Regulation of expression of the stress response gene, *Osp94*: identification of the tonicity response element and intracellular signalling pathways

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Osp94 (osmotic stress protein of 94 kDa) is known to be up-regulated by hypertonic and heat-shock stresses in mouse renal inner medullary collecting duct (mIMCD3) cells. To investigate the molecular mechanism of transcriptional regulation of the Osp94 gene under these stresses, we cloned and characterized the 5′-flanking region of the gene. Sequence analysis of the proximal 4 kb 5′-flanking region revealed a TATA-less G/C-rich promoter region containing a cluster of Sp1 sites. We also identified upstream sequence motifs similar to the consensus TonE/ORE (tonicity-response element/osmotic response element) as well as the consensus HSE (heat-shock element). Luciferase activities in cells transfected with reporter constructs containing a TonE/ORE-like element (Osp94-TonE; 5′-TGGAAAGGACCAG-3′) and HSE enhanced reporter gene expression under hypertonic stress and heat-shock stress respectively. Electrophoretic gel mobility-shift assay showed a slowly migrating band binding to the Osp94-TonE probe, probably representing binding of TonEBP (TonE binding protein) to this enhancer element. Furthermore, treatment of mIMCD3 cells with MAPK (mitogen-activated protein kinase) inhibitors (SB203580, PD98059, U0126 and SP600125) and a proteasome inhibitor (MG132) suppressed the increase in Osp94 gene expression caused by hypertonic NaCl. These results indicate that the 5′-flanking region of Osp94 gene contains a hypertonicity sensitive *cis*-acting element, Osp94-TonE, which is distinct from a functional HSE. Furthermore, the MAPK and proteasome systems appear to be, at least in part, involved in hypertonic-stressmediated regulation of Osp94 through Osp94-TonE.

Key words: heat-shock element, heat-shock stress, hypertonic stress, Osp94 gene, signal transduction, tonicity response element.

INTRODUCTION

Cells in the mammalian kidney inner medulla are routinely exposed to drastic changes in osmolarity by virtue of the urine-concentrating and -diluting mechanisms. The cells, however, adapt to the hyperosmotic environment by accumulating non-perturbing compatible organic osmolytes such as sorbitol, betaine, myo-inositol and taurine, leading to the restoration of the intracellular volume and a decrease in harsh ionic strength [1]. In recent years, several groups isolated cDNAs of the metabolic enzyme, AR (aldose reductase) [2] and transporter genes [3–5] responsible for intracellular accumulation of such organic osmolytes, and identified a *cis*-acting element [known as TonE (tonicity-responsive element) or ORE (osmotic response element)] responsive to hypertonicity in the 5′-flanking region of AR genes in rabbit [6], mouse [7], rat [8] and human [9], as well as the canine betaine transporter gene, BGT1 (betaine-γ-aminobutyric acid transporter) [10] and the bovine Na^+/myo-inositol transporter gene, SMIT [11].

Previous reports showed that in renal epithelial cells, MAPK (mitogen-activated protein kinase) cascades including ERKs (extracellular-signal-regulated kinases) [12,13], JNKs (c-Jun N-terminal kinases) [13–15] and p38 MAPK [13–15], are involved in the intracellular signal transduction of hypertonic stress. Recently, a cDNA was isolated encoding TonEBP (TonE-binding protein), a member of the nuclear factor of an activated transcription family of transcription factors, which activates the expression of BGT1 gene in response to hypertonicity [16]. Most recently, the ubiquitin–proteasome proteolytic system was found to be required for the osmotic induction of BGT1 and SMIT genes and nuclear translocation of TonEBP in response to hypertonicity [17]. These observations have greatly enriched our understanding of the molecular mechanisms of cellular accumulation of the compatible organic osmolytes and intracellular signalling to adapt to the hyperosmotic environment.

In addition to accumulating osmolytes, renal inner medullary cells also protect themselves from the deleterious effects of hypertonicity by expressing a specific class of highly conserved stress proteins, known as heat-shock proteins (HSPs) [18]. HSPs are characterized as molecular chaperones and participate in numerous biological functions. They interact transiently with unfolded or partially folded proteins and thereby assist in the correct folding of proteins, participate in the assembly of proteins into oligomeric structures, transport proteins to specific intracellular locations, interact with cytoskeleton components and contribute to the refolding and degradation of misfolded proteins [19]. As is well known, the expression of HSPs under heat-shock stress is regulated by binding of HSF (heat-shock factor) to an HSE (heat-shock element) localized in the 5′-region of HSP genes [20]. In the native as well as cultured renal epithelial cells, HSP genes are highly expressed under heat-shock and hypertonic stress

Abbreviations used: AR, aldose reductase; BGT1, betaine-γ-aminobutyric acid transporter; DTT, dithiothreitol; EMSA, electrophoretic mobility-shift assay; ERK, extracellular-signal-regulated kinase; HSE, heat-shock element; HSF, heat-shock factor; HSP, heat-shock protein; IMCD3, inner medullary collecting duct cell line; mIMCD3, mouse renal IMCD3; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; ORE, osmotic response element; RT, reverse transcriptase; TonE, tonicity-responsive element; TonEBP, TonE-binding protein; UTR, untranslated region.

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conditions [21,22]. However, the molecular basis of regulation of the expression of HSP genes in response to hypertonicity is poorly understood.

Osp94 (osmotic stress protein of 94 kDa) was recently identified as a new member of the HSP110 family of HSPs known to be induced by hyperosmotic stress [23]. The expression pattern of Osp94 mRNA in the kidney parallels the tissue solute concentration gradient associated with the concentrating ability of the inner medulla [23]. Osp94 mRNA is increased in response to both hypertonic and heat-shock stresses in the cultured mIMCD3 (mouse renal inner medullary collecting duct cell line) cells [23,24]. However, the molecular mechanisms responsible for increased expression of Osp94 mRNA are unclear.

In the present study, to clarify the molecular basis of transcriptional regulation of the Osp94 gene, we isolated the 5′-flanking region of the gene and identified and functionally characterized the specific promoter/enhancer elements. In addition, we sought to distinguish whether thermal and hyperosmotic stresses activate transcription through the same or different enhancer elements, and we also examined the intracellular signalling pathways involved in the regulation of Osp94 gene expression.

MATERIALS AND METHODS

Cloning of the murine *Osp94* gene

To isolate the promoter region of the *Osp94* gene, a mouse genomic DNA library (Clontech, Palo Alto, CA, U.S.A.) was screened with ^{32}P-labelled 5′-UTR (5′-untranslated region) fragment (250 bp) derived from the Osp94 cDNA by digestion with *Bam*HI/*Xho*I. Several genomic clones were isolated, digested with a number of restriction enzymes and electrophoresed on 0.8 % agarose gel, and transferred to a nylon membrane (Duralon-UV membrane; Stratagene, La Jolla, CA, U.S.A.). Southern-blot hybridization analysis with the ^{32}P-labelled 5′-UTR fragment allowed us to create a restriction map of the 5′-flanking region and to orient correctly the clones with regard to the 5′-UTR. On the basis of our restriction map, we isolated a *Sal*I/*Sac*I-digested 8 kbp fragment, which hybridized to the 5′-UTR fragment probe. This 8 kbp fragment was subcloned into Bluescript II KS (+) vector (Stratagene). The 8 kb genomic fragment was further digested with *Sac*I and the resultant 4 kb fragment, still containing the 5′-UTR of Osp94, was completely sequenced (Children's Hospital Sequencing Core Service).

Reporter gene construction

The 4 kbp fragment described above was digested with *Tth*111I, and the resultant 2.8 kb fragment was used for the preparation of reporter gene constructions. Each fragment resulting from the digestion of the 2.8 kb fragment with *Hin*cII, *Apa*I, and *Eag*I was inserted into Bluescript II KS(+), digested with *Sac*I–*Xho*I, and directionally inserted into pGL3-basic vector (Promega, Madison, WI, U.S.A.) upstream of the luciferase coding sequence. In separate experiments, reporter constructs containing TonE enhancer sequences, which were previously reported in canine BGT1 gene [10] and rabbit AR gene [6], were prepared by annealing synthesized sense and antisense oligonucleotides containing the TonE, 5′-CGGAAAATCACCAG-3′ for BGT1 and 5′-TGGAAAAGTCCAG-3′ for AR, flanked by *Kpn*I–*Bgl*II, followed by ligation into the pGL3-promoter vector. The putative tonicity response element (5′-TGGAAAGGACCAG-3′), which spans from -919 to -907 of the *Osp94* gene, was also inserted into the pGL3-promoter vector at *Kpn*I–*Bgl*II. In addition, the HSE (mHSE, 5′-CGCGTCTGGAAGATTCCTGC-3′) located in the mouse *Hsp70* gene was also used to evaluate heat response of the putative TonE of the *Osp94* gene.

