MOLECULAR AND CELLULAR BIOLOGY, Dec. 1988, p. 5486–5494
0270-7306/88/125486-09$02.00/0
Copyright © 1988, American Society for Microbiology
Vol. 8, No. 1988

The Major Inducible Heat Shock Protein hsp68 Is Not Required for Acquisition of Thermal Resistance in Mouse Plasmacytoma Cell Lines

L. AUJAME†* AND H. FIRKO

Department of Biology, Queen's University, Kingston, Ontario, Canada K7L 3N

Received 23 November 1987/Accepted 16 August 1988

In mouse cells, the major inducible heat shock protein is a protein of 68,000 daltons (hsp68). We have previously shown that mouse plasmacytomas do not express hsp68. We have now made use of these natural mutants to assess the contribution of hsp68 to acquired thermotolerance. An endpoint limiting dilution assay was used to quantify cell survival to lethal stresses. Two test plasmacytoma cell lines (C1.18.1 and J558) and an hsp68-positive myeloma, XC1.1/51, used as a control, were examined. All showed recovery when pretreated for 10 min at 44°C 2 h before exposure to otherwise lethal stresses of 1 to 4 h at 43°C. Similar results were obtained with the Friend erythroleukemia line D1B, which we have also shown not to express hsp68. These results indicate that hsp68 is not required for protection against thermal stresses in mouse cells.

When cells are exposed to temperatures above their growth temperatures, a small set of genes is generally activated. The products of these genes, the so-called heat shock proteins (HSPs), have now been identified, and genomic clones have been obtained from a variety of organisms, including bacteria, *Drosophila melanogaster*, humans, mice, *Xenopus laevis*, and maize. The heat shock response appears to be universal, and at least one class of HSPs, the hsp70 family, is highly conserved throughout evolution (see references 2, 13, and 33 for recent reviews).

The *hsp* genes can be activated by heat as well as other stresses ranging from amino acid analogs to heavy metals and inhibitors of oxidative phosphorylation. Most induction conditions are known to lead to intracellular accumulation of abnormal proteins. Recently, Ananthan et al. (1) demonstrated that the introduction of denatured proteins into frog oocytes led to HSP expression, suggesting that abnormal proteins serve as a signal to *hsp* gene activation. The function of HSPs may therefore be to protect cells by removing these abnormal proteins. It has been suggested (see reference 39 for a review) that HSPs participate in the disassembly of hydrophobic protein complexes in an ATP-dependent manner. Consistent with this hypothesis, the bovine 72-kilodalton HSP, a constitutively expressed protein, has recently been shown to be the clathrin ATPase (9, 50). Furthermore, the same or other HSPs are known to be associated with a variety of cellular complexes (32, 38, 43). Indeed, more recent results suggest that some of the HSPs are involved in the proper folding, translocation, and assembly of proteins and protein complexes (see reference 40 for a brief review; 12, 15, 21a).

Such cellular protection by HSPs may come into play during acquired thermal resistance. This phenomenon may be defined as the induced capacity of cells to survive an otherwise lethal challenge after exposure to a nonlethal stimulus (17). Independently of the underlying mechanism of resistance (induced capacity to repair sublethal damage by the whole cell population or true thermotolerance of a subpopulation), pretreatment usually stimulates the synthesis of HSPs, and a relationship between this synthesis and thermotolerance has been suggested (18, 25, 29, 34, 36, 48). A direct cause-effect relationship has yet to be clearly demonstrated, except possibly in *Escherichia coli* (55). Indeed, a number of recent studies have questioned the previous assumption that the synthesis of HSPs in eucaryotes is a requirement for the acquisition of thermotolerance (19, 20, 46, 51, 53).

A genetic approach to the dissection of HSP function has been undertaken by a variety of groups (see, for example, references 14, 18, 24, and 42) with limited success. As a step in this direction, we have attempted to assess the contribution of the mouse major inducible HSP, hsp68, to thermotolerance in mouse plasmacytomas. In most mouse cells examined to date, *hsp68* genes are stringently controlled; that is, they are essentially inactive except under stress. In mouse plasmacytomas, these genes cannot be activated, although other HSPs remain inducible (3, 4). We have begun to make use of this property to try to identify the function of this HSP.

To analyze thermotolerance in myelomas, we used an endpoint limiting dilution assay first employed in immunology to determine the proportion of immunologically competent cells in a test population (49). In this report, we show that both a control myeloma and independently derived plasmacytomas display heat resistance after a pretreatment which activates hsp68 in control cells but not in plasmacytomas. We have extended this study with similar results to a mouse erythroleukemia cell line that is also hsp68 nonresponsive. These results indicate that hsp68 is not required to protect cells from thermal stresses.

MATERIALS AND METHODS

Cells and culture conditions. The origins of most cell lines used in this study were described previously (3). The C3H XC1.1/51 myeloma (referred to here as XC1) is an immunoglobulin-nonproducing derivative of the immunoglobulin G2a-producing plasmacytoma C1.18.4 (referred to here as C118). J558 is a BALB/c plasmacytoma secreting immunoglobulin A. D1B is a DBA/2 Friend virus-induced mouse

* Corresponding author.
† Present address: Institut Mérieux, Unité de Recombinaison Génétique, 1541 Avenue Marcel Mérieux, Marcy l'Etoile 69752, France.

erythroleukemia cell line (10, 11), obtained from the American Type Culture Collection (Rockville, Md.). Cells were generally cultured in α-minimum essential medium (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 10% fetal calf serum at 37°C in a humidified 5% CO_2 incubator. D1B cells were grown in a medium supplemented with 5×10^{-5} M β-mercaptoethanol.

