PROTEIN FACTOR REQUIREMENT FOR BINDING OF MESSENGER RNA TO RIBOSOMES

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The attachment of messenger RNA to ribosomes must be an early step in the translation of the genetic message. Our inability to detect physical association of various synthetic and viral messenger RNA's to ribosomes which had been washed in 1M NH_4Cl led us to investigate the minimum requirements for this process. As a consequence we have been able to identify and purify a protein factor from the 1M NH_4Cl wash of E.coli ribosomes which is required for the attachment of synthetic messenger RNA to ribosomes. This factor, which we call Factor 3, also appears to be required for the binding of R17 viral RNA to E.coli ribosomes. Factor 3 has at least some of the same physical and biological properties as Factor C of Revel and Gros (1967): however, it does not have the same functional activity as either F1 or F2 of Stanley et al. (1966).

Materials and Methods

Tests for the binding of the oligonucleotide $ApUp^{H^3}G(pA)_{\overline{40}}$ and R17 RNA to ribosomes were done using the same protocol described below for the assay of Factor 3 except that the Mg^{++} concentration was 0.01M. Complete reaction mixtures also included 25μg of Factor 3, 1.4 A_{260} units of ribosomes and either 35μμmoles of $ApUp^{H^3}G(pA)_{\overline{40}}$ or 7μμmoles of C^{14}-labelled R17 RNA chains (the gift of Miss Sigrid Stumpp). Incubation time was 20 minutes at 37°. The substrate $ApUp^{H^3}G(pA)_{\overline{40}}$ (1.6 c/mmole of oligonucleo-

tide chains) was prepared according to the method of Sundararajan and Thach (1966).

DNA-dependent RNA synthesis was done using the procedure of Revel and Gros (1967) except that calf thymus DNA and RNA polymerase from M. lysodeikticus (the gift of Dr. Robert Novak) were used. Factor 3 and ribosomes were added as indicated. Radioactive label was supplied as H^3-GTP (38mc/mmole).

Polypeptide synthesis assays were done according to Thach et al. (1967) except that ApUpG$(pU)_{\overline{30}}$ was used as messenger and separated F1 and F2 were used as well as crude initiation factors. F1 and F2 activities were separated by the procedure of Salas, Hille and Wahba (1967) although the hydroxylapatite step was omitted. All protein concentrations were determined by the Biuret method. (Zamenhof, 1957).

Results and Discussion

Our goal in studying Factor 3 was to separate it from other cellular components using a simple assay to detect its presence. The ability of purified Factor 3 to affect the more complex processes of transcription and translation was then investigated.

1. Purification of Factor 3

During purification Factor 3 activity could be assayed conveniently by taking advantage of the fact that the oligonucleotide ApUp^{H^3}G$(pA)_{\overline{40}}$ is retained on Millipore filters only in the presence of extracts containing Factor 3 activity. Hence Factor 3 must be capable of binding to both oligonucleotide and filter. Standard assays were carried out in a total volume of 0.1 ml containing 0.1M KCl, 0.03M Tris-acetate pH 7.8, 10^{-4}M magnesium acetate, 6 x 10^{-3}M β-mercaptoethanol, 0.1μmoles of a mixture of GTP, GDP and GMP, 35μμmoles ApUp^{H^3}G$(pA)_{\overline{40}}$ and 5-100μg of protein fraction to be assayed. Reaction mixtures were incubated for ten minutes at room temperature, diluted with buffer and filtered on Millipore filters. Figure 1 shows that the amount of substrate bound to filters is proportional to the amount of a crude Factor 3 added over a wide range of protein concentration.

