CHEMISTRY AND METABOLISM OF MACROMOLECULES:

Studies on the Role of Guanosine Triphosphate in Polypeptide Chain Initiation in *Escherichia coli*

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Studies on the Role of Guanosine Triphosphate in Polypeptide Chain Initiation in *Escherichia coli**

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SUMMARY

1. The formation and properties of the 30 S initiation complex and its subsequent conversion to the 70 S initiation complex have been studied. The 30 S polypeptide chain initiation complex contains fMet-tRNA and intact GTP in equimolar amounts. Incorporation of both species into the complex requires mRNA (f2 RNA, poly(U, G), or A-U-G) and large amounts of initiation factor IF 2. Initiation factor IF 1 stimulates 30 S complex formation.

2. Addition of 50 S subunits to the isolated 30 S complex results in the hydrolysis of all bound GTP to GDP and P_i. In the absence of 50 S subunits, no hydrolysis occurs. Concurrently a 70 S couple is formed and fMet-tRNA in the complex becomes available for peptidyl transfer.

3. Under appropriate conditions the 30 S complex can be depleted of its complement of GTP while retaining a full complement of fMet-tRNA. GTP released in this manner remains intact. Such a GTP-deficient 30 S complex can still accept 50 S subunits to form a 70 S couple and can still donate fMet into peptide linkage.

4. At the low (catalytic) levels of factor IF 2 necessary for GTP-dependent 70 S initiation complex formation using a mixture of ribosomal subunits, 5'-guanylyl methylene diphosphonate (GMP-PCP) does not replace GTP. At the high (stoichiometric) levels of IF 2 necessary for GTP-dependent 30 S initiation complex formation, GMP-PCP can substitute for GTP in promoting 30 S complex formation. The GMP-PCP-stimulated 30 S complex can then accept a 50 S subunit to form a 70 S couple. However, this complex is inactive in subsequent peptidyl transfer.

5. A model for the initiation process is presented which suggests that a GTP-dependent translocation of fMet-tRNA does not occur. Rather, it is postulated that fMet-tRNA binds directly to the donor site equivalent on the 30 S subunit. Upon 50 S addition, a 70 S couple is formed. GTP hydrolysis serves to release IF 2 and GTP from the initiation complex. Released IF 2 recycles to catalyze another round
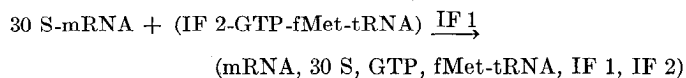
of initiation. Release of GTP and IF 2 also serves to "unblock" fMet-tRNA for peptidyl transfer.

An obligatory intermediate in the initiation of protein synthesis in bacteria is an mRNA-30 S subunit-fMet-tRNA complex (30 S initiation complex). A 50 S subunit joins this complex to form a 70 S initiation complex (1). The initiation process requires GTP and several protein factors which can be extracted from ribosomes by treatment with 1 M NH_4Cl solution. Three such initiation factors have been purified (2-6).[1]

A first step in 30 S complex formation may be attachment of a 30 S subunit to an initiation signal on mRNA to form a 30 S-mRNA complex (7). This would be followed by binding of fMet-tRNA and GTP. Recent evidence suggests that this binding may be mediated by IF 2 (8, 9).

$$IF\ 2 + GTP + fMet\text{-}tRNA \rightarrow (IF\ 2 - GTP - fMet\text{-}tRNA)$$
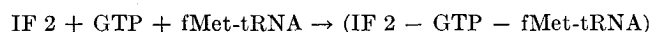
This ternary complex transfers both fMet-tRNA and GTP to the 30 S-mRNA complex in the presence of factor IF 1 to form the 30 S initiation complex (9).

$$30\ S\text{-}mRNA + (IF\ 2\text{-}GTP\text{-}fMet\text{-}tRNA) \xrightarrow{IF\ 1}$$
$$(mRNA,\ 30\ S,\ GTP,\ fMet\text{-}tRNA,\ IF\ 1,\ IF\ 2)$$

Recent reports have also indicated the presence of both GTP and IF 1 in the 30 S initiation complex (10, 11).

These observations suggest that the function of IF 2 in initiation may be analogous to that of factor T in peptide chain elongation. T factor catalyzes the binding of AA-tRNA to the ribosomal A site via the intermediate formation of a factor T-GTP-AA-tRNA complex (12-14). Concomitant with the binding of AA-tRNA to the 70 S ribosome GTP is hydrolyzed to GDP + P_i (14-16). Similarly, during the over-all initiation process, GTP is hydrolyzed to GDP + P_i. It is presently uncertain at which step in initiation complex formation this hydrolysis of

[1] In accordance with a recent nomenclature agreement, this paper employs the designations IF 1, IF 2, and IF 3 for these factors. They correspond to FI, FIII, and FII as described in previous reports from our laboratory (2-4), to F 1, F 2, and F 3 as described in reports from Ochoa's laboratory and to A, C, and B as designated by Revel *et al.* (6).

GTP occurs. According to one report the cleavage of GTP takes place during the formation of the 30 S initiation complex (17). Other results indicate that GTP hydrolysis occurs together with or immediately following upon addition of the 50 S subunit to the 30 S complex (18).

IF 2 has been shown to possess a ribosome-dependent GTPase activity which is maximal in the presence of the other components of initiation, *i.e.* mRNA, fMet-tRNA, and IF 1 (18–19). Both ribosomal subunits are required for this hydrolysis. IF 2 thus appears to be responsible for hydrolysis of GTP during initiation. The hydrolysis of GTP by IF 2 during initiation has also led to the suggestion that IF 2 may function in a manner analogous to that of G factor in polypeptide chain elongation, and may catalyze the GTP-dependent translocation of fMet-tRNA into the P site of the ribosome from some other ribosomal site.

The experiments described in this paper are designed to elucidate further the requirements for 30 S complex formation, its subsequent transformation to the 70 S initiation complex and the role of GTP in these processes.

Evidence is presented that GTP, which is required for 30 S initiation complex formation, remains intact in the 30 S complex. Hydrolysis occurs only upon addition of 50 S subunits. Conditions have been found which permit the selective removal of intact GTP from the 30 S complex. fMet-tRNA in such a GTP-deficient 30 S complex is still available for peptidyl transfer upon addition of 50 S subunits. These results indicate that only the removal and not the hydrolysis of GTP is required for correct positioning of fMet-tRNA in the donor site. Thus, it is likely that an energy-dependent translocation event does not occur during initiation. A model for the initiation process compatible with our data and that of others is presented.

EXPERIMENTAL PROCEDURE

Materials

Various nucleotides, tRNA species, initiation factors, and ribosomes were obtained as described previously (4). f2 phage was prepared by the method of Webster *et al.* (20). f2 RNA was prepared according to the method of Roufa and Leder (21). Puromycin hydrochloride was purchased from Nutritional Biochemicals. A-U-[³H]G was prepared from A-U (Miles Laboratories) and [³H]GDP using purified polynucleotide phosphorylase in the presence of T1 ribonuclease. The final specific activity was 105 cpm per pmole of A-U-[³H]G. Purified polynucleotide phosphorylase was a gift of Dr. Maxine Singer of the National Institutes of Health.

Methods

Assay of fMet-tRNA Binding to Ribosomes—Reaction mixtures (0.11 ml) contained 50 mM Tris-HCl (pH 7.8), 2 mM 2-mercaptoethanol, 0.1 M NH₄Cl, 2 mM GTP, 1 A_{260} unit of tRNA charged with [*methyl*-³H]methionine in the presence of formyl donor (20 to 30 pmoles of fMet-tRNA, specific activity 1500 to 1800 cpm per pmole of methionine), initiation factors, and salt-washed ribosomes (2 A_{260} units of unfractionated ribosomes or 0.7 A_{260} unit of 30 S subunits, and 1.4 A_{260} units of 50 S subunits). For poly(U, G)-directed binding, 0.04 A_{260} unit of poly(U, G) was added and 10 mM Mg(OAc)₂ was present. For f2 RNA or A-U-G codon-directed binding, 100 pmoles of f2 RNA or 0.04 A_{260} unit of A-U-G were used and 5 mM Mg(OAc)₂ was present. In addi-

tion the GTP concentration was lowered to 0.2 mM unless otherwise specified. Incubation was for 15 min at 25°. After incubation the mixtures were chilled and diluted with 3 ml of cold buffer of the same composition as was present in the reaction mixture. The diluted solutions were filtered through nitrocellulose membranes under reduced pressure. The membranes were washed with 6 ml of buffer, dried, and counted in a liquid scintillation counter.