Cell labeling. For control and single heat shock labeling, the protocol described previously was used (3, 4). Cells were suspended in 100 μl of methionine-minus α-minimal essential medium and immersed at 44°C for 10 min. They were then left to recover for 1 h at 37°C before addition of L-[^{35}S]methionine (specific activity, 1,100 Ci/mmol; Dupont, NEN Research Products, Boston, Mass.) to 100 μCi/ml and recovery for another hour. For double heat shock experiments, approximately 10^5 cells were resuspended in 100 μl of methionine-minus α-minimal essential medium and warmed to 44°C for 10 min. They were then left to recover for 2 h at 37°C and incubated at 43°C for 30 min. After 1 h at 37°C, L-[^{35}S]methionine was added to 100 μCi/ml. At the end of a 1-h labeling period, cells were washed twice with phosphate-buffered saline, suspended in 100 μl of Laemmli buffer (27), and boiled for 5 min; lysates were then analyzed on 7.5% sodium dodecyl sulfate-acrylamide gels with a 3% stacking gel in the discontinuous buffer system of Laemmli (27). About 10^5 trichloroacetic acid-precipitable counts were loaded per lane. Gels were run overnight at 150 V and then immersed into excess 1 M sodium salicylate (pH 5.0) for 5 min before exposure to Cronex 6-Plus X-ray film (E. I. du Pont de Nemours & Co., Inc., Wilmington, Del.) with Quanta III intensifying screens at −70°C.

RNA extraction and S1 nuclease assay. The protocols described previously were used (3, 4). Cells were heat shocked at 44°C for 10 min and left to recover either for 1 h at 37°C, after which time RNA was recovered, or for 2 h; in the latter case, the cells were submitted to a second heat shock of 2 h at 43°C and again left to recover for 1 h at 37°C, and RNA was extracted.

For S1 nuclease assays, the probe was a 1.4-kilobase *Eco*RI-*Bgl*II *hsp68* genomic fragment that includes the start of transcription of the cloned mouse *hsp68* gene (4, 41). RNA samples (30 μg) were hybridized overnight at 59°C to 1×10^5 to 2×10^5 cpm of the [γ-^{32}P]ATP end-labeled probe in 80% formamide–40 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)] (pH 6.4)–1 mM EDTA–0.4 M NaCl, followed by a 1-h digestion at 37°C with 100 to 200 U of S1 nuclease, phenol extraction, and precipitation. Digested samples denatured in 30% dimethyl sulfoxide were analyzed on 8 M urea–4% acrylamide sequencing gels together with *Hae*III-digested [γ-^{32}P]ATP-end-labeled φX174 DNA as a marker.

Endpoint limiting dilution assay. The endpoint limiting dilution assay has been described in detail elsewhere (28, 49). It assumes that in a dilution series, the frequency of surviving cells in a test population follows a Poisson distribution. Given that x is the mean number of cells inoculated per well or plate (based on cell counting) and f is the frequency, to be determined, of viable cells in the suspension, the probability of finding y positive wells (that is, displaying cell growth or colony formation) is given by the standard Poisson equation:

$$P(y) = ((fx)^y \cdot e^{-(fx)})/y!$$

When $y = 0$, corresponding to the endpoint of growth in the dilution series, then

$$P_o = e^{-fx}$$

or

$$\ln P_o = fx \quad (1)$$

Therefore, f can be estimated by plotting $\ln P_o$, where P_o is the frequency of negative wells determined from the assay (i.e., number of negative wells per total number of wells), versus cell number. The data may be plotted as the logarithm of the fraction of negative wells (no growth) versus initial cell input (see Fig. 3 and 4), the slope of the straight line passing through the origin providing the survival frequency. Frequency values, standard error, chi square, and confidence intervals were calculated from the data by using the Poisson program of M. Dosch (Hospital for Sick Children, Toronto, Ontario, Canada) or in some cases by using the GLIM program through Queens' University Statslab. The Poisson program evaluates survival frequencies by using the minimum-chi-square, weighted-mean, or maximum-likelihood method. Taswell (49) has shown that the most accurate estimate of f may be obtained by the method of minimum chi square. Only the values obtained by this method were considered and are presented here (see Table 2) (the other two methods gave essentially identical results).

Thermotolerance assay. Cells were plated in twofold serial dilutions into 96-well plates (eight replicates per plate) at an initial input of either 10,000 or 50,000 cells (distributed over two plates). Plates were sealed with Parafilm and incubated in a water bath at 43 or 45°C for the indicated time periods with or without pretreatment (44°C for 10 min, 2 h before) as specified. Colonies were scored visually after 10 to 14 days, wells with single colonies being considered positives.

RESULTS

Experimental design. Our experimental approach was guided by our objective of assessing the contribution of hsp68 to thermal protection. Thus, three initial questions had to be resolved. First, what quantifiable (by the endpoint limiting dilution assay) test conditions resulted in thermal protection in control cells? Second, what was the heat shock response of plasmacytomas under such conditions and, specifically, was the expression of hsp68 still repressed? And finally, how reliable was the assay itself?

In general agreement with previous observations (8; M. D. Perry, W. D. Fulford, and L. A. Moran, unpublished observations), we found that the apparent synthesis of HSPs was maximal in lymphoid cells when measured after a 2- to 3-h recovery at 37°C following a 10-min heat shock at 44°C. As assayed on the T lymphoma S49.1, a cell line which expresses hsp68 (3), adaptation to heat was positively related to increasing temperatures of the initial stimulus. For a given cytotoxic challenge (see below), pretreatment of 44 or 43°C gave greater thermal protection than did pretreatment of 42°C (Fig. 1). These assays provided pretreatment conditions (44°C, 10 min) as well as the time of onset of the second hyperthermic treatment (we chose a 2-h, 37°C recovery). They also demonstrated the sensitivity and reliability of the approach.

To determine the temperature of the second treatment, assays were again conducted on S49.1 cells with challenges of 43 to 46°C for 20 to 60 min. Recoveries were maximal with 43°C treatments and were sharply reduced above that temperature (44 to 46°C). Treatments at 42°C or below were insufficiently injurious except for long durations and were not further examined. A temperature of 43°C for the second lethal shock was therefore used in most subsequent experiments, the duration of the shock depending on the thermal

