Association between the Mammalian 110,000-dalton Heat-Shock Protein and Nucleoli

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ABSTRACT A rabbit antiserum has been prepared using as antigen the 110,000-dalton mammalian heat-shock protein. This protein was purified for injection by two-dimensional PAGE of heat-shocked Chinese hamster ovary cells. Characterization by immunoautoradiography and immunoprecipitation reveals that the antiserum is specific for the 110,000-dalton protein. Both techniques also reveal that the protein against which the antiserum is directed is induced by heat shock. Indirect immunofluorescence shows that the antigen is primarily localized at or near the nucleolus in cultured cells and numerous murine tissues. Treatment of cultured cells with deoxyribonuclease destroys the organization of staining within the nucleus while ribonuclease appears to completely release the antigen from the nucleus. A binding of the antiserum to cytoplasmic structures is also observed by immunofluorescence. This association with nucleoli may have implications in the regulatory aspects of the heat-shock response.

A hyperthermic challenge to a cell can result in a reorganization at translational and transcriptional levels (7, 23) and at the same time protect the cell from additional applications of the same stress (8), probably through the intervention of heat-shock proteins (14, 15, 17, 18, 27, 28). Despite the implications of this, it is important to recognize that the function of heat-shock proteins remains a mystery. However, reports indicate that at least some heat-shock proteins my localize in the nucleus (2, 3, 13, 16, 31, 32), suggesting a direct role in the regulatory changes associated with this response.

To learn more regarding the function of heat-shock proteins and their role in regulation and protection, we investigated mammalian heat-shock proteins using an immunologic approach. We report here on the characterization of an antiserum against the major 110,000-dalton mammalian heat-shock protein (HSP 110).[1] It is demonstrated by indirect immunofluorescence that this protein is localized at or near the nucleolus and is released from the nucleus by treatment with ribonuclease. This suggests that aspects of nucleolar function may be important in the protective and regulatory properties of the heat-shock response.

MATERIALS AND METHODS

Cells: CHO cells (Chinese hamster ovary fibroblasts) obtained originally from Los Alamos National Laboratory were maintained as monolayer cultures at 37°C in Hams F-10 Media (Gibco Laboratories, Grand Island, NY) supplemented with 15% heat-inactivated newborn calf serum. In heat-shock experiments, flasks were immersed horizontally into a constant temperature water bath (Haake FK-2) for the indicated times at 45°C ± 0.1°C. 10T½ mouse embryo fibroblasts were originally obtained from Dr. John Bertram of this Institute. These cells are grown in Eagles basal medium with Earle's salts (Gibco Laboratories) supplemented with 10% heat-inactivated fetal calf serum. Frozen sections were obtained from tissues of BALB/c CR mice supplied by the West Seneca Laboratories of this Institute.

Preparation of Antigen and Immunoautoradiography: Heat-shock proteins were identified by autoradiography of two-dimensional O'Farrel gels, as previously modified and described (10, 19), by applying a [^{35}S]methionine pulse during the peak induction period following a 12 min/45°C heat shock as discussed previously (26). This procedure focuses proteins in pH range of approximately 4–7.

In this study, samples were treated with ribonuclease A (Sigma Chemical Co., St. Louis, MO) before isoelectric focusing. The major heat-shock protein identified at 110,000 daltons was cut from Coomassie-Blue-stained two-dimensional gels with a razor blade and brought to neutral pH in 0.15 M sodium phosphate. Protein from 20 gels (per injection) was homogenized in a motor driven Teflon and glass homogenizer (Potter-Elvehjem) and emulsified with Freund's complete adjuvant for injection as previously described (9), except that Borditella pertussis vaccine was included with the first antigen injection as described (30). Booster injections contained incomplete adjuvant only and were administered at 4-wk intervals. The rabbit was injected subcutaneously (and in some cases intradermally) at approximately 20 sites per immunization. Blood was taken 8 d after each injection and before the first injection. Serum from the third bleed was used in this study. Serum from bleeds 4 and 5 produced results similar to those presented here for bleed 3. The IgG fraction was partially purified with 50% ammonium sulfate and dialysed against buffer as previously described (9). The antiserum was divided into aliquots, quick-frozen in ethanol dry ice and stored at −80°C. Immunoautoradiography (5) was performed as described previously (8, 19), except that only 1 μCi of ^{125}I-Protein A (Amersham Corp., Arlington Heights, IL) was used. Exposure times with a DuPont cronex lighting-plus intensifying screen (DuPont Instruments, Wilmington, DE) for Fig. 1 *b* was 96 h and 48 h in Fig. 1 *d*.

[1] *Abbreviations used in this paper:* CHO, Chinese hamster ovary; HSP 110, 110,000-dalton heat-shock protein.

