Purification of a Factor that Restores Translation of Vesicular Stomatitis Virus mRNA in Extracts from Poliovirus-Infected HeLa Cells

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Purification of a factor that restores translation of vesicular stomatitis virus mRNA in extracts from poliovirus-infected HeLa cells

(translational control/eukaryotic initiation factor 4B/cap-binding protein/translation *in vitro*)

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ABSTRACT **It was previously shown that the poliovirus-induced inhibition of translation of capped mRNAs can be reversed by a protein found in preparations of the eukaryotic initiation factor eIF-4B [Rose, J. K., Trachsel, H., Leong, K. & Baltimore, D. (1978) *Proc. Natl. Acad. Sci. USA* 75, 2732–2736]. This "restoring factor" has now been purified from a high-salt wash of rabbit reticulocyte ribosomes by taking advantage of its tight association with factor eIF-3 at low salt concentrations. It did not copurify with the major M_r 80,000 polypeptide of eIF-4B preparations but did copurify with a M_r 24,000 polypeptide previously shown to bind to the cap structures of mRNAs [Sonenberg, N., Rupprecht, K. M., Hecht, S. M. & Shatkin, A. J. (1979) *Proc. Natl. Acad. Sci. USA* 76, 4345–4349]. Both the electrophoretic mobility and the tryptic peptide pattern of the restoring factor were indistinguishable from those of the cap-binding protein, and the restoring factor could be crosslinked to the 5′-terminal cap on mRNA. Thus, it appears that poliovirus inhibits cellular protein synthesis by inactivation of some crucial property of the cap-binding protein.**

Picornavirus infection inhibits translation of host cell mRNA (1). This inhibition has been shown to occur at the level of translation initiation (2) and not at the level of mRNA degradation (3) or mRNA modification (4). Furthermore, it was shown in cell-free protein-synthesizing systems that the ribosomal salt wash from poliovirus-infected cells does not stimulate the translation of host mRNAs from uninfected cells (5) but stimulates the translation of poliovirus RNA (6).

Vesicular stomatitis virus (VSV) mRNAs can be used as analogs of host mRNA because their translation is inhibited by superinfection of VSV-infected cells with poliovirus (7, 8). By using nuclease-treated cell-free extracts it has been shown that preparations of the eukaryotic initiation factor 4B (eIF-4B)†† can restore VSV mRNA translation in extracts from poliovirus-infected HeLa cells (10). It was therefore suggested that loss of eIF-4B activity could be responsible for the inhibition of VSV mRNA translation. One feature whereby the factor could discriminate between poliovirus RNA and VSV mRNAs (or host mRNAs) lies in the 5′-terminal cap structure (m^7GpppN) present on all known animal cell and viral mRNAs except those of picornaviruses (11). Although there has been some indication that eIF-4B interacts with the cap (12), more recent evidence has shown that eIF-4B (and eIF-3) preparations contain a M_r 24,000 protein that specifically binds to the 5′-terminal cap structure of eukaryotic mRNAs (13). This finding raises the possibility that some of the properties of eIF-4B preparations (10, 14) can be accounted for by the presence of the M_r 24,000 polypeptide in eIF-4B preparations. We therefore undertook purification of the activity that restores the activity of extracts from poliovirus-infected HeLa cells (hereafter referred to as "restoring factor") in order to clarify its nature and its relationship to the M_r 24,000 polypeptide. Here, we present evidence that it is identical to the cap-binding protein (13, 15).

MATERIALS AND METHODS

Buffers. Buffer A: 20 mM Tris·HCl, pH 7.6/0.1 mM EDTA/5 mM 2-mercaptoethanol. Buffer B: 20 mM Tris·HCl, pH 7.6/0.1 mM EDTA/5 mM 2-mercaptoethanol/10% (vol/vol) glycerol.

