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Presence of the Cap-Binding Protein in Initiation Factor Preparations from Poliovirus-Infected HeLa Cells

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Crude preparations of initiation factors from mock-infected and poliovirus-infected HeLa cells were analyzed for the presence of proteins which could be cross-linked to the 5′ cap group of mRNA. A protein having an apparent molecular weight of 26,000, similar to the cap-binding protein in rabbit reticulocytes described by Sonenberg and Shatkin (Proc. Natl. Acad. Sci. U.S.A. 75:4843-4847, 1978), was found in the ribosomal salt wash from both uninfected and infected cells. Cross-linking of this polypeptide was inhibited by the cap analog m^7GMP. In addition, cross-linking of a protein having an approximate molecular weight of 60,000 was similarly inhibited by cap analog. The smaller cap-binding protein fractionated in a 0 to 40% ammonium sulfate precipitate of ribosomal salt wash; the larger protein was found in the 40 to 70% ammonium sulfate fraction. Although the cap-binding proteins were present in both mock-infected and poliovirus-infected ribosomal salt wash, only preparations from uninfected HeLa cells were able to restore translation of capped vesicular stomatitis virus mRNA by extracts prepared from poliovirus-infected cells.

Shortly after infection of HeLa cells with poliovirus, host cell protein synthesis is markedly inhibited. The inhibition results from a failure to form initiation complexes with cellular mRNA's (10, 15, 16), although host cell mRNA remains structurally intact and appears to be biologically functional when used to direct protein synthesis in vitro (9, 11, 15). Vesicular stomatitis virus (VSV) mRNA has frequently been used as a model for cellular mRNA in studies of poliovirus-induced inhibition of translation, since superinfection of VSV-infected cells with poliovirus results in inhibition of VSV protein synthesis in a manner apparently similar to that observed with host cell protein synthesis (8, 9). (Recent work by Jen et al. [14] has suggested that poliovirus and encephalomyocarditis virus employ different mechanisms for inhibiting host cell protein synthesis. For this reason, we have dealt in this report only with data obtained by studies of poliovirus infection and have omitted discussion of other picornavirus infections.)

Several laboratories have presented evidence for some alteration(s) in the activity of initiation factors (IFs) from poliovirus-infected cells (5, 12-15, 19). Specifically, crude IFs from poliovirus-infected cells stimulate translation of poliovirus RNA in vitro, but they fail to stimulate translation of cellular or VSV mRNA's. In addition, the failure of extracts from poliovirus-infected cells to translate VSV mRNA can be overcome by the addition of reticulocyte IFs (19). This "restoring activity" has been attributed to the presence in IF preparations of a cap-binding protein (CBP) (26). First detected by Sonenberg and Shatkin (23), the CBP was chemically cross-linked to radiolabeled cap structures at the 5′ terminus of reovirus mRNA, and cross-linking was prevented by the presence of cap analogs such as m^7GMP. Most recently, the CBP has been shown to specifically stimulate in vitro translation of capped mRNA in preference to uncapped mRNA (24).

The lack of a cap group on poliovirus mRNA stands in striking contrast to virtually all other eucaryotic mRNA's (1). Since IF preparations from poliovirus-infected cells appear able to discriminate between polio RNA and cellular mRNA's, a plausible model for a mechanism of host cell "shutoff" might include the inactivation of some factor which recognizes the cap structure on cellular mRNA and enhances initiation complex formation. This hypothesis has led to the suggestion that poliovirus infection results in inactivation of the CBP, causing inhibition of cellular protein synthesis (24, 26).

In this report, we have analyzed IF preparations from poliovirus-infected and mock-infected HeLa cells for the presence of CBPs. We show that a protein analogous to that found in rabbit reticulocytes is present in both uninfected and infected cells at times when host cell protein

synthesis is completely inhibited. In addition, we show that uninfected HeLa cell IFs contain restoring activity for translation by infected-cell extracts, but that poliovirus-infected cell IFs do not.

MATERIALS AND METHODS

Cells and virus. The growth of HeLa cells and the growth and purification of poliovirus were as previously described (5) except that the cells were grown in 8% calf serum which had been heated at 56°C for 1 h. VSV was purified from infected HeLa cells as described earlier (2) except that the potassium tartrate-glycerol gradient was replaced by a 5 to 20% sucrose (wt/wt) gradient, centrifuged at 18,000 rpm for 50 min at 4°C in an SW27 rotor.

