Requirement of Initiation Factor 3 in the Initiation of Polypeptide Synthesis with N-Acetylphenylalanyl-tRNA
(protein synthesis/initiator codon selection)

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ABSTRACT Initiation factor 3 is required, along with initiation factors 1 and 2, for the incorporation of N-acetylphenylalanine into polypeptides and the formation of N-acetylphenylalanyl puromycin. Initiation factor 3 also strongly stimulates the binding of N-acetylphenylalanyl transfer RNA to isolated 30S ribosomal subunits. Phosphocellulose fractions of initiation factor 3 were found to catalyze N-acetylphenylalanine incorporation differentially with different synthetic messenger RNAs not containing any codons for N-formylmethionine. The results suggest that ribosomes recognize the initiator codon only through the initiator transfer RNA.

Much information has been accumulated on the initiation of protein synthesis in bacteria (1–3), but the critical question of how the initiator codon of the mRNA is selected remains unanswered. It is implicit in a generally held view that this selection occurs with the binding of ribosomes to mRNA. This binding is assumed to be specific and to take place only at the initiation site before initiator tRNA binding (Fig. 1, pathway a) (4). We proposed an alternative pathway in which the binding of the ribosomes to mRNA is postulated not to be specific and the initiator codon is selected by the subsequent binding of an initiator tRNA to ribosomes bound to the proper site (Fig. 1, pathway b) (5).† A third, more recent, view, for which compelling evidence has been obtained in eukaryotes (6–8), postulates that a messengerless complex of initiator tRNA and the small ribosomal subunit forms, and that this complex then binds to the appropriate site on mRNA (Fig. 1, pathway c) (9).

Selection of the initiator codon by specific binding of ribosomes to mRNA would require initiation signals to be recognized independently of initiator tRNA participation, whereas selection after nonspecific binding of ribosomes to mRNA or formation of a 30S ribosome–initiator tRNA complex would not require such recognition. In the two latter instances, the initiation signals would only be recognized, wholly or in part, through the initiator tRNA.

Studies that have been carried out so far do not establish clearly whether or not precise initiation signals exist that are recognized by ribosomes. The sequences of six initiation sites of bacteriophage RNA have been determined, but no common nucleotide sequence other than the initiator codon has been detected (10, 11). The observation that initiation factor 3 (IF-3) is required in the translation of natural mRNAs, but not of synthetic RNAs, suggested IF-3 involvement in ribosome recognition of an initiation signal that is more complex than a single codon (12). The selective translation of natural mRNAs in cell-free systems also supported this view (13–16). Others, however, have observed a requirement for IF-3 in the translation and ribosomal binding of fMet-tRNA with synthetic mRNAs containing random base sequences (17–19).

In the present study, we have approached the problem of initiator codon selection by examining the initiation of polypeptide synthesis with N-acetylphenylalanyl-tRNA (N-AcPhe-tRNA), which has been shown to require IF-1 and IF-2 and to display other characteristics of the natural system with fMet-tRNA (20–23). We have found on careful examination of this system that IF-3, in addition to IF-1 and IF-2, is required for the initiation of polypeptide synthesis with N-AcPhe-tRNA. The results of this study are reported in the present communication and implications of the observations on IF-3 function and initiator codon selection are discussed. A requirement for IF-3 in the poly(U)-directed binding of N-AcPhe-tRNA to 70S ribosomes has been observed independently by Parker-Suttle et al. (24). A summary of the work discussed here has been presented previously (25).

MATERIALS AND METHODS

Materials. Poly(U), poly(C,U), poly(A,C,U), and poly(A,G,U) were purchased from Miles Laboratories; Escherichia coli Q13 cells in mid-logarithmic phase were obtained from General Biochemicals; dextran sulfate (molecular weight

Fig. 1. Possible pathways for initiator codon selection.
5 × 10^9) from Pharmacia; N^4-formyltetrahydrofolate (Calcium Lecovorin Injection), from Lederle Laboratories. Poly(G,U), poly(A,G,U), and poly(A,G,G,U) were kindly provided by Dr. Mohan Koka, and tobacco yellow mosaic viral RNA, by Dr. Robert Haselkorn.

Methods. N-Ac[14C]Phe-tRNA^Phe, NH_4Cl-washed ribosomes, 30S ribosomal subunits, and protamine-treated supernatant were prepared as described (26).

