Proc. Natl. Acad. Sci. USA
Vol. 76, No. 9, pp. 4345–4349, September 1979
Biochemistry

Eukaryotic mRNA cap binding protein: Purification by affinity chromatography on Sepharose-coupled m^7GDP

(7-methylguanosine/eukaryotic initiation factors/protein synthesis)

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Communicated by John M. Buchanan, June 11, 1979

ABSTRACT **A 24,000-dalton polypeptide that binds strongly and can be specifically crosslinked to the 5′-terminal cap structure m^7GpppN in eukaryotic mRNAs has been detected in protein synthesis initiation factor preparations [*Proc. Natl. Acad. Sci. USA* (1978) 75, 4843–4847]. This polypeptide has been purified to apparent homogeneity by one chromatographic passage through an affinity resin prepared by coupling the levulinic acid $O^{2',3'}$-acetal of m^7GDP to AH-Sepharose 4B. Translation, in HeLa cell extracts, of capped mRNAs including Sindbis virus, reovirus, and rabbit globin mRNAs was stimulated by the cap-binding protein under conditions that did not increase translation of noncapped RNAs of encephalomyocarditis virus and satellite tobacco necrosis virus.**

A striking structural feature of most eukaryotic mRNAs that distinguishes them from prokaryotic mRNAs is the 5′-terminal "cap," m^7GpppN (1). This modification occurs on nascent pre-mRNA chains and is conserved as a stabilizing element in mature mRNA molecules (2–5). Eukaryotic and prokaryotic mRNAs are also functionally distinct. In contrast to prokaryotic protein synthesis, which initiates at multiple internal AUG codons in polycistronic mRNA, translation of many if not all eukaryotic mRNAs begins at a single site, the 5′-proximal AUG triplet. Recently, a scanning mechanism has been proposed to explain how eukaryotic ribosomes select the initiator codon (6, 7). In this model the small ribosomal subunit binds initially at or near the 5′ terminus and only subsequently repositions at the adjacent AUG as the large subunit joins to begin peptide bond synthesis.

Results of numerous studies have shown that the 5′-terminal cap promotes translation by facilitating initiation complex formation (1). It has been suggested further that the cap is recognized as part of the mechanism of ribosome attachment to mRNA (8). Consistent with this suggestion, eukaryotic initiation factor preparations contain a polypeptide of apparent molecular weight 24,000 that interacts specifically with the cap and can be crosslinked to the 5′-m^7G of oxidized mRNA (9). This cap-binding protein was found in less than stoichiometric amounts in purified eIF-3 and eIF-4B (9), factors involved in the binding of natural mRNAs to initiation complexes (10). The same factors added in excess to reticulocyte lysate increased the weak translational activity of chemically decapped vesicular stomatitis virus mRNAs by severalfold to a level of about 50% of that of the corresponding capped mRNAs (11). Sucrose gradient sedimentation separated this stimulatory activity from eIF-3 and eIF-4B but it copurified with the cap-crosslinking 24,000-dalton polypeptide (11). These findings as well as other reports (12–15) suggest a regulatory role for eIF-4B and possibly other eukaryotic initiation factors. However, because the purified eIF-4B used in previous studies contained, in addition to the main component of M_r 80,000, small amounts of the 24,000-dalton cap-binding polypeptide, the discriminatory properties could have been due to the presence of the cap-binding minor constituent. This uncertainty emphasizes the need to purify the cap-binding protein for comparative functional studies on capped and uncapped RNAs. For this purpose, we have synthesized the levulinic acid $O^{2',3'}$-acetals of m^7GDP and GDP and utilized these species as ligands for affinity chromatography after coupling to AH-Sepharose 4B. Homogeneous cap-binding protein was obtained from rabbit reticulocyte initiation factors by a single chromatographic step.

