Elucidation of a promoter activity that directs the expression of acetyl-CoA carboxylase α with an alternative N-terminus in a tissue-restricted fashion

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Previous studies in rats and humans have demonstrated that acetyl-CoA carboxylase α (ACC- α), the principal ACC isoenzyme in lipogenic tissues, is transcribed from two promoters, PI and PII, that operate in a tissue-specific fashion. Each promoter gives rise to ACC- α mRNA isoforms that differ in their 5' untranslated regions but essentially encode the same protein product. In the present study we demonstrate that such a pattern of promoter usage is evident in sheep tissues but in addition we have detected the expression of a novel ACC- α mRNA isoform that is expressed in a variety of tissues including kidney, lung, liver and mammary gland, where it is markedly induced during lactation. This novel transcript differs from the previously described ACC- α mRNA in that exon 5, the primary coding exon in both PI and PII transcripts, is replaced by a 424-nt sequence that seems to represent the 5' terminus of the mRNA. The 424-nt sequence

encodes a 17-residue N-terminal region as the N-terminal residue in the deduced sequence is a methionine flanked by several in-frame stop codons. The 5' terminal 424 nt are present as a single exon, which we have termed exon 5A, in the sheep ACC- α gene and this is located approx. 15 kb downstream of exon 5 and 5 kb upstream of exon 6. A 1.5 kb *HindIII*–*BglII* fragment encompassing the 5' terminus and sequence immediately upstream of exon 5A demonstrates promoter activity when transiently transfected into HepG2 cells and HC11 mouse mammary cells and this is markedly enhanced when insulin is present in the culture medium. Promoter activity is also evident in primary sheep mammary epithelial cells. These results demonstrate the presence of a third promoter, PIII, in the ACC- α gene that results in the tissue-restricted expression of an ACC isoenzyme.

INTRODUCTION

Acetyl-CoA carboxylase [EC 6.4.1.2] (ACC), the flux-determining enzyme in the regulation of fatty acid synthesis, exists in isoenzymic forms that can be ascribed to transcription from two distinct genes, ACC- α and ACC- β [1–8]. The ACC- α gene gives rise to the 265 kDa isoenzyme, which is expressed in all cell types but demonstrates elevated expression and is the major form in the lipogenic tissues of liver and adipose tissue and also mammary gland during lactation [4,5,9,10]. Expression of the ACC- α isoenzyme is regulated in a complex fashion both in the short term, through allosteric mechanisms [11] and reversible phosphorylation on a number of critical serine residues [12–16], and chronically through the regulation of transcription of the gene [17]. ACC- β , corresponding to the 275–280 kDa isoenzyme, is the major form in heart and skeletal muscle, where it is implicated in the regulation of fatty acid β -oxidation in mitochondria [6,8,18].

Transcription of the ACC- α gene is initiated from two promoters, promoter I (PI) and promoter II (PII), such that transcripts demonstrate heterogeneity in the 5' untranslated region (UTR) in a tissue-specific fashion [1,2]. Exon 1 or exon 2, the primary exons in PI or PII transcripts respectively, are spliced on to combinations of exons 3 and 4 before exon 5, which is the primary coding exon of the 9 kb ACC- α mRNA [1–3]. PI transcripts are exclusively expressed in the adipose tissue of rats fed *ad libitum*, whereas PII transcripts exhibit a ubiquitous tissue distribution [9,19]. The physiological significance of this tissue-specific promoter usage and transcript diversity is unclear at present but might relate in part to the specialization of adipocytes for fatty acid storage, a function distinct from the synthesis of

fatty acids for incorporation into cell membranes. Such considerations raise the question of physical and metabolic compartmentalization of ACC molecules. This point is further emphasized by the characterization of an ACC- α variant in which an eight-residue domain upstream of the Ser-1200 critical phosphorylation site is deleted and confers an enhanced susceptibility to phosphorylation at Ser-1200 by cAMP-dependent protein kinase *in vitro* [20]. This ACC- α variant is widely expressed but is actually the dominant species in lactating mammary gland [20,21]. This raises the possibility of the existence of homopolymers and heteropolymers of active ACC with the potential for differential catalytic characteristics.

