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DNA Binding of Heat Shock Factor to the Heat Shock Element Is Insufficient for Transcriptional Activation in Murine Erythroleukemia Cells

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The heat shock response is among the most highly conserved examples of regulated gene expression, being present in all cellular organisms. Transcriptional activation of heat shock genes by increased temperature or other cellular stresses is mediated by the binding of a heat shock factor (HSF) to a conserved nucleotide sequence (the heat shock element) present in the promoter of heat-inducible genes. Despite the high degree of conservation of this response, embryonic stages of development are characterized by the absence of a heat shock response. Murine erythroleukemia (MEL) cells also lack this response, and we report here a detailed characterization of this defect for one of the most highly conserved of these genes, *hsp70*. Surprisingly, heat-induced transcriptional activation of this gene does not occur, despite the induction of a protein with the binding specificity of murine HSF. However, the MEL HSF differs slightly in apparent size from the HSF in 3T3 cells, which exhibit a normal heat shock response. These data suggest that activation of mammalian HSF by heat requires at least two separate steps: an alteration of binding activity followed by further modification that activates transcription. MEL cells do not respond to heat shock because they lack the ability to perform this secondary modification. These cells provide a useful system for characterizing heat shock activation in mammals.

The selective increased synthesis of a small number of proteins (termed heat shock proteins) following exposure to an increase in temperature or other stresses is a response characteristic of all cellular organisms (21). The functions of most of these proteins are unknown, but their induction by a variety of stresses has led to the proposal that their increased expression serves to protect the cell from damage due to accumulation of denatured proteins (28). However, these proteins must have critical roles in normal cellular physiology as well, since for at least two of the heat shock genes, deletion is lethal even under nonstressed conditions of growth (7; D. Finkelstein and F. Farrelly, Fed. Proc. **43:** 1499, 1984). Recent evidence suggests that some of these proteins may have roles in protein trafficking (6, 8), as well as in regulation of protein-protein interactions (4). The relationship of these housekeeping functions to the role of the proteins during stress remains speculative.

The nearly universal nature of this stress response has made it a model system with which to analyze the regulation of gene expression. Previous studies have demonstrated the complexity of this regulation, with transcriptional, posttranscriptional and translational mechanisms all having a role in controlling the expression of these proteins (21). The increased transcription rate of the heat shock genes following stress has been shown to require a conserved DNA sequence (the heat shock element [HSE]) located within the promoter region of the genes (27, 29). A protein that binds specifically to this sequence (the heat shock factor [HSF]) has been detected in extracts of heat-shocked cells (26, 37). Purified *Drosophila* HSF is capable of stimulating transcription of heat shock genes when microinjected into *Xenopus* oocytes (38), and both *Drosophila* and human HSFs have been shown to stimulate transcription in vitro (13, 36). The HSF is phosphorylated, and although this modification is not necessary for binding the target DNA sequence, both phosphorylation and possibly other unidentified modifications of the protein may be necessary for transcriptional activation by the HSF (19, 33).

We have recently determined that a decrease in the rate of synthesis of the constitutively expressed isoform of the 70-kilodalton (kDa) heat shock protein, hsc70, occurs prior to the onset of differentiation of inducer-exposed murine erythroleukemia (MEL) cells (16). To better understand the significance of this change, we began to characterize the heat shock response of these cells. We report here that MEL cells do not exhibit a normal response to heat stress, and we have characterized this response for the heat-inducible form of the 70-kDa heat shock protein, hsp70. MEL cells fail to transcriptionally activate and accumulate mRNA for this gene in response to heat. Furthermore, the HSF induced by heat in MEL cells has an abnormal mobility by gel electrophoresis relative to the HSF from another mouse cell line (3T3), which does transcriptionally activate this gene following thermal stress. This altered mobility may reflect, in part, differences in phosphorylation or other modifications of these two HSFs. These data demonstrate that induction of a DNA-binding form of the HSF is insufficient for the transcriptional activation of mammalian heat shock genes and

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that further posttranslational modifications of the HSF are necessary for this protein to activate transcription.

MATERIALS AND METHODS

Cell culture and heat shock. MEL cells (clone 745 PC4-B1-2A$_{17}$) were grown in Dulbecco modified Eagle medium supplemented with L-glutamine (2 mM) and 15% fetal bovine serum and maintained at densities that permitted logarithmic growth (1.0×10^5 to 12×10^5/ml). BALB/c 3T3 cells were grown in Dulbecco modified Eagle medium supplemented with L-glutamine and 10% calf serum and passaged when confluency was attained. L1210 cells were grown in Fischer medium supplemented with 10% horse serum. All experiments were performed with cells in the logarithmic phase of growth. Cell stocks were maintained at 37°C under 5% CO_2. For heat shock experiments, similar CO_2 incubators were used at the temperatures (43 to 45°C) and times indicated below. As indicated below, in some experiments a brief incubation at 37°C followed the heat exposure. For experiments with sodium arsenite or cadmium sulfate as an inducer, cells were incubated for 3 h with the inducer at a concentration of 100 μmol/liter.

Two-dimensional protein gel electrophoresis. Following the indicated heat exposures, cells were placed in prewarmed medium lacking methionine. [^{35}S]methionine was added to a concentration of 500 μCi/ml, and the cells were incubated for 20 min at 37°C. Incorporation of [^{35}S]methionine was terminated by washing with ice-cold phosphate-buffered saline. The cells were lysed and prepared for two-dimensional gel electrophoresis as described by Garrells (10). Isoelectric focusing in 3% polyacrylamide–0.16% bisacrylamide–2.2% Ampholytes–8 M urea gels was performed for a total of 10 kV-h. Second-dimension gels were 10% polyacrylamide–0.27% bisacrylamide–375 mM Tris (pH 8.8)–0.1% sodium dodecyl sulfate gels (SDS). The gels were saturated with EnHance (Du Pont, NEN Research Products), dried, and developed by fluorography.

