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Characterization of the mouse promoter region of the *acyl-CoA synthetase 4* gene: Role of Sp1 and CREB

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ABSTRACT

Acyl-CoA synthetase 4 (Acsl4) is involved in several cellular functions including steroidogenesis, synaptic development and cancer metastasis. Although the expression of Acsl4 seems to be regulated by tissue- and cell-specific factors as well as pituitary hormones and growth factors, the transcriptional mechanisms involved remain unknown. We demonstrated hCG and cAMP regulation of *Acsl4* mRNA in mouse steroidogenic MA-10 Leydig cells. We characterized the transcription initiation site and promoter of the *Acsl4* mouse gene and identified three alternative splice variants present in MA-10 cells. Sequence analysis of a 1.5-kb fragment of the *Acsl4* promoter revealed the absence of a TATA box and the presence of many putative binding sites for transcription factors including Sp1 and CREB. Functional characterization revealed that the specificity protein/Krüppel-like factor Sp1 binding site in the proximal promoter is involved in basal activity and that the cAMP response element-binding site is involved in cAMP stimulation of Acsl4 transcription.

1. Introduction

Intracellular levels of arachidonic acid (AA) are dynamically regulated in response to various physiological agonists (Khan et al., 1995). AA may act directly or may be converted into metabolites that act as potent mediators of cellular responses in a wide variety of tissues (Harizi et al., 2008). Differences in abundance and activity of AA-converting enzymes may result in variations of cellular content of eicosanoids. Therefore, and in view of potential effects exerted by AA and derivative eicosanoids, intracellular distribution and concentration of AA are all under rigorous control within cells (Maloberti et al., 2010).

Acyl-CoA synthetases (ACSs) are enzymes that catalyze the synthesis of acyl-CoA from fatty acids, ATP, and CoA, a reaction involved in mammalian fatty acid metabolism and phospholipid remodeling. ACSs can be divided into five sub-families based on the chain length of their preferred acyl groups (Soupene and Kuypers, 2008). Among the ACSs, acyl-CoA synthetase 4 (Acsl4) prefers AA as a substrate among the long-chain fatty acids. Acsl4 plays an important role in the regulation of AA cellular levels and in AA metabolism (Kang et al., 1997; Lewin et al., 2002). Acsl4 is expressed particularly in steroidogenic tissues including the adrenal gland, ovary, and testis (Kang et al., 1997; Soupene and Kuypers, 2008). *In vivo*, Acsl4 is induced by ACTH and suppressed by glucocorticoids (Cho et al., 2000). The induction of Acsl4 by steroidogenic hormones and epidermal growth factor has been demonstrated in steroidogenic cells in culture (Cano et al., 2006; Castilla et al., 2008; Cornejo Maciel et al., 2005; Maloberti et al., 2005a; Mele et al., 2012). Also, Acsl4 expression is elevated in cancer cells, and over-expression of this enzyme promotes an aggressive phenotype associated with dysregulated production of eicosanoids (Cao et al., 2001; Maloberti et al., 2010; Monaco et al., 2010; Orlando et al., 2012; Sung et al., 2003).

The structure and location of the gene encoding Acsl4 in the genome of mice and humans has been previously determined. In humans, this gene is localized in chromosome Xq22.3-q23, spans approximately 90 kb and consists of 16 exons (Cao et al., 1998; Cho et al., 2001; Minekura et al., 2001). Analysis of the 5′ flanking region revealed potential DNA elements including putative binding sites for transcription factors p300, activating enhancer binding protein 4 (AP-4), SRY, cAMP response element-binding (CREB),

Abbreviations: AA, arachidonic acid; Acot2, mitochondrial acyl-CoA thioesterase 2; ACTH, adrenocorticotrophic hormone; ACSL4, acyl-CoA synthetase 4; CRE, cAMP-responsive element; CREB, cAMP response element-binding factor; hCG, human chorionic gonadotrophin; LH, luteinizing hormone; PGE_2, prostaglandin E2; Sp/KLF, specificity protein/Krüppel-like factor.

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MyoD, and GATA family proteins (Minekura et al., 2001). One, two and three Acsl4 spliced isoforms have been identified in rats, humans and mice, respectively (Soupene and Kuypers, 2008). Mouse variants 2 and 3 encode the same short isoform with only a difference of three bases at a splice site in the first exon. This exon contains two in-frame AUGs. AUG1 is the initiator codon for the long isoform 1 and can be removed by using an alternatively spliced acceptor site present in this exon between the two AUGs. The downstream in-frame AUG2 is used for the shorter isoform. The three nucleotides, AAG, missing in variant 3, indicate the presence of a second alternatively spliced acceptor site, AAGAAG/AAA, which is located three nucleotides downstream of the site used in variant 2: AAG/AAGAAA.

It is well established that Acsl4 plays a crucial role in the regulation of AA metabolism in steroidogenic cells. AA release in mitochondria is due to the concerted action of mitochondrial acyl-CoA thioesterase 2 (Acot2) and Acsl4, since the increase or decrease of Acot2 and/or Acsl4 expression results in the concomitant modification of intramitochondrial content of AA. This, in turn, modifies Steroidogenic Acute Regulatory protein (StAR) expression and steroidogenesis (Maloberti et al., 2005b; Wang et al., 2000, 2003). The expression of *Acsl4* mRNA is regulated through a cAMP-dependent pathway in the acute and chronic phases of steroid production by adrenal and testicular Leydig cells (Cano et al., 2006; Cornejo Maciel et al., 2005; Schimmer et al., 2007). The evolutionarily preserved cAMP signaling system is an important regulator of general and cell-type-specific effects (e.g., metabolism, cell proliferation, cell death, learning and memory) (Skalhegg and Tasken, 2000), and greatly impacts on the expression of genes. For example, treatment of murine S49 lymphoma cells with a PKA-selective cAMP analog alters the expression of 4500 of 13600 unique genes including *Acsl4*, as determined by microarray analysis (Zambon et al., 2005).

Recently, several groups have reported functions of Acsl4 in different physiological/pathological processes. In human arterial smooth muscle cells, Acsl4 modulates prostaglandin E2 release (Golej et al., 2011). In *Drosophila melanogaster*, it regulates axonal transport of synaptic vesicles and is required for synaptic development and function (Liu et al., 2011). The *Acsl4* gene was found to be deleted in a family with Alport Syndrome, elliptocytosis, and mental retardation (Piccini et al., 1998). In addition, there are several reports highlighting a role for Acsl4 in carcinogenesis (Cao et al., 2001; Maloberti et al., 2010; Monaco et al., 2010; Orlando et al., 2012; Sung et al., 2003).

As mentioned above, although the regulation of Acsl4 expression is important in the regulation of several cellular processes, the transcription factors that contribute to the control and regulation of its expression have not been characterized. The overall objective of this study was to determine the factors implicated in the regulation of Acsl4 expression at the transcriptional level, using a cAMP signaling system such as steroidogenic MA-10 Leydig cells, and to characterize transcriptional regulation at the mouse promoter. Our studies succeeded in identifying the transcription initiation site of the *Acsl4* gene in mouse MA-10 Leydig cells as well as in establishing the minimal functional sequence in the promoter necessary for basal and cAMP-stimulated activity. Moreover, we demonstrated a role of cAMP in the expression of Acsl4 at the promoter level, and showed that the transcription factors Sp1 and CREB are involved in the transcriptional regulation of the gene.

2. Materials and methods

2.1. Materials

Waymouth's MB/752, Ham-F10 and 8Br-cAMP were purchased from Sigma–Aldrich (St. Louis, MO, USA). ACTH was provided by ELEA Laboratories (Buenos Aires, Argentina). Purified hCG was kindly provided by Dr. Parlow (National Hormone and Pituitary Program, National Institute of Diabetes & Digestive & Kidney Diseases (NIDDK, NIH, Bethesda, MD, USA). Gentamicin, penicillin–streptomycin, horse serum and PCR primers were purchased from Invitrogen (Carlsbad, CA, USA). Fetal bovine serum was from PAA (Austria). Anti-phospho-CREB (06-519), anti-Sp1 (07-645) and the EZ-ChIP assay kit were purchased from Millipore (Temecula, CA, USA). Polyclonal rabbit antibody anti-Acsl4 was generated in our laboratory (Castillo et al., 2004).

