Association of cap binding protein-related polypeptides with cytoplasmic RNP particles of chick embryonic muscle

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Cap binding protein (CBP)-related polypeptides were identified in different cytoplasmic RNP particles of embryonic chick muscles using monoclonal antibody to purified CBP. A single immunoreactive peptide (M_r 78000) was present in preparations of both free mRNP particles and a novel 10 S translation inhibitory RNP particle. In contrast, proteins isolated from these particles showed two new low-M_r immunoreactive peptides (M_r 43000 and M_r 29000). No CBP related protein could be detected in polysomal mRNP, although an immunoreactive M_r 43000 CBP-related protein was present in polysomes. The relevance of the association of different CBP-related polypeptides with cytoplasmic RNP particles and polysomes are discussed.

Cap binding protein *Monoclonal antibody* *Cytoplasmic RNP particle*

1. INTRODUCTION

The cap structure, m^7GpppN, which is present at the 5′-terminus of eukaryotic cellular mRNAs, is required for efficient translation of mRNAs and functions during polypeptide chain initiation (review [1,2]). Several polypeptides, M_r 24×10^3, 28×10^3, 50×10^3 and 80×10^3, recognize the cap structure (referred to as cap binding proteins or CBPs) [3–6]. The M_r 24×10^3 CBP has been purified to apparent homogeneity and shows biological activity in vitro [4, 5]. Monoclonal antibodies directed against CBPs, which specifically inhibits translation of capped mRNAs in a cell-free system [7], were shown to crossreact with a protein, M_r 28×10^3 and several higher-M_r polypeptides. These immuno-crossreacting polypeptides and the M_r 24×10^3 share common peptides [7], suggesting that they may be structurally related.

In eukaryotic cells, mRNAs are complexed with proteins to form messenger ribonucleoprotein particles [8,9]. Two types of mRNP particles, the polysome-derived and the free mRNP have been described in a variety of cell types [9]. The mRNA-associated proteins have been postulated to be involved in the cellular regulation of mRNA metabolism [9]. However, their biological function remains to be understood. Although several initiation and elongation factors have been reported to be present among the cytoplasmic pool of RNA-binding proteins [10,11], their biochemical characterization and precise role in mRNA function have not been defined.

The isolation and characterization of various classes of cytoplasmic RNP particles from chick embryonic muscle have been reported in [12–14]. These include a novel class of translation inhibitory 10 S RNP (iRNP) containing a 4 S RNA

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Abbreviations: CBP, cap binding proteins; mRNP, messenger ribonucleoprotein; 2D-PAGE, two-dimensional polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; iRNP, translation inhibitory ribonucleoprotein

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(iRNA) [12–14]; the 20–40 S free mRNP particles [15,16]; and the polysomal mRNP [8,17,18]. Using a monoclonal antibody to purified CBP isolated from rabbit reticulocytes [7], we report here that there is specific association of CBP-related polypeptides in certain types of RNP particles. Furthermore, some of the low-M_r CBP-related polypeptides present in the RNP particles may arise due to proteolytic cleavage of an immunoreactive RNP-associated protein M_r 78×10^3.

2. MATERIALS AND METHODS

The 10 S iRNP containing 4 S iRNA, and the 20–40 S free mRNP particles were isolated from the postpolysomal supernatant of 14-day chick embryonic leg and breast muscle by a combination of ultracentrifugation and sucrose gradient fractionation, as in [12,13]. The 10 S iRNP was further purified by gel filtration on ultrogel ACA34 [13]. The polysomal mRNP and its poly(A) protein segment was isolated from the 0.5 M KCl–sucrose-washed EDTA-dissociated polysomes by oligo-(dT)-cellulose chromatography [17,18].

