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Ribosome Recycling
AN ESSENTIAL PROCESS OF PROTEIN SYNTHESIS

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A preponderance of textbooks outlines cellular protein synthesis (translation) in three basic steps: initiation, elongation, and termination. However, researchers in the field of translation accept that a vital fourth step exists; this fourth step is called ribosome recycling. Ribosome recycling occurs after the nascent polypeptide has been released during the termination step. Despite the release of the polypeptide, ribosomes remain bound to the mRNA and tRNA. It is only during the fourth step of translation that ribosomes are ultimately released from the mRNA, split into subunits, and are free to bind new mRNA, thus the term "ribosome recycling." This step is essential to the viability of cells. In bacteria, it is catalyzed by two proteins, elongation factor G and ribosome recycling factor, a near perfect structural mimic of tRNA. Eukaryotic organelles such as mitochondria and chloroplasts possess ribosome recycling factor and elongation factor G homologues, but the nature of ribosome recycling in eukaryotic cytoplasm is still under investigation. In this review, the discovery of ribosome recycling and the basic mechanisms involved are discussed so that textbook writers and teachers can include this vital step, which is just as important as the three conventional steps, in sections dealing with protein synthesis.

Keywords: Protein synthesis; ribosomes; ribosome recycling; RRF; translation.

DISCOVERY

Once the double helix structure of DNA and the implications of that structure became apparent, a wide range of research in the 1960s was set forth to investigate how DNA encoded proteins. One of the milestones of this research was the breaking of the genetic code [1]. Protein synthesis, with few exceptions, is initiated with methionine-encoding AUG and terminated by one of three stop codons, UAA, UAG, or UGA. The basic mechanisms of translational initiation, elongation, and termination were worked out early on, although these models are still under constant modification by current research. These three basic steps of translational initiation, elongation, and termination are the ones that most undergraduate students in the biological sciences become familiar with through textbooks and lectures.

In this review, we describe the long neglected fourth step of translation, recycling of the post-termination complexes. Nascent polypeptide is released during the termination step due to the action of release factors [2]. Some sources, including many textbooks, give the wrong impression that the release of the nascent polypeptide ultimately causes the release of mRNA and spent tRNA from ribosomes as well. In fact, mRNA and tRNA remain bound to ribosome after the nascent polypeptide has been released. It is obvious that the components of the post-termination complex have yet to be recycled for the next round of translation, yet for unknown reasons, this important step had been

overlooked for as long as 30 years. The concept that this step must occur stems from the original finding made in 1963 that tRNA is bound to the complex of ribosome and mRNA (reviewed in Ref. [3]). In addition to the fact that this finding played a key role in deciphering the genetic code, this suggested that ribosome-bound tRNA must be released by some mechanism. Since release of tRNA during the elongation cycle was shown to be catalyzed by elongation factor G (EF-G)[1] [4,5], attention was focused on the release of the last tRNA. This led to studies of the disassembly of the post-termination complexes.

