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Transcriptional regulation of the major zinc uptake protein hZip1 in prostate cancer cells

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Abstract

hZip1 has been characterized as the major zinc uptake transporter regulating the accumulation of zinc in prostate cells. The mechanisms regulating expression of hZip1 have not been described. To explore the mechanisms of transcriptional regulation of the hZip1 gene, we determined the putative promoter sequence for hZip1 and identified the potential transcription start site within the predicted hZip1 promoter region. To further characterize the promoter region for basal hZip1 transcription, 3' and 5' deletion constructs and constructs with mutated binding sites for putative transcription factors were generated by PCR amplification and assessed for transcriptional activity with a luciferase reporter assay in PC-3 prostate cancer cells. The ability of the specific transcription factors to bind the hZip1 core promoter was confirmed by EMSA, Gel Supershift and ChIP assays. Our experiments identified the core promoter region responsible for constitutive expression of hZip1 and demonstrated critical roles for SP1 and CREB1 in transcriptional regulation of the hZip1 gene in prostate cancer cells.

Keywords

core promoter; SP1; CREB; basal transcription

1. INTRODUCTION

The normal human prostate accumulates the highest levels of zinc of any soft tissue in the body. In adenocarcinoma of the prostate, zinc levels decrease markedly. This consistently occurs early in the course of malignancy and levels of zinc decline further during progression to hormone-independent growth. Recent studies suggest down-regulation of the zinc uptake transporter hZip1 as the mechanism for the loss of zinc accumulation in prostate cancer (Franklin et al., 2003; Rishi et al., 2003; Franklin et al., 2005). Indeed, expression of the hZip1 gene and transporter protein is markedly down-regulated in adenocarcinomatous glands and in prostate intra-epithelial neoplasia (PIN) foci when compared with normal peripheral zone glandular epithelium and benign prostatic hyperplasia (BPH) tissue (Franklin et al., 2005).

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Moreover, prostate cancer in high-risk patient populations, such as African-Americans, also is associated with down-regulation of hZip1 (Rishi et al., 2003). In light of these findings, hZip1 has been proposed to function as a tumor suppressor gene in prostate cancer.

Zinc transporters are largely assigned to two metal-transporter families: the ZIP family, which imports zinc, and the ZnT family, which functions in releasing zinc or sequestering zinc internally. Expression of ZnT family members is controlled predominantly by metal-activated transcription factor 1 (MTF1) (Hasumi et al., 2003; Aydemir et al., 2006). While recent studies reveal that the expression of hZip1 is controlled by progesterone and prolactin (Costello et al., 1999), the molecular mechanisms regulating transcription of the hZip1 gene are poorly understood.

In the present study we reveal the core promoter and transcription start site for hZip1 and demonstrate critical roles for SP1 and CREB1 in the transcriptional regulation of the hZip1 gene in prostate cancer cells.

2. MATERIALS AND METHODS

2.1 Cells and culture conditions

Androgen-independent human PC-3 prostate cancer cells were obtained from ATCC (Rockville, MD) and cultured in RPMI 1640 (Bio-Whittaker, Walkersville, MD) supplemented with 10% FCS (Hyclone, Logan, UT), gentamicin (50 mg/l), sodium pyruvate (1 mM), and non-essential amino acids (0.1 mM) under the conditions indicated in the figure legends.

2.2 Computer analysis of the promoter region

Promoter prediction and gene analysis was performed using the EIDorado and Gene2Promoter online programs (www.genomatix.de). Analysis of potential transcriptional factor binding sites was carried out using the AliBaba 2.0, TFSearch, Match (www.gene-regulation.com), and MatInspector (www.genomatix.de) online programs.

2.3 Mapping of hZip1 transcription start site by 5'RACE

Mapping was performed using the GeneRacer kit (Invitrogen, Carlsbad CA) according to the manufacturer's instructions. cDNA was obtained using SuperScript III reverse transcriptase and an oligo(dT₁₆) primer. The products of reverse transcription were amplified with GeneRacer 5' Nested primer and hZip1 specific reverse primer 5'-CTCCTTGTAAGCCAGTGTGATCT and then sequenced in the Fox Chase Cancer Center Automated DNA Sequencing Facility.

