Movement of the Ribosome along the Messenger Ribonucleic Acid during Protein Synthesis*

Sohan L. Gupta, John Waterson, Mohan L. Sopori, Sherman M. Weissman, and Peter Lengyel†

ABSTRACT: Studies are presented demonstrating that: (1) The movement of the ribosome along the mRNA during protein synthesis is triggered by S_2 factor (also designated G factor or translocase) and guanosine 5′-triphosphate. (2) One step of the ribosome along the mRNA is (as expected) three nucleotides long. The experiments on which these conclusions are based include the following. A single ribosome was bound to f2 bacteriophage RNA in the presence of the chain initiator, fMet-tRNA under conditions in which it is bound to the coat protein initiation site (initiation complex). An aliquot of this preparation was converted into a "pretranslocation complex" in a reaction with the appropriate factors (S_1 and S_3, also designated as T_s and T_u), guanosine 5′-triphosphate, and Ala-tRNA. (Alanine is the second amino acid of the coat protein.) In this complex the fMet-Ala-tRNA is bound at the A site, and uncharged tRNA at the P site of the ribosome. An aliquot of this preparation was converted into a "posttranslocation complex" in a reaction with S_2 factor and guanosine 5′-triphosphate. In this complex the fMet-Ala-tRNA is bound at the P site of the ribosome and the A site is vacant. Each of the three complexes was treated with ribonuclease to digest those parts of the f2 RNA which were not protected by the attached ribosome, and centrifuged to separate the ribosomes with the protected f2 RNA fragments from other components. Each of the three isolated f2 RNA fragment–ribosome complexes was examined in two ways. (a) The f2 RNA fragments were isolated from each and the nucleotide sequences at the 3′-terminal regions of the fragments were determined. The main f2 RNA fragment in the posttranslocation complex (3′ end ---UUUACU) extended three nucleotides further toward the 3′ end than that present in both the initiation and pretranslocation complex (3′ end ---UUU). (b) Each f2 RNA fragment–ribosome complex was suspended in a reaction mixture including labeled amino acids, and the f2 RNA fragment in it was translated into oligopeptides. The oligopeptide from the posttranslocation complex had a C-terminal threonine residue (corresponding to ---UUUACU), whereas those from the initiation and pretranslocation complexes had C-terminal phenylalanine residues (corresponding to ---UUU). The pretranslocation complex was found to sediment faster in sucrose gradients than either the initiation or posttranslocation complex. This may indicate that the shape or compactness of the ribosome undergoes cyclic changes during protein synthesis.

During the translation of mRNA into polypeptide the ribosome moves along the mRNA in the 5′ to 3′ direction (Lengyel and Söll, 1969). Each aminoacyl residue in the polypeptide is specified by three adjacent nucleotides (codons) in mRNA (Speyer, 1967). Consequently, it is expected that the ribosome should move the length of three nucleotides when adding one aminoacyl residue to the growing polypeptide. In this communication we present experiments bearing out this expectation.

The addition of each aminoacyl residue to the polypeptide is a cyclic process (chain elongation cycle) (Lipmann, 1969; Lengyel and Söll, 1969). Three amino acid polymerization factors (S_1, S_2, and S_3) and GTP are known to be involved in this cycle in addition to mRNA, ribosomes, and AA-tRNA.[1] S_1, S_2, and S_3 are factors from *Bacillus stearothermophilus* (Skoultchi *et al.*, 1968). These are analogous to factors from *Escherichia coli* and *Pseudomonas fluorescens*. S_1 corresponds to to T_s and F_{Is}, S_2 to G and F_{II}, and S_3 to T_u and F_{Iu} (Lucas-Lenard and Lipmann, 1966; Shorey *et al.*, 1969). Subsequently when discussing a factor from these two microorganisms we shall note in parentheses the designation of the corresponding factor from *Bacillus stearothermophilus*. In the first phase of the cycle (*AA-tRNA* binding) an S_3–GTP–AA-tRNA complex is bound to the AA-tRNA binding site (or A site) of the ribosome–mRNA complex which has peptidyl-tRNA bound at the peptidyl-tRNA binding site (or P site) (Skoultchi *et al.*, 1969, 1970; Lucas-Lenard *et al.*, 1969; Brot *et al.*, 1970; Ravel, 1967; Gordon, 1968; Ertel *et al.*, 1968a,b; Shorey *et al.*, 1969). In the second phase (*peptide-bond formation*) the GTP from the S_3–GTP–AA-tRNA complex is cleaved, an S_3–GDP complex and P_i are released from the ribosome (Shorey *et al.*, 1969; Haenni *et al.*, 1968., Ono *et al.*, 1969a,b; Gordon, 1969; Skoultchi *et al.*, 1970; Waterson *et al.*, 1970), and the peptidyl residue of the peptidyl-tRNA is released from its linkage to tRNA and forms a peptide bond with the α-amino group of the AA-tRNA in the A site (Monro *et al.*, 1969; Skoultchi *et al.*, 1969). In the third phase (*peptidyl-tRNA translocation*) S_2 bound to the ribosome triggers the cleavage of further GTP, the discharged tRNA is released from the P site and the peptidyl-tRNA (which has just been extended by one aminoacyl residue) is shifted from the A site to the P site (Traut and Monro, 1964; Brot *et al.*, 1968; Haenni and Lucas-Lenard, 1968; Kuriki and Kaji, 1968; Pestka, 1968; Erbe *et al.*, 1969; Lucas-Lenard and Haenni, 1969). Finally, S_1 promotes the release of GDP from the S_3–GDP complex and a new S_3–GTP–AA-tRNA complex is formed (Miller and Weissbach, 1970; Waterson *et al.*, 1970; Weissbach *et al.*, 1970a,b). This finishes the process and the stage is set for at-

* From the Department of Molecular Biophysics and Biochemistry, and the Department of Medicine, Yale University, New Haven, Connecticut 06520. *Received May 14, 1971.* This study was supported by research grants from the National Institutes of Health (GM-13707, CA-10137) and the American Cancer Society (ACS-E456). Paper VII in this series.

† To whom to address correspondence.

[1] Abbreviations used are: AA-tRNA, aminoacyl-tRNA; fMet-$tRNA_f^{Met}$, formylmethionyl-$tRNA_f^{Met}$; Ala-tRNA, alanyl-tRNA; fMet-Ala-tRNA, formylmethionylalanyl-tRNA; DTT, dithiothreitol.

taching another AA-tRNA (Skogerson *et al.*, 1971) to the peptide chain by repeating the cycle.

During some phase of the cycle the ribosome moves along the mRNA. The energy required for this movement is presumably obtained by GTP cleavage. For some time peptidyl-tRNA translocation triggered by S_2 was the only phase of the cycle in the cell-free system from *E. coli* in which GTP cleavage was known to occur (Conway and Lipmann, 1964; Nishizuka and Lipmann, 1966a,b). It was assumed, therefore, that ribosome movement must also be triggered by S_2 and must take place in the peptidyl-tRNA translocation phase (Conway and Lipmann, 1964; Nishizuka and Lipmann, 1966a,b). This assumption became less safe with the recent finding that apparently GTP is also cleaved after the binding of the S_3–GTP–AA-tRNA complex to the ribosome, but preceding peptide-bond formation (Arlinghaus *et al.*, 1964; Haenni *et al.*, 1968; Ono *et al.*, 1969a,b; Gordon, 1969; Skoultchi *et al.*, 1970). It became conceivable that ribosome movement is triggered by the cleavage of the GTP from the S_3–GTP–AA-tRNA complex and takes place prior to S_2 action. We attempted, therefore, to establish experimentally in which phase of the cycle the ribosome movement takes place. In this communication we present data indicating that ribosome movement is elicited by S_2 and GTP occurs in the same phase in which peptidyl-tRNA is translocated. We found, furthermore, that the pretranslocation complex sediments faster in a sucrose gradient that either the initiation or the posttranslocation complex. This probably reflects a change in the shape or compactness of the ribosome during protein synthesis and confirms and extends the predictions and finding of Spirin (1969) and Schreier and Noll (1970, 1971). Some of these results were presented at the 8th International Congress of Biochemistry in Montreux in September of 1970.

Experimental Section

Preparation of High-Speed Supernatant Fraction (S100) and Ribosomes. Ten grams (wet weight) of *E. coli* Q13 cells in middle-log phase (from General Biochemicals) were ground with 25 g of alumina (Alcoa A305), suspended in 25 ml of buffer I (60 mM NH_4Cl–10 mM Tris-HCl (pH 7.4)–10 mM magnesium acetate–6 mM 2-mercaptoethanol), and treated with 3 μg/ml of DNase (RNase free) (Worthington) at 2° for 20 min. The alumina and the cell debris were removed by centrifuging the suspension at 5000*g* for 10 min, and centrifuging the resulting supernatant fraction twice at 20,000*g* for 20 min (S20). S20 (9 ml), supplemented with 1 ml of a solution containing 50 mM phosphoenolpyruvate, 30 mM ATP, 2 mM GTP, as well as 1 mg of "leucovorin" (5-formyltetrahydrofolate; Lederle), and 0.2 mg of pyruvate kinase was incubated at 37° for 30 min to allow for the degradation of endogenous mRNA (Kuechler and Rich, 1970) and was centrifuged at 105,000*g* at 2° for 4 hr to sediment the ribosomes. The top two-thirds of the supernatant fraction was removed and passed over a Sephadex G-25 column which had been equilibrated with buffer II (60 mM NH_4Cl–10 mM Tris-HCl (pH 7.4)–10 mM magnesium acetate–1 mM DTT). The fractions eluting in the void volume were pooled, frozen in small aliquots, and stored at −50° (S100).

The sedimented ribosomal fraction was suspended in 2 ml of buffer I and centrifuged at 20,000*g* for 10 min. The resulting supernatant fraction was layered over 8 ml of 1 M sucrose solution in buffer III (60 mM NH_4Cl–10 mM Tris-HCl (pH 7.4)–10 mM magnesium acetate–1 mM DTT–1 mM EDTA) and centrifuged at 230,000*g* for 16 hr (Kuechler and Rich, 1970). The pellet was suspended in buffer I, the concentration of the suspension was adjusted to 560 A_{260} units/ml, and it was frozen in small aliquots and stored at −50° (ribosomes).

