Attenuation of the heat shock response in HeLa cells is mediated by the release of bound heat shock transcription factor and is modulated by changes in growth and in heat shock temperatures

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When HeLa S3 cells are subjected to a continuous 42°C heat shock, activation of heat shock transcription factor (HSF) and transcriptional activation of the heat shock genes *hsp70*, *hsp89α*, and *hsp60* is transient, peaking at 40–60 min of heat shock, and then attenuating. We have used in vivo genomic footprinting to demonstrate that attenuation of *hsp70* transcription is mediated by release of bound HSF from the heat shock element (HSE) of the *hsp70* gene promoter. Release of bound HSF in vivo occurs at a higher rate than would be predicted from in vitro measurements of dissociation. Attenuation of HSF activation and heat shock gene transcription occurs only when mild heat shock temperatures are employed (42°C); increasing the heat shock temperature by 1°C elicits a much higher level of activation, which does not attenuate during a 4-hr heat shock. Surprisingly, altering the temperature at which cells are grown prior to heat shock modulates the magnitude and temporal pattern of the response to a given heat shock temperature. This finding suggests that HSF does not sense temperature directly but, instead, may be responsive to the magnitude of the difference between growth and heat shock temperatures.

[Key Words: Heat shock; HeLa cells; HSF; HSE; transcriptional control]

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The rapid induction of heat shock genes that occurs in all organisms upon exposure to elevated temperatures or other stresses has been studied intensively, both as a paradigm for gene regulation and because of its apparent importance to the survival of the organism under stressful conditions. Early studies of the heat shock response also revealed that expression of these genes decreases upon a return to pre-heat shock temperatures (recovery); moreover, attenuation of the response also occurs during prolonged exposure to elevated temperatures, especially if mild heat shock temperatures are employed (DiDomenico et al. 1982a,b; Mosser et al. 1988; Straus et al. 1990). Although this aspect of the response has received less attention than the activation component, several laboratories focusing on a diverse group of organisms have investigated the phenomenon of down-regulation, exploring the possibility that the products of the heat shock genes themselves autoregulate the response (DiDomenico et al. 1982b; Boorstein and Craig 1990; Stone and Craig 1990; Straus et al. 1990).

While an alteration in heat shock gene transcription in

response to upward and downward shifts in the ambient temperature appears to be the primary component of the heat shock response, there are fundamental differences between organisms in the mechanisms by which this transcriptional regulation is achieved. In *Escherichia coli*, transcriptional induction of heat shock genes is mediated by an alternative σ -factor, σ^{32} , that binds to the RNA core polymerase, thereby enabling the holoenzyme to recognize selectively the heat shock promoters (Grossman et al. 1984). The levels of this factor increase transiently during heat shock, as a result of increased synthesis and stability, allowing a transient burst of heat shock gene transcription (Grossman et al. 1987; Straus et al. 1987; Tilly et al. 1989). The synthesis and stability of σ^{32} appear to be regulated negatively by *E. coli* heat shock proteins, resulting in a shutoff of the response despite maintenance of the cells at an elevated temperature (Tilly et al. 1989; Straus et al. 1990).

In all of the eukaryotes examined so far, transcriptional activation of heat shock genes relies on a specific heat shock transcription factor (HSF). This factor recognizes a consensus element (the heat shock element; HSE) in the promoter of these genes (Pelham 1982; Parker and Topol 1984; Sorger and Pelham 1987; C. Wu

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et al. 1987; Goldenberg et al. 1988). A regulatory feature that distinguishes *Saccharomyces cerevisiae* from higher eukaryotes is that in this species of yeast, HSF is bound to its recognition site prior to heat shock and apparently acquires enhanced ability to stimulate transcription upon heat shock (Sorger et al. 1987; Jakobsen and Pelham 1988). In higher eukaryotes, HSF is present in a non-DNA-binding form prior to heat shock and is activated by heat shock to bind DNA (Wu 1984; Kingston et al. 1987; Sorger et al. 1987; Mosser et al. 1988; Thomas and Elgin 1988; Abravaya et al. 1991).

