A Novel Heat Shock Response in Prolactin-dependent Nb2 Node Lymphoma Cells*

(Received for publication, August 28, 1995)

Michael J. Blake, Arthur R. Buckley, Mingyu Zhang, Donna J. Buckley, and Kathleen P. Lavoi
From the Department of Pharmacology and Toxicology, University of North Dakota School of Medicine, Grand Forks, North Dakota 58202

Virtually all cells respond to heat stress by increased expression or induction of one or more of the highly conserved cellular stress response proteins, heat shock proteins (HSPs). Here, we report the unusual property of rat Nb2-11 cells, a prolactin-dependent pre-T-cell line, to display reduced HSP expression following exposure to elevated temperature. After heat stress (41 °C, 1 h), there was no evidence of inducible members of the 70 kDa HSP family, a response common to other cell culture and tissue systems. Moreover, expression of constitutive members of the HSP70 and HSP90 families decreased during the heat stress, apparently reflecting a decrease in mRNA stability. Gel shift assays revealed that heat shock factor (HSF) was activated in spite of the lack of expression of inducible HSP70 transcripts, although its DNA binding rapidly deteriorated. Immunoblotting, using an antibody specific to HSF1, indicated that proteolysis of HSF1 may be responsible for this rapid termination of heat shock element binding. CCAAT binding, a component of constitutive HSP70 expression, was also reduced by heat stress in Nb2-11 cells and may account for the decline in constitutive HSP70 expression. Prolactin pretreatment prevented the fragmentation of HSF1, protected heat shock element and CCAAT binding, prevented the decline in constitutive HSP70 and HSP90 expression, and restored a modest expression of inducible HSP70 following heat treatment. Results of this study describe a unique regulatory defect in HSP expression in Nb2-11 cells, possibly a common characteristic of other hormone-dependent tumors.

At both the cellular and organismic level, heat and other metabolic and environmental stressors are known to cause a rapid and massive synthesis of a set of highly conserved proteins termed heat shock proteins (HSPs); for review, see Refs. 1 and 2). HSPs have been identified in evolutionarily diverse organisms ranging from bacteria and plants to humans and are categorized based on their molecular masses (1). The four major families, each of which is comprised of multiple genes, include proteins of approximately 110, 90, 70, and 27 kDa.

Some members of HSP families are expressed constitutively in the absence of stress (3, 4). Other HSPs are stress-inducible; they are expressed only after the cells have been exposed to an appropriate stimulus (1, 2). While the precise function of HSPs may depend on the context in which they are studied, they are believed to mediate protection or adaptation in response to cellular stress and to aid in the translocation of metabolically important proteins to their proper intracellular compartments.

The molecular regulation of heat shock protein expression has been studied extensively in cultured cells. Expression of the human constitutive HSP70 gene(s) is cell cycle regulated (5) and is induced by both serum (6) and the adenovirus E1a protein (7). Constitutive HSP70 transcription is regulated by a "basal" promoter, whose activity is predominantly controlled by the CCAAT element at position −70 relative to the transcription initiation site (8, 9) but also involves other cis-acting elements such as Sp1 and activating transcription factor (10). The regulation of stress-inducible HSPs is controlled by preexisting protein factors (heat shock factors (HSFs)) that can be activated by heat shock or treatment with specific chemical agents (11, 12). In mammalian cells, the exact mechanism of this activation appears to involve the association of two or more HSF proteins. The multimerization of HSF confers DNA binding activity, which allows the complex to bind to a conserved and repetitive DNA sequence (heat shock element (HSE)) that has been identified in the promoter region of the HSP70 gene (13, 14). DNA-bound HSF then becomes activated, resulting in an increased transcription of the HSP70 gene. In murine and human cells, two genes (HSF1 and HSF2) encoding unique forms of HSF have been identified (15, 16). HSF1 and HSF2 are activated by distinct mechanisms (17) but may be simultaneously activated in vivo (14), suggesting that mammalian cells possess complex forms of heat shock gene regulation (14). Presumably, HSP expression in all organisms is regulated by similar molecular response elements.

In this report, we demonstrate that defects in HSP transcriptional regulatory mechanisms and mRNA stability may be responsible for an atypical response of Nb2 cells to heat stress. The prolactin (PRL)-dependent rat Nb2 lymphoma cell line was originally derived from a lymph node from an estrogen-treated Noble rat (18) and is dependent upon lactogens (PRL, placental lactogens, or human growth hormone) for proliferation. A partially synchronous growth arrest in the early G1 phase of cell cycle can be induced in exponentially-growing cultures by an 18–24-h incubation in lactogen-deficient medium. The addition of picogram quantities of PRL to such stationary cells reinitiates cell cycle progression (19). Thus, this line has proven invaluable for the study of PRL receptor coupling to a growth response.

