

# Role of an $\alpha$ -Helical Bulge in the Yeast Heat Shock Transcription Factor

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The heat shock transcription factor (HSF) is the master transcriptional regulator of the heat shock response. The identity of a majority of the genes controlled by HSF and the circumstances under which HSF becomes induced are known, but the details of the mechanism by which HSF is able to sense and respond to heat remains an enigma. For example, it is unclear whether HSF senses the heat shock directly or requires ancillary interactions from a heat-induced signaling pathway. We present the analysis of a series of mutations in an  $\alpha$ -helical bulge in the DNA-binding domain of HSF. Deletion of residues in this bulged region increases the overall activity of the protein. Yeast containing the deletion mutant HSF are able to survive growth temperatures that are lethal to yeast containing wild-type HSF, and they are also constitutively thermotolerant. The increase in activity can be measured as an increase in both constitutive and induced transcriptional activity. The mutant proteins bind DNA more tightly than the wild-type protein does, but this is unlikely to account fully for the increase in transcriptional activity as yeast HSF is constitutively bound to its binding site *in vivo*. The stability of the mutant proteins to thermal denaturation is lower than wild-type, though their native-state structures are still well-folded. Therefore, the mutants may be structurally analogous to the heat-induced state of HSF, and suggest that the DNA-binding domain of HSF may be capable of sensing heat shock directly.

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## Introduction

All organisms have evolved systems for responding to universal stresses such as heat shock. In eukaryotes, response to heat shock is regulated at the transcriptional level by the heat shock transcription factor (HSF). HSF is responsible for directing expression of the heat shock proteins and an array of other factors required for responding to varied physiological stresses and for programmed development. Promoters for genes controlled by HSF contain upstream DNA

sequences, called heat shock elements (HSEs), which are composed of multiple, inverted, tandem repeats of the consensus sequence nGAAn, where n can be any nucleotide (Amin *et al.*, 1988; Xiao & Lis, 1988). Because of the alternating nature of the repeats, there are two possible orientations for a two repeat site: head-to-head (nGAAnTTCn) and tail-to-tail (nTTCnnGAAn). HSF binds with different affinities to HSEs depending on the number, sequence, and orientation of the repeats, with number (i.e. length) being the primary consideration for strength of HSF binding *in vitro*.

In the budding yeast *Saccharomyces cerevisiae*, HSF is required for both constitutive growth and the ability to survive stress. Yeast HSF is constitutively bound at heat shock promoters, especially at strong HSEs within the promoter. After heat shock, the occupancy of HSF increases at some of these promoters, particularly on the secondary, weaker HSE sites (Erkine *et al.*, 1999; Giardina & Lis, 1995a; Gross *et al.*, 1990; Jakobsen & Pelham, 1988, 1991; Santoro *et al.*, 1998). Some of these differences

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Abbreviations used: HSF, heat shock transcription factor; HSE, heat shock element; DBD, DNA-binding domain; TFA, trifluoroacetic acid.

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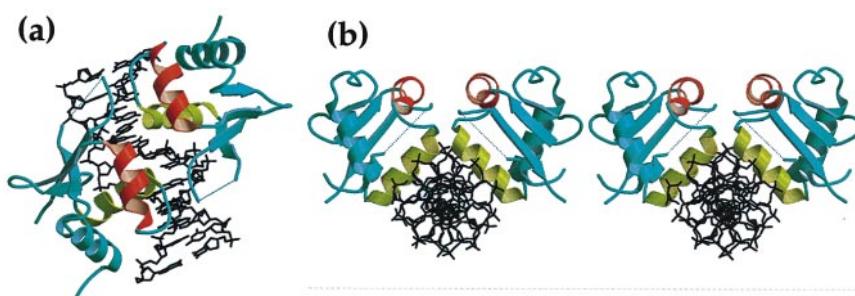
between promoters are due to the positioning of nucleosomes or other factors, but many of them are related to the number and arrangement of the nGAAn repeats within the HSE. Nevertheless, HSF appears to regulate transcription by different mechanisms on different promoters. For example, the *HSP82* promoter is constitutively bound by HSF at its primary HSE and has a low, but detectable level of constitutive transcription. After heat shock, HSF also binds to two secondary HSEs, and there is a 20-fold increase in the level of transcription (Erkine *et al.*, 1999; Giardina & Lis, 1995a). The *HSP26* promoter, on the other hand, is constitutively bound by HSF with no detectable levels of transcript prior to stress. After heat shock, there is no increase in the extent of HSF binding, but there is a greater than 50-fold increase in transcription (Chen & Pederson, 1993). These studies, along with those at other promoters, indicate that the degree of DNA binding does not correlate with transcription levels before or after heat shock. Other mechanisms, such as an increased activity of the activation domains, must be invoked to describe the heat inducibility of HSF.

The manner by which HSF's activity is induced by heat shock, or any other stress to which it can respond, is unknown. Some evidence suggests that HSF may be able to sense the heat shock directly and that induction by different stresses occurs by distinct pathways. For example, induction of yeast HSF by glucose starvation requires functional Snf1 and Snf4; however, these factors are not required for HSF induction by heat shock (Tamai *et al.*, 1994). Point mutations and deletions in any number of regions within HSF can cause an increase in the constitutive transcriptional activity (Bonner *et al.*, 1992; Hoj & Jakobsen, 1994; Jakobsen &

Pelham, 1991; Morano *et al.*, 1998; Morimoto, 1998; Morimoto & Santoro, 1998; Nieto-Sotelo *et al.*, 1990; Sorger, 1990). This suggests that much of HSF's transcriptional activity appears to be under negative regulatory control in the absence of stress.

The domain structure of HSF has been well studied. The most highly conserved region of yeast HSF is the central core, consisting of the DNA-binding and trimerization domains. The trimerization domain forms a three-stranded coiled coil and the presence of this domain is prerequisite for high affinity DNA binding (Peteranderl & Nelson, 1992; Sorger & Nelson, 1989). The crystal and solution structures of the HSF DNA-binding domain (DBD) from the milk yeast *Kluyveromyces lactis* (Damberger *et al.*, 1994, 1995; Harrison *et al.*, 1994), as well as the solution structure of the *Drosophila melanogaster* (Vuister *et al.*, 1994) and *Lycopersicon peruvianum* (Schultheiss *et al.*, 1996) DBDs all show HSF to be a member of the winged helix-turn-helix family of DNA-binding proteins. The conserved central core region is flanked by amino and carboxyl-terminal activation domains. Regulatory regions have been identified in the amino and carboxyl-terminal activation domains, as well as in the DNA-binding and trimerization domains.

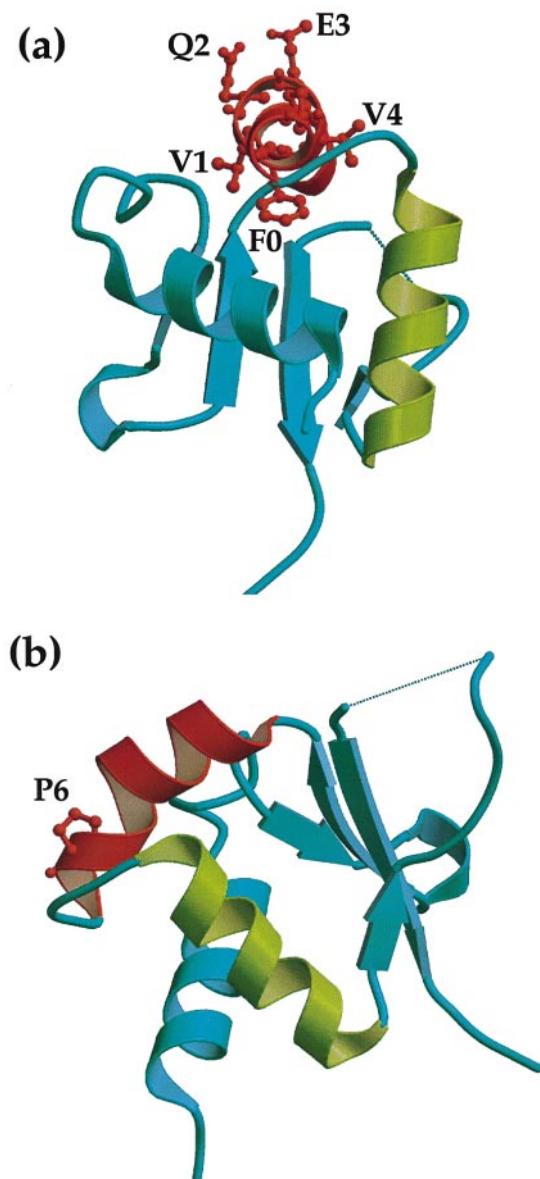
Recently, we have determined the structure of the *K. lactis* DBD in complex with a tail-to-tail two-repeat HSE (Littlefield & Nelson, 1999), and this structure, shown in Figure 1, aids in the interpretation of the role of the mutations within the DBD that affect transcriptional activity. As expected, mutations in several of the residues in the DBD of *S. cerevisiae* HSF, especially those that interact directly with the DNA, can render the yeast inviable or temperature sensitive (Hubl *et al.*, 1994). Intriguingly, several mutations increase HSF's



