cognate factors cross-reacts with that of the other species supports the view that these proteins are conserved in structure.

Acknowledgments

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Immunochemical Characterization of Mammalian Protein Synthesis Initiation Factors†

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ABSTRACT: Immunochemical techniques were used to investigate the molecular weight forms and levels of protein synthesis initiation factors (eIF) in crude cell lysates. Antisera were obtained from goats immunized with purified rabbit reticulocyte eIF-3 and human HeLa eIF-2, eIF-4A, and eIF-4B. The antisera were characterized by double immunodiffusion and immunoblotting techniques. Rabbit reticulocytes or HeLa cells were prepared and lysed rapidly into denaturing buffer containing sodium dodecyl sulfate. The lysates were analyzed by polyacrylamide gel electrophoresis and immunoblotting. With HeLa lysate and anti-eIF-4B, one intense band developed which corresponded in molecular weight with purified eIF-4B. Anti-eIF-2 and anti-eIF-4A produced much less intense bands corresponding to their cognate factors, and all three antisera produced minor bands as well. For improvement of the power of the analyses, antibodies specific for eIF-2, eIF-4A, and eIF-4B were affinity purified by extraction of the antibodies adhering to gel-purified initiation factors blotted onto aminobenzyloxymethyl paper. Each of these antibodies produced a single intense band with HeLa lysates. The eIF-4A and eIF-4B bands corresponded exactly with those against the purified factors, indicating that the isolated factors are the same size as those found in crude lysates. The eIF-2β band in the lysate was slightly higher than that of the isolated factor, which means that the β subunit in this eIF-2 preparation is partially degraded and is not identical with the in vivo form. The analyses further showed that the three factor proteins have no major precursor or degradation forms in lysates and that they are antigenically distinct from other abundant cellular proteins. Analyses of rabbit and HeLa lysates with crude anti-eIF-3 gave rather weak bands whose patterns were simpler than those obtained with purified eIF-3 preparations. The results are consistent with the view that many of the polypeptide subunits of eIF-3 are generated by proteolysis of high molecular weight precursor forms. The levels of eIF-3 in rabbit and HeLa lysates roughly estimated by rocket immunoelectrophoresis and immunoprecipitation are about 0.5 eIF-3 molecule per ribosome.

The initiation phase of protein synthesis in mammalian cells is promoted by a large number of proteins called initiation factors. The factors have been isolated and purified to near-homogeneity in order to identify the initiation factors and elucidate their mechanism of action. Up to 10 different initiation factors have been purified and studied (Schreier et al., 1977; Merrick, 1979; Benne & Hershey, 1978; Sonenberg et al., 1979; Dasgupta et al., 1979; Amesz et al., 1979). At least three of the eukaryotic initiation factors (eIF),[1] eIF-2, eIF-3, and eIF-5, may be isolated in multiple forms which appear

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[1] Abbreviations: eIF, eukaryotic initiation factor; BSA, bovine serum albumin; $NaDodSO_4$, sodium dodecyl sulfate; IgG, immunoglobulin G; Tris, tris(hydroxymethyl)aminomethane.

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to be generated by limited proteolysis (Meyer et al., 1981). If proteolysis of these proteins occurs, especially during the isolation process, it is possible that the initiation factors purified are different from the proteins which function in the intact cell. We lack evidence concerning in vivo forms of the factors and whether precursor forms exist. Furthermore, we need to know the cellular levels of the initiation factors, how their levels are coordinated with ribosome levels, and whether their concentrations depend on the physiological state of the cell.

In order to study the structure and levels of initiation factors without prior purification by classical techniques, we raised antibodies against a number of these proteins. We report here the preparation of goat antisera specific for rabbit reticulocyte eIF-3 and for human HeLa eIF-2, eIF-4A, and eIF-4B. Using a modification (Howe & Hershey, 1981) of the immunoblotting procedure of Towbin et al. (1979), we were able to identify the major forms of the proteins in cells lysed rapidly in sodium dodecyl sulfate ($NaDodSO_4$) buffer. The level of eIF-3 was estimated by rocket immunoelectrophoresis and by immunoprecipitation. These studies establish the efficacy of immunochemical approaches to studying initiation factors in crude cell lysates.

Materials and Methods

Materials. Initiation factors eIF-2, eIF-3, eIF-4A, and eIF-4B were prepared from rabbit reticulocytes (Benne et al., 1979) and human HeLa cells (Brown-Luedi et al., 1982) as described previously. Reticulocyte lysates were prepared as described by Woodward et al. (1974); the HeLa cell lysates used here are described in the preceding paper (Brown-Luedi et al., 1982). Alternatively, both cell types were lysed by sonicating cells in about 2 volumes of $NaDodSO_4$ gel sample buffer (Laemmli, 1970). Protein concentration was determined by the methods of Bradford (1976), Lowry et al. (1951), or Schaffner & Weissmann (1973). Ribosome levels were measured by extracting lysates with perchloric acid at 70 °C, quantitating ribose by the orcinol method (Schneider, 1957), and assuming that 80% of the RNA is ribosomal.

