

Sequence of Events in Initiation of Translation: A Role for Initiator Transfer RNA in the Recognition of Messenger RNA

(fMet-tRNA^{fMet} binding/messenger RNA binding/initiation factors/edeine)

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ABSTRACT It is shown that initiation of translation involves several steps. (i) Binding of fMet-tRNA^{fMet} to the bacterial 30S ribosomal subunit in the absence of messenger RNA, yielding a 34S complex. This binding is rapid and dependent on initiation factor 2 but not on initiation factor 3. (ii) Binding of messenger RNA to the 34S complex. This binding is slower and depends on initiation factor 3. If R17 RNA is used as messenger, the resulting complex sediments at 46 S. (iii) Joining of a 50S subunit to yield a complete initiation complex. Binding of fMet-tRNA^{fMet} not only precedes, but is necessary for, correct binding of messenger RNA to ribosomes. Thus, initiator tRNA may play an active role in the selection of initiation sites in messenger RNA.

Two fundamental aspects of the initiation of translation are the mechanism of attachment of ribosomes to messenger RNA, and the molecular basis for the selection of initiation sites in messenger RNA. According to current thinking (1-3), initiation factor 3 (IF-3) directs the binding of the 30S ribosomal subunit to a specific initiation sequence in messenger RNA. The appropriate AUG initiation codon then specifies the IF-2-dependent attachment of fMet-tRNA^{fMet} (fMet-tRNA) and GTP to this preformed 30S-messenger RNA complex, and is followed by junction of the 50S subunit to complete the process of initiation. This sequence would imply that binding of initiator tRNA is a consequence of, rather than a prerequisite for, the selection of initiation sites in messenger RNA. Instead, we now report that binding of fMet-tRNA not only precedes, but is necessary for, correct binding of messenger RNA to prokaryotic ribosomes. These data suggest that initiator tRNA plays an active role in the selection of initiation sites in messenger RNA.

RESULTS

Isolation of Distinct Initiation Intermediates. Incubation of purified, salt-washed, 30S ribosomal subunits of *Escherichia coli* with [³⁵S]fMet-tRNA, GTP, IF-2 and IF-3 results in the formation of 34S complexes containing ³⁵S label (Fig. 1A). Under the ionic conditions used, these complexes cannot be detected by retention on nitrocellulose filters, and they are detected in sucrose gradients only after fixation with 1% glutaraldehyde. Fixation leads to the formation of a small proportion of 30S dimers, sedimenting at 50 S, that also contain ³⁵S label (Fig. 1A).

Upon addition of R17 bacteriophage RNA, the ³⁵S label at 34 S is converted into complexes sedimenting at 46 S (Fig. 1B).

Abbreviations: fMet-tRNA, *N*-formylmethionyl-tRNA^{fMet}; IF, initiation factor.

Unlike the 34S complexes, 46S complexes are fully retained on nitrocellulose filters and can be detected in sucrose gradients without prior glutaraldehyde fixation. Because of this increased stability, the amount of fMet-tRNA that can be bound at equilibrium is greater when R17 RNA is present. Subsequent addition of 50S ribosomal subunits results in quantitative conversion of the 46S complexes into complete initiation complexes, sedimenting at 71 S (Fig. 1C).

Initiation Factor Requirements for Formation of 34S and 46S Complexes. No 34S complexes are detected when IF-2 is omitted (Fig. 2A). When IF-2 is present, however, 34S complexes are formed (Fig. 2B). The extent of 34S complex formation is stimulated little, if any, by addition of IF-3 (Fig. 2C). Thus, IF-2, but not IF-3, is absolutely required for the binding of fMet-tRNA to purified 30S subunits in the absence of added messenger RNA.

When R17 RNA is present, addition of IF-3 fails to support binding of ³⁵S label (Fig. 2D), but addition of IF-2, without IF-3, induces extensive complex formation (Fig. 2E). Most of the bound ³⁵S sediments at 34 S, however, suggesting that the 34S complex is unable to bind R17 RNA in these conditions. Indeed, in contrast to the formation of 34S complexes, the formation of 46S complexes is dependent upon IF-3: in the presence of this factor, label in 34S complexes is converted nearly quantitatively into 46S complexes (Fig. 2F). The small amount of 46S complex formed in the absence of added IF-3 (Fig. 2E) may be due to traces of IF-3 on the 30S subunits. We conclude that binding of fMet-tRNA to 30S ribosomal subunits occurs in the absence of added messenger RNA and requires IF-2; subsequent attachment of R17 messenger RNA is completely dependent on IF-3.

