Initiation factor eIF-4F, a multiprotein cap binding protein complex, was purified from HeLa cells by m7G affinity chromatography and independently by phosphocellulose column chromatography. The m7G affinity-purified sample contains three major proteins, p220, eIF-4A, and p28 (also known as CBP-I or eIF-4E). The abundances of these proteins are roughly 2, 10, and 0.8 x 10^6 molecules/cell, respectively. Two-dimensional isoelectric focusing/sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the eIF-4F samples shows that p28 comprises two isoelectric variants, one of which labels with phosphate and disappears when samples are treated with alkaline phosphatase. The 45,900 Dalton protein in eIF-4F appears to be identical to eIF-4A. The p220 subunit rarely produces discrete spots on two-dimensional gel electrophoresis; in purified samples it usually forms 3 closely spaced streaks. eIF-4F fractionated by phosphocellulose chromatography separates into forms containing either phosphorylated or unphosphorylated p28. However, both fractions possess similar specific activities in in vitro translation assays for eIF-4F activity. The phosphorylation of p28 decreases upon heat shock when protein synthesis is repressed. The correlation of dephosphorylation of p28 with the inhibition of protein synthesis and the relatively low abundance of the eIF-4F complex suggest that eIF-4F plays a role in the translational control of mRNA binding. Limitations of the in vitro assay system may account for the failure to detect phosphorylation-dependent activity differences.
lytic recycling of the factor (18–20). We have used IEF/SDS-PAGE analysis to precisely quantify the extent of eIF-2α modification in intact cells and correlate this with the inhibition of protein synthesis (21–23). In this report, we identify and quantitate eIF-4F proteins and provide evidence that the 28-kDa protein subunit (eIF-4E) is phosphorylated. When protein synthesis is inhibited by heat shock, the extent of p28 phosphorylation decreases substantially and may play a pivotal regulatory role.

**EXPERIMENTAL PROCEDURES**

**Materials—**[35S]Methionine (1000 Ci/mmol), inorganic [32P]phosphate (1000 Ci/mmol), and N-acetyl-l-cysteine (10 mM) were purchased from New England Nuclear. Acrylamide and bisacrylamide were obtained from Serva. Ampholytes were purchased from LKB Instruments, Inc. Initiation factors were prepared from HeLa cells as described (24).

**Cell Culture—**HeLa (SS) cells were propagated in spinner culture. For the preparation of eIF-4F cells were pelleted and frozen at −70 °C until the beginning of the purification protocol. For labeling and immunoblotting of unfraccionated cell samples, suspension cultured cells were transferred to a 56-mm tissue culture dish at about 1.5 × 10⁶ cells/plate. After allowing 1–2 h for the cells to attach, the old medium was removed, and 3 ml of fresh MEM containing 10% calf serum were added. The abundance and covalent modification status of eIF-4E and eIF-4G have been characterized in a monolayer culture versus suspension culture, and amounts, modifications, and responses have been indistinguishable.

**Protein Labeling and Analysis by PAGE and Immunoblotting—**[35S]Methionine protein labeling, extraction, and gel electrophoresis in two dimensions have been described in Duncan and Hershey (14). Silver staining was performed by the procedure of either Wray et al. (25) or Morrissey (26). β-Mercaptoethanol was omitted from the second dimension equilibration buffer in samples to be silver stained. One-dimensional gels were run as described for the second dimension of the two-dimensional IEF/SDS-PAGE. Details of the immunoblotting techniques have been described elsewhere (14–16).

**Alkaline Phosphatase Analysis—**About 5 μg of eIF-4F (phospho-cellulose column purified) in 50 μl was added with 1 μl of 1 M Tris-HCl, pH 8.0. Soybean trypsin inhibitor (1 mg/ml) and benzamidine (10 mM) were added to inhibit proteases in the phosphatase solution. 20–45 units of alkaline phosphatase (400–900 units/ml) (Sigma) were added and incubated for 30 min at 37 °C. The sample was then mixed with an equal weight of urea and analyzed by two-dimensional IEF/SDS-PAGE.

**Phosphocellulose Purification of eIF-4F—**eIF-4F was purified from about 200 g of HeLa cells initially following our standard procedures for the purification of eIF-3 from the 0–40% ammonium sulfate A cut of the ribosomal salt wash (24). The A cut was centrifuged on a 10-s linear gradient of KCl, 100–500 mM in buffer A. The elution of eIF-4F activity from the column was monitored by analyzing the fractions for eIF-4F activity and for the presence of p220 antigens by immunoblotting. The eIF-4F eluted at about 175 mM KCl. Active fractions were pooled, diluted to 100 mM KCl, and applied to the m7G-Sepharose 4B column. The column was eluted with a linear gradient of KCl from 200 to 500 mM in buffer A in which 20 mM potassium phosphate, pH 7.4, was substituted for the Tris-HCl. Column fractions were assayed as above for the presence of eIF-4F activity and p220 antigens, which were found to elute at about 250 mM KCl in a single broad peak.