**Figure 1.** The crystal structure of two DBDs of *K. lactis* HSF in complex with a tail-to-tail two repeat DNA (Littlefield & Nelson, 1999). The helix-turn-helix region is colored pink for helix 2 (residues 227-238), and yellow for helix 3, the DNA recognition helix (residues 244-256). The bulk of the "wing" was disordered in the structure and is indicated by the dotted cyan line. (a) A view of the complex highlighting the arrangement of the proteins with respect to one another. The amino-terminal ends of the second helices from adjacent DBDs point toward each other at the center of the DNA, and make van der Waals contact. The carboxyl-terminal ends of helix 2 point outward and it has been suggested that they may be capable of interacting with the wing(s) from adjacent HSF molecules on longer DNA binding sites which include a head-to-head repeat (Littlefield & Nelson, 1999). (b) A stereo diagram showing a view down the DNA helical axis, highlighting the contacts made to the DNA. The majority of the HSF-DNA interactions are mediated by the DNA recognition helix, which is shown docked in the major groove of the DNA. No direct or water-mediated contacts to DNA are made by helix 2. This view provides a misleading representation of the proximity of the second helices. It is critical to note that in stereo the helix 2 from the protein on the left is forward, out of the plane of the paper, and the analogous helix from the protein on the right lies behind.

activity. Some of these mutations, such as R206S and V203A in *S. cerevisiae* HSF (residues 228 and 225 in the *K. lactis* sequence), which increase DNA binding and transcriptional activity at the *CUP1* promoter, can be explained by their presence at the interface between adjacent DBDs at the amino terminus of helix two (Sewell *et al.*, 1995; Silar *et al.*, 1991; Yang *et al.*, 1991). Others, such as the M232V mutation (residue 254 in *K. lactis*), which has a constitutively activating phenotype (Bonner *et al.*, 1992), or the P214Q mutation (residue 237 in *K. lactis*), which has reduced activity (Halladay & Craig, 1995), are more difficult to explain based on the co-crystal structure. These and other studies have suggested that the DBD might play an important regulatory role, in addition to its obvious role in mediating DNA binding (Bonner *et al.*, 1992; Nieto-Sotelo *et al.*, 1990; Sorger, 1991).

One of the most notable features of the DBD, which has been observed in all of the structures, is an unusual geometry in the first helix of the helix-turn-helix motif (Damberger *et al.*, 1994, 1995; Harrison *et al.*, 1994; Littlefield & Nelson, 1999; Schultheiss *et al.*, 1996; Vuister *et al.*, 1994). This helix, the second helix of the DBD, contains both an  $\alpha$ -helical bulge and a proline-centered kink (Figure 2), and does not contact DNA (Figure 1).  $\alpha$ -Helical bulges are characterized by the presence of five amino acid residues in a single turn of the helix, whereas canonical  $\alpha$ -helical turns contain only four. Although  $\alpha$ -helical bulges are infrequently observed, the  $\alpha$ -helical bulges found in mercuric ion reductase and iron-free ribonucleotide reductase both appear to be important for activity (Aberg *et al.*, 1993; Schiering *et al.*, 1991).  $\alpha$ -Helical bulges can also be generated synthetically. The insertion of one, two, or three extra amino acid



**Figure 2.** The crystal structure of the DBD of *K. lactis* HSF in two orientations (Harrison *et al.*, 1994). The bulged-kinked helix 2 is colored pink, and the DNA recognition helix is yellow. The dotted cyan line connects two ends of a loop that was disordered and unobserved in the crystal structure. Residues are labeled by their one-letter abbreviation and numbered according to their position in the bulge. (a) The side-chains of residues in the bulged turn of helix 2 are represented in ball and stick form. (b) The proline side-chain at the center of the 29° kink is also represented in ball and stick form.

residues into the longest  $\alpha$ -helix in lysozyme (Heinz *et al.*, 1993, 1994; Vetter *et al.*, 1996) or staphylococcal nuclease (Keefe *et al.*, 1993) resulted in either an  $\alpha$ -helical bulge or a shift in the register of the helix. The bulge-inducing insertions had moderate effects on activity, but were invariably accompanied by a decrease in the thermal stability of the resultant protein (Heinz *et al.*, 1993, 1994). Insertion of an extra glutamate into the conserved 3<sub>10</sub> helix in hemoglobin Catonsville, also results in the formation of a bulge that is associated with the disease state (Kavanaugh *et al.*, 1993). Proline-centered kinks are found in 3-4 % of helices (Barlow & Thornton, 1988), while the combination of an  $\alpha$ -helical bulge and kink, as observed in HSF's DBD, has been reported in only one other protein, GreA, the transcript cleavage factor (Stebbins *et al.*, 1995). When one amino acid residue in the  $\alpha$ -helical bulge in the coiled-coil region of GreA was deleted, the protein was still capable of dimerization mediated by the coiled coil. Although the dimerization constant was actually higher for the deletion mutant, the mutant GreA was no longer competent for transcript cleavage (S. Darst, personal communication).

The presence of a bulged-kinked helix in the DBD of all eukaryotic HSFs suggests that, as observed for GreA, this helix is likely to play an important and conserved role in the function and/or structure of HSF. In an attempt to elucidate the function of this region of HSF, while concomitantly exploring the structural basis of this bulged-kinked helix, we are studying the activity of mutant HSFs. Here we report the characterization of a series of deletion mutations within the bulge of yeast HSF's DBD. The mutations cause an increase in overall activity of the full-length protein, although the mutant domains are destabilized relative to the wild-type DBD. Our studies suggest both a functional requirement for the conserved unusual helical geometry and an insight into the mechanism by which HSF senses heat shock and is induced to a transcriptionally active state.

## Results and Discussion

### Rationale for the mutational design

The bulge and proline-centered kink have been observed in the crystal structure of *K. lactis* HSF DBD (Harrison *et al.*, 1994) and in the solution structures of the HSF DBD from *K. lactis* (Damberger *et al.*, 1994, 1995), *D. melanogaster* (Vuister *et al.*, 1994), and *L. peruvianum* (Schultheiss *et al.*, 1996). A sequence alignment for helix 2, the region of the DBD spanning the helical bulge for the 25 cloned HSFs, is shown in Figure 3(a). This alignment predicts that the bulge and the adjacent proline-centered kink are present in all known HSFs. The high level of sequence conservation in the DBD of HSF suggests a conserved function may be associated with the bulged-kinked helix.

Species	Position in Helix								
	0	1	2	3	4	5	6	7	8
<i>K. lactis</i>	<b>F</b>	V	Q	E	<b>V</b>	<b>L</b>	<b>P</b>	K	Y
<i>S. cerevisiae</i>	<b>F</b>	V	H	Q	I	<b>L</b>	<b>P</b>	K	Y
<i>S. pombe</i>	<b>F</b>	A	K	L	<b>V</b>	<b>L</b>	<b>P</b>	K	Y
<i>H. sapien</i> 1	<b>F</b>	A	K	E	<b>V</b>	<b>L</b>	<b>P</b>	K	Y
<i>H. sapien</i> 2	<b>F</b>	A	K	E	I	<b>L</b>	<b>P</b>	K	Y
<i>H. sapien</i> 4	<b>F</b>	A	K	E	<b>V</b>	<b>L</b>	<b>P</b>	Q	Y
<i>M. musculus</i> 1	<b>F</b>	A	K	E	<b>V</b>	<b>L</b>	<b>P</b>	K	Y
<i>M. musculus</i> 2	<b>F</b>	A	K	E	<b>V</b>	<b>L</b>	<b>P</b>	K	Y
<i>G. domesticus</i> 1	<b>F</b>	A	K	E	<b>V</b>	<b>L</b>	<b>P</b>	K	Y
<i>G. domesticus</i> 2	<b>F</b>	A	K	E	I	<b>L</b>	<b>P</b>	K	Y
<i>G. domesticus</i> 3	<b>F</b>	A	K	E	L	<b>L</b>	<b>P</b>	K	Y
<i>D. melanogaster</i>	<b>F</b>	A	K	E	<b>V</b>	<b>L</b>	<b>P</b>	K	Y
<i>X. laevis</i>	<b>F</b>	A	K	E	<b>V</b>	<b>L</b>	<b>P</b>	Q	Y
<i>A. thaliana</i> 1	<b>F</b>	S	R	D	L	<b>L</b>	<b>P</b>	K	Y
<i>A. thaliana</i> 4	<b>F</b>	A	K	D	L	<b>L</b>	<b>P</b>	R	F
<i>L. peruvianum</i> 8	<b>F</b>	A	K	D	L	<b>L</b>	<b>P</b>	K	Y
<i>L. peruvianum</i> 24	<b>F</b>	A	K	D	L	<b>L</b>	<b>P</b>	R	Y
<i>L. peruvianum</i> 30	<b>F</b>	S	T	T	L	<b>L</b>	<b>P</b>	K	F
<i>G. max</i> 5	<b>F</b>	A	R	D	L	<b>L</b>	<b>P</b>	N	Y
<i>G. max</i> 21	<b>F</b>	S	A	D	I	<b>L</b>	<b>P</b>	R	Y
<i>G. max</i> 29	<b>F</b>	A	R	D	L	<b>L</b>	<b>P</b>	K	Y
<i>G. max</i> 31	<b>F</b>	A	R	N	L	<b>L</b>	<b>P</b>	N	Y
<i>G. max</i> 33	<b>F</b>	A	K	D	L	<b>L</b>	<b>P</b>	K	Y
<i>G. max</i> 34	<b>F</b>	A	K	D	L	<b>L</b>	<b>P</b>	K	Y
<i>S. mansoni</i> 1	<b>F</b>	A	K	E	L	<b>L</b>	<b>P</b>	L	Y

b)

Mutant Abbreviation	<i>K. lactis</i> mutation	<i>S. cerevisiae</i> mutation
$\Delta V1$	$\Delta V232$	$\Delta V210$
$\Delta Q2$	$\Delta Q233$	$\Delta H211$
$\Delta E3$	$\Delta E234$	$\Delta Q212$
$\Delta V4$	$\Delta V235$	$\Delta I213$

**Figure 3.** (a) Sequence alignment for helix 2 of the DBDs from the 25 cloned HSFs. Numbering is relative to the bulge and kink as defined in Materials and Methods. Residues 0-5 comprise the bulged turn of the helix, while the conserved proline at residue 6 is at the center of the conserved kink. Invariant residues are shaded gray, and residues identical to those in the *K. lactis* sequence are in bold. Listed sequences are *K. lactis* (Jakobsen & Pelham, 1991), *S. cerevisiae* (Wiederrecht *et al.*, 1988), *S. pombe* (Gallo *et al.*, 1993), *Homo sapien* 1 (Rabindran *et al.*, 1991), *H. sapien* 2 (Schuetz *et al.*, 1991), *H. sapien* 4 (Nakai *et al.*, 1997), *Mus musculus* 1 and 2 (Sarge *et al.*, 1991), *G. domesticus* 1, 2 and 3 (Nakai & Morimoto, 1993), *D. melanogaster* (Clos *et al.*, 1990), *Xenopus laevis* (Stump *et al.*, 1995), *A. thaliana* 1 (Hubel & Schoffl, 1994), *A. thaliana* 4 (Prandl *et al.*, 1998), *L. peruvianum* (Scharf *et al.*, 1990), *G. max* 5, 21, 29, 31, 33, and 34 (Czarnecka *et al.*, 1995), and *Schistosoma mansoni* 1 (Lantner *et al.*, 1998). (b) Nomenclature of mutations made for this study. Mutations of residues at positions within the bulge are numbered as in (a) and are listed with their residue number for both the *K. lactis* and *S. cerevisiae* HSF sequences.