Extraction and analysis of RNA and DNA. Total cellular RNA was extracted from cells by the guanidinium isothiocyanate method (5). Purified RNA (20 μg) was fractionated in 1.0% agarose–2.2 M formaldehyde gels and transferred to GeneScreen Plus membranes (Du Pont, NEN) by capillary blotting. Filters were hybridized overnight at 65°C in 1 M NaCl–1% SDS–10% dextran sulfate–100 μg of salmon sperm DNA per ml with a ^{32}P-labeled 1,271-base-pair (bp) *Bgl*II-*Xho*I fragment of the mouse hsp70 gene (spanning amino acids 119 to 543) (C. R. Hunt and S. K. Calderwood, Gene, in press). Washes were carried out to a final stringency of 0.2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) 1% SDS at 65°C.

Genomic DNA was prepared by proteinase K-SDS cell lysis. The DNA was digested with the indicated restriction enzyme, fractionated, and transferred to membranes as described above. Labeling, hybridization, and wash conditions were as described above. The probes used for Southern analysis were an 804-bp *Bgl*II-*Nco*I fragment that spanned the 5′ untranslated leader and the promoter regions of the hsp70 gene (Hunt and Calderwood, in press).

Determination of transcription rate by nuclear runoff analysis. Preparation of nuclei and [^{32}P]UTP labeling of runoff transcripts were performed as described by Greenberg and Ziff (14). The labeled transcripts were purified by centrifugation in guanidinium isothiocyanate-CsCl and hybridized with an excess of linearized, denatured plasmid DNA which had been previously blotted to nitrocellulose membranes. Hybridizations were performed at 65°C in 300 mM NaCl–0.2% SDS–10 mM EDTA–10 mM TES [*N*-Tris-(hydroxymethyl)methyl-2-aminoethanesulfonic acid] (pH 7.4) for 48 h. Washes were carried out to a stringency of 1× SSC at 65°C, with a final wash in RNase A (10 μg/ml). Autoradiographic exposure times were adjusted to allow for approximately equal intensity of glyceraldehyde phosphate dehydrogenase transcription. Plasmids used in these experiments included a hamster cDNA clone of GRP78 (20), a human cDNA corresponding to HSP90 (31), a rat glyceraldehyde phosphate dehydrogenase cDNA (9), a mouse hsc70 cDNA clone (nucleotides 104 to 1343) (11), and a mouse hsp70 genomic clone that corresponded to the 3′ untranslated region of the transcript (Hunt and Calderwood, in press).

Gel mobility shift and methylation interference assays. Whole-cell extracts were made from cells as described by Manley et al. (22). Heat shock extracts were made immediately following a 1-h exposure to 43°C. The probes used in these experiments have been described elsewhere and included both a synthetic oligonucleotide containing the HSE (19) and a 74-bp fragment of the human hsp70 promoter that contained the HSE (15). Probes were ^{32}P labeled with either T4 kinase or Klenow fragment. A 10- to 33-fold molar excess of unlabeled oligonucleotide that contained the consensus sequence either for the CAAT box or for the glucocorticoid-responsive element was used as the competitor DNA. Binding reactions were performed as described by Greene et al. (15). The complexes were electrophoresed in 5% acrylamide–0.0625% bisacrylamide nondenaturing gels (50 mM Tris, 280 mM glycine, 2 mM EDTA). Gels were developed by autoradiography.

Methylation interference was performed as described by Gilman et al. (12). Briefly, an end-labeled fragment of the human HSE was partially methylated by dimethyl sulfate, and then binding and mobility shift assays were performed. The bands corresponding to bound and to free probe were excised, eluted from the gel, and cleaved at methylated sites by hydrazine. The DNA fragments were separated by electrophoresis in DNA sequencing gels and analyzed by autoradiography.

UV cross-linking and phosphatase treatment. A ^{32}P-labeled, bromodeoxyuridine-substituted HSE oligonucleotide was cross-linked to the binding activity present in the extracts by a method described previously (19). The band representing the bound complex was separated by electrophoresis in low-melting-point agarose gels, excised, boiled in Laemmli sample buffer, and loaded on a 10% acrylamide Laemmli gel for determination of the approximate size of the binding protein. Phosphatase treatment of the extracts prior to the binding reaction was performed as previously described (19).

Transfection and electroporation of an HSE-CAT construct into 3T3 and MEL cells. The plasmid used in these studies was constructed as described previously (15). Briefly, a human hsp70 gene promoter was inserted 5′ to the bacterial chloramphenicol acetyltransferase (CAT) gene. The promoter was then digested with BAL 31 exonuclease, leaving intact nucleotides −1 to −34 of the original promoter. Thus, of the previously described regulatory sequences, only the TATAA element was left intact. A synthetic oligonucleotide containing an HSE dimer, CtgGAAtaTTCccGAAtaTTC ccGA (consensus HSE sequence is shown in capital letters), was inserted at position −34. Purified HSE-CAT plasmid was linearized and cotransfected into 3T3 cells in a 20:1 ratio with a linearized pRSVNeo plasmid by using standard calcium phosphate precipitation. The plasmids were introduced

into MEL cells by electroporation (250 V/1,180 μF). Cells which had stably integrated the plasmids were selected by growth in G418 at a concentration of 0.4 μg/ml (specific activity). No spontaneously resistant calls were detected at this concentration.

