Fast recycling of *Escherichia coli* ribosomes requires both ribosome recycling factor (RRF) and release factor RF3

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A complete translation system has been assembled from pure initiation, elongation and termination factors as well as pure aminoacyl-tRNA synthetases. In this system, ribosomes perform repeated rounds of translation of short synthetic mRNAs which allows the time per translational round (the recycling time) to be measured. The system has been used to study the influence of release factor RF3 and of ribosome recycling factor RRF on the rate of recycling of ribosomes. In the absence of both RF3 and RRF, the recycling time is ~40 s. This time is reduced to ~30 s by the addition of RF3 alone and to ~15 s by the addition of RRF alone. When both RF3 and RRF are added to the translation system, the recycling time drops to <6 s. Release factor RF3 is seen to promote RF1 cycling between different ribosomes. The action of RRF is shown to depend on the concentration of elongation factor-G. Even in the presence of RRF, ribosomes do not leave the mRNA after termination, but translate the same mRNA several times. This shows that RRF does not actively eject mRNA from the terminating ribosome. It is proposed that terminating ribosomes become mobile on mRNA and ready to enter the next translation round only after two distinct steps, catalysed consecutively by RF3 and RRF, which are slow in the absence of these factors.

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Introduction

The release of completed polypeptide chains from both prokaryotic and eukaryotic ribosomes has been under study for more than two decades (for reviews, see Craigen and Caskey, 1987; Tate and Brown, 1992; Janosi *et al.*, 1996; Nakamura *et al.*, 1996). It is well established that the prokaryotic codon-specific release factors RF1 and RF2 recognize a messenger-encoded stop signal (UAA or UAG for RF1 and UAA or UGA for RF2) and promote the hydrolysis of peptidyl-tRNA in the P-site of the ribosome, releasing the polypeptide chain (Craigen and Caskey, 1987; Tate and Brown, 1992). A single factor eRF1 performs this role at all stop codons in the cytoplasm of eukaryotic cells (Frolova *et al.*, 1994). Both prokaryotes

and eukaryotes employ a further factor (RF3 or eRF3, respectively) that affects the efficiency of the termination process (Milman *et al.*, 1969; Grentzmann *et al.*, 1994; Mikuni *et al.*, 1994; Zhouravleva *et al.*, 1995). Following polypeptide release, another protein, ribosome release (or recycling) factor (RRF), is involved in ribosome re-utilization in prokaryotes (Hirashima and Kaji, 1970; for a review, see Janosi, 1996). Whether a eukaryotic cytoplasmic equivalent to RRF exists is unknown.

The exact roles of such factors as RF3 and RRF remain obscure (Grentzmann *et al.*, 1994; Janosi *et al.*, 1996; Matsumura *et al.*, 1996). *In vitro* studies have led to the suggestions that RF3 (first called S or α) stimulates the formation of termination complexes in the absence of GTP, stimulates the dissociation of the same complexes in the presence of GTP and catalyses the cycling of RF1 and RF2 between terminating ribosomes (Goldstein and Caskey, 1970; Goldstein *et al.*, 1970; Klein and Capecchi, 1971; Mikuni *et al.*, 1994). Recent measurements of Adamski *et al.* (1994) suggest that in *Escherichia coli*, RF1 and RF2 are present in concentrations comparable with the concentration of the ribosomes and, therefore, fast cycling of RF1 and RF2 between ribosomes does not seem essential *per se*. This seems compatible with the finding that, unlike the equivalent protein in yeast, RF3 in *E.coli* is not an essential protein (Grentzmann *et al.*, 1994; Mikuni *et al.*, 1994). It has been shown, however, that under normal conditions of rapid growth, the presence of RF3 is required for an optimal rate of growth (Grentzmann *et al.*, 1994; Mikuni *et al.*, 1994).

Measurements of nonsense codon readthrough have been interpreted in terms of RF3 promoting the association of RF1 and RF2 with the terminating ribosomes, thereby increasing the rate and efficiency of termination (Matsumura *et al.*, 1996, and references therein). However, as first proposed by Goldstein and Caskey (1970), RF3 might instead promote the release of RF1 or RF2 from the ribosome after peptidyl-tRNA hydrolysis, increasing the effective concentration of these factors in the cell. In either case, an important function of RF3 might be to accelerate the overall termination reaction and avoid interference, such as queuing, between ribosomes.

