Heat Shock Resistance Conferred by Expression of the Human HSP27 Gene in Rodent Cells

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Abstract. Heat shock induces in cells the synthesis of specific proteins called heat shock proteins (HSPs) and a transient state of thermotolerance. The putative role of one of the HSPs, HSP27, as a protective molecule during thermal stress has been directly assessed by measuring the resistance to hyperthermia of Chinese hamster and mouse cells transfected with the human HSP27 gene contained in plasmid pHS2711. One- and two-dimensional gel electrophoresis of [^{3}H]leucine- and [^{32}P]orthophosphate-labeled proteins, coupled with immunological analysis using Ha27Ab and Hu27Ab, two rabbit antisera that specifically recognize the hamster and the human HSP27 protein respectively, were used to monitor expression and inducibility of the transfected and endogenous proteins. The human HSP27 gene cloned in pHS2711 is constitutively expressed in rodent cells, resulting in accumulation of the human HSP27 and all phosphorylated derivatives. No modification of the basal or heat-induced expression of endogenous HSPs is detected. The presence of additional HSP27 protein provides immediate protection against heat shock administered 48 h after transfection and confers a permanent thermoresistant phenotype to stable transfectant Chinese hamster and mouse cell lines. Mild heat treatment of the transfected cells results in an induction of the full complement of the endogenous heat shock proteins and a small increase in thermoresistance, but the level attained did not surpass that of heat-induced thermotolerant control cells. These results indicate that elevated levels of HSP27 is sufficient to give protection from thermal killing. It is concluded that HSP27 plays a major role in the increased thermal resistance acquired by cells after exposure to HSP inducers.

EXPOSURE to temperatures slightly above normal induces in virtually all cells a transient state of resistance to killing by subsequent severe hyperthermic treatments (Gerner and Schneider, 1975; Henle and Dethlefsen, 1978; Subjeck and Shyy, 1986; Landry et al., 1987). The nature of the biochemical changes responsible for this induced thermotolerant state is not known. The finding that thermotolerance-inducing treatments also induce new transcriptional activities and the synthesis of a highly conserved group of proteins named heat shock proteins (HSPs),[1] has led to the proposal that HSPs may be involved in this process (McAlister and Finkelstein, 1980; Landry et al., 1982, 1987; Subjeck and Sciandra, 1982; Li and Werb, 1982; Craig, 1985; Lindquist, 1986; Subjeck and Shyy, 1986). However, a direct demonstration that the accumulation of any of these proteins can protect cells from thermal killing has not yet been made.

The evidence that HSPs are involved in thermoprotection relies mainly on correlation made between the presence of the major HSPs, mainly HSP70, and resistance. For example, it was shown that HSP accumulation after mild triggering heat shock closely parallels development of thermotolerance and furthermore, that HSPs return to basal level at the same rate as thermotolerance decays (Landry et al., 1982). Moreover, known chemical inducers of HSPs were found, in most cases, to be good inducers of thermotolerance, and reciprocally, inducers of thermotolerance also induce HSP synthesis (reviewed in Landry et al., 1987). Attempts have been made to directly evaluate the role of individual HSP in thermoresistance. In cos cells, it was found that an increased level of presynthesized HSP70, obtained after transfection of *Drosophila* HSP70, accelerates recovery of nucleoli after heat shock, suggesting that HSP70 may have the property of catalyzing the reassembly of ribonucleoproteins (Pelham, 1984; Lewis and Pelham, 1985). In studies performed with rat and Chinese hamster cells, it was found that inactivation of HSP70 by injection of specific antibodies (Riabowol et al., 1988) or reducing its expression by genetic means (Johnston and Kucey, 1988) increased heat sensitivity. In contrast, inactivation of HSP70 genes in yeast yielded cells with altered growth properties at elevated temperature, but cells that were even more resistant than control cells to an acute heat treatment and were capable of developing thermotolerance (Craig and Jacobsen, 1984). The role of the other HSPs in thermotolerance is also unclear. Mutants of *Dictyostelium* defec-

1. *Abbreviations used in this paper*: HAT, hypoxanthine aminopterin thymidine; HSP, heat shock protein; HST, hamster stable transfectant cells; MST, mouse stable transfectant cells; TK, thymidine kinase.

tive for synthesis of the small HSPs were found to be unable to develop thermotolerance, despite induction of the other HSPs (Loomis and Wheeler, 1982). Also, selective induction of the small HSPs by ecdysone in a hormone-sensitive *Drosophila* cell line was found to bring about the thermotolerant phenotype in the absence of heat shock (Berger and Woodward, 1983). In yeast, however, inactivation of what appears to be a unique gene encoding the single HSP26 protein had no detectable phenotypic effect, and did not prevent attainment of thermotolerance (Petko and Lindquist, 1986).

Recent studies in mammalian cells have suggested a role of a HSP with M_r 27,000 (HSP27) in thermotolerance. Stable heat-resistant variants were isolated after mutagenesis of Chinese hamster cells (Chrétien and Landry, 1988). Four independently derived clones showed constitutive over-expression of HSP27 with no apparent modification in the level of the other HSPs. To further investigate the role of HSP27 in thermotolerance, we have studied the effect of increasing the amount of HSP27 produced by cells upon transfection with a recombinant plasmid containing a human HSP27 gene (Hickey et al., 1986). Here we report that constitutive expression of the human HSP27 protein in transfected Chinese hamster or mouse cell lines confers protection from thermal killing.

Materials and Methods

Cells and Culture Media

023 cells are an anchorage-independent and tumorigenic subclone of the Chinese hamster lung fibroblast cell line CCL39 (American Type Culture Collection, Rockville, MD) (Pouysségur et al., 1980). 023 cells and their transfectants were propagated at 37°C in a 5% CO_2 humidified atmosphere in DME containing glucose (4.5 g/liter) and $NaHCO_3$ (2.2 g/liter) and supplemented with 5% fetal bovine serum. LTA cells are a mouse tk^- $aprt^-$ cell line first described by Wigler et al. (1979). They were cultured in alpha MEM lacking nucleosides and supplemented with 5% fetal bovine serum. The cells were checked monthly for mycoplasma infection and found negative.

Plasmids

The structure of the various plasmids used in this study is shown schematically in Fig. 1. pHS2711 has been described previously (Hickey et al., 1986). It consists of a 3.1-kb Bam HI-Hind III fragment containing the complete human HSP27 with 600 bp of 5′-flanking sequence cloned into pUC12. pHS727A contains the HSP27 gene from pHS2711 fused at the Aat II site within the 5′-untranslated leader with the 5′-leader and regulatory sequences of a human HSP70 gene (Voellmy et al., 1985). pHS727B is a control plasmid containing the human HSP70 promoter fused to the middle of HSP27 exon III. pHS27B-A is a "promoter-only" construct containing the upstream region of the HSP27 gene and a part of the 5′-leader sequence up to site Aat II.