During the characterization of ACC cDNA in sheep tissues we isolated an ACC- α cDNA from a sheep mammary gland cDNA library in which exon 5 encoded sequences and those upstream of exon 5 in the ACC- α cDNA described previously [1–3] were replaced by a 424 nt sequence at the 5' terminus of the transcript that encoded an ACC- α with a variant N-terminus. This variant ACC- α is under the control of a unique promoter, PIII, lending further weight to the concept of compartmentalization of ACC isoenzymes within cells.

EXPERIMENTAL

Animals

Sheep were Finn–Dorset cross-bred animals, fed on hay and cereals for at least 4 weeks before slaughter [22]. Animals were used either as control (non-pregnant, non-lactating), pregnant or lactating ewes, or wethers (6-month-old castrated males). Female sheep were all 3–5 years old and multiparous. Pregnant animals

Abbreviations used: ACC, acetyl-CoA carboxylase; RACE, rapid amplification of cDNA ends; UTR, untranslated region.

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were used between days 100 and 105 of gestation, and lactating ewes, suckling at least two lambs, were used at about day 18 of lactation. Animals were anaesthetized and exsanguinated, then samples of tissue were removed, snap-frozen and stored in liquid nitrogen.

cDNA library screening

A sheep lactating mammary gland cDNA library was constructed in λ gt11 with cDNA prepared by reverse transcription of lactating mammary gland poly(A)⁺ RNA by Moloney murine leukaemia virus reverse transcriptase with random hexamers as primers. The cDNA was then double-stranded and ligated to *Eco*R1/*Not*I adaptors before ligation to *Eco*R1-restricted and dephosphorylated λ gt11 vector by standard methods [23]. The library was amplified by plating out on Y1089 host bacteria [23]. For screening, portions of the library were plated out on Y1088 host bacteria and transferred to nitrocellulose filters. Filters were then hybridized with a 1.6 kb sheep ACC cDNA insert (p4133), corresponding to the 5' end of the coding region [3], radioactively labelled with [α -³²P]dCTP by the random priming method [24]; positive plaques were identified by autoradiography. After several rounds of screening, positive recombinant plaques were purified to homogeneity and the resulting bacteriophage DNA was isolated by standard methods [25].

Rapid amplification of cDNA ends (RACE)-PCR

Total RNA was isolated from lactating sheep mammary gland by using guanidinium thiocyanate and centrifugation through CsCl [26]. cDNA was prepared from 2 μ g of this RNA by using Moloney murine leukaemia virus reverse transcriptase (Gibco BRL) and the synthesis was primed with an oligonucleotide (5'-CTTTCGGTCTCGACCTTG-3') anti-sense to nt +579 to +595 of the sheep ACC cDNA [3]. Owing to the high GC content (greater than 80%) found at the 5' end of the previously cloned rat mammary gland ACC cDNA [1], RNA was denatured at 90 °C for 2 min before annealing of the primer and extension of the cDNA at 42 °C under the conditions specified by the manufacturer. Single-stranded cDNA was purified through Sephadex G-50 and precipitated. After centrifugation and drying, the cDNA was 3' tailed with dATP with terminal transferase. Approximately one-quarter of the cDNA was used to amplify a region of DNA with a downstream primer corresponding to nt +464 to +491 of the sheep ACC cDNA [3] (5'-TCTGAGCTGACAGAGGCTGGTGACAG-3'), a T-adaptor primer (5'-GACTCGAGTCGACATCGATTTTTTTTTTTTTTTTTTTT-3') and an adaptor primer (5'-GACTCGAGTCGACATCG-3') [27]. 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 55 °C for 30 s, and extension at 72 °C for 30 s. One-tenth of the product was then resolved on a 1% (w/v) agarose/Tris/borate/EDTA gel and revealed by staining with ethidium bromide. The remainder of the product was purified with a Promega Wizard PCR clean-up kit, ligated into the pDK101 cloning vector [28] and used to transform competent JM109 cells [29]. Plasmids were purified from recombinant colonies and sequenced by the dideoxy chain-termination method [30] with *Taq* DNA polymerase. The resulting ACC cDNA comprised 21 bp of exon 2, 47 bp corresponding to exon 4 and 196 bp of exon 5.

