Panemangalore, M., & Brady, F. O. (1978) *J. Biol. Chem. 253*, 7898–7904.
Perry, R. P., & Kelley, D. E. (1970) *J. Cell. Physiol. 76*, 127–140.
Piscator, M. (1964) *Nord. Hyg. Tidskr. 45*, 76–82.
Richards, M. P., & Cousins, R. J. (1975a) *Bioinorg. Chem. 4*, 215–224.
Richards, M. P., & Cousins, R. J. (1975b) *Biochem. Biophys. Res. Commun. 64*, 1215–1223.
Rudd, C. J., & Herschman, H. R. (1978) *Toxicol. Appl. Pharmacol. 44*, 511–521.
Rudd, C. J., & Herschman, H. R. (1979) *Toxicol. Appl. Pharmacol. 47*, 273–278.
Shaikh, Z. A., & Lucis, O. J. (1971) *Experientia 27*, 1023–1025.
Shaikh, Z. A., & Smith, J. C. (1976) *Chem.-Biol. Interact. 15*, 327–336.
Shapiro, S. G., Squibb, K. S., Markowitz, L. A., & Cousins, R. J. (1978) *Biochem. J. 175*, 833–840.
Squibb, K. S., & Cousins, R. J. (1977) *Biochem. Biophys. Res Commun. 75*, 806–812.
Squibb, K. S., Cousins, R. J., & Feldman, S. L. (1977) *Biochem. J. 164*, 223–228.
Steinberg, R. A., Levinson, B. B., & Tomkins, G. M. (1975a) *Cell 5*, 29–35.
Steinberg, R. A., Levinson, B., & Tomkins, G. M. (1975b) *Proc. Natl. Acad. Sci. U.S.A. 72*, 2007–2011.
Thompson, E. B., Tomkins, G. M., & Curran, J. F. (1966) *Proc. Natl. Acad. Sci. U.S.A. 56*, 296–303.
Tomkins, G. M., Gelehrter, T. D., Granner, D., Martin, Jr., D., Samuels, H. H., & Thompson, E. B. (1969) *Science (Washington, D.C) 166*, 1474–1480.
Tomkins, G. M., Levinson, B. B., Baxter, J. D., & Dethlefsen, L. (1972) *Nature (London), New Biol. 239*, 9–14.
Webb, M. (1972 *Biochem. Pharmacol. 21*, 2751–2765.
Webb, M., & Daniel, M. (1975) *Chem.-Biol. Interact. 10*, 269–276.
Winge, D. R., Premakumar, R., & Rajagopalan, K. V. (1975) *Arch. Biochem. Biophys. 170*, 242–252.
Zähringer, J., Baliga, B. S., & Munro, H. N. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 857–861.

Mechanism of Translocation: Effect of Cognate Transfer Ribonucleic Acids on the Binding of $AUGU_n$ to 70S Ribosomes†

Karl Holschuh, Jutta Bonin, and Hans Günter Gassen*

ABSTRACT: We try to mimic the unidirectional sliding-type movement of the PP-tRNA·mRNA complex with respect to the ribosome by looking at the effect of different combinations of cognate tRNAs on the stability of the 70S·$AUGU_n$ complex. The association constant for the binary complex 70S·$AUGU_3$ was determined as 6.8×10^5 M^{-1}. Addition of $tRNA_f^{Met}$ resulted in a 67-fold increase in the association constant, which with both cognate tRNAs is revised to $K_{assoc} = 2.2 \times 10^8$ M^{-1}. Increasing the chain length of the oligonucleotide from $AUGU_3$ to $AUGU_{13}$ did not further raise the association constant. The data indicate that the stability of the 70S ribosome·mRNA interaction is governed by the presence of the cognate tRNAs and is topographically restricted to the decoding domains. Since a peptidyl group in the tRNA increases the affinity of $AUGU_3$ for the ribosome by up to 15-fold, we conclude that the affinity of the peptidyl transfer center for the peptidyl moiety pulls the PP-tRNA·mRNA complex from the A (aminoacyl-tRNA) site to the P (peptidyl-tRNA) site. EF-G·GTP or EF-G·GMPPCP 5′-(β,γ-methylene)triphosphate] displace $tRNA_f^{Met}$ from the quaternary complex 70S·$AUGU_n$·$tRNA_f^{Met}$·$tRNA^{Phe}$ (n = 3 and 6) at $Mg^{2+} < 25$ mM. From the amount of EF-G·GTP bound to a 70S ribosome, it follows that the elongation factor replaces the deacylated tRNA in a stoichiometric way. These data indicate that the EF-G·GTP-dependent release of the deacylated tRNA from the P site, followed by removal of EF-G·GDP from the 50S subunit, is sufficient to trigger the translocation of the mRNA·PP-tRNA complex.

The EF-G-[1] and GTP-promoted translocation of the mRNA in ribosome-dependent protein synthesis represents a fascinating mechanistic problem and is still one of the unsolved problems of mechanochemical reactions (Lucas-Lenard & Lipmann, 1971; Haselkorn & Rothman-Denes, 1973; Leder, 1973; Brot, 1977; Weissbach, 1979). During this process, the mRNA is translocated by three nucleotides, i.e., 10 Å per GTP hydrolyzed. Translocation involves the following changes in going from the pre- to the posttranslocational ribosomal state: the release of the deacylated tRNA from the P site (Kuriki & Kaji, 1968; Lucas-Lenard & Haenni, 1969; Ishitsuka et al., 1970; Roufa et al., 1970; Skogerson et al., 1971), the movement of the peptidyl-tRNA to the P site, the site at which the peptidyl moiety is reactive with puromycin, and the translocation of mRNA by one codon bringing a new codon into the A site (Erbe & Leder, 1968; Haenni & Lucas-Lenard, 1968; Pestka, 1968; Erbe et al., 1969).

The movement of the mRNA along the ribosome during translocation can be demonstrated experimentally. It was shown that the mRNA fragment which is protected against ribonuclease by the attached ribosome extends three nucleotides further toward the 3′ end in the mRNA·ribosome com-

† From the Fachgebiet Biochemie, Institut für Organische Chemie und Biochemie, Technische Hochschule Darmstadt, D-6100 Darmstadt, Federal Republic of Germany. *Received March 13, 1980.*

[1] Abbreviations used: EF-G, bacterial elongation factor EF-G; GMPPCP, guanosine 5′-(β,γ-methylene)triphosphate; *N*-AcMet-$tRNA_f^{Met}$, methionyl-tRNA in which the α-amino group is acetylated; ME, β-mercaptoethanol; A site, aminoacyl-tRNA binding site of the ribosome; P site, peptidyl-tRNA binding site.

0006-2960/80/0419-5857$01.00/0

plex that has been treated with EF-G and GTP (Thach & Thach, 1971; Gupta et al., 1971). Frame-shift suppressor tRNAs such as $tRNA^{Gly}_{sufD}$, which bind to a tetranucleotide codon, move the mRNA by four rather than three nucleotides (Riddle & Carbon, 1973).

