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A short element required for turning off heat shock transcription factor: evidence that phosphorylation enhances deactivation

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Transcriptional activation of heat shock genes is mediated by a presynthesized nuclear protein, the heat shock factor (HSF), which transiently converts from an inactive to an active form in response to hyperthermia. It has been suggested that hyperphosphorylation of HSF upon heat shock triggers activation through the induction of a conformational change unmasking transcriptional activator domains. Here we report that a short conserved element is involved in returning yeast HSF to the inactive state after heat shock and show that deactivation can be enhanced by phosphorylation of adjacent serine residues. These results suggest that phosphorylation of HSF in yeast serves as a regulatory mechanism to deactivate HSF, rather than being involved in its activation.
Key words: conformational change/heat shock factor/phosphorylation/transcription factors/transcriptional regulation

Introduction

When exposed to hyperthermia, cells transiently induce transcription from a characteristic set of 'heat shock' genes. Induction is mediated by a universally conserved promoter element, the heat shock element (HSE), which is the binding site for a transcriptional activator protein known as the heat shock factor, HSF (Pelham, 1982; Wu, 1985; Sorger *et al.*, 1987).

It has been shown that in yeast, insect and human cells, transcriptional induction of heat shock genes is not prevented by blocking *de novo* protein synthesis, indicating that activation occurs by modification of pre-existing HSF (Kingston *et al.*, 1987; Sorger *et al.*, 1987; Zimarino and Wu, 1987). The regulation of HSF activity, however, differs somewhat between these cell types. In higher eukaryotes, activation of HSF involves acquisition of DNA affinity by trimerization (Clos *et al.*, 1990; Westwood *et al.*, 1991; Rabindran *et al.*, 1993), but in yeast, trimeric HSF complexes are bound to HSEs at all temperatures (Sorger *et al.*, 1987; Jakobsen and Pelham, 1988, 1991; Perisic *et al.*, 1989; Sorger and Nelson, 1989; Peteranderl and Nelson, 1992). Despite these differences, it has been suggested that hyperphosphorylation upon heat shock is the modification involved in activation of HSF in yeast as well as in animal cells (Sorger *et al.*, 1987; Larson *et al.*, 1988; Sorger and Pelham, 1988).

Isolation of HSF genes from several species, and analyses of HSF mutants, have contributed to an understanding of the fundamental organization of HSF. All HSF proteins have an evolutionarily conserved core consisting of a basic DNA binding domain and a coiled-coil trimerization domain. In yeast, the core of HSF is required to restrain a potent transactivator domain in the C-terminus, the C-terminal activator, CTA (Nieto-Sotelo *et al.*, 1990; Sorger, 1990; Jakobsen and Pelham, 1991; Chen *et al.*, 1993). Furthermore, a yeast specific heptapeptide, termed CE2, is also required to restrain the activity of the CTA (Jakobsen and Pelham, 1991; for review see Sorger, 1991).

Here we report that CE2 is involved in controlling the sustained but not the transient response of *Kluyveromyces lactis* HSF to changes in temperature. A conserved stretch of serines adjacent to CE2 appears to become phosphorylated upon heat shock as their mutation abolishes a characteristic phosphorylation-dependent conformational change in HSF. The serine positions furthermore influence regulation of HSF with an impaired CE2 element in a manner which is consistent with their phosphorylation being involved in controlling deactivation. These results suggest that phosphorylation of HSF upon heat shock serves to enhance the return of activated HSF to the inactive state, rather than being involved in its activation, as previously suggested.

Results

HSF activity can be partly deregulated by mutations in CE2

To assess whether the CE2 region is involved in the HSF response to temperature changes, we introduced single alanine substitutions into CE2 of *K.lactis* HSF and assayed their effects on activity in yeast (*Saccharomyces cerevisiae*) using an HSE–*lacZ* reporter plasmid (Table I). Single substitutions of Leu453 or Leu454 deregulated HSF, producing high activity at 30°C and showing no increase upon heat shock. Dual substitution of Leu452 and Leu454 resulted in even higher constitutive activity. In contrast, substitutions of Asn456 or Arg457, neither individually nor in combination, significantly affected HSF regulation. Substitutions of Arg451 or Lys455 had intermediate effects on HSF regulation, producing an increased activity level at 30°C, but retaining an ~2-fold induction upon heat shock.

These results indicate that CE2 is involved in the mechanisms that regulate HSF activity, rather than being merely required to maintain overall structure. The most important residues for CE2 regulation appear to be the central leucines (residues 452–454), with a less stringent requirement for the two flanking basic residues (Arg451 and Lys455).