Alternatively, binding could be measured by sucrose gradient centrifugation. After incubation, 0.1-ml aliquots of reaction mixtures were layered on 5.3 ml of preformed 5 to 20% sucrose gradients containing reaction buffer. The tubes were then centrifuged at 5° for 85 min at 55,000 rpm in an SW 65 rotor. Under these conditions, 70 S, 50 S, and 30 S ribosomes were resolved from one another. Fractions of 0.2 ml were collected and counted in Bray's solution.

Assay of Initiation Complex Formation by Sephadex G-100 Gel Filtration—Reaction mixtures for initiation complex formation were set up as described above. After incubation 0.1-ml aliquots of the mixtures were applied to columns (20 × 0.5 cm) of Sephadex G-100 equilibrated at 5° with the reaction buffer (22). The column was developed with the same reaction buffer. Fractions of 0.15 ml each were collected and counted in Bray's solution.

Assay of GTP Hydrolysis—Hydrolysis of GTP was determined by a modification of the method of Conway and Lipmann (23) as described in the preceding paper (18).

Assay of fMet-Puromycin Formation—Puromycin (50 nmoles) was added to 0.11 ml of initiation reaction mixture and the mixture was incubated 5 min at 37°. Formation of fMet-puromycin was measured by the method of Leder and Bursztyn (24).

RESULTS

Formation of 30 S Initiation Complex

Binding of fMet-tRNA in 30 S Complex—Sucrose density gradient centrifugation analysis was employed in order to study the formation of the intermediate in the initiation reaction, the (fMet-tRNA-30 S-mRNA) complex, since this method gave more reproducible results than Millipore filtration in experiments with isolated 30 S subunits. Fig. 1*A* shows sucrose gradient analyses of reaction mixtures containing 30 S or 50 S subunits alone, and both subunits in combination, all of which also contained the three initiation factors, GTP, poly(U, G), and f[³H]Met-tRNA. When 30 S subunits were used alone, a small peak of bound fMet-tRNA was observed in the 30 S region of the gradient. The 50 S subunits alone were completely inactive. When both subunits were added, bound fMet-tRNA appeared in the 70 S region, in a markedly greater amount than was present at 30 S in the absence of 50 S particles.

If IF 3 was omitted (Fig. 1*B*), binding at 70 S in the presence of 30 S and 50 S subunits was markedly reduced, in agreement with results obtained using unfractionated ribosomes. Again, no binding was seen with 50 S subunits alone. However, when 30 S subunits alone were used, more fMet-tRNA was bound at 30 S than was observed in the presence of IF 3. Binding at 70 S in the presence of both subunits was also dependent upon IF 1, GTP, and poly(U, G) (not shown) in agreement with results obtained using unfractionated ribosomes.

The experiments described above employed the amounts of factors used in standard binding assays, namely 0.15 μg each of IF 1 and IF 3 and 0.05 μg of IF 2. When considerably larger
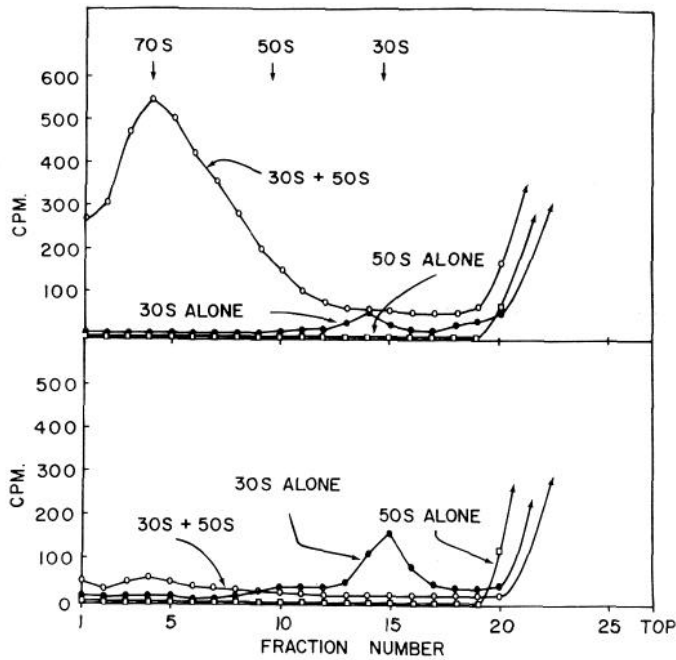
FIG. 1. Binding of fMet-tRNA to ribosomal subunits. Reaction mixtures were as described in "Experimental Procedure" for fMet-tRNA binding assays. Substituted for unfractionated salt-washed ribosomes were 0.7 A_{260} unit of 30 S ribosomal subunits and 1.4 A_{260} units of 50 S ribosomal subunits. Binding of f[³H]Met-tRNA was determined by sucrose gradient centrifugation as described in "Experimental Procedure." *Upper panel*, complete system (0.15 μg of IF 1 + 0.15 μg of IF 3 + 0.05 μg of IF 2); *lower panel*, omit IF 3.

quantities of each factor were tested separately IF 2 promoted a marked increase in the 30 S peak of bound fMet-tRNA (Table I, Experiment A). IF 1 was inactive when tested alone, and in the presence of IF 3 less fMet-tRNA was bound than occurred without any factor. The IF 2-promoted increase in fMet-tRNA bound at 30 S was dependent upon the presence of poly(U, G) and of GTP (Table I, Experiment B). This binding was somewhat stimulated by IF 1 (Table I, Experiment C). IF 3 appeared to be inhibitory, with 0.3 μg bringing about a decrease of bound fMet-tRNA and with 0.7 μg abolishing the peak almost completely (Table I, Experiment C). The presence of IF 1 (0.5 μg) did not influence the apparent inhibition by IF 3. The apparent destabilization of the binding of fMet-tRNA to 30 S subunits by IF 3 will be discussed in a subsequent section.

The binding reaction at 70 S required catalytic amounts of IF 2 (Table II). For example 0.55 pmole of IF 2 catalyzed the binding of 5.7 pmoles of fMet-tRNA in the presence of 30 S and 50 S subunits. The reaction was a time-dependent process at 37° (not shown). By contrast, the binding reaction with isolated 30 S subunits required much more IF 2 and catalytic function could no longer be inferred. Binding to 30 S appeared to require stoichiometric or greater amounts of IF 2. For example 8 pmoles of IF 2 promoted the binding of only 2.2 pmoles of fMet-tRNA to 30 S subunits (Table II). The 30 S binding reaction was complete after 1 min of incubation (not shown) with the quantity of IF 2 determining the yield of bound fMet-tRNA (Table II).

There is an indication, therefore, that IF 2 might act stoichiometrically in binding fMet-tRNA to 30 S subunits and be released to act catalytically upon the addition of 50 S subunits.

Binding of GTP in 30 S Initiation Complex—The requirement for GTP for the IF 2-promoted binding of fMet-tRNA to the

TABLE I

Requirements for binding fMet-tRNA to 30 S subunit

Reaction mixtures were as described in "Experimental Procedure" for poly(U, G)-directed fMet-tRNA binding to 30 S subunits. Factor additions or omissions of other components were as indicated in the table. Binding of f[³H]Met-tRNA (specific activity 1500 cpm per pmole of methionine) to 30 S subunits was determined by sucrose density gradient centrifugation as described in "Experimental Procedure."

