A New Factor from Escherichia coli Affects Translocation of mRNA*

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Reconstitution of protein synthesis from purified translation factors on ribosomes from Escherichia coli has revealed the requirement for a protein, W, that affects chain elongation and is essential to reconstitute the process (Ganoza, M. C., Cunningham, C., and Green, R. M. (1985) Proc. Natl. Acad. Sci. U.S.A. 82, 1648–1652).

We report that W has no effect on initiation complex formation by 30 or 70 S ribosomes or on the association of ribosomal subunits, peptide bond synthesis, or binding Ala-tRNA, which is the second amino acid of the coat protein of the MS2 RNA virion.

W has a pronounced effect on tripeptide synthesis, and is obligatory for the synthesis of the coat protein of Escherichia coli MS2 or f2 RNA coat protein to ribosomes bearing fMet-Ala-tRNA, as well as for the ejection of deacyl-tRNA from ribosomes, which occurred concomitant with the binding of the Ser-tRNA.

We propose that W functions by ejecting tRNAs from ribosomes in a step that precedes the movement of mRNA during translocation.

Our understanding of the mechanisms underlying translation depends, in large part, on the biochemical reconstitution of this process (1–6). Specific factors that affect the initiation, elongation, and termination of translation have been isolated and have been used to examine the mechanism of each reaction on ribosomes programmed with homo- or heteropolymeric mRNAs.

In contrast, only two studies have been published on the reconstitution of translation using native mRNA templates. In one of these studies, the lacZ gene was transcribed by RNA polymerase and pure translation factors and aminoacyl-tRNA synthetases were used to translate these transcripts on Escherichia coli ribosomes (7). The requirements for the N-formyl-methionyl-tRNA, fMet, transformylase and for the RR-F (1) ("ribosome recycling factor") (8, 9) were established by these means.

In other studies, reconstitution of translation was examined by programming synthesis with amber mutants of the coat protein gene of f2 bacteriophage. The requirement for aminoacyl-tRNA synthetases was bypassed by using the appropriate aminoacyl-tRNAs in the reactions (10, 11). This simpler system enabled identification of a set of proteins which occur bound to 70 S ribosomes (12–15).

These proteins have been purified to homogeneity, and the sequence of two of their genes has been established (15). In this communication we examine the site of action of one of these proteins called W, by studying stepwise synthesis of the coat protein's N-terminal six amino acids, examining the intermediates. We propose that W stimulates translocation by accelerating the ejection of cognate tRNAs from ribosomes.

MATERIALS AND METHODS

[^55]Met (550 Ci/mmol), [^3H]Ser (38 Ci/mmol), [^3H]Ala (82.7 Ci/mmol), [^3H]Thr (5.3 Ci/mmol), [^3H]Phe (18.0 Ci/mmol), and [^3H]Lys (100 Ci/mmol) were purchased from Amersham Corp. or Du Pont NEN. The E. coli K12 tRNA and the trinucleotide codon AUG came from Sigma. Poly(U), poly(A), and MS2 RNA were from Boehringer Mannheim.

E. coli K12 mid-log or MRE 600 cells were purchased from the Miles Chemical Corp. or from the University of Alabama Fermentation Center (University of Alabama, Birmingham, AL). MRE 600 and Q13 cells were obtained from B. Bachmann (Yale University, New Haven, CT). Mutants of fusA were obtained from Tocchini-Valentini, G.P. (International Laboratory of Genetics and Biophysics, Naples, Italy). MRE 600, Q13, and the fusA mutant cells used to prepare ribosomes were grown to mid-log phase as described previously (16).

[^55]Met-tRNA[^55]Met,[^3H]Ala-tRNA,[^3H]Phe-tRNA,[^3H]Thr-tRNA,[^3H]Ser-tRNA, and[^3H]Lys-tRNA were acetylated as described previously using unfractionated E. coli K12 tRNA (16). When appropriate, the incubations contained a 10^{-4} M solution of each####

_acid. The source of the enzyme used to prepare these fully charged aminoacyl-tRNAs was an S100 from MRE 600 cells (containing about 10 mg/ml protein) or the S100 purified through DEAE-cellulose columns (16). The later partially purified synthetases enabled the labeling of each specific aminoacyl-tRNA without apparent contamination. This was done by using the appropriate single amino acid or amino acid mixture. The fractions were assayed for each of 20 labeled amino acids prior to use to ensure that each aminoacyl-tRNA was fully charged. The extent of formylation was assessed on aliquots of the[^55]Met-tRNA[^55]Met after hydrolysis in 0.3 N NaOH followed by extraction of[^55]Met from ethyl acetate at 0.11 Ci/HCl (16).