Entry of R17 RNA into 46S Complexes Is Dependent Upon fMet-tRNA. We have just seen that complexes between 30S ribosomal subunits and fMet-tRNA can be formed in the absence of R17 RNA. As demonstrated in Fig. 3A and B, the formation of fast-sedimenting complexes between ³²P-labeled R17 RNA and 30S subunits is absolutely dependent on added fMet-tRNA (labeled with ³⁵S). When fMet-tRNA is present (Fig. 3B), extensive entry of both ³²P and ³⁵S labels into 46S complexes is observed; these R17 RNA-containing complexes are converted quantitatively into 71S initiation complexes upon addition of 50S subunits (Fig. 3C).

Selective Inhibition by Edeine. The oligopeptide antibiotic edeine (6) inhibits quantitatively the formation of complete initiation complexes at a concentration of 10 μM.

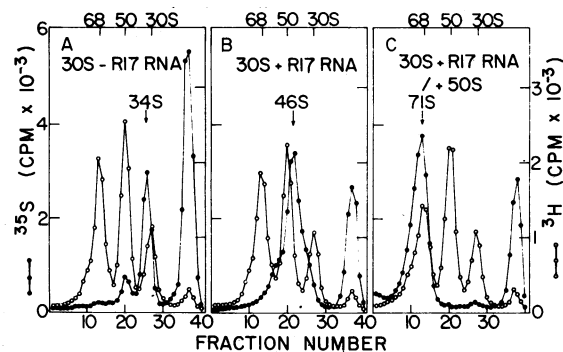


FIG. 1. Formation of [^{35}S]fMet-tRNA-containing complexes. Preparation of fMet-tRNA, R17 RNA, and purified IF-3 was as before (4). IF-2 was purified (5) to more than 95% electrophoretic homogeneity. Ribosomal subunits were obtained from salt-washed ribosomes (4) by twice-repeated centrifugation through sucrose gradients, 1 mM in Mg acetate. (A) To form 34S complexes, a reaction mixture (final volume 0.025 ml) containing 50 mM Tris-HCl (pH 7.8), 50 mM NH_4Cl , 5 mM Mg acetate, 1 mM GTP, 16 mM 2-mercaptoethanol, 0.53 A_{260} unit of 30S subunits (40 pmol), 3.8 μg of IF-2 (42 pmol), 1.34 μg of IF-3 (60 pmol), and 11.2 pmol of [^{35}S]fMet-tRNA was incubated for 12 min at 37°. (B) To form 46S complexes, 0.9 A_{260} unit of R17 RNA (40 pmol) was included. (C) Complete 71S complexes were formed in an additional incubation for 4 min at 37° with 1.1 A_{260} units of 50S subunits (50 pmol). Before centrifugation for 2.5 hr at 41,000 rpm through 12 ml, 5–20% exponential sucrose gradients (4), samples were fixed for 5 min at 0° by addition of 0.1 ml of 50 mM Tris-HCl (pH 7.8)–50 mM KCl–5 mM Mg acetate–1.25% (w/v) glutaraldehyde. In this experiment, ^3H -labeled marker ribosomes were included after fixation.

In the absence of R17 RNA and 50S subunits, however, edeine at this concentration has no effect on the formation of 34S complexes between 30S subunits and fMet-tRNA (Fig. 4A and B). Instead, edeine inhibits the subsequent attachment of these complexes to R17 RNA, preventing their conversion to 46S complexes (Fig. 4C and D). Once they are formed, however, 46S complexes are not disrupted by edeine (Fig. 4E).

