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Transcriptional regulation of the human acetoacetyl-CoA synthetase gene by PPARγ

Francesca Aguiló, Nuria Camarero[1], Joana Relat[1], Pedro F. Marrero and Diego Haro[2]

Department of Biochemistry and Molecular Biology, School of Pharmacy and Institute of Biomedicine of Barcelona University (IBUB).

1 These authors contributed equally to this work

2 Corresponding author: Departamento de Bioquímica y Biología Molecular, Facultad de Farmacia, Universidad de Barcelona, Diagonal 643, 08028 E-08028 Barcelona, Spain. Tel.: (+34) 934034500; Fax: (+34) 934024520; E-mail: dharo@ub.edu

Short title: AACS is a PPARγ target gene

Abbreviations: AACS, acetoacetyl-CoA synthetase; PPAR, peroxisome proliferator-activated receptor; C/EBP, CCAAT/ enhancer-binding protein; DAPA, DNA affinity precipitation assay; ChIP, chromatin immunoprecipitation; ORL, oxidized LDL receptor; FAS, Fatty Acid Synthase; ACAC, Acetyl-CoA Carboxylase; ACLY, ATP-Citrate Lyase; Ap2, Fatty acid binding protein 4.

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ABSTRACT

In the cytosol of lipogenic tissue, ketone bodies are activated by acetoacetyl-CoA synthetase (AACS) and incorporated into cholesterol and fatty acids. AACS gene expression is particularly abundant in white adipose tissue, as it is induced during adipocyte differentiation. In order to elucidate the mechanism controlling the gene expression of human AACS and to clarify its physiological role, we isolated the human promoter, characterized the elements required to initiate transcription and analyzed the expression of the gene in response to PPARγ, an inducer of adipogenesis. We show that the human AACS promoter is a PPARγ target gene and that this nuclear receptor is recruited to the AACS promoter by direct interaction with Sp1.

Key Words: acetoacetate, ketone body utilization, adipogenesis, gene expression, Sp1.

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INTRODUCTION

Acetoacetyl-CoA synthetase (AACS, acetoacetate-CoA ligase, EC 6.2.1.16) is a cytosolic ketone body (acetoacetate)-specific ligase found in several lipogenic tissues [1]. It might play an important role in the provision of Acetyl-CoA for lipogenesis [2]. Transfer of acetyl as acetoacetate, from the mitochondria to the cytosol, would operate in parallel with transfer via citrate and ATP-citrate lyase [3].

AACS activity is highly regulated. It exhibits a ten-fold diurnal variation in liver of adult rats [4] and varies in parallel with changes in liver lipogenesis during development and starvation [2, 4]. Its activity is regulated by modulators that affect the activity of 3-hydroxy-3-methylglutaryl-CoA reductase and/or cholesterol biosynthesis in the liver and adrenals of adult rats. Thus, AACS activity was depressed by feeding cholesterol or by mevalonate administration and was enhanced by the addition to the diet of mevinolin and/or cholestyramine [5]. Furthermore, the activity of AACS was substantially decreased in rat hepatocytes treated with 25-hydroxycholesterol [6]. The expression of the AACS gene is transcriptionally regulated by SREBP, as deduced from a global analysis of gene expression by oligonucleotide arrays hybridized with RNA from livers of three lines of mice (transgenic for SREBP-1a, transgenic for SREBP-2, and knockout for SCAP) [7].

The activity of cytosolic AACS was decreased in livers from streptozotocin-induced diabetic rats [8, 9]. Lowering AACS activity by 80% partially inhibited glucose-induced insulin release, indicating that formation of short chain acyl-CoA from acetoacetate in the cytosol is important for insulin secretion [10].

The tissue-specific expression pattern of the AACS gene is precisely controlled. Human AACS mRNA is high in kidney, heart and brain, but low in liver [11]. In rat, the AACS mRNA is particularly abundant in male subcutaneous white adipose tissue after weaning. In this tissue, AACS mRNA was preferentially detected in mature adipocytes, but not in preadipocytes, and increased during adipocyte differentiation [12]. The mouse gene is also expressed in adipose tissue, and GC box and C/EBPα motifs seem to be crucial for AACS promoter activity in 3T3-L1 adipocytes [13]. These results indicate that AACS contributes to the utilization of ketone bodies in fatty acid-synthesis during adipose tissue development.

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To elucidate the mechanism controlling the gene expression of human AACS and to clarify its physiological role, we isolated the human promoter, characterized the elements required to initiate transcription and analyzed the expression of the gene in response to PPARγ, a known master of adipogenesis. We show that the human AACS promoter is a TATA-less promoter with an Inr sequence and several GC boxes that are determinant for both its basal and PPARγ-stimulated activity. The binding of Sp1 to the GC boxes has been confirmed *in vivo* by chromatin immunoprecipitation (ChIP) and *in vitro* by DNA affinity precipitation assay (DAPA) experiments. We also show that PPARγ transcriptionally induces the expression of the AACS gene by a non-canonical mechanism, since it is recruited to the AACS promoter by direct interaction with Sp1.

MATERIALS AND METHODS

Reagents

All cell culture reagents were from Gibco BRL (Grans Island, NY). Insulin, dexamethasone, isobutyl methylxanthine, and mithramycin A were from Sigma Chemical Co. (St. Louis MO). Rosiglitazone was from Alexis Biochemical (Grunberg, Germany).

Plasmids constructs. Reporter plasmids are named by designating the 5'- and 3'- ends of the AACS DNA fragment relative to the transcription start site. The human AACS promoter region spanning -1425 to + 79 bp was amplified by PCR using the oligonucleotides AACSF (5'-gagctcGAGATCAGGGAGGCTCTGG-3'; positions -1425 to -1407) and AACSR (5′- aagcttACCTGGGACCGGAGGAAG-3; positions +62/+79). Nucleotides in lowercase were added to provide SacI and HindIII sites at the 5' and 3' ends, respectively. The PCR product was cloned into the p-GEM-T vector and subsequently subcloned into SacI/HindIII restriction sites of the pGL3 basic luciferase reporter (Promega, Madison, WI, USA). Deletions in the 5′-flanking regions of the AACS promoter were constructed by PCR. The PCR primers used were as follows: 5′ primers: p-97 (5′- gagctcGGGAGGGCGGGGCCTGGAAG-3′; positions -97/-77), p-79 (5′- gagctcAAGGGGGCGGGGATTCGAG -3′; positions -79/-61), p-66 (5′- gagctcTTCGAGGCGGGGCCTGCGG-3'; positions -66/-48), p-54 (5′- gagctcCCTGCGGGGCGGGGCCTGG-3′; positions -54/-36), p-33 (5'-

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gagctcCAAGCGGCCCGCAG-3'; positions -33/-20) (nucleotides in lowercase were added to provide a SacI site at the 5') and 3′ primer (AACSR). Mutant p-97(M-Inr) was generated from p-97 in which the Inr site was mutated by site-directed mutagenesis using an overlap extension PCR [14]. Briefly, 100 ng of p-97 plasmid was used as a target in two independent PCRs. The first PCR used a forward primer, 5'-gagctcGGGAGGGCGGGGCCTGGAAG-3', which corresponds to co-ordinates -97 to -77 (nucleotides in lowercase were added to provide SacI site at the 5'), and a reverse primer 5'-GCGGCGGCGGGGAACAAGctcgagtACGGCCGCCGCCGCCTCC-3', which corresponds to co-ordinates -20 to +23 (nucleotides designated in underlined lower case correspond to those that were changed from the wild-type sequence). The second PCR used a forward primer, 5'-GGAGGCGGCGGCGGCCGTactcgagCTTGTTCCCCGCCGCCGC-3', which corresponds to co-ordinates -20 to +23, and reverse primer AACSR. In the subsequent overlap extension reaction, 200 ng of each PCR product was used as a target and the primer corresponding to coordinates -97 to -77 and AACSR were used as primers. The PCR product was cloned in the p-GEM-T vector and subsequently subcloned into pGL3 basic, thus generating p-97(M-Inr).

