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Ribosome release factor RF4 and termination factor RF3 are involved in dissociation of peptidyl-tRNA from the ribosome

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Peptidyl-tRNA dissociation from ribosomes is an energetically costly but apparently inevitable process that accompanies normal protein synthesis. The drop-off products of these events are hydrolysed by peptidyl-tRNA hydrolase. Mutant selections have been made to identify genes involved in the drop-off of peptidyl-tRNA, using a thermosensitive peptidyl-tRNA hydrolase mutant in *Escherichia coli*. Transposon insertions upstream of the *frr* gene, which encodes RF4 (ribosome release or recycling factor), restored growth to this mutant. The insertions impaired expression of the *frr* gene. Mutations inactivating *prfC*, encoding RF3 (release factor 3), displayed a similar phenotype. Conversely, production of RF4 from a plasmid increased the thermosensitivity of the peptidyl-tRNA hydrolase mutant. *In vitro* measurements of peptidyl-tRNA release from ribosomes paused at stop signals or sense codons confirmed that RF3 and RF4 were able to stimulate peptidyl-tRNA release from ribosomes, and showed that this action of RF4 required the presence of translocation factor EF2, known to be needed for the function of RF4 in ribosome recycling. When present together, the three factors were able to stimulate release up to 12-fold. It is suggested that RF4 may displace peptidyl-tRNA from the ribosome in a manner related to its proposed function in removing deacylated tRNA during ribosome recycling.

Keywords: protein synthesis/release factors/RF3/RF4/ribosome

Introduction

The normal process of protein synthesis involves the formation of initiation complexes on mRNA, repeated cycles of elongation as the mRNA is decoded, and release of the polypeptide chain by hydrolysis of peptidyl-tRNA when the ribosome encounters a termination signal on mRNA (for a review, see Hershey, 1987). However, the protein synthetic machinery generates peptidyl-tRNA molecules not attached to ribosomes at a rate that is far from negligible, as a result of dissociation from the ribosome at some point during protein synthesis (Manley, 1978; Jørgensen and Kurland, 1990). During the translation of *lacZ*, aborted intermediates are ~40% as frequent as completed chains. For a protein of average size, a similar frequency of 'drop-off' would give rise to truncated chains ~10% as often as the full-length protein. Such failures in processivity represent an enormous energetic loss for the cell, but are believed to be an unavoidable consequence of optimizing the level of translational accuracy (Kurland and Ehrenberg, 1985). The dissociation events accumulate amino acids in useless polypeptides that subsequently must be degraded, and furthermore sequester tRNAs in a form that renders them unavailable for protein synthesis.

The sequestered tRNA molecules are released in bacteria (Cuzin *et al.*, 1967; Kossel and RajBhandary, 1968; Chapeville *et al.*, 1969) and yeast (Kossel and RajBhandary, 1968; Jost and Bock, 1969) by the esterase activity of peptidyl-tRNA hydrolase (Pth; EC 3.1.1.29), though in higher eukaryotes a phosphodiesterase performs this function (Gross *et al.*, 1992). The bacterial enzyme is essential for cell growth, since a mutant form of Pth results in thermosensitive growth and cell death at non-permissive temperatures (Atherly and Menninger, 1972; Menninger, 1979; Garcia Villegas *et al.*, 1991). The isolation of extragenic suppressors of this Pth thermosensitive mutant has shown that the inhibition of growth in Pth-deficient cells results from starvation for $tRNA^{Lys}$, sequestered as peptidyl-$tRNA^{Lys}$ (Heurgué-Hamard *et al.*, 1996), an isoacceptor species previously shown to accumulate as peptidyl-tRNA particularly rapidly in the thermosensitive mutant at a non-permissive temperature (Menninger, 1978). The particular propensity of $tRNA^{Lys}$ for drop-off may be related to an unusual conformation of the anticodon loop due to an interaction involving modified nucleotides (Agris *et al.*, 1997).

Termination signals on mRNA are recognized by two release factors in *Escherichia coli*, RF1 and RF2 (Scolnick *et al.*, 1968), encoded respectively by the genes *prfA* and *prfB* (Caskey *et al.*, 1984; Weiss *et al.*, 1984). A third factor, RF3 (Caskey *et al.*, 1969; Milman *et al.*, 1969; Grentzmann *et al.*, 1994; Mikuni *et al.*, 1994), catalyses the dissociation of RF1 and RF2 from the ribosome in a process which requires GTP hydrolysis (Freistroffer *et al.*, 1997). In a further sequence of reactions that remains poorly understood, the release of ribosomes from the termination site is catalysed by a fourth factor, RF4 (previously called RRF) (Hirashima and Kaji, 1972), acting together with elongation factor EF2 (previously called EF-G) (Pavlov *et al.*, 1997b), whose normal function is to catalyse ribosome translocation during translation elongation (for revised translation factor nomenclature, see Grunberg-Manago *et al.*, 1996). The factor RF4 is essential to cell viability (Janosi *et al.*, 1994); however, inactivation of the gene *prfC* encoding RF3 is not lethal,