In addition to the hormone-dependent lines, several PRL-independent Nb2 sublines have been developed by lactogen starvation and cloning of surviving cells (20). One such line,
designated Nb2-SFJCD1, is completely autonomous but retains a partial sensitivity to the mitogenic effects of PRL (19). Compared with parent PRL-dependent Nb2 cultures, which are relatively indolent when transplanted into Noble rats, Nb2-SFJCD1 cells are highly metastatic; they rapidly develop into lethal lung, kidney, liver, and heart malignancies (21). Therefore, the PRL-dependent Nb2 cells, together with the autonomous Nb2-SFJCD1 subline, provide a useful model system to investigate the molecular consequences of tumor progression from hormone (or growth factor)-dependence to tumor cell autonomy (21).

We have determined that exposure of lactogen-dependent Nb2 cells to heat shock does not result in expression of inducible members of the HSP70 gene family and actually reduces the level of constitutively expressed members of the HSP70 and HSP90 families. This unusual heat-stress response appears to reflect a decreased mRNA stability and a defect in HSP transcriptional regulatory mechanisms that is partially restored by pretreating the cells with PRL. This novel defect in HSP gene regulation in Nb2 cells may have important consequences in the progression of hormone-dependent tumors.

MATERIALS AND METHODS

Cell Culturing—The PRL-dependent rat T-lymphoma cell line, Nb2–11, originally donated by Dr. H. G. Freisen (Winnipeg, Canada) and the PRL-independent subline, Nb2-SFJCD1, developed from the parental line by lactogen starvation and cloning of surviving cells, was obtained from Dr. P. W. Gout (Vancouver, Canada). Nb2–11 cells were maintained at 37°C in Fischer’s medium containing 10% fetal bovine serum (BioWhittaker, Walkersville, MD) as a source of lactogen, 10% horse serum (BioWhittaker), 2-mercaptoethanol (2-ME, 10−4 M), penicillin (50 units/ml), and streptomycin (50 μg/ml) as described by Gout et al. (18). For various experiments, cells were grown arrested in the early G1 phase of cell cycle by an 18–24-h preincubation in lactogen-free medium, i.e. Fischer’s medium supplemented with 2-mercaptoethanol, antibiotics, and 10% nonmitogenic gelation serum (ICN, Irvine, CA) (assay medium). When utilized for comparative purposes, Nb2-SFJCD1 cultures were similarly preincubated in assay medium for 18–24 h prior to commencement of the experimental procedures.

Heat Stress Treatments—Growth-arrested Nb2–11 or similarly pre-treated Nb2-SFJCD1 cells were suspended in pregassed assay medium (1 × 106 cells/ml) in a 50-ml conical culture tubes immersed in a water bath maintained at 41°C for 60 min. Subsequent to heat exposure, the cultures were returned to 37°C. The cultures were harvested at various time points through 4 h after commencement of heat exposure for use in Northern and immunoblotting of HSPs and gel-shift assays. Additionally, an 18-24 h preincubation in lactogen-free medium was apparent in unstressed cells of both lines (zero time point). Ten μg of total RNA were hybridized with cDNA probes that recognize HSP70, HSP89α, and HSP89β by Northern blotting. Molecular weight standards are indicated on the left.

RNA Isolation and Northern Hybridization Analysis—Following exposure conditions, Nb2–11 and Nb2-SFJCD1 cells were disrupted in RNAzol (Tel-Test, Inc.) using a polytron tissue homogenizer (Brinkman Inst.) at high speed for 10 s. Total cellular RNA was isolated using the RNAzol (Tel-Test, Inc.) using a polytron tissue homogenizer (Brinkman Inst.) according to the manufacturer’s recommended conditions in ice-cold buffer containing 20 mM HEPES (pH 7.5), 1.5 mM MgCl2, 0.2 mM EDTA, 0.2 mM dithiothreitol, 0.4 mM NaCl, 20% glycerol, 0.5 mM phenylmethylsulfonyl fluoride, and 0.5 mM leupeptin at 4°C. Insoluble material was removed by microcentrifugation (5 min), and protein was determined spectrophotometrically (Bio-Rad) in the supernatant. Individual protein samples (20 μg/lane) were fractionated on 12% SDS-polyacrylamide gels. Following electrophoresis, the proteins were transferred to Immuno-Lite blotting membrane (Bio-Rad). Membranes were blocked in 5% nonfat dry milk, and immunoblotting was performed using rabbit polyclonal antibodies that recognize HSF1 or HSF2 (graciously provided by Dr. R. I. Morimoto). A goat anti-rabbit IgG conjugated to alkaline phosphatase (1:3000) was used as a secondary antibody. Proteins were visualized utilizing a chemiluminescent reaction (Immun-Lite assay kit, Bio-Rad) followed by exposure to x-ray film.

Probes and Labeling Reactions—A cDNA for HSP70, isolated from a Chinese hamster ovary cell line (23), was provided by Dr. Albert J. Fornace, Jr. HSP89α and HSP89β cDNAs were obtained from Stress Gen (Victoria, BC, Canada). Purified inserts were labeled with 32p-labeled dCTP (DuPont NEN) using the random primer method (24).

