The Dynamic State of Heat Shock Proteins in Chicken Embryo Fibroblasts

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Abstract. Subcellular fractionation and immunofluorescence microscopy have been used to study the intracellular distributions of the major heat shock proteins, hsp 89, hsp 70, and hsp 24, in chicken embryo fibroblasts stressed by heat shock, allowed to recover and then restressed. Hsp 89 was localized primarily to the cytoplasm except during the restress when a portion of this protein concentrated in the nuclear region. Under all conditions, hsp 89 was readily extracted from cells by detergent. During stress and restress, significant amounts of hsp 70 moved to the nucleus and became resistant to detergent extraction. Some of this hsp 70 was released from the insoluble form in an ATP-dependent reaction. Hsp 24 was confined to the cytoplasm and, during restress, aggregated to detergent-insoluble perinuclear phase-dense granules. These granules dissociated during recovery and hsp 24 could be solubilized by detergent. The nuclear hsps reappeared in the cytoplasm in cells allowed to recover at normal temperatures. Sodium arsenite also induces hsps and their distributions were similar to that observed after a heat shock, except for hsp 89, which remained cytoplasmic. We also examined by immunofluorescence the cytoskeletal systems of chicken embryo fibroblasts subjected to heat shock and found no gross morphological changes in cytoplasmic microfilaments or microtubules. However, the intermediate filament network was very sensitive and collapsed around the nucleus very shortly after a heat shock. The normal intermediate filament morphology reformed when cells were allowed to recover from the stress. Inclusion of actinomycin D during the heat shock – a condition that prevents synthesis of the hsps – did not affect the intermediate filament collapse, but recovery of the normal morphology did not occur. We suggest that an hsp(s) may aid in the formation of the intermediate filament network after stress.

A brief exposure of most cells to a heat shock initiates a set of events that ultimately leads to the selective synthesis of a small set of highly conserved proteins. A similar response is elicited when cells are exposed to other types of stress agents such as heavy metals, oxidants, amino acid analogues, etc. (For reviews of the heat shock or stress response, see references 4, 13, 31, 38.) The major stress proteins identified in all cells can generally be divided into three groups based on their subunit molecular masses of 80,000–90,000, 65,000–75,000, and 20,000–30,000 D. We know rather little about the function of these proteins, but there is strong evidence that their presence can protect cells from injury or repair damage imposed by the stress. Pretreatment of cells and intact organisms to a mild stress confers a tolerance to a second more intense stress, producing a very profound effect on survival rates (see reference 26). In addition, the degradation of the major heat shock proteins (hsps)[1] correlates with the decay of thermotolerance (22), and the Drosophila 70 kD hsp is implicated in repair of nucleolar morphology after heat shock (34).

Our laboratory has been studying the heat shock response in primary cultures of chicken embryo fibroblasts (CEF) and we partially characterized and raised monospecific antibodies against three major chicken stress proteins with subunit molecular masses of 89, 70, and 24 kD (17, 18, 37). We wish to know how these proteins could function in the recovery of cells from heat shock and other stresses. To gain possible clues to their putative role in repair and protection, we carried out a series of experiments designed to determine their cellular location during the initial stress and to follow their movement after recovery from stress and during a second restress event. We wished also to relate these findings to the localization of recognized cellular structures. Numerous studies of Drosophila and mammalian tissue culture cells (24, 34, 45, 48) have established that the subcellular location of hsp 70 is dependent on the stressed state of the cell. Upon stress, hsp 70 is concentrated in the nucleus and/or nucleoli; during recovery hsp 70 returns to the cytoplasm until another stress is encountered. A similar cytoplasmic and nuclear location of the Drosophila small hsps has been reported (1, 3,

1. *Abbreviations used in this paper*: FCS, fetal calf serum; hsp(s), heat shock protein(s); MEM, Eagle's minimal essential medium; NP-40, Nonidet P-40; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone.

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40, 42). In contrast to the intensive work on hsp 70, very few studies have addressed the location of the large (80–90 kD) and small (20–30 kD) hsps in species other than Drosophila.

In this report we have combined subcellular fractionation and immunofluorescence microscopy of heat-shocked CEF to examine both the location and possible changes in the hsp 89, hsp 70, and hsp 24 of the CEF. We also examined the effects of heat shock on the morphology of the major cytoskeletal systems – microfilaments, intermediate filaments, and microtubules. We found that the distributions of CEF hsp 89, hsp 70, and hsp 24 are dynamic, and vary with the stress state of the cell. Both hsp 70 and hsp 89 move from the cytoplasm to the nucleus upon stress, whereas hsp 24 aggregates into phase-dense granules. Minor differences in the morphology of CEF microfilaments and microtubules were detected upon a heat shock; however the intermediate filament network was dramatically altered upon heat shock, losing its normal splayed pattern and relocating to the perinuclear region of the cell. Formation of the normal intermediate filament network was rapid upon removal of the stress agent, but this recovery appeared to be related to induction of hsps.

Materials and Methods

Cell Culture

Secondary CEF from 11 d embryos were prepared as previously described (17). Cells were plated in culture dishes and grown to confluency overnight in Eagle's minimal essential medium (MEM) supplemented with 3% fetal calf serum (FCS). Before heat shock, the medium was replaced with MEM supplemented with 1% FCS and 10 mM Hepes (pH 7.4) to maintain a constant pH. Cells were placed in a 45°C thermostated shaking water bath for the times indicated in the text. Control cells were held at 37°C. For recovered cells, medium was again replaced with fresh MEM supplemented with 1% FCS and cultures were held at 37°C for 15–20 h. For labeling, cell cultures were incubated for 30 min in MEM lacking methionine but supplemented with 1% FCS and 10 mM Hepes (pH 7.4) followed by the addition of 15 μCi of L[^{35}S]methionine (1485 C/mmol, Amersham Corp., Arlington Heights, IL)/10^7 cells for 3 h at the temperature indicated.

Cell Fractionation

Cells in monolayers were washed twice with ice-cold PBS (137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, and 1.5 mM KH_2PO_4) after removal of the medium. Cells were scraped into PBS and centrifuged at 4000 rpm for 10 min at 4°C. The pelleted cells were resuspended in 146 mM sucrose, 100 mM KCl, 10 mM Hepes (pH 7.0), and 1.5 mM $MgCl_2$ (Buffer A), and centrifuged. The washed cells were lysed in Buffer A (1 ml/10^7 cells) containing 0.25% Triton X-100, 0.1% deoxycholate, and 1 mM phenylmethylsulfonyl fluoride and the nuclei removed by centrifugation at 4000 rpm for 10 min at 4°C. The soluble or cytoplasmic fraction was collected and the nuclear-cytoskeletal fraction washed with Buffer A containing Triton X-100 and deoxycholate and then with 146 mM sucrose, 100 mM KCl, 10 mM Tris-Cl (pH 7.5), 0.5 mM $CaCl_2$, and 0.5 mM phenylmethylsulfonyl fluoride (0.3 ml/10^7 cells, 28). The nuclear-cytoskeletal fraction was solubilized in Laemmli (21) sample buffer (250 mM Tris-Cl [pH 6.8] 2% SDS, 10% glycerol, and 5% 2-mercaptoethanol with bromophenol blue as a dye marker) before radioimmune precipitation and SDS PAGE.

Immunofluorescence

Secondary CEF were grown at reduced confluency on ethanol cleaned coverslips in MEM containing 3% FCS. Heat shock conditions were identical to those described above. CEF on coverslips were washed three times quickly with PBS prewarmed to 37°C. Cells were fixed with 3% paraformaldehyde in PBS for 30 min at room temperature. The coverslips were washed three times with PBS and cells permeabilized with 0.1% Triton X-100 in PBS for 10 min. Lysis of cells with 0.5% Nonidet P-40 (NP-40) yielded the same results. Lysed cells were washed twice with PBS, incubated with PBS containing 1 mg/ml glycine for 10 min, and washed with PBS containing 0.1% BSA before incubation with antibody. For some samples, cells were lysed before fixing by washing first with PBS containing 5 mM $MgCl_2$ and 3 mM EGTA, and then gently swirling for 1 min with Buffer B (70 mM KCl, 3 mM Hepes, [pH 7.0], 0.5 mM $MgCl_2$, 3 mM EGTA, 1 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride) containing 0.5% NP-40. The material remaining on the coverslip was gently washed twice with Buffer B and fixed with formaldehyde as described above.