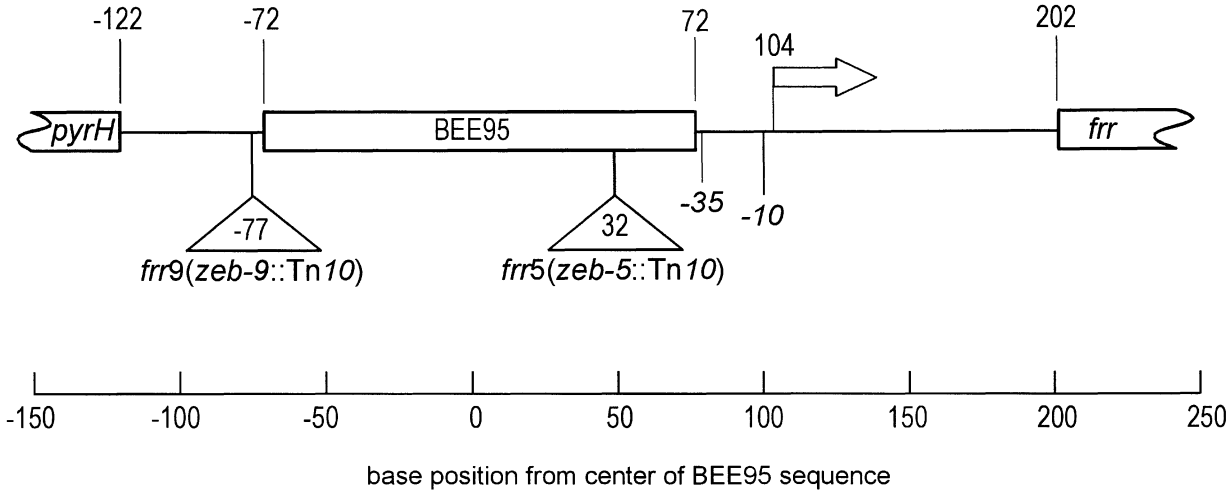

Fig. 1. Mini-transposon insertions upstream of the *frr* gene that restore growth at 42°C to a thermosensitive *pth*(ts) mutant. The BEE95 element is described by Bouché (1995). Nucleotide numbers are with respect to the centre of symmetry of the BEE95 element. Suppression of thermosensitivity was observed with mini-Tn*10*kan insertions at either position –77 [*frr*9(*zeb-9*::Tn*10*kan)] or position 32 [*frr*5(*zeb-5*::Tn*10*kan)].

though cell growth is affected (Grentzmann *et al.*, 1994; Mikuni *et al.*, 1994).

The experiments presented here show that two classes of mutation, resulting respectively in reduced *frr* expression and *prfC* inactivation, act as suppressors of thermosensitive growth in a Pth mutant strain. We argue that this effect is due to reduced levels of peptidyl-tRNA dissociation from the ribosome in the mutant strains. This interpretation is supported by *in vitro* experiments, showing that RF3, RF4 and EF2 provoke the release of peptidyl-tRNA from the P-site of ribosomes. The finding is unexpected, since drop-off generally has been considered to be a passive event, rather than one directly catalysed by protein factors (Kurland and Ehrenberg, 1985). We conclude that the factor-induced drop-off we describe here competes with the normal steps in translation and must account for a significant part of the total peptidyl-tRNA drop-off in the cell.

Results

Isolation of transposon insertion suppressor mutations restoring growth to a thermosensitive pth(ts) mutant

Suppressor mutations that restore growth at non-permissive temperature to the thermosensitive *pth*(ts) strain previously have been isolated by selecting for spontaneous chromosomal mutations (Anderson and Menninger, 1987; Heurgué-Hamard *et al.*, 1996), by selecting for wild-type genes which suppress when overexpressed from a multicopy plasmid (Heurgué-Hamard *et al.*, 1996) or, in one instance, by selecting random insertions of transposons which give rise to suppression (Atherly, 1979). Adopting the latter approach, random insertions of a mini-Tn*10* carrying a kanamycin resistance marker were isolated and suppressor mutations were selected by plating at 42 or 43°C. Following the observation of Atherly (1979) that 12 randomly selected insertions suppressing *pth*(ts) were all co-transducible with *argA* at 60 minutes on the chromosome, 31 of our suppressor insertions were screened for co-transduction with an *argA*::Tn*10* marker. Surprisingly, no insertion near 60 minutes was found. Inverse PCR was employed to group different mutants into classes of probably similar loci, and to generate templates for nucleotide sequencing of the regions adjacent to the transposon. Six classes of mutant were distinguished on the basis of inverse PCR fragment size, and nucleotide sequencing showed that two classes (with one and at least three members respectively) represented insertions upstream of the gene *frr*, encoding the ribosome release factor RF4 (see Figure 1). The insertions fall in or just upstream of a repeated sequence element of unknown function found at several locations in the chromosome of *E.coli* and other prokaryotic organisms (Bouché, 1995). The suppression activity due to these mini-transposon insertions on the growth of streaked colonies of the *pth*(ts) strain and the suppressed mutants is shown in Figure 2A and B (sections b and c).

Two pth(ts) transposon insertion suppressors reduce frr expression

The two mini-transposon insertions shown in Figure 1 fall well upstream of the –35 region of the *frr* promoter characterized by Shimizu and Kaji (1991). Nevertheless, it seemed possible that the primary effect of the insertions was to reduce *frr* expression. To test this hypothesis, the suppressed *pth*(ts) mutant strains were transformed by a plasmid pFRR4 derived from pACYC177 (Chang and Cohen, 1978) carrying the *frr* gene on a 787 bp *Hin*dIII–*Eco*RI fragment. Transformation with this plasmid restored thermosensitivity to the suppressed strain, whereas a derivative pFRR4-12 of pFRR, with a frameshift in the *frr* coding sequence, had no effect. This observation strongly supported the notion that a reduction in the cellular concentration of RF4 was responsible for the suppression of thermosensitivity. Furthermore, *pth*(ts) cells were clearly more thermosensitive when plasmids carrying the *frr* gene were present than they were with control plasmids (Figure 2C). Thus, no growth of the *pth*(ts) strain VH733 was observed at 37°C when transformed by pFRR4 expressing RF4 (except for revertants where a high density of cells was plated), whereas transformation with plasmid