To assay for heat induction of CAT activity, we made extracts by repeated freeze thawing of heat-shocked (43.5°C for 1 h) cells. A 2-h recovery period (at 37°C) following heat shock was used to allow for translation of induced CAT mRNA. CAT activity of extracts was standardized for protein content and assayed by using a two-phase separation system (25). Chloramphenicol which had been acetylated by [^{3}H]acetyl coenzyme A was detected in a Packard Tricarb liquid scintillation spectrometer.

RESULTS

To characterize the heat shock response in MEL cells, we attempted to identify the range of proteins synthesized in these cells in response to heat. When these cells were exposed to temperatures of 43.5°C for 1 to 5 h, the overall pattern of proteins synthesized (as determined by pulse-labeling with [^{35}S]methionine) was unaltered, despite the expected overall decrease in the rate of protein synthesis. This suggested that MEL cells were unable to increase the synthesis of heat shock proteins following heat stress. This finding was consistent with that of Aujame (2) with an independently isolated clone of Friend leukemia virus-transformed murine erythroleukemia cells. To examine this more closely, we focused on the best characterized of these proteins, the 70-kDa heat shock protein. This protein exists in several isoforms, as demonstrated on two-dimensional protein gels. The more acidic isoforms (referred to here as hsc70) are constitutively expressed in all cells examined. While these constitutive forms are expressed at higher levels following thermal and other stresses, the more basic isoforms of the protein (referred to here as hsp70) are detectable only in stressed cells. We therefore examined two-dimensional protein gels from [^{35}S]methionine pulse-labeled MEL cells before and after a 43.5°C heat stress for the appearance of these more basic hsp70 isoforms. Following thermal stress for up to 5 h, no expression of hsp70 was detected (Fig. 1, left). In contrast, in another murine cell line, 3T3, expression of this protein was induced after 1 h at 43.5°C (data not shown); after 5 h of heat exposure, hsp70 was the predominant protein synthesized (Fig. 1, right). These data demonstrated that MEL cells do not respond to a heat stress by inducing hsp70 expression.

Since heat shock genes are subject to translational control, we determined whether mRNA for the heat-inducible forms of hsp70 accumulated in heat-stressed MEL cells. In murine cells, a distinct 3.3-kilobase (kb) mRNA species encoding hsp70 accumulates only after stress (Hunt and Calderwood, in press). The nucleotide sequence of the coding region of this mRNA has extensive homology with that of the constitutively expressed 2.3-kb hsc70 mRNA. By using a cloned gene fragment corresponding to amino acids 119 to 543 of the heat-inducible 3.3-kb hsp70 mRNA, both of these species of mRNA can be detected by hybridization. To determine whether this 3.3-kb hsp70 mRNA was inducible in MEL cells, we stressed these cells by exposure to temperatures up to 45°C. hsp70 mRNA was not detectable in MEL cells under these conditions (Fig. 2). These results confirm those of Aujame, obtained with a genomic hsp70 clone (2). The inability to induce this mRNA was not limited to increased temperature, since both cadmium sulfate and sodium arsenite (two other inducers of stress protein synthesis) also failed to induce accumulation of this mRNA. In contrast, in two other murine cell lines, 3T3 and L1210 leukemia cells, exposure to 43°C for 1 h is sufficient to induce expression of this mRNA. Thus, the inability of MEL cells to demonstrate heat-induced synthesis of hsp70 is associated with a failure to accumulate hsp70 mRNA.

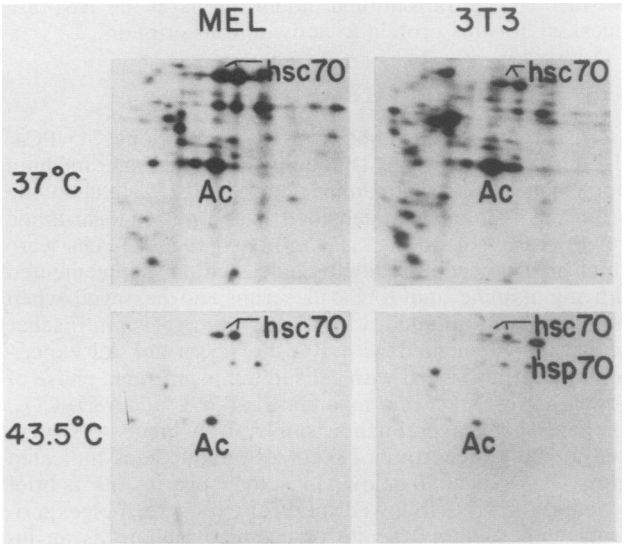

FIG. 1. The heat-inducible isoforms of hsp70 are not expressed in MEL cells. The pattern of proteins synthesized in MEL and 3T3 cells following incubation at 43.5°C was determined by two-dimensional gel analysis of [^{35}S]methionine-pulse-labeled proteins. The gels are labeled to indicate control growth (37°C) or heat shock conditions (43.5°C for 5 h). Proteins with more acidic isoelectric points are to the left. Actin (Ac) and the constitutively expressed (hsc70) and heat-induced (hsp70) isoforms of the 70-kDa heat shock protein are indicated.