Introduction of a TATA box motif into p-33 construction p-33(TATA) was performed by PCR using the forward primer gagctcaataattaaCGCAGGAGGCGGCGGCGGCCGTTCAG (nucleotides designated in lower case correspond to a SacI site and the ones designated in underlined lower case correspond to the TATA box motif) and the reverse primer AACSR. To verify all constructs, automatic sequencing was performed using the fluorescent terminator kit (Perkin–Elmer). pSVSport-PPARγ and pJCXRα contained the cDNAs for mouse PPARγ and human RXRα, respectively. *pRL-CMV* (Promega) contained the *Renilla* luciferase gene under the control of the cytomegalovirus (CMV) intermediate-early enhancer/promoter. pMSV-C/EBPα and pCMV-LIP were kindly provided by Dr. M. Giralt. PPARγ-HA was kindly provided by Dr. C. Caelles.

Cell culture

All cells were grown at 37ºC and 5% CO_2 and all media were supplemented with 100 U/mL penicillin and 100 mg/mL streptomycin. 3T3L1 preadipocytes were grown in

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Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS. For adipocyte differentiation, 2 day post-confluent cells (designated day 0) were exposed for 48 h to DMEM supplemented with 10% FBS, 0.5 mM IBMX, 1 mM dexamethasone and 5 mg/mL insulin, followed by a 48-h incubation in DMEM supplemented with 10% FBS and 5 μg/ml insulin [15]. Thereafter, cells were maintained in DMEM supplemented with 10% FBS. Fat accumulation was visualized by staining with Oil red O (Sigma). The H1B-1B brown preadipocytes were cultured in DMEM/F12 containing 10% FBS as described previously [16].

Transient transfections

HIB-1B cells were seeded at a density of 0.25 x 10^5 cells/well in 12-well plates 1 day before transfection. Cells, were transiently transfected using FuGENE-6 reagent, according to the manufacturer's instructions (Roche Diagnostics Corporation, Indianapolis, IN). For the characterization of the human AACS promoter, HIB-1B cells were transfected with 1 µg of the reporter gene construct and 30 ng of plasmid pRL-CMV, included as internal control. 48 h after transfection, cell lysates were collected for the luciferase assay. For the study of the PPARγ-dependent activation of the human AACS gene, HIB-1B cells were cotransfected with 750 ng of the reporter gene construct and, when indicated, with 150 ng of pSVSport-PPARγ and pJCXRα, and 300 ng of pMSV-C/EBPα and/or pCMV-LIP. The plasmid pRL-CMV (30 ng) was included as an internal control and pBluescript KS(+) was used to keep the amount of transfected DNA constant. Cells were incubated for 48 h after transfection and, when indicated, were treated for 24 h before harvest with 10 μM rosiglitazone, 100 nM of mithramycin A or vehicle (DMSO or ethanol).

Luciferase assays

The cell extracts were prepared using the passive lysis buffer method (Promega) and firefly and *Renilla* luciferase activities were determined in a TD-20/20 Luminometer (Turner Designs) using the Dual-Luciferase Reporter Assay System (Promega), according to the manufacturer's recommendations.

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Animal treatment

For *in vivo* gene expression studies, animals were housed in a temperature-controlled room under a 12-hour light/12-hour dark cycle and were fed *ad libitum*. C57/BL6 received a gavage with rosiglitazone (30 mg/kg) or vehicle (carboxymethylcellulose 1.6%), once a day for 10 consecutive days. Thereafter, mice were anesthetized with isofluorane and adipose tissue (epididymal fat pads) and liver were excised and frozen in liquid nitrogen. All experimental protocols were approved by the Animal Care Research Committee of the University of Barcelona.

RNA analysis

Trizol reagent (Life Technologies, Inc.) was used to isolate RNA. 1 μg of total RNA was reverse transcribed using M-MLV reverse transcriptase (Invitrogen) for first strand cDNA synthesis and random p(dN)6 primers (Roche Diagnostics), according to the manufacturer's protocol. The resultant cDNA was subjected to real-time quantitative PCR, in which a specific primer and probe mix of mouse Aacs (Mm 00513427_m1), Pparg (Mm 01184322_m1), Ap2 (Mm 00445878_m1), Fas (Mm 00662319_m1), Acac (Mm 01304257_m1), and Acly (Mm 00652520_m1) were used (Taqman Gene Expression Assay, Applied Biosystems). Murine differentiation mRNA expression data was normalized to 18S (4319413E, Applied Biosystems). PCR assays were conducted with the ABI 7700 amplification system (Applied Biosystems).

DNA precipitation assays

DNA precipitation assays of HIB-1B cells were performed as described previously [17]. Cells were transfected with 10 μg of the HA-PPARγ construct and, when indicated, were treated for 24 h before harvest with 10 μM rosiglitazone. DNA-bound proteins collected with Tetralink Tetrameric Avidin Resin were separated on an SDS-polyacrylamide gel and identified by Western blotting. Anti-HA monoclonal antibody was used to detect HA epitope-tagged PPARγ. Sp1 was detected using PEP2 antibody from Santa Cruz Biotechnology, Santa Cruz, CA. Biotinylated oligonucleotides comprising the Sp1 sites of AACS (5'-CGGG**AGGGCGGGGC**CTGGAAG**GGGGCGGGGA**TTC**GAGGCGGGGC**CTGC**GGGGCGGGGC**CTGGGC-3': the consensus Sp1-binding sites are shown in bold

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capital letters) and the mutated Sp1 sites (5'-CGGG**agtctagagc**CTGGAAG**ggtctagaga**TTC**gatctagagc**CTGC**ggtctagagc**CTGGGC-3´: mutated nucleotides are shown in bold lowercase letters) were synthesized by Sigma.

Chromatin immunoprecipitation assay

ChIPs assays were modified from a published protocol [18] as follows. 3T3-L1 adipocytes on day 6 were treated with 10 μM rosiglitazone for 24 hours. In order to cross-link proteins to DNA, adipocytes were treated with formaldehyde to a final concentration of 1% and incubated at 37°C for 10 min. Cells were washed with PBS and 1 mM PMSF, scraped and collected by centrifugation. For Sp1 ChIP assays, cells were resuspended in lysis buffer (85 mM KCl, 0.5% NP-40, 1 mM PMSF, 1 μg/ml leupeptin and aprotinin at pH 8) and homogenized. Nuclei were isolated by centrifugation at 250 x *g* for 10 min, then resuspended in sonication buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl at pH 8.1) and sonicated for four cycles of 10 s each. The extracts were then centrifuged at 13 000 r.p.m. for 10 min at 4°C. The cross-linked preparations were diluted 10-fold in ChIP dilution buffer (1% Triton X-100, 1.2 mM EDTA, 167 mM NaCl, 16.7 mM Tris-HCl plus the aforementioned proteases inhibitors, at pH 8.1). The chromatin solution was pre-cleared with 80 μl of protein A agarose/salmon sperm DNA (Upstate Biotechnology) for 1 h at 4°C. An aliquot (10 μl) was removed as a control (input). Immunoprecipitation was performed overnight with 20 μg of the antibody against Sp1 (Santa Cruz Biotechnology) or preimmune serum (IgG). Following immunoprecipitation, 60 μl of protein A agarose/salmon sperm DNA was added, and incubation continued for 4 h. Agarose beads were then collected and washed 4 times for 5 min in Sp1 low salt buffer (0.1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, 150 mM NaCl at pH 8.0) and twice with Tris-EDTA buffer. For PPARγ ChIP assays, cells were resuspended in lysis buffer (1% SDS, 5 mM EDTA, 50 mM Tris-HCl, 1 mM PMSF, 1 μg/ml leupeptin and aprotinin at pH 8.1) and, following a 20-min incubation on ice, samples were sonicated at 15-s pulses three times. The extracts were then centrifuged at 13 000 r.p.m. for 10 min at 4°C, and the collected supernatant was diluted 10-fold in ChIP dilution buffer (1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris-HCl plus the above indicated proteases inhibitors, at pH 8.1). Samples were precleared with 80 μl of protein A agarose/salmon sperm DNA (Upstate Biotechnology) for 1 h at 4°C. An aliquot (10 μl) was removed as a control (input).

