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Activation of the DNA-Binding Ability of Human Heat Shock Transcription Factor 1 May Involve the Transition from an Intramolecular to an Intermolecular Triple-Stranded Coiled-Coil Structure

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Heat stress regulation of human heat shock genes is mediated by human heat shock transcription factor hHSF1, which contains three 4-3 hydrophobic repeats (LZ1 to LZ3). In unstressed human cells (37°C), hHSF1 appears to be in an inactive, monomeric state that may be maintained through intramolecular interactions stabilized by transient interaction with hsp70. Heat stress (39 to 42°C) disrupts these interactions, and hHSF1 homotrimerizes and acquires heat shock element DNA-binding ability. hHSF1 expressed in *Xenopus* oocytes also assumes a monomeric, non-DNA-binding state and is converted to a trimeric, DNA-binding form upon exposure of the oocytes to heat shock (35 to 37°C in this organism). Because endogenous HSF DNA-binding activity is low and anti-hHSF1 antibody does not recognize *Xenopus* HSF, we employed this system for mapping regions in hHSF1 that are required for the maintenance of the monomeric state. The results of mutagenesis analyses strongly suggest that the inactive hHSF1 monomer is stabilized by hydrophobic interactions involving all three leucine zippers which may form a triple-stranded coiled coil. Trimerization may enable the DNA-binding function of hHSF1 by facilitating cooperative binding of monomeric DNA-binding domains to the heat shock element motif. This view is supported by observations that several different LexA DNA-binding domain–hHSF1 chimeras bind to a LexA-binding site in a heat-regulated fashion, that single amino acid replacements disrupting the integrity of hydrophobic repeats render these chimeras constitutively trimeric and DNA binding, and that LexA itself binds stably to DNA only as a dimer but not as a monomer in our assays.

Transcriptional regulation of heat shock protein (hsp) genes is dependent, typically, on multiple arrays (heat shock elements [HSEs]) of the sequence motif NGAAN. HSEs constitute binding sites for heat shock transcription factor HSF (2, 27, 29, 45) which is present in both unstressed and stressed cells (22, 37). Budding yeast contains a single species of trimeric HSF that constitutively binds DNA (37–39, 44). hsp gene expression in this organism appears to be regulated through changes in the transcriptional ability of HSF. The control of HSF activity is more complex in higher eukaryotes (6, 31, 34, 42), many of which express multiple HSF species. Human cells express at least two HSF species (32, 35), only one of which, human HSF1 (hHSF1), is involved in the stress regulation of hsp genes (6, 34).

Sequence comparison revealed conservation in the amino-terminal region of HSF that includes the DNA-binding domain as well as in two regions containing overlapping, hydrophobic heptad repeats or leucine zippers (LZs) of a type known as the 4-3 repeat (LZ1 and LZ2 [20, 24]). Studies with yeast cells have indicated that these LZs are required for trimerization of HSF (30, 38). HSFs from higher eukaryotes share an additional conserved LZ region near the carboxy terminus (LZ3 [32, 35]), and vertebrate HSF species also contain a 12-residue-long region of homology (CTR [33]) which is located downstream from LZ3.

Mammalian HSF1 and *Drosophila* HSF are normally unable to bind to their target DNAs (37, 46) and can be found largely in a monomeric form in extracts from unstressed cells (6, 34, 42, 43). Upon exposure to heat or other forms of stress, HSF forms homotrimers and acquires the ability to bind to HSE DNA (6, 29, 34, 42). Thus, in these organisms HSF activity appears to be primarily regulated at the level of DNA-binding ability.

A general model for the regulation of animal HSF activity by stress has been emerging from recent studies: exposure of cells to amino acid analogs results in increased hsp gene expression (18, 21), suggesting that accumulation of nonfolded proteins may be part of the mechanism triggering HSF activation. This hypothesis has been verified by the demonstration that chemically denatured but not native, purified proteins injected into *Xenopus* oocytes induce hsp gene expression (4). Earlier reports (13, 40) have hypothesized that hsp genes may be subject to negative feedback regulation by one of the hsps, most likely a member of the hsp70 family. hsp70-related proteins appear to interact generically with nonfolded proteins (15, 26, 28), including nascent polypeptides (7). Thus, these hsps, whose availability is expected to change with the level of nonfolded proteins in the cell, appear to be ideally suited to serve as negative regulators of HSF. Indirect evidence for the involvement of hsp70-related proteins in HSF regulation has come from studies with hHSF1 by Abravaya et al. (1) and Baler et al. (5). We hypothesize that inactive, monomeric hHSF1 assumes a metastable, condensed structure formed by specific intramolecular interactions that is stabilized by reversible binding of hsp70. This complex is incapable of trimerization and of acquiring DNA-binding ability. As suggested by an earlier study, LZ3, the CTR element, or both may participate in the formation of the intramolecularly complexed conformation of monomeric hHSF1 (33). During stress, because of the accumulation of unfolded proteins competing for hsp70 bind-

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ing, hsp70 may no longer be readily available for binding to the hHSF1 monomer, which, in the absence of hsp70, may unfold and undergo trimerization, resulting in the acquisition of DNA-binding ability.

Xenopus oocytes injected with RNA encoding hHSF1 and kept at 20°C express hHSF1 as a non-DNA-binding monomer (6). As in human cells, a variable fraction of hHSF1 appears in a relatively unstable complex that may correspond to an hHSF1-hsp70 complex (47). Incubation of oocytes expressing hHSF1 at 37°C, at which temperature injected hsp genes are activated (41), causes hHSF1 to trimerize and to acquire DNA-binding ability (6). Thus, by these criteria, activation of hHSF1 in *Xenopus* oocytes accurately mimics that in human cells. The only obvious difference between activation of hHSF1 in human cells and that in *Xenopus* oocytes is the temperature at which it occurs, i.e., 41 to 44°C in human cells and 33 to 37°C in *Xenopus* oocytes (6). An analogous observation was made recently when hHSF1 was expressed in *Drosophila melanogaster* (10). We interpret these data as further support for the view that hHSF1 is regulated by a cellular protein such as hsp70, whose availability depends on the thermal stability of important groups of cellular proteins that may differ from organism to organism. That the *Xenopus* oocyte is capable of heat-regulating trimerization and acquisition of DNA-binding ability of hHSF1 and that it has a low level of endogenous HSF which is not recognized by anti-hHSF1 antibody render it particularly useful for the identification of structural elements involved in the maintenance of the monomeric conformation of hHSF1. In addition, because of the much lower basal temperature of expression of hHSF1 in *Xenopus* cells (20°C) than in human cells (37°C), protein-protein interactions involving hHSF1 may be more stable in *Xenopus* oocytes than in human cells. Conceivably, intramolecular interactions may be sufficiently stable to alone maintain hHSF1 in its monomeric conformation in *Xenopus* oocytes, i.e., without supporting interactions with cellular factors. Hence, it is conceivable that the complexity of results of a mutational analysis with *Xenopus* cells may be lower than that of a comparable study with human cells.

MATERIALS AND METHODS

Constructions. An hHSF1 cDNA gene, into which an *Nco*I site had been introduced at codon 1, was inserted between the *Hin*dIII and *Eco*RI sites of pGEM-3Zf(+) (6). Internal deletions were constructed by combining appropriate restriction fragments (see Fig. 1), using, where needed, linkers and/or fragments generated by PCR. LZ fragments containing small deletions or substitutions were prepared by a PCR procedure with appropriate primers spanning regions to be altered. $LexA_{87}$-$hHSF1_{79}$ was constructed by inserting between the *Hin*dIII (vector site upstream from the hHSF1-coding sequence) and the filled *Bsp*EI sites of hHSF1 (centered on codon 79) a *Hin*dIII-*Xmn*I fragment from pEG202 (16a), including the first 87 codons of the LexA repressor. $LexA_{87}$-$hHSF1_1$ was prepared by joining the LexA fragment described above to the filled *Nco*I site at codon 1 of hHSF1. $LexA_{87}$-$hHSF1_{79}$-E_{189} and -E_{391} were constructed by combining appropriate restriction fragments from $LexA_{87}$-$hHSF1_{79}$ and hHSF1 mutants E_{189} and E_{391}. To obtain $LexA_{87}$-$hHSF1_{422}$, a *Hin*dIII-*Xmn*I LexA fragment from pEG202, and to prepare $LexA_{202}$-$hHSF1_{422}$, a *Hin*dIII-*Bam*HI (filled) fragment including the entire LexA-coding sequence were ligated to the filled *Bst*EII site of hHSF1 at codon 422. DNA from at least two copies of each mutant was prepared and analyzed by extensive restriction digestion and nucleotide sequencing (in the case of small deletions and substitutions in the hHSF1 gene).

