Methylation-associated Transcriptional Silencing of the Major Histocompatibility Complex-linked hsp70 Genes in Mouse Cell Lines*

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The MHC-linked hsp70 locus consists of duplicated genes, hsp70.1 and hsp70.3, which in primary mouse embryo cells are highly heat shock-inducible. Several mouse cell lines in which hsp70 expression is not activated by heat shock have been described previously, but the basis for the deficiency has not been identified. In this study, genomic footprinting analysis has identified a common basis for the deficient response of the hsp70.1 gene to heat shock in four such cell lines, viz., the promoter is inaccessible to transcription factors, including heat shock transcription factor. Southern blot analyses reveal extensive CpG methylation of a 1.2-kilobase region spanning the hsp70.1 transcription start site and hypermethylation of the adjacent hsp70.3 gene, which is presumably also inaccessible to regulatory factors. Of four additional, randomly chosen mouse cell lines, three show no or minimal hsp70.3 heat shock responsiveness and CpG methylation of both hsp70 genes, and two of the three lines exhibit a suboptimal hsp70.1 response to heat shock as well. In all three lines, the accessibility of the hsp70.1 promoter to transcription factors is detectable but clearly diminished (relative to that in primary mouse cells). Our results suggest that the tandem hsp70 genes are concomitantly methylated and transcriptionally repressed with high frequency in cultured mouse cells.

The heat shock response, the increased transcription of a set of genes in response to heat or other environmental stresses, is a highly conserved biological response, occurring in all organisms so far examined. In eukaryotes, the response is mediated by heat shock transcription factor (HSF), which is present in a monomeric, non-DNA binding form in unstressed cells (except in certain yeast) and is activated by stress to an oligomeric species which can bind to promoters of heat shock genes (2-6). The target sequence for activated HSF, the heat shock element (HSE), consists of inverted repeats of the pentanucleotide sequence NGAA(A,T) (7, 8). In mouse, where two distinct HSF genes have been cloned, antisera specific for HSF1 and HSF2 were used to show that the response to stress was mediated solely by HSF1 (9, 10).

Of the heat shock genes, hsp70 is the most highly stress-inducible. It was therefore quite surprising to find several rodent cell lines where this gene exhibited minimal, if any, activation in response to stress. This finding was initially reported in mouse erythroleukaemia (MEL) cells (11), a mouse plasmacytoma line (MPC-11, Ref. 12), and two mouse embryonal carcinoma (EC) lines (PCC4-AzaR1, PCC7-S-1009, Ref. 13). More recently, the same deficiency was found in a glucocorticoid-resistant rat hepatoma clone (14) and in the murine lymphoma line CH1, where, interestingly, hsp70 heat shock responsiveness was restored when the cells were grown as a tumor and heated in situ (15). Based on results of gel shift analyses and/or transfection studies, a trans-acting defect, possibly in HSF, was proposed as the cause for the lack of hsp70 responsiveness in the MEL, MPC-11, PCC4, and PCC7 mouse lines (11-13). In the case of the rat hepatoma clone, a defect specific to the hsp70 gene itself was one of the possibilities considered (14), and, in the case of CH1 cells, a requirement for additional regulatory factors to activate hsp70 expression was suggested (15). None of the previous studies has identified the exact nature of the defect, however.

In mouse (as well as in human), duplicated genes comprise the hsp70 locus, which is located in the MHC class III region (16, 17). The murine hsp70.1 and hsp70.3 genes are highly homologous, although the nucleotide sequences completely diverge in the 3′-untranslated regions (UTR), encode identical proteins, and are both expressed only in response to stress (18, 19). The genes are in the same transcriptional orientation and are approximately 7 kb apart in mouse (20). Methodology used in some of the previous studies of mouse lines lacking an hsp70 heat shock response would have detected expression of either gene; one can therefore surmise that in these lines, both hsp70 genes are completely unresponsive to heat shock. Using probes which distinguish transcription from each of the hsp70 genes, we formally demonstrate that in MEL, MPC-11, and PCC4 cells, neither hsp70 gene responds to heat shock. Furthermore, examination of additional, randomly selected mouse lines reveals that transcriptional repression of one or both hsp70 genes is very widespread. The defect appears to reside in the hsp70 locus itself, as we find that HSF1 is activated normally by heat shock in all lines deficient for activation of one or both hsp70 genes and that another heat shock gene, hsp86, exhibits normal heat shock induction.

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In the case of the hsp70.1 gene, we have used genomic footprinting and Southern blot analyses to demonstrate that unresponsiveness to heat shock is consistently associated with a complete loss of promoter accessibility and extensive CpG methylation; these alterations apparently constitute the common basis for the nonresponsive phenotype. We also find detectable but reduced (relative to primary mouse cells) hsp70.1 promoter accessibility and partial hsp70.1 gene methylation in three additional mouse cell lines. Two of these three cell lines show a clearly attenuated hsp70.1 transcriptional response to heat shock. In every cell line which exhibits hsp70.1 methylation, the adjacent hsp70.3 gene is also methylated and shows no or a very weak response to heat shock. The tandem hsp70.1 promoter— the adjacent hsp70.3 gene is also methylated and shows a clearly attenuated hsp70.1 transcriptional response to

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**MATERIALS AND METHODS**

Cell Culture, Generation of Primary Mouse Embryo Cells, Heat Shock—Mouse F9 and PCC4.aza.R1 embryonal carcinoma (EC) cells were grown on gelatinized dishes in DME supplemented with 10% FBS; NIH3T3 fibroblasts were grown in the same medium. Mouse erythroleukemia cells were grown on gelatinized dishes in DME supplemented with 10% horse serum and DME supplemented with 20% heat-inactivated FBS. CH27 (mouse B-cell lymphoma) and LK35.2 (mouse plasmacytoma) cells were obtained from ATCC and grown in Fischer’s medium supplemented with 10% FBS, and the cells were pooled and cryopreserved. For heat shock, plates were sealed with parafilm and immersed in a 43°C water bath for 40 min.