Using *in vitro* assays, it was determined that two protein factors in the bacteria *Escherichia coli* were required to release mRNA and tRNA from the post-termination complexes. These two factors were identified as EF-G, a factor that was already known to work in the elongation phase of translation, and an unknown protein called ribosome *releasing* factor (RRF), later renamed to ribosome *recycling* factor [6-9]. Below, the assay first worked out in 1970 [6] and still in wide use today is described.
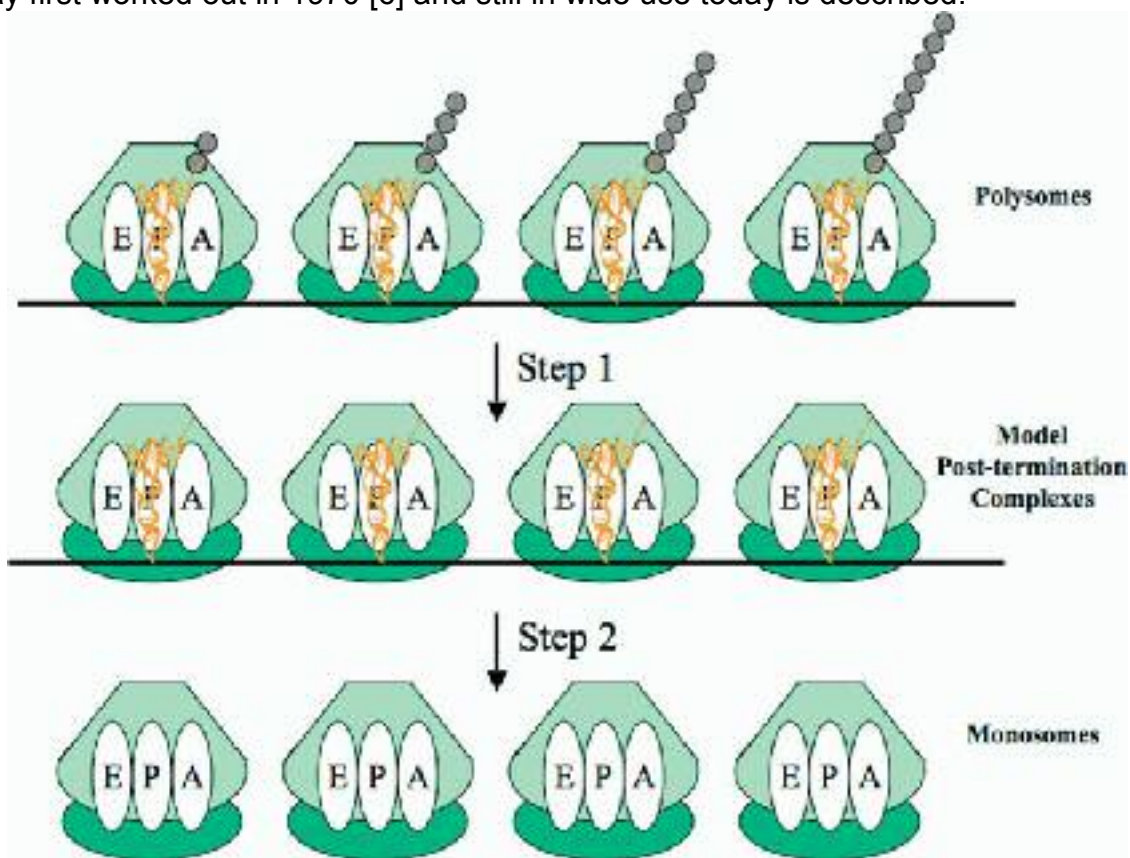
Fig.1. **Model post-termination complexes and disassembly by RRF, EF-G, and GTP**. Model post-termination complexes are created from polysomes treated with puromycin, which releases growing peptide chains (chains of gray spheres) from the P-site bound tRNA (Step 1). The model complexes are converted to monosomes only in the presence of RRF, EF-G, and GTP (Step 2). The final product of the reaction is 70 S monosomes when IF-3 is absent and Mg^{2+} ions are present (>3–5 mM).

[1]The abbreviations used are: EF-G, elongation factor G; RRF, ribosome recycling factor; IF, initiation factor.

BASIC ASSAY REACTION FOR BACTERIAL RIBOSOME RECYCLING

In bacteria, transcription of mRNA and translation of that mRNA occur in conjunction with one another such that many ribosomes can be translating a single mRNA at once. These complexes are called polysomes and can be readily isolated from rapidly growing *E. coli* cells using sucrose density gradients [7, 10]. Once a ribosome reaches a termination codon (UAA, UGA, or UGG), the nascent protein is released, and the mRNA and spent tRNA remain bound to the 70 S ribosome in a complex termed a post-termination complex. Model post-termination complexes can be obtained by isolating polysomes and treating with the antibiotic puromycin that releases polypeptide whether a ribosome has reached a termination codon or not. Thus, the puromycin-treated polysomes contain multiple ribosomes in a post-termination-like state (Fig. 1). Most assays of ribosome recycling have used these model post-termination complexes.

