A RIBOSOME-CATALYZED REACTION BETWEEN N-FORMYLMETHIONYL-TRNA AND PUROMYCIN

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Communicated by Ephraim Katchalski, September 20, 1966

The puromycin reaction is considered to be a prototype of the reaction by which peptide bonds are formed during protein biosynthesis.[1-3] The antibiotic is an analogue of the adenosine-amino acid terminus of aminoacyl-tRNA.[4] When added to a ribosomal system during polypeptide synthesis, it is linked by a peptide bond to the carboxyl end of the growing polypeptide, which then becomes detached from the ribosome, no longer being bound to it by a molecule of tRNA.

When aminoacyl-tRNA is employed as substrate in such a system, the puromycin reaction requires all the factors needed for the polymerization of amino acids: ribosomes, supernatant factors, mRNA, and GTP.[5, 6] However, nascent polypeptidyl-tRNA is an effective substrate when it is bound to the isolated 50*S* ribosomal subparticle; and in this case the supernatant factors, mRNA, and the 30*S* ribosomal subparticles are not required for the puromycin-mediated release of nascent polypeptide, i.e., for the formation of a peptide bond.[7, 8] This suggests that the peptide-forming enzyme may be bound to or even built into the 50*S* ribosome; the other factors and structural elements, while required for the sustained synthesis of specific protein chains, may not participate in the actual synthesis of the peptide bond. It has therefore been evident for some time that the puromycin reaction may offer a means of isolating and studying the peptide-forming step of protein synthesis.

We have been interested in the puromycin reaction for this reason, and have felt that it would be more useful for the purpose if the reaction could be made to occur directly with aminoacyl-tRNA without prior peptide synthesis, in analogy to chain initiation. Our attempts to accomplish this failed. Puromycin apparently does not react directly with aminoacyl-tRNA but only with peptidyl-tRNA.[3] An obvious chemical difference between the two substrates is the presence of a charged α-amino group next to the active ester bond of aminoacyl-tRNA. N-carbobenzyloxyphenylalanine-tRNA was thus prepared, and indeed found to undergo a slow but possbly significant puromycin reaction under conditions where the nonblocked species did not react.

The appearance of reports which strongly implicate N-formylmethionyl-tRNA in the synthesis of the first peptide bond in protein chains[9-12] indicated that our considerations may have been correct, but that the formation of an initial peptide bond might be restricted to a particular amino acid (methionine) and a particular blocking group (formyl). Accordingly, we have tested F-met-tRNA and met-tRNA in the puromycin reaction. Our results, presented here, indicate that the formylated species reacts readily with puromycin in the absence of the supernatant factors required for polypeptide synthesis, while the nonformylated species reacts only weakly, if at all, under these conditions. Similar studies have been recently reported by Bretscher and Marcker.[14]

In an earlier paper it is shown that met-tRNA is actually bound to the ribosomes

more effectively than is F-met-tRNA in the presence of the met codon ApUpG, and it is suggested that the formylation of met-tRNA may occur after binding,[13] providing added impetus for studying the effect of formylation on the step which follows binding, the synthesis of the initial peptide bond.

Materials and Methods.—Preparations used in the experiments with phe-tRNA and cbz-phe-tRNA: *E. coli* MRE-600 (RNase I-deficient[15]) cells were dispersed in 2 ml of buffer A (0.01 *M* Tris-HCl, pH 7.4; 0.01 *M* magnesium acetate; 0.06 *M* KCl; 0.006 *M* mercaptoethanol) per gm of cell paste, broken in a French pressure cell, and centrifuged 40 min at 30,000 *g*, and again for 2.5 hr at 78,000 *g*. The high-speed supernatant was used as such for the puromycin reaction and for the aminoacylation of tRNA. The high speed ribosomal pellet was rinsed twice with buffer A, dispersed in 0.5 *M* NH_4Cl–0.001 *M* Tris-HCl, pH 7.8,[16] shaken gently overnight, clarified by a 15-min centrifugation at 13,000 *g*, and centrifuged for 3.5 hr at 105,000 *g*. The ribosome pellet was again treated with NH_4Cl-Tris in the same way, suspended in and dialyzed overnight against buffer A, and stored at −20° in small aliquots at 50 mg/ml. Transfer RNA was prepared from *E. coli* W according to Holley[17] and aminoacylated with H^3-phe according to von Ehrenstein and Lipmann.[18]

Preparations used in the experiments with met-tRNA and F-met-tRNA: The preparation of ribosomes and supernatant fraction, aminoacylation of tRNA, enzymatic formylation, and analysis of F-met-tRNA, and synthesis and characterization of ApUpG were according to Leder and Bursztyn.[13] The preparation of C^{14}-met-tRNA (51.7 $\mu\mu$moles/A_{260} unit) contained less than 5% of the N-formyl derivative; that of F-C^{14}-met-tRNA (74.1 $\mu\mu$moles/A_{260} unit) contained 27% of nonformylated material.