Expression in *Xenopus* oocytes. *Xenopus laevis* females were purchased from Xenopus I, and oocytes were prepared as described previously (41). hHSF1-containing plasmid DNA [in vector pGEM-3Zf(+)] was linearized at a site several hundred base pairs 3′ of the inserted gene, and cDNA genes were transcribed in vitro by an SP6 RNA polymerase-based reaction. Quality and quantity of transcripts were assessed by gel electrophoresis. Batches of stage VI oocytes were given microinjections in the cytoplasm with aliquots of the transcription reaction mixtures. After 2 days of incubation at 20°C and heat treatment for 30 to 45 min at 36 to 37°C or 60 min at 30°C when applicable, extracts from sets of 5 to 10 oocytes were prepared as described elsewhere (11).

Gel shift assays. Extract (2 to 4 μl) was added to 10 μl of a 2× reaction buffer (2× reaction buffer is 24 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES; pH 7.9], 120 mM KCl, 4 mM $MgCl_2$, 0.24 mM EDTA, 0.6 mM phenylmethylsulfonyl fluoride, 0.6 mM dithiothreitol, and 24% glycerol [22]) and 1 μl of a mixture of sonicated salmon sperm DNA (1 mg/ml) and poly(dI-dC) · poly(dI-dC) (1 mg/ml), and the volume was brought to 19 μl with water. After 15 min of preincubation on ice, 1 μl of ^{32}P-labeled DNA probe (approximately 10,000 cpm/μl) was added, and incubation was continued for 15 min at room temperature. Binding reaction mixtures were electrophoresed on 4.5% native polyacrylamide gels in TGE (40 mM Tris, 200 mM glycine, 2.4 mM EDTA) for 4 h at 4°C or, in one experiment, on a limiting-pore-size gel (see Fig. 6). The gels were dried and autoradiographed. For measurements of hHSF1 DNA binding, an HSE probe (6) was used. The LexA probe was 5′ CTGCTGTATATAAAACCAGTG GTTATATGTACAGTACT.

Immunochemical detection of hHSF1 in Western blots (immunoblots) and blots of limiting-pore-size gels. Protein samples (up to 30 μg per lane) were separated on standard sodium dodecyl sulfate (SDS)-polyacrylamide gels (3% stacking gel and 10% separation gel). Prestained molecular weight standards (BRL; high range) were routinely run in parallel. Polypeptides were transferred electrophoretically at 4°C in a solution containing 50 mM Tris, 380 mM glycine, 0.1% SDS, and 20% methanol onto unsupported nitrocellulose membranes (BA-S 85; Schleicher & Schuell) at 12 V for 4 h. For immunodetection, membranes were washed for 10 min in TBS (25 mM Tris-HCl [pH 8.0], 0.136 M NaCl, 2.7 mM KCl), air dried, and blocked in TBS with 4% nonfat dry milk (TN) for 1 h at room temperature. The membranes were then incubated at 4°C with anti-hHSF1 antiserum (usually at a 2,000-fold dilution), which was prepared against recombinant hHSF1 as described previously (6), in TN containing 0.1% Tween 20 overnight with gentle rocking. The membranes were washed extensively in TBS–0.05% Triton X-100, reblocked as before, and incubated at room temperature with a second antibody (alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G) at a 2,000-fold dilution in TN with 0.1% Tween for 2 h. After several washes with TBS–0.05% Triton X-100, the blots were developed in 100 mM Tris-HCl (pH 9.0)–100 mM NaCl–5 mM $MgCl_2$ with a 5-bromo-4-chloro-3-indolylphosphate toluidinium–nitroblue tetrazolium color detection system (Boehringer Mannheim). For electrophoresis on limiting-pore-size gels, extract samples in DNA-binding reaction mixtures (without or, in one experiment in Fig. 6, with LexA DNA probe) were applied to native gradient polyacrylamide gels (4% stacking gel and 5 to 20% separation gel) in TGE and were separated at a constant voltage (13 V/cm) for 20 h at 4°C. Native molecular weight markers used were apoferritin

(880,000 and 440,000), thyroglobulin (669,000), urease (480,000 and 240,000), β-amylase (200,000), alcohol dehydrogenase (150,000), and hemoglobin (64,000). The gels were transferred as described above, except for a preequilibration step in SDS-polyacrylamide gel electrophoresis running buffer for 30 min. After the transfer, lanes containing molecular weight markers were stained with amido black. Immunodetection of hHSF1 employed the procedure described above. It was noted that the amounts of protein synthesized from a particular hHSF1 construct varied from one injection to another probably because of differences in the fraction of oocytes injected successfully, the volume of RNA delivered by injection, and/or the efficiency of protein synthesis of the oocytes. However, careful inspection of results indicated that the amount of hHSF1 wild-type or mutant protein synthesized had no influence on its regulatory phenotype whether it was examined at the level of DNA binding or of oligomerization. The experiments whose results are shown in Fig. 6 were performed analogously, except that Immobilon P membranes were used, and detection was by the enhanced chemiluminescence (Amersham) procedure applied as described by the manufacturer.

RESULTS

Three discrete regions responsible for maintaining hHSF1 in the inactive, non-DNA-binding state. *Xenopus* oocytes contain low levels of endogenous HSF activity as estimated by gel shift assays with extracts from heat-treated (37°C for 45 min) oocytes using an HSE DNA probe (6). When they are injected with hHSF1 RNA synthesized in vitro in a reaction with SP6 RNA polymerase, oocytes express hHSF1 in quantities readily detectable by anti-hHSF1 Western blots of extracts from single oocytes. Extracts prepared from hHSF1 RNA-injected oocytes that have been heat treated after 2 days of incubation at 20°C produce a gel shift signal 1 to 2 orders of magnitude more intense than that of extracts from uninjected oocytes. Extracts from injected, non-heat-treated oocytes do not show DNA-binding activity above background level.

A systematic deletion analysis was carried out to map regions involved in the suppression of hHSF1 DNA-binding ability in unstressed oocytes (Fig. 1). RNA was prepared as described before, and the HSE DNA-binding abilities of hHSF1 wild-type and mutant proteins synthesized in the oocytes were assessed by the gel shift assay. The protein product of hHSF1 mutant E-X was undetectable by anti-hHSF1 Western blotting. As expected, hHSF1 mutant E-SC, which lacks the DNA-binding domain, did not show DNA-binding ability. Mutant B-X was similarly inactive, which may be explained by the observation that although present at a near-normal level in untreated oocytes, the mutant protein was rapidly degraded during heat treatment. Mutant B-P was also incapable of DNA-binding, even though the protein was detected in both heat-treated and untreated oocytes. The lack of DNA-binding ability of this mutant containing a large deletion between the DNA-binding domain and leucine zippers LZ1 and LZ2, which are presumed to be required for trimerization, may be explained either by inappropriate positioning of the DNA-binding domain or by formation of abortive or unstable trimers (see below). Mutant X-P showed weak but detectable HSE DNA binding in unstressed oocytes. As illustrated by the particular DNA-binding assay result presented in Fig. 1 for mutant X-P, DNA-binding activity in heat-treated oocytes of many constitutively DNA-binding (i.e., DNA binding in unstressed oocytes) mutants was variable because of the reduced stability of the mutant proteins; low levels of DNA-binding activity in heat-treated oocytes were noted to correlate with low amounts of mutant protein detected by Western blotting. The result obtained with X-P indicates that removal of amino acid residues 133 to 145, i.e., of a region including the amino-terminal end of LZ1, results in deregulation of HSE DNA-binding ability. Although they are present in oocytes in nearly normal amounts, mutant proteins P-H and HF-N were unable to bind to DNA, presumably because they failed to form sufficiently stable trimers. A second region involved in suppression of DNA-binding ability was uncovered by mutant H-BH, which lacked most of the LZ2 sequences and constitutively bound DNA. For reasons explained in Discussion, a large number of mutants with different deletions between LZ2 and LZ3 was tested. DNA-binding ability was essentially normally heat regulated in all of these mutants. Mutant SA-SA located a third region playing a role in suppression of DNA-binding ability between residues 385 and 442; this region includes LZ3. Results obtained with mutants N-4Z and 4S-P (the latter mutant protein is stable in untreated but not in heat-treated oocytes) tentatively mapped this region between residues 379 and 409, i.e., overlapping LZ3. Deletion of sequences further downstream had no effect on the heat regulation of DNA binding under the assay conditions used here. Thus, three discrete sequence elements overlapping the three LZ regions appear to be required for maintaining hHSF1 in the inactive, non-DNA-binding state in 20°C oocytes (oocytes that were not heat treated). Sequences separating LZ2 and LZ3, those following LZ3, and apparently also those preceding LZ1 do not play a role in the suppression of DNA-binding ability in unstressed oocytes.