Published procedures were used in obtaining the following preparations: S_1, S_2 (each 20-fold purified), and S_3 (actually an S_3–GDP complex, 70-fold purified, a homogeneous protein) all from *Bacillus stearothermophilus* (Skoultchi *et al.*, 1968). ^{32}P-labeled bacteriophage f2 RNA (10^7 cmp/A_{260} unit) (Gupta *et al.*, 1970), unlabeled bacteriophage f2 RNA (Eoyang and August, 1968; Gupta *et al.*, 1970), f-[^{14}C]Met-tRNA$_f^{Met}$ from *E. coli* (260 Ci/mole) (Kondo *et al.*, 1968), and [^3H]Ala-tRNA from *E. coli* (4600 Ci/mole) (Muench and Berg, 1966). The unfractionated *E. coli* B tRNA (General Biochemicals) used in all experiments, was discharged by incubation in 1.8 M Tris-HCl (pH 8.0) at 37° for 90 min.

Preparation of S_3–GTP–Ala-tRNA Complex Solution and S_3, GTP, and tRNA Solution. The reaction mixture (1 ml) contained the following components: 50 mM Tris-HCl (pH 7.4), 50 mM NH_4Cl, 10 mM magnesium acetate, 6 mM 2-mercaptoethanol, and 0.01 mM GTP, as well as 100 μg of S_1, 100 μg of S_3, and either 50 A_{260} units of Ala-tRNA carrying 1560 pmoles of Ala residues (unlabeled or labeled with ^3H) (for S_3–GTP–Ala-tRNA complex solution) or 52 A_{260} units of tRNA (discharged) (for S_3, GTP, and tRNA solution). After incubation at 37° for 5 min the reaction mixtures were kept at 0°.

Initiation Complex Preparation. The reaction mixture (1 ml) contained the following components: 50 mM Tris-HCl (pH 7.4), 50 mM NH_4Cl, 6 mM 2-mercaptoethanol, 5 mM magnesium acetate, 0.4 mM GTP, 5×10^{-4} mM fMet-tRNA$_f^{Met}$ (unlabeled or labeled with [^{14}C]methionine), 60 A_{260} units of *E. coli* ribosomes, and 60 A_{260} units of f2 RNA (^{32}P labeled for nucleotide sequence studies, unlabeled for translation). After incubation at 37° for 10 min the reaction mixture was cooled to 0° (initiation complex solution I). The initiation complex preparations intended for nucleotide sequence analysis (either as initiation complexes or after conversion into pre- or posttranslocation complexes) were supplemented with 1.5×10^{-4} M aurintricarboxylic acid (Grollman and Stewart, 1968). This reagent was added to prevent further attachment of ribosomes to ^{32}P-labeled f2 RNA or f2 RNA fragments during subsequent steps. The initiation complex solution I was further treated in one of two ways: (a) the Mg^{2+} was increased to 10 mM (to increase the stability of the complex) (initiation complex solution II); (b) 0.3 ml of initiation complex solution I was added to 0.2 ml of S_3, GTP, and tRNA solution, and the reaction mixture was incubated at 0° for 20 min and thereafter the Mg^{2+} was raised to 10 mM (initiation complex solution III). Initiation complex solution III was supposed to serve as a control in proving that any difference between the analyses of initiation complex and pretranslocation complex is not due to the mere presence of S_3, GTP, and tRNA in the reaction mixture, but depends on the conversion of initiation complex to pretranslocation complex (which can take place only if Ala-tRNA is present). It should be noted that no difference was found between initiation complexes II and III in either nucleotide sequence analysis or translation.

Pretranslocation Complex Preparation. This was obtained in one of the following ways.

I. At 0°. Initiation complex solution I (0.3 ml) was added to 0.2 ml of S_3–GTP–Ala-tRNA complex solution (in some experiments, the alanyl residue of the Ala-tRNA was labeled with ^3H) and incubated at 0° for 20 min to allow the fMet-

Ala-tRNA formation. Subsequently, the Mg^{2+} was raised to 10 mM, and the reaction mixture was incubated at 0° for 10 min (pretranslocation complex solution I).

II. At 37° after removal of G(S_2) factor and GTP. For the experiment, in which the dependence of ribosome movement along the mRNA on both S_2 and GTP was demonstrated, pretranslocation complex solution I (5 ml) was passed through a 1.8 × 40 cm Sephadex G-200 column. The column had been equilibrated previously and was eluted with a buffer containing 50 mM Tris-HCl (pH 7.4), 50 mM NH_4Cl, 10 mM magnesium acetate, and 6 mM 2-mercaptoethanol. The fractions containing the excluded A_{260} peak (*i.e.*, the pretranslocation complex, but neither free G (S_2) factor, nor free GTP) were pooled (pretranslocation complex solution II). The conditions of incubation at 37° of aliquots of this preparation are described in Table III.

III. At 37° in the presence of fusidic acid. Fusidic acid was added to 0.3 ml of initiation complex solution I at a final concentration of 1.6 mM and incubated at 37° for 5 min. Thereafter 0.2 ml of S_3–GTP–Ala-tRNA complex solution was added and the reaction mixture incubated at 37° for 20 min. The Mg^{2+} was raised to 10 mM, and the reaction mixture was incubated at 37° for 20 min. The Mg^{2+} was raised to 10 mM, and the reaction mixture was incubated at 0° for 10 min (pretranslocation complex solution III).

Posttranslocation Complex Preparation. This was obtained according to procedure I for pretranslocation complex preparation except that after increasing the Mg^{2+} to 10 mM, 50 μg of S_2 was added, and the reaction mixture was incubated at 37° for 10 min and then cooled to 0°.

Methods of Characterizing the Complex Preparations. I. Initiation complex. To establish the ratio of fMet-$tRNA_f^{Met}$ capable of undergoing peptide-bond formation (*i.e.*, bound in the P site) to total fMet-$tRNA_f^{Met}$ bound, one aliquot of the initiation complex preparation containing f-[^{14}C]Met-$tRNA_f^{Met}$ was incubated with 2 mM puromycin at 0° for 10 min. This treatment results in the release from the ribosomes of all formylmethionyl residues from the fMet-$tRNA_f^{Met}$ (or in general all peptidyl residues from the peptidyl-tRNA) bound in the P site. The puromycin-treated aliquot and an untreated control sample were filtered through a Millipore filter. The ^{14}C counts retained on the filter were determined. (Millipore filters retain only AA-tRNA or peptidyl-tRNA bound to ribosomes (Nirenberg and Leder (1964).)

II. Pretranslocation complex. A portion of the initiation complex preparation containing f-[^{14}C]Met-$tRNA_f^{Met}$ was reacted with S_3–GTP–[^{3}H]Ala-tRNA and the resulting pretranslocation complex preparation was examined in the following way. (a) To determine the ratio of formylmethionyl residues to alanyl residues bound on the ribosome an aliquot of the preparation was either filtered through a Millipore filter, and the ^{14}C and ^{3}H label on the filter was counted, or was centrifuged through a 5–20% (w/v) sucrose gradient (for details, see the following section) and the fractions sedimenting in the 70S region were counted for ^{14}C and ^{3}H. Essentially identical results were obtained in the two procedures. (b) To determine the proportion of the alanyl residues, which are part of fMet-Ala-tRNA to total alanyl residues bound the Millipore filters obtained in procedure a were extracted with 0.4 M triethylamine. The extracts were evaporated to dryness and incubated in 0.1 M KOH at 37° for 15 min to discharge the AA-tRNA and peptidyl-tRNA. The reaction mixture was neutralized and aliquots of it analyzed by electrophoresis at pH 3.5 on Whatman No. 3MM paper (Lodish, 1969). (c) To establish the distribution of fMet-Ala-tRNA, fMet-$tRNA_f^{Met}$, and Ala-tRNA between A site and P site an aliquot of the pretranslocation complex preparation was incubated with puromycin (see I) and examined according to both procedures described in part a.

III. Posttranslocation complex. An aliquot of the double-labeled pretranslocation complex preparation was converted into posttranslocation complex preparation. This was examined as outlined in part II.

Processing of the Initiation, Pretranslocation, and Posttranslocation Complex Preparations for Nucleotide Sequence Analysis

Treatment with Pancreatic Ribonuclease and Isolation of the Protected f2 RNA Fragment. The reaction mixture containing one of the complex preparations (*i.e.*, initiation complex II, III, pretranslocation complex I, III, or posttranslocation complex) was supplemented with 50 μg/ml of pancreatic ribonuclease and incubated at 0° for 60 min. The reaction mixture was then centrifuged through a 5–20% (w/v) sucrose gradient (prepared in 50 mM Tris-HCl (pH 7.4), 50 mM NH_4Cl, 16 mM magnesium acetate, 6 mM 2-mercaptoethanol, and 1 mM DTT) in a Spinco SW 41 rotor at 40,000 rpm at 2° for 3 hr. The fractions sedimenting in the 70S region were pooled, supplemented with 0.5% sodium dodecyl sulfate, and the RNA was isolated by phenol extraction. The f2 RNA fragments protected against ribonuclease cleavage by the attached ribosome were separated from the bulk of the RNA by electrophoresis in 12.5% polyacrylamide gels containing 8 M urea at pH 8.5 (Gupta *et al.*, 1969). The ^{32}P-labeled RNA band was located by autoradiography. The gel was sliced and the slice containing the ^{32}P label was homogenized in 1–2 ml of a buffer containing 1 M NaCl, 100 mM Tris-HCl (pH 7.7), 10 mM EDTA and extracted by phenol treatment. The RNA in the aqueous phase was precipitated by adding 160 μg of *E. coli* tRNA as carrier and three volumes of ethanol. The precipitate was washed once with ethanol and further processed as described in the next section and in the legend to Figure 2.

