Separation of Specific Initiation Factors Involved in the Translation of Myosin and Myoglobin Messenger RNAs and the Isolation of a New RNA Involved in Translation

(muscle/reticulocytes/globin synthesis/translational control)

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ABSTRACT Two messenger specific factors, phosphocellulose fractions 3 and 4, have been isolated from the initiation factor 3 fraction of red muscle initiation factors by chromatography on phosphocellulose. When added to a reticulocyte cell-free system containing both myoglobin and myosin mRNAs, phosphocellulose fraction 3 is found to specifically stimulate the synthesis of myoglobin while phosphocellulose fraction 4 is found to specifically stimulate the synthesis of myosin. In addition, a new RNA, isolated from the initiation factor 3 fraction, is shown to specifically inhibit the translation of heterologous mRNAs.

Myosin and myoglobin mRNA have been translated in heterologous cell-free systems (1, 2). In these systems the mRNAs to be tested have competed with endogenous mRNAs for the various constituents of the cellular apparatus involved in protein synthesis. Under these conditions, the addition of specific initiation factor 3 (IF-3) fractions has been required to make this competition more favorable and allow for more efficient synthesis of these proteins. Red muscle cells contain specific factors for the translation of both myosin and myoglobin, whereas white muscle cells lack the specific initiation factor for myoglobin synthesis (2). These results have led us to suggest that muscle cells contain a multiplicity of specific initiation factors. Recently, several reports utilizing globin and viral mRNAs have also suggested that messenger specific initiation factors are found in eukaryotic cells (3, 4). In addition to specific protein factors involved in the translational control of protein synthesis, a number of investigators have raised the possibility that an RNA molecule may also be involved in translational control by directly effecting the initiation of protein synthesis (5-8).

We report here the separation of myosin and myoglobin specific factor activity by chromatography of red-muscle IF-3 on phosphocellulose. Multiple protein fractions are obtained, and two of these fractions are found to have different specificities in translating myosin and myoglobin mRNA in a reticulocyte cell-free system. In addition, a new RNA species, isolated from IF-3 fraction, is shown to specifically inhibit the translation of heterologous mRNAs. This RNA has been termed translation control RNA (tcRNA).

MATERIALS AND METHODS

Both highly purified myosin and myoglobin as well as their mRNAs were prepared as described (1, 2). The mRNA prep-

arations contain other RNA species and, therefore, the amount of individual mRNA present is not known. Initiation factors (IF-1, IF-2, and IF-3) were prepared from 100 to 150 g of 19-day embryonic chick red muscle. The muscle was homogenized in 0.15 M KCl, 5 mM MgCl₂, 20 mM Tris·HCl (pH 7.4), 18 mM 2-mercaptoethanol, and 10% (v/v) glycerol. Subsequent separation of the initiation factors was performed as described (11) with the exception that the IF-3 fraction was eluted from DEAE-cellulose with 0.28 M KCl containing buffer. The IF-3 fraction, containing 8 mg of protein, determined by the method of Lowry *et al.* (12), was dialyzed against 10 volumes of 50 mM K⁺ phosphate buffer (pH 6.8), 6 mM 2-mercaptoethanol, and 5% glycerol for 4 hr and finally fractionated by chromatography on a 1.0 × 8.0-cm phosphocellulose column (Fig. 1). After 8 mg of IF-3 was loaded on the phosphocellulose column, the column was washed with 6.0 ml of 50 mM K⁺ phosphate buffer (pH 6.8), 6 mM 2-mercaptoethanol, and 5% glycerol, resulting in the elution of phosphocellulose fraction 1 (PC-1) (Fig. 1). Subsequently the column was eluted with a 40-ml gradient (0.1 M K⁺ phosphate to 0.5 M K⁺ phosphate containing 6 mM 2-mercaptoethanol and 5% glycerol).

The dialysate of the IF-3 fraction was used as a source of tcRNA. It was passed through a 0.6 × 8-cm DEAE-cellulose column equilibrated with phosphate buffer. After the column was washed with 10 ml of 50 mM K⁺ phosphate (pH 6.8), the column was eluted with 0.1 M NaCl, 0.1 M Tris·HCl (pH 7.4), and 5 mM EDTA followed by 1.0 M NaCl in the Tris buffer as shown in Fig. 3. The material eluting with 0.1 M NaCl buffer has a ratio of A_{280}/A_{260} of 0.5-0.7, while the material eluted with 1.0 M NaCl buffer has a ratio of A_{280}/A_{260} of 1.9-2.0. This second peak, containing tcRNA, was subsequently precipitated with 3 volumes of cold ethanol. Normally 6-8 mg of IF-3 yield 2-4 μ g of tcRNA. Occasionally insoluble material was present in the tcRNA preparation, which was removed by centrifugation at 10,000 × *g* for 5 min.

