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Effects of Polyunsaturated Fatty Acids and Clofibrate on Chicken Stearoyl-CoA Desaturase 1 Gene Expression

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In chicken, adiposity is influenced by hepatic stearoyl-CoA desaturase (SCD) 1. This gene is up-regulated by low-fat high-carbohydrate diet and down-regulated by addition of polyunsaturated fatty acids (PUFA). In this study, we present evidence for an inhibition of chicken SCD1 expression by PUFA using reporter gene constructs in transient transfection assays. This inhibition does not involve the peroxisome proliferator-activated receptor pathway, in contrast with what has been observed in rodents. We were able to localise a PUFA as well as an insulin response element within the −372/+125 bp region of the promoter. Sequence analyses of this region allowed identification of several *cis*-regulatory elements: A sterol regulatory element (SRE) and a juxtaposed NF-Y element which have been shown to be involved in the regulation of mouse SCD genes by PUFA. In addition, we identified an overlapping Sp1/USF motif, which was described to play a role in insulin/glucose and PUFA regulation of fatty synthase, ATP-citrate-lyase, and leptin genes. These data provide the first characterisation of the chicken SCD1 promoter and putative *cis*-sequences involved in the regulation of this gene by PUFA and insulin.

Key Words: **stearoyl-CoA desaturase 1; polyunsaturated fatty acids; clofibrate; chicken; regulation of expression.**

Stearoyl-CoA desaturase (SCD) (EC 1.14.99.5) is involved in the synthesis of monounsaturated acyl-CoA by the insertion of a *cis*-double bond in the Δ9 position. The preferred substrates are palmitoleyl- and stearoyl-CoA which are converted to palmitoyl- and stearoyl-CoA, respectively (1–4). The fatty acids are further esterified to form triacylglycerol (TG) and phospholipid and are finally secreted as very low density lipoproteins (VLDL).

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Several reports suggest that SCD could play an important role in the regulation of lipid secretion by the liver, as hypothesized by Jeffcoat (5). Experiments conducted in lean and fat chickens divergently selected for low and high abdominal adipose tissue weight (6) have shown that hepatic SCD activity was higher in fat birds compared to lean ones and positively correlated to VLDL and VLDL-TG plasma concentrations (7). Hepatic SCD1 mRNA levels have been correlated to adiposity in these chickens (8), being higher in the fat ones (9). Furthermore, inhibition of SCD1 activity and mRNA expression impairs TG secretion in cultured chicken cells (10, 11). Genetic linkage analyses performed on lean and fat animals indicated that SCD1 gene as well as some genes involved in lipid synthesis and secretion (malic enzyme, ATP citrate lyase, acetyl-CoA carboxylase, and fatty acid synthase) were not associated to the adiposity trait, although some of them were shown to be differentially expressed between lean and fat chickens (unpublished results and 9). These data suggest that the difference of expression observed between lean and fat chickens could be a consequence of genetic differences in factors involved in *trans*-regulation of these genes.

The above described genes as well as several other genes involved in lipid synthesis and secretion and glycolysis are regulated by both nutritional and hormonal stimuli. Starvation represses the expression of these genes and glucagon mimicks this effect. Expression of these genes is induced by refeeding starved animals with a low-fat high-carbohydrate diet, this mechanism involves the glucose/insulin pathway. Polyunsaturated fatty acids (PUFA) also repress the expression of these genes (12–16).

Two highly homologous genes, SCD1 and 2, have been cloned and sequenced from mouse and rat (17–19). In these species, most tissues express both SCD1 and 2 with the exception of the liver, which expresses mainly the SCD1 isoform (17–19). In the liver, under normal dietary conditions, mouse and rat SCD1 mRNA levels are very low, but they are dramatically increased by a fat-free high-carbohydrate diet (17–20). This up-

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regulation is caused by increased gene transcription (21). In the diabetic mouse, the increase in SCD1 mRNA levels after insulin administration is mainly achieved by an increase in gene transcription, although posttranscriptional events have been implied as well (22). SCD1 transcription is repressed by dietary polyunsaturated fatty acids (PUFA) while peroxysome proliferators increase it (21, 23, 24).

