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# The structural and functional analysis of the human *HSPA2* gene promoter region★

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***HSPA2* is a human counterpart of the testis-specific rodent *Hst70/Hsp70.2* gene. In contrast to the latter, the expression of the human *HSPA2* gene is not limited to the testis, and recent data show that human tumor cells can express this gene at significant levels. The characteristics of *HSPA2* expression suggests that it can influence the phenotype and survival of cancer cells similarly as overexpression of major members of the *HSP70* gene family. Until now, neither the structure of the transcription unit of the human *HSPA2* gene has been established nor a functional analysis of its promoter performed. In this study we established that the human *HSPA2* gene, in contrast to its rodent counterparts, is intronless and has a single transcription start site. We also show that the same type of *HSPA2* transcripts are synthesized in the testes and in cancer cell lines. In order to perform a functional study of the *HSPA2* promoter, we used a transient transfection assay and found that the 392 bp fragment upstream of the ATG codon was a minimal region required for efficient transcription, while a 150 bp deletion from the 5′ end of this region dramatically reduced the promoter activity. Delineation of the minimal promoter is a basic step toward identifiying the *cis* and *trans* elements involved in the regulation of the *HSPA2* gene expression in cancer cells.**

**Keywords**: *HSPA2*, transcript structure, expression regulation, cancer cell lines

## INTRODUCTION

*HSPA2*, a member of the *HSP70* family of heat shock genes is a human counterpart of mouse and rat *HspA2* genes (called previously *Hsp70.2* in mouse and *Hst70* in rat), which are specifically and highly expressed in spermatocytes (Krawczyk *et al.*, 1988a; 1988b; Zakeri *et al.*, 1988; Bonnycastle *et al.*, 1994). Rodent *HspA2* genes encode a molecular chaperone essential for dissociation of synaptonemal complexes, progression of meiosis and male fertility (Dix *et al.*, 1996; 1997; Zhu *et al.*, 1997).

Human *HSPA2* gene is highly expressed in spermatids and tails of mature spermatozoa, suggesting its involvement in late stages of spermatid maturation (Huszar *et al.*, 2000). The HSPA2 protein was identified as a testis-specific creatinine kinase type M (CK-M), the decreased activity of which has been connected with male infertility (Yesilli *et al.*, 2005; Huszar *et al.*, 2006; Cedenho *et al.*, 2006). Relatively high levels of *HSPA2* transcripts were found in various human non-testicular tissues (Bonnycastle *et al.*, 1994), but the corresponding protein was essentially undetectable (Son *et al.*, 1999). In contrast to normal tissues, a sig-

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**Abbreviations**: CAT, chloramphenicol acetyltransferase; RLM-RACE, RNA ligase-mediated rapid amplification of cDNA ends.

nificant expression of the HSPA2 protein was found in various human cancer cell lines and primary non-small-cell lung cancer tissues (Ścieglińska *et al.*, unpublished). Since another group (Rohde *et al.*, 2005) reported that silencing of the *HSPA2* gene in cancer cells led to growth arrest, *HSPA2* has been considered important for cancer cell survival.

In order to study the mechanism regulating expression of the *HSPA2* gene in cancer cells it is essential to establish the structure of the gene transcription unit and the minimal promoter sequence. In our previous papers we reported the structure of rodent *HspA2* genes and their promoter regions. We established that their transcription is initiated at two major start sites, T1 and T2, (the latter being localized within the intron) that are found approx. 350 bp (T1 site) and 115 bp (T2 site) upstream of the ATG codon. Transcription initiated at the T1 site generates transcripts containing intronic sequences that are subsequently spliced out, whereas transcripts initiated at the T2 site are not spliced and probably are not translated (Ścieglińska *et al.*, 2001; 2004). We also determined that major regulatory sequences responsible for efficient expression of the rat *HspA2* (*Hst70*) gene are present within a 306 bp DNA fragment (–368/–62) upstream of the ATG translation start codon (Widłak *et al.*, 1994; 1995; Ścieglińska *et al.*, 2001). The regulatory elements indispensable and sufficient for spermatocyte-specific activity of the rat *HspA2* (*Hst70*) gene are localized within a 165 bp fragment between the T1 and T2 transcription start sites, encompassing exon 1 and the 5′ part of the intron (Ścieglińska *et al.*, 2004). It seems that an octamer sequence, localized directly downstream of the T1 transcription start site, is responsible for regulating the developmental activation of the gene (Ścieglińska *et al.*, 2004).

