Heat Shock Effects on Phosphorylation of Protein Synthesis Initiation Factor Proteins eIF-4E and eIF-2α in *Drosophila*†

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ABSTRACT: Heat shock of mammalian cells causes changes in initiation factor phosphorylation that likely contribute to or cause the translational reprogramming characteristic of heat shock. In these investigations we have carried out a parallel analysis of *Drosophila*, focusing on eIF-4E and eIF-2α. eIF-4E plus associated proteins was purified from lysates by m^{7}GTP-Sepharose chromatography. A minor fraction (<10%) of eIF-4E is phosphorylated under normal growth conditions, and phosphorylation decreases during heat shock. *Drosophila* eIF-2α has been identified by *in vitro* translation of T7 RNA polymerase-transcribed mRNA, and immunoblotting with anti-*Drosophila* eIF-2α antiserum. ^{32}P-labeling analysis (unfractionated cell lysates and immunoprecipitates) detects phosphorylated eIF-2α, whose amount increases approximately 2−3-fold upon heat shock. Immunoblotting analysis of two-dimensional gel-resolved proteins to determine the mass fraction of eIF-2α phosphorylated detects a single eIF-2α spot in both normal temperature and heat shocked cells, indicating less than 5% phosphorylation after and before heat shock. Staining quantification is consistent with this low prevalence. A major phosphoprotein which copurifies with eIF-4E on m^{7}GTP-Sepharose shows decreased overall phosphorylation and decreased association with eIF-4E following heat shock. Several distinctive characteristics of this phosphoprotein suggest it is *Drosophila* eIF-4B.

Raising the temperature of cells or organisms several degrees above their optimal growth temperature, commonly referred to as heat shock, causes a battery of metabolic changes termed the "heat shock response" (for reviews, see Lindquist (1986) and Morimoto et al. (1990)). Major changes in gene expression occur at several steps in the pathway. Transcription of most genes is repressed, as is their splicing and transport. Concurrently, a small set of genes, the heat shock genes, are transcribed very actively. They escape splicing inhibition by virtue of lacking introns. In the cytoplasm, translation of virtually all non-heat shock mRNAs is severely repressed, while at the same time the heat shock mRNAs are very efficiently translated. The pre-existing mRNAs are neither degraded nor substantially modified, as demonstrated by *in vitro* translation of total mRNAs extracted from heat shocked cells (Lindquist, 1981). This remarkable discriminatory translation has been the subject of extensive investigation, but the molecular basis for the discrimination remains unclear.

Many situations where translational discrimination occurs are known—for example, during oogenesis and early development, and following amino acid limitation (Richter et al., 1990; Hinnebusch, 1990). Perhaps the best examples occur during viral infections, where host cell translation is repressed while viral mRNAs translate efficiently (Schneider & Shenk, 1987). This precisely parallels what occurs during heat shock. Investigations of the mechanisms of preferential viral translation have uncovered a surprising common pattern: alterations in initiation factor activities are the molecular basis. Specifically, alterations in eIF-2α[1] phosphorylation, and in eIF-4E phosphorylation and activity, lie at the heart of translational control mediated by adenovirus (O'Malley et al., 1989), poliovirus (Etchison et al., 1982; Jackson et al., 1991), rhinovirus (Etchison & Fout, 1985), foot-and-mouth-disease virus (Lloyd et al., 1988), and likely influenza virus (Katze et al., 1986), reovirus (Samuel et al., 1984), mengo virus (DeStefano et al., 1990), and vesicular stomatitis virus (Dratewka-Kos et al., 1984). Several of the best studied nonviral translational control systems also appear to be mediated via initiation factor activity changes. Activation of translation at fertilization in echinoderms likely involves eIF-2 (Winkler et al., 1985; Colin et al., 1987), and perhaps eIF-4F (Huang et al., 1987), activity increases; and in yeast general control based on amino acid levels is determined by eIF-2α phosphorylation and eIF-2 activity (Hinnebusch, 1990).

This raises the obvious question: are initiation factor modifications the molecular basis for preferential translation during heat shock? Because preferential translation of heat

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[1] Abbreviations: HSP, heat shock protein; eIF, eukaryotic protein synthesis initiation factor; 2DGE and IEF/SDS-PAGE, two-dimensional gel electrophoresis using isoelectric focusing in the first dimension, SDS−polyacrylamide gel electrophoresis in the second; 1DGE, one-dimensional SDS-PAGE; eIF-4E(P) and -(PP), phosphorylated forms of eIF-4E. The nomenclature for eIF-4 initiation factor proteins has recently been revised. The protein referred to in this text as eIF-4E has been renamed eIF-4α in the revised nomenclature.

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shock protein (HSP) mRNAs is dramatically evident in *Drosophila*, this has become a model investigatory system. The study of translational control in *Drosophila* has been impeded by the lack of characterization of their initiation factors. In these investigations we have taken advantage of the recent cloning of *Drosophila* eIF-2α and affinity chromatography media for eIF-4E to characterize changes in these factors following heat shock. eIF-2α and eIF-4E are the principal translational control proteins identified to date in organisms ranging from yeast to sea urchins to mammals.

MATERIALS AND METHODS

Materials. m^{7}GTP-Sepharose and ampholines were purchased from Pharmacia. Sepharose CL-6B (a Pharmacia product), alkaline phosphatase, acid phosphatase, RNase A, T1, benzamidine, aprotinin, and amphotericin/penicillin/streptomycin antibiotic mix were purchased from Sigma Biochemical Corp. Acrylamide and Triton X-100 were purchased from Serva. Urea was purchased from Fisher (Biotechnology grade). *Drosophila* tissue culture media (Schneider's formulation), Dulbecco's MEM lacking {methionine, leucine, and lysine} or phosphate (powdered form), and fetal calf serum were purchased as liquid from BRL/GIBCO. ECL Western blotting kit (including horseradish peroxidase-conjugated antibodies), ECL detection reagents, and HYPERfilm were purchased from Amersham. Immobilon P PVDF membrane was purchased from Millipore. [^{35}S]Methionine (translation grade and protein express) and [^{32}P]inorganic phosphate were purchased from New England Nuclear. Pansorbin was purchased from CalBiochem.

Preparation of S2 Cell Protein Lysates. Drosophila Schneider S2 cells were grown in 10% fetal calf serum at 22−25 °C (lab benchtop, room temperature) in either T150 culture flasks (propagation and some experimental) (Corning) or 150 mm petri dishes (for experimental brief-growth conditions) to a density of (6−10) × 10^6 cells/mL. Heat stressed cells were incubated at 37 °C for 30 min unless otherwise indicated. Incubation was carried out in a circulator-driven water bath, with temperature monitored by a digital thermometer to ±0.1 °C. The water bath contained a stirrer underneath, and cells were incubated in the presence of a stir bar or stir flea to maintain agitation during the course of the experiment (note: in initial control analyses constant agitation was found to promote cell metabolic activity). In all the comparative lysate preparations described below and in the Results section, equal cell numbers (and amounts of protein) were compared in non-heat shocked and heat shocked lysates. Equivalency was determined by Bio-Rad assay of protein and/or quantitative staining of gel-resolved proteins.

(i) Lysates for Column Affinity Chromatography. About 75 mL of cells was pooled for most column purification preparations. Control or heat stressed cells were poured over crushed, loosely-packed ice made from wash buffer (50 mM KCl, 15 mM $MgSO_4$, 4 mM $CaCl_2$, 3 mM K_2HPO_4, 10 mM glucose, 20 mM HEPES, pH 6.8, Phenol Red to tint) and then washed thrice by centrifugation. The final cell pellets were resuspended in 5 mL of 4 °C m^{7}GTP column buffer (100 mM KCl, 20 mM HEPES, pH 7.6, 7 mM β-mercaptoethanol, 0.2 mM EDTA, 10% glycerol) to which the following components were added for lysis: Triton X-100 to 0.1%, aprotinin to 0.3 TIU/mL, benzamidine to 10 mM, leupeptin to 10 μg/mL, SBTI to 100 μg/mL, and PMSF to 2 mM. Some lysates also were prepared in the presence of 50 mM β-glycerophosphate, 50 mM NaF, and 100 μM $NaVO_4$ to block phosphatase activity, but phosphorylation amount was not increased by their inclusion. Cell suspensions were homogenized with 7−10 strokes in a stainless steel Dounce homogenizer and then centrifuged for 15 min at 12 000 rpm, 4 °C (SS34 rotor, Sorvall RC-5B centrifuge). The supernatant was recovered and either directly applied to m^{7}GTP-Sepharose or stored at −80 °C until analysis.

