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Heat-resistant variants of Chinese hamster fibroblasts altered in expression of heat shock protein

(thermal resistance/cell survival/70-kDa heat shock protein)

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ABSTRACT **Heat-resistant variants of the Chinese hamster HA-1 line have been isolated after repeated heat treatments. The heat-resistant phenotype has been stable for over 70 passages. One of the members of the 70-kDa heat shock protein family was found to be synthesized at greater levels in the heat-resistant variants under normal growth conditions. Mild heat treatment of the variant lines induced a transient thermotolerance that was accompanied by additional increase in the synthesis of the 70-kDa heat shock proteins. Cell-free translation of total cellular RNA revealed greater amounts of 70-kDa heat shock protein mRNA in both control and heated variant cells. The greater levels of 70-kDa heat shock protein synthesized in the variant cells presumably are a reflection of altered levels of its messenger mRNA. In addition, we found that translational control plays a role in the elevated expression of heat shock proteins in heat-shocked HA-1 cells and their heat-resistant variants. The association of the heat-resistant phenotype with increased levels of a 70-kDa heat shock protein suggests strongly that this gene product plays a role in protecting cells from damage inflicted by elevated temperatures.**

Mammalian cells, when exposed to a nonlethal heat shock, acquire the transient resistance to a subsequent, otherwise lethal heat challenge, a phenomenon that has been termed thermotolerance (1, 2). Recently, it has been demonstrated that the appearance of thermotolerance in mammalian cells correlates in time with the increased synthesis of heat shock proteins (hsp) (3, 4). There is a good correlation between the ability of agents other than heat to simultaneously induce hsp and thermotolerance (5, 6). These observations suggest that at least one of the functions of the hsp may be to protect cells from heat-induced damage.

A genetic approach would be useful to further understand whether the hsp play such a role in conferring heat resistance to cells. Primarily, it would be of interest to determine whether alterations in the structure of hsp and/or the regulation of their expression lead to concomitant modifications in the cells' response to elevated temperatures. While there have been reports demonstrating the existence of stable heat-resistant (HR) variants derived from mammalian cell lines (7–9), none of these reports have investigated the conditions of hsp synthesis or the relationship of hsp to heat resistance in detail.

We report here on the isolation and characterization of HR variants from the Chinese hamster fibroblast HA-1 line. We have chosen this cell line because the phenomenon of thermotolerance has been thoroughly investigated in it (10, 11). Furthermore, extensive work has been done to correlate the levels of hsp and the degree of thermotolerance in this cell line (3, 5, 6, 12).

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MATERIALS AND METHODS

Cells and Culture Conditions. Exponentially growing Chinese hamster fibroblasts, designated HA-1 (13) were grown in Eagle's minimal essential medium supplemented with 15% fetal calf serum and antibiotics (50 μg of gentamycin per ml). After various treatments, cells were treated with trypsin, counted, and plated at appropriate dilutions. After 10 days of incubation at 37°C, colonies were fixed, stained, and counted. Trypsin treatment after heating had little effect on survival. Plating efficiencies were 70–90%.

Heating. Cell monolayers were heated in hot waterbaths located in CO_2 incubators as described (11, 12).

Labeling and Gel Electrophoresis. Cells were labeled with 20–30 μCi/ml (1 Ci = 37 GBq) (for one-dimensional gels) or 200–400 μCi/ml (for two-dimensional gels) of [^{35}S]methionine (specific activity >1200 Ci/mmol, Amersham) in methionine-free Eagle's minimal essential medium. At the end of the labeling period, cells were washed three times with ice-cold phosphate-buffered saline and dissolved in sample buffer (14, 15). One-dimensional analysis in the presence of sodium dodecyl sulfate was performed as described (3), except that the running gels contained 10% acrylamide. Two-dimensional gel electrophoresis was performed as described (16). Autoradiograms of one-dimensional gels were quantitated with an LKB model 927 laser densitometer. Spots on the autoradiograms of the two-dimensional gels were quantitated using a Leitz model image analyzer at the Cell Analysis Facility at the University of California at San Francisco, using an integration program described (17).