2.4 The reporter vectors design

Genome DNA from PC-3 cells was amplified with forward 5'-ATCTTGGGTACCCTTGTCTTTTCCTTGTGTGGGTG and reverse 5'-ATCTTGCTCGAGGGTAAGTTTGGGTGCTGGATCGCTC primers, and the 840bp PCR product was cloned into the Kpn1/Xho1 restriction sites of pGL3-basic luciferase reporter vector (Promega, Madison WI). This fragment was used for subsequent generation of 5'- and 3'- deleted constructs as well as to generate the hZip1 promoter and site-directed mutagenesis (primers listed in Supplementary, Table 1A).

Transcription factor binding sites inside the hZip1 core promoter were mutated by PCR using modified reverse primers spanning the targeted binding site (Supplementary, Table 1B). The product was subsequently ligated with a 3'-flanking region, re-amplified, and cloned. All reporter pGL3 vectors we generated have been sequenced using GL2 primer (Promega, Madison WI) in the Fox Chase Cancer Center Automated DNA Sequencing Facility.

2.5 Transient transfection and luciferase reporter assay

6×10^4 PC-3 cells were placed into 24-well plates and incubated for 24 hours in complete medium as described before. Then the cells were transfected with 0.5 μ g of either pGL3 reporter vectors with various hZip1 promoter inserts, pGL3-basic vector, or pGL3-promoter vector. 0.01 μ g of phRL-TK plasmid was additionally transfected in each well to normalize for transfection efficiency. Transfection was carried out using TransIT Prostate transfection kits (Mirus, Madison WI) according to manufacturer instructions. Firefly and Renilla luciferase activities were measured in cell lysates 24 hours after transfection using the DualGlo Luciferase Assay System (Promega, Madison WI). All experiments were repeated three times and results were normalized using Renilla luciferase activity.

2.6 Knockdown of SP1 and CREB1 mRNA

Transfection of PC-3 cells with 250 pmol (per well) of siRNA duplexes targeting human SP1 (sc-29487) and/or CREB1 (sc-29281) (Santa Cruz Biotechnology Inc., Santa Cruz CA) mRNA was performed in 6-well plates using DharmaFECT-1 (Dharmacon, Lafayette, CO) transfection reagent according to the manufacturer instructions. siRNA duplexes which do not bind with RISC complex (siControl RISC-free siRNA Dharmacon, Lafayette, CO) were used as a negative control for siRNA transfection. 48 hours after transfection cells were collected for protein and RNA extraction.

2.7 Western blot analysis

Whole cell lysates were prepared by boiling in lysis buffer containing 66mM Tris-HCl pH-7.6 and 2% SDS. Protein concentrations were determined by Bradford assay (BioRad, Hercules CA). Western blot analysis was then carried out using specific anti-SP1 (E-3) or anti-CREB1 (C-21) antibodies (Santa Cruz Biotechnology Inc., Santa Cruz CA) as described previously (Crispen et al., 2007).

2.8 RT-PCR analysis of hZip1 expression

Total RNA was isolated from cells using a MINI RNA isolation II Kit (Zymo-Research, Orange, CA) and purified using a DNA-Free RNA Kit (Zymo-Research). Reverse transcription (RT) of 1 μ g total RNA was subsequently carried out in total volume of 20 μ l, using 200 units of SuperScriptIII reverse transcriptase (Invitrogen) and 25pM of oligo(dT)₁₆ primer. Then cDNA was amplified with forward 5'-CCCTGAGCCTAGTAAGCTGTTTC and reverse 5'-CTCCTTGTCAGCCAGTGTCTATCT primers, and 1 μ l of cDNA from the RT reaction. Amplification of the GAPDH gene was used as an internal control for all RT reactions. The primer pair specific for GAPDH was 5'-ATGGGGAAGGTGAAGGTC (forward) and 5'-TCAGGCATTGCTGATGATCTT (reverse).

