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Thermal response of rat fibroblasts stably transfected with the human 70-kDa heat shock protein-encoding gene

(stable expression/cell survival/thermal resistance/nucleus)

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ABSTRACT **The major heat shock protein hsp70 is synthesized by cells of a wide variety of organisms in response to heat shock or other environmental stresses and is assumed to play an important role in protecting cells from thermal stress. We have tested this hypothesis directly by transfecting a constitutively expressed recombinant human hsp70-encoding gene into rat fibroblasts and examining the relationship between the levels of human hsp70 expressed and thermal resistance of the stably transfected rat cells. Successful transfection and expression of the gene for human hsp70 were characterized by RNA hybridization analysis, two-dimensional gel electrophoresis, and immunoblot analysis. When individual cloned cell lines were exposed to 45°C and their thermal survivals were determined by colony-formation assay, we found that the expression of human hsp70 conferred heat resistance to the rat cells. These results reinforce the hypothesis that hsp70 has a protective function against thermal stress.**

When exposed to nonlethal heat shock, a variety of organisms and cell lines acquire transient resistance to subsequent exposures to elevated temperatures (1, 2). This phenomenon has been termed thermotolerance (3, 4). The mechanism for thermotolerance is not well understood, although several studies suggest that the heat shock proteins (hsps) may be involved in their development (5–7). Qualitative evidence exists for a causal relationship between hsp synthesis and thermotolerance (5–9): (*i*) heat shock induces transiently enhanced synthesis of hsps that correlates temporally with the development of thermotolerance; (*ii*) the persistance of thermotolerance correlates well with the stability of hsps; (*iii*) agents known to induce hsps induce thermotolerance; (*iv*) conversely, agents known to induce thermotolerance induce hsps; and (*v*) stable heat-resistant variant cells express high levels of hsps constitutively.

One notable exception to the correlation summarized above is that amino acid analogues have been shown to induce hsps but not thermotolerance; HA-1 cells treated with such compounds are more sensitive to elevated temperatures (10). This apparent lack of correlation can be attributed, however, to the dysfunction of analogue-substituted hsps: the nonfunctional, analogue-substituted hsps would not be expected to protect cells from thermal stress. In support of this notion, Li and Laszlo (10) found that the incorporation of amino acid analogues into cellular proteins inhibits the development of thermotolerance and that thermotolerant cells or permanently heat-resistant cells are more resistant to the thermal-sensitizing action of amino acid analogues.

Quantitatively, of the many hsps preferentially synthesized after heat shock, the concentration of the 70-kDa heat shock proteins (hsp70s) appears to correlate best with heat resistance, either permanent or transient (6, 8, 9). Recently, good correlation between a 27-kDa heat shock protein (hsp27) and thermal resistance also has been reported (11).

In mammalian cells, three types of experiments have been performed to vary the intracellular concentration of hsp70 and to correlate this change with thermal-stress response. (*i*) Microinjection of affinity-purified anti-hsp70 antibodies into rat cells appeared to prevent the nuclear and nucleolar accumulation of hsp70 after a test heat shock and greatly increased the lethality of a 45°C, 30-min heat treatment (12). (*ii*) The 5′-control region of the hsp70-encoding gene was inserted into a plasmid containing the dihydrofolate reductase gene; this recombinant plasmid was then introduced into a Chinese hamster ovary (CHO) cell line, and a 20,000-fold elevation in its copy number was achieved by selection of cells with methotrexate. These copies of the hsp70 regulatory region presumably competed with the endogenous hsp70-encoding gene(s) for factors that activate hsp70 expression to reduce heat-inducible expression from the intact endogenous gene for hsp70 by at least 90%. It was found that cells containing the amplified regulatory sequences display increased thermosensitivity (13). (*iii*) Human hsp70 microinjected directly into CHO cells increased the resistance of cells to 45°C heating (14).

A technique likely to be superior to microinjection is the expression of a cloned mammalian gene for hsp70 in cells. Transient expression of heterologous hsp70s has been used previously to show that Drosophila hsp70 accelerates the recovery of monkey COS cell nucleoli after heat shock (15) and to dissect the domains of human hsp70 responsible for nucleolar localization and for ATP binding (16). In the present communication, we report the construction of rat cell lines stably expressing different levels of a cloned hsp70-encoding human gene. Studies of these lines provide additional evidence that functional hsp70 improves the survival of mammalian cells during and after thermal stress.