Transfection

The cells were transfected by the calcium phosphate precipitate method of Graham and van der Eb (1973) as modified by Wigler et al. (1979). Briefly, exponentially growing cells plated the day before at a concentration of 1×10^4 cells per cm^2 in 75-cm^2 flasks, were incubated overnight in the presence of a precipitate obtained by adding 500 μl of TE/$CaCl_2$ solution (10 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 0.25 M $CaCl_2$) containing up to 40 μg of plasmids to 500 μl of 2× HBSS (280 mM NaCl, 50 mM Hepes, 1.5 mM Na_2HPO_4). The transfected cultures were then trypsinized and plated in normal medium at 10^6 cells/25 cm^2. Stable transfectants of 023 cells (hamster stable transfectant [HST] cells) were derived as a pooled population of colonies that emerged after treating 023 cells transfected with 40 μg of pHS2711 with three 44°C heat shocks of 3.5–4 h at 5, 18, and 48 d after transfection. A similar sequence of hyperthermic treatment yielded no colonies when applied to untransfected cultures. The mouse cell line of stable

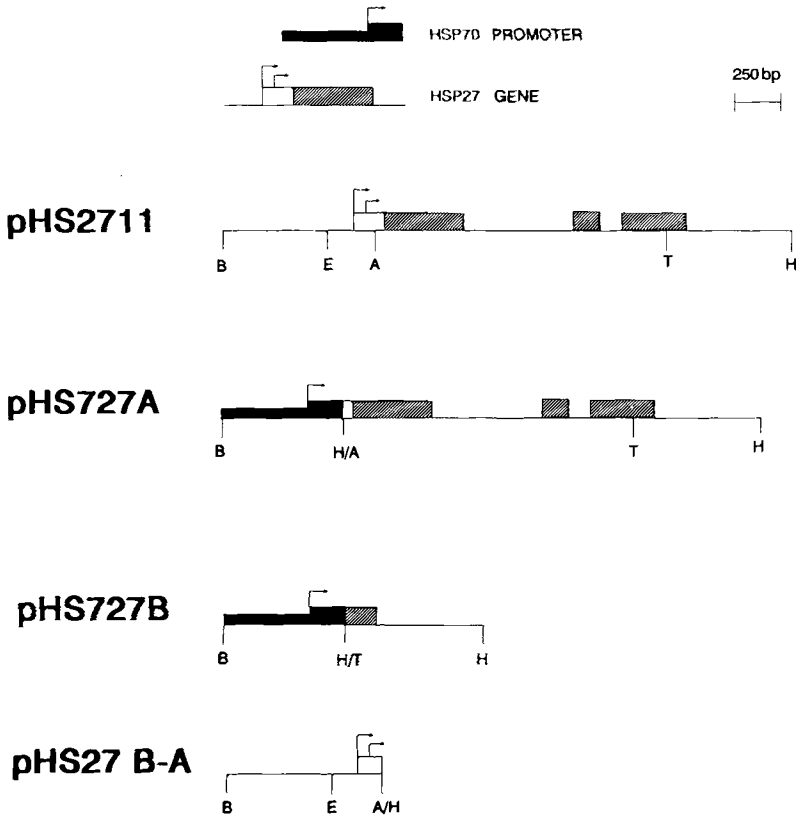

Figure 1. Schematic representations of the plasmid inserts used in this study. The mRNA coding regions of the HSP27 gene are shown as boxes and the 5′-untranslated leader region as an open box. Arrows indicate the start sites for transcription. Nontranscribed and intervening sequence regions are indicated as thin lines. Restriction enzyme cleavage sites: B, Bam HI; E, Eco RI; A, Aat II; T, Taq I; H, Hind III. H/A and H/T are fused, blunt-ended Hind III–Aat II and Hind III–Taq I sites, respectively. See Materials and Methods section for details of each construction.

transfectants (mouse stable transfectant [MST] cells) was obtained after cotransfecting the LTA cells with 10 μg of pHS2711 and 10 μg of paPSl, which contains the herpes simplex thymidine-kinase (TK) gene (Chandler et al., 1983), followed by a selection in HAT medium (alpha MEM lacking nucleosides and containing 100 μM hypoxanthine, 0.4 μM aminopterin, and 16 μM thymidine). The MST cell line consists of a pool of 208 HAT-resistant colonies. Transfection with paPSl only has no effect on cell thermoresistance. Clonal isolates were obtained after plating cells at limit dilution in 96-well dishes. Cell lines were established from the clones and frozen at low passage numbers. In this study, the cells were used at passage number between 3 and 12. In three HST clones studied, human HSP27 and increased thermoresistance were still detected after 40 passages (~240 doubling after first confluence was reached).

Heat Shock Treatment

Heat treatments were administered by immersing culture flasks into a water-bath thermoregulated at 43°C or 44 + 0.05°C. For cell survival determination, the cells were trypsinized immediately after the treatment and plated at appropriate concentrations at 37°C. Relative survival was determined from the number of cells capable of forming colonies containing >~50 cells within 8–10 d. All survival data were corrected for the plating efficiency of the appropriate control group.

Metabolic Labeling of Proteins

[^{3}H]Leucine (110 Ci/mmol) and $H_3{}^{32}PO_4$ (285 Ci/mg of phosphorus) were purchased from ICN Biomedicals (Montréal, Québec). [^{3}H]Leucine was used in regular medium at a concentration of 50 or 100 μCi/ml, depending on whether proteins were to be analyzed by one- or two-dimensional electrophoresis. Labeling with $H_3{}^{32}PO_4$ was achieved with a concentration of 25 μCi/ml or 100 μCi/ml in Earle's balanced salt solution lacking phosphate and containing sodium pyruvate (110 mg/liter), glutamine (290 mg/liter), and BSA (0.8 g/liter). After labeling, the cells were washed three times in PBS (sodium chloride, 8 g/liter; potassium chloride, 400 mg/liter; sodium phosphate monobasic, 530 mg/liter; sodium phosphate dibasic, 1.41 g/liter; glucose, 2 g/liter; phenol red, 10 mg/liter; pH 7.2) at 4°C and the proteins were extracted for electrophoresis.

Protein Electrophoresis

One-dimensional SDS-PAGE. Proteins were extracted in SDS sample buffer (62.5 mM Tris-HCl, pH 6.8, 2.3% SDS, 10% glycerol, 5% beta-mercaptoethanol, 0.005% bromophenol blue, 10 mM sodium fluoride, 1 mM PMSF), passed through a 26-gauge syringe needle and heated for 3 min at 100°C. Electrophoresis was carried out in 10% acrylamide gel slab following the method of Laemmli (1970).

