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# Functional characterization of the mouse melanocortin 3 receptor gene promoter

Keisuke Okutsu [a], Fumiya Ojima [a], Naoto Shinohara [a], Shusuke Taniuchi [a], Yasusyo Mizote [a], Kenji Aoki [a], Toshiyuki Kudo [b], Maho Ogoshi [a], Sakae Takeuchi [a], Sumio Takahashi [a,*]

[a] Department of Biology, Graduate School of Natural Science and Technology, Okayama University, Tsushima, Kita-ku, Okayama 700-8530, Japan
[b] Department of Biological Pharmacy, School of Pharmacy, Shujitsu University, Kita-ku, Okayama 703-8516, Japan

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ABSTRACT

Melanocortin receptor 3 (MC3R) is expressed in the hypothalamus and pituitary in humans and rodents, and is involved in the control of feeding, energy metabolism, and pituitary function. In the mouse pituitary, MC3R is detected in mammotrophs. This study aimed to clarify the regulatory mechanism for *Mc3r* expression in the mouse pituitary. The promoter activities of reporter constructs for the MC3R gene 5′-flanking region up to −4000 bp (transcription initiation site designated as +1) were analyzed. The promoter activity significantly increased in the −86/+109 construct, but decreased in the −38/+109 construct, indicating that the minimal promoter required for basal expression of *Mc3r* is located in the −86/+109 region. Putative binding sites for transcription factors AP-1 and ATF4 were found in the 5′-flanking region of *Mc3r*. Site-directed mutation or deletion of these sites affected the promoter activities. In gel-shift assays with a nuclear extract of mouse anterior pituitary cells, band-shifts were detected for both sites after the addition of the nuclear extract, and were decreased in the presence of excess unlabeled probe competitors. These results indicated that both sites were involved in the regulation of *Mc3r* expression in anterior pituitary cells. Estradiol-17β treatment increased the *Mc3r* promoter activity, indicating that the gene is regulated by estradiol-17β. In conclusion, we have demonstrated the minimum promoter region required for *Mc3r* expression, and identified two binding sites for AP-1 and ATF4 and in the 5′ upstream-flanking region of *Mc3r* that are essential for *Mc3r* expression.



## 1. Introduction

Adrenocorticotropic hormone (ACTH), and α- β- and γ-melanocyte stimulating hormone (α-, β- and γ-MSH) are derived from the precursor protein proopiomelanocortin (POMC), and are known as melanocortins (Smith and Funder, 1988; Hol et al., 1995). These hormones play various physiological roles in central and peripheral tissues. Melanocortin peptides exert their effects via five distinct melanocortin receptors (MC1R–MC5R), which have been cloned and characterized. All MCRs belong to seven transmembrane domain G-protein-coupled receptor groups and stimulate adenylate cyclase.

In the mouse and rat, MC3R consists of 323 amino acid residues, and the homology is 93% (Roselli-Rehfuss et al., 1993; Desarnaud et al., 1994). MC3R is coupled to cAMP- or inositol phospholipid/$Ca^{2+}$-mediated post-receptor signaling system (Konda et al., 1994). Overall, α-, γ-MSH have the strongest binding affinities for MC3R among the melanocortins, and act as its ligands (Roselli-Rehfuss et al., 1993). In CHO-K1 cells that were transfected with MC3R, α-, β-, γ- MSH and ACTH increased cAMP levels, and γ-MSH was the most potent with respect to cAMP-producing ability, followed by β-MSH, α-MSH, and ACTH (Desarnaud et al., 1994).

MC3R is known to be involved in the regulatory mechanism of various body functions. In the brain, MC3R is expressed in the cerebral cortex, thalamus, hypothalamus, hippocampus, and medulla oblongata (Gantz et al., 1993; Roselli-Rehfuss et al., 1993; Desarnaud et al., 1994; Daniels et al., 2005). In addition, MC3R is also expressed in the anterior pituitary of mice (Morooka et al., 1998) and rats (Roudbaraki et al., 1999). *Mc3r*-deficient mice exhibit obesity with increased fat mass and reduced lean mass, suggesting that MC3R is involved in the regulation of energy homeostasis (Butler et al., 2000; Chen et al., 2000). In addition, MC3R is required for the expression of anticipatory feeding behavior during restricted feeding periods in mice (Sutton et al., 2008), suggesting the possibility that MC3R regulates the tuning

