Mutational analysis of human heat-shock transcription factor 1 reveals a regulatory role for oligomerization in DNA-binding specificity

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INTRODUCTION

In response to protein-damaging stresses, including heat shock, eukaryotic HSF (heat-shock transcription factor) induces the transcription of HSP (heat-shock protein) genes. HSPs function as molecular chaperones and maintain the vital homeostasis of protein folds [1,2]. HSFs contain evolutionarily conserved functional domains, including the DBD (DNA-binding domain), which consists of a helix-turn-helix motif and HRs (hydrophobic repeat regions), which have a coiled-coil structure. The HRs facilitate HSF trimerization and the DBDs of a HSF trimer bind to the HSE (heat-shock element), which consists of at least three inverted repeats of the 5-bp sequence nGAAn. Yeast HSF is able to recognize discontinuous HSEs that contain gaps in the array of the nGAAn sequence; however, hHSF1 (human HSF1) fails to recognize such sites in vitro, in yeast and in HeLa cells. In the present study, we isolated suppressors of the temperature-sensitive growth defect of hHSF1-expressing yeast cells. Intragenic suppressors contained amino acid substitutions in the DNA-binding domain of hHSF1 that enabled hHSF1 to regulate the transcription of genes containing discontinuous HSEs. The substitutions facilitated hHSF1 oligomerization, suggesting that the DNA-binding domain is important for this conformational change. Furthermore, other oligomerization-prone derivatives of hHSF1 were capable of recognizing discontinuous HSEs. These results suggest that modulation of oligomerization is important for the HSE specificity of hHSF1 and imply that hHSF1 possesses the ability to bind to and regulate gene expression via various types of HSEs in diverse cellular processes.

Key words: heat-shock element (HSE), heat-shock transcription factor (HSF), heat-shock protein (HSP), heat-shock response, protein–DNA interaction.

Abbreviations used: DBD, DNA-binding domain; DR, direct repeat; EGS, ethylene glycol bis-(succinimidylsuccinate); EMSA, electrophoretic mobility-shift assay; HA, haemagglutinin; hHSF1, human heat-shock transcription factor 1; HR, hydrophobic repeat region; HSE, heat-shock element, HSF, heat-shock transcription factor; HSP, heat-shock protein; RT, reverse transcription; SV40, simian virus 40; VP16, viral protein 16; yHSF, Saccharomyces cerevisiae heat-shock transcription factor.

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**EXPERIMENTAL**

**Plasmids**

Plasmid pK255 (YEp-LEU2-Padow-HA3-hHSF1-Tadow) is a derivative of pK250 containing hHSF1 cDNA downstream of three copies of the influenza HA (haemagglutinin) tag sequence [8]. To create plasmid pYT31, hHSF1 cDNA was subcloned into pcDNA3.1(+) (Invitrogen). Plasmids pK578 [hHSF1–VP16 (viral protein 16)] and pK732 (hHSF1–HA) are derivatives of pYT31 containing sequences of the herpes simplex virus VP16 activation domain (amino acid residues 413–490) and one copy of the HA tag respectively at the 3′ end of the hHSF1 open reading frame. For overexpression analysis, the EN02, HSC82, HSK82, NUS1, UGP1 and YDJ1 genes were amplified by PCR from *S. cerevisiae* genomic DNA and cloned into the high-copy-number plasmids YEp195 (YEp-UARA3) and YEplac112 (YEp-TRP1) [18]. The luciferase reporter gene HSE-SV40p-LUC contains HSE oligonucleotides upstream of the SV40 (simian virus 40) promoter–firefly luciferase fusion gene (SV40p-LUC) of the pG3-3-promoter vector (Promega) [18]. The nucleotide sequences of HSE oligonucleotides are as follows: HSE4Ttc, tcgacTTCtaGAAgcTTCcaGAAattagtgc-tactcga; HSEgap, tcgacTTCtaGAAgctagcaGAAattagtgctactcga; and HSEstep, tcgacTTCactagcTTCcactaatTTCtgctactcga (GAA and its inverted TTC sequence are presented in upper-case letters). The reporter genes HSP27-LUC and c-fos-LUC contained the promoter regions of human HSP27 (~247 to ~16 relative to the translation initiation site) and c-fos (~663 to ~71) upstream of the firefly luciferase gene of the pG3-basic vector (Promega) respectively.