CE2 controls sustained HSF activity

To distinguish whether substitutions in CE2 affect HSF regulation by altering the activation temperature or by

Table I. Deregulatory effects of point mutations in CE2

		Activity	
		30°C	hs
	449 ↑ 468 ↑		
pKlHSF-wt	R P R L L L K N R S M S S S S S S N L N	12	110
pKlHSF-R_{451}→A	- - A L L L K N R - - - - - - - - - -	70	130
pKlHSF-L_{453}→A	- - R L A L K N R - - - - - - - - - -	290	290
pKlHSF-L_{454}→A	- - R L L A K N R - - - - - - - - - -	330	345
pKlHSF-K_{455}→A	- - R L L L A N R - - - - - - - - - -	75	140
pKlHSF-N_{456}→A	- - R L L L K A R - - - - - - - - - -	6	140
pKlHSF-R_{457}→A	- - R L L L K N A - - - - - - - - - -	7	170
pKlHSF-R_{451}/K_{455}→A	- - A L L L A N R - - - - - - - - - -	60	105
pKlHSF-L_{452}/L_{454}→A	- - R A L A K N R - - - - - - - - - -	930	980
pKlHSF-N_{456}/R_{457}→A	- - R L L L K A A - - - - - - - - - -	6	65

Mutations introduced in CE2 and their effects on KlHSF activity at 30°C and after heat shock for 45 min at 39°C. The first line shows the amino acid sequence of KlHSF between residues 449 and 466 covering the CE2 region; amino acids that are conserved between *K.lactis* and *S.cerevisiae* HSF are printed in bold. In the following lines only the heptapeptide originally defined as the CE2 and in which mutations were introduced, is shown in one-letter code; amino acid substitutions are underlined.

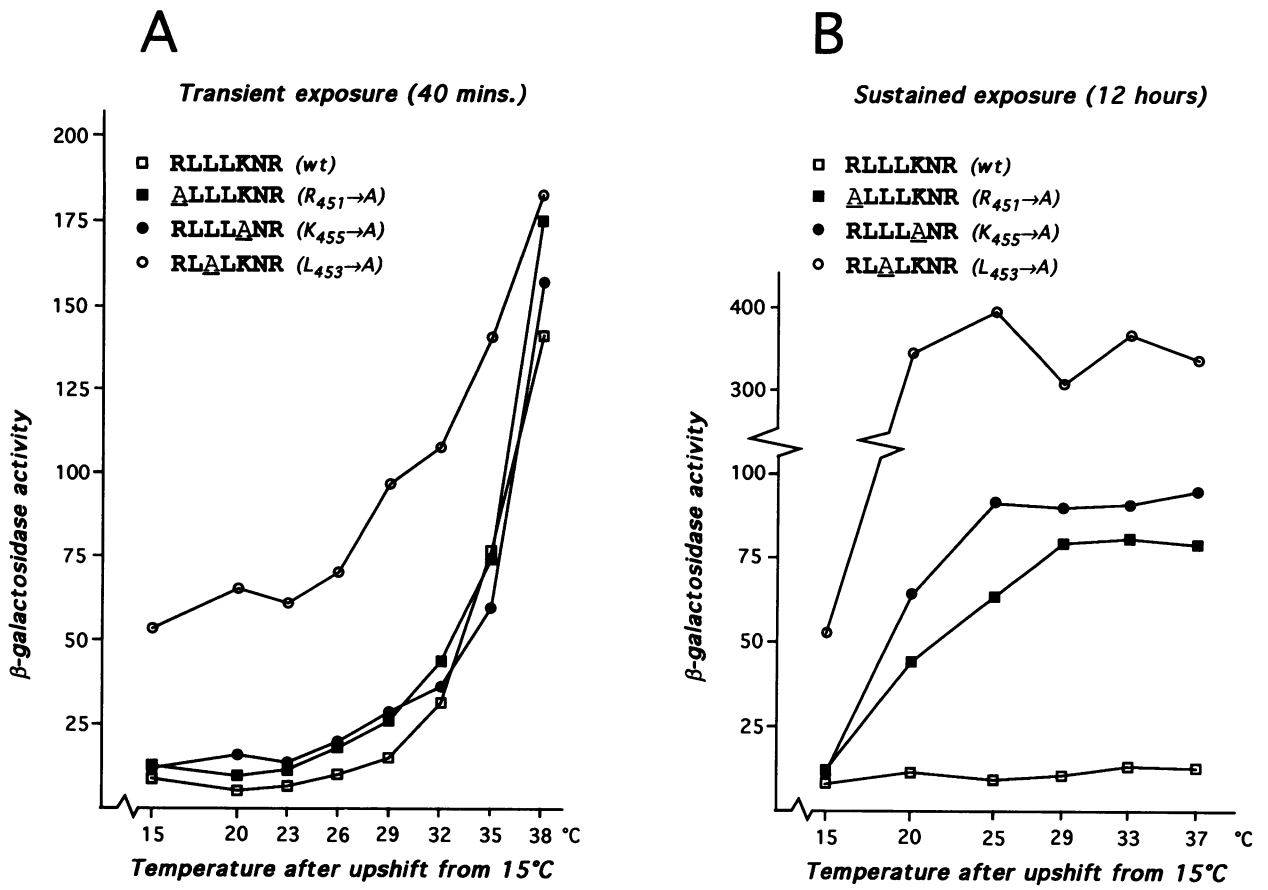

Fig. 1. Point mutations in CE2 affect sustained not transient HSF activity. (**A**) Effects of three selected mutations on HSF activity after growth at 15°C and heat shock for 45 min at the indicated temperatures. (**B**) Effects of the same mutations as in A but after growth at 15°C and upshift for 12 h to the indicated temperatures.

