TRANSLATION OF THE GENETIC MESSAGE: FACTORS INVOLVED IN THE INITIATION OF PROTEIN SYNTHESIS*

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We have observed that whereas some synthetic polynucleotides (poly A, poly U) promote polypeptide synthesis in cell-free systems derived from *Escherichia coli* equally well with crude or purified[1] ribosomes, this is not the case with natural messengers such as MS2 phage RNA. Natural mRNA's are effective in systems containing crude ribosomes, but their effect in stimulating the incorporation of various amino acids is markedly reduced when purified ribosomes are used. This suggests that translation of a natural messenger cannot be initiated in the absence of some factors(s) present in crude ribosome preparations.

We should like to report the isolation and partial purification of two factors which are usually removed during the purification of ribosomes. When supplemented with these factors, purified ribosomes are highly efficient in promoting the incorporation of amino acids in the presence of either MS2, Q_β, or TMV-RNA. By using synthetic oligonucleotides with an AUG codon at or near the 5′-terminus, we have shown that these factors are involved in the initiation of polypeptide synthesis. The new factors specifically stimulate the transfer of methionine from one of the two methionine-specific transfer RNA's into peptide linkage. Clark and Marcker[2] have shown that methionine, when esterified to this particular tRNA (met-$tRNA_2$), is capable of being formylated, and, when transferred into peptide linkage, is found only in an amino terminal position.

The results presented in this paper establish that there exists an initiation signal for protein synthesis and that factors other than the transfer enzymes[3] are required for the translation of this signal.

Materials and Methods.—These were as in previous work[1] except as otherwise noted.

Ribosomes and supernatants: *E. coli* Q13 was obtained from W. Gilbert of Harvard University and was grown as described by Haruna and Spiegelman.[4] Ribosomes from *E. coli* Q13 and from *E. coli* W were purified as in previous work by elution from DEAE-cellulose of 1.0 meq/gm with 1.0 *M* ammonium chloride,[1] or from DEAE-cellulose of 0.79 meq/gm with 0.5 *M* ammonium chloride.[5] Ribosomes eluted with 1.0 *M* ammonium chloride were relatively free of the factors F_1 and F_2 (see below); however, ribosomes prepared with 0.5 *M* ammonium chloride still contained appreciable amounts of both F_1 and F_2. Supernatant fractions were prepared from *Lactobacillus arabinosus*[1] as well as from *E. coli* Q13.

Factors from ribosomes: In the preparation of purified ribosomes, the ribosomes are sedimented from a solution containing 0.5 *M* ammonium chloride.[1,5] The supernatant fluid obtained from this high-speed centrifugation was used for the preparation of both factors. By ammonium sulfate fractionation, followed by DEAE-cellulose chromatography, two factors, F_1 and F_2, were resolved. F_2 was further purified by chromatography on hydroxylapatite. Both F_1 and F_2 were free of nuclease activity. The details of the purification and the properties of the factors will be reported elsewhere.

Transfer RNA: Unfractionated tRNA was prepared from *E. coli* W. *E. coli* B tRNA, obtained from General Biochemicals, was fractionated by countercurrent distribution[6] to yield two methionine-accepting species. These fractions were kindly provided by R. W. Chambers of this department. Acylation of tRNA was carried out according to the procedure of von Ehrenstein and Lipmann.[7]

Polynucleotides: RNA was isolated from the phage MS2 by the method of Strauss and Sinsheimer.[8] Q_β RNA and TMV-RNA were obtained from C. Weissmann and J. H. Schwartz, respectively, of this University. Ribosomal RNA was prepared from *E. coli.*[9] The preparation and characterization of the oligonucleotides used in this work will be described in detail elsewhere.[10] The following is an example of the procedures followed.

