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DECREASED HEAT SHOCK RESPONSE UPON ADIPOSE DIFFERENTIATION OF 3T3-L1 CELLS

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SUMMARY In order to gain a better understanding of the regulation of heat shock gene (hsp) expression in terminal cell differentiation, we evaluated the effects of heat shock on the synthesis of HSPs, the abundance of $mRNA^{hsp}$, and the heat shock transcription factor (HSTF) DNA-binding activity in the 3T3-L1 fibroblasts and adipocytes. We showed that the heat shock (42°C) induction of synthesis of HSPs was significantly greater in the undifferentiated fibroblast than the differentiated adipocyte cultures. In particular, the heat shock induced synthesis of HSP 72 was at least 10 times greater in the fibroblasts than in the adipocytes. Analysis of mRNA of hsp 89α, hsp 89β, hsp 70, and hsp 25 by Northern blot hybridization showed that the expression of these mRNAs was very, if not strictly, dependent on heat shock of the cells; the abundance of these heat inducible mRNAs was significantly higher in fibroblasts than in adipocytes. Quantitation of the HSTF DNA-binding activity by gel retardation assay demonstrated a specific decrease in this activity in the differentiated cells. These results provide evidence of a decreased transcriptional activation of heat shock genes upon adipose cell differentiation.

Prokaryotes and eukaryotes respond to heat and other environmental stimuli by synthesizing a set of heat shock proteins (HSPs) (Atkinson and Walden, 1985; Lindquist, 1986). Cross hybridization and sequence analyses revealed that the genes and proteins of the major HSPs are highly conserved (Hunt and Morimoto, 1983; Kelley and Schlesinger, 1982; Voellmy et al., 1983). These features suggest that HSPs may be important to survival of cells and organisms.

We have been interested in the regulation of heat shock gene expression as it relates to cell aging and terminal differentiation. In previous studies, we showed that the heat shock induction of hsps is attenuated in aging IMR-90 human diploid fibroblasts (Liu et al., 1989a and 1989b). A framework connecting this decreased heat shock response to the loss of dividing potential in aging cells has yet to emerge.

The 3T3-L1 cell line is a clonal derivative of Swiss mouse embryo fibroblasts that undergoes adipose conversion under appropriate experimental conditions (Green and Kehinde 1974, 1975, 1976). The conversion of 3T3-L1 cells from fibroblasts to adipocytes represents a spontaneous and heritable phenotype, and can be facilitated by treating cells with various hormones, drugs, or nutrients. Once the cells are committed to differentiate and begin to accumulate fat droplets in the cytoplasm, the process is irreversible and the adipocytes represent a population of terminally differentiated end-cells. The 3T3-L1 cell line provides a useful model system for studying events related to adipogenesis; in addition, they are useful for delineating biochemical and molecular events associated with terminal cell differentiation. In this study, we evaluated the heat shock induction of hsps in the 3T3-L1 fibroblasts and adipocytes.

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MATERIALS AND METHODS

Materials. [^{35}S]Trans-label, [α^{32}P]dCTP and [γ^{32}P]ATP were from ICN, Irvine, CA. Nick translation kit was from Bethesda Research Lab., Gaithesburg, MD. Tissue culture supplies were obtained from GIBCO, Grand Island, N.Y. The plasmids pHS 801 , 811 and 208 were provided by Drs. Hickey and Weber of the University of South Florida (Hickey et al., 1986). The pH 2.3 genomic DNA clone of human hsp 70 gene (Wu et al., 1985) was from Dr. Morimoto of Northwestern University. Restriction maps of the DNA probes have been published (Hickey et al., 1986; Wu et al., 1985).

Cell culture and Pulse-labeling of Cells with [^{35}S]Trans-label. 3T3-L1 cells were cultured according to conditions previously described (Liu, 1982). To promote differentiation of the 3T3-L1 fibroblasts to adipocytes, confluent monolayers (designated as day 0) were treated for 2 days with 0.5 mM 3-isobutyl-1-methyl xanthine and 0.25 μM dexamethasone, followed by replenishing the cells with fresh medium supplemented with 10% fetal bovine serum and 10 μg/ml insulin. Approximately 60-80% of the cell population appeared "fatty" on day 8 of the treatment program.

