Expression of the human melanocortin-4 receptor gene is controlled by several members of the Sp transcription factor family

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Abstract

The melanocortin-4 receptor (MC4-R) plays a key role in the hypothalamic control of food intake, lending importance to the understanding of the mechanisms that regulate its expression. To identify factors controlling the expression of the human (h) *MC4-R* gene, a fragment containing 1253 bp of the 5'-flanking region of the *hMC4-R* gene was isolated. A series of hMC4-R luciferase constructs were developed and used to transiently transfect HEK293 and GT1-7 cell lines, both expressing endogenous MC4-R mRNA. Deletion analysis of the 1253 bp fragment showed that the basal promoter activity is mainly restricted to the 179 bp upstream of the transcription start site in both cell types. Mutation of a putative Sp1-binding site located at position -76 bp resulted in a dramatic reduction of the luciferase activity in HEK293 and GT1-7 cells by 87 and 80% respectively. Both *in vitro* and *in vivo* studies (gel shift and chromatin immunoprecipitation analyses) revealed binding of both Sp1 and Sp3 to this site in HEK293 cells. Cotransfection with an Sp1 expression vector in *Drosophila* cells that do not express Sp1, in conjunction with treatment of HEK293 cells with mithramycin A, a specific inhibitor of Sp1, confirmed the role of Sp1. For the first time, we have demonstrated that the constitutive activity of the hMC4-R promoter is dependent upon Sp transcription factors.

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Introduction

The melanocortins (adrenocorticotrophic hormone, and α -, β - and γ -melanocyte stimulating hormone (MSH)) (Gee *et al.* 1983) are derived from the precursor protein pro-opiomelanocortin (POMC). The *POMC* gene is expressed in the hypothalamic arcuate nucleus and in the nucleus of the solitary tract (Jacobowitz & O'Donohue 1978, Joseph *et al.* 1983, Palkovits *et al.* 1987). These hormones mediate their effects through G-protein coupled receptors by stimulating adenylate cyclase (Mountjoy *et al.* 1992). To date, five melanocortin receptor subtypes with different patterns of expression in brain and peripheral tissues have been cloned and characterized (Chhajlani & Wikberg 1992, Chhajlani *et al.* 1993, Gantz *et al.* 1993a,b, Mountjoy *et al.* 1992). Melanocortins are known to have a broad spectrum of physiological actions including regulation of melanocyte pigmentation (De Wied & Jolles 1982), thermoregulation (Feng *et al.* 1987), regulation of obesity (Fan *et al.* 1997), control of the cardiovascular system (Li *et al.* 1996) and learning and memory (De Wied & Croiset 1991) as well as immunomodulatory effects.

Activation of melanocortin-4 receptor (MC4-R) by α -MSH increases energy expenditure and decreases food intake. Studies involving targeted gene deletion in mice demonstrate that loss of *MC4-R* results in obesity,

hyperphagia and hyperinsulinaemia (Huszar *et al.* 1997, Marsh *et al.* 1999). In addition, treatment of the paraventricular nucleus or the fourth ventricle with an MC4-R receptor agonist (MTII) suppresses food intake (Giraudou *et al.* 1998). At the central level, an opioid-induced reduction of 125 I-labelled NDP- α -MSH binding in the ventrolateral striatum has been reported (Alvaro *et al.* 1996), as well as food restriction-induced binding of 125 I-labelled Nle⁴, D-Phe⁷(NDP)- α -MSH in several hypothalamic nuclei (Harrold *et al.* 1999) of the rat.

MC4-R +/– mice display an intermediary obese phenotype between that of wild-type mice and MC4-R –/– mice, indicating that the presence of only one mutated allele is associated with obesity (Huszar *et al.* 1997). In the human, an inactivating mutation present in only one allele of the human (h) *hMC4-R* gene-coding sequence induces a morbid obesity (Vaisse *et al.* 1998, Yeo *et al.* 1998). Because 50% expression of the normal *hMC4-R* gene is not sufficient to obtain a normal phenotype, the constitutive expression of the *MC4-R* gene must be strongly controlled, although there are few data available regarding this regulation (Alvaro *et al.* 1996, Harrold *et al.* 1999).

The mouse *MC4-R* (*mMC4-R*) and *hMC4-R* gene promoters have been cloned but the factors involved in their basal expression are unknown (Dumont *et al.* 2001,

Lubrano-Berthelie *et al.* 2003). Herein, we have demonstrated that the maximal basal promoter activity is retained by the proximal 179 bp of the promoter and one Sp1-binding site is absolutely required for the transcriptional activation of the *hMC4-R* gene.

Materials and methods

Materials

Penicillin, streptomycin, fetal calf serum (FCS), Dulbecco's Modified Eagle's Medium/Ham's nutrient mixture (DMEM/F-12, 15 mM Hepes), Dulbecco's Eagle Medium, Schneider insect medium and restriction enzymes were purchased from Invitrogen. Oligonucleotides were prepared by Sigma-Genosys (Cambridge, UK). Mithramycin A was purchased from Cogener (Paris, France).

