



Analysis of expression and structure of the rat *GH-secretagogue/ghrelin receptor (Ghsr)* gene: Roles of epigenetic modifications in transcriptional regulation

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ABSTRACT

In the current study, to elucidate the molecular basis of cell type-specific expression of the *GH-secretagogue/ghrelin receptor type 1A (GHSR1A)*, we characterized the structure and putative promoter region of the rat *Ghsr* gene. We identified an alternative 5'-untranslated first exon that contains multiple transcription start sites, and confirmed a 200-bp sequence proximal to this exon to be sufficient for basal promoter activity. A promoter-associated CpG island conserved across different species was found to be hypomethylated in *Ghsr1a*-expressing cell lines, while being heavily methylated in non-expressing cells. In cells with low or absent *Ghsr1a* expression, treatment with demethylating agents activated *Ghsr1a* transcription. Chromatin immunoprecipitation assays demonstrated *Ghsr1a*-expressing cells to display active histone modifications, whereas repressive modifications were present exclusively in other cell types. These results suggest epigenetic modifications at *GHSR* to play important roles in determining *GHSR1A* expression and abundance, and therefore the consequent sensitivity of cells to ghrelin.

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1. Introduction

The growth hormone (*GH*)-secretagogue/ghrelin receptor (*GHSR*) is a member of the ghrelin receptor subfamily within class A of rhodopsin-like G protein-coupled receptors (GPCRs), originally discovered as the target of a family of small synthetic molecules, GH secretagogues (GHSs), that are capable of stimulating GH release from the pituitary (Howard et al., 1996; Smith et al., 1997). Later, ghrelin, an *n*-octanoylated gastric peptide of 28 amino acids, was

Abbreviations: 5-aza-dC, 5-aza-2-deoxycytidine; ChIP, chromatin immunoprecipitation; ERK1/2, extracellular signal-regulated kinase 1/2; DMEM, Dulbecco's modified Eagle's medium; DNMT, DNA methyltransferase; EST, expressed sequence tag; FBS, fetal bovine serum; GH, growth hormone; GHS, growth hormone secretagogue; GHSR, growth hormone secretagogue receptor; GPCR, G protein-coupled receptor; HDAC, histone deacetylase; IP1, inositol monophosphate; IP3, inositol triphosphate; MAPK, mitogen activated protein kinase; MSP, methylation-specific PCR; PMSF, phenylmethylsulfonyl fluoride; qRT-PCR, quantitative real-time PCR; RACE, rapid amplification of cDNA ends; RLM, RNA ligase-mediated; SP-analog, [D-Arg¹, D-Phe⁵, D-Trp^{7,9}, Leu¹¹]-Substance P; TM, transmembrane; TSA, Trichostatin A; TSS, transcription start site; UCSC, University of California, Santa Cruz; UT, untranslated.

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identified as the natural ligand of this receptor (Kojima et al., 1999). There is now emerging evidence that ghrelin acts through GHSR to exert pleiotropic effects, which include not only the induction of GH secretion but also stimulation of appetite, regulation of energy metabolism, control of gastro-intestinal motility, cardiovascular and hemodynamic effects, as well as having anti-inflammatory and immunomodulating properties (van der Lely et al., 2004; Kojima and Kangawa, 2005; Davenport et al., 2005; Tritos and Kokkotou, 2006; Dixit and Taub, 2005). On the other hand, it has also been proposed that at least some of the effects of ghrelin are likely to be mediated through an as yet uncharacterized, GHSR-independent pathway (Baldanzi et al., 2002; Toshinai et al., 2006; Thielemans et al., 2007).

GHSR mRNA is predominantly expressed in the hypothalamus and pituitary (Howard et al., 1996), consistent with the important role of ghrelin in regulating GH release. On the other hand, lower endogenous expression has also been documented in other central and peripheral tissues (Gnanapavan et al., 2002; Zigman et al., 2006; Sun et al., 2007). The widespread distribution of *GHSR* may support and correlate with the diversity of ghrelin–GHSR system functions. However, it should be noted that several conflicting findings have been reported regarding *GHSR* expression and distribution in normal and tumor tissues, as well as in cell lines;

thus, caution is needed in their interpretation. These inconsistencies might be attributable to the use of different experimental techniques with variable sensitivities, but could also be linked to the presence of a variant *GHSR* transcript, designated *GHSR1B* (Howard et al., 1996; Gnanapavan et al., 2002; Jeffery et al., 2005). *GHSR1A*, the primary full-length gene transcript, produced by splicing of the two coding exons, encodes a typical GPCR with a seven-transmembrane (7TM) domain core. In contrast, the intron is not removed from *GHSR1B*; therefore, an alternative stop codon and a polyadenylation signal within the intron are used to produce a C-terminal truncated, non-functional GPCR form that is unable to bind to GHSs (Howard et al., 1996). Recent studies have, however, demonstrated that *GHSR1B* might modulate the function of *GHSR1A* or other 7TM GPCRs by interfering their cellular expressions and ligand binding properties (Chan and Cheng, 2004; Chu et al., 2007; Leung et al., 2007; Takahashi et al., 2006), yet the precise roles of *GHSR1B* under normal physiological condition are not well understood. In addition, several rodent and human cell lines reportedly express *GHSR* mRNA; however, in many of these cells, the level of *GHSR1A* expression is apparently too low to exert ligand-induced *GHSR1A* activation (Murata et al., 2002; Thielemans et al., 2007; Adams et al., 1998). Nevertheless, these cells have been shown to retain some ability to respond to ghrelin, providing evidence for the existence of an alternative ghrelin receptor.

For many plasma membrane receptors including GPCRs, their density on the cell-surface is finely controlled by various transcriptional, post-transcriptional and post-translational mechanisms, and is often a determinant of overall receptor function in a cell. To date, the transcriptional regulation and molecular basis for tissue and cell type-specific expression of *GHSR* have not yet been fully elucidated. There have been relatively few studies, in a limited number of species including human, chicken and fish (Kaji et al., 1998; Petersenn et al., 2001; Tanaka et al., 2003; Yeung et al., 2004), characterizing the 5'-flanking region of the *GHSR* gene. Previous studies of the human *GHSR* gene have shown that it contains a TATA-less promoter, similar to most other GPCR promoters, and suggested an alternative splicing in the 5'-untranslated (5'-UT) regions (Kaji et al., 1998; Petersenn et al., 2001). These studies also demonstrated that the minimal promoter is mapped to within a relatively small segment (~300-bp) of the 5'-flanking region of *GHSR*. In addition, sequence comparison of human and fish *GHSR* promoters identified putative binding sites for shared transcription factors, such as AP-1, NF-1, Oct-1 and USF (Yeung et al., 2004); however, the functional significance of these transcription factors in transcriptional regulation awaits further investigation.

Herein, we report data on the structural and functional characterization of the 5'-flanking region of the rat *Ghsr* gene. In addition, we demonstrated a highly restricted expression of *Ghsr1a* among endocrine cell lines of rodent origin, and replicated previous findings that original and purified RC-4B/C rat pituitary tumor cell lines express a high level of functional *Ghsr1a* (Falls et al., 2006; Perdonà et al., 2011). Furthermore, using newly established RC-4B/C subclones with either a high or a low level of *Ghsr1a* mRNA, we investigated cell type-specific expression and transcriptional regulation of *Ghsr1a*. Our results indicate that epigenetic changes through DNA methylation and chromatin/histone modifications make significant contributions to determining the level of *Ghsr1a* transcription.

2. Materials and methods

2.1. Cell culture

All cell lines used in this study are listed in [Supplemental Table 1](#). Rat RC-4B/C (pluripotential), GH3 (somatotrophic), MMQ

(lactotropic), AR42J (pancreatic acinar), L6 (myoblastic), and murine AtT20 (corticotrophic) cells were purchased from American Type Culture Collection (Rockville, MD, USA). Rat MtT/S (somatotrophic), murine α T3-1 (gonadotrophic) and MIN6 (insulinoma) cells were kindly provided by Professors Kinji Inoue (Saitama University), Pamela Mellon (University of California at San Diego) and Junichi Miyazaki (Osaka University), respectively. Human embryonic kidney (HEK) 293A cells were obtained from Invitrogen (Tokyo, Japan). GH3, MMQ and MtT/S cells were maintained in Dulbecco's modified Eagle's medium (DMEM)/F-12 (1:1) supplemented with 10% horse serum, 2.5% fetal bovine serum (FBS) and antibiotic solution (100 U/ml penicillin and 100 μ g/ml streptomycin). The remaining cell lines were cultured in high glucose (HG)-DMEM medium containing 10% FBS and antibiotic solution. Cell culture medium, serum and antibiotics were purchased from Invitrogen. All cell lines were certified to be mycoplasma-free by the suppliers, and maintained in a standard humidified incubator at 37 °C and 5% CO₂. To obtain RC-4B/C subclones, the parental cells were counted and plated in a limiting dilution titration in four 96-well cell culture plates (Iwaki, Tokyo, Japan). Clones established from single cells were transferred to new plates for expansion and used for gene expression analysis as described below.

2.2. Animal tissues

All animal experiments were approved by the Animal Research Ethics Committee at the University of Tokushima. Rat tissues (hypothalamus, pituitary, heart and liver) from male Fischer 344 (F344; F344/DuCrIj) rats of three different ages (3, 6 and 18 months; $n = 4$) were purchased from Charles River Inc. (Yokohama, Japan). Tissues were frozen at -80 °C until needed. Each tissue was divided into two equal parts, one each for RNA and DNA extraction, before being processed separately.

2.3. RNA isolation, conventional and real-time RT-PCR

Isolation of total RNA from cell lines and tissues was performed using an RNeasy Mini Kit (QIAGEN, Tokyo, Japan) following the manufacturer's protocol including the on-column DNase digestion step. To remove residual genomic DNA, purified RNA was further treated with Turbo-DNase (Ambion, Austin, TX, USA), using a starting amount of up to 10 μ g of RNA in a 50 μ l reaction. Subsequently, first-strand cDNA was synthesized from 100 ng of total RNA using a SuperScript III First-Strand Synthesis System (Invitrogen). Negative control reactions without reverse transcriptase (RT-minus) were included to monitor genomic DNA contamination in the template RNA. The resulting 2 ng of cDNA (assuming 100% conversion of RNA to cDNA during the reverse transcription step) were amplified using AmpliTaq Gold 360 Master Mix (Applied Biosystems, Foster City, CA, USA) and standard buffer conditions. The reactions were performed in a 20 μ l volume for each well on a MicroAmp 96-well reaction plate using the GeneAmp PE 9700 thermal cycler (Applied Biosystems). RT-PCR primers were designed to amplify the following rat target genes: *Ghsr1a*, *Ghsr1b*, *Gh1* (encoding growth hormone), *Prl* (prolactin), *Tshb* (thyroid-stimulating hormone- β subunit), *Lhb* (luteinizing hormone- β subunit), *Fshb* (follicle-stimulating hormone- β subunit), *Pomc2* (proopiomelanocortin- β or Pomc2), *Cga* (glycoprotein hormone [GSU]- α subunit), *Pou1f1* (Pit-1), *Aff4* (AF5q31) and *Gapdh* (a housekeeping gene). Primer sets were selected to span at least one intron of the genomic sequence whenever possible, to ensure that the RT-PCR products were from cDNA and not genomic DNA. PCR conditions were optimized and the linear amplification range was determined for each primer set by varying the annealing temperature and cycle number. Primer sequences and optimized conditions are summarized in [Supplemental Table 2](#). All PCR experiments

included RT-minus as well as no template negative controls to detect genomic contamination. The RT-PCR products were analyzed by electrophoresis in a 2% agarose gel and by ethidium bromide staining.