To ensure that MEL cells had not deleted this gene during passage in tissue culture, we isolated DNA from MEL and 3T3 cells and analyzed it for the presence of the gene corresponding to the 3.3-kb hsp70 mRNA. To allow for more

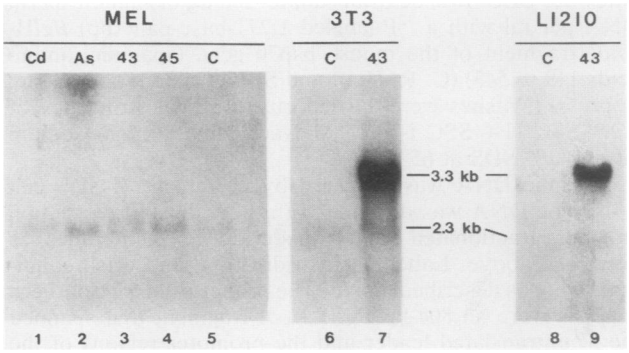

FIG. 2. hsp70 mRNA does not accumulate in MEL cells exposed to heat or heavy metals. The murine cell lines MEL, 3T3, and L1210 were maintained under normal growth conditions (C), or exposed for 1 h to the temperatures (43 or 45°C) or heavy metals (sodium arsenite [As] or cadmium sulfate [Cd]) indicated in the figure. Total RNA was isolated, electrophoretically separated, and transferred to membranes as described in the text. A ^{32}P-labeled hsp70 probe was used to detect both the 3.3-kb heat-inducible hsp70 mRNA and the 2.3-kb hsc70 mRNA.

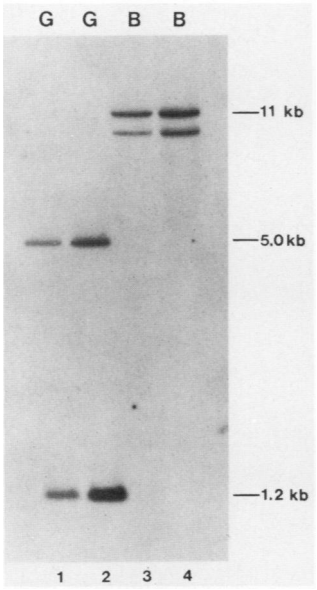

FIG. 3. The hsp70 gene is present in MEL cells. DNA was extracted from MEL and 3T3 cells and digested with *Bgl*II (G) or *Bam*HI (B). The digested DNA was electrophoretically separated, transferred to nitrocellulose membranes, and hybridized with a ^{32}P-labeled DNA fragment that spanned the 5′ untranslated leader and promoter region of the mouse hsp70 gene. Lanes: 1 and 3, 3T3 cell DNA; 2 and 4, MEL cell DNA.

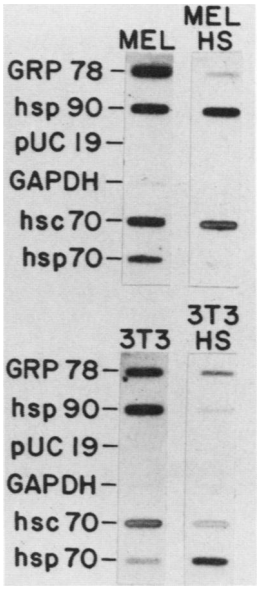

FIG. 4. The MEL hsp70 gene is not transcriptionally activated by heat shock. Nuclei were isolated from 3T3 and MEL cells grown under normal conditions or exposed to a 1-h heat shock (HS) at 43.5°C. Runoff transcripts were ^{32}P labeled and isolated as described in the text. The labeled transcripts were hybridized with an excess of denatured plasmid DNA which had been bound to nitrocellulose. The plasmids represented the 78-kDa glucose-responsive protein (GRP 78), glyceraldehyde phosphate dehydrogenase (GAPDH), the 70-kDa heat-inducible heat shock protein (hsp70), and the constitutively expressed 70- and 90-kDa heat shock proteins (hsc70 and hsp90, respectively). DNA from pUC19 was included as a control for nonspecific hybridization. Exposure times of autorads were adjusted to allow for approximately equal exposure of glyceraldehyde phosphate dehydrogenase.

accurate detection of any deletions in the promoter region, a DNA probe spanning the promoter region and the 5′ untranslated region of the hsp70 gene was used. This probe should hybridize with an 11-kb *Bam*HI fragment extending 7 kb 5′ and 2 kb 3′ to the gene and with a 1.2-kb *Bgl*II fragment spanning the proximal promoter region and 5′ coding sequences. Hybridization with fragments of the predicted sizes was detected in both 3T3 and MEL cells (Fig. 3). Thus, the failure of MEL cells to express this hsp70 mRNA is not due to gross deletions or to rearrangements of the corresponding genetic loci.

The accumulation of hsp70 mRNA in stressed cells is due to both transcriptional activation of the gene and stabilization of the mRNA (21, 30, 35). To determine whether differences in transcription rate were responsible for the expression of this mRNA in heat-shocked 3T3 cells and not in MEL cells, we determined the relative transcription rate of hsp70 in both of these cells by using a nuclear runoff assay. For these experiments a DNA fragment corresponding to the 3′ untranslated region of hsp70, which showed no cross-hybridization with hsc70 (C. R. Hunt, unpublished observations), was used. A cDNA fragment corresponding to the coding region from amino acids 14 through 413 of the murine hsc70 protein, which hybridized with both constitutive and heat-inducible transcripts, was included for comparison. The results are shown in Fig. 4. In 3T3 and MEL cells, transcripts of both the hsp70 and hsc70 genes were detected prior to heat shock. However, only hsc70 transcripts accumulated in the cells under normal growth conditions (Fig. 2). The active transcription of the hsp70 gene with no detectable accumulation of hsp70 mRNA suggests that hsp70 transcripts are specifically degraded during normal growth conditions. When 3T3 cells were exposed to temperatures of 43.5°C for 1 h, a significant increase in the rate of transcription of the hsp70 gene was observed. The transcription rates of the other genes examined here decreased under heat shock conditions. In contrast, in MEL cells the level of hsp70 transcripts decreased dramatically despite the persistence of transcription of both the hsc70 and hsp90 genes. Thus, in heat-shocked 3T3 cells the accumulation of hsp70 mRNA results from a selective increase in transcription of the gene and stabilization of the transcript. In heat-shocked MEL cells the transcription of this gene is neither selectively increased nor maintained, but, rather, decreases dramatically. Furthermore, in MEL cells hsp70 transcripts are not stabilized following heat shock, since no hsp70 mRNA accumulates in these cells despite low but detectable levels of transcription of the gene.

