Poliovirus infection of HeLa cells results in cleavage of the p220 subunit of eukaryotic initiation factor eIF-4F and inhibits cap-dependent initiation of protein synthesis. To examine the effect of virus-induced inhibition on the structure of initiation factor complexes involved in cap binding, the polypeptide compositions of cap affinity-purified complexes from uninfected and poliovirus-infected HeLa cells were analysed. Monoclonal antibodies directed against p220 and an eIF-3 subunit, p170, were utilized to localize eIF-3 and eIF-4F on sucrose gradients and in fractions eluting from cap analog columns. This approach resulted in the purification of several different cap-binding complexes from different cellular substractions and revealed significant differences in their composition after infection. The results indicate that eIF-3 and eIF-4F bind to the cap structure, possibly in the form of a complex, and that a modified form of eIF-3 alone has some cap-binding activity in the complete absence of p220, eIF-4A, and eIF-4E. Ribosome-derived complexes containing cleaved p220 are no longer associated with eIF-3 or eIF-4A, and a significant amount of cleaved p220 is associated with a unique cytoplasmic cap-binding complex. The cytoplasmic complex also contains M, = 170,000 and 80,000 polypeptides, neither of which are major components of eIF-4F. These results demonstrate significant variation in the composition of cap-binding complexes from both infected and uninfected cells. They indicate that eIF-3 might play a direct role in cap binding and suggest that poliovirus-induced cleavage of p220 results in the release of the eIF-4A subunit from eIF-4F and abolishes an association between eIF-4F and eIF-3 which may function during the multifactor steps involved in initiation of cap-mediated translation.

Initiation of protein synthesis in eukaryotic cells generally proceeds by a m'GTP cap-mediated mechanism of mRNA recognition (reviewed in Pelletier and Sonenberg, 1987; Rhoads, 1988; Sonenberg, 1988). After poliovirus infection, however, there is inhibition of cap-dependent initiation and a cap-independent mechanism characterized by internal initiation functions exclusively (Bienkowski-Szewczyk and Ehrenfeld, 1968; Jang et al., 1985; Pelletier et al., 1986; Trono et al., 1986). Thus, cellular capped mRNA is not translated by infected cells while viral mRNA, which is not capped, is readily translated. The specific alteration leading to inhibition of cap-mediated initiation has not yet been determined. The only structural alteration in infected cells which has so far been identified among initiation factors involved in cap recognition is cleavage of p220 (Etchison et al., 1982), a subunit of the cap-binding complex eIF-4F" (Edery et al., 1983; Grifo et al., 1983). Cleavage of p220 correlates with the onset of inhibition of translation of host mRNA after infection, and initiation factors containing cleaved p220 fail to stimulate translation of capped mRNA (Buckley and Ehrenfeld, 1987; Etchison et al., 1984). As a subunit of eIF-4F, p220 may play a role in processes leading to the appropriate recognition of capped mRNA, discrimination between different mRNAs, or steps in initiation which follow that of cap recognition. In this report, we begin to sort out interactions between p220 and other polypeptides involved in the cap-binding step of initiation and the changes in those interactions which occur in infected cells.

The primary cap-binding activity involved in protein synthesis in HeLa cells is associated with the cap-binding protein, eIF-4E (Sonenberg et al., 1978; Sonenberg and Shatkin, 1977). This M, = 25,000 polypeptide binds to caps in structures of the absence of other polypeptides (Rychlik et al., 1986; Sonenberg, 1981) and a possible cap-binding domain has been identified by sequence comparison with other cap-binding proteins (Rychlik et al., 1987). Cap-binding activity of eIF-4E can be affected by post-translational modification. On two-dimensional IEF/SDS gels, eIF-4E separates into two to five spots which differ at least partially in their degree of phosphorylation and focus with pI values ranging from 5.7 to 6.5 (Buckley and Ehrenfeld, 1986; Duncan et al., 1987; Hiremath et al., 1988; Rychlik et al., 1986, 1987). A kinase which is relatively specific for eIF-4E has been identified in rabbit reticulocytes (McMullin et al., 1988), and eIF-4E phosphorylation in vivo can be stimulated by treating cells with phorbol ester (Morely and Traugh, 1989). Phosphorylation does not affect the ability of eIF-4E to cross-link to oxidized cap structures in vivo (Hiremath et al., 1989) but is associated with changes occurring after heat shock (Duncan and Hershey, 1984). Neither crossing linking activity nor degree of phosphorylation as judged by mobility on IEF/SDS polyacrylamide gels are affected by poliovirus infection, however (Buckley and Ehrenfeld, 1987).
Cap-binding complexes containing eIF-4E and p220 cleavage products (p220a) can be purified from infected cells (Buckley and Ehrenfeld, 1987; Lee and Sonenberg, 1982); thus, eIF-4E retains the ability to bind to cap structures and to p220 polypeptide fragments after infection.