*Abbreviations:* ACTH, adrenocorticotropic hormone; AP-1, activating protein-1; ARE, androgen responsive element; ATF4, activating transcription factor 4; CREB, cAMP-responsive element binding protein; DC-FBS, dextran-coated charcoal-treated fetal bovine serum; DIG, digoxigenin; DMEM, Dulbecco's modified Eagle's medium; E2, estradiol-17β; ER, estrogen receptor; ERE, estrogen responsive element; FBS, fetal bovine serum; LB, Luria-Bertani medium; MC3R, melanocortin receptor 3; MSH, melanocyte stimulating hormone; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; POMC, proopiomelanocortin; PRE, progesterone responsive element; PRL, prolactin; RT, reverse transcription; Sp1, specificity protein 1; 3′-RACE, rapid amplification of cDNA 3′- end; 5′-RACE, rapid amplification of cDNA 5′- end.

* Corresponding author.
*E-mail address:* stakaha@cc.okayama-u.ac.jp (S. Takahashi).

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**Table 1**
Primer list for probes of gel-shift assay.

| | Primer | Sequence | Position |
|---|---|---|---|
| AP-1 probe | AP-1 forward | 5′-ACCCAGTGCAGACCTGACCCATACCAACCCACAAA-3′ | −190/−156 |
| | AP-1 reverse | 5′-TTTGTGGGTTGGTATGGGTCAGGTCTGCACTGGGT-3′ | −190/−156 |
| | AP-1 mut forward | 5′-ACCCAGTGCAGACCTAGTCCATACCAACCCACAAA-3′ | −190/−156 |
| | AP-1 mut reverse | 5′-TTTGTGGGTTGGTATGGACTAGGTCTGCACTGGGT-3′ | −190/−156 |
| ATF4 probe | ATF4 forward | 5′-GTGAGCAGTCCGGACTGCAGGGGGCGCCAATCTGC-3′ | −82/−48 |
| | ATF4 reverse | 5′-GCAGATTGGCGCCCCCTGCAGTCCGGACTGCTCAC-3′ | |
| | ATF4 mut forward | 5′-GTGAGCAGTCCGGACTATCGGGGGCGCCAATCTGC-3′ | −82/−48 |
| | ATF4 mut reverse | 5′-GCAGATTGGCGCCCCCGATAGTCCGGACTGCTCAC-3′ | −82/−48 |
| | ATF4 del forward | 5′-GTGAGCAGTCCGGGGGGCGCCAATCTGC-3′ | −82/−48 |
| | ATF4 del reverse | 5′-GCAGATTGGCGCCCCCCGGACTGCTCAC-3′ | −82/−48 |

of meal periods (Sutton et al., 2008). Moreover, *Mc3r*-deficient mice displayed an exacerbated inflammatory arthritis. The MC3R system may be involved in anti-inflammatory circuits in arthritic joints (Patel et al., 2010). Our previous studies suggest that MC3R takes part in the regulation of anterior pituitary functions. MC3R is expressed in mouse pituitary glands (Morooka et al., 1998; Matsumura et al., 2003). α-MSH directly stimulates prolactin (PRL) release through MC3R expressed on mammotrophs (Matsumura et al., 2003). Interestingly, pituitary MC3R is more highly expressed in adult female mice than in adult male mice (Matsumura et al., 2003).

The regulatory mechanisms for three MCR genes (*Mc1r*, *Mc2r* and *Mc4r*) have been analyzed (Marchal et al., 1998; Naville et al., 1999; Dumont et al., 2001; Rouzaud and Hearing, 2005; Noon et al., 2006; Zwermann et al., 2007; Miccadei et al., 2008; Wankhade and Good, 2011). On the other hand, the regulatory mechanism for *Mc3r* expression remains unclear, although the physiological functions of MC3R have been well studied. Therefore, the present study is aimed to clarify the regulatory mechanism for *Mc3r* expression in the mouse.

Mouse MC3R is expressed in mammotrophs and somatotrophs of the anterior pituitary glands. In mammotrophs, MC3R is associated with the regulation of cell division and PRL release (Matsumura et al., 2003). In the female mouse anterior pituitary *Mc3r* mRNA expression appears from around 20 days of age. The *Mc3r* mRNA levels in female pituitaries are higher than in male pituitaries. In mouse pituitaries, in the female mouse anterior pituitary *Mc3r* mRNA expression is regulated by estrogen (Matsumura et al., 2004). However, it is not clear whether estrogen directly or indirectly regulates *Mc3r* transcription. Therefore, we examined whether estrogen affected the regulation of *Mc3r* expression at the transcription level.

