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BREAKTHROUGHS AND VIEWS

Disassembly of the Post-termination Complex and Reduction of Translational Error by Ribosome Recycling Factor (RRF)—A Possible New Target for Antibacterial Agents

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In this paper, we briefly review RRF (ribosome recycling factor, previously called ribosome releasing factor) (for recent reviews covering historical background see (1, 2)).

REACTIONS CATALYZED BY RRF

The ribosome which has just released the completed peptide is situated on the mRNA with the termination codon at the A-site and deacylated tRNA on either P-site or E (exit) site (3, 4) (Fig 1B). In the presence of RRF, GTP, 5 mM Mg^{++}, and either EF-G (elongation factor G) (5) or RF3 (release factor 3) (4), the complex is disassembled into mRNA, tRNA (5, 6) and the ribosome. The ribosome may be dissociated into subunits during the reaction (7). This step is the fourth step of protein biosynthesis following the third step, release of completed peptide from peptidyl tRNA (8-10). RRF is also involved in the elongation step (2) by presumably acting at the ribosomal A site to assure that cognate aminoacyl tRNA is placed on this site (2).

PROPERTIES OF RRF

E. coli RRF is a basic protein with a molecular weight of approximately 20 kDa and consisting of 185 amino acids (11). Purification from the crude extract consists of five steps (12) and RRF free *E. coli* crude extract can be prepared with the use of one of the purification steps (13). *E. coli* RRF is expressed well from the cloned gene (11). It is coded for by the gene named *frr* situated at the 4 min region, 1.1k bases downstream from the gene coding for EF-Ts (14). The gene *frr* has a very strong promoter (15) with minimal expression in the laboratory condition (14) but the expression is elevated in some pathogens infecting animals (16, 17). The molar amount of RRF in a cell is approximately one half of the total number of the ribosome and approximately 30 % of total cellular RRF is bound to ribosomes (18). RRF is an essential protein for *E. coli* (19) and is present in every prokaryote (2) except for archaebacteria (Archaea).

ASSAY METHODS FOR THE RRF ACTIVITY

Assays with the Post Termination Complex (4, 6)

R17 amB2 phage RNA (20, 21) has an amber codon (UAG) at the seventh codon of the coat cistron. The N-terminal amino acid sequence of the coat protein is fMet · Ala · Ser · Asn · Phe · Thr Gln Phe Val Leu Val-- (22). In the assay for RRF activity, ribosomes were bound to the 7th codon of the ^{3}H labeled R17 amB2 phage RNA together with the hexapeptidyl tRNA. After release of the hexapeptide from this complex by RF1, release of ribosomes from the post termination complex by RRF was monitored by centrifugation (6). Ribosomes have to be at the termination codon for the release, because no significant release of the mRNA was observed from the ribosomes with fMet Ala Ser tRNA at the fourth codon of the coat cistron (6). In a simpler assay, using the complex of fmet tRNA, ribosome and the labeled synthetic oligonucleotides (UUC AUG UAA (or UAG or UGA)) we recently showed that release of the mRNA depends on RRF and either EF-G or RF3 (4).

Assay with Puromycin Treated Natural Polysomes—A Model System (23)

For routine assay of RRF, puromycin treated naturally occurring polyribosomes can be used (12, 24).

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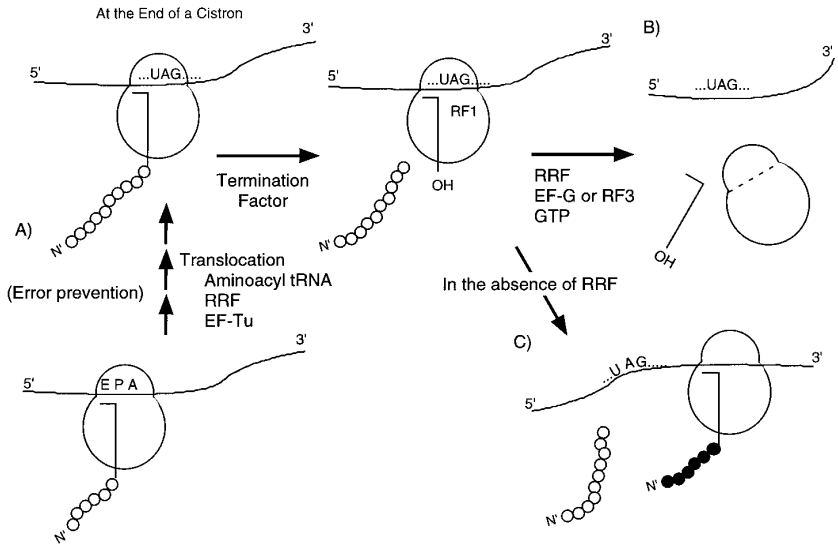

