Down-regulation of the mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase gene by insulin: the role of the forkhead transcription factor FKHRL1

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Normal physiological responses to carbohydrate shortages cause the liver to increase the production of ketone bodies from the acetyl-CoA generated from fatty acid oxidation. This allows the use of ketone bodies for energy, thereby preserving the limited glucose for use by the brain. This adaptative response is switched off by insulin rapidly inhibiting the expression of the mitochondrial 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) synthase (HMGCS2) gene, which is a key control site of ketogenesis. We decided to investigate the molecular mechanism of this inhibition. In the present study, we show that FKHRL1, a member of the forkhead in rhabdosarcoma (FKHR) subclass of the Fox family of transcription factors, stimulates transcription from transfected 3-hydroxy-3-methylglutaryl-CoA synthase promoter-luciferase reporter constructs, and that this stimulation is repressed by insulin. An FKHRL1-responsive sequence AAAAATA, located 211 bp upstream of the HMGCS2 gene transcription start site, was identified by deletion analysis. It binds FKHRL1 *in vivo* and *in vitro* and confers FKHRL1 responsiveness on homologous and heterologous promoters. If it is mutated, it partially blocks the effect of insulin in HepG2 cells, both in the absence and presence of overexpressed FKHRL1. These results suggest that FKHRL1 contributes to the regulation of HMGCS2 gene expression by insulin.

Key words: gene expression, ketogenesis.

INTRODUCTION

Mitochondrial 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) synthase (HMGCS2), which catalyses the condensation of acetyl-CoA with acetoacetyl-CoA to form HMG-CoA and CoA in mitochondria, is a key control site of ketogenesis in various hormonal and metabolic situations [1–4]. Thus its mRNA levels rapidly vary according to cAMP, insulin and dexamethasone and greatly increase both in response to starvation, fat-feeding and diabetes and during the early neonatal period [5–9].

To explore the control of the HMGCS2 gene, we examined its transcriptional regulation. Transient transfection experiments and testing of transgenic mice have revealed that a 1149 bp fragment of the 5′-flanking region contains the elements responsible for the multi-hormonal regulation of transcription of this gene [10,11]. The HMGCS2 gene promoter contains a peroxisome proliferator-activated receptor-responsive element, and the activation of HMGCS2 gene expression by fatty acids may be mediated by this receptor [12]. In addition, the enhanced expression of the gene in the liver of starved rats, newborn rats, suckling rats and fat-fed rats may result from the high plasma glucagon and the low plasma insulin levels that prevail in these situations.

In Type I diabetes, stimulated gluconeogenesis consumes most of the available oxaloacetate, but breakdown of fat produces large amounts of acetyl-CoA. This increased acetyl-CoA is normally directed into the tricarboxylic acid cycle, but since oxaloacetate is in short supply, it is used for excess production of ketone bodies. We thus focused on the molecular mechanism by which insulin inhibits the HMGCS2 gene.

This process is only partially understood. Members of the FKHR (forkhead in rhabdosarcoma) subclass of the Fox family of transcription factors, such as FKHRL1, are involved in the regulation of gene expression by insulin. This family has been the subject of research after genetic complementation studies of *Caenorhabditis elegans* identified the Daf-16 gene as a target of the protein kinase B (PKB)/Akt kinase [13]. The Daf-16 gene belongs to the forkhead family of transcription factors and FKHR proteins are the closest mammalian homologues of the Daf-16 gene product [13–15]. They are phosphorylated by PKB/Akt and regulated by phosphorylation in an insulin-dependent or insulin-like growth factor I (IGF-I)-dependent manner [16,17]. FKHR binds to forkhead-binding sites within the previously identified insulin response element (IRE) of the IGF-binding protein 1 (IGFBP-1) promoter and stimulates promoter activity. This stimulation is suppressed by insulin via phosphorylation of FKHR by PKB [18–23]; furthermore, it has been found that the kinase mammalian target of rapamycin (mTOR) is required for full insulin regulation of IGFBP-1 gene transcription [24]. Signalling by PKB to forkhead proteins can also partially account for the regulation of glucose-6-phosphatase [25] or phosphoenolpyruvate carboxykinase [26] promoter activity by insulin.

In the present study, we identify an FKHRL1-responsive sequence located 211 bp upstream of the HMGCS2 gene transcription start site. This sequence binds to FKHRL1 both *in vivo* and *in vitro* and confers FKHRL1 responsiveness on homologous and heterologous promoters, and its mutation in the context of the HMGCS2 promoter partially abolished the insulin-mediated inhibition of the transcriptional activity of this promoter in HepG2 cells. We also demonstrate that the HMGCS2 gene repression by insulin involves the participation of the phosphoinositide 3-kinase (PI-3K)/PKB transduction pathway.

Abbreviations used: CAT, chloramphenicol acetyltransferase; ChIP, chromatin immunoprecipitation; EMSA, electrophoretic mobility-shift assay; FKHR, forkhead in rhabdosarcoma (a transcription factor); HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; HMGCS2, mitochondrial HMG-CoA synthase; IGF, insulin-like growth factor; IGFBP, IGF-binding protein; IRE, insulin response element; IRS, insulin response sequence; mTOR, mammalian target of rapamycin; PI-3K, phosphoinositide 3-kinase; PKB, protein kinase B; TKLUC, thymidine kinase luciferase.