Fixed cells were incubated for 30 min at room temperature with an immunoglobulin fraction of rabbit anti-chicken hsp serum (hsp 24 at 0.4 μg/μl, hsp 70 at 0.56 μg/μl, or hsp 89 at 0.72 μg/μl), rabbit anti-chicken vimentin serum (5, as provided by A. Laszlo, Washington University Medical School, St. Louis, MO) rabbit anti-tubulin serum, or rabbit anti-actin serum (Miles Scientific Div., Miles Laboratories Inc., Naperville, IL). Properties of antibodies specific for each of the chicken hsps has been previously described (18). The cells were washed with PBS containing 0.1% BSA and then incubated 30 min with rhodamine-conjugated goat anti-rabbit IgG at a 1:50 dilution (~0.36 μg/μl; Cappel Laboratories, Cochranville, PA). Before use, the second antibody was incubated twice for 1 h each with methanol-fixed (−20°C, 15 min) nonstressed CEF. Coverslips were washed three times with PBS containing 0.1% BSA and mounted in 65% PBS/35% glycerol. A Leitz-Ortholux microscope equipped with epifluorescence optics and a Phaco 40X/0.65 lens was used for obtaining photographs on Kodak Tri-X film.

ATP-dependent Release of Proteins from the Nuclear–Cytoskeletal Fractions

The nucleotide-dependent release of proteins from nuclear-cytoskeletal fractions was performed essentially as described by Lewis and Pelham (25) as follows. CEF were heat shocked, labeled with [^{35}S]methionine, and restressed as described above. Monolayers of confluent cells (on a 4.9-cm^2 dish) were lysed with 1% NP-40 in TBS (25 mM Tris-Cl, pH 7.4, 140 mM NaCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonylfluoride). The cellular material solubilized by the detergent was aspirated and the nuclear-cytoskeletal fraction that remains on the dish was washed twice with TBS. TBS containing appropriate nucleotides (50 μl/dish) was added to the nuclear-cytoskeletal fraction and incubated for 10 min at room temperature unless otherwise noted. The incubation fluid was collected, cells washed once with TBS (25 μl), and the solutions combined. The nonreleased material was solubilized with Laemmli sample buffer (21). Equal amounts of samples from both fractions were analyzed by SDS PAGE and fluorography. Similar results were obtained from cells treated immediately after restress or allowed to recover at 37°C for 30 min.

Other Methods

Radioisotope incorporation was measured by precipitation of extracts with 10% TCA with 5 μg/ml carrier BSA. Radioimmune precipitations were performed on SDS-denatured samples as described previously (16). Proteins were analyzed by SDS PAGE according to Laemmli (21), and gels processed for fluorography (6, 23). For quantitation, gel slices from fluorographed gels were placed in ScintiVerse E (Beckman Instruments, Inc., Fullerton, CA) and counted on a Packard Liquid Scintillation counter. *N*-Tosyl-L-phenylalanine chloromethyl ketone–treated trypsin (TPCK-trypsin; Sigma Chemical Co., St. Louis, MO) activity was assayed with *p*-toluene-sulfonyl-L-arginine methyl ester (Sigma Chemical Co.). The activity of immobilized TPCK-trypsin (Sigma Chemical Co.) was assayed in a similar manner except the enzyme was removed by centrifugation before assay.

Results

Distribution of hsps in Subcellular Fractions of CEF before and after Stress

To determine if CEF hsps might change their cellular location as a result of stress, cultures were initially induced for hsp formation by a 45°C heat shock, radiolabeled at 37°C, and allowed to recover overnight. This regimen led to an accumulation of radiolabeled hsp 89, hsp 70, and hsp 24 in the

recovered cells. Minimal degradation of these proteins was detected over the course of the experiment. Cell extracts prepared by detergent lysis were separated into a soluble cytoplasmic and a nuclear-cytoskeletal fraction by centrifugation and polypeptides in both fractions were examined by SDS PAGE and fluorography (Fig. 1, lanes *1* and *2*). The nuclear-cytoskeletal fraction contained a number of radiolabeled proteins; the two most prominent proteins were actin (M_r 43,000) and vimentin (M_r 52,000). Other methods of cell fractionation based on hypotonic swelling and mechanical disruption (9, 20) yielded essentially the same results. The presence of these two cytoskeletal elements has been previously established in isolated nuclear fractions from tissue culture cells (41). Hsp 70 was also a prominent radiolabeled protein in the nuclear-cytoskeletal fraction but was more concentrated in the cytoplasmic fraction. A low level of hsp 24 was detected in the nuclear-cytoskeletal fraction. The percentage of the hsp population in each fraction was determined by excising bands corresponding to hsp 70 and hsp 24 and measuring their radioactivity. Greater than 90% of the total hsp 24 and hsp 70 was in the cytoplasmic fraction. Hsp 89 appeared to be totally solubilized.

Other samples of recovered cells that had accumulated labeled hsps were again heat shocked at 45°C for 3 h and detergent lysates separated into cytoplasmic and nuclear-cytoskeletal fractions (Fig. 1, lanes *3* and *4*). Clearly, hsp 24 has now become a major radiolabeled component of the nuclear-cytoskeletal fraction. Quantitation of bands corresponding to hsp 24 and hsp 70 showed that hsp 24 increased 3.3-fold and hsp 70 increased 1.2-fold in the nuclear-cytoskeletal fraction as a result of the stress, constituting 1.1 and 0.8% respectively, of the total radiolabeled protein of this fraction. The amounts of hsp 70 and hsp 24 in the nuclear-cytoskeletal fraction varied slightly in repeated trials but they always increased in restressed cells, up to twofold for hsp 70 and up to fivefold for hsp 24. These results showed that hsp 70 and hsp 24 in CEF were resorting in response to heat shock. Hsp 89 remained in the soluble fraction after restress, but its cellular location changed (see below).

The kinetics of this change in hsp 70 and hsp 24 cell localization upon heat shock were examined. Recovered radiolabeled CEF were restressed at 45°C for various lengths of time, extracts fractionated, and the nuclear-cytoskeletal fractions analyzed by radioimmune precipitation using anti-CEF hsp 70 and hsp 24 antibodies. The immune precipitates were separated by SDS PAGE and the radiolabeled bands were excised from the gel and quantitated. In this experiment, the amount of hsp 70 in the nuclear-cytoskeletal fraction increased about twofold after restressing for 1 h at 45°C and remained at that level for up to 4 h at 45°C. The amount of hsp 24 in the nuclear-cytoskeletal fraction also increased about twofold after 1 h of heat shock and gradually rose between 3 and 4 h of heat shock with about half of the total hsp

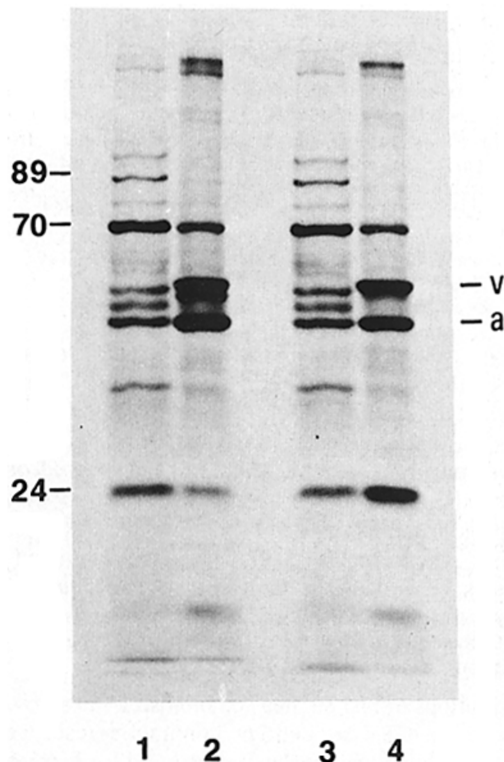


Figure 1. SDS PAGE analysis of subcellular fractions of CEF. CEF were heat shocked at 45°C for 3 h, labeled with [^{35}S]methionine at 37°C for 3 h, and allowed to recover overnight at 37°C. Cells were fractionated into a soluble cytoplasmic fraction (lanes *1* and *3*) and a nuclear-cytoskeletal fraction (lanes *2* and *4*) after recovery (37°C, 18 h; lanes *1* and *2*) or immediately after restress (45°C, 3 h; lanes *3* and *4*). Approximately 10^5 cpm of each fraction were analyzed on 11% SDS-polyacrylamide gels that were processed for fluorography. *v*, vimentin; *a*, actin.

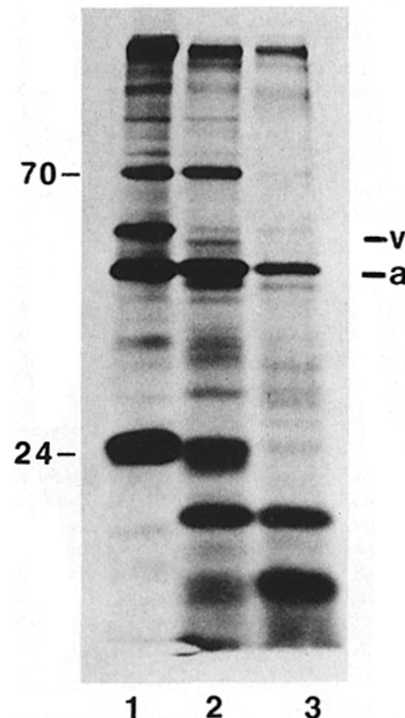


Figure 2. SDS PAGE analysis of protease-treated nuclear-cytoskeletal fraction. The nuclear-cytoskeletal preparation from restressed CEF (1.5×10^5 cpm, lane *1*) was treated with immobilized TPCK-trypsin in the absence (lane *2*) or in the presence (lane *3*) of 0.1% SDS as described in text. After incubation fractions were analyzed on 11% SDS-polyacrylamide gels and processed for fluorography. *v*, vimentin; *a*, actin.

