# Heat shock transcription factor 1 down-regulates spermatocyte-specific 70 kDa heat shock protein expression prior to the induction of apoptosis in mouse testes

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**Expression of constitutively active heat shock transcription factor 1 (HSF1) in mouse spermatocytes induces apoptosis and leads to male infertility. We report here that prior to the onset of massive apoptosis caused by expression of active HSF1 in spermatocytes a marked reduction in spermatocyte-specific Hsp70.2 mRNA and protein levels occurs. In addition, HSP70.2 protein relocalizes from a predominant cytoplasmic to a nuclear position in developing spermatocytes that express active HSF1. Later in the developmental stages, cells undergoing HSF1-induced apoptosis essentially lack the HSP70.2 protein. The down-regulation of *Hsp70.2* gene expression by HSF1 is paradoxical because HSF1 is the prototypical activator of HSP genes. Furthermore, HSF1-mediated repression neither involved a heat shock element (HSE)-like sequence adjacent to the *Hsp70.2* gene nor were *Hsp70.2* promoter sequences associated directly with HSF1. Interestingly, other spermatocyte- and spermatid-specific transcripts are also down-regulated in testes of transgenic mice expressing active HSF1, suggesting involvement of a putative HSF1-dependent block of development of spermatogenic cells. Importantly however, transcription of the *Hsp70.2* gene is down-regulated in testes of wild-type mice subjected to a hyperthermia that induces transient activation of HSF1, indicating that the spermatocyte-specific activity of HSF1 might misdirect a network of transcription factors required for proper regulation of *Hsp70.2*.**

## Introduction

One important response to a cellular stress is heat shock induction, which corresponds to the increased expression and accumulation of heat shock proteins (HSPs) (Lindquist & Craig 1988). HSPs function as molecular chaperones in regulation of cellular homeostasis and promoting survival. In mammalian cells there are five major HSP families, namely HSP100, HSP90, HSP70, HSP60 and small HSP (exemplified by HSP27). Proteins within each of these families have very high sequence homology yet differ by their pattern of expression and intracellular localization. HSP70 family members are the most abundant and most highly conserved (Hunt & Morimoto 1985). Besides inducible HSPs, other members of HSP families are expressed constitutively in the absence of stress, frequently in a tissue-specific and developmentally regulated manner (Sarge & Cullen 1997; Christians *et al.* 2003). Constitutive and inducible HSPs exhibit distinct physiological functions for cellular maintenance and adaptation to stress, respectively. Generally, HSPs prevent inappropriate protein aggregation and mediate transport of immature (or damaged) proteins to target subcellular compartments for final packaging, degradation or repair. They are also involved in antigen presentation, steroid receptor function, intracellular trafficking, nuclear receptor binding and apoptosis (Beere 2004; Binder *et al.* 2004; Pratt *et al.* 2004; Soti *et al.* 2005).

The mouse *Hsp70.2* gene and its rat counterpart *Hst70* are constitutively expressed specifically in spermatocytes and spermatids (Krawczyk *et al.* 1988; Zakeri *et al.* 1988). The genes encode proteins with 99.7% identity. Also organization of the promoters is very similar (87%

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identity in the most essential for regulation fragment: up to 380 bp upstream of the translation start codons). HSP70.2 protein expression appears in leptotene and is largely accumulated in pachytene primary spermatocytes (Dix *et al.* 1997). This is a molecular chaperone that is required for the assembly of a functional Cdc2/cyclin B1 complex in pachytene spermatocytes, which is necessary for completion of meiotic division (Zhu *et al.* 1997). The protein is also essential for desynapsis of synaptonemal complexes (Dix *et al.* 1997). In *Hsp70.2*(-/-) male mice synaptonemal complexes fail to disassemble, and primary spermatocytes arrest in meiosis I and undergo apoptosis, which leads to infertility (Dix *et al.* 1996). The other testis-specific *Hsp70* gene, termed *Hsc70t*, is activated in round spermatids. However, this gene is less abundant compared to *Hsp70.2*, and its regulation and functions remain largely elusive (Matsumoto & Fujimoto 1990).

The major regulators of heat shock genes are heat shock transcription factors (HSFs), which in vertebrates comprise a multimember protein family (Pirkkala *et al.* 2001). Most probably different HSFs have specialized functions in response to distinct stimuli under normal or stress conditions. HSF1 is the primary transcription factor responsible for the transcriptional response to different forms of cellular stress (e.g., heat shock) (Baler *et al.* 1993). Under non-stress conditions, HSF1 exists in an inactive form as a monomer, while in response to cellular stress HSF1 forms homotrimers. Trimers of HSF1 activate transcription of *Hsp* genes by binding to *cis*-acting DNA elements termed heat shock elements (HSEs), which are present in the promoters of heat shock genes (Fernandes *et al.* 1994). The HSE consensus is composed of several contiguous inverted repeats of the 5-base pair sequence nGAAn (Perisic *et al.* 1989). Although three repeats are thought to be required for heat regulation *in vivo*, the binding of HSF to HSEs is cooperative and some deviations from the canonical sequence are functionally tolerated (Santoro *et al.* 1998). The HSE-like element present in the promoter of *Hsp70.2/Hst70* gene consists of two such pentameric repeats. Because HSF1 can recognize variations in the HSE consensus sequence (Kroeger & Morimoto 1994), one cannot exclude potential functional interactions between HSF1 and the HSE-like element in the promoter of the *Hsp70.2/Hst70* gene, which we aimed to investigate in this study.

In male gonads HSF1 acts as a cell-survival factor only in somatic and premeiotic germ cells, but not in meiotic and postmeiotic ones (Izu *et al.* 2004). Primary spermatocytes are the most sensitive germ cells to heat stress (Yin *et al.* 1997). In the majority of mammals male gonads are located outside the main body cavity so as to provide a lower testicular temperature, which is required for correct spermatogenesis and fertility. Transcription of inducible *Hsp70* genes is permanently blocked in spermatocytes and expression of constitutively active HSF1 in such cells leads to caspase-3 dependent apoptosis and male infertility (Nakai *et al.* 2000; Widlak *et al.* 2003a; Vydra *et al.* 2006). Because this apoptotic phenotype is very similar to that exhibited by *Hsp70.2*(-/-) mice, we investigated here the effects of expression of constitutively active HSF1 on *Hsp70.2/Hst70* gene expression in spermatocytes. We found that prior to the onset of HSF1-induced apoptosis in spermatocytes a marked reduction in HSP70.2 protein and mRNA levels occurs. Germ cells in the final stages of apoptosis are essentially deficient in HSP70.2, which leads us to propose a cause-vs.-effect relationship between down-regulation of *Hsp70.2/Hst70* and the degeneration of seminiferous epithelium caused by active HSF1.

