Morphological Study of the Mammalian Stress Response: Characterization of Changes in Cytoplasmic Organelles, Cytoskeleton, and Nucleoli, and Appearance of Intranuclear Actin Filaments in Rat Fibroblasts after Heat-Shock Treatment

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ABSTRACT   Using both electron microscopy and immunological methods, we have characterized a number of changes occurring in rat fibroblasts after heat-shock treatment. Incubation of the cells for 3 h at 42°–43°C resulted in a number of changes within the cytoplasm including: a disruption and fragmentation of the Golgi complex; a modest swelling of the mitochondria and subtle alterations in the packing of the cristae; and alterations in cytoskeletal elements, specifically a collapse and aggregation of the vimentin-containing intermediate filaments around the nucleus. A number of striking changes were also found within the nuclei of the heat-treated cells: (a) We observed the appearance of rod-shaped bodies consisting of densely packed filaments. Using biochemical and immunological methods, these nuclear inclusion bodies were shown to be comprised of actin filaments. (b) Considerable alterations in the integrity of the nucleoli were observed after the heat-shock treatment. Specifically, there appeared to be a general relaxation in the condensation state of the nucleoli, changes in both the number and size of the granular ribonucleoprotein components, and finally a reorganization of the nucleolar fibrillar reticulum. These morphological changes in the integrity of the nucleoli are of significant interest since previous work as well as studies presented here show that two of the mammalian stress proteins, the major stress-induced 72-kD protein and the 110-kD protein, localize within the nucleoli of the cells after heat-shock treatment. We discuss these morphological changes with regards to the known biological and biochemical events that occur in cells after induction of the stress response.

In an effort to understand the many changes occurring in mammalian cells after physiological stress, we have been examining the stress response at both the cellular and biochemical levels. Previous studies from numerous laboratories have defined many of the basic parameters governing the stress response in a variety of different organisms (for reviews see references 3, 34, and 43). Briefly, in almost all organisms studied to date, physiological stress is characterized by the rapid and preferential transcription of genes encoding the stress proteins and a concomitant decrease in the transcription and/or processing of those genes that were active before the environmental insult. These changes at the transcriptional level are accompanied by alterations in translational events occurring in the stressed cell. Specifically, two classes of polysomes are observed: a very active class in which high levels of the stress-induced mRNAs are being translated, and a somewhat inactive class containing mRNAs that were translationally active before the insult. The mechanisms by which these transcriptional and translational changes occur in the stressed cell remain unknown. The net result of these new events is the increased synthesis and accumulation of a small number of proteins referred to as the stress proteins and a decreased production of most other cellular proteins. While considerable effort has been directed at understanding the

biological role of the stress proteins, the exact function of any one of the individual stress proteins still remains unknown. There is evidence, however, that collectively these proteins appear to serve a protective role within the stressed cell. This follows from the observation that the prior induction of the stress proteins appears to confer enhanced protection to the cells (as assayed by cell survival) upon subsequent stress situations (17, 22, 25, 26, 41).

The proteins synthesized at elevated levels in various mammalian cell lines after physiological stress are referred to in our laboratory according to their apparent size in SDS polyacrylamide gel electrophoresis as the 28-, 72-, 73-, 80-, 90-, 100-, and 110-kD stress proteins (43, 49). All of these proteins, with the exception of the most highly induced 72-kD species, are synthesized at modest levels in normal cells (46, 49). Using cell fractionation and immunological methods, the location of most of the mammalian stress proteins has been determined. Briefly, many of the proteins including the 28-, 72-, 73-, and 110-kD species have been shown to localize, in part, within the nucleus (18, 42, 46). Interestingly, the 72- and 110-kD proteins also show a distribution within phase-dense structures in the nucleus, including the nucleolus (42, 46). The 80-, 90-, and 100-kD stress proteins, whose syntheses are also sensitive to extracellular levels of glucose and/or calcium, are present primarily in the cytoplasm (45, 46, 49). The 80- and 100-kD proteins are associated with intracellular membranes, specifically the endoplasmic reticulum and Golgi complex, respectively (24, 49).

Along with our studies aimed at determining the function of the stress proteins, we have been examining changes that occur in the cytoplasm and nucleus of cells after physiological stress. In the present study we describe a number of interesting morphological alterations that occur in rat embryo fibroblast cells after heat-shock treatment. Evidence is presented for changes occurring in cytoplasmic organelles, including the mitochondria, endoplasmic reticulum, and Golgi complex, as well as rearrangements in certain cytoskeletal elements. In addition, a number of changes occurring within the nucleus and nucleolus of cells after heat-shock treatment are described.

MATERIALS AND METHODS

Cell Culture: Established rat embryo fibroblasts (REF-52) or primary rat embryo fibroblasts (isolated from 19-d-old rat embryos) were used in all experiments. Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum on either 35-mm plastic dishes (Falcon Labware, Oxnard, CA) or on glass coverslips. Heat-shock experiments were performed by heating the cells in a water bath (42°–43°C) for 5–10 min and then placing the cells in a humidified incubator at 42°C for 3 h.

Indirect Immunofluorescence Studies: For the analysis of the distribution of actin, REF-52 cells on glass coverslips were fixed with 3.7% formalin and extracted with cold absolute acetone. Anti-actin staining was performed using a mouse monoclonal antibody (JLA20) diluted 1/1,000 in phosphate-buffered saline (PBS) containing 2 mg/ml bovine serum albumin (BSA) and a second antibody, fluorescein-conjugated goat anti–mouse antibody diluted 1/40 in PBS containing BSA. For the distribution of microtubules (anti-tubulin staining) and intermediate filaments (anti-vimentin staining), the cells were fixed and permeabilized by incubation in cold absolute methanol. Anti-tubulin staining was done with a mouse monoclonal antibody specific for alpha-tubulin (DM1A) (7) diluted 1/150, and anti-vimentin staining was performed with a rabbit polyclonal antibody against vimentin (7) diluted 1/40 in PBS containing BSA as described. Double-label indirect immunofluorescence, comparing the distribution of tubulin and vimentin in the same cell, was done by using two second antibodies: fluorescein-conjugated goat anti–rabbit and rhodamine-conjugated goat anti–mouse antibody (Cappel Laboratories, Cochranville, PA), diluted 1/40 and preabsorbed with methanol-fixed rat embryo

fibroblast cells. For the analysis of the intracellular distribution of the 72- and 110-kD stress proteins, rabbit polyclonal antibodies specific for either protein were used. Anti-72-kD serum was diluted 1/40, and anti-110-kD serum (a generous gift of Dr. John Subjeck) was diluted 1/40. The cells were fixed with formalin and extracted with acetone as described above. Rabbit antibodies were detected after incubation with a fluorescein-conjugated goat anti–rabbit antibody. Before their use, all diluted antibodies were clarified using a Beckman Airfuge (Beckman Instruments, Inc., Palo Alto, CA).

Electron Microscopy: Heat-shock and control cultures were fixed in 2% glutaraldehyde in 75 mM cacodylate buffer supplemented with 4.5% sucrose for 30 min at room temperature. Cultures were washed in three changes of 100 mM cacodylate supplemented with 6% sucrose for 8 min, followed by two changes of 100 mM cacodylate without sucrose for 2 min, and postfixed in 1% OsO_4 in 50 mM cacodylate for 30 min at room temperature. After washing in three changes of distilled H_2O, the cultures were en bloc stained in saturated aqueous uranyl acetate for 20 min. The cultures were washed in three changes of distilled water for 5 min and dehydrated in a graded series of ethanol. Infiltration was in 100% EPON overnight, with a fresh change of EPON the next day. The cultures were polymerized at 60°C for 24 h. Polymerized EPON was removed from the petri dish by breaking the dish away from the EPON disk. Small pieces of the disk were glued to a blank EPON block. Thin (silver) en face sections were cut with a diamond knife on an LKB III ultramicrotome, picked up on 200-mesh copper hex grids, and stained with lead citrate. Electron micrographs were taken on a Philips 201 at 80 kV (Philips Electronic Instruments, Inc., Mahwah, NJ).