**mG Affinity Chromatography of eIF-4F—**For this purification we followed the protocol described by Edery et al. (11) without any modifications. eIF-4F has been purified using mG-Sepharose 4B purchased from Pharmacia L-Biochemicals as well as mG-agrose kindly provided by I. Edery and N. Sonenberg (McGill University) with essentially identical proteins selected. In a typical purification, about 30 μg of A cut (24) were passed over a 1-ml column.

For the analysis of [32P]-labeled eIF-4F, about 1.5 × 10⁶ suspension cultured HeLa cells were concentrated 3-fold (to 10 ml) by centrifugation and resuspension in phosphate-free MEM containing 10% calf serum. Cells were labeled for 40 min with 3 mCi of [32P]phosphate (333 μCi/ml). Some cell samples were heat shocked in a water bath at 44 °C for the final 20 min of labeling. Harvested cells were washed three times with 4 °C MEM and homogenized in 1 ml of buffer (LCB: 100 mM KCl, 20 mM HEPPS, pH 7.5, 0.2 mM EDTA, 10% glycerol, 7 mM β-mercaptoethanol) containing 0.5% Triton X-100, 0.2 mM phenylmethylsulfonyl fluoride, 2 units/ml aprotinin, and 50 mM NaF. After nuclei and cell debris were removed by centrifugation (5 min, Beckman Microfuge) the supernatant (S10) sample was directly applied to the mG-Sepharose 4B column.

For purification of eIF-4F from the S10 of control and heat-shocked cells, about 500 ml of culture medium containing HeLa cells (2.5 × 10⁶ cells/ml) were used. Heat-shocked cells were transferred to a 44 °C water bath (with stirring) for 45 min. Cells were poured over crushed frozen MEM, collected by centrifugation, and washed three times with 4 °C MEM. The cell pellets were resuspended in 5 ml of LCB containing 0.5% Triton X-100 and proteinase inhibitors (as described above) and homogenized with 5 strokes of a glass Dounce homogenizer. The lysate was spun for 10 min at 8000 rpm (SS-34 rotor, Sorvall Instruments), and the supernatant (about 55 A₂₈₀ units) was removed and directly applied to a 2 ml mG-Sepharose 4B column. The column was washed with about 50 ml of LCB prior to elutions with (i) 100 mM GDP in LCB (15 ml) and (ii) 75 mM mGTP in LCB. 4 ml of the GDP eluate were combined, precipitated with 4 volumes of acetone overnight at −20 °C, and finally resuspended in two-dimensional gel loading buffer (14). The mGTP eluate was similarly processed and analyzed by IEF/SDS-PAGE.

**Assay for eIF-4F Activity in the Fractionated in Vitro Globin Synthesis System—**In vitro globin synthesis assays using fractionated components of the translational machinery were performed as described previously (24). Most of the assays were performed using an eIF-3 preparation which did not contain contaminating eIF-4F activity, thus allowing the assay of eIF-4F in samples. Some assays were also evaluated with eIF-3 purified from poliovirus-infected HeLa cells (kindly provided by Dr. D. Etchison) which likewise lacks eIF-4F activity (27). Both assay systems gave similar results.

**RESULTS**

**Composition of eIF-4F Examined by One- and Two-dimensional Gel Electrophoresis—**Cap binding proteins and/or multiprotein cap binding protein complexes were isolated from a 0.5 M KCl wash fraction of HeLa cell ribosomes by affinity binding to an mG column (see "Experimental Procedures" for details). The fraction eluted with mGTP was examined by one-dimensional SDS-PAGE (Fig. 1A, left lane). Three major protein bands are observed, of masses 220, 43, and 28 kDa. The protein composition closely resembles that of eIF-4F (6) and cap binding protein II (7) which are likewise isolated by mG affinity chromatography. We have observed minor bands at about 55, 100, and 130 kDa in some eluates, whose possible homologies with the previously described 70- and 90-kDa minor proteins observed by others (6) have not been pursued. The complex that we isolate possesses restoring activity for poliovirus-infected lysates and increases protein synthesis by 2-fold or more in a fractionated in vitro globin synthesis assay (see "Experimental Procedures") (data not shown). The multiprotein complex that we isolate appears to be structurally and functionally homologous with eIF-4F, and we can show both the same large multiprotein complex possessing eIF-4F activity by phosphocellulose column chromatography (see "Experimental Procedures" for details). This preparation contains the 28- and 220-kDa proteins, but little if any eIF-4A (Fig. 1A, right lane). The identities (or absence) of p220 and eIF-4A in these purified preparations were verified by immunoblotting. Note that because purified eIF-4A is present in the in vitro assay for eIF-4F activity, we cannot evaluate whether the two-protein form of eIF-4F is active by itself.

