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Programmed frameshifting in the synthesis of mammalian antizyme is +1 in mammals, predominantly +1 in fission yeast, but –2 in budding yeast

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ABSTRACT

The coding sequence for mammalian ornithine decarboxylase antizyme is in two different partially overlapping reading frames with no independent ribosome entry to the second ORF. Immediately before the stop codon of the first ORF, a proportion of ribosomes undergo a quadruplet translocation event to shift to the +1 reading frame of the second and main ORF. The proportion that frameshifts is dependent on the polyamine level and, because the product antizyme is a negative regulator of intracellular polyamine levels, the frameshifting acts to complete an autoregulatory circuit by sensing polyamine levels. An mRNA element just 5′ of the shift site and a 3′ pseudoknot are important for efficient frameshifting. Previous work has shown that a cassette with the mammalian shift site and associated signals directs efficient shifting in the budding yeast *Saccharomyces cerevisiae* at the same codon to the correct frame, but that the shift is –2 instead of +1. The product contains an extra amino acid corresponding to the shift site. The present work shows efficient frameshifting also occurs in the fission yeast, *Schizosaccharomyces pombe*. This frameshifting is 80% +1 and 20% –2. The response of *S. pombe* translation apparatus to the mammalian antizyme recoding signals is more similar to that of the mammalian system than to that of *S. cerevisiae*. *S. pombe* provides a good model system for genetic studies on the mechanism of at least this type of programmed mammalian frameshifting.

Keywords: antizyme; frameshifting; ornithine decarboxylase antizyme; recoding; *S. pombe*

INTRODUCTION

Because eukaryotic gene functions are so remarkably conserved, the sophisticated genetics of the budding yeast, *Saccharomyces cerevisiae*, and of the fission yeast, *Schizosaccharomyces pombe*, are particularly useful tools for analysis of some higher eukaryotic mechanisms. One phenomenon being investigated in this manner is programmed ribosomal frameshifting. Signals in a minority of mRNAs in probably all organisms cause a substantial proportion of ribosomes to shift reading frame and synthesize an additional product. (These frameshifting examples are a subset of recoding events in which decoding rule changes are programmed in mRNAs). The nature of the mRNA signals is being investigated by site-directed mutagenesis and structural studies. However, classical genetics can play an important role in identifying translation components that recognize these signals and affect altered decoding.

Mutations have been characterized that alter programmed ribosomal frameshifting involved in expression of genes endogenous to *S. cerevisiae* that require either −1 frameshifting (Dinman & Wickner, 1994, 1995; Cui et al., 1996, 1998) or +1 frameshifting (Xu & Boeke, 1990). Because programmed −1 frameshifting at the *gag pol* junction of mouse mammary tumor virus (MMTV) functions reliably in *S. cerevisiae*, mutants have also been isolated that effect frameshifting for this heterologous gene (Lee et al., 1995; Cui et al., 1996; see review, Dinman, 1995). These results suggest that it might be valuable to isolate yeast mutants that affect the +1 programmed frameshifting required for decoding mammalian antizyme, the only known case of programmed frameshifting for expres-

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sion of chromosomal genes of animals. Introduction of a cassette containing antizyme frameshifting signals into *S. cerevisiae* causes high-level (16%) frameshifting at the expected site. However, the shift is −2 instead of +1, suggesting that in this case the budding yeast is not a good model system. Here, we test the possibility that fission yeast, *S. pombe*, may be a better model for analyzing mammalian antizyme frameshifting.

Antizyme is a negative regulator of intracellular polyamine concentrations in mammalian cells (see review, Hayashi et al., 1996). It binds to ornithine decarboxylase (ODC) (see Li & Coffino, 1994). a key enzyme for polyamine biosynthesis, and targets it for degradation by 26S proteasomes. The initial antizyme cDNA clone (Matsufuji et al., 1990) could be engineered to produce functional antizyme, but it lacked an in-frame translation initiator. Subsequent work with a longer cDNA clone showed that the initiator is in a different, partially overlapping, reading frame and that full-length antizyme is expressed by translation from one frame into the other (Matsufuji et al., 1995). Ribosomes shift to the +1 frame at the end of the short ORF1 and enter ORF2, which encodes the functional part of antizyme (Fig. 1). As discovered by Matsufuji and colleagues, the frameshifting is autoregulatory because the shift is modulated by polyamines (see review, Gesteland et al., 1992). Efficiencies of 20–30% have been detected in both reticulocyte lysates (Rom & Kahana, 1994; Matsufuji et al., 1995) and mammalian tissue culture cells (S. Matsufuji, unpubl.). As shown by sequencing of radioactive product made in reticulocyte lysates, the shift occurs at UCC UGA. The hypothesis for the mechanism of frameshifting is that pairing of peptidyl $tRNA^{Ser}$ with UCC somehow occludes the next nucleotide, U, the first base of ORF1 terminator, competing with termination and resulting in the +1 codon, GAU, being the next available codon. Because the mammalian antizyme shift cassette in *S. cerevisiae* shifts is −2 rather than +1, an extra amino acid is found in the product at the transframe junction (Matsufuji et al., 1996). Rather than the shift tRNA:mRNA pairing being deduced to cause occlusion of the next 3′ base, it appears that the shift codon dissociates from the tRNA anticodon, the mRNA

