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

Luke A Noon, Artem Bakmanidis, Adrian J L Clark, Peter J O'Shaughnessy¹ and Peter J King

Endocrinology Centre, William Harvey Research Institute, Bart's and The London, Queen Mary, University of London, London EC1A 7BE, UK

¹Institute of Comparative Medicine, University of Glasgow Veterinary School, Glasgow G61 1QH, UK

(Requests for offprints should be addressed to P J King who is now at Molecular Endocrinology Centre, William Harvey Research Institute, John Vane Science Building, Charterhouse Square, London EC1M 6BQ, UK; Email: p.j.king@qmul.ac.uk)

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.

Journal of Molecular Endocrinology (2006) **37**, 415–420

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 (Cammass *et al.* 1997) where expression is regulated by peroxisome proliferator-activated receptor γ (PPAR γ) 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 (Cammass *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 (Cammass *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 µM 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 (CTTGCCGAGAAAGATCCT (exon 1):GGATCTGGCTTAGAAGGG (exon 4/5)), upper (CCACTCATCAGTTGATGG (exon 3)):GGATCTGGCTTAGAAGGG glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (TGCACCACCAACTGCTTAG:GGATGCAGGGATGATGTTTC). All RT-PCR experiments were performed at least with equivalent results. Representative experiments are shown.

Quantitative (Q) RT PCR

Q-PCR was performed using SYBR green (Molecular Probes, Eugene, Oregon, USA) and an MX4000 real-time PCR machine (Stratagene, Amsterdam, Netherlands).

SYBR green fluorescence was quantified using a serial dilution of template-containing plasmid or PCR product of known concentration and relative abundance of transcript was normalised against 18S RNA levels (primers from Eurogentec). The following primer sequences (SigmaGenosys) were used (Forward:Reverse); MC2-R lower (GAGCTGAAGCCAGCAAGC (exon 1/4):GGATCTGGCTTAGAAGGG), middle (TACCCTCAAC-CAGCAAGC (exon 2/4):GGATCTGGCTTAGAAGGG), upper (CCACTCATCAGTTGATGG (exon 3):GGATCTGGCTTAGAAGGG), lipoprotein lipase (CAACATTGGA-GAAGCCATCC:CTACAACCTCAGGCAGAGCCC), ALBP (GATCATCAGCGTAAATGGGG:TTGTGGTTCGACTTTCCATCC) PPAR γ 2 (GAGATTCTCCTGTTGACCC:A GCTTCAATCGGATGGTTC).

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

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.

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 PPAR γ 2. 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

Figure 2 Quantitative RT-PCR analysis of the different MC2-R splice variants during adipogenesis. The cDNAs derived from a time course of 3T3-L1 differentiation were amplified using forward primers specific for the lower band (exon 1/exon 4, upper left panel), the middle band (exon 2/exon 4, middle left panel) and the upper band (exon *, lower left panel) and the reverse primer used in Fig. 1. For comparison, three differentiation-specific genes were also analysed: lipoprotein lipase (upper right panel), ALBP (middle right panel) and PPAR γ 2 (lower right panel). The relative expression of the transcripts was normalised to 18S RNA levels and the error bars show the standard deviation. Each experiment was performed at least.

targeted to the 5' UTR of the mMC2-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.

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.

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 $\beta 2$ isoform (RAR $\beta 2$) 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

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 in Fig. 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 (omental fat).

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 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.

Acknowledgements

This work was funded by the Research Advisory Board of St Bartholomew's and The Royal London Charitable Foundation. The sequence of exon 3 has been entered into the Genbank database (Accession number AF510739). The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