Other polypeptides co-purify with cap-binding complexes and are capable of cross-linking to cap structures in vitro. These include eIF-4A and eIF-4B (Edery et al., 1983; Grifo et al., 1983; Sonenberg, 1981), both of which may play roles in ribosome scanning, functions which may be initiated upon eIF-4E-mediated positioning of these polypeptides near the cap structure (Abramson et al., 1987). eIF-4A functions as an ATP-dependent helicase, relaxing mRNA secondary structure, presumably to permit ribosome movement (Ray et al., 1985; Sarkar et al., 1985). eIF-4B is associated with a ribosome-dependent ATPase which is thought to aid in ribosome scanning (Hughes et al., 1988), it stimulates release of eIF-4E from the mRNA cap (Ray et al., 1986) and the ATPase activity of eIF-4A and eIF-4F (Abramson et al., 1987; Goyer et al., 1989; Ray et al., 1985). Under some conditions, eIF-4B can be purified together with eIF-4E (Lax et al., 1985) and with eIF-4F (Grifo et al., 1983) by cap affinity chromatography. Only a portion of the eIF-4A present in a cell lysate co-purifies with the eIF-4F complex, the remainder can be purified as a separate factor which stimulates globin mRNA translation in a fractionated in vitro system (Brown-Luedi et al., 1982). Two different genes encoding eIF-4A have been identified in mice, but there is at present no evidence that the protein products are functionally distinct (Nielson and Trachsel, 1988). Thus, eIF-4A and eIF-4B have defined independent activities which may be stimulated by the effect of eIF-4E-mediated alignment at the cap structure as well as other possible functions which might be contributed by p220.

A fourth initiation factor which may be involved in the cap-binding step of initiation is eIF-3. A large (M, = 500,000 to 700,000), multisubunit factor, eIF-3 could potentially contain a number of independent activities (for reviews, see Moldave, 1985; Pain, 1986). Only a few functions have thus far been defined. Purified eIF-3 binds to 40 S ribosomal subunits, possibly effecting dissociation from the 60 S subunit, stimulates initiator methionyl-tRNA binding, and is required for formation of mRNA-associated initiation complexes. The interaction of eIF-3 and one or more subunits of eIF-4F has been described or implicated in a number of reports. A polyclonal antiserum against p220 was raised in goats by immunizing with purified eIF-3 (Meyer et al., 1982). An activity associated with eIF-4F could be affinity-purified on eIF-3-Sepharose (Trachsel et al., 1980). eIF-4E with cap cross-linking activity was found to co-sediment with eIF-3 on sucrose gradients (Hansen et al., 1982). The occasional co-purification of eIF-4F subunits with eIF-3 suggests that this interaction may be functional.

In an effort to define the various complexes with which p220 is associated, we have followed its purification by reactivity with a monoclonal antibody and its association with eIF-4E by cap analog affinity chromatography. As a control, and to identify eIF-3, we have also used a monoclonal antibody directed against the p170 eIF-3 subunit. The results identify at least three different types of cap-binding complexes in uninfected cells, two or more ribosome-associated complexes, and one cytoplasmic complex. After infection, there are significant changes in the structure of the ribosome-associated eIF-4F complex and its association with other factors and a redistribution of p220, now in the form of cleavage products, between ribosome-associated and cytoplasmic cap-binding complexes. These virus-induced changes may reflect structural alterations in initiation factors which contribute to the switch from cap-dependent to cap-independent translation.

EXPERIMENTAL PROCEDURES

Materials—m7GTP (7-methylguanosine 5′-triphosphate sodium salt), mGTP-Sepharose, and IEF Ampholines were purchased from Pharmacia LKB Biotechnology Inc.; horseradish peroxidase- and alkaline phosphatase-conjugated goat anti-mouse F(ab)2 fragments from Organon Teknika; [35S]iodide was purchased from Du Pont-New England Nuclear; pretransitioned molecular weight SDS PAGE and Western markers fromsubtype.

Cells and Viruses—Purification type I (Maloney strain) stocks were prepared from low multiplicity infections of HeLa S3 suspension cells and purified on CsCl gradients as previously described (Etchison and Ehrenfeld, 1980). HeLa S3 cells were grown in suspension culture in Joklik's modified minimal essential medium supplemented with 6% calf serum and 2 mM glutamine. Cells were generally grown to a volume of 10 liters, harvested, and washed three times with cold serum-free medium. Cell pellets were resuspended in 2 pellet volumes of HMKD (20 mM HEPES, pH 7.4, 1.5 mM magnesium acetate, 10 mM potassium chloride (KCl), 2.5 mM dithiothreitol) and frozen at −100 °C. For infected cells, 8–10 liters of cells were harvested and resuspended in 1/3 volume of serum-free medium and infected with poliovirus at a multiplicity of 50 plaque-forming units per cell. After an 18-hr adsorption at 37 °C, prewarmed serum was added to 5%. Infected cells were harvested 4 hr after infection, and the cells were washed, resuspended in HMKD, and frozen. Several such preparations, either infected or uninfected, were combined for purification of initiation factors.