**Two-dimensional IEF/SDS-PAGE analysis of eIF-4F purified by mG affinity chromatography (Fig. 1B) reveals three major spots, two migrating at 28 kDa with pIs of ~5.9 and ~6.3.
Heat Shock Effects on eIF-4F

**FIG. 1. One- and two-dimensional electrophoresis of eIF-4F proteins.** eIF-4F purified by mG-affinity chromatography or phosphocellulose column chromatography as described under "Experimental Procedures" was analyzed by one- and two-dimensional gel electrophoresis. Initial affinity purifications were on mG-agarose, kindly supplied by I. Edery and N. Sonenberg; subsequently eIF-4F has been purified with mG-Sepharose 4B with very similar results. A, one-dimensional SDS-PAGE and silver stain to detect protein bands (25). Positions where molecular weight marker proteins migrate are given in kDa to the right. Left lane, mG-agarose-purified eIF-4F (100 ng); right lane, phosphocellulose purified eIF-4F (4 μg). B, two-dimensional IEF/SDS-PAGE of mG-agarose-purified eIF-4F (200 ng) and silver stain (25). The forms of p28 are labeled a (presumably unmodified) and b (phosphorylated p28). The minor spot migrating directly to the acidic side of eIF-4A has not been observed in other isolations of eIF-4F. The streaks of stain from about 55 to 60 kDa are artifacts seen frequently. The apparent lower Mr, of eIF-4A in A, left lane (where it migrates with the 43-kDa marker), relative to the two-dimensional analysis reflects slightly different gel running conditions and is characteristic of the one-dimensional system used.

~6.2, and the third at 45 kDa, pI 5.8. The 28-kDa proteins are two forms of the protein which has been termed eIF-4E (also known as CBP-I or CBP-24K) and are described in detail below. The more basic form of p28 usually accounts for roughly 65-75% of the staining. The 45-kDa protein is eIF-4A. It comigrates with purified eIF-4A and with the HeLa cell cytoplasmic protein previously identified as eIF-4A (14) and reacts with anti-eIF-4A antisera (data not shown). This is the only eIF-4A protein detected in our isolates of eIF-4F. The 220-kDa protein fails to focus in most IEF runs, producing more or less elongate streaks centered around pI 5.8-5.9 (Fig. 1B, bracket). Usually we observe two or more streaks of identical pI range (Fig. 1B) separated by 5-10 kDa, which correspond to the multiple p220 bands detected on one-dimensional SDS-PAGE (see Fig. 3). Infrequently, we have detected a single p220 spot at a pI of about 6.55. The factors which allow p220 to infrequently produce a focused spot are unclear. An intriguing possibility is that p220 is an mRNA binding protein which is usually isolated as an RNA-protein complex and fails to focus for this reason. However, RNase treatment of eIF-4F fails to convert streaky p220 into a singlet spot.

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**Identification of 35S-Labeled Lysate Proteins Corresponding to eIF-4F Proteins—**Purified eIF-4F was mixed with a [35S] methionine-labeled cell lysate, and the radioactive spots comigrating in two-dimensional gels with Coomassie-stained eIF-4F proteins were identified (Fig. 2). Two labeled p28 spots could be detected, but only after quite long exposures, suggesting that these two proteins are of low abundance in the cytoplasm (see below for a quantitative estimate). We have designated these two eIF-4F proteins as p28a (pI ~ 6.2) and p28b (pI ~ 5.9) to facilitate reference in the descriptions that follow. The eIF-4A in eIF-4F is indistinguishable from the total eIF-4A in lysates as described above. p220 has not been detectable in crude lysates. No 35S-labeled spots are observed in the region where p220 infrequently focuses into a spot. Similarly, and most mysteriously, lysates containing abundant p220, as detected by one-dimensional SDS-PAGE immunoblotting, contain no detectable p220 when subsequently analyzed by either two-dimensional IEF/SDS-PAGE or nonequilibrium pH gradient electrophoresis SDS-PAGE immunoblotting; not even streaks are detected. Thus, the identification and characterization of a p220 spot appears to be a formidable problem and as yet an unattained goal.

**Abundance of eIF-4F Proteins—**The concentration of p220 was estimated by quantitative immunoblotting (15). The amount of 125I bound to a range of p220 concentrations was compared to the amount of 125I bound to p220 in cytoplasmic lysates (Fig. 3). By densitometry of the autoradiograms, we measure that p220 comprises about 0.33% of the cytoplasmic protein mass and about 0.071% of the cytoplasmic protein molecules (Table I); the calculated p220 concentration differs by about 30% depending on which lysate input is analyzed, which is likely due in part to a nonlinearity of this form of assay as higher inputs are used. This corresponds to about 6.2, and the third at 45 kDa, pI 5.8. The 28-kDa proteins are two forms of the protein which has been termed eIF-4E (also known as CBP-I or CBP-24K) and are described in detail below. The more basic form of p28 usually accounts for roughly 65-75% of the staining. The 45-kDa protein is eIF-4A. It comigrates with purified eIF-4A and with the HeLa cell cytoplasmic protein previously identified as eIF-4A (14) and reacts with anti-eIF-4A antisera (data not shown). This is the only eIF-4A protein detected in our isolates of eIF-4F. The 220-kDa protein fails to focus in most IEF runs, producing more or less elongate streaks centered around pI 5.8-5.9 (Fig. 1B, bracket). Usually we observe two or more streaks of identical pI range (Fig. 1B) separated by 5-10 kDa, which correspond to the multiple p220 bands detected on one-dimensional SDS-PAGE (see Fig. 3). Infrequently, we have detected a single p220 spot at a pI of about 6.55. The factors which allow p220 to infrequently produce a focused spot are unclear. An intriguing possibility is that p220 is an mRNA binding protein which is usually isolated as an RNA-protein complex and fails to focus for this reason. However, RNase treatment of eIF-4F fails to convert streaky p220 into a singlet spot.