Ribosomes were isolated from E. coli Q13 or MRE 600 as described (16). Ribosomes were washed using 10 mM HCl-Tris, pH 7.4, containing either 1 M NH4Cl or 2 mM MgCl2, in the first wash or 0.5 M NH4Cl and 10 mM MgCl2 in the second two washes. The cells used to prepare the ribosomes were grown to 4 × 10^{12} viable cells/ml (16) and were routinely tested for RNAse revertants as described (17). Sucrose density gradient analysis of ribosomes was carried out using 5–30% sucrose in 5 mM MgCl2, 10 mM Tris-HCl buffer, pH 7.6, 50 mM NH4Cl. Two-dimensional electrophoretic analysis of ribosomal proteins was performed by the method of Kaltschmidt and Wittmann (18).

The f2am3 bacteriophage, encoding the N-terminal hexapeptide of the f2 RNA coat protein, was isolated, and the RNA was extracted as described (17) and used to program synthesis as in Refs. 10 and 11.

The elongation factors EF-Tu, EF-Ts, and EF-G were assayed with poly(U) and Phe-tRNA as well as by exchange of[^3H]GDP (19) and were purified as described (20, 21). EF-P was assayed and purified as in Ref. 14. The release factor, RF-1, was assayed and purified as described in (22).

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1The abbreviations used are: RR-F, ribosome recycling factor; IF, initiation factor; EF, elongation factor.

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Ref. 22. The “rescue” protein was assayed by complementation of thermolabile synthesis in extracts of strain N4316, supplemented with Asn-tRNA synthetase. The rescue protein was purified as described (13).

If-3, IF-2, and IF-3 were isolated from E. coli K12 cells and assayed with AUG or MS2 RNA (23, 24) using Millipore filters to collect the translocation product of [3H]fMet-tRNA (or AUG-tRNA) in the presence of EF-G and EF-Tu (25, 26). S1 and S1A were detected with antibodies specific for S1.

SDS-polyacrylamide electrophoretic analysis of the proteins was performed as described (26), and the proteins were visualized by staining with silver (27).

Protein concentration was determined as described (28).

Assays for W Using Translocated mRNA—W can be scored with MS2, f2, or f2am3 RNA to program synthesis by using aminoacylated tRNAs and purified ribosomes supplemented with pure initiation and elongation factors (10, 11). However, the efficiency of the reactions is greatly enhanced by using aminoacyl-tRNA synthetases (27). Routinely the following protocol was used to specifically score for W in the absence of pure aminoacyl-tRNA synthetases. Reagents were added in the following order with final concentrations of: 35 mM Tris-HCl, pH 7.4, 0.5 mM GTP, 1.0 mM ATP, 2.8 mM phosphoenolpyruvate, 32 mM N-acetylglutamate (in a 12-fold concentrated mix), 10 mM MgCl₂, 8 mM dithiothreitol, 90 μM amino acid mixture without Lys (or any other amino acid used as a label), 0.75μg of Ca²⁺-leucovorin, 1.0μg of pyruvate kinase, 7.5μg of E. coli B tRNA, 1.0μg of purified EF-Tu (5.4 μg), IF-2 purified through DEAE and P11 (4.25 μg), EF-Tu, EF-Ts, and EF-G fractions purified through one or two A-50 columns (1.2–2.5 μg each). MRE 600 ribosomes (washed one to three times) (10 pmol), MRE 600 S100 (1.7 μg), W purified through two DEA-E A-50 columns (9 μg), and IF-3 purified from DEA-E (0.2 μg). The reactions (30 μl final volume) were incubated for 10 min at 37°C, and then MS2 RNA (8 μg) and [35S]Met-tRNAf (8 μc) were added. The tubes were incubated again for 15 min at 37°C, precipitated with 1 ml of cold 10% trichloroacetic acid, heated at 90°C for 10 min, and washed with 10% trichloroacetic acid on Whatman GF/A glass filters prior to measuring the radioactivity.

