A FACTOR FROM E. coli REQUIRED FOR THE TRANSLATION OF NATURAL MESSENGER RNA

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Both natural and synthetic polynucleotides can serve as templates for polypeptide synthesis in bacterial cell free systems (Nirenberg and Matthaei, 1961). With unfractionated cell extracts it is found that natural messenger RNAs stimulate amino acid incorporation as efficiently as do artificial polynucleotides and even can direct the synthesis of a specific protein as shown with F2 RNA (Nathans et al, 1962). RNA enzymatically synthesized in the presence of DNA can also mediate polypeptide synthesis as demonstrated by Wood and Berg (1962) with systems in which transcription is coupled to translation. In a search for specifying the nature of this coupling in T4 DNA dependent protein synthesis we have found that fractionation of the cell free system yields preparations which are able to support endogenous or poly U directed polypeptide synthesis but remain practically inactive in the translation of T4 messenger RNA. This has led us to the identification of a cellular protein required specifically for the reading of exogenous natural messenger RNA. Similar results have been also very recently reported by Brawerman and Eisenstadt (1966) and by Stanley et al (1966).

MATERIAL AND METHODS

C^{14}-valine (1.2×10^7 cpm/µm) and C^{14}-phenylalanine (2.1×10^7 cpm/µm) were purchased from the CEA. T4 DNA was prepared from purified phages by the method of Berns and Thomas (1965). RNA from T4 infected E. coli B was isolated 6 minutes following infection according to Monier et al (1962). TYMV was a gift from Dr L. Hirth. Poly U was from Takamine. Purified RNA polymerase was a gift from Dr J. Richardson. DNase was from Worthington.

Charged s-RNA was prepared according to Nathans et al (1961) using a C^{14} Chlorella hydrolysate (5×10^6 cpm/µm).

E. coli strains B or CR 34 were grown to the end of the log phase. Cells were opened by sonication with a Mullard desintegrator in 3 volumes of cold buffer containing Tris-HCl pH 7.6,0.01M, NH_4Cl 0.06M, $MgCl_2$ 0.01M and mercaptoethanol 0.006M. "S-30" (Nirenberg and Matthaei, 1961) was then prepared without the use of DNase and "110,000 xg ribosomes"(R-110) separated by centrifugation at 45,000 rpm in Spinco rotor 50. To prepare the "80,000 xg ribosomes"(R-80),S-30 was centrifuged 2 hours at 35,000 rpm in Spinco rotor 40 and the particles washed once in the above buffer. The upper part of the supernatant (S-80) was then centrifuged at 37,500 rpm in SW39 rotor to obtain the active 110,000 xg fraction (crude factor) and the S-110 which were dialysed overnight against the same buffer.

DNA dependent amino acid incorporation was measured in 0.125 ml incubation mixture containing Tris pH 7.6 5µm, $MgCl_2$ 1.9 µm, NH_4Ac 24 µm, mercaptoethanol 0.75 µm, ATP 0.25 µm, GTP CTP UTP 0.05 µm,C^{14}valine 0.01 µm, and cold 19 aminoacids 0.025 µm, pyruvate kinase 5 µg, T4 DNA 9 µg, S-110 0.5 OD_{280}units, purified RNA polymerase 5 µg; ribosomes and Factor as indicated. Incubation was for 30 minutes at 34°C. The reaction was stopped with TCA 5%, the samples heated at 90°C for 15 minutes, filtered through Millipores and counted in a Tracerlab gas flow counter. For RNA directed incorporation RNA polymerase was omitted and 100 µg of RNA from T4 infected cells or 60 µg TYMV-RNA were used. For poly U translation 0.005 µm C^{14} phenylalanine, 40 µg poly U and 2.5 µm $MgCl_2$ were added.

In binding studies the incubation mixture was doubled and P^{32}-T4 DNA (25 x10^6cpm/mg) and C^{12} aminoacids were used. After incubation for 15 min the mixture was diluted with an equal volume of Tris pH 7.6 0.01M, NH_4Cl 0.06M and $MgCl_2$ 0.02M and then layered on a 5-20% sucrose gradient in the same buffer. Centrifugation in the SW25 rotor was for 90 minutes at 23,000 rpm and fractions were collected,precipitated with TCA and counted.

