Characterization of the Thermotolerant Cell.
II. Effects on the Intracellular Distribution of Heat-Shock Protein 70, Intermediate Filaments, and Small Nuclear Ribonucleoprotein Complexes

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Abstract. Here we further characterize a number of properties inherent to the thermotolerant cell. In the preceding paper, we showed that the acquisition of the thermotolerant state (by a prior induction of the heat-shock proteins) renders cells translationally tolerant to a subsequent severe heat-shock treatment and thereby results in faster kinetics of both the synthesis and subsequent repression of the stress proteins. Because of the apparent integral role of the 70-kD stress proteins in the acquisition of tolerance, we compared the intracellular distribution of these proteins in both tolerant and nontolerant cells before and after a severe 45°C/30-min shock. In both HeLa and rat embryo fibroblasts, the synthesis and migration of the major stress-induced 72-kD protein into the nucleolus and its subsequent exit was markedly faster in the tolerant cells as compared with the nontolerant cells. Migration of

preexisting 72-kD into the nucleolus was shown to be dependent upon heat-shock treatment and independent of active heat-shock protein synthesis. Using both microinjection and immunological techniques, we observed that the constitutive and abundant 73-kD stress protein similarly showed a redistribution from the cytoplasm and nucleus into the nucleolus as a function of heat-shock treatment. We show also that other lesions that occur in cells after heat shock can be prevented or at least minimized if the cells are first made tolerant. Specifically, the heat-induced collapse of the intermediate filament cytoskeleton did not occur in cells rendered thermotolerant. Similarly, the disruption of intranuclear staining patterns of the small nuclear ribonucleoprotein complexes after heat-shock treatment was less apparent in tolerant cells exposed to a subsequent heat-shock treatment.

A LTHOUGH the proteins synthesized in response to heat-shock treatment or other forms of environmental stress have been identified and partially characterized, their exact function remains to be established. An important clue regarding their possible function has come from studies examining the phenomenon of thermotolerance. Specifically, cells given a mild heat-shock treatment, sufficient to induce the synthesis of the stress proteins, exhibit considerably higher survival rates after a second and what would otherwise be a lethal heat-shock treatment (references 5, 7 and reviewed in 26). In the preceding study, we demonstrated that cells made thermotolerant also exhibited markedly less inhibition of overall translational activity after a severe heat-shock challenge as compared with nontolerant cells. Similar to previous cell survival studies, our results indicated a relationship between the acquisition of translational tolerance and the synthesis and accumulation of the 70-kD family of stress proteins. Here we have further characterized some of the properties inherent to the thermotolerant cell. We show that, in general, lesions that occur in cells after heat-shock treatment can be prevented, or at least repaired much faster, if the cells are first made thermotolerant. These studies along with those presented in the preceding pa-

per provide new information that may explain, in part, how cells can exhibit increased survival in response to a severe and otherwise lethal heat-shock event.

Materials and Methods

Indirect Immunofluorescence

The intracellular distribution of the 72-kD stress protein, the 73-kD stress protein, the vimentin-containing intermediate filaments, and the small nuclear ribonucleoprotein complexes (snRNPs)[1] were determined using indirect immunofluorescence. In all cases, the cells were fixed and permeabilized by incubation with −20°C absolute methanol for 2 min. Antibodies used included: mouse monoclonal C92F3A-5 (for the 72-kD stress protein [39]); mouse monoclonal V-9 (for vimentin staining, obtained from Boehringer Mannheim, Indianapolis, IN); a rabbit polyclonal antibody, which recognizes both 72- and 73-kD (obtained from T. Chappell and J. Rothman, Stanford University [3]); and a mouse monoclonal anti-Sm antibody, which recognizes proteins of 29 and 16 kD associated with the U-1, U-2, U-4, U-5, and U-6 snRNAs (13) obtained from D. Spector, Cold Spring Harbor Laboratory and Joan Steitz, Yale University). All antibodies were diluted in PBS containing 5 mg/ml BSA. Primary antibodies were visualized after incubation with a fluorescein-conjugated goat anti-rabbit or goat

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1. *Abbreviations used in this paper:* hnRNA, heteronuclear RNA; snRNA, small nuclear ribonucleoprotein complexes.
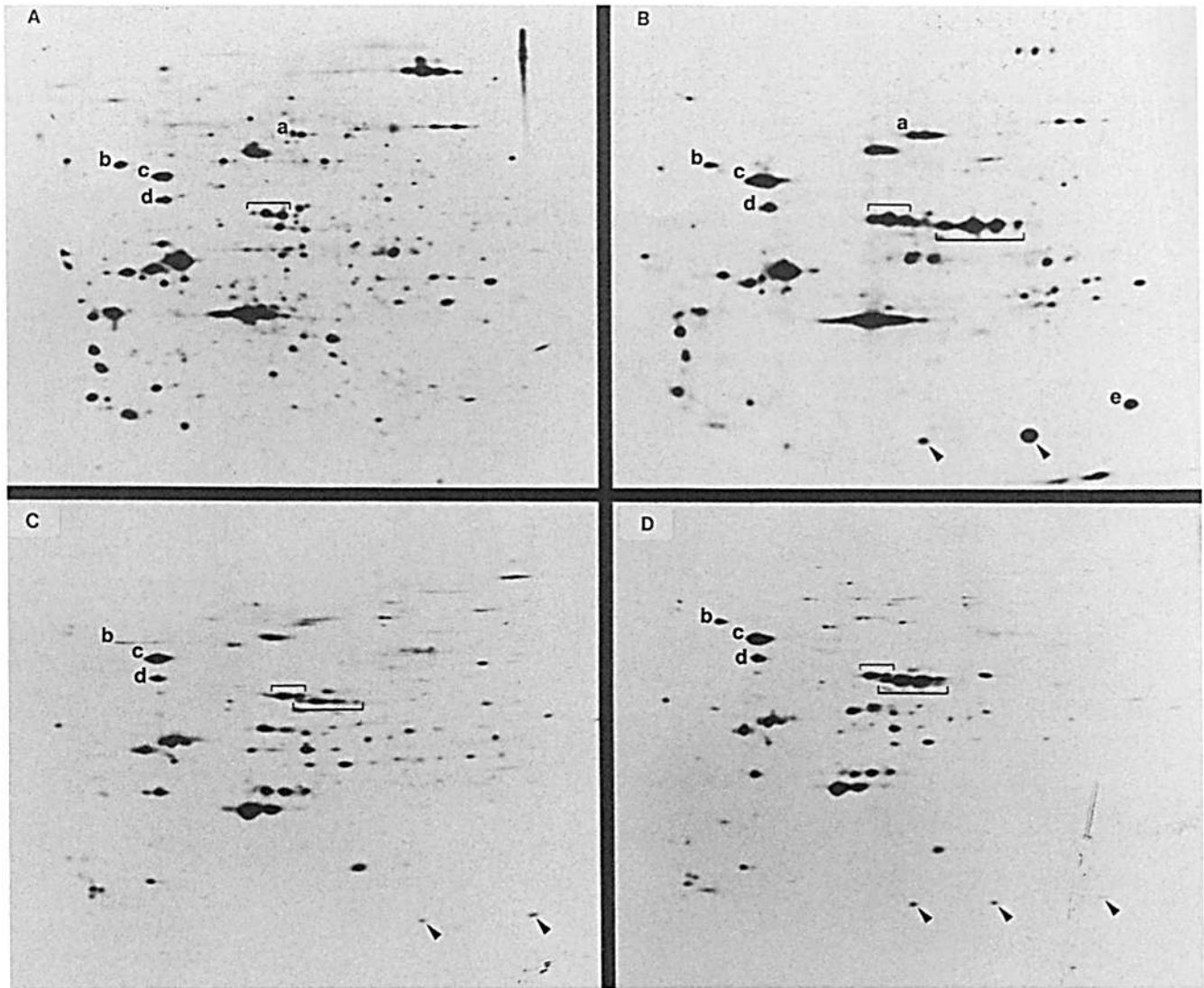
Figure 1. Two-dimensional gel analysis of [³H]leucine-labeled proteins synthesized in normal or heat-shock treated HeLa and rat embryo fibroblasts. HeLa cells or rat embryo fibroblasts (REF-52) growing on Falcon dishes were incubated at either 37° or at 43°C for 1.5 h. The cells were returned to 37°C and labeled with [³H]leucine for 4 h. The cells were solubilized and the labeled proteins analyzed by isoelectric focusing followed by SDS-PAGE on 15% gels. Shown are fluorograms of the gels. Acid end is to the left. The positions of the stress proteins are indicated as: (*a*) 110 kD; (*b*) 100 kD (*c*) 90 kD; (*d*) 80 kD; (*e*) 32 kD. The constitutive 73 kD is indicated by a downward bracket and the position of the inducible 72 kD by an upward pointing bracket. Finally, isoforms of the 28-kD stress protein are indicated by arrowheads. (*A* and *B*) REF-52 cells labeled at 37°C (*A*) or after heat shock (*B*). (*C* and *D*) HeLa cells labeled at 37°C (*C*) or after heat shock (*D*).

anti–mouse antibodies diluted 1:70 (Cooper Biomedical, Malvern, PA). The cells were examined on a Zeiss photomicroscope using a Planapo 63× (1.3 NA) lens. Fluorescent images were photographed with Kodak Tri-X film and phase-contrast images photographed with Kodak technical pan film.