(ii) Lysates for [^{32}P]Inorganic Phosphate Labeling Analysis. (a) Pulse Labeling (Figure 5). Cells (0.5 mL per condition) were centrifuged in a clinical centrifuge (IEC) at speed 4 for 3 min. The normal medium was removed and replaced with Dulbecco's MEM without phosphate containing 20 mM HEPES, pH 6.8, and 10% fetal calf serum. Cells were resuspended by gentle Pasteur pipetting and then transferred to 13 × 100 mL glass test tubes. Cell suspensions were manually shaken throughout the experiment. Following 15 min adaptation, heat shock cells were transferred to a 37 °C water bath. After a further 15 min, 1 mCi of [^{32}P]-orthophosphate was added to each, and incubation continued another 15 min. Cells were mixed with 5 mL of 4 °C wash mix and washed (as described above). Cells were lysed in 0.1 mL of m^{7}GTP column buffer lysis buffer (as described above) or LSB/TXC buffer (25 mM HEPES pH 7.2, 65 mM KOAc, 0.5 mM MgOAc, 0.5% Triton X-100, with protease inhibitors as described above), using only repetitive pipetting and vigorous vortexing (no homogenization). Nuclei and debris were removed by 5 min centrifugation in a microfuge, and the lysates were stored or used immediately. These labeling conditions routinely yield a protein concentration of ~2 μg/μL, and radioactive labeling of 1750 cpm/μL (total TCA precipitable counts). For lysates which were to be used exclusively for IEF/SDS-PAGE analyses, prior to freezing $^1/_{10}$ volume of RNase cocktail (RNase A 1 mg/mL, RNase T1 2500 units/mL) was added and incubated 1−5 min on ice, followed by 1 mg/μL solid urea. The urea was dissolved by vigorous vortexing. RNase treatment has recently been essential for proper protein focusing.

(b) "Steady-State" Labeling (Figures 2 and 7). Cells (5 mL) were collected by centrifugation as described above, then transferred into an equal volume of Dulbecco's MEM without phosphate containing 10% fetal calf serum, and divided into two equal cultures. Following 15 min adaptation, 2−5 mCi of [^{32}P]orthophosphate was added to each, and incubation continued for 3−5 h. During the last 30 min of labeling, one culture (heat shock) was transferred to 37 °C (water bath). Cells were washed as described above and then lysed in 0.5 mL of m^{7}GTP column lysis buffer described above, using only repetitive pipetting and vigorous vortexing (no homogenization). Nuclei and debris were removed by 5 min centrifugation in a microfuge, and the lysates were stored or used immediately for eIF-4E purification. These labeling conditions yield approximately 350 000 cpm/μL (by TCA).

(iii) Degradation of eIF-4E. In most of our column analyses two prominent 28−34 kDa spots are detected. Several lines of evidence suggest that they are degradation products (rather than phospho variants, for example). They are labeled as *deg1* and *deg2* in Figure 1 to indicate this fact. The evidence includes the following: (i) They are of lower molecular weight. (ii) Their abundance is not affected by treatment with phosphatases. (iii) The intensity of the

two *deg* spots varies in equivalent cell state samples, ranging from relatively minimal representation in some lysates to virtual complete conversion of intact eIF-4E to the degradation products in other runs. Furthermore, there was a clear precursor−product relationship, with the amount of authentic eIF-4E being inversely proportional to the amount of the *deg* forms. (iv) More extensive degradation occurred when the initial steps in the purification were carried out slowly, or when exogenous protease was inadvertently introduced onto the m^{7}GTP-Sepharose column (as a contaminant in phosphatase). (v) The extent of "degradation products" was usually less in heat shocked samples. We interpret this to result from an inhibition of proteolytic enzymes by heat shock, as has been observed for other proteins in *Drosophila* (Duncan & Lefrere, 1994; Li & Duncan, submitted). Numerous efforts were made to prevent this degradation, to no avail. Complex protease inhibitor cocktails had no detectable effect, as did varying salt or inclusion of various chelators. The basic extraction buffer contains a fairly extensive protease inhibitor cocktail. This failure may be due in part to the necessity of working with relatively large cell numbers/volumes to allow recovery of significant amounts of eIF-4E.

(iv) Lysis Buffer. In the course of these studies we have made the surprising observation that the apparent abundance of several of the proteins discussed herein is quite sensitive to the extraction buffer. To be specific, eIF-2α was most efficiently extracted with TXC/LSB, whereas the phosphoprotein tentatively identified as "eIF-4B" was virtually undetectable in TXC/LSB extracts. To visualize eIF-4B, we have used a hot SDS extraction buffer (0.3% SDS, 1% β-mercaptoethanol, 50 mM Tris, pH 8.0; following lysis, samples are treated with 50 μg/mL DNase and RNase in 5 mM $MgCl_2$).

m^{7}GTP-Sepharose Column Chromatography. All purifications described in this report were carried out using 1.0 mL (swollen) resin in a 10 mL polypropylene disposable column (Bio-Rad Laboratories) at 4 °C. Several independent columns were used. The effluent tubing was routed through a peristaltic pump set at ~0.5 mL/min. Samples were bound to m^{7}GTP-Sepharose by batch incubation, with rocking, for 2 h at 4 °C. Following binding the slurry was loaded onto the column, allowed to flow through by gravity until lysate had reached the meniscus, and washed 2× with 2 mL of m^{7}GTP column buffer (see above). Then 5 mL of buffer was added, the pump tubing was attached to the bottom and buffer resevoir tubing attached to the top, and washing was continued for 3−5 h (90−150 mL of buffer). Analysis of column fractions during this washing (1DGE and silver staining) demonstrated that there was undetectable eluting protein after 90 mL washing. The column was next washed with 10 mL of 100 μM GDP in m^{7}GTP column buffer and eluted with 10 mL of 75−100 μM m^{7}GTP in m^{7}GTP column buffer. The first 2 mL of eluate was discarded; the next 5 mL was combined in a 15 mL Corex tube to which 500 μL of 3 M Na OAc and 10 mL of 100% EtOH were added, mixed, and incubated at −20 °C overnight. Precipitated proteins were recovered by centrifugation at 12 000 rpm, 4 °C, 30 min (HB-4 rotor). The ethanolic supernatant was poured off, inverted for 5 min, and wiped dry, and proteins were resuspended in 200 μL of AMPHOLYSE (9.8 M urea, 2% Triton X-100, 0.5% 3.5−10 ampholines, and 0.1% β-mercaptoethanol). For the analysis of ^{32}P-labeled samples, the radioactive lysate was mixed with cold carrier lysate from 75 mL of cell culture (combined volume 5.5 mL) prior to batch incubation.

Pre-Affinity Chromatography Purifications. (i) Sepharose CL-6B. As an alternative to sucrose density gradient centrifugation, 100 mL cell cultures were lysed in 2.5 mL of m^{7}GTP-Sepharose column buffer plus protease inhibitors and 0.1% Triton X-100 (see above), clarified, and chromatographed over a Sepharose CL-6B column (28 × 1 cm) at 4 °C in the same buffer. Samples were collected at ~0.3 mL/min, with peristaltic pump. Aliquots from fractions were visualized by 1DGE, and the first 4 and last 4 fractions containing significant Coomassie staining protein were combined. The Coomassie staining pattern followed the predicted profile, with smaller proteins eluting in later fractions. The principal exception was that in the excluded material a range of protein sizes including some quite small species were detected.

(ii) Fractionation by High Speed Centrifugation in the TL-100 Centrifuge. Cell cultures were lysed in m^{7}GTP column buffer plus 0.1% Triton X-100 and protease and phosphatase inhibitors and clarified by low speed centrifugation. Each lysate was split into four tubes and centrifuged in the TL-100 benchtop ultracentrifuge (Beckman Instruments) for 30 min at 100 000 rpm. The supernatant was removed, brought to 500 mM KCl, incubated at 4 °C for 15 min, dialyzed 2 h against 2 L of m^{7}GTP column buffer, and applied to the m^{7}GTP-Sepharose column. The ribosome pellets were resuspended in a total volume of 1000 μL of 500 mM KCl/m^{7}GTP column buffer by exhaustive pipetting, incubated on ice for 30 min, and then recentrifuged in the TL100 as above. The supernatant ("high salt wash") was removed, diluted with 0 M KCl m^{7}GTP column buffer to 0.1 M final K^+, and applied to the m^{7}GTP-Sepharose column. The pellet ("washed ribosomes") was resuspended as above in 5 mL of m^{7}GTP column buffer and applied to the m^{7}GTP-Sepharose column.

Two-Dimensional IEF/SDS-PAGE. First dimension gels contained 9.8 M urea, 2% Triton X-100, 3.45% acrylamides (17.2:1 acrylamide:bis(acrylamide)), 3.75% 5−7, and 1.25% 3.5−10 ampholines. They were routinely poured to a length of ~16 cm in standard 4 mm outer diameter glass tubing. Gels were overlayed with AMPHOLYSE and pre-run for 400 V·h (usually 1 h at 400 V) using degassed upper (0.2% NaOH) and lower (0.2% phosphoric acid) tank buffers. The overlay was removed, and sample was applied (usually ~70 μL) and overlaid with 10−20 μL of 80% AMPHOLYSE containing bromphenol blue tracking dye. The first dimension gels were routinely run 12 h × 1000 V, or 16 h × 800 V (by which time the tracking dye usually forms a thin yellow band at the bottom of the gel). First dimension gels were extruded with air pressure into 5 mL of equilibration buffer (63 mM Tris, pH 6.8, 2.4% SDS, 10.5% glycerol; β-mercaptoethanol, which is sometimes added at 0.05%, was omitted from most equilibrations described herein because it produces silver staining aberrations) in a 13 × 100 mm glass tube and rocked for 10−15 min. The liquid was pipetted off, and the first dimension gel was transferred to the second dimension gel, which is a standard Laemmli-formulation SDS gel (10.5%, 36.5:1 Acr:bis-Acr) with a 1 cm stacking gel extending to the bottom of the bevel in the beveled plate. The first dimension gel was affixed to the gel with 1% agarose/equilibration buffer, and second dimension electrophoresis was carried out at 20 mA/gel, until the tracking dye reached the gel bottom. Gels were fixed in fixing solution (50% methanol, 10% acetic acid).

