*RELEASE OF TRANSFER RNA DURING PEPTIDE CHAIN ELONGATION**

By Jean Lucas-Lenard and Anne-Lise Haenni†

THE ROCKEFELLER UNIVERSITY

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Abstract.—In amino acid polymerization, the tRNA donating its peptidyl moiety to the neighboring aminoacyl-tRNA must be released from the ribosome for further growth of the polypeptide chain. It was not known at what stage of peptide elongation this tRNA is released. To study this question, we prepared ac-C^{14}-Phe-H^3-tRNA using H^3-tRNA isolated from *E. coli* strain 15THU grown in the presence of H^3-uracil, and followed the fate of the H^3-tRNA during chain growth. Our results indicate that donor tRNA is released during the translocation step, mediated by the soluble factor G and GTP.

Three elongation factors, T_u, T_s, and G, are required for ribosome-dependent amino acid polymerization in bacteria.[1-3] Using acPhe-tRNA‡ as a model chain initiator in poly U-directed phenylalanine polymerization, we found that $T_u + T_s$ function in the binding of Phe-tRNA to ribosomes.[4] AcPhe-Phe-tRNA is formed as a result of this reaction.[5] It was further observed that G[6] is involved in the translocation of the newly formed dipeptidyl-tRNA from the acceptor to the donor site,[4, 7-9] thus freeing the acceptor site for another cycle of $T_u + T_s$-directed binding of Phe-tRNA.

During the elongation process, the tRNA donating the initiator or peptidyl moiety must be released from the donor site on the ribosome. It has so far not been determined at what stage this tRNA is released. Seeds and Conway[10] have suggested that a heat-stable fraction and GTP are required for removal of inhibitory tRNA during polymerization. Recent experiments by Kuriki and Kaji[11] also indicate that a heat-stable factor and GTP are involved in tRNA release from ribosomes.

We have prepared ac-C^{14}-Phe-H^3-tRNA using H^3-tRNA isolated from *E. coli* strain 15THU grown in the presence of H^3-uracil, and have examined whether the tRNA bearing the acPhe is released during acPhe-Phe-tRNA synthesis or during the translocation step. Our results indicate that the donor tRNA is released by G and GTP, and that translocation of peptidyl-tRNA is necessary to displace this tRNA from the ribosome.

Preparation and Purification of ac-C^{14}-Phe-H^3-tRNA.—*E. coli* cells, strain 15THU (a gift of Dr. M. Schweiger), were grown to $A_{450} = 0.6$ in 10 ml of the medium described by Stern *et al.*,[12] supplemented with 1% vitamin-free casamino acids. An aliquot was then transferred to 10 ml of the same medium containing 40 μg of H^3-uracil (2.0 mc) in place of the cold uracil. The cells, having incorporated 1.0–1.2 mc of H^3-uracil, were pelleted, resuspended in 0.5 ml of a solution containing 0.001 *M* Tris-HCl, pH 7.2, and 0.01 *M* magnesium acetate (buffer *A*), and extracted for 60 min at 0° with 0.5 ml of phenol saturated with buffer *A*.[13] The emulsion was centrifuged for 20 min at 30,000 × *g*, and the lower phenol phase was re-extracted with 0.5 ml of buffer *A*. The aqueous phases were combined and the nucleic acids were precipitated by addition of 0.1 ml of 20% K-acetate pH 5.5 and two volumes of cold ethanol. In one of two preparations, 70 μg of

phenol-extracted tRNA were added as carrier prior to the addition of ethanol. The precipitate was resuspended in 0.25 ml of 0.1 *M* Tris-HCl, pH 8.2; the resulting solution was incubated for 60 min at 37° to strip the tRNA, extensively dialyzed against water, and lyophilized. The preparation was charged in a total volume of 0.1 ml containing 0.1 *M* Tris-HCl, pH 7.4, 0.01 *M* magnesium acetate, 0.002 *M* ATP, 0.02 *M* dithiothreitol, 140 μg of an S-150,000 fraction, and 0.5 μc of C^{14}-phenylalanine (409 mc/mM), and was incubated for 20 min at 37°. A few crystals of electrophoretically purified DNase were added, and incubation was continued for another 5 min. It was advantageous to have carrier tRNA in the preparation since it enabled us to follow the incorporation of C^{14}-phenylalanine into the H^3-tRNA. The resulting mixture was acetylated, as described previously,[14] and extensively dialyzed against 0.05 *M* acetate buffer, pH 5.