**Yeast strains and RNA analysis**

Yeast cells were grown in YPD medium consisting of 1% yeast extract, 2% polypeptide and 2% glucose. Cells expressing yHSF (strain HS170L; ade2 ura3 leu2 his3 trp1 can1 hsf1::HIS3 YCp-LEU2-yHSF) and HA−–hHSF1 (strain HS255; isogenic with HS170L, except containing pK255 instead of YCp-LEU2-yHSF) were used in the analysis [18,18,25]. For construction of the hsc82-4P mutation, the gap-type HSE of the chromosomal HSC82 promoter was changed to the 4P-type HSE by the pop-in/pop-out replacement method (see Figure 1D) [26]. Intragenic suppressor mutations were isolated as follows. hHSF1 cDNA was amplified by PCR in the presence of 0.1 mM MnCl2 to introduce random nucleotide alterations [27]. The amplified fragments were cloned into the expression vector pK250. The resulting mutant library was introduced into strain TH2670 (his3 leu2 met15 URA3::CMV-tTA kan8-eto-TATA-HSF1) (Open Biosystems), in which yHSF is expressed under the control of the teto promoter [28]. Transformants were grown at 35°C on medium consisting of 0.67% yeast nitrogen base, 2% glucose, 20 μg/ml histidine, 20 μg/ml methionine, 10 μg/ml doxycycline and 2% agar. Plasmids recovered from two heat-tolerant colonies were sequenced. These plasmids contained several amino acid alterations throughout the hHSF1-coding region, but mutations of leucine to phenylalanine at amino acid 25 (L25F) and isoleucine to valine at amino acid 35 (I35V) conferred improved growth.

Total RNA prepared from cells was subjected to RT (reverse transcription)–PCR analysis as described previously [8,18]. The amounts of PCR products were compared after normalizing RNA samples to the levels of control ACT1 mRNA (encoding actin). The experiments were performed at least three times with similar results.

**Mammalian cell culture and luciferase assay**

HeLa cells (cell number: RCB0007, RIKEN Bio Resource Center, Ibaraki, Japan) grown in 12-well plates were transfected by HilyMax (Dojindo Laboratories) with DNA mixtures consisting of firefly luciferase reporter plasmid (50 or 100 ng), pRL-TK control plasmid (10 ng) containing the Renilla luciferase gene driven by the HSV-TK promoter (Promega), hHSF1 expression plasmid (10 ng) and carrier pcDNA3.1(+)-DNA (to bring the total amount of DNA to 1.6 μg) [18]. Cells were cultured at 37°C for 20–24 h after transfection, and firefly and Renilla luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega). The Renilla luciferase activity of each sample was used to normalize firefly luciferase for transfection efficiency. Expression of hHSF1 derivatives in the cells was verified by immunoblot analysis with anti-HA (Invitrogen) and anti-VP16 (Abcam) antibodies, as described previously [18].

**In vitro polypeptide synthesis, chemical cross-linking and EMSA (electrophoretic mobility-shift assay)**

hHSF1 polypeptides were synthesized in the presence or absence of [35S]methionine, using the TNT® T7 Coupled Reticulocyte Lysate System (Promega) and pcDNA3.1(+) derivatives containing hHSF1 cDNA as templates. Upon SDS/PAGE and phosphoimaging, the synthesized polypeptides were detected as a single band of the calculated molecular mass. The amount of hHSF1 polypeptides was determined by immunoblotting with an anti-hHSF1 antibody (Stressgen) using purified recombinant hHSF1 as a reference [18]. The amounts of hHSF1 derivatives were normalized by the levels of incorporated [35S]methionine.

For chemical cross-linking with EGS [ethylene glycol bis-(succinimidylsulfonate)], 35S-labelled hHSF1 polypeptides (0.3–0.4 μl) were incubated in 4 μl of buffer containing 10 mM Tris/HCl (pH 7.6), 2 mM EDTA and 5% glycerol at 37°C for 20 min, treated with EGS at room temperature (23°C) for 20 min, electrophoresed on a SDS/4–10% polyacrylamide gel, and subjected to phosphoimaging [18]. The experiments were performed at least twice with similar results.

