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Competition between frameshifting, termination and suppression at the frameshift site in the *Escherichia coli* release factor-2 mRNA

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ABSTRACT

Competition between frameshifting, termination, and suppression at the frameshifting site in the release factor-2 (RF-2) mRNA was determined *in vitro* using a coupled transcription-translation system by adding a UGA suppressor tRNA. The expression system was programmed with a plasmid containing a *trpE-prfB* fusion gene so that each of the products of the competing events could be measured. With increasing concentrations of suppressor tRNA the readthrough product increased at the expense of both the termination and the frameshifting product indicating all three processes are in direct competition. The readthrough at the internal UGA termination codon was greater than that at the natural UGA termination codon at the end of the coding sequence. The results suggest that this enhanced suppression may reflect slower decoding of the internal stop codon by the release factor giving suppression a competitive advantage. The internal UGAC stop signal at the frameshift site has been proposed to be a relatively poor signal, but in addition the release factor may be less able to recognise the signal with the mRNA in such a constrained state. Consequently, the frameshifting event itself will be more competitive with termination *in vivo* because of this longer pause as the release factor is decoding the stop signal.

INTRODUCTION

Expression of polypeptide release factor-2 (RF-2) in *E.coli*, requires a +1 frameshift during translation of the mRNA to circumvent the 26th codon which is an in-frame stop codon (1). Such frameshifting events are believed to occur with slippage of the ribosome on the mRNA at the site, following a pause or slowing of the translational rate (2). Frameshifting is achieved by ribosomal slippage over a run of uracils, spanning a CUU leucine codon and a UGA stop codon in the RF-2 mRNA (3). The tRNA ^{leu} would recognise the UUU in the +1 frame (4). Elements important for the frameshifting event have been identified (3–6). Critical is a Shine-Dalgarno element at a particular spacing upsteam of the frameshift codon, allowing interaction of the mRNA with the 16S rRNA (4). The stop codon itself contributes significantly to the efficiency (2). Both of these features may be major contributors to slowing or pausing at the site to facilitate frameshifting.

For frameshifting to occur elements favoring the event must compete with translational termination at the stop codon. This codon is part of what has been postulated to be an inefficiently decoded stop signal (UGAC) (7). An elaborate mechanism for RF-2 autoregulation is suggested since, if factor concentration changes, the efficiency of decoding the UGAC as stop would also change, affecting frameshifting at the site and thereby modulating the cellular concentration of release factor. Experimental evidence supports this concept (8,9).

Pausing at the RF-2 frameshift site was investigated indirectly by Curran and Yarus using an *in vivo* approach (5). Their elegant system used the RF-2 frameshift window at the beginning of a β galactosidase reporter gene. Surprisingly a UGA suppressor tRNA, expected to decrease the length of a putative pause at the site and thereby decrease frameshifting, gave the opposite result by increasing the activity of b galactosidase, the indicator of frameshifting. However, expression of this tRNA may have altered the cell physiology, thus producing an apparent increase in frameshifting efficiency. Indeed when the UGA at the RF-2 frameshift site was replaced with UAG the expected decrease in frameshifting mediated by a UAG suppressor tRNA was observed. The UAGC stop signal is rarely used and may be relatively poorly decoded (7, 9). It remained somewhat ambiguous therefore whether an extended pause was occurring at the frameshift site. In these studies (5), of the three possible events at the frameshift site; frameshifting, translational termination or translational suppression of the stop codon, only the frameshift product could be measured.

In the current study an *in vitro* transcription/translation assay was devised where each of the products of termination, suppression or frameshifting could be measured. Since the small

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termination product (25 amino acids) is rapidly degraded *in vitro* (10) a *trpE-prfB* gene fusion, which produces a stable termination product, was used. The effect of a UGA suppressor tRNA on the frameshifting and termination events could be assessed directly, and the dependence of the frameshifting event on the termination and/or suppression events determined.