2.9 Electrophoretic mobility shift (EMSA) and Supershift assays

Double-stranded DNA oligonucleotides were generated by annealing of complementary single-stranded oligos (Supplementary, Table 2). Fifty micrograms of each complementary single-stranded oligos were mixed in 250 μ l of annealing buffer containing 20mM Tris-HCl (pH-7.6), 50mM NaCl, and 10mM MgCl₂, and then warmed to 95°C for 5 minutes and slowly cooled overnight. One microgram of each annealed DNA oligos was labeled with 20 μ Ci of [γ -³²P]ATP using T4 Polynucleotide kinase (New England Biolabs, Ipswich MA) and purified on a Sephadex G25 MicroSpin column (GE Healthcare Ltd, Buckinghamshire UK).

Four micrograms of nuclear extract proteins (nuclear extract preparation in Supplementary) were equilibrated for 20 minutes at 25°C in 20 μ l of binding buffer containing 25mM HEPES (pH-7.6), 30mM KCl, 5mM MgCl₂, 5% Glycerol, 1 $\times$ Protease Inhibitors Cocktail with 1 μ g of poly(dIdC) and 500 μ g/ml bovine serum albumin. Twenty nanograms (10–20,000 Cpm)

of ^{32}P -Labeled double-stranded DNA oligos spanning the hZip1 core promoter were added and incubated for another 20 min at 25°C. In competition assays nuclear extract proteins were pre-incubated with binding buffer and 100 fold molar excess of unlabeled double-stranded DNA oligos identical to the labeled probe. For the gel-supershift assay 2µg of anti-SP1 (E-3) or 2µg of anti-CREB1 (C-21) antibodies (Santa Cruz Biotechnology Inc., Santa Cruz CA) were added to the binding reaction after 20min of pre-incubation with poly(dIdC) and/or specific competitor oligo. Incubation was continued 20min at 25°C.

Reaction mixtures were separated using a 6% polyacrylamide gel in 0.5×TAE for 4h at 140V. Separated complexes were detected by autoradiography after the gels were dried onto filter paper.

2.10 Chromatin immunoprecipitation (ChIP) assay

ChIP experiments were performed according to the protocol provided with the EZ-ChIP assay kit (Upstate USA, Inc., Charlottesville, VA). Briefly, 10^6 cells were cross-linked with 1% formaldehyde, collected, and washed twice with ice-cold PBS containing protease inhibitors provided in the kit. Cells were re-suspended in 100 µl of sodium dodecyl sulfate (SDS) lysis buffer on ice and then sonicated with 4 sets of 10 second pulses by a Kontes Ultrasonic Cell Disrupter sonicator to an average DNA size of 200–400 bp. The chromatin was pre-cleared with salmon sperm DNA protein G-agarose beads for 1 h, followed by an overnight incubation at +4°C with 2µg of anti-SP1 (E-3) antibodies or 2µg anti-CREB1 (H-74) antibodies (Santa Cruz Biotechnology INC, Santa Cruz CA). Antibodies against normal mouse IgG and RNA-Polymerase II were provided with the EZ-ChIP assay kit and used to control immunoprecipitations. Chromatin-antibody complexes were collected by re-incubation with protein G-agarose beads for 1 hour. Chromatin was eluted from the beads and cross-links were reversed at 65°C for 4 h. 4% of ChIP and 4% of input DNA were used for PCR. PCR was performed using primer pairs specific for hZip1 promoter 5'-AGGGTCTCACTACATGGTCTCCGC (forward) and 5'-GCTCTCGCTCACTCTCCTCAGGTC (reverse). PCR products were run in 2% agarose gel and visualized by ethidium bromide staining.

3. RESULTS

3.1 Bioinformatics analysis of the hZip1 promoter

Promoter region prediction and gene analysis were performed using the EIDorado and Gene2Promoter online programs. Two 600bp Genomic DNA fragments were predicted as hZip1 promoter regions in the *Homo sapiens* chromosome 1 genomic contig (GenBank accession number NT_004487). These two promoter regions correspond to two different mRNA types (GenBank accession numbers AK074943 and AK075257) and have a 250bp common region. Therefore, we focused our study on the entire length of the two overlapping promoter regions. The hZip1 promoter region appears to be a TATA-less, G/C-rich region without a canonical transcription initiation site. As a C/G-rich promoter, the hZip1 promoter region has 16 potential binding sites for SP1, as well as binding sites for other transcription factors, including AP1, NF-κB, CREB/ATF, GATA1, C/EBP-alpha, and v-Myb. The alignment of predicted human and mouse Zip1 promoter regions demonstrates a high degree of similarity and conservation, especially near the transcription start site (TSS) (Fig. 1). This suggests that transcription of Zip1 may be regulated by similar mechanisms across mammalian species.