Antisera. Antisera against rabbit reticulocyte eIF-3 and HeLa eIF-2, eIF-4A, and eIF-4B were prepared in mature female goats. Purified initiation factors (300 μg of each protein) in Freund's complete adjuvant were injected intramuscularly at four sites, followed by one or two booster injections of 150 μg of protein at about 6–8-week intervals. Highest titers were observed 4–6 weeks following the booster injections. Blood was collected and allowed to clot and retract, and the sera were clarified and stored in aliquots at −20 °C.

Immunochemical Methods. Double immunodiffusion plates (1.5 mm thick) contained 0.8–1.0% agarose (Bio-Rad) in 10 mM potassium phosphate, pH 7.2, 150 mM KCl, and 0.05% sodium azide. Wells (4-mm diameter) separated by 6 mm were cut as indicated in the figures. Diffusion was performed at room temperature for 3–6 days, and precipitin bands either were visualized directly against a dark field or were washed with 0.5 M NaCl and stained with Coomassie blue by the procedure of Laurell (1972).

Rocket immunoelectrophoresis (Laurell, 1972) was performed in a continuous buffer system (10 mM barbital, 25 mM Tris base, 0.2 mM calcium lactate, and 0.2% sodium azide, final pH 8.55). This buffer was made up 5 times concentrated by boiling the barbital in three-fourths the final volume of water and adding 1 M Tris base before cooling. After the buffer was cooled, calcium lactate and sodium azide were added, and the pH was adjusted. Agarose (Bio-Rad) was dissolved in hot buffer to 0.8% and cooled to 65 °C; anti-eIF-3 (2–4%) was added, and the gel was cast 2 mm thick on a 9 × 12 cm glass plate. Samples were applied in 3-mm wells after electrophoresis was started, and the gels were covered for the duration of the run. Gels were electrophoresed 16–18 h at 10 °C with a potential of 2–3 V/cm of gel width. They were washed and stained as described (Laurell, 1972).

Immunoblotting. Protein preparations were subjected to polyacrylamide gel electrophoresis according to the method of Laemmli (1970) in slab gels (110 × 150 × 1.5 mm) containing 10% acrylamide and 0.27% bis(acrylamide) or 15% acrylamide and 0.09% bis(acrylamide). Following electrophoresis, the proteins were electrotransferred to nitrocellulose sheets (Schleicher & Schuell) by the method of Towbin et al. (1979) in buffer containing 25 mM Tris base, 192 mM glycine, and 20% methanol, pH 8.3, for 3 h at 300 mA. The blot was stained with amido black (0.1% in 45% methanol and 10% acetic acid), destained with 90% methanol–2% acetic acid, and photographed. The nitrocellulose blot then was soaked for 60 min at 37 °C in 3% bovine serum albumin (BSA), 0.9% NaCl, and 10 mM Tris-HCl, pH 7.4, washed 3 times with blotting buffer (10 mM Tris-HCl, pH 7.4, 0.9% NaCl, 0.5% BSA, 0.2% $NaDodSO_4$, and 0.5% Triton X-100), and incubated for 8–12 h at room temperature with 20–40 mL of goat antiserum diluted 1:40 in blotting buffer. The blot was washed 5 times over the course of 30 min with blotting buffer and then incubated 8–12 h at room temperature with 20–40 mL of blotting buffer containing (5–10) × 10^5 cpm/mL ^{125}I-labeled rabbit anti-goat IgG [sp act. (2–5) × 10^7 cpm/μg]. The blot was washed again with 5 changes of blotting buffer. All incubations and washes were carried out with constant gentle rocking of the nitrocellulose sheets in the buffers. The washed blot was air-dried and exposed to SB-5 film for 1–7 days.

Affinity Purification of Antibodies. Antibodies specific for initiation factor proteins were purified by elution of the antibody from immunoblots essentially as described by Olmsted (1981). About 10 μg of purified eIF-4A or eIF-4B was subjected to electrophoresis in 10% acrylamide slab gels (110 × 150 × 1.5 mm); 25 μg of eIF-2 was fractionated into its three subunits on a 15% acrylamide gel as described in Figure 3A. Proteins in the gel were electrotransferred onto aminobenzyloxymethyl paper (ABM paper), and the ABM paper was incubated with 10% ethanolamine, 0.5% BSA, and 0.1 M Tris-HCl, pH 8.8, for 2 h at room temperature and washed with blotting buffer. To locate the factor band, we cut thin strips from the edges of the blot and incubated them with antiserum and then with ^{125}I-labeled rabbit anti-goat IgG as described above. Horizontal strips (about 2–4 mm wide) corresponding to eIF-2β, eIF-4A, or eIF-4B were excised, cut into small pieces, and incubated with the appropriate antiserum. The ABM paper pieces were washed with blotting buffer and then with PBS (10 mM potassium phosphate, pH 7.2, and 150 mM NaCl) containing 0.5% BSA. Antibodies were extracted from the ABM paper pieces by stirring the pieces for 3 min at room temperature with 250 μL of 4 M guanidinium chloride in PBS in a 1-mL Teflon beaker. The solution was removed, and the pieces were extracted again for 2 min with 200 μL of buffer. The combined extracts containing the purified antibodies were dialyzed overnight against PBS.