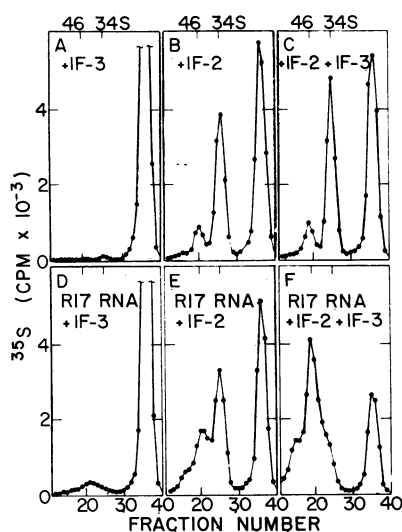


FIG. 2. Initiation factor dependence. Incubation and analysis were as for Fig. 1B, except that IF-2, IF-3, and R17 RNA were present only where indicated.

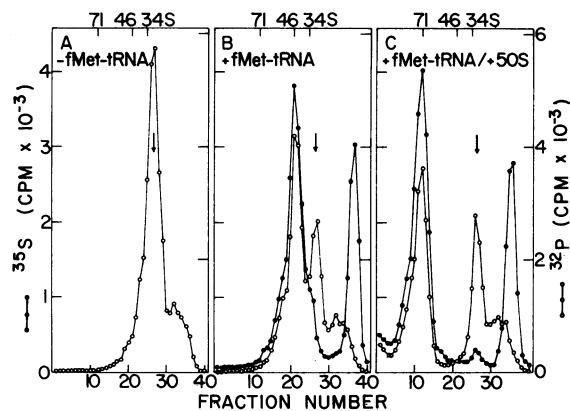


FIG. 3. fMet-tRNA-dependent binding of R17 [^{32}P]RNA. Complex formation was allowed to occur as in Fig. 1B, with R17 [^{32}P]RNA and (A) omitting fMet-tRNA; (B and C) including [^{35}S]fMet-tRNA; (C) adding 50S subunits in a second incubation, as for Fig. 1C. Analysis was as in legend of Fig. 1. Arrow, position of free R17 RNA.

That the 46S complexes remain fully functional in the presence of edeine is shown in Fig. 5. In this experiment, 46S complexes were formed by incubating a complete reaction mixture lacking only 50S subunits (Fig. 5A); upon subsequent addition of 50S subunits, the 46S complexes are converted quantitatively into 71S initiation complexes (Fig. 5B) sensitive to puromycin (Fig. 5C). Edeine does not prevent this formation of 71S initiation complexes (Fig. 5D) nor subsequent release of [^{35}S]fMet label by puromycin (Fig. 5E). The puromycin effect can be eliminated by sparsomycin (Fig. 5F), an inhibitor of transpeptidation and a competitive inhibitor of puromycin, strongly suggesting that the release of fMet label is a direct result of fMet-puromycin formation, rather than a nonspecific disruption.

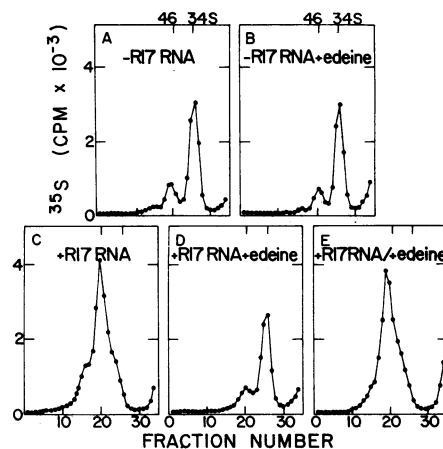


FIG. 4. Effect of edeine on formation of 34S and 46S complexes. To form 34S complexes, mixtures (see Fig. 1A) were incubated for 4 min at 37°, in the absence (A, C, D, and E) or presence (B) of 10 μM edeine. Samples C, D, and E were then incubated for 10 min with R17 RNA (see Fig. 1B) and edeine (D only). Sample E was prepared as C, but then incubated another 5 min with edeine. Analysis as in legend of Fig. 1. The amount of 46S complex formed is identical whether R17 RNA is added to preformed 34S, as in C, or simultaneously with fMet-tRNA, as in Fig. 1B.