2.2. Cell culture and treatments

The mouse MA-10 Leydig tumor cell line was a gift from Dr. M. Ascoli (University of Iowa, Iowa City, IA, USA). MA-10 cells were cultured in Waymouth's MB/752 medium supplemented with 15% heat-inactivated horse serum and 40 μg/ml gentamicin sulfate. Treatment of MA-10 cells with 8Br-cAMP was performed in serum-depleted Waymouth's MB/752 for the indicated time points. Murine Y1 adrenocortical tumor cells, generously provided by Dr. Bernard Schimmer (University of Toronto, Toronto, Canada), were maintained in Ham-F10 medium, supplemented with 12.5% heat-inactivated horse serum and 2.5% heat-inactivated fetal bovine serum, 1.2 g/l $NaHCO_3$, 200 IU/ml penicillin, and 200 μg/ml streptomycin sulfate and maintained in a 5% CO_2 humidified atmosphere.

2.3. RNA extraction and semiquantitative RT-PCR

Total RNA was extracted using TriReagent following the manufacturer's instructions (Molecular Research Center, Inc., Cincinnati, OH, USA). Reverse transcription was performed on 2 μg of total RNA and cDNAs generated were further amplified by PCR under optimized conditions using the primer pairs listed below. Specific primers were used for amplification of both splice *Acsl4* variants (variant 1, 2 and 3 amplicon size: 419 bp). For normalization purposes we used the housekeeping gene *L19* ribosomal protein (Chan et al., 1987). Primers specific for a 405-bp segment of L19 ribosomal protein are detailed in Table 1 (Cooke et al., 2011; Eisenberg and Levanon, 2003). Reaction conditions were as follows: one cycle of 94 °C for 5 min, followed by 25 cycles of 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 60 s. The number of cycles was optimized for each gene to fall within the linear range of PCR amplification. PCR products were resolved on a 1.5% agarose gel containing 0.5 μg/ml of ethidium bromide to determine the molecular sizes of the *Acsl4* and *L19* amplicons. Gel images were acquired in digital format and levels of *Acsl4* and *L19* mRNA were quantified using a computer-assisted image analyzer (GelPro, IPS). PCR results of *Acsl4* for each sample were normalized to *L19* mRNA as internal control.

2.4. Rapid amplification of 5′ cDNA ends (5′ RACE)

MA-10 total RNA, isolated with the RNeasy kit (Qiagen), was subjected to RNA ligase-mediated 5′ rapid amplification of cDNA ends (RLM-RACE) using the GeneRacer kit (Invitrogen) according to manufacturer's instructions. Briefly, 1 μg total RNA was reverse-transcribed with Superscript RT II (Invitrogen). cDNA was subjected to two successive rounds of PCR with Accuprime *Taq* polymerase (Invitrogen) using primers R1 and R2 (Table 1) located in exon 3 of *Acsl4* mRNA. PCR products were separated by electrophoresis in 1.5% agarose containing 0.5 μg/ml ethidium bromide gels and visualized under UV. Purified amplimers were cloned in pGEMT-easy, and 20 clones from RLM-RACE were sequenced.

Table 1
Primers used in the expression and functional analysis of the *Acsl4* promoter.

Reaction	Primer	Primer sequence (5′–3′)
Semiquantitative RT-PCR	Acsl4 forward	GCCCACTTCAGACAAACCTGG
	Acsl4 reverse	ACAGCTTCTCTGCCAAGTGTGG
	L19 forward	GAAATCGCCAATGCCAACTC
	L19 reverse	TCTTAGACCTGCGAGCCTCA
Promoter cloning	Acsl4 promoter forward	ACTCGAGTATCTGTTTCTGGCATTACAACAAGG
	Acsl4 promoter reverse	AAAAGCTTAAGGTGCTGCCTCGCTCGCTA
5′RLM-RACE	R1	CGAAGTGTGTGACAGAGCGATATGGACTTCCAGGT
	R2	GTGGACAGGCAGCAATATAATGGTGAGCACATT
Site-directed mutagenesis	Sp1.2,3 forward	CCCCCGCTCGATCGGCCCCTC
	Sp1.2,3 reverse	GAGGGGCCGATCGAGCGGGGG
	Sp1.1 forward	GCCGGACCCGATCGCCGCTGG
	Sp1.1 reverse	CCAGCGGCGATCGGGTCCGGC
	CREB forward	TTTTGGCGGACTCGTCCTTAGTGAGCG
	CREB reverse	CGCTCACTAAGGACGAGTCCGCAAAA
	CREB2 forward	TTGCTGAGACTCGCGCAAAAC
	CREB2 reverse	GTTTTGCGCGAGTCTCAGCAA
	Sf1 forward	GGGAATCTTAATACAGCTAAGAGCAACAGATAAAAGGATAGAAGG
	Sf1 reverse	CCTTCTATCCTTTTATCTGTTGCTCTTAGCTGTATTAAGATTCCC
ChIP	Proximal promoter forward	CGAGGCAGTCCAAAGATAGG
	Proximal promoter reverse	AGCTGCTGCAAAGGAGGAG
	Distal promoter forward	GGGGATAGGAAGTGGAGAGG
	Distal promoter reverse	GCACAAACCTAAGCAAATGGA
EMSA	Sp1.1 forward	GCCGGACCCCGCCTCCGCTGG
	Sp1.1 reverse	CCAGCGGAGGCGGGGTCCGGC
	Sp1.2/3 forward	CCCCCGCTCCGCCCGCCCCTC
	Sp1.2/3 reverse	GAGGGGCGGGCGGAGCGGGGG
	Creb forward	TTTTGGCGGTGACGCCCTTAGTGAGCG
	Creb forward	CGCTCACTAAGGGCGTCACCGCCAAAA

2.5. Promoter cloning and generation of luciferase reporter constructs

The mouse *Acsl4* promoter was cloned using GC Rich PCR—Advantage® GC 2 Polymerase Mix & PCR Kit (Clontech, Mountain View, CA, USA). Genomic DNA was extracted from MA-10 cells using a commercial kit (Qiagen, Valencia, CA, USA). For amplification of the *Acsl4* promoter sequence, specific primers containing *Xho*I and *Hind*III restriction sites were used. For the generation of the luciferase reporter construct, the PCR product was purified and subsequently digested using *Xho*I and *Hind*III restriction enzymes, and then cloned into the corresponding restriction sites of pGL3-Basic (Promega Corp.). This plasmid was named pGL3-1553. Plasmids pGL3-1414, -997, -675, -530, -453, -264 and -23, which contain' unidirectional deletions of the promoter, were generated with the Erase-a-Base system (Promega Corp., Madison, WI) using pGL3-1553 as a template. This deletion mutagenesis uses the exonuclease III to digest insert DNA from a 5′ protruding restriction site. Reporter constructs pGL3-Sp1.1, pGL3-Sp1.2,3 and pGL3-CREB are modified versions of pGL3-264 carrying mutations in the corresponding putative transcription factor binding sites, and were generated with the megaprimer PCR mutagenesis method (Ling and Robinson, 1997) using *Pfu* polymerase from Promega. All plasmids were sequenced for verification.

2.6. Transfection and luciferase reporter assay

Cells were plated at a density of 1.2×10^5 cells/well in 24-well plates and 24 h later transfected with the different plasmids using Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions. All reporter plasmids were used in equimolar amounts, and pBluescript DNA was used as needed to keep the total amount of DNA constant. The plasmid pRL-SV40 (Promega Corp.) expressing the Renilla luciferase gene was used for normalization. Twenty-four hours after transfection, cells were washed with PBS and total lysates prepared using passive lysis buffer (Promega Corp). Samples were processed with the Dual-Luciferase Reporter system (Promega Corp.) and luciferase activity measured using an automated plate reader (Synergy HT, BioTek). Firefly luciferase activity was normalized to Renilla luciferase activity and expressed as arbitrary units.

2.7. EMSA assay

MA-10 cells cultured in T75 flasks were rinsed twice with ice-cold PBS, scraped, collected in PBS and centrifuged at 1000*g* for 5 min. The pellet was resuspended in buffer A containing protease inhibitors (10 mM HEPES pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol, 0.1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 2 mg/l leupeptin, 2 mg/l aprotinin). After centrifugation, the crude nuclear pellet was resuspended and incubated for 20–30 min in a rotator at 4 °C in buffer C (10 mM HEPES pH 7.9, 0.2 mM NaCl, 0.2 mM EDTA, 25% glycerol). After removing debris by centrifugation at 12,000*g* for 5 min, nuclear extracts were assayed directly or stored at −80 °C. The protein concentration was determined with the Bio-Rad protein assay.