Results

Preparation of Antisera. When this work began, there were no reports of antibodies prepared against mammalian initiation factors, and it was not clear whether an antigenic response could be obtained from commonly used antibody producers. In order to test the feasibility of preparing such antisera, we selected rabbit reticulocyte eIF-3 as the immunogen, since this factor is readily prepared in milligram quantities (Benne et

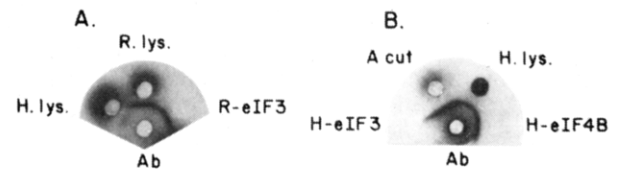

FIGURE 1: Double immunodiffusion analyses with anti-eIF-3 and anti-eIF-4B antisera. Plates were prepared and analyzed as described under Materials and Methods. (A) Anti-eIF-3 serum (20 μL) was placed in the center well; peripheral wells contained 18 μL of a clarified HeLa lysate (H. lys.), 20 μL of a clarified rabbit reticulocyte lysate (R. lys.), and 10 μg of eIF-3 purified from rabbit reticulocytes (R-eIF3). (B) Anti-eIF-4B serum was concentrated 2-fold by precipitation with 45% saturated ammonium sulfate and dialysis against 20 mM potassium phosphate, pH 8.0; 20 μL was added to the central well. Peripheral wells contained 8 μg of eIF-3 purified from HeLa cells (H-eIF3), 150 μg of protein in the 0–40% saturated ammonium sulfate fraction of the high-salt ribosomal wash from HeLa cells (A cut), 20 μL of a clarified HeLa cell lysate (H. lys.), and 10 μg of eIF-4B purified from HeLa cells (H-eIF4B). The figure shows photographs of the plates stained with Coomassie blue.

al., 1979). Goats were chosen for inoculation because they provide large quantities of serum which are needed for many of the techniques to be employed, e.g., rocket immunoelectrophoresis and immunoblotting. Highly purified eIF-3 was injected into a female goat as described under Materials and Methods; after a few weeks, the goat serum gave a weak immunoprecipitation reaction. Following two booster injections, a strong precipitin line was observed by Ouchterlony double immunodiffusion analysis, as shown in Figure 1A. Encouraged by the positive response with rabbit eIF-3, we immunized goats with human HeLa initiation factors eIF-2, eIF-4A, and eIF-4B, purified as described in the preceding paper (Brown-Luedi et al., 1982). Following booster inoculations, a strong immunoprecipitin line formed with anti-eIF-4B (Figure 1B), but precipitin lines formed with antisera against eIF-2 and eIF-4A were weak and inconsistent. In all four experiments, up to a liter of serum was obtained between 4 and 6 weeks after the last booster injection.

Characterization of Antisera. The precipitating antisera made against eIF-3 and eIF-4B were tested by Ouchterlony double immunodiffusion with their cognate factors and with whole cell lysates (Figure 1). Anti-eIF-3 forms a single strong line with rabbit reticulocyte lysate and a much weaker line with HeLa lysate. The fusion of the lines against eIF-3 and the reticulocyte lysate is consistent with the presence of common antigens in the two preparations. Similar results are seen with anti-eIF-4B. The single, fused precipitin lines with eIF-4B, HeLa lysate, and the crude ammonium sulfate fraction A (0–40% saturation) from the ribosomal high-salt wash suggest that common antigens are being recognized in these preparations. Anti-eIF-4B does not cross-react with HeLa eIF-3 by this analysis.

Because the eIF-3 antigen is so complex, we characterized the antiserum by rocket immunoelectrophoresis. During the preparation of eIF-3, sucrose density gradient centrifugation or chromatography on Sepharose 4B is employed to separate eIF-3 from proteins of smaller molecular weight (Benne & Hershey, 1976). When the fractions from a Sepharose 4B column were analyzed, distinct rockets were observed in fractions containing eIF-3 as detected by the classical protein synthesis activity assay (Figure 2), whereas much less intense and distinct rockets were observed with fractions toward the top of the gradient. The intense rockets also corresponded with the presence of eIF-3 polypeptides determined by NaDodSO$_4$–polyacrylamide gel electrophoresis (results not shown). These results suggest that the eIF-3 particle is the major