Purification of Stress Proteins and Microinjection

The constitutive 73-kD protein was purified from rat brain essentially as described before for the 72/73-kD proteins from HeLa cells (35). Briefly, rat brains were solubilized in 1% Triton in 10 mM Tris, pH 7.4, and 0.1 mM EDTA via dounce homogenization. A 15,000 *g* supernatant was prepared, applied to a DEAE-52 cellulose column, and the proteins eluted as described previously (35). Peak fractions containing 73-kD proteins were directly applied to an ATP-agarose column and the proteins further purified as described previously (35).

HeLa 72, 73, 75, and 80 kD were purified from the 12,000 *g* pellet of heat-shock treated and hypotonically lysed HeLa cells exactly as described previously (35).

The purified rat brain 73-kD stress protein was conjugated with tetramethylrhodamine isothiocyanate, and the protein was separated from the free dye and microinjected into rat fibroblasts exactly as described previously (35).

Metabolic Labeling and Two-Dimensional Gels

REF-52 cells or HeLa cells growing on plastic dishes were incubated at either 37° or at 43°C for 1.5 h After the heat-shock treatment, the cells were returned to 37°C and labeled with [³H]leucine as described previously (32). After a 4-h labeling, the cells were solubilized and analyzed by two-dimensional gel electrophoresis (32). Visualization of the proteins was done by fluorography.

Results

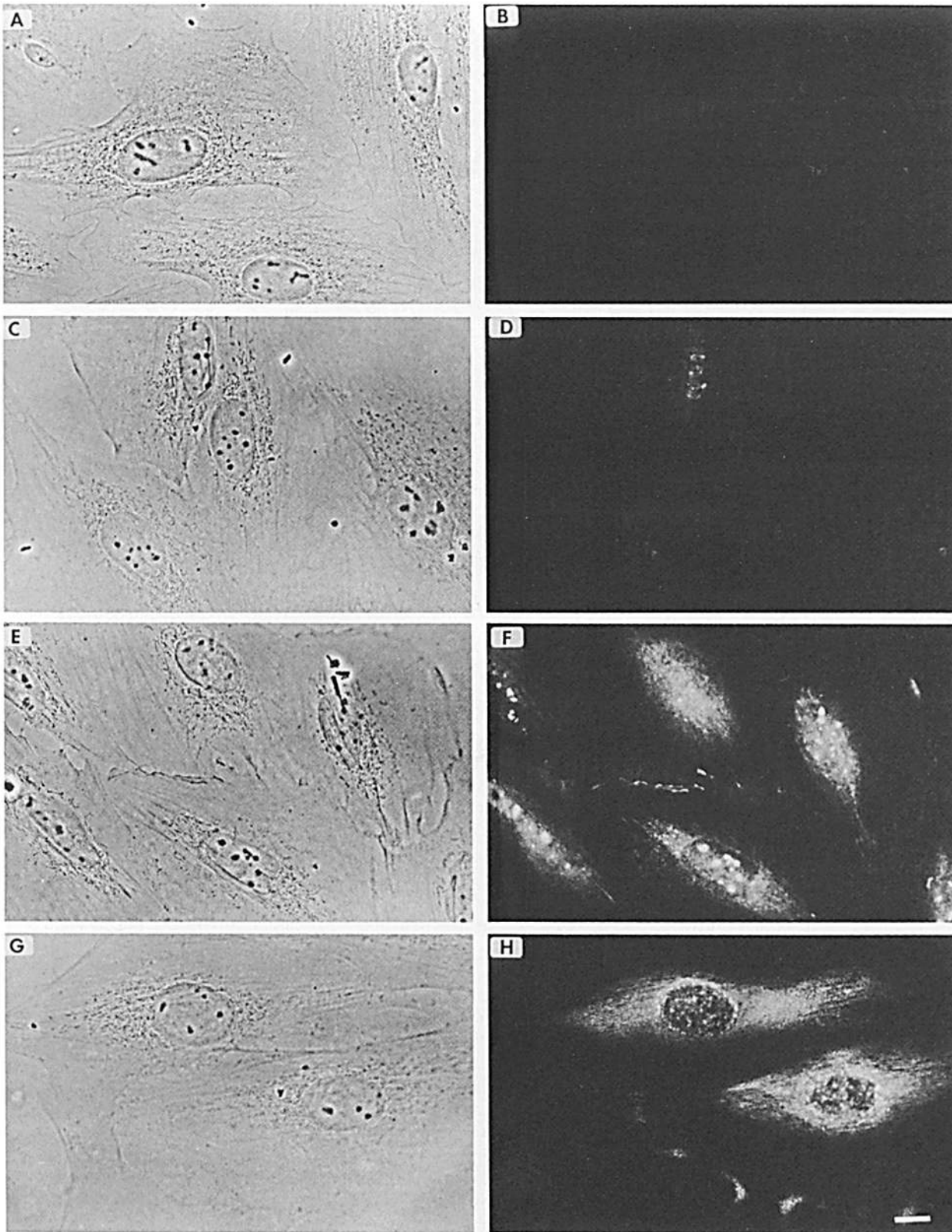The 70-kD family of stress proteins consists of two major

