Antizyme frameshifting as a functional probe of eukaryotic translational termination

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ABSTRACT

Translation termination in eukaryotes is mediated by the release factors eRF1 and eRF3, but mechanisms of the interplay between these factors are not fully understood, due partly to the difficulty of measuring termination on eukaryotic mRNAs. Here, we describe an in vitro system for the assay of termination using competition with programmed frameshifting at the recoding signal of mammalian antizyme. The efficiency of antizyme frameshifting in rabbit reticulocyte lysates was reduced by addition of recombinant rabbit eRF1 and eRF3 in a synergistic manner. Addition of suppressor tRNA to this assay system revealed competition with a third event, stop codon readthrough. Using these assays, we demonstrated that an eRF3 mutation at the GTPase domain repressed termination in a dominant negative fashion probably by binding to eRF1. The effect of the release factors and the suppressor tRNA showed that the stop codon at the antizyme frameshift site is relatively inefficient compared to either the natural termination signals at the end of protein coding sequences or the readthrough signal from a plant virus. The system affords a convenient assay for release factor activity and has provided some novel views of the mechanism of antizyme frameshifting.

INTRODUCTION

The termination of translation occurs on ribosomes in response to one of the three stop codons, UAG, UGA or UAA. Extraribosomal proteins, the release factors, are essential components of this step (1–4). Both prokaryotic and eukaryotic release factors are composed of two classes, namely class-I and -II factors. Prokaryotes generally have two codon-specific class-I factors with overlapping specificity, RF1 (for UAG/UAA) and RF2 (for UGA/UAU), while eukaryotes and archaea have only one omnipotent factor, eRF1 and aRF1, respectively (1,2). A basic mechanism for the codon-specific recognition by the ‘peptide anticodon’ of Escherichia coli RF1 and RF2 has been proposed recently (5). In addition, the crystal structure of eRF1 has been solved (6) and a region defining stop codon recognition has been suggested (6–10).

Class-II factors are RF3 and eRF3 in prokaryotes and eukaryotes, respectively, and are not found in small-genome bacteria or archaea. Class-II factors belong to the guanine nucleotide-binding protein superfamily and share a homology with other translation factors, such as EF1A, EF2 and eEF-1A. The termination of protein synthesis is achieved by the recognition of the stop codon in mRNA by class-I factors, which then trigger the hydrolysis of the ester bond linking the nascent polypeptide to peptidyl-tRNA on the ribosome. RF3 is not essential for bacterial cell growth and is known to recycle class-I factors (11). In contrast, eRF3 is an essential cell component, forms a stable complex with eRF1 and shares a stronger homology to bacterial EF1A rather than to EF2. It has therefore been proposed that eRF3 acts as a vehicle for eRF1, mimicking the carrier function of EF1A for tRNA (12). However, the exact mechanism of action of eukaryotic release factors, in particular the role of eRF3 and GTP hydrolysis, remains unclear. In the literature, the in vitro eukaryotic polypeptide-release assay has been based on the classical Caskey’s model (13). In this system, release of formyl-[35S]methionine (f[35S]Met) from the ribosomal complex composed of f[35S]Met-tRNAfMet, an initiator triplet (AUG) and a stop codon-containing tetranucleotide (i.e. UAAA, UAGA or UGAA) is measured upon the addition of release factors (eRF1 and eRF3). However, the lack of a convenient assay system of eukaryotic termination that is dependent on natural mRNA has been an obstacle to the functional study of the release factors.

A release signal—a stop codon with its flanking regions—can be alternatively decoded in some genes causing...
translational frameshifting, readthrough or selenocysteine incorporation, referred to as 'recoding' for re-programmed genetic decoding (14). The association of recoding with termination may reflect the fact that termination is a relatively slow process compared to the elongation step (11,15). It has been shown that the efficiency of a stop signal is affected by its context and that the stop signal at the recoding site is often in an unfavorable context for efficient termination (16). Each ribosome that reads a stop codon within a recoding site would select either a termination event or, alternatively, a recoding event. Therefore an increase in the frequency of one of these events should result in a decrease in the frequency of the other event. In the case of prokaryotic regulatory frameshifting of E.coli RF2 mRNA, an increase in the RF2 protein decreases the frameshift efficiency in vitro (17). A three way competition between termination, suppression and +1 frameshifting at the RF2 frameshift site was demonstrated by the addition of a suppressor tRNA (18). In eukaryotes, addition of Xenopus eRF1 to the rabbit reticulocyte lysate translation system has been shown to reduce stop codon readthrough of a plant virus mRNA in the presence of suppressor tRNAs (19). Thus, competition between termination and recoding could be utilized to measure the activities of release factors.