Heavy Meromyosin Decoration: Heavy meromyosin decoration of actin filaments was modified from a protocol described by Schloss et al. (35). Heat-shock and control cultures were removed from the CO_2 incubator and washed with three changes of standard salt solution (SSS) buffer (50 mM KCl, 5 mM $MgCl_2$, 7 mM Na-phosphate, pH 7.0, 0.002% azide, and 2 mM EGTA) for 5 min at room temperature. The cultures were incubated in a series of 5, 12, and 25% glycerol/SSS extraction buffer, held in 50% glycerol/SSS extraction buffer plus 0.2% Triton for 30 min, and incubated in a descending series of 25, 12, and 5% glycerol/SSS extraction buffer. After washing in five changes of SSS buffer without EGTA for 30 min, the cultures were incubated in 1 mg/ml heavy meromyosin in SSS buffer for 30 min at room temperature. Control cultures were incubated in SSS buffer without heavy meromyosin. The cultures were washed in five changes of SSS buffer, followed by fixation in 1% glutaraldehyde in 0.1 M Na-phosphate, pH 7.0, plus 2% tannic acid (4) for 30 min at room temperature. After washing in three changes of Na-phosphate for 10 min, the cultures were postfixed in 1% OsO_4 in 0.1 M Na-phosphate for 30 min at room temperature. Additional processing was identical to the electron microscopy procedures described above.
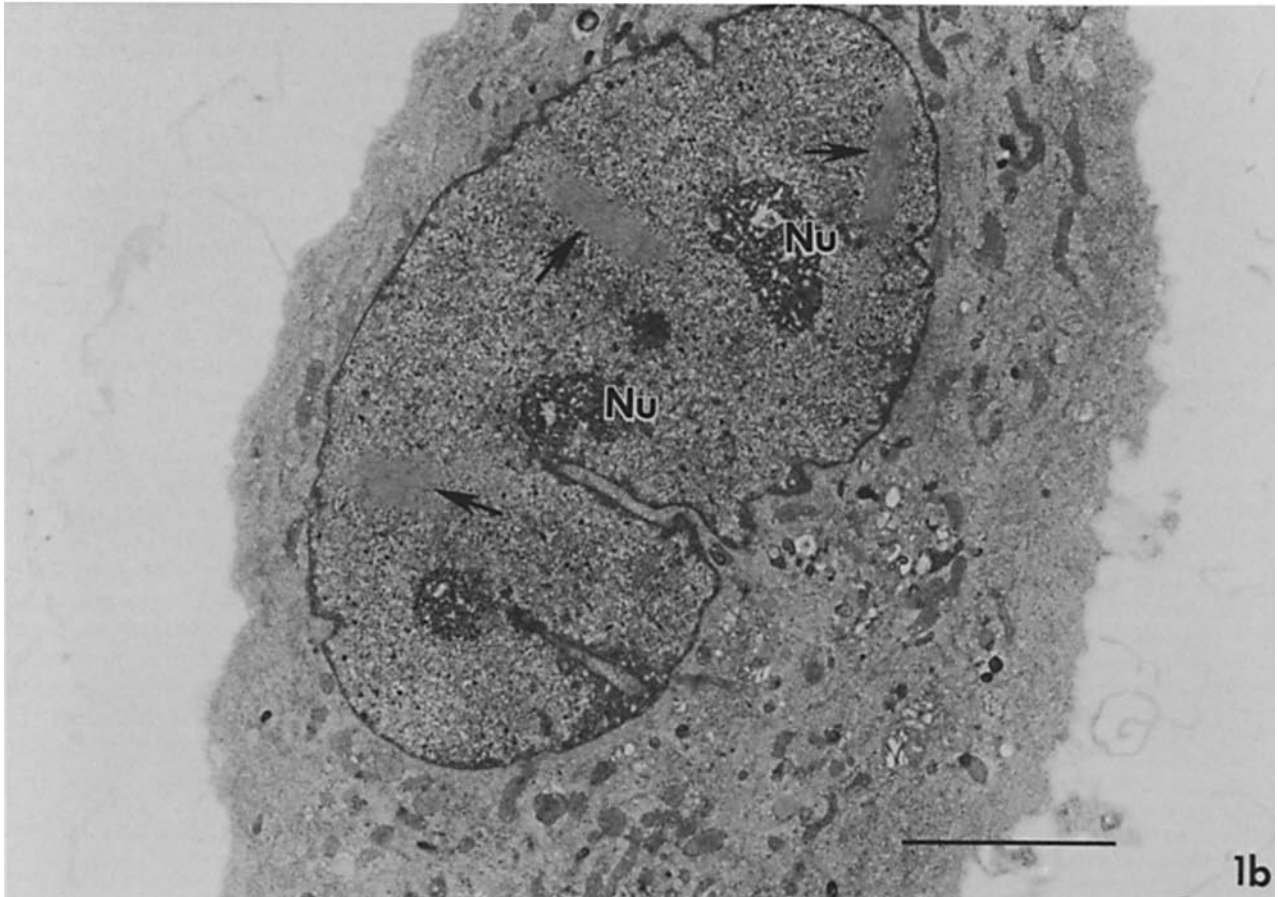
RESULTS

Shown in Fig. 1 are electron micrographs of established rat embryo fibroblasts (REF-52) incubated at the normal temperature (i.e., 37°C) or after heat-shock treatment (3 h at 42°C). At this level of magnification, no obvious changes were observed within the cytoplasm of the cells incubated at the higher temperature (however, see below). In contrast, a number of differences were apparent in the nuclei of the heat-shock–treated cells. (a) We have indicated a number of unusual inclusions within the nucleus of the heat-treated cells (indicated by arrows in Fig. 1b). These rod-like structures appear to consist of closely packed filaments, and we will return to these structures later. (b) Careful examination of the nucleoli revealed considerable alterations after the heat-shock treatment. Specifically, there appeared to be a general swelling of these structures with a number of changes particularly evident within the granular region. In what follows, we will examine closely these changes occurring in the nuclei of the heat-treated cells and in addition will present evidence indicating a number of changes occurring with respect to cytoplasmic membranous organelles and various cytoskeletal elements.

Morphological Changes in the Cytoplasm

Numerous agents that interfere with mitochondrial function, including uncouplers of oxidative phosphorylation and
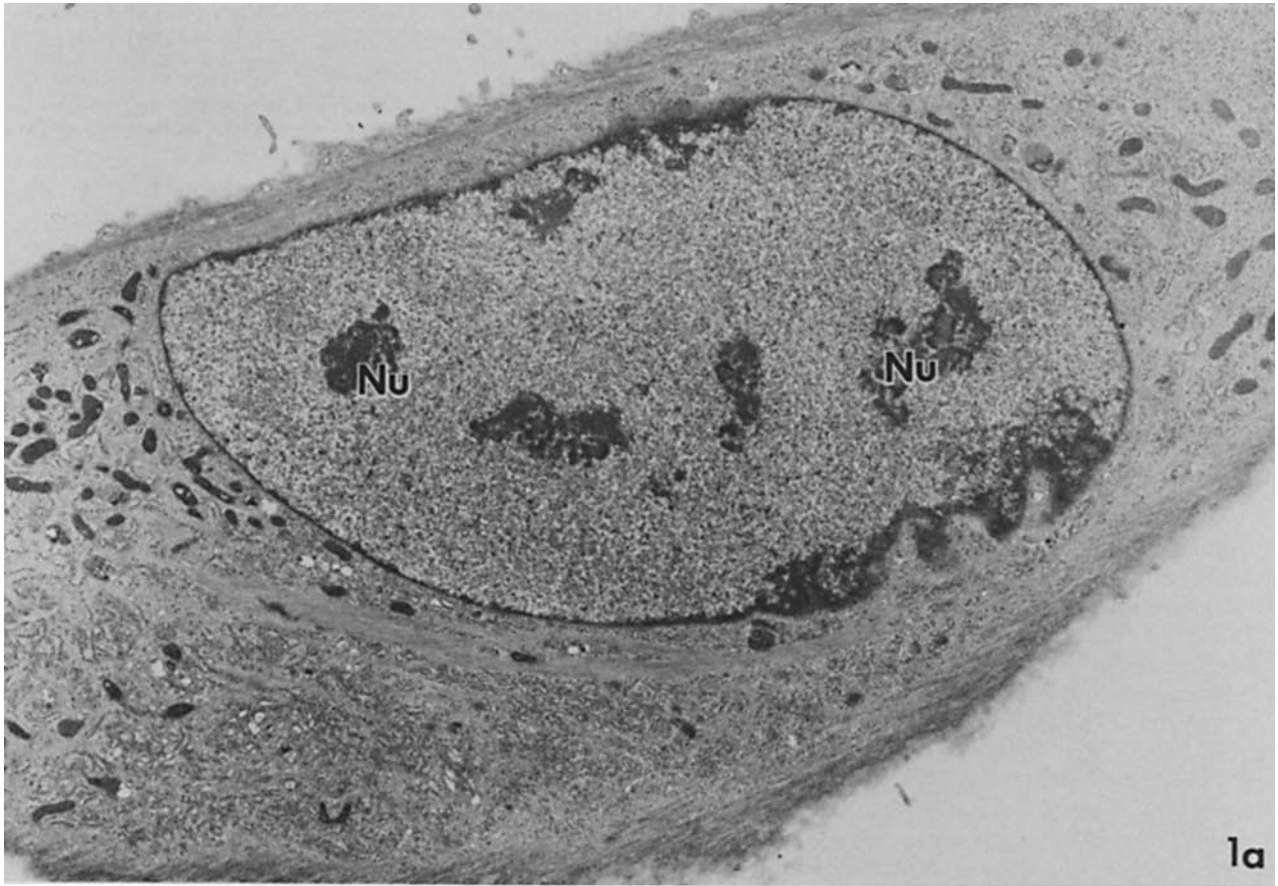
inhibitors of electron transport, also result in the induction of the stress proteins in *Drosophila melanogaster* cells (reviewed in reference 3). These and other observations have led some investigators to suggest that the mitochondria may be the primary target of the many different stimuli that induce the stress response (see reference 3 for review). Therefore, we examined whether any morphological changes occurred within the mitochondrion of mammalian cells after heat-shock treatment. In Fig. 2 are shown electron micrographs of mitochondria present in the unheated control cells (Fig. 2a) or in the heat-shock–treated cells (Fig. 2b). Mitochondria of the 37°C cells displayed a typical cylindrical shape with well-packed and organized cristae. Heat treatment of the cells resulted in a number of structural changes within the mitochondria. Specifically, the mitochondria appeared swollen, the cristae more prominent, and the intracristal spaces appeared enlarged. A number of small phase-dense structures were observed in and around the mitochondria of the 37°C cells (Fig. 2a). These structures are presumably free ribosomes and/or polysomes. In contrast, very few of these structures were evident around the mitochondria in the heat-shocked cells. This may be due to changes in the intracellular distribution of the mitochondria after heat shock. For example, we found an increase in the number of the mitochondria located near and around the nucleus and fewer present out near the cell periphery after heat-shock treatment of the cells (data not shown). This relocalization of the mitochondria may be due to a collapse and aggregation of the intermediate filaments around the nucleus in the heat-treated cells (e.g., see Figs. 4 and 5).