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EXPERIMENTAL

Plasmids

Reporter chloramphenicol acetyltransferase (CAT) construct pSMpCAT1 contains 1148 bp of the 5′-flanking region and the first 28 bp of exon 1 of the rat HMGCS2 gene in the pCAT-BASIC vector (Promega), and has been described previously [10]. PGL3SM1 was constructed by subcloning the −1148 to +28 fragment of pSMpCAT1, obtained by *Sal*I digestion and fill-in, into the pGL3-BASIC firefly luciferase reporter gene (Promega), digested by *Sma*I. pSMpCAT3, pSMpCAT5, pSMpCAT116 and pSMpCAT62 contained progressively smaller fragments of the 5′-flanking region and the same 3′-end point in exon 1 (+28) of the gene as reported elsewhere [12,27]. Mutant pGL3SM1-IRSmut (where IRS is the insulin response sequence) was generated from pGL3SM1, in which the IRE element was mutated by site-directed mutagenesis using overlap-extension PCR [28]. pGL3SM1 (100 ng) was used as a target in two independent PCR reactions (94 °C, 1 min; 55 °C, 1 min; 72 °C, 1 min; 10 cycles). For the first PCR, 5′-CCACAGATGCATACACATAGG-3′, which corresponds to co-coordinates −828 to −808, was used as a forward primer and 5′-TTGTTAGCCTctgcagTGGCTAGCCATGCAAG-3′, which corresponds to coordinates −226 to −195 was used as a reverse primer; here, the nucleotides in lower-case letters correspond to those that have been changed from the wild-type sequence. For the second PCR, the forward primer was 5′-ATGGCTAGCCActgcagAGGCTAACAAGGAAG-3′, which corresponds to coordinates −221 to −190 and the reverse primer was 5′-CTTAGCGAGGCCTCTGCCTG-3′, which corresponds to coordinates −59 to −79. In the subsequent overlap-extension reaction, 200 ng of each PCR product was used as a target and the oligonucleotides corresponding to coordinates −828 to −808 and −59 to −79 were used as forward and reverse primers respectively (94 °C, 1 min; 55 °C, 2 min; 72 °C, 1 min; 10 cycles). The PCR product was cloned in the p-GEM-T vector and subsequently digested by Mph1103I (*Ava*III) and *Stu*I, thus generating an 800 bp fragment, which was subcloned into pGL3SM1Δ800, which corresponded to the plasmid pGL3SM1 previously digested by the same endonucleases, generating the pGL3SM-IRSmut. The IRS–thymidine kinase luciferase (TKLUC) plasmid was constructed by cloning the oligonucleotide 5′-agcttATGGCTAGCCAAAAATAAGGCTAACAAG-3′ (corresponding to coordinates −221 to −195 of the rat HMGCS2 gene) annealed to 5′-tcgacTTGTTAGCCTTATTTTTGGCTAGCCATA (corresponding to coordinates −221 to −195). The nucleotides designated in lower case were added to provide cohesive *Hin*dIII–*Sal*I ends at the 5′- and 3′-termini respectively. pcDNA3-FKHRL1 and pcDNA3-FKHRL1TM (FKHRL1TM, a mutant version of FKHRL1 in which the residues Thr^{32}, Ser^{253} and Ser^{315} have been changed to Ala) were constructed by subcloning the *Hin*dIII–*Xba*I fragments from the pECE HA-FKHRL1 wild-type and pECE HA-FKHRL1TM plasmids [16] into pcDNA3. Both strand sequences of all plasmids were confirmed by the dideoxynucleotide chain-termination method [29] using an automated fluorescence-based system (Applied Biosystems, Warrington, Cheshire, U.K.).

Cell culture and transfections

HepG2 cells were cultured and transfected in Minimal Essential Medium supplemented with 10% foetal calf serum; typically, 5×10^5 cells were co-transfected with 5 μg of the reporter HMGCS2–CAT or luciferase gene constructs, 0.5 μg of an expression vector for the insulin receptor B and, where indicated, 1 μg of effector plasmid expressing full-length cDNA for the FKHRL1 (pECE-FKHRL1) or FKHRL1TM (pECE-FKHRL1TM) transcription factors, or the wild-type or a kinase-inactive (kinase dead) form of PKB. In all luciferase experiments, 50 ng of pRL plasmid (cytomegalovirus promoter-*Renilla* luciferase) was included as an internal control. Transfection experiments were performed by the calcium phosphate method as described elsewhere [30,31]. After 8–10 h, the calcium phosphate–DNA precipitate was removed and cells were incubated with serum-free Dulbecco's modified Eagle's medium supplemented with 1 g/l fatty acid-free bovine albumin (A-9205; Sigma) and harvested for 18–20 h after refeeding. When necessary, bovine pancreas insulin (I-6634; Sigma), LY294002 (440202; Calbiochem, Bad Soden, Germany) or wortmannin (681675; Calbiochem) were added after removing the precipitate.

Luciferase, Bradford and CAT assays

For CAT assays, extracts of harvested cells were prepared by liquid nitrogen freeze–thaw disruption (three times) after resuspension in 50 μl of 0.25 M Tris/HCl (pH 7.5). All samples assayed for CAT activity were first incubated at 65 °C for 5 min. CAT assays were performed for 60 min by the method described previously [30]. Radioactivity of the samples was measured on an LKB-1217 liquid-scintillation counter. For luciferase assays, the cells were harvested by passive lysis and then luciferase assays were performed using the Dual Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. Luciferase activity was measured using TD-20/20 Luminometer (Turner Designs, Inc., Sunnyvale, CA, U.S.A.). Protein content was quantified following the method of Bradford [32]. Both protein quantification and *Renilla* luciferase activity analysis were used to normalize transfection efficiency.

In vitro transcription/translation

cDNA for FKHRL1 was transcribed and translated directly (in rabbit reticulocyte lysate) from 1 μg of pCDNA3-FKHRL1 by using a kit (Promega) according to the manufacturer's instructions. To obtain an unprogrammed lysate as a negative control for the electrophoretic mobility-shift assay (EMSA), a translation reaction was performed with 1 μg of pcDNA3.

EMSA

FKHRL1 (5 μl) synthesized *in vitro* was preincubated on ice for 10 min in 10 mM Tris/HCl (pH 7.5), 80 mM KCl, 4% glycerol, 1 mM 2-mercaptoethanol, 1 mM EDTA and 2 μg of poly(dI/dC). The total amount of reticulocyte lysate (5 μl) was kept constant in each reaction by the addition of unprogrammed lysate. IRS refers to a pair of oligonucleotides containing the fragment corresponding to coordinates −221 to −195 of the rat HMGCS2 gene; IRSmut is the same fragment but with the proposed IRS mutated. Next, 4 ng of either IRS or IRSmut, ^{32}P-labelled by fill-in with the Klenow polymerase, was added, and the incubation was continued for 20 min at room temperature. For competition experiments, a 50-, 100- and 200-fold molar excess of IRS, relative to the labelled probe, was included during preincubation. For the supershift assay, 1 μl of specific FKHRL1 antibody (sc-9813; Santa Cruz) was added to the reaction mixture 10 min after the labelled probe. The final volume for all reactions was 20 μl. Samples were electrophoresed at 4 °C on non-denaturing 4.5% (w/v) polyacrylamide gel in 0.5% TBE buffer (45 mM Tris/45 mM boric acid/1 mM EDTA; pH 8.0).

