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FUNCTIONAL ANALYSIS OF RAT LIVER CITRATE CARRIER PROMOTER: DIFFERENTIAL RESPONSIVENESS TO POLYUNSATURATED FATTY ACIDS

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Short title: Functional characterization of rat citrate carrier promoter

Citrate carrier (CiC), a mitochondrial membrane protein, plays an important metabolic role by transporting acetyl-CoA into the cytosol for fatty acid and cholesterol synthesis. Several studies showed that CiC activity and expression is regulated by dietary fatty acids. Here, we report data on the structural and functional characterization of the 5'-flanking region of rat *Cic* gene. By transient transfection assays in H4IIE cells, a PUFA response region has been identified within the CiC promoter. A cluster of putative binding sites for several transcription factors, composed of a NF-Y site, an E-box like site, a SRE1 like site and four Sp1 sites was localized in the promoter region. Luciferase reporter gene and gel mobility shift assays indicated that a functional E-box like, essential to the basal CiC promoter activity, confers responsiveness to activation by SREBP-1c. This study provides evidence for SREBP-1c as a principal target for PUFA regulation of CiC transcription. In H4IIE cells overexpression of nSREBP-1c overrides arachidonic acid (C_{20:4}, n-6) suppression but does not prevent the repression by docosahexaenoic acid (C_{22:6}, n-3). ChIP assay in H4IIE cells showed that docosahexaenoic acid affects the binding of NF-Y, Sp1 and SREBP-1 to PUFA response region of CiC promoter whereas arachidonic acid alters only the binding of SREBP-1. Our data show that PUFA inhibition of hepatic *Cic* gene transcription is mediated not only by the nuclear level of SREBP-1c, but also might involve a reduction in Sp1 and NF-Y DNA binding, suggesting differential mechanisms in the *Cic* gene regulation by different PUFA.

Key words: citrate carrier, gene expression, lipogenesis, PUFA, rat liver, SREBP-1.

INTRODUCTION

Citrate carrier (CiC), (also known as tricarboxylate carrier), a mitochondrial inner membrane protein catalyzes electroneutral exchange of a tricarboxylate for either another tricarboxylate, a dicarboxylate or phosphoenolpyruvate. CiC has been purified, reconstituted into liposomes, kinetically characterized, cloned and over-expressed in *E. coli*. *Cic* gene, also named *slc25a1*, belongs to the SLC25 gene family coding for mitochondrial carriers. The cDNA sequences of man, cow, rat and yeast are known [for review see 1].

CiC plays an important role in the intermediary metabolism. It exports acetyl-CoA, mainly deriving from sugar sources, from mitochondria to the cytosol, thus providing carbon units for fatty acid synthesis. In this shuttle, NAD⁺ and NADPH, that support glycolysis and lipid biosynthesis respectively, are also supplied [2]. Moreover, CiC participates in metabolic processes other than lipogenesis, such as cholesterol synthesis and the gluconeogenic pathway [1].

The characteristics of this transporting system have been extensively investigated [1], but little is known about its regulation. It has been reported that CiC activity is enhanced in hyperthyroidism [1], reduced significantly during starvation [3], hypothyroidism [4] and type-1 diabetes [1] and can be regulated by insulin [1]. Reports from our laboratory showed that, in parallel with lipogenic enzyme activities, CiC activity and expression is controlled by various nutritional states [3, 5-7]. While saturated and monounsaturated fatty acid-enriched diet administration to rats was practically without effect, dietary n-6 and n-3 polyunsaturated fatty acids (PUFA) regulate CiC gene expression at both transcriptional and post-transcriptional level, being n-3 more effective than n-6 PUFA [6,7]. The molecular mechanisms underlying the transcriptional regulation of *Cic* gene by PUFA are presently unknown because of the lack of information on the CiC promoter.

Lipogenic enzymes are coordinately regulated at transcriptional level during different metabolic states [8]. *In vivo* studies demonstrated that sterol regulatory element-binding protein-1 (SREBP-1) plays a crucial role in the dietary regulation of some hepatic lipogenic genes, such as acetyl-CoA carboxylase (ACACA), fatty acid synthase (FASN), stearoyl-CoA desaturase 1 (SCD1) [9]. These studies include investigations on the effects of the absence or overexpression of SREBP-1 on hepatic lipogenic gene expression [10,11]. SREBPs are transcription factors synthesized as precursor proteins that remain bound to the endoplasmic reticulum and to the nuclear envelope in the presence of sufficient sterol concentrations [12,13]. SREBP-1 and SREBP-2 are encoded by separate genes, and SREBP-1 is expressed as two isoforms, 1a and 1c, which arise from different promoter and exon usage. In liver SREBP-1c is the predominant isoform, whereas SREBP-1a is prevalently expressed in nonhepatic tissues and in most cell lines [13]. Upon sterol deprivation, the precursor protein undergoes a sequential two-step cleavage process to release the NH₂ terminal portion [14]. Then, the mature SREBP enters the nucleus and activates the transcription of genes involved in cholesterol and fatty acid synthesis by binding to sterol regulatory elements (SREs) or to palindromic sequences called E-boxes, within their promoter regions [15,16]. Moreover, it has been reported that PUFA regulate the nuclear abundance of SREBP-1 [17-19]. Given the importance of CiC as a key step in lipogenesis and due to the poor knowledge about the transcriptional regulation of *Cic* gene, we decided to undertake a detailed study of *Cic* gene expression in response to fatty acids. As the transcription of *Cic* gene is markedly inhibited by dietary PUFA [6,7], we hypothesized that some factors such as SREBP-1c, Sp1 and NF-Y regulate this gene by binding its promoter and we tested this hypothesis in this report. To this aim, the structural and functional characterization of the rat *Cic* gene promoter was first of all investigated.

EXPERIMENTAL

Animal treatments and RNA isolation from rat liver

Male Wistar rats (150–200 g) were randomly assigned to one of four different groups. The four groups received for 3 weeks a laboratory chow diet enriched with 15% (w/w) beef tallow (BT), olive oil (OO), safflower oil (SO) or fish oil (FO), respectively. Fatty acid composition of the dietary lipids was reported in [6,7]. The experimental design was in accordance with local and national guidelines covering animal experiments.

Isolation of RNA from rat liver and H4IIE cells and Real-Time qPCR analysis

Total RNA from rat liver and rat hepatoma cells H4IIE was isolated using the SV Total RNA Isolation System kit (Promega), following manufacturer's instructions. RT reaction (20 μ l) was carried out using 5 μ g of total RNA, 100 ng of random hexamers and 200 U SuperScript™ III RNase H- Reverse transcriptase (Invitrogen). Quantitative gene expression analysis was performed (SmartCycler System, Cepheid) using SYBR Green technology

(FluoCycle, Euroclone) and 18S rRNA, for normalization. The primers used for real time PCR analysis were the following: SREBP1for (5'-AGGAGCCACAATGAAGACCG-3'); SREBP1rev (5'-TAGTCGGTGGATGGGCAG-3'); FASNfor (5'-CTCTGGTGGTGTCTACATTTC-3'); FASNrev (5'-GAGCTCTTTCTGCAGGATAG-3'); CICfor (5'-GCCTCAGCTCCTTGCTCTA-3'); CiCrev (5'-ACTACCACTGCCTCTGCCA-3').

