Heat stress response and heat stress transcription factors

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Expression of heat shock protein (HSP)-coding genes is controlled by heat stress transcription factors (Hsfs). They are structurally and functionally conserved throughout the eukaryotic kingdom. In addition to the DNA-binding domain with the helix-turn-helix motif essential for DNA recognition, three functional parts in the C-terminal activator domain were characterized: (i) the HR-A/B region is responsible for oligomerization and activity control, (ii) the nuclear localizing signal (NLS) formed by a cluster of basic amino acid residues which is required and sufficient for nuclear import and (iii) short C-terminal peptide motifs with a central Trp residue (AHA elements). These three parts are indispensible for the activator function. A peculiaritiy of plants is the heat shock-inducible new synthesis of Hsfs. In tomato HsfA1 is constitutively expressed, whereas Hsfs A2 and B1 are heat shock-inducible proteins themselves. We used Hsf knock-out strains of yeast and transient reporter assays in tobacco protoplasts for functional analysis of Hsf-coding cDNA clones and mutants derived from them. HsfA2, which in tomato cell cultures is expressed only after heat shock induction, tends to form large cytoplasmic aggregates together with other HSPs (heat stress granules). In the transient expression assay its relatively low activator potential is evidently due to the inefficient nuclear import. However, the intramolecular shielding of the NLS can be released either by deletion of a short C-terminal fragment or by coexpression with HsfA1, which forms hetero-oligomers with HsfA2.

1. The heat stress response

In 1962, the pioneering work of the Italian developmental biologist F Ritossa led to one of the most seminative discoveries in modern cell biology. After a fortuitous increase of the temperature of the incubator with the *Drosophila* cultures, he observed remarkable changes in puffing, i.e., gene activity patterns, in polytene chromosomes in larval salivary glands (Ritossa 1962). Surprisingly enough, the same reprogramming of transcription was also observed with chemical stressors like salicylate, 2,4-dinitrophenol and azide. About 10 years later Tissieres *et al* (1974) identified the newly formed heat stress proteins (HSPs), and McKenzie and Meselson (1977) characterized the corresponding mRNAs. Due to the favourable properties, i.e., heat stress-inducible mass formation of new mRNAs and proteins, the *Drosophila* heat shock (hs) genes were among the first amenable to cloning procedures (for early summaries see Ashburner and Bonner 1979; Schlesinger *et al* 1982). Soon, the rapidly developing field included investigations of other eukaryotic organisms and bacteria. It turned out that Ritossa in fact had discovered the central parts of a general stress response system conserved throughout the living world (Schlesinger *et al* 1982; Lindquist and Craig 1988; Nover *et al* 1990; Nover 1991).

To present an overview of 35 years of molecular cell biology researeh in this field, we will use a model of a stressed eukaryotic cell (figure 1). The essential elements of this model can be summarized as follows (for references see Nover 1991; Nover and Scharf 1997):

- Besides hs, a large number of chemical stressors cause activation of heat stress genes.
- Very likely the stress sensing system in cells are deviations of protein homeostasis, i.e., of the equilibrium

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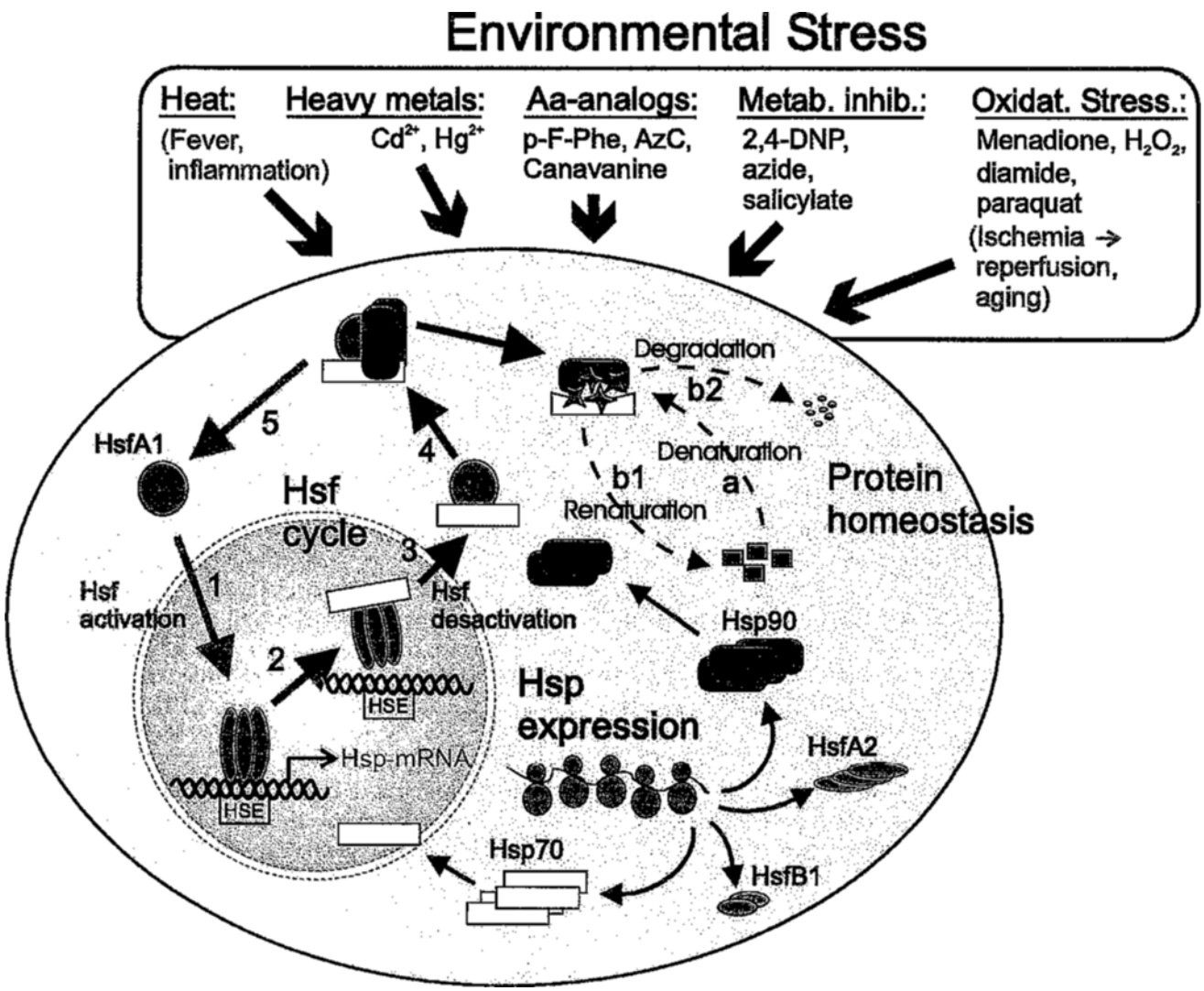


Figure 1. Autoregulation of the heat stress response in a model eukaryotic cell (from Morimoto 1993, modified).
Besides heat stress, a considerable number of seemingly unrelated chemical stressors (examples in the box at top) are able to trigger heat stress gene transcription. Very likely, they all cause accumulation of denatured or immature proteins, which tend to form aggregates and bind molecular chaperones of the Hsp70 and Hsp90 family. Thus, the transient disruption of protein homeostasis results in a deficiency of free chaperones in the cytoplasm with subsequent activation and nuclear import of Hsf. In a type of autoregulatory circuit, hs gene expression is assumed to restore the initial state including inactivation of Hsf (steps 2–4 of the Hsf cycle) and protein renaturation (b1) or degradation (b2). A peculiarity of plants is the synthesis of new Hsfs, e.g., Hsfs A2 and B1, as a result of the hs response (see text).

between new synthesis, folding, intracellular targeting, biological function and degradation of proteins.