Isolation of RNA and *in Vitro* Translation. Total cellular RNA was isolated by using a guanidium thiocyanate/hot phenol procedure (18). The RNA was translated at subsaturating conditions in a rabbit reticulocyte translation kit (Bethesda Research Laboratories), according to the supplier's instructions, except that the total reaction volume was 15 μl. The translation products were analyzed by gel electrophoresis using procedures described above.

RESULTS

Isolation of HR Variants. Initially, two groups of exponentially growing HA-1 cells were treated with heat doses that resulted in a relative survival of approximately 10^{-5}: one for 5 hr at 43°C and the other for 45 min at 45°C. Surviving colonies were isolated, grown to confluency, passaged twice, and tested for survival after a 45 min, 45°C heat challenge. While the clones selected from the 43°C original treatment all exhibited increased resistance to the 45°C challenge, those derived from the 45°C treatment did not. One clone from the 43°C original treatment was then subjected to a further

Abbreviations: HR, heat-resistant; hsp, heat shock protein(s).

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selection of 4 hr at 43°C. Two surviving colonies, designated 331 and 332, were isolated and grown to confluency. Initial survival studies indicated that these two lines were significantly more resistant to prolonged heating than the parental HA-1 cells. Clones from the 332 line that have survived a further selection at 44°C or 45°C were then isolated and again grown to confluency, generating a series of lines, 191, 3011, 3012, and 3911 (survivors isolated after treatment at 45°C) and 2241, 2242, 2245, and 2248 (survivors isolated after treatment at 44°C). During the course of our work, the original 332 cell line became unstable and lost most of its heat resistance. However, the derivative cell lines described above are stable. Some have maintained their intrinsic heat resistance for over 6 months in culture at 37°C (more than 70 passages). In this report we describe the characterization of HR cell lines 3011, 3012, and 2242.

Response of HR Variants to Elevated Temperatures. Exposure of exponentially growing HA-1 cells to elevated temperatures for various lengths of time resulted in the survival curves illustrated in Fig. 1. The shape of these curves is characteristic for most mammalian cell lines; after an initial shoulder, cell survival decreases exponentially (1, 2, 10). The survival curves of the three variants, 3011, 3012, and 2242, after similar treatments are also presented in Fig. 1. It is apparent that, at all temperatures tested, the HR lines are more resistant to thermal stress than their parent line is.

Protein Profiles of the Variant Lines. The profiles of proteins synthesized in HA-1 and HR lines under normal growth conditions and after a mild heat shock were then examined by two-dimensional gel electrophoresis of total cellular extracts (Fig. 2). Several salient features emerge from the comparison of these patterns. First, the 70-kDa region, in which one of the major heat-induced proteins is seen, is more complex than indicated by one-dimensional gel analysis (3, 12). We can identify at least three major polypeptides whose expression is altered by exposure to heat: ≈72 kDa (a), 70 kDa (b), and 68 kDa (c). Both the 70-kDa hsp-a and the 70-kDa hsp-b are found in heated and nonheated HA-1 cells. The 70-kDa hsp-c appears to be found exclusively in the heat-shocked cells. Within the limits of the resolution of our techniques, we could not detect the expression of this polypeptide in either HA-1 or HR variant cells under normal growth conditions (at 37°C). Second, there is a significant increase in the synthesis of the 70-kDa hsp-b in the HR 3012 cells under normal culture conditions (37°C) when compared with their HA-1 counterparts. Thus, the constitutive expression of the major form of the 70-kDa hsp—i.e., 70-kDa hsp-b—is significantly increased in the HR 3012 cells. Since the isoelectric point and molecular weight of this polypeptide are not changed in the HR 3012 cells as indicated by its migration properties, it is likely to be encoded by the same structural gene. Third, the exposure of both HA-1 and HR 3012 cells to a mild heat treatment leads to an increased expression of 70-kDa hsp-b in addition to the induction of synthesis of 70-kDa hsp-c. Thus, although the constitutive levels of the 70-kDa hsp-b are higher in the untreated HR variants, they still respond to heat shock by an increased expression of this gene product. Similar observations were obtained with HR 3011 and 2242 cells (data not shown). Further work has indicated that other hsp inducers, such as amino acid analogues or sodium arsenite, while enhancing the synthesis of 70-kDa hsp-b, do not induce the synthesis of 70-kDa hsp-c (unpublished observation).