3.2 Mapping of the hZip1 transcription start site

To identify the TSS we performed 5'RACE by ligation of the RNA-oligo to decapped mRNA with subsequent amplification of the 5' ends of hZip1 cDNA. Then, by comparing sequences

of the PCR product with the 5'-region of AK075257 cDNA from GenBank, we identified the TSS in position -836 upstream from the hZip1 translation start site (ATG) (Supplementary, Fig. 1). In light of this finding, we refer to TSS -836 as the +1 position in all described experiments.

3.3 Transcription activity of the hZip1 promoter region

To construct a hZip1 luciferase reporter system, a -366/+472 relative to transcription start site fragment of PC-3 DNA representing the hZip1 promoter region was amplified and cloned into a pGL3 reporter vector upstream from the firefly luciferase gene. This vector was used as a template to create various 5'- and 3'-deletion constructs as illustrated in Figure 2.

The hZip1 promoter -224/+167 region demonstrated high transcriptional activity in the luciferase reporter assay (Fig. 2A), comparable to the SV40 promoter. After further 3'- and 5'-deletions were performed, a -224/+82 fragment demonstrated the highest transcriptional activity. This region was considered the core promoter (Figures 2B and 2C).

Computer analysis of the hZip1 -224/+82 core promoter region using the TFSEARCH, AliBaba2.1 and MatInspector programs revealed putative binding sites for several transcription factors (Fig. 3). SP1, GATA1, CREB/ATF1 and NF- κ B had high match scores and were evaluated further. To determine the actual contribution of each transcription factor to regulation of hZip1 promoter basal activity, we created constructs with mutated binding sites for these transcription factors within the hZip1 core promoter (Fig. 4A). Mutation of the GATA1 consensus sequence at positions -118/-125 did not affect transcriptional activity of the hZip1 core promoter. In contrast, mutation of the CREB binding site at position -104 reduced transcriptional activity by 50% (Fig. 4B). Since several SP1 binding sites were identified within the hZip1 core promoter region, we initially mutated three SP1 sites in closer proximity to the transcription start site—specifically positions -8, -12, and -30 (Fig. 4B). Simultaneous mutations of all three sites reduced transcriptional activity of the hZip1 core promoter by approximately 75%, as demonstrated in Figures 4B and 4C. Concomitant mutations of the CREB and SP1 binding sites produced an even more profound effect on hZip1 transcriptional activity (Fig. 4B).

To further characterize the relative contributions of the three SP1 sites toward regulation of hZip1 basal transcription, we generated mutations that would maintain only one intact SP1 binding site (Fig. 4A). A luciferase reporter assay (Fig. 4C) revealed that the SP1 binding site in position -8 plays the most important role in hZip1 promoter activation, as mutation of this particular site dramatically reduced transcriptional activity of the hZip1 promoter. We also evaluated the contribution of the SP1 binding site at the -80 position. Although this binding site is oriented in the opposite direction of the consensus sequence, it proved equally critical to transcriptional activation of the hZip1 promoter (Fig. 4C). Thus, our experiments demonstrate that both SP1 and CREB proteins serve as important transcription factors in the regulation of hZip1 basal promoter activity.

Because the NF- κ B binding site included the TSS and mutation of this binding site might impair transcription initiation, mutation of this site was not performed. Stimulation of NF- κ B with TNF- α did not produce increased expression of hZip1, and the NF- κ B inhibitor BAY-1185 also had no effect on hZip1 expression (data not shown). Further evaluation of the role of NF- κ B was performed by EMSA (below).