Additions	[³H]Met-tRNA bound to 30 S subunit
	pmoles
Experiment A	
None	0.4
IF 1 (0.4 μg)	0.4
IF 2 (1.0 μg)	2.0
IF 3 (0.15 μg)	0.2
Experiment B	
Complete (1.0 μg IF 2)	1.8
Omit GTP	0.4
Omit poly(U, G)	0.4
Experiment C	
IF 2 (1.0 μg) alone	2.1
+ IF 1 (0.75 μg)	3.6
+ IF 3 (0.15 μg)	0.8
+ IF 3 (0.75 μg)	0.2
+ IF 1 (0.15 μg) + IF 3 (0.75 μg)	0.3

TABLE II

Binding of fMet-tRNA to ribosomal subunits

Reaction mixtures were as described in "Experimental Procedure" for standard poly(U, G)-directed fMet-tRNA binding mixtures except that 0.7 A_{260} unit of 30 S ribosomal subunits and 1.4 A_{260} units of 50 S ribosomal subunits were substituted for unfractionated ribosomes. Factor additions were as indicated. Binding of f[³H]Met-tRNA was determined by the Millipore filtration method as described in "Experimental Procedure." A 1.0-μg portion of IF 2 is equivalent to approximately 11 pmoles based on a molecular weight of 80,000 and a purity of approximately 90% (4, 9, 11).

Factor additions	f[³H]Met-tRNA bound	
	30 S	30 S + 50 S
	pmoles	
None	0.8	0.5
IF 3 (0.5 pmole)	0.8	0.7
IF 1 (0.15 μg) + IF 3 (0.15 μg) + IF 2 (0.55 pmole)	1.0	5.7
IF 2 (1.65 pmoles)	1.0	
IF 2 (8.8 pmoles)	2.2	
IF 2 (16.5 pmoles)	4.2	

30 S subunit suggested that GTP might also be present in the 30 S initiation complex. [γ-³²P]GTP (2 nmoles) of high specific activity (8000 cpm per pmole) was incubated with IF 2 (1 μg), purified f[³H]Met-tRNA (20 pmoles), 30 S subunits (0.8 A_{260} unit), and poly(U, G) (0.05 A_{260} unit). Initial attempts to detect GTP binding to 30 S subunits by sucrose gradient analysis were unsuccessful; although fMet-tRNA was bound, no 30 S peak of bound [γ-³²P]GTP was detected (not shown). However, when the reaction mixture was analyzed by Sephadex G-100 gel filtration, a peak of ³²P radioactivity was detected in the void
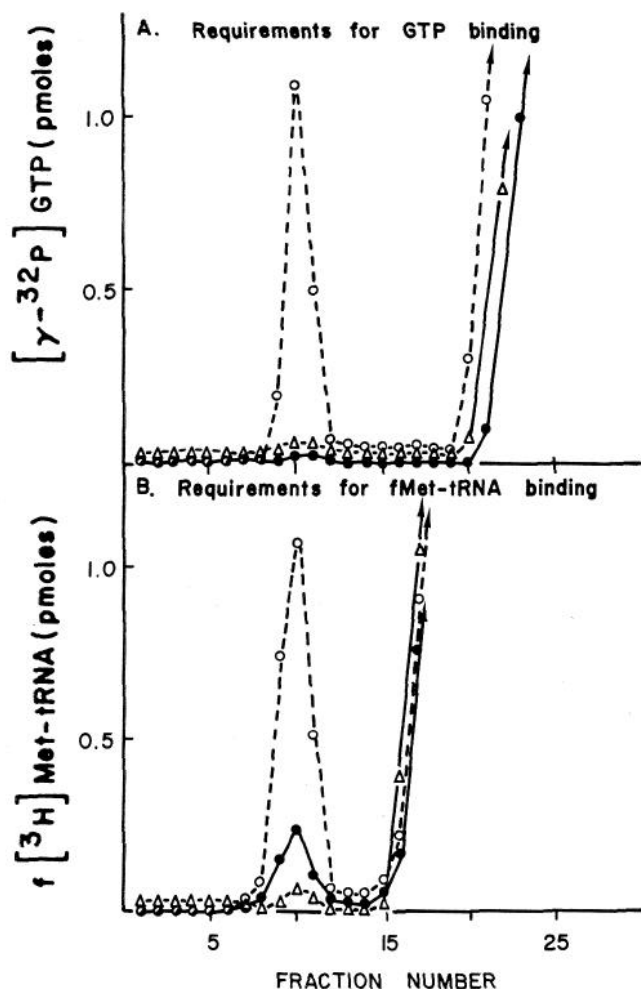
FIG. 2. Requirements for IF 2-promoted binding of GTP and fMet-tRNA to the 30 S subunit. Reaction mixtures for poly-(U, G)-directed fMet-tRNA binding to 30 S subunits were as described in "Experimental Procedure" except that 1.5 μg of IF 2 were used. For measurement of GTP binding to 30 S, 1.8 μmoles of [γ-^{32}P]GTP (5000 to 7000 cpm per pmole) and 15 pmoles of purified fMet-tRNA replaced unlabeled GTP and labeled fMet-tRNA. Omissions were as indicated below. After 10 min at 25°, reaction mixtures were chilled and applied to columns (20 × 0.5 cm) of Sephadex G-100 which had been previously equilibrated with the Tris-Mg^{++}-NH$_4^+$ buffer described above. The columns were developed with this buffer at 5°. Fractions of 0.15 ml were collected and counted in Bray's solution. *A*, ○– – –○, complete system; ●——●, omit fMet-tRNA; △——△, omit poly(U, G). *B*, ○– – –○, complete system; ●——●, omit GTP; △——△, omit poly(U, G).

volume (Fig. 2*A*). The appearance of this peak was dependent upon the presence of fMet-tRNA and poly(U, G). Phe-tRNA could not substitute for fMet-tRNA in promoting the binding of [γ-^{32}P]GTP. Initiation factor IF 2 was also required for GTP binding.

In these experiments a peak of f[^{3}H]Met-tRNA was detected in the void volume coincident with the peak of ^{32}P. The requirements for the appearance of f[^{3}H]Met-tRNA in the void volume were the same as those for the appearance of [γ-^{32}P]GTP (Fig. 2*B*). From the amount of excluded radioactivity, it could be calculated that the IF 2-promoted increase in bound fMet-tRNA was stoichiometric with the increase in bound GTP (2.7 pmoles of ^{3}H and

TABLE III

Factor and ribosomal subunit specificity for GTP binding

Reaction mixtures were prepared and assayed for [γ-^{32}P]GTP bound to the 30 S complex by Sephadex gel filtration as described in the legend to Fig. 2. In Experiment A, all reaction mixtures contained 0.7 A_{260} unit of 30 S ribosomal subunit and factor additions were as indicated. In Experiment B, reaction mixtures contained 1.5 μg of IF 2 and ribosomal subunit additions were as indicated in the table.

Additions	[γ-^{32}P]GTP, bound
	pmoles
Experiment A	
None................................	<0.1
IF 1 (1.5 μg) + IF 3 (1.5 μg).............	<0.1
IF 2 (0.4 μg).........................	0.5
IF 2 (1.5 μg).........................	2.4
Experiment B	
No ribosomes........................	<0.1
30 S subunits (0.7 A_{260} unit).............	2.1
50 S subunits (1.4 A_{260} units)............	<0.2
30 S + 50 S subunits.................	0.4

2.8 pmoles of ^{32}P). These results are in agreement with those of Thach and Thach (10).

Further studies on requirements for [γ-^{32}P]GTP and f[^{3}H]Met-tRNA binding to the 30 S complex have been carried out. These experiments are summarized in Table III. Only the results for GTP binding are depicted; the results for fMet-tRNA binding were qualitatively identical with these and are not shown. IF 2 was specifically required for the binding of GTP to 30 S ribosomes (Table III, Experiment A). The amount of [γ-^{32}P]GTP bound was proportional to the amount of IF 2 added. In the absence of IF 2, neither IF 1 nor IF 3 promoted binding of GTP. Adding IF 1 (0.7 μg) stimulated the IF 2-promoted binding of both GTP and the fMet-tRNA about 2-fold while IF 3 (0.7 μg) was markedly inhibitory (70 to 95%) (not shown), in agreement with the results described above for the sucrose gradient analysis of bound fMet-tRNA. This effect of IF 3 will be discussed in a subsequent section. We could not substitute 50 S subunits for 30 S subunits in promoting complex formation with [γ-^{32}P]GTP (Table III). Addition of 50 S together with 30 S subunits brought about a marked decrease in the amount of bound ^{32}P (Table III, Experiment B).

