Title:
A Peroxisome Proliferator-Response Element In the Murine MC2-R Promoter
Regulates its Transcriptional Activation During Differentiation of 3T3-L1 Adipocytes

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Running Title:
A PPRE regulates MC2-R transcription in the murine adipocyte
Summary

Adrenocorticotropic hormone (ACTH) can stimulate lipolysis and suppress leptin expression in murine adipocytes. These effects are mediated via the melanocortin 2 Receptor (MC2-R), which is expressed when 3T3-L1 cells are induced to undergo adipogenesis. In this study we have characterised the MC2-R promoter in the murine adipocyte, one of the few extra-adrenal sites of expression and a cell type that lacks steroidogenic factor 1 (SF-1), a transcription factor that is required for MC2-R expression in adrenal cells.

Transcriptional regulation of the MC2-R in the absence of SF-1 was investigated by 5' deletion analysis of the murine MC2-R promoter in both undifferentiated and differentiated 3T3-L1 cells. The results revealed the presence of a 59 base pair regulatory region within the promoter, containing an adipocyte specific enhancer. The ability of this region to confer enhanced activity in the adipocyte was mapped to a peroxisome proliferator response element (PPRE)-like sequence which bound to PPARγ and its heterodimeric partner RXRα in adipocyte nuclear extracts. Co-transfection of PPARγ2/RXRα with the pMC2-R[-112/+105]GL3 reporter resulted in transcriptional activation in preadipocytes and this response required an intact PPRE. Mutation of the PPRE so as to prevent PPARγ/RXRα binding resulted in a complete abrogation of the pMC2-R[-112/+105]GL3 reporter activity in day 3 differentiated 3T3-L1 cells, demonstrating key role played by this site in regulating MC2-R expression in the murine adipocyte. These data highlight a novel mechanism for MC2-R transcription, which may have significance in both adrenal and extra-adrenal sites of expression.
Introduction

The melanocortin 2 receptor (MC2-R) is a seven transmembrane G-protein coupled receptor best known for its role in the adrenal cortex, where it couples the actions of adrenocorticotropic hormone (ACTH) to steroidogenesis and increased glucocorticoid output (1). Although the key role of MC2-R in the hypothalamo-pituitary-adrenal (HPA) axis has been the focus of the majority of publications to date, it is also expressed in a number of extra-adrenal sites including the murine adipocyte, fetal testis, human skin and sympathetic ganglia (2-5).

Early studies using the murine 3T3-L1 cell line, a widely used model of adipogenesis, demonstrated the appearance of high affinity ACTH binding sites following treatment of growth-arrested cells with a mixture of adipogenic agents including insulin, dexamethasone and 3-isobutyl-1-methyl-xanthine (IBMX) (6). Subsequent characterisation of the melanocortin receptors expressed by 3T3-L1 adipocytes revealed the presence of both the MC2 and MC5-R. However a pharmacological analysis showed that the actions of ACTH were mediated solely through the MC2-R in this cell line (2). ACTH exerts a potent lipolytic effect on the murine adipocyte via the cAMP pathway (7;8) and has more recently been shown to suppress the expression and secretion of the appetite regulating hormone leptin in differentiated 3T3-L1 cells (9). MC2-R expression may therefore be important in providing feedback between the HPA axis and peripheral leptin production by adipose tissue.

Studies to date on the transcriptional regulation of the murine and human MC2-R genes have focused solely on expression in adrenal-derived cell lines such as murine Y1 and human H295R cells (10-13). Such studies have demonstrated a role for the
transcription factor steroidogenic factor-1 (SF-1), an orphan nuclear hormone receptor that is widely expressed by steroidogenic tissues and which is important for the expression of key regulators of steroidogenesis in both the adrenal and gonadal systems (14). However, while heterologous expression of SF-1 can induce MC2-R promoter activity in non-expressing, non-adrenal cell lines such as JEG3 and L cells (15;10), MC2-R is not expressed by all SF-1 positive tissues and therefore SF-1 is not sufficient for MC2-R gene expression. In contrast, 3T3-L1 adipocytes have been shown not to express SF-1 (16). In the absence of SF-1, MC2-R gene expression must be regulated via an alternative transcriptional mechanism in this cell line and the elucidation of such a mechanism would greatly improve our understanding of the tissue specific expression of this receptor.