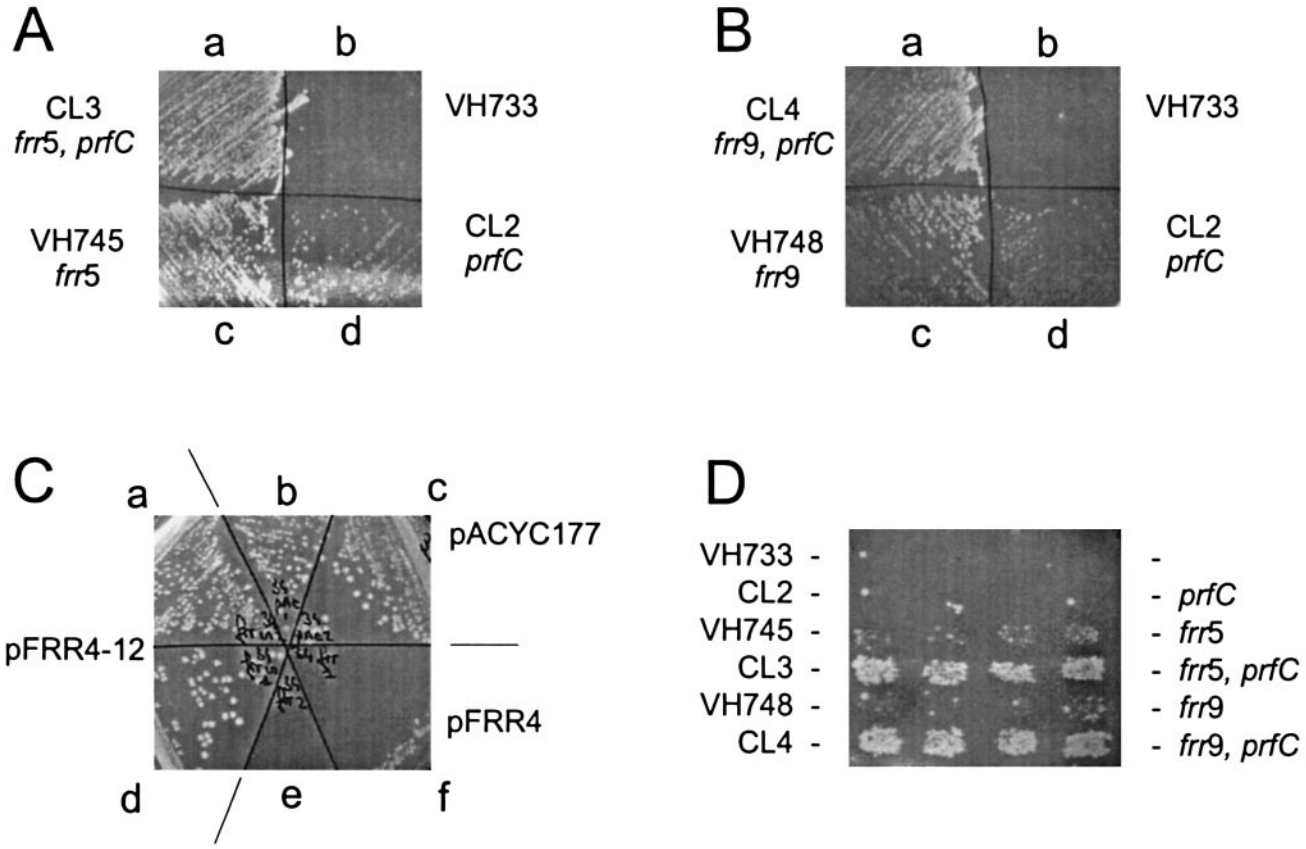

Fig. 2. Suppression of thermosensitive growth of a *pth*(ts) mutant strain by transposon insertion mutations upstream of *frr* or in *prfC*. (**A**) Growth at 42°C on LB plates of *pth*(ts) strain VH733 and suppressed derivatives of this strain, CL2 (*prfC*::tet), VH745 [*frr*5(*zeb-5*::Tn*10*kan)] and CL3 [*prfC*::tet, *frr*5(*zeb-5*::Tn*10*kan)]. (**B**) As in (A) but with the *frr*9 insertion mutant in place of the *frr*5 mutant. (**C**) Growth at 37°C on glucose minimal-Met-ampicillin plates of *pth*(ts) strain VH733 transformed by plasmid pFRR4 expressing RF4 (sections e and f), plasmid pFRR4-12 expressing an RF4 mutant truncated after 67 amino acids (sections a and d) and control plasmid pACYC177 without insert (sections b and c). (**D**) Patch growth at 44°C on LB plates of four sets of similar but independent colonies of *pth*(ts) strain VH733 and suppressed derivatives of this strain, CL2 (*prfC*::tet), VH745 [*frr*5(*zeb-5*::Tn*10*kan)], CL3 [*prfC*::tet, *frr*5(*zeb-5*::Tn*10*kan)], VH748 [*frr*9(*zeb-9*::Tn*10*kan)] and CL4 [*prfC*::tet, *frr*9(*zeb-9*::Tn*10*kan)].

pFRR4-12 in which the coding sequence for RF4 is disrupted by a frameshift, or with the parent plasmid pACYC177, allows normal growth under these conditions (see Figure 2C). This result may be readily understood if RF4 plays a direct role in peptidyl-tRNA drop-off, in terms of the higher cellular concentration of the factor in cells transformed with plasmid pFRR4. The observation also argues that the suppression of thermosensitivity in the *pth*(ts) strain is not due to some small negative effect of reducing *frr* expression on the general level of protein synthesis in the cell.