Synthesis of $A(pA)_3pU^*pG^*(pA)_{\overline{10}}$:[11] $A(pA)_3U^*p$ was prepared by the addition of one single uridylic acid residue (from H^3-labeled UDP) to ApApApA with *Micrococcus lysodeikticus* polynucleotide phosphorylase in the presence of pancreatic ribonuclease. This was followed by removal of the 3′-terminal phosphate with phosphomonoesterase. A single guanylic acid residue (from H^3-labeled GDP) was added to $A(pA)_3pU^*$ with polynucleotide phosphorylase in the presence of T_1 ribonuclease, and was followed by the removal of the terminal phosphate. The $A(pA)_3pU^*pG^*$ was used as primer for the addition of adenylic acid residues from ADP with polynucleotide phosphorylase, essentially by the procedure of Thach and Doty,[12] and the resulting $A(pA)_3pU^*pG^*(pA)_n$ polymers were fractionated by exclusion chromatography on Sephadex G-100 at 25° in 8.0 *M* urea, 0.5 *M* ammonium bicarbonate, pH 8.6.[13] Polymers of the desired molecular weight were recovered by rotary evaporation after exhaustive dialysis against distilled water. The base sequence of the polymer was confirmed by the identification of the H^3-labeled products ApApApApU*p and G*p following digestion of the polymer with pancreatic and T_1 ribonucleases.

Amino acid incorporation: Components of the *in vitro* system, with the exception of ribosomes and supernatants as noted above, were as previously described.[1] Incubations with synthetic oligonucleotides were terminated by the addition of an equal volume of 1 *M* KOH. After 16 hr at 37°, the samples were neutralized and the acid-insoluble radioactivity was precipitated with either 5% TCA containing 0.25% sodium tungstate (reactions with A-rich polymers) or with 5% TCA (reactions with U-rich polymers). Incubations with viral or ribosomal RNA were terminated by the addition of 5% TCA and heating at 90° for 15 min. Precipitated material was collected on Millipore filters, and radioactivity was measured in a window gas-flow counter. Radioactive amino acids were obtained from New England Nuclear with the exception of S^{35}-methionine, 42.9 μc/μmole, which was purchased from Schwarz BioResearch.

Results.—Translation of oligonucleotides of specified base sequence: Table 1 presents a summary of incorporation results with oligonucleotides of specified base sequence. With the exception of AUG, the initial triplet in these polymers is infrequently read; however, as already reported for ACA and AAC,[14] the second triplet is translated specifically. The following codon assignments can be made based on these results: AAC and AAU, asparagine;[1, 14] ACA, threonine;[14] AUA, isoleucine;[15] GAA, glutamic acid;[15] GCA, alanine; and AUG, methionine.

TABLE 1

SUMMARY OF AMINO ACID INCORPORATION RESULTS WITH OLIGONUCLEOTIDES OF SPECIFIED BASE SEQUENCE AS MESSENGERS

Polymer	Triplet First	Triplet Second	Triplet Last	Amino acids incorporated
$(A)_nC$	AAA	AAA	**AAC**	Lys, **Asn**
$(A)_nU$	AAA	AAA	**AAU**	Lys, **Asn**
$C_3(A)_n$	CCC	AAA	AAA	Lys
$A_2C(A)_n$	AAC	AAA	AAA	Lys
$A_4C(A)_n$	AAA	**ACA**	AAA	Lys, **Thr**
$A_5C(A)_n$	AAA	**AAC**	AAA	Lys, **Asn**
$A_4U(A)_n$	AAA	**AUA**	AAA	Lys, **Ile**
$A_3G(A)_n$	AAA	**GAA**	AAA	Lys, **Glu**
$A_3GC(A)_n$	AAA	**GCA**	AAA	Lys, **Ala**
$AGC(A)_n$	AGC	AAA	AAA	Lys
$AGU(A)_n$	AGU	AAA	AAA	Lys
$G(U)_n$	GUU	UUU	UUU	Phe
$GGU(A)_n$	GGU	AAA	AAA	Lys
$GG(U)_n$	GGU	UUU	UUU	Phe
$A_4UG(A)_n$	AAA	**AUG**	AAA	Lys, **Met**
$AUG(A)_n$	**AUG**	AAA	AAA	Lys, **Met**
$AUG(U)_n$	**AUG**	UUU	UUU	Phe, **Met**