The following study investigates the transcriptional activation of the MC2-R gene during 3T3-L1 differentiation. We show that murine MC2-R promoter constructs are activated in adipocytes and that the region of activation maps to a peroxisome proliferator response element (PPRE)-like sequence in the proximal promoter. This is a binding site for the nuclear hormone receptor peroxisome proliferator-activated receptor γ (PPARγ), a key regulator of adipogenesis that has been shown to directly regulate the expression of genes such as adipocyte P2 (aP2) (17) and lipoprotein lipase (18). We show that deletion or mutation of this sequence renders MC2-R promoter constructs uninducible during differentiation into adipocytes or by co-transfection with expression vectors for PPARγ2 and its heterodimeric partner retinoid X receptor α (RXRα).
Experimental Procedures

Plasmids and Constructs

5’ deletions were created by linearising pMC2-R[-1805/+105]GL3, which contains mouse MC2-R sequences from -1805 to +105 cloned into the Sma I site of the promoterless luciferase vector pGL3 (Promega), with Mlu I and treating with exonuclease III. Deleted products were blunt-ended, ligated to Mlu I linkers and ligated back into pGL3 as MluI/BglII fragments to create a 5’ deletion series with the structure pMC2-R[-x/+105]pGL3. Expression vectors for RXRα (pCMX-hRXRα) (19) and PPARγ2 (pCMX-mPPARγ2) (20) were the kind gifts of Prof. R. Evans (Salk Institute, San Diego, USA) and Prof. M. Lazar (University of Pennsylvania, Philadelphia, USA), respectively. Site directed mutagenesis was performed using the QuickChange protocol (Stratagene).

Cell Culture

3T3-L1 preadipocytes (American Type Culture Collection) were maintained in DMEM, 10% FCS (Sigma) at 37°C with 5% CO2 and differentiated by treating 2-day post-confluent cells (day 0) with media containing 0.5mM IBMX, 0.25μM dexamethasone and 1μg/ml insulin for two days. On day 2 media was replaced with insulin only containing media (1μg/ml) for a further 48hrs before returning the cells to normal cell culture conditions (day 4).

Transfections

Cell lines stably harbouring the pMC2-R[-1805/+105]GL3 reporter or the empty vector pGL3 were created by calcium phosphate precipitation transfection. Ten micrograms of each plasmid were co-transfected with 0.5μg of pcDNA3.1
(Invitrogen), which confers resistance to G418S (Geneticin, Invitrogen). Cells were selected and maintained in media containing 500µg/ml G418S 24hrs after transfection and the resulting colonies were pooled to produce polyclonal cell cultures. Transient transfections were performed by lipofection using Fugene 6 (Roche) according to the manufacturer’s instructions. Briefly, cationic lipid was combined in a 2:1 ratio with 2µg of plasmid DNA and then incubated with the cells under serum free conditions for 1hr. An equal volume of DMEM/20% FCS was then added to the cells, which were subsequently harvested for luciferase assay after 24hrs. When differentiating cells were transfected, insulin was included in the media for the 24hrs after transfection. All transient transfections for luciferase reporter assays included 200ng of the pRL-CMV renilla control vector (Promega). For transfections with expression vectors for PPARγ and RXRα the ratio of reporter: expression vector was kept constant by adding the pCMX empty vector.

**Reporter Assays**

Luciferase was measured using the Dual Luciferase reporter assay (Promega). Briefly, cell lysates were prepared according to the manufacturer’s instructions and luciferase activity was measured using a BioOrbit 1253 luminometer (LabTech International, Sussex, UK). Reporter activity for transient transfections was calculated by normalising the reporter luciferase value with that of the renilla control vector. The activity of the pMC2-R[-1805/+105]GL3 reporter in stable cell lines was calculated by normalising the luciferase values to those of the corresponding pGL3-containing cells.

**cAMP assay**
Cells grown in triplicate wells of a 6-well plate were washed twice with DMEM and then stimulated for 30 minutes in the presence of 1mM IBMX with or without ACTH (10^{-8}M). The cells were then scraped on ice before being boiled for 5 minutes. After centrifugation the supernatant was assayed for cAMP using a competitive binding assay (21). The response of differentiating 3T3-L1 cells to ACTH was calculated for each time point by dividing stimulated by unstimulated cAMP values.

