Methylation of Bacterial Release Factors RF1 and RF2 Is Required for Normal Translation Termination in Vivo

Liliana Mora, Valérie Heurguè-Hamard, Miklos de Zamaroczy, Stephanie Kervestin, and Richard H. Buckingham

From the CNRS, UPR 9073, Institut de Biologie Physico-Chimique, 13 rue Pierre et Marie Curie, Paris 75005, France

Bacterial release factors RF1 and RF2 are methylated on the Gln residue of a universally conserved tripeptide motif GGQ, which interacts with the peptidyl transferase center of the large ribosomal subunit, triggering hydrolysis of the ester bond in peptidyl-tRNA and releasing the newly synthesized polypeptide from the ribosome. In vitro experiments have shown that the activity of RF2 is stimulated by Gln methylation. The viability of Escherichia coli K12 strains depends on the integrity of the release factor methyltransferase PrmC, because K12 strains are partially deficient in RF2 activity due to the presence of a Thr residue at position 246 instead of Ala. Here, we study in vivo RF1 and RF2 activity at termination codons in competition with programmed frameshifting and the effect of the Ala-246 → Thr mutation. PrmC inactivation reduces the specific termination activity of RF1 and RF2(Ala-246) by ~3- to 4-fold. The mutation Ala-246 → Thr in RF2 reduces the termination activity in cells ~5-fold. After correction for the decrease in level of RF2 due to the autocontrol of RF2 synthesis, the mutation Ala-246 → Thr reduced RF2 termination activity by ~10-fold at UGA codons and UAA codons. PrmC inactivation had no effect on cell growth in rich media but reduced growth considerably on poor carbon sources. This suggests that the expression of some genes needed for optimal growth under such conditions can become growth limiting as a result of inefficient translation termination.

Termination codons in mRNA are recognized on the ribosome by proteins called class 1 release factors (RFs)2 (1, 2). RFs bind to the ribosome when a stop codon is presented in the A-site and trigger hydrolysis by the peptidyl transferase center of the ester bond between the polypeptide and tRNA of peptidyl-tRNA bound to the P-site. Two class 1 RFs are used in eubacterial cells to recognize the three stop codons UAG, UAA, and UGA with overlapping specificity: RF1, encoded by prfA, recognizes UAG and UAA, and RF2, encoded by prfB, recognizes UAA and UGA. In contrast, a single RF is sufficient for termination at all three stop codons in eukaryotic or archaebacterial cells. The region of RFs that interacts with the peptidyl transferase center has been identified and contains a universally conserved tripeptide motif, GGQ (3, 4). The presence of this interaction has recently been confirmed by x-ray crystallography of RFs bound to Thermus thermophilus 70 S ribosomes (3). The GGQ motif is the only part of RFs that is conserved between factors of eubacterial, eukaryotic, and archaebacterial origin. Although archaebacterial and eukaryotic RFs are homologous, they have apparently evolved independently of the eubacterial RFs. The GGQ tripeptide has been shown to be post-translationally modified by methylation on the side-chain amide group of the Gln residue. This was first shown in bacteria (5), where RFs are modified by the methyltransferase (MTase) PrmC (6, 7), but it has since been demonstrated that methylation occurs also in eukaryotic cells (8, 9), despite the different evolutionary origin of the eubacterial and eukaryotic RFs. Whether archaebacterial RFs are methylated is unknown, but homologues of the RF MTase exist in these organisms (6).

Although the overlapping specificity of stop codon recognition has not been called into question, genetic experiments in Escherichia coli have indicated that certain UAA codons are read almost exclusively by RF1, despite the fact that the intracellular concentration of RF2 is several times higher than that of RF1 (10). Thus, mutations affecting RF1 could suppress UAA nonsense codons, indicating that the presence of RF2 did not compensate for reduced RF1 activity (11, 12). This was in contrast to the situation in Salmonella typhimurium, where RF2 read UAA codons efficiently and RF1 mutants did not show UAA suppressor activity (13). Uno et al. (14) showed that the difference between E. coli and S. typhimurium can be attributed to 1 of the 16 differences in sequence between RF2 in the two organisms: the presence of Thr at position 246 in place of Ala. Residue 246 and the GGQ motif are only four residues apart in the primary sequence.