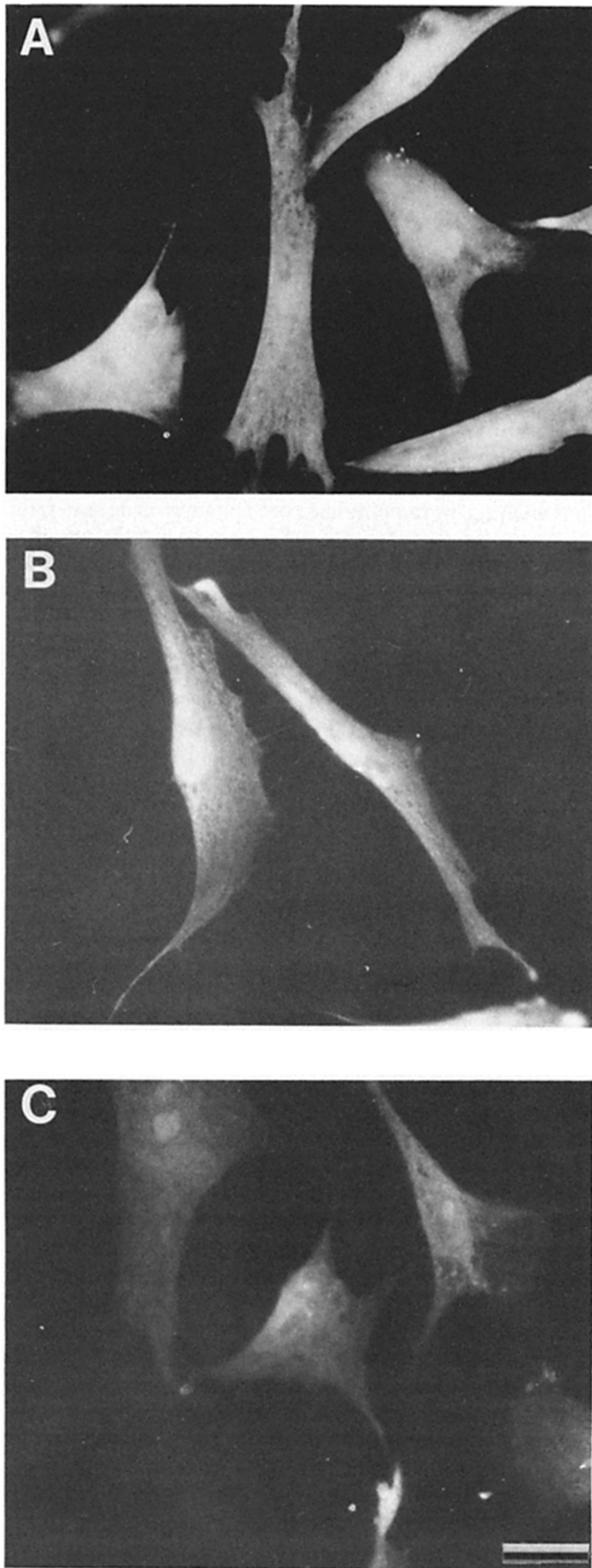


Figure 3. Immunofluorescent staining of nonstressed CEF. Secondary CEF grown at 37°C were fixed with 3% paraformaldehyde, permeabilized with 0.1% Triton X-100, and stained with rabbit anti-CEF hsp 89 antibodies (*A*), anti-CEF hsp 70 antibodies (*B*), or anti-CEF hsp 24 antibodies (*C*). Lengthy exposures were required to visualize the distribution of hsp 70 (*B*, twofold increase over that used for heat-shocked CEF) and especially hsp 24 (*C*, 4.5-fold increase over that used for heat-shocked CEF). Bar, 10 μm.

24 in the nuclear-cytoskeletal fraction. This final level represented a fivefold increase over the amount initially found in this fraction of the cells before restress.

Differential Distributions of hsp 70 and hsp 24 in the Nuclear-Cytoskeletal Fractions

To determine if the hsps segregating with the nuclear-cytoskeletal fraction were inside the nucleus or bound to a structure pelleting with the nucleus, we incubated the nuclear-cytoskeletal fraction from restressed cells with TPCK-trypsin immobilized on agarose. We reasoned that soluble trypsin could enter the nucleus and, indeed, incubation of radiolabeled nuclear-cytoskeletal fractions with soluble trypsin (5.8 U/10^5 cpm of [^{35}S]methionine-labeled cell extract, 20 min, 22°C) resulted in overall degradation of polypeptides in this fraction (data not shown). However, in the presence of immobilized TPCK-trypsin (12.6 U/10^5 cpm of [^{35}S]methionine-labeled cell extract, 20 min, 22°C) two major protein components of the nuclear-cytoskeletal fraction, hsp 24 and vimentin, were readily degraded (Fig. 2, lane *2*) indicating that these two proteins were extranuclear. Actin appeared to be resistant to proteolysis by trypsin. About 19% of hsp 24 was recovered and 50% of hsp 70 were recovered under these conditions as determined by quantitating gel bands. Increasing the concentration of immobilized trypsin to 25.2 U/10^5 cpm of [^{35}S]methionine-labeled cell extract did not significantly change the extent of hsp 70 degradation. This suggests that about half of hsp 70 protein in the nuclear-cytoskeletal fraction was inside the nucleus. When SDS (0.1%) was included with immobilized trypsin, hsp 70 as well as most other protein components were readily degraded (Fig. 2, lane *3*). Prolonged incubation (>60 min) of nuclear-cytoplasmic fractions in the absence of SDS with immobilized trypsin (12.6 U/10^5 cpm) led to an overall degradation of protein.

Re-sorting of hsp 89, hsp 70, and hsp 24 in the Intact Cell after Stress

The experiments described above showed that changes occur after heat shock that influence the distribution of hsp 70 and hsp 24 in subcellular fractions of the CEF cell. The distribution of hsp 89 was not influenced by the stressed state of the cell and remained in the soluble fraction of recovered or heat-shocked cells. However, the assignment of hsp 89 as a cytoplasmic protein and hsp 70 and hsp 24 into the cytoplasmic and nuclear-cytoskeletal fractions based on cell fractionation techniques was inherently tentative; redistribution of proteins during fractionation would lead to erroneous interpretations of results. In addition, these categories were necessarily broad. To fine tune our assignments, we examined the distributions of hsp 89, hsp 70, and hsp 24 in recovered and restressed CEF by indirect immunofluorescence microscopy using polyclonal antibodies specific for hsp 89, hsp 70, and hsp 24 (18).

In cells not given any type of stress, a uniform light staining pattern was detected with anti-hsp 70 antibodies indicative of a basal level of hsp 70 or an immunologically related