Cell culture and DNA transfection

IMCD3 cells [25] were grown to confluence in plastic dishes (10 cm diameter) in Dulbecco's modified Eagle's medium/Ham's F12 (DMEM/F12; Invitrogen, Gaithersburg, MD, U.S.A.) (1:1), supplemented with 10 % foetal bovine serum (Invitrogen) and 2 % penicillin–streptomycin (Invitrogen). For transfection experiments, cells were seeded into 6-well plates (35 mm well diameter) and grown for 24 h in isotonic medium to 60 % confluency. DNA constructs were purified with Qiagen plasmid Midi kits, according to the manufacture's protocol. IMCD3 cells were co-transfected with 2 μg of luciferase reporter construct and 1 μg of pSV-β-galactosidase reporter vector (Promega) using LIPOFECTAMINE™ plus (Invitrogen) in serum-free medium. After incubation at 37 °C for 6 h, the cells were incubated in isotonic (310 m-osmol/kg water) medium for 18 h, and then the cells were treated with either fresh isotonic or hypertonic (200 mM mannitol; 510 m-osmol/kg water) medium [24]. After incubation for 6 h, the cells were harvested by addition of 150 μl of lysis buffer (Promega). To assess the effect of heat shock on reporter gene expression, cells were incubated at 42 °C for 3 h [23], and the cell extracts were prepared and analysed for luciferase activity.

Luciferase assays

Cell lysates were analysed for luciferase activity with luciferase assay reagent (Promega) in accordance with the manufacturer's instructions, and the light emitted was measured by a Lumat LB 9507 luminometer (Berthold, Nashua, NH, U.S.A.). β-Galactosidase activity was determined using standard methods. The luciferase activity in relative light units was normalized to β-galactosidase activity and osmotic response was calculated as the hypertonic/isotonic ratio. The average of each activity from three wells of 6-well plates was used and the experiment was repeated at least three times, and the means $\pm$ S.D. were calculated for statistical analysis.

EMSA (electrophoretic mobility-shift assay)

Nuclear extracts were prepared by slightly modifying the methods described previously [9]. Briefly, mIMCD3 cells were cultured in plastic dishes (10 cm diameter), then treated with isotonic or hypertonic medium for 6 h, and the cells were scraped into 1.2 ml of PBS and centrifuged at 1500 ***g*** for 2 min at 4 °C. The pellet was resuspended in 300 μl of Buffer A [10 mM Hepes/KOH, pH 7.9/1.5 mM $MgCl_2$/10 mM KCl/0.5 mM DTT (dithiothreitol)/0.5 mM PMSF]. The cells were pelleted and resuspended in 300 μl of Buffer A containing 0.5 % Nonidet P40. The resuspended cells were placed on ice for 5 min and centrifuged for 2 min. The resultant nuclear pellet was resuspended in 150 μl of Buffer B (20 mM Hepes/KOH, pH 7.9/0.42 M NaCl/1.5 mM $MgCl_2$/0.2 mM EDTA/0.5 mM DTT/0.5 mM PMSF/25 % glycerol), incubated for 45 min at 4 °C with gentle agitation, and the nuclear debris was removed by centrifugation at 18 000 ***g*** for 30 min at 4 °C. The supernatant was used for EMSA and a measurement of protein concentration was made with a Bio-Rad protein assay kit using BSA as the standard.

The putative TonE enhancer element (13 bp fragment) in the 5′-flanking region of the *Osp94* gene was end-labelled with [α-^{32}P]ATP using T4 polynucleotide kinase, and then the probe was cleaned using a spin column (Probe Quant; Amersham Biosciences, Piscataway, NJ, U.S.A.). The probe (30 000 c.p.m.) was

incubated with 4 μg of nuclear extract in 20 μl of reaction buffer containing 10 mM Tris/HCl, pH 7.5, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM DTT, 50 mM NaCl, 2 μg of poly(dI-dC) and 4 % glycerol. After incubation at room temperature (25 °C) for 20 min, the reaction products were separated on 6 % polyacrylamide gel with 0.5 × TBE at 4 °C and visualized by autoradiography. For competition experiments, 100-fold excesses of unlabelled oligomers were added to the reaction mixture prior to the addition of labelled probe. In addition, 13 bp fragments with mutations of individual nucleotides at different sites were used for EMSAs to evaluate the capability of Osp94-TonE to bind TonEBP.

Western-blot analysis of MAPK activation

IMCD3 cells were grown to subconfluence in DMEM/F12 (1:1), supplemented with 10 % foetal bovine serum and 2 % penicillin–streptomycin. After treatment with serum-free medium for 24 h, cells were exposed to hypertonic stress for 3 h by the addition of 100 mM NaCl. At 15, 30, 60 and 180 min after NaCl treatment, cells were washed twice with ice-cold PBS (−), scraped and the cell suspension was centrifuged at 3300 ***g*** for 2 min. The pelleted cells were treated with a lysis buffer containing 25 mM Hepes/KOH, pH 7.9, 150 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 50 mM NaF, 1 mM Na_3VO_3, 0.5 % Triton X-100, 20 mM β-glycerophosphate, 1 mM PMSF, 5 mM DTT, protease inhibitor (Roche), followed by centrifugation at 5200 ***g*** for 10 min. The supernatant was collected, and the protein content was measured by the Bradford assay (Bio-Rad, Hercules, CA, U.S.A.) using BSA as the standard. The cell lysate (50–100 μg) was subjected to SDS/PAGE and electroblotted on to polyvinylidene difluoride membrane (Millipore, Bedford, MA, U.S.A.). The membrane was blocked for 2 h in 3 % skim milk with TBS (Tris-buffered saline: 20 mM Tris/HCl, pH 7.0/150 mM NaCl), washed with TBS, and then incubated with anti-rabbit polyclonal antibodies against phospho-p38 MAPK, phospho-ERK or phospho-JNK at a dilution of 1:1000 (Cell Signaling Technologies, Beverly, MA, U.S.A.) in blocking solution overnight at 4 °C. The membrane was then washed three times in TBS-0.1 % Tween 20 (TBST), incubated for 1 h with horseradishperoxidase-conjugated donkey anti-rabbit IgG (Amersham Biosciences, Arlington, IL, U.S.A.), washed with TBST, and the signal was detected using ECL® (Amersham Biosciences). For detection of the levels of total MAPKs and β-actin, antibodies (for MAPKs, 1:1000, Cell Signaling Technologies; for β-actin, 1:500, Sigma, St. Louis, MI, U.S.A.) were used after treatment of the same nylon membrane with stripping buffer (Restore, Pierce, Rockford, IL, U.S.A.) at 37 °C for 15 min.

Inhibitors of MAPKs and proteasome

IMCD3 cells were grown as described above. Cells in serum-free medium were pretreated with p38 MAPK inhibitor, SB20385 (Calbiochem, La Jolla, CA, U.S.A.), ERK inhibitor, PD98059 (Calbiochem) and/or U0126 (Calbiochem) and/or the JNK inhibitor, SP600125 (Calbiochem) at indicated doses for 1 h, and then the cells were exposed to hypertonic stress for 6 h by the addition of 100 mM NaCl and/or heat shock (42 °C) for 3 h. For the proteasome inhibition study, MG132 (Calbiochem) was added to mIMCD3 cells at the indicated doses for 1 h, followed by the hypertonic and/or heat-shock stresses. After the treatments, cells were washed twice with ice-cold PBS (−) and total RNA was isolated using the RNAzol B method (Tel-Test, Friendswood, TX, U.S.A.), in accordance with the manufacturer's instructions. Isolated RNA was used for Northern blot and RT (reverse transcriptase)–PCR analyses.