changing the deactivation rate, we compared the activities of wild type and three CE2-mutated HSFs after a series of transient (Figure 1A) or sustained (Figure 1B) temperature upshifts beginning at 15°C. At 15°C (lowest permissible growth temperature), mutations of Arg451 or Lys455 do not affect HSF activity, and the activity profiles after transient temperature changes are identical to that of wild type HSF (Figure 1A). When higher temperatures are maintained for 12 h, however, the activities of these mutants are clearly increased relative to wild type HSF (Figure 1B). Mutation of Leu453 has a stronger effect since constitutive activity even at 15°C is higher than with wild type HSF. When

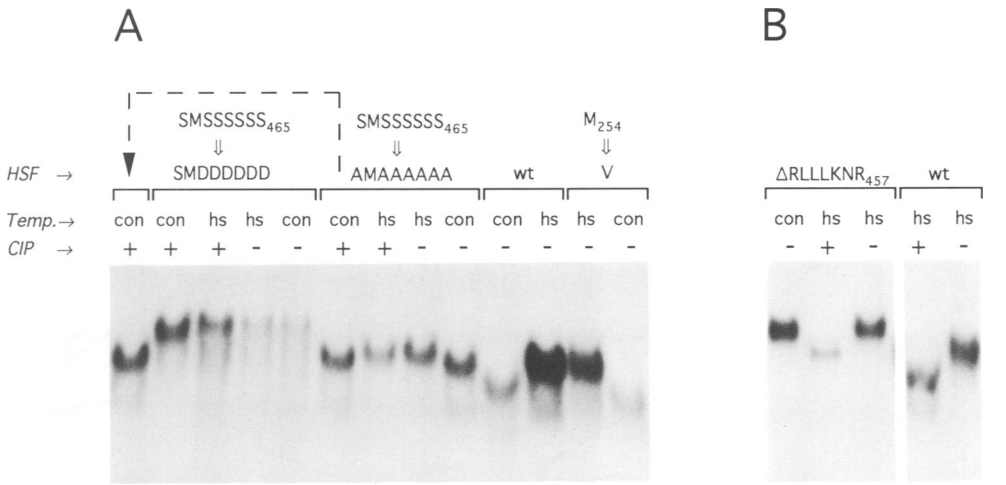

Fig. 2. The phosphorylation dependent mobility shift of HSF upon heat shock is abolished by mutation of serines adjacent to CE2 but not by a deregulating mutation in the DNA binding domain. Complexes formed between a radioactively labelled dimeric HSE probe (HSE2) and wild type or mutant KlHSF were electrophoresed in a native polyacrylamide gel (4%, 0.25 × TBE) at 250 V for ~5 h. Only the sections of the gels containing HSF–HSE complexes are shown. Extracts containing HSF were prepared from cells grown at 30°C, without (con) or with subsequent heat shock at 40°C for 20 min (hs). Extracts were either used directly (−) or after treatment for 20 min with 400 U/ml calf intestine phosphatase (+). The mutations introduced in HSF are indicated above the brackets spanning the corresponding lanes. The arrow signifies that one sample, $AMAAAAAA_{465}$/con/CIP+, was loaded in two lanes in order to facilitate comparison between the mobility of this and the $SMDDDDDD_{465}$ samples. The M_{254}→V mutation is in the conserved DNA binding domain of HSF and renders HSF highly active at all temperatures.

grown at the low temperature this mutant still responds to heat shock (Figure 1A), but when maintained for 12 h at 20°C or higher temperatures, complete deregulation with high constitutive activity is observed (Figure 1B).

Thus, the three mutations tested in CE2 altered the sustained but not the transient temperature response, indicating that they had caused irreversible activation of HSF. These observations suggest that CE2 serves a regulatory role in controlling deactivation of HSF.

Mutation of a conserved stretch of serines adjacent to CE2 affects a phosphorylation-dependent conformational change induced in HSF upon heat shock

An intriguing feature of CE2 is the presence of an adjacent stretch of serines which are conserved between yeast HSFs. Hyperphosphorylation of HSF upon heat shock mainly occurs on serine residues (Sorger, 1990), but in previous experiments no effects on transient HSF activation could be detected by mutations of the CE2 serines (Jakobsen and Pelham, 1991). However, as CE2 is involved in returning HSF from the active to the inactive form the possibility cannot be excluded that the adjacent serines play a more subtle role in regulation of deactivation. We therefore sought to establish whether phosphorylation of the CE2 serines is involved in deactivation of HSF.

Hyperphosphorylation of *S.cerevisiae* HSF upon heat shock can be detected as a mobility shift in native gel electrophoresis which is reversed when the protein is treated with phosphatase (Sorger *et al.*, 1987; Sorger, 1990). As shown in Figure 2A, wild type *K.lactis* HSF also shifts mobility when exposed to heat shock, but heat shock results in no mobility difference when the adjacent serines are mutated to aspartates. Furthermore, phosphatase treatment clearly affects mobility of wild type HSF (Figure 2B, compare CIP− and CIP+ lanes) but also has no effect when the serines are mutated to aspartates (Figure 2A). When the serines are mutated to alanines the mobility shift is significantly reduced but not completely abolished, and only a small effect is observed after phosphatase treatment (Figure 2A). Moreover, it is noteworthy that HSF with the serines substituted for aspartates migrate much more slowly than when the serines are substituted for alanines, even when both proteins have been dephosphorylated (compare the first two lanes in Figure 2A).

