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Demonstration In Vitro that Eucaryotic Initiation Factor 3 Is Active but that a Cap-Binding Protein Complex Is Inactive in Poliovirus-Infected HeLa Cells

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Protein synthesis initiation factor preparations from poliovirus-infected HeLa cells have reduced ability to initiate translation on capped mRNA. The defect in initiation factors has been variously attributed to inactivation of eucaryotic initiation factor 3 (eIF3), eIF4B, or a cap-binding protein (CBP) complex. We have developed a series of in vitro protein synthesis assays to show that eIF3 is active but a CBP complex activity is inactivated after poliovirus infection. eIF3 activity, when determined in the presence of purified CBP complex, is present in sucrose gradients of factors from both infected and uninfected cells. CBP complex activity, determined in the presence of eIF3 from poliovirus-infected cells, is present in uninfected cells only and comigrates on sucrose gradient with an activity which restores the ability of crude initiation factors from infected cells to translate capped globin mRNA. This is the first demonstration by a fractionated translation system that an activity which is attributable to CBP complex is inactivated in poliovirus-infected cells. The results also indicate that eIF3 is undetectable or has greatly reduced activity in the absence of CBP complex.

Poliovirus infection of HeLa cells results in an inhibition of cellular capped mRNA translation. Early work has indicated that protein synthesis initiation factor preparations are defective in infected cells, since crude factors are unable to promote initiation of translation on polysomes containing capped cellular mRNA (13). Fractionation of the initiation factors and comparison of activities in vitro have led to the identification of eucaryotic initiation factor 3 (eIF3) as the inactivated factor in infected cells (12). However, an eIF4B preparation has been found to restore translation of capped mRNA in infected cell lysates (16). Subsequent discovery of a protein or protein complex which is able to recognize mRNA caps and which contaminates the eIF3 and eIF4B preparations (23) has suggested the possibility that this previously unrecognized factor is responsible for the initiation defect in poliovirus-infected cells. Indeed, a cap recognition factor, referred to as cap-binding protein (CBP) II or eIF4F, has since been shown to restore capped template function in poliovirus-infected cell lysates (9).

The first CBP to be identified by a chemical cross-linking assay was a 24-kilodalton (Kd) protein, CBP I (18–20). It was the only protein among crude initiation factors to specifically cross-link to oxidized, labeled, capped mRNA in the absence of ATP. When ATP was added to the reaction, however, several additional cap-binding polypeptides were identified (17), and the cross-linking ability of these polypeptides was found to be inhibited after infection of HeLa cells with poliovirus (14). Although CBP I recognizes cap structures, it cannot alone restore the ability of infected lysates to translate capped mRNAs. Instead, this activity has been found to reside in a complex (CBP II) consisting of CBP I plus higher-molecular-weight polypeptides (22). Purified CBP II (eIF4F), consisting of three or four polypeptides (210, 49, 24 Kd and possibly one of 73 Kd), has been shown to be required for translation of globin mRNA in vitro (9).

A CBP complex used in the experiments described here consists of only three polypeptides, p220, p49, and p24-28 (6). The subunits of CBP complex have previously been the subject of separate studies. We have recently reported that a 220-Kd polypeptide which is immunologically related to p220 of CBP complex is degraded in poliovirus-infected cells and that the kinetics of degradation roughly correlate with the onset of host cell inhibition of protein synthesis (8). The smallest polypeptide of CBP complex, CBP I, has been identified in two forms in uninfected cells, a heavy-sedimenting form and a light-sedimenting form (22). The 49-Kd subunit of CBP complex has been found to comigrate on two-dimensional gels with eIF4A (9) and to have many, but not all, tryptic peptides in common with eIF4A (6), an initiation factor which can be purified independently (3). In addition, antisera prepared against independently purified eIF4A also react with the 49-Kd subunit of CBP complex (6).

That translation of capped mRNAs in poliovirus-infected cell extracts can be restored by the CBP complex (CBP II or eIF4F) purified from rabbit reticulocytes supported the hypothesis that this complex was inactivated or defective in poliovirus-infected cells. Hansen et al. (11) have established that the rapidly sedimenting CBP I-containing complex was absent or dissociated in poliovirus-infected cells, and several investigators have demonstrated that infected cells contain no restoring activity. However, no direct demonstration that this complex is inactive in poliovirus-infected cells has been reported.

