Tomato heat stress transcription factor HsfB1 represents a novel type of general transcription coactivator with a histone-like motif interacting with HAC1/CBP

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Abbreviation index

aa	amino acid residue
AD	activation domain
AHA	aromatic, hydrophobic and acidic amino acid residues containing motif
At	*Arabidopsis thaliana*
ATP	adenosine triphosphate
bp	base pair
CaMV	cauliflower mosiac virus
CBP	CREB binding protein
cDNA	complementary DNA
CLIP	cross-linking immunoprecipitation of DNA-protein complexes
Co-IP	coimmunoprecipitation
CTAD	C-terminal activation domain
CTD	C-terminal domain
CMV	cytomegalo virus
DBD	DNA binding domain
DNA	deoxyribonucleic acid
EMSA	electrophoretic mobility shift assay
EST	expressed sequence tag
Gm	*Glycine max*
GST	Glutathione-S-transferase
GUS	β-Glucuronidase
HAC1	Homologous to acetyltransferase CBP
HAT	Histone acetyl transferase
HTH	helix turn helix
HR-A/B	heptad repeat-A/B
hs	heat stress
HSE	heat stress element
Hsf	Heat stress transcription factor
HSG	heat stress granule
Hsp	Heat stress protein
kDa	kilo Dalton
Le	*Lycopersicon esculentum*

Lp	*Lycopersicon peruvianum*
luc	Luciferase
NES	nuclear export signal
NLS	nuclear import signal
Nt	*Nicotiana tabacum*
ntd	nucleotide
N-terminus	amino terminus of a protein
ORF	open reading frame
Os	*Oryza sativa*
PCAT	p300/CBP acetyltransferase related protein
PCR	polymerase chain reaction
PIC	pre-initiation complex
SRC-1	Steroid receptor coactivator-1
rfu	relative fluorescence units
RNA	ribonucleic acid
RT-PCR	reverse transcriptase-polymerase chain reaction
TAFs	TATA associated factors
TBP	TATA binding protein

One letter code for amino acid residues:

A	Alanine	M	Methionine
C	Cysteine	N	Aspargine
D	Aspartic acid	P	Proline
E	Glutamic acid	Q	Glutamine
F	Phenylalanine	R	Arginine
G	Glycine	S	Serine
H	Histidine	T	Threonine
I	Isoleucine	V	Valine
K	Lysine	W	Tryptophan
L	Leucine	Y	Tyrosine

1 Introduction

1.1 The heat stress response

The pioneering work of the Italian developmental biologist F. Ritossa (Ritossa 1962) led to one of the most seminative discoveries in the field of molecular cell biology. After a mistaken increase in the temperature of chamber with *Drosophila* cultures, a serendipitous discovery showed extraordinary changes in the gene activity pattern of the polytene chromosomes in larval salivary glands. It took another 10-15 years before this unusual gene activity was related to protein synthesis of heat stress proteins (Hsps, Tissieres et al. 1974) and the corresponding mRNAs were identified (McKenzie and Meselson 1977). It soon became clear that Ritossa infact had discovered the most central part of heat stress (hs) response, the unusually strong inducibility which is inherent to induction of heat stress proteins. Further on it was shown that the principles of heat stress inducibility of Hsp genes and the Hsp protein families are conserved from prokaryotes to eukaryotes (for earlier ref. see Ashburner and Bonner 1979; Nover et. al. 1989; Nover 1991). Originally the names of different Hsp families were derived from their apparent molecular sizes (Nover and Scharf 1997; Forreiter and Nover 1998). A gene based nomenclature derived from the compilation of related open reading frames (ORFs) encoding members of *Arabidopsis* Hsp families can be found in a special issue of Cell Stress and Chaperones (Nover and Miernyk 2001).

Hsps act as cellular guard under sub-optimal conditions (e.g. temperature, heavy metals, toxins, oxidants, viral and bacterial infections), both to prevent and repair the damage caused in the cellular homeostasis. In addition these proteins play important role in the house-keeping functions by acting as molecular chaperones, participating in protein folding, topogenesis, translocation and degradation, mostly as multichaperone machines (Vierling 1991; Parsell and Lindquist 1993; Morimoto et al. 1994; Hartl 1996; Rutherford and Lindquist 1998; Bharti and Nover 2001; Queitsch et al. 2002). Heat stress results in a decreased pool of free chaperones, due to their increased demand in maintaining protein homeostasis during a cellular insult. This decreased pool is replenished by the new synthesis of Hsp´s, which is attributed to a conserved regulatory protein, the heat stress transcription factor (Hsf).

1.2 Basic structure and classification of Hsfs

Hsfs, the terminal components of heat stress signalling cascade are the direct inducers of Hsp genes (Bharti and Nover 2001). Similar to many other transcription factors Hsfs have a modular structure, which is more or less conserved among eukaryotes. The basic plan of Hsf structure is exemplified for HsfA2, the best studied plant Hsf (Fig. 1).

Hsf DNA binding domain (DBD) is the most conserved part of the protein, present at the N-terminus end. The DBD can be classified as helix turn helix (HTH) type, and the central H2-T-H3 motif is responsible for specific recognition of HSEs in the promoter regions of Hsp genes. The structural studies from yeast (Harrison et al. 1994), *Drosophila* (Vuister et al. 1994) and plant (Schultheiss et al. 1996) Hsfs DBD showed not only the highly conserved 3-dimensional structure but also highlighted the fact that the whole conformation of DBD is infact stabilized by interactions among bulky hydrophobic and large aromatic amino acids. Details about Hsf binding to the DNA were elaborated by crystal structure analysis of the DBD of *Kluyveromyces lactis* Hsf (Littlefield and Nelson 1999). It was shown that the two monomer DBDs have contacts after binding to the DNA, which are mediated by the 10 amino acid residues of the loop (wing) between β3 and β4 strands. This observation offers an opportunity to elaborate differences between DNA binding preference of Hsfs from plants and other organisms, because the wing region is lacking in all plant Hsfs.

The oligomerization domain (HRA/B region) is separated from the DBD by a linker with varying length in different Hsfs (Nover et al. 2001). This linker is most distant if compared among different Hsf classes but contains some highly conserved motifs when compared with in the same subclass. Initial experiments have showed that this region might specifically affect the oligomerization potential of some Hsfs, therefore has been suggested as the identity region of Hsfs (Nover and Bharti, unpublished). The observation with plant Hsfs are supported by studies done with yeast and mammalian Hsfs, where the linker has been shown to contribute to the oligomerization state of Hsfs (Flick et al. 1994; Liu and Thiele 1999). The presence of arrays of hydrophobic heptad repeats in the HRA/B region suggest a coiled-coil structure which is prototype of leucine-zipper-type protein interaction domains (Peteranderl and Nelson 1992; Peteranderl et al. 1999). The two heptad repeats in the oligomerization domain of Hsfs are separated by an amino acid linker of varying length, which was used as a criteria for the classification

of plant Hsfs into three different classes: A, B and C (Fig. 2), containing 21, no and 7 amino acid residues respectively in the linker (Nover et al. 2001).

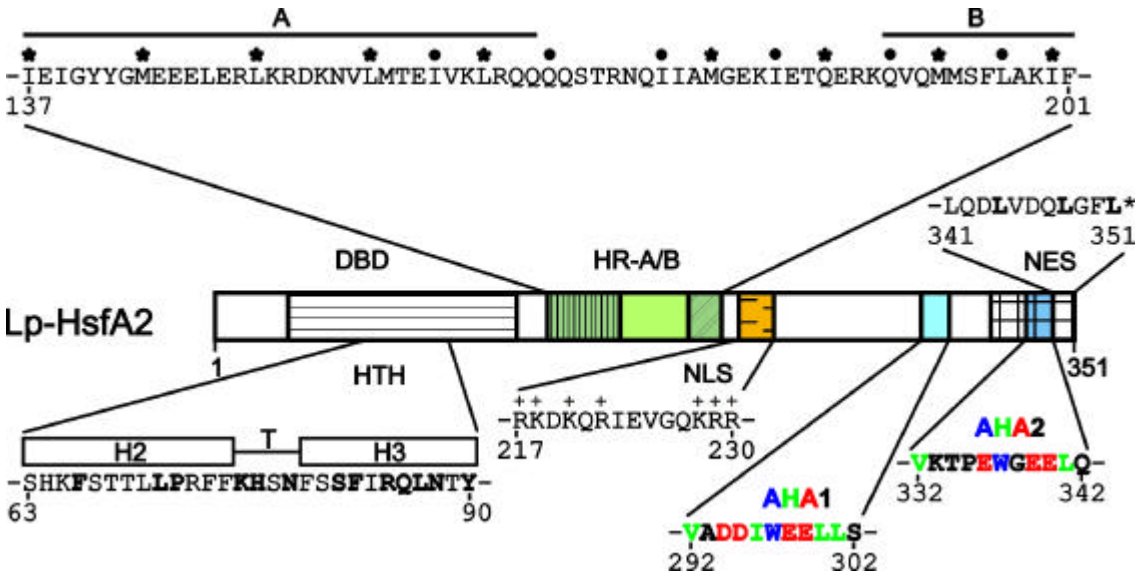


Figure 1. **Basic structure of plant Hsfs.**
Structural details of an Hsf are exemplified for tomato HsfA2. The central H2-T-H3 motif in the DNA binding domain (DBD), which directly contacts the HSE in the DNA is shown. The oligomerization domain (HRA/B region) is characterized by pattern of heptad repeats (dots and asterisks). The linker between heptad repeat A and B, which is used as the classification criteria for plant Hsfs is shown in green. Nuclear localization signal (NLS) is a bipartite cluster of basic amino acids, present immediately C-terminus to HRA/B region. AHA motifs are rich in aromatic (W, Y, F), hydrophobic (L, I, V), and acidic amino acid residues (D, E). The leucine rich motif at the C-terminus functions as a nuclear export sequence (NES).

Immediately C-terminal to the HRA/B region are mono/bipartite clusters of basic amino acid residues which serve as the nuclear localization signals in case of most Hsfs (Fig. 1; Lyck et al. 1997; von-koskull Döring, unpublished). Interestingly, not all Hsfs have a permanent nuclear localization, in many cases the nucleocytoplasmic distribution of proteins can be markedly influenced by nuclear export. The signal for nuclear export was extensively studied for tomato HsfA2, where a leucine rich sequence in the C-terminus was shown to have potential for export of the protein from the nucleus (Fig. 1; Scharf et al. 1998; Heerklotz et al. 2001). Similar peptide motifs have been found in the C-terminal part of several *Arabidopsis* Hsfs, especially HsfA8 has been shown to be a shuttling protein similar to LpHsfA2 (von-koskull Döring, unpublished).

The C-terminal domain of Hsfs is the least conserved part in terms of sequence and size. In case of plant A Hsfs it contains some conserved motifs embedded in an acidic hydrophilic surrounding, the AHA motifs (Döring et al. 2000; Nover et al. 2001). These

short peptide motifs are characterized by the presence of aromatic, large hydrophobic and acidic amino acids. It has been shown that these motifs are responsible for the activation potential of class A Hsfs (Döring et al. 2000; Bharti et al. 2000).They represent the sites for interaction with components of transcriptional machinery (Yuan and Gurley 2000; von-koskull Döring, unpublished).

In contrast to the acidic activation domains of class A Hsfs, the C-terminal domains of class B and C Hsfs are enriched in basic amino acids (Nover et al. 2001). The AHA motifs are lacking not just from the sequence comparison but it has been shown that these Hsfs do not have activation potential characteristic of class A Hsfs (Treuter et al. 1993). This is supported by the observations that only class A Hsfs can restore thermotolerance in protoplasts prepared from HsfA1 knockout plants (Mishra et al. 2002; Mishra, unpublished). Similarly, only class A Hsfs could complement the yeast Hsf for survival functions (Boscheinen et al. 1997). Moreover, another report showed that class B Hsfs even act as repressors for the activation potential of class A Hsfs (Czarnecka-Verner et al. 2000).

1.3 Multiplicity and complexity of plant Hsfs

The sequencing of the first plant genome (The *Arabidopsis* Genome Initiative 2000) led to the identification of 21 members of Hsf family in *Arabidopsis* (Fig. 2). Based on sequence homology in the highly conserved DBD and class specific oligomerization domain, an extensive database search for the expressed sequence tags (ESTs) and partially completed genomes showed for the first time that the Hsf family in plants is much bigger than in other organisms. At least 17 different members have already been identified from tomato and 23 from rice genomes (Fig. 2). In contrast the vertebrates have four whereas yeast, *Drosophila* and *C.elegans* have only one Hsf each (Nover et al. 1996; Nakai 1999; Pirkkala et al. 2001).

Such a unique multiplicity in case of plant Hsf family is worth discussing. Because of the sessile nature of plants they are most challenged to environmental extremes and hence need a more elaborate network of proteins to survive under such conditions. This hypothesis is endured by existing evidences which suggest that the multiplicity of Hsfs does not reflect redundancy but attributes to functional diversity. Following explanations supported the concept of functional diversity of plant Hsfs:

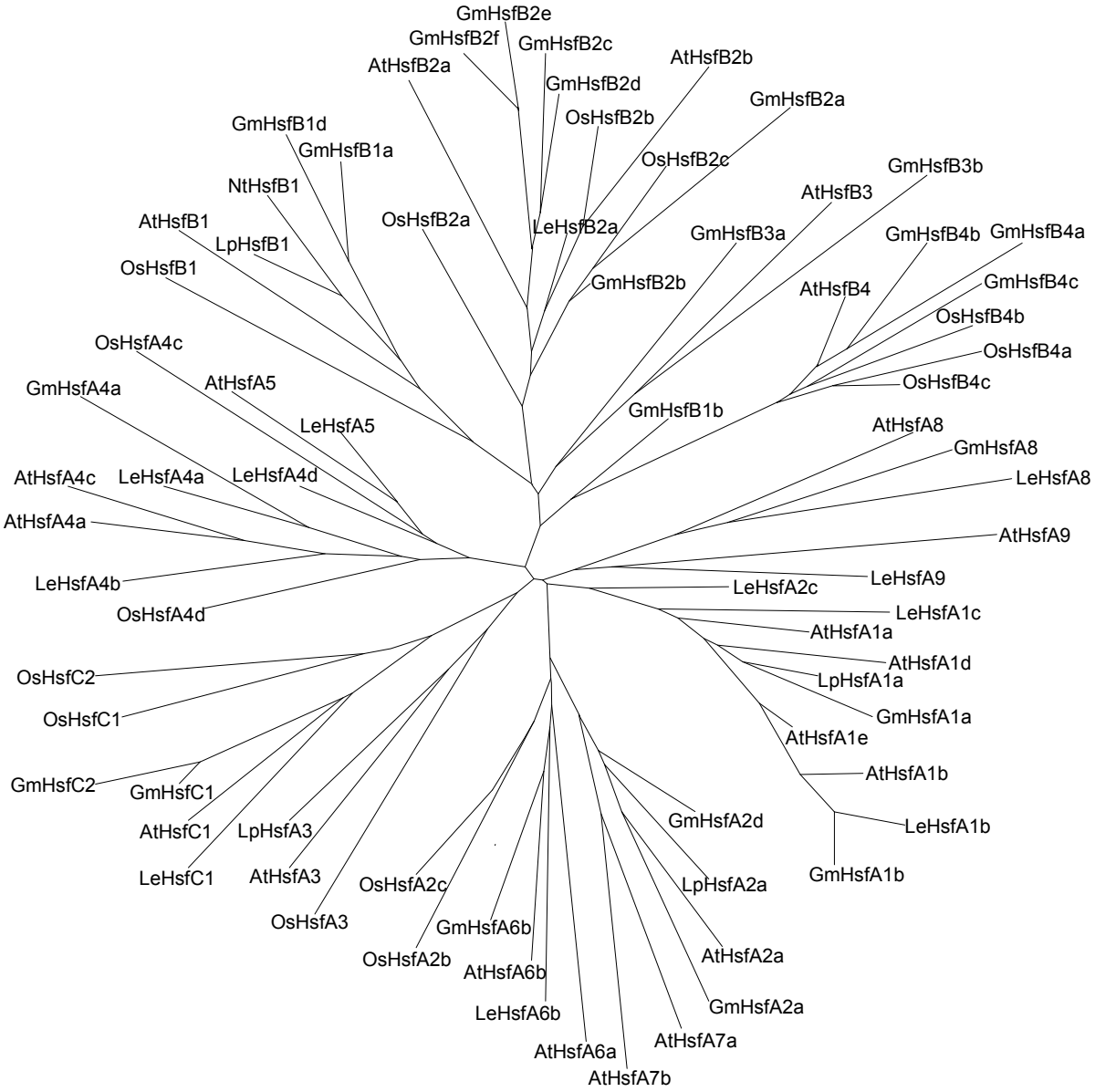


Figure 2. **Phylogenetic tree of plant Hsfs, showing the three basic classes: A, B and C.** Classification of Hsfs is based on the amino acid sequence of DBD and HRA/B regions. The phylogenetic tree for all Hsfs was drawn using the Clustalx 1_8-msw and Tree view softwares. At-*Arabidopsis thaliana*, Gm-*Glycine max*, Lp-*Lycopersicon peruvianum*, *Le-Lycopersicon esculentum*, Nt-*Nicotiana tobacum*, Os-*Oryza sativum*.

1) Tomato HsfsA1, the constitutively expressed member of class A Hsf family has been shown to be the master regulator of thermotolerance in plants. By using functional knock out approach, it was shown that the expression of heat stress inducible transcription factors and Hsps is solely dependent on expression and activity of HsfA1 (Mishra et al. 2002). Although many other members of class A Hsf family rescued the thermotolerance in transient expression system of protoplasts, the same Hsfs are not competent enough to complement the lacking HsfA1 in the whole plant. This may be due to their heat stress inducibility, tissue specific expression, very low expression levels or so far unknown mechanisms. This already suggests that there is a hierarchy in Hsf action.

2) The heat stress inducible member of tomato Hsf family, HsfA2 is unique in itself. Due to a strong NES it is a shuttling protein and requires interactions with other members of class A Hsf family for an efficient nuclear retention (Scharf et al 1998; Heerklotz et al. 2001, and Chan, unpublished). Additionally, this protein has been found to be associated with the cytoplasmic heat stress granule (HSG) complexes, via its interactions with a member of Hsp17-CII family. The interaction might have consequences for regulating the activity of Hsf (Scharf and Port, unpublished).

3) Tomato HsfA3, although constitutively expressed in cell culture (Bharti et al. 2000), might have a tissue specific expression in the plants. Moreover, it is shown be regulated by a heat-activated MAP kinase, which further enlarges the concept of functional diversity of plant Hsfs (Link et al. 2002).

4) Another level of complexity among plant Hsf members is added by the fact that there are sub-class specific interactions observed among different members. The most prominent example is the heterooligomerization among Hsfs A1, A2 and A3. All three Hsfs have been shown to interact with each other, thus synergistically enhance their DNA binding and target gene activation (Bharti and Scharf, unpublished).

5) In contrast to synergistic interactions stated above, Hsfs A4 and A5 seem to form inhibitory complexes. It has been shown recently that a very specific interaction between HsfA4 and HsfA5 leads to complete inhibition of the activation potential of HsfA4. Although the exact molecular mechanism is not clear yet, it is tempting to speculate that HsfA5 might recruit a corepressor complex (Baniwal, unpublished). These observations are particularly interesting because HsfA4 is evidently involved in pathogen induced defence and apoptosis in plants (Yamanouchi et al. 2002).

6) Similar to sub-class A1, A2 and A3 group, Hsfs A6 and A7 form heterooligomers with synergistic outcomes, still the mechanistic details are largely unknown.

7) HsfA9 is also an interesting sub-class, because of its tissue specific expression and role during embryogenesis (Almoguera et al. 2002; Ganguli, unpblished).

8) In contrast to a relatively detailed analysis of class A Hsfs, little is known about the roles of class B and C Hsfs. But their totally different C-terminal domains, lack of any activator function, lacking potential to form hetrooligomers and present of repressor members already proves that they at least to do not have redundant functions with class A Hsfs. Another unique feature of class B Hsfs might be reflected by the fact that in rice and soybean the B2 and B4 groups are specially enlarged (see Fig. 2), arguing that class B Hsfs might mediate plant and growth conditions specific roles. The initial analysis of HsfB1 overexpressing and knock out tomato plants shows that it might have developmental roles (Mishra and Scharf, unpublished). In this study evidences have been presented about the novel coactivator function of sub-class HsfB1 members.

Although incomplete, the above mentioned arguments prove that members of the plant Hsf family may fulfill different functions during plant development and stress-tolerance. It has been suggested that changing combinations of Hsfs during the ongoing heat stress and their differential potentials to form heterooligomers might contribute to promoter specific or coregulator specific complexes of Hsfs.

1.4 Heat stress element (HSE) containing promoters

Probably intimately connected with the complexity of plant Hsf family are the peculiarities of Hsf dependent promoters. Sequence of Hsf binding site, the heat stress element (HSE) is absolutely conserved among all eukaryotes. The consensus sequence is a palindromic module formed by a purine rich head motif (H) and a pyrimidine rich tail motif (T): 5'-aGAAnnTTCt-3' (Pelham 1982; Nover 1987). In addition, Hsp promoters contain binding sites for other activators as well. These sites are mostly associated either with a developmental stage, tissue specific or a different stress induced expression of these proteins (Fernandes et al. 1994; Haralampidis et al. 2002). However, the particular pattern defines the regulatory finger print of hs inducible genes (Nover 1987; Nover 1991). Although the functional significance was not clear at that

time, the existence of elaborate clusters of HSEs in promoter regions of hs inducible genes of plants was recognized earlier (Schöffl et al. 1984; Czarnecka et al. 1985; Nagao et al. 1985).

The HSE clusters present in plant Hsp promoters contain a combination of active and inactive HSE motifs. The existence of such clusters is defined by the exact pattern of the nucleotides and the presence of the underlined G and C residues, which are essential for a HSE motif to be active. In addition, for an active motif at least one of the two nucleotides indicated by large case letters (AA, TT) must be present. The flanking adenosine and thymine residues (a, t) are frequently found but are not essential. Clusters of HSE modules, which are in the focus of this study are defined by an uninterrupted pattern of several motifs. The pattern must be precise, but not all motifs of a cluster need to be functional.

An extensive sequence analysis of several sHsp and some constitutive promoters done during this study showed the presence of such HSE clusters in all these cases. It has been revealed in this study that the presence of HSEs in constitutive promoters is not an irrelevant observation, it demonstrates that the regulatory potential of Hsfs during heat stress is not only limited to Hsp genes but might extend to constitutive genes too.

1.5 Coregulators of Hsf activity

Activation of an Hsp gene requires several steps: including heat stress induced activation of Hsfs, promoter occupancy, assembly of preinitiation complex (PIC) and correct initiation of transcription. The latter two steps need some additional coregulators, which are recruited to the promoters by their specific contacts with Hsfs. These coregulators on the one hand modulate the chromatin structure by acetylation and or by remodelling of histones to facilitate the binding and recruitment of RNA polymerase II holoenzyme complex. On the other hand, they might directly interact with transcriptional machinery to stabilize its assembly at the right promoter (Kadonaga 1998; Workman and Kingston 1998; Glass and Rosenfeld 2000; Lemon and Tjian 2000; Hochheimer and Tjian 2003). TBP-associated factors (TAFs), Histone acetyl transferases (HATs) and ATP dependent chromatin remodelling complexes are the three potential coregulator complexes, which are required for the efficient initiation of transcription. Although these

coregulators are general in their action, their recruitment to a particular promoter is mediated by sequence specific activators.

1.5.1 TBP-associated factors (TAFs)

Binding of TATA binding protein (TBP) to the TATA element in the core promoter is a prerequisite for assembly of the RNA-Polymerase II transcriptional machinery. TBP usually exists as a subunit of large complexes, which is termed TFIID complex in case of RNA-Pol II. TFIID complex consists of TBP and 10-12 TBP-associated factors (TAFs) most of which are highly conserved among eukaryotes. The hypothesis that TAFs are essential mediators of transcription is supported by the fact that they interact both with transcriptional activators and general transcription factors associated with RNA-Pol II holoenzyme (Hahn 1998; Hampsey 1998). It was initially assumed that TAFs on the one hand relay information from activators to RNA Pol II, on the other hand stabilize the assembly of Pol II holoenzyme complex itself. These initial reports about the coactivator function of TFIID complex were overshadowed by the observation that some of the TAFs have structural homology to histone proteins. The biochemical studies showed without doubt that components of TFIID complex indeed assemble like histone octamers and have similar quaternary structure. Although the exact mechanism of action of these histone-fold containing complexes is not known, several arguments exist which suggest that these complexes are either responsible for protein-protein interactions and or protein-DNA interactions, thus substituting the nucleosome (Burley and Roeder 1996).
The importance of TFIID complex is underscored by another fact that several of its subunits are common to SAGA complex, a known histone acetyl transferase (HAT) complex and that some components have HAT activities. Thus TAFs are integral components of TFIID complex needed for efficient transcription initiation.

1.5.2 Histone acetyl transferases (HATs)

The initial observation about association of histone acetylation with transcriptional activity were made in 1964, when pioneering work done by Vincent Allfrey proposed the role of histone acetylation and methylation in transcriptional regulation (Allfrey et al. 1964). Subsequently several studies have linked acetylation of histones to transcriptionally active chromatin (for ref. see Struhl 1998). Acetylation has been shown to occur at N-terminal tails of histones, which are positively charged, have flexible structure (Luger et al. 1997) and were suggested to stabilize nucleosomes by interaction

with the negatively charged DNA. Acetylation of histone tails neutralizes the positive charge, thus destabilizing the nucleosome structure and making it accessible for the transcriptional machinery (Mizzen and Allis 1998; Struhl 1998; Workman and Kingston 1998; Sterner and Berger 2000).

The type A HATs with nuclear localization can be divided in to several superfamilies, e.g. GNAT, MYST, nuclear receptor coactivator, $TAF_{II}110$, TFIIIC and p300/CBP. In the context of this thesis, attention has been focussed on a special HAT family, the p300/CBP family of mammals.

Both p300 and CBP (CREB binding protein) are large proteins with 300 kDa size, originally identified as E1A and CREB binding proteins respectively (Harlow et al. 1986; Chrivia et al. 1993). Initially the two proteins were considered as coactivators of transcription perhaps by linking activators with components of transcriptional machinery (Eckner et al. 1994; Arany et al. 1995; Chan and La Thangue 2001). Their histone acetyl transferase (HAT) activity was discovered in 1996 (Bannister and Kouzarides 1996; Ogryzko et al. 1996). The two proteins are ubiquitously expressed and share high degree of homology especially in domains needed for structural and functional integrity. The homologous domains include three Zinc finger domains (C/H1-C/H3), where C/H2 constitutes the HAT domain. The bromo, CREB, C/H1 and C/H3 domains are known for their high potential for protein-protein interactions (Fig. 3). So far several different proteins have been shown to interact with these domains of p300/CBP proteins, which include transcriptional activators, coactivators, components of Pol II holoenzyme complex and other house-keeping proteins (Fig. 3; Goodman and Smolik 2000; Chan and La Thangue 2001; Vo and Goodman 2001). As a result of this flexibility in protein interactions, these two proteins have been called as global transcriptional coactivators.

Different mechanisms are discussed for the action of CBP/p300:

1) **A bridge with the transcriptional machinery**: Since on the one hand CBP/p300 proteins are recruited to particular promoters by sequence specific transcriptional activators. On the other hand these proteins interact with components of the transcriptional machinery. Therefore they are considered as a bridge between activator and pre-initiation complex (see Fig. 3; ref. by Goodman and Smolik 2000; Chan and La Thangue 2001).

2) **A scaffold for the assembly of multiprotein complexes**: As mentioned above CBP/p300 proteins might nucleate the assembly of diverse cofactors into

multicomponent co-activator complexes, thus increasing their local concentration. Additionally, they might provide a scaffold to stabilize the binding of different activators to the promoter (reviewed by Goodman and Smolik 2000; Chan and La Thangue 2001).

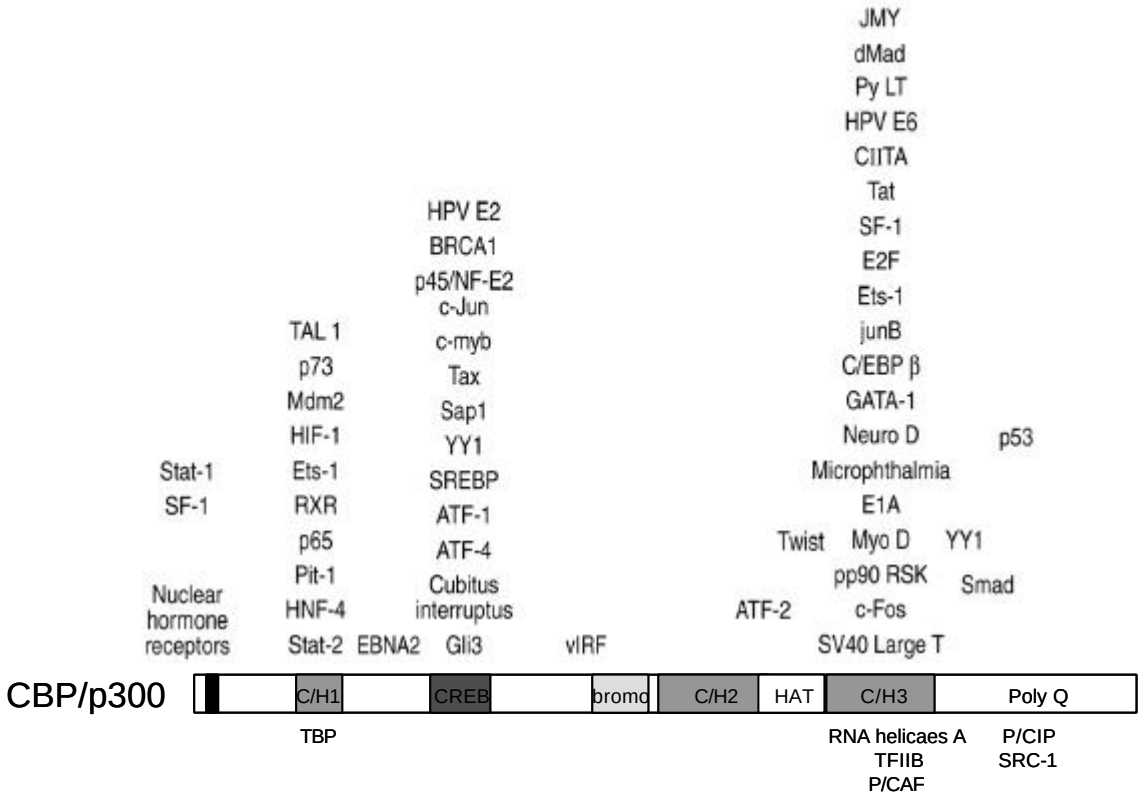


Figure 3. **Structure of mammalian CBP/p300 proteins and interacting partner proteins (Goodman and Smolik 2000).**
Different domains of CBP are shown, namely the three Zinc finger domains (C/H1-C/H3), CREB binding or KIX domain, bromo domain, histone acetyl transferase (HAT) domain and a large poly Q stretch at the C-terminus. The names below show the components of transcriptional machinery which interact with zinc finger three and poly Q stretch of the protein. On the top are the other proteins interacting with CBP/p300, including both transcription factors and transcriptional coactivators. Note that different proteins are interacting all over the body of the protein.

3) As acetylate transferase and ubiquitinase: As discussed above acetylation of histones tails is a prerequisite for efficient transcription. CBP/p300 proteins can acetylate the histones, with a special preference for H2A and H4 tails containing the –GRGK motif (Bannister and Kouzarides 1996; Ogryzko et al. 1996; Kimura and Horikoshi 1998).

The wide potential of CBP/p300 proteins to regulate transcription is enlarged by their ability to acetylate non-histone substrates as well (especially transcription factors; reviewed by Sterner and Berger 2000) and by their ability to ubiquitinate (Grossman et al. 2003).

1.5.3 ATP dependent chromatin remodelling complexes

In addition to the above discussed function of HATs in alteration of nucleosome structure, there are chromatin modelling complexes which utilize ATP to remodel chromatin by changing the location or conformation of nucleosomes. This changed nucleosome positioning might facilitate the access of transcriptional machinery to the promoter. Moreover the ATP-dependent remodelling complexes can remodel the chromatin architecture to a closed state as well. Many different classes of these complexes are known by now but the two best studied are SWI/SNF and ISWI families. All these remodelling proteins exist as hugh multisubunit complexes with several unique polypeptides, but the ATPase subunits are quite conserved among different families (Workman and Kingston 1998).

1.6 Enhanceosomes: the combinatorial units of gene expression

It is fascinating to see how a limited number of transcription factors and interacting coactivators in a genome control such a vast variety of developmental, tissue, environmental and pathogenic cue specific expression, it is still mysterious how such a unique and differential gene activation pattern is achieved? This is assumed that both the transcription factors and their coactivators act in concert, in several combinations to regulate the expression of different genes under varied conditions. This is the basis of the concept of enhanceosomes, i.e. of the existence of combinatorial (unique) units of defined combinations of DNA (enhancer elements) and proteins (activators, co-activators). The combinatorial theory predicts here that gene responding to a single signal would assemble the corresponding enhanceosome only in response to that signal, whereas genes responding to different stimuli would assemble multiple, but signal-specific enhancesomes. Each enhanceosome would be unique in itself with respect to its location relative to the core promoter, number and kind of transcription factors binding to it, regulation by architectural proteins and the type and order of interacting coactivators (Wolberger 1998; Merika and Thanos 2001).

Certain prerequisites need to be full filled for a transcription regulatory mechanism to be called enhancesome mediated.

1) The transcription activators must occupy the enhancer module in a cooperative manner. Direct physical interactions between these transcription factors are not necessary but they might stimulate each other in promoter binding by changing the DNA conformation (bending). Actually, it has been shown in many cases that transcription factors have contacts after their binding to the DNA, which further stabilizes the whole complex. This would ensure that only those promoters are activated under given conditions which contain the right collection of binding sites, i.e. have the appropriate promoter architecture.

2) A supporting argument for enhanceosome function is that under physiological concentrations none of the activators could bind to the DNA with similar affinity.

3) The end result of the cooperative binding of activators is not just the stability of enhanceosome but it displays a second form of cooperativity too. The enhanceosome exposes a unique activating surface, greatly facilitating the recruitment of the coregulators discussed above (see part 1.5). These in turn counteract the repressive chromatin environment, leading to efficient assembly of pre-initiation complex (PIC).

4) This type of synergy cannot be achieved by tandem repeats of activator because the special activating surface needed for the recruitment of coactivators requires multiple types of activation domains in close vicinity.

1.7 Aims of this study

Experiments described in my thesis were performed to study the function of tomato HsfB1 as a novel coactivator cooperating with acidic activator proteins in recruitment of the plant CBP ortholog HAC1. I will deal with six major aspects of coactivator function of HsfB1:

1) The first part of the study proves the synergistic interactions between tomato HsfA1 and HsfB1, observed only on natural promoter fragments of sHsp genes. It will be shown that the presence of natural HSE cluster and the full length Hsf proteins are prerequisite for synergism.

2) Natural Hsp promoter fragments were analyzed to understand the importance of promoter context and the cluster of HSE modules in mediating synergism. Dissection of HSE cluster reveals the need for co-existence of active and inactive HSE motifs for optimum synergistic interactions between HsfA1 and HsfB1.

3) The importance of different domains of HsfA1 and HsfB1 were highlighted by mutational analysis. Especially for HsfB1 it was shown that a single lysine residue in the C-terminus of the protein, embedded in the –GRGK motif is indispensable for synergistic interactions with HsfA1.

4) The coactivator potential of HsfB1 is neither limited to Hsp promoters, nor to cooperation with HsfA1. It acts as a synergistic partner on a subset of house-keeping and viral promoters also. This general coactivator function of HsfB1 can be extended to direct analysis with other acidic activators and similar HsfB type coactivators have been identified from other plants as well.

5) The mechanism behind the synergistic effects of HsfB1 were explored by using *Arabidopsis* orthologs of mammalian CBP, HAC1. HAC1 not only mediates the synergistic reporter gene activation, via the same lysine residue in the –GRGK motif of HsfB1 but also interacts directly both with HsfA1 and HsfB1.

6) Finally, it was shown that HsfB1 forms ternary complexes both on Hsp and viral promoter fragments and this complexing is enhanced and stabilized in the presence of HAC1. These observations confirmed the coactivator function of HsfB1 as part of an enhanceosome-like complex together with HAC1/CBP.

2 Materials and Methods

2.1 General materials and methods

Standard procedures were used for gene technology work (Ausubel et al. 1993; Sambrook and Russell, 2001). For cloning, oligonucleotides were synthesized by Biospring (Frankfurt, Germany) and MWG-Biotech (Ebersberg, Germany). PCR fragments were amplified with Taq Plus Precision System (Stratagene) and purified by "QIAquick gel extraction kit" (Qiagen, Germany). Restriction digestions were performed according to manufacturers protocol (MBI Fermentas and Roche Diagnostics). Ligation was performed with T4-DNA ligase (Roche Diagmostics) according to the recommended protocol. GUS reporter assays and analysis of protein expression from tobacco protoplasts were described previously (Döring et al. 2000; Scharf et al. 1998). For immunoblot analysis proteins were transferred to 45µm nitrocellulose membrane (Schleicher and Schuell) and processed further for chemiluminescence detection following the manufacturer's protocol (NEN). For single cell reporter assays with Gfp and DsRed as reporters, tobacco protoplasts were processed as described by Heerklotz et al. (2001).