Plasmid and reporter vector construction

Nine DNA fragments of rat CiC promoter, (sizes from -1484 to +35, -1114 to +35, -469 to +35, -393 to +35, -314 to +35, -267 to +35, -201 to +35, -147 to +35 and -42 to +35 bp, relatively to the transcription start site) were obtained by polymerase chain reaction (PCR) using a rat genomic cosmidic clone p5B8 as template and one of the forward primers listed in Table 1. The common reverse primer was Co23rev 5'-GCGTCGACCTCGGGTCCGAG-3'. The amplification products were digested with KpnI and SalI, then subcloned into the KpnI and XhoI sites of pGL3 basic vector (Promega). The corresponding constructs are listed in Table 1. Constructs with the mutated Sp1 sites at -129, -118, -92 and -62 bp, E-box like site at -72 bp, Sre1 like site at -67 bp and NF-Y site at -43 bp of the rat CiC promoter were created by site-directed mutagenesis PCR. Mutations were obtained by using the pCiC1484 as template for PCR reactions and two end primers 1484For and Co23rev; the pairs of the complementary mutagenic primers used to generate the mutant constructs are listed in Table 2. Luciferase reporter gene for rat FASN (pFASN-Luc) was constructed as follows. A fragment of rat FASN promoter from -250 to +65 bp relatively to the transcription start site was obtained by PCR using rat genomic DNA as template. The primers used were Fasnpromfor 5'-GAATTCGGTACCGGAGGCCGGGGGGCGGGG-3' (-250) and Fasnpromrev 5'-AAGCTTGTCTGACTGGAGGCAGACGACAAGCGA-3' (+65). The amplification product was digested with KpnI and SalI, then subcloned into the KpnI and XhoI sites of pGL3 basic vector (Promega). All the constructs were sequenced to confirm the accuracy, by using BigDye™ Terminator cycle sequencing kit (Applied Biosystem).

Cell culture and transient transfection assay

H4IIE cells were maintained in Dulbecco's modified Eagle's medium (Euroclone) supplemented with 10% (v/v) fetal bovine serum (FBS), penicillin G (100 units/ml) and streptomycin (100 µg/ml) at 37°C under 5% CO₂ atmosphere. 2 × 10⁵ cells were plated onto 6-well cell culture plates. After 48h cells were co-transfected with one of CiC promoter-luciferase reporter vectors (1.5 µg/well), and a *Renilla* luciferase reference plasmid, pGL4.73 (0.02 µg/well), a control for transfection efficiency, by using FuGENE 6 transfection reagent (Roche Diagnostics). Following eight hours transfection period, the medium was changed to fresh DMEM supplemented with 10% (v/v) of delipidated FBS as reported by Hannah *et al.* [20] and cells were incubated for 24 h. Cells were then lysed and luciferase activity was measured using Dual Luciferase Reporter Assay System (Promega).

The effect of different fatty acids on rat CiC promoter activity was determined after eight hours transfection period, incubating the cells in DMEM medium supplemented with 10% (v/v) delipidated FBS and containing either fatty acid-free BSA (12.5 µM) or one of the following albumin-bound fatty acids (50 µM): stearic acid (C_{18:0}, SA), oleic acid (C_{18:1}, OA), arachidonic acid (C_{20:4, n-6}, AA) or docosahexaenoic acid (C_{22:6, n-3}, DHA) for 24 h. The molar ratio of fatty acids and albumin was 4:1. For transcriptional activation by SREBP-1c, H4IIE cells were transiently co-transfected with pCiC147 or its mutants described above and pGL4.73 reference plasmid, together with either pCMV-SREBP-1c encoding the mature form of SREBP-1c [21], or an empty control vector (pSG5). After transfection the cells were incubated in DMEM with 10% (v/v) of delipidated FBS, cholesterol (10 µg/ml) and 25-hydroxycholesterol (1 µg/ml) for 24 h in order to suppress endogenous SREBP activation.

Purification of Recombinant SREBP-1a Fusion Protein

Recombinant human SREBP-1a was expressed in *Escherichia coli* BL21(DE3). SREBP-1a expression vector pET-SREBP-1a was generated by inserting the cDNA fragments from pCMV-SREBP-1a (a generous gift from Dr. T. Osborne, Department of Molecular Biology and Biochemistry, University of California, Irvine, CA) between the EcoRI and SalI sites of pET-21a (Novagen) and then used for bacteria transformation. The recombinant SREBP-1a was induced for 4 h with 1 mM isopropyl- β -D-thiogalactopyranoside. Following bacteria sonication, the recombinant protein containing COOH-terminal polyhistidine (His 6) tag was purified to homogeneity by Ni-NTA-agarose (Qiagen) chromatography. The purity and concentration of the recombinant proteins were verified by SDS-PAGE followed by Coomassie Brilliant Blue staining.

Chromatin Immunoprecipitation Assay (ChIP assay)

The ChIP assay was performed as in [22]. Chromatin complexes were immunoprecipitated for 12–18 h at 4 °C with 10 μ g of NF-YA antibody (sc-7712), Sp1 antibody (sc-59), SREBP-1 antibody (sc-8984; Santa Cruz Biotechnology), or goat IgG or rabbit IgG overnight at 4 °C on a rotating wheel. After immunoprecipitation with non-specific IgGs or with antibodies against NF-YA, Sp1, and SREBP-1, initial PCRs were conducted with serial dilutions of input material from each immunoprecipitation to establish the appropriate cycling conditions to accurately compare template content across treatments (results not shown). Primers were designed to amplify a 185 bp fragment (– 147 to +38 bp) of the proximal promoter region in the rat *Cic* gene. Primer sequences used in PCR reaction were the following: CiCchipFor (5'-TCAGTTTCCCGGCTGGCACG-3') and CiCchipRev (5'-GGCGTCGACCTCGGGTCC-3'). The PCR reaction was performed with 2 μ l of immunoprecipitate, in a final volume of 25 μ l, 10 μ M of each primer, and 1 $\times$ PCR mix (Sigma) in a Thermal Cycler (Applied Biosystem). Samples were incubated for an initial denaturation at 94 °C for 60 sec, followed by 30 cycles of 94 °C for 20 s, 60 °C for 20 s, and 72 °C for 20 s. PCR products were separated on a 1.5% (w/v) agarose gel and analysed by ethidium bromide staining. A fragment corresponding to FASN proximal promoter was obtained as reported in [23]. The stained gel was visualized and the PCR products from three individual experiments were quantified using a Versadoc imaging system (Bio-Rad Laboratories).

Western analysis

20 μ g of H4IIE nuclear protein prepared in the absence of detergent [15] was separated by SDS-PAGE. After electrophoretic transfer to nitrocellulose, blots were probed with antibodies directed against NF-YA (sc-7712), NF-YB (sc-7711), NF-YC (sc-7114), Sp-1 (sc-59) and anti-SREBP-1 (sc-13551) (Santa Cruz Biotechnology). The detection system employed was the ECL Plus™ Western Blotting Reagents (GE Healthcare).

Electrophoretic Mobility Shift Assay (EMSA)

The wild-type and mutant oligonucleotides, containing the SRE1 like and E-box like of rat CiC promoter from – 80 to –52, were synthesized by Sigma-Genosys. SRE1 FASN probe, used as a positive control, corresponds to –150/–141 of the rat FASN promoter [24]. Double-stranded oligonucleotides were radiolabelled by Klenow filling-in. Recombinant SREBP-1a (100 ng) was mixed with 20000 c.p.m. of ³²P-DNA in 25 mM Tris/HCl (pH 7.5), 40 mM KCl, 1 μ g of poly(dI-dC), 1mM MgCl₂, 10% (v/v) glycerol, incubated for 20 min at room temperature, and electrophoresed in 5% (w/v) acrylamide:bisacrylamide (19:1) with 45 mM Tris/borate (pH 8.3), 1 mM EDTA. Supershift studies were carried out using 0.4 μ g of SREBP-1 antibody (Santa Cruz Biotechnology). Antibody was preincubated with the binding reaction for 2 h on ice prior to addition of labelled DNA. The samples were

electrophoretically separated (200 V for 2 h at 4 °C), the gel was dried and exposed to x-ray film.

Statistical analysis

All data are presented as means $\pm$ S.D. for the number of experiments indicated in each case. Statistical analysis was performed using one-way ANOVA, followed by a post hoc Tukey's B test. Values sharing a different letter differ significantly. Differences were considered statistically significant at $P < 0.05$.

