*TRANSLATION OF THE GENETIC MESSAGE, V. EFFECT OF Mg^{++} AND FORMYLATION OF METHIONINE IN PROTEIN SYNTHESIS**

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Two factors removed during purification of *Escherichia coli* ribosomes are required for translation of natural messenger RNA.[1] Their specific involvement in the transfer of methionine from formyl-met~$tRNA_F$ into peptide linkage, with oligonucleotide messengers having an AUG codon at the 5′-terminus, indicates that they are concerned with chain initiation.[2] These factors act, at least in part, by enhancing the AUG-mediated binding of formyl-met~$tRNA_F$ to ribosomes.[2]

The effect of Mg^{++} and of formylation of met~$tRNA_F$ on the translation of oligonucleotides containing an AUG codon at the 5′-end is described in this paper. Translation of polyadenylic acid (poly A) and other synthetic polynucleotides which do not contain an initiation codon is not influenced by the initiation factors and requires relatively high (18 mM) Mg^{++} concentrations. On the other hand, oligonucleotides having the AUG codon at or near the 5′-end, e.g., AUGAAA...AAA ($AUGA_n$) are efficiently translated at 14 mM and lower Mg^{++} concentrations, provided the initiation factors are present. We have further found that, at low Mg^{++} concentrations (5–8 mM), formylation of met~$tRNA_F$ is essential both for binding to ribosomes and for the transfer of methionine into peptide linkage.

Materials and Methods.—These were as in previous work[2] unless otherwise stated.

Ribosomes, supernatants, and transfer enzymes: Ribosomes from *E. coli* Q13 were purified by *O*-(diethylaminoethyl) cellulose (DEAE-cellulose) chromatography as in previous work.[2] Supernatant fractions were prepared from *Lactobacillus arabinosus*[3] as well as from *E. coli* Q13. Transfer enzymes free of transformylase activity were prepared from *E. coli* Q13 supernatant by ammonium sulfate precipitation between 35 and 65% saturation followed by dialysis and DEAE-cellulose chromatography.[4] The fraction eluted between 0.1 and 0.2 *M* potassium phosphate, pH 7.4, was concentrated by precipitation with ammonium sulfate at 80% saturation and dialyzed overnight against 0.01 *M* potassium phosphate, pH 7.4. Phosphate buffers contained 0.01 *M* 2-mercaptoethanol.

Partial purification of initiation factors: All operations were carried out at 0–4°. A suspension of ribosomal pellets in 0.5 *M* NH_4Cl, 2 mM magnesium acetate, 20 mM Tris-HCl, pH 7.8, was stirred for 14–18 hr. Ten ml of buffer was used for each ribosomal pellet. The supernatant which results from the centrifugation of the ribosomes for 4 hr at 100,000 × *g* was used for the preparation of the factors. Ammonium sulfate was added to this supernatant (21 gm per 100 ml) and the precipitate discarded. Additional ammonium sulfate (18.4 gm per 100 ml) was added to the resulting supernatant and the precipitate, containing the factors, was dissolved in 0.01 *M* potassium phosphate, pH 7.5, 0.01 *M* 2-mercaptoethanol (buffer A) and dialyzed overnight against the same buffer. Approximately 100 mg of protein from the ammonium sulfate fraction, in 10 ml of buffer A, were applied, at 0.4 ml/min, to a column (1.1 × 36 cm) of DEAE-cellulose (0.93 mEq./gm, Bio-Rad) equilibrated with buffer A. The column was washed with 50 ml of 0.025 *M* NH_4Cl in buffer A in order to elute F_1. A linear gradient was then started; the mixing chamber contained 100 ml of 0.1 *M* NH_4Cl and the reservoir 100 ml of 0.3 *M* NH_4Cl, both in buffer A. F_2 was eluted at about 0.18 *M* NH_4Cl. Both factors were concentrated by precipitation with ammonium sulfate to 90% saturation and dialyzed overnight against 0.01 *M* Tris-HCl, pH 7.8, 0.01 *M* 2-mercaptoethanol (buffer B). Approximately 10 mg of F_2 in 1 ml of buffer B were applied, at 0.1 ml/min, to a column of hydroxylapatite (0.6 × 9 cm) equilibrated with buffer A. The

