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 purified1 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, Q₆, 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 Marcker2 have shown that methionine, when esterified to this particular tRNA (met-tRNA₂), 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 are required for the translation of this signal.

**Materials and Methods.**—These were as in previous work1 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 F₁ and F₂ (see below); however, ribosomes prepared with 0.5 M ammonium chloride still contained appreciable amounts of both F₁ and F₂. 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.⁴,⁵ 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, F₁ and F₂, were resolved. F₂ was further purified by chromatography on hydroxylapatite. Both F₁ and F₂ 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 distribution⁶ 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 Sin-
sheimer.\textsuperscript{6} Q\textsubscript{8} RNA and TMV-RNA were obtained from C. Weissmann and J. H. Schwartz, re-
spectively, of this University. Ribosomal RNA was prepared from E. coli.\textsuperscript{9} The preparation
and characterization of the oligonucleotides used in this work will be described in detail else-
where.\textsuperscript{8} The following is an example of the procedures followed.

Synthesis of A\textsubscript{2}G\textsubscript{2}A\textsubscript{2}U\textsubscript{2}pG\textsubscript{2}(pA)\textsubscript{2} was prepared by the addition of one single
uridylic acid residue (from H\textsuperscript{3}-labeled UDP) to ApApApA with Micrococcus lysodeikticus poly-
nucleotide phosphorylase in the presence of pancreatic ribonuclease. This was followed by re-
moval of the 3'-terminal phosphate with phosphomonoesterase. A single guanylic acid residue
(from H\textsuperscript{3}-labeled GDP) was added to A(pA)\textsubscript{2}pU\textsubscript{2}pG\textsubscript{2} with polynucleotide phosphorylase in the pres-
ence of T\textsubscript{2}, ribonuclease, and was followed by the removal of the terminal phosphate. The A(pA)\textsubscript{2}pU\textsubscript{2}pG\textsubscript{2} was used as primer for the addition of adenyl acid residues from ADP with polynucleo-
tide phosphorylase, essentially by the procedure of Thach and Doty,\textsuperscript{12} and the resulting A(pA)\textsubscript{2}pU\textsubscript{2}pG\textsubscript{2}(pA)\textsubscript{2} polymers were fractionated by exclusion chromatography on Sephadex G-100 at
25\textdegree\ in 8.0 M urea, 0.5 M ammonium bicarbonate, pH 8.6.\textsuperscript{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\textsuperscript{3}-labeled products

Amino acid incorporation: Components of the in vitro system, with the exception of ribosomes
and supernatants as noted above, were as previously described.\textsuperscript{1} Incubations with synthetic
oligonucleotides were terminated by the addition of an equal volume of 1 M KOH. After 16 hr
at 37\textdegree, 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\textdegree 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\textsuperscript{35}methionine, 42.9 \textmu c/\mu mole, 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,\textsuperscript{14} the second triplet is translated specifically. The following codon assignments can be made based on these results: AAC, Asp; AAA, asparagine;\textsuperscript{14} ACA, threonine;\textsuperscript{14} AUA, isoleucine;\textsuperscript{15} GAA, glutamic acid;\textsuperscript{15} GCA, alanine; and AUG, methionine.

\begin{table}[h]
\centering
\caption{Summary of Amino Acid Incorporation Results with Oligonucleotides of Specified Base Sequence as Messengers}
\begin{tabular}{|c|c|c|c|}
\hline
Polymer & First & Second & Last & Amino acids \hline
(A)\textsubscript{3}C & AAA & AAA & AAC & Lys, Asn \hline
(A)\textsubscript{3}U & AAA & AAA & AAU & Lys, Asn \hline
C\textsubscript{3}A & CCC & AAA & AAA & Lys \hline
A\textsubscript{3}C\textsubscript{3}A & AAC & AAA & AAA & Lys, Thr \hline
A\textsubscript{3}C\textsubscript{3}A & AAA & ACA & AAA & Lys, Asp \hline
A\textsubscript{3}A\textsubscript{3}A & AAA & AAC & AAA & Lys, Ile \hline
A\textsubscript{3}G\textsubscript{3}A & AAA & GAA & AAA & Lys, Glu \hline
A\textsubscript{3}G\textsubscript{3}C\textsubscript{3}A & AAA & GCA & AAA & Lys, Ala \hline
AGC\textsubscript{3}A & AGC & AAA & AAA & Lys \hline
AGU\textsubscript{3}A & AGU & AAA & AAA & Lys \hline
G\textsubscript{3}U & GGU & AAA & AAA & Lys \hline
GGU\textsubscript{3}A & GGU & UUU & UUU & Phe \hline
GGG\textsubscript{3}U & GGU & UUU & UUU & Phe \hline
AUG\textsubscript{3}A & AUG & AAA & AAA & Lys, Met \hline
AUG\textsubscript{3}U & AUG & UUU & UUU & Phe, Met \hline
\hline
\end{tabular}
\end{table}
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)34. 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, Qφ, and TMV-RNA, purified ribosomes are quite inefficient in promoting protein synthesis. As shown in Table 2, factors (F₁ and F₂), 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, 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) 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)34, is markedly
TABLE 2