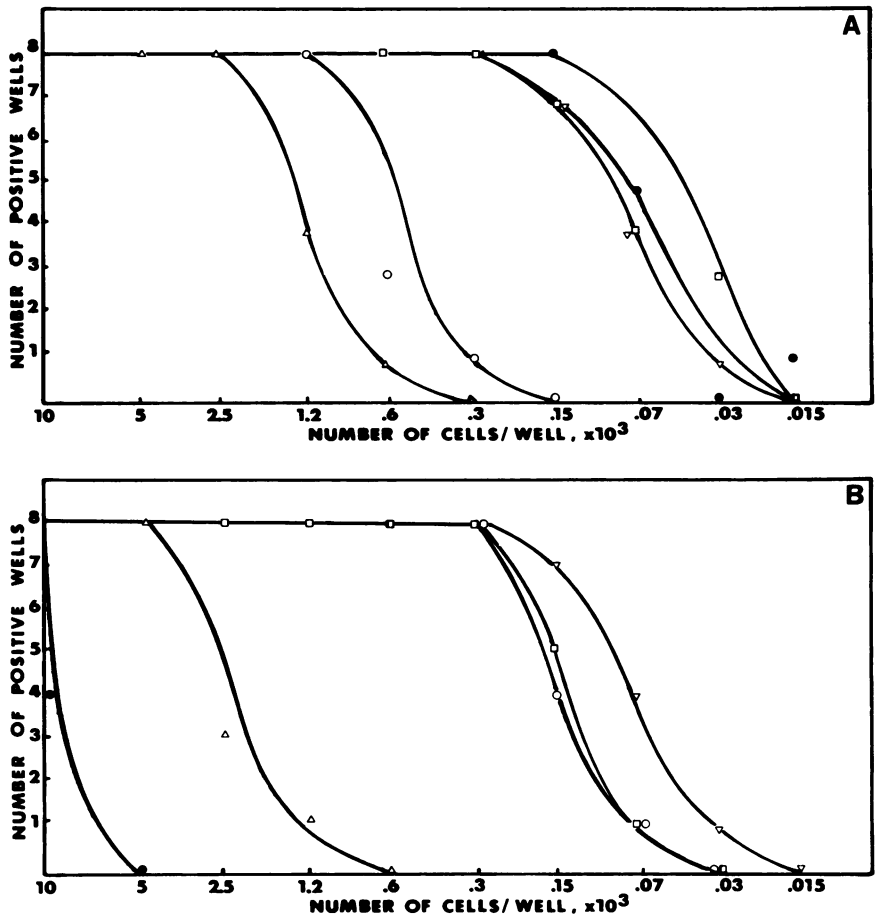

FIG. 1. Thermal resistance of the hsp68-expression S49.1 T lymphoma as assayed by the endpoint limiting dilution assay. Raw data points from single experiments (one 96-well plate) are presented. Curves are first approximations and are not meant for quantification. (A) Cytotoxic treatment of 30 min at 43°C. Symbols: ▽, 37°C control; △, 43°C, 30 min; ○, with 42°C, 10-min pretreatment; □, with 43°C, 10-min pretreatment; ●, with 44°C, 10-min pretreatment. (B) Cytotoxic treatment of 60 min at 43°C. Symbols: ▽, 37°C control; ●, 43°C, 60 min; △, with 42°C, 10-min pretreatment; ○, with 43°C, 10-min pretreatment; □, with 44°C, 10-min pretreatment.

sensitivity of each cell line. Assay conditions used were therefore the following:

44°C, 10 min → 37°C, 2 h → 43°C, 0 to 4 h
pretreatment recovery cytotoxic lethal treatment

Under these conditions, there was no evidence of hsp68 expression in any of the plasmacytomas tested (Fig. 2). In Fig. 2A, XC1, an hsp68-expressing myeloma, and C118, a plasmacytoma, were labeled with [^{35}S]methionine after the first (lane c) or second (lane d) heat treatment, and lysates were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. No band corresponding to hsp68 was apparent in C118 (lane d) after pretreatment and a 43°C, 30-min heat challenge, in contrast to the situation with XC1, in which induction of hsp68 was maximal at 44°C (lane c; compare with lane b, in which cells were submitted to a single 44°C heat shock) and was maintained after the second treatment (lane d). We had previously shown that no hsp68 was synthesized in these plasmacytomas upon longer recoveries (4), and this remained the case under the conditions of double heat shocks used here (data not shown).

Since lethal stresses of 2 to 4 h were to be considered (see below), we also examined hsp68 expression under these longer cytotoxic treatments. However, because incorporation of labeled amino acids into proteins was too low for detection by gel analysis under the conditions of analysis described above, we resorted to the more direct S1 nuclease assay (7) of hsp68 mRNA synthesis. A band corresponding to protected hsp68 mRNA was present in XC1 samples after a second heat shock at 43°C for 2 h but was totally absent in both plasmacytoma samples (C118 and J558) (Fig. 1B). In XC1, there was clearly more hsp68 mRNA after a single 44°C stimulus than after a second 2-h stress at 43°C. We had previously demonstrated the absence of hsp68 mRNA in both plasmacytoma cell lines under steady-state conditions and after a single 44°C heat shock (3).

Thermotolerance assay. The endpoint limiting dilution assay of Taswell (49) seemed particularly well suited for the analysis of thermal resistance of nonadherent cells in that the response to stress could be easily quantified. This proved to be the case with the heat-responsive T-lymphoma S49.1 cell line initially chosen to test the assay (data analysis using the GLIM program is not shown, but see Fig. 1 for actual data points allowing a first approximation).

The reproducibility of the assay was further confirmed by results obtained from separate experiments with either the hsp68-expressing XC1 myeloma or the nonexpressing J558 plasmacytoma. Table 1 presents the data points obtained for two separate experiments with XC1 and three with J558 under similar assay conditions (43°C cytotoxic shocks of 3

