Cellular and Biochemical Events in Mammalian Cells during and after Recovery from Physiological Stress

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Abstract. We have examined and compared a number of cellular and biochemical events associated with the recovery process of rat fibroblasts placed under stress by different agents. Metabolic pulse-labeling studies of cells recovering from either heat–shock treatment, exposure to sodium arsenite, or exposure to an amino acid analogue of proline, L-azetidine 2-carboxylic acid, revealed interesting differences with respect to the individual stress proteins produced, their kinetics of induction, as well as the decay in their synthesis during the recovery period. In the initial periods of recovery, the major stress-induced 72-kD protein accumulates within the altered nucleoli in close association with the pre-ribosomal–containing granular region. During the later times of recovery from stress, the nucleoli begin to regain a normal morphology, show a corresponding loss of the 72-kD protein, and the majority of the protein now begins to accumulate within the cytoplasm in three distinct locales: the perinuclear region, along the perimeter of the cells, and finally in association with large phase-dense structures. These latter structures appear to consist of large aggregates of phase-dense material with no obvious encapsulating membrane. More interestingly we show, using double-label indirect immunofluorescence analysis, that much of the perinuclear and cell perimeter–distributed 72-kD protein coincides with the distribution of the cytoplasmic ribosomes. We discuss the possible implications of the presence of the 72-kD stress proteins within the pre-ribosomal–containing granular region of the nucleolus as well as its subsequent colocalization with cytoplasmic ribosomes in terms of the translational changes which occur in cells both during and after recovery from physiological stress.

ALL organisms from the simplest prokaryotes, yeast, plants, on up to higher eukaryotes respond in a remarkably similar manner to abrupt changes in their environmental circumstance. This apparent defensive mechanism, classically termed the heat–shock response, is now more commonly referred to as the stress response owing to the wide variety of different agents that elicit basically the same cellular changes as those observed after hyperthermic treatment (for reviews see references 3, 8, and 25). Interestingly, the most obvious commonality amongst the various agents that induce the stress response is their ability to promote the accumulation in the cell of abnormal or denatured proteins. The general theme of the stress response in all organisms is the rapid and almost exclusive synthesis of a small number of proteins, the so-called heat–shock or stress proteins, and a corresponding decreased production of most other cellular polypeptides. While considerable work has resulted in identifying the stress proteins in terms of their apparent molecular size, little has been established regarding their mode of action in the cell. However, a recent burst of activity from many laboratories working at the molecular, cellular, and biochemical levels has recently begun to shed new light regarding the major cellular lesions resulting from physiological stress and the possible functions of the stress proteins both in the normal cell as well as in cells experiencing physiological stress.

In mammalian cells placed under stress (for example, in our laboratory we use heat–shock treatment or exposure to either heavy metals or amino acid analogs) a significant increase in the synthesis of ~6–8 proteins is observed. In the absence of a defined function, we refer to these proteins in terms of their apparent molecular mass in SDS polyacrylamide gels as the 28-, 32-, 72-, 73-, 80-, 90-, 100-, and 110-kD proteins (29, 34). Synthesis of almost all of these proteins can be detected easily in the normal, unstressed cell, thereby implicating their probable participation in cellular processes seemingly distinct from physiological stress. For example, synthesis and/or phosphorylation of some of the stress proteins (e.g., the 28-kD, 80-kD, 90-kD, and 100-kD proteins) appear sensitive to various growth factors, tumor promoters, and extracellular levels of glucose or calcium (11, 32, 33, 40). The highly abundant and heavily phosphorylated 90-kD stress protein has been reported to participate in steroid hormone receptor functions as well as displaying transient associations with a number of tyrosine kinases (5, 6, 22, 24). Recent studies have also described a 70-kD protein involved in the ATP-dependent uncoating of clathrin-coated vesicles and which appears to be homologous to one of the members of the 70-kD stress protein family (7, 30).

Owing to their extremely high abundance in the stressed cell as well as their apparent evolutionary conservation amongst different organisms, considerable attention has been

paid to the 70-kD stress protein family. In mammalian cells, there appears to be two major members of the 70-kD family: an abundant, constitutive member we refer to as the 73-kD protein and a highly stress-inducible form, the 72-kD protein. Nucleotide sequence analysis as well as biochemical and immunological analysis has demonstrated that although these proteins are structurally related they are in fact distinct gene products (11, 12, 16, 19, 31, 35, 36). The 72-kD protein is comprised of at least 3–8 distinct isoforms, the exact number differing depending upon the cell type examined, the agent used to induce the response, and finally the severity of the stress treatment. We have previously shown that both the 72-kD and 73-kD proteins exhibit an affinity for ATP in vitro, and we have exploited this property to develop a rapid purification of the proteins using affinity chromatography on ATP–agarose columns (36). Using immunological procedures, we have found that the highly induced 72-kD protein rapidly accumulates within the nuclei and nucleolus after heat–shock treatment of the cells (35). The presence of the 72-kD protein within the nucleolus is always associated with drastic alterations in the integrity of the nucleolus, and we and others have therefore suggested that the 72-kD protein somehow facilitates the recovery of normal nucleolar morphology and function during recovery from stress (15, 20, 35, 37).

In this report, we present new information regarding some of the cellular and biochemical events occurring in rat fibroblast recovering from physiological stress as induced by three different agents: heat–shock treatment, exposure to sodium arsenite, or exposure to an amino acid analog of proline, L-azetidine 2-carboxylic acid (Azc).[1] We show, by metabolic pulse-labeling, similarities and differences with respect to the proteins induced by these different agents as well as major differences in both the kinetics of their induction and decay in their synthesis. We have also analyzed the subcellular distribution of the 72-kD stress protein in the recovering cells and show that early during the recovery period, the 72-kD protein localizes predominantly within that region of the nucleoli involved in ribosomal assembly. In addition, it is shown that the nucleolar distribution of the 72-kD protein always is correlated with marked alterations in the integrity of the individual nucleoli and that as the nucleoli regain normal morphology there is a corresponding exit of the protein from the nucleolus. During the latter periods of recovery, the majority of the 72-kD protein begins to accumulate within the cytoplasm and exhibits, in part, a colocalization with cytoplasmic ribosomes. We discuss these results with regard to the possible function of the 72-kD protein in cells recovering from physiological stress.

Materials and Methods

Cell Culture

Established cultures of rat embryo fibroblasts (REF-52) were used in all experiments (32). Cells were grown in Dulbecco's modified Eagle's medium (DME) supplemented with 10% fetal bovine serum on either plastic dishes (Falcon Labware, Oxnard, CA) or on glass coverslips. Cells were placed under stress by either (a) heat–shock treatment at 42.5°C for 1–3 h (cells were brought quickly to 42.5°C by heating in a water bath for 10 min, followed by incubation in a 42.5°C incubator), (b) exposure to 5 mM Azc (Sigma Chemical Co., St. Louis, MO) for 10 h at 37°C, or (c) exposure to

1. *Abbreviation used in this paper:* Azc, L-azetidine 2-carboxylic acid.

80 μM sodium arsenite for 1.5 h at 37°C (Sigma Chemical Co.). For the recovery experiments, the cells were either placed back at 37°C; or in the case of Azc and arsenite treatment, the culture medium containing the stress agent was removed, the cells were washed twice in DME containing 10% fetal bovine serum, and then further incubated in the same at 37°C.

Metabolic Labeling Studies

REF-52 cells growing on 35-mm dishes were placed under stress as described above. During the last hour of the stress treatment, the culture medium was removed, the cells were washed with leucine-free DME (Gibco, Grand Island, NY), and then further incubated in the leucine-free medium containing 50 μCi of [³H]leucine (L-[4,5-³H] leucine, 50 Ci/mmol; Amersham Corp., Arlington Height, IL) either at 42.5°C or at 37°C in the presence of Azc or sodium arsenite as described above. After a 1-h labeling period, the medium was removed, the cells were washed with phosphate-buffered saline (PBS), lysed in Laemmli sample buffer, and analyzed by one-dimensional SDS PAGE as described previously (33). For the recovery experiments, the stressed cells were returned to normal growth conditions as described above and then pulse-labeled at varying times during the recovery period with [³H]leucine as described above.

One-dimensional and Two-dimensional Gel Electrophoresis

One-dimensional SDS PAGE using a 12.5% polyacrylamide gel was performed as described previously (35).

Two-dimensional gel electrophoresis using isoelectric focusing in the first dimension (pH 5–7 ampholytes) followed by SDS PAGE in the second dimension (12.5% polyacrylamide) was performed as described previously (35).

Characterization of C92 Monoclonal Antibody

A monoclonal antibody was produced from mice immunized with the purified HeLa 72-kD/73-kD stress proteins and characterized by immunoprecipitation analysis as described previously for our polyclonal 72-kD antibody (35).

Indirect Immunofluorescence

Analysis of the intracellular distribution of the 72-kD protein was done by indirect immunofluorescence using either a polyclonal antibody specific for the 72-kD protein (described and characterized previously in reference 35) or a new mouse monoclonal antibody (C92) specific for the 72-kD protein. Cells were fixed and permeabilized by exposure to −20°C absolute methanol for 2 min. Rabbit polyclonal antibody was diluted 1:60 and the mouse monoclonal antibody diluted 1:100. Fluorescein-conjugated goat anti-rabbit or goat anti–mouse antibodies (diluted 1:70) were used to detect the primary antibodies.

For double-label indirect immunofluorescence, the cells were fixed and permeabilized with −20°C absolute methanol. The cells were incubated first with a mixture of the mouse anti–72-kD serum and the human autoimmune anti–ribosomal antibody. Primary antibodies were visualized using a mixture of fluorescein-conjugated goat anti–human antibody (diluted 1:70) and rhodamine-conjugated goat anti–mouse antibody (diluted 1:100). Before use, the secondary antibodies were preabsorbed with methanol-fixed rat embryo fibroblast cells and clarified using a Beckman Airfuge.

Electron Microscopy

Our procedure for electron microscopy was described previously (37).

Immunohistochemistry

72kD. REF-52 cells were heat–shock treated for 1 h at 42.5°C and then reversed to 37°C for 1.5 h. The cells were fixed for 5 min at room temperature in PBS containing 2% paraformaldehyde (pH 7.4), followed by a 10-min room temperature fixation in 100 mM sodium bicarbonate containing 2% paraformaldehyde (pH 10.2). The cells were washed with three changes of PBS, and then incubated for 5 min in PBS containing 50 mM ammonium chloride. The cells were then washed three times with PBS and extracted for 8 min at room temperature with 0.3% Triton X-100 in PBS, and again washed with three changes of PBS containing 2 mg/ml bovine serum albumin (PBS/BSA). The 72-kD monoclonal antibody was diluted 1:50 in PBS/BSA, and the cultures were incubated with the antibody for 90 min at

37°C. (Control cultures were incubated with preimmune serum.) After incubation, the cells were washed with numerous changes of PBS. The cells were then incubated for 60 min at 37°C with affinity-purified peroxidase-conjugated goat anti-mouse antibody (Cappel Laboratories, Cochranville, PA), diluted 1:20 in PBS/BSA. After an overnight wash with PBS at 4°C, the cells were again fixed for 5 min at room temperature with a solution containing 2% glutaraldehyde, 4.5% sucrose, and 75 mM cacodylate. After extensive washing with PBS, the cells were incubated in 300 mM glycine in PBS (pH 10), and then washed for 5 min using three changes of 100 mM Tris-hydrochloride (pH 7.6). The cultures were then incubated for 45 min at room temperature in a solution containing 1 mg/ml diaminobenzidine, 50 mM Tris hydrochloride (pH 7.6), and 0.015% H₂O₂ (added immediately before incubation). The cultures were briefly washed in 100 mM Tris-HCl (pH 7.6) followed by a washing with three changes of PBS. The cells were then postfixed for 10 min in a solution containing 1% OsO₄ and 50 mM cacodylate (pH 7.4). Additional processing for electron microscopy was identical to the protocol described above.

Autoantibody (AA). The protocol for the immunostaining with the human autoantibody specific for the 60S subunit of eukaryotic ribosomes was similar to the 72-kD immunoelectron microscopy staining as described above but with the following changes.

The cultures were fixed in 1% glutaraldehyde in PBS. The autoantibody was diluted 1:400 with PBS/BSA. A bridge antibody, rabbit anti-human, was diluted 1:800 in PBS containing 2 mg/ml BSA, and the cells were incubated for 60 min. After extensive washing with PBS, the cells were incubated with affinity-purified peroxidase-conjugated goat anti-rabbit antibody (Cappel Laboratories) diluted 1:200 with PBS/BSA.