Characterization of the *hMC4-R* cDNA 5'-end

Total RNA (3 µg) from human brain (Clontech) was analysed to obtain the complete cDNA sequence of the *hMC4-R* gene using rapid amplification of the 5'-cDNA end (5'-RACE). This analysis was performed using the GeneRacer kit (Invitrogen) according to the instructions provided. Human brain cDNA was amplified using the specific primer MC4-2 and the GeneRacer primer 1 (5'-cgactggagcagcaggacactga-3'). The primary PCR product was used as template for a secondary PCR using the nested specific primer MC4-3 (5'-ctcactggcattgctgtc agtctgt-3') and the GeneRacer nested primer 2 (5'-ggacactgacatggactgaaggagta-3'). The PCR products were cloned into pCR4-Topo using the TOPO TA cloning kit (Invitrogen). The different clones were obtained from two independent cDNA syntheses. DNA from individual colonies was sequenced using a T7 sequencing kit (Amersham Biosciences).

Reporter-vector constructions

Luciferase reporter plasmids containing *hMC4-R* gene promoter were constructed by cloning deleted fragments of the 5'-flanking sequence of the *MC4-R* gene into pGL3 basic. For the longer constructs, PCR reactions were performed using Taq Extra Polymerase II (Eurobio, Les Ulis, France) a sense primer containing an NheI restriction endonuclease site at the 5'-end, and an antisense primer complementary to exon 1 (5'-gacc agatctccagcctgagcgttgct-3') containing a BglII site and human genomic DNA as a template. The resulting fragments were subcloned into the NheI-BglII digested pGL3 basic vector. The sequence was confirmed to ensure that there were no PCR-generated mutations. Vector p(-279/+26)luc was digested with PvuII enzyme to generate the p(-20/+26)luc vector.

Construction of vectors containing mutated fragments were obtained using the Quick Change site-directed mutagenesis kit from Stratagene (Ozyme, Montigny-le-Bretonneux, France). The presence of the desired mutation inside the site was confirmed by sequencing.

Cell culture and transfection

The mouse hypothalamic GT1-7 and human embryonic kidney 293 (HEK293) cells were used to study the promoter activity of the different constructs. The day before transfection, cells were plated in six-well dishes at 200 000 cells per well in DMEM/F-12 supplemented with 7.5% FCS, penicillin (100 U/ml), streptomycin (0.1 mg/ml), glutamine (100 U/ml) for HEK293 cells and in Dulbecco's Eagle Medium supplemented with 10% FCS, penicillin (100 U/ml), streptomycin (0.1 mg/ml) and glutamine (100 U/ml) for GT1-7 cells.

Transient transfections were performed in serum-free medium without antibiotics using the LipofectAmine Plus reagent (Invitrogen) in the presence of 1 µg of the *hMC4-R* gene construct and 1 µg pRV-βGal per well. Cells were incubated with DNA-LipofectAmine Plus complexes for 3 h at 37 °C under 5% CO₂. After 3 h the serum-free medium was replaced with complete medium. The luciferase and β-Gal activities were measured 3 days after transfection using the appropriate substrates (Promega). Because the transfection efficiency was constant, pRV-βGal was not used systematically in all experiments.

In some experiments, the cells were serum starved 24 h prior to transfection, and mithramycin A was added to a final concentration of 100 nM. Cells were harvested and analysed for luciferase activity 24 h after transfection.

Schneider Line 2 cells (SL-2) were maintained at 25 °C in Schneider medium supplemented with 10% FCS and antibiotics. SL-2 cells were transfected by the calcium phosphate method (Graham & van der Eb 1973). Briefly, cells were seeded in six-well dishes at 300 000 cells per well 24 h before transfection. Each well received 1 µg promoter-reporter gene construct and various amounts of expression plasmids for Sp1 as indicated in Results (pPacSp1; a gift from R Tjian, University of California, Berkeley, CA, USA). Luciferase activity, normalized to the protein concentration of the extract, was measured. Protein concentrations were determined with the BCA protein assay system (Pierce Chemical Co., Montluçon, France).

Electrophoretic mobility shift assay (EMSA)

Nuclear extracts from GT1-7 and HEK293 cells were prepared as previously described (Schreiber *et al.* 1989). Synthetic oligonucleotide probes (Table 1) were prepared by 5'-end labelling of double-stranded

Table 1 Sequences of the double-stranded oligonucleotides used for the EMSA experiments and for site-directed mutagenesis studies (the sense strand only is shown). Mutations sites are in bold

