Proceedings of the National Academy of Sciences
Vol. 67, No. 4, pp. 1782–1788, December 1970

Specificity of mRNA Binding Factor in Eukaryotes*

Stuart M. Heywood

GENETICS AND CELL BIOLOGY SECTION, BIOLOGICAL SCIENCES GROUP,
UNIVERSITY OF CONNECTICUT, STORRS, CONN. 06268

Communicated by Severo Ochoa, September 30, 1970

Abstract. It has been shown previously that ribosomal factors removed from chick muscle ribosomes by a high salt wash are required for both the binding of muscle mRNA to ribosomes and mRNA-directed synthesis of myosin on reticulocyte ribosomes. These factors can be separated into several components on DEAE-cellulose. A factor eluting between 0.18 and 0.25 M KCl is responsible for binding mRNA to the ribosome. This binding factor shows specificity in the recognition of mRNA, as has been demonstrated by the preferential binding of muscle and globin mRNA to ribosomes which contain their respective binding factors. Similarly, globin mRNA is preferentially translated when reticulocyte factors are present on the ribosome.

In the presence of myosin mRNA and reticulocyte ribosomes, formation of an initiation complex and, therefore, the subsequent synthesis of the high molecular weight subunit of myosin requires the presence of initiation factors removed from embryonic chick muscle ribosomes by a high salt wash.[1,2] These studies suggested that in eukaryotes the factors involved in binding mRNA to the small ribosomal subunit during formation of the initiation complex recognize only those mRNAs that are derived from the same cell type as the binding factors.

Recently, several reports have been published that suggest a messenger selection process also operates during polypeptide chain initiation in viral-infected prokaryotes.[3–5] Therefore, it seems likely that protein synthesis in both prokaryotes and eukaryotes may involve a specific messenger recognition factor involved in binding mRNA to the ribosomes during the formation of the initiation complex.

The present studies, undertaken to elucidate further the mechanism by which this messenger selection process operates in eukaryotes, suggest that a factor, EF_3, is responsible for selectively binding mRNA to ribosomes.

Results and Discussion. Our previous studies, involving the muscle initiation factor (EFm)-dependent synthesis of myosin on heterologous ribosomes and the specific binding of muscle mRNA to nitrocellulose filters did not rule out the possibility that these factors removed from reticulocyte ribosomes were inactive after isolation. Therefore, a cell-free amino acid incorporating system using muscle ribosomes was devised that would synthesize hemoglobin. This cell-free amino acid incorporating system was completely dependent upon the addition of globin mRNA (9–13 S) and reticulocyte initiation factors (EFr). As shown in