Subsequent experiments, and the accumulation of genomic sequence data for eubacteria, showed that the presence of Thr-246 in RF2 is a peculiarity of E. coli K12 strains. Other laboratory strains, such as E. coli B and C6 (MRE600), have Ala-246, as do all other E. coli isolates for which genomic data are available (5). This raises the possibility that A246T is a mutation acquired during the extensive laboratory mutagenesis to which early K12 strains were subjected (15). All other bacteria have Ala or Ser in both RF1 and RF2 in the position corresponding to 246 in E. coli RF2. We will therefore consider RF2 Ala-246 as the wild-type form in E. coli, to which K12 strains are an exception.

One consequence of the presence in E. coli K12 strains of Thr-246 in RF2 is that lack of RF2 methylation almost completely inhibits cell growth. Revertants of K12 prmc mutant strains to normal growth in rich media show mutations T246A or T246S in RF2 (5–7). This suggests that the A246T mutation and lack of RF2 methylation both result in defects in RF2 activ-
the point mutation in codon 119 was first introduced into \textit{prmC} cloned in \textit{pLV(hemK)} (19), changing the GGU Gly codon to the frameshift site and variants, were expressed from ampicillin (200 $\mu$g/ml), tetracycline (12.5 $\mu$g/ml), kanamycin (50 $\mu$g/ml), chloramphenicol (15 $\mu$g/ml) (21). Minimal medium was as described by Vogel and Bonner (22) with 0.2% glucose, 0.4% succinate, or 0.4% acetate as carbon source (23). Solid media were similar but contained 12 g/liter agar (Difco). In experiments on bacterial growth, to minimize lag effects due to adaptation to solid media, strains were streaked from liquid media containing similar nutrients to the plates and were in growth phase before streaking.

\textbf{Measurement of Termination Efficiency in Vivo—MalE-LacZ$\alpha$ fusion proteins, separated by a window containing the RF2 frameshift site and variants, were expressed from ampicillin.}

\textbf{Eubacterial Translation Release Factor Methylation}

\textbf{TABLE 1}

\textbf{E. coli strains used in this work}

| Designation | Short name/background | Genotype | Reference |
|-------------|----------------------|----------|-----------|
| VH1039      | A + K12              | Xac, prfB(Ala246) | This work |
| LMG119D     | A − K12              | Xac, prfB(Ala246), prmC(Asp119) | This work |
| Xac         | T + K12              | D(lac-pro), argE, ara, gyrA, rpoB, thi | (45) |
| BLM607      | A + B                | ompT, hsdS$\delta$, dcm, lon, hemA41, zyg: Tn10tet | This work |
| BLM608      | A − B                | ompT, hsdS$\delta$, dcm, lon, prmC(Asp119) | This work |
| BLM612      | T + B                |ompT, hsdS$\delta$, dcm, lon | This work |
| Bl21        |                      | Xac, prfA(His)$_{12}$ − Tc, DprmC, prfB(Ala246) | (6) |
| SC8         |                      | Xac, prfA1, zyg: Tn10tet | (47) |
| US477       |                      | hemA41, relA1, spoT1, mcb1, rmb2, mcrB1 | B. Bachmann, \textit{E. coli} |
| CGSC4806    |                      | prfA, prfB | Genetic Stock Center |
| CAG18579    |                      | Xac, prfA(His)$_{12}$ − Tc, DprmC, prfB(Ala246), zyg: Tn10kan (93% linked to prfB) | V. Huregouri-Hamard, unpublished |
| VH11033     |                      | prfA, prfB | This work |
| VH1125      |                      |prfA, prfB, lacZ$\alpha$ | | |

\textbf{EXPERIMENTAL PROCEDURES}

\textit{Strains—} \textit{E. coli} strains used in this study (Table 1) were derived from the K12 strain Xac or the B strain BL21. VH1039 was constructed from SC8 (6) by transduction with a P1 lysate on strain CAG18579, introducing \textit{trp}::Tn10kan and creating the \textit{prmC}+ strain. The \textit{trp}::hemA region was then restored to wild type by transduction to \textit{trp}+ with a P1 lysate on a wild-type K12 strain. To inactivate \textit{prmC} in strain LMG119D,
Eubacterial Translation Release Factor Methylation