To assay for ribosome recycling, both purified RRF and purified EF-G are added along with GTP, which is hydrolyzed by EF-G bound to ribosome. The assay is monitored by the use of sucrose density gradients. When the mRNA is released from the ribosomes in the polysome complexes, the ribosomal subunits are converted to monosomes (Fig. 1). As described later, the ribosomes are split by EF-G and RRF but reassociate because of the absence of initiation factor 3 (IF-3). Ribosomes, whether as polysomes or monosomes, are detectable spectroscopically at 254–260 nm. The monosomes, however, are easily distinguished from polysomes since monosomes and polysomes migrate at different rates through the sucrose gradients, which can easily be fractionated.

There is an absolute requirement for both RRF and EF-G in this reaction [7]. The requirement for the hydrolysis of GTP by EF-G has also been demonstrated [10]. When non-hydrolyzable analogues of GTP are used, mRNA is not released. Thus, ribosome recycling does not occur unless GTP is hydrolyzed by EF-G.

STRUCTURE OF RRF AND ITS FUNCTION

How RRF and EF-G catalyze the conversion of post-termination complexes to ribosomes remained a mystery until recently. In 1999, collaboration between the laboratories of Anders Liljas and Akira Kaji resulted in the determination of the crystal structure of RRF [11]. The surprising finding was that RRF is nearly identical to tRNA in both shape and size (Fig. 2). Other translation factors exhibit some tRNA mimicry, but none are as close to the near perfect mimicry as shown by RRF. This near perfect mimicry of tRNA was thought to provide clues in elucidating a possible mechanism of action for RRF.
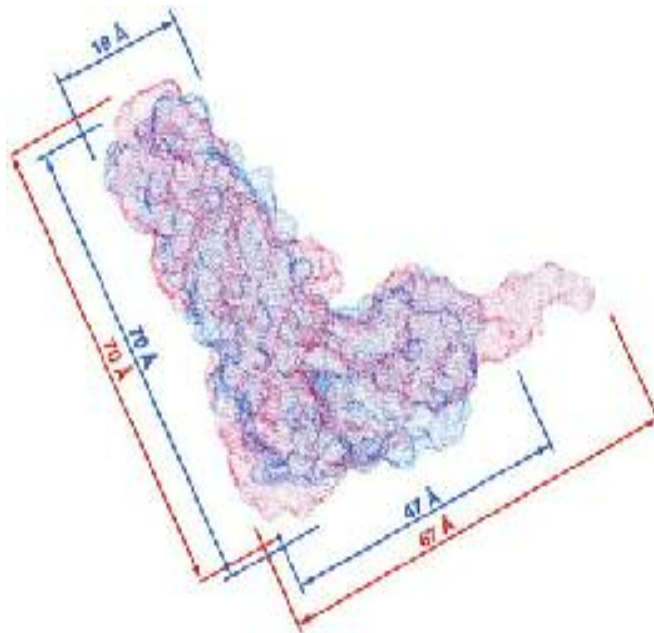
Fig. 2. **RRF is a near perfect structural mimic of tRNA**. The crystal structure of RRF is sown 9in blue), overleapping the structure of tRNA (in red) [11].

tRNA moves through the ribosome in stepwise fashion, from the A-to P-to E-sites. From the structural similarity, it does not take much imagination to postulate that RRF, with the help of EF-G, may move on the ribosome from its initial binding site during catalysis of the disassembly of post-termination complexes. This has been called translocation of RRF. The translocation or movement of RRF by EF-G is now supported by data on antibiotic inhibition [10] and thermodynamic studies [12]. Additional evidence has demonstrated that EF-G not only causes the movement but also the release of RRF itself from ribosomes, events that are required for the disassembly of post-termination complexes [12, 13].