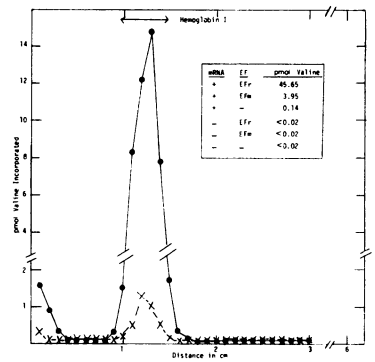

FIG. 1. Acrylamide gel analysis of the products of cell-free synthesis of chicken hemoglobin I. The amino acid incorporating system contained in a final volume of 0.5 ml: 150 mM KCl, 5 mM $MgCl_2$, 20 mM Tris·HCl (pH 7.6), 6 mM β-mercaptoethanol, 0.5 mM ATP, 0.12 mM GTP, 5 mM creatine phosphate, 75 μg of creatine kinase, 5 μCi [^{3}H]valine (2.5mCi/125 μg), 125 μg of initiation factors, 0.5 mg of 14-day-old embryonic-chick muscle ribosomes (see ref. 12), and 5 mg of undialyzed S-200 reticulocyte supernatant protein (equivalent to 0.05-ml packed reticulocyte cells). Reticulocytes were prepared from phenylhydrazine-treated chickens as described by Laver *et al.*[13] After lysis, initiation factors were prepared from reticulocytes and muscle as previously described,[1] except that the 1.0 M KCl wash of the ribosomes was precipitated at 80% saturation of $(NH_4)_2SO_4$ prior to dialysis against incubation buffer. This factor preparation was found not to contain ribosomal particles. Globin mRNA was prepared by resuspending reticulocyte polysomes in 20 mM sodium acetate–40 mM Tris-acetate (pH 7.6)–5 mM EDTA, and 0.5% sodium dodecyl sulfate. Approximately 10 A_{260} units of material was sedimented at 23,000 rpm for 17 hr on a 27-ml, 10–30% sucrose, density gradient containing the Tris buffer, EDTA, and sodium dodecyl sulfate. The gradients were analyzed on a continuously recording Gilford spectrophotometer. An absorbance peak sedimenting from 9–13 S was collected and subsequently used for globin mRNA.[14,15] Sodium dodecyl sulfate in the 9–13 S fraction was removed by precipitation with equimolar amounts of KCl and centrifugation. Finally, two volumes of ethanol and 50 μg of (unstripped) chicken tRNA were added to the supernatant and the RNA precipitated at −20°C. 10 μg of globin mRNA was added to each incubation mixture, which was incubated at 37°C for 30 min. After incubation the mixtures were centrifuged at 240,000 × g for 1 hr. Hemoglobin I was purified from a 45–80% $(NH_4)_2SO_4$ fraction of the supernatant, by the method of Moss and Thompson,[16] and subsequently analyzed on acrylamide gels.[17] The gels were sliced into 1-mm slices and assayed for radioactivity by the method of Terman.[18] Only the first 3 cm of gel, where the hemoglobin I was located, was analyzed for radioactivity. The position and width of the hemoglobin I band is indicated by the horizontal arrow. ●, complete system with reticulocyte initiation factors and globin mRNA. ×, complete system with muscle initiation factors and globin mRNA. *Insert* indicates the total amount of [^{3}H]valine migrating with hemoglobin I under the different experimental conditions.

Fig. 1, when globin mRNA and EFr are added to the incubation medium, a substantial amount of [^{3}H]valine is incorporated into hemoglobin I. Less than 10% of this amount of [^{3}H]valine is incorporated into hemoglobin I when EFr is replaced by EFm. When initiation factors are omitted only a very small amount of [^{3}H]valine is found in hemoglobin I; negligible amounts of [^{3}H]valine are found to migrate with hemoglobin I on acrylamide gels when globin mRNA is omitted from the incubation mixture (*insert*, Fig. 1). The fact that EFr supports hemoglobin synthesis indicated that the initiation factors from reticulocytes are not exceptionally labile to isolation procedures as compared to EFm. Further, the dependence of hemoglobin synthesis on EFr suggests that initiation factors from reticulocytes are specifically required in order to recognize and subsequently translate globin mRNA. This is similar to the specificity previously reported for EFm and muscle mRNA (see ref. 1). The requirement of these factors for the synthesis of hemoglobin indicates that it is unlikely that they are involved with the elongation of the polypeptide chain, i.e., that they are the transfer factors found in the 200,000 × g supernatant.

The selectivity of these factors for particular mRNAs may also be demonstrated by the specific binding of [^{32}P]mRNA to ribosomes on nitrocellulose

filters. Globin mRNA preferentially binds to either muscle or reticulocyte ribosomes when EFr is present on the ribosomes (Table 1). Similarly, muscle mRNA only binds to ribosomes when EFm is present. Some binding of globin mRNA is observed when EFm is used in combination with either muscle or reticulocyte ribosomes. As can be seen in Fig. 1, a small amount of hemoglobin is synthesized when EFm is added to the cell-free amino acid incorporating sys-

TABLE 1. *mRNA binding specificity of EF to salt washed ribosomes.*

Ribosomes	Factors	mRNA	Cpm	% bound
Reticulocyte	—	globin*	27	4.0
Reticulocyte	EFr	globin	166	25.0
Reticulocyte	EFm	globin	85	12.8
Reticulocyte	—	muscle†	6	5.0
Reticulocyte	EFr	muscle	8	6.6
Reticulocyte	EFm	muscle	40	33.2
Muscle	—	globin	41	6.1
Muscle	EFr	globin	160	24.0
Muscle	EFm	globin	75	11.3
Muscle	—	muscle	9	7.5
Muscle	EFr	muscle	6	5.0
Muscle	EFm	muscle	62	51.5
—	EFr	globin	48	7.2
—	EFm	muscle	10	8.4
—	EFm	28S rRNA‡	29	9.7
Muscle	EFm	28S rRNA	26	8.7
Muscle	—	28S rRNA	30	10.0