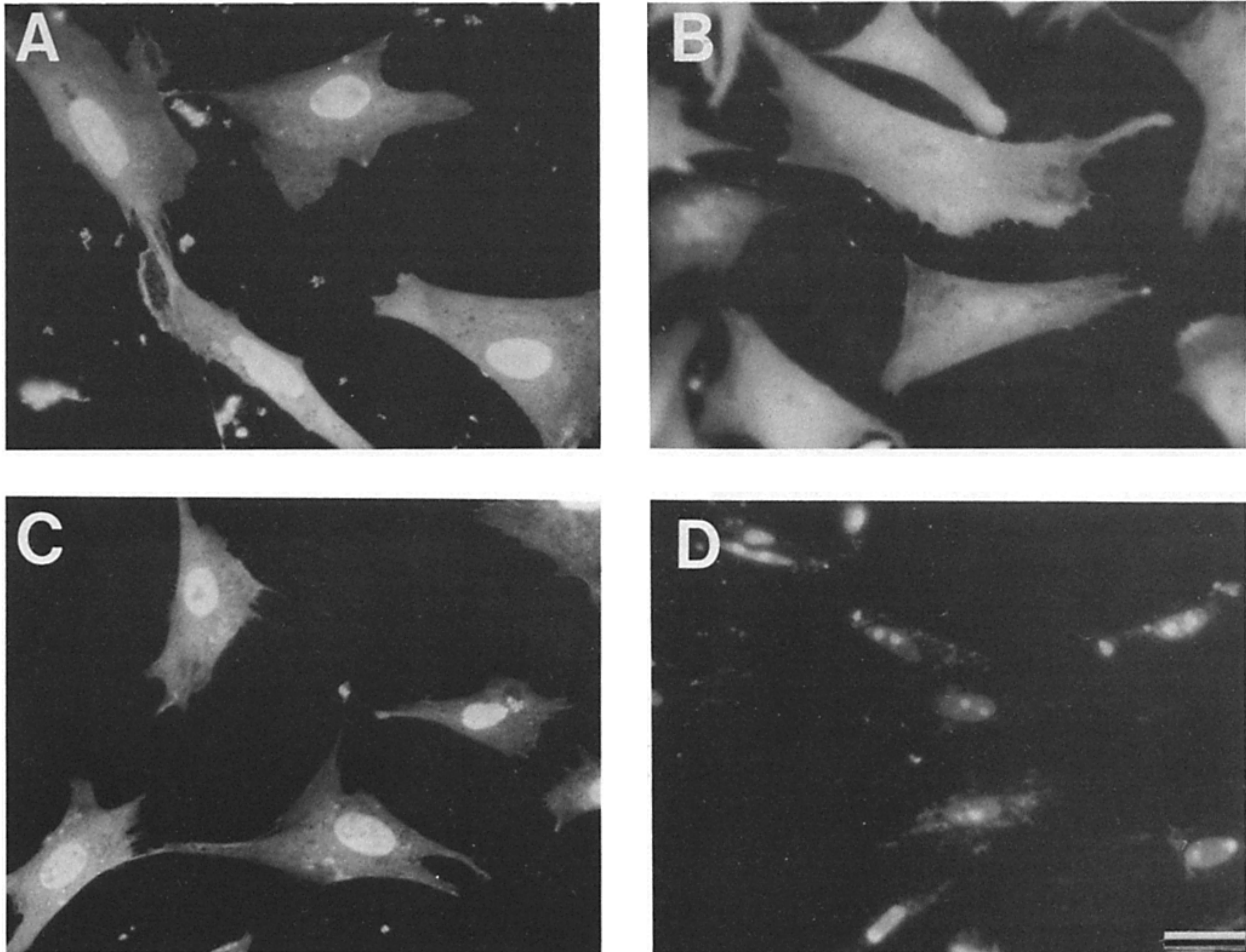
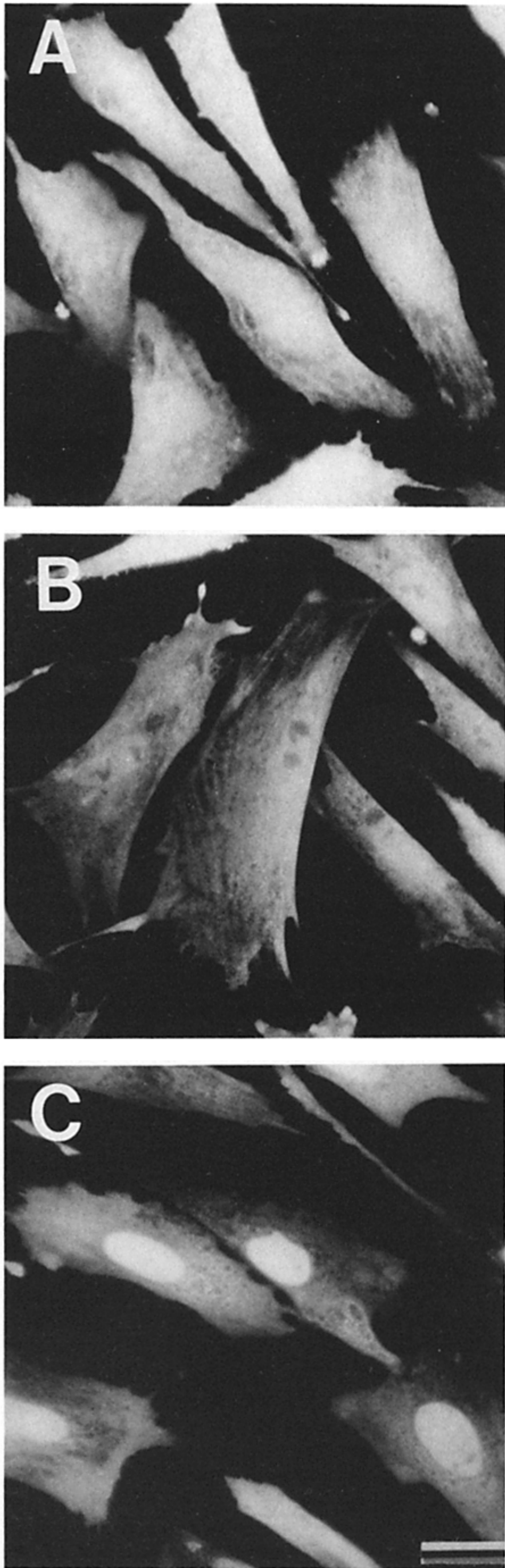


Figure 4. Distribution of hsp 70 in heat-shocked, recovered, and restressed cells. Secondary CEF were heat shocked at 45°C for 3 h (*A*), allowed to recover at 37°C overnight (*B*), or after recovery subjected to a second heat shock at 45°C for 3 h (*C*). Cells were fixed with 3% paraformaldehyde, permeabilized with 0.1% Triton X-100, and stained with rabbit anti-CEF hsp 70 antibodies followed by rhodamine-conjugated anti-rabbit IgG antibodies. The distribution of hsp 70 was also examined in recovered cells subjected to a second heat shock but permeabilized with 0.5% NP-40 before fixation and antibody staining (*D*). Bar, 10 μm.

cognate protein in CEF (Fig. 3 *B*). In contrast, bright immunofluorescent patterns for hsp 70 were seen in CEF that had been heat shocked, demonstrating the vigorous induction of this protein upon stress (Fig. 4 *A*). Hsp 70 appeared concentrated in the nucleus of heat-shocked CEF. Similarly, a uniform anti-hsp 89 staining was detected in nonstressed cells (Fig. 3 *A*) and increased in cells that were heat shocked (Fig. 5 *A*). As expected from radiolabeling and immunoblot analysis, anti-hsp 24 staining was very weak in nonstressed cells and much longer exposures are required to detect any signal (Fig. 3 *C*); however, bright immunofluorescent patterns for hsp 24 were observed in CEF that had been heat shocked (Fig. 6 *A*).

In heat-shocked cells that were allowed to recover at 37°C for 15–20 h (the same regimen as described above for our biochemical analysis), hsp 89 (Fig. 5 *B*), hsp 70 (Fig. 4 *B*), and hsp 24 (Fig. 6 *B*) appeared distributed throughout the cell and appeared fibrous toward the less dense edges of cells. To further investigate any intranuclear accumulation of antigen and the possible association of the hsps to any filamentous systems, recovered cells were permeabilized before fixation to remove soluble components. Most hsp 70 was soluble under these conditions; a low but detectable level of hsp 70 remained in the nucleus (data not shown). Hsp 24 and hsp 89 were completely extracted by mild detergent lysis, indicating that the antigens were not tightly bound to any cellular structure (data not shown).

When recovered CEF containing preinduced hsps were subjected to a second 45°C heat shock for 3 h, we observed greatly depleted amounts of hsp 89 in the cytoplasm and an accumulation of hsp 89 in the nucleus (Fig. 5 *C*). Nuclear staining appeared uniform and nucleoli were not preferentially stained. This pattern contrasted with the distribution of hsp 89 after an initial stress where the protein appeared to be distributed throughout the cell with some concentration in the nuclear region (Fig. 5 *A*). The binding of hsp 89 to a nuclear structure was surprising since mild detergent lysis released most hsp 89, indicating a low affinity or a membrane-mediated structure. In accordance with our biochemical analysis of hsp 89 in these cells, most hsp 89 was released from cells permeabilized with detergent before fixation (data not shown). A small but detectable level of nuclear staining



was retained, however. This nuclear staining was not uniform with small areas of punctate accumulation.

In recovered CEF subjected to a second 45°C heat shock for 3 h, hsp 70 clearly accumulated in the nucleus with detectable levels distributed throughout the cytoplasm, with some areas of concentration (Fig. 4 *C*). An identical localization was noted after just 30 min at 45°C (data not shown). The distribution of hsp 70 within the nucleus was not uniform, with some areas of patchy accumulation. Staining of nucleoli was highly variable; most cells showed no preferential staining of the nucleolus and appeared to exclude antibody from this organelle. Some hsp 70 antigen was stably retained in the nucleus of heat-shocked cells that had been permeabilized with detergent before fixation and immunofluorescent staining (Fig. 4 *D*), but the staining intensity of nuclei was less after mild detergent lysis than that seen on fixed whole cells. In those cells lysed before fixation, nucleoli were brightly stained, indicating an accumulation of hsp 70. Occasionally nucleoli themselves did not appear to contain hsp 70, but seemed to be surrounded by a hsp 70–containing ring. The nucleolar location of hsp 70 that was detected by permeabilizing cells before fixation may result from (*a*) increased permeability of antibody into this organelle, (*b*) removal of loosely or peripherally bound hsp 70 which unmasked an existing structure, or (*c*) detergent-induced modification of the hsp 70 protein. Some hsp 70–containing cytoplasmic components were also retained resulting in a light patchy non-nuclear pattern (Fig. 4 *D*). Thus, hsp 70 exhibited at least four cellular locations upon heat shock: soluble cytoplasmic, insoluble cytoplasmic, nuclear, and nucleolar.