The regions that suppress DNA-binding ability also prevent trimerization of hHSF1. To test the different hHSF1 mutants for their ability to undergo heat-induced trimerization, extracts were prepared from heat-treated or untreated oocytes expressing wild-type or mutant hHSF1 and were analyzed by electrophoresis on native, limiting-pore-size gels and anti-hHSF1 blots. Selected results are shown in Fig. 2A, illustrating the five trimerization phenotypes observed (see legend to Fig. 1). A complete list of results is presented in the last column of Fig. 1. Extracts from untreated oocytes expressing wild-type hHSF1 produce a major hHSF1 signal at a molecular weight of about 100,000, corresponding to a monomeric factor (apparent SDS gel molecular weight, 70,000 to 75,000), and a weak signal of variable intensity at about 200,000, presumably representing an unstable hHSF1-hsp70 complex (Fig. 2). These signals were reduced or absent in extract from heat-treated oocytes. The major signal in the latter extract, previously shown to correspond to trimeric hHSF1 by cross-linking experiments (6), was typically very broad and had an average apparent molecular weight of about 700,000. This signal was much wider than that produced by standard proteins and was unaffected by preincubation of extract with ATP expected to release any bound hsp70. Perhaps this result reflects the existence of multiple, interchangeable conformations of trimeric hHSF1. Mutants E-SC and B-X produced monomer signals with intensities only slightly lower than that of wild-type hHSF1 in untreated oocytes. Thus, residues 7 to 131 may not include elements necessary for suppression of hHSF1 trimerization. hHSF1 signals that were distributed, albeit not monotonously, over the entire molecular weight range from 100,000 to 700,000 were observed with mutants B-P and X-P in extracts from both heat-treated and untreated oocytes. Since Western blots failed to provide any indication of degradation (not shown), this suggests that these mutant proteins were capable of constitutive trimerization but that the resulting trimers were less stable than those formed by the wild-type protein. A fraction of these trimers may have dissociated, and some of the released mono-

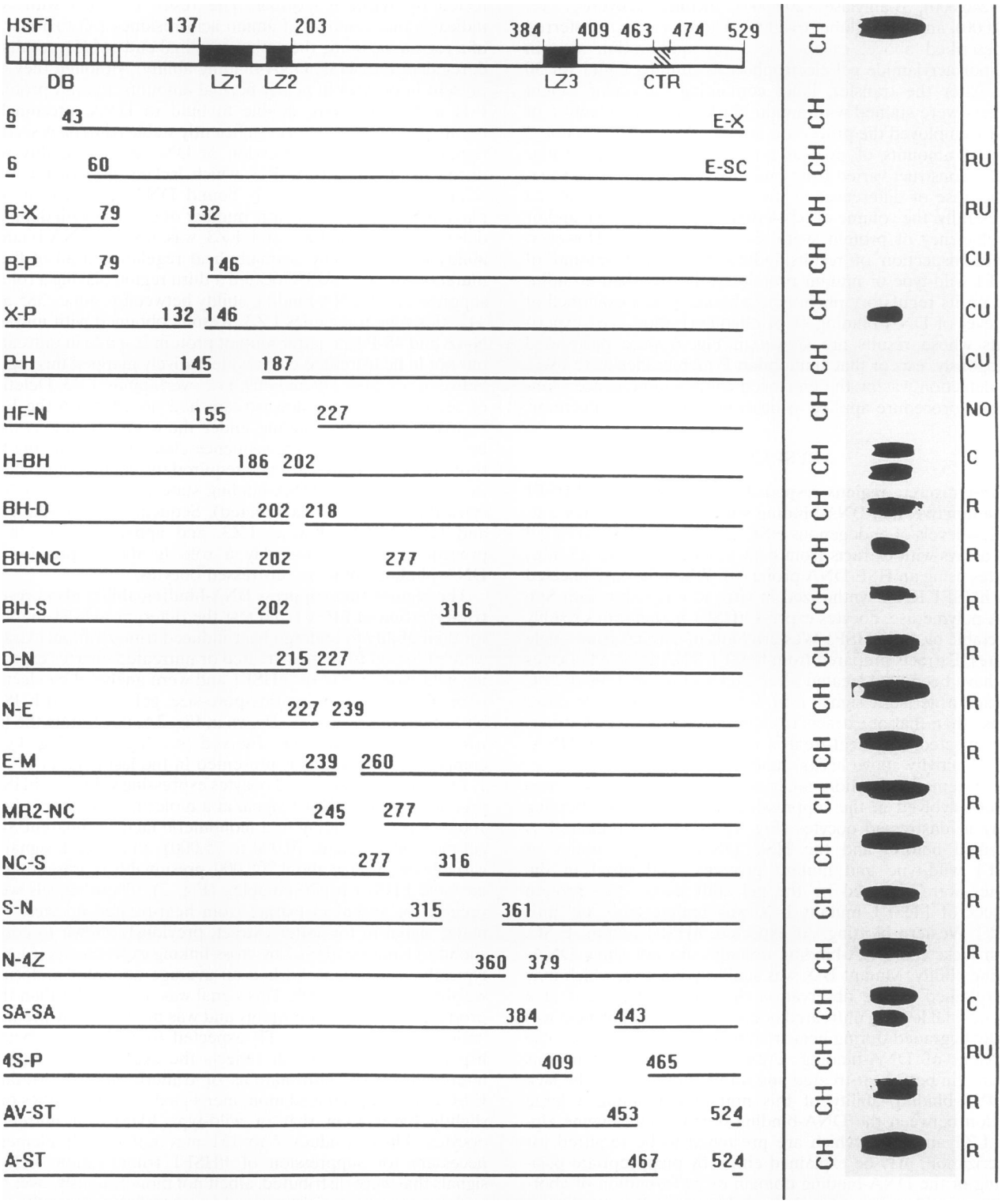

FIG. 1. Structure of internal deletions in hHSF1 and heat regulation of DNA-binding ability and oligomerization. The top line shows known structural features of hHSF1. DB, region forming the DNA-binding domain; CTR, carboxy-terminal mammalian homology region. Numbers refer to the positions of amino acid residues in the deduced protein sequence of hHSF1. Below are the structures of internal deletions; numbers indicate amino acid residues at the deletion endpoints. Results of gel shift assays using an HSE probe (see Materials and Methods) with extracts from heat-treated (H; 37°C for 45 min) or untreated (C) oocytes expressing hHSF1 wild-type or mutant proteins are presented on the right. Only regions containing specific hHSF1-HSE complexes are shown. Trimerization phenotypes are indicated on the far right. RU, regulated/unstable (near-normal level of monomeric hHSF1 in untreated oocytes but no protein detectable in heat-treated oocytes; thus, the term regulated indicates