MATERIALS AND METHODS

Construction of Plasmids Expressing Human hsp70. The 2.3-kilobase (kb) *Bam*HI–*Hin*dIII fragment of the human gene for hsp70 (17, 18) was excised from pHHsp70, a plasmid containing the entire transcribed portion of the hsp70 locus plus 5′ sequences required for heat-inducible expression (obtained from R. Morimoto, Northwestern University), by first cutting with *Hin*dIII, making the end blunt with mung bean nuclease, and then cutting with *Bam*HI. This fragment was subcloned between the *Bam*HI and *Sma* I sites in the polylinker region of pSVSP65 [a pSP65 (Promega) derivative

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Abbreviations: hsp, heat shock protein; hsp70, 70-kDa heat shock protein; hsp27, 27-kDa heat shock protein; IEF, isoelectric focusing; mAb, monoclonal antibody.

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containing the *Pvu* II–*Hin*dIII portion of simian virus 40 early promoter and the origin of replication]. In the resulting plasmid, pSV-hsp70, transcription of the human gene for hsp70 in eukaryotic cells is driven by the simian virus 40 early promoter and enhancer and ends with termination signals in the 3′ region of the hsp70 locus.

Cell Cultures and DNA Transfection of Rodent Cells. Rat fibroblast, designated Rat-1, was used. Cultures were grown in Dulbecco's modified medium (DME-H21) supplemented with 10% fetal bovine serum and appropriate antibiotics. Exponentially growing Rat-1 cells were cotransfected with appropriate plasmids containing the human gene for hsp70 and a gene conferring neomycin-resistance by the calcium phosphate precipitation method as described by Graham and van der Eb (19). Neomycin-resistant cells were selected in medium containing G418 (400 μg/ml), and colonies were isolated, trypsinized, and grown to confluent monolayers for further characterization. In parallel experiments, about 100 drug-resistant colonies were pooled, and each pool was subjected to six cycles of heating (twice at 45°C for 60 min and four times at 45°C for 75 min) over a period of 50 days; these resultant pooled populations, after the repeated heat treatments, were named HR-1_p, HR-2_p, HR-3_p, etc. Individual colonies were then isolated from these pooled populations, trypsinized, grown to monolayers, and were designated HR-21, 23, 24, 25, and 26 (derived from HR-2_p) or HR-33, 34, and 35 (derived from HR-3_p). All cell lines used in this study were derived from individual colonies.

For control experiments, Rat-1 cells were transfected with neomycin-resistance gene alone. Neomycin-resistant colonies were pooled and subjected to similar hyperthermic treatments. From six T-75 flasks of pooled populations tested, five had no surviving colonies. The sixth flask contained only a few colonies; they were trypsinized and grown to monolayers, designated as HR-0 cells, and used as controls.

All transfected cell lines were routinely maintained in DME-H21 medium supplemented with 10% fetal bovine serum, Gentamycin (25 μg/ml), and antibiotics G418 (200 μg/ml). For thermal-survival studies, protein labeling, immuno- and Northern (RNA) hybridization analysis, monolayers of cells were plated on day 0 in medium containing no antibiotics G418, grown exponentially, and used on day 3. All cell lines are stable. For example, after 6-mo culture, the levels of human hsp70 in HR-21, HR-24, and HR-35 remained relatively constant, and the degree of thermal resistance was identical to that of the early passages. Plating efficiencies were 80–90%, 80–90%, 50–60%, 25–35%, and 45–65%, and the doubling times were 12, 12, 20, 25, and 25 hr for Rat-1, HR-0, HR-21, HR-24, and HR-35, respectively.

Preparation of RNA and Northern Hybridization Analysis. RNA was prepared according to the procedure of Laski *et al.* (20). RNA (10–20 μg) was denatured with glyoxal (21), size-fractionated on 1% agarose gels, transferred to Hybond -N membrane (Amersham) in 10× SSC (1× SSC is 0.15 M sodium chloride/0.015 M sodium citrate, pH 7), and probed with the 2.3-kb *Bam*HI–*Hin*dIII fragment of the hsp70-encoding human gene labeled by the random primer method (22). After hybridization, the membranes were washed and autoradiographed with Kodak SB-5 x-ray film and Lightning Plus intensifier screens at −75°C.

Protein Labeling and Gel Electrophoresis. Monolayers of cells were labeled with [^{3}H]leucine (specific activity 120 Ci/mmol; 1 Ci = 37 GBq; Amersham) at concentrations of 200–400 μCi/ml for two-dimensional gels. After labeling at 37°C for 8 hr, cells were washed twice with ice-cold phosphate-buffered saline and lysed in isoelectric focusing (IEF) sample buffer. Two-dimensional gel electrophoresis was done as described (9, 23).