RESULTS

Identification of transcription initiation sites and analysis of the 5' flanking region of the rat liver *Cic* gene

In order to define the transcription start site, primer extension analysis was carried out. As shown in Figure 1, two transcription start sites were detected 59 and 61 nucleotides upstream the AUG translation initiator codon, respectively. Two ESTs reported in GenBank database (NCBI) corresponding to *CiC* mRNA (CB746596 and CD567906) have the 5' end coincident with the transcription start sites identified in this work. In GenBank database there are other ESTs having different 5' ends located upstream the two start sites -59 and -61 bp. This is in agreement with the presence of very weak signals observed in the primer extension experiment (data not shown). A computer-assisted search (Match and P-match programs at www.gene-regulation.com based on the TRANSFAC data base) [25] of the *CiC* promoter revealed some elements that have potential roles in transcriptional regulation of *Cic* gene. The canonical polymerase II transcription element TATA is lacking in the *Cic* gene promoter. There are clusters of GC-rich domains with six sequence elements at positions -187, -160, -129, -118, -92 and -62, which conform to the Sp1-binding site consensus sequence [26] in six to ten out of ten nucleotides. Adjacent to the downstream Sp1 site there was a sequence (ACCACCCCGC) similar to one of classical sterol regulatory elements, SRE1 (ATCACCCAC), which was found in the FASN promoter and was extensively analyzed in terms of sterol regulation [24]. Interestingly, the SRE1-Sp1 sequence was downstream-adjacent to an E-box like (CTGACGTGAC). A similar organization has been reported for mouse SREBP-1c promoter [27]. Downstream the Sp1 site was the NF-Y-binding site (inverted CCAAT box) at -43 bp (Figure 1).

Effect of different exogenous fatty acids on the *CiC* mRNA levels in rat liver and in H4IIE hepatoma cells

To investigate the effect of fatty acids on the expression of the *Cic* gene, Real Time PCR analysis was carried out on total RNA isolated from livers of rats fed on BT- or OO- or SO- or FO- supplemented diet, rich in saturated fatty acids, monounsaturated fatty acids, n-6 PUFA, n-3 PUFA respectively. Quantitation of *CiC* mRNA level (Figure 2A) showed that SO- and FO- administration caused a reduction of the hepatic *Cic* gene expression by approx. 35% and 40% respectively, when compared either to BT- and OO-enriched diet [6,7]. This figure also showed a remarkable reduction of SREBP-1 and FASN mRNAs, upon SO and FO dietary administration. In particular, SO reduced FASN and SREBP-1 mRNA abundances by approx. 65% and 75% respectively, while FO treatment caused a similar marked decrease of both FASN and SREBP-1 expression (Figure 2A). The comparative effects of exogenously added fatty acids on *CiC*, FASN and SREBP-1 expression were investigated also in H4IIE cells. After plating, 50 μ M SA, OA, AA or DHA were added to H4IIE cells and the cultures were incubated for 24 h. The results (Figure 2B) indicated that when compared to SA, used as a control, OA did not determine any statistically significant variations of *CiC*, FASN and SREBP-1 mRNA levels. On the contrary, the *CiC* mRNA content was reduced (approx. 50%)

by AA and (approx. 70%) by DHA addition to the cells. Moreover, AA decreased similarly FASN and SREBP-1 expression (approx. 70%), whereas the strongest inhibitory effect was observed with DHA addition to the cells, upon which FASN and SREBP-1 mRNA abundances were reduced by approx. 80% and 90% respectively.

PUFA affect CiC promoter activity

We compared the CiC promoter activity in H4IIE cells incubated with 50 μ M exogenous fatty acids: the saturated SA, the monounsaturated OA, the n-6 AA and the n-3 DHA. To this aim, the construct pCiC1484 containing the sequences between -1484 and +35 of the *Cic* gene fused to the reporter gene luciferase, was synthesized. H4IIE cells were transiently transfected with pCiC1484 and with a *Renilla* luciferase reference plasmid, pGL4.73 used as a control for transfection efficiency. As shown in Figure 3A, incubation of H4IIE cells with SA and OA was practically without effect, whereas PUFA-supplementation to H4IIE cells significantly reduced (approx. 35% - 55%) luciferase expression from CiC promoter -1484/+35 construct, with respect to the control cells supplemented with only BSA. In particular, DHA was more effective than AA in inhibiting CiC promoter activity.

Inhibition of CiC promoter activity by different concentrations, ranging from 50 to 200 μ M, of AA and DHA was examined in H4IIE cells, transiently transfected with the pCiC1484 construct. The effect exerted by AA and DHA was dose-dependent (Figure 3B). At the concentrations and time used, the effect of AA and DHA on cell viability was analyzed by MTT test. Neither AA nor DHA significantly affected cell viability for concentration up to 50 μ M and for incubation time up to 24 hours (data not shown), ruling out that inhibitory effect exerted by AA and DHA on CiC promoter could be ascribed to a general alteration of cell metabolism or could be a generic "toxic" effect. Therefore, this concentration (50 μ M) has been used in the next experiments.

Localization of PUFA Response Region in the CiC Promoter

To define DNA sequences responsible for the down-regulation of the *Cic* gene by AA and DHA, a series of nested deletion constructs within the 5'-flanking region of the *Cic* gene fused to the luciferase (Luc) gene was used. The activities of each construct were compared in the H4IIE cells cultured at subconfluency in DMEM containing delipidated FBS with or without AA and DHA. Shorter versions of the CiC promoter showed, in some cases, a reduced but yet significant basal activity. The deletion of sequences between -147 and -42 completely abolished the promoter activity. These results suggest that regulatory elements important for the CiC promoter basal activity are present within the -147/-42 region (Fig. 4). Both AA and DHA reduced Luc expression by approx. 45% and 65% respectively, compared to the control, when plasmids containing progressive 5' deletions from position -1484 to -147 of the CiC promoter were transiently transfected into H4IIE cells. These results indicated that the cis-acting DNA sequences that mediate PUFA-responsiveness in H4IIE cells may be located between -147 and +35 of the *Cic* gene.

CiC expression is activated by SREBP-1c in H4IIE cells

To determine whether CiC promoter can mediate transactivation by SREBP-1, transient transfections of H4IIE cells with the promoter-reporter construct pCiC147 together with increasing concentrations of a plasmid encoding for the constitutive SREBP-1c active protein were carried out. The rat CiC promoter (-147/+35) is activated in a dose-dependent manner by SREBP-1c in H4IIE cells (Figure 5). A CiC promoter induction (approx. 12 fold) was observed at 100 ng of pCMV-SREBP-1c. An higher induction (approx. 15 fold) of CiC promoter was observed by SREBP-1a (data not shown). In the figure it has been reported promoter activation of FASN, a well known SREBP-1 target gene [18], which showed a remarkable induction by SREBP-1c (approx. 27 fold).

Localization of candidate regulatory sequences in rat CiC promoter from -147 to +35

Sequence analysis of the CiC promoter region between -147 and +35 bp by the Match and P-Match programs (www.gene-regulation.com) identified candidate transcription factor binding sites, including two SREBP binding sequences (Figure 1) at -72 bp and -67 bp, respectively. The upstream putative SREBP binding site (E-box like at -72 bp) has 1.0 of core similarity and 0.796 of matrix similarity with the E-box containing SREBP-1 binding element described by Kim *et al.* [16], while the other SREBP binding site (SRE1 like at -67 bp) shows a lower similarity (0.8 of core similarity and 0.79 of matrix similarity) to the classical SRE1. The two SREBP binding sites are partially overlapping. In addition, four putative Sp1 binding sites were identified at -129 bp, -118 bp, -92 bp and -62 bp. The latter overlaps with the SRE1-like site. Moreover, a potential site for NF-Y was localized at -43 bp. This cluster consisting of the NF-Y, E-box like, SRE1 like and Sp1 sites has a very high probability of being SREBP-binding and activation site (Figure 1).