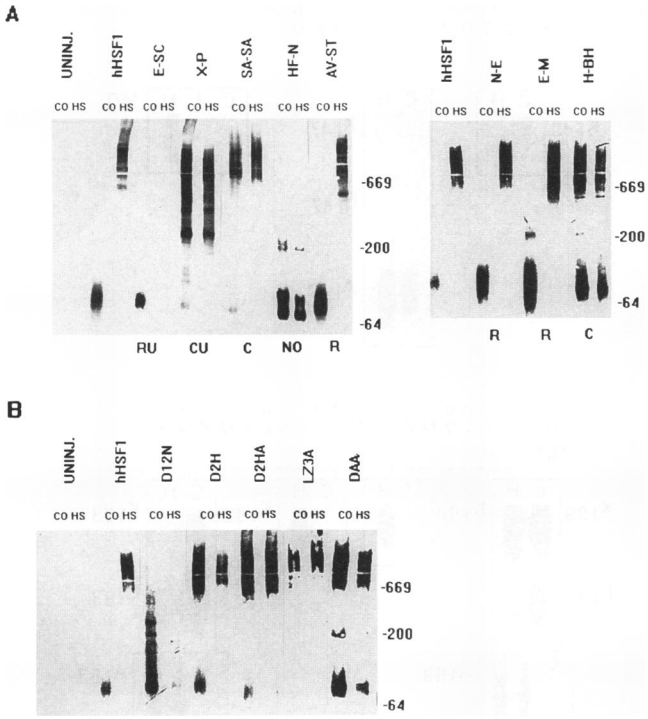

FIG. 2. Anti-hHSF1 blots of limiting-pore-size gels separating extracts from heat-treated (HS) and untreated (CO) oocytes expressing representative hHSF1 mutants with large deletions (A) or with specific, small deletions in LZs (B). Mutant names above the lanes correspond to those in Fig. 1 and 3, respectively. Uninj, extracts from uninjected oocytes. Trimerization phenotypes as defined in the legend to Fig. 1 are indicated below the blots in panel A. Positions of marker proteins (in thousands) are indicated on the sides of the panels. In contrast to the experiment whose results are shown in Fig. 1, extracts from heat-treated and untreated oocytes expressing mutant X-P that produced similar levels of HSE DNA-binding activity in the gel shift assay were used here.

mers may have participated in the formation of aberrant homologous and/or heterologous complexes in the oocytes or in the extract. Further dissociation may have occurred during electrophoresis. (Similar distributions of hHSF1 signals were observed for mutants K_{147} and E_{147}. See text below and Fig. 5.) Thus, the region including the amino-terminal end of LZ1 (residues 133 to 145 are deleted in both B-P and X-P) that plays a role in the suppression of DNA-binding ability also functions to stabilize hHSF1 trimers. Mutant H-BH, which lacks LZ2 and constitutively binds DNA, also constitutively formed trimers. Note that a fraction of H-BH protein was found to have monomer size even in extract from heat-treated oocytes, suggesting that trimerization of this mutant is somewhat less efficient than that of wild-type hHSF1. Heat regulation of trimerization was not affected by deletions in the intervening region between LZ2 and LZ3. In agreement with a previous report (33), constitutively DNA-binding mutant SA-SA, which lacks LZ3, was found to be constitutively trimeric. Normal amounts of monomeric factor were detected in non-heated oocytes expressing mutant 4S-P, AV-ST, or A-ST, suggesting that none of the sequences carboxy terminal of LZ3 are required for suppression of trimerization. In summary, the three regions, overlapping LZ1, LZ2, and LZ3, that prevent the acquisition of DNA-binding ability are also critical for the maintenance of the monomeric conformation of hHSF1. Thus, acquisition of DNA-binding ability and trimerization of hHSF1 are tightly correlated.

That mutants X-P and B-P were capable of forming trimers, albeit unstable ones, suggests that the amino-terminal end of LZ1 (residues 137 to 145) is not absolutely required for oligomerization. Most of the LZ2 sequences could also be deleted without drastically impairing the ability of hHSF1 to trimerize (mutant H-BH). However, removal of the carboxy-terminal half of LZ1 and of all LZ2 sequences (residues 156 to 226; mutant HF-N) rendered the factor incapable of trimerization. Interestingly, deletion of residues 146 to 186 had a less dramatic effect on trimerization, since the respective mutant protein (P-H) was still capable of forming unstable trimers (in non-heat-treated oocytes only). These results suggest that an extended region consisting of LZ1 and LZ2 sequences may participate in trimerization and that the stability of trimers may be determined largely by the length of this region.

Further evidence that the three LZ regions play an essential role in regulation of hHSF1 DNA binding and trimerization. To test whether the deregulation of hHSF1 DNA-binding and trimerization ability observed above was indeed due to the incidental removal of parts of the LZ regions, we deliberately changed the structure of individual LZ regions. We either replaced three residues in the amino-terminal end of LZ1 with a single His residue (D12N), deleted residues 188 to 190 (D2HA) or 189 to 191 (D2H) in LZ2, or replaced residues 395 to 397 in LZ3 with a single Lys residue (LZ3A; Fig. 3). The LZ2 and LZ3 mutants as well as an LZ2-LZ3 double mutant showed high-level, constitutive HSE DNA-binding activity (Fig. 3). All mutants formed trimers constitutively (Fig. 2B). Note, however, that oligomers of D12N appeared to be unstable, which may explain the inability of this mutant to bind DNA (Fig. 2B; the distribution of hHSF1 signals observed for this mutant is consistent with dissociation of trimers during electrophoresis). Thus, structural changes in the amino-terminal end of LZ1 or in LZ2, both of which appear to participate in trimerization of hHSF1, result in constitutive trimerization rather than in the abolishment of trimerization and, in the case of the LZ2 deletion, also in constitutive DNA-binding ability. Similarly, disruption of LZ3 causes constitutive trimerization and DNA binding. Thus, in unstressed cells, all three LZ regions participate in interactions that prevent the association of LZ1-LZ2 regions of different monomers necessary for the formation of the triple-stranded helix of activated, trimeric hHSF1 (see Discussion).

Effects of substitution of hydrophobic residues in heptad repeats. LZ1 and LZ3 contain 4-3 hydrophobic heptad repeats. LZ2 includes four overlapping hydrophobic heptad repeats, pairs of which may also constitute 4-3 hydrophobic repeats. This type of repeat, which occurs in a number of proteins, including fibrous proteins and several transcription factors, is

apparent suppression of trimerization in untreated oocytes, and unstable refers to the occurrence of protein degradation); R, regulated (trimerization characteristics similar to those of wild-type hHSF1); CU, constitutive/unstable (hHSF1 complexes with molecular sizes covering the entire range from monomeric to trimeric hHSF1 in non-heat-treated and, in some cases, also in heat-treated oocytes; in this context, the term unstable refers to instability of trimers, not to protein degradation); C, constitutive (trimers with sizes comparable to those of wild-type hHSF1 are formed in heat-treated as well as untreated oocytes); NO, no trimerization. Results of representative assays of trimerization are shown in Fig. 2.

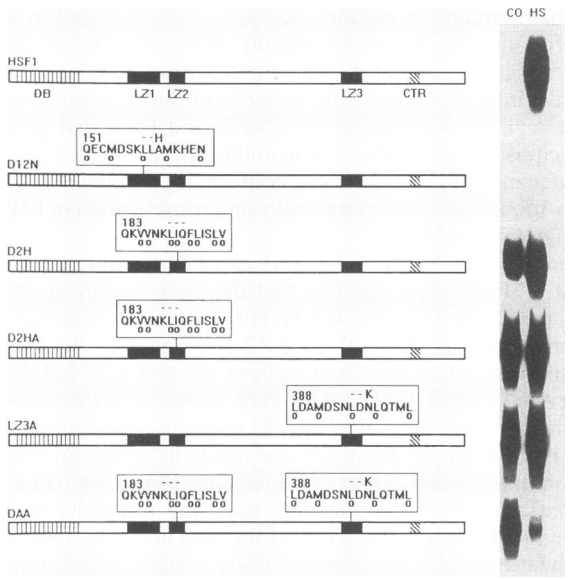

FIG. 3. Heat regulation of DNA-binding abilities of hHSF1 mutants with small deletions in LZ regions. Relevant amino acid sequences, positions of hydrophobic amino acids (circles below the sequences), and changes introduced (above the sequences) are shown in boxes. hHSF1-HSE complexes formed in gel shift assays with extracts from heat-treated (HS) or untreated (CO) oocytes are shown on the right.