1.0 M KCl-washed ribosomes from 14-day-old embryonic chick muscle and from reticulocytes were prepared.[1] Initiation factors were prepared by the modified procedure described in Fig. 1. [^{32}P]RNA from muscle was prepared[1] as a combined 26S and 10–15S RNA sample from the different size polysomes. [^{32}P]mRNA for globin was prepared as described in Fig. 1, after injecting a chicken with 10 mCi of ^{32}P 14-hr before bleeding. The nitrocellulose filter assay for mRNA binding was performed as follows: To each 1.3-cm filter (Millipore Corp., 0.45 μm, only those that are rapidly wetted are used) 200 μg of 1.0 M KCl-washed ribosomes (dialyzed against buffer) were added. The filter was then saturated with bovine serum albumin and washed to remove unbound albumin, then 50 μg of initiation factors was added, followed by an additional wash. Finally [^{32}P]mRNA was added. The filter was washed with 2 ml of buffer, dried, and counted in a low background (less than 2 cpm) gas-flow counter. All solutions were in incubation buffer (150 mM KCl–5 mM $MgCl_2$–20 mM Tris (pH 7.4)–6 mM β-mercaptoethanol). The surface of the filter was always wetted with about 0.10 ml buffer; the filtering rate did not exceed 0.3 ml/min. All solutions used were kept at 0°C. The mRNA preparations were resuspended in buffer containing 0.5 μmol GTP and 50 μg tRNA/ml just prior to using. Previous experiments have shown GTP and tRNA to be required for the formation of the intiation complex.[2] The binding assay was performed at 25°C.

* 665 cpm/assay.

† 120 cpm/assay.

‡ 300 cpm/assay.

tem. This may be a result of a greater heterogeneity of cell types, messengers, and factors present in muscle tissue as compared to reticulocytes. In the absence of ribosomes, slight binding occurs with both mRNA and rRNA preparations. Ribosomal RNA also binds slightly when ribosomes are used in the assay, with or without the addition of factors. This nonspecific binding of rRNA to ribosomes has been reported.[1,2] When the binding of [^{32}P]mRNA to unwashed ribosomes is measured, similar results are obtained (Table 2); the only difference observed is that increased factor-independent binding occurs, presumably due to endogenous factors present on the ribosomes. Again, it is noted that EFm has a greater affinity for globin mRNA than EFr has for muscle mRNA.

TABLE 2. *mRNA binding specificity of EF to ribosomes not washed with salt.*

Ribosomes	Factors	mRNA	Cpm	% bound
Reticulocyte	—	Globin*	74	11.1
Reticulocyte	EFr	Globin	189	28.4
Reticulocyte	EFm	Globin	100	15.0
Reticulocyte	—	Muscle†	4	3.3
Reticulocyte	EFr	Muscle	6	5.0
Reticulocyte	EFm	Muscle	29	24.1
Muscle	—	Globin	130	19.5
Muscle	EFr	Globin	245	36.8
Muscle	EFm	Globin	142	21.4
Muscle	—	Muscle	40	33.3
Muscle	EFr	Muscle	25	20.8
Muscle	EFm	Muscle	80	66.6

Ribosomes from muscle and reticulocytes were prepared as described, except that the 1.0 M KCl wash was omitted.

* 665 cpm/assay.

† 120 cpm/assay.

Recently, Prichard *et al.*[6] have reported that a labile factor necessary for the initiation of globin synthesis in rabbit reticulocyte extracts is excluded from Sephadex G-200. When EFm is chromatographed on Sephadex G-200, the material eluting in and immediately after the excluded volume is responsible for the binding of mRNA to ribosomes (Fig. 2). These results suggest that

