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Association of Cap-Binding Protein with Eucaryotic Initiation Factor 3 in Initiation Factor Preparations from Uninfected and Poliovirus-Infected HeLa Cells

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Extracts from poliovirus-infected HeLa cells are unable to translate vesicular stomatitis virus or cellular mRNAs in vitro, probably reflecting the poliovirus-induced inhibition of host cell protein synthesis which occurs in vivo. Crude initiation factors from uninfected HeLa cells are able to restore translation of vesicular stomatitis virus mRNA in infected cell lysates. This restoring activity separates into the 0 to 40% ammonium sulfate fractional precipitate of ribosomal salt wash. Restoring activity is completely lacking in the analogous fractions prepared from poliovirus-infected cells. The 0 to 40% ammonium sulfate precipitates from both uninfected and infected cells contain eucaryotic initiation factor 3 (eIF-3), eIF-4B, and the cap-binding protein (CBP), which is detected by means of a cross-linking assay, as well as other proteins. The association of eIF-3 and cap binding protein was examined. The 0 to 40% ammonium sulfate precipitate of ribosomal salt wash from uninfected and infected cells was sedimented in sucrose gradients. Each fraction was examined for the presence of eIF-3 antigens by an antibody blot technique and for the presence of the CBP by cross-linking to cap-labeled mRNAs. From uninfected cells, a major proportion of the CBP cosedimented with eIF-3; however, none of the CBP from infected cells sedimented with eIF-3. The results suggest that the association of the CBP with eIF-3 into a functional complex may have been disrupted during the course of poliovirus infection.

Inhibition of host cell protein synthesis is one of the earliest apparent effects of poliovirus infection in HeLa cells. The inhibition of synthesis of cellular proteins is evident at 2 h postinfection, and after 3 h only synthesis of poliovirus proteins can be detected (9, 10, 15, 22). Cellular mRNA is intact (13, 19) and functional when extracted from the infected cell and used to program translation in vitro (11, 17, 33).

It has been demonstrated that after poliovirus infection, initiation complexes no longer form on cellular mRNA, but do form on poliovirus RNA (12, 19). These observations suggested that some change in the protein synthesis machinery had taken place during poliovirus infection so that initiation of translation of cellular mRNA could no longer occur.

Since the inhibition of host cell protein synthesis was due to the failure to initiate on cellular mRNA, several investigators examined the possible role of initiation factors in this phenomenon (6, 15, 17). Rose et al. (23) reported that purified rabbit reticulocyte eucaryotic initiation factor 4B (eIF-4B) could restore the translation of vesicular stomatitis virus (VSV) mRNA in a cell-free extract prepared from poliovirus-infected cells, but using a different assay, Helentjaris et al. (16) found that eIF-3 purified from poliovirus-infected HeLa cells was inactive compared with similar preparations from uninfected cells. Both eIF-3 and eIF-4B have been shown to function in initiation complex formation (3, 31). A possible resolution of this discrepancy was suggested by the work of Sonenberg et al. (24, 26), who described a reticulocyte cap-binding protein (CBP) that specifically recognizes the cap group of most eucaryotic mRNAs and is present in preparations of both eIF-3 and eIF-4B. Subsequently, Trachsel et al. (32) showed that purified reticulocyte CBP, when added to lysates of poliovirus-infected cells, was able to restore translation of VSV mRNA. This result suggested that it was the CBP which was defective in poliovirus-infected cell lysates (and hence in the infected cell), and also that the previously described activity of eIF-3 and eIF-4B might be due to the presence of CBP in these initiation factor preparations (32). Indeed, removal of the

CBP from eIF-3 preparations diminished the ability of eIF-3 to stimulate translation of capped mRNAs (28).

We began a study of the CBP in initiation factor preparations from HeLa cells, since we could then directly compare results from uninfected and poliovirus-infected cells. Previously we showed that HeLa cells contained a CBP analogous to that in reticulocytes, and that this CBP was present in poliovirus-infected cells after the inhibition of host cell protein synthesis was complete (14). During initial purification steps we were unable to detect any major differences between the CBPs isolated from uninfected or infected cells. The CBP from reticulocytes has been reported to copurify with eIF-3 (24); indeed, an affinity column of eIF-3 coupled to Sepharose has been used to purify the CBP (32), and it is possible that this association may represent a functional one. In this report, we have examined the association of the HeLa cell CBP with eIF-3. We have found that CBP from uninfected HeLa cells does associate with eIF-3, but that association has apparently been disrupted in poliovirus-infected cells.