TABLE 2
Intracellular concentrations of RF1 and RF2 during exponential growth in rich media in K12 strains T+, A+, and A−
The quantities of RF1 and RF2 in strain T+ (line 1) were measured by the specific antibodies as described in Fig. 3 and under “Experimental Procedures” using purified RFs as standards. The values for strains A+ and A− were obtained by quantitative Western blot with respect to those in strain T+, normalized to elongation factor EF-Tu and measured by quantitative Western blot in the same way. The values for strain BL21 were obtained by quantitative Western blot with respect to those in strain T+, normalized with respect to A600 of the cell cultures. Values are expressed as molecules per cell, supposing that 1 A600 = 8 × 10^6 cells, and are reproducible to ±25%.

| Strain         | RF1 | RF2 |
|----------------|-----|-----|
| T+             | 3,600 | 26,000 |
| A+             | 5,200 | 13,500 |
| A−             | 4,700 | 15,500 |

lin-resistant pBR-derived plasmids as described by Poole et al. (24). Transformants of A+, A−, and T+ strains (Table 1) were grown to an A600 of 0.5 and induced with isopropyl 1-thio-β-D-galactopyranoside at 1 mM for a further period of 2 h. Cell proteins were solubilized in SDS-gel loading buffer and separated by SDS-PAGE electrophoresis as described by Poole et al. (24). Transfer to nitrocellulose membranes (Hybond C Super, GE Healthcare) and Western blotting with anti-MalE antibodies (Anti-MBP-antiserum, New England Biolabs) diluted 1:10,000 and Western blotting with anti-MalE antibodies (Anti-MBP-antiserum, New England Biolabs) diluted 1:10,000 were performed as described by Sambrook et al. (25), using 125I-labeled protein A (GE Healthcare). Radioactivity was measured using a PhosphorImager (Amersham Biosciences).

Western Blotting for RF Measurement— Cultures were grown in LB medium and induced with 1 mM isopropyl 1-thio-β-D-galactopyranoside as described for in vivo termination efficiency experiments. Western blot experiments were performed using rabbit anti-RF1, anti-RF2, and anti-EF-Tu antibodies commercially prepared from pure factors. For measurement of RF1, preparations were normally used undiluted; for RF2 they were diluted 1:20, and aliquots of 10–20 μl were applied to polyacrylamide gels. Pure non-His-tagged RFs as standards in Western blots were prepared as described by Mora et al. (26). Aliquots of 1 ml of culture were centrifuged, and the cells were lysed for 10 min at 100 °C in 200 μl of lysis buffer (50 mM Tris-HCl, pH 6.8, 100 mM dithiothreitol, 2% SDS, 0.1% bromphenol blue, 10% glycerol). Proteins were separated by electrophoresis on 10% polyacrylamide gels as described by Laemmli (27).

Transfer to nitrocellulose membranes, Western blotting with antibodies (diluted 1:5000), and quantification were performed as described above.