RESULTS

The Heat Shock Response in Nb2–11 and Nb2-SFJCD1 Cells—Nb2–11 and Nb2-SFJCD1 cultures in lactogen-free medium were heat stressed for 1 h as described above. The cells were harvested at 0, 15, 30, 60, and 70 min after the initiation of the heat stress. Cells harvested at 75 and 90 min had been returned to 37°C. Ten μg of total RNA were hybridized with cDNA probes that recognize HSP70, HSP89α, and HSP89β by Northern blotting. Molecular weight standards are indicated on the left.

A Novel Heat Shock Response in Nb2–11 Cells

A novel heat shock response in Nb2 cells. Nb2–11 and Nb2-SFJCD1 cell cultures were exposed to 41°C for 1 h. Cells were harvested at 0, 15, 30, 60, 75, and 90 min after the initiation of the heat treatment. Cells harvested at 75 and 90 min had been returned to 37°C. Ten μg of total RNA were hybridized with cDNA probes that recognize HSP70, HSP89α, and HSP89β by Northern blotting. Molar weight standards are indicated on the left.

FIG. 1. A novel heat shock response in Nb2 cells. Nb2–11 and Nb2-SFJCD1 cells were exposed to 41°C for 1 h. Cells were harvested at 0, 15, 30, 60, and 75 min after the initiation of the heat treatment. Cells harvested at 75 and 90 min had been returned to 37°C. Ten μg of total RNA were hybridized with cDNA probes that recognize HSP70, HSP89α, and HSP89β by Northern blotting. Molecular weight standards are indicated on the left.

GAACCTTTGC-3′ or to the CCAAT element (5′-CTCGGTGATTTGCT-CAAAGG-3′) on ice for 30 min in incubation buffer containing poly(dI-dC)-(dI-dC) (Sigma). Specificity of the binding activity was determined by incubation of reaction lysates in the presence of competing unlabeled HSE or CCAAT (L. S. and 25 ng) or in the presence of 25 ng of unlabeled SP-1 and AP-1 oligonucleotides. Incubation reactions were fractionated on SDS-polyacrylamide gels, dried, and then apposed to x-ray film for visualization.

Protein Extraction and Western Blotting—Cellular protein was extracted from Nb2–11 and Nb2-SFJCD1 cells by homogenization in ice-cold buffer containing 20 mM HEPES (pH 7.5), 1.5 mM MgCl2, 0.2 mM EDTA, 0.2 mM dithiothreitol, 0.4 mM NaCl, 20% glycerol, 0.5 mM phenylmethylsulfonyl fluoride, and 0.5 mM leupeptin at 4°C. Soluble material was removed by microcentrifugation (5 min), and protein was determined spectrophotometrically (Bio-Rad) in the supernatant. Individual protein samples (20 μg/lane) were fractionated on 12% SDS-polyacrylamide gels. Following electrophoresis, the proteins were transferred to Immuno-Lite blotting membrane (Bio-Rad). Membranes were blocked in 5% nonfat dry milk, and immunoblotting was performed using rabbit polyclonal antibodies that recognize HSF1 or HSF2 (graciously provided by Dr. R. I. Morimoto). A goat anti-rabbit IgG conjugated to alkaline phosphatase (1:3000) was used as a secondary antibody. Proteins were visualized utilizing a chemiluminescent reaction (Immun-Lite assay kit, Bio-Rad) followed by exposure to x-ray film.

Probes and Labeling Reactions—A cDNA for HSP70, isolated from a Chinese hamster ovary cell line (23), was provided by Dr. Albert J. Fornace, Jr. HSP89α and HSP89β cDNAs were obtained from Stress Gen (Victoria, BC, Canada). Purified inserts were labeled with 32p-labeled dCTP (DuPont NEN) using the random primer method (24).

RESULTS

The Heat Shock Response in Nb2–11 and Nb2-SFJCD1 Cells—Nb2–11 and Nb2-SFJCD1 cells in lactogen-free medium were heat stressed for 1 h as described above. The cells were harvested at 0, 15, 30, 60, 75, and 90 min after the initiation of the heat stress. Subsequent to heat exposure, some cells were replaced in a 37°C incubator for an additional 15 or 30 min before harvesting. Therefore, the 75 and 90 min time points represent 15 and 30 min of recovery at 37°C. Total RNA was extracted from cells at each time point, and HSP70, HSP89α, and HSP89β mRNA levels were determined by Northern blotting (Fig. 1).