MATERIALS AND METHODS

Cells and virus. Growth of HeLa cells and growth and purification of poliovirus were done as described earlier (7, 14). VSV was produced as described previously (1, 14).

Cell-free translation. Preparation of extracts from either uninfected, mock-infected, or poliovirus-infected HeLa cells was done as previously described (4, 14). All extracts were treated with micrococcal nuclease by the method of Pelham and Jackson (21) to make translation dependent on exogenous mRNA. VSV mRNA was synthesized in vitro (14). Purification of poliovirus RNA from virion particles was as described previously (5). In vitro translation reactions (50 μl) contained 25 μl of nuclease-treated lysate and the following: 20 μM each of 19 amino acids (minus methionine), 1.0 mM ATP, 0.2 mM GTP, 25 mM creatine phosphate, 3.12 μg of creatine phosphokinase (Sigma Chemical Co.), 2.0 mM magnesium acetate, 5 μCi of [^{35}S]methionine (Amersham Corp., 1,100 Ci/mmol), 5 μg of tRNA (Sigma), 2 mM dithiothreitol, 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (pH 7.5), and 60 mM KCl. Additions of mRNA, ribosomal salt wash (RSW), or ammonium sulfate (AS) fractions are indicated in the figure legends.

Fractionation of RSW. RSW was prepared from either mock-infected or poliovirus-infected cells at 3.5 h postinfection as described by Hansen and Ehrenfeld (14). Fractionation of RSWs into 0 to 40% and 40 to 70% AS precipitates was as described previously (16).

Velocity sedimentation analysis. A 0 to 40% AS precipitate from approximately 2×10^9 uninfected HeLa cells (total protein, 12 mg, by absorbancy at 280 nm) or from poliovirus-infected HeLa cells (total protein, 12.5 mg) was each separately applied to a linear 5 to 20% (wt/wt) sucrose gradient prepared in IF buffer (20 mM Tris hydrochloride [pH 7.5], 100 mM KCl, 0.2 mM EDTA, 7 mM β-mercaptoethanol, 5% glycerol [wt/vol]). Sedimentation was in a Beckman SW40 rotor at 4°C at 32,000 rpm for 17 h. Each fraction was concentrated by precipitation with 70% AS by the methods used above. After resuspension in 170 μl of IF buffer, each fraction was dialyzed against a large volume of that buffer.

Immunoblot analysis. Samples (10 μl) of each sucrose gradient fraction were mixed with gel sample buffer, boiled for 3 min, and subjected to electrophoresis on a 10% sodium dodecyl sulfate (SDS)-polyacrylamide slab gel. The protein was electrophoretically transferred from the slab gel to a nitrocellulose sheet essentially by the method of Towbin et al. (30). The gel was placed onto wetted nitrocellulose paper supported by Whatman 3 MM paper and scouring pads on each side and mounted in an electrophoresis chamber so that the nitrocellulose paper was on the anode side and the gel was on the cathode side of the apparatus. The chamber buffer consisted of 25 mM Tris hydrochloride (pH 8.3), 192 mM glycine, and 20% methanol. After electrophoresis for 6 h, the nitrocellulose paper was stained with 0.1% amido black in 45% methanol–10% acetic acid and destained with 90% methanol–2% acetic acid. The paper was rinsed with water and then incubated with 3% bovine serum albumin in 10 mM Tris (pH 7.4)–0.15 M NaCl for 1 h at 37°C. After rinsing three times with antibody blotting buffer (10 mM Tris [pH 7.4], 0.15 M NaCl, 0.2% SDS, 0.5% Triton X-100, 0.5% bovine serum albumin) the paper was incubated for 6 h with anti-eIF-3 antiserum diluted in antibody blotting buffer. Anti-eIF-3 antiserum was prepared by injecting purified eIF-3 from rabbit reticulocytes into goats (L. Meyers, S. Milburn, and J. Hershey, manuscript in preparation). This antiserum does not react with the 24K CBP, but reacts with most of the larger components of HeLa cell eIF-3. After incubation with antiserum, the nitrocellulose paper was washed three times with antibody blotting buffer and incubated for 12 h with ^{125}I-labeled rabbit anti-goat immunoglobulin G. After washing again with antibody blotting buffer, the paper was air dried and exposed to Kodak X-OmAT SB film.