The preparation was made 0.4 *M* in NaCl and 0.01 *M* in magnesium acetate, and applied to a 2-ml BD-cellulose[15] (obtained from Schwarz) column equilibrated with 0.05 *M* acetate buffer, pH 5, 0.01 *M* magnesium acetate (buffer *B*) and 0.04 *M* NaCl. More than 90% of *E. coli* $tRNA^{Phe}$ is eluted by 1 *M* NaCl in buffer *B*.[15] We determined that Phe-tRNA is eluted by 1 *M* NaCl and 4.9% ethanol in buffer *B*, and acPhe-tRNA by 1 *M* NaCl and 8.5% ethanol in buffer *B*. The column was first washed with 0.4 *M* NaCl in buffer *B*, then successively with 1 *M* NaCl in buffer *B*, followed by 1 *M* NaCl and 6% ethanol in buffer *B*, and finally with 1 *M* NaCl and 12% ethanol in buffer *B*, each time until no further radioactivity was recovered in the eluates. Fractions of 1 ml were collected and 10-μl aliquots removed for radioactivity determinations. The radioactive fractions eluted in the final ethanol wash were pooled, extensively dialyzed against water, and lyophilized. After resuspension in 0.2 ml of H_2O, the radioactive product was found to have a high specific activity. It was therefore diluted with ac-C^{14}-Phe-tRNA purified by BD-cellulose chromatography. The specific activity of the diluted material was determined using the poly U-dependent binding assay, under conditions of limiting ac-C^{14}-Phe-tRNA.

Preparation of ribosomes and supernatant factors: *E. coli* B cells were harvested in mid-log phase[6] and were used to prepare ribosomes as reported previously.[5] The supernatant factors T and G were isolated from *Ps. fluorescens* by chromatography on a DEAE-cellulose column as already described.[16] The unseparated T factor was used in these experiments.

Results.—Fate of donor H^3-tRNA during polypeptide elongation: In a first series of experiments, ac-C^{14}-Phe-H^3-tRNA was prebound to ribosomes, followed by the $T(T_u + T_s)$ and GTP-promoted binding of C^{12}-Phe-tRNA.[5] From previous work, it is known that a consequence of this binding reaction is the formation of ac-C^{14}-Phe-Phe-tRNA.[5] Table 1 shows that the donor H^3-tRNA was not released in spite of extensive ac-C^{14}-Phe-Phe-tRNA synthesis.

These ribosomes carrying ac-C^{14}-Phe-Phe-tRNA and the donor H^3-tRNA were isolated and incubated with G and GTP. The translocation of peptidyl-

TABLE 1. *Fate of donor tRNA during transpeptidation.*

Additions	H^3-tRNA remaining ($\mu\mu$moles)	ac-C^{14}-Phe-Phe-tRNA formed ($\mu\mu$moles)
None	1.7	—
T + GTP + C^{12}-Phe-tRNA	1.7	1.3

Ribosomes (1.2 A_{260}) were incubated with 0.05 *M* Tris-HCl, pH 7.4, 0.01 *M* magnesium acetate, 0.16 *M* NH_4Cl, 0.01 *M* dithiothreitol, 5 μg of poly U, and 0.7 μg of ac-C^{14}-Phe-H^3-tRNA charged with 7 $\mu\mu$moles of C^{14}-phenylalanine, in a total volume of 0.125 ml for 20 min at 30°. The samples were cooled to 0° and, where indicated, were supplemented with 0.2 μg of T, 0.05 μmole of GTP, and 15 μg of C^{12}-Phe-tRNA. The mixtures were incubated for 20 min at 0°. The H^3-tRNA bound was determined by the Millipore filter technique.[17] In a parallel experiment, the products formed during the T reaction and bound to ribosomes were analyzed by paper electrophoresis;[4] in addition to 1.3 $\mu\mu$moles of acPhe-Phe-tRNA, 0.4 $\mu\mu$mole of acPhe-tRNA were recovered.

tRNA from the acceptor to the donor site presumably occurs during this step, with simultaneous movement of mRNA.[7] Table 2 shows that in the presence of G and GTP there was a decrease in the amount of donor H^3-tRNA bound to the filter, suggesting that this tRNA had been released from the ribosomes. Fusidic acid[4, 18] inhibited this reaction, and GMP-PCP[19] could not substitute for GTP. No release was observed at 0° in the presence of G and GTP.

An experiment was then designed to determine if G and GTP would also remove the donor H^3-tRNA in the absence of translocation. Ribosomes charged with ac-C-^{14}Phe-H^3-tRNA on the donor site were incubated with puromycin and the effect of G and GTP was studied. As shown in Table 3, even though an extensive transpeptidation of ac-C^{14}-Phe to puromycin had occurred liberating the donor H^3-tRNA, this tRNA was not released by added G + GTP.