## 2. Materials and methods

### 2.1. Animals

Mice of the ICR strain (CLEA Japan Inc., Osaka, Japan) were used at 2–3 months of age. They were kept in a temperature-controlled animal room (20–22 °C; lights on, 07:00–21:00 h) and given a commercial diet (CE-7; CLEA Japan Inc.) and tap water ad libitum. All animal care and experiments were performed in accordance with the Guidelines of Animal Experimentation, Okayama University, Japan.

### 2.2. Cell line culture

GH3 cells and HEK293 cells were obtained from the Health Science Research Resources Bank (Osaka, Japan) and maintained in Dulbecco's modified Eagle's medium without phenol red (DMEM; Sigma-Aldrich, St. Louis, MO, USA) containing 10% heat-inactivated fetal bovine serum (FBS) at 37 °C in a humidified atmosphere of 5% $CO_2$ and 95% air.

### 2.3. Isolation and culture of anterior pituitary cells

Anterior pituitary glands from male mice were dissociated with 0.5% trypsin (0.5% w/v; Invitrogen, Carlsbad, CA, USA) as previously described (Oomizu et al., 2000). The dissociated pituitary cells were suspended in a 1:1 mixture of phenol red-free DMEM and Ham's F-12 medium (DMEM/F12; Sigma-Aldrich) containing 10% (v/v) dextran-coated charcoal-treated fetal bovine serum (DC-FBS; Sigma-Aldrich), and seeded into 24-well tissue culture plates at a density of $0.5 \times 10^5$ cells/well. The plates were incubated at 37 °C in a humidified atmosphere of 5% $CO_2$ and 95% air.

### 2.4. 5′-RACE and 3′-RACE analyses

To determine the transcription start site of the 5′-end and 3′-end of mouse *Mc3r* mRNA obtained from female mouse anterior pituitaries, 5′-RACE and 3′-RACE analyses were performed using a GeneRacer™ kit (Invitrogen) according to the manufacturer's instructions. First-strand cDNA was synthesized from 3 μg of total RNA using an *Mc3r*-specific primer (5′-TAGCAGATGCAGTAGGGATTGGTGG-3′) for 5′-RACE analysis. The PCR was carried out with a thermal cycler using the gene-specific primer and Gene Racer™ 5′ primer. The resulting PCR products were also amplified using a nested primer (5′-CGCAGCAGAC CCAGATGACCCC-3′) and Gene Racer™ 5′ Nested Primer. For 3′-RACE analysis, first-strand cDNA was synthesized from 2 μg of total RNA using the oligo(dT) adaptor primer provided in the kit (Gene Racer™ 3′ Primer). The PCR was carried out using a primer (5′-CATCACTATCCT GCTGGGTGTT-3′) and Gene Racer™ 3′ primer. Both PCR products were electrophoresed in a 2% agarose gel, and stained with ethidium bromide, and photographed under ultraviolet illumination. The sequence of the PCR products were determined and compared with the mouse genomic sequence.

### 2.5. Preparation of mouse genomic DNA

Livers were collected from 6 week-old mice of the Balb/c strain, rapidly frozen with liquid nitrogen, and completely fractured in a chilled mortar. An aliquot of the cell lysate (50 mg of frozen tissue) was treated with 2 ml of proteinase K solution (4 mg/ml) at 55 °C for 16 h, and then treated with a phenol–chloroform solution to isolate mouse genomic DNA.

### 2.6. Preparation of mouse MC3R gene promoter constructs

Parts of the 5′-flanking region of the mouse *Mc3r* were synthesized by PCR using mouse genomic DNA as a template. The PCR was performed using a specific primer pair consisting of a forward primer with Kpn I site (5′-GGGGTACCGTCAGCAAAGTGCTTATT-3′, −4000/−3979,) and a reverse primer (5′-TTGTGGGACTTGACGCTAGATGTG-3′) designed in +86/+109 region. The PCR was carried out using Pfx polymerase (Invitrogen). The PCR program was as follows: 2 min at 95 °C;