RNAse protection analysis

RNAse protection assays were developed with a 309 bp *Pst*I/*Nsi*I fragment of clone 2RP, obtained from screening the lactating

mammary gland cDNA library, and a 264 bp sequence obtained from RACE-PCR. These were subcloned into pGEM5zf(+) and pGEM7zf(+) respectively; anti-sense transcripts were generated with SP6 and T7 RNA polymerases respectively and [α -³²P]CTP [31]. To map the start site of exon 5A a probe (p3A7z) corresponding to the 5' portion of clone 2RP and 219 nt of upstream flanking genomic sequence was generated by PCR of clone λ 3A with the primers 5'-GAAATTCAGTCTTGTGTGA-3' and 5'-ACAGCATCAGCTGAATTTCCCA-3' and cloned into plasmid pGEM 7zf(+); anti-sense and sense transcripts were generated with T7 and SP6 RNA polymerases respectively. Total RNA was isolated from all sheep tissues except adipose tissue by using guanidinium thiocyanate and centrifugation through CsCl [26]. RNA was isolated from adipose tissue by a modification of Chomczynski and Sacchi's procedure [32,33]. Aliquots of total RNA (20 μ g in all cases except adipose tissue, from which 5 μ g of total RNA was used or 100 μ g for start-site mapping) were co-precipitated with 1.5 ng of the labelled anti-sense probe. Similarly 20 or 100 μ g of tRNA (for start-site mapping), with various amounts of sense transcript generated with T7 or SP6 RNA polymerases, was co-precipitated with 1.5 ng of the labelled anti-sense transcript as positive controls. All samples were resuspended in 30 μ l of hybridization buffer [80% (v/v) formamide/40 mM Pipes (pH 6.7)/0.4 M NaCl/1 mM EDTA], overlaid with mineral oil and incubated first at 85 °C for 5 min and then overnight at 45 °C [31]. 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 8% (w/v) polyacrylamide/7 M urea gels with Tris/borate/EDTA buffer. Gels were then dried and exposed to Kodak phosphor screens. The resulting images were then scanned with a Molecular Dynamics phosphorimager 445 SI and the volume of the individual bands was obtained by using IMAGE-QUANT software. DNA was assayed by a modification of the method of Labarca and Paigen [34], in which the tissue was homogenized in 5 M guanidinium isothiocyanate/100 mM EDTA (pH 7.0) and extracted with 4 vol. of water-saturated chloroform before being assayed. The yield of RNA/g of tissue, together with the amount of DNA/g for each sample, was used to calculate the amount of each type of transcript in arbitrary units/mg of DNA.

Genomic library screening

High-molecular-mass genomic DNA was isolated from sheep spleen by standard methods [35]. The DNA was partly digested with *Sau*3AI restriction endonuclease and a fraction corresponding to a size of 15–23 kb was isolated by sucrose-density gradient centrifugation [36]. The resulting DNA was then ligated into the λ GEM-11 vector (Promega) and packaged with Giga-pack II gold extracts (Stratagene). 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 [24] sheep ACC cDNA (p4133) as above. After several rounds of screening, positive recombinant plaques were purified to homogeneity and the resulting bacteriophage DNA was isolated by standard methods [25].

