JOURNAL OF CELLULAR PHYSIOLOGY 149:560–566 (1991)

10/91

Molecular Events Involved in Transcriptional Activation of Heat Shock Genes Become Progressively Refractory to Heat Stimulation During Aging of Human Diploid Fibroblasts

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We examined the induction, by heat shock, of heat shock transcription factor (HSTF) DNA-binding and hsp 70 gene promoter activities during aging of the IMR-90 human diploid fibroblasts. Cells with population doubling level (PDL) ranging from 15–48 were heat shocked at temperatures of 39, 42, and 45°C for various time periods; the binding of HSTF to its consensus DNA was determined by gel retardation assay and the promoter activity of the human hsp 70 gene was analyzed by transient expression of reporter gene activity. We observed that the induction of HSE-binding activity was inversely related to the PDL of the cells used. Importantly, as cells progress through their life span, a higher temperature and a longer period of heat shock were needed to evoke an optimal increase in HSE-binding activity. A substantial and rapid (within 30 min) increase in HSE-binding activity was observed when PDL 20 cells were heat shocked at 39, 42, or 45°C. However, PDL 35 cells did not respond to 39°C, and PDL 48 cells responded slowly to heat shock at 45°C, but not 39 or 42°C. Experiments on the heat induced increase in hsp 70 promoter driven reporter gene expression provided similar information on the age-dependent decrease in transcriptional activation of hsps. These results were further corroborated by quantitation of the abundance of mRNA of hsp 70. Analysis of the cAMP induced expression of the rat somatostatin promoter driven CAT gene provided evidence that the decrease in transcriptional activation of hsps in aging diploid cells was not a reflection of a generalized dysfunction of signal transduction. We conclude that functional changes in the heat shock response occur before cells lose their capacity to replicate, and we suggest that these changes are likely to have a central role in the expression of the aging phenotype.

A characteristic feature of aging is a progressive impairment in the ability to adapt to environmental changes. At the organismic level, this may be manifested as increased morbidity and mortality with age when confronted with adverse situations (Driscoll, 1971; Oechsli and Bueckley, 1970; Shock, 1977). At a cellular level, this may be expressed as modification in the capacity for adaptive regulation of enzyme activities (Adelman, 1979).

Stress induces distinctive biochemical changes in living cells. One of the most profound and best studied biochemical indices of stress is the rapid induction of a class of proteins known as the heat shock proteins (HSPs) or stress proteins. This heat shock response was first described by Ritossa (1962) in *Drosophila*. It has since been shown that the response is ubiquitous and can be elicited by a variety of physiological as well as non-physiological stimuli (Lindquist, 1986; Morimoto et al., 1990).

We have been interested in studying the regulation of heat shock gene expression in cell aging and differentiation (Choi et al., 1990; Liu et al., 1989a, 1990). In our previous studies, we showed that the heat shock induction of HSPs is attenuated in aging human diploid fibroblasts. A variety of evidence suggest that this attenuation is due to a transcriptional mechanism. We proposed that there is an age-associated dysfunction in the heat shock signal transduction mechanism (Choi et al., 1990; Liu et al., 1989a,b).

In this study, we determined if there was an age-dependent difference in temperature profile or time course of the heat shock response, and if the attenuation of the response was progressive and occurred before cells lost their ability to replicate or if the phenomenon is limited to cells at the end of their replicative life span. We also evaluated if the decreased heat shock response simply reflected a generalized

Received July 1, 1991; accepted August 13, 1991.

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down regulation of signal transduction in aging cells or if it is specific to heat shock.

MATERIALS AND METHODS

Materials

The construction and use of pHBCAT, a chimera linking 2.4 kb upstream sequence of a human hsp 70 gene to the coding sequence of bacterial chloramphenicol acetyltransferase, were as previously described (Liu et al., 1989a; Wu et al., 1985). The cAMP responsive reporter gene construct, pD$_3$SSCAT, was from Dr. R. Goodman of Tufts-New England Medical Center, Boston, MA; this is a construct linking a 750bp *Hind*III restriction fragment of the cAMP-responsive rat somatostatin gene promoter to the CAT reporter gene (Montminy et al., 1986). Expression vector of the protein kinase inhibitor gene (pRSVPKI) was from Dr. R. Maurer of the University of Iowa, Iowa City, IO (Day et al., 1989). [^{14}C] chloramphenicol and Gene Screen Plus membrane were from Dupont-New England Nuclear. Calcium phosphate mammalian cell transfection kit was from 5 Prime—3 Prime, Paoli, PA.