Preparation of postmitochondrial supernatant (S10), ribosomal salt wash (RSW), and 0’–40’ and 40’–70’ ammonium sulfate fractions of RSW (RSW A and RSW B, respectively) have been previously described (Etchison and Milburn, 1987). To cleanly separate ribosomal pellets from supernatants, ribosomes and salt-washed ribosomes were pelleted through 20% glycerol, ribosome buffer cushion, and the cushion and interface were discarded after centrifugation. HeLa cell initiation factors eIF-3 and eIF-4A were prepared from uninfected cells by modifications of the methods of Brown-Leudi et al. (1982). To purify eIF-3, an RSW A preparation was loaded onto a 10–50% sucrose gradient in buffer A-500 and centrifuged for 32 h at 26,000 rpm in an SW 27 rotor. Gradient fractions were analyzed for eIF-3 activity in a fractionated translation system (Etchison et al., 1984), and fractions containing eIF-3 activity were pooled and chromatographed on DEAE-cellulose followed by phosphocellulose. eIF-3 eluted at salt concentrations of 160 to 225 mM from DEAE and 250 to 350 mM from phosphocellulose. Pooled fractions from the phosphocellulose column were concentrated by vacuum dialysis, eIF-4A was purified by chromatography on Ultrogel ACA 34 followed by passage through a phosphocellulose column and chromatography on DEAE-cellulose. eIF-4A eluted from DEAE at salt concentrations of 120 to 170 mM. Its identity was confirmed by two-dimensional IEF/SDS gel analysis and comigration with the M, = 45,000 subunit of eIF-4F.

Cap Analog Affinity Chromatography—Cap-binding complexes were purified by chromatography on a 2-ml mGTP-Sepharose column as described by Webb et al. (1984). The column was equilibrated with buffer A-100 (buffer A-KCI concentration: 5% glycerol, 10 mM Tris-HCl, pH 7.4, 2 mM MgCl2, 2 mM mercaptoethanol, 100 mM potassium acetate) to which sample was added to 100 mM salt and applied with a flow rate of 10 ml/hr. The column was then successively washed with 50 ml of A-100 followed by 8 ml of 70 mM GDP in buffer A-100, and 4 ml fractions were collected. Cap-binding complexes were eluted with 8 ml of 70 mM mGTP in A-100 and collected as 0.5-ml fractions. The column was then washed with A-1000 and recycled by re-equilibrating with A-100. Re-equilibrated columns were reused a number of times without a significant loss of binding capacity. With reuse, however, the 43-kDa non-cap-specific binding polypeptide tended to accumulate and could be removed by elution with 5 mM dithiothreitol or washing with 0.1% SDS. Portions of each fraction (25 μl) were transferred to microtiter plates for each ELISA analysis, 30 μl of microtiter plates were precipitated by SDS-PAGE, and gels were visualized by silver staining as described previously (Etchison and Etchison, 1987). ELISAs were developed according to standard procedures with horseradish peroxidase-conjugated goat anti-mouse IgG second antibodies and an o-phenylenediamine color development. Color was quantified by an Titertek automated ELISA plate reader using an A,exc filter.
Monoclonal Antibodies—Preparation and use of anti-p220 and anti-p170 antibodies have been described previously (Etchison and Etchison, 1987). Monoclonal antibodies directed against p220 (25 A C2) and the p170 subunit of eIF-3 (20 F D5) were prepared in the form of mouse ascites fluid. The ascites fluid was used without further purification for immunoblotting with alkaline phosphatase-conjugated goat anti-mouse IgG second antibodies followed by p-nitro blue tetrazolium chloride dye development. For iodinated antibody, the anti-p220 antibody was first purified by chromatography on DEAE-cellulose and then iodinated by a chloramine-T-mediated reaction.

RESULTS

Cap-binding Preparations Purified from the Postribosomal Supernatant of Poliovirus-infected and Uninfected Cells—To investigate the polypeptide interactions of p220 and the changes in those interactions which occur after poliovirus-induced cleavage, cap-binding complexes were purified by cap analog affinity chromatography from various subfractions of infected or uninfected cells as outlined in Fig. 1. An initial comparison of the subcellular distribution of p220 antigen had indicated that in uninfected cells most of the antigen was found in the ribosomal salt wash (RSW), and little to none was found in the postribosomal supernatant (PRS). By contrast, a significant fraction of p220, from infected cells was found in the PRS (data not shown). When cap-binding complexes (CBCs) were isolated by m7GTP-Sepharose chromatography from this infected cell subfraction, polypeptides which were not typical of eIF-4F were found to specifically elute with cap analog. Fig. 2 shows silver-stained gel analysis of selected chromatography fractions (top) and the corresponding fractions analyzed by immunoblot with an anti-p220 monoclonal antibody (bottom). p220, polypeptides were readily detected as 100–130-kDa stained bands which specifically eluted with cap analog. Their identity was confirmed by immunoblot which also detected a small portion of the cleavage products in flow-through fractions (bottom). The major polypeptide identified by silver stain which specifically eluted with cap analog was eIF-4E, but two additional polypeptides, eIF-4B and eIF-4G, were also specifically detected. The larger polypeptide identified by silver stain which specifically eluted with cap analog was eIF-4E, but two additional polypeptides, eIF-4B and eIF-4G, were also specifically detected. The larger polypeptide identified by silver stain which specifically eluted with cap analog was eIF-4E, but two additional polypeptides, eIF-4B and eIF-4G, were also specifically detected.