**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. 2µg of RNA was then DNase treated at 37°C for 15 minutes prior to RT. RT was performed at 37°C for 1hr using MMLV-RT and random hexamers (Promega). PCRs were then performed using the cDNA equivalent of 50ng cytoplasmic RNA. The following primer sequences (SigmaGenosys) were used (Forward: Reverse); MC2-R (GAGCTGAAGCCAGCAAGC: GGATCTGGCTTAGAAGGG), SF-1 (ATGGAAATGCATCGAATCC: AATGCTTGTTGTTCTGGAC), SCD1 (ACATGCTCCAAGAGATCTC: GAGCCTTGTAAGTTCTGTG), GAPDH (TGCACCACCAACTGCTTAG: GGATGCAGGGATGATGTTC), PPARγ2 (GAGATTCTCCTGGTTGACC: AGCTTCAATCGATGTTTC).

**Electrophoretic Mobility Shift Assay (EMSA)**

Probes were created by filling in the 5’ overhangs of the annealed oligonucleotides with Klenow DNA polymerase using a mixture of dATP, dGTP, dTTP and α^{32}P dCTP. Nuclear extracts were prepared by NP40-mediated cytoplasmic lysis (22) and EMSA was performed using 10µg extract per reaction as previously described (23)
using 1µg poly(dI.dC).poly(dI.dC) as competitor (Pharmacia). If antibody was included in the reaction, the initial incubation was extended to 1 hour on ice in the presence of 2µl of either rabbit pre-immune serum, PPARγ antibody (SantaCruz) or RXRα antibody (Kind gift of Prof. R. Evans, Salk Institute, San Diego, USA). Complexes were electrophoresed on a 5% native acrylamide gel, dried and visualised by autoradiography. Oligonucleotide sequences (SigmaGenosys) used were (overhang in lower case): PPRE wt upper strand, gatcTCCCCTTTGGCCTCTCT. PPRE mut upper strand, gatcTCCCGATAGGCCTCTCT

**Statistical Analysis**

ANOVA was performed where appropriate, followed by Student’s *t*-test. *P*-values <0.05 were considered significant.
Results

Transcriptional activation of the MC2-R in the absence of SF-1

Prior to any detailed transcriptional analysis of the MC2-R promoter, it was first necessary to document the timing of MC2-R up-regulation in differentiating 3T3-L1 cells. This was achieved by measuring three relevant aspects of MC2-R expression: receptor activity, promoter activity and mRNA expression (Figure 1).

Due to the absence of a suitable MC2-R antibody it was not possible to measure protein expression directly. Whilst MC5-R has also been shown to be expressed during 3T3-L1 cell adipogenesis ACTH has been shown to act solely through the MC2-R in this cell line (2) and therefore the cAMP response to ACTH was measured as a surrogate for MC2-R protein expression. Figure 1A shows a 4.8 fold increase in cAMP production following ACTH treatment on day 2. This response peaked on day 4 and was completely absent in cells on day 5 that had not received the adipogenic treatment (5u). These data are therefore consistent with previous studies, which have shown the ACTH response to be differentiation dependent (7; 8).

The second aspect of MC2-R expression to be measured was promoter activity. 3T3-L1 preadipocytes were stably transfected with the pMC2-R[-1805/+105]GL3 reporter. These cells were differentiated and the luciferase activity at each time point was compared with that of cells expressing the empty vector pGL3. Promoter activity was inferred from these two measurements and the results show a 2-fold rise in promoter activity in the immediate 24 hours following hormonal induction (Figure 1B). The MC2-R promoter was activated maximally on day 3 and beyond this time point the activity was found to diminish. A 24-hour time lag was observed between changes in
the MC2-R promoter and detectable changes in the response to ACTH stimulation. For example, the onset of a functional response to ACTH on day 2 was preceded by promoter activation on day 1 and the peak response to ACTH on day 4 was preceded by peak promoter activity on day 3. In subsequent experiments in which reporter activity was used as a measure of promoter activity in adipocytes the day 3 time point was chosen as the optimum time to harvest cell lysates following transient transfection.