The EMSA was carried out using unlabelled hHSF1 polypeptides and 32P-labelled HSE oligonucleotides, as described previously [18]. Band intensity was quantified using MultiGauge software (Fujifilm). The intensity of the hHSF1-bound band was divided by the sum of the total intensity, and the binding ratios obtained were used to calculate relative binding affinities. For the trypsin-sensitivity assay, hHSF1 polypeptides (1.2 ng) were incubated with HSE3P oligonucleotides in an 8 μl reaction mixture. Samples were treated with 1 μl of 20 μg/ml trypsin at room temperature for the times indicated, and the reaction was terminated by adding 1 μl of 200 μg/ml soya bean trypsin inhibitor before gel electrophoresis. Band pattern was analysed using GelPro Analyzer software (Media Cybernetics). The experiments were performed at least four times with similar results.

**RESULTS**

**Phenotypes of *S. cerevisiae* cells expressing HA3–hHSF1**

Although hHSF1 fails to substitute for the cell-viability function of yHSF in *S. cerevisiae* [22], HA−–hHSF1, which contains three copies of an HA epitope tag at the N-terminus, was successful in supporting cell growth at 28°C, but not at 33°C or higher temperatures (Figure 1A). When yHSF control cells grown at
25°C were heat-shocked at 39°C, the mRNA levels of yHSF target genes containing 4P-type HSE (perfect-type HSE with four ngAAn units, BTN2, HSP42 and HSP78), 3P-type HSE (perfect-type HSE with three ngAAn units, HSP10, HSP60 and SSA2), gap-type HSE (CPR6, HSC82 and HSP82), step-type HSE (SGT2 FSH1 and YDJ1) or DR-type HSE (AHPI and TIP1) were robustly increased, as judged by RT–PCR analysis (Figure 1B). In HA3–hHSF1 cells, genes containing continuous 4P-, 3P-, or gap-type HSEs were expressed properly, as they were in yHSF cells; however, those containing discontinuous gap-, step- or DR-type HSEs were not. This is consistent with previous results obtained using different hHSF1 derivatives [8] and shows that hHSF1 expressed in S. cerevisiae is defective in regulating genes that contain discontinuous gap-, step- or DR-type HSEs.

Suppression of temperature-sensitivity of HA3–hHSF1 cells by elevated expression of HSC82, HSP82 and YDJ1

We analysed the relationship between gene expression and temperature-sensitivity of HA3–hHSF1 cells. Discontinuous HSEs are found in approximately half of yHSF target genes [8], and several of these genes, including ENO2, ERO1, HSC82, HSP82, MDJ1, NUS1, SSSC1, UGP1 and YDJ1, are necessary for cell viability at normal and/or elevated temperatures. The yHSF-mediated transcription of ERO1, MDJ1 and SSSC1 was not associated with temperature-sensitivity, as judged from the normal growth at 37°C of cells that contain mutations in the HSEs of these genes ([26] and S. Matsuda, A. Sakaguchi and H. Sakurai, unpublished work). The remaining six genes were cloned into a high-copy-number plasmid and introduced into HA3–hHSF1 cells. As shown in Figure 1(C), overexpression of HSC82, HSP82 and YDJ1 rescued growth of HA3–hHSF1 cells at 33°C. These results reinforce the requirement of Hsp90 for growth at elevated temperatures and show that HSE architecture is an important determinant of the requirement of Hsp90 for growth at elevated temperatures.

Isolation of hHSF1 mutations that support S. cerevisiae cell growth at elevated temperatures

To isolate hHSF1 mutations that enable S. cerevisiae cells to grow at elevated temperatures, tetO-yHSF cells were transformed with a plasmid library of HA3–hHSF1 genes containing random nucleotide alterations, and transformed cells were grown at

![Image](https://example.com/image.png)
35°C in the presence of doxycycline. In tetO-yHSF cells, the promoter region of yHSF has been replaced with the tetracycline-regulated tetO promoter, and addition of a tetracycline derivative, doxycycline, leads to repression of yHSF transcription, depletion of yHSF protein and lethality of the cells [28]. Two candidates enabled tetO-yHSF and yHSF-deficient cells to grow at 35°C (Figure 1E, and results not shown). The amino acid substitutions responsible for growth (L25F and I35V) were determined to be located in the DBD of hHSF1 (Figure 1F).