## Results

### Active HSF1 down-regulates Hsp70.2 expression

To directly assess the role of HSF1 in regulating the *Hsp70.2/Hst70* gene we have analyzed the levels of *Hsp70.2* gene transcripts (by RT-PCR) and HSP70.2 proteins (by Western blot) in testes of either wild-type mice or transgenic mice expressing a constitutively active form of HSF1 (the pHST-HSF1ΔRD transgene). Because the *Hsf1* transgene is under the control of the rat *Hst70* promoter (Widlak *et al.* 2003a; Vydra *et al.* 2006), both endogenous *Hsp70.2* and the active form of HSF1 are expressed simultaneously during postnatal development, starting from prophase of the first meiotic division (leptotene spermatocytes). It has been previously observed that expression of this constitutively active form of HSF1 in spermatocytes of transgenic mice induces massive apoptosis (Nakai *et al.* 2000; Widlak *et al.* 2003a; Vydra *et al.* 2006). Considering that, we have analyzed *Hsp70.2* expression in testes of 15-, 18- and 22-day-old animals before the seminiferous epithelium becomes destroyed. We have unexpectedly found that the levels of *Hsp70.2* transcripts (Fig. 1A) and HSP70.2 protein (Fig. 1B) were markedly reduced in the presence of co-expressed active HSF1. Densitometry quantification of Western-blots revealed at least 10-fold reduction of HSP70.2 protein level in 18- and 22-day-old HSF1 transgenic animals as compared to control ones. Pachytene spermatocytes and round spermatids, where HSP70.2 protein normally accumulates, are predominant spermatogenic cells in testes of wild-type 18- and 22-day-old mice, respectively. Importantly however, the *Hsp70.2* gene was not down-regulated in testes of 15-day-old mice (Fig. 1A) where


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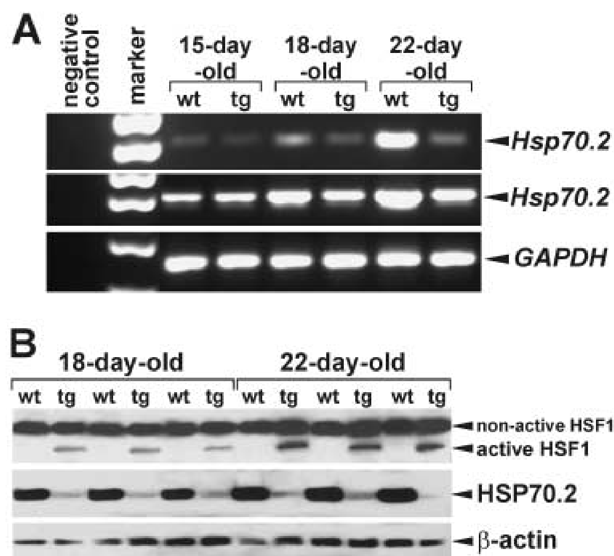


**Figure 1** Expression of constitutively active HSF1 results in the down-regulation of spermatocyte-specific *Hsp70.2* expression. (A) Expression of *Hsp70.2* mRNA in testes of 15-, 18- and 22-day-olds, either wild-type (wt) or expressing active HSF1 (tg), detected by RT-PCR. To better visualize differences in the amounts of the transcripts, reactions were performed either on 0.5 μg of total RNA template (20 cycles; top panel) or 1 μg of total RNA template (35 cycles; middle panel). Control reactions were performed with primers specific to *GAPDH* transcripts (bottom panel). (B) Western analysis of the level of HSP70.2 protein in testes of 18- and 22-day-old animals, either wild-type (wt) or transgenic animals expressing HSF1 (tg). The levels of the transgene product (active HSF1; top panel) and actin control (bottom panel) are also shown. Independent analyses for three animals in each case are shown.

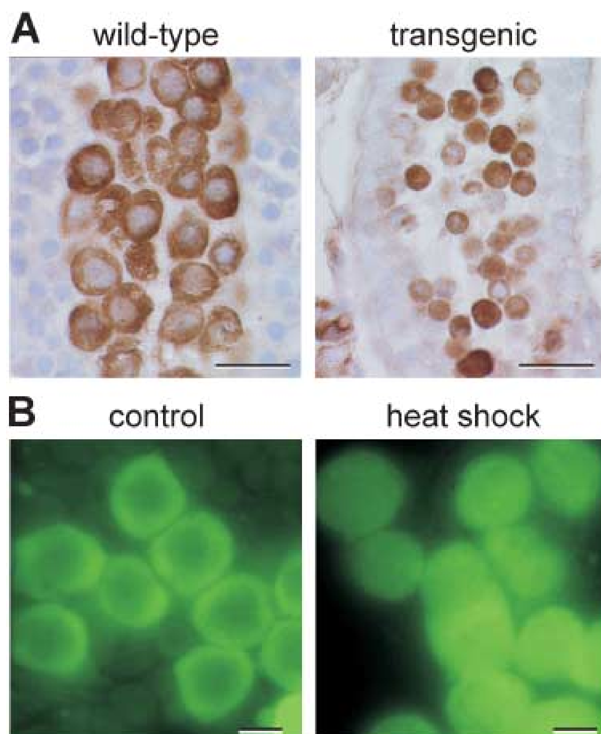


**Figure 2** Active HSF1 induces nuclear retention of HSP70.2 protein in spermatocytes. (A) Localization of HSP70.2 (immunostaining) in spermatocytes of 18-day-old mice testes either wild-type or expressing active HSF1 (transgenic). Scale bars: 25 μm. (B) Cellular localization of a EGFP-HST70 fusion protein in isolated spermatocytes from testes of mice not expressing transgenic HSF1, either at physiological temperature (control) or after 1 h of incubation at 42 °C (heat shock). Scale bars: 10 μm.

leptotene/zygotene spermatocytes are the predominant spermatogenic cells (Bellve *et al.* 1977).

### Expression of active HSF1 induces re-localization of HSP70.2 protein in developing spermatocytes

We have analyzed by immunohistochemistry the localization of HSP70.2 protein in mouse testes during postnatal development and observed that in the presence of constitutively active HSF1, endogenous, HSP70.2 had a different subcellular distribution (Fig. 2A). In seminiferous tubules of 18-day-old wild-type males, HSP70.2 protein was localized mostly in the cytoplasm (up to 95% of HSP70.2–positive cells). In such animals the majority of HSP70.2–positive cells are pachytene spermatocytes. In spermatocytes of HSF1 transgenic mice the HSP70.2 protein was detected in both the cytoplasm and the nucleus (up to 75% of HSP70.2–positive cells). Additionally, HSP70.2–positive cells of 18-day-old transgenic males were markedly smaller, and HSP70.2–negative cells could also be detected centrally within the seminiferous tubules of transgenic males (Fig. 2A). Consequently, the presence of co-expressed active HSF1 induced a subcellular redistribution of HSP70.2 protein and apparently affected the development of spermatocytes.