For this study, confluent quiescent monolayer cultures of the 3T3-L1 fibroblasts and adipocytes were heat shocked at 42°C for time periods specified. To determine the pattern of protein synthesis, cells were pulse labeled with 100 μCi/ml [^{35}S]Trans-label in DM-met$^-$ (Dulbecco's medium without methionine) during the last hr of incubation immediately prior to harvesting. Cells were homogenized in 0.35 ml of a buffer of 10 mM Tris-HCl (pH 7.5) with 1 mM EDTA and 50 μg/ml phenylmethylsulfonyl fluoride. Aliquots of the cell homogenates containing an equal amount of trichloroacetic acid precipitable radioactivity (100,000 - 500,000 cpm) were subjected to analysis by SDS-polyacrylamide gel electrophoresis and autoradiography according to methods described (Culpepper and Liu, 1983).

Northern blot quantitation of mRNA of hsps. Cytoplasmic RNA was isolated from 3T3-L1 cells according to methods described (Liu et al., 1989a). RNA, size fractionated on a 1% agarose-formaldehyde gel, was transferred to Gene Screen Plus membrane. The membrane was processed and probed with denatured, nick-translated plasmid DNA (Liu et al., 1989a); pHS 801 and 811 cDNA probes were used for hybridization to mRNAs of hsp 89α and hsp 89β, respectively, the pH 2.3 genomic DNA probe was used for hsp 70, and pHS 208 cDNA probe was used for hsp 25 (Hickey et al., 1986; Wu et al., 1985). Hybridization was done at 42°C for overnight. The membrane was then washed two times with 2X SSC at room temperature for 5 min each, two times with 2X SSC containing 1% SDS at 50°C for 15 min each, and two times with 0.1X SSC at room temperature for 30 min each. The membrane was air-dried and exposed to X-Omat film with intensifying screen at -80°C.

Quantitation of HSTF DNA-binding activity by Gel retardation assay. Gel retardation assay was performed essentially as previously described (Ausubel et al., 1987; Mosser et al., 1988) using a double stranded synthetic consensus HSE (upper strand 5'-GCCTCGAATGTTCGCGAAGTTTCG-3'; Goldenberg et al., 1988). DNA was labeled by T-4 kinase catalyzed 5' end-labeling with [γ^{32}P]ATP. A typical protein-DNA binding assay mixture (total volume 25-40 μl) contained 10-40 μg whole cell extract protein, 0.5-1 μg poly(dI-dC).poly(dI-dC), in the presence of a buffer of 10 mM Tris (pH 7.8)/50 mM NaCl/1 mM EDTA/0.5 mM DTT/5% glycerol. Reaction was started by the addition of ^{32}P-DNA (1 ng, 10,000 cpm) and was carried out at 25°C for 20 min. Samples were immediately subjected to electrophoresis in low-ionic strength 4% polyacrylamide gel with buffer recirculated between the lower and upper reservoir compartments (Ausubel et al., 1987).

For competition experiments, a 200 X molar excess non-radioactive DNA was added to the cell extract and incubated for 10 min at 25°C. ^{32}P-HSE was then added and incubated at 25°C for 20 min as described above. The double-stranded synthetic CAP DNA used in the competition experiments was from Dr. R. Ebright of Waksman Institute, Rutgers University; the sequence of this DNA (upper strand) was: 5'-GCAACGCAATAAATGTGATCTAGATCACATTTTAGGCACCC-3'.