The fact that RRF moves during its function does not mean that it does so exactly like tRNA. In fact, RRF binds nearly orthogonal to the position of tRNA in the A-site of the ribosome, as determined by chemical analysis and cryo-electron microscopy (Fig. 3) [14, 15]. It now seems more reasonable to suggest that ribosomal structure is such that it may only allow molecules with shapes similar to tRNA to move within the inter-subunit space. EF-G, for example, also has a very mildly tRNA-mimicking domain [16] that may aid its binding and movement in the inter-subunit space to translocate the A-site bound peptidyl tRNA during elongation. Thus, the high degree of structural mimicry between RRF and tRNA is likely due to the necessity of movement of RRF in the inter-subunit's space, although the binding mode is quite different from that of tRNA. In other words, the only functional mimicry is the movement of RRF and tRNA in the inter-subunit space [14, 17].

Although there appears to be some controversy as to the exact *order* of events during this recycling step, close analysis of all the available data indicates that the data from all laboratories are consistent with the interpretation that the action RRF and EF-G releases

mRNA and tRNA. The most significant recent finding regarding the function of RRF and EF-G is that cooperative action of these two proteins splits 70 S ribosomes into subunits [18]. The dissociation of 70 S ribosomes into subunits is a well accepted concept, and every biochemistry textbook describes it. However, only in 2005 has this fundamental reaction of biology been described as being catalyzed by RRF and EF-G. In all the textbooks currently available, it is described as a reaction catalyzed by another protein, IF-3. However, in 2005, two other laboratories, in addition to Hirokawa *et al*. [18] as described above, presented unequivocal evidence to indicate that subunit dissociation is catalyzed by RRF and EF-G [19, 20]; IF-3, in turn, acts to keep the subunits dissociated (Fig. 4, *step 4b*). Thus, again, all laboratories agree on this. Ribosome recycling can therefore be viewed as a direct bridge from termination of protein synthesis to initiation of protein synthesis, long described as beginning from 30 S ribosomal subunits.

Fig. 3. **Binding position of RRF in the 70 S ribosome relative to tRNA**. P-site bound tRNA (left), A-site bound tRNA (middle), and RRF (right) are shown [14,15].

RRF IN BACTERIA AND ORGANELLES

RRF protein was first discovered in *E. coli* cells. The gene encoding RRF was discovered in 1989 [21], and it has been shown that *E. coli* cells do not survive without functional RRF [22]. Many genomes have now been completely sequenced, and all bacteria, except the archaebacteria, possess an RRF-encoding gene [23]. *E. coli* has remained the model organism, but RRF from *Mycobacterium tuberculosis* [24–26], *Thermus*

thermophilus [27], *Aquifex aeolicus* [28], *Thermotoga maritima* [11], and *Vibrio parahemeolyticus* [29] have also been studied.