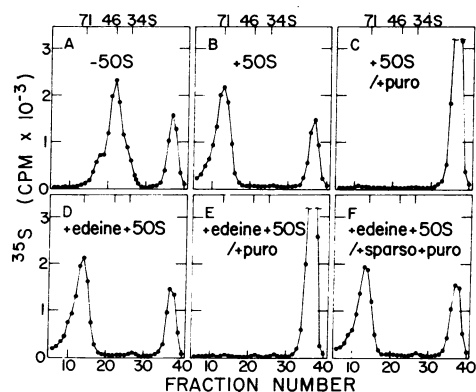


FIG. 5. Edeine fails to inhibit conversion of 46S into 71S complexes. All samples were incubated as for Fig. 1B and, except for A, subsequently were incubated with 50S subunits (as for Fig. 1C) with edeine (10 μ M) present as shown. Samples C, E, and F were then incubated for another 5 min at 37° with puromycin (1.3 mM) and sparsomycin (0.1 mM; F only). Analysis as in legend of Fig. 1.

Edeine, therefore, blocks attachment of R17 RNA to fMet-tRNA \cdot 30S complexes, but does not interfere with preceding or subsequent steps in the initiation process.

34S Complexes Do Not Contain Messenger RNA. In the absence of added messenger RNA, [35 S]fMet-tRNA is bound to 30S subunits in complexes sedimenting at 34 S (Fig. 6A). Like R17 RNA, the addition of ApUpG triplets stimulates significantly the amount of fMet-tRNA bound (Fig. 6C). However, the resulting complexes do not appear at 46 S, as with R17 RNA, but sediment only slightly faster than 34 S (Fig. 6A and C).

The stimulation by ApUpG is the direct result of its binding to the fMet-tRNA \cdot 30S complex, for 35 S label in the resulting complex can be retained on nitrocellulose filters, while in the absence of ApUpG it is not retained (data not shown). Upon addition of 50S subunits, the complexes of Fig. 6C are converted to 71S initiation monosomes (Fig. 6D), fully sensitive to puromycin (Fig. 6E). When ApUpG is omitted, however, and 50S subunits are added, little 35 S label enters the 70S

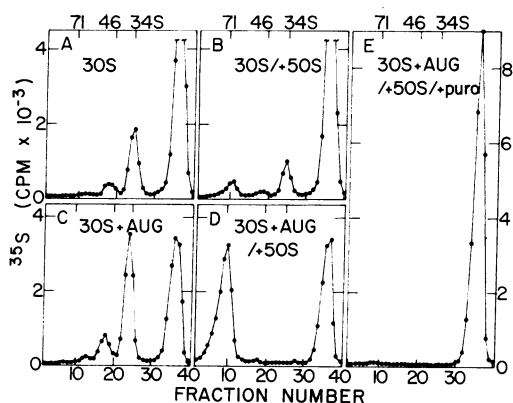


FIG. 6. Effect of ApUpG on complex formation. All samples were incubated as for Fig. 1A, with ApUpG (0.1 A_{260} unit) present as shown. Samples B, D, and E were then incubated with 50S subunits, as for Fig. 1C. Sample E was incubated an additional 5 min with 1.3 mM puromycin. Analysis as in legend of Fig. 1.

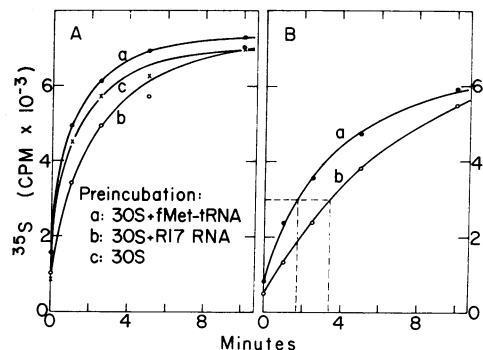


FIG. 7. Effect of preincubation on kinetics of fMet-tRNA binding. Mixtures of 0.075 ml (see Fig. 1) containing reactivated 30S subunits (15 min at 37° in 0.5 M NH_4Cl buffer; ref. 7) (A) or unreactivated 30S (B) were preincubated for 5 min at 37° with fMet-tRNA (curve a), R17 RNA (curve b), or neither (curve c) before addition, at 37°, of R17 RNA (curves a and c) or fMet-tRNA (curves b and c). Samples of 10 μ l were removed at intervals, diluted into 1 ml of 50 mM Tris \cdot HCl (pH 7.8)–50 mM NH_4Cl –10 mM Mg acetate at 0°, and passed without delay through Millipore HA 0.45- μ m filters, washing extensively with the same buffer. Filters were dried and analyzed for radioactivity. A lapse of 10 sec occurred between addition of the component omitted during preincubation and removal of the 0-min sample.

region (Fig. 6B). Comparison of Fig. 6B and D shows clearly that formation of initiation complexes is almost completely dependent upon the addition of ApUpG. The small amount of label near 71 S in Fig. 6B could represent nonspecific complexes containing fMet-tRNA and 30S and 50S subunits, rather than true initiation complexes.