So far, neither the structure of human *HSPA2* gene promoter region nor its functionality have been established. In this study, using 5′ RACE and RT-PCR, we found that human *HSPA2* gene is intronless and that its transcription is initiated from a single start site. We also found that transcription of the *HSPA2* gene is initiated at the same site in cancer cells and in testes. Using transient transfection of cancer cell lines with diffent *HSPA2* transcription levels we also performed functional studies of the *HSPA2* promoter and determined that a 392 bp fragment upstream of the ATG codon functions as a minimal promoter of *HSPA2*.

## MATERIALS AND METHODS

**RNA isolation.** Total RNA was prepared using the guanidine isothiocyanate method (Chomczyński & Sacchi, 1987) from human normal testes and cancer cell lines indicated below. RNA samples were purified from contaminating DNA by on-column treatment with RNase-free DNase I (Qiagen). After inactivation of the enzyme, an aliquot of the RNA sample was digested with RNase A and control PCR for DNA contamination was performed using *GAPDH*, as described earlier (Ścieglińska *et al.*, 1997). The integrity of purified DNase I-treated RNA was analyzed using Bioanalyzer (Ambion). In RT-PCR only RNA with the RNA Integration Number (RIN) higher than 8 was used. RNA concentration was measured using NanoDrop ND1000 (NanoDrop Technology).

**5′ RACE analysis.** The 5′ RACE analysis of *HSPA2* transcripts was done using GeneRacer™ Kit (Invitrogen) and total testicular RNA as a template. This kit enables RNA ligase-mediated rapid amplification of 5′ cDNA end (RLM-RACE) and ensures amplification of only full-length mRNA *via* elimination of truncated molecules from the amplification process. To accomplish this, total RNA was treated with calf intestinal phosphatase (CIP) to remove the 5′ phosphates. As CIP has no effect on full-length capped mRNA, the digestion eliminates truncated mRNA and non-mRNA from subsequent ligation with an RNA oligonucleotide linker. The procedure was performed according to the manufacturer's protocol. An outline of the RLM-RACE procedure is shown in Fig. 1. To increase specificity of RACE amplification for reverse transcription, an *HSPA2*-specific oligonucleotide (Nested) complementary to the *HSPA2* coding region was used. The first round of RACE-PCR was performed with the following primers: Gene Racer 5′ (supplied with the kit) and *HSPA2*-specific primer (Nested). The second round of PCR was performed with the following primers: Gene Racer 5′ Nested (supplied with the kit) and *HSPA2*-specific primer (2-as). The sequences of the *HSPA2*-specific oligonucleotides and their positions in the *HSPA2* gene are given in Table 1. The conditions of the RACE-PCR reactions were as in the manufacturer's protocol; primer annealing temperatures optimal for *HSPA2*-specific primers were used and the reaction mixture contained 1 M betaine and the proofreading DNA polymerase *Pfu* (2 U, Fermentas). To confirm the specificity of RLM-RACE, products of the second round of RACE-PCR were electrophoretically resolved in 2% agarose and transferred onto Hybond-N membrane (Amersham) by capillary blotting. Hybridization was performed overnight at 58°C with 20 pM oligonucleotide probe (T2, Table 1) 5′ labeled with [$\gamma$-$^{32}$P]dATP and T4 polynucleotide kinase (Roche), as described previously (Ścieglińska *et al.*, 2001). The amplification products were also cloned into pCR-Blunt vector using PCR-Blunt-Topo Cloning Kit (Invitrogen), according to the manufacturer's protocol, and the insert was sequenced with