To confirm that RF4 mRNA levels were depressed in the transposon insertion strains, Northern blots were performed on RNA extracted from the suppressed and non-suppressed *pth*(ts) strains, using a probe hybridizing to part of the coding region of *frr*. In the non-suppressed strain, a transcript was visible of a size consistent with the observations of Shimizu and Kaji (1991), but which decreased to low, barely detectable levels in the suppressed strains (results not shown).

prfC inactivation suppresses pth(ts)

According to the current model of polypeptide chain termination, RF4 binds to the ribosome following the departure of RF1 or RF2 from the ribosome, a step catalysed by the G protein RF3 (Pavlov *et al.*, 1997a,b). Though not isolated by using the selection method described above, mutants inactivating the gene *prfC* encoding RF3 have also been found to suppress *pth*(ts) thermosensitivity. The re-establishment of growth at 42°C on inactivation of *prfC* is shown in Figure 2A and B (sections b and d). Neither the *frr* nor the *prfC* mutation when alone were able to restore growth of the *pth*(ts) strain at 44°C to any significant extent. However, the two mutations together do restore growth at this temperature, as shown clearly by the growth of patches after replica plating of the mutant strains (Figure 2D).

Effect of suppressor mutations and RRF overproduction on growth in liquid medium of pth(ts) strains

The suppression of thermosensitive growth of the *pth*(ts) mutant by transposon insertions affecting *frr* expression (see Figure 2) was examined further in experiments on growth rate in solution (Table I). In these experiments, the thermosensitive *pth*(ts) mutant and the suppressed mutant strains were grown at the permissive temperature of 30°C to a density of ~5×10^7 cells/ml before shifting

Table I. Suppression of thermosensitive growth of the *pth*(ts) strain by mutations affecting RF4 and RF3: growth rates (generations/h) of the *pth*(ts) strain and suppressed mutants following an increase from 30°C to the indicated temperature

Strain	Relevant genotype	Temperature		
		42°C	43°C	44°C
VH733	*pth*(ts)	0.0	0.0	0.0
VH745	*pth*(ts), *frr*5	1.03	0.73	0.17
VH748	*pth*(ts), *frr*9	0.74	0.60	0.11
CL2	*pth*(ts), *prfC*	0.95	0.16	0.0
CL3	*pth*(ts), *prfC*, *frr*5	1.38	1.08	0.71
CL4	*pth*(ts), *prfC*, *frr*9	1.28	1.00	0.70

Table II. Inhibition of growth (generations/h) of *pth*(ts) mutant strain VH733 and control strain FTP4993 at 37°C resulting from overproduction of RF4

Plasmid designation	Insert	Host strain	
		pth(ts)	pth^+
pACYC177	none	0.68	0.90
pFRR4	*frr*	0.01	0.86
pFRR4-12	*frr*(frameshift)	0.67	0.89

to a temperature of 42, 43 or 44°C. Growth of the *pth*(ts) strain ceased rapidly at any of these temperatures. The cell density of cultures of the suppressed strains was monitored for periods of up to 9 h after the temperature shift to allow doubling rates during the exponential phase to be quantitated. Both Tn*10* insertions restored growth in liquid culture at 42°C, and to lesser extent at 43 and 44°C; the *frr*5 insertion, closer to the *frr* promoter, was significantly more efficient as a suppressor than the *frr*9 insertion (see Table I). Growth in liquid media demonstrated clearly the suppressor activity of the *prfC* mutation: growth was largely restored at 42°C, to a small extent at 43°C and not at all at 44°C (Table I). As observed on solid media, combination of the two suppressor mutations, *prfC* and *frr*5 or *frr*9, restored growth at 44°C far more efficiently than either suppressor alone.

The effect of overproducing RF4 from plasmid pFRR4 on the growth of *pth*(ts) strains at the permissive temperature of 37°C (see Figure 2C) was also re-examined in liquid cultures (Table II). A shift in temperature from 30 to 37°C completely arrested the growth of *pth*(ts) strains carrying plasmid pFRR4, but not of strains carrying plasmid pFRR4-12 with a frameshift in the RF4 coding sequence, or the parent plasmid pACYC177 with no insert. The presence of plasmid pFRR4 did not affect growth of the control pth^+ strain (Table II).

Peptidyl-tRNA is released from ribosomes in vitro by RF3, RF4 and EF2

The isolation of mutants affecting *frr* and *prfC* that suppress the thermosensitive growth of the *pth*(ts) strain strongly suggests that RF4 and RF3 are involved in peptidyl-tRNA drop-off *in vivo*. In order to determine whether their effect on elongation complexes is direct, peptidyl-tRNA drop-off was studied *in vitro*, using peptidyl-tRNA·mRNA·ribosome complexes separated from other components required for translation. A system for protein synthesis *in vitro* using purified translation factors and synthetic mRNAs possessing a Shine–Dalgarno sequence and encoding short peptides has been described recently (Freistroffer *et al.*, 1997; Pavlov *et al.*, 1997b). An mRNA with the coding sequence AUG-UUC-*UUG*-UAA was employed in this system to synthesize the tripeptide fMet–Phe–Leu (fMFL) in the absence of termination factors, and the stalled release complex was isolated by gel filtration as previously described (Freistroffer *et al.*, 1997). The rate of fMFL-tRNA release from such ribosomal complexes may be studied readily in the presence of Pth, since the tripeptide released from tRNA by the enzyme is soluble in cold trichloroacetic acid (TCA). No detectable fMFL was released from tRNA in the absence of Pth. The rate of fMFL-tRNA drop-off in the absence of added factors was 4.5×10^{-2}/min, but was found to be stimulated 10-fold by the addition of RF3, RF4 and EF2 (1 μM each; see Figure 3A). Higher concentrations of RF3 and RF4 increased the drop-off rate to ~2.0/min (see Figure 4). An inverse plot of drop-off rate and factor concentration (not shown) indicates that higher rates can be attained. Elongation factor EF2 was added with the other factors because it is known to be required for RF4 function in ribosome recycling (Hirashima and Kaji, 1973; Janosi *et al.*, 1996; Pavlov *et al.*, 1997b). The presence of the three proteins was necessary to achieve a high rate of peptidyl-tRNA release: other combinations of factors did not produce more than a 3-fold stimulation of drop-off (Figure 3A and B).