RESULTS

For experiments described in this study, a heat shock temperature of 42°C was used. We noted that higher heat shock temperatures (e.g. 45°C) did not give a significantly greater heat shock response (measured by synthesis of HSPs) but did reduce total cellular protein syntheis and caused cell death. Figure 1 is an autoradiogram illustrating the induction of heat shock protein (HSP) synthesis

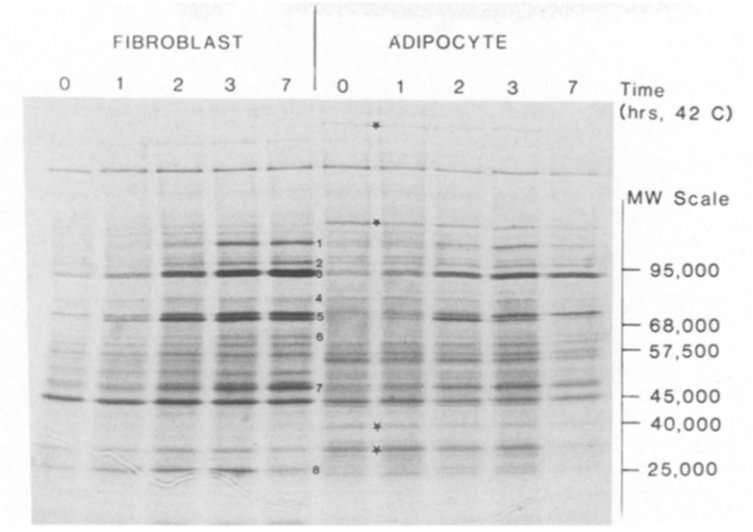

Figure 1. **Autoradiogram illustrating the time course of induction of heat shock protein synthesis in 3T3-L1 fibroblasts and adipocytes.** 3T3-L1 fibroblast and adipocyte cultures were heat shocked at 42°C for time periods as indicated (t=0, i.e. 37°C). Cells were labeled with 100 μCi/ml of [^{35}S]Trans-label during the last hr of incubation prior to harvesting. Aliquots of the cell homogenate containing 200,000 cpm of radioactivity were analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. Bands 1 through 8 on the autoradiogram represent HSPs 110, 98, 89, 78, 72, 64, 50, and 25, respectively. The star symbols (★) identify proteins whose synthesis were increased in the adipose cells; they are (from top to bottom): 230 KDa fatty acid synthetase, 130 KDa pyruvate carboxylase, 40 KDa aldolase, and 37 KDa glyceraldehyde phosphate dehydrogenase.

in 3T3-L1 fibroblasts and adipocytes as a function of time of heat shock at 42°C (t=0, i.e. 37°C). This result showed that heat shock markedly and specifically increased the synthesis of proteins with apparent molecular weights of 110,000, 98,000, 89,000, 78,000, 72,000, 64,000, 50,000, and 25,000 in the 3T3-L1 fibroblasts, designated as bands 1 through 8 in the autoradiogram in Figure 1. Comparison of the induction of HSPs in the 3T3-L1 fibroblasts and adipocytes showed a significant decrease in the magnitude of induction in the adipocytes; there was no major difference in the number of HSPs being induced. We noted that cellular protein synthesis, as determined by the amount of radioactivity incorporated per mg protein, was not substantially affected over this time course of heat shock. Also, as shown in Figure 1, differentiation of the 3T3-L1 cells was associated with increases in the synthesis of several cellular proteins (indicated by star symbols), including the 230 KDa fatty acid synthetase, 130 KDa pyruvate carboxylase, 40 KDa aldolase, and 37 KDa glyceraldehyde phosphate dehydrogenase. Synthesis of the 45 KDa actin in the adipocytes was reduced when compared to that of the fibroblasts, a result consistent with that of previous studies (Spiegelman and Green, 1980).

HSPs often are consisted of a family of proteins with related sequence. As an example HSP 70 can be resolved into three isoforms known as HSP 70, HSX 70, and HSC 70, based on their isoelectric point and basal level of expression (Pelham, 1986). Analysis of the synthesis of HSP 70 in 3T3-L1 cells by 2-dimensional gel electrophoresis showed that it was resolved into at least two major spots (Figure 2). The more basic HSP 72 (identified by an upward arrow) had no measurable basal expression; heat shock markedly increased its synthesis and this increase was at least 10 times greater in the