We next examined two other organelles present in the cytoplasm: the endoplasmic reticulum and Golgi complex. Previous indirect immunofluorescence studies from our laboratory have shown that the 80- and 100-kD stress proteins are present in the endoplasmic reticulum and Golgi complex, respectively (49). After heat-shock treatment of various mammalian cells, we have observed what appears to be a fragmentation and/or disappearance of these two membranous organelles. Consistent with these previous immunofluorescent studies is the data shown in Fig. 3. Fig. 3a shows a micrograph of a thin section near the perinuclear region of control REF-52 cells, and in Fig. 3b a micrograph of a similar section from the heat-treated cells. From these pictures it was difficult to make firm conclusions regarding the structure of the endoplasmic reticulum in either the 37° or 42°C cells. The well-packed and organized Golgi complex (G), however, was easily
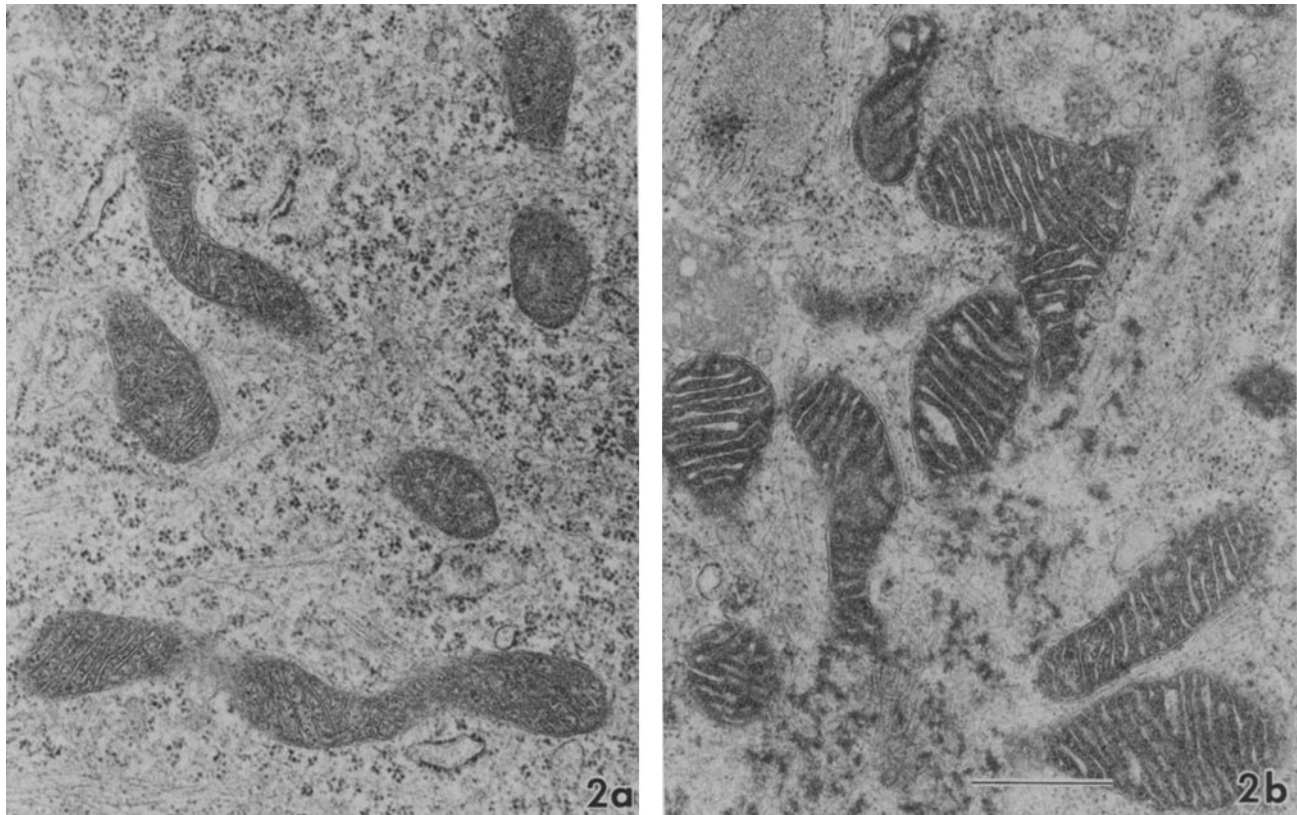

FIGURE 2   Effects of heat-shock treatment on the mitochondria of rat embryo fibroblasts. Rat fibroblasts incubated at 37°C or at 42°C for 3 h were analyzed by electron microscopy. Shown are thin sections of the cytoplasm illustrating the mitochondria from either the 37°C cells (a) or the 42°C cells (b). Bar, 0.5 μM.

FIGURE 1   Effects of heat-shock treatment in rat embryo fibroblasts. Rat embryo fibroblasts growing on 35-mm dishes were incubated at either 37°C (a) or at 42°C (b) for 3 h. The cells were fixed and then stained with aqueous saturated uranyl acetate and lead citrate and analyzed by electron microscopy as described in Materials and Methods. (All of the following electron micrographs were processed in the same manner unless otherwise stated.) Arrows indicate intranuclear fibrous structures; Nu, nucleolus. Bar, 5 μM.
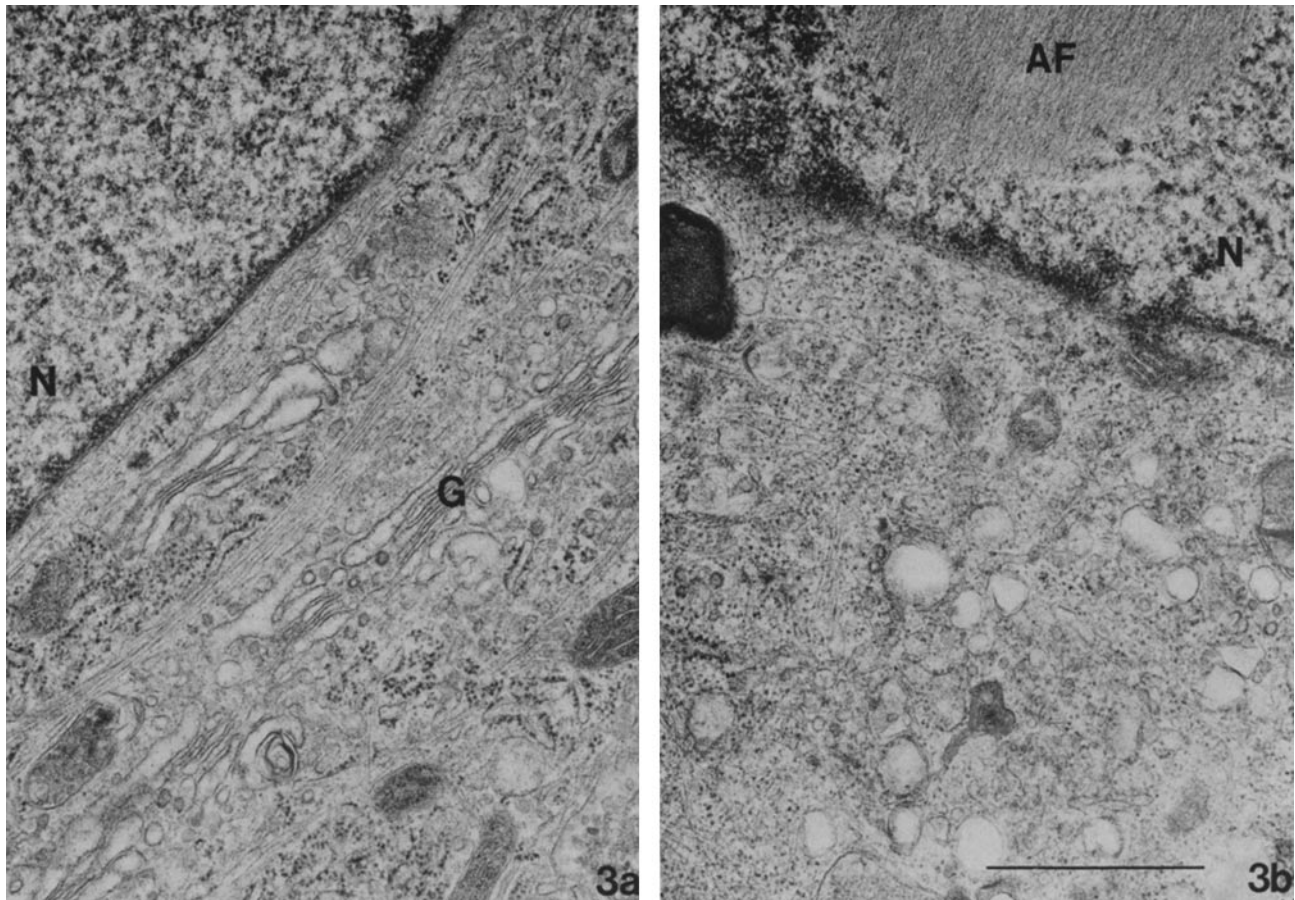
FIGURE 3 Heat-shock treatment results in the fragmentation of the Golgi complex. Rat embryo fibroblasts incubated at either 37°C (a) or 42°C (b) for 3 h were analyzed by electron microscopy. Note the well-organized Golgi complex (G) in the 37°C cells. After heat-shock treatment, the Golgi complex can no longer be discerned. N, nucleus; AF, intranuclear inclusion bodies (described further in Fig. 7). Bar, 1 μM.
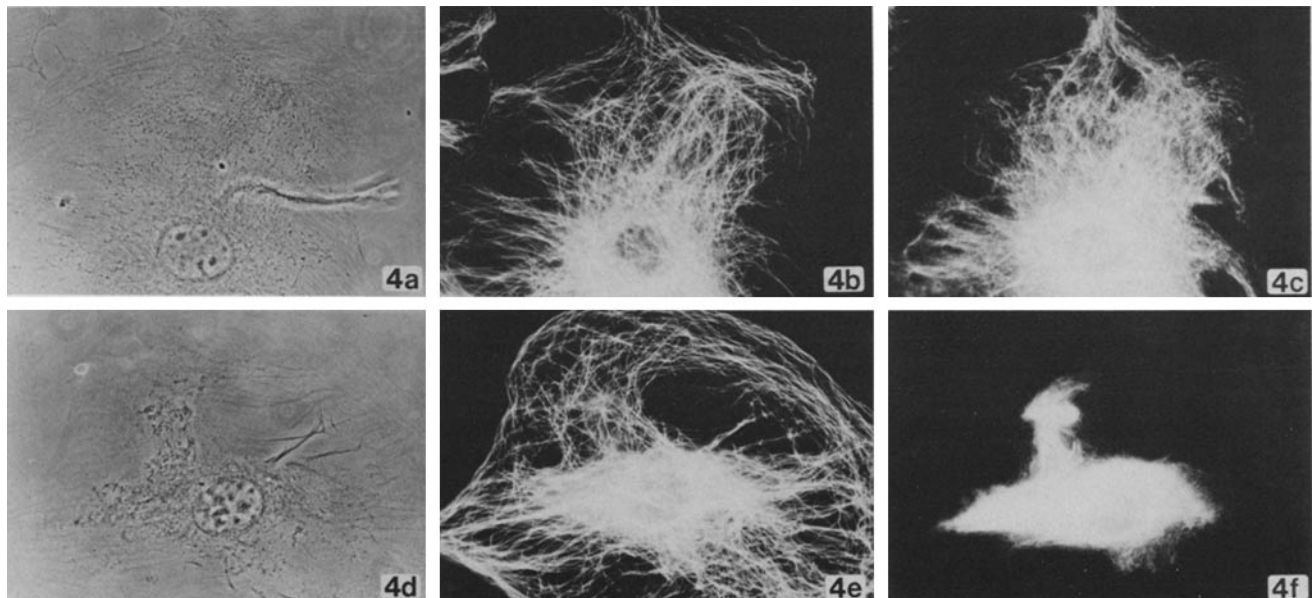

