9
Termination and Ribosome Recycling

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9.1
Introduction

The translation-elongation cycle is brought to an abrupt halt by the appearance of a stop codon in the ribosomal A site, rather than the usual sense codon of the mRNA. If the mRNA being translated is thought of as a sentence then the stop codons generally act as a "full-stop", i.e. they signal to the ribosome that the protein has been fully translated and must now be released from the ribosome. The process of polypeptide release is supposed to be the third (and generally the final) stage of translation, with initiation and elongation (see Chaps. 7 and 8) being the first and second, respectively. However, it should be acknowledged that the process of recycling the ribosomes following release of the polypeptide is often referred to as a post-termination event and, therefore, can be thought as the fourth stage of translation. There are numerous reviews attempting to keep up with the rapid pace that the termination researchers have attained in the past few years and demonstrating the unexpected discoveries that are hiding around every corner (for a recent review see [1]).

So how does the presence of a stop codon in the ribosomal A site trigger entry into the termination and ribosome recycling stages of the translation cycle? The answer to this question relates directly to the make-up of the genetic code; usually all codons, but three (although this can sometimes be one or two for particular organisms or organelles, see later), correspond to a particular amino acid and therefore with an aminoacylated or charged tRNA. Clearly, in the situation where a ribosome bears a stop codon (or any codon for that matter) in the A site for which there is no corresponding aminoacyl-tRNA, the elongation cycle cannot continue. This type of reasoning led to the proposal for the existence of a "terminator-tRNA", i.e., a special (uncharged) tRNA that recognized the stop codons and somehow mediated release of the completed polypeptide. In light of what we now know about the interaction between the anticodon of the tRNA and the codon of the mRNA and how the stereochemistry of this interaction is monitored by the ribosome to ensure its correctness, the idea of a terminator-tRNA would seem like an obvious choice to initiate termination events. However, as so often seen before, nature had evolved another quite distinct mechanism, which does not rely on RNA–RNA interaction at all.

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No terminator tRNAs were discovered, instead it turned out that stop codons or termination signals are recognized by protein factors, termed termination release factors (RFs), so named because they mediate the *release* of the nascent polypeptide from the ribosome. However, it soon became clear that there were two sets of RFs involved in the termination process. The original factors, which decode the stop codons and actually release the polypeptide from the ribosome, were therefore termed class I or decoding release factors. In bacteria, such as *Escherichia coli*, there are two decoding factors, RF1 and RF2. The class II RFs operate after the decoding factors (and perhaps more important for factor nomenclature were also identified subsequent to the decoding factors) and are therefore termed RF3s. RF3 is involved in removal of the decoding RFs from the ribosome and therefore stimulates release of the polypeptide by recycling of the decoding factors. In eukaryotes (and archea), there is only ever one class I decoding RF, termed eRF1 (aRF1) and by analogy the class II factor is termed eRF3 (aRF3). The class II factors are G-proteins and therefore their affinity for the ribosome is regulated by the guanine nucleotide state of the factor (see Chap. 8). For bacterial RF3, the GDP form of RF3 has been shown to have a low affinity for the ribosome following release of the decoding factor and therefore falls off the ribosome. This post-termination ribosome, however, still bears the mRNA it was translating, a deacylated or uncharged tRNA at the P site (the tRNA from which the nascent polypeptide was released) and probably an additional deacylated tRNA at the E site. These components need to be removed and the ribosome dissociated into its component subunits in preparation for the next round of translation. This essential process is termed ribosome recycling. In bacteria, three factors are involved in this process, one that is specific for this stage termed the ribosome recycling factor (RRF) and the others, which are active during elongation and initiation, namely elongation factor G (EF-G) and initiation factor 3 (IF3), respectively. There is some debate at present as to the exact details of the steps mediated by these three factors and this is discussed in more detail in Sect. 9.5. Since RRF is not found in eukaryotes (or archaea), except in mitochondria or chloroplasts, there must be an alternative system operating to fulfill or circumvent the need for this process in the cytoplasm.

9.2 Stop Codon Recognition and Release of the Nascent Polypeptide Chain

The importance of the termination phase of protein synthesis is emphasized by the universal presence of class I decoding release factors, i.e., all archea and eukaryotic genomes sequenced so far have the presence of aRF1 and eRF1, respectively, and all bacterial genomes have at least one of the two decoding factors RF1 or RF2. For example, in the genome of *Mycoplasma genitalium*, the *prfA* gene encoding RF1 is present whereas *prfB*, the gene encoding RF2, is absent. Since *M. genitalium* is often referred to as the bacteria containing the "minimal complement" of genes necessary for survival, its small genome size arising from having dispensed with most of the

non-essential genes [2, 3], this illustrates the importance of the decoding termination factors. Indeed, although eukaryotic and archeal RF1 perform the same role as the bacterial RF1 (and RF2), there is no obvious sequence or structural homology between the two proteins, suggesting that they have arisen independently and thus represent examples of functionally convergent evolution. Despite this evolutionary independence, some remarkable similarities between the two factors have emerged, suggesting that their mechanism of action may also be similar. When one considers the extreme conservation in the target or substrate of their reaction, namely, the ribosome with a peptidyl-tRNA in the P site and a stop codon in the A site, it is not unforeseeable that the similarities in the constraints imposed by the ribosome in terms of binding site are reflected by similarities within the bacterial and eukaryotic factors. With regard to the mechanism of action of the bacterial decoding factors on the ribosome, a number of surprises have recently been brought to light and it remains to be seen whether the eukaryotic factors really operate through a similar mechanism.

9.3
The Bacterial Class I Decoding Release Factors

The eubacterial decoding factors, RF1 and RF2, exhibit overlapping specificities with regard to stop-codon discrimination: for example, in *E. coli*, RF1 decodes the stop codons UAG and UAA, whereas RF2 decodes UGA and UAA [4, 5]. Organisms such as *M. genitalium*, which have dispensed with the *prfB* gene, do not require the corresponding RF2 factor because UGA is not regarded as a stop codon, being decoded by Trp-tRNA^{ACU} and thus incorporating tryptophan at these codons [6]. In organelles, where codon reassignment is common (reviewed in Refs. [7, 8]), there is almost always a loss of the *prfB* gene, for example, yeast mitochondria also decode UGA by tryptophan and therefore have only a single RF of the RF1-type [9]. This raises the intriguing question of why it is always the *prfB* gene and not the *prfA* gene that is continuously lost from these genomes?

9.3.1
The Structure of RF2 and Translational Mimicry

A comparison of the *E. coli prfA* and *prfB* genes [10, 11] and in particular the protein products revealed extensive sequence homology. Based on secondary-structure predictions, the decoding factors have been discussed in terms of five [12] or seven [13] domain models. However, on the basis of functional data, Tate and co-workers [14, 15] proposed a simple two-domain "tRNA-analogue model", where the decoding RFs were thought to span from the stop codon in the decoding site of the 30S subunit to the PTF center on the 50S subunit. In this model, each domain of the RF was associated with a function, one domain with stop codon recognition and the other with release of the polypeptide, in analogy with a tRNA where the anticodon stem loop is associated with codon recognition and the acceptor stem of the tRNA with peptide-bond formation. This tRNA mimicry concept was brought to another level with the

arrival of the structure of the ternary complex of EF-Tu•GTP•Phe-tRNA^{Phe} [16], which had striking overall similarity with the translation elongation factor EF-G [17, 18]. In particular, the tertiary structure of domains III-V of EF-G resembled that of the tRNA in the ternary complex (see Figs. 8.6A and B). This suggested that the A site of the ribosome, the binding site for the tRNA, but also for the translation factors such as EF-G and the EF-Tu ternary complex, imposed constraints on the factors to take-on the shape of tRNA to bind within A-site region. Since the initiation factors IF1 and IF2, as well as the termination factors RF1, RF2, RF3 and RRF, were also known to bind within this region, the idea of macromolecular mimicry of tRNAs by protein factors was extended to encompass all phases of the translation cycle (reviewed by Nissen et al. [19]). Indeed Sprinzl and co-workers [20] proposed, based on sequence alignments, that IF2 would have a structure similar to EF-G except that the missing region of complementarity in IF2, namely of domain IV of EF-G, would be compensated for by homology found in IF1, i.e. together IF2 and IF1 would also mimic the shape of the aa-tRNA•EF-Tu•GTP ternary complex. However, the recent crystal structure of the IF1 bound to the 30S subunit [21] suggests that the concept of molecular mimicry may have been overextrapolated in this case, since the structure and binding position of IF1 bears little resemblance to that of the anticodon stem loop of a tRNA, nor to that of domain IV of EF-G (see Chap. 7.1).