The mechanisms underlying the shutoff of the heat shock response in eukaryotes are not well understood. By using yeast transformed with various HSE-containing promoter- β -gal fusion constructs, it has been demonstrated that the response to a prolonged heat shock can be either transient or sustained, depending on the nature of the promoter (Sorger 1990). These two types of responses have been shown to be a consequence of two distinct transcriptional activation domains in yeast HSF (Sorger 1990). Early studies in *Drosophila* suggested that the kinetics of recovery are affected by the level of heat shock proteins at the time of temperature downshift (DiDomenico et al. 1982b).

Our laboratory has used a different approach to study the attenuation of the heat shock response in human cells. We were interested in examining the changes in HSF-HSE interactions that might account for the decline in transcription of the endogenous *hsp70* gene during the attenuation phase of the heat shock response. To that end, we have used in vivo genomic footprinting to determine that attenuation of *hsp70* transcription is correlated closely with the release of HSF from HSE. Furthermore, we show that dissociation of HSF from the HSE of the *hsp70* promoter occurs at a rate faster than predicted from in vitro measurements of dissociation. We also demonstrate that attenuation of the heat shock response is regulated by both the temperature of heat shock and, more surprisingly, by the temperature at which the cells are grown prior to heat shock.

Results

HSF levels and transcription rates of three distinct heat shock genes decline with similar kinetics during the attenuation phase of the heat shock response

When HeLa cells are exposed to a prolonged 42°C heat shock, transcriptional induction of the *hsp70* gene and activation of HSF occur only during the initial phase of heat shock and then attenuate, despite the continued exposure of the cells to the elevated temperature (Mosser et al. 1988). To examine the attenuation phase of the heat shock response in more detail and to analyze HSF-HSE interactions during this period, HeLa S3 cells were shifted from 37°C to 42°C and samples were withdrawn at selected times for transcription run-on assays, for gel-shift analysis to measure levels of activated HSF, and for in vivo genomic footprinting. As shown in Figure 1A, there was a very rapid induction of *hsp70* transcription,

with activation apparent even at 1 min following temperature upshift. After 20 min of heat shock, transcription of *hsp70* was elevated 50-fold, and transcription was maintained at this elevated level until the 60-min time point. At this point, a gradual attenuation of transcription occurred, with transcription returning to its basal level after 4 hr of continuous exposure to 42°C. The transcription rates of two other heat shock genes, *hsp89 α* and *hsp60*, were also monitored. Although neither gene was induced to the same extent as *hsp70*, their patterns of transcriptional activation, maintenance, and attenuation were identical to that of *hsp70*. The transcription of the non-heat-shock-responsive β -actin gene, which shows a transient increase in response to the pelleting of the cells prior to temperature shift (Fig. 1A, cf. lanes 1' and C), returns to basal levels by 5 min of heat shock and diminishes gradually, consistent with previous observations of the behavior of this gene during heat shock (Abravaya et al. 1991).

In parallel with the transcriptional response of the heat shock genes, levels of activated HSF rise quickly to a peak, remain at the peak level during a 40-min period, and attenuate gradually (Fig. 1B). A graphic comparison of activated HSF levels and the fold of transcriptional induction of *hsp70* reveals a virtual coincidence in the temporal patterns of these two parameters during the entire 4-hr heat shock time course (Fig. 1C). These results establish that the tight correlation between HSF levels and transcriptional induction of *hsp70*, which is seen during the activation phase of the heat shock response (Abravaya et al. 1991), is maintained during the attenuation phase as well.