The L25F and I35V substitutions dramatically improved the heat-shock response of target genes (Figure 1B). Heat-induced transcription via continuous HSEs was transiently activated in HA₃-hHSF1-L25F and HA₃-hHSF1-I35V cells, as it was in yHSF cells. Furthermore, both substitutions enabled HA₃-hHSF1 to properly regulate basal and heat-induced transcription of HSC82, HSP82, YDJ1 and most of the other genes containing discontinuous gap-, step- and DR-type HSEs. It should be noted that hHSF1-L25F and hHSF1-I35V lacking the HA tag regulated transcription similarly to those containing the HA tag and suppressed the growth defect at 33°C (results not shown). Therefore growth at elevated temperatures is correlated with transcriptional activation of yHSF target genes.

Changes in HSE specificity of hHSF1-L25F and hHSF1-I35V in HeLa cells

To analyse the transcriptional effect of hHSF1 in HeLa cells, we employed reporter genes containing oligonucleotides of 4Ptt-, 3P-, gap- and step-type HSEs upstream of the SV40 promoter–luciferase gene fusion (HSE-SV40p-LUC). When HeLa cells were transfected with SV40p-LUC or HSE-SV40p-LUC and were cultured under normal conditions, the presence or absence of HSE did not significantly change the luciferase activity, which suggests that endogenous hHSFs do not affect the constitutive expression of the reporter gene [18]. However, it has been shown that ectopically expressed hHSF1 forms a trimer and binds to HSE at physiological temperatures [11]. Because co-transfected hHSF1 only slightly increased the constitutive luciferase activity of HSE-SV40p-LUC reporters (see Figure 5D), hHSF1 was fused to a viral activator, VP16 (hHSF1–VP16). Fusion of the VP16 activation domain did not significantly affect the hHSF1–HSE interaction, as judged by an EMSA using in vitro synthesized hHSF1 and hHSF1–VP16 [18]. Similarly to previous results [18], hHSF1–VP16 markedly induced the expression of reporter genes containing continuous HSEs (HSE4Ptt-SV40p-LUC and HSE3P-SV40p-LUC), but not of those containing discontinuous HSEs (HSEGap-SV40p-LUC and HSEStep-SV40p-LUC) (Figures 2A and 2B). Notably, hHSF1-L25F–VP16 induced the expression of the reporter gene containing HSEGap 2.9-fold and of that containing HSEStep 2.5-fold, without significantly affecting the expression of reporter genes containing continuous HSEs. The I35V substitution also enabled hHSF1–VP16 to induce the expression of reporter genes containing discontinuous HSEs. The human HSP27 promoter contains an HSE consisting of continuous nGAAn inverted repeats (Figure 2C). As shown in Figure 2A, expression of HSP27-LUC, a luciferase gene driven by the HSP27 promoter, was activated by co-transfection with hHSF1–VP16, and the presence of the L25F or I35V substitution did not affect the expression. In contrast, these substitutions significantly induced expression of c-fos-LUC, which has the c-fos promoter with a discontinuous HSE consisting of gap- and step-type repeats, relative to the wild-type control (Figures 2A and 2C). These results show that L25F and I35V alter the specificity of hHSF1 for HSEs, enabling transcription via discontinuous HSEs.

L25F and I35V substitutions affect hHSF1–hHSF1 and hHSF1–HSE interactions

Oligomerization of hHSF1 was analysed using in vitro synthesized polypeptides (Figure 3A). When polypeptides were incubated at 37°C, treated with the chemical cross-linker EGS, and subjected to SDS/PAGE, most of the wild-type hHSF1 migrated at a position of 70 kDa, the molecular mass of the monomeric form (Figure 3B). In addition to this 70 kDa band, hHSF1 polypeptides containing the L25F and I35V substitutions were detected at positions of 140 and 210 kDa, which correspond to a dimer and trimer respectively. Therefore these substitutions enhance oligomerization of hHSF1.