Very little is known about the mode of regulation of SCD1 expression in chicken liver. We have recently shown that insulin and glucagon have opposite transcriptional effect in chicken liver cells (25). However, no data do yet lying the *cis*-regulatory sequences and *trans*-acting factors involved in the regulation of SCD1 expression and the role of PUFA and peroxisome proliferators.

This prompted us to define the sequences involved in the regulation of SCD1 expression in the chicken liver. Our goal was to identify *cis*-regulatory elements and transcription factors which could be genetically linked to adiposity and be responsible for the overexpression of lipogenic genes in fat birds. In particular, we studied insulin/glucose and polyunsaturated fatty acid which represent major nutritional regulators of SCD1 expression (25, 26).

In a first series of experiments LMH cells were transfected with several luciferase reporter constructs containing different deletions of the chicken SCD1 5′ promoter. This allow us to evidence a SCD1 gene transcriptional inhibition by PUFA and to localise *cis*-regulatory elements involve in both PUFA and insulin-mediated regulation within the proximal region of the promoter. This region has been sequenced and compared with rodents SCD promoter region. In addition, we have shown in transient transfection and Northern blot analyses that clofibrate does not affect chicken SCD1 gene expression in contrast with what has been observed in rodents.

MATERIALS AND METHODS

Plasmid preparation and amplification. Fragments −1975/+125, −1610/+125, −1269/+125, −904/+125, −569/+125, and −372/+125 (1 to 6, respectively) corresponding to nested deletions of the 5′ part of the SCD1 promoter were prepared by PCR amplifications using specific primers and a SCD1 genomic DNA cosmid clone (27). These fragments were cloned in the pGL2 basic vector (Promega), upstream the luciferase reporter gene. The SCD1 luciferase plasmid including the −372/+125 promoter fragment 6 was further deleted by *Kpn*I/*Nru*I and *Kpn*I/*Cpo*I to produce fragments 7 and 8, −292/+125 and −159/+125, respectively. The +1/+125 fragment 9 and the −327/+125 fragment were prepared by PCR amplification from plasmid including fragment 6. The pGL2-Promoter vector (Promega) including the simian virus 40 T antigen promoter was used as a control. All constructs were amplified in DH5α *E. coli* strain and purified using a Plasmid Maxi kit (Qiagen).

Cell culture and transfection procedures. The chicken LMH hepatoma cells (28) were grown in Williams' medium (Life Technologies) supplemented with 10% foetal calf serum (FCS), 100 IU/ml penicillin (Life Technologies), and 100 μg/ml streptomycin (Life Technologies). Cultures were incubated at 37°C in an humidified atmosphere containing 5% CO_2 and media were renewed daily.

Transfections were carried out essentially according to Baum *et al.* (29). Confluent cells of a 75 cm^2 culture flask were pelleted and resuspended in 600 μl of culture medium without serum. These cells (400 μl) were subjected to electroporation (180 V, 1050 μF) in the presence of plasmid DNA (10 μg). Immediately after electroporation the cells were resuspended in 8 ml of culture medium and seeded in duplicate in two 25 cm^2 culture flasks. After 24 h, cells were washed in the presence of HEPES buffer and medium without serum was added for a 24 h culture time. Cells were finally cultured in the presence of insulin (10^{-6} M), clofibrate (dissolved in dimethyl sulfoxide), ETYA, and arachidonic acid (dissolved in ethanol), as described in each experiment. Control cells were cultured in the presence of the vehicle alone.

Luciferase activity and protein assays. Treated cells were collected, resuspended in 100 μl of luciferase lysis Buffer (Promega), and incubated 15 min at room temperature. Cell lysates were stored at −80°C before analyses. After centrifugation 2 min at 500*g*, supernatants (20 μl) were incubated 5 s in the presence 50 μl of luciferase assay buffer (Promega) including luciferin (470 μM) and luciferase activity was determined using a luminometer (Dynatec). Activities were expressed as relative light units per mg of protein.

Cellular protein concentrations (means ± S.D.) were determined from triplicates by the method of Bradford (30) using the Biorad protein assay reagent (Biorad Laboratories, Hercules, CA).

RNA purification and analyses. RNA were extracted from transfected cells in 25 cm^2 culture flasks according to Chomczynski and Sacchi (31) with a solution of 4 M guanidium thiocyanate, 0.5% *N*-lauryl sarcosine, 25 mM sodium citrate, and 0.1 M 2-mercaptoethanol. Total RNA were then extracted by phenol-chloroform after addition of acetate and then ethanol precipitated.