Chromatin immunoprecipitation (ChIP) assays

Cross-linking between transcription factors and chromatin was achieved in H4IIE cells by adding formaldehyde (final concentration of 1 %) to the culture medium for 10 min. The reaction was stopped by adding glycine at a final concentration of 0.125 M, and the cells were washed three times with cold PBS. Cells were then scraped in PBS, centrifuged and washed twice with PBS containing 1 mM PMSF. The pellets were re-suspended in 2 ml of cell lysis buffer [5 mM Pipes (KOH), pH 8.0/85 mM KCl/0.5 % (v/v) Nonidet P40] in the presence of protease inhibitors (100 mg/ml leupeptin, 100 ng/ml aprotinin and 1 mM PMSF). Samples were incubated on ice for 10 min and subjected to 10 strokes in a Dounce homogenizer. Nuclei were collected by centrifugation at 250 ***g*** for 10 min, and the supernatant was discarded. Nuclear pellets were re-suspended in 0.2 ml of nuclear lysis buffer [50 mM Tris (pH 8.0)/10 mM EDTA/1 % (w/v) SDS + protease inhibitors] and sonicated at full power for four 10 s pulses to shear the chromatin to an average length of < 2 kb. Samples were then diluted by 10-fold with the immunoprecipitation dilution buffer [1 % (v/v) Triton X-100/16.7 mM Tris (pH 8)/1.2 mM EDTA/167 mM NaCl plus the protease inhibitors]. To reduce the non-specific binding, samples were incubated with 80 μl of salmon sperm DNA/Protein G–Sepharose slurry (Sigma) at 4 °C for 1 h on a rotating wheel, and beads were collected by centrifugation at 500 ***g*** for 1 min. Precleared chromatin solutions were incubated either with 15 μg of human FKHRL1 antibody (Santa Cruz) or with no antibody and rotated overnight at 4 °C. Immune complexes were collected after the addition of 80 μl of salmon sperm DNA/Protein G–Sepharose slurry (Sigma) for 4 h with rotation. Samples were then washed four times with 1 ml of wash buffer [0.1 % (v/v) Triton X-100/20 mM Tris (pH 8.0)/150 mM NaCl/2 mM EDTA] and eluted by performing three successive 5 min incubations with 150 μl of elution buffer [1 % (v/v) SDS/50 mM $NaHCO_3$]. The eluates were pooled, NaCl was added at a final concentration of 0.3 M and the samples were incubated at 65 °C for 4 h to reverse the formaldehyde-induced cross-linking. Digestion buffer [10 μl of 2 M Tris (pH 6.8)/10 μl of 0.5 M EDTA/2 μl of proteinase K (20 mg/ml)] was added, and the samples were placed at 45 °C for 2 h. Chromatin DNA was extracted with phenol/chloroform followed by ethanol precipitation. DNA was re-suspended in 50 μl of sterile water, and a 5 μl aliquot was used for PCR analysis. Primers for the amplification of the 291 bp fragment from coordinates −346 to −55 of the HMGCS2 promoter, containing the IRS, were: (1) 5′-ACCACCAGGCAGTACAAGTG-3′ and (2) 5′-TCCACTTAGCGAGGCCTCTG-3′. Following amplification, the PCR products were electrophoresed in a 2 % agarose gel and visualized by ethidium bromide staining.

Western-blot assays

The total extract of control, insulin- and LY294002-treated HepG2 cells was separated by SDS/PAGE (10 % gel) and transferred on to a nitrocellulose membrane. Phospho-PKB (Ser^{473}) (Cell Signaling Technology) or PKB-specific antibodies (Santa Cruz) were used at a dilution of 1:2000. Proteins were detected using the ECL® chemiluminescence system (Amersham Pharmacia Biotech).

Animals

Sprague–Dawley rats weighing 120 g were used. After 24 h of starvation, the rats were intraperitoneally injected with wortmannin in a 4 % methanol–saline solution (1.4 mg/kg) or with vehicle. After 15 min, the rats were injected intraperitoneally with human insulin (100 UI/kg) (Actrapid; Novo-Nordisk, Copenhagen, Denmark) or with saline solution and they were killed 1 h later.

RNA-blot analysis

Total RNA was prepared from rat liver by extraction with guanidine isothiocyanate and centrifugation through a CsCl cushion [31]. Aliquots (15 μg) of RNA (determined at A_{260}) were fractionated on 1 % (w/v) agarose/formaldehyde gels, transferred on to Nytran-N membranes (Schleicher & Schuell) and UV cross-linked. Hybridizations were performed as described previously [31], with a 1.1 kb-specific fragment of the rat HMGCS2 cDNA or with a 600 bp-specific fragment of human β-actin cDNA. Washes were performed either at 65 or 55 °C with 0.1 × SSC and 0.1 % SDS respectively. A 1 h exposure of this blot was analysed with a STORM 840 laser scanning system (Amersham Pharmacia Biotech).

RESULTS

The rat HMGCS2 gene is inhibited by insulin

We aimed to measure the effect of insulin on the transcriptional activity of the HMGCS2 gene. Therefore we transiently co-transfected HepG2 cells with the reporter plasmid pGL3SM1 (a reporter construct containing the luciferase gene under the control of the 5′-flanking region of the HMGCS2 gene promoter from −1148 to +28), together with an expression vector for the insulin receptor B. Cells were incubated for 18–20 h with 10 nM insulin, and luciferase activity was determined as indicated in the Experimental section. As shown in Figure 1(A), insulin halves the activity of HMGCS2. On incubation with increasing amounts of insulin, this transcriptional repression was found to increase until reaching a maximum at 10 nM insulin but did not increase at higher concentrations (Figure 1B). Therefore we used 10 nM insulin for the rest of the experiments.