Evaluation of the sequence and associated structure of the bulged-kinked helix in HSF's DBD has provided a rational basis for mutations that could be made to probe the structure and function of this helix. The bulge is characterized by an invariant phenylalanine residue at the zero position and an invariant leucine at the fifth position. Both the phenylalanine and the leucine are completely buried in the core of the crystal structure of *K. lactis* HSF. The invariance of the residues at these two

positions, as well as the consistent spacing of four residues between these invariant large hydrophobic residues, seems to be the required motif for the formation of this bulge. The first position in the bulge is usually occupied by a small hydrophobe, which is followed by two charged or polar residues at the second and third positions in the bulge. In the crystal structure of HSF's DBD, the valine at the first position is 37% solvent exposed, while the glutamine and glutamate at the second and third positions are completely solvent exposed. The identity of the residues on the exposed face of the bulge are not conserved, suggesting that spacing and the presence of hydrophilic residues are the important factors at positions two and three. The fourth residue is typically a  $\beta$ -branched amino acid residue or leucine. In the *K. lactis* structure, the fourth position is occupied by a valine that is completely protected from the solvent. Immediately following the bulge is the invariant proline at the sixth position, which is at the center of the observed kink. The proline-centered kink is undoubtedly an important component of the unusual structure observed in this region. The mutations we describe are aimed to redesign the conserved  $\alpha$ -helical bulge while maintaining the kink.

The design of our mutations was also strongly influenced by previous studies on the effect of insertion of residues into  $\alpha$ -helices. An  $\alpha$ -helix can accommodate an extra amino acid residue by formation of an  $\alpha$ -helical bulge, so we reasoned that by analogy an  $\alpha$ -helical bulge might be removed by deletion of one of the residues contained within the bulge. Typical  $\alpha$ -helices have hydrogen bonds between the  $i$  and  $i + 4$  residues. The residues in the bulge exhibit  $i$  to  $i + 5$  hydrogen bonding, making it possible to delete any one of the solvent-exposed residues in the helix without compromising the overall helicity of that region, yet potentially completely removing the bulge. With this design principle in mind, we generated a series of mutant HSFs by sequentially deleting each of the residues at positions one through four in the  $\alpha$ -helical bulge of the DBD. We designated these deletion mutants  $\Delta V1$ ,  $\Delta Q2$ ,  $\Delta E3$ ,  $\Delta V4$  (see Figures 2(a) and 3(b)). The phenylalanine at position 0 and leucine at position 5 were not deleted because their invariant presence and fully buried nature suggests that they are essential to the proper packing of this helix against the remainder of the hydrophobic core.

We constructed mutant HSFs in three different contexts in order to best take advantage of the strengths of the *K. lactis* and *S. cerevisiae* HSFs. The HSFs from *S. cerevisiae* and *K. lactis* are highly homologous throughout the conserved central core. The *K. lactis* HSF is even able to functionally substitute for the *S. cerevisiae* HSF *in vivo*, although the regulation is not entirely the same (Jakobsen & Pelham, 1991). Therefore, we analyzed the *in vivo* function of HSF in *S. cerevisiae*, with mutations introduced into the full length *S. cerevisiae* HSF

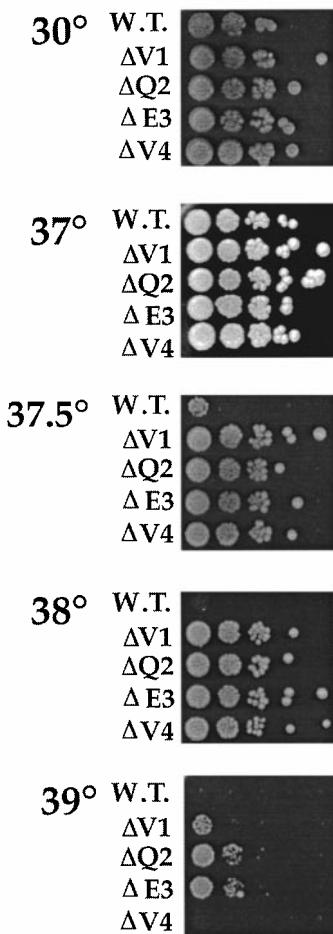
gene. Our previous structural studies on yeast HSF have focused on HSF from the *K. lactis* because of its amenable solubility and overall suitability for structural characterization. Therefore, we used a fragment of *K. lactis* HSF extending from the beginning of the DNA-binding domain through the trimerization domain (amino acid residues 192-394) for the DNA-binding studies of mutant HSFs. For all other biophysical analyses, the mutations were introduced to the isolated DBD (amino acid residues 193-282) from *K. lactis* HSF. The nomenclature of the mutations is shown in Figure 3(b).

### Temperature-dependent growth of yeast containing mutant HSFs

HSF is an essential gene in yeast (Nieto-Sotelo *et al.*, 1990; Sorger, 1990), so it is possible to measure the effect of mutations in the gene on both viability and growth. Some point mutations in the DBD of HSF are lethal, some render yeast temperature sensitive, and other mutations have no negative effect on growth. When conserved residues involved in contacts to DNA are mutated, lethality or temperature sensitivity is often observed (Hubl *et al.*, 1994; Littlefield & Nelson, 1999). The structural conservation of the bulged helix led us to ask whether mutations in the bulge would have a similar effect on viability.

Wild-type and mutant versions of full-length *S. cerevisiae* HSF, under the control of the wild-type promoter on a low copy CEN/ARS plasmid, were expressed as the only HSF in a tester strain that has been deleted for the wild-type chromosomal copy of HSF. As shown in Figure 4, wild-type and all of the deletion mutants are capable of supporting growth at both 30 and 37°C. At temperatures greater than 37°C, yeast containing wild-type HSF are incapable of growth at any dilution. On the other hand, the  $\Delta V1$ ,  $\Delta Q2$ ,  $\Delta E3$ , or  $\Delta V4$  mutant versions of HSF are able to support growth at temperatures up to 38°C, and  $\Delta V1$ ,  $\Delta Q2$ ,  $\Delta E3$  are capable of growth at 39°C. None of the strains are capable of growth at 40 or 41°C (data not shown). Western blots were performed on yeast strains containing mutant or wild-type versions of HSF to ensure that the differences in growth were due to inherent differences in the function of the protein rather than differences in the levels of the  $\Delta V1$ ,  $\Delta Q2$ ,  $\Delta E3$ , or  $\Delta V4$  proteins present in the cell relative to wild-type HSF. This analysis indicates that the plasmid-expressed mutant and wild-type HSFs are present at comparable levels (data not shown). Furthermore, the increase in viability that we observe appears to be dependent on deletion of the residues in the bulge. Mutation of V1, Q2 or E3 to alanine does not increase the viability of cells at higher temperatures relative to cells with wild-type HSF (data not shown).

One possible explanation for the ability of the mutant HSFs to grow at higher temperatures than wild-type HSF is that expression of HSF from the CEN/ARS plasmid results in a higher level of HSF



**Figure 4.** Growth of yeast strains at various temperatures. Yeast strains containing wild-type HSF or  $\Delta V1$ ,  $\Delta Q2$ ,  $\Delta E3$ , or  $\Delta V4$  as the only source of HSF were grown at 30°C to mid-log phase. These cells were then serially diluted into sterile media (tenfold dilution at each step), and spotted onto agar plates to measure growth at 30, 37, 37.5, 38, or 39°C after three days.

expression than from the chromosome, and this might be deleterious to the cell. If that were the case, then the ability of yeast containing  $\Delta V1$ ,  $\Delta Q2$ ,  $\Delta E3$ , or  $\Delta V4$  to survive at higher temperatures might indicate that mutant HSFs have a diminished function that relieves the over-expression induced temperature sensitivity. To test this possibility, the yeast strain W303, which is the parent of the tester strain and contains an intact chromosomal copy of wild-type HSF, was transformed with a plasmid containing wild-type HSF or the empty vector. The strain W303 is normally capable of growth at temperatures up to 39°C. The presence of the empty vector or the HSF-containing plasmid allowed identical growth at both 37 and 39°C under selective conditions, indicating that the presence of the plasmid copy of HSF is not sufficient to induce temperature sensitivity (data not shown). The presence of a deletion in the bulge of the DBD of HSF expressed as the only HSF in

yeast reverses the inability of the tester strain to survive at 39°C, suggesting that the deletion mutations have genuinely increased HSFs activity.