In the present study, we attempted to utilize translational frameshifting of mammalian antizyme to assay translation termination. Antizyme regulates the cellular level of polyamines (putrescine, spermidine and spermine) in a wide variety of eukaryotes (20±23). There are three members of the mammalian antizyme family, antizyme-1, 2 and 3 (22,23). The initiating open reading frame (ORF1) of antizyme-1 mRNA has a UGA at codon 68 where the majority of translating ribosomes terminate translation, while the rest of the ribosomes undergo a +1 frameshift event to decode UCC UGA U sequence (where the terminator of ORF1 is underlined) to serine-aspartate and continue elongation along the second open reading frame (ORF2) (24–26). Frameshifting is induced by polyamines and, in the presence of an optimal concentration of polyamines, the frameshift efficiency reaches >20% (26). A pseudoknot structure 3' to the shift site and an upstream GC-rich sequence are cis-acting elements that stimulate the frameshifting event (26,27). For frameshifting to occur, the UGA codon must occupy the ribosomal A-site. Therefore the system represents a promising means by which translation termination can be measured. We examined the activities of homologous rabbit eRF1 and eRF3 in this assay system.

MATERIALS AND METHODS

Cloning of rabbit eRF3

Rabbit eRF3 cDNA was cloned in this study by hybridization screening of the rabbit muscle λZap II cDNA library (Stratagene) using human eRF3 (kindly provided by Dr S. Hoshino) (28) as a probe. The hybridization and detection of positive clones were carried out with ECL kit (Amersham) according to the instructions. For positive clones, in vivo excision of the pBluescript phagemid from the λZap II vector was carried out using ExAssist/SOLR system (Stratagene). A clone containing the longest insert (2203 bp) was sequenced on both strands with Taq DyeDeoxy terminator cycle sequencing kit (Applied Biosystems). The cDNA contained an open reading frame of 1767 bp where a part of the 5' terminal sequence corresponding to the amino terminal 32 amino acid residues of human eRF3 (NP_060564) is missing. The coding sequence shares 86.4 and 88.6% identity with human eRF3 on the nucleotide and the amino acid levels, respectively. The cDNA sequence has been deposited to DDBJ/EMBL/GenBank accession number AB035256. It is known that the N-terminal part of the mammalian eRF3 sequence is functionally dispensable in vitro and in vivo (29) and in fact, the cloned N-terminally truncated cDNA sequence was functionally active in the complementation test in vivo (see below). Hence we used this cDNA clone in further analysis.

The functional integrity of the rabbit eRF3 clones was tested by complementation of Saccharomyces cerevisiae sup35 (eRF3) mutant as described previously (30). Both untagged and a C-terminal His6-tagged form of eRF3 complemented the temperature-sensitive sup35 mutation at 37°C to the same extent.

The site-directed mutant of rabbit eRF3 was prepared using Altered Sites II in vitro Mutagenesis System (Promega) with the corresponding oligonucleotides and the sequence was verified.

Expression and purification of rabbit eRF1 and eRF3

The untagged form of full-length rabbit eRF1 was overexpressed in E.coli BL21 (DE3) cells via pET-3(a) vector encoding the cDNA of full-length rabbit eRF1 (DDBJ/EMBL/GenBank accession number AB029089) as reported previously (31). Cells transformed with this construct were also transformed with an additional plasmid, pUBS520, encoding a tRNAArg that is cognate to the rare arginine codons of E.coli (kindly provided by Dr R. Mattes) (32). Synthesis of eRF1 was induced when the cell density at OD600 reached 0.5 with 1 mM isopropylthio-D-galactoside (IPTG) for 4 h at 37°C. The soluble fraction of bacterial lysate (S100) was precipitated with ammonium sulphate (25–50%) and dialyzed against buffer A (50 mM Tris–HCl, pH 7.8, 50 mM NaCl and 2 mM DTT). Next, chromatography by a Heparin Toyopearl column (Takara) using a linear gradient of 0.05–1 M NaCl in buffer A was performed. After dialysis, eRF1 was further purified on a MonoQ column (Pharmacia) by FPLC using a linear gradient of 0.15–1 M KCl in buffer A. Fractions containing eRF1 were combined, dialyzed against 50 mM Tris–HCl, pH 7.5, 50 mM KCl, 2 mM DTT and 5% glycerol, and frozen at −80°C.

