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Translocation of Messenger RNA and "Accommodation" of fMet-tRNA

(GMP-PCP/elongation factors/initiation factors/oligonucleotides/ribosomes)

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ABSTRACT **Messenger RNA is moved a distance of approximately three nucleotides in the 5′ direction relative to the ribosome during the translocation of peptidyl-tRNA from the A to the P site. This movement is catalyzed by G factor and is dependent on the hydrolysis of GTP. In contrast, mRNA is not moved during the f_2-catalyzed hydrolysis of GTP that is involved in the activation of ribosome-bound fMet-tRNA. This second type of GTP-dependent reaction has been named "Accommodation".**

In spite of recent advances in our understanding of the initiation of protein synthesis, several important problems remain. Among these is the question of the role of GTP and initiation factor f_2. Is the f_2-catalyzed hydrolysis of GTP used to "translocate" fMet-tRNA, in a reaction analogous to that catalyzed by G factor (1–5)? Or does this reaction have an entirely different function (6–9)? This problem has been placed in a new perspective by the discovery that transfer factor T_u has GTPase activity, and that GTP hydrolysis is required for the activation of bound aminoacyl-tRNA before peptide-bond formation (10–12). This discovery raised the possibility that f_2 might function in a manner analogous to T_u, using the energy from GTP hydrolysis to activate the bound fMet-tRNA for peptide synthesis (6, 7, 9).

Before these questions about the role of GTP and f_2 could be settled, it was necessary to develop a new assay for the translocation reaction. The best operational definition of translocation has always been "a GTP- and enzyme-dependent reaction that converts ribosome-bound peptidyl-tRNA from an unreactive to a reactive state for peptide bond synthesis". Unfortunately, this definition cannot distinguish between translocation *per se* (as envisioned in the more popular models, refs. 13–16) and other types of activation mechanisms. Thus, a new type of operational definition, or assay, for translocation was necessary which was independent of the peptide synthesis reaction. For this reason, we turned our attention to the movement of mRNA with respect to the ribosome. It is generally assumed that during translocation the mRNA is moved a distance of three nucleotides in the 5′ direction (13–16). This presumably allows the ribosome to translate successive codons in the message. We felt that if one could demonstrate that such movement does in fact accompany the translocation of peptidyl-tRNA, then the movement reaction itself could be used as a new assay for translocation. With such an assay, we could then proceed to determine whether the mRNA (and hence the fMet-tRNA) is translocated as a result of GTP hydrolysis during initiation.

Our experimental approach to this problem was based upon recent work (17–19) which has demonstrated that when peptidyl-tRNA is bound to the ribosome in a puromycin-reactive state (i.e., in the conventional P site), its codon is located a distance of 12–14 nucleotides from the "edge" of the mRNA binding site (as defined by the limit of protection of mRNA against pancreatic RNase digestion). On the basis of current models, this suggests that when the same peptidyl-tRNA is bound in the unreactive state (the A site) before translocation, its codon should be located only 9–11 nucleotides from the edge. Moreover, addition of G factor and GTP to the latter complex should bring about an increase in the distance between the codon in question and the edge of the mRNA binding site by a length of three nucleotides.

In this way we sought to define the physical movement of the mRNA. Having accomplished this, we then used this movement as an assay for the translocation of mRNA (and hence of fMet-tRNA) during the initiation reaction sequence.

METHODS

Ribosomes, charged tRNA, initiation factors, and synthetic messengers were prepared as described (1, 2, 20–22). Transfer factors G and T_u were prepared by the method of Ertel *et al.* (23). Binding assays were conducted as described (2), and details of reaction conditions are given in the table legends. The assay of RNase-resistant messenger fragments is described in the legend of Fig. 1.