To investigate which element in PUFA response region (PUFA-RR) was responsible for mediating the activation by SREBP-1c and the suppression by PUFA and whether other auxiliary sites were required, we generated a series of reporter constructs containing the -1484/+35 of the rat CiC promoter with mutation of each element of the PUFA-RR, linked to the luciferase reporter gene. H4IIE cells were transfected with these promoter-reporter constructs and then incubated with either AA or DHA for 24 hours to test their response to PUFA (Figure 6). Furthermore, to test SREBP-1c activation H4IIE cells were transfected with the same promoter-reporter constructs and incubated with either a nuclear SREBP-1c expression vector or the empty vector pSG5 (Figure 6). As shown in this figure, almost all analyzed mutations did not alter basal activity of promoter. When compared to transcriptional activity of wild type pCiC1484, the activity of p92Sm mutant construct was impaired, but it was still approx. 20 fold higher compared to pGL3 basic empty plasmid control. The construct p72Em that contained a mutated E-box like element caused loss of either SREBP-1c induction and PUFA response. Almost all the mutations at the Sp1 binding sites significantly decreased, but did not totally block, the activation by SREBP-1c and the inhibition by AA, being the more effective those at the Sp1 sites closer to the E-box element. Mutation of the putative NF-Y-response element also completely abolished the induction by SREBP-1c and significantly reduced only the inhibition by AA. On the other hand, mutation of the putative SRE1 like, which is partially overlapped with one of the Sp1 sites and the E-box, caused a reduction (approx. 40%) of only the SREBP-1c transactivation. By contrast, mutations at the E-box like site and at Sp1 sites closer to the E-box showed minimal, but yet statistically significant, differences with respect to the control, in affecting DHA inhibition. Similar results were obtained with an analogous series of pCiC147 constructs containing the same point mutations (data not shown). Therefore, the mutations did not have a general effect on the basal activity of the promoter, although they affected PUFA responsiveness and SREBP-1c transactivation.

Specific binding of nSREBP-1 to CiC promoter

To demonstrate the direct binding of SREBP-1 to the PUFA-RR, gel mobility shift assay (EMSA) was performed. Purified, recombinant SREBP-1a formed a shifted complex with double-stranded DNA corresponding to the sequence from -80 bp to -52 bp in the CiC promoter (Figure 7, lane 2).

In competition assays, the shifted band disappeared after addition of excess amount (100x) of the unlabelled PUFA-RR probe (CiC competitor) (lanes 4), or unlabelled FASN SRE1 motif (FASN SRE1 competitor) (lane 5), or PUFA-RR probe with E-box like wild type/SRE1 mutant version (E-boxwt/SRE1 mut competitor) (lane 7). By contrast, addition of the same excess amount of PUFA-RR probe with a mutation in E-box like and SRE1 wild type (E-box

mut/SRE1wt competitor) did not compete the binding of SREBP-1a to native PUFA-RR probe (lane 6). These data clearly indicated that SREBP-1a binds to the E-box in the PUFA-RR, activating CiC promoter. FASN SRE1 probe used as a positive control (lane 3) for SREBP-1a binding also formed a shifted complex. Specificity of SREBP-1a binding to the PUFA-RR probe was confirmed by a supershift after the addition of SREBP-1 antibody (lane 8).

Overexpression of nSREBP-1c abrogates AA inhibition and causes a minimal reduction of DHA suppression of CiC-Luc activity

nSREBP-1 binds and functionally interacts with the CiC promoter at the E-box like (-74/-65). This region is within the cis-regulatory target for PUFA control. Moreover, PUFA suppress the nuclear level of SREBP-1. Taken together, these results suggest that PUFA control *Cic* gene transcription by regulating the nuclear content of the factor binding this region. If this occurs, then overexpression of nSREBP-1c should eliminate the PUFA inhibition of CiC-Luc activity. To test this hypothesis, H4IIE cells were co-transfected with pCMV-SREBP-1c and pCiC1484 and incubated with either 50 μ M AA or DHA (Figure 8). In the absence of co-transfected nSREBP-1c, AA and DHA suppressed CiC-Luc activity by approx. 45% and 65%, respectively. Transfecting H4IIE cells with pCMV-SREBP-1c, a reduction of AA inhibitory effect on CiC activity was observed, so that AA inhibition was almost abrogated in the presence of pCMV-SREBP-1c at 1 μ g/plate (Figure 8). By contrast, overexpression of nSREBP-1c caused only a minimal reduction of DHA inhibition, suggesting that the inhibitory effect exerted by DHA may involve also a SREBP-1 independent mechanism. Similar results were obtained with the pCMV-SREBP-1a (data not shown).

Specific binding *in vivo* of NF-Y, Sp1 and nSREBP-1 to CiC promoter

To investigate whether NF-Y, Sp1, and nSREBP-1 binding to the proximal promoter of the endogenous *Cic* gene is modulated by PUFA *in vivo*, ChIP assay was performed. Chromatin from H4IIE cells incubated for 24 h in DMEM supplemented with BSA (control), AA or DHA was isolated. After immunoprecipitation with antibodies against NF-YA, Sp1, and SREBP-1, or with non-specific IgGs, PCRs were carried out, as described in "Experimental". A 185 bp fragment of the proximal CiC promoter was amplified only when immunoprecipitation was performed with specific anti-NF-YA or anti-Sp1 or anti-SREBP-1 antibodies (Figures 9A and 9B). The treatment of the H4IIE cells with DHA inhibited the binding of NF-YA and Sp1 to the proximal promoter, whereas AA did not exert any statistically significant effect. On the contrary, ChIP assay experiment carried out by using the SREBP-1 antibody showed that both AA and DHA treatment suppressed the SREBP-1 binding to the CiC proximal promoter (Figures 9A and 9B). These results suggested a co-ordinated mechanism responsible for the decreased binding of these factors by DHA. To assess the specificity of the PUFA-dependent decrease in binding of NF-Y, Sp1, and SREBP-1 to the CiC promoter, their binding to promoter of FASN, a well known PUFA-target gene [18,28] was evaluated. DHA supplementation to H4IIE cells decreased the binding of NF-YA, Sp1 and SREBP-1 to FASN promoter by approx. 50%, in agreement with the reduction observed by Teran-Garcia *et al.* in FO-diet fed rats [23]. On the contrary, the treatment of the H4IIE cells with AA caused a reduction of only SREBP-1 binding to FASN, in a similar extent to that observed to CiC promoter. To determine whether the observed reduction in the binding of SREBP-1, NF-Y and Sp1 could be ascribed to a decrease in their nuclear content, Western blotting analysis was performed by using crude nuclear extracts from cultured H4IIE cells, treated with either no fat or 50 μ M AA or DHA for 24 h. A representative Western blot and quantitative results from three separate experiments are illustrated in Figure 10. The content of SREBP-1 mature form was approx. 20% and 35% in nuclei from AA- and DHA-

treated cells, respectively, when compared to nuclei from control cells. By contrast, AA and DHA treatment did not alter the nuclear content of Sp1 or any of the NF-Y isoforms.

DISCUSSION

To our knowledge this is the first study in which rat CiC promoter in any type of cells has been deeply investigated. We characterized structurally and functionally the 5'-flanking regulatory region of the rat *Cic* gene and identified the PUFA-RR.

Sequence analysis of the *Cic* promoter (Figure 1) revealed absence of the canonical polymerase II transcription element TATA box and the presence of GC-rich stretches with the consensus sequence [29] for several Sp1 transcription factors, characteristics reported for the promoter of several TATA-less genes. Many of these genes code for enzymes catalyzing metabolic reactions and are housekeeping genes [30,31]. In fact, CiC is expressed in liver, kidney, pancreas, lung, heart and brain [1]. However, unlike other housekeeping genes, CiC mRNA abundance decreased in the liver of starved [3] and PUFA-fed rats [6,7] and it is inducible in liver by refeeding previously fasted rats [3]. There is convincing evidence, *in vitro* [17] as well as *in vivo* [9], implicating a role for PUFA in inhibiting SREBP-1 mRNA, or SREBP-1 maturation, or in accelerating SREBP-1 nuclear form degradation through a 26S proteasome-dependent process [32], thereby repressing lipogenic gene expression. The unsaturated fatty acid inhibition of CiC promoter activity seems to be dependent on chain length and degree of unsaturation, being DHA more effective than AA, whereas SA and OA do not exhibit any effect (Figure 3A). Interestingly, these data perfectly fit with CiC activity reduction observed in rats fed on PUFA-supplemented diet or in PUFA-treated H4IIE cells (Figure 2). The reduced promoter activity was already evident at 50 μ M of either AA or DHA (Figure 3B). At this concentration luciferase activity was reduced by more than 50% with respect to the control (i.e. in absence of the fatty acid). Transfection reporter assays with H4IIE cells revealed that the PUFA response sequences for rat *Cic* gene reside within the proximal promoter region of -147/+35 (Figures 4 and 6). Alignment of PUFA-RR of the rat CiC promoter with the corresponding portion of the mouse *Cic* gene showed a high sequence homology (approx. 94%), whereas a lower similarity (approx. 76%) was observed aligning rat versus human CiC promoter (not shown). NF-Y and SREBP-1 sites are lacking in human CiC promoter, the first as consequence of a deletion and the second because of difference in the nucleotide sequence. This sequence divergence could explain the different data about the PUFA inhibition of human CiC promoter reported by Infantino *et al.* [33].