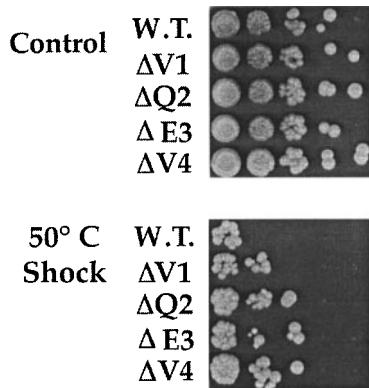
### Thermotolerance of mutant HSFs

HSF responds to heat by becoming induced and subsequently directing the increased expression of heat shock proteins and other genes involved in the stress response. Pretreatment of cells with mild doses of heat or other stimulators of the stress response induces the production of an array of heat shock proteins that provide a protective effect against a subsequent severe heat shock. Specifically, yeast can be induced to thermotolerance by a mild heat shock, usually at 37°C for one hour, which enables them to survive a lethal 50°C heat shock better than uninduced cells (Nicolet & Craig, 1991). Yeast in which this protective mechanism has been induced are termed "thermotolerant." Yeast with mutant HSFs can also become constitutively thermotolerant, that is able to survive a substantial heat shock in the absence of pre-treatment (Halladay & Craig, 1995; Sewell *et al.*, 1995).

The previous growth experiments showed that yeast containing deletion mutations in the bulged helix of HSF are better able to survive growth at elevated temperatures. This suggested that  $\Delta V1$ ,  $\Delta Q2$ ,  $\Delta E3$ , and  $\Delta V4$  might also be constitutively thermotolerant. To test this, cells were grown at 30°C, then either treated with a lethal heat shock at 50°C for five minutes, or maintained at 30°C as a control. Heat-shocked and control cells were then serially diluted, plated on selective plates, and grown at 30°C. As shown in Figure 5, yeast with deletions in the bulge of HSF are 10-100 times more able to survive a lethal heat shock than cells containing wild-type HSF. The well-documented role of HSF in transcriptional regulation of heat shock proteins and thermotolerance suggests that HSFs with deletions in the bulged region are more transcriptionally active than wild-type HSF, and it is this increase in activity which renders cells thermotolerant.

### Transcriptional ability of mutant HSFs

In order to confirm that the deletion mutations  $\Delta V1$ ,  $\Delta Q2$ ,  $\Delta E3$ , or  $\Delta V4$  cause an increase in HSF's transcriptional activity, we assayed the transcriptional activity directly, using a reporter construct, pHSE2-lacZ, which has the *lacZ* gene under the control of an HSF-regulated promoter. The HSE-containing promoter in the reporter plasmid is considered to be a strong promoter and, like promoters studied previously, is predicted to be fully occupied by HSF under constitutive conditions (Giardina & Lis, 1995b; Gross *et al.*, 1990; Jakobsen & Pelham, 1988, 1991). Cells were grown at 30°C and either maintained at 30°C to assay constitutive HSF activity, or heat-shocked for 20 minutes at either 37 or 42°C to measure inducible activity.



**Figure 5.** Thermotolerance of yeast containing wild-type or mutant HSF. Yeast strains containing wild-type HSF or  $\Delta V1$ ,  $\Delta Q2$ ,  $\Delta E3$ , or  $\Delta V4$  as the only source of HSF were grown at 30°C to mid-log phase and either maintained at 30°C or shocked at 50°C for five minutes. All cells were serially diluted in sterile media (tenfold dilution at each step), and spotted onto agar plates to measure growth.

Cell extracts were assayed for the amount of the expressed  $\beta$ -galactosidase.

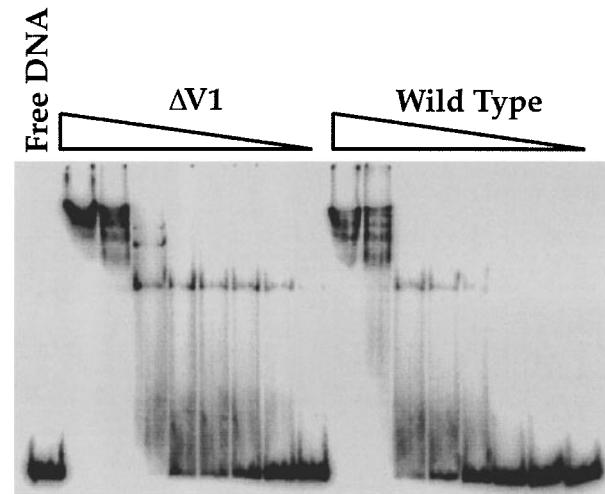
Deletion of any of the residues in the bulge increases the expression of the reporter gene approximately three- to sevenfold under constitutive conditions. It is intriguing that the constitutive transcriptional activity of  $\Delta V1$ ,  $\Delta Q2$ ,  $\Delta E3$ , or  $\Delta V4$  is mirrored by the ability of the yeast containing mutant HSFs to grow at elevated temperature and by their constitutive thermotolerance. The increase in constitutive transcriptional activity seems to be specifically related to deletion of residues from the bulge. Mutation of V1, Q2 or E3 to alanine results in levels of constitutive transcriptional activity that are similar to wild-type HSF (Hubl, 1995).

The transcriptional activities for yeast heat shocked at 37 or 42°C are consistently greater for the mutant HSFs than wild-type, but the magnitude of the induction is similar for  $\Delta V1$ ,  $\Delta Q2$ ,  $\Delta E3$ , and wild-type. When yeast containing wild-type HSF are heat shocked at 37°C, a 3.3-fold increase in  $\beta$ -galactosidase activity is observed. Heat shock at 42°C results in a 15-fold increase in wild-type  $\beta$ -galactosidase activity. The deletion mutants  $\Delta V1$ ,  $\Delta Q2$ , and  $\Delta E3$  are induced to approximately the same extent as the wild-type, showing increases in activity of 2.8, 4.2, and 2.8-fold, respectively, after heat shock at 37°C, or increases of 9, 11, or 8-fold, respectively, after a 42°C heat shock. The deletion mutant  $\Delta V4$  is significantly more induced than the other mutants, showing an increase in transcriptional activity at 37°C of 8.2-fold and a 29-fold increase in activity over constitutive levels after a 42°C heat shock. Mutation of V1, Q2 or E3 to alanine, on the other hand, does not change the induction profile relative to wild-type HSF (Hubl, 1995). Therefore, changing the chemical nature of the residues in the

bulge does not appear to be sufficient for any increase in induction, while deletion of any of the residues in the bulge confers constitutive induction over wild-type HSF, and deletion of the fourth residue in the bulge raises the induced transcriptional activity significantly more than the other deletions or wild-type HSF.

### DNA-binding ability of mutant HSFs

Although the recent co-crystal structure of the DBD in complex with DNA showed that the bulged-kinked helix does not make any contacts to DNA, it is still possible that mutations in this region might affect DNA-binding affinity. To investigate this possibility, we purified truncated versions of *K. lactis* HSF containing the DNA-binding and trimerization domains. Gel mobility shift assays were performed using  $\Delta V1$ ,  $\Delta Q2$ ,  $\Delta E3$ , or  $\Delta V4$  and wild-type HSF protein and a  $^{32}$ P-labeled DNA fragment containing an HSE composed of three perfect nGAAn repeats. A representative assay is shown in Figure 6. The overall  $K_d$  values for the binding of the mutant and wild-type HSF fragments, listed in Table 1, show that mutations in the bulged region yield protein that can bind to this HSE two- to sixfold more tightly than wild-type. These increases may be due to an increase in the protein-protein interactions between adjacent DBDs. For example, the *S. cerevisiae* mutant R206S (residue 228 in the *K. lactis* crystal structure) shows an increase in the DNA binding affinity. It has been proposed that this increase in DNA binding affinity may be the result of an increase in the protein-protein interaction mediated by the



**Figure 6.** DNA-binding assay. A representative gel mobility shift assay of wild-type and  $\Delta V1$  bound to a labeled DNA site is shown. The concentrations of  $\Delta V1$  or wild-type protein in lanes 2-9 and 10-17, respectively, are 2600, 864, 288, 96, 32, 10.6, 3.6, 1.2, 0.4 nM. The first band above the free DNA band is formed from a single trimer of HSF bound to the HSE. Higher-order complexes are formed when additional trimers of HSF bind to the same HSE.

**Table 1.** Macroscopic  $K_d$  values for wild-type and mutant HSF DNA binding and trimerization domains, calculated from gel mobility shift experimental data

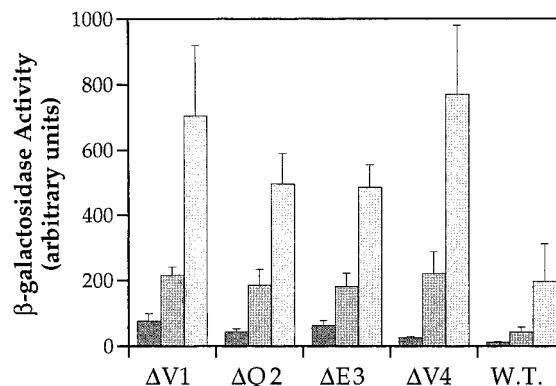
Mutation	$K_d$	$\chi^2$
Wild-type	$6.0 \times 10^{-8} \pm 1.3 \times 10^{-8}$	0.02
$\Delta V1$	$1.6 \times 10^{-8} \pm 0.3 \times 10^{-8}$	0.02
$\Delta Q2$	$2.4 \times 10^{-8} \pm 0.2 \times 10^{-8}$	0.005
$\Delta E3$	$1.0 \times 10^{-8} \pm 0.2 \times 10^{-8}$	0.03
$\Delta V4$	$2.7 \times 10^{-8} \pm 0.3 \times 10^{-8}$	0.01

amino-terminal ends of the bulged-kinked helix (Littlefield & Nelson, 1999). Deletion of residues in the bulge may similarly affect a protein-protein interface formed by this helix and the following turn with the analogous helix and turn and the wing(s) from adjacent proteins on longer DNA binding sites.