Immunoprecipitation: For immunoprecipitation studies radiolabeled ([^{35}S]methionine) heat-shocked or control CHO cells were prepared (28). 10^7 cells were resuspended in 1 ml of lysis buffer (1% Triton X-100 [Sigma Chemical Co.]; 1% Nonidet P-40 [Sigma Chemical Co.]; 0.1% SDS [Accurate Chemical and Scientific Corp., Westbury, NY]; 150 mM sodium chloride; 5 mM Tris-Cl, pH 7.2; 1 mM tosyl-L-arginine methyl ester [Sigma Chemical Co.]). Cells were then sonicated and centrifuged at 15,000 *g* for 30 min. 100 μl of the supernatant was incubated with 100 μl of a 1:10 dilution of the partially purified serum at 25°C for 30 min. 100 μl of formalin-treated 10% *Staphylococcus aureus* (Pansorbin, Calbiochem-Behring Corp., San Diego, CA) (6) suspension was then added for 20 min. The antigen-antibody-*Staphylococcus* complex was then pelleted at 1,000 *g* for 5 min and washed three times with lysis buffer following which the pellet was resuspended in SDS sample buffer and run on a gel as previously described (28).

Immunofluorescence: For immunofluorescence experiments, cells were seeded on glass coverslips and incubated in normal growth media for at least 24 h. Petri dishes containing coverslips were then either heat shocked or used as controls and postincubated as required. Coverslips containing cells were rinsed in growth media without serum and fixed in 2% formaldehyde in Hanks' balanced salt solution for 15 min without calcium and magnesium. They were then rinsed with buffer (130 mM sodium chloride, 5 mM sodium azide, 5 mM magnesium chloride, 1 mM ethyleneglycol-*bis*-N,N^1 tetraacetic acid, 10 mM Tris-Cl, pH 7.5) and then immersed in the buffer containing 0.5% Triton X-100 for 20 min. The coverslips were then rinsed in a buffer containing 0.1% bovine serum albumin, pH 8.2, as previously described (35). Coverslips were then removed and a 1:40 dilution of antisera was added to each and incubated at 37°C for 45 min. They were then rinsed again in buffer following which a 1:150 dilution of fluorescein conjugated goat anti-rabbit antibody (Miles-Yeda, Rehovot, Israel) was added and incubated at 37°C for 30 min. Coverslips were again rinsed and mounted in Elvanol (21) for examination using a Zeiss photomicroscope II. For digestion experiments with deoxyribonuclease (Sigma Chemical Co.) or ribonuclease A (Sigma Chemical Co.), cells were fixed in 95% ethanol and briefly permeabilized in the Triton X-100 buffer described above. Following this the cells were digested with the enzyme (50 μg/ml) for 45 min at 37°C, rinsed, and reacted with the antiserum for immunofluorescence. Frozen sections of mouse tissues were prepared using an IEC Cryotome model CTD-1 (IEC Electronics Corp., Newark, NY).

Immunoadsorption: Adsorptions of immunoprecipitation and immunofluorescence was performed using tropomyosin as a control protein and with the HSP 110. In these studies the proteins were cut from one-dimensional gels following which the protein was eluted in a buffer containing 1% SDS. The resultant solution was then dialysed for 3 d against 0.1 M phosphate buffer and