Our data indicated a sensitivity of the CiC promoter for overexpressed nSREBP-1 (Figure 5). In fact, the recombinant SREBP-1a strongly bound to the region between -80 and -52 (Figure 7, lane 2). Furthermore, mutation at E-box like site lowered either SREBP-1c induction and PUFA response (Figure 6), implying that this site is responsible for both SREBP-1c transactivation and PUFA inhibition of CiC promoter. The fact that only mutation at E-box like, and not at SRE1 like site, prevented the binding of SREBP-1a to PUFA-RR of CiC promoter (Figure 7, lanes 6 and 7) suggested that the E-box plays an important role in SREBP-1 transactivation. Moreover, the observation that the PUFA-RR of the rat CiC promoter contains a functional E-box like and NF-Y and Sp1 sites (Figure 6), might suggest that SREBP-1 activates the promoter in synergy with NF-Y and Sp1. SREBP-1 and NF-Y have been implicated in the PUFA inhibition of transcription of several lipogenic genes, including Δ -6 desaturase, SCD1, ATP citrate-lyase and FASN [34-37]. Dietary PUFA exert their inhibitory influence by lowering the nuclear content of mature SREBP-1 protein and by interfering with the transactivation action of NF-Y [35-37]. On the other hand, in FASN proximal promoter, Osborne and co-workers [38] reported that all isoforms of SREBP were dependent on NF-Y and only SREBP-1c showed a strong dependence on Sp1. The

importance of the Sp1 binding sites for the action of SREBPs has been reported in other promoters, such as low density lipoprotein (LDL) receptor, and ACACA [9]. Moreover, sterol inhibition of human FASN promoter activity appears to involve SREBP-1 as well as NF-Y and Sp1 [39], thus suggesting that the requirements for auxiliary factors are promoter-dependent. CiC promoter contains a Sp1 site very close to the E-box, therefore it could probably be easier for Sp1 and SREBP-1 to communicate together when bound at these sites. SREBP-1 seems to amplify its positive influence on *Cic* gene transcription by enhancing interaction of NF-Y and Sp1 with their respective recognition sites, located at -43 bp and -118/-92/-62 bp, respectively.

In fact mutations at Sp1 and NF-Y sites caused a reduction of SREBP-1c transactivation (Figure 6). Furthermore these mutations decreased (by approx. 50%) AA inhibition of CiC promoter activity (Figure 6). However, as AA lowered the nuclear content of only SREBP-1 (Figure 10), taken together these data suggest that: i) the inhibition of CiC promoter caused by AA depends mainly on this factor, ii) SREBP-1 requires auxiliary NF-Y and Sp1 factors. This hypothesis can be also supported by the observation that overexpression of nSREBP-1c overrides AA inhibition (Figure 7). By contrast, mutations at Sp1 and NF-Y sites decreased minimally DHA inhibition of CiC promoter activity (Figure 6), and SREBP-1c overexpression did not restore CiC promoter activity when H4IIE cells were treated with DHA (Figure 8). Therefore, DHA seems to inhibit CiC promoter activity not only down-regulating SREBP-1c expression, but also affecting Sp1 and NF-Y binding to CiC promoter. ChIP assay results strongly support this hypothesis. Indeed, in H4IIE cells treated with AA, reduction of SREBP-1 but not of Sp1 and NF-Y binding was observed, whereas, when DHA was added to the cells, a decrease not only of SREBP-1 but also of Sp1 and of NF-Y binding was found (Figure 9). Given the absence of PUFA-dependent changes in nuclear content of NF-Y and Sp-1 (Figure 10), these findings raise the possibility that DHA altered NF-Y and Sp-1 DNA binding activity, through post-translational mechanisms. In fact, it has been reported that n-3 PUFA enriched diet post-translationally regulates Sp-1 through a phosphatase 1A-dependent site [23]. Previous studies examined the effect of long chain PUFA on the murine hepatic transcriptome and distinct transcriptomic, signaling cascades and predicted affects on liver metabolism have been elucidated for dietary oils rich either in AA or DHA [40], but mechanisms were not defined. In this work for the first time differential inhibitory effects on promoter activity in response to AA relative to DHA were observed, providing unique mechanistic insight into transcriptional regulation by these fatty acids. Further focused studies will need to dissect the transcription factor signaling pathways to fully explain how DHA and AA achieve their specific effects.

Unlike other strictly lipogenic genes, such as FASN, that works exclusively in fatty acid synthesis, CiC participates in metabolic processes other than lipogenesis, such as cholesterol synthesis and the gluconeogenic pathway [1], thus regulation of *Cic* gene transcription by PUFA might be reflected also on the modulation of other metabolisms.

Abbreviation used: ACACA, acetyl-CoA carboxylase alpha; AA, arachidonic acid; BT, beef tallow; CiC, citrate carrier; ChIP assay, Chromatin immunoprecipitation assay; DHA, docosahexaenoic acid; FASN, fatty acid synthase; FBS, fetal bovine serum; FO, fish oil; NF-Y, nuclear factor-Y; OO, olive oil; PUFA, polyunsaturated fatty acids; SCD1, stearoyl-Coenzyme A desaturase 1; Sp1, stimulatory protein 1; SO, safflower oil; S14, Spot 14 protein; SREBP, sterol regulatory element-binding protein.

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FIGURE LEGENDS

Figure 1 DNA sequence of the 5'-flanking region of the rat *CiC* gene and identification of transcription initiation sites

The sequence shown contains 250 nt upstream the transcription start site of the longest *CiC* mRNA transcript and 61 nt 5' to the ATG translation start codon, which is at 3' end of the sequence. Putative transcriptional and regulatory motifs are underlined. The two guanosine at the transcription start sites are designed +1 and +3 and are indicated by bent arrows.

Figure 2 Fatty acid regulation of *CiC* expression

(A) The histograms represent *CiC*, FASN and SREBP-1 mRNA levels, determined using RT-quantitative PCR and expressed as relative amounts (18S rRNA as a reference) in liver from BT- (control group) or OO- or SO- or FO-supplemented diet fed rats; (B) *CiC*, FASN and SREBP-1 mRNA levels determined in H4IIE cells, as in A. H4IIE cells were treated as reported in "Experimental" with the indicated albumin-bound fatty acids. Different letters indicate significant differences ($P < 0.05$, $n=6$).

Figure 3 Modulation of *CiC* promoter activity by AA and DHA

(A) H4IIE cells were transiently co-transfected with p*CiC*1484 and pGL4.73 control plasmid. After transfection, cells were incubated with BSA (12.5 μ M) alone (control) or with 50 μ M of the indicated albumin-bound fatty acid, for 24 h. Normalized luciferase activity was expressed as percentage of values obtained in the control cells incubated with BSA. Values are means $\pm$ S.D. ($n=6$). (B) Dose-dependent suppression of the *CiC* promoter by PUFA. Increasing concentrations of AA or DHA were added to the H4IIE cells, following transfection of the p*CiC*1484 construct and of the *Renilla* reference plasmid pGL4.73. After 24-h incubation, luciferase activity was measured and normalized to *Renilla* luciferase activity and to protein concentration. Values are means $\pm$ S.D. Different letters indicate significant differences ($P < 0.05$, $n=6$).

Figure 4 Suppression of the *CiC* promoter activity by AA and DHA

H4IIE cells were transiently co-transfected with the *CiC* promoter-luciferase constructs together with *Renilla* luciferase reference plasmid pGL4.73. After 24 h incubation in medium containing 10% delipidated FBS and 50 μ M AA or DHA, firefly luciferase activity was measured and normalized to *Renilla* luciferase activity and to protein concentration. The inhibition of promoter activity by AA or DHA was expressed as percentage relative to the cells treated with BSA alone. Values are means $\pm$ S.D. Different letters indicate significant differences ($P < 0.05$, $n=6$).