We have shown that both the rates of synthesis and constitutive levels of actin are similar in HA-1 and HR variant cells (12). Thus, we could estimate the relative levels of each member of the 70-kDa hsp family by using actin as our internal standard of comparison. The results are summarized in Table 1. The data indicate that the majority of the increase in 70-kDa hsp in heated HR variant cells is due to the increased levels of 70-kDa hsp-b, although increased levels of 70-kDa hsp-c also contribute. Similar results were obtained from the analysis of lysates of cells labeled continuously for 24 hr after a mild heat shock.

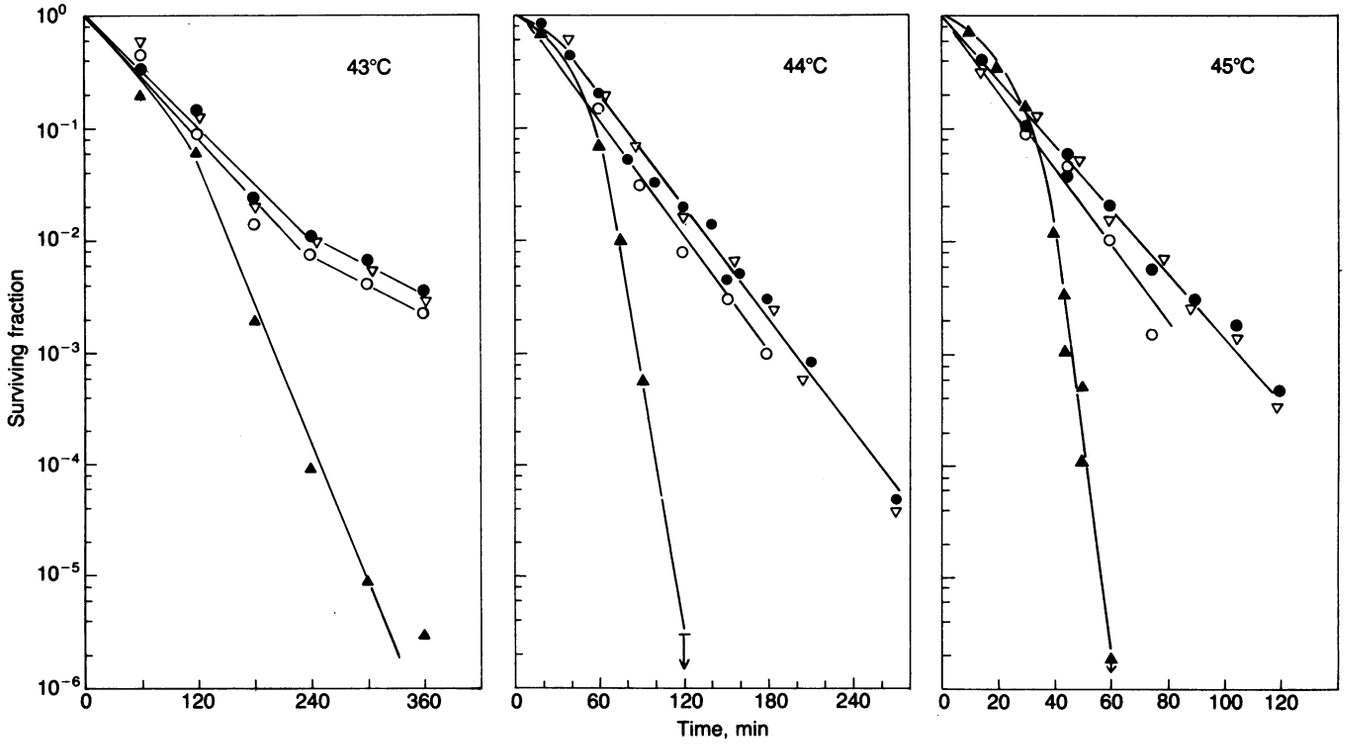

FIG. 1. Response of Chinese hamster HA-1 cells and their HR variants to elevated temperatures. Exponentially growing HA-1 cells (▲) and the HR lines 3011 (●), 3012 (▽), and 2242 (○) were exposed to 43°C, 44°C, or 45°C for various lengths of time. Survival assays were then performed.