Antibodies, Preparation of Cell Lysates, Immunoblot, and Immunofluorescence. Monoclonal antibody (mAb) against hsp70 used in these studies, C92F3A-5 and N27F3-4, was obtained commercially (Amersham, StressGen, Sidney, BC, Canada). Second antibodies and color reagents (for antibody–enzyme-coupled detection) were purchased from Vector Laboratories. mAb C92F3A-5 reacts only with the inducible form of mammalian hsp70 and is of a similar reactivity to the antibody reported by Welch and Suhan (24). Its reactivity with normal, 37°C, unstressed cells was only seen in human cell lines (24). mAb N27F3-4 is specific against both the constitutive and inducible form of mammalian hsp70.

Because boiling or reduction of samples might destroy epitopes (25), we used a modification of the more gentle procedure of Swok *et al.* (26). Briefly, cells were lysed in Nonidet P-40 lysis buffer (50 mM Tris·HCl, pH 6.8/150 mM NaCl/5 mM EDTA/0.5% Nonidet P-40/1 mM phenyl methylsulfonyl fluoride) at 2×10^6 cells per ml, incubated at 4°C for 30 min, and centrifuged at 4°C for 20 min. Cell lysates were brought to a final concentration of 2% SDS/5% glycerol/0.001% bromophenol blue and incubated at room temperature for 15 min before loading onto gels. Immunoblotting was done as described by Towbin *et al.* (27). Immunofluorescence studies were done with the protocols developed by Welch and coworkers (28, 29).

Flow Cytometric Analysis of hsp70. Fluorescein isothiocyanate-conjugated anti-hsp70 antibodies and flow cytometry were used to quantify the level of hsp70. Cells were fixed in 70% ethanol and stained with anti-hsp70 mAbs (1:500 dilution). Fluorescein isothiocyanate-conjugated anti-mouse immunoglobulin (Amersham, 1:100 dilution) was then added to the cell samples, and immunofluorescence was analyzed by flow cytometry (Becton Dickinson, FACS 440).

Heating and Cell Survival. Monolayers of cells were heated in hot water baths in specially designed incubators (6, 8). Thermal-survival studies were done as described (6, 8). Surviving fractions were always normalized by the plating efficiency. All experiments were done at least three times and yielded consistent results.

RESULTS

Human hsp70 Can Be Stably Expressed in Transfected Rat-1 Cells. Expression of human hsp70-encoding gene in transfected rat cells was first verified by Northern hybridization analysis. In Fig. 1, cytoplasmic RNA from Rat-1 cells, Rat-1

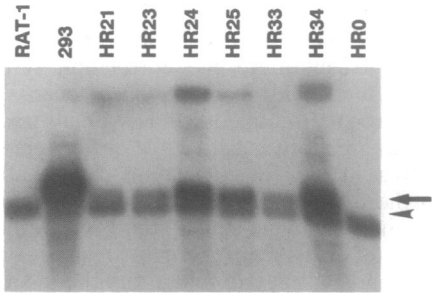

FIG. 1. Northern hybridization analysis of cellular RNA from Rat-1 cells and Rat-1 cells transfected with the human gene for hsp70. Northern blot of cytoplasmic RNA was probed with the human hsp70 gene. Successful expression of the human gene for hsp70 yields mature RNA of ≈2.3 kb. Rat-1: control wild type; HR-21, -23, -24, -25, -33, and -34: different lines derived from Rat-1 cells transfected with human hsp70-encoding gene; HR-0: Rat-1 cells transfected with only neomycin-resistance gene; 293: human cell line 293. The endogenous rat hsp70 message is indicated by an arrowhead. The human hsp70 message (indicated by an arrow) is only present in cells transfected with human hsp70 gene. Human cell line 293 is shown for comparison.

cells transfected with only the neomycin-resistance gene (HR-0), and HR clones derived from Rat-1 cells cotransfected with plasmids containing human hsp70 and neomycin-resistance gene were hybridized with a human hsp70 probe. Expression of the human hsp70 gene yields mature RNA of ≈2.3 kb. The human hsp70 mRNA is clearly separated from the endogenous rat hsp70 mRNA, and the human hsp70 message is only present in cells transfected with human hsp70 gene.

The identity and integrity of the human hsp70 expressed in transfected Rat-1 cells were assessed by two-dimensional gel electrophoresis of proteins extracted from transfected cells (e.g., HR-24 clone), human 293 cells, untransfected Rat-1 cells, and Rat-1 cells transfected with only the neomycin-resistance gene (HR-0 cells).

