Identification of a novel melanocortin 2 receptor splice variant in murine adipocytes: implications for post-transcriptional control of expression during adipogenesis

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Abstract

The ACTH receptor melanocortin 2 receptor (MC2-R) is a G-protein-coupled receptor principally expressed in the adrenal cortex and the adipocyte, where it stimulates steroidogenesis and lipolysis respectively. The coding region of the murine gene is encoded by a single exon, although three upstream non-coding exons have been documented, one of which is incorporated by alternative splicing in adrenal cells. We have detected a novel transcript in adipocytes, which includes a previously unidentified 86 bp exon upstream of the coding region. This transcript appears with slower kinetics during a time course of differentiation of 3T3-L1 cells and is much more highly expressed in these cells and murine adipose tissues than in the Y1 murine adrenocortical cell line, also it is undetectable in murine foetal testes. Inclusion of this exon extends the 5' UTR to 468 bp and introduces three upstream open reading frames. These are typical features of mRNAs under translational control and imply that the MC2-R gene is regulated both transcriptionally and post-transcriptionally during adipogenesis.

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Introduction

The melanocortin 2 receptor (MC2-R) is a seven transmembrane G-protein-coupled receptor that signals through the cAMP pathway. The MC2-R exclusively binds adrenocorticotrophic hormone (ACTH) and regulates steroid output from the adrenal cortex in the hypothalamo–pituitary–adrenal axis (Simpson & Waterman 1983). MC2-R expression has also been described in a growing number of locations in which the role of the receptor has been less well characterised. In the adult, the main site of MC2-R expression outside the adrenal gland is the murine adipocyte (Cammas et al. 1997) where expression is regulated by peroxisome proliferator-activated receptor γ (PPARγ) and transcription of the MC2-R gene is induced within 24 h treatment of 3T3-L1 cells with adipogenic agents (Noon et al. 2004). It has also been shown that there is detectable MC2-R expression in murine foetal testes and ACTH is able to stimulate testosterone production in this tissue (O’Shaughnessy et al. 2003). Testicular expression declines markedly after birth in the mouse and is barely detectable in the adult. Other reported sites of extra-adrenal expression, include human skin (Slominski et al. 1996), sympathetic ganglia (Nankova et al. 2003), the pituitary (Morris et al. 2003), bone marrow (Gondo et al. 2004), osteoblasts and osteoclasts (Zhong et al. 2005).

The murine MC2-R gene (mMC2-R) is located on chromosome 18 (Cammas et al. 1995, Kubo et al. 1995). The entire coding region of the murine MC2-R was reported to be contained within a single exon with two untranslated exons 5' to it (Cammas et al. 1997). Subsequently, a fourth exon was identified, which was alternately spliced in between the two previously described 5' exons in a minority of adrenal transcripts (Shimizu et al. 1997). These exons are referred to as exons 1–4, with exon 4 containing the coding sequence. Alternative splicing within the 5' untranslated region (5' UTR) has also been reported for the human gene (Kubo et al. 2000).

In this study, we report the expression of novel mMC2-R transcripts containing a previously unidentified exon in the 5' UTR included in transcripts from both the 5' promoter and also the recently identified adipocyte-specific promoter (Kubo et al. 2004). This exon is more highly expressed in the murine adrenal gland and adipose tissue than adrenocortical Y1 cells and murine foetal testes data indicate the complexity of tissue-specific alternative promoter usage and post-transcriptional processing.
Materials and methods

Animals

Normal mice, derived from F1 hybrids of C3H/HeH and 101/H strains, were bred as described previously (O’Shaughnessy et al. 2003). To time foetal development, males were caged with females overnight and the morning was designated as embryonic day (E) 0.5. Tissues were recovered from adult animals and from foetuses at E17.5 and stored in liquid N₂.

Cell culture

3T3-L1 pre-adipocytes (American type culture collection) were maintained in Dulbecco’s modified Eagle medium (DMEM), 10% foetal bovine serum (Life Technologies) at 37°C with 5% CO₂ and differentiated by treating 2 day post-confluent cells (day 0) with media containing 0.5 mM 3-isobutyl-1-methyl-xanthine, 0.25 mM dexamethasone and 1 μg/ml insulin for 2 days. On day 2 media was replaced with insulin only containing media (1 μg/ml). Y1 cells were grown in a 1:1 mixture of DMEM:Ham’s F10 supplemented with 12.5% horse serum and 2.5% foetal bovine serum (all from Life Technologies).