The fate of the ribosome after release of the nascent peptide has received relatively little attention (reviewed by Janosi *et al.*, 1996). The ribosome recycling factor RRF and elongation factor G (EF-G) have both been implicated in the release of the terminating ribosome from mRNA (Janosi *et al.*, 1996). RRF was shown to be an essential protein in *E.coli* (Janosi *et al.*, 1994). According to the current model, RRF and EF-G actively eject mRNA from the ribosome (Hirashima and Kaji, 1973; Janosi *et al.*, 1996). However, an obligatory mRNA ejection seems to contradict the experimental data of Adhin and

van Duin (1990), who have shown that the terminating ribosome may not detach from mRNA at a stop signal but, instead, migrates along mRNA in either direction until reaching a nearby initiation site on a polycistronic mRNA or spontaneously dissociating from mRNA. Whether certain messenger sequences favour ribosome scanning as opposed to dissociation, as seems to be the case at some yeast translation termination sites (Grant and Hinnebusch, 1994), remains to be determined.

One reason for the relatively slow progress in unravelling the details of the termination mechanism has been the absence of an adequate *in vitro* system to measure the parameters of termination under conditions comparable with those *in vivo*. The termination assay generally employed *in vitro* (Tate and Caskey, 1990; Grentzmann *et al.*, 1994; Mikuni *et al.*, 1994) uses trinucleotides to model the termination codons in mRNA and is performed at ~30 mM Mg^{2+} . It is well documented, however, that the concentration of free Mg^{2+} as well as the ionic composition of the buffer is crucial for the rate and accuracy of the elongation of translation (Jelenc and Kurland, 1979; Ehrenberg *et al.*, 1990). The concentration of free Mg^{2+} in *E. coli* has been estimated experimentally to be between 1 and 5 mM (Alatossava *et al.*, 1985), and optimized *in vitro* translation systems perform best at Mg^{2+} concentrations between 3 and 5 mM (Ehrenberg *et al.*, 1990). Therefore, the relevance of observations made using the standard termination assay (Tate and Caskey, 1990) for the situation *in vivo* is not obvious.

To overcome the disadvantages of current methods to study termination, we have constructed a complete *in vitro* translation system which is a further development of the recently described system for translation of natural mRNAs (Pavlov and Ehrenberg, 1996) with *in vivo*-like characteristics for elongation rate and accuracy. The main development is the addition of two purified release factors, RF1 and RF3, and a purified RRF to the system; this has allowed us to study ribosomes in recycling mode where they go through multiple rounds of translation, generating several completed polypeptides per ribosome. The system is used here to translate two short artificial mRNAs that code for the tripeptide fMet-Phe-Leu (fMFL) and for the tetrapeptide fMet-Phe-Thr-Ile (fMFTI), with termination occurring at ochre (UAA) and amber (UAG) stop codons, respectively. Measuring the rate of polypeptide synthesis in this system has allowed us to study the influence of release factors and RRF on the rate of ribosome recycling. As well as clarifying the roles of RF3 and RRF, these experiments show that the terminated ribosome almost always slides back to the initiation site of mRNA even in the presence of RRF.

Results

Ribosome recycling in the absence of RRF and RF3 is slow

An *in vitro* translation system was assembled with purified components necessary for the initiation and elongation of protein synthesis (see Materials and methods). Purified termination factor RF1 was added and the system was used to translate the short synthetic mMFL mRNA encoding the tripeptide fMet-Phe-Leu (fMFL). The accumulation of fMFL was measured by HPLC either directly as the

Fig. 1. The recycling of ribosomes in the translation system with RF1 but without RF3 and RRF. The rate of recycling was measured as the rate of fMFL peptide accumulation by HPLC (+) or as the rate of fMet-tRNA consumption by HPLC (○) and by cold TCA precipitation (×) as described in Materials and methods. The linear regression fit gives the rates 0.074 and 0.076 pmol/s for fMFL accumulation and fMet-tRNA consumption, respectively. The amount of active ribosomes, 3.1 pmol (per 12 μ l of the translation mix), was determined from the y-intercept of the linear regression line for fMFL peptides.