Cell-free translation. Preparation of HeLa cell-free extracts (S_{10}) was essentially as described by Brown and Ehrenfeld (4) except that the lysis buffer contained 2.5 mM dithiothreitol and did not contain hemin. Furthermore, extracts were prepared and stored in lysis buffer without further adjustment of salts. Extracts were prepared from either mock-infected or poliovirus-infected HeLa cells, harvested at 4 h postinfection. Extracts from both mock-infected and poliovirus-infected cells were treated with micrococcal nuclease by the method of Pelham and Jackson (18). VSV mRNA synthesized in vivo was purified as described earlier (5). Likewise, the purification of poliovirus RNA from virions was as described (5). In vitro translation reactions (50 μl) contained 25 μl of nuclease-treated lysate and the following: 20 μM of each of 19 amino acids (minus methionine), 1.0 mM ATP, 0.2 mM GTP, 25 mM creatine phosphate, 62.5 μg of creatine phosphokinase (Calbiochem) per ml, 2.0 mM magnesium acetate, 5 μCi of [^{35}S]methionine (Amersham, 1,100 Ci/mmol), and 5 μg of tRNA (Sigma). Additions of mRNA, ribosomal salt wash (RSW), or ammonium sulfate (AS) fractions are indicated in figure legends.

In vitro RNA synthesis. VSV mRNA was synthesized in vitro (2) in the presence of *S*-adenosyl-[*methyl*-^{3}H]methionine (specific activity, 60 to 85 mCi/ml; Amersham). Each reaction was supplemented with 0.6 U of pyrophosphatase per ml, 50 U of pyruvate kinase per ml, 10 mM phosphoenolpyruvate, and 1 mM *S*-adenosyl[*methyl*-^{3}H]methionine. The labeled mRNA sedimented between 12S and 18S on sucrose gradients. On methylmercury hydroxide agarose gels, primarily three bands were obtained, which were judged to be 12S, 14S, and 17S as compared with the mobility of marker RNAs (2). Periodate oxidation of mRNA was carried out as described (19).

Preparation of IFs. IFs were prepared from poliovirus-infected and mock-infected HeLa cells at 4 h postinfection. At this time, synthesis of host cell proteins has been completely inhibited in infected cells, and translation of viral proteins is maximal in extracts prepared from these cells (12). Preparations were essentially as described by Brown and Ehrenfeld (4). Briefly, HeLa cell S_{10} extracts were centrifuged at 49,000 rpm for 90 min at 4°C in a Spinco Angle 50.1 Ti rotor. Ribosomes were suspended to a concentration of 200 to 250 units of absorbancy at 260 nm per ml in lysis buffer. KCl (4 M) was added to a final concentration of 0.5 M, and the solution was stirred on ice for 15 min. After centrifugation at 49,000 rpm for 90 min, the supernatant (RSW) was either dialyzed overnight at 4°C against 20 mM Tris (pH 7.4)–100 mM KCl–0.2 mM EDTA–7 mM β-mercaptoethanol–5% glycerol or fractionated with AS before dialysis. Crude IFs from rabbit reticulocytes were prepared according to the methods of Schreier and Staehelin (20) and Sundkvist and Staehelin (25). Fractionation of crude IFs into 0 to 40% and 40 to 70% AS precipitates was as described previously (13).

Cross-linking of IFs to mRNA. RSW and 0 to 40% or 40 to 70% AS fractions were incubated with periodate-oxidized, *methyl*-^{3}H-labeled VSV mRNA, and complexes were stabilized by reduction with $NaCNBH_3$, as described (21, 23), except that the incubation mixture was treated directly with RNase, omitting any protein precipitation step. Each reaction contained approximately 1 μg of VSV mRNA made in vitro and between 100 and 150 μg of total protein (as determined by E_{280}/E_{260} measurements). The labeled components were then analyzed on sodium dodecyl sulfate (SDS)-polyacrylamide gels which were fluorographed in sodium salicylate (7).