Our analysis of the pattern of hsp70 in Rat-1 cells using two-dimensional gel electrophoresis reveals that the rat hsp70 identified in Rat-1 cells represents at least three major components of ≈73 kDa (rat hsp70-a), ≈72 kDa (rat hsp70-b), and ≈70 kDa (rat hsp70-c). Rat hsp70-b is found in cells under normal growth condition at 37°C, and its expression is enhanced after heat shock (compare Fig. 2 *a* and *b*). Rat hsp70-c is not detectable in Rat-1 cells under normal growth condition at 37°C (Fig. 2*a*) and is induced upon heat shock (Fig. 2*b*). Rat hsp70-a is probably not a heat shock-inducible protein (see Fig. 2 *a* and *b*).

When the protein-synthesis profile of HR-24 cells was examined, an additional protein with molecular mass ≈70 kDa (70_h) was found under normal growth condition at 37°C (Fig. 2*c*) or after 10-min heating at 45°C (Fig. 2*d*). This protein is clearly separated from the constitutive rat hsp70-b and the heat-inducible rat hsp70-c in Rat-1 cells, and its electrophoretic mobility and isoelectric point are identical to those of the human hsp70 in 293 cells (Fig. 3*a*).

All HR-cell lines express human hsp70 constitutively at 37°C. The levels of human hsp70 in various clones are different. Their expression is stable for at least 6 mo in culture. Introducing the human hsp70 gene into Rat-1 cells, as well as the heat-selection procedures employed, did not affect the synthesis profiles of other endogenous rat hsps, e.g., rat hsp27 or other high-molecular-size rat hsps (data not shown).

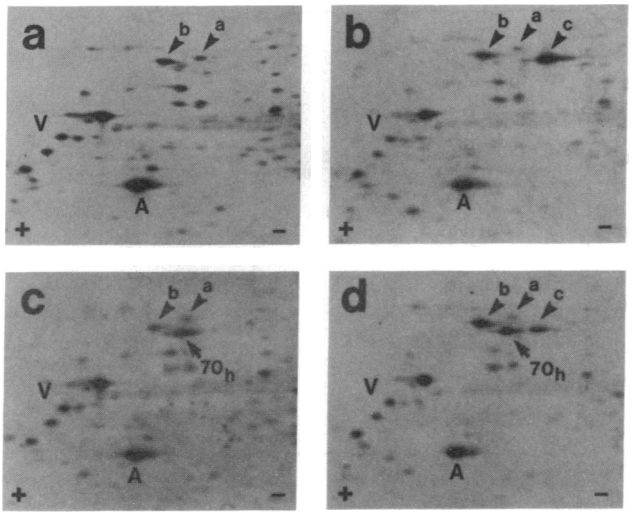

FIG. 2. Autoradiograms showing the successful expression of human hsp70 in transfected rat HR-24 cells. Monolayers of cells were heated at 45°C for 10 min and then labeled with [^{3}H]leucine for 8 hr at 37°C. Cellular proteins from control unheated or heat-shocked cells were analyzed by two-dimensional gel electrophoresis and autoradiography. (*a*) Rat-1 cells at 37°C. (*b*) Rat-1 cells at 45°C for 15 min. (*c*) HR-24 cells at 37°C. (*d*) HR-24 cells at 45°C for 15 min. The endogenous rat hsp70s are indicated by a downward arrowhead. The human hsp70 expressed in HR-24 cells is indicated by an arrow (70_h). Molecular size decreases from top to bottom; isoelectric point increases from left to right. A, actin; V, vimentin.