Northern-blot and RT–PCR analyses

Northern-blot analysis was performed as described previously [23]. Briefly, total (20 μg) RNA was fractionated on a 1 % agarose, 0.7 % formaldehyde denaturing gel and transferred overnight on to a nylon membrane. Blots were prehybridized and hybridized with [α-^{32}P]dCTP-labelled cDNA probes overnight at 42 °C in 40 % formamide, 10 % dextran sulphate, 4 × SSC (1 × SSC consisted of 150 mM NaCl and 15 mM sodium citrate, pH 7.0), 7 mM Tris/HCl (pH 7.6), 0.8 × Denhardt's solution, 20 μg/ml salmon sperm DNA and 0.5 % SDS. After hybridization, the blots were washed twice at room temperature (2 × SSC/0.1 % SDS, 20 min), once at 60 °C (0.2 × SSC/0.1 % SDS, 20 min) and then autoradiographed. Full-length probe of Osp94 cDNA was isolated by digesting the Osp94 cDNA [23] with *Not*I–*Sal*I. For mouse Hsp70 cDNA probe, it was cloned from mIMCD3 cells, identified by sequencing and processed for a preparation of its probe. Rehybridization of the membrane was performed by reprobing the same Northern membrane with each cDNA probe after washing at highly stringent conditions (80 °C, 10 mM Tris/HCl, pH 7.0/0.01 % SDS).

For quantification of mRNA level, RNA levels were assessed using RT–PCR. The RT and PCR reactions were performed using SuperScript II RNase H$^-$Reverse Transcriptase (Invitrogen) and HotStart DNA polymerase (Qiagen) respectively in accordance with the manufacturer's instructions. In brief, 2 μg of total RNA was reverse-transcribed using oligo(dT)primer in 20 μl of total volume and 50 μl of PCR mixture contained 2 μl of cDNA reaction mixture, 1 × PCR buffer, 1.5 mM $MgCl_2$, 200 μM dNTP, 0.2 μM of each primer and 2.5 units of *Taq* polymerase. The thermocycle procedure was as follows: 95 °C for 15 min, 23–24 cycles of 94 °C for 30 s, 58 °C for 30 s, 72 °C for 1 min, followed by one cycle at 72 °C for 10 min. The following primers were used for the PCR. For Osp94: sense, 5′-GCTTCCTCAACTGCTACATCG-3′ and antisense, 5′-CTCCTTGACTGCAGGAATCC-3′. For Hsp70: sense, 5′-TGCGGGCGGCGTGATGACGG-3′; and antisense, 5′-CCGCACCCTGGTACAGCCCA-3′. For GAPDH: sense, 5′-ATCACCATCTTCCAGGAGCGA-3′; and antisense, 5′-GCCAGTGAGCTTCCCGTTCA-3′. Before the experiment, the cycle number for PCR was determined for a quantitative evaluation of Osp94 expression levels as follows: PCRs were performed at cycles of 18, 21, 24, 27 and 30 for Osp94, and the resultant PCR products were quantified densitometrically using an imaging analyser, GelDoc 2000 system (Bio-Rad) [26] after agarose-gel electrophoresis. Similarly, the expression level of GAPDH mRNA was also determined with cycles of 17, 21, 25, 29 and 33. To evaluate the statistical significance of gene expression levels in the presence of the inhibitors, experiments were repeated at least three times.

Statistical analysis

The data were analysed by the one-way analysis of variance and the Dunnett's multiple-comparison test or non-parametric statistics. Results were expressed as means ± S.D., and the significance was assigned to a $P < 0.05$.

RESULTS

Isolation and characterization of the 5′-flanking region of the mouse *Osp94* gene

On the basis of library screening, a 4-kb mouse genomic fragment was isolated and sequenced for identification of the promoter region of the *Osp94* gene. Sequence of the 3′-end of the genomic fragment was identical to that published for the Osp94 cDNA [23].

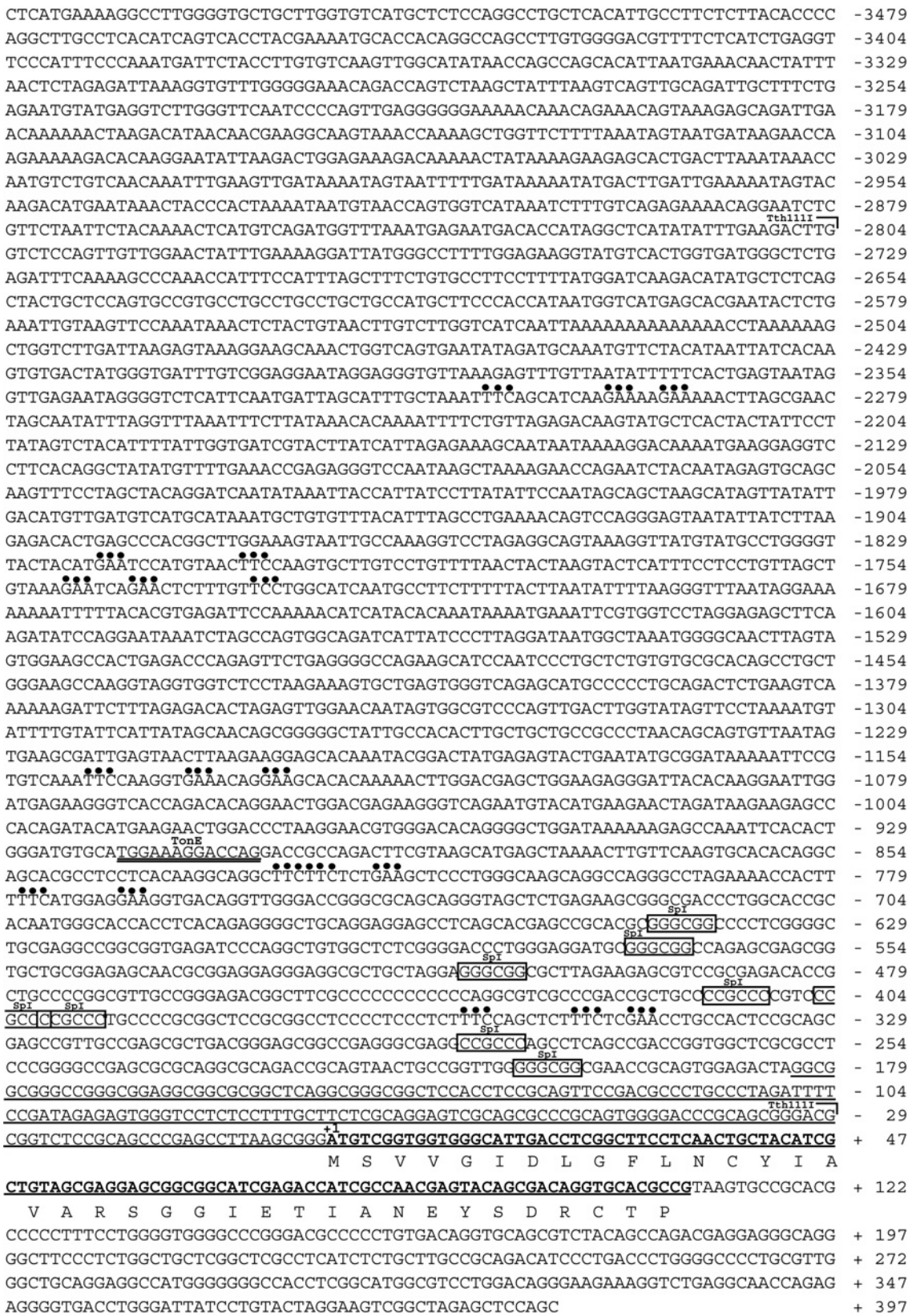

Figure 1 Entire sequence of the 4 kb *Osp94* 5′-flanking region and localization of a putative osmotic element

A mouse genomic DNA library was screened with ^{32}P-labelled 5′-UTR fragment (250 bp) derived from Osp94 cDNA. The isolated clones from library screening were digested with several different restriction enzymes, and digestion products were electrophoresed and subjected to Southern-blot hybridization analysis. Digestion of 8 kb genomic fragment with *Sac*I provided a 4 kb fragment to be subjected to complete sequencing. TonE-like sequence is double underlined. Putative HSE sequences are shown by dots. Boxes indicate Sp1 sites. Nucleotides matching the Osp94 cDNA sequence are underlined. Bold underlined letters show the cording region (+ 1 to + 109), and downstream sequences (+ 110 to + 397) denote an *intron*. Letters under the cording region indicate amino acid sequences. Numbering of nucleotides is from + 1 at the ATG.