The major difficulty in these efforts has been the fact that eIF3 is isolated together with the CBP complex, and thus, independent assays for the two factors have not been available. The restoring factor from reticulocytes has been shown to associate or sediment with eIF3 in sucrose gradients containing low salt, but to sediment more slowly than

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eIF3 in gradients containing high salt (23). However, high-salt-washed eIF3 is inactive in a fractionated translation system with capped globin mRNA as template (21). These results suggest that the activity usually measured in translation assays was actually the combined activities of eIF3 and CBP complex (eIF3-CBP). If this were the case, then the previously reported failure to detect eIF3 activity in poliovirus-infected cells could have been due to the absence of CBP complex activity which resulted in an inhibition of the eIF3-CBP activity. In this report, we demonstrate directly that CBP complex is inactive in infected cell lysates and that eIF3 measured in the presence of purified CBP complex is equally active in infected and uninfected cells.

MATERIALS AND METHODS

Cells and virus. HeLa S_3 cells and poliovirus 1 (Mahoney strain) were grown and virus was purified as previously described (7).

Initiation factor preparations. Poliovirus-infected and uninfected cells were harvested and stored at −70°C until use as described previously (8). Ribosomal salt wash (RSW) and ammonium sulfate fractions, RSW-A (0 to 40% saturation) or RSW-B (40 to 70% saturation), were prepared from infected or uninfected HeLa cells as described previously (12). CBP complex preparation from rabbit reticulocytes was also described previously (8). Purified initiation factors eIF3 and eIF4B from HeLa cells and eIF4A and eIF4C from rabbit reticulocytes were prepared essentially as described previously (1, 4). eIF3 from poliovirus-infected cells was prepared as pooled fractions from a sucrose gradient of RSW-A similar to that shown in Fig. 2. The major eIF3 antigen-containing gradient fractions were pooled, concentrated by 70% ammonium sulfate precipitation, and dialyzed against buffer A (20 mM Tris-hydrochloride [pH 7.5], 0.2 mM EDTA, 7 mM β-mercaptoethanol, 5% glycerol) with 200 mM KCl. This preparation is free of eIF4A, eIF4B, and CBP-p220-related antigens as detectable by immunoblot (5, 8).

In vitro translation systems. Preparation of the fractionated in vitro protein synthesizing system components has been described previously (1). Each reaction contained a total volume of 20 μl and was incubated at 30°C for 60 min. After alkaline degradation of charged tRNA, acid-precipitable counts were determined as previously described (12). Assay conditions were as follows: 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid) (pH 7.5); 20 mM creatine phosphate; 4 U of creatine phosphokinase per ml; 0.8 mM ATP; 0.32 mM GTP; 30 μM each of 19 amino acids except leucine; 20 μCi of [^{3}H]leucine (60 Ci/mmol) per ml; 2.0 mM magnesium diacetate; 100 mM potassium chloride-potassium acetate; 4 mM spermine; 2.5 mM dithioerythritol; 0.5 absorbancy units at 280 nm (A_{280} units) of pH 5 enzymes (from HeLa cells) per ml; 1.5 A_{280} units and 3.8 A_{280} units of purified rat liver ribosomal subunits, 40S and 60S, respectively, per ml; 0.02 mg of purified globin mRNA from rabbit reticulocytes per ml; 0.1 A_{280} units of purified rat liver tRNA per ml; and crude or purified initiation factors as indicated. eIF3 activity was determined in the presence of CBP complex purified from rabbit reticulocytes and eIF4B and RSW-B from HeLa cells. RSW-B provides eIF2, eIF4A, eIF4C, and eIF5. eIF3-CBP activity was determined in the presence of eIF4B and RSW-B purified from HeLa cells. CBP complex activity was determined in the presence of 1 A_{280} unit of eIF3 (sucrose gradient pool) from poliovirus-infected cells, eIF4B, and RSW-B from uninfected cells per ml. Restoring activity was determined in the presence of 2.4 A_{280} units of RSW per ml from poliovirus-infected cells as sole source of initiation factors. All factors were added at saturating levels.