Reverse transcriptase (RT) PCR

Cytoplasmic RNA was harvested from 3T3-L1 cells grown in 6-well plates using the RNeasy miniprep kit (Qiagen) according to the manufacturer’s guidelines. Two microgram RNA was then treated at 37°C for 15 min prior to reverse transcription (RT). The RT reactions were performed at 37°C for 1 h using Moloney murine leukaemia virus-RT and random hexamers (Promega). RNA was extracted from mouse tissues using Trizol (Life Technologies) and reverse transcribed as PCRs were performed using the cDNA equivalent of 50 ng cytoplasmic RNA. The PCR products were then subjected to agarose gel electrophoresis. The following primer sequences (SigmaGenosys) were used (Forward:Reverse); MC2-R (GGATCTGGCTTAGAAGGG), middle (TACCCTCAGCAAGGCAGCAAGGCAG:GAGATCTGGCTTAGAAGGG), upper (CCACTCATGTTGATGG:GGATCTGGCTTAGAAGGG), lipoprotein lipase (CAACATTGGAGAACCCCATCC:CTACAACCTGGCAGAGC), ALBP (GATCATCAGCTAAATTGACC:TTGTGGTGCACATTCCATC:CCATCCC) PPARγ2 (GAGATCTGCTGTGATGCCCAAGCC:AGCTTCAATCCGATGGTTC).

Sequencing

PCR products were excised from agarose gels and the DNA was purified using a Qiagen gel extraction kit. PCR products were then sequenced in both directions using the MC2-R forward and reverse primers with the ABI Prism dye terminator DNA sequencing kit. Sequencing reactions were run on an ABI Prism 377 sequencer. The resulting sequences were analysed using a basic local alignment search tool (BLAST) search of the Genbank database to identify and locate the sequences in the mouse genome.

Results

RT-PCR was performed on RNA harvested from 3T3-L1 cells over a time course of differentiation using a forward primer in exon 1 and an intron skipping reverse primer spanning the exon 3/4 junction. As previously demonstrated (Noon et al. 2004), MC2-R expression was rapidly upregulated following hormonal induction of 3T3-L1 cells (Fig. 1A). However, as well as detecting the two bands observed in the Y1 murine adrenocortical cell positive control, a larger band was observed, which appears later in the differentiation time course. These products were isolated and sequenced and all three were found to contain MC2-R sequences. The two smaller products corresponded to the splice products described previously (Cammas et al. 1997, Shimizu et al. 1997) in Y1 cells. However, the largest product has not been described previously and contains an 86 bp insertion between exons 2 and 3 (Fig. 1B). To confirm that this novel product was upregulated later during differentiation and that its delayed appearance was not a consequence of competitive PCR effects, the individual transcripts were analysed by Q-PCR. Splice variant-specific forward PCR primers were used, together with the same reverse primer as used in Fig. 1A, to amplify the products individually.
over a 3T3-L1 differentiation time course up to 8 days post-induction (Fig. 2). The two shorter splice products were upregulated at approximately the same time during the time course, being detectable by 2 days post-induction, whereas the novel transcript was not detectable until day 3. These results mirror what was seen by conventional RT-PCR (data not shown).

Interestingly, the exon 1-specific transcript is produced transiently, whereas transcripts containing exons 2 and 3 accumulate throughout the time course. The kinetics of production of the splice variants were compared with three markers of adipogenesis, the early gene lipoprotein lipase, the late gene adipose lipid-binding protein, also referred to as adipose P2 and fatty acid binding protein 4 (ALBP, aP2 and FABP4), and the primary transacting factor and determinant of adipogenesis PPARγ2. These data once again confirm that the upregulation of MC2-R mRNA expression, particularly as judged by the expression of exon 1-specific transcripts, is a rapid event following the induction of differentiation.

Searching the Genbank database for the novel 86 bp sequence in the largest PCR product resulted in an exact match on mouse chromosome 18 located within the MC2-R gene between exons 2 and 3. This 86 bp novel exon is flanked on chromosome 18 by dinucleotide AG and GT sequences at its 5’ and 3’ ends respectively (Fig. 3A), which give intron/exon junctions consistent with the conserved AG/GT rule for efficient splicing (Breathnach et al. 1978, Mount 1982). The revised structure of the murine MC2-R gene is shown in Fig. 3B, and the exons have been renamed to include the novel exon. Figure 3A shows that exon 3 contains three ATGs, which could behave as active upstream initiation codons (uAUGs) upstream of the initiation codon in exon 5. The two previously described transcripts also contain uAUGs, and the upstream open reading frames (uORFs) they initiate are shown in Fig. 3C. Inclusion of the novel exon introduces three more uORFs, the longest of which, 159 bp long, starts from the end of exon 3 and terminates in exon 5 before the initiating methionine codon of the MC2-R.