RESULTS

Alterations in IF activity in poliovirus-infected cells. Preparations of IFs from poliovirus-infected cells failed to stimulate translation of cellular or VSV mRNA's in vitro, although they did stimulate translation of poliovirus RNA. An example of this specificity of IF activity is shown in Fig. 1. Lane 1 shows the background translation of a micrococcal nuclease-treated HeLa cell S_{10} extract in the absence of added mRNA. Addition of an RSW as a source of IFs from either mock-infected or poliovirus-infected cells had no effect on the endogenous translation products (lanes 2 and 3), thus demonstrating that these crude IF preparations were not contaminated with mRNA. Addition of VSV mRNA resulted in the detectable synthesis of VSV proteins N, NS, and M, shown in lane 4. Production of VSV proteins could be stimulated by the addition of RSW from reticulocytes (not shown) or from mock-infected HeLa cells (lane 5). The RSW from poliovirus-infected cells, however, did not stimulate VSV translation above the levels observed in the absence of added IFs (lane 6). These results are consistent with those previously reported (6, 12–15), showing that crude IF preparations from poliovirus-infected cells are unable to stimulate translation of other mRNA's.

Translation of poliovirus RNA in HeLa cell extracts was stimulated by RSW from reticulocytes (not shown) and mock-infected or poliovirus-infected HeLa cells (Fig. 1, lanes 8 and 9). Some differences in the resulting polypeptide

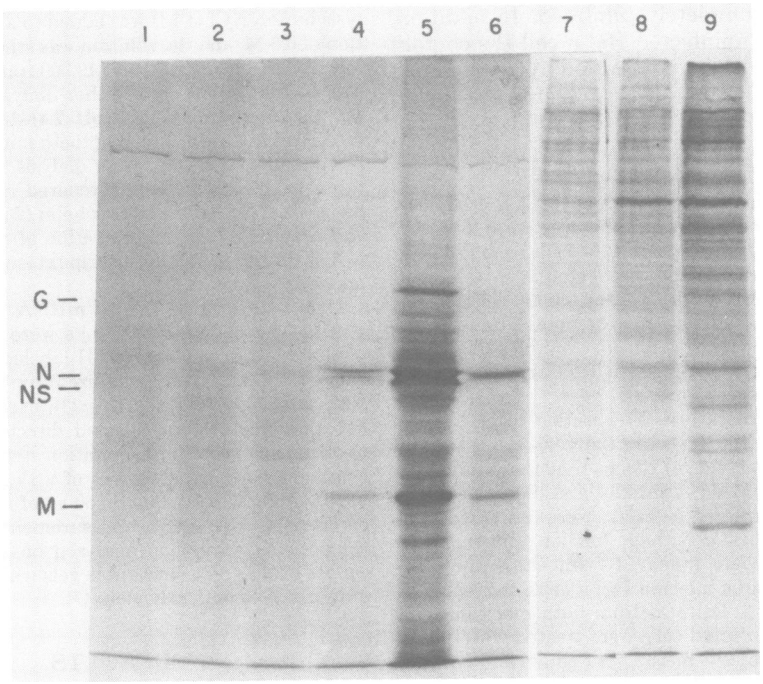

FIG. 1. *Effect of crude IFs on translation in extracts from mock-infected HeLa cells. [^{35}S]methionine-labeled proteins synthesized in vitro were separated by electrophoresis and detected by autoradiography. Reaction mixtures (50 μl) contained micrococcal nuclease-treated S_{10} (25 μl) from mock-infected cells and the following: lane 1, 6 μl of IF buffer only; 2, 6 μl of RSW (125 μg of protein) from mock-infected cells; 3, 6 μl of RSW (110 μg of protein) from poliovirus-infected cells; 4, 2 μg of VSV mRNA; 5, VSV mRNA and mock-infected RSW; 6, VSV RNA and poliovirus-infected RSW; 7, 6 μg of poliovirus RNA; 8, poliovirus RNA and mock-infected RSW; 9, poliovirus RNA and poliovirus-infected RSW. Labeled proteins resulting from in vitro translation of poliovirus RNA do not correspond well to those seen in labeled cytoplasmic extracts from infected cells. The spectrum of proteins made in response to poliovirus RNA is a characteristic of the S_{10} used. This phenomenon has been observed before (6, 19).*

cleavage patterns were observed, as has been previously reported (4), but it was clear that crude preparations of IFs from poliovirus-infected cells will stimulate synthesis of poliovirus proteins although they are defective for stimulating translation of VSV proteins.

