ISOLATION OF PROTEIN FACTORS FROM OVIDUCT POLYSOMES WHICH STIMULATE PROTEIN SYNTHESIS

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SUMMARY

A soluble protein fraction (AvF) was isolated from oviduct polysomes. AvF stimulates by 6-10 fold both rate and extent of protein synthesis on washed ribosomes directed by either natural mRNA or by polyuridylic acid. AvF is inactivated by heat, pronase and N-ethylmaleimide but not by RNase. AvF is distinct from chain elongation factors T_1 and T_2. The fraction stimulates protein synthesis subsequent to acylation of tRNA since equal stimulations are observed using ^{14}C-val or ^{14}C-leu-tRNA. Addition of AvF results in a distinct lowering of Mg^{+2} concentration for optimal protein synthesis using natural message or poly U. Finally analysis by affinity chromatography of peptides synthesized in the AvF-dependent natural message system suggests that ovalbumin comprises 14% of the released proteins.

INTRODUCTION

We have previously described the isolation and characterization of a polyribosomal system from the oviduct of the estrogen stimulated chick which is capable of cell-free protein synthesis (1). This system is capable of synthesizing and releasing polypeptides in a time-dependent manner. A portion of the released peptides are immunologically similar to the tissue-specific protein, ovalbumin (2). It was recognized that this synthesis may have involved only the addition of a few amino acids to previously existing polypeptide chains; that is, ovalbumin chains may not have been initiated *in vitro*. Furthermore, the polysomal system did not respond to addition of the exogenous messenger, polyuridylic acid. It has

been demonstrated that washing rabbit reticulocyte ribosomes with high salt yields a fraction containing protein factors which are required for the initiation of polypeptide synthesis in vitro (3-5). Therefore, we began to further define our protein synthesis system in order to prepare materials capable of both initiating protein synthesis and translating natural endogenous message with fidelity and also capable of translating exogenous mRNA.

METHODS

Seven day old female Rhode Island Red chicks were given daily subcutaneous injections of diethylstilbestrol (5 mg) for 10-12 days. Polyribosomes and a 35-70% ammonium sulfate fraction (AS_{70}) of a 105,000 g supernate were prepared as previously described (1). Isolated polyribosomes were then washed with a solution containing 0.25M sucrose, 0.1 mM Na_2EDTA, pH 7.0, 1.0 mM dithiothreitol, and 0.5M KCl as first reported by Miller and Schweet (3) for the rabbit reticulocyte. The suspension was separated into washed ribosomes and a supernatant fraction (AvF) by centrifugation at 105,000 g. Protein concentration of the AvF fraction was always 3 to 4 mg/ml (6). This fraction was stored in liquid nitrogen in 200 µl aliquots and showed no appreciable loss of activity over a 3 month period. Ribosomes were resuspended in the sucrose-EDTA-dithiothreitol solution to a final concentration of $120A_{260}$/ml. Again 200 µl aliquots were stored in liquid nitrogen. Sucrose gradient analysis of the washed ribosomes revealed that more than 95% of the material consisted of monomers and subunits. Protein content of the ribosome preparation was 7-10 mg/ml and RNA (7) was 7-8 mg/ml.

RESULTS AND DISCUSSION

The KCl-ribosomal-wash fraction which we have labeled AvF

for its avian origin was found to be active in stimulating both the rate and the extent of polymerization when compared to the system incubated with saturating amounts of the 30 to 70% ammonium sulfate fraction (AS_{70}) containing transfer factors and aminoacyl synthetases. Incorporation in the complete system is linear for 30 min and the cell-free ribosomal system exhibits linear dependence upon both the numbers of ribosomes and the amount of AvF protein. Moreover this protein-synthesizing system exhibits the usual requirements for maximal polymerization activity, that is, the AvF-dependent reaction is highly dependent upon ATP, an energy-

TABLE 1

DEPENDENCE OF PROTEIN SYNTHESIS ON VARIOUS COMPONENTS OF THE REACTION MIXTURE

RIBOSOMES (R)	RIBOSOMAL WASH FRACTION (AvF)	SUPERNATANT ENZYME FRACTION (AS_{70})	PMOLES ^{14}C-VAL INCORPORATED
R	AvF	AS_{70}	15.8
R	-	-	0.3
-	AvF	-	0.1
-	-	AS_{70}	0.1
-	AvF	AS_{70}	0.1
R	-	AS_{70}	2.4
R	AvF	-	9.5

