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Indirect regulation of translational termination efficiency at highly expressed genes and recoding sites by the factor recycling function of *Escherichia coli* release factor RF3

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Prokaryotic release factor RF3 is a stimulatory protein that increases the rate of translational termination by the decoding release factors RF1 and RF2. The favoured model for RF3 function is the recycling of RF1 and RF2 after polypeptide release by displacing the factors from the ribosome. In this study, we have demonstrated that RF3 also plays an indirect role in the decoding of stop signals of highly expressed genes and recoding sites by accentuating the influence of the base following the stop codon (+4 base) on termination signal strength. The efficiency of decoding strong stop signals (e.g. UAAU and UAAG) *in vivo* is markedly improved with increased RF3 activity, while weak signals (UGAC and UAGC) are only modestly affected. However, RF3 is not responsible for the +4 base influence on termination signal strength, since *prfC*– strains lacking the protein still exhibit the same qualitative effect. The differential effect of RF3 at stop signals can be mimicked by modest overexpression of decoding RF. These findings can be interpreted according to current views of RF3 as a recycling factor, which functions to maintain the concentration of free decoding RF at stop signals, some of which are highly responsive to changes in RF levels.

Keywords: recoding/release factors/RF3/ribosome/translational termination

Introduction

Termination of protein synthesis in prokaryotes requires two codon-specific protein release factors (RFs) that recognize messenger-encoded stop signals and facilitate release of the completed polypeptide from the ribosome (Tate *et al*., 1995). In 1994, the gene for RF3, first identified in 1969 as a stimulatory factor for *in vitro* termination (Capecchi and Klein, 1969; Milman *et al*., 1969; Goldstein and Caskey, 1970), was identified simultaneously by two groups and designated *prfC* (Grentzmann *et al*., 1994; Mikuni *et al*., 1994). Mutations in *prfC* cause suppression of all three stop codons *in vivo* (Mikuni *et al*., 1994), supporting the hypothesis that RF3 interacts functionally with both RF1 and RF2 during translation termination.

RF3 contains a consensus sequence for a GTP-binding site and shares sequence homology with elongation factors EF-G and EF-Tu (Grentzmann *et al*., 1994; Mikuni *et al*., 1994; Kawazu *et al*., 1995). The role of guanine nucleotides in modulating the termination activity of RF3 recently has been confirmed as that for classical translational G proteins like EF-Tu and EF-G (Freistroffer *et al*., 1997; Pel *et al*., 1998). It originally had been reported that both GDP and GTP inhibited the stimulatory effect of nucleotide-free RF3 on termination *in vitro* (Capecchi and Klein, 1969; Goldstein and Caskey, 1970). An explanation for this apparently paradoxical finding is that nucleotide-free RF3 mimics a structural transition state, and the different guanine nucleotide states of RF3 have different affinities for binding to the ribosome (Pel *et al*., 1998).

How does RF3 stimulate translation termination? The apparent absence of equivalent EF-G domains IV and V from RF3 has led to the suggestion that RF3 might bind to the ribosome simultaneously with a decoding RF, a form of molecular mimicry of the tRNA complexes that decode sense codons during the elongation phase of protein synthesis (Moffat and Tate, 1994; Nakamura *et al*., 1995; Ito *et al*., 1996; Nakamura *et al*., 1996). Indeed, co-operative binding of RF3 and RF2 has been demonstrated, but only with the non-physiological guanine nucleotide-free form of RF3 (Pel *et al*., 1998). Recently, compelling evidence was obtained *in vitro* that RF3 affected dissociation rather than association of the decoding factors, accelerating neither the rate of association of RF1 and RF2 with the ribosome nor the catalytic rate of peptide release (Freistroffer *et al*., 1997; Pavlov *et al*., 1997a). A model was proposed in which RF3 binds after peptidyl-tRNA hydrolysis, displacing the decoding factors from the ribosome.

To clarify further the function of RF3 *in vivo*, we have investigated how it affects termination efficiency at stop signals of different strengths. The efficiency of termination signals is strongly influenced by the nucleotide immediately following the stop codon (+4) (Poole *et al*., 1995). Highly expressed genes in *Escherichia coli* predominantly use the strongest signals, particularly UAA with U as the base following (Tate *et al*., 1995). We show that RF3 co-operates with both RF1 and RF2 *in vivo* to enhance significantly the efficiency of decoding signals when they are to specify stop, but not when they are naturally weak signals and form part of a recoding site.