2 Previous immunoblot analyses of eIF-4A (14) revealed 2-3 immunoreactive spots. Recent antibody preparations have reproducibly reacted with only one eIF-4A spot (21, 22). We presume that the variants observed formerly were contaminating antibodies in the affinity-purified preparations. The minor more acidic spot next to eIF-4A in Fig. 1B has not been detected in other purified samples.

3 A. A. Stewart and J. W. B. Hershey, unpublished observations.
The abundance of p28 could not be estimated by immunoblotting because antibodies are not available. The concentration of the more basic p28a spot was estimated by counting the radioactivity in the p28a spot and by comparative autoradiography of labeled lysate proteins. HeLa cells were labeled with a mixture of 14C-aminocids, extracted, and the protein analyzed by two-dimensional IEF/SDS-PAGE in the presence and absence of exogenous p28. A labeled spot comigrating with p28a was detectable after 268 h of exposure (Fig. 4B). The p28a spot was excised and counted. The p28a spot averaged about 30 cpm above background, whereas eIF-2a (0.089% of the protein radioactivity) and eIF-3p36 (0.072% of the protein radioactivity) averaged 165 and 115 cpm above background, respectively (Table I). Thus, p28a comprises about 0.015% of the protein radioactivity. Reasoning that p28a comprises 70% of the total p28, then total p28 is 0.021%. This corresponds to about 0.86 × 10^6 molecules/cell or 0.26 molecules/ribosome. To confirm these spot counts, which are subject to some uncertainty due to the low cpm found in p28, gels such as shown in Fig. 4B were also exposed for several times to establish a time at which the darknesses of eIF-2a (0.11% of the cytoplasmic protein) and eIF-3p36 (0.094% of the cytoplasmic protein) were roughly equivalent to p28a at 268 h. A 48-h exposure produced the best matches by visual estimation (Fig. 4A). Exposures 0.5 (24 h) or 1.5 (72 h) times that long gave detectably different and less well matched spot darknesses. Thus, the radioactivity in p28a is about 1/5.5 (48/268) that of eIF-2a and eIF-3p36 (14). This is a quite similar conclusion as was reached by direct spot counting and, thus, provides confirmation for that value.

The qualitative conclusion that p28 is substantially less abundant than p220 seems inescapable. The quantitative uncertainty introduced by estimating isoelectric variant abundances and autoradiographic spot darknesses means that there is a degree of uncertainty in the proposed p28 molecular abundance.

### Table I

| eIF-4F protein | Cell protein | Cytoplasmic protein molecules | Molecules/cell | Factor/ribosome |
|---------------|-------------|-------------------------------|---------------|----------------|
| p220          | 0.330       | 0.071                         | 1.73          | 0.52           |
| eIF-4A        | 0.375       | 0.400                         | 9.76          | 2.96           |
| p28a          | 0.021       | 0.035                         | 0.86          | 0.26           |

Values were calculated as described in the text: for p220, comparative densitometry of immunoblots; for eIF-4A, values are from Ref. 14 and were determined by spot counting; for p28, values are from comparative autoradiography and spot counting.

This conversion was achieved by multiplying the "percent input" cell protein value by a molecular weight factor, which was calculated by dividing the molecular weight of the average size HeLa cell protein, 47,000, by the individual factor's molecular weight.

Calculated using the average cytoplasmic protein content of an exponentially growing HeLa cells as 150 pg (14).

The value for ribosomes/cell is 3.3 × 10^6 (14).

For spot excision and counting of p28, eIF-2a, and eIF-3p36, these three spots were excised, hydrated, and digested as previously described (14). In two independent analyses of 14C-labeled cell lysates, p28a contained 83 and 90 cpm, while 2 nearby spot-free gel regions averaged 220 and 268 cpm. eIF-2a measured 225 and 220, while eIF-3p36 measured 117 cpm. The amount of p28a is thus 28/164, or 1/6, of eIF-2a (0.089% of input radioactivity), and 28/117, or 1/4.5 of eIF-3p36 (0.072% of the input radioactivity), corresponding to 0.015% of input radioactivity. Since p28a is roughly 70% of the total mass, the total p28 abundance is (100/70) × 0.015% or 0.021%.

1.7 × 10^6 molecules/cell and about 0.5 molecule of p220/ribosome (Table I).

We have previously quantitated eIF-4A (14); there are about 10 × 10^6 molecules of eIF-4A/cell or about 3 copies/ribosome (Table I).

The qualitative conclusion that p28 is substantially less abundant than p220 seems inescapable. The quantitative uncertainty introduced by estimating isoelectric variant abundances and autoradiographic spot darknesses means that there is a degree of uncertainty in the proposed p28 molecular abundance.