Ribosomes (110 µg rRNA; 1.2 A_{260}) were incubated in the complete cell-free system for 20 min at 37° C. Concentrations of constituents in a final volume of 100 µl were as follows: TRIS-HCl (pH 7.2 at 23° C), 30 mM; ATP, 1.0 mM; GTP, 0.5 mM; phosphoenolpyruvate, 7.5 mM; pyruvic kinase, 1.5 E.U.; $MgCl_2$, 4.0 mM; dithiothreitol, 1.0 mM; ^{14}C-val, 1.0 µM (0.1 µCi; 572 dpm/p mole); (2.0 µM each of) 19 C^{12}-amino acids; avF fraction (70 µg protein); and AS_{70} enzyme fraction (180 µg protein). Samples were prepared for counting as previously described (12).

generating system and Mg^{+2}. Finally the protein nature of the AvF fraction has been demonstrated by its sensitivity to heat, pronase and N-ethyl-maleimide but not to ribonuclease (8).

Dependencies of the system are shown in Table 1. The complete system containing 110 µg ribosomal RNA, 70 µg AvF protein and 180 µg of AS_{70} protein incorporated 15.8 p moles of ^{14}C valine. Less than 0.3 p moles were incorporated with ribosomes alone and less than 0.1 p moles with the AvF fraction, the AS_{70} fraction or both of these together. The system with the ribosomes and the AS_{70} fraction incorporated 2.4 p moles or 15% of the activity of the complete system. Ribosomes and AvF fraction alone incorporated 9.5 p moles or 60% of the activity of the complete system. This would indicate that AvF fraction is not saturating for transfer factors and/or aminoacyl synthetases.

The Mg^{+2} optimum for the AvF dependent system was of critical importance since a lowering of the Mg^{+2} optimum has been associated with chain initiation and fidelity of translation in both bacterial and reticulocyte systems (3,4,9,10). In our system utilizing endogenous natural message and ^{14}C-valine incorporation, the Mg^{+2} optimum in the absence of the AvF fraction is 8 mM (Table 2). In the presence of the AvF fraction there is a lowering of the Mg^{+2} requirement to 4 mM. AvF stimulates the rate (and extent) of protein synthesis using ^{14}C-leucyl-tRNA as the radioactive substrate. Moreover the lower Mg^{+2} optimum is also found under these conditions (Table 2, Column 4). This indicates that the acylation of tRNA is neither responsible for the AvF stimulation of protein synthesis nor for the lower Mg^{+2} optimum.

The next problem was to determine whether the AvF fraction was distinct from transfer factors. For these experiments we utilized natural message deficient rabbit reticulocyte ribosomes

TABLE 2

^{14}C-VAL AND ^{14}C-LEU-tRNA INCORPORATION INTO PROTEIN AS A FUNCTION OF Mg^{+2} CONCENTRATION

Mg^{+2} (mM)	p moles ^{14}C-val/mg RNA		p moles ^{14}C-leu-tRNA/mg RNA
	-AvF	+AvF	
0	0	0	0
2	2	54	38
4	12	132	72
6	23	120	63
8	28	93	56
10	17	55	38
12	12	29	21

Conditions for incorporation of ^{14}C-val are exactly as described in Table 1. When ^{14}C-leu-tRNA was used as radioactive substrate the following conditions were employed: TRIS-HCl (pH 7.2), 30 mM; GTP, 0.5 mM; phosphoenol pyruvate, 7.5 mM; pyruvate kinase, 1.5 E.U.; dithiothreitol, 1.0 mM; $MgCl_2$, 5.0 mM; KCl, 100 mM; ribosomes, 35 µg RNA; AvF, 60 µg protein; AS_{70}, 180 µg protein; and 8.0 p moles (5470 dpm) ^{14}C-leu-tRNA and 19 C^{12}-aminoacyl tRNA's. Incubation was for 3 min at 37° C (incorporation was linear for 5 min). Counting efficiency was 85-88%. Blanks of 1.3 p moles ^{14}C-val and 1.8 p moles ^{14}C-leu-tRNA/mg RNA were obtained for each assay in the absence of Mg^{+2} and have been subtracted from the values shown.

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and partially purified reticulocyte elongation factors, T_1 and T_2. This assay employs the poly U-directed polymerization of ^{14}C-phe-tRNA assayed in the presence of saturating amounts of T_1 and T_2. Again, AvF fraction stimulates the rate of synthesis and lowers the Mg^{+2} optimum from 10 mm to 6 mm (Table 3). These data then, demonstrate that the stimulatory proteins present in AvF fraction are distinct from T_1 and T_2.