RESULTS: 1. Isolation of the translation factor

Addition of T4 DNA to an E. coli (non DNase treated)"S-30" results in a 6 fold stimulation of valine incorporation into protein (Table 1). Fractionation of the S-30 by centrifugation at 110,000 xg yields a ribosomal preparation (R-110) which when supplemented with the corresponding soluble fraction has all the activity present in the original extract. This preparation is however heavily contaminated by cell DNA and DNA-bound RNA polymerase which were found to sediment at about 25 S. To reduce this contamination by DNA, ribosomes were prepared from sonicated cell extracts by centrifugation at 80,000 xg. Table 1 shows that these particules (R-80) which constitute up to 75 p. cent of the total ribosomes in the extract are very

active for poly U dependent polyphenylalanine synthesis but cannot support the T4 DNA directed valine incorporation even when supplemented with purified RNA polymerase. All the activity for translating the T4 DNA product or RNA purified from T4 infected cells is present in the fraction of the 80,000 xg supernatant (S-80) sedimenting at 110,000 xg. This fraction is rich in DNA and contains about 25 p. cent of the total ribosomes.

The factor whose presence is required for T4 mRNA translation could be isolated free of ribosomes by centrifugation of the upper 1/3 of the S-80 at 110,000 xg for 2 hours. Centrifugation for longer periods of time did not yield more activity. Table 2 shows that this fraction stimulates 40 fold T4 DNA directed valine incorporation by inactive ribosomes (R-80) while poly U dependent polyphenylalanine synthesis is stimulated only by a factor of 2. Larger amounts of the factor tend to inhibit valine incorporation. All the activity was lost by heating at 80°C for 10 minutes. This preparation was particularly rich in DNA and RNA polymerase.

Purification of the factor from DNA and ribosomes can be achieved (Table 2) by chromatography on a DEAE cellulose column from which the factor is eluted between 0.2 and 0.3 M NH_4Cl. Ribosomes still remaining in this fraction can be removed by precipitation with protamine sulfate. The behaviour of the factor during purification supports the conclusion that it is a protein.

The presence of DNA in the active cell fractions suggested that the factor sedimented at 110,000 xg because it is attached to the bacterial DNA fragments. To test this hypothesis we have treated crude extracts with DNase (10µg/ml at 4°C) prior to centrifugation at 110,000 xg. Table 1 shows clearly that the ribosomal fraction (R-110) obtained from DNase treated extracts is much less active in the T4 mRNA dependent system than the ribosomes isolated from non-DNase treated extracts. Incorporation of phenylalanine in response to poly U is comparable with both preparations. Thus after digestion of DNA the factor is no more sedimentable.

2. Function of the factor

The factor increases the rate as well as the extent of T4 DNA dependent protein synthesis which is linear for 60 minutes at 34°C.(Fig.1a). The transfer of aminoacids from charged s-RNA to protein is strongly stimulated by the presence of the factor (Fig.1b). In its absence there appears to be a lag before the onset of the transfer. Little transfer is observed when no soluble fraction is present in addition to the factor. A comparison of various mRNAs (Table 3) indicates that the factor stimulates the translation of TYMV-RNA as well as that of RNA from T4 infected bacteria. In contrast endogenous and poly U dependent polypeptide synthesis are only slightly increased

