

## The GC-box is critical for high level expression of the testis-specific *Hsp70.2/Hst70* gene

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The *Hsp70.2/Hst70* gene, which belongs to the 70 kDa heat-shock protein (HSP) family, is expressed specifically in primary spermatocytes and spermatids. The regulatory elements required for a high level of testis-specific expression of the gene are placed between the two major transcription start sites T1 and T2 (approximately 350 and 115 bp upstream of the starting ATG codon). Here we have shown that sequences proximal to the exon1/intron splicing site in the 5' untranslated region of the *Hsp70.2/Hst70* gene, which include a highly conserved element called box B, are required for efficient expression of the chloramphenicol acetyltransferase reporter gene in testes of transgenic mice. However, in spite of the drastically reduced overall activity, the stage-specific expression pattern of the transgene was preserved after removal of these sequences. We have also shown that GC-box located downstream of the box B (approximately 210 bp upstream of the starting ATG codon) is indispensable for efficient expression of the *Hsp70.2/Hst70* gene promoter in spermatogenic cells. The GC-box specifically binds proteins present in nuclear extracts from testes (putatively Sp1-like factors). A change in the pattern of such GC-box-interacting factors corresponds to activation of the *Hsp70.2/Hst70* gene, confirming the importance of this regulatory element.

**Keywords:** regulation of gene expression, heat shock protein, spermatogenesis, GC-box, Sp1

### INTRODUCTION

Spermatozoa develop in testes from spermatogonial stem cells in a process involving a series of mitotic/meiotic divisions and differentiation events. Male germ-cell differentiation requires stage- and cell-specific regulation of gene expression that is achieved by unique chromatin remodeling, transcriptional control and expression of testis-specific genes or isoforms. One of the testis-specific genes is a 70 kDa heat shock protein gene, named *Hsp70.2* in mouse, *Hst70* in rat and *HSPA2* in human (Zakeri *et al.*, 1988; Krawczyk *et al.*, 1988a; Bonnycastle *et al.*, 1994). The rodent *Hsp70.2/Hst70* gene is expressed

almost exclusively in spermatocytes and spermatids (Krawczyk *et al.*, 1988b; Allen *et al.*, 1988). The gene codes for a molecular chaperone protein necessary for completion of meiotic division. 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 *Hsp70.2* and *Hst70* genes have an identical organization in their promoter regions. Transcription of both genes is initiated at two major start sites, T1 and T2, localized approx. 350 and 115 bp upstream of the starting ATG codon, respectively. Transcripts generated at the T1 site contain a

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**Abbreviations:** CAT, chloramphenicol acetyltransferase; DTT, dithiothreitol; FBS, fetal bovine serum; HSP, heat shock protein; *Hsp70*, 70 kDa multigene family of heat-shock or 'stress' genes; mut, mutated; Oct, octamer; PMSF, phenylmethylsulfonyl fluoride; Sp1, transcription factor named according to the original purification scheme that included Sephacryl and phosphocelulose columns; TBE buffer, Tris/borate/EDTA buffer; T1 and T2, transcription start site 1 and 2; tk, thymidine kinase.

239 bp-long intronic sequence that is subsequently spliced out (Ściegłińska *et al.*, 2001). The T2 site located in the intron is preceded by a canonical TATA box (diagram in Fig. 1). However, only the T1 site is functionally important in cells that actually produce the HSP70.2/HST70 protein. Activation of the T1 site is associated with the release of a putative repressor from the Oct sequence, localized directly downstream of the T1 site (Ściegłińska *et al.*, 2004).

Functional analysis of the *Hsp70.2/Hst70* gene promoter revealed that all regulatory elements required for a high level of testis-specific expression are located between the T1 and T2 transcription start sites (Widłak *et al.*, 1994; 1995; Ściegłińska *et al.*, 2001). Two regions of high homology between the *Hsp70.2* counterparts from different species, boxes A and B, are of particular interest. We showed previously that removal of the T1 site and proximal box A totally abolishes the activity of the *Hsp70.2/Hst70* promoter (Ściegłińska *et al.*, 2001). In this work we aimed to assess the role of sequences located in box B, placed around the exon1/intron splicing site, in the regulation of the *Hsp70.2/Hst70* gene expression.