Cell culture

IMR-90 human diploid lung fibroblasts, obtained from the Institute of Medical Research, Camden, NJ, were grown in 100 mm dishes essentially as previously described (Liu et al., 1989a). Cells were subcultured at confluence using 0.25% trypsin in Ca^{2+} and Mg^{2+}-free Earle's balanced salt solution at a 1:4, 1:8, or 1:16 split ratio. In general, cells with PDL 15–25 were considered "young," PDL 25–40 were considered "intermediate," and PDL > 45 were considered "old." In our hands, the IMR-90 cells generally have a life span of 50 + 5 PDL.

Gel retardation assay of the binding of HSTF to HSE

Confluent quiescent cultures of the IMR-90 cells at defined population doubling levels (PDLs) were used. Cells were heat shocked at temperatures ranging from 39 to 45°C for specified time periods; control cells were maintained at 37°C. Cells were harvested by scraping and then pelleted by centrifugation. The procedures for preparing whole cell extract and assaying for the HSTF DNA-binding activity by standard gel retardation method were as described previously (Choi et al., 1990). The sequence of the HSE used was: (upper strand) 5'-GCCTCGAATGTTCGCGAAGTTTCG-3'.

Transient expression of reporter gene activity

In most experiments, DNA transfection was done using trypsinized suspended adherent cells according to the protocol provided by 5 Prime—3 Prime, Inc. Typically, cells from one 150 mm plate were trypsinized, pelleted, washed once with growth medium, and resuspended in 2.5 ml of calcium phosphate/DNA precipitate mixture (20 μg of pHBCAT) and incubated at room temperature for 15 min. Growth medium was then added and cells were plated into individual 60 mm dishes. This procedure ensured that the plates of cells used in a given experiment were transfected under identical conditions and abrogated the need for using a reference gene as an internal control. In other experiments, when different combinations of DNAs were used, each plate was transfected individually with a 1 ml solution of the DNA-calcium phosphate co-precipitate and 9 ml of growth medium; pRSVβgal (Edlund et al., 1985) was co-transfected and the value for CAT activity was normalized against that of the β-galactosidase activity. After transfection, cells were incubated in growth medium at 37°C for 24–48 hr prior to heat shock. CAT and β-galactosidase activities present in a 16,000g supernatant of cell homogenates were assayed as described previously (Liu et al., 1989a).

Isolation of cellular RNA and Northern hybridization

Total RNA was isolated from 1–5 of 100 mm dishes of confluent cells according to methods previously described (Chomczynski et al., 1987). For Northern analysis, aliquots of 10–20 μg of RNA samples were size fractionated on a 1% agarose-formaldehyde gel and then transferred to Gene Screen Plus membrane. The membrane was probed with nick translated and denatured genomic DNA probe of the human hsp 70 gene, pH 2.3 (Liu et al., 1989a; Wu et al., 1985).

RESULTS

In this study, we evaluated if the age-dependent decrease in activation of heat shock transcription factor (HSTF) DNA-binding activity may be due to different requirements, either in the temperature or the duration of heat shock required to activate HSTF. In the two independent experiments illustrated in Figure 1, young and old IMR-90 cells were heat shocked at temperatures of 39, 42, and 45°C for 0.5 (Fig. 1A) or 1 hr (Fig. 1B), and cell extracts were prepared and used to assay for HSE-binding activity. In Panel A, we observed a distinct increase in HSE-binding activity when PDL 25 cells were heat shocked at 39°C for 30 min; heat shock at 42°C or 45°C for 30 min gave a comparable and maximal increase in HSE-binding activity. For PDL 45 cells, there was little or no increase in HSE-binding activity when cells were heat shocked at 39 or 42°C for 30 min; an increase in the HSE-binding activity was observed when these cells were heat shocked at 45°C, and even then, the magnitude of increase was no where near that observed in the young cells. Similar results on the difference in temperature profile for the induction of HSE-binding activity in young and old cells was observed for PDL 20 and 40 cells heat shocked for 1 hr (panel B).

