INITIATION FACTOR 3 REQUIREMENT FOR THE FORMATION OF INITIATION COMPLEXES WITH SYNTHETIC OLIGONUCLEOTIDES

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SUMMARY

Initiation factor IF-3 is required for the poly (U)-directed binding of N-acetyl-Phe-tRNA to 70S ribosomes as well as for the binding of fMet-tRNA directed by poly (U,G), AUG, and bacteriophage f_2 RNA. The formation of the 70S initiation complex is dependent upon IF-2 and is stimulated by IF-1. The requirement for IF-3 is not alleviated by high concentrations of the synthetic templates.

Early investigations on the factors required for the initiation of polypeptide synthesis in prokaryotic systems indicated that two initiation factors, IF-1 and IF-2, were required for the formation of initiation complexes with synthetic oligonucleotides, and that a third initiation factor, IF-3, was essential for chain initiation with natural mRNAs (1). These data suggested that IF-3 was needed for the recognition of specific nucleotide sequences in natural mRNA. Subsequent investigations have shown that IF-3 stimulates the binding of fMet-tRNA to 70S ribosomes at low concentrations of AUG (2, 3), and that IF-3 is essential for the poly (U,G)-directed binding of fMet-tRNA (2). In previous investigations in this laboratory crude DEAE preparations of IF-1 and IF-2 were used to catalyze the poly (U)-directed binding of N-acetyl-Phe-tRNA (AcPhe-tRNA) to Escherichia coli ribosomes (4); however, on further investigation we found that the fraction designated "IF-1" contained not only IF-1 but also IF-3.

In this investigation we have separated IF-1 and IF-3 by a modification of previously described procedures (5, 6, 7, 8) and find that IF-3 is essential not only for the binding of fMet-tRNA to 70S ribosomes directed by AUG,

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poly (U,G) and bacteriophage f_2 RNA, but also for the poly (U)-directed binding of AcPhe-tRNA. In each case the formation of the 70S initiation complex is dependent upon the presence of IF-2 and is stimulated by IF-1. These data indicate that IF-3 is nonspecific with regard to the recognition of nucleotide sequences, thus raising the question of the function of IF-3 in the initiation process.

EXPERIMENTAL

Materials: Ac-[^{14}C]Phe-tRNA was prepared as previously described (4). To prepare f-[^{3}H]Met-tRNA the reaction mixture contained in a total volume of 10 ml: Tris-HCl, pH 7.5, 0.1 M; $MgCl_2$, 10 mM; potassium ATP, 5 mM; 2-mercaptoethanol, 20 mM; E. coli B tRNA, 10 mg; 0.5 ml of a freshly dialyzed sonic extract of E. coli W containing approximately 5 mg of protein; folinic acid, 0.6 mM; and [^{3}H]Met, 10 μM, specific activity 500 C_i/mole. After 20 min of incubation at 37°, the charged tRNA was isolated as previously reported (4). Poly (U) and poly (U,G) (U:G=1), and AUG were purchased from Miles Laboratories. Bacteriophage f_2 RNA was isolated by a modification of the procedure of Webster et al. (9). Salt-washed ribosomes from E. coli W were prepared as previously described (7). E. coli Q 13 cells (mid-log) were purchased from General Biochemicals.

Assays: fMet-tRNA Binding. The standard reaction mixture contained in a total volume of 0.15 ml: Tris-HCl, pH 7.7, 50 mM; dithiothreitol (DTT), 5 mM; NH_4Cl, 0.1 M; $MgCl_2$, 7.5 mM; salt-washed ribosomes, 2.8 A_{260} units; tRNA, 0.08 A_{260} units, charged with 40 pmoles of [^{3}H]Met of which approximately 50% were formylated; poly (U,G), 0.1 A_{260} units; and GTP, 2 mM. The reaction mixture was supplemented with phosphocellulose preparations of IF-1, 0.5 μg protein; IF-2, 0.2 μg protein and IF-3, 0.5 μg protein as indicated. After 10 minutes of incubation at 37° the reaction mixture was diluted with cold Tris buffer, pH 7.7, containing 0.1 M NH_4Cl and 7.5 mM $MgCl_2$, and the ribosomes were collected on a Millipore filter (0.45 μ pore size). The filter

was washed with cold buffer, and the amount of radioactivity retained on the filter was measured as previously described (4).

AcPhe-tRNA Binding. The reaction mixture described above was increased to 0.2 ml and modified to contain: poly (U), 10 μg; $MgCl_2$, 5 mM; GTP, 0.05 mM; and 2.0 A_{260} units of tRNA charged with 60 pmoles of Ac[^{14}C]Phe. Incubation was for 15 min at 25°.