3.4 Analysis of transcription factors ability to bind the hZip1 promoter

To investigate the ability of transcription factors to bind the hZip1 core promoter, EMSA and Gel Supershift assays were performed. For EMSA we created several 30bp double-stranded

DNA oligos covering the hZip1 core promoter region, which contained putative transcription factor binding sites around the transcription start site as illustrated in Figure 3. The ability of the nuclear extract proteins to bind [γ - 32 P]-labeled oligos was examined by EMSA using specific competitors. The nuclear extract proteins bound specifically to DNA-oligos #1, #2, #3, #4, and #5 (Figures 5 and 6). In the cases of DNA-oligos #6 and #7 there was no specific binding with nuclear extract proteins (Fig. 5D). To determine which transcription factors could bind to the oligos, we used specific competitors in the form of double-stranded DNA-oligos containing consensus binding sites for SP1, CREB, GATA1, and NF- κ B transcription factors and mutated analogs. The EMSA results demonstrate that only SP1 and CREB proteins were able to bind corresponding DNA-oligos at their consensus binding sites (Figures 5A and 6). We also performed Gel Supershift, whereby six SP1 binding sites located in the hZip1 -224/+82 core promoter were able to interact with SP1. Likewise, CREB1 transcription factor was allowed to interact with its binding site on oligo #2 (Fig. 5B). We observed specific binding between SP1 and its corresponding binding sites at the -8, -80, and -140 positions (Fig. 6). We did not identify SP1 binding with oligo #4 which contained SP1/-12 and SP1/-30 binding sites. These GelSupershift results confirm the luciferase assay findings that SP1 at the -8 and -80 positions and CREB1 play a critical role in hZip1 promoter activity but that binding sites at -12 and -30 are less involved in regulating transcription.

3.5 Specific binding to hZip1 promoter of SP1 and CREB1 transcription factors

To identify if SP1 and CREB1 specifically bind to the hZip1 promoter *in vivo*, a ChIP assay was performed. Sonicated chromatin samples were pre-cleared with Protein-G agarose beads and then precipitated overnight with the specific antibodies against SP1 and/or CREB1, or control antibodies against normal mouse IgG or RNA-polymerase II. After 1 hour incubation with Protein-G agarose beads, washing and elution followed by reverse crosslinking, DNA samples were purified and analyzed by PCR with primers to the -174/+51 region of the hZip1 core promoter. As expected, we observed the specific 225 bp PCR product when chromatin had been precipitated using anti-SP1 or anti-CREB1 antibodies, as well as in the positive control (anti-RNA-polymerase II antibodies); antibodies against normal mouse IgG as a negative control following PCR did not reveal any positive signal (Supplementary Fig. 2).

3.6 Knockdown of the SP1 and CREB1 proteins has a negative effect on hZip1 transcription

After we identified crucial roles for SP1 and CREB1 proteins in transcriptional activation of the hZip1 core promoter, their roles in transcription regulation of the hZip1 gene were evaluated. Knockdown of SP1 and CREB1 was performed by transfection of the siRNA duplexes targeting their mRNAs. As it was supposed, we found comparable hZip1 mRNA levels in PC-3 cells after 48 hours of treatment with RISC-free siRNA duplexes or non-transfected PC-3 cells. However, we observed low levels of hZip1 mRNA in PC-3 cells after 48 hours of anti-SP1, anti-CREB1, and combination siRNA treatment (Fig. 7).

4. DISCUSSION

Studies have shown that the transformation of benign prostate epithelial cells into adenocarcinoma is consistently associated with loss of the ability to accumulate intracellular zinc through down-regulation of the hZip1 zinc transporter (Franklin et al., 2003; Rishi et al., 2003; Franklin et al., 2005). To further understand this process, we explored the mechanisms regulating hZip1 gene expression.

hZip1 is a housekeeping gene essential to normal cell function and appears to be evolutionarily conserved (Dufner-Beattie et al., 2003). With the conservation of the protein and nucleotide structure, there also may be common mechanisms of transcriptional regulation across species.

Indeed, our comparison of human and mouse Zip1 gene 5'-flanking region sequences revealed a high degree of similarity, especially near the transcription initiation sites.

To locate the core promoter in PC-3 human prostate cancer cells, we obtained an 830-bp DNA fragment representing the predicted promoter region and first exon of the hZip1 gene. The minimal hZip1 core promoter region was identified through luciferase assays as a -224/+82 fragment relative to the transcription start site. This fragment is responsible for basal hZip1 promoter activity, which is comparable to that of the SV40 promoter. To our knowledge, the core promoter region has not been previously identified in the literature.