For the isolation of protein components of RNP, three methods were used. The RNP particles were deproteinized by extraction with phenol/$CHCl_3$/isoamyl alcohol and the proteins were precipitated with acetone from the phenol layer [13]. Alternatively the RNP particles were digested with RNase T_2 in 50 mM sodium acetate buffer, pH 5.0 at 37°C for 1 h and dialyzed against 10 mM Tris–HCl (pH 7.4), 1 mM EDTA, 5 mM $MgCl_2$ and 50 mM NaCl, and the material was lyophilized. The proteins of 10 S iRNP were isolated by DEAE-cellulose chromatography of the RNP under dissociating conditions, as in [19]. The proteins were analyzed by one- and two-dimensional SDS–polyacrylamide gel electrophoresis (SDS–PAGE), as in [13].

For the detection of immunoreactive polypeptides, the proteins following SDS–PAGE, were blotted to nitrocellulose sheet (Millipore) as in [20]. The blot was preincubated for 1 h at 25°C with 2.5% bovine serum albumin and 5% horse serum in buffer A (10 mM Tris (pH 7.4) 0.15 M NaCl). After washing with buffer A the blot was incubated overnight with a solution containing purified anti-CBP monoclonal antibody [7] at 1–2 μg/ml, 1% bovine serum albumin and 0.5% horse serum in buffer A. After washing the blot with buffer A it was reacted with horseradish peroxidase conjugated goat anti-mouse IgG fraction and the immunoreactive bands were stained with a mixture of diaminobenzidine (1 mg/ml), imdiazole (1 mg/ml) and 0.01% H_2O_2 [20].

For peptide mapping, the protein bands were sliced from stained SDS–polyacrylamide gels, the protein was labeled with $Na^{125}I$ (0.4 mCi/slice) and digested with trypsin in gel slices [21]. The resulting peptides were eluted from the gel and analyzed on Polygram-cellulose 300 thin-layer chromatographic plate. The first dimension was electrophoresis in pyridine/acetic acid/acetone/water (1:2:8:40, by vol.) at pH 4.4 for 75 min at 800 V. The second dimension was chromatography in *n*-butanol/acetic acid/water/pyridine (15:3:12:10, by vol.) for 5–6 h. The thin-layer plates were exposed to Kodak XR-5 films for autoradiography.

3. RESULTS AND DISCUSSION

Fig. 1 shows the Coomassie blue-stained SDS–PAGE band patterns of proteins present in different RNP particles of chick embryonic muscle, and the corresponding immunostaining patterns obtained after reaction with anti-CBP monoclonal antibody. In agreement with [12,13,15,16], a complex set of proteins (M_r 15 000–150 000) is present in both 20–40 S free mRNP particles (A, gel 1) and the 10 S iRNP particles (B, gel 1). The polysomal mRNP particles (C, gel 1), with a relatively simple protein pattern (M_r 35 000–78 000) [8,18] gave no detectable immunostaining after reaction with anti-CBP antibody (C, gel 2). Various preparations of polysomal mRNP isolated by different techniques such as oligo(dT)-cellulose chromatography of EDTA-dissociated polysomes and differential elution of the bound mRNP particles at 25°C and 45°C to yield subpopulations of polysomal mRNP, as in [22] were also tested in order to confirm the absence of CBP-related polypeptides in these particles. None of these preparations showed any immunostained band (not shown).

These results suggest that the CBP-related protein (M_r 78 000) identified in free mRNP and the 10 S iRNP is distinct from the poly(A)-bound protein (M_r 78 000), which is present in polysomal mRNP particles of a wide variety of eukaryotic cells [9, 17, 23]. This view was further confirmed