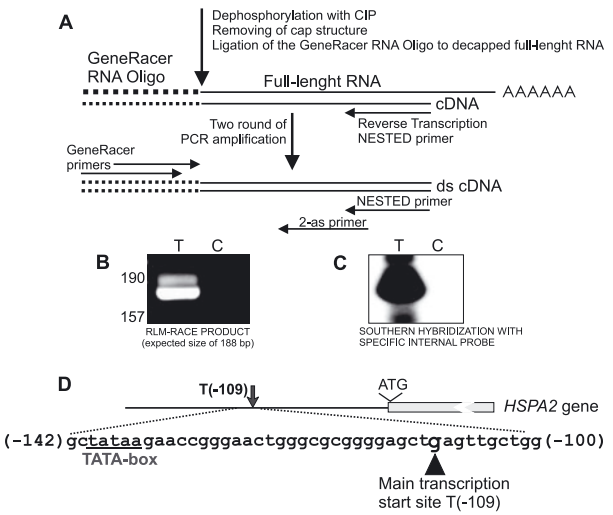


**Figure 1. Detection of the *HSPA2* gene transcription start site by RLM-RACE method.**
(**A**) Scheme of procedure using GeneRacerTM Kit (Invitrogen) described in more detail in Materials and Methods. (**B**) RLM-RACE products obtained after second round of PCR amplification and separated in 2% agarose gel. Legend: T, RLM-RACE reaction with testicular RNA as template; C, control RLM-RACE reaction without template. Numbers on the left-hand side of photograph indicate the size (in bp) of DNA marker. (**C**) Southern hybridization of RLM-RACE products (from panel B) with *HSPA2*-specific T2 oligonucleotide probe (for its nucleotide sequence and position in *HSPA2* gene see Table 1). (**D**) Scheme showing structure of the *HSPA2* gene promoter region and localization of the main transcription start site marked T(-109). The position of the T(-109) start site was identified by determining the nucleotide sequence of RLM-RACE product cloned into pCR-Blunt vector (Invitrogen).

BigDye Terminator v3.1 Cycle Sequencing Kit (PE Applied Biosystem).

**RT-PCR.** The RT-PCR assays were done essentially according to Ścieglińska *et al.* (1997). At the reverse transcription step, the reaction mixture (final volume 50 μl) contained 1 μg of total RNA, PCR reaction buffer, 0.2 mM each dNTPs, lower primer (0.4 μM), RNA (1 μg), ribonuclease inhibitor (40 U, Fermentas), MMLV reverse transcriptase (50 U, Gibco BRL) and *Taq* polymerase (2 U, Qiagen). Samples were incubated in a thermal cycler (Perkin Elmer, type 3200) for 10 min at 50°C and then reverse transcriptase was inactivated for 5 min at 94°C. Immediately after addition of the upper primer (0.4 μM), 35 cycles of PCR were performed (94°C, 30 s; 58–65°C, 30 s; 72°C, 45 s). RT-PCR products were analyzed by electrophoresis in 2% agarose gels containing ethidium bromide. Sequence of primers and their positions in *HSPA2* gene are given in Table 1.

**Recombinant plasmids.** The *HSPA2* promoter fragments were amplified by PCR using human genomic DNA as the template and subcloned into pCR-Blunt vector (Invitrogen). Construction of the pHST-HSPA2 plasmids (Fig. 4B) involved inserting, in front of a promotorless *CAT* reporter gene, fragments of the 5′ flanking region from the *HSPA2* gene (GenBank Acc. No. L26336). The recipient pBLCAT6 plasmid (GenBank Acc. No. M80484) was a gift from Dr. M. Boshart. The pHSPA2(-868/-4) CAT6, pHSPA2(-868/-247)CAT6 and pHSPA2(-868/-351)CAT6 plasmids contain *HSPA2* fragments from nucleotide –868 to nucleotide –4, –247, and –351 (Van95I restriction site), respectively. The pHSPA2(-530/-4)CAT6, pHSPA2(-392/-4)CAT6 and pHSPA2(-279/-4)CAT6 plasmids contain *HSPA2* fragments from nucleotide –530, –392 and –279, respectively, to nucleotide –4. Coordinates of the restriction sites refer to the A(+1) in the ATG codon of the *HSPA2* gene. Successful construction of pHSPA2-CAT expressing vectors was confirmed by their nucleotide sequences determined with BigDye Terminator v3.1 Cycle Sequencing Kit (PE Applied Biosystem). Details on plasmid construction are available on request. Plasmids used for transfection were purified using QIAfilter Plasmid Kit (Qiagen) according to the manufacturer's instruction.

**Cell culture, transient transfection and 5-azacytidine treatment.** Human cancer cell lines: HCT116 (colon carcinoma), NCI-H1299 (non-small-cell lung

**Table 1. Oligonucleotides for the *HSPA2* and *GAPDH* genes.**

Lower-case letters indicate nucleotides not complementary to the *HSPA2* gene sequence.

| | Oligonucleotides sequence | Position in the *HSPA2* gene (GeneBank Acc. No. L26336) |
|---|---|---|
| 1-s | 5′-GAACCTCATTTACATAACGGCCG-3′ | –301 to –323 |
| 2-s | 5′-CtCgAGCCAGTCACTCCGACCTAG-3′ | –237 to –219 |
| Int2-s | 5′-TGCCCGTGGTGCTTGGTTCGAG-3′ | –75 to –96 |
| T2 | 5′-GTGGTGCTTGGTTCGAGGTGGCC-3′ | –69 to –91 |
| 2-as | 5′-AATAGGTGGTGCCCAGGTCGATGCCGATAG-3′ | +20 to +49 |
| Nested | 5′-GATGAGGCGCTCGGTGTCCGTGAA-3′ | +133 to +156 |
| 3′-as | 5′-GgAtcCTTAGTCCACTTCTTCGATGGT–3′ | +1900 to +1926 |
| | | Position in the *GAPDH* gene (GeneBank Acc. No. NM_002046) |
| GAPDH-s | 5′-CGTCTTCACCACCATGGAGA-3′ | 402 to 421 |
| GAPDH-as | 5′-CGGCCATCACGCCACAGTTT-3′ | 628 to 701 |