Results

The experimental system

Physiologically important differences in the decoding of stop signals are not easily studied *in vivo* at natural termination sites, where the competitive advantage of termination over non-cognate events is overwhelming. At a recoding site, however, where the termination codon

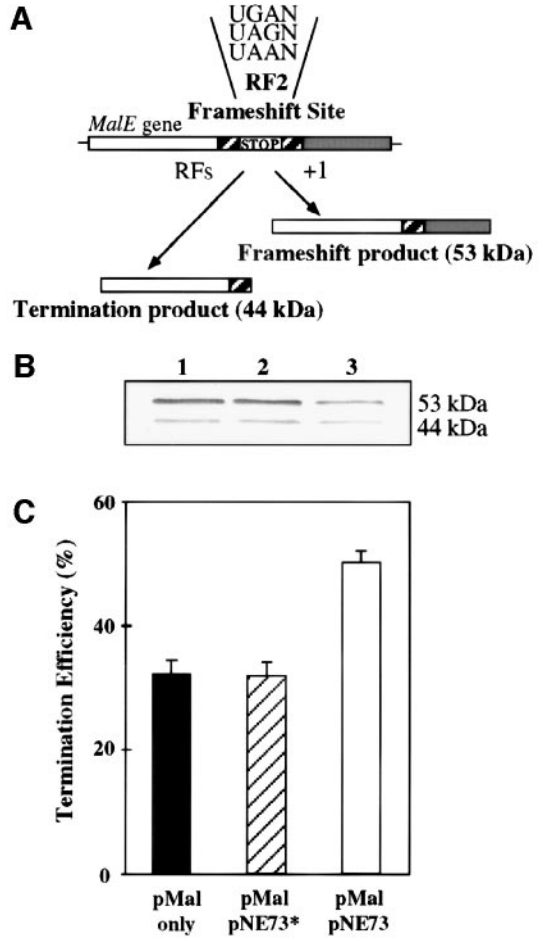

Fig. 1. An experimental system for investigating the effect of RF3 on translation termination *in vivo*. (**A**) Schematic diagram of the protein products produced in the translational termination–frameshift assay system (Poole *et al*., 1995). (**B**) A Western blot probed with an antibody to MBP, demonstrating the relative intensities of the frameshift (53 kDa) and termination (44 kDa) protein products in the presence of endogenous levels of RF3 (pMal only, lane 1), and overexpression of disabled RF3 (pMal and *prfC* deletion construct pNE73*, lane 2) and functional RF3 (pMal and pNE73, lane 3) at a UGA-containing stop signal. (**C**) The intensities of the protein bands shown in (B) were quantitated using laser densitometry and the termination efficiency calculated as described in Materials and methods; pMal only (filled bar), pMal and pNE73* (hatched bar) and pMal plus pNE73 (open bar). The means of three experiments (plus standard errors) are shown.

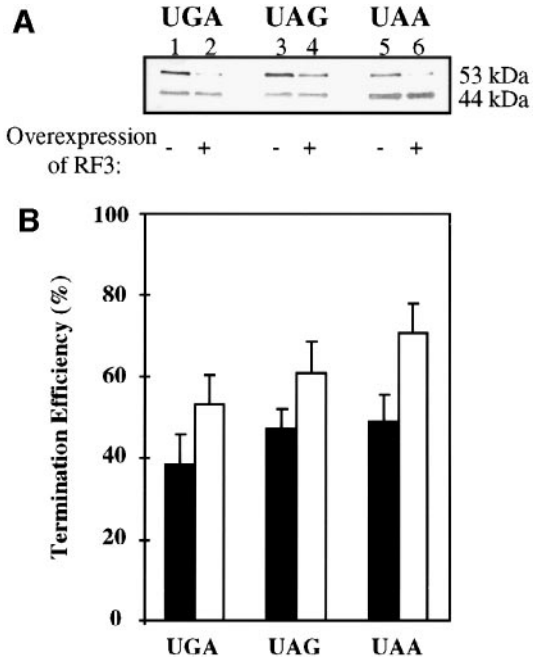

Fig. 2. Effect of overexpression of RF3 on termination efficiency at UGA, UAG and UAA stop codons. (**A**) Western blot probed with anti-MBP showing the relative levels of the frameshift (53 kDa) and termination (44 kDa) products with (lanes 2, 4 and 6) and without (lanes 1, 3 and 5) overexpression of RF3 at UGA (lanes 1 and 2), UAG (lanes 3 and 4) and UAA (lanes 5 and 6). (**B**) The mean termination efficiency (plus standard errors) for each stop signal expressed as a percentage; endogenous RF3 (filled bars), overexpression of RF3 (open bars).