For rabbit eRF3 expression, E.coli BL21 (DE3) was transformed with a PET 30-a(+) vector expressing the eRF3 cDNA with a His6-tag at the C-terminus. The transformants were grown to an OD600 of 0.5, induced with 0.1 mM IPTG and incubated at 25°C overnight. The recombinant eRF3 was purified from the soluble fraction of bacterial lysate by a Ni-NTA agarose column (Qiagen) using a linear gradient of 15–250 mM imidazole in 50 mM NaH2PO4, pH 8.0, 300 mM NaCl and 1 mM DTT. After dialysis against 50 mM Tris–HCl, pH 7.8, 5 mM KCl and 1 mM DTT, eRF3 was then subjected to chromatography using a Heparin Toyopearl column under the same conditions as for eRF1.
Transcription and translation in vitro

The plasmid C3.UGA (referred to as C3NE in the reference) and its derivatives C3.UAG and C3.UAA were described previously (26). Sense and antisense oligonucleotides of constructs containing readthrough or natural termination signals were annealed and cloned into the BstEII and KpnI sites of the pGB vector (Table 1) (26). In vitro transcription and translation were performed as described previously (26). Briefly, translation mixture (15 μl) contained 0.6 pmol of the mRNA, 15 μCi of [35S]methionine (>1000 Ci/mmol, New England Nuclear) and 10.5 μl of ribonuclease-treated rabbit reticulocyte lysate (Promega). All of the translation reactions in this study were supplemented with 0.5 mM spermidine for optimal antizyme frameshifting. After translation reaction at 30°C for 45 min, the labeled products were separated by SDS–PAGE on a 10% acrylamide gel and quantified by scanning the dried gel with a BAS 2000 Image Analyzer (Fuji).

Suppressor tRNA

An artificial amber suppressor tRNA gene was prepared by modification of S.cerevisiae tRNA55r gene (33). It was overexpressed in E.coli and purified by ion-exchange column chromatography as described (34).

RESULTS

Exogenous eRF1 and eRF3 decrease antizyme frameshift efficiency in rabbit reticulocyte lysates

The antizyme frameshifting assay was carried out in the rabbit reticulocyte lysate translation system using recombinant rabbit eRF1 (31) and eRF3 (cDNA cloned in this study) proteins. Translation was carried out with mRNAs carrying brome mosaic virus (BMV) coat gene fused to the rat antizyme-1 ORFs in the presence of 100 μg/ml alone or together with eRF1 (Fig. 1B, lanes 5–8 and Fig. 2A). Addition of eRF3 enhanced the inhibitory effect of eRF1 and lowered the effective dose of eRF1. In a control experiment, heat-denatured eRF1 and eRF3 (Fig. 1C) or purified recombinant green fluorescent protein (data not shown) did not affect antizyme frameshift efficiency. In the absence of exogenous spermidine, antizyme frameshift efficiency was 4.5 times less, but the effects of the addition of eRF1 and eRF3 was identical to those with spermidine (data not shown). These results indicate that in a competing system of translation events and antizyme frameshift events, eRF1 and eRF3 function synergistically to favor the termination events.

eRF1 and eRF3 affect frameshifting at all three stop codons

Although all of the known antizyme frameshift signals, from Schizosaccharomyces pombe to mammals, exclusively contain a UGA codon at their frameshift site (22), it has been shown that experimental substitution of the UGA by either UAG or UAA only caused a mild decrease in the frameshift efficiency (26,27). We tested the effect of eRF1 and eRF3 on these mutant frameshift sites. Addition of eRF1 with or without eRF3 decreased the frameshift efficiency at either the UAG or UAA codon. The effect of eRF1 and eRF3 together was synergistic and showed a similar dose-dependent curve to that for the wild type mRNA (Fig. 2B and C), confirming the omnipotency of codon recognition by eRF1 (19) and showing that eRF3 functions in a cooperative manner at all three stop codons by roughly the same extent.