amount of fMFL peptide in the HCOOH-soluble fraction of the reaction mixture or indirectly as a decrease in the amount of fMet-tRNA in the HCOOH-insoluble fraction. Alternatively, fMet-tRNA consumption was followed by cold trichloroacetic acid (TCA) precipitation (Materials and methods). Figure 1 shows that the rate of fMFL accumulation measured by either of these methods was almost constant with time until most of the substrate, fMet-tRNA, had been consumed. The amount of active ribosomes in the translation system can be estimated from the HPLC data from the initial burst of the synthesis of tripeptide by extrapolation of the HPLC data to zero time (Figure 1). This amount corresponds to ~30% of the total added ribosomes and it is less, by almost an order of magnitude, than the initial amount of fMet-tRNA in the translation system. This implies a steady-state situation where each active ribosome carries out several rounds of translation, each round comprising initiation, elongation and termination. The time per round of translation can be found as the reciprocal of the slopes of the linear part of the experimental curves in Figure 1 multiplied by the amount of active ribosomes. This gives a time of 41 s for ribosome recycling in the absence of RF3 and RRF. The recycling time of 41.4 s obtained from HPLC data for tripeptides and that of 40.4 s measured with HPLC or TCA precipitation for fMet-tRNA consumption (Materials and methods) are very similar. Thus, the direct HPLC measurement of the amounts of released peptides provides us with essentially the same information about ribosome recycling as TCA measurements of the amount of fMet-tRNA left. The latter approach is technically simpler and was therefore used in most experiments reported here.

HPLC elution profiles (not shown) also demonstrated that the main product of translation was the released tripeptide fMFL. No fMFL-tRNA, that might have resulted from drop off competing with RF1-promoted hydrolysis of fMFL-tRNA on the ribosome, was detected

Fig. 2. Ribosomal recycling in the MFL mRNA translation system with 1 μM RF1 containing 300 pmol of [^3H]fMet-tRNA and 80 pmol of total ribosomes per 110 μl of translation mixture. The times per translational cycle were calculated from the slopes of the corresponding linear regression lines, assuming that 30% of total amount of ribosomes are active in translation. **(A)** The system contained 1 μM RF3 and different RRF concentrations: 0 (○), 0.2 (×), 0.5 (+), 2 (*) and 5 μM (□). The corresponding recycling times were 28, 15, 11, 5.5 and 5.1 s. **(B)** The system contained no RF3 and RRF (○), 1 μM RF3 was added but no RRF (×), 1 μM RRF was added but no RF3 (+) and, finally, both 1 μM RF3 and 1 μM RRF (*) were added; the calculated recycling times were 39, 28, 14 and 5.8 s.

(see Materials and methods for details). Furthermore, no peptidyl-tRNA hydrolysis was observed in the absence of RF1 or one or more of the aminoacyl-tRNA synthetases, judging by the constant level of TCA-precipitable tritiated material under these conditions (data not shown).

The presence of both RRF and RF3 is necessary for fast ribosome recycling

The addition of RRF together with RF3 causes a pronounced acceleration of the rate of ribosome recycling (see Figure 2A). The shortest recycling time at the highest concentration of RRF used in this experiment can be estimated to be <6 s, almost an order of magnitude faster than the rate with only RF1 present. This recycling time of 6 s corresponds to the total time of one translation round and includes the times spent by the ribosome in

the initiation, elongation and termination phases of the translation cycle. Taking into account a fast elongation rate of about eight codons per second measured in our translation system (Pavlov and Ehrenberg, 1996), we may estimate that in the presence of both RF3 and RRF the elongation time for the tripeptide fMFL should be <1 s, leaving 5 s for termination and initiation.

Figure 2B shows that simultaneous addition of RF3 and RRF to the system has a much more pronounced effect on ribosome recycling than the addition of either RF3 or RRF alone. The recycling time measured with RF3 alone is 28 s, while that with RRF alone is 14 s. A comparison of these times with the recycling time of 39 s obtained in the absence of both factors shows that RRF is relatively more important for fast ribosomal recycling than is RF3. A similar effect of RF3 and RRF addition is seen in translation of MFTI mRNA, encoding the tetrapeptide fMet-Phe-Thr-Ile, with a UAG stop codon (Table I, last row). This shows that the pattern seen in Figure 2 is not a special case related only to translation of mMFL mRNA.

RF3 catalyses cycling of RF1 between different ribosomes

Using a simple *in vitro* assay based on release of labelled fMet from [fMet-tRNA* AUG *ribosome] complexes it has been shown previously (Goldstein and Caskey, 1970) that RF3 stimulates RF1 cycling between different ribosomes. Figure 3 demonstrates that this conclusion also holds in our 'more natural' translation system. Here, a low RF1 concentration (one RF1 molecule per seven active ribosomes) was used, which results in a very low rate of fMFTI-tRNA hydrolysis (measured as rate of fMet-tRNA consumption) in the absence of RF3. Addition of RF3 considerably accelerates this rate and, since the amount of RF1 is much less than the amount of active ribosomes, the high rate of peptidyl-tRNA hydrolysis in the presence of RF3 can only be explained by RF3-promoted fast cycling of RF1 between different active ribosomes. The estimated time for the RF1 catalytic cycle is 24 s in the absence and ~4 s in the presence of RF3 (see the legend to Figure 3 for details).