Transcriptional activation of the HMGCS2 gene by FKHRL1: localization of the target site

As FKHRL1 binds to the IRE in various promoters, we tested whether it affects the HMGCS2 gene in transient transfection experiments. HepG2 cells were co-transfected with an FKHRL1 expression plasmid and an HMG-CoA promoter–CAT reporter plasmid (pSMpCAT1, a reporter construct containing the CAT gene under the control of the 5′-flanking region of the HMGCS2 gene promoter from −1148 to +28). FKHRL1 increased the HMGCS2 promoter activity by 4–5-fold. To locate the control elements that mediate the effects of FKHRL1, we used several chimaeric constructs carrying progressively larger 5′-deletions of the 5′-flanking region of the mitochondrial synthase gene promoter upstream of the CAT gene. The transcriptional activity of these constructs was tested in the presence or absence of the FKHRL1 expression vector. As shown in Figure 2(A), deletion of nucleotides up to position −333 did not compromise the response to FKHRL1, which was greatly diminished by further deletion up to nucleotide −116. Therefore the promoter region between −333 and −116 from the transcription start site was essential for FKHRL1-induced transcription. When this region was analysed, the nucleotide sequence from −211 to −205 showed a perfect match with a binding site for

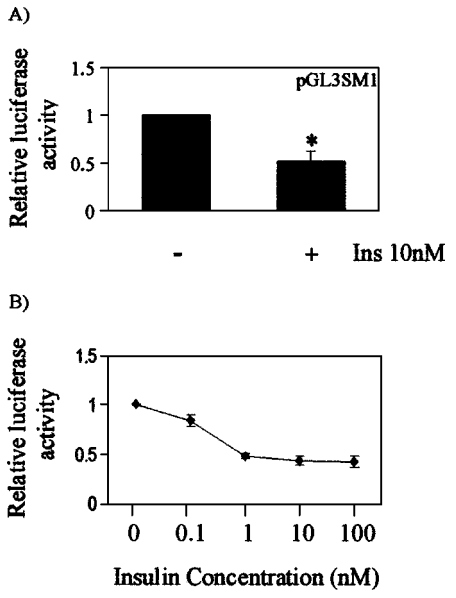

Figure 1 Transcriptional repression of the HMG-CoA synthase gene by insulin and insulin concentration dependence

(**A**) HepG2 cells were transiently co-transfected with 3 μg of the reporter plasmid pGL3SM1 and 0.5 μg of an expression vector for the insulin receptor B and incubated either in the presence or absence of 10 nM insulin (Ins). The results of normalized *Renilla* luciferase activities (means $\pm$ S.D.), from at least three independent experiments with two plates each, are expressed as the relative luciferase activity, whereas the activity in the absence of insulin was defined as 1. (**B**) HepG2 cells were transfected as described above and incubated for 18–20 h in the absence or presence of increasing concentrations of insulin. The results are expressed as the ratio of luciferase activities, corrected for *Renilla* luciferase activities, in insulin-treated versus control cells, and they represent the means $\pm$ S.D. for three independent experiments with two plates each. Statistical analysis of the data confirmed that the repression in transcriptional activity caused by the addition of insulin was significant ($^*P < 0.05$ compared with the luciferase activity in the absence of insulin).

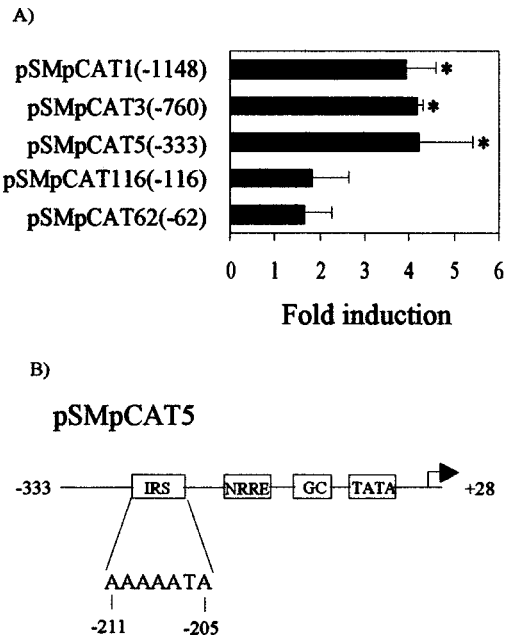

Figure 2 Transcriptional activation of the HMGCS2 gene by FKHRL1 and localization of the FKHRL1 response element by progressive deletion analysis

(**A**) CAT reporter constructs containing the indicated position of the 5′-flanking region of the HMGCS2 gene were transiently co-transfected into HepG2 cells, in the absence or presence of 1 μg of an expression vector for FKHRL1. The results of CAT activities (means $\pm$ S.D.), corrected for the protein concentration in the cell lysate, from at least three independent experiments with two plates each are expressed as fold induction relative to the activity of each construct in the absence of FKHRL1 ($^*P < 0.05$ compared with the basal CAT activity of each construct). (**B**) Scheme of the 5′-flanking region of the HMGCS2 gene from -333 to $+28$.

members of the forkhead family of transcription factors. These results are consistent with previous 'fingerprint' data on the binding of nuclear liver proteins in the same region (results not shown).

The -211 to -205 region of HMGCS2 gene promoter binds to members of the forkhead family of transcription factors

The binding of FKHRL1 to the HMGCS2 gene promoter was confirmed by EMSA. FKHRL1 transcribed and translated *in vitro* was incubated with a labelled fragment containing the putative IRS. Under these conditions, a major complex was observed (Figure 3, lanes 3 and 10). Its specificity was assayed by competition with a 50-, 100- and 200-fold molar excess of unlabelled IRS (Figure 3, lanes 11–13). The identity of the protein in the complex was confirmed by supershift experiments with an antibody raised against FKHRL1, which clearly decreased the formation of the specific complex (Figure 3, lane 4). Mutation of the proposed FKHRL1 binding site obliterates the binding of this transcription factor to the probe (IRSmut) (Figure 3, lanes 5–7).

Endogenous FKHRL1 binds to the HMGCS2 IRS gene promoter

To study whether endogenous FKHRL1 binds to the proposed HMGCS2 IRS, we performed a ChIP assay in which chromatin was precipitated with a specific antibody raised against FKHRL1, and the precipitated fragments were PCR amplified. As shown in Figure 4, a 300 bp fragment was amplified only from chromatin precipitated with the specific antibody (Figure 4, lane 3), whereas it is absent in chromatin in the absence of either antibody (Figure 4, lane 2) or chromatin (Figure 4, lane 4). The results of this experiment, performed twice with two different chromatin preparations, strongly confirms the binding of endogenous HepG2 FKHRL1 to HMGCS2 promoter in its natural context.