Measurement of Transcription Rates—Nuclei were isolated as described previously. Briefly, unstrressed and heat-shocked cells were lysed in hypotonic buffer (10 mM Tris, pH 7.4, 5 mM MgCl₂, 0.4% Nonidet P-40, 0.5 mM dithiothreitol, 0.3 mM sucrose), and nuclei were pelleted through a cushion of the same buffer containing 0.8 mM sucrose. Transcription run-on analysis was done as described previously (22) using 5 x 10⁶ to 1 x 10⁷ nuclei per analysis. The amount of radioactivity incorporated into nascent transcripts was quantified by trichloroacetic acid precipitation and liquid scintillation counting. A probe to specifically measure transcription from the hsp70.1 gene was prepared by subcloning a Stul-BglII fragment of pM9.5 (18), containing sequences from -390 to +150 of the hsp70.1 gene, into the Stu I- and Bam HI sites of pGem-T/Zf (+) (Promega). A probe to specifically measure transcription of the hsp70.3 gene was prepared by subcloning the Scal-SalI fragment of pMH213 (23), containing sequences from the 3'-UTR of the hsp70.3 gene into the Smal and SalI sites of pUC18. These plasmids, as well as pUC801 (human hsp80 as 24) and GAPDH (rat glyceraldehyde-3-phosphate dehydrogenase (23)) were immobilized on filters and hybridized to equivalent amounts of labeled transcripts from each cell line. Results were quantitated with a Fuji PhosphorImager.

Gel Retardation Assays—Whole cell extracts were prepared and gel retardation assays were performed as described previously (22). Briefly, extracts containing 10 µg of protein were incubated with a 32P-labeled oligonucleotide at room temperature for 20 min. To measure Sp1 levels, binding reactions were carried out using a double-stranded oligonucleotide 5'GAGAGTGGGCGGGGCCGGTGAA3' and its complement. To measure levels of HSE binding activity, a double-stranded oligonucleotide 5'ACCGGAAACTGTGAGATCTCCGGCCCTAAA3' and its complement were used. These oligonucleotides corresponded to the proximal elements of the mouse hsp70 promoter (18). The oligonucleotide-binding protein complexes were analyzed on 4% polyacrylamide gels. Antibody perturbation experiments were performed exactly as described previously; dilutions (1 µl) of rabbit antiserum specific for HSF1 or HSF2 were preincubated with whole cell extracts for 20 min at room temperature prior to initiating the binding reactions (10).

Genomic Footprinting—Unstrressed and heat-shocked cells (approximately 2 x 10⁶ cells) were exposed to dimethyl sulfate (DMS), 0.2% final concentration in culture medium, for 3 min at room temperature. For adherent cells, spent medium was removed, DMS was resuspended in 10 ml of this medium by vortexing, and the mixture was added back to the cells. Suspension cells were pelleted and resuspended in 3 ml of spent medium containing DMS. Duplicate samples were exposed to DMS, and replicate experiments were performed with each cell line to ensure reproducibility of results. Genomic DNA was isolated (26), digested with EcoRI, cleaved with piperidine, and used for footprinting. Genomic footprinting was also carried out on DNA which was isolated from NIH3T3 cells, depurinated (naked DNA), and treated with DMS in vitro. Genomic footprinting was analyzed using an ligated-mediated polymerase chain reaction method (27). Primers for footprinting of the coding strand of the hsp70.1 gene were derived from noncoding strand sequences located just downstream of the TATA element (18). The sequence of primer 1 was 5'CTCCGTGTCGACGGTACG3'; the sequence of primer 2 was 5'AGTAGCTGTACGCTGCGCC3'. Primer 3, used for end labeling, had the same sequence as primer 2 but included four additional bases at the 3' end: 5'GCTC3'. The hsp70.3 sequence corresponding to primer 2 is 5'AGTAGCTGTACGCTGCGCC3'- (19); it thus contains both a base change and a base insertion near the 3' end and should not be recognized by footprinting primer 2. In the amplification step of the ligation-mediated polymerase chain reaction procedure, which is carried out at 2°C above the Tₘₐₚ of the primers. We detected labeled product of the expected size (310 bp) in footprinting primers 1 and 2. The footprinting primer strand hsp70.1-specific primer of equivalent Tₘₐₚ (derived from sequences upstream of the distal HSE) were subjected to the same amplification conditions used in the genomic footprinting analysis and then labeled using genomic footprinting primer 3, but we did not detect any labeled product when an opposite strand hsp70.3-specific primer of equivalent Tₘₚ was used instead, thus verifying that, under these conditions, the hsp70.1 footprinting primer 2 does not recognize the hsp70.3 gene.

Amplification, Cloning, and Sequencing of the hsp70.1 Promoter Region—Genomic DNA (100 µg) isolated from MEL, CH27, and MPC-11 cells was amplified with the primer 5'GCTCCGTGTCGACGGTACG3', which overlaps footprinting primer 1, and 5'CCTCCCTCGAATTCGCTCCT3', derived from sequences upstream of the distal HSE of the hsp70.1 promoter. Polymerase chain reaction conditions were 40 cycles of 94°C, 1 min, 64°C, 1 min, and 72°C, 1 min. Each of the DNAs yielded the expected 310-bp amplifier, which was cloned into the pCR1 vector (Invitrogen). Several clones derived from each of the cell lines were subjected to dye sequencing.