RRF abolishes the Mg^{2+} dependence of the ribosome recycling time

The influence of Mg^{2+} concentration on the time of ribosome recycling in the MFL mRNA translation system is summarized in Table I. In the absence of RRF, ribosome recycling is slow and sensitive to the Mg^{2+} concentration. Addition of RRF abolishes the Mg^{2+} dependence of the recycling rate and reduces the recycling times considerably. Thus, RRF catalyses an inherently slow Mg^{2+} -dependent step in the termination process. One reasonable candidate for this slow step with such an Mg^{2+} dependence is the factor-independent dissociation of deacylated tRNA from the P-site of the ribosome after RF1-catalysed peptidyl-tRNA hydrolysis (see Discussion).

RRF-catalysed ribosome recycling depends on the EF-G concentration

The presence of elongation factor EF-G is necessary for RRF catalytic activity (Hirashima and Kaji, 1973; Janosi *et al.*, 1996). We have found that a moderate decrease in EF-G concentration from our standard 0.8 μM to 0.3 μM ,

Table I. Ribosomal recycling times at different Mg^{2+} concentrations

Mg^{2+} concentration (mM)	mRNA	Ribosomal recycling time (s)			
		RF1 only	RF1 + RF3	RF1 + RRF	RF1 with RF3 + RRF
3	mMFL	28	21	15	6.2
4	mMFL	39	28	14	5.8
5	mMFL	64	40	15	5.9
4	mMFL and 0.3 μ M EF-G	—	28	21	—
4	mMFL and 0.8 μ M EF-G	—	28	15	—
4	mMFTI	68	48	16	8

which should not significantly influence the elongation rate (Pavlov and Ehrenberg, 1996), leads to a noticeable increase in the time of RRF-catalysed ribosome recycling from 15 to 21 s. Table I shows (rows 4 and 5) that in the absence of RRF, but with RF3 present, the recycling rate is virtually unaffected by the same decrease in EF-G concentration. Therefore, this result indicates that the action of RRF is stimulated by EF-G, in line with earlier observations by Hirashima and Kaji (1973).

RRF does not actively eject mRNA from the terminating ribosome

The way in which ribosomes re-initiate translation on the MFTI mRNA was studied by competition between this and another mRNA (MFQI coding for fMet-Phe-Gln-Ile) for the re-initiating ribosomes. The experiment shown in Figure 4 demonstrates that ribosomes that terminate either in the presence or in the absence of RRF re-initiate almost exclusively on the same mRNA. In this experiment, a 2-fold excess of the competitor MFQI mRNA over MFTI mRNA was added with the factor mix to the ribosomes pre-initiated with MFTI mRNA. The competitor MFQI mRNA cannot be translated in the MFTI translation system beyond the Phe codon because of the lack of charged Gln-tRNAs. If the ribosomes that have completed translation of MFTI mRNA could re-initiate on MFQI mRNA, this would lead to an effective depletion of the active ribosome pool for the MFTI mRNA translation and an overall reduction in the rate of fMet-tRNA consumption. In a parallel experiment (not shown), it was verified that the pre-initiation of ribosomes with a one to one mixture of MFTI and MFQI mRNAs decreases the rate of fMet-tRNA consumption in the MFTI translation system by a factor of 2. The same was true when translation was started without the pre-initiation of ribosomes. The absence of any appreciable reduction in the fMet-tRNA consumption rate after addition of a competitor mRNA shows (Figure 4) that the ribosome does not switch mRNA either in the presence or in the absence of RRF, at least in the case of the short mRNAs used in this study. We conclude that RRF does not actively eject mRNA from the ribosome since we do not observe any efficient competition between the two mRNA species. We also note that no peptidyl-tRNA hydrolysis is apparent in the MFTI translation system with ribosomes pre-initiated on MFQI mRNA, even after the addition of MFTI mRNA (Figure 4). Thus, under these conditions, ribosomes cannot read through the Gln codon and are effectively trapped on the MFQI mRNA.

Fig. 3. The rate of fMet-tRNA consumption in the MFTI mRNA translation systems containing 350 pmol of [3 H]fMet-tRNA and 200 pmol of total ribosomes per 160 μ l of translation mixture. The system contained 0.06 μ M RF1 and the translation was run with (×) or without (○) 1 μ M RF3. The corresponding rates of fMet-tRNA consumption calculated from the slopes of the regression lines were 0.21 and 0.035 pmol/s. The corresponding cycling time of RF1 was calculated by dividing the amount of RF1 (0.84 pmol in the 14 μ l aliquot) by the rate of fMet-tRNA consumption.