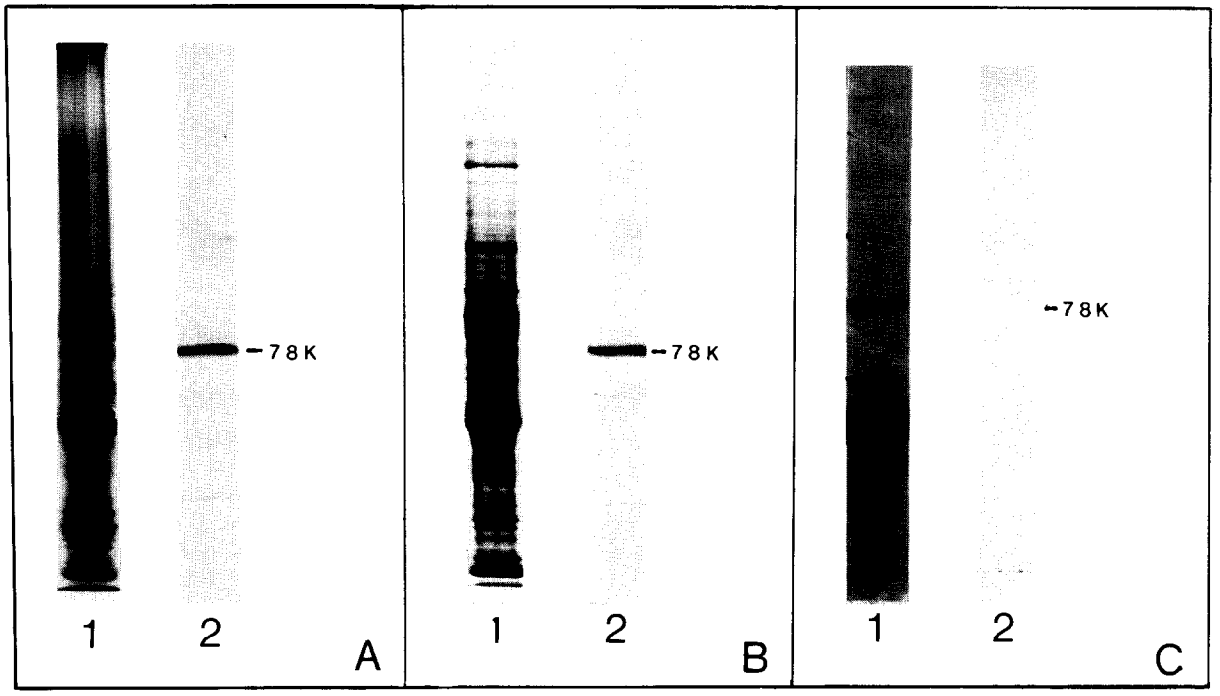


Fig. 1. Reaction of anti-CBP monoclonal antibody with different cytoplasmic RNP particles of chick embryonic muscle. For details see section 2: (A) 20–40 S free mRNP particle, 85 μg protein was used for Coomassie blue staining (gel 1) and immunostaining (gel 2); (B) 10 S iRNP particle, 95 μg protein was used for Coomassie blue staining (gel 1) and immunostaining (gel 2); (C) polysomal mRNP, 60 μg protein was used for Coomassie blue staining (gel 1) and immunostaining.

by our observation that the 10–12 S poly(A)–protein segment isolated from polysomal mRNP [8] and containing the poly(A)-bound M_r 78 000 protein [8] did not give any immunostaining with anti-CBP antibody (not shown).

When the protein components isolated from 20–40 S free mRNP and iRNP were analyzed by immunostaining, the results showed different patterns than those obtained with intact particles. Immunostained components detected in isolated proteins of free mRNP particles corresponded to a major new band, M_r 43 000 and a much less intense band, M_r 78 000 (fig. 2A, gel 2). With the proteins of 10 S iRNP a single immunoreacting component, M_r 29 000 was observed (2B, gel 3). The relative intensities of the M_r 78 000 band and the lower M_r new bands (fig. 1, 2) appear to follow a reciprocal relationship. This implies that the M_r 43 000 and 29 000 bands may have resulted from cleavage of the M_r 78 000 polypeptide during the isolation of the proteins of RNP particles. Interestingly, when proteins of 10 S iRNP were stored in 8 M urea, a single immunostained band of M_r 78 000 (fig. 2B, gel 2), similar to the pattern obtained with intact particles (fig. 1) was observed implying that the presumed proteolytic cleavage of M_r 78 000, CBP-related protein was inhibited under these conditions.