To study further the subcellular localization of the HSP70.2/HST70 proteins we have also expressed an enhanced green fluorescent protein (EGFP) : HST70 fusion protein for analyses. Testes of the pHST(1195/62)-EGFP:HST70 transgenic mice were subjected to a heat shock and the distribution of the EGFP fusion protein was analyzed by fluorescence microscopy. In spermatocytes from control tissue the EGFP : HST70 protein was detected mostly in the cytoplasm, while in

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spermatocytes from heat-shocked testes the protein was equally distributed between the cytoplasm and the nucleus (Fig. 2B). The EGFP protein itself, expressed in spermatocytes from *Hst70* promoter [in testes of pHST(896/62)-EGFP transgenic mice described elsewhere (Widlak *et al.* 2003b)], was evenly distributed in cells irrespective of the heat shock procedure (data not shown). This result suggests that nuclear localization of HSP70.2 in spermatocytes of HSF1 transgenic mice is related to stress conditions mimicked by the transgene rather than to deregulation of spermatocytes' development in testes of HSF1 transgenic mice.

### HSP70.2 protein is not present in the final stages of apoptosis induced by HSF1

Active HSF1 expressed in spermatocytes induces caspase-dependent apoptosis (Vydra *et al.* 2006). Here we aimed to determine the temporal and spatial relationships between HSF1-dependent down-regulation of *Hsp70.2* and the HSF1-dependent apoptosis in spermatocytes. Seminiferous tubules of adult animals expressing active HSF1 contain a large fraction of spermatocytes undergoing the terminal stages of apoptosis, which is evidenced by the TUNEL assay. The presence of HSP70.2 protein was analyzed in the same specimens. We have found that HSP70.2 essentially was not present in such TUNEL-positive cells (Fig. 3). Importantly, the same observation was made in histological preparations from the testes of animals at different ages (18- to 42-day-old, data not shown). These results may suggest a cause-vs.-effect relationship between down-regulation of *Hsp70.2/Hst70* and the degeneration of seminiferous epithelium caused by active HSF1.

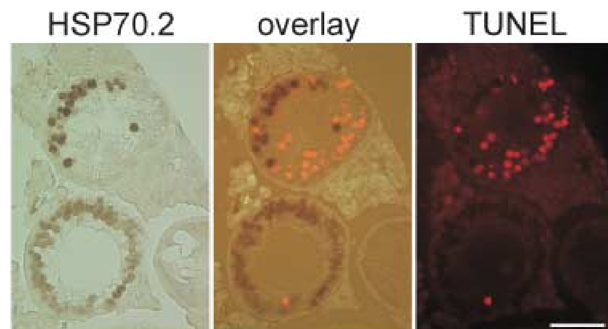


**Figure 3** Spermatogenic cells at final stages of apoptosis do not contain HSP70.2 protein. Co-localization of HSP70.2 (detected by immunohistochemistry) and apoptotic DNA breaks (detected by the TUNEL assay) in seminiferous tubules of adult pHST-HSF1ΔRD transgenic mice (6-week-old). Scale bar: 50 μm.

### The removal of HSE-like element from the Hsp70.2/Hst70 gene promoter does not affect CAT reporter gene expression

Knowing that expression of constitutively active HSF1 results in down-regulation of the *Hsp70.2* gene expression we aimed to identify a putative *cis*-acting element in the *Hsp70.2/Hst70* promoter that might be responsible for interactions with HSF1. The HSE-like element in this gene's promoter was the most obvious candidate. We have previously shown that efficient tissue-specific expression of the *CAT* reporter gene driven by the rat *Hst70* gene promoter requires sequences localized between the T2 transcription start site and box B (see Fig. 4A). A comparison of results obtained with different fragments of the *Hst70* gene promoter, either intact or partially replaced with the minimal thymidine kinase (*tk*) promoter, suggested a functional importance for the HSE-like element localized in the *Hst70* promoter upstream of the TATA box (position −181/−172) (Scieglinska *et al.* 2004). To directly study the role of this HSE-like element in spermatocyte-specific expression of a *CAT* reporter gene, we have generated new lines of transgenic mice carrying a *CAT* reporter gene under the control of the *Hst70* promoter. The HSE-like element (sequence t**TTC**ca**GAA**g) in parental pHST(368/62)-CAT6, which has the highest testis-specific activity (Widlak *et al.* 1995), was replaced by t**GGC**ca**TGG**g, a sequence that could be recognized by *Bal*I or *Nco*I restriction enzymes (Fig. 4A). We have obtained four lines of such pHST (368/62)ΔHSE-CAT6 transgenic mice that express the transgene. Surprisingly, the CAT activity in extracts from testes of mature pHST(368/62)ΔHSE-CAT6 and pHST(368/62)-CAT6 transgenic mice was similar (Fig. 4B).

To further confirm that the *Hst70* gene promoter activity is not altered by the HSE-like sequence, we mated either pHST(368/62)-CAT6 or pHST(368/62)ΔHSE-CAT6 transgenic males with transgenic females expressing constitutively active HSF1 in spermatocytes (both types of transgenes are expressed simultaneously during postnatal development). Double transgenic males 15 and 22 days old were selected for analysis; previous studies have shown that in testes of 15-day-old animals transgenes are expressed but only few "physiological" apoptotic cells are visible, while in testes of 22-day-old animals the expression of HSF1 reaches higher levels and clusters of apoptotic spermatocytes are detected, yet seminiferous epithelium is not completely destroyed (Vydra *et al.* 2006). Although in testes of double transgenic males the CAT activity was markedly lower than in corresponding CAT single transgenic animals, which was in accordance with the finding that HSF1 down-regulates *Hsp70.2/Hst70*,


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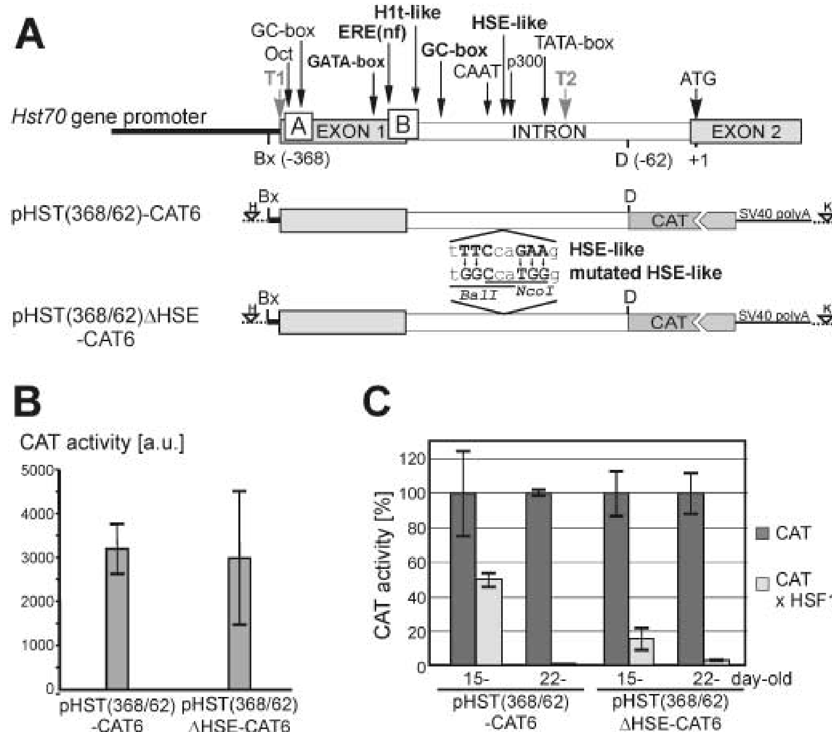