Carbobenzyloxylation of phe-tRNA:[19] Cold carbobenzyloxy chloride (0.2 ml) was added slowly with stirring to 15 mg of H^3-phe-tRNA (14.2 $\mu\mu$moles/A_{260} unit) dissolved in 2 ml of ice-cold 1.0 *M* Tris-HCl, pH 8.0. The mixture was stirred in the cold for 30 min and extracted twice with ether; 0.5 ml of glacial acetic acid and 3 vol of 95% ethanol were added to the aqueous solution. After 15 min in the cold, the precipitated RNA was collected, washed 3 times with cold 95% ethanol, and dried *in vacuo* over $CaCl_2$ and P_2O_5. The degree of carbobenzyloxylation of the final product varied, reaching a maximum of 93%.

The extent of carbobenzyloxylation of the amino group of phe was determined as follows. About 1 mg of the treated RNA was dissolved in 0.3 ml of 1 *M* KOH. After 3 hr at 37°, an aliquot was removed to measure the total radioactivity of the sample. The remaining solution was brought to pH 1–2 with 2 *M* HCl, diluted to 1.0 ml with water, and extracted twice with 2-ml portions of ether. The ether extracts, which contained the N-carbobenzyloxyphenalanine, were combined and evaporated to 1.0 ml in an air stream, and the radioactivity was determined.

The puromycin reaction: The reaction mixtures and incubation conditions for the puromycin reaction are described under the tables The assays were performed as follows. When the substrate was phe-tRNA, cbz-phe-tRNA, or met-tRNA, the puromycin reaction was stopped by the addition of 2 ml of ice-cold 1% potassium acetate, pH 5.3, in 80% ethanol, precipitating tRNA, and unreleased amino acid. After 15 min in ice, the mixtures were centrifuged 20 min in the cold at about 1,000 *g*, and 1.0-ml aliquots of the supernatant fluid were counted in a scintillation counter. The puromycin reaction with F-met-tRNA was assayed according to Leder and Bursztyn[20] as follows: The reaction was stopped by the addition of 1.0 ml of ice-cold water followed by 1.5 ml of ethyl acetate, which, in this case, specifically extracts C^{14}-methionine the amino and carboxyl functions of which are both blocked, such as F-met-puromycin. Met and F-met, being ionized, are essentially insoluble in ethyl acetate. The mixture was shaken vigorously for a few seconds and centrifuged for a few minutes at about 1,000 *g*. The ethyl acetate (upper) phase (1.0 ml) was added to 10 ml of Bray's solution[21] and counted in a scintillation counter.

Characterization of puromycin reaction products: The reaction products were extracted from 1.0-ml volumes of reaction mixture (see Table 2 below) incubated 15 min at 30°. With F-met-tRNA, the supernatant fraction was omitted, and extraction was with 2-ml volumes of ethyl acetate, which were combined and evaporated to dryness *in vacuo*. Met-tRNA was incubated in a complete mixture, after which 3 ml of 1% potassium acetate, pH 5.3, in 80% ethanol were added. After centrifugation the supernatant fluid was taken to dryness in a rotating evaporator. The

TABLE 1

PUROMYCIN REACTION WITH PHE-tRNA AND CBZ-PHE-tRNA

Substrate	Omission	μμMoles H^3-Phe Released: Puromycin Present	Puromycin Absent	Difference due to puromycin
Phe-tRNA	None	11.6	1.2	10.4
	Supernatant	1.9	1.9	0
Cbz-phe-tRNA	None	7.6	6.7	0.9
	Supernatant	1.7	0.2	1.5