Detection of the CBP. The cross-linking assay used to detect the CBP was essentially that of Sonenberg and Shatkin (26), with the modification described in our previous paper (14). For cross-linking, 10 μl of the AS precipitate of each sucrose gradient fraction was used. SDS-polyacrylamide gel electrophoresis (PAGE) on 10% gels and sodium salicylate fluorography were as described previously (8, 14). Tracings of each gel lane were made on a Joyce-Loebl Microdensitometer 3CS, and the area of the CBP peak was determined by integration on a Numonics Graphics calculator 1224.

RESULTS

Differences in initiation factor activity from uninfected and poliovirus-infected cells. Preparations of crude initiation factors from uninfected and poliovirus-infected HeLa cells show marked differences in activity in several in vitro translation assays (5, 6, 15, 23). For example, although crude initiation factor preparations from both uninfected and infected cells actively stimulate translation of poliovirus RNA in a HeLa (or rabbit reticulocyte) cell-free protein-synthesiz-

ing system, translation of other mRNAs is not stimulated by initiation factors from poliovirus-infected cells. Figure 1 illustrates the results of such an assay by comparing the effect of initiation factors on the translation of poliovirus RNA (Fig. 1A) and VSV mRNA (Fig. 1B). Crude initiation factor preparations, in the form of RSW, prepared from uninfected cells will stimulate the translation of both poliovirus RNA (Fig. 1A, lane 4) and VSV mRNA (Fig. 1B, lane 4). The RSW from poliovirus-infected cells will also stimulate translation of poliovirus RNA (Fig. 1A, lane 3). As reported previously, however, the initiation factors from infected cells fail to significantly stimulate translation of VSV mRNA (Fig. 1B, lane 3).

A second assay which illustrates the change in initiation factor activity in poliovirus-infected cells was adapted from that of Rose et al. (23). They showed that cell-free extracts prepared from poliovirus-infected cells were unable to translate VSV mRNA. Purified initiation factors from rabbit reticulocytes were shown to restore the ability of the infected cell extract to translate VSV mRNA. We have subsequently shown (14) that crude initiation factor preparations from uninfected HeLa cells are also able to restore the infected cell extract's translation ability, but that initiation factors from poliovirus-infected cells lack restoring activity. Figure 2 illustrates this result (Fig. 2, lanes 3 and 4) and shows that the restoring activity fractionates in a 0 to 40% AS precipitate of RSW from uninfected HeLa cells (Fig. 2, lane 5). The analogous fraction from poliovirus-infected cells lacks restoring activity (Fig. 2, lane 7); the 40 to 70% AS precipitates contain little or no activity from either source (Fig. 2, lanes 6 and 8).

Restoring activity has been attributed to the CBP, since relatively pure preparations of reticulocyte CBP have restoring activity, although that activity appears to be unstable when the preparation is purified (25, 32). We have previously shown that RSW from both uninfected and poliovirus-infected cells have the CBP, and in both instances, the majority of the CBP separates into the 0 to 40% AS fraction. If restoration activity is a function of the CBP, the CBP present in RSW from infected cells may be in an inactive form since such preparations lack any restoring activity. The CBP is detected by means of a cross-linking reaction, which requires that the CBP will be able to associate with the cap at the 5′ end of mRNAs. In the initial comparison, we could detect no gross differences between the CBP from uninfected and infected cells as to relative mobility on SDS-PAGE and fractionation into the 0 to 40% AS precipitate (14), but there is still the striking difference in restoring activity between the 0 to

