameshift stimulation by the Ty3 stimulator mutants C8U, U9C, U9A, A10C, A11C, C13G, G14A, and U18G agree within experimental error with our previous data (Li et al. 2001). The sole exception is the mutation A10U, which had been reported not to alter stimulation; we find that it reduces stimulation ~ 2.5 -fold. This appears to reflect an error in the original data.

Altering stimulator nucleotides proposed under the model to base pair with Helix 18 should have predictable effects. The Helix 18 pairing model proposes five Watson–Crick base pairs: U9•A580, A11•U578, C12•G577, C13•G576, and G14•C575. Watson–Crick pairs are isomorphic only with other Watson–Crick pairs, because the distance between the sugar-phosphate backbones of Watson–Crick pairs is different from any non-Watson–Crick pair (Gautheret and Gutell 1997). We would predict that mutating any of these putative Watson–Crick pairs should reduce stimulator activity. Replacing Watson–Crick with non-Watson–Crick pairs in verified RNA•RNA helices generally destabilizes pairing. This can be seen most easily in the phylogenetic analysis of rRNA, in which sequence changes within internally paired regions generally restore A•U, G•C, or G•U pairing by introducing compensatory changes in the two-paired regions of the molecule (for example, see Hancock et al. 1988). The model also predicts that A10 is opposite A579, implying the possibility of a non-Watson–Crick A•A pair. As shown in Figure 2, adenines can form non-Watson–Crick base pairs in three ways: Watson–Crick/Watson–Crick (N1:N6 and N6:N1), Watson–Crick/Hoogsteen (the Watson–Crick pairing groups N1 and N6 pairing with the Hoogsteen pairing groups N6 and N7, respectively), and Hoogsteen/Hoogsteen (N6:N1 and N1:N6). None of these potential non-Watson–Crick pairs are

FIGURE 2. Structure of non-Watson-Crick A•A base pairs. Watson-Crick/Watson-Crick (*top*), Watson-Crick/Hoogsteen (*middle*), and Hoogsteen/Hoogsteen (*bottom*). Dotted lines indicated predicted hydrogen bonds. Note the decreasing distance between the phosphodiester backbone of the three pairs and the fact that the Watson-Crick/Hoogsteen pair is parallel rather than antiparallel.

isomorphous (Gautheret and Gutell 1997), so the exact structure of a complex including an A•A pair would depend on the base pair formed. The three A•A pairs, however, are isomorphous with specific A•G, A•C, and G•G pairs, respectively (Gautheret and Gutell 1997). If Helix 18 pairing required a specific conformation at A10 we would expect that one, although not both of the mutants A10G or A10C might mimic that conformation and provide wild-type activity. If, on the other hand, the conformation of the A10 pair were not essential, any Watson-Crick (A•U) or non-Watson-Crick pair might replace A10•A579 and provide activity.

As shown in Figure 3, our data do confirm that region of proposed Helix 18 pairing is important for frameshift stimulation; over half of the mutations reduce frameshift stimulation, with the decrease averaging threefold. The details of the effects of the mutations, however, are not consistent with the model. All mutations of two positions (U9 and G14) reduce stimulation significantly below the wild-type value; at three other positions (A10, A11, and C12) at least one mutation fails to conform to expectations. At two nucleotides predicted to form Watson-Crick pairs (A11 and C12), one or more mutations have activity indistinguishable from wild type, although the model pre-

dicts that all would be defective. In the case of the predicted A10•A579 pair, a specific non-Watson-Crick conformation appears not to be required, since both A10C and A10G have normal activity. On the other hand, the data are inconsistent with the lack of a required conformation, since A10U, which would allow formation of an A•U pair, has reduced activity. We conclude that stimulator activity shows a variable requirement for these six nucleotides, but that the data fail to support the Helix 18 pairing model.

The data show that a 5' domain (C8–A15) of the stimulator is more critical to stimulation than a 3' domain (U16–A21). In the 5' domain, at least one mutation at each position significantly reduced stimulator activity. A total of 79% (19 of 24) of the 5' domain mutations reduced stimulation an average of 2.6-fold. In the 3' domain, a minority of mutations (6 out of 17, or 35%) reduced activity and only two reduce it more than twofold. One mutation, G20U, creates a +1 frame UAA nonsense codon, blocking expression of the *lacZ* reporter. The effect of this mutation on stimulation can therefore not be determined. The overall impression of the data is consistent with the conclusions of our previous work that the 5' domain makes a greater contribution to stimulator activity (Li et al. 2001).