Paper chromatography of oligonucleotides of the type $(Up)_n$ was conducted in a solvent of 1 M NH_4OAc–95% ethanol–glacial acetic acid (70:30:10). In this solvent, R_f depended linearly on n, at least within the size range $6 < n < 15$. For example, in one experiment the distances migrated in 20 hr by the markers $(Up)_8$ through $(Up)_{13}$ were 26.6, 24.7, 22.2, 20.1, 17.2, and 15.2 cm, respectively. The degree of spreading of each oligonucleotide spot due to diffusion is indicated in Fig. 1*b*. Since the relative, as well as absolute, values of the R_f varied slightly from experiment to experiment, several marker oligonucleotides were always included on each chromatogram.

Peptide-bond formation between radioactive amino acids bound to ribosomes was determined as follows. Reaction mixtures were passed through Millipore filters, which were then washed three times with buffer. The bound products were eluted with 1 ml of 0.5 N KOH (30 min at 37°C), neutralized with $HClO_4$, and analyzed by paper chromatography (24).

Abbreviation: GMP-PCP, 5′-guanylylmethylenediphosphonate.

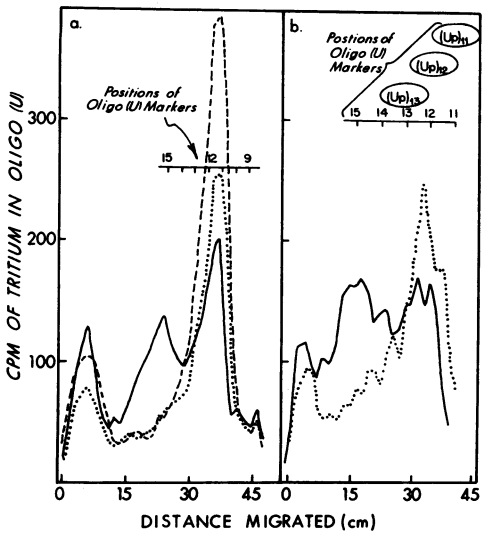

FIG. 1. Chromatograms of oligo(U) portions of RNase-resistant messenger fragments. Reaction mixtures similar to those in Table 1, Expt. 2, where the messenger was [^{3}H]ApU-(pG)$_2$(pU)$_{\bar{30}}$: in (*a*), the "Complete System" product is represented by the *dashed line*, the "Complete System Plus G Factor" by the *solid line*, and the "Complete System Plus G Factor Plus Fusidic Acid" by the *dotted line;* in (*b*) "Complete System Plus G Factor Plus GMP-PCP" is represented by the *dotted line*, whereas the *solid line* represents "Complete System Plus G Factor Plus Excess GTP" (30 nmol of GMP-PCP or GTP were added after the addition of G factor). After incubation the reaction mixtures were treated with RNase (19), followed by addition of 1 μl of 50% diethyl pyrocarbonate in ethanol to stop the RNase reaction. Samples were than fractionated by centrifugation through a sucrose gradient [5–20% sucrose, 15 mM Mg-(OAc)$_2$, 50 mM NH$_4$Cl, 1 mM DTT, and 50 mM Tris·HCl (pH 7.4)], and ribosome-containing peaks were pooled, diluted 1:1 with 0.1 M NH OAc–0.01 M EDTA, and extracted with phenol. To each aqueous phase was added 200 μg of oligo(U) as carrier, followed by 2.5 volumes of ethanol. After 16 hr at −20°C, the precipitates were collected by centrifugation, and dried under reduced pressure for 30 min. Precipitates were dissolved in 0.1 ml of 0.05 M Tris·HCl (pH 7.4)–1 mM EDTA, and treated with 75 units of T$_1$ RNase for 20 min at 25°C in order to split off the ApUpGp or ApUpGpGp moieties from the labeled (Up)$_n$ fragments. These reaction mixtures were then applied to Whatman 3 MM chromatography paper, which were than developed as described in *Methods*. Chromatograms were dried, cut into 1.5-cm squares, eluted with 1 ml of 0.01 M Tris·HCl (pH 7.4) for 3 hrs, and the eluates were added to 20 ml of a toluene–2,5-diphenyloxazole scintillation cocktail containing 10% BioSolv (Beckman Instruments) and counted.