Southern Blot Analysis—One hundred µg of genomic DNA, isolated as for genomic footprinting, was incubated with 5 units/µg of BglII (BRL) for at least 4 h, followed by a second incubation with an additional 2-5 units/µg of enzyme. To specifically analyze hsp70.1 gene methylation, the digests were electrophoresed on 0.8% Seaplague II (BRL) gels, transferred to nitrocellulose membranes, and treated with DMS in DNA was then subjected to further digestion with MspI (New England Biolabs), its methylation-sensitive isoschizomer MspI (New England Biolabs), or Hhal (New England Biolabs), again utilizing two applications of restriction endonuclease to ensure complete digestion. In some experiments, completeness of digestion was checked further by adding 2 ng of the 22.5-kb BamHI fragment of pMSGCAT (Pharmacia Biotech Inc.) to all aliquots of the digestion mixture and using Southern blot analysis to determine that this plasmid fragment was digested to completion. Following electrophoresis of the digested genomic DNA on 1.2% agarose gels, DNA was transferred to Zeta-Probe GT membranes (Bio-Rad) and hybridized with a 32P-labeled probe prepared by random-primed labeling (Life Technologies, Inc.) of a 1.2-kb fragment of the hsp70.1 gene, using protocols recommended by the manufacturers. Bands were visualized by autoradiography.

Transfection and CAT Assay—The plasmids RSVCAT or pHBCAT (28) were introduced into MEL cells by liposome-mediated transfection using Lipofectin (Life Technologies, Inc.). The DNA-liposome complex was incubated with 2.5 x 10⁶ cells overnight in Opti-Mem (Life Technologies, Inc.), and the transfection was stopped by addition of an equal volume of DME, 30% FBS. Eight hours later, cells were pelleted, resuspended in DME, 15% FBS, and aliquoted into two dishes. The next day, one dish was heated to 43°C for 30 min and then cooled, and unstrressed and heat-shocked cells were harvested for CAT assays. CAT assays were performed as described previously (29), and results were quantitated with a Fuji PhosphorImager.
Endogenous hsp70 Genes—mouse cell lines.

In L1210 cells, this response was reproducibly weaker than in shock-responsive, while the hsp70.1 gene consistently exhibits a phenotype: the hsp70.3 gene was weakly or not at all heat shock. NIH3T3 cells, the mouse hybridoma line LK35.2, and neither hsp70 gene showed a transcriptional response to heat shock similar to that seen in primary mouse embryo cells (Fig. 1). The pronounced heat shock inducibility of both hsp70 genes, response of the hsp70 genes to heat shock. Only F9 cells showed an induction (6–13-fold) of promoter activity in the heat-shocked cells. This was true at three levels of introduced plasmid and was not seen when the cells were transfected with RSV CAT. A similar result was obtained with L1210 cells (not shown), suggesting that the heat shock response pathway is functional in this line as well, despite the unresponsiveness to heat shock of the hsp86 gene. These data, coupled with the results of the gel retardation assays, suggest that a feature peculiar to the endogenous hsp70 genes, rather than a deficiency in the overall heat shock response, is responsible for their lack of heat shock responsiveness.

While the levels of HSE binding activity in heat-shocked cells varied considerably among the cell lines (Fig. 2A), there was no correlation between these levels and the presence or absence of an hsp70 heat shock response. For example, MEL cells contain high levels of HSE binding activity after heat shock yet fail to transcribe either hsp70 gene, suggesting, as did our previous findings of similar levels of HSF1 DNA binding activity in heat-shocked PCC4 and F9 cells, that the defect is unrelated to HSF1 levels. Furthermore, there were no cell line-specific differences in the mobilities of the HSE-HSF complexes which might suggest alterations in HSF1 itself or in its oligomeric state.

Another prediction of the tenet that the heat shock response machinery functions normally in cell lines with a deficient hsp70 response is that a transfected heat shock promoter should be activatable by heat shock in these lines. Previous studies where HSE-containing promoters were introduced into such cell lines transiently or permanently have yielded conflicting results (11, 13, 14, 31). In the present study, a CAT reporter gene under the control of the human hsp70 promoter was introduced into MEL cells using liposome-mediated transfection. Two HSEs are present in this promoter, the most proximal of which has previously been shown to be required for stress inducibility (32). Two days post-transfection, the cells were heat-shocked and then harvested for CAT assays. The results (Fig. 3) show a clear induction (6–13-fold) of promoter activity in the heat-shocked cells. This was true at three levels of introduced plasmid and was not seen when the cells were transfected with RSV CAT. A similar result was obtained with L1210 cells (not shown), suggesting that the heat shock response pathway is functional in this line as well, despite the unresponsiveness to heat shock of the hsp86 gene. These data, coupled with the results of the gel retardation assays, suggest that a feature peculiar to the endogenous hsp70 genes, rather than a deficiency in the overall heat shock response, is responsible for their lack of heat shock responsiveness.