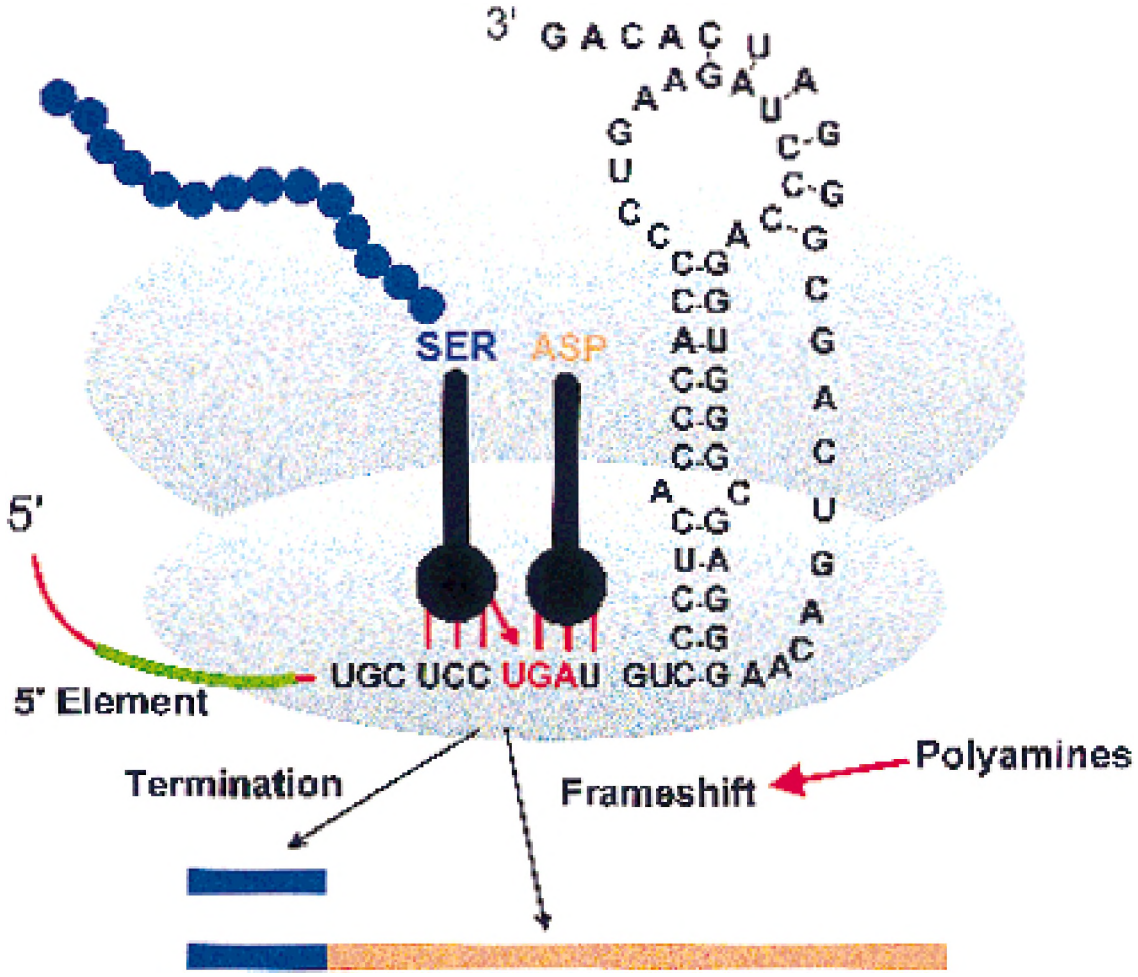


FIGURE 1. Antizyme frameshifting.

slips two bases backward and re-pairs with the anticodon giving −2 frameshifting.

A pseudoknot, starting four nucleotides 3′ of the antizyme shift site, provides a 2.5-fold stimulation of frameshifting in in vitro experiments. A 5′ sequence that stimulates about threefold is currently being characterized (S. Matsufuji, unpubl.) and is conserved to a striking degree in the *Drosophila* homologue (Ivanov et al., 1998b). These signals behave quite differently when tested in *S. cerevisiae*. The 3′ pseudoknot stimulates frameshifting 30-fold and no 5′ stimulator effect has been detected.

Recently, a second mammalian ODC antizyme gene has been discovered (Ivanov et al., 1998a). This gene also relies on translational frameshifting for its regulated expression, but the results presented here deal exclusively with the recoding signals of what is now being termed antizyme 1.

RESULTS

Rat antizyme cassette promotes translational frameshifting in *S. pombe*

A 265-nt fragment of rat antizyme cDNA was inserted between *GST* and *LacZ* genes. This fragment of the antizyme gene contains all but the first codon of ORF1 through the downstream pseudoknot [nt 3–268 in Fig. 1 in Matsufuji et al. (1995)], and includes all known *cis*-acting frameshift stimulatory elements (the 5′ element, the UGA stop codon, and the downstream antizyme pseudoknot). The chimeric gene is driven by an intermediate strength *nmt1*-repressible promoter in the *S. pombe* vector pREP42X. The antizyme ORF1 is fused inframe with *GST*, whereas *lacZ* is fused in the +1 frame so that *lacZ* expression provides a measure of frameshifting.