Expression of *Ghsr1a* was also evaluated by quantitative real-time PCR (qRT-PCR) using TaqMan Gene Expression Assays (Assay ID: rat *Ghsr1a*/Rn00821417_m1) and TaqMan Universal PCR Master Mix without AmpErase UNG on the ABI 7900HT Sequence Detection System (Applied Biosystems) according to manufacturer's recommendations. Assays were carried out in triplicate using 1–2 ng cDNA per well of a 384-well plate, included no template and RT-minus negative control reactions, and repeated at least three times. Relative gene expressions were evaluated by the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001), using 18S ribosomal RNA (Assay ID: Hs99999901_s1) as an internal control gene.

2.4. Receptor signaling assays

Ghrelin and des-acyl ghrelin were purchased from the Peptide Institute, Inc. (Osaka, Japan), and SP-analog ([D-Arg¹, D-Phe⁵, D-Trp^{7,9}, Leu¹¹]-Substance P) from AnaSpec (San Jose, CA, USA). To examine whether exogenous ghrelin can induce *Fos* mRNA (encoding rat c-Fos, an immediate early gene product) expression, subconfluent cells grown in 24-well culture plates were serum-starved for 4 h, stimulated for 30 min with ghrelin (10^{-8} – 10^{-6} M) or vehicle, and harvested for RNA analysis by qRT-PCR with Power SYBR Green PCR Master Mix (Applied Biosystems). Primer sequences for *Fos* amplification, as well as those for *Gapdh* (used for normalization), are shown in Supplemental Table 2. Assays were carried out in triplicate, and included no template and RT-minus negative control reactions. As a negative control for nonspecific effects, cells were also stimulated with des-acyl ghrelin (10^{-6} M) or SP-analog (10^{-6} M). To assess ghrelin-induced phosphorylation of ERK1/2, cells grown in 6-well plates were treated with ghrelin (10^{-7} M) or vehicle for 10 min, and lysed in Cell Lysis Buffer (Cell Signaling Technology, Beverly, MA, USA) containing 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1× Roche complete mini protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN, USA). Protein concentrations were determined using a Bradford protein assay kit (Bio-Rad, Hercules, CA, USA). Western blot analysis was performed according to the standard protocol, using total- or phosphorylation-specific antibodies to ERK1/2 (Cell Signaling Technology). Ghrelin-induced accumulation of inositol triphosphate (IP3) was measured with a cell-based IP-One Tb HTRF Assay Kit (CIS Bio International, Bagnols-sur-Ceze, France) that measures inositol monophosphate (IP1), the stable breakdown product of IP3, following the manufacturer's recommendations. Measurements were performed on a PowerScan 2 microplate reader (DS Pharma Biomedical Co., Osaka, Japan).

2.5. RNA ligase-mediated rapid amplification of 5' cDNA ends (5'-RLM-RACE)

5'-RLM-RACE analysis was performed using rat pituitary total RNA and the Invitrogen GeneRacer Kit, according to the manufacturer's instructions except that KOD-Plus-ver.2 Taq DNA Polymerase (TOYOBO, Tokyo, Japan) was used for PCR amplification. One microgram of total RNA was used as the starting material for two rounds of 5'-RACE PCR (see Supplemental Table 3-1 for primer sequences), carried out on a GeneAmp PE 9700. The resulting 5'-RACE products were purified by Wizard SV Gel and PCR Cleanup System (Promega, Madison, WI, USA), and subcloned using the Zero Blunt TOPO PCR Cloning Kit for Sequencing (Invitrogen). Plasmid DNA was then purified with QIAGEN QIAprep Spin Mini-Prep Kit, and sequenced using a BigDye Terminator v3.1 Cycle Sequencing Kit on an ABI Prism 3130xl genetic analyzer (Applied

Biosystems). Sequence data from 45 independent subclones were analyzed and assembled into contigs using the SeqMan II program (DNASTAR, Madison, WI, USA). To verify the 5'-RACE results, RT-PCR of DNase-treated pituitary RNA was performed using different combinations of a common reverse primer from *Ghsr* exon 2 and forward primers either upstream or downstream from the putative transcription start site (primer sequences are shown in Supplemental Table 3-2, with the locations and directions of the primers in Fig. 3B). RT-PCR was carried out under the same condition as described above, and *Gapdh* mRNA was amplified as a positive control for RT-PCR.

2.6. Promoter reporter assay

A 1549-bp genomic fragment encompassing the 5'-flanking region of the rat *Ghsr* gene was amplified from genomic DNA with KOD-Plus-ver.2 Taq DNA polymerase, and subcloned into the pGL3-Basic luciferase reporter vector (Promega), which contains a firefly luciferase reporter gene (see Supplemental Table 3-3 for primer sequences). The deletion constructs were prepared by performing double digestions with several combinations of restriction enzymes (KpnI-NheI, KpnI-PstI, KpnI-AleI and KpnI-SpeI; New England Biolabs, Beverly, MA, USA) followed by end-filling and re-ligation. These constructs were named based on the distance of the 5'-terminus from the putative transcription initiation site (pGhsr-956, -654, -364, -202, and +194-59). All constructs were transformed into *Escherichia coli* SCS110 (Agilent Technologies, Palo Alto, CA, USA), which lacks methylases (*dcm* and *dam*), to produce unmethylated plasmid DNA preparations. Reporter constructs were transfected into cells using FuGENE HD Transfection Reagent (Roche Applied Science), along with a *Renilla luciferase* reporter plasmid (pTK-RL; Promega) as an internal control for transfection efficiency. Forty-eight hours after transfection, luciferase activity was analyzed using the Dual-Luciferase Reporter Assay System (Promega) on an ARVO SX 1420 multilabel counter (Perkin-Elmer, Boston, MA, USA).

2.7. DNA extraction, methylation-specific PCR (MSP) and bisulfite genomic sequencing

Genomic DNA was extracted from cultured cell lines or tissues using the QIAGEN Blood & Cell Culture DNA Kit. Bisulfite treatment and sample recovery were carried out with either the EpiTect Bisulfite Kit (QIAGEN) or the MethylEasy Xceed Rapid DNA Bisulfite Modification Kit (TaKaRa, Tokyo, Japan) according to the supplier's protocols. Fully-methylated or fully-unmethylated control DNAs were created from rat liver genomic DNA, by either SssI methylase (New England BioLabs) treatment or amplification using a GenomiPhi V2 DNA Amplification Kit (GE Healthcare, Piscataway, NJ, USA), followed by bisulfite-conversion, as described previously (Umetani et al., 2005).

Four different MSP assays (MSP1–4), each using methylated (M) and unmethylated (U) primer pairs, were designed across the 5'-flanking region of rat *Ghsr*. The positions of MSP1–4 are indicated in Fig. 4A, and primer sequences are shown in Supplemental Table 3-5. PCR was performed in 20-μl reaction volume with AmpliTaq Gold 360 PCR Master Mix under the following conditions: 1 cycle of 95 °C for 5 min; 35 cycles of 95 °C for 30 s, 60 °C (M) or 55 °C (U) for 30 s and 72 °C for 30 s; and 1 cycle of 72 °C for 2 min 30 s. The PCR products were analyzed by electrophoresis in a 2% agarose gel and by ethidium bromide staining. In each MSP assay, specificity was verified using methylated or unmethylated control DNAs, and no amplification was observed when a bisulfite-untreated genomic DNA sample was used as the template.

In bisulfite genomic sequencing, the bisulfite-treated DNA was amplified in two overlapping BS1 and BS2 regions (in cell lines)

or in the BS1 region alone (in tissues). The BS1 and BS2 regions are positioned at the 5'-flanking region of rat *Ghsr*, and contain a total of 31 CpG sites (Fig. 4A, bottom). Primer sequences are shown in Supplemental Table 3–6. PCR was performed in a 20- μ l reaction volume under the following conditions: 1 cycle of 95 °C for 5 min; 40 cycles of 95 °C for 15 s, 51 °C for 30 s and 72 °C for 30 s; and 1 cycle of 72 °C for 2 min 30 s. Amplicons were gel purified, and subcloned into a pCR4-TOPO TA vector (Invitrogen). Plasmid DNA, purified using the QIAGEN QIAprep 96 Turbo Miniprep Kit, was then sequenced in both directions, using universal M13 primers. Raw bisulfite sequencing data were analyzed using the web-based program QUMA (QUantification tool for Methylation Analysis: <http://quma.cdb.riken.jp/>; Kumaki et al., 2008). In all experiments, by sequencing 20–48 individual plasmid clones, the percent methylation level at individual CpG sites was determined from the frequency of clones with a methylated CpG at each site, whereas the overall methylation level was calculated by dividing the total number of methylated CpGs by the total number of sequenced CpGs.

2.8. In vitro methylation of reporter plasmids

Two approaches were used to evaluate the effect of DNA methylation on *Ghsr* promoter function. In the first, patch methylation of reporter constructs was performed (Robertson and Ambinder, 1997). Briefly, the pGhsr-654 plasmid was double digested with NheI-XhoI, NheI-AleI or AleI-XhoI restriction enzymes to generate three fragments of the promoter insert: (1) the entire insert, (2) one-third of the 5'-region, or (3) two-thirds of the 3'-region. The fragments were gel-purified using the Wizard SV Gel and PCR Clean-Up System, and 10 μ g of insert DNA were *in vitro* methylated with 12 U SssI at 37 °C for 1 h in the presence of 160 μ M S-adenosyl methionine in a 50 μ l reaction volume. The methylated and mock-methylated (unmethylated) fragments were repurified, ligated back into the reporter constructs with a Ligation High kit (TOYOBO), and used for transfection.

The second approach involved the use of pGhsr-202 to make a CpG-free luciferase reporter construct by replacing the wild-type luciferase gene sequence with a CpG-free sequence. A CpG-free synthetic luciferase cDNA fragment was amplified using the pMOD-lucSh plasmid (InvivoGen, San Diego, CA, USA) as a template, and subsequently cloned into the NcoI-XbaI sites of pGhsr-202. Primer sequences used for amplification are shown in Supplemental Table 3–4. The resulting plasmid designated pCpGfree-Ghsr-202 was methylated with SssI as described above, or treated similarly but without the enzyme. Complete methylation was verified by digestion with BamHI and methylation-sensitive BssHII (New England Biolabs). The methylated and control plasmid DNAs were purified and used for transfection.

2.9. Treatment of cells with 5-aza-2-deoxycytidine (5-aza-dC) and Trichostatin A (TSA)

To evaluate the role of methylation in the inactivation of the *Ghsr* promoter in cell lines, the cells were treated with the DNA methyltransferase (DNMT) inhibitor 5-aza-dC and/or the histone deacetylase (HDAC) inhibitor TSA. Both agents were purchased from Wako Pure Chemical Industries (Osaka, Japan). Cells (GH3, AR42J and L6) were seeded at 50% confluence per well in a 6-well plate 1 day before treatments with various concentrations of 5-aza-dC (0–10 mM) for 4 days and/or TSA (0.5 μ M) for 24 h. The medium with or without 5-aza-dC was changed every other day. At the end of treatment, cells were harvested and subjected to RNA purification and cDNA synthesis as described above. *Ghsr1a* mRNA levels were measured in triplicate by TaqMan qRT-PCR,

normalized to 18S rRNA. Untreated RC-4B/C-H1 cells were used as a positive control for *Ghsr1a* expression.