The experimentally determined by 5'RACE main hZip1 transcription initiation site was -836; whereas the initiation site identified for the AK075257 transcript in the NCBI database is -858. The hZip1 gene appears to lack canonical CCAAT, TATA box, and initiator elements in its GC-rich promoter region. Such promoters containing CpG islands are known to exhibit multiple transcription initiation sites that can be alternatively active (Swick et al., 1989; Geng and Johnson, 1993; Liu et al., 2006; Sandelin et al., 2007). In the case of PC-3 prostate cancer cells, our findings suggest an alternative main transcription initiation site for hZip1. Transcription continued when the main initiation site was inactivated. We found that transcription of a hZip1(-224/-30) fragment lacking the main transcription initiation site remained 30% active compared to baseline. The continued transcription of a housekeeping gene despite disruption of its main initiation site is highly important for cell survival.

Our computer analysis, functional luciferase reporter assays, mutations analysis, GelSupershift, and ChIP experiments identified SP1 as an important transcription factor in the control of hZip1 expression, particularly at the -8 and -80 positions. Previous in vitro studies have shown that SP1 can direct transcription initiation from heterogeneous TSSs in core promoters that lack both TATA and initiator elements (Smale and Kadonaga, 2003; Muckenfuss et al., 2007; Yang et al., 2007). Moreover, GC-rich promoters are characterized by multiple SP1 binding sites, and their activity is often controlled by SP1 (Kingsley and Winoto, 1992; Toonen et al., 1996; Seyed and Dimario, 2007; Yu et al., 2007). The SP1 family of proteins has been implicated in a host of essential biological processes, including apoptosis, cell growth inhibition, differentiation, and carcinogenesis (Lee et al., 2005).

Gel Supershift, luciferase, and ChIP assays also identified CREB1 as an important regulator of hZip1 promoter activity. Signaling pathways through CREB proteins are known to be important for cell survival and proliferation (Mayr and Montminy, 2001). In general, CREB binding sites are located between the -50 and -150 positions relative to a start site and can produce both basal and inducible transcription (Quinn, 1993; Felinski and Quinn, 1999; Mayr and Montminy, 2001).

Furthermore, our data suggest an additive transcriptional regulation of the hZip1 promoter by SP1 and CREB1 transcription factors. Concomitant SP1 and CREB binding site mutation led to an 80% loss of hZip1 promoter transcriptional activity; whereas isolated mutations of SP1 or CREB binding sites resulted in loss of approximately 75% and 50% of activity, respectively.

Previous studies have demonstrated interactions between SP1 and various proteins and transcription factors that proved crucial to regulation of many genes (Kavurma et al., 2002; Lee et al., 2002; Lee et al., 2005). In fact, investigators have reported concomitant activity of SP1 and CREB1 in other core promoters (Mahapatra et al., 2006; Gee et al., 2007; Piera-Velazquez et al., 2007).

The data suggesting that SP1 and CREB1 additively regulate hZip1 promoter activity was supported by ChIP experiments, which demonstrated that these transcription factors specifically bind with the hZip1 core promoter -174/+51 region.

Additionally, we have shown that specific knockdown of SP1 and CREB1 proteins using siRNA duplexes leads to impairment of hZip1 gene transcription.

To avoid the anti-tumor effects of intracellular zinc, malignant prostate cells silence hZip1 gene expression (Rishi et al., 2003; Franklin et al., 2005). Re-establishment of normal intracellular zinc levels is an attractive target for prostate cancer therapy. This study begins to identify the mechanisms responsible for constitutive expression of the hZip1 gene. Increased understanding of the regulatory mechanisms behind hZip1 gene expression could provide the basis for further research to produce therapeutic up-regulation of hZip1 expression in prostate adenocarcinoma.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

BPH	Benign Prostatic Hyperplasia
ChIP	Chromatin Immunoprecipitation
EMSA	Electronic Mobility Shift Assay
GAPDH	Glyceraldehyde 3-phosphate Dehydrogenase
MTF1	Metal-activated Transcription Factor 1
PIN	Prostate Intra-epithelial Neoplasia
RACE	Rapid Amplification of cDNA Ends
TSS	Transcription Start Site
ZIP	Zrt/IRT-like Protein

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Fig. 1. Alignment of the human and mouse Zip1 promoter regions around the transcription start site (TSS, marked by arrow) demonstrates highly conservative consensus sequences for transcription factor binding sites (marked by squares).