Interactions between hHSF1 and various types of HSEs were analysed by EMSAs (Figure 3C). Incubation of wild-type hHSF1 with oligonucleotides of 4Ptt-type and 3P-type HSEs caused protein–DNA complexes to form. In the binding experiments using discontinuous gap-type and step-type HSEs, however, the amounts of complexes formed were 5- and 15-fold lower respectively than the amount formed when using HSE3P. When compared with wild-type hHSF1, hHSF1-L25F exhibited a 1.5- and 1.4-fold higher affinity for HSE4Ptt and HSE3P respectively, and a 2.5- and 3.0-fold higher affinity for HSEGap and HSEStep respectively (Figure 3C). The I35V substitution increased the binding affinity for continuous HSEs 1.7–1.9-fold.
Effect of C103S substitution on oligomerization and HSE-binding of hHSF1

In heat- and oxidative-stress-activated HSF1, two cysteine residues, at positions 36 and 103 (see Figure 1F), are engaged in disulfide bond formation, which regulates HSF1 oligomerization and HSE binding [29,30]. To examine the roles of cysteine residues in HSE specificity, Cys103 was changed to serine (C103S) in hHSF1 (we did not use the C36S substitution, because Cys103 is in close proximity to Leu29 and Ile83, and may thus alter the effects of L25F and I35V independently of disulfide bond formation). The L25F and I35V substitutions enhanced hHSF1 oligomerization, even in combination with the C103S substitution (Figure 4A). When hHSF1–VP16 derivatives were co-transfected with reporter genes into HeLa cells, the C103S substitution slightly reduced the luciferase activity of the reporter genes; however, L25F and I35V still enhanced expression via discontinuous HSEs (Figure 4B). These results suggest that Cys103 does not modulate the effects of the L25F and I35V substitutions on HSE specificity.

Oligomerization of hHSF1 affects HSE specificity

We analysed the HSE specificity of two oligomerization-prone derivatives of hHSF1 that contained the L189E substitution in the HR-B and the M391E substitution in the HR-C (Figure 5A) [31]. Enhancement of oligomerization by these substitutions was confirmed by cross-linking analysis (Figure 5B). As shown by EMSAs using 3P-type HSE, L189E and M391E greatly increased the binding affinity of hHSF1 and caused slower migration of protein–DNA complexes (left-hand panel of Figure 5C). Note that incubation of wild-type hHSF1 and HSE3P at 43°C led to an increase in the amount of protein–DNA complex, but did not affect the mobility of the complex. Importantly, these substitutions enhanced binding to discontinuous HSEs, and the affinities for gap-type and step-type HSEs were only 1.7- and 3.3-fold lower respectively than the affinity for 3P-type HSE (right-hand panel of Figure 5C). When C-terminally HA-tagged hHSF1 was introduced into HeLa cells, wild-type hHSF1–HA failed to activate HSE-SV40p-LUC expression; however, the presence of the L189E substitution caused activation of reporter genes containing discontinuous HSEs, as well as continuous HSEs (Figures 5D and 5E). In yeast cells, the L189E substitution raised the restrictive temperature of HA3–hHSF1–HA induced the expression of reporter genes containing discontinuous HSEs, as well as continuous HSEs in vitro and in vivo. hHSF1–M391E–HA was produced in HeLa cells, but was unable to activate reporter gene expression regardless of the type of HSE present (results not shown). The transcriptional defect of this derivative has been reported previously, and was explained by the involvement of HR-C in the transcriptional activation domain [11,32].