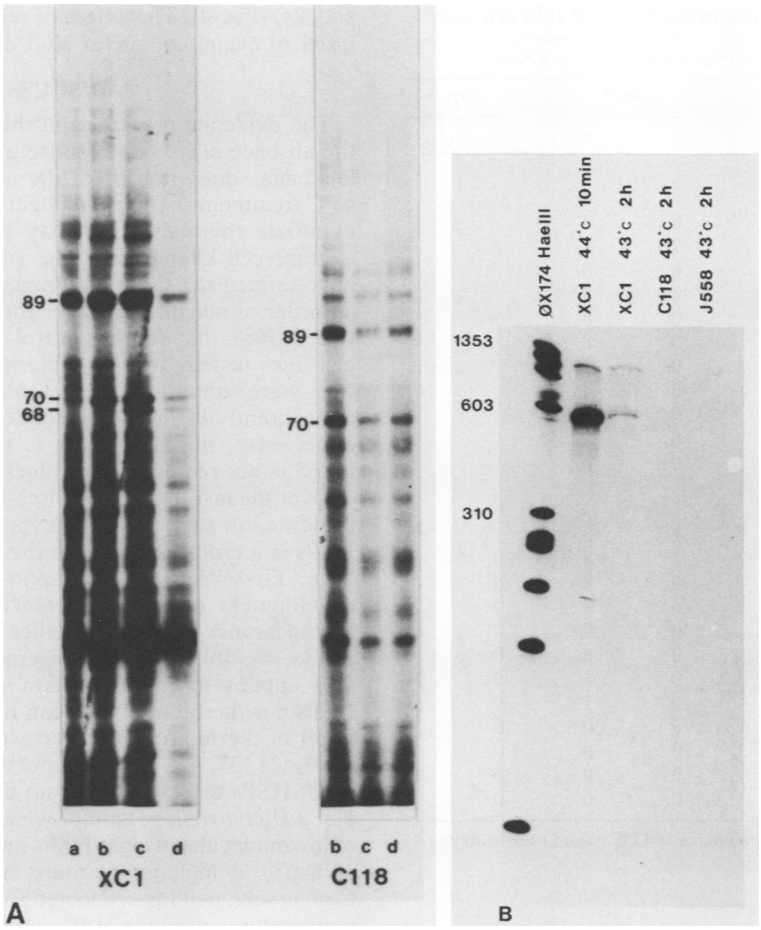

FIG. 2. Evidence that hsp68 is not expressed in plasmacytomas under thermal resistance assay conditions. (A) Fluorograph of 7.5% sodium dodecyl sulfate-polyacrylamide gel of [^{35}S]methionine-labeled polypeptides synthesized at 37°C (lane a), 42°C (lane b), 44°C (lane c), and 44°C for 10 min, followed by a 2-h recovery at 37°C and a second thermal stress of 30 min at 43°C (lane d) in XC1 (left) and C118 (right). A constant amount of counts (approximately 100,000 cpm) that could be precipitated with trichloroacetic acid was loaded in each lane. (B) S1 nuclease analysis of hsp68 mRNA in mouse plasmacytomas (C118 and J558) and the control myeloma XC1 under different stress conditions. Samples marked 43°C, 2 h were pretreated at 44°C for 10 min at 2 h before the 43°C, 2-h treatment, and RNA was extracted after a 1-h recovery at 37°C. S1 nuclease-treated samples were separated on an 8 M urea–4% acrylamide gel for 6 h at 400 V. The gel was exposed to Dupont Cronex 6-Plus film for 48 h.

h). In each case, recoveries were clearly within the same order of magnitude. Variations could reflect the state of the cells at the time of the experiment (they were generally taken in log phase) or the water bath temperature (±0.2°C).

Table 2 summarizes the computer-generated analysis of the data for the control (myeloma XC1) and the two plasmacytomas (C118 and J558) from single sets of experiments (for XC1 and J558, experiments 2 and 3, respectively, of Table 1). Figure 3 shows the data plotted according to equation (1), presented in the Materials and Methods, in which enhanced resistance to heat results in a decreased slope of the curves as the frequency of survivors increases. In Table 3, the same data are presented in the more conventional form of percent survival.

Although the thermal sensitivities of the cell lines differed considerably (C118 being the most sensitive), the data indicate that all cell lines displayed induced thermal resistance irrespective of the lack of hsp68 synthesis. Since the pretreatment conditions (44°C, 10 min) alone had no effect on cell survival in any of the cell lines tested (not shown), our data are a measure of thermal adaptation of the whole cell population (a point made more evident by the observation that some treatments resulted in 100% recoveries; Table 2). In general, results were reproducible within the confidence interval (Tables 1 and 2). In cases where they were not, induced heat tolerance was nevertheless observed. The high chi-square values obtained in two cases (C118, assay 4, and J558, assay 6, in Table 1) correspond to high frequencies of survival and cannot be taken as disproving the hypothesis. Note that, as previously indicated for J558 (Table 1), the assay is reproducible.

The most significant values are therefore not the survival frequencies per se but the thermotolerance index calculated for each cell line (defined as the ratio of percent survival for a given challenge with or without pretreatment) (Table 3). The value obtained for J558 (index, approximately 200) was about 10 times that obtained for XC1, although both lines had relatively similar thermal sensitivities (compared with C118). This result further emphasizes the conclusion that the development of thermal resistance does not require the synthesis of hsp68.

To further demonstrate this point, we also examined thermoprotection in the mouse erythroleukemia cell line D1B. This cell line has been found not to express hsp68 (3a).

TABLE 1. Thermal resistance in the XC1 and J558 cell lines[a]

Initial no. of cells/well	No. of positive wells (n = 8)/lane					
	Expt 1		Expt 2		Expt 3	
	−	+	−	+	−	+
XC1						
10,000	8	8	8	8		
5,000	8	8	8	8		
2,500	3	8	7	8		
1,250	0	8	6	8		
625	0	8	4	8		
312	0	8	0	8		
156	0	5	0	8		
78	0	2	0	5		
37	0	1	0	4		
18	0	0	0	2		
9	0	0	0	1		
4	0	0	0	0		
J558						
10,000	4	8	1	8	1	8
5,000	0	8	1	8	0	8
2,500	0	8	0	8	0	8
1,250	0	8	0	8	0	8
625	0	8	0	8	0	8
312	0	8	0	8	0	6
156	0	1	0	4	0	6
78	0	4	0	1	0	3
37	0	0	0	0	0	1
18	0	0	0	0	0	0
9	0	0	0	0	0	0
4	0	0	0	0	0	0

[a] Data are from separate 3-h experiments at 43°C, with (+) or without (−) pretreatment (44°C, 10 min).

Under assay conditions similar to those used for the myelomas, D1B cells displayed thermotolerance (frequencies shifted from 1/7,500 to 1/1,100 with pretreatment for 3 h and 43°C cytotoxic shocks and from 1/1,800 to 1/250 for 2-h shocks) (Fig. 4). The extent of recovery was within the same order of magnitude as for XC1 cells.

DISCUSSION

The evidence presented in this report demonstrates that the absence of hsp68 in mouse plasmacytomas and erythroleukemias does not affect the adaptation of these cells to 43°C treatments. The main effect of increased resistance to a cytotoxic thermal stress may be seen as an increase in average cell killing time. The value of this shift, which we have termed the thermotolerance index (Table 2), is in fact an order of magnitude higher for one of the plasmacytomas tested, J558, than for the control myeloma XC1 and the other cell lines tested. It is possible that the assay conditions of XC1 were suboptimal or that, alternatively, J558 cells have an inherently higher degree of acquired thermotolerance. In either case, the conclusion is the same: the synthesis of hsp68 is not required for protection to a lethal challenge.

