Promoter I of the ovine acetyl-CoA carboxylase-α gene: an E-box motif at -114 in the proximal promoter binds upstream stimulatory factor (USF)-1 and USF-2 and acts as an insulin-response sequence in differentiating adipocytes

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Acetyl-CoA carboxylase-α (ACC-α) plays a central role in co-ordinating *de novo* fatty acid synthesis in animal tissues. We have characterized the regulatory region of the ovine ACC-α gene. Three promoters, PI, PII and PIII, are dispersed throughout 50 kb of genomic DNA. Expression from PI is limited to adipose tissue and liver. Sequence comparison of the proximal promoters of ovine and mouse PIs demonstrates high nucleotide identity and that they are characterized by a TATA box at -29, C/EBP (CCAAT enhancer-binding protein)-binding motifs and multiple E-box motifs. A 4.3 kb ovine PI-luciferase reporter construct is insulin-responsive when transfected into differentiated ovine adipocytes, whereas when this construct is transfected into ovine preadipocytes and HepG2 cells the construct is inactive and is not inducible by insulin. By contrast, transfection of a construct corresponding to 132 bp of the proximal promoter linked to a luciferase reporter is active and inducible by insulin in all three cell systems. Insulin signalling to the -132 bp construct in differentiated ovine adipocytes involves, in part, an E-box motif at -114. Upstream stimulatory factor (USF)-1 and USF-2, but not sterol regulatory element-binding protein 1 (SREBP-1), are major components of protein complexes that bind this E-box motif. Activation of the 4.3 kb PI construct in differentiated ovine adipocytes is associated with endogenous expression of PI transcripts throughout differentiation; PI transcripts are not detectable by RNase-protection assay in ovine preadipocytes, HepG2 cells or 3T3-F442A adipocytes. These data indicate the presence of repressor motifs in PI that are required to be de-repressed during adipocyte differentiation to allow induction of the promoter by insulin.

Key words: adipose tissue, HepG2 cells, insulin signalling, lipogenesis, preadipocyte.

INTRODUCTION

Acetyl-CoA carboxylase-α (ACC-α; EC 6.4.1.2), is the flux-determining enzyme in the regulation of fatty acid synthesis [1–5]. The ACC-α gene gives rise to a 265 kDa enzyme which is expressed in all cell types but demonstrates elevated expression in the lipogenic tissues of liver and adipose tissue and also mammary gland during lactation [4–7]. Expression of ACC-α is regulated in a complex fashion both in the short term, through allosteric mechanisms [8] and reversible phosphorylation on a number of critical serine residues [9–13], and chronically, through regulation of transcription of the gene [14]. An isozyme, ACC-β, is transcribed from a distinct but related gene, and gives rise to a 275–280 kDa protein that is the major form in heart and skeletal muscle where it is implicated in the regulation of fatty acid β-oxidation in mitochondria [15–18].

Transcription of the ACC-α gene is initiated from three promoters; promoter I (PI), II (PII) and III (PIII) [1,2,19]. Transcripts from PI and PII give rise to the same protein. In addition, alternative exon splicing results in transcripts that include or exclude a 47 nt sequence corresponding to exon 4 such that E(1/4/5) and E(1/5) transcripts are derived from PI, and E(2/4/5) and E(2/5) transcripts are derived from PII. Transcription from PIII results in a transcript that give rise to an N-terminal variant of ACC-α that demonstrates a tissue-restricted mode of expression [19]. This mRNA diversity is found in all mammalian species studied, with the exception that to date PIII transcripts have only been characterized in ovine tissues [1–3,19,20]. In rodents PI transcripts have been demonstrated to be exclusively expressed in the adipose tissues of *ad libitum*-fed animals [6]. In sheep PI transcripts comprise approx. 65% of total ACC-α transcripts in adipose tissue [21]. Expression of PI transcripts in adipose tissue of both species is directly related to the insulin status of an animal or the insulin sensitivity of adipose tissue. In rats streptozotocin-induced diabetes markedly suppresses PI transcripts in adipose tissue and administration of insulin increases levels of PI transcripts to those of non-diabetic animals within 6 h [6,22]. In sheep, lactation markedly suppresses the rate of fatty acid synthesis and levels of ACC-α mRNA in adipose tissue; this is associated with a state of hypoinsulinaemia

Abbreviations used: ACC-α, acetyl-CoA carboxylase-α; BAC, bacterial artificial chromosome; bHLH, basic helix-loop-helix; C/EBP, CCAAT enhancer-binding protein; IRS, insulin-response sequence; PI, PII, PIII, promoters I, II and III; RT-PCR, reverse transcriptase PCR; SREBP, sterol regulatory element-binding protein; USF, upstream stimulatory factor; FAS, fatty acid synthase.

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The nucleotide sequence data reported here will appear in the EMBL, Genbank and DDBJ Nucleotide Sequence Databases under the accession numbers AJ292285 and AJ308471.

and a decline in adipose tissue insulin sensitivity [23,24]. Adipose tissue repression of the ACC-α gene in lactation is largely promoter-specific, in that levels of PI transcripts are potently repressed whereas those of PII are diminished to a lesser extent [21].

In view of the marked sensitivity of PI transcripts to insulin *in vivo* and a deficit of knowledge of the molecular mechanisms of how insulin regulates expression from the PI promoter we have attempted to identify motifs that are responsible for tissue-specific and insulin-regulated expression. In this study we have used a recently characterized ovine adipocyte culture model [25] in which the pattern of expression of PI and PII transcripts is similar to that observed in ovine adipose tissue *in vivo*, and where expression of PI transcripts is differentiation-dependent, in order to delineate these motifs in the ovine PI promoter.

EXPERIMENTAL

Genomic library screening

An ovine genomic library, comprising 15–23 kb of partially digested *Sau*3AI restriction endonuclease fragments ligated into bacteriophage vector λGEM-11 (Promega), was hybridized with DNA fragments corresponding to exons 1 and 5 of the ovine ACC-α cDNA [3], as described previously [19]. Briefly, the library corresponding to 1.8×10^6 individual recombinants was plated on the KW-251 host. Aliquots of the library were replica-plated and screened with radioactively labelled [26] ovine ACC-α cDNA as above. After several rounds of screening, positive recombinant plaques were purified to homogeneity and the resulting bacteriophage DNA was isolated by standard methods [27]. An ovine genomic clone (ovBAC1; where BAC is bacterial artificial chromosome) in the vector pBeloBAC 11 [28] was isolated using a PCR strategy in which clones were selected on the basis that DNA from progressively diluted pools of clones contained sequence corresponding to exons 1 and 5. The sequences of the primers were as follows: exon 1 upstream primer, 5′-CCCTGGACAAACCTGCCGGCTGAGCA-3′; exon 1 downstream primer, 5′-CTGCTTGTCCGTCTTCAGGTCGCGTC-3′; exon 5 upstream primer, 5′-CAAACCTCTGGAGCTGAACCAGCACT-3′; exon 5 downstream primer, 5′-TCTGAGCTGACAGAGGCTGGTGACAG-3′. After an initial denaturation at 94 °C for 5 min, 30 cycles of amplification were performed as follows: denaturation at 92 °C for 30 s, annealing at 58 °C for 30 s and extension at 72 °C for 30 s. A murine (strain 129) BAC containing exon 1 was isolated from a commercial library (Research Genetics, Huntsville, AL, U.S.A.) using a similar strategy. The sequences of the primers were as follows: exon 1 upstream primer, 5′-CTCTGGATAAACCTGAATGCTTGACCA-3′, exon 1 downstream primer, 5′-TCAGTCTTCACTTTGCCTTTGGGAGGC-3′; PCR was performed as above. BAC DNA was isolated using a modification of the basic plasmid alkaline lysis procedure [27], with the exception that the phenol/chloroform step was omitted. DNA restriction endonuclease fragments from bacteriophage and BAC clones were subcloned into plasmid vectors (pGEM-7zf+; Promega) using standard protocols [27]. DNA was sequenced either manually using standard protocols [29] or commercially by automated means (MWG Biotech AG, Ebersberg, Germany).