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Immunoprecipitation was performed overnight with 10 μg of the antibody against PPARγ (Santa Cruz Biotechnology) or preimmune serum (IgG). Samples were then incubated with 60 μl of protein A agarose/salmon sperm DNA for 1 h followed by 5-min sequential washes in low salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, 150 mM NaCl at pH 8.1), high salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, 500 mM NaCl at pH 8.1) and LiCl buffer (0.25 M LiCl, 1% NP-40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris-HCl at pH 8.1) and Tris-EDTA buffer. Precipitates were then extracted by incubating with elution buffer (1% SDS, 0.1 M $NaHCO_3$) at room temperature for 15 min. Eluates were pooled and heated at 65°C for 4 h to reverse the cross-linking. The chromatin-associated proteins were digested with proteinase K and samples were extracted with phenol/$CHCl_3$ followed by precipitation with ethanol. Pellets were resuspended in water and subjected to PCR. The primers used for amplification were 5′- GAATGAACGAACGAACGAGGG-3′ and 5'-CCACAGCGCGAGACTGAAC-3', located at positions -264 to -243 and -4 to +15 of the mouse AACS promoter, respectively. Primers used for exon 17 amplification were 5'-GAAGCCTTCGATGAGGTGG-3' and 5'-GGAATGCCTCGGGTCTCC-3'. ChIP analysis of PPARγ association with the *OLR1* promoter (forward, 5′-TGAACAAACAAGTCGAACCATC-3′ and reverse, 5′-TGTGGGTGGGGAGAATTATATC-3′ located at positions -382 to -361 and -213 to -192, respectively) was used as a positive control [19]. Following amplification, the PCR products were electrophoresed in a 2% (w/v) agarose gel and visualized by staining with ethidium bromide.

5′ RACE

The 5' end of the adipose human AACS cDNA was determined using the SMART RACE cDNA Amplification Kit (Clontech). This approach generates full-length cDNAs in RT reactions [20]. To this end, 1 μg of total RNA from human adipose tissue (Applied Biosystems) was converted into cDNA using PrimeScript^{TM} RT (Takara Bio Inc.) following the manufacturer's instructions. 5'-RACE ready cDNA was obtained using the SMART II A and DH 1114 oligonucleotides (CATTCTCTTTGTGCCGCA, positions +455/+472). Then a touchdown PCR was conducted with the Universal Primer Mix A from the kit and DH 1115 oligonucleotides (GAGGTTTTCTGCATAGTTGAGC, positions +433/+453), with Advantage 2 PCR

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Enzyme System (Clontech). Five initial cycles were carried out, with 30 sec of denaturation at 94 ºC, 30 sec of annealing at 72 ºC, and 3 min of extension at 68 ºC, followed by five cycles with 30 sec of denaturation at 94 ºC, 30 sec of annealing at 70 ºC, and 3 min of extension at 68 ºC, and 27 cycles with 30 sec of denaturation at 94 ºC, and 3.5 min of annealing and extension at 68 ºC. A nested PCR was performed with the Nested Universal Primer from the kit and DH 782 oligonucleotides (ACCTGGGACCGGAGGAAG, positions +62/+79). Twenty cycles were carried out, with 30 sec of denaturation at 94 ºC, and 3.5 min of annealing and extension at 68 ºC. PCR products were resolved in a 1.5% agarose gel, purified and ligated into pGEM-T Easy vector (Promega). Different clones were sequenced using the primer SP6 (5′-GATTTAGGTGACACTATAG).

Statistical analysis

Statistical differences between experimental groups were determined by Student's *t* test using SPSS statistical software (SPSS Worldwide Headquarters, Chicago, IL). Values of $p < 0.05$ were considered statistically significant.

RESULTS

The human AACS gene has a TATA-less promoter with an Inr sequence and several GC boxes that are determinant for its activity.

BLAST alignment of a genomic sequence of the human AACS gene, containing the 5' end of the annotated cDNA sequence, with an expressed sequence tags (ETS) database showed the abundance of ETSs starting 43 nucleotides upstream of the end of the published cDNA [11]. This main position is occupied by an A that is included in a consensus transcription start site (PyrPyrA+1NT/APyrPyr) (Figure 1A). In order to define the transcriptional start site of the AACS gene in adipose tissue, we performed 5' RACE using a pool of RNAs isolated from human white adipose tissue. As a result of this experiment we found two main start sites flanking the putative Inr sequence of the AACS gene. We sequenced 36 clones; 19 of these clones start 11 nucleotides upstream of the A in the Inr and 16 of them 8 nucleotides downstream of this position.

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As expected, the human AACS promoter (from positions -1425 to +79) was able to drive luciferase reporter expression, over the promoter-less pGL3basic construct, when transiently transfected in HIB-1B cells (Figure 1B).

The analysis of the 5' region of the AACS gene showed that it is a TATA-less promoter with the described consensus Inr and several GC boxes. To identify the elements that contribute to the core promoter activity, several reporter constructs were analysed, including a scrambling mutation of the Inr and deletion mutants of the GC boxes. Figure 2A shows that both the Inr and the GC boxes are needed to promote maximal transcription in HIB-1B cells. Furthermore, the introduction of a recombinant TATA box (30 nucleotides upstream of the transcription start site) does not drive AACS promoter-mediated transcription. To further investigate the role of the putative Sp1 binding sites, several deletion mutants were constructed, eliminating each one of these GC boxes. Figure 2B shows that each one of these sequences contributes to the basal activity of the promoter.

The AACS promoter therefore, seems to be a focused promoter with a cluster of transcription start sites in a short region of nucleotides; this organization is partially conserved between the rat, mouse and human AACS promoters (Figure 1C).

Sp1 bound to the GC boxes of the AACS promoter both *in vivo* and *in vitro*.

The capacity of Sp1 to interact *in vitro* with the GC-rich region of the human AACS promoter was analyzed by pull-down experiments with biotinylated wild-type or mutant probes and HIB-1B cells protein extracts. Figure 3A shows the presence of Sp1 in complexes corresponding to the wild type, but not those corresponding to the mutant probe. The *in vivo* interaction of Sp1 on the AACS promoter was analyzed by ChIP experiments with formaldehyde cross-linked chromatin from 3T3L1 cells. Figure 3B clearly shows the presence of Sp1 proteins in the AACS promoter, while this interaction was absent in the coding region of the gene (exon 17).

AACS gene expression was induced by PPARγ ligands.

AACS mRNA expression was high, particularly in white adipose tissue [12] (data not shown). Furthermore, AACS mRNA increased during adipocyte differentiation [12, 13]. Figure 4 shows that in 3T3-L1, AACS gene expression showed the highest levels at day six of differentiation and occurred in parallel with the induction of PPARγ expression. According to this, the expression of the AACS gene was induced in

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white adipose tissue (Figure 4B) or liver (Figure 4C) from mice treated with the PPARγ agonist rosiglitazone. These figures show also that a similar induction was observed for other PPARγ target lipogenic genes.

PPARγ transcriptionally induces the expression of the human AACS gene. To investigate the effect of PPARγ expression on the transcriptional activity of the AACS gene, a human AACS promoter luciferase-reporter construct was transiently cotransfected in HIB-1B cells with or without the expression vectors for PPARγ and RXRα, and 10μM rosiglitazone was added when indicated. Figure 5A shows that PPARγ/RXRα expression increased luciferase activity in the presence of rosiglitazone. To identify the regulatory sequences mediating this activation, the experiment was repeated with a series of deletion mutants of the promoter. As shown in Figure 5B, the response to PPARγ/RXRα was maintained as long as a GC box of the AACS promoter was present in the construct.

Adipocyte differentiation is a complex process involving a cascade of expression of many transcription factors, including CCAAT/ enhancer-binding proteins (C/EBPs). Recently, C/EBPα [13] has been identified as a transactivator of the mouse AACS promoter working through a consensus C/EBP binding site which is not conserved in the human AACS promoter. Also, it has been reported that the transcription factor C/EBP family and Sp1 cooperatively activate a number of genes promoters including CYP2D [21], human CD11c [22], lactoferrin [23], mouse aldose reductase-like gene [24], and mouse IL-10 [25]. As shown in Figure 5B, the GC-box containing sequence in the proximal human AACS promoter is sufficient for C/EBPα–mediated transactivation, suggesting that the binding of Sp1 to the GC-box rich sequence is required not just for the basal human AACS gene expression but also for the PPARγ and C/EBPα-stimulated activity.