- HSPs and other members of the 11 conserved Hsp families were characterized as molecular chaperones essential for maintainance or restoration of protein homeostasis (see Forreiter and Nover 1998). Denaturation of proteins and problems in the processing of newly formed proteins during stress are assumed to result in a decrease of the pool of free chaperones.
- The regulatory proteins (heat stress transcription factors, Hsfs) exist as inactive proteins mostly found in the cytoplasm. Stress causes its activation with oligomerization and recompartmentation to the nucleus and its binding to the promoter target sequences (HSE) triggers transcription of the hs genes.
- New formation of HSPs is assumed to replenish the pool of free chaperones. There is good evidence that in a kind of autorepression of some of the chaperones (e.g., Hsp70, Hsp90) is involved in the second part of the Hsf cycle leading to restoration of its inactive state.
- A peculiarity of plants is the existence of hs-induced forms of Hsfs, which may play a major part in the

modulation of transcription in the course of a long-term hs response.

2. The unfolded protein response

Though not rigorously proven, the concept that accumulation and aggregation of denatured proteins are in the center of the stress sensing system (figure 1) is broadly accepted. It is based on a number of circumstantial evidence compiled in table 1. Details of the mechanism were elaborated in two cases, (i) control of chaperone levels in the ER of yeast and mammalian cells and (ii) control of chaperone synthesis in *E. coli*.

(i) The unfolded protein response in eukaryotes is responsible for the adjustment of ER-bound chaperone levels to the need of protein processing in this compartment. Details of the signalling mechanism were recently elaborated (Cox and Walter 1996; Sidrauski *et al* 1996; Kawahara *et al* 1997; Sidrauski and Walter 1997). Due to the continuity of the ER membrane with the nuclear envelope, a transmembrane receptor kinase (Ire1p) directly connects the lumen of the ER with the nucleoplasmic compartment. Sensing of excess of unfolded proteins in the ER by the N-terminal domain of the receptor leads to its activation connected with autophopsphorylation and oligomerization (Mori *et al* 1992). Upon phosphorylation, the C-terminal domain of the receptor in the nucleoplasm acquires site-specific endonuclease acivity required for the generation of the mature mRNA of the Hac1p transcription factor. By a hitherto unique splicing mechnism the Ire1p endonuclease domain removes a small intron near the 3′ end of the Hac1 pre-mRNA. The two exons are subsequently religated by tRNA ligase. Hac1p binds to the unfolded protein response elements (UPRE) of genes encoding ER-associated chaperones. There is evidence that a similar mechanism is operative in mammalian cells as well (Nikawa *et al* 1997).

(ii) The unfolded protein response in *E. coli* involves two regulons controlled by two promoter-specific sigma factors. Denatured proteins accumulating in the cell affect the availability of the DnaK/DnaJ/GrpE chaperone machinery, which controls the activity and cellular level of sigma 32 (RpoH, Bukau 1993; Gamer *et al* 1996; Yura 1996; Kanemori *et al* 1997; see chapter by Nakahigashi *et al* 1998). On the other hand, misfolded proteins in the periplasmic space and outer membrane cause activation and increased synthesis of sigma 24 (RpoE). Transmembrane signalling in this case is mediated by the RseB/RseA/RseC complex. The sensor subunit (RseB) in the periplasmic space is bound to the RseA transmembrane subunit. Binding of unfolded proteins to the RseB/A/C complex evidently releases sigma 24 from its binding site at the intracellular domain of RseA (DeLasPenas *et al* 1997; Missiakis *et al* 1997). In addition

Table 1. Evidence for the role of denatured or malformed proteins in hs signal transduction and autoregulation of the hs response (from Nover and Scharf 1997)

1. Heat and chemical stressors cause protein denaturation/aggregation; newly synthesized proteins are particulary affected (Lee and Hahn 1988; Bensaude *et al* 1990; Dubois *et al* 1991; Pinto *et al* 1991; Beckman *et al* 1992; Kampinga 1993; Stege *et al* 1994). The efffect may be intensified by ATP depletion during stress (Beckman *et al* 1992; Nguyen and Bensaude 1994).

2a. Injection of denatured proteins into *Xenopus* oocytes leads to Hsf activation (Ananthan *et al* 1986; Mifflin and Cohen 1994a,b).

2b. Hsf activation and hs gene expression results from the generation of abnormal proteins *in situ*, e.g., by synthesis of recombinant or mutant/defective proteins (Goff and Goldberg 1985; Hiromi and Hotta 1985; Lee and Dewey 1987; Grant *et al* 1989), by incorporation of amino acid analogues (Kelley and Schlesinger 1978; Hightower and White 1981; Thomas and Mathews 1984), by oxidative stress with formation of non-native disulfide bonds (McDuffee *et al* 1997) or by inhibition of the proteasome pathway (Bush *et al* 1997; Lee and Goldberg 1998).

3. Unfolded abnormal or denatured proteins interact with the Hsp70/DnaK system (Dubois *et al* 1991; Palleros *et al* 1991; Baler *et al* 1992; Langer *et al* 1992; Hendrick *et al* 1993; Schroeder *et al* 1993).

4. Genetic and biochemical evidence indicate that the level of free Hsp70 controls the extent of hs response (DiDomenico *et al* 1982; Werner-Washburne *et al* 1987; Boorstein and Craig 1990; Stone and Craig 1990; Mosser *et al* 1993; Rabindran *et al* 1994; Kim *et al* 1995; Xiao and DeFranco 1997); Hsp70/Hsp40 directly interact with the activation domain of human Hsf1 (Shi *et al* 1998).

5. Hsfs physically interact with Hsp70 (Abravaya *et al* 1992; Rabindran *et al* 1994; Baler *et al* 1996). Large complexes of both proteins are found in cytoplasm of non-stressed NIH-3T3 cells (Nunes and Calderwood 1995).

6. Hsf activity in non-stressed tobacco protoplasts is repressed by co-expression of Hsf with Hsp70 and Hsp90 (C Kirchner and K D Scharf, unpublished). Overexpression of mammalian HSFs in mammalian cells and *Xenopus* oocytes results in unregulated activity (Baler *et al* 1993; Sarge *et al* 1993). On the other hand, overexpression of Hsc/Hsp70 in mammalian and *Drosophila* cells was found to accelerate the deactivation of Hsf (Mosser *et al* 1993; Rabindran *et al* 1994; Kim *et al* 1995; Baler *et al* 1996; Shi *et al* 1998).

to this general stress response system, there is another transmembrane phosphorelay system with the CpxA as sensor histidine kinase, which is activated by unfolded proteins in the periplasmic space and phosphorylates the intracellular CpxR transcription factor (Danese and Silhavy 1997; Pogliano *et al* 1997; Missiakis and Raina 1997a,b).

3. Basic structure of Hsfs

Generally, selective transcription of eukaryotic genes requires interaction of sequence specific transcription factors with the highly complex basal transcription machinery (transcriptosome) assembled from about 70 subunits around the start site of a gene (Struhl 1996; Kadonaga 1998; Struhl and Moqtaderi 1998). Similar to many other proteins regulating gene activity, heat stress transcription factors (Hsfs) have a clearly defined modular structure (Wu 1995; Nover *et al* 1996). Despite a considerable variability in size and sequence, their basic structure is very similar (figure 2).