In addition to requiring a higher temperature, the time course for the induction of HSE-binding activity was delayed as cells age. In Figure 2 we showed that the peak of increase in HSE-binding activity of PDL 20 cells was at 30 min (if not earlier) after heat shock at 39, 42, or 45°C; 42 and 45°C gave comparable, maximal increase in HSE-binding activity. For PDL 35 cells, the optimal induction of HSE-binding activity required heat shock at 45°C for 1 hr, and the magnitude of this increase was approximately 50% of that of the PDL 20 cells; 42°C gave a somewhat smaller increase in HSE-binding activity at 2 hr, and 39°C led to very little increase in HSE-binding activity. For PDL 48 cells, the optimal induction of HSE-binding activity was observed when cells were heat shocked at 45°C for 2 hr, and the magnitude of this increase was approximately

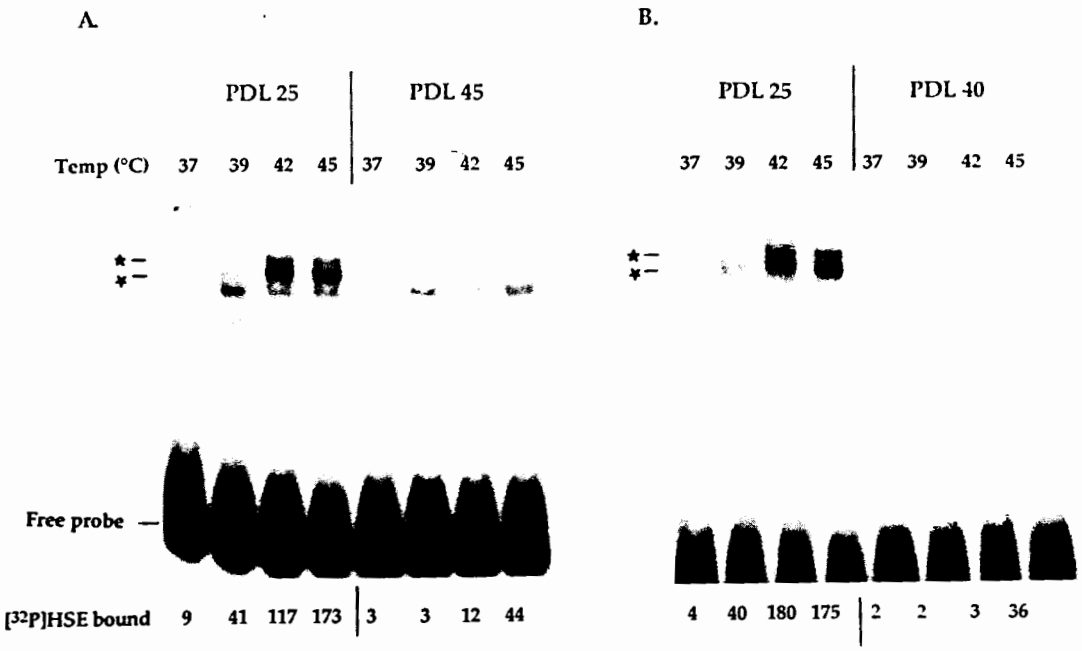

Fig. 1. Differences in the temperature profile of heat shock induction of HSE-binding activity in young and old IMR-90 cells. **Panel A:** PDL 25 and 45 cells were heat shocked at 39, 42, and 45°C for 0.5 hr. **Panel B:** PDL 20 and 40 cells were heat shocked for 1 hr. Aliquots of whole cell extract were used to assay for HSE-binding according to methods described previously (Choi et al., 1990). The positions of the specific HSTF-HSE complex are indicated by asterisks and the relative amounts of [32P]HSE bound are indicated at the bottom of the autoradiograms.