Initiation factors from E. coli Q13 cells were purified by the method of Dubnoff and Maizra (27), with some modifications. All solutions used contained 10 mM imidazole-HCl (pH 7.4) as buffer, as well as 5 mM 2-mercaptoethanol and 5% glycerol. Crude IF-1 was collected from the void volume and from the 25 mM NH_4Cl wash of the initial step of DEAE-cellulose fractionation. The active fractions containing both IF-2 and IF-3 were eluted with 0.6 M NH_4Cl. After dialysis against 0.04 M NH_4Cl, the pooled active fractions were passed through a phosphocellulose column, and the factors were eluted with a 0.1–0.8 M NH_4Cl gradient. Activity of IF-2 was detected between 0.2 and 0.3 M NH_4Cl and IF-3 activity, between 0.5 and 0.6 M NH_4Cl. The factors were initially assayed by measurement of N-formylmethionine or N-acetylphenylalanine incorporation into polypeptide; subsequently the assay was used as the binding of N-AcPhe-tRNA to 30S ribosomal subunits during a 10-sec incubation (see below). Further purification was done according to the procedures of Dubnoff and Maizra. The purified preparations were concentrated on phosphocellulose columns, made 0.5 M in NH_4Cl, and stored at -60° in polyallomer tubes. In the refractionation of IF-3 on phosphocellulose, elution was carried out with a 0.4–0.8 M NH_4Cl gradient. Individual fractions were assayed against a buffered solution containing 0.5 M NH_4Cl and stored at -60°.

Assay for N-acetylphenylalanine incorporation into polypeptide: The reaction mixture contained, in a final volume of 0.25 ml, 50 mM imidazole-HCl (pH 7.4), 4 mM 2-mercaptoethanol, 0.2 mM GTP, 3 mM ATP, 8 mM MgCl_2, 80 mM NH_4Cl, 0.1 mM phenylalanine, 2 mM phosphoenolpyruvate, 2 μg of phosphoenolpyruvate kinase, 1.44 A_260 units of E. coli tRNA, 15 μl of protamine-treated supernatant, 40 pmol of N-Ac[14C]Phe-tRNA of specific activity 495 μCi/mmol, and 1.44 A_260 units of salt-washed ribosomes. Polynucleotides and saturating amounts of initiation factors, usually about 0.5 μg of IF-1 and 1 μg of IF-2 and of IF-3, were added as indicated. The reaction was started with 3 ml of 5% trichloroacetic acid after a 3-min incubation at 37°. The samples were then heated for 10 min at 95°, washed on a Millipore filter, and measured for radioactivity in a gas flow counter.

Assay for N-AcPhe-tRNA binding to 30S ribosomal subunits: The reaction mixture contained, in a final volume of 0.25 ml, 50 mM imidazole-HCl (pH 7.4), 4 mM 2-mercaptoethanol, 1.2 mM GTP, 8 mM MgCl_2, 80 mM NH_4Cl, saturating levels of initiation factors, 0.46 A_260 unit of 30S subunits preincubated at 37° for 15 min in a medium with 0.5 M NH_4Cl and 0.01 MgCl_2 (28–30), 40 pmol of N-Ac[14C]Phe-tRNA, and 10 μg of poly(U). The latter two components were always added last to initiate the reaction after the reaction mixture was equilibrated at 15°. The reaction was stopped after a 10-sec incubation at 15° by the rapid addition of 3 ml of ice-cold buffer containing 10 mM imidazole-HCl (pH 7.4), 1 mM 2-mercaptoethanol, 80 mM NH_4Cl, 8 mM MgCl_2, and 1 μM dextran sulfate. (To remove free sulfate and phosphate ions, dextran sulfate at a concentration of approximately 10 μM was dialyzed against 0.1 M NH_4Cl.) The ribosomal complexes were then adsorbed on a Millipore filter, washed, and counted in a liquid scintillation system.

RESULTS

When the initiation of polypeptide synthesis with N-AcPhe-tRNA was measured by the incorporation of N-acetylphenylalanine into poly(phenylalanine), a strong requirement was observed not only for IF-1 and IF-2, but for IF-3 as well. The requirement of IF-1 seen with saturating amounts of IF-2 or IF-3, or with both IF-2 and IF-3, is shown in Fig. 2a. The requirement of IF-2 with IF-1 and IF-3 is shown in Fig. 2b, and that of IF-3 with IF-1 and IF-2, in Fig. 2c. Omission of any one factor reduced incorporation of N-acetylphenylalanine significantly. The strong dependence of the reaction on IF-3 (Fig. 2c) should be noted particularly.