Attenuation of hsp70 transcription is mediated by release of bound HSF from the HSE of the promoter

The tight correlation between HSF levels and *hsp70* transcription, coupled with the previous demonstration that during a 1-hr heat shock, the intensity of HSF-HSE interactions in vivo was closely coupled to these two parameters (Abravaya et al. 1991), raised the question as to whether attenuation of transcription was mediated by a parallel attenuation of HSF-HSE interactions in vivo. This would imply that HSF bound to the HSE in vivo could be released readily so that occupancy of the HSE would be rapidly reflective of falling HSF levels. However, an alternative possibility, in which HSF remains bound to the HSE but loses its ability to stimulate transcription, must also be considered. This possibility is not necessarily excluded by the overall drop in activated HSF levels measured by gel-shift assay of samples taken during the attenuation phase, because HSF that is bound to functional HSE in vivo may represent a small fraction of the total activated HSF population. In the second scenario, a step in which bound HSF is inactivated would have to be postulated. To distinguish between these two possibilities, a genomic footprinting analysis of HSF-HSE interactions during the attenuation phase was carried out.

The nature and organization of the elements that com-

Figure 1. Analysis of transcription rates and HSF levels during a continuous 42°C heat shock. (A) Transcription rates of *hsp70*, *hsp60*, *hsp89α* and β -actin genes in HeLa S3 cells were measured by run-on assays prior to heat shock (lane C) and at the indicated times of heat shock. (B) HSF levels were measured by gel-shift assays, by using whole-cell extracts prepared from HeLa S3 cells prior to heat shock (C) and at the indicated times of heat shock. Complexes due to nonspecific (NS) DNA-binding proteins and HSF are shown by arrows. (C) Graphic comparison of HSF levels and the transcription rate of the *hsp70* gene at the indicated times of heat shock. The filters from A and the dried gel from B were scanned and quantified with a Molecular Dynamics 400A Phosphorimager. Values were machine counts, plotted on an arbitrary scale.

prise the human *hsp70* promoter are depicted in Figure 2. In addition to consensus-binding sites for the factors CTF, CBF, Sp1, and TFIID, the promoter contains two HSEs (Morgan et al. 1987; B. Wu et al. 1987; Morgan 1989; Williams et al. 1989; Lum et al. 1990). The proximal HSE, which has been shown by transfection studies to be sufficient for heat shock induction (Wu et al. 1986; Williams and Morimoto 1990), consists of five contiguous alternating NGAAN units, three perfect (sites 1, 3, and 4) and two imperfect (sites 2 and 5) matches to the consensus sequence (Amin et al. 1988; Xiao and Lis 1988). Previous genomic footprinting analysis of this region during a 1-hr 42°C heat shock revealed that the consensus G residue in each of these units (G-94, G-97, G-104, G-107, G-114) was protected from methylation during heat shock. There was also protection of adjacent G residues at two of these sites (G-96, G-105) and hypersensitivity to methylation in three guanine residues

flanking the HSE (G-89, G-95, G-116) (Abravaya et al. 1991). This alteration in the methylation pattern can be seen in Figure 3A by comparing lanes 40' (sample taken at 40 min of heat shock) and C (sample from control, i.e., non-heat-shocked cells) in both the coding and noncoding strand methylation patterns. It can also be seen that, consistent with the analysis published previously (Abravaya et al. 1991), no factor is bound to the HSE region prior to heat shock [cf. the patterns for naked DNA (lane N) and control DNA (lane C)]. During the attenuation phase of the heat shock response (lanes 120', 180', 240'), there is a gradual weakening of the protections and hypersensitivities seen in lane 40', so that in samples taken after 4 hr of continuous heat shock, the pattern resembles that of samples from non-heat-shocked cells. The changes in the methylation pattern during the attenuation phase were quantitated by densitometric scanning of individual bands representing

Figure 2. Schematic of the human *hsp70* promoter from -20 to -200 . Consensus sequences recognized by known transcription factors are circled, and the transcription factors that bind to these sites are indicated. The sequences of the distal (-190) and proximal (-100) HSF-binding sites (HSEs) are shown, and the GAA units, comprising an array of repeated inverted units characteristic of HSEs, are underlined and numbered.