MATERIALS AND METHODS

Strains and plasmids

The plasmids pRFTH2 and pRFTH4 have been described previously (9). *E.coli* strain CP79 (*SupE* 44), and this strain harbouring pPY1001, were kind gifts from Robert Weiss (11).

Extraction and purification of tRNA from *E.coli*

Cultures (30 mL) of CP79 (for control tRNA) and CP79 harbouring pPY1001 (for tRNA enriched with the UGA suppressor) were grown to A_{600} of 0.4 in M9 media (12) containing 0.2% casamino acids, and 18 mg/mL chloramphenicol in the latter culture only. To a 5 ml sample of each culture IPTG and cAMP were added (final concentrations of 2 mM and 5mM respectively). After incubating for 2 hours at 37°C with vigorous shaking, the harvested cells (1000×g, 7 min.) were washed with STE (13) and resuspended in 1ml lysis media (10% sucrose, 10 mM Tris-HCl (pH 7.6), 5 mM EDTA, 25 mM NaCl and 5 mg/ml lysozyme). After incubating at 4°C for 10 min., 1 ml of 2% SDS was added and the cells were vortexed for 2 min. The RNA was extracted twice with an equal volume of phenol and then with phenol:chloroform (1:1). RNA from 500 ml was then concentrated 5 fold by precipitation with ethanol. Yield of RNA was approximately 100 mg with a ratio of A_{260}/A_{280} of 2.0. The tRNA was further purified using a 5–20% (w/v) sucrose gradient as described by Clemans (13). The final concentration of purified tRNA was 100 mg/ml.

The *in vitro* transcription-translation assay

The S30 extract used in the assay was prepared from logarithmic growth phase cultures of strain MRE600 (14). *In vitro* transcription-translation assays involving expression of plasmid-borne genes, in the presence of the tRNA preparations, were as described by Pratt (15). Products were labelled with 1 mCi [^{35}S]-methionine (Amersham) per 10 mL assay. A sample of the assay was resolved on a 10% polyacrylamide SDS gel (0.8×80×100mm)(16). Gels were processed for fluorography and resulting exposures on preflashed Cronex X-ray film quantitated on an LKB Ultroscan XL laser densitometer. All quantities were corrected for the frequency of methionine in their products; RFTH2 and RFTH4 contain 14 methionines, the prematurely terminated RFTH2 product and the product from readthrough of the in frame stop codon contain 6 methionines per molecule. Products from stop signal readthrough of RFTH2 and RFTH4 were assumed to contain 14 methionines per molecule.

Western blot analysis

After electrophoresis of one eighth of the assay on a 10% polyacrylamide SDS gel, protein was transferred to nitrocellulose membrane as described (11). Antibody raised against a TrpE-RF-1 fusion protein (including only RF-1 sequence not homologous to RF-2) was a kind gift of John Moffat. The ECL method (Amersham) was used to detect sites of primary antibody binding.

RESULTS

The RF-2 frameshifting site

The frameshift site in the RF-2 gene is shown in Figure 1 with the relevant features affecting the efficiency of the event, the UGA stop codon, the upstream Shine–Dalgarno sequence, and the run of uracils. Furthermore the 7 codons immediately upstream of the frameshift site have a very low codon adaptation index (CAI) (9), in contrast to regions further upstream or downstream of the site . A low CAI correlates with a slow elongation rate (17), suggesting that in the RF-2 mRNA these 7 codons may contribute to a decrease in the rate of translation.