Sp-C/EBP β -MC4-R	5'-gtctcacagactcccattgcattgg-3'
C/EBP β mut	5'-gtctcacaga att acattgcattgg-3'
Consensus C/EBP β	5'-ctaggcgcttgcgcaatctatattcg-3'
Sp1-MC4-R	5'-ggaagcagaggaggagccaccgtg-3'
Mut Sp1-MC4-R	5'-ggaagcagac at agagccaccgtg-3'
Consensus Sp1	5'-attcgatcggggcgggcgagc-3'

oligonucleotides using [γ - 32 P]ATP and T4 polynucleotide kinase (Invitrogen). For EMSA, nuclear extracts (10 μ g) were incubated at 4 °C for 15 min in a binding reaction that contained 10 mM Hepes (pH 8), 50 mM KCl, 0.1 mM EDTA, 2 mM dithiothreitol, 5 mM MgCl₂, 50 mM NaCl, 4 mM spermine, 8% Ficoll and 1 μ g poly dIdC-poly dIdC (Amersham), with or without ZnCl₂ (0.1 mM). For the competition assays, a 200-fold molar excess of unlabelled double-stranded oligonucleotide used as competitor was added to the reaction mixture. The radiolabelled DNA probe (10 000 c.p.m.) was then added and the incubation continued for 15 min at 4 °C. Samples were electrophoresed on a 5% non-denaturing polyacrylamide gel in 0.5 $\times$ Tris-borate-EDTA at 15 °C. The gels were dried under vacuum and analysed by autoradiography.

In some experiments, nuclear extracts were preincubated (overnight at 4 °C) in the presence of polyclonal antibodies against either the human Sp1 (Active Motif Europe, Rixensart, Belgium) or the human Sp3 (Santa Cruz, Tebu, Le Perray-en-Yvelines, France) before adding the labelled probe.

Western blotting

Thirty micrograms of nuclear extracts prepared from HEK293 or GT1-7 cells were separated by SDS-10% polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane. Immunoblotting was performed using anti-Sp1 antibodies (Santa Cruz) and the Envision System Labelled-Polymer-HRP anti-Rabbit (DakoCytomation, Trappes, France). Blots were revealed using the ECL Plus Western Blotting Detection System (Amersham).

Chromatin immunoprecipitation assay (ChIP)

In vivo molecular interaction between Sp1 (or Sp3) and the hMC4-R gene promoter was investigated using the ChIP-IT kit (Active Motif). Briefly, cells were fixed with 1% formaldehyde at room temperature for 10 min. After washing with cold PBS, the cross-linking was stopped by addition of a glycine-containing buffer and incubation at

room temperature for 5 min. Cells were then scraped and lysed in a buffer containing phenylmethylsulphonyl fluoride and a protease inhibitor cocktail (Complete; Roche). The lysate was sonicated 15 $\times$ 20 s to shear the DNA to a length between 200 and 1000 bp. After pre-clearing in the presence of salmon sperm DNA/Protein G agarose beads, the sheared chromatin was incubated with polyclonal antibodies against Sp1 (Active Motif), Sp3 (Santa Cruz) or non-immune IgG, at 4 °C overnight on a rotator. Before incubation an aliquot of DNA (corresponding to the input DNA) was removed. The DNA/Sp/Sp antibody complex was recovered by addition of salmon sperm DNA/Protein G agarose beads and incubation for 1.5 h at 4 °C on a rotator. After extensive washes of the beads, the DNA was eluted from the Protein G. The cross-link was then reversed and RNA removed after incubation in the presence of NaCl and RNase A at 65 °C for at least 4 h (the input DNA was also subjected to this treatment and the following). Proteins were then digested in the presence of EDTA and proteinase K before the DNA purification.

For PCR, 5 μ l of immunoprecipitated DNA or one-to-ten diluted input DNA were used. Four sets of PCR primers were used: a first primer pair flanked and amplified the hMC4-R Sp1-76 site and a second (negative control) primer pair amplified part of the hMC4-R-coding sequence. The third and fourth primer pairs amplified a sequence containing the mMC4-R Sp1-87-binding site and part of the mMC4-R-coding sequence (negative control) respectively.

RT-PCR

Semi-quantitative RT-PCR was used to study the expression of the endogenous MC4-R gene expression in HEK293 and GT1-7 cells. Total RNA was extracted from the cells after a 24 h treatment with or without mithramycin A. To rule out the possibility that PCR products could result from the amplification of genomic DNA contaminating the RNA samples, RNA samples were treated by DNase before the reverse transcription. First-strand cDNA was prepared with 1 μ g RNA using MMLV reverse transcriptase (Invitrogen). PCR reactions were performed in the presence of 2 μ l of reverse transcribed cDNA using EuroblueTaq (Eurobio). The number of cycles used to amplify hMC4-R or mMC4-R cDNA, cyclophilin and GAPDH cDNA (used for normalization in HEK293 and GT1-7 cells respectively) was first determined to ensure that the end of the reaction for the control samples was in the middle of the exponential curve of amplification.

Data analysis

Data are expressed as means $\pm$ S.E.M. of at least three independent experiments performed in triplicate using

two different preparations of vector to rule out plasmid preparation inequalities. Statistical analysis was performed using one-way ANOVA analysis followed by post hoc testing with Fisher's protected least square difference, except for mithramycin A experiments where the Student's *t*-test was used. Differences were considered significant when $P < 0.05$.