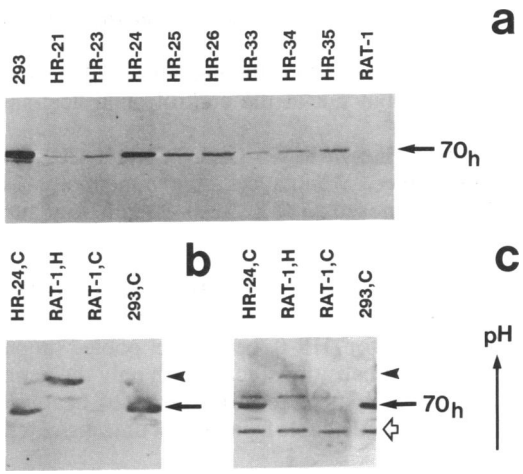

FIG. 3. Immunoblot analysis of cellular proteins from Rat-1 cells and Rat-1 cells transfected with human hsp70 gene. (*a*) Cells, grown at 37°C, were lysed in Nonidet P-40 lysis buffer, omitting boiling in the presence of SDS and treatment of 2-mercaptoethanol. Equal amounts of cellular proteins were separated by gel electrophoresis, transferred to nitrocellulose membrane, and probed with mAb C92F3A-5. 293: human 293 line; Rat-1: wild type Rat-1 cells. HR-21, -23, -24, -25, -26, -33, -34, and -35: clonal lines derived from Rat-1 cells transfected with human hsp70 gene. Human hsp70 (indicated as 70_h) is clearly expressed constitutively in the HR cells but not in wild-type Rat-1 cells. Human 293 line, which constitutively expresses human hsp70, is shown for comparison. Note that mAb C92F3A-5 does not recognize the rat hsp70 in control unstressed Rat-1 cells. (*b* and *c*) Cells grown at 37°C or after a 45°C heat-shock treatment were lysed in IEF lysis buffer. Equal amounts of cellular proteins were separated by IEF slab gel, transferred to nitrocellulose membrane, and probed with mAbs C92F3A-5 (*b*) and N27F3-4 (*c*). Nomenclature is identical to that in *a*. HR-24, C; 293, C; and Rat-1, C: cells grown at 37°C; Rat-1, H: Rat-1 cells heat shocked at 45°C for 15 min and then incubated for 8 hr at 37°C. The human hsp70 gene product is indicated by an arrow. The heat-inducible rat hsp70-c is indicated by a filled arrowhead, and the constitutive rat hsp70-b is indicated by an open arrowhead. Human hsp70 is clearly expressed in the HR-24 cells but not in wild-type control Rat-1 or in heat shocked Rat-1 cells.

Expression of human hsp70 in transfected rat cells was further confirmed by immunoblot analysis using anti-hsp70 mAb C92F3A-5 (specific against the inducible form) and N27F3-4 (specific against the constitutive and inducible form). The immunoblot analysis of cellular proteins separated either on SDS/polyacrylamide gel (Fig. 3*a*) or on IEF gel (Fig. 3 *b* and *c*) demonstrated immunologically that the additional 70-kDa polypeptide in Rat-1 cells transfected with pSV-hsp70 is the human hsp70.

Results depicted in Fig. 3*a* show that human hsp70 is expressed constitutively only in the HR cells but not in the wild-type Rat-1 cells. The level of expression of human hsp70 in the various HR cell lines follows the order: HR-24 (highest amount) ≥ HR-25, HR-26 > HR-35 > HR-23, HR-33, HR-34 > HR-21 (lowest amount).

The mAb C92F3A-5 recognized several isoforms of HeLa hsp72 (the inducible form) and also reacts with the inducible form of hsp70 in heat-shocked rat cells (24). Because human and rat hsp70s were located at almost the same place in a one-dimensional SDS/polyacrylamide gel, it was difficult to exclude the possibility that a rat hsp70-encoding gene was turned on by the process of transfection itself and/or by the heat-selection procedure. We therefore performed additional immunoblot analysis of cellular proteins separated by slab gel IEF (Fig. 3 *b* and *c*). When the results of immunodetection of

human hsp70, inducible rat hsp70, and constitutive rat hsp70 in HR-24, Rat-1, and human 293 cells were compared, again, our data clearly showed that the human hsp70 is expressed only in HR cells but not in the control or in heat-shocked Rat-1 cells.

Expression of Human hsp70 Gene in Rat-1 Cells Confers Thermal Resistance. Monolayers of exponentially growing HR cells were exposed to 45°C for 0–90 min, and survivals were determined by colony-formation assay. Fig. 4*a* shows that when the surviving fraction of HR cells is plotted as a function of heating time at 45°C, HR-24 cells, in which the level of human hsp70 is highest, are most thermal resistant. Thermal survival for HR-0 cells, the pooled population derived from Rat-1 cells transfected with only the neomycin-resistance gene and subjected to a repeated heat-selection protocol, is indistinguishable from that of untransfected Rat-1 cells.

To evaluate the correlation between the level of human hsp70 and thermal sensitivity, the survival of various transfected cell lines after a heat-shock treatment is plotted against their intracellular concentration of human hsp70. Results from one set of experiments are shown in Fig. 4*b*. Here, monolayers of exponentially growing HR cells were exposed to 45°C for 75 min, and survivals were determined. In parallel experiments, the relative levels of human hsp70 expressed in HR cells were measured by flow cytometric analysis with mAb against human hsp70. Clearly, the higher the level of expression of human hsp70, the more resistant the cells were to 45°C thermal stress.