So far, three basic theories have been proposed to explain the mechanism of the translocation reaction. In one model, the driving mechanism for translocation is provided entirely by conformational changes of the ribosome, the other elements such as tRNA maintaining their structure (Spirin, 1969; Chuang & Simpson, 1971; Schreier & Noll, 1971; Waterson et al., 1972; Yourno & Kohno, 1972; Kaziro, 1978). In contrast to this explanation, another model has been proposed which makes use of a conformational change in the tRNA to advance the mRNA by a single codon (Woese, 1970). Finally, the inchworm theory suggests that during the enzymatic binding of aminoacyl-tRNA to the A site of the ribosome a kink is created in the mRNA. The effect of EF-G and GTP is then envisioned as straightening out the kink, and when this is done PP-tRNA is moved from the A to the P site (Hardesty et al., 1969). For one translocational round, i.e., the synthesis of a dipeptidyl-tRNA, hydrolysis of GTP to GDP is not required since GTP can be replaced by the noncleavable analogue GMPPCP (Brot et al., 1972; Inoue-Yokosawa et al., 1974; Modolell et al., 1975; Belitsina et al., 1975; San-Millan et al., 1975). For the synthesis of a tripeptidyl-tRNA, however, hydrolysis of GTP has to occur. Fusidinic acid, a translational inhibitor, blocks translocation by tightening the binding of EF-G·GDP to the ribosome (Brot et al., 1969; Bodley et al., 1970; Kuriki et al., 1970). Thus, release of the elongation factor from the ribosome as EF-G·GDP represents a further critical step in translocation. Experiments using other translational inhibitors also point to EF-G·GTP-dependent removal of deacylated tRNA as the initiating step in translocation (Gavrilova et al., 1976; Cabanas et al., 1978a,b). Furthermore, translocation is blocked by increased Mg^{2+} concentrations (>20 mM), which may either tighten the ribosome structure or bind the deacylated tRNA too strongly to the P site (Spirin, 1969; Belitsina & Spirin, 1979).

In an excellent review on the elongation cycle in protein synthesis, Leder stresses the importance of the affinity of the peptidyl transferase center for both its substrates—peptidyl-tRNA (P site) and aminoacyl-tRNA (A site). After peptide transfer, the A site holds the product, the PP-tRNA, with low affinity while the P site binds the second reaction product, the deacylated tRNA. Thus, the ribosome as an enzymelike system favors the formation of the more stable intermediate—the enzyme·substrate complex instead of the enzyme·product complex—by moving the mRNA·PP-tRNA complex from the A to the P site. If one follows Leder's hypothesis, translocation is governed by the EF-G·GTP-dependent release of the deacylated tRNA from the P site. The second part of the translocation reaction should be spontaneously caused by the affinity of the P site for the peptidyl-tRNA.

In the following part of this publication, we present experiments which support the hypothesis put forward by Leder (Leder, 1973). We give evidence that the binding of an oligonucleotide to the 70S ribosome is not governed by its chain length, but by the presence of the cognate tRNAs, and is mainly topographically restricted to the two decoding sites of the ribosome. The presence of either two tRNAs or one peptidyl-tRNA at the P site guarantees a stable type of ribosome–oligonucleotide interaction. Stoichiometric amounts of EF-G·GTP or EF-G·GMPPCP may replace the deacylated tRNA from the P site. Then the affinity of the peptidyl moiety of the PP-tRNA for the P site results in spontaneous translocation as soon as the P site is free from both tRNA and EF-G·GDP.

Materials and Methods

Materials

AUG was prepared from ApU and ppG by the combined use of polynucleotide phosphorylase (EC 2.7.7.8) and ribonuclease T1 (EC 3.1.4.22) as described (Mohr & Thach, 1969). $AUGU_n$'s were synthesized with primer-dependent polynucleotide phosphorylase from *Thermus thermophilus* (Hishinuma et al., 1977). With this enzyme, the incubation temperature was raised to 65 °C, which reduced the time required for oligonucleotide synthesis to 15 min (A. Kunst and H. G. Gassen, unpublished experiments). The products were separated by DEAE-cellulose chromatography and converted to their NH_4^+ salts with Dowex 50-X4 (NH_4^+). Homogeneity was checked by paper chromatography in 1 M CH_3COONH_4 (pH 5.4) and ethanol (1:1). Nucleoside composition was verified with the nucleoside analyzer (Gassen & Leifer, 1970). Tritium-labeled oligonucleotides were prepared accordingly by using [^{3}H]uridine 5′-diphosphate (specific activity 40 Ci/mol). Ribosomes (70S; "tight couples") were isolated from *E. coli* MRE 600 by zonal centrifugation (Leifer & Kreuzer, 1971; Noll et al., 1973). These ribosomes were completely dependent upon added EF-G when tested for poly(Phe) synthesis in the presence of poly(U), EF-Tu, and an appropriate polymerization mixture (Linde et al., 1979). Elongation factor G was isolated from a 10^5g supernatant of a ribosome preparation by the method of Arai et al. (1972). A sample of EF-G was kindly given to us by Dr. W. Möller, Biochemical Department, University of Leiden, and was used as a control in all EF-G-dependent reactions. Tritium-labeled EF-G was prepared by the following procedure: 100 μg of EF-G was incubated for 30 s at 0 °C in 150 μL of a solution containing 70 mM Na_3BO_3, pH 9.2, 10 mM $MgCl_2$, 15 mM KCl, and 50 mM aqueous formaldehyde. Then 50 μL of [^{3}H]$NaBH_4$ (100 mM in 10 mM NaOH, specific activity 5 Ci/mmol) was added and incubation continued at 0 °C for 15 min. The labeled protein was freed from excess [^{3}H]$NaBH_4$ by dialysis against 2 L of 50 mM Tris-HCl, pH 7.5, 150 mM NH_4Cl, 10 mM $MgCl_2$, and 10 mM mercaptoethanol at 4 °C for 12 h by using a flow-through, 200-μL capacity, dialysis cell. The estimated specific activity of the labeled EF-G was 11 Ci/mmol. $tRNA_f^{Met}$ (1.5 nmol/A_{260} unit), $tRNA^{Phe}$ (1.2 nmol/A_{260} unit), and benzoylated DEAE-cellulose were purchased from Boehringer, Mannheim. $tRNA_m^{Met}$ (1.4 nmol/A_{260} unit) was a gift from Dr. R. Buckingham, Institut de Biologie Physico-Chemique, Paris. [^{3}H]CCA-$_f^{Met}$ (1.5 nmol/A_{260} unit, specific activity 135 Ci/mol) was given to us by Dr. H. Sternbach, Max-Planck-Institut für experimentelle Medizin, Göttingen (Sternbach & Sprinzl, 1978). Membranes highly permeable to oligonucleotides, for equilibrium dialysis, came from Rhône-Poulenc, Paris.

Methods

Equilibrium Dialysis. Equilibrium dialysis was performed as described (Uhlenbeck et al., 1970). Technical details and the method of data calculation have been reported previously (Lipecky et al., 1977). The reaction mixture contained 20 pmol of 70S ribosomes, 150 pmol of tRNA, 10–300 pmol of $AUGU_n$, and a buffer solution with 50 mM Tris-HCl, pH 7.5, 150 mM NH_4Cl, 20 mM $MgCl_2$, and 10 mM mercaptoethanol. One chamber of the dialysis cell contained buffer alone. Equilibrium was reached after 12 h at 0 °C. Asso-

ciation constants and the number of binding sites were calculated from Scatchard plots as shown in Figure 3.

Adsorption of Ribosomal Complexes to Nitrocellulose Filters. The binding of various combinations of oligonucleotides and tRNAs to 70S ribosomes (Figures 6 and 7) was performed in the following 200-μL reaction mixtures: 50 mM Tris-HCl, pH 7.5, 150 mM NH_4Cl, 10 mM $MgCl_2$, 10 mM mercaptoethanol, and 450 pmol of 70S ribosomes. The respective amounts of oligonucleotides and tRNAs were the following: $AUGU_3$, 900 pmol; $AUGU_6$, 1 nmol; [^{3}H]-$tRNA_f^{Met}$, 500 pmol; [^{3}H]fMet-$tRNA_f^{Met}$, 550 pmol; $tRNA^{Phe}$, 600 pmol; [^{3}H]Phe-$tRNA^{Phe}$, 330 pmol; [^{3}H]-$AUGU_3$, 850 pmol; [^{3}H]$AUGU_6$, 1.1 nmol. The mixtures were incubated at 37 °C, and a 20-μL sample was withdrawn from the solution every minute and filtered over nitrocellulose membranes in the usual way (Leder, 1968). After 3 or 4 min, 15 μg of EF-G and 100 μM of the respective guanosine nucleotide, in 10 μL of the above-listed buffer, were added.