Genomic DNA from *Arabidopsis* cell suspension culture was prepared with the CTAB buffer (Ausubel et al. 1993). Total RNA from *Arabidopsis* leaves was isolated by the guanidinium thiocyanate method (Ausubel et al. 1993). cDNA was prepared using MMLV Reverse transcriptase according to the manufacturer's protocol (MBI Fermentas).

Rabbit antisera against tomato HsfA1, HsfA2, HsfA3 and HsfB1 were described (Lyck et al. 1997; Bharti et al. 2000; Mishra et al. 2002). Myc and Flag antisera were obtained from Babco and Sigma respectively. Secondary antibodies against rabbit/mouse immunoglobulins conjugated with horse radish peroxidase were obtained from Sigma.

2.2 Expression and reporter constructs

A complete survey of all the constructs made during this study, with cloning strategy and primer sequence is given in the appendix. Plant expression and reporter constructs are based on the pRT and pBT series of vectors respectively (Döring et al. 2000; Töpfer et al. 1988), whereas animal expression and reporter vectors are based on pcDNA3 and

pluc respectively (Heerklotz et al. 2001). For sequence details and predicted HSEs of sHsp promoter fragments, see Scharf et al. 2001 and for constitutive promoter constructs see Table. 9 in Appendix. His-tagged fusion constructs were created in the pJC series of vectors (Clos and Brandau 1994). GST-tagged vectors were obtained from Promega and are based on pGEX series of vectors. For in vitro transcription-translation, pcDNA3 vectors were used for HsfA1wt and HsfA1-A7 mutant, whereas HsfB1wt and HsfB1-? were cloned into pßstop vector, containing the ß-globin translation enhancer (kind gift from K. Melcher). Important HsfA1 and HsfB1 mutants are defined below (for further details see block diagram in Fig. 1 and 5:

HsfA1: HsfA1-M5(R93>D); HsfA1?HRA/B (deletion of aa 164-238); HsfA1mutNLS (KR253/4>NS), HsfA1?C394 (deletion of C-terminal aa 395-527); HsfA1-A7 (HsfA1 with 451-IDWQSGLL 12aa DPFWEKFL- >451-ID AQSGAA 12aa DPAAEKAA-).

HsfB1: HsfB1-M4 (KH54/5>EL); HsfB1?HRA/B (deletion of aa 145-213); HsfB1?NLS (deletion of aa 214-268); HsfB1?C198 (deletion of C-terminal aa 199-301); HsfB1-? (deletion of aa 272-279 with GRGK motif); HsfB1-R (HsfB1 with 272-GRGK>GKGR).

2.3 Culture and transfection of COS7 cells

COS7 cells were maintained in Nutrient mixture Dulbecco´s modified eagle medium supplemented with 10% fetal bovine serum and 1% Penicillin/Streptomycin (both Life Technologies). Cells were kept in an incubator with 5% CO_2 and 95-99% relative humidity at a temperature of 37°C. Transfections were done with polyfect (Qiagen), according to manufacturer's protocol.

2.4 Luciferase assays

Luciferase assays were performed as described before (Heerklotz et al. 2001). In brief, 200ng of each HsfA1 and HsfB1 or their mutants (see above and appendix for the details of the mutants) encoding plasmids were transfected together with 400ng of CBP and 800ng of reporter plasmid (phsp17**luc*). Samples in triplicate were transfected in six well plates. After overnight expression, cells were harvested in cell lysis buffer

(Promega). 100µl of soluble cell extract was used for luciferase measurement as explained by Heerklotz et al. 2001.

2.5 Purification of recombinant proteins and GST-fused peptides

His tagged HsfA1, HsfB1, NTD-HAC1 and CTD-HAC1 were expressed in BL21RIL (Stratagene) cells. Expression and enrichment with Ni-NTA beads (Qiagen) was done according to manufacturer's protocol. Ni-NTA sepharose beads bound protein was either directly used for in vitro pull down assays or eluted with 250mM imidazole. For GST-fused histone tail peptides, similar protocol for enrichment with glutathione beads (Pharmacia) as recommended by manufacturer was used. GST-peptides were eluted from the beads using 200mM of soluble glutathione.

2.6 In vitro pull-down assays

HsfA1, HsfA1-A7, HsfB1, HsfB1-? were transcribed and translated in vitro in the presence of ^{35}S-methionine using the TNT^{TM}-coupled reticulocyte lysate (Promega), using manufacturer's protocol. Pull down assays were performed as described previously (Kaufmann et al. 2000). His tagged fused protein was immobilised on Ni-NTA beads (see protein purification). Beads were blocked for 20 min with 20% skimmed milk powder dissolved in NETN buffer (100mM NaCl, 20mM Tris HCl, pH 8, 1mM EDTA, 0.5% NP40 and protease inhibitors). Beads were washed twice with NETN buffer and once with TWB (20 mM HEPES, pH 7.9, 60 mM NaCl, 6 mM $MgCl_2$, 8.2% Glycerin, 0.1mM EDTA, +1mM DTT) incubation buffer at 800 rpm for 5 min. 100 µl of fresh TWB was added to the beads along with 5 µl of the radioactively labelled translate. The mixture was then incubated for 2h at RT with gentle intermittent shaking. The beads were washed 5 times with NETN buffer at 1000 rpm for 5 min each. The pulled down pellet was analyzed by SDS-PAGE and signals for the bound Hsfs were detected by autoradiography.

2.7 Coimmunoprecipitation

Protocol by Gingras et al. (1999) was followed for coimmunoprecipitation. The indicated combinations (Fig. 26C) of plasmids were cotransfected in 10 cm radius cell culture dishes. 1µg of each HsfA1 and HsfB1 encoding plasmids or the corresponding mutants were cotransfected either with 2µg of Flag-CBP or 2µg of empty plasmid. After overnight expression, cells were harvested in NETN buffer (100mM NaCl, 20mM Tris HCl, pH 8, 1mM EDTA, 0.5% NP40 and protease inhibitors). 4mg of soluble cell extract was pre-cleared with protein-A sepharose beads for 1 hour. 1:100 dilution of a-Flag antibody was used for coimmunoprecipitation for 2 hours. Antibodies were pulled down by 1:10 dilution of protein A sepharose beads for another 2 hours. After five washings with NETN buffer, precipitates were analyzed by SDS-PAGE and immunoblotted with antibodies against Flag, HsfA1 and HsfB1.

2.8 Electrophoretic mobility shift assay (EMSA)

Probes for EMSA were labelled with ^{32}P by PCR using primers F: 5´-tacgccaagcttggatccgtcg-3´ and R: 5´-ccttatatagaggaagggtcttgcg-3´ for the shsp promoter fragment and primers F: 5´-aaccacgtcttcaaagcaagtgg-3´and R: 5´-agaggaagggtcttgcgaagg-3´ for the CaMV 35S promoter fragment. 10^5 counts of ^{32}P-labelled probes were incubated for 30 min at 25°C with indicated combinations of recombinant proteins (Fig. 27A and 28) or whole cell extracts of tobacco protoplasts (Fig. 27B). 20 µl of the EMSA buffer contained 20mM Hepes, pH7.5, 50mM KCl, 5mM $MgCl_2$, 2mMDTT, 50mM imidazole, 10% glycerol, 2% Ficoll and 2µg polydIdC and 4µg acetylated BSA. After adding 2µl of DNA running buffer, the samples were loaded on to 5% polyacrylamide gels and were run in 0.5xTBE buffer. Signals from the dried gels were detected by autoradiography.

3 Results

3.1 Synergistic interactions between tomato HsfB1 and HsfA1

In spite of the broad in-silico knowledge about tomato Hsfs (see Fig. 2), the functional analyses is basically limited to a few class A Hsfs. Among the four full length Hsfs cloned so far, the only class B Hsfs (HsfB1) was hardly investigated. HsfB1 does not interact physically with any of the class A members. It is weakly expressed in leaves but has a substantial expression in cell culture and fruit pericarp. Most interesting is the transient expression pattern of HsfB1, as observed in cell culture. In contrast to all the class A Hsfs of tomato, HsfB1 protein is transiently but strongly induced by a heat pulse but the level rapidly declines during recovery (Scharf et al. 1998; Fig. 4). This already suggests that HsfB1 might have an important function during the heat stress. It is definitely not required during normal temperature or under recovery conditions. One possibility is that HsfB1 acts as a functional partner of class A Hsfs and somehow modulates their activity during heat stress.

To study the effect of coexpression of HsfA1 and HsfB1 on natural promoter fragments derived from sHsp genes, reporter assays were performed using transient transfections in tobacco mesophyll protoplasts. The hsp17* reporter used in this assay was cloned from the promoter of soybean Hsp17.3B-CI gene (Fig. 5A; Schöffl et al. 1984). This is relatively a weak reporter, with low basal activity (Fig. 5B, sample 1). The reporter activity increases moderately in presence of HsfA1 (sample 2) or HsfB1 (sample 8). Most interesting is the combination containing constant amount of reporter with decreasing and increasing amounts of HsfA1 and HsfB1 encoding plasmids respectively. In all cases the GUS activity obtained was much higher than the basal activity (compare sample 1 with samples 3-7) or even higher than the activities of HsfA1 and HsfB1 alone. Sample 3 shows that even a minute amount of HsfB1 is able to stimulate the activity of HsfA1 by a factor of at least two. However, the best results were obtained by coexpression of 0.25µg of HsfA1 and 0.75µg of HsfB1 encoding plasmids, resulting in 8 fold increased GUS activity as compared to the activity with HsfA1 alone (samples 2 and 6). Immunoblots at the bottom of the figure show the changing expression levels of both Hsfs. Clearly, the signal for HsfA1 is not decreasing proportionally to the decreasing plasmid amounts used for transformation. Although the

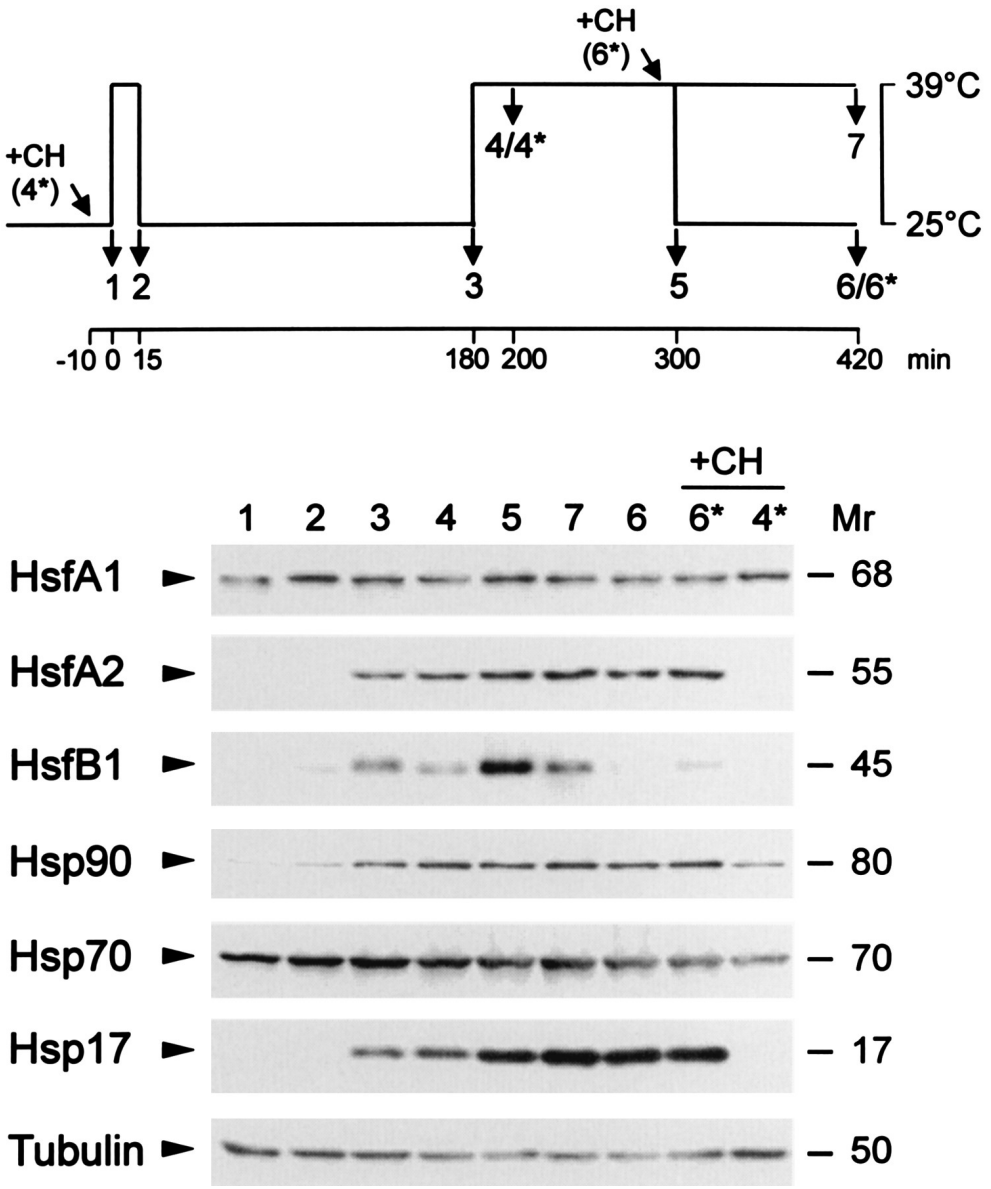


Figure 4. **Heat stress induced expression of Hsfs and Hsps in tomato cell culture (taken from Scharf et al. 1998).**
Heat stress regime, temperature used, time points and different samples taken are shown in the pictograph above. Expression of different Hsfs and Hsps was monitored using corresponding antibodies for immunoblot analysis. Note that HsfB1 is transiently expressed and rapidly disappears in the recovery (samples 3-7).

amount of plasmid was decreased from 1µg to 0.125µg, the signal on immunoblot was decreased only four fold. Evidently, HsfB1 is not only modulating the activity of HsfA1 but is also affecting its expression level, which is controlled by the cauliflower mosaic virus 35S promoter.

To show that the two effects seen in Fig. 5B are based on the same function of HsfB1 as a novel coactivator of HsfA1, different type of titration experiments were performed. In the first case the phsp17**gus* was used as reporter. Increasing amount of HsfB1 encoding plasmid was coexpressed with constant amount of HsfA1 (0.25µg) encoding plasmid (Fig. 6A). Although the increasing amounts of both Hsfs A1 and B1 alone caused a moderate increase in activity (samples 2-5 and 12-17 respectively, Fig. 6A), coexpression of both Hsfs strongly induced the activity of hsp17* promoter fragment (samples 6-11).

The two effects seen in Fig. 5B are confirmed by this experiment: (i) Even minute quantities of HsfB1 induced the activity of HsfA1, without affecting the HsfA1 expression (Fig. 6A, compare sample 2 with 6). (ii) The expression of HsfA1 increased in presence of increasing amounts of HsfB1 (Fig. 6A, compare sample 2 with 6-11). It is also interesting to note that the mere increase of the HsfA1 level does not result in a comparable increase in GUS activity. Comparing samples 2 and 5 (Fig. 6A), it can be clearly seen that although the expression of HsfA1 increases at least 8 fold (corresponding to 8 times increase in HsfA1 encoding plasmid) but the GUS activity increases only 2-3 fold. It shows that high expression of HsfA1 is not automatically related with high GUS activity. In contrast, the activity increases 16 fold in sample 11 (in presence of HsfB1) as compared to sample 2 (Fig. 6A), whereas the expression of HsfA1 increases only 4 fold. This once again confirms the two effects caused by HsfB1. The 16 fold increase in GUS activity is achieved by 4 fold increased HsfA1 expression and 4 fold increase in GUS activity caused by cooperation of HsfA1 with HsfB1 (compare samples 2 and 11, Fig. 6A).

In the second type of titration experiment, the DNA binding domains (DBDs) of HsfA1 and HsfB1 were replaced by the yeast Gal4 transcription factor´s DBD and tested for their activity on a *gus* reporter containing Gal4 binding sites (pgal4DBS*gus*, Fig. 6B). As compared to the weaker hsp17* promoter the gal4DBS promoter shows a much higher activity with Gal4DBD-HsfA1CTD alone (Fig. 6B, samples 2-5) but no increase in GUS

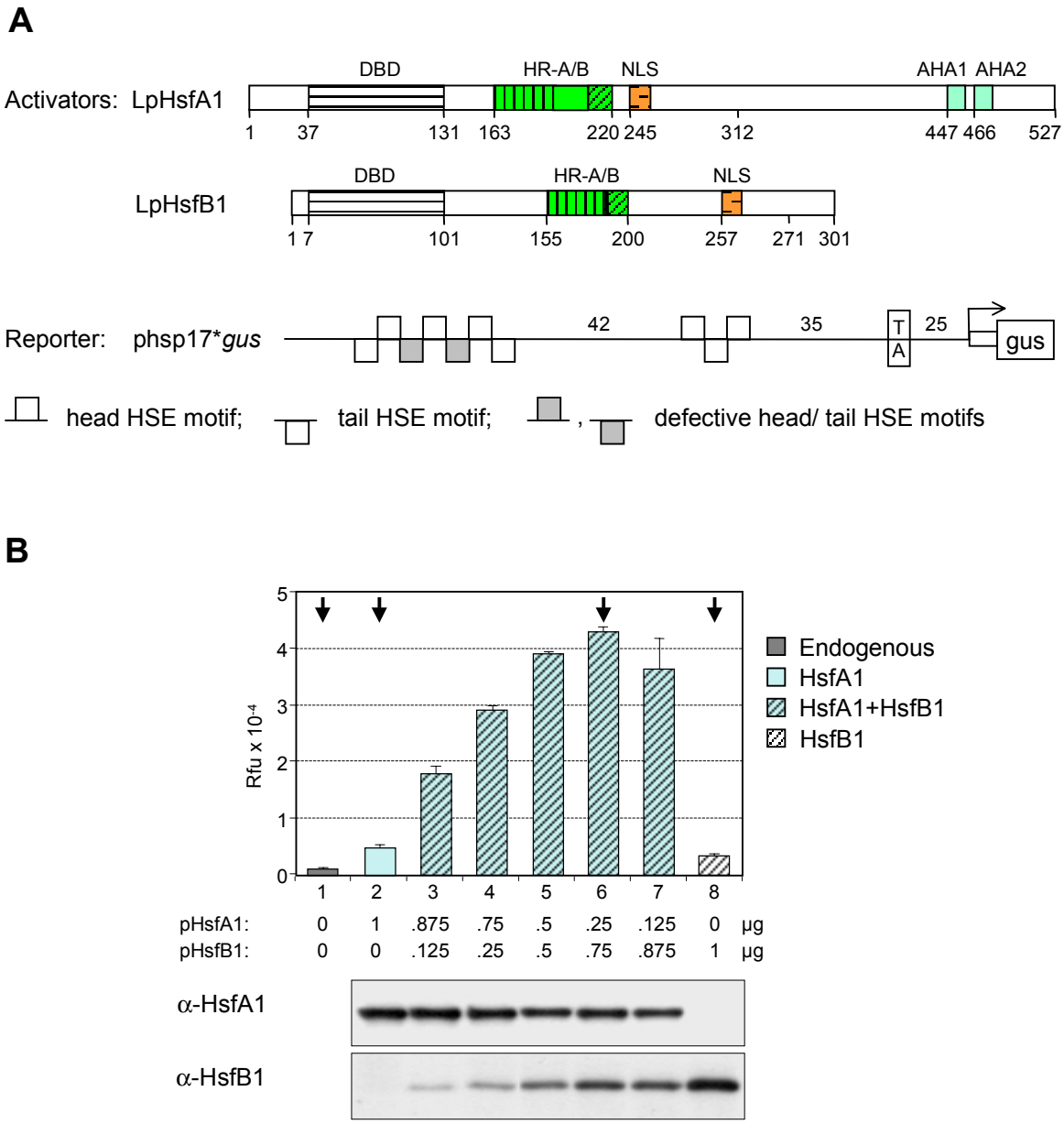


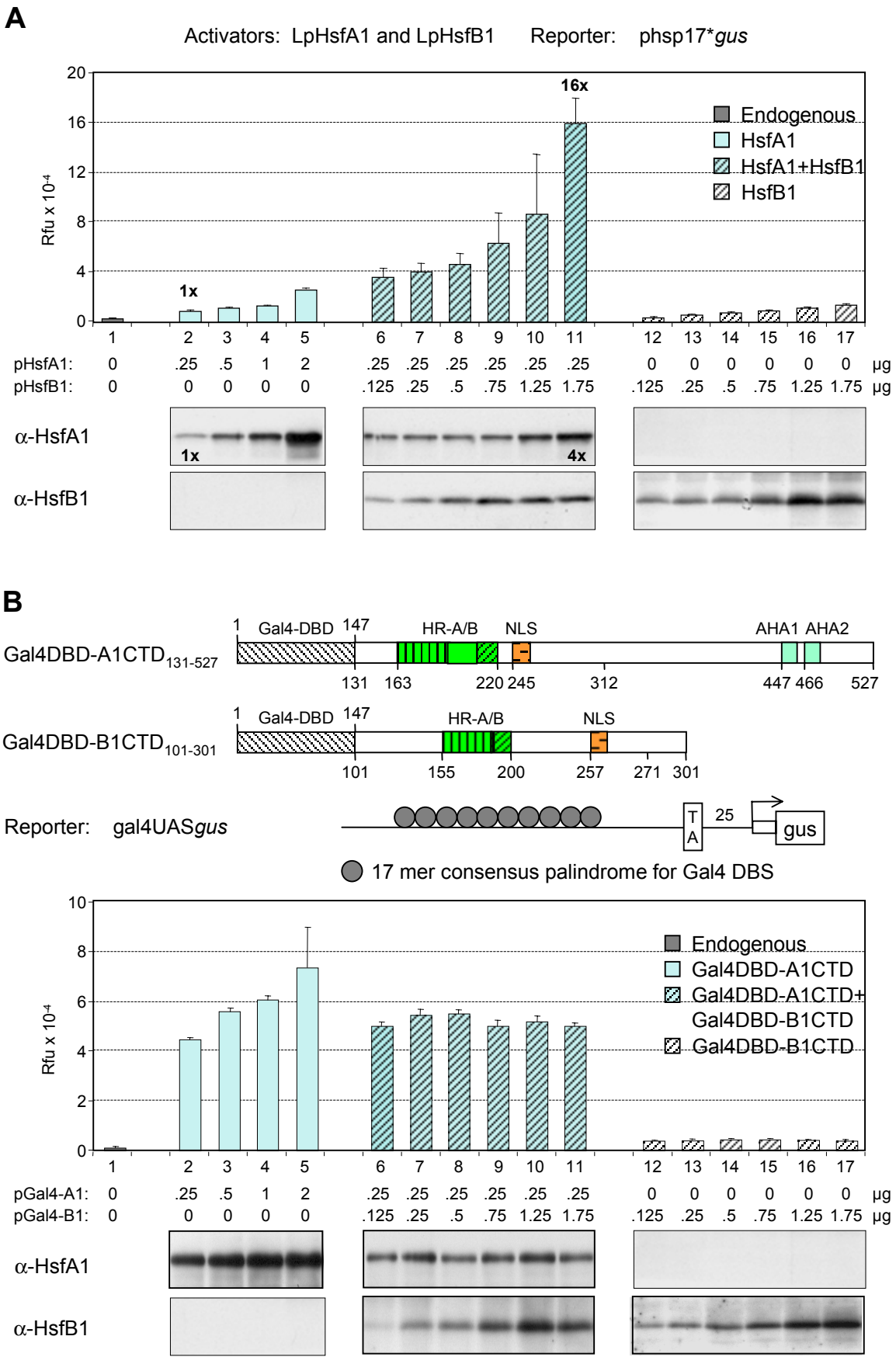
Figure 5. **Influence of synergistic interactions between HsfA1 and HsfB1 on reporter gene activation in tobacco protoplasts.**
(A) Block diagrams showing the basic structure of both Hsfs, with their DNA binding domains (DBD), oligomerization domains (HRA/B), nuclear localization signal (NLS) and the two C-terminal activation motifs known only in case of HsfA1 (AHA1/2). The hsp17*_gus_ reporter contains a promoter fragment from soybean Hsp17.3BCI gene with TATA proximal HSE trimer (HTH) and a TATA distal HSE cluster (THtHtHT) as potential Hsf binding sites. Numbers indicate the distance in bp. WT and defective head/tail motifs are designated as empty and full boxes respectively.
(B) GUS reporter assay showing the expression of phsp17*_gus_ reporter tested in protoplasts transformed with the indicated amounts of HsfA1 and HsfB1 encoding plasmids. Arrows mark samples used as standards for plasmid concentrations in the following experiments. Immunoblots at the bottom indicate the expression levels of both Hsfs A1 and B1.

activity is seen in presence of constant amount of Gal4DBD-A1CTD fusion protein and increasing amounts of HsfB1 fusion protein (Fig. 6B, samples 6-11). In addition, no significant increase in HsfA1 fusion protein expression was observed with increasing HsfB1 fusion protein. This suggests that both the effects of HsfB1 (increased GUS activity and increased HsfA1 expression seen in Fig. 5) require the native state of Hsfs and the natural HSE containing promoters. The gal4DBS promoter fragment, containing tandem repeats of 10 Gal4 binding sites is excellently suited for activity analyses of Hsfs (Döring et al. 2000; Bharti et al. 2000) but is not appropriate to explore certain details about the fine interactions among Hsfs.

Another interesting observation can be made from this experiment. The Gal4DBD-HsfB1CTD protein does not have any activity at all, even if very high amounts of HsfB1 fusion construct encoding plasmid were used for transformation (Fig. 6B, samples 12-17). This emphasizes the fact discussed earlier that HsfB1 CTD has no activator potential of its own. The low activity observed in Fig. 6A, samples 12-17 is probably due to interactions with endogenous class A Hsfs, observed only on natural promoter fragments from sHsp genes.

A similar experiment was done with different type of Gal4 DBD fusion constructs of Hsfs A1 and B1 CTDs (Fig. 7A). These constructs are different from those used in Fig. 6B, because the former ones lack the oligomerization domains of both Hsfs (compare fusion points Fig. 7A and 6B). It is expected that the smaller fusion constructs of both the Hsfs have similar oligomerization potential and hence similar DNA binding affinities. So, the coexpression of these constructs with the pgal4DBS*gus* reporter should lead to a competitive rather than synergistic binding of the two Hsf fusion proteins. A titration experiment similar to Fig. 5B showed that this is indeed the case. The high activity of Gal4DBD-HsfA1CTD alone (sample 2, Fig. 7B) is inhibited even by small quantities of Gal4DBD-HsfB1CTD (sample 3, Fig. 7B).

Figure 6. **Influence of synergistic interactions between HsfA1 and HsfB1 on reporter gene activation in tobacco protoplasts.**
(A, B) GUS reporter assay with phsp17**gus* and pgal4DBS*gus* as reporters respectively. Full length tomato Hsfs A1 and B1 were used as activators in part A. In part B fusion constructs containing the DBD from yeast activator Gal4 (aa 1-147) fused to the CTDs (including HRA/B) of HsfA1 (aa 131-527) and HsfB1 (aa 101-301) were used. As shown identical plasmid concentrations were used in both cases. Expression of full length HsfA1/B1 in part A and fusion constructs in part B are shown by corresponding immunoblots. Numbers in part A indicate the relative increase in GUS activity (16-fold) and the expression of HsfA1 (4-fold) in sample 11 as compared to sample 2.


A
Activators: LpHsfA1 and LpHsfB1
Reporter: phsp17*gus
Rfu x 10-4
16x
1x
Endogenous
HsfA1
HsfA1+HsfB1
HsfB1
pHsfA1:
pHsfB1:
μg
α-HsfA1
α-HsfB1
1x
4x
B
Gal4DBD-A1CTD131-527
Gal4DBD-B1CTD101-301
Gal4-DBD
HR-A/B
NLS
AHA1 AHA2
Reporter: gal4UASgus
TA
gus
17 mer consensus palindrome for Gal4 DBS
Endogenous
Gal4DBD-A1CTD
Gal4DBD-A1CTD+
Gal4DBD-B1CTD
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pGal4-A1:
pGal4-B1:
α-HsfA1
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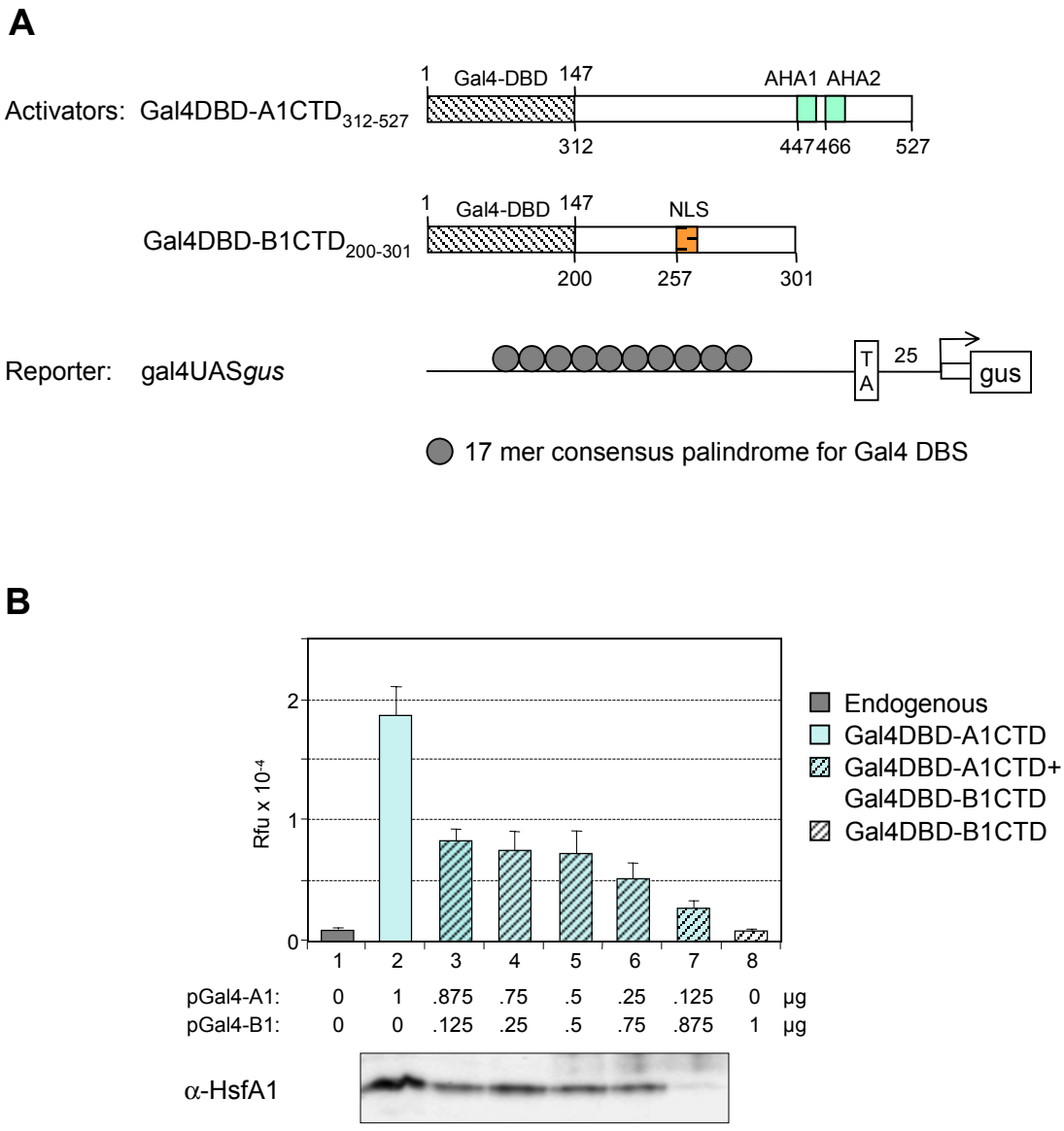


Figure 7. **Effect of promoter architecture on synergistic interactions between HsfA1 and HsfB1.**
(A) Block diagrams showing the fusion constructs of both Hsfs. DBD of yeast activator Gal4 (aa 1-147) fused to the CTDs of HsfA1 (aa 312-527) and HsfB1 (aa 200-301). The *gus* reporter used in this case contains a tandem repeats of a 17mer consensus binding site for Gal4.
(B) GUS reporter assay showing the expression of pgal4DBS*gus* reporter tested in tobacco protoplasts transformed with the indicated amounts of HsfA1 and HsfB1 encoding plasmids. Immunoblot at the bottom indicates the expression levels of HsfA1 fusion construct. HsfB1 fusion construct could not be detected with the available antiserum.

From the above experiments (Fig. 5-7), I would like to draw the following conclusions:

1) Only HsfA1 is a bonafide activator, showing substantial activator potential as wild type or Gal4-fusion protein (samples 2-5, Fig. 6A and B; sample 2, Fig. 7B).
2) HsfB1 has no activation potential of its own (samples 12-17, Fig. 6A and B; sample 8, Fig. 7B).
3) HsfB1 acts as a synergistic partner of HsfA1. Coexpression of HsfB1 with HsfA1 results in an enhanced GUS expression level (Fig. 5B; samples 6-11, Fig. 6A).
4) HsfB1 enhances the expression of HsfA1 cassette connected with CaMV35S promoter (Fig. 5B and 6A).
5) Both above mentioned effects of HsfB1 require the native Hsp promoter and the full length Hsf proteins (Fig. 6B and 7B). They are not observed with the Gal4DBD fusion proteins.

3.2 Concept of synergism: cooperative binding of HsfB1 with HsfA1

Carey M. (1998) and Wang et al. 1999 proposed a mathematical model for synergistic transcriptional activation (Fig. 8A). The model takes into account the relative transcription of a gene analyzed with increasing amounts of plasmid encoding non-cooperative and cooperative activators. The results are plotted as a graph with relative transcription on vertical axis and increasing plasmid concentrations (as log scale) on horizontal axis. According to the model, a standard parabolic curve (curve 1, Fig. 8A) is followed by a single activator binding non-cooperatively to the promoter and being responsible for the recruitment of transcriptional machinery. Whereas cooperatively binding multiple activators give a sigmoidal curve (curve 2, Fig. 8A), because multiple interacting interfaces for the recruitment of transcriptional machinery are involved. The steepness of curve 2 would be affected by reciprocal cooperative effects of the transcriptional machinery and the activators. The enhanced sensitivity imparted by cooperative binding is emphasized by the smaller difference in activator concentrations needed to increase the transcriptional activity from 10% to 90% or near maximal levels. For curve 1, 100 times more activator is required for this increase, whereas only 10 times more activator is needed for the same effect in case of cooperative binding (curve 2).

The same hypothesis was applied to interactions between HsfA1 and HsfB1, i.e. coexpression of both should follow sigmoidal curve if they bind cooperatively to the promoter. To this aim, a reporter assay was performed, where GUS activity from phsp17*_gus_ reporter was measured in presence of either increasing amounts of HsfA1 and HsfB1 encoding plasmids alone or a combination of both in a ratio of 1:3 (standard combination found to give best synergism, see sample 6, Fig. 5B). The total activity obtained was plotted against the increasing plasmid concentration in nanogram (Fig. 8B). Note the different scale used for activity measurements in samples with Hsfs A1 and B1 alone or samples coexpressing Hsfs A1 and B1. It is interesting to see that the GUS activity continues to increase in case of both HsfB1 alone and HsfB1 coexpressed with HsfA1 (although overall scale is different for them). The fact that under the conditions investigated the activity never reaches saturation implies a very dynamic turn over of transcriptional complexes initiated by HsfA1 and HsfB1. In contrast, results with HsfA1 alone clearly give a different curve. Initially, the activity increases with increasing plasmid concentrations but then declines rapidly. This repressive effect of HsfA1 can be explained by the non-cooperative binding of this single activator to the promoter and interacting with transcriptional machinery. Till a certain plasmid concentration, enough HsfA1 is expressed to occupy the promoter and to recruit putative coactivators to the transcriptional machinery. As that threshold concentration is exceeded, free HsfA1 molecules compete with the DNA-bound HsfA1 for the limiting coactivators, resulting in a decrease in reporter gene activation.

Converting the total activity to relative activity (taking maximum activity in each case as 100%) and the net plasmid concentrations to log scale, a graph (best fit curve) was obtained comparable to that proposed by Carey, 1998 (Fig. 8C). Because of the squelching effect of excess HsfA1 protein, it clearly does not follow a sigmoidal curve. Instead a hyperbolic curve was obtained, where several times more activator would be needed to achieve near maximum level of transcription. As expected from the synergistic interactions between HsfA1 and HsfB1, the results obtained by coexpression of both followed a sigmoidal type curve, further impressing upon their cooperative binding to the promoter. Since the curve did not reach saturation, the exact nature of the sigmoidal curve could not be determined but clearly the activator concentrations required to attain a maximum level of transcription is much lower as compared to HsfA1 alone.