Fractionation of Ribonuclease T_1 Digest of the f2 RNA Fragments Isolated from Initiation, Pretranslocation, and Posttranslocation Complex Preparations Treated with Pancreatic Ribonuclease. The RNA fragments in the precipitate were digested with ribonuclease T_1 (Brownlee and Sanger, 1969). Pairs of the digests were spotted on a single Cellogel strip (Colab) about 3 in. apart. Electrophoresis was carried out in the first dimension at pH 3.5 at 50 V/cm for 20 min. The samples were then transferred to a thin-layer plate coated with a 9:1 mixture of cellulose and DEAE-cellulose. The second dimension was developed by homochromatography using homomixture b (Brownlee and Sanger, 1969). The spots were located by autoradiography (Figure 2).

Determination of Nucleotide Sequences at the 3′ Termini of the f2 RNA Fragments. The spots were scraped off the homochromatographic plates and the resulting powders were transferred to disposable syringes and washed repeatedly with ethanol to remove urea. The oligonucleotides were eluted from the powder with triethylamine saturated with CO_2 (pH about 9) (Brownlee and Sanger, 1969). The eluates were evaporated to dryness and digested with either (i) 0.5 mg/ml of pancreatic ribonuclease at 37° for 60 min or (ii) with 5 units/ml of ribonuclease U_2 at 37° for 90 min. The digests were fractionated by electrophoresis on DEAE-cellulose paper at pH 3.5. The products of the digests were located by autoradiography and identified by their electrophoretic mobility relative to xylene cyanol dye (R_b) (Adams *et al.*, 1969; Ohe *et al.*, 1969). The identifications were confirmed by base analy-

sis whenever there was sufficient radioactivity. The R_b values of the relevant ribonuclease U_2 digestion products were the following: CU 1.4, CUUU 0.19, and CUUUA 0.07. The base composition of CUUUA could not be analyzed in this experiment because of too few counts in it. The U_2 ribonuclease digests of oligonucleotide IIIb contained an additional product with an R_b of 0.15. There was not enough radioactivity in it for further analysis. Such a product could have resulted from a cleavage adjacent to the 3′ side of a pyrimidine of a larger oligonucleotide.

Processing of the Initiation, Pretranslocation, and Posttranslocation Complex Preparations for Translation

Treatment with Pancreatic Ribonuclease. The reaction mixture containing one of the complex preparations (*i.e.*, initiation complex II, III, pretranslocation complex I, II, or III or posttranslocation complex) was supplemented with 10 μg/ml of pancreatic ribonuclease, incubated at 0° for 60 min, and centrifuged through a 6-ml layer of 1 M sucrose in a buffer (50 mM Tris-HCl (pH 7.4)–50 mM NH_4Cl–16 mM magnesium acetate–1 mM DTT) in a Spinco 65 rotor at 60,000 rpm at 2° for 5 hr. The resulting pellet was rinsed with and suspended in 1 ml of the same buffer except containing 10 mM magnesium acetate (*suspension T*).

Translation. The translation of the f2 RNA fragments in the ribonuclease-treated complexes and the analysis of the olipeptides formed was performed by a procedure modified from that of Kuechler and Rich (1970). A reaction mixture (1 ml, containing 50 mM Tris-HCl (pH 7.4), 50 mM NH_4Cl, 10 mM magnesium acetate, 10 mM DTT, 4 mM ATP, 0.08 mM of each of the unlabeled amino acids (alanine, asparagine, aspartic acid, glutamic acid, glutamine, leucine, serine, and valine), 4 μCi of [^{14}C]phenylalanine (455 Ci/mole), 20 μCi of [^{3}H]threonine (2250 Ci/mole), 2.4 mg of S100 protein, and 100 A_{260} units of stripped *E. coli* B tRNA) was incubated at 37° for 10 min to allow formation of AA-tRNAs. (In some experiments [^{14}C]glutamine (165 Ci/mole) was substituted for unlabeled glutamine and unlabeled phenylalanine for [^{14}C]phenylalanine.) The reaction mixture was cooled to 0°, supplemented with 8 mM GTP, and 0.1 ml was added to 0.5 ml of suspension T prepared from the various complexes. The reaction mixture was incubated at 37° for 30 min to obtain the translation of the f2 RNA fragments into oligopeptides and the reaction was terminated by adding 2.5 ml of cold 5% trichloroacetic acid.

Characterization of the Oligopeptides. The precipitate formed was isolated by centrifugation at 1000*g* for 5 min, dissolved in 0.25 ml of 0.1 N NaOH, and incubated at 0° for 1 hr to allow the release of the oligopeptides from their linkage to tRNA. Thereafter 0.25 ml of cold 10% trichloroacetic acid solution was added to precipitate the tRNA and protein, and the resulting suspension was centrifuged at 1000*g* for 5 min. The supernatant fraction was processed and the oligopeptides purified according to the procedure of Capecchi (1967) by extraction with cresol–ethyl acetate and ion-exchange chromatography on a Dowex 50 (H^+) column. The eluate of the Dowex 50 column containing the oligopeptides was evaporated to dryness, dissolved in 50 μl of H_2O and fractionated by paper electrophoresis.

Results

Outline of Experiments on Ribosome Movement

A. Sequencing of a Ribosome Binding Site of f2 Bacteriophage RNA in an Initiation Complex. The RNA of the RNA bacteriophage f2 serves as a messenger which directs the synthesis of three proteins (phage coat protein, replicase enzyme, and maturation protein) in infected *E. coli* or in an *E. coli* cell-free system (Viñuela *et al.*, 1967; Eggen *et al.*, 1967; Lodish, 1968). We established earlier that a ribosome binds to f2 RNA forming a 70S initiation complex in the presence of formylmethionyl-$tRNA_f^{Met}$ (fMet-$tRNA_f^{Met}$), GTP, and peptide-chain initiation factors (Kondo *et al.*, 1968). Ribosomes are known to protect the mRNA region to which they are bound against cleavage by nucleases (Takanami *et al.*, 1965). In a previous study we obtained the 70S initiation complex with f2 RNA, treated it with ribonuclease T_1, and isolated the f2 RNA fragment which was protected against ribonuclease cleavage by the attached ribosome (Gupta *et al.*, 1969). The protected f2 RNA fragment was a unique RNA segment that was 61 nucleotides long. Its nucleotide sequence was determined (Gupta *et al.*, 1970). The 21 nucleotides adjacent to its 3′ end were found to specify the first 7 amino acids of f2 coat protein (Weber and Konigsberg, 1967) (fMet-Ala-Ser-Asn-Phe-Thr-Gln).

The availability of purified amino acid polymerization factors (S_1, S_2, and S_3) enabled us to extend the above study to an examination of ribosome movement.

B. Sequencing the 3′ Ends of Ribosome-Binding Sites on Bacteriophage f2 RNA in Initiation, Pretranslocation, and Posttranslocation Complexes. As in the previous study, we first prepared an initiation complex on f2 RNA (Gupta *et al.*, 1970). An aliquot of this was converted into a pretranslocation complex in a reaction with the appropriate factors (S_1 and S_3), GTP, and Ala-tRNA (alanine is the second amino acid in the coat protein). Part of the pretranslocation complex was converted into a posttranslocation complex in a reaction with the appropriate factor (S_2) and GTP (Ono *et al.*, 1969b) (Figure 1).

Each of the three complexes was treated with pancreatic ribonuclease to cleave those parts of the f2 RNA which were not protected by the ribosome (Takanami *et al.*, 1965). Thereafter each was centrifuged in order to separate the ribosomes with the bound, protected f2 RNA fragments from other components of the reaction mixtures (Gupta *et al.*, 1970). Each of the three isolated f2 RNA fragment–ribosome complexes was examined in two ways: (i) the f2 RNA fragments were isolated from each and the nucleotide sequences at the 3′-terminal regions of the fragments were determined; (ii) each was suspended in a reaction mixture containing labeled amino acids and other necessary ingredients, and the f2 RNA fragments in it were translated into oligopeptides which were characterized (Kuechler and Rich, 1970) (Figure 1).

The idea prompting these experiments was the following. In initiation complexes (digested with ribonuclease T_1) the protected f2 RNA fragment extends to the codon specifying the seventh amino acid of the coat protein (Gupta *et al.*, 1970). When the ribosome moves along the f2 RNA toward its 3′ end, then the 3′ end of the protected fragment should also be shifted toward the 3′ end of f2 RNA. Since each amino acid is specified by three adjacent nucleotides, it is presumable that the shift toward the 3′ end should be three nucleotides long when one amino acid is added to the peptide chain. Thus, it would be expected that the cutoff point of the protected f2 RNA fragment should be three nucleotides further toward the 3′ end in the posttranslocation complex than in the initiation complex. In order to find out if the ribosome movement occurs in the course of AA-tRNA binding and peptide bond formation or in the next phase of the chain elongation cycle, *i.e.*, translocation, one has to determine the