2.10. Chromatin immunoprecipitation (ChIP) assay

ChIP was performed for five rat cell lines (L6, GH3, AR42J, RC-4B/C-H1 and -H2). Approximately 1×10^7 cells in a 15-cm dish were used in ChIP assays employing the Simple ChIP Enzymatic Chromatin IP Kit (Cell Signaling Technology), following the manufacturer's protocol. Antibodies specific to active chromatin state markers, histone H3 acetylated at Lys 9/14 (AcH3; Millipore #06-599, Billerica, MA, USA) and trimethyl-histone H3 (Lys4) (H3K4m3; TaKaRa MA304), as well as those specific to heterochromatin markers, trimethyl-histone H3 (Lys9) (H3K9m3; Millipore #07-442) and trimethyl-histone H3 (Lys27) (H3K27m3; Millipore #07-449), were used. Normal rabbit IgG (Cell Signaling Technology #2729) was used as a negative control. Immunoprecipitated DNA, as well as total chromatin (input), was subsequently amplified and quantified by qRT-PCR using the Power SYBR Green PCR Master Mix. The ChIP primers were designed to amplify the rat *Ghsr* distal and proximal promoter (Ghsr-PrDis and PrPrx) regions, as well as the *Gapdh* proximal promoter (Gapdh-PrPrx; as the control, representing transcriptionally active heterochromatin) and the rat *Chromosome 15* microsatellite (Chr15; as the control, representing transcriptionally inactive heterochromatin). Primer sequences are shown in Supplemental Table 3–7. The levels of enrichment of the DNA sequence were determined relative to the total amount of input DNA (percent of input).

2.11. Statistical analysis

Results are expressed as means \pm SD. Data were analyzed with unpaired 2-tailed *t*-tests using GraphPad Instat v3.0 software (GraphPad Software, San Diego, CA, USA). A value of $P < 0.05$ was considered significant.

2.12. Bioinformatics

DNA sequence and annotation data were obtained from National Center of Biotechnology Information (NCBI: <http://www.ncbi.nlm.nih.gov/>) or from University of California Santa Cruz (UCSC) Genome Browser (<http://genome.ucsc.edu/>). Gene/protein names and symbols were used in accordance with the recommendations of the Human Genome Organisation (HUGO) Gene Nomenclature Committee (Povey et al., 2001). The search for putative transcription factors was performed using TESS (Transcription Element Search Software: <http://www.cbil.upenn.edu/tess>; Schug and Overton, 1997) and TFSEARCH (Searching Transcription Factor Binding Sites: <http://mbs.cbrc.jp/research/db/TFSEARCH.html>; Heinemeyer et al., 1998). To predict a CpG island in the rat *Ghsr* gene, we utilized the web-based CpG Island Searcher Program (<http://www.cpgislands.com>; Takai and Jones, 2002) and the CpG islands track from the UCSC Genome Browser. Primers used for RT-PCR experiments were designed with Primer3Plus online software (<http://www.bioinformatics.nl/primer3plus>; Untergasser et al., 2007). Primers for MSP and bisulfite-sequencing PCR were designed using Methyl Primer Express Software v1.0 (Applied Biosystems).

3. Results

3.1. *Ghsr1a* gene expression in rodent cell lines

Expression of *Ghsr1a*, the primary transcription product of the *Ghsr* gene, was first screened with a conventional RT-PCR assay

in RNA preparations from nine rodent cell lines as well as the normal adult rat pituitary (Fig. 1A). Our assay only detected abundant transcripts of endogenous *Ghsr1a* in a pluripotential pituitary cell line, RC-4B/C, at a significantly higher level than in normal pituitary. In the other eight cell lines tested, including those previously reported to express *Ghsr1a* (GH3, AR42J and MIN6 [Murata et al., 2002; Lai et al., 2005; Doi et al., 2006]), *Ghsr1a* mRNA levels were very low and difficult to detect via conventional RT-PCR. Notably, *Ghsr1b* paralleled *Ghsr1a* expression, in that transcripts were detected as a faint amplicon in the pituitary and RC-4B/C cells, but not in other cell lines.

RC-4B/C cells, derived from a rat pituitary adenoma, are reportedly heterogeneous and contain all known anterior pituitary hormone-secreting cell types as demonstrated by immunocytochemistry (Hurbain-Kosmath et al., 1990). Our RT-PCR results showed RC-4B/C cells to have high levels of *Prl* and *Cga* mRNAs, whereas *Gh1* and *Tshb* transcripts were expressed at low-to-moderate levels. We found that RC-4B/C cells did not express detectable levels of *Pomc2*, *Lhb* or *Fshb* (Fig. 1A). Previous studies demonstrated that human *GHSR* promoter activity in monkey kidney COS-7 cells was enhanced by cotransfection of the pituitary-specific transcription factor Pit-1, which encoded by the *POU1F1* gene (Petersenn et al., 2001), while knockout of *Ghsr* in mice resulted in a decrease in pituitary *Pou1f1* mRNA expression, as well as reduced *Gh1* and *Prl* transcripts (Yang et al., 2008). Furthermore, it has also been suggested that *GHSR* expression is negatively regulated by the RNA polymerase II transcriptional repressor AF5q31/MCEF, encoded by the *AFF4* gene (Komori et al.,

2008; Dunn and Bale, 2009). We therefore examined mRNA expression levels of *Pou1f1* and *Aff4* in RC-4B/C cells for comparison with those in other cell lines to ascertain whether there is a relationship with *Ghsr1a* expression levels. However, there was no obvious correlation between the *Ghsr1a* expression pattern and the mRNA levels of *Pou1f1* and *Aff4* genes (Fig. 1A).

Falls et al. (2006) showed that subclones with a robust ghrelin response can be obtained through successful purification of parental RC-4B/C cells. We thus used a similar limiting dilution approach, and selected cells on the basis of their *Ghsr1a* expression levels. Several RC-4B/C subclones with variable levels of *Ghsr1a* mRNA were isolated and established. Results of the TaqMan qRT-PCR assays, using rat-specific primers and probes, indicated that the subclones, RC-4B/C-H1 and RC-4B/C-H2, expressed significantly higher amounts (at least 10-fold) of *Ghsr1a* mRNA than parental cells (Fig. 1B, left), whereas *Ghsr1a* expression was very low or absent in RC-4B/C-L1 and RC-4B/C-L2 subclones from the same cells. Additionally, the high sensitivity and specificity of TaqMan qRT-PCR (Bustin, 2002) allowed the detection of *Ghsr1a* transcripts in cells such as AR42J and GH3, in which *Ghsr1a* expressions were undetectable by conventional RT-PCR (Fig. 1B, right). Yet their expression levels were very low, and the relative expressions were only 2.2% (AR42J) and 0.25% (GH3) of average in normal pituitary.

Receptor functionality of *Ghsr1a* in RC-4B/C-H1 and RC-4B/C-H2 subclonal cells was verified by examining the effects of exogenous ghrelin. In arcuate nucleus neurons, ghrelin administration effectively stimulates the expression of some markers of neural activity, including c-fos (Hewson and Dickson, 2000). Treatment

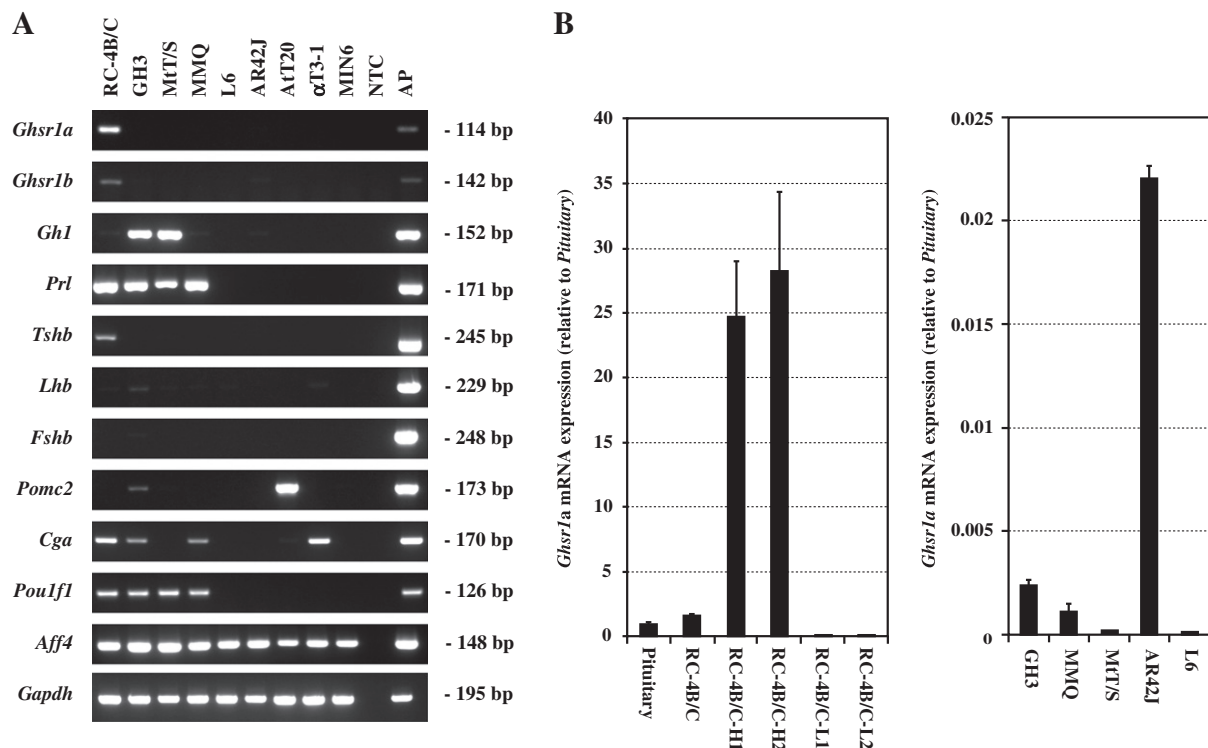


Fig. 1. *Ghsr1a* mRNA expression in rodent cell lines. (A) Conventional RT-PCR analysis. Representative ethidium bromide-stained gel photographs of the indicated RT-PCR products from nine rodent cell lines (RC-4B/C, GH3, MtT/S, MMQ, L6, AR42J, AtT20, α T3-1 and MIN6) and normal rat adult pituitary (AP) are shown. NTC, no template control. All PCR experiments included negative controls without reverse transcriptase, which yielded no PCR products, and the specificity of the RT-PCR products was verified by direct sequencing (data not shown). *Ghsr1a*, (encoding rat growth hormone secretagogue receptor type 1a); *Ghsr1b*, (growth hormone secretagogue receptor type 1b); *Gh1* (growth hormone); *Prl* (prolactin); *Tshb* (thyroid-stimulating hormone- β subunit); *Fshb* (follicle-stimulating hormone- β subunit); *Pomc2* (proopiomelanocortin- β or Pomc2); *Cga* (glycoprotein hormone [GSU]- α subunit); *Pou1f1* (Pit-1); *Aff4* (AF5q31); *Gapdh* (a housekeeping gene). (B) Quantitative real-time RT-PCR analysis. Endogenous *Ghsr1a* mRNA expression levels were determined by TaqMan real-time RT-PCR in RC-4B/C subclones (left panel) and other rat cell lines (right panel). RC-4B/C subclones were selected from parental RC-4B/C cells on the basis of their *Ghsr1a* expression levels (H1 and H2, high *Ghsr1a*-expressing subclones; L1 and L2, low/absent *Ghsr1a*-expressing subclones). Expression levels were normalized to 18S ribosomal RNA, and the data are expressed as the fold mRNA level relative to the average normal pituitary expression (set as 1.0). Note that the graphs have different scales on the y-axis.