RESULTS

RF Methylation Increases Termination Efficiency in Vivo at All Three Stop Codons—Previous studies of the effect of RF methylation on termination efficiency have been restricted to the release of di- and tetrapeptides in an in vitro translation system primed with synthetic mRNAs (5). Under these conditions, termination by RF2 at UAA codons was significantly stimulated when the factor was methylated. However, no effect of methylation on the activity of RF1 was observed. To study the effects on termination efficiency in vivo at each stop codon of termination factor methylation and of the mutation at position 246 in RF2, we adopted the in vivo termination assay developed by Poole et al. (24), in their pioneering work on the effect of downstream context on stop signal efficiency. This system is based on competition between translational termination and ribosomal frameshifting at the frameshift site present in E. coli RF2. At this site, an in-frame UGA stop codon is bypassed by a +1 ribosomal frameshift, stimulated by the presence of a Shine-Dalgarno sequence six nucleotides upstream of the stop codon. In the assay system, the RF2 frameshift window is fused to the malE gene, expressed from a plasmid. Termination, stop codon readthrough (not observed in our experiments) and +1 frameshifting lead to proteins of 43.9, 46.2, and 52.0 kDa, respectively, that are separated by SDS-gel electrophoresis and detected by Western blotting, binding of anti-MalE antibody, and interaction with 125I-labeled protein A (Fig. 1A). The UGA codon may be replaced by UAG or UAA stop codons. The efficiency of stop codons is highly influenced by the surrounding nucleotide sequence (24, 28), the greatest effects being due to the nature of the nucleotide immediately downstream of the stop codon. The three stop codons may be followed by any of the 4 nucleotides, giving 12 “tetranucleotide stop signals.” The efficiency of frameshifting is supposed not to be affected by the nature of the tetranucleotide stop signal (29). The ratio of the frameshift product to the termination product is thus a direct measure of the termination efficiency. This system has been used to compare the efficiency of termination in vivo at the 12-tetranucleotide stop signals, catalyzed by methylated or unmethylated RFs, or the methylated Thr-246 form of RF2 found in K12 strains.
Previous studies of *prmC* inactivation have used strains in which the gene was inactivated by the insertion of a tetracycline-resistant cassette (6), which is likely to increase expression of the downstream genes due to the presence of the active tetA promoter. For this reason we have constructed an inactive point mutant of *prmC* for the experiments we report here in which the most conserved Gly (residue 119) of the major S-adenosylmethionine binding motif (GTG/TG) is replaced by an Asp. Examples of the data obtained at stop signals of widely varying efficiency in each of the three strains, T+ (*prmC*+, RF2(T246)), A− (*prmC−, RF2(A246)), and A+ (*prmC+*, RF2(A246)) are shown in Fig. 1B. MalE-containing products arising from termination and frameshifting were measured as described above, using phosphor storage methods. The rate of termination relative to frameshifting, calculated from such data and obtained at each of the twelve tetranucleotide stop signals UAAN, UGAN, and UAGN is shown in Fig. 2.

The absence of methylation is seen to decrease termination efficiency at all twelve tetranucleotide stop signals, implying that there are significant effects on both RF1- and RF2-dependent termination. A reduction in termination efficiency of approximately similar proportions is seen at each downstream context for all of the three stop codons; thus, comparing the results in strains A+ and A−, the effect of lack of methylation is not significantly greater in some downstream contexts with respect to others. As a corollary, the effects of context in strains A+ and A− are similar to those originally reported by Poole et al. (24) in a K12 strain. Thus, termination efficiency is favored by downstream context in the order U > G > A > C at UAA and UGA codons, and G > U > A > C at UAG codons. Comparing the results in strain T+ to those of Poole et al. (24) in another K12 strain, the observations are qualitatively similar, but the overall levels of termination with respect to frameshifting are lower by a factor of ~3. In our experiments, the frequency of frameshifting at UGAC, the naturally occurring stop signal at the frameshift site in *prfB* is ~60%, comparable to *in vivo* estimations in the *prfB* gene itself (30).

Comparing the efficiency of termination at UGA codons in strains T+ and A+, it can be seen that the reduction in termination efficiency with Thr in place of Ala is considerably greater than the effect of lack of methylation. Unexpectedly, the nature of residue 246 in RF2 was seen to reduce termination efficiency at UAG codons, recognized exclusively by RF1, by ~30%. This will be discussed further below, in terms of changes in the intracellular concentration of the factors.

**Intracellular Concentrations of RFs in Mutant Strains**—The data described above reflect termination efficiency of RF1 and RF2 *in vivo* and are not necessarily a direct measure of $k_{cat}/K_m$, because they do not take into account possible changes in RF concentration in the cell resulting from the mutations in *prmC* and *prfB*. The +1 frameshift required for RF2 synthesis constitutes an autoregulatory mechanism, so that increased RF2 activity will lead to a decrease in the intracellular concentration of the factor (31). Lack of methylation and the mutation A246T in RF2 are therefore expected to increase intracellular RF2 concentrations, which implies that the changes in termination efficiency that we observe *in vivo* will underestimate the changes in $k_{cat}/K_m$. More unexpectedly, the data in Fig. 2 suggest that RF2 activity may affect expression of *prfA*. The concentrations of RF1 and RF2 were therefore measured directly by Western blotting in extracts from cells grown under the same conditions as those used for measurements of termination efficiency. To transform the data on relative concentrations of RFs in the different strains into absolute data, variable quantities of purified RFs were added in some experiments to aliquots of cell extract before electrophoresis on polyacrylamide gel and Western blotting (Fig. 3 and Table 2). To facilitate comparison of different strains, a major protein component in the cell, elongation factor EF-Tu, was chosen as an internal standard, and quanti-
ties of RFs were measured in relation to the concentration of EF-Tu.