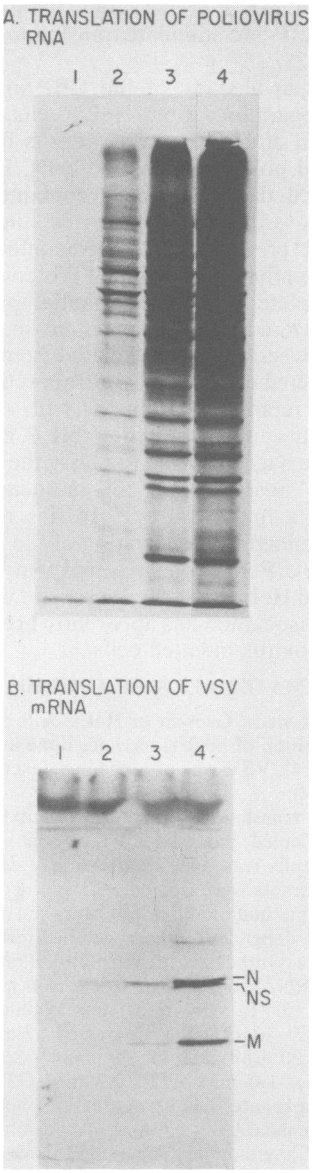

FIG. 1. Stimulatory activity of initiation factors from uninfected and poliovirus-infected HeLa cells. Translation of exogenous mRNAs in micrococcal nuclease-treated extracts from uninfected HeLa cells was performed under the conditions described in the text. (A) Translation of poliovirus RNA. Reactions contained the following: 1, 6 μl of IF buffer only; 2, 5 μg of poliovirus RNA plus 6 μl of IF buffer; 3, 5 μg of poliovirus RNA plus 6 μl of RSW (115 μg of protein) from poliovirus-infected cells; 4, 5 μg of poliovirus RNA plus 6 μl of RSW (125 μg of protein) from uninfected cells. (B) Translation of VSV mRNA. Reactions contained the following: 1, 6 μl of IF buffer only; 2, 1 μg of VSV mRNA plus 6 μl of IF buffer; 3, 1 μg of VSV mRNA plus 6 μl of RSW from poliovirus-infected cells; 4, 1 μg of VSV mRNA plus 6 μl of RSW from uninfected cells.

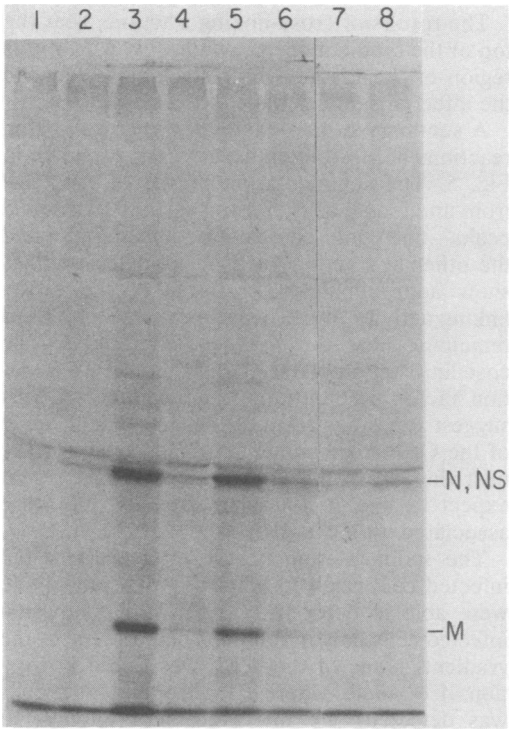

FIG. 2. Restoring activity of initiation factors in extracts from poliovirus-infected HeLa cells. Translation of 1.0 μg of VSV mRNA in a micrococcal nuclease-treated extract from poliovirus-infected cells was performed as described in the text in the presence of the following: 1, no VSV mRNA and 6 μl of IF buffer; 2, VSV mRNA plus IF buffer; 3, VSV mRNA plus 6 μl of RSW (125 μg of total protein) from uninfected cells; 4, VSV mRNA plus 6 μl of RSW (115 μg of total protein) from poliovirus-infected cells; 5, VSV mRNA plus 3 μl of 0 to 40% AS precipitate (95 μg of protein) from uninfected cells and 3 μl of IF buffer; 6, VSV mRNA plus 3 μl of 40 to 70% AS precipitate (36 μg of protein) from uninfected cells and 3 μl of IF buffer; 7, VSV mRNA plus 3 μl of 0 of 40% AS precipitate (110 μg of protein) from poliovirus-infected cells plus 3 μl of IF buffer; 8, VSV mRNA plus 3 μl of 40 to 70% AS precipitate (41 μg of protein) from poliovirus-infected cells plus 3 μl of IF buffer. VSV proteins N, NS, and M are indicated.