The long-awaited structure of a decoding RF was that of the eubacterial *E. coli* RF2 [22], rather than from *Thermus thermophilus* as might have been first thought [23]. Since the vast majority of studies into RFs had come from studies on *E. coli*, this provided the perfect opportunity to correlate structure and function (see the next section). The *E. coli* RF2 structure was solved to 2.3 Å and revealed a four-domain arrangement (Fig. 9.1A). The N-terminal domain (domain I) contains four α-helices that form an α-helical bundle, whereas domains II, III and IV form a very compact

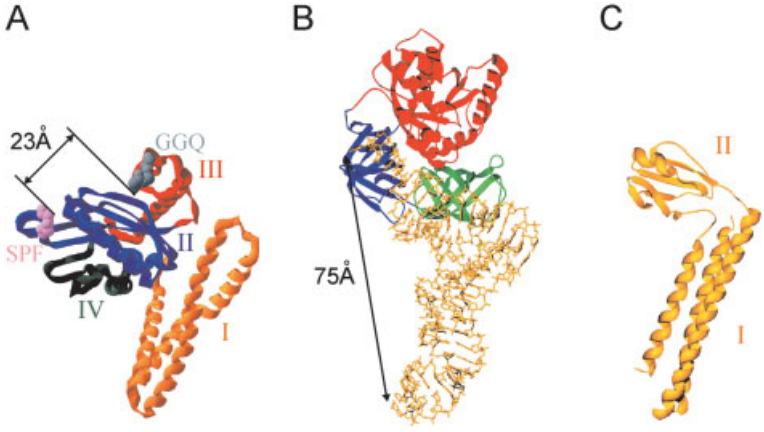

Figure 9.1 Molecular mimicry between tRNA and termination factors. The crystal structures of (A) RF2 [22], (B) tRNA (yellow) in form of ternary complex, (C) RRF [28] are compared illustrating the overall tertiary structure similarities.

structure stabilized by multiple interdomain interactions [22]. Indeed, the structure could be interpreted as having an overall two-domain arrangement, since domain I and domains II–IV are relatively separate from one another. This is supported by the observation that the recent structure of *T. thermophilus* RF2 was almost identical to that of *E. coli* RF2, except that there was a different orientation of domain I with respect to domains II–IV, which similar to the *E. coli* structure, also formed a distinct compact superdomain [24]. Despite the overall two-domain topology, there is little resemblance between the RF2 structures and a tRNA (Fig. 9.1B; reviewed in Refs. [25–27]); however, the previous structures of the ribosome recycling factor (RRF) had reinforced the idea of tRNA mimicry, since this small factor was composed of two domains that were arranged in a definite L-shape with similar dimensions and orientations as a tRNA (Fig. 9.1C; [28]). On the basis of the similarity between domain I of RF2 and domain I of RRF, which was proposed to mimic the anticodon stem loop of the tRNA, domain I of RF2 was docked into the A site. However, this docking, nor in fact any other single orientation, could simultaneously satisfy all the available biochemical data, which had defined particular regions of the decoding RFs as being associated with particular functions, such as codon recognition or peptidyl-tRNA hydrolysis.

9.3.2
The Two-domain Functional Model for RF2

Before the arrival of the RF2 crystal structures, there existed very strong evidence that the decoding factors were intimately involved in stop-codon recognition and peptidyl-tRNA hydrolysis. In fact, distinct regions and even specific amino acids within these regions had been implicated in each function. If the decoding RFs were to mimic tRNAs, then they would be required to interact at one end of the molecule with the stop codon in the A site and at the other end to mediate the transfer of the polypeptide chain from the P-site tRNA to water, thereby releasing it from the ribosome. So what evidence exists for these functional domains in the RFs and how do they relate to the available crystal structure?

9.3.3
Identifying Functional Important Regions within the Decoding RFs

Perhaps the first indication as to which regions of the decoding RFs were functionally significant arose through the use of suppression studies, where competition at stop codons between the decoding RFs and so-called suppressor tRNAs was monitored. The first of these studies analyzed the effect of decoding RFs at UAA stop-codon contexts by utilizing a three-plasmid system, one expressing either RF1 or RF2, one expressing a UAA stop-codon suppressor tRNA and a reporter plasmid expressing a UAA-containing *LacZ* reporter construct [29, 30]. The application of this type of system was used to identify mutant RFs with reduced activity, i.e., the situation that resulted in more efficient competition of the suppressor tRNAs at the stop

signal (see Table 9.1 and Fig. 9.2). Although these genetic screens provided a powerful technique for identifying functional regions in RFs, most of the mutations conferred a recessive phenotype and thus without complementary *in vitro* data provide limited insight into the true nature of the mutation. Indeed, a number of the RF mutants were temperature-sensitive [31–36], suggesting that the defects in these factors may have been due to folding perturbations.

RF fusion proteins were the first demonstration of partly functional release factors, where partial activity in ribosome binding was detected in the absence of peptidyl-tRNA hydrolysis (or release) activity [37]. The separation of the decoding and release activities of the decoding RFs was in agreement with the two-domain model of the RFs. A more directed approach to discovering regions of importance within the decoding RFs was undertaken by generating chimeric RF constructs by exchanging regions between *E. coli* RF1 and RF2 [14]. One of the chimeric constructs generated was identical to RF1 except for the replacement of a small region within domain III by the corresponding region in RF2. Although this in effect yielded only 10 amino acid substitutions, many of which were either conservative or in positions of low conservation, the effect was significant: *in vivo*, the expression of this chimeric factor was toxic to the *E. coli* cell, reproducibly inducing complete cessation of growth upon induction and the strong selection against the plasmid expressing the chimera and often leading to deletions within the plasmid [14]. *In vitro*, this chimera was shown to have significant reduced release activity, whereas the codon-dependent binding activity remained unaffected [14]. This was the first

Table 9.1 Decoding Release Factor Mutations

Factor	Mutation[a]	Reference
RF1 (*E. coli*)	G168D	35
	R137P	36
RF1 (*S. typhimurium*)	G180S	31
	H182Y	
mtRF1 (*S. cerevisiae*)	R190K	9, 12
	P192L	38
RF2 (*E. coli*)	L63F and D79G	151
	E89K	34
	D143N	
	L328F	
	F207T	13
	R213I	
	T246A	63
	E167K	136
RF2 (*S. typh.*)	Y144UGA	33

a All numbering corresponds to the equivalent residue in *E. coli*.

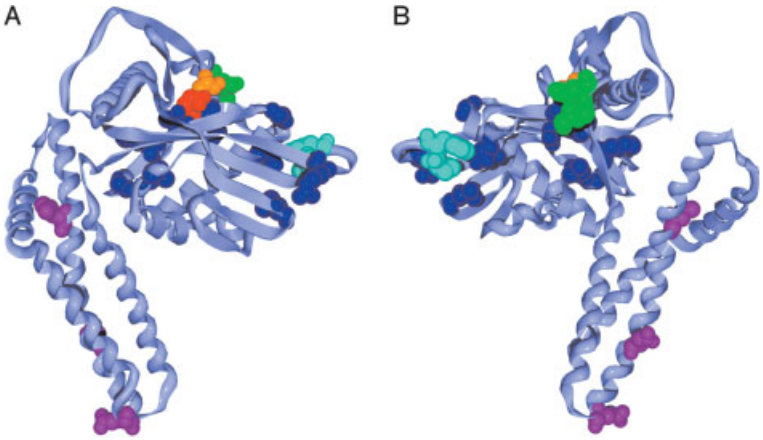

Figure 9.2 The crystal structure of *E. coli* RF2 with mutants from Table 9.1 mapped onto the equivalent positions in *E. coli* RF2. The tripeptide (cyan) and GGQ motif (light green) as well as position 246 (yellow) and the omnipotent E167K (red) are also indicated. Note that most of the mutations map within domains II-IV (dark blue), whereas only few (L63, D79 and E89 colored purple) are in domain I.

evidence implicating domain III as being associated with release of the polypeptide and is discussed in more detail in Sect. 9.2.6.

Around the same time, two mutations in the mitochondrial RF1 gene were shown to be responsible for a splicing defect in the yeast *S. cerevisiae* [9, 12]. The mutations were identified as being in domain II of RF1 and were subsequently shown to impair the factor's ribosome binding ability *in vivo* [38] and *in vitro* [39]. That these sites in domain II were directly associated with ribosome binding was strengthened, when second site suppressors were identified in h44 of the 16S rRNA that restored the binding of the mutant factors. Three of these second site suppressors disrupt a base-pair within h44 and confer resistance to the aminoglycoside antibiotic paromomycin, which is known to bind directly within the decoding site and induce translational misreading (see Chap. 12.3.1.2). Another mutation mapped within the so-called switch region (h27) that has been implicated in translational fidelity [40] and constitutes the binding site for the classic misreading antibiotic streptomycin (see Chap. 12.3.1.2). This is very suggestive of a direct interaction between domain II of the RF and regions within the decoding site, if not the stop codon itself. Indeed, one of the second site suppressor locations (A1408) has been crosslinked from the first position of a UAA stop codon located in the A site [41] and normally base-pairs with A1493, one of the universally conserved residues that is intimately involved in monitoring the correctness of codon–anticodon interaction in the A-Site (see Chap. 8.2.3).