Results

Characterization of the hMC4-R cDNA 5'-end and promoter

Following RACE-PCR on mRNA encoding hMC4-R, a unique band of 600 bp was obtained. After subcloning into pCR4-TOPO vector, 53 different clones were purified and sequenced. All cDNA fragments appeared to contain the same 5'-untranslated sequence of 427 bp in size. This indicated the presence of one major transcription start site located 427 bp upstream from the translation start codon ATG. Comparison between the hMC4-R genomic sequence (accession number Genbank NM_005912) and the cDNA fragment indicated that the *hMC4-R* gene did not contain any intron in the sequenced hMC4-R 5'-flanking region. The DNA sequence upstream of the major transcription start site contains a CAAT box (located at -100 bp in antisense orientation) but lacks a typical TATA box (Fig. 1). The sequence also revealed the presence of several putative binding sites for known transcription factors, in particular three Sp1-binding sites located at nucleotides -76 , -179 and -275 bp from the transcription start site (Fig. 1).

Functional analysis of the 5'-flanking region of the hMC4-R

In order to characterize the regions involved in the regulation of the basal expression of the *hMC4-R* gene promoter, a fragment containing the sequence from -1253 to $+26$ was subcloned into the pGL3 basic luciferase reporter vector. A series of deletion constructs was generated and used to transiently transfect GT1-7 cells, expressing endogenous mMC4-R, and HEK293 cells, expressing endogenous hMC4-R mRNA (Dumont *et al.* 2001). The longest construct, p($-1253/+26$)luc, conferred a strong constitutive luciferase reporter gene expression with 100- and 30-fold increases compared with pGL3 basic in HEK293 and GT1-7 cells respectively (Fig. 2A and B). Truncation of the promoter up to the first 279 bp, induced a slight decrease (10%; $P < 0.05$) of the basal expression in HEK293 cells (Fig. 2A) but this effect is more pronounced in GT1-7 cells (Fig. 2B). No significant decrease was observed upon deletion up to -179 bp. However, subsequent deletions up to -98 bp reduced ($P < 0.05$) the luciferase activity in

Figure 1 Nucleotide sequence of the 5'-flanking region of the *hMC4-R* gene. Nucleotide numbering starts with +1 corresponding to the major transcription start site defined by 5'-RACE. Potential binding sites for transcription factors predicted by the software (AliBaba 2.1) inside the sequence -347 to $+1$ are underlined. The CAAT box is in bold. The first nucleotide of each construct is shown (.). The GenBank accession number for the nucleotide sequence is AY268932.

Figure 2 Functional analysis of the *hMC4-R* gene promoter. Analysis of the luciferase activity relative to pGL3 basic was performed after transient transfection of HEK293 (A) and GT1-7 (B) cell lines, using constructs containing deleted fragments of the *hMC4-R* gene promoter. Values represent means $\pm$ S.E.M. of three different experiments performed in triplicate. 'a' indicates values significantly different from those obtained with the promoterless vector pGL3 basic ($P < 0.0001$); 'b' indicates values significantly different from p(-20/+26)luc vector ($P < 0.001$); 'c' indicates values significantly different from p(-98/+26)luc vector ($P < 0.01$); 'd' indicates values significantly different from p(-1253/+26)luc vector ($P < 0.001$).

HEK293 cells by 35%, which indicated that the region containing the sequence between -179 and -98 bp is partly involved in the basal promoter activity in these cells but not in GT1-7 cells. Deletions up to -20 bp induced a very strong decrease (about 90%) of the remaining luciferase activity in both cell types. These results clearly indicated that a second region, between -98 and -20 bp, contains element(s) essential for the expression of *hMC4-R* gene promoter.

Analysis of the region containing the sequence between -179 and -98 bp by mutagenesis and EMSA studies

In this region, a GC-rich sequence extending from -100 to -109 bp is present corresponding to a putative binding site for Sp-like transcription factors but it is also

Figure 3 Analysis of the region located between -179 and -98 bp of the *hMC4-R* gene promoter: (A) Effects of a mutation in the putative Sp-C/EBP β -binding site on the constitutive *hMC4-R* gene promoter activity. The luciferase activity is expressed as the fold increase over the results obtained with pGL3 basic in transiently transfected HEK293 cells. Values represent the means $\pm$ S.E.M. of three different experiments performed in triplicate. 'a' indicates values significantly different from wild-type p(-279/+26)luc vector ($P < 0.05$). (B): EMSA using oligonucleotide containing the Sp-C/EBP β -hMC4-R-binding site as a probe: The Sp-C/EBP β -hMC4-R oligonucleotide was 5'-end labelled and incubated with nuclear extracts obtained from HEK293 cells, either alone or in the presence of a 200-fold molar excess of unlabelled double-stranded oligonucleotides (Sp-C/EBP β -MC4-R and consensus C/EBP β). EMSA experiments were performed with or without Zn²⁺. U=unbound probe.

listed as a putative C/EBP β -binding site in the software AliBaba 2.1 (www.gene-regulation.com). Site-directed mutagenesis (Table 1) was used to alter this site in the context of the p(−279/+26)luc construct. Results of

transient transfections, performed using HEK293 cells, are shown in Fig. 3A. A significant decrease of the luciferase activity was obtained as compared with the wild-type vector (20%), but no significant difference was observed using the mutant construct compared with the p(−98/+26)luc construct (Fig. 3A). Mutation of this site had no effect when transfections were performed in GT1–7 cells (data not shown), as expected, since deletion of the sequence located between −179 and −98 bp did not modify the luciferase activity (Fig. 2B).