Fifteen micrograms of total RNA were analyzed by Northern blot as previously described (32) using an RNA ladder marker (Promega) as molecular weight marker and radiolabelled specific probes isolated in our laboratory (27; C.D. unpublished results).

RESULTS AND DISCUSSION

Effects of Polyunsaturated Fatty Acids and Clofibrate on SCD1 Expression

LMH cells were transfected with a luciferase reporter construct driven by the chicken SCD1 5′-flanking region (−1975/+125 bp). Subsequently, transfected cells were treated with polyunsaturated fatty acids (PUFA) to determine if the chicken SCD1 promoter responds to fatty acids in a similar fashion as the rodent SCD1 promoter (21, 23).

In the presence of arachidonic acid (AA), transiently transfected LMH cells expressed a lower level of luciferase activity when compared to control cells, incubated with the vector alone (Figs. 1A and 1B). This repression increased with the time of treatment (up to 10 h, Fig. 1A) and was the same magnitude with dosages ranging from 25 μM to 200 μM PUFA (Fig. 1B). These results indicate that the chicken SCD1 gene is down-regulated by PUFA as reported in rodents.

Transiently transfected LMH cells were treated with eicosatetrayonic acid (ETYA), a non-metabolizable acetylenic analog of AA, to determine if PUFA repression needs metabolism of fatty acids. Reduction of luciferase activity was also observed when cells were