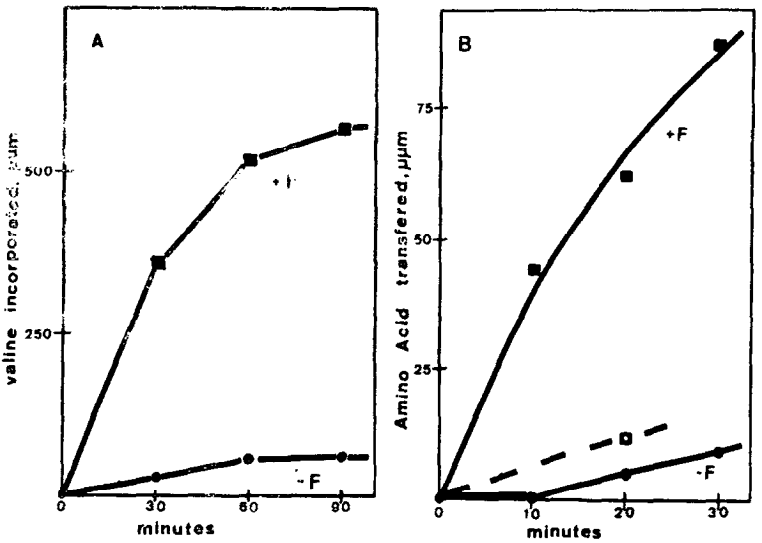

Figure 1. A : Kinetics of T4 DNA dependent aminoacid incorporation into protein with and without factor. B : T4 DNA transfer of aminoacids from charged s-RNA to protein with and without factor. - Conditions as indicated in Methods. 300 µg ribosomes R-80 were used alone (circles) or supplemented with crude factor (squares). Open square represents transfer with the factor in the absence of S-80.

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as compared to natural mRNAs. These result indicate that the factor is not involved in the synthesis of aminoacyl s-RNA and is not one of the known transfer enzymes which are required for poly U translation (Lucas-Lenard and Lipmann, 1966).

Since poly U differs from natural mRNAs in its high affinity for ribosomes (Haselkorn and Fried, 1964) we investigated the possibility that the function of the factor is to promote ribosome binding to natural mRNA. The demonstration by Byrne et al (1964) that even in the absence of protein synthesis the formation of stable DNA-RNA-ribosomes complexes can be observed by sucrose gradient analysis provides a convenient method to detect ribosome attachment to nascent T4 mRNA. For the experiments presented in Fig.2, a P^{32} T4 DNA preparation was used which sedimented at about 30 S when it was free and between 70 and 200 S when aggregates of one to three ribosomes per DNA fragment were made. Fig.2a shows that ribosomes isolated at 110,000 xg are able to bind T4 DNA but that ribosomes prepared at 80,000 xg have lost that capacity. Furthermore addition of the factor to inactive ribosomes (R-80) allowed their attachment to the DNA-RNA complex (Fig.2b) even after the transcription had been interrupted by the addition of actinomycin D. These

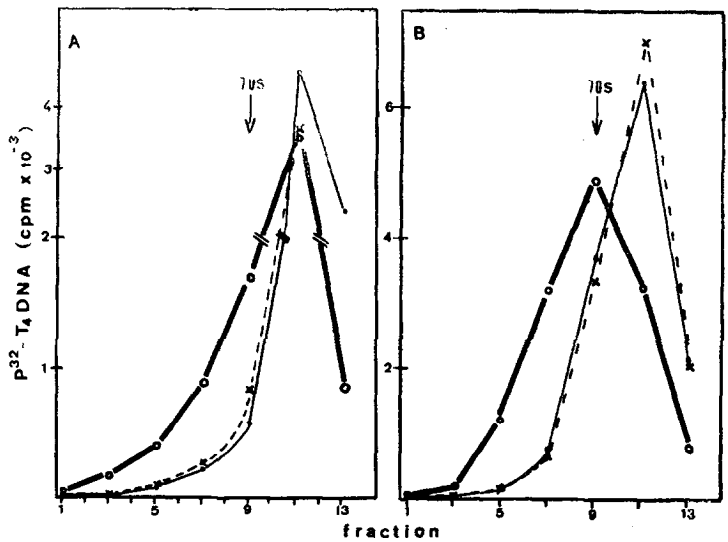

Figure 2. Effect of the factor on the binding of ribosomes to T4 DNA-RNA complexes. - P^{32} labeled T4 DNA was incubated and analysed on sucrose gradient as described in Methods. The arrow shows the position of the ribosome peak. A : •——• no ribosomes added; x - - x with 500 µg R-80 O——O with 260 µg R-110. B : •——• DNA,RNA polymerase no ribosomes added; x - - x with 400 µg ribosomes R-80; O——O with ribosomes R-80 plus crude factor. The factor was added 10 min after the beginning of incubation along with 20 µg Actinomycin D. Total incubation 20 minutes.