When EMSA experiments were performed using an oligonucleotide containing this putative Sp-C/EBP β -binding site in the presence of nuclear extracts prepared from HEK293 cells, a major complex (C1) was present in the absence of Zn²⁺ (Fig. 3B) and formation of this complex was inhibited by the presence of Zn²⁺. This complex was competed by a 200-fold molar excess of unlabeled probe but not by a 200-fold excess of an oligonucleotide containing a C/EBP β consensus sequence. All these results indicate that this element is not a binding site for C/EBP β transcription factor. Moreover, the complex C1 did not contain a zinc-finger domain protein and thus the binding protein did not belong to the Sp transcription factor family.

Analysis of the region containing the sequence −98/−20 bp by mutagenesis and EMSA studies

Sequence analysis revealed the presence of one Sp1-binding motif in this region, located at position −76 bp. Site-directed mutagenesis (Table 1) altering this Sp1-binding site in the context of the p(−279/+26)luc vector resulted in a very strong inhibition of the promoter activity in HEK293 cells (87%) as well as in GT1–7 cells (80%) compared with the wild-type construct (Fig. 4A and B). These results indicated that an intact Sp1-76-binding site is required for the basal promoter activity of the *hMC4-R* gene and confirmed above results showing that the region located between

Figure 4 Analysis of the region located between −98 and −20 bp of the *hMC4-R* gene promoter by mutagenesis. (A and B) Effects of a mutation inside the putative Sp1-binding site on the constitutive *hMC4-R* gene promoter activity. The luciferase activity is expressed as fold increase over the result obtained with pGL3 basic in transiently transfected HEK293 (A) and GT1–7 (B) cells. Values represent means $\pm$ S.E.M. of three different experiments performed in triplicate. 'a' indicates values significantly different from wild-type p(−279/+26)luc vector ($P < 0.05$). (C) Effect of the insertion of three Sp1-binding sites upstream of the p(−20/+26)luc vector. The luciferase activity is expressed as fold increase over the result obtained with pGL3 basic in transiently transfected HEK293 cell lines. Values represent means $\pm$ S.E.M. of three different experiments performed in triplicate. 'a' indicates values significantly different from p(−20/+26)luc vector ($P < 0.001$); 'b' indicates values significantly different from 3Sp1-p(−20/+26)luc vector ($P < 0.01$).

–98 and –20 bp is highly involved in the promoter activity.

To further confirm the involvement of this binding site, a construct containing three Sp1–76 elements inserted upstream of the p(–20/+26)luc vector was used to transfect HEK293 cells. Using this last construct, the basal promoter activity was increased by 10-fold compared with the p(–20/+26)luc vector alone in the same order of magnitude as the induction obtained using the p(–98/+26)luc vector (18-fold) (Fig. 4C).

Using nuclear cell extracts prepared from either HEK293 or GT1–7 cell lines and a fragment of the *hMC4-R* gene promoter containing the Sp1-binding site as probe, EMSA revealed that a major complex (C2) was formed (Fig. 5A). This DNA–protein complex was zinc-dependent (Fig. 5A), suggesting the binding of a zinc-finger protein. As shown in Fig. 5B, the complex formation was inhibited by a 200-fold molar excess of cold native oligonucleotide. In contrast, there was no competition using unlabelled oligonucleotides bearing either an unrelated sequence (CRE-binding site) or a mutated Sp1-binding sequence. A second complex (C3), which migrated more rapidly than the complex C2, appeared to be specific as its formation was inhibited by an excess of unlabelled probe.

To identify the protein(s) bound to both complexes, we used antibodies prepared against either Sp1 or Sp3. A supershifted complex (ss2) as well as a decreased intensity of the complex C2 was observed after preincubation of the nuclear proteins prepared from HEK293 cells in the presence of the Sp1 specific antiserum (Fig. 6A). This complex was not affected by the presence of Sp1 antibodies using GT1–7 cell nuclear extracts (Fig. 6A). In a previous report, Belsham & Mellon (2000) did not detect Sp1 supershift using the Sp1 consensus oligonucleotide as a probe in the presence of GT1–7 nuclear extract. This result could not be explained by an absence of Sp1 expression in these cells as Sp1 was expressed in HEK293 cells but also (at a lower level) in GT1–7 cells, as demonstrated by Western blot analysis (Fig. 6B).