Fig. 1. Summary of experimental approach. Uninfected and poliovirus-infected cells were fractionated according to the procedure outlined here and under "Experimental Procedures." Post-mitochondrial supernatants (S10e) were separated into ribosomal pellets and postribosomal supernatants (PRS). The PRS was applied directly to m7GTP-Sepharose columns. Ribosomal pellets were fractionated into a ribosomal salt wash (RSW) and salt-washed ribosomes. The RSW was further fractionated by ammonium sulfate precipitation and sedimentation on sucrose gradients in the presence of high (500 mM) or low (100 mM) salt buffers. Gradient fractions from the rapidly or slowly sedimenting regions of the gradient which contained p220 and/or p170 antigen were pooled for m7GTP-Sepharose chromatography.

Fig. 2. Cap affinity chromatography of postribosomal supernatants from poliovirus-infected cells: silver stain and anti-p220 immunoblot analysis. PRS from infected cells was prepared and m7GTP-Sepharose chromatography was conducted as described under "Experimental Procedures." Fifty ml of PRS derived from approximately 4 × 10⁸ cells were applied to a 2-ml m7GTP-Sepharose column. Selected column fractions were analyzed by SDS-PAGE (8% acrylamide-0.2% bisacrylamide gels). Flow-through, GDP wash, dGTP-eluted, and 1 M salt-eluted fractions are indicated. A, thirty μl of each fraction were applied to the gel, and, following electrophoresis, the gel was silver-stained as previously described (Ehchison and Etchison, 1985). Prestained markers (M) include α₂-macroglobulin (180 kDa), fructose-6-kinase (84 kDa), β-galactosidase (116 kDa), pyruvate kinase (58 kDa), fumarase (49 kDa), acetate dehydrogenase (37 kDa), and triose-phosphate isomerase (27 kDa). A separately purified cap-binding complex from a similar preparation (CBC PRS INF), representing 0.8 pg of protein, was run for comparison. B, proteins in a gel similar to that in A were analyzed by immunoblot as described under "Experimental Procedures." The immunoblot was incubated with iodinated anti-p220 monoclonal antibody and exposed to x-ray film.

with cap analog was eIF-4E, but two additional polypeptides, M₁ = 170,000 and M₂ = 80,000 (indicated as eIF-4B, as described below), also specifically eluted. The larger polypeptide is not likely to be a variant of p220 because it does not
react with the monoclonal antibody. The $M_r = 45,000$ eIF-4A subunit of eIF-4F was not present in this cap-binding preparation. Instead, there was a nonspecifically eluting polypeptide of $M_r = 43,000$ which may be a GTP-binding protein since we have noted its ability to bind to GTP-agarose columns (data not shown).

On purification of the cytoplasmic CBC from uninfected cells, the same set of polypeptides was found to specifically elute with cap analog with the exception of the p220 polypeptides. These complexes were then further analyzed on SDS/IEF two-dimensional polyacrylamide gels (Fig. 3). The most intensely staining polypeptides present in both preparations were those of eIF-4E, which focused as five spots migrating with an expected $M_r$ of 25,000 and a $p_I$ range of 5.7 to 6.3. The $M_r = 80,000$ polypeptide (p80) migrated as a series of spots with the same $p_I$ range as eIF-4E. This pattern of migration on two-dimensional gels is characteristic of eIF-4B, an 80-kDa polypeptide which frequently co-purifies with cap-binding complexes (Duncan et al., 1987). This polypeptide is therefore tentatively identified as eIF-4B. The $M_r = 170,000$ polypeptide (p170) failed to focus but appeared as a streak from $p_I$ 5.7 to 6.6. The $M_r = 43,000$ polypeptide which eluted nonspecifically focused with a $p_I$ of 5.7. Spots unique to the cytoplasmic complex from infected cells were those of p220, which focused as a set of spots with $p_I$ values of 4.2 to 4.9. Their identity was confirmed by an immunoblot of a similar gel developed with the iodinated anti-p220 monoclonal antibody (data not shown). Although eIF-4E appears to be in molar excess to the p80 and p170 polypeptides, at least a portion of the cytoplasmic form of eIF-4E may be associated with a complex which is distinct from eIF-4F. While the cap-binding preparation from the infected cell PRS contains p220, that from uninfected cells is not associated with intact p220. Thus, intact and cleaved p220 may differ in their affinities for eIF-4E or other components of initiation complexes.

**CBCs Isolated from the RSW of Uninfected Cells**—To isolate ribosome-associated CBCs, initiation factors were prepared as a ribosomal salt wash and precipitated with 40% ammonium sulfate (RSW A fraction). This fraction of the RSW contained virtually all of the p220 and p170 antigen (not shown). The RSW A was further fractionated on sucrose gradients. We had previously noted that sedimentation of p220 is affected by salt concentration which suggested the existence of several forms of eIF-4F (Etchison and Etchison, 1987). We therefore fractionated half the RSW A preparation on a gradient containing high salt (500 mM) buffer and half on a gradient with low salt (100 mM) buffer. Gradient fractions containing eIF-3 were identified using an anti-p170-eIF-3 monoclonal antibody, and those containing p220 were identified by using the anti-p220 monoclonal antibody described previously (Etchison and Etchison, 1987). The anti-p170 antibody reacts strongly with a $M_r = 170,000$ polypeptide present in purified active eIF-3 preparations. A minor antigen of $M_r = 140,000$ is generally found as well and is probably a variant of p170. Fig. 4 shows detection of the two antigens by quantification of color development by ELISA and detection by immunoblot. The p220 antigen separated into at least two forms under low salt buffer conditions. Some of the p220 antigen co-sedimented with the p170 eIF-3 antigen. In the presence of high salt, however, p220 no longer co-sedimented with p170 and instead was found as a slowly sedimenting form.