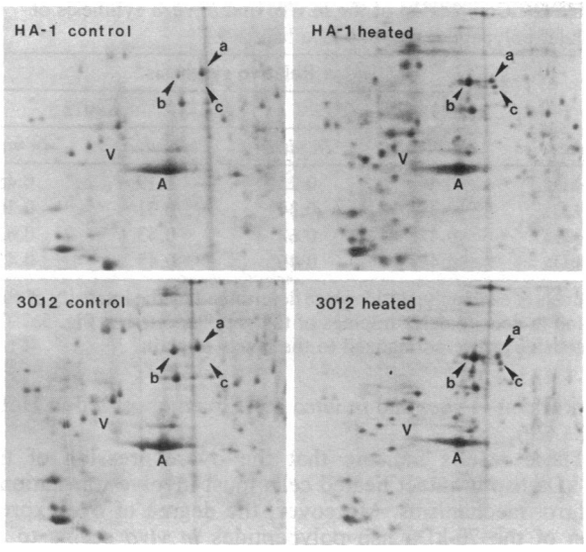

FIG. 2. Two-dimensional gel electrophoretic analysis of proteins synthesized in HA-1 cells and in 3012 HR cells. Exponentially growing cells were labeled with [^{35}S]methionine for 4 hr. Two-dimensional gel electrophoresis, with migration in the first dimension from right to left and migration in the second dimension from top to bottom, was performed as described (15). Heated HR 3012 and HA-1 cells were exposed to 45°C for 15 min and labeled from 4 to 8 hr later. Only a section of each autoradiogram is shown. The locations of 70-kDa hsp-a (a), 70-kDa hsp-b (b), and 70-kDa hsp-c (c) and the locations of two major cellular polypeptides, actin (A) and vimentin (V), are identified.

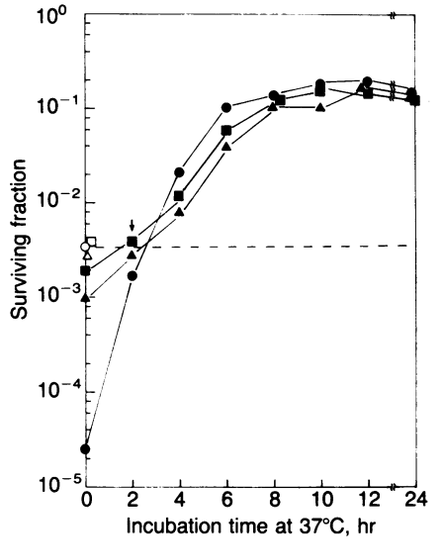

FIG. 3. Development of thermotolerance in Chinese hamster HA-1 cells and HR cells. Exponentially growing cultures were first exposed to a 45°C, 15-min treatment and then incubated at 37°C for various times. After such incubations, cells were challenged with a second heat treatment, 45 min at 45°C for HA-1 cells (●) or 75 min at 45°C for the 3011 (▲) and 3012 (■) HR lines. Survival curves after the combined heat treatments were plotted as a function of 37°C incubation time between the two heat treatments. Survival curve after the second challenge treatment alone for HA-1 (○), 3011 (△), and 3012 (□) is indicated by the dashed line.

Development of Thermotolerance in the HR Variants. The observations that mild heating of the HR lines resulted in an additional increase in the expression of the 70-kDa hsp led us to examine whether thermotolerance could be induced in the HR cell lines. HA-1 and HR cells were first exposed to 45°C for 15 min, then incubated at 37°C for various times, and their survival after a subsequent heat challenge was determined. The second heat treatment was chosen to give isosurvival levels at 45°C for all cell types receiving no preheat treatment: 45 min exposure for HA-1 cells and 75 min for the 3011 and 3012 HR variants (see Fig. 1). Thermotolerance develops in the HA-1 line and the HR variant lines 3011 and 3012 after an identical priming treatment (Fig. 3). Thermotolerance reached maximum 6–8 hr after the priming dose. In these experiments, we could not detect any significant differences in the kinetics of development of thermotolerance between the HA-1 and HR lines.