Cell-Free Translation in Extracts from Poliovirus-Infected HeLa Cells. The growth of HeLa cells, poliovirus infection, and the preparation of the micrococcal nuclease-treated translation system were as described (10). The cell-free translation system was made with an extract from poliovirus-infected HeLa cells (3 hr after infection). The reaction mixtures (12.5 μl) were as described (10) with 0.5 μg of VSV mRNA and a supplement of 0.5 μg of eIF-2. After incubation (60 min at 35°C), 5-μl aliquots were spotted on filter papers, soaked in cold 5% trichloroacetic acid, and then heated for 5 min at 90°C. The filters were rinsed in 5% trichloroacetic acid, absolute ethanol, and acetone and dried under a heat lamp. Incorporation of [^{35}S]methionine (1150 Ci/mmol, Amersham; 1 Ci = 3.7 × 10^{10} becquerels) was measured in 2,5-diphenyloxazole/1,4-bis[2-(5-phenyloxazolyl)]benzene/toluene in a liquid scintillation counter.

One unit of restoring factor was defined as the amount of factor that restored [^{35}S]methionine incorporation in the standard reaction mixture to 50% of the value obtained in the presence of 0.5 μl of step 5 preparation (the same preparation was used as the standard throughout the purification). The linearity of the assay is shown in Fig. 1.

$NaDodSO_4$/Polyacrylamide Gel Electrophoresis. Electrophoresis in slab gels was according to Laemmli (16). The separating gel contained 15% acrylamide (wt/vol) and 0.086% bisacrylamide (wt/vol).

Assay for Initiation Factor (eIF-3, eIF-4B) Activity. Fractions obtained by sucrose density gradient sedimentation were tested for initiation factor activity in the globin mRNA-de-

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Abbreviations: VSV, vesicular stomatitis virus; eIF, eukaryotic initiation factor.

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†† Factor nomenclature according to Anderson *et al.* (9).

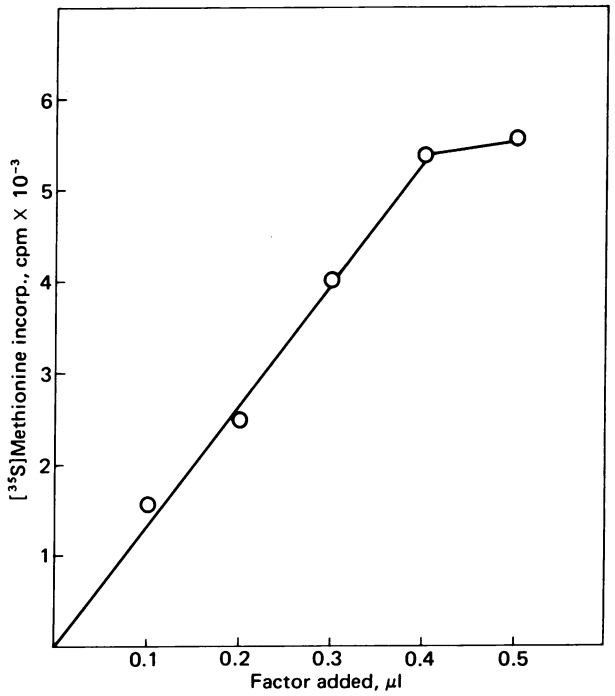

FIG. 1. Effect of restoring factor on VSV mRNA translation. Step 5 restoring factor preparation (9 μg/μl) was titrated in the standard poliovirus-infected HeLa incubation mixture for cell-free translation. Background incorporation in the absence of added factor (1300 cpm) was subtracted.

pendent polypeptide synthesis *system b* with the omission of either eIF-3 or eIF-4B (according to which factor was being assayed) as described by Schreier *et al.* (17).

Tryptic Peptide Analysis. Protein bands from fixed and stained $NaDodSO_4$/polyacrylamide gels were cut out and the protein was labeled with ^{125}I (1 mCi/band) and digested with trypsin, and the tryptic digests were analyzed. The procedure of Elder *et al.* (18) was followed.

Crosslinking of Protein to mRNA. Restoring factor was incubated with periodate-oxidized [^{3}H]methyl-labeled reovirus mRNA, and complexes were stabilized by reduction with $NaBH_3CN$, treated with RNase, and analyzed as described (13, 19).

