Characterization of the Thermotolerant Cell.
I. Effects on Protein Synthesis Activity and the Regulation of Heat-Shock Protein 70 Expression

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Abstract. Exposure of mammalian cells to a nonlethal heat-shock treatment, followed by a recovery period at 37°C, results in increased cell survival after a subsequent and otherwise lethal heat-shock treatment. Here we characterize this phenomenon, termed acquired thermotolerance, at the level of translation. In a number of different mammalian cell lines given a severe 45°C/30-min shock and then returned to 37°C, protein synthesis was completely inhibited for as long as 5 h. Upon resumption of translational activity, there was a marked induction of heat-shock (or stress) protein synthesis, which continued for several hours. In contrast, cells first made thermotolerant (by a pretreatment consisting of a 43°C/1.5-h shock and further recovery at 37°C) and then presented with the 45°C/30-min shock exhibited considerably less translational inhibition and an overall reduction in the amount of subsequent stress protein synthesis. The acquisition and duration of such "translational tolerance" was correlated with the expression, accumulation, and relative half-lives of the major stress proteins of 72 and 73 kD. Other agents that induce the synthesis of the stress proteins, such as sodium arsenite, similarly resulted in the acquisition of translational tolerance. The probable role of the stress proteins in the acquisition of translational tolerance was further indicated by the inability of the amino acid analogue, L-azetidine 2-carboxylic acid, an inducer of nonfunctional stress proteins, to render cells translationally tolerant. If, however, analogue-treated cells were allowed to recover in normal medium, and hence produce functional stress proteins, full translational tolerance was observed. Finally, we present data indicating that the 72- and 73-kD stress proteins, in contrast to the other major stress proteins (of 110, 90, and 28 kD), are subject to strict regulation in the stressed cell. Quantitation of 72- and 73-kD synthesis after heat-shock treatment under a number of conditions revealed that "titration" of 72/73-kD synthesis in response to stress may represent a mechanism by which the cell monitors its local growth environment.

A LL organisms studied to date respond to elevations of some 5°C or more above their normal physiological temperature with the rapid and preferential synthesis of a small number of highly conserved proteins, the heat-shock proteins (for reviews, see 4 and 24). A heat-shock–like response also can be induced in cells by exposure to a wide variety of other environmental insults, such as amino acid analogues, heavy metals, ethanol, and certain metabolic poisons (1, 13, 17, 33). Consequently, the response is often referred to more generally as the stress response, and the proteins induced referred to as the stress proteins.

Collectively, the stress proteins appear to function in the protection and enhanced survival of the cell experiencing stress. For example, it has been demonstrated that mammalian cells exposed to a mild, sublethal temperature elevation acquire resistance to a subsequent, lethal heat-shock challenge. This phenomenon, termed acquired thermotolerance (7, 11), appears dependent upon the production of the stress proteins. For example, a multiplicity of agents, having in common the ability of eliciting stress protein synthesis,

also confers the thermotolerant state (8, 18). Conversely, production of nonfunctional stress proteins, via exposure to amino acid analogues, or inhibiting the synthesis of the stress proteins with agents such as cycloheximide (CHX)[1] prevents the acquisition of thermotolerance (12, 20, 25). Finally, the kinetics of thermotolerance induction and decay are correlated with that of stress protein synthesis and degradation, respectively (15, 21, 32). While collectively these findings demonstrate a strong correlation between stress protein expression and the development of thermotolerance, they do not clearly define which stress proteins are responsible for this protective effect, or furthermore, how these proteins might function to enhance cell survival. Indeed, while some studies on thermotolerance have pointed to an involvement of the 70-kD stress proteins in this phenomenon (e.g., 19, 21, 32), other experiments support the notion that the small stress proteins are important (2, 26). In addition, the actual

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1. *Abbreviations used in this paper:* Azc, L-azetidine 2-carboxylic acid; CHX, cycloheximide.

involvement of the stress proteins has been questioned by recent reports describing the acquisition of thermotolerance in the presence of agents that inhibit the synthesis of the stress proteins (9, 10, 16, 37).

In view of the current literature on thermotolerance, we undertook the present study to develop an experimental approach, distinct from cell survival assays, to study the thermotolerant cell. A number of studies have indicated that the acquisition of thermotolerance is associated with protection of translational activity after heat-shock treatment (28, 30, 31). Furthermore, immunological studies have demonstrated an association of the 70-kD stress proteins with the translational machinery in cells recovering from stress (36). Therefore, we have carefully examined the cellular response to stress, at the level of translation, as a means of defining some alternative, biochemical parameters of the thermotolerant cell.

Materials and Methods

Cell Culture

Continuous lines of baby hamster kidney cells (BHK), Chinese hamster ovary cells (CHO), rat embryo fibroblast cells (REF-52 [34]), HeLa cells, and human 293 cells were grown in DME supplemented with 10% fetal bovine serum (complete DME) on plastic dishes (Falcon Labware, Oxnard, CA).

Cells were placed under stress by either (a) exposure to 5 mM L-azetidine 2-carboxylic acid (Azc; Calbiochem Biochemicals, San Diego, CA) for 12 h at 37°C, (b) exposure to 80–240 μM sodium arsenite (Sigma Chemical Co., St. Louis, MO) for 1.5 h at 37°C, or (c) heat-shock treatment outlined as follows. Cells were stressed using either a single heat-shock (at 43° or 45°C) or a double heat-shock regime, whereby cells initially stressed at 43°C were allowed to recover at 37°C for varying times before a second stress at either 43° or 45°C. Duration of heat-shock treatment for each protocol is given in the figure legends. Cells were heated in waterbaths placed inside of humidified incubators maintained at the desired temperature. For recovery from stress, the cells were either returned to 37°C, or in the case of Azc and arsenite treatment, the culture medium was removed, the cells washed three times in DME, and then incubated at 37°C in cell-conditioned complete DME.

For treatment of cells with cycloheximide (Sigma Chemical Co.), a stock solution was added to the culture medium at a final concentration of 80 μg/ml, and the cells incubated at 37°C for 30 min before experiments were begun. After the indicated times of treatment, CHX was removed from the cells using the washing procedure described above.

Preliminary experiments were performed to ascertain that the experimental protocols used did indeed result in the acquisition of thermotolerance. REF-52 cells (5 × 10⁵ cells/35-mm dish) were stressed by either a 43°C/1.5-h shock, a 45°C/30-min shock, or a 43°C/1.5-h shock followed by a 37°C recovery period (8–12 h) and a subsequent 45°C/30-min shock. Cell viability was then determined 24, 48, and 72 h later by trypsinization and cell counting, and growth curves compared with the 37°C, nonshocked cells. While the 43°C/1.5-h shock resulted in a delay of growth, little or no loss in cell viability was observed. In contrast, the 45°C/30-min treatment resulted in a significant delay of cell growth and ∼70–80% cell killing. Cells first made thermotolerant (43°C/1.5 h) and heat-shock treated 8–12 h later at 45°C/30-min exhibited considerably less delay of cell growth and a viability of >90%.

Metabolic Labeling Studies

Cells growing on 35-mm dishes were placed under stress (either Azc, arsenite, or heat shock), or CHX treated in combination with heat stress, and then recovered at 37°C for varying periods of time as indicated in the figure legends. For pulse-labeling, the culture medium was removed and the cells were washed with DME lacking the particular amino acid being replaced by radioisotope. Cells were labeled for 1 h at 37°C with [³⁵S]methionine (New England Nuclear, Boston, MA; specific activity, >800 Ci/mmol) or [³H]leucine (New England Nuclear; specific activity, 70 Ci/mmol) in 0.5 ml of methionine-free or leucine-free DME (Gibco, Grand Island, NY) sup-

plemented with 2% dialyzed calf serum. After labeling, the medium was removed, the cells washed with cold PBS, lysed in Laemmli sample buffer (14), and analyzed by one-dimensional SDS-PAGE as described previously (35). In all cases, before gel analysis, samples were equalized on the basis of protein concentration as determined by the Bradford assay (3).

Determination of Protein Half-Lives

Cells were pulse-labeled at 37°C for 15 min with [³⁵S]methionine under the following conditions: (a) at 37°C (control); (b) after a 43°C/1.5-h heat-shock treatment and a further 1-h incubation at 37°C; (c) in the presence of 5 mM Azc after a prior 12-h exposure period; (d) after a 12-h Azc exposure, removal of the analogue, and a further 12-h recovery period in normal culture medium; and (e) after exposure to 100 μM sodium arsenite for 1.5 h, removal of the heavy metal, and a further 1-h incubation at 37°C in normal culture medium. After pulse-labeling, radiolabel was removed by extensive washing, and the cells incubated at 37°C in complete DME supplemented with excess nonradioactive methionine (90 ng/ml). Cells were then split into two groups. One group of cells was further incubated at 37°C for only 15 min (to allow for polysome run off) and then immediately harvested (i.e., "pulse" cells). A second group of cells was further incubated at 37°C for progressively longer intervals, encompassing a 96-h period, before harvesting (i.e., "chase" cells). All determinations were performed in triplicate. An equal portion of the total cell lysate was analyzed in each case, and the individual proteins of interest were excised and their radioactivity determined by liquid scintillation counting as described below. The values shown in Table I represent an average of three values determined for each cell lysate.