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Messenger</th>
<th>Factor additions</th>
<th>(\text{Amino Acid Incorporation}^\dagger)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Poly A (10 (\mu)g)</td>
<td>None</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(F_1) (14.5 (\mu)g) + (F_2) (36 (\mu)g)</td>
<td>4518</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(F_1) (14.5 (\mu)g)</td>
<td>64</td>
</tr>
<tr>
<td>2</td>
<td>MS2 RNA (90 (\mu)g)</td>
<td>None</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(F_1) (14.5 (\mu)g) + (F_2) (36 (\mu)g)</td>
<td>138</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(F_1) (14.5 (\mu)g)</td>
<td>174</td>
</tr>
<tr>
<td></td>
<td>Q(\alpha) RNA (96 (\mu)g)</td>
<td>None</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(F_1) (14.5 (\mu)g) + (F_2) (17 (\mu)g)</td>
<td>504</td>
</tr>
<tr>
<td></td>
<td>TMV-RNA (90 (\mu)g)</td>
<td>None</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(F_1) (14.5 (\mu)g) + (F_2) (17 (\mu)g)</td>
<td>1200</td>
</tr>
<tr>
<td></td>
<td>Ribosomal RNA (80 (\mu)g)</td>
<td>None</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(F_1) (14.5 (\mu)g) + (F_2) (17 (\mu)g)</td>
<td>1120</td>
</tr>
</tbody>
</table>

*Conditions as described under “Amino acid incorporation.” Supernatant fluid and ribosomes were from \(E.\)\(\text{coli} Q3.\) For purification, the ribosomes were eluted from DEAE-cellulose with 1.0 \(M\) ammonium chloride. Samples in a final vol of 0.25 \(\text{ml.}\) were incubated for 40 min at 37°. Samples with poly A had 2.4 \(\mu\)g of tRNA, 0.018 \(M\) magnesium acetate, and no unlabeled amino acids. All other samples had 0.5 \(\mu\)g of tRNA, 0.014 \(M\) magnesium acetate, and 19 unlabeled amino acids (each at 0.1 \(\mu\)mole/ml). The specific radioactivity of the labeled amino acids, in \(\mu\)c/\(\mu\)mole, was lysine (C)\(\text{4}), 10;\) leucine (C)\(\text{6}), 10; histidine (C)\(\text{4}), 20;\) and methionine (S)\(\text{4}), 42.9.*

† Net values (blanks without polynucleotide subtracted from values with polynucleotide) in \(\mu\)moles/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 new factors, and 27, 85, 13, and 17, with addition of \(F_1 + F_2.\) The lysine blank (expt. 1) with addition of \(F_2\) was 12, and with addition of \(F_0, 17.\)

TABLE 3

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Messenger</th>
<th>Factor additions</th>
<th>(\text{Amino Acid Incorporation}^\dagger)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>AUG(A)(\text{13}) (21 (\mu)g)</td>
<td>None</td>
<td>413</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(F_1) (8 (\mu)g)</td>
<td>362</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(F_2) (4 (\mu)g)</td>
<td>525</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(F_1) (8 (\mu)g) + (F_2) (4 (\mu)g)</td>
<td>984</td>
</tr>
<tr>
<td>2</td>
<td>AUG(A)(\text{13}) (21 (\mu)g)</td>
<td>None</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(F_2) (13 (\mu)g) + (F_2) (13 (\mu)g)</td>
<td>916</td>
</tr>
<tr>
<td></td>
<td>AUG(A)(\text{15}) (23 (\mu)g)</td>
<td>None</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(F_2) (13 (\mu)g) + (F_2) (13 (\mu)g)</td>
<td>741</td>
</tr>
<tr>
<td>3</td>
<td>AUG(A)(\text{13}) (21 (\mu)g)</td>
<td>None</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(F_2) (8 (\mu)g) + (F_2) (7 (\mu)g)</td>
<td>1220</td>
</tr>
<tr>
<td></td>
<td>GGU(A)(\text{19}) (19 (\mu)g)</td>
<td>None</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(F_1) (8 (\mu)g) + (F_2) (7 (\mu)g)</td>
<td>41</td>
</tr>
<tr>
<td></td>
<td>AGU(A)(\text{19}) (26 (\mu)g)</td>
<td>None</td>
<td>45</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(F_1) (8 (\mu)g) + (F_2) (7 (\mu)g)</td>
<td>30</td>
</tr>
</tbody>
</table>

