Location of promoter elements necessary and sufficient to direct testis-specific expression of the *Hst70/Hsp70.2* gene

Dorota ŚCIEGLIŃSKA, Natallia VYDRA, Zdzisław KRAWCZYK and Wiesława WIDŁAK[1]

Department of Tumour Biology, Centre of Oncology, Maria Skłodowska-Curie Memorial Institute, Wybrzeże Armii Krajowej 15, 44-101 Gliwice, Poland

The rat *Hst70* gene and its mouse counterpart *Hsp70.2* are expressed specifically in pachytene primary spermatocytes and spermatids. Here we demonstrate that a 165 bp fragment of the *Hst70* gene promoter, containing the T1 transcription start site region, entire exon 1 and 42 bp 5′ region of the intron, is sufficient to drive testis-specific expression of the chloramphenicol acetyltransferase reporter gene in transgenic mice with the same developmentally regulated pattern as the endogenous *Hsp70.2* gene. We show further that high-level tissue-specific gene expression requires additional sequences localized upstream of the T2 transcription start site. Electrophoretic mobility-shift assay analysis revealed that only testes of juvenile rats, when *Hst70* gene expression is repressed, contain proteins that specifically bind to the Oct (octamer) sequence localized directly downstream of the T1 site.

Key words: gene regulation, heat shock protein, Oct sequence, spermatogenesis, transcription start sites, transgenic mice.

INTRODUCTION

Spermatogenesis is a process by which immature male germ cells go through a complex series of differentiation steps, involving mitotic and meiotic cell divisions, which finally lead to the formation of mature spermatozoa. Spermatogenic cells exhibit specific and complex patterns of gene expression. The rat *Hst70* gene and its mouse counterpart, the *Hsp70.2* gene, which are specifically expressed in pachytene spermatocytes, belong to the *Hsp70* multigene family of heat shock or 'stress' genes [1–4]. Both genes code for molecular chaperone proteins crucial for development of male germ cells. In *Hsp70.2*(−/−) male mice, synaptonemal complexes fail to disassemble, primary spermatocytes arrest in meiosis I and undergo apoptosis, which leads to infertility. On the other hand, *Hsp70.2*(−/−) female mice are fertile [5].

Molecular mechanisms responsible for regulation of the *Hst70* and the *Hsp70.2* transcription are not clear at present. In our previous work we determined transcription start sites and 5′ splicing patterns for the rat *Hst70* gene transcripts [6]. The 5′ region of this gene contains an intron that has a similar location in the mouse *Hsp70.2* gene [7]. Transcription of both genes is initiated at two major start sites: T1 and T2, localized approximately 350 bp and 115 bp upstream of the ATG codon respectively. Only the T2 site is preceded by a canonical TATA box. In addition to these two major start sites, several minor sites are localized between the T1 and T2 site. Transcription initiated at the T1 site generates transcripts containing the 239 bp intronic sequences that are subsequently spliced out. Transcription initiated at the T2 site generates non-spliced mRNA molecules. Consequently, the transcription of *Hst70*/*Hsp70.2* genes generates two main populations of mRNA molecules with similar sizes but differing in the 5′ untranslated region [6].

Alignment of promoter sequences from the rat *Hst70* gene, the mouse *Hsp70.2* and the human *HspA2* genes [3,8,9] reveals two short conserved regions, which have been termed box A and box B [6]. Box A is localized directly downstream of the T1 site (within the exon 1) and contains an octamer (Oct) consensus sequence for binding Oct family transcription factors and a putative Sp1 binding site. Box B (localized around the exon 1/intron splicing site) consists of sequences having strong similarity to the *cis*-acting element termed TE1 that resides in the promoter of a testis-specific histone H1 gene, which binds testis-specific factors [10]. In addition, the 5′ part of box B contains a non-functional ERE (oestrogen-responsive element)-like sequence that differs by only one nucleotide from the consensus sequence [11].

Functional analysis of the *Hst70* gene promoter, using a transgenic-mouse model, has provided evidence that the 306 bp DNA fragment (− 368/− 62 upstream of the ATG translation start codon) is indispensable for testis-specific expression of the *Hst70* gene [12]. Truncating 42 bp from its 5′ end (which removes the T1 transcription start site and box A) almost totally abolishes activity of this promoter [6]. Intronic sequences localized downstream of the T2 transcription start site are not required for the testis-specific expression of the *Hst70* gene [12]. Collectively it can be deduced from these results that DNA sequences essential for testis-specific expression of the *Hst70* gene should be localized within exon 1 and the intron, between T1 and T2 transcription start sites. Here we demonstrate that this is indeed the case, by showing that a 165 bp fragment of the *Hst70* gene promoter, containing only boxes A and B, is sufficient to activate the testis-specific transcription. Furthermore, we have found testis-specific developmental changes in nuclear proteins that bind specifically to the boxes A and B, and these may be candidates for regulating the *Hst70* gene.

Abbreviations used: CAT, chloramphenicol acetyltransferase; DTT, dithiothreitol; EMSA, electrophoretic mobility-shift assay; ERE, oestrogen-responsive element; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GTF, general transcription factor; *H1t*, gene encoding a testis-specific variant of the histone H1; HSE, heat-shock element; HSF, heat-shock transcription factor; *Hsp70*, multigene family of heat shock or 'stress' genes; Oct, octamer; POU, Pit-1, Oct-1/2 and Unc-86; probe A-Sp1$_{mut}$, oligonucleotide probe with a partially mutated putative Sp1-binding site; probe A-Oct$_{mut}$, oligonucleotide probe with a partially mutated Oct sequence; RT, reverse transcriptase; T1 etc., transcription start site 1 etc.; TE1, *cis*-acting element that resides in the promoter of a testis-specific histone H1 gene, which binds testis-specific factors; tk, thymidine kinase.

[1] To whom correspondence should be addressed (e-mail wwidlak@io.gliwice.pl).

MATERIALS AND METHODS

Recombinant plasmids

The pHST(368/203)-CAT5 plasmid (see Figure 1A) was constructed by inserting the *Bst*XI(− 368 nt)–*Sma*I(− 203 nt) fragment of the rat *Hst70* gene (GenBank® accession no. X15705) in front of a minimal *tk* (thymidine kinase) gene promoter cloned in the plasmid pBLCAT5 (GenBank® accession no. M80483; a gift from Dr M. Boshart, German Cancer Research Centre, Heidelberg, Germany). The pBLCAT5 plasmid contains a fragment of the herpes simplex virus *tk* gene promoter (spanning from nt − 113 to + 57) ligated to the chloramphenicol acetyltransferase (CAT) reporter gene. The pHST(368/62)-CAT6 and the pHST(279/62)-CAT6 plasmids were constructed by inserting defined fragments [*Bst*XI-(− 368 nt)–*Dra*II (− 62 nt) and *Eco*RV (− 279 nt)–*Dra*II (− 62 nt) respectively] of the 5′-flanking region of the rat *Hst70* gene in front of the promoterless CAT gene cloned in the plasmid pBLCAT6 (GenBank® accession no. M80484). The construction and structure of these plasmids have been described previously [6,12]. Coordinates of the restriction sites refer to the A (+ 1 nt) in the ATG codon (in the case of the *Hst70* gene promoter) and to the transcription start site (in the case of the *tk* gene promoter).

Generation of transgenic mice

Constructs used for microinjection (pHST(368/203)-CAT5 and pBLCAT5) were digested with *Hin*dIII and *Kpn*I. Appropriate restriction fragments were recovered from agarose gels by electroelution, then purified with Elutip-D (Schleicher and Schuell, Dassel, Germany), precipitated with ethanol, washed, dissolved, filtered using a 0.02 μm Anotop filter and diluted to 1–5 ng/μl in 10 mM Tris/HCl, pH 7.4, and 0.1 mM EDTA for microinjection. The DNA was microinjected into the pronuclei of zygotes from FVB/N (inbred mouse strain) females. Transgenic founders were screened by PCR using genomic DNA isolated from tail biopsies, and primers complementary to the *Hst70* promoter and CAT sequences. All animal experiments were approved by the Committee of Ethics and Animal Experimentation.