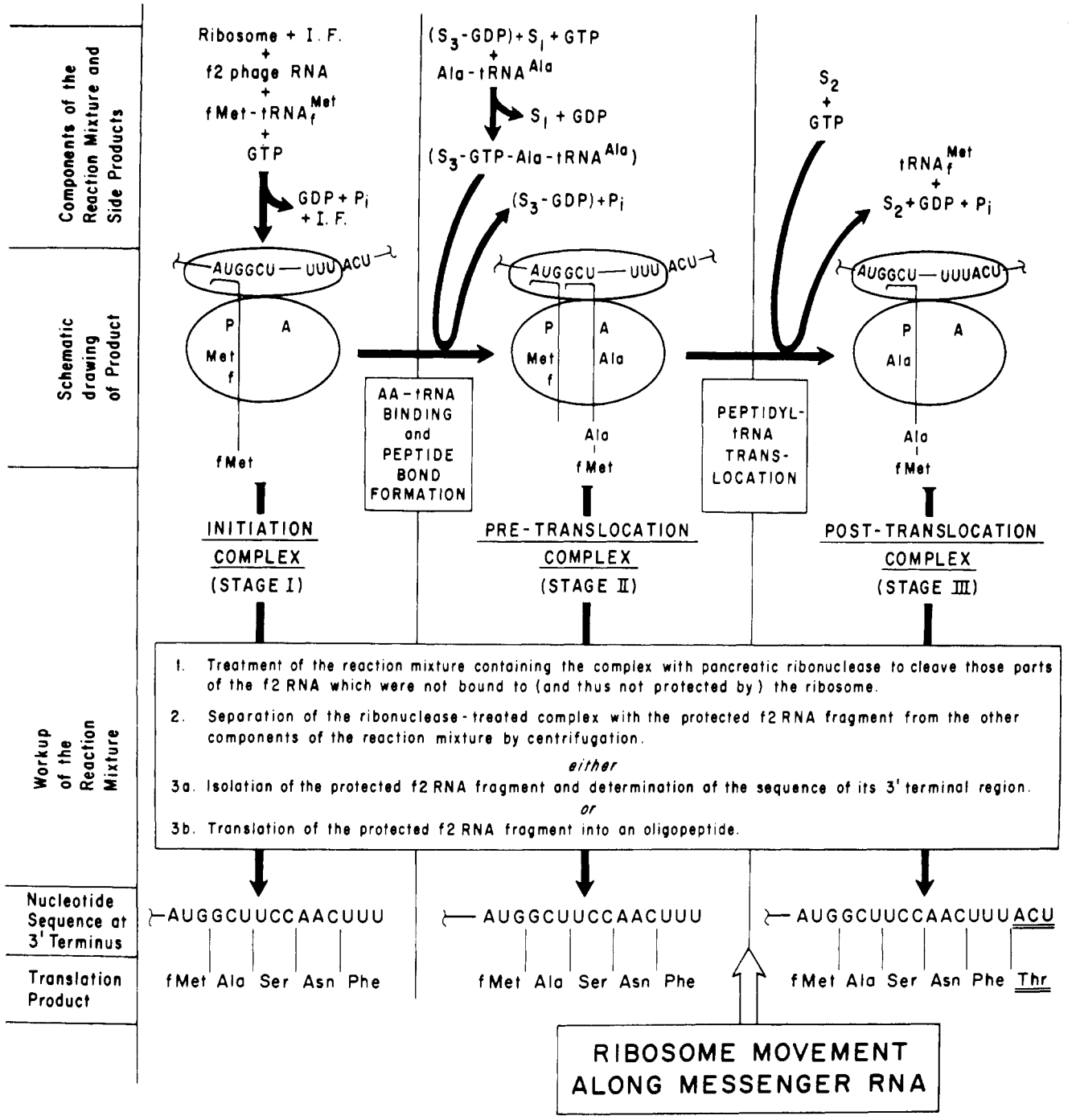

FIGURE 1: Schematic outline of experiment indicating that ribosome movement is triggered by S_2 factor and GTP and occurs in the same phase as peptidyl-tRNA translocation. The small oval shape represents the 30S ribosomal subunit; the large oval shape the 50S ribosomal subunit. P and A indicated in the 50S subunit stand for hypothetical tRNA binding sites. The gallows shape represents tRNA. The curved line bisecting the 30S subunit represents a segment of f2 RNA; only some of the codons are shown. Details are discussed in the text.

cutoff point at the 3′ end of the protected f2 fragment in the pretranslocation complex.

C. Results Obtained in Outline and Conclusions Derived (Figure 1). Identical f2 RNA fragments were found to be protected against cleavage by ribonuclease in the initiation and the pretranslocation complexes. However, the main f2 RNA fragment protected against cleavage by ribonuclease extended three nucleotides further toward the 3′ end in the posttranslocation complex (---UUUACU) than in either the initiation complex or the pretranslocation complex (---UUU).

These results indicate that (a) as expected, the ribosome moves the length of three nucleotides when adding one aminoacyl residue to the peptide chain, and (b) the ribosome movement takes place in the same phase in which peptidyl-tRNA is translocated, and this movement is triggered by S_2 factor and GTP.

The nature of the oligopeptides translated from the various protected f2 RNA fragments provides further support for these conclusions. Thus, *e.g.*, the oligopeptides translated from the main f2 RNA fragment in the ribonuclease-treated

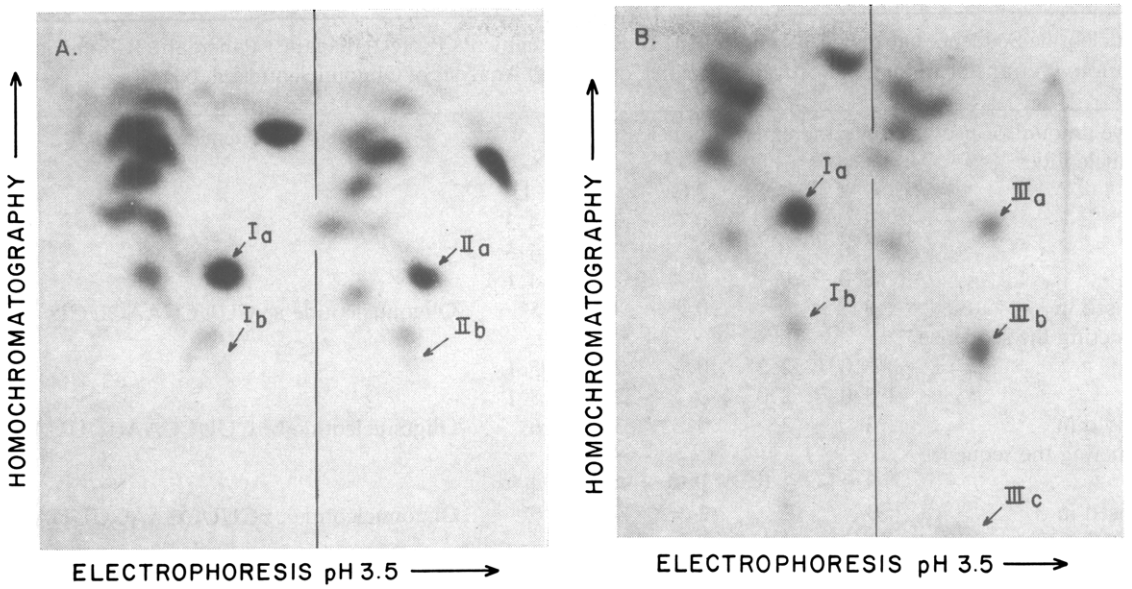

FIGURE 2: Fractionation of ribonuclease T_1 digests of the the f2 RNA fragments isolated from initiation, pretranslocation and posttranslocation complex preparations treated with pancreatic ribonuclease. Part A shows the fractionation of digests of f2 RNA fragments from (I) initiation complex preparation III and (II) pretranslocation complex preparation II. Part B shows the fractionation of f2 RNA fragments from (I) initiation complex preparation II and (III) posttranslocation complex preparation. The spots indicated with the arrows are oligonucleotides from the 3′-terminal regions of the protected f2 RNA fragments. Among the spots in the digests of the f2 RNA fragments from some of the posttranslocation complex preparations was one designated as IIIc moving even more slowly in homochromatography than IIIb. Its location on the homochromatogram is indicated by the arrow. IIIc is an oligonucleotide, larger than a or b, which is derived from the 3′-terminal region of the f2 RNA fragment in the posttranslocation complex. The spots next to Ib and IIb were digested by pancreatic ribonuclease, the products obtained from the two were the same, and were those expected to arise from the AUUACCCAUG oligonucleotide from the replicase initiation site (Steitz, 1969b). The amount of this varied from experiment to experiment, but never exceeded 27% of Ia or IIa calculated on a molar basis. (For further details, see the corresponding part in the Experimental Section.)

initiation and pretranslocation complexes had a C-terminal phenylalanine residue (corresponding to ---UUU), whereas the oligopeptides translated from the main f2 RNA fragment from the ribonuclease-treated posttranslocation complex had a C-terminal threonine residue (corresponding to ---UUU-ACU). The details of these and other experiments are presented in the subsequent sections.

Preparation of Initiation, Pretranslocation, and Posttranslocation Complexes

Three [^{32}P]f2 RNA–ribosome complex preparations were obtained.

I. INITIATION COMPLEX PREPARATION. This was obtained as indicated above by incubating ribosomes which had initiation factors bound, ^{32}P-labeled f2 RNA, GTP, and fMet-$tRNA_f^{Met}$ at 5 mM Mg^{2+} concentration (Gupta *et al.*, 1969).

II. PRETRANSLOCATION COMPLEX PREPARATION. One aliquot of the initiation complex preparation was supplemented with S_3–GTP–Ala-tRNA complex in excess and further incubated to allow for the formation of fMet-Ala-tRNA bound at the A site of the ribosome. The *E. coli* ribosome preparation used was contaminated with small amounts of $G(S_2)$. To avoid translocation we either added fusidic acid, an inhibitor of translocation (Tanaka *et al.*, 1968; Pestka, 1968; Ono *et al.*, 1969a), to the reaction mixture, or performed the incubation at 0° instead of at 37°. AA-tRNA binding and peptide-bond formation take place in this system at 0°, $G(S_2)$ is, however, inactive at this temperature (Ono *et al.*, 1969a). In some cases, after having formed the pretranslocation complex preparation at 0°, the GTP and the contaminating $G(S_2)$ factor were removed by gel filtration and the pretranslocation complex was incubated at 37°.

III. POSTTRANSLOCATION COMPLEX PREPARATION. An aliquot of the reaction mixture containing the pretranslocation complex preparation was supplemented with S_2 and GTP in excess and further incubated at 37° in order to trigger the release of the discharged $tRNA_f^{Met}$ and the translocation of the fMet-Ala-tRNA from site A to site P (Kuriki and Kaji, 1968; Lucas-Lenard and Haenni, 1969).