(i) Close to the N-terminus, the highly structured DNA-binding domain is the most conserved part of Hsfs. It is formed of a three-helical bundle (H1, H2, H3) and a four stranded antiparallel β-sheet (β1, β2, β3, β4). The hydrophobic core of this domain ensures the precise positioning of the central helix-turn-helix motif (H2-T-H3, figure 2B) required for specific recognition of the heat stress promoter elements (Damberger *et al* 1994; Harrison *et al* 1994; Vuister *et al* 1994; Schultheiss *et al* 1996). This palindromic binding motif (HSE: 5′-AGAAnnTTCT-3′) was originally deduced from studies with *Drosophila* heat stress genes (Pelham 1982; Pelham and Bienz 1982), but was found later on to be conserved in all eukaryotes (Bienz and Pelham 1987; Nover 1987, 1991).
(ii) The oligomerization domain (HR-A/B region) is connected to the DNA-binding domain by a flexible linker of variable length (15 to 80 amino acid residues). The heptad pattern of hydrophobic amino acid residues in the HR-A/B region suggest a coiled-coil structure charateristic of Leu-zipper type protein interaction domains (Crick 1953; Lovejoy *et al* 1993). Unfortunately, precise structural data are lacking for Hsf as total protein. But Peteranderl and Nelson (1992) demonstrated the formation of a tripple-stranded coiled coil structure for the HR-A/B fragment of the yeast Hsf1.

In plants, there are two subfamilies of Hsf clearly separated by peculiarities of their HR-A/B regions (Scharf *et al* 1994; Nover *et al* 1996). Hsfs of the B-type are similar to all non-plant Hsfs (see figure 2). In contrast to this, Hsfs of the A-type have an extended HR-A/B region due to an insertion of 21 amino acid residues between the A and B part (see example for HsfA2 in figure 2B). The structural significance of this is unclear. But there is evidence that oligomerization needs the homologous HR-A/B region.
(iii) The nuclear localization signals (NLS) of Hsfs are so-called bipartite clusters of basic amino acid residues (Dingwall and Laskey 1991). They are marked by NLS in the block diagrams in figure 2. These positively charged recognition motifs serve the assembly of a nuclear import complex built of the NLS receptor (importin α) and two other subunits, importin β and Ran(GTP) (Görlich and Mattaj 1996; Hurt 1996). Very likely, nuclear import needs cooperation with the Hsp70 chaperone system (Imamoto *et al* 1992; Shi and Thomas 1992; Shulga *et al* 1996; Yang and DeFranco 1994).
(iv) The C-terminal parts of the Hsfs with the activator function are the least conserved in sequence and size. But they are usually hydrophilic and enriched in proline, serine, threonine, glutamic acid and aspartic acid residues. Their function as transcription activators evidently resides in short peptide motifs found in or close to the HR-C regions. These activator motifs (AHA elements, see Nover and Scharf 1997) are chracterized by aromatic (W, F, Y), large hydrophobic (L, I, V) and acidic (E, D) amino acid residues (see example given in figure 2B). Similar AHA elements are found in the center of the activation domains of human (Newton *et al* 1996), *Drosophila* (Wisniewski *et al* 1996) and yeast Hsfs (Chen *et al* 1993) as well as in many other transcription factors of yeast and mammals, e.g., VP16, RelA, Sp1, Fos, Jun, Gal4, Gcn4 as well as the steroid and retinoic acid receptors (see summary in Nover and Scharf 1997). Most likely, they represent the essential sites of contacts with subunits of the basal transcription complex (figure 5).

There is good evidence that these contacts involve a conformational transition of the mostly unstructured activation domain to a more structured form (Frankel and Kim 1991). In support of this, a transition from a random coil to a helical conformation was reported for the VP16 viral activator when binding to the human TAF 31 (Uesugi *et al* 1997). Interestingly, the large subunit (LSU) of RNA polymerase II also contains AHA-like motifs in its C-terminal domain (Xiao *et al* 1994), suggesting that the activator-induced release from transcriptional arrest with transition to the elongation mode of the transcription complex may depend on the effective competition for binding sites between AHA motifs of the activator proteins and similar structures of the LSU.

4. Multiplicity of Hsfs

The initial cloning and characterization of the yeast Hsf (Sorger and Pelham 1988; Wiederrecht *et al* 1988) was followed two years later by cloning of the corresponding genes from *Drosophila* (Clos *et al* 1990) and tomato (Scharf *et al* 1990). Analysis of the tomato Hsf system

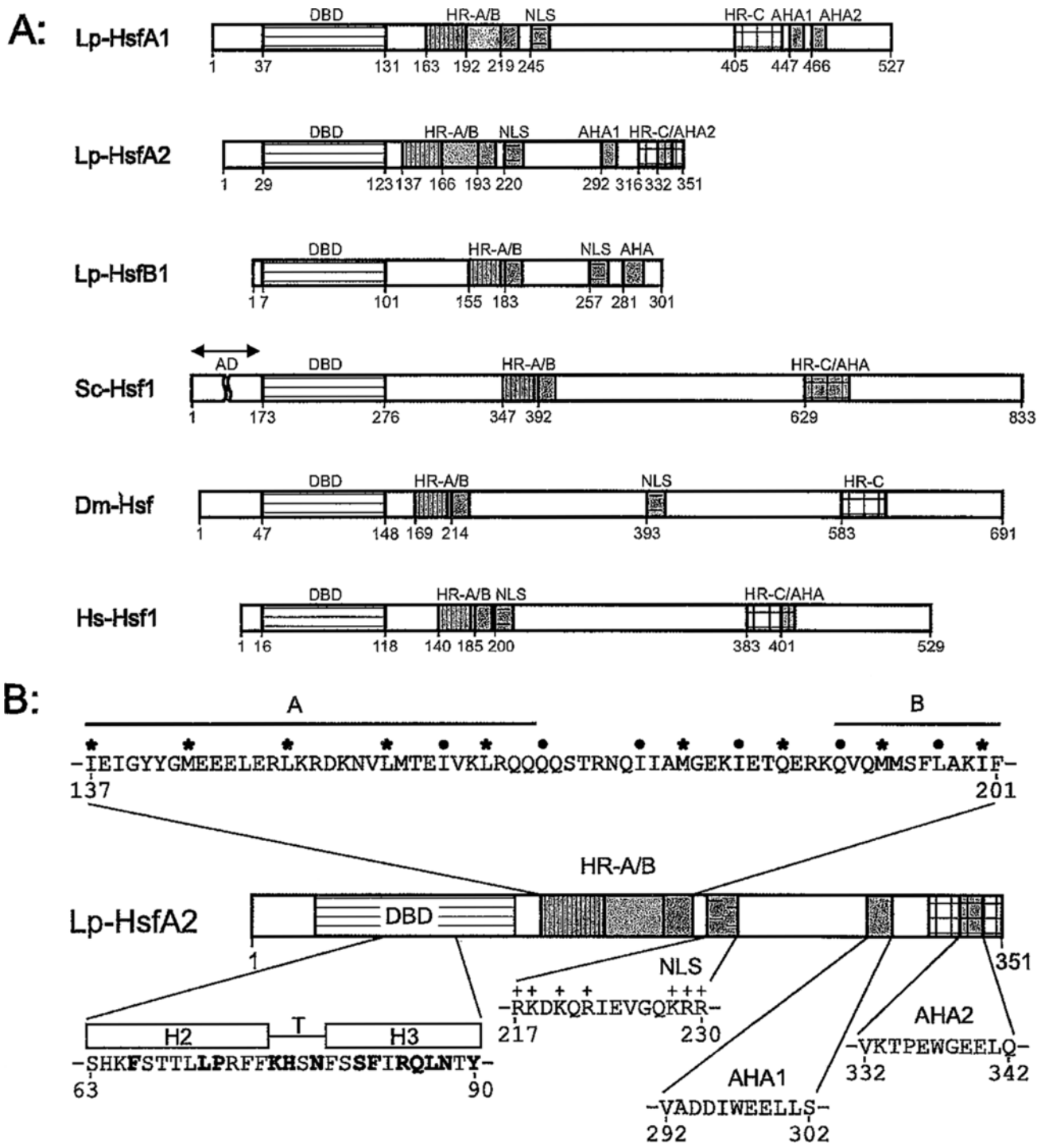


Figure 2. Basic structure of heat stress transcription factors (Hsf).

