MOLECULAR AND CELLULAR BIOLOGY, Dec. 1988, p. 5059–5071
0270-7306/88/125059-13$02.00/0

Vol. 8, No. 12

Dynamic Changes in the Structure and Intracellular Locale of the Mammalian Low-Molecular-Weight Heat Shock Protein

ANDRE-PATRICK ARRIGO, JOSEPH P. SUHAN, AND WILLIAM J. WELCH†*
Cold Spring Harbor Laboratory, P.O. Box 100, Cold Spring Harbor, New York 11724

Received 7 June 1988/Accepted 30 August 1988

Mammalian cells grown at 37°C contain a single low-molecular-weight heat shock (or stress) protein with an apparent mass of 28 kilodaltons (kDa) whose synthesis increases in cells after exposure to elevated temperatures or other forms of physiologic stress. Herein we present data demonstrating that heat shock protein 28 exists in a number of dynamic states depending upon the physiologic state of the cell. Biochemical fractionation of 37°C cells in the absence of nonionic detergent revealed that the 28-kDa protein partitioned approximately equally between the soluble and insoluble fractions. The addition of detergent in the fractionation procedure resulted in all of the protein distributed within the soluble phase. In contrast, in cells first heat shocked and then fractionated in the presence of detergent, most of the 28-kDa protein was found within the insoluble fraction. These biochemical results appeared entirely consistent with indirect immunofluorescence experiments, demonstrating that the 28-kDa protein resided within the perinuclear region of 37°C cells in close proximity to the Golgi complex. After heat shock treatment, the 28-kDa protein relocalized within the nucleus and resisted detergent extraction. The extent of 28-kDa protein redistribution into the nucleus and its detergent insolubility increased as a function of the severity of the heat shock treatment. With time of recovery from the heat treatment there occurred a gradual return of the 28-kDa protein into the detergent-soluble phase. Concomitant with these changes in 28-kDa protein solubility was a corresponding change in the apparent size of the protein as determined by gel filtration. While at 37°C cells the protein exhibited a mass of 200 to 800 kDa; after heat shock the protein assumed sizes of 2 MDa or greater. Using immunoelectron microscopy, we show an accumulation of these aggregates of 28-kDa protein within the nucleus. Finally, we show that the heat-dependent redistribution of the 28-kDa protein from the cytoplasm into the nucleus was greatly diminished when the cells were first rendered thermotolerant, and we suggest that this simple assay (i.e., 28-kDa protein detergent solubility) may prove useful in evaluating the thermotolerant status of a cell or tissue.

Within the past few years there has been tremendous progress in our understanding of the events that occur in cells after heat shock and the biochemistry of the individual heat shock proteins (HSPs). It is clear that most of the mammalian HSPs (or stress proteins) are present in cells maintained under normal growth conditions and participate in events essential to the life-style of the cell. For example, the 83- to 90-kilodalton (kDa) stress protein has been shown to be a component of most steroid receptors (12, 45) as well as involved in the transport and/or regulation of various protein tyrosine kinases, such as $pp60^{src}$ (11). In the case of the major stress protein of ≅70 kDa it has been shown that there exist various forms of the protein, all of which appear to be compartmentalized differently within the cell. All of the related members of the 70-kDa protein family exhibit binding to ATP (54) and are thought to somehow mediate protein folding-unfolding events. Specifically, recent biochemical and genetic data implicate the cytoplasmic form of HSP 70 in facilitating the transfer of proteins from the cytoplasm across the membrane of either the endoplasmic reticulum or mitochondria (13, 19).

Considerably less information is available concerning the structure and function of the low-molecular-weight (MW) HSPs. Unlike the HSP 70 family, considerable sequence divergence of the low-MW HSPs has occurred during evolution. Moreover, although there appear to be multiple low-MW HSPs in *Drosophila melanogaster* and plants (3, 8, 17, 40, 51), in avians, yeasts, or mammals there occurs only a single low-MW HSP (6, 23, 24, 26, 27, 37). The only obvious common property of these proteins from different organisms is their homology to the α-crystallin proteins present in the lens (24, 25, 43). Because both the α-crystallins and the low-MW HSPs are isolated as rather large aggregates (i.e., 200 to 800 kDa from normal, nonheated cells) (1–3, 4, 6, 7, 14, 15, 46), we suspect that their observed homology resides in those domains responsible for the self-assembling properties of the proteins.

We recently described the characterization and purification of the low-MW HSP from HeLa cells (6). The protein, with an apparent mass of 28 kDa, is comprised of at least three related isoforms, two of which contain phosphate. Interestingly, phosphorylation of the protein occurs at 37°C in cells that are treated with various mitogens, calcium ionophores, or tumor promoters (53). In the present study we demonstrate, via biochemical and immunological criterion, that the 28-kDa protein is present primarily within the perinuclear region, in close proximity to the Golgi complex, in cells maintained at 37°C. We show that the 28-kDa protein exists in a variety of dynamic states, all of which appear to be dependent upon the physiologic status of the cell. Finally, we present a new and simple method, based on the relative solubility of the 28 kDa protein, which appears to be useful in correlating the thermotolerant status of the cell.

MATERIALS AND METHODS

Cell culture, radiolabeling, and gel electrophoresis. HeLa cells growing on 35-mm Falcon dishes were either incubated at 37°C or heat shock treated at the temperatures and times

* Corresponding author.
† Present address: Departments of Medicine and Physiology, University of California, San Francisco, CA 94110.

indicated in the figure legends. The control or heat-shocked cells were labeled at 37°C in Dulbecco modified Eagle medium lacking leucine and supplemented with L-[4,5-^{3}H] leucine (50 Ci/mmol; Amersham Corp.) for 1 to 2 h. After labeling, the cells were washed with phosphate-buffered saline (PBS), harvested in Laemmli sample buffer (10), and boiled at 100°C before gel electrophoresis. One-dimensional and two-dimensional gel electrophoresis was performed as described previously (6).

Cell fractionation. (i) Detergent lysis. HeLa cells growing on 35-mm Falcon dishes were washed with PBS and scraped from the dish with a rubber policeman in ice-cold PBS. The cells were pelleted at 500 × *g* for 10 min and lysed by vortexing at 4°C in a buffer containing 10 mM Tris (pH 7.4), 5 mM $MgCl_2$, 10 mM NaCl, 0.1 mM phenylmethylsulfonyl fluoride, and 0.5% Triton X-100. The lysates were centrifuged at 20,000 × *g* for 10 min, and the supernatants and pellets were suspended in Laemmli sample buffer (5× and 1×, respectively) such that the final volume was the same.

(ii) Hypotonic lysis. In other experiments, HeLa cells growing in suspension in F-13 medium supplemented with 5% calf serum (5 × 10^5 cells per ml) were collected by centrifugation, washed in PBS, incubated in cold hypotonic buffer consisting of 10 mM Tris (pH 7.4)–0.1 mM EDTA, and lysed by Dounce homogenization. $MgCl_2$ was then added to a final concentration of 5 mM, and the broken cell lysate was clarified at 2,000 × *g* for 10 min. The 2,000 × *g* supernatant was then spun for 10 min at 20,000 × *g*, and the resultant supernatant was spun at 100,000 × *g* for 1 h. The resulting pellets and supernatants were suspended in Laemmli sample buffer, and equal portions of each were then analyzed by gel electrophoresis and Western blotting (immunoblotting).

For gel filtration analysis, the 20,000 × *g* supernatant (prepared by the hypotonic or detergent lysis procedure) from either normal or heat-shocked lysates was applied to an Ultrogel AcA34 gel filtration column (1.0 by 100 cm) equilibrated in 20 mM Tris (pH 7.4)–20 mM NaCl–0.1 mM β-mercaptoethanol, and the column was developed in the same buffer. Calibration of the column was performed by using the following standards (native molecular mass): blue dextran (>2 MDa), proteasome (800 kDa) (5), ferritin (440 kDa), and catalase (232 kDa).

Immunoblotting. Immunoblotting was performed essentially as described by Bowen et al. (10) but with electrophoretic transfer described by Zeller et al. (57). Anti-28-kDa protein serum (6) was diluted 1/100 in PBS containing 1% bovine serum albumin, and detection of the primary antibody was performed with goat anti-rabbit antiserum (diluted 1/500) containing conjugated horseradish peroxidase. Incubation with each antibody was performed for 1 h at room temperature. After incubation with the second antibody, the immunoblots were washed extensively with PBS and PBS–1% Tween 20.

Indirect immunofluorescence analysis. Human foreskin fibroblasts, monkey CV-1 cells, or HeLa cells growing on glass cover slips were fixed with 2% paraformaldehyde (pH 7.0) in PBS for 20 min and permeabilized with 0.1% Triton X-100 in PBS. Anti-28-kDa protein serum used for staining was diluted 1/100 in PBS containing 1 mg of bovine serum albumin per ml. Goat anti-rabbit antibody coupled with fluorescein isothiocyanate and diluted 1/100 in PBS-bovine serum albumin was used as the second antibody. All incubations were performed for 1 h at room temperature. Double-label immunofluorescence comparing the distribution of 28-kDa protein and the Golgi complex was performed with the anti-28-kDa protein serum and a rhodamine-conjugated wheat germ agglutinin (diluted 1/500) specific for Golgi-associated proteins (Sigma Chemical Co.). Incubations were performed for 1 h at room temperature. The anti-28-kDa protein antibody was detected by using a second, fluorescein-conjugated goat anti-rabbit antibody as described above. The cells were then examined and photographed with the appropriate filters with a Zeiss inverted photomicroscope.

Electron and immunoelectron microscopy. A sample of the purified fraction containing the 28-kDa stress protein (6) or purified bovine lens α-crystallin was adsorbed onto freshly glow-discharged, carbon-coated grids. The grids were rinsed with water and stained with 0.1% uranyl acetate before being air dried. Electron micrographs were taken with a Philips 201 electron microscope (Philips Electronic Instruments, Inc., Mahway, N.J.) at 50 kV. The size of the 28-kDa particles was measured on ×200,000 enlarged prints.