Silver Staining to Detect the Nucleolar Organizer Region. Silver staining of the nucleolar organizer region was performed using a modification of the procedure described by Ploton et al. (21). The cultures were fixed for 10 min at room temperature in phosphate-buffered 2% paraformaldehyde (pH 7.4), washed for 5 min in three changes of PBS, and postfixed for 10 min in Carnoy's fixative (1:3, acetic acid/ethanol). After rehydration, the cultures were stained for 10 min at 70°C in a solution containing 1% formic acid, 2% gelatin, and 50% silver nitrate. The cultures were washed for 5 min in three changes of distilled H₂O followed by a 10-min wash in 5% sodium thiosulfate. A final dH₂O wash was followed by dehydration in ethanol, and embedding for electron microscopy was as described above.

Combined Immunohistochemical and Silver Stain. The cultures were processed for 72-kD immunohistochemical staining as described above. The processing was stopped after the 45-min diaminobenzidine incubation, and small squares surrounding the 72-kD-positive cells were scratched into the plastic petri dish. The cultures were fixed in 2% glutaraldehyde in PBS and silver stained as described above. After the silver nitrate incubation, the cells were washed in dH₂O and postfixed in 1% OsO₄ in 50 mM cacodylate, pH 7.4. Additional processing for electron microscopy was done as described previously (37).

Results

To determine how long rat embryo fibroblast (REF-52) cells continue synthesizing the stress proteins after removal of the stress agent, a simple time course experiment using [³H]leucine pulse-labeling was performed. Cells were placed under stress by three different protocols: (*a*) a 3-h, 42.5°C heat-shock treatment; (*b*) exposure to 80 μM sodium arsenite at 37°C for 1.5 h; and (*c*) exposure to 5 mM Azc, the amino acid analog of proline, for 10 h at 37°C. During the last hour of each stress treatment, the cells were pulse-labeled with [³H]leucine for 1 h. In parallel, the cells placed under stress were returned to normal growth conditions and similarly pulse-labeled with [³H]leucine at 1–2 h, 4–5 h, 7–8 h, and 23–24 h post-reversal. Equal numbers of the labeled cells were then analyzed by SDS PAGE and the labeled proteins visualized by fluorography. As can be seen in Fig. 1, overall protein synthesis was greatly diminished in those cells labeled during the last hour of the particular stress treatment (lane *1*, in each case; heat, arsenite, and Azc). The reduction in the overall patterns of protein synthesis was greatest in the arsenite and heat-shock-treated cells. In the

case of the Azc-treated cells, inhibition of translation was less and synthesis of the stress proteins was easily observed. After return of the stressed cells back to normal growth conditions, the relative level of overall protein synthesis slowly began to increase. Maximal stress protein synthesis was observed at 7–8 h post-reversal in the case of the heat-treated cells, 4–5 h post-reversal in the arsenite-treated cells, and between 2–4 h post-reversal in the Azc-treated cells. The elevated synthesis of the stress proteins continued for as long as 12 h after return of the cells to normal growth conditions. After 24 h of recovery, the increased synthesis of the stress proteins, in general, had markedly subsided with the cells now displaying extremely high levels of normal translation patterns. (Again, note that in every case, equal numbers of cells were applied to the gel.)

A number of other points deserve mention regarding the data presented in Fig. 1. First, in the case of both heat–shock and arsenite treatment, the major induced proteins were the classically defined heat–shock proteins: the 28-kD, 72-kD, 73-kD, 90-kD, and 110-kD stress proteins. Treatment of the cells with the proline analogue, Azc, resulted in an increased production of these same proteins as well as two additional proteins with apparent molecular masses of 80 kD and 100 kD. These latter two proteins are identical to the proteins synthesized at high levels in cells either deprived of glucose, treated with calcium ionophores, or in cells after removal of extracellular calcium (i.e., the so-called "glucose-regulated proteins" [26]). Hence, we classify these two glucose-regulated proteins as members of the stress protein family due to the fact that their synthesis appears sensitive to certain treatments (i.e., Azc) which also affect the synthesis of the classically defined heat–shock proteins. Second, only in the case of the arsenite-treated cells did we observe the increased production of a 32-kD protein. The increased synthesis of this protein appears unique to cells treated with various heavy metals (14, 38). Third, both the extent and duration of inhibition in synthesis of other cellular proteins (e.g., what we refer to as 37°C proteins) differed somewhat depending upon the particular stress agent used. In both the heat–shock- and Azc-treated cells, 37°C translation patterns begin to return by 7–8 h of recovery. In contrast, the restoration of 37°C translation patterns appeared faster (e.g., 2–4 h) in the cells recovering from arsenite treatment. Lastly, we observed that in general, repression of 72-kD synthesis always appeared to precede the repression in synthesis of the other stress proteins. For example, in the Azc-treated cells 7–8 h post-reversal (Fig. 1, *Azc*, lane *4*) the synthesis of the 72-kD protein had markedly subsided while production of the other stress proteins still remained relatively high.

Previous studies in our laboratory had shown that the major stress-induced protein, the 72-kD protein, localized within the nucleus and more prominently the very phase-dense nucleoli after a 3-h heat–shock treatment (35). In contrast, however, we observed little or no nucleolar distribution of 72-kDa in cells placed under stress by either Azc or arsenite treatment. Now knowing that synthesis of the stress proteins continues for at least 8–10 h after reversal of the stress treatment, we decided to reinvestigate the subcellular distribution of the 72-kD protein during the recovery period. REF-52 cells were placed under stress by exposure to either heat–shock, sodium arsenite, or Azc, the particular stress agent was removed, and the cells were then allowed
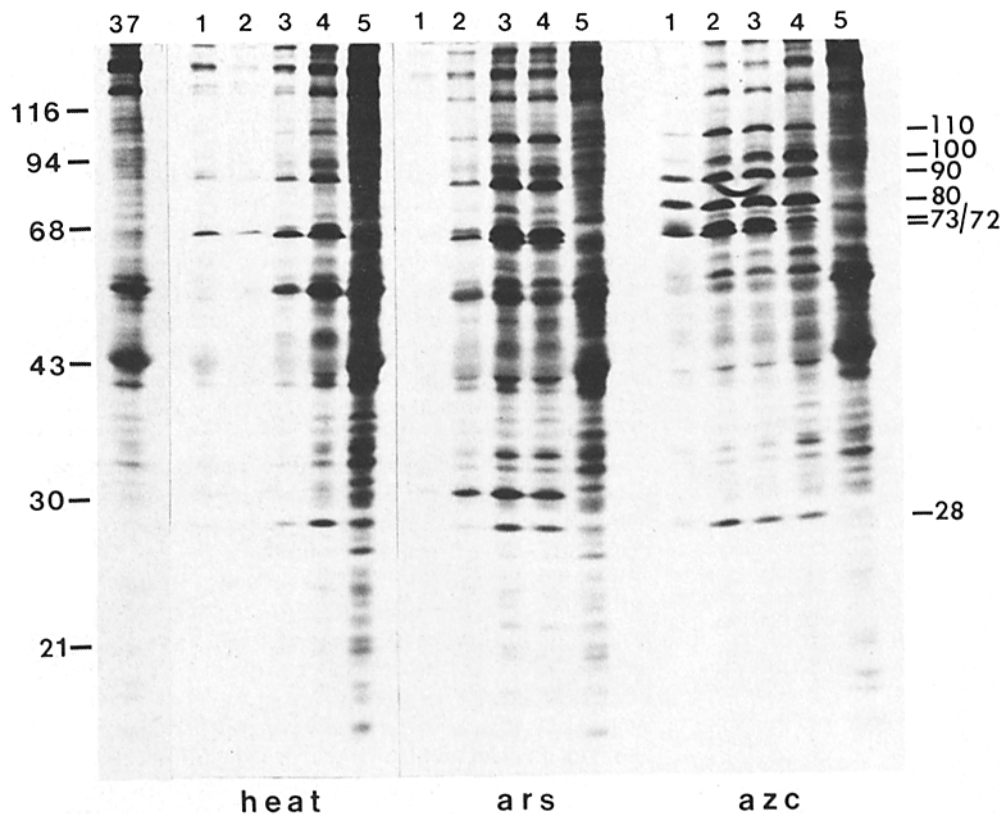
Figure 1. Time course of stress protein synthesis during recovery from heat–shock, sodium arsenite, and amino acid analog treatments. Rat embryo fibroblast (REF-52) cells growing on 35-mm plastic dishes were placed under stress by either (*a*) a 3-h, 42.5°C heat–shock treatment, (*b*) exposure to 80 μM sodium arsenite for 1.5 h at 37°C, or (*c*) exposure to 5 mM Azc for 10 h at 37°C. During the last hour of each stress treatment, the cells were pulse-labeled with [³H]leucine for 1 h and the cells harvested as described in Materials and Methods. In parallel, the heat–shock-, arsenite-, and azetidine-treated cells were returned to normal culture conditions (i.e., removal of the stress agents and/ or further incubation at 37°C) and the cells allowed to recover for 1, 4, 7, or 24 h. At each time point during the recovery period, the cells were pulse-labeled with [³H]leucine for 1 h and the cells harvested. The [³H]leucine–labeled proteins were then examined by SDS PAGE on a 12.5% polyacrylamide gel. An equal number of cells were analyzed in every case. Shown is a fluorograph of the gel with molecular mass markers indicated on the left and the position of the stress proteins indicated on the right. In the first lane on the left are cells labeled at 37°C. To the right, in groups of five, are the cells analyzed after heat–shock treatment (*heat*), sodium arsenite treatment (*ars*), or treatment with the proline analogue (*azc*). Cells pulse-labeled in the last hour of the particular stress treatment (lanes *1*); recovering cells pulse-labeled 1–2 h (lanes *2*), 4–5 h (lanes *3*), 7–8 h (lanes *4*), and 23–24 h (lanes *5*) post-reversal.

to recover for 4 h at 37°C. The distribution of the 72-kD protein in the recovering cells was analyzed by indirect immunofluorescence using the previously well-characterized rabbit polyclonal antibody specific for the 72-kD stress protein (35). Similar to our previous observation examining the distribution of the 72-kD protein immediately after heat-shock treatment, we found a prominent nucleolar locale of the 72-kD protein after the 4-h recovery period (Fig. 2, *A* and *B*). In addition, considerable fluorescence was also observed within the cytoplasm of these cells. In the case of the cells recovering from Azc treatment for 4 h, an intense nuclear and nucleolar distribution of 72-kD was observed. Finally, in the case of the cells recovering from arsenite treatment, fluorescence was observed within the nucleus, the perinuclear region, and along certain points near the cell perimeter. No nucleolar staining, however, was apparent in those cells recovering from arsenite treatment. It should also be noted that in both the heat-treated and Azc-treated cells containing nucleolar-distributed 72-kD, the corresponding phase-contrast micrographs revealed very phase-dense nucleoli. Arsenite treatment, in contrast, did not result in such a significant change in the integrity of the nucleoli; and as discussed later, this probably accounts for the lack of 72-kD nucleolar staining in these cells.

