

# A Peroxisome Proliferator-response Element in the Murine *mc2-r* Promoter Regulates Its Transcriptional Activation during Differentiation of 3T3-L1 Adipocytes\*

Received for publication, February 20, 2004

Published, JBC Papers in Press, March 17, 2004, DOI 10.1074/jbc.M401861200

Luke A. Noon, Adrian J. L. Clark, and Peter J. King‡

From the Molecular Endocrinology Center, William Harvey Research Institute, Bart's and the London, Queen Mary University of London, London, EC1A 7BE, United Kingdom

**Adrenocorticotrophic hormone 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 characterized 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 that bound to peroxisome proliferator-activated receptor  $\gamma$  (PPAR $\gamma$ ) and its heterodimeric partner retinoid X receptor  $\alpha$  (RXR $\alpha$ ) in adipocyte nuclear extracts. Co-transfection of PPAR $\gamma$ /RXR $\alpha$  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 to prevent PPAR $\gamma$ /RXR $\alpha$  binding resulted in a complete abrogation of the pMC2-R(-112/+105)GL3 reporter activity in day 3 differentiated 3T3-L1 cells, demonstrating a 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.**

The melanocortin 2 receptor (MC2-R)<sup>1,2</sup> is a seven-transmembrane G-protein coupled receptor best known for its role in the adrenal cortex where it couples the actions of adrenocorti-

cotropic hormone (ACTH) to steroidogenesis and increased glucocorticoid output (1). Although the key role of MC2-R in the hypothalamo-pituitary-adrenal 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 (6). Subsequent characterization 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 hypothalamo-pituitary-adrenal axis and peripheral leptin production by adipose tissue.

Studies to date on the transcriptional regulation of the murine and human *mc2-r* 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 that is important for the expression of key regulators of steroidogenesis in both the adrenal and gonadal systems (14). However, although heterologous expression of SF-1 can induce *mc2-r* promoter activity in non-expressing, non-adrenal cell lines such as JEG3 and L cells (10, 15), MC2-R is not expressed by all of the 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* 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  $\gamma$  (PPAR $\gamma$ ), a key regulator of adipogenesis that has been shown to directly reg-

\* This work was funded by the Joint Research Board of St. Bartholomew's Hospital (London, United Kingdom). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed. Tel.: 44-20-7601-7444; Fax: 44-20-7601-8468; E-mail: p.j.king@qmul.ac.uk.

<sup>1</sup> The abbreviations used are: MC2-R, melanocortin 2 receptor; ACTH, adrenocorticotrophic hormone; MC5-R, melanocortin 5 receptor; SF-1, steroidogenic factor-1; PPRE, peroxisome proliferator-response element; m, murine; h, human; RXR, retinoid X receptor; EMSA, electrophoretic mobility shift assay; ANOVA, analysis of variance; RT, reverse transcriptase; SCD-1, stearoyl-CoA desaturase-1; STAT, signal transducer and activator of transcription.

<sup>2</sup> L. A. Noon, A. J. L. Clark, and P. J. King, unpublished results.

ulate the expression of genes such as adipocyte P2 (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 PPAR $\gamma$ 2 and its heterodimeric partner retinoid X receptor  $\alpha$  (RXR $\alpha$ ).

#### EXPERIMENTAL PROCEDURES

**Plasmids and Constructs**—5' deletions were created by linearizing pMC2-R(-1805/+105)GL3, which contains mouse MC2-R sequences from -1805 to +105 cloned into the SmaI site of the promoterless luciferase vector pGL3 (Promega) with MluI and treated with exonuclease III. Deleted products were blunt-ended, ligated to MluI 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 RXR $\alpha$  (pCMX-hRXR $\alpha$ ) (19) and PPAR $\gamma$ 2 (pCMX-mPPAR $\gamma$ 2) (20) were the kind gifts of Prof. R. Evans (Salk Institute, San Diego, CA) and Prof. M. Lazar (University of Pennsylvania, Philadelphia, PA), respectively. Site-directed mutagenesis was performed using the QuikChange protocol (Stratagene).