Figure 2. Intracellular distribution of the 72-kD stress protein in nontolerant rat fibroblasts after a 45°C/30-min heat-shock treatment. REF-52 cells, growing on glass coverslips, were subjected to a 45°C/30-min heat-shock treatment and then returned to 37°C for either 0, 3, 7, or 9 h. After the indicated recovery times, the cells were fixed and analyzed for the distribution of the 72-kD stress protein via indirect immunofluorescence as described in the Materials and Methods. Shown in *A*, *C*, *E*, and *G* are the phase-contrast micrographs and in *B*, *D*, *F*, and *H* are the corresponding fluorescent micrographs. (*A* and *B*) Cells heat-shock treated, no recovery. (*C* and *D*) Cells heat-shock treated and recovered at 37°C for 3 h. (*E* and *F*) Cells heat-shock treated and recovered at 37°C for 7 h. (*G* and *H*) Cells heat-shock treated and recovered at 37°C for 9 h. Bar, 10 μm.
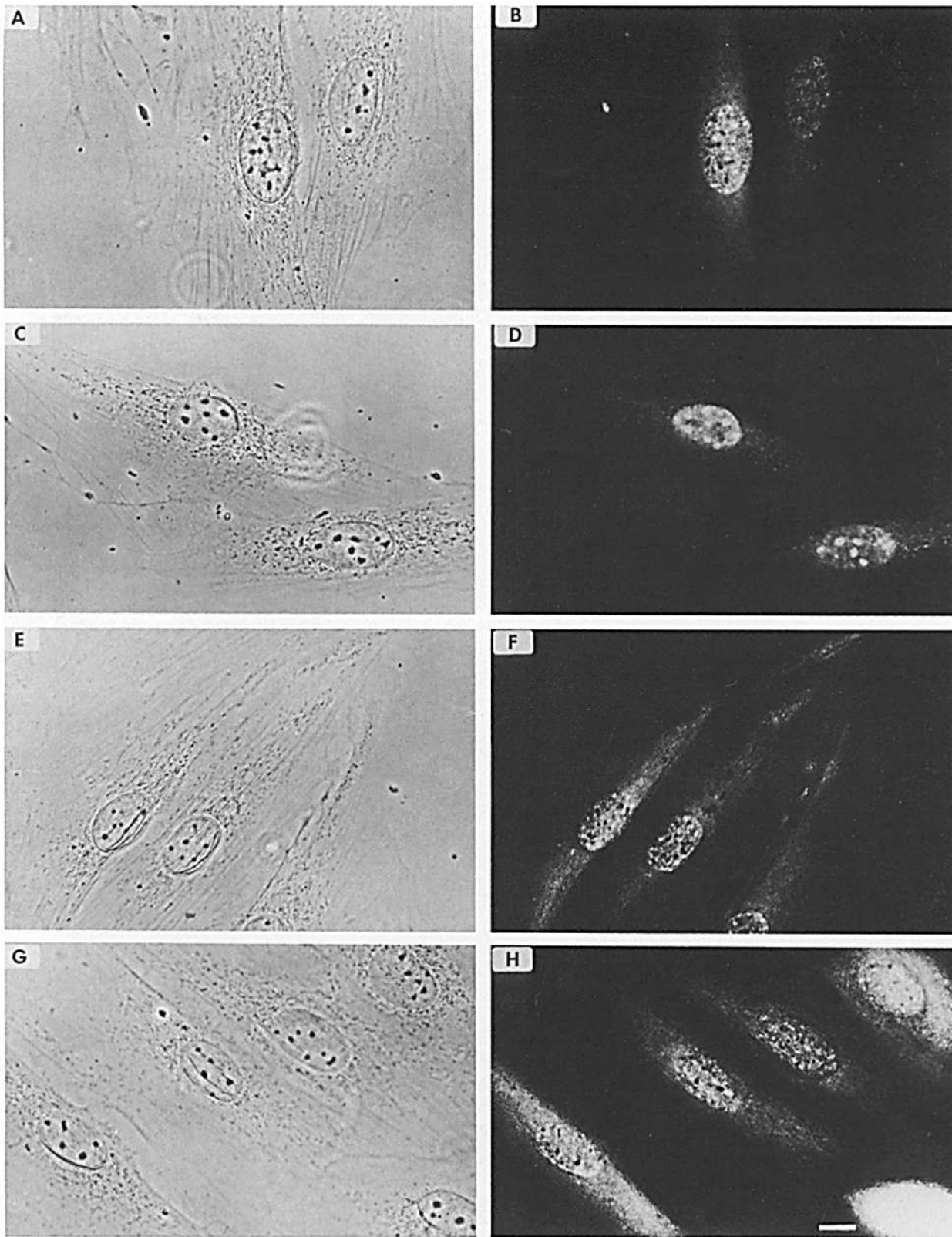
Figure 3. Intracellular distribution of the 72-kD stress protein in thermotolerant rat fibroblasts after a 45°C/30-min heat-shock treatment. REF-52 cells, growing on glass coverslips, were made thermotolerant by a 43°C/1.5-h heat-shock treatment. After 12 h of recovery at 37°C, one coverslip was removed and fixed with cold methanol. The remaining coverslips were given a second, 45°C/30-min heat-shock treatment and the cells either fixed immediately or returned to 37°C and incubated further for 3 or 7 h. These cells were then fixed with methanol and the distribution of the 72-kD stress protein analyzed by indirect immunofluorescence. Shown in *A, C, E,* and *G* are the phase-contrast micrographs and in *B, D, F,* and *H* the corresponding fluorescent micrographs. (*A* and *B*) Cells heat-shock treated at 43°C/1.5 h and recovered at 37°C for 12 h. (*C* and *D*) Thermotolerant cells given a 45°C/30-min heat-shock treatment. (*E* and *F*) Thermotolerant cells given a 45°C/30-min heat-shock treatment and returned to 37°C for 3 h. (*G* and *H*) Thermotolerant cells given a 45°C/30-min heat shock and returned for 37°C to 7 h. Bar, 10 μm.

forms: a constitutive member, 73 kD, and a highly stress inducible member, 72 kD, each comprising multiple isoforms. This is illustrated in Fig. 1 where both rat fibroblasts and HeLa cells were steady-state labeled with [³H]leucine at either 37°C or after a 43°C/1.5-h heat-shock treatment and the labeled proteins analyzed by two-dimensional gel electrophoresis. Rat fibroblasts cultured at 37°C synthesized significant levels of the 73- and 90-kD heat-shock proteins (Fig. 1 A). This relatively high steady-state synthesis of the stress proteins is accompanied by their significant accumulation in the cell (as demonstrated by Coomassie Blue staining of the gels [data not shown]). After heat-shock treatment, synthesis of the 73- and 90-kD heat-shock proteins increased significantly (Fig. 1 B). Moreover, there was a marked induction of the 72-kD heat-shock protein, which consisted of multiple isoforms. Note that the synthesis of the 72-kD protein was not discernable in the 37°C rat fibroblasts. In the case of HeLa cells, a similar but slightly more complicated pattern of heat-shock protein synthesis is observed. Along with the production of the 73- and 90-kD heat-shock proteins, HeLa cells grown at 37°C also synthesized appreciable levels of the 72-kD protein (Fig. 1 C). After heat-shock treatment, increased synthesis of all three heat-shock proteins occurs (Fig. 1 D). Although beyond the scope of this report, this constitutive production of the highly inducible 72-kD stress protein in the 37°C HeLa cells also has been observed in a number of different human cell lines but not in most rodent cell lines grown in culture (references 33, 34; unpublished observations).

The relative subcellular distribution of the most highly induced stress protein, 72 kD, was compared in both tolerant and nontolerant cells given a severe 45°C/30-min heat-shock treatment. Both nontolerant and tolerant rat embryo fibroblasts were presented a 45°C/30-min heat shock, the cells returned to 37°C, and the distribution of the 72-kD heat-shock protein examined at various time points during the recovery period via indirect immunofluorescence. A monoclonal antibody, C92, was used for these studies and has been shown previously to be specific for only the 72-kD stress protein (39). In the case of nontolerant cells, for the first 3 h after the 45°C/30-min shock, little or no 72-kD fluorescence was observed (Fig. 2, A-D), consistent with the metabolic labeling studies demonstrating a complete inhibition of overall protein synthesis for a number of hours after this rather severe heat-shock challenge (see preceding paper). By 7 h of recovery, 72-kD staining was observed, with most of the protein present within the nucleus and nucleolus and to a lesser extent the cytoplasm (Fig. 2, E and F). With increasing recovery time, much of the protein began to accumulate in the cytoplasm with a portion of the protein observed within discrete areas of the nucleus but excluding the nucleolus (Fig. 2, G and H).

In the case of cells first made thermotolerant (via a 43°C/1.5-h heat shock and subsequent 12 h recovery at 37°C) and then presented the 45°C/30-min heat-shock treatment, the migration of 72 kD into and out of the nucleus/nucleolus was greatly accelerated. Analysis of the tolerant cells before the second heat-shock treatment revealed that much of 72 kD made after the priming heat-shock treatment was now distributed within the nucleus and to a lesser extent the cytoplasm with little or none of the protein present within the nucleolus (Fig. 3, A and B). Immediately after the 45°C/

30-min heat-shock treatment of the tolerant cells, most of 72 kD was now observed within the nucleolus (Fig. 3, C and D). We interpret this nucleolar staining to reflect a redistribution of the preexisting 72-kD protein since our metabolic labeling studies had demonstrated there to be little or no new synthesis of 72-kD immediately after this rather severe heat-shock treatment (see preceding paper). After only 3 h of recovery, the tolerant cells no longer exhibited a nucleolar distribution of the protein. Instead, most of the 72-kD protein was present throughout the nucleus and the cytoplasm (Fig. 3, E and F). By 7 h of recovery, the cytoplasmic staining in some, but not all, of the cells had markedly increased (Fig. 3, G and H).