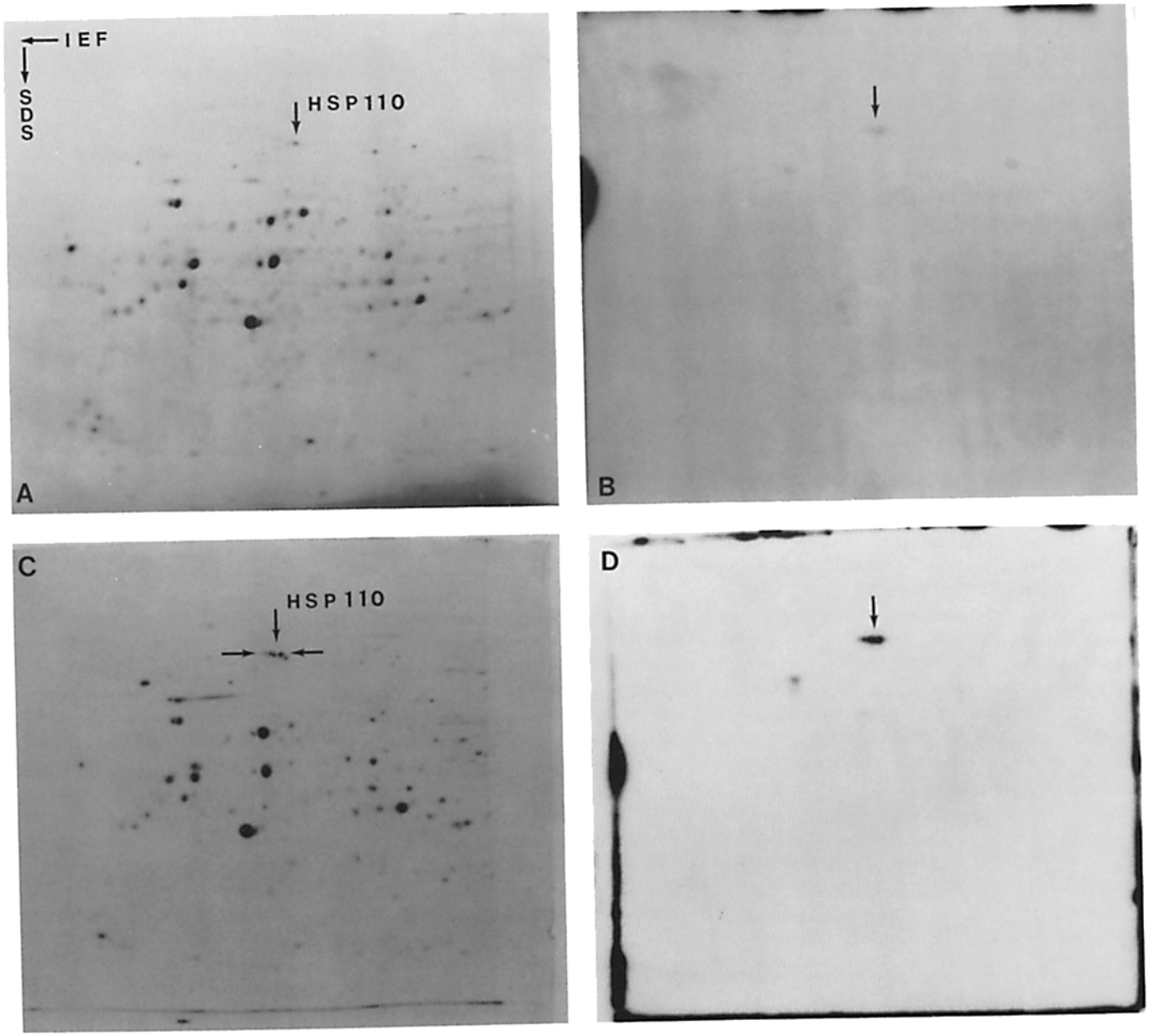

FIGURE 1 Two-dimensional immunoautoradiographic characterizations of anti-HSP 110, *A* and *C* are Coomassie-Blue-stained gels of control (*A*) and heat-shocked (C) CHO cells. *B* and *D* are autoradiograms of *A* and *C* respectively after reaction with antiserum and ^{125}I-protein A. The position of HSP 110 is indicated by arrow. Equal amounts of protein were loaded in *A* and *C*.

for one additional day against distilled water and lyophilized. Protein at a concentration of ~1 mg/ml was then mixed and incubated with the antisera before adding radiolabeled heat-shocked cell extract and subsequent immunoprecipitation, or before incubation of the antisera with cells for immunofluorescence. For immunofluorescence the protein extract-antibody mixture was incubated at 37°C for 60 min following which the specimens were precipitated at 175,000 *g* for 10 min in a Beckman air-fuge.

RESULTS

CHO cell heat-shock proteins have previously been identified by a two-dimensional gel analyses of [^{35}S]methionine-labeled cells before and after heat shock (26). The induced proteins can also be visualized by direct Coomassie Blue staining of gels of control and heat-shocked cells (Fig. 1, *A* and *C*). For preparation of the immunogen, the major heat-inducible protein of 110,000 daltons (delineated by arrows, Fig. 1 *C*) was excised directly from two-dimensional gels of heat-shocked cells to obtain maximum purity. A positive antiserum was detected following the third injection and yielded a single precipitin line after double immunodiffusion with urea extracts of CHO cells. The specificity of this antiserum was determined by immunoautoradiography, a procedure by which antibodies are used to detect antigens in polyacrylamide gels (5). Fig. 1, *B* and *D* show the resulting immunoautoradiograms following reaction of gels shown in Fig. 1, *A* and *C* with this antiserum and ^{125}I-protein A. The antiserum binds with the 110,000-dalton heat-inducible protein used as the immunogen. The reaction of the antiserum with the immunogen in Fig. 1 *D* appears as three closely associated isoelectric variants, all of which are heat-inducible. Upon over exposure of the autoradiogram, a faint reaction of the antiserum for a 92,000-dalton polypeptide is also observed. This has been identified as a probable proteolytic fragment of the immunogen as indicated by the enhanced intensity of this spot with concomitant reduction of the 110,000-dalton polypeptide when the specimen is prepared in the absence of protease inhibitors.