Purification of the Restoring Factor. All operations were done at 0–4°C. Rabbit reticulocyte polysomes were prepared as described by Schreier *et al.* (17); 53,000 A_{260} units of polysomes were diluted to 400 ml. The final ionic conditions were 20 mM Tris·HCl, pH 7.6/500 mM KCl/6 mM $Mg(OAc)_2$/5 mM 2-mercaptoethanol/250 mM sucrose. The polysomes were pelleted by centrifugation in a Beckman type 35 rotor at 30,000 rpm for 8.5 hr at 2°C.

Step 1. Ammonium sulfate fractionation. The supernatant fraction (ribosomal wash) was precipitated by the addition of crystalline $(NH_4)_2SO_4$ to 40% saturation, and the precipitate was collected by centrifugation, dissolved in buffer A containing 100 mM KCl, and dialyzed against this buffer overnight. The protein concentration was determined according to Warburg and Christian (20).

Step 2. Sucrose gradient centrifugation. Eighteen milliliters of step 1 preparation (414 mg of protein) was loaded on six 10–40% exponential sucrose gradients in buffer A containing 100 mM KCl and centrifuged in a Beckman SW 27 rotor at 25,000 rpm for 42 hr at 2°C. The gradients were fractionated (1.5 ml per fraction) and corresponding fractions from the six gradients were pooled. The restoring factor activity sedimented

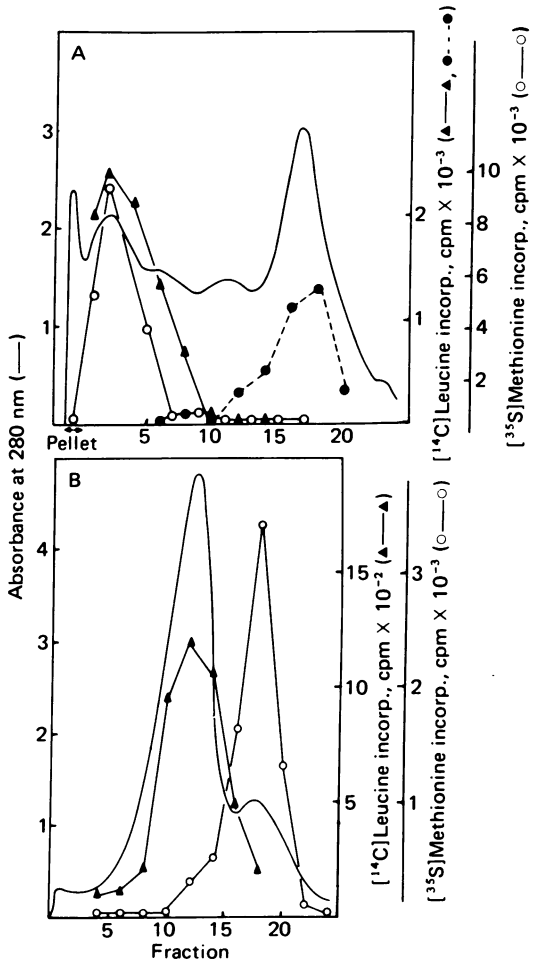

FIG. 2. Sucrose gradient centrifugation. The samples, centrifugation conditions (*A*, low salt; *B*, high salt), and assays for factor activities are described in *Materials and Methods* (steps 2 and 3). Sedimentation is from right to left. Aliquots of fractions (volumes indicated in parentheses) were tested for factor activity: ○, restoring factor (*A*, 4 μl; *B*, 3 μl); ▲, eIF-3 (*A*, 3 μl; *B*, 2 μl); or ●, eIF-4B (*A*, 5 μl).

with eIF-3 (Fig. 2*A*). Fractions 1–7 were pooled, precipitated by the addition of $(NH_4)_2SO_4$ to 50% saturation, resuspended in 5 ml of buffer A containing 100 mM KCl, and dialyzed against this buffer overnight.