Even in combination with C103S, the L189E and M391E substitutions enhanced hHSF1 oligomerization (Figure 5B), and L189E induced the expression of reporter genes containing 3P-, gap- and step-type HSEs in HeLa cells (Figure 5F). Therefore Cys103 is not essential for the recognition of discontinuous HSEs. The central region of hHSF1 has negative regulatory roles, and deletion of this region (Figure 5A) results in a constitutive activator of transcription [11]. A derivative lacking the central region from amino acids 203 to 315 (hHSF1–ΔR) affected neither oligomerization nor HSE binding (Figures 5B and 5C). In HeLa cells, hHSF1–ΔR–HA induced the expression of reporter genes containing continuous HSEs, but not of those containing...
Figure 4  Analysis of hHSF1 derivatives containing the C103S substitution

(A) Cross-linking analysis of hHSF1-C103S derivatives. Labelled polypeptides were incubated at 37°C for 20 min, treated with 0 or 2.0 mM EGS (indicated by − and + respectively) for 20 min, and analysed as described for Figure 3(B). (B) Expression of reporter genes by hHSF1-C103S–VP16 derivatives. HeLa cells were transfected with DNA mixtures containing the indicated HSE-SV40p-LUC reporter genes (100 ng) and hHSF1-C103S–VP16 derivatives (10 ng). Luciferase activity (fold activation) was expressed as described for Figure 2(A), left-hand panel. Results are means±S.D. for at least five experiments. Asterisks indicate significant differences (P < 0.02) when hHSF1-C103S–VP16 was compared with hHSF1-L25F,C103S–VP16 and hHSF1-I35V,C103S–VP16, as determined using Student’s t test.

Figure 5  Analysis of hHSF1 derivatives containing oligomerization-inducing substitutions

(A) Schematic representation of hHSF1 derivatives. The DBD, linker, HR-A/B and HR-C are shown. Numbers indicate amino acid positions. Vertical bars show the positions of amino acid substitutions. hHSF1-ΔR lacks amino acid positions 203–315. (B) Cross-linking analysis of hHSF1 derivatives. Labelled polypeptides were incubated at 37°C for 20 min, treated with 0 or 1.0 mM EGS (indicated by − and + respectively) for 20 min, and analysed as described for Figure 3(B). (C) EMSA of hHSF1–HSE complexes. Left: increasing amounts of unlabelled polypeptides (1.2, 2.4 and 4.8 ng of hHSF1 polypeptides; indicated by open triangles above) were incubated with 32P-labelled HSE3P oligonucleotides at 37°C (and 43°C; wild-type hHSF1) for 20 min. The binding affinities of hHSF1 derivatives relative to wild-type hHSF1 are shown below. Right: polypeptides (2.4 ng) were incubated with oligonucleotides containing 3P-, gap (G)- and step (S)-type HSEs at 37°C for 20 min. The binding affinities for HSEgap and HSEstep relative to HSE3P are shown below. (D) Expression of reporter genes by hHSF1–HA derivatives. HeLa cells were transfected with DNA mixtures containing the indicated HSE-SV40p–LUC reporter genes (100 ng) and hHSF1–HA derivatives (10 ng). Luciferase activity (fold activation) was expressed as described for Figure 2(A), left-hand panel. Results are means±S.D. for at least five experiments. (E) Immunoblot analysis of hHSF1–HA derivatives. HeLa cells were transfected without (indicated by −) or with the indicated hHSF1–HA derivatives (10 ng). Extracts prepared from the cells were subjected to immunoblot analysis using an anti-HA antibody. (F) Expression of reporter genes by hHSF1-C103S–HA derivatives. HeLa cells were transfected with DNA mixtures containing the indicated HSE-SV40p–LUC reporter genes (100 ng) and hHSF1-C103S–HA derivatives (10 ng). Luciferase activity (fold activation) was expressed as described for Figure 2(A), left-hand panel. Results are means±S.D. for at least five experiments.

Changes in protease-sensitivity of hHSF1–HSE complex

The gross structure of hHSF1–HSE complex was analysed using a protease-sensitivity assay. After binding of hHSF1 polypeptides to HSE3P, the complex was treated with trypsin for various periods of time, and the resulting complex was analysed by EMSA (Figure 6). Trypsin treatment converted the complex containing wild-type hHSF1 and HSE3P into faster-migrating two species, T1 and T2. Incubation of the binding reaction mixture at 43°C did not affect the formation of these complexes. When hHSF1 contained the L25F and I35V substitutions, the T1 and T2 complexes were observed; however, the amounts of the discontinuous HSEs (Figures 5D and 5E). Therefore not all of the constitutively active hHSF1 derivatives are able to mediate expression via discontinuous HSEs. WT, wild-type.
T2 complexes increased relative to those of the T1 complexes. In contrast, the L189E and M391E substitutions inhibited the formation of the T2 complex. The differences in the digestion pattern would reflect differences in the accessibility of trypsin to proteolysis sites in these complexes. Although it is unknown why the DBD mutations (L25F and I35V) and the HR mutations (L189E and M391E) oppositely affect the T2 complex formation, these results strongly suggest that these substitutions affect conformation of the hHSF1–HSE complex.