We also examined the possibility that CBP-related polypeptides may remain associated with polysomes. Fig. 3 shows the Coomassie blue-staining patterns of proteins of polysomes washed with 0.25 M or 0.5 M KCl. Whereas the staining intensity of most proteins was found to be similar, some of the protein bands are present in decreased amounts in 0.5 M KCl-washed polysomes suggesting that these proteins are not integral ribosomal components (cf. lane 1 *vs* 2). A single immunostained protein, M_r 43 000, is found in both polysomal preparations (lanes 3, 4) but its intensity is less in 0.5 M KCl-washed polysomes (lane 4). Since no immunostained band is detected in

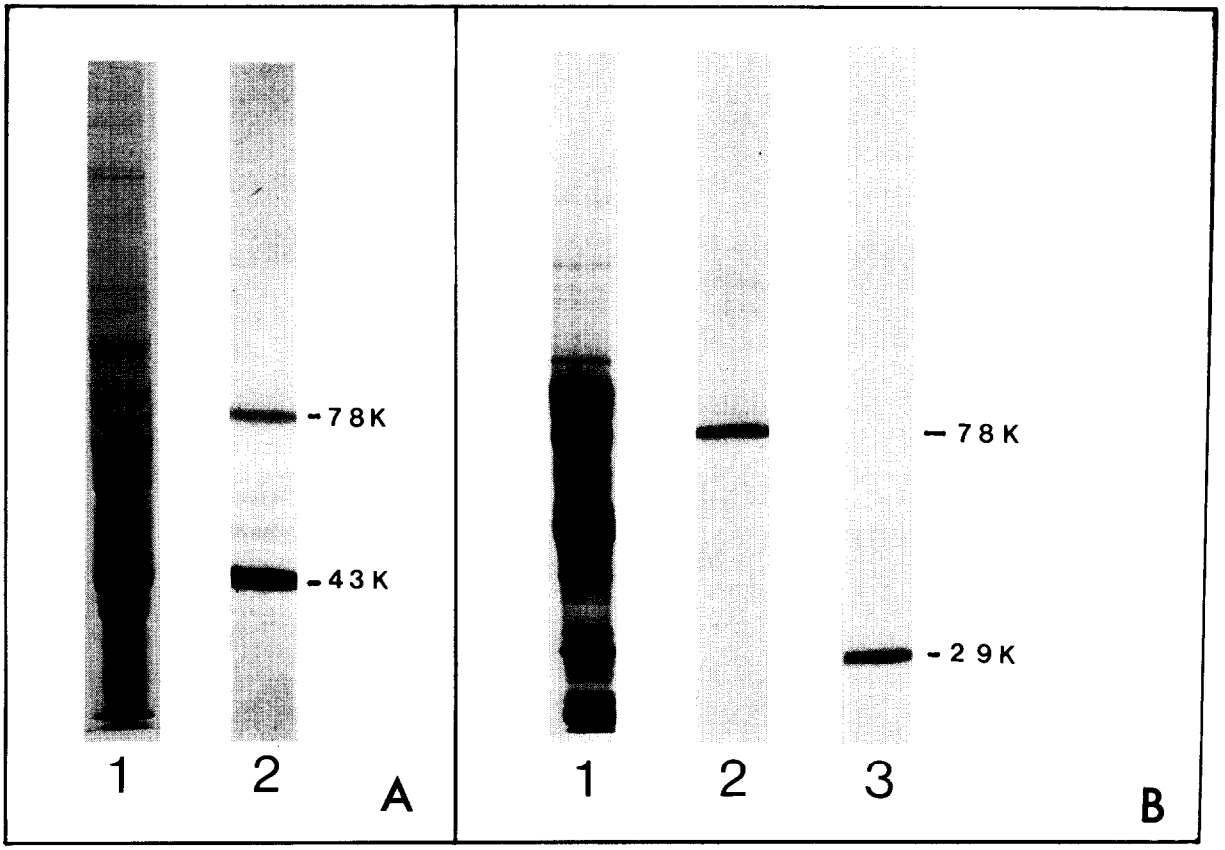
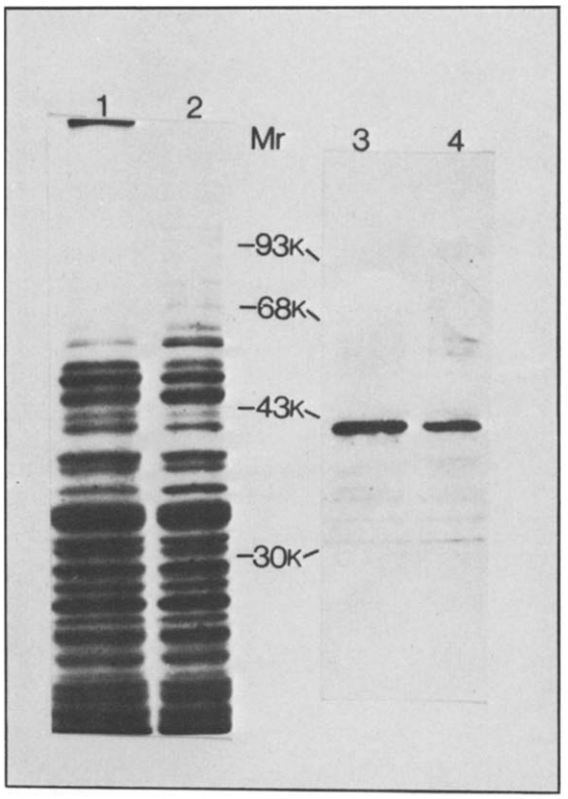