FIG. 1. Schematic representation of reactions involving RRF; ●●● designates polypeptides reinitiated from the codon immediate 3′ of the termination codon; ○○○ designates peptide released at the termination codon UAG; A) cognate aminoacyl tRNA binding; B) Disassembly of the post termination complex; C) Unscheduled reinitiation due to absence of RRF.

Each ribosome in this model substrate has a configuration similar to the post termination complex except that the ribosome is not at the termination triplet. With RRF, GTP, and EF-G, ribosomes are released from the mRNA resulting in conversion of polysomes to monosomes which is monitored by centrifugation.

Indirect Assay Based on Stimulation of Protein Synthesis through Enhanced Ribosome Recycling

The RRF activity can also be measured by stimulation (7 fold) of *de novo* synthesis of lysozyme (25). Earlier, Kung *et al.* reported stimulation by RRF (four fold) of β-galactosidase synthesis in a system consisted of completely purified factors (26). Synthesis of an oligopeptide, fmet-phe-thr-ile, coded for by a synthetic mRNA, AUG UUU ACG AUU has recently been used to observe the stimulation by RRF (7 fold) (27, 28). With such short ORF with a strong SD (Shine and Dalgarno) sequence nearby, however, no released ribosome were observed because ribosomes are in contact with mRNA at least 25 nucleotides upstream from the P-site (29). This may have bearing with the translational coupling (30).

MECHANISM OF THE RRF ACTION

The molecular mechanism of the RRF action is not known except that RRF appears to bind to ribosomal A site judging from two indirect observations. 1) RRF reduces translational error probably interfering with the binding of non cognate aminoacyl tRNA (31) to A-site (2). 2) With a short synthetic mRNA, the inhibitory effect of an excess of RF1 (presumably binds to A-site) on oligopeptide synthesis was reversed by addition of an excess of RRF (32).

A number of questions remain to be answered. Does the post termination complex contain RFs? If so, which RF at what ribosomal site? Are they removed by RF3 and RRF? Which factor, EF-G or RF3, functions *in vivo?* How two factors, RRF and G-protein (EF-G (33) or RF3 (4)) simultaneously (5) act to disassemble the post termination complex? How many GTP molecules at what step are consumed? Which ribosomal proteins and what part of ribosomal RNA interact with RRF? How does RRF recognize mRNA? What is the molecular mechanism of the error reduction by RRF? Why does the release of peptidyl tRNA take place by an excess RRF (34)? These are questions to be answered by future studies.

SERIOUS CONSEQUENCES RESULTING FROM THE ABSENCE OF RRF

Unscheduled Reinitiation without the Initiation Sequence Downstream from the Termination Codon

In the absence of RRF, what would happen to the ribosome remaining on the mRNA? To answer this question, we used the *in vitro* system programmed by amB2 phage RNA as described in the preceding section. Normally, because of UAG at the 7th codon, the hexapeptide corresponding to the NH_2 terminal of the amB2 coat protein is synthesized. Upon removal of RRF, however, the hexapeptide synthesis decreased and the coat protein missing the seven NH_2-terminal amino acids (with the NH_2-terminal sequence, Phe Val Leu Val--), was synthesized (35). Thus, in the absence of RRF, the

ribosome started unscheduled translation from the 8th codon (Fig. 1C).

To demonstrate this reinitiation *in vivo*, we used the temperature sensitive (ts) mutant, LJ14 (36). We placed in this strain a plasmid which carries a short ORF upstream of the reporter gene β-galactosidase without the initiation signal and missing the eight NH_2-terminal amino acids. Upon shift up to the non-permissive temperature, we observed appearance of the β-galactosidase activity. This indicates that ribosomes, upon denaturation of RRF, stay on the mRNA and initiate the unscheduled translation without any initiation signal downstream from the termination codon. The *in vivo* reinitiation is different from that of *in vitro* in that the former took place in all reading frames (36) while the latter was mostly limited to the in frame reinitiation next to the termination codon (35). *In vivo*, ribosomes (perhaps as 70S ribosomes (37)) can slide downstream as many as 10 to 45 nucleotides before it randomly starts translation of the mRNA.