A similar time course experiment comparing the intracellular distribution of 72 kD in both tolerant and nontolerant cells was performed with HeLa cells. This analysis was of interest because HeLa cells inherently appear more thermotolerant than do REF-52 cells (accompanying manuscript) and as shown in Fig. 1, HeLa cells synthesize the 72-kD stress protein in a constitutive fashion (i.e., at 37°C). Therefore, the distribution of 72-kD was compared in both nontolerant and tolerant HeLa cells before and after a 45°C/30-min heat-shock treatment (nontolerant cells in the left column and tolerant cells in the right column). The untreated, nontolerant HeLa cells exhibited a modest staining of 72 kD with the protein present within the nucleus and to a lesser extent the cytoplasm (Fig. 4 A). Cells previously made thermotolerant (i.e., a 43°C/1.5-h shock followed by a 12-h recovery period at 37°C) exhibited, overall, more intense staining than did the nontolerant cells, with such staining present in both the cytoplasm and nucleus but not the nucleolus (Fig. 4 B). After the 45°C/30-min shock, both the nontolerant (Fig. 4 C) and the tolerant (Fig. 4 D) cells exhibited increased staining within the nucleus and nucleolus. Interestingly, there occurred a "ring-like" staining of the nucleolar perimeter in the tolerant cells, whereas in the nontolerant cells 72-kD staining was apparent throughout all of the nucleolus. By 3 h of recovery, intense nuclear and nucleolar staining was observed in the nontolerant cells while the tolerant cells no longer exhibited any prominent nucleolar staining (Fig. 4, E and F). The nontolerant cells continued to show increases in 72-kD staining after 5 h of recovery with the protein distributed within the nucleus, nucleolus, and now the cytoplasm (Fig. 4 G), while the staining pattern had begun to diminish in the tolerant cells (Fig. 4 H). Presently we do not completely understand this apparent diminishment of staining in the tolerant cells. We think it likely that although the protein is still present at appreciable levels, the antigenic site recognized by the monoclonal antibody is no longer accessible at this point in the recovery period. For example, using other monoclonal antibodies that apparently recognize different epitopes, intense staining of 72 kD was observed (data not shown).

The rapid appearance of 72 kD within the nucleolus after a second heat-shock treatment was indicative of there being a redistribution of the preexisting protein. That is, 72 kD made after the initial priming shock migrated into the nucleus/nucleolus, returned to the cytoplasm during recovery, and then after a second shock apparently would return again to the nucleolus. To test this directly, the tolerant cells were given a 45°C/30-min shock, either in the presence or absence of the protein synthesis inhibitor cycloheximide. In both cases, the 72-kD protein was observed to distribute
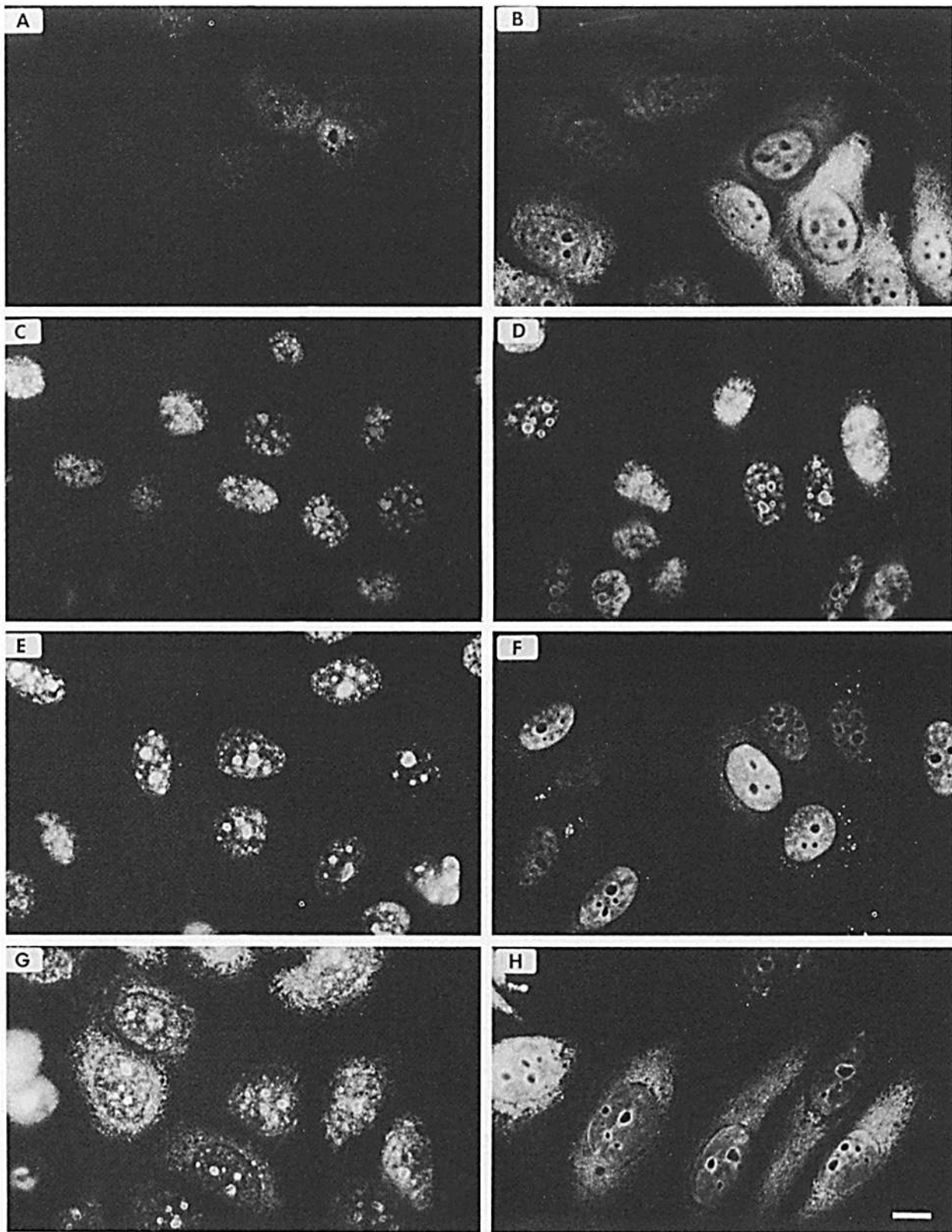
Figure 4. Comparison of 72-kD intracellular locale in tolerant vs. nontolerant HeLa cells exposed to a 45°C/30-min heat-shock treatment. HeLa cells, growing on glass coverslips, were made thermotolerant via a 43°C/1.5-h heat-shock treatment followed by 12-h recovery at 37°C. The thermotolerant cells as well as nontolerant cells (i.e., no prior heat-shock treatment) were then subjected to a 45°C/45-min heat-shock treatment. Both groups of heat-shocked cells, tolerant and nontolerant, were returned to 37°C and further incubated for either 0, 3, or 5 h. At the appropriate time the cells were fixed and analyzed for the distribution of the 72-kD stress protein by indirect immunofluorescence. Shown only are the fluorescent micrographs. In *A*, *C*, *E*, and *G* are shown the nontolerant cells and in *B*, *D*, *F*, and *H* the tolerant cells. (*A* and *B*) Nontolerant and tolerant cells, respectively, 37°C. (*C* and *D*) Nontolerant and tolerant cells, respectively, after the 45°C/45-min heat shock. (*E* and *F*) Nontolerant and tolerant cells, respectively, after the 45°C/45-min heat shock and subsequent recovery at 37°C for 3 h. (*G* and *H*) Nontolerant and tolerant cells, respectively, after the 45°C/45-min heat shock and subsequent recovery at 37°C for 5 h. Bar, 10 μm.
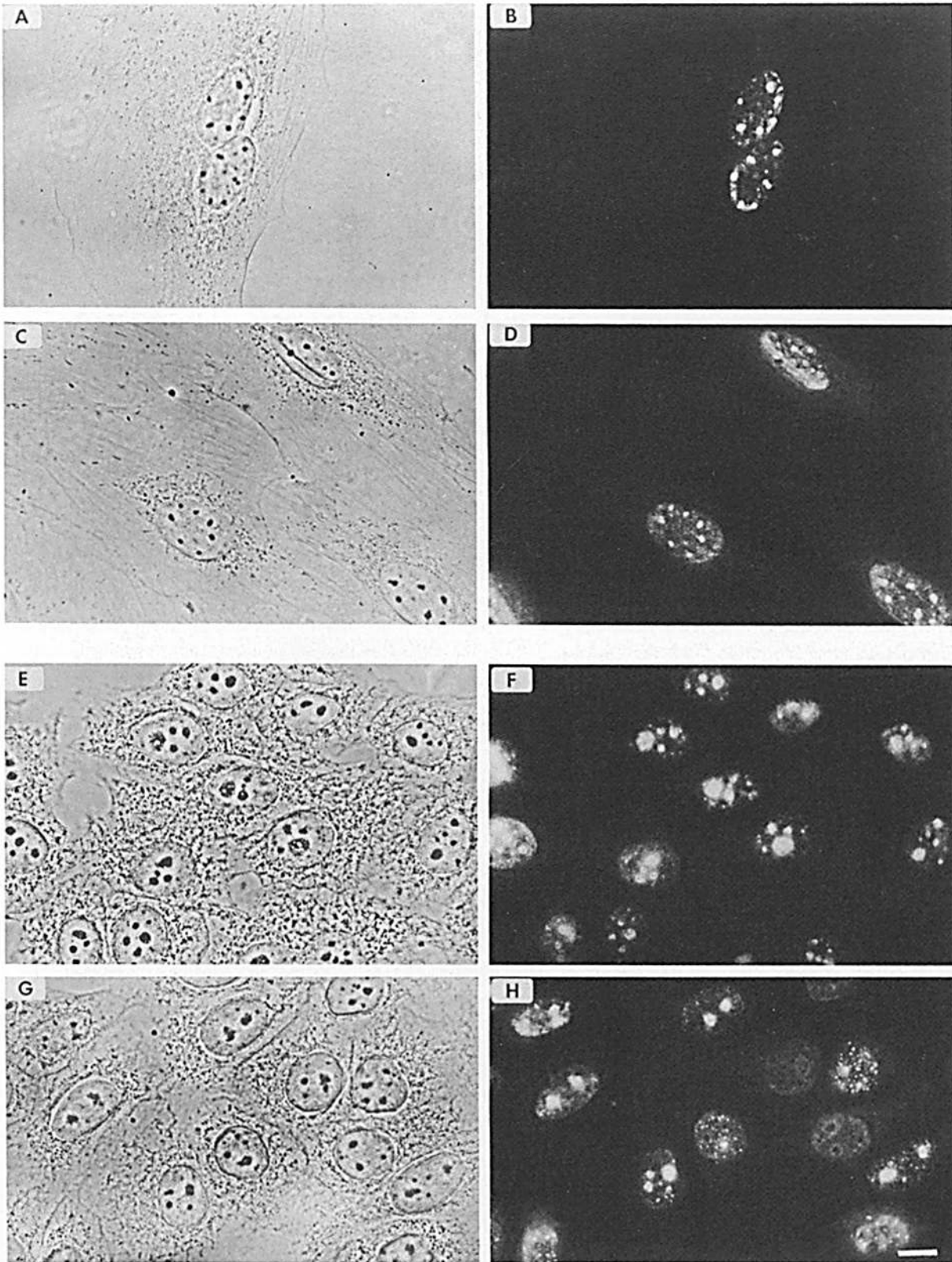
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Figure 5. Preexisting 72-kD stress protein relocates to the nucleus/nucleolus in cells after heat-shock treatment. REF-52 cells, growing on glass coverslips, were made thermotolerant by a 43°C/1-h heat-shock treatment and subsequent recovery at 37°C for 12 h. The thermotolerant REF-52 cells, either in the presence or absence of cycloheximide, were given a second 45°C/30-min heat-shock treatment, then fixed and analyzed for the distribution of the 72-kD stress protein via indirect immunofluorescence. In parallel, normal HeLa cells (i.e., nontolerant) growing on glass coverslips, were subjected to a 45°C/30-min heat-shock treatment, either in the presence or absence of cycloheximide, then fixed and analyzed for the distribution of the 72-kD stress protein using indirect immunofluorescence. Shown in *A*, *C*, *E*, and *G* are the phase-contrast micrographs and in *B*, *D*, *F*, and *H*, the corresponding fluorescent micrographs. (*A–D*) Thermotolerant REF-52 cells after a 45°C/30-min heat-shock treatment in the absence (*A* and *B*) or the presence (*C* and *D*) of cycloheximide. (*E–H*) HeLa cells after a 45°C/30-min heat-shock treatment in the absence (*E* and *F*) or presence (*G* and *H*) of cycloheximide. Bar, 10 μm.