The Ty3 stimulator is located at the mRNA entry channel of the ribosome

The mRNA bound to bacterial ribosomes is well ordered in high-resolution structures only in the decoding center and for two nucleotides in the 5' direction (Schuwirth et al.

FIGURE 3. Phenotype of mutants of the Ty3 stimulator in exponentially growing cells. Each bar represents β -galactosidase activity ($\pm$ SEM) expressed from a reporter involving a wild-type (WT) or missense mutant Ty3 stimulator. Each wild-type nucleotide appears below the graph with a subscript indicating the position; the identity of each mutant tested appears just above in groups of three. Black bars represent results judged by ANOVA as different from wild type ($P \leq 0.05$). The asterisk indicates a mutation (G20U) that introduces an in-frame nonsense codon, which strongly reduces expression of *lacZ*. A21G, which was not included in this set, previously was shown to stimulate 60% the activity of wild type (Li et al. 2001), a significant reduction in activity.

2005; Selmer et al. 2006). Yusupova et al. (2001, 2006) mapped the mRNA path, showing that downstream of the decoding center the mRNA enters the ribosome through protein and rRNA layers of a 20 Å diameter entrance tunnel (Fig. 4). The first four nucleotides downstream from the A site (positions 8–11) are within an rRNA layer of the tunnel made up of parts of Helix 2 (the “central pseudoknot”), 18, 28, and 34 (Yusupova et al. 2001). The next five nucleotides (positions 11–15) pass through a second layer, mostly composed of basic residues from three ribosomal proteins, rpS3, rpS4, and rpS5 (Yusupova et al. 2001). These residues constitute the ribosomal helicase responsible for unwinding mRNA secondary structures in advance of the ribosome (Takyar et al. 2005). These three ribosomal proteins, however, also play a role in ribosomal accuracy; *ram* mutations (ribosomal ambiguity) affecting rpS4 and rpS5 (Birge and Kurland 1970; Deusser et al. 1970; Stoffler et al. 1971) reduced the stringency of decoding (Thompson et al. 1981; Andersson and Kurland 1983) and a mutation in the rpS3 homolog in *S. cerevisiae* increased suppression of frameshift mutations (Hendrick et al. 2001). Past position 15, the mRNA resides outside of the entrance tunnel, but was too disordered to be localized (Yusupova et al. 2001).

Although these data derive from studies of bacterial ribosomes, comparison of high-resolution X-ray crystallo-

graphic structures of bacterial 70S ribosomes (Schwirth et al. 2005; Selmer et al. 2006) and a lower-resolution cryo-electron microscopy structure of a eukaryotic 80S ribosome (Spahn et al. 2001, 2004) demonstrates large-scale similarity in structure and function. The central structures of these ribosomes appear to be conserved. Differences are confined to regions distant from the decoding center. In particular, bacterial and eukaryotic ribosomes each include an entrance tunnel apparently composed of homologous structures.

Comparison of our missense mutations and the predicted path of the mRNA demonstrates that the 5′ (C8–A15) and 3′ (U16–A21) domains of the Ty3 stimulator described above correspond to the regions within and outside of the entrance tunnel. The 5′ domain is divided between nucleotides predicted to be within the rRNA (C8–A11) or the ribosomal protein layer (C12–A15). At least one mutation at each position of the C8–A11 region strongly reduces stimulation with the greater effect at C8 and U9. Yusupova et al. (2001) identified a possible interaction between two rRNA nucleotides that point into the entrance channel, C1397 and U1196, and the mRNA at positions 7 and 9, respectively. They propose that these interactions might position the mRNA downstream of the A-site codon (Yusupova et al. 2001). We have not modified U7 of the Ty3 site in these studies, since this nucleotide forms part of the frameshift heptamer and therefore affects frameshifting directly. It is attractive to suggest, however, that 5′ domain mutations might disrupt interactions with C1397 and U1196, which would implicate them in reading frame maintenance. If these nucleotides interact with the region immediately past the A site, their normal function could be to block reading out of frame. Mutations in the region of C1397 (O’Connor et al. 1995, 1997) and U1196 (Goringer et al. 1991; Moine and Dahlberg 1994; O’Connor et al. 1995, 1997) are known to affect translational accuracy.