CBP in IF preparations. The IF preparations described above were analyzed for the presence of the CBP by a modification of the cross-linking assay developed by Sonenberg and Shatkin (23). This modification yielded a more reproducible recovery of cross-linked protein. Briefly, *methyl*-^{3}H-labeled VSV mRNA synthesized in vitro was periodate oxidized and then incubated with crude IFs. Any complexes formed were stabilized by reduction with $NaCNBH_3$. This mixture was treated directly with RNase and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE), instead of prior isolation of the cross-linked proteins by acetone precipitation. A control sample of labeled RNA incubated in the absence of any proteins and digested with RNase yielded a strong doublet band migrating near the dye front (Fig. 2, lane 1), which we assume to be the residual 5′ oligonucleotide of VSV mRNA's. This doublet consequently appeared in all gels of cross-linked material.

RSW from rabbit reticulocytes contained a number of proteins which could be cross-linked to the cap structure on VSV mRNA (Fig. 2, lane 6). One of these, with an apparent molecular weight (mol. wt.) of 26,000 (26K), was prevented from cross-linking by the inclusion of 1 mM m^7GMP in the reaction mixture of lane 7. This characteristic defined the CBP, originally described by Sonenberg and Shatkin (23). The significance of other proteins which bound to the cap group but which were not prevented from cross-linking by cap analogs is not known. A comparable CBP was present in the RSWs from mock-infected HeLa cells (lanes 2 and 3) and from poliovirus-infected HeLa cells (lanes 4 and 5). The mobility of this protein appeared to be the same in mock-infected and virus-infected

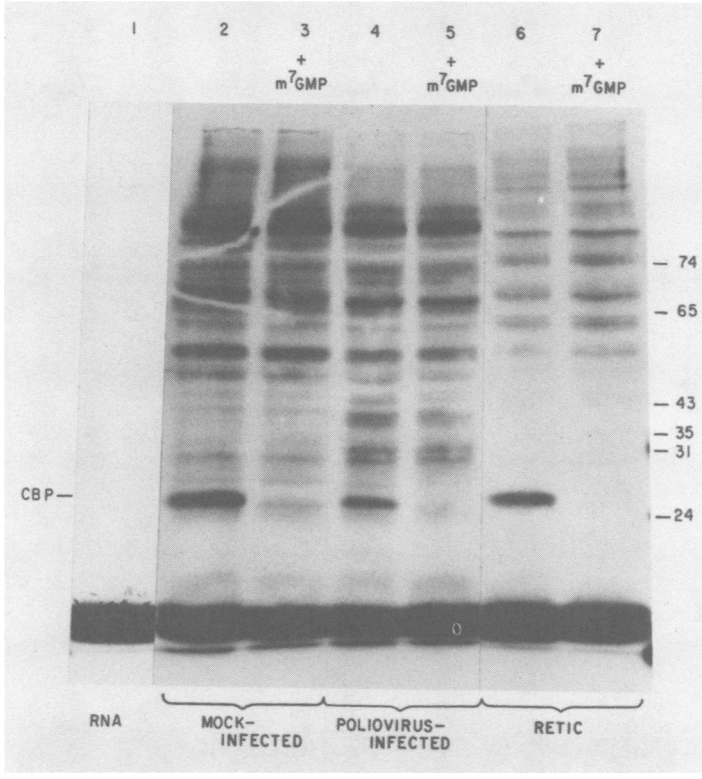

FIG. 2. *SDS-PAGE analysis of cross-linked proteins from RSW. Oxidized methyl-*^{3}*H-labeled VSV mRNA was cross-linked to proteins in RSW and analyzed by SDS-PAGE. Lane 1, No RSW added; 2, mock-infected RSW (104 μg of protein); 3, mock-infected RSW and 1 mM* m^7GMP*; 4, poliovirus-infected RSW (110 μg of protein); 5, poliovirus-infected RSW and 1 mM* m^7GMP*; 6, reticulocyte RSW (125 μg of protein); 7, reticulocyte RSW and 1 mM* m^7GMP*. Approximate* M_r *is indicated on right.*

cells, but the HeLa cell CBP migrated slightly faster than that from rabbit reticulocytes. In some experiments, there appeared to be slightly less cross-linked CBP in RSW from infected cells. The differences, however, were small, and quantitation was uncertain due to variation in protein recovery from the RSW as well as variation in the efficiency of the cross-linking reaction.