is under competition from an alternative event, these differences become more apparent. The RF2 frameshift site constructed in a *MalE* reporter system (Poole *et al*., 1995) has been used as an indicator of stop signal decoding efficiency (Figure 1A). At the recoding site, there is direct competition between termination at the internal stop signal and frameshifting for expression of an extended product. The ratio of the termination (44 kDa) to frameshift (53 kDa) protein products provides a measure of decoding efficiency at the particular stop signal. Changes in termination efficiency at the recoding site, due to an interaction of exogenously expressed RF3 with endogenous RF1 and RF2 proteins, were then measured as a function of the different termination signals.

Initial experiments indicated that overexpression of RF3 in *E.coli* had a specific effect on termination efficiency in *prfC*$^+$ strains. A representative Western blot is presented in Figure 1B, showing results obtained with a UGA-containing termination signal, probed with an antibody to maltose-binding protein (anti-MBP; detects both termination and frameshift protein products). Figure 1C shows the termination efficiency calculated from Western results with endogenous levels of RF3 (pMal only), overexpressed disabled RF3 (pMal, pNE73*) and overexpressed functional RF3 (pMal, pNE73).

RF3 expression affects termination at UAA, UAG and UGA stop codons

Grentzmann *et al*. (1995) provided evidence that RF3 predominantly affected termination at UGA-containing stop signals, although earlier *in vitro* (Goldstein and Caskey, 1970) and suppression studies (Mikuni *et al*., 1994) had not indicated selectivity. To address this question, changes in the ratios of termination to frameshift products with overexpression of RF3 were determined for each of the three stop codons UGA, UAG and UAA in the same sequence context (Figure 2B). The intensity of the frameshifting band (53 kDa) decreased with overexpression of RF3 (Figure 2A, lanes 2, 4 and 6), indicating a relative increase in termination efficiency at all three stop codons. This finding supports the favoured hypothesis that RF3 can interact functionally with both RF1 and RF2 in translational termination (Freistroffer *et al*., 1997; Pavlov *et al*., 1997a; Pel *et al*., 1998).

RF3 accentuates the strength of the stop signal specified by the +4 base

The influence of RF3 on the contribution of the +4 base to termination signal strength was determined. The results