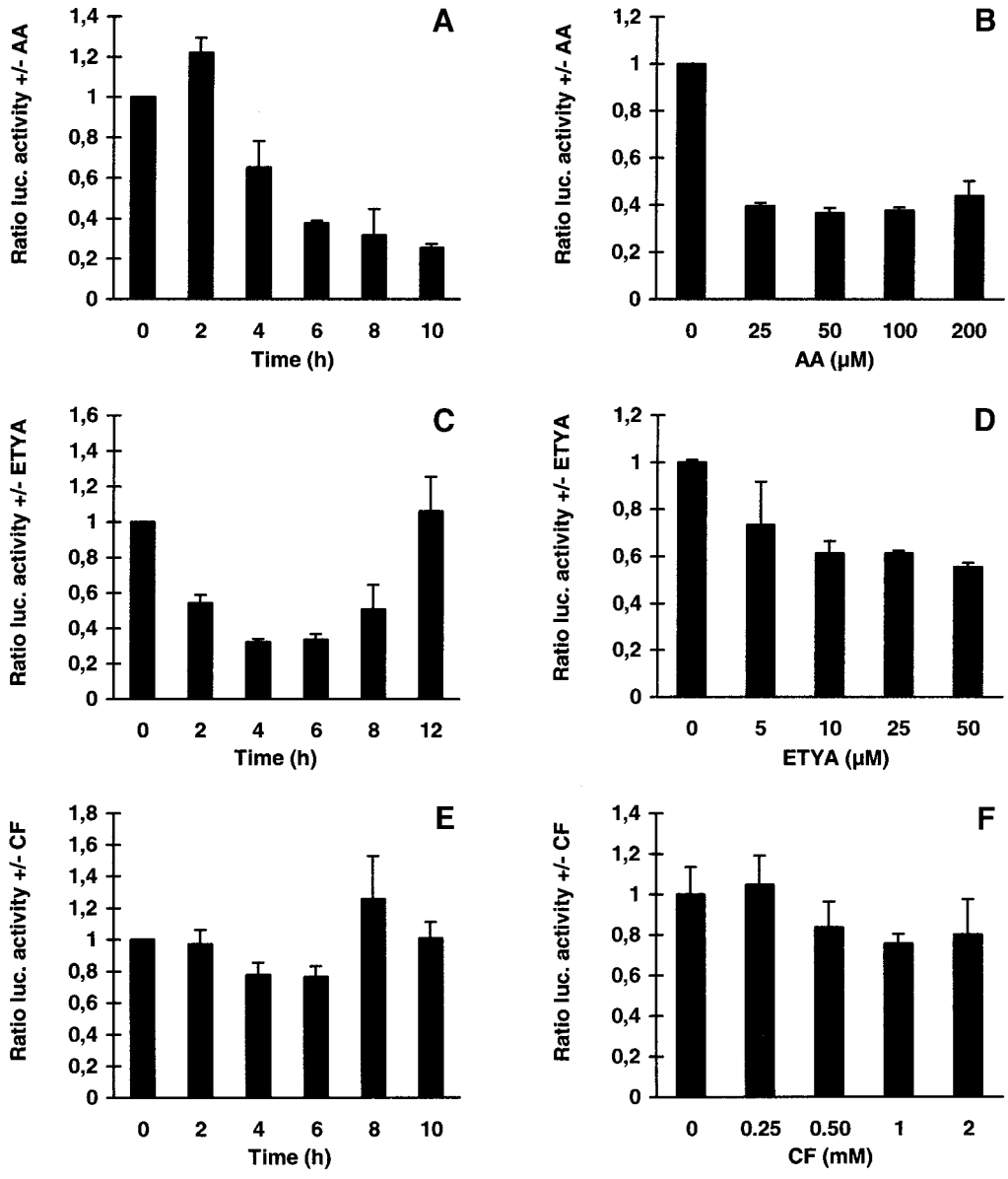

FIG. 1. Time- and dose-response of the chicken SCD1 5′-flanking region to arachidonic acid, eicosatetrayonic acid, and clofibrate. LMH cells were transiently transfected by electroporation with a plasmid including the SCD1 5′ region (−1975/+125 bp) upstream the luciferase reporter gene. Two days after transfection, transfected cells were incubated in the presence of arachidonic acid (AA), eicosatetrayonic acid (ETYA), and clofibrate (CF). Luciferase activities were determined as relative light units at different time of treatment with 100 μM AA (A), 50 μM ETYA (C), and 1 mM CF (E) or after a 6 h treatment with different doses of AA (B), ETYA (D), and CF (F). Results are expressed as a ratio of luciferase activities in the presence and absence of effector (means ± S.D., $n = 3$). All activities were normalized to total protein in cell extracts. Experiments were repeated at least three times with similar results.

treated with ETYA (Figs. 1C and 1D). However, the highest repression (+/− luciferase activity ratio near 0.32) was observed after 4 h of treatment and was decreased thereafter. A clear dose response inhibitory effect was observed, increased from 0.73 to 0.55 when cells were treated with 5 to 50 μM ETYA, respectively.

These results indicate that metabolizable and non-metabolizable PUFA down-regulate SCD1 gene expression. Non-metabolizable molecule has got a transitory action in contrast with metabolizable one for which inhibition appears later but for a longer term. This also suggest that AA is quickly transformed in the cells, and the level of non-metabolised "transcriptional active form" remaining, not significant enough to inhibe SCD1 gene transcription within the two first hours as observed with ETYA. More data are now needed before to draw a conclusion but data concerning glut4 regulation strengthen this hypothesis. Indeed, glut4 gene expression in adipocytes is also down-regulated by AA and ETYA (33). However, when adi-

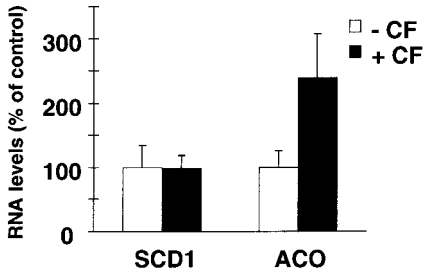

FIG. 2. Northern blot analysis of SCD1 and acyl-CoA oxydase (ACO) mRNA levels in LMH cells incubated in the presence or absence of clofibrate. Total RNA were extracted from LMH cells incubated in the presence (+) or absence (−) of 1 mM clofibrate (CF). They were analyzed by Northern blot using chicken SCD1 and ACO specific probes. RNA levels were determined as relative units (means ± S.D., $n = 3$) and are expressed as a ratio to the SCD1 untreated cells level (−CF). Experiments were repeated three times with similar results.