within the nucleolus of the rat fibroblasts as a function of the heat-shock treatment (Fig. 5, A–D). In the case of HeLa cells, a similar result was obtained. In this case we simply examined the 37°C cells (rather than the tolerant cells) since 72 kD is expressed constitutively in the HeLa cells. After heat-shock treatment, again either in the presence or absence of cycloheximide, 72-kD staining was apparent within the nucleoli (Fig. 5, E–H). However, the number of cells exhibiting nucleolar staining was markedly less in those cells first treated with the protein synthesis inhibitor. These results, in summary, indicate that the preexisting 72 kD will return again to the nucleolus after a second heat-shock treatment and that this nucleolar redistribution is independent of de novo protein synthesis.

As was described in the accompanying paper, recent controversy has arisen concerning the hypothesis that the heat-shock proteins are directly involved in the acquisition of thermotolerance. One major basis for this controversy comes from observations that cells can be made thermotolerant (as assayed by cell survival) by a prior heat-shock treatment and subsequent recovery in the presence of agents that prevent the synthesis of the heat-shock proteins (6, 11, 41). In addition to the complicated and pleiotropic effects of these protein synthesis inhibitors (as discussed in the preceding paper and in reference 12), these studies often overlook the fact that many of the stress proteins are already present at significant levels in the cells grown at 37°C (e.g., see Fig. 1). Hence, might the constitutive stress proteins be involved in the acquisition of thermotolerance? Moreover, might their involvement require an activation event such as that provided by the initial priming heat-shock treatment? To address this question, we examined whether the constitutive 73-kD stress protein, like the inducible 72-kD protein, might show changes in its intracellular distribution as a function of heat-shock treatment. Therefore, to examine its subcellular distribution, we elected to purify the 73-kD protein, conjugate it with a fluorescent chromophore, and microinject it back into living cells and thereby determine its intracellular locale before and after heat-shock treatment. The 73-kD protein was purified from rat brain and the purified protein first analyzed by two-dimensional gel electrophoresis. The purified protein migrated as a single species and in mixing experiments with proteins purified from HeLa cells, the rat brain protein was observed to co-migrate with only the HeLa 73-kD protein (Fig. 6, a–c). The purified bovine 73-kD protein was then conjugated with tetramethylrhodamine isothiocyanate and the protein further purified to remove excess free chromophore. The fluorescently labeled protein was microinjected into the cytoplasm of 37°C rat fibroblasts and the cells then further incubated at either 37°C or given a 43°C/1.5-h heat-shock treatment. In the microinjected cells maintained at 37°C, the fluorescently labeled 73 kD was present within the cytoplasm and the nucleus (Fig. 6, A and B). After heat shock, much of the protein was observed to redistribute into the nucleolus. Hence, the constitutive 73-kD protein, similar to that of the highly induced 72-kD stress protein, localizes to the nucleolus but only in response to heat-shock treatment. A similar result was observed when the analysis was performed using immunological techniques. For these studies an antibody that recognizes both the 73- and the 72-kD proteins was used in the analysis. To be certain that we analyzed the fate of only the constitutive 73-kD protein, the rat fibro-

blasts were first incubated with cycloheximide (to prevent synthesis of the heat-inducible 72-kD species), then heat-shock treated and analyzed by indirect immunofluorescence. The addition of cycloheximide allowed us to examine only the preexisting, constitutive 73-kD protein since rat fibroblasts, at 37°C, contain only 73 and not 72 kD (e.g., see Fig. 1). In those cells heat-shock treated in the absence of the drug, intense nucleolar staining was observed (Fig. 6, E and F). Such staining, however, may reflect both the constitutive (73 kD) as well as the inducible (72 kD) forms of the protein. Cells heat-shock treated in the presence of cycloheximide similarly showed bright nucleolar staining (Fig. 6, G and H). We conclude then that the constitutive 73-kD protein, like its related and highly inducible 72-kD counterpart, relocalizes into the nucleus and nucleolus as a result of heat-shock treatment.