The complete reaction mixture (0.2 ml) contained: 0.05 *M* Tris-HCl, pH 7.2; 0.015 *M* Mg acetate; 0.16 *M* NH_4Cl; 0.0006 *M* GTP (Pabst); 0.005 *M* phosphoenolpyruvate (cyclohexylamine salt, Sigma); 5 μg polyuridylic acid (Miles); 4.8 A_{260} units of ribosomes; 0.001 *M* puromycin; supernatant fraction (80 μg protein); 17.9 μμmoles of H^3-phe as phe-tRNA or cbz-phe-tRNA. The cbz-phe-tRNA was 93% carbobenzyloxylated. Incubation was 15 min at 30°. The assay is described in *Materials and Methods.*

dry residues were dissolved in 0.1 ml 10% acetic acid and added to a glass-stoppered flask containing 50 mg cyanogen bromide (CNBr) in 3 ml of 10% acetic acid. This reagent specifically cleaves peptide bonds next to met residues, converting them to homoserine residues.[22] After 20 hr at room temperature, the solution was lyophilized and the residue dissolved in 0.1 ml of water and analyzed by paper chromatography in *n*-butanol-acetic acid-water (the upper phase of a 10:1.2:10 mixture, v/v). Control substances run on the same chromatograms were: L-methionine (Calbiochem); DL-homoserine (N.B.C.); F-met (chemically formylated[13]); and the CNBr products of met (homoserine and presumably a trace of homoserine lactone), F-met (F-homoserine and a trace of homoserine); F-met amide (the same products as with F-met plus an additional spot assumed to be F-homoserine lactone). Spots were located by spraying according to Zahn and Rexroth,[23] and radioactive compounds were located with a paper-strip scanner (Vanguard).

The puromycin product obtained with F-C^{14}-met-tRNA was chromatographed on paper, with authentic F-C^{14}-met-puromycin[20] as a control in the following solvents (v/v): 2-butanol:10% NH_3, 85:15; *n*-butanol:pyridine:glacial acetic acid:H_2O, 30:20:6:24; *n*-butanol:glacial acetic acid:H_2O, 5:1:1.

Results.—The puromycin reaction with phe-tRNA and cbz-phe-tRNA: The unblocked substrate, phe-tRNA, gave the usual rapid puromycin reaction in a complete reaction mixture suitable for polypeptide synthesis (Table 1). Omission of the supernatant fraction abolished this puromycin reaction, leaving only the background hydrolytic release of phe from phe-tRNA which is also observed in the absence of puromycin. With cbz-phe-tRNA, in the complete system, a slow puromycin reaction occurred, nearly obscured by a high hydrolytic background. In this case, however, the puromycin reaction persisted in the absence of the supernatant fraction, with a greatly reduced background. This was seen more clearly in the experiments with F-met-tRNA.

The puromycin reaction with met-tRNA and F-met-tRNA: In a complete reaction mixture containing the methionine codon ApUpG[24, 25] in place of added mRNA, both met-tRNA and its N-formyl derivative reacted with puromycin, and at nearly

TABLE 2

PUROMYCIN REACTION WITH MET-tRNA AND F-MET-tRNA

Omission	μμMoles C^{14}-met Released*: F-met-tRNA	Met-tRNA	Relative Rates: F-met-tRNA	Met-tRNA
None	1.47	1.63	100	100
Supernatant	1.39	0.24	95	15
Ribosomes	0	0	0	0
ApUpG	0.75	0.58	51	36
GTP	1.45	1.55	99	95

The complete reaction mixture (0.1 ml) contained: 0.1 *M* Tris-HCl, pH 7.2; 0.01 *M* Mg acetate; 0.05 *M* K acetate; 0.001 *M* puromycin; 0.0006 *M* GTP; 0.77 A_{260} units of ApUpG; 5.0 A_{260} units of ribosomes; 10 μl supernatant fraction (80 μg protein); 5 μμmoles of C^{14}-met as F-met-tRNA or met-tRNA. Incubation was for 10 min at 30°. The assay is described in *Materials and Methods.*

* The background hydrolytic release of the complete mixture has been subtracted; it is 0.08 (F-met-tRNA) and 0.67 (met-tRNA). The difference is due both to the different substrates and the different assay procedures.