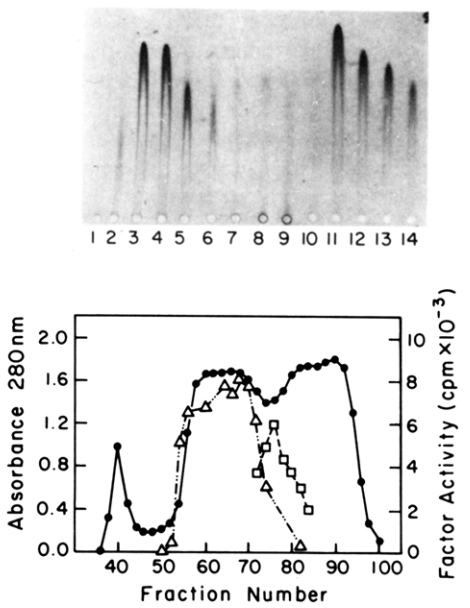

FIGURE 2: Rocket immunoelectrophoretic analysis of eIF-3. The 0–40% saturated ammonium sulfate fraction (A cut) prepared from the high-salt ribosomal wash of rabbit reticulocytes (Benne et al., 1979) was fractionated by molecular sieve chromatography on a Sepharose 4B column (100 cm long, 2.5 cm in diameter) in buffer containing 10 mM potassium phosphate, pH 7.5, 100 mM KCl, 0.2 mM EDTA, 7 mM 2-mercaptoethanol, and 5% (v/v) glycerol. (Upper panel) Column fractions (4.7 μL) were analyzed by rocket immunoelectrophoresis with 2% anti-eIF-3 serum as described under Materials and Methods. Wells 1–10 contained 7 μL each of column fractions 50, 54, 58, 62, 68, 72, 76, 80, 84, and 90. Wells 11–14 contained 2, 1.5, 1, and 0.5 μg of eIF-3 from rabbit reticulocytes. (Lower panel) Column fractions were analyzed for the absorbance at 280 nm (●), and aliquots (5 μL) were assayed for eIF-3 (△) and eIF-4B (□) activities in the globin synthesis assay (Benne et al., 1979).

antigen recognized by the antiserum. However, a precise correlation of rocket height with polypeptide components or eIF-3 activity was not possible. Since the component or components in the eIF-3 complex required for activity are not known, we are not able to conclude with certainty that the rockets represent active eIF-3 complexes. In other experiments, electrophoresis of reticulocyte lysate in agarose at pH 8.6, followed by immunodiffusion with anti-eIF-3, resulted in a single precipitin line in the same position as eIF-3 (results not shown). The results, along with those from immunoblotting described below, support the view that the antiserum recognizes primarily the eIF-3 particle.

Because antisera against eIF-2 and eIF-4A did not form precipitins and because of the difficulty in analyzing eIF-3, we characterized the four antisera by immunoblotting. Immunoblotting is a technique which involves electrotransfer of proteins from polyacrylamide gels to nitrocellulose paper, followed by treatment of the paper with specific antibodies. The technique is particularly powerful, since the binding of antibody to antigen requires only a single antigenic determinant. We used the procedure of Towbin et al. (1979) modified in the following ways: the time of electrotransfer was increased to allow proteins of high molecular weight to transfer quantitatively; the buffers used for antibody treatment contained 0.2% NaDodSO$_4$ and 0.5% Triton X-100 to reduce background binding; and ^{125}I-labeled rabbit IgG against goat IgG was used to detect specific antibody binding. Details of the procedure and the modifications are given under Materials and Methods and elsewhere (Howe & Hershey, 1981). When antisera against eIF-2, eIF-3, eIF-4A, and eIF-4B were tested, all four sera reacted with their cognate factors (Figure 3). Anti-eIF-2, which failed to form a precipitin line with eIF-2,