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MATERIALS AND METHODS

Preparation of Affinity Resins. Synthesis of levulinic acid acetals of m^7GDP and GDP was carried out by a modification of the procedure of Seela and Waldek (16). The products were lyophilized and stored at −70°C in 5.6-μmol aliquots corresponding to 50 A_{258} units and 75 A_{253} units for the m^7GDP and GDP derivatives, respectively. Immobilization of the nucleotide derivatives by coupling to resin was done as described (16). AH-Sepharose 4B (0.125 g) was swollen in 10 ml of 0.5 M NaCl and washed on a fritted glass filter with 25 ml of 0.5 M NaCl followed by 25 ml of H_2O. Ligand (5.6 μmol dissolved in 1 ml of H_2O) was added to a 0.5-ml sample of wet resin, and the pH was adjusted to 5.5–6.0 with $NaHCO_3$. After gentle mixing for 10 min, 29 mg of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride was added (0.1 M final) and the suspension was shaken at pH 5.5–6.0 at room temperature for 24 hr. The resin was collected by filtration, washed successively with 25 ml each of 0.1 M $NaHCO_3$ (pH 8), 1 mM HCl (pH 3), 0.5 M NaCl, H_2O, and, finally, buffer A [20 mM Hepes, pH 7/1 mM dithiothreitol/0.2 mM EDTA/10% (vol/vol) glycerol] containing 0.1 M KCl. The ligands bound covalently to the resin to the extent of 30–45% of the starting material. The resins were packed in 1-ml syringes and could be stored in buffer A for about 1 month at 5°C before loss of binding activity became apparent.

Initiation Factors. High-salt wash of reticulocyte ribosomes was fractionated by ammonium sulfate precipitation as described (17). The 0–40% saturation ammonium sulfate cut was sedimented in a 10–40% glycerol gradient (SW 41 rotor, 40,000 rpm, 20 hr) containing 0.1 M KCl in buffer A. Fractions from the top half of the gradient were pooled for affinity chromatography.

Chromatography. Fractions were loaded onto a 1 × 0.7 cm column of m^7GDP-Sepharose or GDP-Sepharose equilibrated with 0.1 M KCl in buffer A. The columns were washed with 50 ml of the same buffer and eluted as described.

Crosslinking and Gel Analysis. Reovirus mRNA *methyl*-^3H-labeled in the 5′-terminal cap m^7GpppG^m was synthesized

Abbreviation: $NaDodSO_4$, sodium dodecyl sulfate.

and periodate-oxidized to convert the 5′-linked m^7G to the dialdehyde. Protein fractions and oxidized mRNA were incubated, and the complexes formed were stabilized by reduction with $NaBH_3CN$. After treatment with RNase, the polypeptides that were radioactive as a consequence of crosslinked ^3H-labeled caps were analyzed by sodium dodecyl sulfate ($NaDodSO_4$)/polyacrylamide gel electrophoresis and fluorography as described (9, 18).

Iodination of the Purified Cap-Binding Polypeptides. Iodination was done by a modification of the Hunter–Bolton technique (19). The M_r 24,000 polypeptide which was labeled to a specific activity of 3×10^6 cpm of ^{125}I per μg of protein was analyzed as described (18, 20).

Cell-Free Translation. Protein-synthesizing extracts of mock-infected and type 1 poliovirus-infected HeLa cells were prepared by procedures similar to those of Rose *et al.* (15).

RESULTS

Previously it was shown (9) that one polypeptide in eukaryotic initiation factor preparations binds specifically to the m^7G cap of mRNA with high affinity. Because affinity chromatography has already proven to be a powerful technique for identifying and purifying polypeptides and other molecules with specific binding properties, we decided to use this approach to obtain pure cap-binding protein for functional studies. The choice of affinity resin was made on the basis that alterations in the sugar moiety did not abolish cap activity—e.g., cap-binding protein recognizes and can be crosslinked to the m^7G-2′,3′-dialdehyde of oxidized mRNA (9) and oxidized m^7GDP and 2′-deoxy-m^7GDP inhibit protein synthesis initiation complex formation as effectively as does m^7GDP (unpublished results; ref. 21). m^7GDP and GDP were converted to their levulinic acid $O^{2',3'}$-acetals and then coupled through the free carboxylates to the aminocaproyl spacer chains of Sepharose 4B by carbodiimide-mediated peptide bond formation.