carcinoma), NCI-H358 (non-small-cell lung carcinoma), NCI-H292 (lung mucoepidermoid carcinoma), A549 (lung carcinoma), MCF7 (breast epithelial adenocarcinoma), Me45 (melanoma), HepG2 (hepatocellular carcinoma) and human epithelial cell lines: HBL-100 (breast myoepithelial tumor) MCF-10A (immortalized human mammary epithelial cells) and BEAS-2B (virus-transfected normal bronchial epithelium) were used. The cell lines were grown in RPMI 1640 medium with the exception of HepG2, Me45, HBL-100 and MCF-10A cell lines, which were grown in DMEM medium, and HCT116 cells, which were grown in McCoy medium. All growth media were supplemented with 10% fetal bovine serum (ICN) and 40 μg/ml gentamycin. The growth medium for MCF-10A cells was additionally supplemented with 10 μg/ml insulin, 10 ng/ml EGF and 100 ng/ml hydrocortisone, whereas that for BEAS-2B cells with 0.5 ng/ml EGF, 500 ng/ml hydrocortisone, 0.005 mg/ml insulin, and for HBL-100 with 250 ng/ml insulin and 20 ng/ml EGF.

For transient transfection, A549 and HepG2 cells were seeded at 1–2.5 × $10^4$ cells per 35 mm culture dish 24 h before transfection. The cells were incubated with 2.5 μg of pHSPA2-CAT plasmids mixed with 50 ng of pGL3-Control plasmid (Promega) and 10 μl of lipofectin (Invitrogen) in serum-free growth medium according to the manufacturer's protocol. The transfection mixtures were removed after 8 h of incubation and replaced by growth medium. Cells were harvested 48 h after transfection and lysed in reporter lysis buffer (Promega) according to the manufacturer's protocol. Crude lysates were clarified by centrifugation (14000 rpm, 5 min at 4°C) and total protein content was determined using a Protein Assay Kit (BioRad).

For 5-azacytidine (5-aza) treatment, HepG2 cells were seeded at 30% confluence in 60 mm culture dishes and after 24 h normal growth medium was replaced by medium containing 2 μM 5-azacytidine (Sigma). The medium supplemented with 5-aza was replenished at 2-day intervals. Cells were harvested after 10 days of culture and RNA or proteins were isolated.

**Luciferase and CAT reporter gene assay.** To perform CAT assays supernatants were heated at 60°C for 10 min and centrifuged again (14000 rpm, 10 min at 4°C). An aliquot of the clarified cell extract containing 10–20 μg of protein was added to the same volume of reaction mixture containing 0.25 M Tris/HCl (pH 7.8), 1 mM EDTA, 4 mM acetyl-CoA (Sigma) and 2.5 μl of [$^{14}$C]chloramphenicol (2.5 μCi/ml, ICN). The samples were incubated for 2 h at 37°C. The acetylated forms of chloramphenicol were separated by thin-layer chromatography as described earlier (Widłak *et al.*, 1995). CAT activity was expressed as the percentage of acetylated products formed per one hour per milligram of protein, as described previously (Widłak *et al.*, 1995), normalized according to the activity of an internal control (luciferase acivity) and expressed in relative activity units.

To perform the luciferase assay, 1 μl of non-heated protein extract was diluted in reporter lysis buffer (Promega) and 20 μl was added to 100 μl of luciferase assay substrate (Promega) prepared according to the manufacturer's instruction. Luciferase activity was measured using a Berthold Lumat LB95d instrument and calculated per 1 mg of protein. For estimating the transfection efficiency the luciferase activity was taken as internal control.

## RESULTS AND DISCUSSION

### The structure of *HSPA2* transcripts

To determine the structure of the 5′ region of the *HSPA2* gene we initially assumed that the general transcription unit structure is similar in the rodent and human homologues, i.e. the mouse *HspA2* (*Hsp70.2)*, the rat *HspA2* (*Hst70)* and the human *HSPA2* genes. We found this assumption justified by the fact that all of these genes share high nucleotide sequence similarity, not only within the coding region but also upstream of the ATG codon, including the splicing donor and acceptor sites (Bonycastle *et al.*, 1994; Widłak *et al.*, 1995; Ścieglińska *et al.*, 2001). The basic issue to resolve was to find out if the *HSPA2* gene transcription was initiated at two sites and whether the gene contained an intron upstream of the ATG codon.