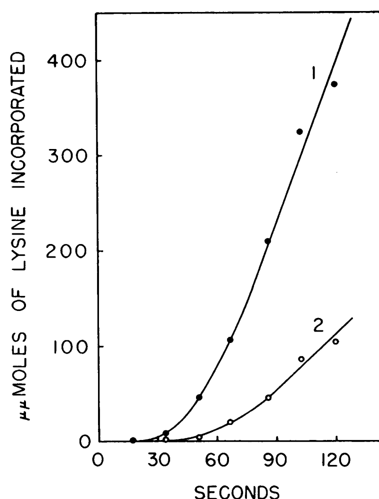

FIG. 1.—Kinetics of lysine incorporation into peptide chains with AUG-$(A)_{24}$ (curve *1*) or AGU$(A)_{24}$ (curve *2*) as messenger. Conditions as described under *Amino acid incorporation*. The supernatant fluid was from *L. arabinosus*. The ribosomes, from *E. coli* W, were purified by elution from DEAE-cellulose with 0.5 *M* ammonium chloride. Samples (final vol, 0.1 ml) had 0.014 *M* magnesium acetate, 19 unlabeled amino acids, and C^{14}-labeled lysine (2.0 μc/μmole). The concentration of each amino acid was 0.2 μmole/ml; that of each polynucleotide, 107 μg/ml. Incubation at 37°.

The translation of the polymers having AUG in the first or second position is much faster than that of the others. This is illustrated in Figure 1 which shows a kinetic analysis of the incorporation of lysine in the presence of either AUG$(A)_{24}$ or AGU$(A)_{24}$. It is clear that an AUG codon at the 5′-terminus of an oligonucleotide facilitates the translation of the remaining messenger. The results suggest that this codon is concerned with the initiation of polypeptide synthesis.

Translation of natural messengers: With natural messengers, such as MS2, Q_β, and TMV-RNA, purified ribosomes are quite inefficient in promoting protein synthesis. As shown in Table 2, factors (F_1 and F_2), normally discarded during the purification of ribosomes, enhance the incorporation of amino acids. Addition of both factors results in a five- to tenfold stimulation of incorporation; either factor alone produces only a marginal effect. On the other hand, the incorporation of lysine promoted by poly A proceeds rapidly in the absence of added factors and their addition has no effect whatsoever on the reaction. In the case of MS2 RNA, it is to be noted that histidine incorporation is stimulated to the same relative extent as other amino acids, indicating that the synthesis of polypeptides other than MS2 coat protein, which contains no histidine,[16] is also increased.

Effect of factors on the translation of oligonucleotides of specified base sequence: In order to elucidate the role of the factors in protein biosynthesis, a series of synthetic polynucleotides of both random and specified base sequence was tested. In the presence of poly UG (5:1) and of poly UAG (6:1:1), the factors gave a two- to threefold stimulation of both phenylalanine and methionine incorporation. Results with oligonucleotides of specified base sequence, summarized in Table 3, clearly indicate that the effect of the factors may be correlated with an AUG (methionine) codon at or near the 5′-terminus of the messenger. Control polymers containing the triplet GGU (which strongly induces the binding of glycine tRNA to ribosomes)[17] or the triplet AGU (which has the same base composition as AUG) gave no response to the factors.

Transfer of methionine from methionyl∼tRNA into peptide linkage: As seen in Table 4, the transfer of labeled methionine from (mixed) met-tRNA into peptide linkage, in the presence of unlabeled lysyl∼tRNA and AUG$(A)_{13}$, is markedly

TABLE 2

Effect of Factors on Polypeptide Synthesis with Poly A and Natural Messengers*

Experiment	Messenger	Factor additions	Amino Acid Incorporation† Lys	Leu	Met	His
1	Poly A (10 μg)	None	4898	—	—	—
		F_1 (14.5 μg) + F_2 (36 μg)	4518	—	—	—
	MS2 RNA (90 μg)	None	64	68	15	10
		F_1 (14.5 μg)	138	—	—	—
		F_2 (36 μg)	174	—	—	—
		F_1 (14.5 μg) + F_2 (36 μg)	**606**	**767**	**155**	**92**
2	MS2 RNA (90 μg)	None	84	—	—	—
		F_1 (14.5 μg) + F_2 (17 μg)	**504**	—	—	—
	Qβ RNA (96 μg)	None	332	—	—	—
		F_1 (14.5 μg) + F_2 (17 μg)	**1200**	—	—	—
	TMV-RNA (90 μg)	None	348	—	—	—
		F_1 (14.5 μg) + F_2 (17 μg)	**1120**	—	—	—
	Ribosomal RNA (80 μg)	None	0	—	—	—
		F_1 (14.5 μg) + F_2 (17 μg)	1	—	—	—