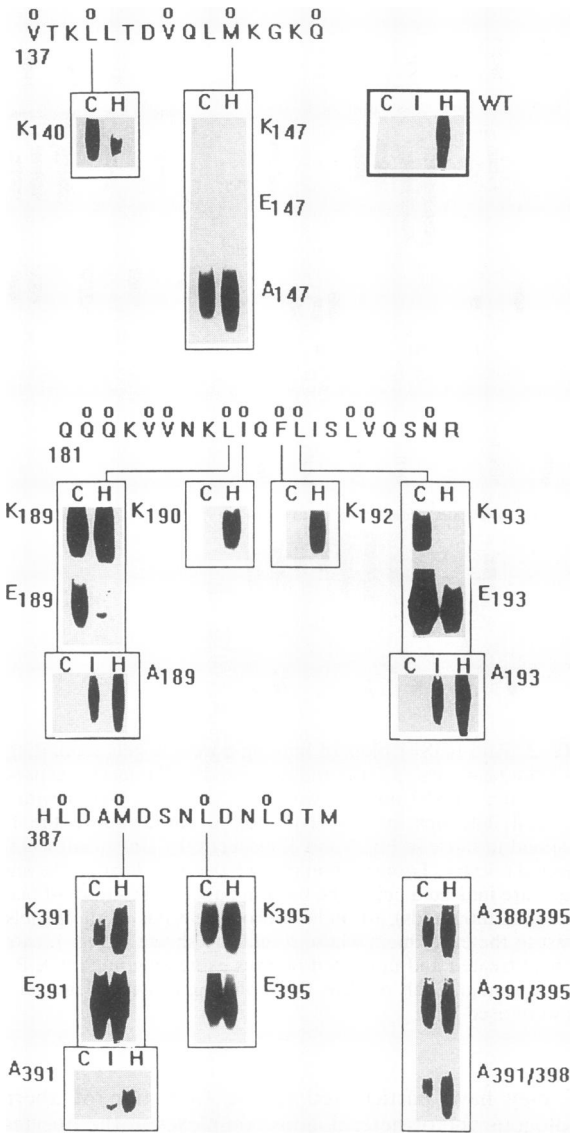

FIG. 4. Heat regulation of DNA-binding abilities of hHSF1 mutants with substitutions of hydrophobic amino acids in LZ1 (top panel), LZ2 (middle panel), or LZ3 (bottom panel) heptad repeats. hHSF1-HSE complexes formed in gel shift assays with extracts from oocytes heat treated for either 30 min at 37°C (H) or for 60 min at 30°C (I) or from untreated oocytes (C) expressing wild-type (WT) or mutant hHSF1 proteins (named according to the nature and position of the amino acid replaced) are shown in boxes below regions of relevant amino acid sequence of hHSF1. Positions of hydrophobic amino acids are indicated by circles.

known to participate in the formation of either double- or triple-stranded coiled coils (14, 23, 25). Both configurations involve simultaneous hydrophobic contacts between residues at both the a and d positions of interacting 4-3 heptad repeat units. To obtain evidence that maintenance of the inactive hHSF1 conformation is predicated on hydrophobic interactions of the three LZs as well as to test whether these interactions involve residues corresponding to both the a and the d positions of 4-3 repeat units, hydrophobic residues in individual heptad repeats in the three LZs were replaced with Ala or hydrophilic amino acids known to be compatible with α-helical structure (Fig. 4). To assess heat regulation of the DNA-binding abilities of substitution mutants, oocytes expressing mutant proteins were heat treated either at 37°C or, in some cases, to detect partial deregulation, at 30°C or were kept at 20°C (Fig. 4), and gel shift assays were performed with oocyte extracts as described above. Replacement of Leu-140 with Lys rendered hHSF1 constitutively DNA binding (Fig. 4, top panel). Mutants in which Met-147 had been replaced with either Lys or Glu lacked DNA-binding ability, presumably because of insufficient stability of the resulting trimers (see below). As expected, because of the small size of this amino acid, substitution with Ala at the same position had a less drastic effect on trimer stability, and the mutant protein showed constitutive DNA-binding ability.

Substitutions were also made in each of the four heptad repeats in LZ2 (Fig. 4, middle panel). Replacement of a hydrophobic residue (Leu-189 or Leu-193) in two of these heptad repeats with Lys or Glu resulted in constitutive DNA binding, and replacement with Ala resulted in DNA-binding activity at 30°C. Analogous substitutions in the other two repeats (Ile-190 and Phe-192) failed to similarly deregulate hHSF1 DNA-binding ability. These results suggest that the former but not the latter two repeats participate in hydrophobic interactions. It is interesting to note that the two repeats affected by the substitutions are part of a 4-3 repeat that is in frame with the LZ1 4-3 repeat. Analogous results were obtained with substitutions in the two hydrophobic repeats of LZ3 (Fig. 4, bottom panel). Replacement of Met-391 or Leu-395 with either Lys or Glu resulted in a constitutively DNA-binding factor. The regulatory changes caused by Ala substitutions tended to be somewhat less drastic than in LZ2; only double but not single Ala substitutions in LZ3 were capable of altering drastically the regulation of DNA-binding ability. Analyses of trimerization by limiting-pore-size gel electrophoresis and anti-hHSF1 blotting, examples of which

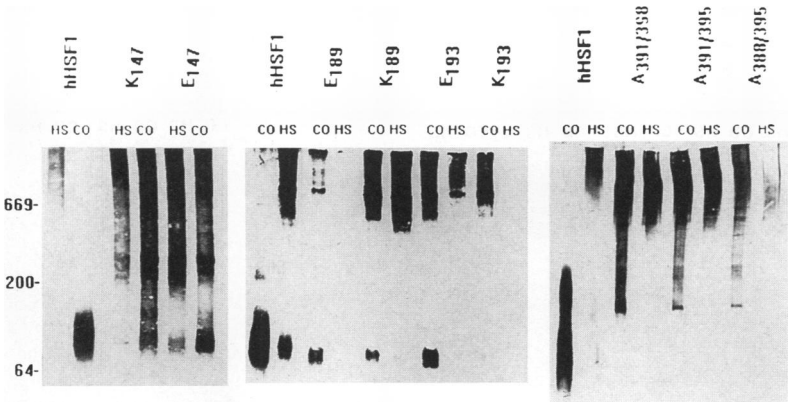

FIG. 5. Analysis of trimerization by limiting-pore-size gel electrophoresis and anti-hHSF1 blotting of mutants containing replacements of hydrophobic amino acids in heptad repeats. See the legend to Fig. 2 for other details and the legend to Fig. 4 for the names of mutants.

are shown in Fig. 5, revealed that each substitution that resulted in constitutive DNA-binding ability also caused constitutive trimerization. These findings indicate that trimerization of hHSF1 and acquisition of DNA-binding ability are prevented by hydrophobic interactions involving heptad repeats of all three LZs. That all heptad repeats whose alteration resulted in deregulation form part of 4-3 repeats supports the idea that the inactive, monomeric hHSF1 conformation may be maintained by coiled-coil interactions of the three LZs. Note that the non-DNA-binding mutants containing substitutions in LZ1 produced patterns similar to those seen with deletions in the same region (mutants B-P and X-P in Fig. 1 and 2), suggesting instability of trimers and formation of aberrant homologous and/or heterologous, hHSF1-containing complexes. Unlike B-P and the LZ1 substitution mutants described here, deletion mutant X-P is capable of binding DNA, albeit less efficiently than wild-type hHSF1. We suspect that this difference in DNA-binding ability is due to some difference in the stability or structure of trimers that was not detected by our native gel blot assays. To a minor extent, hHSF1 complexes of various intermediate apparent sizes were also observed with the double Ala substitutions in LZ3, but only in extracts from non-heat-treated oocytes. Heat treatment may have either caused degradation of mutant protein in the aberrant complexes or resulted in the reformation of these complexes.

hHSF1 LZ interactions can control the DNA-binding ability of a linked, heterologous DNA-binding domain: heat regulation of LexA DNA binding. Several observations support the view that hHSF1 DNA-binding ability is predicated on cooperative effects between the three DNA-binding domains present in the trimer and that LZ interactions serve to prevent these cooperative effects in unstressed cells and to enhance them in stressed cells. First, trimerization induced by stress or by mutational changes affecting the integrity of any of the three LZs enables the HSE DNA-binding function of hHSF1. These findings are compatible with the view that acquisition of HSE DNA-binding ability requires arrangement of three hHSF1 DNA-binding domains in a way that permits cooperative DNA-binding interactions. Second, mutants P-H, HF-N, and B129 (lacking residues 130 to 200 [data not shown]), which fail to properly trimerize because of extensive deletion of LZ1-LZ2 sequences, are incapable of binding effectively to HSE DNA. Thus, disruption of LZ interactions alone does not activate the DNA-binding function of hHSF1; trimerization appears to be required. Third, Harrison et al. (17) overexpressed a short polypeptide containing the DNA-binding domain of yeast HSF but lacking sequences required for trimerization. Although it was capable of residual binding to HSE DNA, the binding affinity of this polypeptide was much lower than that of intact yeast HSF.