Figure 5 Transcriptional activation of the p*CiC*147 construct by SREBP-1c

H4IIE cells were transiently co-transfected with the p*CiC*147 construct containing the PUFA-RR of *CiC* promoter together with increasing amounts of pCMV-SREBP-1c as indicated. Cells were harvested after 24 h and the luciferase activity was normalized to *Renilla* luciferase activity. Results are expressed as fold induction of the luciferase activity by SREBP-1c, with respect to control (cells transfected with pSG5, the empty vector). Values are means $\pm$ S.D., $n=6$.

Figure 6 Effects of mutations in the E-box like, SRE1 like, Sp1 and NF-Y sites on the SREBP-1a activation and PUFA inhibition of the *CiC* promoter activity

For each construct containing various mutations (symbolized by cross in the respective element), the basal promoter activity was reported (white bars). The sequence of wild type and mutants at the Sp1, E-box like, SRE1 like and NF-Y sites are shown. For PUFA

suppression, H4IIE cells were transiently transfected with CiC reporter constructs containing various mutations and treated with 50 μ M albumin-bound AA, DHA or with BSA (12.5 μ M) alone. The inhibition of promoter activity by AA or DHA was expressed as percentage relative to the cells treated with BSA alone. For SREBP-1c transactivation, H4IIE cells were transiently transfected with reporter constructs described above, together with pCMV-SREBP-1c or pSG5, the empty vector (control). The reporter gene activity was measured as in "Experimental". Values are reported as fold induction of luciferase activity by SREBP-1c, with respect to the control empty vector. Values are means $\pm$ S.D. Within the same experiment, groups bearing different letters differ significantly ($P < 0.05$, $n=6$).

Figure 7 SREBP-1a binds to the E-box like sequence in the rat CiC promoter

(A) The sequence of wild type and mutants CiC probes (nucleotides -80 to -52) utilized for the gel shift are shown. The E-box like and SRE1 like binding sites are underlined. Oligonucleotide named E-box mut/SRE1wt contains mutations at E-box like site and is wild type at SRE1 like site, whereas oligonucleotide named E-boxwt/SRE1mut contains mutations at only SRE1 like site. (B) Gel mobility shift assays were performed with the 32 P-labelled wild type CiC or SRE1-FASN probe (20000 c.p.m., 1.5 fmol) and recombinant SREBP-1a (100 ng) (lanes 2 and 3). In the competition assays, unlabelled oligonucleotides CiC competitor (lane 4), SRE1-FASN competitor (lane 5), the mutants E-boxmut/SRE1wt competitor (lane 6) and E-Boxwt/SRE1mut competitor (lane 7) were added at 100-fold molar excess. Specificity of SREBP-1 binding (indicated by arrow) to the CiC probe, was confirmed by a super-shift, after the addition of SREBP-1 antibody (lane 8).

Figure 8 Overexpression of SREBP-1c abrogates AA inhibition and reduces DHA suppression of CiC-Luc activity

H4IIE cells were co-transfected with pCiC1484 (1.5 μ g), pCMVSREBP-1c (at 0, 0.1, 1 μ g/plate) together with *Renilla* luciferase reference plasmid pGL4.73. The total amount of co-transfecting plasmid was kept constant by supplementing with pSG5 empty vector. After transfection, cells were treated with either AA or DHA acids for 24 h. Control cells were treated with BSA (12.5 μ M) alone. Cells were harvested and assayed for luciferase activity. CiC-Luc activity in the presence of AA or DHA was expressed as percentage with respect to the control. Values are means $\pm$ S.D. Within the same experiment, groups bearing different alphabets differ significantly ($P < 0.05$, $n=6$).

Figure 9 Differential effect of AA and DHA on NF-Y, Sp1 and SREBP-1c binding to CiC promoter

(A) ChIP analysis of the CiC promoter was conducted using H4IIE cells treated with 50 μ M of albumin-bound AA or DHA, or BSA (12.5 μ M) used as a control. Chromatin fragments immunoprecipitated with anti-NF-Y, anti-Sp1 and anti-SREBP-1 were amplified by PCR with primers spanning the PUFA-RR of the CiC and FASN promoter. Samples incubated with aspecific preimmune IgG or no antibodies were used as negative controls. The results shown are representative of six individual experiments. (B) results were quantified by measuring the density of the PCR products separated on agarose gels. The values were derived from the average density of the PCR products from AA or DHA treated H4IIE cells, compared with the average density of those from H4IIE cells treated with only BSA (12.5 μ M), which are set to 100% (control). Different letters indicate significant differences ($P < 0.05$, $n=6$).

Figure 10 Effect of PUFA on the SREBP-1, Sp1 and NF-Y isoform contents

(A) Cells were incubated for 24 h in DMEM medium without or with AA or DHA. Cells were then harvested for preparation of a crude nuclear fraction. Proteins (20 μ g) were separated by SDS-PAGE and immunodecorated with antisera against SREBP-1, Sp1, NF-

YA, NF-YB and NF-YC. **(B)** The content of SREBP-1 mature form, Sp1, NF-YA, NF-YB and NF-YC in PUFA-treated cells was quantified by densitometric analysis of the blot and expressed as percentage of the protein levels in control cells. Values are means $\pm$ SD, $n=4$.

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Table 1. Oligonucleotides used for CiC promoter deletion constructions

Constructs	Primer	Oligonucleotide sequence
pCiC1484	1484For	5' - GAATTCGGTACCATTGCATGGAACAAGCCCC - 3'
pCiC1114	1114For	5' - GAATTCGGTACCTGTAGGCTCTCCTCTGCTGC - 3'
pCiC469	469For	5' - GAATTCGGTACCAAGCTCTGTGATTACAATG - 3'
pCiC393	393For	5' - GAATTCGGTACCTCAGAGAATCCGGTCACTC - 3'
pCiC314	314For	5' - GAATTCGGTACCATAGAACTGCACCTTGACCC - 3'
pCiC267	267For	5' - GAATTCGGTACCTTTGCTGTCCCGAGACCACG - 3'
pCiC201	201For	5' - GAATTCGGTACCGGTTGATCTCAGCTCTCCGC - 3'
pCiC147	147For	5' - GAATTCGGTACCTCAGTTTCCCGGCTGGCACG - 3'
pCiC42	42For	5' - GAATTCGGTACCTTGGTCGCGGTCTCTGTAGC - 3'

Table 2. Oligonucleotides used to obtain mutant constructs by site-directed mutagenesis. Mismatched nucleotides are underlined.

Mutant	Primers	Oligonucleotide sequence
p129Sm	129Sp1mFor	5' - ACGGAATT <u>CGG</u> CGTGGGCGG - 3'
	129Sp1Rev	5' - CGCCGAATTCCGTGCCAGC - 3'
p118Sm	118Sp1mFor	5' - CGTGAATT <u>CGG</u> CTCAGCTCA - 3'
	118Sp1mRev	5' - AGCCGAATTCACGCCACGC - 3'
p92Sm	92Sp1mFor	5' - GCGGAATT <u>CAG</u> CCGGGGAGC - 3'
	92Sp1mRev	5' - CGGCTGAATTCCGCGTGGCC - 3' ;
p72Em	72EboxmFor	5' - AGCTTTTGTGACCACCCCGC - 3'
	72EboxmRev	5' - TGGTCACAAAAGCTCCCCG - 3'
p67Srm	67SremFor	5' - GATACCCAATCCTCCGGGACCAGAA - 3'
	67SremRev	5' - GAGGATTGGGTATCACGTCAGCTCC - 3'
p62Sm	62Sp1mFor	5' - ACCGAATTCCCGGGACCAG - 3'
	62Sp1mRev	5' - CCGGGAATTCCGGTGGTCAC - 3'
p43Nm	43NFYmFor	5' - CAGAGATCTTCGCGGTCTCTG - 3'
	43NFYmRev	5' - CCGCGAAGATCTCTGGTCCCCG - 3'