### Fig. 3. Quantitation of p220 by immunoblotting.

Phosphocellulose-purified eIF-4F (lacking eIF-4A) was analyzed at several input concentrations by one-dimensional SDS-PAGE and immunoblotting (15) using affinity-purified anti-p220 (47) and 35S-labeled rabbit anti-goat IgG. A lane of eIF-4F first was stained with Coomassie Blue and scanned; the fraction of p220 protein in the preparation was 50% (result not shown). The pg of p220 present in each lane are shown. Samples of cell lysates were similarly analyzed on the same gel (panel B). Cell lysates have been prepared from monolayer cells; suspension-cultured cells gave indistinguishable results. Cell lysates were prepared by lysis in IEF/SDS-PAGE lysis buffer (14); lysis into SDS-containing buffer gave indistinguishable results. Two independent samples were analyzed at two input concentrations, 30 and 60 µg.

The autoradiograms were scanned in an automated densitometer to provide the quantitations described in the text.

### Fig. 4. Quantitation of p28a by comparative autoradiography.

Cells were plated in one well of a 24-well tissue culture plate. 50 µCi of a mixture of 15C-aminocids were lyophilized and resuspended in 200 µl of MEM containing 10% calf serum. The cell medium was replaced with the labeling medium, and cells were incubated for 24 h. A cell lysate was analyzed by two-dimensional IEF/SDS-PAGE in the presence and absence of exogenous eIF-4F. The stained gel was used to identify the location of the labeled p28a spot. A, 48-h exposure of the gel; B, 268-h exposure of the same gel. Two other initiation factor protein spots are identified. The gel sector shown is from the sample run in the absence of eIF-4F. Thus, the radioactivity in p28a is not due to radioactivity "trapping" that might occur in a comigration sample due to the relatively large amount of eIF-4F subunit proteins.

A. p220 STANDARDS

B. LYSATES

| Protein | Cell Protein | Cytoplasmic Protein | Molecules/cell | Factor/ribosome |
|---------|--------------|---------------------|---------------|----------------|
| p220    | 0.330        | 0.071               | 1.73          | 0.52           |
| eIF-4A  | 0.375        | 0.400               | 9.76          | 2.96           |
| p28a    | 0.021        | 0.035               | 0.86          | 0.26           |

Values were calculated as described in the text: for p220, comparative densitometry of immunoblots; for eIF-4A, values are from Ref. 14 and were determined by spot counting; for p28a, values are from comparative autoradiography and spot counting.

This conversion was achieved by multiplying the "percent input" cell protein value by a molecular weight factor, which was calculated by dividing the molecular weight of the average size HeLa cell protein, 47,000, by the individual factor's molecular weight.

Calculated using the average cytoplasmic protein content of an exponentially growing HeLa cells as 150 pg (14).
concentration, but we estimate that the maximum p28 concentration consistent with all our gel analyses would still be at least 2-fold lower than p220 and most other initiation factor proteins.

Phosphorylation of p28: IEF/SDS-PAGE Characterization—The most intriguing observation arising from the IEF/SDS-PAGE characterization of eIF-4F is that p28 occurs in two isoelectric forms (proof follows). Because the most common post-translational covalent modification producing multiple isoelectric forms is phosphorylation, we examined whether the acidic p28b variant was phosphorylated by comigration in gels of eIF-4F with [32P]phosphate. IEF/SDS-PAGE analysis showed that a prominent [32P]-labeled spot (Fig. 5A) migrates about 1–2 mm above the acidic p28b form. On some gels this [32P] spot precisely comigrates with p28b, and this initially led us to suspect it corresponded to eIF-4E (28). The [32P] spot has been characterized by others as a phosphorylated variant of the 28-kDa heat shock protein (HSP 28b) (29). Two variants of HSP 28 can be identified in crude lysates as moderately abundant spots by staining or amino acid labeling (29). The more acidic variant precisely comigrates with the [32P]-labeled spot (29). Whereas p28b is similar to HSP 28b in isoelectric point (and molecular weight), p28a is displaced about 5 mm (approximately 0.1 pH unit) to the acidic side of HSP 28a. Partial V8 protease digestion mapping of [32P]-labeled phosphorylated HSP 28a (HSP 28b and HSP 28c; silver stain and radiolabeled) versus p28 (silver stain) indicates no homology by this test (Fig. 6).

We have tried to detect a minor [32P]-labeled spot that comigrates with p28b. Most long exposures of [32P] autoradiograms of protein from cells grown at 37 °C show little evidence for a [32P]-labeled p28b. In the gel shown in Fig. 5A a faint [32P]-labeled spot migrating to the coordinates of p28b can be detected. The input to this gel was 1/100 of the sample used to purify p28, as described in the next paragraph. Since it was necessary to analyze the total m7G-Sepharose 4B-selected sample to detect [32P]-labeled p28b, it seems unlikely that [32P]-labeled p28b can be readily detected in unfractionated lysates. Though we cannot directly ascertain the identity of faint [32P]-labeled spots in total lysates that migrate to coordinates indistinguishable from p28b, these abundance considerations suggest they are other proteins.