The block diagrams in (**A**) represent Hsfs of different organisms with their conserved functional domains. Lp, *Lycopersicon peruvianum* (tomato); Sc, *Saccharomyces cerevisiae* (yeast); Dm, *Drosophila melanogaster*; Hs, *Homo sapiens* (human). (**B**) Essential structural details are exemplified for the tomato HsfA2. (i) The central part of the DNA-binding domain (DBD) is the helix-turn-helix motif (H2-T-H3) with a considerable number of amino acid residues invariant between different organisms (bold letters). (ii) The oligomerization domain (HR-A/B is marked by the heptad pattern of hydrophobic residues (dots, asterisks). In the class A Hsfs of plants this region is extended between (**A**) and (**B**) by an insertion of 21 amino acid residues. (iii) The bipartite nuclear localization signal (NLS) represents a cluster of basic residues (K, R) recognized by the NLS receptor. (iv) Central elements of the activator region are short motifs (AHA elements) rich in aromatic (W, Y, F), hydrophobic (L, I, V) and acidic residues (D, E). For the tomato HsfA2 the two tryptophane residues are crucial for the activator function (P Döring, E Treuter, K-D Scharf and L Nover, in preparation).

revealed two interesting peculiarities. (i) There are at least three different Hsfs (see table 2) which belong to two subfamilies (Hsfs A1 and A2 vs. HsfB1). (ii) Two of the three Hsfs are hs-inducible proteins themselves. Though with few exceptions, multiple Hsfs or Hsf-related proteins were found in other organisms as well (table 2), the peculiarities of an extended HR-A/B region in the subfamily A (figure 2) and the hs-dependent expression are unique features of the plant Hsf system.

The multiplicity of Hsfs prompts questions about functional differences between the isoforms. Pertinent data are summarized in table 2. The following examples are worth being emphasized:

(i) The unique Hsfs in yeast and *Drosophila* are required not only for the hs response. *hsf* gene disruption in yeast is lethal even at normal growth temperatures (Sorger and Pelham 1988; Wiederecht *et al* 1988). This provides the basis for testing heterologous Hsfs in yeast (see table 3 and Boscheinen *et al* 1997; Liu *et al* 1997). In *Drosophila*, strains with a conditional lethal *hsf* allele survive, but they have abnormalities in oogenesis and early larval development (Jedlicka *et al* 1997).
(ii) Though the yeast *Saccharomyces cerevisiae* contains three additional genes coding for Hsf-like proteins with the typical conserved DNA-binding domain, they are not able to functionally replace the yeast Hsf. One of the Hsf-like proteins (Skn7 = Pos9) represents a transcription factor essential for the oxidative stress response (Brown *et al* 1993; Krems *et al* 1995; Morgan *et al* 1997). Considering the role of heat and oxidative stressors for the induction of the heat stress response (see figure 1), this structural relatedness of Hsf and Pos9 represents an intriguing aspect of evolution currently being investigated by our group.
(iii) The major vertebrate Hsf responsive to stress induction is Hsf1, whereas Hsf2 activity is controlled by developmental signals, e.g., during spermatogenesis (Sarge *et al* 1994; Fiorenza *et al* 1995) or erythroid cell differentiation (Sistonen *et al* 1992, 1994; Nakai *et al* 1995). Mouse Hsf1 knock-out strains are defective in thermotolerance and fertility, but constitutive synthesis of most chaperones is unaffected (McMillan *et al* 1998). So far the Hsf3-type was only found in chicken. It is only weakly activated by a moderate temperature increase, where Hsf1 is already fully active. In contrast to this, Hsf3 becomes the dominant form under severe and long lasting stress when Hsf1 activity declines (Tanabe *et al* 1997). Remarkably, *hsf*3 gene disruption lines of avian lymphoid cells exhibited a severe reduction in the hs response connected with deficiency in Hsf1 activation indicating an interesting interdependency of the two Hsfs (Tanabe *et al* 1998). In addition, Hsf3 was shown to interact with the *c-myb* proto-oncogene. The Hsf3/c-myb complex is evidently responsible for the cell cycle-specific expression of Hsp70 (Milarski and Morimoto 1986; Kamano and Klempnauer 1997; Kanei-Ishii *et al* 1997). A fourth type of Hsf (Hsf4) was characterized from human cells. Its expression is tissue-specific and, in contrast to the other vertebrate Hsfs, it has no activator function. It may act as repressor Hsf reducing the basal level expression of hs-inducible genes (Nakai *et al* 1997). An interesting peculiarity of the mammalian Hsfs 1 and 2 is the coexistence of two forms (α and β). The β-forms contain an additional miniexon of 22 (Hsf1) and 18 (Hsf2) amino acid residues close to the HR-C region (Fiorenza *et al* 1995; Goodson *et al* 1995)

5. Systems for Hsf expression and functional analysis

Selection of an increasing number of clones potentially coding for Hsfs (table 2) urges the need for appropriate expression and test systems. For evident reasons generation of bacterial expression vectors for synthesis of recombinant Hsfs in *E. coli* is a valuable starting point. The recombinant Hsfs, frequently connected with affinity tags, can be easily purified and used for raising antisera. Furthermore, they are tools for studies on specific DNA binding by electrophoretic mobility shift assays and for *in vitro* protein interaction assays using protein extracts obtained from the native cells (pull-down experiments).

In a few cases, generation of stable transgenic material was used, e.g., a ts-mutant form of the *Drosophila* Hsf (Jedlicka *et al* 1997) or the ectopic expression of HsfA1xGus fusion proteins in *Arabidopsis* (Lee *et al* 1995). Unfortunately, the elaborate selection procedures connected with such experiments limit their application. Many aspects of Hsf function and interaction with other proteins cannot be tested in a reasonable time. However, a more convenient test system is baker's yeast (*Saccharomyces cerevisiae*). The only Hsf-coding gene in this organism can be disrupted and functionally replaced by plasmid-borne variants of the homologous or even by a number of heterologous Hsfs. The basic procedure is demonstrated in figure 3. Hsfs of different organisms and mutant forms of them were tested successfully in yeast (see refs. given in table 3). The dual function of the yeast Hsf, i.e., survival function under normal temperatures vs. role as activator for hs gene expression, allows multifold tests with heterologous Hsfs in this system (Boscheinen *et al* 1997; Liu *et al* 1997; Yuan *et al* 1997).

Two examples may demonstrate the potential of recombinant yeast strains with *hsf* gene disruption. (i) Using different forms of tomato Hsfs, Boscheinen *et al* (1997) reported that class A Hsfs are fully functional in yeast but not class B Hsfs. By using fusion constructs, they demonstrated that the defect was not due to differences in the DNA-binding domain but to hitherto

Table 2. Survey of heat stress transcription factors (see summaries by Scharf and Nover 1994; Nover *et al* 1996).