Quantitation of [³⁵S]Methionine and [³H]Leucine-labeled Proteins

After one-dimensional SDS-PAGE gel analysis, the regions of the dried gel containing the radiolabeled proteins of interest were visualized by autoradiography and excised. Individual gel slices were placed in glass scintillation vials and swollen in 100-μl water for 1 h before addition of 500 μl Protosol (New England Nuclear) and incubation at 37°C for 24 h with constant agitation. Aquasol scintillant (New England Nuclear) was then added to each vial to a final volume of 3.0 ml and the [³⁵S]methionine or [³H]leucine-associated radioactivity determined by scintillation counting. For each determination, protein samples from experiments done in triplicate were analyzed on 10, 12.5, or 15% polyacrylamide gels to give maximal resolution of the desired protein(s).

Results

A comparison of the protein synthesis patterns in rat embryo fibroblast (REF-52) cells recovering from a mild (43°C) or severe (45°C) heat-shock treatment is shown in Fig. 1. Immediately after the 43°C/1.5-h heat-shock treatment (A), induction of the major heat-shock (or stress) proteins (of 110, 90, 73, and 72 kD), as well as a minor stress protein (32 kD), was observed. After return of the cells to 37°C, there was a slight but transient inhibition of overall cellular protein synthesis. While the elevated synthesis of the 110- and 90-kD stress proteins continued for several hours, synthesis of the 72- and 73-kD stress proteins began to decline within 4–6 h and were no longer detected by 6–8 h, respectively, post-heat shock. Apart from the slightly elevated synthesis of the 100- and 90-kD stress proteins, by 9 h of recovery the cells had returned to translation patterns similar to that of the non-stressed, 37°C cells.

In contrast, cells presented a 45°C/30-min heat-shock treatment exhibited a complete inhibition of protein synthesis for as long as 5 h after recovery back at 37°C (Fig. 1, B). Upon return of translation, there was a marked induction of stress protein synthesis. By 13 or 14 h of recovery, stress protein synthesis had declined, and the cells had returned to essentially normal 37°C translation patterns. As was observed
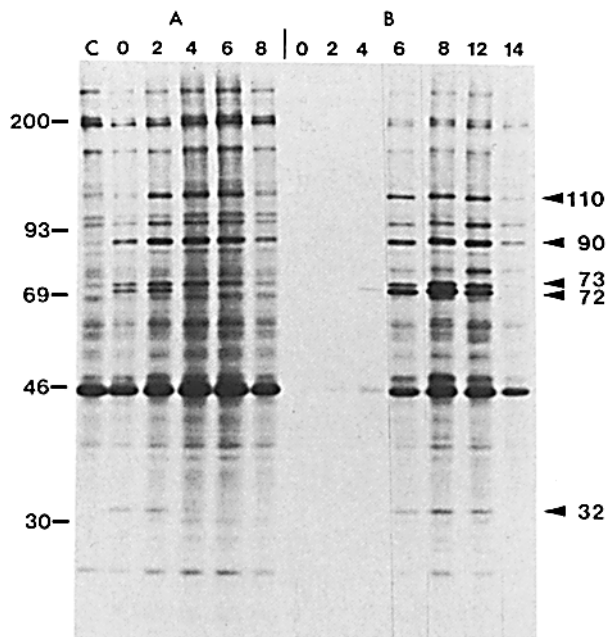
Figure 1. Time course comparison of stress protein synthesis in rat fibroblasts heat-shock treated at either 43° or 45°C. Rat embryo fibroblast (REF-52) cells, growing on 35-mm plastic dishes, were heat-shock treated at either 43°C for 1.5 h or 45°C for 30 min. The cells were returned to 37°C, and at 2-h intervals thereafter were pulse-labeled for 1 h with [^{35}S]methionine. The [^{35}S]methionine-labeled proteins were examined by SDS-PAGE on a 12.5% poly-acrylamide gel. An equivalent amount of total protein was analyzed in every case. Shown is a fluorograph of the gel with molecular mass markers indicated on the left and the position of the major stress proteins indicated on the right. The first lane on the left shows the proteins synthesized in cells incubated at 37°C (*C*). *A* and *B* represent cells pulse-labeled after heat-shock treatments of 43°C for 1.5 h or 45°C for 30 min, respectively, at 0–1 h (*0*), 2–3 h (*2*), 4–5 h (*4*), 6–7 h (*6*), 8–9 h (*8*), 12–13 h (*12*), and 14–15 h (*14*) post-reversal.

during recovery from a 43°C/1.5-h heat-shock treatment, repression of 73- and 72-kD stress protein synthesis preceded that of the other stress-induced proteins in the cells recovering from the 45°C/30-min heat-shock treatment.

Previous studies have shown that cell killing by a severe heat-shock treatment can be reduced by a prior exposure of cells to a sublethal heat-shock treatment, a phenomenon termed acquired thermotolerance (7, 11). In addition, it has been demonstrated in a number of different systems, including those of plant (22), insect (23, 28, 30), and vertebrate (31) origin, that such pre–heat-shock treatments can afford the cellular translational machinery a significant measure of thermoprotection. Therefore, as a means of studying the thermotolerant cell by a more direct, biochemical approach, we characterized the development of thermotolerance at the translational level.

To examine in detail the temporal development of such "translational tolerance," REF-52 cells were given a 43°C/1.5-h heat-shock treatment, allowed to recover at 37°C for varying time periods, and then subjected to the severe 45°C/30-min heat-shock challenge (Fig. 2). Preliminary cell survival studies confirmed that this protocol did indeed re-

sult in the acquisition of thermotolerance (see Materials and Methods). In general, cells first made thermotolerant recovered translational activity more rapidly than did the non-tolerant cells after the 45°C/30-min challenge. In addition, the extent of such translational recovery after the 45°C/30-min challenge appeared dependent upon the length of the 37°C recovery period after the initial 43°C/1.5-h priming treatment. For example, cells allowed to recover for only 4 h after the priming heat treatment recovered translational activity 4 h after the second heat-shock treatment (Fig. 2 *A*) as compared with the 6 h required for the nontolerant cells (Fig. 1 *B*). Moreover, cells allowed to recover for either 8, 12, or 24 h after the priming heat treatment recovered translation patterns by 2 h after the 45°C/30-min challenge (Fig. 2, *B–D*, respectively). In every case, the tolerant cells responded to the 45°C/30-min challenge with the synthesis of the stress proteins for some 8 h or longer, and in general exhibited a faster return to normal (37°C) translation patterns than did the nontolerant cells (e.g., compare Fig. 2, *A–D* with Fig. 1 *B*). With recovery periods in excess of 8 h, synthesis of the 72-kD/73-kD stress proteins was extended during recovery from the 45°C challenge (i.e., compare lane *6* in each of Fig. 2, *A–D*).

Having established the parameters of translational tolerance in the rat fibroblast system, we wanted to demonstrate the generality of these results in a variety of other mammalian cell lines. For these experiments, we used a standard protocol in which the cells were made tolerant by a 43°C/1.5-h heat shock and subsequent 8 h recovery at 37°C before the 45°C challenge. Shown in Fig. 3 is a comparison of the translational profiles of nontolerant vs. tolerant CHO, BHK, HeLa, and 293 cells recovering from the 45°C challenge. In every case, the recovery of translational activity after the 45°C heat shock was accelerated in the thermotolerant cells (right half of Fig. 3, *A–E*) as compared with their nontolerant counterparts (left half of Fig. 3, *A–E*). Again, similar to the rat fibroblasts, once protein synthesis had resumed, induction of the major stress proteins was observed in both the tolerant and nontolerant cells. Although not shown, but similar to the situation with the rat fibroblasts, the duration of stress protein synthesis was notably reduced in the tolerant cells. A comparison of the four mammalian cell lines by this translational assay indicates that each cell line possesses a unique, intrinsic level of translational tolerance to 45°C challenge. In this regard, it should be noted that the duration of the 45°C heat-shock treatment was adjusted for each cell line to produce comparable results in the translational assay. For example, the length of the standard 30-min, 45°C heat-shock treatment used previously for REF-52 cells was reduced to 20 min for CHO cells (Fig. 3 *A*), kept at 30 min for BHK cells (Fig. 3 *B*), or increased to 40 min for HeLa and 293 cells (Fig. 3, *D* and *E*). For comparison, shown in Fig. 3 *C* are the results obtained in HeLa cells given the standard 30-min, 45°C heat-shock treatment showing their relatively high level of intrinsic tolerance. Therefore, relative to REF-52 cells one can define cell lines that display reduced (CHO), similar (BHK), or enhanced (HeLa and 293) translational tolerance to heat-shock treatment.