Immunoblotting. Gels were transferred to ImmobilonP at 300 mA for 5 h in a liquid transfer tank (Hoeffer Scientific Corp.) using Laemmli-style SDS buffer with SDS reduced to 0.01% and 10% methanol added. Gels were stained with 0.1% amido Schwartz in destaining solution (80% methanol, 2% acetic acid) for ~5 min, destained with three rinses of destaining solution, and preblocked with 5% nonfat milk (Carnation) in Tris-buffered saline. Subsequent steps followed guidelines provided in the ECL Western blotting kit (chemiluminescence detection using HYPERfilm). First antibody was used at 1:5000 dilution for 2 h with rocking at room temperature in small plastic boxes. Second antibody was used at 1:30000 dilution for 1 h.

In Vitro Transcription/Translation. Transcription reactions contained, in a 10 μL volume, 200−400 ng of linearized DNA or PCR template, 400 μM each ATP, CTP, and UTP (Phamacia), 80 μM GTP, 800 μM m^7GpppG, 10 mM DTT, 15 nCi of [^{32}P]ATP (3000 Ci/mmol; ICN), 40 mM Tris-HCl, pH 7.5, 10 mM NaCl, 6 mM $MgCl_2$, 2 mM spermidine, 4 units of RNase inhibitor (Amersham), and 4 units of T7 polymerase (Promega). Reactions were incubated at 37 °C for 2 h and then digested for 15 min, 37 °C, with 100 μg/mL deoxyribonuclease I (Worthington Biochemicals). RNAs were extracted once with phenol/chloroform/isoamyl alcohol (25:24:1) and further purified by Sephadex G50 spin column chromatography (Sambrook et al., 1989). RNAs were brought to 2.5 M ammonium acetate and precipitated with 2.5 volumes of ethanol overnight at −20 °C. Samples were centrifuged in a microfuge for 30 min at 4 °C. The ethanolic supernatant was removed, 75% ethanol added, and the pellet agitated and then recentrifuged for 5 min in a microfuge. The ethanol was removed by pipetting, the residual ethanol was removed by vacuum drying, and the RNA was resuspended in autoclaved, glass distilled water. Yield and concentration were determined by precipitation in 10% TCA in the presence of 50 ng of carrier DNA. A specific activity of 6×10^3 cpm/μg is produced with these conditions. The RNA integrity was analyzed by 8 M urea-PAGE (standard procedures as described in Sambrook et al. (1989)). mRNA-dependent rabbit reticulocyte lysate (RRL) was prepared by adding 20 μM hemin, 50 mg/mL creatine phosphate kinase, and 1 mM $CaCl_2$ to the RRL and then treating with 90 units/mL micrococcal nuclease for 20 min at room temperature (22−24 °C). The digestion was stopped with 2 mM EGTA and supplemented with 135 μg/mL tRNA. Translation reactions contained, per 10 μL, 4 μL of nuclease-treated lysate, 100 mM KCl, 0.5 mM $MgCl_2$, 10 μM creatine phosphate, 100 μM each unlabeled amino acid (minus methionine), 50 μCi of [^{35}S]methionine (translation grade, New England Nuclear), and 50 ng of transcripts. After 30 min incubation at 30 °C, a sample was diluted to 1× Laemmli sample loading buffer and analyzed by 10.5% SDS-PAGE. Gels were dried and visualized by autoradiography at room temperature without screens.

Immunoprecipitation. Cells were pulse-labeled with [^{32}P]-orthophosphate for 15 min (see above). A mix containing 50 μL of lysate, 25 μL of eIF-2α antisera, and 125 μL of RIPA buffer (10 mM HEPES, pH 7.2, 70 mM NaCl, 0.1% Triton X-100, 1% sodium desoxycholate, 0.1% SDS, 0.05% β-mercaptoethanol) was assembled and incubated with rocking for 8−12 h at 4 °C. A parallel incubation contained 1 mL of Pansorbin, 500 μL of *Drosophila* lysate (~1 mg), and 2.5 mL of RIPA. After the binding incubation, the Pansorbin was washed 3× with 1 mL of RIPA by repeated microcentrifugation and pipetting, and resuspended in 1 mL of RIPA. It was added to the immunoprecipitation tubes at 10:1 Pansorbin:neat antiserum (v/v). Following incubation for 1 h on ice (with periodic mixing), the mixture was pelleted by microcentrifugation for 1 min, resuspended in 1 mL of RIPA, and washed three times. The final pellet was resuspended in 100 μL of 2× Laemmli-formulation SDS sample buffer, heated to ~95 °C for 4 min, and re-microcentrifuged as above. The supernatant was removed and the elution repeated. Supernatants were combined, and an aliquot was removed for immunoblot analysis. To the remainder was added ~10 μg of unlabeled *Drosophila* lysate (to provide marker proteins). Next, 40 μL of 3 M NaOAc, 260 μL of m^7GTP column buffer, 10 μL of 10% Triton X-100, and 1000 μL of EtOH were added and incubated at −20 °C overnight. The precipitate was recovered by microcentrifugation for 30 min at 4 °C, resuspended in 80 μL of AMPHOLYSE, and analyzed by IEF/SDS-PAGE, silver staining, and autoradiography (2 week exposure, −80 °C, with screens). Initial experiments analyzed by 1DGE revealed no specific bands at ~40 kDa on short exposures, and too much general background to detect faint bands after long exposures.

Silver Staining. Gels to be silver-stained were incubated in fixing solution overnight (see above) and then in 10% glutaraldehyde for 30−60 min. Next they were washed 6−10 times with water over 5 h. Ammoniacal silver solution was prepared: 800 mg of silver nitrate was dissolved in 4 mL of water and added dropwise to a solution of 20 mL of 0.36% NaOH + 1.4 mL of NH_4OH, with stirring. H_2O (75 mL) was added, and the gel was incubated for 20−30 min in the silver solution. The gel was washed twice, 5 min each, with water, and then transferred into a glass baking dish containing 0.005% citric acid and 0.05% neat (37%) formaldehyde. Staining was continued to the desired darkness, at which point the gel was removed and placed in fixing solution again (stop bath). After overnight incubation, fixing solution was removed, and the gel was incubated in 50% concentrated Kodak photographic fixer for 1 min (silver destain), washed 2 times over 1 min with H_2O, incubated in 5× concentration hypoclear (Kodak) (silver destaining stop bath) for 30 min, in H_2O for 15 min, and finally dried. The incubation intervals in the destaining procedure were sometimes varied empirically to best display the spot or banding pattern.

Protein Kinase C Treatment in Vitro. m^7GTP-Sepharose-purified proteins (100 ng) were incubated with protein kinase C (25 ng; Promega) and 10 μCi of [γ-^{32}P]ATP in a final volume of 12 μL. The incubation buffer was 20 mM HEPES (pH 7.4), 0.34 mM EDTA, 0.34 mM EGTA, 1.67 mM $CaCl_2$, and 10 mM $MgCl_2$, 1 mM DTT. Reactions were carried out for 30 min at 37 °C.

RESULTS

Relatively little is known about the physical characteristics and regulatory influence of protein synthesis initiation factors in *Drosophila*, relative to mammalian cells. *Drosophila* eIF-2 has been purified by Sierra and colleagues (Mateu & Sierra, 1987a,b). It appears to be a three protein complex, as in mammalian cells. Guanine exchange factor (GEF or eIF-2B) activity seems required for recycling (catalytic activity), but phosphorylation-dependent inhibition of GEF activity could not be observed. *Drosophila* eIF-4E has been

purified by Sierra and colleagues as well (Maroto & Sierra, 1989) using m^{7}GTP-Sepharose affinity chromatography. It is reported to be a three protein complex, again mirroring its composition in mammalian cells. Zapata et al. (1991) suggest that subunit dissociation during heat shock may block eIF-4F activity and account for preferential translation. We have further characterized eIF-2 and eIF-4E using novel and parallel strategies, focusing on alterations caused by heat shock. For eIF-2, we have specifically focused on the α subunit and its phosphorylation using specific antisera, since this is the principal regulatory subunit of this factor. For eIF-4E, purification has been performed, followed by a characterization of its phosphorylation state. The analysis of eIF-4E is presented in the first section, followed by eIF-2α characterization, followed by an analysis of an eIF-4E-associated phosphoprotein with characteristics suggesting it is *Drosophila* eIF-4B.