HSF class[a]	Ref.[b]	Size/properties[c] mRNA (kb)	Protein (aa)	MW (kDa)	M_r (kDa)	Expression/Remarks
Plant, class A						
1. Lp-HsfA1 (Hsf8)1	1	2·0	527	57·5	68	Constitutive
2. At-Hsf1	2	2·1	491	54·0	80	Constitutive
3. Zm-Hsfa	3	2·3	trunc.			Constitutive
4. Lp-HsfA2 (Hsf30)	1	1·4	351	40·2	55	Hs-induced; functional interaction with HsfA1
5. Gm-HsfA2 (Hsf21)	4	ND	trunc.	ND	ND	Hs-induced
6. Zm-Hsfb	3	1·6	308	35·3		Hs-induced
7. Zm-Hsfc	3	1·5	> 364	ND	ND	Hs-induced
8. At-HsfA2 (Hsf21)	5	ND	> 400	46·3	ND	
Plant, class B						
9. Lp-HsfB1 (Hsf24)	1	1·5	301	33·3	45	Hs-induced
10. Gm-HsfB1 (Hsf34)	4	ND	282	31·2	37·4	Hs-induced
11. At-HsfB1 (Hsf4)	5	ND	284	31·4	ND	
12. Gm-HsfB2-1 (Hsf29)	4	ND	trunc.	ND	ND	Hs-induced
13. Gm-HsfB2-2 (Hsf5)	4	ND	370	42·1	ND	Constitutive
14. Gm-HsfB2-3 (Hsf31)	4	ND	trunc.	ND	ND	
Yeast						
16. Sc-Hsf1	6		833	93·2	130	Single, constitutively expressed genes; essential for survival
17. Kl-Hsf	7		677	75·4	–	
18. Sp-Hsf	8		609	67·0	108	
Drosophila						
19. Dm-Hsf	9		691	77·3	110	Single, constitutively expressed gene
Xenopus						
20. Xl-Hsf1	10		451	49·5	67	Constitutive
Vertebrates						
21. Hs-Hsf1[d]	11	2·3	529	57·3	83	Constitutive
22. Hs-Hsf2[d]	12	2·7	536	60·3	87	Tissue-specific
23. Hs-Hsf4	13	2·5	463	50·0	55	Tissue-specific
24. Mm-Hsf1[d]	14	2·0	503/525	54·8	75	Constitutive
25. Mm-Hsf2[d]	15	2·1	517/535	58·2	71	Tissue-specific
26. Gd-Hsf1	16	1·9	491	53·6	65	Constitutive
27. Gd-Hsf2	16	2·8	564	62·8	80	Tissue-specific
28. Gd-Hsf3	16	4·5	467	51·9	64	Constitutive

[a]Species are abbreviated as follows: Lp, *Lycopersicon peruvianum* (wild tomato); At, *Arabidopsis thaliana*; Zm, *Zea mays* (maize); Gm, *Glycine max* (soybean); Sc, *Saccharomyces cerevisiae*; Kl, *Kluvieromyces lactis*; Sp, *Schizosaccharomyces pombe*; Dm, *Drosophila melanogaster*; Xl, *Xenopus laevis*; Hs, *Homo sapiens* (human); Mm, *Mus musculus* (mouse); Gd, *Gallus domesticus* (chicken).

[b]1. Scharf *et al* 1990, 1993; Treuter *et al* 1993; 2. Hübel and Schöffl 1994; 3. Gagliardi *et al* 1995; 4. Czarnecka-Verner *et al* 1995; 5. P Barros and E Czarnecka-Verner, unpublished; 6. Sorger and Pelham 1988; Wiederrecht *et al* 1988; 7. Jakobsen and Pelham 1991; 8. Gallo *et al* 1993; 9. Clos *et al* 1990; Orosz *et al* 1996; Zandi *et al* 1997; 10. Stump *et al* 1995; Mercier *et al* 1997; 11. Rabindran *et al* 1991; 12. Schuetz *et al* 1991; Sistonen *et al* 1994; 13. Nakai *et al* 1997; 14. Sarge *et al* 1991, 1993; Fiorenza *et al* 1995; Goodson *et al* 1995; Rallu et al 1997; 15. Nakai and Morimoto 1993; Nakai *et al* 1995; Tanabe *et al* 1997.

[c]Size of the protein is given as number of amino acid residues (aa), the sequence-derived mol. weight (MW in kDa) and the apparent size in SDS-PAGE (M_r).

[d]For the mammalian Hsfs 1 and 2 two forms (α and β) were found. Form β contains an additional miniexon of 22 (Hsf1) and 18 (Hsf2) amino acid residues inserted close to the HR-C region (see text for further explanations).

unnoticed differences in the activator function. (ii) Important new insights came also from tests with the two human Hsfs (Liu *et al* 1997). In this case Hsf2 was functional and Hsf1 was not, i.e., yeast cells with the *hsf1* plasmid did not survive the FOA treatment (figure 3). This defect of Hsf1 could be alleviated by removing part of its C-terminal domain which evidently blocks Hsf1 function in yeast but not in mammalian cells. Both negative systems can be used to screen for auxiliary proteins from plants (HsfB1) or human cells (Hsf1), which are lacking in yeast but are evidently required for the normal function of these Hsfs in their native cells.

A convenient plant transient expression system based on the PEG-mediated transformation of tobacco protoplasts (figure 4) was established by our group (Treuter *et al* 1993; Lyck *et al* 1997; Scharf *et al* 1998). It is particularly suited for rapid functional testing of homologous and heterologous Hsfs (Treuter *et al* 1993) as well as for analysis of Hsf mutants (Lyck *et al* 1997). Most important, however, is the possibility for combination of different expression plasmids to study functional interaction between Hsfs or between Hsfs and chaperones (see below).

Additional examples for other expression systems are summarized in table 3. Though more or less artificial, they are all characterized by the ease of manipulation of the test conditions and by the low endogenous background, especially when using appropriate reporter constructs. These systems have considerably contributed to

Table 3. Heterologous test systems used for expression and functional analyses of Hsfs.

Origin of hsf gene/cDNA	Test system[b]	Test[c]/results	Ref.
Plant Hsf			
Arabidopsis HsfA1	*Drosophila* SL2 cells (t), Human 293 cells (t)	Constitutively active form (EMSA, CAT reporter assay)	Hübel *et al* 1995
Tomato HsfA1, HsfA2, HsfB1[a]	Yeast strains with disruption of hsf1 gene (p)	Survival, growth at elevated temperature induced thermotolerance; expression of lacZ reporter	Boscheinen *et al* 1997
Drosophila Hsf			
	Schizosaccharomyces pombe, hsf disruption strains (p)	Strains with Dm-Hsf survive, reduced growth (13 h doubling time)	Gallo *et al* 1993
	Human 293 cells (t)	Hsf constitutively active and trimerized (EMSA, PEC)	Rabindran *et al* 1993
	Xenopus oocytes (microinjection of Hsf-mRNA) (t)	Hsf synthesized in inactive form, but activated at 37°C (DNA binding)	Clos *et al* 1990
	Tobacco protoplasts (t)	Expression of hs-dependent gus reporter gene	Treuter *et al* 1993
Human			
Hsf1[d]	*Drosophila* SL2 cells (t)	Hs-inducible at 37°, induced by ethanol (EMSA, PEC)	Rabindran *et al* 1993
Hsf1[d]	*Xenopus* oocytes, (microinjection of Hsf1-mRNA) (t)	Hsf1 synthesized in an inactive form, but activated at 37°C (DNA binding)	Baler *et al* 1993; Zuo *et al* 1994
Hsf1[d]	Tobacco protoplasts (t)	Expression of gus reporter gene, hs-inducible at 35°C	Treuter *et al* 1993
Hsf1[e], Hsf2	Yeast with disruption of hsf1 gene (p)	Survival, growth at elevated temperature, expression of lacZ reporter	Liu *et al* 1997; Yuan *et al* 1997

[a]Only HsfB1 fusion proteins with C-terminal parts of HsfA2 are functional in yeast.
[b]Test systems are classified with (p) for permanent and (t) for transient expression.
[c]EMSA, electrophoretic mobility shift assay; PEC pore exclusion chromatography.
[d]Human Hsf1 activity is hs-inducible in all heterologous systems tested, but the temperature threshold (35–37°C) corresponds to the normal hs temperature of the expression system (see text).
[e]In contrast to Hsf2, only Hsf1 mutant forms with deletion of HR-C region are active in yeast.