of parental RC-4B/C cells with ghrelin led to a significant dose-dependent induction of *Fos* mRNA, and the level of induction was much greater in RC-4B/C-H1 and RC-4B/C-H2 subclonal cells (Fig. 2A). Unlike ghrelin, neither des-acyl ghrelin (the unacylated

ghrelin form unable to bind to GHSR1A) nor SP-analog (a known antagonist of GHSR1A) increased *Fos* mRNA. Signal transduction pathways coupled to GHSR1A activation include the ERK1/2 activation and phospholipase C/IP3 pathways (Baldanzi et al., 2002;

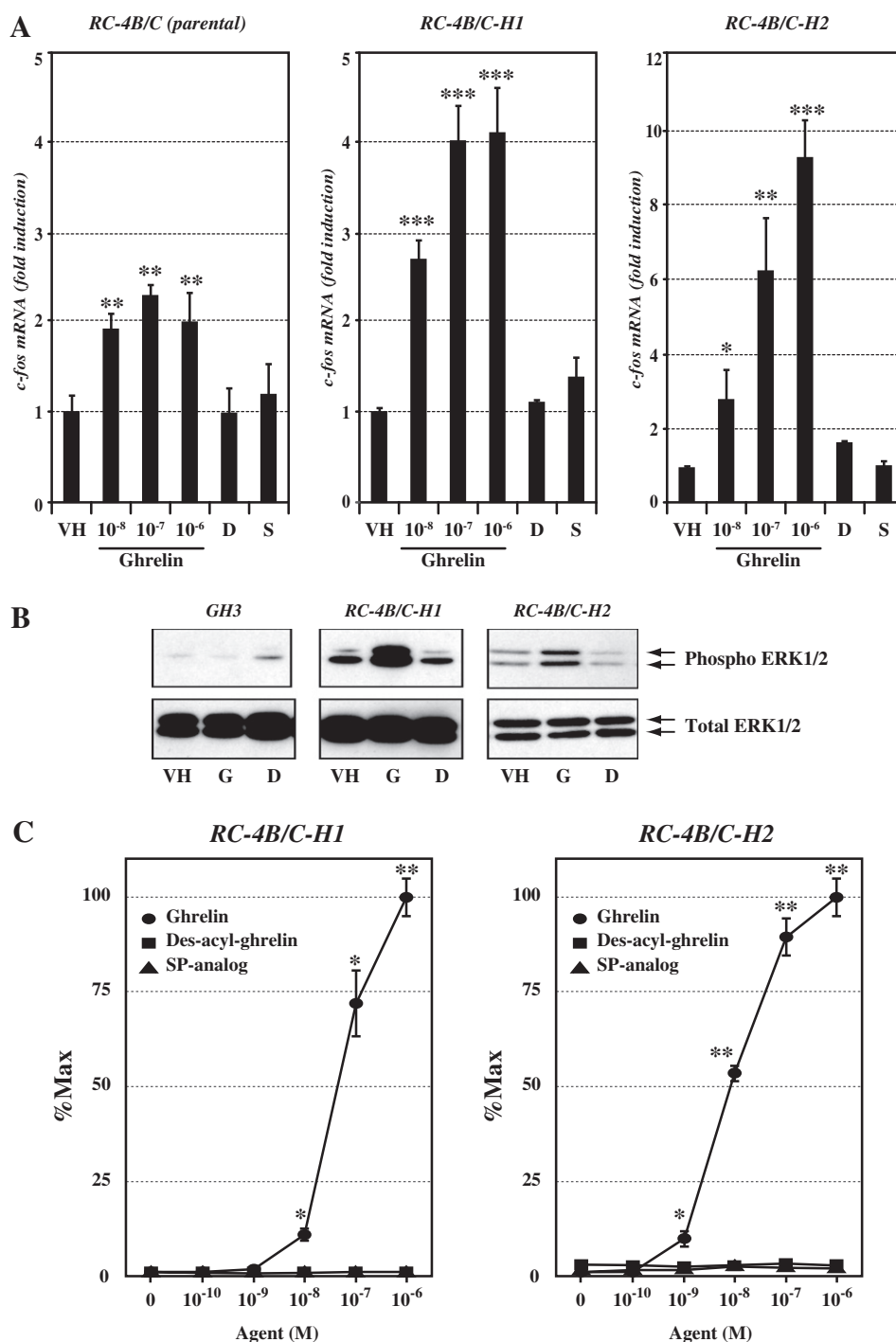


Fig. 2. Characterization of *Ghrelin* receptor activity in RC-4B/C-H1 and H2 cells. The effects of ghrelin on *Fos* mRNA induction (A), ERK1/2 phosphorylation (B) and inositol phosphate accumulation (C) were investigated in the high *Ghrelin* receptor-expressing RC-4B/C subclones, H1 and H2. (A) Parental RC-4B/C cells, RC-4B/C-H1 and H2 subclones were treated with either vehicle (VH), 10^{-8} – 10^{-6} M ghrelin, des-acyl ghrelin (D; 10^{-6} M) or SP-analog (S; 10^{-6} M). *Fos* mRNA expression levels were measured by SYBR-green based real-time RT-PCR and normalized to *Gapdh* expression. Results are expressed as the mean \pm SD of triplicate determinations from one representative experiment, repeated three times with similar results. * P < 0.05, ** P < 0.01, *** P < 0.001 versus VH-treated cells (unpaired *t*-test). (B) GH3 cells, RC-4B/C-H1 and H2 cells were treated with either vehicle (VH), ghrelin (G; 10^{-7} M) or des-acyl ghrelin (D; 10^{-7} M). Cell lysates were analyzed for phospho-ERK1/2 and total ERK1/2 by Western blot. (C) RC-4B/C-H1 and H2 cells were treated with various doses (0– 10^{-6} M) of ghrelin, des-acyl ghrelin or SP-analog, and intracellular inositol phosphate accumulations were measured. Results are expressed as a percentage of the maximum accumulation of inositol monophosphate (IP1), a downstream metabolite of inositol triphosphate (IP3), obtained in the presence of 10^{-6} M ghrelin. Results are expressed as the mean \pm SD of triplicate determinations from one representative experiment, repeated three times with similar results. * P < 0.001, ** P < 0.0001 versus untreated cells (unpaired *t*-test).

Mousseaux et al., 2006). Consistently, RC-4B/C-H1 and RC-4B/C-H2 cells exhibited specific and robust responses to ghrelin, in terms of either ERK1/2 phosphorylation levels (Fig. 2B) or inositol phosphate production (Fig. 2C). These results indicated that RC-4B/C-H1 and RC-4B/C-H2 subclonal cells express the functional Ghslr1a protein properly on the cell surface, and are capable of responding to ghrelin.

3.2. Genomic organization of 5'-flanking region of rat Ghslr and mapping of the transcription start site

We conducted 5'-RLM-RACE analysis using total RNA from normal rat pituitaries. Gel electrophoresis of the final 5'-RACE products showed a major band of approximately 400-bp as well as a smear of larger and smaller bands (Fig. 3A, lane 2). Subcloning and sequencing analysis of the RACE products followed by a comparison with the rat genomic sequence revealed that the majority

of subclones (37 out of 45) contained a 5'-untranslated (UT) first exon followed by a 221-bp intron and an exon 2 with a 120-bp UT sequence immediately preceding the translation initiation codon (Fig. 3B, left).

5'-RACE clones that contained exon 1, corresponding to the major broad band on agarose gels, varied at their 5'-ends, indicating heterogeneous use of the transcription start site (TSS). Putative TSSs were evenly scattered, but almost exclusively mapped within an approximately 100-bp region at the 5'-end. Therefore, the 5'-most position within this cluster was tentatively assigned as +1, which was the second upstream TSS among the clones. Notably, five clones lacked the first exon sequence, and transcription initiated from alternative TSSs at -85 and -22 bp upstream and +52 bp downstream from the 5'-end of exon 2.

Confirmatory RT-PCR analysis using various sense primers located around exon 1 and a common antisense primer showed results generally consistent with 5'-RACE (Fig. 3B, right). Abundant