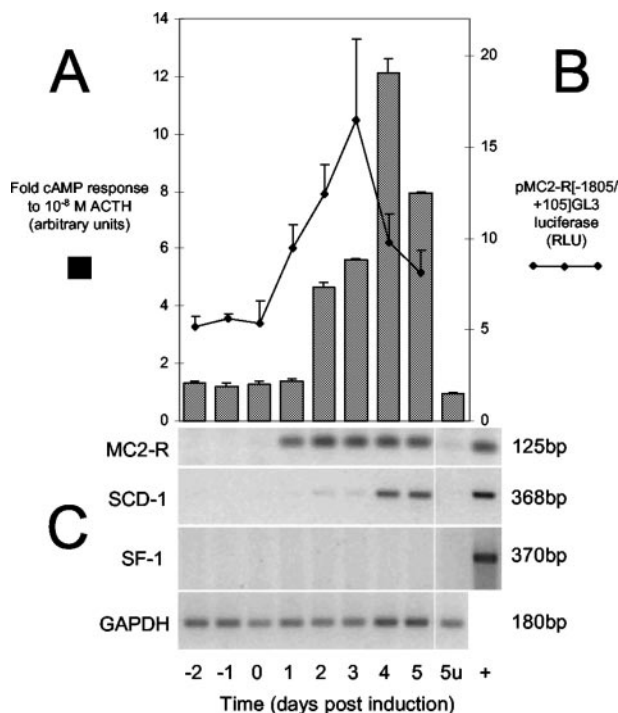
**Cell Culture**—3T3-L1 preadipocytes (American Type Culture Collection) were maintained in Dulbecco's modified Eagle's medium, 10% fetal calf serum (Sigma) at 37 °C with 5% CO<sub>2</sub> and differentiated by treating 2-day post-confluent cells (day 0) with medium containing 0.5 mM 3-isobutyl-1-methyl-xanthine, 0.25  $\mu$ M dexamethasone, and 1  $\mu$ g/ml insulin for 2 days. On day 2, medium was replaced with insulin only containing medium (1  $\mu$ g/ml) for a further 48 h before returning the cells to normal cell culture conditions (day 4).

**Transfections**—Cell lines stably harboring 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 0.5  $\mu$ g of pcDNA3.1 (Invitrogen), which confers resistance to G418S (Geneticin, Invitrogen). Cells were selected and maintained in medium containing 500  $\mu$ g/ml G418S 24 h after transfection, and the resulting colonies were pooled to produce polyclonal cell cultures. Transient transfections were performed by lipofection using FuGENE 6 (Roche Applied Science) according to the manufacturer's instructions. Cationic lipid was combined in a 2:1 ratio with 2  $\mu$ g of plasmid DNA and then incubated with the cells under serum-free conditions for 1 h. An equal volume of Dulbecco's modified Eagle's medium, 20% fetal calf serum was then added to the cells, which were subsequently harvested for luciferase assay after 24 h. When differentiating cells were transfected, insulin was included in the media for the 24 h after transfection. All of the transient transfections for luciferase reporter assays included 200 ng of the pRL-CMV *Renilla* control vector (Promega). For transfections with expression vectors for PPAR $\gamma$  and RXR $\alpha$ , 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). Cell lysates were prepared according to the manufacturer's instructions, and luciferase activity was measured using a BioOrbit 1253 luminometer (LabTech International, Sussex, United Kingdom). Reporter activity for transient transfections was calculated by normalizing 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 normalizing 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 Dulbecco's modified Eagle's medium and then stimulated for 30 min in the presence of 1 mM 3-isobutyl-1-methyl-xanthine with or without ACTH (10<sup>-8</sup> M). The cells were then scraped on ice before being boiled for 5 min. 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 cAMP values by unstimulated 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. 2  $\mu$ g of RNA was then DNase-treated at 37 °C for 15 min prior to RT. RT was performed at 37 °C for 1 h using Moloney murine leukemia virus-RT and random hexamers (Promega). PCRs were then performed using the cDNA equivalent of 50 ng of cytoplasmic RNA. The following primer sequences (Sigma) were used: MC2-R (forward, 5'-GAGCTGAAGCCAGCAAGC-3', and reverse, 5'-GGATCTGGCTTAGAAGGG-3'); SF-1, (forward, 5'-ATGGAATGCATCGAATCC-3', and reverse, 5'-AATGCTTGTGTTCTGGAC-3'); stearoyl-CoA desaturase-1 (SCD-1) (forward, 5'-ACATGCTCCAAGAGATCTC-3', and reverse, 5'-GAGCCTTGTAAGTTCTGTG-



**FIG. 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<sup>-8</sup> M 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 normalizing reporter luciferase to that of cells stably expressing empty vector pGL3 on each day. RLU, relative light units. Data are presented as mean  $\pm$  S.E. 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 under "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).