Discussion

The *in vitro* experiments reported here demonstrate that the simultaneous presence of RF3 and RRF in a translation system is necessary for fast ribosome recycling. We have found that ribosome recycling in the absence of RF3 and RRF takes ~40 s and that this time is reduced to ~15 s by the addition of RRF alone or to 28 s by the addition of RF3 alone. The recycling time in the presence of both factors is less than 6 s.

The effects of RF3 and RRF appear to be additive; each factor catalysing a separate rate-limiting step. This may be seen as follows. If one subtracts the recycling time in the presence of both RF3 and RRF (6 s) from the recycling time in the presence of only RRF (15 s), a difference of 9 s is obtained. This difference gives an estimate of the uncatalysed reaction time for the step that normally involves RF3. A similar calculation leads to an estimate of ~22 s for the uncatalysed reaction time of the RRF step. Now, if one adds the sum of these rate-limiting steps (9 s + 22 s) to the recycling time in

Fig. 4. fMet-tRNA consumption in the translation system for MFTI mRNA containing 2.5 μ M MFTI mRNA in the absence (○) or after addition of 5 μ M MFQI mRNA (×); fMet tRNA consumption in the translation system for MFTI mRNA containing 2.5 μ M MFQI mRNA in the absence (+) or after addition of 5 μ M MFTI mRNA (*). (A) The translation system contains 1 μ M RF1, 1 μ M RF3 and 1 μ M RRF. (B) The same as (A) except that RRF was omitted.

the presence of both factors (6 s) one obtains an estimate of 37 s for the recycling time in the absence of RF3 and RRF. This calculated value fits very well with the measured recycling time of 40 s.

A hypothetical model for the termination process, which accounts for all experimental data, is given in Figure 5. The first step in the model is the hydrolysis of peptidyl-tRNA by RF1, leaving a deacylated tRNA in the P-site and RF1 in the A-site of the ribosome. The second step is the RF3-catalysed dissociation of RF1, which opens the A-site for subsequent binding of RRF. The third step is the dissociation of deacylated tRNA from the P-site, which is catalysed by RRF and EF-G. This last step allows the ribosome to move in relation to the mRNA.

The model is not unique, and other interpretations of the experimental data deserve to be mentioned. First, the model implies that the main role of RF3 is to promote dissociation of RF1 from the A-site, rather than to catalyse the association of RF1 with the ribosome or to accelerate the hydrolysis rate of peptidyl-tRNA. Any of these alternatives could, in principle, account for the experimental results in Figure 4 and also for the *in vivo* data of

Matsumura *et al.* (1996), but we favour the first option for several reasons. First, there is direct *in vitro* evidence both of Goldstein and Caskey (1970) and Mikuni *et al.* (1994) showing that, in the presence of GTP, RF3 promotes dissociation of a complex between the ribosome, RF1 and the termination trinucleotide UAA. Second, no direct experimental data demonstrating the stimulatory effect of RF3 on RF1/2 association with terminating ribosomes at physiological GTP concentrations are available. Finally, our own *in vitro* data (Freistroffer *et al.*, 1997) show that RF3 enhances neither the rate of RF1 association with the ribosome, nor the rate of peptidyl-tRNA hydrolysis. These experiments show, instead, that RF3 only promotes dissociation and recycling of RF1/2 in a GTP-dependent manner.

Stimulation of RF1 dissociation is one of the mechanisms of action of RF3 first suggested by Goldstein and Caskey (1970). However, in our model, the main role of RF3 catalysis is to ensure the fast clearance of the RF1-binding site on the ribosome for subsequent RRF binding, while in the model of Goldstein and Caskey (1970) RF3 ensures the fast re-utilization of supposedly scarce RF1 and RF2.

The idea that RRF (together with EF-G) promotes ejection of the deacylated tRNA from the P-site is based on the observation that ribosome recycling with RF3 in the absence of RRF is slow and Mg^{2+} dependent and that the addition of RRF makes the recycling fast and Mg^{2+} independent (Table I). In support of this, the recycling times in the absence of RRF and the dissociation times of fMet-Phe-tRNA from the ribosomal P-site are similar and exhibit a similar Mg^{2+} dependence (Karimi and Ehrenberg, 1996; and our unpublished results). These recycling times also depend on the nature of the deacylated tRNA in the P-site: thus, we find 28 s for tRNA_{4^{Leu}} and 48 s for tRNA_{1^{Ile}} (Table I, column 'RF1 + RF3').