The Common Basis for the Lack of Heat Shock Responsiveness Is the Failure of HSF1 to Bind to the hsp70.1 Promoter—Genomic footprinting studies of the human hsp70 promoter have shown dramatic changes in the regions corresponding to the HSEs during heat shock, suggesting interaction of a factor, presumably HSF1, with this site (33). This technique, which detects the binding of regulatory factors to endogenous sequences because bound factors alter the susceptibility of guanines within these sequences to methylation by DMS, was used to examine the occupancy of the HSEs in the murine hsp70.1 promoter during heat shock. A 200-nt region of the coding strand of the proximal hsp70.1 promoter was examined; this region contains two consensus HSEs, a proximal HSE approximately 80 bp upstream of the TATA element and a more distal HSE located 80 bp further upstream of the proximal HSE (Fig. 4A). Just downstream of each HSE is a GC box (GGGCCG), a putative binding site for the transcription factor Sp1; the most proximal of these GC boxes is part of a decanucleotide, 5'TGCCCCGGGC3', which constitutes an especially good binding site for Sp1 (34). A CCAAT box is located between the proximal HSE and Sp1 sites.

For primary mouse embryo cells and for each cell line, the methylation pattern in DNA isolated from heat-shocked cells was compared to that in DNA isolated from unstressed cells as well as to the pattern in protein-free (naked) genomic DNA which had been methylated in vitro. Similar to their assignment with regard to hsp70 heat shock responsiveness, primary mouse cells and the eight cell lines could be grouped into the same three categories with respect to their methylation pat-
TABLE I

| Transcriptional response to heat shocka | Occupancy of hsp70.1 promoter sites | CpG methylation (5’ end of gene)b |
|---------------------------------------|-------------------------------------|----------------------------------|
| hsp70.1 | hsp70.3 | HSF1 | Sp1 | hsp70.1 | hsp70.3 |
| MEL | 4.6 | 2.8 | -- | -- | High | High |
| MPC-11 | 1.4 | 1.3 | -- | -- | High | High |
| CH27 | 2.6 | 2.1 | -- | -- | High | High |
| PCC4 | 5.5 | 5.2 | -- | -- | High | High |
| NIH | 161.5 (99.2) | 13.6 (7.4) | + | + | Moderate | High |
| LK35.2 | 55.3 (20.2) | 7.0 (3.5) | + | + | Moderate | High |
| L1210 | 9.0 (8.2) | 2.3 (2.2) | + | + | Moderate | High |
| F9 | 89.6 (66.0) | 76.2 (62.7) | +++ | +++ | ND | ND |
| MEF | 100.0 (100.0) | 100.0 (100.0) | +++ | +++ | ND | ND |

a Transcription rates, in arbitrary units, as determined by PhosphorImager scanning of filters from transcription run-on assays. Values are normalized to those in MEFs. Numbers in parentheses are from a second, independently performed experiment.

b ND, not detectable.

Fig. 2. Analysis of HSE binding activity in mouse cell lines after heat shock. A, gel retardation assay using an HSE oligomer derived from the mouse hsp70.1 promoter and whole cell extracts from unstressed (37°C) and heat-shocked (43°C, 40 min) cells. HSF denotes specific HSE-HSF complexes; NS denotes a nonspecific complex, i.e. not competed by excess unlabeled oligonucleotide. B, antibody recognition analysis as in A.

Fig. 3. CAT assay of MEL cells transfected with an hsp70 promoter-CAT construct. MEL cells transfected with the indicated constructs were maintained at 37°C (control, C) or heat-shocked (HS) at 42°C for 40 min and allowed to recover for 8 h prior to harvesting. Cell extracts were incubated with [14C]chloramphenicol and acetyl-CoA, and the acetylated products were separated by TLC.

4C, suggesting that the HSEs are not occupied by factors either prior to or during heat shock. The same result was obtained with DNA isolated from unstressed and heat-shocked MPC-11 and PCC4 cells (not shown). These results suggest a common basis for the lack of heat shock responsiveness of the hsp70.1 gene in these four cell lines, viz. HSF1, although activated, does not bind to the hsp70.1 promoter during heat shock.

In DNA isolated from L1210 cells, where the hsp70.1 gene shows a low level of heat shock responsiveness, there were only subtle differences in the HSE methylation pattern following heat shock (Fig. 4C). The guanine residues at both boundaries of the proximal HSE were hypersensitive to methylation after heat shock, although not nearly to the extent seen in F9 and mouse embryo cells, and, of the intervening guanines, only the most distal one showed any protection from methylation. Similarly, in the distal HSE, the only definitive change in the L1210 methylation pattern following heat shock was a minor hypersensitivity in the middle NGAAN binding site (which in the distal HSE is NGAGN). Thus, consistent with the weak transcriptional response of the hsp70.1 gene in L1210 cells relative to F9 and mouse embryo cells, occupancy of the HSEs during heat shock was much less apparent.

Similar differences in methylation patterns were also seen in DNA isolated from unstressed and heat-shocked NIH3T3 and LK35.2 cells, which, like L1210 cells, show a defective transcriptional response of one or both hsp70 genes (Table I). Following heat shock, a hypersensitive guanine at the upstream boundary of the proximal HSE was readily apparent in both lines, although in both lines, the guanine at the downstream

TABLE I

Transcriptional responsiveness to heat shock, accessibility to transcription factors, and methylation status of the hsp70 genes in primary mouse embryo cells and murine cell lines.