Does the increased DNA-binding affinity of the mutant proteins explain the increased transcriptional activity? To a first approximation, the increased affinities (Table 1) are correlated with the increased constitutive activity (Figure 7). However, this correlation weakens when considering the increased heat shock induced activity, especially of  $\Delta V4$ . In addition, the transcriptional activity was assayed using a strong HSE with four perfect (nGAAn) repeats and two imperfect (nGAGn or nGGAn) repeats. It is likely that the HSE for the transcriptional assay is constitutively bound by HSF, as a similar HSE, used in a competition assay, was found to be constitutively bound by HSF and no increase in binding was observed after heat shock (Jakobsen & Pelham, 1988).

Finally, it has been shown that DNA binding alone does not appear to be sufficient for activation of transcription by HSF. The *CUP1* promoter, for example, is constitutively bound by HSF, but not actively transcribed under non-shock conditions. Strong promoters, like those used in these DNA binding and transcriptional activity experiments, are expected to be fully occupied by wild-type HSF. Therefore, an increase in the binding affinity is not likely to alter the HSF occupancy of HSEs on these strong promoters. We suggest that an increase in the transcriptional activity cannot be fully explained by a decrease in the dissociation constant alone, and instead is best explained by binding of an induced form of HSF.

On the other hand, we cannot rule out that this increase in the binding constant plays a role in constitutive thermotolerance or in the ability of yeast containing  $\Delta V1$ ,  $\Delta Q2$ ,  $\Delta E3$ , or  $\Delta V4$  to grow at elevated temperatures. Hsp104, one of the principal proteins required for thermotolerance (Lindquist & Kim, 1996), has several non-consensus repeats in the HSE in its promoter. The details of the regulation of this promoter have not been reported. However, since the HSE is similar to the secondary HSEs in the *HSP82* promoter, which are not fully occupied by HSF under constitutive conditions, it is possible that wild-type HSF does

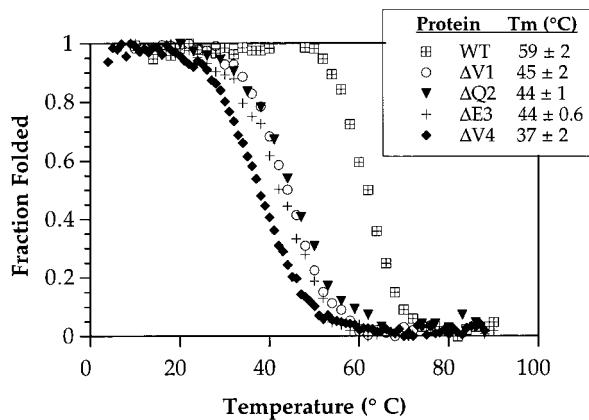


**Figure 7.**  $\beta$ -Galactosidase expression from an HSE-*lacZ* fusion. The constitutive (dark gray), 37 °C heat shock (medium gray) and 42 °C heat shock (light gray) levels of expression for strains bearing the indicated mutations in the DBD of HSF are shown.

not fully occupy the *HSP104* HSE under similar conditions. In that case, the increase in the strength of the binding of  $\Delta V1$ ,  $\Delta Q2$ ,  $\Delta E3$ , and  $\Delta V4$  may contribute to the increased ability to survive a lethal heat shock. Given that the increases in survivability are ten- to 100-fold, while the increase in DNA-binding affinity is two- to sixfold, it is unlikely that the increase in DNA-binding affinity can entirely explain the increase in constitutive thermotolerance.

### Mutations alter thermal stability of the DBD

HSF is known to respond to heat shock, so if the DBD is important in sensing and modulating this response, a change in the thermal stability of this domain may lead to differential regulation of the HSF controlled genes. We produced versions of the DBD containing the mutations  $\Delta V1$ ,  $\Delta Q2$ ,  $\Delta E3$ , and  $\Delta V4$  to determine whether these mutations to redesign the bulge affect the stability of the DBD against thermal melting. All of the circular dichroism spectral features of the wild-type protein were maintained and observed in the mutant spectra. Thermal denaturation of these proteins was followed as loss of a CD signal at 222 nm. Representative thermal denaturation curves for each of the protein fragments are shown in Figure 8. The thermal melting temperature ( $T_m$ ) was assigned for each of the proteins and is listed in Figure 8. Deletion of the first three partially or fully solvent exposed residues in  $\Delta V1$ ,  $\Delta Q2$ , or  $\Delta E3$  results in similar destabilization against thermal unfolding of about 15 °C. The deletion of the fully buried fourth residue, V4, destabilizes the protein even more substantially. This might explain why  $\Delta V4$ , which is at least as active as the other mutants as measured by transcriptional activity, cannot support growth at as elevated a temperature as the other mutants.



**Figure 8.** Thermal denaturation profiles. The thermal melting of wild-type and mutant proteins was followed by circular dichroism at 222 nm. Melting temperature ( $T_m$ ) is assigned to the temperature at which 50% of the protein is folded and are listed in the box in the upper right-hand corner.

Formation or deletion of bulges has been shown to affect thermal stability. For example, when  $\alpha$ -helical bulges are synthetically generated by insertion of an amino acid residue in T4 lysozyme, decreases in thermal stability of 2.0 to 28.8 °C are observed depending on the location and degree of solvent exposure at the site of insertion (Heinz *et al.*, 1993, 1994; Vetter *et al.*, 1996). Deletions of single residues from  $\alpha$ -helices result in thermal destabilization from 7.1 to 19.4 °C (Vetter *et al.*, 1996). The decreases in  $T_m$  of the HSF deletion mutants are similar to the decreases introduced by either insertion of an amino acid residue to generate a synthetic bulge or deletion of a residue from within an  $\alpha$ -helix (Heinz *et al.*, 1993, 1994; Vetter *et al.*, 1996). This suggests both that the structural effects of removal of HSF's bulge may be similar to generation of a bulge and that the overall architecture of the HSF has been optimized around incorporation of the bulge.

#### Structural perturbations rendered by the DBD mutations

The insertion of amino acid residues into  $\alpha$ -helices has yielded  $\alpha$ -helical bulges, or translocations of a segment of the helix, but has never been reported to result in the unfolding of a protein (Heinz *et al.*, 1993, 1994; Keefe *et al.*, 1994; Vetter *et al.*, 1996). Similarly, we expected that deletion of single residues from the bulge would not prevent formation of a single, uniquely folded state. We used NMR spectroscopy to probe the nature and extent of the structural changes introduced by mutation of the bulged-kinked helix in the isolated DBD.

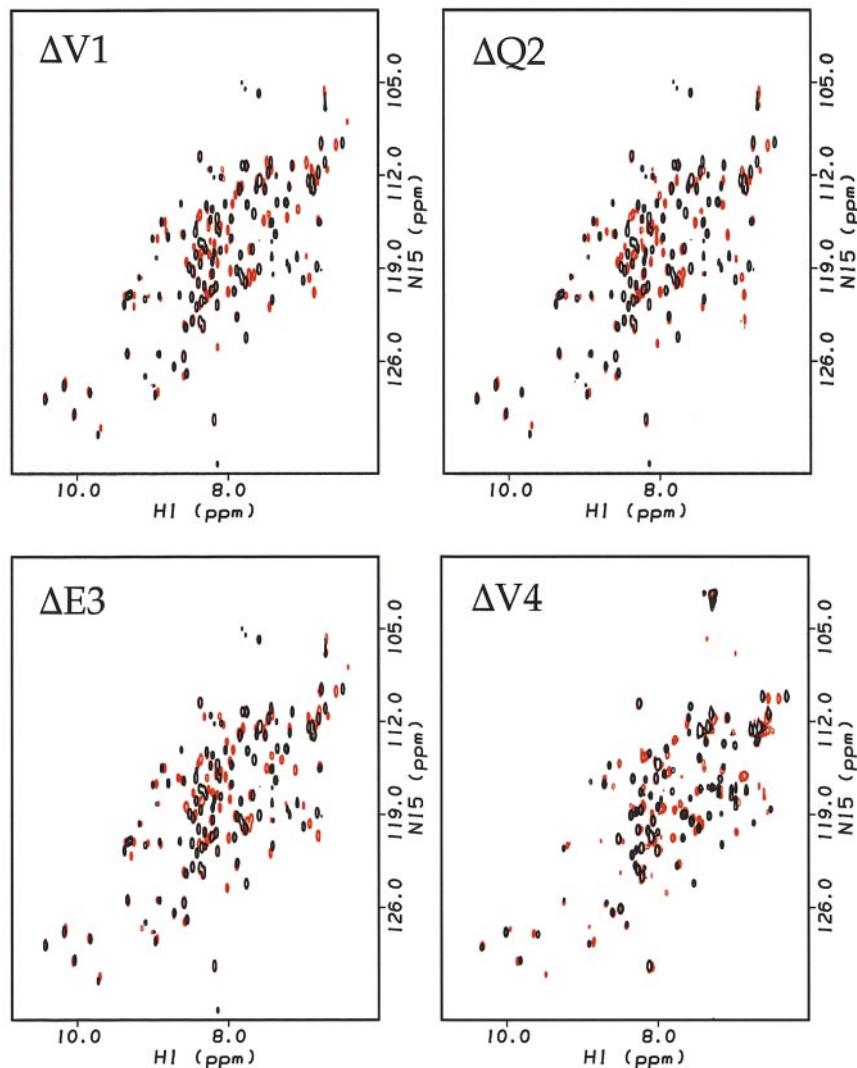
The one-dimensional NMR spectra of all of the isolated mutant DBDs have characteristics of well-folded proteins and are qualitatively similar to the

wild-type spectrum (data not shown). The presence of strongly upfield-shifted methyls and similar peak spacings and peak widths throughout the spectra indicate not only that the structures of the mutant proteins are native-like, rather than molten globule-like, but also that they maintain a fold that is very similar to the wild-type.