Both the differences and similarities in the subcellular distribution of the 72-kD protein in cells recovering from physiological stress as induced by different agents, as well as the

fact that synthesis of the 72-kD protein proceeded for as long as 12 h throughout the recovery period, prompted us to more thoroughly investigate the distribution of the 72-kD protein throughout a 24-h recovery period. Therefore, the distribution of the 72-kD protein was examined (*a*) immediately after removal of the stress agents, (*b*) 4 h post-reversal, (*c*) 8 h post-reversal, and (*d*) 24 h post-reversal. For these studies we elected to use a new mouse monoclonal antibody (C92) since it seemed to recognize the cytoplasmic distribution of the 72-kD protein more effectively than did the polyclonal antibody. The specificity of the C92 antibody for only the 72-kD stress protein was demonstrated by immunoprecipitation from [³⁵S]methionine–labeled heat-shock HeLa cell lysates and analysis of the immunoprecipitate by two-dimensional gel electrophoresis (Fig. 3). The monoclonal antibody immunoprecipitated most all of the various 72-kD isoforms. Using the C92 antibody the distribution of the 72-kD protein was analyzed in cells immediately after the heat–shock treatment as well as in cells at various times during the recovery period (Fig. 4). After the 3-h heat treatment, most of the 72-kD protein localized within both the nucleus and nucleolus. With increasing times of recovery, much of the protein began to accumulate within the cytoplasm and by 8 h of recovery only ∼20% of the cells still exhibited a nucleolar distribution of the protein. Moreover, only those recovering cells which still displayed visibly altered nucleoli exhibited a nucleolar deposition of the 72-kD protein (e.g., compare
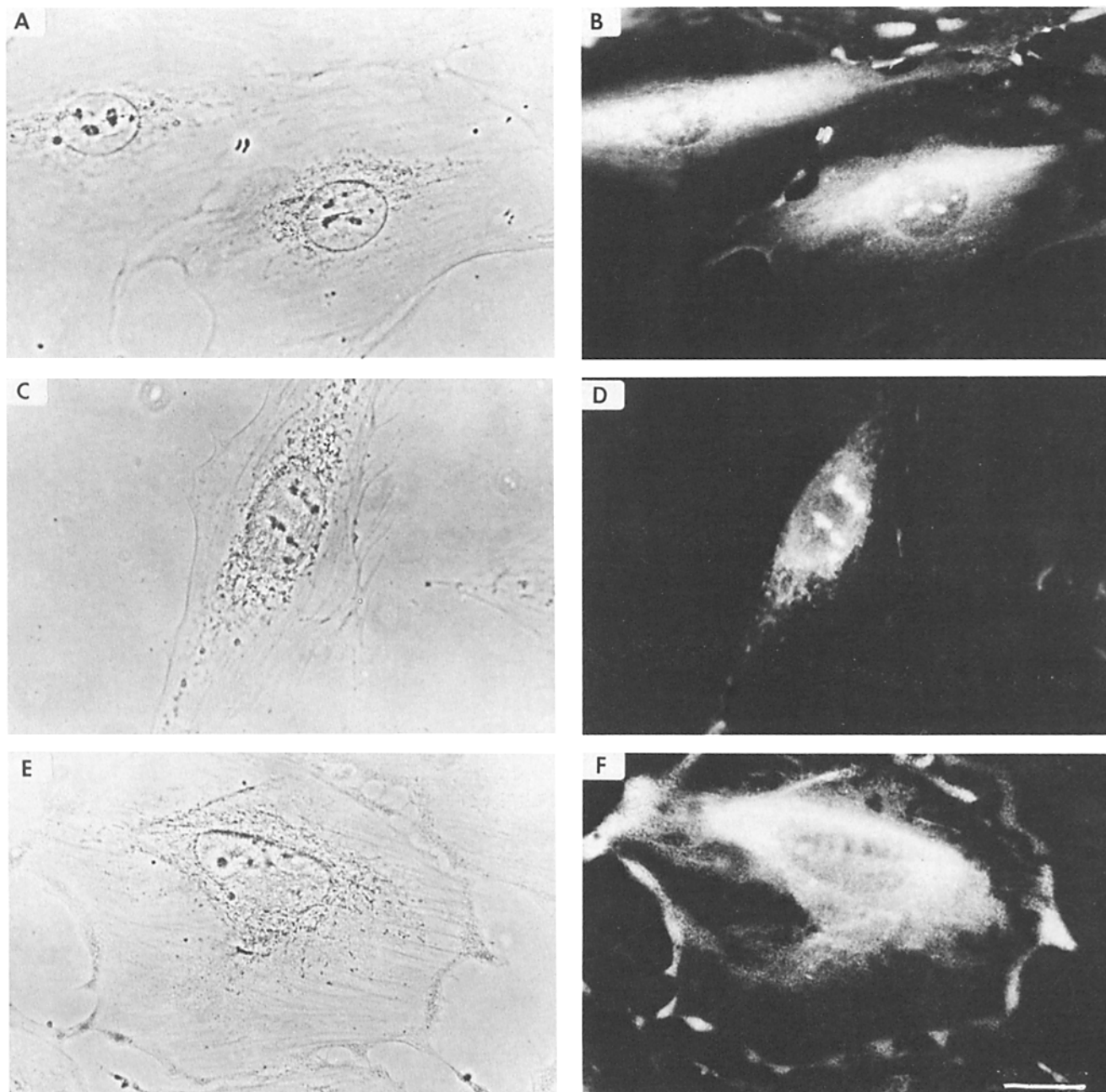
Figure 2. Intracellular location of 72-kD stress protein in cells recovering from physiological stress as determined by indirect immunofluorescence using a polyclonal antibody. REF-52 cells growing on glass coverslips were subjected to either a 42.5°C, 3-h heat-shock treatment, exposure to 80 μM sodium arsenite for 1.5 h at 37°C, or exposure to 5 mM Azc for 10 h at 37°C. The cells were then returned to normal growth condition (e.g., return to 37°C and/or removal of the arsenite or azetidine), further incubated for 4 h in complete DME at 37°C, and then fixed and analyzed by indirect immunofluorescence using a rabbit polyclonal antibody specific for the 72-kD protein (Materials and Methods). *A*, *C*, and *E* are phase-contrast micrographs; *B*, *D*, and *F* are the corresponding fluorescent micrographs. (*A* and *B*) Heat–shock-treated cells recovered for 4 h. (*C* and *D*) Azc-treated cells recovered for 4 h. (*E* and *F*) Arsenite-treated cells recovered for 4 h. Bar, 20 μm.

phase-contrast and fluorescent micrographs in Fig. 4, *C–F*). Conversely, those cells whose nucleoli had regained a normal appearance contained no nucleolar 72-kD protein. By 24 h of recovery, only half of the cells still exhibited 72-kD staining, and such staining was confined to the nuclear and perinuclear region. Little or none of the cells after 24 h displayed altered nucleoli and correspondingly no nucleolar 72 kD stress protein.

A similar induction and reversal experiment analyzing the distribution of the 72-kD protein in cells placed under stress by exposure to the amino acid analog of proline, Azc, was performed (Fig. 5). After the 10-h Azc treatment, low level fluorescence was observed, with most of the protein being present within the cytoplasm. Consistent with our previous studies, little of the protein was detected within the nucleoli (35). After 4 h of recovery, intense fluorescence was now ob-
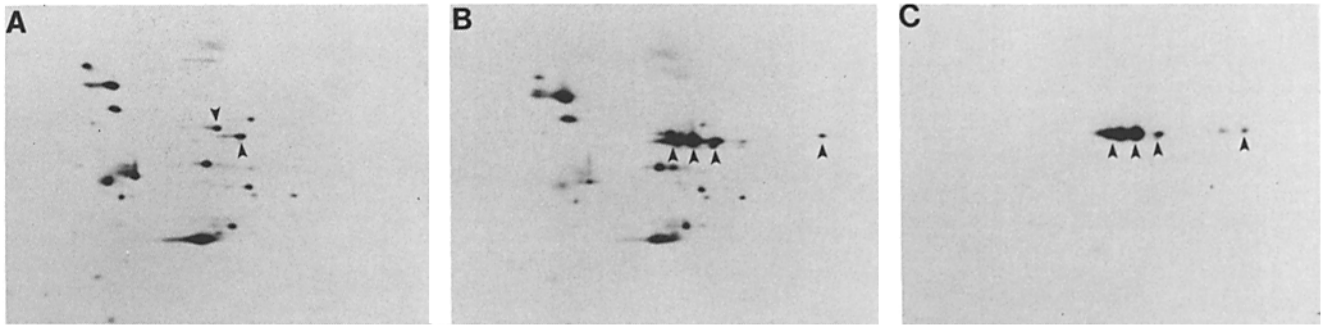
Figure 3. Specificity of C92 monoclonal antibody for the 72-kD protein. HeLa cells, growing on 10-cm plastic dishes, were labeled with [^{35}S]methionine at either 37°C or at 37°C after a 1.5-h, 42°C heat-shock treatment. The cells were solubilized and immunoprecipitation was performed using the C92 monoclonal antibody (using the method described in reference 35). The 37°C and heat-shock-treated cell lysates and the immunoprecipitation product were then analyzed by two-dimensional gel electrophoresis. (*A*) The [^{35}S]methionine-labeled 37°C HeLa cells. (*B*) The [^{35}S]methionine-labeled heat-shock-treated cells. (*C*) The C92 immunoprecipitation product from the heat-shock-treated cells. Downward-pointing arrow in *A* indicates the 73-kD stress protein while upward-pointing arrows in *A–C* indicate the multiple isoforms of the highly stress-induced 72-kD protein. Note that the C92 antibody is specific for only the 72-kD stress protein.

served within the nucleoli. Again, the nucleolar distribution of the 72-kD protein occurred only in those cells which displayed unusually phase-dense and disrupted nucleoli (e.g., Fig. 5, *C* and *D*). In addition, large "patches" of cytoplasmic fluorescence were observed, and in most cases these patches were easily visualized in the corresponding phase-contrast micrographs (e.g., *C* and *D*). By 8 h post-reversal, most of the cells exhibited a cytoplasmic locale of the 72-kD protein and only a few of the cells still displayed a nucleolar distribution of the protein. By 24 h of recovery, the 72-kD protein showed a general distribution throughout the cell with the only exception now being the nucleoli.

To complete our study, we analyzed the distribution of 72-kD in cells recovering from sodium arsenite treatment (Fig. 6). Consistent with the low-to-negligible levels of 72 kD being synthesized during the arsenite treatment (Fig. 1, lane *1*), little or no 72-kD fluorescence was observed immediately after the arsenite treatment (Fig. 5, *A* and *B*). After 4 h and 8 h of recovery, 72-kD staining was observed primarily within the cytoplasm; some nuclear fluorescence was detected as well. However, we did not observe obvious nucleolar staining or visible alterations in the integrity of the nucleoli. The cytoplasmic staining was also accompanied by the appearance of unusual phase-dense bodies within the cytoplasm (Fig. 6, *C–F*). We will return to these structures later. By 24 h, most of the cells exhibited low-to-negligible levels of 72-kD staining (Fig. 6, *G* and *H*) with the residual staining associated almost exclusively with these cytoplasmic phase-dense structures.

Using electron microscopy, we examined more closely the very phase-dense cytoplasmic structures present in cells recovering from heat shock, Azc, or sodium arsenite treatment (Fig. 7). In the case of those cells recovering from heat-shock treatment, unusual linear arrays of electron-dense material were observed (*A*). In most cases, this material was found to run along the actin-containing stress fibers. In those cells recovering from Azc treatment, the phase-dense material generally appeared similar to the structures observed after recovery from heat-shock treatment (*B*). Again, in many instances the material was closely aligned along the stress fibers. Interestingly, we never detected any organelles and/or well-defined mono- or polyribosomes

present within these phase-dense structures. In Fig. 7, *C–E* we present three successive higher magnification electron micrographs of the structures which developed in cells recovering from sodium arsenite treatment. The arsenite-induced structures appeared as circular bodies further out in the cytoplasm or in some cases very near the nucleus. The material present within these bodies had a ribbon-like appearance, and in some cases appeared somewhat similar to the structures found in the Azc-treated cells. Interestingly, these arsenite-induced structures persist within the cells for as long as 36–48 h after reversal of the arsenite treatment (data not shown). Current efforts are being directed at trying to purify these structures and to ascertain their complete molecular composition.

Next, we examined the phase-dense structures containing the 72-kD protein which were present along the perimeter of the cell directly underneath the plasma membrane. Sections were taken near the cell border of cells which had recovered for 8 h after the Azc or sodium arsenite treatment and analyzed by the electron microscope (Fig. 8). Structures present near the plasma membrane in cells recovering from the Azc treatment are shown in *A* and then, in a higher magnification micrograph of the same section, in *B*. These structures consist of finely packed and electron-dense particles. Similar structures were observed in those cells recovering from sodium arsenite treatment (Fig. 7, *C* and *D*). We will show below that these phase-dense particles consist, in part, of ribosomes.