RNase-protection assays

Use of riboprobes p1AEX and p3E2 to measure transcripts comprising exon 1 and exon 2 respectively as leader exons have been described previously [21]. To map the start site of exon 1, a probe (p1st) corresponding to the 5′ portion of clone N9 [3] and 216 nt of upstream flanking genomic sequence was generated by PCR with primers (5′-AAGGGAGGCTCTAGTCATAA-3′ and 5′-AGATAGGACAGCTAGGCCTT-3′) and cloned into plasmid pGEM-7zf+; antisense and sense transcripts were generated with SP6 and T7 RNA polymerases respectively. A human E(1/4/5) cDNA was generated by PCR of cDNA prepared from HepG2 cells; the upstream primer was 5′-GAAGCTGCTCGTGGATGAACCAGACTG-3′, and the downstream primer was 5′-TCTGAGCCAACAGAAGCAGGTGACAA-3′. Similarly, a murine E(1/4/5) cDNA was generated by PCR of cDNA prepared from mouse adipose tissue RNA. Total RNA was isolated from various sheep tissues by using guanidinium thiocyanate and centrifugation through CsCl [30]. Samples (10–30 μg) of total RNA were precipitated with 1.5 ng of labelled antisense cRNA, and then resuspended in 40 μl of 5 M guanidinium thiocyanate/100 mM EDTA, pH 8.0. Similarly 30 μg of tRNA were precipitated with various amount of sense transcript and treated as above, and used as positive controls. The samples were incubated first at 70 °C for 5 min and then overnight at room temperature. After RNase treatment to remove unhybridized RNA, samples were extracted with phenol/chloroform and precipitated with ethanol. After centrifugation and drying, samples were resuspended and resolved on 6 % (w/v) polyacrylamide/7 M urea gels with Tris/borate/EDTA buffer. Gels were dried and exposed to Kodak phosphor screens. The resulting images were then scanned with a Molecular Dynamics PhosphorImager 445 SI and the volume of individual bands was obtained by using IMAGEQUANT software.

Cell culture, transfection and luciferase assays

Fragments corresponding to $-4359/+138$ and $-132/+15$ of PI generated by PCR with *Pfu* DNA polymerase were subsequently sequenced and ligated into the pGL3basic vector (Promega). Site-directed mutagenesis was performed with *Pfu* DNA polymerase using a method based on the QuikChange mutagenesis kit (Stratagene). All mutant plasmids were sequenced to confirm the mutation. All culture media and supplements were from Gibco-BRL unless otherwise stated. A human hepatoma cell line, HepG2, was grown in minimal essential medium with Earle's salts supplemented with 2 mM glutamine, non-essential amino acids and 10 % (v/v) foetal calf serum.

Cells were plated into 24-well plates at a density approximating 50 % confluence 24 h before transfection. Murine 3T3-F442A cells, an embryonic preadipocyte cell line, were grown in Dulbecco's modified Eagle's medium supplemented with 10 % (v/v) foetal calf serum, 2 mM glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin. At 2 days post-confluence cells were induced to differentiate by incubation in the above medium with the addition of 170 nM insulin (Sigma). Stromovascular (preadipocyte) cells were isolated from sheep adipose tissue as described previously [25] and grown in M199 supplemented with 2 mM sodium acetate, 20 % (v/v) newborn calf serum, 100 units/ml penicillin and 100 μg/ml streptomycin. Cells were either plated at 50 % confluence for transfection the following day or, after 2 days at confluence, cells were rinsed twice with PBS and differentiated in Dulbecco's modified Eagle's medium/Ham's F12 supplemented with 2 mM sodium acetate, 280 nM insulin, 2 nM 3,3′,5-tri-iodo-L-thyronine ('T_3'; Sigma), 10 nM dexamethasone (Sigma) and 10^{-7} M BRL 49653 (a peroxisome-proliferator-activator receptor-γ which was a gift from SmithKline Beecham) for 5 days before transfection. All cells were transfected in 24-well plates with 1 μg of plasmid and a 2:1 charge ratio of Tfx-50 reagent/well using the manu-

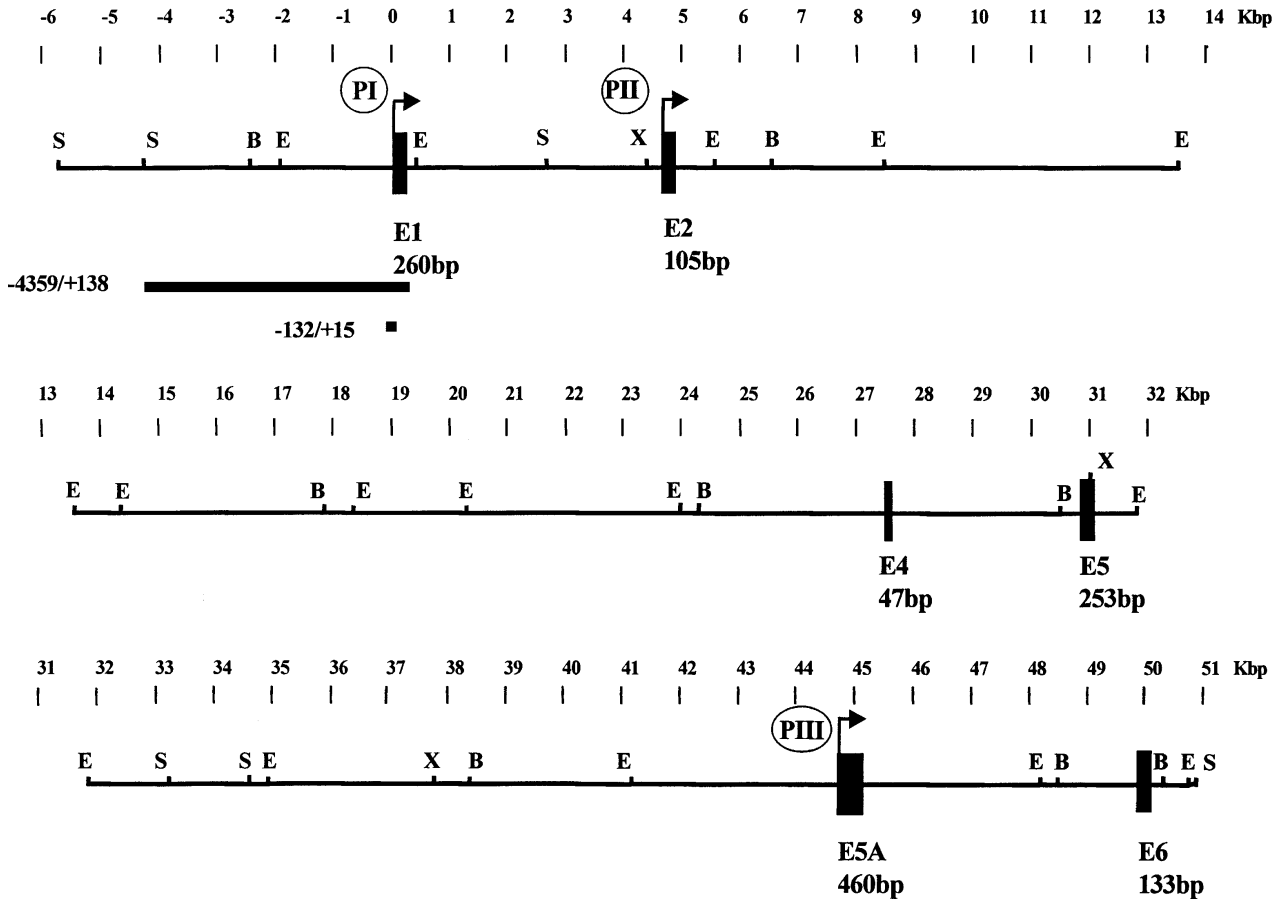

Figure 1 Structure of the 5′ region of the ovine ACC-α gene

The figure shows a restriction map of the region encompassing exon 1 and exon 6 of the ovine ACC-α gene, demonstrating the relative positions of the exons (E) and the promoters PI, PII and PIII. The thick black lines denote the location of sequences within the −4359/+138 and −132/+15 PI-luciferase reporter constructs. B, *Bam*HI; E, *Eco*RI; S, *Sac*I; X, *Xho*I.

facturer's protocol for the transfection of adherent cells (Tfx-50 is a liposome-mediated transfection reagent obtained from Promega). Transfected cells were maintained in culture for a further 48 h in the presence or absence of insulin (280 nM). Cell extracts were then prepared and assayed for luciferase activity with the Promega Luciferase Assay System. The luciferase activity for each well was then normalized for the relative transfection efficiency of plasmid DNA in each well, determined by dot-blotting a portion of the cell extract on to Biotrans nylon membrane (ICN) and hybridizing this to the pGL3 basic vector [31]. In brief, a 15 μl portion from a total of 100 μl of cell extract was boiled for 5 min, diluted with 20 × SSC (3 M NaCl/0.3 M sodium citrate) and dot-blotted on to nylon membrane. The DNA was UV-fixed to the membrane and hybridized to the pGL3basic vector labelled with [^{32}P]dCTP with random primers [26]. The filter was then washed and exposed to a Kodak phosphor screen overnight. The resulting images were then scanned and the volumes of individual dots determined as described above. The luciferase activity was then expressed as the luciferase activity/well divided by the relative amount of plasmid DNA/well (in arbitary units).