Increased antizyme frameshifting by the G-domain variant of eRF3

It has been reported that eRF3 stimulates the activity of eRF1 in the presence of GTP (29) and that eRF3 exhibits an eRF1- and ribosome-dependent GTPase activity (35). To test whether the inhibitory activity of eRF3 on antizyme frameshifting depends on its GTP/GDP binding ability, we substituted an essential amino acid residue within the frameshifting efficiency. The BMV coat-antizyme fusion mRNA directed frameshifting at an efficiency of ~25% in the reticulocyte lysate supplemented with 0.5 mM spermidine. The addition of increasing amounts of purified eRF1 decreased the frameshift efficiency in a dose-dependent manner (Fig. 1B, lanes 1–4 and Fig. 2A). Next, purified eRF3 was added at a concentration of

<table>
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<th>Signal</th>
<th>Sequence</th>
<th>GenBank accession no.</th>
</tr>
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<tr>
<td>SIND</td>
<td>Sindbis virus readthrough</td>
<td>CCAGTTGACTAGACGGTGACAGCG</td>
<td>J02363</td>
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<tr>
<td>BNYVV</td>
<td>BNYVV readthrough</td>
<td>CCGGACAAATAGCAATTAGGCG</td>
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<td>ATGTTGACAGACACG</td>
<td>M16650</td>
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<tr>
<td>ODC-C</td>
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<td>ATGTTGACAGACAGACG</td>
<td>M16650</td>
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<tr>
<td>ODC-T</td>
<td>Human ornithine decarboxylase termination</td>
<td>ATGTTGACAGACACG</td>
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<tr>
<td>La</td>
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<td>GAGACGAGTTGTTAGTAA</td>
<td>J04205</td>
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<tr>
<td>FCRB</td>
<td>Human low-affinity IgG Fc receptor termination</td>
<td>GTATTGACAGACACG</td>
<td>M28696</td>
</tr>
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*The stop codon is underlined.*

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Table 1. Constructs containing natural readthrough or termination signals used in this study
of 100 μg/ml increased the antizyme frameshifting from 15 to 26%. The increase could be reduced to the control level by the addition of 40 μg/ml of purified eRF1. The opposite effect of the mutant eRF3 strongly suggests that the mutant eRF3 acts as a dominant negative inhibitor that retains eRF1-binding capacity (discussed below). This result also suggests that the effect of eRF3 to enhance termination is dependent on its GTPase activity.

**Competition between frameshifting, readthrough and termination at the antizyme frameshift site**

The termination assay was extended by adding a suppressor tRNA to the translation reaction so that termination, frameshifting and readthrough at the antizyme frameshift site could be monitored simultaneously. We used an amber suppressor tyrosine tRNA (33) and coordinated the mRNA with the altered antizyme frameshift signal with UAG stop codon. Addition of the suppressor tRNA to the translation reaction reduced the amount of the frameshift and termination products, and concomitantly, two additional bands appeared between the frameshift and termination bands (Fig. 4A). The size of the newly appeared small product (RT1) coincided to the calculated molecular mass (30 367 Da) yielded by UAG codon readthrough at the frameshift site (codon 258) followed by termination at the next in-frame UAG stop (codon 282). The other, larger, product (RT2) is likely to be caused by readthrough of both UAGs at codons 258 and 282, followed by termination at the next in-frame UAA at codon 299 (expected molecular mass 32 127 Da). The nucleotides 3′ to UAG at codon 282 and UAA at codon 299 are both ‘C’, and UAG-C is known to be an inefficient terminator (see below). The sum of intensities of the two readthrough bands (RT1 + RT2), as well as the RT2/RT1 ratio, was increased by the suppressor tRNA (Fig. 4B). The decrease of the termination and frameshift products by the suppressor tRNA was almost proportional, unchanging the ratio of the frameshift to the termination products, that was 0.19 without the suppressor tRNA and 0.17 with 10 μg/ml of the suppressor tRNA.

In the presence of 0.2 μg/ml of the suppressor tRNA, ~10% of the frameshift product and 25% of the readthrough product were observed. Under these conditions, we measured the effect of the release factors on the two recoding events. As shown in Figure 5, the efficiencies of frameshifting and readthrough were reduced by the addition of exogenous
release factors in a similar manner. The results described in this section are consistent with a model that the three alternatives at the antizyme frameshift site, termination, frameshifting and readthrough, are in direct competition at the same instant.