A system to detect termination, frameshifting, and suppression products

A gene fusion was utilised to study the events at the RF-2 frameshift site (Figure 2). The *prfB* gene was fused downstream of the 5′ region of the *trpE* gene. The fusion gene encodes all of the RF-2 protein except the first 12 amino acids, and expression of the full-length RFTH2 fusion protein requires a +1 frameshift over the UGAC stop signal at the RF-2 frameshift site. Expression of both the product of the frameshift event (M_r of 76 000) and the termination event (M_r of 38 000) was measured using a coupled *in vitro* transcription/translation assay (9). In the assays conducted the frameshifting efficiency was approximately 20%. A fusion gene lacking the RF-2 frameshifting site (RFTH4 – M_r 70 000) was used as the control (see Figure 2). If suppression at the stop signal in the RF-2 frameshift site were occurring then a product of 41 000 M_r was expected. Hence this assay allows all three products of the competing events to be separated on the basis of their molecular weight differences and individually measured.

Isolation of UGA suppressor tRNA

Transfer RNA was prepared from cells containing or lacking the suppressor tRNA, Su7UGA, to give samples of suppressor tRNA or control tRNA respectively. The Su7UGA was expressed in cells harbouring the plasmid pPY1001 by induction of transcription using IPTG and cAMP as described previously (12). This $tRNA^{Trp}$ has a change in its anticodon from CCA to UCA compared with the wild type $tRNA^{Trp}$. It can also read inefficiently the normal tryptophan codon, UGG, as well as UGA, as expected from Watson–Crick wobble base pairing. The charging specificity is not altered by the change in the anticodon (18). Crude tRNA was isolated and then further purified on a 5–20% (w/v) sucrose gradient to remove DNA and other RNA.

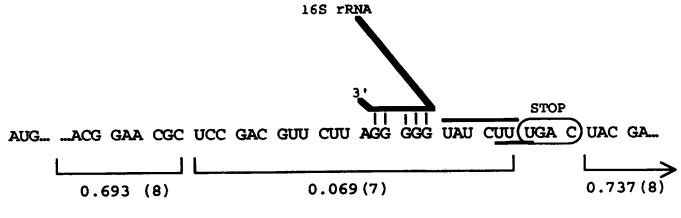

Figure 1. Features of the RF-2 mRNA that contribute to frameshifting. The putative 'poor' stop signal, and the Shine–Dalgarno interaction, between the 16S rRNA and RF2 mRNA, are indicated. Sub-optimal spacing between the site of the Shine–Dalgarno interaction and the leucine codon is indicated by an overline. The run of uracils is underlined. CAI values for specified regions around the frameshift site are indicated and the average of the number of codons are shown in brackets.

The purified tRNA was resuspended at a final concentration of ~100mg/ml.

Suppression at the internal stop codon of the RF-2 frameshift site

The suppressor and control tRNAs were titrated into *in vitro* transcription/translation assays directed by the plasmid encoding the fusion protein, RFTH2 (Figure 3A). A product of suppression was observed increasing in intensity (arrow RP1) when the suppressor tRNA was titrated (lanes 2–5) but not in the titration of the control tRNA (lanes 6–9). There is suggestion of a small amount of readthrough at this UGA codon even in the translations where the tRNA containing the Su7UGA was not present (for example Fig. 3A, lane 6). Where might this be coming from? The readthrough of UGA codons is known to occur at a low level through recognition of UGA by the wild type $tRNA^{Trp}$ (19); in the UGA context under study at the RF-2 frameshift site, as discussed below, such readthrough may be particularly favoured. The upper prominent band in all lanes is the frameshift product (76 000M_r), and the band just below the suppression product (RP1) is the termination product (38 000Mr). Suppression of the natural stop signal (UGAG), though less pronounced, was also observed at the higher concentrations of suppressor tRNA leading to a second readthrough product of slightly higher relative mass than the frameshift product (RP1 —lanes 4,5). The lowest band on the gel is the plasmid-encoded ampicillin resistance gene product (β-lactamase).

A Western blot of a sample of the assays was probed with an antibody reactive against the trpE portion of the fusion proteins. The antibody recognised the readthrough product at ~41 000M_r, demonstrating it contained the trpE amino acid sequences. The readthrough of the UGA at the frameshift site in the RF-2 mRNA means that translation proceeds until the next in frame stop codon (UAA) is encountered. This is 32 codons past the UGA (1) so that the product is extended by 32 amino acids (the readthrough amino acid and then another 31 until the next stop signal).