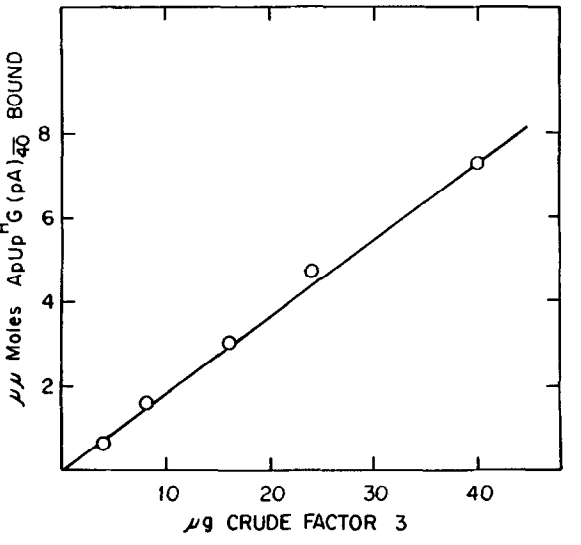

Figure 1: Factor 3 dependent binding of $ApUp^{H^3}G(pA)_{40}$ to Millipore filters. Crude Factor 3 used in this experiment was from fractions 39-44 of the TEAE Cellulose column shown below.

The source of Factor 3 for these experiments was the 1M NH_4Cl wash of E.coli K12 ribosomes. All operations were carried out at 0°-4°C. Crude ribosomal wash was prepared according to Hershey and Thach (1967). Material precipitating between 40% and 60% saturated $(NH_4)_2SO_4$ was re-dissolved and dialyzed against Buffer 1 (0.05M NH_4Cl, 0.01M Tris-HCl pH 7.4, 6mM β-mercaptoethanol).

Further purification of Factor 3 was obtained by column chromatography on TEAE Cellulose. A 1cm x 50cm column of TEAE Cellulose (Bio-Rad Laboratories) was equilibrated with Buffer 1. 115 mg of the protein precipitated between 40% and 60% saturated $(NH_4)_2SO_4$ was applied to the column in a volume of 23 ml Buffer 1. The column was eluted with a 1000 ml linear gradient of between 0.1M NH_4Cl and 0.4M NH_4Cl in Buffer 1. Figure 2 shows the OD_{280} and Factor 3 activity profiles obtained. Material from fractions 39-44 was used for further purification of Factor 3.

Factor 3 used in the experiments reported here was purified

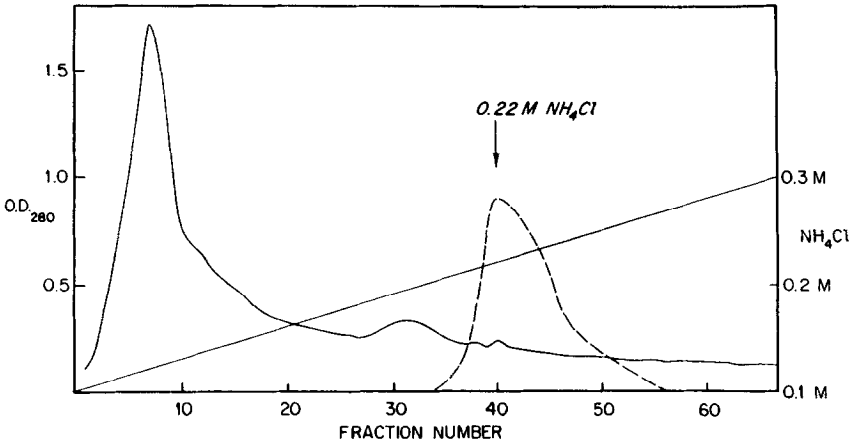

Figure 2: TEAE Cellulose chromatography of 40% to 60% ammonium sulfate fraction of crude ribosomal wash. OD_{280} (————) and Factor 3 activity (--------) profiles are shown.