| Cell Line | hsp70.1 Transcriptional Response | hsp70.3 Transcriptional Response | HSF1 Occupancy | Sp1 Occupancy | CpG Methylation |
|-----------|---------------------------------|---------------------------------|----------------|--------------|----------------|
| MEL       | 4.6                             | 2.8                             | --             | --           | High           |
| MPC-11    | 1.4                             | 1.3                             | --             | --           | High           |
| CH27      | 2.6                             | 2.1                             | --             | --           | High           |
| PCC4      | 5.5                             | 5.2                             | --             | --           | High           |
| NIH       | 161.5 (99.2)                    | 13.6 (7.4)                      | +              | +            | Moderate       |
| LK35.2    | 55.3 (20.2)                     | 7.0 (3.5)                       | +              | +            | Moderate       |
| L1210     | 9.0 (8.2)                       | 2.3 (2.2)                       | +              | +            | Moderate       |
| F9        | 89.6 (66.0)                     | 76.2 (62.7)                     | +++            | +++          | ND             |
| MEF       | 100.0 (100.0)                   | 100.0 (100.0)                   | +++            | +++          | ND             |

a Transcription rates, in arbitrary units, as determined by PhosphorImager scanning of filters from transcription run-on assays. Values are normalized to those in MEFs. Numbers in parentheses are from a second, independently performed experiment.

b ND, not detectable.
FIG. 4. Genomic footprinting of the coding strand of the mouse hsp70.1 promoter in cell lines and mouse embryo fibroblasts before and after heat shock. A, schematic representation of the proximal 200 bp of the mouse hsp70.1 promoter and the sequence of the region shown in the autoradiogram (this region starts just upstream of the TATA element). The sequences of the CCAAT, HSE, and Sp1 sites are boldfaced and underlined. DMS methylation patterns of the guanine residues in genomic DNA which was isolated from heat-shocked (43°C, 40 min) or unstressed (37°C) MEF and F9 cells (B) or from L1210, CH27, MEL, NIH3T3, and LK35.2 cells (C). N lanes show the pattern for protein-free (naked) DNA which was DMS-treated in vitro. The correspondence between bands and Gs in the proximal Sp1 and HSE sites is shown adjacent to the autoradiogram in B. For the HSE, arrows indicate guanine residues which are protected from methylation by DMS in heat-shocked cells as compared to unstressed cells or naked DNA, and asterisks denote guanines which are hypersensitive to methylation. For the Sp1 sites, where cell line-specific differences in occupancy are seen both in the stressed and unstressed states, the arrows and asterisks depict differences in sensitivities of guanines to DMS in DNA isolated from either stressed or unstressed cells as compared to naked DNA. The bracketed regions at the tops of the autoradiograms correspond to the distal Sp1 and HSE sites, where differences in methylation patterns can also be seen.
boundary of the proximal HSE was only slightly hypersensitive (Fig. 4C). Several intervening guanines were protected after heat shock in NIH3T3 DNA, while in LK35.2 DNA, only slight protection of the most distal guanine could be seen. The minor hypersensitivity in the distal HSE which is seen in heat-shocked L1210 cells is also seen in heat-shocked NIH3T3 and LK35.2 cells. Thus, as in L1210 cells, the differences in the NIH3T3 and LK35.2 methylation patterns before and after heat shock were considerably more subtle than those seen in F9 and mouse embryo cells, but they were seen at the same guanine residues and in replicate experiments, validating their authenticity.

Although stronger than in L1210 cells, the hsp70.1 transcriptional response to heat shock in LK35.2 cells is reproducibly lower than in F9 and mouse embryo cells (Table I), consistent with the observed reduction in HSE occupancy. However, NIH3T3 cells consistently show a very robust hsp70.1 response to heat shock. It is thus quite surprising that occupancy of the HSEs of the hsp70.1 promoter in this cell line is clearly (and reproducibly) submaximal. One possibility is that NIH3T3 cells have a higher hsp70.1 gene copy number than the other cell lines, which would counterbalance the reduced HSF1 binding and allow retention of a robust transcriptional response; this and other possible explanations are detailed further under “Discussion.”

Occupancy of Sp1 Sites in the hsp70.1 Promoter prior to or during Heat Shock Correlates with HSF1 Occupancy during Heat Shock—Significantly, mouse embryo cells and the eight murine cell lines could be grouped into the same three categories with regard to occupancy of the Sp1 sites in the hsp70.1 promoter (Table I). In unstressed F9 and mouse embryo cells, one guanine in the proximal Sp1 site was extremely hypersensitive to methylation (compared to the same residue in naked DNA), while five flanking guanines were strongly protected from methylation (Fig. 4B). (Heat shock did not affect Sp1 occupancy, in agreement with previous footprinting data from the human hsp70 promoter (33).) A hypersensitive guanine in the proximal Sp1 site could also be seen in DNA isolated from L1210, NIH3T3, and LK35.2 cells, although the hypersensitivity was not nearly as striking as in F9 cells (Fig. 4C). Of the five guanines which were clearly protected from DMS in F9 cells and MEFs, only the most distal was protected in NIH3T3 cells, and no protected guanines in the proximal Sp1 site were visible in the L1210 and LK35.2 DNA. The band pattern in the proximal Sp1 site in DNA from CH27 and MEL cells was identical with that in naked DNA, however, suggesting that this site was unoccupied in these cells (Fig. 4C). Likewise, no occupancy of the proximal Sp1 site of the hsp70.1 promoter was seen in MPC-11 or PCC4 cells, the other two lines in which no occupancy of the HSEs was seen after heat shock (not shown). The same cell line-specific differences in occupancy were seen at the distal GGGCGG hexanucleotide. One of the downstream guanines was very hypersensitive to methylation in mouse embryo and F9 cells, less so in L1210, LK35.2, and NIH3T3 cells, and not at all in CH27, MPC-11, MEL, and PCC4 cells (Fig. 4B and C, not shown). Thus, in those lines where HSF1 does not bind or binds submaximally to the hsp70 promoter during heat shock, the binding of at least one additional transcription factor, Sp1, is similarly diminished; this is apparent both prior to and during heat shock. Gel retardation assays using an oligomer corresponding to the proximal Sp1 site revealed no significant differences in the levels of Sp1 binding activity in the various cell lines (not shown), eliminating this as a possible reason for the differences in Sp1 site occupancy. Our results thus suggest that the differences among cell lines in HSF1 binding to the hsp70.1 promoter during heat shock reflect differences in overall promoter accessibility. (It is also noteworthy that these differences in promoter accessibility do not affect basal hsp70.1 transcription, which is undetectable in primary mouse embryo cells (not shown) and in all of the cell lines.)