In order to localize the transcription start site of the *HSPA2* gene, we performed 5′ RACE analysis using mRNA isolated from normal human testes. For selective detection of full length 5′ UTR ends of the *HSPA2* transcripts we choose the RLM-RACE method (described in Materials and Methods). To enhance the detection specificity for cDNA synthesis and subsequent RACE-PCR reactions, the *HSPA2*-specific Nested and the 2-as antisense oligonucleotide were used (Fig. 1A; for nucleotide sequences and positions of the oligonucleotides see Table 1). The nucleotide sequences of the RLM-RACE products indicate that the majority (eleven out of twelve) of amplified 5′ ends of the *HSPA2* transcripts start from nucleotide 109 upstream of the ATG codon (Fig. 1D).

To characterize the 5′ UTR structure of the *HSPA2* transcripts synthesized in testes we performed RT-PCR reactions according to the schedule shown in Fig 2A. As expected, the RT-PCR reactions gave products of the same length (145 bp)

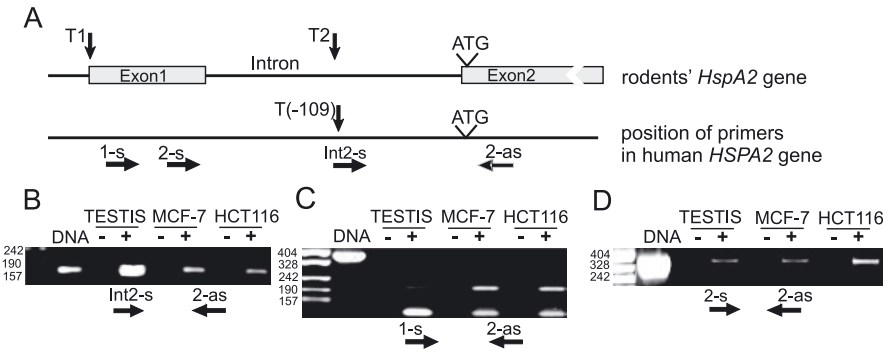


**Figure 2. Detection of *HSPA2* transcripts synthesized in testis and in selected cancer cell lines.**
(**A**) Upper part shows structure of rodent *HspA2* genes. Vertical arrows indicate the position of two main transcription start sites marked T1 and T2. Lower part shows position of oligonucleotides (marked as horizontal arrows) used in the analysis of *HSPA2* transcripts by PCR and RT-PCR (for their nucleotide sequence and position in the *HSPA2* gene see Table 1). Vertical arrow indicates position of *HSPA2* main transcription start site marked T(-109). (**B**) Examples of RT-PCR results showing transcripts initiated at the T(-109) main transcription start site of the *HSPA2* gene. (**C**) RT-PCR analysis showing that *HSPA2* transcription is not initiated in region corresponding to T1 initiation site of rodent *HspA2* gene. Only unspecific bands are visible; their nucleotide sequence was verified by sequencing and is complementary to gene coding for 28S rRNA. (**D**) RT-PCR analysis showing transcript initiated at an additional minor transcription start site from which intronic sequences are not spliced out. DNA, products of PCR reaction obtained on DNA matrices using indicated primers. "–" denotes control RT-PCR reactions with RNA digested with DNase I and RNase A (for details see Materials and Methods); "+" denotes RT-PCR reactions with total RNA as template. Numbers on the left-hand side of each photograph indicate the sizes (in bp) of DNA markers. Under each photograph are indicated primers used in RT-PCR analysis.

as the PCR products obtained from the total DNA template using the sense primer (Int-2) complementary to sequences directly downstream of the detected *HSPA2* transcription start site T(-109) and the antisense primer matching a region downstream of the ATG codon (2-as) (Fig. 2B). By RLM-RACE we did not detect transcripts initiated at the transcription start site corresponding to the T1 site of rodent *HspA2* genes. No specific amplified product was obtained by RT-PCR using the sense primer (1-s) complementary to sequences directly downstream of the putative T1 transcription start site and the antisense primer (2-as) complementary to a region downstream of the ATG codon (Fig. 2C). Only unspecific products were present, the nucleotide sequences of which were complementary to those of the gene coding for 28S rRNA (Fig. 2C). These data indicate that transcription of human *HSPA2* gene is not initiated in the region corresponding to the T1 transcription start site of rodent *HspA2* (*Hst70/Hsp70.2)* genes. Thus, unlike rodent *HspA2* genes, the 5′ region of human *HSPA2* gene is devoid of intronic sequences. The *HSPA2* transcripts detected by us are initiated at a transcription start site which corresponds to the T2 transcription start site of rodent *HspA2* genes (compare Figs. 1D and Fig. 2A).