TABLE 1

Effect of Arachidonic Acid (AA, 100 μM), Eicosatetrayonic Acid (ETYA, 50 μM), and Insulin (10^{-6} M) Supplementation on Levels of Reporter Plasmid Expression in LMH Cells

Reporter plasmids	Effector		
	AA	ETYA	Insulin
	Ratio luciferase activities ± effector		
C	0.92 ± 0.12	1.16 ± 0.53	1.42 ± 0.29
1	0.6 ± 0.09	0.42 ± 0.11	3.55 ± 0.39
2	0.64 ± 0.06	0.51 ± 0.26	3.76 ± 0.21
3	0.6 ± 0.1	0.65 ± 0.16	3.73 ± 0.94
4	0.62 ± 0.05	0.43 ± 0.12	3.01 ± 0.5
5	0.64 ± 0.01	0.44 ± 0.11	4.12 ± 0.53
6	0.53 ± 0.06	0.51 ± 0.06	3.79 ± 0.38

pocytes are treated with a strong cyclooxygenase inhibitor, AA down-regulation is no more observed. Furthermore, prostaglandin E2 mimicks the AA effect on glut4 expression. Thus, PUFA effects could involve two pathways, one of which needing PUFA metabolization.

Fatty acids as well as peroxisome proliferator hypolipidemic drugs have been shown to activate peroxisome proliferator-activated receptors (15). Presence of chicken PPARα mRNA in LMH cells has been recently demonstrated (34). In order to analyse the putative involvement of the peroxisome proliferator activated receptor (PPAR) α in the regulation of chicken SCD1 expression, transiently transfected LMH cells were incubated in the presence of clofibrate, a potent peroxisome proliferator (Figs. 1E and 1F). No significant effect was observed on SCD1 promoter activity, neither after a 24 h treatment with 1 mM clofibrate (Fig. 1E) nor with a 6 h treatment with the higher dosage of 2 mM (Fig. 1F). No effect was also observed on endogenous SCD1 RNA levels in LMH cells (Fig. 2) and in chicken hepatocytes in primary culture (data not shown). Control experiments revealed that acyl-CoA oxydase (ACO) RNA level was increased (2.5-fold) in LMH cells treated in the presence of clofibrate (Fig. 2). These data indicate that the PPARα pathway is effective in LMH cells but is not involved in the regulation of SCD1 expression, even after recruitment with a

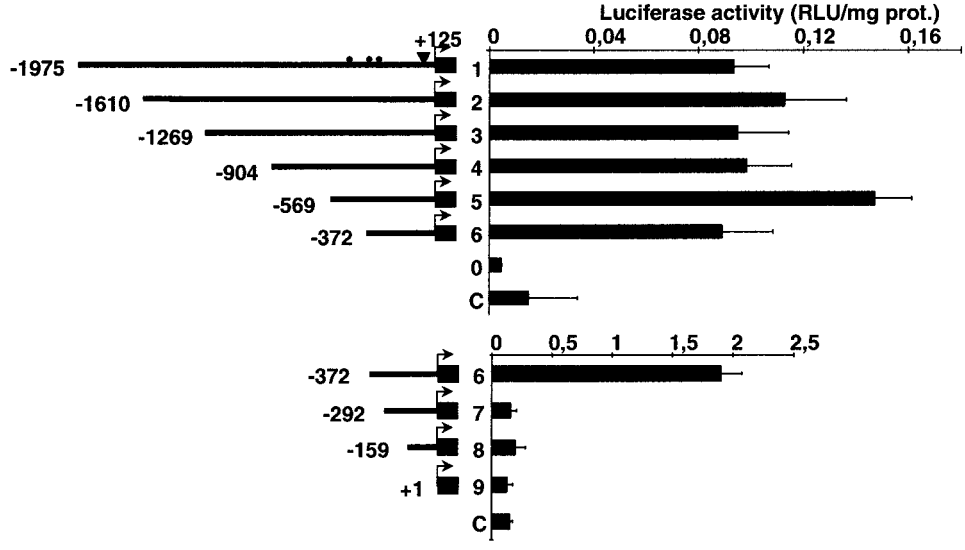

FIG. 3. Analysis of chicken SCD1 5′-flanking region. Using restriction enzyme sites and PCR amplifications, −1975/+125, −1610/+125, −1269/+125, −904/+125, −569/+125, −372/+125, −292/+125, −159/+125, and +1/+125 fragments were cloned in the pGL2 basic vector, upstream the luciferase reporter gene. LMH cells were transfected with the luciferase vectors and then incubated in the presence of fetal calf serum (10%, v/v). Luciferase activities were normalized to total protein in cell extracts and are expressed as relative light units/mg protein (means ± S.D., $n = 3$). Experiments were repeated at least three times with similar results. 0: pGL2 basic vector without SCD1 promoter sequence (negative control); C: pGL2 basic vector including the SV40 early promoter (inactive in LMH cells).