When a preincubation in the presence of Sp3 antibodies was performed using nuclear proteins prepared from either HEK293 or GT1–7 cells, a supershifted complex (ss3) was also observed in parallel with the disappearance of the complex C3 (Fig. 6A). We therefore concluded that the complex C2 contained the Sp1 protein in HEK293 cells and an Sp1-related protein in GT1–7 nuclear extract, but Sp3 was involved in the formation of the complex C3 in both cell types.

To further confirm the binding of Sp1 (HEK293 cells) or an Sp1-related protein (GT1–7 cells) to the *hMC4-R* gene proximal promoter region, we used a 200-fold molar excess of unlabelled Sp1 consensus sequence to compete the Sp1-MC4-R fragment used as labelled

Figure 5 Analysis of the region located between –98 and –20 bp of the *hMC4-R* gene promoter by EMSA. (A). EMSA was performed using nuclear extracts from HEK293 and GT1–7 cells and the end-labelled Sp1-*hMC4-R* oligonucleotide as probe in the presence or not of Zn²⁺. The arrow indicates the position of the Sp1-*hMC4-R* (C2) complex. U=unbound probe. (B). The Sp1-*hMC4-R* oligonucleotide was 5'-end labelled and incubated with nuclear extracts obtained from HEK293 and GT1–7 cells, either alone or in the presence of a 200-fold molar excess of unlabelled double-stranded oligonucleotides (Sp1-MC4-R, mut Sp1-MC4-R, CRE).

probe. This Sp1 consensus oligonucleotide completely abolished the appearance of the specific complex C2 (Fig. 6C). Moreover, when the labelled oligonucleotide

Figure 6 The Sp1 and Sp3 proteins bound the Sp1-76-binding site. (A) The Sp1-hMC4-R fragment was 5'-end labelled and incubated with nuclear extracts obtained from HEK293 and GT1-7 cells, in the presence of antibodies directed against Sp1 (Sp1 Ab) or Sp3 (Sp3 Ab). Autoradiographs were exposed overnight except for the Sp3-Ab(2) band, which was exposed for 3 days. U=unbound probe. (B) Immunodetection of Sp1 in GT1-7 and HEK293 cell nuclear extracts. (C) Oligonucleotides containing either Sp1-hMC4-R (1) or consensus Sp1-binding site (2) were 5'-end labelled and incubated with nuclear extracts obtained from HEK293 and GT1-7 cells, either alone or in the presence of a 200-fold molar excess of unlabelled double-stranded oligonucleotides (Sp1-MC4-R and consensus Sp1).

containing the Sp1 consensus sequence was used as probe, one major complex was formed and migrated at the same position as the complex C2 (Fig. 6C) indicating that this complex contained the Sp1 transcription factor or related factor in HEK293 and GT1-7 cells respectively.

The Sp1 and Sp3 proteins interact with the *hMC4-R* gene promoter *in vivo*

The *in vivo* interaction between the Sp1-76-binding site and the Sp1 and Sp3 proteins was demonstrated by ChIP assay using HEK293 cells (Fig. 7A). PCR products obtained using the Sp1-76 primer pair and the negative control primers were 155 and 157 bp in size respectively. Input genomic DNA and Sp1 and Sp3 antibody ChIP DNA gave a strong PCR amplification using the Sp1-76 primer pair, but there was only a slight amplification using the negative control ChIP DNA (IgG) as a template (Fig. 7A). On the contrary, amplifications were equivalent for both Sp1 or Sp3 antibody ChIP and negative control ChIP (IgG) DNAs when the negative control primers were used. These data indicate that Sp1

and Sp3 bind *in vivo* (as well as *in vitro*) to the Sp1-76 site present on the *hMC4-R* gene promoter.

The major role of the Sp1-binding site inside the *hMC4-R* gene promoter might be extended to the mouse gene as we found the same site in the published mouse promoter sequence at position -87 bp upstream of the transcription start site (Fig. 8), which permitted us to perform ChIP assays using Sp3 antibody and GT1-7 cells. The specific PCR amplified a sequence containing this Sp1-87 site present in the mouse promoter. We demonstrated an *in vivo* interaction between the mMC4-R Sp1-87-binding site and the Sp3 protein in GT1-7 cells (Fig. 7B).

Control of the basal activity of the *hMC4-R* gene promoter by Sp1 transcription factor

The effects of Sp1 on basal transcriptional activity of the *hMC4-R* gene promoter were examined using *Drosophila* SL-2 cells (Fig. 9A). This cell line is devoid of endogenous Sp family members (Courey & Tjian 1988). Cells were cotransfected with p(-279/+26)luc and different amounts of pPacSp1. Overexpression of

Figure 7 ChIP assays with Sp1 and Sp3 antibodies. (A) ChIP of the *hMC4-R* gene promoter. Cross-linked chromatin prepared from HEK293 cells was immunoprecipitated in the presence of a specific Sp1 antibody, Sp3 antibody or non-immune IgG. Input DNA corresponds to the chromatin fragments before immunoprecipitation. PCR (32 cycles) was performed using primers that amplified an Sp1–76-containing fragment (MC4-R) or primers designed to amplify a fragment which did not contain any putative Sp1-binding site (negative control). (B) ChIP of the mouse *MC4-R* gene promoter. Cross-linked chromatin prepared from GT1–7 cells was immunoprecipitated in the presence of either specific Sp3 antibody or non-immune IgG. Input DNA corresponded to the chromatin fragments before immunoprecipitation. PCR (36 cycles) was performed using primers that amplified an Sp1–87-containing fragment (MC4-R) or primers designed to amplify a fragment which did not contain any putative Sp1-binding site (negative control).