Cell culture and transient transfection

Rat hepatoma cell line FTO-2B was grown in Dulbecco's modified Eagle's medium/Ham's medium (1:1, v/v; Gibco BRL) supplemented with 10 % (v/v) foetal calf serum (Gibco BRL). Cells were transfected by electroporation using a Bio-Rad Gene Pulser (Bio-Rad Laboratories, Hercules, CA, U.S.A) in combination with a Bio-Rad capacitance extender (260 V and 950 μF). For transfection, 10 μg of plasmid DNA was used per 5×10^6 of cells. After electroporation, cells were grown at 37 °C for 48 h before harvesting and then the CAT assay was performed as described below.

Analysis of CAT activity

Tissue homogenates were prepared according to [13]. Briefly, excised tissues were frozen in liquid nitrogen and then homogenized in 5–10 vol. 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 (dithiothreitol), and 0.4 mM PMSF. Cell culture extracts were prepared by the Tris buffer freeze–thaw protocol as described previously [6]. Crude lysates were clarified by centrifugation (15 000 ***g***, 5 min at 4 °C), supernatants were heated at 60 °C for 10 min, and centrifuged again (15 000 ***g***, 10 min at 4 °C). To perform CAT assays, an aliquot of 100 μl of extract was added to 100 μl of the 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, Irvine, CA, U.S.A.). The CAT assay mixtures contained 75–300 μg of protein and samples were incubated for 1–5 h at 37 °C. In the case of cell extracts, the mixtures contained 10 μg of protein and samples were incubated for 2 h. The acetylated forms of chloramphenicol were separated by TLC as described previously [12]. CAT activity was expressed as a percentage of acetylated products formed per hour per milligram of extract protein, as described previously [13]. Total protein content of the supernatant after heating of extracts was determined using a Protein Assay Kit (Bio-Rad).

Preparation of nuclei and nuclear extracts

Freshly dissected tissues were homogenized in ice-cold low-salt buffer containing 10 mM KCl, 0.25 M saccharose, 1.5 mM $MgCl_2$, 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 pelleted by centrifugation (5 min, 900 ***g*** at 4 °C). Pelleted nuclei were washed in low-salt buffer, resuspended in the same buffer supplemented with 100 mM KCl and then used in a DNaseI hypersensitivity assay, or stored in 50 % (v/v) glycerol at − 70 °C. Purified nuclei were extracted by incubation for 30 min at 4 °C in a buffer consisting of 0.4 M NaCl, 10 mM Hepes/NaOH (pH 7.9), 1.5 mM $MgCl_2$, 0.1 mM EGTA, 0.5 mM DTT, 5 % (v/v) glycerol and a protease inhibitor mixture (Complete™, Roche). Insoluble remnants of the nuclei were pelleted by centrifugation for 30 min at 20 000 ***g*** at 4 °C. Resulting supernatants were referred to as nuclear extracts. The protein concentration was assayed using the Protein Assay Kit (Bio-Rad).

EMSA (electrophoretic mobility-shift assay)

Complementary single-stranded oligonucleotides were annealed in aqueous solutions. Overhanging ends of double-stranded oligonucleotides were labelled with [γ-^{32}P]dCTP using Klenow enzyme. The sequences of the resulting DNA probes are shown in Figures 3(A) and 4(A). Labelled probes were purified from non-denaturating polyacrylamide gels. Probes (20 ng) were incubated with nuclear extracts (5 μg of protein) for 30 min at 4 °C in the binding buffer, consisting of 20 mM Tris/HCl, pH 7.6, 5 mM $MgCl_2$, 0.5 mM EDTA, 1 mM DTT, 5 % (v/v) glycerol and 150 mM NaCl, in a final volume of 20 μl. Incubation was in the presence of non-radioactive poly(dI-dC) double stranded co-polymer (0.5 μg and 1.0 μg) used as a non-homologous competitor, and then nucleoprotein complexes were resolved by electrophoresis on 6 % nondenaturing polyacrylamide gels in a 0.5 × TBE running buffer. Gels were dried and autoradiographed.

RNA extraction and RT (reverse transcriptase)-PCR

Total RNA was prepared using the guanidine isothiocyanate method [14]. RNA samples were purified from DNA contamination by digestion with DNaseI [15]. After inactivation of the enzyme, a control PCR for DNA contamination was performed using GAPDH (glyceraldehyde-3-phosphate dehydrogenase) primers. The RT-PCR assays were performed essentially according to [16]. At the reverse transcription step the reaction mixture (final volume 50 μl) contained PCR reaction buffer, 0.2 mM of each of the dNTPs, reverse primer (0.4 μM), RNA (1 μg), ribonuclease inhibitor (40 units; MBI Fermentas, Vilnius, Lithuania), MMLV reverse transcriptase (50 units; Gibco BRL) and Taq polymerase

Table 1 Primers used in RT-PCR analyses

-s, sense; -as, antisense; Ex, exon; Int, intron.

Primer pair	Primer sequence	Position in the *Hsp70.2* gene*	Product length (bp)
3′-s	5′-AGGGCCCACCATCGAGGAAGTG-3′	2505 to 2526	343
3′-as	5′-TCAAGCAAATCTCCATGTACATAC-3′	2825 to 2848	
Ex1-s	5′-AAGCGCCTCACCCAACTA-3′	332 to 349	108
Ex2-as	5′-AGTAAGTGGTGCCCAGGTCGATGCCGATAG-3′	650 to 679	
Int-s	5′-CGCTCGTGGAGAGTTGTGAGA-3′	524 to 544	155
Ex2-as	5′-AGTAAGTGGTGCCCAGGTCGATGCCGATAG-3′	650 to 679	
		Position in the *GAPDH* gene†	
GAPDH-s	5′-TGTCTTCACCACCATGGAGA-3′	340 to 359	299
GAPDH-as	5′-CGGCCATCACGCCACAGCTT-3′	620 to 639	

* GenBank® accession no. M20567.
† GenBank® accession no. M32599.

(2 units; MBI Fermentas). Samples were incubated in a thermal cycler (PerkinElmer, type 3200) for 10 min at 50 °C and then inactivation of reverse transcriptase was performed for 5 min at 94 °C. Immediately after addition of forward primer (0.4 μM), 35 cycles of the PCR were performed (94 °C, 30 s; 55–58 °C, 30 s; 72 °C, 45 s). RT-PCR products were analysed on 2 % agarose gels containing ethidium bromide. The sequences of primers used in PCR and RT-PCR as well as length of the products are listed in Table 1.

Protein extraction and Western blotting

Excised tissues were frozen on dry ice and then homogenized in 5 vol. of buffer containing 50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 0.1 % Nonidet P-40, 1 mM DTT, 1 mM PMSF, a protease inhibitor mixture (Complete™, Roche), and centrifuged at 15000 ***g*** for 10 min at 4 °C. Total protein content of the supernatant was determined using a Protein Assay Kit (Bio-Rad). Proteins (100 μg) were separated by SDS/PAGE on 8 % polyacrylamide gels and blotted on to nitrocellulose (BA85; Schleicher and Schuell). The filter was blocked for 60 min in 5 % non-fat milk in TTBS (0.25 M Tris/HCl, pH 7.5, 0,1 % Tween-20, 0.15 M NaCl). Anti-rabbit antibody against HSP70-2 (1:2500; a gift from Dr E. M. Eddy, National Institutes of Health, NC, U.S.A. [17]) was used. The primary antibody was detected by appropriate secondary antibody conjugated with horse-radish peroxidase (1:5000; Roche) and visualized by enhanced chemiluminescence (Pierce, Rockford, IL, U.S.A.).