The wild-type antizyme cassette directs efficient (9%) +1 translational frameshifting compared with the in-frame control, comparable to the level of rat antizyme translational frameshifting in mammals (6–30%) and in *S. cerevisiae* (16%). Addition of the polyamines, putrescine, or spermidine to the media (up to 1 mM) did not measurably increase the efficiency of frameshifting (data not shown).

Amino acid sequence of the transframe peptide in *S. pombe*

A 79-nt fragment of antizyme mRNA that does not contain the 5′ stimulatory element, but includes the last four codons of ORF1 and the downstream pseudoknot directs ~6% frameshifting in reticulocyte lysate and ~16% in *S. cerevisiae*. This fragment, inserted between *GST* and *lacZ* flanked by factor Xa protease cleavage sites, was used to determine the sequence of the transframe protein made in *S. cerevisiae* (Matsufuji et al., 1996). We inserted this *GST-lacZ* fusion cassette into pREP3 vector in which the transcription of the fusion protein was driven by a full-strength *nmt1* promoter. It directed ~2.5% frameshifting in *S. pombe* when compared to an in-frame control. The fusion protein was purified and the transframe peptides were prepared and analyzed as described by Matsufuji et al. (1996). Two peaks (Fig. 2A) were observed after the transframe peptides were separated on reverse-phase HPLC. Edman degradation of the peptide in the major peak gave the sequence W-?-S-D-V-P-H-P-P-L-K-I (see Fig. 2B). W and S are from the end of ORF1. (The cysteine from ORF1 in position #2 was not detected because the cysteines of the peptides were not derivatized before sequencing.) The sequence D-V-P-H-P-P-L-K-I is derived from +1 frameshifting at the codon preceding the UGA stop codon. This exactly matches the frameshift product from translation of rat antizyme in reticulocyte lysate. The peptide from the minor peak revealed two sequences (see Fig. 2C). One seems identical to the peptide in the major peak and probably results from contamination from that peak. However, Asp was not detected at position 4. The other one has the sequence W-?-S-P-D-V-?-P-P-L-K-I (the amount of protein in this preparation was limiting and histidine was not detected). This peptide has an additional proline residue (position 4) and results from −2 frameshifting at the UCC serine codon and is identical to the major transframe product produced by expression of this antizyme sequence in *S. cerevisiae*. The ratio of heights of the two peaks is 2.5:1 in favor of the peak containing only the +1 transframe peptide. By estimating the areas of the two peaks, we conclude that, with the rat antizyme frameshift site in *S. pombe*, 80% of the ribosomal frameshifting is +1 and 20% is −2. In subsequent sections, frameshifting efficiencies are presented. Assays were performed on β-galactosidase expression from a +1 frame lacZ gene downstream of the recoding signals. The assay values reflect the efficiencies of shifting to the +1 frame and do not distinguish between the contributions of +1 and −2 shifting.

Effect of antizyme 5′ elements in *S. pombe*

One of the three known *cis*-acting elements that stimulate antizyme frameshifting is an as yet uncharacterized 5′ sequence. It stimulates frameshifting threefold in an in vitro mammalian system. By contrast, Matsufuji et al. (1996) have shown that the 5′ element is completely inactive in *S. cerevisiae*. We utilized the serial deletion constructs of antizyme ORF1, described by Matsufuji et al. (1995). to determine the importance of this region for antizyme frameshifting in *S. pombe*. With a construct having only the last five codons of ORF1, frameshifting is decreased ~3.5-fold (see Fig. 3) compared to the wild-type construct. This result is consistent with the 5′ element being functional in *S. pombe*.