TABLE 2. *Release of H^3-tRNA during translocation.*

Additions	H^3-tRNA remaining ($\mu\mu$moles)
None	1.1
G + GTP	0.5
G + GTP + fusidic acid	1.1
G + GMP-PCP	1.2

Ribosomes were charged with ac-C^{14}-Phe-H^3-tRNA and C^{12}-Phe-tRNA, as described under Table 1, and pelleted.[4] The resuspended ribosomes, carrying 1.3 $\mu\mu$moles of H^3-tRNA, were incubated for 5 min at 30° in a 0.125-ml mixture containing 0.05 *M* Tris-HCl, pH 7.4, 0.01 *M* magnesium acetate, 0.16 *M* NH_4Cl, 0.01 *M* dithiothreitol, and, where indicated, 2.5 μg of G, 0.4 mM GTP, 0.6 mM fusidic acid, and 0.4 mM GMP-PCP. The Millipore binding assay[17] was used to determine the level of H^3-tRNA that remained bound.

TABLE 3. *Stability of donor tRNA during ac-C^{14}-Phe-puromycin formation.*

Additions	H^3-tRNA remaining ($\mu\mu$moles)	ac-C^{14}-Phe-tRNA remaining ($\mu\mu$moles)
None	1.2	1.1
Puromycin	1.2	0.6
G + GTP + puromycin	1.1	0.6

Ribosomes were charged with ac-C^{14}-Phe-H^3-tRNA as described in Table 1. After the 20-min incubation at 30°, 2.5 μg of G, 0.05 μmole of GTP, and 0.2 μmole of puromycin were added where indicated, and the reaction mixture was incubated for 5 min at 30°. The Millipore binding assay[17] was used to measure the extent of donor H^3-tRNA and ac-C^{14}-Phe-puromycin release.

Because donor H^3-tRNA appeared to be released in the G + GTP step, an attempt was made to correlate this release with acPhe-Phe-tRNA translocation as measured by acPhe-Phe-puromycin formation.[4] To this end, ribosomes containing ac-C^{14}-Phe-Phe-tRNA and donor H^3-tRNA were prepared as described in Table 2. Figure 1*A* shows the kinetics of donor tRNA release in the presence of G and GTP, and Figure 1*B* the rate of translocation of ac-C^{14}-Phe-Phe-tRNA, as determined by acPhe-Phe-puromycin formation. The rate of the puromycin reaction is fast as compared with the rate of translocation of acPhe-Phe-tRNA,[20] and thus the kinetics of acPhe-Phe-puromycin formation (Fig. 1*B*) should represent the G and GTP reaction. The results of this experiment indicate that the ratio of donor H^3-tRNA released to ac-C^{14}-Phe-Phe-puromycin formed approximates one to one.

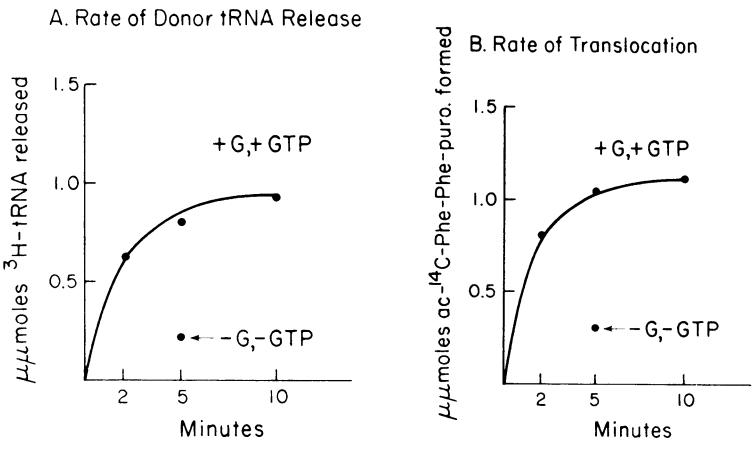

FIG. 1.—Correlation of H^3-tRNA release and ac-C^{14}-Phe-Phe-tRNA translocation. Ribosomes were charged with ac-C^{14}-Phe-H^3-tRNA and C^{12}-Phe-tRNA, pelleted, and an aliquot analyzed electrophoretically as described in Table 1. Aliquots of the resuspended ribosomes carrying 1.3 μμmoles of ac-C^{14}-Phe-Phe-tRNA and 0.4 μμmole of ac-C^{14}-Phe-tRNA were incubated at 30° in a 0.125-ml incubation mixture containing 0.05 *M* Tris-HCl, pH 7.4, 0.01 *M* magnesium acetate, 0.16 *M* NH_4Cl, 0.01 *M* dithiothreitol, and, where indicated, 2.5 μg of G, 0.4 mM GTP, and *only* in *B*, with 1.6 mM puromycin. At the time intervals indicated, the reactions were stopped: in *A*, by Millipore filtration;[17] in *B*, by addition of sodium acetate pH 5 and extraction of the puromycin products by ethyl acetate.[21] In *B*, the actual experiment yielded both ac-C^{14}-Phe-puromycin and ac-C^{14}-Phe-Phe-puromycin. For the purposes of this experiment, however, the amount of ac-C^{14}-Phe-puromycin formed was subtracted from the total puromycin products.

