In Vivo Growth of a Murine Lymphoma Cell Line Alters Regulation of Expression of HSP72

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We have identified a murine B-cell lymphoma cell line, CH1, that has a much-diminished capacity to express increased levels of heat shock proteins in response to heat stress in vitro. In particular, these cells cannot synthesize the inducible 72-kDa heat shock protein (HSP72) which is normally expressed at high levels in stressed cells. We show here that CH1 fails to transcribe HSP72 mRNA after heat shock, even though the heat shock transcription factor, HSF, is activated correctly. After heat shock, HSF from CH1 is found in the nucleus and is phosphorylated,imerized, and capable of binding the heat shock element. We propose that additional signals which CH1 cells are unable to transduce are normally required to activate hsp72 transcription in vitro. Surprisingly, we have found that when the CH1 cells are heated in situ in a mouse, they show normal expression of HSP72 mRNA and protein. Therefore, CH1 cells have a functional hsp72 gene which can be transcribed and translated when the cells are in an appropriate environment. A diffusible factor present in ascites fluid is capable of restoring normal HSP72 induction in CH1 cells. We conclude that as-yet-undefined factors are required for regulation of the hsp72 gene or, alternatively, that heat shock in vivo causes activation of hsp70 through a novel pathway which the defect in CH1 has exposed and which is distinct from that operating in vitro. This unique system offers an opportunity to study a physiologically relevant pathway of heat shock induction and to biochemically define effectors involved in the mammalian stress response.

The heat shock proteins (HSP) are a group of proteins, many of which are essential cellular proteins and act as molecular chaperones (13). They are highly conserved in all organisms from bacteria to humans. After heat shock or a range of other stresses, such as exposure to arsenite, ethanol, transition metal ions, amino acid analogs, and some sulfhydryl reagents and viruses, the levels of some of these constitutively expressed proteins are increased while other new isoforms are induced (24). Two families of HSP, HSP70 and HSP60, are crucial for the translocation and folding of newly synthesized cellular proteins (22). The protein that is the focus of this study is one of the HSP70 family of proteins, for which multiple isoforms exist. In addition to the cognate cytoplasmic isoform, HSP73, mammalian cells express a highly inducible isoform, HSP72, after stress. Other HSP70 isoforms are found in the mitochondria and endoplasmic reticulum (37). HSP73 has been shown to act as a chaperone for other cellular proteins, especially during synthesis, folding, and translocation to organelles (13).

Recently, we have identified a cell line with a defective heat shock response. The murine B-cell lymphoma CH1 is unable to synthesize detectable amounts of heat-inducible proteins after a mild heat shock sufficient to induce thermotolerance (11). Subsequently, we have shown that severe heating or treatment with the amino acid analogue azetidine-2-carboxylic acid can induce small increases in HSP90 and HSP73 synthesis but that no HSP72 is synthesized. The lack of expression of HSP72 is unexpected since it is a major heat-inducible protein in all mammalian species. The transcriptional activation of HSP72 has been well studied and is regarded as a model system for inducible gene regulation (27).

Transcriptional regulation in response to heat shock or other stresses is mediated by transcription factors which bind to a repeated 5-base sequence (NGAAN) in the promoter region (27). This sequence is known as the heat shock element (HSE). At least three heat shock transcription factors (HSF) are known to exist in mammalian cells, with HSF1 the factor responsible for binding to the HSE in response to stress. HSF2 appears to be active during development and differentiation of cells (34), and the normal function of HSF3 is unknown (29). HSF1 is present as a monomer in both the cytoplasm and nucleus in control cells and has no DNA-binding activity. After stress, it forms a trimer, accumulates in the nucleus, and binds to the HSE (6, 31, 33). There is also evidence of heat-induced phosphorylation of HSF, but the importance of this for HSF activation is unknown. For example, the proline analogue azetidine-2-carboxylic acid induces HSP72 synthesis in human cells without apparent phosphorylation of HSF1, although HSF1-HSE complexes are detected (33). Other experiments indicate that the above-described model is incomplete and that, in fact, HSF binding to the HSE is not sufficient for transcriptional activity. Sodium salicylate, as well as two different oxidants, has been shown to cause HSF to bind to the HSE but fail to induce transcription of mRNA for HSP72 (8, 20). The regulation of HSP72 varies between species since the protein is expressed constitutively in primate cells but only after stress in rodent cells (38), and, at least for Saccharomyces cerevisiae, evidence has been obtained which suggests that in addition to HSF, other factors can mediate heat-induced activation of transcription (21).