These experiments demonstrate that the mobility of HSF in native gel electrophoresis is greatly influenced by amino acid identity at residues 458–465, and that imitation of phosphorylation through introduction of negative charge (Ser→Asp) at these residues abolishes the mobility shift normally displayed upon heat shock. Ablation of potential phosphorylation by the Ser→Ala mutation furthermore reduces the mobility shift. These results are consistent with the hypothesis that phosphorylation of the serines adjacent to CE2 is involved in bringing about the conformational change detected in HSF upon heat shock. Thus, as it has previously been demonstrated that mutation of the serines has no observable effect on HSF activation (Jakobsen and Pelham, 1991), phosphorylation and the conformational change appear not to be required for the ability of HSF to respond to heat shock.

To verify that the phosphorylation-induced conformational change is not a prerequsite for high HSF activity we also tested the mobility of HSF containing a mutation in the DNA binding domain (Met254→Val) which was reported to deregulate a *S.cerevisiae* HSF–VP16 fusion protein (Bonner *et al.*, 1992). This mutation, although it also renders *K.lactis* HSF constitutively active at all temperatures (data not shown), does not abolish its mobility shift upon heat shock (Figure 2A). Thus phosphorylation, as detected by the mobility shift, is not required for increased HSF activity in yeast. This is consistent with observations by Sorger (1990) who found that yeast HSF remains hyperphosphorylated after termination of the transient heat shock response. In humans,