Over the last few years, three lines of evidence have been presented in support of the hypothesis that the synthesis of HSPs is a crucial element in the acquisition of thermotolerance. First, there is a temporal association between the development of thermotolerance and the appearance and disappearance of HSPs, whether induced by heat (18, 25, 29, 34, 36, 44, 48) or by other agents (30, 45). This association also applies to preblastoderm embryos in which neither HSPs nor thermotolerance can be induced and in which the onset of thermotolerance corresponds to the inducibility of HSPs (21, 37, 47). Second, mutants defective in the synthesis of HSPs display little or no thermotolerance. For example, a *Dictyostelium* mutant which does not synthesize a set of low-molecular-weight HSPs and displays decreased levels of hsp70 at high temperature is not thermoresistant (35). Conversely, cell lines selected for increased survival at high temperatures produce HSPs constitutively (24, 31). Third, a block of HSP synthesis by cycloheximide during the recovery period inhibits tolerance in a dose-related manner in a number of systems (23, 34, 45).

More recent results, however, suggest that the synthesis of HSPs may not be a requirement for the development of

TABLE 2. Minimum chi-square estimates of frequency of survival in thermotolerance assay of mouse myelomas

Cell line	Assay	1/f	SE (× 0.001)	Chi square	P	Confidence interval
XC1	1. Control[a]	4				
	2. 43°C,2 h	46	5.79	1.554	0.4597	30–97
	3. 43°C, 3h	1,018	0.2493	0.203	0.8682	680–2,026
	4. 43°C, 4h[b]	40,000				
	5. 44°C, 10 min, then 43°C, 3 h	68	4.1998	0.254	0.9684	44–156
	6. 44°C, 10 min, then 43°C, 4 h	1,112	0.2842	0.254	0.8808	687–2,918
C118	1. Control	6	41.63	0.612	0.9617	4–11
	2. 43°C, 1 h	1,490	0.2849	0.558	0.7566	813–2,867
	3. 43°C, 90 min[c]	47,303	0.071	0.897	0.6399	21,502–138,970
	4. 44°C, 10 min, then 43°C, 1 h[c]	59	2.3272	12.59	0.0275	47–91
	5. 44°C, 10 min, then 43°C, 90 min[c]	1,676	0.1778	2.847	0.2482	1,058–4,030
J558	1. Control[a]	4				
	2. 43°C, 1 h	14	21.16	0.990	0.6097	9–31
	3. 43°C, 2 h	785	0.6063	0.484	0.4868	406–1,743
	4. 43°C, 3 h[c]	40,045	0.0066	0.835	0.814	26,368–83,395
	5. 44°C, 10 min, then 43°C, 2 h[a]	4				
	6. 44°C, 10 min, then 43°C, 3 h[c]	169	0.9792	8.365	0.2126	128–250

[a] All wells were positive.
[b] Only one data point per plate.
[c] Initial cell input was 5×10^4. All other assays were done with 10^4 cells.

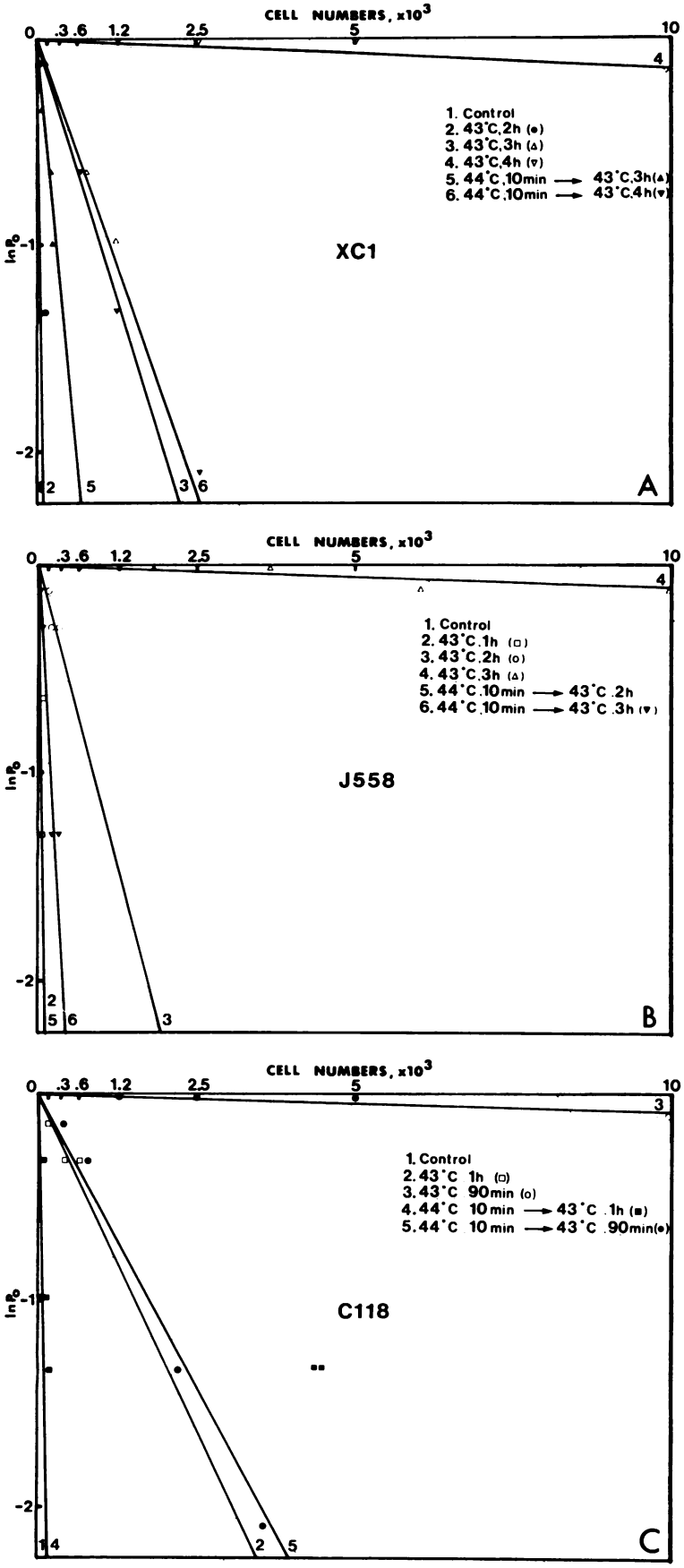

FIG. 3. Response curves of thermal resistance of mouse myelomas. Curves represent the data of Tables 1 (experiments 2 and 3 for XC1 and J558, respectively) and 2 plotted according to equation (1) in see Materials and Methods.