Isolation of 70S·$AUGU_3$·tRNA Complexes by Sepharose 6B Column Chromatography. In order to demonstrate the existence of stable 70S·$AUGU_3$·tRNA complexes, the appropriate reaction mixtures were chromatographed on a Sepharose 6B column (0.8 × 20 cm), equilibrated, and eluted with 50 mM Tris-HCl, pH 7.5, 150 mM NH_4Cl, 20 mM $MgCl_2$, and 10 mM mercaptoethanol at 0–4 °C; the flow rate was 0.5 mL/min. Samples (80 μL) containing 110 pmol of 70S ribosomes, 180 pmol of [^{3}H]$AUGU_3$, 230 pmol of $tRNA^{Phe}$, and/or 200 pmol of $tRNA_f^{Met}$ were incubated in elution buffer for 5 min at 37 °C prior to column chromatography. From the column effluent, 500-μL aliquots were collected and tested as follows: (a) the concentration of 70S ribosomes was determined from their absorbance at 260 nm; (b) total $AUGU_3$ concentration was found by applying a 50-μL sample to a glass fiber filter and then determining the radioactivity; (c) concentration of $AUGU_3$ within the 70S·$AUGU_3$·tRNA complex was calculated by first applying a 400-μL sample to nitrocellulose membranes and then counting the radioactivity after the removal of non-ribosome-bound $AUGU_3$ by washing the filters with 15 mL of buffer.

Mg^{2+}-Dependent Binding of [^{3}H]$tRNA_f^{Met}$ to the 70S·$AUGU_3$·$tRNA^{Phe}$ Complex. The Mg^{2+} dependence of the binding of $tRNA_f^{Met}$ to $AUGU_3$-programmed 70S ribosomes was followed in a volume of 100 μL containing 50 mM Tris-HCl, pH 7.5, 150 mM NH_4Cl, 10 mM mercaptoethanol, 0–50 mM $MgCl_2$, 10 pmol of 70S, ribosomes 3 nmol of $AUGU_3$, 20 pmol of $tRNA_f^{Met}$, and 20 pmol of $tRNA^{Phe}$. The mixture was incubated at 37 °C for 3 min and filtered over nitrocellulose membranes. In the second experiment, 15 μg of EF-G and 100 μM GTP were added after 3 min, and incubation was continued for 1 min.

Binding of the Elongation Factor EF-G to 70S Ribosomes. A Sepharose 6B column (0.8 × 20 cm) was equilibrated and eluted with 50 mM Tris-HCl, pH 7.5, 150 mM NH_4Cl, 10 mM $MgCl_2$, 10 mM mercaptoethanol, and the respective guanosine nucleotide (100 μM GDP, GTP, or GMPPCP). A sample (20 pmol) of 70S ribosomes in 100 μL of elution buffer was incubated with 2 μg of [^{3}H]EF-G with or without the guanosine nucleotides for 10 min at 37 °C and applied to the column. Elution and assay conditions were the same as described in the preceding section.

Results

Binding of Phe-tRNA to the 70S·$AUGU_3$·$tRNA_f^{Met}$ Complex. At a Mg^{2+} concentration above 30 mM, two tRNA molecules can be bound to a 70S ribosome, albeit with a high degree of poly(U)-independent binding of the tRNA (A. Möller, M. Schmitt, D. Riesner, and H. G. Gassen, unpublished experiments). However, in polyuridylate-dependent binding of Phe-tRNA at 12–16 mM Mg^{2+}, only 30–50% of the ribosomes can be saturated with Phe-tRNA. The quantity of ribosomes active in the binding of Phe-tRNA is further decreased when polyuridylate ($n > 30$) is replaced by U_3–U_6 (Linde et al., 1979). This indicates that critically important for the ability of ribosomes to bind an elongator tRNA is the efficient binding of the mRNA analogue to the ribosome. If the binding of the ternary complex Phe-$tRNA^{Phe}$·EF-Tu·GTP to the 70S initiation complex, 70S·$AUGU_4$·fMet-$tRNA_f^{Met}$, is followed, stoichiometric binding of the elongator tRNA can be achieved (figure 1). Even in the absence of initiation factors and EF-Tu, the ribosomes can be saturated with Phe-$tRNA^{Phe}$ by increasing the concentration of the tRNA (Figure 2A,B). Plotting the reciprocal of the concentration of active ribosomes vs. the reciprocal of free Phe-$tRNA^{Phe}$ concentration gives a straight line, intercepting the ordinate at 1. This indicates that at [Phe-$tRNA^{Phe}$] → ∞ 100% of the 70S ribosomes are active in the binding of Phe-$tRNA^{Phe}$ to

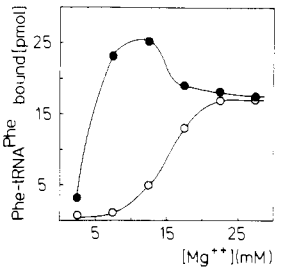

FIGURE 1: Mg^{2+} dependence of the binding of Phe-$tRNA^{Phe}$ to the 70S·$AUGU_4$·fMet-$tRNA_f^{Met}$ complex with or without EF-Tu·GTP. The 100-μL reaction mixture contained 50 mM Tris-HCl, pH 7.5, 150 mM NH_4Cl, 10 mM ME, 1 mM GTP, 7.6 μg of EF-Tu·GDP, 1 μg of IF-1/3, 1.8 μg of IF-2, 25 pmol of 70S, 750 pmol of $AUGU_3$, 30 pmol of fMet-$tRNA_f^{Met}$, 30 pmol of [^{3}H]Phe-$tRNA^{Phe}$, and Mg^{2+} as indicated on the abscissa. Incubation was for 3 min at 37 °C, followed by filtration of the mixture over nitrocellulose filters. (●) With EF-Tu; (○) without EF-Tu. The real situation in the above experiment is represented by $tRNA_f^{Met}$ in the P site and fMet-Phe-$tRNA^{Phe}$ in the A site; i.e., peptide transfer should occur with the release of EF-Tu·GDP from the ribosome. The decrease in the amount of Phe-$tRNA^{Phe}$ bound at elevated Mg^{2+} comes from the competition of $tRNA^{Phe}$ with [^{3}H]Phe-$tRNA^{Phe}$ for the A site.