**Figure 4** The lack of a HSE-like element does not affect CAT reporter gene expression driven by the *Hst70* gene promoter. (A) Structure of the *Hst70* gene promoter and corresponding transgenes that were used to generate transgenic mice. Vertical arrows denote positions of the two main transcription start sites (T1 and T2) and putative transcription factor binding sites. Exons and conservative boxes A and B are shown as rectangles. *Bst*XI, *Dra*II, *Hind*III and *Kpn*I restriction sites are abbreviated as Bx, D, H, K, respectively; coordinates are related to A (+1) in the ATG codon. Open triangles indicate the polylinker restriction sites used to excise the hybrid genes for microinjection. (B) Comparison of the CAT activity in testes of pHST(368/62)-CAT6 and pHST(368/62)ΔHSE-CAT6 transgenic mice. Four different lines were analyzed for each transgene. Analyses were performed in triplicate for each line. The CAT activity (mean values and standard deviations) is expressed in arbitrary units defined as amounts of acetylated products formed per 1 h per milligram of extract protein. (C) Comparison of CAT activity in testes of 15- and 22-day-old pHST(368/62)-CAT6 or pHST(368/62)ΔHSE-CAT6 transgenic (CAT) and double transgenic (CAT × HSF1) mice, which express both CAT and constitutively active HSF1. The values are expressed as a percent of CAT activity observed in the testes of animals with the CAT transgene only. Shown are the mean values from three independent experiments. Error bars indicate standard deviations.

there was not a significant difference between these levels in the [pHST(368/62)-CAT6]x[pHST-HSF1ΔRD] and [pHST(368/62)ΔHSE-CAT6]x[pHST-HSF1ΔRD] animals (Fig. 4C). In other words, expression of constitutively active HSF1 prevents the stage-dependent activation of the *Hsp70.2/Hst70* gene irrespective of the presence of the HSE-like element. These results demonstrate directly that *Hst70* promoter sequences other than the HSE-like segment are those which respond to HSF1-mediated repression.

### The HSF1 is not associated with the Hsp70.2 gene promoter *in vivo*

The results obtained with the *CAT* reporter gene (Fig. 4) show that the HSE-like sequence does not affect *Hsp70.2/Hst70* gene expression. However, to determine further whether HSF1 interacts with this element and/or any other sequences within the promoter, we have assayed for HSF1 association *in vivo*. Testes of 15-day-old males, either wild-type or pHST-HSF1ΔRD transgenic, were selected for chromatin immunoprecipitation experiments (Fig. 5). The promoter of the *Hsp105* gene, which has been shown to be up-regulated in the testes of HSF1 transgenic mice (Vydra *et al.* 2006), was used as a positive control. Sequences specific for the *Hsp70.2* gene promoter were not immunoprecipitated with anti-HSF1 antibody (the PCR primers used would amplify sequences from −299 to +34, covering almost the entire promoter tested in transgenic studies). In marked contrast, the fragment of the *Hsp105* gene promoter containing a HSE was highly enriched in the

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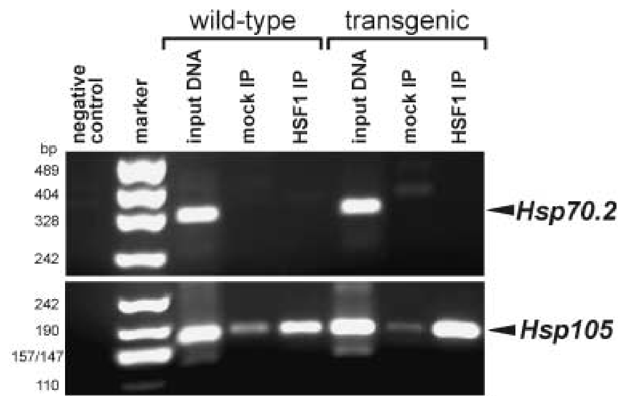


**Figure 5** HSF1 is not associated with the *Hsp70.2* gene promoter. *In vivo* interactions between HSF1 and the *Hsp70.2* or *Hsp105* gene promoters were tested by chromatin immunoprecipitation in testes of either wild-type or transgenic mice expressing active HSF1 (15-day-old). Shown are PCR products specific for *Hsp70.2* and *Hsp105* gene promoters immunoprecipitated with anti-HSF1 antibody (HSF1 IP) or preimmune controls (mock IP) (an excessive number of PCR cycles resulted in the appearance of weak signals in the mock IP for the *Hsp105*).

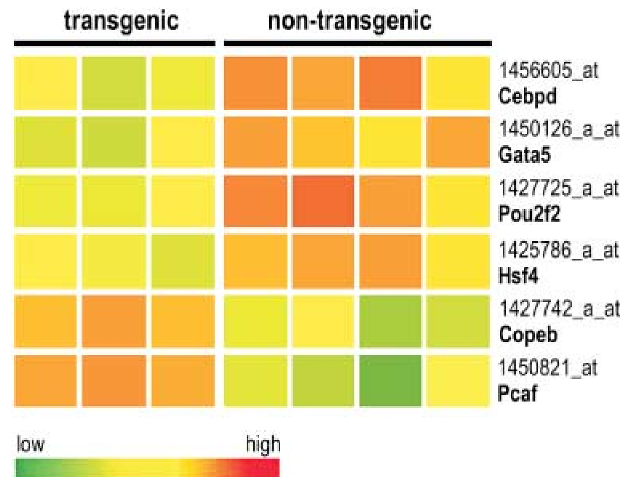


**Figure 6** Expression levels of selected transcription factors in testes of three pHST-HSF1ΔRD transgenic and four non-transgenic 15-day-old mice assessed using the Affymetrix DNA microarrays.

HSF1-immunoprecipitated fraction, in either wild-type or transgenic HSF1-expressing animals. These results conclusively demonstrate that HSF1 does not interact with the HSE-like element or any other sequences within the *Hst70* promoter.