In order to demonstrate fidelity of translation in a cell-free system, one must show that this system is capable of de novo

TABLE 3

^{14}C-PHE-tRNA POLYMERIZATION AS A FUNCTION OF Mg^{+2} CONCENTRATION

Mg^{+2} (mM)	p moles ^{14}C-phe-tRNA/mg RNA -AvF	+AvF
2	0.3	0.7
4	0.6	2.0
6	1.0	3.4
8	1.2	3.1
10	1.5	2.9
12	1.3	2.6

Each 50 µl reaction mixture contained 0.2 A_{260} of twice washed (0.5M KCl) rabbit reticulocyte ribosomes (incorporation was linear to 0.35A 260 units), 0.5A 260 units poly U, 5.5 p moles of chick oviduct ^{14}C-phe-tRNA (840 cpm/p mole), and 30 µg of AvF protein. Saturating amounts of partially purified rabbit reticulocyte T_1 protein (20 µg) and T_2 protein (3.0 µg) were used in all tubes. The reaction mixture contained Tris-HCl (pH 7.2), 30 mM; GTP (pH 7.0), 0.5 mM; phosphoenol pyruvate (pH 7.0), 3.0 mM; pyruvate kinase, 1.5 E.U.; dithiothreitol, 1.0 mM; and KCl, 100 mM (partially supplied by AvF fraction). Incubation was for 2 min at 37°(incorporation was linear for 3 min). A blank of 0.24 p moles obtained in the absence of Mg^{+2} was subtracted from each point. Incorporation without the addition of poly U was 0.10 p moles in the presence of AvF and 6 mM Mg^{+2}. Rabbit materials were kindly supplied by Dr. W. French Anderson of the N.I.H.

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synthesis of a specific protein molecule. Table 4 is an outline of the initial steps which we are employing in an attempt to analyze the product synthesized in the AvF-dependent natural message system. A large reaction mixture is incubated and then separated into ribosomes and supernatant by ultracentrifugation. Incorporated radioactivity is determined in aliquots of each fraction. Under these conditions 27% of the incorporated counts are released into the supernatant. The supernatant is then passed through an affinity column to which highly specific anti-

TABLE 4

AvF-DEPENDENT SYNTHESIS OF IMMUNOLOGICALLY REACTIVE OVALBUMIN ON OVIDUCT RIBOSOMES IN VITRO

FRACTION	ACID-INSOLUBLE RADIOACTIVITY (cpm)
Ribosomal pellet	74,000
Released peptides	26,860
Immunologically reactive released peptides	3,740

The reaction mixture (2.0 ml) on which the analysis was carried out contained 2.0 mg ribosomal RNA (20.0 A_{260} units), 400 µl of AvF (1.2 mg protein), 3.6 mg of AS_{70} protein, and other components as described in Table 1. Following incubation of 60 min at 37° the reaction mixture was centrifuged at 105,000 g (Rav) for 60 min. The supernatant was carefully removed. Duplicate aliquots were prepared for counting and the remainder applied to an antiovalbumin affinity column. The bound counts were collected in 2 ml following elution with 6M quanidine-HCl, dialyzed against H_2O and 1 ml counted in Spectrafluor (Amersham/Searle):toluene: Triton X-100 (42 ml:958 ml:500 ml). The ribosomal pellet was resuspended in H_2O and counted as hot acid insoluble material (1,12).

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ovalbumin is coupled (11). The column is washed thoroughly and then eluted with 6M guanidine-HCl. Fractions are collected, pooled and dialyzed. It can be seen that 14% of the released peptides are immunologically similar to ovalbumin.

Thus, we have described the properties of a new protein synthesis system derived from a hormone-sensitive target tissue. The system is dependent upon a high salt wash (AvF) of oviduct polyribosomes. The system shows linear kinetics for time, ribosomes, and the AvF fraction. The AvF fraction lowers the Mg^{+2} optimum of the reaction under a variety of assay conditions. Finally, 14% of the released peptides synthesized in this system have been shown to possess ovalbumin immuno-reactivity. Our data would

suggest this AvF-dependent oviduct system may be capable of chain-initiation and demonstrates a high degree of fidelity in translation. We hope to utilize this system to investigate directly changes in the content of specific mRNA's which occur during estrogen-mediated differentiation of the oviduct (13).

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