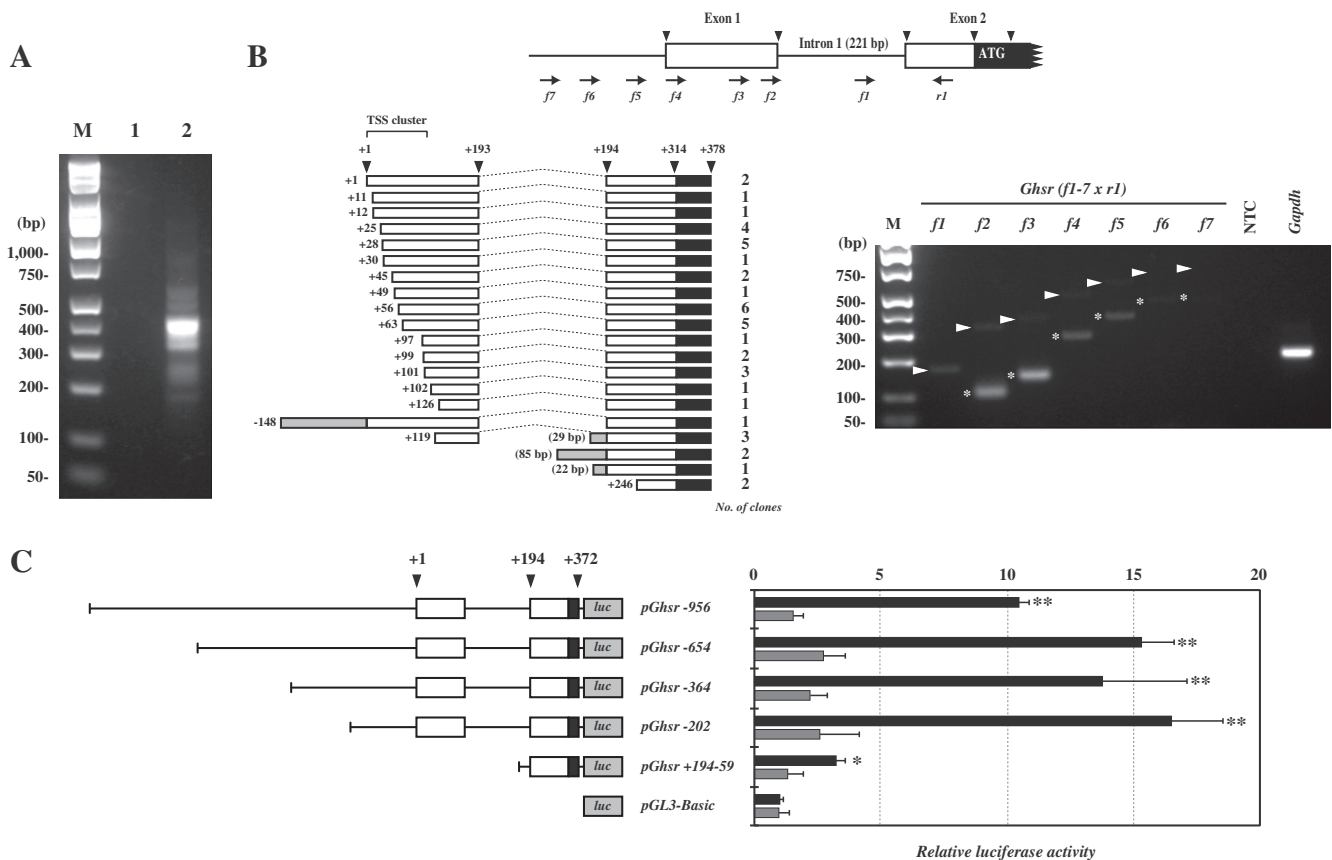


Fig. 3. Analysis of 5'-RLM-RACE and mapping of the Ghslr transcription start site (TSS). (A) 5'-RLM-RACE analysis. 5'-RACE was performed using total RNA isolated from rat pituitaries, and analyzed by running on a 1.5% agarose gel followed by staining with ethidium bromide. RACE products were not detected after the first round of amplification (lane 1), whereas the second round of nested PCR generated a major band of approximately 400-bp, as well as a smear of larger and smaller bands (lane 2). The second round PCR products were subsequently purified, cloned and analyzed by sequencing. Lane M, DNA size marker. (B) Sequence analysis of cloned RACE products and confirmatory RT-PCR. The top panel shows a schematic of the 5'-end and upstream region of the rat *Ghslr* gene. The open box represents the 5'-untranslated (UT) region; lines, 5'-flanking and intronic sequences; the closed box, a protein-coding sequence. On the lower left, 45 5'-RACE clones are diagrammed along with the number of clones isolated with each sequence. Position +1 was assigned to the most 5'-position in the TSS cluster. Note that one clone contains a longer 5'-UT region that extends to -148 bp upstream from the purported TSS, and three differ in the usage of an alternative splice acceptor site 29-bp upstream from exon 2. In addition, three forms of cDNA clones shown at the bottom lack exon 1 and use alternative TSSs near the 5'-end of exon 2. On the lower right, is RT-PCR confirmation of the first UT exon. RT-PCR of DNase-treated pituitary RNA was performed using sense primers located in intron 1 (f1) and exon 1 (f2–f4), as well as upstream from the purported TSS (f5–f7), in combination with the common antisense r1 primer (the locations and directions of the primers are shown at the top). Asterisks indicate bands corresponding to the spliced products, while bands predicted to be unspliced products with retention of the 221-bp of intron 1 are denoted by white triangles. *Gapdh* mRNA was used as a positive control. NTC, no template (negative) control. (C) Promoter reporter gene constructs and their relative promoter activity. Schematic diagrams of the rat *Ghslr* promoter deletion fragments fused to a luciferase gene (*luc*) are shown to the left with luciferase activity on the right. The promoter-reporter constructs were named according to the nucleotide positions of their 5'-termini, using the TSS (position +1, closed triangle) as the reference. The open box indicates the 5'-UT sequence; black box, coding sequence; gray box, luc gene; lines, 5'-flanking and intronic sequences. All constructs were transiently transfected into RC-4B/C-H1 (black bars) or HEK293A (gray bars) cells. Luciferase assays were repeated four times (RC-4B/C-H1) and three times (HEK293A); each performed in quadruplicate with similar results. The mean luciferase activity (\pm SD, $n = 3-4$) of each construct is given relative to the activity of the promoter-less pGL3-Basic vector (set as 1.0). Asterisks indicate promoter activity significantly greater than that of pGL3-Basic. * $P < 0.01$, ** $P < 0.001$ (unpaired *t*-test) versus pGL3-Basic.

RT-PCR products were obtained in the reaction with sense primers located proximal to the purported TSS, with sizes corresponding to the expected spliced products. However, a reduction in the product levels could be detected even when the sense primers distal to the TSS were used. In addition, for each set of primers, we also detected a weak second band corresponding to the unspliced form that retains the 221-bp intron 1. These data thus demonstrate the complexity found at the 5'-end of rat *Ghsr* mRNA.

3.3. Functional characterization of the rat *Ghsr* promoter

Within the proximal promoter region of the rat *Ghsr*, no apparent TATA- or CCAAT-box sequences were found (Supplemental Fig. 1). The region contained a number of putative transcription factor-binding sites, including binding sites for tissue-specific transcription factors, such as Pit-1, Oct-1 and Pitx1. However, a multiple sequence alignment of the corresponding rat, mouse and human genomic sequences suggested these binding sites to be less conserved, and no common element was identifiable (data not shown).

As previous studies suggested that a relatively short (<1-kb upstream from the initiating ATG start codon) genomic fragment encompassing the human *GHSR* promoter is sufficient for basal promoter activity (Kaji et al., 1998; Petersenn et al., 2001), we prepared a luciferase reporter gene construct containing an

approximately 1.3-kb sequence of rat *Ghsr* (positions –956 to +372 relative to the TSS), as well as four sequential 5'-deletion constructs, and tested their activities *in vitro*. We used RC-4B/C-H1 cells for transient transfection because they express a high level of endogenous *Ghsr*, and show a high degree of adherence to culture dishes with relatively high transfection efficiency. As shown in Fig. 3C, the longest pGhsr-956 construct, with a 956-bp of the proximal promoter sequence, displayed significantly elevated luciferase activity in RC-4B/C-H1 cells as compared to the promoter-less pGL3-Basic vector. In contrast, pGhsr-956 showed no significant activity in HEK293A that lack endogenous *GHSR* expression. The *Ghsr* promoter-driven luciferase activity was increased by additional 5'-deletions up to –202 (pGhsr–202), whereas a significant drop in activity was observed with deletion of the entire 5'-flanking sequence and the 5'-UT first exon and its 3'-flanking intronic sequence (pGhsr+194–59). Thus, the proximal region of the promoter from –202 to TSS, and possibly its downstream sequences, contains the regulatory elements responsible for basal promoter activity.

3.4. DNA methylation status of 5'-flanking region of rat *Ghsr*

Annotations from the UCSC Genome Browser indicated the presence of a CpG island in the rat *Ghsr* locus, and a subsequent detailed analysis revealed that the island extended approximately

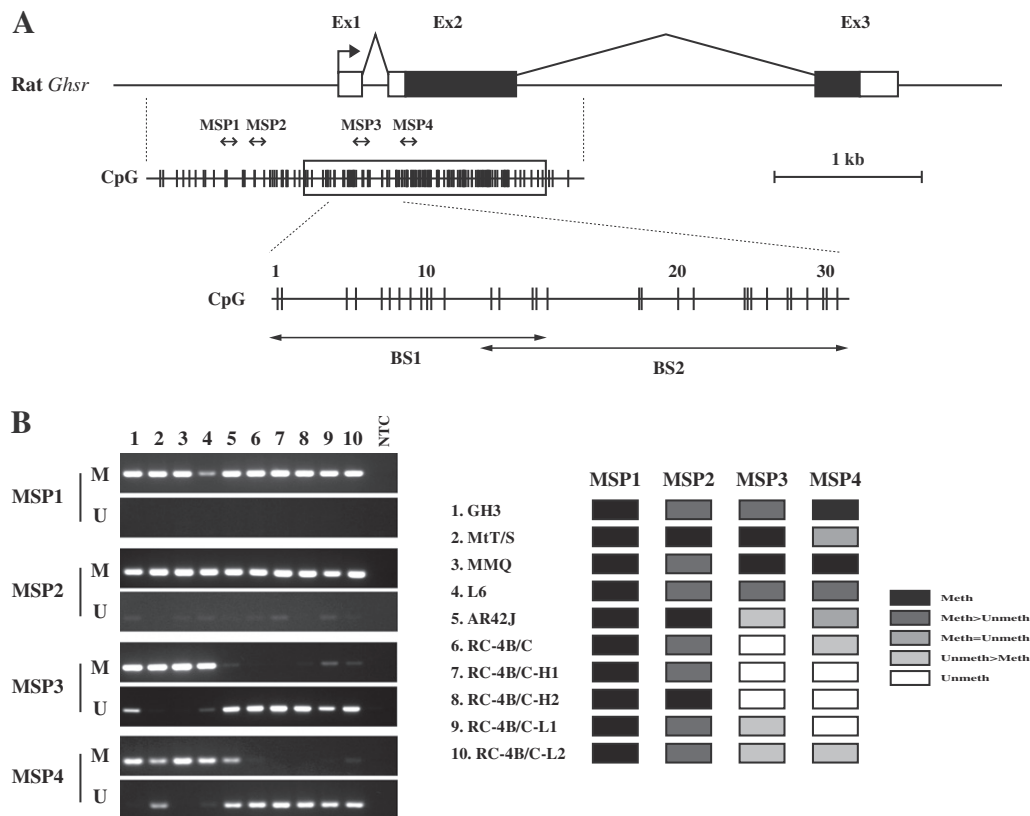


Fig. 4. Location and methylation status of the CpG island at the rat *Ghsr* locus. (A) Schematic representation of the genomic structure of the rat *Ghsr* gene and an adjacent CpG island. The genomic organization is shown at the top. The open boxes indicate untranslated regions; black box, coding sequence; arrow, the site of initiation of transcription (TSS). In the middle, a graphical map of CpG dinucleotide distribution, generated using a web-based CpG Island Searcher Program (Takai and Jones, 2002), is shown. The short vertical bars indicate the locations of CpG sites, and the boxed area the predicted CpG island that extends approximately 3.5-kb in length, spanning the sequence from the proximal promoter to the second intron. The double arrowhead denotes the locations analyzed by methylation-specific PCR assay (MSP1–4). The genomic region analyzed by bisulfite sequencing is enlarged at the bottom, in which two overlapping BS1 and BS2 fragments, containing a total of 31 CpG sites (numbers on top), were analyzed. (B) Methylation-specific PCR (MSP) analysis of rat cell lines. In each cell line, the DNA methylation status of four distinct sites (MSP1–4) within the *Ghsr* 5'-flanking region was assessed. Representative ethidium bromide-stained gel photographs are shown on the left. M, PCR product amplified by methylated-specific primers; U, PCR product amplified by unmethylated-specific primers. Lanes 1–10 show cell lines GH3, MtT/S, MMQ, L6, AR42J, RC-4B/C (parental), RC-4B/C-H1, RC-4B/C-H2, RC-4B/C-L1 and RC-4B/C-L2, respectively. NTC, not template control. On the right, the level of methylation at each site was scored based on the relative intensity of the M- and U-bands, and given as a heat map in black/white coding (black: complete or high level, gray: intermediate or low, white: no methylation).