The downstream half of the 5′ domain (C12–A15) is predicted to interact with rpS3, rpS4, and rpS5. Our data imply that the residues of these proteins that constitute the ribosomal helicase (Takyar et al. 2005) may have a second role in maintaining translational reading frame. We find that nearly all mutations of C12 to A15 strongly reduce frameshift stimulation. It is attractive to suggest that the nucleotide sequence in this region (5′-CCGA-3′) interferes with a function of the ribosomal protein residues in preventing +1 frameshifting. Although genetic evidence already identifies these three proteins in translational accuracy, available mutations do not implicate the helicase center directly in that process. The *ram* mutations, however, were selected to enhance missense errors and compensate for hyperaccurate mutations of rpS12, the product of the *strA* gene (Gorini 1974). It may be that mutations in the helicase center do not affect this phenotype, but only affect frame maintenance. We are testing these predictions by directed mutagenesis of the helicase center encoded in the *rpsC* (rpS3), *rpsD* (rpS4), and *rpsE* (rpS5) genes.

FIGURE 4. Pathway of the mRNA through the ribosome’s entrance channel. The figure shows a rendering based on the X-ray structure of the *Thermus thermophilus* 70S ribosome in the “post-initiation” state (Yusupova et al. 2006). The multicolored tube represents the mRNA from the P site to position 13. The colors represent the P site codon (orange), A site codon (green), position 7 (white), positions 8–11 (red) corresponding to the rRNA layer of the entrance tunnel, and positions 12–13 (pink) corresponding to the first two nucleotides of the ribosomal protein layer. The gray tubes represent the 16S rRNA; the two nucleotides proposed to interact with the mRNA at positions 7 and 9, C1397 and U1196 appear in the center of the image. Ribosomal proteins S3 (blue), S4 (green), and S5 (yellow) are shown in cartoon format at left. Residues in front of the entrance tunnel have been made partially transparent in the image for clarity. The coordinate file used to construct the figure, 2HGP, was obtained from the Protein Data Bank; the image was created using VMD 1.8.6 (Humphrey et al. 1996).

Most mutations to the 3' domain of the Ty3 stimulator (U16–A21) have no significant effect on stimulation. The exceptional mutations that cause a significant decrease include U16A and mutations to the 3' end of the stimulator, U19, G20, and A21. Most of these mutations reduce stimulation less than twofold. The fact that a portion of the 3' domain does appear to affect stimulation suggests that the Ty3 stimulator interacts with target(s) outside of the ribosome's entrance tunnel, which could include elements of the solvent face of the 40S subunit or some nonribosomal factor(s). The region is unlikely to interact directly with either elongation factor (eEF-1A or eEF2); cryoelectron microscopic analysis of EF2 bound to 80S ribosomes (Gomez-Lorenzo et al. 2000) and of the eEF-1A homolog EF-Tu bound to 70S bacterial ribosomes (Stark et al. 1997) show that these factors bind at a site at the interface between the 40S and 60S subunits far from the solvent side of the entrance tunnel.

Comparison of two frameshift stimulators suggests conserved interactions with the ribosome

We recently characterized a stimulator sequence immediately downstream of the programmed +1 frameshift site in the *S. cerevisiae* *EST3* gene that is almost twice the length of the Ty3 stimulator, with which it shares no primary sequence similarity (Taliaferro and Farabaugh 2007a). The *EST3* stimulator, like the Ty3 stimulator, increases frameshifting eightfold. Progressive 3' deletions of the *EST3* stimulator suggest that it comprises three separable domains, each responsible for about twofold stimulation. The downstream boundaries of these domains are at positions 13, 25, and 34, numbered from the P site codon (Taliaferro and Farabaugh 2007a). The data of Yusupova et al. (2001) suggest that the first of these domains is mainly in the rRNA layer of the entrance tunnel and the second and third are in the space beyond the entrance tunnel. Saturation mutagenesis of the *EST3* stimulator showed that 35 of 86 mutations tested significantly reduced stimulation relative to wild type, and in one it was significantly increased. These mutations are scattered, but only 11 were predicted to lie in the ribosomal entry tunnel (positions 8–15). The strongest clustering of defective mutations were between position 18 and 29, all predicted to lie outside of the tunnel.