To confirm that the readthrough product was dependent upon the presence of the frameshift site in RFTH2 mRNA, the suppressor tRNA was titrated into transcription /translation assays directed by the plasmid encoding RFTH4 from which the frameshift site had been deleted (see Figure 2). As shown in Figure 3B (lanes 5–8) no product of the size of the readthrough product was observed, compared with the reactions directed by the RFTH2 encoding plasmid (lanes 1–4). The major products seen in the reactions directed by RFTH4 were the expected fusion polypeptide of ~70 000M_r, and the b lactamase, but a readthrough of the natural stop signal was observed with a band above the fusion polypeptide.

The amounts of the suppression, termination and frameshifting products were quantitated over the titration of suppressor tRNA in assays expressing RFTH2 or RFTH4. The signals were corrected for the number of methionines in each product (6 for the shorter termination and readthrough products, and 14 for the larger frameshift and readthrough products).The percentage of each product calculated for each concentration of the suppressor tRNA is shown in the Table. Fluorographs of various exposures of the experiment detailed in figure 3b, were used to determine the relative levels of the proteins expressed *de novo*. Percentage of readthrough at each stop codon resulting in the expression of RP1, RP2 and RP3 was calculated for the titration of suppressor tRNA into the assays. The product of termination at the frameshift stop codon and RP1 both have 6 methionines. RFTH2 and RFTH4 both have 14 methionines. RP2 and RP3 were assumed to have 14 methionines. The significant conclusion from this analysis was that as suppressor tRNA concentration increased the percentage of *both* the frameshifting and termination products decreased simultaneously. Concomitant with these changes was

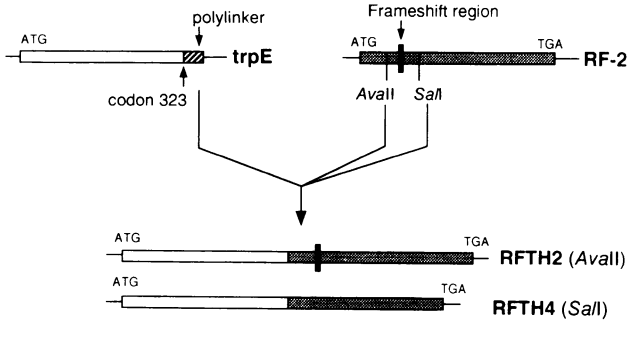

Figure 2. The *trpE-prfB* gene fusions. Fragments of the gene for RF2 were created by enzyme restriction with *Ava*II (pRFTH2) and *Sal*I (pRFTH4). The fragment ends were repaired with the Klenow fragment before ligation into the *Sma*I site of pATH10 and pATH11, respectively, as shown.