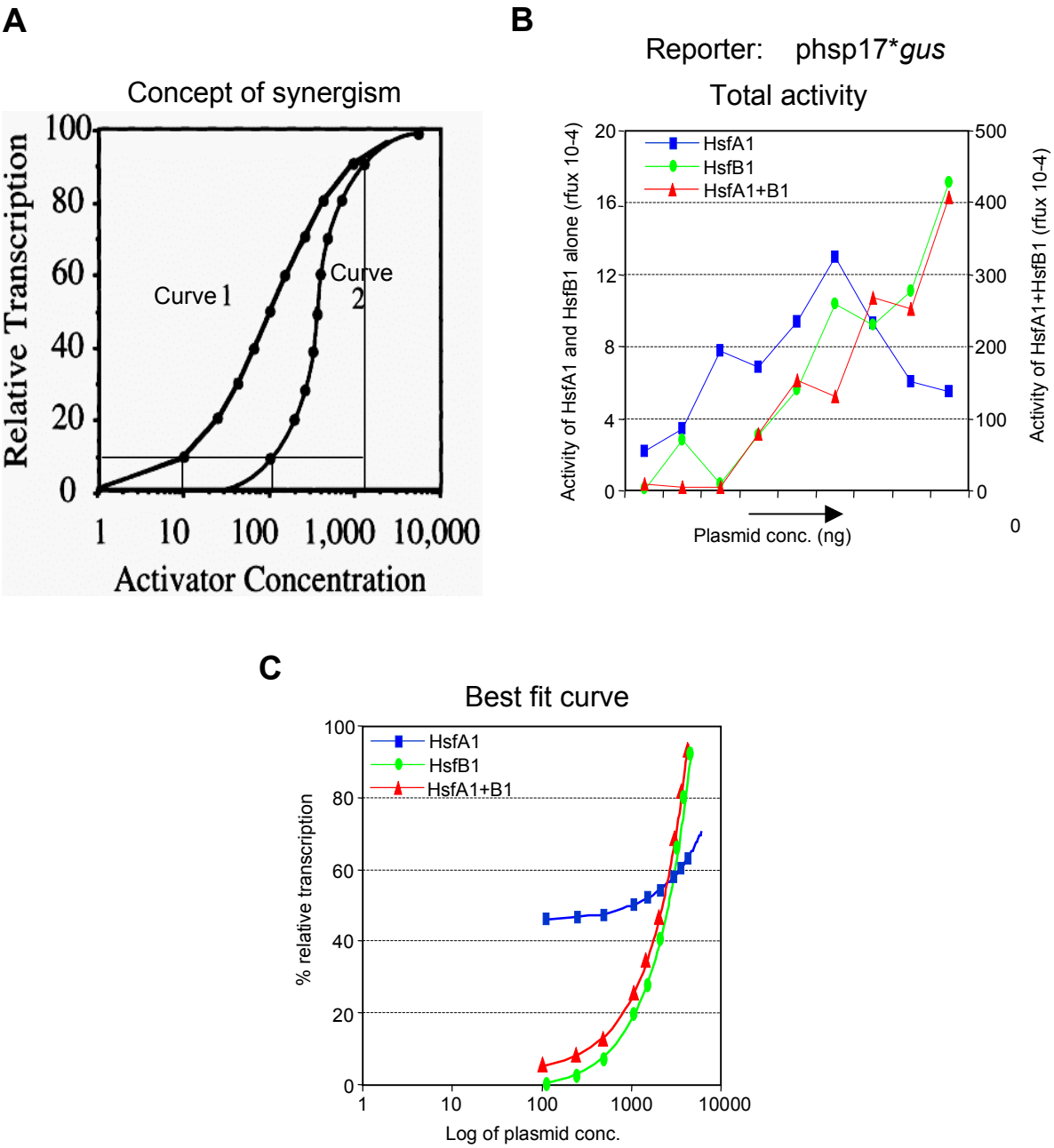


Figure 8. **Concept of synergism (Carey, M. 1998) and results with Hsfs A1 and B1.**
(A) The concept behind synergistic/cooperative interactions between two/more transcriptional activators. Relative transcriptional activity is plotted against log of activator concentrations. In case of curve 1, 100 times more activator is needed to achieve maximum transcription, whereas only 10 times activator is required for curve 2 with cooperatively binding activators.
(B) Total activity obtained using phsp17*gus* as reporter with HsfA1 (blue), B1(green) alone and coexpressed (red). Increasing amounts of plasmid concentration for activators is plotted against the activity. Note the different scale for activity of HsfA1, HsfB1 alone (left Y-axis) and HsfA1+B1 (right Y-axis).
(C) Activity from part B is plotted as % relative transcription (calculated by setting the maximum value to 100% for the respective cases) against log of plasmid concentrations of activator. A best fit curve, taking into account the most probable values for the defined plasmid concentrations is drawn.

Most interesting is the case with HsfB1 alone. Although the overall activity achieved is much less as compared with coexpression of Hsfs A1 and B1 (16 rfu and 400 rfu respectively, Fig. 8B), the GUS activities still follow a sigmoidal curve, similar to the results of samples with coexpression of HsfA1 and HsfB1 (Fig. 8C). This once again suggests that HsfB1 shows synergistic interactions with the endogenous Hsfs from tobacco, and this effect is based on the same cooperative principle. However, due to lower expression of endogenous Hsfs, the overall activity never reaches the HsfA1/B1 coexpressed state (also see the samples 12-17, Fig. 6A and 6B).

Altogether, the above results can be summarized by saying that cooperative binding tendency was observed for HsfA1 and HsfB1 coexpressed with hsp17* promoter fragment, and this cooperativity might be the reason for the transcriptional synergy.

3.3 Importance of HSE clusters as independent units for synergistic interactions between Hsfs A1 and B1

As shown in Fig. 5-7 the promoter context is absolutely required for synergistic interactions between HsfA1 and HsfB1. In order to explore this effect further, different natural promoter fragments (including the 5´upstream regulatory regions, TATA box and the leader sequences) from sHsp genes were amplified and cloned upstream of a *gus* reporter cassette. Fig. 9 represents a collection of such natural promoters derived reporters from soybean Hsp17.3B-CI gene (construct 1, also used in previous experiments), genes encoding the indicated members of the *Arabidopsis* Hsp20 family (constructs 2-5, Scharf et al. 2001) and a sunflower Hsp17-CI encoding gene (construct 6, Rojas et al. 1999). The classification of *Arabidopsis* sHsp genes is based on their localization in the nucleo-cytoplasmic compartment (classes CI and CII) or in the mitochondria (M), plastids (P) and endoplasmic reticulum (ER). Expression of all selected genes is strongly hs-inducible in their native tissues (Siddique, unpublished). The block diagrams in Fig. 9 show that, basically, all promoters look similar with respect to the arrangements of HSE modules. They all contain HSE clusters containing the combinations of both active and inactive HSE modules.

The reporters were tested with the four standard samples indicated by arrows in Fig. 5B, i.e. endogenous, HsfA1 alone, HsfA1/B1 coexpressed and HsfB1 alone. Although all

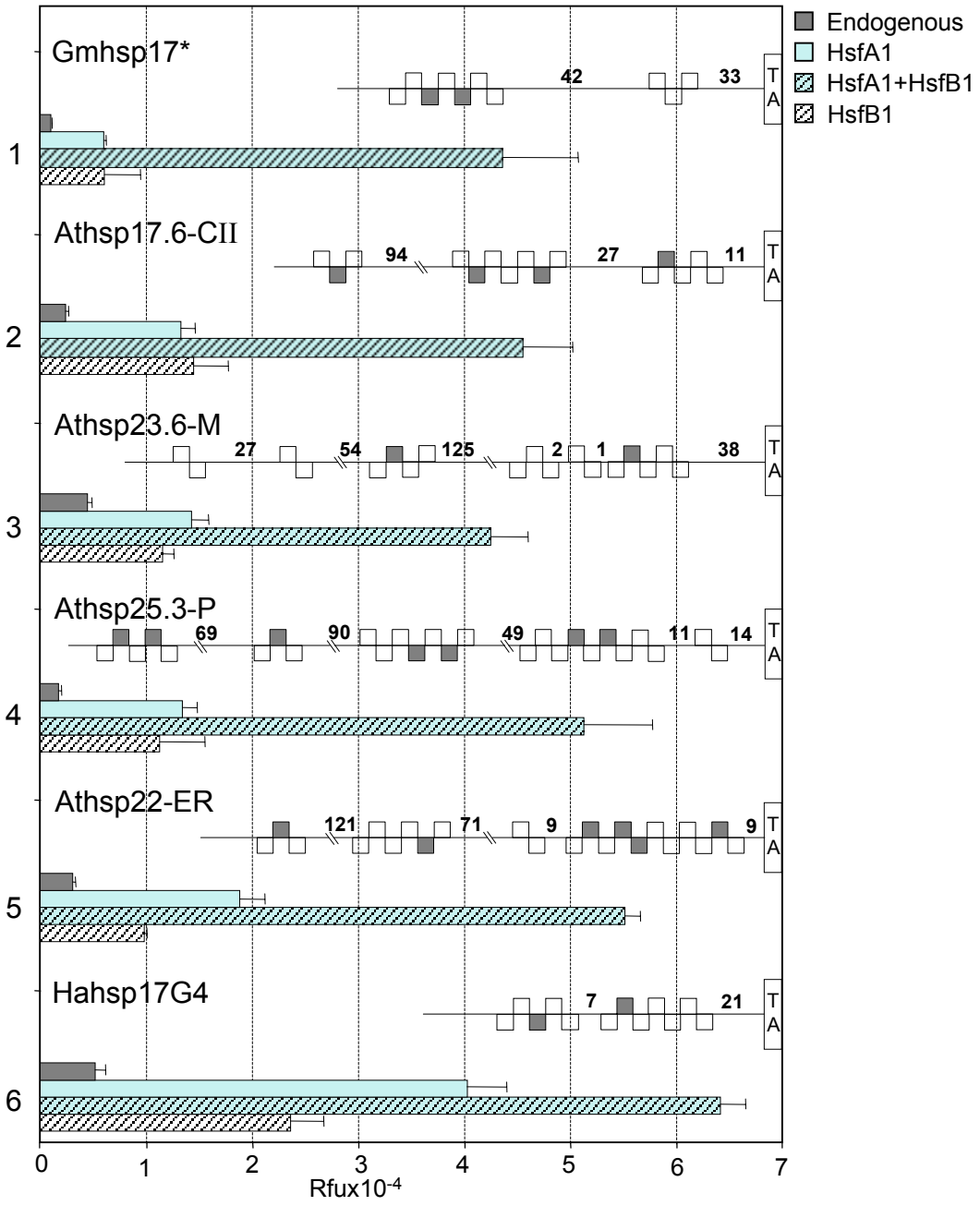


Figure 9. **Synergistic interactions between HsfA1 and HsfB1 on different sHsp gene promoters.**
GUS reporter assay using promoter/leader fusions of promoter fragments of sHsp genes from soybean (construct 1), *Arabidopsis* (constructs 2-5) and sunflower (construct 6) as reporters. Four standard samples (shown by arrows in Fig. 5B) were taken for each reporter, i.e. endogenous (empty plasmid-1.0µg), HsfA1 (1.0µg), HsfA1+HsfB1 (0.25µg+0.75µg) and HsfB1 (1.0µg). *Arabidopsis* promoters used are from different classes of sHsps, CII-nuclear/cytoplasmic, M-mitochondrial, P-plastidial and ER-endoplasmic reticulum (Rojas et al. 1999; Scharf et al. 2001).

promoter fragments turned out to be strong Hsf dependent reporters, the synergistic effects were much less pronounced as compared to the relatively weak promoter fragment of soybean Hsp17.3B-CI gene, the hsp17**gus* reporter (construct 1). The sunflower Hsp17G4 gene derived reporter (construct 6) was an extreme case. There was no synergistic effect at all, the activity observed by coexpression of HsfA1 and HsfB1 was just an addition of the two activities alone. The other constructs 2-5 showed results similar to each other with almost identical basal and synergistic activities of Hsfs A1 and B1. Unfortunately, no direct correlations between the observed GUS activities and HSE configuration of a given promoter could be derived from the set of reporters tested in Fig. 9. In order to better understand the importance of HSE clusters, a smaller set of promoters was further dissected and the corresponding reporter constructs were investigated for their capability to respond to synergistic gene activation. Three different promoter fragments were selected for detailed analysis: the soybean hsp17.3B-CI (construct 1, Fig. 10), *Arabidopsis* hsp17.4-CI (construct 4, Fig.10) and *Arabidopsis* hsp17.8-CI (construct 7, Fig. 10). The HSE cluster modules were cloned into the neutral background of a minimal promoter, 50 nucleotides upstream of TATA box. Evidently, these HSE clusters represent the minimal and sufficient requirement for synergistic interactions between HsfA1 and HsfB1 (Fig. 10). The HSE cluster module of Gmhsp17.3B-CI promoter (construct 3) is the best example. It seems that this is the most important module of the promoter required for both activity and synergism. The two HSE clusters isolated from the Athsp17.4-CI promoter (constructs 5 and 6) not only look similar to construct 3 but also behave in a similar fashion, i.e. have low over all activities but due to lower basal activities for HsfA1 and HsfB1 alone, the synergism is quite strong. In contrast to this, the HSE cluster modules isolated from Athsp17.8-CI promoter (constructs 8-10) behave totally different. With the exception of construct 10, the other two constructs do not show any synergism. But keeping in mind the HSE architecture of constructs 3, 5 and 6, the outcome can be expected. It seems that in some cases, e.g. Athsp17.8-CI promoter, the activity obtained from the whole promoter is a composite effect of several individual parts.

Following conclusions can be drawn from this analysis: HSE clusters with a combination of active and inactive HSE motifs represent the individual unit required for synergistic interactions between HsfA1 and HsfB1. In natural promoters with more than one unit together, the effects get complicated. The HSE clusters do behave as separate units when tested in a neutral background but might behave different if investigated in their

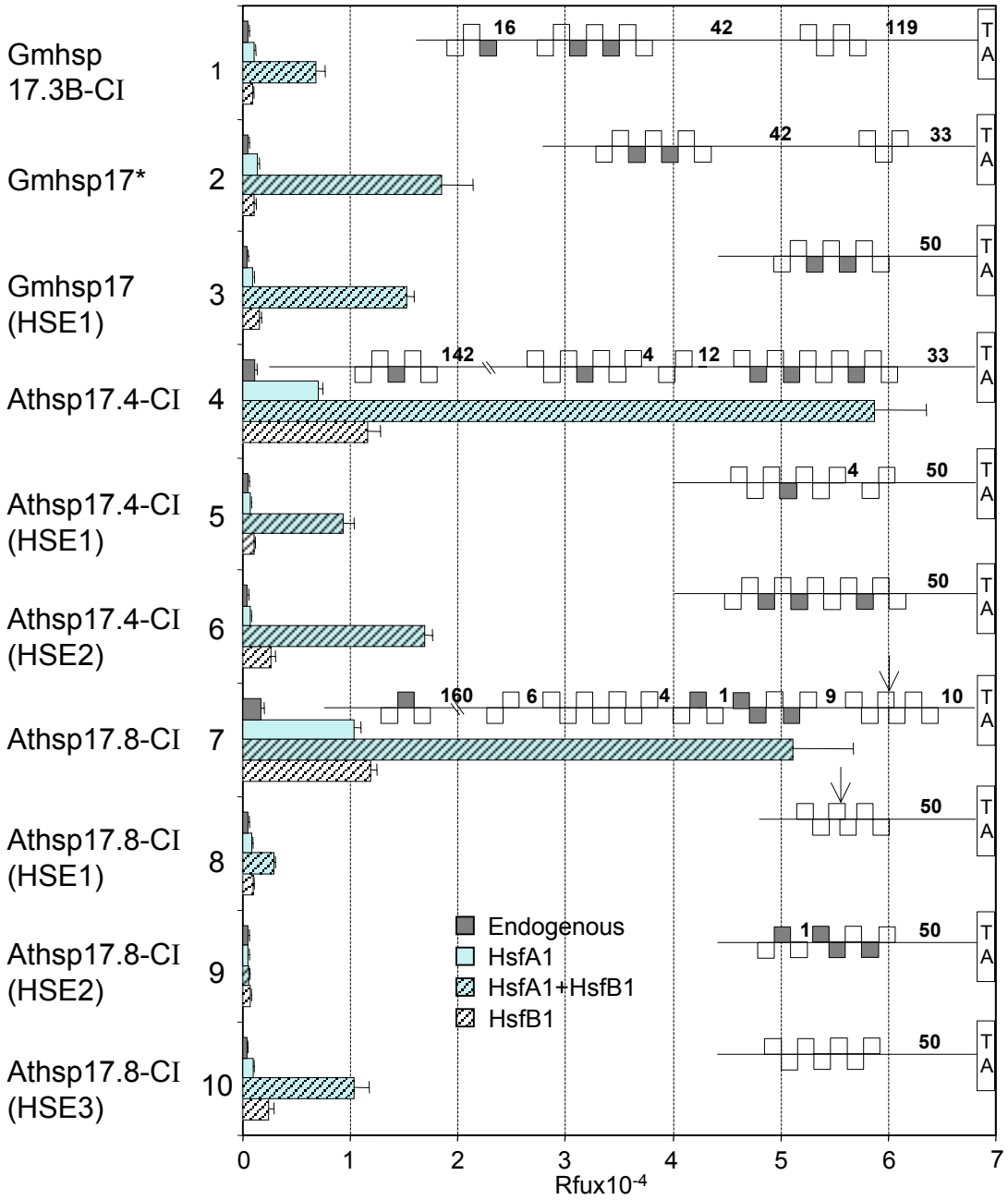


Figure 10. **HSE clusters as independent units for synergism.**
The indicated parts of sHsp gene promoters were fused 50 nucleotides upstream of TATA box in a minimal promoter and tested for synergism in a GUS reporter assay with the four standard samples (arrows in Fig. 5B). Only one HSE cluster was isolated from Gmhsp17.3B-CI promoter (constructs 1-3), two from Athsp17.4-CI (constructs 4-6) and three from Athsp17.8-CI (constructs 7-10). Arrows mark HSE motifs which are overlapping by one nucleotide.

natural context, depending upon their distance from the TATA box or their accessibility in the chromatin structure.

3.4 Functional dissection of HSE cluster modules

Although the presence of HSE clusters containing the combination of active and inactive HSE motifs was noted much earlier (Czarnecka et al. 1985; Nagao et al. 1985; Nover 1991), their functional significance was not known at that time. The evidences presented above show for the first time the importance of such HSE clusters in mediating cooperative DNA binding of Hsfs A1 and B1. Therefore the most urgent requirement to understand the molecular mechanism behind synergism was to study the architecture of HSE clusters in more detail. For this purpose, construct 3 (Fig. 10) was selected. In spite of a simple composition containing THtHtHT HSE cluster, with 3rd and 5th inactive motifs it gave the best synergism as compared to other such HSE clusters (compare constructs 3, 5, 6, 8 and 10, Fig. 10). Different types of mutations were made to dissect the wild type cluster (construct 1, Fig. 11). In the first set the active HSE motifs were inactivated by mutation of the invariant G/C residues, resulting in an increase in number of inactive HSE motifs (constructs 2-6, Fig. 11). Evidently, the wild type cluster (construct 1, Fig. 11) with only two inactive HSE motifs is the minimum requirement for optimal synergism. Any further increase in inactive HSE motifs results in strong reduction in both activity and synergism (bold faced numbers on top of the bars show fold synergism; Fig. 11). In the second set of mutation distance between the 5´ or 3´ functional HSE dimers was changed with the rest of the cluster by introducing 3 nucleotides (constructs 7 and 8). The aim was to disrupt the continuous palindrome pattern of active and inactive HSE motifs. As expected even these mutations resulted in a drastically reduced activity and synergism. Not only the sequence of HSE cluster is important but their proper positioning with respect to each other is also must for the cooperative binding of Hsfs A1 and B1.

A peculiar property of these HSE clusters is the presence of inactive motifs present in a continuous pattern in between the active ones. So, the third set of mutations was directed towards this particular property, i.e. the HSE cluster module was formally improved by mutating one or both of the inactive tail HSE motifs to active HSE motifs or

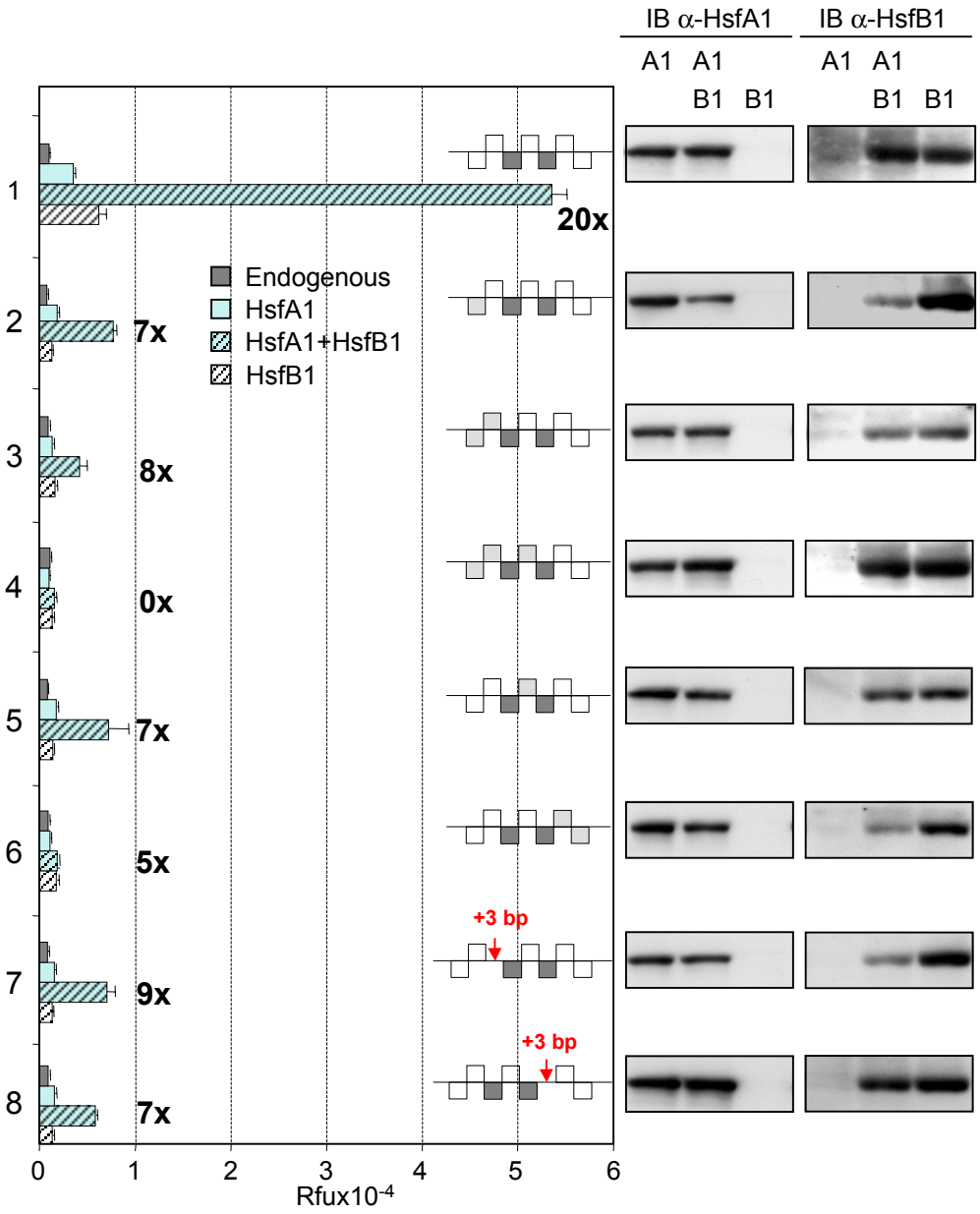


Figure 11. **Dissection of HSE clusters: importance of distinct patterns of functional HSE motifs for synergism.**
HSE cluster module was mutated either by exchange of invariant G/C residues in the functional HSE motifs (light grey boxes, constructs 2-6) or by insertion of 3 nucleotides between 5´or 3´ functional HSE dimer motifs and the rest of the cluster to disrupt the palindrome pattern of continuous HSE motifs (constructs 7 and 8). All the mutant versions of HSE cluster were fused 50 nucleotides upstream of TATA box in a minimal promoter and tested with the standard samples (arrows in Fig. 5B). Expression controls for HsfA1 and HsfB1 are shown. Numbers show the fold synergism, calculated by the following formula; (GUS activity of HsfA1+B1-endogenous GUS activity)/(GUS activity of HsfA1-endogenous GUS activity).

addition of a new active head motif (Fig. 12). Although there was a moderate increase in activities of Hsfs A1 and B1 alone, no increase in total activity was observed, and hence the extent of synergism was reduced considerably (compare fold synergism in construct 1 with 2-5). This finally proves that the wild type cluster (construct 1) is indeed the best platform for cooperative binding of HsfA1 and HsfB1. The inactive HSE motifs act as insulators to weaken the binding of individual Hsfs at the promoter in such a way that they do not compete with each other for the same site. The Hsfs bind to defined positions as trimer (HsfA1) and dimer (HsfB1) respectively. Mutations of the inactive HSE motifs to active ones increase the strength of the promoter, thus Hsfs tend to bind stronger alone and hence compete with each other during coexpression.

To further characterize the essential role of the insulator HSE, a modified version of wild type HSE cluster (construct 2, Fig. 13) was used for this purpose. In construct 2 the marked tail HSE was altered to introduce insulators of varying lengths, flanked by a trimer (5´) and dimer (3´) binding sites (constructs 3-7, Fig. 13). The most interesting results were obtained with construct 4 and 7, where the trimer and the dimer binding sites are separated by 5 and 15 nucleotides respectively. Both reporters show the highest overall activity and synergism. It is intriguing to note the differences between these two reporters, by increasing the distance between trimer and dimer binding sites from 5 to 15 nucleotides the reporter gets weaker, therefore the basal activities with Hsfs A1 and B1 go down (compare GUS activities obtained with Hsfs A1 and B1 alone in construct 4 and 7). At the same time the reporter becomes more responsive to cooperative binding of the two proteins (compare the fold synergism obtained with construct 4 and 7, i.e.12 and 17 fold respectively). The other three constructs with disturbed palindrome patterns either by addition of 3 (construct 3), 5 (construct 6) or 8 (construct 5) nucleotides are neither improved in overall activity nor in extent of synergism (as compared to construct 2).

It can be concluded from above results that prerequisites for optimum interaction of the two binding sites within such a cluster are both the combination and the positioning of head and tail motifs. Insulating (inactive) motifs can improve the efficiency of cooperative binding if they do not interrupt the continuity of head and tail motifs within the cluster.

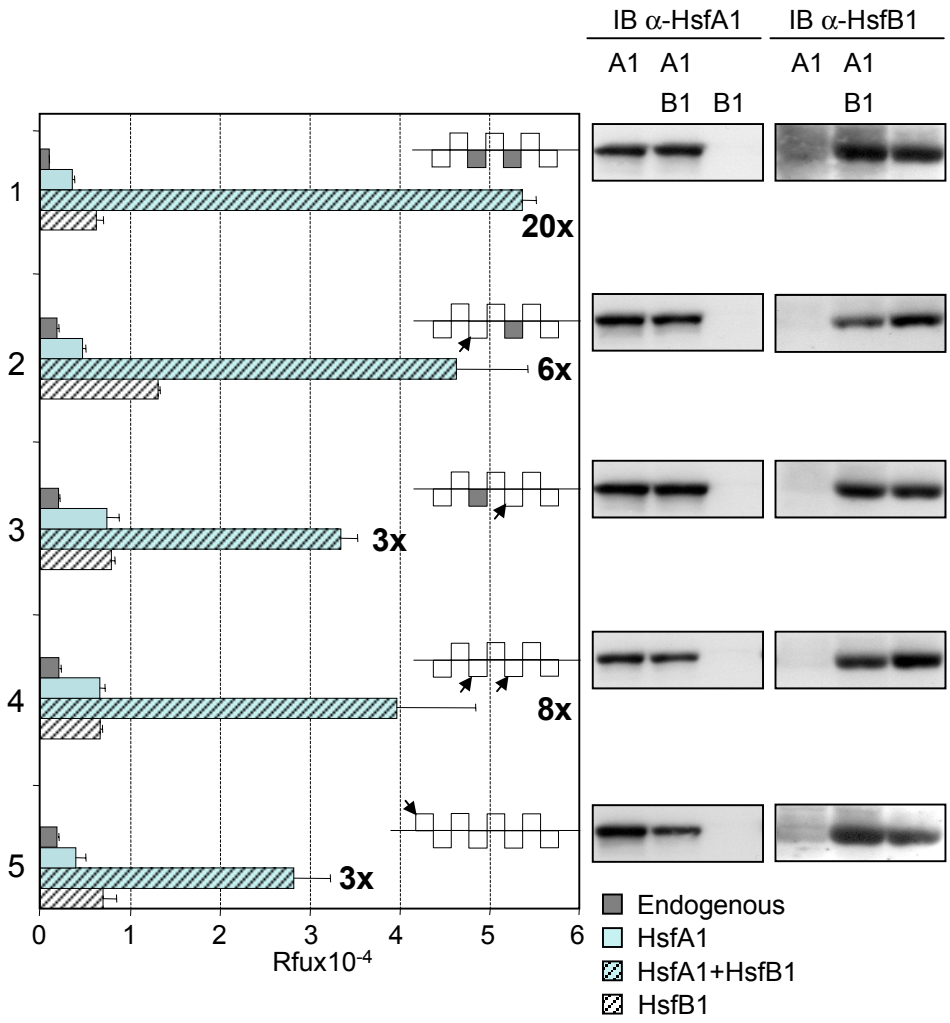


Figure 12. **Dissection of HSE clusters: importance of inactive HSE motifs for synergism.** Naturally inactive HSE motifs were made active by introducing the invariant G/C residues in the sequence of inactive motifs (see open boxes marked with arrows). Cloning and activity analyses were done similar to Fig. 11. Numbers show the fold synergism, for calculation see Fig. 11.

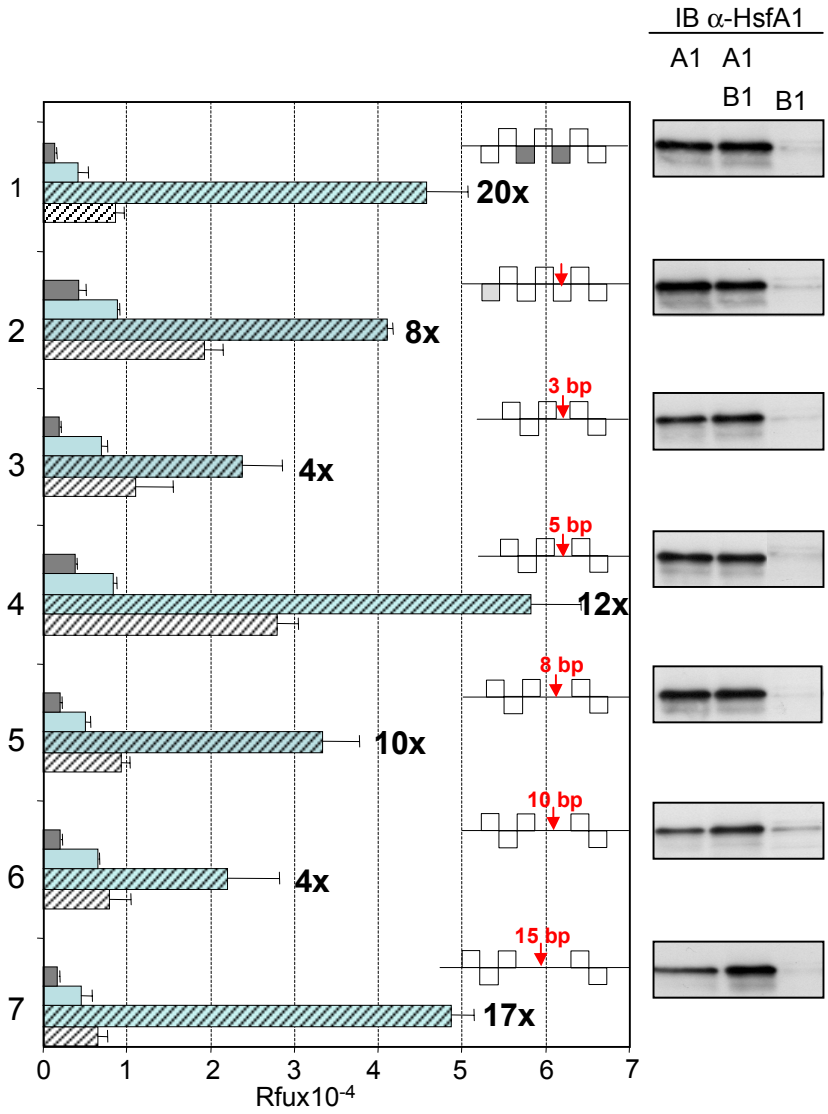


Figure 13. **Dissection of HSE clusters: creation of "ideal" cluster.**
Construct 2 obtained by mutational analysis of wild type cluster construct 1 was further modified (at marked HSE motif) by changing the distance between 5´ trimer and 3´dimer HSE motifs (see respective distances introduced; constructs 3-7). Cloning and activity analyses were done similar to Fig. 11. Numbers show the fold synergism, for calculation see Fig. 11.

3.5 Structural requirements of Hsfs A1 and B1 for synergistic activation of phsp17**gus* reporter

Having analyzed the prerequisites of promoter architecture for synergistic interactions of Hsfs A1 and B1, the next aim was to focus on the heat stress transcription factors themselves. What are the structural features of both Hsfs which are not only needed for cooperative binding on the promoter but also for synergistic induction of transcription? As can be seen in the block diagrams in Fig. 5A, the two Hsfs have similar modular structures, so the same types of mutations were used in both cases. Part A of Fig. 14 shows the four standard reference samples (shown by arrows in Fig. 5B). Sample 4 with coexpression of HsfA1 and HsfB1 represents the reference sample for GUS activities obtained with the samples in parts B and C. In part B HsfA1 mutants were combined with wild type HsfB1, whereas in part C HsfB1 mutants were combined with wild type HsfA1. The mutants of the two Hsfs used were defective in DNA binding (sample 1, part B and C; Boscheinen et al. 1997; Littlefield and Nelson, 1999), deletion forms lacking their oligomerization domain (sample 2, part B and C), defective in NLS function (sample 3, part B and C; Lyck et al. 1997; Lyck, unpublished) and, finally, Hsfs with C-terminal deletions (sample 4, part B and C; Döring et al. 2000; Treuter, unpublished). In contrast to the combination of wild type Hsfs A1 and B1 (sample 4, part A), no synergism was observed for any of the mutant combinations in part B and C. This shows that for synergistic interactions both Hsfs need not only their functional DNA binding or nuclear localization domains. But they must also be in their native oligomeric state, i.e. trimer for HsfA1 and dimer for HsfB1 (Calligaris, unpublished). Last but not least, the two types of C-terminal domains (CTDs) are also essential for synergistic reporter gene activation. For HsfA1 the presence of AHA motifs (aromatic, large hydrophobic and acidic amino acids containing peptide motifs) were shown to be important for transcriptional activity (Treuter et al. 1993; Döring et al. 2000). Details about the importance of HsfB1 CTD analyzed during this study are shown by the following experiment.