Double stranded oligonucleotides covering Sp1 sites of the *Acsl4* promoter were end-labelled with [γ-^{32}P]ATP using T4 polynucleotide kinase (Promega Corp). Unincorporated ATP was removed by column purification (Quiagen). Nuclear proteins (10 μg) were incubated with binding buffer (4% glycerol, 10 mM HEPES pH 7.5, 1 mM dithiothreitol, 1 mM EDTA, 1 μg poly(dI-dC), 1 μg/μl BSA and a radiolabelled probe for 20 min at room temperature. Corresponding nuclear extracts were incubated for 20 min at room temperature with 3 μg of antibody against Sp1 previous to the probe addition. Competition studies were carried out in the presence of 50-fold molar excess unlabelled probes. DNA–protein complexes were resolved by electrophoresis in 5% non-denaturing polyacrylamide gel electrophoresis (PAGE) using 0.5 × Tris–borate–EDTA buffer. Gels were dried and subjected to autoradiography.

2.8. Chromatin immunoprecipitation (ChIP) assay

ChIP assays were performed with modifications of the manufacturer's protocol with the ChIP assay kit purchased from Millipore. Briefly, 9×10^6 MA-10 cells were plated in a T75 flask. Thirty-six hours after seeding, cells were incubated in the absence or presence of 1 mM 8Br-cAMP for 2 h. After treatment, 1% formaldehyde was added and cells were incubated for 10 min at 37 °C. Cells were washed twice with ice-cold PBS containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, and 1 μg/ml pepstatin A, pelleted, resuspended in SDS-lysis buffer, and sonicated using a Branson Sonifier 250 (VWR Scientific Company) for 7 cycles of 15-s pulses. Chromatin fragments were determined to be less than 1000 bp in size. Sheared chromatin was immunoprecipitated overnight with rotation at 4 °C with anti-phospho-CREB, anti-Sp1 or control IgG. Immunocomplexes were collected using salmon sperm/protein A-agarose slurry and subsequently washed once each with low salt, high salt, LiCl, and Tris/EDTA buffers following manufacturer's instructions. Protein–DNA complexes were eluted from protein A-agarose beads with a 1% SDS, 0.1 M $NaHCO_3$ buffer and rotation at room temperature. Formaldehyde cross-links were reversed by the addition of 200 mM NaCl and heating at 65 °C for 5 h. DNA was purified using RNAse and proteinase K treatment followed by column purification. PCR was performed using 2 μl input chromatin sample and 2 μl immunoprecipitated DNA sample with primer pairs specific for the proximal and distal region of the *Acsl4* promoter (Table 1). PCR was carried out at 95 °C for 5 min, followed by 32 cycles of 95 °C for 30 s, 59 °C for 30 s, and 72 °C for 30 s. PCR products were separated in a 2% agarose gel containing 0.5 μg/ml of ethidium bromide.

The region containing both Sp1 and CREB sites is rich in GC content. A set of primers designed to hybridize within Sp1 and CREB sites failed to amplify any genomic DNA even after several technical approaches. For that reason, we designed primers that provided the best amplification probability according to established bioinformatic tools. ChIP studies rely on amplification of DNA obtained after specific immunopreciptation. As shown in Fig. 6, the set of primers we used yielded effective PCR amplification whereas the use of a non-related antibody for immunoprecipitation resulted in no PCR amplification. We also performed an additional control using primers that recognize a sequence distal to Sp1 and CREB sites that yielded negative results, thus confirming that the specific antibody is not precipitating a non-specific DNA fragment. This also confirms that the length of DNA fragments obtained during the procedure was correct.

2.9. Western blot analysis

Total proteins (20 μg) were separated on SDS–PAGE and electro-transferred to poly(vinylidene difluoride) membranes (Bio-Rad Laboratories). Membranes were incubated with 5% fat-free powdered milk in 500 mM NaCl, 20 mM Tris–HCl pH 7.5, and 0.5% Tween 20 (TTBS) for 60 min at room temperature with gentle shaking. Membranes were then rinsed twice in TTBS and incubated overnight at 4 °C with primary antibodies (1:1000 rabbit polyclonal anti-Acsl4 and 1:5000 mouse monoclonal anti-β-tubulin). After incubation with the corresponding horseradish peroxidase-conjugated secondary antibody, immunoreactivity was detected by chemiluminescence. Immunoblots were quantified using a Gel-Pro Analyzer (IPS).

2.10. Sequence and statistical analysis

Sequence analysis was performed with the Vector NTITM Suite 8 software (InforMax, Inc.). Putative transcription-factor binding sites were identified using MatInspector version 2.2 (Genomatix, Inc.). Statistical analyses were performed with Instat version 3.0 (GraphPad, Inc.).

3. Results

3.1. Effect of hCG and cAMP on Acsl4 mRNA levels

To examine the regulation of *Acsl4* mRNA levels by the cAMP-dependent pathway, we first carried out a time-course analysis of the effects of hCG or 8Br-cAMP, the permeable analog of cAMP, on *Acsl4* mRNA levels in MA-10 Leydig cells by semiquantitative RT-PCR (Fig. 1). To determine *Acsl4* mRNA levels, we amplified a region common to all variants of *Acsl4*, resulting in a 419-bp amplicon (*Acsl4* forward and *Acsl4* reverse primers). Basal levels of *Acsl4* mRNA were detected in MA-10 untreated cells. The MA-10 cells stimulated withhCG (20 ng/ml, Fig. 1A) or 8Br-cAMP (1 mM,

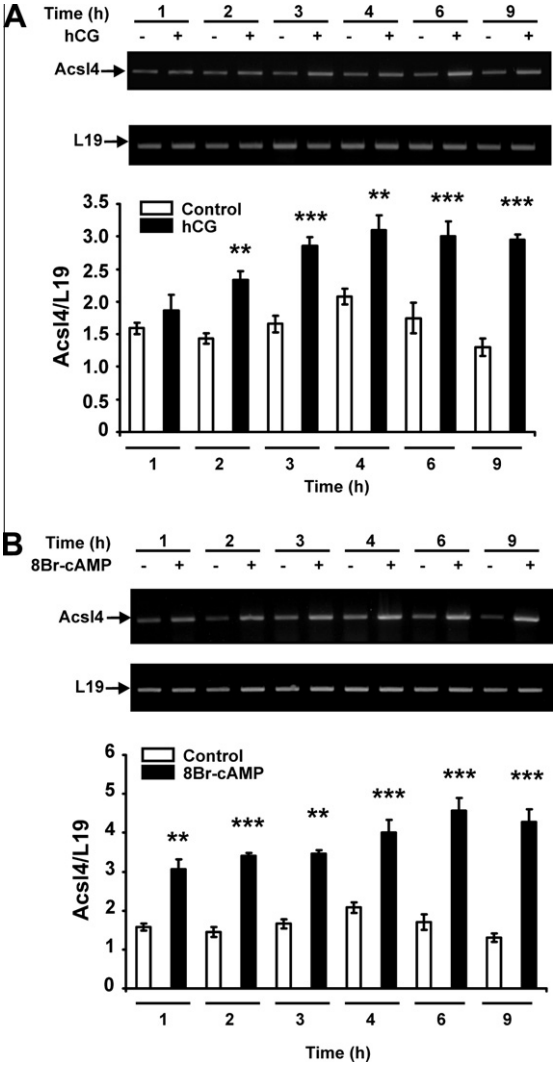

Fig. 1. Evaluation of Acsl4 mRNA levels in MA-10 Leydig cells. Total RNA was extracted from MA-10 Leydig cells following 20 ng/ml hCG (A) or 1 mM 8Br-cAMP (B) treatment for times indicated, reverse-transcribed and Acsl4 and ribosomal protein L19 (L19) mRNAs amplified by PCR. RT-PCR products were resolved in ethidium bromide-stained agarose gels, specific bands detected by transillumination and quantified by densitometry. The figure shows representative images of four independent experiments and the corresponding quantification of the integrated optical density of Acsl4 normalized against L19 mRNA bands. Data represent the mean ± SEM arbitrary units. $^{**}P < 0.01$; $^{***}P < 0.001$ *vs* non-treated cells.