column was washed successively with 10 ml of 0.05 and 0.075 *M* potassium phosphate, pH 7.5, in 0.01 *M* 2-mercaptoethanol. F_2 was eluted with 0.125 *M* potassium phosphate, pH 7.5, 0.01 *M* 2-mercaptoethanol, concentrated by precipitation with ammonium sulfate to 90% saturation, and dialyzed overnight against buffer B. For the routine assay of the activity of the factors the stimulation of lysine incorporation into acid-insoluble material directed by MS2 RNA was followed.[1] Protein was determined spectrophotometrically.[5]

After the DEAE-cellulose step both F_1 and F_2 can be kept frozen for several months without loss of activity. However, after the hydroxylapatite step, F_2 loses activity on freezing. It can be kept at 4° for about 2 weeks. When heated in buffer B, F_2 loses over 90% of its activity in 5 min at 50°; F_1 loses about 90% of its activity in 5 min at 70°. However, when heated at higher salt concentrations (0.08 *M* NH_4Cl, 0.06 *M* Tris-HCl, pH 7.8), F_1 loses only 10–20% of its activity in 5 min at 85°. Both factors have very low nuclease activity.

Transfer RNA: The formylmethionine-accepting species of tRNA ($tRNA_F$) was obtained from *E. coli* B as previously described.[1] Formyl-met $\backsim$ $tRNA_F$ and met $\sim$ $tRNA_F$ were prepared by acylation[6] of the $tRNA_F$ fraction with C^{14}-methionine (specific radioactivity 187 $\mu c/\mu$mole) in the presence or absence of N^{10}-formyltetrahydrofolic acid, respectively. The methionyl-tRNA synthetase and transformylase were prepared from *E. coli* Q13 supernatant essentially as described by Marcker.[7] The fraction eluted from a DEAE-cellulose column between 0.05 and 0.15 *M* potassium phosphate, pH 7.4, free of formyl donor, was used. The methionine residue in formyl-met $\sim$ $tRNA_F$, after release of the formyl methionine by hydrolysis at pH 11.5 for 20 min at 37°, was shown by electrophoresis[8] to be 95% formylated. In the same way, met $\backsim$ $tRNA_F$ was found to be 98% unformylated. H^3-labeled lysyl $\sim$ tRNA (specific radioactivity 168 $\mu c/\mu$mole) was prepared by acylation of unfractionated tRNA from *E. coli* B using *L. arabinosus* supernatant as the source of lysyl $\backsim$ tRNA synthetase.

Amino acid incorporation: Samples contained the following components in a final volume of 0.125 ml: Tris-HCl buffer, pH 7.8, 60 mM; NH_4Cl, 50 mM; 2-mercaptoethanol, 16 mM; magnesium acetate, as indicated; adenosine 5′-triphosphate (pyro) (ATP), 1.2 mM; guanosine 5′-triphosphate (pyro) (GTP), 0.3 mM; phosphocreatine, 18 mM; creatine kinase, 9 μg; *E. coli* W tRNA, 1.1 mg; purified *E. coli* Q13 ribosomes, 7 A_{260} units; *L. arabinosus* supernatant, containing formyl donor, with 0.4 mg of protein; 20 amino acids (one of them with radioactive label), each 0.2 mM; with or without messenger; with or without addition of both factors F_1 and F_2. The specific radioactivity of the labeled amino acids, and the amount of each factor and of polymer used is given in Table 1 and Fig. 1. After incubation for 40 min at 37° the reaction was terminated by addition of KOH (final concentration 0.5 *M*). The mixtures were kept at 37° for 3 hr followed by neutralization and the acid-insoluble radioactivity determined as previously described.[1]

Binding of aminoacyl $\sim$ tRNA to ribosomes: Samples contained, in a volume of 0.05 ml: Tris-HCl buffer, pH 7.2, 100 mM; NH_4Cl, 50 mM; magnesium acetate, 5 or 10 mM; purified ribosomes, 3 A_{260} units; labeled formyl-met $\sim$ $tRNA_F$, 13 $\mu\mu$moles (0.69 A_{260} units) or met $\sim$ $tRNA_F$, 11.5 $\mu\mu$moles (0.55 A_{260} units); with or without the trinucleotide ApUpG (AUG), 0.2 A_{260} units; with or without F_1 (6 μg) and F_2 (10 μg). Binding was determined as described by Nirenberg and Leder.[9] The radioactivity was determined as previously described.[2]