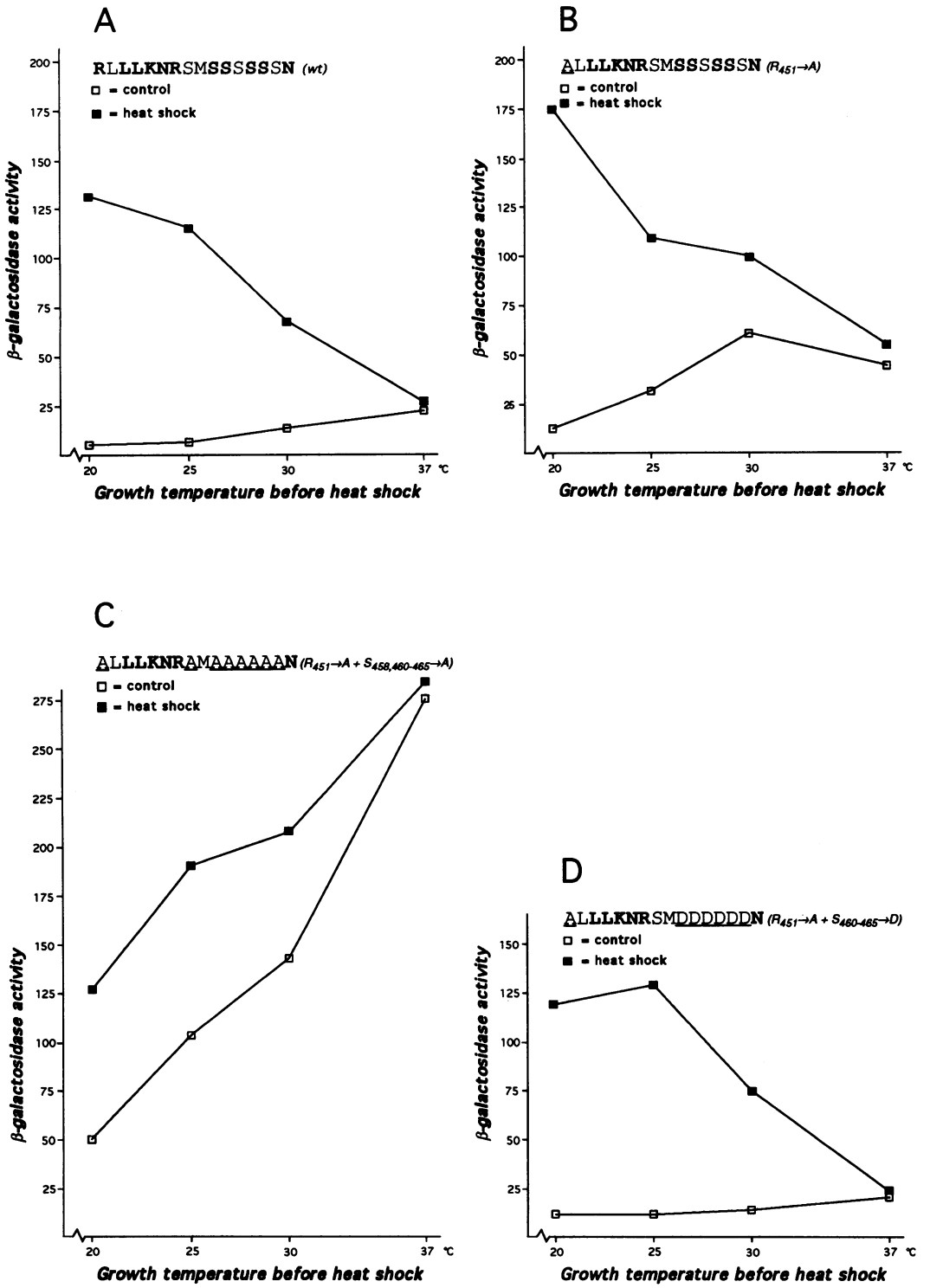

Fig. 3. Effects on transcription when combining mutation of Arg451 with mutation of the serines. β-galactosidase levels were determined after growing cultures for at least 12 h at four different temperatures (open squares), and after shifting cultures from these temperatures to 39°C for 45 min (heat shock; solid squares). Activities were determined from yeast cells expressing wild-type HSF (**A**), HSF with Arg451 changed to alanine (**B**), HSF with mutation of Arg451 to alanine combined with mutation of Ser460–465 to alanine (**C**), or HSF with mutation of Arg451 to alanine combined with mutation of serines 458 and 460–465 to aspartic acid (**D**).

observations on HSF1 suggest that activation of this factor can occur without hyperphosphorylation (Sarge *et al.*, 1993).

How could phosphorylation of the serines adjacent to CE2 be induced upon heat shock? To approach this question, we observed the mobility of HSF from which CE2, but not the serines, has been deleted. As shown in Figure 2B, little or no mobility shift is observed with this protein upon heat shock. However, a significant change is observed after phosphatase treatment. Thus, in the ΔCE2 mutant the serines adjacent to CE2 appear to be constitutively phosphorylated and, therefore, phosphorylation upon heat shock is unlikely to be the result of a temperature activated kinase. Instead

Table II. Mutation of Ser460 abolishes phosphorylation control of HSF deactivation

		con	hs
R L L L K N R S M S S S S S S	wt	12	80
A L L L K N R S M S S S S S S	R451→A	47	82
A - - - - - - P - ● ● ● ● ●	S_{1-1}	58	110
A - - - - - - A - ● ● ● ● ●	S_{1-2}	58	105
A - - - - - - ● - P ● ● ● ●	S_{1-3}	340	450
A - - - - - - ● - T ● ● ● ●	S_{1-4}	280	330
A - - - - - - ● - ● P ● ● ●	S_{1-5}	31	79
A - - - - - - ● - ● A ● ● ●	S_{1-6}	70	125
A - - - - - - ● - ● T ● ● ●	S_{1-7}	42	72
A - - - - - - ● - ● ● A ● ●	S_{1-8}	65	98
A - - - - - - ● - ● ● T ● ●	S_{1-9}	52	86
A - - - - - - ● - ● ● D ● ●	S_{1-10}	85	124
A - - - - - - ● - ● ● ● N ●	S_{1-11}	72	115
A - - - - - - ● - ● ● ● ● P	S_{1-12}	51	94
A - - - - - - ● - ● ● ● ● A	S_{1-13}	80	115
A - - - - - - ● - ● ● ● ● ● P	S_{1-14}	20	67
A - - - - - - ● - ● ● ● ● ● A	S_{1-15}	37	83
A - - - - - - ● - ● ● ● ● ● T	S_{1-16}	42	86
A - - - - - - P - ● T ● ● ●	S_{2-1}	55	105
A - - - - - - A - ● T ● ● ●	S_{2-2}	180	240
A - - - - - - ● - P ● P ● ●	S_{2-3}	13	37
A - - - - - - ● - A P ● ● ●	S_{2-4}	330	300
A - - - - - - ● - A T ● ● ●	S_{2-5}	240	265
A - - - - - - ● - ● A P ● ●	S_{2-6}	330	315
A - - - - - - ● - ● A ● T ●	S_{2-7}	140	160
A - - - - - - ● - ● C ● ● ● T	S_{2-8}	100	120
A - - - - - - ● - ● ● P P ● ●	S_{2-9}	60	150
A - - - - - - ● - P P P ● ● ●	S_{3-1}	280	280
A - - - - - - ● - A P A ● ● ●	S_{3-2}	240	335
A - - - - - - ● - ● T P ● ● T	S_{3-3}	27	67

The first line shows the amino acid sequence of CE2 and adjacent serines; in the following lines deviations from this sequence are highlighted as underlined single letter codes. Amino acids that have not been affected by mutation are shown as dashes, or in the case of serines, as dots.

it seems likely that CE2 under non-shock temperatures is involved in forming a structure which shields the serines from phosphorylation but which, after heat shock, changes conformation so that the serines become exposed. This observation also indicates that the CE2 sequence is not required for phosphorylation of the serines.

Mutations of the serines can enhance or neutralize deregulatory mutations in CE2

To determine whether the serines adjacent to CE2 serve a function in deactivation of HSF which is not observed in the presence of the normal CE2 (Jakobsen and Pelham, 1991), we combined mutation of Arg451, which has an intermediate effect on HSF regulation, with mutations of the serines. In the context of the Arg451 mutation, substitution of the serines to alanines resulted in dramatically higher activity, although a slight increase was still observed in response to heat shock (compare Figure 3B with C). By contrast, mutation of the serines to aspartates, which with respect to charge mimics phosphorylation, neutralizes the effect of the Arg451 mutation, resulting in regulation similar to that of wild type HSF (compare Figure 3D with A and B). These results strongly suggest that the phosphorylation state of the serines can influence the rate of deactivation mediated by CE2.