Two-dimensional Isoelectric Focusing (IEF)–SDS-PAGE. Proteins were extracted in lysis buffer (9.5 M urea, 2% 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfate, 2% ampholines [75% Bio-lyte 5–7, 25% Bio-lyte 3–10], 10% glycerol, 5% beta-mercaptoethanol, 10 mM sodium fluoride, 1 mM PMSF) and vortexed until complete solubilization was obtained. IEF was carried out following the method of O'Farrell (1975). At the end of the run, the gel rods were equilibrated for 20 min in the SDS sample buffer and transferred on top of the SDS-PAGE gels for the second dimension. Chemicals for electrophoresis were purchased from Bio-Rad Laboratories (Mississauga, Ontario) and ICN Biomedicals. Gels containing ^{3}H-labeled proteins were impregnated with En^{3}Hance (New England Nuclear, Lachine, Québec) and autoradiographed at −80°C on Kodak X-OMAT-AR radiographic films. Gels containing ^{32}P-labeled samples were autoradiographed at −80°C using an intensifying screen (Lightning Plus, DuPont Co., Wilmington, DE).

Antibodies and Immunoblot Analyses

To prepare Hu27Ab and Ha27Ab, two rabbit antisera recognizing specifically the human and hamster HSP27 respectively, HSP27 was purified to homogeneity based on the method described by Arrigo and Welch (1987). Briefly, a 10,000-*g* supernatant cellular fraction from 023 or pHS2711-transfected 023 cells was enriched for HSP27 sequentially on hydroxylapatite and Superose-12 columns and the fractions containing HSP27 submitted to preparative two-dimensional gel electrophoresis. Hamster or human HSP27 species A were cut from the Coomassie blue-stained gels with a razor blade, lyophylized, homogenized, and emulsified with complete (first two injections) or incomplete Freund's adjuvant. Approximately 20–40 μg of HSP27 (10–15 gels) was used for each injection. Booster injections were given at 4-wk intervals and blood was collected 7–10 d after each injection. The specificity of the antisera was tested on Western blots of proteins extracted of whole cells from various organisms. Ha27Ab was found to react exclusively against hamster HSP27 and gave no reaction against mouse, human, monkey, bovine, or *Drosophila* cells. Hu27Ab reacts against human HSP27 present in human cells or in pHS2711-transfected rodent cells, but not against the hamster or mouse proteins. Immunoblots were prepared as described by Towbin et al. (1979) with minor modifications. Proteins were separated by one- or two-dimensional gel electrophoresis, electroblotted on nitrocellulose papers, and stained with Red Ponceau. For immunodetection of HSP27, the blots were soaked for 1.5 hours in 5% skim milk in TBS (10 mM Tris-HCl, 150 mM NaCl, pH 7.4) and then allowed to react for 1 h with the immunoserum diluted at appropriate concentration (1:500 to 1:1,000) in TBS containing 1% skim milk. Antigen-antibody complexes were revealed with a ^{125}I-labeled goat anti-rabbit IgG.

Results

Expression of the human HSP27 gene cloned in plasmid pHS2711 is highly dependent upon heat shock in both human cells and *Xenopus* oocytes (Hickey et al., 1986; Pfielsticker, J., and L. A. Weber, unpublished results). In contrast, when this gene was introduced into Chinese hamster 023 cells we observed at 48 h after transfection high constitutive levels of the HSP27 transcripts (data not shown) and a high expression of the human HSP27 protein in ~25% of the cells when visualized by indirect immunofluorescence using a specific antibody to HSP27 (see below). The expression of the protein was confirmed by SDS-PAGE analysis of total cell proteins 48 h after transfection. In transfected cells incubated in the presence of [^{3}H]leucine for 24 h, a new protein band migrating slightly faster than the homologous hamster protein was detected at a concentration that correlated linearly with the amount of transfected pHS2711 DNA (Fig. 2). The presence of the transfected protein had no effect on the basal or heat shock–induced level of HSP70 or actin. Heat shock of the transfected culture caused an increase in the level of total (hamster plus human) HSP27, which could be fully explained by a normal induction of only the endogenous hamster HSP27.

The identity and integrity of the human HSP27 protein expressed in hamster cells was assessed by two-dimensional gel electrophoresis, comparing proteins extracted from the transfected cells to proteins extracted from human WI-38 fibroblasts (Fig. 3). Proteins were visualized by autoradiography after incubating the cells for 30 h in the presence of [^{3}H]leucine or 2 h with [^{32}P]orthophosphate. In hamster cells, two ^{3}H-labeled HSP27 species (*a* and *b*) are detected at 37°C and accumulate to high levels during an 8-h period that follows a 20-min heat shock. As shown previously (Chrétien and Landry, 1988; Landry et al., 1988), heat shock induces a rapid phosphorylation of HSP27. Whereas the *b* species and an additional *c* species are detected upon ^{32}P-labeling at 37°C, a fourth species (*d*) becomes visible and the *b* and *c* species show a marked increase in phosphorylation during the first 20 min at 44°C. Except for a slightly more acidic isoelectric point, human HSP27 in WI-38 cells is qualitatively similar to hamster HSP27. It is also composed of four distinct isoforms (*A*, *B*, *C*, and *D*), of which all but the *A* species are phosphoproteins. The protein accumulating in the hamster cells after transfection is indistinguishable from HSP27 expressed in human WI-38 cells; it is also composed of four distinct isoforms, three of which

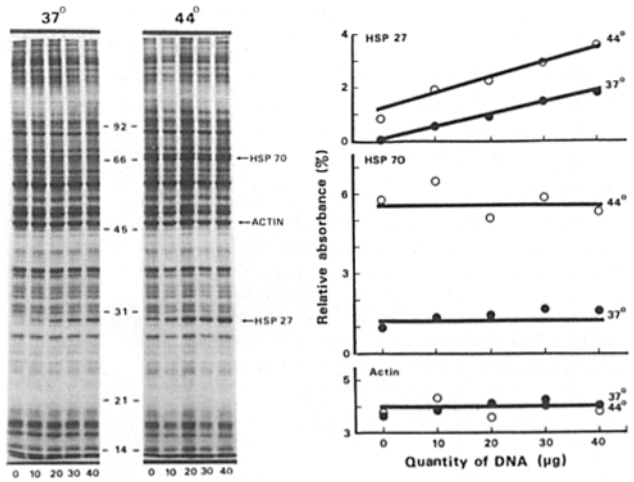

Figure 2. Accumulation of human HSP27 in Chinese hamster cells transfected with pHS2711. 023 cells transfected with 0–40 μg of plasmid pHS2711 (complemented to 40 μg with the insert-free plasmid) were incubated for 24 h (starting 24 h after transfection) in the presence of [^{3}H]leucine at 37°C, interrupted (44°C) or not (37°C) at the 16th hour by a heat shock of 20 min. Proteins were extracted and processed to obtain the SDS-PAGE fluorogram. The relative levels of actin, HSP70 (doublet), and HSP27 (hamster plus human) was determined from a densitometric scan of the fluorogram.