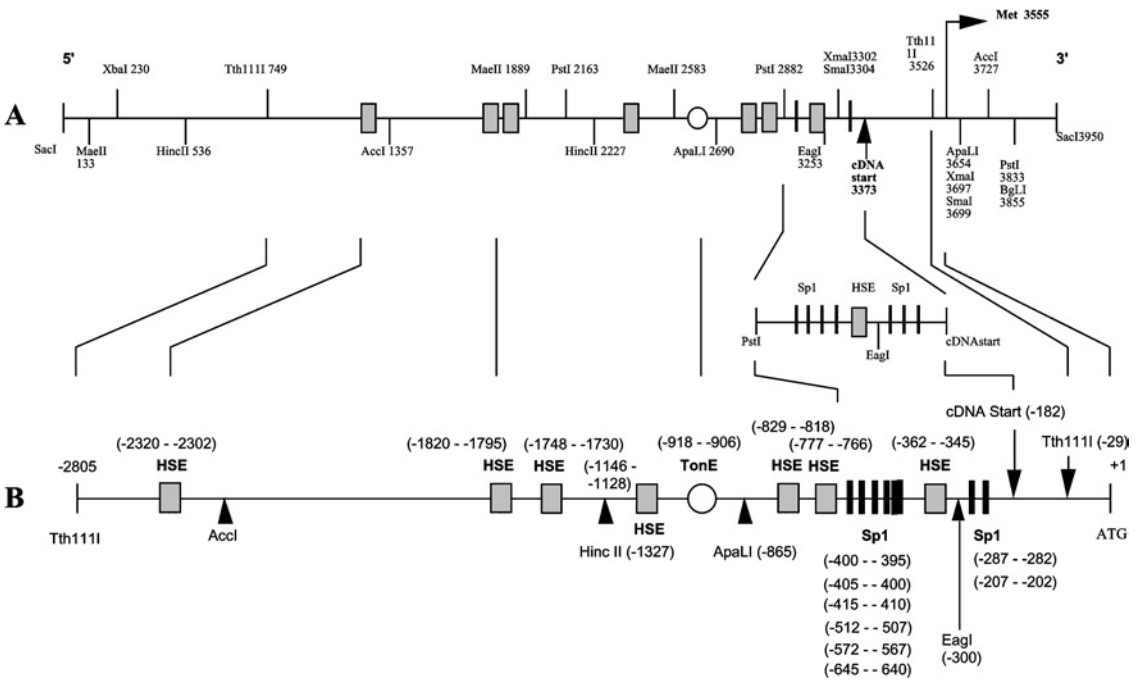

Figure 2 Schematic representation of the location of potential regulatory elements within a 2.8 kb *Osp94* genomic fragment

The 4 kb fragment described above was digested with *Tth*111I, and the resultant 2.8 kb fragment was used for the preparation of reporter gene constructions. (**A**, **B**) The 4 and 2.8 kb fragments respectively.

In addition, the sequence (from +51 to −677) of the obtained *Osp94* gene showed a sequence identical with that reported for the Apg-1 gene [27], which is known as a gene identical with Osp94. The translation start site (ATG) was defined as +1 to locate the position of potential regulatory sites in the present study. As shown in Figures 1 and 2, complete sequencing of the 4-kb 5′-flanking region of *Osp94* gene revealed a cluster of Sp1 sites at positions −172, −208, −288, −401, −406, −416 and −513 with some of them overlapping (−401, −406, −416). This 342 bp region contained seven Sp1 sites (−172 to −513) and was G/C-rich (77 %). Sequence motifs similar to HSE were also identified at positions −363, −791, −1749 and −2312. There is no TATA box in the promoter region and, as is typical of TATA-less promoters, multiple transcription start sites of *Osp94* were identified by primer extension experiments (results not shown). The most interesting feature of the promoter region was the presence, at positions −919 to −907 bp upstream of the translation start site, of a sequence (5′-TGGAAAGGACCAG-3′) similar to the TonE (5′-CGGAAAATCACCAG-3′) previously reported in the canine BGT1 gene responsible for the uptake of betaine [10].

Response of the *Osp94* gene to hypertonic and heat-shock stresses

We next sought to identify the *cis*-regulatory enhancer element(s) within the *Osp94* gene that are responsive to hypertonic and heat-shock stresses. Using the 2.8 kb upstream fragment, a series of truncation constructs were created for luciferase reporter assays. Figure 3(A) schematically illustrates these constructs. As shown in Figure 3(B), transient transfection assays in cultured mIMCD3 cells revealed that the TonE-like element was required for hypertonic stress (+200 mM mannitol) to enhance reporter gene expression; the deletion mutant constructs (pGL3-*Apa*LI and pGL3-*Eag*I) resulted in a loss of the transcriptional activation by hypertonicity. The construct (pGL3-*Apa*LI) containing the HSEs, without the putative TonE failed to respond to hypertonic stress. On the other hand, when the transfected cells were exposed to heat-shock stress at 42 °C for 3 h, the reporter constructs with HSE showed higher luciferase activity than that observed in control vector (pGL3basic) and the construct (pGL3-*Eag*I) without HSE. These results suggest that the TonE-like sequence at position −919 to −907 acts as TonE, Osp94-TonE, in the *Osp94* gene. Interestingly, the pGL3-*Apa*LI construct with the HSE was not responsive to hypertonic stress.

Identification of the TonE of Osp94

To examine further the capacity of Osp94-TonE to behave as a TonE, an Osp94-TonE oligonucleotide (5′-TGGAAAGGACCAG-3′) was ligated into an SV40 promoter-driven luciferase reporter construct. Similar to previous studies of the canine BGT1-TonE [10] and rabbit AR-ORE-driven reporter constructs [6], Osp94-TonE was transcriptionally responsive to hypertonic stress (Figure 4A). A reporter construct with multiple concatenated copies of Osp94-TonE (Osp94-TonE ×3) showed higher transcriptional activity when compared with that in the construct with a single copy (Osp94-TonE ×1). On the other hand, a point mutation from adenine to thymine (Osp94-TonEmAT) or from guanine to adenine (Osp94-TonEmGA) at nucleotide position 9 or 7 respectively abolished the osmotic response of the Osp94-TonE (Figure 4A). Thus, the experiments with base substitution in the Osp94-TonE indicate that guanine and adenine at positions 7 and 9 respectively of the 13 bp fragment, are at least required for eliciting the functional activity of Osp94-TonE.

To clarify whether Osp94-TonE responds to heat-shock stress, Osp94-TonE-transfected cells were incubated at 42 °C for 3 h. As shown in Figure 4(B), no significant increase in luciferase activity was observed in the Osp94-TonE-transfected cells under heat shock, although mHSE showed increased level of luciferase activity. These results indicate that the Osp94-TonE is a hypertonic, but not heat-shock, stress-responsive element.