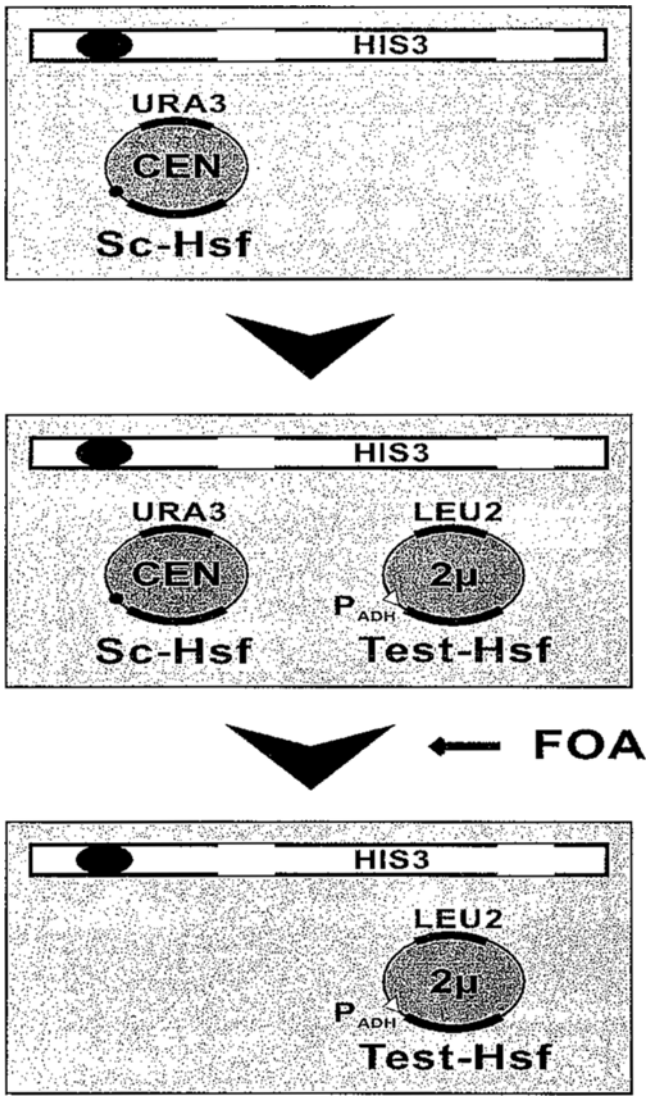


Figure 3. Hsf gene disruption and functional replacement in yeast.

Using a histidine-auxotrophic strain, the yeast *hsf* gene is disrupted by site-specific insertion of a *his3* gene. Because the Hsf in yeast is essential, haploid strains can only survive if the defect is complemented by a plasmid-borne copy of the *hsf* gene. The *ura-3* selection marker on this plasmid allows growth on uracil-free medium. On the other hand, it can be used to eliminate the *ura3* plasmid by selection on media with fluoro-orotic acid (FOA). The procedure allows simultaneous replacement of the yeast *hsf* gene by a heterologous *hsf* expression cassette, e.g., derived from the corresponding tomato, *Drosophila* or human genes (see table 3).

our understanding of Hsf function and regulation. The comparison of results obtained with homologous and heterologous expression systems help to recognize artifacts and to search for additional proteins required for the normal function and regulation of Hsfs.

6. Control of Hsf activity

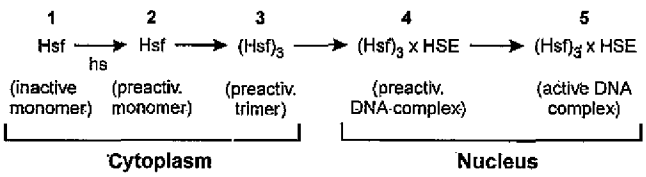
The two dimensional presentation of Hsfs with their functional domains/modules (figure 2) is convenient and reflects part of the reality derived from mutational analyses and testing of appropriate fusion proteins. But it is also misleading. It should be kept in mind that essential details of the Hsf function and in particular of its activity control depend on ill-defined intramolecular interactions between the highly flexible C-terminal domain and other parts of the molecule. Unfortunately, in contrast to the well structured DNA-binding domain (see § 3), no information on the 3-D organization of the C-terminal part of the Hsfs is available at present.

The importance of the homologous molecular context is best illustrated by the divergent results observed with fusion proteins containing the C-terminal activator domains (CTAD) of Hsfs linked to different heterologous DNA-binding domains. Using the human or mouse Hsf1 CTADs, fully active and hs-regulated hybrid activator proteins were obtained (Zuo *et al* 1994; Green *et al* 1995a,b; Shi *et al* 1995). Evidently, the CTAD contains all information necessary for the regulated phenotype. In contrast to this, corresponding fusion proteins with the CTADs from the yeast (Nieto-Sotelo *et al* 1990) and *Drosophila* Hsfs (Wisniewski *et al* 1996) were active but unregulated.

In the following, we will summarize important experimental evidence from different systems illustrating this problem in more detail.

6.1 *Stress-induced changes of Hsf activity and intracellular distribution*

Based on experimental data from vertebrates and *Drosophila* (Westwood and Wu 1991; Baler *et al* 1993; Rabindran *et al* 1993; Sheldon and Kingston 1993; Zuo *et al* 1994, 1995) the following five step activation process for Hsf can be defined:



Upon heat stress activation, the inactive monomer in the cytoplasms (state 1) undergoes a confomational change (state 2) with subsequent oligomerization (state 3). The Hsf trimer, transported to the nucleus, is able to bind to the hs promoter elements (HSE, states 4, 5). Using transcription of *in vitro* reconstituted chromatin templates Sandaltzopoulos and Becker (1998) present evidence that Hsf-mediated reinitiation without the slow reassembly of the TFIID/TFIIA complex may be essential for the efficiency of hs gene transcription.