The partially purified initiation factor preparation that was used as starting material for affinity chromatography contained many different polypeptides as measured by $NaDodSO_4$/polyacrylamide gel electrophoresis and Coomassie blue staining (Fig. 1, lane 1). Aliquots of the protein mixture were loaded onto columns of m^7GDP-Sepharose and GDP-Sepharose. Most (>85%) of the proteins were not retained, and the gel profiles of the unbound material from both resins and the starting material were similar (lanes 1, 2, and 4). The proteins retained by the resins were eluted by increasing the KCl concentration from 0.1 M to 1.0 M. By stained gel analysis, the spectrum of proteins eluted from the GDP-Sepharose differed somewhat from the unbound sample, and the retained polypeptides were enriched for material in the M_r range ≈75,000 (lane 5). The material bound by m^7GDP-Sepharose included polypeptides of the same M_rs as in the fraction retained on GDP-Sepharose but was also enriched for several other polypeptides. Most notable was the appearance of a new stained band of apparent M_r 24,000 (lane 3).

To determine if this polypeptide corresponded to the 24,000-dalton cap-binding protein described previously (9), the

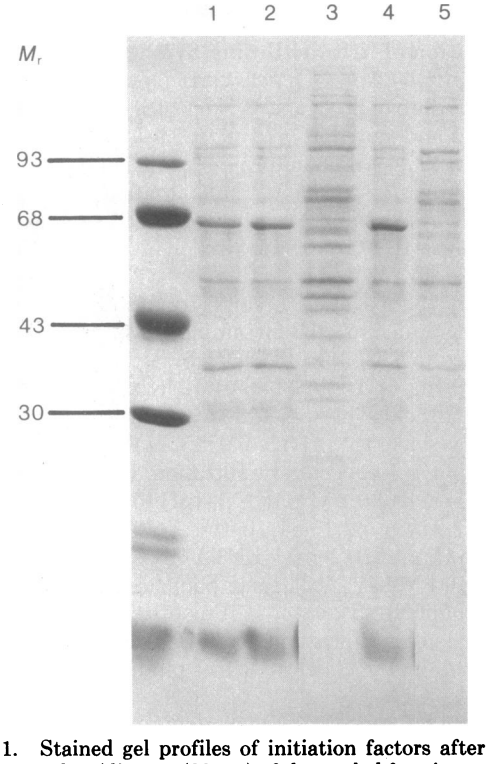

FIG. 1. Stained gel profiles of initiation factors after affinity chromatography. Aliquots (38 mg) of the pooled fractions of factors from the upper half of a glycerol gradient were loaded onto m^7GDP-Sepharose and GDP-Sepharose in buffer A containing 0.1 M KCl. After the flow-through fraction was collected, the column was washed with 50 ml of the same buffer. Bound material was then eluted in buffer A containing 1.0 M KCl. Samples were analyzed by electrophoresis for 14 hr at 60 V on 10 cm $NaDodSO_4$/10–18% polyacrylamide gels and stained with Coomassie blue. Marker proteins included rabbit muscle phosphorylase a (93,000), bovine serum albumin (68,000), ovalbumin (43,000), and carbonic anhydrase (30,000). Lanes: 1, 14 μg of protein loaded onto each resin; 2, 15 μg from a total of 33 mg of protein not retained by m^7GDP-Sepharose; 3, 10 μg of 3 mg of protein bound to m^7GDP-Sepharose; 4, 16 μg of 32 mg of protein not retained by GDP-Sepharose; 5, 6.5 μg of 3 mg of protein bound to GDP-Sepharose. M_r is shown $\times 10^{-3}$.