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further by $ZnCl_2$ precipitation. Tubes containing Factor 3 activity after TEAE Cellulose chromatography were pooled, concentrated by $(NH_4)_2SO_4$ precipitation and dialyzed against 0.2M NH_4Cl, 0.01M Tris-HCl pH 7.1, 6mM β-mercaptoethanol. The protein concentration was adjusted to 0.3mg/ml and the solution was warmed to room temperature. 0.1M $ZnCl_2$ was added dropwise to a final concentration of 2mM. The solution was stirred for 30 minutes at room temperature and the precipitated material removed by centrifugation and discarded. The supernatant was then made 8mM in $ZnCl_2$ and the pH raised to pH 7.5 by addition of Tris base. After stirring for 30 minutes the precipitate containing most of the Factor 3 activity was collected by centrifugation, re-dissolved and dialyzed against Buffer containing 5×10^{-5}M EDTA. The ultraviolet spectrum of this material showed $A_{280}/A_{260}=1.61$ suggesting that it contains very little nucleic acid. It migrated as a single component during electrophoresis on 7% polyacrylamide gels at pH 8.6.

2. Biological Activity of Factor 3

The first clue to the fact that a third component may be required for the binding of messenger RNA to ribosomes came

from the observation that ribosomes tended to lose their ability to interact with natural and synthetic mRNA after washing with 1M NH_4Cl. The extent of the binding of the synthetic substrate $ApUp^{H^3}G(pA)_{\overline{40}}$ to well washed ribosomes was determined by the technique already described for the routine assay of Factor 3 activity except that ribosomes were included in the incubation. The Mg^{++} concentration was 0.01M and incubation was for 20 minutes at 37°. Oligonucleotide bound either to Factor 3, to ribosomes or to both was expected to be retained by the Millipore filter. The experiment described in Table I was designed to test whether the substrates $ApUp^{H^3}G(pA)_{\overline{40}}$ and R17 viral RNA would be bound by excess ribosomes in the presence of Factor 3. The data suggest that both polynucleotides are bound more effectively by ribosomes in the presence than in the absence of Factor 3. Sucrose gradient analysis of complete reaction mixtures confirmed the fact that the substrate was bound to ribosomes (presumably in the form of a ternary complex involving messenger, ribosome and Factor 3) and not to Factor 3 alone. In the absence of Factor 3 R17 RNA bound more effectively to ribosomes than did $ApUp^{H^3}G(pA)_{\overline{40}}$.

TABLE I

Incubation	μμ Moles $ApUp^{H^3}G(pA)_{\overline{40}}$ Bound	μμ Moles R17 RNA Bound
Complete	1.64	2.28
Omit Ribosomes	0.49	0.42
Omit Factor 3	0.09	0.93
Omit Ribosomes and Factor 3	0.08	0.21

Careful studies of the mechanism of DNA-dependent RNA synthesis by RNA polymerase have demonstrated that the product RNA remains physically bound to the template DNA (Bremer and Konrad,

1964). The addition of ribosomes to these reactions depresses apparent product inhibition and increases the production of RNA. Sucrose gradient analysis of the product of such reactions has been used to support the conclusion that some of the newly synthesized RNA has been bound to ribosomes and released from the DNA template, Shin and Moldave (1966). Recently Revel et al. (1968) showed that a protein fraction isolated from the ribosomal wash is necessary for the attachment of nascent T4 mRNA to E. coli ribosomes. Without this factor ribosomes did not affect the rate or amount of RNA synthesized by RNA polymerase using T4 DNA as template. In order to test whether Factor 3 would show this same activity transcription reactions were carried out in the presence and absence of ribosomes with varying amounts of Factor 3. It was expected that in the absence of ribosomes Factor 3 would have little effect on the amount of RNA synthesized whereas in the presence of ribosomes the amount of RNA synthesized should depend on the amount of Factor 3 present over a suitable range. The results shown in Figure 3 support this conclusion: Factor 3 stimulates transcription only in the presence of ribosomes. Con-