Perhaps our most striking observation was the immunofluorescent-staining pattern in restressed cells of the anti–hsp 24 antibodies (Fig. 6 *C*). The hsp 24 was distributed in cytoplasmic aggregates concentrated in perinuclear caps. We had noted that restressed cells were more phase-dense than recovered or nonstressed cells and that most cells contained phase-dense cytoplasmic granules surrounding the nucleus (Fig. 6 *D*). The hsp 24–containing cytoplasmic aggregates corresponded precisely with these phase-dense granules surrounding the nucleus. Hsp 24 aggregates were in close apposition to the nucleus but often a clear nuclear boundary was apparent. The perinuclear distribution of hsp 24 was not uniform from cell to cell; in some cases the clusters of aggregates completely surrounded the nucleus and in others they appeared as one or more perinuclear caps. When cells were permeabilized before fixation, these rings and caps of hsp 24–containing aggregates were retained and were intensely stained with anti–hsp 24 antibodies (Fig. 7). No overall nuclear staining was detected, but occasionally we observed a light staining that corresponded to the phase-dense nucleoli.

Figure 5. Distribution of hsp 89 in heat-shocked, recovered, and restressed cells. Secondary CEF were heat shocked at 45°C for 3 h (*A*), allowed to recover at 37°C overnight (*B*), or after recovery subjected to a second heat shock at 45°C for 3 h (*C*). Cells were fixed with 3% paraformaldehyde, permeabilized with 0.1% Triton X-100, and stained with rabbit anti–CEF hsp 89 antibodies followed by rhodamine-conjugated anti-rabbit IgG antibodies. Bar, 10 μm.

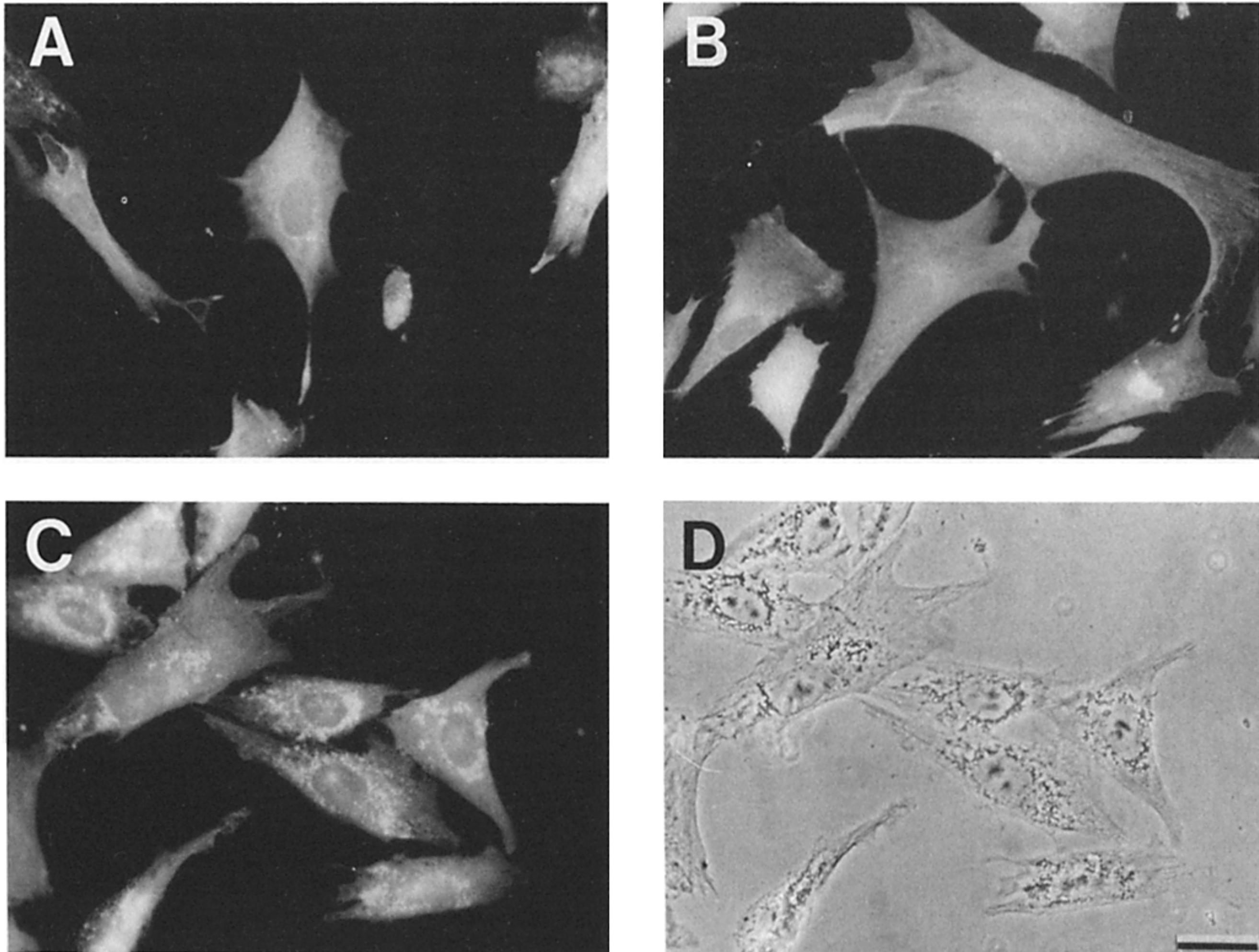


Figure 6. Distribution of hsp 24 in heat-shocked, recovered, and restressed cells. Secondary CEF were heat shocked at 45°C for 3 h (*A*), allowed to recover at 37°C overnight (*B*), or after recovery restressed by a second heat shock at 45°C for 3 h (*C*). Cells were fixed with 3% paraformaldehyde, permeabilized with 0.1% Triton X-100, and stained with rabbit anti-CEF hsp 24 antibodies followed by rhodamine-conjugated anti-rabbit IgG antibodies. The corresponding phase-contrast image of restressed CEF is shown (*D*). Bar, 10 μm.

When cells were allowed to recover at 37°C for 4 h after a restress, these perinuclear particulates disappeared and an overall cytoplasmic staining of hsp 24 antigen increased. Thus the aggregation of hsp 24 in phase-dense perinuclear granules as well as the concentration of hsp 70 in the nucleus were reversible processes.

ATP-dependent Release of hsp 70

An ATP-dependent release of hsp 70 from the nuclei of restressed mammalian cells has recently been reported by Lewis and Pelham (25). We examined restressed CEF to determine if the hsp 70 retained in the nuclear-cytoskeletal fraction could be solubilized by ATP. In a control experiment, 40% of hsp 70 was released after incubating the nuclear-cytoskeletal fraction of restressed CEF for 10 min at 23°C. In the presence of 1 mM ATP, however, 75% of hsp 70 was released. We could also detect qualitatively this ATP-dependent loss of hsp 70 from the restressed cell's nuclear-cytoskeletal fraction by immunofluorescence. Incubation with 1 mM ADP or AMP failed to show this increased release of hsp 70. The nonhydrolyzable ATP analogues, AMP-PNP and ATP γ-S, were also ineffective in releasing hsp 70. No release of hsp 24 was detected under these conditions.