Results and Discussion.—Effect of initiation factors and Mg^{++} on the translation of synthetic oligonucleotides: In Table 1 it may be seen that, at 14 mM Mg^{++}, the incorporation of lysine and methionine in the presence of $AUGA_{\overline{18}}$ is completely dependent on the presence of initiation factors. In contrast, the amino acid incorporation with poly A and with $GGUA_{\overline{24}}$, is small and is not stimulated by the factors. At 18 mM Mg^{++}, in the presence of factors, lysine and methionine incorporation directed by $AUGA_{\overline{18}}$ is only slightly better than at 14 mM Mg^{++}. However, at this Mg^{++} concentration, there is good lysine incorporation with poly A and $GGUA_{\overline{24}}$ in the absence of factors which have no effect on the translation of these polymers. It is apparent from these results that polymers which do not contain an initiation codon can be translated only at higher Mg^{++} concentrations and that the initiation factors do not enhance their translation. It may be also

TABLE 1

EFFECT OF INITIATION FACTORS AND Mg^{++} ON THE TRANSLATION OF $AUGA_{\overline{18}}$, $GGUA_{\overline{24}}$, AND POLY A

Messenger	Mg^{++} concentration (mM)	Factor additions	Amino Acid Incorporation* Lysine	Methionine	Glycine
$AUGA_{\overline{18}}$	14	None	0	0	—
"	"	$F_1 + F_2$	704	115	—
$GGUA_{\overline{24}}$	14	None	91	—	3
"	"	$F_1 + F_2$	32	—	0
Poly A	14	None	262	—	—
"	"	$F_1 + F_2$	81	—	—
$AUGA_{\overline{18}}$	18	None	378	23	—
"	"	$F_1 + F_2$	1280	175	—
$GGUA_{\overline{24}}$	18	None	1346	—	2
"	"	$F_1 + F_2$	1018	—	2
Poly A	18	None	4436	—	—
"	"	$F_1 + F_2$	4106	—	—

Conditions as described under *Amino acid incorporation*. The specific radioactivity of the labeled amino acids (in $\mu c/\mu mole$) was C^{14}-lysine, 2; C^{14}-methionine, 20; C^{14}-glycine, 20. F_1 and F_2 with 12 and 20 μg of protein, respectively. The amount of polymer used was, $AUGA_{\overline{18}}$, 22 μg, $GGUA_{\overline{24}}$, 15 μg, and poly A, 5 μg.

* Net values (blanks without polynucleotide, subtracted from values with polynucleotide) in $\mu\mu$moles/sample. The blanks (essentially the same without or with the addition of factors and at 14 or 18 mM Mg^{++}) averaged 54, 22, and 23 for lysine, methionine, and glycine, respectively.

seen in Table 1 that with $GGUA_{\overline{24}}$, containing a glycine codon at the 5'-terminus, there is essentially no incorporation of this amino acid. Previous results showed that the initial triplet (at the 5'-end) in synthetic oligonucleotides is not read unless it is a formyl-methionine codon.[1, 10] The efficiency of translation of $AUGA_n$ and AAAACAAAA...AAA (A_4CA_n) is illustrated in Figure 1. It may be seen that the rate of the factor-dependent lysine and methionine incorporation with $AUGA_{\overline{18}}$