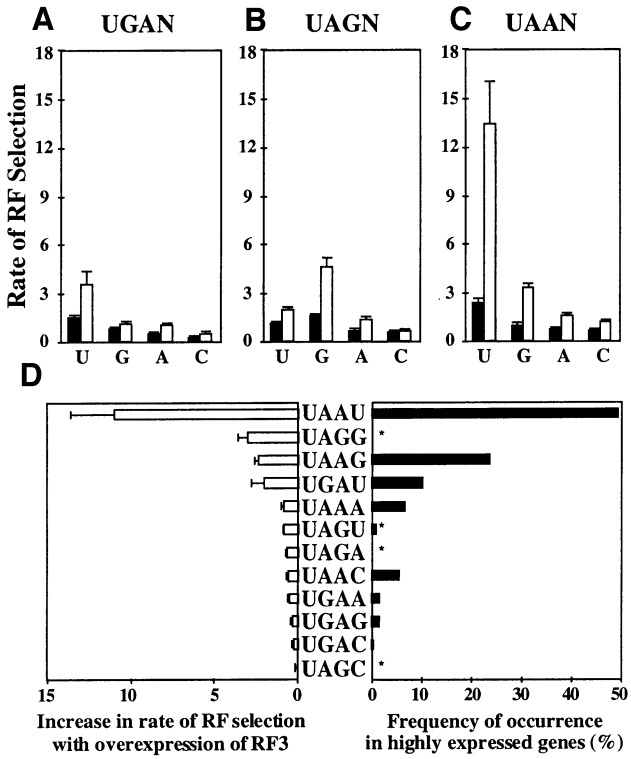

Fig. 3. Influence of endogenous RF3 (filled bars) and overexpression of RF3 (open bars) on the rate of RF selection at (**A**) UGAN, (**B**) UAGN and (**C**) UAAN tetranucleotide stop signals. Average RF selection rates (see Materials and methods) and standard errors from three experiments with multiple isolates of each clone are shown. (**D**) Increase in the rate of RF selection with overexpression of RF3 at the 12 tetranucleotide stop signals (left-hand panel) versus their frequency of occurrence in highly expressed genes of *E.coli* (right-hand panel). The stop signals are listed according to their stimulation by RF3. The relative usage of each stop signal is expressed as a percentage of those tetranucleotide signals used in highly expressed genes of the *E.coli* genome. Note that UAGN signals are rarely used for termination in *E.coli* (indicated with asterisks).

are presented as RF selection rates (Figure 3) so that sensible comparisons can be made of the effects of RF3 among signals of different strengths (see Materials and methods). The ratio of the termination product to frameshift product changed significantly when the fourth base of the signal was altered (Figure 3A–C, filled bars), as found previously. With overexpression of RF3 (Figure 3A–C, open bars), the effect of the +4 base on stop signal strength was accentuated, but significantly the greatest increase in RF selection rate occurred at the strongest stop signals, particularly UAAU. Indeed, the ability of RF3 to stimulate termination activity at UGAN and UAAN tetranucleotide stop signals strongly correlated with how frequently these signals are used at natural termination sites of the most highly expressed genes in *E.coli* (those whose expression predominates at fast growth rates; Figure 3D). The weaker stop signals (UGAC, UGAA and UGAG), i.e. those used infrequently by highly expressed genes, were considerably less responsive to increased RF3 activity. The rate of RF1 selection at UAGN signals with overexpression of RF3 did not correlate with usage (indicated with asterisks) as previously observed in the absence of RF3 overexpression. Despite UAG-containing signals being efficient terminators, they are used very infrequently in *E.coli* genes.

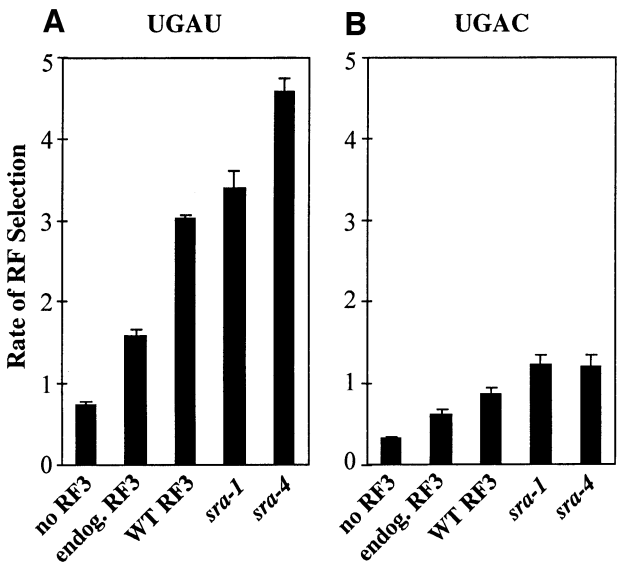

Fig. 4. Relative rates of RF selection at (**A**) UGAU and (**B**) UGAC with different levels of RF3 activity. Experiments were carried out in the absence of *prfC* expression (no RF3), and in the presence of endogenous RF3 (endog. RF3), overexpressed wild-type RF3 (WT RF3) and overexpressed *sra-1* and *sra-4* RF3 variants. The average rates of three experiments and standard errors are shown.

Does RF3 activity alone determine stop signal strength?

Is RF3 directly involved in the signal recognition process and the +4 base effect, or are the above observations an indirect effect of its factor recycling function? If RF3 were the sole determinant of stop signal strength, the differential effect of the +4 base on termination efficiency would disappear in the absence of *prfC* expression. The experiments were therefore repeated in a strain of *E.coli* (RM745) in which the *prfC* gene had been disrupted by insertion of a kanamycin resistance cassette.