To unambiguously identify phosphorylated p28b, cytoplasmic lysates of HeLa cells labeled with [32P]phosphate were prepared, and p28 (and any complexed proteins) was affinity purified from the lysates by m7G-Sepharose 4B chromatography. The bound and eluted proteins were examined by IEF/SDS-PAGE, silver staining (Fig. 5B), and autoradiography (Fig. 5C). A small amount of p28 is detected on the silver-stained gel (Fig. 5B); p28b is detected as a very faint spot; p28a likewise forms a light, though obviously detectable, silver-stained spot. The p28a and p28b “spots” appear as doublets in the molecular weight dimension, which is characteristic of many of our preparations of p28. Presumably p28 is sensitive to limited terminal proteolysis, even though protease inhibitors were present throughout the purification procedures. Alternatively, migration rate may be affected by its sulfhydryl oxidation state (30). The autoradiogram of the gel shows distinctly labeled protein co-migrating with both molecular weight forms of p28b (Fig. 5C). This strongly indicates that p28b is a phosphoprotein.

Further evidence showing that p28b is a phosphorylated variant comes from alkaline phosphatase treatment of purified eIF-4F containing both forms of p28. Phosphatase elim-
In each fraction, the amount of eIF-4F proteins was estimated by immunoblotting with anti-p220 antibody (Fig. 9A, right lane), because eIF-4A is not retained in the complex under these purification conditions (12).

To examine whether phosphorylation of p28 affects eIF-4F activity we have employed an in vitro assay for eIF-4F function using eIF-3 preparations which lack eIF-4F activity, based on an assay described by Grifo et al. (6). In vitro globin protein synthesis assays using this eIF-3 (plus all the other components of the translation assay) are relatively inactive unless supplemented with eIF-4F. The activity profile of the separate column fractions is shown in Fig. 9A. Both early and late eluting fractions possessed eIF-4F activity.

To correlate the activities with the concentration of eIF-4F in each fraction, the amount of eIF-4F proteins was estimated by immunoblotting with anti-p220 antibody (Fig. 9A, direct

complex, as previously has been described (33). In the 37°C cells about 30% of the p28 is in the phosphorylated spot, as described above. However, in the heat-shocked cells the amount of phosphorylated p28 has diminished to less than 5% of the total indicating that a substantial dephosphorylation has occurred. The appearance that the absolute amount of p28 is increased in heat-shocked cells is due to darker overall staining of this panel and was not detected in other analyses. Dephosphorylation was also shown by labeling cells with inorganic [32P]phosphate at 37 and 44°C, selecting p28s from the S10s by mGTP-Sepharose 4B chromatography, and autoradiography of gel-resolved proteins (Fig. 8, C and D). The data show that labeling of p28 is significantly reduced at 44°C to barely detectable levels (compare 8C and 8D). The dephosphorylation of p28 correlates with the inhibition of protein synthesis caused by heat shock. In addition, we note that in the heat shock sample there is a marked reduction in the apparent amount of eIF-4A and p220 recovered (Fig. 8, A and B), suggesting that the eIF-4F complex has been dissociated. The effect of stress on initiation factor complexes will be considered in more detail in a future study.

Translational Activity of eIF-4F (p28b) versus eIF-4F (p28a)—Phosphocellulose-purified eIF-4F is eluted from the column using a KCl gradient. Usually all fractions containing eIF-4F activity and antigens are pooled. However, when individual gradient fractions are examined by two-dimensional IEF/SDS-PAGE, we find that the first eluting fractions containing eIF-4F activity contain predominantly the more acidic phosphorylated p28b form whereas the late eluting fractions are highly enriched for p28a (Fig. 9, B and C). All fractions contain only two eIF-4F proteins, p28 and p220 (see Fig. 1A, right lane), because eIF-4A is not retained in the complex under these purification conditions (12).

To examine whether phosphorylation of p28 affects eIF-4F activity we have employed an in vitro assay for eIF-4F function using eIF-3 preparations which lack eIF-4F activity, based on an assay described by Grifo et al. (6). In vitro globin protein synthesis assays using this eIF-3 (plus all the other components of the translation assay) are relatively inactive unless supplemented with eIF-4F. The activity profile of the separate column fractions is shown in Fig. 9A. Both early and late eluting fractions possessed eIF-4F activity.

To correlate the activities with the concentration of eIF-4F in each fraction, the amount of eIF-4F proteins was estimated by immunoblotting with anti-p220 antibody (Fig. 9A, direct...
**Heat Shock Effects on eIF-4F**

**FIG. 8.** Isolation of p28 from control (37 °C) and heat-shocked (44 °C) cells. Control and heat-shocked cells were prepared and p28 isolated by m'G affinity chromatography as described under “Experimental Procedures.” Proteins eluted with 75 μM m'GTP were precipitated with acetone and analyzed by IEF/SDS-PAGE. Panels A and B, silver staining. A, control; B, heat shock. The locations of the two p28 forms are indicated in panels A and B. The locations of the other eIF-4F subunits, eIF-4A and p220, are also indicated in these panels with lines; the identification of p220 is tentative but likely correct, being based on migration characteristics which are somewhat variable (see text). Panels C and D, autoradiography. A different cell culture was used. 2 × 10⁶ cells were used in each labeling, performed as described under “Experimental Procedures.” Labeled cells were mixed with 1.2 × 10⁶ unlabeled cells (37 °C with 37 °C, 44 °C with 44 °C) and processed as described. C, control; D, heat shock. The spot spreading seen on the autoradiogram is due to the long exposure (10 days with screen) and the presence of the cold carrier protein which promotes large spots (see p28, panels A and B). Gel sectors between 20 and 40 kDa are shown.