FIGURE 4 Double-label indirect immunofluorescence analysis of the distribution of the vimentin-containing intermediate filaments and of the microtubules. Rat embryo fibroblasts growing on glass coverslips at either 37°C or at 42°C for 3 h were fixed and processed for indirect immunofluorescence analysis as described in Materials and Methods. Double-label staining using a rabbit polyclonal specific for vimentin and a mouse monoclonal antibody specific for alpha-tubulin was performed as described in the Materials and Methods. Shown in a and d are the phase contrast micrographs, and in b, c, e, and f the corresponding fluorescent micrographs. In a, b, and c are shown the same cell incubated at 37°C and stained with anti-tubulin (b) and anti-vimentin (c). In d, e, and f are shown the same cell incubated at 42°C for 3 h and stained with anti-tubulin (e) and anti-vimentin (f).

discerned in the 37°C cells (Fig. 3a). After heat-shock treatment it was difficult to detect any remaining organized Golgi complex (Fig. 3b). Instead there appeared to be an increase in the number of vesicularized membranes found throughout the perinuclear region.

Changes in Cytoskeletal Elements after Heat-Shock

We have examined the integrity of the three major cytoskeletal networks—actin filaments, vimentin-containing intermediate filaments, and the microtubules—in cells after physiological stress. In the case of the actin-containing microfilaments, physiological stress resulted in a general flattening of the cells and an increased number of actin-containing stress fibers spanning the cell, changes characteristic of the growth-arrested state of the cells (43). The distribution of microtubules and intermediate filaments in both normal cells and in cells after heat-shock treatment was analyzed by double-label indirect immunofluorescence. In the 37°C cells (Fig. 4a), the microtubules (Fig. 4b) and intermediate filaments (Fig. 4c) showed a well-spread and often coincident distribution. After the heat-shock treatment (Fig. 4d), no obvious change in the distribution of the microtubules was observed (Fig. 4e). In the case of the intermediate filaments, however, the heat-shock treatment resulted in a collapse and aggregation of the filaments in and around the nucleus (Fig. 4f). We have previously observed a similar collapse of the intermediate filaments in cells exposed to either amino acid analogues (43) or to heavy metals (our unpublished observation), two other agents that strongly induce in cells the stress response. Removal of any of these stress agents and incubation of the cells under normal conditions resulted in a gradual restoration of the normal distribution of the intermediate filaments (43; and

our unpublished observations).

We have also analyzed this interesting rearrangement of the intermediate filaments using the electron microscope. While only few of the filaments were observed near the nucleus in the control, 37°C cells (Fig. 5a), large bundles of the 10-nm filaments were found very near the nuclear membrane in the heat-shock–treated cells (Fig. 5b). Higher magnification pictures of these closely packed intermediate filaments are shown in Fig. 5c. The collapsed filaments appeared to run parallel to one another and occupied an area as much as 1 μm in diameter. Along with the collapse of the intermediate filaments was a similar redistribution of the mitochondria toward the cell center, some of which appeared in close proximity to the aggregated intermediate filaments (data not shown).