Heat-shocked and control CV-1 cells grown on 35-mm Falcon dishes were fixed for 15 min in 2% paraformaldehyde in PBS. After several rinses in PBS, the cells were incubated overnight in a 1:100 dilution (PBS–1 mg of bovine serum albumin per ml) of anti-28-kDa protein antibody at 4°C. After extensive washing in PBS, the cells were incubated overnight with a 1:40 dilution (PBS–1 mg of bovine serum albumin per ml) of peroxidase-conjugated goat anti-rabbit $F(ab^1)^2$ (Organon Teknika) at 4°C. After cells were washed for 5 h in several changes of PBS, they were fixed in 2% glutaraldehyde in 75 mM cacodylate and 4.5% sucrose for 5 min, rinsed in several changes of PBS, and then rinsed briefly in 100 mM Tris hydrochloride (pH 7.6). The cells were incubated for 45 min in a solution containing 1 mg of diaminobenzidine (DAB-4HCL) per ml–0.015% H_2O_2–50 mM Tris hydrochloride (pH 7.6), briefly washed in 100 mM Tris hydrochloride (pH 7.6), and washed in several changes of PBS. The cells were fixed in 1% OsO_4 in 75 mM cacodylate for 30 min, followed by several changes of distilled water. The cells were dehydrated in a graded series of ethanol and embedded in Epon. Sections were cut on a diamond knife, and electron micrographs were taken with a Hitachi 7000 electron microscope at 50 kV.

RESULTS

Unlike *Drosophila* cells, which contain 4 low-molecular-mass (e.g., 20- to 30-kDa) HSPs (3, 17, 51), mammalian cells contain only a single small HSP (28 kDa) which is comprised of multiple isoforms (6, 23, 24, 27, 53). HeLa cells were incubated at 37°C or heat shocked at 43°C for 90 min and labeled with [^{3}H]leucine, and the labeled proteins were then analyzed by two-dimensional gel electrophoresis (Fig. 1). In addition to the increased synthesis of the 70- and 90-kDa HSPs, elevated synthesis of the three different isoforms (designated a, b, and c) of the 28-kDa stress protein was observed in the cells after heat shock. The observed heterogeneity with respect to the isoelectric point appears due to phosphorylation: isoform a is unphosphorylated, whereas both isoforms b and c are known to contain phosphate (6, 27, 53). Notice that isoform c often is observed as two closely migrating isoforms and appears even more intense in cells exposed to heavy metals. That all three of the indicated proteins are indeed members of the 28-kDa family was confirmed by Western blot analysis with a polyclonal anti-28-kDa protein serum (Fig. 1A and B). In the case of the 37°C HeLa cells, isoform a, and to a lesser extent isoform b, exhibited reactions with the antibody. After heat shock, the relative levels of the protein increased and the third more acidic isoform, c, was observed.

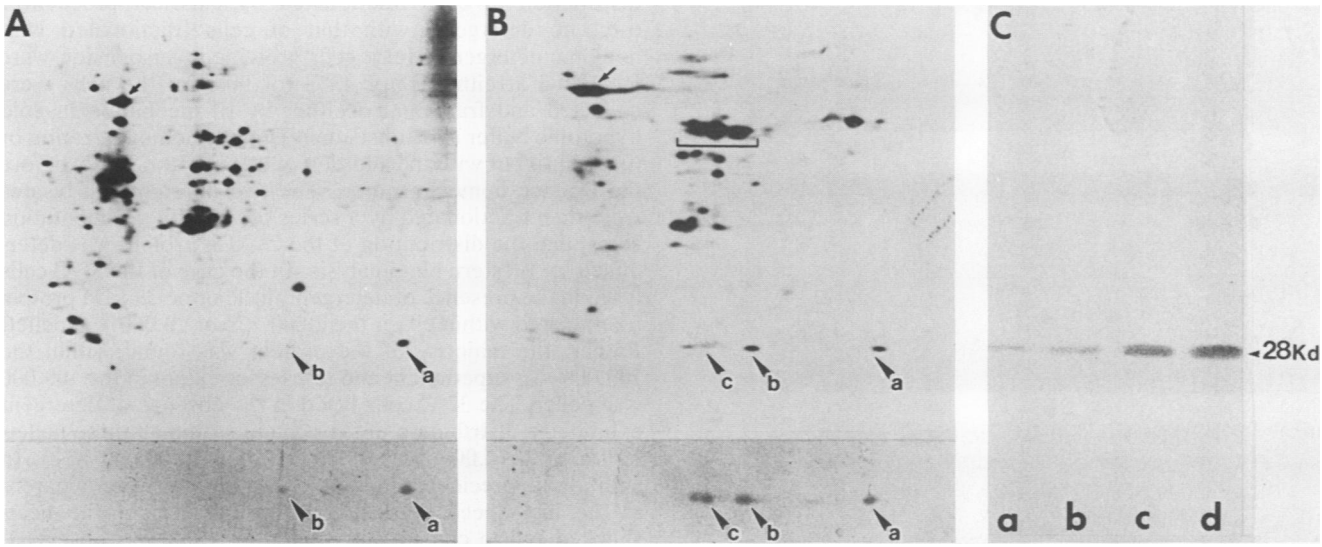

FIG. 1. The 28-kDa stress protein is comprised of multiple isoelectric forms and accumulates in cells after heat shock. (A and B) HeLa cells growing on 35-mm Falcon dishes were either kept at 37°C (A) or heat treated at 43°C for 90 min (B). After the heat shock treatment, the cells were returned to 37°C and then labeled for 1 h with [^{3}H]leucine. The labeled proteins were analyzed in the first dimension by isoelectric focusing (pH 5 to 7), followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on a 15% polyacrylamide gel. The acidic end is to the left. The arrow indicates the position of the 90-kDa stress protein; the bracket indicates the position of the 72- and 73-kDa stress proteins; and the arrowheads, designated a, b, and c, indicate the three major isoforms of the 28-kDa stress protein. In parallel experiments with the same samples, the two-dimensional gels were transferred onto nitrocellulose and the 28-kDa stress protein isoforms were detected by Western blot analysis by using the rabbit polyclonal antibody specific for 28 kDa (6). Only that portion of the immunoblots (between 20 and 35 kDa) is shown. (C) HeLa cells growing on 35-mm Falcon dishes were kept at 37°C or heat shocked at 43°C for 90 min. The heat-treated cells were returned to 37°C and allowed to recover for 12 h. One plate of the cells was then exposed to a second heat shock treatment at 43°C for 90 min. The cells, after the various treatments, were lysed in Laemmli sample buffer, and an equal amount of total protein was then analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. After transfer to nitrocellulose, the relative amount of 28-kDa protein was determined by Western blot analysis. Lanes: a, 37°C; b, 43°C for 90 min; c, 43°C for 90 min, cells allowed to recover at 37°C for 12 h; d, as in c and exposed to a second 90-min heat shock at 43°C.

The 28-kDa protein was present in the cell before stress and increased significantly after stress (Fig. 1C). Although low levels of the protein are observed in cells at 37°C (Fig. 1C, lane a), the protein increased with time of recovery after heat shock, reaching a maximal level approximately 12 to 14 h after the heat stress (lanes b, c, and d).

Previous studies in both *Drosophila* and avian cells have indicated a change in the solubility of the low-MW HSPs, depending upon the physiologic state of the cell (1–4, 14, 15). Specifically, whereas the majority of low-MW HSPs is present primarily within the detergent-insoluble fraction of the cell during and immediately after heat shock, these proteins slowly redistribute back into the detergent-soluble fraction during recovery from the heat treatment (1–4, 6, 14, 31, 47). Using a rather simple assay, we had observed a similar situation with respect to the mammalian low-MW HSP, and we extended these previous studies to show that this redistribution is dependent upon both the severity of the stress response and the prior physiologic state of the cell. The assay involved lysis of the cells with 0.5% Triton X-100 and subsequent velocity sedimentation at 20,000 × *g* of the lysed cells to obtain supernatant and pellet fractions. In each case equal portions of the supernatant and pellet were applied to the gel, and the relative distribution of the 28-kDa proteins was determined by Western blot analysis. The vast majority of 28-kDa protein partitioned within the soluble phase of cells maintained at 37°C (Fig. 2A, panel 1). With increasing severity of the heat stress, more of the protein was recovered in the insoluble phase (panels 2, 3, and 4). In the case of the cells exposed to either the amino acid analog of proline (azetidine [10 mM] for 12 h; panel 5) or to 100 μM sodium arsenite for 2 h (panel 6), two other inducers of the stress response, the 28-kDa protein partitioned into both the soluble and insoluble phase. Thus, as the temperature was increased there was a corresponding increased redistribution of the 28-kDa protein into the insoluble fraction, similar to the situation for the low-mw *Drosophila* hsps (1, 3, 31, 47).

In addition to the heat dependence of 28-kDa protein solubility, we observed that the prior state of the cells greatly affected the distribution of the 28-kDa protein after the heat shock treatment. Specifically, the heat-dependent redistribution of the 28-kDa protein into insoluble fraction was significantly reduced when cells were first made thermotolerant. Thermotolerance is a phenomenon in which cells that have received a mild sublethal heat shock treatment exhibit significantly higher survival rates after a second heat shock challenge that would otherwise be lethal (18, 21, 22, 28, 32–35, 36, 38, 39, 41). For our studies here, both nontolerant (i.e., no prior heat treatment) and thermotolerant cells (i.e., a prior 90-min heat [43°C] shock treatment and subsequent recovery at 37°C) were challenged with a 30-min heat (44°C) shock treatment. The cells were then returned to 37°C; at various times thereafter the cells were fractionated into detergent-soluble and -insoluble fractions as described above, and the distribution of the 28-kDa protein in each fraction was determined by Western blot analysis. In the case of both the nontolerant and tolerant cells maintained at 37°C (Fig. 2B, panels a and g, respectively), the majority of the 28-kDa protein again was found distributed within the soluble phase. Immediately after 30-min heat (44°C) shock treatment, most of the 28-kDa protein was redistributed into the pellet of the nontolerant cells (panel b) whereas signifi-