Immunoblot analysis. A 30-μl amount of each sucrose gradient fraction was subjected to electrophoresis on sodium dodecyl sulfate–10% polyacrylamide slab gels. The proteins were electrophoretically transferred to nitrocellulose paper sheets and immunoblotted as previously described (8). Antiserum used against purified eIF3-CBP was made in goats (15). The second antibody was rabbit anti-goat immunoglobulin radiolabeled with ^{125}I (to a specific activity of 5×10^7 cpm/μg). The immunoblot was exposed to Kodak X-Omat SB5 film for 3 days.

RESULTS

Detection of eIF3 and CBP complex activities. To assay separately for both eIF3 and CBP complex activities, it was necessary to obtain a purified eIF3 preparation that was free of CBP complex. Cross-linking assays showed that our eIF3 preparations from uninfected HeLa cells contained a 24- to 28-Kd cross-linking polypeptide which is presumably the CBP I subunit of CBP complex (Fig. 1). This result was not unexpected, since similar eIF3 preparations from rabbit reticulocytes have been previously shown to contain CBP I (18), and antisera prepared against eIF3 has been shown to react with at least one other CBP complex polypeptide, p220 (8). Crude initiation factor preparations from both uninfected and poliovirus-infected cells were sedimented through sucrose gradients and each fraction was assayed in a transla-

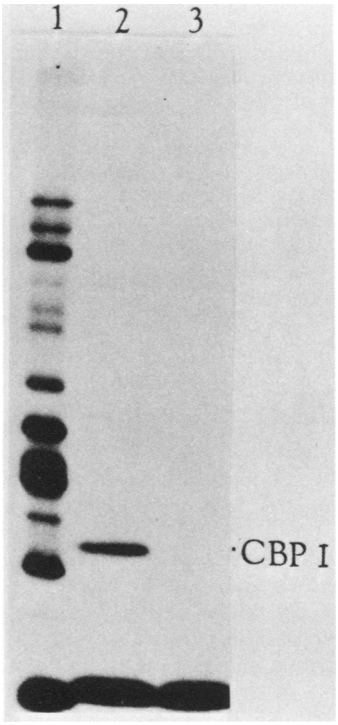

FIG. 1. Analysis of CBPs in purified eIF3 preparations. eIF3 (10 μg) from HeLa cells was incubated with *methyl*-^{3}H-labeled vesicular stomatitus virus mRNA, plus (lane 3) or minus (lane 2) 1 mM m^7GDP, as indicated. Cross-linking assay conditions are described in detail elsewhere (10). Proteins cross-linked to the 5′ end of mRNA were separated on sodium dodecyl sulfate–10% polyacrylamide gels, and the dried gel was exposed to X-ray film. Lane 1 is a cell lysate from poliovirus-infected cells labeled with [^{35}S]methionine for 1 h, starting 3 h after infection. The gel position of CBP I is indicated on the right.