Table 1. Quantitation of the 70-kDa hsp in two-dimensional gels

Cells	Relative synthesis of 70-kDa hsps		
	a	b	c
HA-1			
Control	0.07	0.01	ND
Heated, labeled at hr 0–4	0.03	0.22	0.03
Heated, labeled at hr 4–8	0.04	0.23	0.04
3012			
Control	0.09	0.08	ND
Heated, labeled at hr 0–4	0.06	0.47	0.03
Heated, labeled at hr 4–8	0.07	0.48	0.07

Cells were heated at 45°C for 15 min and labeled for 4 hr at 37°C immediately after heating or 4 hr later. Two-dimensional gels of total cell extracts were electrophoresed and analyzed as described. Control cells were labeled for 4 hr at 37°C without heating. The numbers represent the ratio of the intensity of the 70-kDa hsp spots to the intensity of actin, which has been shown to be constant under these experimental conditions (12). The data represent the average of two determinations. ND, not detectable.

Altered Expression of the 70-kDa hsp mRNA in the HR Variants. The data presented in Fig. 2 and Table 1 indicate that the rate of synthesis of the 70-kDa hsp-b polypeptide may be altered in the HR variants. This aspect was further explored by comparing the levels of mRNA coding for these polypeptides in HA-1 and HR cells. Total cellular RNA was isolated from control and heated HA-1 and HR 3012 cells and translated in a rabbit reticulocyte system. Total cellular RNA was analyzed because of the reported difficulties in fractionating RNA in heated cells (19). Two-dimensional analysis of the translation products indicated that the relative levels of mRNA coding for 70-kDa hsp-b were higher in control and heated HR 3012 cells than in their HA-1 counterparts (Fig. 4). The patterns of proteins synthesized *in vivo* and *in vitro* in

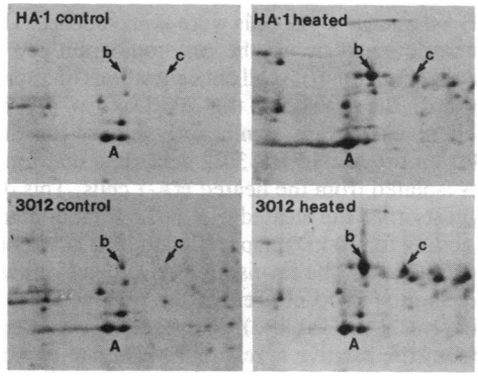

FIG. 4. Analysis of *in vitro* translation products of total cellular RNA isolated from control and heated HA-1 and HR 3012 cells. In the case of the heated cells, cells were first exposed to 45°C for 15 min and RNA was isolated after 6 hr of incubation at 37°C. The locations of actin (A), 70-kDa hsp-b (b), and 70-kDa hsp-c (c) are indicated.