Since a multiplicity of stress agents other than heat can induce stress protein synthesis (reviewed in 4, 24) and, by cell survival criteria, can also induce thermotolerance (e.g., 8, 18), we were interested in determining whether thermopro-
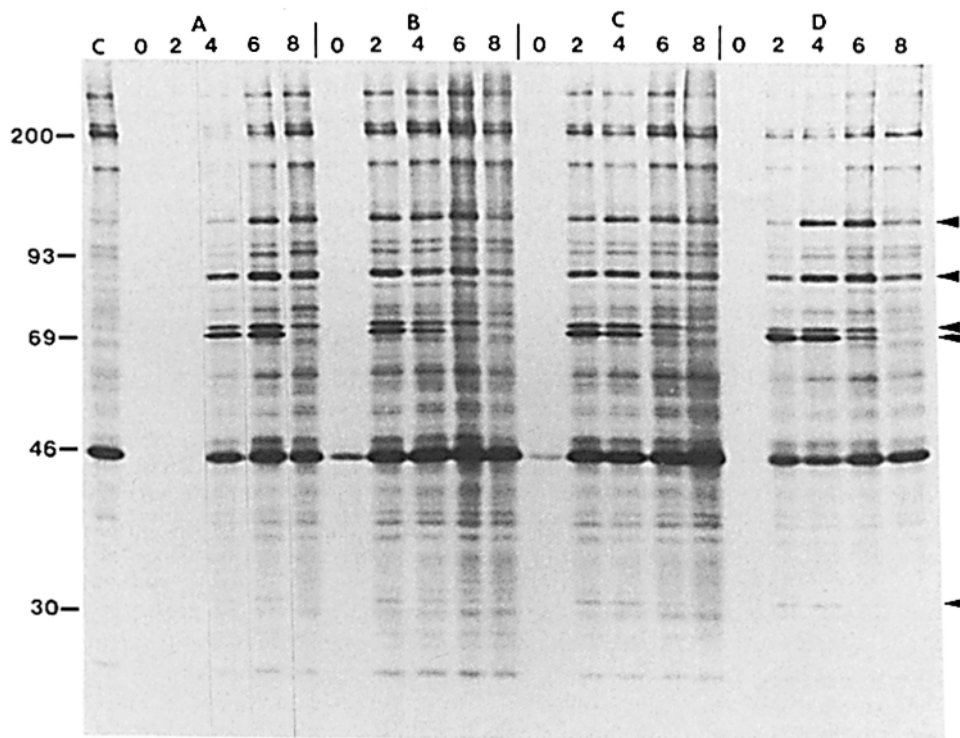
Figure 2. Temporal development of translational tolerance to heat-shock challenge. REF-52 cells were subjected to an initial, mild heat-shock treatment (43°C/1.5 h), allowed to recover for varying times at 37°C (e.g., 4, 8, 12, or 24 h), and then given a second, more severe heat-shock challenge (45°C/30 min). Upon return to 37°C, the cells were pulse-labeled with [^{35}S]methionine at 2-h intervals, the cells harvested, and the proteins analyzed by SDS-PAGE. Shown is a fluorograph of the gel with molecular mass markers indicated on the left and on the right, in descending order, arrows marking the positions of the 110-, 90-, 73-, 72-, and 32-kD stress proteins (as in Fig. 1). *A–D* represent cells made tolerant and provided a recovery interval of either 4, 8, 12, or 24 h between the first and second heat-shock treatments, respectively. After the 45°C/30-min shock the cells were returned to 37°C and pulse-labeled at 0–1 h (*0*), 2–3 h (*2*), 4–5 h (*4*), 6–7 h (*6*), and 8–9 h (*8*) post-reversal.
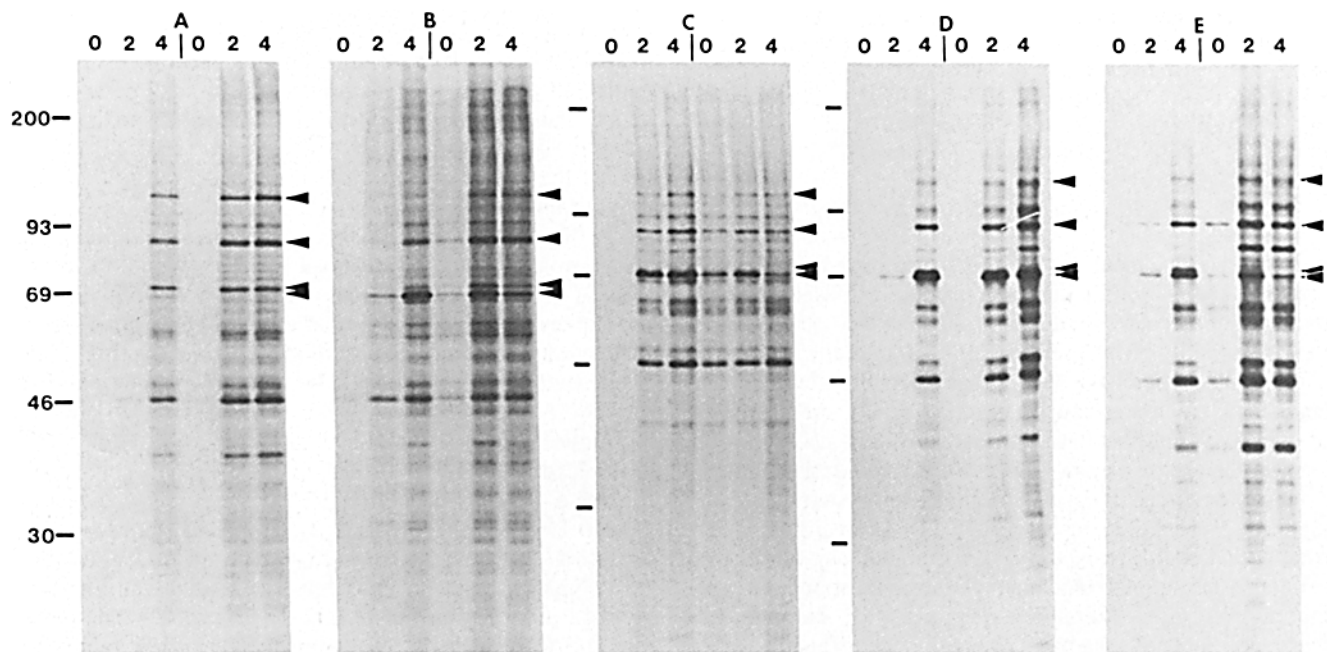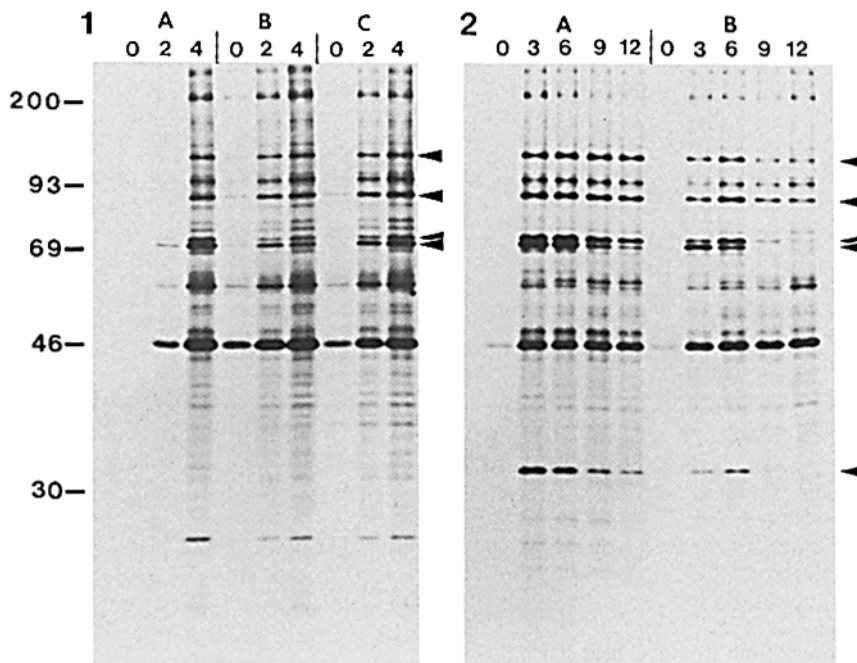