The rabbit reticulocyte lysate was prepared by the method of Evans and Lingrel (9). The lysate was not preincubated before use. Salt conditions were adjusted to be optimal for myosin and myoglobin synthesis in this system [0.1 M KCl, 3 mM MgCl₂, and 20 mM Tris·HCl (pH 7.4)]. Although globin synthesis was slightly less than optimal under these conditions, the inhibitory response of globin synthesis to tcRNA was unaffected. Each cell-free system, in a final volume of 0.25 ml, contained 0.20 ml of lysate containing 0.6 mg of ribosomes, 20 μ g of chick tRNA (1), 2 μ g of a myosin

Abbreviations: IF-3, initiation factor 3; tcRNA, translation control RNA; PC, phosphocellulose fractions.

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FIG. 1. The chromatography of IF-3 fraction on phosphocellulose. IF-3 was prepared and fractionated on phosphocellulose as described in *Methods*. After 1.5-ml fractions were collected, the two fractions containing the highest 280-nm absorbance in peaks PC-1-PC-4 were concentrated against dry Sephadex G-200 and dialyzed against 0.15 M KCl, 20 mM Tris (pH 7.4), 5 mM MgCl₂, 6 mM 2-mercaptoethanol, and 10% (v/v) glycerol. Samples containing 1 mg/ml of protein were stored at -80° for up to 3 weeks before they were used.

mRNA preparation, 2 μ g of a myoglobin mRNA preparation, 20 μ g of IF-1 and IF-2, 20 μ g of IF-3 or PC-1, PC-2, PC-3, or PC-4 prepared from IF-3 where indicated, and an ATP-generating system as described (1). Unless otherwise indicated, 0.25 nmoles each of 20 amino acids containing 5 μ Ci 3 H-labeled amino-acid mixture (New England Nuclear Corp.) was added to each incubation mixture. After incubation for 45 min at 35° , 0.5 mg of carrier myosin and 1.0 mg of carrier myoglobin were added. Myosin was partially purified by precipitation as described (1) and subsequently analyzed by Na dodecyl sulfate-acrylamide gel electrophoresis (1). Myoglobin was purified from the supernatant of the myosin precipitation as described (2). Final purification of myoglobin was accomplished by 6 M urea-acrylamide gel electrophoresis to assure no contamination of hemoglobin (2). The radioactivity migrating with myosin and myoglobin was determined by liquid scintillation counting after the acrylamide gels were sliced (2). The amount of globin synthesis was estimated as the total acid-precipitable radioactivity of the incubation mixture less myosin and myoglobin radioactivity.

RESULTS

Previous results have suggested that red muscle may contain a number of message specific factors (1). In order to separate these factors into fractions specific for either myosin or myoglobin, the IF-3 fraction from red muscle was chromatographed on phosphocellulose. As shown in Fig. 1, four distinct peaks are observed, PC-1-PC-4. These fractions were collected, concentrated, and tested in a cell-free system, containing both myosin and myoglobin mRNA, for their ability to specifically stimulate either myosin or myoglobin synthesis (Table 1). The results shown in Table 1 summarize the results obtained after measurement of the radioactivity migrating with myosin and myoglobin during acrylamide gel electrophoresis as illustrated in Fig. 2. In the absence of added

FIG. 2. Electrophoretogram of radioactive myosin and myoglobin synthesized *in vitro*. The horizontal arrows represent the position of carrier protein after purification of myosin (A) and myoglobin (B) from the cell-free system. (A) Na dodecyl sulfate-acrylamide gel electrophoresis of partially purified myosin. (B) Urea-acrylamide gel electrophoresis of purified myoglobin. Procedures of protein purification and electrophoresis have been described (1, 2). O—O, PC-3 (Fig. 1) added to cell-free system; ●—●, PC-4 (Fig. 1) added to cell-free system. Values for cpm should be multiplied by 10^{-2} , as indicated.