The transcriptional activation of the MC2-R gene was also inferred by RT-PCR analysis of RNA harvested from differentiating 3T3-L1 cells using intron-spanning primers (Figure 1C). Consistent with the observed increase in promoter activation, MC2-R message was detectable 24hrs after hormonal induction and this appearance preceded the up-regulation of the adipocyte marker stearoyl-CoA desaturase-1 (SCD-1) (24) by 72 hours. MC2-R transcriptional activation therefore precedes the attainment of terminal characteristics of differentiation and the accumulation of lipid, which was not apparent until day 3 post induction, as measured by Oil-Red O staining. RT-PCR was also used to measure SF-1 expression in 3T3-L1 cells. The results confirmed the absence of mRNA for this factor throughout the time course of differentiation and this result prompted a search for an alternative positive acting factor(s) driving the basal activity of the promoter in adipocytes.

A 59bp region of the proximal promoter enhances MC2-R transcription in the adipocyte

In an attempt to characterise regions of the MC2-R promoter that confer enhanced activity in the adipocyte a 5’ deletion series was transiently transfected into both
undifferentiated and differentiated cells and the activities of the constructs was compared (Figure 2A). Consistent with the results obtained using stable cells, the activity of the full length pMC2-R[-1805/+105]GL3 reporter was significantly higher in day 3 adipocytes compared to undifferentiated cells and similar significantly enhanced levels of luciferase activity were observed for all of the deletion constructs between –1805 and –112. This suggested that each of these constructs contained within their sequence an adipocyte-specific enhancer. Further deletion of the promoter between –112 and –53 effectively abolished this enhanced activity indicating that the enhancer sequence(s) resided between these two markers. When this 59bp region of sequence was analysed using MatInspector (25) a putative PPRE was identified between –95 and –83 relative to the transcription start site (Figure 2B). The 13bp sequence shared a 77% similarity with a consensus Direct Repeat-1 (DR1) site to which PPARγ is known to bind as a heterodimer with RXRα (26). PPARγ2 is the adipocyte-specific PPARγ isoform and it is up-regulated during 3T3-L1 differentiation (27). RT-PCR was used to compare the mRNA profile of PPARγ2 with that of the MC2-R in a time course of differentiation of 3T3-L1 cells. Both mRNAs were up-regulated within the first 24 hours following hormonal induction and therefore an increase in PPARγ2 levels might explain the activation of the MC2-R gene (Figure 2C). This coincidence of PPARγ2/MC2-R expression together with the presence of a PPRE in the regulatory region of the MC2-R promoter highlighted PPARγ2 as a potential candidate regulator of MC2-R expression.

The -95 PPRE binds to PPARγ/RXRα in adipocyte nuclear extracts

A series of experiments were carried out in order to test the ability of the –95/-83 sequence to bind to endogenous PPARγ/RXRα in 3T3-L1 adipocytes. A radiolabelled
double stranded oligonucleotide probe corresponding to the −95/-83 sequence was prepared and used in EMSA with both preadipocyte and day 3 adipocyte nuclear extracts (Figure 3). No factors binding to this sequence were observed in preadipocyte nuclear extracts but a DNA-protein complex was seen when adipocyte nuclear extracts were used (lanes 1 and 2). This result was consistent with the hypothesis that the transcription factor regulating the expression of the MC2-R is expressed as the cells begin to differentiate. The binding observed with adipocyte nuclear extract could be abolished by mutating 3 of the 4 core bp (28) within the DR1 sequence and this inactivation of the site formed the basis of later mutational analysis (lanes 3 and 4). When in vitro translated RXRα and PPARγ2 were combined with the −95/-83 probe, three bands were observed, the largest of which had the same mobility as that formed with day 3 adipocyte nuclear extracts (lane 8). Antibodies to both RXRα and PPARγ were able to supershift the complex formed with adipocyte nuclear extracts (lanes 6 and 7) as well as the upper band formed with in vitro translated protein (lanes 9 and 10) confirming the presence of these two proteins in the gel shift complexes. The lower complex is retarded with the RXR antibody and represents RXR homodimers which are known to bind to a DR1 site (lane 9) (29;30).