Characterization of the Complex Preparations

Tests were performed to determine what fraction of each complex preparation consisted of the complex wanted. In the initiation complex preparation 75% of the fMet-$tRNA_f^{Met}$ was reactive with puromycin indicating that at least this much was bound at the P site (Traut and Monro, 1964). In the pretranslocation complex preparation 50% as many alanyl residues as formylmethionyl residues were bound to ribosomes; 85% of the alanyl residues and 45% of the formylmethionyl residues were part of fMet-Ala-tRNA. Furthermore, 85% of the alanyl residues and 49% of the formylmethionyl residues were not reactive with puromycin under our assay conditions. These data indicate that *at least 40% (80% of 50%) of the ribosome complexes in the pretranslocation complex preparation* had fMet-Ala-tRNA bound in the A site, *i.e., were in the form of pretranslocation complexes.* The rest consisted presumably of initiation complexes (see also the section on Differences in Sedimentation Rates...). In the posttranslocation complex preparation 60% as many alanyl residues as formylmethionyl residues were bound to ribosomes. Moreover, 90% of the alanyl residues and formylmethionyl residues were reactive with puromycin. These data reveal that *about 54%* (90% of 60%) *of the ribosome complexes in the posttranslocation complex preparation* had fMet-Ala-tRNA bound in the P site, *i.e., were in the form of posttranslocation complexes.* The rest consisted presumably mainly of initiation complexes and of

TABLE I: Nucleotide Sequences at the 3′ Termini of the f2 RNA Fragments Protected against Pancreatic Ribonuclease Cleavage in the Initiation, Pretranslocation, and Posttranslocation Complexes: Analysis of Oligonucleotides a, b, and c.[a]

i. Relative amount of products in pancreatic ribonuclease digest of oligonucleotides	AAC	AC	AG	C	U	Sequence deduced
Ia	3.0	0	0	2.9	4.3	
IIa	3.0	0	0	2.9	4.5	
IIIa	3.0	0	0	2.8	4.1	
Integer used in constructing the sequence	3	0	0	3	5*	Oligonucleotide a: CUUCCAACUUU
Ib	3.0	2.2	0	2.6	5.1	
IIIb	3.0	2.0	0	2.7	5.1	
Integer used in constructing the sequence	3	2	0	3	6*	Oligonucleotide b: CUUCCAACUUUACU
IIIc	3.0	2.0	1.9	4.0	4.9	
Integer used in constructing the sequence	3	2	2	4	6*	Oligonucleotide c: CUUCCAACUUUACUCAG

ii. Relevant products in ribonuclease U_2 digest of oligonucleotides
IIa CUUU (no CUU)
IIIa CUUU (no CUU)
IIIb CUUUA, blurred spot in position of CU (no C, CUC, CUUU, or CUCA)

[a] Ia, Ib, IIa, IIIa, IIIb, and IIIc are among the oligonucleotides obtained by treating f2 RNA–ribosome–peptidyl-tRNA complexes with pancreatic ribonuclease, isolating the protected f2 RNA fragments, digesting them with ribonuclease T_1 and fractionating the digests by electrophoresis and homochromatography (see the Experimental Section). I refers to initiation complex preparation, II to pretranslocation complex preparation, III to posttranslocation complex preparation. a, b, and c refer to spots corresponding to those indicated by the arrows in Figure 2. The spots were scraped off the homochromatographic plates and the labeled substances eluted from the resulting powder were characterized as described in the Experimental Section. (i) The relative amounts were calculated from the counts in each product; the counts in AAC were taken as a standard representing three phosphate residues. The sequence proposed for oligonucleotide c is identical with that of the 3′-terminal region of an f2 RNA fragment from the coat protein initiation site sequenced previously (the sequence is presented in Figure 3). This sequence and those presented in (ii) served as the basis for using the integers for U (marked by an asterisk) and in part for deducing the sequences presented for oligonucleotides a, b, and c.

less than 10% (if any) pretranslocation complexes. It should be recalled that the reaction mixture, in which the initiation complex preparation was converted into the pretranslocation complex preparation, contained the components needed for the conversion in excess. Similarly, the reaction mixture, in which the pretranslocation complex preparation was converted into the posttranslocation complex preparation, also contained the components needed for the conversion in excess. Thus, those portions of the preparations which did not undergo the wanted conversion consisted presumably of complexes unable to undergo the conversion or at least sluggish in it.

Determination of the Nucleotide Sequences at the 3′-Terminal Regions in the f2 RNA Fragments from the Complex Preparations Treated with Pancreatic Ribonuclease (Gupta *et al.*, 1969, 1970; Steitz, 1969a,b; Hindley and Staples, 1969)

A. Initiation and Pretranslocation Complex. The protected f2 RNA fragments were isolated from each of the three complex preparations by (a) treatment with pancreatic ribonuclease (to trim away the unprotected parts of the f2 RNA), (b) isolation of the 70S complex by centrifugation through sucrose gradient, and (c) isolating the fragment from the complex by phenol extraction and acrylamide gel electrophoresis (Gupta *et al.*, 1969).

The f2 RNA fragments obtained were digested with ribonuclease T_1 and the digests were fractionated by electrophoresis and homochromatography (Brownlee and Sanger, 1969) (Figure 2). Each of the oligonucleotides detected on the homochromatograms was eluted and digested with pancreatic ribonuclease. Some of the oligonucleotides were also digested with ribonuclease U_2 (Arima *et al.*, 1968). The resulting products were characterized by base analysis and by electrophoretic mobility (Adams *et al.*, 1969). The analyses revealed among others the oligonucleotides originating from the 3′ ends of the f2 RNA fragments. These are indicated by the arrows in Figure 2. There are the same two 3′-terminal oligonucleotides (a and b) in the f2 RNA fragments from the initiation and pretranslocation complex preparation, and three 3′-terminal oligonucleotides (a, b, and c) in those from the posttranslocation complex preparations. The identification of these oligonucleotides was aided by the following. The f2 RNA fragments were obtained from the complexes by cleavage with pancreatic ribonuclease. Hence, they must be terminated at their 3′ ends with pyrimidine nucleoside 3′-phosphate residues. The f2 RNA fragments were further cleaved with ribonuclease T_1. This enzyme is known to cleave internucleotide linkages producing guanosine 3′-phosphate residues (Uchida and Egami, 1966). Consequently, oligonucleotides which result from the cleavage of the f2 RNA fragments with ribonuclease T_1 and are free of guanosine residues must originate from the 3′ ends of the f2 RNA fragments. As shown in Table

I the oligonucleotides a and b contain no guanosine residues. These oligonucleotides are consequently taken to originate from the 3′ ends of the f2 RNA fragments protected by the ribosome against nuclease cleavage. (A sequence similar to a was found at the 3′ end of a protected RNA fragment from bacteriophage R17 in an initiation complex by Steitz (1969b).)

The first 11-nucleotide long sequence (counting from the 5′ end) is identical in a and b (Table I). In 91–97% of the initiation and pretranslocation complexes the ribosome protects against pancreatic ribonuclease cleavage an f2 RNA fragment terminating in oligonucleotide a. In 3–9% of these complexes the protected fragment extends three nucleotides further toward the 3′ end and terminates in b (Table II). The fact that the length of the 3′-terminal oligonucleotides of the protected f2 RNA fragments is not uniform in either initiation or pretranslocation complexes (*i.e.*, the ends are frayed) may indicate that the protection against pancreatic ribonuclease cleavage is not uniform at the 3′ end of the protected fragment. It is also conceivable that the fraying results from the conversion of a small fraction of the complexes into posttranslocation complexes in both preparations. Such a conversion may be made possible by traces of endogenous Ala-tRNA and some residual S_2 activity in the conditions of the experiment. The latter explanation of the fraying could account for the fact that no 3′-terminal oligonucleotides were detected which are less than three nucleotides longer than a. But whatever the cause of the fraying is, its extent is small.

Moreover, most significantly the set of f2 RNA fragments (over 90% ending in a and less than 10% in b) protected against pancreatic ribonuclease cleavage by the ribosome in initiation and pretranslocation complex preparations is identical. This indicates that ribosome movement along the mRNA does not occur during the formation of the pretranslocation complex from the initiation complex.

B. Posttranslocation Complex. If the ribosome movement does not take place in the phase of AA-tRNA binding and peptide-bond formation, then it must occur in the phase in which peptidyl-tRNA is translocated. It is in accord with this expectation that in one experiment 47%, and in another 40% of the 3′-terminal oligonucleotides of the f2 RNA fragments from posttranslocation complex preparations extended further toward the 3′ end than the major 3′-terminal oligonucleotide a from the f2 RNA fragment in initiation or pretranslocation complex preparations (Tables I and II). Taking into consideration that only about half of the complexes in the posttranslocation complex preparation are actually posttranslocation complexes (see the section on the characterization of the complex preparations), this indicates that the ribosome moves along the f2 RNA during the conversion of the pretranslocation complex into the posttranslocation complex. Moreover, oligonucleotide b that comprises the large majority of the longer 3′-terminal oligonucleotides from the posttranslocation complex (41% out of 47% in expt 2 and 29% out of 40% in expt 4) extends three nucleotides further toward the 3′ end than the major 3′-terminal oligonucleotide a in initiation and pretranslocation complex preparations. This indicates that, as expected, a "step" of the ribosome is three nucleotides long.

The minor fraction of the longer 3′-terminal oligonucleotides (oligonucleotide c) from the posttranslocation complex preparations extends further toward the 3′ end than oligonucleotide b. Since the 3′-terminal oligonucleotides were prepared by cleaving the f2 RNA fragment with ribonuclease T_1, which cleaves after guanosine 3′-phosphate and the 3′-terminal of oligonucleotide c is guanosine 3′-phosphate, it is not clear how far the f2 RNA fragment giving rise to oligonucleotide c extended in the posttranslocation complex. Since the f2 RNA fragment was obtained by pancreatic ribonuclease cleavage that results in hydrolysis after a pyrimidine residue and the nucleotide adjacent to the 3′ side of the terminal guanosine of oligonucleotide c must be a pyrimidine (as deduced from the known amino acid sequence of the coat protein (Weber and Konigsberg, 1967), it is conceivable that this was the terminus of the protected f2 RNA fragment. It is reasonable to assume that the fraying at the 3′ end of the f2 RNA fragments in the posttranslocation complex preparation is due to causes similar to those in the case of the initiation and pretranslocation complex preparations.