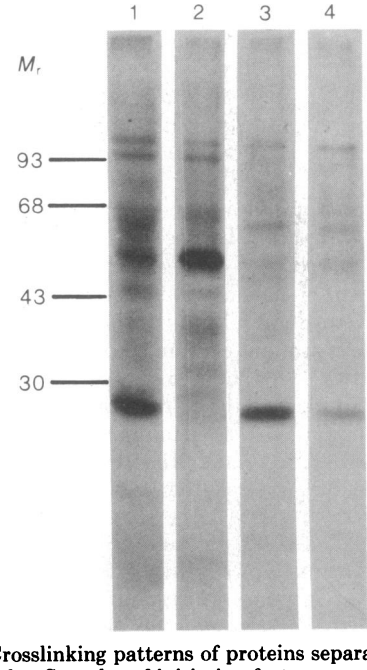

FIG. 2. Crosslinking patterns of proteins separated by affinity chromatography. Samples of initiation factors were separated into bound and unbound fractions by affinity chromatography on m^7GDP-Sepharose and GDP-Sepharose as for Fig. 1. The fractions were crosslinked to *methyl*-^3H-labeled oxidized reovirus mRNA and analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis and fluorography. Lanes: 1, 62 μg of protein not retained by GDP-Sepharose; 2, 96 μg of protein not retained by m^7GDP-Sepharose; 3, 6 μg of protein bound to m^7GDP-Sepharose and eluted in 1 M KCl; 4, 6 μg of protein bound to GDP-Sepharose and eluted in 1 M KCl. M_r is shown $\times 10^{-3}$.

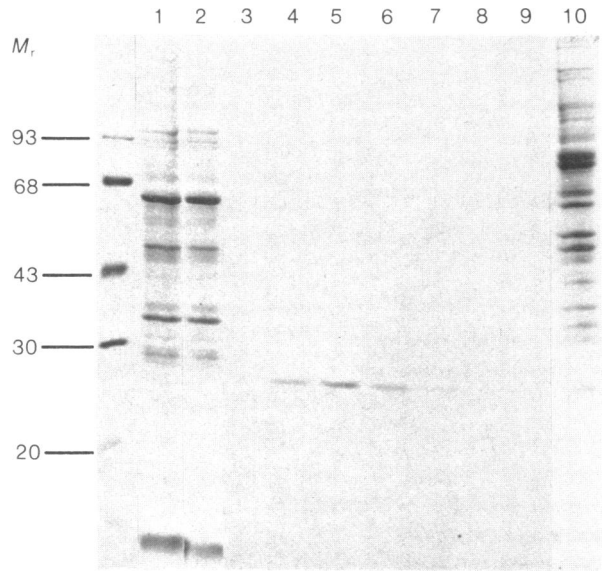

FIG. 3. Stained gel profile of 24,000-dalton polypeptide purified by binding to m^{7}GDP-Sepharose and elution with m^{7}GDP. Partially purified initiation factors from the upper half of a glycerol gradient were pooled, and 38 mg of protein was loaded onto m^{7}GDP-Sepharose in buffer A containing 0.1 M KCl. After a wash with 50 ml of the loading solution, the column was eluted with 3 ml of buffer A plus 0.1 M KCl plus 0.07 mM m^{7}GDP, and 10 equal fractions were collected. Finally, the column was eluted with 3 ml of buffer A plus 1.0 M KCl. Samples were analyzed by NaDodSO$_4$/polyacrylamide gel electrophoresis with marker proteins including soybean trypsin inhibitor (20,000). Lanes: 1, 19 μg of loaded sample; 2, column flow-through fraction, 17 μg from 30 mg of total protein; 3–9, 30-μl aliquots of each of the m^{7}GDP-eluted fractions; 10, 1 M KCl eluate, 10 μg from 4 mg. Pooled fractions 4–9 yielded 40 μg of 24,000-dalton polypeptide after dialysis against buffer A plus 0.1 M KCl. M_r is shown $\times 10^{-3}$.