Mutation of the HMGCS2 IRS partially suppresses the insulin-mediated repression of basal and FKHRL1-activated transcription

Next, we studied whether the insulin effect was mediated by the IRS element described in the HMGCS2 promoter. HepG2 cells were co-transfected with an expression vector for the insulin receptor B and the reporter plasmids pGL3SM1 or pGL3SM1-IRSmut (a construct in which the IRS was altered by mutation) and incubated with 10 nM insulin. This mutation partially inhibited the response to insulin (Figure 5A). It also repressed the activation of the HMGCS2 gene by FKHRL1 (Figure 5B). Given that insulin repression and FKHRL1 activation were localized at the same responsive element, we studied whether insulin modifies the activity of FKHRL1. The effect of FKHRL1 on the HMGCS2 promoter was inhibited by incubating transfected cells with 10 nM insulin (Figure 5B), indicating that both signals belong to the same pathway.

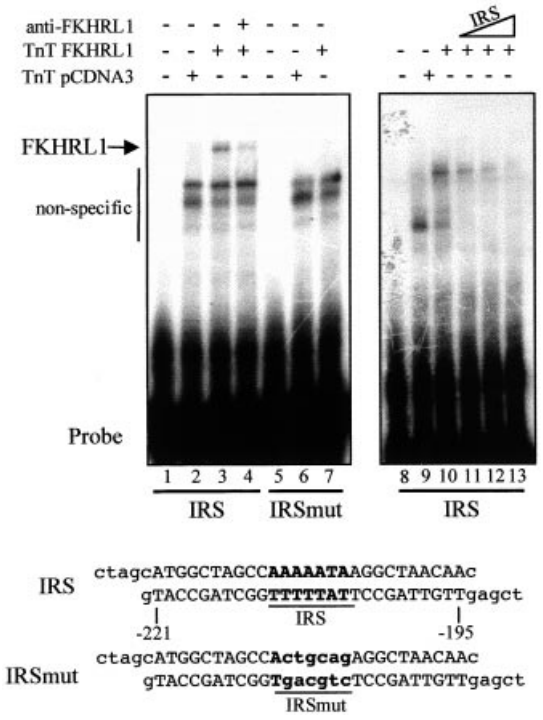

Figure 3 EMSA of the HMGCS2 FKHRL1-responsive element

Two labelled annealed complementary oligonucleotides containing the proposed FKHRL1-responsive element (coordinates −221 to −195 of the mitochondrial synthase promoter) (IRS) were incubated with water (lanes 1 and 8), unprogrammed reticulocyte lysate (lanes 2 and 9) and FKHRL1 translated *in vitro* (lanes 3, 4 and 10–13), and they were then analysed by EMSA. Lane 4 contains an anti-FKHRL1 antibody (1 μl), whereas lanes 11, 12 and 13 contain a 50-fold, 100-fold and 200-fold molar excess of the unlabelled probe respectively. In lanes 5–7, a probe containing a mutation of the proposed element (IRSmut) was incubated with water (lane 5), unprogrammed reticulocyte lysate (lane 6) and FKHRL1 translated *in vitro* (lane 7). The mutated bases are shown at the bottom of the Figure.

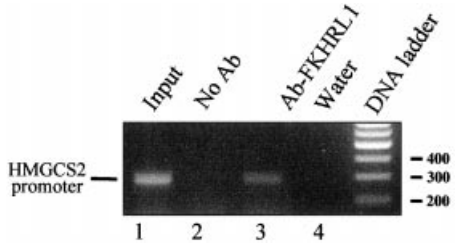

Figure 4 ChIP assay to demonstrate the binding of endogenous FKHRL1 to HMGCS2 gene IRS

A 291 bp fragment of the HMGCS2 gene promoter from coordinates −346 to −55 was amplified by PCR using chromatin immunoprecipitated with an anti-FKHRL1-specific antibody as a template (lane 3). The other lanes show results of the PCR using as a template water (lane 4), a fraction (1%) of the input chromatin without immunoprecipitation (lane 1), or precipitated in the absence of antibody (lane 2).

The proposed IRS confers FKHRL1 and insulin responsiveness to a heterologous promoter

A pair of oligonucleotides containing one copy of the mitochondrial synthase IRS was inserted into TKLUC, a plasmid containing the luciferase gene under the control of the thymidine kinase gene promoter. As shown in Figure 6, this element

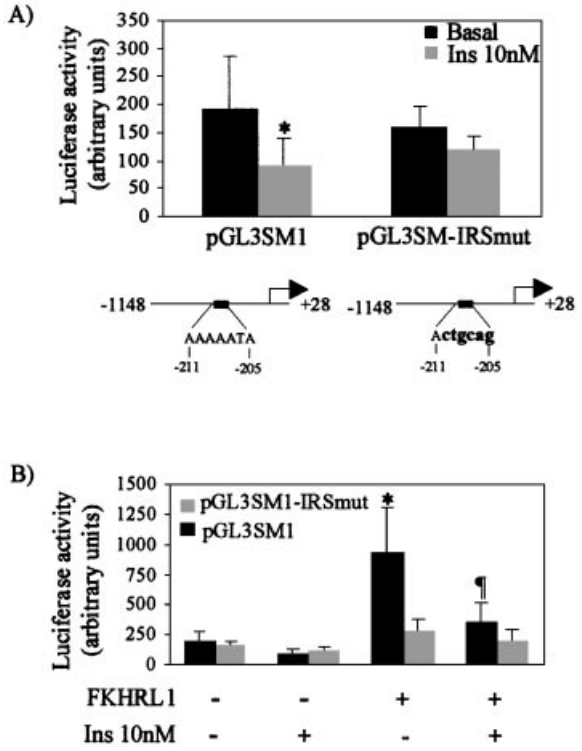

Figure 5 Influence of mutation of the proposed IRS on the effects of insulin and FKHRL1

(**A**) HepG2 cells were co-transfected with 0.5 μg of an expression vector for the insulin receptor B and 3 μg of either the pGL3SM1 or pGL3SM1-IRSmut plasmid (containing a mutated version of the proposed IRS) and incubated in the presence or absence of 10 nM insulin (Ins). The results of normalized *Renilla* luciferase activity (means ± S.D.), from at least three independent experiments with two plates each, are expressed as arbitrary units of luciferase. (**B**) HepG2 cells were co-transfected with 0.5 μg of an expression vector for the insulin receptor B, 3 μg of the pGL3SM1 or pGL3SM1-IRSmut plasmid and 1 μg of an expression vector for FKHRL1 and incubated in the presence or absence of 10 nM insulin, as indicated at the bottom of the Figure. The results of luciferase activities (means ± S.D.), corrected for the protein concentration in the cell lysate, from at least three independent experiments with two plates each, are expressed as arbitrary units of luciferase activity. Statistical analysis of the data confirmed that the increase in transcriptional activity caused by FKHRL1 and the repression caused by insulin on the wild-type construct were significant (*$P < 0.05$ compared with the luciferase activity of the wild-type construct; ¶$P < 0.05$ compared with the activation produced by the FKHRL1 transcription factor).

conferred FKHRL1 and insulin responsiveness on the otherwise unresponsive thymidine kinase gene promoter, confirming that it is essential for the inhibition of the FKHRL1-stimulated HMGCS2 promoter by insulin.