An HSP70 transcript that migrated at approximately 2.3 kb was apparent in unstressed cells of both lines (zero time point). The magnitude of expression of this transcript was consistently greater in Nb2-SFJCD1 cells. This transcript corresponds to a constitutively expressed HSP70 gene observed in other cells and tissues that hybridized with our cDNA probe, as it is similar in size and present in unstressed cells. Surprisingly, expression of this transcript declined after 60 min of heat exposure in Nb2–11 cells and remained depressed during the 30-min recovery period. In addition, there was no evidence of...
levels of inducible HSP70 transcripts (2.8 kb) were detectable in Nb2–11 and Nb2-SFJCD1 cells during the exposure conditions, and low levels of constitutive HSP70 transcripts did not change in any sample. In contrast, expression of constitutive HSP70 genes was reduced after exposure to 43°C and 45°C for 30 min, although expression was limited to the constitutive transcript and was not increased by heat exposure. HSP89α mRNA expression was in-creased by 30 min of heat exposure in Nb2–11 and Nb2-SFJCD1 cells. Additionally, several smaller bands were also present at 30 min and later time points that may represent HSP89α fragments. HSP89β expression during the exposure conditions was quite different from that observed for HSP89α, but very similar to the changes observed in constitutive HSP70 expression. HSP89β levels were greatly reduced at 60, 75, and 90 min in Nb2–11 cultures, whereas expression did not change noticeably in Nb2-SFJCD1 cells.

Characterization of the Heat Shock Response in Nb2–11 and Nb2-SFJCD1 Cells—Virtually all cells respond to heat stress by increased expression of HSP70 mRNA transcripts. Therefore, it was unusual that HSP70 was not induced by heat in the Nb2–11 line. Even more remarkable was that constitutive HSP70 mRNA levels were actually reduced by heat shock. However, the heat shock response is known to be dependent upon the nature and relative severity of the stress that elicits it. Thus, several experiments were performed to further characterize the heat shock response in Nb2–11 and Nb2-SFJCD1 cells.

Initially, Nb2–11 and Nb2-SFJCD1 cells were exposed to 37, 39, 41, 43, or 45 °C for 30 or 60 min in a water bath. Control samples were taken from cultures maintained at 37 °C in an incubator. No inducible HSP70 transcripts were expressed at any temperature/time point in Nb2–11 cultures (Fig. 2). Consistent with previous experiments, constitutive HSP70 was reduced after a 60-min exposure to 41 °C. Constitutive HSP70 expression was also reduced after exposure to 43 °C and 45 °C for 30 and 60 min. There was a small amount of inducible HSP70 transcripts in Nb2-SFJCD1 cells exposed to 41 °C for 60 min as observed previously. Constitutive HSP70 in Nb2-SFJCD1 cells was unaffected at any temperature after 30 min, although constitutive levels dropped slightly after 60 min at 43 and 45 °C.

Since both cell lines displayed an unusual sensitivity to heat stress, we determined the viability of Nb2–11 and Nb2-SFJCD1 cells after the respective treatments described above. Very few nonviable cells were detected in either cell line at temperatures of 41 °C or below (Fig. 3). Although there were generally more nonviable Nb2–11 compared with Nb2-SFJCD1 cells in any given population, the percentage of nonviable cells from either line was typically 10% or less at these temperatures. The number of nonviable cells in each line increased after 30 min of exposure to 43 and 45 °C. Even greater increases were observed in cultures treated at these temperatures for 60 min. Thus, exposure to 41 °C is sufficient to induce the response in the Nb2 lines but does not significantly affect cell viability.

To determine the time course for generation of the heat shock response in Nb2–11 and Nb2-SFJCD1 cells, both lines were exposed to 41 °C followed by the determination of HSP70 expression at time points to 6 h. In Nb2–11 cultures, HSP70 expression was limited to the constitutive transcript and was again reduced after 1 h of heat exposure (Fig. 4, upper panel). Importantly, levels of expression began to increase notably by 4 h and returned to near control levels by 6 h after initiation of the heat shock. In un-stressed Nb2-SFJCD1 cells (Fig. 4, lower panel), levels of constitutive HSP70 actually showed a slight decrease over time. Constitutive HSP70 expression was not reduced by heat shock in this line, and again there were larger, heat-inducible HSP70 transcripts evident at 1, 2, 4, and 6 h after heat exposure.

Heavy metals are known to induce an HSP response in many systems. Therefore, cadmium was used to provide additional characterization of the HSP response in Nb2–11 and Nb2-SFJCD1 cells. Cadmium (25 μM) increased constitutive HSP70 mRNA expression after 3 h of exposure, but no increases in inducible transcripts were detected at any time (data not shown). Collectively, these results indicated that at least two separate events were responsible for the heat-induced decline of HSP70 transcripts in Nb2–11 cells: a decline in constitutive mRNA and a lack of expression of inducible transcripts.

Stability of HSP70 Transcripts in Nb2–11 Cells—The regulation of expression of constitutive and inducible HSP70 genes is mediated by distinct mechanisms. Thus, it was likely that several factors contributed to the decline in constitutive HSP70
and the lack of inducible transcripts in Nb2–11 cells. Initially, it was important to determine whether the cause of these observations resulted from reduced transcription of HSP70 genes or from a decreased stability of HSP70 messages in this cell line.