Reverse transcriptase PCR (RT-PCR)

Total RNA was isolated as above. cDNA was prepared from 2 μg of this RNA by using Moloney murine leukaemia virus reverse transcriptase (Gibco-BRL) and the synthesis was primed with an oligonucleotide (5′-CAGAGGTTTGGCCAAGGAA-GAAGGTTCACCC-3′) antisense to nt +322 to +352 of the ovine ACC-α cDNA [3]. Single-stranded cDNA was purified through a Sephadex G-50 column and precipitated. Approx. one-quarter of this cDNA was used to amplify a region of DNA with a downstream primer (5′-TGTTCCTTCAAAACGAGC-CCTCAGAG-3′) antisense to exon 5 of the ovine ACC-α cDNA and an upstream primer (5′-GGACGCTGCCGCCGCCGC-CTTGAG-3′) lying within exon 2. After an initial denaturation at 94 °C for 5 min, 35 cycles of amplification were performed as follows: denaturation at 92 °C for 30 s, annealing at 60 °C and extension at 72 °C for 30 s. The reaction products were ligated into the pCR-Blunt II TOPO (Invitrogen) cloning vector. Plasmids were purified from recombinant colonies and sequenced as above.

Gel mobility-shift assays

Nuclear extracts were prepared from cultured ovine adipocytes on day 7 of the differentiation procedure. To prepare nuclear extracts, cells (adipocytes, preadipocytes, HepG2 cells) were resuspended in two packed cell vol. of solution I (10 mM Hepes, pH 7.9, 0.3 M sucrose, 10 mM KCl, 1.5 mM $MgCl_2$, 0.1 mM EGTA, 0.5 mM dithiothreitol and 0.5 mM PMSF) on ice. Cells were lysed in a Dounce homogenizer using 15 strokes. The

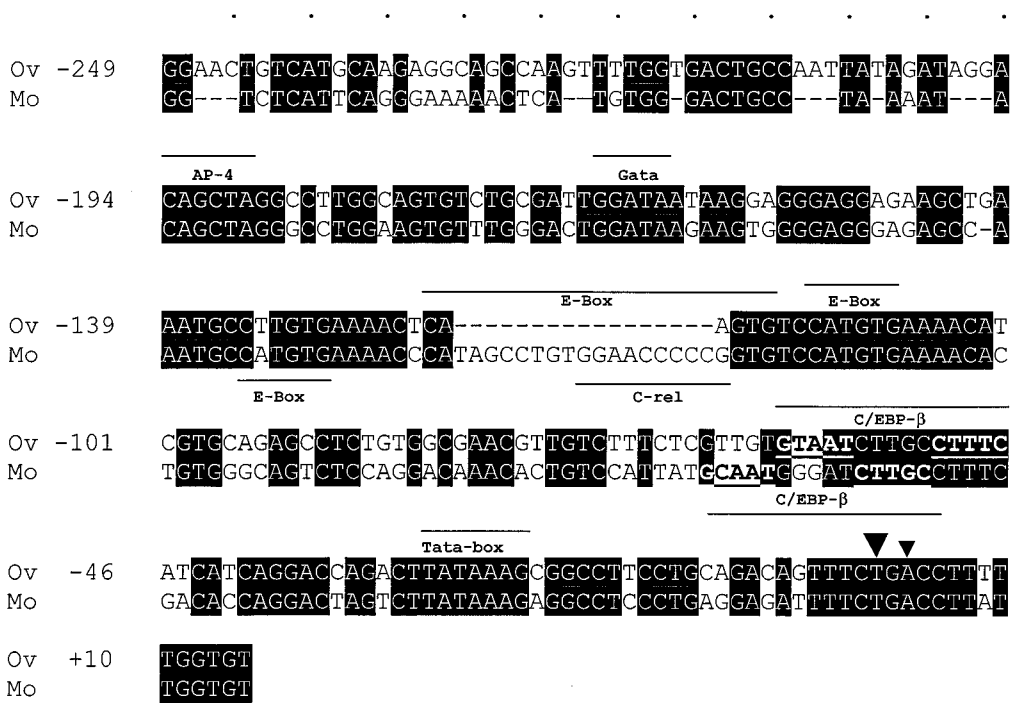

Figure 2 Multiple alignment of the proximal region of ovine and mouse PI

The nucleotide sequence from −249 to +15 nt of the ovine PI aligned with the corresponding region in the murine ACC-α gene, illustrating the position of the start sites (▼) mapped by the RNase-protection assay (see Figure 3). Binding sequences for various transcription factors are indicated either above (sheep) or below the alignment (mouse); AP-4, activator protein 4.

homogenate was centifuged at 1600 ***g*** for 7.5 min at 4 °C. The resultant nuclei-enriched fraction was resuspended in 2.5 vol. of solution II (10 mM Hepes, pH 7.9, 400 mM NaCl, 1.5 mM $MgCl_2$, 0.1 mM EGTA, 0.5 mM dithiothreitol, 0.5 mM PMSF and 5 %, v/v, glycerol) and extracted at 4 °C for 30 min with continuous stirring. The extract was centifuged at 16000 ***g*** for 30 min at 4 °C and the protein content determined (protein assay reagent; Bio-Rad, Hemel Hempstead, Herts., U.K.). Gel mobility-shift assays were performed with adipocyte nuclear extract as follows. Nuclear-extract protein (3 μg) was added to a 20 μl binding reaction containing 15 mM Hepes, pH 7.9, 3 mM $MgCl_2$, 60 mM KCl, 1 mM EDTA, 5 mM dithiothreitol, 8 % (v/v) glycerol, 2.5 μg of BSA, 0.1 μg of poly(dI-dC) and 3×10^4 c.p.m. of ^{32}P-labelled oligonucleotide (1 ng), with or without competitors at a 100× molar excess. The total concentration of monovalent cation was 105 mM. The following complementary pairs of single-stranded oligonucleotides were synthesized by MWG-Biotech AG: ACC-PI IRS (insulin-response sequence), 5′-CTCAAGTGTCCATGTGAAAA-3′ and 3′-GAGTTCACAGGTACACTTTTGTAG-5′; ACC-PI IRS mE2, 5′-CTCAAGTGTCGAATTCAAAACATC-3′ and 3′-GAGTTCACAGCTTAAGTTTTGTAG-5′; ACC-PI IRS mE1, 5′-CTACAGGTTCCATGTGAAAACATC-3′ and 3′-GATGTCCAAGGTACACTTTTGTAG-5′; ACC-PI IRS mE1E2, 5′-CTACAGGTTCGAATTCAAAACATC-3′ and 3′-GATGTCCAAGCTTAAGTTTTGTAG-5′; ACC-PI IRS mdn, 5′-CTCAAGTGAGCATGTGAAAACATC-3′ and 3′-GAGTTCACTCGTACACTTTTGTAG-5′; rat FAS IRS (where FAS is fatty acid synthase), 5′-AGCTGTCAGCCCATGTGGCGTGGCCGC-3′ and 3′-AGTCGGGTACACCGCACCGGCGTCGAC-5′; rat FAS sterol regulatory element, 5′-CGCGCGCGGGCATCACCCCACCGACGG-3′ and 3′-GCCCGTAGTGGGGTGGCTGCCGCCG-5′.

Probes were made by labelling with [α-^{32}P]dCTP, or non radioactive dNTPs, and Klenow fragment of *Escherichia coli* DNA polymerase to create blunt-end probes as required. After 1 h of incubation at room temperature the binding reactions were applied to a 4 % non-denaturing polyacrylamide gel run in 1× gel running buffer (25 mM Tris, pH 8.3, 190 mM glycine and 1 mM EDTA) at 20 mA constant current for 1 h. For antibody 'supershift' assays, 2 μl of 2 μg/μl anti-USF-1 (C-20), anti-USF-2 (C-20; where USF is upstream stimulatory factor) or anti-SREBP-1 (H-160; where SREBP is sterol regulatory element-binding protein) antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) were added to the binding reaction in the last 30 min; antibodies cross-reacting with the corresponding proteins in ovine tissues were identified by Western blotting. Gels were dried and exposed to Kodak phosphor screens. The resulting images were scanned with a Molecular Dynamics PhosphorImager 445 SI.