In this study, we demonstrate that CH1 cells have a functional HSP72 gene and that the capacity to synthesize HSP72 and other inducible HSP is revealed if all the appropriate regulatory factors are present. These unidentified factors are not provided in vitro but are provided within the environment of mice in which the CH1 cells are grown and exposed to heat. We describe the development of an in vitro-in vivo system...
ences in heat sensitivity between the RIF-1 and the CH lines (15). To conserve
lines. The difference in the severity of the heat treatment allows for the differ-
ent to steriletubes. The cellswere washed with sterilephosphate-buffered saline
which offers a unique approach for the identification of these factors.

MATERIALS AND METHODS

Cell culture. The CH1 and CH12/4927 cells were derived from murine B-cell
lymphomas isolated by Lanier et al. (23) and were maintained at 37°C in sus-
pension culture in RPMI 1640 containing 5% fetal calf serum (FCS), 1 mM
sodium pyruvate, and 50 μM β-mercaptoethanol. RIF-1 cells, a murine fibro-
sarcoma (36), were maintained in RPMI 1640 containing 10% FCS.

Tumor growth. Both C57BL/6J-nt mice (The Jackson Laboratory, Bar Har-
bors, Maine) and BALB/c-nu mice (Animal Resources Centre, Perth, Australia)
were used for tumor growth. The use of nude mice with a defective immune
response allowed the growth of nonsyngeneic tumor cells. Female mice, 6 to 8
weeks old, were injected intraperitoneally with 5 x 10⁶ CH1 cells. Tumors developed
over a period of 6 to 14 days postinjection. In somo
experiments, cells were grown in diffusion chambers placed in the peritoneal
cavity of the mouse. The chambers were prepared by sealing 0.22-μm-pore-size
filters over both sides of a 14-mm-diameter Plexiglas ring (Millipore) to create a
chamber 2 mm deep. After gas sterilization, the chambers were filled with a
suspension of 5 x 10⁶ cells, the entry port was sealed, and the chambers were
surgically implanted in the peritoneal cavity of BALB/c mice. After 5 days, some
of the mice were heated at 45°C for 15 min (see below). The chambers were
removed immediately from the mice and opened, and the cells were transferred
into sterile tubes. The cells were washed with sterile phosphate-buffered saline
(PBS), resuspended in culture medium, and radiolabelled with [³⁵S]methionine
as described below.

Incubation of tissue culture cells in ascites fluid. For experiments in which
ascites fluid was added to cells grown in vitro, the fluid was removed by syringe
from mice bearing CH1 tumors and placed in a heparin-treated tube to avoid
clotting. The fluid was centrifuged to pellet cells, and the supernatant was
sterilized by passage through a 0.22-μm-pore-size filter. The filtrate was mixed
with complete medium plus 20 mM HEPES (N-2-hydroxyethylpiperazine-N’-2-
ethanesulfonic acid) to buffer against the acidity of the fluid. The final concen-
tration of the filtered ascites fluid in culture medium was 10 or 50%. Overnight
culture in this medium had no visible adverse effect on CH1 cells.

Hyperthermia treatments. Cells in tissue culture were heated by replacement
of the medium with fresh medium preheated to the required temperature and
immersion of the cells in a water bath for the times given in the figure legends.
Suspension-cultured cells were always heated at a cell density of 5 x 10⁷/ml.
Adherent cells were heated whilst in the logarithmic growth phase. Mice with
ascites tumors or implanted with diffusion chambers containing CH1 cells were
anesthetized with pentobarbitol sodium (Nembutal) and placed in a jig in a 43°C
water bath so that only the head protruded above water level. The rectal tem-
perature was monitored by using a thermistor probe, and heating times were
measured from the time when core temperature reached 43°C, usually after
about 10 min. Following the heat treatment, the mice were killed by cervical
dislocation and the ascites cells and fluid were removed from the peritoneal
cavity and placed on ice. Nonheated mice were given pentobarbitol sodium while
the other mice were being heated.