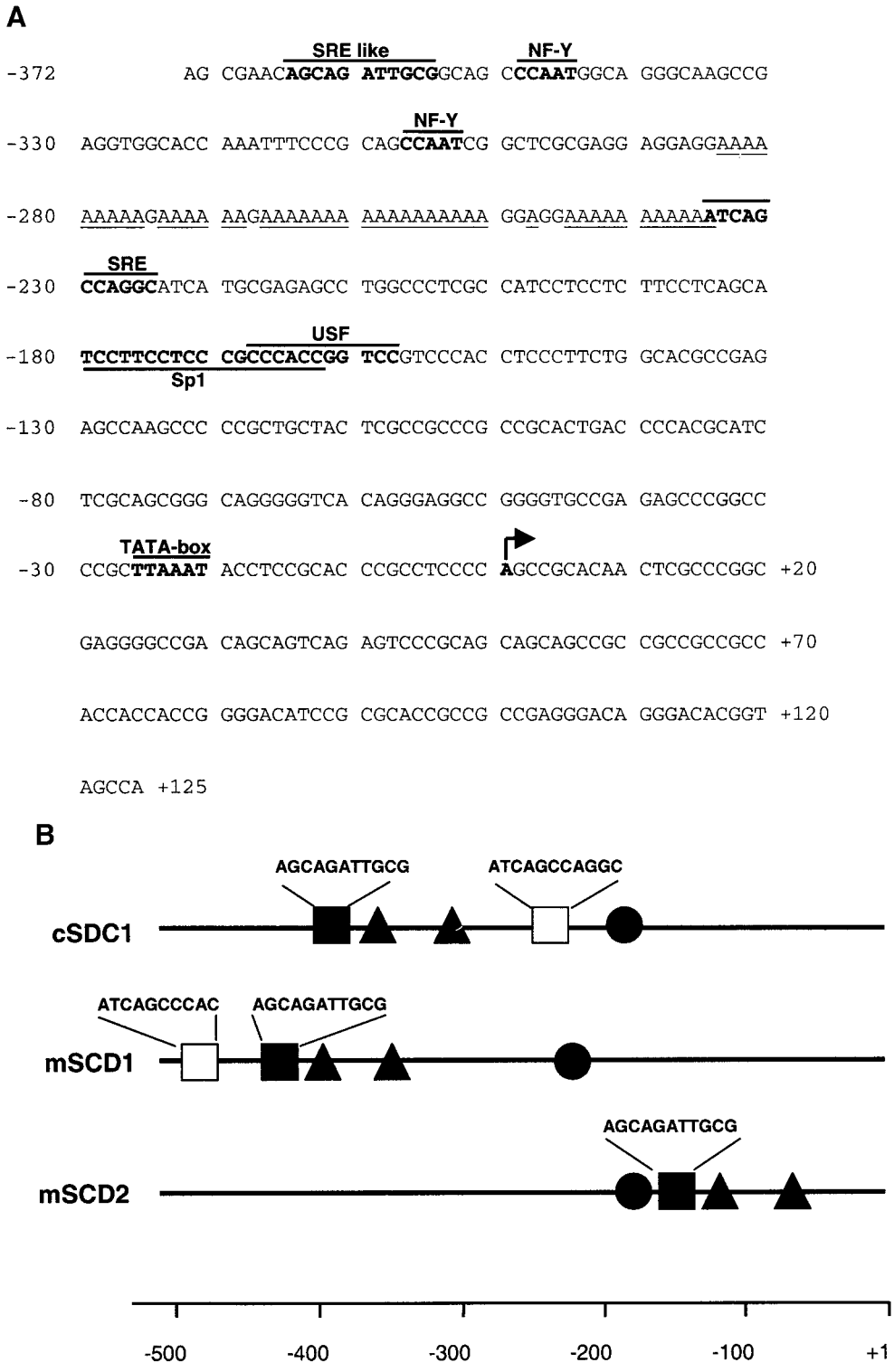

FIG. 4. Sequence analysis of the proximal promoter of the chicken SCD1 gene. The nucleotide sequence of the chicken SCD1 promoter was determined from nucleotide −372 to +125 and analyzed for the presence of putative response elements (A). The numbering is given relative to the transcription start site that is designated as +1 and is indicated by a broken arrow. A schematic representation (according to Tabor *et al.*, 1999) of chicken SCD1 (cSCD1), mouse SCD1 and 2 (mSCD1 and 2) promoters is shown (B). Putative elements that correspond to a classical SRE (□), novel SRE (■), NF-Y (▲), and Sp1 (●) sites are indicated. The poly(A) rich region is also indicated as underlined.