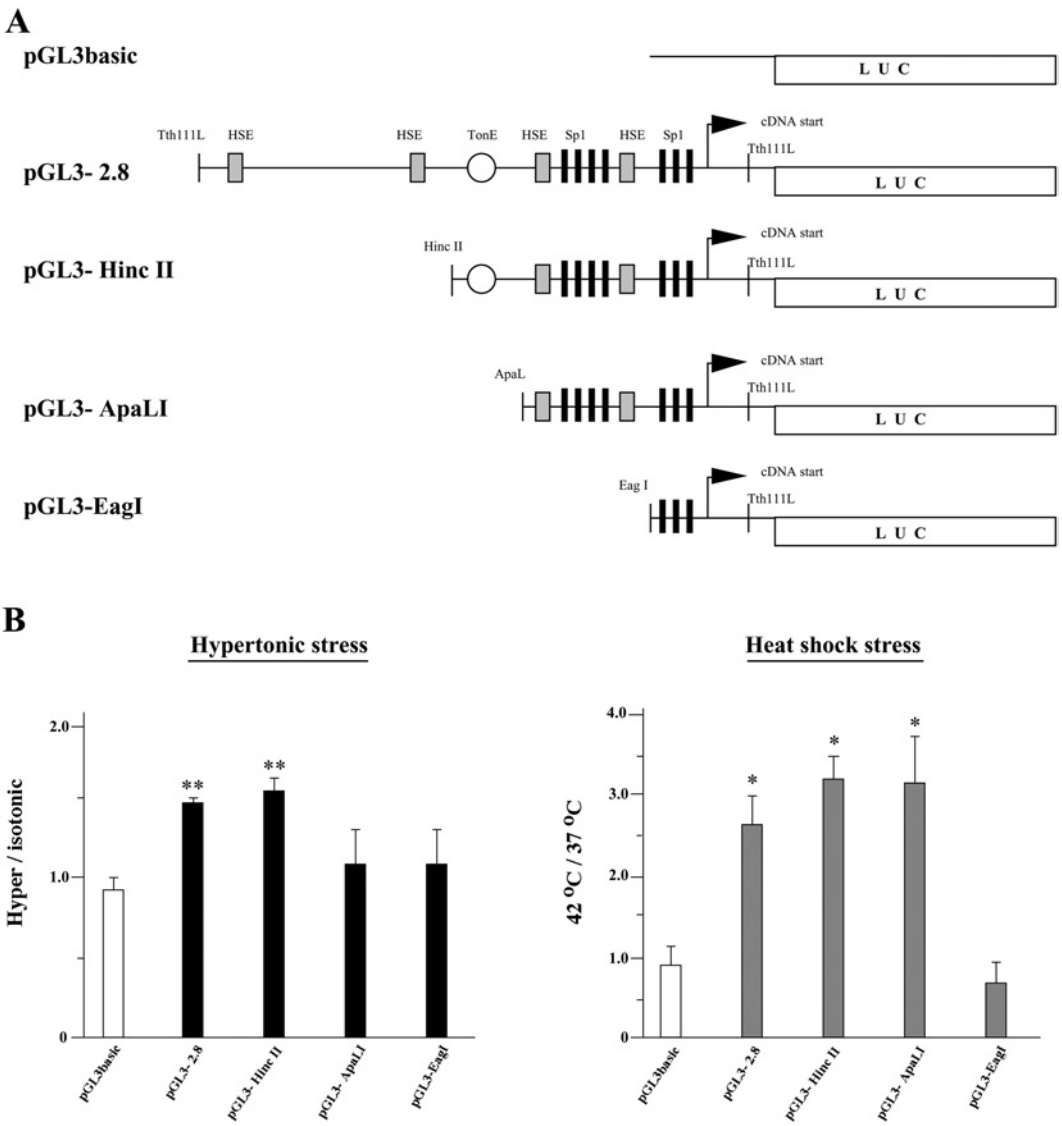

Figure 3 Luciferase reporter constructs and luciferase reporter analysis of the *Osp94* promoter activity in response to hypertonic and heat-shock stresses

(**A**) Schematic representation of luciferase reporter constructs used for the transfection in mIMCD3 cells. (**B**) Relative luciferase activity of the five reporter constructs in the cells exposed to hypertonic or heat-shock stress. Each fragment resulting from digestion of the 2.8 kb fragment with *Hinc*II, *Apa*II, and *Eag*I was inserted into Bluescript II KS(+), digested with *Sac*I/*Xho*I, and directionally inserted into pGL3-basic vector. DNA fragments of different sizes after enzymic digestion were inserted into pGL3-basic vector and transfected into the mIMCD3 cells. To assess the transfection efficiency, cells were co-transfected with pSV-β-galactosidase plasmid. Cells were treated with either the isotonic or hypertonic (200 mM mannitol) medium. In a separate experiment, transfected cells were exposed to heat-shock stress (42 °C for 3 h). Then, the cells were harvested, and the luciferase and β-galactosidase activities were determined as described in the Materials and methods section. Results are expressed in terms of luciferase activity relative to β-galactosidase and normalized to basal control level. Results in each panel are means $\pm$ S.D. and the number of independent transfections for each construct was $\geqslant 3$. $^{*}P < 0.05$, $^{**}P < 0.01$ versus pGL3-basic.

Tonicity-induced protein binding to Osp94-TonE

To examine whether hypertonicity enhances binding of a transcription factor, presumably TonEBP, to Osp94-TonE, EMSAs were performed using Osp94-TonE as a probe. As shown in Figure 5, a slowly migrating band (arrow) was evident when Osp94-TonE was incubated with nuclear extracts from mIMCD3 cells. With hypertonic treatment, there was an increase in the amount of protein bound to Osp94-TonE. This gel-shifted protein binding was eliminated by the addition of excess unlabelled probe, indicating the specificity of the interaction of Osp94-TonE and its binding protein. Thus, under hypertonic conditions, there is enhanced binding of a protein, perhaps TonEBP [16], to the Osp94-TonE element.

Signalling pathways involved in Osp94 gene expression under hypertonic and heat-shock stresses

Figure 6 presents the time course of activation of MAPKs after hyperosmotic stimuli. NaCl-treated mIMCD3 cells resulted in the time-dependent activation of the three MAPKs, namely, p38, ERK and JNK with their highest activation at 30 min after the treatment, indicating that MAPK signalling pathways participate in cellular response to hypertonic stress.

Treatment of mIMCD3 cells with SB203580, a p38 MAPK inhibitor, showed a marked decrease in Osp94 mRNA level induced by hypertonic stress in a dose-dependent manner (Figure 7A) and RT–PCR analysis substantiated the result of Northern-blot analysis in Figure 7 (Figure 8C). PD98059, a MEK (MAPK/ERK

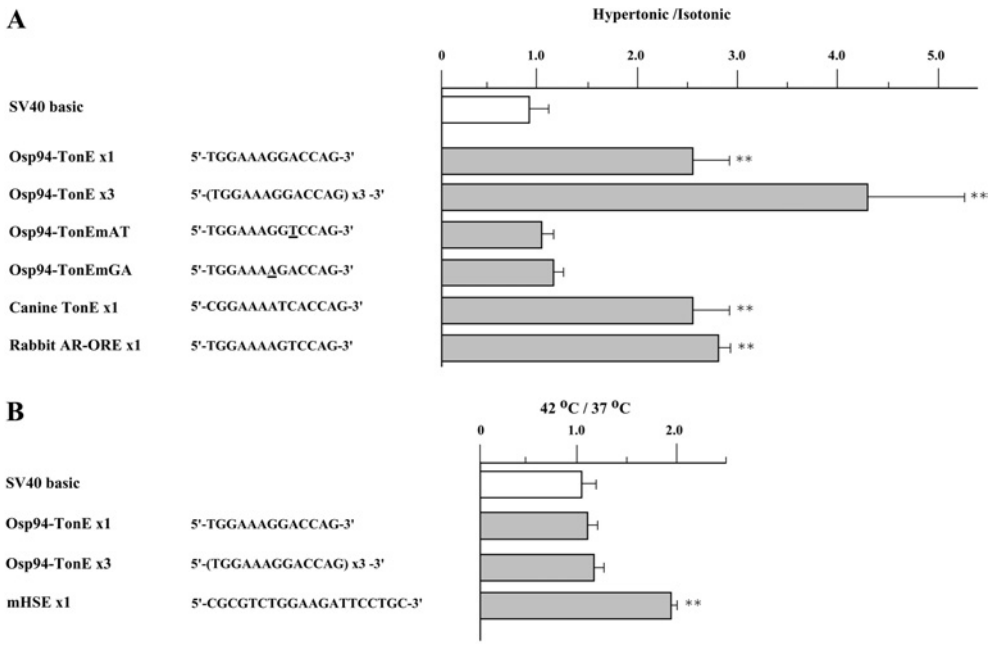

Figure 4 Functional activity of Osp94-TonE under hypertonic stress

(**A**) To confirm the location and functional activity of the osmotic-responsive element in the 5′-flanking region of the Osp94 gene, TonE-like element, 5′-TGGAAAGGACCAG-3′ (Osp94-TonE), was inserted into the pGL3-promoter vector. Point-mutated Osp94-TonE fragments, and TonE in the canine BGT1 gene and AR-ORE in rabbit AR gene were also used as positive control. Luciferase activity, under hypertonic stress in mIMCD3 cells transfected with the reporter plasmids, was determined, as described in the Materials and methods section. (**B**) To examine whether Osp94-TonE responds to heat-shock stress, mIMCD3 cells with Osp94-TonE reporter plasmid were exposed to heat shock, as described in the Materials and methods section. Underlines represent the location of point mutations of the Osp94-TonE. Osp94-TonE × 1, one copy of Osp94-TonE; Osp94 × 3, three copies of Osp94-TonE; Osp94-TonEmAT, point mutation A → T at position 9 bp; Osp94-TonEmGA, point mutation G → A at position 7 bp; canine TonE × 1 and rabbit AR-ORE × 1, one copy of TonE in canine BGT1 gene and rabbit AR gene respectively; mHSE × 1, one copy of HSE of mouse Hsp70 gene. Results in each panel represent means ± S.D. and the number of independent transfections for each construct was ⩾ 3. $^{**}P < 0.01$ versus SV40 basic.