PI-3K mediates the inhibition of HMGCS2 transcription by insulin

Since many of the metabolic activities of insulin can be mediated by a protein kinase cascade that lies downstream of PI-3K, we studied whether the PI-3K transduction pathway was involved in the insulin-mediated transcriptional repression of the HMGCS2 gene. HepG2 cells were transfected with the reporter plasmid pGL3SM1 and incubated with insulin and the PI-3K-specific inhibitors, LY294002 (25 μM) and wortmannin (200 nM), as indicated in Figures 7(A) and 8(A). The presence of the PI-3K inhibitors abolishes the response to insulin, revealing the PI-3K participation in the insulin repression of HMGCS2. We aimed at showing that the concentration of inhibitor used to block the transcriptional repression of the HMGCS2 gene by

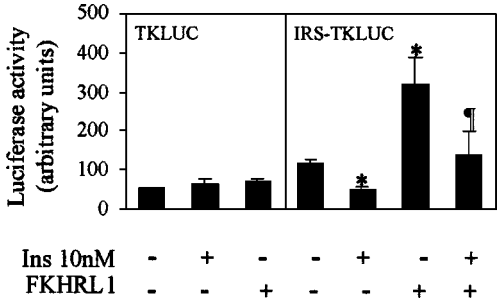

Figure 6 Effect of insulin and FKHRL1 on transcriptional activity of the IRS–TKLUC reporter construct

HepG2 cells were co-transfected with 0.5 μg of an expression vector for the insulin receptor B and the TKLUC or IRS–TKLUC plasmid (containing the luciferase gene under the control of the thymidine kinase promoter and one copy of the FKHRL1 response element of the HMGCS2) in the presence or absence of 1 μg of an expression vector for FKHRL1 and insulin (Ins), as indicated at the bottom of the Figure. The results of luciferase activities (means $\pm$ S.D.), corrected for the protein concentration in the cell lysate, from at least three independent experiments with two plates each, are expressed as arbitrary units of luciferase activity (*$P < 0.05$ compared with the basal luciferase activity of each construct; ¶$P < 0.05$ compared with the activation caused by FKHRL1).

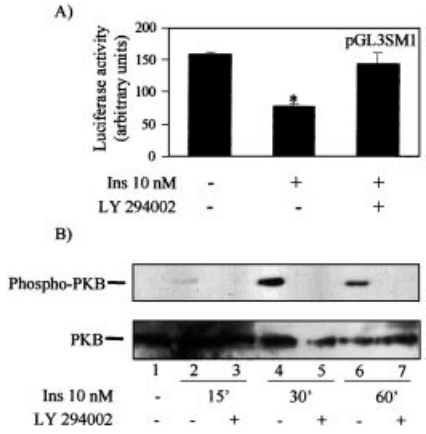

Figure 7 LY294002, a PI-3K-specific inhibitor, obliterates the insulin-mediated transcriptional repression of the HMGCS2 gene

(**A**) HepG2 cells were transiently co-transfected with 3 μg of the reporter plasmid pGL3SM1 and 0.5 μg of an expression vector for the insulin receptor B and incubated in the presence or absence of 10 nM insulin (Ins) and 25 μM LY294002, which was added 30 min before insulin, as indicated at the bottom of the Figure. The results of *Renilla* luciferase activities (means $\pm$ S.D.), from at least three independent experiments with two plates each, are expressed as arbitrary units of luciferase activity (*$P < 0.05$ compared with the luciferase activity in the absence of insulin). (**B**) Western-blot analysis of insulin- and LY294002-treated HepG2 cell extracts (bottom of the Figure) using phospho-PKB (Ser^{473}) or PKB antibodies.

insulin was able to inhibit effectively insulin-induced activation of PI-3K/Akt. Therefore we performed Western-blot assays with a phospho-PKB (Ser^{473}) antibody on HepG2 cell extracts incubated with insulin in the presence or absence of LY294002, as indicated in Figure 7(B). To study the *in vivo* role of the PI-3K transduction pathway in the insulin-mediated transcriptional repression of the HMGCS2 gene, starved rats were treated with insulin or insulin plus wortmannin and the total liver mRNA was analysed by Northern blotting. As shown in Figures 8(B) and 8(C), insulin represses HMGCS2 mRNA expression *in vivo*, whereas this repression is abolished in the presence of wortmannin.

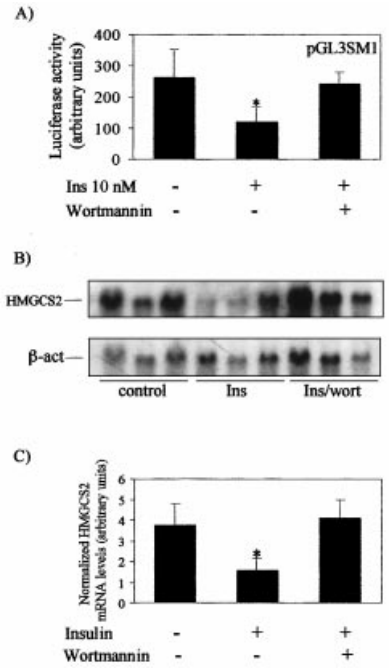

Figure 8 Wortmannin, a PI-3K-specific inhibitor, obliterates the insulin-mediated transcriptional repression of the HMGCS2 gene *in vitro* and *in vivo*

(**A**) HepG2 cells were transiently co-transfected with 3 μg of the reporter plasmid pGL3SM1 and 0.5 μg of an expression vector for the insulin receptor B and incubated in the presence or absence of 10 nM insulin (Ins) and 200 nM wortmannin, which was added 30 min before insulin, as indicated at the bottom of the Figure. The results of normalized *Renilla* luciferase activities (means $\pm$ S.D.), from at least three independent experiments with two plates each, are expressed as arbitrary units of luciferase activity (*$P < 0.05$ compared with the luciferase activity in the absence of insulin). (**B**) Northern-blot analysis of HMGCS2 mRNA levels in control, insulin and wortmannin-treated rats. Total mRNA was also hybridized with a β-actin-specific probe (β-act) for normalization. (**C**) Quantification of the Northern-blot experiment. The blot was analysed with a STORM 840 laser scanning system, and the results (means $\pm$ S.D.) from three rats in each group are expressed as arbitrary units (*$P < 0.05$ compared with the control mRNA levels).