The requirement of IF-3 in the initiation of polypeptide synthesis with N-AcPhe-tRNA was also demonstrated by use of the formation of N-acetylphenylalanyl-uracil as a measure of initiation. The results obtained with four different mRNAs, including two natural mRNAs, are presented in Table 1. It is clear from the data that the codon for phenylalanine can serve as an initiator triplet even in the presence of natural initiator codons in the RNA. RNA of tobacco yellow mosaic virus was fairly active as messenger with N-AcPhe-tRNA as initiator tRNA. MS2 RNA, on the other hand, was only slightly active, indicating that codons for phenylalanine in the RNA were not readily accessible to the ribosomes. The inability of Phe-tRNA to substitute for N-AcPhe-tRNA assures us that N-AcPhe-tRNA participates directly in the initiation reaction without undergoing deacetylation.

The same three factors were necessary for the incorporation of N-formylmethionine into polypeptide. The effect of increasing concentrations of IF-3 on N-formylmethionine incorporation directed by poly(A,G,U) in the presence of saturating amounts of IF-1 and IF-2 is shown in Fig. 3. The quantity of factors needed to produce maximum incorporation was similar to that required in N-acetylphenylalanine incorporation. These results, together with the fact that the initiation factors were purified according to published procedures, verify that the three initiation factors required for the initiation of polypeptide synthesis with N-AcPhe-tRNA are the same.
ones required with fMet-tRNA. A similar requirement for the three initiation factors was observed in the amino-acid incorporation directed by MS2 viral RNA (data not presented).

The dependence of N-acetylphenylalanine incorporation on IF-3 was seen over a broad range of poly(U) concentrations (Fig. 4). The reaction was dependent on IF-3 even in the presence of a large excess of poly(U). This observation is not in agreement with the suggestion that the IF-3 requirement with synthetic RNA can be seen only at low concentrations of the polymer (31, 32).

Since it was possible that IF-3 stimulated N-acetylphenylalanine incorporation by merely acting as a dissociating agent and providing free subunits from 70S ribosomes, the requirement of IF-3 in the binding of N-AcPhe-tRNA to isolated 30S subunits was tested. When incubation was carried out for a brief period so that the initial rate of binding could be measured, a strong requirement for all three initiation factors, including IF-3, was observed in N-AcPhe-tRNA binding to the 30S subunits (Fig. 5). The reaction was quite rapid even at 15°, especially when the ribosomes were preincubated in high salt concentration (28–30). The binding reaction was usually complete in 30–40 sec; therefore, incubations were done for 10 sec. Prolonged incubations obscure IF-3 requirement because there occurs a moderately fast IF-2-stimulated binding, as well as a lower, factor-independent binding. The poly(A,G,U)-directed binding of fMet-tRNA to the 30S ribosomal subunit responds similarly, a strong requirement of IF-3 being observed only with short incubations (data not presented). Perhaps, because of the stability of the complex formed between synthetic RNA and ribosomes (5, 33), some binding of the initiator tRNA occurs without the full complement of initiation factors, or even in their complete absence.

As in the case of polypeptide synthesis initiated with fMet-tRNA, the incorporation of N-acetylphenylalanine into polypeptide, when directed by different mRNAs, was differentially stimulated by IF-3 fractionated on phosphocellulose (Fig. 6). The profiles shown in the upper panel of Fig. 6 represent N-acetylphenylalanine incorporation directed by poly(U) and poly(C,U). The two profiles are obviously different, indicating that fractions of IF-3 exhibited different specificities toward the two polymers. These same phosphocellulose fractions also differentially stimulated N-formylmethionine incorporation. The results of N-formylmethionine incorporation directed by poly(A,G,U) and poly(G,U) are shown in the lower portion of Fig. 6.

The pattern of N-acetylphenylalanine incorporation stimulated by the IF-3 fractions was also different from that of the amino-acid incorporation directed by MS2 RNA. In Fig. 7, a comparison of the profiles obtained for N-acetylphenylalanine incorporation with poly(U) and poly(A,C,U) and amino-acid incorporation with MS2 RNA is presented. Again, IF-3 fractions clearly exhibited differential activity toward all three mRNAs.

**DISCUSSION**

The present study strongly suggests that ribosomes do not recognize the initiator codon directly, but do so only through the initiator tRNA. Because N-AcPhe-tRNA was sufficiently similar to fMet-tRNA and was compatible with all three initiation factors, including IF-3, the codon for phenylalanine
was "recognized" as the initiation triplet in our model system. This would not be the case if the initiator codon were recognized directly since a totally different triplet would not then be able to substitute for the natural initiator codon. Thus, the results of our study are more in accord with pathways b and c of Fig. 1 than with pathway a.