RESULTS

Translocation of mRNA

In order to develop an assay for the translocation of mRNA, it was first necessary to find a system in which this could be studied conjointly with the conventional translocation of peptidyl-tRNA. For this we chose a highly purified system containing 70S ribosomes (dissociated to 50S and 30S subunits, as in ref. 2), f$_1$, f$_2$, T$_u$, mixed tRNA charged with fMet and Val, ApU(pG)$_2$(pU)$_{\bar{30}}$, GTP, and salts (Table 1). When these components are mixed and incubated at 25°C, fMet-tRNA binds to 44% of the ribosomes, whereas Val-tRNA binds to 20%. Analysis of this bound radioactivity (see *Methods*) has shown that 85% of the [^{14}C]Val and about 40% of the [^{3}H]fMet is present as the dipeptide fMet-Val, which is presumably esterified to tRNA^{Val}. The fact that fMet-tRNA binds to so much greater an extent than Val-tRNA is consistent with the observation of Erbe *et al.* (25) that A-site binding is much less stable than P-site binding. None of the bound [^{14}C]Val is released from ribosomes by puromycin (Table 1, Expt. 2), implying that all the fMet-Val-tRNA must be present in the A site. However, addition of G factor to this complex converts about 65% of the T$_u$-dependent valine to a puromycin-reactive site. This translocation reaction is partially inhibited by fusidic acid, which is consistent with its specific dependence on G factor.

TABLE 1. *Conventional assay for translocation of fMet-Val-tRNA*

Expt.	Reaction components	Amount bound to ribosomes (pmol) fMet	Val
1	Complete*	24.4	11.2
	− T$_u$	20.6	1.2
	+ G	22.6	10.5
	+ G + Puromycin	10.0	4.4
	+ G + Fusidic Acid	22.2	10.1
	+ G + Fusidic Acid + Puromycin	14.3	8.3
2	Complete†	—	14.6
	− T$_u$	—	2.1
	− AUG$_2$U$_{\bar{30}}$	—	2.8
	+ Puromycin	—	15.4

* Reaction mixtures (50 μl) contained 55 pmol of 70S ribosomes, 37 μg of charged tRNA (containing 31 pmol of [^{3}H]fMet-tRNA, 3490 Ci/mol, and 50 pmol of [^{14}C]Val-tRNA, 228 Ci/mol), 5 μg of ApU(pG)$_2$(pU)$_{\bar{30}}$, 0.2 μg of f$_1$, 4 μg of f$_2$, 5 μg of T$_u$, 0.2 mM GTP, 50 mM Tris·HCl (pH 7.4), 5 mM Mg(OAc)$_2$, 100 mM NH$_4$Cl, and 1 mM dithiothreitol. Reactions were incubated 15 min at 25°C, diluted, filtered, and counted (2). Where indicated, 7 μg of G factor, 40 nmol of fusidic acid, or 100 nmol of puromycin were added after the 15-min incubation; incubation was continued for another 5 min at 25°C before filtering.

† Reaction mixtures were identical to those of Expt. 1, except that the tRNA was charged with cold fMet and [^{14}C]Val, and the APU(pG)$_2$(pU)$_{\bar{30}}$ contained a tritium label in the (pU)$_{\bar{30}}$ moiety (100 Ci/mol uridylate residues).

With this system we next sought to see if the messenger ApU(pG)$_2$(pU)$_{\bar{30}}$ is translocated at the same time as fMet-Val-tRNA. To do this, reaction mixtures similar to those described in Table 1, Expt. 2, containing H^3-labeled messenger were treated with RNase and analyzed for the chain length of the ribosome-bound messenger fragments. Typical results are shown in Fig. 1, where the chromatographic distribution of the ^{3}H-labeled (Up)$_n$ portion of the ApU(pG)$_2$(pU)$_n$p fragments is compared with oligo(U) markers. Thus, in Fig. 1*a*, the major radioactive product corresponds to (Up)$_{11-12}$, indicating that in this case most of the messenger protected from RNase was ApU(pG)$_2$(pU)$_{11-12}$p. The second small peak of radioactivity near the origin, corresponding to ApU(pG)$_2$-(pU)$_{20-25}$p, is thought to represent nonspecific binding of messenger to ribosomes, as originally observed by Takanami *et al.* (26). Unlike the major peak, its binding is not dependent on GTP (Fig. 2*a*) or charged tRNA (data not shown).