This result confirms the correlation between the *in vitro* results and the *in vivo* situation.

PKB is able to repress both basal and FKHRL1-induced transcriptional activities of the HMGCS2 gene

To confirm the role of PKB activation in the control of HMGCS2 gene expression, we co-transfected HepG2 cells with a luciferase construct, together with an expression vector for an active or a kinase dead form of PKB and FKHRL1. As shown in Figure 9, PKB represses both basal and FKHRL1-induced transcriptional activities of the HMGCS2 gene, whereas the expression of the kinase dead form of PKB does not affect either basal or induced promoter activity. This result confirms the role of PKB in the insulin-induced repression of HMGCS2 gene transcription.

A non-phosphorylatable mutant version of FKHRL1 binds to IRS and transactivates the HMGCS2 gene but is less responsive to insulin than the wild-type form

We aimed at verifying whether the repression of the HMGCS2 promoter by insulin involves the phosphorylation of FKHRL1 by PKB/Akt. Therefore we co-transfected HepG2 cells with the reporter pGL3SM1 and an expression vector for FKHRL1

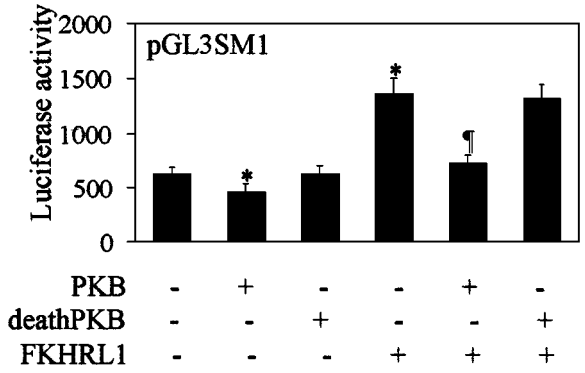

Figure 9 Coexpression of active PKB suppresses the basal promoter activity and FKHRL1-induced transcriptional induction of the HMGCS2 gene

HepG2 cells were transiently co-transfected with 3 μg of the reporter plasmid pGL3SM1 and 0.5 μg of an expression vector for either FKHRL1, the wild-type PKB or the kinase dead form of PKB, as indicated at the bottom of the Figure, and incubated for 24 h. The results of normalized luciferase activities (means $\pm$ S.D.), from at least three independent experiments with two plates each, are expressed as arbitrary units of luciferase activity (*$P < 0.05$ compared with the basal luciferase activity of the construct; ¶$P < 0.05$ compared with the transcriptional activation caused by FKHRL1).

or a non-phosphorylatable mutant of this transcription factor (FKHRL1TM) and incubated the cells either in the presence or absence of insulin. In cells transfected with the mutant FKHRL1, the response to insulin was partially decreased, suggesting that phosphorylation of FKHRL1 by PKB mediates the transcriptional response to insulin (Figure 10A). This mutant can bind to the same sequence as the wild-type factor (Figure 10B).

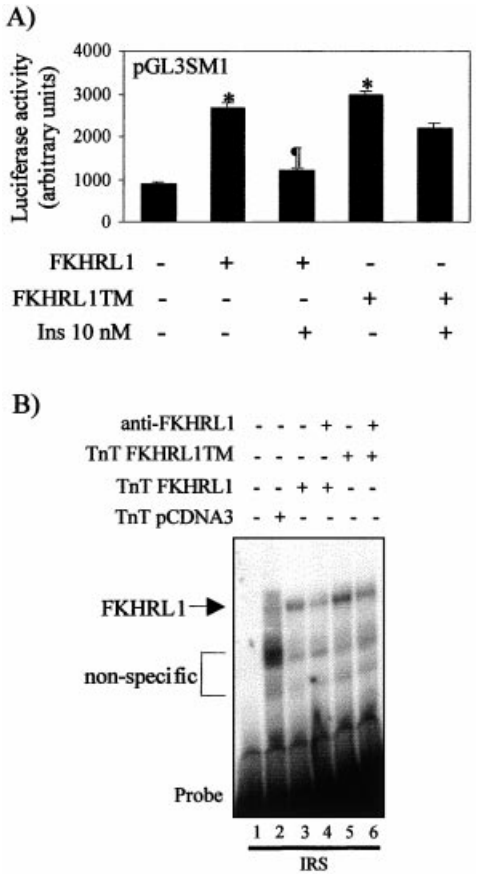

Figure 10 A non-phosphorylatable mutant version of FKHRL1 binds to IRS and transactivates the HMGCS2 gene but is less responsive to insulin than the wild-type form

(**A**) HepG2 cells were co-transfected with 0.5 μg of an expression vector for the insulin receptor B, 3 μg of the pGL3SM1 plasmid and 1 μg of an expression vector for the wild-type FKHRL1 or a version mutated in the PKB phosphorylation sites (FKHRL1TM), and the cells were then incubated in the presence or absence of 10 nM insulin, as indicated at the bottom of the Figure. Results of normalized luciferase activities (means $\pm$ S.D.), corrected for the protein concentration in the cell lysate, from at least three independent experiments with two plates each, are expressed as arbitrary units of luciferase activity (*$P < 0.05$ compared with the basal luciferase activity of the construct; ¶$P < 0.05$ compared with the FKHRL1 transcriptional activation). (**B**) The labelled proposed FKHRL1-responsive element (coordinates -221 to -195 of the mitochondrial synthase promoter) (IRS) was incubated with water (lane 1), unprogrammed lysate (lane 2) and FKHRL1 translated *in vitro* (lanes 3 and 4) or mutated FKHRL1 (lanes 5 and 6) and analysed by EMSA. Lanes 4 and 6 contain an anti-FKHRL1 antibody (1 μl).