**CBCs Isolated from the RSW of Infected Cells**—To isolate cytoplasmic-associated CBCs, initiation factors were prepared from the infected cell PRS of HeLa cells (Duncan et al., 1987). This polypeptide is therefore tentatively identified as eIF-4B. The $M_r = 170,000$ polypeptide (p170) failed to focus but appeared as a streak from $p_I$ 5.7 to 6.6. The $M_r = 43,000$ polypeptide which eluted nonspecifically focused with a $p_I$ of 5.7. Spots unique to the cytoplasmic complex from infected cells were those of p220, which focused as a set of spots with $p_I$ values of 4.2 to 4.9. Their identity was confirmed by an immunoblot of a similar gel developed with the iodinated anti-p220 monoclonal antibody (data not shown). Although eIF-4E appears to be in molar excess to the p80 and p170 polypeptides, at least a portion of the cytoplasmic form of eIF-4E may be associated with a complex which is distinct from eIF-4F. While the cap-binding preparation from the infected cell PRS contains p220, that from uninfected cells is not associated with intact p220. Thus, intact and cleaved p220 may differ in their affinities for eIF-4E or other components of initiation complexes.

**Fig. 3.** IEF/SDS two-dimensional gel analysis of cap-binding complexes from PRS of infected cells. Two-dimensional gel electrophoresis was conducted according to procedures described by Duncan and Hershey (1984). Cap-binding complexes from PRS of uninfected (A) or infected (B) cells were prepared, and 500 ml of pooled m7GTP-eluted fractions were dialyzed against 10 mM Tris, 10 mM NaCl and lyophilized. The residue was resuspended in IEF gel sample buffer and loaded onto tube gels (130 x 2 mm). A third blank gel was run for $p_I$ gradient determination. On completion of electrophoresis focusing, the gels were removed from the tubes, and the blank gel was sliced into 10-mm lengths. Each slice was placed in 1 ml of 0.1 M KCl, and the pH was determined after equilibration. The other two gels were incubated with SDS gel sample buffer, aligned on the top of a 130 x 100-mm SDS-polyacrylamide gel (10% acrylamide-0.27% bisacrylamide), and proteins were separated by electrophoresis and visualized by silver staining. Prestained markers were co-electrophoresed.
FIG. 4. Sedimentation of p220 and p170-eIF-3 antigens from RSW A on sucrose gradients under different ionic conditions: ELISA and immunoblot detection. RSW A from 65 ml of packed uninfected cells (approximately $1 \times 10^{9}$) was prepared as described under "Experimental Procedures." Half was loaded onto a 10–30% sucrose gradient in buffer A-100. The other half was loaded onto a gradient in buffer A-500. The gradients were centrifuged in an SW 27 rotor at 26,000 rpm for 32 h. Thirty 1.2-ml fractions were collected. For ELISA analysis, 25 µl of each fraction were tested for reactivity with a 1:1,000 dilution of the anti-p220 ascites (triangles) and for reactivity with a 1:2,000 dilution of the anti-p170 ascites (circles) as described under "Experimental Procedures" (Panels A and B). For immunoblot analysis (Panels C–F), 40 µl of each fraction were run on each of two SDS-polyacrylamide gels (8% acrylamide-0.21% bisacrylamide), the proteins were electroblotted onto nitrocellulose, and the blots were developed by incubation with either a 1:1,000 dilution of the anti-p220 ascites (Panels C and D) or a 1:2,000 dilution of the anti-p170 ascites (Panels E and F). Antibody reactivity was detected by incubation with alkaline phosphatase-conjugated goat anti-mouse second antibodies followed by dye development as described under "Experimental Procedures." A standard p220-containing marker was a sucrose gradient-purified eIF-3.4F preparation. The standard p170-containing marker was purified eIF-3. Prestained markers (M) are on the left of each blot. The direction of sedimentation is indicated by arrows. Fractions pooled for subsequent chromatography on m'GTP-Sepharose are indicated by bars. Low salt sucrose gradient, A, C, E; high salt sucrose gradient, B, D, F.

partially flowed through. Flow-through p220 represented overloading since re-application to a fresh column resulted in binding (data not shown), therefore, the amount of antigen bound from the p220 HS pool is an indication of column capacity for the p220-associated CBC. Because of the effect of antigen conformation on ELISA quantification, it is not possible to determine the proportion of antigen bound from ELISA data. These data measure exposed antigenic epitope only. However, a comparison of the relative amount of antigen detected in cap analog-eluted fractions, which is expected to represent a single epitope conformation, should be valid. Thus, the relative amount of detectable p220 antigen found in CBCs from the two gradient pools from the low salt gradient are roughly equivalent to that found as a single sedimenting form on the high salt gradient. The rapidly sedimenting p220 from the low salt gradient is therefore likely to be a component of a larger structure.