In Figure 4, it can be seen that the rate of selection of the decoding RF is lower for both a strong (UGAU) and weak (UGAC) stop signal in the *prfC*$^-$ strain (no RF3), compared with the *prfC*$^+$ strain (endogenous RF3). However, the influence of the base following the stop codon on termination efficiencies is maintained in the absence of *prfC* expression; compare RF selection rates at UGAU (Figure 4A) and UGAC (Figure 4B) with no RF3. The rate of RF selection at UGAG and UGAA is intermediate to these (data not shown). This suggests that RF3 is not involved directly in stop signal recognition.

Two mutant *prfC* genes, *sra-1*, containing a C to T substitution (serine to leucine) between GTP-binding subdomains G3 and G4, and *sra-4*, containing a G to A substitution (alanine to threonine) in a region proposed to be equivalent to a ribosome/RNA-binding region in domain III of EF-G (Kawazu *et al.*, 1995), express variant RF3 proteins that have greater activity than wild-type RF3 (Matsumura *et al.*, 1996). At the relatively strong stop signal UGAU, the two variant RF3 proteins were able to increase the competitiveness of termination even higher than wild-type RF3 in the frameshift–termination assay system (Figure 4A). At the strongest stop signal, UAAU, frameshifting was completely eliminated (data not shown). In contrast, weak signals (such as UGAC) were virtually

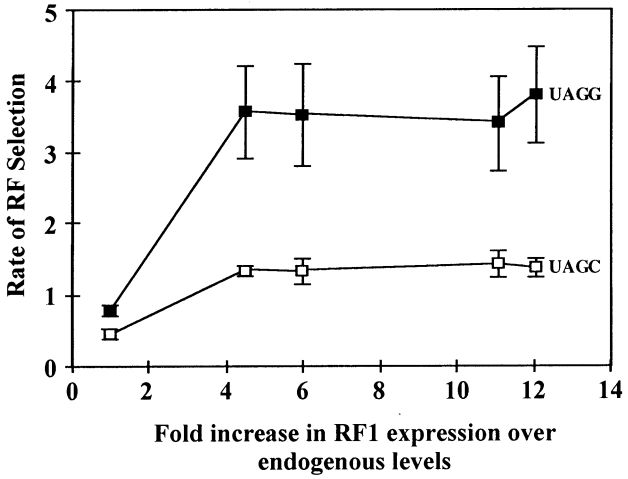

Fig. 5. The effect of increases in cellular RF1 concentration on RF selection rates at UAGG (■) and UAGC (□) stop signals in the absence of *prfC* expression. Experiments were carried out in the *E.coli* strain RM745, transformed with an RF1 expression vector pTGRF1. RF1 expression from pTGRF1 was controlled by varying the concentration of tryptophan in the M9 media (see Materials and methods). The average rates of three experiments and standard errors are shown.

unresponsive to the more active variant proteins (Figure 4B).

Strong stop signals are more sensitive to small increases in the level of decoding factor than weak signals

Is the differential effect of RF3 on strong and weak stop signals an indirect effect of its ability to recycle decoding RF? To address this question, the level of the decoding factor RF1 was increased in the absence of RF3 expression in the *prfC* null strain. RF1 was chosen because it is not subject to the same autoregulatory control as RF2 (high levels of RF2 are toxic to the cell; Craigen *et al*., 1985) and our test system contained strong and weak UAG-containing signals (UAGG and UAGC, respectively). The Trp promoter of the RF1 expression vector, pTGRF1, is repressed by tryptophan, so by altering the levels of tryptophan it was possible to control with some precision the level of RF1 expressed from this vector. At endogenous levels of RF1, termination complexes are not saturated with the decoding factor, and a differential termination efficiency between UAGG and UAGC was observed (Figure 5). With an ~5-fold increase in RF1 expression over endogenous levels, the rate of RF selection at UAGC increased from 0.4 to 1.3 units. The same level of RF1 at UAGG, however, increased the rate of RF selection from 0.8 to 3.6 units. With a 12-fold increase in RF1 expression, the same effect was observed. These results suggest that strong stop signals are more sensitive to increases in the level of decoding factor than weak signals. Despite the apparent plateau, at much higher concentrations of RF1 (~50-fold), 100% termination was measured (data not shown).