**Comparison of the strength of termination signal at the antizyme frameshift site and those in other contexts**

During this study, we noticed that the effective concentrations of eRF1 to inhibit antizyme frameshifting was higher than for the stop-codon readthrough of the plant RNA virus (beet necrotic yellow vein virus, BNYVV) reported earlier (19). We tested whether termination at the antizyme frameshift site is less efficient than those at the viral recoding signal or natural termination signals at the end of various protein coding regions. For this purpose, we first examined the effects of the suppressor tRNA in different terminal contexts. Three UAG terminator signals from human genes encoding ornithine decarboxylase (ODC), autoantigen ribonucleoprotein La (La) and low-affinity IgG Fc receptor (FCRB) were chosen based on the diversity of their respective coding contexts. The stop codons of these genes, as well as 5–9 flanking nucleotides on both sides, were inserted into the cloning site between two reporter genes [glutathione-S-transferase (GST) gene at the 5’ end and rabbit β-globin gene at the 3’ end] of the plasmid vector, pGB (26). Thus, only when the insert directed readthrough, could the β-globin reporter be translated as a fusion with GST. The mRNAs were synthesized from the constructs and translated in the reticulocyte lysate in the absence or the presence of varying concentrations of the suppressor tRNA. As shown in Figure 6A, UAG in the antizyme frameshift context was not suppressed in the absence of suppressor tRNA, while the addition of the suppressor tRNA caused a marked increase in readthrough products. On the other hand, the BNYVV signal caused a significant level of readthrough even in the absence of suppressor tRNA, but was less sensitive to the suppressor tRNA than the antizyme signal. The ‘true’ termination signals required even higher concentrations of the suppressor tRNA to be readthrough.

It has been reported that the identity of the base adjacent to the stop codon at the 3’ end is highly biased (16,38) and that it influences the efficiency of termination or suppression in experimental systems (39–41). For instance, UAG-A is a more efficient terminator than UAG-U or UAG-C (40,41). The base adjacent to the stop codon at the 3’ end used in our experiments was ‘U’ in the antizyme, La and FCRB termination signals, ‘C’ in the BNYVV, and ‘A’ in the ODC termination signal. We tested the effects of the suppressor tRNA for readthrough efficiency on derivatives of the ODC construct in which the base 3’ adjacent to the UAG codon was substituted by either C or U. As shown in Figure 6B, the effects of changing the 3’ adjacent base were much smaller than the differences for the dose–responses between the recoding and termination contexts (see Fig. 6A). Therefore the termination efficiency of a stop codon is not merely determined by the 3’ base adjacent to the stop codon, but by a broader context.

We then compared the effects of the release factors on the recoding event at the stop codons of varying contexts by the addition of amber suppressor tRNA to translation reactions directed by the antizyme, BNYVV or ODC constructs (Fig. 7). The effects of the release factors were almost identical for frameshifting and readthrough in the context of the antizyme stop signal (with or without suppressor tRNA) (Fig. 7A–C). The natural termination signal of ODC was much more sensitive to the release factors (Fig. 7F) while readthrough of the BNYVV termination signal showed an intermediate
sensitivity to the addition of release factors (Fig. 7E). We also tested a UGA readthrough signal from an animal virus, Sindbis (SIND), which can be readthrough in the absence of exogenous suppressor tRNA (42,43). The efficiency of stop codon readthrough in the SIND context was ~7%, and this was reduced by the addition of release factors to a similar extent to that of frameshifting or readthrough at the antizyme recoding site (Fig. 7D).

DISCUSSION

We developed an in vitro assay system to monitor the activity of eukaryotic release factors in translation termination by measuring the relative frequency of competing frameshift events at the antizyme termination signal. Ribosomes decoding along the antizyme frameshift site will either terminate translation at the UGA codon, or undergo a +1 frameshift event and continue elongation. Therefore reduction in frameshift events would correspond to an increase in translation termination activity, and vice versa. The relatively high occurrence of antizyme frameshifting in rabbit reticulocyte lysates provides a convenient, quantitative assay to monitor release factor activity. In this system, it is not necessary to supplement suppressor tRNA in the basic assays, and termination at all three stop codons can be quantified by simply changing the codon in the termination signal of the tester mRNA.