Our data to this point (this and preceding paper) had demonstrated that cells first made thermotolerant and then subjected to a severe heat-shock challenge exhibited differences with respect to (a) the extent of translational inhibition, (b) the kinetics of synthesis and subsequent repression of the stress proteins, and (c) the kinetics of redistribution of the 72-kD stress protein as compared with the nontolerant cell. Hence, we were interested to determine whether the acquisition of the thermotolerant state might have manifestations in other cellular events that are known to be perturbed after heat shock. In previous studies we and others had demonstrated that one of the early events after heat shock was the collapse of the vimentin-containing intermediate filament network (2, 4, 27, 36, 37). For example, within minutes after temperature elevation, or after exposure of cells to other agents that induce the stress response, the intermediate filaments rapidly redistribute from their normal well-spread cytoplasmic array into a tight cage around the nucleus. This is illustrated in Fig. 7, A and B, where the intermediate filaments have collapsed in and around the nucleus of the rat fibroblasts after a 43°C/1.5-h heat-shock treatment. In contrast, cells first made thermotolerant (e.g., 43°C/1.5-h shock followed by a 12-h recovery) exhibited little or no collapse of the intermediate filaments after a second and identical 43°C/1.5-h heat-shock treatment (Fig. 7, C and D). Using this same approach, we examined whether intermediate filament integrity would be protected in cells exposed to an even more severe heat-shock treatment than that used to make the cells tolerant. That is, nontolerant cells along with cells previously made tolerant by a 43°C/1.5-h shock were now given a more severe 45°C/30-min heat shock and the distribution of the filaments analyzed. As before, the nontolerant cells showed a rapid and complete collapse of the intermediate filaments (Fig. 7, E and F). In the case of the tolerant cells, there occurred only a partial collapse of the filaments (Fig. 7, G and H). Finally, recovery of normal intermediate filament integrity occurred significantly faster in the tolerant as compared with the nontolerant cells upon return of the cells to 37°C (data not shown).