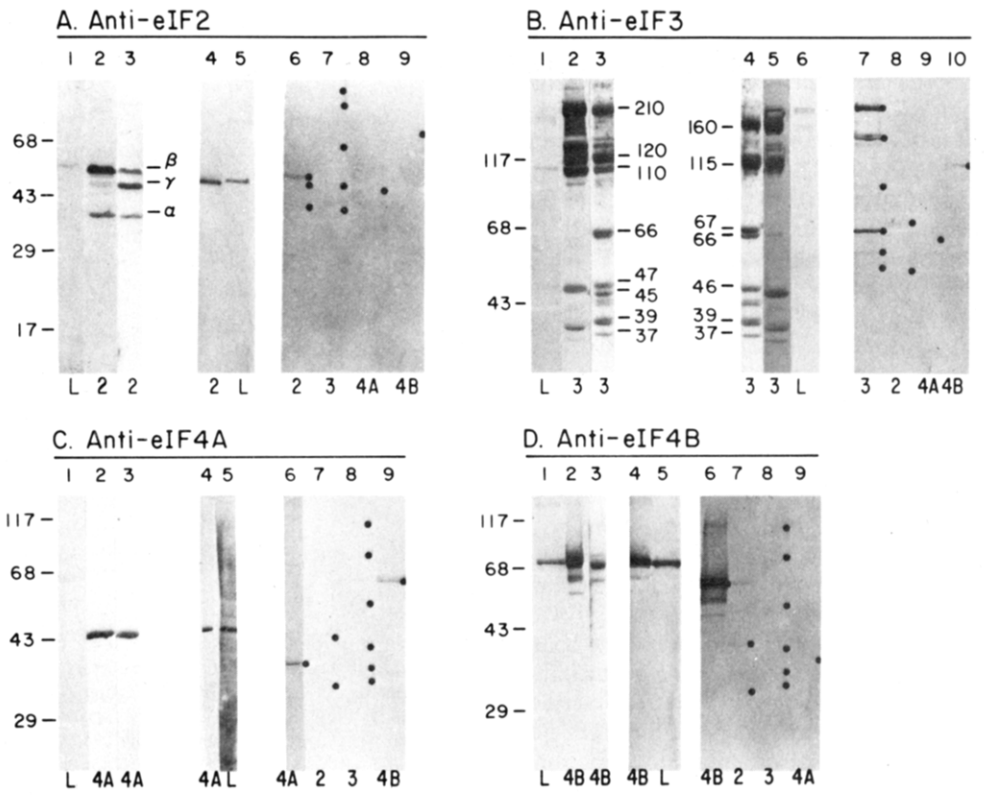

FIGURE 3: Immunoblot analyses of initiation factors and lysates. Purified initiation factor preparations or cells lysed directly into $NaDodSO_4$ buffer were analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis and immunoblotting with the four antisera, as described under Materials and Methods. All gels in panels B–D were composed of 10% acrylamide and 0.27% bis(acrylamide); those in panel A contained 15% acrylamide and 0.09% bis(acrylamide), which better separates the subunits of eIF-2 (Meyer et al., 1981). The figure shows photographs of autoradiograms or stained blots (lane 3 in all panels and lane 4 in panel B). Four molecular weight markers were included, and migration positions are shown by dashes to the left of each panel: β-galactosidase, 117 000; bovine serum albumin, 68 000; ovalbumin, 43 000; carbonic anhydrase, 29 000. The positions to which initiation factor proteins migrated are shown by dots on the right side of some of the gel lanes. The antiserum used is identified at the top of each panel. In panels A, C, and D, affinity-purified antibodies were used for lanes 4 and 5. Gel lanes are identified by numbers at the top of each lane; the initiation factors analyzed are identified at the bottom by their number; lysates are denoted L. In panels A, C, and D, lanes 6–9, and panel B, lanes 7–10, the gels contained the following amounts of HeLa factors: 2 μg of eIF-2, 5 μg of eIF-3, 1 μg of eIF-4A, and 1 μg of eIF-4B, except for the cognate factor in each panel, which was half the amount above. Other lanes contained the following amounts of proteins from HeLa cells unless otherwise noted. Panel A: lane 1, 50 μg of lysate; lane 2, 4.5 μg of eIF-2; lane 3, photograph of stained blot of lane 2; lane 4, 200 μg of eIF-2; lane 5, 50 μg of lysate. Panel B: lane 1, 60 μg of rabbit reticulocyte lysate; lane 2, 8.7 μg of rabbit eIF-3; lane 3, photograph of stained blot of lane 2; lane 4, photograph of stained blot of lane 5; lane 5, 8.7 μg of HeLa eIF-3; lane 6, 60 μg of HeLa lysate. Panel C: lane 1, 40 μg of lysate; lane 2, 1 μg of eIF-4A; lane 3, photograph of stained blot of lane 2; lane 4, 200 μg of eIF-4A; lane 5, 50 μg of lysate. Panel D: lane 1, 40 μg of lysate; lane 2, 1 μg of eIF-4B; lane 3, photograph of stained blot of lane 2; lane 4, 200 μg of eIF-4B; lane 5, 50 μg of lysate.

reacted strongly with the β subunit of eIF-2 and moderately with the α subunit, but only weakly with the γ subunit (Figure 3A, lane 2). The pattern of bands formed with anti-eIF-3 (prepared against rabbit reticulocyte factor) is more complex and reflects in part the eIF-3 preparation analyzed (Figure 3B). Rabbit eIF-3 (lane 2) formed strong bands from 210 000 to 110 000 daltons, corresponding to stained proteins (lane 3) in the preparation. The antiserum also reacted with a few, but not all, proteins in the group between 35 000 and 50 000 daltons but reacted very poorly with the protein doublet at about 66 000 daltons. As shown in lane 5, anti-eIF-3 also cross-reacted with the HeLa eIF-3 preparation, recognizing both the high molecular proteins (160 000 and 115 000 daltons) and those at 37 000–46 000 daltons. Anti-eIF-4A reacted strongly with eIF-4A (Figure 3C, lane 2), although it failed to form a precipitin line by double immunodiffusion. Anti-eIF-4B bound to the major (80 000 dalton) component of eIF-4B (Figure 3D, lane 2) and also to a number of minor impurities of lesser molecular weight.

We tested the four antisera for cross-reaction with the other initiation factors. As shown in the last four gel lanes of each section of Figure 3, each antiserum recognized most strongly its cognate factor, which was present in about half the molar quantity as the other three factors. The only substantial cross-reactivity encountered was with anti-eIF-4A and eIF-4B (Figure 3C, lane 9). Slight cross-reactivity was seen with anti-eIF-3 and eIF-2β,γ and eIF-4B (Figure 3B, lanes 8 and 10), with anti-eIF-4A and eIF-2β,γ (Figure 3C, lane 7), and with anti-eIF-4B and eIF-2β,γ (Figure 3D, lane 7). The minor cross-reactivities observed are likely due to nonspecific binding to the large amounts of protein on the blots, or to low levels of contaminating antibodies in the antisera, but not to shared antigenic determinants, as discussed below.