The mMC2-R responds to RXRα/PPARγ2 in pre-adipocytes and this response maps to the −95 PPRE

Having determined that endogenous RXRα and PPARγ in adipocyte nuclear extracts could bind to the −95/-83 sequence the ability of these factors to transactivate the MC2-R promoter was tested. 3T3-L1 preadipocytes were transfected with pMC2-R[−112/+105]GL3 and expression constructs for PPARγ2 and RXRα. Consistent with previous studies of PPRE-containing promoters (27;31), transfection of either
PPARγ2 or RXRα expression vectors alone had no effect on the activity of the pMC2-R[-112/+105]GL3 reporter in 3T3-L1 preadipocytes. However, when both PPARγ2 and RXRα expression vectors were transfected together with the pMC2-R[-112/+105]GL3 reporter, a 9-fold induction of luciferase activity was observed (Figure 4A). This response to PPARγ2/RXRα was mapped by co-expressing the two factors with a series of fine deletion constructs prepared between –112 and –53, the two markers that had previously been shown to contain the adipocyte specific enhancer (Figure 3B). This analysis showed that deletion between –104 and –86, which effectively removes the putative PPRE, resulted in a dramatic reduction in the promoter’s ability to respond to PPARγ2/RXRα. Mutating the –95/-83 sequence in the context of the pMC2-R[-112/+105]GL3 reporter by introducing, by site directed mutagenesis, the 3bp mutation that had previously been shown to abolish PPARγ2/RXRα binding in day 3 differentiated cell extracts (Figure 3 lane 4) reduced the PPARγ2/RXRα response in preadipocytes to 20% that of the wild type reporter. These results indicated the requirement of the –95 PPRE in mediating the effects of heterologously expressed PPARγ2/RXRα in undifferentiated 3T3-L1 cells. However, in order to assess whether or not endogenous PPARγ2/RXRα contribute to the basal activity of the mMC2-R promoter during adipogenesis, the wild-type and –95 PPRE mutated pMC2-R[-112/+105]GL3 reporter constructs were transfected into differentiating 3T3-L1 cells and their activity was compared on day 3 post induction (Figure 4D). These data demonstrate the complete abrogation of reporter activity in adipocytes following mutagenesis of the –95 PPRE in the context of the pMC2-R[-112/+105]GL3 construct.
Discussion

ACTH exerts its effects upon the murine adipocyte via the MC2-R, which is expressed in 3T3-L1 cells when they are induced to differentiate into adipocytes. In this study we have used a range of techniques to map precisely the dynamics of receptor expression during adipogenesis, and the mechanism of transcriptional activation has been elucidated through mutational analysis of the murine promoter. MC2-R expression is limited to a small number of tissues and previous work has implicated SF-1, a key regulator of steroidogenic genes, in the basal expression of the MC2-R in adrenal cells. One possible mechanism for MC2-R expression in the SF-1 non-expressing adipocyte was by SF-1 homologues acting through the same sites in the promoter as those thought to regulate expression in adrenal cells. However a minimal promoter construct in which both SF-1 sites, at –31 and +31, were mutated (32) was not impaired during adipocyte differentiation². It was therefore hypothesised that a novel SF-1-independent mechanism controls the expression of the MC2-R gene in the adipocyte. This study set out to explore MC2-R expression in the 3T3-L1 cell line and the results highlight a key role played by a previously uncharacterised PPRE located between positions −95/-83 upstream of the transcription start site.

Whilst the up-regulation of MC2-R in murine adipocytes has been documented (33), the precise timing of this event was unknown. We have shown that MC2-R gene transcription is activated within the first 24hrs following hormonal induction, several days prior to the expression of mature adipocyte markers such as SCD-1 (24) and the morphological changes associated with lipid accumulation. The early nature of MC2-R expression raises the possibility that this gene plays a role in the process of differentiation itself, and the effect of ACTH treatment during adipogenesis is
currently being investigated. Also, in addition to its early expression, the transcriptional activation of the MC2-R promoter appears to be transient, with peak activity on day 3 and a peak ACTH response on day 4. PPAR\(\gamma\) levels remain elevated throughout the time course of differentiation and it is therefore possible that the MC2-R promoter is subject to transcriptional repression after day 3. The mechanism of this down-regulation of promoter activity is the focus of ongoing studies.