Figure 3. Translational thermotolerance is observed in a variety of human and rodent cell lines. CHO cells, BHK cells, HeLa, and human 293 cells were subjected to a 45°C heat-shock treatment after either no pretreatment (nontolerant) or a prior mild heat-shock (43°C/1.5 h) and subsequent recovery for 8 h at 37°C (tolerant). The cells were incubated at 45°C for 20 min (CHO, *A*), 30 min (BHK, *B*; HeLa, *C*), or 40 min (HeLa, *D*; 293, *E*), then returned to 37°C and pulse-labeled with [^{35}S]methionine at 2-h intervals thereafter. The labeled proteins were analyzed by SDS-PAGE. Shown are fluorographs of the gels obtained from CHO (*A*), BHK (*B*), HeLa (*C* and *D*), and 293 (*E*) cells, with molecular mass markers indicated to the left. To the right of each fluorograph are arrows indicating, in descending order, the 110-, 90-, 73-, and 72-kD stress proteins. Nontolerant (left half of each panel) and tolerant (right half of each panel) cells were labeled at 0–1 h (*0*), 2–3 h (*2*), and 4–5 h (*4*) during recovery from the 45°C heat shock.

tection of translational activity (i.e., translational tolerance) could be induced in cells by priming treatments using different stress agents and second, whether the state of tolerance induced by one agent conferred protection of translational activity against challenge by another. Toward this end, heat and the heavy metal, sodium arsenite, were used as both the priming and challenging agents in the translational assay. As shown in Fig. 4, cells receiving either heat-shock or sodium arsenite pretreatments acquired an equivalent degree of translational tolerance to the 45°C challenge (Fig. 4, panel *1, B* and *C*), as compared with the nontolerant cells (panel *1A*). In a reciprocal experiment (Fig. 4, panel *2*), exposure of either tolerant (heat-shock primed) or nontolerant cells to high concentrations of sodium arsenite (240 μM) resulted in a similar short-lived (∼2 h) inhibition of translation. However, as before, the duration of subsequent stress protein synthesis was markedly reduced in the tolerant cells (panel *2B*) versus the nontolerant cells (panel *2A*).

To this point we had established (*a*) that a severe 45°C/30-min heat-shock treatment could render REF-52 cells translationally inactive for some 5 h and (*b*) that the recovery of translational activity was accelerated in cells first made tolerant by pretreatment with either heat or sodium arsenite, two agents that induce the synthesis of the stress proteins. To more carefully examine the possible role of the stress proteins in the acquisition of such translational tolerance, we next examined the effects of another stress protein inducer, the amino acid analogue of proline, Azc. In this case, however, we and others have shown that while synthesis of the stress proteins will occur in the presence of Azc, the proteins produced appear "nonfunctional" as assayed by their inability to correctly localize within the cell (5, 36). Therefore, we

compared translational activity after a 45°C challenge in cells pretreated with heat, pretreated with a continuous exposure (12 h) to Azc, or pretreated with Azc for 12 h and then incubated (12 h) in complete medium lacking the analogue (to allow for production of "functional" stress proteins). As shown in Fig. 5, in those cells continuously treated with Azc for 12 h, then given a 45°C challenge, no translational tolerance was observed (Fig. 5 *C*). In contrast, in Azc-treated cells allowed to recover in the absence of the analogue before heat-shock challenge (thereby resulting in the production of functional stress proteins [36]), full translational tolerance was observed (Fig. 5 *D*). For comparison, the results obtained for thermotolerant (panel *B*) and nontolerant cells (*A*) using the standard heat-shock protocol are also shown.

The data presented in Fig. 2 indicated that maximal translational tolerance was observed in cells allowed to recover at 37°C for 8 h after the initial priming heat-shock treatment. A considerable degree of tolerance persisted even in cells allowed to recover up to 24 h before a second heat-shock challenge. Hence, if the heat-shock proteins are somehow involved in the acquisition of translational tolerance, one would expect their half-lives to be relatively long. To determine the half-lives of the stress proteins, pulse-chase analyses were performed in cells after heat-shock, sodium arsenite, and Azc treatments, as described in Materials and Methods. Approximate half-lives of the 72/73-kD and 90-kD stress proteins, as well as actin are shown in Table I. In general, the stress proteins induced by the 43°C/1.5-h priming heat-shock treatment possessed rather long half-lives, on the order of 48 h. Interestingly, actin half-life (74 h at 37°C) was reduced after the heat-shock treatment (54 h). In the case of proteins synthesized in the presence of Azc, half-lives were dramati-

Figure 4. Exposure of cells to sodium arsenite results in the acquisition of translational thermotolerance. In panel *1*, REF-52 cells were exposed to a 45°C/30-min heat-shock treatment after (*A*) no pretreatment (nontolerant), (*B*) prior exposure to a 43°C/1.5-h heat-shock and subsequent recovery for 8 h at 37°C (tolerant), or (*C*) prior exposure to 80 μM sodium arsenite for 1.5 h, removal of the metal, and subsequent recovery for 8 h at 37°C. After the 45°C/30-min shock, the cells were returned to 37°C and pulse-labeled with [35S]methionine at 0–1 h (*0*), 2–3 h (*2*), or 4–5 h (*4*). In panel *2* both nontolerant (*A*) and tolerant (*B*) REF-52 cells (made tolerant by a prior 43°C/1.5-h heat-shock and subsequent 8-h recovery at 37°C) were exposed to 240 μM sodium arsenite for 1.5 h, the metal then removed, and the cells pulse-labeled with [35S]methionine at 0–1 h (*0*), 3–4 h (*3*), 6–7 h (*6*), 9–10 h (*9*), and 12–13 h (*12*) during the recovery period. The labeled proteins were analyzed by SDS-PAGE. Shown are fluorographs of the gels with molecular mass markers on the left and on the right of each panel, in descending order, the position of the 110-, 90-, 73-, 72-, and 32-kD stress proteins.
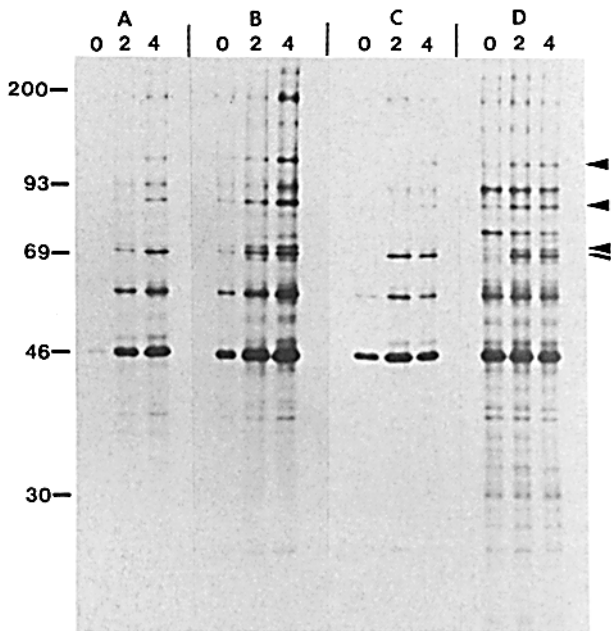
Figure 5. Acquisition of translational thermotolerance requires the prior synthesis of functional proteins. REF-52 cells were exposed to a severe heat stress, 45°C/30 min, either after no pretreatment (nontolerant); a prior 43°C/1.5-h heat-shock treatment and 8-h recovery at 37°C (tolerant); a prior and continued exposure to the amino acid analogue of proline, Azc; and a prior exposure to Azc, removal of the analogue, and subsequent recovery in normal culture medium for 12 h at 37°C. After the 45°C/30-min shock, the cells were returned to 37°C and pulse-labeled with [³⁵S]methionine at 2-h intervals. The labeled proteins were analyzed by SDS-PAGE. Shown is a fluorograph of the gel with molecular mass markers indicated on the left and on the right, in descending order, arrows marking the positions of the 110-, 90-, 73-, and 72-kD stress proteins. From the left are represented cells which received, before the 45°C/30-min heat-shock treatment, no pretreatment (A); a 43°C/1.5-h heat-shock treatment and 8-h recovery at 37°C (B); incubation with 5 mM Azc for 12 h at 37°C (C); and incubation with Azc as in C followed by a 12-h recovery at 37°C in the absence of Azc (D). Cells were incubated at 0–1 h (0), 2–3 h (2), and 4–5 h (4) during recovery from the 45°C/30-min shock.

Table I. Approximate Half-lives of Selected Mammalian Stress Proteins during Recovery from Heat-Shock, Azc, and Sodium Arsenite Treatment*

	37°C	43°C	Azc	Azc recovery	Arsenite
			Half-lives, h		
Protein					
72-kD/73-kD	—	48	3	64	38
90 kD	—	42	6	59	36
Actin	74	54	3	58	49

* See Materials and Methods for experimental protocol.

cally reduced (to ~3–6 h). However, when protein labeling was performed after recovery from Azc exposure (i.e., removal of the analogue), a striking increase in the stability of the stress proteins as well as actin was observed (half-lives of 58–64 h). Finally, the stress protein half-lives in arsenite-treated cells (36–38 h), although reproducibly less, were roughly similar to those observed in the heat-shock treated cells (42–48 h).