3'); glyceraldehyde-3-phosphate dehydrogenase (forward, 5'-TGCACC-ACCAACTGCTTAG-3', and reverse, 5'-GGATGCAGGGATGATGTTTC-3'); and PPAR $\gamma$ 2 (forward, 5'-GAGATTCTCCTGTTGACCC-3', and reverse, 5'-AGCTTCAATCGGATGGTTC-3').

**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 [ $\alpha$ -<sup>32</sup>P]dCTP. Nuclear extracts were prepared by Nonidet P-40-mediated cytoplasmic lysis (22), and EMSA was performed using 10  $\mu$ g of extract per reaction as described previously (23) using 1  $\mu$ g of poly(dI-dC):poly(dI-dC) as competitor (Amersham Biosciences). If antibody was included in the reaction, the initial incubation was extended to 1 h on ice in the presence of 2  $\mu$ l of rabbit pre-immune serum, PPAR $\gamma$  antibody (Santa Cruz Biotechnology), or RXR $\alpha$  antibody (a kind gift of Prof. R. Evans, Salk Institute, San Diego, CA). Complexes were electrophoresed on a 5% native acrylamide gel, dried, and visualized by autoradiography. Oligonucleotide sequences (Sigma) used were (overhang in lowercase): PPRE wild type upper strand, 5'-gatcTCCCCTTTGGCCTCTCT-3', and PPRE mutant upper strand, 5'-gatcTCCCGATAGGCCTCTCT-3'.

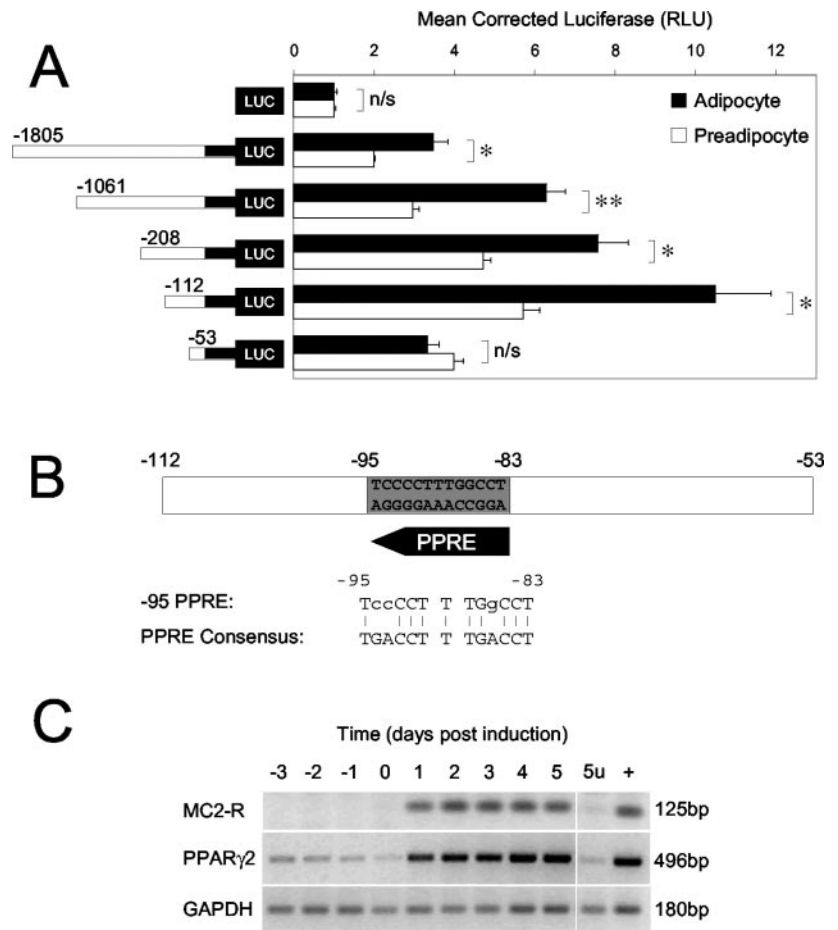
**Statistical Analysis**—ANOVA was performed where appropriate followed by Student's *t* test. *p* values <0.05 were considered significant.