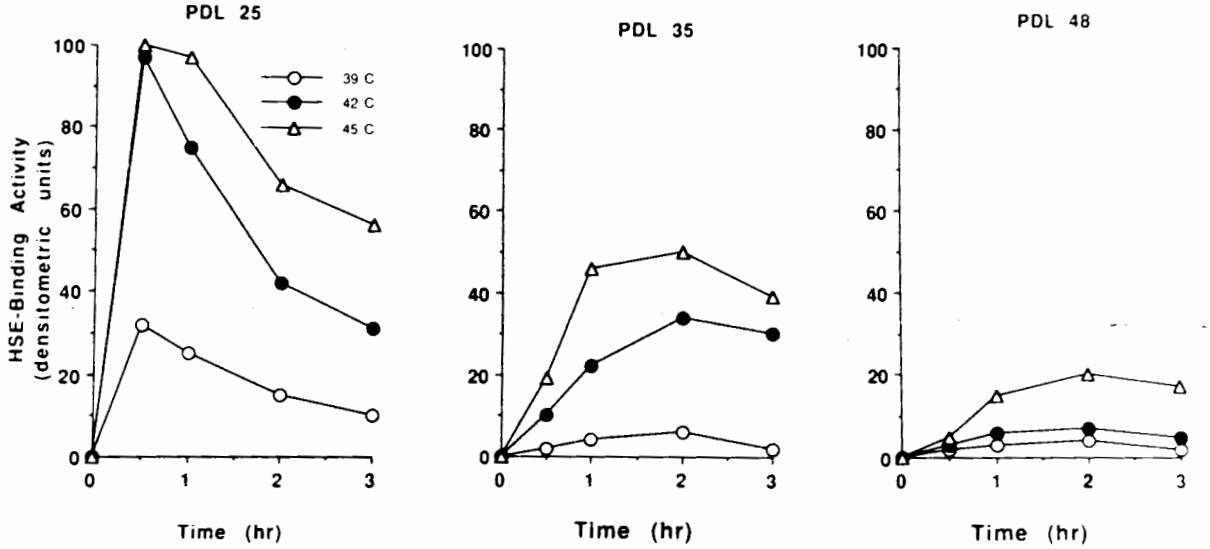

Fig. 2. Time course of induction of HSTF DNA-binding activity upon heat shock at 39, 42, and 45°C. PDL 20, 35, and 48 IMR-90 cells were heat shocked at the temperature and time as indicated; 37°C sample being the control (t = 0). Whole cell extracts were used to assay for [32P]HSE binding activity. The relative amount of [32P]HSE bound, determined by densitometric tracing of the autoradiograms, was plotted against time of heat shock at 39 (○), 42 (●), and 45°C (△).

20% of that observed in PDL 20 cells; heat shock at 39 and 42°C for up to 3 hr resulted in little or no increase in HSE-binding activity in PDL 48 cells. In other words, an early and robust response to heat shock at 39°C appeared to be a feature of the young cells, whereas a protracted and blunted response to heat shock at 45°C, but not 42 or 39°C, appeared to be characteristic of cells at the end of their life span. The ability or lack of ability to mount a heat shock response at 39°C may be of particular physiological relevance; 39°C (102°F) is within the physiological range of body temperature in humans.

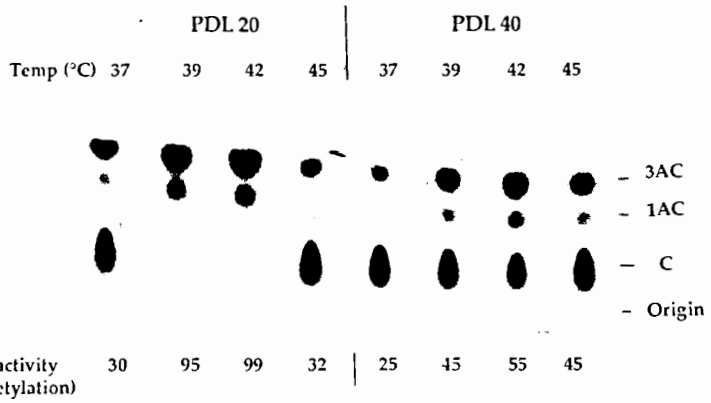

Fig. 3. Transient expression of the hsp 70 promoter driven reporter gene, pHBCAT, in young and old IMR-90 cells. PDL 20 and 40 cells were transfected with pHBCAT according to the protocol described in the text. Thirty-six hours after the transfection procedure, cells were heat shocked at temperatures of 39, 42, and 45°C for 6 hr; 37° being the control. Aliquots of cell extracts containing 50 μg protein were used to assay for CAT activity. Result is expressed as the % of [^{14}C]chloramphenicol acetylated (determined by liquid scintillation counting of appropriate portion of the TLC).