Ser460 is required for phosphorylation-enhanced deactivation

To establish whether any individual serine is important for enhancing deactivation of HSF, we combined substitution of Arg451 with random mutagenesis of the serines (Table II). Of the single substitutions we characterized, only those affecting Ser460, i.e. S_{1-3} and S_{1-4}, have a significant deregulatory effect, causing high constitutive HSF activity. All other positions only have deregulatory effects when two or three serines are mutated simultaneously, i.e. S_{2-2}, S_{2-4} to S_{2-8}, and S_{3-1} and S_{3-2}. In S_{2-1}, S_{2-3}, S_{2-9} and S_{3-3}, several substitutions of serines do not cause deregulation. The differences between these and some mutations that do cause deregulation are subtle (e.g. compare S_{1-3} and S_{2-3}), but an explanation for their different effects may be that proline residues introduce additional rigidity which, at certain positions or in certain combinations, can stabilize CE2 structure. In conclusion, Ser460 appears to be of primary importance for phosphorylation-enhanced deactivation of HSF, though multiple adjacent serines are also likely to be involved.

Discussion

CE2 is a regulatory element involved in turning off active HSF

Three conserved regions in yeast HSF, the DNA binding domain, the helical trimerization surface, and a short conserved element, CE2, are required to restrain transcriptional activity during normal growth temperatures. We have investigated the restraining role of CE2 by analysing the effects of point mutations in this element. In some mutants, the activity after sustained exposure to higher temperature is increased, although the initial activation upon temperature upshift was unaffected. Thus, CE2 is not involved in triggering the initial heat shock response but in mediating the sustained activity level of HSF.

This conclusion is supported by earlier observations on HSF mutants from which the strong activator domain in the C-terminus was deleted. Such mutants retain the ability to respond to heat shock, although with reduced activity supplied through an N-terminally located activator domain (Nieto-Sotelo *et al.*, 1990; Sorger, 1990; Jakobsen and Pelham, 1991). CE2, in contrast to the helical trimerization region, is not required for maintaining such deleted HSF proteins inactive during normal temperatures, or for their heat shock activation (Jakobsen and Pelham, 1991; Chen *et al.*, 1993). This shows that the ability to mediate the transient response upon heat shock is a property of the N-terminal part of HSF, and does not involve CE2.

These observations also show that CE2 only regulates activity supplied through the C-terminal activator domain. Therefore, HSF mutants from which the C-terminal activator is deleted are not capable of compensating for the loss of sustained activity by using the N-terminal activator. This explains the paradox that C-terminally deleted HSF mutants are not capable of growing at high temperature, although they possess sufficient activator potential in their N-termini to provide a normal sustained activity level (for description