G-116, G-105, G-104, and G-94 on the coding strand and G-96, G-97, and G-107 on the noncoding strand. After 120 min of heat shock, there was a 50–70% decrease in the degree of protection from methylation of these G residues as compared to the maximal protections in the 40-min samples. This corresponds well to the 50% decrease from the maximal values in both levels of activated HSF and the rate of *hsp70* transcription that was seen at this same time point (Fig. 1C). This result supports the suggestion that attenuation of *hsp70* transcription during a prolonged 42°C heat shock is mediated by the release of bound HSF from the HSE of the promoter and that occupancy of the HSE *in vivo* is not maintained when cellular levels of activated HSF decline.

Although the intensity of bands representing protected guanine residues in samples taken at 240 min of heat shock resembled that in non-heat-shocked cells, hypersensitivities at the two guanines flanking the HSE on its proximal side (G-89, G-95) were still apparent (Fig. 3A). The persistence of these hypersensitivities, which have been postulated to result from locally increased dimethylsulfate concentrations in hydrophobic pockets created by protein–DNA interactions (Becker and Schutz 1988) and which are very susceptible indicators of HSF binding, may reflect a very low level of residual HSF binding at this time point. Alternatively, hypersensitivities have been suggested to result from changes in chromatin structure related to HSF binding (Abravaya et al. 1991), and such changes may persist despite release of the bound factor.

The release of bound HSF from the hsp70 promoter in vivo occurs at a rate higher than that predicted from in vitro measurements of dissociation

Although both HSF levels and transcriptional activation attenuate during a sustained 42°C heat shock, this attenuation occurs more rapidly if cells are returned to 37°C after a 40-min, 42°C heat shock (Figs. 4 and 5). Such a difference presumably reflects an ongoing, although constantly diminishing, signal for activation of HSF during the last 3 hr of a prolonged heat shock, whereas a return of cells to 37°C would be expected to extinguish this

signal more rapidly. During recovery at 37°C, when activation of HSF is no longer occurring or has been reduced substantially, a measurement of the rate with which protections at the NGAAN sites diminish should allow an estimate of the *in vivo* rate of dissociation of the HSF–HSE complex.

Samples withdrawn at selected times during recovery at 37°C were analyzed by genomic footprinting (Fig. 6A,B). Within 15 min of the return of cells to 37°C, protections at all five NGAAN sites were reduced markedly (60–100%), and after 1 hr at 37°C, the methylation pattern (protections and hypersensitivities) was indistinguishable from that of control cells. Thus, in agreement with the analysis of cells maintained at 42°C, the rapid decline of HSF levels is mirrored in a rapid release of bound HSF, and quantitation of the rate at which these protections diminish allows us to estimate the half-life of the HSF–HSE complex *in vivo* as 10–15 min (Fig. 7).

Because of the rapidity with which HSF is released during recovery, we wanted to determine whether dissociation of a HSF–HSE complex *in vitro* occurred equally as fast. The dissociation half-time of the HSF–HSE complex *in vitro* was determined previously in our laboratory (Mosser et al. 1988). This analysis was performed prior to our *in vivo* footprinting studies and employed an HSE oligomer that contained sites 3, 4, and 5 only (reflecting the view accepted of that time of the consensus HSE as a 14-bp sequence, CNNGAANNTTCNNG) and a dissociation temperature of 25°C. To analyze the rate of dissociation *in vitro* so that a more appropriate comparison to the *in vivo* rate could be made, we utilized an HSE oligomer that included all five NGAAN sites, a whole-cell extract from the same sample that was taken at 40 min of heat shock for use in genomic footprinting, and a dissociation temperature of 37°C. The binding reaction was carried out as for a gel-shift assay, and a preformed, radiolabeled HSF–HSE complex was incubated with a 200-fold excess of unlabeled oligomer. The decrease in the levels of radiolabeled complex with time was then determined. This determination yielded a value for the half-time of dissociation *in vitro* of 90 min, substantially longer than the 10–15 min *in vivo* half-time (Fig. 7). The discrepancy between *in vivo* and *in vitro* rates of disso-

ciation suggests that in vivo, release of bound HSF may be a facilitated event.