#### 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 (Fig. 1).

Because of the absence of a suitable MC2-R antibody, it was not possible to measure protein expression directly. Although

**FIG. 2. Identification of PPAR $\gamma$  as a candidate regulator of the *mc2-r* 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. *RLU*, relative light units. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; *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 PPAR $\gamma$ 2 mRNA in a time course of adipocyte differentiation compared with glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*).



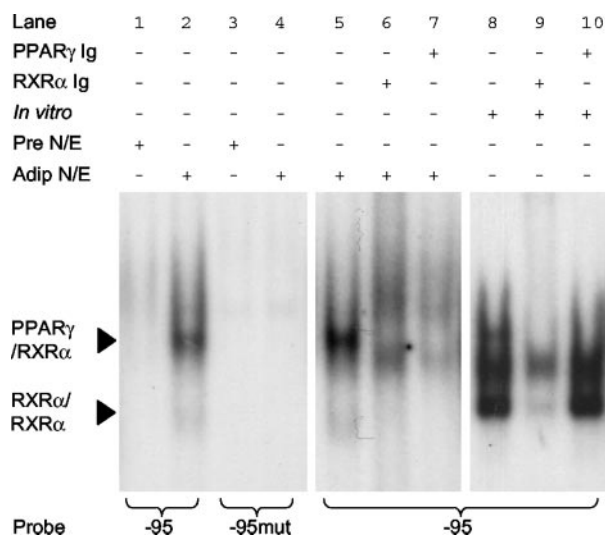
MC5-R has also been shown to be expressed during 3T3-L1 cell adipogenesis, ACTH has been shown to act solely through the MC2-R in this cell line (2) and, therefore, the cAMP response to ACTH was measured as a surrogate for MC2-R protein expression. Fig. 1A shows a 4.8-fold increase in cAMP production following ACTH treatment on day 2. This response peaked on day 4 and was completely absent in untreated cells on day 5 (5u). Therefore, these data are 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 h following hormonal induction (Fig. 1B). The *mc2-r* promoter was activated maximally on day 3, and beyond this time point, the activity was found to diminish. A 24-h 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* was also inferred by RT-PCR analysis of RNA harvested from differentiating

3T3-L1 cells using intron-spanning primers (Fig. 1C). Consistent with the observed increase in promoter activation, MC2-R message was detectable 24 h after hormonal induction and this appearance preceded the up-regulation of the adipocyte marker SCD-1 (24) by 72 h. 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.<sup>2</sup> 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 59-bp Region of the Proximal Promoter Enhances *mc2-r* Transcription in the Adipocyte**—In an attempt to characterize 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 were compared (Fig. 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 with 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 59-bp region of sequence was analyzed using MatInspector (25), a putative PPRE was identified between -95 and -83 relative to the transcription start site (Fig. 2B).



**FIG. 3. The -95 PPRE binds to PPAR $\gamma$ /RXR $\alpha$  in adipocyte nuclear extracts.** The ability of the -95 PPRE to bind to protein factors in both preadipocyte (*Pre*) (lanes 1 and 3) and adipocyte (*Adip*) (lanes 2 and 5-7) nuclear extracts was compared with *in vitro* translated mPPAR $\gamma$ 2/hRXR $\alpha$  protein (lanes 8-10) using EMSA. Antibodies to both RXR $\alpha$  (lanes 6 and 9) and PPAR $\gamma$  (lanes 7 and 10) were included to supershift the DNA/protein complexes formed. The bands corresponding to PPAR $\gamma$ /RXR $\alpha$  heterodimers and RXR $\alpha$  homodimers are *high-lighted*. The mutated -95 (-95mut) PPRE was used as a probe in combination with preadipocyte (lane 3) and adipocyte (lane 4) nuclear extracts (N/E), respectively.