Stimulation of drop-off occurs from both elongation and termination complexes

The specificity for recognition of termination signals is thought to reside exclusively in termination factors RF1 and RF2 in *E.coli*. Nevertheless, since the only known functions of factors RF3 and RF4 are related to polypeptide chain termination and ribosome recycling, it was of interest to determine whether the factor-catalysed drop-off seen in the experiments of Figure 3A was dependent on the presence of a stop codon in the ribosomal A-site. Similar experiments were therefore performed with ribosomal complexes bearing the same fMFL nascent peptide but paused at an AUC sense codon instead of a stop codon. As shown in Figure 3B, the rate of spontaneous drop-off was lower from these ribosomal complexes (2.6×10^{-2}/min) than from the complexes paused at the UAA stop codon (4.5×10^{-2}/min), indicating that the sequence downstream of the Leu codon influences this rate. However, RF3, RF4 and EF2 added together stimulated the drop-off rate by approximately the same factor (12-fold) as found previously. The drop-off rate for both complexes was found to increase steadily with the concentration of RF4 within the range 0.2–2 μM under conditions of constant RF3 and EF2 concentration (1 μM each, see Table III). The fact that similar observations can be made when ribosomal complexes are paused at sense codons as opposed to stop codons is also an indication that the factor-stimulated peptidyl-tRNA release cannot be accounted for in terms of traces of release factors in the preparations of RF3, RF4, EF2 or Pth.

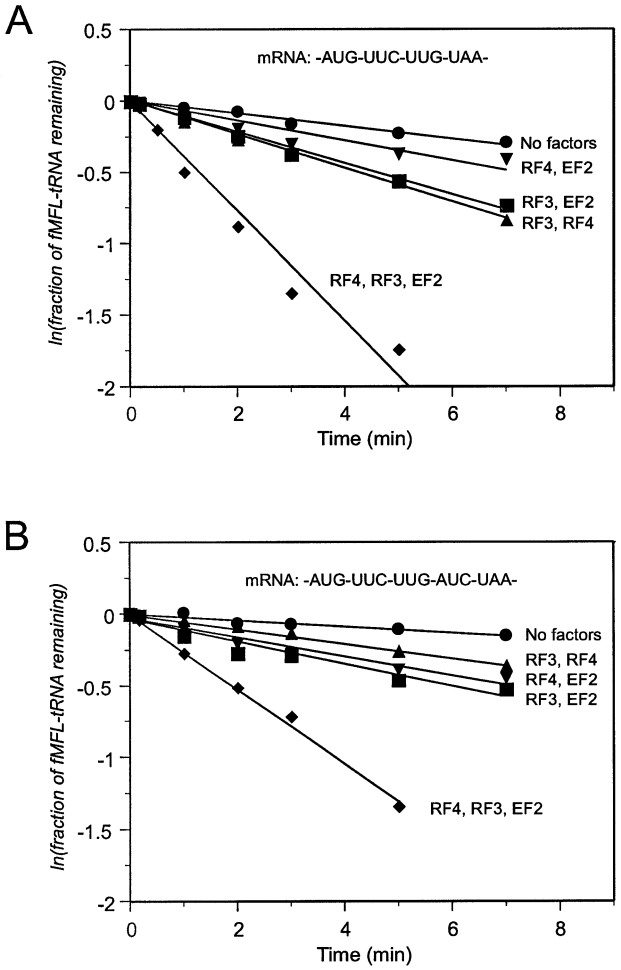

Fig. 3. Factor-catalysed release of peptidyl-tRNA from ribosomal complexes *in vitro*. Purified ribosomal complexes containing [^{3}H]fMet-Phe-Leu-tRNA$_4^{Leu}$ paused with a stop codon (**A**) or a sense codon (**B**) in the A-site were incubated with RF3, RF4 and EF2 (◆), RF3 and RF4 (▲), RF3 and EF2 (■), RF4 and EF2 (▼) or no factors (●). Dissociation of peptidyl-tRNA was measured as TCA-soluble tripeptide released by Pth in the reaction mixture. Each factor was present at a concentration of 1 μM. The natural logarithm of the fraction of fMFL-tRNA remaining ribosome bound is plotted as a function of time.

Dependence of factor-stimulated drop-off on guanine nucleotide

The replacement of GTP by GDP was found to abolish the stimulation by RF3, RF4 and EF2 of peptidyl-tRNA drop-off (see Table IV). However, in the presence of the non-hydrolysable GTP analogue GDPNP, stimulation was still observed and was indeed somewhat more marked than with GTP. This was also true in the case of RF3 added alone or when RF4 and EF2 were added without RF3 (Table IV). This may be due to the prolonged presence on the ribosome of factors RF3 or EF2 unable to undergo a conformational change needed for release, and indicates that GTP hydrolysis is not necessary for the effect on drop-off. Experiments using the antibiotic fusidic acid, which is thought to maintain EF-2 in the GTP conformation even after the hydrolysis of GTP, indicated that the rate of peptidyl-tRNA drop-off in the presence of RF3, RF4, EF2 and GTP was unaffected by the addition of fusidic acid.