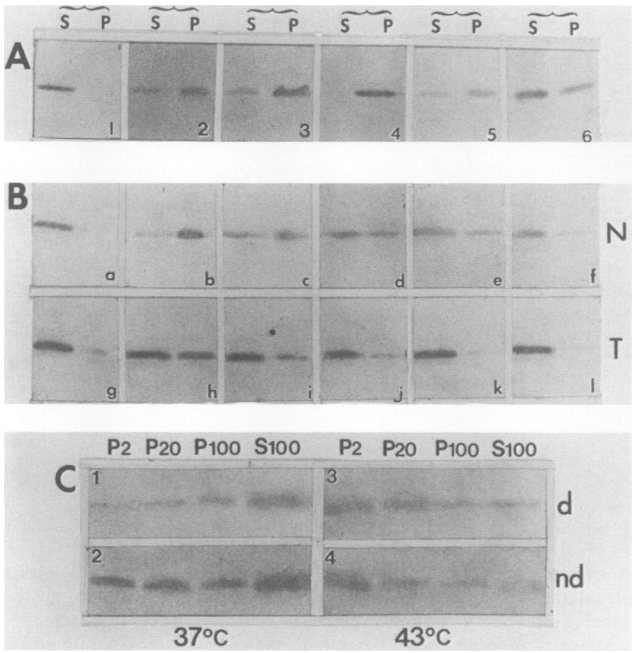

FIG. 2. Effects of stress on 28-kDa protein solubility. HeLa cells growing on 35-mm Falcon dishes were exposed to various heat shock treatments, sodium arsenite, or the amino acid analog azetidine, as detailed below. In some cases the cells were first made thermotolerant by a prior 90-min shock at 43°C and subsequent recovery at 37°C for 12 h. Except where indicated, the cells were fractionated into a low-speed pellet (P) or supernatant (S) by detergent lysis of the cells and centrifugation at 20,000 × *g* for 10 min as described in Materials and Methods. Equal portions of the supernatant or pellet were analyzed on a sodium dodecyl sulfate-polyacrylamide gel, the proteins were transferred to nitrocellulose, and the amount of 28-kDa protein was determined by Western blotting. (A) Effects of different stresses. Panels: 1, 37°C; 2, 43°C for 90 min; 3, 44°C for 30 min; 4, 45°C for 30 min; 5, 10 mM azetidine for 12 h; 6, 100 μM arsenite for 2 h. (B) Nontolerant (N) and tolerant (T) cells were given a 30-min heat (44°C) shock and then returned to 37°C for either 2, 4, 12, or 24 h. Panels: (a and g) 37°C; (b and h) 44°C for 30 min; and (c and i) 44°C for 30 min, recovery for 2 h; (d and j) 44°C for 30 min, recovery for 4 h; (e and k) 44°C for 30 min, recovery for 12 h; (f and l) 44°C for 30 min, recovery for 24 h. (C) Comparison of 28-kDa protein solubility in detergent-extracted cells versus cells extracted without detergent. HeLa cells growing in suspension were kept at 37°C or heat shocked at 43°C for 90 min and then fractionated by a series of successive velocity sedimentation steps (2,000 × *g*, 20,000 × *g*, and 100,000 × *g*) as described in the Materials and Methods. Equal portions of each fraction were then analyzed by Western blotting with the anti-28-kDa protein serum. Abbreviations: P_2, 2,000 × *g* pellet; P_{20}, 20,000 × *g* pellet; P_{100}, 100,000 × *g* pellet; S_{100}, 100,000 × *g* supernatant; d, detergent; Nd, no detergent.

cantly less of the total protein was observed in the pellet of the heat-treated tolerant cells (panel h). Moreover, with time of recovery from the heat shock treatment, in the tolerant cells that portion of the 28-kDa protein which did redistribute into the insoluble fraction exhibited faster kinetics of return into the soluble fraction as compared with that of the nontolerant cells (e.g., compare panels i, j, k, and l with panels c, d, e, and f in Fig. 2B). In summary, markedly less of the 28-kDa protein became insoluble in the tolerant cells after heat shock, with that portion of the protein in the insoluble phase returning to the soluble phase with faster kinetics as compared with that of the nontolerant cells.

In an effort to understand the true subcellular distribution of the 28-kDa protein in HeLa cells, we compared its relative distribution in cells fractionated by classical procedures (i.e., no detergent) with that of cells fractionated with nonionic detergent. HeLa cells growing in suspension were incubated at either 37 or 43°C for 90 min; the cells were collected and fractionated either by (i) incubation in cold hypotonic buffer and subsequent Dounce homogenization or (ii) as in (i) but with the addition of 0.5% Triton X-100 before the Dounce homogenization. The two different cell lysates were then fractionated by a series of velocity sedimentation steps, and the distribution of the 28-kDa protein was determined by Western blot analysis. In the case of the 37°C cells lysed in the presence of detergent, little or no 28-kDa protein fractionated within either the 2,000 × *g* or 20,000 × *g* pellet. Rather, the majority of the protein was found within the 100,000 × *g* supernatant and to a lesser extent in the 100,000 × *g* pellet. The 37°C cells lysed in the absence of detergent exhibited a distribution of the protein within all three pelleted fractions (2,000 × *g*, 20,000 × *g*, and 100,000 × *g*). In addition, appreciable amounts of the protein were apparent in the high-speed supernatant. In the case of the heat-shocked cells a considerably different result was observed. Regardless of the presence or absence of detergent in the lysis buffer, the majority of the 28-kDa protein was isolated in the 2,000 × *g* pellet and to a lesser extent within the 20,000 × *g* pellet. Very little of the protein was found in the high-speed supernatant.

Workers in a number of laboratories have observed that shortly after heat shock the vimentin-containing intermediate filaments redistribute from their normal cytoplasmic array into a tight cage around the nucleus (9, 14, 50, 55). Because the low-MW HSPs similarly exhibit a rapid redistribution into the low-speed insoluble fraction after heat shock, it has been suggested that 28-kDa protein redistribution is a consequence of the collapsed intermediate filament network, perhaps via entrapment of the protein within the collapsed vimentin filament-nuclear fraction (30). To examine this possibility, we analyzed the distribution of the 28-kDa protein in detergent-lysed 37°C cells first exposed to a number of agents which promote the collapse of the intermediate filaments. In 37°C cells containing a collapsed intermediate filament network, the 28-kDa protein remained within the detergent-soluble fraction (Fig. 3). However, when the drug-treated cells were subsequently given a heat shock treatment, much of the 28-kDa protein again redistributed into the insoluble fraction. These results clearly indicate that (i) the redistribution of the 28-kDa protein into the insoluble fraction of cells is not simply a consequence of the collapse of the intermediate filaments, and (ii) cells that already contain a collapsed intermediate filament network still exhibit a redistribution of the 28-kDa protein into the insoluble fraction after heat shock treatment.

Using our relatively simple assay of the distribution of the 28-kDa protein in detergent-lysed cells, we performed numerous experiments analyzing a number of parameters influencing the intracellular distribution of the 28-kDa protein. Although too extensive to discuss in detail here, these results are presented in Table 1. It should be noted that the relative distributions of the 28-kDa protein (e.g., supernatant versus pellet) listed in Table 1 were arrived at by visible inspections of the Western blots (in each case done in duplicate or triplicate) and are not meant to be absolutely quantitative but rather provide a more relative qualitative description of the data. The pertinent points to be noted in these studies are as follows. (i) The 28-kDa protein showed an increasing redistribution from the soluble phase into the insoluble phase as a function of the severity of heat shock

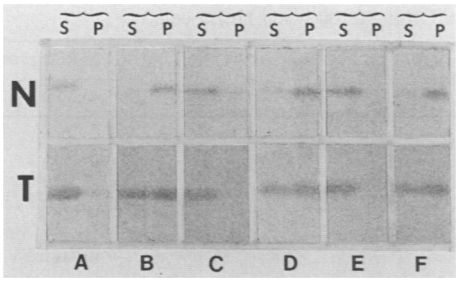

FIG. 3. Redistribution of the 28-kDa protein into the insoluble fraction is not simply a consequence of collapsed intermediate filaments but is dependent upon heat shock treatment. HeLa cells growing on 35-mm Falcon dishes were incubated at 37°C (N) or made thermotolerant (T) by a incubation at 43°C for 90 min and subsequent recovery at 37°C for 12 h. The normal or thermotolerant cells were then incubated at (A) 37°C, (B) 45°C for 30 min, (C) 37°C in the presence of 0.1 mM 8-bromo-cyclic AMP and 0.1 mM methylisobutylxanthine for 30 min, (D) as in (C) and then heat shocked at 45°C for 30 min, (E) 37°C in the presence of 0.1 mM colcemide, or (F) as in (E) and then heat shocked at 45°C for 30 min. After the appropriate incubation the cells were then lysed in the presence of 0.5% Triton X-100 and fractionated into a 20,000 × *g* supernatant (S) or pellet (P). Equal portions of the supernatant and pellet were then analyzed for the distribution of the 28-kDa protein by Western blotting.

treatment. (ii) With time of recovery from the heat stress the protein slowly redistributed back into the soluble phase; in cells first made thermotolerant (i.e., a prior heat shock treatment and recovery period) and then heat shocked, significantly less of the total 28-kDa protein partitioned into the insoluble phase. Moreover, the portion of 28-kDa protein that did fractionate within the insoluble phase exhibited a faster return to the soluble phase in the tolerant cells as compared with the nontolerant cells during recovery from the heat shock treatment. (iii) The addition of cycloheximide during the initial heat shock and recovery period caused significantly more of the 28-kDa protein to partition into the insoluble phase after a second heat shock treatment, indicating a requirement for protein synthesis for this protective effect of 28-kDa protein solubility. (iv) Redistribution of the 28-kDa protein into the insoluble phase was not simply a consequence of intermediate filament collapse. (v) Cells that already contained collapsed intermediate filaments still showed a redistribution of the 28-kDa protein into the insoluble fraction after heat shock. (vi) Return of the 28-kDa protein into the soluble fraction after heat shock could still occur in cells containing a collapsed intermediate filament network. (vii) Inhibition of either transcription or translation either before or after the heat shock treatment (and/or subsequent recovery) did not greatly affect either the migration of the 28-kDa protein into the insoluble pellet or the kinetics of return of the 28-kDa into the soluble phase. (viii) Exposure of cells to either sodium arsenite or the amino acid analog azetidine and subsequent recovery in the absence of the drugs resulted in the cells exhibiting tolerance as assayed by the reduction of the 28-kDa protein in the insoluble fraction after a heat shock treatment.

When examining these various properties of the 28-kDa protein, it is important to remember that the protein appears to exist in a number of dynamic states and, as shown by cDNA sequence analyses, exhibits considerable homology with the α-crystallin proteins present within the lens (24, 25, 43). Although no known biological activities have been described for the α-crystallins, the fact that these proteins exhibit such high sequence homology suggests a yet-to-be-defined functional similarity with the low-MW HSPs. Similar to the lens α-crystallins (7, 46), the 28-kDa protein exists as a high-MW structure in cells after recovery from heat shock (6). In an effort to better understand the possible relationship between the α-crystallins and the 28-kDa hsp, we analyzed purified preparations of both proteins by electron microscopy. These two related proteins exhibited a remarkably similar appearance; both exhibited a spherical structure, each with a relatively constant diameter of ≅15 to 20 nm (Fig. 4). The structures were resistant to disruption by RNase, high salt, or nonionic detergent (data not shown).