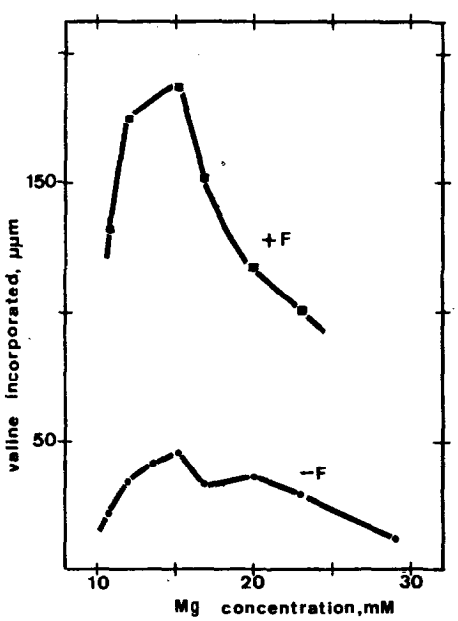

Figure 3. Effect of Mg concentration on T4 DNA dependent aminoacid incorporation in the presence and absence of the factor. - The factor was prepared by chromatography on DEAE cellulose as described in the text. squares : with factor; circles : no factor.

results indicate that the factor promotes the binding of T4 mRNA to ribosomes by a mechanism which is not involved in poly U translation.

The concentration of Mg ions optimal for poly U translation is 20 mM and is much higher than that required with natural mRNA (Willson and Gros,1964; Davies et al, 1964; Capecchi and Gussin, 1965). We have previously shown that this particularly high Mg concentration was actually required for the initiation of polyphenylalanine synthesis (Revel and Hiatt, 1965). The effect of Mg concentration on T4 DNA dependent protein synthesis is presented in Fig.3. When no factor is present we have repeatedly found two optimal Mg concentrations at 15 and 20 mM but in the presence of the factor a unique peak at 15 mM is observed. Thus in the translation of natural mRNA the initiation step in high Mg which is observed with poly U must be replaced by a mechanism involving the action of the factor.

TABLE 1

T4 DNA, T4 RNA and Poly U dependent amino acid incorporation with various preparations of ribosomes

Ribosomal preparation	Incorporation of Valine amino acid per 300 µg ribosomes µµmoles Endogenous	T4 DNA dependent	T4 RNA dependent	Phenylalanine ** POLY U dependent
1. 30,000xg supernatant	126	750	-	-
ribosomes isolated at 110,000xg *	84	610	-	-
2. ribosomes isolated at 80,000xg *	28	13	6	780
fraction from S-80 sedimenting at 110,000xg *	35	510	234	1250
3. ribosomes isolated at * 110,000xg without DNase	-	-	266	810
ribosomes isolated at 110,000xg with DNase *	-	-	65	690

* Supplemented with 110,000xg supernatant. Conditions as in Methods.
** Endogenous incorporation has been substracted from the template stimulated values. DNA dependent valine incorporation was completely inhibited by Act D while RNA dependent synthesis was not affected by Act D.

TABLE 2

Partial Purification of the Translation Factor

1. Factor isolated from the upper 1/3 of the S-80	T4 DNA dependent valine incorporation	Poly U dependent phenylalanine incorporation
	μμ moles per 300 μg ribosomes	
Complete with factor*	510	1450
2x factor	445	
1/2 factor	344	1140
no factor	12	680
with factor, no ribosomes	14	86

2. Factor purified on DEAE cellulose and by protamine sulfate		Optical density (260mμ) of the factor used
Complete no factor	13	-
Crude Factor ** :		
complete	175	6.3
omit RNA polymerase	146	
omit ribosomes	159	
DEAE fraction (0.25M NH_4Cl):		
complete	96	2.5
omit RNA polymerase	4	
omit ribosomes	19	
Protamine sulf. supernatant :		
complete	38	0.04

* amount of Factor corresponding to 150 μg of cells.
** amount of Factor corresponding to 15μg of cells.