Numerous cell survival studies have demonstrated a positive correlation between synthesis of the stress proteins and the acquisition of thermotolerance (e.g., 15, 21, 25, 32). However, some recent studies have indicated that thermotolerance can develop in the presence of agents that prevent the increased synthesis of the stress proteins (9, 10, 16, 37). Hence, we examined the effects of CHX on the development of translational tolerance. Shown in Fig. 6, panel 1 for reference, are the translational profiles after a 45°C challenge of both nontolerant cells (A) and cells made tolerant (B) by the standard heat-shock pretreatment protocol (i.e., 43°C/1.5 h plus 8 h at 37°C). As before, heat pretreatment rendered the

cells translationally tolerant. In panel 2 are shown the results obtained in parallel when CHX was included during various intervals of the heat-shock pretreatment protocol. When just the priming 43°C/1.5-h heat-shock treatment was performed in the presence of CHX and the cells then recovered for 8 h at 37°C in the absence of the drug (panel 2A), the degree of translational tolerance to the 45°C challenge was similar to that of cells made tolerant in the absence of the drug (panel 1B). Note, however, that in these CHX-treated cells, 72/73-kD synthesis was prolonged during recovery from the 45°C challenge as compared with the control, tolerant cells (compare 2A to 1B). In the case of cells treated with CHX either during the entire heat-shock pretreatment protocol (panel 2B) or only during the 37°C recovery period before the 45°C/30-min shock (panel 2C), again translational tolerance was observed, but in this case the translation patterns were somewhat different from those of cells made tolerant in the absence of the drug. Specifically, while there was no inhibition of translation after the 45°C/30-min shock in the CHX-treated cells, the immediate return of translation was not accompanied by 72/73-kD stress protein synthesis. Rather these cells responded by an increased production of the 90- and 110-kD stress proteins along with the continued production of proteins normally synthesized at 37°C. Only after 4 h of recovery did the CHX-treated cells begin to synthesize high levels of the 72-kD and to a lesser extent the 73-kD stress proteins. Moreover, while cells made tolerant in the absence of the drug exhibited a decline in 72/73-kD production by 6 h of recovery from the 45°C/30-min shock (1B), cells made tolerant in the presence of the drug were still synthesizing 72/73 kD for as long as 10–12 h after the 45°C/30-min shock (data not shown but compare lane 6 in 1B with that in 2B or 2C). As will be discussed later (Discussion), an analysis of the polysome profiles of the cells made tolerant in the presence of CHX can fully account for the differences in the translation patterns observed.

As has been noted by others (5) and in our own preliminary quantitation studies, we observed that the absolute amount of the 72/73-kD stress proteins produced in REF-52 cells varied as a function of heat-shock severity. That is, cells treated at 43°C/1.5 h produced relatively less 72/73 kD than those treated at 45°C/30 min. Moreover, tolerant cells given a 45°C challenge produced significantly less 72/73 kD than did nontolerant cells exposed to the same treatment. In what follows, we demonstrate that cells respond to defined amounts
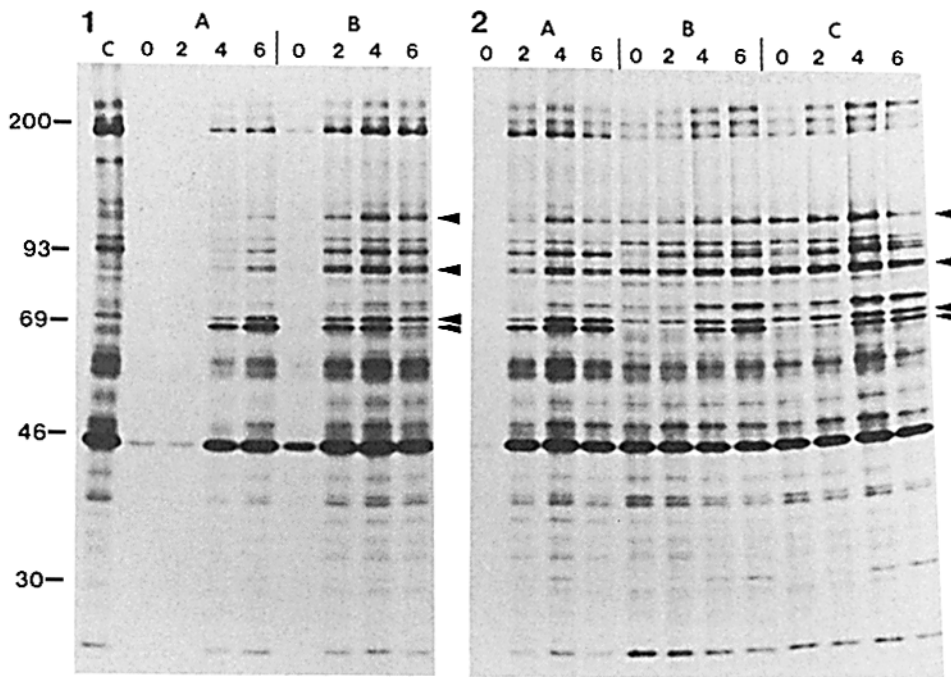
Figure 6. Effects of CHX on the development of translational tolerance. In panel *1*, REF-52 cells, either nontolerant or tolerant (i.e., given a prior 43°C/1.5-h heat-shock followed by an 8-h recovery period at 37°C), were subjected to a 45°C/30-min shock, the cells returned to 37°C, and pulse-labeled with [³⁵S]methionine at 2-h intervals during the recovery period. The labeled proteins synthesized in the nontolerant (*A*) and tolerant (*B*) cells were analyzed by SDS-PAGE. Shown is a fluorograph of the gel analyzing proteins synthesized in the 37°C control cells (*C*), or in cells labeled at 37°C 0–1 h (*0*), 2–3 h (*2*), 4–5 h (*4*), or 6–7 h (*6*) after the 45°C/30-min shock. In panel 2 the effects of CHX on the development of translational thermotolerance were examined. Cells were exposed to a 43°C/1.5-h heat-shock and subsequent recovery for 8 h at 37°C. In one case, 80 μg/ml of CHX was included during the 43°C/1.5-h shock, then removed, and the cells further incubated at 37°C for 8 h (*A*). In a second case, 80 μg/ml CHX was included during both the 43°C/1.5-h shock and subsequent recovery at 37°C for 8 h (*B*). In the third case, 80 μg/ml CHX was present only during the 8-h recovery period at 37°C after the 43°C/1.5-h shock (*C*). In all three cases the cells were then subjected to a 45°C/30-min shock (in the absence of CHX) and then returned to 37°C and pulse-labeled with [³⁵S]methionine at 2-h intervals. Labeling periods are the same as that described above in panel *1*. Shown is a fluorograph of the gel. Molecular mass markers are indicated at the left, and on the right, in descending order, the positions of the 110-, 90-, 73-, and 72-kD stress proteins.

of stress by apparently titrating the levels of the induced 72/73-kD stress proteins with such titration also depending upon the preexisting levels of the proteins in the cell. As a first approach, induction of stress protein synthesis was compared in cells after a single 43°C/2-h shock, or in cells made tolerant by this same pretreatment, then rechallenged with an identical 43°C/2-h shock. In this case, [³H]leucine labeling was done to ensure detection of the smaller 28-kD stress protein, which contains little or no methionine (34). In contrast to cells given a single 43°C/2-h shock (Fig. 7 *A*), tolerant cells rechallenged by an identical 43°C/2-h shock synthesized very little new 72/73-kD stress proteins. In contrast, modest induction of the 28-, 90-, and 100-kD synthesis was observed (Fig. 7 *B*).

As a second approach, we examined stress protein synthesis as a function of sodium arsenite concentration. As shown in Fig. 8, panel *1*, in cells exposed for 1.5 h to increasing concentrations of arsenite (80 μM, panel *A*; 160 μM, panel *B*; 240 μM, panel *C*), there occurred a corresponding increase in translational inhibition (Fig. 8, panel *1*, lane *1* in each case). In addition, the induction of stress protein synthesis increased in proportion to arsenite concentration. In particular, among the stress proteins, synthesis of the 72/73-kD proteins appeared the most responsive to increasing arsenite concentration. Hence, similar to heat induction, 72/73-kD synthesis was proportional to the degree of arsenite stress. In Fig. 8, panel *2*, using a protocol similar to that of Fig. 7, we compared stress protein induction in cells after a single arsenite treatment (160 μM) with that observed in cells primed by, and then challenged with, the same arsenite in-

sult. Analogous to the results obtained using isothermic heat-shock treatments (Fig. 7), in arsenite-primed cells subsequently challenged with an identical arsenite treatment, only minor induction of 72/73-kD synthesis was observed, in contrast to the significant induction of the other 110-, 90-, 32-, and 28-kD stress proteins (Fig. 8, panels *2A* and *2B*). These results (Figs. 7 and 8) indicate that cells primed with a given stress treatment, allowed to recover, and then challenged with exactly the same stress treatment respond by synthesizing little or no new 72/73-kD stress proteins.