Purification and Analysis of eIF-4E. eIF-4E (and associated proteins) was purified from control, nonheated, and heat shocked cells by chromatography of unfractionated *Drosophila* cell lysates on m^{7}GTP-Sepharose. Following extensive washing with loading buffer and GDP/loading buffer, proteins were eluted with m^{7}GTP, concentrated by ethanol precipitation, and analyzed by one-dimensional SDS-PAGE and two-dimensional IEF/SDS-PAGE. The major band purified from control and heat shocked cells migrates at ~35 kDa (Figure 1, panel A). This corresponds to the molecular weight of the *Drosophila* eIF-4E identified by Maroto and Sierra (1989) and is substantially larger than mammalian or yeast eIF-4E (~25−28 kDa). The amount of eIF-4E recovered from heat shocked cells is similar to non-heated cells when equivalent total protein is applied to the column (Figure 1, panel A, lane 1 vs 2; and panels B vs C), indicating there is little if any overall reduction in binding activity due to heat shock (in some purifications a modest decrease, never exceeding 50%, was observed). The gel in panel A was developed in silver for a prolonged interval to visualize any coselected proteins. The most prominent have molecular masses of ~43, 48, 53−55, ~70, and 83 kDa, and their identities are described in the Discussion section. Using shorter development times, only eIF-4E was clearly detectable. The eIF-4E-associated proteins were recovered in approximately equal amounts from the heat shocked and control samples in this paired set (compare lanes 1 and 2). A decrease in the associated proteins was observed in other comparative analyses (data not shown; a modest decrease is seen by two-dimensional analysis: panels B and C). The extent of dissociation of eIF-4E-associated proteins we observe is less than reported by Zapata et al. (1991). The open arrowheads in panel A mark unavoidable degradation products of eIF-4E (these are detected more clearly as spots, labeled *deg1* and *deg2*, in panel B). Various characteristics indicate that they are degradation products of eIF-4E, including a direct inverse correlation between the amount of intact eIF-4E remaining and the amount of the degradation forms (see Materials and Methods and text below for additional bases and interpretation).

eIF-4E fractions from control (panel B) and heat shocked (panel C) cells were also examined by two-dimensional IEF/SDS-PAGE and silver staining to assess the covalent modification status of eIF-4E, and the effects of heat shock upon it. A representative comparison is shown in Figure 1; the quantified densitometric results of 8 independent paired analyses may be summarized as follows: in most (5/8) nonheated, purified samples there was a single eIF-4E spot at about p*I* 6.2 (very similar to mammalian eIF-4E's p*I*), with virtually no acidic-focusing variant (less than 5% of the total eIF-4E; discussed below). In panel B, two minor acidic forms of eIF-4E can be observed (labeled 4E(P) and 4E(PP)). The 4E(P) was detected in 3/8 non-heat shocked analyses, and the 4E(PP) form in 2/8, at an abundance of >5% of the total eIF-4E. In these 3 samples containing modest amounts of the phosphorylated forms, 5%, 8%, and 12% of the total eIF-4E was in the summed (P) and (PP) forms. Proof that 4(E)P is phosphorylated eIF-4E is presented in the next section. Careful scrutiny of the heat shock sample (panel C, on which an equal amount of cell lysate was loaded) shows that the most phosphorylated form is reduced in amount (approximately 2-fold, based on densitometric quantification). In 5/8 analyses, the most phosphorylated form was reduced by 2−6-fold (including samples where eIF-4E(P) represented less than 5% of the total eIF-4E); in 2/8 analyses phosphorylated forms were below the level of detection in both the nonheated or heat shocked purifed preparations; and in 1/8 the amount of heat shock eIF-4E(P) was 1% and undetectable in the non-heat shocked sample. This pattern of heat-induced dephosphorylation of eIF-4E in *Drosophila* parallels results obtained in mammalian cells (Duncan et al., 1987; Lamphear & Panniers, 1990, 1991) but on a much reduced scale. Two putative degradation products of eIF-4E can be readily detected on the 2DGE analyses. The initial impression that these are the phosphorylated forms of eIF-4E is inconsistent with their smaller apparent size. Furthermore, they are not affected by alkaline or acid phosphatase treatments and were most abundant in lysates retaining little intact eIF-4E. A modest loss of associated proteins can be observed in the heat shock purified sample (comparing panels B and C).

To further characterize the phosphorylation state of eIF-4E, and its possible influence on regulating protein−protein interactions (Joshi-Barve et al., 1990; Lamphear & Panniers, 1990, 1991), cell lysates were subjected to preparatory fractionations prior to affinity chromatography. Two approaches were investigated: (i) separation into ribosome-associated vs free eIF-4E by a high speed centrifugal pelleting; and (ii) selection of multiprotein aggregates from monomeric eIF-4E by Sepharose CL-6B size exclusion chromatography. In both cases, there was no difference between multimeric and free eIF-4E, in the extent of phosphorylation (all were very low as depicted in Figure 1), indicating that the phosphorylated eIF-4E is not preferentially recovered in ribosome-bound or multiprotein complexed forms.

The failure to detect a relatively abundant acidic (i.e., phosphorylated) form of eIF-4E in normal, exponentially growing cells was surprising, since in growing mammalian cells ~50% of the eIF-4E is phosphorylated and required for factor activity. However, it is unlikely that the low phosphorylation state of eIF-4E results from an active phosphatase artifactually reducing the detected level. Substantial amounts of phosphatase inhibitors were included during eIF-4E purification. Likewise, other proteins known to contain significant amounts of phosphate in nonheated cells were shown to be in their predicted phosphorylated state in the same lysate with the very low eIF-4E phosphorylation levels—for example, the phosphoprotein tentatively identified as eIF-4B (identified in Figures 2 and 6) and GRP78. Other reasons are discussed below.

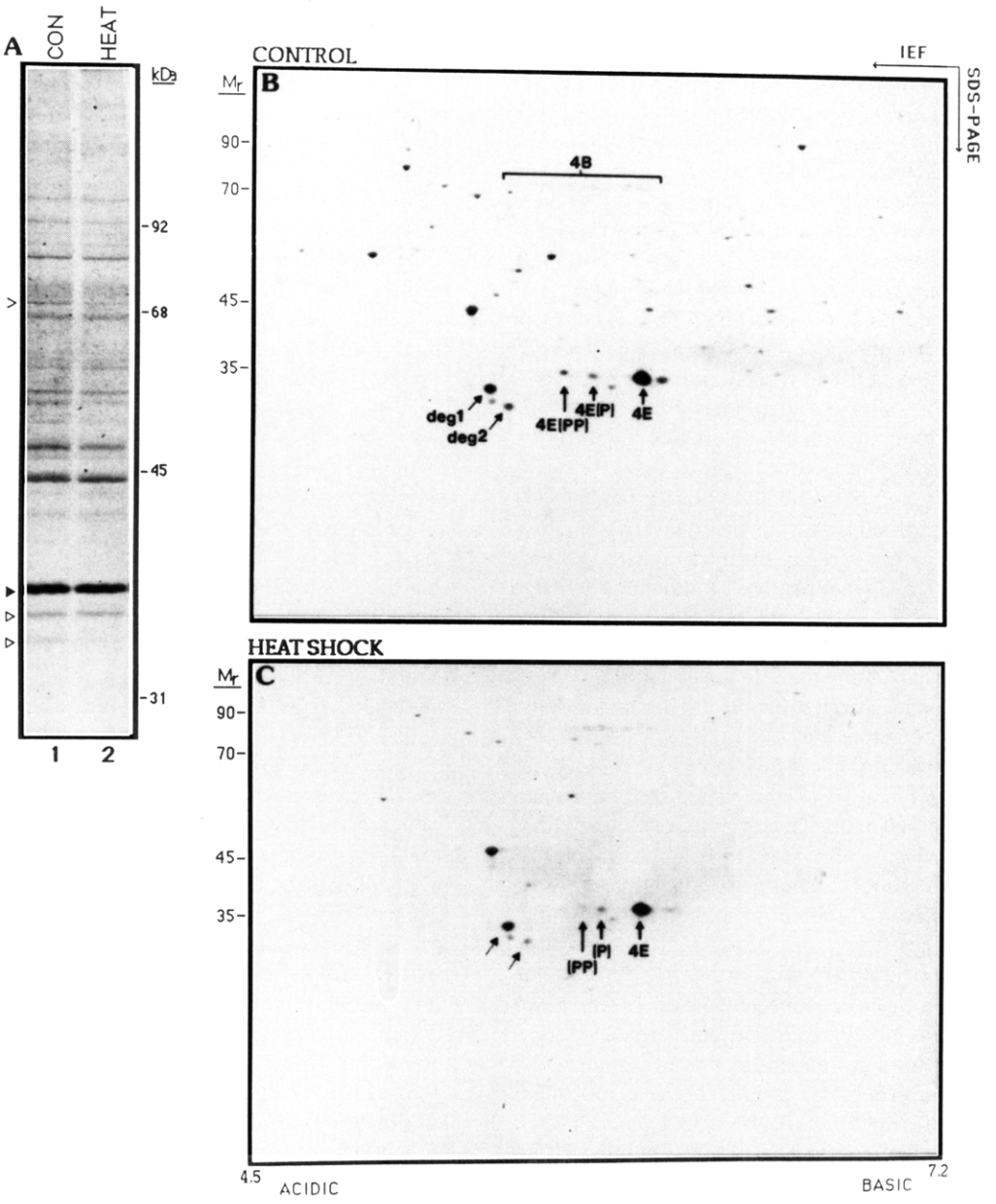

FIGURE 1: eIF-4E$^+$ from control and heat shocked cells. eIF-4E plus associated proteins was purified from equal amounts of control and heat shocked (30 min, 37 °C) *Drosophila* tissue culture cell lysate protein, as described in the Materials and Methods. Panel A: Equal amounts of column-purified samples were analyzed by one-dimensional SDS-PAGE and silver staining. *Lane 1*: control; *lane 2*: heat shock. eIF-4E is indicated by the bold arrowhead, and the degradation products of eIF-4E by open arrowheads. Molecular masses of marker proteins, in kDa, are shown to the right. No associated proteins of lower molecular weight were detected in other analyses. Panels B and C: Equal amounts of column-purified samples were analyzed by two-dimensional IEF/SDS-PAGE and silver staining. Panel B: normal temperature (control) purification. The positions of eIF-4E and its phosphorylated variants are indicated, as are the positions of the degradation products and putative eIF-4B (downpointing brackets; M_r ~80K). The basis for identifying these as degradation products is discussed in the text and Materials and Methods. Numerous associated proteins are also detectable. The major ones are considered in detail in Figure 3. The many minor proteins in panel A are due in part to the loading of a relatively large amount of purified eIF-4E$^+$ on this gel and were not observed in other purified samples or at lower loadings. Panel C: 30 min, 37 °C purification. Labeling as in panel A. The eIF-4E forms were slightly compressed in the p*I* dimension in this panel's gel analysis.