9.3.4 Codon Recognition Domain of Bacterial RFs: the Termination Signal

It seems very probable that the decoding release factors recognize a stop codon in the A site of the ribosome by directly interacting with it, as opposed to recognizing the presence of a stop codon indirectly through putative conformational changes that occur specifically in the ribosome during the termination phase. The latter seems less probable – it would require conformational changes that are specific for the stop codon, since RF1 and RF2 discriminate between UAG and UGA, although such changes have been proposed [42]. In addition, close proximity between stop codon and RF is evident from the zero-length crosslinks observed from the first position of a stop codon (using thiouridine) to RF2 [41, 43]. Moreover, it has been shown that the context of the stop codon played an important role in the efficiency of the termination reaction (reviewed in Refs. [44–46]). This was perhaps first evident from the markedly differing levels of stop codon readthrough by suppressor tRNAs depending on the context of the stop codon, both upstream and downstream. Tate and co-workers went on to demonstrate that the position immediately following (3′ to) the stop codon (termed the +4 position) had the most significant effect on termination efficiency [47], but additional influences were also seen as far downstream as the +6 position [48]. Since RF decoding of stop codons involves protein–RNA interactions, rather than RNA–RNA (tRNA–mRNA) interaction, and requires no further reading frame maintenance, there is no reason to restrict the interaction to triplet stop codon. Instead RFs can be thought to decode termination signals consisting of the stop codon and its surounding context. Evidence for such a proposal comes from striking correlation between the relative efficiency of the termination signals seen with *in vivo* reporter assays, used by Tate and co-workers for example, and from analysis of the termination signals present in the genome (Fig. 9.3). This is especially true in the case of UGAN and UAAN signals utilized by *highly expressed genes*, where in the +4 position (N), pyrimidine is preferred to purine, with uridine usually the strongest. The presence of tandem stop codons, UGAUGA for example, was thought to be advantageous, since it appeared to be over-represented in the genomes of bacteria, however, from analysis using the reporter systems, it seems that tandem stop signals confer no additional advantage other than having the +4 position uridine [49]. Further support for a direct interaction between RFs and the extended termination signal came when crosslinks from the +4 position [50] and weaker crosslinks from the +5 and +6 positions (but not +7 to +10 positions) [48] with the RF were demonstrated. Attempts to identify the region within the RF to which the crosslink was attached were largely unsuccessful, although there was some hint that domain II was involved [51].

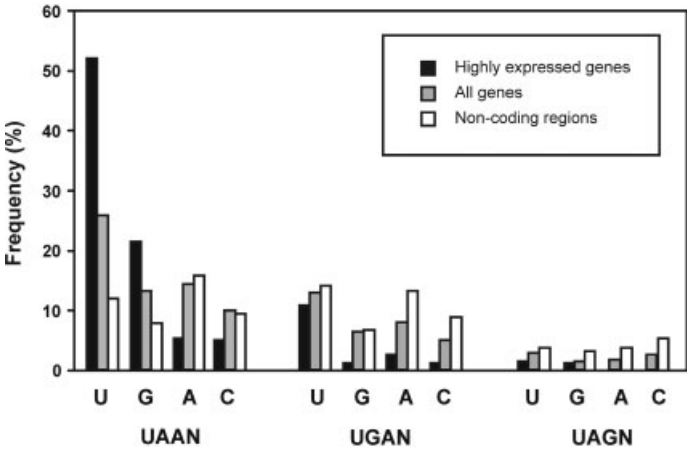

Figure 9.3 The frequency (in percentage) of occurrence for base (N is either U, G, A or C) following each of the three stop codons UAA, UGA and UAG in the higly expressed genes (black bars), all genes (grey bars) and within non-coding regions (white bars) of the *Escherichia coli* genome.

9.3.5
Codon Recognition Domain of Bacterial RFs: the "Tripeptide Motif"

To identify the domain within decoding RFs involved in codon recognition, genetic screening of mutant RF2s that could complement both conditional-lethal RF1 and RF2 mutants was undertaken. One of the factors identified had a mutation E167K and could terminate translation at all three stops, much similar to an eRF1, although the specificity of the interaction was questionable, since termination at some sense codons was also observed [52]. Following this, Nakamura and co-workers performed an elegant series of experiments identifying tripeptide motifs, PAT and SPF, within domain II of the decoding RFs, RF1 and RF2 respectively, which discriminate specifically the three different stop codons ([53], reviewed in [54, 55]). The first and third positions of the tripeptide motif discriminate the second and third positions of the stop codon (Fig. 9.4, [55]), such that the motif SXT is omnipotent (including UGG) and PXF is UAA restricted [53]. From a biochemical point of view, the location of the tripeptide motif within domain II was consistent with many other mutations located within this region that were identified using various readthrough assays. In this respect, it is noteworthy that one of the locations of one of the mitochondrial RF1 mutants corresponded with the first position of the tripeptide motif. Furthermore, the so-called "charge-flip" changes at multiple Glu (E) residues located in close proximity to the tripeptide motif also interfered with codon recognition [56].

However, from a structural point of view, it was surprising that the tripeptide motif was located within domain II and not domain I. Because domain I of RF2

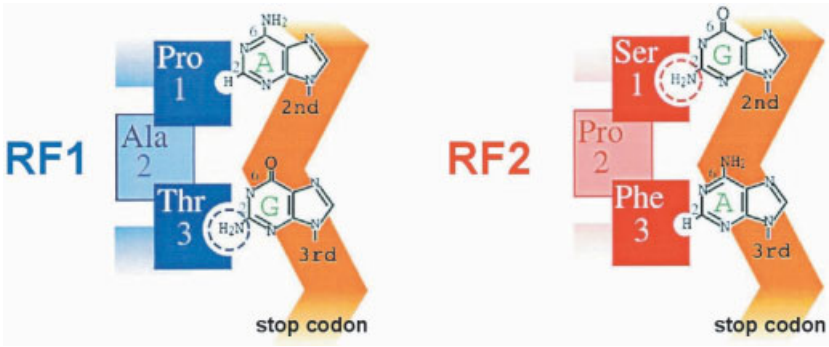

Figure 9.4 A model showing how the tripeptide motif PAT in RF1 and SPF motif in RF2 decode the second and third positions of the stop codon. Reprinted with permission from Ref. [55].

resembled domain I of RRF, which in turn based on a proposed tRNA mimicry was equivalent to the anticodon stem of a tRNA (see Fig. 9.1), the thinking at the time was that the region associated with stop-codon recognition should be located within the loop located between α3 and α4. Although there was little support biochemically for this presumption, a model was presented where the RF2 structure was docked into the programed 70S crystal structure with the tip of domain I in the decoding site. This model necessitated that the tripeptide motif function indirectly to decode the stop codon; however it made contact with h44 and therefore was supposed to monitor stop-codon recognition indirectly through conformational changes induced within h44 [22]. In support of this model was that the so-called GGQ motif (see following sections), located in domain III was located in close proximity to the PTC in accord with biochemical data and there were no spatial clashes. In contrast, when the tripeptide motif was placed in the decoding site, there were numerous clashes between other regions of the RF2 and components of the ribosome. Furthermore, in this orientation, the GGQ motif was located far from the PTC.

9.3.6
Peptidyl-tRNA hydrolase function of bacterial RFs: domain III and the GGQ motif

The ultimate role of the class I RFs is to mediate the release of the polypeptide from the peptidyl-tRNA bound at the P site of the ribosome. During elongation, the peptidyl moeity of the P-site tRNA is transferred to the α-amino group of the aminoacyl-tRNA bound at the A site (Fig. 9.5A; see Chap. 8.3 for more details). By analogy with this situation, the termination reaction was proposed to involve the transfer of the peptidyl moeity to a water molecule, as illustrated in Fig. 9.5(B) [57–59]. The question is to what extent do the class I RFs play a role in this reaction? Are the decoding factors simply messengers passing on a signal from the decoding site to

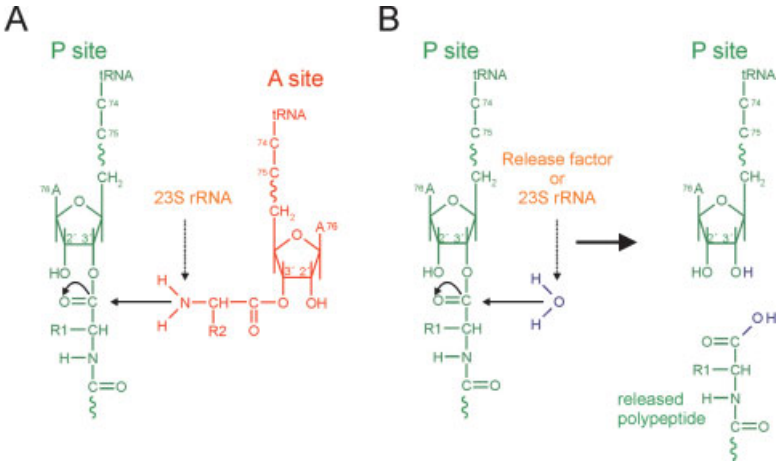

Figure 9.5 Scheme for (A) peptidyl-transferase and (B) termination reactions on the ribosome. In (A) there is a nucleophillic attack by the α-amino group of the A site tRNA on the carbonyl group of P site peptidyl-tRNA (indicated by solid arrow). There is controversy over the extent that components of the ribosome, such as bases of the 23S rRNA, contribute to this reaction (see Chapter 8). In (B) the water molecule replaces the A site ligand to make a nucleophillic attack on P site ligand. The question has been raised as to whether this water molecule is co-ordinated by the termination release factor or some component of the 23S rRNA (indicated by dashed arrow).

the PTF center that mediates peptide release or does a region of the decoding factor play an active role in the release of the peptide by, for example, co-ordinating the water molecule needed for the reaction?