If trimerization indeed enhances hHSF1 DNA-binding ability by facilitating cooperative binding interactions of monomeric DNA-binding domains, it should be possible to create hybrid transcription factors capable of heat-regulated DNA binding by replacement of the HSE DNA-binding domain in hHSF1 with domains from other DNA-binding proteins known to bind DNA as dimers but not as monomers. This could be achieved, of course, only if closeness of DNA-binding domains were alone sufficient to allow cooperative binding interactions. If hybrid factors displaying this property could be obtained, this would constitute corroborating evidence for the mechanism of regulation of hHSF1 DNA binding suggested above.

A bacterial LexA repressor was chosen as the donor of the heterologous DNA-binding domain. The 202-residue LexA protein binds DNA in a dimeric configuration. The LexA DNA-binding domain is located between residues 1 and 87, and the dimerization domain is located between residues 88 and 202. We prepared constructs in which a segment containing the first 87 codons of LexA was linked in frame to codon 1 ($LexA_{87}$-$hHSF1_1$) or codon 79 ($LexA_{87}$-$hHSF1_{79}$) of hHSF1 and expressed the chimeric proteins in the oocyte. Gel shift assays revealed heat-regulated binding of both types of chimeric proteins to a LexA DNA probe (Fig. 6A). Anti-hHSF1 blots of native limiting-pore-size gels showed that the proteins were largely monomeric in 20°C oocytes but underwent oligomerization upon heat treatment (Fig. 6B). To demonstrate that heat activation of the LexA DNA-binding ability of the chimeric proteins was tightly correlated with their oligomerization, the DNA-binding and oligomerization properties of derivatives with substitutions in either LZ2 ($LexA_{87}$-$hHSF1_{79}$-E_{189}) or LZ3 ($LexA_{87}$-$hHSF1_{79}$-E_{391}) were analyzed. As predicted from our deletion and substitution studies showing that LZ interactions were involved in maintaining hHSF1 in the monomeric conformation, these derivatives were constitutively oligomeric as well as constitutively binding to the LexA DNA probe.

To rule out the possibility that heat regulation of LexA DNA binding was entirely predicated on masking of the LexA DNA-binding domain rather than on the absence of cooperative interactions between multiple LexA DNA-binding domains in the LexA-hHSF1 monomer, an additional set of LexA-hHSF1 chimeric genes was prepared by linking either

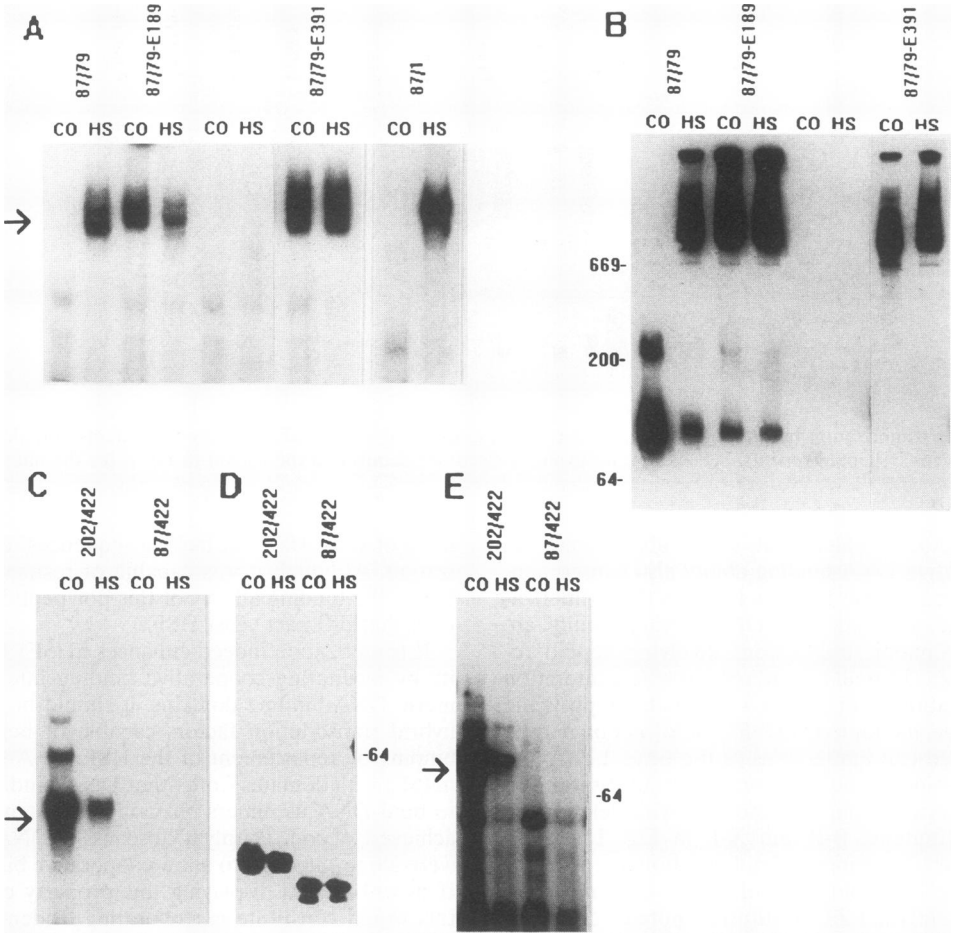

FIG. 6. DNA-binding and oligomerization abilities of LexA-hHSF1 chimeras. (A) Gel shift assay utilizing an end-labeled oligonucleotide probe containing a LexA-binding site of extracts from control (CO) and heat-treated (HS) oocytes that had been injected with RNA prepared in vitro from constructs encoding different LexA-hHSF1 chimeras or that had not been injected (unnamed lanes). Abbreviated names of constructs are indicated above the panels. 87/79, $LexA_{87}$-$hHSF1_{79}$; 87/79-E189, $LexA_{87}$-$hHSF1_{79}$-E_{189}; 87/79-E391, $LexA_{87}$-$hHSF1_{79}$-E_{391}; 87/1, $LexA_{87}$-$hHSF1_{1}$. The position of major protein-DNA complexes is indicated by the arrow. (B) Anti-hHSF1 blot of a native limiting-pore-size gel analyzing some of the extracts listed above. The positions of marker proteins (in thousands) are indicated on the left. (C) Gel shift assay as in panel A. 202/422, $LexA_{202}$-$hHSF1_{422}$; 87/422, $LexA_{87}$-$hHSF1_{422}$. (D) Anti-hHSF1 blot of a native limiting-pore-size gel analyzing the extracts tested for DNA binding in panel C. The position of hemoglobin (64,000) is indicated on the right. (E) Same as in panel D, except that extracts were incubated with radiolabeled LexA DNA probe under binding assay conditions prior to electrophoresis on a native limiting-pore-size gel. The gel was dried and exposed for autoradiography.

the complete LexA-coding sequence or the sequence encoding residues 1 to 87 to a carboxy-terminal segment of hHSF1. Because they lacked the hHSF1 LZs, the chimeras encoded by these constructs, with subunit M_rs of 33,000 and 22,000, respectively, could neither trimerize nor assume a monomeric conformation stabilized by intramolecular LZ interactions. The chimeras, which are referred to as $LexA_{202}$-$hHSF1_{422}$ and $LexA_{87}$-$hHSF1_{422}$, were expressed in the oocytes as described above. Gel shift assays with extracts from heat-treated as well as untreated oocytes using the LexA DNA probe showed that the chimera containing the LexA dimerization domain was highly effective in DNA binding, whereas the one lacking this domain was incapable of DNA binding (Fig. 6C), suggesting that dimerization of the LexA DNA-binding domain was required for effective DNA binding in our assay system. Anti-hHSF1 blotting of a limiting-pore-size gel revealed that the two types of chimeras accumulated to similar levels in the oocytes (Fig. 6D). That the $LexA_{202}$-$hHSF_{422}$ proteins appeared to migrate as a monomer under the conditions in these assays suggested that dimers may form only on the target DNA. To show that this was the case, the $LexA_{202}$-$hHSF1_{422}$ protein-LexA DNA complex was analyzed on the same type of gel and was found to migrate somewhat more slowly than hemoglobin, which was compatible with dimerization of the chimeric protein (Fig. 6E).