eIF-4E Dephosphorylation Detected by [^{32}P]Phosphate Labeling. An alternate approach to investigating whether eIF-4E is a phosphoprotein, and its response to heat shock, is to label cells with [^{32}P]orthophosphate, purify eIF-4E, and display purified proteins by IEF/SDS-PAGE. This analysis is shown in Figure 2, comparing control (top panel) and heat shocked (bottom panel) cells. A major 35 kDa spot is detected in control cells which comigrates with eIF-4E(P) (as marked in Figure 1B). [^{32}P]eIF-4E(P) decreases dramatically following heat shock (Figure 2, bottom panel). While the magnitude of change is clear, the very low absolute amount of eIF-4E(P) (cf. Figure 1) must temper any interpretation of its significance vis a vis translational reprogramming. No labeling of 4E(PP) was observed in this sample. However, as mentioned above, 4E(PP) was also not detected in most silver stain analyses of control cell eIF-4E$^+$. Among the heat shock-affected, ^{32}P-labeled proteins purified by m^{7}GTP-Sepharose chromatography is a phosphoprotein which shares several characteristic features with eIF-4B; a comprehensive discussion is presented below. The

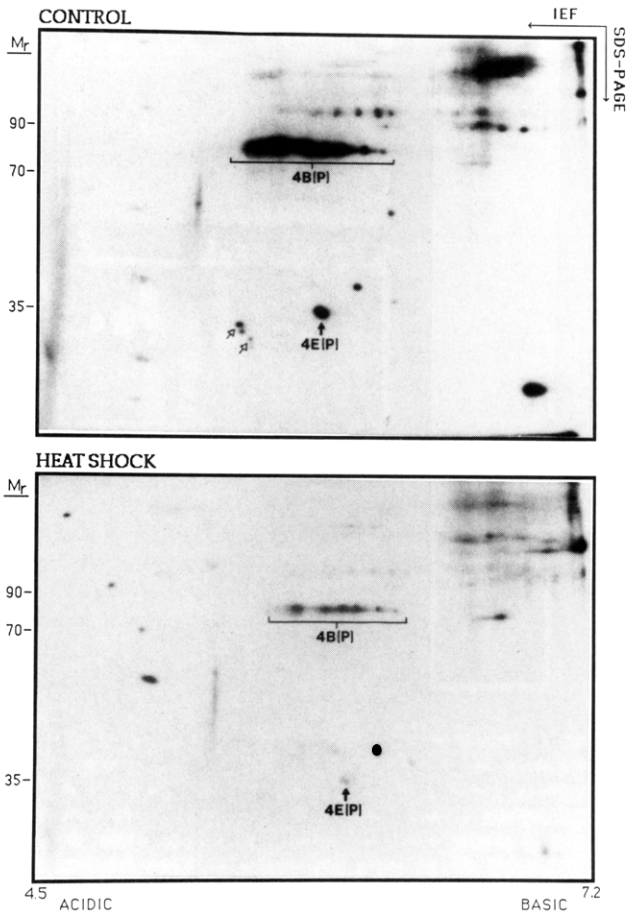

FIGURE 2: ^{32}P-Labeled eIF-4E^{+} from control and heat shocked cells examined by two-dimensional IEF/SDS-PAGE. ^{32}P-Labeled eIF-4E plus associated proteins was purified from [^{32}P]orthophosphate-labeled equivalent amounts of control and heat shocked *Drosophila* tissue culture cell lysate, as described in the Materials and Methods. Equal amounts of column-purified samples were analyzed by two-dimensional IEF/SDS-PAGE and autoradiography. Top panel: normal temperature (control) purification. Bottom panel: 30 min, 37 °C purification. The position of eIF-4E(P) is indicated. The positions of the degradation products are indicated by open arrows in top panel, and putative eIF-4B is labeled. Other copurifying proteins whose phosphorylation is heat-affected are evident, but their identities cannot be assigned with certainty at this time.

regulated decrease in eIF-4E(P) due to heat shock is not consistent with artifactual phosphatase activity producing apparent low extents of phosphorylation. Indeed, in [^{32}P]-phosphate pulse-labeling experiments, overall proteins incorporate twice as much ^{32}P during heat shock. Also, the major copurified phosphoprotein (putative eIF-4B) is substantially phosphorylated in both the nonheated and heat shocked sample (based on acidic shift distance in the IEF dimension), though its copurification with eIF-4E markedly decreases upon heating. Furthermore, other proteins such as MAP kinase isoforms show large increases in phosphorylation upon heat shock in parallel lysates (Chen et al., unpublished observations), indicating that there is not a pervasive phosphatase activity induced by heat shock.

Basic Characterization of eIF-2α Based on the Cloned cDNA. *Drosophila* eIF-2α has been cloned (Qu & Cavener, 1994). It contains a serine-50 residue, in a region that is very similar to that surrounding mammalian Ser51 where regulated phosphorylation controls eIF-2, and overall protein synthetic, activity. To identify its protein product in *Drosophila* cells, two types of analyses were performed. First, the cDNA was transcribed and translated *in vitro*, using a T7 promoter-driven vector followed by rabbit reticulocyte translation of the mRNA. IEF/SDS-PAGE analysis of ^{35}S-labeled *in vitro* translated protein detected one to three closely spaced protein spots, p*I* ~5.1, M_r ~40 000 (data not shown). In translation reactions where several spots were detected, the p*I* shift of the most acidic form (~3 mm) suggests it results from phosphorylation due to reticulocyte lysate activities, but this has not been pursued. The *in vitro* synthesized eIF-2α was mixed with *Drosophila* lysate, and the lysate protein that comigrated with the *in vitro* synthesized one was identified. eIF-2α in *Drosophila* lysates was also identified by immunoblotting with eIF-2α-specific antisera, raised against a synthetic peptide antigen (data not shown). eIF-2α is detected a single spot (in exponentially growing cells), located at identical coordinates to the *in vitro* synthesized eIF-2α. The *Drosophila* lysate protein spot which colocalizes antigenically-detected or *in vitro* synthesized eIF-2α can be identified in IEF/SDS-PAGE separations by either silver stain or ^{35}S-labeling and autoradiography (data not shown). The p*I* and M_r of *Drosophila* eIF-2α are very similar to those of mammalian eIF-2α.

Phosphorylation Changes on eIF-2α following Heat Shock. Several complementary approaches were undertaken to assess whether eIF-2α phosphorylation changes occur during heat shock, including ^{32}P-labeling/IEF/SDS-PAGE, immunoprecipitation of ^{32}P-labeled lysate protein, and IEF/SDS-PAGE immunoblotting.

Figure 3 depicts IEF/SDS-PAGE autoradiograms of [^{32}P]-phosphate pulse-labeled proteins from control (top panel) and heat shocked (bottom panel) cells. eIF-2α spots are marked. These migrate very slightly to the acidic side of eIF-2α, consistent with an acidic shift due to phosphorylation. The actual shift distance was slight but discernible, as noted above regarding reticulocyte lysate-induced eIF-2α phosphorylation. The intensity of the ^{32}P-labeled eIF-2α increases 2−3-fold following heat shock, suggesting increased phosphorylation is induced by heat shock. The putative phosphorylated form can also be faintly detected as a silver-stained spot upon careful examination of IEF/SDS-PAGE proteins (data not shown); it appears to comprise less than 5% of the total protein mass, an estimate corroborated by the immunoblot analysis presented below.

To confirm that heat modestly increases eIF-2α phosphorylation, ^{32}P-labeled lysates were incubated with eIF-2α antiserum to immunoprecipitate eIF-2α. Immunoselected proteins from control (Figure 4B) and heat shocked (Figure 4C) cells were analyzed by immunoblotting (panel A; to verify equivalent recoveries) and IEF/SDS-PAGE/autoradiography. A single labeled spot was immunoprecipitated from control and heat shocked cells. Its intensity increased slightly greater than 2-fold following heat shock (based on densitometric scans of films). Its location was indistinguishable from the ^{32}P-labeled spot identified in unfractionated lysates (Figure 3; see Materials and Methods for details of this analysis). The increased phosphorylation detected in Figures 3 and 4 could result from increased turnover rather than increased overall phosphorylation. This is further addressed in the next section.