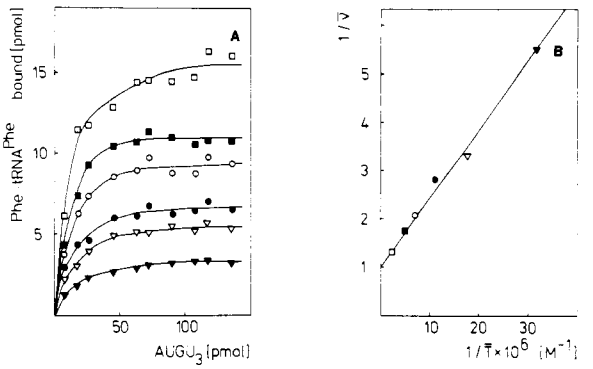

FIGURE 2: (A) Binding of Phe-$tRNA^{Phe}$ to a 70S·$AUGU_3$·$tRNA_f^{Met}$ complex for different ratios of Phe-$tRNA^{Phe}$ over 70S ribosomes. The binding of Phe-$tRNA^{Phe}$ was examined in a 100-μL volume containing 50 mM Tris-HCl, pH 7.5, 150 mM NH_4Cl, 10 mM $MgCl_2$, 10 mM ME, 21 pmol of 70S, 150 pmol of $tRNA_f^{Met}$, increasing amounts of $AUGU_3$, and the following ratios Phe-$tRNA^{Phe}$/70S: (▼) 0.33; (▽) 0.58; (●) 0.80; (○) 1.16; (■) 1.50; (□) 3.00. (B) Plot of the reciprocal of the active ribosome concentration ($1/\bar{\nu}$) vs. the reciprocal of the free Phe-tRNA concentration ($1/\bar{T}$) for [$AUGU_3$] → ∞. At Phe-$tRNA^{Phe}$ → ∞) the straight line intercepts the ordinate at 1, indicating that 100% of the ribosomes are active in the binding of the tRNA.

Table I: Effect of the Chain Length and of Cognate tRNAs on the the Formation of the 70S·AUGU$_n$ Complex[a]

complex	tRNA present	K_{assoc} (M^{-1})	no. of binding sites
70S·AUGU$_3$		6.8×10^5	1.3
70S·AUGU$_6$		8.7×10^5	1.3
70S·AUGU$_{13}$		9.3×10^5	1.4
70S·AUGU$_3$	tRNA$_f^{Met}$	4.6×10^7	1.0
70S·AUGU$_3$	tRNA$_m^{Met}$	3.9×10^6	1.0
70S·AUGU$_3$	tRNA^{Phe}	4.1×10^6	1.2
70S·AUGU$_3$	tRNA$_f^{Met}$, tRNA^{Phe}	2.2×10^8	1.1
70S·AUGU$_6$	tRNA$_f^{Met}$, tRNA^{Phe}	2.1×10^6	1.0
70S·AUGU$_{13}$	tRNA$_f^{Met}$, tRNA^{Phe}	2.2×10^8	1.1
70S·AUGU$_3$	tRNA$_m^{Met}$, tRNA^{Phe}	0.8×10^7	1.0

[a] The association constants as measured by equilibrium dialysis (see Methods) were derived from a Scatchard plot as shown for one specific complex in Figure 3. The standard error of K_{assoc} was calculated to be ±5%.

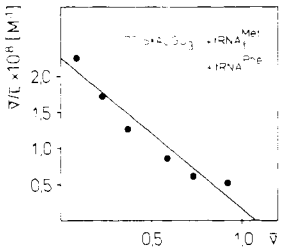

FIGURE 3: Scatchard plot for the formation of the 70S·AUGU$_3$ complex in the presence of excess tRNA$_f^{Met}$ and tRNA^{Phe}. For the estimation of the best straight line, the method of least squares (unweighted) was used: $\bar{\nu}$ = AUGU$_3$ bound per 70S ribosome; L = concentration of free AUGU$_3$; n = number of binding sites for AUGU$_3$ on the 70S ribosome.

the aminoacyl site. The stimulatory effect of tRNA in the peptidyl site of the ribosome—which in the classical poly(U)-Phe-tRNA^{Phe} assay system is the deacylated tRNA^{Phe} present in the reaction mixture—is greater than the influence of oligonucleotide chain length on the ability of ribosomes to bind an elongator tRNA (Table I).

Effect of Cognate tRNAs on the Stability of the 70S·AUGU$_3$ Complex. The effect of cognate tRNAs (the initiator tRNA$_f^{Met}$ and two elongator tRNAs, tRNA$_m^{Met}$ and tRNA^{Phe}) on the binding of AUGU$_n$ to 70S ribosomes was followed by equilibrium dialysis (Table I). Association constants and the number of binding sites were in each case calculated from a Scatchard plot as documented for one individual complex in Figure 3. The data listed in Table I demonstrate the dominating influence of the initiator tRNA on the formation of the binary 70S·AUGU$_3$ complex. The presence of the tRNA$_f^{Met}$ increases the association constant for the 70S·AUGU$_3$ complex more than 60-fold. Elongator tRNAs like tRNA$_f^{Met}$ and tRNA^{Phe} have a positive influence, but to a much weaker extent (6-fold). The data for the aminoacylated tRNAs are in a comparable range, but are not listed in the table, since about 50% of the aminoacyl moiety is lost during the 12-h equilibrium dialysis. The presence of both cognate tRNAs results in a further stabilization as compared to the 70S·AUGU$_3$·tRNA$_f^{Met}$ complex. Again, the elongator tRNA, tRNA$_f^{Met}$, is less efficient as compared to the initiator tRNA. The chain length of the oligonucleotide has a small effect on binary complex formation, whereas no chain length effect can be detected in the presence of two cognate tRNAs. Thus, the binding of an oligonucleotide to the 70S ribosome is governed

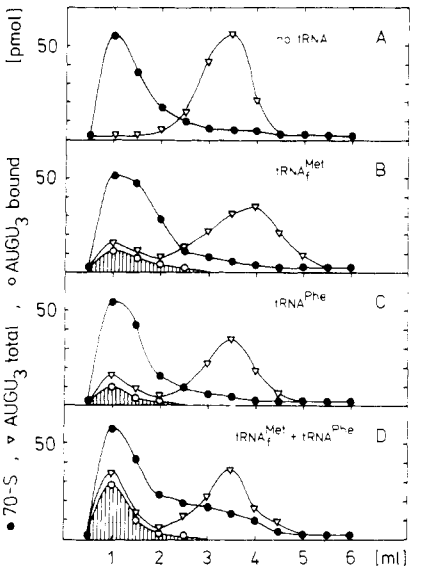

FIGURE 4: Isolation of 70S·AUGU$_3$ complexes by Sepharose 6B column chromatography in the presence of cognate tRNAs. Details of the experiments are described under Methods. (A) No tRNA; (B) plus tRNA$_f^{Met}$; (C) plus tRNA^{Phe}; (D) plus both tRNAs. (●) 70S ribosomes; (▽) total amount of AUGU$_3$; (○) amount of AUGU$_3$ in the complex.

Table II: Increasing Affinity of AUGU$_3$ toward the Ribosome by the Peptidyl Moiety of tRNA[a]

complex	tRNA present	K_{assoc} (M^{-1})	no. of binding sites
70S·AUGU$_3$	tRNA$_f^{Met}$	4.6×10^7	1.0
70S·AUGU$_3$	fMet-tRNA$_f^{Met}$	1.1×10^8	1.1
70S·AUGU$_3$	tRNA$_m^{Met}$	3.9×10^6	1.0
70S·AUGU$_3$	*N*-AcMet-tRNA$_m^{Met}$	5.3×10^7	1.1

[a] About 50% of the respective tRNA contained a blocked aminoacyl residue, 10% of which was hydrolyzed during the 12-h dialysis. Thus, the true differences between the N-blocked AA-tRNA and free tRNA should be even greater than indicated by the experimental data in the table.

mainly by the presence of one or two cognate tRNAs, and is topographically restricted to the two decoding domains of the ribosome.

Since the interplay of the "one tRNA state" as compared to the "two tRNA state" is of critical importance for the proposed model of translocation, we used gel permeation chromatography as a second method to examine the influence of cognate tRNAs on the stability of the 70S·AUGU$_3$ complex. The presence of tRNA$_f^{Met}$ increases the lifetime of the 70S·AUGU$_3$ complex such that it partially elutes in the void volume of a Sepharose 6B column (Figure 4). Addition of the second tRNA increases the amount of complex by a factor of two. These data further support the dominating effect of the cognate tRNAs on the mRNA·ribosome association.