The results show that RF2 levels in strains T+, A−, and A+ vary in the way expected from the autocontrol mechanism: RF2 is present in strain A+ (A246, prmC+) at ∼60% the concentration compared with strain T+; the level in strain A− is intermediate, as found for the release activity at UGA codons. In the case of RF1, a level ∼30% less was found in strain T+ compared with strain A+; an intermediate level was found in strain A−. This finding is consistent with the expectation that the specific activity of RF1 for peptide release is similar in strains T+ and A+. The reason for the change in RF1 level is not clear, but control experiments in which RF levels were measured in strains T+, A−, and A+ that were not transformed by plasmids expressing MalE, or that were transformed by control plasmids lacking the MalE insertion, did not show these differences in the level of RF1 (results not shown), suggesting that they are an indirect effect of MalE overproduction.

Previous studies (13, 32) show that E. coli cells are far from being saturated with respect to release factors; the rate of termination should therefore be approximately proportional to RF concentration. Whereas the data in Fig. 2 reflect termination activity in cells, a more accurate measure of RF specific activity may therefore be obtained by normalizing these values with respect to the concentration of the RF (Fig. 4). Because the results in Fig. 2 showed that downstream context did not selectively affect the effect of lack of methylation or the A246T change, the results from the set of four contexts for each stop codon and by correcting for changes in the cellular concentration of RF1 and RF2, as measured by Western blot analysis. Data for strains A− and T+ are expressed relative to strain A+.

![Graph showing intracellular concentrations of RF1 and RF2 in K12 strain T+](image)

**FIGURE 3.** Intracellular concentrations of RF1 and RF2 in K12 strain T+. A culture of strain T+ was grown to an A600 of 1.6 in LB medium. Top, aliquots corresponding to 0.16 A600 units of cell culture (1.3 × 10^8 cells) for RF1 determination (open squares), or 0.008 A600 units of cell culture (6.5 × 10^7 cells) for RF2 determination (closed squares), solubilized in SDS sample buffer, were loaded on to SDS gels alone or mixed with increasing amounts of purified RF1 or RF2, separated, and visualized by Western blotting using anti-RF1 and anti-RF2 antibodies. Bottom, similar amounts of purified RF1 (open circles) or RF2 (closed circles) without added cell extract were analyzed also. Bound antibody was measured with 125I-labeled protein A and phosphorimaging (arbitrary units).
ity of the factors, whereas the A246T change reduces the specific activity of RF2 \(\sim 10\)-fold.

**Effects on Cell Growth of prmC Inactivation**—The RF MTase PrmC is universally found in eubacteria and has been classed among the minimum set of genes required for bacterial viability (33, 34). Nevertheless, except in the special case of K12 strains where the activity of RF2 is low because of the A246T change, previous experiments in rich media, using either liquid or solid media, have not shown any appreciable effect on cell growth of the inactivation of PrmC.\(^3\) We have therefore studied cell growth in a variety of poorer media, in the expectation of finding conditions under which RF methylation is important for cell growth. The growth of the same three strains, derived from the K12 background, as used above in termination efficiency experiments, T\(^+\), A\(^-\), and A\(^+\), was studied in four different liquid and solid media: LB, glucose minimal medium with required supplements, and two minimal media with poorer carbon sources, succinate and acetate (23). The growth rates in logarithmic phase of the three strains were not significantly different in LB or glucose minimal liquid medium, but in succinate and acetate the MTase-deficient grew only at \(\sim 65\) and 72% of the rate of either of the MTase-containing strains (Table 3). This implies that only \(~30\) generations would be required to dilute the PrmC-deficient cells 1000-fold with respect to normal cells. Little significant difference was seen between strains T\(^+\) and A\(^+\) during exponential growth, although strain T\(^+\) grew slightly more slowly at higher cell densities, and significantly more slowly on solid LB medium (Fig. 5). The lesser effect of the mutation A246T in RF2 compared with PrmC inactivation is consistent with the fact that, although RF2(T246) is generally less active than non-methylated RF2(A246), as reported above, the lack of MTase PrmC in cells affects the activity of both RF1 and RF2. When similar experiments were performed with strains derived from *E. coli* B (which is naturally RF2(A246)), slower growth of the PrmC-deficient strain was also seen on glucose minimal medium.