resin-bound and unbound fractions were crosslinked to 5′-*methyl*-^{3}H-labeled, oxidized mRNA and analyzed by gel electrophoresis and fluorography. The material that passed through the GDP-Sepharose contained a radioactive band of M_r 24,000 and several crosslinked polypeptides of higher M_r (Fig. 2, lane 1). These larger polypeptides were also found in the m^{7}GDP-Sepharose flow-through fraction, but the 24,000-dalton polypeptide was completely retained on the resin and was missing from this fraction (lane 2). When the salt concentration was increased to 1 M KCl, the cap-binding polypeptide was eluted and was readily detected by crosslinking (lane 3). A fraction of this polypeptide was also retained by GDP-Sepharose and eluted in high salt (lane 4). In other experiments (not shown), crosslinking of the 24,000-dalton polypeptide but not the higher M_r polypeptides was prevented by 0.4 mM m^{7}GDP and not by GDP, consistent with the described (9) specific interaction of this protein with the m^{7}G cap of mRNA. The polypeptides that crosslinked nonspecifically included a prominent band of M_r ≈50,000 (lane 2) which corresponds to EF-1 (9), a factor that binds strongly to RNA (22).

In order to improve the specificity of the affinity procedure, samples were loaded onto m^{7}GDP-Sepharose in 0.1 M KCl but were eluted with 0.07 mM m^{7}GDP before addition of 1 M KCl. Again, more than 75% of the proteins in the factor preparation did not bind to the column, and stained gel analysis indicated that the load and flow-through fractions were also qualitatively similar (Fig. 3, lanes 1 and 2). The fractions that eluted in buffer containing m^{7}GDP contained exclusively a 24,000-dalton polypeptide (Fig. 3, lanes 3–9). When the salt level was increased to 1 M KCl, the residual bound proteins were released. They included a spectrum of stained bands and only a small proportion of the 24,000-dalton material (lane 10).

The fractions eluted with m^{7}GDP (corresponding to lanes 4–9 in Fig. 3) were pooled, dialyzed against buffer A containing 0.1 M KCl to remove the m^{7}GDP, and assayed for ability to crosslink to the 5′-terminal m^{7}G of oxidized mRNA. Consistent with cap-binding activity, the purified 24,000-dalton polypeptide was crosslinked, and the interaction was blocked by m^{7}GDP (Fig. 4). To test its purity further, the 24,000-dalton polypeptide was analyzed in one- and two-dimensional gel systems after iodination with ^{125}I by the Bolton–Hunter technique (19). A single radioactive polypeptide of the expected M_r was obtained in the NaDodSO$_4$/polyacrylamide gel (Fig. 5). In the two-dimensional system all the radioactivity again migrated as a single band with a M_r of 24,000. However, by isoelectric focusing the iodinated protein was partially resolved

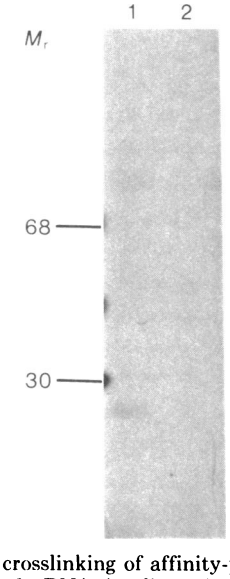

FIG. 4. Specific crosslinking of affinity-purified 24,000-dalton polypeptide to 5′-cap of mRNA. An aliquot (≈20 ng) of the pooled and dialyzed fractions corresponding to the material analyzed in lanes 4–9 of Fig. 3 was crosslinked to *methyl*-^{3}H-labeled oxidized reovirus mRNA in the absence (lane 1) or presence (lane 2) of 0.4 mM m^{7}GDP. Samples were analyzed by NaDodSO$_4$/polyacrylamide gel electrophoresis and fluorography as described (9, 18) except that 5 μg of bovine serum albumin was added as carrier after the crosslinking reaction. M_r is shown $\times 10^{-3}$.