Metabolic labelling and antibody binding. Following the heat treatment, cul-
tured CH1 cells were centrifuged at 80 x g for 3 min; resuspended in methi-
onine-free RPMI containing 5% dialyzed FCS, 1 mM pyruvate, 50 μM β-mer-
kaptoethanol, and 50 μC of [³⁵S]methionine per ml (specific activity, 1,200
Ci/mmol; NEN Du Pont); and incubated at 37°C for 4 h. Cells removed from
ascites tumor-bearing mice were rinsed twice in culture medium, counted, re-
suspended in the radioactive medium described above, and incubated at 37°C for
4 h. Bone marrow cells were flushed from the femurs of BALB/c mice, heated for
10 min at 43°C, and then incubated in the radioactive medium for 4 h at 37°C.
Tissue-cultured cells were harvested directly for electrophoresis. Suspension cells
from ascites tumors were removed from the petri dish, washed twice in PBS
containing 5% calf serum (PBS-CS), and incubated for 30 min on ice with
anti-H-2Kk antibody (Becton Dickinson). The cells were washed twice in PBS-CS
and incubated for 30 min on ice with fluorescein isothiocyanate (FITC)-conju-
gated anti-mouse immunoglobulin G (IgG3) (Silenus). Cells were washed again
in PBS-CS and sorted on the basis of FITC fluorescence. In parallel with treatment

FIG. 1. Radiographs of two-dimensional gels of proteins from control (con) and heated murine cells grown in tissue culture. Panels: A, CH1; B, CH12/4927; C, RIF-1. Proteins were separated first in tube gels according to their isoelectric point, with more acidic proteins on the left, and then in slab gels according to their subunit molecular mass. Molecular mass markers (in kilodaltons) are shown on the left of panel A. The upward- and downward-pointing arrows show the positions of HSP72 and HSP73, respectively. The arrowhead marks HSP90, and “A” refers to actin. The RIF-1 cells were heated for 10 min at 45°C, and the CH1 and CH12/4927 cells were heated for 15 min at 43°C. This was followed by a 6-h incubation at 37°C in [³⁵S]methionine-containing medium for all three cell lines. The difference in the severity of the heat treatment allows for the differences in heat sensitivity between the RIF-1 and the CH lines (15). To conserve space, only part of each gel is shown.

FIG. 2. Northern analysis of total RNA from CH1 and CH12/4927 cells before (Con) or 90 min after (Heat) treatment for 15 min at 43°C. A 10-μg amount of total RNA was loaded in each lane of the agarose gel. The membranes were probed with digoxigenin-labelled cDNA probes encoding hsp72, hsp73, hsp90, and gapdh as indicated.
of cells obtained from an ascites tumor, a sample of CH1 cells from tissue culture was tagged with antibodies to measure H-2Kk levels.

**Flow cytometry.** A FACStar Plus Cell Sorter (Becton Dickinson) with a 5-W argon laser (Coherent Innova 90A) and a 340 Hewlett Packard computer was used for flow cytometric analysis and sorting. The excitation wavelength was 488 nm with 200-mW laser power, and the instrument was aligned with 3.41-μm-diameter fluorescent beads. Forward angle light scatter and 90° scatter were collected by using 488-nm bandpass filters. FITC fluorescence emission was collected by using a 530/30-nm bandpass filter and a 4-decadelogarithmic amplification. Cellswere acquired for data analysis. Lysys II software was used for data acquisition and analysis and for setting of sort regions. Flow-sorted cells were analyzed by two-dimensional gel electrophoresis.