To assess HSP70 mRNA stability, Nb2–11 and Nb2-SFJCD1 cells were subjected to a 1-h heat stress (41°C) and then treated with actinomycin-D (5 μg/ml). Control cultures received no heat treatment. HSP70 mRNA expression was then determined in all cultures at 0, 30, 60, 90, and 120 min after actinomycin-D treatment. Constitutive HSP70 transcripts appeared stable since actinomycin-D treatment produced no inducible HSP70 transcripts in heat-stressed Nb2–11 and Nb2-SFJCD1 cells (Fig. 5). However, in heat-stressed Nb2–11 cells, constitutive HSP70 mRNA levels were reduced and continued to decline during the 2 h after treatment. Additionally, smears were evident below 2.3 kb in these cells, indicating that the stability of this message was reduced by heat. In Nb2-SFJ CD1 cells, the signal intensity of the 2.3-kb transcript did not decline as dramatically compared with Nb2–11 cultures, but degraded products were again evident. Inducible HSP70 transcripts were present early after heat treatment but declined during the 2 h after actinomycin-D treatment.

Transcriptional Regulation of the Heat Shock Response in Nb2–11 and Nb2-SFJ CD1 Cells—The preceding experiment indicated that reduced stability of constitutive HSP70 transcripts contributed to their heat-induced decline but did not rule out the possibility that changes in transcriptional regulation were also involved, nor did it address the cause of the lack of expression of inducible HSP70 transcripts. Expression of inducible HSP70 genes by heat is mediated by activation of HSF (13). Concomitant with the decline in constitutive HSP70 mRNA, no inducible transcripts were apparent in heat-stressed Nb2–11 cells (Figs. 1, 2, and 4). To determine whether the lack of inducible HSP70 transcripts could be attributed to a reduced capacity to activate HSF in Nb2–11 cells, HSE binding was assessed by gel mobility shift assay in both cell lines exposed to the identical heat shock conditions described in Fig. 1.

Prior to heat exposure (zero time point), no HSE binding was observed in either line (Fig. 6). However, after 15 min, substantial HSE binding was evident in both cell lines. In Nb2–11 cells, this binding was still distinct after 30 min, but it appeared to deteriorate with continued exposure. Only not did the magnitude of the binding decline, but the bands also became less distinct and began to smear below the size of the bands present at 15 and 30 min. The magnitude of HSE binding also declined in Nb2-SFJ CD1 cells during the heat exposure, but the overall signal at each time point was greater in these cells compared with the Nb2–11 line. Moreover, the bands did not appear to become degraded or smeared in the Nb2-SFJ CD1 cells, as observed in Nb2–11 cultures.

These results indicated that HSF was activated by heat in Nb2–11 cells, yet no inducible HSP70 transcripts were expressed. Although HSE binding was induced by heat exposure, this activity was short lived and appeared to degrade after 1 h. The smearing of the HSE binding signal in the gel shift assays led us to suspect that HSF may be adversely affected by heat stress in the Nb2–11 line. To determine the effects of heat exposure on HSF, the identical protein samples used in the gel shift assays were immunoblotted with an antibody that specifically recognizes HSF1.

A single band migrating at about 90 kDa was recognized by the HSF1 antibody in unstressed Nb2–11 and Nb2-SFJ CD1 cells (Fig. 7, zero time point). By 0.5 h after heat exposure, a second protein band was evident in Nb2–11 lysates that was smaller in molecular weight. Additional smaller protein bands were detected in Nb2–11 cells at 1, 2, and 4 h, suggesting that HSF may undergo proteolysis subsequent to heat exposure. In contrast, no such fragmentation of HSF1 was apparent in Nb2-SFJ CD1 cells at any time during or after the exposure. Identical samples of heat-treated Nb2–11 and Nb2-SFJ CD1 cells were immunoblotted with an antibody that recognizes HSF2. However, no HSF2 immunoactivity was detected in any sample from either cell line (data not shown).

CCAAT response element has been identified in the regulatory region of constitutively expressed HSP70 genes and is thought to contribute to their transcriptional regulation (25, 26). Since constitutive levels of HSP70 mRNA decreased in Nb2–11 cells during heat exposure, CCAAT binding was deter-
are induced by exposure to different stressors, the HSP70 family includes heat-induced HSP70, HSP89, and HSP90 (Fig. 9).

Expression in Nb2–11 cells (19). To determine the effect of PRL pretreatment on the heat shock response in Nb2–11 and Nb2-SFJCD1 cells, heat-induced HSP70, HSP89α, and HSP89β mRNA expression was assessed in each cell line in the presence or absence of PRL (Fig. 9).