The 13-bp sequence shared a 77% similarity with a consensus direct repeat-1 site to which PPAR $\gamma$  is known to bind as a heterodimer with RXR $\alpha$  (26). PPAR $\gamma$ 2 is the adipocyte-specific PPAR $\gamma$  isoform, and it is up-regulated during 3T3-L1 differentiation (27). RT-PCR was used to compare the mRNA profile of PPAR $\gamma$ 2 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 h following hormonal induction, and therefore, an increase in PPAR $\gamma$ 2 levels might explain the activation of the *mc2-r* (Fig. 2C). This coincidence of PPAR $\gamma$ 2/MC2-R expression together with the presence of a PPRE in the regulatory region of the *mc2-r* promoter highlighted PPAR $\gamma$ 2 as a potential candidate regulator of MC2-R expression.

**The -95 PPRE Binds to PPAR $\gamma$ /RXR $\alpha$  in Adipocyte Nuclear Extracts**—A series of experiments were carried out to test the ability of the -95/-83 sequence to bind to endogenous PPAR $\gamma$ /RXR $\alpha$  in 3T3-L1 adipocytes. A radiolabeled 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 (Fig. 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 direct repeat-1 sequence, and this inactivation of the site formed the basis of later mutational analysis (lanes 3 and 4). When *in vitro* translated RXR $\alpha$  and PPAR $\gamma$ 2 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 RXR $\alpha$  and PPAR $\gamma$  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 anti-

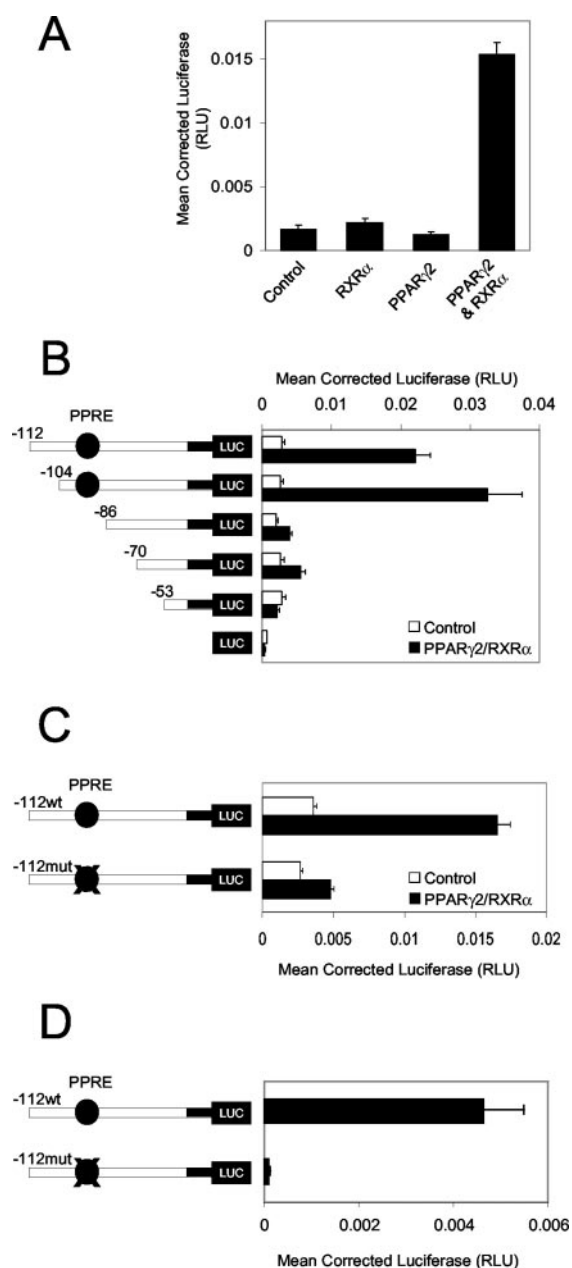
body and represents RXR homodimers that are known to bind to a direct repeat-1 site (lane 9) (29, 30).