**FIG. 9.** Two-dimensional IEF/SDS-PAGE showing separation of eIF-4F containing or lacking p28b. A, eIF-4F (lacking eIF-4A) was purified by elution from phosphocellulose by a 100–500 mM KCl gradient. Fractions were assayed for eIF-4F activity (○) as described under “Experimental Procedures.” The relative concentrations of the p220 subunit of eIF-4F were measured by immunoblotting as described in the legend to Fig. 3; densitometric scans were made and the areas (○) are plotted in arbitrary units. A portion of the elution profile is shown. Fractions possessing eIF-4F activity were analyzed by two-dimensional IEF/SDS-PAGE. B, analysis of an early eluting fraction (no. 76); C, analysis of a late eluting fraction (no. 82). Proteins were detected by silver staining (25); only a portion of the gel is shown. The heavily stained smear at the top is bovine serum albumin used as carrier. It provides a rough pH orientation marker. Silver staining intensity is not strictly proportional to protein mass in the gels, since efforts were not made to precisely equalize staining times. probes for p28 abundance are not available to us). The abundance of p220 roughly parallels the activity profile (Fig. 9A). It is clear that fractions containing predominantly p28b (Fraction 76, Fig. 9B) or p28a (Fraction 82, Fig. 9C) have very similar specific activities (Fig. 9A).

**DISCUSSION**

This report presents a characterization and quantitative analysis of the subunits of eIF-4F, using principally the technique of two-dimensional IEF/SDS-PAGE. The results complement and extend our previous analyses of eIF-2, eIF-3, eIF-4A, and eIF-4B using two-dimensional IEF/SDS-PAGE (14). Two central findings stand out. First, the smallest subunit of eIF-4F, p28, is sensitive to heat shock. The spot spreading seen on the autoradiogram is due to the long exposure (10 days with screen) and the presence of the cold carrier protein which promotes large spots (see p28, panels A and B). Gel sectors between 20 and 40 kDa are shown.
unit of eIF-4F, p28, is less abundant than any other initiation factor protein thus far quantitated. Though the p220 and eIF-4A subunits are more abundant, the level of p28 determines the maximum amount of eIF-4F which can form in cells. Second, and most intriguing, is the observation that p28 is partially phosphorylated in exponentially growing cells and that the extent of phosphorylation is substantially decreased during translational inhibition caused by heat shock. These observations suggest that the level and/or modification state of p28 and hence eIF-4F play a role in translational control.

The abundancies of the 3 major subunits of eIF-4F are quite different, spanning about a 12-fold range. For eIF-4A, p220, and p28, approximate molecules/cell are 10, 2, and 0.8 × 10^6, respectively. Thus, only a small fraction of the more abundant subunits, p220 and eIF-4A, can actually be present in eIF-4F if its stoichiometry is 1:1:1. The quantitations of p220 and p28 can only be estimated to about ±30%, but the data reported here and our estimated recoveries during protein purification strongly suggest that p220 is at least twice as abundant as p28. Though our reported p220 abundance is based on semi-quantitative gel scans, the analytical methods used are the most precise currently available and have provided us with the first tangible basis for relating p220 abundance to that of the other eIF-4F subunits and to the initiation factor proteins as a whole. p28 levels are low, and some uncertainty necessarily follows from this. In protein samples labeled with 14C-aminoacids, p28 is not detectable at an exposure time when the other identified eIF proteins clearly can be (14). Samples labeled with [³⁵S]methionine require even longer exposures than the 14C-labeled samples to detect p28, suggesting this protein is relatively deficient in methionine. Amino acid composition analysis of eIF-4E indicates it contains 1.6% methionine (30), which compares with a methionine composition in other factor proteins ranging between 1.5 and 3.3% (10). A low abundance for p28 (0.02% protein) in rabbit reticulocytes has been measured by quantitative immunoblotting (31). A low level of p28, and hence eIF-4F, supports reports suggesting that limitation in the availability of eIF-4F plays a role in regulating the ratio of α- and β-globin chains synthesized (13) and in the translational control of other mRNAs that compete least efficiently for eIF-4F binding (12).