Increase of Translational Error

Removal of RRF in the polyphenylalanine synthesis system programmed by polyuridylic acid (poly U) caused misincorporation of amino acids other than phenylalanine (3 to 10 fold more (2)), indicating that RRF reduced the translational error. To support this notion further, we demonstrated that β-galactosidase synthesized *in vivo* by LJ14 at the non permissive temperature was much more heat labile than the regular β-galactosidase (unpublished observation). This suggests that the enzyme has many errors in the amino acid sequence resulting in heat lability.

Bactericidal and Bacteriostatic Effect

We observed bactericidal and bacteriostatic effect of reversible inactivation of ts RRF in the lag phase and in the logarithmic phase (36) respectively. In contrast, irreversible inhibition of the RRF activity by expressing plant RRF homologue caused bacterial death regardless of the physiological phase of bacteria (unpublished observation).

Fate of Ribosomes in Vivo at the Physical End of mRNA

We showed that in the absence of RRF the ribosomes reinitiate translation downstream from the termination codon. What happens then to these ribosomes at the end of mRNA? In answer to this question, we recently showed that ribosomes with the peptidyl tRNA is pushed out of the 3′ end of mRNA in LJ14 exposed to the non permissive temperature (unpublished observation). Recent studies indicated that 10Sa RNA (also termed trans-mRNA, or tmRNA (38)) binds to ribosomes at the end of mRNA (for review see (39)). 10Sa RNA codes for a peptide added to the C-terminus of the nascent polypeptide chain of the peptidyl tRNA carried by the ribosome at the 3′ end of the truncated mRNA (40). In the absence of RRF, however, this function of 10Sa RNA may be completely abolished because the ribosomes will reach the physical end of 10Sa RNA.

RRF AS A POSSIBLE TARGET OF NEW ANTIBACTERIAL AGENTS

Bactericidal and bacteriostatic effects of removal of RRF *in vivo* suggest that RRF may be an ideal target for antibacterial agents. Even the reversible inactivation of temperature sensitive RRF caused bacterial death when they are in lag phase (36). The *frr* homologues are found in yeast (41), human (GenBank accession #T19688, #AA004407), carrot (GenBank accession #585565), and spinach (Rolland *et al.* in preparation). RRF homologue in spinach is localized in organelles such as chloroplast (Rolland *et al.* in preparation) and presumably mitochondria. In yeast, the RRF homologue is not essential because the strain without the *frr* homologue grows well in glucose (Dr. Wek, Indiana University). Hence, inhibition of eukaryotic RRF should not influence cytoplasmic protein synthesis. In support of this concept, *M. Jannaschii*, a prokaryote without *frr* homologue, has a protein synthesis machinery similar to that of eukaryotes (42). One may expect that the proposed RRF inhibitor may harm the cellular respiration. Indeed, erythromycin, tetracycline, and chloramphenicol inhibit mitochondria protein synthesis (43, 44). But these side effects are minimal judging from the wide usage of these antibiotics. For rational drug design against RRF, we need 3D structure of RRF. Toward this goal, we recently completed assignment of all NMR spectrum peaks corresponding to 185 amino acids of RRF (with Dr. Kobayashi of Osaka University), and obtained crystals of RRF (with Dr. Liljas of Lund University).

DISASSEMBLY OF THE POST TERMINATION COMPLEX IN EUKARYOTES

We postulate that at the end of ORF, 80S ribosomes are dissociated into 40S and 60S subunits (45) by yet unidentified soluble factors. The 40S subunits remain on the mRNA and scan through the rest of mRNA. The presence of spent 40S subunits scanning downstream does not do much harm because most of mRNA of eukaryotes are monocistronic (46) and ribosomes do not generally enter mRNA in the middle except for the special case with IRES (47). Eukaryotic mRNA often carry short ORFs upstream of the ORF of the cistron (for example, (48)). Although the exact reason for the presence of short upstream ORFs is not clear, this may add additional means of controlling the translation by

manipulating the yet undiscovered process of the fourth step of protein synthesis in eukaryotes.