Discussion

Recently, it has been discovered that some genes have evolved novel translational mechanisms at special sites in the mRNA to regulate their expression (Atkins *et al*., 1990; Farabaugh, 1993). Many of these classic recoding sites contain relatively weak stop signals and can 'escape' termination, allowing the alternative event with varying degrees of efficiency. In highly expressed genes, however, where the emphasis is on efficient gene expression, and presumably at translational termination, on the rapid decoding of stop signals, the strongest termination signals can be found (Tate and Brown, 1992).

RF3, in enhancing termination efficiency, would assist the decoding of signals in highly expressed genes, but an enhanced decoding of signals at recoding sites would be counter to the desired relatively inefficient termination at these sites. It was significant then, to find that within each stop signal series, the ability of RF3 to enhance termination efficiency was dependent on the identity of the +4 base, the determinant of stop signal efficiency (Figure 3). For example, within the UGAN series, RF3 stimulated termination activity most efficiently at the strong signal UGAU (found in a number of highly expressed genes), whereas the weak UGAC signal (found at recoding sites but not at termination signals of highly expressed genes) was significantly less responsive to increased RF3 activity (Figure 3). This difference in specificity was particularly evident in studies with variant RF3 proteins with greater activity (Figure 4). Since stop signal recognition is thought to reside exclusively in the decoding release factors RF1 and RF2, the critical questions raised by these findings were how RF3 increased the termination strength of signals, and, particularly, why some signals were influenced more than others.

The results presented in Figure 5 provide evidence that strong stop signals are more sensitive to changes in the concentration of decoding factor than weak signals. Although this would almost certainly be mediated by RF3 *in vivo*, it can be mimicked, as we have shown, simply by increasing the cellular level of decoding factor in the absence of *prfC* expression. These results can be interpreted in terms of a faster rate of association of the decoding RF with ribosomes programmed with strong stop signals (Poole *et al*., 1995; Figure 3). If the decoding RF has a higher affinity for strong stop signals than weak signals, and is present at limiting concentrations in the cell, then a small increase in the cellular level of free decoding RF would give the observed increase in termination activity at such signals (Figure 5). Vector-based expression systems such as the one used in this study can make up a significant proportion of the protein synthesis of the cell. In this case, we estimate it to be consistently ~10% for each of the stop signals in the test constructs. The relative concentrations of ribosomal-bound and free RF have been examined in cells expressing the test sytem with a strong or weak signal and compared with cells growing normally without the system. Multiple studies have not shown significant differences in the concentration of free RF and the percentage of ribosome-bound RF.

In the presence of RF3 and non-limiting decoding RF *in vitro*, Pavlov *et al*. (1997b) have found that the translation recycling times for mRNAs containing strong and weak stop signals are similar. In the absence of RF3, high RF1 concentrations have been shown to reduce the rate of ribosomal recycling *in vitro* (Pavlov *et al*., 1997b). The extent of this inhibition was highest for the strong

stop signal UAAU and minimal for the weaker signal UAAC. In other words, in the absence of RF3, the decoding RFs have a high rate of association with strong stop signals and a correspondingly low rate of dissociation, the opposite being true for weak signals. This would explain a narrowing of the differences seen in our studies between termination efficiencies at strong and weak signals in the *prfC* null bacteria (Figure 4). Although the decoding RF will associate (or mediate the hydrolysis event) with strong signals faster than with weak signals, hence giving rise to higher termination efficiencies, it will not be released rapidly for further rounds of termination in the absence of RF3. It may be argued that both the *in vivo* and *in vitro* model systems under study by us and by Ehrenberg and co-workers (Freistroffer *et al*., 1997; Pavlov *et al*., 1997a,b), respectively, are not strictly physiologically representative of natural termination sites. Nevertheless, it is gratifying that data derived from both approaches relevant to the decoding of strong and weak termination signals are quite consistent (Poole *et al*., 1995; Pavlov *et al*., 1997b).

Translational termination in *E.coli* has evolved to balance efficiency and processivity (Jørgensen *et al*., 1993). This is supported by the strong correlation between the relative usage of stop signals in highly expressed genes of *E.coli* and the ability of RF3 to accentuate termination activity at these signals (Figure 3). The results described here suggest strongly that this is achieved in part through the ability of RF3 to increase the cellular levels of free RF1 and RF2 (resulting in an indirect enhancement of the function of the +4 base in regulating the efficiency of translation termination *in vivo*), rather than by a direct involvement of RF3 in codon recognition. Organisms such as *E.coli* which have the *prfC* gene would therefore have an enhanced potential to regulate their gene expression, as compared with organisms such as *Mycoplasma genitalium* (Fraser *et al*., 1995) which lack the gene.