40% AS fraction from uninfected versus infected cells. Therefore, we began to look at the CBP further by exploring the association of the CBP with other initiation factors. Since the CBP from reticulocytes is reported to copurify the eIF-3 (24), we chose to look at the possible association of the CBP with eIF-3 in HeLa cells by sedimentation analysis.

Sedimentation of eIF-3. Ribosomal salt washes from uninfected and poliovirus-infected cells were fractionally precipitated with AS and the 0 to 40% precipitates were subjected to velocity sedimentation on 5 to 20% sucrose gradients as described above. Portions of the AS precipitate of each gradient fraction were separated by SDS-PAGE, and the gels were blotted onto nitrocellulose paper and incubated with antiserum prepared against reticulocyte eIF-3 as described above in detail. The autoradiographs of these blots are shown in Fig. 3. Equal amounts of purified reticulocyte eIF-3 was included as markers on each gel. As is evident from the reduced intensity of bands in the marker lane, the efficiency of transfer of protein to the nitrocellulose filter was unequal for the two gels. Nevertheless, the position of the majority of eIF-3 antigens is identical in the gradients from both infected and uninfected cells and is localized in fractions 4, 5, and 6. Some eIF-3 antigenic material trails into lighter fractions of the gradient, possibly representing degradation products of eIF-3 polypeptides (20) or the presence of cross-reacting antigens. The antiserum to eIF-3 has been used to follow the purification of eIF-3, from both reticulocytes and HeLa cells, and a good correlation between antigens and eIF-3 activity exists (Etchison and Hershey, unpublished results).

Sedimentation of the CBP. The AS precipitate of each sucrose gradient fraction was also tested for the presence of the CBP by means of the cross-linking assay. The assay involves incubation of proteins to be tested for cap-binding activity with oxidized *methyl*-^{3}H-labeled VSV mRNA and subsequent reduction to establish a covalent linkage between the RNA and associated protein. The cross-linked product is then digested with RNase, resulting in a transfer of the radiolabeled cap residue to the protein, and the polypeptides are displayed on a 10% SDS-polyacrylamide gel. Representative results from different regions of the gradients are depicted in Fig. 4. Figure 4A shows the cross-linking assay results for the comparable fractions 5 from both the uninfected (U5) and the poliovirus-infected (P5) samples. This fraction was chosen as representative of fractions which also contain eIF-3 (see above). The CBP is clearly present in this fraction from the uninfected cell sample. The band labeled CBP is specifically inhibited by the presence of the unlabeled cap analog m^7GDP in the cross-linking reaction. In contrast to the uninfected cell sample, the homologous fraction (P5) from the gradient containing samples from poliovirus-infected cells lacks any detectable CBP. Both the uninfected and infected samples contain a second band, approximately 68K M_r, indicated by the arrow, whose cross-linking is inhibited by the presence of m^7GDP. The significance of this band is not known. It might be related to some of the higher-molecular-weight forms of CBP seen by others (27), or it may be