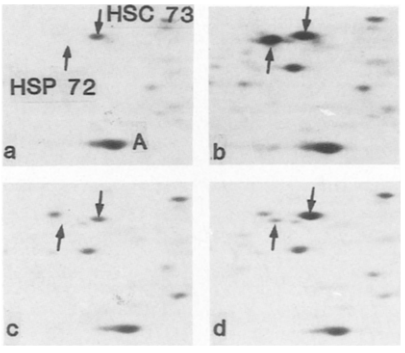

Figure 2. Two dimensional gel electrophoresis analysis of the HSP 70 protein family in 3T3-L1 fibroblasts and adipocytes. Control (37°C) and heat shocked (42°C, 6 hr) 3T3-L1 fibroblasts and adipocytes were pulse labeled with [^{35}S]Trans-label according to procedures described in the legend of Figure 1. Aliquots of the cell homogenate containing 200,000 cpm of radioactivity were subjected to 2-dimensional gel electrophoresis analysis (Liu et al., 1989a). Panels (a) and (b) represent control and heat shocked fibroblasts; panels (c) and (d) represent control and heat shocked adipocytes. The upward and downward arrows indicate, respectively, the positions of HSP 72 and HSC 73. A: actin.

fibroblasts than in the adipocytes. The more acidic HSC 73 (downward arrow), by comparison, had a basal level of expression that was not significantly different between the fibroblasts and adipocytes; heat shock increased the synthesis of HSC 73 and this increase was about 2-3 X greater in the fibroblasts than in the adipocytes.

To determine if a transcriptional mechanism may account for the difference in heat shock induction of HSPs in the 3T3-L1 fibroblasts and adipocytes, we quantitated the amount of mRNA^{hsps} by Northern blot hybridization; we also determined the heat shock gene transcription factor (HSTF) DNA-binding activity by gel retardation assay. The results of these experiments are shown in Figures 3 and 4. In the autoradiogram illustrated in Figure 3A, the membrane was simultaneously probed with pHS 801 (hsp 89α) and pHS 208 (hsp 25). We noted a low basal level of expression of the 2.95 kb mRNA of hsp 89α; heat shock at 42°C for 3 hr increased the abundance of the hsp 89α mRNA by 7.1 fold in the fibroblasts and by about 3 fold in the adipocytes. The 0.95 kb mRNA of hsp 25 had a barely detectable basal level of expression; heat shock increased the abundance of this mRNA, and the increase was substantially greater in the fibroblasts than the adipocytes. Analysis of the mRNA of hsp 89β showed that this mRNA was expressed in the control cells (Figure 3B). Heat shock had a small but reproducible effect in increasing the abundance of hsp 89β mRNA; the increase was approximately 2.5 fold in the fibroblasts and about 1.3 fold in the adipocytes. Quantitation of the mRNA of hsp 70 by hybridization to the pH 2.3 genomic DNA probe gave no evidence of a basal level of expression; heat shock significantly increased the expression of mRNA^{hsp70}, and the increase was at least 10 times greater in the fibroblasts than the adipocytes (Figure 3C). The size of the mRNA^{hsp70} in 3T3-L1 cells was determined to be 2.8 kb; this differs from the 2.6 kb size of human mRNA^{hsp70} as previously reported (Liu et al., 1989a; Wu et al., 1985).

Heat shock induction of hsps involves primarily transcriptional activation of heat shock genes. The molecular events that subserve this transcription activation has been examined; it has been shown