DISCUSSION

Previous work had suggested that HSF oligomerization and acquisition of DNA-binding ability are negatively regulated (32, 33). Analysis of deletions across the entire hHSF1-coding region as well as of small deletions within LZs identified three regions that apparently function to maintain the factor in the inactive, monomeric conformation: the first at the amino-terminal end of LZ1 (note that the remainder of LZ1 could not be examined since it is essential for trimerization), the second corresponding to LZ2, and the last corresponding to LZ3. The LZ1 and LZ3 regions contain two and the LZ2 region contains

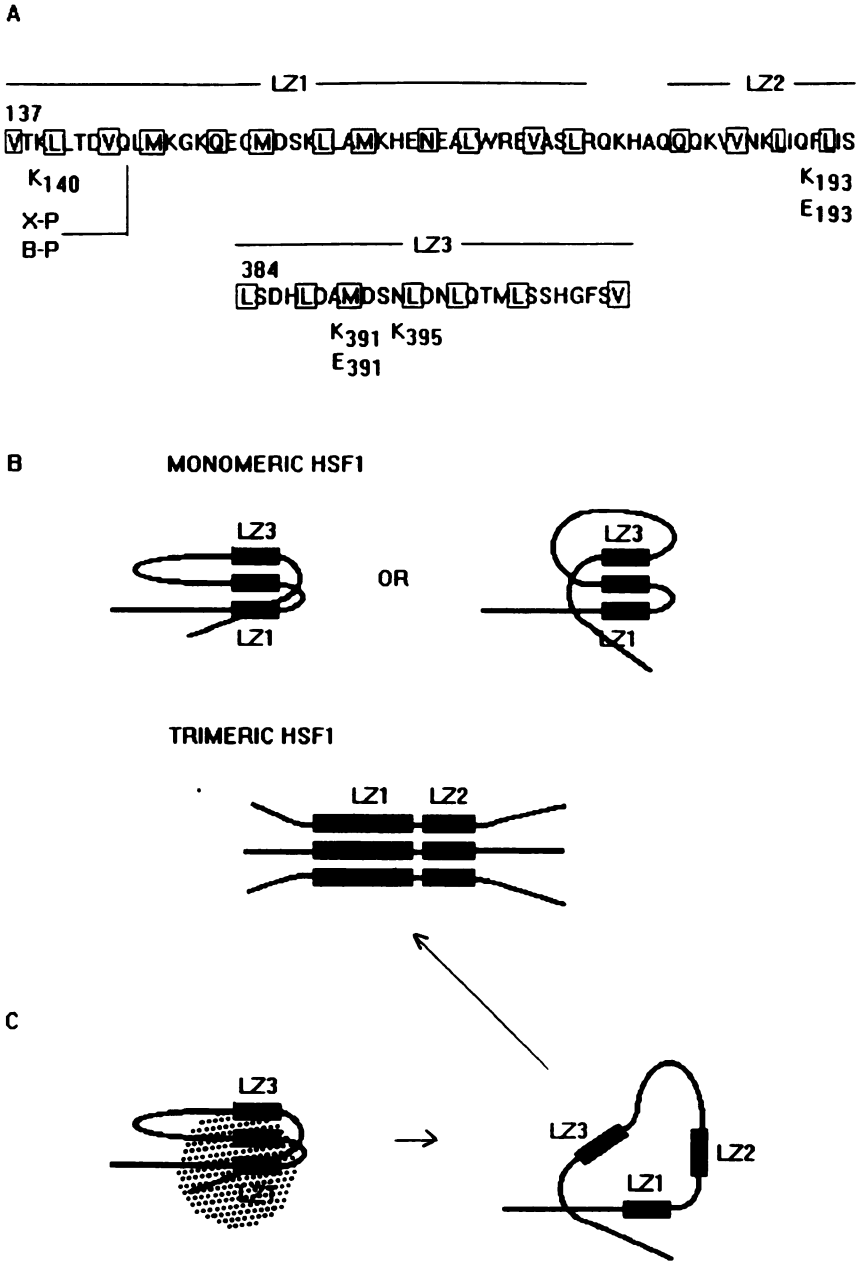

FIG. 7. (A) Deduced amino acid sequences of the LZ1-LZ2 (top sequence) and LZ3 (bottom sequence) regions and locations of mutations analyzed. Hydrophobic amino acids of heptad repeats are boxed. Numbers refer to positions of residues relative to the hHSF1 start codon. (B) Current model of hHSF1 structures analyzed in the oocyte system. (C) Proposed mechanism of regulation of hHSF1 trimerization and DNA-binding ability in mammalian cells; upon heat treatment, hHSF1-hsp70 complex dissociates, which causes the condensed hHSF1 structure to unfold, leading to trimerization of the factor and acquisition of DNA-binding ability.

four overlapping hydrophobic repeats. To obtain evidence that hydrophobic interactions involving these repeats are critical in maintaining the inactive monomer, we replaced single hydrophobic amino acids in individual repeats with charged amino acids. With the exception of two of the four repeats in LZ2, all substitutions in hydrophobic repeats caused constitutive oligomerization and DNA binding. Analogous observations were made with single Ala substitutions in LZ1 and LZ2, whereas only double substitutions in LZ3 produced a similar effect. These observations, therefore, support the view that the monomeric conformation of hHSF1 is maintained by hydrophobic interactions involving all three LZ regions. As shown experimentally for LZ2 and LZ3, each LZ region may provide two independent contact surfaces. These conclusions are reinforced by the finding that substitutions in two of the four LZ2 repeats did not yield a constitutively oligomeric and DNA-binding factor, suggesting that not every mutation within a general region involved in interaction but rather only substitutions of residues lying in the interacting surface cause deregulation of hHSF1.

How do the three repeats interact to form the inactive hHSF1 conformation? Results obtained from our analysis of

substitutions of hydrophobic residues in the three LZs of hHSF1 suggest that these LZs may participate in coiled-coil interactions that may be of either a double- or a triple-stranded nature. That sequence changes in any one of the three LZs cause deregulation of hHSF1 appears to rule out models invoking the formation of double-stranded coiled coils that would involve only two of the LZs. Nevertheless, the proximity of LZ1 and LZ2 raises the question of whether a region including parts of both LZ1 and LZ2 may serve as an extended target for interaction with LZ3. However, assuming that the interacting LZ regions are mostly in an α-helical conformation, our results are difficult to reconcile with this type of model; LZ3 appears to be too short to make simultaneous contact with the regions within both LZ1 and LZ2 that were shown to be critical by our experiments with deletion and substitution mutants (Fig. 7A). In a variant model, the carboxy-terminal portion of LZ1 may form a loop that allows the amino-terminal ends of LZ1 and LZ2 to each interact with part of the short LZ3 region. Although this model is theoretically feasible, the interactions which it implicates may be of only marginal stability (19, 25). These considerations lead us to propose that the core of the condensed monomer structure of inactive hHSF1 consists of a triple-stranded coiled coil formed by interactions among all three LZs (Fig. 7B). In an attempt to characterize the trimeric conformation of yeast HSF, which may closely resemble that of trimeric, active hHSF1, Peterand-erl and Nelson (30) examined the oligomerization properties of a peptide spanning yeast LZ1 and LZ2 and concluded that this peptide oligomerizes and forms a triple-stranded coiled coil. If correct, these models of the conformations of inactive, monomeric and active trimeric hHSF1 would imply that the process of activation and/or deactivation of hHSF1 is remarkably symmetrical and involves the interconversion of intramolecular and intermolecular coiled-coil structures.