We also tested the antiserum by immunoprecipitation using total protein extracts of CHO cells shocked at 45°C for 12 min, incubated for 8 h at 37°C and then labeled for 1 h (the time of peak induction [27]). Fig. 2, lane *A*, is an autoradiogram of the immunoprecipitation of the antiserum with *S. aureus*. The precipitation of a 110,000-dalton protein is evident. A 68,000-dalton protein is also co-precipitated. The precipitation of both proteins is significantly enhanced in heat-shocked preparations relative to control samples (data not shown). To examine the basis for this co-precipitation, we prepared a control protein (tropomyosin) without radiolabel as a lyophilized powder. When immunoprecipitation is performed in the presence of an excess of this unlabeled protein, precipitation of the 68,000-dalton protein appears diminished while precipitation of the 110,000-dalton protein appears unchanged (Fig. 2, lane *B*). Lane *C* in Fig. 2 is the identical study as shown in lane *B*, except unlabeled HSP 110 is eluted as just described and added to the antiserum. The preimmune serum also precipitates a 68,000-dalton peptide, although to a reduced degree when compared with the immune serum. The other lower molecular weight bands observed in Fig. 2, lane *A*, are also precipitated, with an equal intensity by the preimmune serum. This data suggests that in part, the co-precipitation of the 68,000-dalton protein is a result of nonspecific binding, although other mechanisms must also be considered. A similar co-precipitation of a 68,000–70,000-dalton protein has been observed by others during the characterization of antiserum against heat-shock proteins (11, 34).

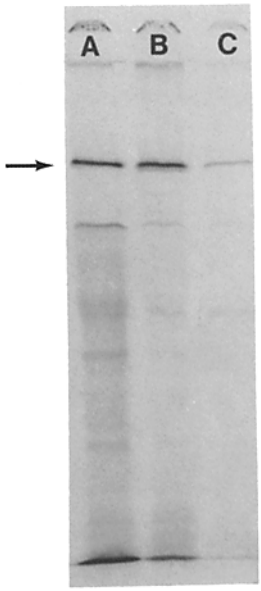

FIGURE 2 Immunoprecipitation of total protein extract of heat shocked and [^{35}S]methionine-labeled CHO cells. Lane *A*: standard immunoprecipitation (arrow indicates HSP 110). Lane *B* indicates precipitation in the presence of an unlabeled nonheat shock protein. Lane *C* indicates precipitation in the presence of unlabeled HSP 110. Intensity of HSP 110 is not diminished in lane *B* although secondary bands are reduced. Equal masses of precipitated protein were loaded.

Indirect immunofluorescence together with phase contrast was used to examine the distribution of the HSP 110 in cultured cells. Fig. 3 shows the immunofluorescence pattern of a subconfluent culture of CHO cells grown at 37°C. Fig. 3 *A* shows phase-contrast and Fig. 3 *B* the fluorescence pattern of staining of the same field of CHO cells. Fig. 3 *C* again shows phase contrast and Fig. 3 *D* fluorescence of another field following absorption of the antiserum with purified HSP 110 before reacting the antiserum with the cells. The specimens represented in Fig. 3, *A* and *B*, are prepared in an identical manner to the adsorption shown in Fig. 3, *C* and *D*, except that HSP 110 was replaced by tropomyosin. By fluorescence a heterogeneous nuclear staining indicative of a nucleolar association is observed. This pattern is seen to precisely correspond to nuclear phase-dense bodies. The prior adsorption of the antiserum with HSP 110 completely abolishes this staining. The preimmune serum as well as serum from bleeds following injections one and two (which were negative by all of the criteria described above) also did not exhibit any specific staining of nuclear structures. An analogous localization is observed in subconfluent C3H 10T½ cells and an absorption study in this cell line indicates as well that the nucleolar fluorescence is completely abolished by preabsorption of the immune serum with purified HSP 110 (data not shown). Fig. 4 shows the immunofluorescence pattern following reaction of the antiserum, with frozen sections of murine liver (Fig. 4 *A*) and brain (Fig. 4 *B*) indicating an analogous staining pattern. All mouse tissues examined indicated a similar nucleolar affinity. Fig. 5 shows the reaction of the antiserum with subconfluent CHO cells before (Fig. 5 *A*) and after treatment with deoxyribonuclease (Fig. 5 *B*), or ribonuclease (Fig. 5 *C*). Deoxyribonuclease destroys the organization within the nucleus while ribonuclease appears to completely release the antigen from the nucleus. The results with ribonuclease suggests that this heat-shock protein may bind RNA directly or indirectly via another protein or protein assembly.