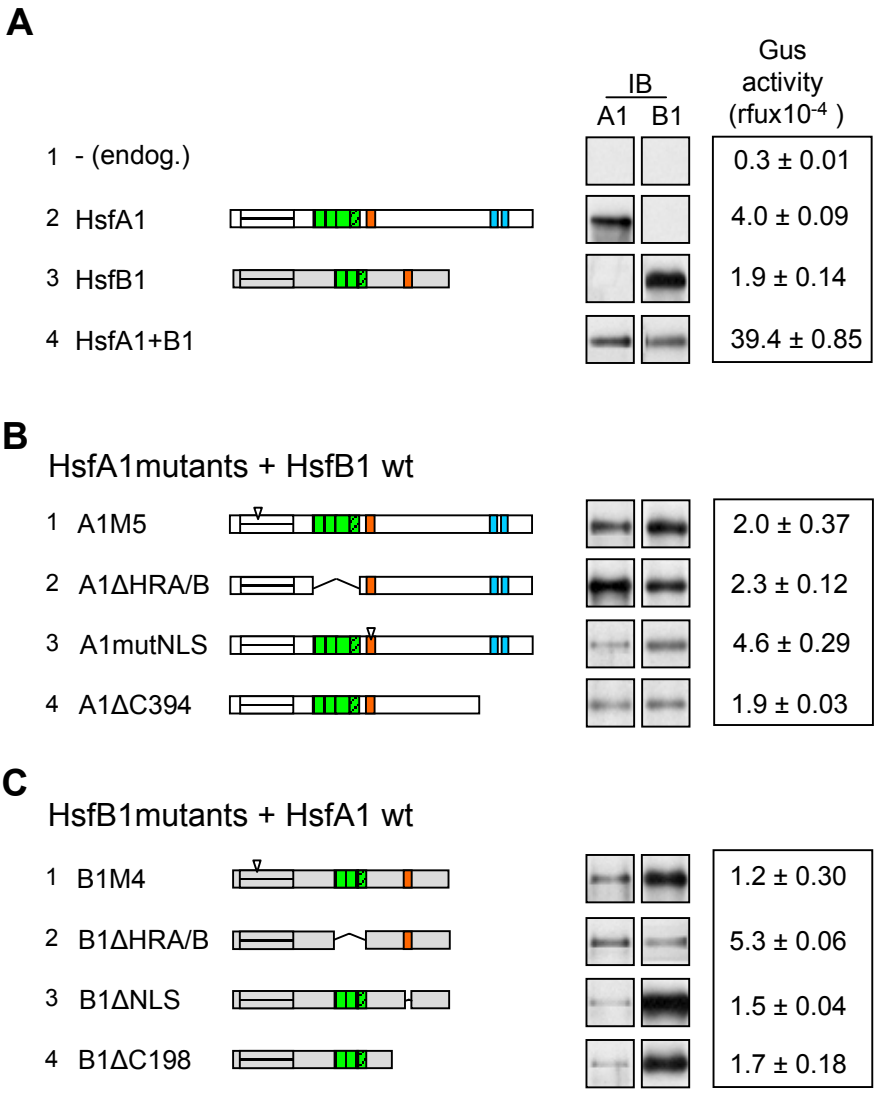


Figure 14. **Structural requirements of Hsfs A1 and B1 for synergistic activation of phsp17****gus* **reporter.**
Block diagrams represent the wild type and different mutants forms of HsfA1 and HsfB1. Immunoblots confirm the expression of different mutant versions of both Hsfs. GUS activities measured are given as absolute numbers, ± standard deviation.
(A) GUS activities were measured with the four standard samples, i.e. endogenous (sample 1), HsfA1 alone (sample 2), HsfB1 alone (sample 3) and HsfA1+HsfB1 (sample 4). Sample 4 containing 0.25µg of HsfA1 and 0.75µg of HsfB1 serves as a reference for all the samples in parts B and C.
(B) GUS activities measured by using indicated HsfA1 mutants coexpressed with wild type HsfB1, namely A1M5-DBD mutant, A1ΔHRA/B-oligomerization deletion mutant, A1mutNLS-nuclear localization signal mutant and A1ΔC394-C-terminal deletion mutant (see Materials and Methods for details).
(C) Same mutants were taken for HsfB1 and coexpressed with wild type HsfA1, namely B1M4-DBD mutant, B1ΔHRA/B-oligomerization deletion mutant, B1ΔNLS-nuclear localization signal deletion mutant and B1ΔC198-C-terminal deletion mutant (see Materials and Methods for details).

3.6 Importance of a single lysine residue in the CTD of HsfB1 for synergistic gene activation

Earlier studies with C-terminal deletion forms of HsfB1 have indicated that the synergistic activity obtained with HsfB1 resides in the last 30 aa residues of the protein (Treuter et al. 1993). Thus our attention was focused on this part of HsfB1 CTD. Construct 1, Fig. 15 shows the GUS activity obtained from synergistic reporter gene activation by coexpression of wild type HsfB1 and HsfA1. This sample was used as a reference for the rest of the samples with mutant CTDs. Mutation of the only tryptophan residue to serine (construct 2) or tyrosine residue to alanine (construct 3) did not affect the HsfB1 activity in presence of HsfA1. This situation was in contrast to the observations made for class A Hsfs, where the aromatic amino acid residues in AHA motifs were shown to be important for transcriptional activation (Döring et al. 2000). C-terminal deletions of 6 and 15 amino acids (constructs 4 and 5 respectively) also had no effect. But deletion of 27 amino acids (construct 6) led to a complete loss of synergistic GUS activity obtained by coexpression of HsfB1 with HsfA1. This shows that the activation core of HsfB1 lies in these 12 amino acid residues.

Tests with a small internal deletion of amino acid residues 272-279 narrowed the core sequence to seven important residues (construct 7). Decisive insights about the role of lysine 275 came from analysis of different point mutants, i.e. construct 8 with GRGK(272-275)>LWTT, construct 9 with GK(274-275)>VD and construct 10 with K275>Q. In all cases, the only common residues mutated was K275 and this led to a loss of synergistic interactions between HsfA1 and HsfB1. Moreover, importance of the lysine residue itself and not just positive charge of amino acid 275 was further underscored by construct 11, where a mutual exchange between lysine-275 and arginine-273 did not rescue the synergistic effect (construct 11, GRGK>GKGR). The specificity for the importance of this lysine comes form the mutation of adjacent lysine 278 to arginine (construct 12, K278>R), which also has no effect on GUS activity observed by coexpression of HsfA1 and HsfB1.

From this mutational analyses I conclude that the lysine residue embedded in the core sequence –GRGKMMK is indispensable for the function of HsfB1 as a coactivator of HsfA1. This lysine residue can neither be replaced by an equally charged residue arginine (K275>R), nor by the naturally adjacent lysine residue (K278) and nor by a lysine residue introduced at position 273 (R273>K).

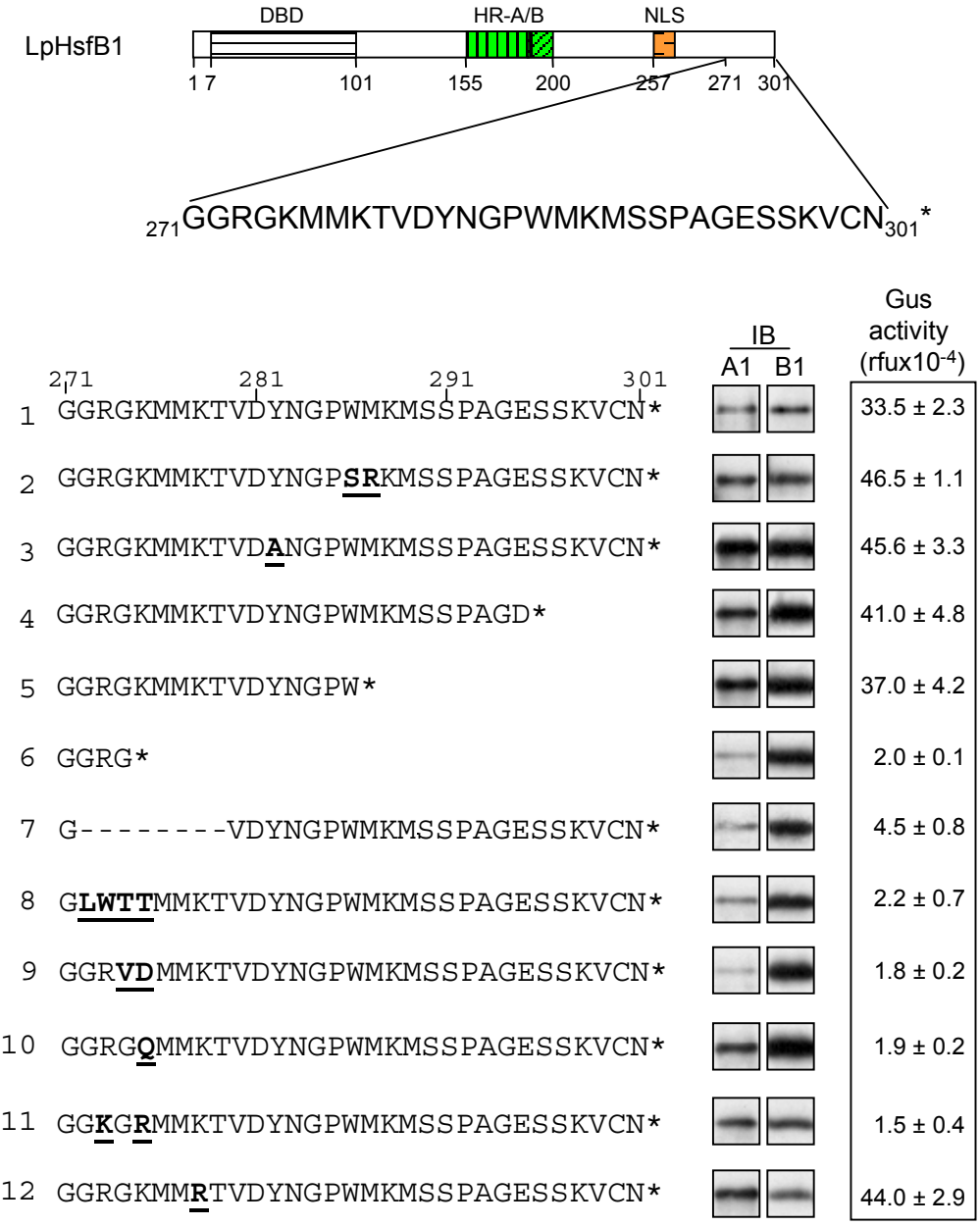


Figure 15. **Importance of HsfB1 CTD for synergistic reporter gene activation in coexpression with HsfA1.**
Block diagram on top shows the basic structure of HsfB1, sequence of important residues in the CTD (aa 271-301) is given. HsfB1 CTD was functionally dissected by point mutations and deletion analyses. All the mutated amino acids are underlined and bold faced. In all cases the GUS activities were measured by coexpression with wild type HsfA1, in presence of phsp17**gus** as reporter (see sample 4, Fig. 14A). Immunoblots depict the expression levels of HsfA1 and HsfB1 in respective samples.

3.7 HsfB1 as a coactivator for cauliflower mosaic virus 35S (CaMV35S) promoter

As shown in Fig. 5B and 6A, HsfB1 not only acts as a synergistic partner of HsfA1 on the GmHsp17.3B-CI gene reporter, but it also increases the expression of plasmid borne copy of HsfA1. The later effect must be the result of a so far not described activity of HsfB1 on CaMV35S promoter, used to drive the expression of HsfA1. CaMV35S promoter is one of the most widely accepted plant promoter, used to drive the constitutive expression of desired cDNAs in different transient or stable expression assay systems (Jefferson et al. 1987; Fang et al. 1989; Odell et al. 1985; Lamm 1994). The assumption of HsfB1 regulating the expression of 35S promoter was tested in Fig. 16. A closer inspection of the sequence of CaMV35S enhancer region led to the identification of putative Hsf binding sites, HSE motifs (Fig. 16A). Evidently the Hsf binding sites are intermingled between the binding sies for 35S enhancer binding proteins (TGA2.1/2.2) and even overlapping in some cases (Lamm 1994).

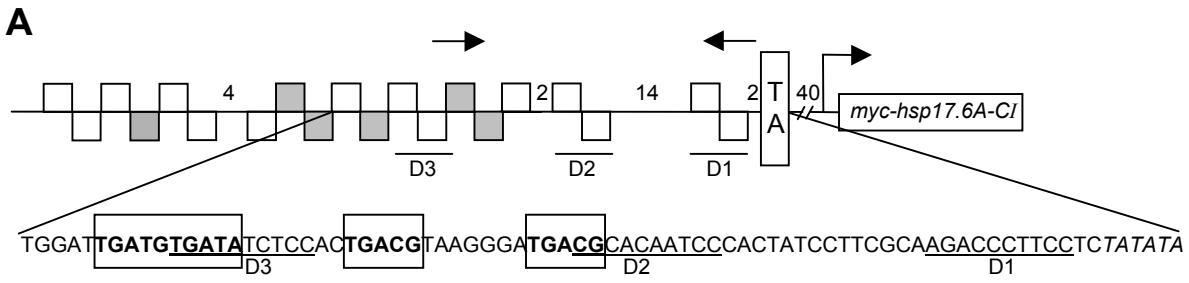
In the following experiments Myc-Hsp17.6 was used as a reporter and its expression was monitored by performing immunoblots with a-Myc antibody. So, using a CaMV35S-*myc-hsp17.6* expression cassette, effect of HsfB1 was tested on the induction of 35S promoter. The plasmid ratios used for cotransfections were identical to the four standard samples used in Fig. 5B, i.e. 2µg of reporter was cotransfected with empty plasmid or 1µg of HsfA1 alone, 0.25µg of HsfA1+0.75µg of HsfB1 and 1µg of HsfB1 alone. As can be expected from predicted synergistic interactions between HsfB1 and TGA proteins, there was a strong stimulation of 35S promoter activity, lanes 3 and 4, Fig. 16B. HsfA1 did not show any effect (lane 2). Moreover, heat stress did not affect the outcome of this experiment (compare lanes 3-4 with 6-8, Fig. 16B).

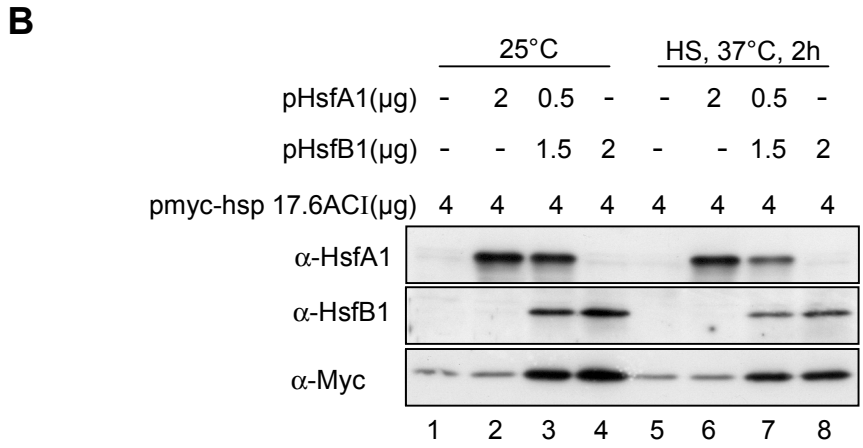
Reverse transcriptase-polymerase chain reaction (RT-PCR) was performed to confirm that the effect seen in Fig. 16B is valid at transcription level as well. CaMV35S-*myc-hsp17.6* reporter was transfected either alone (sample II, Fig. 16C) or with 5 and 10 times HsfB1 encoding plasmid (samples III and IV respectively). RT-PCR was standardized by taking different dilutions of the template in each case so that finally similar signals can be seen on ethidium bromide stained gels. On the basis of template dilution and intensity of the PCR fragments, it can be estimated that the transcription from plasmid borne copy of CaMV35S promoter can be increased by about 40 times (see legend Fig. 16) in presence of HsfB1. RT-PCR has served here as a semi-

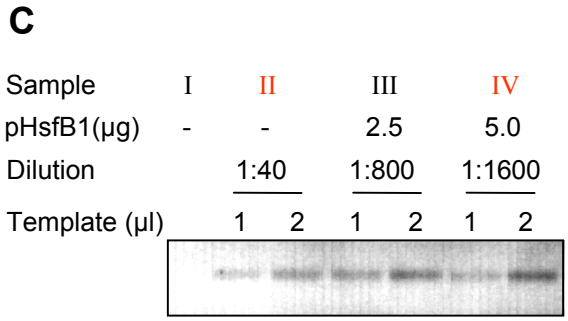
quantitative measure to find out the level of induction of 35S promoter in presence of HsfB1. However, the actual quantitative measurements can only be done by real time PCR or nuclear run on assay.

So far there are some hints that the coactivator function of HsfB1 works on the same principle both in case of Hsp promoters with HsfA1 and CaMV35S promoter, perhaps in cooperation with TGA factors. To prove the equality of both effects, similar mutants of HsfB1 (those tested in Fig. 14C and 15) were tested for their capability to mediate the 35S promoter enhancer effect. As can be seen in the immunoblot Fig. 16D, none of the mutants, namely the DBD mutant (M4), the oligomerization mutant (HR), the GRGK>GKGR mutant (R) and the Δ272-279 mutant (Δ), are capable to enhance 35S promoter activity comparable to wild type HsfB1 (compare lane 1 with 3-6). Incapability of DBD mutant (M4, lane 3) once again confirms the observation about the role of the HsfB1 binding sites in 35S enhancer region. The fact that Δ and R, the two CTD mutants of HsfB1 can not synergize on both types of promoters (see Fig. 15 and 16D) indicates that both coactivator functions of HsfB1 are dependent on the same as yet unidentified coregulator protein.

The results presented above demonstrate an intriguing function of tomato HsfB1, as a coactivator of both Hsp and viral promoters. On Hsp promoters, HsfB1 gives synergism with expressed HsfA1, whereas it gives synergistic gene activation on CaMV35S promoter together with endogenous activators of this promoter, i.e. TGA proteins.







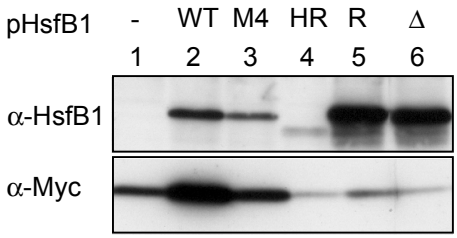
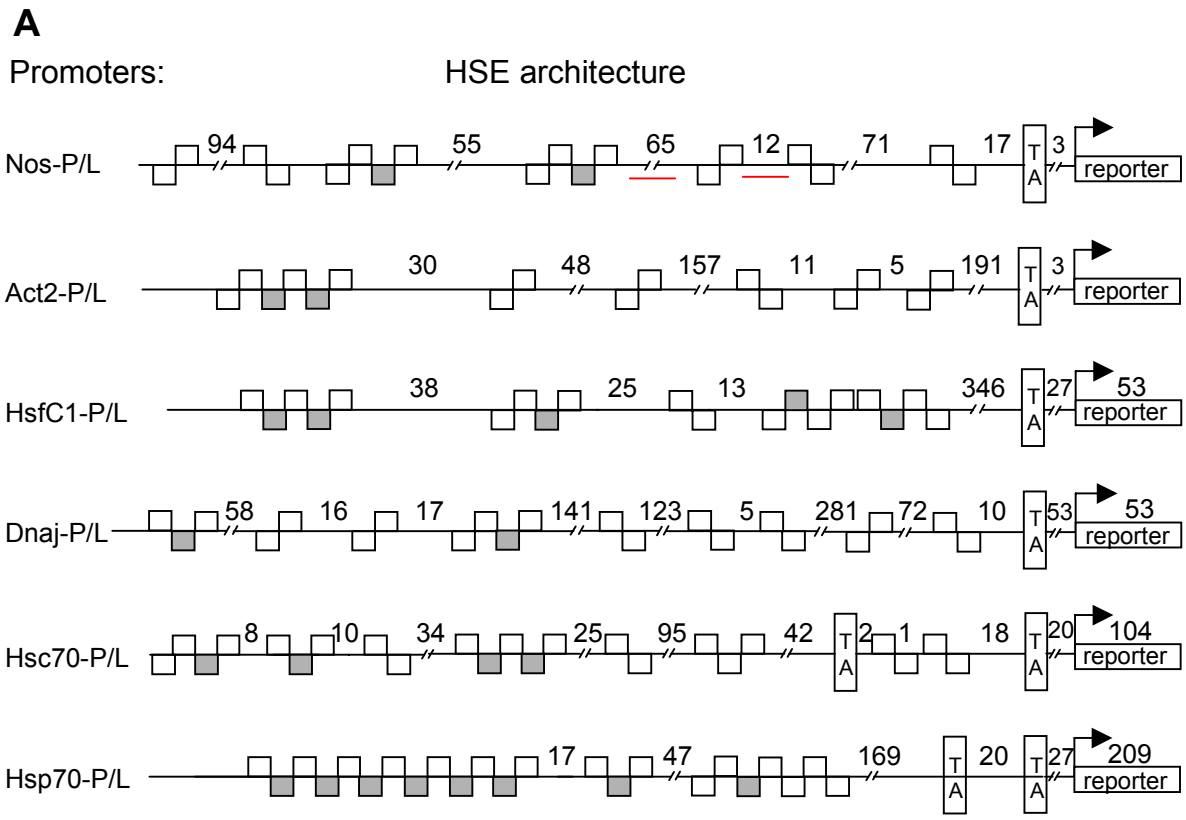


Figure 16. **HsfB1 as a coactivator of cauliflower mosaic virus 35S promoter.**
(A) Schematic diagram of CaMV35S promoter-reporter fusion, showing putative HSEs (as boxes, see Fig. 5A for details), underlined in the sequence as D1, D2 and D3. Known TGA binding sites are boxed and boldfaced.
(B) Induction of CaMV35S promoter-*myc-hsp17.6A-CI* reporter expression in presence of HsfA1, HsfB1 and Hsf A1+B1 (standard samples similar to Fig. 5B were taken), under control (25°C) and heat stress (37°C) conditions. Plasmid concentrations used and control expression blots for Hsfs A1 and B1 are shown. MycHsp17.6A-CI expression was monitored by using a-Myc antibody.
(C) RT-PCR analysis to evaluate the induction of 35S promoter by HsfB1 at RNA level. Sample I-mock transformed, sample II-pCaMV35S-*myc-hsp17.6A-CI* (0.5µg) alone, sample III-pCaMV 35S-*myc-hsp17.6A-CI* (0.5µg)+pHsfB1 (2.5µg) and sample IV- pCaMV35S-*mychsp17.6A-CI* (0.5µg)+pHsfB1 (5µg). Different dilutions of the template were taken, so that similar signals are detected on the ethidium bromide stained DNA gels. By comparing the template dilutions in samples II and IV, i.e. 40 and 1600 respectively and similar signals, it can be calculated that the transcription of 35S promoter in presence of HsfB1 increases about 40 fold.
(D) Effects of HsfB1 mutants on induction of 35S promoter. M4-DBD mutant, HR-oligomerization domain deletion mutant, R-GRGK(272-275)>GKGR, Δ-internal deletion of amino acid residues 272-279 (for details see Fig. 14C, 15 and Materials and Methods).

3.8 HsfB1 as a general coactivator for house-keeping promoters

The results with the CaMV35S promoter prompt the question: Is HsfB1 a general coactivator of a subset of house-keeping genes? To test this concept different plant specific bacterial or house-keeping promoters were amplified. The Myc-Hsp17.6 based reporters contained the promoter/leader regions of Agrobacterium T-DNA Nos gene (construct 1, Fig. 17C) and of *Arabidopsis* Actin2, HsfC1, Dnaj, Hsp70 and Hsc70 genes (constructs 2-6, Fig. 17C). Fig. 17A shows the block diagrams of all these reporter constructs, with the predicted HSE motifs shown as boxes. Interestingly all promoters contain multiple Hsf binding sites spread all over the promoter. In case of Nos promoter, the binding site for the known activators is underlined. In all other cases the detailed functional anatomy of the promoters as well as the constitutive activators needed for their normal expression are not known yet. Except Hsp70, which is strictly heat stress inducible, all other promoters were shown to be constitutively expressed (Actin2: Kandasamy et al. 2002; Laval et al. 2002; HsfC1: Nover et al. 2001, Dnaj: Miernyk 2001; Hsp/c70: Lin et al. 2001). The standard conditions used to test this set of reporters are shown in Fig. 17B, with expression control for HsfA1 and HsfB1. With an exception of hsp70 promoter (construct 5, Fig. 17C) all other promoters were strongly induced in the presence of HsfB1 (compare lanes 3, 4 with 1). Similar to the situation with the 35S promoter, HsfA1 either had no effect (lane 2) or it reduced the induction caused by HsfB1 (compare lane 3 with 4). Most intriguing are the differences between the hsp70 and hsc70 promoters (constructs 5 and 6). The former one is strictly heat stress inducible (Lin et al. 2001), similar to sHsp gene promoters (Fig. 9). In this test, it is also strongly induced only by the combination of Hsfs A1 and B1. The latter one, hsc70 promoter is constitutively expressed and similar to other constitutive promoters tested (constructs 1-4), it is also strongly induced by HsfB1 alone. This confirms the concept that HsfB1 is indeed a general coactivator that acts together with HsfA1 on heat stress inducible promoters and with yet unidentified activators on constitutive promoters.



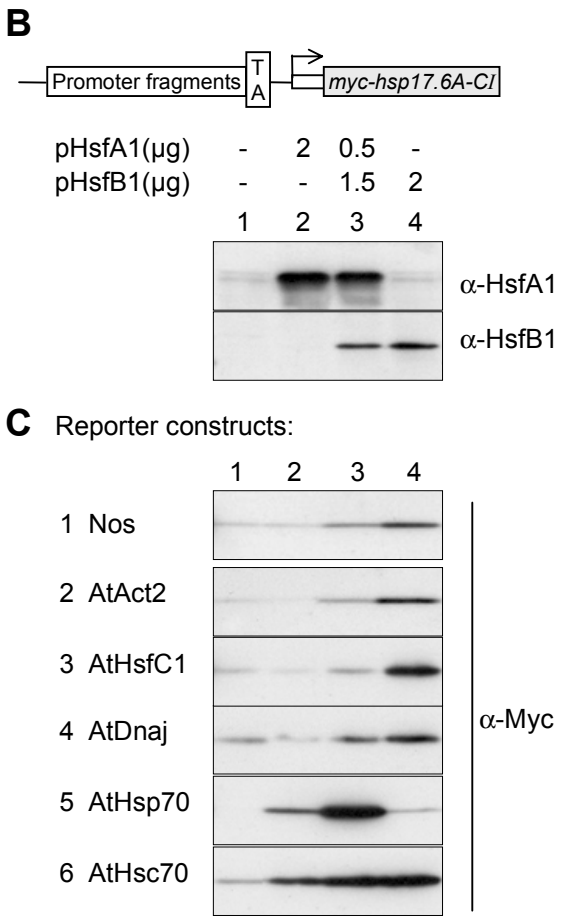


Figure 17. **Activation of plant promoters by HsfB1 in a transient reporter assay.**
(A) Schematic diagram of promoter/leader fragments from different house-keeping/bacterial genes fused to reporters. Putative HSEs are shown as boxes.
(B) Block diagram of promoter-*myc-hsp17.6A-CI* reporter used in part B and C. Plasmid concentrations and expression controls for HsfA1 and HsfB1 are shown.
(C) Expression levels of reporter constructs containing promoter/leader fusions of indicated genes, in presence of HsfA1 (lane 2), HsfB1 (lane 4) and HsfA1+HsfB1 (lane 3).

3.9 In vivo role of HsfB1

The two contrastingly behaving hsp70 and hsc70 promoters were used to demonstrate the effect of changing Hsf combinations inside the cell during an ongoing heat stress. For this purpose, hsp70 promoter/leader fragment was fused to Gfp and hsc70 promoter/leader fragment was fused to DsRed as reporters (block diagrams, Fig. 18). Both reporters were coexpressed in the same cell with and without Hsfs A1 and B1. In this “single cell reporter assay” (Blackwood and Kadonaga 1998), reporter activity was measured in the same cell as green or red colour for hsp/c70 promoters activity respectively (Fig. 18). The red fluorescence reflecting the activity of constitutive hsc70 promoter was present in all samples, albeit strongly enhanced in the presence of HsfB1. In contrast to this the green fluorescence showing the activity of heat stress inducible promoter was very weak in general, except in samples coexpressing HsfA1 and HsfB1. This reflects the situation at the onset of heat stress, when the master regulator of heat stress response HsfA1 (Mishra et al. 2002) induces the expression HsfB1. The newly synthesized HsfB1 becomes not only a part of heat stress enhanceosome together with HsfA1 but also synergizes with some other activators.

To confirm that the synergistic gene activation caused by HsfA1 and HsfB1 was not restricted to plasmid borne reporters, endogenous sHsp genes were investigated. Different plasmid amounts of HsfA1 and HsfB1 (similar to Fig. 6A) were transfected into tobacco protoplasts and the expression of endogenous Hsp-CI was monitored by specific antibodies. As can be seen in Fig. 19, the mild induction of Hsp17-CI expression, caused by HsfA1 alone (lanes 1-3), could be strongly stimulated by HsfB1 (lanes 4-7), whereas HsfB1 alone had no effect (lanes 8-11). This result has strengthened the findings of previous experiments, by showing that synergism between HsfA1 and HsfB1 is not only restricted to plasmid borne reporters but can also be detected on endogenous promoters. This has proved that at least for this case the chromatin configuration is favourable for synergistic interactions observed between HsfA1 and HsfB1.

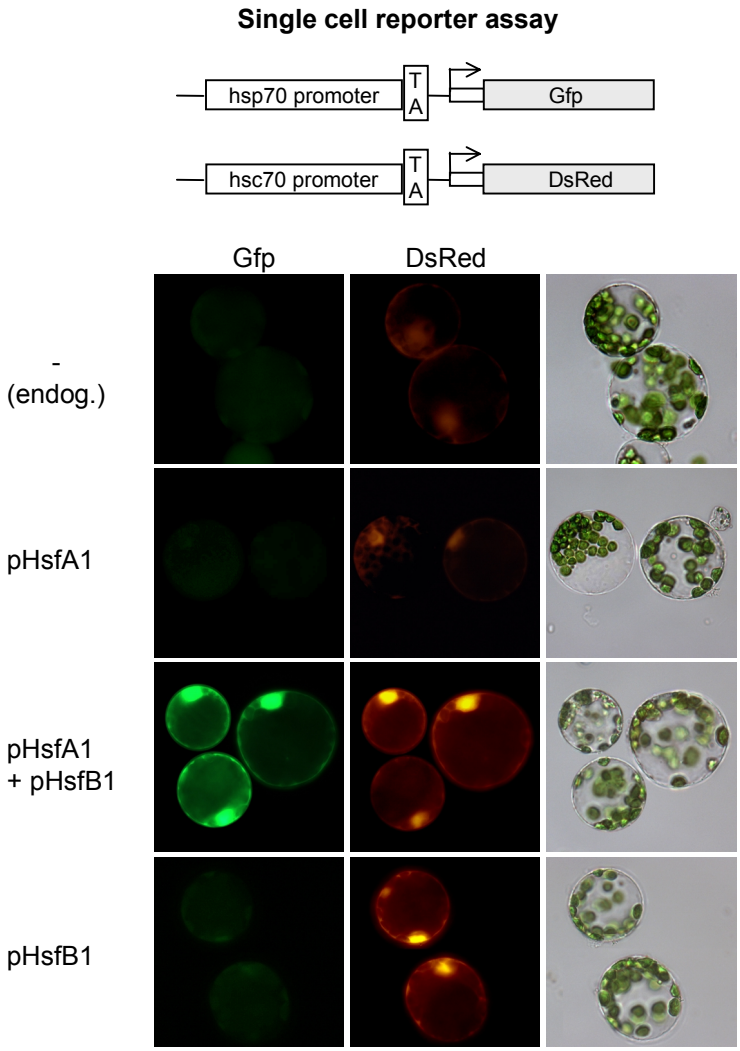


Figure 18. **Activation of plant promoters by HsfA1 and HsfB1 in a single cell reporter assay.**
Single cell reporter assay with tobacco protoplasts cotransformed with indicated Hsf expression plasmids (left) and two reporters with promoter/leader fragments from hsp70/hsc70 genes, fused to Gfp and DsRed as reporters (see block diagrams on top).

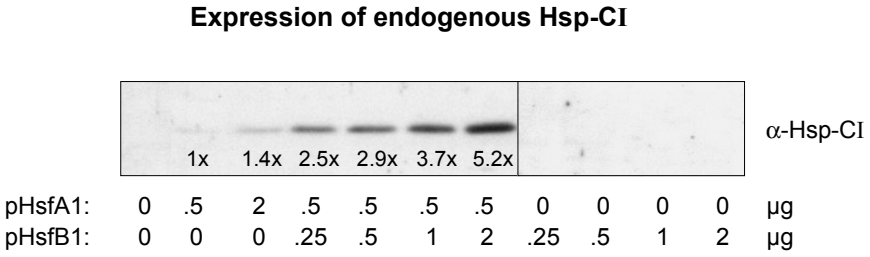


Figure 19. **Synergistic activation of endogenous Hsp-CI promoters by HsfA1 and HsfB1.**
Effect of coexpression of HsfA1 and HsfB1 on induction of endogenous Hsp-CI gene. Plasmid concentrations for HsfA1 and HsfB1 are given. Expression of endogenous Hsp-CI was detected on immunoblots with corresponding antibodies.

3.10 Synergistic interactions between acidic activators and class B Hsfs from different plants

Based on the observation that HsfB1 acts as a general coactivator on different Hsp and constitutive promoters, it was hypothesized that tomato HsfB1 cooperates with acidic activators in general. To verify this hypothesis, four different well known acidic activators were tested for their capability to give synergistic transcriptional activation in the presence of HsfB1 (Fig. 20). Acidic activation domains of two other members of tomato Hsf family, HsfA2 and HsfA3 were well characterized before (Döring et al. 2000 and Bharti et al. 2000). In addition two non-plant acidic activation domains were selected, i.e. 125 amino acids from an extended acidic activation domain of yeast transcription factor Gal4 (Melcher and Johnston 1995; Melcher 2000) and 78 amino acids from herpes simplex virus activator VP16. Infact, VP16 activation domain was among the first examples for acidic activation domains shown to interact with components of transcriptional machinery and the importance of large hydrophobic and aromatic amino acid residues for the activation potential of the protein was highlighted (Goodrich et al. 1993; Regier et al. 1993). Since the synergism was tested on a Hsp promoter fragment, the later two activation domains were fused to a C-terminally deleted HsfA1 still containing the DBD, oligomerization and NLS functions (construct 4, Fig. 15B). All four acidic activators, i.e. LpHsfA2, LpHsfA3, LpHsfA1(aa 23-394)xGal4AD and LpHsfA1(aa 23-394)xVP16AD (see block diagrams in Fig. 20 for details), were titrated against LpHsfB1 encoding plasmid, similar to Fig. 5B.

Although differing in details, all four activation domains gave synergistic activation of GUS activity in presence of HsfB1 (Fig. 20, parts A-D). It is tempting to speculate that depending on the fine structure of their activation domains, some on the contacts with the components of transcriptional machinery are different and this gives rise to a slightly different outcome of the test. Expression controls for all proteins used are shown by immunoblots.

It can be concluded from the above results that tomato HsfB1 can give synergistic reporter gene activation with acidic activators in general. Whatever components of transcriptional machinery are needed for synergism, require the presence of an acidic activation domain on one hand and HsfB1 on other hand.

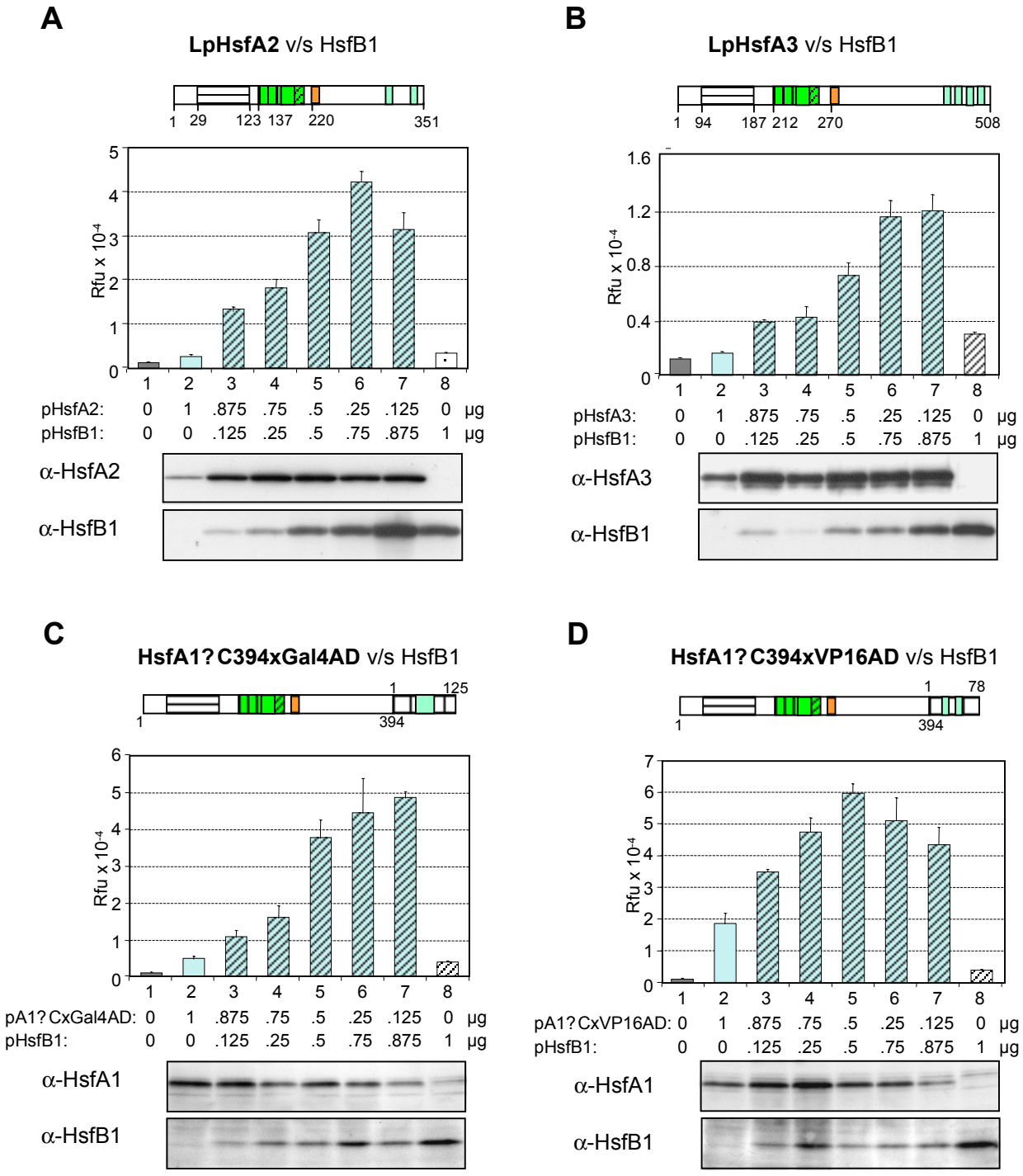


Figure 20. **Synergistic interactions between acidic activators and LpHsfB1.**
GUS reporter assay showing the expression of phsp17*_gus_ reporter tested in protoplasts transformed with the indicated amounts of LpHsfA2 **(A)**, LpHsfA3 **(B)**, fusion construct LpHsfA1 (aa 1-394)xGal4AD (aa 1-125) **(C)** and fusion construct LpHsfA1 (aa 1-394)xVP16AD (aa 1-78) **(D),** with indicated amounts of LpHsfB1 encoding plasmids. Immunoblots at the bottom indicate the expression levels of corresponding constructs. Note that the fusion constructs in parts C and D could be easily detected by a-HsfA1 antibody.