Another possibility for the step catalysed by RRF is the dissociation of mRNA from the ribosome, since the rate of this event also has a strong Mg^{2+} dependence (Dahlberg and Haselkorn, 1967). This alternative is, however, rendered less likely by our observation (Figure 4) that the ribosome preferentially re-initiates on the same mRNA either in the absence or presence of RRF. This means that complete dissociation between mRNA and ribosome takes place only occasionally and therefore does not influence the recycling times significantly.

The finding that the ribosome stays on the same mRNA during several translational rounds even in the presence of RRF (Figure 4) shows that RRF does not actively eject mRNA from the ribosome as assumed in the current model of RRF action (Janosi *et al.*, 1996). However, this finding is not that surprising when taken together with the experimental observation *in vivo* (Adhin and van Duin, 1990) that a ribosome terminated at the end of the coat cistron of fr phage can re-initiate 34 nucleotides upstream on the overlapping lysis cistron. One obvious difference between our *in vitro* system and the *in vivo* experiments with respect to re-initiation is that for fr phage the ribosome has to slide 34 nucleotides upstream to reach a re-start position, whereas in the case of MFTI mRNA the sliding distance is only 15 nucleotides (Figure 6). A second difference is that MFTI mRNA possesses a stronger Shine-Dalgarno sequence than the phage fr lysis protein

Fig. 5. The kinetic model of the termination process leading to the mobility of the ribosome on mRNA.

cistron. Finally, the presence of upstream elongating ribosomes in the polysome *in vivo* may interrupt the scanning process. These differences may explain why we observe almost 100% re-initiation efficiency, whereas the re-initiation efficiency on phage fr RNA is only ~5%.

It is tempting to speculate that RRF, in its catalytic action, may mimic peptidyl-tRNA bound to the A-site of the ribosome and that EF-G may translocate RRF to the P-site, effectively ejecting deacylated tRNA (Nakamura *et al.*, 1996). This is supported by our observation (see Table I) that EF-G stimulates the RRF-catalysed ribosomal recycling in the absence of RF3. The positioning of RRF in the P-site may subsequently promote ribosomal opening and dissociation of the 50S subunit. The observed dissociation of mRNA from the ribosome (Hirashima and Kaji, 1973; Janosi *et al.*, 1996) after prolonged incubation with RRF and EF-G may result from an intrinsic instability of the 30S-mRNA complex after tRNA depletion. Whether the 30S subunit alone or the 70S ribosomal complex slides along mRNA and for how long a distance before dissociation occurs awaits further experimentation.

Unlike RF3, RRF is an essential protein for bacterial growth (Janosi *et al.*, 1994), and our data readily suggest why the factors may differ in this respect. While the RF3-dependent step in our model takes ~9 s in the absence of RF3, the RRF-dependent step takes 22 s or more without RRF. Thus, the absence of RRF may retard termination more severely than the absence of RF3. However, while superficially satisfactory, a more quantitative comparison between our model *in vitro* system and the living cell raises potential problems. The calculations of Bremer and Dennis (1996) suggest that, on average, in rapidly growing bacteria, the centre to centre distance between successive ribosomes on mRNA is ~41 nucleotides, and translation proceeds at 63 nucleotides/s. Thus both initiation and termination occur on an mRNA molecule at intervals of 0.7 s. The sum of these periods (1.4 s) is considerably shorter than our estimate of 6 s for the ribosome recycling

Fig. 6. Comparison of coat lysis overlapping cistrons of phage fr with MFTI mRNA. Note that the initiation codon for the LYSIS cistron is UUG.

time with both RF3 and RRF present in the system. Although the *in vivo* estimate of 1.4 s depends critically on a precise determination of the mRNA level in the cell, we think that there may still be a significant difference between the recycling rates *in vivo* and *in vitro*. We further suggest that this difference may be caused partially by different pathways for both initiation and recycling in the two cases. While ribosomes *in vivo* mainly initiate on free 5' ends of mRNA, the ribosomes in our *in vitro* experiments initiate preferentially on mRNA by scanning upstream from the termination site. This difference in mechanism may account for the longer recycling times that we observe.

When RF3 is removed from the bacterial cell, the cell generation time increases by between 44 (Mikuni *et al.*, 1994) and 71% (Grentzmann *et al.*, 1994). At the same time, our *in vitro* experiments show an increase of 9 s in the recycling time on removal of RF3, which we attribute

to a prolonged residence time of RF1 in the A-site. The question arises as to how such a delay in termination should be expected to influence the bacterial generation time.