## MATERIALS AND METHODS

**Recombinant plasmids.** The pHST(368/62)-CAT6 plasmid was constructed by inserting the *Bst*XI(-368)-*Dra*II(-62) fragment of the rat *Hst70* gene (GenBank accession no. X15705) in front of the pro-

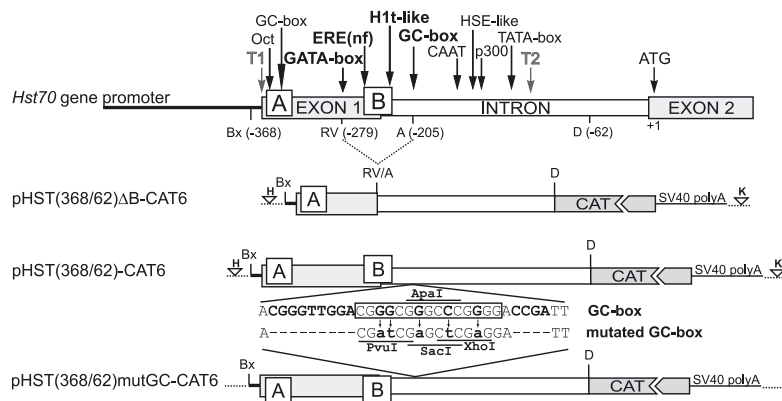
moterless *CAT* gene cloned in the pBLCAT6 plasmid (GenBank accession no. M80484), as described earlier (Widłak *et al.*, 1995). To obtain pHST(368/62) $\Delta$ B-CAT6 plasmid the *Eco*RV(-279)-*Apa*I(-205) fragment of the rat *Hst70* gene promoter was removed from the pHST(368/62)-CAT6 plasmid. The pHST(368/62)mutGC-CAT6 plasmid was obtained from pHST(368/62)-CAT6 plasmid by PCR-mediated site-directed mutagenesis. The primers used were: sense - 5'-ACG ATC GAG CTC GAG GAT TG; antisense - 5'-TCC TCG AGC TCG ATC GTC CA. The original GC-rich sequence was replaced by a sequence containing *Pvu*I, *Sac*I and *Xho*I restriction sites, which was confirmed by sequencing (Fig. 1). Coordinates of the restriction sites refer to the A(+1) in the starting ATG codon of the *Hst70* coding sequence.

**Generation of transgenic mice.** Transgenic mice were engineered as described in details elsewhere (Widłak *et al.*, 1995). Plasmids used for microinjection were digested with *Hind*III and *Kpn*I in order to remove vector sequences, and then appropriate 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. All experiments were approved by the Committee of Ethics and Animal Experimentation.

**Analysis of CAT activity.** Analysis of the chloramphenicol acetyltransferase (CAT) activity was performed as described previously (Ściegłińska *et al.*, 2001). CAT assay mixtures contained 10–150  $\mu$ g of protein and samples were incubated for 15 min to 5 h at 37°C. CAT activities were expressed in arbitrary units defined as the percentage of chloramphenicol converted into acetylated products during one hour in a reaction involving one milligram of protein extract (Pothier *et al.*, 1992). Total protein content was determined using a Protein Assay Kit (BioRad).

### Transfection of somatic cells.

Plasmid DNA was transiently transfected into human HCT116 cells with Dharmafect reagent (Dharmacon) according to the manufacturer's protocol. Briefly, cells were seeded at  $2 \times 10^4$  cells per 35 mm culture dish 24 h before transfection. Two micrograms of pHST-CAT plasmids and 4  $\mu$ l of Dharmafect in appropriate volume of medium were used for single transfection. Cells were incubated with trans-