C3H mouse embryo 10T½ cells are preferable for morphologic analysis since they are large, well spread cells, compared

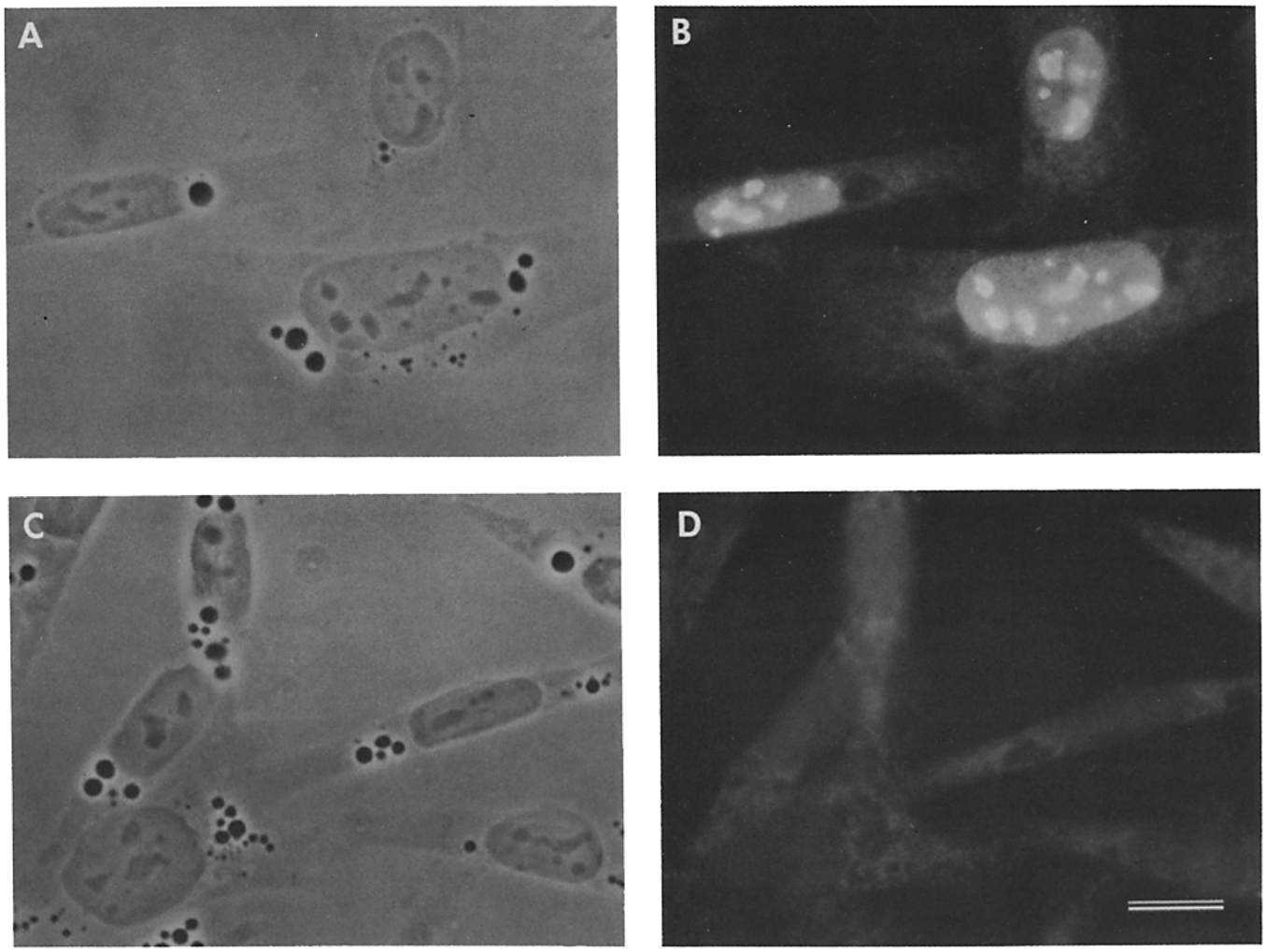

FIGURE 3 Phase-contrast (*A* and *C*) and indirect immunofluorescence of CHO cells reacted with anti-HSP 110 (*B*) and with anti-HSP 110 after prior adsorption with purified HSP 110 (*D*). Bar, 10 μm. × 1,390.