Resorting of hsps Induced by Sodium Arsenite

To determine if the resorting of the hsps was restricted to heat shock, CEF were stressed with sodium arsenite (50 μM for 3 h at 37°C) and allowed to recover overnight. The distributions of the hsps were examined in recovered and restressed (50 μM sodium arsenite, 3 h, 37°C) cells. The distributions of hsp 70 and hsp 24 were indistinguishable from that observed under heat shock conditions except that hsp 70 was partially retained in the nucleus in recovered cells (data not shown). Hsp 70 was excluded from nucleoli in most cells except that, as noted in heat-shocked cells, when cells were permeabilized before fixation, hsp 70 antigen was detected in nucleoli. Hsp 89 exhibited an overall cytoplasmic distribution in sodium arsenite–treated cells and, even at sodium arsenite concentrations up to 100 mM, was only slightly concentrated toward the nuclear region after the initial stress and

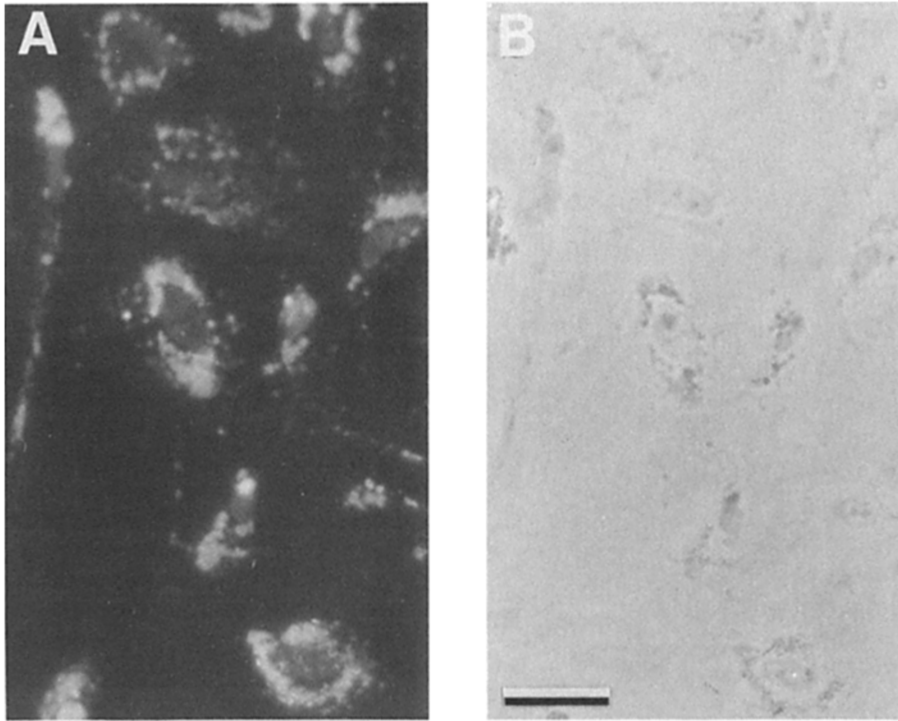


Figure 7. Distribution of hsp 24 in restressed cells. Secondary CEF were heat shocked at 45°C for 3 h, allowed to recover at 37°C overnight, and restressed by a second heat shock at 45°C for 3 h. Cells were permeabilized with 0.5% NP-40, fixed with 3% paraformaldehyde, and stained with rabbit anti-CEF hsp 24 antibodies followed by rhodamine-conjugated anti-rabbit IgG antibodies. (*A*). The corresponding phase-contrast image is shown (*B*). Bar, 10 μm.

after restress (data not shown); thus its distribution differed from that observed in heat-shocked cells.

Response of the CEF Cytoskeleton to Heat Shock

The dramatic resorting of the hsps that occurred when recovered cells were subjected to a second stress prompted us to examine stress-induced changes in the cytoskeleton, and, in particular, to determine any modulation by hsps. The effect of an initial heat shock on the morphology of microfilaments, intermediate filaments, and microtubules in CEF was examined by indirect immunofluorescence microscopy using antibodies specific for actin, vimentin, and tubulin, respectively. To examine the effects of hsps on stress-induced cytoskeletal changes, recovered cells containing previously induced hsps were subjected to a second heat shock. The morphology of the cytoskeleton during the restress was then compared with that of the initial heat shock.

Both the microtubule and microfilament network remained intact during the initial heat shock or restress, and only minor alterations in their morphology were observed when compared with nonstressed cells. Within 5–15 min and up to 3 h of heat shock microtubules were somewhat more concentrated in the perinuclear region of the cell (Fig. 8 *A*) compared with that of nonstressed CEF. This perinuclear concentration disappeared upon recovery overnight at 37°C. A second heat shock resulted in a slightly concentrated peri-

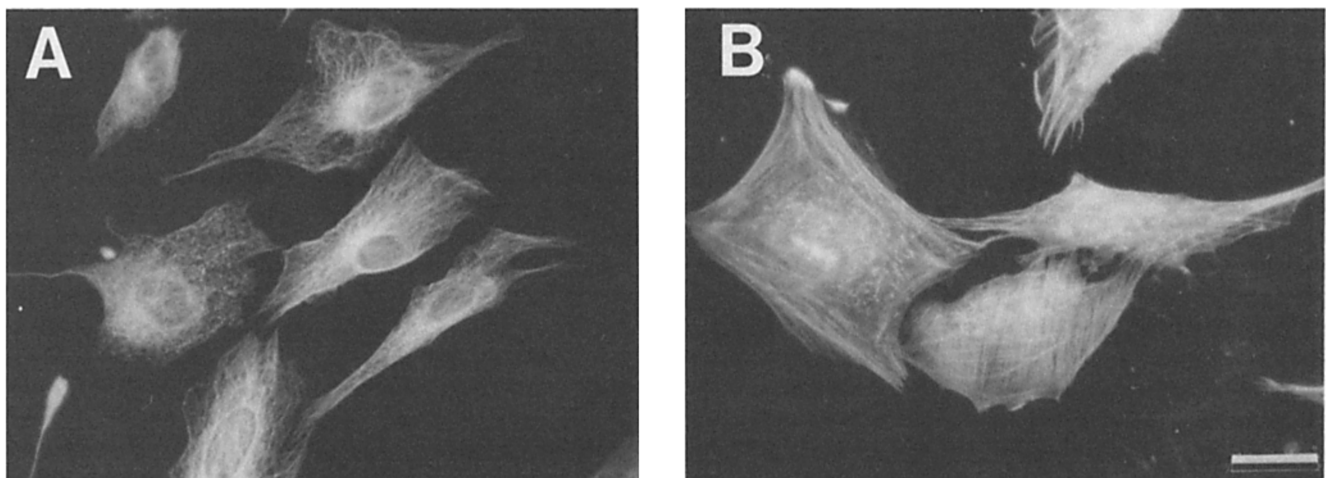


Figure 8. Morphology of microtubules and microfilaments in restressed CEF. Secondary CEF were heat shocked at 45°C for 3 h, allowed to recover at 37°C overnight, then subjected to a second heat shock at 45°C for 3 h. The distribution of microtubules (*A*) and microfilaments (*B*) were examined in cells fixed with 3% paraformaldehyde, permeabilized with 0.1% Triton X-100, and stained with rabbit antibodies against tubulin or actin followed by rhodamine-conjugated anti-rabbit IgG antibodies. Bar, 10 μm.

nuclear distribution similar to that seen in cells subjected to an initial heat shock. Microfilaments or stress fibers were more defined in CEF heat shocked for 5–15 min and up to 3 h (Fig. 8 *B*) or in cells subjected to a second heat shock. Microfilament morphology in recovered cells was similar to that of nonstressed cells. In contrast, the intermediate filament morphology was dramatically altered in heat shock cells. The network of wispy, well spread filamentous structures (Fig. 9 *A*) disappeared in ~20% of the cells after only a 5-min heat shock, a time before cells could effectively synthesize significant quantities of hsps. After 15 min of heat shock almost 50% of cells had lost the typical intermediate filament network and by 30 min and up to 3 h 70% of the cells showed altered structures (Fig. 9 *B*). In these cells a thick fibrouslike ring formed around the nucleus, indicating that the intermediate filament structure of the cell had reorganized or collapsed during the heat shock. The remaining 30% cells showed intermediate filament with various degrees of collapse. The heat shock–induced perinuclear intermediate filament structure was morphologically distinct from the capped perinuclear ribbonlike pattern seen in CEF after treatment with colcemid (5 μM, 16 h, 37°C). When cells were allowed to recover at 37°C overnight, the normal intermediate filament morphology reappeared (Fig. 9 *C*).

To examine if preinduced hsps might affect the reorganization of intermediate filaments induced by a heat shock, recovered cells were subjected to a second heat shock and the intermediate filament pattern examined. Within 30 min and up to 3 h of a second heat shock, the typical intermediate filament network again reorganized into a perinuclear ring (Fig. 9 *D*). This vimentin-containing ring structure was indistinguishable from that elicited during the first heat shock. Thus hsps, present in the cell as a result of the initial heat shock, did not prevent the collapse of the intermediate filament network.