**Figure 1. Structure of the *Hst70* gene promoter and transgenes used in analyses.**

Vertical arrows denote positions of the two main transcription start sites (T1 and T2) and putative *cis*-regulatory elements. Exons and regions of perfect homology between rat, mouse and human counterparts (boxes A and B) are shown as rectangles. *Bst*XI, *Eco*RV, *Apa*I, *Dra*II, *Hind*III and *Kpn*I restriction sites are abbreviated as Bx, RV, A, D, H, K, respectively, and their coordinates are related to A(+1) in the starting ATG codon. The ligation between *Eco*RV and *Apa*I restriction sites (blunted with T4 DNA polymerase) is marked as RV/A. Open triangles indicate the polylinker restriction sites used to cut out the hybrid gene for microinjection. A boxed sequence represents the most likely location of the functional GC-box. The bolded bases in the GC-box were changed as indicated to obtain the mutated GC-box.

fection mixture for 4 h, then it was removed and replaced by medium McCoy containing 10% FBS. Cells were harvested 48 h after transfection for analyses of CAT activity.

**Electroporation of testis.** Electroporation was performed as described in details elsewhere (Widłak *et al.*, 2003). Fifty microliters of a solution containing 80 µg of the pHST-CAT plasmid and 10 µg of the control pEGFP-GL3 plasmid (the enhanced green fluorescent protein gene under control of the SV40 promoter used to monitor the electroporation efficiency) was injected directly into seminiferous tubules of each testis. Electric pulses were applied to each testis 8 times at 40 V for 50 ms. Testes were dissected and processed for the CAT activity assay 24 h after electroporation.

**Preparation of nuclear extracts.** Freshly dissected tissues were homogenized in ice-cold low salt buffer consisting of 10 mM KCl, 0.25 M saccharose, 1.5 mM MgCl<sub>2</sub>, 0.5 mM EGTA, 1 mM DTT, 0.5 mM PMSF, 10 mM Tris/HCl, pH 7.4, and a protease inhibitor mixture (Complete™, Roche), supplemented with 0.5% Nonidet P-40. Homogenates were filtered using gauze and nuclei were sedimented by centrifugation (5 min, 900×g at 4°C), washed in the low salt buffer then in the same buffer containing 100 mM KCl. Nuclear proteins were extracted by incubation for 30 min at 4°C in a high salt buffer consisting of 0.4 M NaCl, 10 mM Hepes/NaOH (pH 7.9), 1.5 mM MgCl<sub>2</sub>, 0.1 mM EGTA, 0.5 mM DTT, 5% glycerol and a protease inhibitor mixture (Complete™, Roche). Insoluble remnants of the nuclei were pelleted by centrifugation for 30 min at 16000×g at 4°C. The resulting supernatants were referred to as nuclear extracts. The protein concentration was assayed using the Protein Assay Kit (BioRad).

**Electrophoretic mobility-shift assay.** Complementary single stranded oligonucleotides were annealed in aqueous solutions to obtain double-stranded DNA probes. The sequences of the probes were as follows: Sp1-cons – 5'-GGT GCT CGC CCC GCC CCG ATC GAA TGA TTG; GC-box – 5'-GGT TGG ACG GGC GGG CCC GGG GAC CGA TTG; mutGC – 5'-CGC TGG ACG ATC GAG CTC GAG GAT TGG CTC. The DNA probes were labeled with [ $\gamma$ -<sup>32</sup>P]dATP using T4 polynucleotide kinase and purified from nondenaturing polyacrylamide gels. The probes (20 ng) were incubated with nuclear extracts (5 µg of protein) for 30 min at 4°C in a binding buffer consisting of 20 mM Tris/HCl (pH 7.6), 5 mM MgCl<sub>2</sub>, 1 mM EDTA, 1 mM DTT, 5% glycerol and 200 mM NaCl, in the presence of a ten-fold excess of non-radioactive poly(dI-dC) double-stranded alternating copolymer used as a non-homologous competitor, in a final volume of 20 µl. Nucleoprotein complexes were resolved by electrophoresis on