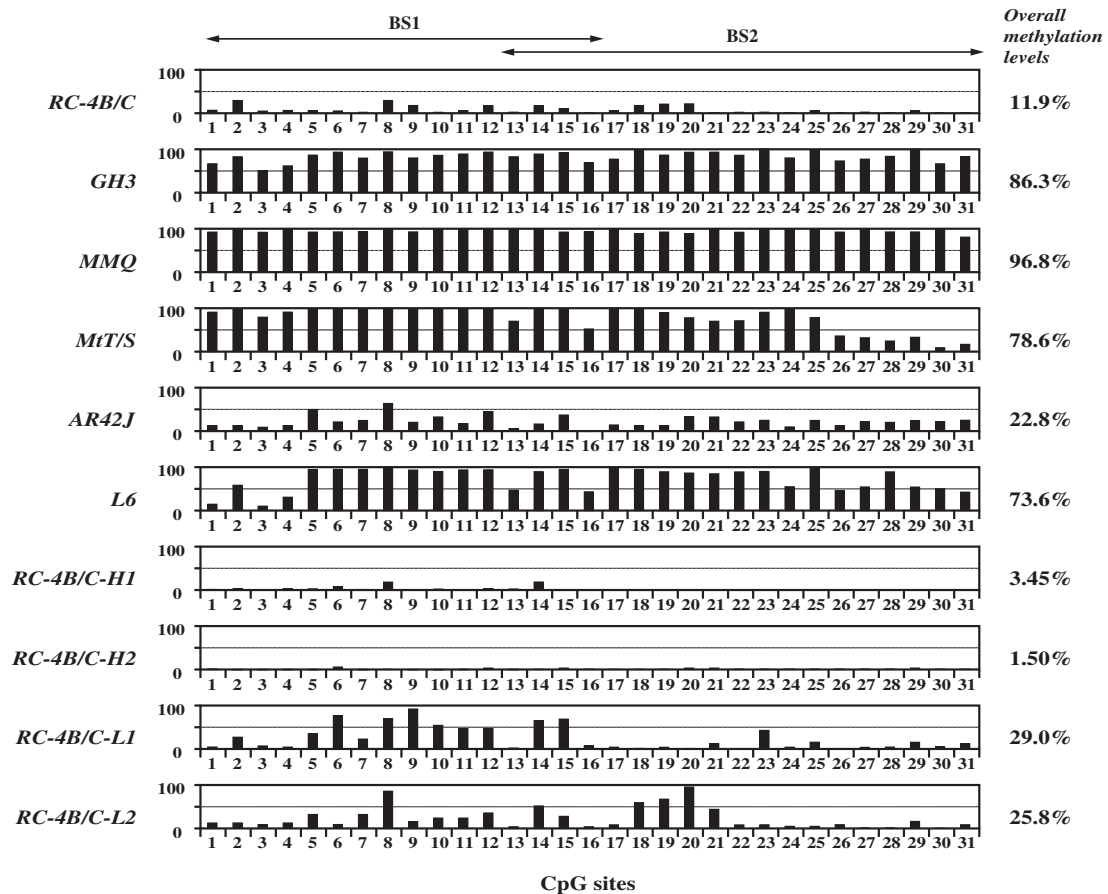


Fig. 5. Bisulfite sequencing analysis in rat cell lines. In ten rodent cell lines, bisulfite genomic sequencing was performed in two overlapping regions (BS1 and BS2), mapped at the 5'-flanking region of rat *Ghsr*. Cell line names are indicated on the left. Numbers on the x-axis represent CpG sites that correspond to those shown in Fig. 4A. In each graph, black bars indicate percent methylation (0–100%) at each CpG site. The overall methylation levels (%), shown on the right, were determined by dividing the total number of methylated CpGs by the total number of sequenced CpGs (unmethylated + methylated) and then multiplying by 100.

3.5-kb, encompassing the region from the proximal promoter to the second intron (Fig. 4A). In addition, this CpG island was found to be conserved in, at a minimum, several mammalian species including mice and humans (Supplemental Fig. 2). To measure the methylation level in this CpG island in rat cell lines, we performed MSP assays, utilizing four distinct MSP primer sets (MSP1–4; Fig. 4A, middle) that target different sites within the 5'-flanking region of *Ghsr*. Our MSP results indicated the MSP3 region neighboring the TSS to be unmethylated in cell lines showing a high level of *Ghsr1a* mRNA expression (RC-4B/C, and H1 and H2 subclones), with a similar tendency in the downstream MSP4 region, whereas the MSP3 and MSP4 regions were both predominantly methylated in cells without *Ghsr1a* expression (Fig. 4B). The more distal 5'-flanking regions of the promoter, MSP1 and MSP2, were hypermethylated in all cell lines tested.

Methylation patterns of the MSP3 region were mapped more precisely with bisulfite sequencing of 31 CpG sites within a 571-bp region around the TSS. For this mapping, the corresponding region was amplified as two partially overlapping fragments (BS1 and 2; Fig. 4A), and at least 20 subclones were sequenced for each individual cell line. As shown in Fig. 5, we observed a clear association between heavy methylation of CpG sites in this region and a lack of *Ghsr1a* mRNA expression (GH3, MMQ, MtT/S and L6; overall methylation levels >70%), and *vice versa* (RC-4B/C, H1 and H2; <12%). AR42J, RC-4B/C-L1 and L2 cells displayed intermediate overall methylation levels (>20%). In addition, it is noteworthy that each cell line displayed a different methylation profile, even those

cells that did not express *Ghsr1a*, and CpGs at positions 5–15 (Fig. 5) appeared to be the most stable indicators of mRNA expression levels.

3.5. Effects of 5-aza-dC and TSA on *Ghsr1a* expression

We next examined the effects of the DNMT inhibitor 5-aza-dC and/or the HDAC inhibitor TSA on *Ghsr1a* transcription in cells expressing a very-low or undetectable level of endogenous mRNA (GH3, AR42J and L6). As shown in Fig. 6, *Ghsr1a* transcription was induced in all three cell lines tested; however, response patterns to these agents varied considerably among cell lines. In GH3 cells, treatment with 5-aza-dC for 4 days induced a significant dose-dependent increase in *Ghsr1a* expression whereas treatment with TSA alone had no effect. Combination treatment with 5-aza-dC plus TSA produced no augmentation of 5-aza-dC effects, instead decreasing the expression of *Ghsr1a*, possibly because the combination of these two agents influenced cell viability. In contrast, *Ghsr1a* mRNA in AR42J cells was unaffected by 5-aza-dC, while there was a significant induction (approximately 4-fold) with TSA treatment; no synergism with 5-aza-dC was observed. In L6 cells, up-regulation of mRNA expression by either 5-aza-dC or TSA alone appeared to be minimal, but there was a dramatic synergistic effect in response to combined treatment with these two agents. These results strongly suggest that, in cultured cell lines, DNA methylation and histone modification influence *Ghsr1a* gene silencing in a cell type-specific manner.

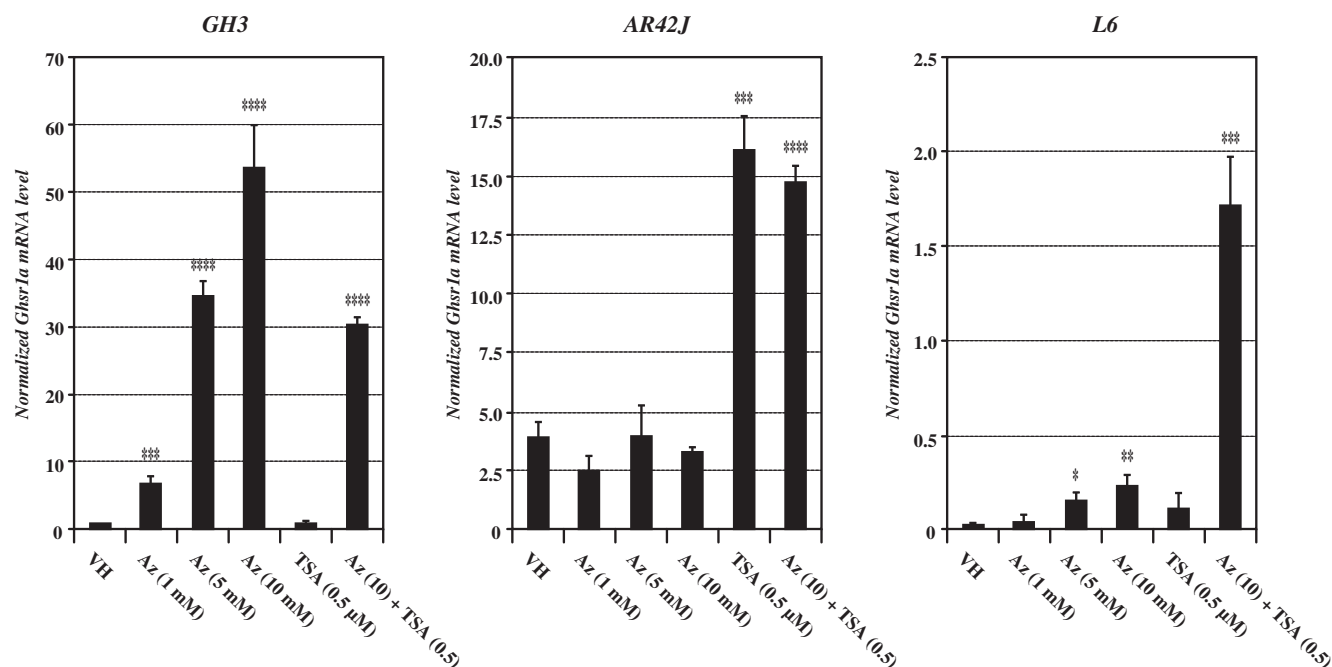


Fig. 6. Effects of 5-aza-dC and TSA on *Ghr1a* mRNA expression. Three rat cell lines (GH3, AR42J and L6) were treated with various concentrations of 5-aza-dC (Az; 1–10 mM) for 4 days and/or TSA (0.5 μM) for 24 h, or with vehicle (VH). RNA was extracted from treated cells, converted to cDNA, and subjected to TaqMan quantitative real-time RT-PCR. The bar graphs show the expression level of *Ghr1a* mRNA measured after normalization for 18S ribosomal RNA, relative to control cells (untreated RC-4B/C-H1) set as 100%. The data, presented as mean ± SD, are the most representative of the 3–4 independent experiments performed in each case, all yielding similar results. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ versus VH-treated cells (unpaired t -test, $n = 3–4$).

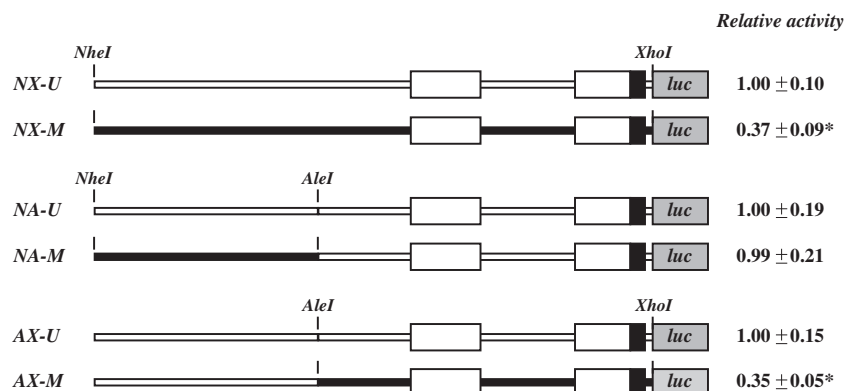


Fig. 7. Effect of CpG methylation on *Ghr* promoter activity. Regional methylation was performed on the *luciferase* reporter construct pGhr-654, by double-restriction digestion with NheI–XhoI (NX), NheI–AclI (NA) or AclI–XhoI (AX) and *in vitro* methylation. Promoter-reporter gene assays were conducted using *Ghr1a*-expressing RC-4B/C-H1 cells. In the reporter constructs, shown on the left, narrow white and black boxes indicate unmethylated (U) and fully-methylated (M) regions, respectively. The open box indicates the 5'-untranslated sequence; black box, coding sequence; gray box, *luciferase* (*luc*) gene. Note that the *luc* gene itself and other vector sequences were not methylated in all constructs. The relative luc activity (means ± SD) of each construct, normalized to activities of the mock-treated constructs (set as 1.0), is shown on the right. The results from three independent experiments are presented. * $P < 0.001$ versus mock-treated constructs, unpaired t -test.