In the absence of RRF, terminated ribosomes initiate unscheduled translation downstream of the stop codon [30–32]. This event ultimately wastes energy and deprives the cell of ribosomes that should be initiating mRNA properly; thus, this event may be a primary reason that cessation of cell growth follows the loss of RRF. It should be pointed out that the loss of RRF leads to cell death if it occurs during the lag phase [32].
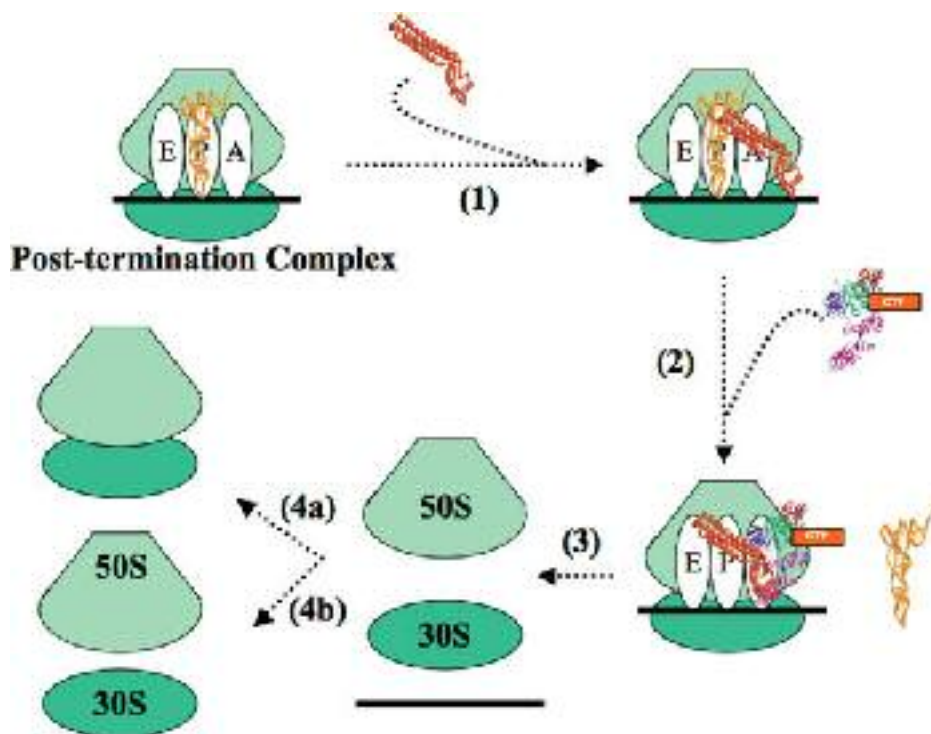

FIG.4. **The current model for post-termination complex disassembly**. In step 1, RRF binds to the A-site region of ribosome but orthogonal to tRNA. In step 2, EF-G binds, causing a change in RRF binding position and releasing tRNA. By the action of EF-G and RRF, mRNA is released in step 3 as the 70 S ribosome splits into its subunits, 30 S and 50 S. In the presence of IF-3 (*step 4b*), the subunits remain split. In the absence of IF-3 but in the presence of Mg2+ (>3–5 mM), the subunits are free to rejoin as 70 S ribosomes (*step 4a*).

Eukaryotic organelles that contain DNA are thought to have evolved from early, endosymbiotic bacteria. These organelles possess their own ribosomes that are closely related to bacterial ribosomes. From this, one may correctly predict that the organelles such as mitochondria and chloroplasts possess RRF. Both mitochondria and chloroplasts have functional RRF homologues, although the genes are nuclear-encoded with N-terminal sequences to allow entry into the organelles [33, 34].

A temperature-sensitive mutation of the mitochondrial RRF has been generated [34]. At the non-permissive temperature, cells exhibit respiratory incompetence, and the mitochondrial genome becomes unstable. Thus, RRF is essential to the maintenance of

mitochondria. Studies on chloroplastic RRF have been limited, but the chloroplastic RRF protein has been shown to bind to chloroplastic ribosomes at an ~1:1 ratio [35].

EUKARYOTIC CYTOPLASMIC PROTEIN SYNTHESIS AND RRF

No obvious eukaryotic RRF homologues can be found by a search of published eukaryotic genome sequences, except for the organelle-localized RRF. This suggests that cytoplasmic protein synthesis is missing RRF. How ribosomes are recycled in eukaryotic cytoplasm is still unclear. However, knowing that the basic steps of translation (initiation, elongation, and termination) occur on eukaryotic cytoplasmic ribosomes, ribosome recycling (i.e. the release of ribosomes from the mRNA after termination and splitting of the ribosome) must occur as well. It is possible that this is an intrinsic property of the ribosomes in the cytoplasm of eukaryotes, or some currently undescribed factor(s) may be involved.

CONCLUSION

Ribosome recycling has been an overlooked cellular process in most published textbooks that cover translation. In the past decade, it has gained wide acceptance and prominence among researchers in the field of translation. This is particularly true since it is vital to bacteria and at least mitochondria as well. It is essential that textbooks used to teach new generations of scientists include this essential step of protein synthesis, as outlined in Fig. 4. For more specific details of the process and current research questions, readers can consult [36].

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