All the sequences of the f2 RNA fragments protected against cleavage by ribonuclease in initiation, pretranslocation, and posttranslocation complexes are presented in Figure 3. One feature in this figure, which was not discussed in previous sections, concerns the 5′ end of the various fragments. An analysis of all the products in the ribonuclease T_1 digest of the fragments revealed that there is an extensive fraying at the 5′ end. (Such fraying at the 5′ end of R17 bacteriophage RNA fragments was described by Steitz (1969b).) Though we

TABLE II: Nucleotide Sequences at the 3′ Termini of the f2 RNA Fragments Protected against Pancreatic Ribonuclease Cleavage in the Initiation, Pretranslocation, and Posttranslocation Complexes: Comparison of the Amounts of Oligonucleotides a, b, and c.[a]

	Relative Amount (%)								
	I			II			III		
Expt	a	b	c	a	b	c	a	b	c
1	91	9	0	97	3	0			
2				92	8	0	53	41	6
3	97	3	0	94	6	0			
4				97	3	0	60	29	11

[a] The spots were obtained from initiation (I), pretranslocation (II), and posttranslocation (III) complex preparations produced, digested, and fractionated as described in the Experimental Section and in the legend to Figure 2. Translocation was inhibited in preparing pretranslocation complex (II) in expt 1 and 2 by incubation at 0° and in expt 3 and 4 by treatment with fusidic acid prior to incubation at 37° (see the Experimental Section). Ia, Ib, IIa, IIb, IIIa, IIIb, and IIIc refer to spots corresponding to those indicated by the arrows in the homochromatograms in Figure 2. The relative amounts of oligonucleotides in the spots were determined by scraping the spots from the plates, counting the radioactivity, and calculating the relative molar amounts of oligonucleotides in spots a, b, and c resulting from the digestion and fractionation of the same complex preparation. The different size of the oligonucleotide in a, b, and c was taken into account in the calculations. As discussed in the section on Differences in the Sedimentation Rates... pre- and posttranslocation complex preparations also contain apparent initiation complexes that failed to undergo further conversion. In evaluating the data in this table it should be taken into consideration that these apparent initiation complexes would be expected to give rise principally to oligonucleotide a.

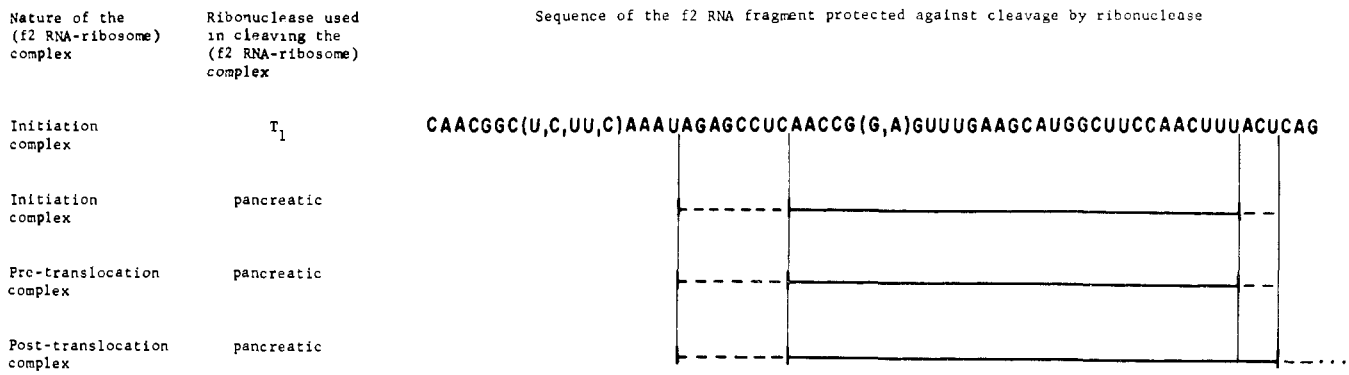

FIGURE 3: Sequences of the f2 RNA fragments protected against cleavage by ribonuclease in initiation, pretranslocation, and posttranslocation complex preparations. The sequence of the fragment protected against ribonuclease T_1 cleavage in the initiation complex was determined in a previous study (Gupta *et al.*, 1970). The continuous horizontal line demonstrates the main oligonucleotide fragment produced in digesting a complex with pancreatic ribonuclease. The broken horizontal lines indicate that additional fragments were found in which a longer sequence had been protected (fraying). The evidence for the 3′ ends of the fragments a, and b is presented in Table I. Fraying at the 3′ ends is presented in Tables I and II. The 3′ end of the longest f2 RNA fragment from the posttranslocation complex preparation was not established (see the discussion of oligonucleotide c in the text). This is indicated by the three adjacent dots in the figure. Other portions of the sequences were established by analyzing all products in the ribonuclease T_1 digests of the fragments (data not shown).

expected to find that the 5′ ends of the f2 RNA fragments protected against cleavage by pancreatic ribonuclease in intiation, and pretranslocation complexes would be different from those protected in posttranslocation complexes, we did not detect any such difference (data not shown). It is conceivable that such differences exist, but are obscured by the fraying.

It should be noted that the nucleotide sequence data obtained in this study corroborate the sequence earlier proposed for this fragment (Gupta *et al.*, 1970).

Translation of the f2 RNA Fragments in Ribonuclease-Treated Initiation, Pretranslocation, and Posttranslocation Complexes into Oligopeptides

Kuechler and Rich (1970) reported recently that the bacteriophage R17 RNA fragment in ribonuclease-treated initiation complex can be translated into the N-terminal pentapeptide of the R17 coat protein. Following the procedure of these authors we studied the translation into oligopeptides of the protected f2 RNA fragments in our nuclease-treated initiation, pretranslocation, and posttranslocation complex preparations. The oligopeptides formed were characterized by paper electrophoresis. The results obtained in the cases of initiation complex preparation, pretranslocation complex preparations I and III, and posttranslocation complex preparation, are shown in Figure 4. In the case of each complex preparation the oligopeptides obtained were those expected to be translated from the f2 RNA fragments known to be present in the complex. Thus, a large majority (about 81–93%) of the oligopeptides translated from both initiation and pretranslocation complex preparations was the N-terminal pentapeptide of the coat protein (fMet-Ala-Ser-Asn-Phe) corresponding to the fact that the large majority (91–97%) of the f2 RNA fragments in these complex preparations have a 3′-terminal ---AUGGCUUCCAACUUU sequence. Moreover, a small portion (7–19%) of the oligopeptides formed was the N-terminal hexapeptide of the coat protein (fMet-Ala-Ser-Asn-Phe-Thr), and we saw in the previous section that the minority (3–9%) of the f2 RNA fragments in these complex preparations has the 3′-terminal sequence ---AUGGCUUCCAACUUUACU which specifies the formation of this hexapeptide. Finally, the amount of N-terminal heptapeptides (fMet-Ala-Ser-Asn-Phe-Thr-Gln) translated from either initiation or pretranslocation complex preparations, if any, was less than 4%.

As noted earlier, the characterization of the posttranslocation complex preparation revealed that only about half of the ribosome complexes in it are posttranslocation complexes and the rest consist mainly of initiation complexes and to a very small extent, pretranslocation complexes. Furthermore, the nucleotide sequence studies revealed that in the posttranslocation complex preparations 29–41% of the f2 RNA fragments have a 3′-terminal sequence specifying the first six amino acids of the coat protein, 6–11% specifying the first seven amino acids and about 53–60% (presumably those present in the initiation complexes in this preparation) the first five amino acids. Translation of the f2 RNA fragments in the posttranslocation complex preparation resulted in more N-terminal hexapeptides (about 82%) and heptapeptides (about 12%) of the coat protein and less N-terminal pentapeptides (about 6%) than expected from the relative amounts of the f2 RNA fragments specifying each. It is conceivable that the same defect of the initiation complexes in the posttranslocation complex preparation that inhibited their conversion into posttranslocation complexes also made them inefficient in translating the f2 RNA fragment in them.

Demonstration that Both S_2 Factor and GTP Are Required for Triggering Ribosome Movement along the mRNA

In the experiments presented so far the conversion of the pretranslocation complex to the posttranslocation complex was inhibited either by fusidic acid, an inhibitor of translocation, or by preparing and keeping the pretranslocation complex at 0° at which temperature translocation does not occur. We wanted (a) to verify that both of the reactants needed for the conversion of a pretranslocation complex into posttranslocation complex (*i.e.*, S_2 and GTP) are also required for ribosome movement and (b) to exclude the hypothetical possibility that ribosome movement, though independent of either S_2 or GTP or both, is, on the one hand, inhibited by fusidic acid and, on the other hand, does not occur at 0°. For these purposes we performed the following experiments (see Table III). We prepared pretranslocation complex at 0°, removed as far as possible GTP and contaminating G (S_2) factor by gel filtration at 0°, and incubated the complex

TABLE III: Dependence of Ribosome Movement along the mRNA on S_2 and GTP: Data from Electrophoretic Analyses of N-terminal Oligopeptide Fragments of f2 Coat Protein Translated from f2 RNA Fragments in a Pretranslocation Complex Preparation Incubated with: (a) S_2, (b) GTP, and (c) Both S_2 and GTP (*i.e.*, Posttranslocation Complex Preparation.)[a]

Aliquot	Final Incubation At °C	With Added	[^{14}C]Phe Incorpd into Peptide (pmole)	[^{3}H]Thr Incorpd into Peptide (pmole)	pmole of Thr:pmole of Phe
1	0		0.25	0.05	0.20
2	37		0.25	0.05	0.20
3	37	S_2	0.24	0.08	0.33
4	37	GTP	0.22	0.08	0.36
5	37	S_2, GTP	0.81	0.74	0.91