RESULTS

Structural features of the 5′ region: species comparisons

The restriction map of a contiguous 50 kb DNA sequence encompassing promoters PI, PII and PIII of the ovine ACC-α gene is shown in Figure 1. The map was derived from genomic DNA libraries cloned in BAC (ovBAC1) and bacteriophage lambda vectors. OvBAC1 was isolated on the basis that it contained sequence corresponding to exon 1 and exon 5. It did not comprise sequences corresponding to exon 5A or exon 6. Portions of the map in Figure 1 downstream of exon 5 were derived from λGEM-11 clones that have been described previously [19]. Also, portions of the map (6 kb upstream of exon 1 and downstream to exon 2, and around exon 5) were derived from both lambda and BAC subclones and were identical with

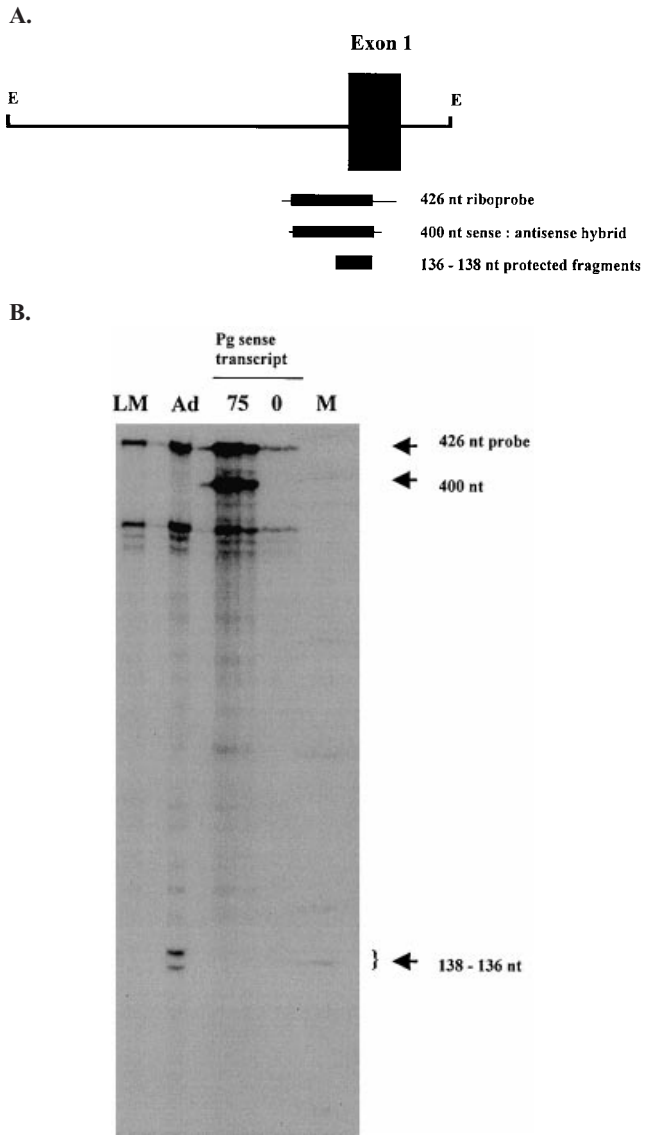

Figure 3 Mapping the 5′ end of exon 1

(**A**) An RNase-protection assay was established to determine the position of the 5′ end of exon 1 by using an antisense probe corresponding to nt −216 to +124 relative to the 5′ end of clone N9 [3]. The 426 nt riboprobe is shown relative to the position of exon 1 in a 2.3 kb *Eco*RI fragment (see Figure 1). 75 ng of sense transcript was included in the assay as a hybridization control, resulting in a 400 nt protected fragment. (**B**) Total RNA (20 μg) was isolated from lactating mammary gland (LM) and adipose tissue (Ad) and used in the RNase-protection assay. The positions of the antisense probe (426 nt), sense-antisense hybrid (400 nt) and the principal protected fragments in the RNA samples (138 and 136 nt) are indicated. M, ^{32}P-labelled pGEM-7zf(+)-*Hpa*II markers; the slowest and fastest markers correspond to 485 and 110 nt respectively. The location of the 5′ end of exon 1 is shown in Figure 2.

each other. The ovine ACC-α gene 5′ region is similar in overall size and structure to the previously published structure for the rat ACC-α gene [1]. For example, the distance from exon 1 to exon 5 is approx. 30 kb in both species (29.9 kb in the ovine gene and 29.3 kb in the rat). However, a notable difference between the two species is the distance between exon 1 and exon 2; in the ovine gene this is 4.5 kb whereas in the rat gene the distance is 12.3 kb.

To date no comparative sequence data for PI from any species exists in the databases. To aid in the identification of functionally conserved regions in the ovine PI we cloned the homologous region of the mouse ACC-α gene from a BAC genomic library. Sequence comparison of 873 bp upstream of the predicted 5′ terminus of exon 1 in both species demonstrated that the proximal 236 bp had 61.8 % identity, with the distal 637 bp having only 24.4 % identity. Sequence comparison of the proximal promoter in both species is shown in Figure 2. An experiment was conducted to delineate the 5′ extent of exon 1 more precisely as a means of identifying upstream sequence elements involved in transcription initiation in the ovine PI promoter. This analysis was performed by RNase-protection assay with a probe derived from genomic DNA that extended 216 nt upstream of the 5′ extent of previously isolated cDNA clones comprising exon 1 (clone N9) [3]. Figure 3 shows two protected bands in ovine adipose tissue RNA, a major species of 138 nt and a minor species of 136 nt. Fragments in this size range were not detected in lactating-mammary-gland RNA. This is consistent with the tissue-restricted expression of transcripts containing exon 1 (Figure 4, see below). The 138 nt protected fragment indicates that the 5′ extent of exon 1 lies 15 nt upstream of the 5′ terminus of cDNA clone N9 [3]. By reference to Figure 2, delineation of the 5′ extent of exon 1 reveals that the ovine PI promoter exhibits a TATA transcription initiation motif at −29 relative to the transcription start site with a CCAAT box motif, in a context to potentially bind CCAAT enhancer-binding protein (C/EBP) family members, at −61. These motifs are conserved in the murine PI, together with an E-box motif, potentially able to bind basic helix-loop-helix (bHLH) transcription factor family members, at −114. Interestingly, in ovine PI an E-box type motif is present 8 bp upstream of the −114 E-box. This sequence is interrupted in the murine PI by an 18 bp insertion comprising a consensus site for the nuclear factor κB (NF-κB) transcription factor family member, c-rel. Another E-box motif is present in the proximal region of murine PI at −152 (−134 on alignment with the ovine PI).

PI transcripts are restricted to ovine adipose tissue and liver

The tissue distribution of PI and PII ACC-α mRNA was determined by RNase-protection assay in a number of ovine tissues. Two probes were utilized in this analysis. Plasmid p1AEX contains cDNA comprising exon 1, exon 4 and exon 5; full-length protected fragments (509 nt) correspond to E(1/4/5), whereas E(1/5) transcripts are represented by the exon 1 (242 nt) plus exon 5 (220 nt) portions of the probe in equimolar amounts. PII transcripts are represented by the exon 4 plus exon 5 [E(2/4/5); 267 nt], and the exon 5 [E(2/5)]-protected fragments minus those derived from E(1/5). To confirm the indirect calculation of PII transcripts in this fashion, direct measurement of E(2/4/5) transcripts was performed using a plasmid (p3E2) comprising a 264 nt sequence corresponding to exons 2, 4 and 5. Using p3E2, hybrid formation with E(1/4/5) mRNA results in the protection of the exon 4 plus exon 5 portion of the riboprobe (243 nt); protection of the exon 5 portion of the riboprobe only (196 nt) arises from both E(2/5) and E(1/5) mRNA. Figure 4(A) shows that protected fragments corresponding to E(1/4/5) and E(1/5) are only found in adipose tissue and in liver; PI transcripts in these tissues account for approx. 60 % and 40 % of total ACC-α transcripts respectively. By comparison, expression of PI transcripts in livers of rats fed *ad libitum* is low, accounting for approx. 1 % of total ACC-α transcripts. Expression of PI transcripts in ovine skeletal muscle probably arises through association of muscle fibres with adipocytes. All tissues contained transcripts that resulted in the protection of the exon 4 plus exon 5 portion of the probe. Using p3E2 (Figure 4B) all tissues