Cell culture, transfection and luciferase assays

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. Cells were

transfected in 24-well plates with 0.5 μ g of plasmid DNA (purified through CsCl gradients) and a 2:1 charge ratio of Tfx-50 reagent (Promega) per well, with the manufacturer's protocol for the transfection of adherent cells. HC11 cells were maintained and differentiated as described previously [37]; cells were transfected with 1.0 μ g of plasmid DNA and a 3:1 charge ratio of Tfx-50 reagent. Primary sheep mammary epithelial cells were prepared as described previously [38] and maintained on plastic in 24-well plates in a medium comprising Ham's F-12/Medium 199 (1:1, v/v), 20% (v/v) newborn calf serum, 5 μ g/ml insulin, 1 μ g/ml cortisol and 10 ng/ml epidermal growth factor; cells were transfected under similar conditions to the HC11 cells. Transfected cells were maintained in culture for a further 48 h in the media described above and either in the presence or absence of insulin (5 μ g/ml). 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 Biotrans nylon membrane (ICN) and hybridizing this to the pGL3 basic vector [39]. In brief, a 15 μ l portion from a total of 100 μ l of cell extract was boiled for 5 min, diluted with 20 $\times$ SSC (3 M NaCl/0.3 M sodium citrate) and dot-blotted on nylon membrane. The DNA was UV-fixed to the membrane and hybridized to the pGL3 basic vector labelled with [32 P]dCTP with random primers [24]. 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 per well divided by the relative amount of plasmid DNA (in arbitrary units) per well.

RESULTS

Isolation of a cDNA corresponding to a novel ACC- α mRNA isoform from a lactating sheep mammary gland cDNA library

Previously we isolated clones from an adipose tissue cDNA library corresponding to the sheep homologue of rat ACC- α mRNA [3]. These cDNA species encompass the 7 kb coding region, the 3' UTR and most, if not all, of the 5' UTR; the 5' UTR of these cDNA species were homologous with exons 1, 4 and 5 (E1/4/5- and E1/5-type mRNA species) of the rat ACC- α gene [1]. In an attempt to isolate clones corresponding to ACC- α transcripts derived from the sheep homologue of PII, i.e. containing exon 2 as the primary exon in the mature mRNA (E2/4/5- and E2/5-type mRNA species), we screened a lactating sheep mammary gland random-primed cDNA library. approx. 8×10^5 recombinants were screened with a 1.6 kb cDNA (p4133) [3] corresponding to the N-terminal region of the protein; three recombinants were isolated. Two of these were identical (0.85 kb) and corresponded to the coding sequence only (positions +730 to +1578 nt relative to the start of the E1/4/5-type mRNA). The third clone (2RP) of 1.8 kb corresponded to position +1994 nt at its 3' end; positions +545 to +1994 nt were 100% identical to the sequence that we had described previously [3]. The 424 bp at the 5' end of clone 2RP were completely distinct from the sequence in the E1/4/5-type mRNA (Figure 1). Deduction of the translated product due to the inclusion of the 424 nt sequence in ACC- α mRNA results in the replacement of the 76-residue terminus encoded by exon 5 in the E1/4/5-type mRNA by a 17-residue sequence in the variant transcript. The N-terminal residue in the deduced sequence of the E5A-type transcript is a methionine flanked by several upstream in-frame stop codons (Figure 1). Because the two transcripts differ at the 5' boundary of the sequence corresponding to exon 6 (Figure 1),

we have termed the variant transcript E5A-type mRNA as the 424 bp sequence probably represents one or more exons spliced to exon 6 owing to alternative splicing.

Expression of the E5A-type ACC- α mRNA demonstrates a tissue-restricted distribution

The tissue distribution of the E5A-type transcript was determined by RNase protection assay with a 309 bp *PstI*–*NsiI* fragment of 2RP as a probe template. The basis for the RNase protection assay is shown in Figure 2. Figure 3 demonstrates that a 309 nt protected fragment corresponding to the E5A-type transcript is detectable in RNA from a number of tissues, notably lactating mammary gland, kidney, lung and liver. The transcript is also detected in brain tissue (results not shown). The E5A-type transcript does not seem to be detectable in adipose tissue, heart, muscle or spleen. When the DNA content of the homogenate is taken into account it seems that the highest level of expression is in lactating mammary gland, approaching 10-fold greater expression per mg of tissue DNA than in the other tissues in which E5A-type transcripts are detected (results not shown). The 175 nt fragment in Figure 3 corresponds to transcripts containing exon 6 and exon 7 and probably represents transcripts in which exon 5 is spliced to exon 6 owing to transcriptional initiation at PI and/or PII in a tissue-specific fashion. However, at this stage we cannot exclude the possibility that other, as yet undetermined, 5' exons are spliced to exon 6.