**Gel electrophoresis.** Cells were dissolved in sample buffer for isoelectric focusing in tube gels and then separated on the basis of molecular weight by using 10% acrylamide slab gels as described earlier (11). Gels were stained with Coomassie blue, destained, dried, and exposed to X-ray film (Fujifilm) to generate autoradiographs.

**Western blotting (immunoblotting).** After transfer of proteins on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels to nitrocellulose, the membranes were probed with monoclonal antibodies either against HSF1 (33) at a 1:10,000 dilution or against hsp72 (C92) (25) at a 1:1,000 dilution and then with a horseradish peroxidase-conjugated secondary antibody diluted 1:20,000. Antibody complexes were detected by using a chemiluminescence detection system (Amersham).

**Northern (RNA) analysis.** Total RNA was extracted from cells by the acid guanidinium thiocyanate-phenol-chloroform method (9). A 10-μg amount of total RNA was separated on a 1.2% agarose–formaldehyde gel and transferred to Zetaprobe membrane (Bio-Rad) in 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). cDNA probes were labelled by using either [α-32P]dCTP or the Boehringer Mannheim digoxigenin system and were hybridized to the membranes overnight in Church buffer (1% bovine serum albumin, 1 mM EDTA, and 7% SDS in 0.25 M phosphate buffer, pH 7.2) at 65°C (10). Membranes were washed twice for 10 min each time at room temperature in 2× SSC-0.1% SDS and then twice for 15 min each time in 0.5× SSC-0.1% SDS at 65°C before a standard detection procedure using anti-digoxigenin antibodies (Boehringer Mannheim) and the hsp90 probe was a 1,700-bp human cDNA which specifically binds the heat-inducible hsp90 (18). The murine hsp72 probe was from the coding region of the cDNA isolated by Giebel et al. (14) and included nucleotides 814 to 2044.

**Gel mobility shift assay.** Nuclear extracts were prepared by a rapid fractionation protocol (6) from control cells and cells which had been heated at 43°C for 15 min and then allowed to recover at 37°C for 15 min. Cells were pelleted in cold PBS and lysed in 5 ml of lysis buffer (0.5% Nonidet P-40, 5 mM KCl, 10 mM Tris [pH 8.0], 1 mM dithiothreitol [DTT], 2 mM phenylmethylsulfonyl fluoride [PMSF]) for 5 min. After a brief 10,000× g spin, the supernatant containing the cytosolic proteins was discarded and the lysis step was repeated with Triton X-100 added to a final concentration of 1% in lysis buffer. The supernatant was removed, and the pellet was resuspended in 100 μl of high-salt extraction buffer (0.5 M NaCl, 10 mM Tris [pH 8.0], 0.1% Nonidet P-40, 1 mM DTT, 2 mM PMSF) for 30 min at 4°C. The insoluble nuclear fraction was removed by a 10-min high-speed spin in a Beckman Airfuge. Extracts containing 5 μg of protein were incubated at room temperature with a 32P-labelled double-stranded oligonucleotide in DNA-binding buffer [1 mM EDTA, 10 mM Tris (pH 8.0), 50 mM NaCl, 5 mM MgCl2, 0.5 mM PMSF, 1 mM DTT, 0.4 mg of poly(dI-dC) per ml] plus cold competitor DNA in 50-fold excess as appropriate. The oligonucleotide was based on the HSE in the murine hsp70.1 gene promoter and contains almost four copies of the consensus HSF binding site in the sequence 5′-cagacggAaagTcTgaGAgGATCcgc-3′. After a 20-min incubation at room temperature, complexes were separated on a nonradiating 5 to 20% gradient polyacrylamide gel, dried, and exposed to film.

**PCR.** Genomic DNA (0.5 μg) isolated as described elsewhere (32) was amplified with primers specific to hsp72 for 30 cycles (90 s at 94°C, 120 s at 55°C, 120 s at 72°C). The primers were 5′-AAC CTG CAA AAA AGA GGG AGG G-3′ and 5′-GTC TCT GCT GTG CTC TGG ATG-3′. Aliquots of the reaction products were analyzed on 2% agarose gels. A 610-bp product is expected from hsp72. The identity of the product was confirmed by restriction digests.