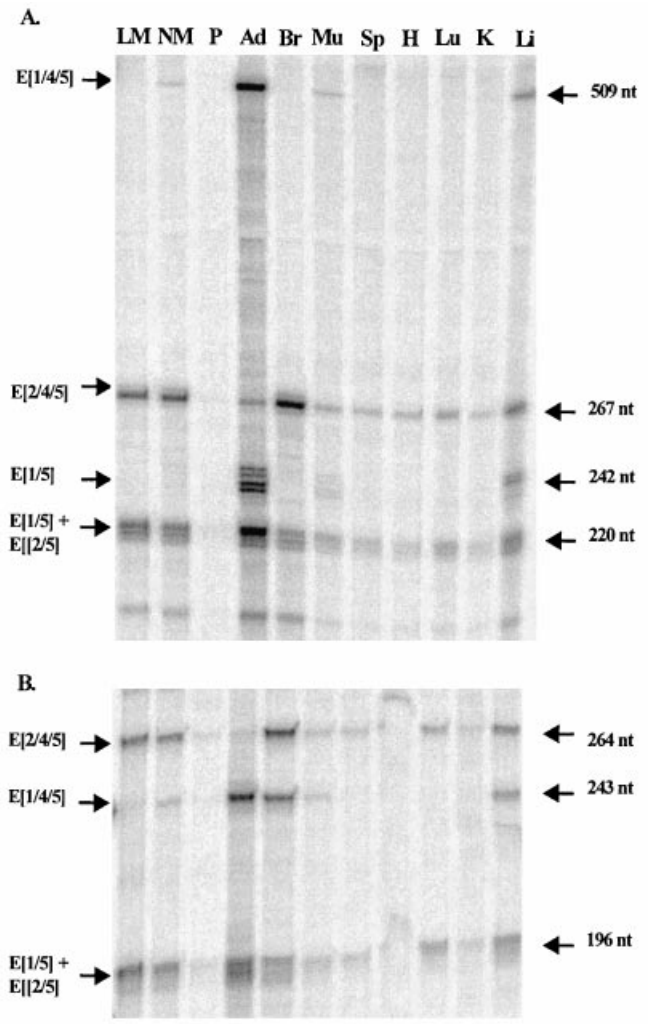

Figure 4 Expression of ACC-α transcripts in various ovine tissues using the p1AEX (A) and p3E2 (B) riboprobe vectors

Samples of RNA from lactating mammary gland (LM), non-lactating mammary gland (NM), pancreas (P), adipose tissue (Ad), brain (Br), muscle (Mu), spleen (Sp), heart (H), lung (Lu), kidney (K) and liver (Li) were hybridized with antisense probes comprising exon 1 (p1AEX) and exon 2 (p3E2) as described in the Experimental section.

Table 1 Analysis of promoter activity in the $-4359/+138$ and the $-132/+15$ PI-luciferase reporter constructs in the presence and absence of insulin

HepG2 cells, ovine preadipocytes and differentiated ovine adipocytes were transiently transfected with 1.0 μg of luciferase reporter gene construct. Constructs transfected were either the $-4359/+138$ or $-132/+15$ PI constructs. Cells were harvested 48 h after transfection and assayed for luciferase. Luciferase activities were normalized by determining the relative transfection efficiencies by filter hybridization of cell homogenates with ^{32}P-labelled pGL3basic vector. Transfections were performed in the absence or presence of 170 nM (HepG2 cells) or 280 nM insulin (adipocytes and preadipocytes). Results are means $\pm$ S.E.M. for three to five individual experiments using duplicate preparations of plasmid DNA. $^{***}P < 0.001$ and $^{**}P < 0.01$ compared with cultures in the absence of insulin.

	Normalized luciferase activity			
Plasmid ...	$-4359/+138$		$-132/+15$	
Cell type	$-$Insulin	$+$Insulin	$-$Insulin	$+$Insulin
HepG2 cells	104 ± 6	190 ± 24	1108 ± 170	$2107 \pm 268^{**}$
Preadipocytes	0.44 ± 0.1	0.59 ± 0.1	2.74 ± 0.6	$7.04 \pm 0.9^{***}$
Differentiated adipocytes	22 ± 6	$141 \pm 23^{***}$	31 ± 13	$119 \pm 28^{**}$

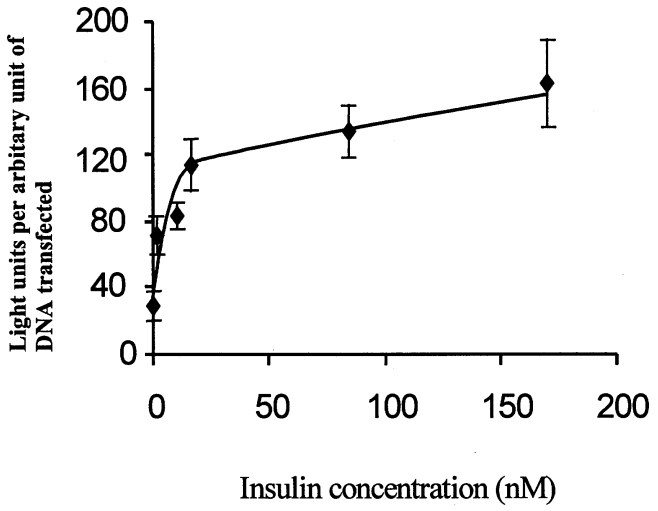

Figure 5 Effect of various concentrations of insulin on the expression of the $-132/+15$ bp construct in transfected adipocytes

Ovine adipocytes were transfected with 1.0 μg of a reporter construct in which promoter I sequences from -132 to $+15$ bp were cloned upstream of the luciferase coding region. After transfection, adipocytes were treated for 48 h in the absence or presence of insulin concentrations ranging from 1.7 to 170 nM. Cells were then harvested and assayed for luciferase activity. Data was normalized against the amount of plasmid DNA taken up by the cells. Data are the means $\pm$ S.E.M. from six determinations from two independent experiments.

contained a protected fragment of 264 nt corresponding to the E(2/4/5) transcript; there was a strong relationship between determination of E(2/4/5) with p1AEX and direct measurement with p3E2, as has been demonstrated previously for adipose tissue [21]. Interestingly, hybridization of brain (cerebral cortex) RNA with p3E2 resulted in a hybrid corresponding to the exon 4 plus exon 5 (243 nt) portion of the probe that could not be explained by the presence of E(1/4/5) transcripts in the mRNA population. Kim and colleagues [32] have shown that a minority of transcripts in rat lactating mammary gland comprise an additional exon, exon 3, in the 5′ untranslated region in the context of E(2/3/4/5) mRNA. Other workers [33] have failed to detect by RT-PCR transcripts containing exon 3 in a variety of rat tissues, including brain, although expression in rat lactating mammary gland was not determined in this study. Therefore, to exclude the possibility that the exon 4 plus exon 5 hybrid detected in cerebral cortex with riboprobe p3E2 could result from transcripts in which an additional exon downstream of exon 2 is present in ACC-α transcripts from brain, we performed RT-PCR, using primers corresponding to exon 2 and exon 5, with RNA from ovine cerebral cortex, pituitary, hypothalamus and lactating mammary gland. Two DNA species were produced for each RNA sample. These corresponded to E(2/4/5) and E(2/5) transcripts (results not shown). Thus there remains the possibility of as-yet uncharacterized exons in the 5′ untranslated region of ACC-α transcripts in brain that do not result from transcription from PI or PII.

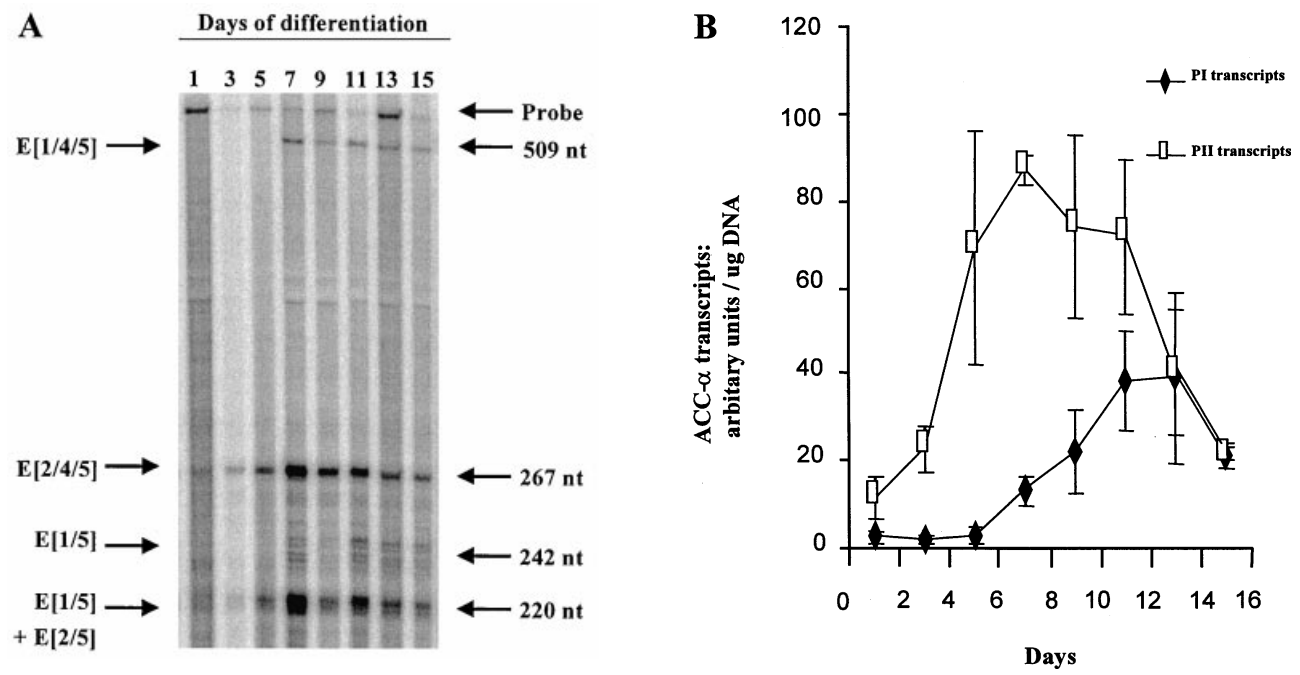

Figure 6 Expression of PI and PII transcripts during the differentiation of ovine adipocytes

(**A**) RNase-protection assay using riboprobe p1AEX with samples of RNA (10 μg) from adipocytes on various days after the differentiation procedure is commenced. Bands corresponding to PI and PII transcripts are indicated. (**B**) Data from (**A**) is quantified using IMAGEQUANT software and represent the means $\pm$ S.E.M. for cells from three individual animals. PI transcripts correspond to E(1/4/5) and E(1/5), and PII transcripts correspond to E(2/4/5) and E(1/5).