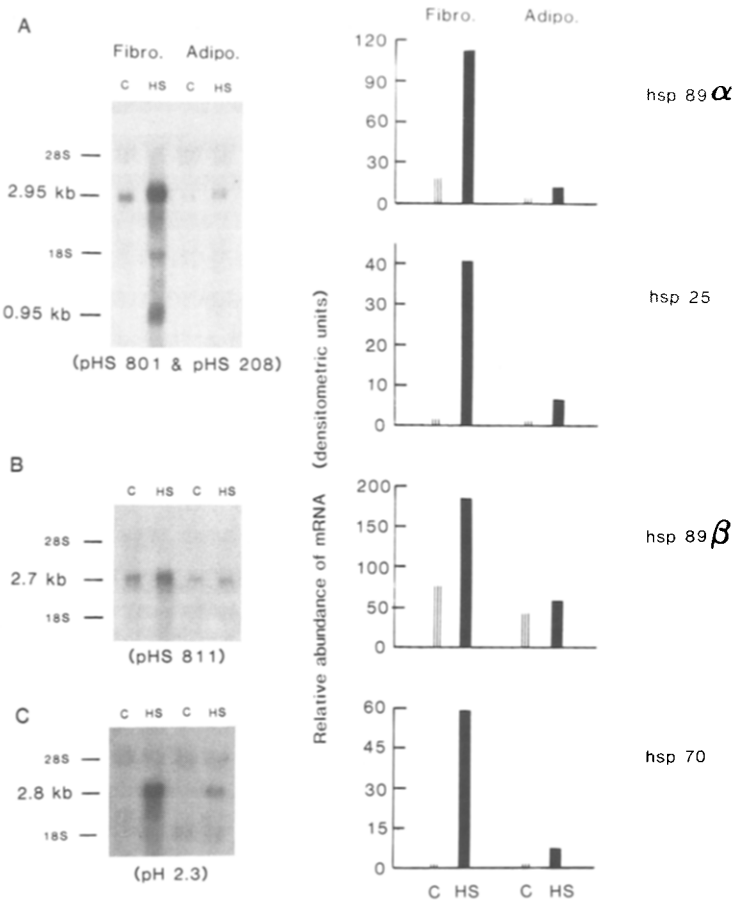

Figure 3. Northern blot analysis of mRNA of (A) hsp 89α and hsp 25, (B) hsp 89β, and (C) hsp 70 in control and heat shocked 3T3-L1 fibroblasts and adipocytes. 3T3-L1 fibroblasts and adipocytes in 100 mm dishes were heat shocked at 42°C for 3 hrs. Total RNA, isolated from 3-5 100-mm dishes, was size fractionated by agarose gel electrophoresis and then transferred onto Gene Screen Plus membrane. The positions on the autoradiograms of the 2.95 kb mRNA of hsp 89α, 0.95 kb mRNA of hsp 25, 2.7 kb mRNA of hsp 89β, and 2.8 kb mRNA of hsp 70 are indicated. Also indicated are the positions of the 18 and 28S rRNA. The relative abundance of the $mRNA^{hsps}$ are depicted in bar-graph forms on the right. Fibro., 3T3-L1 fibroblasts; Adipo., 3T3-L1 adipocytes; C, control; HS, heat shocked.

that it involves activation of heat shock gene transcription factor (HSTF) that binds to the consensus heat shock element (HSE) upstream of heat shock genes (Kingston et al., 1987; Mosser et al., 1988; Wu, 1984; Wu et al., 1987). In this context, we determined the HSE-binding activity present in extracts of control and heat shocked 3T3-L1 fibroblasts and adipocytes. Figure 4 showed that appearance of the HSE-binding activity was very dependent on heat shock of the cells; the HSTF-HSE complex appeared to consist of two closely migrating species. The HSE-binding activity in the heat shocked adipocytes was only a fraction (10-15%) of that of the fibroblasts; this decrease in HSE-binding activity was virtually complete for the faster migrating species. Specificity of the binding was confirmed by the displacement of this binding by a 200 X excess of self (HSE) DNA but not by non-self (CAP) DNA. It should be noted that the decrease in HSTF DNA-binding activity in the adipocytes was not a reflection of a general decrease in transcriptional activity. Analysis of the DNA-binding activity of ATF,