E5A-ACC- α mRNA is markedly induced in mammary gland during lactation

Because the E5A-type ACC- α mRNA was expressed to a relatively high level in the mammary gland of lactating animals, we investigated the developmental regulation of the transcript throughout pregnancy and lactation. Figure 4(A) demonstrates that the abundance of the E5A transcript in mammary homogenates increases markedly from non-pregnant/non-lactating animals, when it approximates to 10% of the total ACC- α transcript, to lactation, when it is nearly one-third of the total. To confirm that the 175 nt protected fragment in Figure 4(A) corresponds to ACC- α transcripts containing exon 5, arising from transcription from the PII promoter, we established an RNase protection assay with a 264 bp 5' RACE-PCR product as a template; this cDNA comprises 21 bp of exon 2, 47 bp corresponding to exon 4 and 196 bp of exon 5. In Figure 4(B) protected fragments of 264 nt correspond to the E2/4/5 transcript and these increase between late pregnancy and lactation; the fragment of 196 nt corresponding to exon 5 also increases and therefore probably represents E2/5-type transcripts because little or no E1/5-type transcript is found in sheep mammary gland (results not shown). When the DNA content of the tissue is taken into account, ACC- α transcripts containing exon 5 increase approx. 3-fold throughout pregnancy and into lactation at a time when the expression of the E5A-type transcript increases 15-fold per mg of tissue DNA (Table 1). A good concordance is observed between the 175 nt protected fragment in Figure 4(A) and results obtained with the E2/4/5 riboprobe (Figure 4B), suggesting that, at least in sheep mammary gland, the 175 nt fragment represents ACC- α transcripts in which exon 6 is spliced to exon 5 as a result of transcription from PII.

The 424 nt sequence corresponding to E5A is present as a single exon and is flanked by upstream promoter activity

The above studies indicated that the 424 nt sequence incorporated into ACC- α transcripts of the E5A type represented one or more

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E145 +1 GAAGTCCCTCAGACTCTTAACCTCGGACAAACCTGCACGGCTGAGCAAAGTCGAAGAACAGTGTCTGATGAAGTCTTCTGTCTCTTTTCTCTTATGACTAGAGCCTCC
E145 +121 GTTCTGGTCGAGGAGTCTCCCTGAAGTCTGTCATCTGTGAAGTATCTCTGTGGAGTTTGTGCTCCCGACGCGACCTGAAGACGGCAAGCAGACATTGGAAGCAGAGAGGTC

E5A +1 CTTTGTCTGACGCTAAGGCAGAAGCTGCTGAGATCTACTTTAGAGTTATACCGCTTTTATATTCTCCCTCTCTCTGTGCCCTTGGGAAATCAGTGTGCTGTGTGGGAGC
***

E145aa M--G--E--P--S--S--L--A--K--P--L--E--L--N--
E145 +241 TCTGAGTCTCTTTTGAAGTGGATATCTGCTCAGACAATAAGAATTATAAGAGCTCTGAGGGCTCGTTTGAAGGAACAATGGGTGAACCTTCTCTCTTGGCCAAACCTCTGGAGCTGAA
E5A +119 CCATTGTAATGGGGGGAGGTGCAAAACAGGAGGGGAAGTATGGAGATGGGGACAGAGTAGACAAAAAGCCTATGGTTTGAAGCCATGAGGAATACTCTACTCTGACTGAAGCAGGGGCCA
***

E145aa -Q--H--S--R--F--I--I--G--S--V--S--E--D--N--S--E--D--E--I--S--N--L--V--K--L--D--L--L--E--E--K--E--G--S--L--S--P--A--S--V--
E145 +361 CCAGCACTCCCGATTCAATAATGGCTCCGTGTCTGAAGATAAATCGGAGGATGAGATTAGCAACCTGGTGAAGCTCGACCTACTGGAGGAGAAGGAGGCTCTCTGTGACCAGCCTCTGT
E5A +239 AGAAGTAGGCAGAAGGCACAGGATCTTTGTCTCTGGGTTTAAAGCACCTGCAGCAGGAGGACGAACCTCCAGCTTGTGTTTCAAGGCCGACAGCTGAAGAGAAAACTCTATTCTCT
***