**Southern analysis.** Genomic DNA (10 μg) isolated as described elsewhere (32) was digested with restriction enzymes overnight and then separated on a 1% agarose gel in TAE (Tris-acetate-EDTA). After two 10-min treatments of the gel in 0.2 M HCl, the DNA was transferred to a Zetaprobe membrane (Bio-Rad) in 20× SSC overnight. The membrane-bound DNA was hybridized overnight in Church buffer containing a 32P-labelled 800-bp probe containing the promoter region of hsp72 (hsp70-1) (19). The membrane was washed twice for 20 min at 1× SSC containing 0.5% sodium pyrophosphate at 65°C and then twice for 25 min at 1× SSC containing 0.1% SDS at 65°C. The membrane was exposed to X-ray film (Fuji) with an intensifying screen for 3 days.

**RESULTS**

Expression of HSP72 is impaired in CH1 cells grown in vitro. Nearly all murine cells grown in culture respond to heat by synthesizing a strictly inducible isofrom of HSP70 called...
HSP72. The typical level of induction of HSP72 after heat shock in murine cells is demonstrated in Fig. 1C for a fibrosarcoma line, RIF-1. Cells of lymphoid origin generally show a less pronounced HSP72 response than do fibroblast lines, but the response is still clear, as demonstrated by the murine B-cell lymphoma CH12/4927 (Fig. 1B). In contrast, although CH1 cells are B-cell lymphoma cells derived from the same strain of mice as are CH12/4927 cells (16), they do not express HSP72 when grown in culture, even when tested with a range of heat treatments (Fig. 1A) (11). The identity of the induced 72-kDa protein was confirmed by two-dimensional Western analysis using the C92 antibody, which is specific to HSP72 (data not shown). Note that the isoelectric point of this protein does vary, being more acidic in the RIF-1 cells than in the CH12/4927 cells.

The lack of HSP72 protein in CH1 cells is paralleled by the absence of HSP72 mRNA, as shown on a Northern blot of total CH1 RNA hybridized with a cDNA probe specific to HSP72 (Fig. 2). In comparison, HSP72 message was clearly present in CH12/4927 cells, but only after heat treatment. gapdh (glyceraldehyde phosphate dehydrogenase) cDNA hybridized to bands in all lanes, and furthermore, the levels of transcripts from two constitutively expressed HSP, HSP73 and HSP90, increased after heat shock (Fig. 2).

**CH1 cells express functional HSF1 and contain the hsp72 gene.** One explanation for the lack of transcription of HSP72 after heat shock in CH1 cells is that HSF1, the factor which normally activates HSP72 transcription, is defective in CH1 cells. This seems unlikely considering that levels of HSP73 and HSP90 mRNA increase after heat shock. Nevertheless, we examined the ability of proteins in nuclear extracts from CH1 and CH12/4927 cells to bind to the appropriate promoter element by using gel mobility shift assays. We found that both lines contain a nuclear protein capable of binding to the HSE from the murine hsp72 promoter (Fig. 3). This protein binds only after heat shock, and its binding is prevented completely by a 50-fold excess of cold oligonucleotide (Fig. 3). We have also determined that a mutant HSE oligonucleotide (with specific guanines changed to cytosine) does not compete with the binding activity (data not shown). Thus it appears that HSF1 in CH1 cells is capable of migrating to the nucleus after heat shock, where it can trimerize and bind to the appropriate sequence, although this does not result in *hsp72* transcription.

Several studies have shown that HSF1 is usually, but not always, phosphorylated prior to activation of transcription (33). The phosphorylation state of HSF1 can be determined on the basis of its migration distance on SDS-PAGE gels. We detected an apparent increase in the molecular weight of HSF1 after heat shock when we used an HSF1 antibody to probe a Western blot of proteins from CH1 and CH12/4927 cells (Fig. 4). The HSF1 from CH1 appeared to be phosphorylated after heat shock to the same extent as HSF1 from CH12/4927, suggesting that lack of phosphorylation does not account for the lack of transcription of *hsp72* in CH1 cells.