mRNA, myosin or myoglobin synthesis is not observed, while only low levels of synthesis of these proteins are seen when IF-3 is omitted from the reaction mixture. PC-1 and PC-2 are ineffective in stimulating myosin or myoglobin synthesis to levels above those obtained in the absence of added IF-3. However, as shown in Fig. 2 and Table 1, when PC-3 is added to the reaction mixture, myoglobin synthesis is increased approximately 7-fold with no effect on myosin synthesis. On the other hand, when PC-4 fraction is added, it is found to specifically stimulate myosin synthesis while having no effect on myoglobin synthesis. In addition, the phosphocellulose

TABLE 1. The synthesis of globin, myosin, and myoglobin using different fractions of muscle IF-3

Exp.	cpm			
	Globin	Myosin	Myo-globin	Myosin/myoglobin
-IF-3	15,650	250	205	1.22
+IF-3	16,090	670	640	1.05
PC-1	16,690	136	205	0.66
PC-2	17,420	228	250	0.91
PC-3	17,200	248	1520	0.16
PC-4	16,210	1056	150	7.00
IF-3 - mRNA	18,000	40	0	—
IF-3 + tcrNA ^a	10,600	1178	8695	1.22

^a tcrNA (0.7 μ g) from muscle added to reaction mixture. Values in *italics* emphasize translational specificity.

FIG. 3. Preparation of tcRNA. The 50 mM K^+ phosphate dialysate of IF-3 (*Methods*) was passed through a 0.6×8 -cm DEAE-cellulose column. After the column was washed free of 260-nm absorbance material with two buffers as described in *Methods*, the tcRNA was eluted with 1.0 M NaCl containing buffer (fractions 9–12). Fractions (0.8-ml) were collected. Muscle, rabbit reticulocyte, and chicken erythroblast tcRNA had similar elution properties and an A_{260}/A_{280} ratio of 2.0.

fractions 3 and 4 have a higher specific activity for myosin or myoglobin synthesis than the unfractionated IF-3 fraction. The large difference in the ratio of myosin to myoglobin radioactivity when the different phosphocellulose fractions are added to the incubation mixture further emphasizes the specificity of these factors. The addition of PC-1 or PC-2 to either PC-3 or PC-4 had no effect on these ratios (unpublished). Therefore, these results confirm the fact that a number of messenger specific factors exist in muscle cells and indicate that they can be separated in active form for further biochemical studies.

When the PC-3 and PC-4 fractions of IF-3 are examined by Na dodecyl sulfate-acrylamide gel electrophoresis, each fraction has six similar protein bands at 50,000 daltons or less. Both PC-3 and PC-4 have four protein bands between 50,000 and 110,000 daltons, two of which are similar in molecular mass and two of which are dissimilar (to be published elsewhere).

Bogdanousky *et al.* (5) have isolated a small RNA molecule from the dialysate of a reticulocyte ribosomal wash that stimulates globin synthesis. When the dialysates of the IF-3 fractions from muscle or erythroblasts are chromatographed on DEAE-cellulose, an RNA fraction is eluted with 1.0 M NaCl buffer (Fig. 3). When this RNA, termed translational control RNA (tcRNA), is added to the incubation mixtures, it is found to specifically inhibit the synthesis of heterologous proteins (Table 2). Chicken erythroblast or rabbit reticulocyte (not shown) tcRNA specifically inhibits both myosin and myoglobin synthesis while having no effect on globin synthesis. However, if tcRNA prepared from muscle IF-3 is added to the reaction mixture, globin synthesis is decreased substantially, while no inhibition of myosin and myoglobin

FIG. 4. The effect of muscle tcRNA on globin synthesis. Cell-free systems were as described in *Methods* except that myosin and myoglobin mRNAs were not added, 20 μ Ci of 3H -labeled amino-acid mixture was added to each reaction mixture, and no additional initiation factors were added. Erythroblast tcRNA ($\bullet$ — $\bullet$), muscle tcRNA ($\bullet$ --- $\bullet$). Radioactivity was determined after precipitation of the reaction mixture with hot trichloroacetic acid.

is observed. The experiment shown in Fig. 4 further illustrates the specificity of the inhibition of hemoglobin synthesis to muscle tcRNA and indicates that only small quantities of tcRNA are required to obtain this inhibition. While a stimulation of protein synthesis is occasionally seen after addition of homologous tcRNA (Table 1 and myosin in Table 2), this effect is not reproducible. Therefore, the results shown here suggest that this new RNA, isolated from IF-3 fraction of initiation factors, does specifically inhibit the utilization of heterologous mRNAs. In so doing, it is likely that this RNA