On solid media, the lack of MTase PrmC has rather more striking effects on cell growth than in liquid media (Fig. 5). As in liquid-rich medium, A\(^+\) and A\(^-\) strains grew similarly, although the T\(^+\)/K12 strain grew perceptibly slower. On glucose minimal medium, as well as on acetate medium, only very slow growth of PrmC-deficient cells was observed, compared with *prmc*\(^+\) cells (either RF2(T246) or RF2(A246), which both grew well). Broadly similar observations were made for strains derived from K12 or B backgrounds (Fig. 5).

\(\frac{\text{TABLE 3}}{\text{Growth rates in liquid media of normal (A\(^+\)), PrmC-deficient (A\(^-\)), and RF2(T246) (T\(^+\)) K12-derived strains}}\)

| Strain | LB | Glucose | Succinate | Acetate |
|--------|----|---------|-----------|---------|
| A\(^+\) | 2.23 | 0.775 | 0.610 | 0.460 |
| A\(^-\) | 2.22 | 0.770 | 0.390 | 0.335 |
| T\(^+\) | 2.13 | 0.775 | 0.710 | 0.460 |

\(\frac{\text{FIGURE 5. Growth defect of prmC mutant strains on glucose or acetate minimal agar plates}}{\text{Small colonies of A\(^+\), T\(^+\), and A\(^-\) strains derived from *E. coli* K12 and *E. coli* B backgrounds growing on LB, minimal glucose, or acetate solid media (with Pro for strain T\(^+\)) were resuspended in liquid minimal medium without carbon source and streaked on similar plates. LB, glucose, and acetate plates are shown after 1, 2, and 3 days, respectively, of incubation at 37 °C.}}\)

**DISCUSSION**

On the basis of current data, RF methylation is a universally conserved modification in eubacteria, affecting a small domain of the factors that interacts with the peptidyl transferase center on the large ribosomal subunit, triggering polypeptide release, and therefore of crucial importance to translation termination. Here we produce quantitative data on the effect of methylation on the termination process. We also measure the effect of an amino acid change present exclusively in RF2 in *E. coli* K12 strains, affecting a residue close to the methylated Gln, that partially inactivates the factor. This is of particular interest, because a substantial majority of published in vivo experiments on translation termination in eubacteria have been conducted in *E. coli* K12 strains and are potentially affected by the fact that these strains suffer from a partial deficiency in RF2-dependent termination.

The results show that lack of methylation reduces the specific activity of both RF1 and RF2(A246) by \(\sim 4\)- to 5-fold at UGA and UAG codons. The change A246T in RF2 reduces the measured termination in cells by \(\sim 5\)-fold at both UGA and UAA codons, which leads us to conclude that termination at UAA in cells carrying RF2(A246) is primarily the role of RF2 rather than RF1. This is the opposite of the situation in K12 strains carrying RF2(T246), where genetic experiments have shown that RF1 plays a greater role at UAA codons than RF2. Thus, *E. coli* prfA mutants suppress UAA, whereas prfB mutants do not (12, 14, 35). The A246T change in RF2 is therefore associated with a switch from RF2 to RF1 as the predominant RF responsible for reading UAA codons. We did not apply the experimental approach described here to measure the role of methylation in RF2(T246) activity, because inactivation of *prmC* almost completely inhibits cell growth in such cells and is of little relevance to understanding why the gene is universally conserved in eubacteria. The unexpected observation by Nakahigashi *et al.* (7), that lack of methylation decreased readthrough of a UAA codon \(\sim 2\)-fold, may be related to the very poor growth of a PrmC-inactivated RF2(T246) strain, or to the fact that the assay used did not allow measurement of the product of termination in addition to the readthrough product.

\(^3\) L. Mora, unpublished data.
Eubacterial Translation Release Factor Methylation

Previous experiments, measuring the release of short peptides from ribosomal termination complexes in vitro, showed that RF2 defective either through lack of methylation or because of the presence of T246, or both, showed a loss of activity, and that the two defects were cumulative. The loss of activity was strongly dependent on the length of the peptide. In these in vitro experiments, the release of dipeptides, tripeptides, and tetrapeptides was studied, but the results gave little indication of what should be expected for translation termination releasing proteins of normal length.