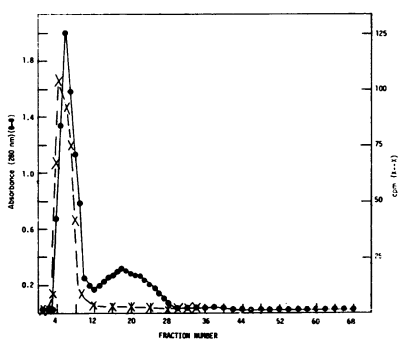

Fig. 2. Sephadex G-200 chromatography of muscle initiation factors. 8 mg of initiation factors was dialyzed against 20 mM Tris·HCl (pH 7.4–5 mM $MgCl_2$–150 mM KCl–6 mM β-mercaptoethanol, and chromatographed on a Sephadex G-200 column 1.5 × 15 cm). 2-ml fractions were collected and tested for their ability to bind muscle mRNA to ribosomes on nitrocellulose filters (see Legend, Table 1). Void volume was 6 ml. 190 cpm [^{32}P]mRNA was used per assay.

the factor responsible for binding mRNA to the ribosomes may be identical to the factor reported by Prichard *et al.*[6] to be required for the initiation of polypeptide synthesis directed by natural mRNA.

Several laboratories have reported that the factors removed from ribosomes by a high salt wash are necessary for the *de novo* synthesis of proteins.[1,7,8] These factors have been reported to be sensitive to pronase, but not to ribonuclease.[7,9] Similarly, pronase completely destroys the ability of EFm to bind muscle mRNA to ribosomes (unpublished results).

In order to identify the factor involved with binding mRNA to ribosomes, EFm was separated into several components by DEAE-cellulose chromatography (Fig. 3). The elution profile of EFr is identical to that shown for EFm. The different absorbance peaks that elute from the DEAE-cellulose column at increasing ionic strengths are called EF_1, EF_3, and EF_2, to correspond with the

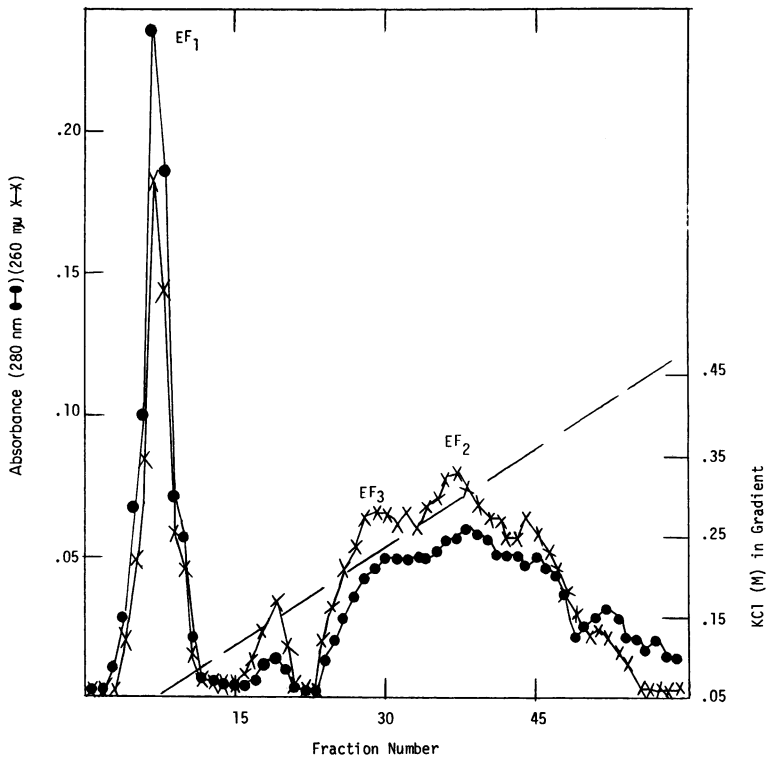

FIG. 3. DEAE-cellulose chromatography of muscle initiation factors. EFm was prepared as in Table 1, except that after $(NH_4)_2SO_4$ precipitation the material was dialyzed against 20 mM Tris·HCl (pH 7.4)–50 mM KCl–1 mM EDTA–12 mM β-mercaptoethanol, and 10% glycerol. The sample, containing 9 mg of protein, obtained from approximately 40 mg of ribosomes was layered on a 1 × 12 cm DEAE-23 (Whatman) cellulose column equilibrated with the dialysis buffer with 5% glycerol. 3-ml fractions were collected and their absorbance recorded at 280 and 260 nm. After 8 fractions had been collected, a linear gradient of KCl was introduced, 0.05–0.5 M, and a total of 60 fractions were collected. All operations were performed at 2°C.