* Conditions as described under "Amino acid incorporation." Supernatant fluid and ribosomes were from *E. coli* Q13. For purification, the ribosomes were eluted from DEAE-cellulose with 1.0 *M* ammonium chloride. Samples, in a final vol of 0.25 ml, were incubated for 40 min at 37°. The samples with poly A had 2.4 mg of tRNA, 0.018 *M* magnesium acetate, and no unlabeled amino acids. All other samples had 0.5 mg of tRNA, 0.014 *M* magnesium acetate, and 19 unlabeled amino acids (each at 0.1 μmole/ml). The specific radioactivity of the labeled amino acids, in μc/μmole, was lysine (C^{14}), 10; leucine (C^{14}), 10; histidine (C^{14}), 20; and methionine (S^{35}), 42.9.

† Net values (blanks without added polynucleotide subtracted from values with polynucleotide) in μμmoles/sample. All values are the average of duplicate runs. The blanks for lysine (in expts. 1 and 2) and those for leucine, methionine, and histidine (in expt. 1) averaged 9, 31, 6, and 15, respectively, with no added factors, and 27, 83, 13, and 17, with addition of $F_1 + F_2$. The lysine blank (expt. 1) with addition of F_1 was 12, and with addition of F_2, 17.

TABLE 3

Effect of Factors on Polypeptide Synthesis Directed by Oligonucleotides of Specified Base Sequence*

Experiment	Messenger	Factor additions	Amino Acid Incorporation† Lys	Met	Gly	Ser
1	$AUG(A)_{\overline{13}}$ (21 μg)	None	413	28	—	—
		F_1 (8 μg)	362	31	—	—
		F_2 (4 μg)	525	48	—	—
		F_1 (8 μg) + F_2 (4 μg)	**984**	**87**	—	—
2	$AUG(A)_{\overline{13}}$ (21 μg)	None	311	24	—	—
		F_1 (13 μg) + F_2 (13 μg)	**916**	**87**	—	—
	$A_4UG(A)_{\overline{10}}$ (23 μg)	None	156	9	—	—
		F_1 (13 μg) + F_2 (13 μg)	**741**	**81**	—	—
3	$AUG(A)_{\overline{13}}$ (21 μg)	None	220	23	—	—
		F_1 (8 μg) + F_2 (7 μg)	**1220**	**115**	—	—
	$GGU(A)_{\overline{13}}$ (19 μg)	None	72	—	1	—
		F_1 (8 μg) + F_2 (7 μg)	41	—	1	—
	$AGU(A)_{\overline{13}}$ (26 μg)	None	45	—	—	0
		F_1 (8 μg) + F_2 (7 μg)	30	—	—	0

* Conditions as described under "Amino acid incorporation." Samples, in a final volume of 0.125 ml, were incubated for 40 min at 37°. The supernatant fluid was from *L. arabinosus*. The ribosomes, from *E. coli* Q13, were purified by elution from DEAE-cellulose with 1.0 *M* ammonium chloride. Each sample contained 19 unlabeled amino acids and one labeled amino acid. The specific radioactivity of the latter, in μc/μmole, was lysine (C^{14}), 2; methionine (S^{35}), 42.9; glycine (C^{14}), 47; and serine (C^{14}), 30.

† Net values (blanks without polynucleotide subtracted from values with polynucleotide) in μμmoles/sample. All values are the average of duplicate runs. The blanks (virtually the same without or with the addition of $F_1 + F_2$) averaged 85 for lysine, 10 for methionine, 18 for glycine, and 17 for serine.

stimulated by the factors. The transfer of methionine from met-$tRNA_1$ or met-$tRNA_2$ was then studied in the presence of either $AUG(A)_{\overline{13}}$ or $A_4UG(A)_{\overline{10}}$. Table 4 shows that transfer from met-$tRNA_1$ occurs with $A_4UG(A)_{\overline{10}}$ but not with $AUG(A)_{\overline{13}}$ as messenger, i.e., only when the AUG codon is in the second (internal) position. On the other hand, transfer from met-$tRNA_2$ takes place with either $AUG(A)_{\overline{13}}$ or $A_4UG(A)_{\overline{10}}$. Moreover, whereas the transfer of methionine from met-