### Expression of constitutively active HSF1 modulates the expression of several transcription factors potentially involved in regulation of the Hsp70.2/Hst70 promoter

We have analyzed global gene expression profiles in testes of 15-day-old males constitutively expressing active HSF1 and identified 713 genes whose expression was repetitively modulated in transgenic animals as compared to non-transgenic controls (Welch *t*-test non-corrected $P < 0.05$) (Vydra *et al*. 2006). Among those the most differentially regulated there were 85 genes known to be involved in different aspects of transcription regulation (61 up-regulated and 24 down-regulated). This included 67 genes encoding known or putative transcription factors; however, none of them contained HSE sequences. Three down-regulated genes (Fig. 6) encoded for transcription factors potentially binding to *cis*-elements present in the *Hsp70.2/Hst70* promoter (Fig. 4A): C/EBPδ (Cebpd) that could possibly bind to CAAT-boxes, GATA5 that could possibly bind to a GATA-box and an Oct -2 family member Pou2f2 that could possibly bind to the Oct sequence. Additionally, HSF4 that could hypothetically bind to the HSE-like sequence was also down-regulated in transgenic males; however, this element is not essential for gene regulation in the CAT assay (Fig. 4). Among the up-regulated transcription factors there was the Sp1 family member Copeb (core promoter element binding protein, also termed Klf6 or Zf9) that could possibly bind to several Sp1-binding sites (GC-boxes) present in the *Hsp70.2/Hst70* promoter, and p300/CBP-associated factor (Pcaf) (a putative p300-binding sequence is present in the analyzed promoter). However, the functional importance of these factors for the *Hsp70.2/Hst70* gene regulation, if any, remains to be verified experimentally.

### Active HSF1 down-regulates expression of different spermatocyte- and spermatid-specific transcripts

A hypothetical HSF1-dependent block of spermatocyte differentiation at early prophase of meiosis I could possibly contribute to down-regulation of HSP70.2 observed in testes of transgenic mice. Assuming this possibility one should expect that other developmental stage-specific genes were down-regulated in testes of HSF1 transgenic animals. To address this question we have analyzed expression patterns of several spermatocyte- and spermatid-specific transcripts in such testes. We have selected genes that become active at the same developmental stages as *Hsp70.2* (histone *H1t*, Drabent *et al*. 1993; pyruvate dehydrogenase E1 alpha 2, Iannello & Dahl 1992; lactate dehydrogenase C, Thomas *et al*. 1990), in late pachytene spermatocytes (proacrosin, Kashiwabara *et al*. 1990; phosphoglycerate kinase 2,


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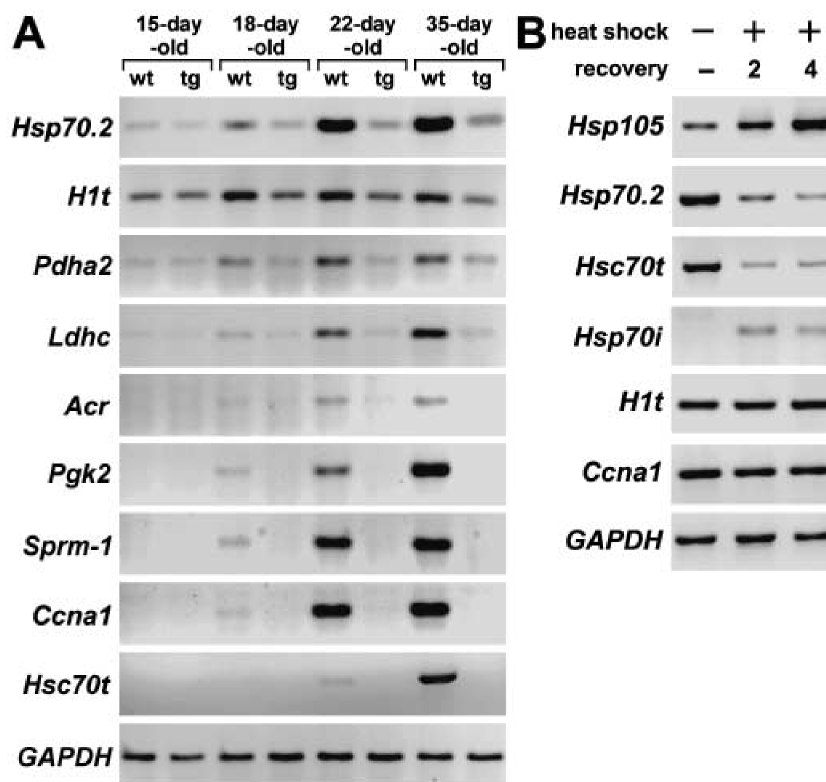


**Figure 7** Expression of genes specific to spermatogenic cells is down-regulated in the presence of active HSF1. (A) Expression of spermatocyte- and spermatid-specific genes in testes of pHST-HSF1ΔRD transgenic mice. RT-PCR reactions were performed on 1 μg of total RNA template, 20 PCR cycles. Transcripts were analyzed in testes of 15-, 18-, 22- and 35-day-old mice, either wild-type or pHST-HSF1ΔRD transgenic. Analyzed genes were histone *H1t* (*H1t*), pyruvate dehydrogenase E1 alpha 2 (*Pdha2*), lactate dehydrogenase C (*Ldhc*), proacrosin (*Acr*), phosphoglycerate kinase 2 (*Pgk2*), sperm 1 POU-domain transcription factor (*Sprm1*), cyclin A1 (*Ccna1*) and *Hsc70t*. Control reactions were performed with primers specific to *GAPDH* transcripts. (B) Expression of selected transcripts in testes of adult wild-type mice subjected to the heat shock. Gene activities were assessed by PCR-mediated (30 cycles) nuclear run-on assay in control animals and in heat shocked animals after 2 and 4 h recovery.

Robinson & Simon 1991; cyclin A1, Sweeney *et al.* 1996; sperm 1 POU-domain transcription factor, Andersen *et al.* 1993) or in spermatids (*Hsc70t*, Matsumoto & Fujimoto 1990). These transcripts were analyzed by semiquantitative RT-PCR in testes of 15-, 18-, 22- and 35-day-old mice, either from control or pHST-HSF1ΔRD transgenic (Fig. 7A). Spermatocytes at early prophase of meiosis I (leptotene, zygotene, early pachytene) and mid- to late pachytene spermatocytes were predominant spermatogenic cells in testes of normal 15- and 18-day-old mice, respectively. In testes of 22-day-old mice round spermatids began to accumulate (Bellve *et al.* 1977). The expression patterns of genes activated in leptotene/zygotene spermatocytes were similar to those of *Hsp70.2*– in 15-day-old mice comparable amounts of transcripts could be detected in either control or transgenic testes, while in testes of older animals markedly lower amounts of transcripts were detected in transgenic tissues (levels of such transcripts in older transgenic animals barely increased compared to 15-day-old mice). More importantly, transcripts of genes activated in late pachytene spermatocytes or spermatids could not be easily detected in testes of HSF1 transgenic mice at any stages. These data indicate that expression of constitutively active HSF1 results in down-regulation of different spermatocyte- and spermatid-specific genes and/or down-regulation of *Hsp70.2* and other testis-specific genes results from a HSF1-dependent blockade of development of spermatogenic cells in testes of transgenic animals.