Fig. 1B) showed a time-dependent increase in levels of *Acsl4* mRNA. Stimulation with 8Br-cAMP yielded a higher increase of mRNA levels compared to hCG.

3.2. Identification of transcription initiation site(s)

RLM-RACE was used to map the 5′ end of the mouse *Acsl4* gene in MA-10 Leydig cells. Antisense primer R1 and nested primer R2 (Table 1), complementary to the sequence of exon 3, were used for two successive rounds of PCR (Fig. 2). This amplification generated a very prominent band of 340 bp and a minor band of 600 bp which corresponds approximately to the alternative splice variants of *Acsl4* mRNA (Fig. 2A). This result suggests that the mRNA expressed in these cells is either variant 2 or 3. The two PCR products were subcloned into the pGEMT-easy vector and isolated, and 20 positive colonies from RLM-RACE products were sequenced. This analysis demonstrated the presence of variant 3 in MA-10 cells but in a low proportion compared to variant 2. Initiation sites cor-

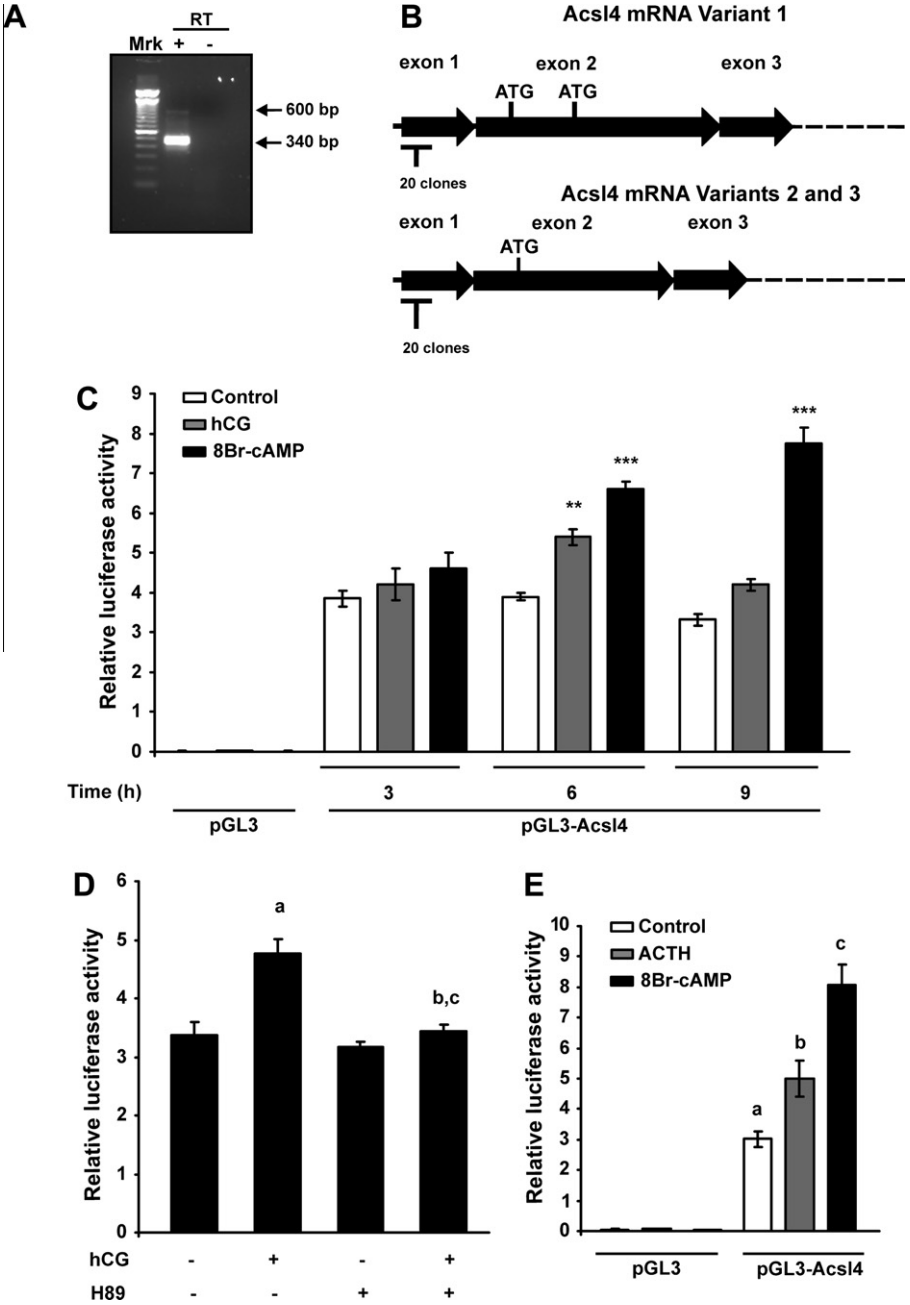

Fig. 2. Mapping of the transcription initiation site using RLM-5́ RACE. (A) Total RNA, extracted from MA-10 Leydig cells, was subjected to RLM-5́RACE as described in Section 2. Amplification products were resolved in ethidium bromide-stained agarose gels and specific bands detected by transillumination. Mrk: molecular weight markers. Numbers on the right indicate expected molecular size in bp. (B) Schematic representation of initiation sites of both Acsl4 mRNA variants obtained from the clones from RLM-5′ RACE. (C) MA-10 Leydig were transiently-transfected with a construct containing a 1.7-kb fragment of the mouse *Acsl4* promoter and the luciferase gene (pGL3-Acsl4) or the empty vector (pGL3) treated with either 20 ng/ml hCG or 1 mM 8Br-cAMP for the times indicated and luciferase activity measured by luminescence. $^{**}P < 0.01$; $^{***}P < 0.001$ *vs* non-treated cells. (D) MA-10 Leydig were transiently-transfected with a construct containing a 1.7-kb fragment of the mouse *Acsl4* promoter and the luciferase gene (pGL3-Acsl4) Transfected cells were stimulated for 6 h with 20 ng/ml hCG or its vehicle following a 30 min preincubation with 20 μM H89. a, $P < 0.05$ *vs* non-treated cells; b, $P < 0.01$ *vs* non-treatred cells. (E) transfected Y1 cells as described in Panel C were stimulated for 6 h with either 20 mIU/ml ACTH or 1 M 8Br-cAMP. Results represent luciferase activity and are expressed as the mean ± SEM firefly/*Renilla* luciferase activity arbitrary units of six independent experiments. a, $P < 0.001$ *vs* pGL3; b, $P < 0.001$ *vs* pGL3-Acsl4 with no treatment; c, $P < 0.001$ *vs* pGL3-Acsl4 with no treatment.

responding to transcript variants 1, 2, and 3 are located in the region of exon 1 (Fig. 2B), arguing that these variants are regulated by the same promoter region and excluding the possibility that an alternative promoter sequence is present. Sequence analysis demonstrated several initiation sites in a region of 100 bp. Alternative splicing determines that the initiation site is located between bases 220 and 118 from the ATG codon of the mRNA in variant 2 and between bases 387 and 331 from the ATG codon in variant 1.

3.3. Cloning and sequence analysis of the mouse Acsl4 promoter

A ~1.7-kb promoter fragment containing exon 1 but not the exon 1-intron 1 junction was amplified from mouse genomic DNA, cloned into the luciferase reporter vector pGL3-Basic. The functional role of the mouse *Acsl4* promoter in the regulation of transcription was assessed by its ability to drive expression of the firefly luciferase gene. MA-10 cells were transiently transfected and 48 h after transfection, cells were treated with either 20 ng/ml hCG or 1 mM 8Br-cAMP for 3, 6 and 9 h, and luciferase activity determined as a readout of transcriptional activity. Transfection of plasmid pGL3-1553 resulted in consistently higher activity (70- to 80-fold) than that of the promoterless plasmid pGL3-Basic (Fig. 2C). Whereas *Acsl4* promoter activity was not changed after 3 h of stimulation, there was a significant increase in luciferase activity 6–9 h after treatment with either hCG or cAMP, ($P < 0.001$). Maximum induction occurred at 6 h for hCG and at 9 h for 8Br-cAMP. This difference may relate to the sustained effect of the non-hydrolysable cAMP analog. Notably, this result agrees

Fig. 3. Nucleotide sequence of the mouse *Acsl4* promoter and of part of exon 1. Acsl4 transcription initiation site is indicated by an *arrow*. The sequence of exon 1 is shown in *bold*. Putative transcription factor binding sites are *boxed*.

with the induction of *Acsl4* mRNA levels by cAMP or hCG in MA-10 cells (Fig. 1), an indication that activation of the cAMP-dependent pathway exerts an action at the transcriptional level. Treatment of MA-10 cells with the cAMP-dependent protein kinase (PKA) inhibitor H89 abolished the effect of hCG on *Acsl4* promoter activity (Fig. 2D). We also analyzed promoter activity in another steroidogenic model: Y1 adrenocortical cells. In this cell line we observed increased activity of the *Acsl4* promoter upon stimulation with ACTH (20 mUI/ml). This effect is considerably greater upon 8Br-cAMP (1 mM) stimulation (Fig. 2E). This finding is in line with previous observations indicating that ACTH induces Acsl4 expression in a cAMP-dependent fashion (Cornejo Maciel et al., 2005).