**DISCUSSION**

In *S. cerevisiae* cells, HA1–hHSF1 properly regulated transcription via continuous HSEs consisting of contiguous inverted nGAAn repeats, but failed to do so via discontinuous HSEs containing gaps in the array. Extragenic suppressor analysis revealed that transcriptional activation of HSC82/HSP82 and *YDJ1* is necessary for growth of HA1–hHSF1 cells at elevated temperatures. Intragenic substitution mutations, L25F and I35V, of hHSF1 greatly improved the heat-shock response of genes containing discontinuous HSEs. In *vitro*, these substitutions enhanced hHSF1 oligomerization, and the known oligomerization-prone hHSF1 derivatives containing the L189E and M391E substitutions were capable of binding to discontinuous HSEs. In HeLa cells, expression of hHSF1–HA derivatives containing the L25F, I35V and L189E substitutions led to the activation of reporter genes containing discontinuous HSEs. These results suggest that hHSF1 possesses an intrinsic ability to recognize discontinuous HSEs as well as continuous HSEs, and that this ability is modulated by oligomerization.

Under unstressed conditions, HR-C maintains HSF1 in an inactive monomeric form by forming a triple-stranded coiled-coil with HR-A/B that facilitates HSF1 oligomerization [11,31]. In contrast, mammalian HSF4 lacks an HR-C-like domain and constitutively forms oligomers [33]. We have recently found that human HSF4 recognizes gap- and step-type HSEs and that efficient oligomerization is a prerequisite for binding to discontinuous HSEs [18]. The HR-A/B of yHSF facilitates oligomerization and regulates HSE specificity [34]. Similarly to HSF4 and yHSF, oligomerized HSF1 is capable of binding to discontinuous HSEs. On continuous HSEs, three DBDs of the HSF1 trimer stably bind to three contiguous inverted nGAAn repeats. On discontinuous gap-type and step-type HSEs, only two of the three DBDs may engage in binding to the nGAAn units, and HSF1 readily dissociates from the HSE sequence. Stable oligomerization may inhibit dissociation and/or stimulate rebinding of HSF1.

However, it is puzzling why heat-activated hHSF1 fails to bind to discontinuous HSEs, even though it forms oligomers [18]. Heat-induced disulfide bonding between Cys36 and Cys103 is important for HSE binding [29,30]. Even in combination with the C103S substitution, the L25F, I35V, L189E and M391E substitutions enhanced hHSF1 oligomerization and binding to various types of HSEs. In EMSAs, the mobility of the hHSF1–HSE complex, which contained hHSF1 with the oligomerization-inducing substitutions, was slower than that of the heat-activated hHSF1–HSE complex (Figures 3C and 5C). The digestion of hHSF1–HSE3P complex by trypsin shows that these substitutions alter the accessibility of trypsin to proteolysis sites in the complex. These results suggest that heat-activated hHSF1 and oligomerization-prone hHSF1 have different conformations and imply that heat-activated oligomers fail to undergo the conformational change required for binding to discontinuous HSEs. We presume that HSF1 binds to discontinuous HSEs under other stress conditions [35,36]. Alternatively, interaction between HSF1 and discontinuous HSEs may be involved in gene expression during cell differentiation and development [37–40].