Figure 1

-250 ACGCCTCCGGCGGTGTTCTCCATCTCCCCGGAACCCAGCACCGGCCATTG
 -200 GTTGATCTCAGCTCTCCGCCCCGACGCGTGCCTCGGTGCATCCCGCCCAA
 -150 GCCTCAGTTTCCCGGCTGGCACGGGGCGTGGCGTGGGCGGGGCTCAGCTC
 -100 AGGCCACGCGGGGCGGAGCCGGGGAGCTGACGTGACACCCCGCCTCCGG
 -50 GACCAGAAATTGGTCGCGGTCTCTGTAGCCTCCGGCCCCGTTGCTTTCCGA
 +1 GTGCCCCGCGCCGGAGGCTCGGACCCGAGGTCGACGCCGGGCGCCCTCC
 +51 TCCTGCCCCGCCATG

Figure 2

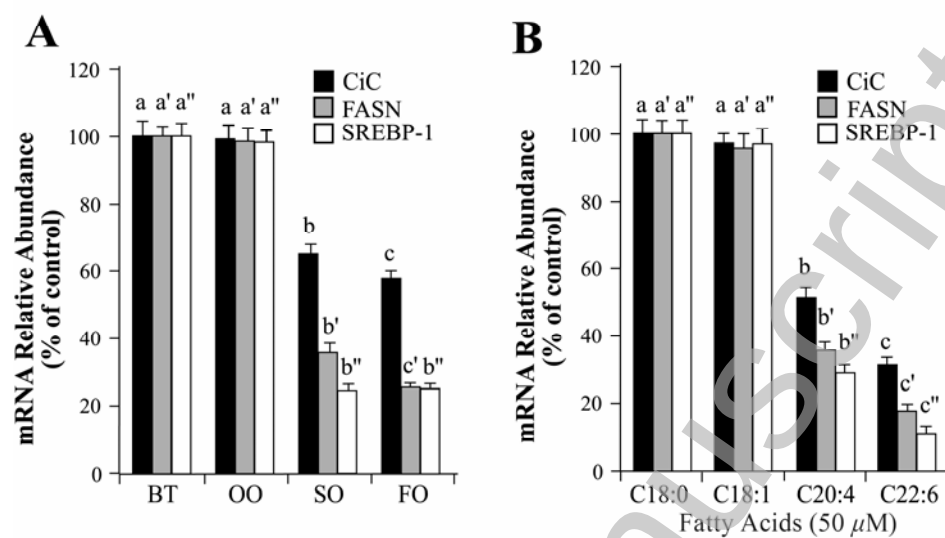


Figure 3

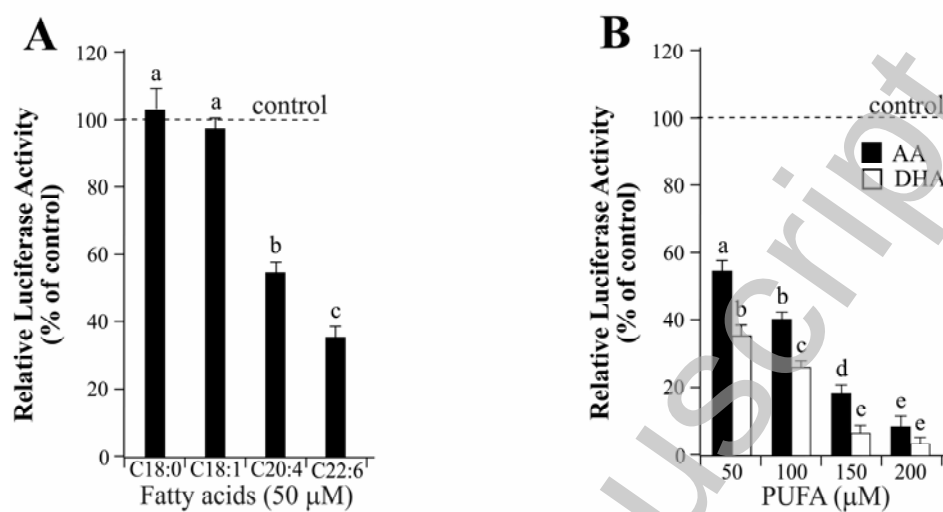


Figure 4

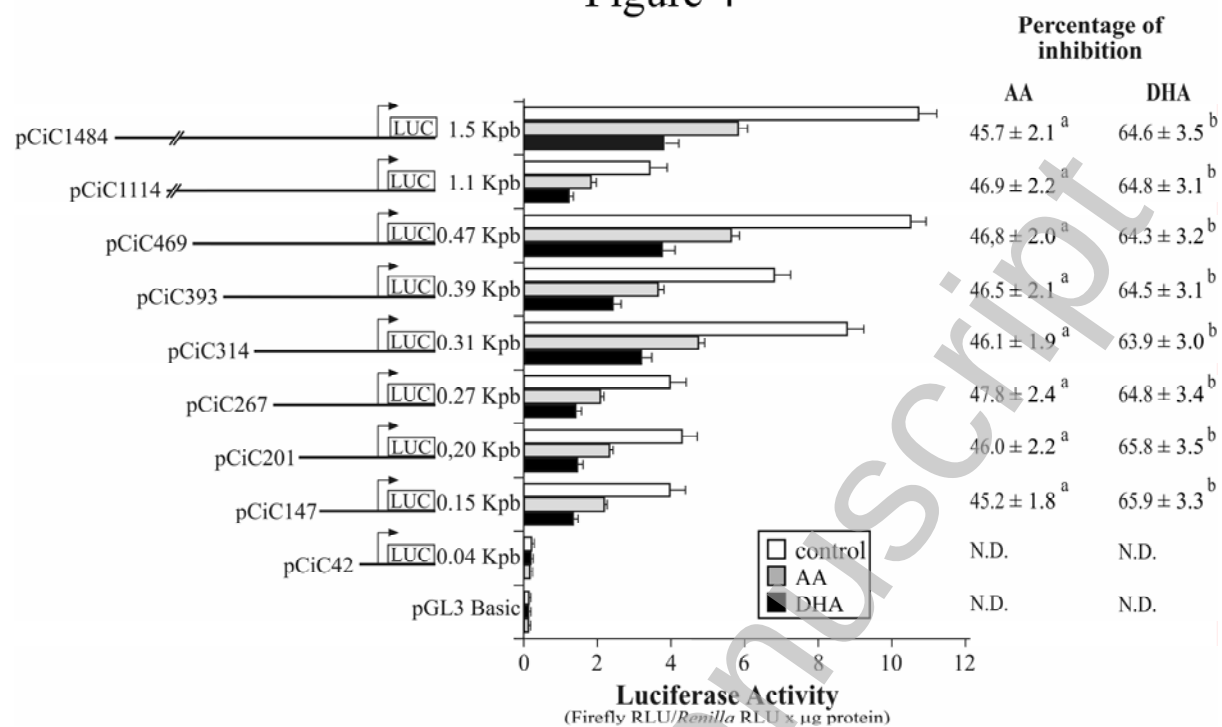


Figure 5

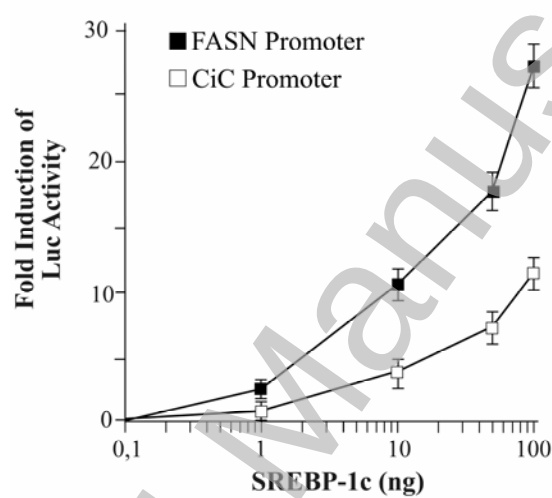


Figure 6

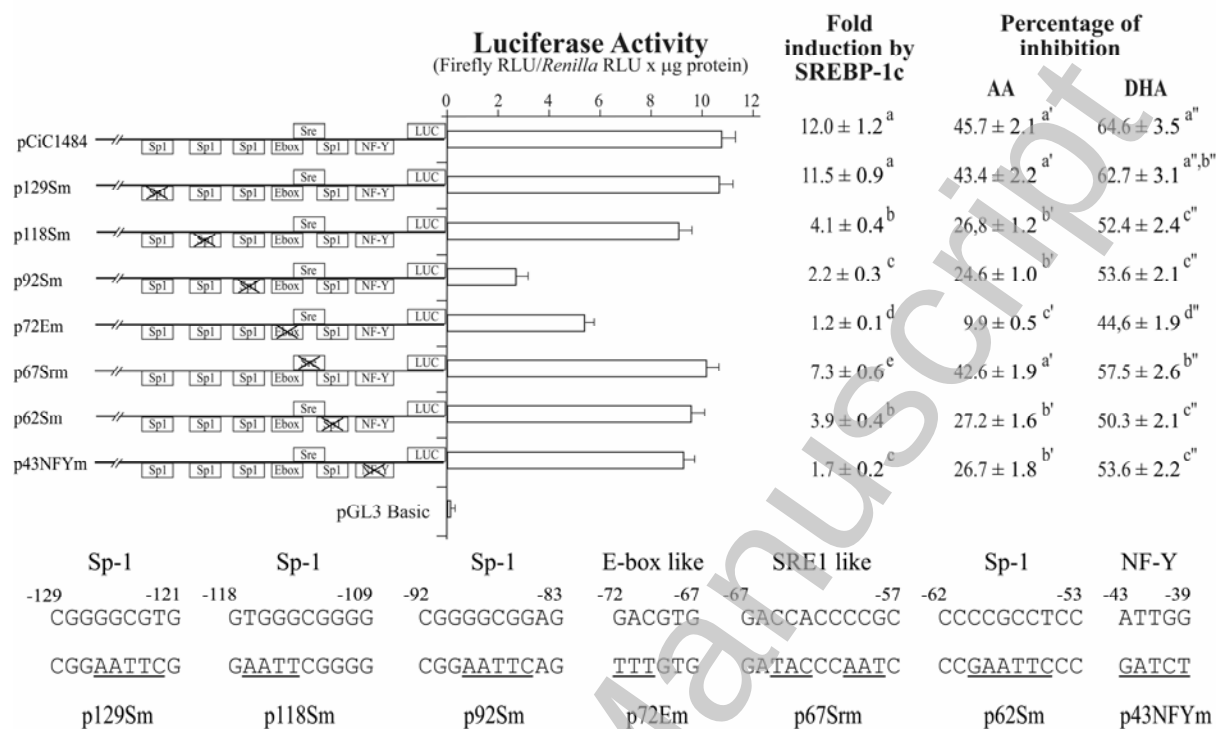