Influence of the Peptidyl Moiety of PP-tRNA on the Stability of the 70S·AUGU$_3$ Complex. It was previously reported that the peptidyl moiety of peptidyl-tRNA contributes to the stability of the 70S·PP-tRNA interaction (Rychlik, 1966). As outlined in the introduction, the higher affinity of the peptidyl-tRNA binding site (P site), as compared to the aminoacyl-tRNA binding site (A site), of the ribosomes for the PP-tRNA may be a prerequisite for a translocation mechanism. Therefore, we investigated the influence of the peptidyl moiety in fMet-tRNA$_f^{Met}$ and in *N*-AcMet-tRNA$_m^{Met}$ on the 70S·AUGU$_3$ association constant (Table II). Replacing

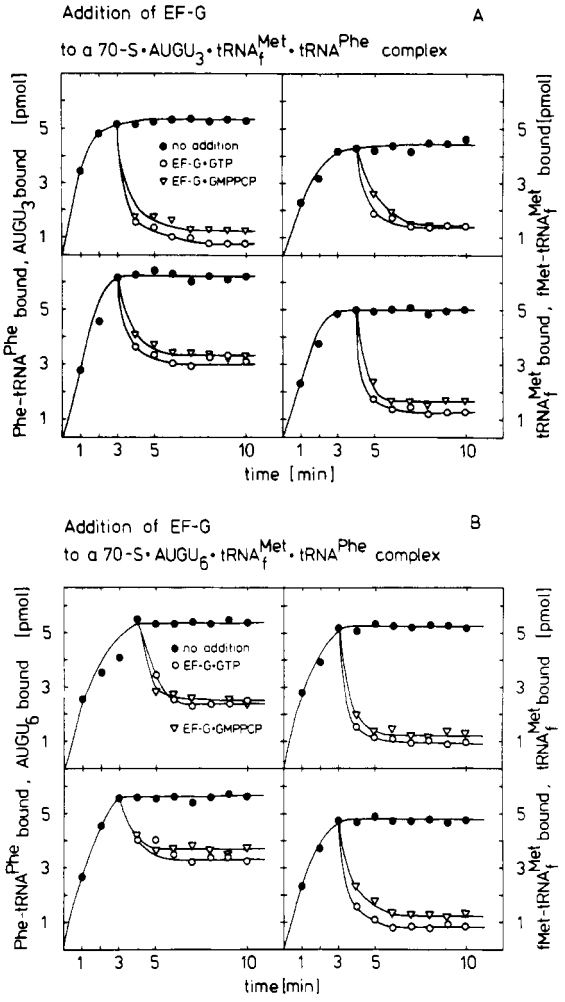

FIGURE 5: Effect of the elongation factor EF-G on the stability of the quaternary complex 70S·$AUGU_3$·$tRNA_f^{Met}$·$tRNA^{Phe}$ and the respective complex with $AUGU_6$. (●) Binding of the labeled component to the ternary complex; (○) release of the labeled component after the addition of EF-G·GTP; (▽) addition of EF-G·GMPPCP. Addition of GTP, EF-G, or EF-G·GDP had no effect on the quaternary complex.

$tRNA_f^{Met}$ by fMet-$tRNA_f^{Met}$ resulted in a 2.4-fold increase in the association constant. A comparison of *N*-AcMet-$tRNA_m^{Met}$ with $tRNA_m^{Met}$ shows that the peptidyl moiety is more effective; namely, a 13.6-fold stimulation was found. It may be characteristic for the initiator tRNA, which is initially attached to the 30S subunit, to bind with high affinity to the 70S ribosome, and, therefore, the effect of the formylmethionyl group is less pronounced.

Release of $tRNA_f^{Met}$ from a 70S·$AUGU_n$·$tRNA_f^{Met}$·$tRNA^{Phe}$ Complex by the Addition of EF-G and GTP. According to the translocation model proposed in the introduction, the removal of the deacylated tRNA from the peptidyl site represents the critical step in the translocation reaction. Therefore, we investigated the effect of EF-G and GTP on the release of $tRNA_f^{Met}$ from the quaternary complex 70S·$AUGU_3$·$tRNA_f^{Met}$·$tRNA^{Phe}$ (Figure 5A,B).

Addition of free EF-G or GTP did not affect the stability of the complex, indicating that the 70S ribosomes were free of intrinsic EF-G activity. The destabilizing effect of EF-G with either GTP or GMPPCP is evident from the release of the labeled compound from the complex (fMet-$tRNA_f^{Met}$ or $tRNA_f^{Met}$ > 80%; $AUGU_3$ > 80–90%; Phe-tRNA ~ 60%).

Experiments were done in addition with the nonanucleotide $AUGU_6$ to guarantee that after one round of translocation a codon still occupies the A site (Figure 5B). A consistent

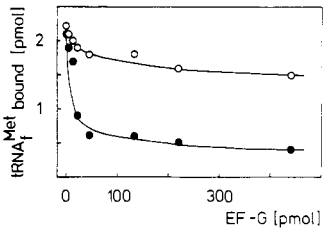

FIGURE 6: Amount of EF-G required to release $tRNA_f^{Met}$ from the 70S·$AUGU_7$·$tRNA^{Phe}$ complex. The reaction mixture contained in 100 μL 10 pmol of 70S, 33 pmol of [^{3}H]$tRNA_f^{Met}$, 1.3 nmol of $AUGU_7$, and in one experiment 36 pmol of $tRNA^{Phe}$. Buffer conditions were as described in Figure 5. Preincubation was 4 min at 37 °C, then EF-G and 10 nmol of GTP were added, and incubation was continued for 5 min. the EF-G was quantitated by its GTP binding capacity. (●) +$tRNA^{Phe}$; (○) −$tRNA^{Phe}$.

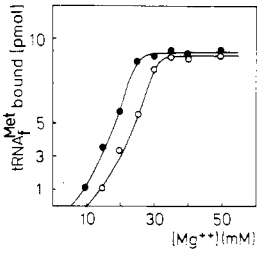

FIGURE 7: Mg^{2+} dependence of the binding of $tRNA_f^{Met}$ to a 70S·$AUGU_3$·$tRNA^{Phe}$ complex. (○) With EF-G·GTP; (●) without EF-G·GTP.

picture is found if $AUGU_3$ is replaced by $AUGU_6$. Yet, a higher amount of $AUGU_6$ remains bound to the ribosome, and only 40% Phe-$tRNA^{Phe}$ is released. This is plausible, since after translocation $tRNA^{Phe}$—always present in the incubation mixture—occupies the P site and Phe-$tRNA^{Phe}$ the A site.

In order to get an estimate of the amount of EF-G necessary to release the $tRNA_f^{Met}$ from the ribosome, we followed the tRNA dissociation with respect to EF-G concentration (Figure 6). At about a 2–5-fold excess of EF-G·GTP (as determined by the GTP binding capacity of the protein) over ribosomes, more than 80% of the bound tRNA is released. In the absence of $tRNA^{Phe}$, only a small percentage of the $tRNA_f^{Met}$ is removed from the ribosome. In agreement with other reports, occupation of the A site is required to release a tRNA from the P site (Lucas-Lenard & Haenni, 1969; Roufa et al., 1970).