TABLE 4

TRANSFER OF METHIONINE FROM METHIONYL~tRNA INTO PEPTIDE LINKAGE*

		Methionine Transferred†		
		Expt. 1	Expt. 2	
Polymer	Factor additions	Met-tRNA (mixed)	Met-tRNA$_1$	Met-tRNA$_2$
None	None	(4.7)	(0.46)	(0.14)
	$F_1 + F_2$	(6.9)	(0.46)	(0.12)
AUG$(A)_{\overline{13}}$ (21 μg)	None	1.3	0.05	1.44
	$F_1 + F_2$	**32.4**	0.04	**2.97**
A_4UG$(A)_{\overline{10}}$ (16 μg)	None	—	1.78	0.72
	$F_1 + F_2$	—	1.82	**1.93**

* Conditions as described under "Amino acid incorporation." Samples, in a final volume of 0.125 ml, were incubated for 40 min at 37°. Factor additions of F_1 and F_2 corresponded to 14.5 and 17 μg, respectively. The supernatant fluid was from *L. arabinosus*. *Expt. 1:* *E. coli* Q13 ribosomes were purified by elution from DEAE-cellulose with 1.0 *M* ammonium chloride. Samples contained unlabeled lysine (25 mμmoles), tRNA (1.2 mg), and 194 μμmoles of C^{14}-methionyl~tRNA (specific radioactivity, in μc/μmole, 198). *Expt. 2:* *E. coli* W ribosomes were purified by elution from DEAE-cellulose with 0.5 *M* ammonium chloride. Samples contained unlabeled lysine and methionine (25 mμmoles each), 0.8 mg tRNA, and either 14.0 μμmoles of S^{35}-methionyl~tRNA$_1$ or 21.0 μμmoles of S^{35}-methionyl~tRNA$_2$ (specific radioactivity of S^{35}-methionine, in μc/μmole, 42.9).

† Net values (blanks without polynucleotide—given in parentheses in the table—subtracted from values with polynucleotide) in μμmoles/sample. All values are the average of duplicate runs.

tRNA$_2$ is stimulated by the factors, transfer from met-tRNA$_1$ occurs in the absence of added factors and is not enhanced by their addition.

In line with the results of the transfer experiments, when assayed by the technique of Nirenberg and Leder,[18] met-tRNA$_2$ is bound to ribosomes in the presence of either A_4UG$(A)_{\overline{10}}$ or AUG$(A)_{\overline{13}}$. However, this binding is not stimulated by the factors.

The above experiments clearly show that the factors specifically affect some reaction involved in the formation of the initial peptide bond between methionine from met-tRNA$_2$ and the next aminoacyl~tRNA.

Discussion.—The recent discovery of a methionine tRNA (met-tRNA$_2$) which is able to transfer an amino acid residue with a formylated amino group into polypeptide linkage,[19, 20] has led to the demonstration that this tRNA is involved in the initiation of protein biosynthesis in *E. coli*. Adams and Capecchi,[21] and Webster *et al.*[22] found that in cell-free *E. coli* systems primed with phage RNA, the residue at the NH_2-terminal end of the polypeptides formed is N-formylmethionine. Binding experiments[2] indicate that the codon corresponding to met-tRNA$_2$ may be either GUG, UUG, or AUG. It has been suggested,[23, 24] also on the basis of binding experiments, that the AUG codon at or near the 5′-terminus promotes "in phase" reading of oligonucleotides.

The results presented in this paper show that factors, normally associated with ribosomes, enhance the translation of natural messengers in an *in vitro* system. Some light on the role played by these factors in protein synthesis was shed by the use of synthetic oligonucleotides containing an AUG (methionine) codon at or near the 5′-terminus. The factors specifically stimulated the transfer into peptide linkage of methionine from met-tRNA$_2$, which is specific for N-formylmethionine, and greatly facilitated the translation of the remainder of the chain yielding an over-all net increase of polypeptide synthesis.

As already mentioned, one of the possible functions of the factors is the formation of the first peptide bond between methionine, esterified to met-tRNA$_2$, and the following amino acid. This is suggested by the fact that the factors exert their stimulatory effect in the presence of an excess of transfer enzymes and are not

involved in the activation or formylation of methionine or in the binding of met-$tRNA_2$ to ribosomes.

Finally, it is to be noted that the coding of an amino acid with a blocked amino group, therefore necessarily amino terminal, by a codon at the 5′-end of a messenger is a direct confirmation of earlier determinations of the direction of reading of the genetic message.[1, 14]

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