Next aim was to test, if synergism a special feature of tomato HsfB1 only or other plant HsfB types can also show this effect. Two known HsfB1 from tobacco (Nt) and soybean (Gm; Czarnecka-Verner et al. 1995) were investigated for this purpose (Fig. 21). For comparison the C-terminal sequence of both Nt and Gm HsfB1 were aligned with the tomato HsfB1 sequence and the important lysine residue is marked (Fig. 21A). In this test, the CTDs of Nt and Gm HsfB1 were fused to a C-terminally truncated construct of LpHsfB1, lacking its own CTD. The resulting constructs are shown as block diagrams in Fig. 21A. Results of the GUS assay show that both the fusion proteins were almost as active as wild type LpHsfB1 (Fig. 21B). Immunoblots confirm the equal expression of all the proteins tested.

These experiments demonstrate for the first time that HsfB1 represents a type of common general coactivator in plants. However, a similar experiment done for *Arabidopsis* class B Hsfs showed that actually such a member is lacking in *Arabidopsis*. In agreement with this result, the HsfB1 ortholog in *Arabidopsis* contains a threonine at the same position, where the important lysine is present in the other HsfB1 types (see sequence alignment in Fig. 21A). This suggests the existence of a naturally occurring mutant version of the synergistically active HsfB1 type and might also explain its repressive behaviour (Czarnecka-Verner et al. 2000).

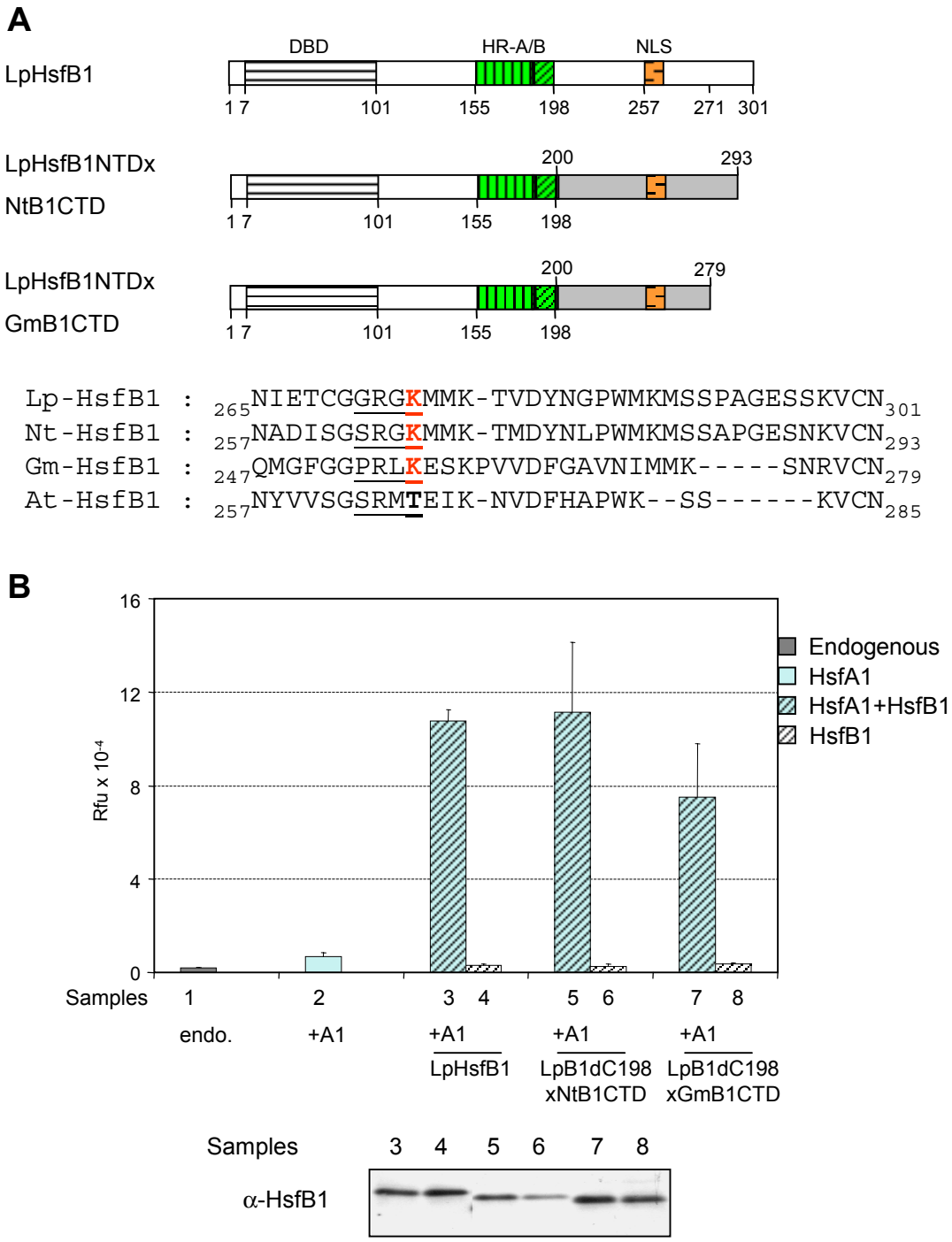


Figure 21. **Synergistic interactions between class B Hsfs from different plants and LpHsfA1.**
(A) Block diagrams showing the full length HsfB1 and the two fusion constructs containing NTD of LpHsfB1 (aa 1-198) with CTD of NtHsfB1 (aa 200-293) and GmHsfB1 (aa 200-279). Lp-*Lycopersicon peruvianum* (tomato), Nt-*Nicotiana tabacum* (tobacco), Gm-*Glycine max* (soybean), At-*Arabidopsis thaliana*. The sequence comparison of all four CTDs is also shown, functionally important lysine is bold faced and marked.
(B) Activity on phsp17**gus* reporter in presence of different fusion constructs of class B Hsfs, coexpressed with LpHsfA1. Standard plasmid concentrations as shown in Fig. 5B were used. Immunoblot with LpHsfB1 antibody detects all the three constructs.

3.11 Similarity between the -GRGKMMK motif of HsfB1 and N-terminal tails of histones: role of CBP/HAC1 in synergism

The demonstration that a single lysine residue at position 275, embedded in the –GRGKMMK motif is crucial for coactivator function of HsfB1 both on Hsp and house-keeping/viral promoters (Fig.15 and 16D), leads to the question about the protein interacting with HsfB1. This motif, although not frequently found in transcription factors, is highly conserved in N-terminal tails of histones, especially histones H2A and H4.

Histone H2A: 1-SGRG**K**QGG**K**

Histone H4: 1-SGRG**K**–GG**K**

Histone H4: 9-**K**GLG**K**GGA**K**

LpHsfB1: 271-GGRGK–MMK

The bold faced lysine residues are known to be acetylated by mammalian histone acetyl transferase (HAT) proteins, including CREB binding protein (CBP), p300 and closely related HATs (see Introduction for details about structure and function of HATs). Based on this intriguing similarity of motifs, the role of human CBP in the synergism assay with Hsfs A1 and B1 was investigated.

In contrast to very extensive studies done with mammalian CBP/p300 proteins, not much is known about their plant orthologs. In fact five CBP-like proteins (HAC1-HAC5) have just been identified from *Arabidopsis* database (Bordoli et al. 2001; Yuan and Giordano 2002). All contain the most conserved central parts of CBP with the three Zn finger (C/H1 to C/H3) and the embedded HAT domains (see block diagrams in Fig. 22A). Sequence alignment of the C/H2-C/H3 region (including the HAT domain) of *Arabidopsis* HAC1, HAC2 with human CBP shows a high degree of conservation in functionally important parts of these proteins (Fig. 22B). Because only HAC1 was shown to have HAT activity (Bordoli et al. 2001), its full length cDNA was cloned from *Arabidopsis* leaf RNA and was used to compare the effect obtained with human CBP in reporter assays with coexpression of HsfA1 and HsfB1. In addition, the N-terminal (NTD) and the C-terminal (CTD) domains were cloned separately in plant expression vectors (see marks for NTD and CTD of HAC1 in Fig. 22A).

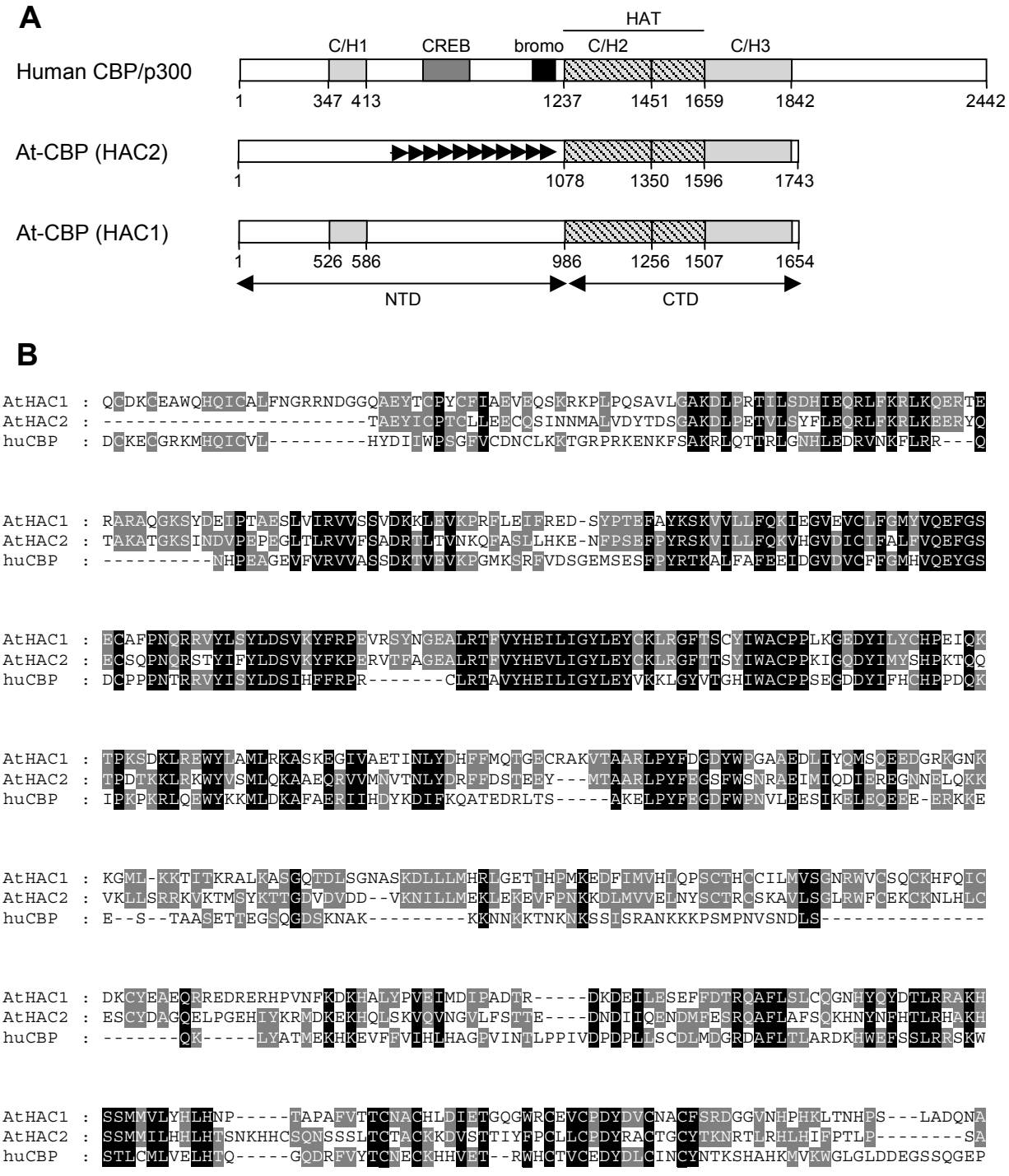


Figure 22. **Basic structure of HAC proteins, CBP/p300 orthologs in *Arabidopsis*** (Bordoli et al. 2001).
(A) Block diagrams of human CBP/p300, *Arabidopsis* HAC1 and HAC2. HAC 4 and 5 are not shown, because structurally they are similar to HAC1. Regions of most homology are shaded grey, HAT (histone acetyl transferase) domain is hatched. Note that *Arabidopsis* HAC proteins lack CREB and bromo domains. HAC2 lacks C/H1 region also and contains 11 copies of a repetitive sequence with no homology to known sequences.
(B) Sequence alignment from C/H2 and C/H3 region of *Arabidopsis* HAC1 (aa 1025-1569), HAC2 (aa 1117-1660) and human CBP (aa 1283-1757). Amino acid residues identical in all three proteins are shaded black, whereas those identical in just two proteins are shaded grey.

3.12 Effect of CBP/HAC1 on reporter gene expression in presence of Hsfs A1 and B1

GUS reporter assay was performed in tobacco protoplasts, cotransformed with phsp17**gus* as reporter and the standard amounts of HsfA1, HsfB1 alone or together. In addition, protoplasts were transfected with 2µg of plant expression plasmid encoding either human CBP, At-HAC1, At-HAC1NTD and At-HAC1CTD (Fig. 23A). The results clearly demonstrate that the expression of above mentioned HAT constructs have no effect on endogenous activity (samples 1-5, Fig. 23A), on activity of HsfA1 alone (samples 6-10) or HsfB1 alone (samples 11-15). However, up to two fold stimulation of GUS activities was observed (samples 17-19) when human CBP, At-HAC1 and its NTD were coexpressed with usual combination of Hsfs A1 and B1 (sample 16). It seems that NTD of At-HAC1 was most efficient (sample 19) whereas the CTD had only a minor effect (sample 20). From the reporter assay it is evident that CBP/HAC1 can modulate the synergistic reporter gene expression caused by HsfA1 and HsfB1. The immunoblots on right confirm that the expression levels of two Hsfs were similar in all cases.

It has been argued repeatedly that HsfB1 acts as a coactivator for HsfA1 as well as for other acidic activators (Fig. 5, 6, 16, 17 and 20). To confirm this, the effect of CBP/HAC1 was investigated on reporter gene expression from two constitutive promoters, namely CaMV35S and hsc70 (Fig. 23B). Again MycHsp17.6 was used as a reporter and its expression was monitored by immunoblotting. The immunoblot signals were scanned and assigned numbers, as compared to the signal without HsfB1 and CBP/HAC1. Although the induction of both promoters by coexpression of HsfB1 is quite similar (lane 2), further stimulation in presence of CBP, HAC1 and NTD of HAC1 is much stronger for the hsc70 promoter than for the CaMV35S promoter (compare lanes 3-5 for both promoters, Fig. 23B). Even the CTD of HAC1 has a strong influence on hsc70 promoter, whereas no effect was found in case of 35S promoter (lane 6). These results indicate that even the CBP/HAC1 effect is promoter specific. If it is assumed the CBP/HAC1 proteins are corecruited to the promoter by both acidic activators and HsfB1. Hence, it can be speculated that the presence of other acidic regulators, their proximity to the HsfB1 binding site and the strength of their activation domain could affect the efficiency of corecruitment and thus alter the outcome of the experiment.

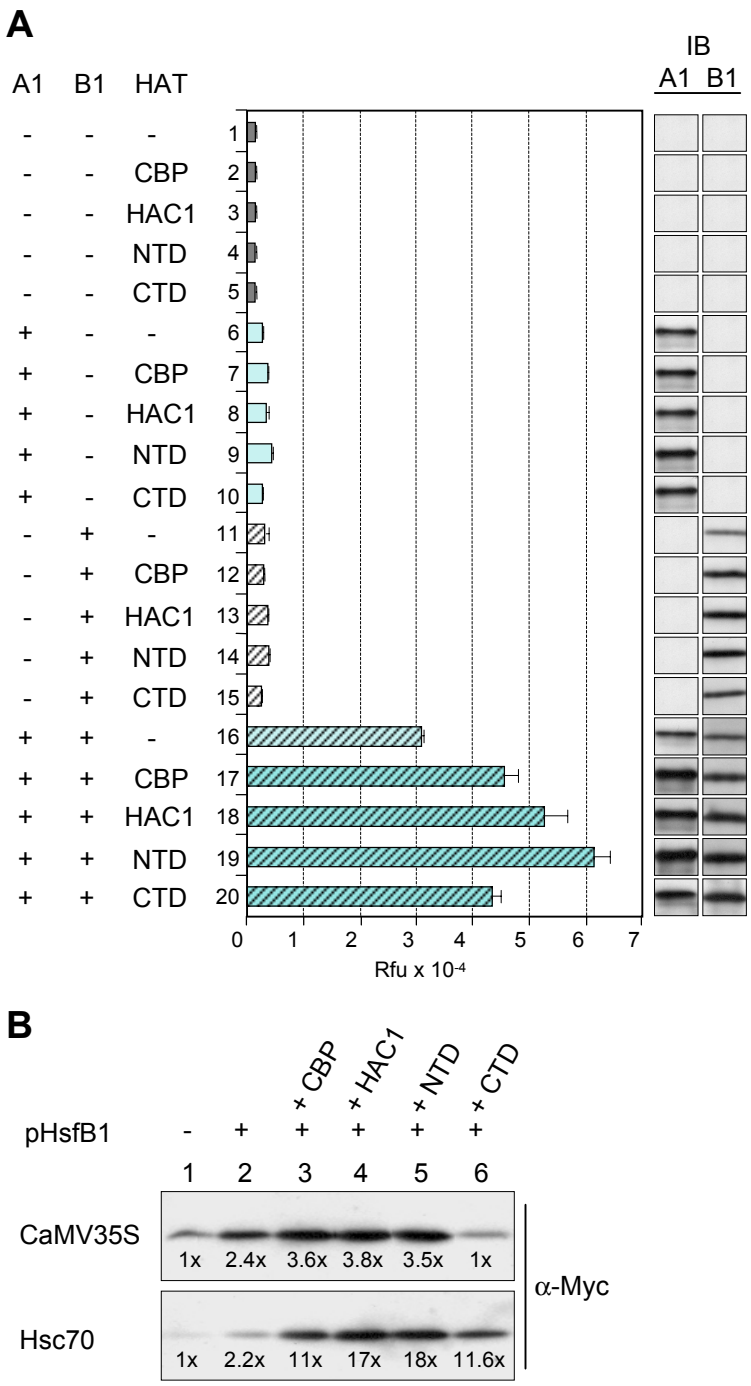


Figure 23. **Effect of mammalian and *Arabidopsis* CBP/HAC1 on the reporter gene expression in presence of HsfA1 and HsfB1.**
(A) GUS reporter activities obtained with phsp17**gus* in tobacco protoplasts transformed with the standard amounts of the indicated plasmids encoding Hsfs A1, B1. For the plasmids encoding mammalian CBP, *Arabidopsis* HAC1, its NTD and CTD, 2µg was used for transformation.
(B) Effect of HsfB1 and CBP/HAC1 on induction of constitutive promoters: CaMV35S and hsc70. Myc-Hsp17.6A-CI was used as reporter, as shown in Fig. 16B. Reporter gene expression was monitored by immunoblot using a-Myc antibody. Plasmid concentrations used correspond to samples 1 and 11-15 of part A. Numbers given on the blots represent the quantification of immunoblot signals, as done by desitometry scan software (Pharmacia).

Mutant forms of Hsfs A1 and B1 were used prove the specificity of stimulation observed above. Standard reporter and plasmid concentrations for WT and mutant Hsfs were taken as shown in Fig. 14 and 15. Sample 3 and 4, Fig. 24A show the synergistic reporter gene activation by coexpression of HsfA1 and HsfB1 and the stimulation by HAC1 respectively (as compared to samples 1, 2 and 5, Fig. 24A). These samples act as reference for rest of the samples with mutant Hsfs. In case of mutant Hsfs the GUS activities obtained with the indicated combinations are compared, i.e. the mutant Hsf alone, mutant Hsf coexpressed with wild type Hsf partner, the two coexpressed with HAC1. As before the mutants used in this test either lack their DNA binding ability, A1-M5 (samples 6-8) and B1-M4 (samples 12-14) or have defective C-terminal domains, A1-A7 (samples 9-11) and B1-? (samples 15-17). In all cases of mutant Hsfs, the reporter gene expression without or with HAC1 is much lower than in samples 3 or 4.

Most interesting are the results from the C-terminal mutant forms of two Hsfs. It is tempting to speculate that the lacking increase in activity in case of samples 9-11 (mutated HsfA1 CTD) and 15-17 (deleted HsfB1 CTD) is due to inefficient corecruitment of HAC1. The motifs which were mutated or deleted in the C-terminus of the two Hsfs might be required for recruitment of HAC1. Even if one of the Hsfs is functioning, the recruitment of HAC1 is not efficient enough to give a substantial reporter gene activation.

As discussed before, the stimulating effect of HsfB1 on transcription from constitutive promoters goes in parallel with its synergistic interactions with HsfA1 on Hsp promoters. Therefore, effect of HAC1 on induction of CaMV35S promoter by different HsfB1 mutants was investigated (Fig. 24B). As expected the three mutant forms of HsfB1 used were inactive in this assay as well (compare lanes 2, 3 with 4-6). Only the wild type HsfB1 could enhance the activity of 35S promoter and was further stimulated by HAC1 (lanes 1, 2 and 3). All the tests done so far have confirmed the idea that the coactivator function of HsfB1 follows the same principle irrespective whether plasmid borne reporters, derived from Hsp and constitutive genes or endogenous heat stress inducible genes are used.

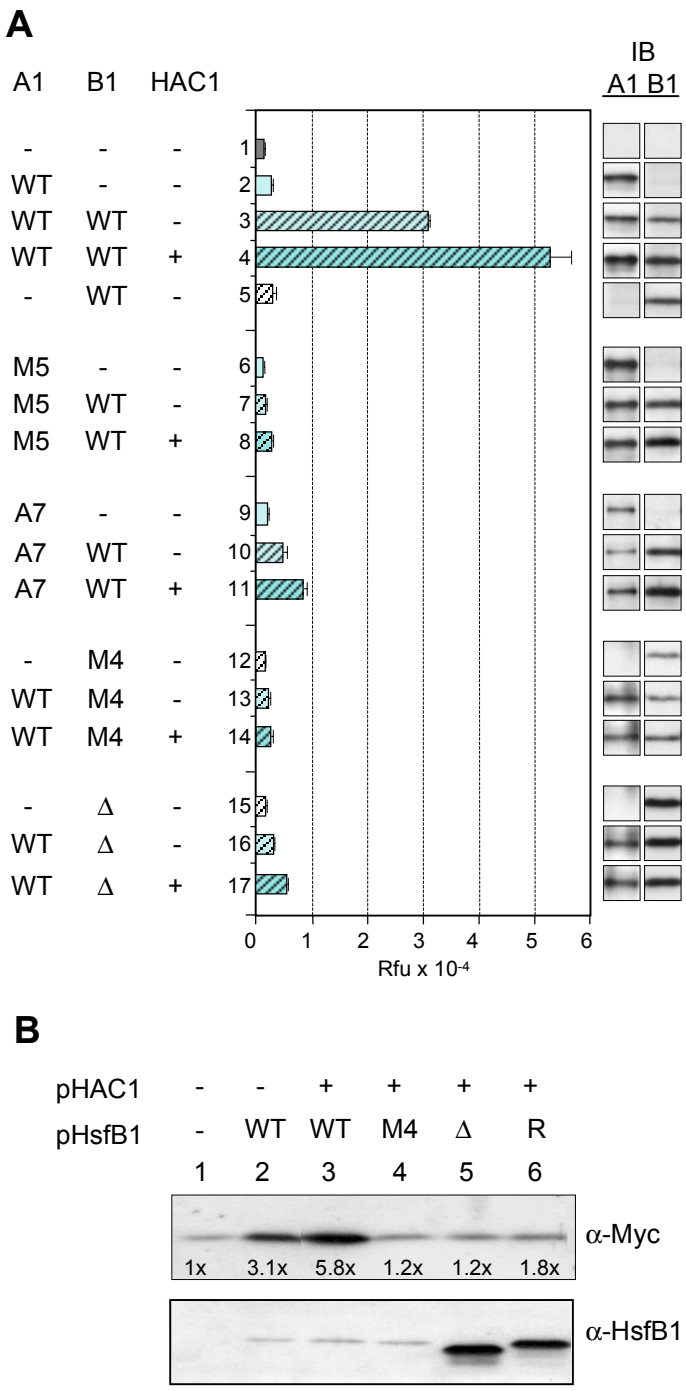


Figure 24. **Effect of *Arabidopsis* HAC1 on the reporter gene expression in presence of HsfA1/B1 mutants.**
(A) Reporter gene expression in presence of HAC1 in combination with wt and mutant forms of Hsfs A1 and B1 was measured. M5 and M4 represent DNA binding mutants of Hsfs A1 and B1 respectively. A7 is HsfA1 mutant with 7 alanine substitutions in AHA motifs and Δ is HsfB1 mutant with internal deletion of GRGKMMK motif (?272-279), for details see Materials and Methods.
(B) Effect of HAC1 on induction of CaMV35S promoter in presence of HsfB1 mutants. For M4 and Δ see part A, R-GRGK (272-275)>GKGR. Reporter and plasmid concentrations are same as in Fig. 23B.

3.13 Conservation of HsfA1/B1 synergism between plant and animal cells

It is remarkable to see that the conservation between mammalian and plant CBP/HAC1 proteins is not restricted to sequences similarity but both proteins show functional conservation also. The mammalian CBP can function in tobacco protoplasts like At-HAC1, at least for the synergistic assay with Hsfs A1 and B1 (Fig. 23A). This provoked an intriguing question, whether the synergistic interactions between HsfA1 and HsfB1 might also work in animal cells, e.g. COS 7 cells (Fig. 25). The reporter used for the assay in animal cells contained luciferase as reporter gene and the same plant promoter fragment hsp17*, responding strongly to the synergism of HsfA1 and HsfB1. Sample 1, Fig. 25 shows that the reporter gene has almost no background activity in COS7 cells but can be nicely stimulated by tomato HsfA1 (sample 2). Interestingly, both the synergistic reporter gene activation by coexpression of HsfA1-HsfB1 and the stimulation of it by CBP could be nicely reproduced in animal cells (samples 3 and 4). Once again the specificity of this effect was confirmed by using HsfA1 and HsfB1 mutants. The mutants of HsfA1 and HsfB1 used in this assay are identical to those used in Fig.25. DNA binding defective mutants, A1-M5 (samples 6-8) and B1-M4 (samples 12-14) and mutants defective in C-terminal domains A1-A7 (samples 9-11) and B1-R (samples 15-17); all of them were inactive in animal cells as well. Clearly, both the HsfA1/B1 synergism and CBP effect have the identical requirement in animal and plant cells.

The results obtained with COS7 cells provide some important information about the mechanism of synergism between HsfA1-B1 and CBP. (i) There is no additional plant specific protein required to mediate the synergistic effect. CBP represents the sole requirement and whatever the mechanism of CBP action is, it is conserved between plant and animal cells. This holds true for other components of the transcriptional machinery as well. (ii) There is no indication for the importance of any plant specific post-translational modification of these proteins for synergism.

Another important point which is worth mentioning here is that since HsfA1 expression in COS7 cells is driven by cytomegalovirus (CMV) promoter, which unlike CaMV35S promoter of plants is not stimulated by HsfB1. The synergism observed is only due to the cooperative binding of HsfA1 and HsfB1 to the promoter. Finally, there are interesting differences in extent of HsfA1-B1 synergism and effect of CBP/HAC1 on this in plant and animal cells (compare lanes 2-5 in Fig. 24A and 25). The high HsfA1-B1

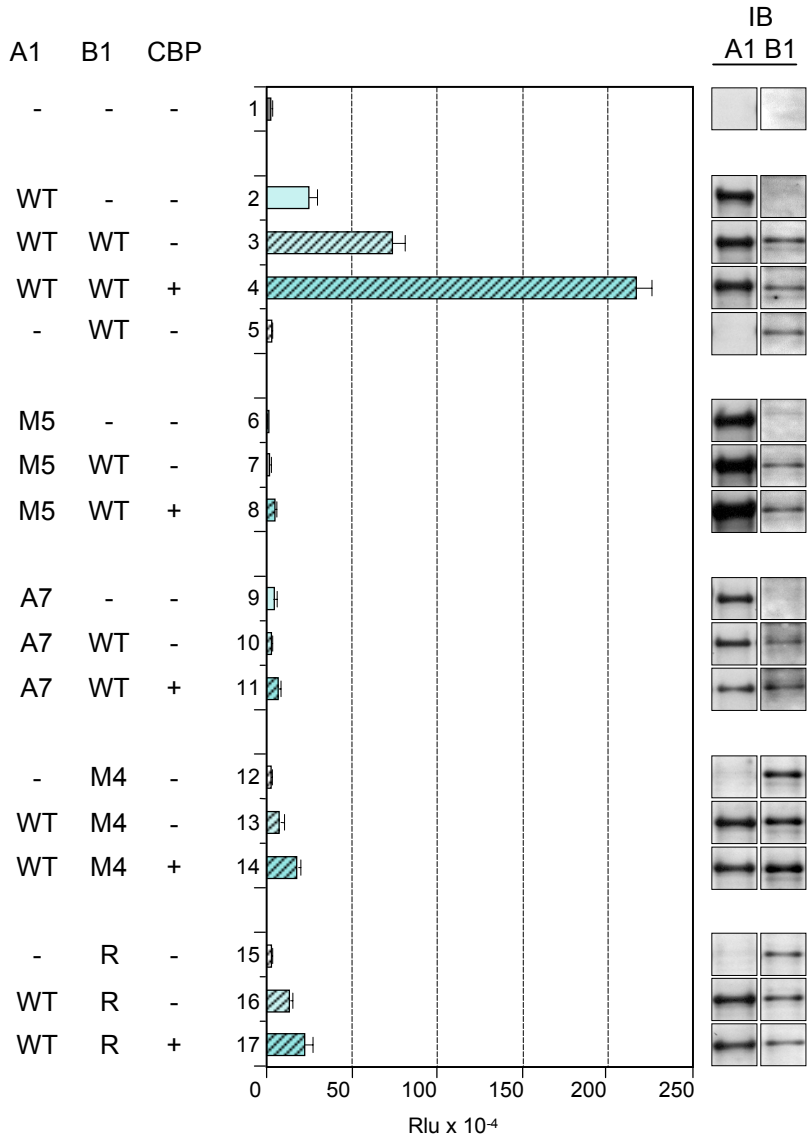


Figure 25. **Synergistic interactions between tomato Hsfs A1, B1 and mammalian CBP in COS7 cells.**
Expression of phsp17**luc* reporter in presence of wild type HsfA1, HsfB1 and CBP and the indicated mutants of HsfA1 and HsfB1. For details of the mutants see Fig. 24 and Material and Methods. 200ng of each HsfA1 and HsfB1 encoding plasmid was transfected into COS7 cells, whereas amount of CBP and reporter were 400 and 800 ng respectively.

synergism and relatively low HAC1 effect in tobacco protoplasts is to be compared with the relatively low HsfA1-B1 synergism but high CBP effect in COS7 cells. These differences may be explained by endogenous levels of CBP. Probably, tobacco protoplasts have sufficient levels of endogenous HAC1, whereas COS7 cells have low endogenous levels of CBP (Nakashima et al. 1999).

3.14 In vitro interactions of HAC1/CBP with HsfA1 and HsfB1

Fairly detailed insights into the structural prerequisites of Hsfs A1 and B1 for synergistic gene activation and corecruitment of HAC1/CBP have been obtained so far. To provide evidence for direct interactions between Hsfs A1, B1 and HAC1/CBP, two different approaches were followed.

In the first approach pull down assays were performed, using recombinant His-tagged NTD and CTD of HAC1 and in vitro translated S^{35} labelled Hsfs. Fig. 26A shows pull down performed using in vitro translated HsfA1 and its mutant HsfA1-A7. A strong binding of HsfA1 was observed only with His-NTD loaded on Ni-NTA beads, as compared to Ni-NTA beads alone (lanes 1 and 2, Fig. 26A). No interaction was observed with CTD of HAC1 (lane 3). Moreover no interaction for HsfA1 mutant, which contains mutated AHA motifs was observed (lanes 5-8). Inputs for both reactions are shown in lanes 4 and 8 respectively. By comparing the input (100%) in lane 4 with signal in lane 2, it can be said that binding of HsfA1 to NTD of HAC1 is quite strong.

A similar pull down assay performed with in vitro translated S^{35} labelled HsfB1 and its mutant form with deleted “histone-like motif” (?) showed that HsfB1 binds both NTD and CTD (lanes 2 and 3, Fig. 26B), although the strength of interaction is much higher for NTD than for the CTD. In contrast to wild type HsfB1, the deletion mutant (?) did not show any binding.

An interesting observation was made during this pull down assay. The binding of HsfB1 to the NTD was strongly enhanced in presence of recombinant HsfA1 (compare lane 2 with 4, Fig. 26B). This observation has far reaching conclusions. It confirms the assumption that both HsfA1 and HsfB1 bind to distinct domains of CBP. The formation of ternary complex between HsfA1, HsfB1 and HAC1 (NTD) does not require DNA binding per se. Somehow a conformational change in the secondary complex (with two proteins) increases the affinity for the formation of ternary complex. These observations

support the result from reporter assays, where NTD alone was sufficient for efficient stimulation of GUS activity (sample 19, Fig. 23A). The lack of interactions between HsfA1-A7 and HsfB1-? mutants with the NTD of HAC1 confirm the predictions of reporter assays that the AHA motifs of HsfA1 and the –GRGK motif of HsfB1 are necessary for interaction with HAC1.

In the second approach, coimmunoprecipitation (Co-IP) was performed using whole cell extracts prepared from COS7 cells, expressing the indicated combinations of Hsf mutants and Flag-CBP (Fig. 26C). As shown in Fig. 25, COS7 cells faithfully represent the whole synergistic reporter gene activation.

The immunoprecipitates obtained with anti-Flag antibody were either probed with Hsf specific antibodies to check for Hsfs bound to CBP or with anti-Flag antibody to control the quality of immunoprecipitation (Fig. 26C). Lane 3 shows that all three proteins could only be coprecipitated when both Hsfs were present with intact CTDs. In case any of the CTD was mutated, A1-A7 (mutation of AHA motifs, lane 4) or B1-R (mutation of lysine residue in “histone-like motif”, lane 5), the whole complex was disrupted and not even the other wild type partner could be coprecipitated with CBP. These results once again confirm the findings of reporter assays (samples 9-11 and 15-17, Fig. 24A and 25), where mutation of one partner disrupted the whole synergism, even if the other wild type partner Hsf was present. As expected no effect on efficiency of coimmunoprecipitation was observed with DNA binding domain mutants of both Hsfs (lane 6). Both proteins bound CBP with similar affinity as their wild type counterparts. These results are in accordance with the in vitro pull down results using labelled HsfB1, where ternary complex formation was strongly enhanced by addition of recombinant HsfA1 (lanes 2 and 4, Fig. 26B). The Co-IP in COS7 cells also solved the problem that due to almost undetectable expression levels of different tagged versions of At-HAC1, no interaction assays could be achieved in tobacco protoplasts.