A human autoimmune antibody that recognizes three proteins of the 60S subunit of eukaryotic ribosomes (4, 10, 17) was obtained (kindly provided by Mike Mathews, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY) and used to determine whether the phase-dense structures accumulating at the cell periphery were in fact comprised of ribosomes. REF-52 cells were treated with Azc and then returned to normal growth conditions for 8 h, the time at which the perimeter-distributed phase-dense structures were most apparent. The cells were then fixed, permeabilized, and analyzed for the distribution of cytoplasmic ribosomes. Thin sections were cut near the cell border and examined by immunoelectron microscopy using the anti–ribosomal antibody (Fig. 9). It can be seen that the phase-dense structures
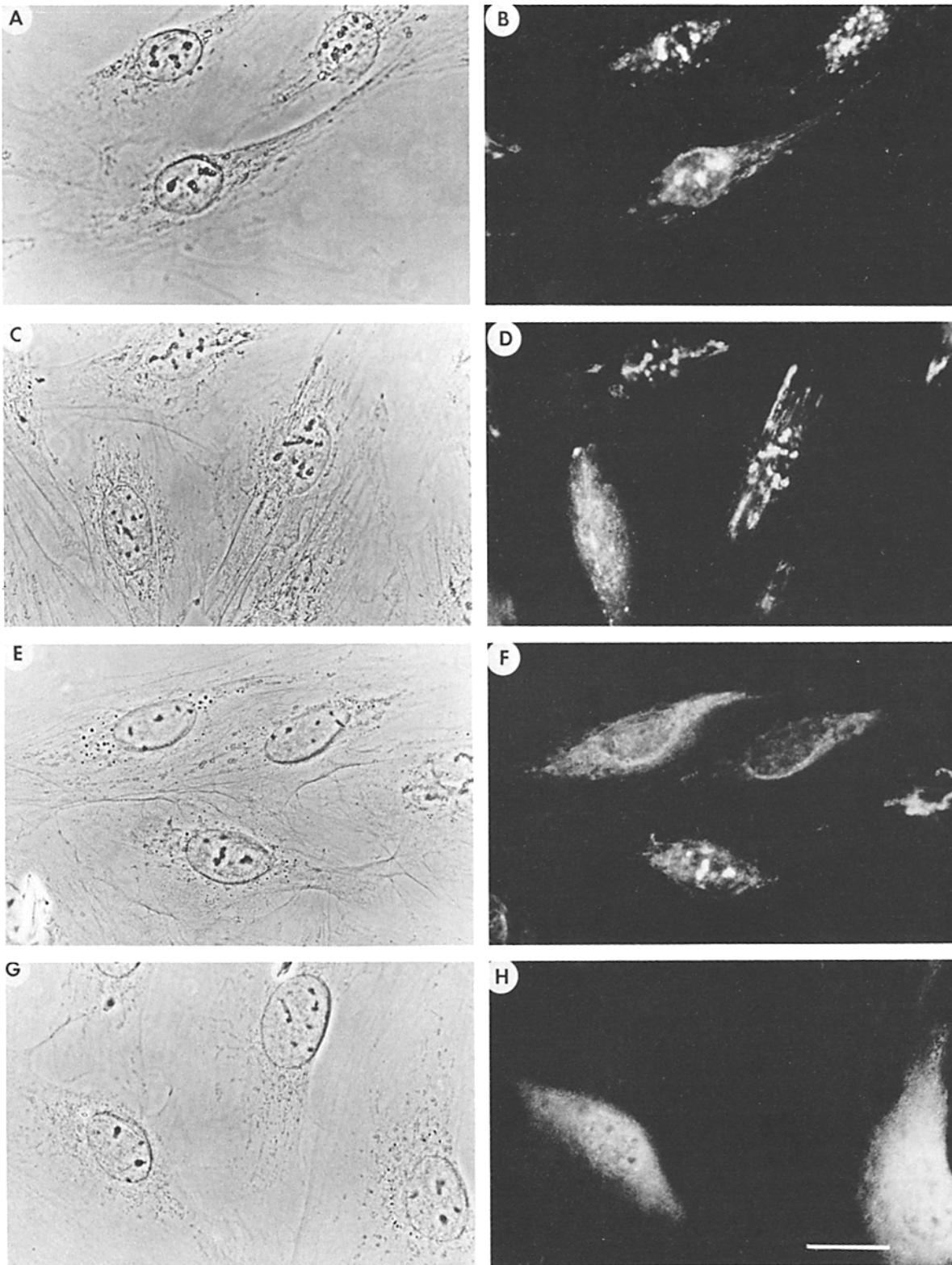
Figure 4. Distribution of the 72-kD stress protein in heat–shock-treated cells and in cells recovering from heat–shock treatment as determined by indirect immunofluorescence using the C92 antibody. REF-52 cells growing on glass coverslips were heat–shock-treated at 42.5°C for 3 h. After the heat treatment, one coverslip was removed, fixed, and analyzed by indirect immunofluorescence using the mouse monoclonal antibody (C92) specific for 72-kD as described in Materials and Methods. The remaining coverslips were removed from the heat–shock incubator, further incubated at 37°C for either 4, 8, or 24 h, and then fixed and analyzed for the distribution of the 72-kD protein as described above. *A*, *C*, *E*, and *G* are phase-contrast micrographs; *B*, *D*, *F*, and *H* are the corresponding fluorescent micrographs. (*A* and *B*) Cells heat–shock treated for 3 h. (*C* and *D*) Cells heat–shock treated for 3 h and returned to 37°C for 4 h. (*E* and *F*) Cells heat–shock treated for 3 h and returned to 37°C for 8 h. (*G* and *H*) Cells heat–shock treated for 3 h and returned to 37°C for 24 h. Bar, 20 μm.
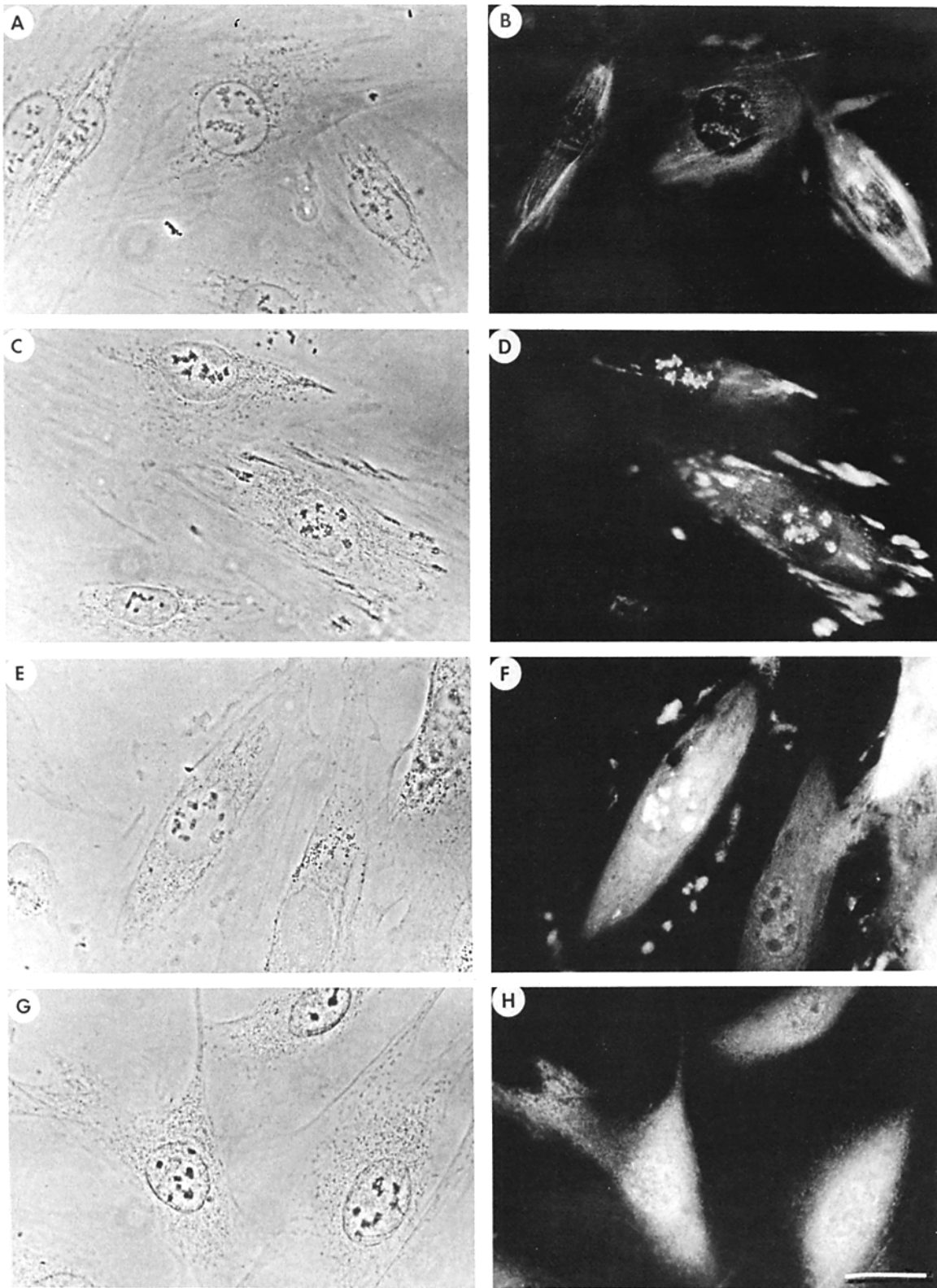
Figure 5. Distribution of the 72-kD stress protein in amino acid analog stressed and recovering cells as determined by indirect immunofluorescence using the C92 monoclonal antibody. REF-52 cells growing on glass coverslips were exposed to the proline analog Azc for 10 h. One coverslip was removed, fixed, and analyzed by indirect immunofluorescence using the C92 monoclonal antibody specific for the 72-kD protein. To the remaining coverslips, the cells were washed with and further incubated in DME containing no Azc for 4, 8, or 24 h at which times the cells were fixed and analyzed for the distribution of 72 kD. *A, C, E,* and *G* are phase-contrast micrographs; *B, D, F,* and *H* are the corresponding fluorescent micrographs. (*A* and *B*) Cells exposed to 5 mM Azc for 10 h. (*C* and *D*) Cells exposed to 5 mM Azc for 10 h and recovered for 4 h. (*E* and *F*) Cells exposed to 5 mM Azc for 10 h and recovered for 8 h. (*G* and *H*) Cells exposed to 5 mM Azc for 10 h and recovered for 24 h. Bar, 20 μm.
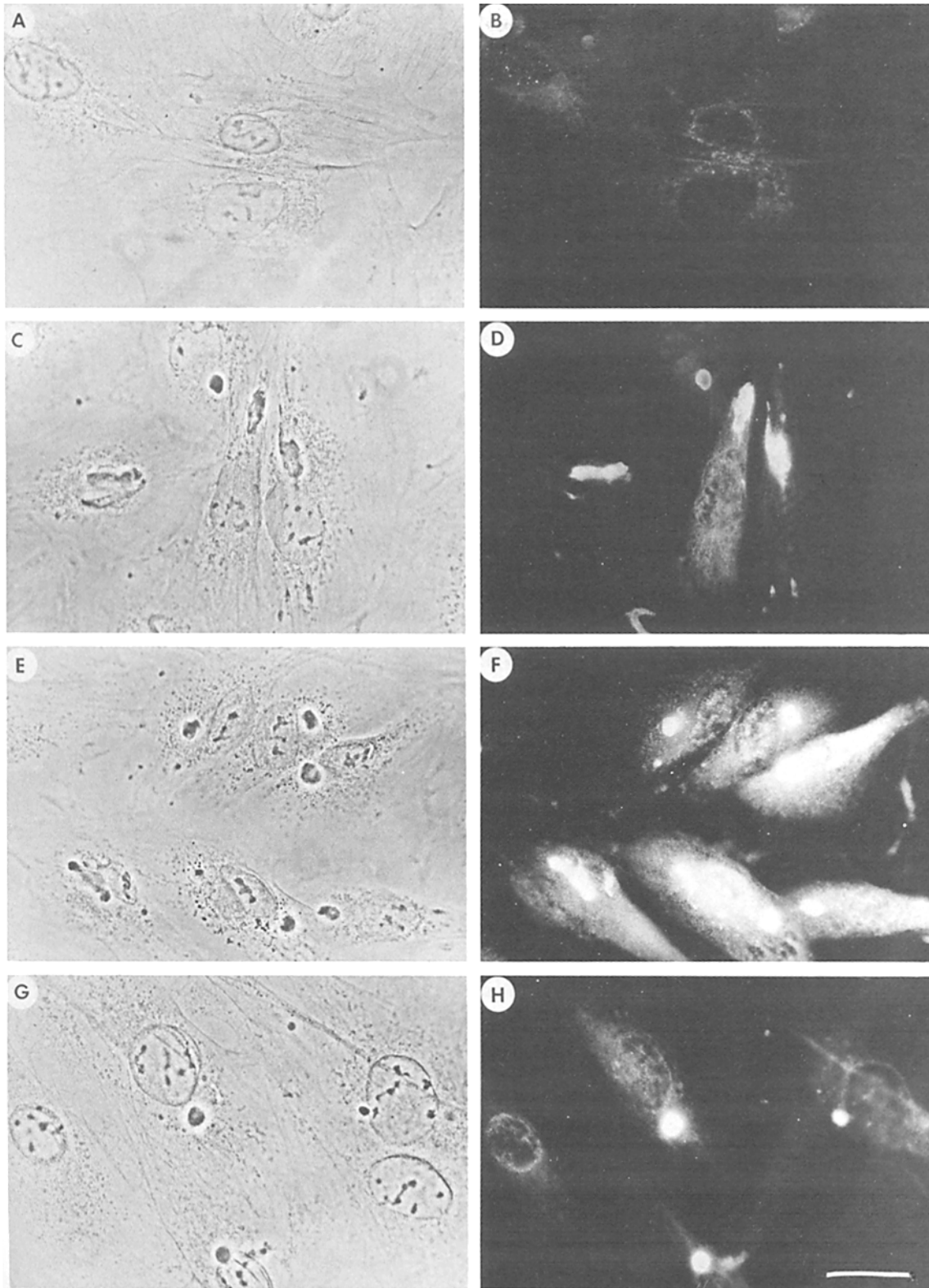
Figure 6. Distribution of the 72-kD stress protein in sodium arsenite stressed and recovering cells as determined by indirect immunofluorescence using a monoclonal antibody. REF-52 cells growing on glass coverslips were exposed to 80 μM sodium arsenite for 1.5 h. One coverslip was then removed, fixed, and analyzed by indirect immunofluorescence using the C92 monoclonal antibody specific for the 72-kD stress protein. To the remaining coverslips, the arsenite-containing medium was removed and the cells were further incubated in normal DME for 4 h, 8 h, or 24 h. At each time, the cells were fixed and analyzed by indirect immunofluorescence using the anti-72-kD antibody. *A, C, E,* and *G* are phase-contrast micrographs; *B, D, F,* and *H* are the corresponding fluorescent micrographs. (*A* and *B*) Cells exposed to 80 μM sodium arsenite for 1.5 h. (*C* and *D*) Cells exposed to 80 μm sodium arsenite for 1.5 h and recovered for 4 h. (*E* and *F*) Cells exposed to 80 μM sodium arsenite for 1.5 h and recovered for 8 h. (*G* and *H*) Cells exposed to 80 μM sodium arsenite for 1.5 h and recovered for 24 h. Bar, 20 μm.
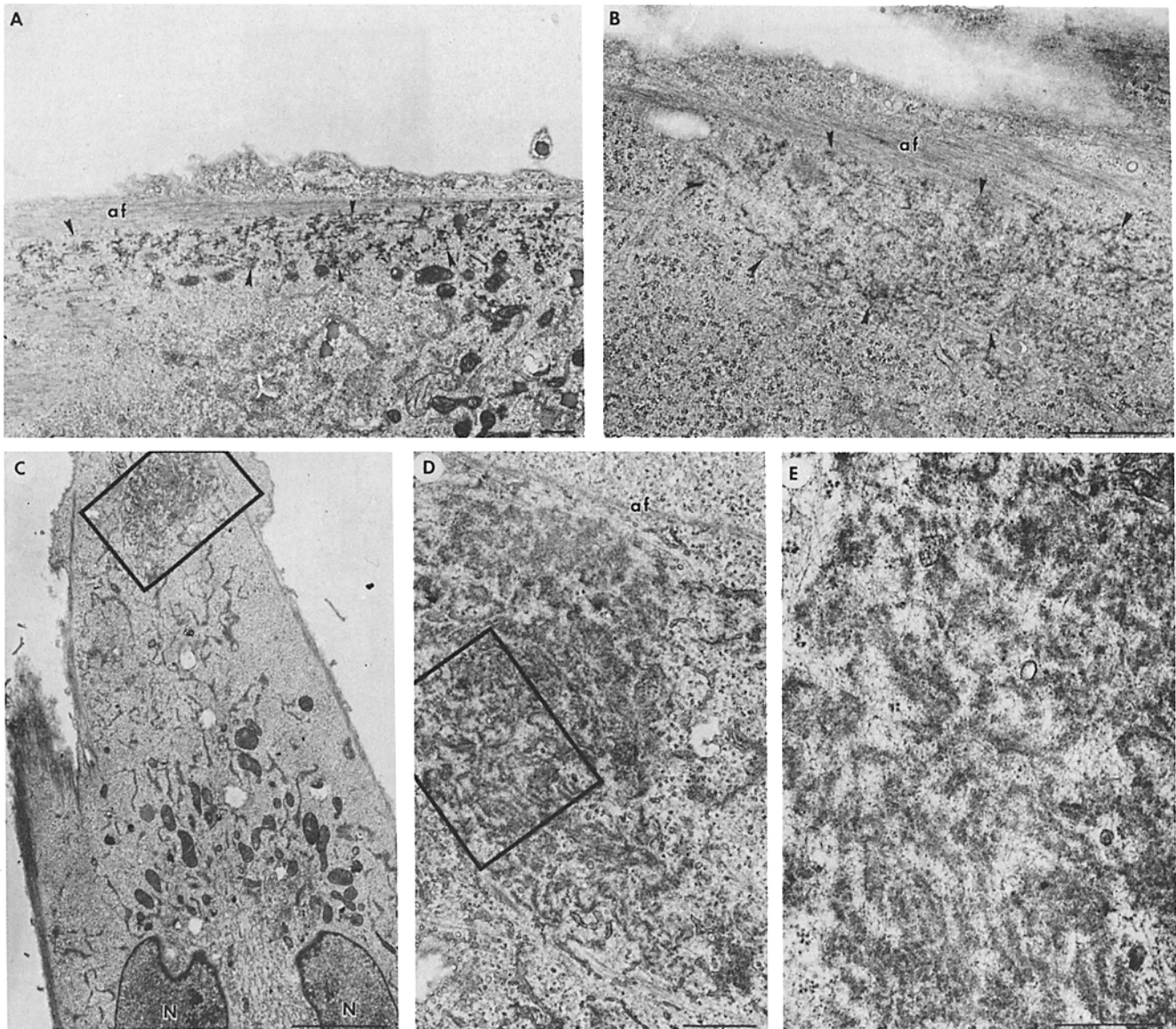
Figure 7. Internal phase-dense structures in cells recovering from heat–shock, Azc, or sodium arsenite treatment as analyzed by electron microscopy. REF-52 cells growing on plastic 35-mm dishes were subjected to either heat–shock treatment (42.5°C for 3 h), Azc treatment (5 mM for 10 h), or sodium arsenite treatment (80 μM for 1.5 h). After induction of the stress response, the cells were returned to normal culture conditions and allowed to recover for either 4 h (heat- or Azc-treated cells) or 8 h (arsenite-treated cells). The cells were then fixed and stained with aqueous saturated uranyl acetate and lead citrate and analyzed by electron microscopy as described in the Materials and Methods. Shown are thin sections of the cytoplasmic region of the cells. Arrowheads denote the unusual phase-dense structures which the anti–72-kD serum recognizes. (*A*) Cells 4 h after heat–shock treatment. (*B*) Cells 4 h after Azc treatment. (*C–E*) Cells recovered for 8 h after sodium-arsenite treatment: (*D*) a higher magnification of the structure enclosed in the boxed region of *C*; (*E*) a higher magnification of the same structure shown in *D*. Bars, (*A* and *B*) 1 μm; (*C*) 5 μm; (*D*) 0.1 μm; (*E*) 0.5 μm.