Owing to the above-described data showing changes in solubility of the 28-kDa protein as a function of heat shock as well as its ability to form high-order structures, we investigated the size distribution of the 28-kDa protein in cells before and after heat shock treatment by using gel filtration analysis of the soluble phase of the cells and the detection of the 28-kDa protein via Western blots. In cells maintained at 37°C, the 28-kDa protein exhibited considerable heterogeneity, varying in size between approximately 200 and 800 kDa (Fig. 5a, forms I and II). After a 90-min heat (43°C) shock treatment, only a portion of the 28-kDa protein remained in the soluble phase; the majority of this soluble form of the protein was approximately 200 kDa in size (Fig. 5b, form I). Consistent with the data presented in Fig. 2 and 3, a severe 30-min heat (45°C) shock resulted in little of the protein remaining in the soluble phase. However, the small amount that did remain soluble appeared quite large and eluted within the void volume of the column (Fig. 5c, form III). When the cells heat shock treated at 43°C for 90 min were allowed to recover at 37°C for 12 h, much of the 28-kDa protein redistributed back into the soluble phase, with the protein exhibiting considerable heterogeneity with respect to its native size (e.g., 200 to 800 kDa; Fig. 5d). Finally, in cells first made thermotolerant and then heat (45°C) shocked for 30 min, the fraction of the 28-kDa protein remaining within the soluble phase (approximately 60% of the total) exhibited little or no change in its apparent size (Fig. 5e). Thus the apparent size of the 28-kDa protein, along with its solubility characteristics, appears dependent upon both the severity of the heat shock treatment and the prior physiologic state of the cell.

As a final approach toward characterizing this apparent dynamic distribution of the 28-kDa protein as a function of heat shock treatment, we examined the intracellular distribution of the protein by using indirect immunofluorescence analysis. For these studies we employed our rabbit polyclonal anti-28-kDa protein serum and examined the distribution of the protein in three different primate cell lines (our antibody does not cross-react with the rodent form of the protein) before and after recovery from heat shock. (Confirmation of the specificity for the 28-kDa HSP was determined by Western blotting and two-dimensional gel analysis in all three cell lines. Moreover, prior incubation of the antibody with the purified antigen resulted in diminished fluorescence.) In the case of human foreskin fibroblasts incubated at 37°C, the antibody decorated a structure within the perinuclear region of the cells (Fig. 6A and B). Little or no nuclear fluorescence was observed in 37°C cells. After a 30-min heat (44°C) shock treatment, much of the well-defined perinuclear staining appeared diminished, and there was a concomitant increased staining within the nucleus (Fig. 6C and D). With time of recovery from the heat treatment the relative intensity of the 28-kDa protein staining began to increase, especially within the perinuclear region.

TABLE 1. Analysis of 28-kDa stress protein solubility in Triton X-100-lysed HeLa cells

Stress conditions	28-kDa protein in:	
	Supernatant	Pellet
Cells maintained at 37°C		
(i) No treatment	++++	+
(ii) Cycloheximide for 1 to 7 h	++++	+
(iii) Colcemid added for 30 min	++++	+
(iv) 8-Bromo-cyclic AMP and MIX added for 30 min	++++	+
(v) Serum starved for 36 h	++++	+
(vi) As in (v), then refed with serum for 2 h	++++	+
Effect with increasing temperature		
(i) 37°C	++++	+
(ii) 43°C for 90 min	++	+++
(iii) 44°C for 30 min	+	++++
(iv) 45°C for 30 min	±	++++
Cells heat treated at 44°C for 30 min		
(i) No other treatment	+	++++
(ii) Cycloheximide added 7 h or 30 min before heat shock	+	++++
(iii) Colcemid added 30 min before heat shock	+	++++
(iv) 8-Bromo-cyclic AMP and MIX added 30 min before heat shock	+	++++
Cells heat treated during recovery period after a first heat shock		
(i) Heat shock at 43°C for 90 min followed by a recovery period of 1 h at 37°C	+++	++
(ii) As in (i) but then exposed to 44°C for 30 min	+	++++
(iii) As in (i) but recovery for 12 h at 37°C	++++	+
(iv) As in (iii) but then exposed to 44°C for 30 min	+++	++
(v) As in (i) but recovery for 24 h at 37°C	++++	+
(vi) As in (v) but then exposed to 44°C for 30 min	++	+++
Cells recovering from heat shock		
(i) Heat shock at 43°C for 90 min, no recovery	++	+++
(ii) As in (i) but recovery for 12 h at 37°C	++++	+
(iii) As in (ii) but cycloheximide added immediately after heat shock	+++	++
(iv) As in (ii) but actinomycin D added immediately after heat shock	+++	++
(v) As in (ii) but colcemid or 8-bromo-cyclic AMP and MIX added during recovery	++++	+
(vi) As in (ii) but exposed to a second heat shock at 44°C for 30 min	+++	++
(vii) As in (vi) but treatment with colcemid or 8-bromo-cyclic AMP and MIX added for 30 min before the second heat shock at 44°C	+++	++
(viii) As in (vi) but cycloheximide added immediately before the first heat shock and kept during recovery and during the second heat shock	++	+++
Effect of arsenite		
(i) Exposure for 2 h	+++	++
(ii) As in (i) but removal of metal and 12-h recovery	++++	+
(iii) As in (ii) but heat treated at 44°C for 30 min	++	+++
Effect of azetidine		
(i) Exposure for 12 h	++	+++
(ii) As in (i) but removal of analog and recovery for 24 h	++++	+
(iii) As in (ii) but heat treated at 44°C for 30 min	+++	++

[a] HeLa cells growing on 35-mm Falcon dishes were either incubated at 37°C or exposed to different types of stress. The cells were lysed in the presence of 0.5% Triton X-100, and the protein content of two subcellular fractions, the supernatant and pellet from centrifugation at 20,000 × *g* for 10 min, were analyzed on sodium dodecyl sulfate-polyacrylamide gels. The 28-kDa stress protein was localized by immunoblot and probed with anti-28-kDa protein serum and goat anti-rabbit serum conjugated with horseradish peroxidase. Equal portions of each supernatant and pellet were analyzed. The level of 28-kDa stress protein in both the supernatant and pellet was visually estimated as follows: ±, barely detectable; +, ≤25%; ++, ≤50%; +++, ≤75%; ++++, >75%. The concentrations of the drugs used in these experiments are as follows: arsenite, 0.1 mM; azetidine, 10 mM; colcemid, 0.1 mM; 8-bromo-cyclic AMP, 0.1 mM; cycloheximide, 20 μg/ml; actinomycin D, 10 μg/ml; methylisobutylxanthine (MIX), 0.1 mM.

However, even after 4 h of recovery the well-defined perinuclear structure observed in the 37°C cells had still not reappeared. Only by 8 to 12 h of recovery did the perinuclear structures seen in the 37°C cells begin to reform. Similar results were observed with either HeLa or monkey CV-1 cells.

The polarized, perinuclear staining exhibited by the antibody against the 28-kDa protein appeared similar to what has been described for a number of proteins situated in or around the Golgi complex. Therefore, a fluorescent lectin (wheat germ agglutinin), specific for a number of glycoproteins present within the mid- and trans-Golgi apparatus (49, 52), was obtained and used in a double-label staining experiment along with the anti-28-kDa protein antibody. Monkey CV-1 cells, either at 37°C or after heat shock treatment, were fixed and analyzed simultaneously for the distribution of the 28-kDa protein and the Golgi complex. Double-label staining of the 37°C cells revealed very similar staining patterns

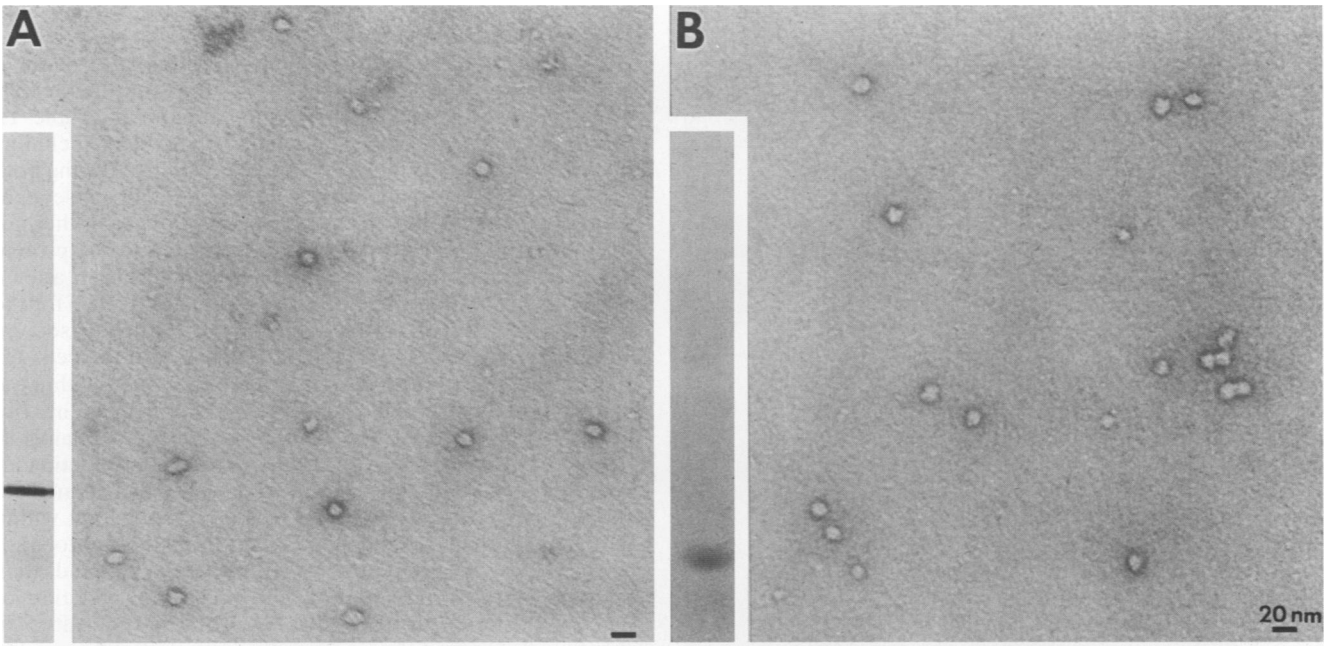

FIG. 4. Comparison of the purified 28-kDa stress protein (form II) (A) and α-crystallin (B) purified from bovine lens by electron microscopy. Both purified 28-kDa protein (form II, see Fig. 5) and α-crystallin from bovine lens were analyzed by electron microscopy by using the uranyl acetate staining method. Similar types of rounded structures of about 10 to 15 nm in diameter were observed for both proteins. Inserts show Coomassie blue-stained sodium dodecyl sulfate gels of the material analyzed by electron microscopy. (Apparent molecular mass of α-crystallin in sodium dodecyl sulfate gel is 19 to 20 kDa [7, 45].)

elicited by the Golgi lectin and the 28-kDa hsp antibody (Fig. 7A, B, and C). In cells that were first heat shock treated, slightly different staining patterns were observed. Although the lectin again decorated a structure on one side of the nucleus, the 28-kDa protein antigen was observed either within the same perinuclear region, diffuse in the cytoplasm, or, as before, within the nucleus (Fig. 7D, E, and F). These results indicate that the 28-kDa protein resides near the Golgi complex in mammalian cells maintained at 37°C and that after heat shock treatment much of the protein redistributes into the nucleus. At the present time, however, it is still not clear whether the 28-kDa protein is actually an integral component of the Golgi apparatus.