Heat Shock Causes a Transient Import of Cytoplasmic Human hsp70 into the Nucleus/Nucleoli of Transfected Rat-1 Cells. Cellular localization of human hsp70 in transfected Rat-1 cells at normal growth temperature or after heat shock was determined by indirect immunofluorescence assay using specific mAb C92F3A-5. Fig. 5 shows the intracellular distribution of human hsp70 in unheated HR-24 cells and in heated HR-24 cells at 0, 1, 2, 4, and 16 hr after a 45°C, 10-min heat-shock treatment. At 37°C, human hsp70 is found exclusively in the cytoplasm (Fig. 5*A*). After 10-min heating at 45°C, the protein rapidly moves into the nucleus and becomes associated with the nucleoli (Fig. 5 *B–E*). By 16 hr after the heat shock, however, most—if not all—of the protein has again disappeared from the nucleus/nucleoli, and the staining pattern of the cells reverts to that of unstressed HR cells at 37°C (Fig. 5*F*). Control experiments with identical immunostaining protocol showed insignificant amount of reaction between the antibody and rat hsp70 expressed in Rat-1 cells grown at 37°C or immediately after a 45°C, 10-min heat-shock treatment (data not shown).

DISCUSSION

hsp70, a major hsp, is synthesized by cells of many organisms in response to thermal or other environmental stresses. It has been hypothesized that the transient induction of hsp70 may enable cells to recover from previous thermal stress and provide cells with a transient degree of protection from subsequent heat challenge. A corollary of this hypothesis is that the overexpression of hsp70 in cells confers permanent heat resistance. By using DNA-mediated transfection, we have obtained transfected rat cell lines stably and constitutively expressing different amounts of human hsp70 and demonstrated that the expression of human hsp70 confers heat resistance to the rat cells. This result lends further support to the hypothesis that one of the functions of hsp70 is to protect cells from thermal damage.

In the work reported here, we have used six cycles of heating to enhance the frequency of selection for colonies that express the human hsp70-encoding gene. Without heat selection, the expression frequency of human hsp70 in transfected Rat-1 cells was found to be <3%. Immunoblot analysis using cell lysates extracted from the unheated pool population showed that these cells expressed an insignificant amount of human hsp70s. The slower growth rate under normal conditions of the transfected rat cells expressing human hsp70 may explain why it was difficult to obtain these

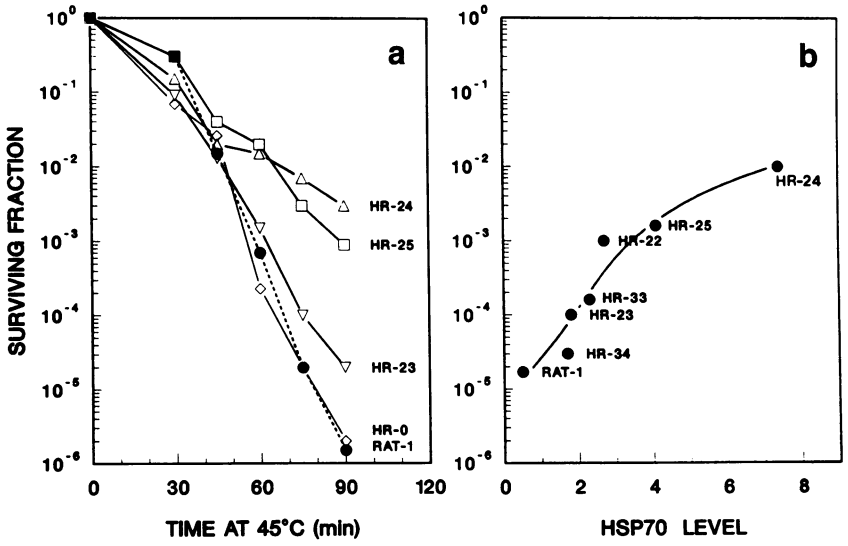

FIG. 4. Expression of human hsp70 gene confers thermal resistance to Rat-1 cells. (*a*) Monolayers of exponentially growing HR cells were exposed to 45°C for 0–90 min, and survivals were determined by colony-formation assay. Each cell line expresses human hsp70 in the following order: HR-24 (highest amount) > HR-25 > HR-23 (lowest amount). Rat-1, control untransfected Rat-1 cells; HR-0, Rat-1 cells transfected with only the neomycin-resistance gene. Thermal survival for HR-0 cells is indistinguishable from that of the control untransfected Rat-1 cells. The thermal resistance seen in HR cells is unlikely to be from the transfection of a human gene *per se*. We have found that Rat-1 cells, transfected with human *MYC* oncogene, singly or in combination with *RAS*, were more sensitive to thermal stress (30). (*b*) Monolayers of exponentially growing HR cells were exposed to 45°C for 75 min, and survival was determined by colony-formation assay. In parallel experiments, the relative levels of human hsp70 expressed in HR cells were measured by flow cytometry using mAb C92F3A-5 against hsp70. Thermal survival of various transfected cell lines is plotted against the relative level of human hsp70 in them. At least 20,000 cells were analyzed per data point for the flow cytometric measurement. Experiments have been repeated and yield nearly identical results.