The assay for W was supplemented with EF-Tu, EF-Ts, and EF-G to insure that these proteins did not limit W-dependent synthesis. The diluted S100 was used as a source of the 20 aminoacyl-tRNA synthetases, the Met-tRNA transformylase, the rescue protein EF-F, the release factors RF-1 and RF-2, and the RR-F protein. S100 fractions had to be titrated for W activity prior to use.

Formation of Pretranslocation Complexes—Pretranslocation complexes were formed from ribosomes, S100 fractions, and elongation factors derived from a fusA mutant that harbors a temperature-sensitive EF-G protein. EF-G was irreversibly inactivated by preincubating the fusA⁺ cells for one generation at 44°C.

Purification of the W Protein—The W protein was purified from ribosomes derived from 200–500 g of mid-log E. coli K12 or MRE 600 cells. Ribosomes were extracted for 5 h at 0°C with 1 M NH₄Cl, 2 mM MgCl₂, in 20 mM Tris-HCl, pH 7.4, containing 1 mM dithiothreitol. Ribosomes were removed by ultracentrifugation, and the ribosomal extracts were concentrated with ammonium sulfate to 80% saturation at pH 7.4. After dialysis, the fractions were chromatographed on a DEAE-A50 column and eluted with a linear 0.15–0.4 M KCl gradient. Fractions having W activity (at 0.13 M KCl) were concentrated and chromatographed on a second DEAE-A50 column, which was eluted with a linear gradient containing 0.1–0.3 M KCl. W eluted at 0.18 M KCl and was further fractionated by Sephacryl S200 and/or by hydroxyapatite chromatography. W eluted with 0.05–0.13 M phosphate buffer from the latter column. W prepared in this manner comprises 90% of the protein observed upon SDS-polyacrylamide gel electrophoretic analysis. The preparations are free of all elongation factors and other proteins involved in translation. The details of this purification scheme will be described in detail in a subsequent publication.

High Voltage Electrophoretic Analysis of the Products—Samples (15 μl) were hydrolyzed with 0.33 N NaOH for 10 min. at 37°C, neutralized with HCl, and electrophoresed on Whatman No. 1MM paper for 2 h and 40 min (4000 V) using pyridine, acetic acid, and water 1:10:189, v/v/v. Strips were cut and counted.

RESULTS AND DISCUSSION

Protein chain elongation entails the cyclical alignment of aminoacyl-tRNAs in response to their specific codons in mRNA, peptide bond synthesis, and movement of mRNA relative to the ribosome (translocation). These mechanisms have been examined predominantly with homopolymeric mRNAs using ionic conditions that obviate the requirements for initiation factors and other proteins that may be essential for operations on natural mRNAs (see below) (1–4). EF-Tu complexed to GTP, possibly as a dimer (1, 5), accelerates the rate of binding of the aminoacyl-tRNA to the mRNA-programmed ribosome (6). GTP hydrolysis is required to proofread aminoacyl-tRNAs that are near-cognate to the mRNA template (29–31). After GTP hydrolysis, EF-Tu-GDP leaves the ribosome and a peptide bond can be formed (1). EF-Ts catalyzes the exchange of GDP in EF-Tu-GDP with free GTP enabling, once again, the formation of the ternary complex (6). The elongation factor, EF-G, then stimulates ribosomes to translocate (1–4).

Although the mechanism of chain elongation has been examined with homopolymeric mRNAs, there is considerable evidence that mRNA structure affects this process, e.g., highly structured mRNAs tend to promote premature release of pretranslocation complexes from ribosomes. It is known that a number of such “processivity” errors result from faulty elongation events (32, 33).