This experiment rules out the possibility that AUG-containing fragments of messenger RNA on 30S subunits are responsible for the binding of fMet-tRNA observed in the absence of added ApUpG or R17 RNA. This binding thus appears to be independent of messenger RNA.

Effect of Preincubation on Kinetics of Complex Formation. To examine further if binding of fMet-tRNA to the 30S subunit precedes the attachment of messenger RNA, we studied

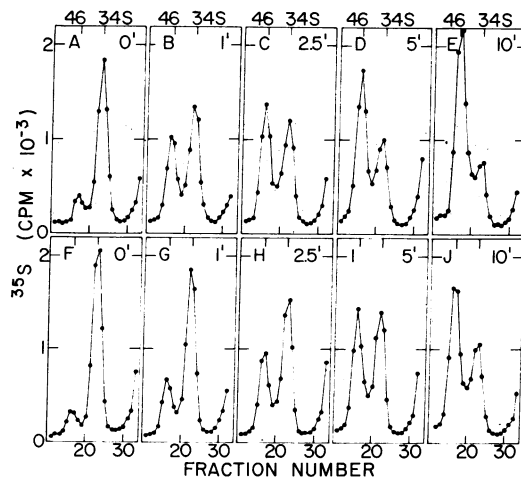


FIG. 8. Effect of preincubation on kinetics of 34S to 46S conversion. Incubation and sampling at time intervals (min) were as for Fig. 7B (curves a and b), but samples were fixed and centrifuged as in Fig. 1. (A–E) Preincubated with fMet-tRNA; (F–J) preincubated with R17 RNA.

the kinetics of formation of R17 RNA-containing 46S complexes. Since 34S complexes are too unstable to be detected on nitrocellulose filters, filtration can be used to study selectively the formation of 46S complexes. As seen in Fig. 7A (curve *c*), the rate of 46S complex formation with reactivated 30S subunits is very rapid, with a half-time of about 0.5 min at 37°. If the 30S subunits are first incubated with fMet-tRNA, GTP, IF-2, and IF-3, and R17 RNA is subsequently added, the rate of 46S complex formation is increased slightly (curve *a*). On the other hand, if the 30S subunits are preincubated with R17 RNA before the addition of fMet-tRNA, the kinetics are significantly slower (curve *b*), particularly when compared to curve *a*. This difference is also observed when unreactivated 30S subunits are used; in that case, the rate of formation of 46S complexes is less rapid (Fig. 7B). Here, the half-time of formation of 46S complexes is consistently about 2-fold shorter when preincubation is with fMet-tRNA (curve *a*) rather than with R17 RNA (curve *b*). This decrease in rate does not seem to be due to degradation of R17 RNA during preincubation, since the same equilibrium level is reached in each case.

To study more directly the rate of formation of 34S and 46S complexes, samples were analyzed on sucrose gradients after fixation. As seen in Fig. 8A-E, preincubation of unreactivated 30S subunits with ³⁵S-labeled fMet-tRNA results in the formation of 34S complexes that are only slowly converted to 46S after addition of R17 RNA. Formation of 34S complexes is almost instantaneous when fMet-tRNA is added to 30S subunits in the absence of R17 RNA (data not shown) or after preincubation with R17 RNA (Fig. 8F). Thus, attachment of R17 RNA is relatively slow when compared to the rate of binding of fMet-tRNA. Consistent with the data of Fig. 7, the rate of appearance of 46S complexes is significantly slower with 30S subunits preincubated with R17 RNA (Fig. 8F-J).