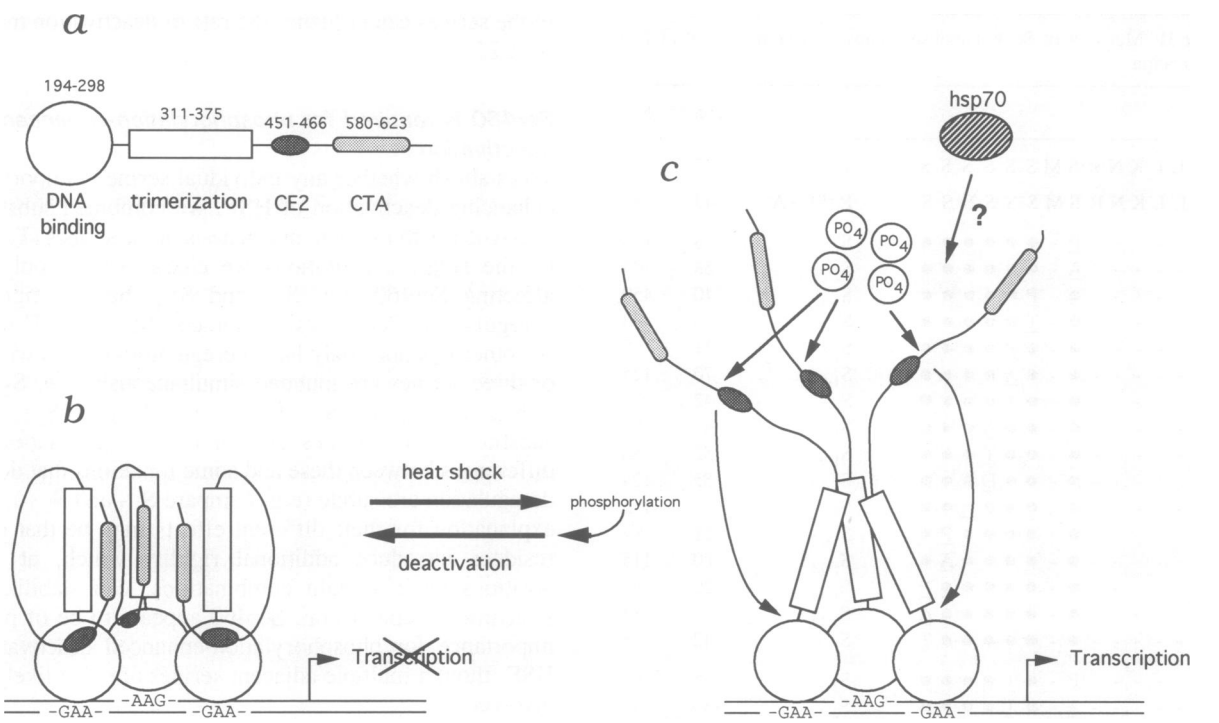

Fig. 4. Schematic model of active and inactive yeast HSF. (**a**) Linear presentation of an HSF subunit showing the symbols used in B and C to illustrate functional domains in HSF. The basic DNA binding domain is depicted as a circle, the coiled-coil trimerization surface as a rectangle, CE2 as a darkly shaded oval, and the C-terminal activator (CTA) as a lightly shaded rod. Numbers above each symbol indicate the residue numbers constituting the respective domains. The N-terminal part of KlHSF which appears to be dispensible for regulation has been omitted for clarity. (**b**) HSF in the inactive state which is predominant during normal temperatures. The CTA is masked by the conserved HSF core, consisting of the DNA binding and trimerization domains, via a contact between the core and CE2. HSF is shown constitutively bound to DNA, each DNA binding domain contacting a unit of the inverted HSE repeat (Pelham, 1982; Perisic *et al.*, 1989) (**c**) Active HSF. When exposed to heat shock a conformational change in the core, illustrated as a change of angles between the HSF subunits, disrupts core interactions with CE2 and the CTA. This unmasks the CTA but also renders the serines adjacent to CE2 accessible for phosphorylation which enhances reestablishment of the CE2−core contact possibly by presenting CE2 or another region of HSF as a substrate for interactions with hsp70 or other heat shock proteins.

of temperature sensitive mutants see Sorger, 1990; Jakobsen and Pelham, 1991; Chen *et al.*, 1993).

The inability of the N-terminus of HSF to mediate sustained activity also suggests that feedback regulation by heat shock proteins (Stone and Craig, 1990) is mediated via the C-terminal activator and thus is likely to be controlled via CE2.

Regulation by heat induced phosphorylation of serines adjacent to CE2

Previous observations of hyperphosphorylation of HSF upon heat shock, together with the finding that CE2 controls sustained rather than transient HSF activity, prompted us to reinvestigate the importance of a conserved stretch of serine residues adjacent to CE2. We have presented two lines of evidence which suggest that phosphorylation of these serines upon heat shock is involved in regulation of HSF activity.

First, we studied a phosphorylation dependent mobility shift in native gel electrophoresis which is induced in HSF upon heat shock. The conformational change which the mobility shift presumably reflects is abolished when the serines adjacent to CE2 are mutated to acidic residues. The very slow mobility of this HSF indicates that change of conformation, as mediated by amino acid identity at the serine positions adjacent to CE2, is induced optimally by the acidic substitutions (Figure 2A). In concordance with this, when the serines are replaced with alanines, the mobility shift upon heat shock is reduced (Figure 2A), suggesting that the optimal conformational change in HSF requires phosphorylation at the serines.

Secondly, we observed the effects on HSF activity of mutating the serines in the context of a CE2 mutation (Arg451→Ala) which, because it produces higher sustained activity, facilitates detection of regulatory effects on the deactivation rate. Remarkably, the change of serines to alanines or aspartates has opposite effects on activity regulation. The conservative change of serines to alanines amplifies the deregulatory effect of the Arg451→Ala mutation leading to high activity. In contrast, the mutation to aspartates, which in respect of negative charge mimics phosphorylation, completely neutralizes the deregulatory effect.

These results suggest that the negative charge which phosphorylation would introduce in the serines adjacent to CE2 would enhance reversal of HSF from the active to the inactive state. However, the charge does not impair initial heat shock activation (Figure 3D).

We have not with these investigations ruled out the possibility that phosphorylation in other regions of HSF may occur upon heat shock and serve different regulatory purposes. However, heat shock-induced phosphorylation does not appear to be required for activation as a mobility shift similar to that in wild type is detected in HSF with a point mutation that makes it highly active at all temperatures. Similar observations have been made on proteins with a

range of mutations in the C-terminal activator or in the region of the N-terminus which is dispensible for regulation (N.Barlev and B.K.Jakobsen, unpublished observations).

Taken together, these lines of evidence strongly suggest that heat shock-induced phosphorylation occurs in the serine residues adjacent to CE2 and helps to decrease sustained HSF activity. We therefore propose that heat-induced phosphorylation of the serines adjacent to CE2 is involved in inducing a change of conformation in HSF which helps to enhance the return to the inactive state.