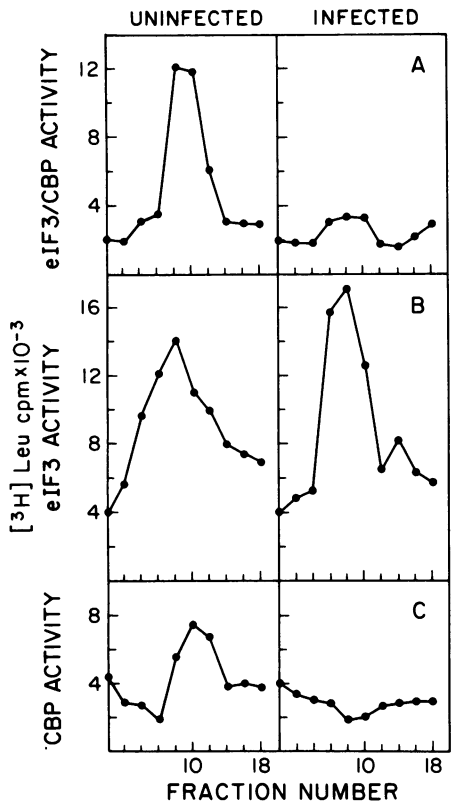

FIG. 2. In vitro activities of sucrose gradient fractions of crude initiation factors from poliovirus-infected or uninfected HeLa cells. RSW 0 to 40% ammonium sulfate fractions (RSW-A) from poliovirus-infected or uninfected HeLa cells were prepared as described in the text. RSW-A fractions derived from ca. 3×10^9 cells (31 A_{280} units of RSW-A from poliovirus-infected cells or 22 A_{280} units of RSW-A from uninfected cells) were applied to two 15 to 30% sucrose gradients in buffer A (20 mM Tris, pH 7.5; 0.2 mM EDTA; 7 mM β-mercaptoethanol; 5% glycerol) containing 200 mM KCl. Centrifugation was for 20 h at 38,000 rpm in a Beckman SW40 rotor. Gradients were fractionated into 18 fractions, and 2 μl of each fraction was assayed for eIF3-CBP, eIF3, or CBP complex activity as described in the text. Sedimentation in this and other figures is from right to left.

tion assay requiring both eIF3 and CBP complex. Uninfected HeLa cells contained an activity which stimulated this translation assay, but poliovirus-infected cells contained almost none (Fig. 2A). This result is consistent with previous reports. When the translation assay was conducted in the presence of added purified CBP complex, the results shown in Fig. 2B were obtained. Both uninfected and infected cells contain active eIF3, whose activity was readily detected in infected cell preparations when functional CBP complex was supplied. The profile of eIF3 activity in uninfected cells was broader than that of the combined eIF3-CBP curve.

Attempts were made to prepare eIF3 free of CBP complex from uninfected HeLa cells to assay for the presence of CBP complex in these gradient fractions. Sedimentation of eIF3 preparations through high-salt sucrose gradients was not successful. There was either residual CBP complex which resulted in high background levels in the absence of CBP complex or there were major losses of eIF3 activity. Since infected cells did contain active eIF3 (Fig. 2B), this material was used in the translation assay to monitor CBP complex activity in the sucrose gradient fractions. CBP complex activity determined in the presence of eIF3 from poliovirus-infected cells was present in sucrose gradients of crude initiation factors from uninfected cells, but not in those of infected cells (Fig. 2C). These data confirm that it is the CBP complex which is inactive in infected cells and that eIF3 is present but its activity is not detectable in the absence of the CBP complex.

Comparison of eIF3-CBP antigens and activity. It is important to note that the sedimentation profiles of the CBP complex and of eIF3 activities did not coincide in these gradients and that the eIF3-CBP activity represented the overlap of the two activities measured independently. Figure 3 demonstrates further evidence that eIF3-CBP activity represents the overlapping region between eIF3 and CBP complex on a sucrose gradient. Crude initiation factors from uninfected cells were sedimented on a sucrose gradient and each fraction was assayed for the presence of eIF3-CBP activity. A second portion of each fraction was run on a polyacrylamide slab gel, electrotransferred to nitrocellulose paper, and reacted with eIF3-CBP antisera. eIF3, which is represented by antigens p170, p115, and p47 together (fractions 9 to 23) and the CBP complex antigen, p220 (fractions 16 to 28), overlapped in the sucrose gradient, and eIF3-CBP activity peaked in the region of antigen overlap. This figure also demonstrates that eIF3 antigens tend to trail toward the top of the gradient. Although eIF3 from rabbit reticulocytes has been shown to have a sedimentation value of about 17S under low-salt-buffer conditions (2), eIF3 from HeLa cells appeared to become partially disrupted at the slightly higher salt concentration (300 mM KCl) used in the gradient shown here. This apparent instability has frequently been observed in eIF3 preparations from HeLa cells.

Comparison of CBP complex and restoring activities. For assurance that the CBP complex activity measured above is identical to the factor which restores the ability of infected cell lysates to translate capped mRNA (restoring factor), fractions from the two gradients shown in Fig. 1 were tested for restoring activity. Grifo et al. have shown that purified CBP II has restoring activity (9). We wished to show here that CBP complex activity and restoring activity have identical sedimentation properties. To measure restoring activity, we utilized a more purified in vitro translation system than the nuclease-treated infected cell lysate of Rose et al. (16). RSW from poliovirus-infected cells was added to essentially the same fractionated translation system used for determination of the activities of individual initiation factors. RSW from infected cells should provide all the necessary initiation factors to the translation system except for the restoring factor. Figure 4A demonstrates the validity of this assay. RSW from infected cells is inactive alone in translation of capped globin mRNA in this system, whereas RSW from uninfected cells is active. This fractionated translation system was compared with that of the nuclease-treated infected cell lysate assay described in detail elsewhere (10) by analyzing fractions of a sucrose gradient of crude factors from uninfected HeLa cells. Restoring activity profiles from both assay systems were identical (J. Hansen and D. Etchison, unpublished data). In addition, purified CBP complex restores activity in this assay (D. Etchison and I. Edery, data not shown).