Changes within the Nucleus

As was shown in Fig. 1, there appeared a number of unusual fibrous-containing, rod-like structures within the nuclei of the heat-treated cells. In addition, changes in the condensation state of the individual nucleoli were readily apparent. High magnification electron micrographs of nuclei from control and heat-treated established rat embryo fibroblasts or from normal and heat-treated primary cultures of rat embryo fibroblasts are shown in Fig. 6. In the control, 37°C cells (a and c), the nuclei appeared quite typical with well-organized and condensed nucleoli. After the heat-shock treatment, however, a number of changes were observed (Fig. 6, b and d). (a) The nucleoli appeared less condensed, and showed signs of unraveling. We will return to this point later. (b) In the heat-treated cells only, there was a large number of very small electron-dense nuclear structures (indicated by arrowheads in Fig. 6, b and d) which may correspond to perichromatin granules. (c)
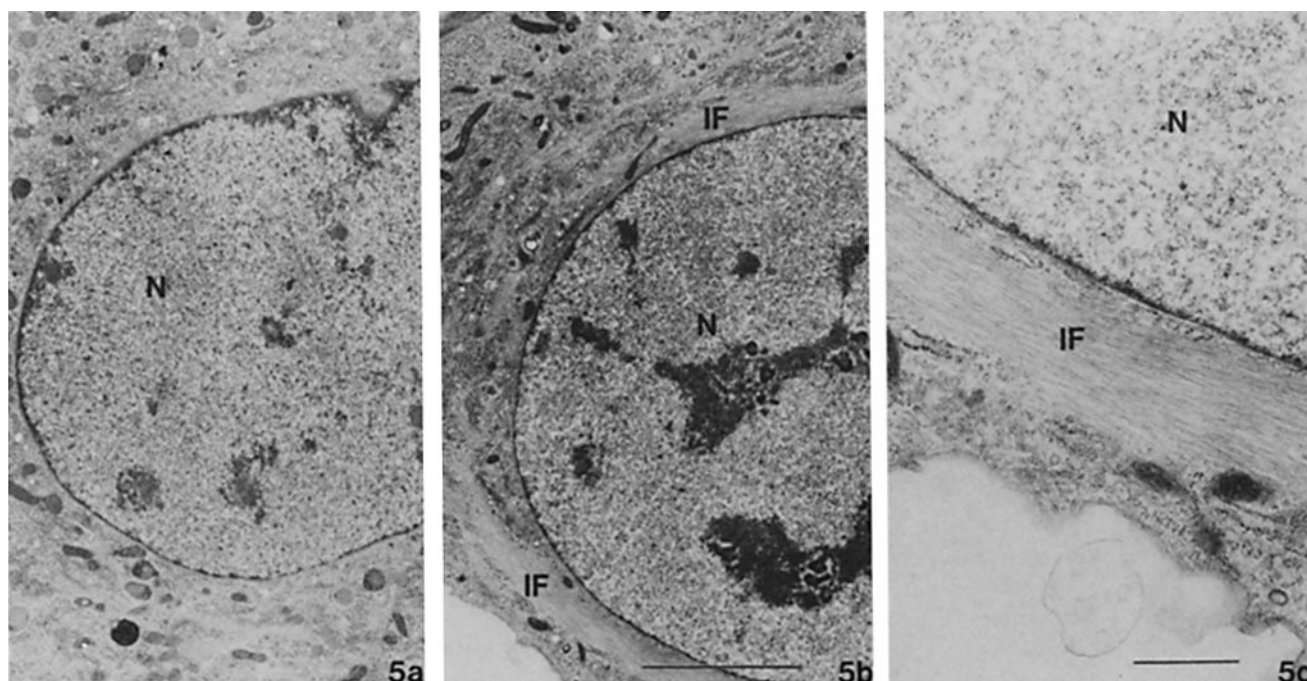
Figure 5   Analysis by electron microscopy of the rearrangement of intermediate filaments after heat-shock treatment. Rat embryo fibroblasts were incubated at either 37°C or at 42°C for 3 h and then analyzed by electron microscopy. Shown in a is a section near the nucleus of the cells incubated at 37°C. In b and c are sections near the nucleus of the cells incubated at 42°C for 3 h. Note the dense bundles of intermediate filaments (IF) running along the border of the cytoplasm and nucleus (N). Bar in a and b, 5 μM; bar in c, 1 μM.
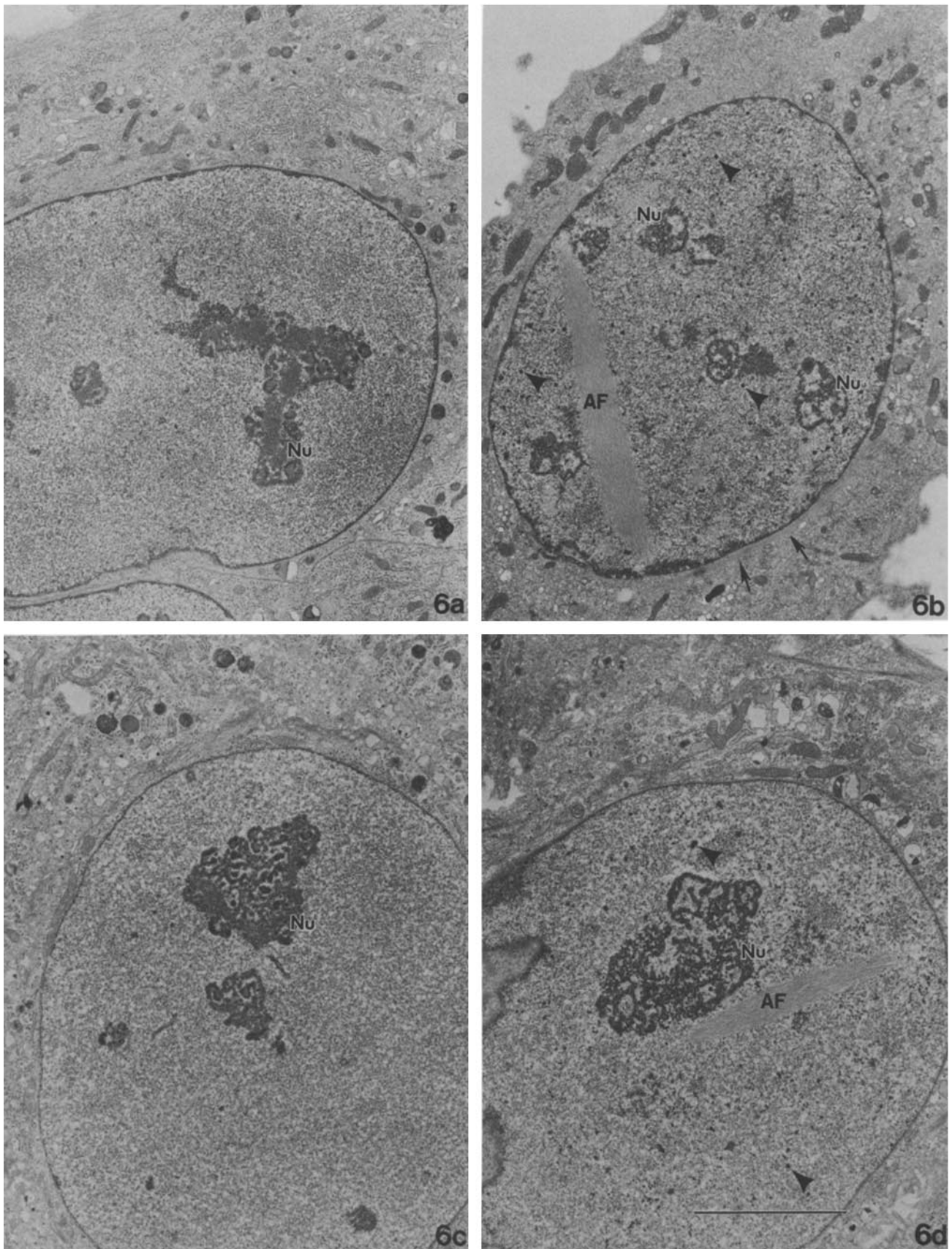
FIGURE 6   Alterations in the nuclei of rat fibroblasts incubated under heat-shock conditions. Established rat embryo fibroblasts (REF-52) or primary cultures of rat fibroblasts derived from 19-d-old embryos were incubated at either 37°C or at 42°C and analyzed by electron microscopy. In a, 37°C REF-52 cells; b, 42°C REF-52 cells; c, 37°C primary rat fibroblasts; d, 42°C primary rat fibroblasts. Indicated are the nucleolus (Nu) and intranuclear inclusion bodies (AF, see below). Arrowheads in b and d indicate small phase-dense structures (perichromatin granules?) which are more abundant in the heat-shocked cells. Arrows in b indicate collapsed intermediate filaments. Bar, 5 μM.

The nuclei of the heat-treated cells contained unusual rod-like inclusion bodies that appeared to be packed with thin parallel filaments (indicated by AF).

Higher magnification electron micrographs illustrate the fibrous nature of these intranuclear inclusion structures in the heat-treated cells (Fig. 7, a and b). In many cases the filaments were found to terminate very near the nuclear envelope (Fig. 7b). We have never observed these structures to extend through the nuclear membrane into the cytoplasm. The structures are often found juxtaposed to one another in an almost perpendicular fashion (Fig. 7a). Because of the small diameter of these nuclear filaments (~4–6 nm), we examined whether they were in fact comprised of actin. Heat-shock–treated cells were extracted and incubated with a solution containing heavy meromyosin as described in Materials and Methods. The nuclear structures were found to bind the meromyosin, thus confirming that they indeed contained actin. We could not ascertain whether the filaments all displayed a uniform directionality (i.e., arrowheads pointing in one direction) similar to that often observed for cytoplasmic microfilaments (5). Further confirmation that these nuclear structures were in fact comprised of actin was provided by indirect immunofluorescence analysis using a monoclonal antibody specific for actin. In Fig. 7d, the phase-dense nuclear structures were easily seen in the phase-contrast micrograph of the heat-treated rat fibroblasts. These nuclear structures were recognized by the actin monoclonal antibody (Fig. 7e). In addition, the cytoplasmic actin microfilaments (or stress fibers) can also be seen spanning the cytoplasm of the cell.

Previous studies from both our laboratory and from Subjeck's group have shown that two of the major mammalian stress proteins, the 72- and 110-kD proteins, localize within the nucleus and nucleolus (42, 46). In the case of the 72-kD stress protein, very little of the protein can be detected in most mammalian cells grown at 37°C. After heat-shock treatment (or after induction of stress by other agents), synthesis of the 72-kD protein represents the major translational activity of the cells (46, 49). Indirect immunofluorescence analyses of rat fibroblasts incubated at either 37° or 42°C for 3 h and stained with a polyclonal antibody specific for 72-kD protein are shown in Fig. 8. Consistent with its low level, little or no fluorescence was observed in the 37°C (Fig. 8b) cells. After heat-shock treatment, however, fluorescence was observed within the nucleus and more prominently within the nucleolus (Fig. 8d). Note also the increase in the phase density of the individual nucloeli after the heat-shock treatment (compare Fig. 8a and 8c). 110 kD is a constitutive protein whose synthesis increases after physiological stress (Shyy, T. T., J. R. Subjeck, R. Heinaman, and G. Anderson, unpublished observations). Indirect immunofluorescence analysis using a rabbit polyclonal antibody specific for 110 kD (kindly provided by John Subjeck) demonstrated the protein to be present within the nucleolus (Fig. 8f) (42). After heat-shock treatment, 110 kD appeared to show a slight relocalization within the nucleolus. Specifically, the majority of the protein now appeared to localize primarily along the perimeter of the now swollen nucleoli (Fig. 8h).