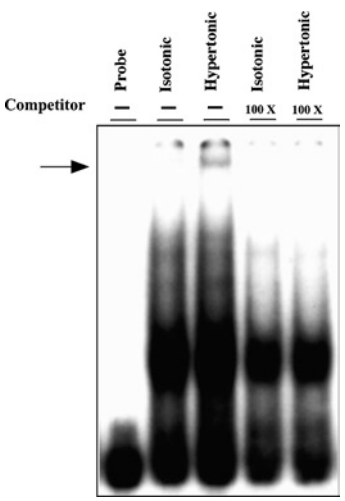

Figure 5 Identification of an Osp94-TonE binding protein by EMSA

A ^{32}P-labelled Osp94-TonE 13 bp oligonucleotide fragment was incubated with 4 μg of nuclear extracts prepared from mIMCD3 cells maintained in isotonic or hypertonic medium in the absence (—) or presence of 100-fold excess of the unlabelled 13 bp oligonucleotide, as described in the Materials and methods section. EMSA was performed with nuclear extracts prepared from cells treated with either isotonic or hypertonic medium. Probe, labelled 13 bp probe only; Isotonic, isotonic medium; Hypertonic, hypertonic medium supplemented with 200 mM mannitol; Competitor, unlabelled oligonucleotide fragment. Arrow indicates a slowly migrating band of an Osp94-TonE/protein interaction.

kinase) inhibitor, suppressed the increase in Osp94 mRNA associated with hypertonic stress (Figures 7B and 8E). Similarly, treatment with U0126, an MEK inhibitor, also resulted in a decrease in Osp94 mRNA elevated by hypertonicity (Figure 8F). Furthermore, expression of Osp94 gene was dose-dependently inhibited by the JNK inhibitor, SP600125 (Figure 8D). However, the induction of Osp94 mRNA by heat-shock stress was insensitive to all of the drug treatments (Figures 7C, 7D and 9).

Furthermore, when the cells were pretreated with MG132, a proteasome inhibitor, MG132 inhibited the increased level of Osp94 mRNA caused by either hypertonic (Figures 10A and 10B) or heat-shock (Figures 10C and 10D) stress.

DISCUSSION

This study examined the gene regulatory elements that underlie transcriptional regulation of the *Osp94* gene in response to hypertonicity and thermal stress, and the signalling pathways of *Osp94* gene expression. The principal observation was that the TonE (Osp94-TonE) was identified in the 5′-flanking region of the *Osp94* gene, an HSP110 family member, that hypertonicity and heat shock may enhance transcription of the *Osp94* gene through independent *cis*-acting elements, and that MAPKs and the proteasome system are integrally involved in *Osp94* gene expression.

Human *Hsp27* [28], mouse *Hsp47* [29], mouse and human *Hsp70* [30,31] and human *Hsp90* α [32] have TATA boxes and Sp1 sites as common sequences in the 5′-flanking region. In comparison, the *Osp94* gene was found to possess a TATA-less promoter. The most proximal region of the *Osp94* gene contains a 342 bp region with a high G/C content and seven partially overlapping Sp1 transcription factor-binding sites (Figure 1). This appears to be the promoter region that underlies the transcriptional activity of the native *Osp94* promoter in reporter assays. This cluster of Sp1 binding sites is a common feature of many genes

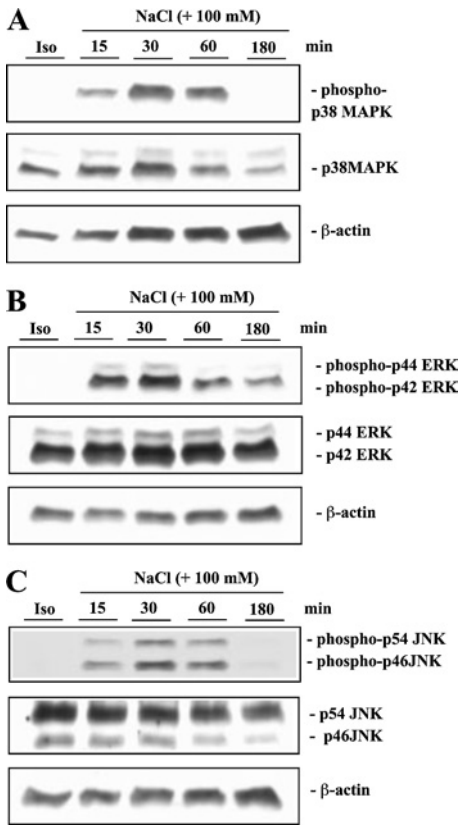

Figure 6 Time course of MAPK activation under hypertonic stress

IMCD3 cells were treated with NaCl (+ 100 mM), and then the cell lysate was subjected to SDS/PAGE and Western-blot analysis, as described in the Materials and methods section. MAPKs showed time-dependent activation caused by hypertonicity, with their peak activity at 30 min after the treatment. (**A**) Phosphorylated/total p38 MAPK, (**B**) phosphorylated/total p44 and p42 ERK, (**C**) phosphorylated/total p54 and p46 JNK.

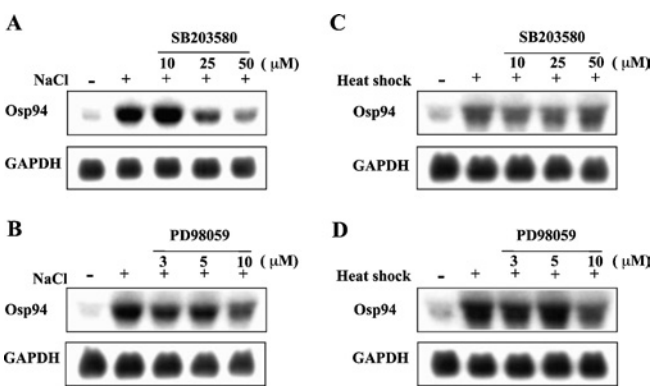

Figure 7 Northern-blot analysis of the effects of SB203580 (p38 MAPK inhibitor) and PD98059 (MEK inhibitor) on Osp94 mRNA expression under hypertonic and heat-shock stresses

IMCD3 cells were pretreated with SB203580 or PD98059 at the indicated doses, and then the cells were exposed to + 100 mM NaCl (**A**, **B**) for 6 h or heat shock (**C**, **D**) for 3 h. Control cells were treated with DMSO as a vehicle. Total RNA isolation and Northern-blot analysis were performed as described in the Materials and methods section.

in the TATA-less promoters. For instance, in the interleukin 1 type I receptor gene [33], cyclin A1 genes [34], and GFI1 proto-oncogene [35], the 5′-upstream regulatory elements lack a TATA box, but contain several Sp1 sites and GC boxes. In the TATA-less promoter region of human Ha-ras gene, the Sp1 site plays an important role as a selector of transcription initiation site [36]. Moreover, it has been reported that clustered Sp1 sites are required for transcriptional activity in the TATA-less promoter of the genes of insulin-like growth factor-binding protein-2 [37] and adenosine deaminase [38]. In the TATA box lacking genes with Sp1 sites and/or high G/C content, multiple transcription start sites were often observed [36,39]. These findings suggest the biological significance of the Sp1 sites, clustered Sp1 sites and high G/C content in the efficient transcription of the genes without a TATA box. Thus, the observed cluster of Sp1 sites and G/C-rich sequence in the *Osp94* gene may also play a role in its efficient transcription, although it must be demonstrated by further experiments.

In the case of hypertonicity, a functional 13 bp TonE was identified in the 5′ flanking region whose sequence (5′-TGGAA-AGGACCAG-3′) matches the TonEBP consensus binding sequence [40]. Furthermore, EMSA analysis revealed that Osp94-TonE binds a nuclear protein, most probably TonEBP [16]. Thermal stress, on the other hand, elevated transcription of Osp94 through a classic HSE (5′-CNNGAANNTTCNNG-3′), presumably via an active HSF. Of particular note, heat stress failed to activate the Osp94-TonE and hypertonicity failed to activate HSE-mediated transcription, underscoring the importance of hypertonicity as a specific physiological modulator of Osp94 expression.