Requirement of Gene Transcription for Recovery of Normal Intermediate Filament Morphology after Heat Shock

To examine the possible participation of hsps in recovery of intermediate filament morphology, CEF were incubated with 1 μg/ml actinomycin D during a 2-h heat shock at 45°C. Inclusion of actinomycin D at this level had no effect on the intermediate filament morphology of cells at 37°C and did not

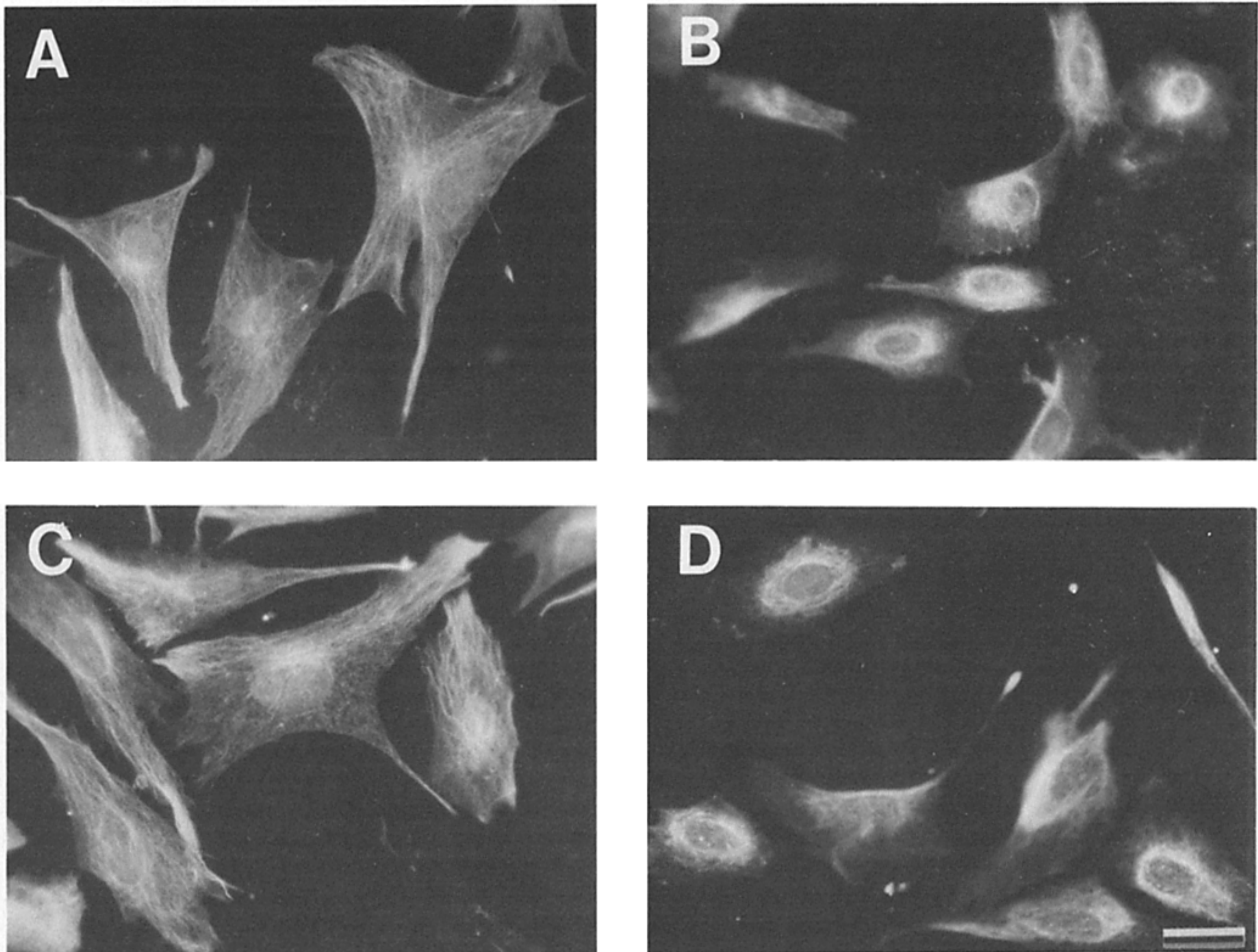


Figure 9. Distribution of intermediate filaments in heat-shocked, recovered, and restressed cells. Secondary CEF were held at 37°C (*A*), heat shocked at 45°C for 3 h (*B*), allowed to recover at 37°C overnight (*C*), or subjected to a second heat shock at 45°C for 3 h (*D*). Cells were fixed with 3% paraformaldehyde, permeabilized with 0.1% Triton X-100, then stained with antibodies against chicken vimentin followed by rhodamine-conjugated anti-rabbit IgG antibodies. Bar, 10 μm.

prevent the heat shock–induced collapse of the filaments. After heat shock, actinomycin D was washed from the cells and recovery of normal intermediate filament morphology was followed in cells at 37°C. Recovery in cells not treated with the drug was rapid and within 2 h at 37°C after a 2-h heat shock, ~85% of the cells had the normal splayed pattern of vimentin-containing intermediate filaments (Fig. 10 *A*). However, actinomycin D–treated cells did not recover their normal intermediate filament morphology after 2 h (Fig. 10 *B*) or even up to 6 h after removal of the drug (Fig. 10 *D*, cf. Fig. 10 *C*). Protein synthesis in cells recovering from heat shock was not affected by including actinomycin D during the stress period. Thus, failure to reform the intermediate filament network cannot be due to a block in protein synthesis. Also, hsp synthesis that is blocked in cells treated with actinomycin D during stress does not occur when cells recover in the absence of the drug. Thus we suggest that the hsps themselves may participate in the recovery of the normal intermediate filament morphology.

Discussion

The immunological and biochemical data presented here show that the hsps in CEF undergo dynamic intracellular reorganization in response to the stress state of the cell. Upon heat shock the hsps preformed by a previous heat shock were distributed to specific cellular locations. Both hsp 70 and hsp 89 sorted to the nucleus upon heat shock, but their binding was distinct. Hsp 89 was readily solubilized by detergents, whereas a portion of hsp 70 remained bound to insoluble nuclear structures. In contrast, hsp 24 was concentrated in distinct insoluble aggregates in the cytoplasm. Recovery of stressed cells led to mobilization of these proteins and they distributed throughout the cytoplasm in a detergent-soluble form.

The nuclear accumulation of hsp 70 in heat-shocked cells and its relocation to the cytoplasm upon recovery is well established for several species (3, 24, 45, 48). The distribution of CEF hsp 70 described here resembles this pattern but the nuclear/cytoplasmic ratio of CEF hsp 70 after heat shock was three to fourfold less than that reported for mammalian and Drosophila cells (3, 48). Generally, a portion of hsp 70 has been localized to the nucleoli by immunofluorescence and biochemical studies (3, 34, 42, 48). However, hsp 70 was excluded from nucleoli in heat-shocked Drosophila salivary glands (45) and in azetidine and sodium arsenite–treated