To examine the tissue distribution of the novel exon, RNA was isolated from mouse tissues previously shown to express MC2-R message and RT-PCR was performed using the same primer combinations used in Fig. 1A and the exon 3-specific primers used in Fig. 2. Figure 4 shows the different splice products detected in these tissues. Using primers complementary to sequences in exons 1 and 4/5, MC2-R-specific products were detected as expected in foetal and adult adrenal tissue, foetal testis, white and brown adipose tissue and 3T3-L1 adipocytes. There was no detectable expression in adult testes. All expressing tissues contained the alternatively expressed exon 2, and exon 3 was also detected in adipocyte tissue and 3T3-L1 cells. Using specific primers, exon 3 could clearly be detected in all the expressing tissues apart from very low expression in the testes. Using a forward primer specific for the alternative first exon within intron 1 (Kubo et al. 2004), it can be seen that the alternative exon was also included in transcripts from this late-onset, adipocyte-specific promoter. Sequencing the PCR products revealed that in these transcripts the novel exon was again included when exon 2 is spliced into the message (data not shown).

The data presented in Fig. 1 suggest that exon 3 is not expressed in Y1 cells although it is clearly expressed in murine adrenal tissue (Fig. 4). RT-PCR using a forward primer specific for exon 3 (Fig. 5) shows that this splice form is detectable in Y1 cells, albeit at a much lower level than in differentiated 3T3-L1 cells and adipocytes from brown adipose tissue and three white adipose depots, s.c., omental and epididymal fat.

**Discussion**

The 5’ UTR of the mMC2-R is alternately spliced and two mRNA transcripts have been demonstrated in adrenal cells (Shimizu et al. 1997). In this study, primers

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**Figure 1** A novel MC2-R transcript is observed during adipogenesis. (A) cDNA from a time course of 3T3-L1 differentiation was analysed by PCR using a forward exon designed to detect exon 1 and a reverse primer spanning the exon 3/4 boundary. Y1 cDNA was used as a positive control (+) and H2O as a negative control. The products are compared against a size marker (M, size indicated in bp to the left of the panels) and GAPDH (lower panel) is used as a loading control. (B) The individual MC2-R products from Y1 and day 5 3T3-L1 cDNA PCRs using the primers in A were gel purified and sequenced. The predicted RNA species are shown with the upper product including a novel exon between exons 2 and 3 (*). The translation start site in exon 4 is shown by an arrow.
targeted to the 5' UTR of the mM2C2-R were used to characterise the splicing of this gene in the 3T3-L1 adipocyte. Our results confirm the rapid upregulation of MC2-R mRNA following hormonal induction (Noon et al. 2004) and demonstrate the expression of a novel third mRNA transcript. This transcript contains an additional 86 nucleotides, which are spliced immediately downstream of the alternate exon 2. We have not detected any sequences, which contain exon 3 and not exon 2, however, searching an expressed sequence tag library for mRNA transcripts containing exon 3 yielded an exact match (Accession number AI_153955) from a cDNA library prepared from 4-week-old mouse mammary gland (presumably expressed in the fat depot), which contained both exons 4 and 5 downstream sequences but with exon 1 sequences directly upstream of exon 3. This indicates that further splicing events may exist in vivo.

The delayed upregulation of the novel transcript with respect to the other two species may suggest that its expression is regulated in a different manner to the two previously reported transcripts. This observation is supported by the apparent relative abundance of the novel transcript in both brown and white adipose tissues. It also seems that while the novel transcript is readily detectable in the foetal murine adrenal gland it is barely expressed in the foetal testis, despite the clear presence of both of the previously identified transcripts in these tissues. Indeed, this study is the first to demonstrate the pattern of MC2-R splicing in the foetal testis, which has a shared embryological origin to the cells of the adrenal cortex in the adrenogenital primordium (Hatano et al. 1996). The novel exon may therefore be useful as a marker for distinguishing adrenal progenitors expressing this sequence from those that develop into the foetal testis.