Sp1 in the presence of the *hMC4-R* gene construct induced a maximal increase of the luciferase activity by 3.5-fold using 1 µg pPacSp1. No effect was obtained when Sp1 was expressed in the presence of Sp1–76 mutated in the context of p(–279/+26)luc construct.

To further validate the importance of Sp1 binding for the activation of the *hMC4-R* gene, the effect of mithramycin A was investigated in HEK293 cells because this drug inhibits binding of Sp1 to its binding sites (Blume *et al.* 1991). When HEK293 cells were treated for 24 h in the presence of 100 nM mithramycin, the endogenous MC4-R mRNA expression decreased dramatically (Fig. 9B). The pretreatment of HEK293 cells with 100 nM mithramycin A, before transfection

with the p(–279/+26)luc construct, induced a strong decrease (68%) of the luciferase activity (Fig. 9C). In contrast, it had no effect when the construct containing the mutated Sp1 was used. These results confirmed that, in HEK293 cells, Sp1 bound to the –76 binding site of the *hMC4-R* gene promoter to activate the transcription of this gene. No effect of mithramycin A was observed using GT1–7 cells (Fig. 9D), which confirms that, in this cell type, Sp1 did not bind to the Sp1–87-binding site present in the mouse promoter.

Discussion

The objective of this study was to better understand the transcriptional regulation of the *hMC4-R* gene in expressing tissues. The *hMC4-R* gene contained a unique exon as observed in the *mMC4-R* gene (Dumont *et al.* 2001). One major transcriptional start site, at position –427 bp from the ATG codon was identified. This is similar to the site at –426 bp found by Lubrano-Berthelier *et al.* (2003). Dumont *et al.* (2001) previously estimated a major transcription start site at 415 nucleotides upstream of the start of translation in *mMC4-R*. The *hMC4-R* gene promoter did not contain a consensus TATA box, but a CAAT box was observed in an antisense orientation as described for the *mMC4-R* gene promoter (Dumont *et al.* 2001). The MC4-R belongs to a family of five melanocortin receptors. Promoter analyses have been mostly performed on two of the encoding genes, *hMC1-R* (Moro *et al.* 1999), *mMC2-R* and *hMC2-R* (Naville *et al.* 1994, 1997, Cammas *et al.* 1997, Shimizu *et al.* 1997) and these genes lack both TATA and CAAT boxes.

Promoter deletion studies allowed us to define two regions involved in the constitutive promoter activity of the *hMC4-R* gene. In the first region, mutation of the putative Sp-C/EBPβ element only slightly decreased the promoter activity in HEK293 cells, suggesting that this element plays only a moderate role in the regulation of the basal *hMC4-R* gene expression. The protein which binds to this binding site is not related to Sp-like proteins or C/EBPβ and it remains to be identified.

We have focused our study on the second region which clearly drives the promoter activity. This region contained a putative Sp1-binding site, the mutation of which dramatically reduced the basal promoter activity, demonstrating that a functional Sp1-binding site was absolutely required for the activation of the *hMC4-R* gene promoter.

Insertion of three Sp1–76 elements in the *hMC4-R* gene promoter fragment –20/+26, significantly increased the luciferase activity in HEK293 cells, demonstrating the essential role of this binding site in the expression of the *hMC4-R* gene. The fact that the

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agactccccattgcattgggattggtcagaaggaagcagaggaggagccaccgtgaacatggt
      . . . . .
tcagaaccactgaatacggattggtcagaaggaagcagaggaggagccattcagaacacccc