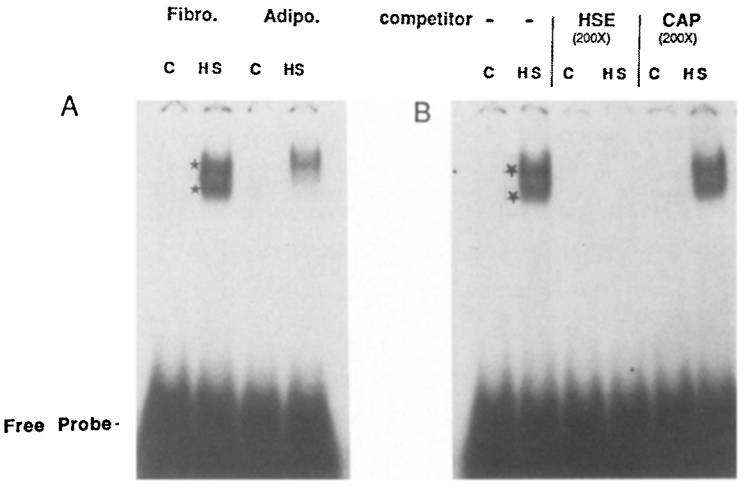

Figure 4. **A. HSTF DNA-binding activity in extracts of control and heat shocked 3T3-L1 fibroblasts and adipocytes.** Cells were heat shocked at 42°C for 1 hr. Aliquots of whole cell extracts containing 30 µg protein were used to assay for binding to the double stranded ^{32}P-labeled consensus HSE according to methods described in the text. Positions of the specific HSTF-HSE complexes are indicated by (*). The relative abundance of the HSTF-HSE complex, determined by densitometry tracing of the autoradiogram, were: 9.5 (fibroblasts, C), 383 (fibroblasts, HS), 5.7 (adipocytes, C), and 54 (adipocytes, HS). **B. Specificity of protein binding to [^{32}P]HSE.** Aliquots of 30 µg whole cell extract protein from control and heat shocked (42°C, 1 hr) 3T3-L1 fibroblasts were preincubated with a 200 X molar excess of the non-radioactive DNA at 25°C for 20 min prior to the addition of [^{32}P]HSE. The positions of the specific HSTF-HSE complexes are indicated (*).

activating transcription factor, showed little difference as a function of heat shock or cell differentiation (data not shown).

DISCUSSION

This study provide evidence of a differentiation-dependent decrease in the induction of heat shock gene expression in 3T3-L1 cells. This conclusion is based on measurements of the heat shock induction of synthesis of HSPs, the abundance of mRNA of hsps, and the DNA-binding activity of the transacting HSTF. Our result adds to a growing list of evidence that the induction of hsps may be regulated in cell aging (Liu et al., 1989a, 1898b) and differentiation (Bensaude and Morange, 1983; Hensold and Housman, 1988; Morange et al., 1984).

The decreased heat shock induction of hsps in the differentiated 3T3-L1 adipocytes appeared to be due to a decrease in the DNA-binding activity of the transactivating HSTF. There is evidence that the heat shock induced increase in HSE-binding activity is due to activation of pre-existing HSTF rather than de novo synthesis. Thus, the DNA-binding activity of HSTF could be induced, de-induced, and re-induced in the absence of protein synthesis, indicating a post-translational mechanism of regulation of HSTF (Mosser et al., 1988; Zimarino and Wu, 1987; Zimarino et al., 1990). The rapid kinetics of the induction of HSE-binding activity and the feasibility of activating this binding activity in an in vitro cell free system (Larson et al., 1988; Mosser et al., 1990) further support the notion of "activation" of HSTF. Indeed, in both yeast (Sorger and Pelham, 1988; Sorger et al., 1987) and human cells (Larson et al., 1988), heat shock induced transcriptional activation is associated with extensive

covalent modification of the HSTF protein as detected by changes in the mobility of HSTF-DNA complex in non-denaturing gel. Phosphatase sensitivity indicates that most if not all of the modification involves phosphorylation of the HSTF protein.

We do not know if and how the decreased heat shock response in the differentiated 3T3-L1 adipocytes relate to a defect/dysfunction of the activation of HSTF. Purification of the HSTF, production of antibodies against HSTF, cloning of the gene for HSTF, as well as analysis of the activation of HSTF in an in vitro system would further clarify the molecular mechanism(s) involved in the differentiation-dependent decrease of heat shock gene induction.

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