to the smaller and more rounded CHO cells. These cells are also highly contact inhibited. Fig. 6 shows phase-contrast and fluorescent (Fig. 6, *A* and *B*, respectively) studies of 10T½ nuclei in 48-h postconfluent cells. The studies indicate that in these quiescent cells the strongest nuclear fluorescence appears as a cap in contact with a phase-dense structure. The phase-dense structure itself apears to exclude the antigen in some cases. Not all phase-dense bodies reacted with the antiserum. When similar confluent cells are heat shocked (45°C/12 minutes) and postincubated for 8 h (37°C), at which time the maximal rate of heat-shock protein synthesis occurs (29), this segregation is lost and the phase-dense (Fig. 6*C*) and fluorescent (Fig. 6*D*) structures now coincide. A similar coincidence also occurs in control log phase cells, suggesting that the segregation of the two bodies may associate with nuclear inactivity. The heat-shock experiment shown in Fig. 6, *C* and *D*, also appears to result in the secondary staining of some cytoplasmic structures.

DISCUSSION

Kelly and Schlesinger (11) have reported on the preparation of three antisera against major avian heat-shock proteins of 24,000, 70,000, and 89,000 daltons. These authors report that the 70,000- and 89,000-dalton heat-shock protein antibodies are widely cross-reactive in several organisms, and it is likely that these proteins correspond to the major heat-shock proteins of CHO cells at 68,000 and 89,000 daltons (on which we have reported previously). In addition, we observed in this system the strong induction of a HSP 110 and have shown that the expression of this protein correlates well with the expression of thermotolerance (27, 28). One-dimensional proteolytic mapping shows that HSP 110 shares no homology with the other major CHO cell heat-shock proteins at 68,000 and 89,000 daltons (data not shown). The 107,000-dalton heat-shock protein studied by Landry et al. (12) in hepatoma cells and the 112,000-dalton heat-shock protein examined by Tomosovic et al. (29) in mammary adenocarcinoma cells appear analogous to HSP 110 discussed here.

This report concerns the preparation of a rabbit antiserum using as antigen the mammalian HSP 110. This protein was purified for injection by two-dimensional gel electrophoresis of heat-shocked CHO cells and characterization of this antiserum by immunoautoradiography and immunoprecipitation reveals that it is highly specific for a 110,000-dalton protein which is induced by heat shock. A comparison of phase-contrast and indirect immunofluorescence shows that the

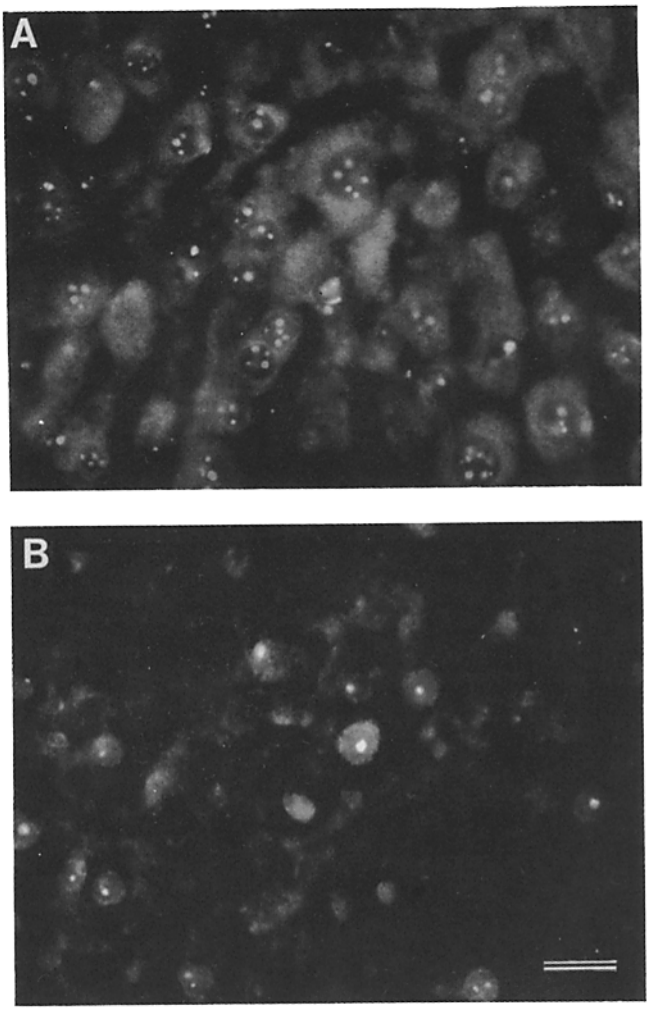

FIGURE 4 Indirect immunofluorescence of frozen sections of murine liver (*A*) and brain (*B*). Bar, 20 μm. × 500.

antigen is localized at or near the nucleolus in cultured cells and a similar observation has been made in several murine tissues. The segregation of antigen from an antigen-excluding phase-dense region in quescent postconfluent nuclei resembles the previously reported segregation of nucleolar fibrillar and granular elements by actinomycin D (25). This segregation was eliminated by heat shock and was also absent in subconfluent actively proliferating cells.