To further clarify this question, we have performed additional experiments to assess the influence of active HSF1 upon transcription of the *Hsp70.2* gene. Using a PCR-based nuclear run-on assay we have analyzed transient changes in transcription of *Hsp70.2* and several other genes in testes of wild-type mice subjected to the heat shock (Fig. 7B). This short-term assay allowed studying of the effects of hyperthermia-mediated HSF1 activation, while the content of different spermatogenic cells should not be disturbed. We have selected four *Hsp* genes for such analyses: *Hsp105*, known to be up-regulated in testes of HSF1 transgenic animals (Vydra *et al.* 2006), two testis-specific *Hsp70* genes—*Hsp70.2* and *Hsc70t*—apparently down-regulated in testes of HSF1 transgenic animals (Fig. 7A), and *Hsp70i*, the major heat shock-induced *Hsps*. Additionally, transcriptional activities of histone H1t, cyclin A1 and *GAPDH* genes were analyzed. We have found that transcriptional activity of *Hsp105* gene increased, while activities of *Hsp70.2* and *Hsc70t* decreased in testes of males subjected to the heat shock, which was in agreement with previous data. We have also observed transcription of *Hsp70i* genes, putatively in somatic testicular cells, that confirmed activation of

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HSF1 by the heat shock. Surprisingly, however, transcriptional activities of histone H1t and cyclin A1 did not decrease after transient activation of endogenous HSF1. Noticeably, no significant heat shock-induced down-regulation was detected for any of selected genes if total cellular RNA pool was used for analyses (not shown). The data presented on Fig. 7B indicated that transcriptional activity of HSF1, but not HSF1-dependent blockade of germ cell differentiation, could be the major cause for down-regulation of testis-specific *Hsp70.2* and *Hsc70t* genes, at least, in testes of HSF1 transgenic mice.

## Discussion

We report here that active HSF1 down-regulates *Hsp70.2/Hst70* gene expression. This is quite surprising because HSF1 is generally responsible for the activation of heat shock genes and activation of HSF1 in somatic cells is a part of a cytoprotective system. It is also interesting that HSE-like sequence localized in the *Hsp70.2/Hst70* promoter does not influence *Hsp70.2/Hst70* gene expression in testes and that repression of the *Hsp70.2/Hst70* gene in the presence of active HSF1 is not due to direct interactions of HSF1 with the gene's promoter.

The *Hsp70.2/Hst70* gene promoter that drives expression of both endogenous HSP70.2 and transgenic, mutated HSF1 becomes active in leptotene spermatocytes yet *Hsp70.2* transcripts and protein accumulate largely in pachytene spermatocytes and later developmental stages (Dix *et al.* 1997). A putative HSF1-dependent block of spermatocyte development at early prophase of meiosis I could possibly contribute to down-regulation of *Hsp70.2* and other testis-specific developmentally regulated genes. Morphological differences visible in seminiferous tubules of 15- and 18-day-old wild-type and HSF1 transgenic mice (Fig. 2A) apparently support this possibility. Alternatively, active HSF1 could misdirect a network of transcription factors required for proper regulation of *Hsp70.2* and other spermatocyte- or spermatid-specific genes, which in turn could disturb spermatogenesis. To further elucidate this question the influence of transiently activated HSF1 upon expression of *Hsp70.2* gene was analyzed in testes of wild-type mice exposed to the heat shock (Fig. 7B). Obtained data suggest that transcriptional activity of HSF1 but not HSF1-dependent blockade of spermatocyte differentiation could be the major cause for down-regulation of *Hsp70.2* gene in testes of HSF1 transgenic mice. Interestingly, in testes of transgenic mice that express constitutively active HSF1 under the control of the actin promoter, whose testicular activity is detected mostly in spermatids but not spermatocytes (Ventela *et al.* 2000), Nakai and coworkers observed down-regulation of spermatid-specific genes, including *Hsc70t*, but not those specific for earlier developmental stages, including *Hsp70.2* (Nakai *et al.* 2000). However, hypothetical HSF1-dependent factor(s) involved in negative regulation of *Hsp70.2/Hst70* and other testis-specific genes remain to be identified. Apparently, elimination of spermatocytes by apoptosis cannot contribute significantly to down-regulation of HSP70.2 detected in 18-day-old pHST-HSF1ΔRD transgenic males because massive HSF1-dependent apoptosis and degeneration of seminiferous epithelium are observed in older animals (Widlak *et al.* 2003a; Vydra *et al.* 2006).

The HSP70.2 protein is functionally important in the nucleus where it takes part in desynapsis of synaptonemal complexes. However, the vast majority of cellular HSP70.2 has a cytoplasmic localization in normal spermatocytes (Dix *et al.* 1997). Here we observed that in spermatocytes expressing transcriptionally active HSF1, a large fraction of HSP70.2 became relocalized to the nucleus. Other HSP70 proteins are also known to exhibit a nuclear redistribution in heat-shocked cells (Milarski & Morimoto 1989). The HSC70 protein, which has 95% similarity to the HSP70.2 protein, possesses three regions functionally responsible for cytoplasm/nucleus trafficking: a nuclear localization signal involved in the binding of cargo proteins (NLS; residues 246–262) (Lamian *et al.* 1996), a nuclear localization-related signal (NLRS; residues 473−472) and a nuclear export signal (NES; residues 394–401) (Tsukahara & Maru 2004). Almost identical sequences are present in other HSP70 proteins, including the HSP70.2, that apparently regulate their cytoplasmic or nuclear localization. Interesting future questions are whether the HSP70.2 protein indeed shuttles between the nucleus and the cytoplasm, and whether HSF1-inducible gene products are involved in down-regulating nuclear export of the HSP70.2 protein. Also the importance of nuclear retention for functions of the HSP70.2 protein remains to be established.

Activation of HSF1 in spermatocytes does not activate inducible HSPs but leads instead to a massive caspase-3-dependent apoptosis and degeneration of seminiferous tubules in testes of 3-week-old and older mice (Widlak *et al.* 2003a; Vydra *et al.* 2006). Here we demonstrate that HSF1-induced apoptosis is preceded by a marked down-regulation in *Hsp70.2* gene expression, and that spermatocytes undergoing the terminal stages of apoptosis (DNA fragmentation) do not contain HSP70.2 protein. Given that this phenotype is very similar to that exhibited by *Hsp70.2*(-/-) knockout mice (Dix *et al.* 1996), the induction of apoptosis by HSF1 might be directly linked to the HSF1-mediated down-regulation


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of the *Hsp70.2* gene. The permanent heat shock induced by cryptorchidism results in both degeneration of germ cells and down-regulation of *Hsp70.2/Hst70* gene expression. However, it was not clear what phenomenon starts earlier and what the functional relationship is between them (Krawczyk *et al.* 1987: Guo *et al.* 2001; Kon & Endoh 2001; Barqawi *et al.* 2004; Chaki *et al.* 2005). Our data suggest that HSP70.2/HST70 protein may provide anti-apoptotic functions in spermatocytes and a cause-vs.-effect relationship between down-regulation of *Hsp70.2/Hst70* and the degeneration of seminiferous epithelium.