Immunoblot Analysis of Cell Lysates. HeLa cells or rabbit reticulocytes were prepared and lysed rapidly in buffer containing $NaDodSO_4$ as described under Materials and Methods. Immediate denaturation of the proteins with $NaDodSO_4$ is desirable in order to minimize possible proteolytic breakdown of initiation factors. The freshly prepared lysates were analyzed immediately by $NaDodSO_4$–polyacrylamide gel electrophoresis and immunoblotting as described above. Anti-eIF-2 formed a single weak band with the HeLa cell lysate, corresponding to the β subunit of eIF-2 (Figure 3A, lane 1). Anti-eIF-4A formed a weak band at the position of eIF-4A and a somewhat stronger band at 68 000 daltons (but not corresponding to eIF-4B at 80 000 daltons). The reactions of lysate with anti-eIF-2 and anti-eIF-4A were so weak that little information could be obtained. In contrast, anti-eIF-4B gave

a strong band with HeLa lysate and a much weaker band of very high molecular weight. The major lysate band corresponds exactly in molecular weight with purified eIF-4B, and no degradation forms of lower molecular weight were detected in the lysate. The pattern with anti-eIF-3 and rabbit reticulocyte lysate (Figure 3B, lane 1) was rather weak, with the major band corresponding to 110000 daltons and a diffuse minor band at about 170000 daltons. Reaction with the 47000-dalton protein is barely detectable, if at all. Similarly, with the HeLa cell lysate (lane 6), anti-eIF-3 bound primarily but weakly to proteins at 115000 and 160000 daltons and somewhat more strongly to a doublet at about 210000 daltons. The patterns observed with cell lysates are simpler than those obtained with purified eIF-3 preparations.

In order to obtain a better signal to noise ratio with lysates, and thus obtain more clearly interpretable results, we affinity purified the antibodies by the method of Olmsted (1981). Purified preparations of eIF-2, eIF-4A, and eIF-4B were subjected to $NaDodSO_4$–polyacrylamide gel electrophoresis, the proteins were electrotransferred to ABM paper, and protein bands corresponding to eIF-2β, eIF-4A, and eIF-4B were excised. The bands were incubated with antiserum, and bound antibodies were extracted into buffer containing guanidinium chloride. The affinity purification procedures are described in detail under Materials and Methods. The three purified antibody preparations were tested by immunoblotting against their cognate initiation factor and with HeLa cell lysate as shown lanes 5 and 4, respectively, of Figure 3A,C,D. The three purified antibodies each produced a single strong band with lysates. No precursor or degradation forms were detectable except for anti-eIF-4B, which formed a very weak band at about 100000 daltons (Figure 3D, lane 5). The positions of migration of eIF-4A and eIF-4B in lysates corresponded exactly with those of the purified factors, indicating that the molecular weights of these factors are unaltered during purification. The lysate band with anti-eIF-2β was slightly higher than that obtained with the purified preparation, which suggests that the β peptide in purified eIF-2 is somewhat shorter than that found in crude lysates. We are in the process of affinity purifying the antibodies against eIF-3 components and will describe such experiments elsewhere.

Cellular Levels of eIF-3. We have employed the anti-eIF-3 antiserum to estimate the concentration of eIF-3 in crude rabbit reticulocyte lysates. Clarified lysates prepared in nondenaturing buffer as described under Materials and Methods were analyzed by rocket immunoelectrophoresis as described in Figure 2. Figure 4 shows that the lengths of the rockets formed with purified eIF-3 (upper panel, lanes 1–5) are proportional to the amount analyzed. A linear standard curve over the range 0.1–1 μg of protein was obtained (lower panel). When 2.6 μL (upper panel, lane 6) or 1.3 μL (lane 7) of a lysate was analyzed, the resulting rocket heights corresponded to about 0.75 μg (lower panel, point a) and 0.38 μg (point b) of eIF-3, respectively. The apparent eIF-3 concentration is therefore about 0.28 mg/mL in this lysate and represents about 0.3% of the protein. The ribosome concentration, determined as described under Materials and Methods, was 1.1 μM, so the molar ratio of eIF-3 [assuming a molecular weight of 410000 (Brown-Luedi et al., 1982)] to ribosomes is about 0.5. This must be considered a crude estimate, for reasons discussed below.

A second method was used to estimate eIF-3 concentrations in rabbit reticulocyte and HeLa cell lysates. We examined the composition of immunoprecipitates formed between the crude lysates and anti-eIF-3 antiserum at equivalence. The

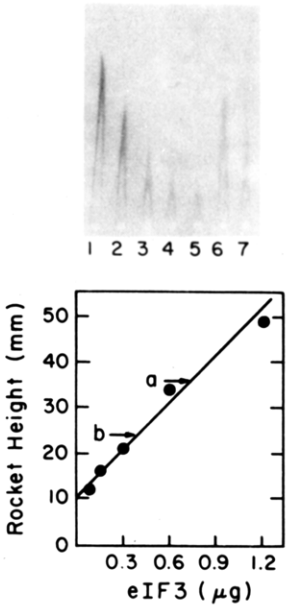

FIGURE 4: Quantitation of eIF-3 level by rocket immunoelectrophoresis. Rocket immunoelectrophoresis with 4% anti-eIF-3 serum was performed as described in Figure 2 and under Materials and Methods. (Upper panel) The figure shows a photograph of the plate stained with Coomassie blue. Wells 1–5 contained 1.2, 0.6, 0.3, 0.15, and 0.08 μg of rabbit eIF-3, respectively. Well 6 contained 2.6 μL and well 7 1.3 μL of a clarified rabbit reticulocyte lysate. (Lower panel) The heights of the rockets shown in the upper panel (wells 1–5) were measured and plotted vs. the amount of eIF-3 analyzed (●). The heights for 2.6 μL of lysate (arrow a) and 1.3 μL of lysate (arrow b) were also measured, and the eIF-3 concentration was calculated from the standard curve.