To ensure that the *hsp72* gene had not been deleted from the CH1 cells, we probed a Southern blot of genomic DNA isolated from CH1 and CH12/4927 cells with the *hsp72* cDNA (Fig. 5). The restriction maps were identical for the two lines, indicating that CH1 cells do contain the *hsp72* gene. To check for the presence of the promoter region, primers specific to this region of the *hsp72* gene were prepared and genomic DNA from CH1 and CH12/4927 cells was amplified by PCR. The anticipated 610-bp fragment was present in both cell lines (Fig. 6).

**CH1 cells express HSP72 if grown and heated in situ in a mouse.** Since the gene and the transcription factors required for its transcription were present in CH1 cells, we decided to test whether growth of the cells in a different environment would activate transcription of the gene after heat shock. To achieve the altered environment, we grew the CH1 cells as tumors in allogeneic mice to facilitate the subsequent separation of tumor and host cells on the basis of surface antigen
expression. CH1 cells, being derived from the double congenic mouse strain B10-H-2aH-4bp/Wts (39), express the H-2Kk antigen on the cell surface. Cells from C57BL/6J-nu and BALB/c-nu mice do not express this antigen, thus providing a means for separating host from tumor cells.

Ascites tumor cells extracted from control BALB/c-nu mice or those that had been subjected to whole-body hyperthermia were incubated in medium containing [35S]methionine. After 4 h, suspension cells were removed from the dish, leaving behind a monolayer of adherent cells, predominantly macrophages and other cells of host origin. The suspension cells were incubated with an antibody against H-2Kk and then with a second-ary anti-mouse IgG antibody conjugated to FITC. Most of the cells were CH1 as judged visually by fluorescence microscopy, but a few smaller and nonfluorescent cells were present. This was confirmed by the fluorescence profiles generated on the flow cytometer, where a shoulder can be seen in the profile of cells grown in vivo (Fig. 7B and 7C) but not in that of cells grown in vitro (Fig. 7A). There was a reduction in fluorescence intensity in the cells from the heated mouse (Fig. 7C) in comparison with the fluorescence in cells from the control mouse (Fig. 7B), reflecting a reduction in H-2Kk concentration on the surface of the heated cells. Those cells falling in the upper part of the spectrum (region 1) were collected as CH1 cells, and the rest were designated host cells, although this fraction (region 2) still contains mainly CH1 cells. Region 1 was deliberately set to collect only the more fluorescent CH1 cells to avoid even low levels of contamination with the minor population of host cells.

Analysis of the CH1 fraction (region 1 shown in Fig. 7B and C) by two-dimensional gel electrophoresis revealed the expression of HSP72 in the cells from the heated mouse but not from the control mouse (Fig. 8). The identity of the inducible protein as a member of the HSP70 family was confirmed by two-dimensional Western blotting of CH1 samples obtained from control and heated mice (data not shown). The antibody used, N6, recognized both the constitutive and inducible isoforms of HSP70 (3). Similar results were obtained when the experiment was repeated with C57BL/6J-nu mice (data not shown).

As a verification of the flow-sorting procedure, bone marrow cells obtained from the femurs of BALB/c mice were heated and then mixed with nonheated tissue-cultured CH1 cells. There was a 40-fold excess of bone marrow cells over CH1 cells in the sample. The mixture was incubated for 4 h in a medium containing [35S]methionine, treated with the anti-H-2Kk antibody followed by the secondary antibody, and then flow sorted. When analysis based on FITC fluorescence was done, two distinct cell populations were obtained (Fig. 9) but only the bone marrow fraction (region 2) contained inducible HSP72 as judged by two-dimensional gel electrophoresis. Thus, despite the large excess of heated bone marrow cells, a CH1 population lacking HSP72 expression could still be obtained by this sorting procedure. This experiment confirmed that the heat-induced HSP72 is derived from the CH1 cells and not from the host cells when CH1 cells are heated in situ in a mouse.