Positive synergy between PPARγ and C/EBPα has been described [26]. However the ectopic expression of both C/EBPα with PPARγ/RXR didn't increase the AACS promoter luciferase activity compared to the cotransfection of either C/EBPα or PPARγ/RXR alone (data not shown).

In order to discard an indirect effect of PPARγ on the AACS promoter through C/EBPs, the AACS promoter luciferase reporter was cotransfected with PPARγ and RXRα with or without LIP, a dominant negative of C/EBPβ able to inhibit the activation of the

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AACS promoter induced by C/EBPα (Figure 5C). Figure 5D shows that this dominant negative does not prevent the activation of the AACS promoter by PPARγ but even increases it, suggesting the existence of a functional antagonism between PPARγ and C/EBPα mediated by competition for a limiting critical cofactor. This phenomenon might be explained by the dimerization of LIP with the endogenous C/EBPα, increasing the accessibility of PPARγ to Sp1. However, the mechanistic regulation of the human acetoacetyl-CoA synthetase gene by C/EBPα requires further investigation.

To confirm the importance of Sp1 in the PPARγ–mediated transactivation, we treated the cells with mithramycin, an antibiotic that inhibits the Sp1 binding to DNA. Figure 5E shows a 50% reduction on the activation of the AACS promoter by PPARγ in the presence of mithramycin.

PPARγ was recruited to the AACS promoter by direct interaction with Sp1. Sp1 and PPARγ interaction has been shown before [27], and can be reproduced by pull-down experiments with a GST-humanPPARγ recombinant protein and Sp1 transcribed and translated *in vitro* (data not shown). To assess whether the Sp1-binding sites present in the AACS gene recruited PPARγ, we performed DNA precipitation assays using HIB-1B cell extracts expressing tagged PPARγ that were incubated with biotinylated GC wild-type or mutant probes. Figure 6A shows the binding of PPARγ when the streptavidin-agarose trapped protein complexes, corresponding to the wild type but not to the mutant probe, were analyzed by Western blot with anti-HA specific antibodies. The interaction of PPARγ with chromatinized DNA was analyzed by a ChIP experiment with formaldehyde cross-linked chromatin from differentiated 3T3L1 cells and a pair of oligonucleotides corresponding to the mouse gene promoter. As shown in Figure 6B, the presence of PPARγ was detected in the AACS promoter, while this interaction was absent in the coding region of the gene (exon 17). The binding of PPARγ to a known target gene, the oxidized LDL receptor (OLR) was used as a positive control [19].

DISCUSSION

Even though the utilization of ketone bodies as a lipogenic substrate in different tissues has been amply documented since the late seventies of the last century, their physiological role remains to be elucidated. The AACS gene presents a tissue-specific expression pattern, as it is expressed in several lipogenic tissues. One of the tissues in

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which the acetoacetyl-CoA synthetase gene is particularly abundant is white adipose tissue. In this tissue, AACS mRNA is preferentially detected in mature adipocytes, but not in preadipocytes, and increased during adipocyte differentiation [12, 13]. This indicates that AACS contributes to the use of ketone bodies for fatty acid-synthesis during adipose tissue development. This is a paradoxical situation, in which ketone bodies produced after the oxidation of fatty acids are used to resynthesize fatty acids in the adipose tissue. This may be an alternative way to transport fatty acid equivalents across different tissues. According to this putative anabolic role, the expression of the AACS gene is under the control of the SREBPs in liver [7] and is decreased in livers from streptozotocin-induced diabetic rats [8, 9].

We studied the mechanisms controlling the gene expression of human AACS. We isolated the human promoter, characterized the elements required to initiate transcription and analyzed the expression of the gene in response to PPARγ, a known master of adipogenesis.

The AACS promoter is a TATA-less promoter whose transcriptional activity is driven by an Inr sequence and several GC boxes. Other significant elements present in the core promoters do not occur in the AACS promoter. The BRE (TFIIB recognition element), an element existing immediately upstream of some TATA boxes, is not present; neither is the DPE (downstream promoter element) typically found in TATA-less promoters. The region between -3 and +5 of the AACS promoter contains a consensus Inr site (PyrPyrA+1NT/APyrPyr), and is both sufficient to drive a weak transcription and necessary for the activity of the promoter when the region up to -93, containing several binding sites for transcription factor Sp1, is added. The sum of these results with those obtained by 5' RACE from human adipose tissue, suggests that this Inr supports high levels of transcription that initiate at a cluster of start sites in a region of several nucleotides.

The 5' flanking region of the human AACS gene contains several Alu repeats. In the approximately 1.5 Kb of the 5' sequence analysed in this paper there are two repeats that contains putative response elements for transcription factors (e.g. LXR or SREBP), which are potentially involved in regulating AACS gene expression. Experiments that are not shown in this paper illustrate that these are non-functional elements.

The expression of the AACS gene in adipose tissue, its appearance after PPARγ during

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differentiation to adipocytes in cell culture, and its putative role in adipogenesis, point to this gene as a target for this nuclear receptor. Effectively, our results show that the expression of the AACS gene is induced in cell culture and in mice by treatment with the PPARγ agonist rosiglitazone. To determine whether PPARγ was directly involved in the AACS gene induction, HIB-1B cells were transfected with the PPARγ and RXRα expression plasmids and an AACS promoter luciferase reporter. Subsequently, the transcriptional activity was measured either with or without 10 μM rosiglitazone. These results show that the activity of the reporter can be induced by rosiglitazone and that this induction is augmented in the presence of cotransfected PPARγ and RXRα.

The analysis of the sequence showed the absence of a consensus PPRE in the 5' flanking region of the human AACS gene up to -1500-bp relative to the transcription start site. This suggests that a PPRE-independent mechanism might be involved in PPARγ-mediated AACS gene expression. To identify the regulatory sequences that are important for AACS gene induction by PPARγ, we prepared deletion constructs from the human AACS promoter and assayed for PPARγ transactivation. Sequence deletions up to -54-bp retained the PPARγ-dependent promoter activity. However, deletion of the region between -54 to -33-bp resulted in a complete loss of the promoter activity, indicating that this region of the AACS promoter was required for PPARγ-mediated transactivation.

The results of this study show by chromatin immunoprecipitation the presence of PPARγ on the region of the AACS promoter that contains several GC boxes. Moreover, we show by DNA affinity precipitation assay (DAPA) experiments that this interaction occurs through the wild type GC boxes, as it is not observed when these sequences are mutated. This suggests that induction of AACS by rosiglitazone is dependent on PPARγ interactions with Sp1 bound to GC-rich sites in the proximal promoter of the AACS gene.

The interaction of PPAR with the Sp1 family has been amply documented. A functional interaction of peroxisome proliferator-activated receptor, retinoid-X receptor, and Spl in the transcriptional regulation of the acyl-coenzyme-A oxidase promoter was shown [28]. RNA interference and protein expression/DNA binding assays suggest that DIM-C-pPhCF3 induced p21 expression through a mechanism that involves PPAR interactions with both Sp1 and Sp4 proteins bound to the proximal GC-rich region of the p21 promoter [29]. The hormone-sensitive lipase gene expression is positively regulated by PPARγ and this upregulation requires the involvement of Sp1 [30]. PPAR

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γ1 enhances KDR gene expression at the transcriptional level, via the enhancement of Sp1-KDR promoter binding in the absence of its ligands in retinal capillary endothelial cells (RCECs). PPAR ligands, however, suppress KDR gene expression via a decreased interaction between PPAR and Sp1/Sp3. PPAR γ1 appears to have bifunctional properties in the regulation of KDR gene expression, mediated via interaction with both Sp1 and Sp3 in RCECs [31].