DNase hypersensitivity assay

DNase I hypersensitivity was assayed as described in [18] with modifications. Purified nuclei were incubated for 15 min at 27 °C with DNase I (Pharmacia; at concentrations ranging from 0 to 25 units/ml; 0 to 10 units/0.5 mg of protein) in a buffer consisting of 4 mM $MgCl_2$, 1 mM $CaCl_2$, 100 mM KCl, 20 mM Tris/HCl, pH 7.4, and 1 mM DTT. Reactions were stopped by addition of EDTA and SDS, samples were incubated with proteinase K and extracted with phenol/chloroform. Ethanol precipitated solubilized DNA was digested with *Hin*dIII restriction endonuclease and subjected to 2 % agarose gel electrophoresis. Separated DNA fragments were blotted onto nylon membranes and probed with a radiolabelled PCR product spanning positions −899 to −704 of the *Hst70* gene.

RESULTS

A 165 bp fragment of the *Hst70* gene promoter is sufficient for testis-specific and developmentally regulated expression of a transgene reporter gene

To determine whether a relatively short *Hst70* promoter gene segment, bearing only a T1 site, could direct appropriate expression of a reporter gene in transgenic mice, a 165 bp *Bst*XI (−368)–*Sma*I (−203) restriction fragment of the gene that contained boxes A and B was inserted in front of the minimal *tk* gene promoter in a CAT reporter vector (pBLCAT5). Because of the lack of TATA box in this fragment, we decided to use a construct containing the heterologous *tk* gene promoter. Schematic structure of the resulting construct, termed pHST(368/203)-CAT5, is shown in Figure 1(A). We obtained three lines of transgenic mice bearing this construct (named Tg1, Tg2, Tg3), as well as two lines of control transgenic mice with the *tk*-CAT sequences only (termed pBLCAT5). CAT activity was detected in testes from all three lines of pHST(368/203)-CAT5 but not from pBLCAT5 control transgenic mice (Figure 1B). In contrast, no CAT activity was detected in liver of transgenic animals. Consistent with the known tissue-specific pattern of endogenous *Hst70*/*Hsp70.2* gene expression [12,15], detectable transgene activity was also found in brain tissue of these animals (Figure 1C). The developmentally regulated pattern of the transgene expression was analysed during postnatal development of transgenic mice derived from the Tg3 founder. CAT activity was detected in testes extracts obtained from mice over 10-days-old (Figure 1D), which corresponds with the beginning of the first meiotic division, consistent with *Hst70*/*Hsp70.2* gene expression in spermatocytes [2,17].

The ability of the −368/−203 DNA sequences to activate transcription was further confirmed in transiently transfected cultured rat hepatoma FTO-2B cell line (Figure 2). The pHST(368/203)-CAT5 construct, containing tested sequences linked to the heterologous *tk* promoter, was twice as active than control pBLCAT5 plasmid. The −368/−203 DNA sequences significantly stimulated transcription also when linked to the *Hst70* promoter fragment containing endogenous general '*cis*' regulatory elements, e.g. TATA box [the pHST(368/62)-CAT6 construct]. It should be noted that, in transfections of hepatoma cells, the efficiency of this 'testicular' promoter was considerably lower than that of a 'somatic' (minimal *tk*) promoter (Figure 2B). In marked contrast, in testes of transgenic mice, the pHST(368/62)-CAT6 construct showed apparently higher activity as compared with the pHST(368/203)-CAT5 construct (Figure 2C; [6,12]). Thus, in

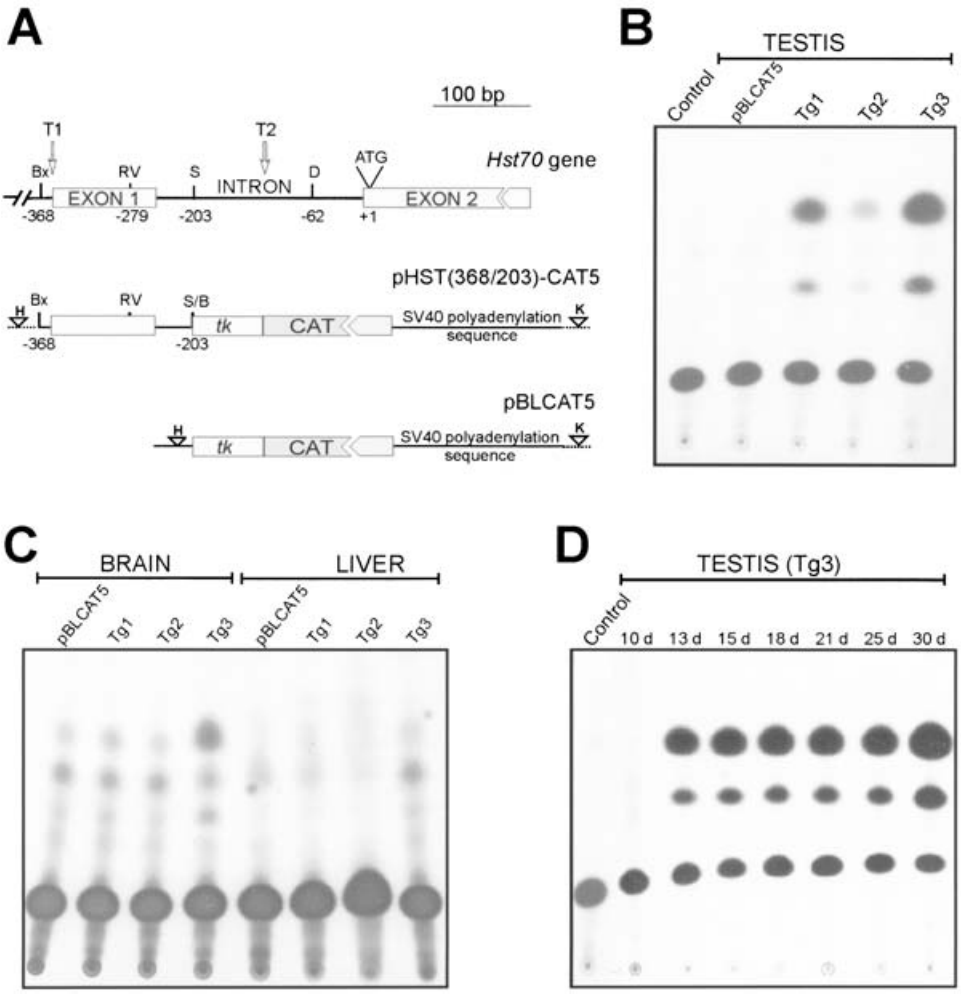

Figure 1 CAT activity in tissues of pBLCAT5 and pHST(368/203)-CAT5 transgenic mice

(**A**) Structure of the *Hst70* gene and the pHST(368/203)-CAT5 and pBLCAT5 transgenes. Vertical arrows show position of the two main transcription start sites (T1 and T2). Bx, RV, S, D, B, H, K indicate restriction sites for *Bst*XI, *Eco*RV, *Sma*I, *Dra*II, *Bgl*II, *Hind*III and *Kpn*I restriction enzymes respectively. Numbers under restriction sites indicate position in relation to A (+1) in ATG codon. S/B is the ligation site at *Sma*I and *Bgl*II (Klenow-filled). Open triangles indicate the polylinker restriction sites used to cut off the hybrid gene for microinjection. *tk* is the minimal thymidine kinase promoter. (**B**) Representative CAT assay performed on testes of pBLCAT5 and Tg1, Tg2 and Tg3 lines of the pHST(368/203)-CAT5 transgenic mice. Each reaction mixture contained 75 μg of protein and the reaction time was 4 h with pBLCAT5 and 1 h with Tg1–Tg3. (**C**) CAT activity in brain and liver of pBLCAT5 and Tg1, Tg2 and Tg3 lines of the pHST(368/203)-CAT5 transgenic mice. Each reaction mixture contained 150 μg of protein (brain) or 300 μg of protein (liver) and the reaction time was 4 h. The autoradiogram was overexposed to visualize a weak CAT activity in the brain of Tg3 line. (**D**) Developmental activation of the pHST(368/203)-CAT5 transgene in the Tg3 line. Each reaction mixture contained 75 μg of protein and the reaction time was 5 h.

spermatogenic cells, the −368/−203 promoter fragment shows much stronger activating potential when linked to its endogenous 'testicular' promoter than to the 'somatic' promoter.