[a] Aliquots of pretranslocation complex solution II were treated in the following way: aliquot 1 was kept at 0°. Aliquots 2, 3, 4, and 5 were first incubated at 37° for 3 min, subsequently aliquot 3 was supplemented with 0.6 μg/ml of S_2, aliquot 4 with 0.2 mM GTP, aliquot 5 with 0.6 μg/ml of S_2, and 0.2 mM GTP. Finally, aliquot 2, 3, 4, and 5 were incubated at 37° for 5 min, and cooled to 0°. The aliquots were further processed according to the procedure described in the section on Processing of Initiation, Pretranslocation, and Posttranslocation Complex Preparations for Translation and the legend to Figure 4. The material eluted from 1-cm wide strips of the electropherograms were counted. The amounts of amino acids incorporated into peptides are those present in the major peak (see Figure 4). Aliquot 5 incorporated more Phe than the other aliquots; it is possible that this reflects that the posttranslocation complex is more protected against damage by ribonuclease under our conditions than the pretranslocation complex.

at 37° without and with S_2 or GTP or both. The resulting complex preparations were treated with pancreatic ribonuclease, the protected f2 RNA fragment in each was translated into oligopeptides and the oligopeptides formed were characterized by paper electrophoresis (see the previous section). The following results were obtained. Incubation of the pretranslocation complex at 37° causes no increase in the ratio of threonine:phenylalanine incorporated which is taken here to reflect the ratio of hexapeptide to pentapeptide formed. Incubation at 37° with either added S_2 or GTP (but not both) causes a 1.8-fold increase in this ratio. This may reflect at least in part a contamination of the pretranslocation complex preparation with small amounts of S_2 and GTP. Incubation at 37° with added S_2 and GTP results in a 4.5-fold increase in this ratio, actually 91% of the oligopeptides formed is hexapeptide in these conditions. The results clearly prove that both S_2 and GTP are required for ribosome movement along mRNA.

In summarizing the results of the translation assay it can be stated that the fact that the major translation product of f2 RNA fragments in posttranslocation complexes is one aminoacyl residue longer than that of f2 RNA fragments in initiation or pretranslocation complexes is fully consistent with the conclusion that one step of the ribosome is three

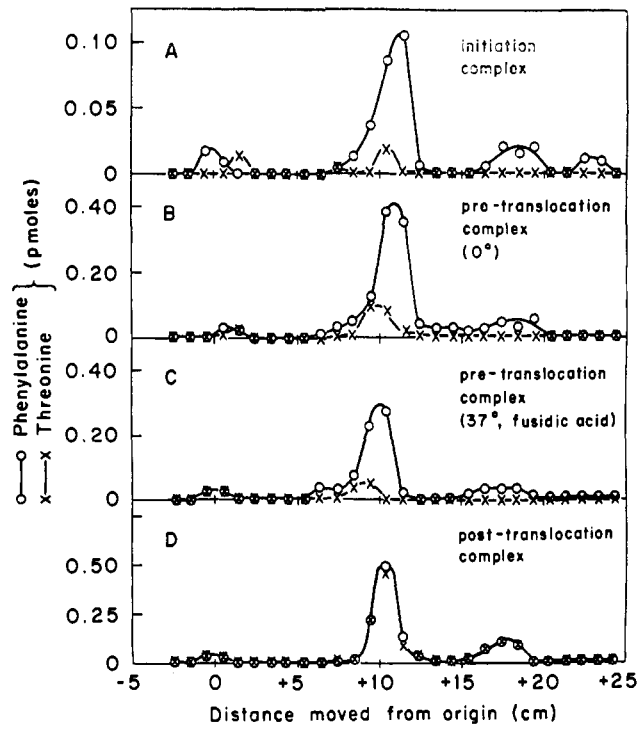

FIGURE 4: Electrophoretic analysis of N-terminal oligopeptide fragments of f2 coat protein translated from f2 RNA fragments in initiation, pretranslocation, and posttranslocation complexes treated with pancreatic ribonuclease. Solutions of the oligopeptide fragments translated from the various complexes (see the part on Processing of the Initiation, Pretranslocation, and Posttranslocation Complex Preparations for Translation in the Experimental Section) were applied to Whatman No. 3MM paper and fractionated by electrophoresis in a buffer containing 12.5 ml of acetic acid and 14 ml of pyridine per l. at 40 V/cm for 90 min. Strips (1 cm) were cut, eluted with 1 ml of H_2O, and counted in 10 ml of Bray's scintillation fluid (Bray, 1960). The nature of the complex preparation used in the experiment, and in the case of pretranslocation complex, the procedure followed to inhibit translocation are specified in the figure. The following results and considerations indicate that the major phenylalanine- and threonine-containing peak (at +10 cm in the electropherogram) corresponds to the N-terminal oligopeptide of the f2 coat protein. (a) The lack of retention on Dowex 50 (H^+) indicates that the α-amino group of the N-terminal aminoacyl residue of the oligopeptide is blocked (formylmethionine) and that the oligopeptide does not contain arginine (*i.e.*, does not correspond to the f2 maturation protein (Lodish, 1969). (b) Threonine incorporation resulting from the translation of the f2 RNA fragment in the posttranslocation complex depends on the presence of f2 RNA, fMet-$tRNA_f^{Met}$, and Ala-tRNA in the reaction mixture in which the posttranslocation complex is formed and on the presence of serine, asparagine, and phenylalanine (*i.e.*, all amino acids preceding threonine in the N-terminal coat oligopeptide) in the reaction mixture in which the f2 RNA fragment is translated (data not shown). It is conceivable that the minor peak (at +17 cm in the electropherogram) containing phenylalanine (if resulting from the translation of the f2 RNA fragment in either the initiation or pretranslocation complex) or both phenylalanine and threonine (if resulting from the translation of the f2 RNA fragment in the posttranslocation complex) may result from the deamidation of a small portion of the asparaginyl residues to aspartyl residues in the oligopeptides. The average ratio of threonyl to phenylalanyl residues in the major peak calculated from the data of three separate experiments is 0.07 in the translation of the f2 RNA fragment of the initiation complex, 0.19 in that of the pretranslocation complex, and 0.94 in that of the posttranslocation complex. The ratio of glutaminyl to threonyl residues in the major peak is 0.19 in the translation of the f2 RNA fragment of the pretranslocation complex and 0.16 in that of the posttranslocation complex.

nucleotides long. Moreover the fact that ribosome movement is triggered by S_2 and GTP, the same components triggering translocation indicates that ribosome movement along the mRNA takes place in the translocation phase.

Differences in Sedimentation Rates among Initiation, Pretranslocation, and Posttranslocation Complexes

It was discussed earlier that to determine the proportion of a complex preparation consisting of the type of complex wanted we obtained initiation complexes with ^{14}C-labeled fMet-tRNA$_f^{Met}$. Aliquots of these were converted into pretranslocation and posttranslocation complexes by reacting them with ^{3}H-labeled Ala-tRNA in the presence of the other required components. An examination of these doubly labeled complex preparations revealed (see the section on Characterization of the Complex Preparations) that the conversion of f-[^{14}C]met-tRNA$_f^{Met}$ in the initiation complex preparation into f-[^{14}C]Met-[^{3}H]Ala-tRNA amounted to about 40% in the pretranslocation complex preparation and to about 54% in the posttranslocation complex preparation. Consequently, in a pretranslocation complex preparation ^{14}C counts were present in two forms: in f-[^{14}C]Met-[^{3}H]Ala-tRNA bound at the A site in the pretranslocation complexes and in f-[^{14}C]Met-tRNA$_f^{Met}$ in the initiation complexes. The ^{3}H counts in the same preparation are present almost exclusively in the form of f-[^{14}C]Met-[^{3}H]Ala-tRNA bound at the A site in the pretranslocation complexes.

The sedimentation profile of labeled pretranslocation complex preparations (I, II, or III) centrifuged through a 5–20% (w/v) sucrose gradient in 10 mM Mg^{2+} revealed the following. The peak of ^{3}H counts sedimented consistently one fraction heavier than the peak of ^{14}C counts or ^{32}P counts (^{32}P counts were present in the f2 RNA in the complex). Since the ^{32}P counts and the ^{14}C counts were contributed jointly by pretranslocation complexes and initiation complexes in the preparation, whereas the ^{3}H counts only by the pretranslocation complexes proper, it follows that the pretranslocation complex sediments slightly faster than the initiation complex (J. Waterson *et al.*, manuscript in preparation).

One aliquot of the triply labeled pretranslocation complex preparation was treated with puromycin to destroy the initiation complexes by releasing their formyl-[^{14}C]methionyl residues (Bretscher, 1966). A study of the sedimentation profile of such a pretranslocation complex preparation (devoid of ^{14}C label in initiation complexes) revealed that the ^{3}H and ^{14}C counts sedimented in an overlapping fashion. Their peak sedimented one fraction heavier than the peak of ^{32}P counts. This result confirms the conclusion that the pretranslocation complex sediments faster than the initiation complex. (It also shows that the puromycin treatment of the initiation complex did not result in the release of the ribosome from the mRNA.)

In posttranslocation complex preparations also ^{14}C counts are present in two forms: In f-[^{14}C]Met-[^{3}H]Ala-tRNA bound at the P site of the posttranslocation complexes and in f-[^{14}C]Met-tRNA$_f^{Met}$ in the initiation complexes present in the preparation.

In the sedimentation profile of the posttranslocation complex preparation, however, ^{3}H, ^{14}C, and ^{32}P counts peaked in one fraction. Moreover, this one fraction was the same as that in which the ^{14}C label and ^{32}P label of an initiation complex preparation (in a separate gradient) peaked.

Discussion

The data presented indicate that the movement of the ribosome along the mRNA during protein synthesis is triggered by S_2 factor and GTP and occurs concurrently with peptidyl-tRNA translocation and one step of the ribosome along the mRNA is (as expected) three nucleotides long.