Heat-induced transcription of heat shock genes is associated with the binding of HSF to a conserved nucleotide sequence (HSE) located within 100 bp 5′ of the transcriptional start site. The sequence of the promoter region in the mouse hsp70 gene includes this conserved sequence (Hunt and Calderwood, in press). To determine whether this HSF is induced in MEL cells following heat exposure, we assayed extracts from heat-exposed MEL cells for the ability to bind specifically to a radiolabeled oligonucleotide containing the HSE and thus retard its mobility during electrophoresis under nondenaturing conditions (gel mobility shift assay). The results of this experiment are shown in Fig. 5A. Extracts from MEL and 3T3 cells grown at 37°C both demonstrated binding that was specifically inhibited by an unlabeled HSE oligonucleotide (Fig. 5A, X). A similar finding has been described by other investigators (24), and methylation inter-

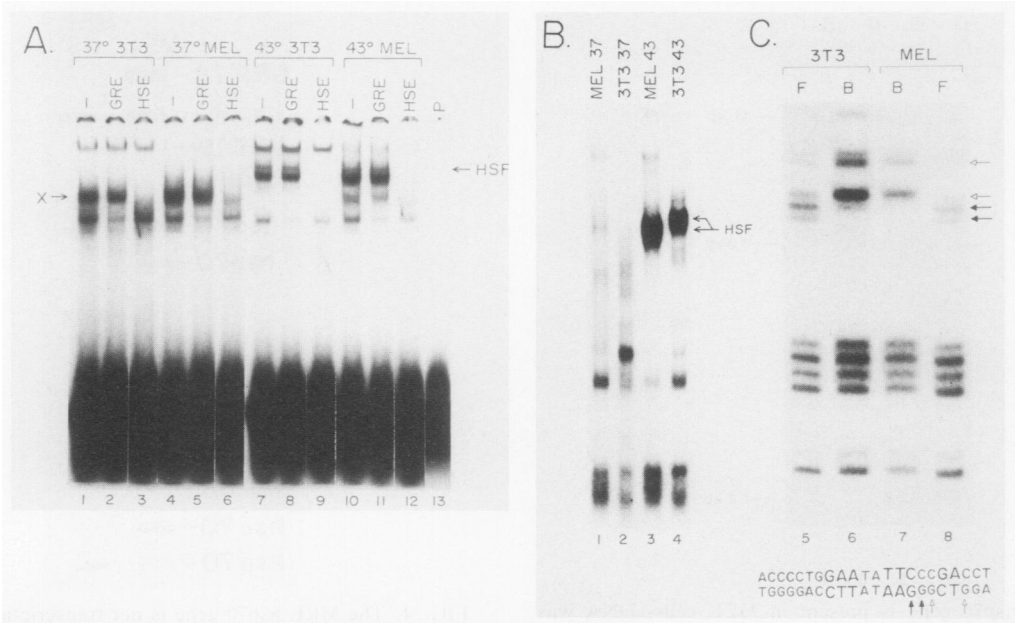

FIG. 5. Heat stress in MEL cells induces a binding activity specific to the HSE, but with altered electrophoretic mobility. Extracts were prepared from control and heat-shocked MEL and 3T3 cells as described in the text. A ^{32}P-labeled oligonucleotide containing a consensus HSE dimer was incubated with the extracts and then electrophoresed in nondenaturing polyacrylamide gels as described in the text. (A) HSE gel mobility shift assay of MEL and 3T3 cell extracts. The band labeled X indicates an HSE-specific band detected in control extracts. The heat-induced binding activity is labeled HSF. The extracts used in each binding reaction are indicated above the lanes (3T3 or MEL extracts prepared after incubation at 37 or 43°C). Binding reactions containing specific unlabeled competitor DNA fragments are indicated above each lane (GRE [glucocorticoid-responsive element] and HSE). (B) HSE gel mobility shift assay demonstrating the different electrophoretic mobilities of binding activities in MEL and 3T3 heat-shocked cell extracts apparent after prolonged electrophoresis. (C) Methylation interference patterns of HSE-binding activity in heat-shocked MEL and 3T3 cell extracts. Binding was performed with an end-labeled, partially methylated DNA fragment containing the HSE and separated by electrophoresis in native gels. The bound (B) and free (F) fragments were identified by autoradiography, excised from the gels, and chemically cleaved and the fragments were separated in sequencing gels. The partial sequence of the DNA fragment used in the binding reactions is indicated below the figure, with the consensus HSE shown in large capital letters. Methylation of the bases indicated by the black arrows interfered with binding to the HSE. Methylation of the bases indicated by the white arrows enhanced binding to the HSE.