We do not yet know if the eIF-4F subunits are present in the complex in a 1:1:1 stoichiometry. A rough estimate of the relative abundancies of the 3 subunits based on recovery and staining intensities after m7G-affinity column chromatography suggests p28 is severalfold more abundant in these preparations than either p220 or eIF-4A (roughly equi-abundant). A similar composition for eIF-4F can be inferred from stained patterns of eIF-4F reported by Tahara et al. (8) and Lee et al. (32). Yet, p28 is the least abundant subunit in total lysates. Its enrichment in affinity-purified eIF-4F presumably occurs because p28 is the only subunit directly bound to the column whereas eIF-4A and p220 binding presumably occur via their intermolecular association with p28. The enrichment for p28 could be due to complex disruption before or during purification; or perhaps, multiple copies of p28 may be present in each eIF-4F complex. We must, however, entertain the possibility that only a fraction of the p28 forms eIF-4F complexes and hence that the number of molecules of eIF-4F complex is substantially less than the number of p28 molecules. Evidence suggesting that p28 occurs in free non-complex form (33) provides further reason to believe that the ratio of eIF-4F to ribosomes is likely <0.1.

Since eIF-4A and p220 are more abundant than p28, they may possess additional functions. eIF-4A has been extensively characterized as a single protein that promotes mRNA binding to the 43 S preinitiation complex (2, 3). Thus, an independent role for eIF-4A appears substantiated in this case. eIF-4A, present in about 10^7 copies/cell (14), is the most abundant initiation factor thus far quantitated, exceeding the number of mRNA molecules and ribosomes by about 20- and 3-fold, respectively.

**Covalent Modification of p28**—Two p28 spots are detected in eIF-4F purified from exponentially growing HeLa cells. eIF-4F purified from rabbit reticulocytes also contains two p28 spots (30, 31). The more acidic variant labels with phosphate and is lost upon phosphatase treatment, indicating that it is phosphorylated p28. Rychlik et al. (30) have recently also determined that p28 is a phosphoprotein. We detect only two forms of p28 in HeLa cells, whereas they observe as many as five variant forms in human and rabbit erythrocytes. The more basic form of p28 fails to label with phosphate (40-min pulse) and is not displaced on gels by phosphatase. We believe that the basic form of p28 represents the unmodified protein, though this has not been proven. Since phosphorylation frequently functions as a modulator of protein activity, the presence of a phosphorylated p28 variant immediately suggests the possibility that eIF-4F-dependent mRNA binding to the translational machinery is regulated.

Heat shock of HeLa cells produces a rapid inhibition of protein synthesis. The p28 subunit of eIF-4F is concurrently dephosphorylated, and the three-protein eIF-4F complex also appears to disaggregate, perhaps in response to dephosphorylation. Pannier et al. (34) have reported that eIF-4F activity is depressed in Ehrlich ascites cells, and we have observed a similar inhibition in HeLa eIF-4F activity in reconstituted in vitro protein synthesis assays (21). The changes in eIF-4F structure reported here provide the first evidence of molecular changes occurring during heat shock (neither eIF-4A nor p220 is altered in detectable ways (21)). eIF-4E dephosphorylation may constitute an exciting new locus of translational control.

The regulated phosphorylation of p28 represents the fourth example of a phosphorylation change affecting a protein of the translational machinery that is correlated with translational regulation, the others being eIF-2α, eIF-4B, and ribosomal protein S6. eIF-4B and S6 are dephosphorylated during heat shock, as well as during other translational repressions (21, 22, 35, 36). eIF-4B and S6 are both phosphorylated in response to phorbol myristate acetate (37) and in vitro by the same purified protein kinase (4).

The effect of phosphorylation on ribosomal protein S6, which is implicated in mRNA binding (38), has been extensively investigated for almost a decade (35, 39, 40). Only meager solid evidence has accrued to suggest that phosphorylation has any influence whatsoever on activity (40-44), very likely due to the difficulty of reproducing in vitro translational controls in relatively inefficient in vitro systems. Phosphorylation of eIF-2α correlates with translational repression in reticulocyte lysates due either to hemin deficiency or treatment with double-stranded RNA (45). Despite extensive subsequent efforts to identify an in vitro lesion in activity, none was detected until recently. Now a consistent elegant explanation has finally emerged wherein eIF-2 phosphorylation inhibits the recycling aspect of eIF-2 function (18, 20, 46). The initial failure of the in vitro systems to detect this lesion directly sprang from their relative inefficiency.

We have begun an investigation of whether p28 phosphorylation alters eIF-4F activity. The results to date are negative. eIF-4F fractions either lacking or containing the phosphorylated p28 variant show similar activities in promoting the synthesis of globin in a reconstituted in vitro assay system.
While disappointing, this result in no way constitutes a conclusive test due to the inadequacies of the in vitro tests. The example of eIF-2, cited above, is a case in point. Our assays of eIF-4F phosphorylation versus activity may suffer from a similar limitation. An alternate possibility is that active p28 is a contaminant of some of the purified components in the assay and can exchange into the eIF-4F complex to render it active in all the assays evaluated. Efforts will be made to evaluate these possibilities using more active or different assay systems.

A final observation also suggests that phosphorylated p28b and nonphosphorylated p28a are both active in cap recognition. The recovery of p28 forms from phosphocellulose chromatography, not involving an m7G affinity step, can be compared with yields from m7G affinity chromatography of an 4F complex or with eIF-4F's associations with other components of the translational machinery.

We will seek to address is whether phosphorylation of p28 alters its intermolecular interactions, either within the eIF-4F complex or with other components of the translational machinery.

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