It is known that the Mg^{2+} dependence of cell-free protein synthesis follows a bell-shaped curve with an optimum Mg^{2+} concentration of 12–18 mM (van Dieijen et al., 1975; Gavrilova et al., 1976). Excess Mg^{2+} may inhibit protein synthesis by reducing the dissociation of the deacylated tRNA from the P site of the ribosome. As can be seen from the data given in Figure 7, the binding of $tRNA_f^{Met}$ becomes stoichiometric with respect to the ribosome at 25 mM Mg^{2+}. The presence of EF-G and GTP causes a shift in the binding isotherm to lower Mg^{2+}. Thus, the addition of elongation factor to the system has a similar effect on the stability of the 70S·$AUGU_3$·$tRNA_f^{Met}$·$tRNA^{Phe}$ complex as when the Mg^{2+} concentration is lowered by approximately 5 mM. EF-G·GTP cannot release $tRNA_f^{Met}$ from the peptidyl site of 70S ribosomes at elevated Mg^{2+} concentrations (>30 mM). If the 70S ribosomes are treated with *p*-chloromercuribenzoate, a higher Mg^{2+} concentration is required to saturate the ribosomes with $tRNA_f^{Met}$ (data not shown).

Binding of the Elongation Factor EF-G to 70S Ribosomes. A stoichiometric displacement of the deacylated tRNA by EF-G·GTP represents the simplest explanation for the function of EF-G·GTP in translocation. In order to test this possibility, we followed the binding of tritium-labeled EF-G to ribosomes

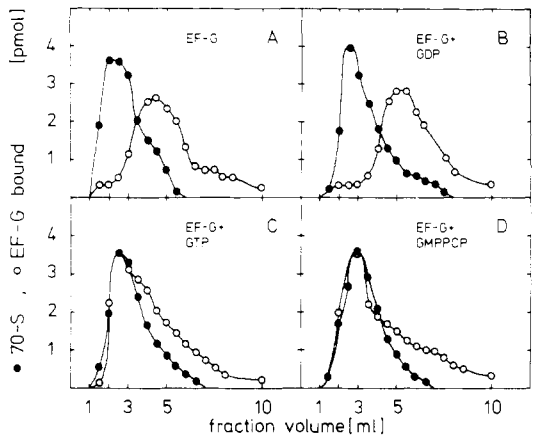

FIGURE 8: Binding of ^{3}H-labeled EF-G to 70S ribosomes as followed by Sepharose 6B column chromatography. The experimental details for the experiment are described under Methods. The elution buffer was made 0.1 mM of the respective guanosine nucleotide to avoid the release of EF-G from the 70S ribosome because of GTP hydrolysis. (●) 70S ribosomes; (○) total amount of EF-G.

by Sepharose 6B gel permeation chromatography. The appropriate nucleotide was always added to the elution buffer to avoid release of EF-G from the ribosome because of the GTPase action. With GTP or GMPPCP, EF-G forms a stable complex with the 70S ribosomes (Figure 8). From the specific radioactivity of the elongation factor, a stoichiometry of 70S to EF-G of ~1 was calculated.

Translocation in the Absence of EF-G. Peptide bond formation, albeit at a lower rate, can be achieved in the absence of elongation factors (Pestka, 1968; Gavrilova et al., 1976). To present evidence that a spontaneous translocation of the PP-tRNA·mRNA complex occurs as soon as the tRNA is released from the P site of the ribosome, we tried to synthesize the tripeptide fMet-Phe-Phe in the absence of the elongation factor EF-G. For tripeptide synthesis, the fMet-Phe-tRNA^{Phe} has to be translocated from the A site to the P site, prior to the binding of a new Phe-tRNA to the A site. EF-G action was replaced by a shift in the Mg^{2+} concentration from 14 to 7 mM, since it is evident from the data presented in Figure 7 that at 7 mM Mg^{2+} very little $tRNA_f^{Met}$ remains bound to the ribosome.

The 70S·AUGU$_7$·$tRNA_f^{Met}$(P)·fMet-Phe-tRNA(A) complex was formed at 14 mM Mg^{2+} (Table III). Next, the Mg^{2+} concentration was lowered to 7 mM by diluting the reaction mixture twofold. Phe-tRNA·EF-Tu·GTP was added, and the incubation was continued for 10 min at 37 °C. The reaction mixture was treated with 0.5 M NaOH at 37 °C, and the amino acids and peptides released from the tRNA were separated on benzoylated cellulose.

Table III: Formation of the Tripeptide fMet-Phe-Phe in the Absence of EF-G[a]

	expt a	expt b	expt c
fMet, Phe (pmol)	97.1	46.8	50.2
fMet-Phe (pmol)	<2	23.1	30.2
fMet-Phe-Phe (pmol)	<2	<2	12.3

[a] The reaction mixture for experiment a contained (in 100 μL) 50 mM Tris-HCl, pH 7.5, 150 mM NH_4Cl, 14 mM $MgCl_2$, 10 mM mercaptoethanol, 0.2 mM GTP, 45 pmol of [^{3}H]fMet-$tRNA_f^{Met}$, and 50 pmol of [^{3}H]Phe-tRNA^{Phe}. Experiment b contained in addition 200 pmol of 70S ribosomes, 6.8 nmol of AUGU$_7$, and 12 μg of EF-Tu·GTP. For experiment c, this reaction mixture was diluted with 100 μL of the above buffer system, containing no Mg^{2+} but 100 pmol of [^{3}H]Phe-tRNA^{Phe} and 12 μg of EF-Tu·GTP. Incubation was 10 min at 37 °C, with an additional 10 min in experiment c after dilution of the Mg^{2+} to 7 mM. AA-tRNA and PP-tRNA were deacylated by treatment with 100 μL of 0.5 M NaOH at 37 °C for 10 min. After neutralization with 100 μL of 0.5 M HCl, the products were separated on a benzoylated DEAE-cellulose column (Pestka, 1971). The amino acids were eluted from the column with 50 mM KOAc, pH 5.7, the fMet-Phe with formamide/ethanol/H_2O (32:30:38) and the tripeptide fMet-Phe-Phe with formamide/ethanol/H_2O (65:30:5).

Discussion

We have been intrigued by the ideas on the mechanism of translocation, as outlined by Leder in a review article several years ago (Leder, 1973). In order to prove his hypotheses, we tried to mimic the stepwise propagation of the mRNA along the ribosome by following the effect of cognate tRNAs on the oligonucleotide–ribosome equilibrium (Holschuh & Gassen, 1980).

The dominating effect of P-site occupation by $tRNA_f^{Met}$ on the binding of Phe-tRNA^{Phe} in the presence and absence of elongation factor is shown in Figures 1 and 2. Besides the stoichiometric binding of the aminoacyl-tRNA, it is noteworthy that only a 7-fold excess of AUGU$_3$ over ribosome is used. In the conventional poly(U)/Phe-tRNA system, a 500–1000-fold excess of poly(U) has to be used to achieve optimum binding of Phe-tRNA (Linde et al., 1979).

Ribosome–mRNA interaction in protein synthesis occurs at the level of the 30S subunit and the 70S ribosome. Structural and biochemical properties of both complexes are widely different. The 30S subunit selects the initiation region of a cistron and frames the mRNA into codons. Besides the initiator tRNA:AUG double strand, the "Shine–Dalgarno" complex (a second RNA·RNA hybrid) guarantees a stable, and with respect to the mRNA region, selective 30S mRNA complex formation (Steitz, 1979; Schmitt et al., 1980).

The construction of 70S ribosomes from two subunits, with the mRNA at the interface, restricts mRNA movement to a one-dimensional motion. According to the data listed in table I, the association between the 70S ribosome and the mRNA, in contrast to the 30S·mRNA complex, is not a function of the chain length of the oligonucleotide. It is governed by the number and types of cognate tRNAs present and topographically is restricted to the decoding domain of the ribosome. In a more provocative way, we can say that the mRNA is not bound to the ribosome, but to the tRNA, which as a mRNA·tRNA complex has a high affinity for the ribosome.