Unlabeled, unfractionated control and heat shocked cell lysates were also analyzed by IEF/SDS-PAGE followed by immunoblotting. In Figure 5 three independent pairs of control and heat shocked lysates are presented (only a portion of the gel was probed and depicted). There is little or no

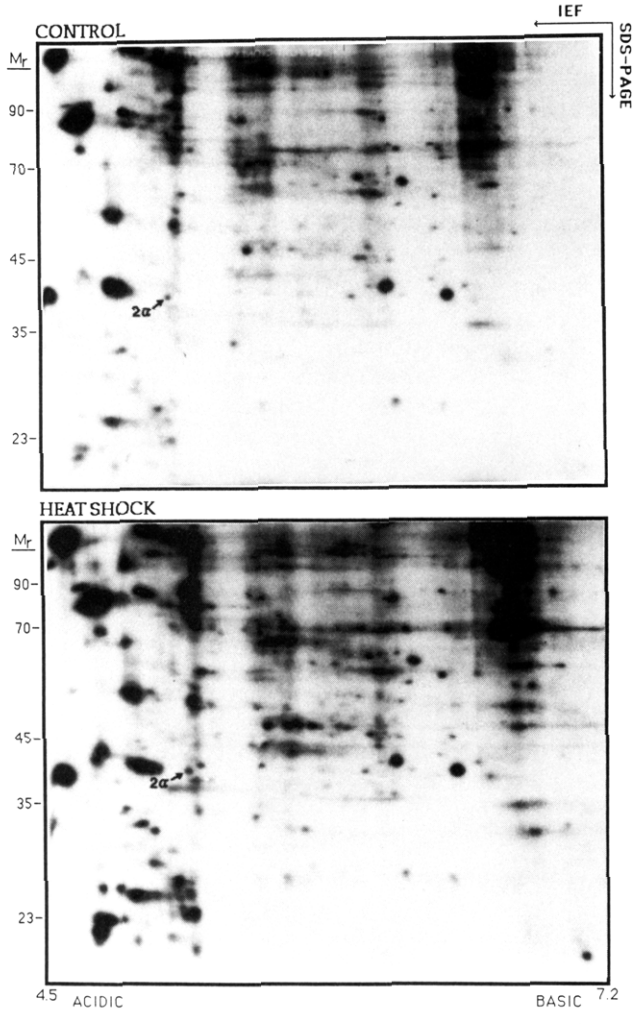

FIGURE 3: ^{32}P-Labeling analysis of phosphorylation changes in eIF-2α. Cells were pulse-labeled with [^{32}P]orthophosphate for 15 min, protein extracted by TXC/LSB buffer, and displayed by IEF/SDS-PAGE/autoradiography. Equal amounts of protein were loaded in each panel. Exposure was for about 4 days (intensifying screens at −80 °C). Top panel: cells grown and labeled at 22−24 °C (normal temperature controls). Bottom panel: cells placed at 37 °C for 30 min and labeled the last 15 min (heat shock). The control labeled cells are overall lighter, reflecting overall reduced ^{32}P incorporation (relative to heat shocked cells; this feature will be discussed in more detail in a separate report).

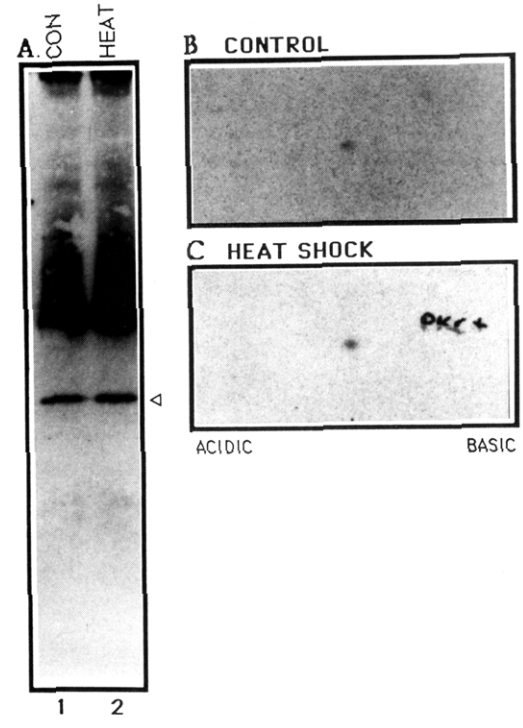

FIGURE 4: Immunoprecipitation analysis of ^{32}P-labeled protein for phosphorylation changes in eIF-2α. Control and heat shocked *Drosophila* tissue culture cells were pulse-labeled with [^{32}P]-orthophosphate as described in the Materials and Methods. Equal amounts of protein from control and heat shocked lysates were used for ^{32}P-labeled eIF-2α immunoprecipitation. Equal fractions of the immunopurified, eluted proteins were resolved by two-dimensional IEF/SDS-PAGE and detected by autoradiography (panels B and C). Panel A: 1D SDS-PAGE/immunoblotting analysis to verify equivalent recovery. The arrowhead marks the position of eIF-2α; the dark reacting area above eIF-2α represents immunopurification antibody that binds HRP-labeled detection antibody. Panel B: normal temperature (control) immunopurification. Panel C: 30 min, 37 °C immunopurification (labeled during the last 15 min).

detectable acidically-focusing eIF-2α(P) in any lysate. In one paired sample (panels E and F) there is a hint of acidically-migrating forms, reflected by a bulge on the acidic side of the main spot. However, (i) the bulge is greater in the nonheated sample, which is inconsistent with heat-induced phosphorylation; (ii) reducing the exposure time in the immunoblot analyses to resolve putatively fused signals reproducibly failed to provide evidence for a detectable phosphorylated form; (iii) horizontal bulges are frequently observed in IEF/SDS-PAGE immunoblots, on both the basic and acidic side of proteins (see panel A for an example); and (iv) parallel analyses using mammalian lysate and anti-eIF-2α easily detected a minor phosphorylated form (representing 10−15% of the total protein mass; data not shown). The failure to detect a phosphorylated eIF-2α in heat shocked *Drosophila* lysates is *not* inconsistent with the ^{32}P-labeling analysis. Rather, it confirms a nonquantitative analysis of silver-stained gels indicating that the *absolute amount* of phosphorylated eIF-2α is always small. That is, an acidically displaced eIF-2α representing ≥10% its mass is not observed either before or during heat shock. The immunoblot analysis cannot accurately detect and quantify phosphorylation extents affecting less than 5% of the molecular mass, so minor changes could easily go undetected. In conclusion, eIF-2α presents a situation analogous to eIF-4E: eIF-2α phosphorylation changes following heat shock, but the magnitude is 5−10 less than occurs in mammalian cells.

Dephosphorylation and Dissociation of an eIF-4E-Associated, 80 kDa Phosphoprotein following Heat Shock. There is no simple, accurate means for characterizing eIF-4B in *Drosophila* currently available. However, mammalian eIF-4B possesses several unique characteristics that facilitate its identification, and which have been used to purify it from distantly related species such as sea urchins (A. Lopo, personal communiciation): (i) It is very efficiently phosphorylated by protein kinase C *in vitro* (Duncan & Hershey, 1987b; Tuazon et al., 1989; Morley & Traugh, 1991; E. Mandley and A. Lopo, personal communication). (ii) It is a major phosphoprotein in cells, based on IEF/SDS-PAGE of unfractionated ^{32}P-labeled cell lysates (Duncan & Hershey, 1984; Duncan et al., 1987). (iii) It presents a unique, unusual spot distribution comprising two closely separated rows of spots that differ by 0.5−2 kDa; and each row contains 5−10 p*I* isoelectric variants which all label with ^{32}P, with the exception of the most basic variant. (iv) It copurifies with eIF-4E on m^{7}GTP-Sepharose, though the amount retained is quite variable suggesting a relatively weak interaction.

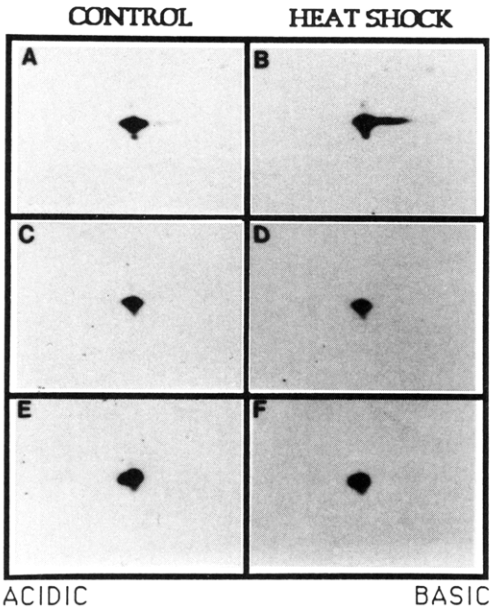

FIGURE 5: Immunoblotting analysis for phosphorylation changes in eIF-2α. Control and heat shocked (30 min, 37 °C) *Drosophila* tissue culture cells were lysed in TXC/LSB extraction buffer as described in the Materials and Methods and analyzed by two-dimensional IEF/SDS-PAGE and immunoblotting using eIF-2α antisera. Equal amounts of protein were loaded within each paired set, verified by Amido Schwartz staining prior to immunodetection. Sections of the complete blot are shown in each panel. Three independent paired sets of samples are shown. Panels A and B: set one, control and heat shock, respectively. Panels C and D: set two, control and heat shock, respectively. Panels E and F: set three, control and heat shock, respectively.