Finally, to quantitate this apparent "titration" of 72/73 kD in response to varying degrees of stress, we examined: (*a*) the amount of these proteins produced as a function of stress severity; (*b*) the amount of new 72/73-kD synthesis in tolerant cells challenged by stress; and (*c*) the amount of 72/73 kD produced in cells during recovery from heat-shock treatment performed in the presence of cycloheximide. With respect to aim *a*, rat fibroblasts were steady-state labeled with [³H]leucine either after a 43°C/45-min, 43°C/1.5-h, or a 45°C/30-min heat-shock treatment. As expected, with increasing stress severity there was a corresponding increase in 72/73-kD production (Fig. 9, panel *A*). In experiment *b*, cells were first made tolerant by either a 43°C/45-min or 43°C/1.5-h shock, allowed to recover at 37°C for 8 h, and then given a 45°C/30-min shock. The cells were then returned to 37°C and steady-state labeling performed. As compared with the nontolerant cells, the tolerant cells produced significantly less 72/73 kD after the 45°C/30-min shock (Fig. 9, panel *A*). More importantly, the sum of 72/73 kD made after the priming shock plus that made after the severe heat-shock treat-
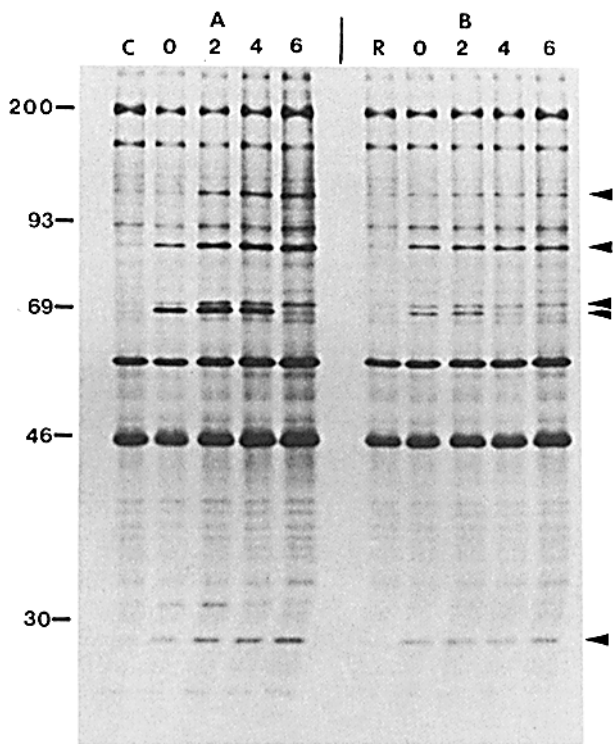
Figure 7. Thermotolerant cells subjected to a second isothermal shock show differential induction of the stress proteins. REF-52 cells were exposed to a 43°C/2-h heat-shock treatment, returned to 37°C, and pulse-labeled with [³H]leucine at 2-h intervals during the recovery period (*A*). In parallel, REF-52 cells were made tolerant by a prior 43°C/2-h heat-shock treatment, allowed to recover at 37°C for 8 h, and then subjected again to the same 43°C/2-h heat shock. The cells were returned to 37°C and pulse-labeled with [³H]leucine at 2-h intervals during the recovery period (*B*). The labeled proteins were analyzed by SDS-PAGE. Shown is a fluorograph of the gel. In *A* are the nontolerant and in *B*, the tolerant cells labeled after the 43°C/2-h shock at 0–1 h (*0*), 2–3 h (*2*), 4–5 h (*4*), and 6–7 h (*6*) during recovery at 37°C. Also shown in *A* are the control (37°C) cells (*C*) and in *B* the cells labeled at 37°C 8 h after the initial 43°C/2-h shock (*R*). Molecular mass markers are on the left and on the right, in descending order, the positions of the 110-, 90-, 73-, 72-, and 28-kD stress proteins.

ment was approximately equivalent to the amount produced in nontolerant cells after the severe heat-shock treatment. In contrast, the overall synthesis of the other major stress proteins (28, 90, and 110 kD) was not regulated in such precise fashion (data not shown). Thus, cells apparently titrate the amount of 72/73 kD produced as a function of both severity of stress treatment and the preexisting levels of the two proteins in the cell prior to the stress event.

In experiment *c*, we examined whether cells heat-shock treated in the presence of CHX would produce equivalent amounts of 72/73 kD as compared with cells heated in the absence of the drug. Therefore, cells were given a 43°C/1.5-h shock or a 45°C/30-min shock in the absence or presence of CHX, the drug then removed, and the cells steady-state labeled at 37°C. In either case nearly equivalent amounts of 72/73 kD were produced (Fig. 9, panel *B*). Finally, we compared the amounts of 72/73 kD produced after stress challenge in cells previously made tolerant in either the absence

or presence of CHX. Specifically, one set of cells was heat-shock treated at 43°C/1.5-h, returned to 37°C for 8 h, then presented a 45°C/30-min shock and the amount of 72/73-kD synthesis determined by steady-state labeling. In parallel, CHX was added to another set of cells, and the cells then given a 43°C/1.5 h shock and allowed to recover at 37°C in the presence of the drug. The drug was then removed, the cells shocked at 45°C/30 min, and the amount of 72/73-kD synthesis determined as before (Fig. 9, panel *B*). As noted above, the amount of 72/73 kD produced in tolerant cells was significantly less than that produced in nontolerant cells after the 45°C/30-min shock. In contrast, cells whose development of tolerance was performed entirely in the presence of the drug produced levels of 72/73 kD equivalent to that of nontolerant cells after the 45°C/30-min shock (Fig. 9, panel *B*).

Discussion

To date, studies examining the phenomenon of acquired thermotolerance have, by definition, been focused primarily at the level of cell survival. Here we have carefully examined the translational response of rat fibroblast (REF-52) cells to stress and demonstrate that (*a*) the acquisition of thermotolerance can be assessed at the level of translation (referred to as translational tolerance); (*b*) an alternative inducer of stress protein synthesis, sodium arsenite, also confers translational tolerance, whereas Azc, an inducer of nonfunctional stress proteins, does not; (*c*) the development of thermotolerance in the presence of CHX can provide a unique form of translational thermoprotection; and (*d*) there is strict regulation of 72/73-kD synthesis in both naive and translationally tolerant cells challenged by stress.