AS fractionation of CBP. CBP from rabbit reticulocytes has been reported to fractionate with eIF-4B and eIF-3 in a 0 to 40% AS precipitate of crude RSW (22, 26). We therefore similarly analyzed the CBP from uninfected and infected HeLa cells. RSW was fractionally precipitated into 0 to 40% and 40 to 70% AS cuts, and the resulting dialyzed samples were separately cross-linked to *methyl-*^{3}H-labeled VSV mRNA's. Figure 3 shows the SDS-PAGE profile of the cross-linked proteins. From both mock-infected and virus-infected HeLa cells, the 26K-mol. wt. CBP was found to be present in the 0 to 40% AS fractions (lanes 1 and 5), and cross-linking of this protein was inhibited by 1 mM m^7GMP (lanes 2 and 6). The 40 to 70% AS fraction contained much less of the 26K-mol. wt. CBP (lanes 3 and 7), and its cross-linking was also inhibited by m^7GMP (lanes 4 and 8). We concluded that the majority of the CBP fractionates into the 0 to 40% AS precipitate from both uninfected and infected HeLa cells. In addition, the 40 to 70% AS fraction also contained a protein of approximately 60K mol. wt. (indicated by arrow), whose cross-linking to the cap group of mRNA was inhibited by m^7GMP (lanes 3 and 4). This protein was also present in IF preparations from infected cells (lanes 7 and 8).

Marked differences in the other cross-linked proteins from both the 0 to 40% and 40 to 70% AS fractions were seen between infected and mock-infected cells. The differences in the 0 to 40% AS fraction were most noticeable in the region of 30K to 40K mol. wt., whereas differences in the 40 to 70% fraction were most apparent from about 25K to 50K mol. wt. Cross-linking of these proteins was not inhibited by