3.6. Inhibition of transcriptional activity by methylation of the rat *Ghr* promoter

The effects of methylation on *Ghr* promoter function were assessed with reporter gene assays using *patch* (or regional) methylated promoter constructs (Robertson and Ambinder, 1997). In this experiment, insert fragments of the pGhr-654 reporter construct were purified, *in vitro* methylated and ligated back into the original vector. The resulting reporter constructs, in which the target fragments alone were methylated and other sequences were not, were transfected into RC-4B/C-H1 cells. Methylation of the full promoter insert significantly suppressed promoter activity (Fig. 7; NX-M), and a similar degree of suppression was observed with methylation of the proximal fragment (AX-M). On the other hand, methylation of the distal region (NA-M) did not have suppressive effects.

Consistent with the *patch* methylation results, significant suppression of *Ghr* promoter activity by methylation was observed using the modified pGhr-202 reporter construct, pCpGfree-Ghr-202, in which the *luciferase* open reading frame was replaced by a CpG-free sequence (Supplemental Fig. 3). Taken together, these data indicate the rat *Ghr* promoter to be methylation sensitive, and that proximal promoter methylation caused a significant suppression of promoter activity.

3.7. Associations between histone H3 modification status and *Ghr1a* expression levels in cell lines

Post-translational modification of histone H3, one of the core histones, is known to play important roles in modulating chromatin structure and regulating the transcriptional activity of specific

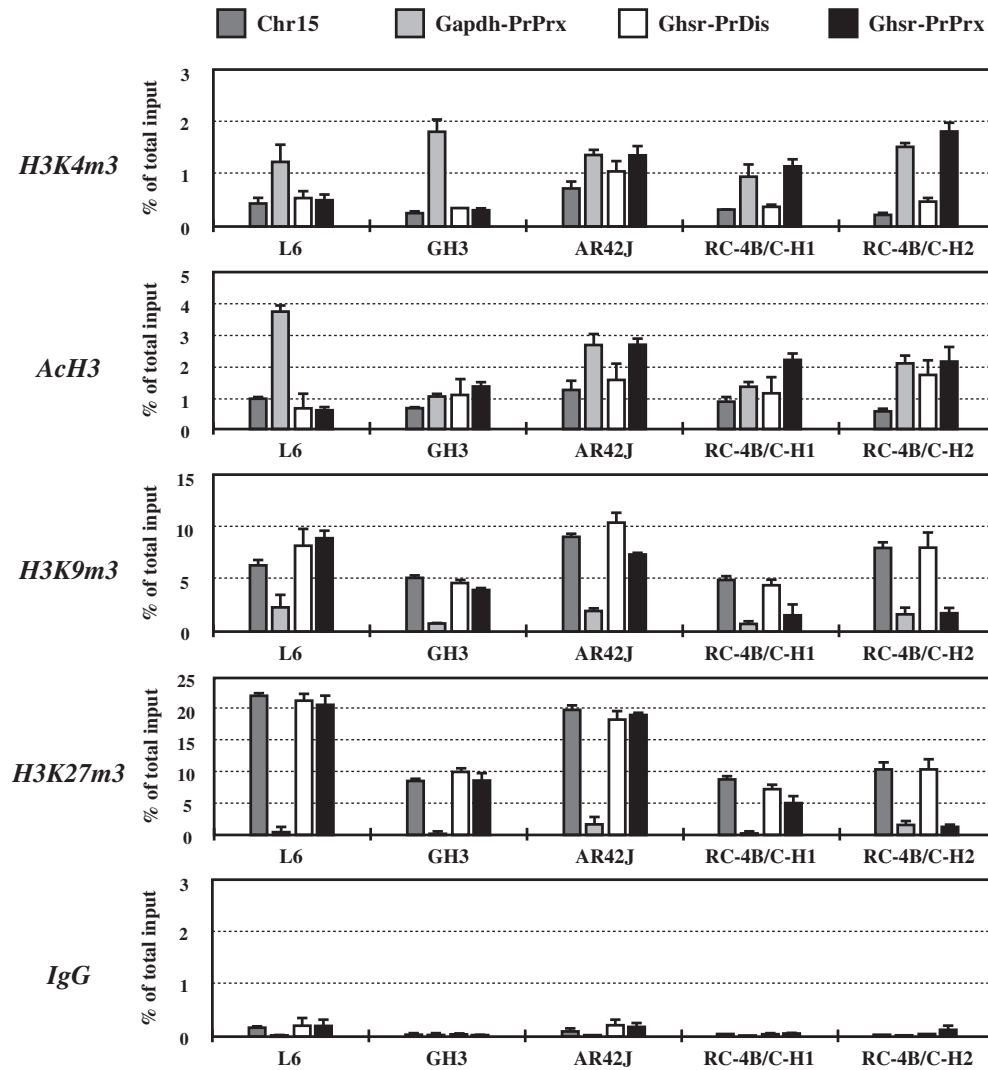


Fig. 8. Histone H3 methylation status analyzed by chromatin immunoprecipitation (ChIP). ChIP was performed for five rat cell lines (L6, GH3, AR42J, RC-4B/C-H1 and -H2), using anti-trimethyl-histone H3 (Lys4) (H3K4m3), anti-histone H3 acetylated at Lys 9 and 14 (AcH3), anti-trimethyl-histone H3 (Lys9) (H3K9m3) and anti-trimethyl-histone H3 (Lys27) (H3K27m3) antibodies. We performed SYBR-green based quantitative real-time PCR of the rat *Chromosome 15* microsatellite (Chr15), *Gapdh* proximal promoter (Gapdh-PrPrx), and *Ghshr* distal and proximal promoter (Ghshr-PrDis and PrPrx) regions. The data are expressed as percent precipitation (means \pm SD) relative to total chromatin input. Note that a control ChIP with a normal rabbit IgG (negative control) showed low overall background levels ($<0.2\%$ of the total input chromatin; bottom panel).

genes (Berger, 2007; Rando, 2007). We thus used ChIP assays to evaluate the status of histone H3 modification in the *Ghshr* promoter region, using chromatin preparations from two cell lines constitutively expressing a high level of endogenous *Ghshr1a* (RC-4B/C-H1 and H2) and three with low or no expression (L6, GH3 and AR42J). The results are shown in Fig. 8. As expected, ChIP results for H3K4m3 (an activation mark) showed higher levels of enrichment in the active *Gapdh* proximal promoter region (Gapdh-PrPrx) for all five cell lines, than in the *Chr15* microsatellite (Chr15) and the distal *Ghshr* promoter (Ghshr-PrDis; located approximately 4.7-kb upstream of the TSS), regions assumed to be transcriptionally inactive. Under the same experimental conditions, the proximal *Ghshr* promoter region (Ghshr-PrPrx; located within a 200-bp region upstream from the TSS) was enriched in RC-4B/C-H1 and H2 cells, whereas in other cell lines this change was minimal (AR42J) or completely absent (L6 and GH3). ChIP results for AcH3 showed a similar pattern, but were more subtle.

ChIP patterns for heterochromatin marks, H3K9m3 and H3K27m3, were essentially the reverse of the H3K4m3 pattern. That is, in all cell lines, Chr15 and Ghshr-PrDis were clearly enriched

for H3K9m3 and H3K27m3, while Gapdh-PrPrx showed near-total depletion. Ghshr-PrPrx was depleted in RC-4B/C-H1 and H2, but significantly enriched in L6 and GH3 cells. AR42J cells showed an intermediate ChIP pattern. Collectively, ChIP analyses revealed Ghshr-PrPrx to display increased levels of activating chromatin marks (H3K4m3 and AcH3) along with reduced levels of suppressive marks (H3K9m3 and H3K27m3) in *Ghshr1a*-expressing cells, whereas this region harbored abundant suppressive marks but low levels of activation marks in cells with low or absent *Ghshr1a* mRNA.

3.8. Lack of correlation between the degree of *Ghshr* promoter-methylation and *Ghshr1a* mRNA expression in normal rat tissues

Our next goal was to examine whether the DNA methylation status of the *Ghshr* gene promoter correlates with tissue-specific *Ghshr1a* expression or age-associated changes in gene expression. We thus evaluated the extents of methylation of 16 CpG sites around the TSS (BS1; Figs. 4A and 5) in tissues from normal male F344 rats of different ages, expressing either high levels