DISCUSSION

These experiments support the concept that the anticodon of fMet-tRNA, present on the 30S ribosomal subunit, plays an active role in the selection of initiation sites on messenger RNA in the presence of initiation factors, and that the recognition of AUG initiation codons is directed by fMet-tRNA (8). They offer no support for the earlier view that initiation sites are selected entirely by 30S ribosomal subunits and initiation factors, and that AUG codons in messenger RNA subsequently direct the binding of fMet-tRNA.

Specifically, we find that binding of fMet-tRNA not only precedes, but is necessary for, correct binding of messenger RNA to bacterial 30S ribosomal subunits. Our experiments lead to the following sequence of events in initiation:

- (1) In the absence of messenger RNA, fMet-tRNA is bound to the 30S subunit, yielding a 34S complex. This binding is absolutely dependent on IF-2, but does not require IF-3. IF-2 may promote binding of fMet-tRNA, or may stabilize fMet-tRNA on 30S subunits once it is bound.
- (2) Messenger RNA is bound to the 34S complex. This binding is dependent on IF-3. If R17 RNA is used as messenger, the resulting complex sediments at 46S.
- (3) Joining of a 50S ribosomal subunit yields a complete initiation complex.

The instability of the fMet-tRNA·30S complex may explain why it has not been detected in earlier studies, though its existence has been postulated (8). This instability, as

opposed to the stability of complexes containing messenger RNA, can also explain why in earlier studies the detection of complexes between fMet-tRNA and 30S subunits showed an apparent absolute requirement for messenger RNA.

Although IF-3 is not required in step 1, its presence on the 30S subunit stabilizes the 34S complex in the presence of 50S subunits and prevents the formation of single ribosomes at the expense of 34S complexes (data not shown). Once messenger RNA is bound, IF-3 may leave the complex. It has been shown that IF-1 is not required to form initiation complexes, but subsequently acts to recycle IF-2 (9).

The proposed sequence is supported by the effect of edeine, which blocks specifically the binding of messenger RNA but does not affect preceding and subsequent steps in initiation. Tetracycline, on the other hand, prevents the formation of both 34S and 46S complexes (data not shown).

The increase in sedimentation coefficient to 46S seen upon binding of R17 RNA to 34S complexes appears not to be caused by a large conformational change in the 30S subunit, for it is not seen when ApUpG is used as template. The contribution of the RNA is sufficient to account for this increase.

While binding of fMet-tRNA to 30S subunits is exceedingly rapid, the subsequent attachment of R17 RNA is a slower process (Fig. 8). One interpretation may be that binding of fMet-tRNA to 30S subunits induces a slow conformational change that must take place before R17 RNA can be bound; alternatively, or in addition, binding of R17 RNA may be limited by the rate at which initiation sites can be selected.

The kinetics of fMet-tRNA binding, illustrated in Fig. 8, show that 34S complexes behave as intermediates in the formation of 46S complexes; the edeine experiments show that the latter complexes are intermediates in the formation of complete initiation monosomes. While preincubation of 30S subunits with fMet-tRNA slightly increases the rate of subsequent binding of R17 RNA into 46S complexes, preincubation with R17 RNA not only fails to stimulate, but actually slows down the formation of these complexes (Figs. 7 and 8), in contrast to earlier measurements (2). This slowing effect may be due to incorrect binding of R17 RNA to the 30S subunit in the absence of fMet-tRNA, altering the conformation of the 30S subunit such that upon dissociation of this complex, fMet-tRNA must first induce a conformational change in the 30S subunit before R17 RNA can be bound and phased correctly. In the absence of fMet-tRNA, we have not been able to detect rapidly sedimenting complexes between R17 RNA and 30S subunits, as reported by others (10), although the shape of the RNA curve in Fig. 3A is perhaps suggestive of a fraction sedimenting faster than free RNA. The material in this shoulder, however, represents an extremely small proportion of the RNA capable of entering 46S complexes in the presence of fMet-tRNA (Fig. 3B).

Our finding that binding of fMet-tRNA to small ribosomal subunits of bacteria precedes the attachment of messenger RNA agrees well with earlier observations in eukaryotic systems (11-14), and suggests that initiator tRNA in all organisms may play an active role in the selection of initiation sites in messenger RNA.

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