One of the first regions implicated in peptide release came from proteolytic studies with the decoding factors. A protease-sensitive site was identified in a similar position in RF1 and RF2 [15], which maps to what we now know is the GGQ-containing loop within domain III (Fig. 9.2). Limited proteolytic cleavage of RF2 with chymotrypsin produced two relatively stable fragments of approximately 24 and 15 kDa. N-terminal sequencing demonstrated that the cleavage occurred between tyrosine and arginine residues at positions 244 and 245, respectively. Interestingly, the chymotryptic fragments of RF2 remained associated, when isolated using anion-exchange and gel-permeation chromatography under non-denaturing conditions and while the nicked RF2 factor retained the ability to bind to the ribosome and discriminate stop codons, it was completely inactive for peptide release [15]. This suggested that this region within domain III was essential for release activity of the factors, but not binding or codon recognition.

At around the same time, a chimera of RF1 was constructed where a small section of domain III (including the proteolytic site) was replaced with the homologous region from RF2 [14]. As mentioned, this chimera was inactive for release activity

and unlike the exogenous expression of RF1 in bacteria, overexpression of the chimera was very toxic to the cell. Indeed, the observed phenotype was remarkably reminiscent to that observed when *E. coli* RF2 is exogenously overexpressed. In addition, the *in vitro* characteristics were also similar, namely that the purified protein was active for codon recognition and ribosome binding but severely impaired for release activity [60]. What was intriguing was that there was a correlation between the level of overexpression and the loss of release activity, such that the higher the expression, the lower the specific activity of the RF2 protein [61, 62]. However, this phenotype seemed to be specific for *E. coli* since overexpression of *S. typhimurium* was not toxic to the cell and this recombinant RF2 protein was fully active *in vitro* (see [63] and references therein). Since there were only 16 differences between *S. typhimurium* and *E. coli* RF2, fragment swap and site-directed mutagenesis experiments were employed to identify which residues were responsible for the characteristic phenotypes. One of the 10 RF2 residues in the RF1 chimera, Thr246, was identified, which is normally serine in *E. coli* RF1 and alanine in *S. typhimurium* RF2 at the equivalent position. Replacing Thr246 in *E. coli* RF2 or in the RF1 chimera with Ser or Ala restored peptide-release activity to the purified factor and alleviated the growth toxicity associated with factor expression [63, 64]. In fact, the class I decoding RFs of most bacteria have Ser or Ala at position 246. The Thr246 seemed to be specific for the K12 strains of *E. coli*, since *E. coli* strains such as MRE600 or BL21 were shown to have Ala246 [65]. Ehrenberg and co-workers [65] went on to show using an *in vitro* system that although the termination efficiency of overexpressed RF2 with Ala246 was considerably improved compared with the RF2 with Thr246, it was still significantly less efficient than the endogenous RF2 factor, which also has Ala246. Characterization of the two proteins revealed that the overexpressed RF2 factor lacked an N^5-methylation on glutamine at position 252 of a universally conserved GGQ motif (see below). Indeed, the presence of the methylation stimulated the termination activity of the RF2 factor regardless of the amino acid at position 246, which suggested that the effects are cumulative [65]. It should be noted that RF1 also undergoes methylation although the effect of the modification of the termination efficiency by RF1 does not seem to be as significant as that seen for RF2. Interestingly, the methylase that modifies RF1 and RF2 was identified independently by two different groups [66, 67] and renamed *prmC* [66] from *hemK*, after its mis-assignment as an enzyme involved in the heme biosynthetic pathway (reviewed in [68]). In *E. coli*, the *prmC* gene is located directly downstream of *prfB*, the gene encoding RF2, such that the AUG of the start codon of the former overlaps the UGA stop codon of the latter. Knock-out of *prmC* gene is not lethal but the growth rate is severely reduced, probably due to defects in translational termination. Both RF1 and RF2 isolated from the *ΔprmC* strain lack the methylation at Gln252 as expected. Suppressor strains that partially overcome the growth defect were characterized as having Thr246Ala mutations in the RF2 gene. One of the questions remaining is how does methylation at Gln252 affect the peptide release activity of the decoding RFs?

If the decoding RFs really directly mediate release of the polypeptide, then some part of the factor needs to interact with the PTF center of the ribosome. Since this region consists entirely of highly conserved rRNA nucleotides made up from domain V of the 23S rRNA, a corresponding region of high conservation between all RFs should exist. Despite the lack of sequence homology between bacterial and eukaryotic decoding RFs, a universally conserved sequence of Gly-Gly-Gln (usually referred to as the "GGQ motif") in domain III was identified [69]. The Gln residue of the GGQ motif is the same residue identified by Ehrenberg and co-workers as being methylated in *E. coli*. The GGQ motif has been demonstrated to be essential for peptide release activity of the factor: mutations in either of the glycine residues abolished activity in bacterial RFs *in vivo* and *in vitro* [70]. Indeed, one of these mutants, RF2-GAQ, was shown to be 4–5 orders of magnitude less efficient in the termination reaction than wild-type RF2-GGQ, despite the fact that the binding of both factors to the ribosome were similar [71]. While mutations of Gln (Q) of the GGQ motif to Gly or Ala yielded RFs that retained some *in vitro* termination activity (~20%), these factors could not however rescue the appropriate thermosensitive RF mutants at the non-permissive temperature *in vivo* [70]. Indeed the RF2-GGA mutants were much more active than the RF2-GAQ mutants, by approximately one order of magnitude [71]. Within the RF2 crystal structure the electron density for the loop containing the GGQ motif was poorly resolved suggesting that this region is highly flexible. In eukaryotes, the GGQ motif was also shown to be important for peptide release and was similarly located within a loop at the end in domain I (NTD) of eRF1 (see Sect. 9.3.1 for more details).

9.3.7
Large Conformational Changes Associated with RF2 Binding to the Ribosome

Now there were two sets of very compelling biochemical data, one associating the codon-recognition function of RF2 with the tripeptide motif in domain II and the other associating the peptide-release function of RF2 with the GGQ motif, both of which were located only 45 amino acids away in sequence from one another. Although theoretically this would be sufficient to span from the decoding site on the 30S subunit to the PTF center on the 50S which is a distance of ~70 Å, in the RF2 crystal structure, these motifs are located only 23 Å apart (as seen in Fig. 9.2). To resolve this paradox, the decoding RFs needed to be analyzed in terms of their interaction with the ribosome.