Fig. 2. Reaction of anti-CBP monoclonal antibody with proteins isolated from cytoplasmic RNP particles of chick embryonic muscle. For details see section 2 and fig. 1. (A) Proteins isolated from 20–40 S free mRNP particle: (1) Coomassie blue staining; (2) immunostaining. (B) Proteins isolated from 10 S iRNP: (1) Coomassie blue staining; (2) immunostaining of proteins stored in 8 M urea; (3) immunostaining of proteins stored in the absence of 8 M urea.

polysomal mRNP samples (fig. 1), it is quite likely that the M_r 43000 CBP-related protein located in polysomes, may have been lost during the isolation of polysomal mRNP. Anti-CBP antibody reacts with M_r 50000 protein located in reticulocyte polysome [7] and in the cytoskeletal fraction in BHK cells [24]. These results imply that the M_r 50000 CBP-related protein may be involved in binding of mRNA to cytoskeleton, which is believed to be a requisite for translation [25]. The M_r 43000 CBP-related polypeptide in chick embryonic muscle polysome (fig. 3) may function in an analogous manner to the above-mentioned M_r 50000 CBP-related protein in [7,24].

The nature of the immunoreactive M_r 78000 polypeptide identified in the 10 S iRNP and the 20–40 S free mRNP particles was further examined by 2D-PAGE and peptide mapping. The major immunostained band obtained by 2D-PAGE in the case of 10 S iRNP and the 20–40 S free mRNP particles had M_r 78000 and pI ~7.0 (fig. 4A, B). Peptide mapping using a sequential combination of electrophoresis and chromatography on thin-layer plate [21] shows that the M_r 78000 polypeptides present in 20–40 S free mRNP (fig. 5A) and 10 S iRNP (fig. 5B) share several common peptides (indicated by arrows). The immunoreactive M_r 78000 polypeptides found in the two types of RNP particles according to these results appear to be structurally related. The presence of CBP-related M_r 78000 polypeptide in the 10 S iRNP is somewhat surprising, since both iRNA and iRNP are potent inhibitors of in vitro translation of capped and uncapped mRNAs [12–14]. Determina-



tion of nucleotide sequence at the 5-terminus of iRNA, which is now in progress in our laboratory, will be helpful in understanding the interaction of CBP-related polypeptides with iRNA.