Using this system, we tested the activities of recombinant eRF1 and eRF3 of rabbit origin. Antizyme frameshifting was inhibited by eRF1 alone in a dose-dependent manner. Although addition of eRF3 alone caused only a marginal to mild inhibition, simultaneous addition of both factors synergistically inhibited antizyme frameshifting. Next we examined the effect of a mutant eRF3 at lysine 216, the residue known to be essential for guanine-nucleotide binding of G proteins (36). The mutant eRF3 increased the frameshift efficiency when added alone and the effect was reversed by eRF1. It is known that the binding of eRF3 to eRF1 does not
require the G domain, which is unlike prokaryotic RF3 (44,45). Therefore our result can be explained by assuming that the mutant eRF3 retains the eRF1-binding capacity and hinders eRF1 function by forming a non-functional eRF1–eRF3 complex or by preventing an eRF1 exchange. However, other possibilities cannot be excluded at present since eRF3 function per se is not yet well understood. Further in vivo and in vitro analyses are necessary to uncover the reason for increased antizyme frameshifting by the G-domain variant of eRF3.

To analyze the relationship between the frameshift and termination events more precisely, we developed an additional assay method where frameshifting, stop codon readthrough and termination at the antizyme frameshift site can be measured with a single reporter system. This was achieved by the addition of an artificial amber suppressor tRNA as well as using the mutant antizyme frameshift signal with the UAG stop codon. Addition of increasing concentrations of the suppressor tRNA lowered both frameshifting and termination proportionally. In the presence of smaller concentrations of the suppressor tRNA, both of the frameshift and readthrough products were reduced by the release factors with a similar dose dependency. Thus, among three alternatives at the antizyme frameshift site, the forced increase of either readthrough or termination caused a concomitant decrease in the other two events. This is consistent with a model that the three events are in direct competition at the same instant of the translation cycle: i.e. the instant at which the A-site codon is recognized. The same conclusion has been drawn for E.coli RF2 frameshifting (18).

Although it is widely accepted that eRF3 acts as a vehicle for eRF1 mimicking EF1A for tRNA, the timing of the GTP hydrolysis is still unclear and this model is not fully consistent with the results of molecular dissection studies of eRF1 and eRF3 (1). In addition, the discrepancy between the apparent dispensability in vitro and indispensability in vivo of eRF3 has led to the hypothesis that eRF3 has an alternative/additional function(s). Two possibilities have been hypothesized. First, by an analogy to prokaryotic RF3, eRF3 may be involved in recycling of the translational complex after the termination process (4,11,46). Second, eRF3 could be involved in either increasing the accuracy of stop codon recognition or perhaps proofreading stop codon recognition by eRF1 (47). Given that termination and frameshifting are competing at the codon recognition step, our results suggest that the primary role of eRF3 is the enhancement of the stop codon recognition by eRF1 and that GTP hydrolysis is necessary before the completion of the codon recognition step. Unfortunately, the alternative possibilities could not be addressed with our system since the recycling event would occur after A-site codon recognition, and termination proofreading cannot be detected at the stop codon. Thus, these alternative events are not in competition with the antizyme frameshifting event used in this system.

The contexts around the stop codon are not random and the efficiency of translation termination is influenced by context in eukaryotes. For ‘true’ termination signals, contexts are biased to preferable sequences for efficient termination and weak termination signals are often used at recoding sites (16,38). It has been demonstrated in mammalian systems that the nucleotide 3′ to the stop codon is a major determinant for the termination efficiency (40,41). By monitoring the effects of the addition of both suppressor tRNA and release factors, we demonstrated that the signal at the antizyme frameshift site is prone to less efficient termination, when compared to the natural termination signals of some protein coding sequences.

Interestingly, the stop signal at the readthrough site of plant virus BNYVV (UAG-C) was much more efficient for termination than those in animal recoding site, antizyme (UGA-U) and Sindbis virus readthrough (UGA-U), even though the UAG-C was shown to be one of the most inefficient tetranucleotide terminators in mammalian cells by others (40,41). Thus, consistent with previous observations in yeast (48,49), our results show that the termination efficiency is determined by a wider context. The antizyme mRNA context that circumvents termination at the shift site must be an important factor for efficient frameshifting and to address this issue further, precise mapping of cis-element(s) in the mRNA context should be performed.

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