Exposure to 41°C resulted in reduced constitutive HSP70 and HSP89β mRNA levels, and an increased HSP89α by 90 min in Nb2–11 cells as previously observed. By comparing the zero time points of treated and untreated Nb2–11 cells, it was evident that PRL pretreatment resulted in elevated levels of constitutive HSP70 and HSP89β. Moreover, there was no reduction in constitutive HSP70 or HSP89β expression in lactogen-treated Nb2–11 cells. Thus, PRL prevented the heat-induced decline in constitutive HSP70 and HSP89β mRNA levels in these cells. The pattern of HSP89α expression in response to heat treatment did not differ between PRL-treated and untreated Nb2–11 cells. In Nb2-SFJ CD1 cells, HSP transcripts were not reduced by heat stress nor did PRL pretreatment affect expression of any HSP species in response to heat.

The effect of PRL on HSE and CCAAT binding was also determined in Nb2–11 and Nb2-SFJ CD1 cells subjected to the treatment conditions described in Fig. 9. A smearing and reduced magnitude of HSE binding was again apparent in heat-shocked Nb2–11 cells at 90 min of exposure (Fig. 10). Pretreatment with PRL prevented the smearing of the HSE binding signal in these cells albeit the magnitude of the signal was still reduced at 90 min. The decline in CCAAT binding activity following heat stress was less apparent in PRL-treated Nb2–11 cells (Fig. 8). Additionally, the hormone appeared to prevent the loss of one of three bands comprising the CCAAT binding signal (see arrows in Fig. 11). PRL did not appear to substantially affect HSE or CCAAT binding in Nb2-SFJ CD1 cells (Figs. 10 and 11).

To determine the effect of PRL pretreatment on the fragmentation of HSF by heat stress in Nb2–11 cells, protein samples from control and PRL-treated cultures subjected to heat stress were probed with HSF1 antibody. PRL treatment greatly reduced the fragmentation of HSF1 in these cells (Fig. 12). Apparently, PRL treatment increases the stability of HSF1 in Nb2–11 cells to that found in Nb2-SFJ CD1 cells.

**DISCUSSION**

Although members of several families of heat shock proteins are induced by exposure to different stressors, the HSP70 family has received the preponderance of investigation in response to heat. Constitutive members of the HSP70 family are expressed in unstressed cells. Following heat exposure, there appears to be a slight increase in constitutive HSP70 expression.
A large portion of the decline in constitutive HSP70 mRNA expression in Nb2-11 cells appears to be attributable to reduced mRNA stability after heat exposure. The levels of this transcript remained stable in unheated actinomycin-D-treated cultures, whereas it was dramatically reduced in heated cultures (Fig. 5). The degradation products observed below the 2.3 kb band further reflected the instability of this message. In Nb2-SFjCD1 cells, the 2.3-kb transcript appeared more stable during heat stress since the 2.3-kb transcript signal intensity did not decline as much as in Nb2-11 cells. Alternatively, Nb2-SFjCD1 cells may be able to replace lost transcript by an increased transcription. Collectively, these data indicate that reduced stability contributes to the heat-induced decline in constitutive HSP70 mRNA transcripts but do not address changes in the transcriptional regulation of these genes that may occur concurrently.

As previously indicated, the molecular regulation of the heat shock response has been the subject of intense investigation yielding a wealth of information regarding the control of HSP expression. Due to the unusual pattern of HSP mRNA expression observed in PRL-dependent Nb2 cells, we initiated experiments addressing the regulation of HSP expression in response to heat in Nb2-11 and Nb2-SFjCD1 cell lines. Gel mobility shift assays were used to determine whether the lack of inducible members of the HSP70 gene family in Nb2-11 cells following heat stress occurred as a result of altered HSE binding (Fig. 3). Strong HSE binding was apparent in extracts from both cell lines 15 min after initiation of heat treatment. By 60 min, the intensity of HSE binding had begun to decline in both lines and displayed a further decline during the 30 min recovery period. While the reduction in HSE binding in Nb2-SFjCD1 cells could be attributed to a decreased signal intensity, the reduced HSE binding in Nb2-11 cells was qualitatively distinct. In addition to a reduced signal intensity, HSE binding in the Nb2-11 cells became diffuse and smeared below the level of the bands observed at the 15 min time point. These data suggest that protein factors capable of interacting with HSE, while retaining their DNA binding activities, were being rapidly altered so as to increase their mobility through the polyacrylamide gels. Based on these results, we concluded that HSF itself was adversely affected by heat stress in Nb2-11 but not in Nb2-SFjCD1 cells.

To determine whether the changes in DNA binding were due to alterations in protein factors binding to the HSE, the identical protein samples used for the gel shift assays were immunoblotted using HSF1 and HSF2 antibodies. Fragmentation of HSF1 was apparent by 30 min of heat exposure in Nb2-11 cells. This apparent degradation intensified throughout the rest of the treatment. In contrast, HSF1 in heat-stressed Nb2-SFjCD1 cells did not appear to suffer the same proteolytic demise, although there did seem to be a gradual loss of signal intensity. These results indicate that the decline in HSE binding activity in Nb2-11 cells may be due to a rapid decay of HSF. The rapid loss of HSF binding may be ultimately responsible for the lack of inducible HSP70 mRNA transcripts in these cells. In Nb2-SFjCD1 cells, there was no fragmentation but HSE binding and HSF1 protein levels declined with time after heat stress. Apparently, these cells maintain a modest ability to respond to heat stress through the expression of inducible HSP70 mRNA transcripts. However, despite their improvement in HSE expression relative to Nb2-11 cells, the heat-stress response of Nb2-SFjCD1 cells is less than that observed in many other cell types (27).