The magnitude and pattern of the heat shock response are modulated by temperature of heat shock and by temperature at which cells are grown prior to heat shock

The experiments presented so far describe and analyze

the attenuation phase of the heat shock response, which is seen in cells heat-shocked at 42°C. We wanted to determine whether the response attenuated regardless of the severity of the heat shock. HeLa cells were therefore subjected to a continuous heat shock at 41°C, 42°C, or 43°C. At selected times, samples were withdrawn for gel-shift analysis and for transcription run-on assays. In cells that were shifted to 43°C, levels of activated HSF were considerably higher than in cells shifted to 42°C,

Figure 4. Analysis of transcription rates and HSF levels during recovery at 37°C. As part of the same experiment shown in Fig. 1, HeLa S3 cells, which had been heat-shocked for 40 min (HS), were allowed to recover at 37°C (Recovery). (A) Transcription rates of *hsp70*, *hsp60*, *hsp89α*, and β -actin genes were measured by run-on assays after 40 min of heat shock and at the indicated times during recovery. (B) Levels of activated HSF were measured at the indicated times by gel-shift assays as described in Fig. 1. Complexes due to nonspecific (NS) DNA-binding proteins and heat shock factor (HSF) are shown by arrows.

and these high levels were maintained during the entire 4-hr heat shock (Fig. 8A). Cells maintained at 41°C for 4 hr showed barely measurable levels of activated HSF (Fig. 8A). Nuclei run-on assays of *hsp70* transcription yielded results that were consistent with HSF levels (data not shown). These data indicate that in human cells, a transient response to heat shock is evoked only at mild heat shock temperatures; a 1°C increase in the heat shock temperature elicits a much stronger response that does not attenuate during a 4-hr period of exposure.

Surprisingly, it is not only the absolute heat shock temperature that dictates the extent of HSF activation and whether an attenuation phase is observed. We found that the magnitude and temporal pattern of the response to a specific heat shock temperature could be modulated by altering the temperature at which the cells were grown prior to heat shock (Fig. 8B). When cells were grown at 35°C for several days prior to heat shock, a continuous 42°C heat shock elicited high levels of activated HSF, which were maintained during the entire 4-hr heat shock period. A transient response, typically evoked

by a 42°C heat shock of cells grown at 37°C, was observed when cells grown at 35°C were shifted to 41°C (a temperature that elicited barely detectable levels of activated HSF in cells grown at 37°C). Transcription run-on assays yielded results compatible with those of the gel-shift assays (data not shown). These data indicate that the absolute temperature of heat shock is not the sole determinant of the magnitude and duration of HSF activation. It appears instead that the response may depend on the magnitude of the difference between the growth temperature and the temperature of heat shock.

Discussion

Our results reveal several noteworthy features of the attenuation phase of the heat shock response in HeLa S3 cells. First, the decline in transcription rates of three distinct heat shock genes follows similar kinetics. Second, in the case of the *hsp70* gene, the decline in transcription is an apparent consequence of the release of bound HSF from the HSE. This release of bound HSF occurs at a higher rate in vivo than would be predicted from in vitro measurements of dissociation rates. Third, the heat shock response attenuates only when temperatures that elicit submaximal enhancement of heat shock gene transcription are employed. Finally, the heat shock temperature at which cells exhibit a multiphasic response is not an absolute value but can be modulated by the growth temperature.

The initial response of HeLa S3 cells to a 42°C heat shock is a rapid increase in the transcription rate of the *hsp70* gene (as well as two other heat shock genes), accompanied by a parallel elevation in the levels of activated HSF. However, despite continued exposure to elevated temperature, after ~40 min of heat shock a switch is flipped, reversing the process of HSF activation, and the transcription of *hsp70* declines, eventually returning to basal levels. Our in vivo footprinting analysis of HSF–HSE interactions demonstrates that transcriptional attenuation during continuous heat shock is mediated by a release of bound HSF from the HSE rather than by events that inactivate the bound factor.