E145aa -S--S--D--T--L--S--D--L--G--I--S--S--L--Q--D--G--L--A--L--H--M--R--S--S--M--S--G--L--H--L--V--K--Q--G--R--D--R--K--K--I--
E5Aaa M--E--G--S--A--E--E--S--K--E--M--R--Y--Y--M--L--Q--R--S--S--M--S--G--L--H--L--V--K--Q--G--R--D--R--K--K--I--
E145 +481 CAGCTCAGACACGCTCTCTGACTTGGGATCTCGAGCCTCCAGGATGGTTTGGCCTTACACATGAGATCCAGCATGTCTGGTTTGACCTAGTCAAGCAAGGTTCGAGACCGAAGAAAAAT
E5A +359 TTGACATCTTGATAGGAGGTTCTGCGGAGGAGAGTAAGGAGATGAGATATTACATGCTTCAAGATCCAGCATGTCTGGTTTGACCTAGTCAAGCAAGGTTCGAGACCGAAGAAAAAT
***
E6Genome tctagactttgggtgattctctgtttttacagATCCAGCATGTCTGGTTTGACCTAGTCAAGCAAGGTTCGAGACCGAAGAAAAAT

E145aa -D--S--Q--R--D--F--T--V--A--S--P--A--E--F--V--T--R--F--G--G--N--K--V--I--E--K--V--L--I--A--N--N--G--I--A--A--V--K--C--M--
E5Aaa -D--S--Q--R--D--F--T--V--A--S--P--A--E--F--V--T--R--F--G--G--N--K--V--I--E--K--V--L--I--A--N--N--G--I--A--A--V--K--C--M--
E145 +601 AGACTCAGCAGGATTTTCACTGTAGCTCGCCAGCAGAAATTTGTACTCGTTTGTGGGAAATAAGTGATTGAGAAAGTTCTCATCGCCCAATGGCATTTGAGCTGTGAATGCAT
E5A +479 AGACTCAGCAGGATTTTCACTGTAGCTCGCCAGCAGAAATTTGTACTCGTTTGTGGGAAATAAGTGATTGAGAAAGTTCTCATCGCCCAATGGCATTTGAGCTGTGAATGCAT
E6Genome AGACTCAGCAGGATTTTCACTGTAGCTCGCCAGCAGAAATTTGTACTCGTTTGTGGGAAATAAGTGATTGAGAAAGTgaagttacaacctgtcaattatatttttcaaaagcccat

E145aa -C--S--I--R--R--W--S--Y--G--M--
E5Aaa -C--S--I--R--R--W--S--Y--G--M--
E145 +721 GAGATCCATCCGCGGTGGTCTTATGAGATG
E5A +599 GAGATCCATCCGCGGTGGTCTTATGAGATG
E6Genome gagatttaataagctcgatttgtagtctaga

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Figure 1 Sequence comparison of the 5' regions of ACC- α transcripts of the E5A type with those of the E1/4/5 type, illustrating the divergence at the 5' boundary of exon 6

Position +1 of both the E1/4/5 [3] and E5A-type ACC- α transcripts (clone 2RP) denotes the 5' nucleotide of cDNA clones of each mRNA species; 100% nucleotide identity between the genomic sequence around exon 6 and cDNA sequence of both E1/4/5-type and E5A-type mRNA is denoted by bold type and illustrates that the divergence of the nucleotide sequence between the two cDNA species occurs at the 5' boundary of exon 6. The deduced amino acid sequence in each cDNA is shown in the one-letter code; *** denotes in-frame termination codons upstream of the N-terminal methionine residue in the deduced translation product of the E5A-type transcript. The underlined sequence at positions +204 to +225 of the E5A-type transcript denotes the possible presence of a small open reading frame.

novel exons. RACE-PCR of lactating mammary gland cDNA with a primer anti-sense to positions +91 to +112 of the E5A transcript failed to generate any further DNA sequence 5' to that already obtained, suggesting that the sequence in Figure 1 represented a close approximation to the 5' extent of the mRNA (results not shown). The location of the 424 bp sequence in the sheep ACC- α gene was investigated. Plasmid p4133 was used to screen a sheep genomic library; three recombinants were isolated and characterized. Southern blotting and DNA sequencing of the exonic regions enabled us to establish a partial contiguous structural map. This is shown in Figure 5 in relation to the known structure of the rat ACC- α gene, including the promoter regions. The intervening sequence between exons 5 and 6 was 18 kbp. By using the 309 bp *Pst*I-*Nsi*I fragment of clone 2RP (Figure 2) as a probe, sequences corresponding to the primary 424 nt of the E5A transcript were located 5 kbp upstream of exon 6 and these were present as a single exon.