NOMENCLATURE OF RRF

An ad hoc IUB committee recently renamed RRF as RF4 (49) which gives wrong impression that the function of RRF is similar to the termination factors (RFs). For this reason, the members of the nomenclature committee, Drs. M. Grunberg-Manago, and J. Hershey, upon consultation with Dr. R. Buckingham of CNRS, Paris, and A. Kaji of University of Pennsylvania, have recently agreed to retain the name RRF, instead of RF4. A note on this matter will be submitted to the journal Biochimie shortly.

REFERENCES

1. Janosi, L., Hara, H., Zhang, S., and Kaji, A. (1996) *Adv. Biophys.* **32,** 121–201.
2. Janosi, L., Ricker, R., and Kaji, A. (1996) *Biochimie* **78,** 959–969.
3. Agrawal, R. K., Penczek, P., Grassucci, R. A., Li, Y., Leith, A., Nierhaus, K. H., and Frank, J. (1996) *Science* **271,** 1000–1002.
4. Grentzmann, G., Kelly, P. J., Laalami, S., Shuda, M., Firpo, M. A., Cenatiempo, Y., and Kaji, A. (1998) *RNA* (in press).
5. Hirashima, A., and Kaji, A. (1973) *J. Biol. Chem.* **248,** 7580–7587.
6. Ogawa, K., and Kaji, A. (1975) *Eur. J. Biochem.* **58,** 411–419.
7. Kaempfer, R. (1970) *Nature* **228,** 534–537.
8. Tate, W. P., and Mannering, S. A. (1996) *Mol. Microbiol.* **21,** 213–219.
9. Nakamura, Y., Ito, K., and Isaksson, L. A. (1996) *Cell* **87,** 147–150.
10. Buckingham, R. H., Grentzmann, G., and Kisselev, L. (1997) *Mol. Microbiol.* **24,** 449–456.
11. Ichikawa, S., and Kaji, A. (1989) *J. Biol. Chem.* **264,** 20054–20059.
12. Hirashima, A., and Kaji, A. (1972) *Biochemistry* **11,** 4037–4044.
13. Ogawa, K., and Kaji, A. (1975) *Biochim. Biophys. Acta* **402,** 288–296.
14. Ichikawa, S., Ryoji, M., Siegfried, Z., and Kaji, A. (1989) *J. Bacteriol.* **171,** 3689–3695.
15. Shimizu, I., and Kaji, A. (1991) *J. Bacteriol.* **173,** 5181–5187.
16. Lowe, A. M., Beattie, D. T., and Deresiewicz, R. L. (1998) *Mol. Microbiol.* **27,** 967–976.
17. Vizcaino, N., Cloeckaert, A., Dubray, G., and Zygmunt, M. (1996) *Infect. Immun.* **64,** 4834–4837.
18. Ryoji, M. (1981) University of Pennsylvania School of Medicine, Ph.D thesis.
19. Janosi, L., Shimizu, I., and Kaji, A. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 4249–4253.
20. Gussin, G. N. (1966) *J. Mol. Biol.* **21,** 435–453.
21. Robinson, W. E., and Frist, R. H. (1969) *Science* **166,** 1291–1293.
22. Weber, K. (1967) *Biochemistry* **6,** 3144–3154.
23. Hirashima, A., and Kaji, A. (1970) *Biochem. Biophys. Res. Commun.* **41,** 877–883.
24. Hirashima, A., and Kaji, A. (1972) *J. Mol. Biol.* **65,** 43–58.
25. Ryoji, M., Karpen, J. W., and Kaji, A. (1981) *J. Biol. Chem.* **256,** 5798–5801.
26. Kung, H-F., Treadwell, B. V., Spears, C., Tai, P-C., and Weissbach, H. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 3217–3221.
27. Pavlov, M.Yu., Freistroffer, D., MacDougall, J., Buckingham, R. H., and Ehrenberg, M. (1997) *EMBO J.* **16,** 4134–4141.
28. Freistroffer, D. V., Pavlov, M.Yu., MacDougall, J., Buckingham, R. H., and Ehrenberg, M. (1997) *EMBO J.* **16,** 4126–4133.
29. Gold, L., Pribnow, D., Schneider, T., Shinedling, S., Singer, B. S., and Stormo, G. (1981) *Ann. Rev. Microbiol.* **35,** 365–403.