*Conditions as described under “Amino acid incorporation.” Samples, in a final vol of 0.125 \(\text{ml.}\) were incubated for 40 min at 37°. The supernatant fluid was from \(L.\)\(\text{arabinose.}\) The ribosomes, from \(E.\)\(\text{coli} Q3,\) 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 \(\mu\)c/\(\mu\)mole, was lysine (C)\(\text{4}), 2;\) methionine (S)\(\text{4}), 42.9;\) glycine (C)\(\text{4}), 47;\) and serine (C)\(\text{4}), 30.*

† Net values (blanks without polynucleotide subtracted from values with polynucleotide) in \(\mu\)moles/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\(\text{1}\) or met-tRNA\(\text{2}\) was then studied in the presence of either AUG(A)\(\text{13}\) or \(AUG(A)\(\text{15}\). Table 4 shows that transfer from met-tRNA\(\text{1}\) occurs with \(AUG(A)\(\text{15}\) but not with AUG(A)\(\text{13}\) as messenger, i.e. only when the AUG codon is in the second (internal) position. On the other hand, transfer from met-tRNA\(\text{2}\) takes place with either AUG(A)\(\text{13}\) or \(AUG(A)\(\text{15}\). Moreover, whereas the transfer of methionine from met-
tRNA₂ is stimulated by the factors, transfer from met-tRNA₁ 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-tRNA₂ is bound to ribosomes in the presence of either A₄UG(A)₁₆ or AUG(A)₁₆. 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-tRNA₂ and the next aminoacyl-tRNA.

**Discussion.**—The recent discovery of a methionine tRNA (met-tRNA₂) 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 NH₂-terminal end of the polypeptides formed is N-formylmethionine. Binding experiments²³ indicate that the codon corresponding to met-tRNA₂ may be either GUG, UUG, or AUG. It has been suggested²³, 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-tRNA₂, 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-tRNA₂, 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

---

**TABLE 4**

**TRANSFER OF METHIONINE FROM METHIONYL-tRNA INTO PEPTIDE LINKAGE***

<table>
<thead>
<tr>
<th>Polymer</th>
<th>Factor additions</th>
<th>Met-tRNA (mixed)</th>
<th>Met-tRNA₁</th>
<th>Met-tRNA₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>None</td>
<td>4.7 (0.46)</td>
<td>0.46 (0.14)</td>
<td></td>
</tr>
<tr>
<td>A₄UG(A)₁₆</td>
<td>None</td>
<td>6.9 (0.46)</td>
<td>0.05 (0.12)</td>
<td>1.44</td>
</tr>
<tr>
<td>A₄UG(A)₁₆</td>
<td>F₁ + F₂</td>
<td>32.4 (0.04)</td>
<td>2.97</td>
<td></td>
</tr>
<tr>
<td>A₄UG(A)₁₆</td>
<td>F₁ + F₂</td>
<td></td>
<td>1.78</td>
<td>0.72</td>
</tr>
<tr>
<td>A₄UG(A)₁₆</td>
<td>F₁ + F₂</td>
<td></td>
<td>1.82</td>
<td>1.93</td>
</tr>
</tbody>
</table>

* Conditions as described under "Amino acid incorporation." Samples, in a final volume of 0.125 ml, were incubated for 40 min at 37°C. Factor additions of F₁ and F₂ corresponded to 14.5 and 17.5 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 μmole), tRNA (1.2 mg), and 194 μmole of C¹⁴-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 μmole each), 0.8 mg tRNA, and either 14.0 μmole of S³⁵-methionyl-tRNA or 21.0 μmole of S¹⁴-methionyl-tRNA (specific radioactivity of S³⁵-methionine, in μu/μmole, 42.9).

† Net values (blanks without polynucleotide—given in parentheses in the table)—subtracted from values with polynucleotide in μmole/sample. All values are the average of duplicate runs.
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.\textsuperscript{1, 14}

We are indebted to Dr. Jesse C. Rabinowitz, University of California at Berkeley, for the formyl-tetrahydrofolate synthetase which allowed us to identify met-tRNA$_2$; to Dr. Merrill B. Hille for the assay and acylation of the met-tRNA$_1$ and met-tRNA$_2$ fractions; and to Dr. Jerold A. Last for the preparation of C$_6$(A)$_n$ oligonucleotides. Our thanks are also due to Mr. Horace Lozina for the preparation of tRNA and of ribosomal and supernatant fractions, and to Miss Maria Pinney and Miss Jana Krausova for skillful technical assistance.

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† International postdoctoral fellow of the National Institutes of Health, U.S. Public Health Service. Permanent address: Instituto Marañon, Centro de Investigaciones Biológicas, C.S.I.C., Madrid, Spain.


11) Shorthand writing of polynucleotides and abbreviations for nucleotides, amino acid residues, etc., are as recommended by \textit{J. Biol. Chem.}, and previously used (refs. 1 and 14).