Experiments with the pHST(279/62)-CAT6 construct (both *in vitro* transfections and analysis of transgenic mice) showed that the presence of box A is indispensable for *Hst70* promoter activity. Thus, we showed that the 165 bp −368/−203 fragment of the promoter region, containing conserved boxes A and B and the T1 transcription start site, is the shortest fragment sufficient to confer general and tissue-specific *Hst70* gene transcription.

The Oct sequence from box A is involved in regulation of the *Hst70/Hsp70.2* gene

In order to identify *trans*-acting factors that interact with *cis*-acting elements responsible for the activity of the −368/−203 promoter fragment, we searched for proteins that specifically bind sequences from boxes A and B. First we analysed binding patterns of nuclear proteins from 27- and 9-day-old rat testes, which either did or did not contain spermatocytes, to DNA sequences from box A. An abundant specific nucleoprotein complex was detected using an EMSA, when a DNA probe corresponding to box A was incubated with nuclear proteins from the testes of 9-day-old rats (complex I, Figure 3B). Another complex with lower abundance and specificity could be also detected with nuclear extracts from either 9- or 27-day-old animals (complex II, Figure 3B). Box A contains a putative Sp1 binding site and the Oct sequence. To identify an element responsible for the formation of complex I, we used oligonucleotide probes with a partially mutated putative Sp1-binding site (probe A-$Sp1_{mut}$) or a partially mutated Oct sequence (probe A-Oct_{mut}) in further analyses. Complex I could not be detected using the A-Oct_{mut} probe (Figure 3C). In contrast, complex II was still detected when this probe was incubated with extracts from either 9- or 27-day-old rats, along with another complex (complex X, Figure 3C). This latter complex may contain proteins that bind to the 'mutated' Oct

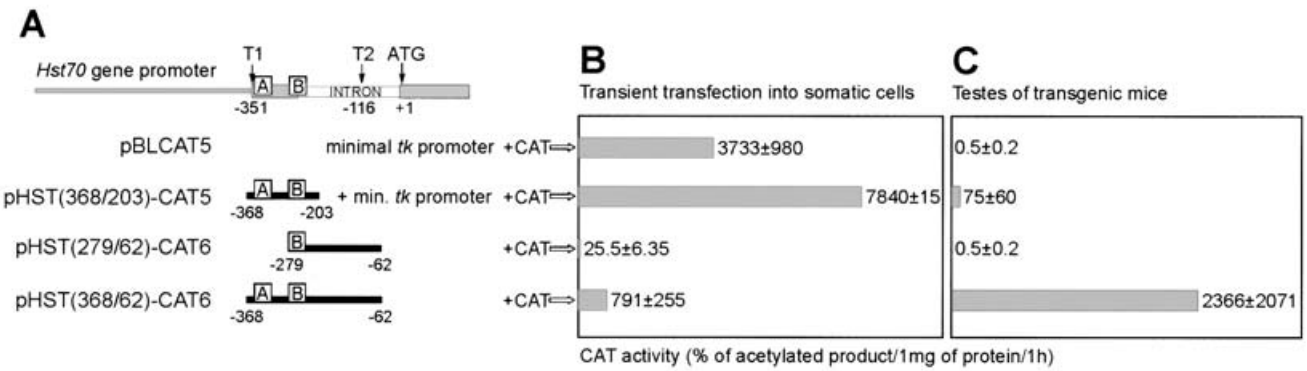

Figure 2 Comparison of CAT expression driven by different fragments of the *Hst70* promoter in somatic FTO-2B cells after *in vitro* electroporation (B), and in testes of transgenic mice (C)

(**A**) Schematic structure of the *Hst70* gene promoter and vectors used for analysis. T1 (−351) and T2 (−116) transcription initiation sites are shown. Numbers indicate position in relation to A (+1) in the ATG codon. The positions of boxes A and B are indicated. Mean values of CAT activity (± S.D.) are shown for three to six independent transfection assays (**B**), or two to three transgenic lines (**C**), with each construct as indicated in (**A**). Details are described in the Materials and methods section.

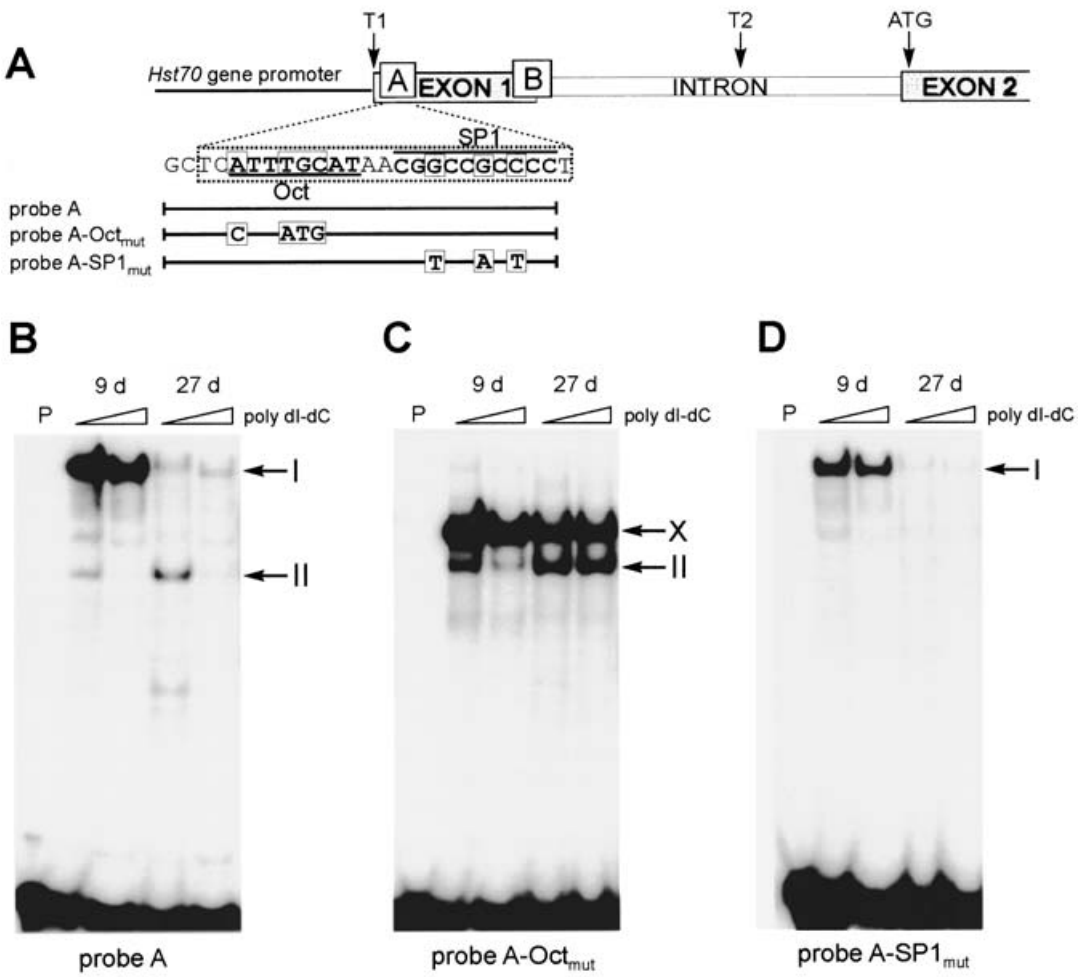

Figure 3 Analysis of protein-binding properties of the Oct and Sp1 sequences localized in box A of the *Hst70* gene promoter