TABLE 3

Comparison of the effect of the factor on various messenger RNAs

	Amino acid incorporation into Protein * amino acid per ribosome			
	Endogenous	stimulated by added RNA **		
		T4 RNA	TYMV RNA	POLY U ***
Without Factor	2.1	6.3	1.7	5.4
With the Factor	2.1	21.3	7.4	7.2
Difference in p.cent	0	+250	+300	+35%

* Conditions as described in Methods. The values obtained for valine with natural messenger RNAs have been multiplied by 20 assuming a uniform distribution of all 20 amino acids in the product formed.
** Endogenous incorporation has been deducted.
*** Phenylalanine was used.

DISCUSSION

The results presented here independently establish the existence of a factor required specifically for the translation of natural mRNA which has also been demonstrated very recently by Brawerman and Eisenstadt (1966) and by Stanley et al (1966). The latter authors have shown that by "stripping" the ribosomes in concentrated salt solutions two factors can be obtained which are both required for chain initiation i.e. the incorporation of formyl methionine by the formylatable met s-RNA in the presence of the initiation codon.

Our experiments are concerned with a factor which is present in cell extracts associated to DNA; most of the ribosomes are found to be naturally free of this factor. It is possible that the second factor of Stanley et al (1966) is present on these ribosomes but alone it is not sufficient to activate them for the translation of natural mRNA. The factor we have studied affects only slightly endogenous aminoacid incorporation (which represents mainly completion of nascent chains) and poly U directed polyphenylalanine synthesis in agreement with the proposed effect on chain initiation. It is not specific to a given messenger RNA but is active with T4 mRNA and with TYMV-RNA while Stanley et al (1966) have shown their factor to be active with MS2-RNA and TMV-RNA.

We have studied the stimulatory effect of the factor on T4 DNA dependent protein synthesis and our results suggest that the factor acts by promoting the attachment of ribosomes to the nascent T4 mRNA chains. It has indeed been reported that while ribosomes bind very efficiently to poly U giving rise to polysomes, their affinity for natural mRNA is much smaller (Barondes and Nirenberg, 1962; Haselkorn and Fried, 1963; Willson and Gros, 1964) presumably because natural mRNAs have specific initiation sites. The mechanism by which this protein factor enhances ribosomal attachment to mRNA is unclear. The formation of DNA-RNA-ribosomes complexes does not require protein synthesis as already shown by Byrne et al (1964). Wether it involves incorporation of formyl methionyl s-RNA is now under study. The slight but reproducible stimulation of poly U translation by the factor might indicate that some effect is observed even when the initiation codon is not present.

Initiation of poly U translation takes place at a Mg concentration so high as to inhibit subsequent chain elongation (Revel and Hiatt, 1965). This step corresponds to the attachment of two phenylalanyl s-RNA molecules on the ribosome. Such a high Mg concentration is not necessary with natural mRNA as F2 RNA, a fact which has been related to the existence of an initiation codon (Capecchi and Gussin, 1965; Sunderarajan and Thach, 1966). With T4 mRNA we

have observed that the factor eliminates any favoring effect of high Mg concentration suggesting that it participates in the mechanism which ensures the initiation of natural mRNA translation at the Mg concentration optimal for chain elongation.

Finally, the existence of a factor necessary for the formation of DNA-RNA-ribosomes complexes is of interest since it has recently been postulated that ribosomes are involved in the liberation of mRNA from DNA (Stent, 1965; Naono et al, 1966). Most of the ribosomes present in cell extracts seem to be incapable of binding to nascent mRNA unless they are activated by this factor. The observation that the factor is in limiting amounts in the extracts and is associated with the DNA fraction suggests that it might regulate the attachement of ribosomes to the newly produced mRNA chains and thereby participate in the control of mRNA production.

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