We also found that release of bound HSF from the *hsp70* promoter in vivo occurs much more rapidly than does release of activated HSF from an oligomer whose sequence matches that of the proximal HSE. This suggests that release in vivo is facilitated, either by modification of bound HSF or by the participation of additional factors in the dissociation process. It is also possible that release of HSF from its endogenous binding site, which is embedded in chromatin and flanked by additional DNA sequences and associated transcription factors, occurs more readily than release of the factor from an isolated fragment of DNA in vitro. Differences in the nuclear and in vitro environments may also modulate the kinetics of release.

An attractive hypothesis is that heat shock proteins themselves may contribute to the deactivation of HSF and/or the release of bound HSF, thereby providing an autoregulatory loop. However, despite evidence for the

Figure 5. Comparison of attenuation of *hsp70* transcription and levels of activated HSF during continuous heat shock and during recovery at 37°C. (■) Samples during continuous heat shock at 42°C; (□) samples during recovery at 37°C. (A) The regions of filters hybridized to labeled *hsp70* transcripts from transcription assays shown in Figs. 1A and 3A were scanned, quantified, and plotted on an arbitrary scale. (B) Regions of the gels containing complexes as a result of HSF from gel-shift assays shown in Figs. 1B and 3B were scanned, quantified, and plotted on an arbitrary scale.

regulation of σ -32 synthesis and stability by *E. coli* heat shock proteins (Tilly 1989; Straus et al. 1990), as yet there has been no demonstration that HSF activity is regulated by heat shock proteins in eukaryotes. Two recent reports from the laboratory of E. Craig have addressed the question of autoregulation in *S. cerevisiae* (Boorstein and Craig 1990; Stone and Craig 1990). In one case, the autoregulation has been shown to be mediated through an HSE (Boorstein and Craig 1990).

The deviation of natural HSE sequences from the consensus, as exemplified by the proximal HSE of the *hsp70* promoter, may represent an essential component of the mechanism that permits the rapid release of bound HSF from the promoter and thus the rapid down-regulation of heat shock gene transcription. Xiao et al. (1991) have demonstrated recently that dissociation of purified *Drosophila* HSF from an array of five perfect NGAAN-binding units occurs extremely slowly. We have also observed that in a HeLa S3 whole-cell extract, dissociation of HSF from an oligomer comprising four perfect NGAAN repeats occurs much more slowly than from an oligomer representing the proximal HSE of the *hsp70* promoter, in which only three of five units are perfect matches to the consensus.

In addition to the HSE centered at -100, the *hsp70* promoter contains a distal HSE located at -200. This HSE consists of six NGAAN repeats, only two of which

are perfect matches to the consensus (Fig. 2). During the attenuation phase, HSF is released from the distal HSE with kinetics identical to that of release from the proximal HSE (data not shown). The HSE of the *hsp89 α* gene (whose transcription attenuates with the same kinetics as the *hsp70* gene) consists of an array of five NGAAN units, only three of which are perfect matches to the consensus, as is the case for the proximal HSE of the *hsp70* promoter (Hickey et al. 1989). Assuming that the attenuation of *hsp89 α* transcription reflects the release of bound HSF from the *hsp89 α* HSE, dissociation of HSF from three distinct HSEs occurs with very similar kinetics. Although one view of the situation in vivo is that HSEs with varying affinities for HSF are competing for the factor (Xiao et al. 1991), it is also possible that within any one species, HSEs in the various heat shock promoters have evolved to possess similar affinities for HSF, allowing a coordinate pattern of response to heat shock, at least at the transcriptional level. Although the synthesis of various heat shock proteins did not occur in a coordinate fashion in *Drosophila* (Lindquist 1980), these results may reflect variations in post-transcriptional regulation between heat shock genes rather than noncoordinate transcription or may signify a basic difference between the heat shock response in *Drosophila* and mammalian species.