However, several studies show an inhibitory effect of PPARγ on Sp1 activation. For example, the follistatin promoter was dramatically regulated by Sp1, and this regulation was inhibited by PPARγ expression. PPARγ was found to interact with Sp1 and repress its transcriptional activation function [32]. Activation of PPARγ by its agonists suppresses thromboxane receptor and type angiotensin II receptor gene expression in vascular smooth muscle cells by direct interaction of PPARγ with Sp1 [33, 34]. Similarly, in human lung carcinoma cells, PPARγ agonists inhibit fibronectin gene expression, preventing Sp1 from binding to its respective sites [35]. These studies coincidently show that PPARγ can regulate the promoter-specific gene expression by interacting with Sp1.

FUNDING

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FIGURE LEGENDS

Figure 1. Cloning of the 5'-flanking region of the human AACS gene. (A) Sequence analysis of human AACS gene (-1425 to +79). Coordinates referring to the transcription start site (+1) are indicated by an asterisk. The transcription start sites determined by 5' RACE with human WAT are indicated by diamonds. Positions of potential regulatory element software are boxed (Yutaka Akiyama: "TFSEARCH: Searching Transcription Factor Binding Sites", http://www.rwcp.or.jp/papia/). Alu sequences are indicated in grey. **(B)** Normalized luciferase activity from HIB-1B cells transfected with: p-1425, or pGL3basic reporter plasmids. Results show the mean ± SEM of at least three independent experiments done in duplicate. The activity of the pGL3basic vector was set at 1. The other data were normalized to this value. (**C**) Multiple sequence alignment of the rat, mouse and human AACS promoters; the conserved Sp1 binding site and Inr are boxed.

Figure 2. Characterization of the human AACS promoter. (A) Normalized luciferase activity from HIB-1B cells transfected with: p-97, p-97(M-Inr), p(TATA), p-33, or pGL3basic reporter plasmids. Results show the mean ± SEM of at least three independent experiments done in duplicate (*$p < 0.001$ vs. p-97). **(B)** Normalized luciferase activity from HIB-1B cells transfected with p-291, p-97, p-79, p-66, p-54, p-33, or pGL3basic reporter plasmids. Results show the mean ± SEM of at least three independent experiments done in duplicate. (*$p < 0.001$ vs. p-291; **$p < 0.05$ *vs*. p-97; ***$p < 0.001$ *vs*. p-79; †$p < 0.001$ *vs*. p-66; ††$p < 0.001$ *vs*. p-54; ‡p not significant *vs*. p-33.

Figure 3. Sp1 binds *in vitro* and *in vivo* to the AACS promoter. **(A)** DAPA of HIB-1B cells extracts incubated with biotinylated oligonucleotides containing the Sp1 sites of human AACS (*Wt*) or a scrambled sequence of these sites (*Mut*) (see *Materials and Methods*). Avidin-purified oligonucleotides were processed for Western blot analysis with Sp1 specific antibodies. **(B)** ChIP analysis of Sp1 association with the AACS promoter in 3T3-L1. Chromatin preparations were immunoprecipitated with antibodies against Sp1 or an unrelated IgG. The immunoprecipitates were then analyzed by PCR using specific primers for the AACS proximal promoter or AACS exon 17. Aliquots of chromatin taken before immunoprecipitation were also analyzed (*Input*).

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Figure 4. AACS gene expression is induced by PPARγ ligands. (**A**) Time course of the AACS and PPARγ mRNA expression during adipocyte differentiation in 3T3-L1 cells; mRNA expression data were determined by real-time quantitative-PCR assays and normalized to 18S. Results show the mean ± SEM of at least two independent experiments. **(B-C)** Regulation of AACS by PPARγ agonist *in vivo*. C57/BL6 were gavaged daily with rosiglitazone (30 mg/kg) or a vehicle for 10 days. At the end of the treatment period, total RNA was isolated from subcutaneous white adipose tissue **(B)** or liver **(C).** Gene expression for individual animals was determined by real-time quantitative-PCR assays. (** $p \leq 0.01$; * $p \leq 0.05$; n=8 or 7 for white adipose tissue control or treated, respectively and n =7 or 6 for liver control or treated, respectively).

Figure 5. PPARγ-dependent activation of the human AACS gene is mediated by the GC boxes. HIB-1B cells were cotransfected with p-1425 AACS reporter gene construct and, when indicated, with the effector plasmids expressing full-length cDNAs for human PPARγ and RXRα. *Statistically significant differences with respect to untreated controls ($P < 0.005$). **(B)** HIB-1B cells were co-transfected individually with p-1425, p-291, p-97, p-79, p-66, p-54, p-33 or pGL3basic reporter plasmids, and in the absence or presence (closed bars) of effector plasmids expressing full-length cDNAs for murine PPARγ and human RXRα plus 10 μM rosiglitazone. *Statistically significant differences with respect to untreated controls ($P < 0.001$). **(C)** HIB-1B cells were cotransfected with p-97 AACS reporter gene construct and, when indicated, with the effector plasmids expressing full-length cDNAs for C/EBPα and/ or LIP. *Statistically significant difference with respect to C/EBPα cotransfection (in absence of LIP) ($P < 0.001$). **(D)** HIB-1B cells were cotransfected with p-97 AACS reporter gene construct and, when indicated, with the effector plasmids expressing full-length cDNAs for PPARγ/RXRα and/ or LIP and, treated with 10 μM of rosiglitazone. *Statistically significant difference with respect to PPARγ/RXRα cotransfection (in absence of LIP) ($P < 0.001$). (E) HIB-1B cells were cotransfected with p-97 AACS reporter gene construct and, when indicated, with the effector plasmids expressing full-length cDNAs for PPARγ/RXRα and treated with 10 μM of rosiglitazone and/or 100 nM of mithramycin. *Statistically significant difference with respect to PPARγ/RXRα cotransfection (in absence of mithramycin) ($P < 0.05$). In all the transfection assays, the amount of plasmid was kept constant by using pBluescript KS(+) vector. Luciferase activity is depicted relative to the respective *Renilla* activities. When cells were

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cotransfected with C/EBPα expression vector, luciferase activity is normalized relative to the respective amount of protein. Results show the mean ± SEM of at least three independent experiments done in duplicate.

Figure 6. PPARγ is recruited to the AACS promoter by direct interaction with Sp1. (A) HIB-1B cells were transfected with the HA-PPARγ plasmid and 24 hours later, cells were treated with 10 mM rosiglitazone. After 24 hours of treatment, cells were harvested and whole-cell extracts were incubated with biotinylated oligonucleotides containing the Sp1 sites of human AACS (W*t*) or a scrambled sequence of these sites (*Mut*). Avidin-purified oligonucleotides were processed for Western blot analysis with antibodies against HA. **(B)** ChIP with antibodies against PPARγ or an unrelated IgG in 3T3-L1 adipocytes (see *Materials and Methods*). The immunoprecipitates were analyzed by PCR using specific primers for the AACS proximal promoter, AACS exon 17 or ORL1. Aliquots of chromatin taken before immunoprecipitation were also analyzed (*Input*).