Previous reports have indicated that *Drosophila* heat-shock proteins are transported into the nucleus soon after synthesis (16, 31). Vincent and Tanguay (32) have also shown in *Chironomous tentans* by microdissection that some heat-shock proteins exhibit a broad intranuclear distribution. However, these authors further indicate that some heat-shock proteins are heavily represented in nucleoli. Other studies using cellular fractionation (2) and autoradiography (31) also suggest that some heat-shock proteins concentrate in nucleoli of *Drosophila melanogaster* cells. While an HSP 110 has not been observed in lower organisms, a mammalian nucleolar protein of ~110,000 daltons and an isoelectric point closely resembling HSP 110 has been studied by others (4, 22). Although a good agreement between the expression of the HSP 110 and survival resistance to a second severe heat challenge has been observed (12, 27, 28) and nucleoli have been shown to be particularly sensitive to supranormal temperatures (1, 24, 33), it is still unclear as to whether this protein is directly involved in thermotolerance or expresses an independent function.

It has recently come to our attention that a rabbit antiserum

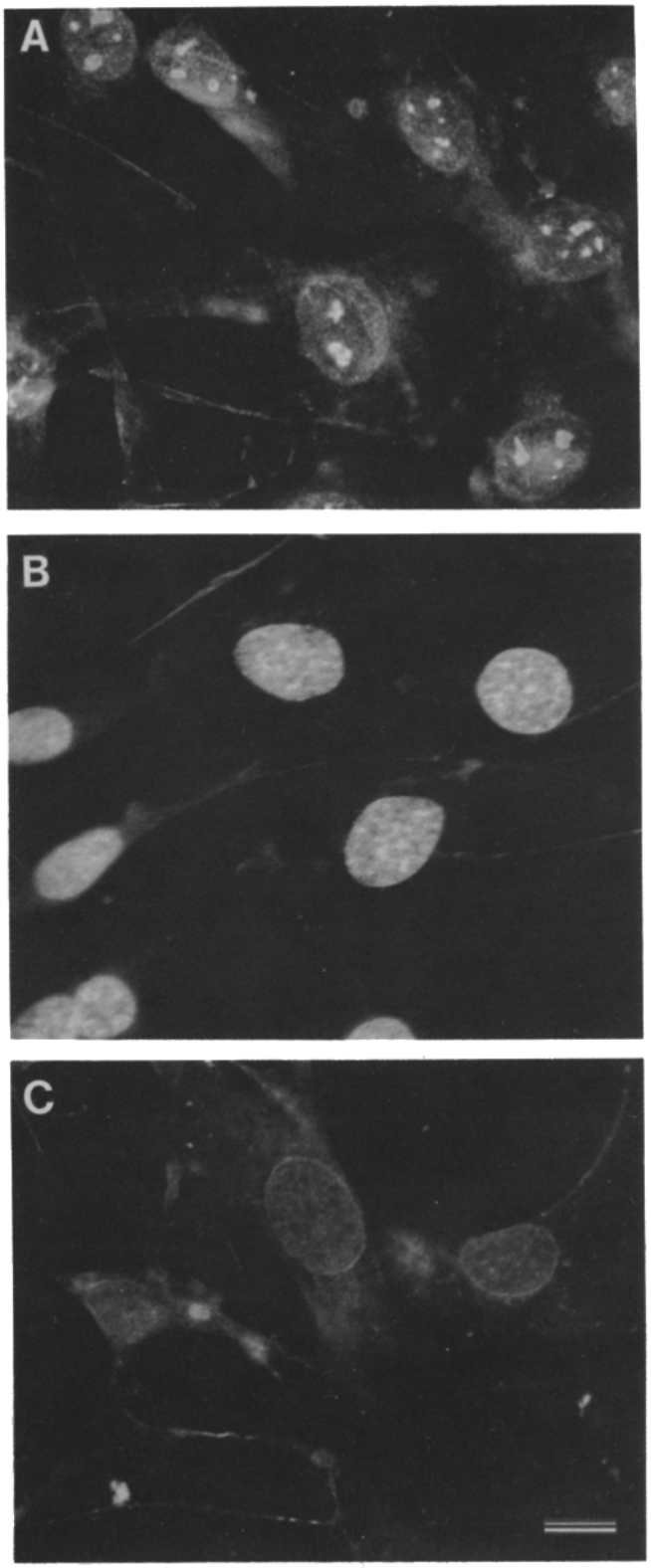

FIGURE 5 Indirect immunofluorescence of CHO cells before (*A*) and after treatment with deoxyribonuclease (*B*), or ribonuclease (*C*). Bar, 10 μm. × 950.