Both the apparent changes in the phase density of the nucleoli and the observation that two of the mammalian stress proteins localize to the nucleolus prompted us to examine carefully those alterations occurring within the nucleoli after heat-shock treatment. In Fig. 9 are shown high magnification electron micrographs of individual nucleoli present in either established rat embryo fibroblasts (Fig. 9a) or freshly prepared primary rat embryo fibroblasts (Fig. 9c) grown at 37°C. These nucleoli exhibited the typical phase-dense granular regions (indicated by g) that consist of preribosomal particles and other ribonucleoprotein complexes. In addition, the fibrillar reticulum (designated f) is easily discerned. After heat-shock treatment of either the established cells (Fig. 9b) or the primary cells (Fig. 9d), considerable changes in the structure of the nucleoli were observed. First, the nucleoli in almost all cells were swollen and appeared to be unraveling. Instead of the finely packed intranucleolar granular components, there now appeared to be larger, very electron-dense granules (Fig. 9b). In addition, the fibrillar reticulum, now difficult to define in most cases, appeared present primarily along the perimeter of the enlarged nucleoli. In some cases we observed almost a complete loss of all of the intranucleolar granular components (Fig. 9d). These alterations in the organization and composition of the nucleoli after heat-shock treatment of the cells were reversible. Removal of the heat treatment and incubation of the cells at the normal temperature resulted in a very gradual restoration of normal nucleolar morphology (Welch, W. J., and J. P. Suhan, manuscript in preparation).

DISCUSSION

Using both electron microscopy and immunological methods, we have characterized a number of morphological changes occurring in mammalian cells after heat-shock treatment. Specifically, we have described changes in the organization of the Golgi complex, alterations in the structure of the mitochondria, an aggregation and collapse of the vimentin-containing intermediate filaments, and the appearance of actin-containing inclusion bodies within the nucleus. Lastly, major alterations with respect to the organization of the nucleolus, the location in the cell of two of the major stress proteins, were observed. These findings are discussed below concerning previous observations of the biology of the stressed cell and the known biochemistry of some of the stress proteins.

Previous indirect immunofluorescence studies have shown that two of the mammalian stress proteins, the 80- and 100-kD proteins, are associated with the endoplasmic reticulum and Golgi complex, respectively, in cells grown under normal conditions (49). After heat-shock treatment, we have observed major changes in the intracellular location of these two proteins. Specifically, both 80 and 100 kD were now found localized throughout the cytoplasm, with a small portion of the two proteins also showing a nuclear locale (24, 49). We suspected that these changes in the intracellular distribution of 80 and 100 kD after heat-shock treatment were most likely due to a fragmentation and/or vesicularization of both the endoplasmic reticulum and Golgi complex. Indeed, we did observe a disruption in the normal organization of the Golgi complex and an accompanying increase in the number of vesicularized membranes within the perinuclear region after a 3-h heat-shock treatment of the rat fibroblasts (Fig. 3). In the case of the endoplasmic reticulum, however, it was not possible to ascertain in these electron micrographs any major changes after heat shock. We are currently analyzing whether a similar disruption in the organization of the Golgi complex occurs in cells placed under stress by exposure to other agents such as sodium arsenite or amino acid analogues. Although the Golgi complex is involved in the glycosylation of most
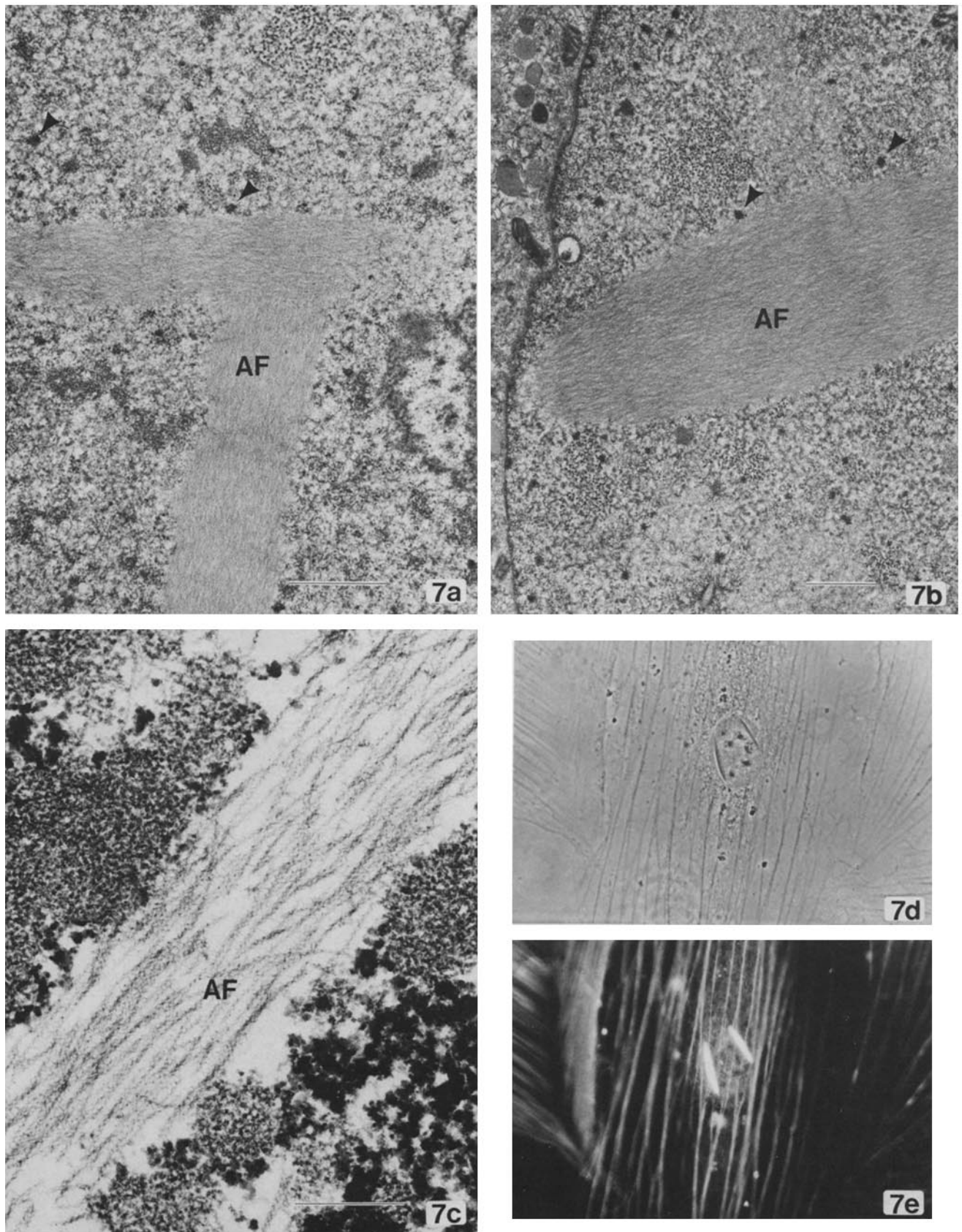
FIGURE 7   Intranuclear inclusions in heat-treated cells are comprised of actin. Rat embryo fibroblasts were grown on plastic dishes at 42°C for 3 h and sections through the nucleus analyzed by electron microscopy (a and b). Similarly, heat-treated fibroblasts were fixed, extracted, and incubated with heavy meromyosin as described in the Materials and Methods (c). Note the typical arrowhead decoration of the actin filaments. In d are shown the phase contrast and corresponding fluorescent micrograph (e) of a heat-treated cell analyzed by indirect immunofluorescence using a mouse monoclonal antibody specific for actin. The phase-dense nuclear inclusions were evident (d) and were stained with the anti–actin antibody (e). AF, actin filaments. Bars in a and b, 5 μM; bar in c, 1 μM.
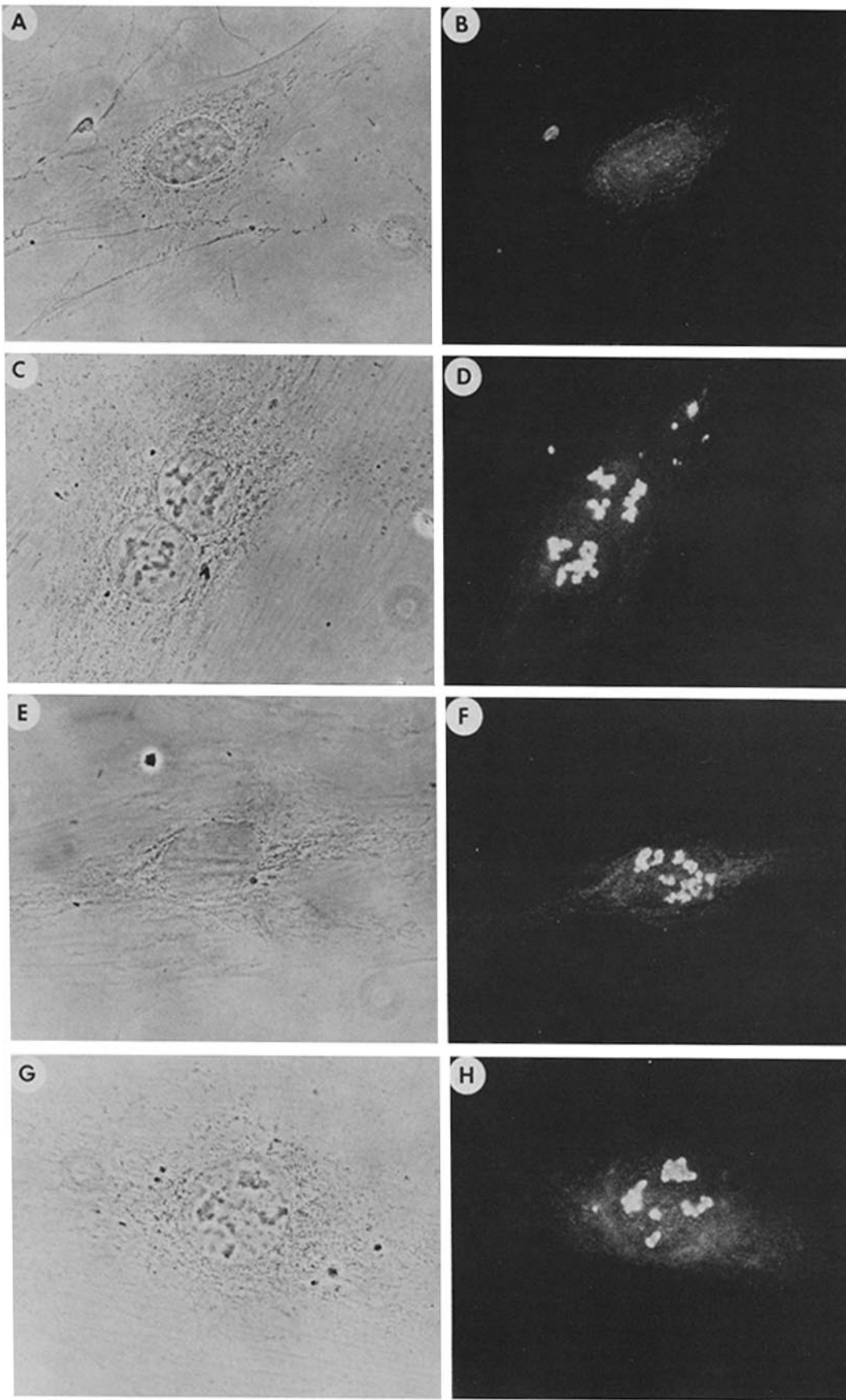
FIGURE 8   Localization of the 72- and 110-kD stress proteins in normal and heat-treated rat embryo fibroblasts. Rat embryo fibroblasts (REF-52) were grown on glass coverslips at either 37°C or at 42°C for 3 h. The cells were fixed and processed for indirect immunofluorescence analysis as described in the Materials and Methods. Analysis was performed using either a rabbit polyclonal antibody specific for the 72-kD stress protein or a rabbit polyclonal antibody specific for the 110-kD stress protein. Shown in panels *a*, *c*, *e*, and *g* are phase contrast micrographs, and in *b*, *d*, *f*, and *h* are the corresponding fluorescent micrographs. (*a* and *b*) 37°C cells stained with the anti–72-kD serum; (*c* and *d*) 42°C cells stained with the anti–72-kD serum. (*e* and *f*) 37°C cells stained with the anti–110-kD serum. (*g* and *h*) 42°C cells stained with the anti–110-kD serum.
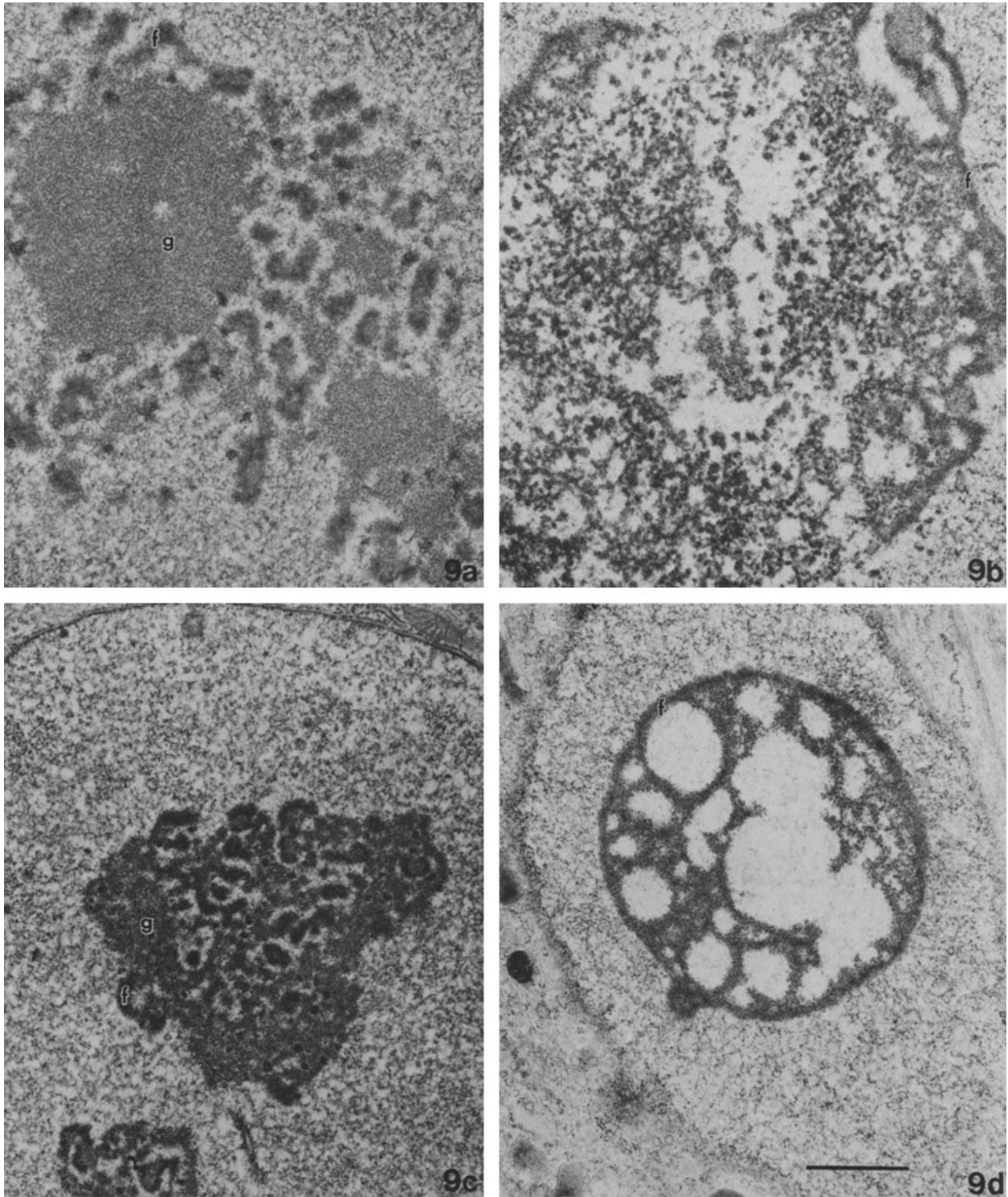
FIGURE 9   Morphological changes occurring in the nucleoli of cells after heat-shock treatment. Established rat embryo fibroblasts (REF-52) or primary cultures of rat fibroblasts were incubated at either 37°C or at 42°C for 3 h and regions within the nucleus analyzed. In a is shown a nucleolus from the 37°C REF-52 cells and in b a nucleolus from the 42°C REF-52 cells. In c is shown a nucleolus from the 37°C primary rat embryo fibroblasts, and in d a nucleolus from the same cells incubated at 42°C. Indicated are the fibrillar region (f) and the granular region (g). Bar in d, 1 μM.