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FIGURE 1

A)

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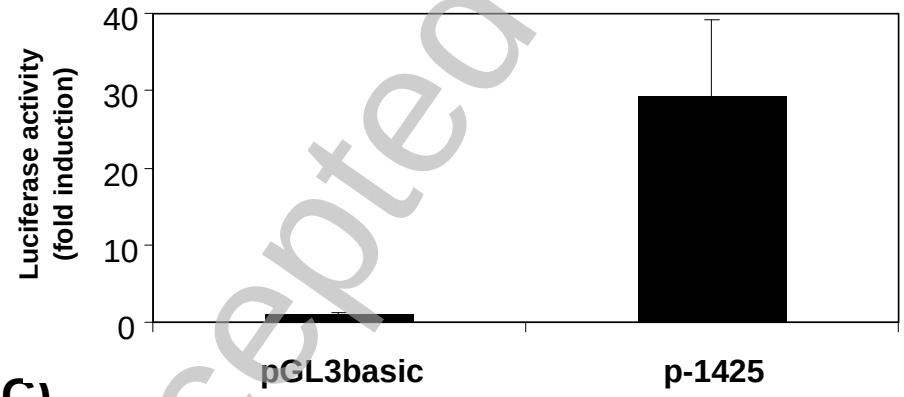
GAGATCAGGG AGGCTCTGGA GGGGGTGCTC TCAGACCTGG GCTCACTCTT GTAATCCCAG CACTTTGGGA GGCCGAGGCG
GGTGGATCAC CTGAGGTCAG GACTTCAAGA CCAGCCTGGC CAACATGGTG AAATCCCGTC TCTACAAAAA TGCAAAAATG
        DR4
AGCCGGGCTC GGTAGCTCAC GCCTGTAATT CCAGCACTTT GGGAGGCTGA GGCGGGCGGA TCACCTGAGG CCGGGAGTTG
GAGACCAGCC TGACCAACAT GGAAAAACCC TGTCTGTACT AAAAATACAA AATTAGCCAG GCGTGGTGGC ACATGCCTGT
AATCCCAGGT ACTCGAGAGG CTGAGGCAGG AGAATTGCTT TAACCTGGGA GATGGAGGTT GCCGTGTGCC GAGATCTCGC
AATTGCACTC CAGCCTGGGT AACAAGAGCG AAACTTCGTC TCAAAAAAAC AAAACAAACA ACAACAACAA CAAAAAAAAA
AACACGAAAA ATTAGCTGGG CATGATGGCG TGTGCCTGTA ATCCCAGCTA CTCAGGAGGC TGAGGCAGGA GAATCGCTTG
AACCTGGGAA GCAGAGGTTA CAGTGAGCTG AGATTGGGAC ATTGCACTCC AGCCTGGGCG ACTGAGTGAG ACTCAAAAAA
AAAAAAAAAG CTAAGGCGGG GAGGGGGCCT TCCAGGTCAT AGGTAGATAA GGGACAAATG GCTGCATTCT TTTGAGTTTT
CAATTAGCCT CTGCAAAGGA GGCAATCAGG TGTGCATTTA ACTCAGCAGA GGGGTGACTT TGAATAGAAT GGAGGCAGGT
TAGCCCTAAG CAGTTCCCAG CTTTTCTTTT CCCTTTAGCT TAGTAGTGGG GCCCCAAGAT TTATTTTCCT TTCACAAATG
TGTATTTTGA TACCCATTGG CATACTATGT ATATTATTGT ATTATACTAT GACATTATCT TGTGTATGTA CACGATGTAC
TTATTTGTTA ATCGTCTCCC GTCTTCCTCA CACCTGTACC CCTCCTCTCC TGTTGACAGA GAACAAGAAT TTTGTTTTGT
TCGCTGCTGT ATCCGCAAAG CTCCCAAAGT CCTCCGAATG CCGCAGGCTC TCAGTAAAGA CCTGCAGGAA GAAATAGCGA
            E box
ATGAATGATG TACACGTGTA GGAAACAGCG GGCTCGGACG CCTGGCTTCT CCGGCTGCCC TAACGCCCTC CAGCTCCAGG
                                                  GC
TGAGGTCGGG GAGCTGGGGC CAAGGCTCGT GAGCGCCCCT CACCCCCACG CCCCCCGGCC GCGGGCGCTG GAGCCGGCGG
                                                          GC                GC
CTGCGAGCAC TCCTTTGAGC GGCGCTGCAC TCAGCGGAGG GTTACAGGCG GGAGGGCGGG GCCTGGAAGG GGGCGGGGAT
    GC          GC                                          ◆          *          ◆
TCGAGGCGGG GCCTGCGGGG CGGGGCCTGG GCCAAGCGGC CCGCAGGAGG CGGCGGCGGC CGTTCAGTCC CTTGTTCCCC
                                                                      Inr
GCCGCCGCCG TCGCTGACCC AGCCCGCCAG GCGCTCCTGA CCGTCGCTTC CTCCGGTCCC AGGT
```

B)



C)

```
rat     CGGCGGAGGGTTACAG--AAGAGGGCGTGGTCTCAAGAGGCGTGGCCTTCTGGAGGCGT
mouse   CGGCGGAGGGTTACAG--AAGAGGGCGTGGTCTCAAGAGGCGTGGCTTTCTGGAGGCGT
human   CAGCGGAGGGTTACAGGCGGGAGGGCGGGGCCTGGAAGGGGGCGG------GGATTCGA

rat     GGCGAAGCCTGCTTGGGGGTGTGACTAGGCCGGATGTCCCGCAGGCCGTGCCGGCGCTG
mouse   GGCGAAGGCTGCTGGGGGTGTGACCAGGCCGGATGTCCCACAGGCCGCGCCGGCGCTGG
human   GGCGGGGCCTGCGGGGCGGG-GCCTGGGCCAAGCGGCCCGCAGGAGGCGGCGGCGGCCG