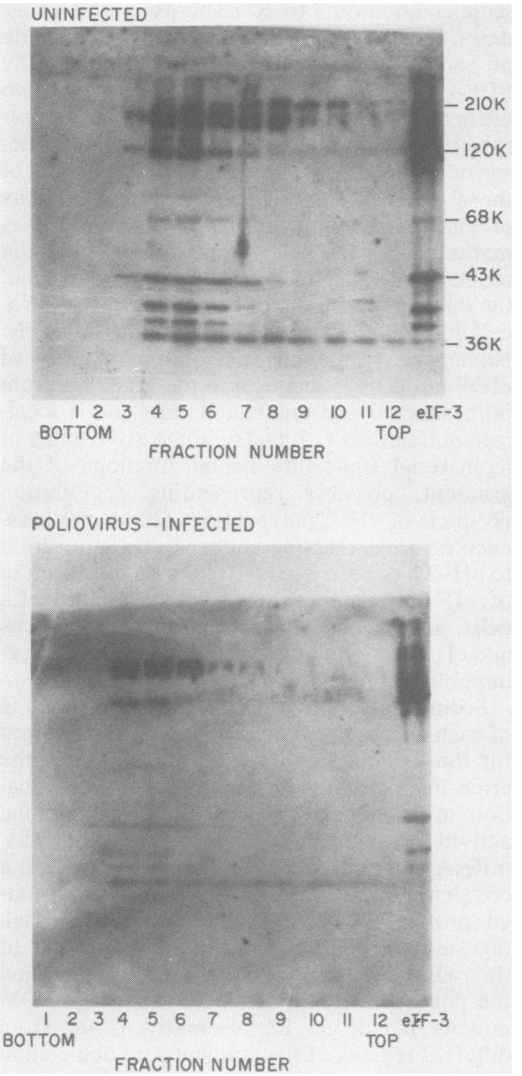

FIG. 3. Immunoblot analysis of eIF-3 antigens in sucrose gradient fractions. A 0 to 40% AS precipitate of RSW from uninfected or poliovirus-infected cells was layered onto a 5 to 20% (wt/wt) sucrose gradient and centrifuged as described in the text. The gradient was divided into fractions. Each fraction was concentrated by AS and dialyzed. Equal portions (10 μl) of each gradient fraction were separated on SDS-PAGE, transferred to nitrocellulose, and then allowed to react with goat antiserum prepared against reticulocyte eIF-3 as described in detail in the text. The paper was then blotted with ^{125}I-labeled rabbit anti-goat immunoglobulin G before autoradiography. Purified rabbit reticulocyte eIF-3 was used as a marker in each blot (2).

related to a polypeptide in the eIF-3 complex of a similar molecular weight (Etchison and Hershey, unpublished observations). It is present in both uninfected and infected samples in fractions 4, 5, and 6 (data not shown).

The results of cross-linking fractions near the top of the gradient are shown in Fig. 4B. In this region of the gradient both the uninfected and the infected samples have a detectable CBP.

A summary of the results of the cross-linking reactions across the entire gradient is shown in Fig. 5. The sedimentation profile of the CBP from uninfected cells shows basically two broad peaks—one centered near fractions 4 and 5 and the other at fraction 10. Densitometer tracings show approximately two-thirds of the cross-linking activity in the heavy peak and one-third remaining near the top of the gradient. The cosedimentation of eIF-3 and the CBP in initiation factor preparations from uninfected cells suggests an association of these proteins. Some of the CBP from uninfected cells remains very near the top of the gradient, where one might expect to find it if it were free or if it were associated with eIF-4B.

The sedimentation profile of the CBP from infected cells showed a striking difference. We were able to detect the CBP from poliovirus-infected cells only in fractions near the top of the gradient. None of this CBP was found in fractions 4, 5, and 6, where eIF-3 from infected cells was detected. We interpret these findings to suggest that the association of eIF-3 with the CBP, normally seen in uninfected HeLa cells, has been disrupted over the course of poliovirus infection.

DISCUSSION

In this report we have compared the sedimentation properties of the CBP from uninfected and poliovirus-infected HeLa cells. CBP in the ribosomal salt wash of uninfected HeLa cells fractionates into a 0 to 40% AS precipitate, as detected by a cross-linking assay (14). Upon fractionation of the AS precipitate on sucrose gradients, about two-thirds of the CBP from uninfected ribosomal salt wash cosediments with eIF-3. An association between CBP and eIF-3 in ribosomal salt washes from rabbit reticulocytes has been previously reported (32). Surprisingly, a similar analysis of CBP from poliovirus-infected HeLa cells showed that none of the CBP from infected cells was detected in gradient fractions that contained eIF-3. Thus, poliovirus infection may result in a disruption of the association of eIF-3 and CBP, or the ability of eIF-3-associated CBP to interact with cap structures may be lost, resulting in our failure to detect it by the cross-linking assay.