Systematic analysis of the MC2-R promoter revealed the presence of an adipocyte-specific enhancer element(s) within a 59bp region of the proximal promoter. In addition to the putative PPRE at -95/-83, a potential signal transducer and activator of transcription (STAT) site was also identified within this region between -68 and -60. STATs 1, 3 and 5 have been shown to be induced during adipogenesis (34;35) and a dominant negative STAT5 construct can down-regulate a number of genes activated during adipogenesis (35). EMSA experiments using a double stranded oligonucleotide probe corresponding to the -68/-60 sequence failed to show binding to either preadipocyte or adipocyte nuclear extracts and this sequence was therefore unlikely to be important for MC2-R expression in these cells. In contrast, the -95/-83 PPRE binds PPAR\(\gamma\)/RXR\(\alpha\) heterodimers in adipocyte nuclear extracts and mediates PPAR\(\gamma\)/RXR\(\alpha\)-induced transcriptional activation. Furthermore, mutation of this sequence renders the promoter inactive in the adipocyte in the presence of endogenous PPAR\(\gamma\)/RXR\(\alpha\) and thereby demonstrates the important role played by the -95 PPRE in regulating the expression of the MC2-R gene. These data explain the ability of the MC2-R to be expressed in the adipocyte in the absence of SF-1.
The question as to whether or not human adipocytes express the MC2-R has not been satisfactorily resolved. Early studies looked for a lipolytic response to ACTH equivalent to that of the murine adipocyte in both human and primate tissues (36;37) and concluded that such a response was absent. Such studies, together with a number of attempts to document the tissue distribution of melanocortin receptor mRNAs (36;38) have concluded that the MC2-R is not present in human adipocytes, and this lack of expression might be explained by the absence of an equivalent PPRE-like sequence in the proximal promoter of the human MC2-R gene. However, analysis of the distal human promoter sequence reveals at least three putative PPRE-like elements and recent work using both human subcutaneous adipose tissue and a human embryonic stem cell line capable of undergoing adipogenesis, demonstrated the expression of MC2-R mRNA in the human adipocyte (39). Interestingly, the cell line used in this study is analogous to the 3T3-L1 cell line, being of embryonic mesenchymal origin. The expression of the MC2-R in human adipocytes may therefore be developmentally regulated in adipose tissue, as it is in the testes, where expression has been shown to be restricted to foetal Leydig cells and down-regulated in the adult (3).

It is interesting to speculate as to the role of PPARγ in the regulation of MC2-R in tissues other than the adipocyte. For example, PPARγ has been shown to be abundantly expressed in the adrenal gland (40), and this may explain the ability of MC2-R expression to be maintained in SF-1 haploinsufficient mice in which adrenal MC2-R expression is higher in mutant mice than in their wild type siblings (41;42) suggesting the existence of compensatory mechanisms. PPARγ expression has also been demonstrated in the human pituitary, where thiazolidinedione (TZD) PPARγ
agonists are a novel therapeutic target for ACTH-secreting adenomas (43;44). The MC2-R is also expressed in the pituitary and has been proposed to function in maintaining a negative feedback loop controlling ACTH secretion (45;46). Our results suggest a possible mechanism by which TZDs might exert their anti-tumorigenic effects upon pituitary adenomas, through activation of MC2-R expression. Increased MC2-R expression would serve to enhance the proposed negative feedback of ACTH on cell growth that has been previously demonstrated in adrenocortical tumour cells (47;48).

In conclusion, these data elucidate a novel mechanism of transcriptional regulation for the murine MC2-R and suggest potentially exciting areas of further study in the adrenal and extra-adrenal sites of expression such as the pituitary.
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Footnotes

This work was funded by the Joint Research Board of St Bartholomew’s Hospital, London.

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1The abbreviations used are: MC2-R, melanocortin 2 receptor; ACTH, adrenocorticotropic hormone; HPA, hypothalamo-pituitary-adrenal; IBMX, 3-isobutyl-1-methyl-xanthine; MC5-R, melanocortin 5 receptor; SF-1, steroidogenic factor-1; PPRE, peroxisome proliferator response element; (m)PPARγ2, (murine) peroxisome proliferator-activated receptor γ2; aP2, adipocyte P2; (h)RXRα, (human) retinoid X receptor α; EMSA, electrophoretic mobility shift assay; DMEM, Dulbecco’s modified Eagle’s medium; FCS, foetal calf serum; ANOVA, analysis of variance assay; RT-PCR, reverse transcriptase-polymerase chain reaction; SCD-1, stearoyl-CoA desaturase-1; bp, basepair; DR1, direct repeat-1; STAT, signal transducer and activator of transcription; TZD, thiazolidinedione; SEM, standard error of the mean; n/s, not significant.