While HSF appears responsible for activation of inducible HSP expression, CCAAT binding factors play a major role in expression of constitutively expressed HSPs. The importance of CCAAT in the regulation of constitutive HSP70 expression prompted us to investigate whether alterations in CCAAT binding may explain the decline in constitutive HSP70 mRNA expression in heat-stressed Nb2-11 cells. CCAAT binding displayed a steady decline in Nb2-11 cells during the heat stress conditions (Fig. 5). By 90 min after initiation of the heat treatment, the intensity of the CCAAT binding signal was reduced by over 80%. There was also an apparent loss of binding by one of three CCAAT binding factors as indicated by the loss of one of the bands comprising the signal. In Nb2-SFjCD1 cells, CCAAT binding also declined but, as with HSE binding, the magnitude of the decline was much less than in Nb2-11 cells. CCAAT was originally identified as a potential promoter element for a number of eukaryotic genes (28). Subsequently, multiple CCAAT binding activities have been reported (29–31). Although it is difficult to assess the relationship among these activities, the current data suggest that different CCAAT elements are recognized in vivo by different factors. While our results do not specifically identify which of the several CCAAT binding factors are affected by heat stress in Nb2-11 cells, they do suggest that their reduced binding may contribute to the...
decline in constitutive HSP70 mRNA expression in these cells. Pretreatment with PRL prevented the heat-induced reduction of constitutive HSP70 in Nb2–11 cells. The hormone also afforded Nb2–11 cells a meager ability to express inducible HSP70 transcripts in response to heat shock. Both HSE and CCAAT binding were improved in PRL-treated Nb2–11 cells. Perhaps more significantly, PRL treatment also prevented the fragmentation of HSF1 in Nb2–11 cells, thus providing a possible explanation for the change in HSE binding characteristics and mRNA expression. Essentially, the effect of PRL pretreatment on Nb2–11 cells was to transform their heat shock response to one similar to that observed in lactogen-independent Nb2-SFJ CD1 cells.

Prolactin binds to its receptor initiating a cascade of intra-cellular events that stimulate proliferation of Nb2–11 cells. Importantly, PRL-provoked Nb2 cell mitogenesis is coupled to the activation of several tyrosine and serine/threonine kinases that most likely participate in the transcriptional regulation of growth-related genes (32–37). Since the transcriptional regulation of the heat shock genes has been reported to involve specific phosphorylation events (9), it is likely that one or more of the kinases activated as a consequence of PRL stimulation is directly involved in restoring the heat shock response in Nb2–11 cells. Conversely, the lack of an appropriate HSP response in untreated Nb2–11 cells may directly contribute to the hormone-dependent phenotype of these cells. Delination of the cellular events responsible for the conversion of the HSP response in Nb2–11 cells to one similar to that of Nb2-SFJ CD1 cells may provide important insights into the nature of oncogenic progression from growth factor dependence to tumor autonomy, a clinical occurrence with disastrous consequences.