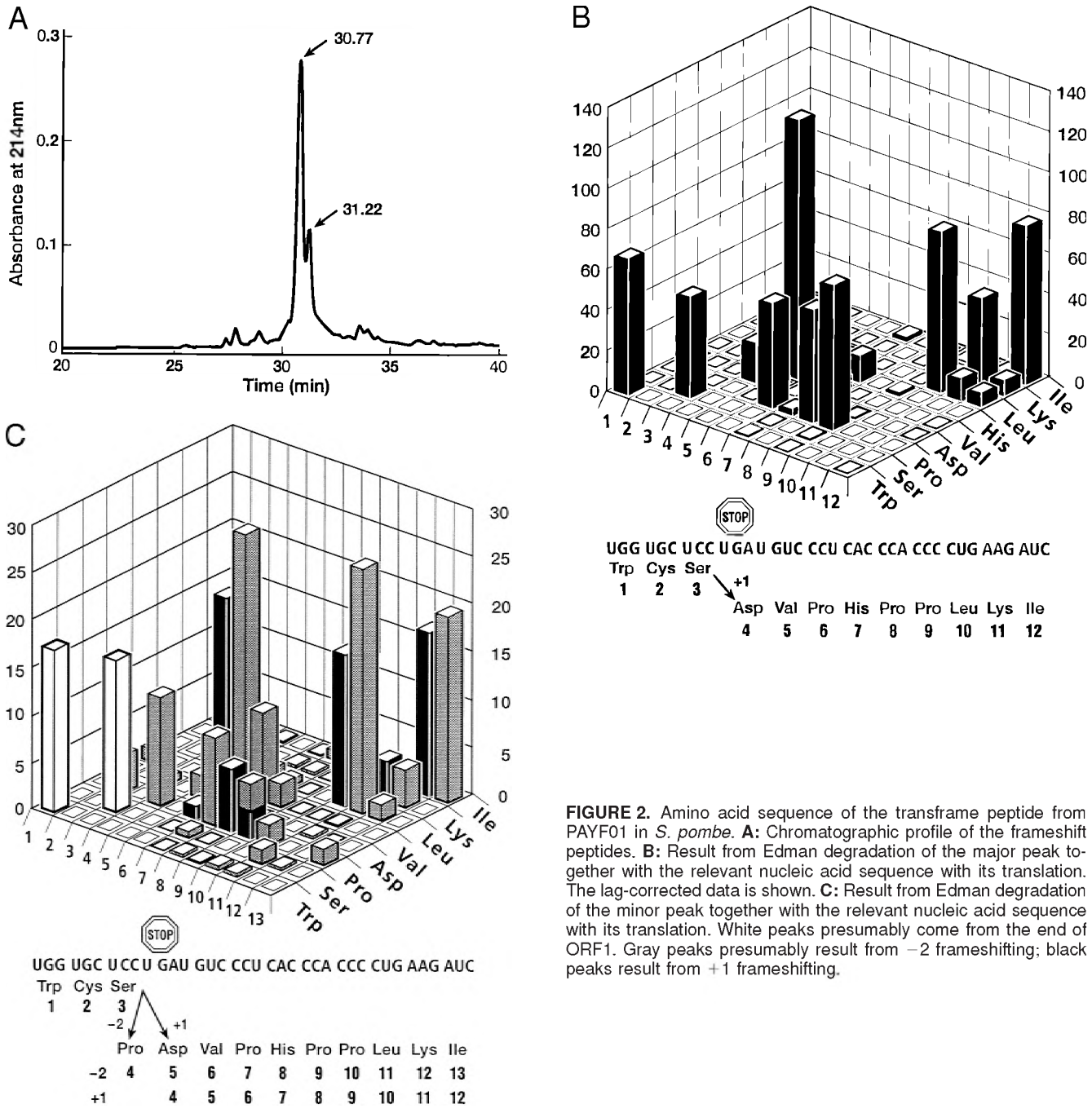


FIGURE 2. Amino acid sequence of the transframe peptide from PAYF01 in *S. pombe*. **A:** Chromatographic profile of the frameshift peptides. **B:** Result from Edman degradation of the major peak together with the relevant nucleic acid sequence with its translation. The lag-corrected data is shown. **C:** Result from Edman degradation of the minor peak together with the relevant nucleic acid sequence with its translation. White peaks presumably come from the end of ORF1. Gray peaks presumably result from −2 frameshifting; black peaks result from +1 frameshifting.

In constructs lacking the downstream pseudoknot, the 5′ element stimulated frameshifting only ~1.7-fold. This result is discussed below.

To address whether, in *S. pombe*, the 5′ element effect is operative at the nucleotide or amino acid level, two mutants were constructed. As detailed in Materials and Methods, each had a single-nucleotide change in each codon from 17 codons before the ORF1 stop codon to 4 codons before the stop codon. One had synonymous codons so that the amino acid sequence was unchanged, whereas the other had all the amino acids changed in a nonconservative manner. With both mutants, the effect was the same and comparable to having a deletion of the 5′ element, but with retention of the last four codons of ORF1 (data not shown). We infer that the 5′ element is operative at the nucleotide level in *S. pombe*.

Mutational analysis in the vicinity of the frameshift site revealed similarities to both reticulocyte lysate and *S. cerevisiae* systems

To compare the mechanism of frameshifting between fission yeast and the other two systems (*S. cerevisiae*

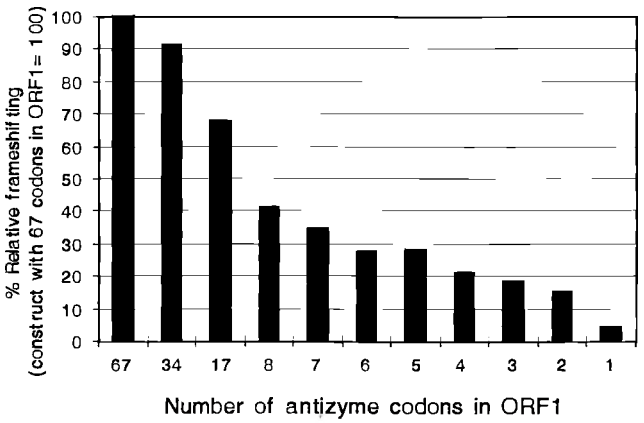


FIGURE 3. Deletional analysis of the 5′ stimulatory elements. Antizyme sequences containing the indicated number of the last ORF1 codon(s) and the pseudoknot region (up to nt 268) were inserted in PIU-LAC vector. Frameshift efficiencies were determined by β-galactosidase activities, relative to the wild-type (PAYNE Δ269) value.

and reticulocyte lysate), we performed a mutational analysis of the shift region. We chose to study mutants that showed the greatest difference in activity between *S. cerevisiae* and reticulocyte lysate, but we used two different mutant contexts because the 5′ element was not functional in *S. cerevisiae*. The *S. cerevisiae* constructs contained only the last five codons of antizyme ORF1 (and therefore lacked most, or all, of the 5′ stimulatory element), whereas the constructs tested in reticulocyte lysates contained the entire ORF1. Because the 5′ element is not functional in *S. cerevisiae*, results from the two sets of constructs could be compared directly to each other. The 5′ element is functional in *S. pombe*; therefore, to make a fair comparison, we made both sets of mutant constructs (with and without the 5′ element) and tested them in *S. pombe*.