We have also identified by 5′ RACE some cDNA clones which possibly represent less abundant *HSPA2* transcript synthesized in testes from some additional transcription start site localized about 200 bp upstream of the main T(-109) initiation site. Its 5′ UTR region corresponds exactly to the nucleotide sequence of DNA, indicating that this less prominent transcript is not subjected to splicing. This transcript was also detected by RT-PCR with the sense primer (2-s) corresponding to the *HSPA2* sequence directly upstream of the putative donor splicing site and the antisense primer (2-as) located downstream of the ATG codon (see Fig. 1C). These data, together with our data presented earlier (Widłak *et al.*, 1995; Ścieglińska *et al.*, 2001) show that, in contrast to its rodent counterparts, human *HSPA2* gene does not contain an intron at its 5′ end and is transcribed from the main transcription initiation site preceded by a canonical TATA-box.

A remarkable feature of mammalian spermatogenesis is that many genes in spermatogenic cells exhibit conspicuously different expression patterns than in somatic cells. A large number of mRNAs in spermatogenic cells differ in size and structure from transcripts of the same genes in somatic cells by usage of spermatogenic cell-specific transcription start sites, as well as alternative splicing and polyadenylation sites. There are multiple reports that the altered testicular transcripts encode proteins of different functions to those performed in somatic cells (Eddy, 2002). To find out whether the structure of the *HSPA2* mRNA synthesized in cancer cell lines differs from that produced in testicular cells we performed an RT-PCR analysis according to the schedule shown in Fig. 2A.

Total RNA was isolated from a panel of cancer cell lines originating from human skin (Me45), colon (HCT116), liver (HepG2), breast (MCF-7), lung (A549, NCI-H1299, NCI-H358, NCI-H292), and non-tumorigenic epithelial cell lines from breast (HBL-100, MCF-10A) and lung (BEAC-2B). Examples of this analysis are shown for total mRNA isolated

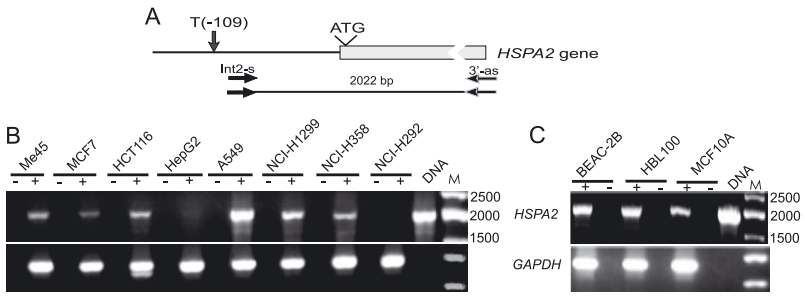


**Figure 3. Analysis of *HSPA2* transcription in various human cell lines.**
(**A**) A scheme showing structure of the *HSPA2* gene (upper part), positions of oligonucleotide primers (horizontal arrows) used in RT-PCR and PCR analysis of *HSPA2* transcripts and expected size (in bp) of RT-PCR product (lower part). Vertical arrow indicates position of the main transcription start site. For nucleotide sequences and positions of the primers see Table 1. (**B**) Detection of *HSPA2* transcripts in cancer cell lines and (**C**) in immortalized epithelial cell lines by RT-PCR analysis. Control RT-PCR reactions were made with primers corresponding to *GAPDH* gene (bottom picture). "–", control RT-PCR reactions with RNA digested with DNase I and RNase A (for details see Materials and Methods); "+", RT-PCR reactions with total RNA as template. Numbers on the right side indicate sizes (in bp) of DNA markers.

from HCT116 and MCF-7 cell lines (Fig. 2B, 2C, 2D). Abundant RT-PCR products were obtained only in the reaction with the sense primer (Int2-s) and total RNA template isolated from the majority of the cell lines tested. By RT-PCR reaction with the sense primer (2-s) we detected also very low amounts of *HSPA2* transcripts beginning probably at minor additional transcription start site. The specificity of the RT-PCR products was confirmed by Southern hybridization with an internal specific probe (not shown). The results obtained by RT-PCR analysis indicate that in the analyzed cancer cell lines transcription of *HSPA2* starts at the same initiation site as in testicular cells.