The solution structure of the DBD of *K. lactis* HSF has been determined by NMR (Damberger *et al.*, 1994, 1995), so the NMR spectra of the mutant proteins could be compared to the wild-type spectrum to quantitate the structural changes introduced by the mutations. A series of  $^{15}\text{N}-^1\text{H}$  heteronuclear single quantum coherence (HSQC) NMR experiments were used to compare resonances from the peptide backbone of the wild-type and mutant HSF DBDs. In Figure 9, the wild-type spectrum in black is overlaid against the spectra of each of the mutant proteins in red. As is the case in the one-dimensional spectra, the mutant spectra are all characteristic of native-like, well-folded proteins. The chemical shifts from the tryptophan residues in the mutant spectra (peaks present at approximately 10 ppm  $^1\text{H}$  and 130 ppm  $^{15}\text{N}$ ), which are buried in the hydrophobic core, are all virtually invariant from wild-type, suggesting that their chemical environment, and by extension the majority of the core, is unperturbed from wild-type. The changes in the chemical shifts for the vast majority of residues are moderate enough that the corresponding peaks for each of the residues in the mutant proteins could be identified unambiguously.

After identification of the peak from each residue, it was possible to calculate a parameter that we termed  $\Sigma\Delta\delta^{15}\text{N}^1\text{H}_W$ , which is indicative of the extent to which the chemical shift of that residue is perturbed relative to the same residue in the wild-type protein. The calculation of  $\Sigma\Delta\delta^{15}\text{N}^1\text{H}_W$ , described in more detail in Materials and Methods, consists of normalizing the range of proton chemical shifts to that of the range of amide chemical shifts, and then calculating the sum for each residue. In Figure 10, the structure of the wild-type *K. lactis* DBD has been color-coded by  $\Sigma\Delta\delta^{15}\text{N}^1\text{H}_W$  for each residue. The residues with the greatest change in chemical shift are colored in red while those with the least change are in black. Deleted residues are shown in white. The clustering of large chemical shift changes indicates a structural change in the proximity. Scattered chemical shift changes may be more indicative of global structural change.

Mutations  $\Delta\text{V1}$ ,  $\Delta\text{Q2}$ , or  $\Delta\text{E3}$  affect the chemical environment in similar ways. In each of these mutants, the most significant chemical changes are concentrated within the bulged-kinked helix. Other residues that produce varied signals are located within the other members of the three-helix bundle, in which the bulged-kinked helix participates. Very few or no changes were observed within the  $\beta$ -sheet, indicating that the structural changes are localized to the bulged helix. It is not surprising

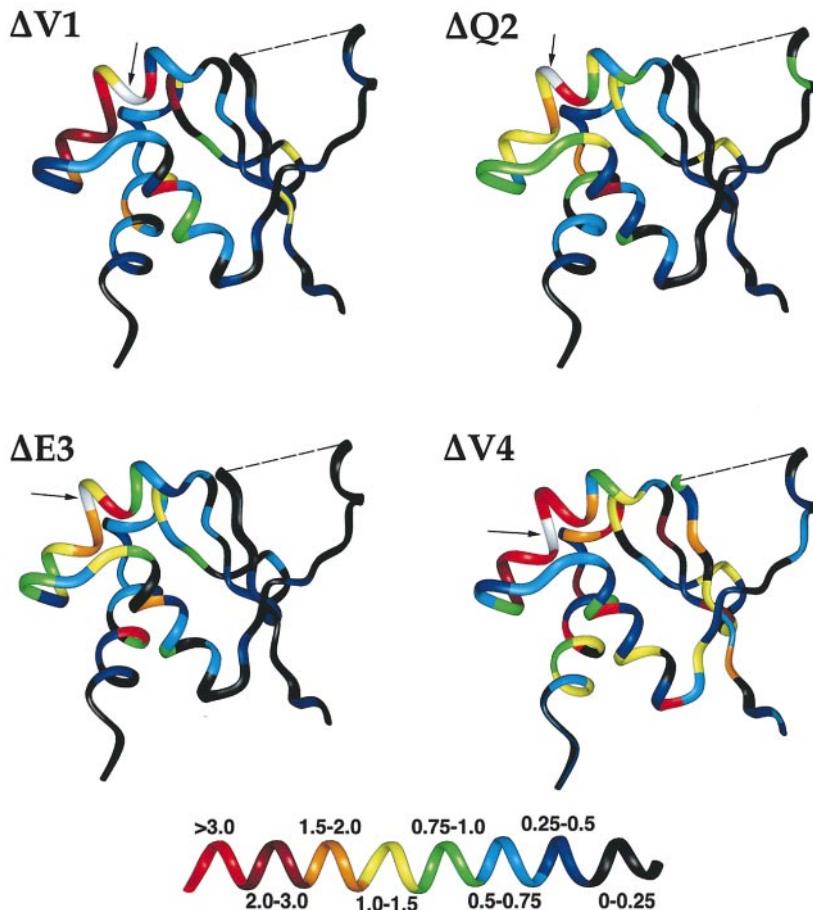


**Figure 9.** Comparisons of wild-type and mutant NMR HSQC spectra. The  $^1\text{H}$ - $^{15}\text{N}$  cross-peaks in the amide region of wild-type (black) and mutant (red) HSF DBDs are shown. Spectra for wild-type,  $\Delta\text{V1}$ ,  $\Delta\text{Q2}$ , and  $\Delta\text{E3}$  were collected at 25 °C, while both the  $\Delta\text{V4}$  spectrum and the wild-type spectrum to which it is compared were collected at 4 °C.

that the deletion of the partially solvent-exposed V1 residue leads to more substantial quantitative changes in the chemical shifts in the bulged-kinked helix than deletion of the fully solvent-exposed Q2 or E3. Again, the localization of major changes to the proximity of the deleted residue suggests that the overall structure of the protein remains intact. A suitable model for the mode of the accommodation of the deletion is simply removal of the bulge, rather than a shift in the register of the remaining residues in the helix. This result can be easily reconciled with the solvent accessibilities of each of these residues: one would predict that deletion of V1, Q2, or E3 would not result in the formation of any large hydrophobic holes in the core of the protein.

Simple removal of the bulge cannot be unambiguously predicted from the spectrum of  $\Delta\text{V4}$ . Deletion of residue V4 has a much more wide-

spread effect on the overall structure of the protein. It was necessary to measure the NMR spectrum for  $\Delta\text{V4}$  at 4 °C rather than 25 °C, which is the temperature at which the wild-type and other mutant spectra were collected, due to the diminished melting temperature of this protein.  $\Delta\text{V4}$  exhibits a somewhat molten globule spectrum at 25 °C (data not shown) but a well-ordered spectrum at 4 °C. Although  $\Delta\text{V4}$  has a native-like spectrum, the number of residues with significant changes in chemical shift is much larger than for any of the other deletion mutants. Moreover, the affected residues are scattered throughout the protein, rather than being localized to the mutated helix or the three-helix bundle. Analysis of the crystal structure indicates that the core of the HSF DBD appears to be quite optimally packed (Harrison *et al.*, 1994), so any mutation which perturbs this packing might have deleterious effects throughout the core. We



**Figure 10.** HSQC assessment of structural changes. The magnitude of the changes in the chemical shifts quantified as  $\Sigma \Delta \delta^{15}\text{N}^1\text{H}_w$  (arbitrary units) of each residue have been calculated and diagrammed on the structure of the wild-type protein. A scale of relative change is from red which indicates the greatest deviation from wild-type chemical shift to black, indicating small or no change from wild-type. Arrows indicate the location of the deleted residues, which have been colored white. The broken line connects the two ends of a disordered loop.

predict that deletion of residue V4 either forces the insertion of a charged residue into a hydrophobic hole or requires a dramatic shift in the packing of the invariant leucine at position 5, and by extension, the entire core. In either case, both from the perspective of a stability or a native-state fold argument, major changes have been rendered by deletion of this residue. Because  $\Delta V4$  is capable of supporting growth and binding to DNA, we believe that the overall fold of the protein has been maintained. We propose that the large number of chemical shifts has occurred due to a major change in the structure of the bulged helix, analogous to the shift in register that has been observed by insertional analysis in lysozyme.

## Conclusions

Previous studies on HSF's activity have suggested that the DBD, in addition to its obvious role in recognizing HSEs, is also involved in the regulation of HSF's activity. For example, point mutations within the DBD can increase transcriptional activity (Bonner *et al.*, 1992). In addition, studies of fusions between fragments of HSF and a heterologous DBD have shown that removal of HSF's DBD increases the activity of both of yeast HSF's transcriptional activation domains (Bonner

*et al.*, 1992; Nieto-Sotelo *et al.*, 1990). Mutation and deletion of other regions of HSF yields similar phenotypes. This has lead to the suggestion that the regulation of HSF is mediated through a complex set of interactions that negatively regulate the activity of the activation domains prior to stress. Our results support the idea that the wild-type DBD negatively regulates or represses transcriptional activity and suggests a mechanism for derepression upon heat shock *via* thermally induced structural or dynamic changes.