REFERENCES

1. Welch, W. J. (1992) Physiol. Rev. 72, 1063–1081
2. Morimoto, R. I., Tissieres, A., and Georgopoulos, C. (eds.) (1994) The Biology of Heat Shock Proteins and Molecular Chaperons, Vol. 26, pp. 1–30, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
3. Söger, P. K., and Polham, H. R. B. (1987) EMBO J. 6, 993–998
4. O’Malley, K., Mauron, A., Barchas, J. D., and Kedes, L. (1985) Mol. Cell. Biol. 5, 3476–3483
5. Miliarski, K., and Morimoto, R. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 9517–9521
6. Wu, B., and Morimoto, R. (1985) Proc. Natl Acad. Sci. U. S. A. 82, 6070–6074
7. Wu, B., Hurst, H., Jones, N., and Morimoto, R. (1986) Mol. Cell. Biol. 6, 2994–2999
8. Greene, J., Larin, Z., Taylor, L., Prentice, H., Gwinn, K., and Kingston, R. E. (1987) Mol. Cell. Biol. 7, 3646–3655
9. Wu, B., Kingston, R., and Morimoto, R. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 629–633
10. Morimoto, R. I., Sarge, K. D., and Abravaya, K. (1992) J. Biol. Chem. 267, 21987–21990
11. Holmgren, R., Corces, V., Morimoto, R., Blackman, R., and Melszeln, M. (1981) Proc. Natl. Acad. Sci. U. S. A. 78, 3775–3778
12. Pellham, H. R. B. (1982) Cell 30, 517–528
13. Wu, C., Clos, J., Giorgi, G., Haroun, R. I., Kim, S.-J., Rabindran, S. K., Westwood, J. T., Winiwarter, J., and Yim, G. (1994) in The Biology of Heat Shock Proteins and Molecular Chaperons (Morimoto, R. I., Tissieres, A., and Georgopoulos, C., eds.) Vol. 26, pp. 395–416, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
14. Morimoto, R. I., Jurivich, D. A., Kroeger, P. E., Mathur, S. K., Murphy, S. P., Dakal, A., Sarge, K., Abravaya, K., and Sistonen, L. T. (1994) in The Biology of Heat Shock Proteins and Molecular Chaperons (Morimoto, R. I., Tissieres, A., and Georgopoulos, C., eds.) Vol. 26, pp. 417–456, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
15. Sarge, K. D., Zimarino, V., Holm, K., Wu, C., and Morimoto, R. I. (1991) Genes & Dev. 5, 1902–1911
16. Schuetz, T. J., Gallis, G. J., Sheldon, L., Tempest, P., and Kingston, R. E. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 6910–6915
17. Sistonen, L., Sarge, K. D., and Morimoto, R. I. (1994) Mol. Cell. Biol. 14, 2087–2099
18. Gout, P. W., Beer, C. T., and Nobid, R. L. (1980) Cancer Res. 40, 2433–2436
19. Buckley, A. R., Buckley, D. J., Gout, P. W., Liang, H., Rao, Y.-P., and Blake, M. J. (1993) Mol. Cell. Endocrinol. 86, 17–25
20. Gout, P. W. (1987) Cancer Res. 47, 1791–1795
21. Gout, P. W., Horsman, D. E., Foc, K., Dejong, G., Ma, S., and Bruchovsky, N. (1994) Anh Cancers Res. 14, 2485–2492
22. Church, G. M., and Gilbert, W. (1984) Proc. Natl. Acad. Sci. U. S. A. 81, 1991–1995
23. Fornace, A. J., Alamo, J., Hollander, M. C., and Lamoreaux, E. (1989) Exp. Cell Res. 182, 61–74
24. Feinberg, A. P., and Vogelstein, B. (1983) Anal. Biochem. 132, 6–13
25. Lum, L. S. Y., Sultzman, L. A., Kaufman, R. J., Linzer, D. I. H., and Wu, B. J. (1990) Mol. Cell. Biol. 10, 6709–6717
26. Morgan, W. D., Williams, G. T., Morimoto, R. I., Greene, R. K., Kingston, R. E., and Tjian, R. (1987) Mol. Cell. Biol. 7, 1129–1138
27. Blake, M. J., Gershon, D., Fargnoli, J., and Holbrook, N. J. (1990) J. Biol. Chem. 265, 15275–15279
28. Efstradiatis, A., Posakony, J., Maniatis, T., Lewn, R., O’Connell, C., Spritz, R., DeRiel, J., Forget, B., Weissman, S., Slightom, J., Blechl, A., Smithies, O., Baralle, F., Shoulders, S., and Proudfoot, N. (1990) Cell 21, 653–668
29. Barberis, A., Superti-Furga, G., and Buslunger, M. (1987) Cell 50, 347–359
30. Chadock, L., Baldwin, A., Carthew, R., and Sharp, P. (1988) Cell 53, 11–24
31. Dorn, A., Bolejens, J., Staub, A., Genoist, C., and Mathis, D. (1987) Cell 50, 863–872
32. Rui, H., Kirken, R. A., and Farrar, W. (1991) J. Biol. Chem. 266, 5364–5368
33. Campbell, B. R., Argetsinger, L. S., Ihle, J. N., Kelly, P. A., Rilem, J. A., and Carter-Su, C. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 5232–5236
34. Cleveger, C. V., and Medagali, M. V. (1994) Mol. Endocrinol. 8, 674–681
35. Cleveger, C. V., Torio, T., and Reed, J. C. (1994) J. Biol. Chem. 269, 5559–5565
36. Buckley, A. R., Rao, Y.-P., Buckley, D. J., and Gout, P. W. (1994) Biochem. Biophys. Res. Commun. 204, 1138–1146
37. Rao, Y.-P., Buckley, D. J., and Buckley, A. R. (1995) Cell Growth & Differ. 6, 1235–1244
A Novel Heat Shock Response in Prolactin-dependent Nb2 Node Lymphoma Cells
Michael J. Blake, Arthur R. Buckley, Mingyu Zhang, Donna J. Buckley and Kathleen P. Lavoi

J. Biol. Chem. 1995, 270:29614-29620.
doi: 10.1074/jbc.270.49.29614

Access the most updated version of this article at http://www.jbc.org/content/270/49/29614

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 37 references, 18 of which can be accessed free at http://www.jbc.org/content/270/49/29614.full.html#ref-list-1