Identification of Intact GTP in 30 S Complex—To demonstrate that the entire GTP molecule was present in the complex, GTP labeled both with ^{3}H in the ring (130 cpm per pmole) and with ^{32}P in the γ position (400 cpm per pmole) was used as substrate. Other conditions were as described in the legend to Fig. 2. Both ^{3}H and ^{32}P radioactivity was detected in the complex, in equimolar quantities (data not shown).

Although both the guanine base and γ-phosphorus atom of GTP were thus shown to be present in the complex, prior hydrolysis of GTP with retention of both moieties was not yet ruled out. GTP was shown to be intact by isolating and characterizing the complex as follows: [γ-^{32}P]GTP (5,500 cpm per pmole) was employed as substrate, and the 30 S complex was synthesized and chromatographed on Sephadex G-100. Aliquots (20 μl) of the resulting fractions were counted in Bray's solution. Fractions containing the isolated complex were pooled, and the resulting solution (13,000 cpm) was divided into two portions.

The ^{32}P radioactivity of one portion was characterized by Norit adsorption (18). More than 90% of the radioactivity was acid soluble and Norit adsorbable. The second portion was made 50 mM in NaOH, kept for 5 min at 0°, and then neutralized. Unlabeled GTP (0.5 nmoles) was added as carrier. The solution was applied to a column (2 × 0.5 cm) of Dowex 1 (Cl) which had been washed previously with water. The column was developed stepwise with increasing concentration of LiCl in dilute HCl (18). Approximately 85% of the applied radioactivity cochromatographed with GTP. The remaining 15% of the radioactivity eluted at the position of P_i.

Nucleotide and tRNA Specificity for 30 S Complex Formation—The nucleotide and tRNA specificity for 30 S complex formation was also studied. Only GTP promoted the binding of fMet-tRNA to 30 S subunits. ATP, UTP, CTP, or GDP could not

FIG. 3. Exchangeability of fMet-tRNA$_f$ and GTP bound to 30 S subunits. *A*, reaction mixtures were set up as described in the legend to Fig. 2 for fMet binding assays except that 15 pmoles of f[^{3}H]Met-tRNA were used. After 10 min of incubation at 25°, 400 pmoles of unlabeled fMet-tRNA were added to one mixture containing the complete system and incubation was continued for 5 additional min. Bound fMet-tRNA was assayed by Sephadex G-100 gel filtration as described in "Experimental Procedure" and was calculated using the specific activity of f[^{3}H]Met-tRNA before the chase. ○– – –○, no additions; ●——●, addition of 400 pmoles of unlabeled fMet-tRNA after 10 min. *B*, two reaction mixtures for GTP binding were set up as described in the legend to Fig. 2 except that 0.8 μg of IF 2 was used. After 10 min of incubation at 25°, 150 nmoles of unlabeled GTP were added to one mixture and incubation was continued for 5 additional min. Bound GTP was assayed by Sephadex G-100 gel filtration as described in the legend to Fig. 5 and was calculated using the specific radioactivity of [γ-^{32}P]GTP before the chase. ○– – –○, no additions; ●——●, addition of 150 nmoles of unlabeled GTP after 10 min.

replace GTP. Furthermore, only GTP was bound to the 30 S subunit. Other nucleotides did not bind. Under conditions of IF 2-promoted 30 S complex formation, only formylated fMet-tRNA was bound to the 30 S subunits. Neither unformylated Met-tRNA$_f$ nor internal tRNAs such as Met-tRNA$_m$ or Phe-tRNA were bound. In these experiments A-U-G or poly(U) replaced poly(U, G) and the magnesium concentration was lowered to 5 mM; fMet-tRNA was still bound in control experiments at 5 mM Mg^{2+}. Sephadex G-100 analysis was carried out as described in "Experimental Procedure."

Exchangeability of fMet-tRNA and GTP Bound in 30 S Initiation Complex—To detect exchange of fMet-tRNA bound to the 30 S subunits, a 30 S complex containing f[^{3}H]Met-tRNA was allowed to form. It was then challenged with a large excess of unlabeled fMet-tRNA. Most of the radioactivity eluting in the void volume of Sephadex G-100 was retained, indicating that bound fMet-tRNA is not exchangeable (Fig. 3A). To detect exchange of GTP bound to the 30 S subunit, a similar experiment was performed except that [γ-^{32}P]GTP and unlabeled fMet-tRNA were used to form the 30 S complex. Challenge was with a large excess of unlabeled GTP. Most of the ^{32}P radioactivity eluting in the void volume of Sephadex G-100 was lost, indicating that bound GTP is exchangeable (Fig. 3B). The stability of GTP binding to the 30 S subunit will be considered in a subsequent section.

Binding of A-U-G in 30 S Initiation Complex—Under conditions of initiation complex formation A-U-[^{3}H]G became bound to the 30 S subunit (data not shown). Omission of fMet-tRNA, initiation factors, or GTP markedly reduce A-U-G binding. This result is in contrast to reports that neither fMet-tRNA nor GTP but only initiation factors are required for messenger binding (25). However, since some residual binding is observed, the possibility exists that A-U-G does bind to ribosomes in the presence of initiation factors alone and that this binding is stabilized by fMet-tRNA and GTP.

Effect of IF 3 on 30 S Complex Formation—As previously discussed, IF 3 is inhibitory to 30 S complex formation. It seemed unreasonable that IF 3, which is required for 70 S complex formation, could actually prevent the formation of the 30 S complex. A more likely explanation would be that IF 3 could destabilize a pre-existing 30 S complex so that it was no longer detectable by sucrose gradient analysis. To examine this possibility, we compared the effect of IF 3 on formation of a 30 S complex as assayed by sucrose gradients, Sephadex G-100 gel filtration, and Millipore filtration (Table IV). When poly(U, G) was used as messenger, 0.7 μg of IF 3 virtually abolished the IF 2-promoted 30 S peak of bound fMet-tRNA detectable by sucrose gradient analysis or the peak of excluded fMet-tRNA detectable by Sephadex gel filtration (Table IV, Experiment A). In contrast, when Millipore filtration was used as an assay, IF 3, instead of inhibiting the binding of fMet-tRNA to 30 S subunits, was now slightly stimulatory.

This suggested that IF 3 was not breaking down the 30 S complex, but was acting to destabilize it to sucrose gradient or gel filtration analysis.

To test whether the destabilization produced by IF 3 represented a discrimination against artificial messenger as has been proposed (25), f2 RNA was substituted for poly(U, G) (Table IV, Experiment B). In this case IF 3 also diminished the 30 S peak of bound fMet-tRNA as measured by sucrose gradients or Sephadex G-100 gel filtration while slightly stimulating fMet-
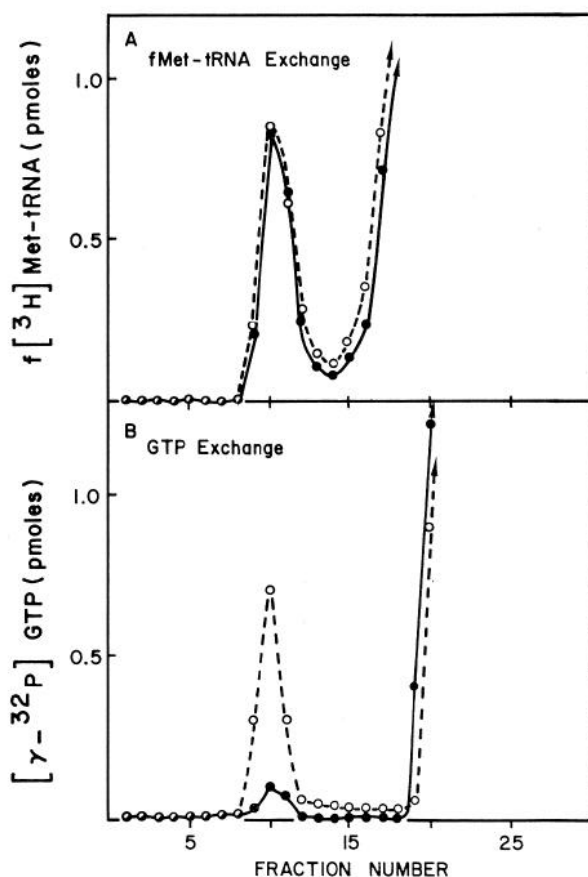
TABLE IV

Influence of factor IF 3 on 30 S initiation complex formation

Reaction mixtures were as described in "Experimental Procedure" for fMet-tRNA binding to 30 S ribosomes except that 15 pmoles of purified f[³H]Met-tRNA were used. Factor additions were IF 1, 0.7 µg; IF 3, 0.7 µg, IF 2, 1.0 µg. In Experiment B, 100 pmoles of f2 RNA were substituted for poly(U, G). The amount of fMet-tRNA bound to 30 S ribosomes as assayed by various techniques was as described in "Experimental Procedure."