rat     CTCAGTCTCGCGCT
mouse   TTCAGTCTCGCGCT
human   TTCAGTCCCTTGTT
```

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FIGURE 2

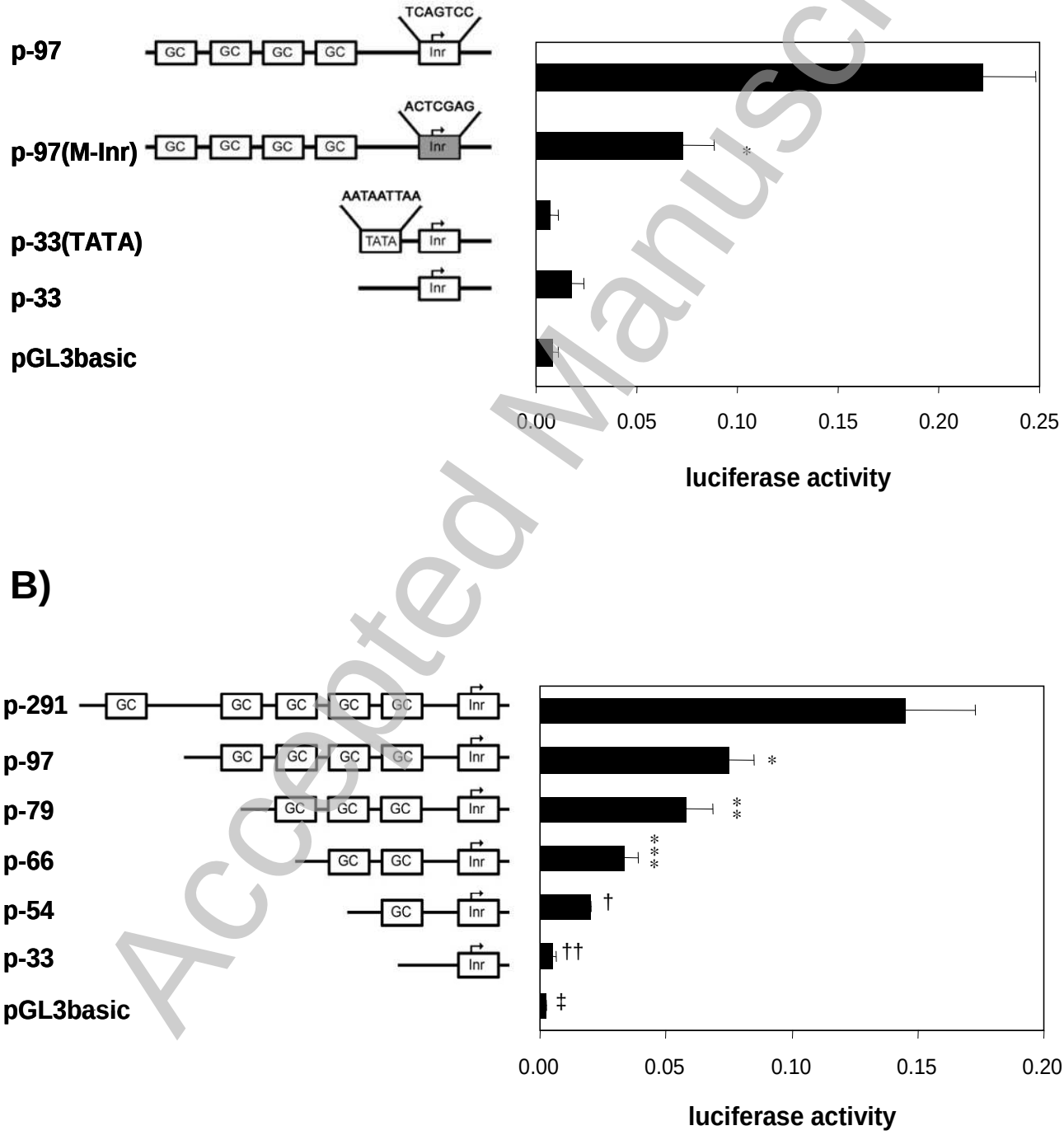

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FIGURE 3

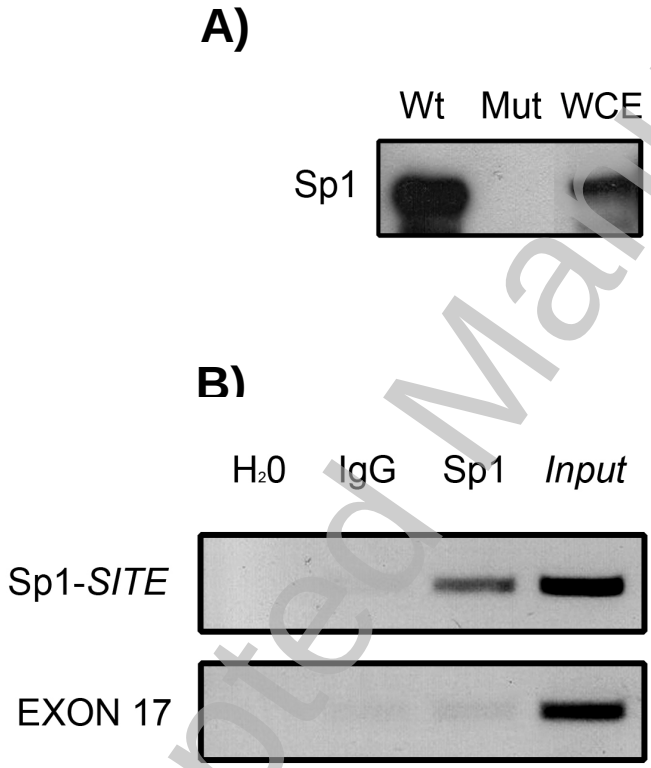

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FIGURE 4

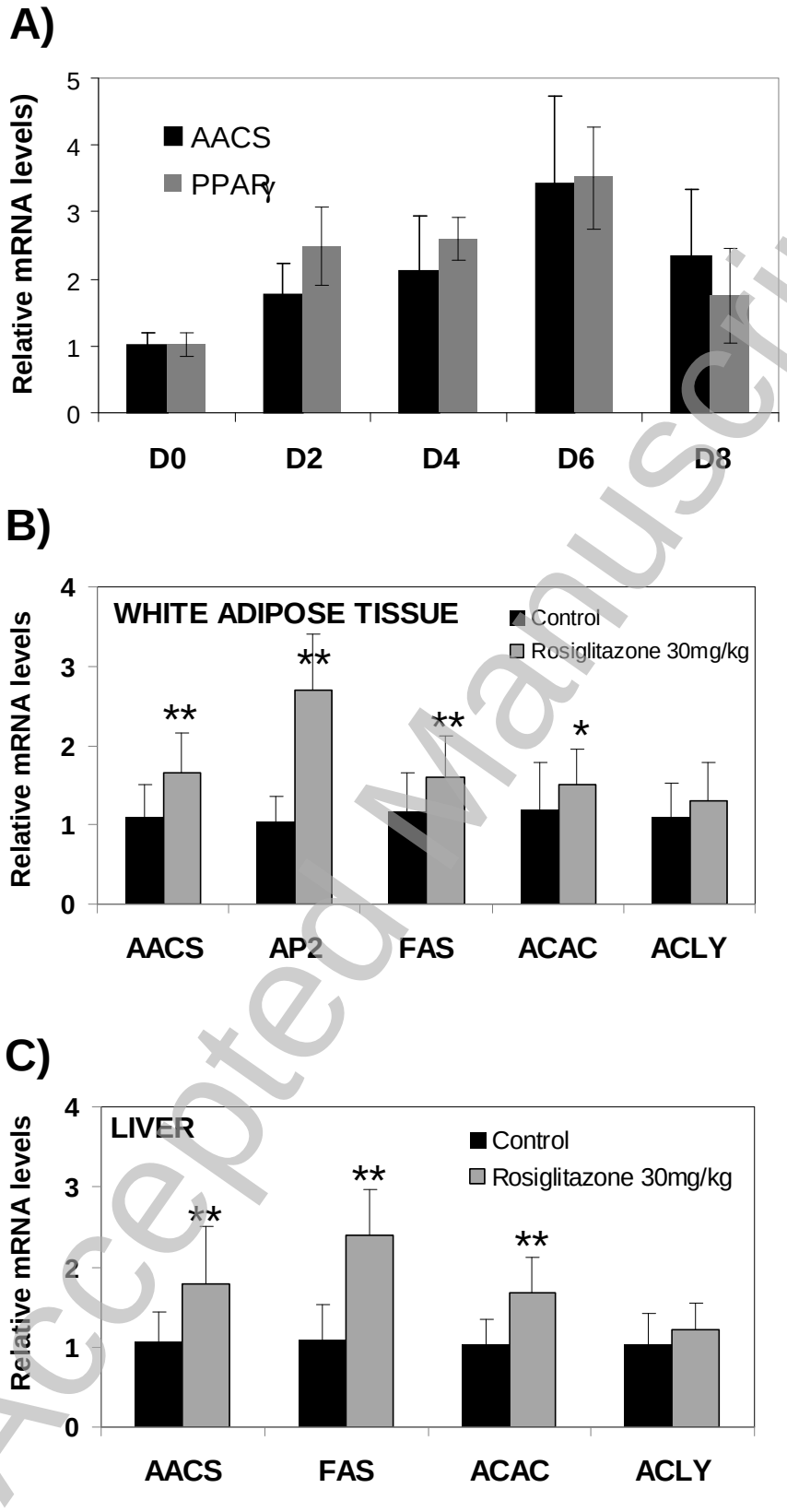