Because the primary 424 nt of the E5A transcript seemed to correspond to the 5' extent of the mRNA, we investigated whether the genomic sequences upstream of the exon exhibited promoter activity. A 1.5 kbp *Hind*III-*Bgl*II fragment corresponding to positions -1441 to +35 relative to the 5' terminal nucleotide of clone 2RP (Figure 1) was cloned into the luciferase reporter vector pGL3basic and used in transient transfection of

a number of cell types. Transfection performed in this way tests the ability of the 'naked' transfected DNA to recruit the RNA polymerase complex and thus says little about the mechanisms that result in the tissue-restricted expression of the E5A transcript, which probably involve the regulation of chromatin condensation. Human hepatoma HepG2 cells were used initially in this analysis, principally owing to their ease of transfection and also because we had demonstrated low-level expression of E5A transcripts in sheep liver (Figure 3). However, to demonstrate the universality of the promoter activity we also transfected mammary cells of murine and sheep origin. Furthermore to assess the affinity of the -1441/+35 construct for the RNA polymerase complex, its activity was compared with the relatively strong simian virus 40 promoter activity encompassed within the pGL3control (promoter plus enhancer sequences) and pGL3-promoter (proximal promoter only) vectors. Table 2 demonstrates that the 1.5 kbp -1441/+35 fragment possesses significant basal promoter activity in all three cell types, with the level of luciferase activity generated being between 10-fold (HC11 cells and sheep mammary cells) and 40-fold (HepG2 cells) that of the pGL3basic vector. Furthermore sequences in the -1441/+35 construct that direct the RNA polymerase complex operate in an orientation-dependent fashion, as the DNA fragment demonstrated little luciferase activity in the reverse orientation in

Figure 2 Diagrammatic representation of the RNase protection assay to determine the expression of E5A ACC- α transcripts in sheep tissues

The 5' region of ACC- α transcripts of the E5A type is compared with a structural representation of the constituent exons of the E1/4/5-type (expressed principally in adipose tissue) transcript. Exon 5 is the primary coding exon in the E1/4/5-type transcript. Boxed regions represent coding regions and shaded lines denote a 5' untranslated sequence. The dotted lines in the representation of the exon 6 genomic region denote an intronic sequence. A 309 nt *PstI*–*NsiI* fragment (black boxed area) of clone 2RP, encompassing part of the sequence upstream of that encoded by exon 6 as well as the common nucleotide sequence between transcripts of the E1/4/5 type and that of the E5A type corresponding to exon 6 and part of exon 7, was cloned into the *PstI* site of pGEM-5zf+. Anti-sense transcripts were generated from the plasmid linearized with *NotI* with SP6 RNA polymerase. Fully protected fragments of 309 nt correspond to transcripts of the E5A type. Protected fragments of 175 nt correspond to transcripts comprising exons 6 and part of exon 7 and thus represent ACC- α mRNA in which exon 5 is the likely exon upstream of exon 6.

Figure 3 Expression of E5A ACC- α transcripts in sheep tissues

RNA was prepared from various sheep tissues. Abbreviation: LMG, lactating mammary gland. With the exception of the mammary tissue, all samples were from castrated male sheep. RNA samples (20 μ g) were used in an RNase protection assay with anti-sense E5A ACC- α transcript resulting in protected fragments of 309 and 175 nt (see the legend to Figure 2). A representative assay is shown.