Step 3. Sucrose gradient centrifugation. Five milliliters of step 2 preparation (70 mg of protein) was adjusted to 0.5 M KCl, loaded on four 10–45% exponential sucrose gradients in buffer A containing 0.5 M KCl, and centrifuged in the Beckman SW 41 rotor at 39,000 rpm for 21 hr at 2°C. Fractions (0.5 ml) were collected and assayed for eIF-3 and restoring factor activity. Under these high-salt conditions, the two activities were separated (Fig. 2*B*). Fractions 16–22 were pooled, precipitated as described above, dissolved in 2.2 ml of buffer B containing 50 mM KCl, and dialyzed against this buffer overnight.

Step 4. DEAE-cellulose chromatography. Of the step 3 preparation, 0.8 ml (3.6 mg of protein, 1000 units) was applied to a DEAE-cellulose column (Whatman DE-52, 1.2 × 4 cm) equilibrated with buffer B containing 50 mM KCl. The column was washed with this buffer and then step-eluted with buffer B containing 150 mM KCl for the first step and 300 mM KCl for the second step. About 85% of the recovered restoring factor activity eluted at 150 mM KCl.

Step 5. Phosphocellulose chromatography. Two milliliters of step 4 preparation (2.5 mg of protein) was diluted to 3 ml

with buffer B to adjust the KCl concentration to 100 mM. This preparation was applied to a phosphocellulose column (Whatman P11, 0.8 × 4.5 cm) equilibrated with buffer B containing 100 mM KCl. The column was washed with this buffer and then step-eluted with buffer B containing 200 mM KCl for the first step and 400 mM KCl for the second step. About 95% of the recovered restoring factor activity eluted at 400 mM KCl. The active fraction (1 ml) was dialyzed against buffer B containing 50 mM KCl for 2 hr at 4°C.

Step 6. Affinity chromatography. The initiation factor eIF-3 (7.5 A_{280} units of protein) was coupled to CNBr-activated Sepharose 4B (1.5 g) as described by the maker (21). One milliliter of step 5 preparation (0.9 mg of protein) was applied to an eIF-3-Sepharose affinity column (0.8 × 4 cm) equilibrated with buffer B containing 100 mM KCl. The column was washed with this buffer and then step-eluted with buffer B containing 500 mM KCl. More than 90% of the restoring factor activity eluted at 500 mM KCl (Fig. 3). The active fractions (2 ml, 0.09 mg of protein) were dialyzed against buffer B containing 100 mM KCl for 2 hr at 4°C.

Step 7. Phosphocellulose chromatography. Step 6 preparation was applied to a phosphocellulose column (0.5 × 2.5 cm) equilibrated with buffer B containing 100 mM KCl. The column was washed and protein was eluted as described for step 5. The restoring factor activity was found in the 400 mM KCl fraction (Table 1).

RESULTS

It was previously shown that a protein in eIF-4B preparations is able to restore VSV mRNA translation in extracts of poliovirus-infected HeLa cells (10). The major polypeptide in eIF-4B preparations has a molecular weight of 80,000 (17, 22). To examine whether the M_r 80,000 polypeptide in eIF-4B preparations was responsible for the restoring activity, the restoring activity was directly purified from the salt wash of reticulocyte ribosomes.

When the 0–40% saturated $(NH_4)_2SO_4$ precipitate derived from a salt wash preparation was sedimented through a sucrose gradient at low ionic strength, the restoring activity sedimented with initiation factor eIF-3 (Fig. 2A). At a high salt concentration, however, the restoring activity could be separated from eIF-3 (Fig. 2B), suggesting that the factor binds to eIF-3 (see below). This result is also consistent with the previous finding that high-salt-purified eIF-3 did not have restoring activity (10). In low ionic strength sucrose gradients, an activity that stimulated an eIF-4B-deficient cell-free protein-synthesizing system was separable both from eIF-3 and from the restoring activity (Fig. 2A).