TABLE 2. *Puromycin reactivity of fMet-tRNA bound to ribosomes with GTP or GMP-PCP*

Reaction components*	Amount of fMet bound to ribosomes (pmol)
Complete (GTP)	25.6
+ Puromycin	5.4
Complete (GMP-PCP)	13.8
+ Puromycin	15.2
Complete (no nucleoside triphosphate)	2.2

* Reaction mixtures were similar to those in Table 1, except that the tRNA was charged only with [^{14}C]fMet, T_u was omitted, the messenger was ApUpG, and the nucleoside triphosphate was varied as indicated.

When G factor is added to the complete system (already containing GTP), the amount of radioactivity in the major peak is reduced and a third peak appears in the chromatogram (Fig. 1*a*), which has an average chain length of about 15 residues. This corresponds to an RNase-resistant fragment of $ApU(pG)_2(pU)_{15}p$. The amount of radioactivity in this peak varies from experiment to experiment, but it is usually roughly equal to that remaining in the 11- to 12-mer peak. We interpret this result as indicating that the addition of G factor has caused the movement (relative to the ribosome) of roughly half of the bound messenger by a distance of about three nucleotides in the 5′ direction. That only about half of the messenger is moved, or translocated, is consistent with the observation (Table 1) that only 45% of the ribosomal complexes formed have fMet-Val-tRNA in the A site; the other 55% have fMet-tRNA in the P site, in which case translocation of the corresponding mRNA should not occur.

It is evident that fusidic acid inhibits the movement of mRNA, since the G-factor dependent appearance of $(Up)_{15}$ is prevented by this drug (Fig. 1*a*). Moreover, this movement requires GTP; excess GMP-PCP inhibits the reaction, as shown in Fig. 1*b*. (The experimental evidence for this differs somewhat from that shown in Fig. 1*a*, since the chromatographic separation of the markers is greater, the average chain length of the main peak is now almost exactly 12, and the proportion of radioactivity in the 15-mer region is greater; nevertheless the major conclusion that GMP-PCP cannot substitute for GTP in this reaction is quite clear.) This suggests that the energy released by hydrolysis of the GTP drives the translocation of mRNA. Thus, at least three different elements of the translocation reaction—the movement of peptidyl-tRNA from the A to the P site (Table 1), the release of deacylated tRNA from the P site (27, 28), and the movement of the mRNA relative to the ribosome—all show a dependence on G factor and GTP, and all are inhibited by fusidic acid and GMP-PCP. Therefore, it seems possible that these elements may be all part of a single concerted reaction; on the other hand, the possibility that there may be several distinct intermediates in this process cannot be ruled out.

It should be noted that these experiments not only shed light on the mechanism of translocation, but also provide new criteria for defining the two tRNA binding sites on the ribosome. Thus, when fMet- or peptidyl-tRNA is in the P site (reactive with puromycin), the distance between the corresponding codon and the limit of RNase protection of the