While the mutagenesis analysis discussed above strongly suggests that the hydrophobic repeats in LZ1 to LZ3 are important to prevent trimerization of the factor, it does not provide direct evidence for direct interactions between the LZs in the hHSF1 monomer. The same applies to recent findings made with hHSF2, a factor regulated very differently from hHSF1 and not able to be activated by stress but related structurally to hHSF1, showing that the intracellular localization of this factor is altered by mutations in the amino-terminal LZs or in LZ3 (36). The cause of uncertainty about the model described above is that hHSF1, although predominantly present as a monomer in extract from unstressed cells, may not be monomeric in the cells. Indeed, as discussed in the introduction, the current model of stress regulation, which is based on a considerable body of indirect evidence, involves hsps, particularly of the hsp70 type, as negative regulators of HSF activation (1, 5, 47). Thus, while we favor a model in which cellular proteins stabilize an intramolecularly complexed hHSF1 monomer (see also below), we cannot currently rule out the possibility that the LZs in hHSF1 interact with a cellular protein(s) rather than with one another. Clearly, verification of the proposed structure of the hHSF1 monomer eventually will require appropriate physical studies.

Since, in the model described above of the structure of the inactive monomer, the region including LZ3 folds back on the molecule to contact LZ1 and a LZ2, it was tempting to speculate that the sequences between LZ2 and LZ3 may play more than merely a structural role and may drive the stress-induced unfolding of the condensed monomer structure that precedes trimerization. To address this possibility, we prepared a large number of mutants by deleting various portions of the LZ2-LZ3 tether region or by reducing its length to different extents. If the tether region indeed guided unfolding, one would predict that some of these deletions, either because they removed the active principle of the mechanism or because they altered the relative positions of the amino- and carboxy-terminal portions of the molecule, would cause incorrect monomer unfolding and trimerization. We failed to observe any drastic change in the oligomerization and DNA-binding properties in any of the deletion mutants. Thus, if the tether region plays an active role in unfolding, it must be functionally redundant to a remarkable degree.

As also alluded to in the introduction, a mutagenesis analysis of the kind described herein might uncover two different types of interacting regions involved in suppression of DNA-binding ability and trimerization of hHSF1, the first type consisting of regions participating in intramolecular interactions such as were postulated for the LZ regions and the second type including target regions for a regulatory cellular protein(s) such as hsp70. The possibility that the latter type of interaction plays an important role in controlling hHSF1 activity is supported by findings that DNA-binding ability and trimerization of hHSF1 are regulated at the *Xenopus*-specific heat shock temperature in oocytes of this organism (6) and at the *Drosophila*-specific temperature in *D. melanogaster* (10). However, depending on differences between the stability of the intramolecular interactions underlying the formation of the inactive monomer, different test systems may differ in their abilities to uncover sequences involved in intermolecular regulatory interactions. Since the *Xenopus* oocyte system operates at 20°C rather than at 37°C, the basal temperature of human cells, one would predict that intramolecular interactions forming the condensed structure of the inactive hHSF1 monomer would be substantially more stable in oocytes than in human cells and may alone be capable of maintaining the monomeric conformation. Thus, sequences affecting intermolecular interactions that may be uncovered in human cells may not be revealed by the *Xenopus* oocyte test system, or, put differently, a mutational analysis of hHSF1 in *Xenopus* oocytes may permit the selective mapping of sequences involved in intramolecular interactions. These considerations may provide an explanation for a discrepancy between our results and those of an earlier study of hHSF1 in human cells (33). While our respective studies are in agreement that gross changes in LZ3 render hHSF1 constitutively trimeric and DNA binding, they differ with respect to the importance of the CTR sequence. A mutant virtually identical to AV-ST herein that lacks the CTR region was found to render hHSF1 constitutively trimeric and DNA binding in human cells. In contrast, this mutant, although perhaps somewhat more sensitive to heat activation than wild-type hHSF1, is incapable of DNA binding and is monomeric in at 20°C oocytes but acquires DNA-binding ability and trimerizes upon heat treatment. Thus, analysis of 37°C human cells maps an additional element not detected by the 20°C *Xenopus* oocyte assay that may play a role in an interaction between hHSF1 and a regulatory protein such as hsp70.

As discussed above, although the coiled-coil structure of the hHSF1 monomer is perhaps stable in 20°C *Xenopus* oocytes, it may not be able to survive the conditions prevailing in human cells (high basal temperature, etc.) without stabilization by a regulatory protein(s). Thus, binding and release from binding of this protein(s) would regulate the trimerization and DNA-binding ability of hHSF1 in human cells (Fig. 7C). On the basis of a recent study (47) that demonstrated the existence of a major inactive hHSF1-hsp70 complex in human cells, we suggest that the coiled-coil structure of the hHSF1 monomer may be stabilized by binding of a protein(s) of the hsp70 family. It is interesting to note that events somewhat analogous to

those postulated to occur in the course of hHSF1 activation may take place during the maturation of trimeric influenza virus hemagglutinin, including binding of an hsp70-like protein to a monomeric hemagglutinin precursor, release of the hsp70-like protein upon its trimerization (12, 16), and reformation of triple-stranded coiled-coil structures upon conversion of the trimer to the fusogenic state (8).

Our studies revealed a tight correlation between hHSF1 trimerization and acquisition of DNA-binding ability. The relationship between these two processes is unknown. Several mechanisms by which trimerization may result in activation of the DNA-binding function of hHSF1 can be envisaged. First, the DNA-binding domain may be masked in the condensed hHSF1 monomer. The large-scale structural change resulting from trimerization may render the DNA-binding domain accessible to DNA. Second, the functional DNA-binding domain of hHSF1 may be trimeric. Third, each hHSF1 monomer contains a DNA-binding domain that may have a relatively weak affinity for HSE, the target DNA element. Trimerization concentrates three DNA-binding domains within a narrow space, which may facilitate their cooperative binding to the target DNA sequence. The observations that deletion of the first two LZs renders HSF incapable of DNA binding and that a segment containing only the DNA-binding domain of yeast HSF binds DNA with a much lower affinity than intact yeast HSF (17) are most readily explained by the third mechanism. We attempted to obtain positive evidence for this third mechanism by analyzing the properties of LexA-hHSF1 chimeras. LexA was chosen because this protein normally binds its target DNA as a dimer and because the location of its dimerization domain is known, permitting its separation from the DNA-binding domain. To demonstrate that dimerization of the LexA protein is required for efficient DNA binding, we fused either the entire LexA-coding sequence or an amino-terminal segment encoding only the DNAbinding domain to a carboxy-terminal segment of hHSF1. The resulting constructs, $LexA_{202}$-$hHSF1_{422}$ and $LexA_{87}$-$hHSF1_{422}$, were expressed in *Xenopus* oocytes, and the DNA-binding abilities of the chimeras were assessed by gel shift assays. As predicted, only the chimera containing the entire LexA sequence bound to its target DNA. Analysis of the chimeric protein-DNA complex on a limiting-pore-size gel suggested that the protein bound DNA as a dimer. Note that although this experiment showed the importance of dimerization, it did not reveal whether LexA DNA binding required exact alignment of monomers to assemble a dimeric DNA-binding domain or whether it was due to cooperative binding interactions facilitated by dimerization. When the LexA DNA-binding domain segment was linked to various positions near the amino terminus of hHSF1 (results with two of these constructs, $LexA_{87}$-$hHSF1_1$ and $LexA_{87}$-$hHSF1_{79}$, are reported herein), the resulting chimeras were heat regulated for oligomerization as well as for binding to a LexA target DNA sequence. Experiments with mutant derivatives containing either a nonfunctional LZ2 or LZ3 region indicated that oligomerization of the chimeras and their LexA DNA-binding abilities are tightly linked. That oligomerization, presumably trimerization, was required for activation of LexA DNA-binding and that this activation was largely independent of the relative position of the LexA DNA-binding domain with respect to hHSF1 sequences support the view that LexA DNA binding by the chimeras was controlled by the third mechanism discussed above. Oligomerization of the chimeras appears to have served to confine multiple LexA DNA-binding domains to a narrow space, increasing the probability of cooperative DNA-binding interactions. Since it functioned to control LexA DNA binding by the LexA-hHSF1 chimeras, it is likely that the same mechanism also operates to regulate the DNA-binding ability of the hHSF1 DNA-binding domain.

We have not yet attempted to test whether DNA-binding domains other than that of LexA can be brought under the control of hHSF1. Nevertheless, the relative positional independence of the LexA DNA-binding domain in functional, heat-regulated hHSF1 chimeras suggests that it is possible to subject to heat shock control other DNA-binding domains whose binding abilities depend on oligomerization. If this is the case, hHSF1 chimeras may provide a useful, novel tool for studies of gene regulation.

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