DISCUSSION

HMGCS2 is a key enzyme that controls ketogenesis in certain metabolic situations, thus the mRNA and protein levels of HMGCS2 are increased during streptozotozin-induced diabetes and starvation, and these changes are counteracted by insulin or refeeding [5].

We have shown that insulin suppresses HMGCS2 promoter activity. This effect requires a forkhead-binding site in the 5′-flanking region of the gene, in which a mutation partially impairs the inhibition of both basal and FKHRL-induced transcriptional activities by insulin. This sequence binds both *in vivo* and *in vitro* to FKHRL1. The present study has also shown that both a mutated form of FKHRL, which cannot be phosphorylated by PKB, and the PI-3K inhibitors LY294002 and wortmannin abolish the regulation of the HMGCS2 promoter by insulin. Our data provide further evidence of the role of the phosphorylation of the forkhead proteins by PKB in the regulation of glucose homoeostasis, which can also be applied to ketogenesis.

We first showed that insulin inhibits HMGCS2 gene transcriptional activity in a dose-dependent manner to a maximum of approx. 50 % of the activity observed in the absence of insulin. Similar repression levels for other insulin-regulated genes have been reported previously [19].

Members of the FKHR subclass of the Fox family of transcription factors are involved in the regulation of gene expression by insulin [21–23,25]. Therefore we studied whether they also participate in the transcriptional repression of the rat HMGCS2 gene by insulin. FKHRL1 stimulates transcription from transfected HMGCS2 promoter–luciferase reporter constructs. We mapped the sequence responsible for this induction; by deletion analysis and mutation experiments, we showed that the sequence AAAAATA, located 211 bp upstream of the HMGCS2 gene transcription start site, binds to FKHRL1 *in vitro* and that it confers FKHRL1 responsiveness on the homologous and heterologous promoters. The same results were obtained with FKHR, another member of this subfamily of the forkhead family of transcription factors (results not shown). This stimulation is repressed by insulin. Mutation of this sequence partially abolishes the effect of insulin both in the absence or presence of overexpressed FKHRL1. Moreover, ChIP demonstrates that in HepG2 cells without overexpression of FKHRL1, it binds to the HMGCS2 gene promoter in its natural context. This result supports all *in vitro* experiments and includes physiological evidence for the role of forkhead transcription factors in the transcriptional regulation of the HMGCS2 gene. All the above results suggest

that FKHRL1 contributes to the regulation of HMGCS2 gene expression by insulin, although the participation of other factors and the presence of other *cis*-acting sequences cannot be ruled out.

We used different approaches to address the contribution of the phosphorylation of forkhead proteins through the PI-3K/Akt transduction pathway to the effects of insulin on the transcriptional activity of the HMGCS2 gene. Firstly, the presence of different PI-3K-specific inhibitors, such as LY294002 and wortmannin, completely block the effect of insulin on HMGCS2 gene transcription in HepG2-transfected cells, and intraperitoneally injected wortmannin also blocks the insulin-induced repression of HMGCS2 mRNA. The involvement of PI-3K/Akt is reinforced by the results of Western-blot experiments showing that LY294002, at the concentration used in the present study, effectively blocks insulin-induced activation of PI-3K/Akt, parallel with the inhibition of insulin-mediated transcriptional repression of the HMGCS2 gene. Secondly, overexpression of an active form of PKB/Akt, which is a downstream PI-3K target in the insulin-signalling pathway, significantly represses both basal and FKHRL1-induced transcriptional activities of the HMGCS2 gene, whereas a kinase dead form of PKB does not. Thirdly, overexpression of a mutant FKHRL1 that cannot be phosphorylated by PKB partially suppresses inhibition by insulin, which supports the idea that phosphorylation of FKHRL1 by insulin-activated PKB mediates the inhibition of HMGCS2 gene transcription by insulin. Together, these results suggest that signalling via the PI-3K/PKB/forkhead transcription factor pathway is involved in the regulation of this gene by insulin. However, as the activation produced by the overexpression of this non-phosphorylable mutant is still partially suppressed by insulin, other pathways may also be involved. Forkhead proteins regulate the transcription of several genes associated with the homoeostasis of glucose (this activity is suppressed by insulin), e.g. the IGFBP-1 gene [33,34], which is thought to maintain glucose homoeostasis by inhibiting the insulin-like effects of IGF-I and IGF-II, and the gluconeogenic genes phosphoenolpyruvate carboxykinase (PEPCK) [35] and glucose-6-phosphatase [36]. All these genes contain an IRE that binds forkhead proteins [19,21–23,25,26]. Overexpression of FKHR proteins in cells leads to a stimulation of the reporter constructs driven by the promoter of these genes, and this transactivation is blocked by insulin [21,23,25,26]. Although the forkhead proteins are involved in all cases, it has been found that in the case of IGFBP-1, but not glucose-6-phosphatase or PEPCK, mTOR is required for the full insulin regulation of gene transcription [24]. The role of FKHRL1 in the insulin-mediated inhibition of gene transcription has been challenged by experiments with PEPCK and IGFBP-1 IREs [26]. These experiments have shown that although FKHRL1 binds *in vitro* to these sequences and activates transcription through these elements, the specific point mutations in these IREs that abolish insulin repression of transcription do not affect the binding of FKHRL1 to these sequences *in vitro*. Still, it is possible that FKHRL1 is the IRE-specific factor, and differences between *in vivo* and *in vitro* binding to DNA could explain those results. Nevertheless, the *in vivo* binding of FKHRL1 to the IRS sequence is well demonstrated in the present study. The ChIP assay confirms that endogenous HepG2 FKHRL1 occupies the HMGCS2 gene promoter in its natural context.

The present study supports the role of forkhead proteins in the regulation of another gene associated with glucose homoeostasis, namely HMGCS2. This enzyme has been proposed as a key control site in the ketogenesis pathway, which produces ketone bodies that can be used as a metabolic fuel during catabolic states such as starvation and diabetes. We have identified an IRS in the 5′-flanking region of this gene that contributes to the repression of transcription induced by insulin in HepG2 cells, although we cannot rule out the participation of other *cis* elements or the binding of other *trans*-acting factors. Also, since our expression studies were performed in the HepG2 cell line, further studies in hepatocytes may be required to investigate the physiological significance of these findings.

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