A diffusible factor potentiates HSP72 expression in CH1 cells. To determine whether the potentiation of HSP72 induction was due to a diffusible factor present in the peritoneal cavity of the mice or was due to direct cell-to-cell contact, we
placed CH1 cells inside diffusion chambers and surgically implanted these within the peritoneum of BALB/c mice. The chambers permitted transfer of soluble factors but not cells through the 0.22-μm-pore-size membrane, and this was sufficient to allow the CH1 cells to respond to hyperthermia treatment of the mouse by inducing HSP72. Northern analysis of RNA extracted from CH1 cells in the chambers from control and heated mice is shown in Fig. 10. HSP72 mRNA was present only in the heated sample. Two-dimensional gel analysis revealed a protein with the same mobility as HSP72 (Fig. 11A) which by Western blotting reacted with an antibody specific to HSP72 (C92 from W. Welch) (Fig. 11B).

To initiate the purification of the factor, we tested whether ascites fluid from tumor-bearing mice could potentiate heat shock induction of HSP72 when added to CH1 cells in culture. The ascites fluid was sterilized (see Materials and Methods) and added to cultured CH1 cells at a final concentration of 10 or 50% for an overnight incubation. Cells were rinsed in fresh medium not containing the ascites fluid, heated, incubated for 4 h in medium containing [35S]methionine, and then separated by two-dimensional electrophoresis. The presence of either 10 or 50% ascites fluid in the medium was sufficient to permit low-level, but definite, heat-induced expression of HSP72 in CH1 cells (Fig. 12). Immunostaining of two-dimensional Western blots of the cells treated with 50% ascites fluid with the C92 antibody confirmed the identity of the induced protein as HSP72 (Fig. 12). Thus, we have shown that one or more components of the ascites fluid can potentiate HSP72 expression.

**DISCUSSION**

The induction of HSP is often described as a ubiquitous event in all organisms. However, there are a few exceptions, the CH1 line being one of these (11). The lack of HSP72 expression is not typical of all B-cell lines, since two other lines derived in a similar way from the same strain of mice, the CH12/4927 cells reported here and CH34 cells (unpublished data), can be induced to synthesize HSP72 in vitro. We have also shown that other inducers of the stress response such as arsenite, ethanol, and an amino acid analogue are equally ineffective in inducing HSP72 expression in CH1 cells (unpublished data).

The induction of HSP72 by stress is associated with the development of thermotolerance, a transient resistance to heat shock. The amount of HSP72 expressed correlates with the...
FIG. 12. Radiographs and Western blots of two-dimensional gels of proteins from cells incubated in ascites fluid before heat shock. The cells were treated overnight with either 10 or 50% ascites fluid in culture medium. (A) Cells incubated in 50% ascites fluid but not heated. The cells treated with 10% ascites fluid (B) and one-half of those treated with 50% ascites fluid (C) were heated for 15 min at 43°C in fresh medium and then incubated for the next 4 h in medium containing [35S]methionine. Only the 70-kD regions of the gels are shown. For an explanation of the arrows, see the legend for Fig. 1. The radiographs are shown on the left, and the Western blots probed with the C92 antibody are shown on the right.

extent of thermotolerance (4), suggesting a protective role for HSP72. However, there is still debate about whether HSP expression constitutes the only mechanism for thermotolerance, and we have shown that the CH1 cells develop moderate thermotolerance in the absence of increased levels of HSP11.

We have shown here that CH1 cells contain an HSF that is present in the nucleus, is phosphorylated, and binds to the HSE in response to heat shock. Despite this apparently normal behavior, there appears to be a defect at the level of transcription, since no mRNA for HSP72 is detectable after heat shock. The absence of message explains our initial observation that CH1 cells are unable to synthesize the HSP72 protein after heat shock (4, 11).

In a recently proposed model for the regulation of the heat shock response, the HSP70 that accumulates after heat shock interacts with HSF1, resulting in feedback inhibition of hsp70 transcription (1, 28). It is possible that in the CH1 cells, unusually high constitutive levels of HSP73 cause it to bind to the HSF and repress transcription. However, we consider this unlikely as we have assayed the levels of HSP73 by an enzymelinked immunosorbent assay and find them to be in the normal range (4, 11), and the regulation of other heat shock genes, such as hsp73 and hsp90, appears normal.