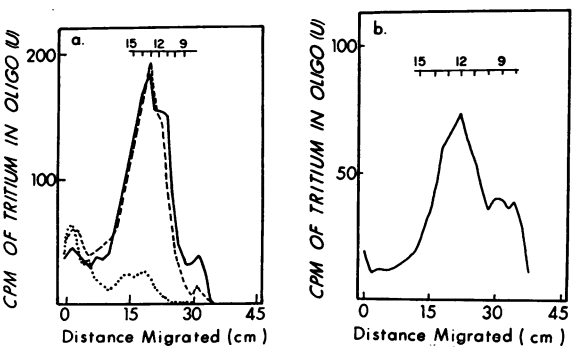

FIG. 2. Chromatograms of oligo(U) portions of RNase-resistant messenger fragments. Reaction mixtures in (*a*) were similar to those in Table 1, except that the tRNA was charged only with [^{14}C]fMet, T_u was omitted, the messenger was [^{3}H]-$ApUpG(pU)_{\bar{30}}$, and the nucleoside triphosphate was: GTP, *solid line;* GMP-PCP, *dashed line;* none, *dotted line.* The reaction mixture in (*b*) was similar to those in (*a*), except that the messenger was [^{3}H]$ApU(pG)_2(pU)_{\bar{30}}$, GTP was used, and 30S particles (10 pmol in 50 μl) were used instead of 70S ribosomes.

mRNA is an average of 13 nucleotides. On the other hand, when peptidyl-tRNA is in the A site (unreactive with puromycin), the distance between its codon and the limit of RNase protection is about 10 nucleotides.

Does translocation occur during initiation?

Having demonstrated that the messenger is indeed moved a distance of one codon during translocation, we can now proceed to use this reaction as an assay to detect translocation of mRNA during the initiation process. In particular, we want to know if the f_2-dependent hydrolysis of GTP during the junction step (9, 29, 30) causes translocation of the messenger. One experimental approach to this problem is to substitute GMP-PCP for GTP in the initiation reaction, and to look to see if the messenger fragment obtained in this case is shorter by three nucleotides than when GTP is used. If this were so, then we would conclude that translocation normally does occur, and that the prevention of GTP hydrolysis (by using GMP-PCP) inhibits it. On the other hand, if the protected messenger fragments have the same length with GTP and GMP-PCP, then it implies that GTP hydrolysis is not used for translocation of messenger. When this experiment was performed, the latter result was obtained, as shown in Fig. 2*a*. Thus, when fMet-tRNA is bound to ribosomes with [^{3}H]-$ApUpG(pU)_{\bar{30}}$ as mRNA in the presence of f_1, f_2, and GTP, the major fragment of protected messenger is $ApUpG(pU)_{13}p$, with a minor component (about 7%) of $ApUpG(pU)_{10-11}p$. When GMP-PCP is substituted for GTP, however, a virtually identical distribution is seen. Thus, we conclude that the hydrolysis of GTP is not used to translocate messenger during the initiation reaction; moreover, this suggests that GTP is not used to translocate fMet-tRNA either, since our previous results with G factor indicate that translocation of both mRNA and peptidyl-tRNA occurs simultaneously. Thus, GTP must serve some different function in initiation.

An indication of what this function might be is seen in Table 2, which shows that in our present system, as previously (2, 30), fMet-tRNA bound to ribosomes with GMP-PCP is completely unreactive with puromycin. When GTP is used, on the other hand, about 80% of the fMet is released with