TABLE 2. The effect of muscle and erythroblast tcRNA on protein synthesis

Exp. *	cpm			
	Globin	Myosin	Myo- globin	Myosin/ myoglobin
—PC-3, —PC-4, +mRNA	74,290	315	300	1.04
PC-3, PC-4, —mRNA	78,510	20	0	—
PC-3	75,000	648	1747	0.37
PC-4	73,580	1200	600	2.00
PC-3 + tcRNA _m †	50,200	527	1696	0.31
PC-4 + tcRNA _m	46,350	1655	489	3.38
PC-3 + tcRNA _{er} ‡	75,100	91	42	—
PC-4 + tcRNA _{er}	72,530	54	115	—

* 3H -Labeled amino-acid mixture (20 μ Ci) in each experiment.

† Muscle tcRNA (1.0 μ g) added to incubation mixture.

‡ Erythroblast tcRNA (1.0 μ g) added to reaction mixture.

TABLE 3. The effect of RNase on tRNA activity

Exp.	cpm
—	74,150
2 μ g of tRNA	17,910
2 μ g of tRNA + RNase*	65,130
2 μ g of tRNA + RNase*	63,520

* tRNA and tRNA were heated with 2 μ g/ml of pancreatic RNase at 37° for 10 min. tRNA was re-extracted with phenol-chloroform-isoamylalcohol (25:24:1).

species is involved in the translational control of protein synthesis in eukaryotes.

The tRNA has an A_{260}/A_{280} ratio of 1.9–2.0, is tightly bound to DEAE-cellulose, and has solubility characteristics of a nucleic acid. In order to confirm its identity as an RNA molecule and to partially characterize it, a number of tests were performed. When muscle tRNA is treated with pancreatic RNase and subsequently reextracted with phenol-chloroform, the digested material does not substantially inhibit globin synthesis, while intact muscle tRNA inhibits globin synthesis about 75% (Table 3). An equal amount of RNase-treated tRNA inhibits globin synthesis to the same extent as RNase-treated tRNA. These results suggest that the active factor resulting in inhibition of specific synthesis is an RNA molecule. The absorbance spectrum of tRNA is shown in the insert of Fig. 5, further identifying the molecule as a nucleic acid. The molecular weight of muscle tRNA is approximately 6500, as determined by its elution properties on Sephadex G-25 (Fig. 5). Taken together, these results identify the active molecule as a new species of RNA.

DISCUSSION

The translational control of protein synthesis in eukaryotes has been shown to include, in part, protein factors that stimulate the utilization of specific mRNAs. This may be accomplished by a sequestering of nonspecific components of the initiating system by these specific factors, resulting in their utilization in translation of specific proteins. We show here the separation of two such factors (myosin and myoglobin specific factors) possibly still complexed to other initiation factors. Neither the precise manner by which these factors demonstrate messenger specificity nor the role they play in the formation of the complete initiation complex is known. However, it is likely that such factors are important in determining the utilization of the long-lived mRNAs found in eukaryotic cells.

The finding of a new RNA molecule, presumably involved with initiation of protein synthesis, that specifically inhibits the translation of heterologous mRNAs introduces yet another mode by which protein synthesis can be regulated in eukaryotic cells. It is not yet known how this translational control RNA (tRNA) itself recognizes mRNAs and inhibits their translation. However, since double-stranded RNA is known to block protein synthesis (10), supposedly by inactivating IF-3 (11), it is possible that tRNA, in conjunction with heterologous mRNAs, blocks synthesis in a similar manner.

The results reported here have demonstrated the specific inhibition of protein synthesis using tRNA isolated from different cell types. This suggests a tissue-specific class of tRNA. However, tRNA may be a number of distinct short-lived RNA molecules inhibiting the synthesis of specific

FIG. 5. Molecular weight determination and ultraviolet absorbance spectrum of tRNA. Superfine Sephadex G-25 (Pharmacia) was calibrated with tRNA, vitamin B₁₂, and cytochrome c. The tRNA shows an approximate molecular weight of 6500.

proteins within the cell. In this case, inhibition of synthesis of tRNA may result in a "superinduction" of enzyme synthesis such as that observed by Tomkins and others (13). In addition, tRNA could play an important role in blocking the utilization of foreign mRNAs such as those of viral origins. Nevertheless, the translational control of protein synthesis in eukaryotes, utilizing both positive (protein-specific factors) and negative (tRNA) control elements, appears to be a complex mechanism.

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