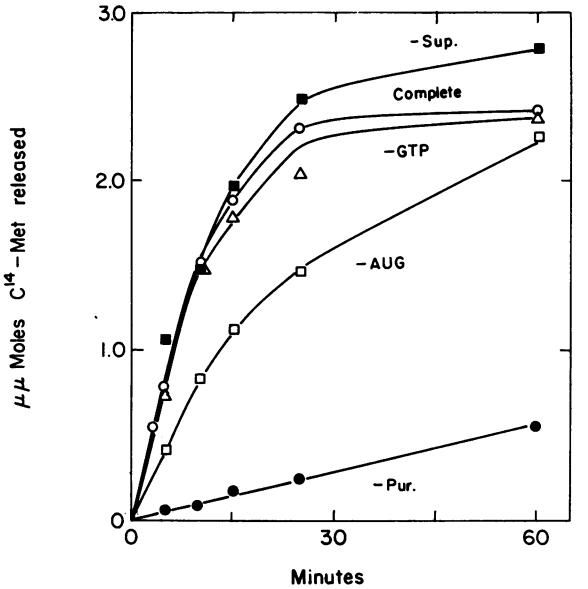

FIG. 1.—Time course of the puromycin reaction with F-C^{14}-met-tRNA. Complete mixture, ○; minus puromycin, ●; minus GTP, △; minus ApUpG, □; minus supernatant fraction, ■. Aliquots of 0.1-ml were assayed. See Table 2 for details.

equal rates (Table 2). However, the two reactions differ strikingly in that formylation abolished the requirement for the supernatant fraction. There was only a partial requirement for ApUpG in both cases, somewhat more pronounced with met-tRNA. Both reactions required ribosomes; neither required added GTP, perhaps owing to contaminating GTP on the ribosomes.

Figures 1 and 2 show the time course of the puromycin reactions and the effects of various omissions on both the rate and extent of the reactions. In the case of F-met-tRNA (Fig. 1), it is again seen that the reaction is independent of added supernatant fraction which, in fact, was slightly inhibitory. The effect of omitting ApUpG was to reduce the rate of the reaction but not its extent. A requirement for GTP did not appear, even after prolonged incubation. With met-tRNA (Fig. 2), both the rate and extent of the reaction were reduced in the absence of either the supernatant fraction or ApUpG. The reaction in the absence of supernatant was slow but sustained and may have resulted from contamination of the ribosomes with supernatant factors. The reaction in the absence of ApUpG leveled off at about 50 per cent of the maximal value obtained in the complete system. Again, no significant requirement for added GTP could be detected.

The nature of the puromycin reaction products with F-met-tRNA and met-tRNA: In the assay employed with F-met-tRNA, the puromycin reaction product was extracted at neutral pH with a solvent in which charged molecules are only slightly soluble, indicating that its terminal amino and carboxyl groups were both blocked. Thus, the product is likely to be either the dipeptide F-met-puromycin or oligopeptides of the type F-met-(amino acid)n-puromycin. Such oligopeptides would be

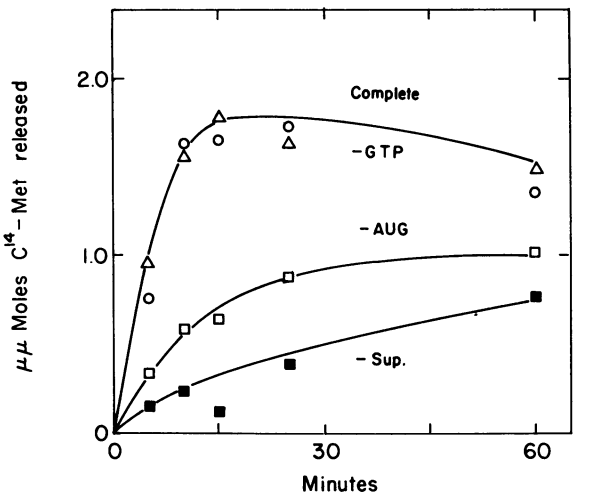

FIG. 2.—Time course of the puromycin reaction with C^{14}-met-tRNA. See Fig. 1 for details.

expected to contain nonformylated C^{14}-met in internal positions, since the preparation of F-C^{14}-met-tRNA employed contained 27 per cent of the nonformylated species. Consequently, these two types of product could be distinguished by testing for internal methionine. For this purpose we have used CNBr, a reagent which specifically cleaves peptides next to met residues and converts the met to homoserine residues.[22] Preliminary control experiments showed that the same treatment converts F-met to F-homoserine nearly quantitatively and without significant deformylation.