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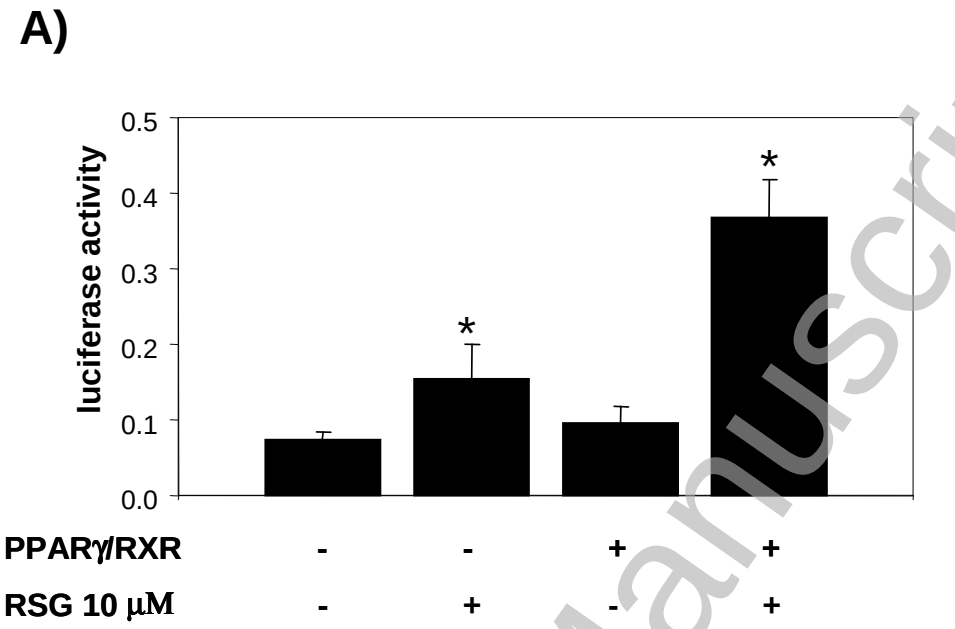
FIGURE 5



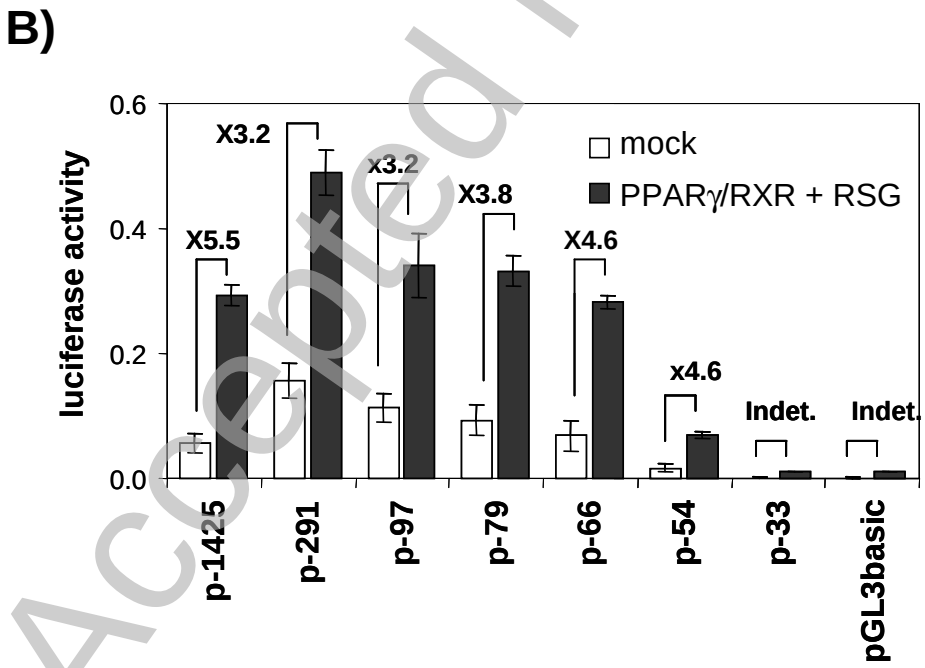


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FIGURE 5

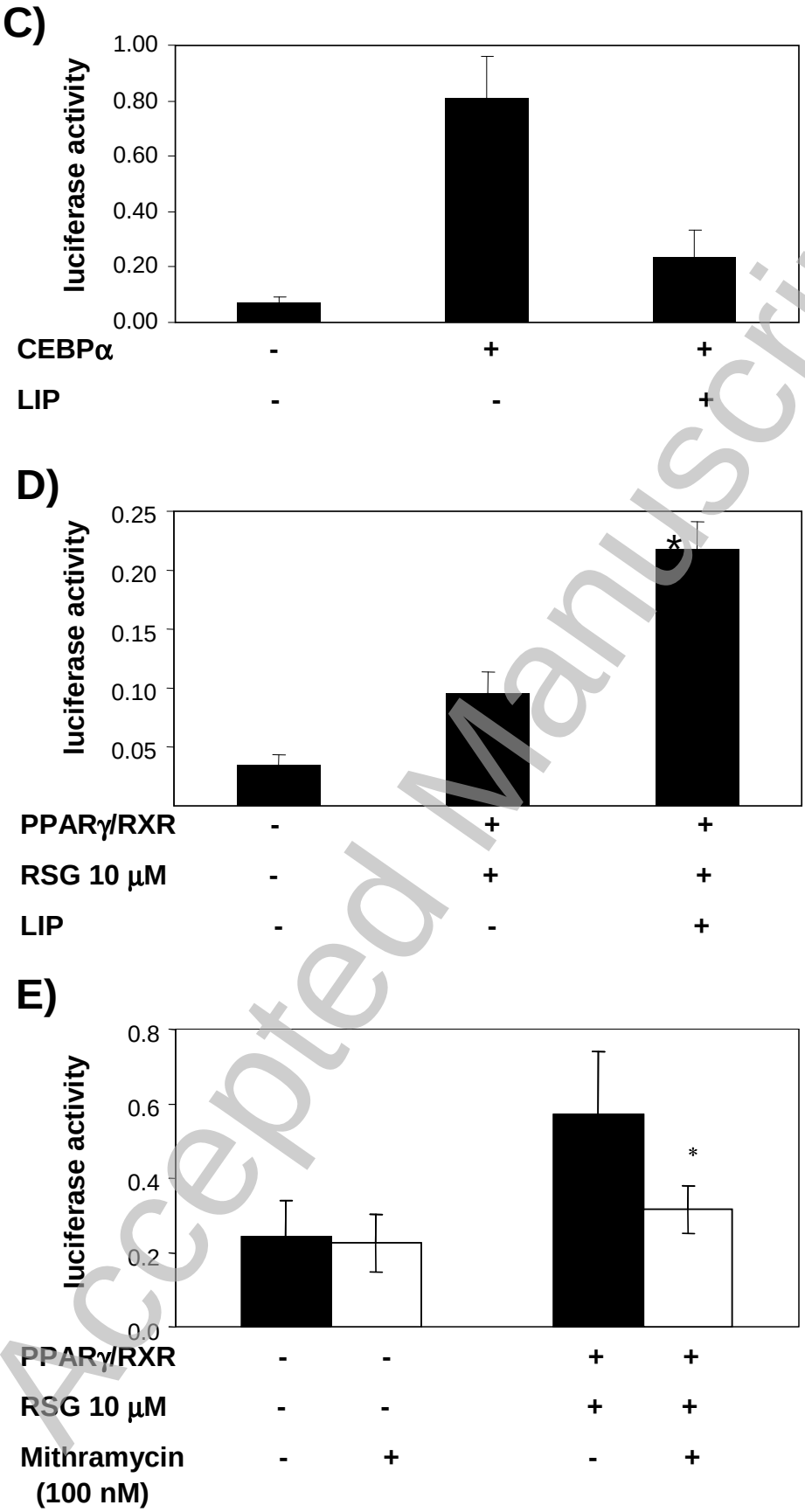

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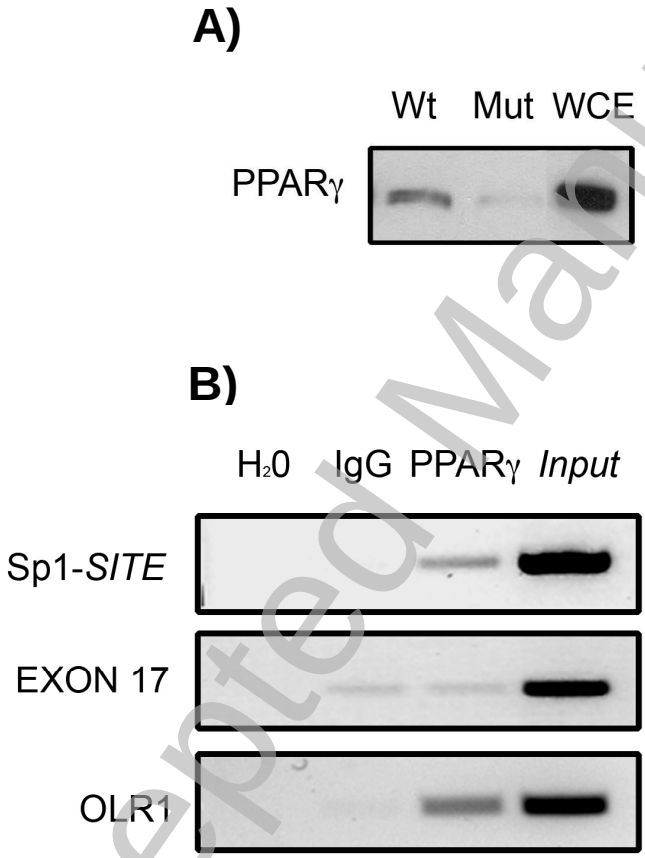
FIGURE 6



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