Analysis to identify regulatory elements in the Acsl4 promoter region was performed by the MatInspector program (http://www.genomatrix.com) with a cutoff value over 90%. This computer analysis revealed the absence of a TATA box close to the transcription initiation site. In addition, we identified several binding sites for the Sp1 transcription factor: one site located in position −64, and two overlapped sites at −112 and GC-rich sequences near the initiation site. Analysis with EMBOSS CpGPlot/CpGReport/Isochore revealed one CpG island region between −411 to +89. We also found that the promoter region contains sites for GATA, Elk-1, calcium phospholipid-binding protein (CPBP), Krüppel-like factor 3 (KLF3), CREB, AP-4, and steroidogenic factor 1 (SF1). It has been demonstrated that GATA, SF1, and CREB are regulators of the expression of proteins involved in steroidogenesis (Ozisik et al., 2003; Rosenberg et al., 2002; Tremblay and Viger, 2003). Relevant for our studies, the presence of a consensus site for the CREB factor in *Acsl4* promoter is consistent with the fact that levels of Acsl4 enzyme are regulated by cAMP in steroidogenic tissues (Cano et al., 2006; Cornejo Maciel et al., 2005; Cho et al., 2000). In addition to the consensus sites mentioned above, the promoter sequence has consensus sites for transcription factors related to many other functions (nuclear respiratory factor 1, Myc-associated zinc finger protein, Wilms tumor suppressor, Sp/basic Krüppel-like factor 3) (Fig. 3).

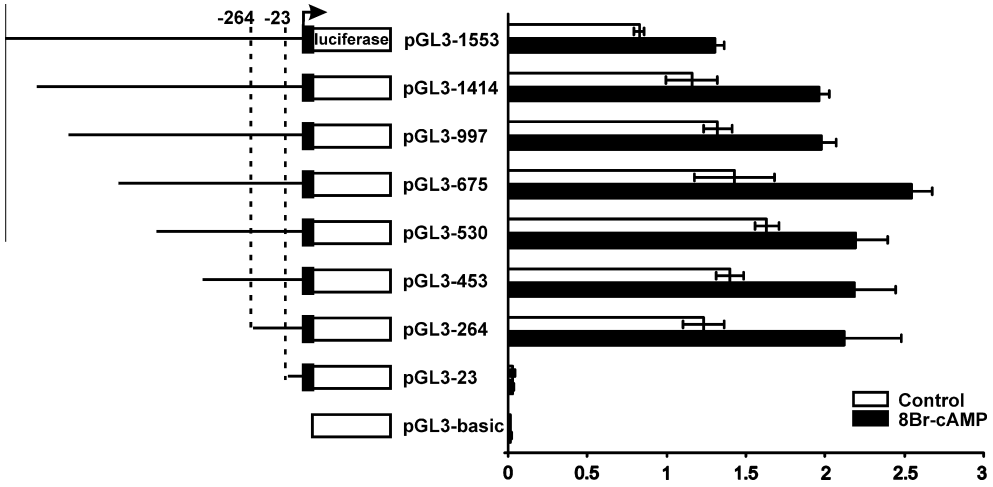

Fig. 4. Functional characterization of the mouse *Acsl4* promoter. A series of plasmids containing 5′ unidirectional deletions of the promoter region of the Acsl4 gene (pGL3-1553, -1414, -997, -675, -530, -301, -453, -264 and pGL3-23 and PGL3) fused in frame to the luciferase gene were transfected into MA-10 cells. Cells were allowed to recover for 48 h and luciferase activity was then determined by luminescence following 6 h stimulation with 1 mM cAMP. Firefly luciferase activity was normalized against Renilla luciferase activity and results are expressed as the mean ± SD arbitrary units of three independent experiments. Positions −264 and −23 bp of the promoter are also shown.

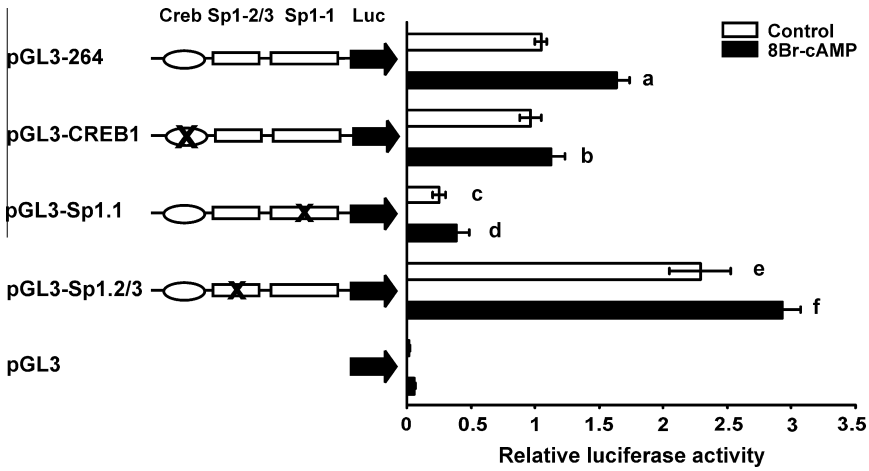

Fig. 5. Analysis of Sp1 and CREB binding sites by site-directed mutagenesis. A series of mutants were generated by site-directed mutagenesis of the Sp1.1 (pGL3-Sp1.1) and Sp1.2/3 (pGL3-Sp1.2/3) and of CREB (pGL3-CREB) binding sites present in the pGL3-264 construct of the *Acsl4* promoter. The different constructs were transiently-transfected into MA-10 cells by lipofection. Cells were allowed to recover for 48 h, stimulated for 6 h with 1 mM cAMP and luciferase activity was measured by luminescence. Results are expressed as the mean ± SEM arbitrary units of Firefly luciferase activity normalized against Renilla luciferase activity of three independent experiments. a, $P < 0.01$ pGL3-264 *vs* pGL3-264 + 8Br-cAMP; b, $P < 0.05$ pGL3-CREB + 8Br-cAMP *vs* pGL3-264 + 8Br-cAMP; c, $P < 0.001$ pGL3-Sp1.1 *vs* pGL3-264; d, $P < 0.001$ pGL3-Sp1.1 + 8Br-cAMP *vs* pGL3-264 + 8Br-cAMP; e, $P < 0.001$ pGL3-Sp1.2/3 *vs* pGL3-264; f, $P < 0.05$ pGL3-Sp1.2/3 + 8Br-cAMP *vs* pGL3-Sp1.2/3; g, $P < 0.001$ pGL3-Sp1.2/3 + 8Br-cAMP *vs* pGL3-264.

Alignment of the mouse sequence with the human promoter revealed approximately 85% homology in the first 230 bp and approximately 71% homology in the total cloned sequence, with many of the sites for transcription factors preserved between the mice and humans (Minekura et al., 2001).

3.4. Functional characterization of the mouse Acsl4 promoter

To measure the activity of potential cis-acting elements and determine the minimum sequence required for activity, a series of eight reporter constructs with progressively larger deletions from the 5′ end of the promoter were generated. The effects of these modifications were evaluated upon transfection of the corresponding luciferase reporter plasmids into MA-10 Leydig cells (Fig. 4). These studies revealed that deletion of the sequence from −1553 to −530 bp increased basal promoter activity approximately twofold ($P < 0.01$), suggesting that this region may contain a negative regulatory element. A further deletion to −264 bp resulted in an approximately 30% loss of basal promoter activity. Interestingly, deletion between −264 and −23 bp abolished transcriptional activity, indicating that this fragment contains elements that positively regulate the basal expression of the gene. In all the reporter constructs except that with deletion to −23 bp, 8Br-cAMP significantly increased transcriptional activity relative to non-treated cells.