Materials and methods

Materials

Rabbit anti-RF3 antibodies, the *prfC* null strain RM745, wild-type (pNE73) and mutant (pSRA1 and pSRA4) RF3 expression vectors were as described previously (Matsumura *et al*., 1996). The RF1 expression plasmid pTGRF1 was produced by J.Mansell, and pMal clones containing the RF2 frameshift window were constructed by E.Poole (Poole *et al*., 1995). Anti-MBP was purchased from New England Biolabs. Plasmids were purified using a Bio-Rad Miniprep kit and were electroporated into bacteria using an Electro Cell Manipulator® 600 (BTX).

Computer sequence analysis

Statistical analyses of nucleotide sequences were performed on whole genome *E.coli* data obtained from the 1997 release of the TransTerm database (Dalphin *et al*., 1998). This database contains the sequence contexts around *E.coli* stop signals for 100 nucleotides before and after the stop codon. Highly expressed genes were classified as those falling within the top 10% of codon adaptation index (CAI) values.

Growth media and bacterial strains

Expression studies were carried out in the *E.coli* strain FJU_{112} [Δ*(lac-pro) gyrA ara recA56*^Tn*10*, F′*lacI*^{Q1}]. This strain has wild-type ribosomes and no suppressor tRNAs which could compete with the termination or frameshifting events. Subsequent experiments were carried out in the *prfC* null strain RM745 and its parental strain W3110. RM745 bacteria were maintained in 50 μg/ml kanamycin. Bacteria were grown in LB medium and/or M9 medium supplemented with glucose and thiamine as described previously (Poole *et al*., 1995; Matsumura *et al*., 1996).

Expression and analysis of RF3 and MBP fusion proteins

Analysis of plasmid-encoded proteins expressed *in vivo* was essentially as previously described (Poole *et al*., 1995). For detection of RF3, blots were incubated with anti-RF3 serum diluted 1:1000 in TBS buffer [40 mM Tris–HCl pH 7.6, 150 mM NaCl, 0.05% (v/v) Tween-20] and with anti-rabbit and anti-S5 (ribosomal protein) serum. Termination efficiency is calculated from the ratio of the termination product (S) to the sum of the termination (S) and frameshift (F) products: termination efficiency = [(S/(S + F)) × 100%]. The formula used by Pedersen and Curran (1991) to calculate the relative rates of RF1 selection at UAG has been adapted for these experiments. In the Pedersen and Curran study, the rate of RF selection was defined as [((S + F)/F) – 1] where S + F and F were measured in separate experiments. In our study, S and F were determined in a single experiment; therefore, the rate of RF selection can be calculated from S/F. This analysis allows comparisons of the efficiency of decoding of multiple signals to be made under conditions in which the starting efficiencies are quite different. For example, it is difficult to compare a termination efficiency change from 80 to 90%, with one that increases from 10 to 20%. The 2-fold increase in termination efficiency from 10 to 20% corresponds to a change of only 0.15 rate units (rate of RF selection relative to the rate of shift), whereas the 80 to 90% (1.1-fold) increase corresponds to a change of 5 rate units.

Induction of RF1 expression from pTGRF1

Expression from pTGRF1 is repressed by tryptophan so all experiments involving this vector were carried out in M9 media. Aliquots of 250 μl of overnight cultures of RM745 containing pTGRF1 and pMal (supplemented with 100 μg/ml ampicillin, 10 μg/ml gentamicin and 20 μg/ml tryptophan) were inoculated into 5 ml of the same media. Bacteria were grown to an OD_{600} of 0.5, then isopropyl-β-D-thiogalactopyranoside (IPTG) added (final concentration 1 mM) to induce expression of MBP fusion proteins and 3-β-indoleacrylic acid (IAA) for expression of RF1. To regulate the level of RF1 expressed from pTGRF1, tryptophan concentrations were varied between 20 and 150 μg/ml and IAA concentrations between 0 and 50 μg/ml. Bacteria were harvested and cellular proteins fractionated by SDS–PAGE. For Western analysis of RF1, a 1:2000 dilution of the sheep anti-RF1 antibody α163 was used.

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