**The *mmc2-r* Promoter Responds to RXR $\alpha$ /PPAR $\gamma$ 2 in Pre-adipocytes and This Response Maps to the -95 PPRE**—Having determined that endogenous RXR $\alpha$  and PPAR $\gamma$  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 PPAR $\gamma$ 2 and RXR $\alpha$ . Consistent with previous studies of PPRE-containing promoters (27, 31), transfection of either PPAR $\gamma$ 2 or RXR $\alpha$  expression vectors alone had no effect on the activity of the pMC2-R(-112/+105)GL3 reporter in 3T3-L1 preadipocytes. However, when both PPAR $\gamma$ 2 and RXR $\alpha$  expression vectors were transfected together with the pMC2-R(-112/+105)GL3 reporter, a 9-fold induction of luciferase activity was observed (Fig. 4A). This response to PPAR $\gamma$ 2/RXR $\alpha$  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 (Fig. 3B). This analysis showed that deletion between -104 and -86, which effectively removes the putative PPRE, resulted in a dramatic reduction in the ability of the promoter to respond to PPAR $\gamma$ 2/RXR $\alpha$ . Mutating the -95/-83 sequence in the context of the pMC2-R(-112/+105)GL3 reporter by introducing, by site-directed mutagenesis, the 3-bp mutation that had previously been shown to abolish PPAR $\gamma$ 2/RXR $\alpha$  binding in day 3 differentiated cell extracts (Fig. 3, lane 4) reduced the PPAR $\gamma$ 2/RXR $\alpha$  response in preadipocytes to 20% of that of the wild type reporter. These results indicated the requirement of the -95 PPRE in mediating the effects of heterologously expressed PPAR $\gamma$ 2/RXR $\alpha$  in undifferentiated 3T3-L1 cells. However, to assess whether or not endogenous PPAR $\gamma$ 2/RXR $\alpha$  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 (Fig. 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 differentiation.<sup>2</sup> Therefore, it was hypothesized that a novel SF-1-independent mechanism controls the expression of the *mc2-r* 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 uncharacterized PPRE located between positions -95 and -83 upstream of the transcription start site.

Although the up-regulation of MC2-R in murine adipocytes has been documented (33), the precise timing of this event was



**FIG. 4. The  $-95$  PPRE mediates the MC2-R response to PPAR $\gamma$ 2/RXR $\alpha$  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 PPAR $\gamma$ /RXR $\alpha$  to induce reporter activity was assessed after 24 h. *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 PPAR $\gamma$ /RXR $\alpha$  co-transfection was assayed after 24 h. *C*, the  $-95$  PPRE was mutated in the context of the pMC2-R(-112/+105)GL3 reporter and the effect of PPAR $\gamma$ /RXR $\alpha$  co-transfection was compared between the wild type and mutant constructs. *D*, the wild type and mutant pMC2-R(-112/+105)GL3 ( $-112wt$  and  $-112mut$ , respectively) constructs were transiently transfected into day 2 adipocytes, and basal activity was compared 24 h later (day 3). All of the data are presented as average  $\pm$  S.E. LUC, luciferase. RLU, relative light units.

unknown. We have shown that *mc2-r* transcription is activated within the first 24 h 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 receptor 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; therefore it is 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 59-bp 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.<sup>2</sup> In contrast, the  $-95/-83$  PPRE binds PPAR $\gamma$ 2/RXR $\alpha$  heterodimers in adipocyte nuclear extracts and mediates PPAR $\gamma$ 2/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*. However, an analysis of the distal human promoter sequence revealed the presence of at least three putative PPRE-like elements,<sup>2</sup> and recent work using both human subcutaneous adipose tissue and 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 fetal Leydig cells and down-regulated in the adult (3).

It is interesting to speculate as to the role of PPAR $\gamma$  in the regulation of *mc2-r* in tissues other than the adipocyte. For example, PPAR $\gamma$  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. PPAR $\gamma$  expression has also been demonstrated in the human pituitary gland where thiazolidinedione PPAR $\gamma$  agonists are a novel therapeutic target for ACTH-secreting adenomas (43, 44). The MC2-R is also expressed in the pituitary gland 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 thiazolidinediones 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 tumor 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 gland.