ttttttctctccacacatcataaatcagcagctggcgccctacagcccct +1 human
      . . . . .
ccacccccggccccccctccgtctaaccataagaaatcagcagcccgac -15 mouse

```

Figure 8 Sequence alignment of the *hMC4-R* and *mMC4-R* gene promoter. The Sp1-binding sites are in bold and the C/EBP β -binding site is underlined.

induction obtained with this construct was slightly lower than that obtained using p(-98/+26)luc suggested that sequences present in the vicinity of the Sp1-binding site were also necessary.

In HEK293 cells, the Sp1 transcription factor was able to bind the Sp1-76 site as demonstrated by EMSA using an antibody directed against Sp1 (complex C2). This result was confirmed using ChIP analyses. After cotransfection experiments with expression plasmid for Sp1 into SL-2 cells, which lack endogenous transcription factors of the Sp family and then cannot compensate for the absence of Sp1 by another factor of the same family, the promoter activity was significantly induced by expression of Sp1, which confirms the role of Sp1 in *hMC4-R* gene transcription. The importance of Sp1 binding was also confirmed by using mithramycin A, a drug that specifically blocks Sp1 binding (Blume *et al.* 1991, Huang *et al.* 1991, Ryuto *et al.* 1996, Zhao *et al.* 2001). The physiological relevance of Sp1 binding transcriptional activity of the *MC4-R* gene was further confirmed by the strong decrease of the endogenous *MC4-R* gene expression in HEK293 cells after treatment with mithramycin. In addition to Sp1, the transcription factor Sp3 bound the same Sp1-binding site as demonstrated by EMSA and ChIP analyses. The binding of both Sp1 and Sp3 to the same site has already been reported for other gene promoters, as in the case of the lutropin receptor (Chen *et al.* 2000).

Sp1 and Sp3 were able to bind the *hMC4-R* Sp1-binding site both *in vitro* and *in vivo* in HEK293 cells, although this site did not correspond to the classic Sp1 consensus sequence. Indeed, it is known that Sp1, one member of the Sp/XKLF family of transcription factors, is able to recognize different sequences including GC boxes, GT boxes and CACC motifs (Gloss & Bernard 1990, Li *et al.* 1991, Yu *et al.* 1991). Using GT1-7 cell

nuclear extracts, we showed that the DNA-protein complex C2 did not contain Sp1, because there was neither competition nor supershift by Sp1 antibodies (Fig. 6A). The absence of supershift by Sp1 antibody using GT1-7 nuclear proteins has been reported (Belsham & Mellon 2000) despite the evidence that Sp1 is present in these cells (Fig. 6B). Therefore, in GT1-7 cells, the absence of Sp1 in the complex C2 was not due to the absence of expression of this factor but may be related to the low expression levels of this factor. This could also be due to the absence of one particular co-factor in GT1-7 cells that may prevent a stable binding of Sp1 to the Sp site. Because several factors can compete for the same site, this could favour the binding of another related factor which may require another co-factor. An oligonucleotide containing a consensus Sp1-binding site was able to competitively inhibit the formation of the C2 complex using GT1-7 cells in the present study. Therefore, an Sp1-related protein could replace Sp1 in these cells.

Sp1 is an ubiquitous transcription factor present in all normal mammalian cells and plays an important role in basal expression of numerous genes lacking TATA boxes, such as those encoding structural proteins, metabolic enzymes, cell cycle regulators, transcription factors, growth factors, surface receptors and others (Braun & Suske 1998, Courey & Tjian 1988, Philipson & Suske 1999, Suske 1999). Sp1 is abundantly expressed in most cell types including those of the hypothalamus (Ren *et al.* 1998), and its level of expression changes during development and varies among different cell types (Lania *et al.* 1997, Suske 1999). Expression of Sp1 protein and mRNA appears to be highest in fetal cells (Saffer *et al.* 1991) and Sp1^{-/-} embryos display growth retardation and die early in gestation (Marin

Figure 9 Role of the Sp1 protein in the transcriptional activity of the *hMC4-R* gene promoter. (A) Cotransfection experiments with an expression plasmid for Sp1, in SL-2 cells. The promoter-reporter gene constructs were the p(-279/+26)luc and the promoter mutated at the -76 Sp1-binding site. The amounts of expression plasmid for Sp1 are indicated. Values represent the average of three independent transfections. 'a' indicates values significantly different from p(-279/+26)luc in the absence of Sp1 ($P < 0.05$). (B): Effect of mithramycin A on endogenous *hMC4-R* gene expression in HEK293 cells. RT-PCR on cell samples treated with or without 100 nM mithramycin A for 24 h. Cyclophilin was used as the reference gene. (C) Effect of mithramycin A on *hMC4-R* promoter activity. The promoter-reporter gene constructs used were the p(-279/+26)luc and the promoter mutated at the -76 Sp1-binding site. Cells were preincubated in the presence or absence of mithramycin A 100 nM 24 h before transfection. Results are expressed as the fold induction over the respective pGL3 basic in the absence or presence of mithramycin A. This figure corresponds to a representative experiment and each data point is the mean $\pm$ s.d. 'a' indicates values significantly different from corresponding control without mithramycin A ($P < 0.05$). (D) Effect of mithramycin A on endogenous *mMC4-R* gene expression in GT1-7 cells. RT-PCR on cell samples treated with or without 100 nM mithramycin A for 24 h. GAPDH was used as the reference gene.

et al. 1997). Similarly, the *MC4-R* gene is expressed during early ontogeny in the central and peripheral nervous systems.

In conclusion, we have shown for the first time that Sp1 (or a closely related factor) is absolutely required for the regulation of the activity of the *hMC4-R* gene; Sp1

could act through interplay with other specific factors, such as Sp3.

More study is needed in order to determine whether mutations of the Sp1-binding site in the *hMC4-R* gene promoter might be linked to obesity.

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