The results from the shift site mutants tested in *S. pombe* are presented in Figure 4. Both sets of mutant constructs yielded distinctive patterns. The mutants without the 5′ element (except U202C) showed frameshift efficiencies not dissimilar to those exhibited by the corresponding mutants in *S. cerevisiae*, yet differences between *S. pombe* and reticulocyte lysate are not as drastic as they are between reticulocyte lysates and *S. cerevisiae*. Mutants of the frameshift site itself, with the 5′ element, tested in *S. pombe* show relative frameshift efficiencies intermediate between those in reticulocyte lysate constructs (+5′ element) and *S. pombe* without the 5′ element: Overall, the data from mutant sets (except for mutant U202C) form a gradient: constructs with the 5′ element in reticulocyte lysates (most like the authentic antizyme frameshift event) > *S. pombe* mutants with 5′ element > *S. pombe* without 5′ element > *S. cerevisiae* without 5′ element (least like the real antizyme frameshift event), The possible significance of this trend is discussed below.

Reticulocyte lysate translation experiments have shown the importance of having a stop codon 3′ adjacent to the shift site (Matsufuji et al., 1995). When the mammalian shift cassette was transferred to *S. cerevisiae*, the stop codon was also important, al-

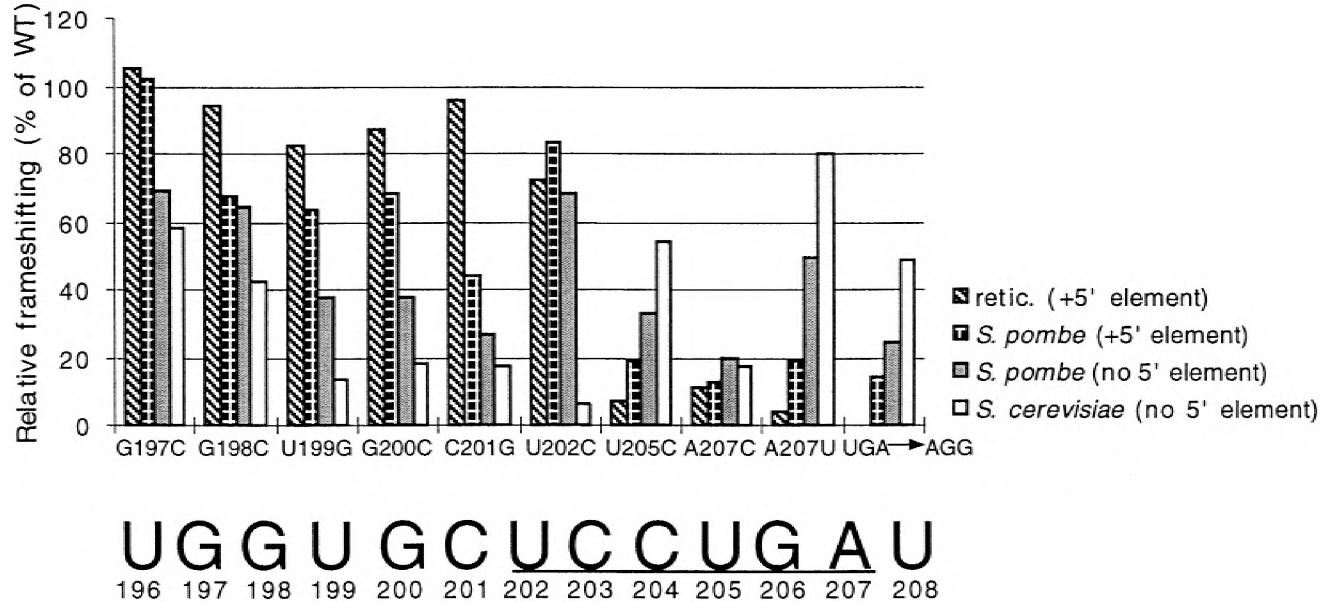


FIGURE 4. Mutational analysis of the frameshift site. Each mutation tested is indicated at the bottom of the figure. The different bars represent the relative efficiencies of the mutants examined. Frameshift efficiencies of the mutants tested in rabbit reticulocyte lysate cell-free system are reproduced from Matsufuji et al. (1995). Results of frameshift efficiencies of the mutants tested in *S. cerevisiae* are reproduced from Matsufuji et al. (1996).

though, in this case, a limited number of sense codons could efficiently substitute for the stop codon (Matsufuji et al., 1996). Four mutants of the stop codon (U205C [UGA to CGA], A207C [UGA to UGC], A207U [UGA to UGU], UGA to AGG) were tested in *S. pombe* for their effect on frameshifting directed from the mammalian antizyme cassette. A207C was chosen because this showed a large diminution in frameshifting levels in both reticulocyte lysates and *S. cerevisiae*. It showed an equivalent large diminution in *S. pombe*. The other three were chosen because they showed the largest difference between reticulocyte lysate translations and *S. cerevisiae*. They showed intermediate values in *S. pombe*. Interestingly, as determined by testing these three mutants, addition of the 5′ element sequence increases the importance of the stop codon (Fig. 4).