Figure 7

A

-80 E-box like SRE1 like -52

CiC probe GGGGAGCTGACGTGACCACCCGCTCCG

E-boxmut/SRE1wt GGGGAGCTTTTGTGACCACCCGCTCCG

E-boxwt/SRE1mut GGGGAGCTGACGTGATACCCAATCCTCCG

B

CiC probe	+	+	-	+	+	+	+	+
SRE1 FAS probe	-	-	+	-	-	-	-	-
CiC competitor	-	-	-	+	-	-	-	-
SRE1 FAS competitor	-	-	-	-	+	-	-	-
E-boxmut/SRE1wt competitor	-	-	-	-	-	+	-	-
E-boxwt/SRE1mut competitor	-	-	-	-	-	-	+	-
SREBP-1a	-	+	+	+	+	+	+	+
Ab-SREBP-1	-	-	-	-	-	-	-	+
Lane	1	2	3	4	5	6	7	8

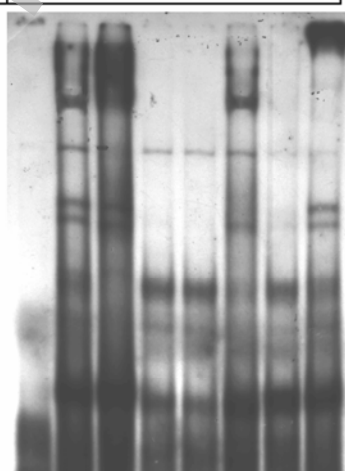


Figure 8

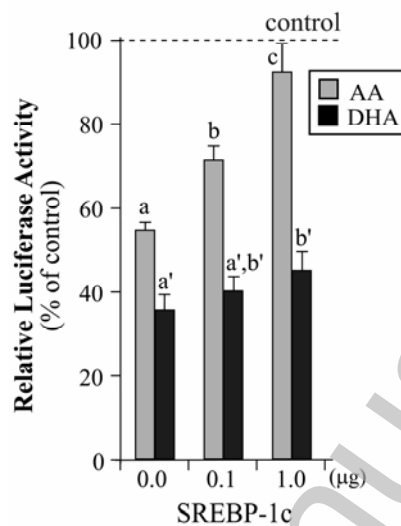


Figure 9

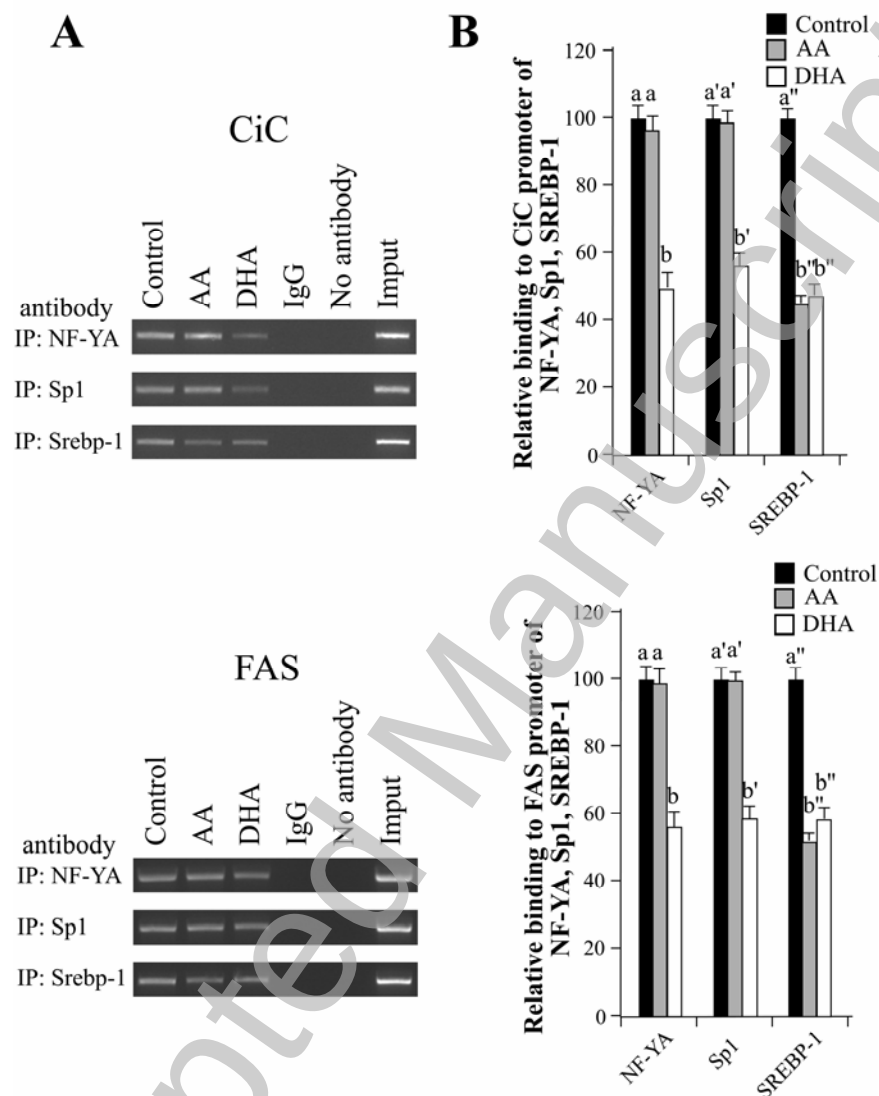


Figure 10