nomenclature for factors from *Escherichia coli*.[10] If the amount of protein chromatographed is decreased to one-half of that shown in Fig. 3, EF_3 and EF_2 separate better, giving a purer preparation of EF_3. The muscle factor EFm_3, which elutes between 0.18–0.25 M KCl, is the only fraction that causes [^{32}P]-mRNA to bind to ribosomes (Table 3). EFm_1, eluted at 0.05 M KCl, enhances the binding reaction of EFm_3; EFm_2 has no effect. The material eluted from the column at 0.15 M KCl was also tested and was not involved with binding mRNA to the ribosomes. In the absence of ribosomes, EFm_3 binds mRNA only minimally. Similar results are obtained if the initiation factors from reticulocytes are separated by DEAE-cellulose chromatography and tested for their ability to bind globin mRNA to ribosomes. It is of interest that EF_3, the factor involved with binding mRNA to ribosomes, seems to function similarly to the F_3 factor found in *E. coli*.[10,11]

Normally, 15 μg of factor protein are used for each assay. Increasing the amount of EFm_3 above this amount results in no further increase in the amount

TABLE 3. *Identification of mRNA binding factor.*

Factor	Cpm	% bound
—	1	0.9
EFm_1	2	1.8
EFm_3	34	30.9
EFm_2	4	3.6
$EFm_1 + EFm_3$	40	36.4
$EFm_3 + EFm_2$	35	31.8
-Ribosomes + EFm_3	8	7.3

The individual factors were separated by DEAE-cellulose chromatography, as in Fig. 3, except that 6 mg of protein was applied to the column and the factors were eluted stepwise—EFm_1 at 0.05 M KCl, unknown material at 0.15 M KCl, EFm_3 at 0.25 M KCl, and EFm_2 at 0.35 M KCl. The peak fractions were precipitated at 80% saturation of $(NH_4)_2SO_4$, resuspended, and dialyzed against the buffer used for the binding assay (Table 1). 110 cpm of [^{32}P]mRNA from muscle, 200 μg of 1 M KCl-washed ribosomes, and 15 μg of each factor was used for each assay.

of [^{32}P]mRNA bound (Fig. 4). This amount of EFm_3 corresponds to approximately 50 μg of unfractionated EF protein, the amount of EF which gives optimal binding to 200 μg ribosomes per nitrocellulose filter.

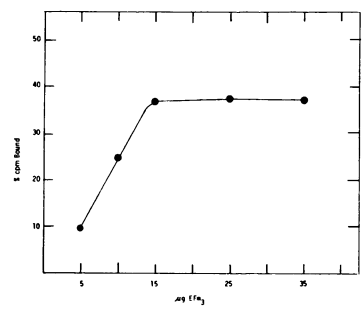

Fig. 4. EFm_3-dependent binding of muscle mRNA. 130 cpm of muscle mRNA was used per assay.

Exchange experiments between reticulocyte and muscle initiation factors were designed to test if EFm_3 from muscle and reticulocyte are responsible for the selectivity of messenger recognition observed in both the binding and cell-free synthesis studies. The factors from reticulocyte (EFr_1, EFr_3, EFr_2) and from muscle (EFm_1, EFm_3, EFm_2) were chromatographed as shown in Fig. 3. EF_3 and EF_2 (EF_{3+2}) from either muscle or reticulocytes were normally eluted from the column in one fraction unless EF_3 was to be tested separately. When either muscle or reticulocyte ribosomes are used, muscle mRNA only binds to the ribosomes when EFm_3 is attached to them (Fig. 5). The binding of globin mRNA preferentially occurs when EFr_3 is present; however, some globin mRNA also binds in the presence of EFm_3. As noted previously, EFm_3 has a higher affinity for globin mRNA than EFr_3 has for muscle mRNA. Nevertheless, the results suggest that the factor (EF_3) that binds mRNA to ribosomes during initiation is also the component that confers specificity to mRNA recognition However, since EF_3 has not yet been completely purified, it is possible that yet another component is involved in the specificity of the binding reaction between mRNA and the ribosome.