The transcriptional regulation of HSPs has been extensively investigated in response to many environmental stresses, especially heat shock [41]. Nonetheless, the molecular mechanisms controlling transcription of HSPs in response to hypertonicity have not been elucidated. Increased osmolality is known to enhance mRNA and protein levels of HSP70, HSP25/27, α B-crystallin and Osp94 in kidney [42,43] and other cell types [44] although the promoter/enhancer elements involved in this response are unknown. Alfieri et al. [45] found that the hypertonicity activates binding of HSF1 to HSE, but this does not enhance HSP70 expression in 3T3 cells. Similarly, Caruccio et al. [46] failed to find an association between the activation of HSF1 and accumulation of HSP70 mRNA in response to hypertonicity in HeLa cells. These observations raised the likelihood that there was a *cis*-acting element distinct from HSE responsible for the hypertonicity-induced gene expression of HSP70, and presumably other HSPs. These observations in concert with those in the present study suggest that transcriptional regulation of HSPs by hypertonicity probably involves activation through TonEs and not through HSEs. In fact, examination of the human or mouse Hsp70 promoter region reveals the presence of a putative TonE (5′-TGGAATATTCC-3′), which was reported by Miyakawa et al. [40]. Whether these are functional remains to be determined.

The 5′-flanking region of Osp94 gene has four motifs matching the consensus HSE (5′-CNNGAANNTTCNNG-3′). Interestingly, in our reporter assays, constructs containing the HSE motifs were responsive to heat shock but not hypertonicity (Figure 3B). The transcription activities were almost equivalent when the reporter contained one or both HSEs (Figure 3B), showing that the most proximal enhancer is sufficient to drive transcription, although both could be involved. In fact, the most proximal HSE has been reported to be responsive to heat-shock stress by gel-shift-assay [27]. There exists much evidence for the role of HSE in cellular responses to heat-shock stress. In cells exposed to high temperature, cellular proteins are denatured causing the release of HSFs bound to those proteins into the cytoplasm. The released HSF is activated by forming a phosphorylated homotrimer complex. Then, in turn, this complex translocates into the cell nuclei and binds to the HSE in the 5′-upstream enhancer region of HSP genes, leading to ‘heat-induced’ gene expression

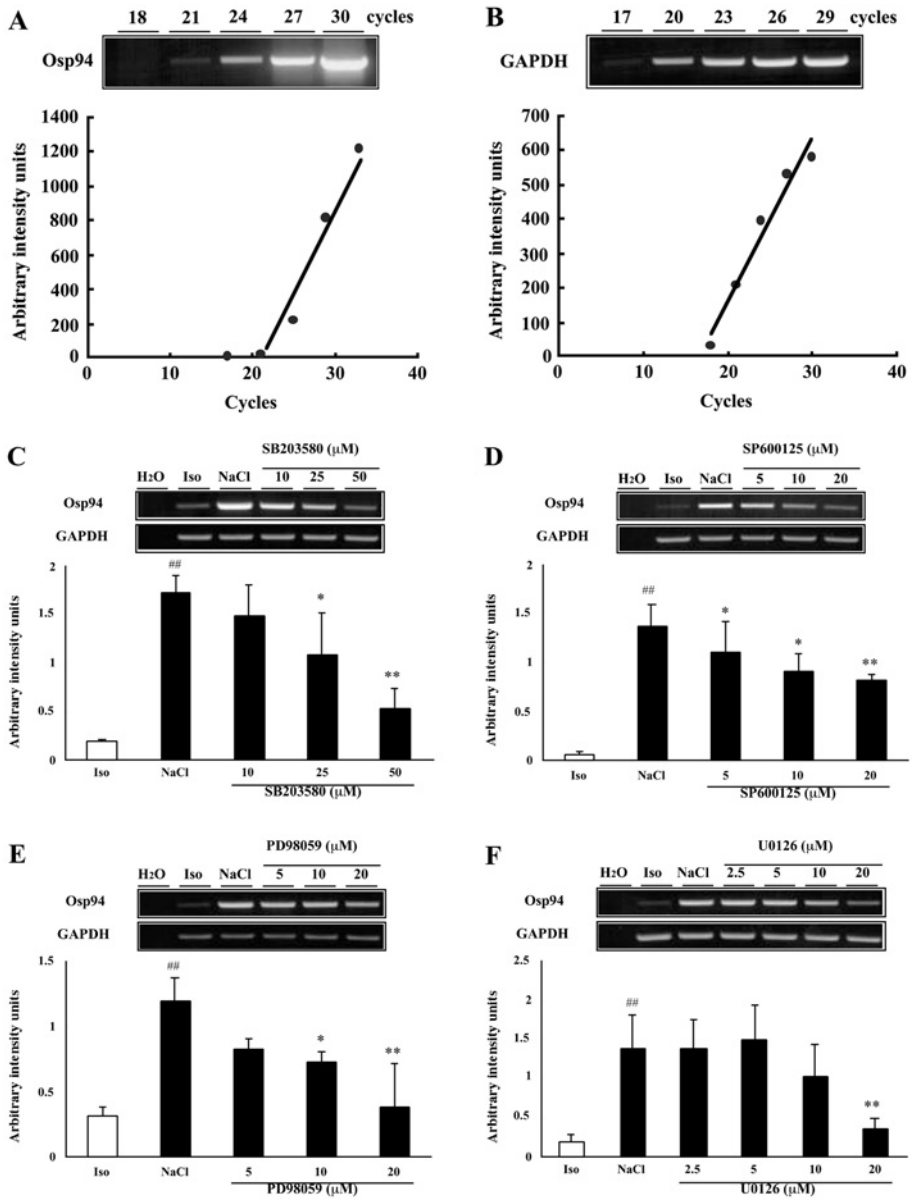

Figure 8 RT–PCR analysis of the effects of MEK inhibitors SB203580, PD98059 and U0126, and JNK inhibitor SP600125 on Osp94 mRNA expression under hypertonic stress

For quantification of mRNA level, RNA levels were also assessed using RT–PCR, as described in the Materials and methods section. The PCR cycle number was determined for Osp94 (**A**) and GAPDH (**B**), and as a result, 24 and 23 cycle numbers were used for Osp94 and GAPDH respectively. Treatment of mIMCD3 cells with SB203580 showed a decrease in Osp94 mRNA level induced by hypertonic stress in a dose-dependent manner (**C**). PD98059 (**E**) and U0126 (**F**) suppressed an increase in the gene expression of Osp94 induced by hypertonic stress. Similarly, expression of Osp94 gene was dose-dependently inhibited by SP600125 (**D**). Results in each panel represent means ± S.D. ($n = 3$). ##$P < 0.01$ versus Iso (Isotonic); *$P < 0.05$; and **$P < 0.01$ versus NaCl.

of HSPs [20]. Indeed, in the kidney cells, heat shock activates HSF1 and promoted the binding of HSF1 to HSE [47]. Thus, it is probable that the functional activity of the HSE observed in the reporter assay may be elicited through those pathways of the cellular heat-shock response. It should be noted, however, that since hyperosmotic stress failed to elicit transcriptional activity through HSEs, there appears to be no role for HSFs in regulating Osp94 and perhaps other HSPs in response to this physiological stimulus.

Using luciferase reporter assays, we were able to map and identify the functional Osp94-TonE enhancer element. This 13 bp sequence (5′-TGGAAAGGACCAG-3′) closely matches TonE seen in the canine BGT1 gene (5′-TACTTGGTCGGAAAA-TCACCAG -3′) and rabbit AR gene (5′-CGGAAAATCACC-3′). Serial base substitution experiments have defined a functional consensus sequence for TonE/ORE. Ferraris et al. [48,49] showed that the sequence of NGGAAA(A/T)(T/A/G)(C/A/T)(A/C)(N) could function as a minimal essential ORE in rabbit renal medulla PAP-HT25 cells. In addition, based on studies of the BGT1 gene, the sequence of (C/T)GGAANNN(C/T)N(C/T) was found to be a consensus *cis*-acting TonE in response to hypertonicity in MDCK cells [40]. Comparison of these sequences to Osp94-TonE, including our analysis of non-functional point mutants (Figure 4A), shows Osp94-TonE to fall within a uniform TonE consensus sequence of (C/T)GGAANNN(C/T)N(C/T) [40] and/or NGGAAA(A/T)(T/A/G)(C/A/T)(A/C)C [49], although slight