A 4.3 kb PI-luciferase reporter construct is inducible by insulin in differentiated ovine adipocytes

To investigate hormonal induction of PI we transfected a $-4359/+138$ bp PI-luciferase construct into a number of cell types. Human hepatoma HepG2 cells were initially chosen as they had been demonstrated to express PI transcripts by RT-PCR. [2]. Table 1 shows that the $-4359/+138$ bp construct is relatively inactive in HepG2 cells and is not induced by insulin. We also transfected a shorter construct of $-132/+15$ linked to luciferase. This construct corresponds to the highly similar region of the proximal promoter of the ovine and murine PI, and includes the conserved TATA box, CCAAT box and E-box at -114 (Figure 2). Interestingly this shorter construct was inducible by insulin in HepG2 cells (Table 1). Also, murine 3T3-F442A and 3T3-L1 adipocytes were initially used in our experiments as they undergo a differentiation process with lipid accretion and associated increases in the expression of lipogenic enzyme genes [34]. Kim and colleagues [20] also demonstrated that a similar cell-line, murine 30A5 adipocytes, expresses PI transcripts during the differentiation process, albeit at a low level and detectable only by PCR. Similar to the HepG2 cells, the $-4359/+138$ bp construct was inactive relative to the $-132/+15$ bp construct in these murine adipocyte cell-lines (results not shown). The $-4359/+138$ bp construct was then transfected into primary cultures of differentiating ovine adipocytes [25]. In ovine adipocytes the $-4359/+138$ bp construct was active and inducible by insulin, as was the $-132/+15$ bp construct. Approx. 50% of the maximal insulin stimulation was observed at a concentration between 1.7 and 10 nM (Figure 5). The $-4359/+138$ bp construct behaved in a similar fashion to varying insulin concentration as the $-132/+15$ bp construct in these cells (results not shown). This demonstrates that physiological concentrations of insulin regulate expression of the ACC-α PI reporter constructs in differentiated adipocytes. The basal and insulin-stimulated activities of the $-4359/+138$ bp and $-132/+15$ bp constructs were similar in the transfected ovine adipocytes, whereas in the HepG2 cells the basal activity of the $-4359/+138$ bp construct was markedly lower than the basal activity of the $-132/+15$ bp construct. A similar pattern was seen when ovine preadipocytes were transfected with the two constructs (Table 1). Similar to the differentiated adipocytes, insulin induction of the $-132/+15$ bp construct in HepG2 cells and preadipocytes occurred at a half-maximal concentration between 1.7 and 10 nM (results not shown), suggesting that in these cells signalling to the reporter construct was largely through the insulin receptor. Overall, these results suggest that the $-4359/+138$ bp construct is subject to active repression in the HepG2 cells, 3T3 adipocyte cell lines and ovine preadipocytes but not in the differentiated ovine adipocytes.

PI transcripts are expressed during differentiation of ovine preadipocytes

The expression of endogenous PI transcripts in the cells in which the transfection of PI-luciferase constructs were performed (Table 1) was investigated. Expression of ovine ACC-α transcripts was determined by RNase-protection assay as described above (Figure 4) using the p1AEX and p3E2 riboprobe vectors. Similar riboprobes corresponding to murine and human ACC-α cDNAs were used for the determination of these transcripts in the 3T3-F442A adipocytes and HepG2 cells. Figure 6(A) shows that no bands corresponding to E(1/4/5) (509 nt) and E(1/5) (220 nt)

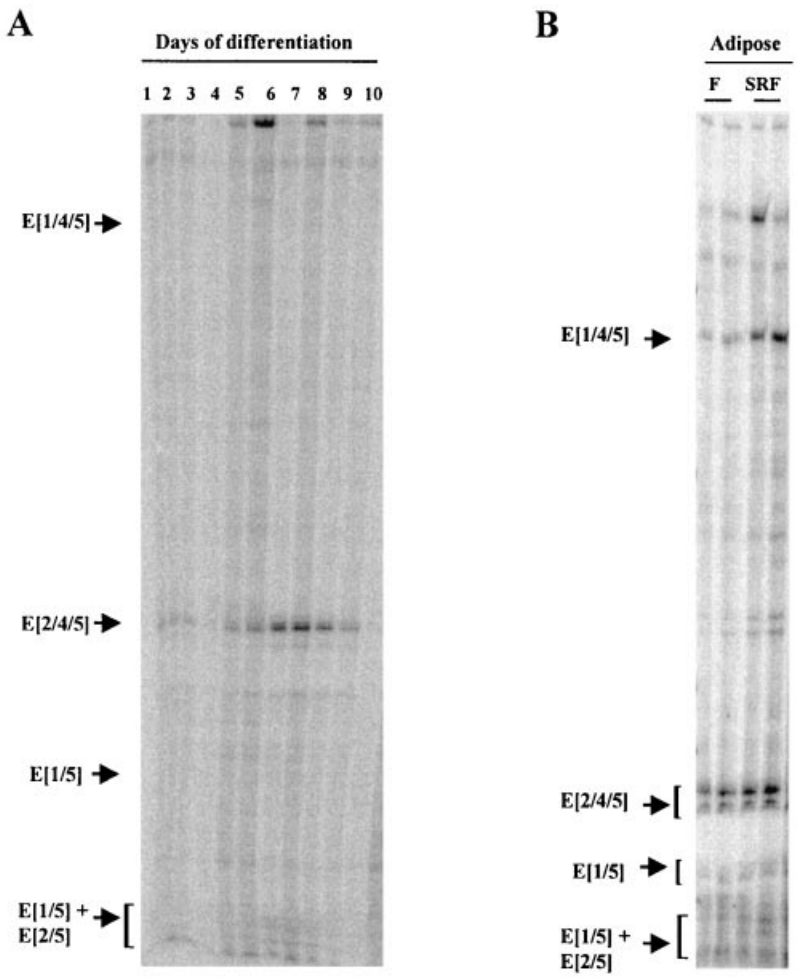

Figure 7 Expression of PI and PII transcripts in mouse adipose tissue *in vivo* and during the differentiation of 3T3-F442A adipocytes

(**A**) RNase-protection assay using a murine E(1/4/5) riboprobe with samples of RNA from 3T3-F442A adipocytes on various days after the differentiation procedure was commenced. The positions of E(1/4/5) and E(1/5) transcripts are indicated by reference to samples of mouse adipose RNA run on the same gel, although they are not shown. (**B**) RNase-protection assay using a murine E(1/4/5) riboprobe with samples (10 μg) of RNA from mice either fed *ad libitum* (F) or after a 24 h fast and then fed a high-carbohydrate diet for a further 24 h (SRF). Data are from two animals.

were detectable in ovine preadipocytes prior to differentiation and indeed only become detectable as differentiation proceeded. Quantification of this data (Figure 6B) shows that PII transcripts [E(2/4/5) plus E(2/5)] are up-regulated by day 3 and increase to a maximum at days 5–11; PI transcripts demonstrate induction after day 5, rising to a maximum at day 13 when they represent approx. 50% of the total ACC-α transcripts.

By contrast, expression of PI transcripts was not detected during the differentiation of 3T3-F442A adipocytes (Figure 7A), although PI transcripts were readily detectable in mouse adipose tissue RNA *in vivo* in both the *ad libitum*-fed and starved refed animals (Figure 7B). Direct measurement of transcripts containing exon 2 suggests that the increase in ACC-α transcripts observed throughout differentiation of 3T3-F442A adipocytes is due to increased expression from PII (results not shown). Also, murine 3T3-L1 adipocytes behave in a similar fashion to 3T3-F442A cells in that PI transcripts are not detectable by RNase-protection assay throughout differentiation (results not shown). Likewise, PI transcripts are not detectable in HepG2 cells (results not shown). These results suggest there is an association between endogenous expression of PI transcripts and the ability of the $-4359/+138$ bp construct to be stimulated by insulin in transfected cells.