F-C^{14}-met-tRNA was reacted with puromycin in the absence of supernatant fraction and the product was extracted, treated with CNBr, and chromatographed as described in *Materials and Methods*. Radioactivity was found either in two spots corresponding to the CNBr products of F-met amide, presumably F-homoserine and its lactone (Fig. 3*a*), or one spot corresponding to the lactone (Fig. 3*b*). No radioactive material corresponding to nonformylated products could be detected. When not treated with CNBr, the puromycin reaction product migrated as a single component, very slightly ahead of the presumed F-homoserine lactone.

When the substrate was C^{14}-met-tRNA, the puromycin reaction mixture contained the supernatant fraction and the products were extracted with a solvent which does not distinguish between formylated and nonformylated products. After CNBr treatment and chromatography, radioactivity was found in one spot corresponding to homoserine; there was no sign of radioactive F-homoserine or its lactone (Fig. 3*c*). This result does not distinguish between the dipeptide met-puromycin and oligopeptides containing met. It does show that the reaction product contains met which is not formylated to a significant extent.

There is additional evidence that F-met-tRNA and met-tRNA form different puromycin products. The radioactive product from F-C^{14}-met-tRNA migrated as a single spot with the mobility of authentic F-met-puromycin, in three solvents in paper chromatography. The solubility of the products in ethyl acetate was not

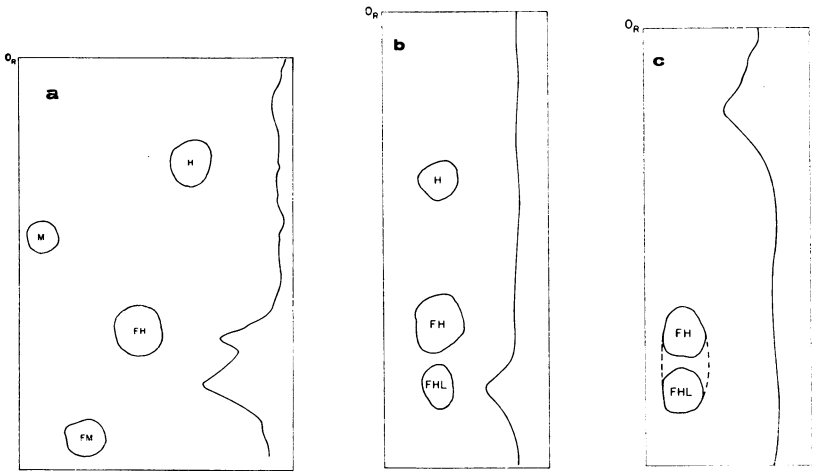

FIG. 3.—Tracings of chromatograms of radioactive puromycin reaction products after degradation with CNBr. The line at the right shows the radioactivity. The spots are control marker compounds; *M*, met; *FM*, F-met; *H*, homoserine; *FH*, N-formylhomoserine; and *FHL*, N-formylhomoserine lactone (produced from F-met amide with CNBr); *Or*, origin. (*a*),(*b*) Product of puromycin reaction with F-C^{14}-met-tRNA. (*c*) Product of puromycin reaction with C^{14}-met-tRNA.

pH-dependent. The radioactive product from C^{14}-met-tRNA showed a marked pH dependence in its solubility in ethyl acetate.[20] Some values observed in a single experiment were: 50 cpm extracted by ethyl acetate from an aqueous phase at pH 5.3, 220 cpm at pH 7.2, 310 cpm at pH 8.8. This behavior is compatible with the presence of a free ionizable α-amino group, and would not be shown by F-met-puromycin. Also, the product of the puromycin reaction with F-met-tRNA is stable in the complete reaction mixture, while experiments not described here have shown that the met-tRNA product is degraded rapidly.

In all, it seems clear that the two substrates form different products with puromycin. With F-met-tRNA, at least a major product is the formylated dipeptide F-met-puromycin. With met-tRNA, the product is not formylated; whether it is a di- or an oligopeptide remains to be shown.

The marked difference in response to the supernatant fraction which distinguishes formylated from nonformylated met-tRNA suggests that the two puromycin reactions proceed by different mechanisms. A trivial alternative—that the supernatant fraction functions by converting met-tRNA to F-met-tRNA through a transformylase reaction—appears ruled out by the demonstration that the puromycin product with met-tRNA is not formylated, and is not produced by the deformylation of F-met-puromycin, since this compound is not deformylated under the same conditions. Thus, while the role of the supernatant fraction is not known, it does not seem to involve formylation.