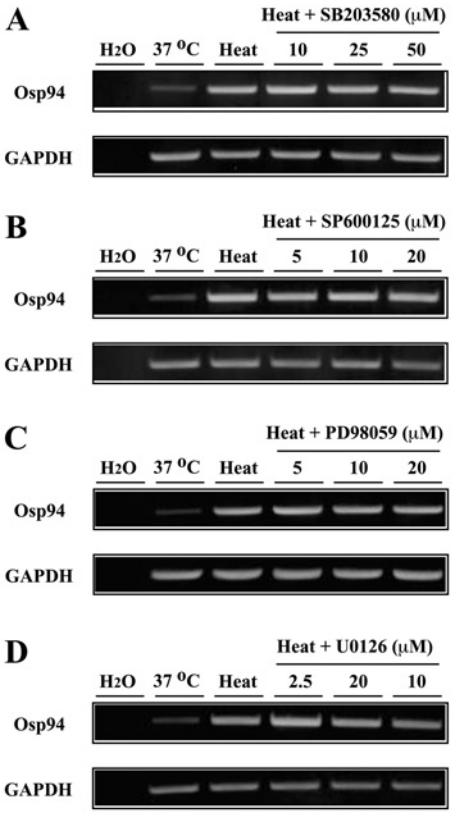

Figure 9 RT–PCR analysis of the effects of SB203580, PD98059, U0126 and SP600125 on Osp94 mRNA expression under heat-shock stress

IMCD3 cells were pretreated with SB203580, PD98059, U0126 and/or SP600125 at the indicated doses, and then the cells were exposed to heat shock for 3 h. The induction of Osp94 gene by heat-shock stress was insensitive to MAPK inhibitors.

differences that might exist among different species may need to be considered. In addition, the results with the point mutants further suggest a significance of the combination of nucleotide sequence of the element in its functional activity. Of interest, our reporter assays showed that the Osp94-TonE was a hypertonic, but not heat-shock, stress-responsive element, as demonstrated in Figure 4(B). This finding, as well as the result in Figure 3(B), suggests that the 5′-flanking region of the *Osp94* gene is independently regulated by tonicity and heat-shock enhancer elements in response to hypertonicity and heat-shock stresses respectively.

To examine the interaction of the Osp94-TonE with putative hyperosmotically activated transcription factors, EMSAs were performed using the 13 bp TonE characterized in reporter assays as a probe. When the nuclear extracts from the cells exposed to hypertonic medium were used for EMSA, a slowly migrating band was observed with a stronger intensity than when exposed to iso-osmotic medium (Figure 5). Miyakawa et al. [16] isolated TonEBP as the transcription factor that binds TonE and regulates transcription of BGT1 and other osmolyte-related genes. Given that TonEBP binds the consensus TonE that encompasses both the BGT1-TonE and the Osp94-TonE, it is probable that TonEBP is associated with Osp94-TonE. TonEBP is a ubiquitously expressed protein that could regulate expression of Osp94 and perhaps other HSPs in many different tissues, opening the possibility that other physiological stimuli beyond hypertonicity may regulate HSP transcription via the TonE enhancer element.

As noted previously, to understand the regulatory mechanisms of *Osp94* gene expression, it is worth determining whether MAPK

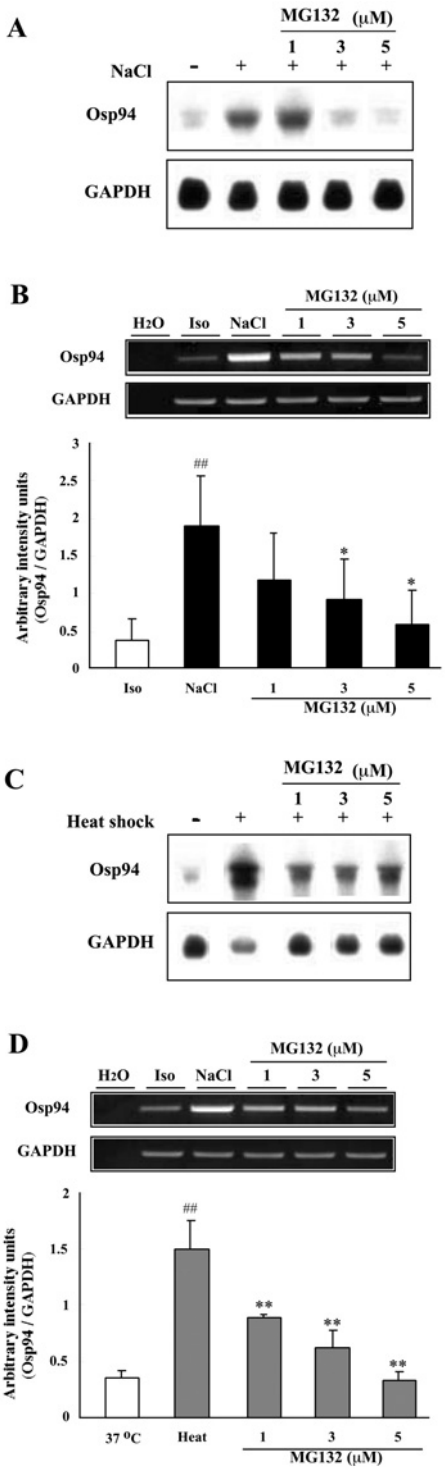

Figure 10 Effects of a proteasome inhibitor, MG132, on Osp94 gene expression under hypertonic and heat-shock stresses

Cells were pretreated with MG132, followed by exposure to hypertonic (**A**, **B**) or heat-shock (**C**, **D**) stresses. Total RNA isolation, Northern-blot and RT–PCR analyses were performed as described in the Materials and methods section. Treatment with MG132 suppressed the gene expression of Osp94 under hypertonic and heat-shock stresses in a dose-dependent manner. Results in each panel represent means ± S.D. ($n = 3$). ##$P < 0.01$ versus Iso or 37 °C; *$P < 0.05$, **$P < 0.01$ versus NaCl or heat.

pathways and the ubiquitin–proteasome system are involved in the *Osp94* gene expression and what intracellular signals lead to Osp94-TonE activation. In hypertonic stress, it has been shown

that MAPK cascades are involved in the intracellular signalling of osmotic shock and the expression of osmotic-sensitive gene. Using SB203580, a p38 MAPK inhibitor, Sheikh-Hamad et al. [50] have demonstrated that osmotic induction of Hsp70 and BGT1 mRNAs is mediated by p38 MAPK in MDCK cells. AR mRNA induced by hypertonicity has been shown to be abolished in HepG2 cells treated with PD098059, an MEK1 inhibitor [51].

In the present study, we found that in mIMCD cells, NaCl-induced hypertonicity activated p38 MAPK, ERK and JNK (Figure 6), and that hypertonic induction of *Osp94* gene expression was regulated by such MAPK pathways (Figures 7 and 8). In contrast, in the case of heat shock, no significant changes were observed in the Osp94 mRNA level when cells were pretreated with the MAPK inhibitors (Figures 7C, 7D and 9). This result further suggests that the Osp94 gene expression is independently regulated by hypertonic and heat-shock stresses through Osp94-TonE and HSE respectively.

Recently, Woo et al. [17] have shown that the ubiquitin–proteasome system participates in the regulation of gene expression of BGT1 and SMIT. They demonstrated the reduced osmotic induction of BGT1 and SMIT mRNAs and nuclear translocation of TonEBP by both MG132 and MG115. Our findings with MG132 suggest that *Osp94* gene expression under hypertonic stress is also regulated by the ubiquitin–proteasome system (Figures 10A and 10B), as well as by the MAPK pathways. Taken together, the present studies suggest that hyperosmolarity-induced *Osp94* gene expression is regulated by signalling pathways through MAPK and TonEBP/TonE pathways by which BGT and SMIT mRNAs are modulated. Of interest, heat-shock-induced *Osp94* gene expression was also blocked by MG132 (Figures 10C and 10D). This suggests that intracellular proteins, which are inactivated by the ubiquitin–proteasome system, participate in the heat-shock response of the *Osp94* gene, as well as hypertonic response. Further studies are needed to define which proteins contribute to such regulatory mechanisms and to understand the special role of the ubiquitin–proteasome system in this process.

In conclusion, the present study indicates that the 5′-flanking region of the *Osp94* gene has functional TonE and HSE enhancer elements that respond independently to hypertonicity and heat stress. *Osp94* gene has a cluster of Sp1 sites and a TATA-less promoter, which is an unusual characteristic of promoter regions among HSPs genes. MAPKs such as p38 MAPK and ERK, and the ubiquitin–proteasome system are involved in the regulation of the *Osp94* expression. Furthermore, the present study suggests that hypertonicity may regulate transcription of other HSP genes such as Hsp70, as well as Osp94 through TonEs, and perhaps TonEBP.

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