TABLE 3. Cell survival estimates in thermal resistance assays of mouse myelomas

Cell line	Assay	% Survival ± SE	Thermotolerance index[a]
XC1	1. Control	100[b]	
	2. 43°C, 2 h	8.6 ± 4.4	
	3. 43°C, 3 h	0.39 ± 0.2	
	4. 43°C, 4 h	<0.01	
	5. 44°C, 10 min, then 43°C, 3 h	5.8 ± 2.2	14.8
	6. 44°C, 10 min, then 43°C, 4 h	0.36 ± 0.23	>36
C118	1. Control	100[b]	
	2. 43°C, 1 h	0.4 ± 0.3	
	3. 43°C, 90 min	0.012 ± 0.01	
	4. 44°C, 10 min, then 43°C, 1 h	10 ± 3.5	40
	5. 44°C, 10 min, then 43°C, 90 min	0.35 ± 0.21	29
J558	1. Control	100[b]	
	2. 43°C, 1 h	28.5 ± 15	
	3. 43°C, 2 h	0.5 ± 0.3	
	4. 43°C, 3 h	0.01 ± 0.005	
	5. 44°C, 10 min, then 43°C, 2 h	100	200
	6. 44°C, 10 min, then 43°C, 3 h	2.3 ± 0.7	230

[a] Ratio of percent survival for a given challenge.
[b] Cloning efficiency was taken as 25% ($1/f = 4$) for XC1 and J558 and as 16.6% ($1/f = 6$) for C118.

thermotolerance. The addition of cycloheximide after the initial pretreatment does not prevent thermotolerance in rats (53), CHO cells (46), Morris hepatoma cells (26), yeast cells (19), and *Tetrahymena* sp. (20). Hallberg (20) has presented a convincing argument that in the latter case no HSP synthesis is required for the induction of thermotolerance at temperatures below 46°C. Also, pretreatments which do not lead to the enhanced synthesis of HSPs or of a subset of HSPs give rise to heat resistance in both CHO (46) and rat-1 (52) cells. In the latter case, Widelitz et al. (52) showed that the synthesis of hsp68 was not a requirement for thermotolerance. Similar conclusions were drawn for the products of two yeast *hsp70* genes (14) as well as the yeast *hsp26* gene (42).

Our results are in agreement with those presented above. In contrast to the approaches of both Przybytkowski et al. (46) and Wildelitz et al. (52), our results do not depend on minimal temperature shifts which do not enhance the synthesis of HSPs. Therefore, although the expression of hsp68 may be dissociated from thermoresistance, a role for other HSPs cannot be ruled out. The dual nature of HSPs as both constitutive and inducible proteins has too often been overlooked. hsp68 is a strictly inducible protein, and the fact that it is not expressed in plasmacytomas conclusively excludes it from any role in heat protection. On the other hand, some other members of the hsp70 family, such as the 72-kilodalton clathrin-uncoating ATPase (9, 52), and a number of other HSPs are also constitutively expressed. Both what we have called hsp70 (3, 4), which could correspond to the protein discussed above, and hsp89 remain expressed or inducible (or both) in plasmacytomas. The maintenance, activation, or redistribution of these constitutively expressed proteins may be sufficient to promote thermotolerance in cycloheximide inhibition studies and in the present case.

The recent demonstration (16) that ubiquitin, a heat-inducible but also constitutively expressed protein (and one present in plasmacytomas; L. Aujame and H. Firko, unpublished observations), is involved in thermotolerance in yeast cells and that hsp70, also expressed in mouse plasmacytomas, is associated with translocation of secretory and mitochondrial proteins and presumably involved in their unfolding and proper folding (12, 15, 21a) strengthens this argument.

Hallberg (20) has suggested that in *Tetrahymena thermophila* the synthesis of HSPs is required at 46 °C for the induction of thermotolerance. We examined this possibility in the plasmacytomas, even though we had observed minimal enhancement of thermotolerance measured at temperatures above 43°C in control cells. Nevertheless, we observed a similar low acquisition of thermotolerance in the plasmacytomas and in the control XC1 cell line at 45 to 46°C (not shown).

The function of hsp68 thus remains to be elucidated. Although our data strengthen the argument made by others (52) that hsp68 is not involved in protection of mammalian

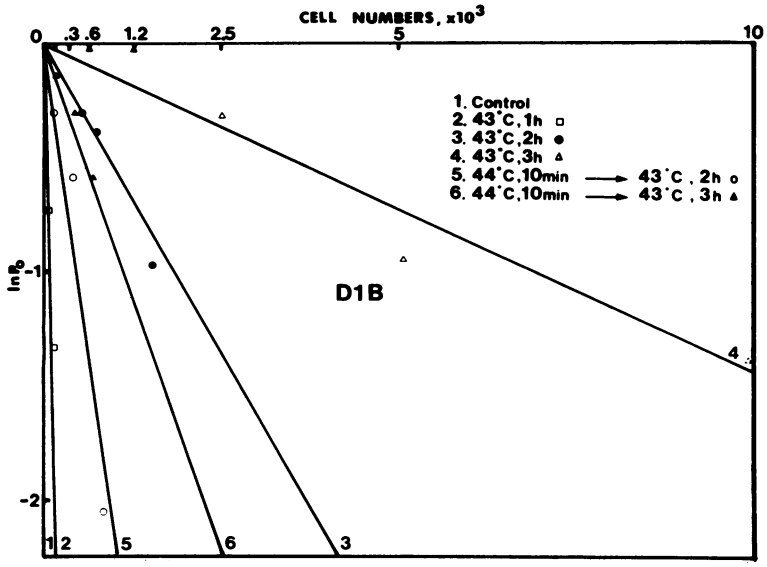

FIG. 4. Acquisition of thermal resistance in the D1B erythroleukemia cell line. Data points are from single experiments plotted according to equation (1) in Materials and Methods.

cells from thermal stress, it does not exclude the possibility that this protein plays a role in the thermal adaptation of cell populations that are under conditions of true thermotolerance (22) or are stressed by as yet undetermined environmental stimuli. Alternatively, the constitutive expression of this protein during early mouse embryogenesis (5, 6, 54) suggests that it may play a role more fundamental than in thermal protection.