3.5. Relevance of Sp1 and CREB binding sites in the proximal promoter of Acsl4

Functional characterization of the promoter indicated that the sequence extending to −264 bp is responsible for most of the transcriptional activity. As mentioned above, one CREB and three Sp1 transcription factor binding sites are present in this region. Putative Sp1 binding sites 2 and 3 partially overlap, and for the purpose of this study were treated as a single site designated Sp1.2/3. Mutations were introduced into each of these three putative binding sites to determine their relevance in the pGL3-264 construct. The basal activity of the pGL3-264 construct was increased by the treatment of cells with 8Br-cAMP (Fig. 5). We observed that mutation of the putative CREB site only affected cAMP-stimulated activity of the *Acsl4* promoter, whereas mutations of the putative regulatory element Sp1.1 abolished both basal and cAMP-stimu-

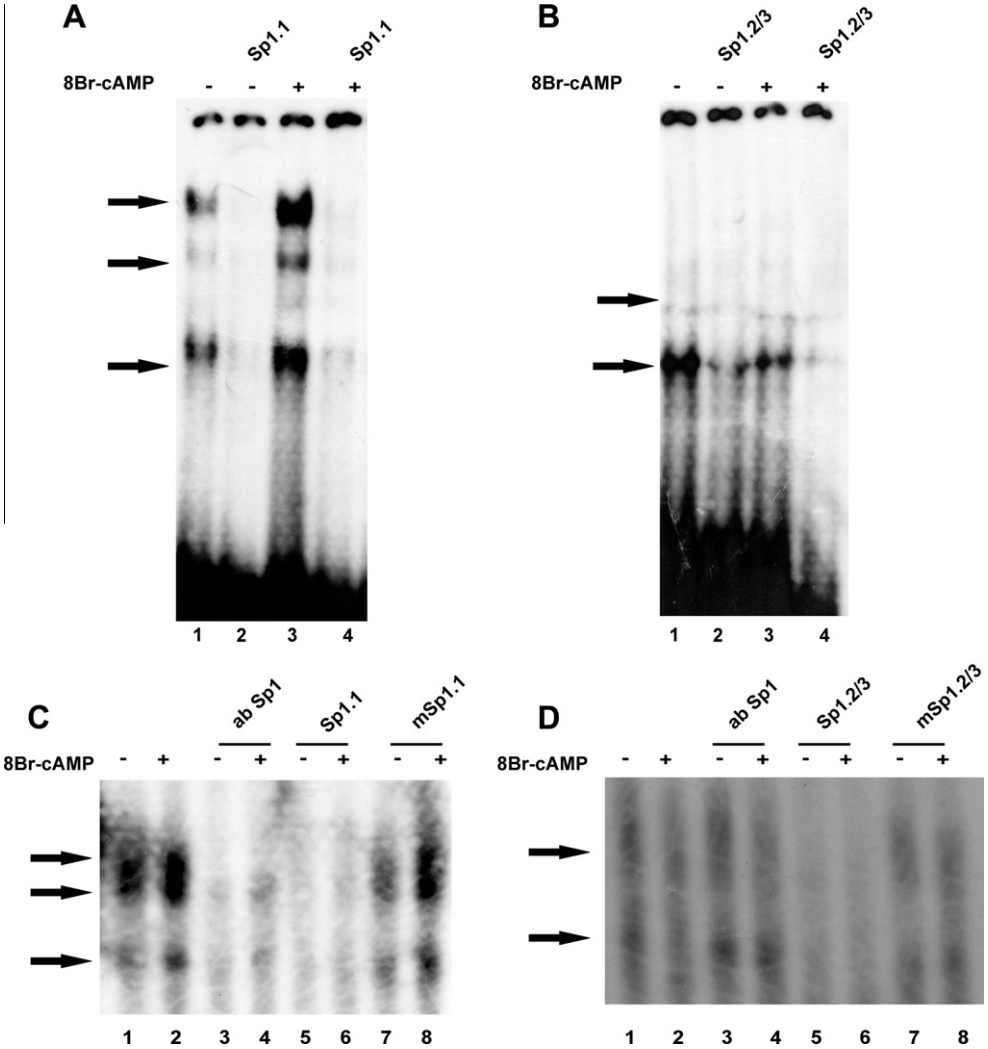

Fig. 6. Analysis of Sp1 binding by EMSA. MA-10 cells were treated for 3 h with 1 mM 8Br-cAMP or its vehicle, homogenized and nuclear extracts (10 μg protein) incubated with ^{32}P end-labelled oligonucleotides (2×10^4 cpm) spanning the Sp1.1 and Sp1.2/3 sites of the Acsl4 gene promoter (sequence shown in Table 1) for 20 min at room temperature. Aliquots were loaded onto 5% acrylamide, 0.5 × TBE gels and electrophoresed at 200 V for 2 h. Specific competition was done by incubation of the EMSA reaction with the unlabelled oligonucleotide probe (Sp1.1 and Sp1.2/3) or with an unlabelled probe containing a mutation of the Sp1 binding site (mSp1.1 and mSp1.2/3) as indicated. For supershift studies of the complexes formed during EMSA, nuclear extracts were preincubated with 3 μg of an anti-Sp1 (ab Sp1) antibody and further incubated for 20 min at room temperature with the ^{32}P-labelled oligonucleotide probe.

lated activity of the promoter. On the other hand, mutation of Sp1.2/3 site resulted in a significant increase in activity compared to pGL3-264 (150%). A second putative CREB site was identified approximately −650 bp, as depicted in Fig. 3. Mutation of the site located at ∼−200 bp abolished the effect of the cAMP analog on the promoter, whereas mutation of the site located at ∼−650 bp did not change cAMP-stimulated promoter activity. Taken together, these results demonstrate the involvement of the ∼−200 bp CREB site in regulation of *Acsl4* promoter activity.

3.6. Analysis of the association between Sp1 and CREB with the proximal promoter of Acsl4

To further investigate the relevance of the Sp1 in the *Acsl4* promoter we used EMSA. As shown in Fig. 6, a probe containing the Sp1.1 motif located at −64 bp formed three prominent complexes (Fig. 6A and C, lane 1) that were competed by an unlabelled probe (Fig. 6A, lane 2 and B, lane 5). Similar results were obtained with extracts of cAMP-treated cells (Fig. 6A, lanes 3 and 4 and C lanes 2 and 6). When the EMSA was performed using a probe containing the Sp1.2/3 motif, located at −112 bp, even though we observed complexes formation, the EMSA analysis for this site showed much weaker signals compared with the Sp1.1 site (Fig. 6B and D). These complexes were specific as they were competed by an unlabelled probe (Fig. 6B, lanes 2 and 4 and D, lanes 5 and 6). Similar results were observed with extracts of cAMP-treated cells (Fig. 6B, lanes 3 and 4; and D, lanes 2 and 6).

Preincubation of the nuclear extracts with a polyclonal antiserum specific for Sp1 blunted complex formation for Sp1.1 (Fig. 6C, lanes 3 and 4). On the other hand, analysis of the Sp1.2/3 site showed that there was no significant inhibition in the complex formation by the anti-Sp1 antibody (Fig. 6D, lanes 3 and 4).

To further analyze the effect of 8Br-cAMP treatment on CREB and SP1 in more physiological conditions, ChIP assays were performed. Cross-linked sheared chromatin from MA-10 cells treated with 8Br-cAMP for 2 h was immunoprecipitated with anti-phospho-CREB or anti-Sp1 antibodies and the DNA recovered was subjected to PCR using primers specific to the proximal region of the *Acsl4* promoter (Fig. 7A). A PCR product specific to the *Acsl4* proximal promoter was amplified from both anti-phospho-CREB- (Fig. 7B) and anti-Sp1-immunoprecipitated (Fig. 7C) DNA samples. In contrast, little or no amplification could be observed with DNA recovered using control IgG for immunoprecipitation (Fig. 7B and C) or when primers for the *Acsl4* distal promoter region were used (Fig. 7A). These data strongly argue for the specific association of CREB and Sp1 to the proximal region of the *Acsl4* promoter. Whereas Sp1 association was modified only slightly or not at all by 8Br-cAMP treatment, phospho-CREB association with the *Acsl4* promoter increased following treatment of MA-10 cells with 8Br-cAMP.