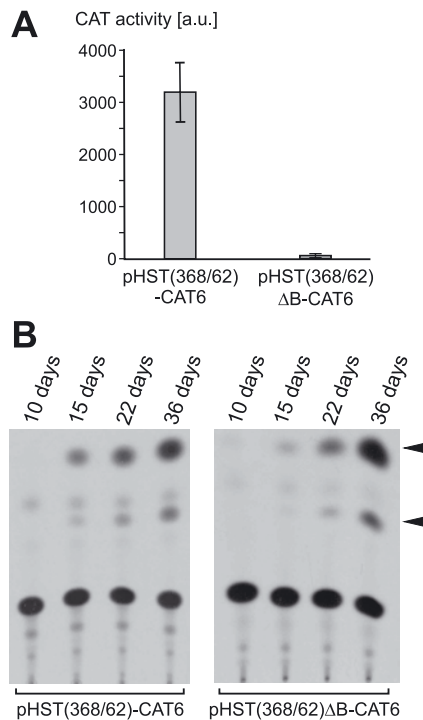
6% nondenaturing polyacrylamide gels in a 0.5×TBE running buffer. Gels were dried and autoradiographed.

## RESULTS AND DISCUSSION

We have aimed to establish the role of the highly conserved DNA sequences localized around the exon1/intron splicing site (box B) in the regulation of the *Hsp70.2/Hst70* gene expression. The 75 bp-long *EcoRV*(-279)-*ApaI*(-205) restriction fragment of the rat *Hst70* gene promoter was removed from the pHST(368/62)-CAT6 reporter transgene, which was previously shown to have the highest specific activity in testes of transgenic mice (Widłak *et al.*, 1995). The resulting pHST(368/62)ΔB-CAT6 construct (Fig. 1) was used to generate new lines of transgenic mice. The average CAT activity was calculated from four such transgenic lines (the transgene expression was completely absent in two other lines). The activity of CAT was assayed in testes of mature mice, carrying either the pHST(368/62)ΔB-CAT6 or the pHST(368/62)-CAT6 transgene. A comparison of the expression of the two transgenes revealed that removal of the *EcoRV*-*ApaI* fragment resulted in an about 50-fold reduction of CAT expression (Fig. 2A), proving the importance of the analyzed sequences for the activity of the *Hsp70.2/Hst70* gene promoter.

The expression of both pHST(368/62)ΔB-CAT6 and pHST(368/62)-CAT6 transgenes was analyzed also in the course of the postnatal development of mice. In spite of the drastically reduced overall activity, the stage-specific expression pattern of the transgene in pHST(368/62)ΔB-CAT6 mice was similar to that observed in pHST(368/62)-CAT6 mice (Fig. 2B). In both groups of animals CAT activity appeared in extracts from testes of 15-day-old mice (when pachytene spermatocytes were fully developed), increased markedly in extracts from testes of 22-day-old mice (when spermatids were formed) and reached the maximum in sexually mature animals.

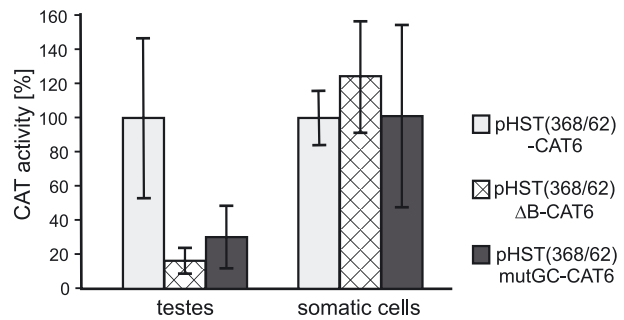
The *EcoRV*-*ApaI* restriction fragment of the rat *Hst70* gene promoter encompasses a sequence that has a strong similarity to the *cis*-acting element from the promoter of a testis-specific histone H1 gene termed TE1 (Grimes *et al.*, 1992). In addition, the 5' part of box B contains a sequence that differs by only one nucleotide from the estrogen responsive element (ERE) consensus sequence that was termed ERE-like. However, our previous experiments with the *Hst70* gene promoter showed that the ERE-like sequence did not respond to hormone stimulation in a transient transfection assay (Krawczyk *et al.*, 1993) and that the TE1-like element did not bind any specific testicular proteins *in vitro* (Ściegłińska *et al.*, 2004). Those data suggest that neither the TE1-



**Figure 2.** Removal of box B and its adjacent sequences from the *Hst70* gene promoter reduces the overall expression of the CAT reporter gene yet the stage-specific expression pattern of the transgene is preserved in testes of transgenic mice.