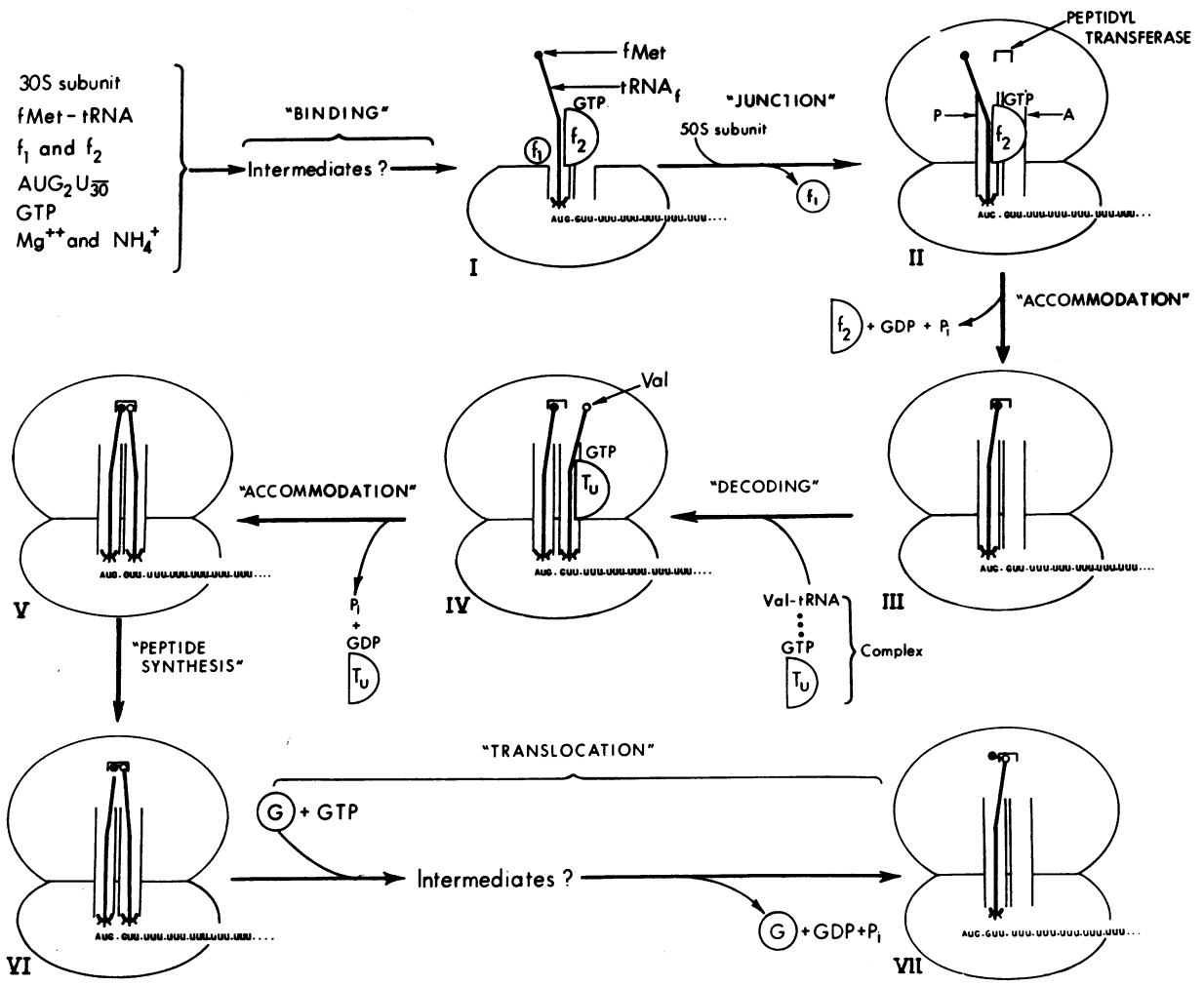

FIG. 3. Mechanism of initiation and elongation reactions in protein synthesis. Between steps VI and VII, $tRNA_f^{Met}$ should appear with Ⓖ and GDP + P_i as a product.

puromycin. This implies that GTP hydrolysis is used for activating the bound fMet-tRNA for peptide synthesis. There is as yet no evidence as to the chemical nature of this activation step, except that it is different from translocation. One possibility (among many) is that it involves movement of the fMet moiety plus the 3′ end of the $tRNA_f$ into the donor site of the peptidyl transferase. This could be accomplished by a conformational change of the $tRNA_f$, or by expulsion of factors (such as f_2) that might otherwise hinder this movement. Another possible interpretation of this activation reaction is that it represents a "half-translocation" step, as originally proposed by Bretscher (4). In any case, whatever the mechanism may be, we prefer to call this reaction the "Accommodation Step", so as to distinguish it from the translocation reaction *per se* (and from the activation of amino acids that precedes esterification to transfer RNA).