Co-sedimentation of the rapidly sedimenting p220-associated complex with p170-eIF-3 antigen suggested that the larger structure might include eIF-3. By ELISA detection, the majority of the p170 antigen sedimented as a single form characteristic of an eIF-3 complex. A minor amount was detectable in CBCs from slowly sedimenting material as well (Fig. 5, bottom panels). This is consistent with the presence of a small fraction of p170 in the corresponding sucrose gradient fractions pooled. This minor form may represent p170 which has dissociated from the eIF-3 complex. In contrast to p220 antigen binding, only a portion of the p170 antigen present in the p220-p170 or p170 HS pools bound to the column. Furthermore, there was a significant difference in the ability of p170 to remain bound to the column in the presence or absence of p220. When p220 antigen was present, p170 binding was stable (Fig. 5, p220-p170). In the absence of p220, however, some of the p170 eluted off during the washing procedures (Fig. 5, p170 HS). Flow-through p170 did not bind when reapplied to the column, indicating that some of the p170 was associated with complexes which are not active in cap binding (data not shown). These results indicate that at least a portion of a p170-associated complex contains cap-binding activity, but that stable binding may require the presence of a p220-associated complex.

To determine the polypeptides associated with these CBCs, column fractions were analyzed by SDS-PAGE, and polypeptides were visualized by silver staining. Cap analog-eluted peak fractions obtained by chromatography of the four sucrose gradient pools are compared in Fig. 6A. CBCs which contained
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Fig. 5. Cap affinity chromatography of sucrose gradient pools: ELISA analysis. Pooled fractions from the sucrose gradients in Fig. 4 were separately chromatographed on a 2-ml m7GTP-Sepharose column and fractions were analyzed by ELISA as described under “Experimental Procedures.” The pools from the HS gradient were first diluted 5-fold with buffer A-O. Top panels are fractions analyzed with a 1:1000 dilution of anti-p170 ascites, and bottom panels with a 1:2000 dilution of anti-p170 ascites. Labels at the top indicate the sucrose gradient pool used as starting material for each chromatography procedure. Pools were labeled according to the presence of buffer changes: A-100, 70 ~o~s, 170, and 8 ~o~s, respectively. Furthermore, while p170 itself partially eluted in wash fractions, the other eIF-3 subunits appeared to be bound nonspecifically since the lower molecular weight polypeptides were equally represented in GDP-eluted fractions as well as in m7GTP-eluted fractions (Fig. 6C). This is in contrast to their cap-specific binding in the presence of eIF-4F (Fig. 6B). Thus, cap-binding by eIF-3 appears to be stabilized by the presence of eIF-4F.

A comparison of the ribosome-associated CBCs with the cytoplasmic complex from the PRS of infected cells (CBC PRS INF) demonstrates that the M, = 80,000 and 43,000 polypeptides were found in the cytoplasmic complex only. Furthermore, the only subunit that the p220 HS and LS complexes had in common with the cytoplasmic complexes was the M, = 25,000 polypeptide, eIF-4E. The p170 subunit of CBC PRS INF co-migrated electrophoretically with the p170 subunit of the complexes which contained eIF-3-like subunits. The anti-p170 antibody generally does not react with the cytoplasmic complexes, however, so they appear not to be identical polypeptides (data not shown).

While cap binding by eIF-3 was an unexpected observation, preliminary results suggest that other factors or conformational aspects may be involved. When eIF-3 was further purified from the p170 HS sucrose gradient pool by DEAE- and phosphocellulose chromatography it failed to bind to m7GTP-Sepharose (data not shown). This would suggest that the complex purified in this manner is conformationally inactive, that some unknown component of the p170 HS pool might be responsible for conformational changes resulting in exposure of the cap-binding domain, or that another factor removed during purification is directly responsible for cap binding.

Ribosome-associated CBCs from Poliovirus-infected Cells—To compare CBCs from poliovirus-infected cells with those from uninfected cells, crude initiation factors were prepared from infected cells by procedures which were identical with those used for factors from uninfected cells described above. The anti-p220 monoclonal antibody does not react by ELISA to nondenatured cleavage products on sucrose gradients; therefore, p220 and p170 were identified on silver-stained SDS-polyacrylamide gels, and their identity was confirmed by immunoblot. Exposure to buffers of different salt concentration had little effect on the sedimentation of p220, which sedimented slowly with respect to the p170 antigen (data not shown). Immunoblot analysis of cleavage products in a low salt sucrose gradient has been shown previously (Etchison and Etchison, 1987). Salt concentration did affect the sedimentation of the p170 antigen, however, in exactly the same manner as that shown for uninfected factor preparations in Fig. 4. Fractions containing p220 antigen and p170 antigen were separately pooled from two sucrose gradients containing high and low salt buffers, and CBCs were purified by m7GTP-Sepharose chromatography. Fig. 7 shows these CBCs directly compared with one another, with eIF-3 and eIF-4A purified by standard procedures, with p170 HS and p220 HS CBCs identical and represented eIF-4F: p220, p145 (eIF-4A), and p25 (eIF-4E). These polypeptides also represented a subset of those found in the p220-p170 CBC, but they were not found in the p170 HS CBC. Thus, the p220-p170 CBC appeared to consist of eIF-4F and eIF-3 (eIF-3.4F), while the p170 HS CBC contained no trace of eIF-4F. Cap binding by the p170-associated complex appeared to be both weak and unstable in the absence of eIF-4F. Less total protein from the p170 HS pool bound to the column as indicated by the reduced stain intensity. Total protein yields in the p220-p170 and p170 HS pooled cap analog-eluted fractions were 25 and 8 ~o~s, respectively. The anti-p170 pools from the 220 pool contained at least eight polypeptides, and these subunits include 160,000, 115,000, 67,000, 66,000, 46,000, 39,000, and 37,000 (Meyer et al., 1982). The CBC from the p170 HS pool contained at least eight polypeptides, and these represented a subset of those found in the p220-p170 CBC. The CBCs from the p220 HS and LS pools were virtually
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The study involves the isolation and analysis of cap-binding complexes (CBCs) from poliovirus-infected HeLa cells. The complexes are isolated from different subcellular fractions of infected and uninfected cells. The presence of two major polypeptides, eIF-4F and eIF-3, is observed, and their association with the complexes is analyzed through sucrose gradient fractionation and protein blotting.