As a final approach, we investigated the intranuclear locale of the 28-kDa protein in heat-shocked HeLa cells by using immunoelectron microscopy. CV-1 cells were given a 30-min heat (45°C) shock and then either fixed and sectioned immediately or allowed to recover at 37°C for 2 h. In most of the cells right after the heat shock there were rather large, localized patches of 28-kDa protein within the nucleus (Fig. 8). These appeared to be randomly distributed within the nucleus and showed no obvious codistribution with nucleoli. If the cells were allowed to recover for 2 h after the heat treatment, many of these intranuclear aggregates of 28-kDa protein began to break up and appeared as a collection of smaller patches throughout the nucleus (Fig. 8). By 4 h of recovery no large nuclear aggregates of the protein were observed (data not shown).

DISCUSSION

The studies presented here extend our previous work on the purification and partial characterization of the 28-kDa HSP of mammalian cells (6). Unlike the situation in plants or *Drosophila* cells, where there exist multiple and in some cases related forms of HSPs between 20 and 30 kDa, in all higher eucaryotes and in avian or yeast cells there appears to be only a single low-MW HSP. The only common property exhibited by the low-MW HSPs from different species is their conserved sequence homology with certain domains of the α-crystallin proteins of the lens (24, 25, 43). The crystallin proteins are the major structural components of the lens, exhibit self-assembly properties, and often are purified as aggregates sedimenting between 15 and 20S (7, 46). As is shown here and as previously shown in mammalian (6), plant (40), *Drosophila* (1–3, 4), and avian cells (14, 15), the low-MW HSPs are similarly capable of forming homo-oligomers; the size of such oligomers varies as a function of the physiologic state of the cell.

The 28-kDa HSP was observed to assume different forms dependent upon the physiologic state of the cell. Hypotonic lysis of 37°C cells (no detergent) caused the protein to partition equally between the particulate and soluble fractions. When the cell fractionation was performed in the presence of nonionic detergent, the majority of the 28-kDa protein was found in the soluble phase. In heat-shocked cells, most of the 28-kDa protein fractionated within the low-speed pellet regardless of whether the cells were lysed in the presence or absence of detergent. This insolubility of the 28-kDa protein after heat shock is analogous to the situation in *Drosophila* cells, where all the low-MW HSPs accumulate within the nuclease-resistant and high-salt-insoluble nuclear fraction after heat shock (1, 31, 47). However, when the cells were first made thermotolerant (i.e., a prior exposure to a mild heat shock treatment), significantly less of the 28-kDa HSP was found in the insoluble phase after a second and more severe heat shock treatment. In addition, the kinetics by which the 28-kDa protein returned to the soluble phase

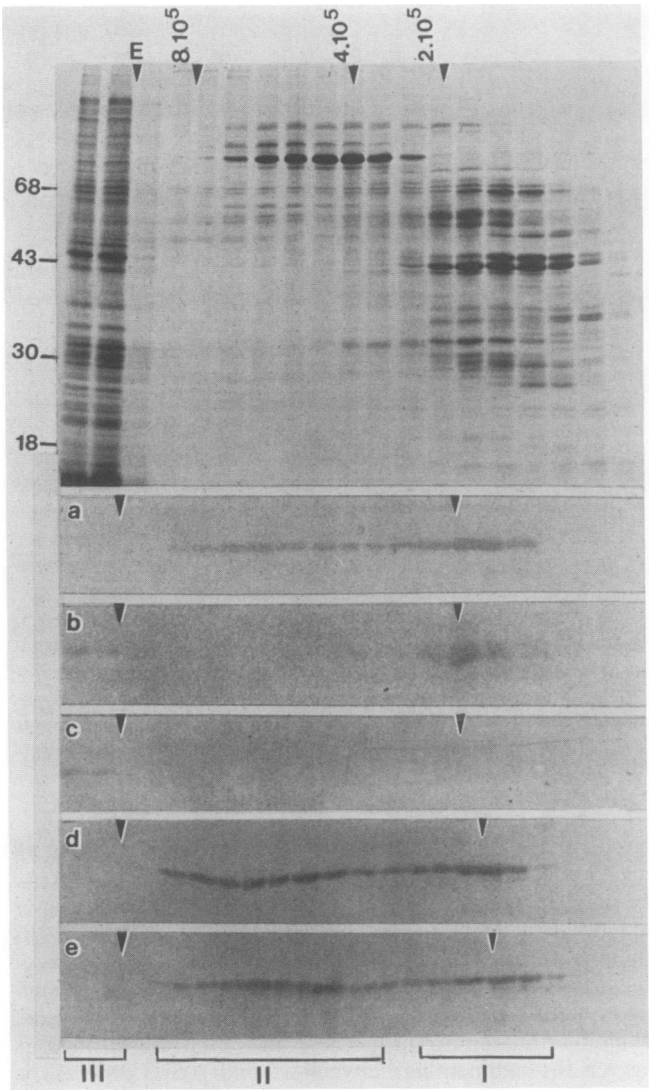

FIG. 5. Changes in the apparent size of 28-kDa protein as a consequence of heat shock treatment and recovery. HeLa cells growing in suspension (5×10^5 cells per ml) were maintained at 37°C, exposed to a 43°C for 90 min, or were first made thermotolerant and then rechallenged with a severe 30-min heat (45°C) shock. After the appropriate incubation, the cells were collected and lysed by Dounce homogenization in hypotonic buffer (no detergent), and a 20,000 × *g* supernatant was prepared and applied to an Ultrogel AcA 34 gel filtration column (1.0 by 100 cm). Similar results were obtained when cells were lysed in the presence of detergent. The column was developed in a buffer containing 20 mM Tris (pH 7.4)–20 mM NaCl–5 mM $MgCl_2$–0.1 mM EDTA–15 mM β-mercaptoethanol, and 100 1-ml fractions were collected. A portion of every third fraction was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (in duplicate). One gel was stained with Coomassie blue; the other was transferred to nitrocellulose, and the distribution of the 28-kDa protein was determined by Western blot analysis. Shown is a representative Coomassie blue-stained gel of the fractions eluting off the column from the 20,000 × *g* supernatant of cells incubated at 37°C. Shown at the top of the gel are the elution profile positions of molecular mass standards applied to the column. In panels a through e are shown the representative portions of the Western blots analyzing the elution of the 28-kDa protein in the following: a, cells maintained at 37°C; b, cells incubated at 43°C for 90 min; c, cells incubated at 45°C for 30 min; d, cells incubated at 43°C for 90 min and allowed to recover for 12 h at 37°C; e, cells made thermotolerant (as in d) and then subjected to a 45°C shock for 30 min. The designations I, II, and III at the bottom indicate the three major, broad peak distributions of the 28-kDa protein (100 to 300 kDa, 400 to 800 kDa, and ≥1 mDa, respectively). E designates the position of the exclusion (void) volume of the column. The two arrowheads in panels a through e indicate the position of the void volume and the elution position of a 200-kDa standard.

was always accelerated in the thermotolerant cells as compared with the nontolerant cells.

We have used this assay to explore a number of parameters governing the differential distribution of the 28-kDa protein as a function of heat shock treatment (Table 1). Briefly, we find a number of interesting phenomena including the following. (i) The inclusion of cycloheximide during both the initial priming heat shock treatment and subsequent recovery period at 37°C (i.e., development phase of thermotolerance) diminished the protective effect, with the protein now partitioning mainly within the insoluble phase after a second heat shock treatment, similar to that observed for the nontolerant cell. (ii) Pretreatment of the cells with sodium arsenite, removal of the metal, and recovery of the cells for 12 h caused most of the 28-kDa protein to remain within the detergent soluble phase after a heat shock treatment. (iii) Pretreatment of the cells with an amino acid analog of proline, azetidine, thereby resulting in the production of nonfunctional HSPs (20, 33, 39, 56), and subsequent heat shock treatment resulted in the protein fractionating within the insoluble pellet, similar to results with the nontolerant cells. However, pretreatment with the analog and then removal of the drug, thereby allowing for production of functional stress proteins (20, 39, 56), now resulted in significantly more of the protein remaining within the soluble phase after heat shock treatment, similar to the effect with thermotolerant cells. These results, overall, are entirely consistent with previous studies examining the phenomenon of thermotolerance. Specifically, a prior induction of the stress response, by whatever agent, results in the cells acquiring a significant, although transient, tolerance to a second and what would otherwise be a lethal heat shock challenge. Moreover, previous studies (22, 33, 35, 39) and those shown here indicate a requirement for the prior synthesis (or activation) of the stress proteins for the development of tolerance. For example, inclusion of cycloheximide during the development phase of tolerance decreases the protective effect. In summary we suggest that the distribution of the 28-kDa protein within either the soluble or the insoluble fraction after heat shock treatment may prove to be a simple and reliable assay by which to ascertain the thermotolerant status of a cell. Whereas nontolerant cells exhibit the protein within the insoluble fraction after heat shock (and a corresponding slow return to the soluble phase during recovery), tolerant cells appear to exhibit markedly less of the protein in the insoluble phase after heat shock (with that portion of the protein which does become insoluble returning to the soluble phase with faster kinetics). Indeed a similar situation has already been described in vivo in *D. melanogaster* (1). In pupae exposed to a drastic heat shock treatment and subsequent detergent lysis of the whole insect, the 23-kDa hsp accumulates with the insoluble fraction; as shown in parallel experiments, the pupae do not develop into mature flies. However, exposure of the pupae to a mild heat shock treatment before the drastic heat treatment (i.e., pupae made thermotolerant) results in considerably less 23-kDa protein accumulating within the insoluble pellet and a considerably higher percentage of the pupae emerging as adult flies (1).