Reconstruction of translation programmed by native templates has revealed the requirement for several factors (10, 11). One of these, the “rescue” protein (12, 13), is required for association of native ribosomal subunits; a second factor, EF-P, is essential for synthesis of the first peptide bond on 70 S ribosomes (15); the third of these factors, W, is involved in chain elongation and is essential to reconstitute translation (10, 11).

W has a relative mass of 47 kDa and a sedimentation coefficient of 4.1 S. The W protein, purified to 90% apparent homogeneity, is free of all initiation, elongation, termination factors as well as other proteins, such as the RR-F (9) required to reconstitute protein synthesis (7, 10, 11).

The gene encoding W has been cloned and mapped on the E. coli chromosome. The requirement for synthesis, chromosome location, and the partial sequence of the protein suggest that it is a new component of translation.

Here we examine the effect of the W protein on synthesis of the full length or of the first six N-terminal amino acids of the MS2 or f2am3 coat protein. Fig. 1A shows a bar graph of the products of synthesis analyzed by SDS-polyacrylamide gel electrophoresis. Synthesis products were formed in response to MS2 RNA in a purified system dependent on the W protein. 90% of the products synthesized correspond to the coat protein of the MS2 virion. W stimulates this synthesis at least 6-fold, but has no effect on synthesis directed by poly(A) or poly(U) (Fig. 1A and B). Synthesis programmed by poly(U) initiated by N-acetyl-[3H]Phe-tRNA also does not depend on W (Fig. 1C). Thus, synthesis directed by a natural mRNA requires W, whereas synthesis directed by these artificial templates does not.

W does not dissociate, associate, or prevent association of 70 S ribosomes and is not required for binding fMet-tRNA, Met to ribosomes nor for peptide bond synthesis, as measured by f[35S]Met-puromycin or f[35S]Met-Ala synthesis (10, 11).

To determine whether W is required for elongation, we examined its effect on synthesis of amino acids 2–6 of the MS2 coat protein by labeling each of the corresponding amino acids. Fig. 2 shows that there is a linear correlation between the requirement for W on synthesis and the position of the amino acid in the coat protein. The synthesis of the entire coat protein is absolutely dependent on W (10, 11), whereas dipeptide synthesis is nearly independent of W, further confirming this relationship (Fig. 2).

The requirement for W on chain elongation was further studied by reconstructing synthesis with extracts of a thermostable mutant in the translase, EF-G, which were freed of
W by purification. Fig. 3A shows that, in such systems, synthesis of the coat protein of the MS2 bacteriophage requires both EF-G and W.

As expected, in the absence of a functional EF-G protein, fMet-Ala accumulates on ribosomes resulting in arrested synthesis. Under these conditions, binding of the second aminoacyl-tRNA, Ala-tRNA, does not require W (data not shown). (W stimulates fMet-Ala synthesis by 50% upon addition of EF-G but not in the absence of EF-G, data not shown.) Synthesis of the N-terminal pentapeptide (examined electropectrophoretically) programmed by MS2 RNA (Fig. 3B, 1) or by f2am 3 RNA (Fig. 3B, 2) requires not only EF-G but also W. W stimulates hexapeptide synthesis about 2-fold. Using more highly purified ribosomes, a 10–20-fold stimulation is observed (data not shown).

As shown in Fig. 4, binding of the third aminoacyl-tRNA, Ser-tRNA
Ser, of the MS2 bacteriophage coat protein to the 70 S-mRNA-fMet-Ala-tRNA-EF-Tu complex depends on addition of both EF-G and W. This experiment suggests that W functions with EF-G in translocation.

The synthesis of tripeptides involves the movement of mRNA relative to the ribosomal complex and requires EF-G. This event is preceded by the release of deacyl-tRNA from ribosomes after peptide-bond synthesis. Translocation is required to move the nascent peptide into a site where it can be active in peptide-bond formation and where recognition of the next codon on the mRNA can occur.

Synthesis of the initial dipeptide is followed by ejection of the deacyl-tRNA (tRNAfMet, in this case) from the ribosome. Therefore, we examined the effect of W on ejection of deacyl-tRNA from the pre-translocation complex (ribosome-MS2 RNA-fMet-Ala-tRNA). The data in Fig. 4 indicate that W indeed ejects [3H]tRNA bound to ribosomes, concomitant with binding of [3H]Ser-tRNA. EF-G does not substitute for W in this reaction (Fig. 4).