Separation of IF-1, IF-2, and IF-3: Ribosomes obtained from 80 g of E. coli Q 13 cells by the procedure described by Remold-O'Donnell and Thach (7) were washed with 1 M NH_4Cl instead of 0.5 M NH_4Cl. The 1 M NH_4Cl was adjusted to 80% of saturation by the addition of solid $(NH_4)_2SO_4$ (51.6 g/100 ml), and the precipitate was collected by centrifugation. The precipitated protein was dissolved in approximately 15 ml of 10 mM potassium phosphate buffer, pH 7.5, containing 0.5 mM EDTA, 10 mM 2-mercaptoethanol and 10% glycerol (Buffer C), dialyzed overnight against 1 liter of the Buffer C, and clarified by centrifugation.

Chromatography on DEAE-cellulose. A modification of the procedures of Iwasaki et al. (5) and Dubnoff and Maitra (6) was used. A portion of the dialyzed ammonium sulfate fraction (12 ml containing 167 mg of protein) was applied to a 1.5 x 40 cm column of DEAE-cellulose (Whatman-52) equilibrated in Buffer C. The column was washed with 130 ml of Buffer C, followed by 170 ml of Buffer C containing 0.025 M NH_4Cl. A 700 ml linear gradient from 0.025 M NH_4Cl to 0.35 M NH_4Cl in Buffer C was then applied to the column. The flow rate was approximately 50 ml per hour, and 10 ml fractions were collected. A typical elution profile is shown in Fig. 1.

Concentration and Purification on Phosphocellulose. A modification of the procedures of Hershey et al. (8) and Remold-O'Donnell and Thach (7) was used. DEAE fractions 8-10, containing IF-1, were pooled and applied to a 3 cc column of phosphocellulose (Whatman P11) equilibrated in 50 mM Tris-HCl, pH 7.5; 0.5 mM DTT; 1 mM EDTA; and 10% glycerol (Buffer D), and 0.02 M NH_4Cl. The column was washed with 10 ml of Buffer D containing 0.02 M

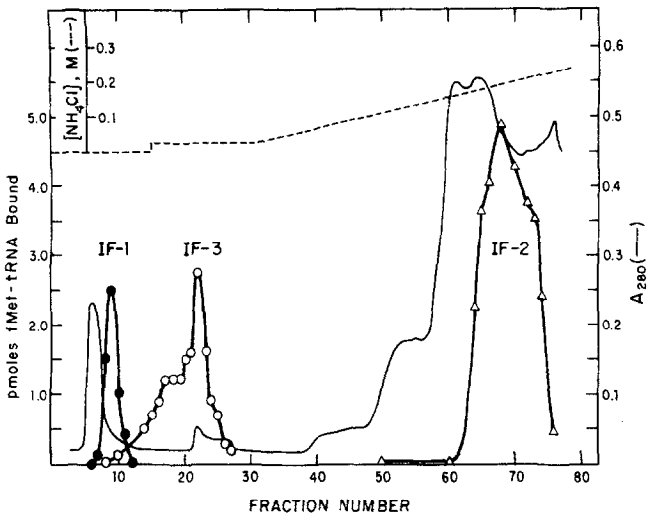

Figure 1. Separation of initiation factors on DEAE-cellulose. The dialyzed ammonium sulfate precipitate of the 1 M NH_4Cl wash of ribosomes was chromatographed on DEAE-cellulose as described in Experimental. 20 μl Aliquots of the fractions were assayed for activity in the poly (U,G)-fMet-tRNA binding assay supplemented with PC preparations of either IF-2 and IF-3, IF-1 and IF-2, or IF-1 and IF-3.

NH_4Cl, followed by 10 ml of Buffer D containing 0.15 M NH_4Cl and 10 ml of Buffer D containing 0.35 M NH_4Cl. The IF-1 was recovered in 2-5 ml of the 0.35 M NH_4Cl buffer. DEAE fractions 17-23, containing IF-3, were pooled and applied to a 3 cc column of phosphocellulose equilibrated and washed as described above. The IF-3 was eluted from the column with 0.6 M NH_4Cl in Buffer D and was recovered in 2-3 ml. DEAE fractions 65-73, containing IF-2, were pooled, dialyzed overnight against Buffer D containing 0.1 M NH_4Cl, and applied to a 10 cc column of phosphocellulose equilibrated in Buffer D containing 0.1 M NH_4Cl. A 120 ml linear gradient between 0.1 M NH_4Cl and 0.4 M NH_4Cl in Buffer D was applied to the column. IF-2 eluted from the column at approximately 0.2 M NH_4Cl and was recovered in 4-5 ml. The phosphocellulose (PC) preparations were stored at -70°.