Discussion.—The results presented indicate that after peptide bond formation has taken place the donor tRNA is not released from the ribosomes. It is only during the translocation step involving G and GTP that the donor tRNA is displaced. Fusidic acid, an antibiotic that inhibits the G reaction, prevents the release of donor tRNA; GMP-PCP will not substitute for GTP.

The finding that the donor tRNA is not released by G and GTP during the acPhe reaction with puromycin suggests that its release must be coupled with translocation.

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† Present address: Service de Biochimie, Département de Biologie du Centre d'Études Nucléaires de Saclay, 91—Gif-sur-Yvette, France.

‡ Abbreviations: acPhe-tRNA, N-acetylphenylalanyl-tRNA; acPhe-Phe-tRNA, N-acetylphenylalanyl-phenylalanyl-tRNA; acPhe-Phe-puromycin, N-acetylphenylalanyl-phenylalanyl-puromycin; acPhe-puromycin, N-acetylphenylalanyl-puromycin; Phe-tRNA, phenylalanyl-tRNA; acPhe, N-acetylphenylalanine; BD-cellulose, benzoylated diethylaminoethyl-cellulose; GMP-PCP, 5′-guanylylmethylene diphosphonate.

[1] Lucas-Lenard, J., and F. Lipmann, these PROCEEDINGS, **55**, 1562 (1966).

[2] Ertel, R., B. Redfield, N. Brot, and H. Weissbach, *Arch. Biochem. Biophys.*, **128**, 331 (1968).

[3] Skoultchi, A., Y. Ono, H. M. Moon, and P. Lengyel, these PROCEEDINGS, **60**, 675 (1968).

[4] Haenni, A-L., and J. Lucas-Lenard, these PROCEEDINGS, **61**, 1363 (1968).

[5] Lucas-Lenard, J., and A-L. Haenni, these PROCEEDINGS, **59**, 554 (1968).
[6] Nishizuka, Y., and F. Lipmann, these PROCEEDINGS, **55**, 212 (1966).
[7] Nishizuka, Y., and F. Lipmann, *Arch. Biochem. Biophys.*, **116**, 344 (1966).
[8] Erbe, R. W., and P. Leder, *Biochem. Biophys. Res. Commun.*, **31**, 798 (1968).
[9] Pestka, S., these PROCEEDINGS, **61**, 726 (1968).
[10] Seeds, N. W., and T. W. Conway, *Biochem. Biophys. Res. Commun.*, **23**, 111 (1966).
[11] Kuriki, Y., and A. Kaji, these PROCEEDINGS, **61**, 1399 (1968).
[12] Stern, J. L., M. Sekiguchi, H. D. Barner, and S. S. Cohen, *J. Mol. Biol.*, **8**, 630 (1964).
[13] von Ehrenstein, G., in *Methods in Enzymology*, ed. L. Grossman and K. Moldave (New York: Academic Press, 1967), vol. 12A, p. 588.
[14] Haenni, A-L., and F. Chapeville, *Biochim. Biophys. Acta*, **114**, 135 (1966).
[15] Wimmer, E., I. H. Maxwell, and G. M. Tener, *Biochemistry*, **7**, 2623 (1968).
[16] Nishizuka, Y., F. Lipmann, and J. Lucas-Lenard, in *Methods in Enzymology*, ed. L. Grossman and K. Moldave (New York: Academic Press, 1968), vol. 12B, p. 708.
[17] Nirenberg, M., and P. Leder, *Science*, **145**, 1399 (1964).
[18] Tanaka, N., T. Kinoshita, and H. Masukawa, *Biochem. Biophys. Res. Commun.*, **32**, 9 (1968).
[19] Hershey, J. W. B., and R. E. Monro, *J. Mol. Biol.*, **18**, 68 (1966).
[20] Lucas-Lenard, J., and A-L. Haenni, unpublished results.
[21] Leder, P., and H. Bursztyn, *Biochem. Biophys. Res. Commun.*, **25**, 233 (1966).