Additions	f[³H]Met-tRNA bound at 30 S region of sucrose gradient	f[³H]Met-tRNA excluded from Sephadex G-100	f[³H]Met-tRNA retained on Millipore filter
	pmoles		
Experiment A (poly(U, G) directed)			
Complete.................	0.2	0.2	2.7
Omit IF 3.................	2.6	2.3	2.3
Experiment B (f₂ RNA directed)			
Complete.................	1.2	1.5	2.2
Omit IF 3.................	2.1	2.2	2.0

RNA binding as measured by Millipore filtration. However, in contrast to the complete abolition of binding observed with poly(U, G), a reduction of only 30 to 40% was observed with f2 RNA as messenger. These results are consistent with the suggestion that IF 3 discriminates against binding of the 30 S subunits to "improper" initiation sequences on messenger RNA of which there would be far more in random copolymers such as poly(U, G) than in f2 RNA.

We also studied the effect of IF 3 on a preformed 30 S initiation complex. The IF 2 promoted 30 S complex was isolated largely free of unbound materials by Sephadex G-100 gel filtration. When this preparation of complex was run on a sucrose gradient, a considerable fraction of the radioactivity sedimented at 30 S (Fig. 4). Addition of IF 3 to the isolated complex brought about a release of radioactivity from the 30 S complex to the top of the gradient. If 50 S subunits were added to the isolated 30 S complex, bound radioactivity was shifted to the 70 S region. If IF 3 was added together with 50 S subunits, the 70 S peak was undiminished.

When an identical experiment was carried out with f2 RNA as messenger (Fig. 4B) a similar pattern emerged. However, in this case, addition of IF 3 to the isolated 30 S complex alone brought about the release of only 40% of radioactivity associated with the 30 S complex to the top of the gradient.

These experiments show directly that IF 3 destabilizes or dissociates the isolated 30 S complex formed with poly(U, G), but is less effective in destabilizing such a complex formed with f2 RNA. However, with either messenger, IF 3 does not destabilize the 70 S complex formed in the presence of the 50 S subunit. No stimulation of 70 S binding by IF 3 was possible in these experiments because the pool of unreacted fMet-tRNA has been removed upon isolation of the 30 S complex by gel filtration.

Reactions of Isolated 30 S Initiation Complex

Fate of fMet-tRNA and GTP upon 50 S Addition—In order to determine whether the 30 S complex isolated by gel filtration is a functional intermediate in 70 S initiation complex formation, the behavior of the complex upon addition of 50 S subunits was
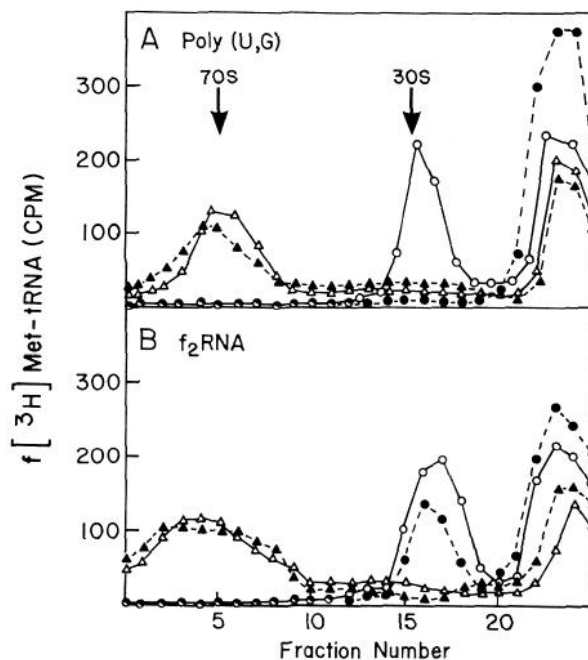

Fig. 4. Effect of IF 3 on fMet-tRNA bound to 30 S subunit. Reaction mixtures (1.1 ml) for fMet-tRNA binding to the 30 S subunit contained 50 mM Tris-HCl (pH 7.0), 5 mM 2-mercaptoethanol, 10 mM Mg(OAc)₂ 0.1 M NH₄Cl, 18 nmoles of GTP, 7 A_{260} units of 30 S ribosomal subunits, 150 pmoles of purified f[³H]Met-tRNA (specific activity 1800 cpm per pmole), 7 µg of IF 1, and 15 µg of IF 2. In Experiment A, 0.5 A_{260} unit of poly(U, G) was included. In Experiment B, 1.0 nmole of f2 RNA replaced poly(U, G) and the magnesium concentration was lowered to 5 mM. After 10 min at 25°, each mixture was passed through a column of Sephadex G-100 (1.5 × 30 cm) as described in "Experimental Procedure." In each case the peak of radioactivity which appeared in the void volume was collected and pooled. Four aliquots of 0.1 ml were taken from each solution; each aliquot contained 2100 cpm of radioactivity. Additions to each aliquot were made as indicated below. After 10 min at 25°, the mixture was centrifuged on sucrose gradients as described in "Experimental Procedure." ○——○, no addition; ●---●, 0.3 µg of IF 3; ▲---▲, 0.3 A_{260} unit of 50 S subunits; △——△, 0.3 A_{260} unit of 50 S subunits and 0.3 µg of IF 3.

studied. Subunits of 50 S can join the isolated 30 S complex to form a 70 S couple. This is illustrated by the experiment shown in Fig. 4 and discussed above. As noted above, if IF 3 was added together with the 50 S subunits, the 70 S peak was undiminished. Addition of IF 1 or IF 2 alone or together with 50 S subunits did not affect the amount of 70 S complex formed (not shown). This experiment shows that additional factors are not necessary for association of the 50 S subunits once the 30 S initiation complex has formed, although IF 1 and IF 2 may still be present in the isolated 30 S complex.

In order to determine the fate of fMet-tRNA and GTP bound in the 30 S complex upon addition of 50 S subunits, the 30 S complex containing bound [γ-³²P]GTP and bound f[³H]Met-tRNA was isolated on Sephadex G-100. Aliquots were incubated with 50 S subunits in the presence or absence of IF 1 and IF 3. Addition of 50 S subunits to the isolated 30 S complex rendered all bound fMet-tRNA reactive to puromycin (Table V). This indicates that all of the fMet-tRNA bound to the 30 S complex moves to the donor site of the 70 S ribosome when 50 S subunits are added. The addition of IF 1 and IF 3 together with 50 S subunits did not substantially affect this result. Approximately 50% of the ³²P radioactivity initially present in the 30 S complex

TABLE V

Reactions of isolated 30 S initiation complex

A large scale reaction mixture was prepared and passed through Sephadex G-100 as described in the legend to Fig. 4A except that 32 nmoles of [γ-^{32}P]GTP were used in addition to labeled fMet-tRNA. The isolated material was divided into 0.2-ml aliquots. Each aliquot contained 1.39 pmoles of f[^{3}H]Met-tRNA and 1.36 pmoles of [γ-^{32}P]GTP. (In a duplicate experiment, shown in parentheses, each aliquot contained 0.66 pmoles of f[^{3}H]Met-tRNA and 0.72 pmoles of [γ-^{32}P]GTP.) Where indicated, 3 A_{260} units of 50 S subunits, 0.7 μg of IF 1, 0.7 μg of IF 3, and 1 μg of IF 2 were added. Retention of fMet-tRNA and GTP in the isolated 30 S complex was assayed by Sephadex G-100 gel filtration; fMet-puromycin formation and [γ-^{32}P]GTP hydrolysis were measured as described in "Experimental Procedure."