The experiments reported here have not defined the manner by which the mRNA–ribosome interaction occurs, nor have they shown if the binding is an enzymatic reaction. Nevertheless, these results and our previous studies suggest that differentiated cells of eukaryotes contain cell-specific, or possibly messenger-specific, factors involved in the initiation of protein synthesis. These factors would exert a positive control on protein synthesis and play an important role in cellular differentiation and the stability of the differentiated state. Such

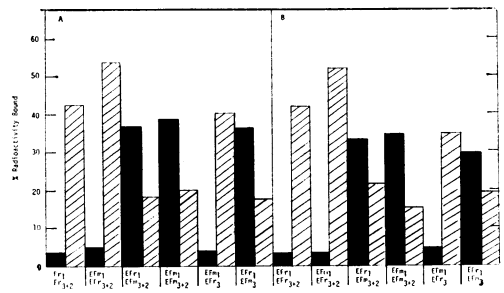

Fig. 5. Specificity of mRNA binding in the presence of EF_3: (*A*) 1 M KCl-washed muscle ribosomes, (*B*) 1M KCl-washed reticulocyte ribosomes. Solid bars represent muscle mRNA (115 cpm [^{32}P]mRNA per assay). Hatched bars represent globin mRNA (750 cpm [^{32}P]mRNA per assay). 15 μg of each individual factor and 25 μg of combined EF_{3+2} was used per assay. Factors were chromatographed as described in the legends of Table 3 and Fig. 3. Under first bar, block *A*, read EFr_1 for Fr_1 and EFr_{3+2} for Fr_{3+2}; under third bar, block *B*, read EFr_1.

control could limit at any given time the proteins that could be synthesized in a cell regardless of the kinds of mRNA it may contain.

Abbreviations: EFm, eukaryotic initiation factor from muscle; EFr, eukaryotic initiation factor from reticulocyte.

* Supported by grant HD 03316 from the National Institute of Child Health and Human Development, NIH, and NIH Career Development Award GM 18904 awarded to S. M. Heywood.

[1] Heywood, S. M., *Cold Spring Harbor Symp. Quant. Biol.*, **34**, 799 (1969).
[2] Heywood, S. M., *Nature*, **225**, 696 (1970).
[3] Hsu, W. T., and S. B. Weiss, *Proc. Nat. Acad. Sci. USA*, **64**, 345 (1969).
[4] Dube, S. K., and P. S. Rudland, *Nature*, **226**, 820 (1970).
[5] Steitz, J. A., S. K. Dube, and P. S. Rudland, *Nature*, **226**, 824 (1970).
[6] Prichard, P. M., J. M. Gilbert, D. A. Shafritz, and W. F. Anderson, *Nature*, **226**, 511 (1970).
[7] Miller, R. L., and R. Schweet, *Arch. Biochem. Biophys.*, **125**, 632 (1968).
[8] Godin, C., J. Kruk, and J. C. Dreyfus, *Biochim. Biophys. Acta*, **182**, 175 (1969).
[9] Cohen, B., *Biochem. J.*, **110**, 231 (1968).
[10] Iwasaki, K., S. Sabol, A. Wahba, and S. Ochoa, *Arch. Biochem. Biophys.*, **125**, 542 (1968).
[11] Revel, M., M. Herzberg, and H. Greenshpan, *Cold Spring Harbor Symp. Quant. Biol.*, **34**, 261 (1969).
[12] Heywood, S. M., and M. Nwagwu, *Biochemistry*, **8**, 3839 (1969).
[13] Laver, W. A., A. Neuberger, Hans S. Udenfriend, *Biochem. J.*, **70**, 4 (1958).
[14] Scherrer, K., and L. Marcaud, *J. Cell. Physiol.*, **72**, 181 (1968).
[15] Lockwood, R., and J. Lingrel, *Biochem. Biophys. Res. Commun.*, **37**, 204 (1969).
[16] Moss, B., and E. O. P. Thompson, *Aust. J. Biol. Sci.*, **22**, 1455 (1969).
[17] Moss, B., and V. Ingram, *J. Mol. Biol.*, **32**, 481 (1968).
[18] Terman, S. A., *Proc. Nat. Acad. Sci. USA*, **65**, 985 (1970).