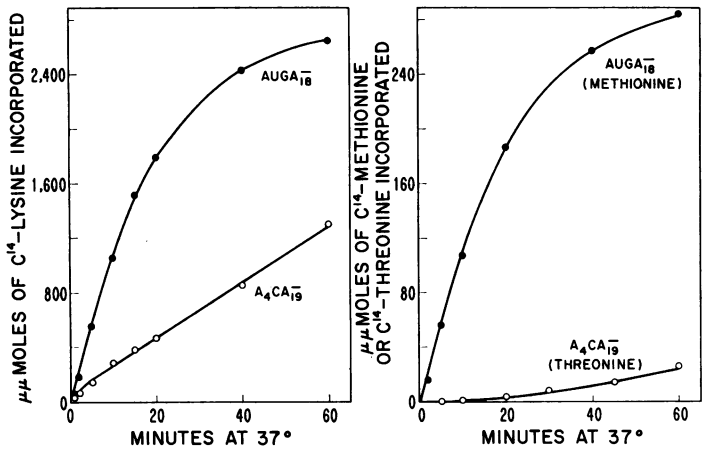

FIG. 1.—Kinetics of amino acid incorporation with $AUGA_{\overline{18}}$ and $A_4CA_{\overline{19}}$. With $AUGA_{\overline{18}}$ (22 μg) the Mg^{++} concentration was 14 mM; F_1 and F_2 with 12 and 20 μg of protein, respectively, were added. With $A_4CA_{\overline{19}}$ (24 μg), the Mg^{++} concentration was 18 mM and no factors were added. The specific radioactivity of the labeled amino acids (in $\mu c/\mu mole$) was C^{14}-lysine, 10; C^{14}-methionine, 20; C^{14}-threonine, 30. The incubation was at 37°. Net values (blanks without polynucleotide subtracted from values with polynucleotide) are given in $\mu\mu$moles/sample. The blank values, essentially the same at different times, averaged 45, 10, and 19 $\mu\mu$moles/sample for lysine, methionine, and threonine, respectively.

is much greater than that of the factor-independent incorporation of lysine and threonine with $A_4CA_{\overline{19}}$. With $AUGA_{\overline{18}}$ the lysine to methionine ratio is close to the value expected from its chain length. However, with $A_4CA_{\overline{19}}$ the ratio of lysine to threonine is far greater than the theoretical value.

Effect of formylation: (*a*) *Binding of formyl-met∼tRNA$_F$ and met∼tRNA$_F$ to ribosomes:* The binding of formyl-met∼tRNA$_F$ and met∼tRNA$_F$ to ribosomes was studied in order to elucidate the role of formylation on this reaction. As previously shown,[2] at 5 mM Mg^{++} the binding of formyl-met∼tRNA$_F$ is completely dependent on the factors (Fig. 2*A*). In contrast, unformylated met∼tRNA$_F$ does not bind to the ribosomes at this magnesium concentration whether in the absence or presence of factors (Fig. 2*A*). On the other hand, at 10 mM Mg^{++} (Fig. 2*B*) there is some binding of formyl-met∼tRNA$_F$ in the absence of factors.

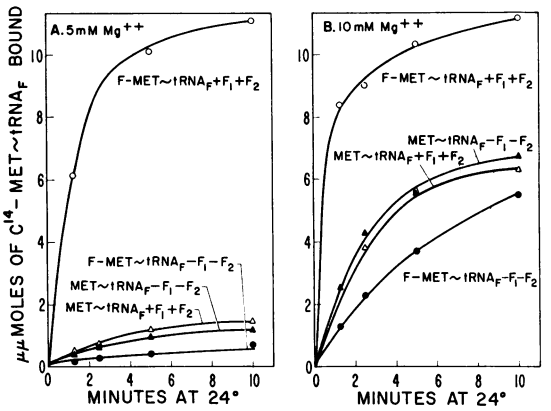

FIG. 2.—Effect of the factors on the binding of formyl-met ∼ tRNA$_F$ or met ∼ tRNA$_F$ to ribosomes. Blanks without AUG (not shown on the figure) were run simultaneously. (*A*) The Mg^{++} concentration was 5 mM. The 10-min blank binding values were (in μμmoles/sample) for formyl-met ∼ tRNA$_F$ 0.09 and 0.84 without and with factors, respectively. The corresponding values for met ∼ tRNA$_F$ were 0.05 and 0.15. (*B*) The Mg^{++} concentration was 10 mM. The 10-min blank binding values were (in μμmoles/sample), for formyl-met ∼ tRNA$_F$ 0.11 and 0.86 without and with factors, respectively. The corresponding values for met ∼ tRNA$_F$ were 0.10 and 0.18. Solid circles (●), formyl-met ∼ tRNA$_F$ without factors; open circles (○) formyl-met ∼ tRNA$_F$ with factors; solid triangles (▲), met ∼ tRNA$_F$ without factors; open triangles (△), met ∼ tRNA$_F$ with factors.