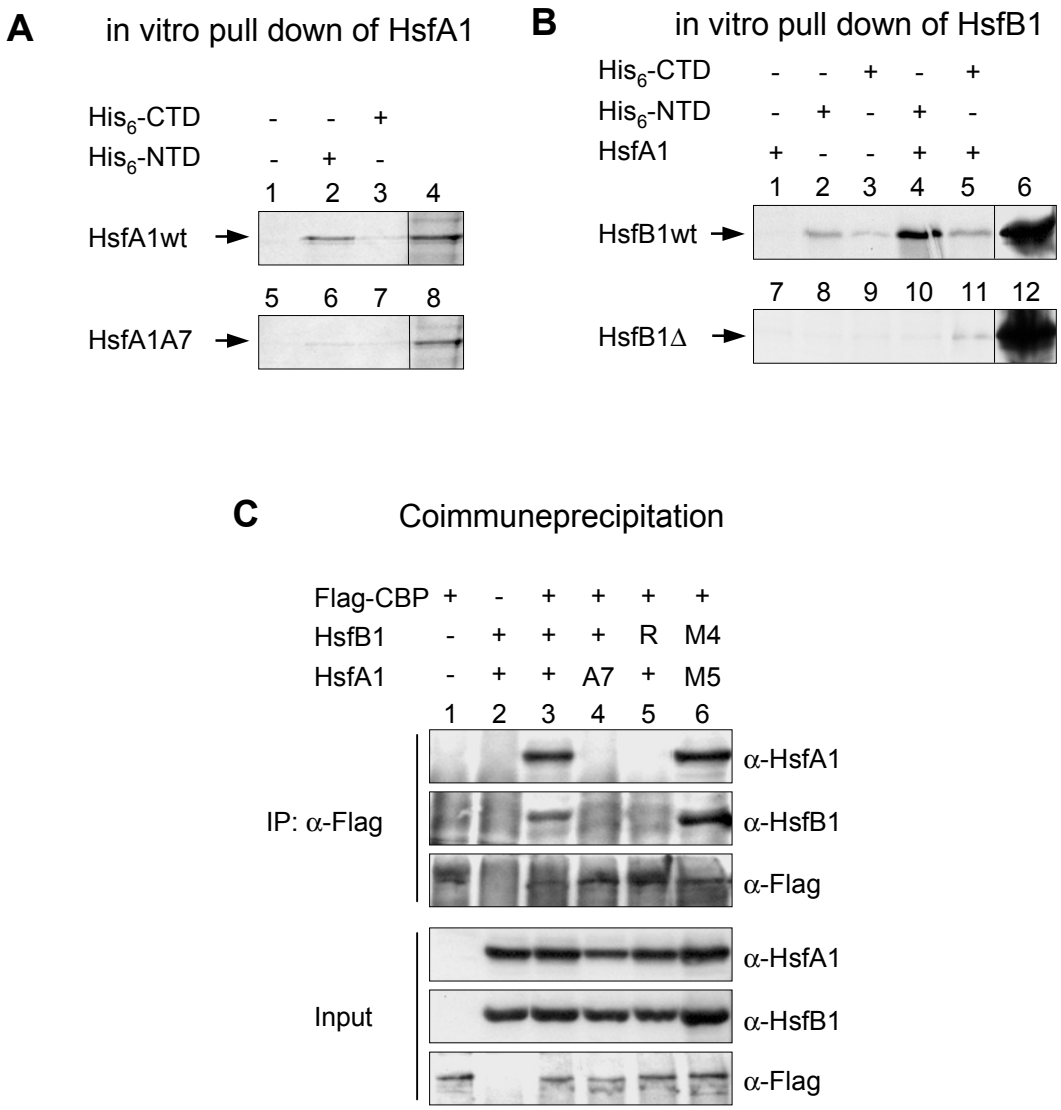


Figure 26. **In vitro interaction of HAC1/CBP with HsfA1 and HsfB1.**
(A, B) In-vitro pull down of HsfA1 or HsfB1 with NTD, CTD of HAC1. HsfA1, HsfA1-A7, HsfB1 and HsfB1-Δ were radioactively labelled by in vitro transcription-translation (see Material and Methods). The labelled Hsfs (for input see lanes 4 and 8 of part A and lanes 6 and 12 of part B) were used for pull-down assay with Ni-NTA beads alone or Ni-NTA beads coated with 20 µg of the His-tagged NTD or CTD of HAC1. In samples 1, 4 and 5 of part B 100 ng of recombinant HsfA1 was added to the reaction before applying it to the Ni-NTA beads.
(C) Coimmunoprecipitation (Co-IP) of CBP with HsfA1 and HsfB1. COS7 cells were transfected with indicated combinations of plasmids. 4 mg of total protein from cell extracts was used for Co-IP. α-Flag antibody was used for coimmunoprecipitation of Flag-CBP with HsfA1 and HsfB1. 4% of the whole cell extracts were used for the input controls.
M5 and M4 represent DNA binding mutants of Hsfs A1 and B1 respectively. A7 is HsfA1 mutant with 7 alanine substitutions in AHA motifs. Δ is HsfB1 mutant with internal deletion of –GRGKM MK motif (?272-279) and R is the HsfB1 mutant with an exchange of -GRGK (272-275) motif to GKGR.

3.15 Cooperative DNA binding of HsfB1 with HsfA1/NTD and TGA transcription factors

Cooperative DNA binding is an important aspect for synergistic gene activation. To provide evidences for this, electrophoretic mobility shift assays (EMSA) were used. First the binding preferences of recombinant Hsfs A1, B1 and NTD of HAC1 to a 160 bp promoter fragment of GmHsp17.3B-CI gene was tested. The P^{32} labelled probe either contained the wild type cluster, i.e. THtHtHT (lanes 1-8, Fig. 27A), with strong synergistic effect in the reporter assays or the mutant cluster, ththtHT (lane 9, Fig. 27A), which was completely inactive. Both recombinant HsfA1 and HsfB1 showed a weak binding to the promoter (lanes 2, closed triangle and lane 4, open triangle; Fig. 27A), which can be several fold enhanced by addition of purified NTD of HAC1 (lanes 3 and 5 for HsfA1 and HsfB1 respectively). NTD itself showed no binding to the DNA (lane 8). Most interesting is the enhanced binding of HsfA1 in presence of HsfB1 (lane 6). Keeping in mind the fact that the two proteins do not interact physically with each other, the enhanced signal seen in lane 6 solely comes from their cooperative binding to the DNA. This is confirmed by a much stronger enhancement in signal of DNP complex by addition of NTD (compare lane 6 with 7). Specificity of ternary complex formation is proven by lacking signal with the mutant probe (lane 9). Unfortunately, HsfA1 signal migrated slowly in the gel, so a supershift in presence of HsfB1 or NTD could not be detected.

In case of the CaMV35S promoter, the putative interaction partners of HsfB1 for the stimulation of transcription are the well-known b-Zip transcription factors TGA 2.1 and 2.2 (Katagiri, et al. 1989; Lamm and Chua 1989). To prove that this stimulation of transcription by HsfB1 also results from the cooperative binding of HsfB1 with TGA transcription factors, band shift assay was performed using a 94bp, P^{32} labelled CaMV35S promoter fragment. Position of primers used to amplify the promoter fragment is shown in Fig. 16A. Whole cell extracts from tobacco protoplasts expressing the indicated mixtures of HsfA1, HsfB1 and TGA2.1/2.2 encoding plasmids were used (Fig. 27B). As shown by previous studies (Katagiri et al. 1989; Niggeweg et al. 2000) two bands were detected for extracts expressing TGA proteins (closed circles, lane 2, Fig. 27B) but no binding was detectable for HsfB1 (lane 3) or HsfA1 (lane 5) alone. However, for HsfB1 a supershift was detected when coexpressed with TGA factors (open arrow,

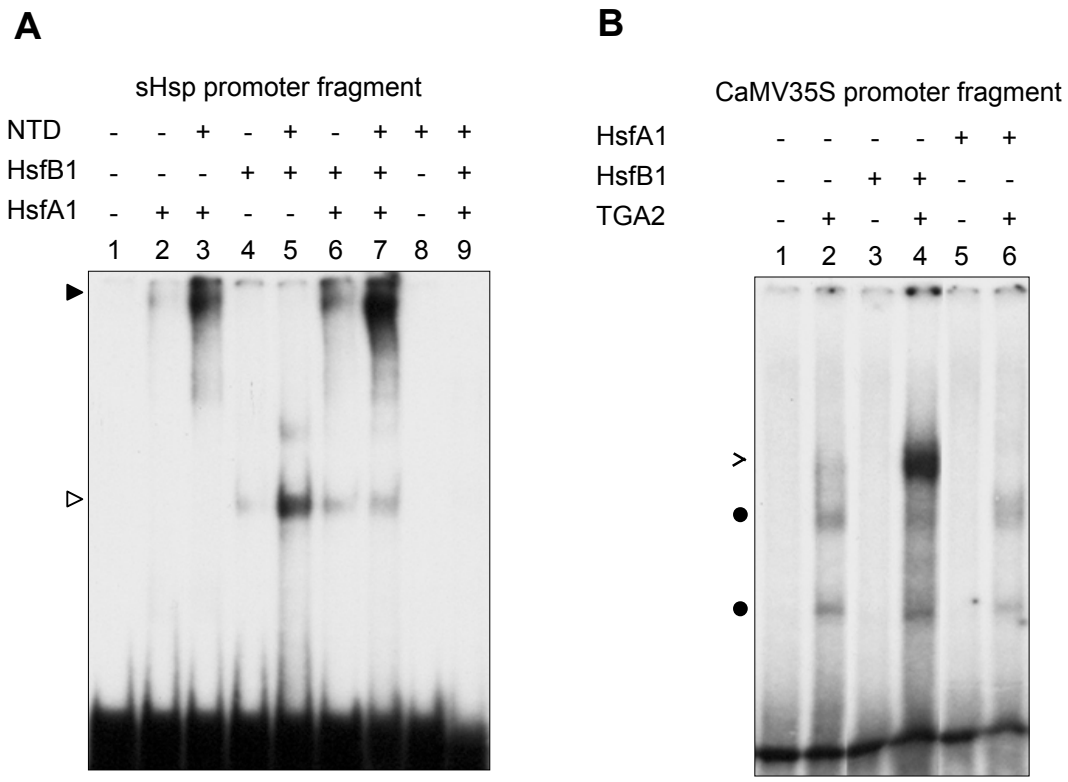


Figure 27. **Cooperative DNA binding of HsfB1 with HsfA1 and TGA transcription factors.**
(A) For the electrophoretic mobility shift assay (EMSA) with sHsp promoter fragment, P^{32} labelled PCR probes (160 bp) containing the active THtHtHT cluster (samples 1 to 8) or the inactive mutant cluster ththtHT (sample 9) were incubated with the indicated recombinant proteins (25 ng NTD, 50 ng HsfA1 and 30 ng HsfB1). The complexes were separated by polyacrylamide gel electrophoresis (Lyck et al. 1997). The closed triangle points to the HsfA1, the open triangle to the HsfB1-specific bands.
(B) EMSA with CaMV35S promoter fragment was performed using a P^{32} labelled PCR probe (94 bp) and the whole cell extracts of tobacco protoplasts expressing either HsfA1, HsfB1, TGA2.1/2.2 or the indicated mixtures of the transcription factors. The two TGA-specific bands are marked by the closed circles. The arrow head marks the supershift band observed only in the presence of TGA2.1/2.2 and HsfB1.

lane 4). No such new signal was observed when HsfA1 was coexpressed with TGA factors (lane 6). This confirms the results obtained with reporter assays (Fig. 16 and 17) that only HsfB1 shows synergistic activity on CaMV35S promoter and in this case HsfA1 has no effect. It can be assumed that the situation would be similar on other constitutive promoters as well.

The two representative results of EMSA confirm that the general coactivator function of HsfB1 resides in its ability to bind cooperatively with promoter specific acidic activators and in the ability of both proteins to cooperatively recruit the CBP/HAC1 protein.

3.16 Presence of a “histone like motif” in the CTD of HsfB1

The final aim of this study was to show the analogy between the –GRGKMMK motif of HsfB1 and the N-terminal tail motif of histones H2A and H4. The analogy focused here is that both motifs should bind to the same site in the NTD of HAC1. To this aim, band shift assay was performed as shown in Fig. 27A, with the THtHtHT wild type cluster as probe. GST fused peptides containing either the wild type sequence of histone H4 tail -SGRGKGGKG or two mutant versions of it, i.e. -SGRG**R**GGKG and -SGRG**R**GG**R**G were used to see the effect on DNP complex formation by recombinant HsfA1 plus NTD and HsfB1 plus NTD. As can be seen in lane 1 and 2, Fig. 28 almost undetectable binding of HsfA1 is strongly enhanced by NTD. Addition of 100 fold excess of GST alone has no effect (lane 3) but GST-fused with histone N-terminal tail peptides dissolve the normal HsfA1-NTD complex (closed circle) and led to a smear with a totally new complex with much higher mobility (open circle, lanes 4-6). On the other hand, the HsfB1-NTD complex (closed triangle, lane 8), is completely competed out by the GST-fusion proteins (lanes 10-12), but not by GST alone (lane 9).

The results from the competition assay clearly demonstrate that both HsfB1 with its –GRGKMMK motif and histone H4 with its N-terminal tail (–SGRGKGGK) bind to the same site in the NTD of HAC1 (compare lane 8 with 10-12). In case of HsfA1-NTD complex, the results are a bit ambiguous but it again suggests that the histone N-terminal tail peptide is at least binding to the NTD at a site different than HsfA1. The binding of this peptide somehow changes the conformation of the whole complex in such a way that it becomes unstable and runs with a totally different mobility (lanes 4-6). From its higher mobility it can be assumed that the complex is more compact now.

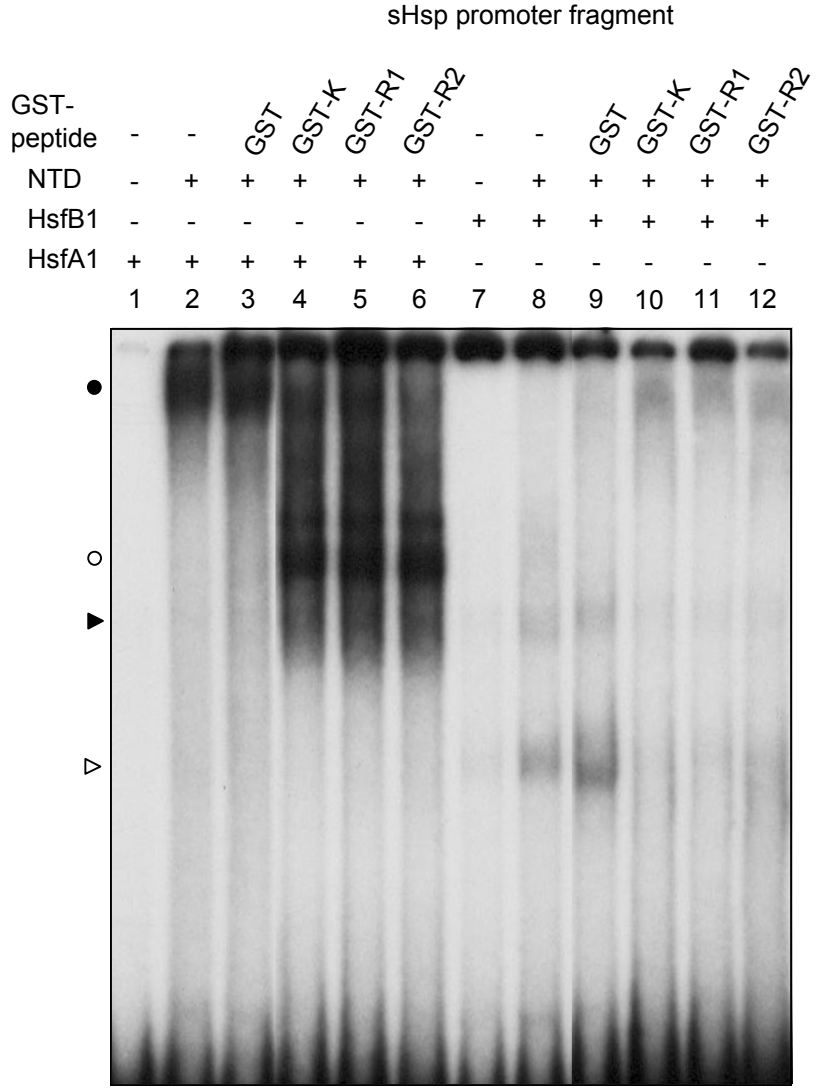


Figure 28. **Competitive band shift assay between HsfB1 and GST-fused H4 tails for interactions with NTD of HAC1.**
A competitive band shift assay was performed using P^{32} labelled PCR probe (160 bp) containing the active THtHtHT cluster and indicated recombinant proteins (see Fig. 27A for details). In addition, the reaction mixture contained 100 fold excess (as compared to HsfB1) of either GST alone or GST fused with part of histone H4 tail, i.e. –SGRGKGGKG (GST-K), –SGRGRGGKG (GST-R1), –SGRGRGGRG (GST-R2). Closed circle shows HsfA1 band formed in presence of NTD, which shifts to a completely different complex in presence of GST-fusion peptides (open circle). Open and closed triangles show HsfB1 bands in presence of NTD, which disappear in presence of GST-fusion peptides.

It can be said that the GST fused histone peptides complement HsfB1´s role to a certain extent. However, the lacking specificity of these peptides is surprising. Both the single and double lysine mutants have the same effect as the wild type peptide. The reason here might be that the in vitro situation is not so stringent in its selectivity, a matter that needs further investigations.

From this study at least one point can be strongly suggested. HsfB1 indeed represents a novel type of activator, which contains “histone-like motif” required for recruitment of CBP/HAC1 protein and it is the first example for any transcription factor containing such a motif required for a special function.

4 Discussion

4.1 Tomato HsfB1: an activator, a repressor or a coactivator?

4.1.1 An activator

There is not much known about the function of class B Hsfs in plants and whatever little knowledge exists is further complicated by seemingly controversial results about the role of this type of Hsf (Treuter et al. 1993; Czarnecka-Verner et al. 2000). The results outlined in this thesis represent the extensive study done on the function of class B Hsfs. The results for tomato HsfB1 and related Hsfs from other plants demonstrate that HsfB1 functions as a coactivator protein. Experiments with full length HsfB1 tested alone in animal cells (Fig. 25) and with Gal4 DBDxHsfB1CTD fusion protein expressed in plant cells (Fig. 6B and 7B) showed that HsfB1 has no activation potential of its own. These results are supported by the failure of HsfB1 to complement for the yeast hsf1, whereas class A Hsfs can do so (Boscheinen et al. 1997; Bharti et al. 2000). Another example for the lack of functional independence came from the studies done with protoplasts from tomato plants with a functional knockout of HsfA1 expression. Again only expression of class A Hsfs but not HsfB1 could restore the thermotolerance in these protoplasts (Mishra et al. 2002; Mishra, unpublished).

4.1.2 A repressor Hsf

Czarnecka-Verner et al. 2000 have discussed the role of HsfB class as a repressor of class A Hsf mediated reporter gene transcription. This possibility can not be ruled out, because, depending upon the test conditions and promoter context, any transcription factor can act as a repressor. In fact during this study two interesting observations were made, where the well known activator HsfA1 acted as a repressor. Fig. 8B shows that increasing amounts of HsfA1 encoding plasmid used for transformation of tobacco protoplasts did not increase the transcriptional activity linearly. Instead after a certain plasmid concentration the GUS activity started to decrease. This effect can be explained by squelching phenomenon. If the amount of a strong activator increases above a threshold concentration and there are no more binding sites on the DNA available. The unbound Hsf can still interact with the limiting components of the transcriptional machinery and compete with the bound Hsfs for the same coregulators. Similarly HsfB1, which has been shown in this study to interact specifically with CBP/HAC1 protein (Fig.

26B and C; Fig. 27A), can act as repressor by squelching HAC1 from the active promoters. So, depending upon the experimental conditions chosen, HsfB1 can be a coactivator or repressor.

The second interesting observation made about the role of HsfA1 as a repressor was while studing the induction of constitutive promoters by HsfB1 (Fig. 16 and 17). In most cases, where HsfB1 acted as a strong activator, HsfA1 either had no effect or acted as a repressor. This seems to be the result of a promoter context problem, because all the constitutive promoters contain scattered HSE motifs (Fig. 16A and 17A). So, basically any Hsf can bind to these sites but only the binding of HsfB1 with its “histone-like motif” is favourable for cooperative recruitment of CBP type coactivators and hence for synergistic gene activation. Binding of HsfA1 might disturb the binding of other activators or of endogenous HsfB1 or might disturb their interactions with the transcriptional machinery and hence act as repressor. In the same way, HsfB1 can act as a repressor if proper positioning on the promoter is not available. Fig. 6, 7 and 9 show that Hsf A1/B1 synergism is clearly promoter context dependent. Gal4 fusion constructs of HsfA1 and HsfB1 do not give synergism.

In spite of these arguments favouring the role of HsfB1 as a coactivator, it is worth mentioning here that AtHsfB1 never showed any synergistic interactions with tomato HsfA1 (von-koskull Döring, unpublished). Infact the important lysine residue in the-GRGK is replaced by a threonine residue in this case.This suggests that AtHsfB1 might indeed act as a repressor, by competing with the binding of an active HsfB type. Curiously enough none of the members of class B Hsfs from *Arabidopsis* were active in synergistic assays with tomato HsfA1. Since promoter context is an important requirement for synergism, it cannot be excluded that *Arabidopsis* class B Hsfs are promoter specific coactivators for other heat stress inducible promoters, for promoters induced under different stress conditions (oxidative, mechanical or biotic stress) or even for defined house-keeping promoters. In other words, they might need different activation partners and or different coregulators. Another intriguing possibility is that AtHsfB proteins might act via long-distance enhancers rather than via short-distance enhancers (distance with respect to TATA box), which were mainly investigated during this study (Seipel et al. 1992). Finally, post-translational modifications affecting DNA binding preference and choice of interaction partner must also be taken into account. In support of this, the DNA binding of human Hsf2 was shown to be modulated by SUMO-1 modification (Goodson et al. 2001), and it was shown for mammalian NF-?B

transcription factor that the phosphorylation state of the protein is decisive for its interactions with CBP/p300 proteins or HDAC-1 (Zhong et al. 2002).

4.1.3 A coactivator

It has been shown in this study that tomato HsfB1 coactivates reporter gene transcription either together with HsfA1 or together with constitutive trans-activators on a subset of house-keeping promoters (see model, Fig. 29). Pull down assays were performed to show that HsfB1 binds to NTD of HAC1. Moreover binding of HsfB1 to the NTD is improved in presence of HsfA1. Finally, gel shift assays were used to demonstrate that the binding of HsfB1 to the DNA is enhanced in presence of the other activator (HsfA1 or TGA). From all these results and the arguments discussed above, it becomes evident that HsfB1 is indeed a coactivator and not an activator or a repressor.

4.2 HsfB1 as a general coactivator for heat stress inducible, viral and house-keeping gene promoters

4.2.1 Heat stress inducible promoters

Different sHsp promoter fragments were investigated to study the function of HsfB1 as a coactivator of HsfA1 on these promoters. The deletion analyses exemplified for Athsp17.4-CI and Athsp17.8-CI promoters confirmed that almost each of sHsp promoters contains cluster HSE modules as independent units for synergism. An extensive mutational analysis of the Gmhsp17.3B-CI HSE cluster illustrated for the first time the role of active and inactive HSE motifs in the promoters of plant Hsp genes. Both type of HSE motifs are important for the formation of heat stress induced enhanceosome on Hsp promoters. (see model Fig. 29A). Still several points need to be clarified with respect to in vivo regulation of these promoters and the role of HSE cluster modules as independent units mediating synergism.

1) Many of the sHsp promoter fragments showed more or less similar response to coexpression of HsfA1 and HsfB1 (Fig. 9 and 10). It would be interesting to see whether at their genomic location, i.e. enpacked in chromatin, these promoters behave similar or depending upon the chromatin conformation and accessibility of promoters the response would change. Although one such example, was provided in Fig. 19 using the endogenous Hsp17-CI genes of tobacco, the individual members of sHsp family could

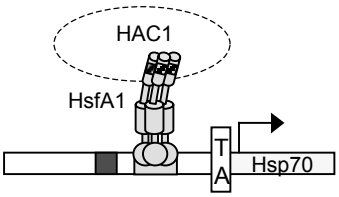
not be analyzed in detail. Moreover, the analysis in this experiment was done at the protein level. Actual changes at the level of transcription can be investigated by nuclear run on assays for individual genes or on the whole genome level by microarray analysis.

2) In this study attention was focussed mainly on synergism between HsfA1 and HsfB1 on sHsp promoters. But there are enough reports about the differential expression of sHsp genes during different developmental stages (Hightower and Nover 1991; Waters et al. 1996; Wehmeyer et al. 1996) or under other stress conditions. Moreover, the developmental expression of sHsp genes in tomato seems to be independent of the master regulator, HsfA1 (Mishra et al. 2002). It should be investigated, whether HsfB1 can also synergize with developmental regulators of sHsp gene expression. Such investigation might also show that, in fact, the role of HsfB1 for the expression of sHsp genes is different depending upon the developmental or stress conditions and hence depending on the set of acidic activators active at a given time.

3) There is no doubt about the role of HSE cluster modules as independent units for cooperative binding of HsfA1 and HsfB1, but in spite of several efforts, no real evidence could be obtained for the exact binding sites for HsfA1 or HsfB1. It is not clear why the two Hsfs do not compete for the same binding sites. Investigations about the inactive insulator motifs present with in the active HSE clusters and analogy with known examples from the literature suggest that the binding of the two Hsfs to these promoters is context dependent (Fry and Farnham 1999), i.e. there is no easily detectable binding site for any Hsf alone but binding of one changes the conformation of DNA in such a way that another Hsf with a slightly different affinity for a near by site will occupy it with much higher affinity (Erkine et al. 1999). Similarly, it was shown that mammalian Hsfs1 and 2, can synergistically regulate hsp70 promoter (Sistonen et al. 1994). Cooperative binding of different Hsf trimers was observed on a HSE array, where binding of one trimer facilitated binding of another trimer on near by HSE motifs, by a factor of 200 (Xiao et al. 1991). Using biochemical approaches like affinity measurements on Biacore (using purified DNA and proteins), DNase foot-print analysis or chromatin immunoprecipitation, these problems can be solved.

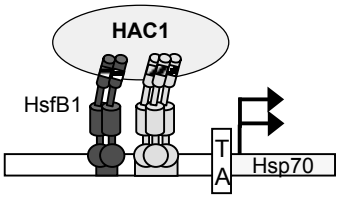
A: Hs-inducible promoter

I: Onset of heat stress



HsfA1 as master regulator triggers expression of HsfB1 and Hsp encoding genes; weak interaction with HAC1

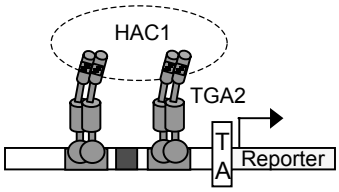
II: Prolonged heat stress



Complementation of the hs enhanceosome by recruitment of newly formed HsfB1; strong interaction with HAC1.

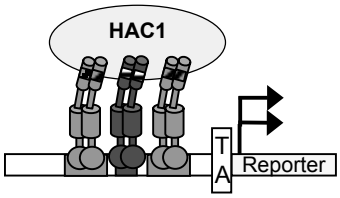
B: CaMV35S promoter

I: Control conditions



TGA2 transcription factors responsible for constitutive activity of 35S promoter; weak interaction with HAC1?

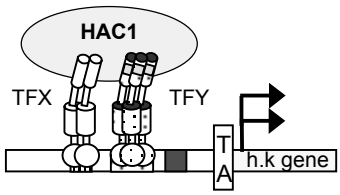
II: Prolonged heat stress



Binding of HsfB1complements the TGA2 complex and leads to strong recruitment of HAC1.

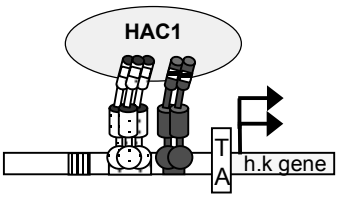
C: House keeping promoters

I: Control conditions



Strong expression of house keeping genes; interaction between TFX and TFY.

II: Prolonged heat stress



TFX inactivated due to hs, maintenance of transcription because HsfB1 can substitute for TFX.

Figure 29. **Model of synergistic interactions of HsfB1 with acidic activators (HsfA1, TGA, TFY) and corecruitment of HAC1.**
(A to C) Three examples of promoters influenced by HsfB1. For further explanations see text.

4.2.2 Viral promoters

The concept about the role of HsfB1 as a general coactivator stems from the observation and findings that HsfB1 affects the transcription of non-Hsp encoding genes as well. Because of the fairly detailed knowledge about the promoter architecture and binding proteins, cauliflower mosaic virus 35S promoter is particularly suitable to illustrate this idea. Based on the RT-PCR analysis performed during this study, stimulation of transcription from CaMV35S promoter by HsfB1 can be as high as 40 fold (Fig. 16C). The binding of HsfB1 to 35S promoter fragment is confirmed by band shift assay (Fig. 16 and 27B) and similar to the case with Hsfs (as discussed above), the binding of HsfB1 to 35S promoter is also context dependent, because: (i) In the band shift assays, binding of HsfB1 to the 35S promoter fragment was not detectable unless coexpressed with TGA factors (Fig. 27B). (ii) An initial mutational analysis of HsfB1 binding sites in CaMV35S promoter showed that mutation of any site leads to loss of binding of the other partner (TGA) as well. The above evidence suggests that virus is actually abusing a strong coactivator to maintain transcription of its key genes during heat stress and thus guarantee survival under stressful situations also. Weak interactions of TGA factors with HAC1 are complemented by binding of HsfB1 resulting in its cooperative and enhanced recruitment (see model, Fig. 29B). Viruses are known for their opportunistic nature. In fact, CaMV35S promoter is known to be activated during different developmental stages, in different tissues and by a number of different stimuli (Fang et al. 1989; Yanagisawa and Izui 1992; Lamm 1994; Qin et al. 1994; Jupin and Chua 1996). This study presents the first evidence that 35S promoter is directly regulated by a specialized Hsf. However, the interplay between HsfB1 and other stimuli which also activate 35S promoter needs to be investigated.

Another interesting approach to emphasize the importance of HsfB1 for viral survival would be to check the virus infection and its propagation during heat stress in HsfB1 transgenic plants. From this analysis, I would predict that in HsfB1 knock out plants, the virus should have problems to infect or to propagate in the host during ongoing heat stress.

4.2.3 House-keeping promoters

Similar to the situation with 35S promoter, a small subset of house-keeping promoters, investigated in this study, was found to be affected by HsfB1. Although the results presented in this study are a starting point for a detailed future investigation, they

nevertheless show that HsfB1 might represent the long searched, heat stress induced factor required for the maintenance of house-keeping transcription. As, shown in the model in Fig. 29C, inactivation or loss of a factor TFX during heat stress might result in reduced expression of constitutive promoters but HsfB1 substitutes for the factor TFX and results in maintenance of transcription.

However, in contrast to the situation with CaMV35S or sHsp promoters, detailed knowledge about the functional parts and their corresponding activators of house-keeping genes is limited. A closer look at the promoter sequence led to the identification of several putative Hsf binding sites. Despite the fact that no binding studies were performed for HsfB1 or other Hsfs, but by comparing results from Hsp and 35S promoter, similar outcome about cooperative binding of HsfB1 with gene-specific activators can be predicted. One critical point must be emphasized once more. This study was made on plasmid borne promoter fragments as part of appropriate reporter constructs. It does not take in to account the constraints or influences posed by chromatin structure. The effects need to be reinvestigated with endogenous genes using, real time PCR, nuclear run on assays or on a whole genome level, using microarray analysis. Different approaches can be used to solve it:

1) Protoplasts transformed with different combinations of HsfB1, HsfA1 and their mutants can be used to prepare samples for microarray analysis to find the genes differentially expressed by HsfA1, HsfB1 or both.This approach should be followed using *Arabidopsis* protoplasts, since the full genome microarrays are commercially available only in this case.

2) Transgenic plants containing knock out of HsfB1 expression or over expressing HsfB1 can also be used to identify the genes (house-keeping or inducible) affected by changed HsfB1 expression.

3) By studying the expression profile of house-keeping genes during different stages of heat stress in HsfB1 knock out plants, the actual role of HsfB1 in maintenance of transcription during heat stress can be evaluated.

4.3 In vivo functions of HsfB1

Due to the lacking availability of an established experimental system for tomato, most of the studies about the coactivator function of tomato HsfB1 were performed either using

transient transfections in heterologous protoplasts system of tobacco or in vitro. This situation certainly limits any interpretations about the in vivo role of HsfB1. Nevertheless, in an independent study HsfB1 transgenic plants were generated and are being analyzed (Scharf and Mishra, unpublished). The following results can be summarized from the initial analysis of the transgenic lines available. As expected from a transcription factor expressed transiently under heat stress, both the lacking expression and the overexpression of HsfB1 are not lethal for the plants, plants grow and propagate normally. However, a few developmental peculiarities were noticed: Plants lacking HsfB1 expression have longer internodes and set fruits at least one week earlier than the wild type plants. On the contrary, plants overexpressing HsfB1 have shorter internodes and are a week delayed in fruit setting (Mishra, unpublished). The molecular details of this phenotype are far from clear at this stage but it can be confirmed that HsfB1 expression affects development. The preliminary biochemical analysis suggests that the level of chaperones in general is disturbed in HsfB1 transgenic plants, but the differences are quantitative rather than qualitative. Keeping in mind the coactivator nature of HsfB1, it would be expected that changing HsfB1 expression level has some quantitative consequences on the level of transcription of other genes rather than all or none effects. It is tempting to speculate that changed chaperones levels (especially Hsc/p 70, 90 and 101) might be the reason behind the developmental phenotypes observed in HsfB1 transgenic plants. In support of above arguments, it has been shown in *Drosophila* and *Arabidopsis* that Hsp90 acts as a buffer for morphological variations, and compromised Hsp90 levels are responsible for producing an array of morphological phenotypes (Rutherford and Lindquist 1998; Queitsch et al. 2002).

Interestingly, HsfB1 transgenic plants are not compromised in thermotolerance, which completely killed the HsfA1 knock out plants (Mishra et al. 2002). First of all it suggests that the quantitative differences in chaperone levels observed in HsfB1 transgenic plants are not sufficient to show any thermosensitive phenotype, at least not under the conditions tested (2 hrs. heat stress at 45°C). Assuming that there are quantitative reductions in transcription of house-keeping genes during heat stress in the absence of HsfB1, a longer but milder heat stress might show a phenotype specific to HsfB1 knock out. The plants should get more sensitive if the transcription of important house-keeping genes is compromised for longer durations. As already mentioned, it would be interesting to use these transgenic plants for studying the propagation of pathogens under stress conditions.

4.4 Regulation of HsfB1 expression and activity: does HsfB1 recruit proteasomal subunits to the promoters?

4.4.1 Role of a single lysine residue for both stability and activity of HsfB1

As shown in Fig. 4, the expression of HsfB1 is transiently induced during heat stress and rapidly declines at recovery. In contrast the other heat stress inducible proteins e.g. HsfA2 or sHsps reach a maximal level of induction in heat stress but persist in the cell for hours during recovery from stress. In addition, inhibition of endogenous Hsp90 by geldanamycin leads to a block in turn over of HsfB1 (Scharf, unpublished). From these observations it becomes apparent that the turn over of HsfB1 protein is under a strict control.

A supporting observation was made during this study, exemplified in Fig. 30. Varying intensities of signals on immunoblots were detected by using different mutants in the CTD of HsfB1. If it is assumed that the mutations have no effect on the rate of transcription, translation or mRNA stability, these mutations can be correlated with the turnover of the protein. The immunoblots in Fig. 30 show the expression of the different constructs, without and with the presence of a reporter (phsp17**gus*). Although no GUS measurements are done in this case, a reporter was added, because a higher signal on immunoblot was observed by addition of any sequence containing Hsf binding sites (HSEs). The wild type construct (no. 1) shows that the level of detectable HsfB1 is increased at least 2 times by addition of the reporter. This gives the first hint that this protein is undergoing high turn over, especially when it is in a non-DNA bound form. Deletion analysis of the C-terminus showed that amino acid residues 296-301 have no role (construct 2), but residues 287-295 (construct 3) and residues 272-279 (constructs 4 and 5) might be important for stability of the protein. The constructs containing point mutations of lysine 275 (construct 6-8) showed beyond any doubt that the same lysine which is important for synergism is also important for stability of protein too. Additionally, lysine residue 288 might also have an important role in protein stability. The above observations are quite intriguing and several speculations about the regulation and functioning of this protein can be drawn.

Lysine is a versatile amino acid, which can be either acetylated, sumoylated or ubiquitinated (Freiman and Tjian 2003). In case of HsfB1 no attempts were made to study ubiquitination or protein stability. However, in spite of several attempts (in vivo/in vitro acetylation assays, anti-lysine antibody immunoblots), no hint for any acetylation of

HsfB1 could be gathered. But a possibility about the role of post-translational modification at the lysine residue in the CTD of HsfB1 can not be ruled out. Especially when CBP/p300 proteins are known to both acetylate (Kouzarides 2000; Sterner and Berger 2000) and ubiquitinate transcription factors (Grossman et al. 2003). It is tempting to speculate that a competitive ubiquitination of an acetylated HsfB1, targets it for degradation at the end of a heat stress. Similar examples are known in the literature, where competitive ubiquitination or acetylation of proteins control their activity and or stability.