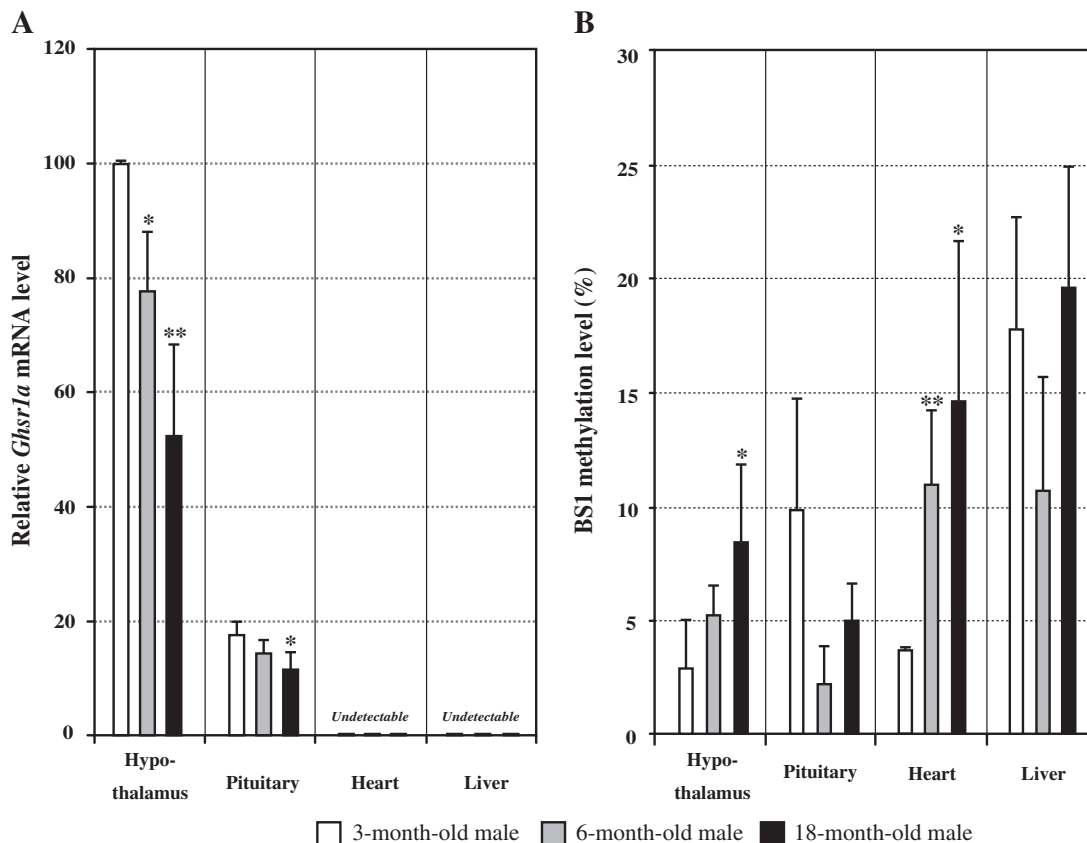


Fig. 9. mRNA expression levels and methylation status of *Ghnr* in normal rat tissues. (A) *Ghnr1a* mRNA expression levels in four tissues (hypothalamus, pituitary, heart and liver) in different age groups (3, 6 and 18 months; $n = 4$) of male F344 rats were determined by TaqMan quantitative real-time RT-PCR. After normalization to 18S ribosomal RNA levels, results are expressed as relative mRNA levels (means \pm SD) compared with the average *Ghnr1a* expression in the hypothalamus of 3-month-old rats (set as 100). * $P < 0.05$, ** $P < 0.01$ versus *Ghnr1a* expression level in the corresponding tissues from 3-month-old rats (unpaired t -test). (B) In each tissue, the methylation state of 16 CpG sites in the BS1 PCR fragment (Fig. 4A) was determined by bisulfite sequencing. The bar graphs show the average methylation level of all 16 CpG sites (\pm SD; $n = 4$), determined by dividing the total number of methylated CpGs by the total number of sequenced CpGs (unmethylated + methylated) and then multiplying by 100. * $P < 0.05$, ** $P < 0.01$ versus overall methylation levels in the corresponding tissues from 3-month-old rats (unpaired t -test).

(hypothalamus and pituitary) or low/absent (heart and liver) levels of *Ghnr1a* mRNA. As shown in Fig. 9A, our qRT-PCR results revealed *Ghnr1a* mRNA to be predominantly expressed in the hypothalamus as well as in the pituitary, while no measurable signal was detected in cardiac or hepatic tissues. In addition, we observed a significant age-dependent decrease in *Ghnr1a* expression in the hypothalamus and, to a lesser extent, in the pituitary.

Bisulfite sequencing showed methylation status to vary among individual tissues (especially the heart and liver; data not shown); however, the calculated average methylation levels of all 16 CpG sites were generally lower ($\sim 10\%$) in the hypothalamus and pituitary (Fig. 9B), with a tendency for increased levels in non-*Ghnr1a* expressing tissues, the heart and liver, particularly in 6- and 18-month-old animals. In the hypothalamus, there was a weak but significant age-dependent increase in methylation level, thus showing an opposite pattern of mRNA change. However, this change was not clearly reproduced in the pituitary. Cardiac tissues showed low methylation in 3-month-old animals and then exhibited a significant age-dependent increase in methylation.

4. Discussion

The ghrelin-GHSR1A signaling system is a pivotal regulator of various physiological and pathological processes. Therefore, it represents one of the most promising targets for the development of new drugs particularly for the treatment of metabolic diseases

such as obesity, diabetes and cancer cachexia (Kojima and Kangawa, 2005; Chen et al., 2009). A better understanding of the molecular mechanisms underlying the cell type-specific expression of *GHSR1A* will not only provide insights into the mechanisms of gene regulation, but may also be useful for the development of new therapeutic strategies. To this end, we first performed gene expression analyses and confirmed that substantial levels of *Ghnr1a* mRNA were detectable in only one cell line of endocrine origin, RC-4B/C, out of nine rodent cell lines examined, data supporting the observation of Falls et al. (2006). Subsequently, we established RC-4B/C-derived subclones (RC-4B/C-H1 and H2), expressing a high level of endogenous *Ghnr1a* mRNA, and demonstrated that they do indeed possess a functional receptor capable of responding to exogenous administration of ghrelin, but not des-acyl ghrelin. We believed these subclones to be valuable tools for studying *Ghnr* gene regulation *in vitro*, and thus used them for all subsequent investigations.

In the present study, we also characterized the upstream structure and regulation of the rat *Ghnr* gene. 5'-RACE using pituitary total RNA revealed a novel 5'-UT first exon characterized by multiple TSSs mapping within a TATA-less, CpG island promoter. A comparison with the transcriptional activity of a series of deletion promoter constructs in RC-4B/C-H1 cells indicated that the minimal promoter region required for basal activity resided downstream from position -202 relative to the TSS. Thus, our findings are consistent with previous studies on the human *GHSR* gene (Kaji et al., 1998; Petersenn et al., 2001), which

implied the presence of an alternative 5'-UT first exon and mapped a minimal promoter within a region less than 1-kb upstream from the ATG translation initiation codon. In addition, a preliminary search using the UCSC Genome Browser identified a mouse EST sequence (GenBank Accession No. AK049671) corresponding to the sequence of the first exons in rats and humans. Thus, the 5'-flanking regions of *GHSR* genes seem to be very similar structurally and to be conserved among these species. Although analyses of the minimal promoter regions in different species predicted a number of transcription factor binding sites, there was no evidence of definitely conserved binding sites shared among these species. To obtain clues to the specific molecular players involved in cell type-specific *GHSR1A* expression, we compared the gene expression profiles of selected genes between *Ghsr1a*-expressing and non-expressing cell lines, but no genes, even those of pituitary hormones and pituitary-specific transcription factors, showed a clear correlation with the *Ghsr1a* expression level (H. Inoue, unpublished observations). Thus, the molecular mechanisms that dictate cell-specific *GHSR1A* gene expression remain obscure.

It is widely acknowledged that CpG methylation of the promoter regions of some genes plays an important regulatory role in transcriptional regulation and in the establishment and maintenance of cell type-specific gene expression (Li, 2002; Jaenisch and Bird, 2003). This study obtained multiple lines of evidence that *GHSR1A* transcription is in fact influenced by methylation of the promoter region. First, a defined CpG island spanning the proximal promoter and exonic regions was identified in the rat *Ghsr* gene. This CpG island is highly structured and conserved in, at a minimum, several mammalian species, including mice and humans. Second, in multiple cell types including RC-4B/C-derived sublines, MSP and bisulfite sequencing revealed a strong inverse correlation between mRNA expression level and methylation status in the promoter region. Third, in a transient transfection reporter assay, *in vitro* methylation of the proximal *Ghsr* promoter sequence resulted in a significant reporter activity decrease. Fourth, in cells with low or absent *Ghsr1a* expression, epigenetic modifications by DNMT and/or HDAC inhibitors effectively induced re-expression of *Ghsr1a* mRNA. Finally, the patterns of histone H3 post-translational modifications occurring at the *Ghsr* proximal promoter were cell type-specific, and an active pattern of histone H3 modifications was obvious in *Ghsr1a*-expressing RC-4B/C subclonal cells and *vice versa*.

It is noteworthy that the effects of 5-aza-dC and TSA differed significantly among cell types. In pituitary GH-secreting GH3 cells, treatment with 5-aza-dC, but not with TSA, resulted in a concentration-dependent re-expression of *Ghsr1a* transcription, whereas TSA alone induced a slight but significant upregulation in pancreatic acinar AR42J cells. In L6 myoblasts, the individual agents had a minimal effect on *Ghsr1a* expression, but a remarkable synergistic effect was demonstrated using a combination of these two agents. These results suggest that DNA methylation and histone acetylation contribute differently to *Ghsr* silencing in different cell types, and that DNA methylation is probably dominant over HDAC activity because the histone deacetylation by TSA alone appeared to be insufficient to “unlock” the *Ghsr* gene from the silenced chromatin state in cells with a densely methylated promoter (GH3 and L6), and 5-aza-dC had no effect on cells which displayed a relatively low level of promoter methylation (AR42J).

In line with previous studies showing that the presence of DNA methylation can modify local histone modification patterns (Jaenisch and Bird, 2003; Lande-Diner et al., 2007), our ChIP results indicated that *Ghsr* gene silencing in cell lines results from increased levels of heterochromatin-associated H3K9 and H3K27 trimethylation in the proximal promoter region of *Ghsr*. In contrast, *Ghsr1a*-expressing RC-4B/C-H1 and H2 subclones showed

depletions of H3K9 and H3K27 trimethylation, but enrichment of H3K4 trimethylation in the same region, thus displaying a modification pattern associated with transcriptionally permissive/active chromatin.

Next, we examined whether the relationship between *GHSR* gene expression patterns and promoter DNA methylation, both of which occur in an essentially all-or-none fashion in cultured cell lines, is reproducibly detectable in normal tissues. However, our methylation analysis results on rat tissues in relation to a tissue-specific pattern or age-associated changes in *Ghsr1a* mRNA expression were apparently inconclusive. We did observe a non-significant trend for more methylation in non-expressing tissues (heart and liver), and found that, in the hypothalamus, an age-related decline in *Ghsr1a* mRNA is accompanied by increased methylation. However, age-related methylation changes were not reproducible in the pituitary, instead being seen in cardiac tissue, in which *Ghsr1a* mRNA was undetectable at all ages examined. These observations raise the possibility of altered methylation status being independent of gene expression changes, although in the hypothalamus they might contribute to or accelerate changes in gene expression. It should be noted that, in this study, only a small number of rat tissues were tested. Thus, there is a clear need to analyze more tissues, preferably at different developmental stages, to determine whether there are clear associations between *Ghsr1a* mRNA expression patterns and promoter DNA methylation. However, there is a major challenge in that the “normal” tissues used in our present experiments were likely comprised of DNA from a heterogeneous cell population with variable *Ghsr1a* mRNA expression levels, as well as from other sources such as connective tissue. Therefore, more elaborate methods analyzing methylation in a pure population of cells expressing *Ghsr11* are also needed.

Taken together, our results show that, at least in the *in vitro* cell line model, methylation within the proximal promoter region of *GHSR* is an important determinant of its expression; however, to determine whether this represents a physiologically and/or pathophysiologically relevant event, further investigations are necessary. In this regard, it is noteworthy that two recent studies indicated that alteration of the level of DNA methylation at the *GHSR* locus might be causally implicated in the pathogenesis of cancer and metabolic diseases. A human study demonstrated that the *GHSR* promoter region is frequently hypermethylated in primary breast tumors, and suggested that the methylation status of this region may be useful as a diagnostic biomarker (Ordway et al., 2007). Another recent study of inbred mice demonstrated that growth and metabolic phenotypes derived from maternal high-fat diet exposure can persist across at least two generations of offspring through putative epigenetic inheritance mechanisms (Dunn and Bale, 2009). Their study also provided evidence for a sex-dependent mechanism as first and second-generation female, but not male, mice displayed significant plasma IGF-I elevation and increased neuronal *Ghsr1a* mRNA expression, the latter of which was accompanied by reduced CpG methylation in the 5'-flanking region of the *Ghsr* gene.

In summary, our data suggest that alterations in DNA methylation and histone modifications regulate *GHSR1A* expression. It is reasonable to speculate that changes in the epigenetic status of the *GHSR* gene play an important role in determining cellular sensitivity to ghrelin, and thus have a significant impact on various disease phenotypes. The data presented herein provide a basis for further research testing of this hypothesis.

Disclosure summary

The authors have no conflicts of interest to declare.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.mce.2011.06.034.

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