Additions	f[^{3}H]Met-tRNA retained in complex	f[^{3}H]Met puromycin formed	[γ-^{32}P]GTP retained in complex	^{32}P$_i$ Released
	pmoles			
30 S complex.	1.18 (0.70)	<0.01 (<0.01)	0.64 (0.34)	0.05 (0.04)
30 S complex + 50 S....	1.26 (0.61)	1.16 (0.66)	0.03 (0.03)	0.70 (0.32)
30 S complex + 50 S + factors....	1.10 (0.64)	1.08 (0.55)	0.03 (0.03)	0.71 (0.36)

TABLE VI

Reactions of GTP-deficient 30 S initiation complex

A large scale reaction mixture was prepared and passed through G-100 as described in the legend to Table V. (Sephadex G-100 analysis of a 0.2-ml aliquot before incubation showed 1.41 pmoles of f[^{3}H]Met-tRNA and 0.87 pmole of [γ-^{32}P]GTP still bound.) After isolation the 30 S complex was incubated for 15 min at 25° to release bound GTP. Aliquots of 0.2 ml were taken and additions of 3 A_{260} units of 50 S subunits, 0.7 μg of IF 1, 0.7 μg of IF 3, and 1.0 μg of IF 2 were made as indicated. Retention of ^{3}H and ^{32}P in the complex was determined by Sephadex G-100 gel filtration as described in "Experimental Procedure." fMet-puromycin formation and GTP hydrolysis were also measured as described in "Experimental Procedure."

Additions	f[^{3}H]Met-tRNA retained in complex	[γ-^{32}P]GTP retained in complex	Ratio of ^{3}H to ^{32}P retained in complex	f[^{3}H]Met-puromycin formed	^{32}P$_i$ released	Ratio of f[^{3}H]Met-puromycin formed to P$_i$ released
	pmoles					
30 S complex...	1.32	0.15	8.8	<0.01	0.02	
30 S complex + 50 S.........	1.38	0.02	69	1.26	0.14	9.0
30 S complex + 50 S + factors.	1.25	0.02	62	1.24	0.13	9.7

was released as ^{32}P$_i$ by addition of 50 S subunits (Table V). Analysis of the isolated untreated 30 S initiation complex on Sephadex G-100 showed that only 50% of the ^{32}P radioactivity was still bound (Table V). The result strongly suggests that all GTP still present in the isolated 30 S complex is hydrolyzed upon 50 S addition. The ^{32}P radioactivity released from the 30 S complex in the absence of 50 S additions eluted from Sephadex G-100 at the position of small molecules. Furthermore, the released ^{32}P was still Norit absorbable and acid soluble and eluted

at the position of GTP on Dowex 1 (Cl), indicating that [γ-^{32}P]-GTP had been released intact. These experiments suggest that (*a*) all GTP which remains bound in the isolated 30 S initiation complex is hydrolyzed upon addition of the 50 S subunit and (*b*) GTP tends to dissociate from the 30 S initiation complex in the absence of 50 S subunits. GTP which is released in this manner remains intact. The instability of GTP binding to the 30 S complex will be discussed subsequently.

Behavior of GTP-deficient 30 S Initiation Complex—As mentioned previously, [γ-^{32}P]GTP binding to the 30 S complex could not be detected by sucrose gradient centrifugation, but was detectable by Sephadex G-100 gel filtration. However, when the 30 S complex isolated by gel filtration was analyzed by either sucrose gradient centrifugation or Millipore filtration, it was observed that [γ-^{32}P]GTP in the complex was almost entirely lost. In contrast, when the isolated 30 S complex was rerun on Sephadex G-100, only about 50% of the nucleotide was lost. The release of GTP as detected by Sephadex G-100 analysis is a function of the time and temperature at which the isolated 30 S complex is maintained. We found that incubation of the 30 S complex for 15 min at 25° resulted in the release of most of the bound GTP without substantially decreasing the level of bound fMet-tRNA. GTP released from the 30 S complex in this manner had not been hydrolyzed, since the released ^{32}P was still Norit adsorbable and acid soluble. The properties of this GTP-deficient complex are summarized in Table VI and closely resemble those of a 30 S complex with a full complement of GTP. All bound fMet-tRNA moves to the 70 S region of sucrose gradients when 50 S subunits are added (not shown). All bound fMet-tRNA is rendered puromycin-reactive upon 50 S addition and no further factor additions are necessary for this reactivity (Table VI). On a molar basis, approximately 10 times as much fMet-puromycin is formed than GTP bound in the 30 S complex. The behavior of this GTP-deficient 30 S complex suggests that once a 30 S complex is formed, the continued presence of GTP and therefore the energy from its hydrolysis is not necessary for positioning of fMet-tRNA in the puromycin-reactive donor site on the 70 S ribosome. However, whatever GTP does remain bound to the 30 S initiation complex is quantitatively hydrolyzed upon addition of 50 S ribosomal subunits (Table VI).

Reactions of fMet-tRNA Bound to 30 S Subunits in Absence of GTP—GTP stimulates the binding of fMet-tRNA to 30 S subunits. However, some residual binding of fMet-tRNA is always observed in the presence of initiation factors and mRNA, but in the absence of GTP. The properties of an isolated 30 S complex formed in the absence of GTP are shown in Table VII. fMet-tRNA bound to the 30 S subunit in the absence of GTP can move to 70 S upon addition of 50 S subunits. Furthermore, this fMet-tRNA is available for reaction with puromycin upon 50 S addition. This data suggests that fMet-tRNA bound to 30 S in the absence of exogenous GTP is bound at a 30 S site corresponding to the donor site on the 70 S ribosome.

Effect of GMP-PCP[2] on 70 S Initiation Complex Formation—We extended our studies on GTP function to an investigation of the effect of GMP-PCP on initiation complex formation. This compound is an analogue of GTP in which the oxygen atom between the β- and γ-phosphorus atoms is replaced by a methylene group. It is not susceptible to enzymatic hydrolysis at that position.

[2] The abbreviation used is: GMP-PCP, 5'-guanylyl methylene diphosphonate.

TABLE VII

Reactions of 30 S initiation complex formed in absence of GTP

A large scale reaction mixture was prepared and passed through Sephadex G-100 as described in legend to Table V, except that GTP was omitted from the reaction mixture. Sephadex G-100 analysis of a 0.5-ml aliquot before incubation showed 0.62 pmoles of f[^{3}H]Met-tRNA still bound. Aliquots of 0.5 ml were taken and additions of 3 A_{260} units of 50 S subunits were made as indicated. fMet-puromycin formation was measured as described in "Experimental Procedure."

Additions	f[^{3}H]Met-tRNA retained in complex	f[^{3}H]Met-puromycin formed
	pmoles	
30 S complex.................	0.62	<0.02
30 S complex + 50 S..........	0.58	0.47

TABLE VIII

Puromycin reactivity of GMP-PCP-stimulated 70 S initiation complex

Reaction mixtures were as described in "Experimental Procedure" for fMet-tRNA binding to 70 S ribosomes except that 1.5 μg of IF 2 was used. Nucleotide additions (300 nmoles each) were as indicated. fMet-tRNA binding was assayed by Millipore filtration. f[^{3}H]Met-puromycin formation was measured as described in "Experimental Procedure."

Nucleotide addition	f[^{3}H]Met-tRNA bound to Millipore filter	f[^{3}H]Met-puromycin formed
	pmoles	
None.................	4.7	4.8
GTP..................	29.8	21.0
GMP-PCP............	11.8	1.8

TABLE IX

Reactions of GMP-PCP-stimulated 30 S initiation complex

A large scale reaction mixture was prepared and passed through Sephadex G-100 as described in legend to Table V except that 2 mM GMP-PCP replaced GTP. Radioactive fractions in the excluded volume were pooled. Aliquots (0.1 ml) containing 0.50 pmole of f[^{3}H]Met-tRNA were taken. Three A_{260} units of 50 S subunits were added where indicated. f[^{3}H]Met-tRNA binding was assayed by sucrose gradients and measurement of fMet-puromycin formation was as described in "Experimental Procedure."