A successful technique for mapping the position of translation factors on the ribosome has been hydroxyl-radical probing. The technique involves the tethering of Fe(II) to sulfydryl groups of cysteine residues on the surface of a protein via a chemical linker and has been applied to define the binding sites of tRNA [72], IF3 [73], EF-G [74], RRF [75] as well as RF1 [76] and RF2 [77]. The results of these latter studies for the RFs demonstrated that tethers placed in close proximity to the tripeptide motif (Fig. 9.6A), cleaved only 16S rRNA in the vicinity of the decoding

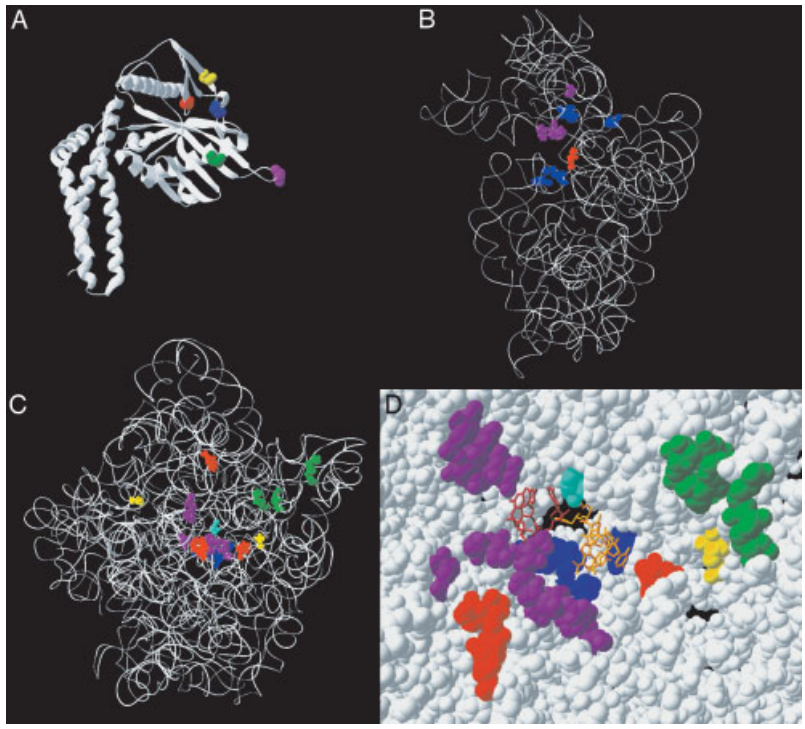

Figure 9.6 The ribosomal binding site of *E. coli* RF2. (A) Identification of Fe(II)-tethering sites on the structure of *E. coli* RF2. Space-fill representation of tethered residues are as follows: Val243 (yellow), Thr246 (purple), Cys274 (red), Leu201 (green) and Ser209 (magenta). (B) Mapping the footprint sites of 201 and 209 of RF2 on the 16S rRNA of the *T. thermophilus* 30S structure: cleavages from position 209 alone are green, whereas those from 201 and 209 are yellow. For reference, the conserved A1492 and A1493 are shown in red. (C) Mapping the footprint sites of 243, 246 and 274 of RF2 on the 23S rRNA of the *H. marismortui:* cleavages from positions 243, 246 and 274 are colored yellow, blue and red, respectively. Where there are cleavages from two positions, the bases are colored green (243 and 246) or purple (246 and 274). (D) The region of the 50S subunit containing the PTF center has been amplified and presented as a space-fill representation with the cleaved residues colored as in (C). For reference, peptide-bond formation products in the A and P sites are colored brown and orange, respectively, and A2451 is colored cyan. Mapping of hydroxyl radical probing data from various tethers placed on RF2 (A) to the 16S rRNA of the 30S (B) and the 23S rRNA of the 50S (C). (D) Close-up of the PTC cleavages (data taken from Scarlett et al. [77]).

site, namely the 790 loop (h24), the 530 loop (h18) and h33 (Fig. 9.6B). These cleavages were similar to those generated from a tether placed at the tip of domain IV of EF-G [74], i.e., the domain that mimics the anticodon stem-loop region of a tRNA and has been shown by cryo-EM to approach the decoding region of the A site (see Chap. 8.4.1). What was surprising was that unlike the probing with EF-G no cleavage in the decoding region (1400 region) was observed. This may reflect the difference in action of the factors, such that RFs physically contact and protect this region during stop signal decoding, whereas EF-G exhibits sequence-independent ribosome binding. Likewise, tethers flanking the $_{250}$GGQ$_{252}$ motif (Fig. 9.6A) cleaved only the 23S rRNA and predominantly within of the PTF center and the so-called GTPase-associated center (GAC; Figs. 9.6C and D). For example, tethers located at either position 246 and/or 273 cleaved position; (i) A2602, a universally conserved bulged nucleotide that becomes protected from chemical probing by the aminoacyl moiety of an A-site-bound tRNA [78]. Cleavage of A2602 was also obtained from a tether placed at the 5′-ACC-end of a deacylated tRNA, (ii) nucleotides 2253–2254 within the P loop of domain V which form Watson–Crick base-pairs with the C74 and C75 positions of a P-site-bound tRNA, and (iii) A2451, which has been proposed to be intimately involved in facilitating peptide-bond formation (see Chap. 8.3.2).

Again, the picture emerged that the tripeptide and GGQ motifs were distinctly associated with the 30S and 50S subunit, respectively, and suggested that RF2 must undergo significant conformational rearrangement upon binding to the ribosome to fulfill this criteria. However, since "seeing is believing", not until the cryo-electron microscopic reconstruction of the RF2-ribosome termination complex was accomplished was the matter finally put beyond reasonable doubt [79, 80]. What these two reconstructions revealed was that the tripeptide motif was in fact located in the decoding site and that domain III came away from domains II/IV such that the GGQ motif located within the loop region now reached into the PTF center (Fig. 9.7). The RF was oriented on the ribosome such that the α-helical bundle of domain I was contacting the GAC, consisting of the base of the stalk region, L11-binding region (H43 and H44 of the 23S rRNA), sarcin-ricin loop (H93) and to some extent L7/L12.

This finding fits nicely with the wealth of biochemical data implicating the GAC as an interaction site for RF1 and RF2. Preliminary footprinting studies performed in the early 1990s using RF2 identified a change in the protection pattern within the 2660 region (H93) of the 23S rRNA (C.M. Brown and W.P. Tate, unpublished results). Cleavages were also identified within this region from tethers located at position 229 in RF1 [76] and 650 of EF-G [74]. In addition, position 229 of RF1 cleaves within the L11-binding region, both in H43 and H44 [76], as do tethers from positions 650 and 655 in domain V of EF-G, which cleaved within the 1095 and 1067 stem-loops, respectively [74]. This region is the target for a family of thiazole antibiotics such as thiostrepton and micrococcin, both of which have been shown to inhibit elongation (see Chap. 12.3.3.1) and termination factor activity [81]. When 70S ribosomes were treated with anti-L11 (and anti-L16), RF-dependent peptidyl-tRNA

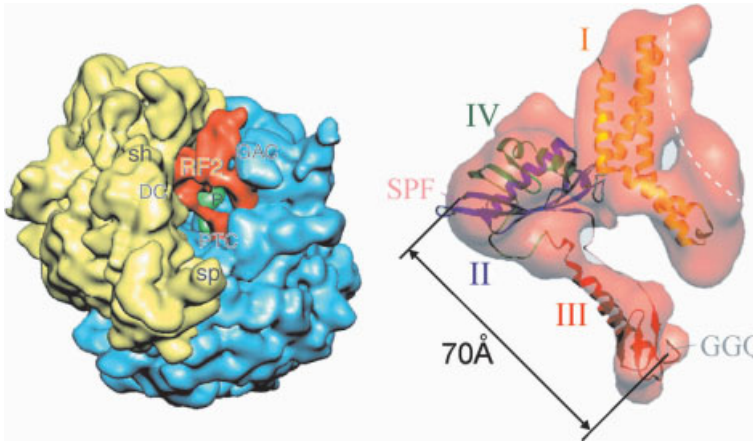

Figure 9.7 Cryo-EM reconstruction of the *E. coli* RF2 termination complex, revealing the significant conformational changes induced in RF2 upon ribosome binding. The approximate distance between the tripeptide motif (SPF in RF2) and the GGQ motif located in the loop of domain III is indicated. Reprinted with permission from Rawat et al. [80].

hydrolysis was partially inhibited whereas binding was not [82–86]. Specifically, the N-terminal region of L11 seems critical for modulating RF function, as antibodies to nucleotides 1–64 as opposed to 65–102 strongly inhibited *in vitro* termination [83].

Although it seems probable that RF1 will also bind analogously to RF2 on the ribosome, it is noteworthy that RF1 is slightly shorter than RF2 and therefore is probably missing the first α-helix of domain I. Consistently, many of the differential effects between RF1 and RF2 have been related to the interaction between the factors with the GAC region. For example, the absence of L11 exhibits a differential effect on RFs, such that RF1 activity is reduced and RF2 activity is enhanced several fold [87, 83]. The presence of L11 is required for RF1 function, specifically the N-terminal domain (NTD) [88], but somehow suppresses RF2 function [85]. Modification of Tyr7 (and negligibly at Tyr61) in the NTD of L11 and reincorporation into 70S ribosomes significantly reduced RF1 but not RF2 termination activity [84]. In contrast, mutations in the 23s rRNA of the L11-binding region seem to influence RF2-mediated termination without affecting RF1 [89]. For example, mutation at G1093A results in UGA-specific suppression [90] and was independent of the disruption of the base pair with A1098 [91]. *In vitro,* this mutation was shown to reduce the association constants of RF1, but more markedly of RF2, with the ribosome [92], suggesting that this region provides more of a binding site for RF2 than RF1. Interestingly, deletion of two nucleotides within domain V of 23S rRNA (G2046 and C2049) could compensate for the conditional lethality caused by the G1093A mutation. These deletions decreased the UGA suppression *in vivo* associated with the G1093A phenotype (reviewed in [93]). Mutations in nucleotides neighboring G1093 also exhibit

UGA-specific suppression, as do substitutions or deletions at A1067 [93]. Taken together, these data suggest that RF1 and RF2 have overlapping binding sites but interact with the ribosomal components in a distinct manner.