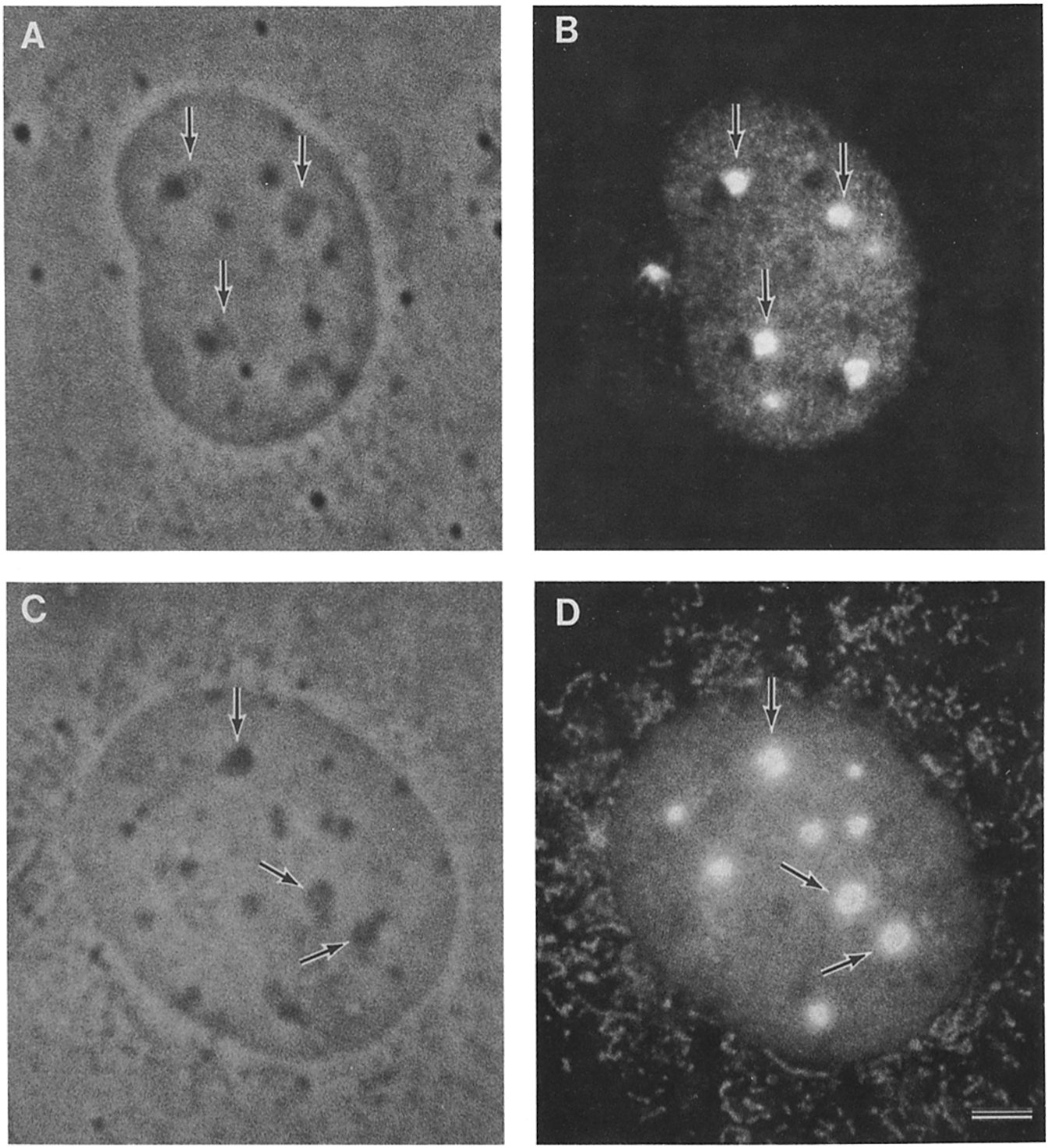

FIGURE 6 Phase-contrast (*A*) and indirect immunofluorescence (*B*) of postconfluent 10T½ cells. Phase-contrast (*C*) and indirect immunofluorescence (*D*) of postconfluent 10T½ cells after heat shock. Arrows indicate some sites where anti-HSP 110 appears to react. Bar, 3 μm. × 3,400.

prepared against a mammalian 72,000-dalton heat-shock protein also exhibits an affinity for nucleoli after heat shock (W. Welch and J. Feramisco, manuscript in preparation).

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