A number of immunoreactive polypeptides (M_r 78 000, 43000 and 29 000) are identified in the free mRNP particles and the 10 S translation inhibitory RNP of chick embryonic muscle by reaction with anti-CBP antibody. Their molecular sizes are similar to those reported for the CBP-related proteins isolated from other eukaryotic cells [6,7,24]. The M_r 78 000 polypeptide appears to be the single immunoreactive species found in intact RNP. In contrast, the lower-M_r proteins (M_r 43 000 and 29 000) appear in preparations of the isolated pro-

Fig. 3. SDS–PAGE patterns of chick embryonic muscle polysomes and the corresponding anti-CBP cross-reacting immunostained band patterns. For details see section 2: (1) Coomassie blue staining of SDS gels of 0.25 M KCl-washed polysomes; (3) immunostaining of the corresponding nitrocellulose blot; (2) Coomassie blue staining of SDS gels of 0.5 M KCl-washed polysomes; (4) immunostaining of the corresponding nitrocellulose blot.

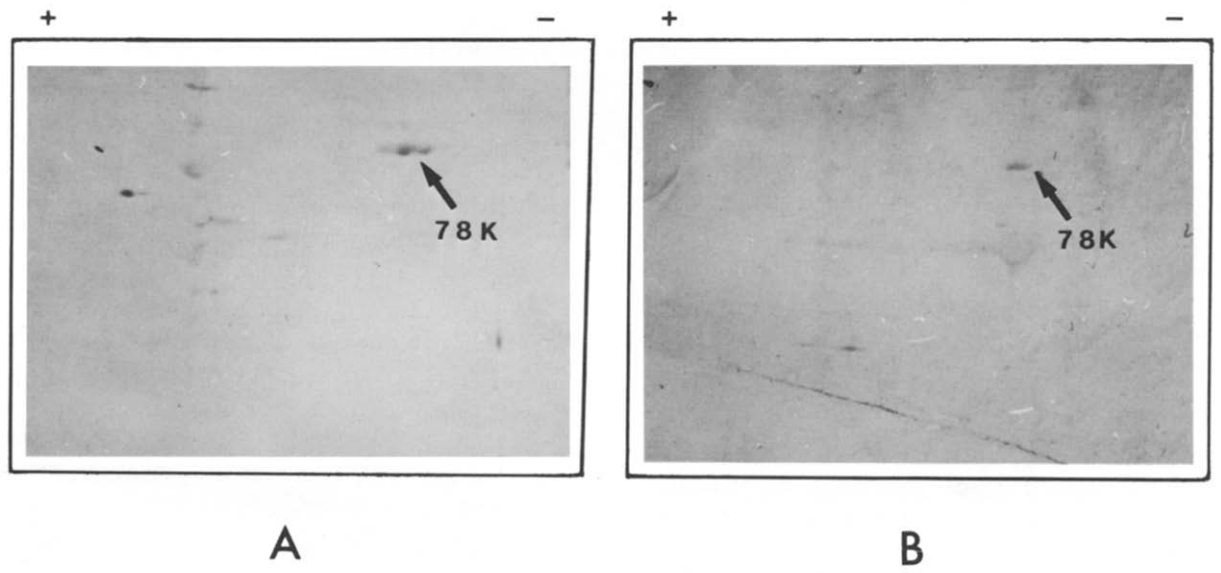


Fig. 4. Immunostained band patterns obtained with two-dimensional gel electrophoresis of cytoplasmic RNP particles of chick embryonic muscle. For details see section 2: (A) 20–40 S free mRNP particle; (B) 10 S iRNP particle.