Pulse labeling studies of REF-52 cells after heat-shock (Fig. 1) or sodium arsenite treatment (Fig. 8) demonstrated qualitatively that the kinetics of stress protein induction and repression and degree of translational inhibition were dependent upon stress severity. Among the stress proteins induced, 72/73-kD synthesis was the most responsive to stress severity and also the first to be repressed during recovery, similar to the situation described in heat-shocked *Drosophila* cells (6). Using protocols that result in the acquisition of thermotolerance by cell survival criteria (i.e., mild heat-shock, 37°C recovery interval, severe heat shock), we have characterized the translational response of REF-52 cells to a severe heat-shock challenge after first being made thermotolerant. Collectively, the pulse-labeling data presented in Figs. 1 and 2 demonstrate that thermotolerance can be characterized, at the level of translation, through a consideration of three parameters: (*a*) duration of translational inhibition; (*b*) kinetics of 72/73-kD synthesis and subsequent repression; and (*c*) recovery kinetics of 37°C translation patterns. With respect to these three parameters, the thermotolerant cell given a severe heat-shock challenge exhibits an acceleration in (*a*) recovery of translational activity, (*b*) kinetics of 72/73-kD synthesis and repression, and (*c*) return to normal 37°C translation patterns, as compared with the nontolerant cell. Using these three criteria, the extent of tolerance appeared dependent upon the recovery interval between the initial priming shock and the subsequent challenging shock. For example, while those cells allowed a 4-h recovery period had acquired some degree of translational tolerance, maximal
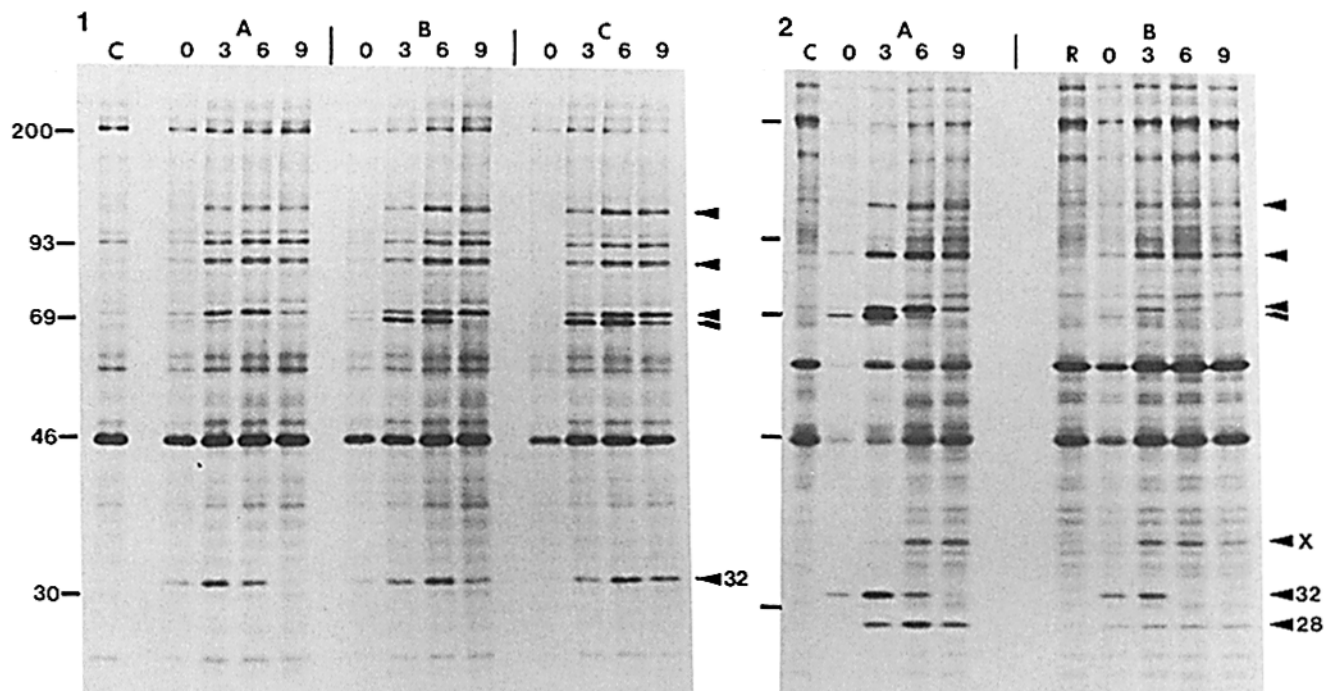
Figure 8. Both the extent and duration of stress protein synthesis are dependent upon the severity of the stress treatment. In panel *1* REF-52 cells were exposed to either 80 μM (*A*), 160 μM (*B*), or 240 μM (*C*) sodium arsenite for 1.5 h at 37°C. The heavy metal was removed and the cells then pulse-labeled with [³⁵S]methionine at 3-h intervals during recovery in normal culture medium. The labeled proteins were analyzed by SDS-PAGE. Shown is a fluorograph of the gel. Cells were pulse-labeled at 0–1 h (*0*), 3–4 h (*3*), 6–7 h (*6*), or 9–10 h (*9*) during recovery from arsenite treatment. In panel *2* cells were made tolerant by exposure to 160 μM sodium arsenite for 1.5 h at 37°C, the heavy metal removed, and the cells further incubated for 14 h at 37°C. These tolerant cells (panel *2B*) and nontolerant cells (i.e., no pretreatment, panel *2A*) were then exposed to 160 μM sodium arsenite for 1.5 h at 37°C, the heavy metal removed, and the cells pulse-labeled with [³H]leucine at 3-h intervals during the recovery period and the proteins analyzed as described above. Lane *C* in panel *1* represents the control (37°C) cells whereas lane *R* in panel *2* represents the cells recovered from the 160 μM/1.5-h arsenite treatment for 14 h. Indicated on the left are molecular mass markers and on the right, in descending order, the positions of the 110-, 90-, 73-, 72-, 32-, and 28-kD stress proteins.

translational tolerance was observed in those cells allowed to recover for at least 8 h before the second heat-shock treatment (Fig. 2). These differences were paralleled by the observation that after 4 h of recovery from the priming heat shock, synthesis of 72/73 kD continued at a significant rate (Fig. 1 *A*) whereas by 8 h of recovery, synthesis of these two proteins had been completed (Fig. 1 *B*). The kinetics of induction and decay of translational tolerance described here are very similar to that described for thermotolerance as assayed by cell survival (15, 19, 21, 32). Moreover, as we have demonstrated for the acquisition of translational thermoprotection, other studies have emphasized a good correlation between cell survival and previous accumulation of the 70-kD stress proteins (19). Finally, the ability of prior heat-shock treatments to confer subsequent translational thermoprotection has been demonstrated in vivo for *Drosophila* larvae (28) and soybean seedlings (22), as well as in both *Drosophila* and mammalian cells grown in vitro (23, 30, 31), and in some instances has been associated with acquisition of thermotolerance (28, 31) or prevention of developmental abnormalities (28). However, because thermotolerance (i.e., cell survival) is likely dependent upon a number of different parameters, we agree with previous investigators that one must exercise caution when attempting to correlate the phenomenon of translational tolerance with overall cellular thermotolerance.

Application of our translational assay to a variety of mammalian cell lines (Fig. 3) confirmed the generality of the results obtained in the rat fibroblasts. In each case, cells given a mild heat-shock pretreatment acquired translational tolerance to a subsequent severe heat-shock challenge. With regard to intrinsic translational tolerance, the human lines, HeLa and 293, were relatively more thermoresistant than the rodent lines. For example, nontolerant HeLa cells displayed less translational inhibition after heat-shock challenge than did the nontolerant CHO, BHK, or REF-52 cells. Previous studies have shown that both HeLa (35) and 293 (29) cells constitutively express both the 72- and 73-kD stress proteins at 37°C. In contrast, rodent cells synthesize only 73 kD and little or no 72 kD at 37°C (35). These observations reinforce the possible correlation between levels of the 70-kD stress proteins and relative intrinsic degrees of thermotolerance. In this connection, heat-resistant variants derived from a CHO cell line were found to possess enhanced constitutive expression of the 70-kD stress protein(s) (19, 21). Hence, intracellular 70-kD levels are correlated with intrinsic heat resistance, as expressed at both the level of translation and cell survival.

Agents other than heat, which induce the synthesis of the stress proteins, could render the cells translationally thermotolerant. For example, treatment with sodium arsenite and recovery in the absence of the metal conferred an equivalent degree of translational thermotolerance as did a prior
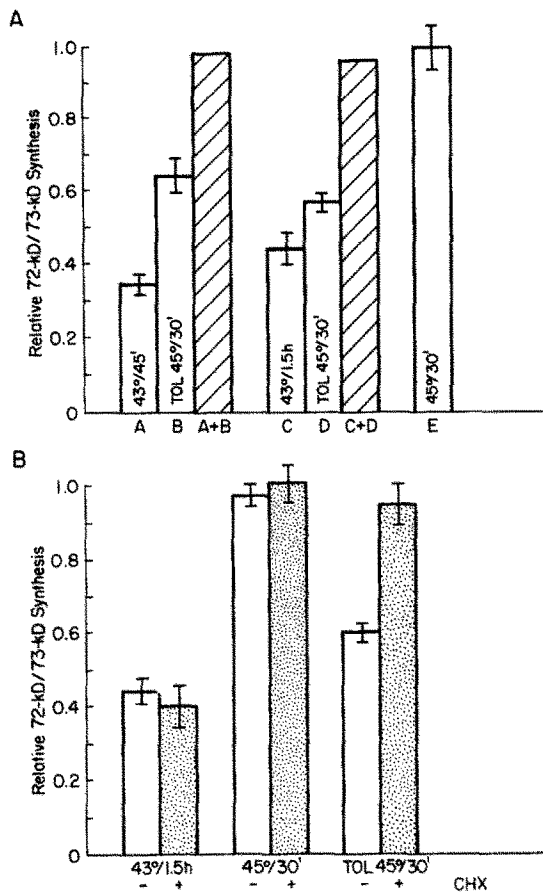
Figure 9. Synthesis of 72-kD/73-kD stress proteins is tightly regulated as a function of both the severity of the stress and the preexisting levels of the proteins in the cell. (panel *A*) REF-52 cells were given a single heat-shock treatment of 43°C/45 min (*A*), 43°C/1.5 h (*C*), or 45°C/30 min (*E*), the cells steady-state labeled with [³H]leucine, and the amount of 72/73 kD synthesized determined as described in Materials and Methods. Note as the severity of the stress treatment increases, there is a corresponding increase in the amount of the stress proteins produced. In parallel, cells were first made thermotolerant by either a 43°C/45-min shock or a 43°C/1.5-h shock, plus 8-h incubation at 37°C, and then reshocked at 45°C/30 min. The amount of 72/73 kD produced after the second shock was then determined. In *B* is shown the amount of 72/73 kD produced in the 43°C/45-min tolerant cells and in *D* the amount of 72/73 kD produced in the 43°C/1.5-h tolerant cells after the second heat-shock treatment. Note that the amount of 72/73 kD produced after the 43°C shock plus that made after the 45°C/30-min shock is equivalent to that made in a nontolerant cell given the 45°C/30-min shock (*A+B* and *C+D*). (panel *B*) REF-52 cells were given a 43°C/1.5-h or 45°C/30-min shock in either the presence or absence of CHX, the cells returned to 37°C, the drug removed and the cells steady-state labeled with [³H]leucine. The amount of 72/73 kD produced in the cells was approximately the same regardless of whether CHX was included during the heat-shock treatment. Lastly, a third set of cells was given a 43°C/1.5-h shock and subsequent 8 h recovery at 37°C in either the presence or absence of CHX. The drug was removed and both sets of cells were then given a 45°C/30-min shock, returned to 37°C, and steady-state labeled with [³⁵S]methionine. Cells whose development of thermotolerance was performed in the presence of CHX produced significantly more 72/73 kD than did those cells whose tolerance was developed in the absence of the drug. (In fact, the levels are equivalent to that of the nontolerant cells given the 45°C/30-min shock). In panels *A* and *B*, quantitations were normalized to values obtained for the 45°C/