A number of investigators have concluded from the available data that the poliovirus-induced inhibition of host cell protein synthesis results from inactivation of the CBP after infection (18, 28, 32). If the association between CBP

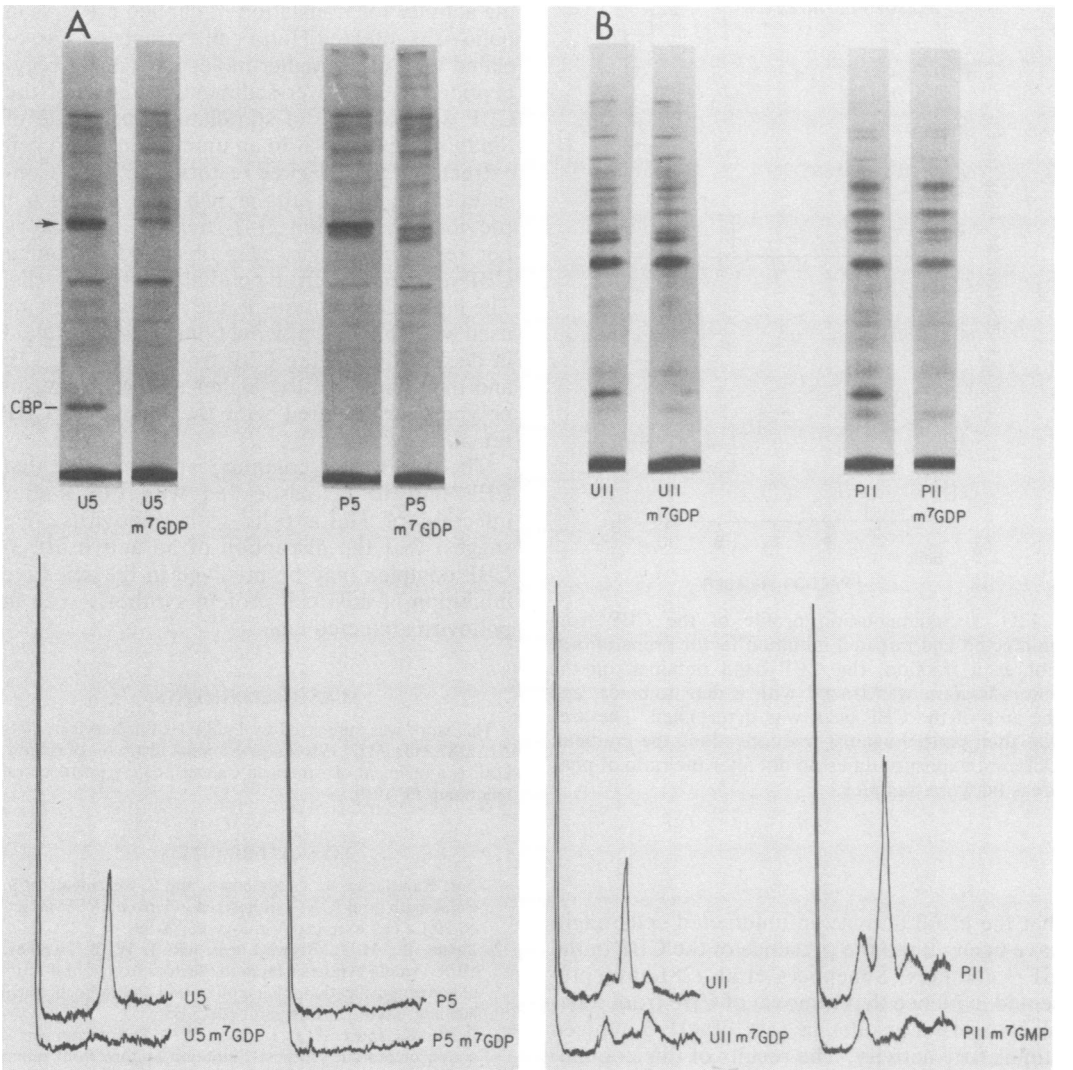

FIG. 4. Detection of the CBP in fractions of sucrose gradient. For cross-linking, 10 μl of the AS precipitate of each fraction from the sucrose gradient analyzed in Fig. 3 was incubated with [*methyl*-^{3}H]VSV mRNA, plus or minus 1 mM m^{7}GDP, as indicated. Proteins cross-linked to the 5′ end of mRNA were separated on 10% SDS-PAGE and fluorographed with sodium salicylate before autoradiography. Densitometer tracings shown are approximately the lower third of each gel. The heavy band at the dye front contains the residual T1 oligonucleotides from [*methyl*-^{3}H] mRNA remaining after RNase digestion (14). The band identified as CBP runs as 26K M_r, whereas the band marked with an arrow is approximately 68K M_r, based on protein markers used previously (14). (A) U5, Fraction 5 from the gradient of a 0 to 40% AS precipitate prepared from uninfected cells; P5, the analogous fraction from a 0 to 40% AS precipitate prepared from poliovirus-infected cells. Fraction 1 is from the bottom of the gradient. (B) U11, Fraction 11 from the uninfected sample; P11, fraction 11 from the infected sample. Fraction 12 is the top of the gradient.