Previous studies by Yost and Lindquist have described the inhibition of heteronuclear RNA (hnRNA) processing in *Drosophila* cells after a severe heat-shock treatment (43). If the cells were first made thermotolerant, however, markedly less inhibition of hnRNA processing was observed. Hence, we examined the intracellular distribution of the small nuclear snRNPs involved in the processing of hnRNA cells af-
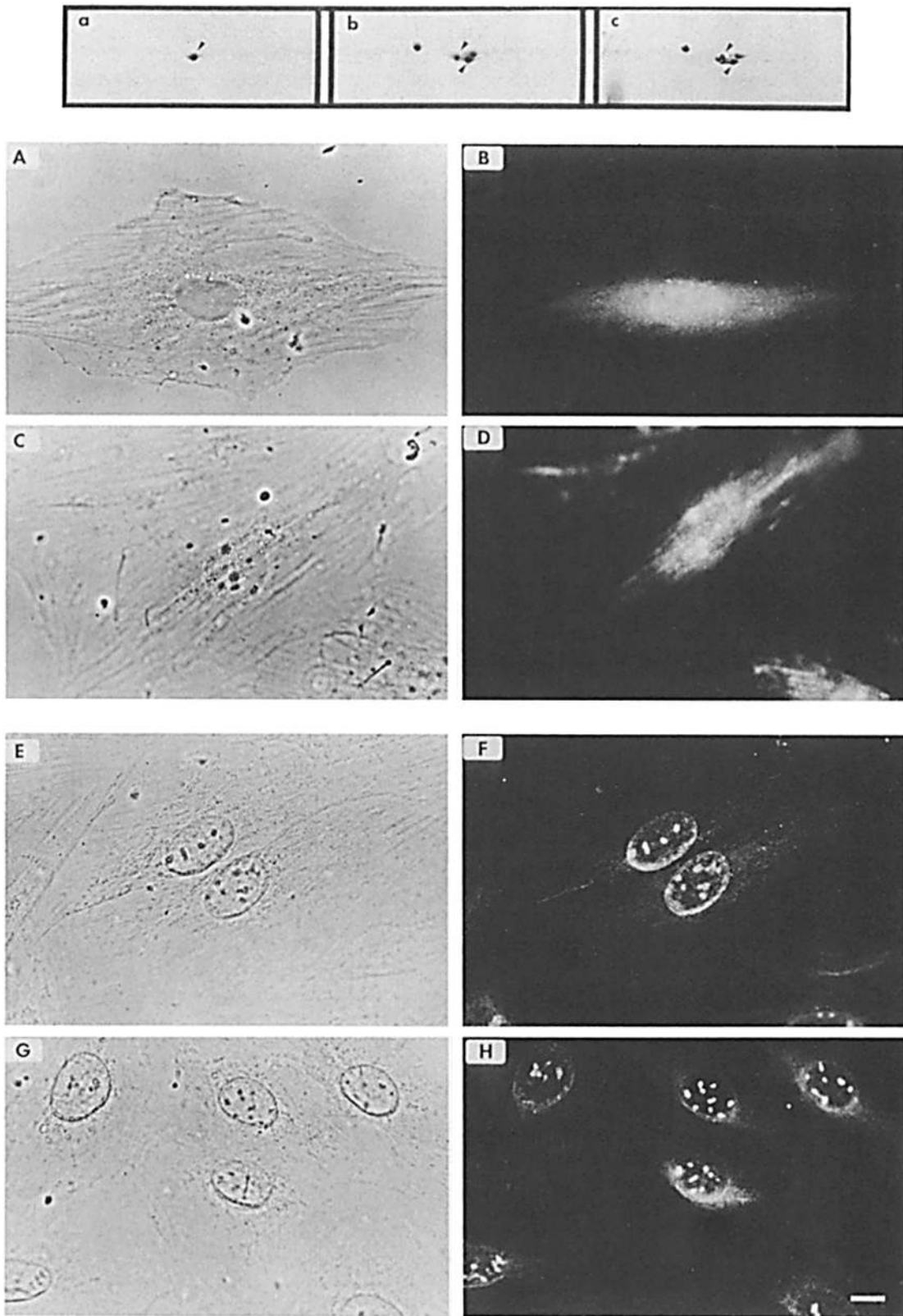
Figure 6. The constitutive 73-kD stress protein localizes to the nucleus/nucleolus after heat-shock treatment. Rat brain 73-kD stress protein and HeLa 72-, 73-, 75-, and 80-kD stress proteins were purified and analyzed by two-dimensional gel electrophoresis as described in the Materials and Methods. Shown in the top panels are: (*a*) rat brain 73 kD; (*b*) HeLa 72-, 73-, 75-, and 80 kD; and (*c*) a mixture of the rat brain 73-kD and the HeLa proteins. The positions of the 73-kD protein are indicated by a downward pointing arrowhead and the 72-kD protein is indicated by an upward pointing arrow. The 73-kD protein, purified from rat brain, was conjugated with a chromophore, tetramethylrhodamine isothiocyanate, and microinjected into the cytoplasm of rat embryo fibroblasts. The injected cells were incubated at either 37°C (*A* and *B*) or at 43°C for 90 min (*C* and *D*) and the distribution of the injected protein visualized by fluorescence microscopy. REF-52 cells, growing on glass coverslips, were heat-shock treated at 43°C for 90 min either in the absence or presence of cycloheximide. After heat shock, the cells were fixed and analyzed using a polyclonal serum that recognizes all of the members of the heat-shock 70-kD family of proteins (see text). Shown in *E* and *F* are the cells heat-shock treated in the absence of cycloheximide and in *G* and *H* the cells heat-shock treated in the presence of cycloheximide. Bar, 10 μm.
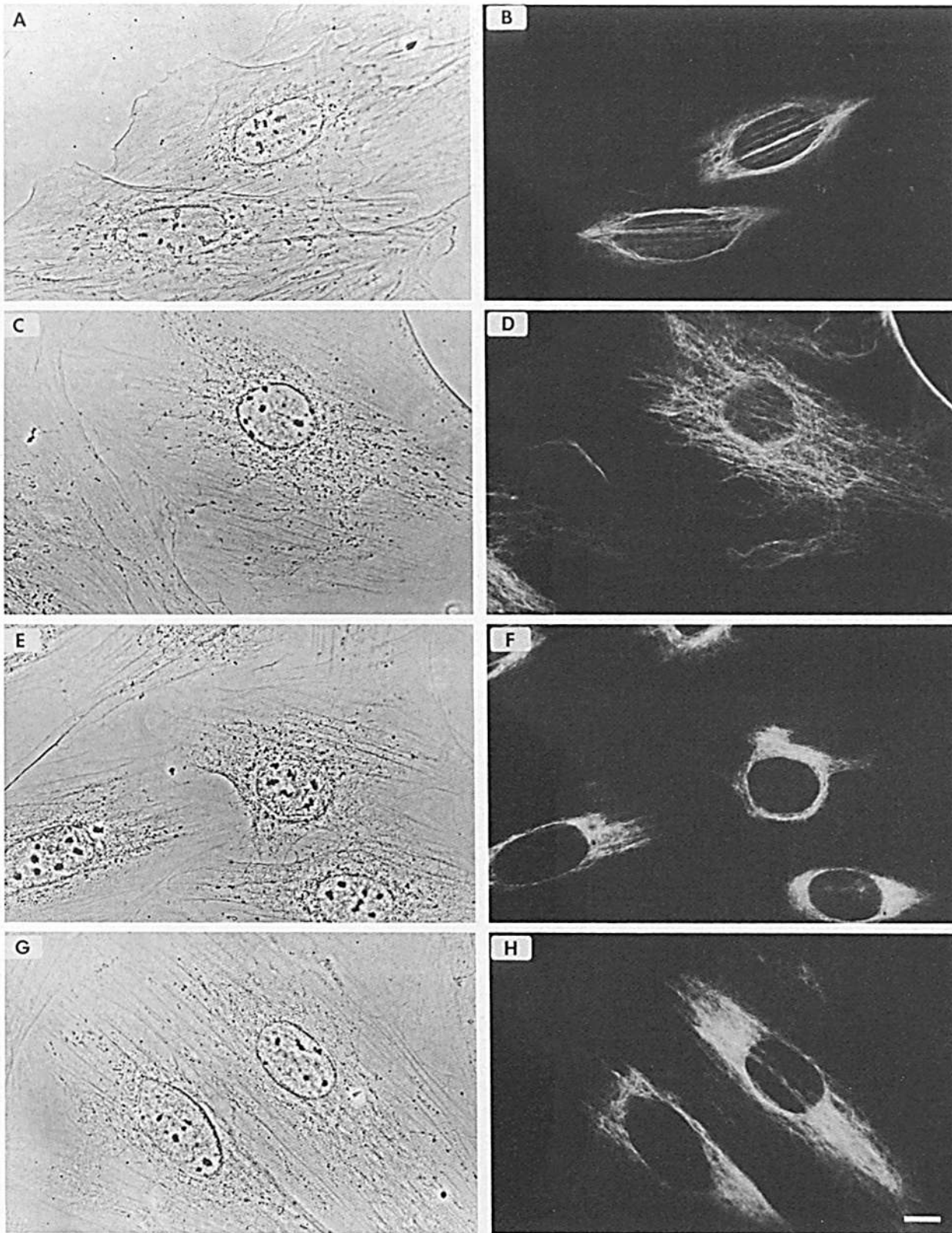
Figure 7. Protection of intermediate filament integrity in thermotolerant cells after a second heat-shock treatment. REF-52 cells, growing on glass coverslips, were made thermotolerant by a 43°C/90-min heat-shock treatment and subsequent recovery at 37°C for 12 h. The tolerant cells along with nontolerant cells (i.e., no prior heat-shock treatment) were then subjected to either a 43°C/90-min heat-shock or a 45°C/30-min heat-shock treatment. The cells were fixed and analyzed for the distribution of the vimentin-containing intermediate filaments using a monoclonal antibody specific for vimentin. Shown in *A*, *C*, *E*, and *G* are the phase-contrast micrographs and in *B*, *D*, *F*, and *H*, the corresponding fluorescent micrographs. (*A* and *B*) Nontolerant cells, 43°C/90-min heat shock. (*C* and *D*) Tolerant cells, 43°C/90-min heat shock. (*E* and *F*) Nontolerant cells, 45°C/30-min heat shock. (*G* and *H*) Tolerant cells, 45°C/30-min heat shock. Bar, 10 µm.
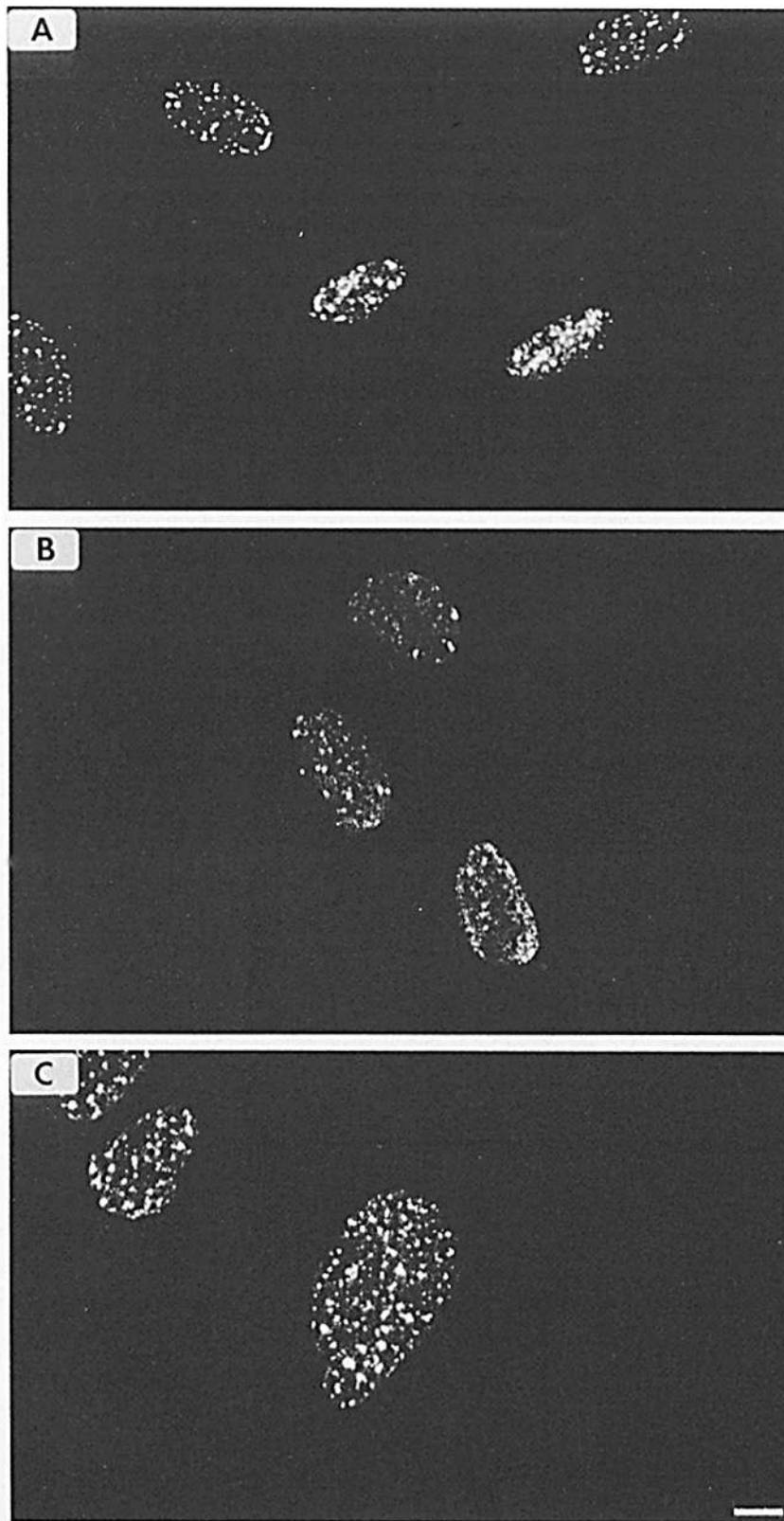
Figure 8. Protection of nuclear snRNP staining in thermotolerant rat fibroblasts. REF-52 cells, growing on glass coverslips, were made thermotolerant as described in Fig. 7. The tolerant cells along with nontolerant cells were subjected to a 45°C/30-min heat-shock treatment, returned to 37°C for 2 h, and then fixed and analyzed for the distribution of snRNPs using a mouse monoclonal antibody. Shown are the fluorescent micrographs only. (*A*) 37°C cells. (*B*) Nontolerant cells after a 45°C/30-min heat-shock treatment and recovery at 37°C for 2 h. (*C*) Tolerant cells after a 45°C/30-min heat-shock treatment and recovery at 37°C for 2 h. Bar, 10 μm.

ter heat-shock treatment. Moreover, owing to the studies of Yost and Lindquist (43), we compared the distribution of the snRNPs in both nontolerant and tolerant cells after a severe 45°C/30-min heat-shock treatment. Similar to what others have shown (20, 24), a distinct "speckled-like" pattern of

staining was observed in rat fibroblasts incubated at 37°C (Fig. 8 *A*). After a 45°C/30-min heat-shock treatment, this pattern of snRNP staining appeared different (Fig. 8 *B*). For example, in some of the cells the overall extent of staining appeared either diminished or in some cases somewhat

diffuse. In cells first made tolerant and then exposed to the same heat-shock treatment, there was little or no change in the pattern of the snRNP staining. Rather, the well-defined speckled pattern of staining, in general, was not perturbed. While these changes are admittedly subtle, over the course of many experiments we have consistently observed the tolerant cells to exhibit considerably less perturbation of snRNP staining as compared with the nontolerant cells after the heat-shock treatment.