present along the cell perimeter exhibited a positive reaction with the anti–ribosomal antibody.

To ascertain whether the cell perimeter staining of the 72-kD stress protein in cells recovering from physiological stress coincided with the distribution of the above-described perimeter ribosomes, we next performed a double-label indirect immunofluorescent experiment using both the anti–72-kD and anti–ribosomal antibodies (see Materials and Methods). The results of such a double-label indirect immunofluorescence experiment analyzing the distribution of both the 72-kD stress protein and ribosomes in the same cell after recovery from stress are shown in Fig. 10. With each

antibody, two distinct regions of staining were observed: a perinuclear distribution of both the 72-kD protein and the ribosomes as well as a distribution of the two along the perimeter of the cells. The two staining patterns were found to be coincident in most every case. Although not shown here, a similar double-label staining experiment using anti–vimentin and anti–ribosomal antibodies has demonstrated that the tight perinuclear distribution observed for the ribosomes was due to a collapse of the vimentin-containing intermediate filaments. In addition, we suggest that those ribosomes found along the perimeter of the cells somehow escaped such a redistribution (see Discussion). Finally, we should point out
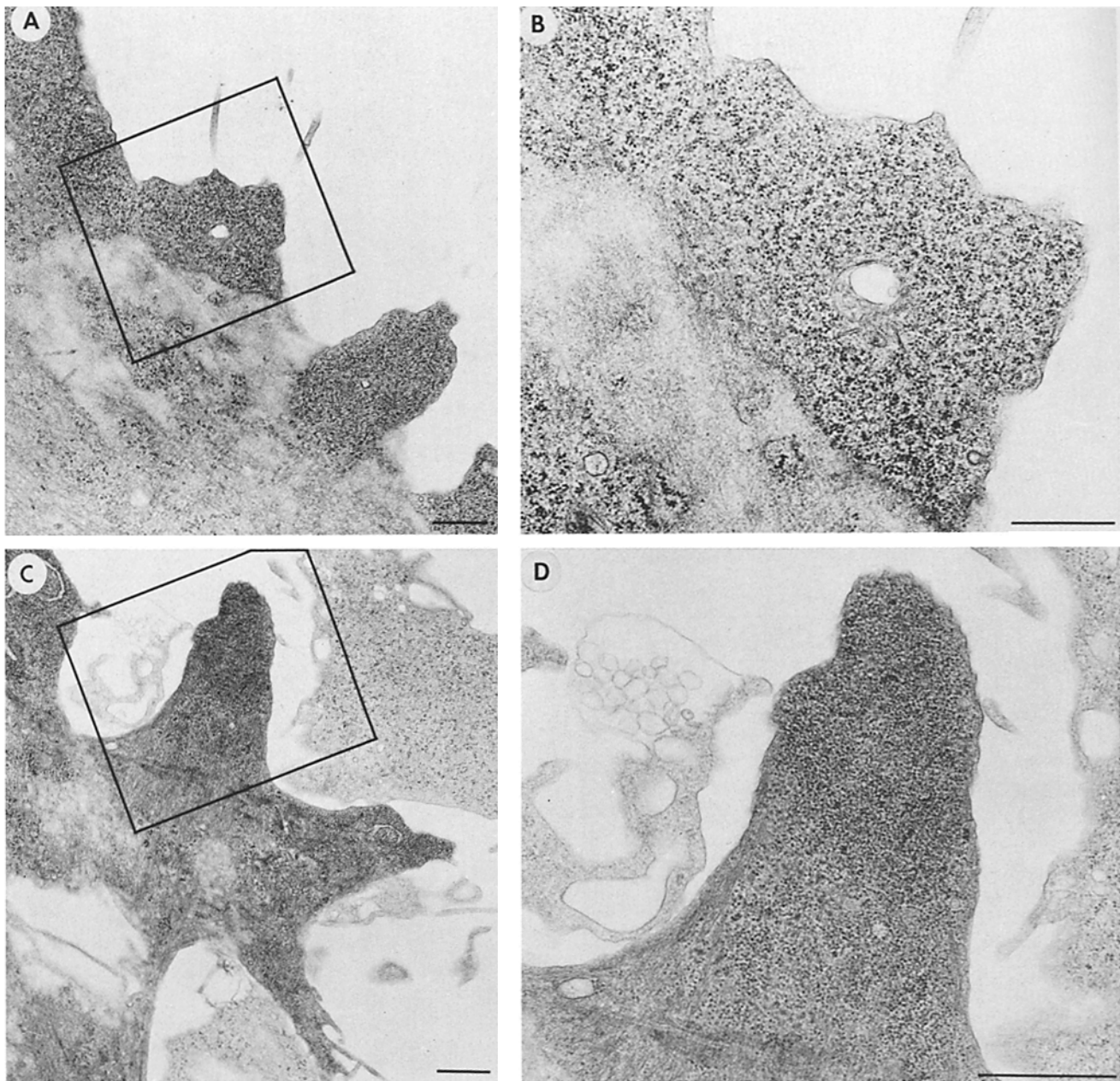
Figure 8. Peripheral phase-dense structures in cells recovering from Azc or sodium arsenite treatment analyzed by electron microscopy. REF-52 cells growing on plastic dishes were placed under stress by exposure to either 5 mM Azc for 10 h or to 80 μM sodium arsenite for 1.5 h at 37°C. The particular stress agent was removed, the cells were washed with DME and further incubated at 37°C for 8 h. The cells were then fixed and stained with aqueous saturated uranyl acetate and lead citrate and analyzed by high voltage electron microscopy. Shown are thin sections very near the cell perimeter. (*A*) A section from the cells recovering from Azc treatment. (*B*) A higher magnification of the boxed area in *A*. (*C*) A section from the cells recovering from sodium arsenite treatment. (*D*) A higher magnification of the boxed area shown in *C*. Bars, (*A, C,* and *D*) 1 μm. Bar, (*B*) 0.5 μm.

that little or no ribosomes were found distributed along the cell perimeter of the unstressed 37°C cells (see below).