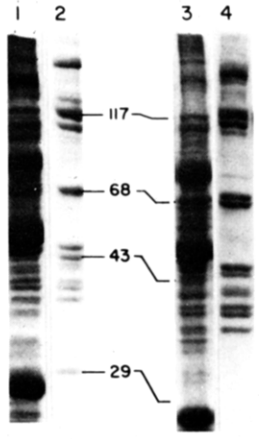

FIGURE 5: Immunoprecipitation of eIF-3 from crude cell lysates. Anti-eIF-3 was mixed with cell lysates at equivalence for eIF-3, the solutions (100 μL) were incubated at 4 °C for 3 days, and precipitates were collected by centrifugation, washed, dissolved in $NaDodSO_4$ buffer, and analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis as described in Figure 3. The figure shows photographs of the gels stained with Coomassie blue. Lane 1, 50 μL of rabbit reticulocyte lysate and 25 μL of anti-eIF-3 serum; lane 2, 10 μg of rabbit eIF-3; lane 3, 20 μL of HeLa lysate and 50 μL of anti-eIF-3 serum; lane 4, 10 μg of HeLa eIF-3.

precipitates were prepared and analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis as described in Figure 5. Precipitates from reticulocyte (lane 1) and HeLa (lane 3) lysates were compared with purified eIF-3 preparations from reticulocytes (lane 2) or HeLa cells (lane 4). In both cases, bands visible in the purified preparations are also found in the immunoprecipitates. This is most apparent for bands at about 68000 daltons and for components between 35000 and 50000 daltons. The eIF-3 polypeptides of high molecular weight also

are found in the immunoprecipitates, but their relative intensities differ, probably due to the action of proteases (Meyer et al., 1981) during the long incubation period used. In addition to the darkly staining bands at 25000 and 50000 daltons which correspond to immunoglobulin chains, other non-eIF-3 proteins are present in the precipitates. Their presence may be due to nonspecific precipitation, to specific binding to the eIF-3 particle, and/or to impurities in the antiserum. Based on the results of rocket immunoelectrophoresis, we estimated the amount of eIF-3 expected to be precipitated from the lysates. Precisely this amount of purified eIF-3 was added for comparison (lanes 2 and 4). The intensities of the eIF-3 bands below 70000 daltons [which are the more constant components of the eIF-3 complex (Meyer et al., 1981)] in the precipitates and the eIF-3 preparations are approximately the same, consistent with the estimate that the eIF-3 to ribosome molar ratio is about 0.5.

Discussion

In the preceding paper (Brown-Luedi et al., 1982), we presented evidence that the structures of initiation factors from rabbits and humans are very similar. There are some differences in primary structures, however, and such differences may be exploited for the production of antibodies in mammals. Our results indicate that goats can be used to raise antibodies which recognize rabbit eIF-3 and human eIF-2, eIF-4A, and eIF-4B. The antisera cross-react to variable extents with the corresponding cognate factors from either rabbit reticulocytes or human HeLa cells (Brown-Luedi et al., 1982). The antisera generally have rather low titers, and only anti-eIF-3 and anti-eIF-4B reliably form precipitates at equivalence. Because goats produce large quantities of antiserum, the titer problem is reduced; analysis by immunoblotting circumvents the need to have precipitating antisera. Other workers also have reported the preparation of antisera against initiation factors. Trachsel and co-workers prepared a mouse antiserum against rabbit reticulocyte eIF-3 and selected a hybridoma cell line which produces an antibody which reacts with the cap binding protein family in rabbits and human HeLa cells (Sonenberg et al., 1981). Using a different strategy, Gupta and co-workers used chickens to prepare polyvalent antisera against rabbit reticulocyte eIF-2 and Co-eIF-2A (Ghosh-Dastidar et al., 1980) but did not identify the subunits of eIF-2 which were recognized.

We have used the antisera and the immunoblotting technique to characterize the molecular weights of the factor proteins in crude cell lysates (Figure 3). This is important because it has not yet been shown that purified initiation factors have in fact the same molecular form as those present in intact cells. Because the crude antisera gave only weak bands and high background with crude cell lysates, we affinity purified the antibodies against eIF-2β, eIF-4A, and eIF-4B. Since each antibody gave a single, moderate to strong band with lysates, we conclude that the purified antibodies do not cross-react significantly with other proteins in the lysate. We also conclude that only one major form of the factor exists in HeLa cells and that no abundant precursor or degradation forms exist. The precise correspondence in migration position of lysate and purified eIF-4A and eIF-4B bands means that the purified factors have molecular weights indistinguishable from the cellular (i.e., lysate) forms. Interestingly, the apparent molecular weight of the β subunit of purified eIF-2 is slightly smaller than that of the lysate protein. We have reached the same conclusion by analysis of the β subunit in lysates and eIF-2 preparations by two-dimensional polyacrylamide gel electrophoresis.[2] Sensitivity of the β subunit to proteases has been reported (Mitsui et al., 1981). These results raise the question whether purified eIF-2 preparations may be significantly altered in activity due to partial degradation of the β subunit. The rather striking difference in immunoblot patterns observed between lysates and eIF-3 preparations suggests that the eIF-3 complex in vivo may be quite different from that isolated and purified. We are now beginning to use affinity-purified antibodies to probe the native structure of eIF-3.