Sequencing of the Proximal Region of the hsp70.1 Promoter—Although genomic footprinting of the hsp70.1 promoter in all of the cell lines showed the expected G ladder, the possibility remained that other bases in this region were mutated in the cell lines which contained heat shock unresponsive hsp70.1 genes and that such mutations might account for the diminished binding of factors to the promoter. To rule this out, PCR was used to amplify a region of 310 bp from MEL, CH27, and MPC-11 genomic DNA; the resulting amplimers were cloned and several clones derived from each cell line were sequenced. These amplimers included the entire region of the hsp70.1 promoter examined by genomic footprinting. Within this region, no deviations from the published sequence were seen in any of the clones (not shown), thus ruling out mutations in the proximal promoter as the reason for the lack of factor binding.

Methylation of CpG Sites in the hsp70.1 Promoter Correlates with Reduced Accessibility to Transcription Factors—Our results indicate that in seven of the eight mouse lines we examined, the hsp70.1 promoter has undergone a loss of accessibility. CpG methylation is commonly associated with promoter inaccessibility; studies of both X-linked and autosomal genes in cultured cells have demonstrated that the promoters are free of CpG methylation when the gene is transcriptionally active, while assembly into an inaccessible conformation and transcriptional silencing is accompanied by promoter methylation (36–38). To determine the methylation status of the hsp70.1 promoter region in the cells analyzed by genomic footprinting, a 1.16-kb (hereafter referred to as 1.2 kb) BglII fragment which encompasses 0.57 kb of promoter sequences and 0.59 kb of transcribed sequences was analyzed, using the methylation-sensitive restriction enzymes HpaI and HhaI and the methylation-insensitive isoschizomer of HpaI, MspI. There are 15 HpaI/MspI and HhaI sites in this BglII fragment (Fig. 5A), which is CpG-rich, especially the transcribed sequences, and meets the established criteria for a CpG island (39).

When genomic DNA was cut with BglII alone and analyzed by Southern blotting, the expected 1.2-kb band was seen in all of the DNAs; an additional, strongly hybridizing 3.0-kb band, corresponding to the hsp70.3 gene, was also seen. To specifically examine methylation of the hsp70.1 gene, we size-fractionated the BglII-cut DNA on an agarose gel, isolated the DNA fragments which were approximately 1.2 kb in size, and confirmed recovery of the hsp70.1 BglII fragment by Southern blotting. This DNA was then further digested with HpaII, MspI, or HhaI. A cloned hsp70.1 BglII 1.2-kb fragment, free of CpG methylation, was also digested with the same three enzymes.

When cut with the methylation-insensitive restriction enzyme MspI, all of the DNAs except NIH3T3 yielded the expected fragments, 220, 380, and 500 bp in size, which comigrated with the products of digestion of the cloned BglII fragment (Fig. 5B). In the case of NIH3T3 DNA, only the 500-bp fragment and one additional, slightly larger fragment were seen; this pattern suggests that in NIH3T3 cells, the MspI/HpaII site in the 5’-UTR of the hsp70.1 gene (Fig. 5A) is not present.

When digested with HpaII, the methylation-sensitive isoschizomer of MspI, F9 and MEF DNA yielded products identical with those obtained from MspI digestion (Fig. 5C), suggesting that the MspI/HpaII sites are completely unmethylated, although this cannot be determined with certainty for the two closely spaced sites just 10 and 20 bp upstream of the BglII site.
in the coding region. F9 and mouse embryo cells showed the 
greatest accessibility of the hsp70.1 promoter to Sp1 and HSF1. In 
contrast, the hsp70.1 BglII fragment was almost completely 
refractory to HpaII digestion in DNA from all four cell lines 
which showed a total lack of accessibility to factors by footprint-
ing analysis (Fig. 5). This suggests that all five Mspl/HpaII sites are 
methylated, with the same caveat as above. Finally, partial 
digestion by HpaII of the hsp70.1 BglII fragment was 
evident in DNA from LK35.2 and L1210 cells, two lines where 

the footprinting analysis showed some, but not full, promoter 
accessibility. Neither the 380- nor the 220-bp fragments was 
detected in these digests, suggesting that neither of these cell 
lines contains an allele where the Mspl/HpaII sites are 
completely unmethylated. The retention of the intact 1.2-kb 
fragment in the LK35.2 and L1210 digests indicates that each of 
these two cell lines contains one allele with methylation at all 
five sites. The NIH3T3 HpaII digest pattern was identical with 
that obtained with Mspl, however, suggesting no methylation 
of the four Mspl/HpaII sites in the proximal promoter in this 
cell line.