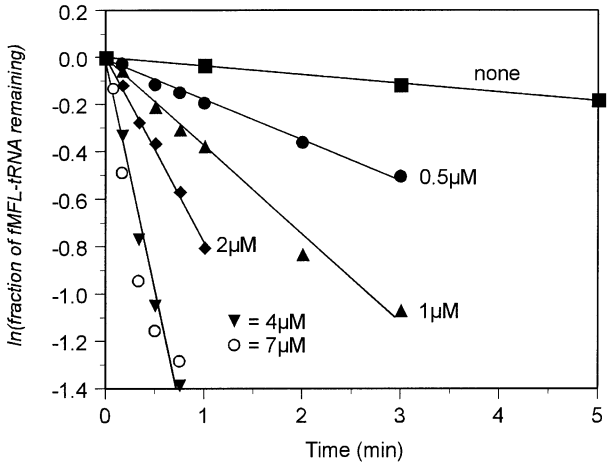

Fig. 4. Dependence on RF3 and RF4 concentration of the release of peptidyl-tRNA from ribosomal complexes *in vitro*. Purified ribosomal complexes resulting from AUG-UUC-*UUG*-UAA translation and containing [^{3}H]fMet-Phe-Leu-tRNA$_4^{Leu}$ in the P-site were incubated with RF3 and RF4 at the equimolar concentrations indicated and EF2 (1 μM). Dissociation of peptidyl-tRNA was measured as TCA-soluble tripeptide released by Pth in the reaction mixture. The natural logarithm of the fraction of fMFL-tRNA remaining ribosome bound is shown as a function of time.

Table III. Dependence on RF4 concentration of fMFL dissociation from ribosomal complexes in the presence of RF3 and EF2

RF4 (μM)	mRNA	Lifetime (min)
–	-AUG-UUC-*UUG*-UAA-	9.2
0.2	-AUG-UUC-*UUG*-UAA-	4.1
0.5	-AUG-UUC-*UUG*-UAA-	3.1
1.0	-AUG-UUC-*UUG*-UAA-	2.3
2.0	-AUG-UUC-*UUG*-UAA-	1.5
–	-AUG-UUC-*UUG*-AUC-UAA-	13.2
0.2	-AUG-UUC-*UUG*-AUC-UAA-	5.8
0.5	-AUG-UUC-*UUG*-AUC-UAA-	4.3
1.0	-AUG-UUC-*UUG*-AUC-UAA-	4.0
2.0	-AUG-UUC-*UUG*-AUC-UAA-	2.4

Table IV. Dependence on guanine nucleotide of factor-stimulated drop-off of tripeptide fMFL translated from mRNA -AUG-UUC-*UUG*-UAA-

Factors (1 μM)	Lifetime (min)		
	GTP	GDP	GDPNP
None	40	35	32
RF3	12.4	35	4.1
RF4, EF2	15.2	35	11.5
RF3, RF4, EF2	3.5	20	1.9

Discussion

Pth is believed to be an essential enzyme because it is necessary to recycle peptidyl-tRNAs that dissociate from the ribosome during mRNA translation, allowing the tRNA species to return to the pool of aminoacyl-tRNAs available for protein synthesis. Previous work has shown that the immediate reason for protein synthesis inhibition when Pth activity is insufficient is starvation for tRNA^{Lys}

(Heurgué-Hamard *et al.*, 1996). Thus, overproduction of this species of tRNA from a plasmid will suppress *pth*(ts) thermosensitivity, whereas overproducing a wide variety of other tRNAs is without effect. Apart from this multicopy suppressor, chromosomal suppressors were isolated which mapped at two locations, and probably also functioned by increasing the intracellular level of $tRNA^{Lys}$ (Heurgué-Hamard *et al.*, 1996).

The selection for temperature-resistant pseudo-revertants of *pth*(ts) should also be expected to produce mutations affecting ribosomal components or factors that associate with the ribosome during protein synthesis, the effect of which would be to reduce drop-off of peptidyl-tRNA from the ribosome. This is the most likely explanation for the mechanism of suppression associated with the *frr* and *prfC* mutants. Direct measurement of peptidyl-tRNA dissociation from the ribosome *in vitro* shows that RF4 and RF3 considerably stimulate the rate of dissociation, strongly supporting this interpretation. This is an unexpected finding, since it has generally been thought that drop-off of peptidyl-tRNA from the ribosomal P-site is a spontaneous event rather than one directly involving translation factors (Kurland and Ehrenberg, 1985). The action of RF4 in dissociating peptidyl-tRNA from the ribosome requires elongation factor EF2, as in the case of ribosome recycling, the normal function of the factor (Janosi *et al.*, 1996; Pavlov *et al.*, 1997b). The involvement of both RF3 and RF4 in stimulating drop-off, seen clearly in the *in vitro* experiments, is corroborated by the observation *in vivo* that combining the suppressor mutations affecting the two proteins restores growth to the *pth*(ts) strain at 44°C, which neither of the mutations alone is able to do.

It has been proposed that the normal action of RF4 is to catalyse the removal of deacylated tRNA from the ribosome (Pavlov *et al.*, 1997a,b). According to this model, RF4 binds to the A-site of the ribosome, or a site overlapping the A-site, and provides a substrate for EF2 in a translocation reaction analogous to that which occurs during elongation. This would displace deacylated tRNA from the ribosomal P-site. Support for this hypothesis has been provided by the demonstration that RF1 and RF4 compete for binding to the 70S ribosome, and that the competition is increased by the absence of RF3 (Pavlov *et al.*, 1997a). Applied to the phenomenon of peptidyl-tRNA drop-off we report here, this model suggests that the factor-catalysed drop-off is due to a related mechanism in which peptidyl-tRNA is displaced instead of deacylated tRNA.