polypeptides, its disruption does not seem to adversely affect the ability of the heat-treated cells to glycosylate a newly synthesized protein. This follows from the fact that the 100-kD Golgi-associated stress protein itself is a glycosylated protein, yet its synthesis and glycosylation continues to occur in the heat-treated cells despite the lack of an organized Golgi

complex (49). It does remain possible, however, that the extent of carbohydrate modification of 100-kD may be altered in the heat-shocked cells due to the fragmentation of the Golgi complex.

Because there exists so many agents that induce the synthesis of the stress proteins, the precise manner by which cells

recognize different environmental insults and then trigger the stress response remains unclear. Previous studies with *Drosophila* cells have demonstrated that many inhibitors of electron transport or uncouplers of oxidative phosphorylation act as potent inducers of the stress response (reviewed in reference 3). It has been suggested, therefore, that perhaps changes in mitochondrial function serve as the primary signal for the induction of the stress response. We observed subtle alterations in the structure of the mitochondria after heat-shock treatment of rat fibroblasts. These changes included a general swelling of the mitochondria, a change in the organization of the cristae, and an increase in the spaces between the individual cristae (Fig. 2). At the present time we do not know whether these subtle alterations in the morphology of the mitochondria may also reflect changes in mitochondrial function. Others, however, have noted very similar alterations in the structure of the mitochondria after exposure of chick myoblasts to various uncouplers of oxidative phosphorylation (10). In addition, numerous studies have implicated a possible relationship between changes in respiratory metabolism and the induction and/or regression of heat-shock puffs in *Drosophila* (reviewed in reference 3). Hence, using biochemical techniques we are continuing to examine whether changes in mitochondrial function occur in mammalian cells after induction (and/or reversal) of the stress response. We also found that in the heat-treated cells many of the mitochondria relocalized toward the cell center in a manner analogous to that of the collapsed intermediate filaments. A similar rearrangement in the location of the mitochondria in cells containing collapsed intermediate filaments has also been observed by others (27). However, it remains to be determined whether such a rearrangement is due to a true association of the mitochondria with the intermediate filaments or whether such a rearrangement is due merely to some type of trapping of the mitochondria as the intermediate filaments collapse around the nucleus.