are phosphoproteins, with a relative molecular mass and isoelectric point identical to the WI-38 HSP27 species. In addition, the transfected proteins was modified similarly to endogenous HSP27 after heat shock. The transfected *B* and *C* species were labeled with ^{32}P at normal temperature in hamster cells. Their levels of phosphorylation increased markedly within 20 min at 44°C and a *D* species became visible. This analysis suggested that the human HSP27 protein is correctly expressed and is functioning normally in hamster cells. Moreover, the results show that the pHS2711 gene encodes all four isoelectric forms of human HSP27, thus confirming previous suggestions that the various HSP27 isoforms represent post-translational modifications of the same protein (Kim et al., 1984; Welch, 1985).

To facilitate further studies, we developed two rabbit antisera, Hu27Ab and Ha27Ab, that react specifically with human and hamster HSP27 respectively. The specificity of the antibodies was demonstrated by Western blot analyses of protein extracted from hamster 023 cells and human HeLa cells (Fig. 4 *a*). In both hamster and human cells, most HSP27 is found in the unphosphorylated form (*A* or *a*) at 37°C. Immediately after a 20-min heat shock and before any increase in total HSP27 content is detected, the phosphorylated forms of HSP27 appear. The most heavily phosphorylated forms of each protein detected by ^{32}P-incorporation (*D* and *d*) could not be detected by Western blot analysis. Although one cannot exclude possible changes in antigenic properties due to extensive phosphorylation, it is probable that this isoform is not abundant enough to be detected, as this isoform is also not visible by silver staining (data not shown). Analyses of interspecies specificity were performed on cells that have accumulated HSP for 8 h after heat shock. The results indicated negligible immunological cross-reaction between hamster and human HSP27 antibodies. The antibodies were then used to probe the same protein samples presented in Fig. 2. The results of the immunoblot analysis (Fig. 4 *b*) again showed that the presence of the human protein had no detectable effect on the basal or induced level of the endogenous hamster protein. In all cases, an ~10-fold increase in hamster HSP27 was measured after heat shock, whereas human HSP27 increased by <1.3-fold. The constitutive expression of pHS2711 and the absence of effect of a high level of human HSP27 protein on the induction of the endogenous HSP27 was confirmed in studies on pooled (see below) or cloned (data not shown) populations of stable pHS2711-transfectants.

The high constitutive expression of pHS2711 in the transfected cells provides a good model system on which to study the role of presynthesized HSP27 in thermotolerance. The putative biological activity of the human HSP27 protein in the hamster cells was tested by examining the effect of hyperthermic treatment on the clonogenic survival of pHS2711-transfected versus control (pUC19-transfected) 023 cells. There was a dose-dependent increase in the number of cells

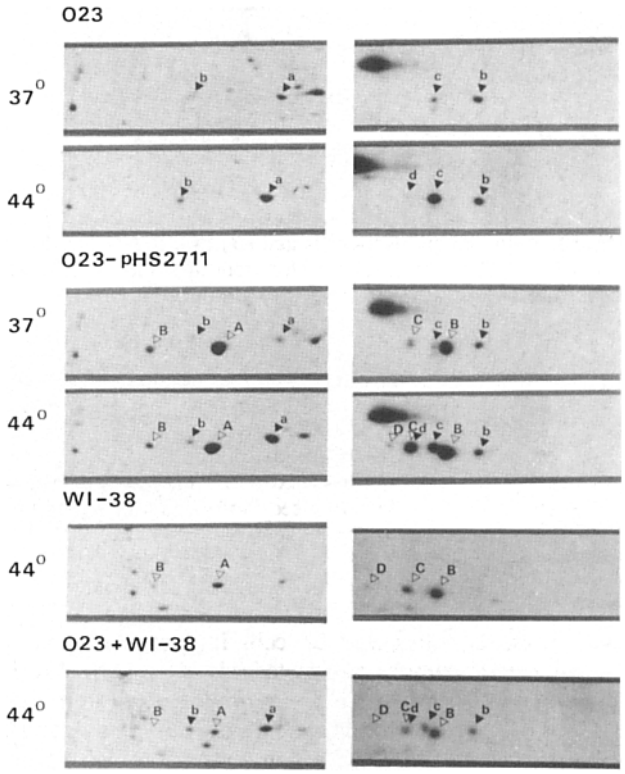

Figure 3. Two-dimensional gel analysis of human HSP27 expressed in pHS2711-transfected 023 Chinese hamster cells and in human WI-38 fibroblasts. Extracts of proteins metabolically labeled with [^{3}H]leucine for 30 h (*left column*) or $H_3{}^{32}PO_4$ for 2 h (*right column*) were analyzed by two-dimensional gel electrophoresis and fluorographed. When indicated, a heat shock of 20 min at 44°C was administered 8 h (^{3}H) or 20 min (^{32}P) before extraction. In the case of transfected cells, proteins were extracted 48 h after transfection. (023, 023-pHS2711) Chinese hamster cells untransfected or transfected with 40 μg of pHS2711; (WI-38) human WI-38 fibroblasts; (023 + WI-38) equal amounts of extracts from each cell type were mixed before electrophoresis. Chinese hamster HSP27 species are labeled *a*, *b*, *c*, and *d*. Human HSP27 species are labeled *A*, *B*, *C*, and *D*.