ference studies suggest that it represents binding to the HSE, but with slightly different protein-DNA contacts. The relationship of this band to authentic HSF has not been established, and therefore it was not further evaluated in the present study. Extracts prepared from heat-exposed 3T3 and MEL cells both demonstrated binding (indicated by HSF) that was specifically inhibited by unlabeled HSE oligonucleotides. On closer examination of these two heat-inducible bands, it became apparent that the band from the MEL extracts migrated slightly faster and was more diffuse than the band detected in extracts of 3T3 cells. These differences were more apparent with prolonged electrophoresis (Fig. 5B) and were reproducible between different gel assays and between two separate MEL extracts. Therefore, to confirm that the binding activities detected in both heat-shocked cell extracts were binding in a similar manner to the HSE, we performed methylation interference studies. This approach depends on the ability of methylation within a recognition sequence to affect the avidity with which a factor binds the sequence. A characteristic pattern of methylation interference that overlaps the HSE has been previously reported with HSF binding (19, 24). By using this approach to analyze binding in the MEL and 3T3 extracts, it was evident that only methylations that overlapped the defined HSE sequence affected binding (Fig. 5C). This confirmed that the binding activity in each extract was specific for the HSE and not another cryptic sequence within the DNA fragment used for these studies. Additionally, the pattern of methylation interference was similar for both extracts, suggesting that each binding activity contacted the HSE in a similar manner. The pattern of methylation interference for both MEL and 3T3 cells was also similar to that previously described for the human HSF. Therefore, it is likely that in each extract, the binding activity detected was the HSF.

Despite similarities in binding activity in both 3T3 and MEL extracts, a slight difference in the mobility of the bound DNA fragment in nondenaturing gels existed between the two extracts. This altered gel mobility could be accounted for by differences in modification of the binding protein, by differences in associated proteins, or by the action of different heat-inducible binding proteins. To determine the approximate size of the protein that bound the HSE in the two extracts, a bromodeoxyuridine-substituted, radiolabeled HSE oligonucleotide was cross-linked to the binding protein by UV photoactivation. The approximate molecular mass of the cross-linked protein was then determined by SDS-polyacrylamide gel electrophoresis. By using this technique, the similarity of the binding proteins was further demonstrated, since in each extract the approximate molecular mass of the cross-linked, heat-inducible binding activity was 92 to 93 kDa (Fig. 6). However, even under these denaturing conditions, the binding activity in MEL cells migrates slightly faster. Thus, the difference in gel mobility appears to be due to an intrinsic change in the binding protein itself, and not to differences in noncovalently associated proteins.

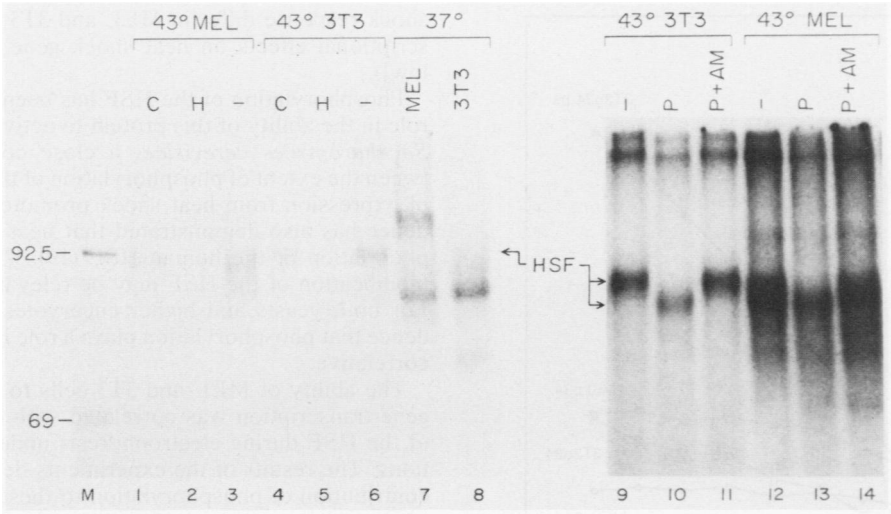

FIG. 6. The mobility of the HSE-binding proteins in MEL and 3T3 cell extracts is affected by treatment with phosphatase. Binding proteins were UV cross-linked to a ^{32}P-labeled, bromodeoxyuridine-substituted HSE. The cross-linked proteins were separated by electrophoresis in agarose and then excised, electrophoresed in denaturing Laemmli gels, and developed by autoradiography. The position of the heat-induced band is indicated (HSF). Extracts (MEL and 3T3) and unlabeled competitor DNA (C [CAAT element], H [HSE], - [no competitor]) are indicated above each lane. Molecular mass markers are present in the autoradiograph on the left, in lane M. The effects of prior incubation with potato acid phosphatase (P) or potato acid phosphatase and the phosphatase inhibitor ammonium molybdate (P+AM) on the mobility of the HSE-binding protein is shown in the autoradiograph on the right.

The HSF is known to be modified by phosphorylation. To determine whether the observed mobility difference was due to absent phosphorylation of the MEL HSF, the extracts were treated with potato acid phosphatase prior to cross-linking and electrophoresis. Phosphatase treatment increased the mobility of both HSFs and eliminated the mobility difference between the 3T3 band and the main band in the MEL extract (Fig. 6). This effect was blocked by simultaneous incubation with micromolar levels of ammonium molybdate, a phosphatase inhibitor. That the rate of migration of the MEL HSF increased following phosphatase treatment suggests that this HSF was at least partially phosphorylated. The diffuseness of the MEL band persisted despite repeated experiments with two separate MEL cell extracts, and therefore it appears to be an inherent characteristic of the HSF in MEL cells. Thus, differences in phosphorylation appear to account only partially for the mobility differences of the binding activities in the two extracts. However, the similarity in size of the main heat-induced band in the MEL extract to that in the 3T3 extract suggests that the binding proteins in the two extracts are the same and that both represent the HSF.