References

- Breathnach R, Benoist C, O'Hare K, Gannon F & Chambon P 1978 Ovalbumin gene: evidence for a leader sequence in mRNA and DNA sequences at the exon-intron boundaries. *PNAS* **75** 4853–4857.
- Cammas FM, Kapas S, Barker S & Clark AJ 1995 Cloning, characterization and expression of a functional mouse ACTH receptor. *Biochemical and Biophysical Research Communications* **212** 912–918.
- Cammas FM, Pullinger GD, Barker S & Clark AJ 1997 The mouse adrenocorticotropin receptor gene: cloning and characterization of its promoter and evidence for a role for the orphan nuclear receptor steroidogenic factor 1. *Molecular Endocrinology* **11** 867–876.
- Gerlitz G, Jagus R & Elroy-Stein O 2002 Phosphorylation of initiation factor-2 alpha is required for activation of internal translation initiation during cell differentiation. *European Journal of Biochemistry* **269** 2810–2819.
- Gondo S, Yanase T, Okabe T, Tanaka T, Morinaga H, Nomura M, Goto K & Nawata H 2004 SF-1/Ad4BP transforms primary long-term cultured bone marrow cells into ACTH-responsive steroidogenic cells. *Genes To Cells* **9** 1239–1247.
- Hatano O, Takakusu A, Nomura M & Morohashi K 1996 Identical origin of adrenal cortex and gonad revealed by expression profiles of Ad4BP/SF-1. *Genes To Cells* **1** 663–671.
- Kubo M, Ishizuka T, Kijima H, Kakinuma M & Koike T 1995 Cloning of a mouse adrenocorticotropin receptor-encoding gene. *Gene* **153** 279–280.
- Kubo M, Shimizu C, Kijima H, Ishizuka T, Takano K, Takano A & Koike T 2000 Alternate exon in the 5'-untranslated region of the human ACTH receptor gene. *Endocrine Journal* **47** 543–548.
- Kubo M, Shimizu C, Kijima H, Nagai S & Koike T 2004 Alternate promoter and 5'-untranslated exon usage of the mouse adrenocorticotropin receptor gene in adipose tissue. *Endocrine Journal* **51** 25–30.
- Meijer HA & Thomas AA 2002 Control of eukaryotic protein synthesis by upstream open reading frames in the 5'-untranslated region of an mRNA. *Biochemical Journal* **367** 1–11.
- Mitani F, Mukai K, Miyamoto H, Suematsu M & Ishimura Y 1999 Development of functional zonation in the rat adrenal cortex. *Endocrinology* **140** 3342–3353.
- Morris DR & Geballe AP 2000 Upstream open reading frames as regulators of mRNA translation. *Molecular and Cellular Biology* **20** 8635–8642.
- Morris DG, Kola B, Borboli N, Kaltsas GA, Gueorguiev M, McNicol AM, Ferrier R, Jones TH, Baldeweg S, Powell M *et al.* 2003 Identification of adrenocorticotropin receptor messenger ribonucleic acid in the human pituitary and its loss of expression in pituitary adenomas. *Journal of Clinical Endocrinology and Metabolism* **88** 6080–6087.
- Mount SM 1982 A catalogue of splice junction sequences. *Nucleic Acids Research* **10** 459–472.
- Nankova BB, Kvetnansky R & Sabban EL 2003 Adrenocorticotrophic hormone (MC-2) receptor mRNA is expressed in rat sympathetic ganglia and up-regulated by stress. *Neuroscience Letters* **344** 149–152.
- Noon LA, Clark AJ & King PJ 2004 A peroxisome proliferator-response element in the murine *mc2-r* promoter regulates its transcriptional activation during differentiation of 3T3-L1 adipocytes. *Journal of Biological Chemistry* **279** 22803–22808.
- Noon LA, Clark AJL, O'Shaughnessy PJ & King PJ 2007 A C/EBP site at 87 is required for the activation of a novel murine melanocortin 2 receptor (MC2-R) promoter at late stages during adipogenesis. *Endocrinology* (in press).
- O'Shaughnessy PJ, Fleming LM, Jackson G, Hochgeschwender U, Reed P & Baker PJ 2003 Adrenocorticotrophic hormone directly stimulates testosterone production by the fetal and neonatal mouse testis. *Endocrinology* **144** 3279–3284.
- Reynolds K, Zimmer AM & Zimmer A 1996 Regulation of RAR beta 2 mRNA expression: evidence for an inhibitory peptide encoded in the 5'-untranslated region. *Journal of Cell Biology* **134** 827–835.
- Shimizu C, Kubo M, Saeki T, Matsumura T, Ishizuka T, Kijima H, Kakinuma M & Koike T 1997 Genomic organization of the mouse adrenocorticotropin receptor. *Gene* **188** 17–21.
- Simpson ER & Waterman MR 1983 Regulation by ACTH of steroid hormone biosynthesis in the adrenal cortex. *Canadian Journal of Biochemistry and Cell Biology* **61** 692–707.
- Slominski A, Ermak G & Mihm M 1996 ACTH receptor, *CYP11A1*, *CYP17* and *CYP21A2* genes are expressed in skin. *Journal of Clinical Endocrinology and Metabolism* **81** 2746–2749.
- Smith SR, Gawronska-Kozak B, Janderova L, Nguyen T, Murrell A, Stephens JM & Mynatt RL 2003 Agouti expression in human adipose tissue: functional consequences and increased expression in type 2 diabetes. *Diabetes* **52** 2914–2922.
- Zhong Q, Sridhar S, Ruan L, Ding KH, Xie D, Insogna K, Kang B, Xu J, Bollag RJ & Isales CM 2005 Multiple melanocortin receptors are expressed in bone cells. *Bone* **36** 820–831.

Received in final form 3 July 2006

Accepted 3 July 2006

Made available online as an Accepted Preprint on 15 August 2006