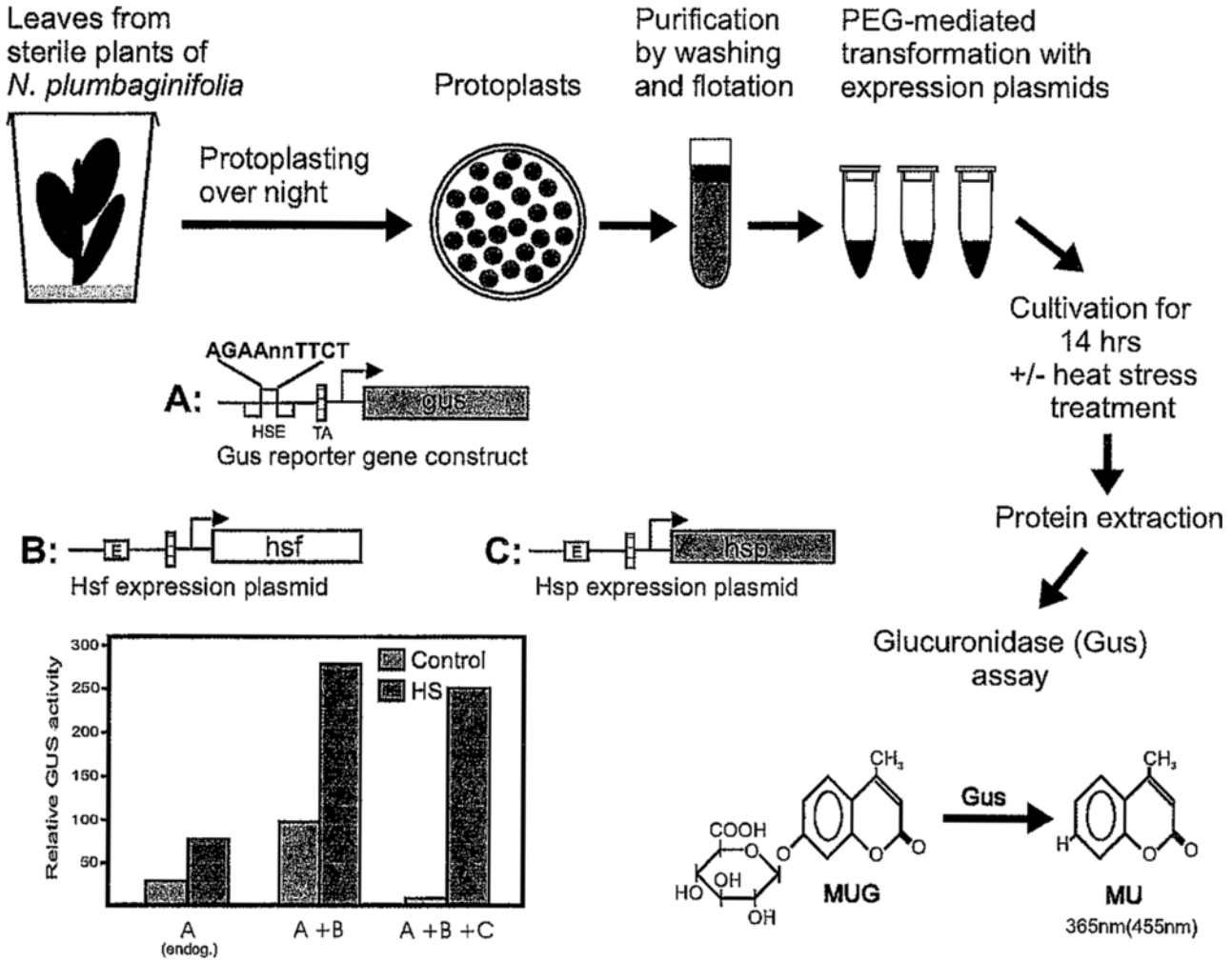


Figure 4. Transient expression assay for testing Hsf function in tobacco protoplasts.
Protoplasts from sterile *Nicotiana plumbaginifolia* plants are transfomed by polyethylene glycol treatment with a *hs* promoter x *gus* reporter plasmid (A) in combination with a Hsf expression plasmid (A+B) or with Hsf plus a chaperone expression plasmid (A + B + C). The principal results of the glucuronidase assay after 20 h of incubation under control and hs conditions are shown for the tomato HsfB1 by the diagram in the lower left corner. The low basal level expression due to the endogenous Hsf system of the protoplasts (A) can be considerably increased by coexpression of reporter with activator (A + B). Interestingly, the tightly regulated phenotype of glucuronidase expression requires coexpression of the Hsf with chaperones of the Hsp70 and/or HSPs90 family (A + B + C).

The discrimination of two separate states in the nucleus depends on the following observations: (i) Salicylate or indomethacine treatments of mammalian cells result in Hsf activation, nuclear transport and DNA-binding (state 4) but no hs gene transcription (Jurivich *et al* 1992; Lee *et al* 1995; Cotto *et al* 1996). (ii) Hsfs in yeasts (Sorger and Pelham 1988; Wiederrecht *et al* 1988; Jakobsen and Pelham 1991) and *Xenopus* oocytes (Mercier *et al* 1997; Bharadwaj *et al* 1998) are always nuclear and DNA-bound, and yet transcription of hs genes needs stress induction. (iii) In mouse embryonic carcinoma cells Hsf2 exists in oligomeric, DNA-bound form without detectable transcription of the *hsp70* and *hsp90* genes (Murphy *et al* 1994).

6.2 *Elements of Hsf activity control*

It is now generally accepted that the major point of stress control is the maintenance of Hsf in an inactive state and/or the regeneration of this state after the stress activation (see figure 1). Depending on the cell type, organism and Hsf investigated, this inactive state can be anything between states 1 and 4. Despite this evident flexibility in detail, there are a number of common features for all or at least for a group of Hsfs.

Repression of Hsf activity results from intramolecular interactions involving C-terminal parts and parts of the HR-A/B and/or DNA-binding domains. Evidence for this stems from the following observations.

(i) As already mentioned, replacement of the homologous DNA-binding domain, e.g., in the yeast and *Drosophila* Hsf, can generate constitutively active transcription factors (Nieto-Sotelo *et al* 1990; Wisniewski *et al* 1996).
(ii) C-terminal deletion forms of the *Drosophila* Hsf (Rabindran *et al* 1993), the chicken Hsf3 (Nakai and Morimoto 1993) or the tomato HsfA2 (Lyck *et al* 1997) are more potent than the wild-type Hsfs but unregulated. Interestingly, only a C-terminal deletion but not the wild-type form of the human Hsf1 is able to functionally replace the yeast Hsf (Liu *et al* 1997).

In this context the properties of the tomato HsfA2 are particularly remarkable (Scharf *et al* 1998). Intramolecular shielding of the NLS in the wild-type protein prevents efficient nuclear transport even under stress conditions. This block can be relieved by deletion of only seven amino acid residues from the C-terminus (Lyck *et al* 1997). The intriguing question about nuclear transport of the wild-type HsfA2 in the native cells (tomato) is answered by coexpression experiments with HsfA1 using tobacco protoplasts or by colocalization studies in tomato cells using double fluorescence labelling (figure 6). We have evidence that HsfA2 forms heterooligomeric complexes with HsfA1, and only in this form, it is able to enter into the nucleus during heat stress (figure 6). HsfA2 accumulating in the cytoplasm during the stress period becomes part of large cytoplasmic aggregates containing small HSPs and HsfA2. They probably represent storage forms of these heat stress proteins (Scharf *et al* 1998).

A frequently discussed model used to explain the inactive state of Hsfs involves intramolecular interactions between the C-terminal HR-C and the central HR-A/B/NLS region (Rabindran *et al* 1993; Zuo *et al* 1994, 1995). This part of the proteins is required for oligomerization and nuclear transport, which are both steps of the activation process. Hence, intramolecular shielding may efficiently block Hsf function as transcription activator. In keeping with this, deletions or point mutations in the HR-C or HR-A/B regions were repeatedly reported to abolish hs regulation (Chen *et al* 1993; Zuo *et al* 1994; Green *et al* 1995a,b; Shi *et al* 1995;

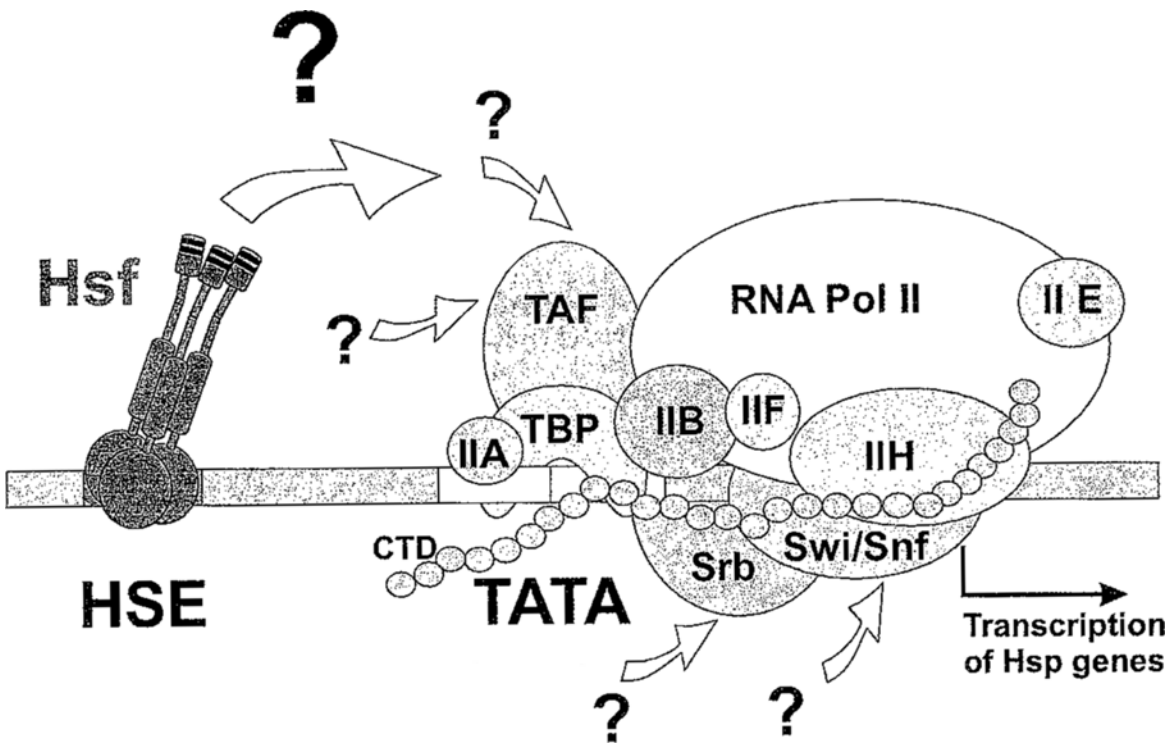


Figure 5. Potential contact sites of the Hsf activator domain with components of the transcriptosome (modified from Struhl 1996).