## REFERENCES

- Simpson, E. R., and Waterman, M. R. (1983) *Can. J. Biochem. Cell Biol.* **61**, 692–707
- Boston, B. A., and Cone, R. D. (1996) *Endocrinology* **137**, 2043–2050
- O'Shaughnessy, P. J., Fleming, L. M., Jackson, G., Hochgeschwender, U., Reed, P., and Baker, P. J. (2003) *Endocrinology* **144**, 3279–3284
- Slominski, A., Ermak, G., and Mihm, M. (1996) *J. Clin. Endocrinol. Metab.* **81**, 2746–2749
- Nankova, B. B., Kvetnansky, R., and Sabban, E. L. (2003) *Neurosci. Lett.* **344**, 149–152
- Grunfeld, C., Hagman, J., Sabin, E. A., Buckley, D. I., Jones, D. S., and Ramachandran, J. (1985) *Endocrinology* **116**, 113–117
- White, J., and Engel, F. (1958) *J. Clin. Investig.* **37**, 1556–1563
- Ramachandran, J., and Lee, V. (1976) *Biochim. Biophys. Acta* **428**, 339–346
- Norman, D., Isidori, A. M., Frajese, V., Caprio, M., Chew, S. L., Grossman, A. B., Clark, A. J., Michael, B. G., and Fabbri, A. (2003) *Mol. Cell. Endocrinol.* **200**, 99–109
- Cammas, F. M., Pullinger, G. D., Barker, S., and Clark, A. J. (1997) *Mol. Endocrinol.* **11**, 867–876
- Naville, D., Jaillard, C., Barjhoux, L., Durand, P., and Begeot, M. (1997) *Biochem. Biophys. Res. Commun.* **230**, 7–12
- Marchal, R., Naville, D., Durand, P., Begeot, M., and Penhoat, A. (1998) *Biochem. Biophys. Res. Commun.* **247**, 28–32
- Naville, D., Penhoat, A., Durand, P., and Begeot, M. (1999) *Biochem. Biophys. Res. Commun.* **255**, 28–33
- Parker, K. L., and Schimmer, B. P. (1997) *Endocr. Rev.* **18**, 361–377
- Sarkar, D., Kambe, F., Hayashi, Y., Ohmori, S., Funahashi, H., and Seo, H. (2000) *Endocr. J.* **47**, 63–75
- Clyne, C. D., Speed, C. J., Zhou, J., and Simpson, E. R. (2002) *J. Biol. Chem.* **277**, 20591–20597
- Tontonoz, P., Hu, E., and Spiegelman, B. M. (1994) *Cell* **79**, 1147–1156
- Schoonjans, K., Peinado-Onsurbe, J., Lefebvre, A. M., Heyman, R. A., Briggs, M., Deeb, S., Staels, B., and Auwerx, J. (1996) *EMBO J.* **15**, 5336–5348
- Mangelsdorf, D. J., Ong, E. S., Dyck, J. A., and Evans, R. M. (1990) *Nature* **345**, 224–229
- Adams, M., Reginato, M. J., Shao, D., Lazar, M. A., and Chatterjee, V. K. (1997) *J. Biol. Chem.* **272**, 5128–5132
- Brown, B. L., Albano, J. D., Ekins, R. P., and Sgherzi, A. M. (1971) *Biochem. J.* **121**, 561–562
- Whiteside, S. T., Visvanathan, K. V., and Goodbourn, S. (1992) *Nucleic Acids Res.* **20**, 1531–1538
- Fowkes, R. C., Sidhu, K. K., Sosabowski, J. K., King, P., and Burrin, J. M. (2003) *J. Mol. Endocrinol.* **31**, 263–278
- Kim, Y. C., and Ntambi, J. M. (1999) *Biochem. Biophys. Res. Commun.* **266**, 1–4
- Quandt, K., Frech, K., Karas, H., Wingender, E., and Werner, T. (1995) *Nucleic Acids Res.* **23**, 4878–4884
- Kliwer, S. A., Umesono, K., Noonan, D. J., Heyman, R. A., and Evans, R. M. (1992) *Nature* **358**, 771–774