treatment with mild heat shock (Fig. 4). A similar cross-over effect of agents that induce the synthesis of the stress proteins (e.g., ethanol, heavy metals) has been shown to result in the acquisition of thermotolerance as assayed by cell survival (18). Our studies using the amino acid analogue of proline, Azc, implicate a role for the stress proteins in the acquisition of translational thermotolerance. Specifically, cells that were incubated in the continued presence of Azc produced high levels of the stress proteins but did not exhibit translational tolerance when challenged with heat. If, however, the Azc-treated cells were provided a recovery period in the absence of the analogue, the cells continued to synthesize the stress proteins and now appeared fully translationally thermotolerant. A likely explanation for these observations is the fact that while continued Azc treatment will result in production of the stress proteins, the proteins containing the analogue are probably rendered "nonfunctional." Support for this notion is threefold: (*a*) 72 kD synthesized in the presence of Azc fails to correctly localize in the nucleus/nucleolus (5, 36); (*b*) the half-life of 72 kD synthesized in the presence of Azc is dramatically reduced (3–6 h) as compared with its half-life after heat shock or in cells Azc treated and allowed to recover in the absence of the analogue (48–64 h); and (*c*) amino acid analogues fail to confer cellular thermotolerance despite the vigorous production of the stress proteins (20). Although we recognize there to be significant pleiotropic effects due to amino acid analogue exposure, we suggest that the simplest explanation as to why amino acid analogues fail to confer thermotolerance (translational or cellular) is their failure to produce functional stress proteins.

Our studies examining the effect of CHX on the acquisition of translational tolerance produced a number of interesting results (Fig. 6). Cells given the 43°C/1.5-h priming heat-shock treatment in the presence of CHX but allowed to recover in its absence displayed essentially full translational tolerance against the subsequent 45°C/30-min challenge. As was shown in the quantitation studies (Fig. 9), under these conditions stress protein synthesis and accumulation occur normally upon removal of the drug, suggesting that full stress protein accumulation may account for the translational tolerance observed. Unexpectedly, preventing stress protein synthesis entirely with CHX during the priming shock and recovery at 37°C did not abolish translational protection against the 45°C/30-min challenge. However, under these conditions the pattern of proteins synthesized after the 45°C/30-min shock was significantly different than in cells made tolerant in the absence of the drug. Specifically, these CHX-treated cells exhibited, overall, translational patterns similar to the unstressed (e.g., 37°C) cells with the only difference being the slightly elevated synthesis of the 90- and 110-kD stress proteins. Furthermore, synthesis of the 72/73-kD proteins, which normally parallels the return of translational activity in the tolerant cells after the 45°C/30-min challenge, did not occur immediately, but rather was delayed for some 4–5 h in the cells made tolerant in the presence of the drug. These somewhat confusing results could be explained, in

30-min heat-shock treatment, which was given an arbitrary value of 1.0. Results are averages from triplicate samples, and the error bars represent the standard deviations observed about the graphed values.

part, by examining the polysome profiles after the 45°C/30-min shock. As would be expected from the translational profiles shown in Fig. 6, nontolerant cells exhibited a complete disruption of polysomes for as long as 5 h after the severe heat-shock treatment. Upon recovery of normal polysome profiles, there occurred an accompanying synthesis of the stress proteins. In cells first made thermotolerant in the absence of CHX, polysomes were disrupted but had returned by 1 h after the 45°C/30-min challenge, and the cells again exhibited a vigorous production of the stress proteins. In contrast, when CHX was included during both the priming shock and subsequent recovery at 37°C, then removed immediately before the 45°C/30-min challenge, there was significant retention of polysome structure upon recovery at 37°C. These results examining polysome profiles after heat-shock treatment are entirely consistent with the previous studies of McCormick and Penman (27). These workers similarly noted a disruption of polysome integrity after heat shock but found that prior addition of CHX prevented such heat-induced disaggregation. Therefore, the ability of CHX to stabilize polysomes against heat-induced disruption probably accounts for these cells displaying translational thermotolerance and also exhibiting translation patterns similar to that of the unstressed 37°C cell after the 45°C/30-min shock. That is, addition of CHX during the priming shock and subsequent recovery period at 37°C resulted in the polysomes to remain loaded with only 37°C mRNAs. Removal of the drug and immediate exposure of the cells to a 45°C/30-min shock resulted in a retention of polysome integrity, subsequent translation of the 37°C mRNAs, and with time of recovery (i.e., 4-5 h) a slow changeover to translation of mRNAs encoding the stress proteins.

Although the cells made tolerant in the presence of CHX displayed a form of "translational thermotolerance," they exhibited considerable differences compared with cells made tolerant in the absence of the drug. For example, and as pointed out above, the CHX-treated cells did not respond to the 45°C/30-min shock with immediate induction of 72/73-kD synthesis, but instead required an additional 4 h of recovery before elevated synthesis of these two proteins was observed. Moreover, the CHX-treated cells continued to synthesize these two proteins for a considerably longer period of time than did the cells made tolerant in the absence of the drug. Finally, the absolute amount of 72/73 kD made in the cells whose tolerance was developed in the presence of CHX equaled that made in a nontolerant cell similarly challenged by a 45°C/30-min heat shock. In contrast, cells made tolerant in the absence of CHX responded to a second heat-shock treatment by producing significantly less 72/73 kD. In summary then, while it would appear that translational thermotolerance can develop in cells previously heat-shock treated and allowed to recover in the presence of CHX, the cells, by a number of other criteria (e.g., kinetics of synthesis and overall amount of 72/73 kD produced) are in fact markedly different than cells whose development of thermotolerance is performed in the absence of the drug. These complex results suggest that alterations in the translational machinery, here manifested as increased thermoresistance, can be elicited by either prior stress protein induction or prolonged incubation with CHX, a finding corroborated in *Tetrahymena* (10). In addition, the ability of CHX to modulate heat-induced changes in translational activity, as described here, may be relevant

to its reported effects on reducing heat-shock lethality (16) and the apparent ability of cells, under certain conditions, to acquire thermotolerance in the absence of induced stress protein synthesis (9, 10, 37).

Finally, our studies consistently indicated that the amount of 72/73 kD produced was dependent upon both the severity of the stress treatment and the preexisting levels of these proteins before the stress event. Similar to what has been observed previously (5), as the severity of the stress treatment increases, there is a corresponding increased production of these two proteins. Moreover, in cells administered two-step heat-shock treatments (e.g., 43°C/1.5 h, 37°C recovery, 45°C/30 min) the overall amount of 72/73 kD produced is the same as that produced in cells given only the single severe heat-shock treatment (e.g., 45°C/30 min). Such a strict correlation was not observed in the case of either the 28-, 90-, and 110-kD stress proteins. Inclusion of CHX had little or no effect on the absolute levels of the 72/73 kD produced. Rather, upon removal of the drug, the cells continued to synthesize the two proteins for a longer period of time to achieve a similar accumulation. Collectively these studies indicate that a cellular "memory" of stress might operate via monitoring the intracellular levels of the 72/73-kD stress proteins. In addition, this apparent strict regulation of their synthesis as a function of the stress severity suggests an integral role for these proteins in providing the cell protection to environmental traumas.

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