Fig. 2.

PC-3 cells were transfected with pGL3 luciferase reporter vectors, and 24 hours after transfection luciferase activity was measured. Transcriptional activity of the hZip1 promoter region fragments is represented by the histograms. The data are presented as the mean ($\pm$ SD) of three independent experiments performed in triplicates. **(A)** Transcriptional activity of the -366/+472 fragment, as well as 3'- and 5'-deleted fragments. The -224/+167 fragment was then subjected to further deletion analysis. **(B)** Generation of 5'- and 3'-deletion constructs from the hZip1 promoter region identified the -224/+82 fragment as having the highest transcriptional activity. This was considered the hZip1 core promoter. **(C)** Schematic illustration of 5'- and 3'-deleted fragments of the hZip1 core promoter with the locations of putative transcription factor binding sites (marked by arrows) cloned upstream of the firefly luciferase gene.

Fig. 3. The hZip1 core promoter sequence around the TSS (marked by arrow), transcription factor binding sites (marked by squares), and the locations of dsDNA oligos used as probes for EMSA (marked by arrows below the sequence).

Fig. 4.

PC-3 cells were transfected with pGL3-hZip1(-225/+85) vectors with mutated transcription factor binding sites. After 24 hours the luciferase assay was performed. The data are presented as the mean ($\pm$ SD) of three independent experiments performed in triplicates. **(A)** Schematic illustration of the -225/+82 fragments with transcription factor binding sites inactivated by mutagenesis (underlined). **(B)** Mutation of the GATA1 consensus sequence does not influence transcriptional activity of the hZip1 core promoter; whereas selective mutation of CREB and SP1 binding sites impaired approximately 50% and 75% of activity, respectively. Concomitant mutation of SP1 and CREB binding sites led to the loss of more than 80% of hZip1 core promoter activity. This suggests an additive effect upon hZip1 promoter activation. **(C)** Of the SP1 binding sites at the -8, -12, -30 and -80 positions the SP1/-8 and SP1/-80 binding sites are the most important in regulating hZip1 core promoter activation.

Fig. 5.

Analysis of the nuclear extract proteins ability to bind dsDNA oligos targeting the hZip1 promoter using ESMA. Nuclear extract proteins were incubated with ^{32}P -labeled dsDNA oligos #2, #5, #6 and #7 with 100-fold molar excess of unlabeled competitors. **(A)** Using specific competitors containing consensus binding sites for GATA and/or CREB, we show that CREB but not GATA proteins specifically bind to oligo #2. **(B)** The GelSupershift assay with specific anti-CREB1 antibodies was performed to detect CREB1 transcription factor binding to oligo #2. **(C)** There was no specific binding of NF-κB with oligo #5. Specific competitors containing the NF-κB consensus sequence did not change the ESMA profile. **(D)** Nuclear extract proteins did not bind to oligos #6 and #7.

Fig. 6.

Characterization of SP1 binding with the hZip1 promoter using a GelSupershift assay. Nuclear extract proteins were incubated with specific ^{32}P -labeled dsDNA oligos #1, #3, #4, and #5 in the presence of 100-fold molar excess of their unlabeled competitors. ^{32}P -labeled SP1-oligo was used as a control. SP1 was shown to bind all examined oligos except oligo #4. Comparison of GelSupershift profiles of SP1 binding with oligos #4 and #5 demonstrates specific binding with only the SP1/-8 binding site.

Fig. 7.

Down-regulation of the SP1 and CREB1 proteins by siRNA leads to impairment of hZip1 gene expression. PC-3 cells were transfected with siRNA duplexes targeting SP1, CREB1, and a combination of both. After 48 hours cells were harvested and lysed for western blot and Reverse Transcription PCR assay. **(A)** Total PC-3 cell lysates were subjected for Western Blot Analysis with the appropriate antibodies to evaluate if siRNA treatment of PC-3 cells specifically down-regulates levels of SP1 and/or CREB1 proteins. **(B)** 1 μ g of PC-3 total RNA was reverse transcribed with oligo(dT)₁₆ primers, and then cDNA was amplified with hZip1 or GAPDH specific primers.