An examination of the response of HeLa S3 cells to

Figure 6. Genomic footprinting of the *hsp70* proximal HSE region during recovery at 37°C. As part of the same experiment shown in Fig. 3A, HeLa S3 cells that had been heat shocked for 40 min (HS) were allowed to recover at 37°C (Recovery). (A) Methylation patterns in genomic DNA isolated from cells at 40 min of heat shock and at the indicated times during recovery. [Lane N] (naked)] Deproteinized DNA, methylated in vitro; [lane C (control)] DNA was isolated from non-heat-shocked cells. The HSE sequence, where the GAA sites are numbered as in Fig. 2., is shown adjacent to the methylation patterns for the coding (left) and noncoding (right) strand. Arrows denote guanine residues protected from methylation; stars denote guanines hypersensitive to methylation. Band intensities were determined by densitometric scanning, and guanines were judged hypersensitive or protected from methylation on the basis of normalization to neighboring bands that did not show any altered reactivity. (B) Summary of quantitations of DMS reactivity patterns of the proximal HSE region at 40 min of heat shock and at the indicated times of recovery. The larger arrows and stars denote two- to threefold differences in reactivities of guanines when compared with the corresponding guanine in DNA from non-heat-shocked cells; the smaller arrows and stars reflect less than twofold differences.

continuous heat shock at different temperatures revealed that increasing the severity of the heat shock virtually abolished the attenuation phase, at least during the 4-hr period monitored. Thus, in HeLa S3 cells, one can alter the pattern of response of heat shock genes from transient to sustained by changing the temperature of heat shock. One interpretation of this finding rests on

the assumption that heat-induced protein damage provides the signal for HSF activation. During a mild heat shock, heat shock protein-mediated repair of protein damage and/or prevention of further damage would diminish this signal, whereas during a more severe heat shock, the capacity of the cell to counter the damage would be exceeded and the signal for HSF activation

Figure 7. Comparison of in vivo and in vitro rates of dissociation of HSF from the HSE. The decrease in protection from methylation of indicated guanines during recovery from a 42°C, 40-min heat shock is denoted by the arrow labeled in vivo. (■) G-114 (1); (□) G-107 (2); (◆) G-105 (3); (◇) G-104 (3); (▲) G-97 (4); (△) G-96 (4); (▽) G-94 (5). The number in parenthesis denotes the number of the NGAAN site to which each guanine corresponds. Densitometric scanning of the autoradiogram shown in Fig. 6A was used to determine the intensities of bands corresponding to these guanines, normalizing to neighboring bands representing guanines whose reactivity to methylation is not affected by heat shock. The intensities of these bands in the 40-min heat-shocked sample were arbitrarily taken to represent maximal binding, and the intensities of these bands in the non-heat-shocked sample were taken to represent the absence of HSF binding. The rate of dissociation of the HSF–HSE complex in vitro, denoted by the arrow labeled in vitro, is also plotted. The whole-cell extract from the 40-min heat-shocked sample taken in the experiment shown in Fig. 6A was incubated with a ³²P-labeled oligomer, whose sequence matched that of HSE of the *hsp70* promoter. After a 20-min incubation at 25°C, a 200-fold excess of unlabeled oligomer was added. (Insert) The autoradiogram indicates the amount of HSF–HSE complex prior to the addition of competitor (–) and at the indicated times after the addition of competitor. The dried gel was scanned and quantitated by using a Molecular Dynamics 400A Phosphorimager, and the results were plotted, with the amount of HSF–HSE complex prior to the addition of competitor being taken to represent 100% binding.

would persist. The recent cloning of two distinct human HSF genes (Rabindran et al. 1991; Schuetz et al. 1991) raises another issue that must be considered in interpreting our data. The relative contribution of each of these factors to transcriptional stimulation during heat shock has not been determined, and it is possible that their relative contributions vary according to the severity of heat shock. In the case of yeast HSF, which is present as a single gene, separate domains that mediate a transient or a sustained response have been described (Sorger 1990). It is possible that in human cells, transient and sustained activation are mediated by two distinct proteins.