The restoring activity was further purified by DEAE-cellulose and phosphocellulose chromatography. The active material was then passed through a column containing eIF-3 that had been immobilized by linkage to Sepharose. Unlike the bulk of the protein, the restoring activity bound quantitatively to the eIF-3 affinity column (Fig. 3). As a final purification step, a second phosphocellulose chromatography was used. A summary of the purification process is shown in Table 2.

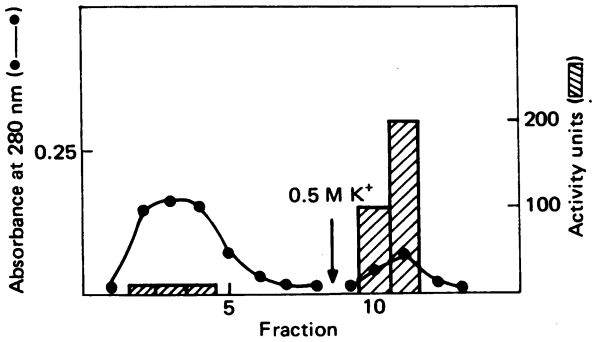

FIG. 3. eIF-3-Sepharose affinity chromatography of restoring factor activity (step 6). One-milliliter fractions were collected and 3-μl aliquots were tested for restoring factor activity.

Table 1. Phosphocellulose chromatography (step 7)

Addition to assay	[^{35}S]Methionine incorp., cpm
None	3,024
Control factor (0.5 μl)	26,523
Void fractions (pooled)	2,561
200 mM KCl	
Fraction 2	2,657
3	2,509
4	2,899
400 mM KCl	
Fraction 1	2,747
2	7,140
3	13,379
4	8,935

Phosphocellulose chromatography: 100-μl fractions were collected (void fractions were pooled) and 3-μl aliquots were assayed for restoring factor activity. Control factor was step 5 preparation (see Fig. 1).

After purification through the first phosphocellulose chromatography step, the restoring activity remained very stable even upon storage at room temperature overnight. After affinity chromatography, however, the activity became labile, and this material or restoring factor that had been purified through a second phosphocellulose column lost most of its activity on storage overnight at −70°C or in liquid nitrogen.

Upon analysis of the various fractions during purification by $NaDodSO_4$/polyacrylamide gel electrophoresis, a selective enrichment of an M_r 24,000 polypeptide was evident (Fig. 4, slots b–g). The cap-binding protein (15), analyzed in slot h (Fig.

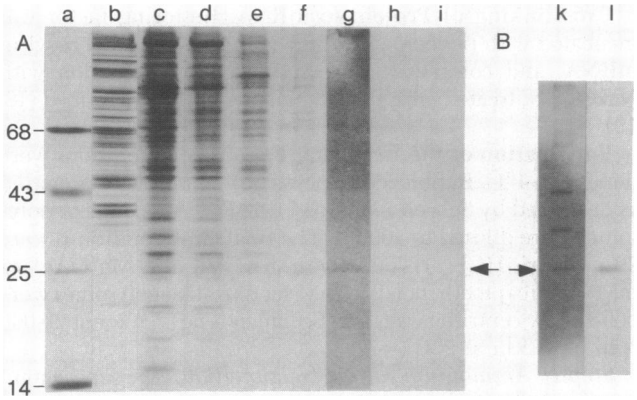

FIG. 4. $NaDodSO_4$/polyacrylamide gel electrophoresis. (*A*) Stained lanes from different gels are shown. Slot a, marker proteins ($M_r \times 10^{-3}$ given on the left): bovine serum albumin (68,000), ovalbumin (43,000), chymotrypsin (25,000), lysozyme (14,000). Slot b, 28 μg (1.3 units) of step 2 factor; slot c, 22 μg (6 units) of step 3 factor; slot d, 15 μg (5 units) of step 4 factor; slot e, 9 μg (5 units) of step 5 factor; slot f, 4 μg (12 units) of step 6 factor; slot g, 0.8 μg (12 units) of step 7 factor; slot h, 2 μg of cap-binding protein purified as described (15); slot i, sample buffer. The faint band in the region of M_r 68,000 (slots g and h) is an artefact also observed in a slot containing only sample buffer (slot i). (*B*) Restoring factor purified through steps 1–5 and 7 was crosslinked to the cap structure of mRNA. Slot k, 7.5 μg of factor, not crosslinked (stained gel); slot l, 2.5 μg of factor, crosslinked (autoradiography). The arrows point to the M_r 24,000 polypeptide.