**DISCUSSION**

To characterize the changes in cap-binding complexes associated with poliovirus infection, the study has analyzed the subunit composition of complexes isolated by m'GTP-Sepharose chromatography from uninfected and poliovirus-infected HeLa cells. The presence of the p220 subunit of eIF-4F and the p170 subunit of eIF-3 was determined by reactivity with monoclonal antibodies. This report has described the isolation of several forms of CBCs from different subfractions of uninfected cells and has shown that complexes isolated from similar subfractions of poliovirus-infected cells are altered in composition.

The study indicates that the eIF-4F equivalent complex from infected cells contains p220 and eIF-4E, but lacks eIF-4A. This could only be seen in the preparation from the low salt gradient because of the large number of contaminating polypeptides present in the corresponding preparation from the high salt gradient (p220, HS INF). The identity of these contaminating polypeptides is unknown, but their binding was not cap-specific (data not shown). These results indicate that the eIF-4F equivalent complex from infected cells consists of eIF-4E and p220. The association between the p220 and eIF-4E subunits of eIF-4F is therefore unaffected by cleavage of p220 or by dissociation of the eIF-4A subunit. Furthermore, the p220 must contain the eIF-4E binding domain of p220. A summary of the results described above is shown in Table I.

In addition to the alteration in eIF-4F after infection, there also appeared to be differences in the p170 CBCs from infected cells compared with that from uninfected cells. Fig. 7 shows that three polypeptides (M, = 170,000, 110,000–120,000, and 60,000) were the more prominent components of the p170 HS CBC preparations from infected and uninfected cells and that one of them appeared to be altered after infection. Also present, but at reduced stain intensity, were lower molecular weight eIF-3 polypeptides, but these were not altered by infection. The p120 polypeptide present in the p170 CBCs from uninfected cells was significantly reduced in amount in the corresponding preparations from infected cells. Instead, the p170 INF preparations contain slightly smaller polypeptides. These could be a modified form of p120 or a completely unrelated polypeptide. Further work will be required to investigate the importance of this observation.

**Table I**

Composition of cap-binding complexes from uninfected and poliovirus-infected cells

<table>
<thead>
<tr>
<th>Source</th>
<th>Uninfected</th>
<th>Infected</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRS CBC PRS</td>
<td>eIF-4E</td>
<td>eIF-4E</td>
</tr>
<tr>
<td>PRS CBC PRS INF</td>
<td>eIF-4E</td>
<td>eIF-4E</td>
</tr>
<tr>
<td>p170</td>
<td>p170</td>
<td>p220cp</td>
</tr>
<tr>
<td>RSW RS LS p220-p170</td>
<td>eIF-3</td>
<td>p170 LS INF</td>
</tr>
<tr>
<td>RSW SS HS p170 HS</td>
<td>eIF-3</td>
<td>p170 HS INF</td>
</tr>
<tr>
<td>RSW RS LS p220 LS</td>
<td>eIF-4E</td>
<td>p220cp</td>
</tr>
<tr>
<td>RSW SS HS p220 HS</td>
<td>eIF-4E</td>
<td>p220cp</td>
</tr>
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*RS and SS, rapidly and slowly sedimenting.
* eIF-3 in which some subunits are more prevalent than others.

The study further undertook a detailed analysis of CBCs from different subcellular fractions of uninfected cells. This resulted in the isolation of several different complexes: three ribosome-associated complexes and a unique cytoplasmic complex. eIF-4F was purified from the slowly sedimenting region of sucrose gradients containing either high or low salt buffers and contained p220 but little p170 antigen. In the presence of low salt buffer, some of the eIF-4F co-sedimented with p170 eIF-3 antigen and CBCs isolated from these pooled fractions consisted of polypeptides characteristic of both eIF-3 and eIF-4F (eIF-3-4F). The p170 eIF-3 antigen separated from p220 in the presence of high salt buffer and CBCs isolated from these fractions consisted of a partially denatured eIF-3 complex in which only a few of the subunits appeared to be specifically bound. Thus, eIF-3 is associated with cap-binding activity which appears to be enhanced or stabilized in the presence of eIF-4F.