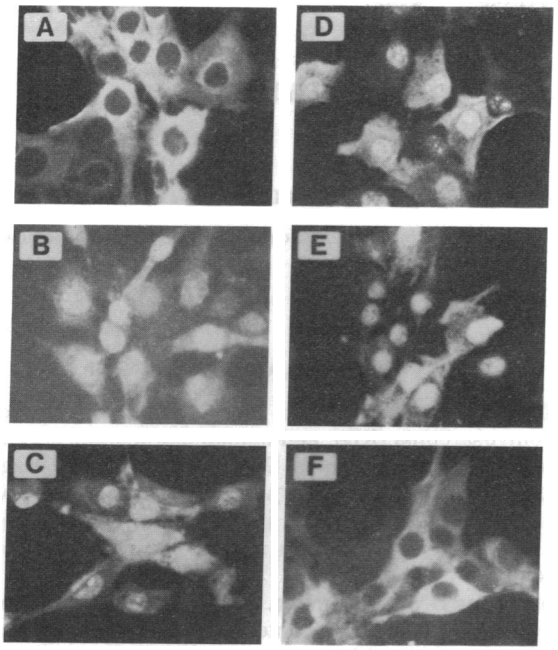

FIG. 5. Intracellular distribution of the human hsp70 expressed in rat HR-24 cells before and after a 45°C, 10-min heat-shock treatment. HR-24 cells, grown on glass coverslips, were heated at 45°C for 10 min and returned to 37°C for 0, 1, 2, 4, or 16 hr. After indicated recovery times, the cells were fixed and stained with anti-hsp70 mAb C92F3A-5. Distribution of human hsp70 was analyzed by indirect immunofluorescence. (*A*) Cells at 37°C. (*B*) Cells heat-shock treated at 45°C, no recovery. (*C*) Cells heat-shock treated, recovery at 37°C for 1 hr. (*D*) Cells heat-shock treated, recovery at 37°C for 2 hr. (*E*) Cells heat-shock treated, recovery at 37°C for 4 hr. (*F*) Cells heat-shock treated, recovery at 37°C for 16 hr. At 37°C human hsp70 is found exclusively in the cytoplasm (*A*). After 45°C, 10-min heating, human hsp70 is rapidly moved into the nucleus and nucleoli (*B–E*). By 16 hr after heat shock, the protein has mostly disappeared from the nucleus/nucleoli, and the staining pattern reverts to that of unstressed HR cells at 37°C (*F*). Control experiments with identical immunostaining protocol showed insignificant amount of reaction between the mAb and rat hsp70 expressed in Rat-1 cells grown at 37°C or immediately after a 45°C, 10-min heat-shock treatment (data not shown).

cell lines without heat selection. We emphasize that the heat resistance demonstrated in HR cells is unlikely to be a consequence of the repeated heat treatments. Rat-1 cells transfected with only the neomycin-resistance gene and subjected to the identical heat treatments—i.e., the HR-0 line—neither expressed elevated levels of endogenous rat hsps (hsp70, hsp27) nor showed increased thermal resistance when compared with untransfected Rat-1 cells. Furthermore, preliminary results in which a retroviral vector was used to more efficiently introduce the human hsp70 gene into Rat-1 cells indicate that relatively uniform and high-level expression of human hsp70 gene can be obtained without the repeated heating cycles; the same correlation between human hsp70 expression and thermal resistance is observed under this condition (unpublished observation).

One way to investigate the function of hsp70 in unstressed and heat-shocked cells is to determine the intracellular localization of the protein and to identify molecules or structures with which hsp70 interacts. Using mAbs specific to human hsp70, we found that at 37°C this protein is mostly cytoplasmic. Immediately after a 45°C, 10-min heat shock, a substantial fraction of the protein rapidly moves into the nucleus and becomes transiently associated with the nucleoli. By 16 hr after the heat shock, the protein has mostly disappeared from the nucleus, and intracellular distribution of hsp70 reverts to that of unstressed HR cells at 37°C. These findings are similar to those of Welch and coworkers (28, 29) on rat hsp70-b and rat hsp70-c. Thus, in response to a heat-shock treatment, the intracellular distribution of this constitutively expressed human hsp70 in Rat-1 cells is similar to that of the endogenous rat hsp70s.