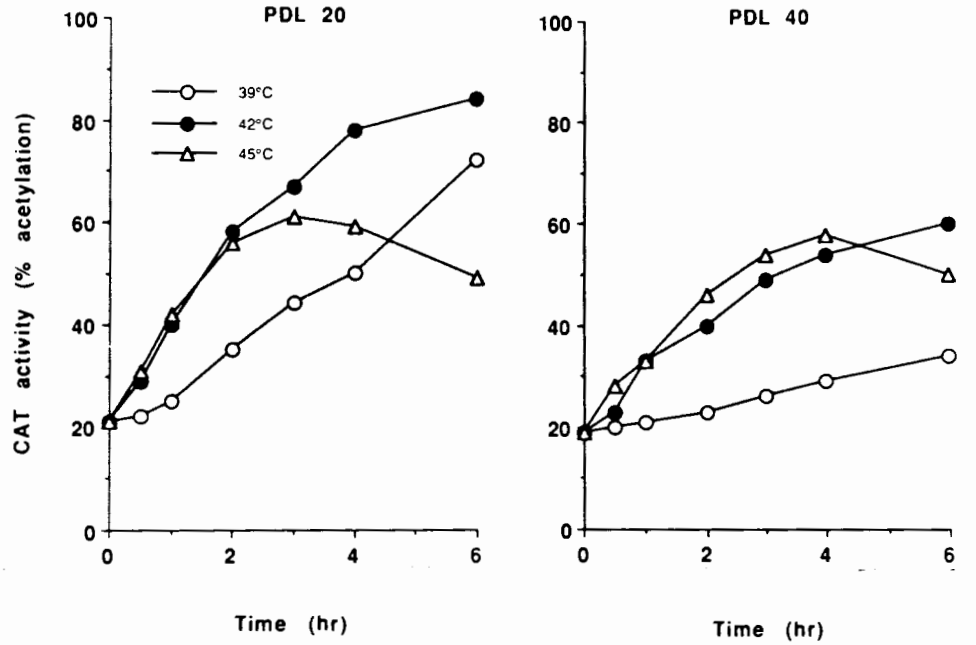

Fig. 4. Time course of heat shock induction of hsp 70 promoter driven CAT gene expression in PDL 20 and 40 IMR-90 cells. Cells were transfected with the hsp 70 promoter linked CAT gene construct, pHBCAT, according to methods described in the text. Cells were heat shocked at 39 (○), 42 (●), and 45°C (△) for time periods of 0.5, 1, 2, 3, 4, and 6 hr; 37°C being the control (t = 0). Aliquots of cell extracts containing 30 μg protein were used to assay for CAT activity. CAT activity (% acetylation) is plotted as a function of time of heat shock.

The DNA-binding activity of HSTF was manifested upon heat shock of the cells. In previous studies, it was shown that this HSE-binding activity and the hsp 70 gene transcription rate are coordinately regulated (Mosser et al., 1988). It was suggested that the control (non-heat shocked) form of HSTF represents a transcriptionally inert form that does not bind DNA and that heat shock promotes the HSTF DNA-binding and transactivating activities. Given that there was an age-dependent modulation of the magnitude, time course, and threshold of the induction of HSTF DNA-binding activity, we wondered if this might translate to differences in the regulation of the heat shock gene promoter activity. In this context, we transfected cells with pHBCAT, a chimera linking the human hsp 70 promoter to the bacterial CAT gene; CAT activity was evaluated in transient expression experiments.

The autoradiogram of a thin layer chromatogram illustrating the CAT activity of PDL 20 and 40 cells that were heat shocked at 39, 42 and 45°C for 6 hr is shown in Figure 3; the time course of induction of CAT activity at these temperatures is shown in Figure 4.