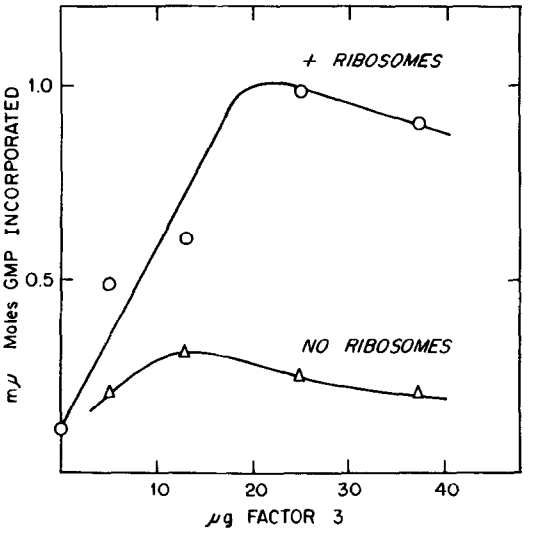

Figure 3: Effect of Factor 3 on DNA-dependent RNA synthesis. 0.05 ml reactions contained 10μg Calf thymus DNA and 5μg RNA polymerase in the presence (—o—o—) or absence (—Δ—Δ—) of 1.4 A_{260} units of ribosomes. Incubation was for 3 hours at 37°.

versely, ribosomes alone show little effect on the amount of RNA synthesized. We conclude that at least part of the activity called Factor C by Revel et al. (1968) is the same as our Factor 3.

Two protein factors found in the 1M NH_4Cl ribosomal wash have been shown by Stanley et al. (1966) to be required in addition to supernatant enzymes for the in vitro translation of viral RNA and synthetic messenger RNA's containing N-formyl-methionine codons. Although the primary function of these factors appears to be the binding of the initiator N-formyl-methionine-tRNA to ribosomes, it seemed possible that our Factor 3 might be identical to one of these activities. Several lines of evidence suggest that they are not. F1 of Stanley et al. (1966) is eluted from columns of DEAE Cellulose at low salt concentration (less than 0.1M NH_4Cl) where Factor 3 remains strongly bound to the column. When F1 is prepared according to Salas et al. (1967), it shows no Factor 3 activity when assayed by the Millipore binding method described above.

In order to test whether our Factor 3 was the same as F2, polypeptide synthesis experiments were carried out using a cell free system which required initiation factors for the incorporation of N-formyl-methionine into the polypeptide product. It was expected that if Factor 3 was the same as F2, then it should be able to substitute for F2 in the translation of $ApUpG(pU)_{\overline{30}}$. Polypeptide synthesis was done according to Thach et al. (1967) using washed ribosomes, salts, ATP generating system, fully formylated N-formyl-H^3Methionine-tRNA and S-100 as a source of supernatant enzymes. The synthetic messenger was $ApUpG(pU)_{\overline{30}}$. Results shown in Table II suggest that the level of activity observed for crude, unseparated initiation factors was approached only when both F1 and F2 were present. Factor 3 could not substitute for F2 in the presence of F1, nor could Factor 3 replace F1 when incubated in the presence of F2. The data suggest that both F1 and F2 contain activities which are required for the translation of $ApUpG(pU)_{\overline{30}}$ and are not possessed by Factor 3. However, the question of whether Factor 3 is also required for translation of $ApUpG(pU)_{\overline{30}}$

TABLE II

Crude Initiation Factor	F1	F2	Factor 3	μμ Moles H^3-F-Met Incorporated
-	-	-	-	0.18
+	-	-	-	1.19
-	+	-	-	0.18
-	+	-	+	0.16
-	+	+	-	1.01
-	+	+	+	1.03
-	-	+	-	0.64
-	-	+	+	0.58

The following amounts of factors were added: 37μg Crude Initiation Factor, 25μg F1, 8μg F2 and 7μg Factor 3.

could not be answered by these experiments since our S-100 supernatant fraction contained Factor 3 activity. We hope that the use of purified Factor 3 and ribosomes will settle this point and be of help in defining the physical and chemical requirements for a ribosomal attachment site in messenger RNA.

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