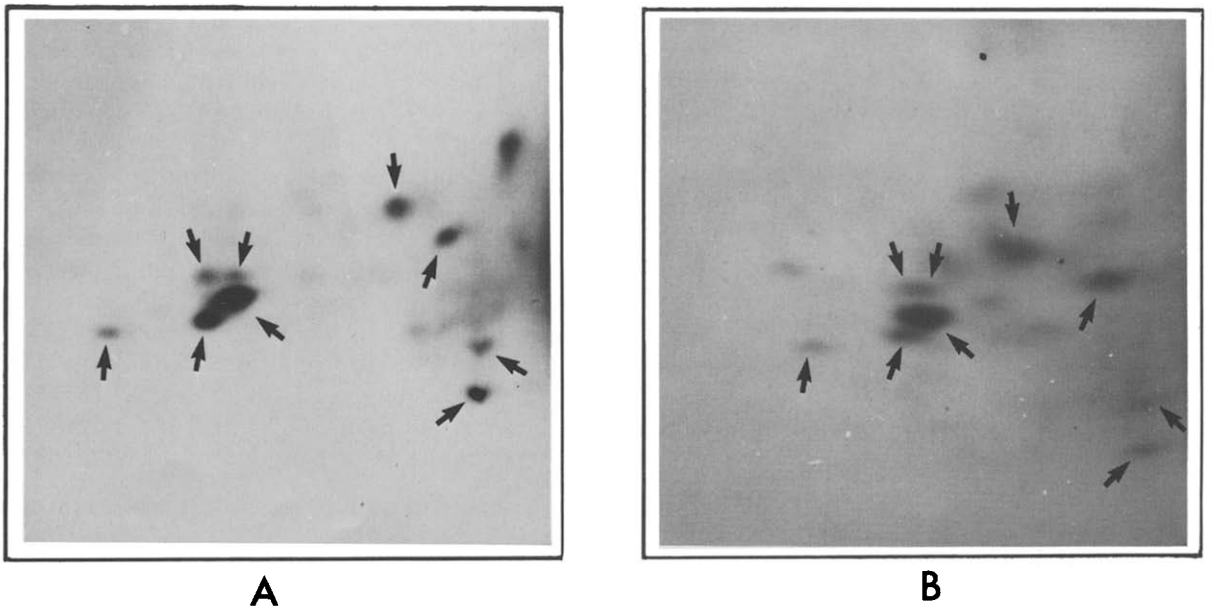


Fig. 5. Tryptic peptide patterns of the M_r 78000 immunoreactive polypeptides isolated from cytoplasmic RNP particles of chick embryonic muscle. For details see section 2. Electrophoresis was from right to left (first dimension) and chromatography was from bottom to top (second dimension). The bottom right corner represents the origin. The autoradiograph is shown: (A) M_r 78000 polypeptide from 20–40 S free mRNP particle; (B) M_r 78000 polypeptide from 10 S iRNP particle.

teins presumably due to cleavage of the M_r 78000 polypeptide. The immunoreactive M_r 78000 protein identified in cytoplasmic RNP is distinct from the poly(A)-bound mRNP-protein of similar M_r-value known to be present in mRNP particles from wide variety of eukaryotic cells [9,17,23]. The observation that polysomes contain a single immunoreactive protein, M_r 43000, in contrast to the M_r 78000 polypeptide present in free mRNP particles suggests that the CBP-related proteins undergo a dynamic exchange during the transit of mRNA from the free mRNP pool to polysomes. Such a dynamic exchange for the majority, if not all of the mRNA-associated proteins, was postulated in [18,26,27] on the basis of subcellular localization and biological activity of eukaryotic mRNAs.

Some of these results agree with those obtained by Drs H. Trachsel and A. Vincent who have independently identified CBP-related polypeptides in cytoplasmic mRNP particles of reticulocytes by reaction with anti-CBP antibody (personal communication).

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