An E-box binding protein is involved in the induction of the $-132/+15$ PI proximal promoter construct by insulin

Results presented above have demonstrated that the $-132/+15$ construct is insulin responsive when transfected into a variety of cell types. Thus it contains an insulin-response sequence. Figure 2 shows that the $-132/+15$ construct contains an E-box motif at position -114 to -109 that is conserved in the murine PI proximal promoter. E-box motifs are able to bind members of the bHLH family of transcription factors, and through the binding of USF family members (USF-1 and USF-2) have been implicated in insulin and glucose signalling to a number of genes involved in energy metabolism [35–37]. We sought to investigate the role of the E-box at -114 in the insulin responsiveness of the $-132/+15$ construct by site-directed mutagenesis. Table 2 shows that mutation of the E-box motif, 5′-CATGTG-3′ to 5′-GAATTC-3′ ($\Delta-114$) reduces the insulin response by approx. 70% in differentiated ovine adipocytes, without effecting basal

Table 2 Analysis of promoter activity in the −132/+15 PI construct and in a Δ−114 E-box mutant when transfected into ovine adipocytes, preadipocytes and HepG2 cells in the presence and absence of insulin

Cells were transiently transfected with 1.0 μg of luciferase reporter gene construct. Constructs transfected were either the −132/+15 PI construct or a mutant (Δ−114) in which the −114 E-box, 5′-CATGTG-3′, is mutated to 5′-GAATTC-3′. Cells were harvested 48 h after transfection and assayed for luciferase. Luciferase activities were normalized by determining the relative transfection efficiencies by filter hybridization of cell homogenates with ^{32}P-labelled pGL3basic vector. Transfections were performed in the absence or presence of 170 nM (HepG2 cells) or 280 nM insulin (adipocytes and preadipocytes). Results are means ± S.E.M. from four to eight individual experiments using duplicate preparations of plasmid DNA. $^{***}P < 0.001$, $^{**}P < 0.01$ and $^{*}P < 0.05$ compared with cultures in the absence of insulin; †$P < 0.001$ when Δ−114 is compared with −132/+15 in the presence of insulin.

	Normalized luciferase activity			
Plasmid ...	−132/+15		Δ−114	
Cell type	−Insulin	+Insulin	−Insulin	+Insulin
Differentiated adipocytes	25 ± 3	166 ± 29***	19 ± 3	67 ± 11*†
Preadipocytes	1.1 ± 0.28	5.7 ± 1.7*	1.3 ± 1.7	8.3 ± 1.8**
HepG2 cells	1000 ± 82	3299 ± 622**	1022 ± 291	2500 ± 434*

activity in the absence of insulin. Conversely, in HepG2 cells and ovine preadipocytes mutation of the E-box motif had no significant effect on the ability of insulin to signal to the reporter construct (Table 2).

As the E-box motif at −114 appeared to confer a partial response to insulin in differentiated adipocytes we sought to identify factors binding to this motif by gel mobility-shift assays. An oligonucleotide (corresponding to −125 to −101 of PI) containing the −114 E-box (E2), together with the E-box-like motif at −122 (E1) was ^{32}P-labelled and incubated with nuclear extract prepared from differentiated ovine adipocytes cultured with insulin. Two major protein–DNA complexes, referred to as band 1 and band 2 in Figure 8(A) were detected by gel mobility-shift assay (lane 1); band 2 resolved into two bands (not shown). These complexes can be effectively competed with by a 100× molar excess of unlabelled oligonucleotide (Figure 8A, lane 2). Mutation of the E2 (5-CATGTG-3′ to 5′-GAATTC-3′; mE2) effectively abolished competition with band 1, and diminished competition with band 2 (Figure 8A, lane 5). Mutation of both E-boxes (E1, 5′-CAAGTG-3′ to 5′-ACAGGT-3′; mE1E2) showed qualitatively similar behaviour (Figure 8A, lane 7) to mutation of E2 alone. Similarly when E1 alone (mE1) is mutated (Figure 8A, lane 6) the oligonucleotide acts as an effective competitor. Mutation of the dinucleotide (mdn) between E1 and E2 also results in effective competition (Figure 8A, lane 8). Taken together, these data suggest that E2 is the functionally important motif. Figure 8(A), lane 3, shows that band 1 is also effectively competed with by an oligonucleotide comprising the −65 to −60 E-box of rat FAS that has been demonstrated to bind USF-1 and act as an insulin-response sequence [35,38]. Likewise, band 1 is not competed with as effectively by an oligonucleotide containing the −150 sterol response element of rat FAS that has been demonstrated to bind SREBP; SREBP has also been shown to bind E-boxes *in vitro* although at lower affinity than the sterol-response element [39,40]. These results strongly suggest that at least one factor that binds the −114 E-box is a bHLH factor family member, akin to USF, rather than SREBP, and this corresponds to band 1. Band 2 would also appear to be specific as it is competed by oligonucleotides containing a non-mutated E2, suggesting that the factor is

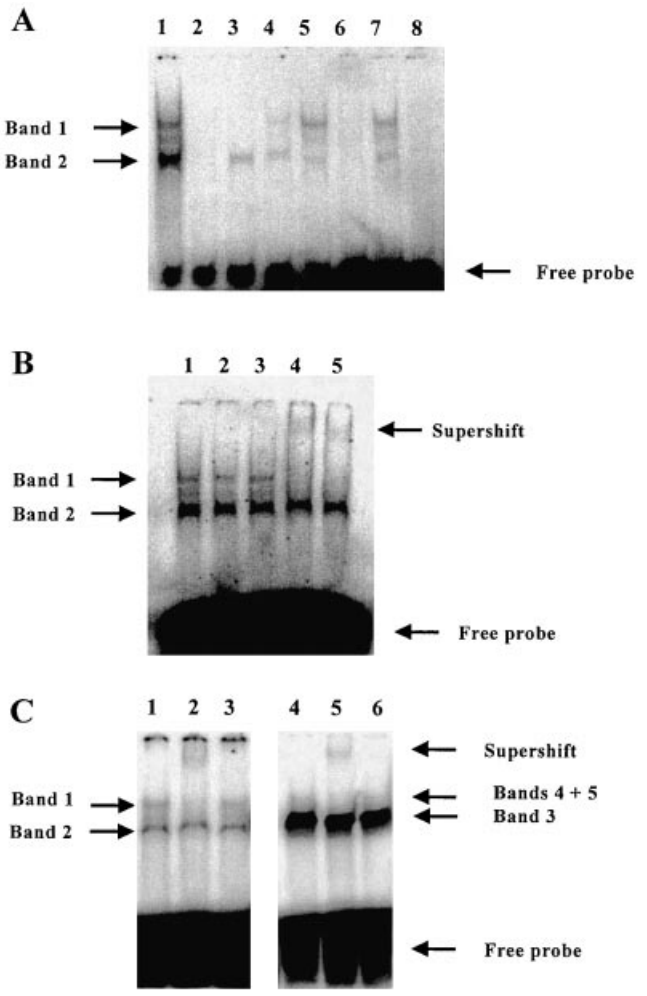

Figure 8 Gel mobility-shift assay of nuclear extract from insulin-stimulated adipocytes, preadipocytes and HepG2 cells with ACC-PI insulin-response sequence

(**A**) Each reaction contained 3 μg of adipocyte nuclear extract and 10 ng of ^{32}P-labelled ACC-PI IRS oligonucleotide and various unlabelled competitors at a 100× molar excess. Lane 1, no competitor; lane 2, ACC-PI insulin-response sequence; lane 3, FAS E-box; lane 4, FAS sterol regulatory element; lane 5, mE2; lane 6, mE1; lane 7, mE1E2; lane 8, mdn. The reaction mixtures were incubated at room temperature for 1 h and applied to a 4% non-denaturing polyacrylamide gel. (**B**) Gel-supershift assay of adipocyte nuclear extract and ACC-PI insulin-response sequence with various antibodies. Lane 1, no antibodies; lane 2, rabbit IgG; lane 3, anti-SREBP-1; lane 4, anti-USF-2; lane 5, anti-USF-1. (**C**) Gel-supershift assay of preadipocyte (lanes 1–3) and HepG2 (lanes 4–6) nuclear extract (3 μg) and ACC-PI insulin-response sequence; lanes 1 and 4, no antibodies; lanes 2 and 5, anti-USF-1; lanes 3 and 6, rabbit IgG.

related to the presence of the E-box. However it is partially competed by the other oligonucleotides which do not have common sequence elements. The reason for this is unclear.