RESULTS AND DISCUSSION

As shown in Fig. 1, when the dialyzed ammonium sulfate precipitate of the 1 M NH_4Cl wash of ribosomes is chromatographed on DEAE-cellulose and the fractions are assayed for the ability to stimulate the poly (U,G)-directed

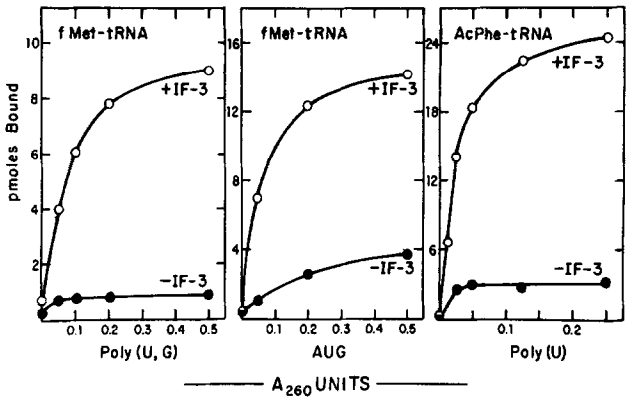

Figure 2. IF-3 requirement at different concentrations of template. The standard reaction mixtures were modified as described in Table 1 and supplemented with PC-IF-1, 0.5 μg; PC-IF-2, 0.2 μg and with poly (U,G), AUG, and poly (U) as indicated.

binding of fMet-tRNA to 70S ribosomes in the presence of two of the initiation factors, three distinct peaks of activity are obtained. IF-1 is recovered in the initial column wash; IF-3 elutes from the column in 0.025 M NH_4Cl; and IF-2 elutes at approximately 0.2 M NH_4Cl. If the ammonium sulfate precipitate of the 1 M NH_4Cl wash of the ribosomes is not dialyzed sufficiently, or if twice the amount of dialyzed ammonium sulfate precipitate is applied to the same size column, appreciable amounts of IF-3 are recovered in the initial column wash with IF-1. In a procedure previously used for the separation of the initiation factors (10), the sample was applied to a DEAE-cellulose column equilibrated in 0.05 M NH_4Cl. Under these conditions essentially all of the IF-3 is recovered in the column wash with IF-1.

When IF-1 and IF-3 were separated as described in Fig. 1, we found that those fractions which had IF-3 activity in the poly (U,G)-fMet-tRNA binding assay also stimulated the poly (U)-directed binding of AcPhe-tRNA to 70S ribosomes in the presence of IF-1 and IF-2. In the experiment described in Table 1, the DEAE-cellulose fractions containing IF-1, IF-3, and IF-2, respectively, were concentrated and purified by adsorption and elution from phosphocellulose and then assayed for their ability to stimulate the binding reaction in the presence of several different templates. Very little enhance-

TABLE 1. Factors Required for the Binding of fMet-tRNA and AcPhe-tRNA to 70S Ribosomes

Additions	fMet-tRNA Bound			AcPhe-tRNA Bound
	poly (U,G)	Aug	f_2 RNA	poly (U)
			(pmoles)	
None	0.7	2.4	0.3	3.0
IF-1	0.7	2.5	0.3	2.6
IF-2	0.7	2.9	0.3	3.1
IF-3	1.7	2.3	1.4	4.1
IF-1 + IF-3	2.3	2.4	2.1	4.4
IF-1 + IF-2	0.9	2.9	0.3	3.1
IF-2 + IF-3	4.1	5.6	2.6	9.5
IF-1 + IF-2 + IF-3	6.5	11.0	5.0	13.7

For AUG-directed binding, the reaction mixture described in Experimental was modified to contain 0.2 A_{260} units of AUG, 5 mM $MgCl_2$, and 0.1 mM GTP. Incubation was for 15 min at 25°. For binding directed by f_2 RNA, the reaction mixture contained 3.0 A_{260} units of f_2 RNA, 5 mM $MgCl_2$ and 0.3 mM GTP. Incubation was for 10 min at 37°. The reaction mixtures were supplemented, as indicated with PC preparations of IF-1 (0.5 μg protein), IF-2 (0.2 μg protein) and IF-3 (0.5 μg protein).

ment of binding is observed upon the addition of IF-1, IF-2 or IF-1 plus IF-2. However, with each of the templates tested, poly (U,G), AUG, poly (U), and f_2 RNA, a marked increase in binding occurs when IF-3 is added in the presence of IF-2 or IF-2 plus IF-1. Although appreciable binding is obtained with IF-3 plus IF-2, IF-1 appears to be required for maximal binding. Increasing the concentration of one of the initiation factors does not obliterate the effects of the other two initiation factors. Similar results were also obtained with poly (A,U,G).

In a previous study it was reported that IF-3 was required only at low concentrations of AUG (3). The data given in Fig. 2 show that the requirement for IF-3 is not alleviated by high concentrations of the synthetic templates. The degree of stimulation obtained with IF-3 is approximately the same at high concentrations of poly (U,G), AUG, and poly(U) as at low concentrations.

The results of this investigation demonstrate that IF-3, IF-2, and IF-1 are required for the maximal poly (U)-directed binding of AcPhe-tRNA to 70S ribosomes, as well as for maximal binding of fMet-tRNA directed by poly (U,G),

AUG, and f_2 RNA. The requirement for IF-3 does not appear to be dependent upon the concentration of synthetic template, nor does it appear to be dependent upon the recognition of specific nucleotide sequences. Further investigation is in progress to ascertain the role of IF-3 in the initiation process directed by synthetic templates.

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