The above conclusions about ribosome movement are based on the difference between the 3′ ends of the mRNA fragments protected against ribonuclease cleavage in initiation, pretranslocation, and posttranslocation complexes. Thus, they reflect the movement along the mRNA of those parts of the ribosome which are protecting the mRNA. The results provide no solution of the problem of the relative positions of the two ribosomal subunits in the various phases of the peptide-chain elongation cycle (Bretscher, 1968; Spirin, 1969; Lengyel and Söll, 1969).

The finding that the pretranslocation complex sediments faster in a sucrose gradient than the posttranslocation complex (or the initiation complex) (see also Spirin, 1969; Schreier and Noll, 1970, 1971) suggests that the compactness of the ribosome changes during the peptide-chain elongation cycle. It is probable that the changes in compactness may involve changes in the relative position of the two ribosomal subunits (Bretscher, 1968; Spirin, 1969; Lengyel and Söll, 1969).

The following considerations make it reasonable to assume that this hypothetical change in the relative positions of the two subunits is a consequence of the conformational change in a single protein. There are indications that the conversion of a pretranslocation complex into a posttranslocation complex may require the cleavage of a single GTP molecule (Nishizuka and Lipmann, 1966b). The simplest assumption is that the cleavage of a single GTP should *directly* affect the conformation of a single protein only. The identification of this protein is of obvious interest. (Similar considerations hold for the conversion of the posttranslocation complex to the pretranslocation complex.)

It should be noted that we know now about the following events occurring during the translocation phase of peptide-chain elongation: (1) release of discharged tRNA (Lucas-Lenard and Haenni, 1969; Kuriki and Kaji, 1968); (2) peptidyl-tRNA translocation (Traut and Monro, 1964; Erbe *et al.*, 1969); (3) GTP cleavage (Haenni and Lenard, 1968; Brot *et al.*, 1968); (4) ribosome movement along the mRNA (this paper); and (5) change in the rate of sedimentation of the ribosomes (Spirin, 1969; Schreier and Noll, 1970, 1971; this paper). One can assay for each of the above events selectively.

It will be revealing to establish the temporal order of these events and the components of the translating machinery involved in each.

Acknowledgments

We thank Professor F. Egami for U_2 ribonuclease.

References

Adams, J. M., Jeppesen, P. G. N., Sanger, F., and Barrell, B. G. (1969), *Nature (London) 223*, 1009.

Arima, T., Uchida, T., and Egami, F. (1968), *Biochem. J. 106*, 609.

Arlinghaus, R., Shaeffer, J., and Schweet, R. (1964), *Proc. Nat. Acad. Sci. U. S. 51*, 1291.

Bray, G. (1960), *Anal. Biochem. 1*, 279.

Bretscher, M. S. (1966), *Cold Spring Harbor Symp. Quant. Biol. 31*, 289.

Bretscher, M. S. (1968), *Nature (London) 218*, 675.

Brot, N., Ertel, R., and Weissbach, H. (1968), *Biochem. Biophys. Res. Commun. 31*, 563.
Brot, N., Redfield, B., and Weissbach, H. (1970), *Biochem. Biophys. Res. Commun. 41*, 1388.
Brownlee, G. G., and Sanger, F. (1969), *Eur. J. Biochem. 11*, 395.
Capecchi, M. R. (1967), *Biochem. Biophys. Res. Commun. 28*, 773.
Conway, T. W., and Lipmann, F. (1964), *Proc. Nat. Acad. Sci. U. S. 52*, 1462.
Eggen, K., Oeschger, M. P., and Nathans, D. (1967), *Biochem. Biophys. Res. Commun. 28*, 587.
Eoyang, L., and August, J. T. (1968), *Methods Enzymol. 12B*, 530.
Erbe, R., Nau, M., and Leder, P. (1969), *J. Mol. Biol. 39*, 441.
Ertel, R., Brot, N., Redfield, B., Allende, J. E., and Weissbach, H. (1968a), *Proc. Nat. Acad. Sci. U. S. 59*, 861.
Ertel, R., Redfield, B., Brot, N., and Weissbach, H. (1968b), *Arch. Biochem. Biophys. 128*, 331.
Gordon, J. (1968), *Proc. Nat. Acad. Sci. U. S. 59*, 179.
Gordon, J. (1969), *J. Biol. Chem. 244*, 5680.
Grollman, A. P., and Stewart, M. L. (1968), *Proc. Nat. Acad. Sci. U. S. 61*, 719.
Gupta, S. L., Chen, J., Schaefer, L., Lengyel, P., and Weissman, S. M. (1970), *Biochem. Biophys. Res. Commun. 39*, 883.
Gupta, S. L., Chen, J., Schaefer, L., Weissman, S. M., and Lengyel, P. (1969), *Cold Spring Harbor. Symp. Quant. Biol. 34*, 630.
Haenni, A., and Lucas-Lenard, J. (1968), *Proc. Nat. Acad. Sci. U. S. 61*, 1363.
Haenni, A., Lucas-Lenard, J., and Gordon, J. (1968), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 27*, 397.
Hindley, J., and Staples, D. H. (1969), *Nature (London) 224*, 964.
Kondo, M., Eggertsson, G., Eisenstadt, J., and Lengyel, P. (1968), *Nature (London) 220*, 368.
Kuechler, E., and Rich, A. (1970), *Nature (London) 225*, 920.
Kuriki, Y., and Kaji, A. (1968), *Proc. Nat. Acad. Sci. U. S. 61*, 1399.
Lengyel, P., and Söll, D. (1969), *Bacteriol. Rev. 33*, 264.
Lipmann, F. (1969), *Science 164*, 1024.
Lodish, H. F. (1968), *Nature (London) 220*, 345.
Lodish, H. F. (1969), *Biochem. Biophys. Res. Commun. 37*, 127.
Lucas-Lenard, J., and Haenni, A. (1969), *Proc. Nat. Acad. Sci. U. S. 63*, 93.
Lucas-Lenard, J., and Lipmann, F. (1966), *Proc. Nat. Acad. Sci. U. S. 55*, 1562.
Lucas-Lenard, J., Tao, P., and Haenni, A. (1969), *Cold Spring Harbor Symp. Quant. Biol. 34*, 455.
Miller, D. L., and Weissbach, H. (1970), *Biochem. Biophys. Res. Commun. 38*, 1016.
Monro, R. E., Staehelin, T., Celma, M. L., and Vazquez, D. (1969), *Cold Spring Harbor Symp. Quant. Biol. 34*, 357.
Muench, K. H., and Berg, P. (1966), *in* Procedures in Nucleic Acid Research, Cantoni, G. L., and Davies, D. R., Ed., New York, N. Y., Harper & Row, p 375.
Nirenberg, M., and Leder, P. (1964), *Science 145*, 1399.
Nishizuka, Y., and Lipmann, F. (1966a), *Arch. Biochem. Biophys. 116*, 344.
Nishizuka, Y., and Lipmann, F. (1966b), *Proc. Nat. Acad. Sci. U. S. 55*, 212.
Ohe, K., Weissman, S. M., and Cooke, N. R. (1969), *J. Biol. Chem. 244*, 5320.
Ono, Y., Skoultchi, A., Waterson, J., and Lengyel, P. (1969a), *Nature (London) 222*, 645.
Ono, Y., Skoultchi, A., Waterson, J., and Lengyel, P. (1969b), *Nature (London) 223*, 697.
Pestka, S. (1968), *Proc. Nat. Acad. Sci. U. S. 61*, 726.
Ravel, J. M. (1967), *Proc. Nat. Acad. Sci. U. S. 57*, 1181.
Schreier, M. H., and Noll, H. (1970), *Nature (London) 227*, 128.
Schreier, H. M., and Noll, H. (1971), *Proc. Nat. Acad. Sci. U. S. 68*, 805.
Shorey, R., Ravel, J., Garner, G. W., and Shive, W. (1969), *J. Biol. Chem. 244*, 4555.
Skogerson, L., Roufa, D., and Leder, P. (1971), *Proc. Nat. Acad. Sci. U. S. 68*, 276.
Skoultchi, A., Ono, Y., Moon, H. M., and Lengyel, P. (1968), *Proc. Nat. Acad. Sci. U. S. 60*, 675.
Skoultchi, A., Ono, Y., Waterson, J., and Lengyel, P. (1969), *Cold Spring Harbor Symp. Quant. Biol. 34*, 437.
Skoultchi, A., Ono, Y., Waterson, J., and Lengyel, P. (1970), *Biochemistry 9*, 508.
Speyer, J. F. (1967), *in* Molecular Genetics, Part II, Taylor, J. H., Ed., New York, N. Y., Academic Press, p 137.
Spirin, A. S. (1969), *Cold Spring Harbor Symp. Quant. Biol. 34*, 197.
Steitz, J. A. (1969a), *Cold Spring Harbor Symp. Quant. Biol. 34*, 621.
Steitz, J. A. (1969b), *Nature (London) 224*, 957.
Takanami, M., Yan, Y., and Jukes, T. H. (1965), *J. Mol. Biol. 12*, 761.
Tanaka, N., Kinoshita, T., and Masukawa, H. (1968), *Biochem. Biophys. Res. Commun. 30*, 278.
Traut, R., and Monro, R. (1964), *J. Mol. Biol. 10*, 63.
Uchida, T., and Egami, F. (1966), *in* Procedures in Nucleic Acid Research, Cantoni, G. L., and Davies, D. R., Ed., New York, N. Y., Harper & Row, p 3.
Viñuela, E., Algranati, I. D., and Ochoa, S. (1967), *Eur. J. Biochem. 1*, 3.
Waterson, J., Beaud, G., and Lengyel, P. (1970), *Nature (London) 227*, 34.
Weber, K., and Konigsberg, W. (1967), *J. Biol. Chem. 242*, 3563.
Weissbach, H., Miller, D., and Hachmann, J. (1970a), *Arch. Biochem. Biophys. 137*, 262.
Weissbach, H., Redfield, B., and Hachmann, J. (1970b), *Arch. Biochem. Biophys. 141*, 384.