Transcriptional activation of the hsp70 gene does not occur in MEL cells despite the induction of a DNA-binding activity which appears identical to the HSF. This suggests that induction of a factor which binds to the HSE is not sufficient in itself to activate transcription of heat shock genes. To exclude the possibility that the HSF was active but that other binding factors inhibited transcriptional activation, or that methylation or point mutations within the MEL hsp70 promoter precluded binding, we assessed the ability of a recombinant HSE promoter to be heat inducible in 3T3 and MEL cells. This promoter contained only a synthetic HSE oligonucleotide inserted at position 34, 8 bp 5′ to the TATAA box of a previously described human hsp70 promoter-CAT fusion gene. Sequences 5′ to this insertion site had been previously deleted. This construct was introduced into MEL and 3T3 cells, and stable populations of cells were selected by growth in the antibiotic G418. Extracts prepared from these cells following a 1-h heat shock and a 2-h recovery period were assessed for CAT activity. This activity was heat inducible in 3T3 cells (Fig. 7). No heat induction of CAT activity was detected in MEL cells, even when the amount of protein assayed was increased fourfold, relative to 3T3 assays. Southern blots (data not shown) confirmed that MEL cells contained the DNA constructs. Therefore, the inability of MEL cells to demonstrate heat-induced transcription of hsp70 is due to an inability of the MEL HSF to activate transcription.

DISCUSSION

The heat shock response is among the most highly conserved examples of regulated gene expression and can be demonstrated in organisms as diverse as bacteria and humans. The transcriptional activation of these genes in response to heat is nearly universal. For these reasons, we were intrigued by the observation that MEL cells lack the ability to induce expression of the major heat shock gene product, hsp70, in response to heat (2). We therefore characterized the heat shock response of these cells in detail to determine how this response was regulated. In MEL cells the failure to express hsp70 is due to both transcriptional and posttranscriptional mechanisms. Surprisingly, despite alteration of its DNA-binding properties by heat, the HSF in MEL cells was incapable of activating the transcription of genes containing an HSE. Thus, in these cells a modification of the HSF necessary for transcriptional activation is regulated in a separate manner from the induction of DNA-binding capability. Furthermore, the normal heat-induced increase in stability of the hsp70 mRNA is not evident in these cells. Understanding this unusual pattern of heat shock gene regulation will provide insight into both the overall regulation of heat shock gene expression and the developmental alterations of this regulation. MEL cells provide a valuable tool for investigating these processes.

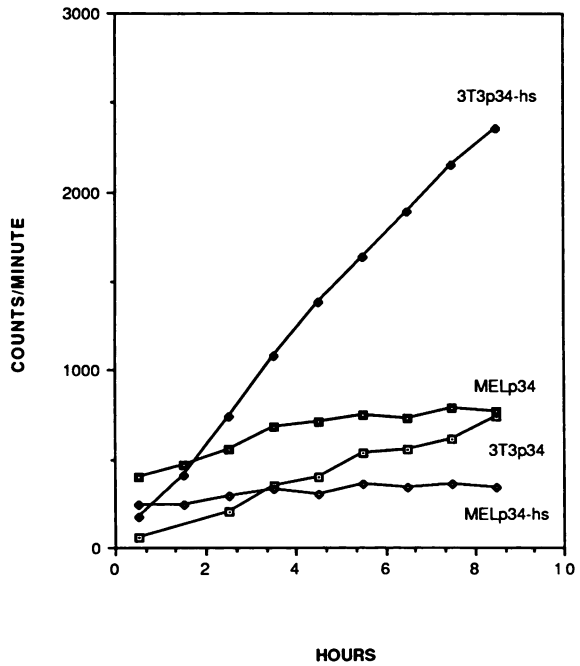

FIG. 7. The HSE does not confer heat inducibility to a CAT gene in MEL cells. Plasmids containing only the HSE and the TATA element in the promoter region of a CAT gene construct were stably introduced into MEL and 3T3 cells. Extracts made from these cells under normal growth conditions or following a 1-h heat shock were assayed for CAT activity as described in the text. The accumulated incorporation of [^{3}H]acetyl coenzyme A into chloramphenicol for the extracts (labeled MELp34, MELp34-hs, 3T3p34, and 3T3p34-hs) is shown.

An important conclusion of the data presented here is that the ability of the mammalian HSF to bind to the HSE is insufficient for activation of heat shock gene expression. Previously, the yeast HSF has been shown to bind equally well to the HSE in extracts made from both normally growing and heat-induced cells, suggesting that binding of yeast HSF to the HSE was independent of transcriptional activation (33). However, previous data have suggested that the mammalian and *Drosophila* HSFs differ from the yeast HSF in this aspect. In these higher eucaryotes, heat does affect the ability of HSFs to bind to the HSE (17, 19, 24, 39). Thus, this is the first evidence that in higher eucaryotes the binding activity of the HSF can be separated from the ability of the factor to activate transcription of heat shock genes.

It is most likely that the inability of the HSF in MEL cells to activate the transcription of genes containing an HSE is due to alterations in posttranscriptional modification of the HSF. Alternatively, such modifications might affect the ability of the HSF to localize to the nucleus. However, on the basis of the data presented here, we cannot absolutely exclude the possibility that a defect exists in the HSF genes or in other undefined genes that participate in posttranslational modification of the HSF in MEL cells. However, the demonstration that activation of heat shock genes can be developmentally regulated (see below) and that a similar defect occurs in an independently isolated clone of MEL cells (the DB1 clone used by Aujame [2] versus the 745 clone used here) argues that a necessary component of the heat shock response is turned off during the transformation process that generates MEL cells. This explanation is further supported by evidence that other facets of the heat shock response differ in MEL and 3T3 cells (i.e., posttranscriptional effects on heat shock gene transcripts [see below]).