Mutational analysis of the downstream antizyme pseudoknot

Mutational analysis in reticulocyte lysates revealed that a pseudoknot starting four nucleotides 3′ of the UGA stop codon of ORF1 stimulates antizyme frameshifting 2.5-fold. By contrast, in *S. cerevisiae*, the downstream pseudoknot stimulates frameshifting 30-fold. To analyze the role of the antizyme pseudoknot in *S. pombe*, we used two sets of constructs. The first set contained exactly the same fused open reading frame that was used in the *S. cerevisiae* experiments, with only the last five codons of antizyme ORF1. The other set of constructs contains the entire ORF1 of antizyme except for the initiating Met. Partial or complete deletion of the pseudoknot region leads to a 6.5-fold decrease in the level of frameshifting if the entire ORF1 is present and 3.2-fold if not.

We also investigated the effect of disrupting the individual stem components of the pseudoknot on the efficiency of antizyme frameshifting in *S. pombe*. For these experiments, we used constructs containing only the last five codons of ORF1 (the same constructs used in the *S. cerevisiae* analysis), lacking most, or all, of the 5′ stimulatory element. Mutants disrupting any one of the three stem components of the pseudoknot (stem 1 bottom, stem 1 top, or stem 2) result in a substantial decrease of frameshifting levels compared to the wild-type construct. Compensatory mutations restoring the pseudoknot structure restored or even exceeded the wild-type level of frameshifting (Fig. 5).

DISCUSSION

The results show that the frameshifting exhibited in decoding the mammalian antizyme shift cassette in *S. pombe* is much more similar to that occurring in reticulocyte lysates than in *S. cerevisiae*. However, the data also show that some aspects of antizyme frameshift signal recognition, peculiar to the *S. cerevisiae* system, are shared by *S. pombe*.

The termination codon of ORF1 plays a very important role in antizyme frameshifting in the mammalian system. In *S cerevisiae*, replacement of the stop codon by at least some sense codons is not incompatible with frameshifting. This was interpreted to reflect the differ-

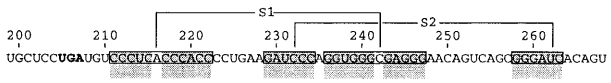
Construct	Sequence	Frameshift efficiecy (% of Wild-type) S. pombe without 5' sequence	S. pombe with 5' sequence	S. cerevisiae without 5' sequence	Reticulocyte lysate with 5' sequence
Wild-type		100.0	100.0	100.0	100.0
ΔPseudoknot		30.9	15.7	3.3	38.4
ΔS2-3'		30.8	13.7	7.5	67.1
S1B5'		25.9		8.5	40.7
S1B3'		31.2		8.3	51.2
S1Bcomp		74.0		79.6	95.5
S1T5'		35.3		7.2	69.0
S1T3'		26.8		7.1	62.5
S1Tcomp		125.3		104.0	93.4
S2-5'		31.0		9.5	53.3
S2-3'		47.9		10.3	57.7
S2comp		91.9		79.1	95.2

FIGURE 5. Pseudoknot mutational analysis. The relevant wild-type antizyme sequence is shown on the top line. Shaded areas indicate the nucleotides involved in base pairing. In the mutant constructs, open bars denote regions identical to the wild-type sequence and solid bars denote deletions. Substitution of nucleotides is indicated. The reticulocyte lysate results are from Matsufuji et al. (1995). The *S. cerevisiae* results are from Matsufuji et al. (1996).

ence in frameshifting mechanisms (+1 versus −2) between the two systems. However, these same sense codons can also partially stimulate frameshifting in *S. pombe* to a degree that exceeds what would be expected from the level of −2 frameshifting (if it is fair to extrapolate the ratios of −2 to +1 frameshifting from WT, because the ratio was not measured with these mutants). This indicates that it is not the direction of frameshifting that is responsible for differences in recognition of this stimulatory element. In the *S. cerevisiae* experiments, four of five sense codons that substituted for the UGA stop codon to support at least 50% of wild-type frameshifting were rare codons. However, the fifth was the major cysteine codon. [Rare codons correspond to sparse tRNAs, limitation of the aminoacylated form of which can stimulate certain types of frameshifting (see review, Farabaugh, 1996).] In *S. pombe*, two of four tested sense codons (and only in the context in which the 5′ element is absent) supported some frameshifting. With this small sample, no correlation between rarity and ability to support frameshifting is evident and other factors must be involved.