(A) Comparison of the CAT activity in testes of adult transgenic mice. Four different lines carrying either the pHST(368/62)-CAT6 or the pHST(368/62)ΔB-CAT6 transgene were analyzed in triplicate. The CAT activities (mean values and standard deviations) are expressed in arbitrary units. (B) The CAT activities were analyzed in extracts from testes of pHST(368/62)-CAT6 and pHST(368/62)ΔB-CAT6 transgenic mice of different age. 25 μg of protein and 15 min of reaction time or 50 μg of protein and 5 h of reaction time were used, respectively. Acetylated forms of chloramphenicol are marked with arrowheads.

like nor the ERE-like sequence present in box B has a functional importance. However, removal of the *EcoRV*–*ApaI* restriction fragment, in addition to the box B elimination, affects adjacent GATA- and GC-boxes (Fig. 1). GC-boxes, alternatively termed Sp1-binding sites, are particularly rich in G and C nucleotides and potentially bind the Sp1-like transcription factor (reviewed in Kaczynski *et al.*, 2003). Our earlier examination of the pHST(368/62)-CAT6 transgene, where the T2 transcription start site and proximal sequences including part of the same putative GC-box [*SmaI*(–203)–*DraII*(–62) fragment] were replaced with the minimal *tk* promoter (Ściegłińska *et al.*, 2004), showed reduction of the CAT activity similar to that observed for the pHST(368/62)ΔB-CAT6 transgene (Fig. 2). Both complementary experiments with deleted the –279/–205 and –203/–62 fragments of the *Hst70* promoter pointed to the potential importance of the GC-box within apparently the –214/–200



**Figure 3.** The GC-box present in *Hst70* promoter is required for its activity in spermatogenic cells.

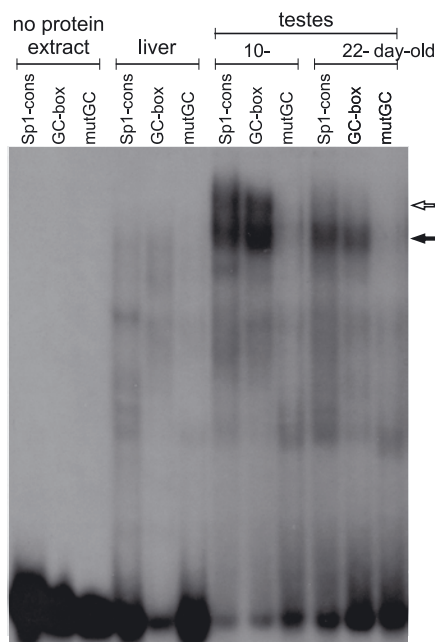
Comparison of the CAT activity in testes electroporated *in vivo* and HCT116 cells transfected *in vitro* (somatic cells) with either pHST(368/62)-CAT6, pHST(368/62)ΔB-CAT6 or pHST(368/62)mutGC-CAT6 plasmid. Values (means from 3–6 independent experiments) are expressed as percentage of CAT activity of the pHST(368/62)-CAT6 construct (taken as 100%).

sequence. Here we aimed to directly verify the potential importance of the GC-box located downstream of box B for regulation of the *Hsp70.2/Hst70* promoter, and analyzed the activity of the promoter with a mutated GC-box.

The whole GC-rich fragment (–223/–196) of the *Hst70* promoter was replaced in the initial pHST(368/62)-CAT6 construct by PCR-mediated mutagenesis and the resulting construct was termed pHST(368/62)mutGC-CAT6 (Fig. 1). Activities of the pHST(368/62)mutGC-CAT6, pHST(368/62)-CAT6 and pHST(368/62)ΔB-CAT6 constructs were analyzed in either testes electroporated *in vivo* or in somatic cells transfected *in vitro* (Fig. 3). Importantly, both the removal of box B with adjacent sequences and mutation of the GC-box reduced the *Hst70* promoter activity in spermatogenic cells to a similar extent (Fig. 3). On the other hand, neither mutation of the GC-box nor removal of box B affected the *Hst70* promoter activity in transiently transfected HCT116 somatic cells. These data indicate that the *trans*-acting regulatory elements that interact with the GC-box and/or the other sequences around exon1/intron splicing site are specific for spermatogenic cells.