The novel finding in this study is that the CH1 cell line can express HSP72 in response to stress when the cells are in the appropriate environment, and we conclude that, unlike most cells, CH1 cells require external factors for the expression of HSP72. We have shown that the gene for HSP72 is still present in CH1 cells, as judged by Southern blotting and PCR, and its induction in vivo proves that its promoter is still capable of responding to heat. Control sorting experiments confirm that it is possible to separate host and tumor cells, thus eliminating the possibility that the observed response is due to host cells. Further, the expression of HSP72 in CH1 cells in a chamber that excludes host cells demonstrates that it is the CH1 cells that are expressing HSP72. There are some other examples of cell lines that cannot express HSP72 in response to heat shock (5, 17, 26, 41), but this is the first study demonstrating the rescue of HSP72 synthesis in such cells. The cause of the defect has not been determined for any of these other deficient lines.

MEL cells show normal heat-inducible binding of the HSF to the HSE, but unlike the situation in CH1 cells, the HSF of MEL cells appears to have a decreased level of phosphorylation (17), although it is unknown whether this reduces its activity as a transcription factor. Aberrant methylation of the hsp72 promoter has been proposed as an explanation for the lack of HSP72 synthesis in MEL, PCC4, CH27, and MPC11 cells (30a).

We have no evidence that the activation of hsp72 transcription in vivo is occurring through the HSF rather than through one of the other elements of the hsp72 promoter. v-Myb can activate the human hsp72 promoter, this activation being dependent on the TATA box (12), while the CCAAT binding factor (CBF) can mediate both transactivation of human hsp72 by the adenovirus protein Ela (40) and repression of hsp72 by the tumor suppressor p53 (2). Preliminary immunoprecipitation experiments suggest that wild-type p53 is not present in CH1 cells (unpublished data).

To explain the induction of HSP72 in vivo we considered the possibility that the cells were being stimulated through surface IgM molecules. Stimulation with anti-IgM antibodies has been shown to induce HSP72 in human peripheral B cells (35), but we were unable to repeat this observation using CH1 or CH12/4927 cells incubated in vitro with anti-IgM antibody (unpublished data). It is known that these two lines express surface IgM molecules (16). The transformed phenotype of the CH lines may account for this difference from the published data.

We have established that the stimulus does not require contact of CH1 cells with host cells present in the tumor, since diffusible factors present in the ascites fluid are adequate. CH1 cells removed from a nonheated mouse, washed in culture medium, and then heated in vitro rapidly lose the ability to express HSP72 (data not shown), suggesting that the factor needs to be present immediately before the time of heating. This result also rules out the possibility that passage through the mouse induces differentiation of the CH1 cells to a state in which they can express HSP72 in response to stress. Overnight culture of the cells in medium containing cell-free ascites fluid from a tumor-bearing mouse is also sufficient to potentiate the heat shock induction of HSP72. We suggest that the complementing factor for HSP72 synthesis may act by correcting a defect in the HSF1-mediated pathway of HSP72 expression or may be acting through a different pathway, for example, through activation of HSF2. Either way, the results presented here add a new level of complexity to the study of stress protein induction and point to physiologically relevant induction pathways which have not yet been addressed in vitro. Interestingly, there is evidence that physiological stress in vivo can be transmitted to cells on a molecular level. For example, stress induced by restraint of rats stimulates the pituitary gland to secrete adrenocorticotropic hormone (ACTH), which is both necessary and sufficient to induce hsp72 transcription in the adrenal cortex (7).

To assist in the purification and identification of the potentiating factor(s), we are preparing CH1 cells transfected with a reporter gene linked to the murine hsp72 promoter. This will enable rapid screening of purified factors or components of the ascites fluid that are active. We believe that this unique in
vitro-in vivo system will reveal further steps of regulation of HSP72 expression in stressed cells.

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