Conclusions regarding methylation status were generally 
supported by Southern blot analyses using HhaI, except for 
NIH3T3 cells, where the HhaI digestion pattern suggested 
partial methylation. There are many closely spaced HhaI sites in 
the 1.2-kb region of the hsp70.1 gene, and, when the cloned 
BglII fragment is digested with HhaI, only three bands, repre-
senting 480-, 175-, and 145-bp fragments, are detected (Fig. 
5D). This same set of fragments is seen in HhaI digests of DNA 
from F9 and MEF cells, suggesting a complete absence of CpG 
methylation at HhaI sites in this 1.2-kb region. Further digestion of 
the 1.2-kb fragment by HhaI is apparent, suggesting methylation 
of all of the HhaI sites, while in the MPE.11 lane, 
roughly equivalent amounts of the undigested 1.2-kb fragment 
and a slightly smaller fragment are seen, suggesting that in 
one hsp70.1 allele in this line, a single HhaI site near one of the 
fragment ends may not be methylated. The 480-bp fragment 
is seen in the NIH3T3, LK35.2, and L1210 digests; however, the 
smaller fragments are not, and a fragment slightly larger than 
480 bp is also present in the NIH3T3 and LK35.2 lanes. The 
intact, undigested 1.2-kb BglII fragment is present in the 
LK35.2 and L1210 lanes but not in the NIH3T3 lane, while 
the slightly smaller fragment which was produced by HhaI diges-
tion of MPE.11 DNA appears to be present in both the NIH3T3 
and LK35.2 lanes. These data suggest that in LK35.2 and 
L1210 cells, one hsp70 allele is fully methylated at CpG sites 
in this region and one is methylated at some sites, while there is 
methylation at some HhaI sites in both alleles in NIH3T3 cells. 
The 1.2-kb region flanking the transcription start site of the 
hsp70.1 gene is therefore hypermethylated in every cell line in 
which promoter accessibility is diminished (Table I).

For all of the cell lines, only a small set of predominant bands 
is seen after HpaII and HhaI digestion, suggesting that the cell 
populations we examined are largely homogeneous with re-
spect to hsp70 methylation patterns. Data supporting this 
suggestion were obtained for the NIH3T3 population, where 
Southern blot analyses of DNA isolated from six clonal isolates 
yielded fragment patterns identical with those seen in DNA 
isolated from the parental population (not shown).

The hsp70.3 Gene Is Methylated in All Cell Lines Exhibiting 
hsp70.1 Gene Methylation—The failure of the hsp70.3 gene to 

respond to heat shock in all cells except MEF and F9 suggested 
that it too had undergone methylation. To examine this pos-

sibility, unfractonated BglII digested DNA from each of the cell 
lines was further digested with Mspl or HpaII. When digested 
with BglII, the hsp70.3 gene generates a fragment approxi-
mately 3 kb in length (the two hsp70 sequences diverge up-
stream of the distal HSE) which hybridizes with the hsp70.1 
probe (Fig. 6A). Both the 1.2- and 3-kb fragments were 
detected in a Southern blot of BglII cleaved DNA from all of 
the cell lines (Fig. 6B). When digested further with Mspl, both 
the 1.2- and 3-kb fragments disappear and are replaced by a set 
of three bands identical in size with those generated by digestion 
of the isolated 1.2-kb hsp70.1 fragment (Fig. 6C). This is the 
expected result: all of the hsp70.1 Mspl sites shown in Fig. 5A
are identically located in the hsp70.3 gene, and there is an MspI site in hsp70.3 which is located upstream of the point of sequence divergence, generating a 360-bp fragment which comigrates with the hsp70.1-derived 380-bp fragment (Fig. 6A). When BglII-digested DNA is further digested with HpaII, the same set of three bands is seen only in DNA from F9 and MEF cells, and complete disappearance of the 1.2- and 3-kb fragments is seen only in DNA from these cells. This result indicates that the hsp70.3 gene is methylated, apparently quite extensively, in all of the remaining cell lines.

**DISCUSSION**

Our results reveal a common basis for the lack of heat shock responsiveness of the hsp70.1 gene in CH27, MEL, MPC-11, and PCC4 cells, viz. the promoter is inaccessible to HSF1 as well as to other transcription factors. This lack of accessibility is associated with extensive methylation of a 1.2-kb region surrounding the transcription start site. While its exact role in gene silencing is not clear, CpG hypermethylation is a hallmark of mammalian genes which have assumed an inaccessible and transcriptionally inert state. First shown for X-linked genes (40), this as well as previous studies (36) document the association of methylation and promoter inaccessibility for autosomal genes as well. We detect no deficits in the overall heat shock pathway in these four mouse cell lines: HSF1 is activated normally by heat shock and is able to stimulate the transcription of another heat shock gene, hsp86. Furthermore, an HSE-containing promoter introduced by transfection into MEL cells is heat shock-responsive.

Previous studies of *Drosophila* heat shock genes have established that, despite being present in a highly condensed region of the chromosome, the regulatory regions of these genes are maintained in a nuclease-sensitive, accessible conformation prior to heat shock, such that the TATA element is occupied and RNA polymerase is already bound (41–43). In *Drosophila*, the binding of the GAGA factor plays a major role in establishing and maintaining this open conformation (44). One could speculate that the transcription factor Sp1 serves a role in mammalian heat shock genes analogous to the GAGA factor in *Drosophila* heat shock genes, since, as exemplified by the murine hsp70 promoter, Sp1 sites are commonly found in proximity to HSEs in mammalian heat shock genes. Methylation of the CpG dinucleotide in the Sp1 binding site could therefore potentially prevent Sp1 binding and the establishment of an accessible state. However, at least in vitro, Sp1 binding to its recognition site is not appreciably affected by CpG methylation (45, 46).