If polysome structure were to remain unchanged in RF3-depleted cells, with a ribosome about every 40 nucleotides on mRNA, this would imply that the protein elongation rate must drop from 21 amino acids in the presence to <1.6/s in the absence of RF3. Such a large reduction in protein elongation rate seems impossible to reconcile with the comparatively moderate increase in generation time observed *in vivo*. It is possible that the stop signals (i.e. the stop codons and their surrounding nucleotide context) employed preferentially in well-translated messengers (Tate and Mannering, 1996) release RF1/2 more rapidly than in the cases we have studied so far. Furthermore, we cannot exclude that another protein can partially fulfil the role of RF3 in cells lacking this factor. An adaptation of cellular metabolism that includes an increase in the amount of mRNA in *prfC*-cells, coupled with an increase in the distance between ribosomes in polysomes, may also be a part of the explanation. Clearly, further study of translation in the absence of RF3 both *in vivo* and *in vitro* will be necessary to resolve this enigma.

Materials and methods.

Chemicals

Phosphoenolpyruvate, putrescine, spermidine, myokinase and non-radioactive amino acids were from Sigma. Pyruvate kinase was from Boehringer (Mannheim). All radioactive amino acids were from Amersham. Other chemicals of analytical grade were from Merck.

General components used in the translation system

Initiation factors were purified from overproducing strains according to Soffientini *et al.* (1994) with minor modifications. Elongation factors EF-Tu, EF-Ts and EF-G, and Leu- and Phe-tRNA synthetases (LeuRS and PheRS) were purified as described (Ehrenberg *et al.*, 1990). tRNA^{Phe}, tRNA^{Ile}, tRNA^{Thr} and tRNA^{Leu} were partially purified following procedures described previously (Ehrenberg *et al.*, 1990, and references therein). Ribosomes were purified from *E. coli* strains 017 (wild-type) according to Jelenc (1980). Ile-tRNA synthetase (IleRS) and termination factors RF1 and RF3 were purified to apparent homogeneity as described (Freistroffer *et al.*, 1997). The purification of RRF is described in J.MacDougall, K.Mortensen, D.Freistroffer, M.Pavlov, M.Ehrenberg and R.Buckingham (in preparation). Thr-tRNA synthetase (ThrRS) was prepared from MRE600 cells according to Brunel *et al.* (1993) with minor modifications.

Heteropolymer mRNAs

The gene for MFTI mRNA with the coding sequence AUG UUU ACG AUU UAG (which translates into fMet-Phe-Thr-Ile-stop) was constructed as follows. First the oligonucleotide A: 5'-ctctctGGTACCG-AAATTAATACGACTCACTATAgggAATTCGGGGCCCTTGTTAACAA-TTAAGGAGG and the oligonucleotide B: 5'-tttttttttttttttCTGCAGctaAATCGTAAACATAGTATACCTCCTAATTGTTAACAAGGG-CCCCG containing complementary sequences (underlined) were synthesized and purified using an 8% sequencing gel. These oligonucleotides were then aligned and 'filled' with *Taq* polymerase. The resulting MFTI gene: 5'-ctctctGGTACCGaaattaatcgaactcactataggAATTCGGGGCCCTTGTTAACAAATTAAGGAGGTATACTATGTTACGATTUAGcT/tg-cagaaaaaaaaaaaaaaaaa contains a promoter for T7 RNA polymerase (underlined bold), and the ribosome binding site (underlined capital boldface) analogous to that of 002 mRNA (Calogero *et al.*, 1988). It also contains a coding sequence (underlined italics) and a poly(A) tail which simplifies purification of mRNAs. This gene was cloned into a derivative of pUC18 vector (M.Pavlov, unpublished), and its sequence was confirmed by sequencing the plasmid. The plasmid was then used as template for PCR amplification with 2 nmol each of the 'forward' (5'-tttttGGTACCGaaattaatcgaactcactatag) and of the 'reverse' (5'-tttttttttttttttCTGCAG) primers. The final yield from the PCR reaction was ~1.5 nmol of the MFTI gene (DNA).

The gene for MFL mRNA with the coding sequence AUG UUC UUG UAA (fMet-Phe-Leu-Stop) and the gene for MFQI mRNA with the coding sequence AUG UUU CAG AUC UAG (fMet-Phe-Gln-Ile-stop) have the same sequences as the MFTI gene outside the coding sequence (underlined italics in the MFTI gene). They were constructed in the same way as the MFTI gene (only oligo B needs to be synthesized *de novo*). Corresponding mRNAs were prepared by *in vitro* transcription as described (Pavlov and Ehrenberg, 1996).

Translation experiments

All mixes were prepared in polymix buffer (Jelenc and Kurland, 1979) containing, at final concentration, 5 mM Mg acetate, 5 mM K phosphate (pH 7.3), 95 mM KCl, 5 mM NH₄Cl, 0.5 mM CaCl₂, 1 mM spermidine, 8 mM putrescine and 1 mM 1,4 dithioerythritol (DTE).