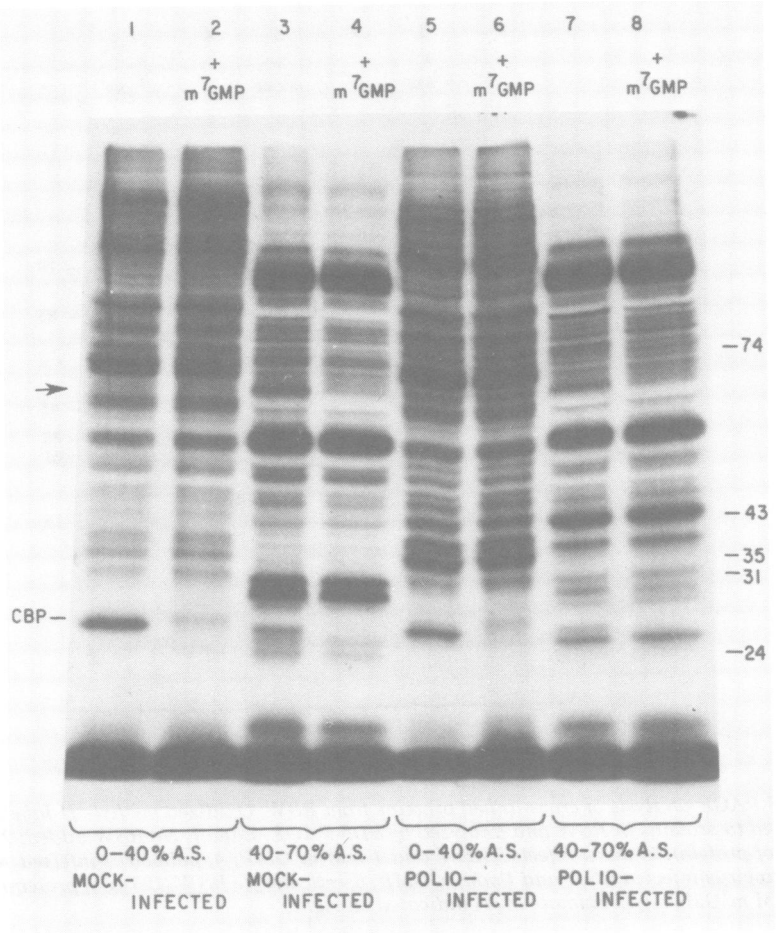

FIG. 3. *Cross-linking pattern of proteins in AS fractions. Cross-linking was performed as described. Lane 1, 0 to 40% AS precipitate (220 μg of protein) from mock-infected cells; 2, mock-infected 0 to 40% AS precipitate and 1 mM m⁷GMP; 3, mock-infected 40 to 70% AS precipitate (83 μg of protein); 4, mock-infected 40 to 70% AS precipitate and 1 mM m⁷GMP; 5, 0 to 40% AS precipitate (226 μg of protein) from poliovirus-infected cells; 6, poliovirus-infected 0 to 40% AS precipitate and 1 mM m⁷GMP; 7, poliovirus-infected 40 to 70% AS precipitate (75 μg of protein); 8, poliovirus-infected 40 to 70% AS precipitate and 1 mM m⁷GMP.*

m⁷GMP, and their significance is not clear.

Restoring activity of HeLa cell IFs. Extracts from poliovirus-infected cells have been shown to be defective for translation of VSV mRNA (19). The ability of these extracts to translate VSV mRNA can be restored by the addition of purified CBP from rabbit reticulocytes (26). Since the CBP is present in crude preparations of IFs from both uninfected and poliovirus-infected HeLa cells, as well as from rabbit reticulocytes, we analyzed these preparations for restoring activity. Extracts from poliovirus-infected HeLa cells were prepared and treated with micrococcal nuclease as described. These extracts had a background of endogenous translation (Fig. 4, lane 1). The addition of RSW from either reticulocytes (not shown) or mock-infected or poliovirus-infected HeLa cells (lanes 2 and 3) did not substantially alter this background. (The background was repeatedly higher and more resistant to micrococcal nuclease treatment than that seen with extracts from uninfected cells. This was somewhat troublesome since one background protein has the same mobility as VSV N protein.) As reported by others (19), when VSV mRNA was added to this extract no VSV proteins were synthesized (lane 4). Addition of reticulocyte RSW (not shown) or RSW from uninfected HeLa cells, however, restored the ability of the infected cell extract to synthesize VSV proteins N, NS, and especially M (lane 5). In contrast,

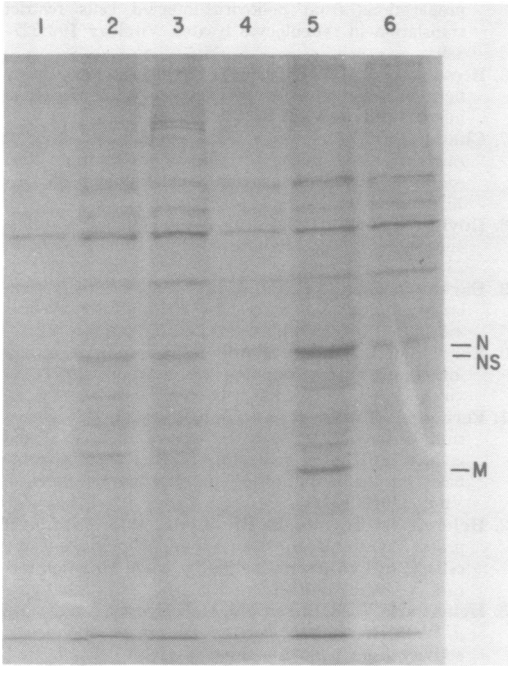

FIG. 4. *Restoration of VSV translation in extracts prepared from poliovirus-infected cells. [^{35}S]methionine-labeled proteins synthesized in vitro were analyzed by SDS-PAGE and autoradiography. Reaction mixtures (50 μl) contained micrococcal nuclease-treated S_{10} (25 μl) from infected cells and the following: lane 1, initiation factor buffer only; 2, 6 μl of RSW (125 μg of protein) from mock-infected cells; 3, 6 μl of RSW (110 μg of protein) from poliovirus-infected cells; 4, 2 μg of VSV mRNA; 5, VSV mRNA and mock-infected RSW; 6, VSV mRNA and poliovirus-infected RSW.*

RSW from poliovirus-infected HeLa cells was unable to restore VSV translation (lane 6). Thus, the ability to restore translational activity in extracts from poliovirus-infected cells was not correlated with the presence of the CBP in IF preparations.