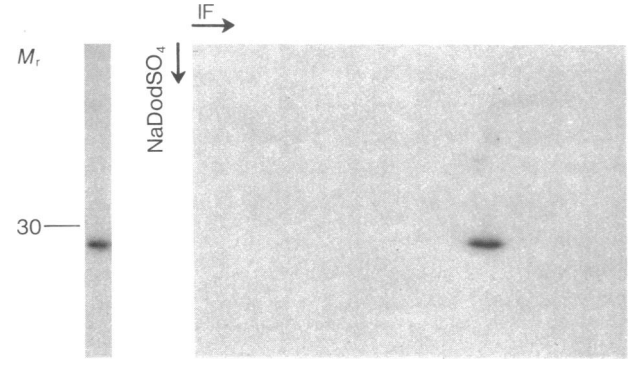

FIG. 5. Autoradiogram of ^{125}I-labeled 24,000-dalton polypeptide after one- and two-dimensional gel analysis. The polypeptide purified by adsorption to m^{7}GDP-Sepharose and elution in m^{7}GDP as in Fig. 3 was iodinated by the Bolton–Hunter procedure (19). Radioactive protein (20,000 cpm; ≈7 ng) was analyzed by electrophoresis in a NaDodSO$_4$/10–18% polyacrylamide slab gel with carbonic anhydrase (30,000) as marker and also by isoelectric focusing (IF) followed by gel electrophoresis as described by O'Farrell (20).

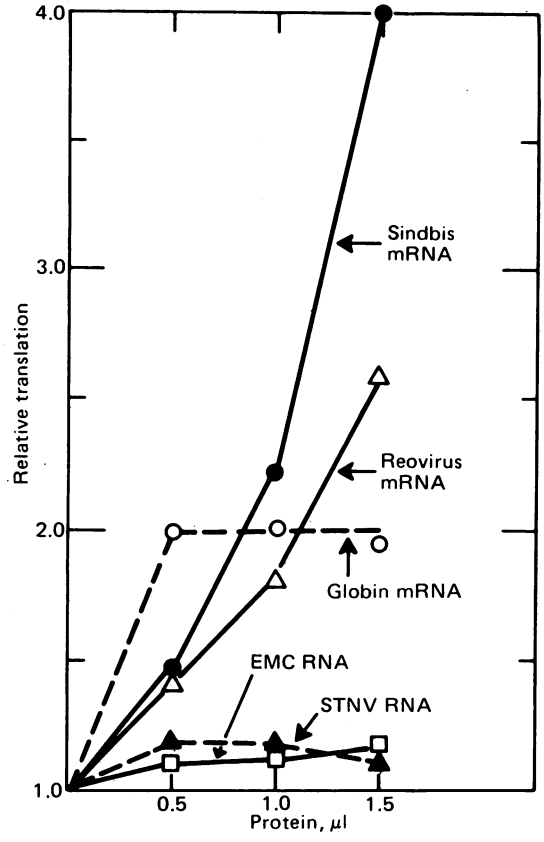

FIG. 6. Effect of purified protein on the translation of capped and noncapped RNAs in HeLa cell extracts. Protein synthesis was done as described (15) except that 25-μl reaction mixtures contained 1–2 μg of mRNA, 1 mM Mg acetate, and 90 mM K acetate in addition to 36 mM KCl contributed by the extract. Incorporation of [^{35}S]methionine (16 μCi; 643 Ci/mmol) into acid-precipitable products was assayed after 60 min at 37°C by spotting 5-μl aliquots on Whatman 3 MM filter paper discs and assaying radioactivity in toluene-based scintillant after successive washing in 7% trichloroacetic acid (20 min at 100°C), 5% trichloroacetic acid at room temperature, ethanol, and ether. Values set at 1.0 were obtained without adding 24,000-dalton polypeptide as follows: Sindbis virus mRNA, 32,471 cpm (● — ●); reovirus mRNA, 18,013 cpm (△ — △); rabbit globin mRNA, 14,143 cpm (○ -- ○); mouse encephalomyocarditis virus (EMC virus) RNA, 89,569 cpm (□ — □); and satellite tobacco necrosis virus (STNV) RNA, 7662 cpm (▲ -- ▲) after subtracting from all values 4680 cpm incorporated minus mRNA. Procedures for preparing Sindbis virus mRNA (23), reovirus mRNA (8), and EMC virus RNA (24) have been described; globin mRNA and STNV RNA were kindly provided by M. Innis (Roche Institute) and J. M. Clark, Jr. (University of Illinois). The concentration of affinity-purified dialyzed 24,000-dalton polypeptide in buffer A/0.1 M KCl/0.01% bovine serum albumin was estimated as 10 ng/μl on the basis of comparing stained gels containing known amounts of 24,000-dalton polypeptide prepared without albumin.