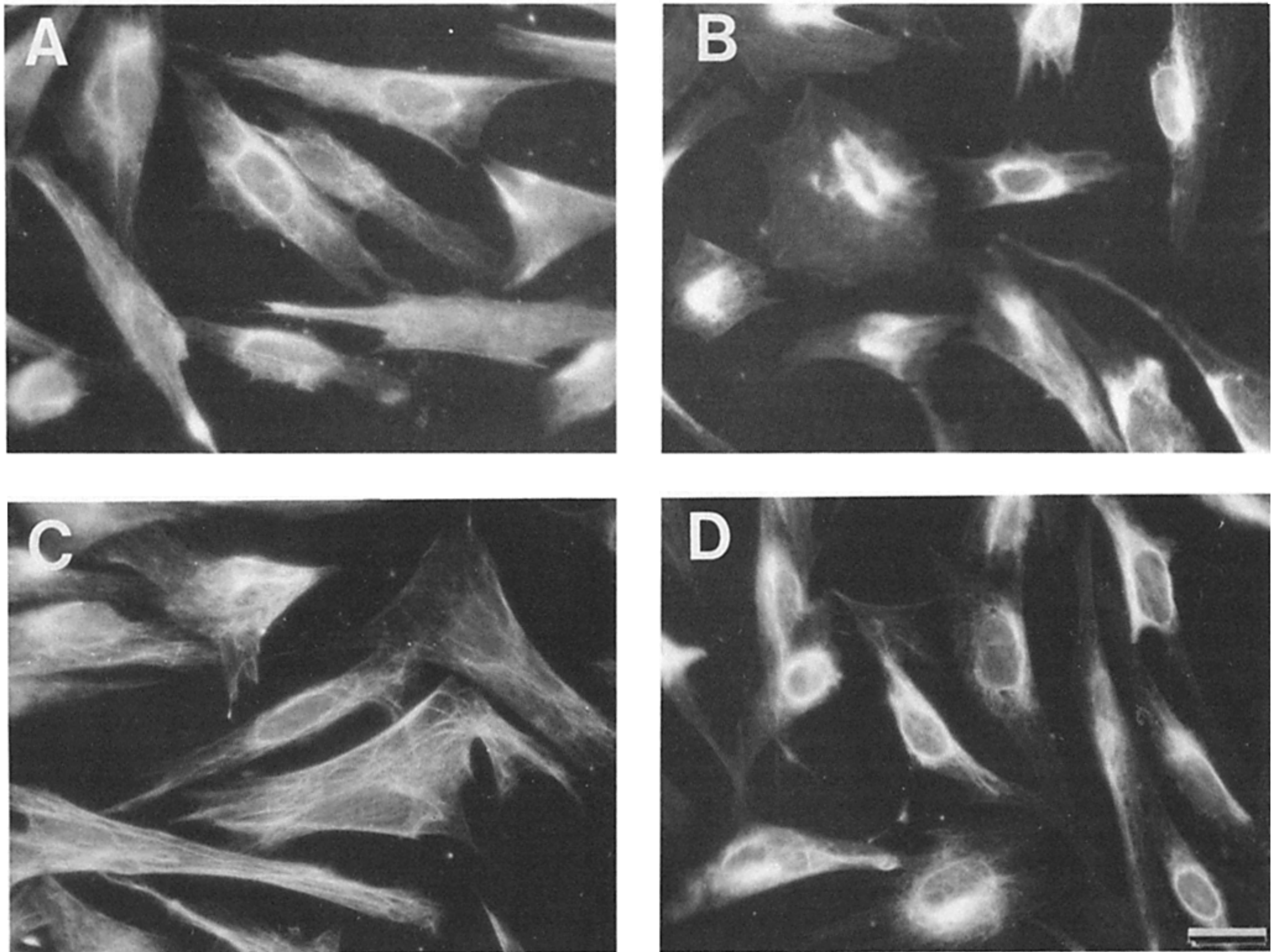


Figure 10. Distribution of intermediate filaments in actinomycin D–treated cells. Secondary CEF were heat shocked at 45°C for 2 h either in the absence (*A* and *C*) or in the presence (*B* and *D*) of 1 μg/ml actinomycin D. The cells were allowed to recover at 37°C in the absence of the drug, then processed for immunofluorescence as described in the legend to Fig. 9. The distribution of the intermediate filament network was examined after 2 h (*A* and *B*) or 6 h (*C* and *D*) recovery. Bar, 10 μm.

mammalian tissue culture cells (48). This disparity of distribution may reflect the intensity of the stress encountered and the developmental state of the cell, but it also suggests multiple functions for hsp 70. We found some hsp 70 in nucleoli of both heat-shocked and sodium arsenite–treated CEF, but its localization was highly variable and dependent on the fixation method.

The functions of hsp 70 are not known but its accumulation in nucleoli suggests participation in RNA processing and/or ribosomal assembly. Pelham (34) recently reported that the presence of hsp 70 correlated with the acceleration of recovery of normal nucleolar morphology and export of ribosomes in heat shock nucleoli. Pelham and coworkers have also found that hsp 70 was released from its binding sites in nuclei of heat-shocked mammalian cells in an ATP-dependent reaction (25). We have shown here a similar activity for CEF hsp 70. In a preliminary experiment, we were unable to detect a phosphorylated hsp 70 during ATP-dependent release of hsp 70 from nuclei using high specific activity ^{32}P-γ-ATP.

The cytoplasmic localization of the homologous high M_R hsp previously reported for CEF (hsp 89), Drosophila (hsp 83), and mammalian (hsp 90) cells after heat shock was based on subcellular fractionation. For the Drosophila hsp 83, immunofluorescence confirmed an exclusive cytoplasmic distribution with concentration toward the plasma membrane under heat shock conditions (42). Our detection of nuclear accumulation of CEF hsp 89 during restress by immunofluorescence was surprising. Its detergent-sensitive binding to nuclear structures suggests a membrane involvement. The hsp 89 has recently been found associated with steroid-receptors (10, 35, 36) and one can envision that the resorting of hsp 89 to the nucleus upon stress and its dissemination throughout the cytoplasm in recovered cells could reflect a shuttling activity among membrane-associated components, similar to its proposed interaction with the Rous sarcoma virus oncogene product, pp60src (8, 33, 51).

Our observations of hsp 24 in subfractionated CEF are similar to studies of Drosophila tissue culture cells (summarized in reference 42), rat embryo fibroblasts (20), and plant cells (19). In each of these systems, a portion of the small hsps becomes associated with a nuclear or nuclear-cytoskeletal fraction upon stress. A cytoplasmic localization of CEF hsp 24 was suggested by its sensitivity to trypsin treatment of the nuclear-cytoskeletal fraction of restressed CEF. But the precise localization of the chicken small hsp was derived from immunofluorescence microscopy of restressed CEF where the distribution of hsp 24 was distinctive and highlighted by a favorable ratio of cytoplasmic to nuclear mass in the fibroblast. Hsp 24 appeared as clusters of aggregates in a equatorial perinuclear ring. A similar distribution of Drosophila hsp 23 in tissue culture cells was recently reported by Tangauy (42) yet appeared localized in the nucleus in heat-shocked Drosophila salivary glands (1).

Hsp 24 was coincident with perinuclear phase-dense particles that formed in restressed CEF. Occasionally we noted hollow particles suggestive of vesicles and hsp 24 appeared to surround the hollow particles. We have found that hsp 24 partially distributed with Triton X-114 upon temperature-induced phase separation (7), an indication that hsp 24 is partially hydrophobic and capable of binding to membranes (data not shown).

The tendency of the small hsps to form aggregates or oligomers appears to be common in plants (32), Drosophila (1, 42), and CEF. The small hsps from Drosophila exist as oligomers or aggregates and have been detected in a 20-S RNA-containing structure (2), similar to the prosome structure found in duck and mouse cells (39). Purified preparations of duck prosomes contain a protein that crossreacts with antibodies to CEF hsp 24 when examined by immunoblot analysis. (Schlesinger, M., and K. Scherrer, unpublished observations). However, until the RNA content of the CEF hsp 24 aggregates is analyzed, the relationship between the particles observed in heat shock of CEF and the 20S structure remains speculative. The hsp 24 granules may be aggregated hsp–ribonucleoprotein particles and similar to the >100-S aggregates observed in nuclear fractions of Drosophila salivary cells (2). Based on these observations, we suggest that hsp 24 and related small hsps may function in protecting or repairing the cells' machinery for mRNA processing and protein synthesis.

The results presented here also show that of the three cytoskeletal networks in CEF, the intermediate filament network appeared most sensitive to the effects of stress. This filament system rapidly collapsed upon heat shock but quickly reformed upon recovery of cells at normal growth temperatures. A similar heat shock–induced collapse of intermediate filaments has been reported in invertebrate and in vertebrate cell lines, both mammalian and avian (29, 43, 46, 50). The recent identification of a plant protein that crossreacts with antibody against intermediate filaments (14) will allow for similar investigation in higher plants. The collapse of the intermediate filament network due to stress was not a heat-induced artifact since incubation with amino acid analogues (43) or with sodium arsenite (Collier, N. C. and M. J. Schlesinger, unpublished experiments) led to a similar redistribution. Our conditions for heat shock of CEF induced relatively minor morphological changes in microtubules despite their close morphological association with intermediate filaments in normal cells. Our stressed CEF showed minimal gross changes in microfilaments, although actin-containing stress fibers increase in stressed mammalian cells (43), and actin-containing nuclear inclusion bodies appear after heat shock (50). Incubation of Chinese hamster ovary cells at high temperature (45°C) results in rapid disappearance of microfilaments (15). These differences can probably be accounted for by variations in the severity of the stress.

A most intriguing observation to emerge from this study was the apparent dependence on stress-induced proteins for recovery of normal intermediate filament morphology after heat shock. Recovery of the intermediate filament morphology, which normally began within minutes after removal of the stress, was blocked when cells were heat shocked in the presence of actinomycin D. This finding strongly suggests that hsps may participate in the repair of normal intermediate filament morphology after heat shock. The colcemid-induced collapse of the intermediate filament network is slowly reversible after removal of the drug (49). Because the drug does not induce synthesis of hsps, the recovery of normal intermediate filament morphology after treatment with colcemid may be affected by an alternate recovery process or by constitutive levels of a cognate form of an hsp.

The cytoskeleton appears to be a target of cellular stress.

Several investigators have reported that hsps, in particular the hsp 70 family and their cognates, may associate with the cytoskeletal elements of the cell (24, 27, 30, 31, 47). In addition there is a recent observation that a cognate of the mammalian 70-kD stress protein family is identical to a protein that binds to and dissociates clathrin-coated vesicles in a ATP-dependent reaction (12, 44). Further investigations are in progress to determine the roles of the hsps in the recovery of the intermediate filament system in CEF.

In summary, CEF hsp 89, hsp 70, and hsp 24 respond to cellular stress and recovery by a dynamic resorting among cellular organelles. The functions of these hsps remain elusive; however, their selective subcellular localization during heat shock and recovery as described here leads us to speculate that they participate in the protection and repair of the cell's organelle structure and, in particular, the cytoskeleton, from the effects of stress. We can only speculate that each hsp plays one or more unique roles reflective of their intracellular distribution and that the protection and recovery of the cell from the effects of stress relies on their combined activities.

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