DISCUSSION

This study has demonstrated that the RSW from uninfected HeLa cells has a restoring activity for translation of VSV mRNA, similar to that ascribed to reticulocyte IFs (19). This restoring activity was not present in the RSW from poliovirus-infected cells, even though preparations from both sources contained the CBP. The CBP has been defined by a chemical cross-linking assay so that detection of the CBP does not depend on any kind of biological activity. The fact that the CBP from poliovirus-infected cells can be detected by cross-linking, however, implies that this protein is present and is still able to associate with the cap structure.

The ability to restore VSV translation in an extract from poliovirus-infected cells was initially attributed to reticulocyte eIF-4B (8) but was determined subsequently to be due to the presence of the CBP (26). All subsequent investigations have utilized preparations of reticulocyte eIF-3 as the source of CBP (22, 26). We find that restoring activity for VSV translation does not correlate simply with the presence of CBP in IF preparations. However, the results reported here for the HeLa cell CBP are consistent with the methods of purification for the reticulocyte CBP, since the majority of the CBP from both mock-infected and poliovirus-infected HeLa cells was found in the 0 to 40% AS fraction, where eIF-3 and eIF-4B also precipitate (13, 20).

Recently, purified CBP has been shown to stimulate translation of capped mRNA, but not uncapped mRNA (24). Sonenberg et al. have suggested that the preferential stimulation of translation of capped mRNA exhibited by eIF-3 may be due entirely to the CBP, since removal of the CBP by purification in high salt reduced the activity of eIF-3 to near background (24). Helentjaris et al. (13) have reported that eIF-3 preparations from poliovirus-infected HeLa cells are inactive for translation of globin, a capped mRNA. Since CBP has a high affinity for eIF-3 (22) it is possible that those authors were measuring CBP activity in their preparations of eIF-3. If this assumption is correct, it suggests that the CBP may indeed be defective in poliovirus-infected cells.

The form of the biologically active CBP remains unclear. An early report suggested that the 24K (or 26K) M_r CBP sedimented on sucrose gradients as if it were a protein of 200K M_r (3). The VSV restoring activity of the purified CBP is reportedly very unstable (26). These two findings suggest that the CBP may be biologically active in a multimeric form or that it may require other components to remain active. We can detect the presence of the CBP, by cross-linking to cap structures, in IF preparations from infected cells that have no VSV restoring activity. This result may indicate that some factor(s) other than, or in addition to, the CBP is responsible for the restoring activity. Another equally acceptable explanation is that the CBP may have at least two activities, cap binding and what we assay as restoring activity. One activity might be inhibited without substantially affecting the other.

In addition to the 26K CBP, we detected a second protein whose cross-linking was also inhibited by the cap analog. This protein, of mol.

wt. approximately 60K, fractionated in the 40 to 70% AS precipitate from both mock-infected and poliovirus-infected cells. This protein may represent a larger form or precursor of the 26K CBP or it may be another "cap-binding protein" altogether.

The gel profiles of cross-linked proteins from mock-infected and poliovirus-infected cells were quite different. These differences were apparent in both the 0 to 40% and the 40 to 70% AS fractions, but the significance of these differences is not known. They may be due to changes in protease activities in infected cells; alternatively, these differences may represent viral proteins or modifications of proteins normally found in the RSW.

The band we identify as the CBP has an apparent mol. wt. of 26K, as opposed to the 24K mol. wt. reported for the reticulocyte CBP by Sonenberg and Shatkin. Since the cross-linking of both proteins is inhibited by 1 mM m^7GMP, we believe the difference in apparent mol. wt. reflects trivial differences in markers or gel systems.

In summary, we have presented results which show that a 26K-mol. wt. CBP is present in both mock-infected and poliovirus-infected HeLa cells. No difference in the apparent mol. wt. of the CBP from either source was detected. The CBP from both kinds of cells separated predominantly into the 0 to 40% AS fraction of the RSW. The CBP was detected, by cross-linking to capped mRNA, in crude IFs from infected cells that do not contain any restoring activity for translation of VSV mRNA. Further studies of the biological function of CBP in infected cells are in progress.

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