3.7. Effect of CREB over-expression and of Sp1 silencing on 8Br-cAMP-stimulated Acsl4 promoter activity and protein levels in MA-10 cells

To further explore the role of CREB on Acsl4 expression, we overexpressed either wild-type or an inactive form of CREB containing a point mutation at Ser133 (mCREB), and analyzed Acsl4 protein expression and promoter activity (Fig. 8A and C). Notably, over-expression of wild-type CREB caused increases both in cAMP-induced Acsl4 protein expression and promoter activity. On the other hand, mCREB impaired the effect of cAMP both on protein and *Acsl4* promoter activity.

Lastly, the involvement of Sp1 in the regulation of Acsl4 expression was studied using RNAi. Fig. 8E shows that Sp1 expression was significantly reduced when cells were transfected with a siRNA plasmid for Sp1. Interestingly, Sp1 silencing by siRNA significantly reduced basal and cAMP-stimulated Acsl4 protein expression and luciferase promoter activity (Fig. 8B and D, respectively), therefore suggesting that Sp1 plays an important role in controlling the expression of this promoter.

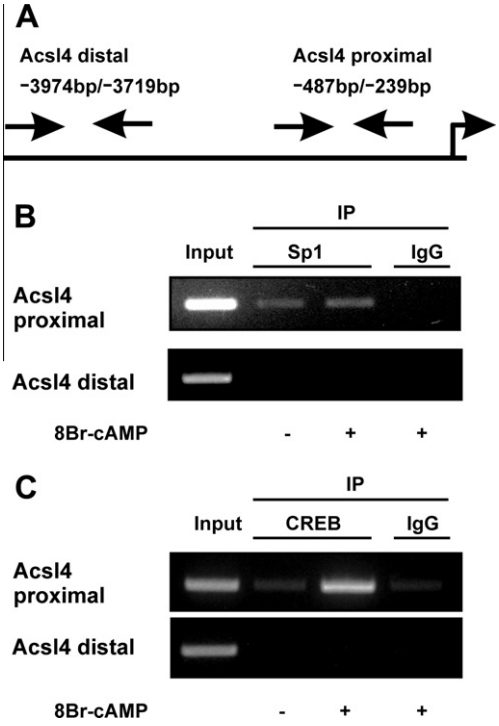

Fig. 7. CREB and Sp1 association with the proximal region of the *Acsl4* promoter. MA-10 cells were treated in the absence or presence of 1 mM 8Br-cAMP for 2 h and a ChIP assay was run. Cross-linked sheared chromatin was immunoprecipitated with control IgG, anti-phospho-CREB, or anti-Sp1 antibodies. Recovered chromatin was subjected to PCR analysis using primers specific for the proximal and distal *Acsl4* promoter regions. (A) A schematic representation of the *Acsl4* promoter with the locations of the primer pairs indicated. (B and C) Ethidium bromide-stained gels of PCR products. Primers for the proximal and distal regions were expected to amplify 249-bp and 255-bp fragments, respectively. A representative ChIP analysis from three independent experiments is shown.

4. Discussion

We report herein the regulation of *Acsl4* mRNA in MA-10 cells by hCG and cAMP determined by semiquantitative RT-PCR. We also show the presence of alternative splice variants 1, 2, and 3 of *Acsl4* mRNAs in MA-10 mouse Leydig cells. The results of RLM-RACE demonstrated that the shortest variant (variant 2) of the *Acsl4* mRNA is the predominant isoform in MA-10 cells. Low levels of variant 1 were evidenced by nested PCR in the RLM-RACE (Fig. 2). This tallies with the fact that this longer variant has been reported to be specific to brain (Piccini et al., 1998). To better understand the regulation of Acsl4 at the transcriptional level, we cloned and functionally characterized the promoter of the *Acsl4* gene. The activity of the cloned promoter was regulated by cAMP or hormone treatment in MA-10 Leydig cells and in the Y1 adrenocortical steroidogenic cells. In this study we performed an comprehensive *in silico* analysis of the 5′ region of the mouse genomic sequence and established the absence of canonical TATA box or CAAT-like sequences and a region rich in GC content. Several potential transcription factor binding motifs including those for Sp1, GATA, Sf1 and CREB were identified in the promoter region. Although the Acsl4 promoter region contains a putative SF-1 and several putative GATA sites, the minimal fragment with promoter activity contains no SF-1 or GATA binding sites. Correspondingly,

deletion or mutation of the SF-1 site within the *Acsl4* promoter did not significantly affect promoter activity (data not shown).

A deletional analysis of the *Acsl4* promoter revealed increased basal transcriptional activity when the sequence between −1553 and −675 bp was deleted, suggesting that this promoter region contains a negative regulator. A similar result has been previously reported for the human Acsl4 promoter (Minekura et al., 2001). Comparative analysis of the mouse and human sequence revealed that this deleted sequence contains a region of 250 bp with high identity. This comparative sequence analysis also demonstrated that the *Acsl4* promoter is highly preserved within the first 230 bp (85% homology). This finding supports the idea that the first 264 bp of the promoter are primarily responsible for most of the transcriptional activity observed in MA-10 cells.