We have demonstrated the relative kinetics of induction of the three splice products and noted that the lower transcript, including exon 1, is rapidly but transiently upregulated. We have shown (Noon et al. 2007) that there is a switch in promoter usage around day 6 from the upstream promoter to a novel CCAAT/enhancer binding protein (C/EBP)-driven downstream promoter, which accounts for both the decline in exon 1-specific mRNA synthesis and the continued MC2-R expression and mRNA synthesis, as observed in the exons 2- and 3-specific Q-PCR figures. The abundant transcription from the novel promoter in adipocytes can be seen in Fig. 4.
The 5' UTR of the majority of mammalian genes is short and free of uORFs (reviewed in Morris & Geballe 2000, Meijer & Thomas 2002). Exon 3 increases the length of the 5' UTR to 468 nt with exon 1 and 500 nt with exon 1* and contains three uAUGs, with the longest uORF potentially encoding a 53 amino acid peptide. All of the uORFs are terminated before the initiating methionine of the MC2-R and therefore do not alter the coding sequence of the receptor. An increasing number of genes is being discovered with uORFs, most often those with important roles in cell growth and differentiation (Morris & Geballe 2000). In most cases studied it appears that these uORFs have a negative control on translatability of mRNA, often by blocking the scanning of ribosomes (Reynolds et al. 1996, Morris & Geballe 2000, Meijer & Thomas 2002), although it has been shown that two uORFs in the retinoic acid receptor β2 isoform (RARβ2) are important for efficient translation of the mRNA (Reynolds et al. 1996). The inclusion of exon 3 could potentially allow the MC2-R to bypass the inhibition of translation observed in terminally differentiated cells (Gerlitz et al. 2002) and the relative abundance of transcripts including exon 3 during adipogenesis may therefore be significant in regulating MC2-R mRNA stability or translatability. A phylogenetic analysis of the mouse, rat and human MC2-R genes indicates the existence of an exon 3 homologue in the rat but not in the human (data not shown). Unlike rodent adipose tissue, human adipose tissue does not express MC2-R, possibly as a consequence of the lack of a region homologous to the alternative promoter and first exon

Figure 3 The novel exon introduces three uORFs when spliced into the 5' UTR of MC2-R. (A) The sequence of the novel exon is shown (upper case). This is flanked by AG and GT dinucleotides (lower case). ATGs are underlined. (B) The genomic organisation of the murine MC2-R is shown (not to scale) incorporating the novel exon (*) now referred to as exon 3. The alternatively spliced exons 2 and 3 are shown as open boxes. The size of the exons is shown in bp and the approximate size of the introns is shown in bp. (C) The open reading frames encoded by each of the transcripts are shown as grey bars and the translation start site of the coding region in exon 5 is shown as an arrow. Two of the uORFs from exon 3 are out of frame with the coding exon and are shown above the others.

Figure 4 Tissue-specific expression of exon 3. To examine the expression of exon 3, cDNAs derived from MC2-R-expressing mouse tissues were amplified by PCR using forward primers specific for exon 1, exon 3 and the alternative exon 1 (exon 1*) (indicated on the right) and the reverse primer used inFig. 1. The structure of the different splice products observed is indicated on the left. GAPDH is used as a loading control (lower panel). BAT, brown adipose tissue; WAT, white adipose tissue (omentum).

Figure 5 Exon 3 is expressed at low levels in Y1 cells. A forward primer specific for exon 3 and the reverse primer used in Figs 1, 2 and 4 was used to amplify cDNA from Y1 cells, day 14 differentiated 3T3-L1 cells and different adipose tissues. GAPDH was used as a loading control.
(Kubo et al. 2004) although MC2-R is expressed during early human adipogenesis (Smith et al. 2003). Perhaps, the absence of an exon 3 homologue in the human gene is a consequence of the lack of expression of MC2-R at later times during adipogenesis in the human.

Exon 3 is expressed at very much lower levels in Y1 cells than in either adrenal tissue or adipocytes and this was confirmed using exon 3-specific primers. Y1 cells are rapidly growing compared to MC2-R-expressing cells within the adrenal cortex, which are more comparable to differentiated adipocytes, having reduced proliferative capacity (Mitani et al. 1999). It is possible that the under-expression of exon 3-containing message in Y1 cells indicates that the inclusion of this exon is under both trophic and tissue-specific control, increasing the likelihood that it plays a functional role in the regulation of MC2-R expression.

In conclusion, we have identified a novel exon in the 5′ UTR of MC2-R transcripts. This exon, which is relatively abundant in adipose tissues and cells, extends the length of the 5′ UTR and introduces three uORFs. This suggests that during adipogenesis the murine MC2-R gene is under translational as well as transcriptional control. Investigation of the roles of splicing and the influence of the 5′ UTRs on translation will be the subject of future investigations.

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