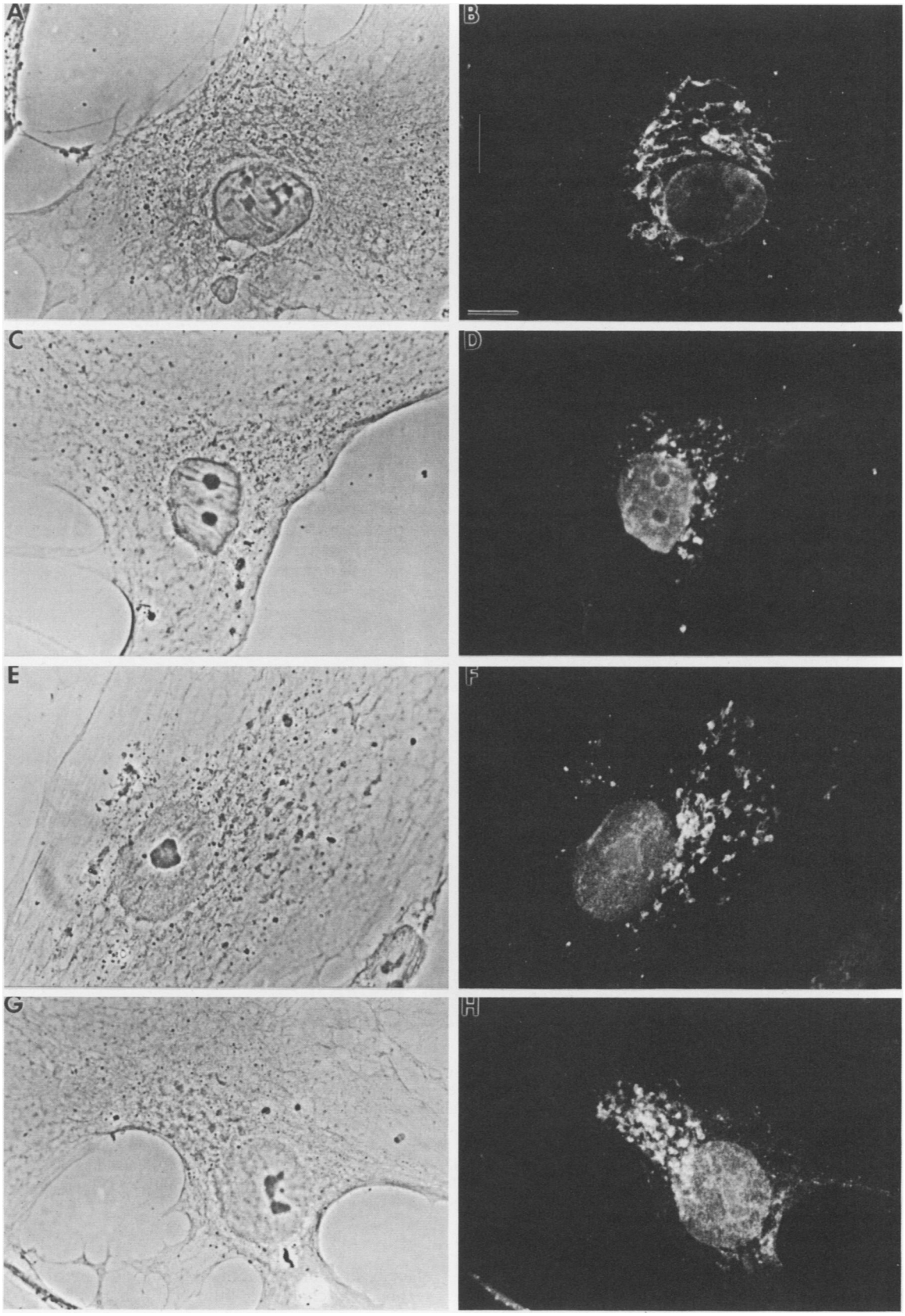

FIG. 6. Intracellular locale of the 28-kDa protein in human foreskin fibroblasts before and after heat shock treatment. Human foreskin fibroblasts (HF) growing on glass cover slips were either (A and B) kept at 37°C, (C and D) heat treated for 30 min at 44°C, or (E through H) heat treated at 44°C for 30 min and allowed to recover at 37°C for either (E and F) 2 or (G and H) 4 h. The cells were fixed with 2% paraformaldehyde and permeabilized with 0.1% Triton X-100 before being processed for indirect immunofluorescence by using anti-28-kDa protein serum and fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin G (B, D, F, and H; corresponding phase-contrast micrographs are shown in A, C, E, and G, respectively). Bar, 10 μm.

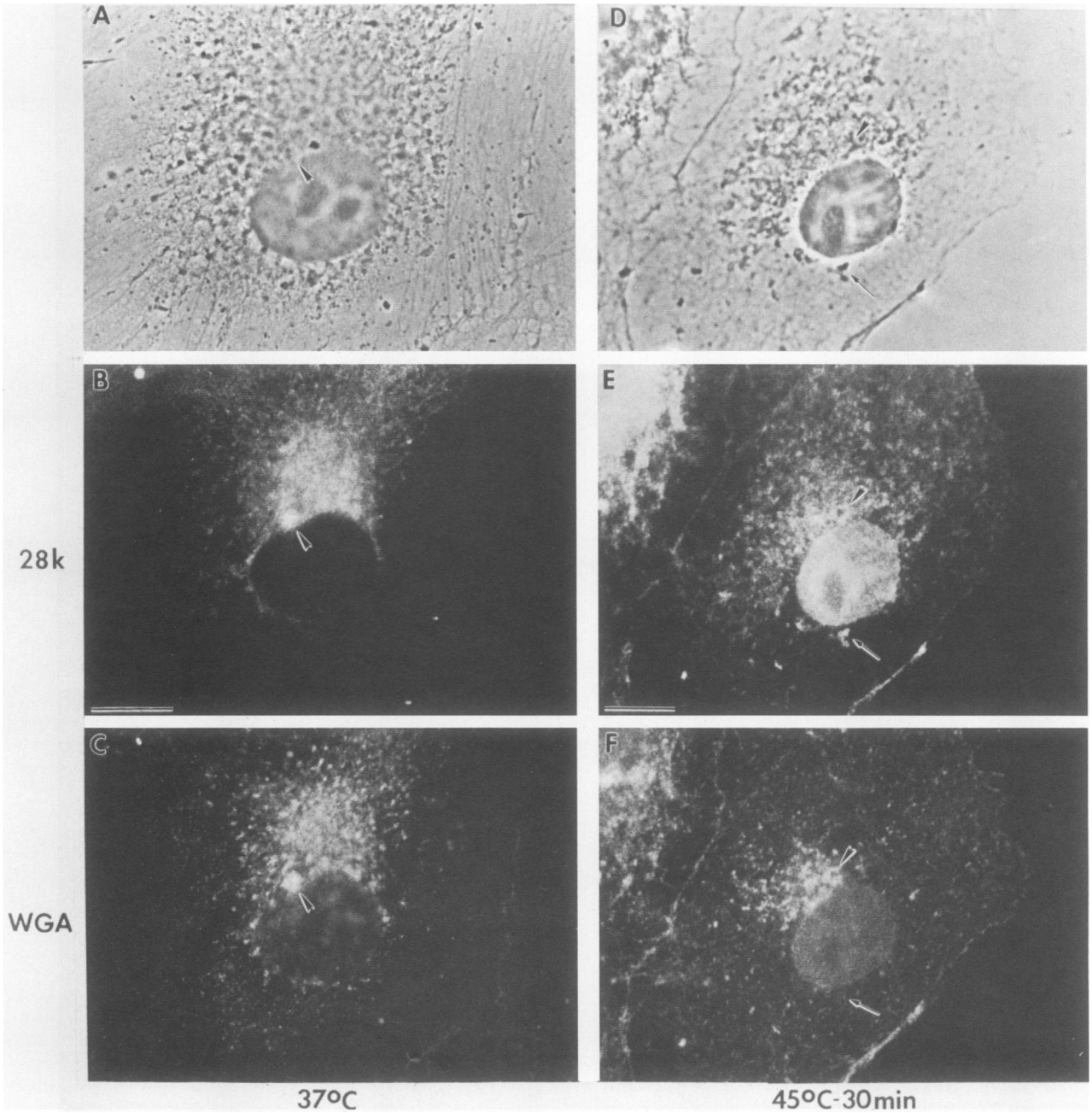

FIG. 7. Distribution of the 28-kDa protein in 37°C cells coincides with the Golgi complex. CV-1 cells growing on glass cover slips were incubated either at 37°C or at 44°C for 30 min. The cells were then fixed, permeabilized, and then analyzed by double-label indirect immunofluorescence with the rabbit polyclonal anti-28-kDa protein antibody and a rhodamine-conjugated wheat germ agglutinin (WGA), which recognizes a number of glycoproteins present within the Golgi complex. Visualization of the rabbit anti-28-kDa protein antibody was performed with fluorescein-conjugated goat anti-rabbit antibody. (A, B, and C), phase contrast, 28-kDa protein staining, and wheat germ agglutinin staining, respectively, in the same cell incubated at 37°C. (D, E, and F), phase contrast, 28-kDa protein staining, and wheat germ agglutinin staining, respectively, in the same cell incubated at 44°C for 30 min. The codistribution of the 28-kDa protein and wheat germ agglutinin is indicated by arrowheads, and the arrows indicate cytoplasmic 28-kDa protein staining not coincident with wheat germ agglutinin staining. Bars, 10 μm.

A possible explanation for this dynamic distribution of the 28-kDa HSP is the fact that the protein appears capable of forming a variety of higher-ordered structures in the cell as a function of heat shock or stress. For example, gel filtration analysis demonstrated that the 28-kDa HSP exists in size between 200 and 800 kDa in the 37°C cells and 800 to 2,000 kDa or greater in cells after heat shock treatment. In the case of cells first made thermotolerant and then exposed to a second heat challenge, little or no change in the size of the protein was observed. Therefore, a possible explanation for the redistribution and/or insolubility of the 28-kDa HSP after heat shock treatment is the apparent aggregation of the protein into higher-MW complexes. These results are reminiscent of the situation with the structurally related α-