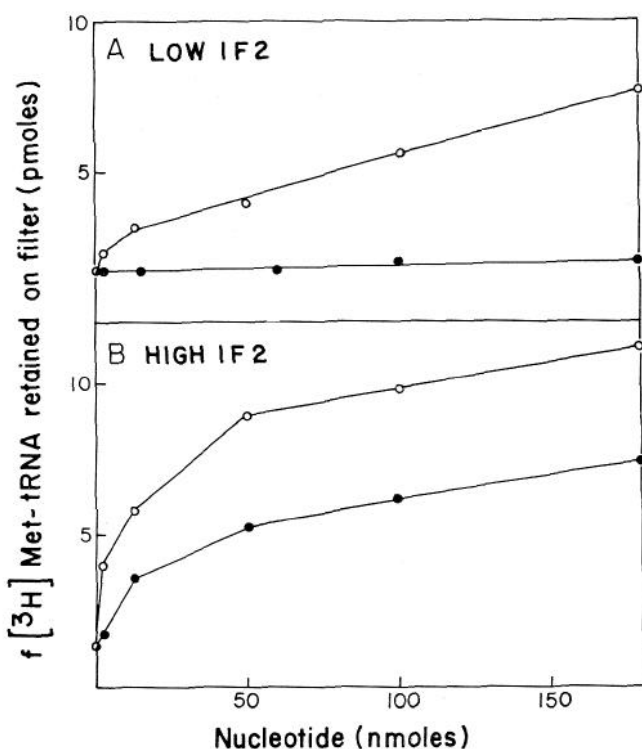| Additions	f[^{3}H]Met-tRNA bound		f[^{3}H]Met-puromycin formed
	30 S region	70 S region	
	pmoles		
30 S complex.............	0.42	0.02	<0.02
30 S complex + 50 S.....	0.02	0.38	<0.02

FIG. 5. Effect of GMP-PCP on fMet-tRNA binding to 70 S ribosomes. Reaction mixtures were as described in "Experimental Procedure" for fMet-tRNA binding except that the type and concentration of nucleotide additions were as indicated. Each reaction mixture contained 0.15 μg of IF 1 and 0.15 μg of IF 3. In addition, mixtures in *A* contained 0.05 μg of IF 2 and mixtures in *B* contained 1.0 μg of IF 2. After 10 min at 37°, the reaction mixtures were assayed for fMet-tRNA binding by Millipore filtration. O——O, GTP; ●——●, GMP-PCP.

Contradictory reports have appeared in the literature concerning the question of whether GMP-PCP can substitute for GTP in initiation complex formation. Thach and coworkers (26) found that GMP-PCP did stimulate 70 S initiation complex formation, but that fMet-tRNA bound at 70 S was not available for fMet-puromycin formation. Chae *et al.* (17) found that GMP-PCP could not replace GTP in stimulating fMet-tRNA binding at 70 S.

We have resolved this anomaly in the following manner. The GTP-dependent incorporation of fMet-tRNA into a 70 S initia-

tion complex required catalytic amounts of IF 2. Under these conditions, GMP-PCP did not replace GTP (Fig. 5A). In contrast, at high levels of IF 2, GMP-PCP did stimulate fMet-tRNA incorporation into the 70 S complex with about 50% the efficiency of GTP (Fig. 5B). Under the conditions used, this level of IF 2 did not additionally stimulate the GTP-dependent binding of fMet-tRNA.

In this case bound fMet-tRNA sedimented at 70 S (data not shown), but was not available for fMet-puromycin formation (Table VIII). In fact, less fMet-puromycin was formed in the presence of GMP-PCP than in the absence of any nucleotide (Table VIII).

These results are consistent with the suggestion that when GTP is hydrolyzed, factor IF 2 is released from the 70 S initiation complex and can act catalytically to promote fMet-tRNA binding. When hydrolysis is prevented by the use of GMP-PCP, IF 2 does not cycle but remains bound to the 70 S ribosome. Stoichiometric levels of IF 2 are therefore required to detect significant fMet-tRNA binding in the presence of GMP-PCP.

Behavior of 30 S Initiation Complex Formed in Presence of GMP-PCP—At the stoichiometric (high) levels of IF 2 required for fMet-tRNA binding to 30 S subunits, GMP-PCP stimulated this binding with about 50% the efficiency of GTP (data not shown). The isolated GMP-PCP-stimulated 30 S complex reacted with 50 S subunits to form a 70 S couple (Table IX). However, fMet-tRNA bound in such a 30 S complex was unable to

react with puromycin in the presence of 50 S subunits (Table IX). In fact, less fMet-puromycin was formed in the presence of GMP-PCP than in the absence of any nucleotide. Furthermore, incubation of the isolated 30 S complex for 20 min at 25°, followed by reisolation of the complex on a column of Sephadex G-100, converted most of the 30 S complex to an active form able to react with puromycin upon 50 S addition (data not shown). This observation is consistent with the idea that incubation and reisolation causes dissociation of GMP-PCP from the 30 S complex. Presumably such release of GMP-PCP is sufficient to convert the 30 S complex to a form active in subsequent peptidyl transfer.

DISCUSSION

This paper has attempted to define the components of the 30 S initiation complex, and the requirements for its formation as well as for its subsequent function as an intermediate in the production of the 70 S initiation complex. The function of GTP in this process has been studied.

We have shown that each 30 S initiation complex contains a 30 S ribosomal subunit, mRNA, and stoichiometric amounts of fMet-tRNA and GTP. GTP bound in the 30 S initiation complex is intact (10). It is likely that initiation factors IF 1 and IF 2 are also present in the 30 S complex. Thach and coworkers (11) have obtained direct evidence for the presence of IF 1 in such a complex, while our observations on the stoichiometry of IF 2 in initiation complex formation, and on GTP hydrolysis (discussed below) suggest the presence of IF 2 in the initiation complex (27).

Binding of fMet-tRNA to the 30 S subunit requires mRNA and GTP. Stoichiometric quantities of initiation factor IF 2 are absolutely required for the binding of fMet-tRNA. This is in contrast to the binding of fMet-tRNA at 70 S, where much smaller amounts of IF 2 are needed. Initiation factor IF 1 stimulates 30 S binding of fMet-tRNA about 2-fold. Only GTP promotes this binding; other nucleotides cannot substitute. fMet-tRNA is the only species tested whose binding to the 30 S subunit is dependent upon initiation factors under conditions of 30 S initiation complex formation. Once fMet-tRNA is bound in the 30 S initiation complex, it is not available for exchange with exogenous fMet-tRNA.

Binding of GTP to 30 S ribosomal subunits requires fMet-tRNA and mRNA. Phe-tRNA cannot substitute for fMet-tRNA. Other nucleotides are not bound. The factor requirements for GTP binding to the 30 S initiation complex are identical with those for fMet-tRNA binding; stoichiometric quantities of IF 2 are needed and IF 1 stimulates about 2-fold. In contrast to bound fMet-tRNA, the GTP bound in the 30 S complex is exchangeable with exogenous GTP.

It has been suggested that initiation factor IF 3 is involved in the specific recognition of natural, as opposed to synthetic, mRNA (5, 6, 25). We find that IF 3 is not required for 30 S initiation complex formation. In fact, IF 3 destabilizes the 30 S complex (with respect to both fMet-tRNA and GTP binding) as measured by sucrose gradient or Sephadex gel filtration analysis. IF 3 does not destabilize the 30 S initiation complex to Millipore analysis, but neither does it stimulate significantly. The destabilization observed in the presence of IF 3 is much less pronounced when complex formation is directed by natural messengers such as f2 RNA than when synthetic messengers such

as poly(U, G) are used. Since synthetic messengers are likely to contain many more erroneous initiation sites than f2 RNA, our observations are in accord with the proposal that IF 3 specifically recognizes correct initiation sites for 30 S binding to mRNA (5, 6, 25). However, we and others have also observed that at the 70 S level, IF 3 is required in addition to IF 1 and IF 2 for maximum incorporation of fMet-tRNA into a 70 S complex (3, 27). This is true with either natural (f2 RNA) or synthetic messengers (A-U-G or poly(U, G)) (28). This apparent discrepancy has yet to be resolved.