Both rocket immunoelectrophoresis and antibody precipitation methods suggest that the level of eIF-3 in rabbit reticulocyte lysates corresponds to 0.5 molecule per ribosome, or 0.3% of the protein in a clarified lysate. Because of the complexity of the factor and the presence of contaminating antibodies in the serum, the rocket gel results are difficult to evaluate, but the results are consistent with the rather crude estimates made by the precipitation experiments. Estimates of cell levels based on purification yields (Benne et al., 1979) give factor to ribosome ratios of 0.3–0.4. Our tentative value of 0.5 for the eIF-3 to ribosome ratio is substantially higher than the initiation factor to ribosome ratios in bacteria, which are 0.15–0.2 in *Escherichia coli* as measured by radioimmune assays (Howe et al., 1978) and by quantitative immunoblotting (Howe & Hershey, 1981). We are now in the process of measuring the levels of initiation factors in crude cell lysates of HeLa cells by using the immunoblotting technique (Howe & Hershey, 1981) and two-dimensional polyacrylamide gel techniques (O'Farrell et al., 1977). These techniques are expected to give measurements for most of the initiation factors which should be more reliable than those determined here for eIF-3.

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Phase Behavior of Mixed Phosphatidylglycerol/Phosphatidylcholine Multilamellar and Unilamellar Vesicles†

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ABSTRACT: The phase behavior of dipentadecanoylphosphatidylglycerol ($DC_{15}PG$)/dimyristoylphosphatidylcholine (DMPC) mixtures has been studied in both small, unilamellar vesicles and large, multilamellar vesicles. We have used both the steady-state fluorescence polarization of 1,6-diphenyl-1,3,5-hexatriene (DPH) and high-sensitivity differential scanning calorimetry to detect temperature-dependent changes in membrane structure. Electron microscopy has demonstrated different fracture face morphologies for large, multilamellar vesicles depending on sample composition and temperature. These data have been interpreted in terms of proposed phase diagrams for this lipid mixture. The shapes of the proposed phase diagrams have led us to conclude that DMPC and $DC_{15}PG$ mix freely in the plane of a lipid bilayer only at less than 50 mol % $DC_{15}PG$. At higher $DC_{15}PG$ content, the data have been interpreted as reflecting substantial compositional inhomogeneities in the plane of the bilayer, if not phase immiscibility, even in the fluid phase. In addition, small vesicles containing greater than 50 mol % $DC_{15}PG$ were unstable in the ordered phase and spontaneously converted to larger vesicles. Finally, the anisotropy of DPH fluorescence was found to be invariant with $DC_{15}PG$ content at temperatures just above the liquidus phase line in small, unilamellar vesicles. This demonstrated that inclusion of negatively charged phosphatidylglycerol does not noticeably affect the order within the acyl chain region of the bilayer, relative to phosphatidylcholine.

Phosphatidylglycerol is a major phospholipid class in plant and microbial membranes (Galliard, 1973) and is present in small amounts in mammalian membranes (McMurray, 1973). The ready availability and chemical stability of synthetic phosphatidylglycerols have made them attractive model compounds for studying the effects of acidic phospholipids on membrane structure and function. The pK_a is sufficiently low (2.9 at physiological ionic strength; Watts et al., 1978) that phosphatidylglycerol maintains a full negative charge at physiological pH. Several biophysical studies have focused on the phase behavior of pure synthetic phosphatidylglycerols (van Dijck et al., 1975; Jacobson & Papahadjopoulos, 1975; Ranck et al., 1977; Watts et al., 1978; Wohlgemuth et al., 1980; Cevc et al., 1980; Harlos & Eible, 1980). Physical techniques employed in these studies have included spin resonance spectroscopy, fluorescence polarization, X-ray diffraction, electron microscopy, and differential scanning calorimetry. Two recent papers report rapid scanning (300 and 560 °C/h) differential calorimetric measurements of phase separations in large, multilamellar vesicles (LMV)[1] composed of phosphatidylglycerol/phosphatidylcholine mixtures (van Dijck et al., 1978; Findlay & Barton, 1978). With the assumption that rapid scanning did not significantly perturb the system from equilibrium, these studies concluded that the glycerol and choline head groups allowed complete mixing of lipid species.

While most determinations of phase behavior have been made with LMV preparations, unilamellar vesicles are more appropriate model membranes for investigating interactions of proteins and lipids at a membrane surface. Small, unilamellar vesicle (SUV) preparations composed of synthetic phosphatidylglycerol/phosphatidylcholine mixtures have been used to study the Ca^{2+}-mediated binding of γ-carboxyglutamate-containing human prothrombin to negatively charged membrane surfaces (Dombrose et al., 1979). This is one of the several protein/lipid interactions that are thought to be crucial to the ability of phosphatidylglycerol/phospha-

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[1] Abbreviations: LMV, large, multilamellar vesicles; SUV, small, unilamellar vesicles; $DC_{15}PG$, 1,2-dipentadecanoyl-3-*sn*-phosphatidylglycerol; DMPC, 1,2-dimyristoyl-3-*sn*-phosphatidylcholine; DPH, 1,6-diphenyl-1,3,5-hexatriene; Tes, 2-[[tris(hydroxymethyl)methyl]amino]-ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

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