9.3.8 The Trigger for RF-mediated Release of the Nascent Chain and the Outcome

If the arrival of the stop codon in the A site is the discriminating factor for decoding RFs, it is logical to presume that the cognate stop codon is the initial signal for the RF to accommodate into the A site by undergoing a conformational transformation. An analogous situation might be the selection process of cognate tRNAs (see Chap. 8.2): here codon–anticodon interaction at the A site presumably transfers a signal to the GTPase center inducing EF-Tu-dependent GTP hydrolysis, which in turn leads to the dissociation of EF-Tu from the ribosome and the release of the aminoacyl-tRNA (aa-tRNA), enabling it to accommodate into the A site on the 50S subunit, i.e., the accommodation of the CCA-end into the PTF center. Recent cryo-EM reconstructions of the EF-Tu•GTP•aa-tRNA stalled with the antibiotic kirromycin reveal that the GAC region moves 7 Å to contact the tRNA [94]. Since the bacterial decoding factors are not delivered to the ribosome and have no GTPase activity, the mechanism to ensure that termination does not occur at non-cognate or sense codons might be a conformational change rather than the factor-mediated GTP hydrolysis. It has been shown that mutations at position 246 in the GGQ-containing loop of domain III could influence both codon-dependent binding as well as peptidyl-tRNA hydrolysis activity of the factor. This led to the suggestion that recognition of the correct stop codon by the RF may induce a conformational change in the factor, a so-called switch [64], so as to bring the GGQ motif into the PTF center. Therefore, the signal, which originates from correct detection of the stop codon in the A site, is relayed through the RF to the tip of domain III. In fact, domain III of RF2 shows structural homology with ribosomal protein S5, the structure of which has been solved for the isolated protein (pdb1pkp, [95]) and on the 30S subunit (1fjf, [96]). The loop of S5, equivalent to the GGQ loop in RF2, adopts a straight β-hairpin upon assembly into the ribosome, i.e., binding to its rRNA segment. By analogy, the tip of the β-hairpin that contains the GGQ motif in RF2 may also insert into the PTF center to modulate release of the polypeptide chain. The question is whether some residue(s) within the decoding RF modulates this reaction, perhaps by co-ordinating a water molecule or whether the RFs mediate this reaction indirectly via residues of the rRNA in the PTF center. It had been proposed that the glutamine residue (Q of the GGQ motif) was involved in co-ordinating the water molecule [69, 97]. However, mutation of GGQ to GGA in both bacterial RF1 and RF2 [70] and in eRF1 [98] produced factors that still retained partial peptidyl-tRNA hydrolysis activity, disproving this notion – indeed, bacterial factors containing GAQ were more severely affected than the GGA mutants [70, 71]. Similarly, mutations of either glycine of the GGQ motif in human eRF1 abolished release activity without affecting ribosome-binding activity of the factor [69]. It should be pointed out that the context of the GGQ motif

in domain III is GxGGx, which is in fact not unique to bacterial RFs, being present also in the loop or linker (α4 and β1) located between domains I and II–IV. Nor is it in fact unique to decoding factors generally, since the GxGGx motif is a well-known turn motif found in, for example, the HIV proteases. This suggests that the GGQ motif may in fact be more important for providing flexibility, i.e., the flexibility between domains I and II–IV could be important for interaction with the GAC and stabilizing the open position of the factor, whereas in domain III, the GxGGx motif may be important for positioning of other residues in this region that directly or indirectly participate in the release reaction. If this is the case and the GGQ motif is not functionally important for the release reaction *per se*, then the focus should be turned to the multitude of highly conserved residues that are found in the region following the GGQ motif, for example, the asparagine (N) residue, located three amino acids C-terminal to the GGQ motif, which is conserved in all bacterial RF sequences known to date [99].

Recently, reconstituted 50S subunits from *T. thermophilus* with 23S rRNA containing mutations at position A2602 within the PTF center were shown to exhibit differential effects with regard to catalysing the peptidyl-transferase reaction in comparison with the termination reaction [100]. In particular, mutations at position A2602, or deletion of this residue altogether, did not significantly affect the former reaction but severely reduced RF1-mediated peptide release, completely abolishing it in some cases. The authors demonstrated that this effect was not due to a decrease in the binding of the decoding factors since (i) increasing the excess of the decoding factors did not compensate for the loss of activity and (ii) the A2602 mutants were inactive under conditions which induce an RF-independent peptidyl-tRNA hydrolysis assay (the presence of 30% acetone [101, 57]) but were active for peptide-bond formation under the same conditions. In the RF-dependent peptidyl-tRNA hydrolysis assay, the authors could demonstrate that the addition of deacylated tRNA cognate to the A site significantly stimulated the reaction. These results suggest that different features of the PTF center are responsible for the PTF reaction during elongation and the peptidyl-tRNA hydrolysis reaction during termination. Furthermore, this was exemplified by the differential effects that a subset of PTF inhibitors had on the two reactions, in particular, the lincosamides, lincomycin and clindamycin completely abolished the PTF reaction at 100 μM, whereas the termination remained unaffected [100]. Thus, this suggests that the release of the peptide by the decoding factors would be indirectly governed through A2602. Since A2602 has been shown to be highly flexible, being in a different conformation in almost every 50S ribosome structure to date, the binding of the RFs may alter the positioning of this residue such that reactive groups can activate a water molecule, thus enabling nucleophilic attack on the carbonyl carbon atom of the peptidyl-tRNA ester bond (Fig. 9.8) [100].

9.4 Eukaryotic Class I Termination Factors

Although a single eRF1 was detected as early as 1971 [102], partially purified [103] and shown to recognize all three stop codons [104], only more recently was the gene

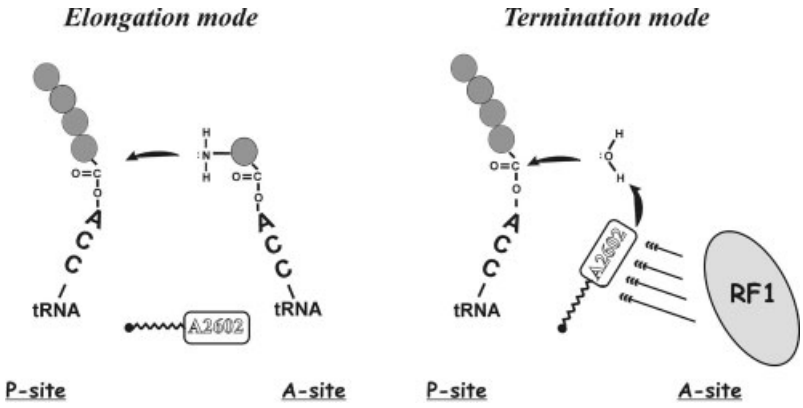

Figure 9.8 The putative conformational switch at A2602 as a trigger for changing the mode of activity of the ribosomal peptidyl transferase center. Orientation of A2602 during translation elongation allows for proper positioning of peptidyl- and aminoacyl-tRNAs in the peptidyl transferase center that makes peptidyl transfer and a new peptide-bond formation possible. Binding of the class 1 release factor (RF1 in the figure) in response to the presence of a stop codon in the decoding site reorients A2602. This places it in a position where its reactive groups can potentially activate a water molecule, facilitating its nucleophilic attack on the carbonyl carbon atom of the peptidyl-tRNA ester bond, thereby accelerating the rate of peptidyl-tRNA hydrolysis. Reprinted with permission from Polacek et al. [100].

correctly identified [105]. Standard methods of sequence comparison showed no significant similarity between the bacterial decoding RFs and the eRF1 family [105]. Although conservation of some sequence elements suggesting a common evolutionary origin has been claimed [106], this study did not even identify the universally conserved GGQ motif associated with peptidyl-tRNA hydrolysis in both prokaryotic and eukaryotic decoding factors ([69]; see Sect. 9.2.6). Furthermore, there is no sign of evolutionary conservation when comparing the crystal structure for *E. coli* RF2 [22] (Fig. 9.2) and human eRF1 (Fig. 9.9; [97]; comment by Kisselev [107]). In contrast, RFs from archeal species (aRF1) show high amino acid sequence homology to eRF1s and have several sequence motifs in common. Indeed, aRF1 from *Methanococcus jannaschii* was shown to be active with mammalian ribosomes, terminating translation at all three stop codons [108], strengthening predictions that aRF1s and eRF1s are descendent from a common evolutionary ancestor [69].