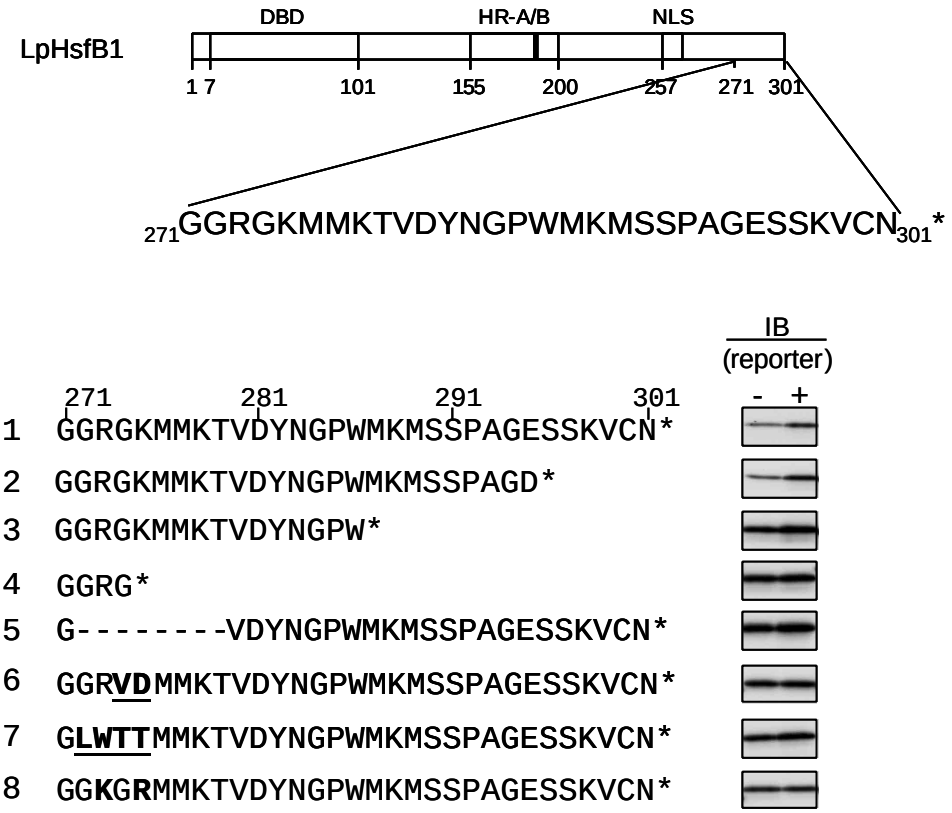


Figure 30. **Importance of lysine residues in the CTD HsfB1 for stability of the protein.**
Block diagram on top shows the basic structure of HsfB1, with the important residues in the CTD (aa 271-301). Expression levels of different HsfB1 mutants, in absence or presence of reporter plasmid are shown.

In case of p53, an external stimuli mediated competitive mono-ubiquitination by MDM2 or acetylation by p300 of the same lysine residues decide, whether the protein is multi-ubiquitinated by p300 itself and targeted for degradation or acetylated and protected from degradation and becomes transcriptionally active (Liu et al. 1999; Oren 1999; Kobet et al. 2000; Nakamuraa et al. 2000; Prives and Manley 2001; Grossman et al. 2003).

4.4.2 Are the lysine residues in the CTD of HsfB1 responsible for recruitment of proteasome subunits to the promoters?

It has been suggested that potent activators are subject to degradation during transcription initiation. These activators recruit ubiquitination machinery to the promoters, which not only ubiquitinates the activator but also the histones and RNA Pol II. The ubiquitinated activator in turn recruits proteasomal subunits to the promoters during transcription. These proteins degrade the activator on one hand but on the other hand help the elongating polymerase, perhaps by providing energy through their ATPase activity associated with the lid (see model in Fig. 31). The group of William Tansey have proposed a general “degron” function to acidic activation domains (Molinari et al. 1999; Thomas and Tyers 2000; Salghetti et al. 2001; Tansey 2001; Ganzalez et al. 2002; Ottosen et al. 2002; Muratani and Tansey 2003). Similar “degron” type motifs were also identified in the activation domain of *Arabidopsis* class A Hsfs and deletion of these motifs stabilized the proteins (von-Koskull Döring, unpublished). Although the above mentioned concept was validated for acidic activators only, because of a special coactivator function and the importance of a single lysine residue important for both synergism and stability of the protein, it is worth testing similar arguments for HsfB1 as well. If the lysine-275 in the CTD of HsfB1 also gets ubiquitinated, thus recruits proteasomal subunits to the promoters?

4.5 Presence of a novel “histone-like motif” in the CTD of HsfB1

Activation domains of eukaryotic transcription factors can be classified into at least three distinct types based on the amino acid composition of the activation domains: acidic, proline-rich and glutamine-rich (Tjian and Maniatis 1994; Goodrich et al. 1996; Ptashne and Gann 1997; Kadonaga 1998).

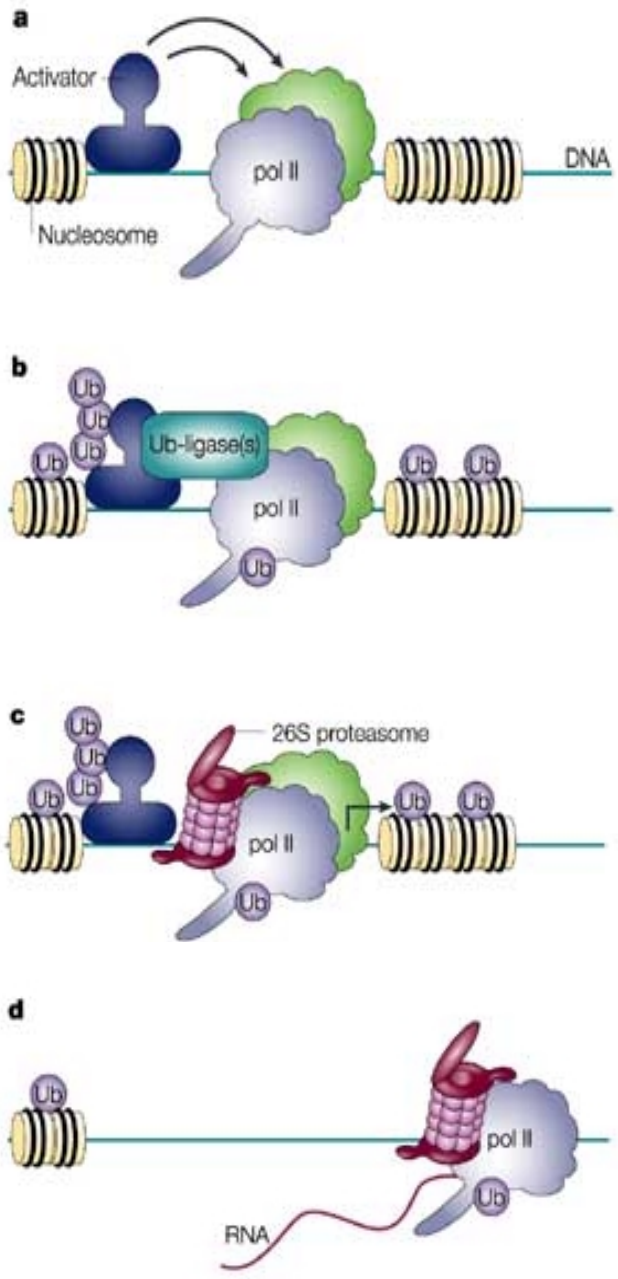


Figure 31. **A unified model for the role of ubiquitin (Ub)–proteasome system to regulate transcription at numerous levels (Muratani and Tansey 2003**).
(a) Interactions of an activator with the general transcriptional machinery (green) functions to **(b)** recruit ubiquitin ligase(s) to the site of transcription and ubiquitinates many factors, including the activator, RNA polymerase II (pol II) and histones. **(c)** These ubiquitination events in turn recruit the 26S proteasome, which **(d)** simultaneously destroys the activator and promotes elongation of transcription by pol II. Importantly, this proposed mechanism limits uncontrolled transcription in two ways — by destroying the activator at each cycle of promoter 'firing' and by ensuring that interactions between pol II and the proteasome are made in an activator- and promoter-dependent manner.

1) The majority of transcription factors can be classified as acidic, this includes most class A Hsfs of plants. Classical examples are VP16 and yeast Gal4 proteins, which act as universal activators (Struhl 1989; Regier et al. 1993).

2) The other two types of activators, i.e. the proline-rich (AP-2, CTF) and the glutamine- rich (Sp1, Oct1/2, HAP1/2) are less common. Their activation potential is usually weaker and needs close proximity to the TATA box (Courey and Tjian 1988; Pugh and Tjian 1990; Kim and Roeder 1993; Xiao et al. 1994; Xiao and Jeang 1998; Escher et al. 2000). Sp1 is usually known for its capabilities as enhancing factor (as discussed above).

By looking at the above criteria of classification, it can be said that HsfB1 does not correspond to any of the three classes. It represents a new type of activator or perhaps coactivator, containing “histone-like motif” in its CTD. So far two more members of this type have been identified in plants (from tobacco and soybean, Fig. 21). Different arguments favour the CTD of HsfB1 to be called a “histone-like motif”.

1) The sequence required for coactivation function, i.e. -GRGKMMK is highly homologous to the N-terminal tail of histones (-GRGKGGK).

2) The histone tail binds to CBP/p300 coactivators and so far the only known interaction partner known for HsfB1 is also CBP/HAC1 protein. Moreover the same motif in case of HsfB1 is required for interaction with CBP/HAC1 (Fig. 27)

3) The ultimate proof for analogy of two motifs came from the competitive band shifts, where only the nine amino acids from histone tail, containing the above mentioned sequence could easily compete for the binding of HsfB1 to the NTD of HAC1 (Fig. 28).

4) However, the controversial point is the binding site of histone motif in CBP/HAC1 protein. From earlier studies it is known that the GRGKGGK motif of histones binds to the CTD of CBP, in the enzymatic pocket of the HAT domain, whereas the “histone-like motif” of HsfB1 interacts with the NTD of HAC1. The specificity of this interaction is proven by pull down assays, Co-IP and competitive band shifts. In fact these results suggest that there might be a novel, so far unidentified binding site for histone tail in the NTD of CBP.

4.6 Synergism among different types of domains: concept of enhanceosome

Nearly all eukaryotic promoters contain binding sites for several different transcription factors, which on a simplified scale was also shown during this study. The most important requirement for synergistic gene activation is the cooperative binding of different transcription factors to the DNA. Cooperative DNA binding by distinct transcription factors can be achieved by two different modes.

In the first mode, transcription factors physically interact with each other. With a few exceptions, this option is usually limited to factors belonging to the same class/family, i.e. should have similar oligomerization domain to form heterooligomers.

In the second mode, transcription factors do not interact with each other per se but binding of one causes such a conformational change in DNA, that facilitates binding of other. This type of favoured binding has been discussed above for Hsfs and also has been shown for distinct transcription factors (as reviewed by Wolberger 1998). Usually allosteric effects of DNA contribute a lot to this type of binding (Lefstin and Yamamoto 1998). Such subtle details are yet not known for cooperative binding of HsfB1 with other activators. However, the evidences presented above suggest that, the distinct C-terminal domains, i.e. acidic for HsfA1 and TGA, “histone-like” for HsfB1 create by the cooperative binding an interface with multiple contact points, so that the components of transcriptional machinery can be synergistically recruited to the promoter, thus forming an enhanceosome like structure. The virus-inducible enhancer of the IFN-β gene provides one of the best understood examples of how combinatorial interactions between transcription factors can lead to a specific gene expression program (Merika and Thanos 2001). In the following arguments, HsfB1 induced enhanceosome is compared with the classical IFN-β enhanceosome.

1) Enhanceosomes are usually inducible and are required for an accelerated gene expression. This is the case with IFN-β enhanceosome, which is induced by virus and is needed for strong expression of interferon β in response to virus infection (Du et al. 1993; Thanos and Maniatis 1995). Similarly HsfB1 enhanceosome induced by heat stress and is required for enhanced Hsp expression.

2) Information about the specificity and regulatory potential of natural enhanceosome sequence came from the investigation of artificially synthesized/modified enhancer units. In both cases the artificial enhancers display

varying levels of transcription, are less inducible and also less specific (Fig. 11-13, this study and Thanos and Maniatis 1995; Merika and Thanos 2001).

3) It was shown for IFN-β enhanceosome that all the different activators (NF-kB, IRF proteins and ATF-2/c-Jun heterodimers) together with architectural and coactivator proteins (see model in Fig. 33) can be assembled as a stable DNP complex and a synergistic reporter gene activation can be measured in vitro (Kim and Maniatis 1997; Kim et al. 1998). Although the studies with Hsfs A1/B1 enhanceosome are limiting in this direction, Fig. 27 shows that all the parts of enhanceosome investigated in this analysis can be assembled on a DNA template in vitro. The stability and synergistic transcription from these in vitro assembled enhanceosome complexes has to be determined.

4) Role of architectural proteins: High mobility group (HMG) proteins have been shown to be important components of an enhanceosome complex (Thanos and Maniatis 1992; Yie et al. 1997). HMG proteins are a set of architectural chromatin bound proteins, which have a non-specific affinity for DNA binding, especially for A/T bases rich regions. The binding of HMG proteins is usually associated with bending of DNA (Bianchi and Beltrame 2000). The importance of HMG proteins for enhanceosome assembly was underscored by findings that the allosteric changes induced in DNA by binding of HMG I and HMG I(Y) proteins is a prerequisite for cooperative recruitment of activator proteins (Falvo et al. 1995; Yie et al. 1999). Furthermore these proteins are not only important for assembly of enhanceosome but their acetylation leads to turn off of the synergistic expression from the enhanceosome and destabilizes it (Munshi et al. 1998).

A similar concept needs to be explored in case of Hsfs A1/ B1 enhanceosome. The role of HMG proteins was never investigated for this purpose. May be the stabilization of complex and cooperative binding of proteins can be enhanced in presence of HMG proteins.

4.7 Role of CBP/HAC1 as a scaffold for HsfA1 and HsfB1

Similar to the results with IFN-β enhanceosome (Merika et al. 1998), CBP ortholog HAC1 was identified as the first target of Hsfs A1 and B1 enhanceosome. Unfortunately, no evidences for histone acetylation and about the importance of HAT domain of HAC1

were obtained in this case. In fact the NTD of HAC1 was alone sufficient for all the effects (Fig. 23-27), but the importance of local histone modifications or of an important transcriptional player can not be ruled out. However, the most prominent effect of NTD of HAC1 was a scaffolding function. It seems that binding of NTD to both HsfA1 and HsfB1 stabilizes binding of the two proteins to DNA (Fig. 27A), thus acting as a scaffold or a bridge rather than a HAT. A similar argument has been shown for mammalian p300 protein, which acts as a bridge to stabilize STAT3-Smad1 complex in fetal brain and the stabilized triple complex has synergistic activation potential (Nakashima et al. 1999; Chan and Lathangue 2001).

The scaffolding function of HAC1 can be seen more clearly in Fig. 32. As expected, in contrast to the wild type reporter (construct 1), the reporter with mutation of 5´ active HSE motifs (construct 2) shows very low synergistic GUS activity by coexpression of HsfA1 and HsfB1 (represented as number of plus). But surprisingly, the effect of HAC1 is reversed on the two constructs, the mutant reporter responds much stronger as compared to the wild type. This suggests that in the absence of part of enhancer elements (mutated 5´ active HSE motifs in construct 2) HAC1 functions as a scaffolding protein by communicating with Hsfs bound to TATA distal and TATA proximal HSEs.

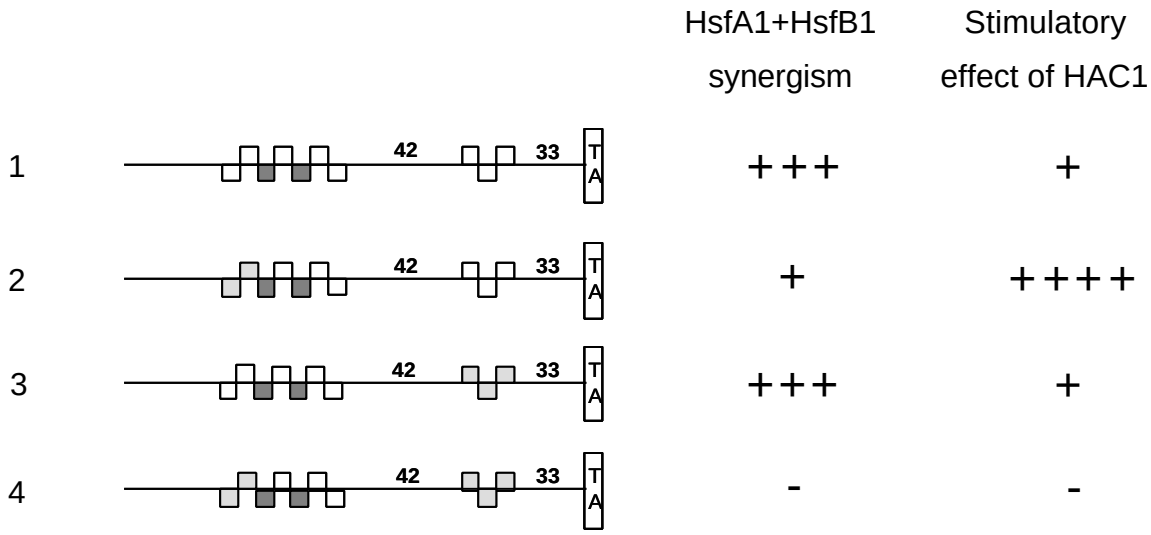


Figure 32. **Scaffolding function of HAC1 on distant and mutant HSE modules.**
GUS reporter activities were measured using phsp17**gus* (construct 1) and a version of it with 5´ HSE motifs mutated (see light grey boxes in construct 2). Both constructs were similarly mutated, i.e. at the TATA proximal HSE module (constructs 3 and 4). Standard concentrations of Hsfs A1, B1 and HAC1 were taken (Fig. 23A, sample 18). Extent of synergistic reporter gene activity and the effect of HAC1 obtained from all these constructs are represented as plus/minus, e.g. construct 1 and 3 show stronger synergism but is weaker in HAC1 effect, whereas construct 2 shows low synergism but has a high stimulatory effect of HAC1. Construct 4 is completely inactive for both effects.

The specificity of this effect is proven by comparison of constructs 3 and 4, where the TATA proximal HSE has been mutated. This mutation neither affects the HsfA1/B1 synergism nor the effect of HAC1 in case of wild type cluster (compare constructs 1 and 3) but totally abrogates the HAC1 effect in case of mutant cluster (compare constructs 2 and 4). Confirming the idea that in case of construct 2, HAC1 acted as a scaffold between TATA proximal HSE and TATA distal HSE cluster.

Additional evidences were presented during this study to support the scaffolding function of HAC1. It was shown that NTD of HAC1 interacts with both Hsfs directly (Fig. 27, 28) but the exact binding sites could not be defined. By looking at the remarkable functional conservation between mammalian and plant CBP, it is tempting to speculate that both Hsfs A1 and B1 bind to the C/H1 region in the NTD. Keeping in mind their entirely different interacting domains, this option is less likely although the C/H1 region is the only sequentially conserved part between two CBP orthologs. However, using phage display libraries, Frangioni et al. (2000) identified a consensus peptide motif WWVYDLLF, as the minimum sequence required for interaction with the CREB binding domain of CBP. This motif closely resembles the AHA motifs, e.g. for HsfA1 AHA1:–DIDWQSGLL- , AHA2:-DPFWEKFL-. Although from the sequence comparison, At-HAC1 lacks a visible CREB binding domain domain (Fig. 22A), this does not exclude that part of the domain are functionally preserved.

From these consideration several interesting experiments can be derived, to prove the specificity of interactions between HAC1 and HsfA1-B1.

1) First of all the binding sites for both HsfA1 and HsfB1 need to be defined more precisely. Using GST-pull down with recombinant proteins fragments of NTD of HAC1, yeast two hybrid or in vitro pull down with radiolabelled proteins can be used to solve this.

2) Competitive binding assays using E1A, a known interaction partner for CBP (binds both to the NTD and CTD) can be used to get more insight into the binding sites of Hsfs A1 and B1 to the NTD of HAC1 (Eckner et al. 1994; Arany et al. 1995; Kurokawa et al. 1998). In fact Bordoli et al. 2001 showed that E1A binds to the C/H3 domain of At-HAC1 as well, it is worth investigating whether it binds to the NTD also and if it competes with the binding of any of the two Hsfs.

3) It has been argued during this study that the synergistic effects of HsfB1 seen by coexpression with HsfA1 or on constitutive promoters are contributed by endogenous HAC1. The best argument to prove this would be a knock out of endogenous HAC1.

This should abolish the synergism mediated by HsfB1, if HAC1 is the sole requirement for this synergistic effect. HAC1 knock out can either be achieved in a transient system, using RNAi technique or in T-DNA insertion lines.The former one leads to a functional knock out of expression, whereas the latter creates a physical knock out of the gene. However, few points have to be kept in mind while doing such an analysis. HAC1 has 4 other isoforms (at least 3-5 are very homologous to HAC1) and none of them has been checked in transient or in vitro studies for its effects on synergism. A functional complementation by another isoforms (in the absence of HAC1) can not be ruled out, at least for the seemingly general effect of HsfB1.

4.8 Order of recruitment of other coactivators and setting up of an histone code for HsfB1 enhanceosome

As shown in Fig. 33, the transcription initiation requires an ordered recruitment of several coactivators, including HAT complexes e.g. CBP/p300, GCN5 and P/CAF, kinases, ATP dependent remodelling complexes and finally the components of holoenzyme complex together with TBP-TFIID complex (Korzus et al. 1998; Glass and Rosenfeld 2000; Lemon and Tjian 2000; Featherstone 2002;). Opening up of the chromatin structure by HATs and ATP dependent remodelling complexes is a prerequisite for proper positioning of TFIID-TBP complex at TATA box and an efficient initiation of transcription by RNA Pol II holoenzyme complex recruited by TFIID complex (Nikolov and Burley 1997; Hahn 1998; Hampsey 1998; Struhl 1998; Kingston and Narlikar 1999; Wolffe and Hayes 1999).

Although different models exist for recruitment of different activators (He and Weintraub 1998; Cosma et al. 1999; Fry and Peterson 2001; Cosma 2002), the sequential model shown for IFN-ß enhanceosome (Agalioti et al. 2000) has been taken as a reference for HsfB1 induced enhanceosome. According to Agalioti et al. 2000 and 2002, the recruitment of initial coactivators to the enhanceosome set a “histone code” by acetylating the N-terminal tails of histones, which is read by subsequent coactivators to initiate a highly specific and efficient transcription. The term “histone code” was coined by Strahl and Allis (2000), who proposed that the binding of activators recruit a certain set of coactivators and these coactivators induce post-translational modifications of N-terminal tails of histones. These modifications are not only required for nucleosome

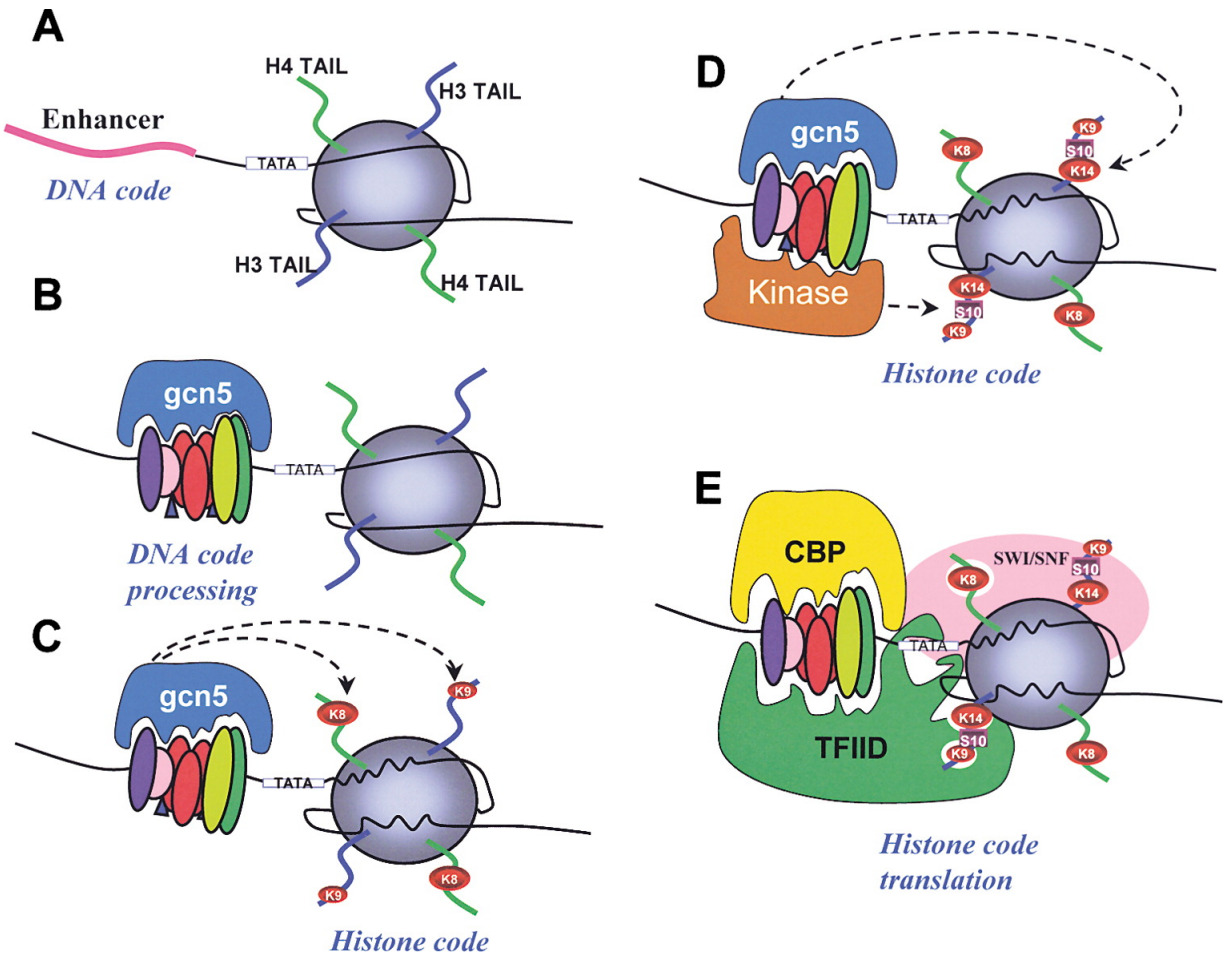


Figure 32. A model depicting the order of recruitment of coactivators at the IFN-β enhanceosome, events involved in setting up and translation of histone code (Agalioti et al. 2002).

(A) The DNA code "enhancer" contains all the information for initiation of enhanceosome assembly, i.e. recruitment of coactivators and setting up of histone code.

(B) DNA code information is processed. Activators and architectural proteins assemble in an enhanceosome complex at the enhancer element. This leads to recruitment of first target coactivator, the GCN5 histone acetyltransferase.

(C) GCN5 starts to print the histone code by acetylation of histone N-terminal tails.

(D) Recruitment of a so far unknown kinase, the second target coactivator phosphorylates the ser residues in the histone tails. An important step for the further printing of histone code at IFN-β enhanceosome.

(E) Continued recruitment of coactivators like CBP and other coactivators (SWI/SNF and TFIID), translate the histone code, via bivalent interactions with enhanceosome on one side and acetylated histone tails on the other side, thus initiating a highly specific and efficient round of transcription.

destabilization but actually set up a histone code. This code is translated by subsequently recruited coactivators and hence is required for a highly specific initiation of transcription. The code is unique for each gene and the uniqueness is determined by the initially bound activators and the first set of coactivators recruited by these activators. Since the choice of activators for a particular gene is dependent on the cis-elements in the promoter of that gene, hence the coactivators choice and the histone code is unique for each gene (Jenuwein and Allis 2001; Gamble and Freedman 2002; Turner 2002).

The above mentioned concept could not be evaluated for HsfA1-B1 enhanceosome because of the lacking availability of chromatin cross linking and immunoprecipitation (CLIP) technique for plants cells. It is the most modern and useful technique to study DNA-protein interactions and most of the work done on IFN-ß enhanceosome with respect to ordered recruitment of coactivators and setting up of the “histone code” during enhanceosome action was performed using this technique (Agalioti et al. 2000; Agalioti et al. 2002). However, a recent paper by Chua et al. 2003, establishes CLIP for plant cells and even shows the acetylation status of genes affected by different stimuli. It is hoped that use of this technique will help in the future to uncover several of the above mentioned mysteries for the heat stress induced enhanceosome discovered in this study.

The role of tomato HsfB1 and related Hsfs from other plants has been suggested in a model in Fig. 29. Because of its coactivator function, HsfB1 complements the heat stress induced enhanceosomes on Hsp (Fig. 29A), viral (Fig. 29B) and house-keeping (Fig. 29C) gene promoters by enhanced trancription. Although, the model shows a general role of HsfB1 in improved recruitment of CBP as the reason behind increased transcription, it can not be applied in general, at least not in this simplified form. The future investigations need to focus on the other aspects of HsfB1 induced enhanceosome, e.g. the role of other coactivators, their order of recruitment to the promoter and last but not least the setting up of an “histone code”. It is tempting to speculate that the promoters affected by HsfB1 have a partially common code, initiated by HsfB1, whereas the uniqueness is contributed by promoter specific activators.

5 Summary

In contrast to the class A heat stress transcription factors (Hsfs) of plants, a considerable number of Hsfs assigned to classes B and C have no evident function as transcription activators on their own. In the course of my PhD work I showed that tomato HsfB1, a heat stress induced member of class B Hsf family, is a novel type of transcriptional coactivator in plants. Together with class A Hsfs, e.g. tomato HsfA1, it plays an important role in efficient transcrition initiation during heat stress by forming a type of enhanceosome on fragments of Hsp promoter. Characterization of promoter architecture of hsp promoters led to the identification of novel, complex heat stress element (HSE) clusters, which are required for optimal synergistic interactions of HsfA1 and HsfB1.

In addition, HsfB1 showed synergistic activation of the expression of a subset of viral and house keeping promoters. CaMV35S promoter, the most widely expressed constitutive promoter turned out to be the the most interesting candidate to study this effect in detail. Because, for most house-keeping promoters tested during this study, the activators responsible for constitutive expression are not known, but in case of CaMV35S promoter they are quite well known (the bZip proteins, TGA1/2). These proteins belong to the acidic activators, similar to class A Hsfs. Actually, on heat stress inducible promoters HsfA1 or other class A Hsfs are the synergistic partners of HsfB1, whereas on house-keeping or viral promoters, HsfB1 shows synergistic transcriptional activation in cooperation with the promoter specific acidic activators, e.g. with TGA proteins on 35S promoter. In agreement with this the binding sites for HsfB1 were identified in both house-keeping and 35S promoter. It has been suggested during this study that HsfB1 acts in the maintenance of transcription of a sub-set of house-keeping and viral genes during heat stress.

The coactivator function of HsfB1 depends on a single lysine residue in the –GRG**K** motif in its CTD. Since, this motif is highly conserved among histones as the acetylation motif, especially in histones H2A and H4,. It was suggested that the –GRGK motif acts as a recruitment motif, and together with the other acidic activator is responsible for corecruitment of a histone acetyl transferase (HAT). So, the effect of mammalian CBP (a well known HAT) and its plant orthologs (HAC1) was tested on the stimulation of synergistic reporter gene activation obtained with HsfA1 and HsfB1. Both in plant and mammalian cells, CBP/HAC1 further stimulated the HsfA1/B1 synergistic effect.

Corecruitment of HAC1 was proven by in vitro pull down assays, where the NTD of HAC1 interacted specifically both with HsfA1 and HsfB1. Formation of a ternary complex between HsfA1, HsfB1 and CBP/HAC1 was shown via coimmunoprecipitation and electrophoretic mobility shift assays (EMSA). In conclusion, the work presented in my thesis presents a new model for transcriptional regulation during an ongoing heat stress.

Zusammenfassung

Im Gegensatz zu den pflanzlichen Hitzestreßtranskriptionsfaktoren (Hsf) der Klasse A zeigen die Vetreter der Klassen B und C keine offensichtliche Funktion als Transaktivatoren in den geläufigen Testsystemen. Im Verlauf der experimentellen Arbeiten zu dieser Promotion ist es aber gelungen, die komplexe Rolle eines wichtigen Vetreters der Klasse B der Tomate (Hsf B1) aufzuklären. Im Zusammen-spiel mit Klasse A Hsfs, z.B. HsfA1, spielt er eine unverzichtbare Rolle für die effizienten Veränderungen der Transkriptionsprogramme im Verlauf der Hitzestreß-antwort. Auf der einen Seite wirkt er als Teil von Hitzestress-Enhanceosomen als synergistischer Verstärker für die Hitzestress-induzierte Genaktivierung durch HsfA1. In umfangreichen Studien zur Promoterarchitektur habe ich die Voraussetzungen für die Assemblierung solcher Enhanceosomen aufgedeckt.

Auf der anderen Seite wirkt HsfB1 als Coaktivator auch an der Aufrechterhaltung bzw. Wiederherstellung der Transkription wichtiger Haushaltsgene während und nach der Stressperiode mit. Von exemplarischen Interesse ist in diesem Zusammenhang die starke Stimulation der Transkription von Konstrukten mit dem Blumenkohlmosaik-virus 35S Promoter, der als starker konstitutiver Promoter für viele pflanzliche Expressionsplasmide genutzt wird. Während für die meisten der von uns getesteten Haushaltsgene die für die konstitutive Expression verantwortlichen Aktivatorproteine nicht bekannt sind, sind die Aktivatoren für den 35S Promoter gut untersucht (bZip Proteine TGA1/2). Sie gehören wie die Klasse A Hsfs zu den sogenannten sauren Aktivatorproteinen, d.h. die fördernde Wirkung von HsfB1 auf die Transcription von Haushalts- und viralen Genen sowie von Hitzestressgenen beruht auf der universellen Fähigkeit, in Kombination mit sauren Aktivatoren die Rekrutierung von Komponenten des Initiationskomplexes für die Transkription zu verstärken. Entsprechende Bindungsstellen für HsfB1 haben wir in allen von uns getesteten Haushaltsgenen und auch im 35S Promoter identifiziert.

Die eingehende Charakterisierung von HsfB1 Mutanten haben gezeigt, dass die Rolle als Coaktivator essentiell mit einem einzigen Lysinrest in der C-terminalen Domäne verbunden ist, der Teil eines Histon-artigen -GRGK- Motifs ist. Da solche Motive die hauptsächlichen Erkennungsstellen für Acetyltransferasen in den N-terminalen Domänen der Histone H2A und H4 darstellen, haben wir die Rolle der in Tieren und Pflanzen konservierten Histonacetyl-Transferase CBP bzw. des orthologen Proteins

HAC1 aus Arabidopsis in unseren Testsystemen untersucht. In der Tat kann die synergistische Aktivierung der Transkription durch HsfA1 und HsfB1 in Gegenwart von HAC1/CBP in pflanzlichen wie in tierischen Zellen noch gesteigert werden. Die unmittelbare Interaktion von HsfA1, HsfB1 und CBP/HAC1 in einem ternären Komplex wurde durch Coimmunpräzipitation, Pull-down Assays und in vitro DNA Bindungsstudien (EMSA, electrophoretic mobility shift assays) belegt. In Zusammenfassung meiner Untersuchungen habe ich ein neues Modell für die Regulation der Transkriptionsprozesse im Verlauf der Hitzestressantwort entwickelt.

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7 Appendix

The following tables summarize different constructs prepared and used during this study. Cloning strategies including primers, cloning enzymes, templates and cloning vectors are mentioned for each case. For the sake of simplicity cloning vector is not mentioned separately, if it is the same as the template. Cloning sites used are underlined in the primer sequences and the mutated nucleotides are bold faced.

Table 1. Plant expression plasmids containing point mutations/deletions in the ORFs of Hsf constructs.

Lab name	Name (remarks)	Primers (cloning enzymes, template, cloning vector)	Date
HsfA1			
KB129	pRT-LpHsfA?71-8	SalI-XbaI, pRTHsfA1.8, pRTHsfA1.71	10/00
KB265	pRT-LpHsfA1.M5 (R93>D) (triple ligation of PCR products from Pr. 50-1084 and Pr. 1083-254 with the vector)	50.F: 5´-gacgcacaatcccacta-3´ 1084.R: 5´-cagctg**g**tcgacaaagctggaaaagttattatgc-3´ 1083.F: 5´-agcttt**g**tcgaccagcttaatacttatgg-3´ 254.R: 5´-cctgaagagtgactcctgaaacacg-3´ (XhoI-SalI-NsiI, pRT-LpHsfA1)	02/02
HsfB1			
KB201	pRT-LpHsfB1.24 (K275>Q)	815.F: 5´-gagaatgtcgacacttgtggtggacgtgg**cc**a**g**atgatgaaaaac-3´ 179.R: 5´- cacacattattctggag-3´ (SalI-BamHI, pRT-LpHsfB1.24)	06/01
KB202	pRT-LpHsfB1.23 (K278>R)	816.F: 5´-acttgtgtcgaccg**c**ggtaaaatgatg**cgt**actgtgg-3´ 179.R: 5´- cacacattattctggag-3´ (SalI-BamHI, pRT-LpHsfB1.23)	06/01

Table 2. Plant expression plasmids containing fusion constructs of HsfA1 and HsfB1 with DNA binding or activation domains from other transcription factors.