Table 2. Summary of purification

Step	Total protein, mg	Specific activity, units per mg	Purification, fold	Recovery, %
1. $(NH_4)_2SO_4$ cut	414	9.5	1	100
2. Sucrose gradient (low salt)	70	46	4.9	82
3. Sucrose gradient (high salt)	10	282	29.8	72
4. DEAE-cellulose chromatography	2.5	344	36.2	62*
5. Phosphocellulose chromatography	0.9	555	58.7	36*
6. Affinity chromatography	0.09	3,233	342	21*
7. Second phosphocellulose chromatography	0.004†	14,500	1526	4*

* Of the step 3 preparation, 3.6 mg was further purified; recovery was corrected for this.
† Estimated from stained $NaDodSO_4$ gel.

4), comigrated during electrophoresis with the major polypeptide in the restoring activity. Furthermore, the M_r 24,000 polypeptide in the restoring activity could be selectively crosslinked to the 5′-terminal cap structure of messenger RNA by using the procedure described (19) (Fig. 4, slots k and l). These results suggest that the cap-binding protein and the M_r 24,000 restoring activity are identical polypeptides.

To compare these two polypeptides structurally, their tryptic digest patterns were examined. Although comparison of the two patterns (Fig. 5 *A* and *B*) showed differences in relative intensity of spots, a mixture of the two preparations showed that the spots overlapped (Fig. 5*C*) and thus the two proteins appear to be identical.

DISCUSSION

Using the restoration of VSV mRNA translation in extracts of poliovirus-infected cells as an assay system, we were able to identify an activity that selectively restored translation. The activity copurified with an M_r 24,000 polypeptide that was identical to the cap-binding protein (13,15) by electrophoretic mobility in $NaDodSO_4$/polyacrylamide gels, crosslinking to the 5′ terminus of mRNA, and tryptic peptide pattern. We therefore assume that the restoring factor and the cap-binding protein are identical. It is not certain, however, that inactivation of the cap-binding protein completely explains the ability of poliovirus infection to inhibit translation of host cell mRNAs. Other factors may interact with cap-binding protein, and until these interactions are understood the action of poliovirus will not be clear. It does appear from these data that one consequence of poliovirus infection is modification of cap-binding protein, but the nature of the modification and its consequence remain to be shown.

Originally, eIF-4B was defined in a fractionated protein-synthesizing system as a factor that stimulated initiation (17); the factor was later shown to be involved in mRNA binding (23). This activity copurified with a M_r 80,000 polypeptide (17, 22). Because all eIF-4B preparations tested recently also contained low levels of the cap-binding protein, and because partially purified preparations of cap-binding protein significantly stimulated the fractionated system (results not shown), it may be that this protein is partially responsible for the eIF-4B activity described in the fractionated system (17, 22–24). Further work will be needed to determine the relative contributions to eIF-4B activity of these two polypeptides. The M_r 80,000 polypeptide separates from eIF-3 during sucrose gradient centrifugation in low salt concentration (22) and may be responsible for the stimulation of the fractionated system by top fractions of our sucrose gradients (Fig. 2*A*).