into two spots of pI = 5.2–5.7, suggesting that some of the molecules were differentially modified in a way that altered the net charge.

Because the cap-binding protein appears to be homogeneous after affinity chromatography on m^{7}GDP-Sepharose, we initiated functional characterization of it. Translation, in HeLa cell extracts, of capped Sindbis virus, reovirus, and rabbit globin mRNAs was stimulated 2- to 4-fold by 24,000-dalton polypeptide (Fig. 6). Gel analyses indicated that the synthesis of authentic products was stimulated (data not shown). Because a crude system was used to detect the stimulatory activity of the purified protein, the stoichiometry is difficult to establish. However, only nanogram amounts of protein were required to elicit stimulatory activity from microgram amounts of mRNA. By contrast, the purified cap-binding protein had little effect on the translation of two naturally noncapped RNAs, from mouse encephalomyocarditis virus (EMC) and satellite tobacco necrosis virus (STNV).

DISCUSSION

Eukaryotic initiation factors previously have been assayed for cap-binding activity by retention of mRNA–protein complexes on nitrocellulose membrane filters. By this method two different factors, eIF-4B and eIF-2, were found to contain cap-binding activity (14, 25). However, we (9, 26) as well as others (22, 27), found that most of the initiation factors have the capacity to bind ribosomal RNAs as well as mRNA. Moreover, by the filter method, m^{7}G nucleotides were found to inhibit nucleoprotein complex formation nonspecifically (26), making it difficult to detect mRNA-specific interactions. As an alternative assay for identifying proteins that interact with the 5′-terminal m^{7}G of capped mRNAs, we developed a cap crosslinking method that depends upon covalent bond formation between protein(s) and *methyl*-^{3}H-labeled m^{7}GpppN in oxidized capped mRNA (9, 18). In this assay, cap-specific crosslinking reactions were inhibited by m^{7}GDP but not by GDP.

With this approach, a cap-specific polypeptide of apparent M_r 24,000 by NaDodSO$_4$/polyacrylamide gel electrophoresis was found in rabbit and mouse initiation factors eIF-3 and eIF-4B (9). The low amount of 24,000-dalton polypeptide relative to the multiple subunits in purified eIF-3 and to the major 80,000-dalton constituent in eIF-4B suggested that the cap-binding activity might be a separate functional entity that copurified by adventitious adsorption to these factors. Consistent with this suggestion, the polypeptide in eIF-3 that crosslinked to ^{3}H-labeled mRNA migrated faster than the stained band corresponding to the high molecular weight factor by polyacrylamide gel electrophoresis under nondenaturing conditions (unpublished results). Furthermore, the bulk of the cap-binding activity sedimented in a sucrose gradient containing 0.4 M KCl together with other proteins at a position between the eIF-3 and eIF-4B activities (11). Sedimentation of the cap crosslinking activity in a position corresponding to a M_r of about 200,000 may be due to adsorption to other proteins in the partially purified factor preparation. Alternatively, the cap-binding activity may exist in the native, functional state as a multimeric protein.