The primers used in the described RT-PCR analysis enabled amplification of only the 5′ region of the *HSPA2* transcripts. Using primers complementary to sequences within the 5′ UTR and to the 3′ UTR regions (primers Int2-s and 3-as; for sequences of primers see Table 1) we show by RT-PCR that the *HSPA2* transcript of the expected length (i.e. testicular one), albeit at different levels, could be detected in the majority of the cell lines studied (Fig. 3), with the exception of HepG2 hepatoma and H292 lung mucoepidermoid carcinoma. These results indicate that in testes and in cancer cell lines the same HSPA2 protein is produced.

**Functional study of the *HSPA2* gene promoter**

To obtain a preliminary characterization of the *HSPA2* gene promoter region we analyzed the regulatory elements required for expression of the *HSPA2* gene localized directly upstream of the transcription start site. Fig. 4A shows the localization of putative regulatory elements in a 860-bp-long 5′-flanking region upstream of the *HSPA2* transcription start site. To confirm its promoter functionality, the pHSPA2(-868/-4)CAT6 reporter plasmid was constructed that contains the *HSPA2* fragment from nucleotides –868 to –4 fused to the chloramphenicol acetyltransferase (CAT) reporter gene. To test this promoter's activity we used A549 cells in which we had previously found high expression of the endogenous *HSPA2* gene (Ścieglińska *et al.*, not shown). As a control we used the HepG2 cell line in which we had found endogenous *HSPA2* gene activity to be fully repressed. The pHSPA2(-868/-4)CAT6 plasmid was transiently cotransfected with the luciferase-encoding pGL3-Control vector (Promega). Surprisingly, in both cell lines we found similar, high levels of the promoter activity. Subsequently, we analyzed expression of two groups of the pHSPA2-CAT plasmids. The first group included pHSPA2(-530/-4)CAT6, pHSPA2(-392/-4)CAT6 and pHSPA2(-243/-4)CAT6 constructs containing promoter fragments shortened from the 5′ end while the second included pHSPA2(-868/-247)CAT6 and pHSPA2(-868/-351)CAT6 constructs with promoter fragments shortened from the 3′ end. The absolute level of CAT activity was normalized to luciferase activity (internal control) and expressed in relative units. The normalized activity of the pHSPA2(-868/-4)CAT6 construct (the longest promoter fragment analyzed) was set as 100% and the activity of the other pHSPA2-CAT constructs was normalized against that value.

Truncation of the *HSPA2* promoter from the 5′ end to nucleotides –530 and –392 (the pHSPA2(-530/-4)CAT6 and pHSPA2(-392/-4)CAT6 constructs, respectively), diminished the reporter gene activity to no more than 60% of those observed for the full-length *HSPA2* promoter in both cell lines studied, presumably due to elimination of three distal CAAT putative regulatory sequences (Fig. 4).

Truncation of the *HSPA2* promoter from the 5′ end to nucleotide –43 (p*HSPA2*(-243/-4)CAT6 construct), which additionally eliminated three consensus elements recognized by the SP1-like transcription factor, dramatically reduced the reporter gene