The strong stimulation of N-AcPhe-tRNA binding to isolated 30S ribosomal subunits by IF-3 and the differential incorporation of N-acetylphenylalanine catalyzed by phosphocellulose fractions of IF-3 observed in our system indicate that (1) IF-3 plays an active role in the formation of the 30S initiation complex with N-AcPhe-tRNA and its function is not limited to dissociating ribosomes, and that (2) this other role of IF-3 in the reaction may involve messenger discrimination. This second role of IF-3 still remains to be rigorously demonstrated, however, since interfering substances not normally associated with protein synthesis may have been responsible for the differential activity of the IF-3 fractions. Nucleases were ruled out as such a group of interfering substances in the case of poly(U) and poly(C,U) by showing that the messenger function of these polymers was not differentially destroyed by the phosphocellulose fractions of IF-3 (unpublished observation).

If some form of messenger recognition actually occurred in the incorporation of N-acetylphenylalanine, the nature of the synthetic mRNAs differentiated would suggest that either RNA structures acting as initiation signals were recognized only preferentially, or they were recognized precisely and were relatively simple. It should be mentioned in this regard that a number of restrictions make the process of initiation in the natural system with fMet-tRNA quite selective even in the absence of special initiation signals. The fact that initiation occurs only with fMet-tRNA eliminates all codons except that of methionine as a starting point of initiation. Secondary structures, not necessarily dependent on precise base sequence, make internal methionine codons inaccessible to ribosomes and unavailable for initiation (23, 24) and probably also make initiation sites differentially active. Out-of-phase codons for methionine in regions of the mRNA accessible to ribosomes may have been avoided by selection of appropriate synonym codons in the course of evolution (5).

Finally, even though the conclusions drawn from the present study are subject to the usual uncertainties of extrapolat-

**Fig. 5.** Factor requirement for N-AcPhe-tRNA binding to 30S subunits. The assay conditions were as described in Methods, with 10 μg of poly(U) added. In each case, the concentration of one factor was varied while the other factors were present in saturating levels.

**Fig. 6.** Differential activity of phosphocellulose fractions of IF-3 in the incorporation of N-acetylphenylalanine and N-formylmethionine with different mRNAs. The curves in the upper panel represent IF-3 activity of phosphocellulose gradient fractions in the incorporation of N-acetylphenylalanine with poly(U) and poly(C,U). The curves in the lower panel represent IF-3 activity in the incorporation of N-formylmethionine with poly(A,G,U) and poly(G,U). The basic assay conditions for N-acetylphenylalanine incorporation were as described in Fig. 2, and for N-formylmethionine incorporation as described in Fig. 3. Ten micrograms of the indicated RNA, 0.5 μg of IF-1, 1 μg of IF-2, and 20 μl of IF-3 fraction were added to each reaction mixture. Maximum incorporation in pmol of N-acetylphenylalanine was 19.4 with poly(U) and 12.1 with poly(C,U), and of N-formylmethionine, 5.8 with poly(A,G,U) and 14.3 with poly-(G,U).

**Fig. 7.** Differential activity of phosphocellulose fractions of IF-3 in N-acetylphenylalanine incorporation and in amino-acid incorporation directed by MS2 RNA. The basic reaction mixture for the amino-acid incorporation directed by MS2 RNA was essentially as described in Methods and in the legend of Fig. 3 for N-formylmethionine incorporation, except for the omission of f[14C]Met-tRNA and the presence of 0.2 mM [14C]phenylalanine of specific activity 513 μCi/μmol, 10 μl of N₅-formyltetrahydrofolate (Calcium Leucovorin Injection), 32 μg of MS2 viral RNA, and 20 μl of the indicated phosphocellulose fraction of IF-3. Incubation was for 10 min at 37°C. The assay procedure for N-acetylphenylalanine incorporation with 10 μg of poly(U) and poly(A,C,U) was as described in the legend of Fig. 6. Maximum incorporation, in picomoles, of N-acetylphenylalanine was 5.5 with poly(A,C,U) and 5.8 with poly(U), and of phenylalanine, 2.2 with MS2 RNA.
ing from a study of a simple model system, we feel that the properties exhibited by IF-3 in this system reflect intrinsic properties of the factor for the following reasons: (1) the model system resembles the natural system in its requirement for Mg\(^{2+}\) (21, 23) and for all three initiation factors; (2) the model system, under identical conditions, is capable of synthesizing yeast with fMet-tRNA in the presence of synthetic RNA containing the codon for N-formylmethionine or of MS2 viral RNA; and (3) the initiation of polypeptide synthesis with N-AcPhe-tRNA can be demonstrated in freshly prepared and unfractionated extracts of E. coli (21).

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