To address further the identity of bands 1 and 2 we tested whether these bands were affected by addition of USF- and SREBP-1-specific antibodies to the gel mobility-shift assay. Figure 8(B) shows that incubating the nuclear extract with the ACC-PI oligonucleotide in the presence of anti-USF-1 (lane 5) and anti-USF-2 (lane 4) antibodies resulted in disruption of band 1 and resulted in a 'supershift'; band 2 was not affected. Addition of anti-SREBP-1 antibodies (Figure 8B, lane 3) or rabbit IgG (Figure 8B, lane 2) did not result in disruption of

either complex. Nuclear extract was also prepared from HepG2 cells and ovine preadipocytes that had been stimulated with insulin, and used to evaluate components in these cells binding to the ACC-PI oligonucleotide. Figure 8(C) shows that in HepG2 cells a major band (band 3, lane 4) was evident, which migrated at a slightly higher position in the gel than band 2 present in the nuclear extract from the differentiated adipocytes. Two minor bands (bands 4 and 5) were also evident in HepG2 cell nuclear extract; these supershifted with anti-USF-1 antibodies (Figure 8C, lane 5). Ovine preadipocytes demonstrated a pattern of component binding in nuclear extracts qualitatively similar to that in differentiated adipocytes (Figure 8C, lane 1); band 2 was composed of USF as it is 'supershifted' with anti-USF-1 antibodies (Figure 8C, lane 2). These results demonstrate that USF factors are components of complexes binding to the IRS corresponding to the ACC-PI oligonucleotide in nuclear extracts from differentiated adipocytes, preadipocytes and HepG2 cells.

DISCUSSION

This study has shown that activation of ACC-α PI-luciferase constructs by insulin when transfected into cells is dependent on both the context of the insulin-response motifs in relation to flanking sequence and to the phenotype of the host cell. Thus the $-4359/+138$ bp construct is relatively inactive and is not inducible by insulin when transfected into HepG2 cells, ovine preadipocytes or 3T3-F442A adipocytes, whereas the shorter $-132/+15$ bp construct demonstrates higher basal activity and is inducible by insulin in these cells. This is consistent with a repressor mechanism which serves to limit basal as well as insulin-stimulated activity. Repression could be mediated through a factor binding to repressor motif(s) that serve to limit the recruitment of activators and the RNA polymerase II complex to the core promoter [41]. A species incompatibility between the transfected ovine DNA and the HepG2 cells and 3T3 adipocytes is unlikely to account for the results observed, as reference to the above model would suggest that incompatibility is most likely to present as a lack of repression. A repressive mechanism would seem likely as endogenous PI transcripts are also repressed in these cell types, most notably with respect to 3T3-F442A adipocytes when compared with mouse adipose tissue *in vivo*. Conversely, when the $-4359/+138$ bp construct is transfected into differentiated ovine adipocytes a similar level of basal and insulin-activated activity as that of the $-132/+15$ bp construct is observed and this is associated with endogenous expression of PI transcripts. Thus, this data is consistent with an active de-repressive mechanism occurring during differentiation of the ovine adipocytes, and acting at the PI promoter, in both the context of the endogenous gene and also the transiently transfected $-4359/+138$ bp reporter construct. Thus PI transcription in adipocytes has at least two modalities: (i) a differentiation-dependent de-repression acting at repressor motif(s) which results in an up-regulation of basal promoter activity and which also confers (ii) a response to insulin acting at separate IRSs, thereby allowing a variable response to the hormone in the context of a differentiated cell.

The repressor motif(s) are probably located 5′ to position -132, although as the $-132/+15$ bp construct has less exon 1-derived sequence in the construct we cannot as yet exclude this region for the repressive effect. Kim and co-workers [20] using a construct corresponding to $-1008/+136$ bp of rat PI mapped a repressive element that suppressed the basal activity of the promoter in murine 30A5 preadipocytes to a $[dCdA]_{28}$ repeat at -220, i.e. just 5′ to the position of high sequence identity observed between the ovine and murine PI proximal promoters in Figure 2. This [dCdA] repetitive element has a unique relationship with the CCAAT box at -61 such that it acts as a repressor motif only in the presence of the CCAAT box. Indeed, ectopic expression of C/EBP, which increases throughout adipocyte differentiation [42] results in a several-fold activation of the PI promoter construct, suggesting that this [dCdA] element serves to repress endogenous PI transcription in the context of the preadipocyte phenotype [20]. However, the rat PI [dCdA] element is not conserved in the region of the ovine PI promoter corresponding to the $-4359/+138$ bp construct, suggesting that other motifs in the transfected DNA must fulfil the repressor function.

Insulin induction of the $-132/+15$ bp construct is greatest in the differentiated adipocytes (6–7-fold) when compared with the preadipocytes or HepG2 cells (2–4-fold). We have demonstrated that an E-box at -114 acts as an IRS in differentiated adipocytes, whereas insulin signalling to the reporter construct in preadipocytes and HepG2 cells is independent of the E-box, thus invoking the presence of other insulin-response elements in the construct. USF proteins, which belong to the family of bHLH-leucine-zipper transcription factors, are components of the protein complexes from all three cell types that bind *in vitro* to the -114 E-box. USF-1 and USF-2 are present in differentiated adipocytes as heterodimers, as the band 1 complex is disrupted by either anti-USF-1 or anti-USF-2 antibodies (Figure 8B), and as such probably bind this motif. Disrupting the -114 E-box in the context of transfected adipocytes reduces insulin induction (from 6-fold to 2.5-fold), although it does not abolish it completely. This suggests that the E-box is a target of an adipocyte-restricted (when compared with preadipocytes and HepG2 cells) component that may contribute to the markedly higher inducibility by insulin in differentiated adipocytes. Whether such a component requires USFs as a co-factor, or acts as an independent factor, will need to be established. Such a component has not been detected by our gel-shift experiments, which used relatively small quantities of unfractionated nuclear extract. Alternatively, USFs may be a direct target of insulin signalling.

A motif comprising two half E-box motifs (5′-CACG-3′ or 5′-CGTG-3′) separated by 7 or 9 bp has previously been shown to be involved in the glucose regulation of a number of genes, including L-pyruvate kinase and S14 [36,37,43,44]. The L-pyruvate kinase glucose-response element binds USFs *in vitro*, and results with gene-knockout mice suggest that USFs, especially USF-2, are important *in vivo* in transactivating the glucose-response element [45–47]. USF family members have also been implicated in the insulin responsiveness of the rat FAS gene, by binding to E-box motifs, notably at -65 and -332 [40,48]. Indeed, ablation of USF-1 and USF-2 in knockout mice severely impairs the induction of the FAS gene in response to feeding after a period of dietary restriction [49]. The -65 E-box of the FAS promoter, which also competes for band 1 in Figure 8(A), also comprises two SREBP motifs flanking the E-box [38]. As mutations of the -65 E-box that reduce insulin responsiveness also result in disruption of the two SREBP sterol-response elements, there has been some debate as to the nature of the factor that confers the insulin response at this site. SREBP-1 is not detected in the two complexes formed with the ACC-PI IRS oligonucleotide with ovine adipocyte nuclear extract. Likewise, SREBP-1 is not present in similar complexes formed with HepG2 nuclear extract (results not shown). Sterol regulatory elements are not present as part of the ACC-PI IRS, suggesting that if SREBP acts in this region it acts through the E-box motif. SREBP-1 is generally present in cells at low abundance, and actually binds the sterol-response element with higher affinity

than E-box motifs *in vitro* [40]. This suggests that SREBP-1 binding is probably not likely to be functionally relevant at the −114 E-box of the ovine PI proximal promoter.

Recently, Towle and co-workers [50] have demonstrated that glucose signalling to a rat ACC-α PI proximal promoter construct in hepatocytes is conferred by a motif comprising two half-E-boxes separated by 7 bp, and is functionally similar to the glucose-responsive motif in the mouse S14 gene [51]. The upstream half E-box in the glucose-response motif corresponds to the −114 E-box. The putative glucose-response factor, ChoRF, appears to require both half-sites for functionality, as mutation of either half-site abolishes the response to glucose, at least in the context of the motif in the mouse S14 gene [51]. Similarly, in the rat ACC-α PI construct, mutation of the downstream half-site (7 bp downstream of the −114 E-box) completely abolishes the response to glucose [50]. Whether a similar factor is responsible for the insulin signalling to the −132/+15 bp construct in differentiated adipocytes remains to be established, although as mutation of the E-box only reduces, but does not abolish, the response to insulin, other components may be involved that remain to be elucidated.

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