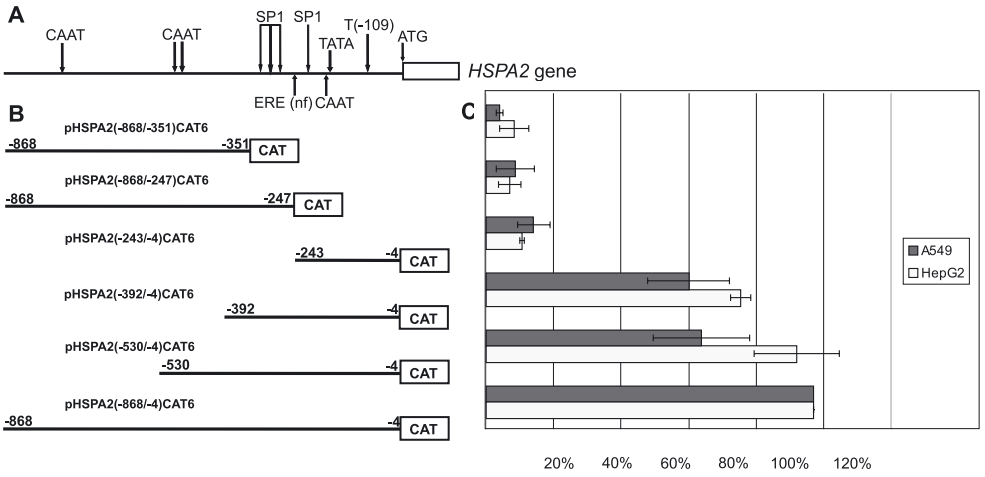


**Figure 4. Functional analysis of the *HSPA2* promoter by transient transfection experiments.**
(**A**) Possible regulatory elements localized upstream of the ATG codon of the *HSPA2* gene; ERE(nf) – estrogen response element found to be non-functional (nf) (Krawczyk *et al.*, 1993), T(-109) — the *HSPA2* gene transcription start site. (**B**) Structure of the pHSPA2-CAT constructs. The open box represents the CAT reporter gene. The thick line represents the promoter of the *HSPA2* gene. Numbers are coordinates of the nucleotide sequence with respect to nucleotide +1 (A in the ATG codon). Details on the construction of expression vectors are available on request. (**C**) CAT activity in transiently transfected A549 (dark gray box) and HepG2 (light gray box) cells. Cells were transfected with a mixture containing 2.5 μg of pHSPA2-CAT plasmid and 50 ng of pGL3-Control plasmid (Promega). CAT reaction mixture contained 20 μg of protein and the reaction time was 2 h. The absolute level of CAT activity was normalized to the activity of the control luciferase activity. CAT activity for individual constructs is expressed as the percentage of the pHSPA2(-868/-4)CAT6 construct (the longest promoter fragment analyzed) and represents the mean of three experiments ± S.D. using independent plasmid preparations. Details in the Materials and Methods.

activity to approx. 10% of that observed with the full-length *HSPA2* promoter. Reduction of the promoter activity to nearly background levels was also observed when sequences surrounding the T(-109) transcription start were removed from the *HSPA2* promoter fragments (p*HSPA2*(-868/-247)CAT6 and p*HSPA2*(-868/-351)CAT6 constructs).

Functional analysis of the deletion mutants revealed that the *HSPA2* gene fragment between nucleotides –392 and –4 contains the regulatory elements indispensable for efficient transcriptional activity of the *HSPA2* promoter. It can be thus speculated that distal Sp1 sequences are necessary for transcription of the *HSPA2* gene.

Despite the lack of endogenous expression of the *HSPA2* gene in HepG2 cells (Fig. 3) a significant activity of pHSPA2-CAT expression construct could be observed after transient transfection (Fig. 4). Thus, in HepG2 hepatoma cells the pHSPA2-CAT transcription units can be efficiently transcribed when present in the episomal form which suggests repression of the chromosomal *HSPA2* gene dependent on the chromatin structure. However, it seems that the mechanism of the *HSPA2* repression is independent of DNA methylation, as treatment of HepG2 cells with the DNA methylation inhibitor 5-azacytidine did not induce expression of the endogenous *HSPA2* gene (not shown).

Our earlier study on the transcription regulation of the rat *HspA2* gene also suggests an involvement of chromatin structure in the repression/derepression of genes highly expressed in the testes (Widłak *et al.*, 2003). We demonstrated that a transgene driven by certain fragments of the promoter region of the rat *HspA2* gene was not transcribed in the testes of transgenic mice, whereas it was highly expressed after transfection into rat FTO-2B hepatoma cells. And, conversely, some other rat *HspA2* promoter fragments were highly active in transgenic mice but significantly less active in transfected FTO-2B cells.

It is well established that certain genes which are highly expressed in spermatocytes and repressed in somatic cells can be activated in tumor cells. Such genes are known as so-called cancer-testis antigen genes (Scanlan *et al.*, 2004). These genes attract significant interest especially if the corresponding protein is expressed on the surface of cancer cells, being a potential target for immunotherapy. Considering the expression pattern of the *HSPA2* gene, i.e. its highest activity in the testis, repression or insignificant activity in somatic cells and enhanced activity in cancer cells it can be proposed that *HSPA2* may encode a novel cancer-testis antigen. It seems significant in this context that a recent global profiling of the cancer cells proteome led to identification of the HSPA2 protein on the surface of several cancer cell lines (Shin *et al.*, 2003). Among the latter were also A549 cells derived from non-small-cell lung carcinoma, in which we found a significant expression of the HSPA2 protein (Ścieglińska *et al.*, unpublished). However, whether the *HSPA2* gene does indeed exhibit all the features attributed to genes belonging to the cancer-testis an-

tigen group requires further study. The mapping of the transcription initiation site and preliminary characterization of the potential promoter region of the *HSPA2* gene performed in this study will enable us to further characterize the mechanisms involved in the activation of the *HSPA2* gene in tumor cells.

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