Discussion.—The experiments reported here indicate that the formylation of the α-amino group of met-tRNA converts it into a substrate for the direct ribosome-catalyzed reaction with puromycin. The reaction does not require added supernatant factors and produces the formylated dipeptide F-met-puromycin. In contrast, met-tRNA shows the usual requirement of aminoacyl-tRNA

for supernatant factors in the puromycin reaction, and forms a product which is different at least to the extent that it is not formylated. Thus, the supernatant fraction appears to fulfill a requirement that is eliminated by prior formylation; however, it does not do so by formylating met-tRNA. The results also suggest, though tentatively, that the effect of blocking the α-amino group may be general, since cbz-phe-tRNA also reacted with puromycin in the absence of supernatant factors while the unblocked species did not.

The observation that the puromycin reaction occurs in the absence of added supernatant factors provides additional support for the view that the peptide-forming enzyme may be located on the ribosome.[7, 8, 26] While the puromycin reaction is analogous to general peptide bond synthesis, the direct puromycin reaction which links puromycin to a single amino acid is analogous to chain initiation, i.e., the synthesis of the first peptide bond in a protein molecule. We therefore believe that the direct puromycin reaction will prove valuable in studying chain initiation as well as the peptide-forming step of protein biosynthesis.

With respect to chain initiation, there is evidence to support the notion that F-met-tRNA may be the normal initiator, at least in *E. coli*.[9-12] The role of the formyl group has not been elucidated. Its effect on the binding of met-tRNA to ribosomes is not to enhance but apparently to depress the binding, and it may not be involved in this step.[13] However, our results make it clear that formylation is involved in a subsequent step, the ribosome-catalyzed synthesis of a peptide bond. For this, either formylation or supernatant factors are required. Our experiments do not clarify the way in which formylation eliminates the need for supernatant factors. A number of possibilities exist, e.g., that the transfer enzyme cannot link puromycin directly to an unblocked aminoacyl-tRNA and the supernatant fraction is required for prior peptide synthesis; or that a supernatant factor interacts with met-tRNA to give it a conformation suitable for chain initiation, with formylation producing an equivalent effect; or others.

The results can also be interpreted more explicitly in terms of a widely considered model which pictures the ribosome as having two binding sites for tRNA.[7, 27, 28] The incoming molecule of aminoacyl-tRNA is bound at one site, accepts the nascent peptide chain from peptidyl-tRNA in the second site with the concomitant formation of a peptide bond, and is then moved to the second site in a translocation reaction which requires supernatant factors and GTP. F-met-tRNA, being an analogue of peptidyl-tRNA, could directly enter the second site, which normally binds peptidyl-tRNA, bypassing the translocation step and its requirement for supernatant factors and GTP.

We did not observe the presumed requirement for added GTP, but the ribosomes may have been contaminated with this compound. The model requires, however, that met-tRNA and F-met-tRNA bind to different ribosomal sites, while a previous report[13] indicates that met-tRNA competes with F-met-tRNA in binding. It is important to determine whether binding, as experimentally observed, involves more than one ribosomal site, and whether binding to one site affects binding to another. These and other aspects of the problem are now under investigation.

Summary.—Ribosomes readily catalyze the formation of the dipeptide N-formylmethionyl-puromycin from N-formylmethionyl-tRNA and puromycin in

the absence of added supernatant factors, while the nonformylated species reacts weakly, if at all. There are indications that in similar conditions N-carbobenzyloxy-phenylalanyl-tRNA reacts, while phenylalanyl-tRNA does not. The results indicate that the peptide-forming enzyme is located on the ribosome under the conditions employed.

We are grateful to Dr. M. Wilchek for advice and aid, Mr. I. Jacobson for materials, Mrs. T. Gabai and Miss H. Bursztyn for assistance, and the U.S. Public Health Service for financial support (grant GM-12588 and Public Law 480 Agreement no. 675133). P. L. was supported by American Cancer Society grant PF 245.

The following abbreviations are used: F, N-formyl; cbz, carbobenzyloxy; met, methionyl, methionine; phe, phenylalanyl, phenylalanine; ApUpG, adenosyl-3′-P-5′-uridylyl-3′-P-5′-guanosine; tRNA, transfer RNA; mRNA, messenger RNA.

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