The effect of varying the growth temperature on the synthesis of *hsp70* protein over a range of heat shock temperatures was investigated in *Drosophila* >10 years ago (Lindquist 1980). In contrast with our findings, it was reported that varying the growth temperatures of *Drosophila* cells between 19°C and 26°C had little effect on the pattern of heat shock response. The present study differs from this earlier work in several important respects, any or all of which could be responsible for the divergent results. Our study examines effects of changes in growth temperature utilizing cells of a homeothermic species, whereas the former study employed cells from a poikilothermic organism. We monitored the activation/deactivation of HSF during the heat shock response, whereas in the *Drosophila* study *hsp70* synthesis was assayed. *Drosophila* cells express *hsp70* during heat shock only, whereas in HeLa S3 cells *hsp70* is constitutively expressed. We observe a dramatic alteration in the response to a given heat shock temperature resulting from a 2°C change in the growth temperature. This suggests that HSF is not sensing temperature directly; rather, it is the

magnitude of the difference between growth and heat shock temperatures that influences the extent and temporal pattern of HSF activation and deactivation. Thus, activation and deactivation are not "hard-wired" responses.

Materials and methods

Cell culture and heat shock conditions

HeLa S3 cells were grown in Joklik's medium supplemented with 5% calf serum. In experiments where effects of growth temperature on the heat shock response were examined, cells were grown in temperature-controlled ($\pm 0.1^\circ\text{C}$) water baths for 96 hr prior to heat shock. Cells were concentrated by centrifugation (600g, 5 min) and resuspended at a density of 8×10^5 cells/ml in spent medium that had been prewarmed to the temperature of heat shock. After 40 min of heat shock, an aliquot of cells was returned to 37°C to recover, whereas the remaining cells were maintained at the heat shock temperature. At selected times, 4×10^6 cells were pelleted and frozen at -80°C for preparation of whole-cell extracts, nuclei from 1.2×10^7 cells were isolated and frozen for use in transcription run-on assays, and 2×10^7 cells were used for genomic footprinting.

Measurement of transcription rates

Isolation of nuclei and transcription run-on analysis were as described previously (Mosser et al. 1988). The following plasmids were immobilized on filters and hybridized to labeled transcripts: pH 2.3 (human *hsp70*; Wu et al. 1985); pUC801, human *hsp89 α* , kindly provided by L. Weber (Hickey et al. 1989); pUCHS601, human *hsp60*, kindly provided by L. Weber (Hickey et al. 1986); and pH β A-1 (human β -actin; Gunning et al. 1983).

Figure 8. Effect of heat shock and growth temperatures on the activation and deactivation of HSF. Levels of activated HSF at the indicated times of heat shock were measured by gel-shift assay. The dried gels were scanned and quantified by using a Molecular Dynamics 400A Phosphorimager. Values are machine counts, plotted on an arbitrary scale. (A) Cells were grown for 4 days at 37°C prior to heat shock at 41°C, 42°C and 43°C. (B) Cells were grown for 4 days at 35°C prior to heat shock at 40°C, 41°C, and 42°C.

Gel mobility-shift assay

Conditions for the gel-shift assay, a description of the ^{32}P -labeled HSE oligonucleotide, and preparation of whole-cell extracts were as published previously (Mosser et al. 1988).

Genomic footprinting analyses

Genomic footprinting of samples taken during heat shock and recovery was performed as described previously (Abravaya et al. 1991) by using a ligation-mediated polymerase chain reaction (Mueller and Wold 1989). Duplicate samples were analyzed at each time point. Primers used for footprinting of the coding and noncoding strands of the proximal HSE region of the *hsp70* promoter were described previously (Abravaya et al. 1991).

Measurement of in vitro dissociation rate of HSF from HSE

To measure the stability of the HSE–HSF complex, binding reactions were incubated at 25°C for 20 min before the addition of a 200-fold molar excess of unlabeled HSE oligonucleotide. This mixture was then incubated at 37°C, and the amount of complex remaining at selected times was determined by gel electrophoresis. The HSE oligonucleotide used in this assay comprises five NGAAN sites and contains the sequences from –120 to –96 of the human *hsp70* promoter.

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