When fractions from the sucrose gradient of factors from uninfected cells shown in Fig. 2 were assayed for restoring activity in the assay described above, the profile of restoring activity coincided with that of the CBP complex activity (Fig. 4B). As expected, there was no restoring activity in the corresponding gradient of factors from infected cells (data

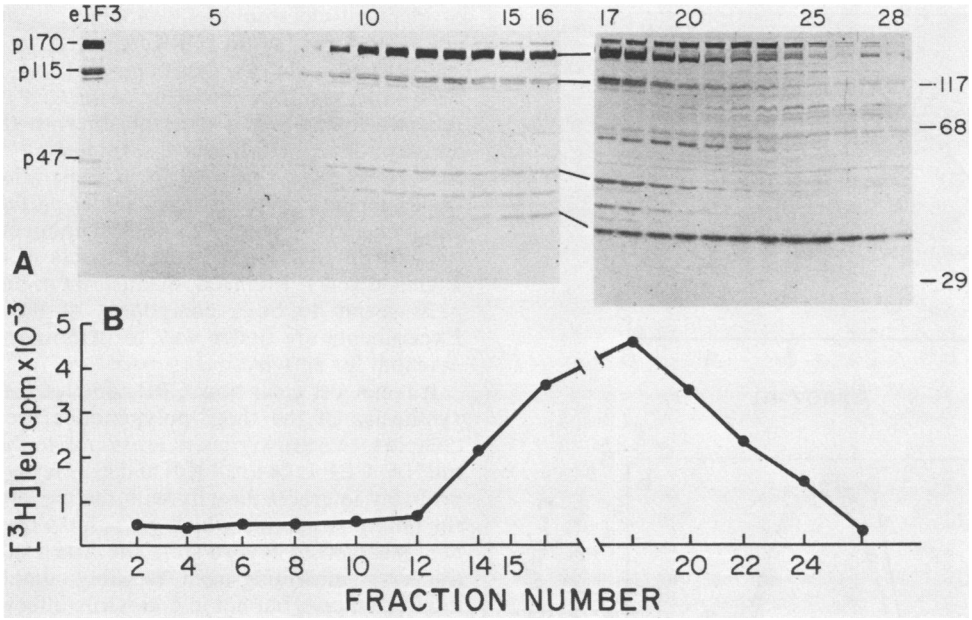

FIG. 3. eIF3-CBP activity and immunoblot analysis of sucrose gradient fractions of crude initiation factors from uninfected cells. Approximately 100 A_{280} units of 0 to 40% ammonium sulfate fraction of RSW from uninfected HeLa cells were applied to a 15 to 30% sucrose gradient in buffer A containing 300 mM KCl. Centrifugation was performed in a Beckman SW27 rotor for 28 h at 26,000 rpm. The gradient was fractionated into 28 fractions. Of each fraction, 2 μl was assayed for eIF3-CBP activity, which is shown in (B). Of each fraction, 30 μl was applied to two sodium dodecyl sulfate–10% polyacrylamide gels, and the separate slab gels were analyzed by immunoblot with eIF3-CBP antisera. The separate autoradiographs (A) of the two immunoblots are shown joined with lines indicating common bands which migrated slightly differently on each gel. The left lane is purified HeLa eIF3. Molecular weight markers on the right were detected as stained bands on the nitrocellulose sheets; β-galactosidase (117 Kd), bovine serum albumin (68 Kd), and carbonic anhydrase (29 Kd).

not shown). This result supports the conclusion that CBP complex is the restoring factor, since the two activities have identical sedimentation properties.