Lab name	Name	Primers (cloning enzymes, template, cloning vector)	Date
KB240	pBI-Gal4DBD (aa 1-141), S, LpHsfB1CTD (aa 101-301)	874.F: 5´-cggaagtcgaccgtgacatcaaccccagc-3´ 568.R: 5´-attagagcggccgctcagttacaaaccttgctg-3´ (SalI-NotI, pRT-LpHsfB1, pBI-Gal4DBD)	08/01
KB241	pBI-Gal4DBD (aa 1-141), ST, LpHsfA1 CTD (aa 131-527)	875.F: 5´-cgtaagtcgacgcctgctcatggacatgctcaac-3´ 487.R: 5´-atatagagcggccgcttgcggactctagatg-3´ (SalI-NotI, pRT-LpHsfA1, pBI-Gal4DBD)	08/01
KB315	pRT-LpHsfA1?C394 (aa 1-394)xGal4AD (aa 768-881)	1151.F: 5´-tgggtgctagccgccaattttaatcaaagtggg-3´ 387.R: 5´-gtatctacgattcatagatctctgc-3´ (NheI-XbaI, pAD-Gal4, pRT-A1?C394)	06/02
KB316	pRT-LpHsfA1?C394 (aa 1-394)xVP16 (aa 412-490)	1152.F: 5´-gactggctagcaccgcccccattaccgacg-3´ 179.R: 5´-cacacattattctggag-3´ (NheI-XbaI, pRT-3HA-LpHsfA2DBDxVP16, pRT-A1?C394)	06/02
KB324	pRT-LpHsfB1?C198 (aa 1-198)xNtHsfB1 CTD (aa 204-292)	1226.F: 5´-tgatcgctagcatcattagccaaggaacctc-3´ 1229.R: 5´-atttggtctagatcttcagttacagaccttgttac-3´ (NheI-XbaI, Nt-cDNA, pRT-B1?C198)	08/02

KB330	pRT-LpHsfB1?C198 (aa 1-198)xGmHsf B1CTD (aa 204-282)	1225.F: 5´-agatcgctagcatcatgcggcaaggaag-3´ 1228.R: 5´-gagcctctagattcagttgcaaaccctgttg-3´ (NheI-XbaI, pGmHsfB1, pRT-B1?C198)	08/02

Table 3. Plant expression plasmids containing human CBP or Arabidopsis HAC1

Lab name	Name (remarks)	Primers (cloning enzymes, template, cloning vector)	Date
KB317	pRT-HsCBP	SacI-NdeI, pcDNA3-HsCBP, pRT101	07/02
KB337	pRT-AtHAC1CTD (aa 984-1654)	1221.F: 5´-caccgtaggagcgtcgaccctaggatgtacttttgtattcc-3´ 1223.R: 5´-aatttcccgggcctaggttacctgcaacgaggtacatggc-3´ (BlnI-XbaI[1], At-cDNA, pRT101)	09/02
KB338	pRT-AtHAC1NTD (aa 1-988)	1220.F: 5´-ggggcaggtgtcgaccctaggactatgtcgggg-3´ 1224.R: 5´-gtcgccccgggattcctaggttatggaatacaaaagt aatg-3´ (BlnI-XbaI[1], At-cDNA, pRT101)	09/02
KB339	pRT-AtHAC1 (two step cloning: 1) A C-terminal fragment amplified by Pr. 1219-1223, cloned into pRT=pRT-fragCTD. 2) The N-terminal fragment amplified with Pr. 1220-1222, fused in frame to CTD in pRT-fragCTD.)	1219.F: 5´-gcctaaggcaagactcgagaaaaag-3´ 1223.R: 5´-aatttcccgggcctaggttacctgcaacgaggtacatggc-3´ (XhoI/BlnI-XbaI[1], At-cDNA, pRT101) =pRT-fragCTD 1220.F: 5´-ggggcaggtgtcgaccctaggactatgtcgggg-3´ 1222.R: 5´-ctttttctcgagtcttgccttaggc-3´ (XhoI, At-cDNA, pRT-fragCTD)	09/02

[1] Isoschizomers were used to cut the vector and the insert

Table 4. Animal expression plasmids containing tomato Hsf mutants

Lab name	Name	Cloning enzymes, template, cloning vector	Date
KB341	pcDNA3-LpHsfA1 heptaA (aa 451-IDWQSGLL 12aa DPFWEKFL- >451-IDAQSGAA 12aa DPAAEKAA-);	XhoI-XbaI, pRT-LpHsfA1hepta A, pcDNA3	01/03
KB342	pcDNA3-LpHsfA1M5 (R93>D)	XhoI-XbaI, pRT-LpHsfA1M5 (R93>D), pcDNA3	01/03
KB343	pcDNA3-LpHsfB1M4 (KH54,55>EL)	XhoI-XbaI, pRT-LpHsfB1M4 (KH54,55>EL), pcDNA3	01/03
KB344	pcDNA3-LpHsfB1 (GRGK272-275>GKGR)	XhoI-XbaI, pRT-LpHsfB1 (GRGK272-275>GKGR), pcDNA3	01/03

Table 5. Bacterial expression plasmids containing tagged versions of Hsfs and HAC1

Lab name	Name	Primers (Cloning enzymes, template, cloning vector)	Date
KB237	pJC40-His10-LpHsfB1 (M1>L)	872.F: 5´-cgagagtcgacaagcttcgcaaagaacagcgcc-3´ 873.R: 5´-gactctcgagttttgcggccgctagatgtcagttagc-3´ (HindIII-XhoI, pRT-LpHsfB1, pJC40-His10)	08/01
KB238	pJC40-His10-LpHsfB1.20 (M1>L, GK273-274>VD)	872.F: 5´-cgagagtcgacaagcttcgcaaagaacagcgcc-3´ 873.R: 5´-gactctcgagttttgcggccgctagatgtcagttagc-3´ (HindIII-XhoI, pRT-LpHsfB1.20, pJC40-His10)	08/01

KB247	pJC-LpHsfB1-HC	926.F: 5´-aagaggcatatgtcgcaaagaacagcg-3´ 927.R: 5´-catagggatccacgcggaaccaagttacaaaccttgctgctttc -3´ (NdeI-BamHI, pRT-LpHsfB1, pJC-B1?C293HC)	06/01
KB350	pJC40-His10-AtHAC1NTD (aa 1-988)	1220.F: 5´-ggggcaggtgtcgaccctaggactatgtcgggg-3´ 1224.R: 5´-gtcgccccgggattccctaggttatggaatacaaaagtaat g-3´ (SalI/XhoI-XmaI[1], pRT-AtHAC1NTD (aa 1-988), pJC40-His10)	01/03
KB353	pJC40-His10-AtHAC1CTD3HA (aa 984-1654)	1221.F: 5´-caccgtaggagcgtcgaccctaggatgtacttttgtattcc-3´ 1223.R: 5´-aatttcccgggcctaggttacctgcaacgaggtacatggc-3´ (SalI/XhoI-XmaI[1], pRT-AtHAC1CTD (aa 1-988), pJC40-His10)	01/03

[1] Isoschizomers were used to cut the vector and the insert

Table 6. Reporter constructs containing mutations in the HSE motifs of hsp17* promoter.

Lab name	Name	Primers (HSE configuration; x,any nucleotide) (cloning enzymes, template, cloning vector)	Date
Derivatives of full length hsp17* promoter			
KB46	phsp17*mut46-gus	592.F: 5´-tctagagtcgaccgcaaaacccttactc-3´ 285.R: 5´-ttcgcgatccagactgaatgcc-3´ (THtHtHT 42x HTH 21x **ht** 2x TATA box) (SalI-NcoI, pBT-hsp17*gus)	06/00
KB55	phsp17*mut55-gus	593.F: 5´-aagcttggatccgtcgaattcgtttaaaatgtttctgaaag-3´ 285.R: 5´-ttcgcgatccagactgaatgcc-3´ (**th**tHtHT 42x HTH 21x **ht** 2x TATA box) (BamHI-NcoI, KB46, pBT-hsp17*gus)	07/00
KB57	phsp17*mut57-gus	604.F: 5´-ccgtcgaattcgtttaaaatgtttaatattgtttcag-3´ 285.R: 5´-ttcgcgatccagactgaatgcc-3´ (**th**t**h**tHT 42x HTH 33x TATA box) (EcoRI-NcoI, KB55)	07/00
KB58	phsp17*mut58-gus	605.F: 5´-ccgtcgaattcgtccagaatgtttaatattgtttcagaaaattc-3´ 285.R: 5´-ttcgcgatccagactgaatgcc-3´ (TH**th**tHT 42x HTH 33x TATA box) (EcoRI-NcoI, KB46, KB55)	07/00
KB59	phsp17*mut59-gus	606.F: 5´- ccgtcgaattcgtccagaatgtttctgaaagtttctaacaatttaagttttg- 285.R: same as in KB46 (THtHt**ht** 42x HTH 33x TATA box) (EcoRI-NcoI, KB46, KB55)	07/00
KB130	phsp17*mut130-gus	678.F: 5´- gcttggatccgtcgaagaagtccagaatgttctagaaagtttcag-3´ 580.R: 5´-gaggaagggtcttgcggtcg-3 (TH**T**HtHT 42x HTH 33x TATA box) (BamHI-PstI, KB46)	12/00
KB131	phsp17*mut131-gus	679.F: 5´- gcttggatccgtcgaagaagtccagaatgtttctgaacgttctagaaaattctag-3´ 580.R: 5´-gaggaagggtcttgcggtcg-3 (THtH**T**HT 42x HTH 33x TATA box) (BamHI-PstI, KB46)	12/00
KB132	phsp17*mut132-gus	680.F: 5´- gcttggatccgtcgaagaagtccagaatgttctagaacgttctagaaaattctag-3´ 580.R: 5´-gaggaagggtcttgcggtcg-3 (TH**T**H**T**HT 42x HTH 33x TATA box) (BamHI-PstI, KB46)	12/00
Derivatives of cluster HSE in hsp17* promoter			
KB47	phsp17*mut47-gus	592.F: 5´-tctagagtcgaccgcaaaacccttactc-3´ 285.R: 5´-ttcgcgatccagactgaatgcc-3´ (THtHtHT 90x TATA box) (SalI-NcoI, KB46, pBT-hsp17*gus)	06/00
KB56	phsp17*mut56-gus	593.F: 5´-aagcttggatccgtcgaattcgtttaaaatgtttctgaaag-3´	07/00

		285.R: 5´-ttcgcgatccagactgaatgcc-3´ (**th**tHtHT 90x TATA box) (BamHI-NcoI, KB47, pBT-hsp17*gus)	
KB74	phsp17*mut74-gus	581.F: 5´-ggaaacctcgaggaccatgattacg-3´ 624.R: 5´-gttatgcgtcgaccggccgtacttctgaaaatctc-3´ (THtHtHT 50x TATA box) (HindIII-Sal, KB46)	08/00
KB75	phsp17*mut75-gus	581.F: 5´-ggaaacctcgaggaccatgattacg-3´ 624.R: 5´-gttatgcgtcgaccggccgtacttctgaaaatctc-3´ (**th**tHtHT 50x TATA box) (HindIII-Sal, KB55, KB46)	08/00
KB76	phsp17*mut76-gus	581.F: 5´-ggaaacctcgaggaccatgattacg-3´ 624.R: 5´-gttatgcgtcgaccggccgtacttctgaaaatctc-3´ (**th**t**h**tHT 50x TATA box) (HindIII-Sal, KB57, KB46)	08/00
KB77	phsp17*mut77-gus	581.F: 5´-ggaaacctcgaggaccatgattacg-3´ 624.R: 5´-gttatgcgtcgaccggccgtacttctgaaaatctc-3´ (TH**th**tHT 50x TATA box) (HindIII-Sal, KB58, KB46)	08/00
KB78	phsp17*mut78-gus	581.F: 5´-ggaaacctcgaggaccatgattacg-3´ 624.R: 5´-gttatgcgtcgaccggccgtacttctgaaaatctc-3´ (THtH**ht** 50x TATA box) (HindIII-Sal, KB59, KB46)	08/00
KB138	phsp17*mut138-gus	581.F: 5´-ggaaacctcgaggaccatgattacg-3´ 624.R: 5´-gttatgcgtcgaccggccgtacttctgaaaatctc-3´ (TH**T**HtHT 50x TATA box) (HindIII-Sal, KB130, KB46)	12/00
KB139	phsp17*mut139-gus	581.F: 5´-ggaaacctcgaggaccatgattacg-3´ 624.R: 5´-gttatgcgtcgaccggccgtacttctgaaaatctc-3´ (THtH**T**HT 50x TATA box) (HindIII-Sal, KB131, KB46)	12/00
KB140	phsp17*mut140-gus	581.F: 5´-ggaaacctcgaggaccatgattacg-3´ 624.R: 5´-gttatgcgtcgaccggccgtacttctgaaaatctc-3´ (TH**T**H**T**HT 50x TATA box) (HindIII-Sal, KB132, KB46)	12/00
KB142	phsp17*mut142-gus	703.F: 5´-agcttggatccgtcgaagaagtccagaattcggtttctgaaagtttcag-3´ 582.R: 5´-ccttatatagaggaagggtcttgcg-3´ (TH **3x** tHtHT 50x TATA box) (BamHI-Sal, KB74)	01/01
KB143	phsp17*mut143-gus	704.F: 5´-agcttggatccgtcgaagaagtccagaatgtttctgaaactag tttcagaaaattctag-3´ 582.R: 5´-ccttatatagaggaagggtcttgcg-3´ (THtH **3x** tHT 50x TATA box) (BamHI-Sal, KB74)	01/01
KB144	phsp17*mut144-gus	705.F: 5´-agcttggatccgtcgaagaagtccagaatgttctagaaac tagtttcagaaaattctag-3´ 582.R: 5´-ccttatatagaggaagggtcttgcg-3´ (TH**T**H **3x** tHT 50x TATA box) (BamHI-Sal, KB138, KB74)	01/01
KB145	phsp17*mut145-gus	706.F: 5´-agcttggatccgtcgaagaagtccagaatgtttcatat ggttctagaaaattctag-3´ 582.R: 5´-ccttatatagaggaagggtcttgcg-3´ (THt**hT**HT 50x TATA box) (BamHI-Sal, KB139, KB74)	01/01
KB209	phsp17*mut209-gus	820.F: 5´-aagcttggatcctgcagaatagtccagaatg-3´ 285.R: 5´-ttcgcgatccagactgaatgcc-3´ (**H**TH**t**h**T**HT 50x TATA) (BamHI-Sal, KB145, KB74)	07/01
KB210	phsp17*mut210-gus	820.F: 5´-aagcttggatcctgcagaatagtccagaatg-3´ 285.R: 5´-ttcgcgatccagactgaatgcc-3´ (**H**TH**t**h**t**HT 50x TATA box) (BamHI-Sal, KB77, KB74)	07/01
KB211	phsp17*mut211-gus	820.F: 5´-aagcttggatcctgcagaatagtccagaatg-3´ 285.R: 5´-ttcgcgatccagactgaatgcc-3´ (**H**TH**T**H**T**HT 50x TATA box) (BamHI-Sal, KB140, KB74)	07/01
KB212	phsp17*mut212-gus	820.F: 5´-aagcttggatcctgcagaatagtccagaatg-3´ 285.R: 5´-ttcgcgatccagactgaatgcc-3´ (**H**THtHtHT 50x TATA box) (BamHI-Sal, KB74)	07/01
KB213	phsp17*mut213-gus	821.F: 5´-aagcttggatccgtcgaagaatattagaatg-3´ 285.R: 5´-ttcgcgatccagactgaatgcc-3´ (**t**HtHtHT 50x TATA box) (BamHI-Sal, KB74)	07/01
KB214	phsp17*mut214-gus	821.F: 5´-aagcttggatccgtcgaagaatattagaatg-3´ 285.R: 5´-ttcgcgatccagactgaatgcc-3´ (**t**H**T**H**T**HT 50x TATA box) (BamHI-Sal, KB140, KB74)	07/01
KB215	phsp17*mut215-gus	821.F: 5´-aagcttggatccgtcgaagaatattagaatg-3´ 285.R: 5´-ttcgcgatccagactgaatgcc-3´ (**t**H**T**HtHT 50x TATA box) (BamHI-Sal, KB138, KB74)	07/01

KB257	phsp17*mut257-gus	931.F: 5´-aatgttctagaaagctagaaaattctagttttg-3´ 285.R: 5´-ttcgcgatccagactgaatgcc-3´ (HTH 3x HT 50x TATA box) (XbaI-NcoI, KB215)	07/01
KB258	phsp17*mut258-gus	932.F: 5´-aatgttctagaaagtttaaacagaaaattctag-3´ 285.R: 5´-ttcgcgatccagactgaatgcc-3´ (HTH 8x HT 50x TATA box) (XbaI-NcoI, KB215)	07/01
KB259	phsp17*mut259-gus	933.F: 5´-aatgttctagaaagtttcccgggagaaaattctag-3´ 285.R: 5´-ttcgcgatccagactgaatgcc-3´ (HTH 10x HT 50x TATA box) (XbaI-NcoI, KB215)	07/01

Table 7. Reporter constructs containing promoters from Arabidopsis sHsp genes with ß-glucoronidase as reporter.

Lab name	Name	Primers (HSE configuration of promoter; x, any nucleotide) (cloning enzymes, template, cloning vector)	Date
beta-glucoronidase as reporter			
KB187	pBT2-Athsp17.8CI-P/L-gus	753.F: 5´-acaacggatcctgtggattagccaaggtatacacc-3´ 754.R: 5´-acattctcgagttgatttcgaaagcgaaagag-3´ (ThT 160x TH 6x HTHTHTH 4x ThT 1x htHtH 9x H**TH**HT 10 TATA box) (BamHI-XhoI, At-gDNA, pBT2-gus)	04/01
KB193	pBT2-Athsp25.3P-P/L-gus	759.F: 5´-atctggatcctagcacacataatggtgacttggtgag-3´ 760.R: 5´-tttgtctcgagtatgagccaaaaatatcgc-3´ (ThThT 69x ThT 90x HTHtHtH 49x THThThTHT 11x HT 14x TATA box) (BamHI-XhoI, At-gDNA, pBT2-gus)	04/01
KB198	pBT2-Athsp17.4CI-P/L-gus	782.F: 5´-tgagaggatcctctagtcttacagggacc-3´ 783.R: 5´-cgatctcgagcgtttcgcttactctgtttgctctg-3´ (THtHT 142x HTHtHTH 4x TH 12x HtHtHTHtHT 33x TATA box) (BamHI-XhoI, At-gDNA, pBT2-gus)	05/01
KB205	pBT2-hsp17.6 CII-P/L-gus	831.F: 5´-attaggatcctgaacttctgatcttgagg-3´ 832.R: 5´-ttgtctcgagtttgctttcctgattctttg-3´ (HtH 94x HtHTHtH 27x ThTHT 11x TATA box) (BamHI-XhoI, At-gDNA, pBT2gus)	07/01
KB207	pBT2-Athsp22 ER-P/L-gus	835.F: 5´-aagaggatccaaactctaaaatgttgtcagaaac-3´ 836.R: 5´-ggttctcgagttggatttattgtagaagg -3´ (ThT 121x THTHtH 71x HT 9x ThThtHTHThT 9x TATA box) (BamHI-XhoI, At-gDNA, pBT2gus)	07/01
KB208	pBT2-Athsp 23.6 M-P/L-gus	837.F: 5´-atcggatcctcaacaccaatatatatttgcc-3´ 838.R: 5´-atttctcgagaaacaggaagctttgttgag-3´ (HT 27x HT 54x ThTH 125x THT 2x HT 1x ThTHT 38x TATA box) (BamHI-XhoI, At-gDNA, pBT2gus)	07/01
Reporter constructs containing promoter fragments of At-sHsp gene promoters. PCR products amplified from corresponding templates were cloned via BamHI-PstI into phsp17*THtHtHT_PstI_50nt_TATAbox-gus			
KB195	pBT2-cluster2 Athsp17.4CI-gus	784.F: 5´-ctgaggatcctggtagcgacactcttgaaagacacg-3´ 785.R: 5´-cgatgtcgacaaatgatggtagagaagaggataagag-3´ (HtHtHTHtHT 50x TATA box) (BamHI-SalI/XhoI[1,3], At gDNA, pBT2-gus)	05/01
KB243	pBT2-cluster1 Athsp17.4CI-gus	918.F: 5´-caaccggatccatagaagcttcttgaagcc-3´ 919.R: 5´-tttctctgcagcatgcctttcaagagtgtcg-3´ (HTHtHTH 4x TH 50x TATA box) template: pBT2-Athsp17.4CI-P/L-gus	06/01
KB244	pBT2-cluster1 Athsp17.8CI-gus	920.F: 5´-cttgaggatccaaggcatctactagtagac-3´ 921.R: 5´-aaaagctgcagaaacagaatccagaaaactc-3´ (HT**HT**HT 50x TATA box) template: pBT2-Athsp17.8CI-P/L-gus	06/01
KB245	pBT2-cluster2	922.F: 5´-ttctggggatccgttttccaatcttttcatcg-3´ 923.R: 5´-aatttctgcagcccaaacttctttaaacttc-3´	06/01

	Athsp17.8CI-gus	(ThT 1x htHtH 50x TATA box) template: pBT2-Athsp17.8CI-P/L-gus	
KB246	pBT2-cluster3 Athsp17.8CI-gus	924.F: 5´-gaagtggatccgccgtaaattctagaagc-3´ 925.R: 5´-atttactgcagtgaagaagaaatctccagagc-3´ (HTHTHTH 50x TATA box) template: pBT2-Athsp17.8CI-P/L-gus	06/01

[1] Isoschizomers were used for cloning

[3] cases where different restriction sites were used for cloning

Table 8. pRT based vectors containing different house-keeping gene promoters.

Lab name	Name	Primers (cloning enzymes, template, cloning vector). See the sequences below for details about putative HSE motifs.	Date
Following four constructs were created by triple ligation of HindIII-XhoI cut PCR products with HindIII cut pRT101 and HindIII-XhoI cut CaMV poly A region.			
KB269	pRT101MCS-nos-P/L	1107.F: 5´-gccgcaagctttcagggcgcaaggg-3´ 1108.R: 5´-cctcgactcgagtcgagatctggattg-3´	03/02
KB270	pRT101MCS-Atact2-P/L	1109.F: 5´-caactaagcttatgcatgcaagagtcagcatatg-3´ 1110.R: 5´-ctttctctcgaggtcttcttccttgttcttctc-3´	03/02
KB309	pRT101MCS-Athsc70.1-P/L	1166.F: 5´-caaataagctttgaaatagaagaaaaagcc-3´ 1167.R: 5´-tttatctcgagatttggaaactacaaggg-3´	06/02
KB310	pRT101MCS-Athsp70-P/L	1168.F: 5´-gagacaagcttaaagcaatcgagttaaaac-3´ 1169.R: 5´-aaaagctcgagtaattgaatgaaattggaag-3´	06/02
Subcloning of Myc-Hsp17.6A-CI as reporter from pRT-myc-hsp17.6A-CI into above four constructs, via XhoI/XbaI.			
KB273	pRT-nos-P/L-myc-hsp17.6ACI	cloning vector: pRT-nos-P/L	03/02
KB277	pRT-Atact2-P/L-myc-hsp17.6ACI	cloning vector: pRT-Atact2-P/L	03/02
KB312	pRT-Athsc70.1-P/L-myc-hsp 17.6ACI	cloning vector: pRT-Athsc70.1 -P/L	06/02
KB314	pRT-Athsp70-P/L-myc-hsp17.6ACI	cloning vector: pRT-Athsp70-P/L	06/02
KB331	pRT-AthsfC1-P/L-myc-hsp17.6ACI	1232.F: 5´-ctagtcagctgttttaagttaaaatctgaatac-3´ 1234.R: 5´-tctctctcgagctctgttttccttatgg-3´ (PvuII-HincII/XhoI, At-gDNA, pRT-myc-hsp17.6ACI)	08/02
KB332	pRT-Atdnaj-P/L-myc-hsp17.6ACI	1233.F: 5´-attcagtcgacttttcttctatttgaatg-3´ 1235.R: 5´-cgttgctcgaggaaacgttttcgagttttg-3´ (HincII/XhoI, At-gDNA, pRT-myc-hsp17.6ACI)	08/02
Gfp as reporter			
KB323	pRT-Athsp70-P/L-gfp	NcoI-XbaI, pBT-Athsp17.8-P/L-gfp, pRT-Athsp70	08/02
Ds-red as reporter			
KB329	pRT-Athsc70.1-P/L-dsred	SalI-XbaI, pHahsp17G4-dsred, pRT-Athsc70.1-P/L-mychsp17.6ACI	08/02

Table 9. Sequence and putative HSEs in different promoters used during this study.

No	Promoter-leader fragment	Putative HSE configuration (underlined; x, any nucleotide) of promoter with **TATA** box and start codon (ATG) (gene accession number, reference); sequence
25	CaMV35S promoter	HT 6x HTHThT 91x TH 42x HTHtHT 4x ThtHtHT 2x htH 2x HT 14x HT 2x **TATA box** aagcttgcatgcctgcaggtcaacatggtggagcacgacactctcgtctactccaagaatatcaaagatacagtctcagaagaccagagggctattgagactttcaacaaagggtaatatcgggaaacctcctcggattccattgcccagctatctgtcacttcatcgaaaggacagtagaaaaggaagatggcttctacaaatgccatcattgcgataaaggaaaggctatcgttcaagaatgcctctaccgacagtggtcccaaagatggacccccacccacgaggaacatcgtggaaaaaagaagacgttccaaccacgtcttcaaagcaagtggattgatgtgatatctccactgacgtaagggatgacgcacaatcccactatccttcgcaagaccttcctctcTATATaaggaagttcatttcatttggagaggacctcgagaattcgagctcggtacccggccgcgagaaagaggggATG
26	T-DNA Nos promoter	98x TH 94x HT 11x THtH 55x THtH 104x TH 11x HT 79x HT **TATA box** aagctttcagggcgcaagggctgctaaaggaagcggaacacgtagaaagccagtccgcagaaacggtgctgaccccggatgaatgtcagctactgggctatctggacaagggaaaacgcaagcgcaaagagaaagcaggtagcttgcagtgggcttacatggcgatagctagactgggcggttttatggacagcaagcgaaccggaattgccagctggggcgcctctggtaaggttgggaagccctgcaaagtaaactggatggctttcttgccgccaaggatctgatggcgcaggggatcaagatcatgagcggagaattaagggagtcacgttatgacccccgccgatgacgcgggacaagccgttttacgttggaactgacagaaccgcaacgttgaaggagccactcagccgcgggttctggagtttaatgagctaagcacatacgtcagaaaccattattgcgcgttcaaaagtcgcctaaggtcactatcagctagcaaatatttcttgtcaaaaatgctccactgacgttccaTAAATtcccctcggtatccaattagagtctcatattcactctcaatccagatctcgactcgagtcgaggtggccacATG

27	Atactin2 promoter	6x THtH 15x HtH 30x HTTH 48x TH 116x TH 31x HT 11x TH 5x TH 191x **TATA box**, (At3g18780) aagcttatgcatgcaagagtcagcatatgtataattgattcagaatc gtttgacgagttcggatgtagtagtagccattatttaatgtacata ctaatcgtgaatagtgatatgatgaaacattgtatcttattgtataa atatccataaacacatcatgaaagacactttctttcacggtctgaat taattatgatacaattctaatagaaaacgaattaaattacgttgaat tgtatgaaatctaattgaacaagccaaccacgacgacgactaacgtt gcctggattgactcggtttaagttaaccactaaaaaaacggagctgt catgtaacacgcggatcgagcaggtcacagtcatgaagccatcaaag caaaagaactaatccaagggctgagatgattaattagtttaaaaatt agttaacacgagggaaaaggctgtctgacagccaggtcacgttatct ttacctgtggtcgaaatgattcgtgtctgtcgattttaattatttt ttgaaaggccgaaaataaagttgtaagagataaacccgcc**TATATA**a attcatatattttcctctccgctttgaattgtctcgttgtcctcctc actttcatcagccgttttgaatctccggcgacttgacagagaagaac aaggaagaagacctcgagtggccacATG
28	AthsfC1 promoter	30x HT 133x THtH 50x THtHTH 17x HT 13x ThTHHtHT 44x TH 167x TH 115x **TATA box** (At3g24520, Nover et al. 2001) cagctgttttaagttaaaatctgaatacacagcatgttccttctaac gtttttaacataattgtaaactaaagaaaaattataatataataaa ttaaattataccctctctattcattgtagaagatttgtttgtttcac caaatgtgttttttttttataagttgctctcactcttgagagttttg gagtattttgaagaggacgaggttcttttgtgaaaaagtgaaagaaa caaatcatgtcaagaattagaaagaaatcatgacaataatttttgtt tgtgcagaaattacaaatgaatcatagatatccttctaattcgggat cggaaaccagttgaaaaatcgatggactaagctttctatattgggac tagcttacccttagaattagtcaagcagcttaaatcatctatgactt aaaattataattaagaaaaaacaatgcctaaatatgcatatatttca aatgtatcacataacttgtgacataagaaaatataaacaaaacaaaa agggcaaaaaagacctgaaagcttagaggcacacctgcataggtccc acagttcactcgtgacaccgtaaaaggcaaaacacgaacccgccacg ttatcacaaaaagcaagccacgtcaatatagtctcactgtcaactac

		acttaacttactattttcacatctcattttcctatctt**TATATA**aac cctccaggctcctctttaatttctttaccaccaccaacaacaaacat ataaaccataaggaaaacagagctcgagtggccaccATG
29	Atdnaj promoter	46x HTH 58x TH 16x THtH 13x TH 118x HT 111x TH 2x HT 5x HT 281x TH 72x HT 10x **TATA box** (At4g36040) gtcgacttttcttctatttgaatgtttagcaaaagaaaaaataaaccgacttt ccttgattataacattgacaaccattagtaatctattataataagatacatttgaaaacatttatattacttgatcatttgtatttatcgtaatctcgtacaaacaccgttagttggtatcatgaaattctgtgagctaagaaaacaatttctcacgaatcaaatttgcagattaccattggctttttatatttccattcctatagtaagaaaataataaagcacataaatattaaaaaaaatgtttgtatggtggaggcaatctttaaatacagtagagacttttctcaatcttctaaaaatctatttctgtcttctcaatatccaacaaacaatacatggtccaaattacgtctccatacaccaatttatatttttataaataaaagaaaaaaaaatctactcgtaataagaagattctaatttcgaatttccaccttaaaatactcttctgctaaagaaaattaaaaaaaaaaaaaaaatgataaataactaaaaaccaaaacgtgattagtatttctgtgcaaattaaatattggattcctttggcattaatatattttgtaagaatatgttaaaatgacaattacagccacagaacaatttggccactatgaataatatcttacgtactacattcttatctcttctgcaattatttccccaactggataagccttttttatctgactcagatcacaggatccgacccgactttacccgaccgtaacttaatcccctatatccgttttagtatgtaattaataaactattcaaaatcttaattaaccaattaatgactaatcgcctcttcgcc**TATAAAT**taaacccctccatacctttcttcttcaatctttccctctcctcctcgaacaaaaacaacaaacgcagagaaactcaaaactcgaaaacgtttcctcgagtggccaccATG
30	Athsc70 promoter	177x THtH 8x HtH 10x HT 43x HT 31x HT 95x HTH 50x HT x HT 18x **TATA box** (At5g02500) aagctttgaaatagaagaaaaagccttttcctttgacaacaacatataaaatcatactcccattaaaaagattttaatgtaaaattctgaatataagatatttttacaacaacaaccaaaaatatttatttttttcctttttacagcaacaagaaggaaaaacttttttttttgtcaagaaaag

		gggagattatgtaaacagataaaacagggaaaataactaaccgaactctcttaattaacatcttcaaataaggaaaattatgatccgcatatttaggaagatcaatgcattaaaacaacttgcacgtggaaagagagactatacgctccacacaagttgcactaatggtacctctcacaaaccaatcaaaatactgaataatgccaacgtgtacaaattagggttttacctcacaaccatcgaacattctcgaaacattttaaacagcctggcgccatagatctaaactctcatcgaccaatttttgaccgtccgatggaaactctagcctcaacccaaaactc**TATATA**aagaaatcttttccttcgttattgcttaccaaatacaaaccctagccgccttattcgtcttcttcgttctctagttttttcctcagtctctgttcttagatcccttgtagtttccaaatctcgagtggccaccATG
31	Athsp70 promoter	129x TH 78x HtHtHthtHtHtH 17x HtH 47x THtHTHT 82x HT 2x HT 108x THTH 54x THT 20x **TATA box** (At1g16030) aagcttaaagcaatcgagttaaaacgagaaattcagtttctttaattctcacagagaacctcagagatgaactatactcaccgagcatttctctgggtttcgtcggaacaagctgtagatgattaccacgatcgggaactcaataatctgaatatcaacatcaaaacaaaaaggctaaaattaactgaaaaatatccactagcaaccaggttatgaagaaagtttagtaccccataggagacgcagagtgagagttggatcagaaatgagatcgacagagtatttgttacggaccacgtgaaatccgaagatcagaaataacccagtaatcacataaacagcaaaagccccccaagttgatatcgtgatactaacggagatttctggattcttcttctccttcgcctctttcatggcttttcctttctcgtcttcgaaatcacagaacaagtgaagaaagaagacgtaaacaaaatattgaaaatcctccagaacttacactgggccttttattctatatacgggcctacaagtttataccatatgggctttaataggcccatttaattatcaagcggtcgccggagataaaatatatcccggtcggtgaatccagaactctcttgtacgtttgcgcgatttctccacctttccacaatcccctgggttgtgccacgacctttttctcgaaatgtctcgttcctctcgtcggattcg**TATATA**tagcttcttccatcgtttccgattcttcatcaaacagataaacaaacaaaagaaatcgaaaaacctcacttccaatttcattcaattactcgagtggccaccATG

8 Curriculum vitae

Kapil Bharti

Born:	09.07.1974	Jagadhri, India
Education:	1979-1990	Mukand Lal School, Yamuna Nagar, India
	1990-1992	Mukand Lal National College, Yamuna Nagar, India
	1993-1996	Bachelors of Science (Hons. School), Biophysics, Panjab University, Chandigarh
	1996-1998	Masters of Science, Biotechnology, M.S. University, Baroda
	1998-1999	Diploma Thesis “Functional interactions of tomato heat stress transcription factors (Hsfs) studied in transgenic yeast” under supervision of Prof. Dr. Lutz Nover
	1999-2003	Ph.D. work in the group of Prof. Dr. Lutz Nover
Honors:	1996	Gold medal in Bachelors of Science
	2000	“Best foreign student prize”, of German Academic Exchange Service (DAAD)

9 Own publications

9.1 Original publications

Bharti, K., Schmidt, E., Lyck, R., Heerklotz, D., Bublak, D., and Scharf, K.-D. (2000). Isolation and characterization of HsfA3, a new heat stress transcription factor of *Lycopersicon peruvianum* . *The Plant Journal* **22:** 355-365.

This publication describes the identification of a novel protein, HsfA3 and its characterization as a Hsf.

Bharti, K., von Koskull-Döring, P., Bharti, S., Kumar, P., Tintschl-Körbitzer, A., Treuter, E., and Nover, L. Tomato heat stress transcription factor HsfB1 represents a novel type of general transcription coactivator with a histone-like motif interacting with At-CBP/HAC1 (submitted).

This paper compiles the work explained in this thesis. HsfB1, a heat stress inducible member of class B Hsf family, is a novel type of transcriptional coactivator in plants. The coactivator function of HsfB1 depends on a single lysine residue in the –GRGK motif in its CTD. The –GRGK motif acts as a recruitment motif, and together with the other acidic activator is responsible for corecruitment of a histone acetyl transferase (HAT).

9.2 Reviews

Nover, L., **Bharti, K.**, Döring, P., Ganguli, A., and Scharf, K.-D. (2001). *Arabidopsis* and the Hsf world: How many heat stress transcription factors do we need? *Cell Stress Chap.* **6:** 177-189.

This report documents for the first time an extensive sequence analysis of the whole Hsf family of *Arabidopsis* and identification of a novel class C, based on sequence homology in the oligomerization domain.

Bharti, K. and Nover, L. (2001). Heat stress-induced signalling. In D. Scheel and C. Wasternack (eds.) *Plant Signal Transduction: Frontiers in Molecular Biology*. Oxford University Press.

This chapter is the most recent compilation of all the different signalling cascades affected during heat stress in plants. Special emphasis has been given to the role of Hsfs and Hsps in protection against heat stress induced apoptosis.

Hiermit erkläre ich an Eides Statt, dass ich die vorliegende Dissertation selbständig und nur unter Verwendung der angegebene Literatur und Hilfsmittel angefertigt habe.

Frankfurt am Main, den 04.09.2003