The current model for antizyme +1 frameshifting in reticulocyte lysates is that the shift tRNA does not dissociate from its initial codon and re-pair to the +1 frame codon, but rather, it somehow occludes the flanking 3′ nucleotide (Matsufuji et al., 1995). In contrast, the −2 frameshifting in *S. cerevisiae* appears to be due to dissociation and re-pairing (Matsufuji et al., 1996), The finding of both +1 and −2 frameshifting in a 4:1 ratio in *S. pombe* suggests that, in this organism, the mechanisms may be similar. Further investigation is required. At the time that sequencing of the product from reticulocyte lysate was performed, no −2 product was detected. The reticulocyte lysate product was detected by its radioactivity and fractions were collected from an HPLC column and portions counted prior to protein sequencing. Prior to HPLC, nonradioactive transframe peptides from both +1 and −2 shift products in *S. cerevisiae* were added to permit continuous monitoring by UV and so serve as position indicators. The fractions identified by the nonradioactive +1 and −2 markers were pooled and sequenced. The sequencing results showed that if −2 frameshifting occurred at the antizyme shift sequence in the reticulocyte lysate, it must have been below a few percent. We conclude that there is a real difference between the reticulocyte lysate and *S. pombe* with regard to at least the amount of −2 frameshifting, Irrespective of the degree of homogeneity of the reticulocyte lysate product, its *S. cerevisiae* counterpart is clearly not homogeneous. In *S. cerevisiae*, although greater than 90% of the product is due to −2 frameshifting, a small amount of +1 product was also detected.

The *S. cerevisiae* experiments indicate that the identities of nt C201 (last nucleotide of the next to last codon of ORF1) and U202 (first nucleotide of the serine codon at which the frameshifting occurs) are crucial for the re-pairing of peptidyl $tRNA^{Ser}$ in the −2 frame. Although this reasoning can also explain the reduction of frameshifting in *S. cerevisiae* with mutants C201 and U202, it is not sufficient to explain the reduced level of frameshift efficiency in *S. pombe* with C201 changed to G, nor the high level of frameshifting when U202 is changed to C (this is in contrast to the level of frameshifting of the same mutation in reticulocyte lysate). These two observations are another example of differences between the *S. cerevisiae* and reticulocyte lysate systems that were thought to reflect differences in the direction of frameshifting, but in fact may reflect some other alteration in the recognition of the antizyme frameshift signal in the yeast systems on one hand and the mammalian system on another hand.

Interestingly, addition of the 5′ stimulatory element leads to more mammalian-like importance of the stop codon for stimulating frameshifting even though this addition is not expected to affect the direction of the frameshifting event [sequencing of a peptide produced in reticulocyte lysate by a construct lacking the 5′ element showed only +1 frameshifting and no detectable −2 product (Matsufuji et al., 1995)]. Addition of the complete 5′ stimulator also reduces the effect of mutations G197C, G198C, U199G, G200C, C201G, and U202C (Fig. 3) on the efficiency of frameshifting. These two observations might suggest that the reduced frameshifting efficiencies seen with mutations G197C, G198C, U199G, G200C, C201G, and U202C in *S. cerevisiae* and *S. pombe* (especially without the complete 5′ element) may not reflect differences in the direction of frameshifting (as was suggested previously by Matsufuji et al., 1996), but may be related in some way to the recognition of the UGA stop codon of ORF1 as a signal for frameshifting stimulation in yeast.

Recognition of a 5′ stimulatory element in *S. pombe* is significant because this signal is not recognized at all in *S. cerevisiae*. This indicates that the interaction and therefore the cellular factors that underlie this recognition are likely present in *S. pombe*.

The importance of the downstream pseudoknot on antizyme frameshifting in *S. pombe* is more similar to that in the mammalian system than to *S. cerevisiae*. This result correlates with the conclusions of Matsufuji et al. (1996) that the −2 direction of the frameshifting in *S. cerevisiae* reflects a difference in some aspect of the recognition of the pseudoknot by the decoding apparatus.

The results argue that the effects of the 5′ stimulatory element and the pseudoknot on ribosomal frameshifting in *S. pombe* are more than additive (the status of this interaction in reticulocyte lysate is not yet known). The downstream pseudoknot stimulates frameshifting 6.5-fold if the entire ORF1 is present, but 3.2-fold if only the last four sense codons of ORF1 are present. Also, the sequence between codon 1 and the last four co-

dons of ORF1 ("the 5′ stimulatory element") stimulates frameshifting 3.5-fold if the downstream pseudoknot is present, but only 1.7-fold if the pseudoknot is not. The synergy between the 5′ stimulatory element and the antizyme pseudoknot was unexpected, but not totally surprising. In fact, there are other examples of interactions between two signals in a frameshift event. Matsufuji et al. (1996) hinted that the combined effects of the antizyme pseudoknot and the UGA stop codon of ORF1, both in reticulocyte lysate and in *S. cerevisiae*, are more than additive.

It is clear that *S. pombe* is a more suitable biological system than *S. cerevisiae* for genetic identification and analysis of cellular factors interacting with the mammalian antizyme frameshifting signals. At the very least, it should be possible to identify factors that interact with two of the three stimulatory elements: the antizyme 5′ stimulatory element and the downstream pseudoknot. The recoding event itself is much more faithfully replicated in *S. pombe* than in *S. cerevisiae*, with most of the frameshifting occurring through +1 rather than −2 ribosomal shifting, The antizyme frameshifting results are consistent with the more mammalian-like nature of the fission yeast than budding yeast first evident with the very different cellular processes of POL II transcription, cell cycle control, pre-mRNA splicing, and signal transduction (Hayles & Nurse, 1992).