The association constant of the 70S·mRNA complex is increased by a factor of 320 in the presence of the two cognate tRNAs. With $tRNA_f^{Met}$, the effect is less pronounced, which indicates the high affinity of the initiator tRNA for the P site. With the elongator tRNA, however, the contribution of the peptidyl moiety *N*-AcMet-$tRNA_f^{Met}$ to the stability of the 70S·AUGU$_3$ complex exceeds that of the formylmethionyl group (Table II). Therefore, we can define two stable intermediates in the 70S·oligonucleotide association: first, the pretranslocational complex with a two-tRNA occupation of the ribosome (Table I); second, the AA-tRNA selection step, the one tRNA state, but with PP-tRNA at the P site (Table II). Here the interaction of the nascent peptide chain with the peptide transfer center additionally stabilizes the ribosome·tRNA complex.

For translocation to occur, the peptidyl moiety has to be transferred to the AA-tRNA at the A site. Since the peptidyl group reacts with puromycin in the absence of elongation factors and GTP and the free energy of hydrolysis of the ester bond exceeds that of the peptide bond by 4 kcal, it is conceivable that this group-transfer reaction occurs spontaneously with the release of EF-Tu·GDP from the ribosome (Monro,

1969; Lucas-Lenard & Lipmann, 1971). This leaves the PP-tRNA at the A site, which according to Leder is its low-affinity site ("enzyme·product relationship") (Leder, 1973). The ribosome·mRNA complex is still of the "locked type" since it is stabilized by the presence of the two tRNAs. The next step is the displacement of the deacylated tRNA from the P site by a stoichiometric amount of EF-G·GTP (see Figures 6 and 8). Displacement of the deacylated tRNA becomes possible since a major contribution to its stable binding came from the peptidyl moiety. Even fMet-tRNA$_f^{Met}$ is replaced by EF-G·GTP with either $AUGU_3$ or the nonanucleotide as a mRNA substitute. Removal of the deacylated tRNA from the P site is coregulated by magnesium ions. At too high Mg^{2+} concentration, the tRNA sticks at the P site, which explains the inhibition of protein synthesis at $Mg^{2+} >$ 25 mM, and at too low Mg^{2+} the regulatory function of EF-G is lost (nonenzymatic translocation). The GTPase, located on the 50S ribosomes, hydrolyzes GTP to GDP, which causes the conformational change in the elongation factors, as a result of which the protein loses its affinity for the ribosome (Brot et al., 1972; Baca et al., 1976; Weissbach, 1979).

With the high-affinity site of the peptidyl transfer center now vacant (Leder's enzyme·substrate relation), the PP-tRNA·mRNA moves from its low- to its high-affinity site. The data listed in Table III, namely, the synthesis of a tripeptide at a low Mg^{2+} concentration, support the concept of a spontaneous movement of the PP-tRNA to the P site. Furthermore, one has to realize that the tRNA governs the stability of the ribosome·mRNA interaction. The displacement of the deacylated tRNA not only makes the P site available for the PP-tRNA but also loosens the ribosome·mRNA interaction at the same time, and thus facilitates the translocation of the PP-tRNA·mRNA complex.

The translational cycle is mainly controlled by the elongation factors, EF-Tu·GTP functioning as the AA-tRNA association factor and EF-G·GTP as the tRNA dissociation factor. The activity of both factors is modulated by the ratio GTP/GDP in the sense that the GTP complex represents the protein with high affinity for the ribosome, whereas the GDP complex is the low-affinity form, and Mg^{2+} acts in all tRNA-dependent reactions as cofactor. The stability of the ribosome·mRNA interaction is controlled by the number and types of tRNAs present. The affinity of the P site for the peptidyl tRNA represents a major factor in the translocation of the mRNA·PP-tRNA by a codon length.

Acknowledgments

We thank E. Rönnfeldt for her help in preparing the manuscript. The work was supported by grants from the Deutsche Forschungsgemeinschaft and the Fonds der Chemischen Industrie. We are grateful to Dr. H. Sternbach, Max-Planck-Institut für Experimentelle Medizin, Göttingen, for providing us with the ^{3}H-labeled tRNA$_f^{Met}$. We thank Dr. W. Möller, Biochemical Department, University of Leiden, for a sample of elongation factor G and Dr. R. Buckingham, Institut de Biologie Physico-Chimique, Paris, for the tRNA$_f^{Met}$.

References

Arai, K. I., Kawakita, M., & Kaziro, Y. (1972) *J. Biol. Chem. 247*, 7029–7037.

Baca, O. G., Rohrbach, M. S., & Bodley, J. W. (1976) *Biochemistry 15*, 4570–4574.

Belitsina, N. V., & Spirin, A. S. (1979) *Eur. J. Biochem. 94*, 315–320.

Belitsina, N. V., Glykhova, M. A., & Spirin, A. S. (1975) *FEBS Lett. 54*, 35–38.

Bodley, J. W., Zieve, F. J., Lin, L., & Zieve, S. T. (1970) *J. Biol. Chem. 245*, 5656–5661.

Brot, N. (1977) *Mol. Mech. Protein Biosynth.*, 375–411.

Brot, N., Spears, C., & Weissbach, H. (1969) *Biochem. Biophys. Res. Commun. 34*, 834–850.

Brot, N., Yamasaki, E., Redfield, B., & Weissbach, H. (1972) *Arch. Biochem. Biophys. 148*, 148–155.

Cabanas, M. J., Vazquez, D., & Modolell, J. (1978a) *Eur. J. Biochem. 87*, 21–27.

Cabanas, M. J., Vazquez, D., & Modolell, J. (1978b) *Biochem. Biophys. Res. Commun. 83*, 991–997.

Chuang, D., & Simpson, M. V. (1971) *Proc. Natl. Acad. Sci. U.S.A. 68*, 1474–1478.

Erbe, R. W., & Leder, P. (1968) *Biochem. Biophys. Res. Commun. 31*, 798–803.

Erbe, R. W., Nau, M. M., & Leder, P. (1969) *J. Mol. Biol. 39*, 441–460.

Gassen, H. G., & Leifer, W. (1970) *Fresenius' Z. Anal. Chem. 252*, 337–343.

Gavrilova, L. P., Kostiashkina, O. E., Kotelinansky, V. E., Rutkewitch, N. M., & Spirin, A. S. (1976) *J. Mol. Biol. 101*, 537–552.

Gupta, S. L., Waterson, J., Sopori, M. L., Weissman, S. M., & Lengyel, P. (1971) *Biochemistry 10*, 4416–4421.

Haenni, A. L., & Lucas-Lenard, J. (1968) *Proc. Natl. Acad. Sci. U.S.A. 61*, 1363–1369.

Hardesty, B., Culp, W., & Mc Keehan, W. (1969) *Cold Spring Harbor Symp. Quant. Biol. 39*, 331–334.

Haselkorn, R., & Rothman-Denes, L. B. (1973) *Annu. Rev. Biochem. 42*, 397–438.

Hishinuma, F., Hirai, K., & Sakaguchi, K. (1977) *Eur. J. Biochem. 77*, 575–583.

Holschuh, K., & Gassen, H. G. (1980) *FEBS Lett. 110*, 169–172.

Inoue-Yokosawa, N., Ishikawa, C., & Kaziro, Y. (1974) *J. Biol. Chem. 249*, 4321–4323.

Ishitsuka, H., Kuriki, Y., & Kaji, A. (1970) *J. Biol. Chem. 245*, 3346–3351.

Kaziro, Y. (1978) *Biochim. Biophys. Acta 505*, 95–127.

Kuriki, Y., & Kaji, A. (1968) *Proc. Natl. Acad. Sci. U.S.A. 68*, 1399–1405.