Although we do not know what changes in chromatin structure have rendered the murine hsp70.1 promoter inaccessible to transcription factors, it is quite possible that changes in nucleosomal packaging or positioning have occurred. Relevant to this possibility, there are two previous in vitro studies demonstrating that HSF is unable to bind to an HSE-containing template after it has been packaged into nucleosomes (47, 48). On the other hand, Sp1 was demonstrated to bind in vitro (although with greatly reduced affinity) to GC boxes which had been reconstituted into nucleosome cores (49), and, at least in yeast, HSF was shown to bind in vivo to a plasmid in which an HSE is packaged into nucleosomes (50). Based on this set of results, it is hard to predict the extent to which the inclusion into nucleosomes of their recognition sites would hinder the binding of Sp1 and/or HSF1 to the hsp70.1 promoter. Further-
more, packaging into nucleosomes is considered to represent only the first step in chromatin condensation; in cell lines where hsp70 fails to respond to heat shock, the hsp70 gene may be present in a highly condensed heterochromatin-like structure.

Ours is only the second reported study in which the methylation status and accessibility to transcription factors of a particular gene have been evaluated in a set of cell lines which show differential expression of that gene. As in the previous study, which focused on the human 6-O-methylguanine DNA methyltransferase gene (51), the relationship between gene expression and accessibility as determined by genomic footprinting was good but not perfect. In the previous study, no transcription factor binding to the 6-O-methylguanine DNA methyltransferase promoter could be detected in cells where the gene was silent, but this was also true for a cell line with reduced but detectable 6-O-methylguanine DNA methyltransferase expression. Three of the lines which we examined in our study exhibited a submaximal and roughly equivalent state of accessibility to transcription factors, yet the transcriptional response to heat shock ranged from quite robust (NIH3T3) to very weak (L2120). There are at least two possible explanations for the disparity. In genomic footprinting analysis, one is visualizing an average of the interactions which are occurring at both, or in the case of gene amplification or polyploidy, all of the alleles in the cell. NIH3T3 cells, for example, may contain one allele which, despite some CpG methylation, is fully accessible to HSF1 and can generate a full transcriptional response. A second consideration is that the mechanism by which HSF1, once bound to the promoter, is able to stimulate transcription of heat shock genes is still unknown; the range of transcriptional responses in the three cell lines may reflect differing levels or activities of a coactivator through which HSF1, although weakly bound in all three cases, contacts the basal transcription complex.

Although methylation-associated transcriptional repression of genes in cultured cells is a frequent and well-documented phenomenon (52), this is the first reported case involving the hsp70 genes, and the first reported instance of concomitant methylation of duplicated genes. The observation that methylation commonly or perhaps, invariably, affects both of the tandem hsp70 genes assumes greater significance in view of the location of these genes in the class III region of the MHC. This region is very gene dense and (C + G)-rich, with an estimated 40 expressed genes occupying approximately 1000 kb. Although the function of most of these genes is not known, many of these genes contain CpG islands (53). Such islands are also found in the MHC class I H-2D/L genes, which encode proteins vital for the presentation of non-self-antigens to cytotoxic T lymphocytes and which lie just telomeric to the class I region (54). These class I genes have also been shown to be susceptible to methylation-associated transcriptional repression (55, 56). The high density of CpG island-containing genes in the MHC region, and the demonstration in this and previous studies that genes in this region are susceptible to methylation, raises the possibility that the MHC region constitutes a “hot spot” for aberrant hypermethylation. In light of previous studies demonstrating that inactive chromatin can spread from an initial focus of methylation (57, 58), it is also possible that a block of DNA encompassing many MHC region genes may become incorporated into an inaccessible structure as a unit.

Interestingly, although diminution or loss of hsp70 heat shock responsiveness has been observed frequently in mouse cell lines, there is only one report so far of a similar phenomenon in human cell lines, viz., the Y79 retinoblastoma line, where the loss of stress responsiveness was also associated with a lack of promoter occupancy (59). Unlike rodent cell lines, there is at least some, and frequently robust, basal expression of the hsp70 gene(s) in most human cell lines, and loss of this basal expression may be disadvantageous for human cells.

The mechanism and kinetics of the process by which endogenous genes or a region encompassing several genes acquire a new methylation pattern are not understood, nor is the relationship between this altered methylation pattern and losses in accessibility to regulatory proteins, which result in transcriptional repression. Because they are intronless, facilitating detection of methylase expression. Three of the lines which we examined in our study exhibited a submaximal and roughly equivalent state of accessibility to transcription factors, yet the transcriptional response to heat shock ranged from quite robust (NIH3T3) to very weak (L2120). There are at least two possible explanations for the disparity. In genomic footprinting analysis, one is visualizing an average of the interactions which are occurring at both, or in the case of gene amplification or polyploidy, all of the alleles in the cell. NIH3T3 cells, for example, may contain one allele which, despite some CpG methylation, is fully accessible to HSF1 and can generate a full transcriptional response. A second consideration is that the mechanism by which HSF1, once bound to the promoter, is able to stimulate transcription of heat shock genes is still unknown; the range of transcriptional responses in the three cell lines may reflect differing levels or activities of a coactivator through which HSF1, although weakly bound in all three cases, contacts the basal transcription complex.

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hsp70 Transcriptional Silencing