A *Drosophila* 80 kDa phosphoprotein detected in these studies shares all these features. It is tentatively identified as *Drosophila* eIF-4B, but further unequivocal characterization will be required to confirm its identity. The ^{32}P labeling pattern of this phosphoprotein before and after heat shock is compared in Figure 6, top and middle panels, where whole cell, unfractionated proteins are displayed. There is a clear shift in labeling intensity toward more basic, less phosphorylated forms, precisely as occurs with mammalian eIF-4B during heat shock (Duncan & Hershey, 1984, 1989). In some samples of m^7GTP-Sepharose-purified eIF-4E, there are a very faint set of silver-stained spots at the same p*I* and M_r as the ^{32}P-labeled spots shown in Figure 6 (Figure 1, panel B, provides an example), consistent with a small (and variable) amount of this protein copurifying with eIF-4E. The m^7GTP-Sepharose-purified sample shown in Figure 1 was treated with protein kinase C in the presence of [^{32}P]-ATP (Figure 6, bottom panel). The phosphoprotein spots label disproportionately with protein kinase C, mirroring the properties of authentic eIF-4B in mammalian and sea urchins. For example, compare the ratios of the 80 kDa phosphoprotein to eIF-4E on the silver-stained gel (Figure 1B) vs the autoradiogram (Figure 6, bottom); while eIF-4E does label, as it is also good protein kinase C substrate (Duncan & Hershey, 1987a; Tuazon et al., 1989), the ratio of signal of the 80 kDa to eIF-4E labeling on the autoradiogram is substantially higher than on the silver stain. Similarly, compared to HSP83 or GRP78 (these copurify with eIF-4E, see Discussion), which are known phosphoproteins, the 80 kDa phosphoprotein is disproportionately labeled.

A second dramatic heat shock-induced alteration in this phosphoprotein can be observed in Figure 2. There is a major ~80 kDa, ^{32}P-labeled protein which dissociates from eIF-4E following heat shock (compare top and bottom panels), which is tentatively identified as eIF-4B based on the preceding discussion. These protein spots have indistinguishable migration properties to the 80 kDa phosphoprotein identified in unfractionated lysates (Figure 6, top and middle panels) or silver stain analysis (Figure 1B) or by protein kinase C labeling (Figure 6, bottom panel). If this 80 kDa phosphoprotein proves to be *Drosophila* eIF-4B, then its loss from eIF-4E constitutes the disruption of a multifactor initiation complex which may have functional significance. The loss of ^{32}P-labeled 80 kDa phosphoprotein corresponds to the loss of protein mass, rather than to dephosphorylation. This can be inferred from the data in Figure 6, top and middle panels: in unfractionated nonheated and heated lysates there is roughly the same amount of total ^{32}P-labeled 80 kDa phosphoprotein, though the relative isoelectric variant abundance is shifted to more basic, less phosphorylated forms. Note also that in the heat shocked, affinity-purified sample (Figure 2, bottom panel) the residual bound 80 kDa phosphoprotein resembles the normal temperature variant distribution, suggesting that the more phosphorylated forms are preferentially retained in the multifactor protein complex.

DISCUSSION

Heat shock blocks virtually all normal protein synthesis in cells ranging from bacteria to yeast to sea urchins to flies to mammals (Schlesinger et al., 1982). A likely molecular basis for the inhibition is initiation factor modifications, since these have been documented to mediate various diverse translationally controlled situations. In this work, we have focused on *Drosophila* initiation factor eIF-2α and eIF-4E modifications during heat shock. In other cell systems, these are the two primary molecular regulators of translation. Prior to this study, investigations of initiation factor-mediated heat shock translational control have been principally the domain of mammalian studies, with a recent foray into the *Drosophila* eIF-4E/4F area by Sierra and colleagues. The results from these studies are equivocal. In both previous studies and those reported here there are evidences that initiation factor changes can be detected under certain conditions, as discussed below, but their significance or generality is uncertain.

Heat-Induced Translational Reprogramming. (i) Mammalian Cells. There are quantitative and qualitative differences in heat-induced translational reprogramming between mammals and insects. The pronounced preferential translation of the heat shock mRNAs characteristic of insect cells is not observed in mammalian cells (Hess and Duncan, unpublished results), whereas mammalian cells can "adapt" to heat shock but *Drosophila* cells cannot (McCormick & Penman, 1969; Duncan & Hershey, 1989; R. Duncan, unpublished observations). In mammalian cells initiation factor eIF-2α shows increased phosphorylation (Duncan & Hershey, 1984, 1989; Rowlands et al., 1988) and eIF-4E, eIF-4B, and ribosomal protein S6 show decreased phosphorylation (Duncan et al., 1987; Lamphear & Panniers, 1990, 1991; Kennedy et al., 1984; Duncan & Hershey, 1984, 1989). Additionally, eIF-4F dissociates into component subunits (Duncan et al., 1987; Lamphear & Panniers, 1990, 1991). These events occur during moderate to severe heat stresses and are sufficient to account for the repression of translation. However, under less severe conditions (41−42 °C), that still produce >80% inhibition of protein synthesis, none of these changes occur (Duncan & Hershey, 1989; except rpS6 has

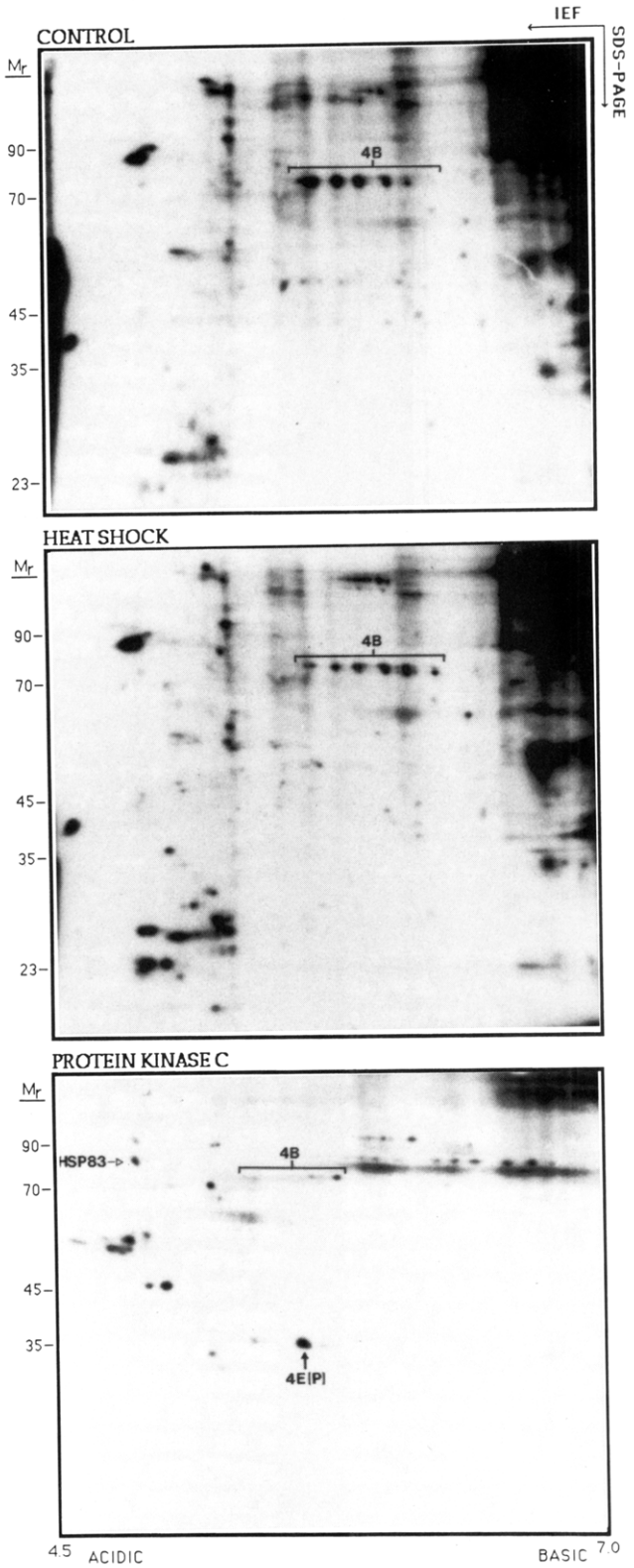

FIGURE 6: Phosphorylation of an eIF-4E-associated, 80 kDa phosphoprotein, *in vivo* and *in vitro*. Control and heat shocked *Drosophila* tissue culture cells were labeled with [^{32}P]orthophosphate. Label was added to both cultures at normal temperature, for 30 min. Heat shocked cells were then transferred to 37 °C for 30 min. ^{32}P-Labeled proteins were extracted with 100 °C SDS buffer, and displayed by IEF/SDS-PAGE/autoradiography. Equal amounts of protein were loaded on each gel. Exposure was for about 2 days (intensifying screens at −80 °C). Top panel: normal temperature control cells. The location of the 80 kDa phosphoprotein tentatively identified as eIF-4B is indicated in this and other panels. Middle panel: heat shocked cells placed at 37 °C for 30 min. Approximately equal amounts of cell protein were loaded on each gel, based on silver (or Coomassie) staining. Bottom panel: the purified eIF-4E (sample as shown in Figure 2) was treated with protein kinase C and [^{32}P]ATP and then displayed by IEF/SDS-PAGE/autoradiography. The locations of labeled 80 kDa phosphoprotein tentatively identified as eIF-4B and eIF-4E are indicated. ^{32}P-Labeled 80 kDa phosphoprotein precisely comigrated with the silver-stained protein grouping labeled "eIF-4B" in Figure 2 (barely detectable there in the photographic reproduction). The PKC treatment gel p*I* range is slightly displaced toward the acidic side.

not been evaluated). This suggests the primary cause of heat-induced repression is an as yet unidentified molecular alteration. No analysis of phosphorylation changes in *Drosophila* has been previously described.