Owing to their somewhat unusual distribution, we examined whether the ribosomes accumulating at the cell periphery of the recovering cells were still capable of translating mRNA. Rat fibroblasts, incubated at 37°C or after recovery from Azc or sodium arsenite treatment for 8 h, were pulse-labeled with [³H]leucine for 2 min at 37°C. The labeling medium was quickly removed, the cells were washed three times with DME containing excess unlabeled leucine, and

then immediately fixed by the addition of −20°C absolute methanol. To ascertain the distribution of the ribosomes, the cells were incubated with the anti–ribosomal antibody followed by a second, fluorescein-conjugated goat anti–human antibody. Next, a photographic emulsion was applied to the fixed cells to determine the sites of active protein synthesis. After a suitable exposure time (∼8 d), the film emulsion was developed, and the cells analyzed under the microscope using both phase and fluorescent optics. Shown in Fig. 11 are the results of such a double-label emulsion autoradiography
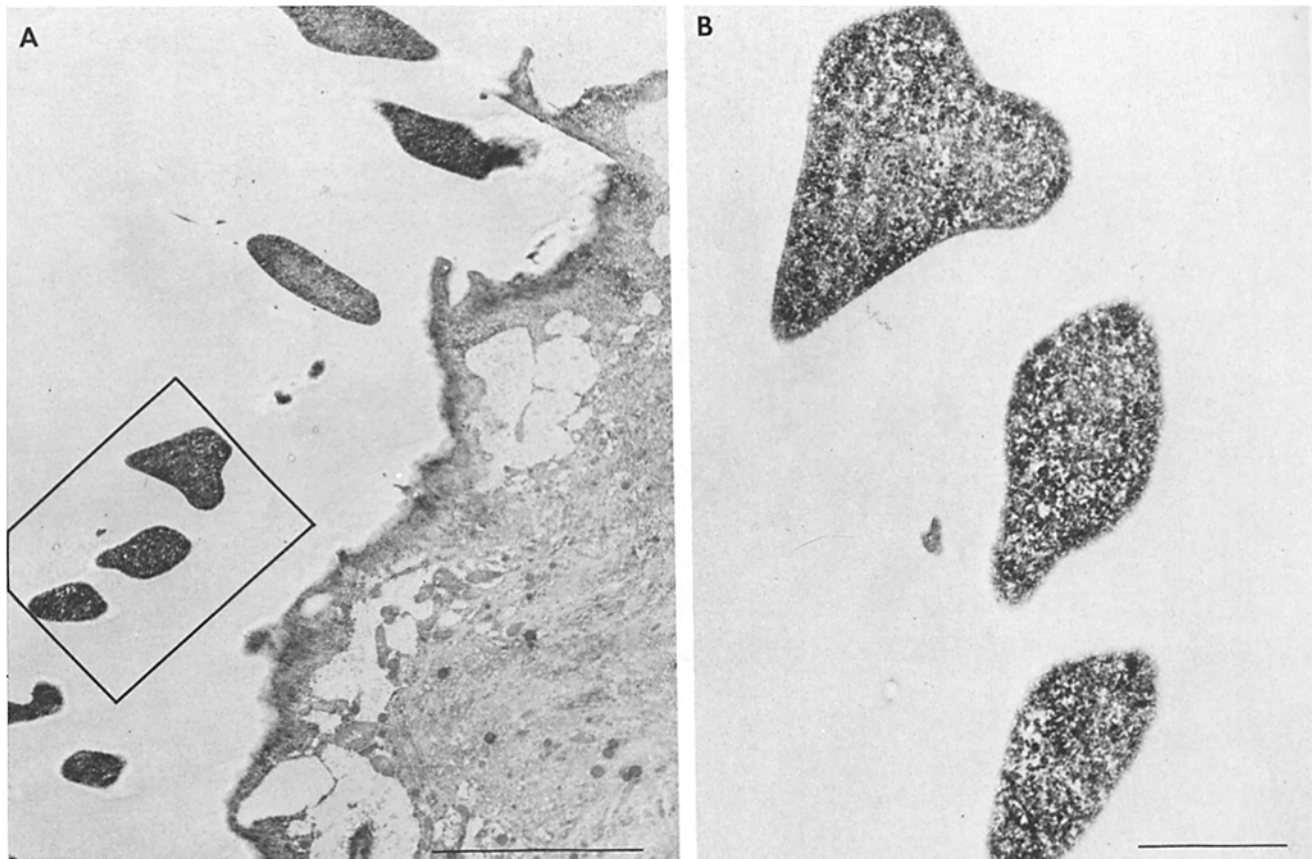
Figure 9. Phase-dense perimeter structures in cells recovering from Azc treatment contain ribosomes. REF-52 cells were placed under stress by exposure to 5 mM Azc for 10 h; the Azc was then removed and the cells were allowed to recover in normal DME for 8 h. The cells were fixed, permeabilized, and incubated with a human autoantibody specific for the eukaryotic 60S ribosomal subunit. The primary antibody was visualized by incubation with a rabbit anti–human antibody followed by incubation with peroxidase-conjugated goat anti–rabbit IgG. *A* is a low magnification micrograph; *B* is a higher magnification micrograph of the boxed area shown in *A*. Bars, (*A*) 10 μm; (*B*) 2 μm.

and indirect immunofluorescent analysis. In the case of the unstressed 37°C cells, the majority of ribosomal staining was observed throughout the cytoplasm with the corresponding silver grains showing a coincident distribution (Fig. 11, *A* and *B*). Note that there was neither staining nor silver grains present along the border of the 37°C cells. Cells recovering from either Azc or arsenite treatment displayed an altered distribution of cytoplasmic ribosomes with considerable fluorescence again being observed along the perimeter of the cells. In addition, numerous silver grains could be detected within these peripherally distributed ribosomes (Fig. 11, *C–F*). These results indicate that those ribosomes accumulating directly underneath the plasma membrane are in fact translationally active.

Because of the apparent co-distribution of both the 72-kD stress protein and ribosomes in cells recovering from stress, we felt compelled to further investigate the distribution of the 72-kD protein within the nucleolus. Specifically, we wanted to determine whether it localized within either of two major regions of the nucleolus: the so-called fibrillar centers involved in ribosomal RNA transcription or alternatively the granular region which is characterized by the presence of large numbers of granular-like particles, most of which consist of pre-ribosomes and/or other ribonucleoprotein complexes. We reasoned that if the 72-kD protein showed an

affinity for the cytoplasmic ribosomes, then it should similarly display a co-distribution with the pre-ribosomes present in the granular region of the nucleolus. To define the nucleolar fibrillar centers, we used a sensitive silver staining procedure developed by others (21). In parallel, we analyzed the subnucleolar distribution of the 72-kD protein using immunoelectron microscopy as described in the Materials and Methods. For these experiments REF-52 cells were heat–shock-treated for only 1 h and then allowed to recover at 37°C for 90 min. Such a short heat–shock treatment was chosen since our earlier studies had shown that a 3-h heat–shock treatment results in a rather significant disruption of nucleolar architecture, thereby rendering it almost impossible to define subnucleolar domains (37). Immunostaining (using the monoclonal anti–72-kD antibody followed by a peroxidase-conjugated second antibody) revealed a segregated distribution of the 72-kD protein within the heat-treated nucleoli (Fig. 12 *B*). Areas of the nucleoli which did not display obvious 72-kD staining appeared identical to those sites which displayed positive silver staining (Fig. 12 *C*). To confirm this segregated distribution of the 72-kD protein we performed simultaneous silver staining and immunostaining of the heat–shock-treated cells (Fig. 12, *D* and *E*). Indeed, it was observed that the two staining patterns complemented one another. We conclude from these experiments that the
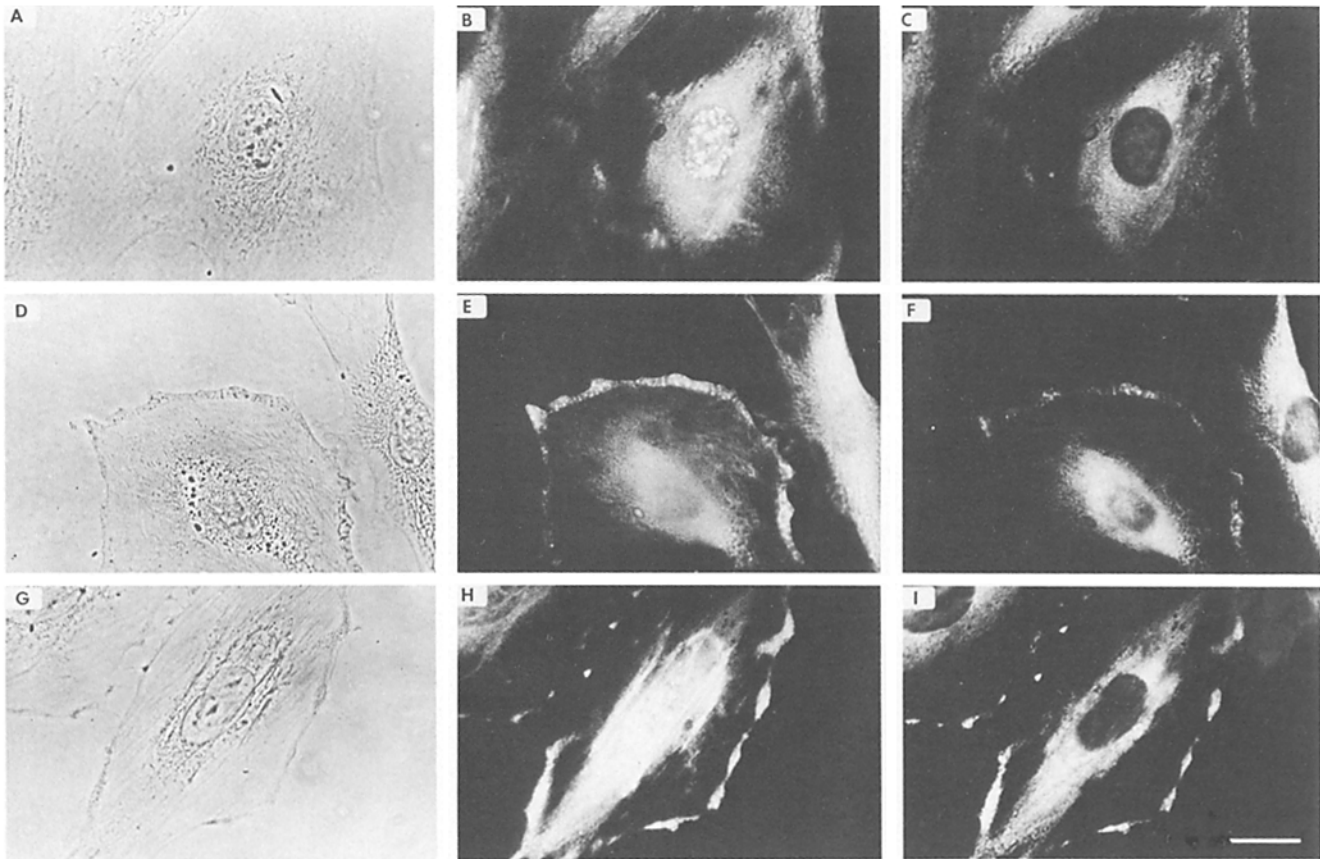
Figure 10. Colocalization of the 72-kD stress protein with ribosomes in cells recovering from physiological stress. REF-52 cells growing on glass coverslips were placed under stress by heat–shock treatment (42.5°C for 3h), exposure to Azc (5 mM for 10 h), or exposure to sodium arsenite (80 µM for 1.5 h). The cells were then returned to normal culture conditions for either 4 h (heat–shock) or 8 h (Azc and arsenite) at which time the cells were fixed and permeabilized as described in Materials and Methods. The cells were then incubated with a mixture of the mouse monoclonal anti–72-kD serum and the human autoimmune anti–ribosomal antibody. The primary antibodies were visualized with a mixture of rhodamine-conjugated goat anti–mouse and fluorescein-conjugated rabbit anti–human antibodies. *A, D,* and *G* are phase-contrast micrographs; *B, E,* and *H* show the anti–72-kD staining; *C, F,* and *I* show the anti–ribosomal staining. (*A–C*) Cells heat–shock treated and recovered for 4 h. (*D–F*) Cells treated with Azc and recovered for 8 h. (*G–I*) Cells treated with sodium arsenite and recovered for 8 h. Bar, 20 µm.

majority of the 72-kD stress protein is accumulating within the pre-ribosomal–containing granular region of the nucleolus.