The crystal structure of human eRF1 presents a three-domain molecule with an asymmetric Y-shaped formation for which each of the three domains can be tentatively assigned a function (Fig. 9.9A; [97]): The stem, domain 1 (N-terminal domain), and one arm, domain 2, are proposed to represent the codon recognition and peptidyl-tRNA hydrolysis domains, respectively, whereas the other arm, domain 3 (C-terminal domain), is the site of interaction with eRF3 (reviewed in [1];

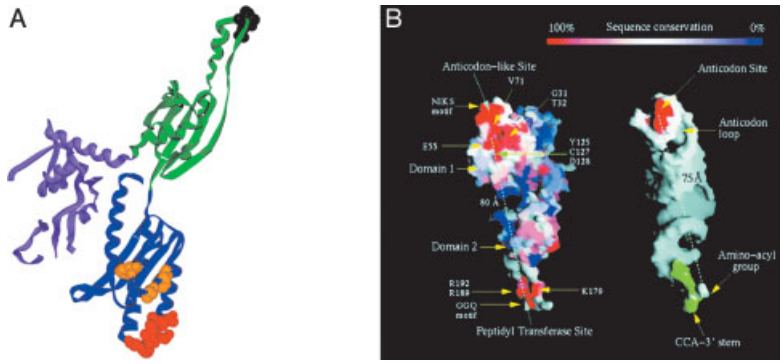

Figure 9.9 The crystal structure of human eRF1 reveals a three-domain structure. (A) Ribbons view of human eRF1 [97] with domain I (blue) with TASNIKS motif (red) and the YxCxxxF (yellow), domain II (green) with GGQ motif in dark green and domain III in purple. (B) Surface representation with sequence conservation: reprinted with permission from Song et al. [97].

see Sect. 9.4.2). The proposed mimicry of a tRNA molecule by eRF1 is certainly not convincing, particular considering the distance between the regions proposed to mimic the anticodon and CCA end, domains I and II, respectively, are approximately ~100 Å apart [22]. This exceeds the 75 Å distance measured from the anticodon stem loop to the CCA end of a tRNA, suggesting that if domains I and II are really associated with the decoding site on the 30S subunit and the PTF center on the 50S subunit, then conformational change upon ribosome binding is necessary to account for this discrepancy. So what is the evidence that associates domains I and II with the aforementioned regions? First, by simply looking at the sequence conservation of human eRF1, it was immediately obvious that there are regions located in domains I and II that are highly conserved (Fig. 9.9B) and also positively charged. These are prime candidates for regions to interact with the decoding and PTF center, since these regions of the ribosome are also highly conserved and composed almost entirely of rRNA, which is negatively charged.

9.4.1 Stop-codon Recognition is Associated with Domain I of eRF1

Reassignment of a stop codon in bacteria can simply involve loss of the appropriate RF, as previously mentioned for the absence of RF2 in *Mycoplasma genitalium*. However, in eukaryotes, there is only one factor that decodes all the stop codons. Therefore, changes in codon reassignment, i.e., the reassignment of a stop codon as sense, should be reflected by changes in the sequences of the eRF1, since this factor should no longer recognize the reassigned stop codon as sense.

In this respect, the use of alternative nuclear genetic codes makes the ciliates perfect for this type of analysis, for example, some ciliate species, such as the *Euplotes*

have reassigned UGA as Cys (C), whereas other species, such as *Tetrahymena*, translate both UAA and UAG with Glu (E) ([109]; reviewed in [110]). Validating this assumption, it was recently demonstrated that, indeed, the ciliate eRF1 does not recognize the reassigned stop codons *in vitro* [111, 112]. A flurry of sequencing activity saw a rapid increase in the number of available ciliate eRF1 gene sequences [100, 113–116]. These types of analyses revealed that most of the convergent changes were indeed associated with domain I; however, the number of positions decreased significantly as the number of ciliate eRF1 gene sequences available increased, challenging the reliability of this method.

Consistent with the assignment of domain I as the codon-recognition domain, random mutagenesis of yeast eRF1 identified numerous locations scattered through domain I, which altered the stop-codon recognition specificity [117]. The mutations located to a groove formed by two helices (α2 and α3), which was proposed to form a binding pocket into which a triplet stop codon was modeled [117]. The involvement of domain I in codon recognition was convincingly demonstrated when hybrid eRF1, containing the domain I from *Tetrahymena* eRF1 (which recognizes only UGA) and domains II and III from *Saccharomyces cerevisiae* eRF1 (which recognizes all stop codons), terminated only at UGA stop codons [111]. Consistently, it was demonstrated recently that a combination of four substitutions in two different regions of domain I had a profound effect on the stop-codon specificity of human eRF1 *in vitro*, such that it only terminated efficiently at UGA stop codons, similar to ciliate eRF1s [118]. This result suggests that in fact two distinct regions within domain I are involved in codon recognition, and that the protein-anticodon mimicry concept [55] may, in contrast with the situation in bacteria where decoding occurs through a simple tripeptide motif, be far too simplistic to describe the situation in eukaryotes and bacteria.

The two prime candidates thought to be involved in stop-codon recognition in eRF1 are two loop regions located in domain I, one containing a heptapeptide sequence $_{58}$TASNIKS$_{64}$ (human eRF1 numbering) and the other a consensus sequence $_{125}$YxCxxxF$_{131}$ (reviewed in [1]). The close proximity of the TASNIKS sequence to the stop codon was confirmed when the Lys (K) residue was found to be crosslinked when synthetic mRNAs containing 4-thiouridine at the first position of the stop codon were used [119]. This suggests that like the situation in bacteria, the protein factor may be directly decoding the stop codon; however, the mechanism may differ significantly.

It is noteworthy that the conditional lethality associated with a mutation in domain I of the yeast eRF1 (P86A) is rescued by compensating mutations A1491G and U1949 located in helix 44 of the decoding region [120]. Interestingly, the mutation G1491 creates a base-pair with C1409 yielding yeast cells that are extremely sensitive to paromomycin. Furthermore, second-site mutations were identified in the switch region at U912C and G886A of the 18S rRNA [120]. Whether this region actually represents a universal switch has recently been brought into doubt by the creation of the equivalent switch mutants in yeast, which did not exhibit the predicted ram or restrictive phenotypes although they did support the involvement of this region in ribosomal fidelity [336]. In any case, the complexity of stop-codon

recognition seems to be a conserved feature between eukaryotes and eubacteria and will require dissection of the (e)RF:termination complex by cryo-EM and crystallization to understand the mechanism fully.

9.4.2
eRF1-mediated Polypeptide Release

As mentioned in Sect. 9.2.6, a universally conserved GGQ motif was identified in all decoding RFs [69]. In eRF1 and aRF1s, the GGQ motif is located in the extremity of domain II, forming a highly exposed minidomain ([97]; Fig. 9.9A). Mutations in the GGQ motif results in loss of peptidyl-tRNA hydrolysis activity, particularly in the first two Gly (G) positions [69, 97], whereas eRF1 with mutations at the Gln (Q) position still retain some activity *in vitro* [98, 121]. If the Lys (K) of the TASNIKS motif is located at the decoding site, then the GGQ motif is separated by 100 Å and therefore is too far apart to fit nicely into the PTF center. Of course, the binding of eRF1 to the ribosome may, in analogy with the bacterial RF2 situation, result in conformational changes in the eRF1 such that the distance between the K and the GGQ is reduced to the optimal 75 Å. This may not be as significant as that observed for RF2 and may simply involve the movement of domain II as proposed in Klaholz et al. [79]. Alternatively, if the YxCxxxF motif, rather than the TASNIKS motif, was directly in contact with the stop codon in the A site, then little or no conformational change in eRF1 would be required since the distance measured between the former motif and the GGQ is precisely 75 Å [1].

9.5
Dissociation of the Post-termination Complex

9.5.1
Eubacterial RF3 Dissociates the Class I Termination Factors

RF3 activity was identified over 30 years ago and the corresponding protein was termed "S", for its ability to stimulate the termination efficiency of RF1 and RF2 [122, 123]. RF3 is not essential for cell survival, since a gene knock-out of *prfC* is viable and the Mycoplasma species have dispensed with this gene. However, the importance of RF3 is illustrated by its necessity for translational fidelity, especially under stress conditions [124]. Utilizing an *in vitro* translation system, RF3 was shown to decrease the recycling time of the decoding RFs [125] by accelerating the dissociation of the decoding RFs from the ribosome [126]. RF3 was shown to have a particularly pronounced effect at strong stop signals [127], where the association rate of the decoding factor for the ribosome is much higher [125]. There is a cost associated with this increased recycling rate, namely a slight reduction in the fidelity of decoding [128].

RF3 contains a GTP-binding motif and thus belongs to the large family of G proteins. It has more sequence similarity with EF-G than to EF-Tu, supporting the contention that RF3 plays a dissociative rather than a delivery role for the decoding RFs.