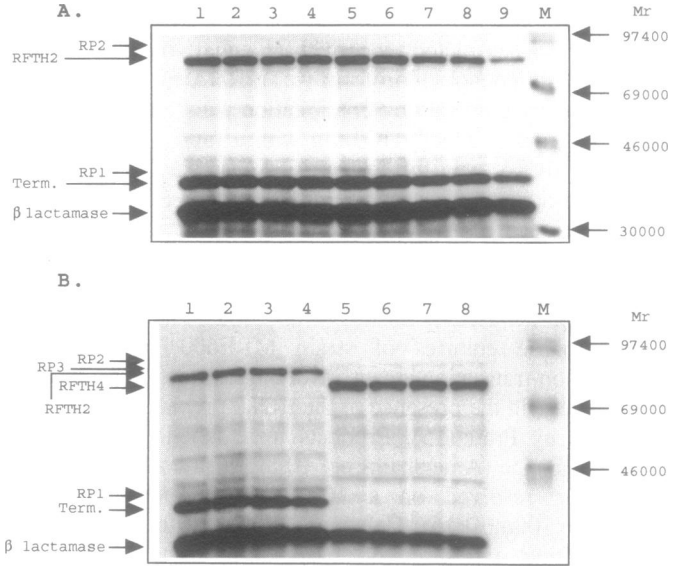

Figure 3. A. Effect of the suppressor tRNA on expression of RFTH2. The tRNA extract containing Su7UGA (lanes 2–5) and control tRNA (lanes 6–9) were titrated (37.5, 75, 125, 150 μg of each) into the *in vitro* transcription-translation assay expressing RFTH2 (0.1 μg of pRFTH2). Lane 1, 0.1 μg pRFTH2 alone. Protein in 1/5 of the assay was fractionated on a 10% SDS PAG and the gel processed to obtain the fluorograph. Novel proteins labelled with [^{35}S]Met are indicated. Term indicates the prematurely terminated RFTH2. RP1 and RP2 are the products of readthrough of the frameshift stop codon and the RF2 stop signal, respectively. M_r, molecular weight markers. **B.** Effect of the suppressor tRNA on *in vitro* expression of RFTH2 and RFTH4. Into *in vitro* transcription-translation assays expressing RFTH2 (0.1 μg of pRFTH2, lanes 1–4) or RFTH4 (0.1 μg of pRFTH4, lanes 5–8), 37.5, 75 and 150 μg of tRNA containing Su7UGA tRNA (lanes 2–4 and 6–8), was titrated. Lanes 1 and 5 contained no added tRNA. Protein from 1/4 of the assay was fractionated on a 10% SDS PAG and a fluorograph obtained. Term indicates the prematurely terminated protein of RFTH2. RP1, RP2 and RP3, are readthrough products of the frameshift stop codon in the RFTH2 gene, and the RF2 stop signal of the RFTH2 and RFTH4 genes, respectively. M_r, molecular weight markers.

Table 1. Competition between frameshifting, termination and suppression, at the internal stop codon of the RF-2 frameshifting site of RFTH2 and between termination and suppression at the natural termination codons in RFTH2 and RFTH4 mRNAs.

Suppressor tRNA (μg)	RFTH2 Frameshift site % frameshift	% term.	% supp.	RFTH2 stop signal % supp.	RFTH4 stop signal % supp.
0	16.4	83.6	nd[1]	nd	1.4
37.5	15.3	75.6	9.1	nd	2.9
75	15.9	73.7	10.3	5.1	3.4
150	14.2	72.1	13.6	7.4	5.1

[1] not detected sufficiently over background

the appearance of the readthrough products. While accurate absolute quantitation of these products is difficult the evidence both from visual observation of the products (see Figure 3A) and from this quantitative analysis is strongly suggestive that suppression is significantly greater at the UGAC in-frame stop signal than at the natural UGAG stop signal. In both the control construct and the construct containing the internal stop signal the readthrough at the natural signal is 2−3 fold lower than that observed at the internal stop signal.

DISCUSSION

Four possible scenarios for competition of the events at the RF-2 frameshift site are shown in Figure 4. Model A assumes all three events are competing at the site and therefore if one event is modulated up or down both of the other two would be expected to respond to this perturbation. This model was predicted by Curran and Yarus (5). Model B assumes a decision to frameshift is dominant and that competition at the stop codon would only affect the termination and suppression events. Model C assumes termination is dominant and that frameshifting or suppression will compete with each other as secondary events. A fourth alternative (D) would allow for frameshifting or suppression events to compete before the mRNA was in a position where decoding of the stop signal would occur.