Discussion

The immunological studies presented here, complementing the biochemical studies presented in the accompanying paper, further characterize and define properties inherent to the thermotolerant cell. These studies, in summary, indicate that a major hallmark of the tolerant cell is the faster kinetics of both the synthesis and repression of the stress proteins and their accelerated intracellular redistribution after a second and more severe heat-shock treatment. In addition, acquisition of the tolerant state results in the prevention of other cellular perturbations, which normally occur in nontolerant cells after heat shock. While these studies present interesting phenomenology, more importantly they point us in a direction by which to directly test the possible function of the stress proteins and, in particular, the role of the complex 70-kD stress protein family in the development of the thermotolerant state.

HeLa cells or rat fibroblasts made thermotolerant exhibited markedly faster kinetics with respect to the changing intracellular distribution of the major and most highly induced stress protein, 72 kD, as compared with the nontolerant cells after a severe heat-shock treatment. For example, entry of 72-kD into the nucleolus and its subsequent exit occurred faster in the tolerant cells. This redistribution of the protein from the nucleus and cytoplasm into the nucleolus appeared independent of active protein synthesis. It is still unclear, however, why the nucleolus acts as a "sink" for 72 kD even after multiple heat-shock treatments. We and others have suggested that the protein might serve in the repair and/or recovery of nucleolar function known to be perturbed after heat-shock treatment (14, 34, 39). One might predict that in tolerant cells the accelerated entry of 72 kD into the nucleolus would result in a faster return of normal nucleolar function as compared with the nontolerant cell. Consistent with this idea is the fact that cells containing high levels of the protein (via transfection of the gene and its constitutive expression) result in an acceleration of normal nucleolar morphology after heat shock (23). Similarly, in plants acquisition of the thermotolerant state renders a protective effect of nucleolar function, as assayed by rRNA processing and ribosomal assembly, after a second heat-shock treatment (21).

Similar to the highly stress-induced 72 kD, the related, constitutive 73-kD stress protein was also observed to redistribute from the nucleus and cytoplasm into the nucleolus as a function of the heat-shock treatment. An obvious question arises then as to why are there two major forms of the 70-kD stress proteins, one constitutive (73 kD) and one highly inducible (72 kD)? Moreover, do these two related, but in fact distinct, proteins serve the same or different functions in the cell? With respect to the constitutive form of the protein, a number of studies have implicated its involvement in a vari-

ety of different cellular phenomena. For example, a portion of 73 kD is observed to co-purify with microtubules prepared from either brain or tissue culture cells by repeated cycles of assembly/disassembly, and therefore has been referred to as a microtubule-associated protein (16, 31, 36). A portion of 73 kD has also been observed to fractionate with the Triton-insoluble intermediate filament enriched cytoskeleton, and accordingly has been referred to as an intermediate filament–associated protein (18, 19, 30). Finally, studies by Chappell et al. (3) and Ungewickel (28) have reported the isolation of a 70-kD protein that mediates the uncoating of clathrin coated vesicles and appears homologous to the constitutive 73-kD stress protein. Suffice it to say then, that there exists some confusion with respect to what function this protein serves in the cell, be it under normal conditions or in the cell experiencing stress. However, it should be noted that the 70-kD stress proteins are a family of proteins consisting of multiple isoforms (and multiple genes [40]) and therefore may have evolved to satisfy a variety of functions, presumably through some common denominator, depending upon the physiological state of the cell.

With respect to its possible role in the stress response, we have demonstrated, for the first time, the redistribution of 73 kD as a function of heat-shock treatment. As was mentioned above, 73 kD, like 72 kD, showed a relocalization from the cytoplasm and nucleus into the nucleolus after the heat-shock treatment. We were not too surprised by this result considering the high degree of homology of 73 kD with that of the highly inducible 72 kD. For example, immunological, biochemical, and DNA sequence analyses have all demonstrated the two proteins to be highly related but nevertheless distinct gene products (9, 10, 17, 22, 29, 33, 42). To date, we have not been able to define any biochemical differences between the two proteins. Both are observed to co-purify throughout various chromatographic steps, bind ATP, and co-precipitate after immunoprecipitation under nondenaturing conditions; and a considerable portion of both proteins are observed to copurify with isolated nuclei after heat-shock treatment (33, 35; unpublished observations). Hence, it seems plausible that the two proteins might serve similar functions in the cell. If so, this might account for some of the controversy regarding the role of the heat-shock proteins in the acquisition of thermotolerance. For example, a number of studies have reported that the presence of protein synthesis inhibitors will prevent the development of the thermotolerant state while other, more recent, studies have described the acquisition of thermotolerance in cells heat-shock treated in the presence of such inhibitors (8, 15, 25; 11, 41). However, in most of these studies the role of the constitutive stress proteins, many of which are rather abundant in the normal cell, has often been ignored. Might these constitutive proteins serve to protect the cell with their protective effect first requiring a priming event, such as that provided by mild heat-shock treatment? In light of what we have observed for the constitutive 73 kD, i.e., its changing intracellular redistribution as a function of heat-shock treatment, we think it likely that the constitutive 73 kD, and perhaps some of the other constitutive stress proteins, may in fact contribute to the acquisition of the thermotolerant state.

Lastly, our studies indicate that other perturbations that occur in cells as a consequence of heat-shock treatment can be minimized (or prevented) by first making the cells ther-

motolerant. For example, we have shown that the heat-induced collapse of the intermediate filament cytoskeleton can be prevented (or minimized) by first making the cells thermotolerant. Even when there did occur perturbations in the intermediate filament network after heat shock, the tolerant cells always exhibited a faster return of normal intermediate filament integrity as compared with the nontolerant cells. Another system protected was the so-called snRNP complexes involved in hnRNA processing. Although the effects were more subtle than those observed for the intermediate filaments, we consistently observed a disruption of the typical nuclear, speckled staining patterns of the snRNP after a severe heat-shock treatment. In contrast, such a perturbation in snRNP distribution was less evident in the thermotolerant cells. These heat-induced changes in the distribution of snRNP staining are paralled by the observations of Yost and Lindquist demonstrating an inhibition of RNA splicing in *Drosophila* cells after a severe heat-shock treatment (43). However, *Drosophila* cells first made thermotolerant exhibited markedly less inhibition of RNA processing after the second heat-shock treatment. Again, a role for the heat-shock proteins was suggested since the inclusion of protein synthesis inhibitors during the development phase of thermotolerance prevented this protection of RNA splicing after the second heat-shock treatment.

In conclusion, our studies have provided new parameters that define the thermotolerant state. In addition to the protection of heat-induced lethality and the prevention of heat-induced developmental abnormalities, the acquisition of the thermotolerant state has manifestations at the level of protein synthesis, in the kinetics of stress protein intracellular redistribution, and the protection of cellular systems normally perturbed after heat-shock treatment. In addition, using the electron microscope, we find that many of the morphological lesions that occur in cells after heat-shock treatment (38) are no longer apparent (or repaired faster) if the cells are first made thermotolerant. Finally, similar to the situation in *Drosophila* pupae (1), we have observed that the low molecular mass 28-kD stress protein of mammalian cells remains in the insoluble fraction of cells after a severe heat-shock treatment while in the heat-stressed thermotolerant cell most of 28 kD remains in the soluble phase (Arrigo, A.-P., J. Suhan, and W. Welch, manuscript in preparation). Hence, it seems reasonable that thermotolerance and increased cell survival are manifested, in part, by the ability of the tolerant cell to prevent and/or quickly repair heat-induced lesions, thereby allowing for the continuation of normal cellular activities that are typically inhibited by high temperatures. Although correlative data indicate a role for the stress proteins in the acquisition of such tolerance, still no definitive data exist demonstrating unequivocably their participation. However, having now established parameters inherent to the thermotolerant cell (distinct from that of cell survival), both a genetic and biochemical approach can be pursued to delineate the role of the stress proteins in the acquisition of the thermotolerant state.

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