(**A**) Structure of the *Hst70* gene promoter, sequence of box A (in broken rectangle) and probes used in the assay. Sequence of the Oct is underlined, Sp1 is overlined; bases in boxes were replaced in probes as indicated to obtain mutated Oct or mutated Sp1 sequences. (**B**–**D**) EMSA analysis of the box A sequence. The radiolabelled double-stranded probes [probe A (**B**), probe A-Oct$_{mut}$ (**C**), probe A-SP1$_{mut}$ (**D**)] were incubated with either 9- or 27-day-old rat testis nuclear extract. The arrows indicate the position of the major complexes (I, II, or X). Lanes P, no extract or competitor.

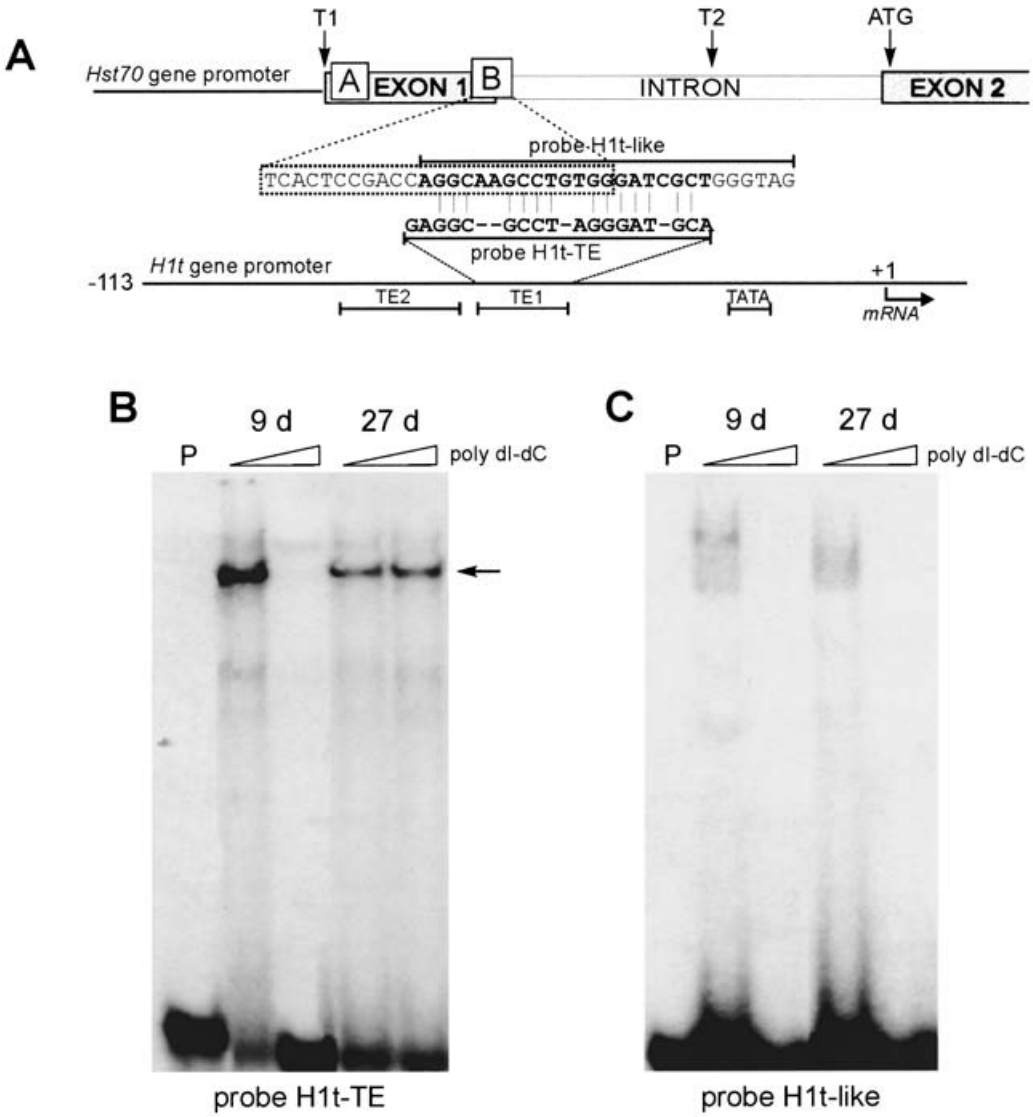

Figure 4 Analysis of protein-binding properties of the *H1t*-like sequence from the *Hst70* gene promoter

(**A**) Structure of the *Hst70* gene promoter, sequence of box B (in broken rectangle) and probe H1t-like. Sequence of the H1t-like element (bold) was aligned to the sequence of the TE1 element, which is essential for testis-specific expression of the histone *H1t* gene. (**B**) EMSA analysis of the TE1 element of the histone H1t promoter. The arrow indicates the position of the major complex. (**C**) EMSA analysis of the H1t-like element of the Hst70 promoter. The radiolabelled double-stranded probes [probe H1t-TE (**B**) and probe H1T-like (**C**)] were incubated with either 9- or 27-day-old rat testis nuclear extract. Lanes P, no extract or competitor.

sequence. Proteins giving rise to complex X were present in testes of either 9- or 27-day-old animals as well as in rat liver (results not shown). When an A-Sp1$_{mut}$ probe was used, only complex I was detected after incubation with testicular extracts from 9-day-old but not 27-day-old rats (Figure 3D), complexes were not detected at all in liver extracts (results not shown). The data show that the testes of juvenile rats contain proteins that specifically bind to the Oct sequence from box A of the *Hst70*/*Hsp70.2* gene (complex I in Figures 3B and 3D). The specificity of such binding was further confirmed in the experiments where complexes between testicular extracts and probe A were formed in the presence of either A-Sp1$_{mut}$ or A-Oct$_{mut}$ as homologous competitors (results not shown). In contrast, proteins that bind to the putative Sp1 site of box A could be detected in testes of either 9- or 27-day-old rats (complex II in Figures 3B and 3C) as well as in liver (results not shown). To summarize, our data indicate that the Oct sequence from box A of the rat *Hst70*/*Hsp70.2* gene specifically binds nuclear proteins from testes of 9-day-old rats, which do not contain spermatocytes. Such proteins cannot be detected in testes of 27-day-old animals, which express the *Hst70*/*Hsp70.2* gene in spermatocytes and spermatids.