Using the technique of Sephadex G-100 gel filtration, we have been able to isolate the 30 S initiation complex free of unreacted fMet-tRNA, unbound initiation factors, and free GTP. With this isolated intermediate, we have shown that once the 30 S complex forms, no subsequent initiation factor additions are required for formation of a 70 S complex which can donate fMet into peptide linkage with puromycin. However, IF 1 and IF 2 are probably present in the 30 S complex. Furthermore, the IF 2-dependent hydrolysis of GTP occurs at the 70 S level, not during 30 S complex formation. Evidence of this includes the demonstration that (a) GTP is incorporated intact into the 30 S initiation complex and (b) joining of the 50 S subunit to the isolated 30 S complex results in the cleavage of all bound GTP. Chae *et al.* (17) have reported previously that GTP is cleaved at the 30 S level. This cleavage may have been due to low level contamination of 30 S subunits with 50 S subunits.

GMP-PCP can substitute for GTP in both 30 S and 70 S initiation complex formation, but only in the presence of stoichiometric levels of factor IF 2. Previous contradictory reports (3, 17, 26) concerning the ability of GMP-PCP to replace GTP would appear to be due to differences in the amount of factor IF 2 used to promote fMet-tRNA binding. We find that (a) at high (stoichiometric) levels of IF 2 fMet-tRNA is bound to 70 S in the presence of GMP-PCP but cannot function in peptidyl transfer (26) and (b) addition of 50 S subunits to an isolated 30 S initiation complex formed in the presence of GMP-PCP results in a 70 S couple inactive in peptidyl transfer.

To examine whether the free energy of GTP hydrolysis is required for movement of fMet-tRNA to the puromycin-reactive donor site, we made use of the observation that incubation of the isolated 30 S complex preferentially releases most of the bound GTP from the complex without hydrolysis while leaving most fMet-tRNA still bound. We found that such a GTP-deficient 30 S complex is still fully active in subsequent 70 S complex formation and peptidyl transfer.

Furthermore, although a 30 S complex formed in the presence of GMP-PCP is inactive in subsequent peptidyl transfer, puromycin reactivity can be restored by first incubating the GMP-PCP-stimulated 30 S complex and then passing it through Sephadex G-100. It is likely that this treatment results in the dissociation of GMP-PCP from the 30 S complex although this has yet to be confirmed directly with labeled GMP-PCP. Presumably, release of GMP-PCP alters the conformation of the 30 S initiation complex so as to permit subsequent peptide bond formation. We suggest that the behavior of 30 S complexes formed either with GTP or GMP-PCP are analogous. In either case, once the 30 S complex forms, the continued presence of the nucleotide prevents fMet-tRNA from participating in subsequent peptide bond formation. Removal of the nucleotide alters the configuration of the complex to place fMet-tRNA in a reac-
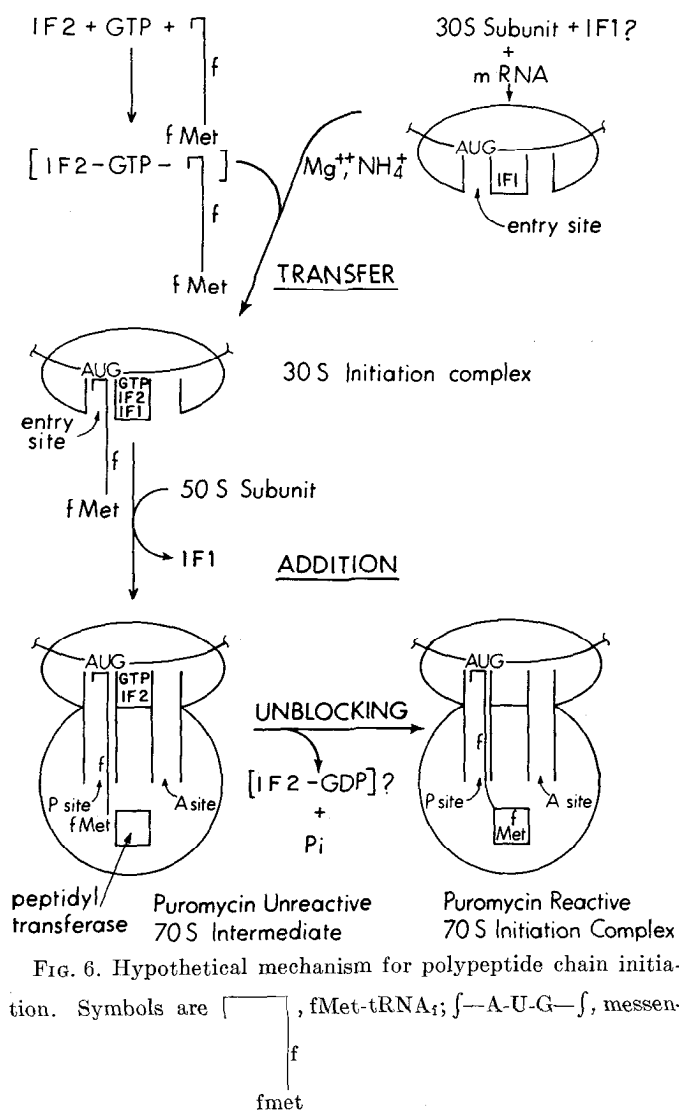
FIG. 6. Hypothetical mechanism for polypeptide chain initiation. Symbols are ⌐⌐, fMet-tRNA$_f$; ʃ—A-U-G—ʃ, messenger RNA showing A-U-G at initiation site.

tive state. GTP is normally removed via hydrolysis upon junction of the 50 S subunit, but it can be eliminated intact at the 30 S level by manipulations described in this paper. GMP-PCP cannot be removed by the normal hydrolytic event. Therefore, 50 S junction yields an unreactive 70 S complex. However, GMP-PCP can be eliminated at the 30 S level in the same manner as GTP. This restores the 30 S complex to an active state.

Therefore, it is likely that a "translocation" of fMet-tRNA, dependent on the free energy of hydrolysis of GTP and catalyzed by IF 2, does not occur during the initiation process. Rather, GTP hydrolysis may be required to "unblock" fMet-tRNA by releasing GTP and IF 2 from the ribosome so that peptide bond formation can occur. In this respect, the function of IF 2 and GTP in initiation would be more analogous to that of T factor and GTP in elongation than to that of G factor. Using a different experimental approach, quite similar conclusions have been reached recently by Thach and Thach (30).

A hypothetical scheme for the initiation process compatible with our results and those of others is shown in Fig. 6. IF 2 initially binds fMet-tRNA and GTP to form a (IF 2-GTP-fMet-

tRNA) complex. This entire complex becomes attached to a 30 S-mRNA complex in the presence of factor IF 1 to form the 30 S initiation complex (an alternative model for 30 S binding has been proposed by Groner and Revel (31)). Initial binding of fMet-tRNA is to a site on the 30 S ribosome which corresponds to or will become part of the peptidyl or donor site on the 70 S ribosome. We tentatively call this the "entry" site. Subsequently a 50 S subunit adds to form a 70 S complex and the "entry" site now becomes part of the donor site. IF 1 is released at this stage. fMet-tRNA bound in this intermediate is not available for peptide bond formation. Together with or immediately following upon addition of the 50 S subunit GTP is hydrolyzed to GDP and P$_i$ by IF 2. This hydrolysis serves two purposes. (a) It releases IF 2, GDP, and P$_i$ from the initiation complex, possibly as an IF 2-GDP complex. IF 2 can then recycle catalytically to promote another round of initiation. (b) The hydrolysis of GTP also permits fMet-tRNA to donate fMet into peptide linkage. This may occur via a conformational change in the donor site or by "unblocking" the site through removal of IF 2. It is uncertain whether release of IF 2 requires the free energy of hydrolysis of GTP or whether hydrolysis simply effects a conformational change which allows release.

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