30. Spanjaard, R. A., and van Duin, J. (1989) *Nucleic Acids Res.* **17,** 5501–5507.
31. Takeda, Y., Suzuka, I., and Kaji, A. (1968) *J. Biol. Chem.* **243,** 1075–1081.
32. Pavlov, M.Yu., Freistroffer, D. V., Heurgue-Hamard, V., Buckingham, R. H., and Ehrenberg, M. (1997) *J. Mol. Biol.* **273,** 389–401.
33. Conway, T. W., and Lipmann, F. (1964) *Proc. Natl. Acad. Sci. USA* **52,** 1462–1469.
34. Heurgue-Hamard, V., Karimi, R., Mora, L., MacDougall, J., Leboeuf, C., Grentzmann, G., Ehrenberg, M., and Buckingham, R. H. (1998) *EMBO J.* **17,** 808–816.
35. Ryoji, M., Berland, R., and Kaji, A. (1981) *Proc. Natl. Acad. Sci. USA* **78,** 5973–5977.
36. Janosi, L., Mottagui-Tabar, S., Isaksson, L. A., Sekine, Y., Ohtsubo, E., Zhang, S., Goon, S., Nelken, S., Shuda, M., and Kaji, A. (1998) *EMBO J.* **17,** 1141–1151.
37. Petersen, H. U., Joseph, E., Ullman, A., and Danchin, A. (1978) *J. Bacteriol.* **135,** 453–459.
38. Atkins, J. F., and Gesteland, R. F. (1996) *Nature* **379,** 769–771.
39. Jentsch, S. (1996) *Science* **271,** 955–956.
40. Tu, G., and Reid, G. (1995) *J. Biol. Chem.* **270,** 9322–9326.
41. Johnston, M., Andrews, S., Brinkman, R., Cooper, J., Ding, H., Dover, J., Du, Z., Favello, A., Fulton, L., Gattung, S., Geisel, C., Kirsten, J., Kucaba, T., Hillier, L., Jier, M., Johnston, L., Langston, Y., Latreille, P., Louis, E. J., Macri, C., Mardis, E., Menezes, S., Mouser, L., Nhan, M., Rifkin, L., Riles, L., St. Peter, H., Trevaskis, E., Vaughan, K., Vignati, D., Wilcox, L., Wohldman, P., Waterston, R., Wilson, R., and Vaudin, M. (1994) *Science* **265,** 2077–2082.
42. Bult, C. J., White, O., Olsen, G. J., Zhou, L., Fleischmann, R. D., Sutton, G. G., Blake, J. A., Fitzgerald, L. M., Clayton, R. A., Gocayne, J. D., Kerlavage, A. R., Dougherty, B. A., Tomb, J. F., Adams, M. D., Reich, C. I., Overbeek, R., Kirkness, E. F., Weinstock, K. G., Merrick, J. M., Glodek, A., Scott, J. L., Geoghagen, N. S. M., Weidman, J. F., Fuhrmann, J. L., Nguyen, D., Utterback, T. R., Kelley, J. M., Peterson, J. D., Sadow, P. W., Hanna, M. C., Cotton, M. D., Roberts, K. M., Hurst, M. A., Kaine, B. P., Borodovsky, M., Klenk, H-P., Fraser, C. M., Smith, H. O., Woese, C. R., and Venter, J. C. (1996) *Science* **273,** 1058–1073.
43. Pious, D. A., and Hawley, P. (1972) *Pediat. Res.* **6,** 687–692.
44. Borst, P., and Grivell, L. A. (1998) *FEBS Lett.* **13,** 73–88.
45. Kozak, M. (1987) *Mol. Cell. Biol.* **7,** 3438–3445.
46. Shih, D. S., and Kaesberg, P. (1973) *Proc. Natl. Acad. Sci. USA* **70,** 1799–1803.
47. Pelletier, J., and Sonenberg, N. (1988) *Nature* **334,** 320–325.
48. Marton, M. J., Vazquez de Aldana, C., Qiu, H., Chakraburtty, K., and Hinnebusch, A. G. (1997) *Mol. Cell. Biol.* **17,** 4474–4489.
49. Clark, B. F. C., Grunberg-Manago, M., Gupta, N. K., Hershey, J. W. B., Hinnebusch, A. G., Jackson, R. J., Maitra, U., Mathews, M. B., Merrick, W. C., Rhoads, R. E., Sonenberg, N., Spremulli, L. L., Trachsel, H., and Voorma, H. O. (1996) *Biochimie* **78,** 1119–1122.