It should be noted that immediately prior to the accommodation step, at least part of the fMet-tRNA molecule must be bound in the conventional P site of the ribosome, even though the fMet moiety is not reactive with puromycin. This conclusion is drawn from the fact that in this state, prior to GTP hydrolysis, the AUG codon is situated 13 nucleotides away from the edge of the mRNA-binding site. These conditions of partial P-site occupancy might be interpreted as defining a new type of ribosomal binding site. As indicated above, this situation has been anticipated by Bretscher (4), who has postulated the existence of a hybrid form of the P and A sites to account for it. In any case, whatever its structural characteristics may be, we prefer to designate this preaccommodation state of fMet-tRNA the "pre-P site".

Location of the 30S binding site

It was of interest to determine whether fMet-tRNA that is bound to free 30S particles is situated the same distance from the RNase defined edge, i.e., occupies a portion of the P site. To do this, we repeated the same basic experiments with 30S particles in place of 70S ribosomes. A typical example is shown in Fig. 2*b*. The majority of the radioactivity migrates as a 12-mer. Since the messenger in this case was $ApU(pG)_2(pU)_{\overline{30}}$, it is clear that the AUG codon is located a distance of 13 nucleotides from the limit of RNase resistance, at least in most of 30S complexes. Thus, it seems likely that most of the fMet-tRNA is bound in that portion of the P site that exists on the 30S particle. Two reservations about this conclusion should be noted. First, there is no guarantee that the 30S and 70S complexes are equally sensitive to RNase, thus the equivalence of protected lengths of message in both cases may be fortuitous. Second, the presence of a significant amount of $(Up)_9$ in the chromatogram of Fig. 2*b* leaves open the possibility that the AUG codon and its corresponding fMet-tRNA

were originally bound in the A site, or at least passed through it on the way to the P site (1, 2, 31, 32).

DISCUSSION

Perhaps the most convenient way to summarize our results is to incorporate them into the standard model of protein synthesis. An attempt at this is shown in Fig. 3. (It should be noted that there are several other models that fit the biochemical data almost as well, and we have chosen to use the most common one purely for the sake of convenience. Indeed, so little is known about the structures of tRNA and the ribosome that models of this type have validity primarily as compendia of biochemical information; whatever structural information they contain is based largely on speculation).

The binding step that results in the formation of the 30S initiation complex (I) probably involves a sequence of many reactions, about which little is known. In complex I, the fMet-tRNA is shown bound in a portion of the P site, although it may have previously passed through the A site to reach this state (31, 32). Complex I has been recently shown to contain GTP, and probably f_2, in addition to the other components (29). The junction step gives rise to a very short-lived complex II, containing fMet-tRNA in the pre-P site. The existence of complex II can only be shown using GMP-PCP in place of GTP and, as noted above, its actual structure may be very different from that shown in Fig. 3. The accommodation step involves hydrolysis of the bound GTP, release of f_2 (6, 21), and the fitting of the top portion of fMet-tRNA into the donor site of the peptidyl transferase (Complex III). The immediate product of the decoding step, complex IV, contains Val-tRNA bound in at least part of the A site. However, if GTP hydrolysis is prevented, this Val-tRNA is unreactive in peptide-bond synthesis (11, 12). This situation seems analogous to that of fMet-tRNA in complex II, and so we propose to describe the Val-tRNA as being bound in the pre-A site. The result of the subsequent GTP hydrolysis step is to activate the Val-tRNA for peptide-bond synthesis; therefore we have also referred to this reaction as an accommodation step. After peptide-bond formation, the stage is set for translocation. As discussed above, this complicated reaction is catalyzed by G factor and driven by energy obtained from GTP hydrolysis. As a result of this step, peptidyl-tRNA is translocated from the A to the P site, a $tRNA_f$ is expelled from the P site, and the mRNA is moved a distance of one codon in the 5′ direction. While there is no evidence for any intermediate states in this reaction, its very complexity prompts us to hold open this possibility.

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