As a first step toward elucidating the structure and possible roles of cap-binding proteins in eukaryotic cells, we have purified to apparent homogeneity the polypeptide from rabbit reticulocyte initiation factors that crosslinks specifically to the 5′-terminal cap of mRNA. A similar protein occurs in the high-salt wash of ribosomes of mouse ascites and L cells. The high affinity of this polypeptide for m^{7}G nucleotides served as the basis of an affinity purification procedure using m^{7}GDP-Sepharose to separate the cap-binding protein from a complex protein mixture. The specificity of the interaction of this polypeptide with the cap was strikingly demonstrated by its unique release from the affinity resin upon elution with the cap analog, m^{7}GDP. To obtain essentially homogeneous 24,000-dalton polypeptide by single passage through the affinity resin, it was necessary to remove eIF-3 from the initiation factor mixture before loading it onto the column. Ammonium sulfate fractions (0–40% saturation) of ribosomal high-salt wash applied directly to the column without removal of eIF-3 by the sucrose gradient step did not yield polypeptides upon elution with 70 μM m^{7}GDP. At a level of 5 mM m^{7}GDP the cap-binding protein was eluted but together with several other proteins. The results suggest that eIF-3 not only binds the 24,000-dalton polypeptide but also adsorbs to m^{7}GDP-Sepharose, requiring

higher levels of m^7GDP for elution of the cap-binding protein. Binding of the cap-binding protein to eIF-3 has also been used as a purification step in an alternative procedure for obtaining 24,000-dalton cap-binding polypeptide from reticulocyte factors (28).

It has been known for some time that poliovirus infection of tissue culture cells results in a redirection of protein synthesis from host cell polypeptide formation to virus-specific products (29). Recently, it was found that the translation of capped mRNAs markedly decreased in extracts of poliovirus-infected HeLa cells whereas poliovirus RNA, which is not capped, continued to function well *in vitro* (15). Addition of purified eIF-4B to infected cell extracts restored capped mRNA translation without increasing poliovirus synthesis. Other eukaryotic initiation factors did not have this "restoring activity." The results suggested that poliovirus infection selectively blocks host cell protein synthesis—i.e., the translation of capped mRNAs—by inactivating eIF-4B (15). We confirmed the differential loss of capped mRNA function by using the RNAs of satellite tobacco necrosis virus and mouse encephalomyocarditis virus as messengers that are not capped and reovirus and Sindbis virus 26S RNAs as capped mRNAs in extracts of polio-infected HeLa cells. Preliminary results (28) indicate that the affinity-purified 24,000-dalton cap-binding protein partially restores the translation of these capped mRNAs *in vitro* without increasing naturally noncapped RNA translation. This suggests that the "restoring activity" and possibly the mRNA discriminatory properties previously observed with purified eIF-4B were due to the presence of the 24,000-dalton polypeptide.

The 24,000-dalton protein eluted from the affinity column by 70 μM m^7GDP was estimated to be several-hundredfold purified over the starting material. However, the crosslinking activity appeared not to be increased to the same extent. In addition, the restoring activity for capped mRNA translation in extracts of poliovirus-infected HeLa cells decreased during storage of the purified 24,000-dalton polypeptide at 4°C or frozen in buffer containing 10% glycerol and 0.1% bovine serum albumin. The decrease was not due to loss by adsorption to the vessel walls but reflected an instability of the purified cap-binding activity. The loss of function may indicate that the cap-binding protein is active as a multimer that dissociates into subunits at low concentrations or association of the 24,000-dalton polypeptide with another protein is required for stable activity.

Previously it was found that in reticulocyte initiation complexes the main polypeptides that can be specifically crosslinked to the 5′-m^7G of oxidized mRNA have higher M_rs than, and do not include, the 24,000-dalton polypeptide (18). This suggests that the 24,000-dalton polypeptide, or a multimer of it, may interact with the m^7G in mRNA before the formation of protein synthesis initiation complexes. Joining of mRNA to 40S ribosomal subunits may be accompanied by the release of the 24,000-dalton cap-binding protein or a conformational change that results in the interaction of other polypeptides with the cap. Additional studies with the purified cap-binding protein should contribute to a better understanding of the initiation and regulation of eukaryotic protein synthesis.

We thank W. C. Merrick and G. Kramer for providing initiation factor preparations, H. Trachsel for valuable discussions, and M. Morgan and A. LaFiandra for assistance.

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