Kuriki, Y., Inoue, N., & Kaziro, Y. (1970) *Biochim. Biophys. Acta 244*, 487–497.

Leder, P. (1968) *Methods Enzymol. 12*, 725–727.

Leder, P. (1973) *Adv. Protein Chem. 27*, 213–242.

Leifer, W., & Kreuzer, T. (1971) *Anal. Biochem. 44*, 89–96.

Linde, R., Khanh, N. Q., Lipecky, R., & Gassen, H. G. (1979) *Eur. J. Biochem. 93*, 565–572.

Lipecky, R., Kohlschein, J., & Gassen, H. G. (1977) *Nucleic Acids Res. 4*, 3627–3642.

Lucas-Lenard, J., & Haenni, A. L. (1969) *Proc. Natl. Acad. Sci. U.S.A. 63*, 93–97.

Lucas-Lenard, J., & Lipmann, F. (1971) *Annu. Rev. Biochem. 40*, 409–448.

Modolell, J., Girbès, T., & Vazquez, D. (1975) *FEBS Lett. 60*, 109–113.

Mohr, S. C., & Thach, R. E. (1969) *J. Biol. Chem. 244*, 6566–6576.

Monro, R. E. (1967) *J. Mol. Biol. 26*, 147–151.

Noll, M., Hapka, B., Schreier, M. H., & Noll, H. (1973) *J. Mol. Biol. 75*, 281–294.

Pestka, S. (1968) *Proc. Natl. Acad. Sci. U.S.A. 61*, 726–733.

Pestka, S. (1971) *Methods Enzymol. 20*, 508–511.

Riddle, D. L., & Carbon, J. (1973) *Nature (London), New Biol. 242*, 230–232.

Roufa, D. J., Skogerson, L., & Leder, P. (1970) *Nature (London) 227*, 567–570.
Rychlik, I. (1966) *Biochim. Biophys. Acta 114*, 425–427.
San-Millan, M. J., Vazquez, D., & Modolell, J. (1975) *Eur. J. Biochem. 57*, 431–440.
Schmitt, M., Manderschied, U., Kyriatsoulis, A., Brinckmann, U., & Gassen, H. G. (1980) *Eur. J. Biochem. 109*, 291–299.
Schreier, M. H., & Noll, H. (1971) *Proc. Natl. Acad. Sci. U.S.A. 68*, 805–809.
Skogerson, L., Roufa, D., & Leder, P. (1971) *Proc. Natl. Acad. Sci. U.S.A. 68*, 276–279.
Spirin, A. S. (1969) *Cold Spring Harbor Symp. Quant. Biol. 39*, 197–207.
Steitz, J. A. (1979) in *Ribosomes: Structure, Function, and Genetics* (Chambliss, G., Craven, G. R., Davies, J., Davis, K., Kahan, L., & Nomura, M., Eds.) pp 479–495, University Park Press, Baltimore.
Sternbach, H., & Sprinzl, M. (1978) *Methods Enzymol. 59*, 182–190.
Thach, S. S., & Thach, R. E. (1971) *Proc. Natl. Acad. Sci. U.S.A. 68*, 1791–1795.
Uhlenbeck, O. C., Baller, J., & Doty, P. (1970) *Nature (London) 225*, 508–510.
van Dieijen, G., van der Laken, C. J., van Knippenberg, P. H., & van Duin, J. (1975) *J. Mol. Biol. 93*, 351–366.
Waterson, J., Sopori, M. L., Gupta, S. L., & Lengyel, P. (1972) *Biochemistry 11*, 1377–1382.
Weissbach, H. (1979) in *Ribosomes: Structure, Function, and Genetics* (Chambliss, G., Craven, G. R., Davies, J., Davis, K., Kahan, L., & Nomura, M., Eds.) pp 377–412, University Park Press, Baltimore.
Woese, C. (1970) *Nature (London) 226*, 817–820.
Yourno, J., & Kohno, T. (1972) *Science (Washington, D.C.) 175*, 650–652.

Productive and Abortive Initiation of Transcription in Vitro at the *lac* UV5 Promoter†

Jay D. Gralla,* Agamemnon J. Carpousis, and James E. Stefano

ABSTRACT: The rates of productive and abortive initiation of transcription in vitro at the *lac* UV5 promoter have been determined and compared to values determined for phage λ and T7 promoters. The rate constants for productive initiation of *lac* transcript are consistently lower over a range of low to moderate concentration of initiating nucleoside triphosphate (ATP). Abortive initiation of *lac* dinucleoside tetraphosphate is also slower at low to moderate concentrations of ATP. These data demonstrate the existence of significant differences in initiation rate among promoters. We suggest that these differences may be a consequence of the initial mRNA sequences and extents of RNA polymerase cycling during initiation of promoter-specific transcription.

The detailed mechanism by which promoter-bound RNA polymerase binds incoming nucleoside triphosphates and initiates productive transcription is still uncertain [for reviews, see Krakow et al. (1976) and Chamberlain (1976)]. Many tentative conclusions concerning the rate and mechanism of initiation must now be reevaluated as a consequence of recent studies. For example, much information was derived from use of the rifampicin challenge assay for initiation (Mangel & Chamberlin, 1974; Rhodes & Chamberlin, 1975) which has now been shown to be based on an over-simplified mechanism of rifampicin action (McClure & Cech, 1978; Carpousis & Gralla, 1980). As a part of this reevaluation, certain conclusions based on this assay have been confirmed by a direct assay (Nierman & Chamberlin, 1979, 1980), but other conclusions remain uncertain. Moreover, it has been shown that initiation may be more complex than previously assumed since steps subsequent to formation of the first phosphodiester bond may be important to the mechanism. Specifically, McClure et al. (1978) showed that formation of the first phosphodiester does not necessarily lead to formation of a full-length transcript on the λ P_R' promoter. Subsequently, in a detailed study of the *lac*[1] UV5 promoter, Carpousis & Gralla (1980) showed initiation to be very complex; RNA polymerase catalyzes several cycles of synthesis accompanied by premature chain termination before escaping to form a fully committed elongation complex.

The *lac* UV5 promoter has been inferred to be a much slower initiator than phage T7 promoters (Stefano & Gralla, 1979). This inference, however, was based on comparing a rate determined by direct assay to one nucleoside triphosphate concentration with rate constants determined by rifampicin challenge assay. Since the T7 rate has now been studied by using a direct assay (Nierman & Chamberlin, 1979, 1980), a direct comparison with *lac* UV5 becomes possible. Below we show that the *lac* UV5 promoter has an initiation rate constant 2 orders of magnitude lower than that reported for T7 over a range of ATP concentration.

An important contributing factor to the initiation rate is assumed to be the rate of formation of the first phosphodiester bond. This rate has been estimated by using an "abortive initiation" assay which measures formation of dinucleoside tetraphosphate under conditions where chain elongation is disallowed (Johnston & McClure, 1976). In order to investigate the potential contribution of this rate to the slow overall rate of productive initiation of the *lac* UV5 promoter, we have determined the abortive initiation rate as a function of ATP

† From the Biochemistry Division of the Chemistry Department and the Molecular Biology Institute, University of California, Los Angeles, Los Angeles, California 90024. *Received May 9, 1980.* Supported by Grant No. CA19941 from the National Cancer Institute and a grant from the UCLA Academic Senate. Both A.J.C. and J.E.S. were supported as trainees on U.S. Public Health Service Grant No. GM 07185.

[1] Abbreviations used: *lac*, lactose operon; Tris, tris(hydroxymethyl)-aminomethane; PPO, 2,5-diphenyloxazole.

0006-2960/80/0419-5864$01.00/0