Using both immunological methods and electron microscopy, we have shown a number of changes with respect to various cytoskeletal elements after physiological stress. As was shown in Fig. 4, and by others, the microtubules and intermediate filaments are closely juxtaposed in cells grown under normal conditions. After heat-shock treatment, no major change was observed in the distribution of the microtubules. In contrast, the heat-shock treatment resulted in a collapse and aggregation of the vimentin-containing intermediate filaments around the nucleus. A similar capping of the intermediate filaments is observed when mammalian cells are placed under physiological stress by exposure to numerous other agents including amino acid analogues or heavy metals (43). Biessmann et al. have also observed a similar redistribution of the analogous intermediate-like filaments in *Drosophila* cells after heat-shock treatment (6). In the case of mammalian cells this redistribution of the intermediate filaments is reversible. Removal of the stress agent and incubation of the cells under normal conditions results in a restoration of the normal distribution of the filaments (43). We should also point out that the collapse of the intermediate filaments is not necessarily a true indicator of the cells being in a stress response. For example, other treatments to cells including certain viral infections (4; and our unpublished studies), microtubule depolymerization agents (48), or microinjection of various antibodies specific for various intermediate filament proteins or microtubule proteins (7, 23) all

result in a collapse of the intermediate filaments but not an induction of the stress proteins.

A number of changes also occur with respect to the third major cytoskeletal component, actin, in cells exposed to heat-shock treatment. In general, mammalian cells placed under physiological stress become growth arrested (as assayed by the lack of DNA synthesis [our unpublished observation]) and display a more flattened morphology. Consistent with their growth-arrested state is the increased number of actin-containing fibers that are found spanning the cytoplasm of the stressed cells (43). More interesting is our observation here demonstrating the presence of actin-containing nuclear inclusion bodies. These structures, consisting of finely packed and parallel fibers, were observed in ~50–60% of the cells after the heat-shock treatment. We have not observed these nuclear inclusion bodies in cells placed under stress by other agents. Other laboratories have observed similar intranuclear fibrous structures that appear similar to those described here. For example, Pekkala et al. recently described bundles of 4-nm filaments within the nucleus of the fungi *Achyla ambisexualis* after heat-shock treatment (29). Similarly, bundles of intranuclear actin filaments (or paracrystals) have been reported in both *Dictyostelium* and in rat kangaroo PtK_2 cells after exposure of the cells to either high levels of dimethylsulfoxide or to calcium ionophores in conjunction with high magnesium (15, 16, 28, 32). Finally, similar intranuclear rod-like structures, although not characterized with respect to their composition, have been described in pancreatic islet B cells (8), in maturing oocytes of the newt *Triturus viridescens* after treatment of cells with Actinomycin D (19), in human endometrium cells after administration of steroids (30), and finally in a number of different neuronal cells (12, 36). It is difficult to ascertain whether there exists some common denominator in the aforementioned examples and the appearance of these nuclear structures. What also remains unclear is both the source of the nuclear actin and the biological significance of these structures. Concerning its source, the intranuclear actin bundles could be derived from either pre-existing nuclear actin or by recruitment of the cytoplasmic actin. Support for the latter possibility has been provided by the previous studies of Sanger et al. (32). First, they observed that in cells treated with high levels of dimethylsulfoxide, there was a simultaneous disappearance of the cytoplasmic actin-containing stress fibers and an appearance of the intranuclear actin bundles. Unlike the cytoplasmic actin filaments, however, the nuclear filaments did not appear to contain other stress fiber–associated proteins such as alpha-actinin, tropomyosin, or myosin (28, 32). Second, microinjection of fluorescently labeled actin into the cytoplasm of cells and subsequent dimethylsulfoxide treatment resulted in a recruitment of the fluorescent actin into the nuclear actin structures (32). These results, then, would be consistent with the idea that the intranuclear actin bundles are in fact derived from a cytoplasmic pool of actin. Even more interesting is the question regarding the function of these intranuclear actin bundles. In the case of heat-shock treatment, a major event occurring within the nucleus is the changeover in transcriptional activities, specifically the increased transcription of genes encoding the stress proteins and the decreased transcription and/or processing of transcripts that were active before the environmental insult. Within this context of transcription processes and nuclear actin, very recent studies by Scheer et al. (33) have shown that after microinjection of antibodies to either actin or other actin-

binding proteins into the nucleus of living oocytes, transcription of lampbrush chromosomes, but not of rRNA genes, becomes inhibited. Moreover, these investigators also showed that when transcription is repressed in cells by treatment with various drugs or when nuclear RNA is digested by microinjection of RNase into oocyte nuclei, an extensive meshwork of actin filaments is now found in association with the isolated lampbrush chromosomes. These studies then indicate that there may be a possible relationship between intranuclear actin and certain transcription processes. We should also point out that actin itself has been shown to be a specific inhibitor of DNase I activity in vitro (20). One wonders then whether the presence of intranuclear actin filaments could perhaps serve some protective capacity within the nucleus of the heat-shocked cell. Further work will be necessary to determine whether these structures play some role in the changeover of transcriptional activities both during and/or after reversal of physiological stress or alternatively whether they function in some sort of protective capacity during the heat-shock treatment.

Two of the major mammalian stress proteins, the 72- and 110-kD proteins, are associated, in part, with the nucleolus. For example, as was shown previously by Subjeck et al. (42; Shyy, T. T., J. R. Subjeck, R. Heinaman, and G. Anderson, unpublished observations), and in Fig. 8 b, the 110-kD protein is present in 37°C cells and localizes within the phase-dense nucleoli. After heat-shock treatment, synthesis of 110 kD increases and the protein continues to remain associated with the nucleoli, however in a slightly different fashion. The nucleoli began to unravel and loosen, and the majority of 110 kD now appeared present along the outermost portion of the nucleolus (which we think corresponds to the fibrillar region). In the case of the 72-kD stress protein, very little or none of the protein is synthesized in most mammalian cells grown under normal conditions (46, 49). Consistent with its low level is the immunofluorescence analysis showing little or no 72 kD staining in cells grown at 37°C. After heat-shock treatment, however, synthesis of 72 kD constitutes the major translational activity of the cell. Using indirect immunofluorescence analysis, the 72-kD stress protein was found to localize predominantly within the phase-dense nucleoli (Fig. 8 h). Unlike the 110-kD protein which localized to the perimeter of the swollen nucleoli after stress, the 72-kD protein was distributed well within the nucleoli. Although not shown here, reversal of the heat treatment and incubation of the cells at normal temperatures resulted in a decline in synthesis of 72 kD and a corresponding gradual exit of the protein from the nucleus and nucleolus (46).

Because of the nucleolar locale of two of the stress proteins, we examined by electron microscopy the morphological changes that occur within the nucleus and nucleolus of cells after heat-shock treatment. As was shown in Fig. 9, there appeared to be considerable alterations in the organization of the nucleoli after the heat-shock treatment. The 37°C cells showed the typical tightly packed and organized nucleoli with both the granular and fibrillar regions easily distinguishable. 3 h of heat-shock treatment resulted in a dramatic loosening of the nucleolus and a corresponding change in the organization of both the fibrillar and granular regions. In some cases there appeared to be a diminishment in the number of granular particles. Furthermore, the remaining granules appeared enlarged and very electron dense (Fig. 9 b). In other cases, there appeared to be a substantial loss in the amount of granular components after the heat-shock treatment (Fig. 9 d). Other laboratories have similarly noted a decrease in the granular ribonucleoprotein components and a disappearance of the nucleolar reticulum after heat-shock treatment (1, 11, 37, 38). We do not as yet understand the basis of these morphological changes occurring within the nucleolus or the significance of the nucleolar localization of either the 72- or 110-kD stress proteins. In general, nucleolar function, specifically ribosomal RNA synthesis and ribosomal assembly, appears diminished in cells after heat-shock treatment (1, 9, 14, 31). After reversal of the shock treatment, the nucleoli very slowly regain their normal appearance, and ribosomal assembly is gradually restored (Welch, W. J., and J. P. Suhan, manuscript in preparation). The 72- and/or the 110-kD proteins could conceivably be involved in the shutdown of nucleolar function during stress or alternatively be involved in the recovery of normal nucleolar function after reversal of the stress state. Another possibility is that for at least the 72-kD stress protein, the protein migrates to the nucleolus in the stressed cell (or the recovering cell) to be assembled into some type of small ribonucleoprotein complex. This suggestion is prompted by our observations and those of others that the 72-kD protein is abundant both in the cytoplasm and nucleus of the stressed cells (2, 18, 21, 39, 44–46) and that the protein shows an affinity for RNA (13, 38, 40, 46). Consistent with its potential affinity for RNA we have recently found that the highly induced 72-kD protein, as well as the constitutive 73-kD stress protein, both show an affinity for various nucleotides in vitro (47). Therefore, we are examining these various possibilities regarding the function of the 72- and 110-kD stress proteins using both in vivo and in vitro assays. In addition, with the use of different monoclonal and polyclonal antibodies specific for either 72 or 110 kD, we are continuing to examine the fine details of the nucleolar location of these two stress proteins using immunoelectron microscopy.

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