Phosphorylation of the HSF has been suggested to play a role in the ability of this protein to activate transcription. In *Saccharomyces cerevisiae*, a close correlation exists between the extent of phosphorylation of the HSF and the level of expression from heat shock promoters (34). Recent evidence has also demonstrated that heat increases the phosphorylation of the human HSF (19). Thus, this conserved modification of the HSF may be relevant to HSF function. For both yeasts and higher eucaryotes, however, the evidence that phosphorylation plays a role in activation remains correlative.

The ability of MEL and 3T3 cells to activate heat shock gene transcription was correlated with mobility differences of the HSF during electrophoresis under denaturing conditions. The results of the experiments designed to assess the contribution of phosphorylation to these gel mobility differences were equivocal. Phosphatase treatment alters the mobility of the HSF from both 3T3 and MEL cell extracts, and the effect appears greater in 3T3 cells. However, persistent indistinct resolution of the phosphatase-treated band in MEL cells makes it difficult to rigorously argue that there are phosphorylation differences between the MEL and 3T3 HSFs. Since the mobility of the MEL HSF is altered by phosphatase treatment (and this effect is blocked by simultaneous incubation with an inhibitor of the phosphatase), it is also difficult to argue that phosphorylation of the mammalian HSF alone is sufficient for the HSF to activate transcription. Thus, it appears that either a differential effect of the site and amount of phosphorylation or other distinct modifications of the HSF (or both) are responsible for the failure of the MEL HSF to activate transcription. Therefore, our data do not clarify the role of phosphorylation in transcriptional activation by the mammalian HSF. However, the alterations in gel mobility of the HSFs in MEL and 3T3 cells make it likely that differential modification of the HSF occurs in these two murine cells. The MEL cell system provides a tool to characterize the pathways involved in the activation of heat shock gene transcription in mammalian cells.

The data obtained from the nuclear runoff experiments provide additional insight into the regulation of hsp70 expression in both MEL and 3T3 cells and exemplify the complexity of the regulation of heat shock gene expression. Although these data demonstrate the characteristic transcriptional activation of the hsp70 gene in response to heat (in 3T3 cells), they also demonstrate the importance of posttranscriptional mechanisms in the expression of this gene. The inability to detect hsp70 mRNA in whole-cell preparations (despite transcription of this gene) excludes the possibility that a block in nucleocytoplasmic transport is responsible for the failure of this mRNA to accumulate in these cells. The lack of accumulation of hsp70 transcripts under normal growth conditions could be explained either by an extremely rapid degradation of hsp70 mRNA or by the presence of a block to mRNA elongation that was relieved when nuclei were made. Rougvie and Lis (32) have shown that RNA polymerase is stalled approximately 25 nucleotides after initiating from a *Drosophila* hsp70 promoter under control conditions. If a similar event occurred in mammalian cells, it might explain our observations. Under nuclear runon conditions, the stalled polymerase could be released to create the observed signal. Alternatively, an extremely rapid degradation of hsp70 mRNA under normal growth conditions would also produce the observed results. Peterson and

Lindquist (30) have reported that a truncated *Drosophila* hsp70 mRNA has a half life of 15 to 30 min under normal growth conditions and that this mRNA is stabilized following heat shock. Similar stabilization of a human hsp70 mRNA has also been reported (35). We could not determine the half-life of our hsp70 transcript because we cannot detect it under control conditions, so we therefore cannot determine whether its stability is altered by heat shock.

Following heat shock, the relative transcription rate of the hsp70 gene in 3T3 cells increases. In contrast, in heat-shocked MEL cells the relative transcription rate of this gene decreases. The decreased rate of hsp70 transcription is consistent with the overall decrease in transcription rate from non-heat shock genes that occurs during this time and is best exemplified by the results of the nuclear runoff data from heat-shocked 3T3 cells. In MEL cells the hsp70 mRNA does not accumulate under either normal growth or heat shock conditions, despite a detectable level of synthesis. Thus, whether transcript stability or release from transcriptional attenuation is responsible for accumulation of hsp70 mRNA in heat-shocked 3T3 cells, the mechanism does not operate in MEL cells. MEL cells therefore are deficient not only in increasing the transcriptional rate of hsp70, but also in some postinitiation event that allows accumulation of the mRNA.

The ability of heat to induce the expression of hsp70 is a developmentally regulated event. In *Drosophila* oocytes, in mouse, *Xenopus*, and *Drosophila* embryos, and in yeasts undergoing sporulation, hsp70 cannot be heat induced (18, 23, 40). Banerji et al. (3) have demonstrated that in contrast to mature chicken erythroid cells, embryonic erythrocytes are incapable of increasing the synthesis of hsp70 in response to thermal stress (although in mature erythrocytes this response is regulated translationally). Therefore, the inability to induce hsp70 expression in response to stress may represent an embryonic form of gene expression. Although MEL cells are not embryonic, it is possible that transformation of these erythroid progenitor cells results in a pattern of gene expression characteristic of embryonic cells (1). However, transformation per se is not associated with inhibition of hsp70 expression, since in L1210 cells, a murine lymphoid leukemia cell line, hsp70 is heat inducible. A more thorough understanding of the mechanisms responsible for regulating expression of these genes will provide a better understanding of the regulation of these developmental events.

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