The transcriptosome is a complex machinery built of RNA polymerase II and the associated initiation factors (TFIIA, B, E, F, H), the Srb and Swi/Snf complexes as well as the TATA-binding protein (TBP) and associated proteins (TAF). Totally an estimated number of 70–80 protein components are involved (Struhl 1996; Drysdale *et al* 1998). An important control element for the subunit composition and functional state of the complex, e.g., the initiation vs. elongation state, is the phosphorylation/dephosphorylation of the C-terminal repeat domain (CTD) of its largest subunit. There are many examples for protein contacts between sequence-specific activator proteins, like Hsf, and different components of the transcription complex (arrows). They may help in the assembly of the complex (recruitment), in the transition to the elongation mode, in CTD phosphorylation or chromatin remodelling, e.g., by histone acetylation/deacetylation (see reviews Kadonaga 1998; Lee and Young 1998; Struhl and Moqtaderi 1998).

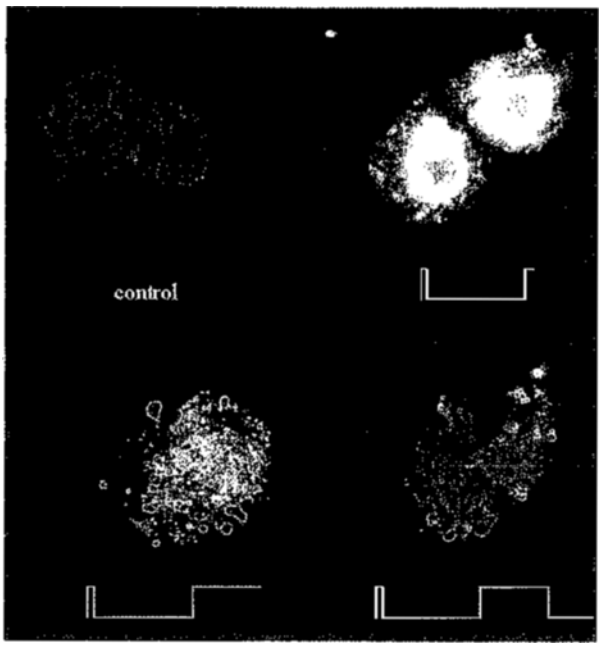


Figure 6. Colocalization of Hsfs A1 and A2 in tomato cell cultures using double immunofluorescence and confocal laserscan microscopy.

Intracellular sites with HsfA1 only are detectable by green, sites with HsfA2 only by red fluorescence. Sites with both Hsfs appear in yellow. The stress conditions are indicated by the pictographs. In control cells (upper left) only HsfA1 is present, distributed between cytoplasm and nucleus. After pulse hs induction (15 min 40°C), HsfA2 is newly synthesized, and in the following hs, migrates to the nucleus together with HsfA1 (yellow nuclear fluorescense in the upper right part). However, with ongoing hs, the accumulating HsfA2 forms large aggregates in the cytoplasm together with other Hsp (heat stress granules, see red clusters in the lower left and right parts). Most of the HsfA1 stays in the nucleus (green label).

Boscheinen *et al* 1997; Farkas *et al* 1998). Particularly interesting examples were reported for the human Hsf1 and the *Drosophila* Hsf. Single point mutations in the HR-B part of Hs-Hsf1 (Zuo *et al* 1994) or the NLS region of Dm-Hsf (Zandi *et al* 1997) create constitutively active and multimeric factors.

The special role of the central part of the Hsf for stress regulation is also supported by investigations on Hsf phosphorylation. Except for plants, change of the phosphorylation state accompanies Hsf activation in yeast, *Drosophila* and vertebrates. Phosphorylation of distinct serine residues is evidently not an essential part of the immediate activation process (Cotto *et al* 1996). But it may be important for sustained Hsf activity under long-term stress (Xia and Voellmy 1997). At any rate, it is essential for the shut-off of Hsf activity, i.e., the re-generation of the inactive state (Hoj and Jakobsen 1994; Chu *et al* 1996; Knauf *et al* 1996; Kline and Morimoto 1997). Deactivation coincides with dephosphorylation and it is blocked by phosphatase inhibitors (Kim *et al* 1995; Xia and Voellmy 1997).

6.3 *Search for corepressors*

Evidently, the concept of intramolecular interactions as basis for the repressed form of Hsf does not exclude the requirement of additional proteins (corepressors) to generate and/or maintain the inactive state (Sarge *et al* 1993; Hoj and Jakobsen 1994; Zuo *et al* 1995). Most probably, Hsp70 is a central player in the control of Hsf activity (see data compiled in table 1): In addition, the Hsp90 complex may be involved as well. Similar to the steroid homone receptors (see Forreiter and Nover 1998), the human Hsf1 can be assembled *in vitro* into a multichaperone complex with all known components of the Hsp70 and Hsp90 machines (Nair *et al* 1996). Moreover, geldanamycin, which specifically interferes with Hsp90 function, was shown to promote Hsf activation (Hegde *et al* 1995). In fact, both types of transcription factors seem to compete for the same type of chaperones. Overexpression of steroid receptors in COS cells induce Hsf activity, and this effect can be abolished by addition of the steroid ligand or by coexpression of the receptors together with Hsp70 (Xiao and DeFranco 1997). These results indicate that both transcription factors compete for the same chaperone systems. Recently, Shi *et al* (1998) reported on the direct interaction of the Hsp70/Hsp40 chaperone system with the C-terminal activator domain of human Hsf1.

In mammalian cells, two Hsfs were identified as potential repressors. (i) Hsf4 has no functional activation domain. Overexpression in HeLa cells does not affect hs inducible gene expression, but it reduces the basal level expression of Hsp90, Hsp70 and Hsp27 (Nakai *et al* 1997). (ii) The intriguing observation of two forms (α vs. β) of Hsfs 1 and 2 in mammals led to investigations about their cell-specific expression levels and possible functions. Overexpressing the Hsf2β in human erythroleukemia cells, Leppa *et al* (1997) observed an inhibition of the hemin-induced cell differentiation. The authors conclude that Hsf2β may act as negative regulator of Hsf2α.

It is tempting to speculate that the plant HsfB2 type so far isolated only from soybean may have a similar repressor function (Czarnecka-Verner *et al* 1998).

7. Perspectives

Following the initial cloning of Hsfs from yeast, *Drosophila*, plants and mammals, the rapid increase of our knowledge in the preceding 10 years provided insights into the modular structure of Hsfs, their multiplicity and into key elements involved in the control of their activity. The elaboration of appropriate homologous and hetero-

logous test systems helped considerably in the functional characterization of Hsfs. Now, all essential tools and methods are available to dissect the complex cellular situation summarized in figure 1. The Hsfs are embedded into a network of protein contacts evidently responsible for their intracellular localization and activity control, which are nicely tuned to the needs of the cell.

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