We attempted to further analyze the presence of putative tissue-specific proteins that may interact with the GC-box from the *Hsp70.2/Hst70* promoter. The electrophoretic mobility-shift assay was used to detect complexes formed *in vitro* between nuclear proteins and radioactive DNA probes (Fig. 4). Three 30 bp-long radioactive probes were used: the consensus sequence for the human Sp1 binding site (Kadonaga *et al.*, 1987), the GC-box from the *Hsp70.2/Hst70* promoter, and the mutated GC-box (the same sequence as in the pHST(368/62)mutGC-CAT6 construct). Nuclear proteins were extracted from mouse liver (where the *Hsp70.2/Hst70* gene expression is





**Figure 4. Spermatogenic cells contain proteins that specifically interact with the GC-box from the *Hsp70.2/Hst70* gene promoter.**

Complexes were formed *in vitro* between DNA probes containing either Sp1 binding site consensus (Sp1-cons), GC-box from the *Hsp70.2/Hst70* gene (GC-box) or mutated GC-box (mutGC), and proteins from nuclear extracts from liver or testes of 10- and 22-day-old mice. Arrows indicate the positions of the major complexes.

permanently blocked), testes of 10-day-old mice (before the appearance of spermatocytes and corresponding activation of the *Hsp70.2/Hst70* gene), and testes of 22-day-old mice (that contain spermatocytes and round spermatids with the active *Hsp70.2/Hst70* gene). We observed that the GC-box from the *Hsp70.2/Hst70* promoter, but not the mutated sequence, efficiently formed complexes with nuclear proteins from testes but not those from liver. Similar complexes were formed between proteins from testes (but not those from liver) and the Sp1 binding site consensus (the specificity of the complexes was verified using the same non-radioactive sequences as homologous competitors; data not shown). This indicates that the GC-box from the *Hsp70.2/Hst70* promoter binds Sp1-like factors, and that the amounts of such proteins are higher in testes as compared to liver extracts. More interestingly, two different nucleoprotein complexes that contained the GC-box were detected with the testicular extracts from 10-day-old mice. However, the lower-electrophoretic-mobility complex could not be detected with testicular extracts from 22-day-old mice, in which the *Hsp70.2/Hst70* gene is activated. Taken together our observations suggest that certain testis-specific proteins that bind to the GC-box might function as transcriptional activators while others might be transcriptional repressors in younger males (when the

*Hsp70.2/Hst70* gene is inactive). A similar repressor function was previously reported for a protein that bound to the Oct sequence in juvenile testes and was released after the *Hsp70.2/Hst70* gene activation in spermatocytes (Ściegłińska *et al.*, 2004).

GC-boxes are among the most common *cis*-regulatory elements widely distributed in promoters, enhancers and locus control regions of numerous genes, either housekeeping or tissue-specific ones. They are also involved in regulation of several genes specifically expressed in differentiating mouse germ cells, such as *Ldh* family members (Bonny *et al.*, 1998; Thomas *et al.*, 2005), *Pgk2* (Gebara & McCarey, 1992), *Pdha2* (Iannello *et al.*, 1993; 1997) and histone *H1t* (Wilkerson *et al.*, 2002). GC-boxes are recognized by a group of transcription factors that contain a highly conserved DNA-binding domain consisting of three zinc fingers (Sp1-like/KLF family) (reviewed in Kaczynski *et al.*, 2003). Among the proteins that are likely to bind to the *Hsp70.2/Hst70* GC-box is the Sp1 transcription factor, which is abundant in testes (as well as in thymus and prostate), while its concentration is low in most somatic tissues (Saffer *et al.*, 1991; Robidoux *et al.*, 1992). Several Sp1 isoforms are expressed in spermatocytes, e.g. 110 kDa, 90 kDa and 60 kDa SP1 proteins (Persengiev *et al.*, 1996; Thomas *et al.*, 2005). However, the identity of the putative transcription factor interacting with the *Hsp70.2/Hst70* GC-box and its exact role in the *Hsp70.2/Hst70* gene regulation remain to be established.

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