The normal function of RF3 is to catalyse the removal from the ribosome of RF1 or RF2 after the release of the polypeptide chain (Freistroffer *et al.*, 1997; Pavlov *et al.*, 1997b), and occurs prior to the involvement of RF4 (Pavlov *et al.*, 1997a). The role that RF3 plays in increasing drop-off is not yet clear. RF3 may induce a conformational change in the ribosome that facilitates the binding of RF4. A direct interaction between the two factors is an interesting possiblity that requires further study. Presumably, the factor-induced drop-off that we have demonstrated here competes with normal steps in translation, namely the binding of ternary complex or RF to the ribosomal A-site, when this is occupied by a sense codon or a stop codon.

The size distribution of peptidyl-tRNAs dissociating from the ribosome has not been well studied, and deserves closer attention in view of current hypotheses concerning the exit route of nascent polypeptides from the ribosome. Recent models for the 50S ribosomal subunit based on cryo-electron microscopy show a tunnel of ~85 Å in length (Frank *et al.*, 1995). Combined with the finding that 30–40 amino acids of the nascent peptide are protected against protease attack under some circumstances, it has been proposed that the large subunit tunnel serves as an exit route for polypeptide from both prokaryotic and eukaryotic ribosomes (Malkin and Rich, 1967; Sabatini and Blobel, 1970; Milligan and Unwin, 1986; Frank *et al.*, 1995). Supposing this to be true, it is difficult to envisage how peptidyl-tRNA could any longer dissociate from the ribosome once the nascent peptide has folded extensively on the exit side of the tunnel, unless the tunnel itself can open as a result of some conformational change to the 50S ribosomal subunit. However, several observations are incompatible with polypeptide export via a tunnel of the dimensions seen by microscopy, and raise severe doubts that the tunnel serves this purpose at all (Kolb *et al.*, 1987; Ryabova *et al.*, 1988; Komar *et al.*, 1997). It is now clear that in eukaryotic organisms, an extra-ribosomal protein is responsible for protection of the nascent peptide from proteolysis and can be cross-linked to it close to the peptidyl transfer centre (Wang *et al.*, 1995).

Published data do not allow us to exclude that most peptidyl-tRNA drop-off involves short peptides. To our knowledge, the only direct attempt to determine the size of drop-off products has been due to Menninger (1976), who observed a broad distribution of peptide size up to ~10^2 amino acids. Larger peptidyl-tRNAs might not have been extracted by the procedures used. The failures in translational processivity measured by Manley (1978) and by Jørgensen and Kurland (1990) did involve long peptides, but it has not been shown conclusively that they were the result of peptidyl-tRNA drop-off. On the other hand, modifications near the 3′ end of long messengers highly expressed *in vivo* can considerably modify the frequency of drop-off of the encoded product, strongly suggesting that drop-off of the almost completed protein as peptidyl-tRNA can occur (J.Menez, V.Heurgué-Mamard and R.H.Buckingham, unpublished results). A better understanding of the relationship between RF4 and RF3 and peptidyl-tRNA drop-off will require the identification of the points of drop-off along an mRNA, and the changes in the pattern of these products in mutant cells.

Materials and methods

Bacterial strains, plasmids and bacteriophage

The *E.coli* K12 strains are listed in Table V. VH733 was obtained by transducing strain FTP4993 with a phage P1 lysate on VH6, selecting for Tet^R and screening for Trp^- and themosensitive growth. The Tn*10* was removed in a second transduction with a P1 lysate on strain Y1, selecting for Trp^+ and screening for Tet sensitivity and themosensitive growth. The *frr* gene was cloned as a 929 bp *Sma*I–*Eco*RI fragment (MacDougall *et al.*, 1997) into pWSK129 (Wang and Kushner, 1991), excised as a 805 bp *Bam*HI–*Hin*dIII fragment and recloned between the sames sites of vector pACYC177 (Chang and Cohen, 1978). A derivative of this plasmid (pFRR4-12) was constructed by digesting pFRR4 at the unique *Xma*III site, filling the ends and religating, which introduced a frameshift, mutated the encoded protein and truncated it to 67 amino acids. The *prfC* gene, cloned as a 3.5 kb *Pst*I fragment (Grentzmann

Table V. *Escherichia coli* K12 bacterial strains

Strain	Genotype	Reference, construction
CL2	*metB, glyV55, prfC::*tet, *pth*(ts)	this work, derivative of VH733, see text
CL3	*metB, glyV55, pth*(ts), (*zeb-5*::Tn*10*kan), *prfC::*tet	this work, VH745 and P1 lysate on CL2
CL4	*metB, glyV55, pth*(ts), (*zeb-9*::Tn*10*kan), *prfC::*tet	this work, VH748 and P1 lysate on CL2
FTP4993	*metB, glyV55, trpA*(AGC233, UGA234)	Frances T.Pagel
GC3366	*thr, pro, leu, recD*1009	Richard d'Ari
IB209	*thi1, metE68, spoT1, Hfr, argA81::*Tn*10*	Jacqueline Plumbridge
VH6	*supE44, thi1, thr1, leuB6, lacY1, tonA21, pth*(ts), (*zcg-3060::*Tn*10*tet)	this work
VH733	*metB, glyV55, pth*(ts)	this work, from FTP4993, P1 transductions from VH6 and Y1
VH745	*metB, glyV55, pth*(ts), (*zeb-5::*Tn*10*kan)	this work, derivative of VH733
VH748	*metB, glyV55, pth*(ts), (*zeb-9::*Tn*10*kan)	this work, derivative of VH733
Y1	*trpR, lacZ*U118	(Yanofsky and Soll, 1977)