ACKNOWLEDGMENTS

We thank Alain E. Lagarde for suggesting the endpoint limiting dilution assay and initial discussions; L. A. Moran for the hsp68 probe; and W. J. MacKillop and G. R. Wyatt for critical reading of early versions of the manuscript.

This work was supported by grants from the Banting Research Foundation and the Queen's University Advisory Research Committee.

LITERATURE CITED

1. **Ananthan, J., M. L. Goldberg, and R. Voellmy.** 1986. Abnormal proteins serve as eukaryotic stress signals and trigger the activation of heat-shock genes. Science **232**:542–544.
2. **Atkinson, B. G., and D. B. Walden. (ed.).** 1985. Changes in eukaryotic gene expression in response to environmental stress. Academic Press, Inc., Orlando, Fla.
3. **Aujame, L.** 1986. Mouse plasmacytomas constitute a class of natural heat-shock variants in which the major heat-shock gene (hsp68) is not expressed. Can. J. Genet. Cytol. **28**:1064–1075.

3a. **Aujame, L.** 1988. The major heat-shock protein hsp68 is not induced by stress in mouse erythroleukemia cell lines. Biochem. Cell. Biol. **66**:691–200.

4. **Aujame, L., and C. Morgan.** 1985. Nonexpression of a major heat shock gene in mouse plasmacytoma MPC-11. Mol. Cell. Biol. **5**:1780–1783.
5. **Bensaude, O., C. Babinet, M. Morange, and F. Jacob.** 1983. Heat-shock proteins: first major products of zygotic gene activity in mouse embryos. Nature (London) **305**:331–333.
6. **Bensaude, O., and M. Morange.** 1983. Spontaneous high expression of heat-shock proteins in mouse embryonal carcinoma cells and ectoderm from day 8 mouse embryos. EMBO J. **2**:173–177.
7. **Berk, A. J., and P. A. Sharp.** 1977. Sizing and mapping of early adenovirus mRNAs by gel electrophoresis of S1 endonuclease digested hybrids. Cell **12**:721–732.
8. **Burdon, R. H.** 1982. The human heat-shock proteins, their induction and their possible intracellular functions, p. 283–288. *In* M. J. Schlesinger, A. Tissieres, and M. Ashburner (ed.), Heat shock: from bacteria to man. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
9. **Chappell, T. G., W. J. Welch, D. M. Schlossman, K. B. Palter, M. J. Schlesinger, and J. E. Rothman.** Uncoating ATPase is a member of the 70kilodalton family of stress proteins. Cell **45**:3–13.
10. **Chesebro, B., K. Wehrly, K. Chesebro, and J. Portis.** 1976. Characterisation of Ia8 antigen, Thy-1.2 complement receptors and virus production in a group of murine virus-induced leukemia cells. J. Immunol. **117**:1267–1274.
11. **Chesebro, B., K. Wehrly, and D. Housman.** 1978. Lack of erythroid characteristics in Ia-positive leukemia cell lines induced by Friend murine leukemia virus: brief communication. J. Natl. Cancer Inst. **60**:239–242.
12. **Chirico, W. J., M. Gerard Waters, and G. Blobel.** 1988. 70K heat shock related proteins stimulate protein translocation into microsomes. Nature (London) **332**:805–810.
13. **Craig, E. A.** 1985. The heat-shock response. Crit. Rev. Biochem. **18**:239–280.
14. **Craig, E. A., and K. Jacobson.** 1984. Mutations of the heat-inducible 70K gene of yeast confer temperature-sensitive growth. Cell **38**:841–849.
15. **Deshaies, R. J., B. D. Koch, M. Werner-Washburne, E. A. Craig, and R. Schekman.** 1988. A subfamily of stress proteins facilitates translocation of secretory and mitochondrial precursor polypeptides. Nature (London) **332**:800–805.
16. **Finley, D., E. Ozkaynac, and A. Varshavsky.** 1987. The yeast polyubiquitin gene is essential for resistance to high temperatures, starvation and other stresses. Cell **48**:1035–1046.
17. **Gerner, E. W.** 1983. Thermotolerance, p. 141–162. *In* F. K. Storm (ed.), Hyperthermia in cancer therapy. G. K. Hall, Boston.
18. **Hahn, G. W., and G. C. Li.** 1982. Thermotolerance and heat-shock proteins in mammalian cells. Radiat. Res. **92**:452–457.
19. **Hall, B. G.** 1984. Yeast thermotolerance does not require protein synthesis. J. Bacteriol. **156**:1363–1365.
20. **Hallberg, R. L.** 1986. No heat shock protein synthesis is required for induced thermostabilization of translocation machinery. Mol. Cell. Biol. **6**:2267–2270.
21. **Heikkela, J. J., M. Kloc, J. Bury, G. A. Schultz, and L. W. Browder.** 1985. Acquisition of the heat-shock response and thermotolerance during early development of Xenopus laevis. Dev. Biol. **107**:483–489.

21a. **Hemmingsen, S. M., C. Woolford, S. M. van der Vies, K. Tilly, D. T. Dennis, C. P. Georgopolous, R. W. Hendrix, and R. J. Ellis.** 1988. Homologous plant and bacterial proteins chaperone oligomeric protein assembly. Nature (London) **333**:330–334.