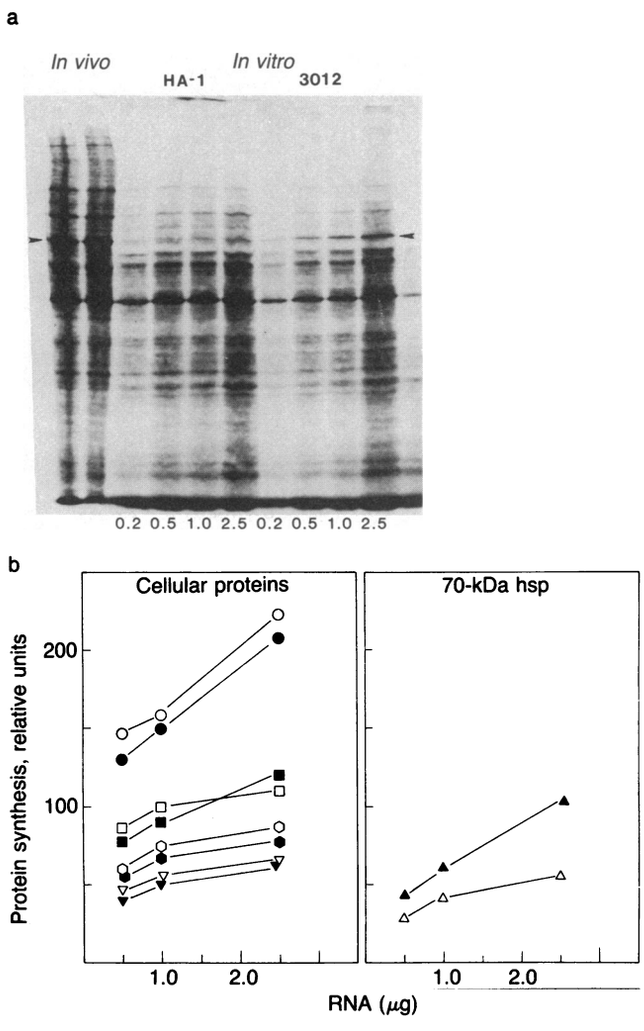

FIG. 5. Comparison of hsp synthesis *in vivo* and *in vitro*. (*a*) HA-1 and HR 3012 cells were first incubated at 45°C for 15 min and then at 37°C incubation for 6 hr. A small aliquot of cells was then pulse labeled with [^{35}S]methionine for 1 hr; the rest of the cells were used for isolation of total cellular RNA. *In vitro* translation was performed with various levels of input RNA. The arrowhead indicates the 70-kDa hsp. (*b*) A summary of the densitometric analysis of the fluorogram in *a*. Proteins from HA-1 cells are represented by open symbols; proteins from HR 3012 cells, by closed symbols. ○ and ●, Actin; □ and ■, 59 kDa; ○ and ●, 62 kDa; ▽ and ▼, 34 kDa.

heated HA-1 and HR 3012 cells were also compared (Fig. 5*a*). Densitometric analysis of the autoradiogram led to two conclusions (Fig. 5*b*). (*i*) At all concentrations of total cellular RNA tested, the levels of the 70-kDa hsp polypeptides translated *in vitro* were significantly higher for the RNA isolated from the heated HR 3012 cells than that found with the RNA isolated from the heated HA-1 cells. This observation suggests that the heated HR 3012 cells contain more mRNA coding for 70-kDa hsp, reflecting the situation found *in vivo* (Fig. 2). (*ii*) The levels of 70-kDa hsp synthesized in heated cells *in vivo* do not reflect the relative level of their respective mRNAs (Table 2). With actin as a standard of comparison, the relative levels of 70-kDa hsp expressed *in vivo* are approximately 2.5-fold higher than the level expressed *in vitro* in both HA-1 and HR 3012 cells. On the other hand, the expression of two cellular polypeptides, 34-kDa and 62-kDa, is identical in both situations, while the expression of the 59-kDa polypeptide is greater *in vitro* than *in vivo*. This polypeptide is vimentin; similar differences between its

Table 2. Comparison of the *in vivo* and *in vitro* synthesis of cellular polypeptides and 70-kDa hsp

	Relative synthesis*			
	HA-1		3012	
hsp	*In vivo*	*In vitro*	*In vivo*	*In vitro*
70-kDa	0.64	0.25	1.19	0.45
34-kDa	0.31	0.34	0.31	0.36
59-kDa	0.47	0.62	0.43	0.62
62-kDa	0.43	0.46	0.45	0.42

*Levels of each polypeptide were determined by the intensity of each band in densitometer tracings of the gel illustrated in Fig. 5*a*. The intensities were normalized to the levels of actin.

expression *in vivo* and *in vitro* have been observed in HeLa cells (20).

These results indicate that the overexpression of the 70-kDa hsp in intact heated cells must involve translational control mechanisms. Moreover, the degree of overexpression of the 70-kDa hsp polypeptides *in vivo* seems to be similar in HA-1 and HR 3012 cells (≈2.5 fold), suggesting that this translational control mechanism(s) is probably not altered in the HR 3012 cells.

Growth Characteristics of the HR Lines. The growth characteristics of the HR lines were of interest because it is well known that heat sensitivity is cell cycle specific (21). We examined the doubling time, cell size, cell-cycle distribution, and chromosome number of HR lines (Table 3). No significant difference in growth characteristics was observed between HA-1 cells and their HR variants.

DISCUSSION

In this report we have described the isolation and partial characterization of stable HR variants from the HA-1 line of Chinese hamster fibroblasts. The HR phenotype has been stable for over 6 months in culture at 37°C; it is not associated with altered growth properties, distributions in the various phases of the cell cycle, or changes in chromosome number.