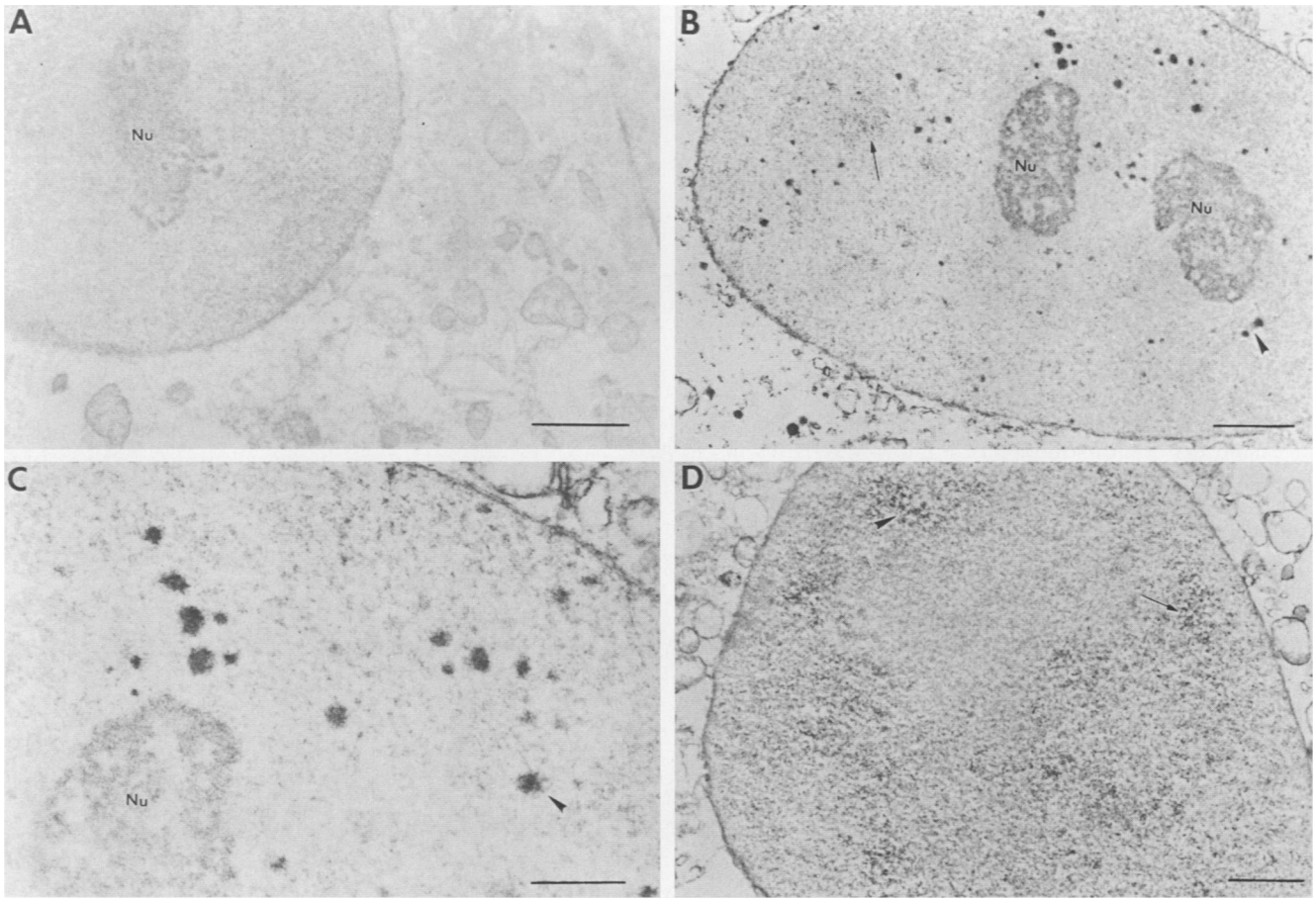

FIG. 8. The 28-kDa protein forms large intranuclear aggregates during heat shock. CV-1 cells were heat shocked at 44°C for 30 min and either fixed immediately (see Materials and Methods) or allowed to recover at 37°C for 2 h and then fixed. The cells were then examined for the distribution of the 28-kDa protein by immunoelectron microscopy with the 28-kDa protein antibody as described in Materials and Methods. Shown are sections of the nucleus. The nucleoli in panels A, B, and C are designated Nu, the arrowheads indicate the aggregates of the 28-kDa protein, and the arrow indicates aggregates of 28-kDa protein which appear to be breaking up during the recovery from heat shock. (A) Cells incubated only with the second peroxidase-conjugated goat anti-rabbit antibody (i.e., control). (B and C) Low- and high-magnification micrographs, respectively, of nuclei from heat-shocked cells. (D) High-magnification micrograph of nuclei from heat-shocked cells recovered for 2 h at 37°C. Bars A, 2 μm; B, 2 μm; C, 1 μm; D, 2 μm.

crystallin proteins, which similarly exhibit heterogeneity in size as a function of environmental factors such as temperature, pH, and ionic strength. Indeed, both the 28-kDa HSP and α-crystallin form ≅15-nm spherical structures that resemble one another in the electron microscope. We suspect that those domains of the 28-kDa HSP which are homologous with the α-crystallin protein are responsible for the self-aggregating properties exhibited by these two seemingly different biological molecules. We are currently examining whether posttranslational modifications of the 28-kDa HSP, such as phosphorylation, may regulate the oligomerization status of the 28-kDa HSP.

The indirect immunofluorescence experiments using our polyclonal antibody to the 28-kDa protein revealed the protein to be distributed in a polarized fashion within the perinuclear region of the cell. Because the Golgi complex is also known to be localized in a polarized fashion, we examined, by double-label staining, the relative distribution of the 28-kDa protein and the Golgi complex. In every primate cell we have examined (our antibody cross-reacts poorly, if at all, with other species), the two staining patterns appeared coincident. In some cells a weak endoplasmic reticulum-like staining of the 28-kDa protein was also observed. Immediately after heat shock we observed a decrease in the perinuclear staining and a concomitant increased staining of the 28-kDa protein within the nuclear region. Although the cytoplasmic staining pattern was no longer observed when the cells were first extracted with detergent, much of the nuclear staining after heat shock appeared resistant to detergent extraction (data not shown); these results are consistent with our biochemical fractionation studies. With time of recovery after heat shock, the nuclear staining gradually decreased and the cytoplasmic staining began to increase. In addition, much of the staining during the recovery period appeared to consist of large aggregates of the 28-kDa protein within the cytoplasm. Similar cytoplasmic aggregates have been observed for the low-MW HSP from avian and plant cells after heat shock (14, 15, 40). By 12 h of recovery from the heat shock treatment, the well-defined Golgi-like staining patterns of the 28-kDa HSP had returned. A second heat shock treatment of the tolerant cells resulted in markedly less nuclear staining and less disruption of the cytoplasmic Golgi-like staining patterns of the 28-kDa protein (data not shown). As a final

point with respect to its intracellular locale, agents that disturb the structure and organization of the Golgi (monensin, colcemide [29, 44, 48]) similarly result in a disruption of perinuclear 28-kDa protein staining. However, in those cells containing a disrupted Golgi complex, we observe different intracellular locales of the 28-kDa protein and the residual Golgi complex. Hence, it is possible that although the 28-kDa HSP resides near the Golgi complex, it may in fact turn out not to be an integral Golgi-associated protein. In this respect, however, it is interesting to note that HSP 25 from maize has been described to colocalize with the endoplasmic reticulum and the Golgi apparatus (16) and that the homologous α-crystallin proteins have been described to interact with membranes (42).

Previous studies have shown that heat shock treatment results in a collapse of the vimentin-containing intermediate filament cytoskeleton around the nucleus (9, 55). It has been suggested that the distribution of the low-MW HSPs in the detergent-insoluble nuclear-cytoskeletal fraction after heat shock is a consequence of such filament collapse (30). Although we cannot rule out this possibility entirely, we have shown here that treatment of cells with various other agents that promote the collapse of the intermediate filaments does not result in the redistribution of the 28-kDa protein into the insoluble fraction. Rather, the protein remains within the soluble phase, similar to the protein in the untreated control cells. Moreover, when the drug-treated cells, which now contain a collapsed intermediate filament network, are then heat shocked, the 28-kDa protein does redistribute into the insoluble fraction. These results indicate that (i) the simple collapse of the intermediate filaments is not sufficient to result in a redistribution of the 28-kDa protein, and (ii) the 28-kDa protein can move into the insoluble fraction regardless of the state of the intermediate filaments. Finally our data also indicated that the return of the 28-kDa protein from the insoluble fraction to the soluble fraction during recovery from heat shock treatment was independent of transcription, translation, or the status (normal or collapsed) of the intermediate filaments.

In summary, the low-MW HSP of mammalian cells appears to be a dynamic protein whose structure and solubility are dependent upon the physiologic state of the cell. The protein is normally present within the perinuclear region of nonstressed cells in proximity with the Golgi. After heat shock, much of the protein relocalizes into the nucleus, assembles into large aggregates, and is resistant to detergent extraction. In current studies we are examining (i) the proteins possibly associated with the 28-kDa protein in 37°C cells or in cells after heat shock, (ii) the possible role of phosphorylation in modulating the dynamic state of the 28-kDa protein, and (iii) whether the 28-kDa protein is in fact a component of the Golgi and somehow plays a role in protein trafficking or secretion.

ACKNOWLEDGMENTS

We are grateful to J. D. Watson for his continued, enthusiastic support of this work. We thank Fiona Giblen (London, United Kingdom) for a gift of purified bovine α-crystallin, Mary Mulcahy, Laura Cipp, and Marie-Christine Arrigo for excellent technical assistance, Philip Renna for photographic work, and Madeline Szadkowski for typing the manuscript. Thanks to Jim Feramisco, Les Mizzen, and Karl Riabowol for helpful discussions.

We acknowledge the Cold Spring Harbor electron microscope facility supported by Public Health Service grants I510 RR03430-01 and IP30 CA34408-01 from the National Institutes of Health, National Science Foundation grant BB5-8604215, and the Fannie H. Rippel Foundation. This work was supported by Public Health Service grants GM33551 and HL23848 from the National Institutes of Health to W. J. Welch and by a Bristol Myers Institutional grant to A.-P. Arrigo.