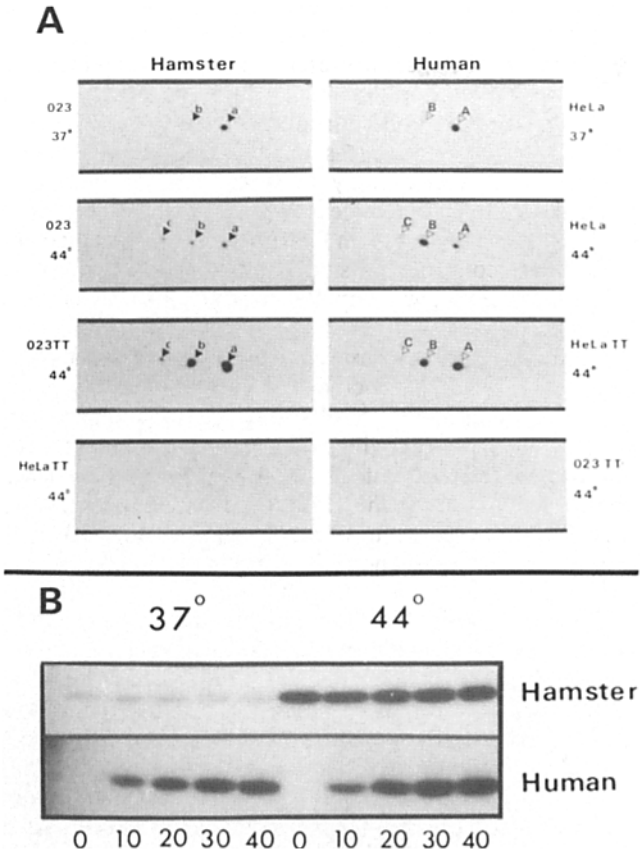

Figure 4. Immunoblot analysis of hamster and human HSP27 expressed (*A*) in 023 and Hela cells and (*B*) in pHS2711-transfected 023 cells. (*A*) Proteins were extracted from 023 cells, HeLa cells, or from cells made thermotolerant by a 20-min heat treatment 8 h before (023TT and HeLaTT), separated by two-dimensional gel electrophoresis, transferred on membranes, and probed with Hu27Ab (human) or Ha27Ab (hamster). When indicated (44°C), the cells were heat shocked for 20-min just before extraction. (*B*) Two aliquots of proteins extracted 48 h after transfection of Chinese hamster 023 cells with 0–40 μg of pHS2711 (samples of Fig. 2) were separated by SDS-PAGE, transferred on membranes, and probed with either antibody. When indicated (44°C), the cells were heat shocked for 20 min 8 h before protein extraction.

that were able to form colonies after a 3.5-h treatment at 44°C, from <1 colony per 10^6 cells in control cultures to 300 colonies in cultures transfected with 40 μg of pHS2711 plasmid (Fig. 5). Less than one surviving colony was also obtained when the 023 cells were transfected with plasmids pHS727B and pHS727B-A, which contain only partial sequences of pHS2711 and failed to generate detectable human HSP27 (Fig. 5). However, a low but significant level of protection was conferred upon transfecting plasmid pHS727A, containing the coding region of pHS2711 under the control of a human HSP70 promoter. Transfection with the pHS727A construct yielded a very low level of expression of HSP27 protein in unstressed cells, but in contrast to pHS2711, the HSP70 promoter of pHS727A gives heat-inducible expression of human HSP27. These combined results indicate that thermoprotection is dependent on HSP27 protein expression at the time of heat shock, and that the transfection process or the sequences contained in pHS2711 DNA have no effect per se on thermosensitivity.

We examined whether HSP27 gene expression can confer a permanent heat resistant phenotype in stable pHS2711 transfectants of Chinese hamster and mouse cells. Chinese HST cells were successfully derived from 023 cell survivors after three hyperthermic treatments administered over a 7-wk period after transfection with pHS2711. Similar hyperthermic treatments administered to the parental untransfected 023 cells yielded no surviving colonies. To rule out the possibility that the heat selection procedure influences the thermoresistant phenotype, stably transformed cells were also obtained by cotransfecting the pHS2711 plasmid along with a selectable marker. As 023 cells proved to be highly resistant to the neomycin derivative G418, mouse TK-deficient LTA cells were used. MST cells were obtained by cotransfecting LTA cells with pHS2711 and a plasmid containing a

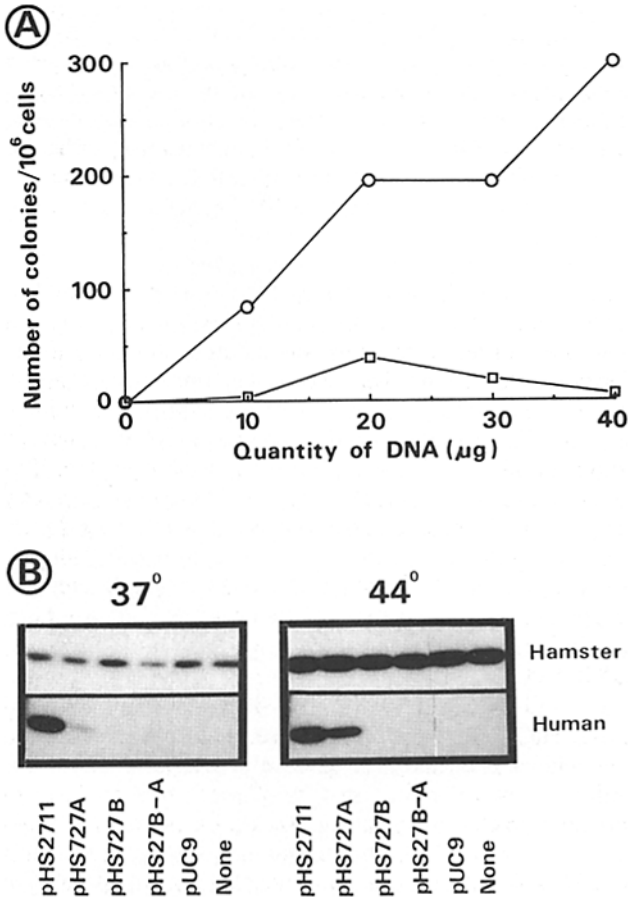

Figure 5. Expression of the human HSP27 confers immediate protection from lethal heat shock. (*A*) 44 h after transfection with 0–40 μg of plasmid, the cells were heat shocked at 44°C for 3.5 h and plated at 37°C for colony formation. Survival was estimated from the number of colonies visible 7 d later. Plasmid pHS2711 (*circle*), plasmid pHS727A (*box*). Transfection with plasmids pHS727B or pHS727B-A yielded less than one colony per 10^6 cells at all concentrations. (*B*) 023 cells were transfected with 40 μg of the indicated plasmids (*None*, no plasmid). In all cases, two aliquots of proteins extracted 48 h after transfection were separated by SDS-PAGE, transferred on membranes, and probed respectively with Hu27Ab (*Human*) or Ha27Ab (*Hamster*). When indicated (44°C), the cells were heat shocked for 20 min at 44°C 7.5 h before protein extraction.