We have used three different assays to show that deletion of a single residue within the bulge of the DBD can cause an increase in activity *in vivo*. While the maximum temperature for growth of yeast containing wild-type HSF is 37°C, cells containing  $\Delta V4$  can grow at 38°C, while cells containing  $\Delta V1$ ,  $\Delta Q2$ , or  $\Delta E3$  can grow at 39°C. This result is specific to the presence of the bulge: substitution of V1, Q2 or E3 to alanine did not change the growth phenotype from wild-type HSF. Cells containing  $\Delta V1$  are ten times more likely than wild-type HSF to survive a lethal heat shock, while cells containing  $\Delta Q2$ ,  $\Delta E3$ , or  $\Delta V4$  are approximately 100 times more likely to survive. Finally, cells containing any of the deletion mutants have constitutive levels of transcriptional activity that are at least equal to the 37°C heat-shock induced level of activity of cells containing wild-type HSF.

Although the ratios of induced to constitutive levels of activity for  $\Delta V1$ ,  $\Delta Q2$ , and  $\Delta E3$  are similar to the ratio for wild-type HSF,  $\Delta V4$  has a ratio of induced to constitutive activity that is twice as high as the others after a 42 °C heat shock. The substitution of alanine at V1, Q2 or E3 leads to wild-type levels of constitutive and heat-shock induced transcription (Hubl, 1995). Therefore, these results support a critical function for the presence of a bulge in HSF, rather than the presence of a specific side-chain. This was predicted by evolutionary conservation, which showed the conserved nature of the bulge, but not a conservation of the sequences within the bulge.

We have also shown that these deletions can increase the DNA-binding activity of a fragment of HSF containing the DNA-binding and trimerization domains. Can the increase in activity *in vivo* be explained by these *in vitro* results? It is possible that at least some of the increase in constitutive thermotolerance is due to increases in DNA-binding affinity. The Hsp104 protein is critical in conveying thermotolerance (Lindquist & Kim, 1996). The regulation of its promoter has not been characterized, but its sole proximal HSE is similar to the secondary HSEs in the *HSP82* promoter, which are known to be only partially occupied under constitutive conditions (Erkine *et al.*, 1999). Nevertheless, the transcriptional activation levels of the HSF mutants were assayed directly from a synthetic promoter with a strong HSE. Previous studies have shown that similar HSEs are constitutively bound *in vivo* (Giardina & Lis, 1995a; Gross *et al.*, 1990; Jakobsen & Pelham, 1988, 1991), making it unlikely that an increase in binding affinity will affect the occupancy at this strong HSE. Therefore, the moderate changes in binding affinity of mutant HSFs cannot fully account for the increases seen in transcriptional activity.

The mutant HSFs differ from wild-type in two other parameters, namely structure and thermal stability, which might explain the role of the bulge in regulating activity. NMR analysis of the mutant DBDs allowed detection of localized and global changes in structure. For V1, Q2, and E3, the deletions of these residues appear to result in removal of the bulge with minimal global rearrangement. Deletion of V4 results in a more global reorganization of the domain, which might have been predicted from the buried location of that residue in the wild-type structure. It is unlikely that changing the side-chain from valine to alanine would have as dramatic an effect. One model to explain our results is that the bulge is directly involved in negative regulatory interactions. Removal of the bulge might prevent some of these critical interactions, resulting in the constitutively activated phenotypes we have observed. Future studies on the deletion mutants will likely shed light on this.

Deletion of single residues within the bulge destabilizes the DBD by 14 to 22 °C. Changes in  $T_m$  are an indication of a change in the thermal response profile of the mutant proteins. Any ther-

mally induced structural and dynamic changes that occur in wild-type HSF will occur at a lower temperature for mutant HSFs. The effect of these mutations on transcriptional activity has been to shift the range of thermal response to lower temperatures, i.e. the mutant HSFs have similar levels of activity at a lower temperature to that of wild-type HSF at a higher temperature. This leads to the intriguing idea that the induction to a transcriptionally active state might occur as a result of thermally induced changes in structure or dynamics within the DBD. It will be interesting to see if, as our results may suggest, other destabilizing mutations within HSF also increase transcriptional activity.

A thermally induced transition is not unprecedented in HSF regulation. In higher eukaryotes, HSF can undergo a thermally induced transition from an inactive, monomeric state to a DNA-binding competent and trimeric state *in vitro* (Goodson & Sarge, 1995; Larson *et al.*, 1995; Mosser *et al.*, 1990; Sarge *et al.*, 1993; Westwood & Wu, 1993; Zhong *et al.*, 1998; Zuo *et al.*, 1994). No analogous transition has been reported for yeast HSF, but since both yeast and higher eukaryotic HSF are known to be responsive to the stress of heat, it is not unlikely that yeast HSF is also capable of sensing heat directly. Our results support a role for the yeast HSF DNA-binding domain to function as a cellular thermometer.

## Materials and Methods

### Protein nomenclature

HSF from either *K. lactis* or *S. cerevisiae* were used in the different experimental procedures. The core regions of HSF from *K. lactis* and *S. cerevisiae* are highly homologous and available data suggest that HSF from these two yeast are conserved both structurally and functionally. *K. lactis* was chosen for biochemical and biophysical analysis because of the structural data available. *S. cerevisiae* was used for *in vivo* analysis because of the ease of genetics in that system. An alignment of the sequences of the second helices of the DBDs from *K. lactis* and *S. cerevisiae* is shown here:

228 RERFVQEVLKYF 240	<i>K. lactis</i>
206 REEFVHQILPKYF 218	<i>S. cerevisiae</i>

For continuity and ease in discussion, the amino acid residues in the bulged-kinked region, highlighted above, have been numbered sequentially from 1-4 and the following abbreviations were used ( $\Delta$  signifies deletion of the indicated residue):

Mutant abbreviation	<i>K. lactis</i> mutation	<i>S. cerevisiae</i> mutation
$\Delta V1$	$\Delta V232$	$\Delta V210$
$\Delta Q2$	$\Delta Q233$	$\Delta H211$
$\Delta E3$	$\Delta E234$	$\Delta Q212$
$\Delta V4$	$\Delta V235$	$\Delta I213$

## Construction of yeast strains and test for viability of mutant strains

The construction of pHN1031, the *S. cerevisiae* full-length HSF expressed from its own promoter on a yeast CEN/ARS plasmid, has been described (Hubl *et al.*, 1994). Mutations in this plasmid were introduced by a mega-primer PCR approach and confirmed by DNA sequencing. These plasmids were introduced into the tester strain PS145, which carries the *HSFΔ2::LEU2* chromosomal deletion, as well as a *URA3*-marked plasmid containing the wild-type HSF gene under the control of a *GAL1* promoter (Sorger & Pelham, 1988). Transformants were plated on media containing dextrose to ensure expression of HSF only from its own promoter (Hubl *et al.*, 1994). Once it was obvious that the deletion mutants would support growth, the *URA3*-marked plasmid containing the *GAL1:HSF* fusion gene was removed from the strain by growth in the absence of its selective marker and subsequent testing to ensure its loss. Further studies of viability and temperature dependence were done on the strains expressing only the wild-type or mutant versions of HSF from the CEN/ARS plasmid. Cultures were grown from a single colony in selective liquid media for eight hours. The  $A_{600}$  was then measured. All strains were diluted to the same cell density, then serially diluted in sterile media, spotted on the agar plates, and grown at 30, 37, 37.5, 38, 39, or 41 °C for three days to measure growth.

The haploid strain of *S. cerevisiae* W303 (Shore & Nasmyth, 1987) was transformed with either pHN1031 or pRS414 (Sikorski & Hieter, 1989), the empty vector. These strains were grown in liquid culture, serially diluted, spotted on the appropriate selective plates, and grown at various temperatures as described above.

## Thermotolerance assay

Strains described above were grown to mid-log phase in selective media at 30 °C, and split into two identical cultures. One culture was maintained at 30 °C in a shaking water bath, while the other culture was heat shocked for five minutes in a 50 °C shaking water bath. The cells were immediately diluted to an  $A_{600}$  of 0.1 in sterile selective media. From this concentration, cells were serially diluted in sterile media, spotted onto the appropriate selective plates, and grown at 30 °C for three days to measure growth.

## $\beta$ -Galactosidase assay

The strains derived for testing viability and thermotolerance were transformed with the plasmid pHSE2-lacZ, which has an HSE inserted into a disabled *CYC1* promoter upstream of the *lacZ* gene (Sorger & Pelham, 1987). The HSE sequence in this promoter is **AGAAGCTTCTA-GAAGCTTCTAGAGGATCCC**, where consensus GAA repeats are shown in bold. Yeast were grown on selective synthetic media at 30 °C to mid-log phase ( $A_{600}$  0.2–0.6) over a 12 to 18 hour period. Three aliquots were dispensed into 15 ml tubes such that each aliquot gave the equivalent of 5 ml of cells at an  $A_{600}$  of 0.2. The control aliquot was maintained at 30 °C. Other aliquots were shocked at 37 or 42 °C for 20 minutes, then were allowed to recover for 90 minutes to allow expression of  $\beta$ -galactosidase. All cultures were harvested by centrifugation at 4 °C.  $\beta$ -Galactosidase activity was determined as described (Hubl *et al.*, 1994). Each reported activity is an

average of four experiments; all strains were assayed on each of four days.

## Western blotting

Yeast strains were grown and harvested in the same manner as those prepared for the  $\beta$ -galactosidase assays. The cell pellet was resuspended in 0.125 M Tris (pH 6.8), 4% (w/v) SDS, 20% (v/v) glycerol, 1% (v/v)  $\beta$ -mercaptoethanol, and lysed by boiling and vortexing in the presence of glass beads. Cell extract was separated from cell debris by centrifugation. Cell extracts were electrophoresed on a 4%–20% (w/v) denaturing gradient gel and electroblotted overnight onto an Immobilon filter (Millipore), which was probed with a rabbit anti-HSF antibody (a gift from David Gross) and then analyzed by chemiluminescence.