In addition, down-regulation of the *Hsp70.2/Hst70* promoter by HSF1 possibly contributed to the lack of Hsp70i-mediated protection against HSF1-induced degeneration of spermatogenic cells observed in transgenic mice expressing the *Hsp70i* gene under control of this promoter (Vydra *et al.* 2006).

## Experimental procedures

### RNA extraction and RT-PCR

Total RNA was prepared using the guanidine isothiocyanate method (Chomczynski & Sacchi 1987). DNA was removed from RNA samples by digestion with RNase-free DNase I (Scieglinska *et al.* 1997). After inactivation of the enzyme, control PCR assays were performed to assess for DNA contamination using primers for a glyceraldehyde-3-phosphate dehydrogenase gene (GAPDH; GenBank accession no. M32599; amplified a 299 bp fragment from position 340 to 639). RT-PCR assays were performed essentially according to Singer-Sam *et al.* (1990). At the reverse transcription step the reaction mixture (final volume 50 μL) contained RNA (0.5–1 μg), dNTPs (0.2 mM each), primers (0.4 μM each), RNase Inhibitor (40 U, Fermentas), MMLV reverse transcriptase (50 U, Gibco BRL) and Taq polymerase (2 U, Fermentas). The reverse transcription step was performed for 10 min at 50 °C and after inactivation of reverse transcriptase at 94 °C for 4 min, 20–35 cycles of the PCR were performed (94 °C for 30 s; 52 °C or 55 °C for 30 s; 72 °C for 45 s). Products were analyzed on 2% agarose gels stained with ethidium bromide. Primers used in the analysis specifically amplified transcripts of the *Hsp70.2* gene (GenBank accession no. M20567; 343 bp fragment from position 2505 to 2848), histone *H1t* (GenBank accession no. X72805; 231 bp fragment from position 2429 to 2659), *Pdha2* (GenBank accession no. NM 008811; 201 bp fragment from position 1555 to 1755), *Ldhc* (GenBank accession no. NM 013580; 218 bp fragment from position 1056 to 1273), *Pgk2* (GenBank accession no. NM 031190; 228 bp fragment from position 1256 to 1483), *Ccna1* (GenBank accession no. NM 007628; 242 bp fragment from position 1010 to 1251), *Sprm-1* (GenBank accession no. AK005948; 228 bp fragment from position 759 to 986), *Acr* (GenBank accession no. NM 013455; 235 bp fragment from position 1025 to 1259), *Hsc70t* (GenBank accession no. D85732; 511 bp fragment from position 38 to 549), *Hsp105* (GenBank accession no. D67016; 326 bp fragment from position 201 to 526) and inducible *Hsp70* (*Hspa1a*—GenBank accession no. NM 010479; 199 bp fragment from position 2098 to 2296; primers complementary also to *Hspa1b*—GenBank accession no. M35021; 208 bp fragment from position 2707 to 2914). Primer's sequences are available upon request.

### Protein extraction and Western blotting

Excised tissues were frozen on dry ice and then homogenized in five volumes of buffer containing 50 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.1% Nonidet P-40, 1 mM dithiothreitol (DTT), 1 mM PMSF, a protease inhibitor mixture (Complete™, Roche). After centrifugation at 15 300 ***g*** for 10 min at 4 °C, total protein content in supernatants was determined using a Protein Assay Kit (Bio-Rad). Protein species (80 μg) were separated by SDS-PAGE on 8% polyacrylamide gels and blotted to nitrocellulose (BA85 Schleicher and Schuell, Dassel, Germany). The filter was blocked for 60 min with 5% non-fat milk in TTBS (0.25 M Tris–HCl pH 7.5, 0.1% Tween-20, 0.15 M NaCl). Anti-HSP70.2 rabbit polyclonal antibody was generated (by Sigma) using the NH2-SKLYQGGPGGGG SSGGPT peptide corresponding to amino acids 611–628 in the HSP70.2 sequence (Rosario *et al.* 1992). The primary antibody was detected by horse-radish peroxidase-conjugated secondary antibody (Roche) and visualized by enhanced chemiluminescence (Pierce).

### Immunohistochemistry and TUNEL assay

Testes from 15-, 18-, 22-, 35- and 42-day-old wild-type and HSF1 transgenic mice were dissected and fixed overnight in 10% buffered formaldehyde at 4 °C, washed in PBS at 4 °C, dehydrated, paraffin-embedded and sectioned. Tissue sections (6 μm) incubated with anti-HSP70.2 antibody (described above) were stained with an ABC Vectastain kit (Vector Laboratories, Burlingame, CA) according to the manufacturer's guidelines. All steps were performed in a humid chamber at room temperature. DAB was used as a chromogen for visualization of immunohistochemical reactions and hematoxylin was used for counterstaining. For simultaneous analysis of HSP70.2 and DNA breaks on the same specimen, an antigen retrieval step in 0.01 M citrate buffer pH 6.0 was performed before the immunohistochemistry procedure. Immediately after staining for HSP70.2 the specimen sections were processed for TUNEL assays. Prepared sections were incubated with a mixture composed of terminal transferase (TdT) and labeled nucleotides (fluorescein dUTP) at 37 °C for 60 min according to the supplier's protocol (Roche). The samples were washed twice with PBS and incubated with DAKO™ fluorescent medium before analysis (counterstaining by hematoxylin was omitted in this case).

### Recombinant plasmids

For the construction of the pHST(1195/62)-EGFP:HST70 transgene, a *Bsp1407I* site was created by PCR at the 5′ end and a *Not*I site was created at the 3′ end of the *Hst70* coding sequence. This modified DNA was ligated through the *Bsp1407I* and *Not*I sites to the pHST(1195/62)-EGFP plasmid containing a *Kpn*I(-1195)-*Dra*II(-62) fragment of the *Hst70* promoter inserted in front of the

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promoterless *EGFP* gene cloned in the pEGFP-1 plasmid (GenBank accession no. U55761; Clontech Laboratories Inc., PaloAlto, CA). A spacer encoding 10 amino acids was introduced between the EGFP and the HST70 genes' sequences to eliminate possible interference between fused proteins.

The pHST(368/62)-CAT6 plasmid was constructed by inserting a *Bst*XI(-368)-*Dra*II(-62) fragment of the rat *Hst70* gene (GenBank accession no. X15705) in front of the promoterless *CAT* gene cloned in the plasmid pBLCAT6 (GenBank accession no. M80484), as described earlier (Widlak *et al.* 1995). The pHST(368/62)ΔHSE-CAT6 plasmid was obtained from the pHST(368/62)-CAT6 plasmid by PCR-mediated site-directed mutagenesis (Fig. 4A). The primers used to introduce *Bal*I and *Nco*I restriction sites in place HSE-like sequence (–180, –171) were sense, 5′-TGA GAG TGG CCA TGG GGC AGG-3′; anti-sense, 5′-CCT GCC CCA TGG CCA CTC TCA-3′. Coordinates of the restriction sites refer to the A[ +1] in the ATG codon downstream of the *Hst70* gene promoter.