Discussion

The aim of the present study was to examine in detail some of the cellular and biochemical events associated with mammalian cells recovering from physiological stress. Particular attention was paid to synthesis and localization of the 72-kD stress protein since this protein is barely detectable in rodent cells grown under normal conditions but represents the most highly induced polypeptide in these cells after physiological stress. The only exception to this rule that we have observed so far occurs in various human cell lines. Here, synthesis of the 72-kD protein occurs at modest or even high levels in various human cells grown under normal conditions (Welch, W. J., and R. I. Morimoto, manuscript in preparation). Moreover, this constitutive expression of the 72-kD protein in the human cells appears to be tightly cell cycle–regulated (16a, 39). Similar to the rodent cells, synthesis of the 72-kD protein in the human cells after heat–shock treatment again

represents the major translational product of the cells. Thus, in general, the increased synthesis of the 72-kD protein appears to be the best indicator of cells experiencing physiological stress.

A comparison of the protein synthesis patterns in the rat fibroblasts recovering from exposure to the different stress agents (heat, arsenite, and the amino acid analog) revealed both interesting similarities and differences. First, all three of the treatments resulted in an induction of the classical heat–shock proteins: the 28-kD, 72-kD, 73-kD, 90-kD, and 110-kD proteins. Only in those cells treated with the proline analog, Azc, did there occur a vigorous production of two other proteins of 80 kD and 100 kD. Interestingly, the synthesis of these same two proteins increases markedly in cells after depletion of either extracellular levels of glucose or calcium or in cells treated with a calcium ionophore (11, 33, 40). Studies are in progress to determine whether the increased production of these two proteins in cells treated with Azc is due to alterations in either glucose or calcium homeostasis. Finally, a protein with an apparent molecular mass of 32 kD was induced to high levels in only the arsenite-treated cells. The elevated production of this 32-kD protein appears
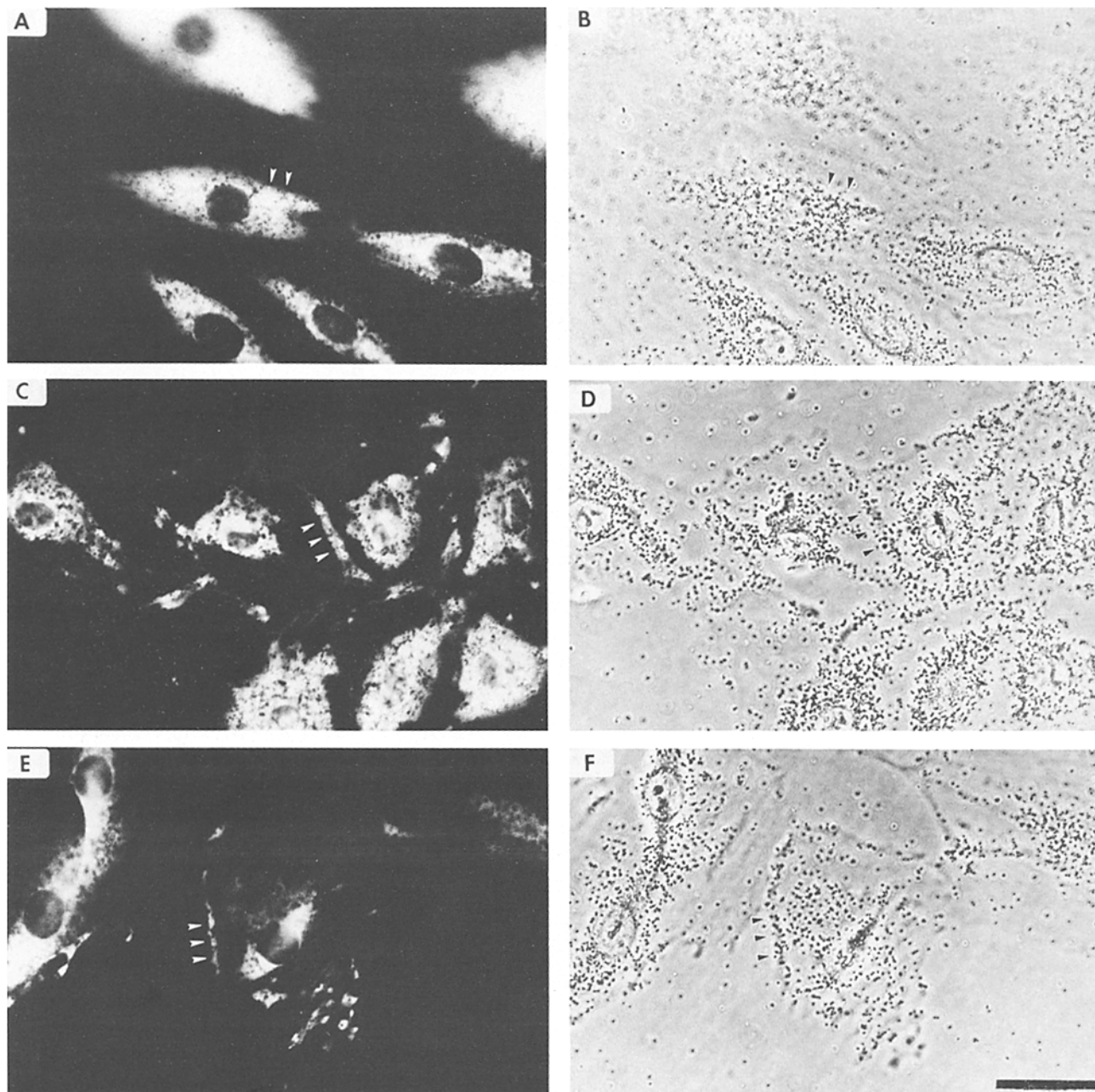
Figure 11. Ribosomes accumulating at the perimeter of cells recovering from physiological stress are translationally active. REF-52 cells growing on glass coverslips were either incubated at 37°C, treated with 80 μM sodium arsenite for 1.5 h at 37°C, or treated with 5 mM Azc for 10 h at 37°C. The stress agents were removed and the cells were further incubated in DME at 37°C. After 5 h of recovery the cells were pulse-labeled with [³H]leucine for 2 min, the labeling medium was removed, and the cells were washed three times with DME containing five times the normal concentration of leucine. (Total time of manipulation was ∼1 min.) The cells were fixed and permeabilized by exposure to −20°C absolute methanol. The cells were then analyzed for the distribution of ribosomes using indirect immunofluorescence as described in Materials and Methods. A thin layer of photographic emulsion was applied to the coverslips and the radiolabeled cells were allowed to expose the film for 8 d. After developing the photographic emulsion, the cells were photographed using both phase-contrast and fluorescent optics to determine the location of both the silver grains and ribosomes, respectively. Arrowheads in each case indicate a coincident distribution of ribosomes and [³H]leucine silver grains. (*A* and *B*) Distribution of ribosomes and silver grains in 37°C cells. (*C* and *D*) Distribution of ribosomes and silver grains in the arsenite-treated cells after recovery for 5 h at 37°C. (*E* and *F*) Distribution of ribosomes and silver grains in the Azc-treated cells after recovery for 5 h. Bar, 40 μm.

unique to cells treated with certain heavy metals including both zinc and cadmium (14, 38; and our unpublished observations).

Differences with respect to both the kinetics of induction as well as the decay in synthesis of the stress proteins were also observed depending upon the stress agent used. In the Azc- and arsenite-treated cells, maximal production of the stress proteins occurred much earlier during the recovery period (3–4 h) as compared to the heat–shock-treated cells (7–8 h). Similarly, the rate by which the synthesis of the
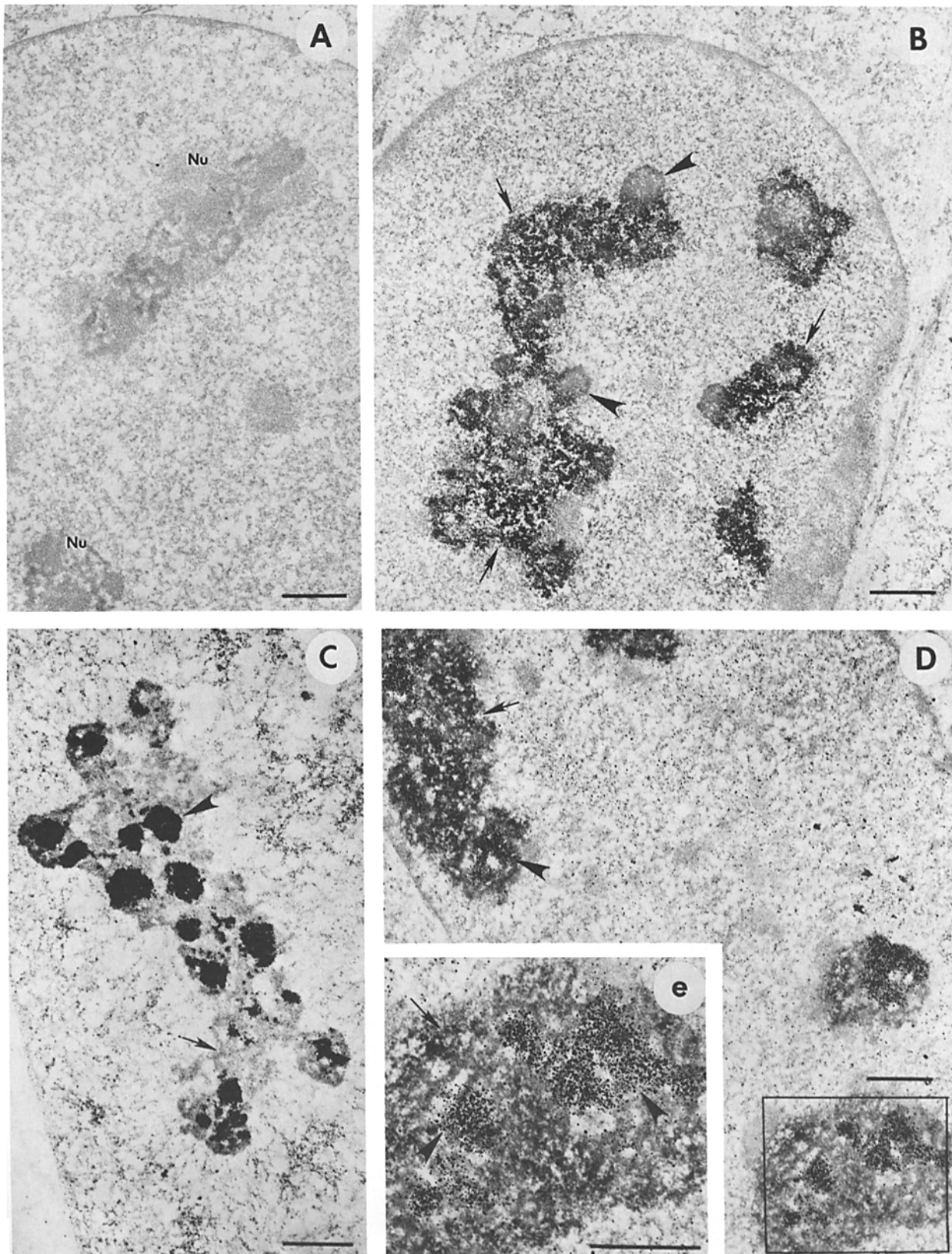
Figure 12. The 72-kD stress protein localizes within the granular region of nucleoli after heat–shock treatment. REF-52 cells were heat–shock treated for 1 h at 43°C, returned to 37°C for 1.5 h, and then fixed and permeabilized as described in Materials and Methods. The cells were then analyzed by either immunostaining with anti–72-kD antibody to determine the intranucleolar distribution of the 72-kD protein, silver staining to detect the nucleolar organizing center (or fibrillar region), or combined immunostaining and silver staining. (*A*) Control immunostaining using a preimmune serum followed by incubation with peroxidase-conjugated goat anti–mouse antibody. (*B*) Intranucleolar distribution of the 72-kD protein as determined by anti–72-kD antibody followed by peroxidase-conjugated goat anti–mouse antibody. (*C*) Fibrillar regions as determined by the silver staining. (*D*) Results of the combined immunostaining and silver staining. (*E*) A higher magnification of the boxed area shown in *D*. Arrowheads in *B–E* indicate the silver staining regions while arrows indicate the granular region where the 72-kD protein predominantly localizes. Bars, 1 μm.

stress proteins subsided and normal translation patterns were restored occurred fastest in the Azc- and arsenite-treated cells and was somewhat slower in the cells which had experienced heat-shock. Finally, the extent of repression of general translation patterns (i.e., synthesis of 37°C proteins) appeared dependent upon the stress treatment used. The heat-treated cells exhibited a rather severe curtailment of 37°C translation patterns, and the Azc-treated cells exhibited a modest inhibition, while the arsenite-treated cells displayed only a slight inhibition in normal translation patterns. Work from a number of laboratories (and our own) indicates that the extent of inhibition of 37°C translation patterns and the rate by which synthesis of the stress proteins subside and normal translation patterns return all appear directly correlated with the subsequent degree of cell survival. For example and not surprisingly, cells which show both the fastest decay in stress protein synthesis and corresponding resumption in normal protein synthesis patterns generally show the highest survival rates (reviewed more extensively in reference 28).