et al., 1994) in plasmid vector pACYC177, was inactivated by insertion of the tetracycline-resistant cassette excised as a *Sma*I–*Bsr*BI fragment from plasmid pCP16 (Cherepanov and Wackernagel, 1995) in place of a *Pvu*II–*Pvu*II fragment in *prfC*. The inactivated gene was recombined with the chromosome in the *recD* strain GC3366 (*recD*1009, *thr, leu, pro, rpsL)* after linearization of the plasmid by digestion with *Xho*I (Shevell *et al.*, 1988). P1 transduction of the interrupted *prfC* gene to VH733 yielded strain CL2.

Chemicals

Nucleotide triphosphates were from Pharmacia. Phosphoenolpyruvate, putrescine, spermidine and non-radioactive amino acids were from Sigma, L-[methyl-^{3}H]methionine was from Amersham. All other chemicals were of analytical grade from Merck. Polymix buffer was prepared according to Jelenc and Kurland (1979) and contained at final concentration 5 mM magnesium acetate, 0.5 mM $CaCl_2$, 5 mM NH_4Cl, 95 mM KCl, 8 mM putrescine, 1 mM spermidine, 5 mM potassium phosphate (pH 7.3) and 1 mM dithioerythritol (DTE).

Growth conditions

Luria Broth (LB) medium (Sambrook *et al.*, 1989) or minimal Vogel–Bonner medium (Vogel and Bonner, 1955) were supplemented according to requirements. Antibiotics were added at the following final concentrations: tetracycline 12.5 μg/ml; kanamycin 50 μg/ml; ampicillin 100 μg/ml. In growth experiments, pre-cultures were grown at 30°C in LB-ampicillin (Table I) or minimal glucose-Met-Trp-ampicillin (Table II). After dilution to a cell density of ~10^7 cells/ml, growth was continued at 30°C to a cell density of ~5×10^7 cells/ml; the temperature of growth was then increased as indicated in Results. Growth was monitored for periods of up to 9 h or until late exponential phase.

Recombinant DNA manipulations and genetic manipulations

General procedures for DNA recombinant techniques, plasmid extraction, etc. were performed as described by Sambrook *et al.* (1989). Purification of DNA fragments on agarose gel was by Jetsorb gel extraction (Bioprobe). Phage P1 lysates, transductions and transformations were performed as described by Miller (1992).

Isolation of pth(ts) suppressors

Random insertion of a mini-Tn*10* Kan^R in strain VH733 was performed by transposition from λNK1316 (Kleckner *et al.*, 1991). Kan^R insertion mutants were selected by plating at 43°C on LB-Kan or glucose minimal-Met-Kan at 42°C. Suppressors of thermosensitive growth due to the Tn*10* insertions were distinguished from other spontaneously arising suppressors by P1 transduction to VH733, selecting for Kan^R. Regions of the chromosome adjacent to the mini-transposons were amplified by inverse PCR after digestion by *Rsa*I or *Taq*I and circularization, and sequenced by thermal cycling using oligonucleotide primers complementary to sequences within the mini-Tn*10*.

Northern blots

Total RNA was extracted from cells grown in LB medium by the hot phenol technique (Regnier and Portier, 1986). The RNA was separated by electrophoresis on 1% (w/v) agarose/formaldehyde gels and transfered to Amersham Hybond N$^+$ membrane for hybridization with ^{32}P-labelled probes as described by Regnier and Grunberg-Manago (1989). Transcripts of *frr* were detected with an anti-RNA probe transcribed by T7 RNA polymerase in the presence of [^{32}P]UTP from a DNA template of 397 nucleotides amplified from chromosomal DNA by PCR. The upstream and downstream primers were respectively 5′-AAATGCGTAGAA-GCGTTCA-3′ and 5′-TAATACGACTCACTATAGCTTTCACTTTGT-CGTTCGC-3′, the latter primer harbouring the sequence of the T7 promoter (Mulligan *et al.*, 1987).

Peptidyl-tRNA drop-off assays

The synthesis of mRNAs and the isolation of ribosomal complexes paused at a nonsense codon were performed as described previously (Freistroffer *et al.*, 1997). The same procedure was used for complexes paused at a sense codon by omitting the corresponding tRNA isoacceptor from the translation system. Peptidyl-tRNA drop-off from the P-site was measured by adding purified Pth to the reactions. The enzyme was prepared from an overproducing strain C600/pBSAE53 (Garcia Villegas *et al.*, 1991) (R.Karimi and M.Ehrenberg, in preparation). For drop-off assays, a factor mix was prepared containing, in 25 μl of polymix buffer: 50 nmol of GTP, 50 nmol of ATP, 0.5 μmol of phosphoenolpyruvate, 2.5 μg of pyruvate kinase, 0.15 μg of myokinase, 16 pmol of Pth and quantities of each of the factors RF3, RF4 and EF2 as indicated in Figure 3 and Table III. The factor mix was incubated for 3 min at 37°C. Then 25 μl of peptidyl-tRNA·ribosome·mRNA complex (also in polymix buffer), pre-incubated for 1 min at 37°C, was added to the factor mix. After incubation for 0–8 min, the reaction was quenched with ice-cold 5% TCA containing 15% casamino acids (Difco) and filtered onto GF/C glass fibre filters (Whatman). The filters were washed with ice-cold 5% TCA, then with propanol, dried and the radioactivity was measured (Ehrenberg *et al.*, 1990).

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