The reduction in the specific activity of RF2 was found to be partially compensated for in cells, as expected, because of the autocontrol mechanism dependent on ribosomal frameshifting, leading to an increase in the intracellular level of RF2. Thus, strain T+ containing about twice as much RF2 as strain A+, and we conclude that the difference in specific activity (k_{cat}/K_m) of RF2(A246) compared with RF2(T246) is ~10-fold. An unexpected but significant change in the level of RF1 was also observed when comparing T+ and A+ strains. However, this effect appears to be an indirect one due to the induction of Male fusion proteins in the experimental system we employed and was not seen in control experiments when Male synthesis was not induced, or when cells were transformed with control plasmids not expressing Male. It has been suggested that E. coli and Salmonella may possess an autocontrol mechanism for RF1 synthesis (37), dependent upon readthrough of the weak UAGC tetranucleotide stop signal of the hemA gene upstream of prfA, coupled with a suboptimal ribosome binding site for prfA expression (38). However, a study of mutations affecting the hemA termination signal or the intergenic hemA-prfA region in E. coli is not in favor of this hypothesis.4

The resolution of the crystal structures of RFs bound to 70 S ribosomes is currently too low to explain directly how RF methylation may affect translation termination (3). However, recent molecular dynamics simulations suggest how Gln methylation may stimulate pepidyl-tRNA hydrolysis (39). Molecular docking procedures predict possible modes of binding of the GGQ/H1 motif in the pepidyl transferase center and serve as the basis for molecular dynamics simulations of termination. An extensive network of hydrogen bonds is vital to the hydrolytic attack, involving the methylated Gln amide side chain and backbone CO and NH groups, the surrounding bases Cys-2452 and Ala-2606 of 23 S RNA, Ala-76 of the P-site tRNA, and the hydrolytic and one further water molecule. Deletion of the methyl group appears to increase mobility of the Gln residue, rendering the network of hydrogen bonding more labile and increasing the activation energy along the reaction path.

Despite the partial compensation resulting from the autoregulatory mechanism for RF2 synthesis, a 5- to 6-fold difference in termination efficiency is seen between T+ and A+ strains at both UAAN and UGAN stop signals. This throws light on a previously puzzling observation, namely, that UAGN stop signals are rarely used in E. coli, although they appear (especially UAGG) to be about as efficient as UAAN and UGAN signals (24). Because we now view the presence of RF2(T246) in E. coli K12 strains as an artifact due to a recent mutation in the prfB gene (5), it is clear that it is of more physiological significance to look for a correlation between stop signal usage and stop signal efficiency in A+ rather than K12 (T+) strains. Thus, in A+ strains, UAGN stop signals are now seen to be much less efficient than UAAN and UGAN stop signals, and it is now more readily understandable why the use of UAGN stop signals should be selected against, particularly in highly expressed genes.

Although in E. coli K12 strains RF methylation is required for normal growth on rich media, and any growth on glucose minimal media (6), this and previous work show that there is no significant effect of the lack of RF methylation in strains containing RF2(A246) or RF2(S246) when grown on rich media, although the experiments did not exclude the possibility that, in competition with each other, the wild-type or the MTase-deficient strain might eventually become dominant (6). The previous observation in a K12 strain that the suppressor mutation RF2(T246A) did not restore normal growth on rich media to a prfA strain (7) may be due to strain differences or to the fact that growth measurements were conducted in a nitrosoguanidine-mutagenized background. The experiments we present here show that, when grown on the poor carbon sources, succinate or acetate, MTase-deficient strains are at a clear disadvantage. Firstly, this suggests that efficient termination is needed for the synthesis of some proteins that are important under poor growth conditions, and that a reduction in termination efficiency due to lack of RF methylation may reduce the synthesis of some proteins to such an extent that they become growth limiting. It should be noted that the substitution A246T in RF2 does not lead to similar slow growth on poor carbon sources, suggesting that it is not termination at UGA codons that is implicated in this phenomenon. Several mechanisms might contribute to reduced protein yield, such as ribosome queuing (18), mRNA degradation, and triggering of transfer messenger RNA action (17, 40).