DISCUSSION

A number of lines of evidence suggest that a CBP complex is inactivated in poliovirus-infected cells, and that this inactivated factor is responsible for the failure of the infected cell to translate capped mRNAs. In this report, we show that a CBP complex activity measured in vitro is detectable in crude initiation factors from uninfected HeLa cells but not in factors from poliovirus-infected cells. To do this, we developed assays designed to measure the separate activities of eIF3 and CBP complex and used these to examine sucrose gradient fractions of crude initiation factors from uninfected and infected cells. The results show that CBP complex activity is present in factors from uninfected cells, but not infected cells, and eIF3 activity is present in initiation factor preparations from both infected and uninfected cells.

It seems likely that a defect in the CBP complex is solely responsible for the poliovirus-induced inhibition in cellular capped mRNA translation. In previous work, we have shown that the levels, molecular weights, and isoelectric forms of eIF4A and eIF4B are unchanged after poliovirus infection (5). In earlier work, we have reported that fractions from infected cells thought to contain eIF3 are inactive in the standard globin synthesis assay for eIF3 (12). In the work reported here, we showed that eIF3 activity is, in fact, not reduced in infected cell fractions when active CBP complex is added to the assay system. The earlier assay measured a combination of eIF3 and CBP complex activities, the latter factor being unrecognized at the time. Thus, infected cells contain active eIF3, eIF4A, and eIF4B, but inactive CBP complex.

The separation of eIF3 and CBP complex in preparations from uninfected HeLa cells is not straightforward. Standard techniques for purification of CBP complex involve separating the two factors on high-salt-containing sucrose gradients (6); however, we have found that the composition of eIF3 as determined by immunoblot is altered after high-salt treatment (8). In addition, such an eIF3 preparation is inactive in a fractionated translation system, even in the presence of CBP complex (unpublished data). In contrast, eIF3 purified through a low-salt sucrose gradient step is active but tends to contain variable amounts of CBP complex. To obtain eIF3 free of CBP complex, we decided to use eIF3 from poliovirus-infected cells, since, in a rather circular argument, we reasoned that infected cells contained no active CBP complex. Although we used a limited purification procedure for this eIF3 preparation, the eIF3 fraction from infected cells prepared as described in this report contains no detectable eIF4A, eIF4B, or p220-related antigens. We have more recently been able to purify CBP-free eIF3 from uninfected cells by selecting only the fastest sedimenting portion of the eIF3 peak which was found to be free of CBP-p220 antigens (unpublished data). This highly purified eIF3 preparation can be substituted in the CBP complex assay for eIF3 from poliovirus-infected cells (unpublished data). Thus, activity in the CBP complex assay does not depend on some contaminant present in eIF3 from infected cells.

Before this report only two assays for CBP which could be directly correlated with poliovirus-induced inactivation had been published. The in vitro cap cross-linking assay depends on the ability of proteins to interact with the cap structure on mRNA in a cap-analog-sensitive way (14, 18); however, this activity resides primarily with the 24-Kd polypeptide, and the quantitation of the cross-linking is difficult to evaluate. A more direct assay for CBP complex is the restoring assay of