A model for HSF regulation

How does CE2 mediate deactivation of HSF after heat shock? The structure of CE2, involving only a short stretch of residues, suggests that CE2 is unlikely by itself to form a structure that can suppress activity and respond to temperature change. More probably, CE2 is involved in forming a dynamic structure through interactions with other regions of HSF. Two observations suggest that CE2 restrains activity through interactions with the conserved core of HSF rather than with the C-terminal activator itself. Firstly, HSF activity cannot be released by mutations in the C-terminal activator (Chen *et al.*, 1993). Secondly, CE2 contributes to restrain heterologous activator domains when these are fused in the C-terminus of HSF (Bonner *et al.*, 1992; Chen *et al.*, 1993).

In the schematic model proposed in Figure 4, the activator is contained in a masked position by the structural core of HSF via a contact between this and CE2 (Figure 4b). Upon heat shock, a conformational change in the core disrupts contact with CE2 and unmasks the activator (Figure 4c). However, as the serines adjacent to CE2 become accessible and are phosphorylated, re-establishment of the core−CE2 contact is enhanced, and HSF is rapidly returned to its inactive state.

How could phosphorylation aid refolding of HSF? An attractive hypothesis would be that phosphorylation induces a conformational change in HSF that enhances contacts with heat shock proteins, thus mediating feedback regulation. Acting as chaperones, the heat shock proteins would facilitate refolding of HSF, and in particular re-establishment of the contact between the core and CE2. Interactions between human hsp70 and HSF have been observed *in vitro* (Abravaya *et al.*, 1992). Furthermore, *hsp70* genes of *S.cerevisiae* and human have been shown to feedback regulate their own transcription (Stone and Craig, 1990; Mosser *et al.*, 1993).

In conclusion our results suggest, in contrast to previous theories, that phosphorylation is not involved in transcriptional activation of HSF upon heat shock, but occurs as a consequence of activation and is involved in regulating deactivation of the molecule.

Materials and methods

Construction of HSF mutants

Mutations in the *K.lactis* HSF (KlHSF) gene were introduced by site-directed mutagenesis as described by Kunkel *et al.* (1987). To obtain random mutations of the serines adjacent to CE2 a redundant oligo with the sequence G CTA TTG TTA AAG AAC AGA XCT ATG XCG XCC XCA XCT XCG XCT AAT CTC AAT CAA AGG CAG was synthesized. In the synthesis, X, which matches the first nucleotide position of each serine codon adjacent to the CE2 region, was applied from a mixed nucleotide solution containing ~76% T and 8% each of A, G and C. From site directed mutagenesis performed with this oligo 72 plasmids were isolated that had lost a *Sac*I site constituted by codons 460−462 in the wild type KlHSF gene. By sequencing the CE2 region of these plasmids, 46 were found to contain mutations of one to three serines; of these 28 were unique (see Table II).

Expression and activity assays of HSF mutants in yeast

To ensure that authentic levels of HSF protein were obtained in yeast, all KlHSF mutants were expressed from the *S.cerevisiae* HSF (ScHSF) promoter. Mutant genes were cloned in a centromere-containing plasmid carrying the *HIS3* gene as a selectable marker (pRS313, Sikorski and Hieter, 1989) and transformed into the haploid strain BJ100, which is a derivative of strain W303 (*ade2 leu2 can1-100 his3 ura3*). This strain carries an *HSFΔ2::TRP1* disruption (Sorger and Pelham, 1988) and expresses the ScHSF gene on a *URA3*-containing vector. The ScHSF gene was removed by counter-selection with 5-fluoro-orotic acid (FOA; Boeke *et al.*, 1984) and the resultant *ura3* cells transformed with a *URA3* plasmid containing an HSE−*lacZ* reporter gene. Determination of HSF activity *in vivo* was monitored by preparation of yeast extracts and determination of β-galactosidase levels as described (Sorger and Pelham, 1988). Cells were heat shocked by shaking for 45 min in a 39°C waterbath and allowed to recover at room temperature for 45 min before extracts were prepared. Transcriptional activities were in all cases determined as average values of four to six experiments.

HSF mobility shift experiments

For mobility shift experiments, KlHSF proteins were expressed in a derivative of the protease-deficient strain BJ5462 (*ura3-52 trp1 leu2Δ1 his3Δ200 pep4::HIS3*) in which the region encoding residues 41−657 of ScHSF was replaced by a fragment encoding the *TRP1* gene, and which contained a *ura3* plasmid carrying the ScHSF gene. KlHSF plasmids carrying the *HIS3* gene were cotransformed with plasmid pRS315 which carries the selectable marker *LEU2*. Although no selection was applied for the presence of the *HIS3* gene this was stable in all transformants that were subjected to counter-selection on 5-FOA.

Mobility shift experiments were performed by incubating yeast extracts with ^{32}P-labelled HSE probe in a buffer containing 20 mM HEPES pH 8.0, 60 mM KCl, 1 mM MgCl, 0.1% NP40, 12% glycerol, 5 mM β-mercaptoethanol, 1 mg/ml poly(dI:dC) and 1 mg/ml supercoiled pUC18 plasmid. HSF−DNA complexes were electrophoresed in a 4% acrylamide, 0.08% *bis*-acrylamide gel containing 0.25 × TBE buffer at 250 V for ~5−6 h.

Cells were heat shocked in a 39°C waterbath for 20 min, whereafter extracts were immediately prepared. Phosphatase treatments were carried out as described (Sorger and Pelham, 1988).

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