LITERATURE CITED

1. **Arrigo, A.-P.** 1987. Cellular localization of HSP23 during *Drosophila* development and subsequent heat shock. Dev. Biol. **122**:39–48.
2. **Arrigo, A.-P., and C. Ahmad-Zadeh.** 1981. Immunofluorescence localization of a small heat shock protein (HSP23) in salivary gland cells of *Drosophila melanogaster*. Mol. Gen. Genet. **184**: 73–79.
3. **Arrigo, A.-P., S. Fakan, and A. Tissieres.** 1980. Localization of the heat shock-induced proteins in *Drosophila melanogaster* tissue culture cells. Dev. Biol. **78**:86–103.
4. **Arrigo, A.-P., and D. Pauli.** 1988. Characterization of HSP27 and three immunologically related polypeptides during *Drosophila* development. Exp. Cell. Res. **175**:169–183.
5. **Arrigo, A.-P., K. Tanaka, A. L. Goldberg, and W. J. Welch.** 1988. Identity of the 19S "prosome" particle with the large multifunctional protease complex of mammalian cells (the proteasome). Nature (London) **331**:192–194.
6. **Arrigo, A.-P., and W. J. Welch.** 1987. Characterization and purification of the small 28,000-dalton mammalian heat shock protein. J. Biol. Chem. **262**:15359–15369.
7. **Augusteyn, R. C., and J. F. Koretz.** 1987. A possible structure for α-crystallin. FEBS Lett. **222**:1–5.
8. **Barnett, T., M. Altschuler, C. N. McDaniel, and J. P. Mascarenhas.** 1980. Heat shock induced proteins in plant cells. Dev. Genet. **1**:331–340.
9. **Biesman, H. F. G., H. Falkner, H. Saumweber, and M. F. Walter.** 1982. Disruption of the vimentin cytoskeleton may play a role in heat-shock response, p. 275–281. *In* M. J. Schlesinger, M. Ashburner, and A. Tissieres (ed.), Heat shock: from bacteria to man. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
10. **Bowen, B., J. Steinberg, and U. K. Laemmli.** 1980. The detection of DNA-binding proteins. Nucleic Acids Res. **8**:1–20.
11. **Brugge, J. S.** 1985. Interaction of the Rous sarcoma virus protein, $pp60^{src}$, with the cellular proteins, pp50 and pp90. Curr. Top. Microbiol. Immunol. **122**:59–81.
12. **Catelli, M. G., N. Binart, I. Jung-Testas,. J. M. Renoir, E. E. Baulieu, J. R. Feramisco, and W. J. Welch.** 1985. The common 90-kd protein component of non-transformed "8S" steroid receptors is a heat-shock protein. EMBO J. **4**:3131–3135.
13. **Chirico, W. J., M. C. Waters, and G. Blobel.** 1988. 70k heat shock related proteins stimulate protein translocation into microsomes. Nature (London) **332**:805–810.
14. **Collier, N. C., and M. Schlesinger.** 1986. The dynamic state of heat shock proteins in chicken embryo fibroblasts. J. Cell Biol. **103**:1495–1507.
15. **Collier, N. C., J. Heuser, M. Aach-Levy, and M. Schlesinger.** 1988. Ultrastructural and biochemical analysis of the stress granules in chicken embryo fibroblasts. J. Cell Biol. **106**:1131–1139.
16. **Cooper, P., and T.-H. David Ho.** 1987. Intracellular localization of heat shock proteins in maize. Plant Physiol. **84**:1197–1203.
17. **Corces, V., R. Holmgreen, R. Freund, R. Morimoto, and M. Meselson.** 1980. Four heat shock proteins of Drosophila melanogaster coded with a 12-kilobase region in chromosome subdivision 67B. Proc. Natl. Acad. Sci. USA **77**:5390–5393.
18. **Craig, E. A., and K. Jacobsen.** 1984. Mutation of the heat inducible 70 kilodalton genes of yeast confer temperature sensitive growth. Cell **38**:841–849.
19. **Deshaies, R. J., B. D. Koch, M. Werner-Washburne, E. A. Craig, and R. Schekman.** 1988. A sub-family of stress proteins facilitates translocation of secretory and mitochondrial precursor polypeptides. Nature (London) **332**:800–805.
20. **DiDomenico, B. J., G. E. Bugaisky, and L. Lindquist.** 1982. The heat shock response is self regulated at both the transcriptional and post-transcriptional levels. Cell **31**:593–603.
21. **Gerner, E. W., and M. J. Schneider.** 1975. Induced thermal resistance in Hela cells. Nature (London) **256**:500–502.

22. **Henle, H. J., and D. B. Leeper.** 1982. Modification of heat response and thermotolerance by cycloheximide, hydroxyurea and lucanthone in CHO cells. Radiat. Res. **90**:339–347.
23. **Hickey, E. D., and L. A. Weber.** 1982. Modulation of heat shock polypeptide synthesis in Hela cells during hyperthermia and recovery. Biochemistry **21**:1513–1521.
24. **Hickey, E. S., E. Brandom, R. Potter, G. Stein, J. Stein, and L. A. Weber.** 1986. Sequence and organization of genes encoding the human 27 kDa heat shock protein. Nucleic Acids Res. **14**:4127–4145.
25. **Ingolia, T. D., and E. A. Craig.** 1982. Four small *Drosophila* heat shock proteins are related to each other and to mammalian alpha-crystallin. Proc. Natl. Acad. Sci. USA **79**:2360–2364.
26. **Kelley, P. M., and M. J. Schlesinger.** 1978. The effect of amino acid analogues and heat shock on gene expression in chicken embryo fibroblasts. Cell **15**:1277–1286.
27. **Kim, Y.-J., J. Shuman, M. Sette, and A Przybyla.** 1984. Nuclear localization and phosphorylation of three 25-kilodalton rat stress proteins. Mol. Cell. Biol. **4**:468–474.
28. **Landry, J., D. Bernier, P. Chretien, L. Nicole, R. M. Tanguay, and N. Marceau.** 1982. Synthesis and degradation of heat shock proteins during development of thermotolerance. Cancer Res. **42**:2457–2461.
29. **Ledger, P. W., N. Uchida, and M. L. Tanzer.** 1980. Immunocytochemical localization of procollagen and fibronectin in human fibroblasts: effect of the monovalent ionophore: monensin. J. Cell Biol. **87**:663–671.
30. **Leicht, B. G., H. Biessman, K. Palter, and J. J. Bonner.** 1986. Small heat shock proteins of *Drosophila* associate with the cytoskeleton. Proc. Natl. Acad. Sci. USA **83**:90–94.
31. **Levinger, L., and A. Varshavsky.** 1981. Heat shock proteins of *Drosophila* are associated with nuclease-resistant, high-salt resistant nuclear structures. J. Cell Biol. **90**:793–796.
32. **Li, G. C.** 1983. Induction of thermotolerance and enhanced heat shock protein synthesis in Chinese hamster fibroblasts by sodium arsenite and by ethanol. J. Cell. Physiol. **115**:116–122.
33. **Li, G. C., and A. Laslo.** 1985. Amino acid analogs while inducing heat shock proteins sensitive CHO cells to thermal damage. J. Cell. Physiol. **122**:91–97.
34. **Li, G. C., and Z. Werb.** 1982. Correlation between synthesis of heat shock proteins and development of thermotolerance in Chinese hamster fibroblasts. Proc. Natl. Acad. Sci. USA **79**: 3218–3222.
35. **Loomis, W. F., and S. Wheeler.** 1980. Heat shock response in Dictyostelium. Dev. Biol. **79**:399–408.
36. **McAlister, L., and D. B. Finkelstein.** 1980. Heat shock proteins and thermal resistance in yeast. Biochem. Biophys. Res. Commun. **93**:819–824.
37. **McAlister, L. V., S. Strausberg, A. Kalaga, and D. B. Finkelstein.** 1979. Altered patterns of synthesis induced by heat shock in yeast. Curr. Genet. **1**:63–74.
38. **Mitchell, H. K., G. Moller, N. S. Petersen, and L. Lipps-Sarmiento.** 1979. Specific protection from phenocopy induction by heat shock. Dev. Genet. **1**:181–192.
39. **Mizzen, L., and W. J. Welch.** 1988. Characterization of the thermotolerant cell. I. Effects on protein synthesis activity and the regulation of HSP70 expression. J. Cell Biol. **106**:1105–1116.
40. **Nover, L., K. D. Sharf, and D. Neumann.** 1983. Formation of cytoplasmic heat shock granules in tomato cell culture and leaves. Mol. Cell. Biol. **3**:1648–1655.
41. **Petersen, N. S., and H. K. Mitchell.** 1981. Recovery of protein synthesis after heat shock: prior treatment affects the ability of cells to translate mRNA. Proc. Natl. Acad. Sci. USA **78**:1708–1711.
42. **Ramaekers, F. C. S., I. Dunia, H. J. Dodemont, E. L. Benedetti, and H. Bloemendal.** 1982. Lenticular intermediate-sized filament: biosynthesis and interaction with plasma membrane. Proc. Natl. Acad. Sci. USA **79**:3208–3212.
43. **Rassnak, R. H., and E. P. M. Candido.** 1985. Locus encoding a family of small heat shock genes in *Caenorhabditis elegans*: two genes duplicated to form a 3.8-kilobase inverted repeat. Mol. Cell. Biol. **5**:1268–1278.
44. **Robbins, E., and N. K. Gonatas.** 1964. Histochemical and ultrastructural studies on Hela cell cultures exposed to spindle inhibitors with special reference to the interphase cell. J. Histochem. Cytochem. **12**:704–711.
45. **Sanchez, E. R., D. O. Toft, M. J. Schlesinger, and W. B. Pratt.** 1985. Evidence that the 90 kDa phosphoprotein associated with the untransformed L-cell glucocorticoid receptor is a murine heat shock protein. J. Biol. Chem. **260**:12398–12405.
46. **Seizen, R. J., J. G. Bindel, and H. J. Hoenders.** 1978. The quaternary structure of bovine alpha-crystallin. Eur. J. Biochem. **91**:387–396.
47. **Sinibaldi, R. M., and P. W. Morris.** 1981. Putative function of *Drosophila melanogaster* heat shock proteins in the nucleoskeleton. J. Biol. Chem. **256**:10735–10738.
48. **Tartakoff, A. M., and P. Vassalli.** 1977. Plasma cell immunoglobulin secretion. J. Exp. Med. **146**:1332–1345.
49. **Tartakoff, A. M., and P. Vassalli.** 1983. Lectin-binding sites as markers of Golgi subcompartment proximal-to-distal maturation of oligosaccharides. J. Cell Biol. **97**:1243–1248.
50. **Thomas, G. P., W. J. Welch, M. B. Mathews, and J. Feramisco.** 1982. Molecular and cellular effect of heat shock and related treatments of mammalian tissue culture cells. Cold Spring Harbor Symp. Quant. Biol. **46**:985–996.
51. **Tissieres, A., H. K. Mitchell, and U. M. Tracy.** 1974. Protein synthesis in salivary glands of *Drosophila melanogaster*. J. Mol. Biol. **84**:389–398.
52. **Virtanen, I., P. Ekblom, and P. Laurila.** 1980. Subcellular compartmentalization of saccharide moieties in cultured normal and malignant cells. J. Cell Biol. **85**:429–434.
53. **Welch, W. J.** 1985. Phorbol ester, calcium ionophore, or serum added to quiescent rat embryo fibroblast cells result in the elevated phosphorylation of two 28,000-dalton mammalian stress proteins. J. Biol. Chem. **260**:3058–3062.
54. **Welch, W. J., and J. R. Feramisco.** 1985. Rapid purification of mammalian 70,000-dalton stress proteins. Mol. Cell. Biol. **5**: 1229–1237.
55. **Welch, W. J., J. R. Feramisco, and S. H. Blose.** 1985. The mammalian stress response and the cytoskeleton: alterations in intermediate filaments. Ann. N.Y. Acad. Sci. **455**:57–67.
56. **Welch, W. J., and J. P. Suhan.** 1986. Cellular and biochemical events in mammalian cells during and after recovery from physiological stress. J. Cell Biol. **103**:2035–2052.
57. **Zeller, R., T. Nyffenegger, and E. M. DeRobertis.** 1983. Nucleocytoplasmic distribution of snRNPs and stockpiled snRNA binding proteins during oogenesis and early development in *Xenopus laevis*. Cell **32**:425–434.