The restoring activity of purified cap-binding protein preparations decreased rapidly during storage. Cruder preparations, however, were stable and in addition were able to reactivate purified cap-binding protein that had lost its restoring

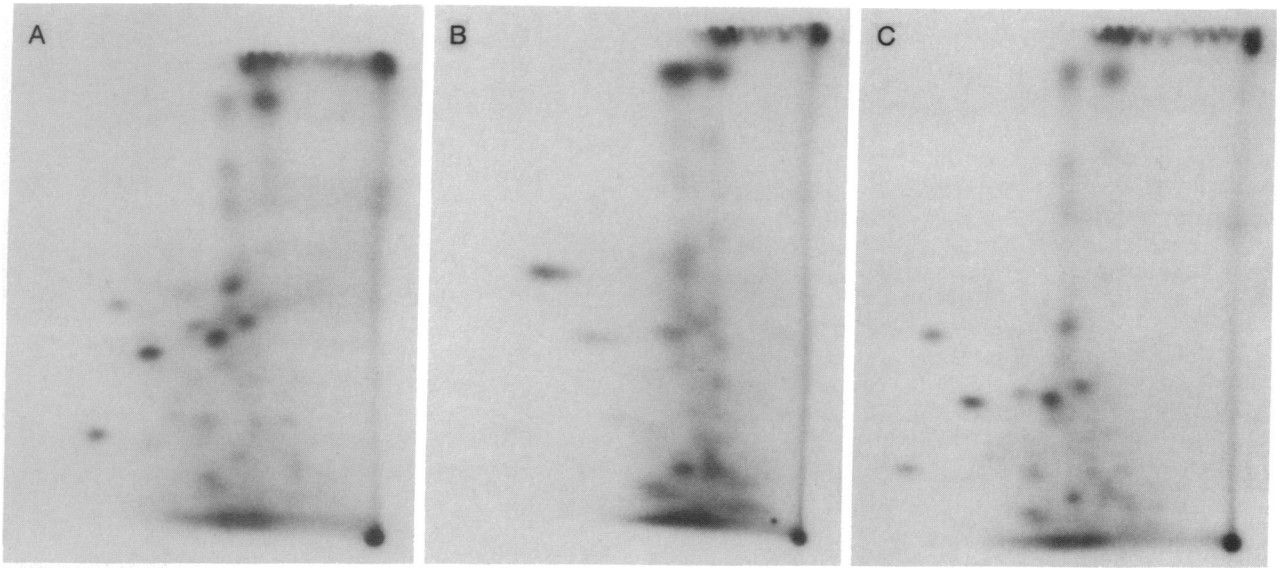

FIG. 5. Comparison of tryptic peptide patterns. The M_r 24,000 polypeptide (Fig. 4, slot f) and the cap-binding protein (Fig. 4, slot h) were excised from the destained wet gel and further processed as described in *Materials and Methods*. The autoradiographs (exposure time 145 hr) are shown. Electrophoresis was from right to left, chromatography from bottom to top. (*A*) M_r 24,000 restoring factor; (*B*) cap-binding protein (15); (*C*) equal amounts of *A* and *B* mixed.

activity (results not shown). This suggests that an additional component may be required to maintain the restoring activity of purified cap-binding protein.

The finding that the restoring activity is identical with the cap-binding protein fits into the scheme (10) whereby poliovirus infection leads to the inactivation of the cap-dependent recognition mechanism of mRNAs during protein synthesis initiation. This results in the inhibition of host mRNA but not poliovirus RNA translation, because the latter lacks the 5′ terminal cap structure (25, 26) and presumably does not need cap-binding protein for initiation. Consistent with this scheme, purified cap-binding protein does not stimulate the translation of poliovirus RNA in extracts from poliovirus-infected or uninfected HeLa cells (results not shown). We find the cap-binding protein associated with the complex initiation factor eIF-3, however, in quite small amounts (Fig. 2): we calculate from Table 2 (1 unit of activity ≈ 70 ng of protein) and Fig. 2*B* (45 A_{280} units of eIF-3 release 2820 units of restoring activity) that the ratio of eIF-3 to cap-binding protein is at least 8:1. It is possible that some cap-binding protein remains associated with mRNA. The cap-binding protein might then serve as the contact site for the recognition of the 5′-terminus of mRNA by [40S ribosomal subunit·eIF-3] complexes, possibly as the first step in mRNA binding.

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