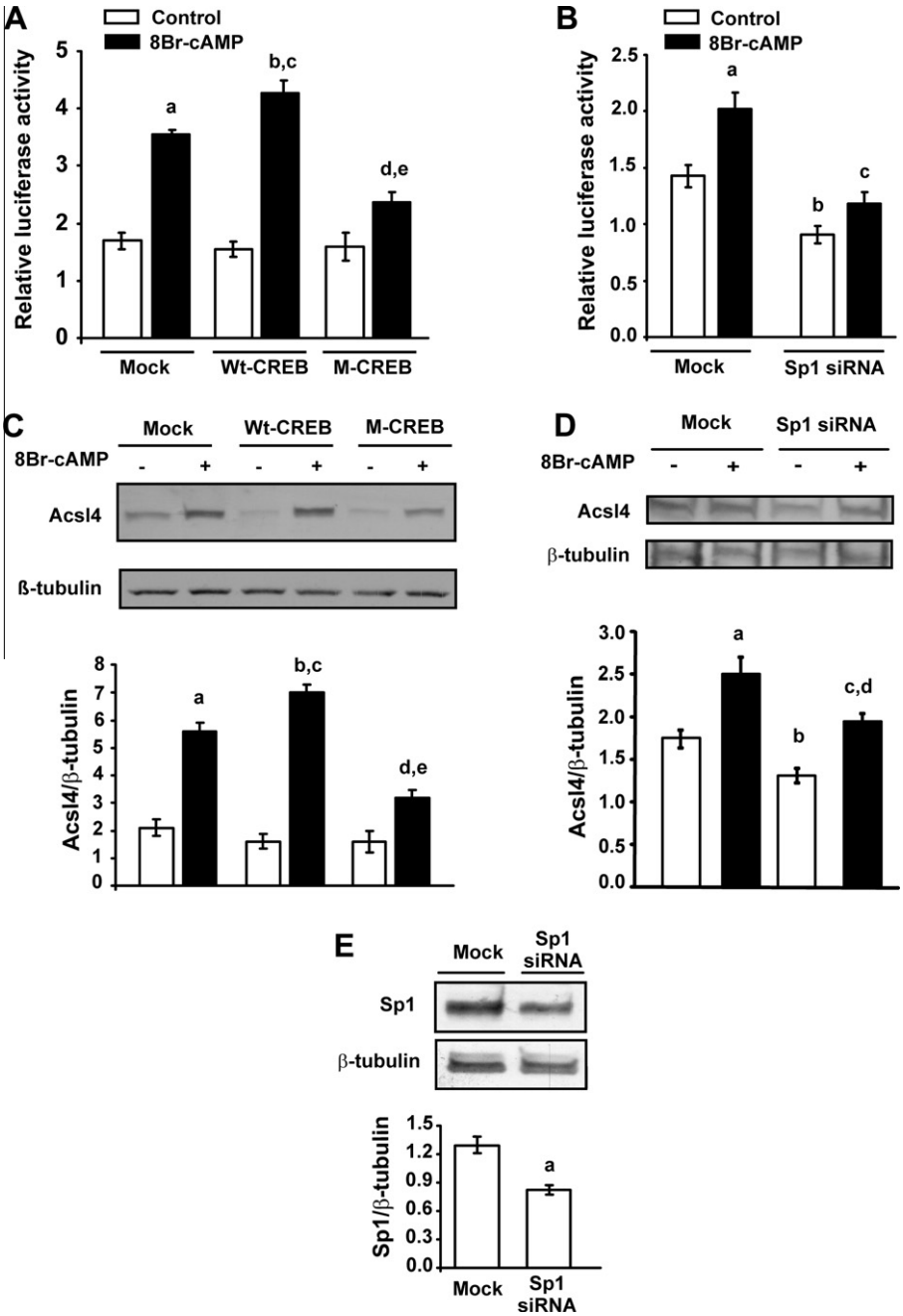

Fig. 8. Effect of expression of WT-CREB and mCREB on 8Br-cAMP-stimulated Acsl4 promoter and Acsl4 protein expression in MA-10 cells. Cells were transiently-transfected with either wild-type (Wt-CREB), mutant CREB (M-CREB) or empty vector (Mock) (A and C) or with an Sp1 siRNA expression plasmid (Sp1 siRNA) or with empty plasmid (Mock) (B, D, and E) as described in Section 2 and allowed to recover for 48 h. Cells were then stimulated for 6 h with 0.5 mM 8Br-cAMP (8Br-cAMP) or its vehicle (Control) and luciferase activity measured by luminescence (A and B) or Acsl4 (C and D) and Sp1 (E) expression analyzed by Western blot and quantified by densitometry. Results represent relative luciferase activity (A and B) and are expressed as the mean ± SEM arbitrary units of Firefly/Renilla luciferase activity of three independent experiments. (A) a, $P < 0.001$ *vs* Mock without treatment; b, $P < 0.001$ *vs* Wt-CREB without treatment; c, $P < 0.05$ *vs* Mock + 8Br-cAMP; d, $P < 0.05$ *vs* M-CREB without treatment; e, $P < 0.001$ *vs* Mock + 8Br-cAMP. (B) a, $P < 0.01$ *vs* Mock without treatment; b, $P < 0.05$ *vs* Mock without treatment; c, $P < 0.01$ *vs* Mock + 8Br-cAMP. (C–E) Figures show representative Western blots and integrated optical density of Acsl4 (C and D) or Sp1 (E) normalized against β-tubulin expression. Values represent the mean ± SEM arbitrary units of three independent experiments. (C) a, $P < 0.001$ *vs* Mock without treatment; b, $P < 0.001$ *vs* Wt-CREB without treatment; c, $P < 0.01$ *vs* Mock + 8Br-cAMP; d, $P < 0.01$ *vs* M-CREB without treatment; e, $P < 0.001$ *vs* Mock + 8Br-cAMP. (D) a, $P < 0.01$ *vs* Mock without treatment; b, $P < 0.05$ *vs* Mock without treatment; c, $P < 0.05$ *vs* Sp1 siRNA; d, $P < 0.05$ *vs* Mock + 8Br-cAMP. (E) a, $P < 0.001$ *vs* Mock without treatment.

Characterization of the promoter sequence involved the identification of transcription initiation sites. Our data indicate that transcription initiates at a number of sites at the 5′ end of the gene, making exon 1 variable in length. This type of behavior is usually exhibited in genes lacking TATA and CCAAT boxes within the proximal promoter, as is the case of *Acsl4* (Valen and Sandelin, 2011). Recent bioinformatic studies have revealed that most mammalian genes do not conform to the simple model in which a TATA box directs transcription from a single defined nucleotide position. Many promoters have an array of closely located transcriptional initiation sites spread over 50–100 bp (Valen and Sandelin, 2011). Approximately 72% of human promoters have CpG islands. These genes often contain the sequence PyPyrA(+1)NT/APyPy, termed an initiator (Inr) sequence, spanning the transcription initiation site (Valen and Sandelin, 2011). In the case of *Acsl4*, the sequence spanning the most frequently used transcription initiation site resembles an Inr sequence without the A in the +1 position. The transcription initiation site of promoters associated with CpG islands may coincide with sequences exhibiting weak homology to the Inr consensus or may be unrelated to this sequence (Smale and Kadonaga, 2003). The transcription initiation site in the human promoter was assigned to a G nucleotide located 210 bp upstream of the initiator methionine codon, which is approximately in the same region as in the mouse promoter and downstream conserved Sp1 sites (Minekura et al., 2001).

Analysis of a region comprising positions −1553 to +156 revealed that the minimal active promoter sequence could be localized to the segment −264 to −23, which contains consensus motifs for transcription factors Sp1 and CREB. Sp1 is of particular interest because it has been reported to participate in the formation of transcription initiation complexes (Bouwman and Philipsen, 2002). Sp1 is a GC box-binding activator of transcription found in many promoters and enhancers. We found that mutation of the Sp1.1 site markedly reduced promoter activity. Since the *Acsl4* promoter lacks a classical TATA box but shows a relatively high level of basal promoter activity, our results strongly indicate a role for Sp1 in the formation of the transcription initiation complex of the *Acsl4* gene. In support of the involvement of Sp1, we demonstrated by EMSA and ChIP assay that this transcription factor was able to bind sequences in the proximal promoter of *Acsl4* in MA-10 cells.

We also observed that mutation of Sp1.2/3 site resulted in an increase in basal and cAMP-stimulated promoter activity, suggesting that this Sp1 site could have an inhibitory function. The EMSA analysis revelead that Sp1 does not bind with high affinity to this sequence. The Sp family of transcription factors consists of at least four members that share a highly preserved DNA-binding domain composed of three zinc finger motifs (Bouwman and Philipsen, 2002). Sp1 (the prototype of the family) and Sp3 are ubiquitously expressed and bind virtually identical DNA sequences (GC boxes) with comparable affinity. Sp1 acts as a transcriptional activator for a large number of genes including structural proteins, metabolic enzymes, cell cycle regulators, transcription factors, and growth factors (Bouwman and Philipsen, 2002). Although Sp1 is an activator, Sp3 can function either as an activator or a repressor (Bouwman and Philipsen, 2002). It has been reported that in promoters containing multiple adjacent binding sites Sp3 may repress transcription driven by Sp1 or other transcription factors (Bouwman and Philipsen, 2002; Yu et al., 2003). We hypothesize that this could be the case for the *Acsl4* promoter, although additional studies would be required to address this possibility.

In the gonads and adrenal gland, trophic hormones stimulate steroid synthesis predominantly through the cAMP-dependent pathway. We previously reported that an elevation in intracellular cAMP levels is accompanied by rapid inductions in *Acsl4* mRNA and protein levels (Cano et al., 2006; Cooke et al., 2011; Cornejo Maciel et al., 2005). Our study demonstrates that the increase of mRNA *Acsl4* levels by cAMP during the acute phase of steroidogenesis is due, at least in part, to transcriptional activation. Transactivation of target genes by the cAMP-PKA pathway involves the binding of cAMP response element-binding protein (CREB) family members to consensus cAMP response elements (CREs) within the promoter. In the canonical cAMP-PKA signaling pathway, activated PKA phosphorylates CREB in Ser133 leading to increased transcription (De Cesare and Sassone-Corsi, 2000). Consistent with the regulation of Acsl4 expression by cAMP, here we report that the proximal promoter sequence activated by cAMP contains a CREB site. Moreover, site-directed mutagenesis of this site impaired cAMP activation of transcription without affecting basal activity. Over-expression of wild-type CREB augments 8Br-cAMP-induced Acsl4 expression, whereas an inactive form of CREB (with a point mutation at Ser133) impaired induction of Acsl4 expression by 8Br-cAMP. The phospho-CREB association with the *Acsl4* proximal promoter was confirmed by ChIP approach, suggesting it is physiologically relevant. Our results demonstrated an increase in phospho-CREB association with the *Acsl4* proximal promoter after 120 min of treatment with 8Br-cAMP. Altogether, these experiments demonstrated that CREB plays a relevant role in the regulation of *Acsl4* transcriptional activity induced by cAMP.

In conclusion, the results presented here describe the role of Sp1 and CREB in the regulation of *Acsl4* gene expression. These results may lead to enhanced understanding of the regulation of Acsl4 expression in other models. Additional elements in the *Acsl4* promoter with an effect on transcription of the gene remain to be identified. These studies will help us to the elucidation of the molecular mechanisms underlying abnormal Acsl4 expression in pathological conditions and potentially to the identification of new modes of regulation.

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