Herpes virus TK gene followed by selection in HAT medium. Thermal resistance of the HST cells was compared with that of the parental 023 line by subjecting the cells to 44°C for increasing periods of time. The more thermosensitive LTA cells were tested at 43°C. The results show that both HST and MST cells are thermoresistant compared to the parental cell lines (Fig. 6). To establish a more direct link between expression of human HSP27 and thermoresistance, Western analyses of human HSP27 content using Hu27Ab and determinations of thermoresistance were performed in a total of 23 cell lines established from clonal isolates of HST and MST cells. Human HSP27 was expressed, albeit at varying levels, in all 16 and all 7 clonal cell lines isolated from HST and MST cells respectively, and all clones studied showed increased thermoresistance as compared with their respective parental cell lines. A positive correlation was found between the relative concentration of the exogenous protein and cell survival to 44°C or 43°C hyperthermia (Fig. 7). Only one MST and two HST clones deviate from the correlation curves, which could be explained on the basis of natural clonal variations. In the hamster cells constitutive expression of human HSP27 has no detectable effect on the expression of hamster HSP27 as determined in parallel experiments using Ha27Ab (data not shown). The results established for both Chinese hamster and mouse cell lines demonstrate a causal relationship between HSP27 content and thermoresistance.

We next examined the basal and heat-induced levels of the human and endogenous rodent HSP27 proteins in MST and HST cells (Fig. 8 *a*). As determined in transient expression experiments (Fig. 4 *b*), the results shown in Fig. 8 *a* also indicate that the transfected protein was not further induced by heat shock treatments in both cell lines. Furthermore, the basal and heat shock–induced expression of the hamster HSP27 protein was not modified in HST vs. 023 cells. The presence of the mouse HSP27 protein was not detected with either antibody. We also evaluated the effect of a high level of human HSP27 on expression of the other HSPs. The protein synthesis pattern of HST cells was compared with that of 023 cells after labeling the cells for 10 h in the presence of [^{3}H]leucine after a heat shock for various periods of time at 44°C. As shown in Fig. 8 *b*, the expression of the human protein in HST cells has no effect on the basal level of expression of HSP70, HSP89, and HSP107 in HST as compared to 023 cells, nor on the heat-induced synthesis of these proteins.

Heat shock treatments as used in Fig. 8 are known to induce in 023 cells not only the accumulation of HSPs, but also the development of a transient state of acquired thermotolerance (Landry et al., 1986). Because HST cells possess, at 10 h after heat shock, the human HSP27 in addition to the heat-induced elevated level of endogenous HSP27, it was of interest to determine whether these cells would develop a higher state of acquired thermotolerance. HST and 023 cells were first heat shocked for 0, 10, 20, or 30 min at 44°C, allowed to recover for 10 h and then subjected to a second hyperthermic treatment of 3 h at 44°C. A period of 10 h was chosen based on previous results indicating that thermotolerance is maximally expressed at this time (Landry et al., 1986). The results show that the basal thermoresistance of HST cells is intermediate between control and fully thermotolerant 023 cells (which is attained after heat treatments

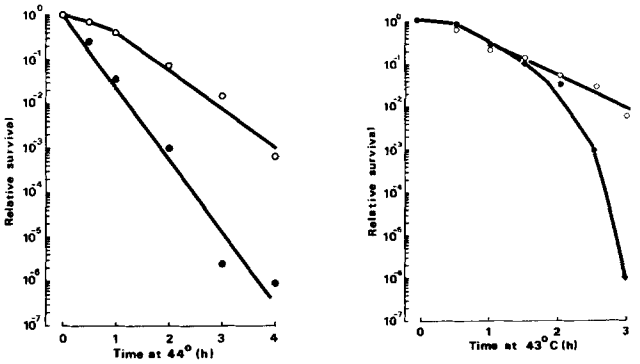

Figure 6. Expression of thermoresistance in stable pHS2711-transfectants. Chinese hamster 023 (*left*) and mouse LTA (*right*) cells were heat shocked at 44°C or 43°C for the indicated periods of time and plated at appropriate dilution for colony formation at 37°C. (*Closed circle*) Parental 023 and LTA cell lines; (*open circle*) stable pHS2711-transfectants of 023 (HST) and LTA (MST) cells.

of 20–30 min) (Fig. 9 *a*). Intriguingly, the presence of the human HSP27 had no influence on the maximal level of thermotolerance attainable after heat shocks of 20–30 min. The basal and maximal induced level of thermoresistance was also evaluated in three distinct clones isolated from HST cells (Fig. 9 *b*). Our results show that, although individual clones express varying degrees of basal thermoresistance, they do not acquire a level of thermotolerance that surpasses that found in induced control cells.

Discussion

This study directly addresses the long-standing hypothesis that HSPs are involved in the phenomenon of thermotolerance induction. Experimental evidence is presented showing that a thermoresistant phenotype can be conferred to Chinese hamster or mouse cells by transfection with the human HSP27 gene contained in pHS2711. This establishes a causal relationship between the presence of a specific heat shock protein, HSP27, and thermoresistance. The results extend earlier studies in *Dictyostelium* and *Drosophila* cells which suggested involvement of the small HSPs in thermotolerance (Loomis and Wheeler, 1982; Berger and Woodward, 1983), and indicate that the presence of HSP27 alone may be sufficient to explain in a large part the increased ability of heat-induced thermotolerant cells to survive hyperthermic treatment.

Transfection of rodent cells with plasmid pHS2711 results in the accumulation of human HSP27, which becomes rapidly phosphorylated during heat shock into three additional isoforms indistinguishable from those present in human WI-38 cells. Based on comparison of peptide distributions obtained after proteolytic digestion of HSP27, Welch (1985) and also Kim et al. (1984) previously suggested that the various isoforms of HSP27 correspond to the same protein but with increasing degree of phosphorylation. A similar conclusion was reached by Chrétien and Landry (1988) who found that in hamster cells, there was an immediate increase in the levels of the phosphorylated forms of HSP27 after a heat shock of 10 min, which was accompanied by a corresponding decrease in the level of the unphosphorylated form *A*. The pres-