The two stimulators may be more similar than this summary implies. Like the Ty3 stimulator, mutations to positions 8 and 9, immediately downstream from the frameshift heptamer, generally strongly reduce frameshifting (Taliaferro and Farabaugh 2007a). It may be that these two sequences interfere with frame maintenance by similar mechanisms. The region of the *EST3* stimulator predicted to occupy the rpS3/rpS4/rpS5 layer of the entrance channel, corresponding to position 11–17, is a run of pyrimidines, 5'-UUUCCCC-3'. This region is poorly conserved in budding yeast *EST3* genes (Taliaferro and Farabaugh

2007a), suggesting that it may not be essential to function. We do not, however, know how these homologous stimulator sequences may affect frameshifting. We do know that some of the missense mutations in this region of the *S. cerevisiae* sequence do significantly reduce stimulation, which suggests that a functional interaction does exist between the *EST3* stimulator and rpS3/rpS4/rpS5. The rest of the mutations that reduce *EST3* stimulation would fall in the solvent-accessible region past nucleotide 15. A very significant cluster of defective mutants falls within the region from A18 to T30. This region partly overlaps with the 3' domain of the Ty3 stimulator, suggesting that the two may also share a target on the ribosome, though they share little primary sequence similarity. The fact that so many mutations in this region have no significant effect on stimulation suggests that the sequence constraints on the function of the region may be weak and that multiple unrelated sequences might be able to function equivalently.

The analyses of these two frameshift stimulators suggest that the interaction between these mRNA elements and the ribosome may largely involve other than Watson–Crick base pairing. A prejudice in favor of base pairing no doubt derives from the example of the function of base pairing of the Shine–Dalgarno site during translational initiation (Shine and Dalgarno 1974). Although Watson–Crick pairing has been assumed in these interactions because it is fully compatible with the A form of the RNA•RNA helix, the assumption has been questioned more recently by many RNA duplex structures showing involvement of non-Watson–Crick pairs (for review, see Leontis and Westhof 1998; Westhof and Fritsch 2000). Previously, we proposed such an interaction to explain the Ty3 stimulator effect (Li et al. 2001). Many others proposed an mRNA•rRNA pairing scheme to explain regulatory mRNA effects (Olins and Rangwala 1989; Thanaraj and Pandit 1989; Ivanov et al. 1995; Resch et al. 1996; Tranque et al. 1998). The validity of these base-pairing interactions has not always been tested explicitly and some have been disproven (for example, the downstream box, O'Connor et al. 1999; La Teana et al. 2000; Moll et al. 2001). It may be that non-base-pairing interactions are much more typical of the interaction of the ribosome with effector RNAs.

MATERIALS AND METHODS

Bacterial and yeast strains

The strains used in this work were the *Escherichia coli* strain DH5 α (F $^{-}$ ϕ 80dlacZ Δ M15 Δ (lacZYA-argF)U169 *deoR* *recA1* *endA1* *hsdR17*(rk $^{-}$ mk $^{+}$) *phoA* *supE44* λ^{-} *thi-1* *gyrA96* *relA1*) (Hanahan 1983) and *S. cerevisiae* strain FY1679–18B (*Mata*, *his3- Δ 200*, *leu2- Δ 1*, *trp1 Δ 63*, *ura3-52*, *GAL2*) (Dujon et al. 1994).

Media and culture conditions

All *E. coli* strains were grown in LB medium: 10 g of Bacto-tryptone (Difco), 5 g of Bacto yeast extract (Difco), 10 g of NaCl, and either

100 µg/mL ampicillin or 30 µg/mL chloramphenicol. All bacterial transformations were performed using the Z-competent protocol (Zymo research). *S. cerevisiae* strains were grown on synthetic medium with 2% glucose (SD) supplemented with amino acids, lacking uracil for plasmid selection. All *S. cerevisiae* transformations were performed using the LiAc method (Ito et al. 1983).

Plasmid constructions and reporter assay

Plasmids for the study of Ty3 frameshifting are derivatives of the shuttle plasmid pMB38, a *lacZ* reporter plasmid that includes the *bla* gene of pBR322 conferring resistance to ampicillin in bacteria and the *URA3* gene of *S. cerevisiae*, a selectable marker for yeast transformation (Belcourt and Farabaugh 1990). Expression of *lacZ* depends on the transcription and translation from the *HIS4* gene, the first 33 codons of which have been fused to *lacZ*. Saturation mutagenesis was performed using Quickchange XL II protocol in DH5α as recommended by the manufacturer (Stratagene). All DNA oligonucleotides were purchased from Integrated DNA Technologies. Assays of β-galactosidase in *S. cerevisiae* were essentially as described (Farabaugh et al. 1989). Triplicate assays were conducted on at least three independent transformants. The statistical significance of the data was calculated using the students T-test with data considered significant at the 95% level.

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