The 5′ part of the box B contains the ERE-like sequence closely resembling the palindromic ERE consensus. However, our previous data showed that this ERE-like sequence did not respond to hormone stimulation in *in vitro* transient transfection assays, suggesting its lack of functional importance [11]. The 3′ part of box B contains sequences that are similar to the TE1 element from the promoter of the *H1t* gene encoding a testis-specific variant of the histone H1 (Figure 4A). Like the *Hst70*/*Hsp70.2* gene, the *H1t* gene is specifically activated in pachytene spermatocytes, and proteins binding to the TE1 element are necessary for testis-specific expression of the *H1t* gene [10]. We

have compared binding of the nuclear proteins with the *H1t* gene TE1 element and with the H1t-like element from the *Hst70* gene box B (details in Figure 4A). As expected, specific nucleoprotein complexes were detected with nuclear extracts from testes of 27-day-old rats when the *H1t* gene TE1 element was tested (Figure 4B). In contrast, no specific nucleoprotein complexes were detected when the *Hst70*/*Hsp70.2* H1t-like probe was tested, with extracts from neither 9- nor 27-day-old animals (Figure 4C). This indicates that the TE1-like element is possibly not involved in the regulation of *Hst70*/*Hsp70.2* gene expression. The high conservation of box B could be related to its localization proximal to the exon 1/intron splicing site.

The T1 transcription start site is functionally important in cells that produce the HST70/HSP70.2 protein

The rat *Hst70* gene and the mouse *Hsp70.2* genes have an identical organization in their promoter regions. In order to determine whether T1 and T2 transcription start sites are used differentially at different stages of spermatogenesis, we have looked for *Hsp70.2* transcripts in testes before the appearance of spermatocytes and spermatids (10-day-old mice), in testes containing spermatocytes in prophase of the first meiotic division (15-day-old mice), round spermatids (25-day-old mice), and spermatids in the final step of differentiation (35-day-old mice). Using RT-PCR primers complementary to the 3′ end of the *Hsp70.2* gene, we have found its transcripts in all tested samples as well as in 10-day-old (Figure 5B) and even 5-day-old (results not shown) animals (specificity of RT-PCR products was confirmed by hybridization with radiolabelled internal primer, data not shown). However, we were not able to detect the HSP70.2 protein in 10-day-old animals, although small amounts could be detected in 15-day-old animals (Figure 5F). We also used RT-PCR primers to differentiate transcripts starting from either T1 or T2 sites (according to [6]). Transcripts present in testes of 10-day-old mice originate exclusively from the T2 site (Figures 5C and 5D). The T1 site signal starts to be detectable in testes of 15-day-old mice.

Sites in chromatin that have already bound transcription factors, or that will allow their binding, are experimentally detected as nuclease hypersensitive sites and are indicators of regulatory regions of genes [19]. We have searched for DNaseI hypersensitive sites within the rat *Hst70* gene promoter in three different tissues: testes of 27-day-old rats that contain spermatocytes, testes of 9-day-old animals that contain only somatic cells and spermatogonia, and rat FTO-2B hepatoma cells where the endogenous *Hst70* gene is repressed. Transcripts and protein products of the *Hst70*/*Hsp70.2* gene are detectable only in testes of 27-day-old animals; both transcription start sites are active in such animals [6]. Data presented in Figure 6 show that two DNaseI hypersensitive sites could be detected in testes of 27-day-old animals but not hepatoma cells or testes of 9-day-old animals; one mapped to the T1 transcription initiation site and another just upstream of this site (Figure 6, asterisks). The results indicate that in cells expressing the *Hst70*/*Hsp70.2* gene the promoter chromatin structure is altered, probably by transcription factors/activators that occupy the T1 transcription initiation site.

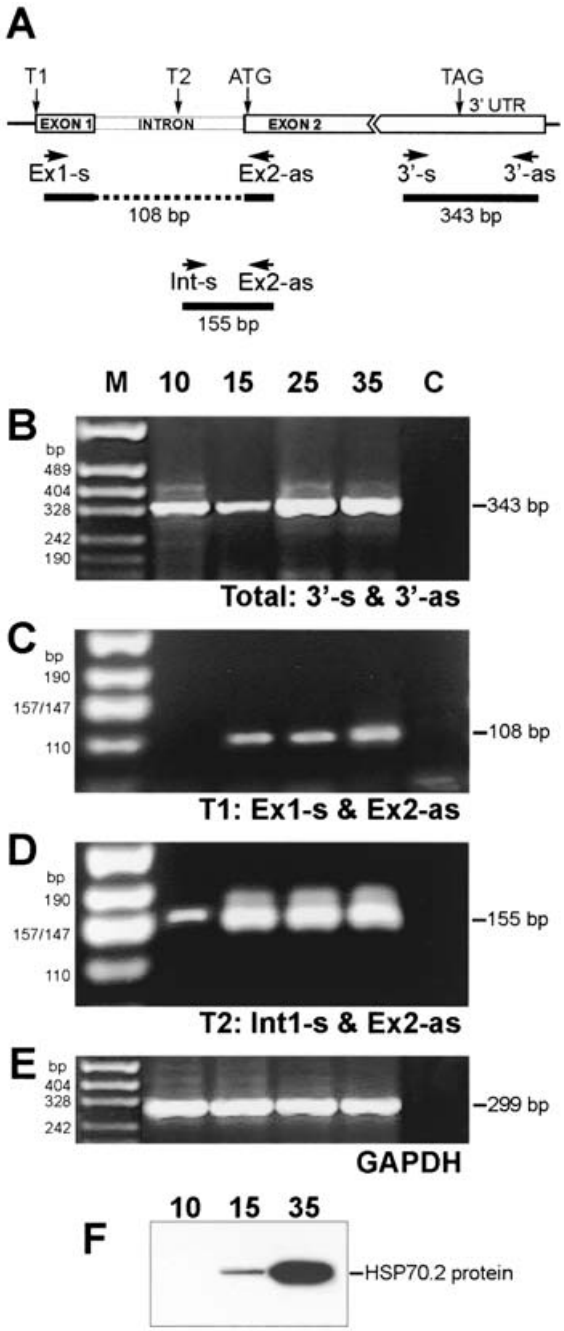

Figure 5 Detection of *Hsp70.2* transcripts and HSP70.2 protein in mouse testes during postnatal development

(**A**) Schematic structure of the *Hsp70.2* gene, localization of primers (horizontal arrows) used for analyses (for nucleotide sequence and the exact position in the gene see Table 1) and predicted RT-PCR product sizes generated from two types of transcripts. RT-PCR analysis was performed on templates from 10-, 15-, 25-, and 35-day-old mice with primer pairs complementary to (**B**) the 3′ part of the gene (detection of both *Hsp70.2* transcript types), (**C**) transcripts initiated at the T1 site, (**D**) transcripts initiated at the T2 site, and (**E**) with primers complementary to the GAPDH gene (for RNA quality control). (**F**) Detection of HSP70.2 protein by Western blotting. M, DNA size markers (bp); C, control reaction without template.

DISCUSSION

We have shown previously that the *Bst*XI(−368)–*Dra*II(−62) fragment of the *Hst70* promoter, containing sequences located between two transcription start sites (T1 and T2), is the shortest promoter fragment that will maintain efficient transgene activity in testes [6,12]. Now we have presented evidence indicating that the 165 bp-long *Bst*XI(−368)–*Sma*I(−203) fragment, containing only the T1 transcription start site and two regions of high homology between rat, mouse and human counterparts (named boxes A and B), is necessary and sufficient to direct testis-specific