MATERIALS AND METHODS

Strains

S. pombe stains *ura4-D18 leu1-32 ade6-M216 h*$^-$ and *leu1-32 h*$^-$ were used in these experiments. Plasmids were prepared from *Escherichia coli* strains SU1675 and DB6507 and were transformed by a standard electroporation protocol.

Construction of plasmids

The *S. pombe* expression vectors pREP3 and pREP42X were supplied by R. Rowley, To construct pREP3-AYF01 (PAYF01). a two-step cloning approach was used. In the first step, pREP3 was digested with *Bal* I and *Bam*H I restriction endonucleases and a *Sma* I/*Bam*H I fragment from AYF01 (Matsufuji et al., 1996), containing the first part of the fusion protein (GST and the antizyme sequence), was ligated into the gap. In the second step, this new construct was digested with *Bam*H I and a *Bam*H I/*Bam*H I fragment from AYF01 containing the *lacZ* gene was inserted. The correct orientation of *lacZ* was verified by restriction digestion. For construction of the other mutants, *Sma* I/*Sal* I fragment of pGL107 containing the GST-*lacZ* fusion was inserted into pREP42X digested with *Sma* I. This new plasmid was named PIU-LAC. Subsequently, *Bst*E II/*Kpn* I fragments bearing the corresponding mutations from the *S. cerevisiae* plasmids were inserted between *GST* and *lacZ* of PIU-LAC. Pseudoknot mutants containing all but the first codon of antizyme ORF1 were constructed using PCR amplification from AYNE Δ269 (Matsufuji et al., 1995). The constructs produced through the use of PCR and construct pREP3-AYF01 were verified by DNA sequencing.

The modified 5′ element sequence constructs contained the 3′ pseudoknot and were made by PCR. The sequence of the sense-strand primer used for the synonymous changes in the 5′ element was: GCATCCGGTCACCAC, GTA, GCA, CTA. CAC, TGT, TGC, TCT, AAT, TTG, GGA, CCT, GGT, CCA, CGG, TGG, TGCTCC, which gives the amino acid sequence: Val, Ala. Leu, His, Cys, Cys, Ser, Asn, Leu, Gly, Pro, Gly, Pro, Arg, Trp. The sense-strand primer used for the nonconservative changes was: GCATCCGGTCACCAC, GAT, GAC, CCT, CAA, TGG, TGG, AGA, AAA, ATG, AGT, ACG, AGG, ACT, GGG, TGG, TGCTCC, which gives the amino acid sequence: Asp. Asp, Pro, Gln, Trp, Trp, Arg, Lys, Met, Ser, Thr, Arg, Thr, Gly, Trp, The antisense primer is complementary to *lacZ* sequence 3′ of the *Kpn* I site of pGL107 (Matsufuji et al.,1996). After amplification [using construct AY103 as template (Matsufuji et al., 1996)], the PCR products were gel purified and digested with *Bst*E II/*Kpn* I and cloned into PIU-LAC. The "wild-type" construct in this set of experiments is the same as the construct with 17 codons in ORF1 in Figure 3.

In vivo frameshifting assay in *S. pombe*

S. pombe cells were transformed using the electroporation method described by Grey and Brendel (1992). Selection was done on MM plates, containing 5 mg/L thiamine, for LEU$^+$ or URA$^+$ colonies. β-galactosidase activities were measured essentially according to Guarente (1983). For assays, several clones of a single mutant construct were grown to saturation in selective medium.

Protein sequencing

For sequencing transframe peptides, a construct equivalent to the *S. cerevisiae* construct AYF01 with the WT shift site was prepared from the antizyme sequence corresponding to nt 193–269 flanked by Factor Xa protease cleavage sites. *S. pombe* strain (leu1-32 h$^-$) carrying this construct was grown to saturation in 4 L MM media (no thiamine). The cells were collected by centrifugation and washed with PBS. The pellet (39 g) was resuspended in 250 mL of PBS containing 5 mM DTT. The cell wall was digested with 78 mg yeast lytic enzyme for 30 min at room temperature. The cells were homogenized in a Bead-Beater (Biospec Products) with 200-mL glass beads. PMSF was added to a final concentration of 1 mM just before homogenizing the cells. The lysate was clarified by centrifugation at 10,000 rpm for 30 min followed by centrifugation at 45,000 rpm for 2 h. GST fusion proteins were purified on glutathione–Sepharose 4B column according to the manufacturer's specifications. Eight milligrams of the GST fusion protein were digested with 4 mg of Factor Xa (New England Biolabs) with addition of $CaCl_2$ (2 mM). NaCl (100 mM), and DTT (5 mM) at room temperature for 13 h. The digested material was fractionated on reverse-phase HPLC column with a linear gradient of 5–50% acetonitrile in 0.1% trifluoroacetic acid/water increasing 1%/min at a flow rate of 0.5 mL/min. The major peaks between 15 and 50% acetonitrile were collected and sequenced on an Applied Biosystems 475A sequencer equipped with a 120A PTH analyzer.

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