Our results strongly support Model A where all three events are competing with each other, since changing the competitive advantage of one event, namely suppression in our experiments has affected the other two events simultaneously. The earlier *in vivo* studies of Curran and Yarus (5) were consistent with model A but could not eliminate model C since addition of suppressor tRNA was seen to affect frameshifting efficiency, but the termination event was not measured. In contrast to the *in vivo* results of Curran and Yarus the *in vitro* system used in this study showed that a UGA suppressor tRNA could compete at the frameshift site and lower the frameshifting efficiency. Indeed when the stop codon in the frameshift site is replaced with a rapidly decoded sense codon (for example, UGG, UUA) then the frameshifting efficiency decreased to about 10%, whereas if replaced by a rare sense codon decoded by a minor species of tRNA the frameshifting efficiency remained high (3). The stop codon, UAA, recognised by both release factors is more competitive than the naturally occurring UGA at the site, reducing the frameshifting efficiency by about half (3). These findings indicate that slow decoding of a poor stop signal in an unfavorable context can significantly contribute to the high efficiency of frameshifting.

Pausing of the ribosome at the RF-2 frameshifting site was postulated as a requirement for efficient frameshifting by Weiss and Gallant (6). The string of rare codons preceding the site, and the Shine-Dalgarno element are likely contributors. One way to obtain a longer pause would be for the release factor to decode the stop signal inefficiently, accentuating what normally appears to be a relatively slow event in protein synthesis. The fact that UGAC has been proposed to be a poor stop signal would be consistent with that hypothesis. The prediction from this scenario is that suppression at the in frame stop signal might be more efficient than at a stop signal which was decoded by release factor more efficiently.

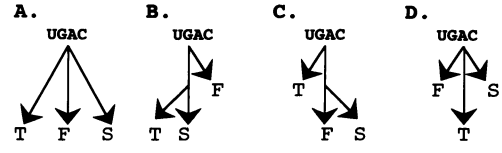

Figure 4. Models of competition at the RF2 frameshift site. **A.** RF2, the frameshift context and suppressor tRNA compete at the stop signal (UGAC) in the RF2 frameshift site for termination (T), frameshifting (F) or suppression (S). **B.** If frameshifting (F) does not occur then termination (T) and suppression (S) will be in direct competition as the default option. **C.** If termination (T) does not occur, the frameshifting (F) and suppression (S) will compete as the default option. **D.** Frameshifting (F) and suppression (S) compete and if neither occur then termination (T) is the default option.

Our study suggests that suppression efficiency at the internal UGA is perhaps two-three fold greater than at the natural stop codon. These results are consistent with the data of Curran and Yarus who demonstrated a high level of UAG suppression in the same context (5). The context of the stop signal at the end of the RF-2 coding sequence, UGAG, would be predicted to be *more easily* suppressed, by several fold, than that at the frameshift site, UGAC, from the studies of Kopelowitz *et al* 1992 (20) and Stromo *et al* 1986 (21). The opposite effect was found in our studies. The two-three fold higher efficiency of suppression found at the internal stop codon in the RF-2 mRNA suggests that the stop signal in this unfavorable context is decoded by the release factor with reduced efficiency. What might that unfavorable context be? The use of suboptimal codons prior to the frameshift site and the Shine-Dalgarno interaction reducing the elongation rate may be significant among those factors determining the efficiency with which the release factor can decode the internal stop signal. It has been determined, for example, that the frameshifting efficiency at the site is enhanced on hyperaccurate ribosomes where the rate of decoding of sense codons is reduced (22). The probability of frameshifting and suppression would increase if frameshifting occurs as the mRNA is in a transition stage (either prior to positioning the mRNA for termination and/or while waiting for RF to decode the stop codon), and if the period

of transition was lengthened as a result of slowing of the elongation rate or pausing.

In this study it has been found that suppression of the stop codon at the frameshift site in the RF-2 mRNA occurs at the expense of both frameshifting and termination, indicating all three events are in competition. The suppressor tRNA could compete more efficiently with the release factor at the frameshift site than at the natural stop signal suggesting that there is an extended pause at the stop signal of the frameshift site. The highly efficient frameshift is likely to be caused in part by a decreased efficiency of decoding of the stop signal by the release factor, and by other features of the context that slow the rate of elongation in this region.

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