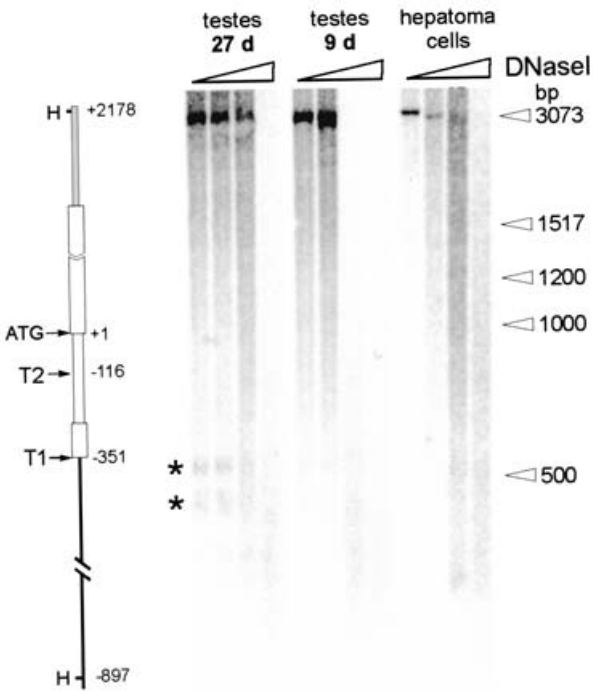

Figure 6 Detection of DNaseI hypersensitive sites in the *Hst70* gene promoter

Nuclei of 27- and 9-day-old rats and rat hepatoma FTO-2B cells were used for analysis. Hypersensitive sites are indicated by asterisks. The *Hst70* gene structure is shown to the left of the footprint. Positions of DNA size markers (bp) are shown on the right-hand side.

gene expression. Multiple transcription initiation sites seem to be a common theme for promoters of genes activated in developmentally regulated patterns [20,21], including genes involved in testicular development and spermatogenesis [22,23].

Tissue-specific and developmentally regulated promoter-binding transcription factors are apparently involved in the regulation of the activities of multiple transcription initiation sites. Recent studies have revealed that several tissue-specific general transcription factors (GTFs) work in concert with RNA polymerase II, raising the idea that pre-initiation complexes with distinct core promoter specificity and/or repressor/activator specificity may be utilized at some promoters. Several GTFs and GTF-related factors display unique patterns of expression in testis. Among them are the TATA-binding protein (TBP)-related factor, TRF2 [24], TAF1L that seems to replace somatic $TAF_{II}250$ (the largest subunit of the general transcription factor TFIID) [25] and TFIIAα/β-like factor [26]. In a search for particular *cis*-acting elements from the $-368/-203$ fragment of the *Hst70* gene that specifically interact with testicular proteins, we have identified an Oct sequence within conserved box A. The Oct sequence binds nuclear proteins from testes of 9-day-old but not 27-day-old rats. The only spermatogenic cells present in seminiferous epithelium of 9-day-old rats are spermatogonia which do not express the *Hst70*/*Hsp70.2* gene, while spermatogonia are only a minor component (approx. 5–10 %) in testis of 27-day-old animals. This suggests that Oct sequence-binding protein(s) could be involved in repression of the *Hst70*/*Hsp70.2* gene in spermatogonia. Among proteins recognizing the Oct sequence are POU transcription factors (representing a homeodomain protein family of which the founder members are Pit-1, Oct-1/2 and Unc-86) that contain bipartite DNA binding domain (POU specific and POU homeodomain). Interestingly, it has been shown that the *Oct-4* gene encoding a POU family transcription factor, associated with potentially totipotent cells, is specifically expressed in spermatogonia [27,28]. Furthermore, its activity as a transcriptional repressor has been demonstrated during bovine embryogenesis [29]. One can speculate that in spermatogonia, Oct-4 transcription factor represses genes that are only expressed in later stages of spermatogenic cell differentiation, including *Hst70*/*Hsp70.2*. Low levels of transcripts initiated from the T2 site are detected in juvenile testes at stages when the Oct sequence could be occupied by a putative suppressor of the *Hst70*/*Hsp70.2* gene transcription. In marked contrast, efficient expression of this gene is restricted to cells at those spermatogenesis stages where the T1 site is active and the Oct sequence would be predicted to be unoccupied (Figures 3 and 5). This suggests that the Oct sequence is involved in regulation of the activity of transcription initiation sites. The release of a putative repressor from the Oct sequence may allow binding of transcription factors (possibly spermatocyte-specific GTFs) to the T1 transcription initiation site, which could be manifested in a form of DNase-hypersenstitive sites detected around this site (Figure 6). Results presented in this paper indicate the functional importance of the T1 site for *Hst70*/*Hsp70.2* gene expression. However, whether only transcripts initiated from this site are translated in spermatocytes remains unclear at the moment. In fact, RNase-protection assays revealed similar amounts of transcripts started from both T1 and T2 sites in testes of adult rats [6].

Although the $-368/-203$ fragment of the *Hst70* gene promoter confers tissue-specific and developmentally regulated patterns of expression, our results suggest that the T2 transcription start site and sequences located directly upstream of this site are required for efficient gene expression in testis. Such sequences, in addition to the TATA box, CAAT box and Sp1-binding site that are present also in the minimal *tk* promoter, contain sequence resembling a heat-shock element (HSE). Replacement of the T2 site and proximal sequences by the minimal *tk* promoter resulted in marked reduction of the transgene activity in testes (Figure 2C). This observation points to a potential functional importance of the HSE-like element localized upstream of the TATA box. HSEs located in the regulatory regions of heat-shock genes activate transcription after binding heat-shock transcription factor (HSF) [30]. Among three members of HSF family identified in mammals, HSF2 is the most abundantly expressed in mouse testes [31]. HSF2 functions as a regulator of *hsp* gene expression under non-stress conditions, particularly in cells involved in process of differentiation and development [32,33]. However, fragmentary and contradictory data exist in the literature concerning the functional importance of HSF2 for *hsp* gene regulation in testis [33,34]. Thus, further studies are required to determine whether the HSE-like element present in the *Hst70*/*Hsp70.2* gene promoter is critical for regulation of its expression.

The authors wish to thank Professor W.T. Garrard (UT Southwestern Medical Center, Dallas, TX, U.S.A.) and Professor P. Widłak, (Maria Sklodowska–Curie Memorial Institute, Gliwice, Poland) for comments and help with preparation of the manuscript. We also thank Mrs K. Klyszcz for skilful technical assistance. This work was mainly supported by a grant from the Polish Ministry of Science and Informatics (Grant KBN 3 P04A 033 23 to W.W.) and by a grant awarded to Z.K. by the Foundation for Polish Science.

REFERENCES

1 Krawczyk, Z., Wiśniewski, J. and Biesiada, E. (1987) An *hsp70*-related gene is constitutively highly expressed in testis of rat and mouse. Mol. Biol. Rep. **12**, 27–34
2 Krawczyk, Z., Mali, P. and Parvinen, M. (1988) Expression of testis-specific *hsp70* gene-related RNA in defined stages of rat seminiferous epithelium. J. Cell Biol. **107**, 1317–1323

3 Zakeri, Z. F., Wolgemuth, D. J. and Hunt, C. R. (1988) Identification and sequence analysis of a new member of the mouse *HSP70* gene family and characterization of its unique cellular and developmental pattern of expression in the male germ line. Mol. Cell. Biol. **8**, 2925–2932

4 Raab, L. S., Polakoski, K. L., Hancock, L. W. and Hamilton, D. W. (1995) Characterization of the heat-shock protein P70 in rat spermatogenic cells. Mol. Reprod. Dev. **40**, 186–195

5 Dix, D. J., Allen, J. W., Collins, W., Mori, C., Nakamura, N., Poorman-Allen, P., Goulding, E. H. and Eddy, E. M. (1996) Targeted gene disruption of *Hsp70-Hsp72* results in failed meiosis, germ cell apoptosis, and male infertility. Proc. Natl. Acad. Sci. U.S.A. **16**, 3264–3268

6 Ścieglińska, D., Widłak, W., Konopka, W., Poutanen, M., Rahman, N., Huhtaniemi, I. and Krawczyk, Z. (2001) Structure of the 5′ region of the *hst70* gene transcrition uni: presence of an intron and multiple transcription initiation sites. Biochem. J. **359**, 129–137