In fact, the homology between RF3 and EF-G was proposed, based on a dot plot and threading analysis, to extend beyond domains I, II and the G domain of EF-G to include domain II and part of domain IV (Fig. 9.10; [129]). By analogy with EF-G, this would suggest that the N-terminal region of RF3 extends towards the 30S subunit and may exert its dissociative effect on the decoding factors through this extension, perhaps by physically levering the decoding factor from the ribosome.

A recent analysis of the role of guanine nucleotides during RF3 action supports a dissociative role for RF3 [130]. These results suggest that it is the RF3•GDP form that binds a post-termination ribosome complex, i.e., a ribosome that has released the nascent chain but still contains a stop codon and corresponding decoding RF at the A site. Nucleotide exchange occurs on the ribosome and is activated by the post-termination complex. The finding that RF3•GDPNP competes for a binding site with the decoding factors suggests that it is the RF3•GTP form that is responsible for dissociating the decoding factors from the ribosome. This implies that it is the nucleotide exchange (GDP for GTP) that dissociates the decoding factors from the ribosome and not hydrolysis of the GTP to GDP. Instead, hydrolysis of

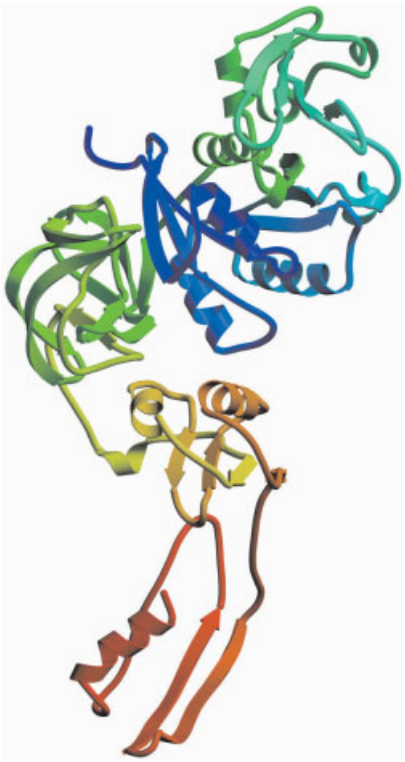

Figure 9.10 Threading model of *E. coli* RF3 based on sequence similarity with EF-G. Reprinted with permission from Wilson et al. [129].

GTP functions to dissociate RF3 since the RF3•GDP form has less affinity for the ribosome due to the absence of the decoding factors, i.e., RF3•GDP has high affinity for ribosome in the *absence* of the peptidyl moiety and the presence of the decoding factors [130]. This ensures that RF3 cannot bind and dissociate the decoding factors until they have completed their job, i.e., release of the nascent polypeptide.

9.5.2 Eukaryotic RF3: Dissociation versus Delivery of eRF1

In contrast with bacterial RF3, eRF3 is an essential gene [131, 132] and has been shown to interact physically and functionally with eRF1 [133, 134]. This interaction involves the C-terminal domain 3 of eRF1, although the exact residues involved appear to differ for different organisms and methods used to determine their interaction [135–137]. Progressive deletion of the C-terminal 6–19 amino acids in *S. cerevisiae* eRF1 [135] and 17 amino acids of *S. pombe* [136] results in a corresponding loss of eRF3 binding. C-terminal deletions disrupt a conserved motif, which with the most recent eRF1 sequences added to the alignment has become GFGGxGG/AxxR and remove a high number of acidic amino acids, mainly glutamic and aspartic acids, which, when mutated to alanine, significantly reduce eRF3 binding [137]. Within the crystal structure for eRF1, the last 15 amino acids, constituting the acidic region, are disordered suggesting some flexibility [97]. These C-terminal residues appear to be dispensable in *Homo sapiens* eRF1 as deletion of the last 22 amino acids (which includes all the acidic residues and part of the conserved motif) did not significantly reduce eRF3-binding capability [138, 137]. Instead, further deletions were necessary to loose eRF3 binding [137]. In any case, the core eRF3-binding region identified for *H. sapiens* eRF1 by these deletion studies (residues 281–415) correlates well with domain 3 from the crystal structure [97].

Another distinction between eubacterial and eukaryotic RF3s is that eRF3 has higher amino acid sequence homology to elongation factor EF-1a (the EF-Tu equivalent present in eubacteria) than to EF2 (the EF-G equivalent), implying that the mode of action of these factors may be different. Heterodimer formation between eRF1 and eRF3 is also suggestive of a delivery mechanism for eRF3, analogous to the delivery of a tRNA to the ribosome by EF-1a. It is probable that this interaction is not strictly necessary as deletion of residues within domain 3 of eRF1 results in the loss of eRF3 interaction, while retaining termination activity and maintaining the viability of the yeast cell [138, 137]. However, it should be noted that these cells exhibit a nonsense suppression phenotype, suggesting a reduction in the efficiency of termination [135, 136]. Although consistent with results where overexpression of both eRF1 and eRF3 was necessary for efficient termination [139], it seems that overexpression of eRF1 alone can also rescue a nonsense suppressor phenotype *in vitro* [140] and *in vivo* [141]. Perhaps the most compelling evidence for the dispensability of eRF3 comes from analyses of the situation in archea. A number of genomes from the archea kingdom have been completely sequenced and all aRF1 genes identified have a shorter C-terminal region of domain 3 lacking the acidic

residues and have a less conserved motif (xFxGxxG/AxLRY/F). Correspondingly, no equivalent gene to eRF3 or bacterial RF3 has been identified in these genomes [142].

Thus, the importance of eRF3 is unclear. Perhaps it derives from another role of eRF3. It has been well documented that the first 114 amino acids of eRF3 are not required for termination activity [143], instead they are implicated with a prion-like form [PSI+] of inheritance (reviewed in [144]). The nonsense suppression phenotype of [PSI+] cells results from the sequestering of eRF1 into large oligomers of eRF3. Under stress conditions, modulation of the cellular levels of solubilized eRFs by chaperones was demonstrated to confer a selective advantage to the yeast cells [145]. Intriguingly, eRF3 has also been shown to play a role in mRNA stability (see Chap. 5.3.2).

9.6 Ribosome Recycling

9.6.1 RRF Mediates Ribosome Recycling in Eubacteria

Following release of the nascent polypeptide and dissociation of the decoding factors by RF3, the cell must recycle the mRNA, deacylated tRNA at the P site and dissociate the 70S ribosome into the constituent subunits, in preparation for the next round of translation. This process is mediated by three factors, a ribosome recycling factor (RRF) that was identified over 30 years ago [146, 147], EF-G and IF3. RRF is an essential gene, the dependence on RRF for cell growth is exemplified again by the Mycoplasma species, which have dispensed with RF3 but retained RRF [2]. RRF and RF3 are both necessary for fast ribosome recycling times. Although their effects are additive, the larger contribution comes from RRF [148]. The exact role of RRF in ribosome recycling is unclear. A model proposed by Ehrenberg and co-workers [149] suggests that RRF, in concert with EF-G, dissociates the 70S subunit but does not release the mRNA or the deacylated tRNA from the P site. Instead, dissociation of the tRNA and mRNA from the 30S subunit is proposed to be a role undertaken by IF3. This model proposes that EF-G has another role to that performed during elongation, namely a dissociative rather than a translocative role, and together with RRF generates a high-energy state necessary for subunit dissociation.

An alternative hypothesis from Kaji and co-workers derives from the remarkable similarity between the crystal structure of RRF and that of a tRNA (see Fig. 9.1C; [28]). In this model, RRF would bind to the ribosomal A site, and EF-G, analogous to its function during elongation, would translocate RRF and the deacylated P site tRNA, from the A and P sites, to the P and E sites respectively. Furthermore, Kaji and co-workers advocate that the role of RRF and EF-G is the removal of the deacylated tRNAs and mRNA from the ribosome but not the dissociation of the 70S ribosome into the component subunits. This latter step is proposed to be mediated by IF3, which is well-known to fulfill this role (see Chap. 7.1). Inhibitors of translocation, such as thiostrepton, aminoglycosides and viomycin, also inhibit ribosome

recycling suggesting that the translocative role of EF-G is also important for the post-termination step [150]. Recently, hydroxyl-radical-probing data [75] have suggested that the mimicry by RRF of a tRNA is in fact misleading, since RRF binds to the ribosome with domain I (the region that was proposed to mimic the anticodon stem loop of a tRNA) extending into the PTF center on the 50S subunit; in fact, RRF makes little contact with the 30S subunit. Since there is some overlap in the position of domain II and the binding site of EF-G, this suggests that EF-G binding may propel RRF through the ribosome to clear out the tRNAs and mRNAs.

RRF is not present in the cytoplasm of eukaryotes (or archaea); the only forms present in eukaryotes are either mitochondrial or plastid in the case of plants; therefore, it will be interesting to see how ribosome recycling is mediated in this case.

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