In addition to the intermolecular hydrophobic interactions of HR-A/B, HSF1 oligomerization is modulated by DBD–DBD and DBD–linker interactions [24,29,30]. The Leu25 and Ile35 residues, located within the first α-helix and first β-sheet respectively of the winged helix–turn–helix motif, constitute the hydrophobic core of the protein, and anchor the helix and sheet to the core (see Figure 1F) [41,42]. In the protein–DNA complex, Leu25 and Ile35 do not participate directly in contact with the DNA or in DBD–DBD interactions [43]; however, it was suggested recently that the hydrophobic core of HSF1 is important for oligomerization [44]. These residues are close to Cys36, which forms a disulfide bond with Cys103 located at the N-terminus of the linker region of HSF1 [29,30]. The linker region regulates HSF1 oligomerization [23]. The L25F and I35V substitutions are presumed to affect the structure of the hydrophobic core, thereby enhancing DBD–linker

**Figure 6** Protease-sensitivity assay of hHSF1–HSE3P complex

Polypeptides (1.2 ng) incubated with 32P-labelled HSE3P oligonucleotides at 37 °C and 43 °C; wild-type hHSF1 were treated with trypsin at room temperature for the times indicated. After terminating the reaction by the addition of soya bean trypsin inhibitor, samples were subjected to PAGE and phosphoimaging. Closed arrowheads (T1 and T2) indicate trypsin-resistant protein–DNA complexes. Densitometric scans of T1 and T2 complexes formed after 12 min of trypsin treatment are shown below. WT, wild-type.
interaction and oligomerization, independently of disulphide bond formation.

The temperature-sensitivity of HA_{1}–hHSF1 cells is suppressed by elevated expression of HSC82, HSP82 and YDJ1. The Hsp90 molecular chaperone encoded by HSC82 and HSP82 is not a general chaperone for newly synthesized proteins, but is required for stabilizing the labile conformations of proteins and for the assembly and disassembly of multiprotein complexes [45]. The Hsp40 chaperone encoded by YDJ1 co-operates with Hsp70 to protect newly synthesized proteins from degradation and to promote polypeptide folding and maturation [46]. During conditions of cellular stress, Hsp90 enhances the rate at which damaged proteins are reactivated [47], and Hsp40 and Hsp70 participate in Hsp104-mediated refolding of aggregated proteins [48]. Mutations of yHSF, HSP82/HSC82 and YDJ1 cause cell-cycle arrest in the G_{2}/M-phase, and the yHSF mutation is suppressed by overexpression of HSC82/HSP82 [45,49,50]. Therefore yHSF is involved in the refolding of heat-inactivated proteins, including proteins involved in cell-cycle progression, through transcriptional activation of HSC82/HSP82 and YDJ1. The heat-induced transcription of other genes containing discontinuous HSEs is dispensable for growth at moderately elevated temperatures (e.g. 33°C), but transcription of several of these genes would be indispensable for growth at higher temperatures (e.g. 37°C).

Continuous HSEs consisting of four or more nGAAn repeats mediate a higher level of transcriptional activation than HSEs with three nGAAn repeats, because of co-operative binding of the trimers [51]. In S. cerevisiae, gap-type HSEs are involved in moderate transcriptional activation, whereas step-type HSEs are involved in basal transcription and in low-level stress-induced moderate transcriptional activation, by elevated expression of HSF1. Therefore, gap-type HSEs are involved in basal transcription and in low-level stress-induced moderate transcriptional activation, whereas step-type HSEs mediate a higher level of transcriptional activation than HSEs with three nGAAn repeats, because of co-operative binding of the HSF trimers [51]. In mammalian cells, discontinuous HSEs may account for differential gene- and stress-specific transcription by HSF1.

**REFERENCES**

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SUPPLEMENTARY ONLINE DATA

Mutational analysis of human heat-shock transcription factor 1 reveals a regulatory role for oligomerization in DNA-binding specificity

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Figure S1 Growth of S. cerevisiae cells expressing HA3–hHSF1-L189E and HA3–hHSF1-ΔR

Cells expressing the indicated HSFs were streaked on to YPD medium and incubated at 28 or 32°C for 2 days. The L189E substitution partially suppressed the temperature-sensitivity associated with HA3–hHSF1. However, HA3–hHSF1-M391E failed to support S. cerevisiae cell growth (results not shown). Cells expressing HA3–hHSF1-ΔR were able to grow at 32°C, implying that deletion of the central repression region from HA3–hHSF1 led to an overall activation of various yHSF target genes in yeast cells and enabled cells to grow at elevated temperatures, even though hHSF1-ΔR exhibited low binding affinity to discontinuous gap- and step-type HSEs in vitro.

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