- Tontonoz, P., Hu, E., Graves, R. A., Budavari, A. I., and Spiegelman, B. M. (1994) *Genes Dev.* **8**, 1224–1234
- Frohnert, B. I., Hui, T. Y., and Bernlohr, D. A. (1999) *J. Biol. Chem.* **274**, 3970–3977
- Mangelsdorf, D. J., Umesono, K., Kliewer, S. A., Borgmeyer, U., Ong, E. S., and Evans, R. M. (1991) *Cell* **66**, 555–561
- Zhang, X. K., Lehmann, J., Hoffmann, B., Dawson, M. I., Cameron, J., Graupner, G., Hermann, T., Tran, P., and Pfahl, M. (1992) *Nature* **358**, 587–591
- Tontonoz, P., Hu, E., Devine, J., Beale, E. G., and Spiegelman, B. M. (1995) *Mol. Cell. Biol.* **15**, 351–357
- King, P. J., and Clark, A. J. (1998) *Endocr. Res.* **24**, 397–402
- Boston, B. A. (1999) *Ann. N. Y. Acad. Sci.* **885**, 75–84
- Gerhold, D. L., Liu, F., Jiang, G., Li, Z., Xu, J., Lu, M., Sachs, J. R., Bagchi, A., Fridman, A., Holder, D. J., Doebber, T. W., Berger, J., Elbrecht, A., Moller, D. E., and Zhang, B. B. (2002) *Endocrinology* **143**, 2106–2118
- Nanbu-Wakao, R., Morikawa, Y., Matsumura, I., Masuho, Y., Muramatsu, M. A., Senba, E., and Wakao, H. (2002) *Mol. Endocrinol.* **16**, 1565–1576
- Kiwaki, K., and Levine, J. A. (2003) *J. Comp. Physiol. B Biochem. Syst. Environ. Physiol.* **173**, 675–678
- Bousquet-Melou, A., Galitzky, J., Lafontan, M., and Berlan, M. (1995) *J. Lipid Res.* **36**, 451–461
- Chhajlani, V. (1996) *Biochem. Mol. Biol. Int.* **38**, 73–80
- Smith, S. R., Gawronska-Kozak, B., Janderova, L., Nguyen, T., Murrell, A., Stephens, J. M., and Mynatt, R. L. (2003) *Diabetes* **52**, 2914–2922
- Kliwer, S. A., Forman, B. M., Blumberg, B., Ong, E. S., Borgmeyer, U., Mangelsdorf, D. J., Umesono, K., and Evans, R. M. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91**, 7355–7359
- Bland, M. L., Jamieson, C. A., Akana, S. F., Bornstein, S. R., Eisenhofer, G., Dallman, M. F., and Ingraham, H. A. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97**, 14488–14493
- Beuschlein, F., Mutch, C., Bavers, D. L., Ulrich-Lai, Y. M., Engeland, W. C., Keegan, C., and Hammer, G. D. (2002) *Endocrinology* **143**, 3122–3135
- Heaney, A. P., Fernando, M., Yong, W. H., and Melmed, S. (2002) *Nat. Med.* **8**, 1281–1287
- Heaney, A. P., Fernando, M., and Melmed, S. (2003) *J. Clin. Investig.* **111**, 1381–1388
- Morris, D. G., Kola, B., Borboli, N., Kaltsas, G. A., Gueorguiev, M., McNicol, A. M., Ferrier, R., Jones, T. H., Baldeweg, S., Powell, M., Czirjak, S., Hanzely, Z., Johansson, J. O., Korbonits, M., and Grossman, A. B. (2003) *J. Clin. Endocrinol. Metab.* **88**, 6080–6087
- Boscaro, M., Sonino, N., Paoletta, A., Rampazzo, A., and Mantero, F. (1988) *J. Clin. Endocrinol. Metab.* **66**, 255–257
- Masui, H., and Garren, L. D. (1971) *Proc. Natl. Acad. Sci. U. S. A.* **68**, 3206–3210
- Lotfi, C. F., Todorovic, Z., Armelin, H. A., and Schimmer, B. P. (1997) *J. Biol. Chem.* **272**, 29886–29891