With respect to the localization of the major, stress-induced 72-kD protein, we again observed both similarities and differences depending upon the agent used to induce its synthesis. Immediately after the heat-shock treatment and over the first few hours of recovery, much of the 72-kD protein was found within the nucleus and nucleolus. In the case of the cells treated for 10 h in the presence of Azc, little or no nucleolar staining of 72-kD was observed even though the cells displayed visibly altered nucleoli. However, as soon as the amino acid analog was removed and the cells were further incubated in normal growth medium, nucleolar staining was observed. Using radiolabeled [^{3}H]azetidine, we have demonstrated that the analog does in fact become incorporated into the 72-kD protein (Thomas, P., unpublished observation). The 72-kD protein containing the analog, therefore, is probably rendered "nonfunctional" and as such may not be able to assume its proper locale within the cell. We suspect that upon removal of the analog, the recovering cells now produce a functional 72-kD protein and the protein correctly moves into the disrupted nucleoli.

In contrast to the Azc- and heat-treated cells, those cells recovering from arsenite treatment exhibited only a nuclear but not a nucleolar distribution of 72-kD. The lack of obvious nucleolar staining in the arsenite-treated cells is probably explained by examining and comparing the phase-contrast micrographs (as well as electron micrographs) of the cells placed under stress by the three different agents. Both heat-shock and Azc treatment resulted in considerable changes in the integrity of the nucleoli. Specifically, one observes in cells recovering from either of these two treatments a disruption in nucleolar architecture, including an apparent relaxation in the condensation state of the nucleolar chromatin and a rather severe change in the organization of the granular region (27, 37). Such a disruption in the nucleoli was always correlated with a corresponding nucleolar distribution of the 72-kD protein. Conversely, cells which had recovered and regained normal nucleolar morphology no longer displayed a nucleolar distribution of the 72-kD protein. In the case of arsenite treatment, however, we did not observe any obvious perturbation in nucleolar structure. Rather, the nucleoli remain condensed but did show a slight increase in their phase density. Others have demonstrated that heat-shock treatment results in an inhibition of both ribosomal RNA synthesis and

ribosomal assembly (1, 23). Hence, perhaps the changes we observed in the organization of the nucleolus after heat-shock or Azc treatment are the cause (or effect) of such an impairment in nucleolar function after the heat-shock treatment. Current studies are in progress to test this idea. Specifically, will Azc treatment, which disrupts the integrity of the nucleolus similar to that observed after heat-shock treatment, also result in an inhibition of nucleolar function? Conversely, will nucleolar function be unperturbed in the arsenite-treated cells owing to the fact that nucleolar integrity is basically unchanged?

To facilitate our understanding of the role of 72-kD within the nucleolus, we examined the fine details regarding its subnucleolar distribution. Using both immunoelectron microscopy to detect the distribution of the 72-kD protein, and a sensitive silver staining technique to define the nucleolar-organizing centers, we concluded that the majority of nucleolar 72-kD protein resided within the granular region. This is the site within the nucleolus in which pre-ribosomes and some other ribonucleoprotein complexes are assembled. Others have described severe perturbations in the organization of the granular region after heat-shock treatment. Specifically, a considerable portion of the granular components appears morphologically altered and/or becomes aggregated in response to the heat treatment (1, 27, 37). Thus, one function of the 72-kD protein within the altered nucleoli may be its involvement in the repair of such heat-induced lesions. Further support for this idea follows from our observation that the Azc-treated cells, similar to the heat-treated cells, displayed altered nucleoli and a corresponding nucleolar distribution of 72 kD while those cells treated with sodium arsenite showed little or no nucleolar disruption and little or no nucleolar 72-kD protein.

During the later periods of recovery from heat shock, Azc, or sodium arsenite treatment, much of the 72-kD protein began to accumulate within the cytoplasm. The cytoplasmic distribution of 72-kD differed somewhat depending upon the particular stress agent used, but basically in all three cases there appeared to be three distinct cytoplasmic domains of the protein. Specifically, we observed the 72-kD protein: (a) in the perinuclear region; (b) further out in the cytoplasm in association with very phase-dense structures; and (c) very near the perimeter of the cells, again in conjunction with phase-dense structures which were easily visualized in the light microscope. As we showed by immunoelectron microscopy, these structures at the perimeter of the cells are comprised, in part, of ribosomes. Moreover, double-label indirect immunofluorescence studies using anti-72-kD and anti-ribosomal antibodies demonstrated a colocalization of the 72-kD protein with the cytoplasmic ribosomes. Thus, in concert with our observation demonstrating the presence of the 72-kD protein within the pre-ribosomal-containing granular region of the nucleolus during the early phases of recovery, we similarly find the protein associated with cytoplasmic ribosomes during the later periods of recovery. These observations then raise two pertinent questions: What is the functional significance, if any, of this colocalization of the 72-kD protein with the translational machinery? And what is the manner by which there occurs two distinct populations of the ribosomes in both the perinuclear region as well as along the perimeter of the cells? Deferring the first question till later in this discussion, we suggest that the two

populations of the ribosomes observed resulted from the redistribution of the vimentin-containing intermediate filaments after induction of the stress response. Indeed, in double-label indirect immunofluorescence studies, using anti–vimentin and anti–ribosomal antibodies, most of the ribosomes are found within the collapsed intermediate filament network after heat–shock. We suspect that a portion of the ribosomes (perhaps monosomes) escape such a redistribution after the heat treatment and instead accumulate along the cell perimeter. Moreover, unlike the ribosomes present within the collapsed intermediate filaments, these ribosomes along the cell border are easily solubilized after treatment with low concentrations of Triton X-100 (Welch, W. J., manuscript in preparation). Finally, as we have shown here using emulsion autoradiography (Fig. 11), the perimeter-distributed ribosomes were perfectly capable of translational activity.

Along with its distribution within the perinuclear region and along the perimeter of the cells, we also observed significant levels of the 72-kD protein within the cytoplasm in close association with unusually phase-dense structures. In the case of the Azc or the heat–shock-treated cells, these structures appeared to be comprised of linear phase-dense aggregates. In those cells recovering from arsenite treatment, the structures appeared even more phase-dense and usually assumed a circular-like appearance. At the present time we do not understand either the composition of these structures (other than the presence of the 72-kD protein) or their functional significance. Other laboratories have similarly described the presence of phase-dense cytoplasmic granules in cells (particularly plant cells) recovering from heat–shock treatment and have shown that such granules are comprised, in part, of high levels of the smaller 20–30-kD stress proteins as well as a portion of the 70-kD stress proteins (18). It should also be pointed out that the small *Drosophila* heat–shock proteins are structurally related to the alpha-crystallins of the lens and that a common property of both groups of proteins is their ability to form large aggregates both in vitro and in vivo (2, 13; Arrigo, P., manuscript in preparation). We suspect that these phase-dense structures described here in the mammalian cells may be analogous to the cytoplasmic granules described in plant cells and/or represent aggregates of denatured protein. We are currently trying to purify these structures (they remain intact in cells after Triton X-100 extraction) and to characterize their molecular composition.

We return then to the question raised earlier regarding the significance of the observed localization of the 72-kD stress protein within the granular region of the nucleolus early during the recovery period as well as its colocalization with cytoplasmic ribosomes during the later periods of recovery from physiological stress. As we and others have previously speculated, the migration of the 72-kD protein into the nucleolus may be due to its (*a*) being assembled into some type of ribonucleoprotein complex and/or (*b*) possible involvement in the repair and/or recovery of normal nucleolar function (15, 20, 35, 37). Over the past year, work from Pelham's laboratory has strongly supported the idea of the 72-kD protein being involved in nucleolar repair. For example, they have shown that cells constructed to synthesize high constitutive levels of the 72-kD protein show a more rapid recovery of normal nucleolar morphology after heat–shock treatment than do the unconstructed cells not constitutively

expressing 72 kD (20). In addition, the ATP-binding properties we have described for the 72-kD protein may be crucial to such a repair process (36). Specifically, Lewis and Pelham have shown that release of the 72-kD protein from the nucleolus occurs rapidly in isolated nuclei incubated with micromolar concentrations of ATP (15). Such a release of the protein in this in vitro assay was not observed when nonhydrolyzable analogues of ATP were used, thereby implicating a requirement of ATP-hydrolysis for the release of the 72-kD protein. Hence, we suspect that 72-kD is somehow functioning within the nucleolus to repair (or possibly remove) denatured and/or aggregated granular components such as newly forming pre-ribosomal particles. Such a repair process may require the energy derived from ATP hydrolysis. Once repaired, the nucleolus regains its normal morphology and the 72-kD protein now exits from the nucleolus.

During the later periods of recovery from stress, the 72-kD protein is observed to accumulate within the cytoplasm with a considerable portion of the protein showing a colocalization with the cytoplasmic ribosomes. Similar to its putative repair role within the pre-ribosomal–containing region of the nucleolus, we suspect that the cytoplasmic 72-kD protein associated with the ribosomes is essential for the proper functioning of the translational machinery. This is suggested since the ribosome, with its numerous protein constituents, would be a large and sensitive target to agents which promote protein denaturation (i.e., agents which induce the stress response). Indeed others have reported the high sensitivity of the translational machinery to various protein denaturants and/or elevated nonphysiological temperatures (9). This point is also illustrated here in Fig. 1 where it can be seen that translation, in general, was greatly inhibited in the cells while exposed to the particular stress agents (e.g., Fig. 1, lane *1*, in each case). As the recovery process proceeds and more 72 kD is made, the absolute degree of translational activity began to increase. For example, the heat–shock-treated cells showed a maximal production of the 72-kD protein after 7–8 h of recovery, and only then did the cells begin to show a return to normal translation patterns (e.g., the return in translation of 37°C proteins). Similarly, in the case of the arsenite-treated cells, maximal synthesis of the 72-kD protein was observed much earlier during the recovery period (4–5 h), and again one could observe at this time a return of 37°C translation patterns.

That the 72-kD protein may be involved in rescuing translational activities is also supported by studies from other laboratories as well as our own (see, for example, review by Subjeck and Shyy [28]). Specifically, if cells are immediately brought to a very high temperature, overall translation patterns (both normal and heat–shock proteins) are severely inhibited. If, however, the cells are first given a mild heat–shock treatment, returned to the normal growth temperature, and then subsequently presented with the very high temperature shock, the cells now display an overall increased ability to translate protein. Furthermore, this regime also results in a much faster production of the heat shock proteins, and a corresponding faster return to normal translation patterns as compared to those cells immediately brought to the high lethal temperature. This experimental protocol (a mild heat–shock recovery at 37°C, and a second more severe heat–shock challenge) has been shown by others to also result in a dramatic increase in cell survival and therefore has

been termed acquired thermotolerance (28). We think the key to thermotolerance resides in the ability of the cells to function normally (e.g., transcription, translation) even when experiencing the toxic and inhibitory effects of the stress treatment. Consistent again with this idea is the recent results of Yost and Lindquist (41) who have shown that a brief but severe heat-shock treatment in *Drosophila* cells results in a block of mRNA processing. However, a mild heat-shock treatment before the more severe treatment rescues the ability of the cells to correctly process new mRNA transcripts. Similar to the rescue of translation, the thermotolerant cell has now apparently regained the ability to process mRNA transcripts. Thus, an attractive hypothesis is that the 72-kD protein, by virtue of both its abundance throughout the cell and its putative ability to hydrolyze ATP, somehow functions to restore, retain, and/or stabilize multi-protein complexes (e.g., transcriptional and translational machinery) whose function is sensitive to protein denaturants (i.e., stress-inducing agents). In the thermotolerant cell, where 72 kD has already been synthesized and associated with such machinery, transcription and translation activities can now proceed normally even in response to a second stress challenge.

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