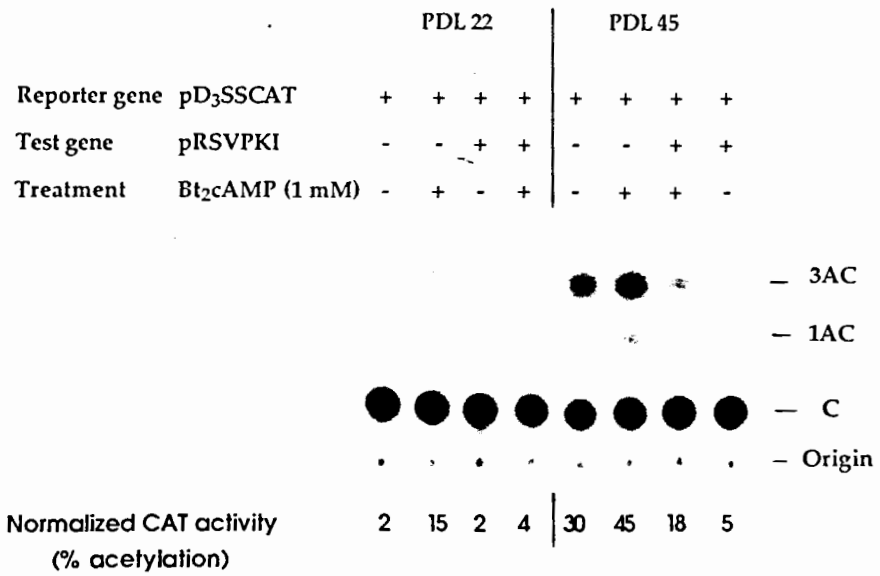

Fig. 6. Effect of dibutyryl cAMP and an expression vector of the protein kinase inhibitor (PKI) on the transient expression of pD_3SSCAT, the cAMP-responsive somatostatin promoter driven CAT gene, in young and old IMR-90 cells. Cells were transfected with pD_3SSCAT (20 μg/100 mm plate) either without or with the expression vector of PKI (pRSVPKI; 13 μg/100 mm plate); pRSVβgal (13 μg/100 mm plate) was co-transfected as a reference gene. To determine the cAMP-inducible expression of reporter gene activity, 1 mM dibutyryl cAMP was added to designated plates of cells immediately after the transfection procedure and cells were incubated in its presence for 36 hr prior to harvesting. Result on CAT activity, normalized against that of the β-galactosidase activity, is shown at the bottom of the autoradiogram.

tial. In addition, we showed that the age-dependent attenuation in response to heat shock was progressive (i.e., as cells go through their life span they become increasingly refractory to heat stimulation). In studies involving heat stress of whole animals, there was a decline in heat-induced hyperthermia and HSP 70 mRNA in aged rats (Blake et al., 1991; Fagnoli et al., 1990).

The induction of HSPs by heat shock and other noxious stimuli can be viewed as a homeostatic mechanism that confers protection on cells for survival under adverse conditions (Landry et al., 1987; Li et al., 1991). In this context, our observation of age-dependent functional changes of the heat shock response could have physiological implications. We would like to suggest that the attenuation or loss of heat shock response in cells before the cessation of division capacity produce physiological decrements in animals and that these changes are likely to have a central role in the expression of aging and to result in increased morbidity and mortality of the individual animal well before its cells fail to divide. We point out that epidemiological studies have shown that death rate from heat stroke rises sharply after age 60 (Driscoll, 1971; Oechsli et al., 1970; Shock, 1977).

We have previously suggested that there is an age-dependent deterioration or dysfunction of the signal transduction mechanism of heat shock. However, we note that not all signal transduction pathways are compromised in the aging cells. We showed that the ability of cAMP to activate the cAMP-responsive somatostatin gene promoter is greater in the old cells than that of the young cells. We have previously demonstrated that there is an increase in the type I cAMP-dependent protein kinase (PKA) during aging of IMR-90 cells (Liu et al., 1986). It seems reasonable to assume that this increased PKA activity is the basis of the increased basal and cAMP-induced expression of pD_3SSCAT in senescent cells.

The molecular mechanism of the defect or dysfunction of the heat shock signal transduction pathway is not known at this time. There are many possibilities that can account for such dysfunction including: (1) a reduced mechanism for sensing heat, (2) a change in the amount/activity of a protein/enzyme involved in the heat shock signal transduction pathway, or (3) a decrease in the abundance of HSTF or a change in the amount/activity of a factor that associates with and modulates the activity of HSTF.

Our analysis of the mechanism of age-dependent attenuation of heat shock gene expression is intimately related to our understanding of the molecular mechanism of heat induced activation of HSTF. We have previously shown that the HSTF DNA-binding activity can be modulated by a "dominant inhibitor" whose amount/activity increases upon aging of the IMR-90 cells (Choi et al., 1990). A recent study from C. Wu's laboratory on the cloning and expression of the *Drosophila* HSTF showed that while the HSTF produced in *E. coli* under nonshock conditions binds DNA and is transcriptional active, the same factor expressed in *Xenopus* oocytes requires heat shock induction for maximal expression of DNA-binding activity (Clos et al., 1990). The authors suggested that HSTF is subjected to negative regulation in higher eukaryotes, either by a change in protein conformation or by

association of HSTF with a specific inhibitor substance (Clos et al., 1990). Other studies provided evidence that heat shock induced transcriptional activation is associated with increased phosphorylation of HSTF (for a review, see Sorger, 1991).

These considerations suggest that the difference in magnitude, temperature threshold, and time of onset of the heat shock response in young versus old cells may be due to age-dependent differences in (1) post-translational modification of HSTF or (2) expression of a specific inhibitor of HSTF. The progressive (as opposed to quantal) nature of the attenuated heat shock response perhaps argues against an age-dependent difference in phosphorylation of HSTF. Rather, it seems more likely to be due to an age-dependent increase in expression of a putative inhibitor of HSTF. This possibility is being investigated in our laboratory.

ACKNOWLEDGMENTS

This work was supported in part by grants from the ACS (CD–406A), NSF and NIA (AG 03578, KYC) and the Busch Memorial Funds.

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