(ii) Drosophila Cells. In *Drosophila* (insect) cells, HSP mRNAs can translate very efficiently at heat shock temperatures (37 °C). The 5′ untranslated region (UTR) contains necessary and sufficient information to cause preferential translation (DiNocera & Dawid, 1983; Bonner et al., 1984; Klementz et al., 1985). The 5′UTR may confer eIF-4F independence, and hence cap-recognition independence, based on its relatively unstructured nature (Lindquist & Petersen, 1990). However, mRNAs with similarly low predicted 5′UTR structure, such as actin, or a synthetic 5′UTR created by maintaining base composition but randomizing order, do not preferentially translate (Lindquist & Petersen, 1990), suggesting a specific-sequence element requirement also exists.

The heat shocked *Drosophila* translational machinery appears lesioned in the initiation factor fraction (Sanders et al., 1986; Zapata et al., 1991), paralleling observations in mammalian cells (Duncan & Hershey, 1984; Panniers et al., 1984, 1985). Some evidence for a ribosomal fraction lesion has also been obtained (Scott & Pardue, 1980), the differences likely resulting from stringency of salt-wash removal of ribosomal-associated proteins. Recent work from Sierra and colleagues strongly implicates alterations in *Drosophila* eIF-4E and eIF-4F activity in preferential translation (Maroto et al., 1989; Zapata et al., 1991, 1994) and supports the model for preferential translation of HSP mRNAs based on cap-independent translation (Lindquist, 1987; Lindquist & Petersen, 1990; Maroto & Sierra, 1988; Zapata et al., 1991, 1994). The *Drosophila* cap binding protein complex (Maroto & Sierra, 1989) may dissociate during heat shock at 37 °C (Zapata et al., 1991; but see discussion below), which likely would result in its inactivation. To address the consequences of inactivation, antibody-mediated inhibition of eIF-4F was demonstrated to repress translation of normal mRNAs *in vitro*, but have relatively little inhibitory effect on HSP mRNAs (*ibid.*). Similarly, blocking eIF-4F by m^7GTP cap analogue inhibition preferentially inhibits normal mRNA translation while HSP mRNA translation is relatively spared (Maroto & Sierra, 1988).

Heat-Induced eIF Phosphorylation Changes and Their Modest Scope. We have detected changes in eIF-2α and eIF-4E phosphorylation during heat shock of *Drosophila* tissue culture cells. The increased eIF-2α phosphorylation and the decreased eIF-4E phosphorylation are characteristically associated with the inactivation of these factors. However, the magnitude of change is very small compared to what has been observed in other systems, suggesting these may be of little functional consequence. For example, translational repression mediated by eIF-2α phosphorylation in mammals and yeast is associated with 20−60% of the protein molecules becoming phosphorylated. Though the extent of phosphorylation required to repress translation is coregulated by the relative abundance of eIF-2B, its recycling factor, and could theoretically begin to have an effect at very low levels of phosphorylation, such a circumstance has not been described. Similarly, in mammalian cells eIF-4E is typically monophosphorylated on approximately 50% of the molecules during active protein synthesis, which drops to <10% during repression. Our preferred interpretation of the phosphorylation changes is that they make at most a minor contribution to translational reprogramming and may be wholly without physiological consequence. However, we continue to entertain the possibility that the *Drosophila* translational machinery is sensitive to small changes in eIF phosphorylation and, when occurring as a concerted response (i.e., eIF-2α, eIF-4E, and putative eIF-4B), may have regulatory consequences. If these changes are not the major regulatory factors, though, then other translational regulatory mechanisms must predominate, and other investigatory avenues pursued. As one potential example, we have recently identified a poly(A)-sensitive step in heat shock translational repression (Duncan, submitted), reminiscent of established embryonic translational control strategies (Richter et al., 1990). eIF-2α and eIF-4E phosphorylation changes likewise do not appear to be the basis for translational represssion in mammalian cells subjected to mild heat stress (Duncan & Hershey, 1989).

It is curious that in nonheated *Drosophila* cells, with <10% eIF-4E in the phosphorylated state, 70−90% of their ribosomes are in polysomes (based on numerous unpublished observations). This is a striking finding, since in other organisms phosphorylation is required for activity. At a phenomenological level, only phosphorylated eIF-4E can promote mRNA binding to form the 48S preinitiation complex (Joshi-Barve et al., 1990). Mutated nonphosphorylatable variants are inactive (Joshi-Barve et al., 1990; DeBenedetti & Rhoads, 1991; Lazaris-Karatzakis et al., 1991). The effect in mammalian cells of decreasing phosphorylation from approximately 50% (exponential growth conditions) to 5−10% has not been directly tested, but this represents the level that is detected in severely heat shocked cells or adenovirus-infected cells, and which has been proposed to render eIF-4F inactive. Thus, quantitative aspects of initiation factor phosphorylation vs function differ dramatically in the *Drosophila* system.

We have carried out a cursory analysis of the proteins associated with eIF-4E following purification by m^7GTP-Sepharose chromatography (as depicted in Figure 1). Based on comigration and antibody recognition, the eIF-4E-associated proteins are principally well-characterized abundant cellular proteins. Several of these are chaperone-like proteins (HSP83, GRP78, HSC70), which participate in multiprotein complex assembly/disassembly, and actin. The association of these proteins may be functional: the chaperones may promote and/or stabilize eIF-4E/4F subunit association/dissociation during catalytic recycling, and actin has been implicated as interacting with eIF-4E in cytoskeletal-eIF-4F interactions (Howe & Hershey, 1984). In the heat shocked sample, HSP70 copurification was evident, potentially suggesting a molecular basis for its role in restoring translational activity during recovery (DiDomenico et al., 1982). We do not believe the copurification of these proteins results solely from their high intracellular abundance: following heat shock the amounts of these identified copurifying proteins were substantially less in most analyses, weighing against artifactual association based simply on prevalence (which does change following heat shock).

We find no evidence for eIF-4F subunits in most of our purifications of eIF-4E plus associated proteins, nor for their heat shock-induced dissociation from eIF-4F heterotrimeric complex. These results contrast with a previous report (Zapata et al., 1991) which suggested eIF-4F subunit dissociation occurs during heat shock. For example, eIF-4Fβ, also known as eIF-4A, can be assigned IEF/SDS-PAGE coordinates (Dorn et al., 1993). No copurified protein at these coordinates is detected (e.g., Figure 1, and many replicate analyses), whereas mammalian eIF-4A is readily detectable as a stained spot in parallel procedures. Both our

m^7GTP-Sepharose purifications and those described by Sierra and colleagues (Maroto & Sierra, 1989; Zapata et al., 1991) contain major associated proteins of ~70 000 Da, and we concur that copurification is reduced in heat shocked samples. They suggest this may be *Drosophila* eIF-4A, but subsequent cloning of eIF-4A has ruled out that possibility (Dorn et al., 1993). Our data suggest the 70 kDa protein is likely HSC70. Similarly, the identity of the largest subunit identified by Zapata et al. (1991) which dissociates during heat shock remains to be clarified. Therefore, while the hypothesis that subunit dissociation is a critical event leading to eIF-4F inactivation during heat shock remains attractive, we conclude that there is little hard evidence to support that idea as yet.

In conclusion, initiation factor phosphorylation-based strategies have been developed by numerous viruses to bypass translational lesions, and to achieve preferential translation. For example, several picornaviruses can translate via cap-independent internal initiation when eIF-4F is inhibited; and adenovirus and influenza virus induce intracellular situations allowing efficient translation of their mRNAs in the presence of high, inhibitory levels of eIF-2α phosphorylation. Whether the heat shock mRNAs and the heat shocked cell constitute a normal (i.e., nonviral) translational control pathway whereby preferential translation is mediated by initiation factor activity inhibition remains an open question, but there is no obvious involvement of either eIF-2α or eIF-4E phosphorylation changes. However, as broached above, a contributory or perhaps even substantial role cannot be ruled out. To directly unequivocally address their import, targeted mutations that should abrogate their regulation can be introduced into these proteins. Subsequently, they can be introduced into cells by transfection and their effects on heat shock translational reprogramming quantified, as has been carried out in mammalian cells (Murtha-Riel et al., 1993).

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