Since W strongly stimulates binding of the third, but not of the second, aminoacyl-tRNA to the fMet-tRNA-EF-Tu-mRNA-ribosome complex, as well as ejection of tRNA from ribosomes, it may increase the affinity of the A site by ejecting tRNAs from the E or P site after they have participated in synthesis. To begin examining this possibility, the kinetics of binding and tRNA ejection were examined using the pre-translocation complex (ribosome-MS2 RNA).

As shown in Fig. 5, W stimulates the rate of binding of [3H]Ser-tRNA to MS2 RNA-ribosome complexes that contain fMet-Ala-tRNA. In the absence of W, very little binding of [3H]Ser-tRNA is observed and the [32P]tRNA is stably bound to the ribosomes. Addition of W results in the ejection of [32P]tRNA from these complexes concomitant with the binding of [3H]Ser-tRNA. Since unfractionated tRNA was used in these experiments, the tRNA released from these complexes was identified by acylation with [35S]Met using Met-tRNA synthetase. Thirty to 40% of the deacyl-[32P]tRNA ejected from the ribosome is tRNAfMet.

Three sites, A, P, and E, are known to be involved in binding, respectively, aminoacyl-tRNA, peptidyl-tRNA, or deacyl-tRNA to ribosomes (2, 3). Two models for the elongation reaction have
been recently proposed. Both are based on analysis of intermediates in polyphenylalanine synthesis programmed with poly(U). In the first model it is suggested that two sites (A and P) exist on the 30S, and three (A, P, and E) on the 50S particle. Hybrid states of the ribosome are proposed to explain the altered "footprinting" pattern observed on addition of the 50S particle, EF-Tu, and EF-G (2). According to this model, the peptidyl-tRNA or fMet-tRNAfMet bind to a P/P site which is

Fig. 3. A, effect of W on pre-translocation intermediates synthesized with a mutant of EF-G. MS2 RNA-programmed synthesis was scored with extracts purified from a temperature-sensitive mutant defective in EF-G (34). The purification of each initiation, elongation factor, W, and aminoacyl-tRNA synthetases as well as the assay of reconstructed synthesis are described in Refs. 10 and 11 and under "Materials and Methods." Each reaction was supplemented with 5.0 μg of W, 5.0 μg of EF-G, or a mixture of these proteins at these concentrations. Aliquots of 25 μl of the reactions were withdrawn after a 15-min incubation at 37 °C. B, effect of W on the N-terminal products of synthesis formed with a mutant of EF-G. High voltage electrophoresis of the acid-soluble products of synthesis was conducted as described in Ref. 35 using factors purified from cells with a temperature-sensitive mutant in EF-G (34). Reactions were programmed with 10 μg of MS2 RNA (1) or with 10 μg f2am3 RNA (2) in the presence (solid bars) or in the absence (clear bars) of W. Reactions programmed with MS2 RNA were treated for 10 min at 37 °C with chymotrypsin in order to cleave the N-terminal pentapeptide of the coat protein. Formylated di- and tripeptides as well as the coat protein's hexapeptide were used as standards. The migration of the penta- and hexapeptides was confirmed in double-label experiments using [35S]Met and [3H]Phe or [3H]Thr, which occur in the first, fifth, and sixth position of the hexapeptide, respectively. The penta- and hexapeptides were purified as described by Capechi (35). Approximately 5,000 dpm were observed without addition of MS2 RNA f2am3 RNA, and this value was subtracted.