Munro and Pelham (15) and Milarski and Morimoto (16) have successfully transfected mammalian cells with hsp70-encoding genes. However, expression of the transfected genes was transient and may not give an accurate picture of hsp70 in an equilibrium condition. Stably transfected cell lines that express human hsp70 should provide a steady-state condition for studying cellular targeting and physiological functions of hsp70 at 37°C, after heat shock or other environmental stresses, or during the development of thermotolerance. In addition, the use of stable cell lines should eliminate the limitations of transient transfection experiments that are imposed by the narrow time window. Detailed thermal-survival studies, studies on the kinetics of thermotolerance development, and nucleus/nucleolar localization, etc., can be performed repeatedly and more reproducibly for the same clonal derivatives. These studies should provide further insights on the mechanisms of thermal killing, thermal resistance, and transient thermotolerance of mammalian cells.

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1. Gerner, E. W. & Schneider, M. J. (1975) *Nature (London)* **256,** 500–502.
2. Henle, K. J. & Leeper, D. B. (1976) *Radiat. Res.* **66,** 505–518.
3. Henle, K. J. & Dethlefsen, L. A. (1978) *Cancer Res.* **38,** 1843–1851.
4. Lindquist, S. & Craig, E. A. (1988) *Annu. Rev. Genet.* **22,** 631–677.
5. Landry, S., Bremier, D., Chretien, P., Nicole, L. M., Tanquay, R. M. & Marceau, N. (1982) *Cancer Res.* **42,** 2457–2461.
6. Li, G. C. & Werb, Z. (1982) *Proc. Natl. Acad. Sci. USA* **79,** 3219–3222.
7. Subjeck, J. R., Sciandra, J. J. & Johnson, R. J. (1982) *Br. J. Radiol.* **55,** 579–584.
8. Li, G. C. (1985) *Int. J. Radiat. Oncol. Biol. Phys.* **11,** 165–177.
9. Laszlo, A. & Li, G. C. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 8029–8033.
10. Li, G. C. & Laszlo, A. (1985) *J. Cell. Physiol.* **122,** 91–97.
11. Landry, J., Chretien, P., Lambert, H., Hickey, E. & Weber, L. A. (1989) *J. Cell Biol.* **109,** 7–15.
12. Riabowol, K. T., Mizzen, L. A. & Welch, W. J. (1988) *Science* **242,** 433–436.
13. Johnston, R. N. & Kucey, B. L. (1988) *Science* **242,** 1551–1554.
14. Li, G. C. (1989) in *Hyperthermic Oncology, 1988*, eds. Sugahara, T. & Saito, M. (Taylor & Francis, London), Vol. 2, pp. 256–259.
15. Munro, S. & Pelham, H. R. B. (1984) *EMBO J.* **3,** 3087–3093.
16. Milarski, K. L. & Morimoto, R. I. (1989) *J. Cell Biol.* **109,** 1947–1962.
17. Wu, B., Hunt, C. & Morimoto, R. I. (1985) *Mol. Cell. Biol.* **5,** 330–341.
18. Hunt, C. & Morimoto, R. I. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 6455–6459.
19. Graham, F. L., van der Eb, A. J. (1973) *Virology* **52,** 456–467.
20. Laski, F. A., Alzner-DeWeerd, B., RajBhandary, U. L. & Sharp, P. A. (1982) *Nucleic Acids Res.* **10,** 4609–4626.
21. Thomas, P. S. (1980) *Proc. Natl. Acad. Sci. USA* **77,** 5201–5205.
22. Feinberg, A. P. & Vogelstein, B. (1983) *Anal. Biochem.* **132,** 6–13.
23. O'Farrell, P. J. (1975) *J. Biol. Chem.* **250,** 4007–4020.
24. Welch, W. J. & Suhan, J. P. (1986) *J. Cell Biol.* **103,** 2035–2052.
25. Cohen, B. B., Moxley, M., Crichton, D., Dean, D. L. & Steel, C. M. (1984) *J. Immunol. Methods* **75,** 99–105.
26. Swok, J. S., Nakatsuji, T., Ito, K. & Tsuju, K. (1987) *Biotechniques* **5,** 564–571.
27. Towbin, H., Staehelin, T. & Gordon, J. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 4350–4354.
28. Welch, W. J. & Mizzen, L. A. (1988) *J. Cell Biol.* **106,** 1117–1130.
29. Welch, W. J., Feramisco, J. R. & Blose, S. H. (1985) *Ann. N.Y. Acad. Sci.* **455,** 57–67.
30. Li, G. C., Ling, C. C., Endlich, B. & Mak, J. Y. (1990) *Cancer Res.* **50,** 4515–4521.