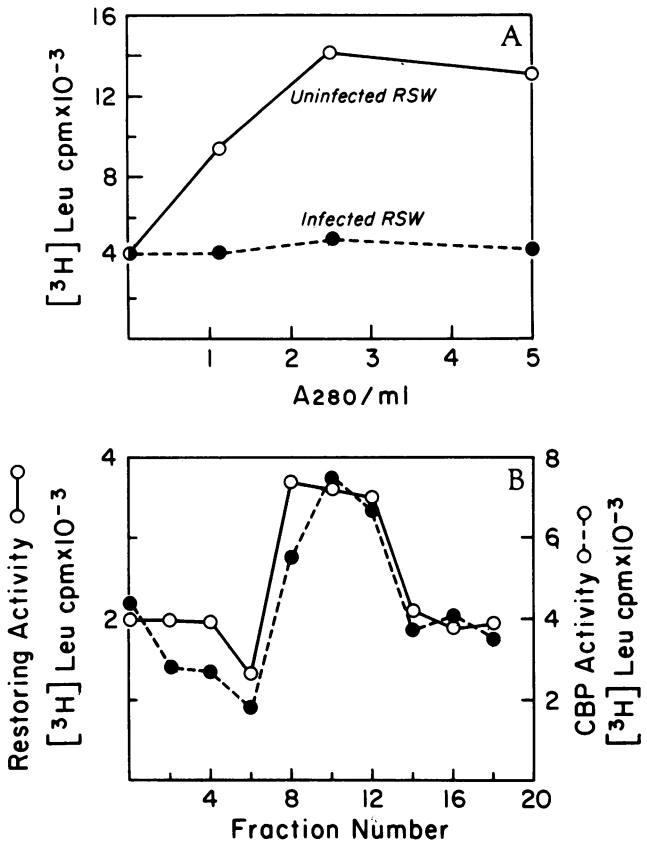

FIG. 4. Stimulation of globin mRNA translation in vitro by RSW from infected or uninfected cells. (A) RSW from uninfected cells or from poliovirus-infected cells harvested 4 h postinfection was prepared as described in the text and dialyzed against buffer A containing 100 mM KCl. The fractionated translation system described in the text was used, except that no purified initiation factors were added. Instead, initiation factors were added in the form of RSW from infected or uninfected cells as indicated. (B) Fractions from the sucrose gradient of RSW-A from uninfected cells shown in Fig. 2 were assayed for the presence of restoring activity and CBP activity as described in the text.

Rose et al. (16). This approach depends on the ability of a preparation to restore capped message translation in infected cell lysates. A highly purified CBP complex, called eIF4F, alone has this activity, but thus far, no direct demonstration that this particular complex is altered in infected cells has been made (9). In this report, we used a more highly purified fractionated translation system in which the only component used that was common to the restoring assay was partially purified eIF3 from poliovirus-infected cells. All other components were derived from uninfected HeLa cells or other cell types. The results are essentially identical to those of the restoring assay in that CBP complex was required for the translation of capped mRNA. Thus, other components of the infected cell lysate, with the possible exception of eIF3, appear not to be directly involved in the capped mRNA discrimination mechanism. Since we showed here that eIF3 was active in infected cells, it is likely that only CBP complex is inhibited by poliovirus infection.

In this report, we showed that a CBP complex was inactivated by poliovirus infection. In a previous report, we have demonstrated that a 220-Kd polypeptide (p220), which shares immunological determinants with a CBP polypeptide of identical electrophoretic mobility, is proteolytically degraded to form 110- to 130-Kd cleavage products in poliovirus-infected cells (8). Although we do not know whether p220 is an essential component of the CBP complex, we have recently found that it does purify with the CBP complex activity described here (D. Etchison, S. Milburn, and J. W. B. Hershey, manuscript in preparation), and monoclonal antibodies prepared against the partially purified CBP complex activity react with p220 in uninfected cells, but only the 110- to 130-Kd cleavage products in infected cells (D. Etchison and J. Etchison, manuscript in preparation). Thus, p220 seems to be a component of the active complex. Experiments are under way to determine whether p220 is essential for activity.

It is not yet clear how CBP complex functions in protein synthesis. Of the three polypeptides present in the CBP complex, two are known to cross-link to the cap structure of mRNA: CBP I (24 to 28 Kd) and eIF4A (49 Kd) (6, 9). CBP I probably interacts directly with the cap structure since it is the only polypeptide which cross-links by an ATP-independent mechanism in vitro (17). The CBP I subunit is present in a heavy-sedimenting form, possibly complex associated, in uninfected cells but not in poliovirus-infected cells (11). This finding suggests that the CBP complex may be disrupted in infected cells, and since p220 is the only detectable structural alteration identified in initiation factors from infected cells, CBP complex disruption might be the result of p220 degradation. Cap cross-linking to eIF4B has also been shown to occur (9), but efficient cross-linking of both eIF4B and eIF4A requires the presence of CBP complex (6). It has been suggested that eIF4A and eIF4B interact with the cap structure, or RNA regions near the cap structure, only after CBP complex has reacted (6). CBP complex may be a part of a multifactor cap recognition complex with eIF4B and possibly the free form as well as the complex-associated form of eIF4A. Proteolysis of p220 as a result of poliovirus infection may prevent formation of the putative cap-recognition complex.

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