Fig. 4. Effect of W on binding of [3H]Ser-tRNA to a pre-translocation complex and on the ejection of [32P]tRNA from ribosomes. Reactions (0.15 ml final volume) were programmed with MS2 RNA using initiation and elongation factors and ribosomes purified from a mutant of EF-G as described in Fig. 2 and under "Materials and Methods." Where indicated, reactions were supplemented with 5 μg of EF-G and 7.1 μg of W (S-200 fraction) and [3H]Ser-tRNA (104,000 dpm). Aliquots (25 μl of each reaction) were withdrawn after 15 min at 37 °C and assayed for [3H]Ser-tRNA bound to ribosomes (A, open bars). Reactions were also supplemented with [32P]tRNA (approximately 80,000 dpm) and the indicated factors (B, solid bars). [32P]tRNA (unfractionated E. coli K12) was labeled as described (36) and was bound to ribosomes using initiation factors of concentration given under the assay for W (see "Materials and Methods"). The amount of [32P] bound to ribosomes was estimated as described under "Materials and Methods." The migration of the penta- and hexapeptides was confirmed in double-label experiments using [35S]Met and [3H]Phe or [3H]Thr, which occur in the first, fifth, and sixth position of the hexapeptide, respectively. The penta- and hexapeptides were purified as described by Capechi (35). Approximately 5,000 dpm were observed without addition of MS2 RNA f2am3 RNA, and this value was subtracted.

Fig. 5. Effect of W on the kinetics of [3H]Ser-tRNA binding to and on the ejection of [32P]tRNA from ribosomes. The [32P]tRNA was labeled with [32P]ATP and T4 polynucleotide kinase as described (36). Reactions (155 μl) were conducted at 37 °C using the buffers, mono- and divalent ions described under "Materials and Methods." Each reaction also contained ribosomes purified from MRE 600 cells, IF-1, IF-2, IF-3, EF-Tu, EF-G, Met-, Ala-, and Ser-tRNA synthetases [3H]Ser, [32P]tRNA and W where indicated. Aliquots of 25 μl were withdrawn at the indicated times, and the amount of radioactive [3H]Ser-tRNA bound in the presence of W (●) or in the absence of W (○) was determined.
equivalent to the P site. The EF-Tu-GTP-aminoacyl-tRNA binds to an A/T state. EF-Tu-GDP is released after hydrolisis of GTP allowing the aminoacyl-tRNA to interact with the 50 S A (AA) site. During peptide bond formation, both the peptidyl and the deacyl-tRNAs move relative to the 50 S subunit such that the deacyl-tRNA moves from the P/P to the P/E site and the peptidyl-tRNA moves from the AA to the A/P site. During translocation, EF-G and GTP promote movement of the peptidyl-tRNA relative to the 30 S subunit from the A/P to the P/E sites. These data are also explained by a scheme involving two steps, where the subunits and not the tRNAs move relative to each other after peptide bond formation and translocation (2).

In the second model, a pre-translocation state is defined by the occupancy of tRNA in the P site and peptidyl-tRNA in the A site. The post-translocation state, the tRNA occupies the E site and the peptidyl-tRNA occupies the P site. EF-G is thought to effect the conversion of the pre- to the post-translocation states (3). Occupation of the E site is known to induce a low affinity sites, and occupation of the A site induces a low affinity E site effecting the release of tRNA (3).

It has been proposed that the filling of the E site with deacyl-tRNA induces a low affinity A site, which enables the tRNA-ribosome complexes to dissociate (3). Since W stimulates Ser-tRNA binding to ribosomes concomitant with the ejection of deacyl-tRNA from the particles, it could be stimulating cognate tRNA ejection by affecting the affinity of the E site. The relative contribution of W to the exit of cognate tRNAs from the E site of the ribosome is under study using suitable heteropolymeric mRNAs. Other experiments are required to establish whether W affects an essential hybrid site utilized during chain elongation.

W has no effect on poly(U)- or poly(A)-programmed synthesis from Phe-tRNA<sup>me</sup> or Lys-tRNA<sup>Lys</sup>. For these cases, a more rapid release of the cognate tRNAs from the ribosome may occur, which may not be detected under our experimental conditions. Alternatively, it is possible that W ejects only certain tRNAs from ribosomes, e.g. deacyl-tRNA<sup>me</sup>. The specificity of W in the binding and unbinding of tRNAs is also under investigation.

The combined results presented here suggest that the W protein could have pronounced effects on the efficiency of translation programmed by native mRNAs by removing tRNAs from ribosomes that are spent during synthesis. The discovery of this protein has enabled reconstitution of translation from pure components permitting the study of these and other reactions that regulate translation of mRNA transcripts.

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