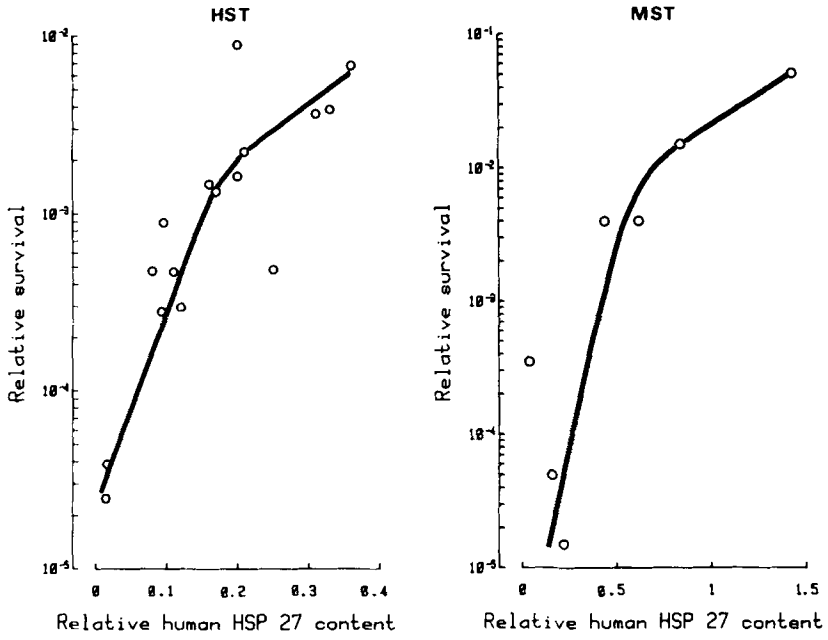

Figure 7. Correlation between levels of human HSP27 and survival to hyperthermia of clonal isolates of Chinese hamster 023 cells and mouse L cells transfected with pHS2711. Survival was determined by colony formation following exposure of the cells to hyperthermia for 4 h at 44°C (*HST*) or 3 h at 43°C (*MST*). Total proteins were extracted from control cultures (no heat shock), separated on SDS gels, and blot-transferred onto nitrocellulose. Levels of human HSP27 were determined by Western analyses using Hu-27Ab. Values were normalized relative to the signal obtained after probing the same immunoblots with an antiserum specific to actin. Analyses were performed in parallel with Ha27Ab to show that the hamster HSP27 does not vary in concentration among the hamster clones (data not shown).

ent results now clearly establish that the four isoforms of human HSP27 are the product of the single gene contained in pHS2711. Furthermore, the stimulation of phosphorus incorporation into HSP27 shortly after heat shock does not depend upon an increase in the total mass of the protein.

The pHS2711 gene product is constitutively expressed at normal temperature in the Chinese hamster and mouse cells and was not appreciably induced further by heat shock. This mode of control of pHS2711 expression was unexpected. The gene cloned in pHS2711 contains promoter sequences ho-

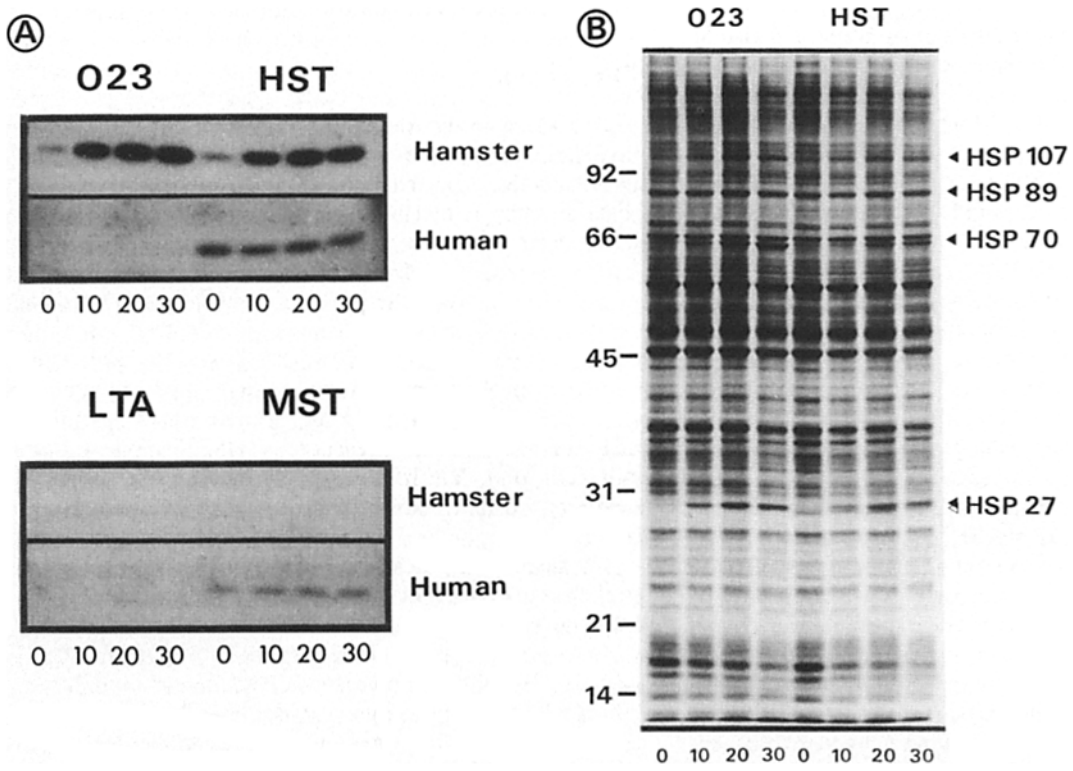

Figure 8. Expression of HSPs in stable transfectants. (*A*) Constitutive and heat-induced expression of human and hamster HSP27 in stable pHS2711-transfectants of Chinese hamster (HST) and mouse (MST) cells. Proteins were extracted from the parental Chinese hamster 023 and mouse LTA cells or their respective HST and MST derivatives, 10 h after a heat shock from 0 (control, constitutive expression) to 30 min at 44°C (hamster cells) or 43°C (mouse cells). The protein samples were processed in duplicates to obtain the Ha27Ab (hamster) and Hu27Ab (human) immunoblots. (*B*) Constitutive and heat-induced expression of HSPs in HST and 023 cells. After a conditioning heat treatment for the time indicated (in minutes) at 44°C, the cells were incubated at 37°C for 10 h in the presence of [^{3}H]leucine. Proteins were then extracted and processed to obtain the electrophoretic fluorogram.