peroxisome proliferator. This is in contrast to results reported by Diczfalusy *et al.* (35) and Miller and Ntambi (24), which demonstrated SCD1 induction by clofibrate in the mouse. This apparent discrepancy is most likely the result of species-specific differences in lipid metabolism observed between rodents and birds.

Localisation of a PUFA Response Region in the SCD1 5′-Flanking Region

To localize *cis*-regulatory elements involved in down-regulation of SCD1 by AA and ETYA, LMH cells were transiently transfected with luciferase reporter constructs carrying chicken SCD1 5′-flanking fragments of decreasing size, i.e., 2100, 1735, 1394, 1029, 694, 497, 417, 284, and 125 bp long. These fragments were cloned upstream the luciferase reporter gene in the pGL2 basic vector and analysed by transient transfection into LMH cells.

In the presence of serum, the basal activity was similar with fragments 1 to 6, including deletions from −1975 to −372 (Fig. 3, upper part). However, when SCD1 5′-flanking sequences were deleted to −292 and further in constructs 7 to 9, luciferase activity was similar to that observed with C and 0 negative controls (Fig. 3, lower part). Another fragment carrying the SCD1 5′-flanking region −327/+125 was prepared and tested in the presence of serum. No luciferase activity was observed (data not shown) suggesting that an important region for SCD1 expression is included within the −372/−327 sequence.

As shown in Table 1, repression by AA and ETYA and activation by insulin were similar using plasmid 1 to 6, suggesting that all elements required for these regulations are included within this short, 496 bp fragment included in plasmid 6.

Promoter Analyses for Putative Response Elements

Fragment 6 was sequenced (Accession No. AJ297918) and compared with rodent SCD1 and 2 promoter sequences (Fig. 4). The results indicated the presence at position −365/−355 of a 5′ AGCAGATTGCG 3′ element similar to that described in mouse SCD1 and 2 genes and shown to be a novel, functional sterol regulatory element (SRE) (36–38).

A CAAT-like, NF-Y element found in the −372/−327 region and shown to play an important role in SCD1 expression in rodents, was also observed at position −349/−345, 5 bp downstream the novel SRE element. The composite NF-Y and SRE motif was similar to that observed in rodent SCD1 and 2 promoters (36–38), and was shown to play a major role in the regulation by PUFA in the mouse (38).

The chicken sequence contains another element which corresponds to an overlapping Sp1 and USF motif at −180/−158. A similar complex element, described in the promoter of fatty synthase, ATP citrate-lyase and leptin rat genes, plays a role in insulin/glucose and PUFA regulation (39–41).

Another element, similar to a classical SRE, already described in mouse SCD1 promoter (38) was also observed in the chicken SCD1 promoter.

Also shown is a poly(A) rich region. The meaning of this region found in the chicken promoter remains to be established.

In conclusion, we demonstrate that SCD1 expression is down-regulated by PUFA by a mechanism which, in contrast to rodents does not involve the PPARα pathway, since the clofibrate peroxisome proliferator was without effect on chicken SCD1 expression. LMH cells represent a good model to analyze the expression of SCD1 in the chicken and to analyze *cis*-regulatory elements involved in PUFA regulation without interference with the PPARα pathway. Two complex regulation motifs for DNA binding proteins, similar to those reported in SCD1 and 2 and other lipogenic genes were found in the chicken SCD1 promoter. They could play a major role in PUFA regulation. Additional investigations will be undertaken to determine the exact role of each component evidenced on the SCD1 promoter in the PUFA and insulin regulations. It will also be interesting to look at sequences upstream the promoter, to identify or not enhancer(s) and or silencer(s) which may be involved in the regulation by these effectors. These may allow us to identify major components of genetic variability in chicken selected in basis of differences in adipose tissue weight.

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