It is well known that E. coli grown on rich media express predominantly a subset of genes that are not only characterized by a bias with respect to the use of synonymous sense codons but also show a strong bias toward the use of efficient tetranucleotide stop signals (24). As carbon sources become poorer, a progressively wider variety of genes becomes induced in a largely hierarchical manner (43). Somewhat surprisingly, the families of genes mobilized appear to be far broader than those strictly required to metabolize the available substrate. Nevertheless, the shift toward poor carbon sources undoubtedly requires expression of additional genes, some of which may have poor termination signals for which efficient RFs are needed. Preliminary experiments show that poor growth of prfA mutants on some poor carbon sources can give rise to fast growing revertants by second site mutations. Identification of suppressor mutations may offer an approach to the identification of proteins whose synthesis is significantly affected by a reduction in termination efficiency.

REFERENCES

1. Kisselev, L. L., and Buckingham, R. H. (2000) Trends Biochem. Sci. 25, 561–566.

4 L. Mora and R. H. Buckingham, unpublished work.
Eubacterial Translation Release Factor Methylation

24. Poole, E. S., Brown, C. M., and Tate, W. P. (1995) *EMBO J.* **14**, 151–158
25. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York
26. Mora, L., Heurgué-Hamard, V., Champ, S., Ehrenberg, M., Kisselev, L., and Buckingham, R. H. (2003) *Mol. Microbiol.* **47**, 267–275
27. Laemmli, U. K. (1970) *Nature* **227**, 680–685
28. Stormo, G. D., Schneider, T. D., and Gold, L. (1986) *Nucleic Acids Res.* **14**, 6661–6679
29. Curran, J. F., and Yarus, M. (1989) *J. Mol. Biol.* **209**, 65–77
30. Craig, W. J., and Caskey, C. T. (1986) *Nature* **322**, 273–275
31. Craigie, W. J., Cook, R. G., Tate, W. P., and Caskey, C. T. (1985) *Proc. Natl. Acad. Sci., U. S. A.* **82**, 3616–3620
32. Weiss, R. B., Murphy, J. P., and Gallant, J. A. (1984) *J. Bacteriol.* **158**, 362–364
33. Gerdes, S. Y., Scholle, M. D., Campbell, J. W., Balazsi, G., Ravas, E., Daugherty, M. D., Somera, A. L., Kyripides, N. C., Anderson, I., Gelfand, M. S., Bhattacharya, A., Kapral, I., D’Souza, M., Baev, M. Y., Grechkin, Y., Mseeh, F., Fonstein, M. Y., Overbeek, R., Barabasi, A. L., Oltvai, Z. N., and Osterman, A. L. (2003) *J. Bacteriol.* **185**, 5673–5684
34. Mushegian, A. R., and Koonin, E. V. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93**, 10268–10273
35. Kawakami, K., Inada, T., and Nakamura, Y. (1988) *J. Bacteriol.* **170**, 5378–5381
36. Singer, M., Baker, T. A., Schnitzler Deischel, S. M., Goel, M., Dove, W., Jaacks, J., Grossman, A. D., Erickson, J. W., and Gross, C. A. (1989) *Microbiol. Rev.* **53**, 1–24
37. Elliott, T. (1989) *J. Bacteriol.* **171**, 3948–3960
38. Dahlgren, A., and Ryden-Aulin, M. (2004) *Biochimie* (Paris) **86**, 431–438
39. Trobro, S., and Asqvist, I. (2007) *Mol. Cell* **27**, 758–766
40. Li, X., Yokota, T., Ito, K., Nakamura, Y., and Aiba, H. (2007) *Mol. Microbiol.* **63**, 116–126
41. Ikemura, T. (1981) *J. Mol. Biol.* **151**, 389–409
42. Gouy, M., and Gautier, C. (1982) *Nucleic Acids Res.* **10**, 7055–7074
43. Liu, M., Durfee, T., Cabrera, J. E., Zhao, K., Jin, D. J., and Blattner, F. R. (2005) *J. Biol. Chem.* **280**, 15921–15927
44. Curran, J. F., and Yarus, M. (1988) *J. Mol. Biol.* **203**, 75–83
45. Coulondre, C., and Miller, J. H. (1977) *J. Mol. Biol.* **117**, 525–575
46. Studier, F. W., and Moffatt, B. A. (1986) *J. Mol. Biol.* **189**, 113–130
47. Kaczanowska, M., and Rydén-Aulin, M. (2004) *J. Bacteriol.* **186**, 3046–3055