CBCs isolated from identical subcellular fractions of poliovirus-infected cells differed from those of uninfected cells in several respects. An eIF-4F analogous complex consisted of p220 and eIF-4E only. There was no evidence for an association between the eIF-4F analogous complex and eIF-3. Thus,
cleavage of p220 appears to result in the dissociation of both eIF-4A and eIF-3 from the p220-containing CBC. Cap binding by the p170-associated partially denatured eIF-3 complex from infected cells was again observed and is therefore not affected by p220-related changes.

Cytoplasmic CBCs isolated from the PRS of infected or uninfected cells were nearly identical except for the presence of p220, in that from infected cells. These CBCs were not similar to eIF-4F because they were not associated with eIF-4A, and the complex from uninfected cells lacked p220. Instead, they contained two additional polypeptides, an M₆ of ~80,000 polypeptide which appears to be eIF-4B and an M₆ of 170,000 polypeptide. While it’s possible that these subunits are separately bound to the column rather than part of a complex, eIF-4B might be directly associated with eIF-4E in the cytoplasm as a consequence of its eIF-4E-releasing activity (Ray et al., 1986). A p80-p28 CBC which may be similar to the cytoplasmic complex we describe here has been isolated from wheat germ and referred to as iso-4F (Browning et al., 1989; Lax et al., 1985). A p80 subunit in CBCs from cytoplasmic extracts of poliovirus-infected cells was described previously (Buckley and Ehrenfeld, 1987). It is likely that this preparation was a mixture of cytoplasmic and ribosome-associated complexes.

Since polypeptides characteristic of eIF-3 were found to bind to the column in the absence of eIF-4F polypeptides, it was not clear whether the p220-p170 CBC contained two complexes which were separately bound, or a single complex consisting of both eIF-3 and eIF-4F which was bound to the column through the cap-binding protein, eIF-4E. Several findings argue for a physical association between the two complexes. First, they co-sedimented under low salt conditions. Second, the protein yields of the p220-p170 CBC eIF-3-4F were several-fold greater than yields of the p170 eIF-3 CBC. Third, binding by eIF-3 polypeptides in the absence of eIF-4F was weak and many of the subunits appeared to be non-specifically eluting. This suggested that the eIF-3 complex was partially dissociated in the absence of eIF-4F, and that those subunits not directly bound to the column were gradually lost during the chromatography procedure. Cap binding by eIF-3 therefore appears to be stabilized by eIF-4F.

The failure of eIF-3 purified by standard techniques to bind to m'GpppG-Sepharose suggests that cap binding by this initiation factor requires other associated components or activities. It is possible that the interaction with eIF-4F during initiation of protein synthesis results in relaxation of the eIF-3 structure and exposure of the cap-binding domain. If this were the case, then a similar conformational relaxation might also be induced by exposure to high salt or other factors. Cap binding by eIF-3 is not simply activated by exposure to high salt buffer conditions during sedimentation, however, since the p170 INF pool from the low salt gradient contained a ~170,000 polypeptide. While it’s possible that these subunits are separately bound to the column rather than part of a complex, eIF-4F might be directly associated with eIF-4E in the cytoplasm as a consequence of its eIF-4E-releasing activity (Ray et al., 1986). A p80-p28 CBC which may be similar to the cytoplasmic complex we describe here has been isolated from wheat germ and referred to as iso-4F (Browning et al., 1989; Lax et al., 1985). A p80 subunit in CBCs from cytoplasmic extracts of poliovirus-infected cells was described previously (Buckley and Ehrenfeld, 1987). It is likely that this preparation was a mixture of cytoplasmic and ribosome-associated complexes.

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The exact mechanism of the cap binding step of protein synthesis is still not understood, and it is not clear whether eIF-4F functions in the form of the tripartite complex or as one or more independent factors. It has been shown that eIF-4E synthesized in vitro from the cloned gene becomes associated with initiation complexes containing mRNA but not with 40 S preinitiation complexes (Hiremath et al., 1989). This would suggest that it binds capped mRNA first and delivers the RNA to the 40 S ribosomal subunit. It is entirely possible that eIF-4E binds cap structures as a free factor or as a complex with the 60-kDa and possibly the p170 polypeptide from the cytoplasmic complex, and that the eIF-4F complex forms on the capped mRNA subsequent to that event. In support of this model, we have evidence which indicates that p220 binds to the 40 S subunit before the addition of mRNA. The identification of a significant amount of active eIF-4E not associated with the other two polypeptides of eIF-4F in the post-ribosomal supernatant argues for the existence of a pool of eIF-4E which is capable of binding capped mRNA in the absence of eIF-4A or p220. Since cleaved p220 is part of complexes which are not associated with eIF-4A or eIF-3, it is possible that the role of intact p220 is to bring these various factors together in a functional complex. Failure of the capped mRNA-eIF-4E-p220 complex to associate with eIF-3 might prevent 40 S ribosomal subunit joining, thereby inhibiting cap-mediated translation. A limited degree of cap binding by eIF-3 in the absence of eIF-4F might explain the observed low level of translation of capped mRNA after virus-induced cleavage of p220 (Bonneau and Sonenberg, 1987).

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D Etchison and K Smith


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