Our analysis of the hsp in HA-1 cells and their HR variants utilizing two-dimensional gel electrophoresis revealed that the 70-kDa hsp identified in HA-1 cells (3, 5, 7) represents several polypeptides, possibly related. These proteins have at least three major components of approximate molecular mass of 68, 70, and 72 kDa, respectively. The complex nature of the 70-kDa hsp is characteristic of avian and mammalian cells (22, 23). The family of 70-kDa hsp found in HA-1 cells is the simplest one found so far in mammalian cells. One member, 70-kDa hsp-b, is found in cells under normal growth conditions at 37°C, and its expression is enhanced after exposure to elevated temperatures. A second member, 70-kDa hsp-c, is found exclusively in heat-shocked cells. The third member, 70-kDa hsp-a, very likely is a mitochondria-associated protein, called Mitcon 3 by Anderson (24). We could not find any evidence for either the synthesis of 70-kDa hsp-c or the presence of translatable mRNA encoding for it in either HA-1 or HR cells grown at 37°C.

Table 3. Comparison of wild type and HR HA-1 cells with respect to several cellular parameters

Cell line	Cell size, μm^3	Fraction in cell cycle			Number of chromosomes	Doubling time, hr
		G1	S	G2		
HA-1	1845	0.277	0.501	0.222	21 ± 1	16
2242	1885	0.245	0.463	0.292	21 ± 1	17
3011	1845	0.235	0.510	0.254	21 ± 1	16
3012	1860	0.262	0.478	0.263	21 ± 1	16

The HR phenotype of all of our variants examined so far is associated with an alteration in the expression of the 70-kDa hsp-b under normal growth conditions (37°C). Pulse labeling experiments indicated an increased rate of synthesis of this polypeptide in the HR variants. We have also demonstrated increased levels of mRNA coding for this polypeptide (70-kDa hsp-b) under normal growth conditions in one of the HR variants, 3012. We could not detect any alterations in the expression of 70-kDa hsp-c in the HR variants under normal growth conditions, neither at the level of protein synthesis in whole cells nor at the level of mRNA translated *in vitro*. Recently, there have been reports describing the localization of inducible forms of 70-kDa hsp (analogous to the 70-kDa hsp-c found in HA-1 cells) in mammalian and *Drosophila* cells (25, 26). The authors of these reports have attempted to correlate the intracellular localization of such polypeptides with their putative function in protection of cells from heat-induced damage. If, indeed, the family of 70-kDa hsp plays a role in protecting cells against heat-induced damage, then our studies demonstrate that increased levels of the constitutive form of the 70-kDa hsp—i.e., 70-kDa hsp-b—are sufficient to alter the cell's response to heat.

Mild heat shock altered the expression of the 70-kDa hsp family in both HA-1 and HR cells and led to the appearance of thermotolerance. This result suggests that although the HR variants are significantly more resistant to heat than HA-1 cells are, they are still capable of developing thermotolerance. It appears that the intrinsic heat resistance of the HR variants described here does not necessarily alter the ability of cells to develop transient thermotolerance. One intriguing observation is that the levels of 70-kDa hsp synthesized by the variants after an exposure to mild heat treatment are higher than those synthesized by HA-1 cells treated similarly. The reason for this difference is not clear at present.

The finding that translational control mechanisms may be responsible for the overexpression of the hsp after a mild heat shock parallels reports of similar observations in heated *Drosophila* and HeLa cells (19, 27–30). The degree of translational control, however, was similar in HA-1 and HR cells.

Recently, HR variants of human melanoma cells have been isolated (9). In these variants, the levels of hsp were found to be identical to those in the wild type under normal growth conditions. However, a distinct change in cholesterol levels was found to be associated with the HR phenotype (31). On the other hand, the cholesterol levels of HA-1 cells and the HR variants described in this report were found to be identical (R. Anderson and G. M. Hahn, personal communication). These results indicate that alterations in parameters other than the expression of hsp can occur during development of thermotolerance or stable heat resistance. Nevertheless, they do not negate our working hypothesis that the hsp also play a role in determining the heat response of the cells.

In summary, the association of increased levels of 70-kDa hsp in the stable HR variants and in transiently thermotolerant HA-1 and HR cells provides additional evidence that this gene product may play a role in protecting cells from damage inflicted by elevated temperatures.

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