and eIF-3 represents a functional form of the CBP, disruption or inactivation of the complex by some virus-induced change in either the CBP or eIF-3 may be the mechanism of host translational inhibition by poliovirus infection.

Previous results from this laboratory showed that ribosomal salt wash prepared from poliovirus-infected cells contained inactive eIF-3, although analogous preparations from uninfected HeLa cells did contain eIF-3 activity. The assay for eIF-3 activity measured stimulation of translation of capped globin mRNA, and it is possible

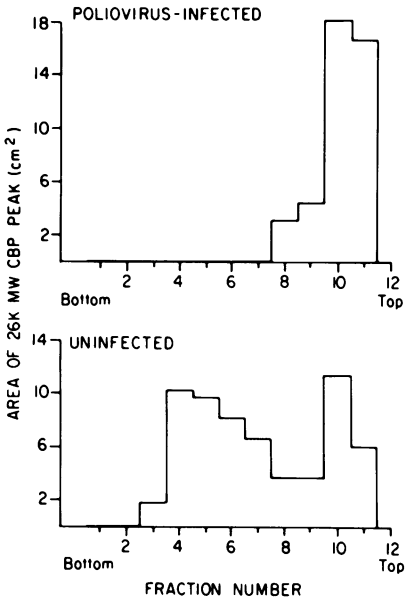

FIG. 5. Sedimentation profile of the CBP from uninfected and infected initiation factor preparations. For each fraction, the CBP band obtained on the autoradiogram was traced with a densitometer, and the area of the CBP peak was determined. The area was then plotted against fractions along the gradient. Different exposure times do not alter the ratio of peak areas between samples.

that the eIF-3 activity in uninfected cells might have been due to the presence of the CBP in the eIF-3 complex. Sonenberg et al. (28) have presented evidence that removal of CBP from reticulocyte eIF-3 results in loss of eIF-3-induced stimulatory activity. The results of this communication suggest that in poliovirus-infected HeLa cells the CBP is not associated with eIF-3, perhaps explaining the lack of eIF-3 activity for infected cells seen earlier.

The eIF-3 complex consists of a number of polypeptides, many of which are related according to partial proteolysis data (20). Other investigators have hinted that a similar situation may exist with the CBP, since monoclonal antibody that reacts with the CBP also reacts with other larger polypeptides which share tryptic and chymotryptic peptides with the CBP of 24 to 26K seen in the gels of cross-linked material (27). These observations lead to the speculation that a larger polypeptide may be involved in binding of capped mRNAs, and that the 24 to 26K band detected in SDS-PAGE gels after cross-linking may be the fragment of a precursor left after a cleavage event. Tahara et al. reported that a reticulocyte CBP which contained stable restoring activity for translation of capped mRNAs in poliovirus-infected HeLa cell extracts was associated with other higher-molecular-weight polypeptides (29). Slower-sedimenting forms of the CBP were capable of stimulating translation of Sindbis virus mRNA in an uninfected HeLa cell extract, but these lacked restoring activity. Consistent with the results shown here and in our previous publication (14), neither stimulatory nor restoring activity was observed in similar CBP preparations from poliovirus-infected cells. The high salt conditions in the sucrose gradients used by Tahara et al. would likely have resulted in dissociation of the CBP from eIF-3 (28, 32), and the identity of the higher-molecular-weight polypeptides isolated with the functional CBP remains unknown.

The data in this communication indicate that CBP fails to be associated with eIF-3 after infection of HeLa cells with poliovirus. We suggest that the disruption of an active eIF-3-CBP complex may be involved in the selective inhibition of host cell protein synthesis seen in poliovirus-infected cells.

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