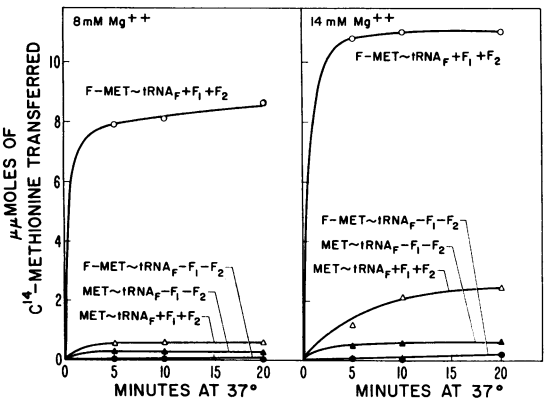

FIG. 3.—Transfer of methionine from formyl-met ∼ tRNA$_F$ or met[3] ∼ RNA$_F$ with $AUGA_{\overline{18}}$. Conditions as described under *Amino acid incorporation* except for the omission of uncharged tRNA, free amino acids, and *L. arabinosus* supernatant. H^3-labeled lysyl ∼ tRNA (103 μμmoles, 10.1 A_{260} units), C^{14}-methionine labeled formyl-met ∼ tRNA$_F$ (23 μμmoles, 1.39 A_{260} units), or met ∼ tRNA$_F$ (22 μμmoles, 1.32 A_{260} units) and 45 μg of *E. coli Q* 13 transfer enzymes prepared as described under *Ribosomes, supernatants and transfer enzymes*, were added. F_1 and F_2 contained 12 and 25 μg of protein, respectively. The Mg^{++} concentration was 8 or 14 mM, as indicated. Incubation at 37°. Net values (blanks without messenger subtracted from values with messenger) are given in μμmoles/sample. The blank values, essentially the same at the various times, were (in μμmoles/sample) for formyl-met ∼ tRNA$_F$, at 8 mM Mg^{++}, 0.07 and 0.25 without and with factors, respectively, and at 14 mM Mg^{++}, 0.09 and 0.42 without and with factors, respectively; for met ∼ tRNA$_F$, essentially the same with or without factors, averaged 0.05 and 0.26 at 8 and 14 mM Mg^{++}, respectively.

It may also be seen in Figure 2*B* that unformylated met∼tRNA$_F$ binds at 10 mM Mg^{++}, but this binding is not stimulated by the factors.

(b) *Transfer of methionine from formyl-met∼tRNA$_F$ or met∼tRNA$_F$ into peptide linkage:* As seen in Figure 3, the transfer of methionine from formyl-met∼tRNA$_F$ is completely dependent on the presence of initiation factors either at 8 or 14 mM Mg^{++}. In contrast, the transfer of methionine from met∼tRNA$_F$ in a system devoid of transformylase is negligible at 8 mM Mg^{++} whether in the absence or presence of factors. At 14 mM Mg^{++} the methionine transferred is higher than at 8 mM^{++} and a small stimulation by factors is observed. However, even after 20 minutes of incubation in the presence of factors, the amount of methionine transferred from met∼tRNA$_F$ is only 20–25 per cent of that transferred from formyl-met∼tRNA$_F$. A further increase in the Mg^{++} concentration to 18 mM did not increase the methionine incorporated from met∼tRNA$_F$.

Recently, it was shown that, at low Mg^{++} concentrations (5–8 mM), the translation of random poly AUG[11] as well as that of natural messengers,[12, 13] is dependent on the presence of a formyl donor. The results presented here indicate that the initiation factors do not function with unformylated met∼tRNA$_F$. At low Mg^{++} concentrations, the binding of met∼tRNA$_F$ to ribosomes as well as the transfer of methionine from met∼tRNA$_F$ into peptide linkage is negligible in the absence or presence of initiation factors.

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