22. **Henle, K. J., J. E. Karamuz, and D. B. Leeper.** 1976. Induction of thermotolerance in Chinese hamster ovary cells by high (45°) or low (40°) hyperthermia. Cancer Res. **38**:670–674.
23. **Henle, K. J., and D. B. Leeper.** 1979. Effects of hyperthermia (45°C) on macromolecular synthesis in Chinese hamster ovary cells. Cancer Res. **39**:2665–2674.
24. **Iada, H., and I. Yahara.** 1984. A heat-shock resistant mutant of Saccharomyces cerevisiae shows constitutive synthesis of two heat-shock proteins and altered growth. J. Cell Biol. **99**:1441–1450.
25. **Landry, J., D. Bernier, P. Chretien, L. M. Nicole, R. M. Tanguay, and N. Marceau.** 1982. Synthesis and degradation of heat-shock proteins during development and decay of thermotolerance. Cancer Res. **42**:2457–2461.
26. **Landry, J., and P. Chretien.** 1983. Relationship between hyperthermia-induced heat-shock proteins and thermotolerance in Morris hepatoma cells. Can. J. Biochem. Cell. Biol. **61**:428–437.
27. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
28. **Lefkowitz, J., and H. Waldmann.** 1984. Limiting dilution analysis of the cells of the immune system. I. The clonal basis of the immune system. Immunol. Today **5**:265–268.
29. **Li, G. C.** 1983. Induction of thermotolerance and enhanced heat-shock protein synthesis in Chinese hamster fibroblasts by sodium arsenite and by ethanol. J. Cell. Physiol. **115**:116–122.
30. **Li, G. C.** 1985. Elevated levels of 70,000 dalton heat-shock protein in transiently thermotolerant Chinese hamster fibroblasts and in their stable heat-resistant variants. Int. J. Radiat. Oncol. Biol. Phys. **11**:115–117.
31. **Li, G. C., and Z. Werb.** 1982. Correlation between synthesis of heat-shock protein and development of thermotolerance in Chinese hamster fibroblasts. Proc. Natl. Acad. Sci. USA **79**: 3219–3222.
32. **Lim, L., C. Hall, T. Leung, and S. Whatley.** 1984. The relationship of the rat brain 68kDa microtubule-associated protein with synaptosomal plasma membrane and with the Drosophila 70kDa heat-shock protein. Biochem. J. **224**:667–680.
33. **Lindquist, S.** 1986. The heat-shock response. Annu. Rev. Biochem. **55**:1151–1191.
34. **Loomis, W. F., and S. A. Wheeler.** 1980. Heat-shock response of Dictyostelium. Dev. Biol. **79**:399–408.
35. **Loomis, W. F., and S. A. Wheeler.** 1982. Chromatin-associated heat-shock proteins of Dictyostelium. Dev. Biol. **90**:412–418.
36. **McAllister, L., and D. B. Finkelstein.** 1980. Heat-shock proteins and thermal resistance in yeast. Biochem. Biophys. Res. Commun. **93**:819–824.
37. **Muller, W. U., G. C. Li, and L. S. Goldstein.** 1985. Heat does not induce synthesis of heat-shock proteins or thermoresistance in the earliest stage of mouse embryo development. Int. J. Hyperthermia **1**:97–102.

38. **Napolitano, E. W., J. S. Pachter, S. S. M. Chin, and R. K. H. Lien.** 1985. b-Internexin, a ubiquitous intermediate filament associated protein. J. Cell Biol. **101**:1323–1331.
39. **Pelham, H. R. B.** 1986. Speculations on the functions of the heat-shock and glucose-regulated proteins. Cell **46**:959–961.
40. **Pelham, H. R. B.** 1988. Heat shock proteins: coming in from the cold. Nature (London) **332**:776–777.
41. **Perry, M. D., and L. A. Moran.** 1987. Isolation of a mouse heat-shock gene (hsp68) by recombinational screening. Gene **51**: 225–234.
42. **Petko, L., and S. Lindquist.** 1986. Hsp26 is not required for growth at high temperatures nor for thermotolerance, spore development or germination. Cell **45**:885–894.
43. **Pinhasi-Kimhi, O., G. Michalovitz, A. Ben Zeez, and M. Oren.** 1986. Specific interactions between the p53 cellular tumor antigen and major heat-shock proteins. Nature (London) **330**: 182–185.
44. **Plefosky-Vig, N., and R. Brambl.** 1985. Heat-shock response of Neurospora crassa: protein synthesis and induced thermotolerance. J. Bacteriol. **162**:1083–1091.
45. **Plesset, J., C. Palm, and C. S. McLaughlin.** 1982. Induction of heat-shock proteins and thermotolerance by ethanol in Saccharomyces cerevisiae. Biochem. Biophys. Res. Commun. **108**: 1340–1345.
46. **Przybytkowski, E., J. H. T. Bates, D. A. Bates, and W. J. MacKillop.** 1986. Thermal adaptation in CHO cells at 40°C: the influence of growth conditions and the role of heat-shock proteins. Radiat. Res. **107**:317–321.
47. **Roccheri, M. C., M. G. Di Bernarda, and G. Guidice.** 1981. Synthesis of heat-shock proteins in developing sea urchins. Dev. Biol. **83**:173–177.
48. **Subjeck, J. R., J. J. Sciandra, and R. J. Johnson.** 1982. Heat-shock protein and thermotolerance; a comparison of induction kinetics. Br. J. Radiol. **55**:579–584.
49. **Taswell, C.** 1981. Limiting dilution assays for the determination of immunocompetent cell frequencies. I. Data analysis. J. Immunol. **126**:1614–1619.
50. **Ungewickell, E.** 1985. The 70kd mammalian heat-shock proteins are structurally and functionally related to the uncoating protein that releases clathrin triskelia from coated vesicles. EMBO J. **4**: 3385–3391.
51. **Watson, K., G. Dunlop, and R. Caviechioli.** 1984. Mitochondrial and cytoplasmic protein synthesis are not required for heat-shock acquisition of ethanol and thermotolerance in yeast. FEBS Lett. **272**:299–302.
52. **Widelitz, R. B., B. M. Magun, and E. W. Gerner.** 1984. Dissociation of 68,000 Mr heat-shock protein synthesis for thermotolerance expression in rat fibroblasts. Radiat. Res. **99**: 432–437.
53. **Widelitz, R. B., B. E. Magun, and E. W. Gerner.** 1986. Effects of cycloheximide on thermotolerance expression, heat-shock protein synthesis, and heat shock protein mRNA accumulation in rat fibroblasts. Mol. Cell. Biol. **6**:1088–1094.
54. **Wittig, S., S. Hensse, C. Keitel, C. Elsner, and B. Wittig.** Heat-shock gene expression is regulated during teratocarcinoma cell differentiation and early embryonic development. Dev. Biol. **96**:504–514.
55. **Yamamori, T., and T. Yura.** 1982. Genetic control of heat-shock protein synthesis and its bearing on growth and thermal resistance in Escherichia coli K12. Proc. Natl. Acad. Sci. USA **79**: 860–864.