7 Dix, D. J., Rosario-Herrle, M. O., Gotoh, H., Mori, C., Goulding, E. H., Voelker, C. R. and Eddy, E. M. (1996) Developmentally regulated expression of *Hsp70-Hsp72* and a *Hsp70-Hsp72/lacZ* transgene during spermatogenesis. Dev. Biol. **174**, 310–321

8 Wiśniewski, J., Kordula, T. and Krawczyk, Z. (1990) Isolation and nucleotide-sequence analysis of rat testis-specific major heat shock (*hsp70*)-related gene. Biochim. Biophys. Acta **1048**, 93–99

9 Bonnycastle, L. L. C., Yu, Ch-E., Hunt, C. R., Trask, B. J., Clancy, K. P., Weber, J. L., Patterson, D. and Schellenberg, G. D. (1994) Cloning, sequencing, and mapping of the human chromosome 14 heat shock protein gene (*HspA2*). Genomics **23**, 85–93

10 Grimes, S. R., Wolfe, S. A. and Koppel, D. A. (1992) Tissue-specific binding of testis nuclear proteins to a sequence element within the promoter of the testis-specific histone *H1t* gene. Arch. Biochem. Biophys. **296**, 402–409

11 Krawczyk, Z., Schmid, W., Harkonen, P. and Wolniczek, P. (1993) The ERE-like sequence from the promoter region of the testis specific *hsp70*-related gene is not estrogen responsive. Cell Biol. Int. **17**, 245–253

12 Widłak, W., Markkula, M., Krawczyk, Z., Kananen, K. and Huhtaniemi, I. (1995) A 252 bp upstream region of the rat spermatocyte-specific *hst70* gene is sufficient to promote expression of the *hst70-CAT* hybrid gene in testis and brain of transgenic mice. Biochim. Biophys. Acta **1264**, 191–200

13 Pothier, F., Ouellet, M., Julien, J. P. and Guerin, S. L. (1992) An improved CAT assay for promoter analysis in either transgenic mice or tissue culture cells. DNA Cell Biol. **11**, 83–90

14 Chomczyński, P. and Sacchi, N. (1987) Single-step method of RNA isolation by acid guanidinium isothiocyanate-phenol/chloroform extraction. Anal. Biochem. **162**, 156–159

15 Ścieglińska, D., Widłak, W., Rusin, M., Markkula, M. and Krawczyk, Z. (1997) Expression of the testis-specific HSP70-related gene (*HST70*) gene in somatic non-testicular rat tissues revealed by RT-PCR and transgenic mice analysis. Cell Biol. Int. **21**, 813–821

16 Singer-Sam, J., Robinson, M. O., Bellve, A. R., Simon, M. I. and Riggs, A. D. (1990) Measurement by quantitive PCR of changes in *HPRT*, *PGK-1*, *PGK-2*, *APRT*, *MTase* and *Zfy* gene transcripts during mouse spermatogenesis. Nucleic Acids Res. **18**, 1255–1259

17 Rosario, M. O., Perkins, S. L., O'Brien, D. A., Allen, R. L. and Eddy, E. M. (1992) Identification of the gene for the developmentally expressed 70 kDa heat-shock protein (P70) of mouse spermatogenic cells. Dev. Biol. **150**, 1–11

18 Sambrook, J. and Russel, D. W. (2001) Molecular Cloning. A Laboratory Manual, 3rd edn., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

19 Gross, D. S. and Garrard, W. T. (1988) Nuclease hypersensitive sites in chromatin. Annu. Rev. Biochem. **57**, 159–197

20 Liu, T. Y., Chen, H. H., Lee, K. H. and Choo, K. B. (2003) Display of different modes of transcription by the promoters of an early embryonic gene, *Zfp352*, in pre-implantation embryos and in somatic cells. Mol. Reprod. Dev. **64**, 52–60

21 Frazar, T. F., Weisbein, J. L., Anderson, S. M., Cline, A. P., Garrett, L. J., Felsenfeld, G., Gallagher, P. G. and Bodine, D. M. (2003) Variegated expression from the murine band 3 (AE1) promoter in transgenic mice is associated with mRNA transcript initiation at upstream start sites and can be suppressed by the addition of the chicken beta-globin 5′ HS4 insulator element. Mol. Cell. Biol. **23**, 4753–4763

22 Kleene, K. C., Cataldo, L., Mastrangelo, M. A. and Tagne, J. B. (2003) Alternative patterns of transcription and translation of the ribosomal protein L32 mRNA in somatic and spermatogenic cells in mice. Exp. Cell Res. **291**, 101–110

23 Yamauchi, M., Itoh, H., Naganuma, S., Koono, M., Hasui, Y., Osada, Y. and Kataoka, H. (2002) Expression of hepatocyte growth factor activator inhibitor type 2 (HAI-2) in human testis: identification of a distinct transcription start site for the *HAI-2* gene in testis. Biol. Chem. **383**, 1953–1957

24 Zhang, D., Penttila, T. L., Morris, P. L. and Roeder, R. G. (2001) Cell- and stage-specific high-level expression of TBP-related factor 2 (TRF2) during mouse spermatogenesis. Mech. Dev. **106**, 203–205

25 Wang, P. J. and Page, D. C. (2002) Functional substitution for TAFII250 by a retroposed homolog that is expressed in human spermatogenesis. Hum. Mol. Genet. **19**, 2341–2346

26 Upadhyaya, A. B., Lee, S. H. and DeJong, J. (1999) Identification of a general transcription factor TFIIAa/b homolog selectively expressed in testis. J. Biol. Chem. **274**, 18040–18048

27 Pesce, M., Gross, M. K. and Schöler, H. R. (1998) In line with our ancestors: Oct-4 and the mammalian germ. Bioessays **20**, 722–732

28 Yoshimizu, T., Sugiyama, N., De Felice, M., Yeom, Y. I., Ohbo, K., Masuko, K., Obinata, M., Abe, K., Scholer, H. R. and Matsui, Y. (1999) Germline-specific expression of the *Oct-4/green fluorescent protein (GFP)* transgene in mice. Dev. Growth Differ. **41**, 675–684

29 Ezashi, T., Ghosh, D. and Roberts, R. M. (2001) Repression of Ets-2-induced transactivation of the tau interferon promoter by Oct-4. Mol. Cell. Biol. **21**, 7883–7891

30 Wu, C. (1995) Heat shock transcription factors: structure and regulation. Annu. Rev. Cell. Dev. Biol. **11**, 441–469

31 Fiorenza, M. T., Farkas, T., Dissing, M., Kolding, D. and Zimarino, V. (1995) Complex expression of murine heat shock transcription factors. Nucleic Acids Res. **23**, 467–474

32 Murphy, S. P., Gorzowski, J. J., Sarge, K. D. and Phillips, B. (1994) Characterization of constitutive HSF2 DNA-binding activity in mouse embryonal carcinoma cells. Mol. Cell. Biol. **14**, 5309–5317

33 Sarge, K. D., Park-Sarge, O. K., Kirby, J. D., Mayo, K. E. and Morimoto, R. I. (1994) Expression of *heat shock factor 2* in mouse testis: potential role as a regulator of heat-shock protein gene expression during spermatogenesis. Biol. Reprod. **50**, 1334–1343

34 Alastalo, T. P., Lonnstrom, M., Leppa, S., Kaarniranta, K., Pelto-Huikko, M., Sistonen, L. and Parvinen, M. (1998) Stage-specific expression and cellular localization of the *heat shock factor 2* isoforms in the rat seminiferous epithelium. Exp. Cell Res. **240**, 16–27

Received 1 December 2003/29 January 2004; accepted 6 February 2004
Published as BJ Immediate Publication 6 February 2004, DOI 10.1042/BJ20031842