Figure Legends

Figure 1. Time course of MC2-R expression during 3T3-L1 adipogenesis. A, 3T3-L1 cells were differentiated and the fold induction of cAMP in response to 10^8M ACTH was measured at daily intervals. B, 3T3-L1 cells stably expressing the pMC2-R[-1805/+105]GL3 reporter were differentiated and promoter activity was calculated by normalising reporter luciferase to that of cells stably expressing empty vector pGL3 on each day. Data are presented as average ± standard error of the mean (SEM). C, Panel of gene expression as determined by RT-PCR. Cytoplasmic RNA was harvested from differentiating 3T3-L1 cells at daily intervals and RT-PCR was performed as outlined in experimental procedures. The PCR product in each panel is indicated on the left. Positive control cDNAs (+) are from adrenocortical Y1 cells (MC2-R and SF-1) or day 14 adipocytes (SCD-1).

Figure 2. Identification of PPARγ as a candidate regulator of the MC2-R gene in the murine adipocyte. A, A 5’ deletion series created from pMC2-R[-1805/+105]GL3 was transiently transfected into preadipocytes (open bars) and adipocytes (closed bars) and the activity of the resulting deletion series was compared. *P<0.05, **P<0.01, n/s = not significant. B, Location of the PPRE-like sequence identified between positions −95 and −83 relative to the transcription start site is shown and the sequence is aligned with a consensus PPRE. C, RT-PCR for MC2-R and PPARγ2 mRNA in a time course of adipocyte differentiation compared to GAPDH.

Figure 3. The −95 PPRE binds to PPARγ/RXRα in adipocyte nuclear extracts. The ability of the −95 PPRE to bind to protein factors in both preadipocyte (lanes 1
and 3) and adipocyte (lanes 2 and 5-7) nuclear extracts was compared with in vitro translated mPPARγ2/hRXRα protein (lanes 8-10) using EMSA. Antibodies to both RXRα (lanes 6 and 9) and PPARγ (lanes 7 and 10) were included to supershift the DNA/protein complexes formed. The bands corresponding to PPARγ/RXRα heterodimers and RXRα homodimers are highlighted. The mutated -95 PPRE was used as a probe in combination with preadipocyte (lane 3) and adipocyte (lane 4) nuclear extracts respectively.

**Figure 4. The –95 PPRE mediates the MC2-R response to PPARγ2/RXRα co-transfection and is essential for the maintenance of basal activity in the adipocyte.** A, 3T3-L1 preadipocytes were transiently transfected with the pMC2-R[-112/+105]GL3 reporter and the ability of co-transfected expression vectors for PPARγ/RXRα to induce reporter activity was assessed after 24hrs. B, A series of deletion constructs spanning the region of the MC2-R promoter between –112 and –53 was transiently transfected into 3T3-L1 preadipocytes and the effect of PPARγ/RXRα co-transfection was assayed after 24hrs. C, The –95 PPRE was mutated in the context of the pMC2-R[-112/+105]GL3 reporter and the effect of PPARγ/RXRα co-transfection was compared between the wild type and mutant constructs. D, The wild type and mutant pMC2-R[-112/+105]GL3 constructs were transiently transfected into day 2 adipocytes and basal activity was compared 24hrs later (day 3). All data are presented as average ± SEM.
Fig 1

A

Fold of cAMP response to 10^(-8) M ACTH (arbitrary units)

B

pMC2-R [1805/+105]GL3 luciferase (RLU)

C

Time (days post induction)

MC2-R 125bp
SCD-1 368bp
SF-1 370bp
GAPDH 180bp
Fig 2

A

Mean Corrected Luciferase (RLU)

-1805
-1061
-208
-112
-53
n/s

Adipocyte
Preadipocyte

B

-112
-95
-83
-53

PPRE

-95 PPRE:
TCCCTTTGgCCT
AGGGGAAACCGGA

PPRE Consensus:
TCCCTTTGGCCT
AGGGGAAACCGGA

C

Time (days post induction)

MC2-R

PPARγ2

GAPDH

125bp
496bp
180bp
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**Fig 3**

![Probe](http://www.jbc.org/)

**Probes:**
- PPARγ /RXRα
- RXRα / RXRα

**Probes:**
-95
-95mut
-95
Fig 4
A peroxisome proliferator-response element in the murine MC2-R promoter regulates its transcriptional activation during differentiation of 3T3-L1 adipocytes
Luke A. Noon, Adrian J. L. Clark and Peter J. King

J. Biol. Chem. published online March 17, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M401861200

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