The ribosome mix (usually 15 µl) contained ribosomes and [³H]fMet-tRNA in amounts indicated for each experiment. The initiation factors and mRNA were present in the mix in the molecular ratio IF1:IF2:IF3: ribosomes:mRNA equal to 1:0.5:1:2. The ribosome mix also contained 1 mM GTP and 4 mM free Mg²⁺. The factor mix (usually 95 µl) contained 0.05 mM each of cold Leu and Phe, 120 pmol each of tRNA^{Phe} and tRNA^{Leu} and 80 U (defined in Ehrenberg *et al.*, 1990) each of PheRS and LeuRS for MFL mRNA translation or 0.05 mM each of cold Phe, Thr and Ile, 120 pmol each of tRNA^{Phe}, tRNA^{Thr} and tRNA^{Ile} and 80 U each of PheRS, ThrRS and IleRS for MFTI mRNA translation. In addition, the mix contained 1.1 mM ATP, 11 mM phosphoenolpyruvate, 1.0 mM GTP, 5 µg of pyruvate kinase (PK), 0.3 µg of myokinase, 5 µM EF-Tu, 1 µM EF-Ts and 0.8 µM EF-G. The concentration of free Mg²⁺ in the factor mix was adjusted to 4 mM with 100 mM Mg acetate, unless indicated otherwise. The concentrations of termination factors RF1 and RF3 and of ribosome recycling factor RRF were as specified in each experiment. The possibility of re-initiation in the absence of RRF at a sense codon that follows the stop codon was reported (Ryoji *et al.*, 1981). This possibility is excluded here since there are no charged tRNAs reading the CUG codon that follows the stop codon in mMFL and mMFTI mRNAs in the translation system.

Ribosome initiation was started by incubating the ribosome mix for 10 min at 37°C. The factor mix was also pre-incubated for 10 min at 37°C. Translation was usually started by adding 95 µl of pre-incubated factor mix to 15 µl of pre-incubated ribosome mix. Aliquots (usually 12 µl) were withdrawn after different incubation times and quenched either in 8 ml of ice-cold 5% TCA or in 12 µl of 25% formic acid. TCA precipitates were collected by filtration through GF-C filters (Whatman) and their tritium content was determined by scintillation counting.

HPLC measurements

Samples quenched with formic acid were processed as follows. First, the precipitate containing aminoacyl- and peptidyl-tRNAs (as well as other components of the translation mix) was pelleted by centrifugation. The supernatant (~20 µl, referred to as S-fraction below) containing released tri- or tetrapeptides was transferred to another Eppendorf tube and 0.2 ml of weak KOH solution was added to obtain a final solution with a pH of ~5. Then 0.15 ml of this solution was used for HPLC analysis. The pellet (called P-fraction of the original sample) was dissolved in 0.22 ml of 0.5 M KOH and incubated for 40 min at 42°C to hydrolyse all peptidyl- and aminoacyl-tRNAs. Subsequently, 11 µl of concentrated formic acid was added to the treated pellet and the precipitated material (also containing deacylated tRNAs) was separated from peptides and amino acids by centrifugation. Following centrifugation, 0.15 ml of the supernatant was used for HPLC analysis.

The peptide content of the S- and P-fractions of the HPLC samples was analysed using a C18 column (Merck), equilibrated and eluted with an H₂O buffer containing 42% methanol and 0.1% trifluoroacetic acid. The amount of peptides in the S-fraction of the HPLC sample corresponds to the amount of peptides released from the ribosome during translation, whereas the amount of peptides and fMet in the P-fraction corresponds to the amount of peptidyl-tRNAs and fMet-tRNA in the aliquot withdrawn from the translation system.

Monitoring the accumulation of released peptides during translation

The HPLC measurements described above allow direct measurement of the amount of peptidyl-tRNA hydrolysed on the ribosomes as a result of RF1 action. In another approach, the released peptides were measured indirectly by counting the tritium content of the cold TCA precipitate. This takes advantage of the fact that radioactive tetrapeptides f[³H]Met-Phe-Thr-Ile (or tripeptides f[³H]Met-Phe-Leu), products of the translation of MFTI and MFL mRNAs, do not precipitate in cold 5% TCA and

hence are not retained on GF-C filters. TCA-precipitated [³H]fMet-tRNA and the [³H]fMet-Phe-Thr-Ile-tRNA are retained. Thus, the reduction of tritium-labelled cold TCA-precipitable material should correspond to the amount of peptidyl-tRNA hydrolysed by RF1 on the ribosome.

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