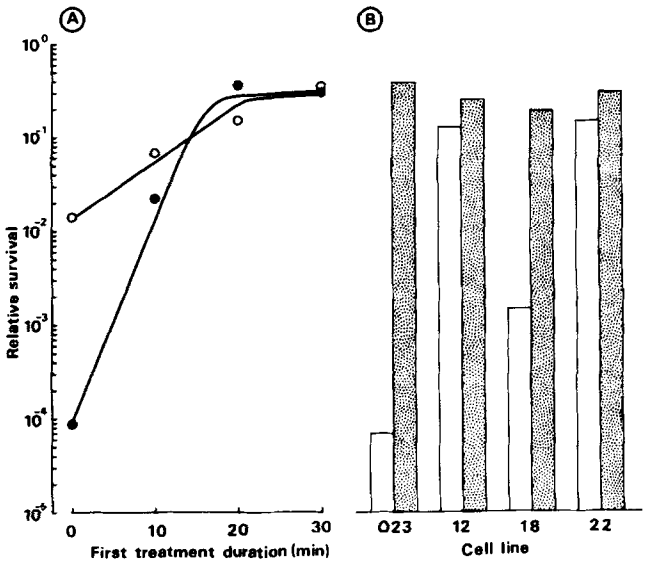

Figure 9. Induction of thermotolerance in parental 023 cells, pooled pHS2711-transfectant HST cells, and three clonal cell lines derived from HST cells. (*A*) HST (*open circle*) and 023 (*closed circle*) cells were exposed to a conditioning treatment of various durations (0–30 min) at 44°C, allowed to recover at 37°C for 10 h, subjected to a test treatment of 3 h at 44°C, and then returned to 37°C for colony formation. (*B*) 023 and clones 12, 18, and 22 from HST cells, were exposed to a first treatment of 0 (no treatment, *open bars*) or 30 min (*dotted bars*) at 44°C, returned to 37°C for 10 h, and then subjected to hyperthermia at 44°C for 3 h.

mologous to the consensus heat shock element, and was found to be heat inducible when injected into the *Xenopus* oocyte (Hickey et al., 1986). Moreover, the regulatory sequences of pHS2711 were found to confer strictly heat-inducible expression to a TK reporter gene upon transfection in HeLa cells (Pfielsticker, J., and L. A. Weber, unpublished results). One possibility that might explain consititutive expression is that uptake of a large quantity of pHS2711 perturbs the normal regulatory mechanisms operating in the hamster cells. In principle, this could result in the activation of the endogenous HSP genes, and this stress response, rather than the expression of the human protein, may be responsible for the acquired thermoresistant phenotype. However, this does not appear to be the case, as neither the basal or heat-induced level of endogenous HSP27 or other HSPs is modified in cells expressing human HSP27. A second possibility is that pUC, the carrier plasmid of pHS2711, contains cryptic promoter sequences that are capable of driving constitutive transcription in rodent cells. This could be easily tested by recloning in a different vector. Note, however, that pHS727A is also cloned in pUC and its expression appears correctly controlled by the HSP70 promoter. Finally, it is possible that there exists major differences in the control of HSP27 gene expression between Chinese hamster, mouse and HeLa cells. This would not be expected considering the high degree of similarity in the control of expression of other HSP genes between various species as divergent as *Drosophila* and human (for review, see Voellmy, 1984). However, the small heat shock protein genes are known to be regulated independently from the other HSP genes during development (Mestril et al., 1986; Thomas and Lengyel, 1986; Hoffman et al., 1987). It is possible that transcription of the human HSP27 gene in the rodent cells is under control of regulatory elements other than the heat shock elements. The human HSP27 gene in pHS2711 is the only mammalian HSP27 gene that has been isolated to date and very little is known concerning the mechanisms controlling expression of the small HSPs in higher organisms.

The high constitutive expression of pHS2711 in 023 cells was quite fortuitous and allowed us to demonstrate a protective role of HSP27 during hyperthermia. It appears that it is the amount of presynthesized HSP27 present in the cell at the time of heat shock rather than the level attained after induction that is the important determinant of cell thermoresistance. This conclusion is supported by the finding that cells transfected with pHS727A, a plasmid that yields a very low level of accumulation of human HSP27 in unstressed cells, are not protected efficiently, although they accumulate a significant amount of human HSP27 after heat shock. The accumulation of HSP27 to high level in wild type cells after heat shock may therefore be responsible for at least part of the increased thermoresistance to a second heat shock observed in induced-thermotolerant cells. Induced-thermotolerant 023 cells possess a level of HSP27 that is ~10-fold higher than preinduced cells. The fold increase in total (human plus hamster) HSP27 in the transfected cells could not be determined because the relative sensitivities of the two antibodies to their respective human and hamster antigen is not known with precision at this moment. However, according to preliminary determinations, the most resistant HST clones would contain somewhere between 5 and 10 times more total HSP27 than parental 023 cells. If one considers that these transfectants have levels of thermoresistance close to that of induced-thermotolerant cells, it is reasonable to propose that HSP27 is a protecting factor in induced-thermotolerant cells. In support to this, we have shown that the presence of the human protein in HST cells and in three clonal isolates provides no additional protection to cells made thermotolerant by a prior heat treatment.

Very little information is available on the biochemical properties of HSP27 so that one can only speculate about the mechanisms of protection. The action of HSP27 may either be direct, by acting at specific heat-sensitive site, or indirect, by affecting a more general process that moves the cells into a less thermosensitive physiological state. HSP27 exhibits sequence homology with the central portion of alpha crystallin of the lens (Ingolia and Craig, 1982; Hickey et al., 1986) and similarly to this protein, exists in a native conformation mostly as large multimeric aggregates of ~500,000 D (Arrigo and Welch, 1987). This has been taken as evidence that HSP27 may have a very stable structure and a function regulated in part by aggregation (Ingolia and Craig, 1982; Wistow, 1985; Wistow and Piatigorski, 1987). Recent results suggest that such a function may be linked to processes regulating cell responses to external stimuli. A number of agents that activate signal transduction pathways, such as serum, growth factors, tumor promoters, and calcium-active products, have been shown to induce a rapid phosphorylation of HSP27 (Welch, 1985; Regazzi et al., 1988). Thus, it is significant that HSP27 is also rapidly phosphorylated in the first minutes of hyperthermic treatment, making this protein a good candidate for acting at an early step of the stress response. It is generally considered that hyperthermic cell kill-

ing depends on a balance between the induction of damages and the repair capacities of the cells. An attractive hypothesis is that the activation of HSP27 by phosphorylation early during heat shock sets up an immediate feedback protective mechanism to limit heat-induced cascade reactions that lead to hyperthermic lesions. The other HSPs may then act at a later time to repair lesions that have escaped the HSP27-dependent protecting mechanism. For example, HSP70 has been shown to be involved in the recovery of heat-damaged nucleoli (Pelham, 1984) and has general biochemical properties consistent with a role in the repair of damaged proteins or structures (Pelham, 1986). Such a complementary role for HSP27 and HSP70 is consistent with the recent studies of Riabowol et al. (1988) and Johnston and Kucey (1988), who found that inactivating HSP70 by microinjection of specific antibodies or blocking heat-induction of HSP70 after heat shock by competitive inhibition at the level of gene transcription makes cells thermosensitive. Current studies aiming at determining whether the many functions and cellular activities protected in heat-induced thermotolerant cells are also protected in the pHS2711-transfected cell lines, should help in deciphering the contribution of the various HSPs to thermoprotection and in determining the site of action of HSP27.

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