## Overexpression and purification of HSF fragments containing the DNA-binding and trimerization domains

The expression plasmid pHN208, previously described as HSF<sub>DT</sub> (Rye *et al.*, 1993), is a derivative of pET-3b (Studier *et al.*, 1990) and contains the coding sequence for *K. lactis* HSF amino acid residues 192–394, which includes the DNA-binding and trimerization domains, generating a 23.7 kDa protein. Mutations in the bulged region of HSF were introduced into pHN208 by the PCR mutagenesis and confirmed by DNA sequencing.

Wild-type and mutant protein were expressed and purified identically. Expression plasmids were transformed into BL21(DE3) cells harboring a derivative of the pAcYc177 containing *lacI*<sup>Q</sup> (Stark, 1987; Studier *et al.*, 1990). Protein expression was induced by the addition of IPTG to a final concentration of 0.5 mM. Cells were harvested by centrifugation and stored as frozen suspensions in 200 mM NaCl, 50 mM Tris (pH 7.5), 10% glycerol, 5 mM MgCl<sub>2</sub>. Cells were then thawed, supplemented with 1  $\mu$ g/ml aprotinin, 2  $\mu$ g/ml pepstatin, and 1  $\mu$ g/ml leupeptin, incubated on ice for 15 minutes with 200  $\mu$ g/ml lysozyme, disrupted by sonication, and centrifuged at 39,000  $g$  for 15 minutes at 4 °C. The high speed supernatant was diluted fourfold with 50 mM Tris (pH 7.5) and loaded onto a heparin-agarose column (Sigma). The column was developed with a gradient from 50 mM to 1250 mM NaCl in 50 mM Tris (pH 7.5). The proteins eluted over a NaCl concentration range from 600 to 1000 mM. Fractions with the highest concentration of protein were diluted with 50 mM Tris (pH 7.5) to a final NaCl concentration of 50 mM, and loaded onto a sulfopropyl column (Waters). The column was developed with a gradient from 50 mM to 600 mM NaCl in 50 mM Tris (pH 7.5). The protein eluted between 200 mM and 250 mM sodium chloride. At this point the proteins appeared to be ~90% pure from Coomassie-stained SDS-PAGE gels. The protein was then stored on ice and used within one week of purification. Protein concentrations were determined with an extinction coefficient of 33,700  $\text{cm}^{-1} \text{M}^{-1}$ , which was calculated from the tyrosine and tryptophan content of the proteins. Samples of all mutant proteins were also analyzed by analytical reversed-phase HPLC to more accurately determine purity; the values, between 86 and 95%, were used to adjust the protein concentrations appropriately.

## DNA-binding assays

Binding of mutant HSF DNA-binding and trimerization domains to DNA was performed as previously described, using the 145 bp HSE-containing fragment from the plasmid pBL2 (Drees *et al.*, 1997). The HSE in the DNA probe is **AGAATATTCTAGAAA**, where GAA repeats are shown in bold. Binding assays were performed at room temperature; however, the results at room temperature and 4°C were the same for wild-type protein. Dried gels were used to expose Molecular Dynamics PhosphorImager plates, which were then scanned on the Molecular Dynamics PhosphorImager. The signal of unbound bands and all bands from bound species were quantified using Molecular Dynamics ImageQuant. To estimate binding affinity, the amount of bound DNA was graphed against protein concentration and fitted to a rectangular hyperbola. The data from four independent experiments was used for the curve fitting. The  $K_d$  value was measured as the protein concentration at which 50% of the DNA was bound. The analysis did not address cooperativity of binding of additional trimers of HSF to the DNA; only global dissociation constants were obtained.

## Overexpression and purification of the DBD

The expression plasmid pHN212 described previously (Harrison *et al.*, 1994), is a derivative of pET-3b (Studier *et al.*, 1990) and contains the coding sequence for *K. lactis* HSF amino acid residues 193 to 282, which includes the DBD, generating a 10.9 kDa protein. pHN212 was mutated to replace arginine codons which are infrequently used in *E. coli*, and which in previous studies were often translated as lysine instead of arginine. Site-directed mutagenesis was used to replace the codons for residues R194, R228, R275, and R282\* (an artifact of the cloning procedure) to the codon CGC, a common *E. coli* arginine codon. This new plasmid, pHN212R, was subsequently mutated by site-directed mutagenesis to sequentially delete each of the residues in the bulged region. All mutations were confirmed by DNA sequencing.

Mutant and wild-type proteins were overexpressed in *E. coli* strain BL21(DE3) containing the pLysS plasmid (Studier *et al.*, 1990). Purification of the wild-type DBD was accomplished by a technique which has been described (Harrison *et al.*, 1994). The mutations introduced for this study rendered the mutant proteins less soluble upon overexpression than wild-type, therefore an alternative purification scheme was developed for the mutant proteins. Cells were harvested by centrifugation, resuspended in a solution of 50 mM Tris (pH 7.5), 200 mM NaCl, 5 mM MgCl<sub>2</sub>, and 10% glycerol, then frozen. Cells were subsequently thawed, supplemented with 1 µg/ml aprotinin, 2 µg/ml pepstatin, and 1 µg/ml leupeptin, incubated on ice for 15 minutes with 200 µg/ml lysozyme, disrupted by sonication, and centrifuged at 39,000 g for 15 minutes at 4°C. The high speed pellet containing the mutant protein was resuspended in 6 M guanidine-HCl, incubated for 30 minutes at room temperature, and dialyzed twice, for two hours at each step, at room temperature against 0.1% trifluoroacetic acid (TFA). The dialysate was applied to a Sephadryl S-100 size exclusion column (Pharmacia) equilibrated in 0.1% TFA. Size fractionation occurred using 0.1% TFA as the mobile phase throughout the separation. Fractions containing the mutant protein were pooled and applied to a reversed-phase C-18 HPLC column. The protein was

eluted with an aqueous TFA/organic acetonitrile gradient and manually collected as approximately 1 ml fractions that were subsequently frozen and lyophilized. All protein concentrations were determined with extinction coefficient of 25,400 cm<sup>-1</sup> M<sup>-1</sup>, which was calculated from the tyrosine and tryptophan content of the proteins.

For NMR analysis, the cells were grown in M9 minimal media either with <sup>15</sup>NH<sub>4</sub>Cl as the sole nitrogen source or with [<sup>15</sup>N]valine and all additional amino acids unlabeled. Labeled wild-type and mutant protein were purified as described above. Purified proteins were suspended at approximately 20 mg/ml in 50 mM d<sub>3</sub>-acetate buffer (pH 5.0) with 10% <sup>2</sup>H<sub>2</sub>O for spectral analysis.

## Circular dichroism spectroscopy

Lyophilized wild-type and mutant HSF DBD constructs were resolubilized in water then diluted to 20 µM in 20 mM citrate/phosphate buffer (pH 4). CD spectra were recorded on an AVIV 60 DS spectropolarimeter at 4°C. Measurements were taken at 1 nm intervals with a one second time constant and 1.5 nm band width. A path length of 0.1 cm was used. The thermal melting of these constructs was followed at 222 nm. The temperature was raised by 1°C at each step, with a five minute equilibration time, ten second averaging time, and 1.5 nm bandwidth. The temperature at the inflection point of the cooperative unfolding curve was assigned as the  $T_m$  value.

## NMR analysis

All spectra were collected at 25°C on a Bruker AMXIII-500 spectrometer. Data for  $\Delta V4$  were also collected at 4°C because the decreased melting temperature of this mutant protein yielded a partially unfolded spectrum at 25°C. Two-dimensional <sup>1</sup>H, <sup>15</sup>N HSQC (Bax *et al.*, 1990) were acquired on uniformly <sup>15</sup>N-labeled wild-type or mutant HSF or [<sup>15</sup>N]valine labeled wild-type HSF in 90% H<sub>2</sub>O/10% <sup>2</sup>H<sub>2</sub>O. <sup>1</sup>H and <sup>15</sup>N carriers were placed at 4.7 ppm and 117 ppm, respectively. The spectral width consisted of 12 ppm in the <sup>1</sup>H dimension (1024 complex points) and 35 ppm in the <sup>15</sup>N dimension (64 complex points.) The raw data were transformed into two-dimensional matrices of 1024 by 1024 points. Suppression of the water resonance was accomplished by presaturation. The quadrature detection in the indirect dimension was achieved using the States TPPI method (Marion *et al.*, 1989). Data were processed and analyzed using the Felix package (Biosym Technologies, San Diego, CA). Assignments of the wild-type spectrum were made based on the published data (Damberger *et al.*, 1994, 1995). To verify our assignments, the spectrum of wild-type HSF, which was uniformly labeled with [<sup>15</sup>N]valine was collected, assigned and compared to the fully <sup>15</sup>N-labeled spectrum. Our wild-type spectrum and the assignment of it, was nearly identical to the published data. Peaks from the mutant spectra were assigned by inspection to give the lowest spectrally localized chemical shift differences, such that a maximum number of peaks could be assigned, in a manner similar to a previously described technique (Farmer *et al.*, 1996). The changes in the observed chemical shifts of the mutant protein compared with the wild-type were quantitated as  $\Delta \delta^{15}\text{N}$  and  $\Delta \delta^1\text{H}$ . For each mutant, the average of the absolute values of avg $\Delta \delta^{15}\text{N}$  and avg $\Delta \delta^1\text{H}$  were calculated. A weighting factor,  $W$ , equal to the ratio

between the average values for  $\Delta\delta^{15}\text{N}$  and the average values of  $\delta^1\text{H}$ , was applied to  $\Delta\delta^1\text{H}$  for each residue to yield  $\Delta\delta^1\text{H}_w$ . Finally,  $\Delta\delta^{15}\text{N}$  and  $\delta^1\text{H}_w$  were summed for each residue to give  $\Sigma\Delta\delta^{15}\text{N}^1\text{H}_w$ .

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