## Generation of transgenic mice

Constructs used for microinjection were digested in order to remove vector sequences. To generate transgenic mice appropriate gel-purified restriction fragments were microinjected into the pronuclei of zygotes from FVB/N females by standard procedures. Transgenic founders were screened by PCR using genomic DNA isolated from tail biopsies and primers complementary to the *Hst70* promoter and *CAT* sequences or *EGFP* and *Hst70* coding sequence. PCR products amplified from DNA of pHST(368/62)ΔHSE-CAT6 founders were digested by *Nco*I or *Bal*I to confirm the existence of the mutated HSE-like sequence. pHST-HSFΔRD transgenic mice that carry the constitutively active mutant form of human HSF1 under control of the rat *Hst70* gene promoter were engineered as described elsewhere (Widlak *et al.* 2003a). All animal experiments were approved by the Committee of Ethics and Animal Experimentation.

## Heat shock of testes and monitoring of EGFP expression

Testes of EGFP-HST70 transgenic males were monitored for EGFP fluorescence under a microscope (ECLIPSE E800 Nicon) without any fixation. For heat shock, whole excised testes were immersed in KSOM medium and exposed to 42 °C for 1 h in a $CO_2$ incubator. Segments of the control or heat shocked seminiferous epithelium were dissected in M2 medium and carefully squashed between a microscope slide and coverslip (Kotaja *et al.* 2004). Pictures were recorded with an image analyzer equipped with a Hamamatsu Color Chilled 3 CCD camera.

## Analysis of CAT activity

Tissue homogenates were prepared according to Pothier *et al.* (1992). Briefly, excised tissues were frozen in liquid nitrogen and then homogenized in five volumes of a solution consisting of 0.15 M Tris–HCl pH 8.0, 60 mM KCl, 15 mM NaCl, 2 mM EDTA, 0.15 mM spermine, 1 mM DTT and 0.4 mM PMSF. Crude lysates were clarified by centrifugation (15 300 ***g***, 5 min at 4 °C), supernatants were heated at 60 °C for 10 min and centrifuged again (15 300 ***g***, 10 min at 4 °C). To perform CAT assays an aliquot of 100 μL of extract was added to 100 μL of a reaction mixture containing 0.25 M Tris–HCl, pH 7.8, with 1 mM EDTA, 4 mM acetyl-CoA (Sigma) and 6.25 μCi of [$^{14}$C] chloramphenicol [2.5 mCi/mL, ICN]. CAT assay mixtures contained 10–100 μg of protein and samples were incubated for 10–60 min at 37 °C. The acetylated forms of chloramphenicol were separated by thin-layer chromatography with a chloroform : methanol mixture in proportion 19 : 1. CAT activity was expressed as a percentage of acetylated products formed per 1 h per milligram of extract protein as previously described (Pothier *et al.* 1992). Total protein content in supernatants after heating of extracts was determined using a Protein Assay Kit (Bio-Rad).

## Chromosomal immunoprecipitation (ChIP) assay

ChIP assays were performed using a kit from Upstate Biotechnology Inc. (Lake Placid, NY) according to the supplied protocol. In brief, testes from 15-day-old wild-type and pHST-HSF1ΔRD transgenic mice were chopped into small pieces and fixed with 1% formaldehyde in PBS. After 15 min of incubation, the pelleted samples were disaggregated in PBS using a dounce homogenizer. The cell pellets were collected by centrifugation and then lyzed on ice in SDS-containing buffer. The samples were sonicated on ice in the presence of glass beads (212- to 330-μm diameter) to generate chromatin fragments with an average DNA length of 1000 bp and then cell lysates were clarified by centrifugation. The lysates were diluted and precleared by incubation with salmon sperm DNA saturated protein A-agarose beads, and then incubated with rabbit anti-HSF1 antibody (Stressgen) or mock-treated. Immunoprecipitation was carried out at 4 °C overnight, and immune complexes were collected with salmon sperm DNA saturated protein A-agarose beads. After extensive washing the immunoprecipitated complexes were eluted with 0.1 M $NaHCO_3$ and 1% SDS, and then protein-DNA cross-links were reversed by incubating at 65 °C for 4 h. DNA was purified using proteinase K digestion, phenol : chloroform extraction and ethanol precipitation. PCR was performed (40 cycles: 94 °C for 45 s, 55 °C for 30 s, 72 °C for 30 s) using primers specific for the murine *Hsp70.2* sequence between −299 and +34 (where +1 is A in the ATG codon): 5′-AAGCGCCTCACCCAACTA-3′ (forward) and 5′-GGTCGATGCCGATAGCCGGG-3′ (reverse). As a positive control, immunoprecipitated DNA was also amplified using primers specific for an HSE-containing, 175-nt fragment of the murine *Hsp105* promoter, nucleotides 907–1081 according to numbering in GenBank (accession no. AB005282): 5′-CTGTCACCATGGCAACTCAG-3′ (forward) and 5′-CCAATCGCTCAGCCTTATGT-3′ (reverse) using the same PCR conditions as for *Hsp70.2*, except for a 50 °C annealing temperature.

## Analysis of expression of transcription factors

A global gene expression profiling was performed on RNA isolated from testes of 15-day-old males, either pHST-HSF1ΔRD


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transgenic or non-transgenic, using the Affymetrix high-density mouse genome 430 A array as described in details elsewhere (Vydra *et al.* 2006). Putative transcription factor binding sites within the *Hsp70.2/Hst70* gene promoter were predicted thanks to the TESS system available at (<http://www.cbil.upenn.edu>).

### Whole body hyperthermia and PCR-based nuclear run-on assay

Adult FVB/N males (10- to 12-week-old, five for each experimental point) were anesthetized with i.p. injection of avertin, and then the lower half of the torso of each animal was submerged in a water bath at 42 °C for 30 min. Testes were collected from control animals and after 2 and 4 h of recovery after the heat shock. Testicular tubules were released by an incision in the tunica albuginea, then individual cells were isolated after mechanical dispersion in ice-cold low salt buffer supplemented with 0.5% Nonidet P-40 by a passage through an 18-gauge needle and nuclei were isolated as described earlier (Scieglinska *et al.* 2004). Nuclei were isolated from equal amounts of tissues (0.75 g in each case; total RNA was isolated from remaining material); completeness of cell lysis and quality of nuclei preparation were controlled under microscope. For additional standardization total protein contents were determined using a Protein Assay Kit (Bio-Rad). Nuclei were processed for PCR-based run-on assay as described in Rolfe *et al.* (1997). Briefly, nuclei suspended in 1 mL of the run-on buffer were incubated for 30 min at 30 °C with 0.5 mM each of rNTP. Transcription was terminated, RNA isolated, then subjected to RT-PCR as described above.

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## Supplementary Material

The following supplementary material is available for this article online:

**Figure S1** Low magnification comparison of HSP70.2 immunostaining in sections of testes from wild-type and HSF1 transgenic mice (18-day-old).

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