all three cell types. The $-1441/+35$ construct was approx. 300-fold more active in the HepG2 cells than in the sheep mammary cells. Such differences were also evident for the pGL3control and pGL3promoter vectors in the three cell types and probably reflected differences in cellular metabolic activity. Interestingly, insulin stimulated the activity of the $-1441/+35$ construct approx. 3-fold in HepG2 cells, whereas the simian virus 40 promoter and enhancer was stimulated only 1.8-fold. In HC11 cells the pGL3control vector was not stimulated by the presence of insulin, whereas the $-1441/+35$ construct was stimulated 2-fold. The pGL3promoter vector exhibited a similar activity to the $-1441/+35$ construct in all three cell types and was not

Figure 4 Induction of ACC- α transcripts in mammary gland during lactation

RNA was prepared from non-pregnant non-lactating (C), 100–105-day pregnant (P) and lactating (L) animals. RNA samples (20 μ g) were used in a RNase protection assay with anti-sense E5A-type ACC- α transcript (A) (see the legend to Figure 3) and with an anti-sense ACC- α transcript corresponding to exons 2/4/5 (B). In (B), protected fragments of 264 nt correspond to E2/4/5 transcripts transcribed from ACC- α PII; fragments of 196 nt correspond to the exon 5 part of the riboprobe.

Table 1 Expression of ACC- α transcripts in sheep mammary gland during pregnancy and lactation

Protected fragments from the samples shown in Figure 4 were quantified with a phosphorimager. Tissue DNA content was determined; E5A-type ACC- α mRNA and ACC- α mRNA incorporating exon 5 (corresponding to E2/4/5 and E5 protected fragments) are expressed in arbitrary units/mg of tissue DNA. Values were analysed by analysis of variance and are means $\pm$ S.E.M for five animals for each stage of mammary gland development. **Significantly different ($P < 0.01$) compared with tissue from non-pregnant/non-lactating animals.

mRNA	Non-pregnant non-lactating	Pregnant	Lactating
Exon 5A	4.2 $\pm$ 2.4	14.1 $\pm$ 1.8	65.3 $\pm$ 14.9**
Exon 5	52.4 $\pm$ 18.2	62.2 $\pm$ 11.3	157.7 $\pm$ 38.0**

stimulated by insulin in either HepG2 cells or HC11 cells. This demonstrates the relative specificity of the insulin response to the $-1441/+35$ construct. However, the physiological significance of such an insulin response is uncertain, especially to a promoter that demonstrates increased activity in the mammary gland during lactation, and might merely suggest that the promoter possesses enhancer elements that are able to result in the up-regulation of the basal promoter activity.

Further experiments were conducted to delineate the 5' extent of exon 5A more precisely as a means of identifying upstream sequence elements involved in transcriptional initiation. This analysis was performed by RNase protection assay with a probe derived from genomic DNA that extended 219 nt upstream of the 5' end of clone 2RP (Figure 6A). The major protected fragment in lactating sheep mammary gland RNA had a size of 141 nt with minor protected fragments 4–6 nt upstream and

Figure 5 Presence of the 424 nt sequence corresponding to E5A as a single exon downstream of exon 5 and upstream of exon 6 of the ACC- α gene

The figure shows a restriction map of the region encompassing exons 5 and 6 of the sheep ('ovine') ACC- α gene, demonstrating the relative positions of the exons and the relationship of this region to the known promoter structure of the rat ACC- α gene [1]. The three λ GEM-11 clones encompassing the sheep genomic region are shown below the composite structure. B, *Bam*HI; E, *Eco*RI; S, *Sac*I; X, *Xho*I.

Table 2 Analysis of promoter activity in a 1.5 kbp *Hind*III–*Bgl*II fragment encompassing the 5' terminus and sequence immediately upstream of exon 5A

HepG2 cells, mouse mammary HC11 cells or primary mammary epithelial cells from sheep were transiently transfected with 0.5–1.0 μ g of luciferase reporter gene constructs. Constructs transfected were either the pGL3control vector (containing the simian virus 40 promoter and enhancer), the pGL3promoter vector (containing the simian virus 40 proximal promoter only), the pGL3basic vector (no promoter upstream of the luciferase gene), or a *Hind*III–*Bgl*II fragment of λ 3A corresponding to the region –1441 to +35 nt relative to the start of exon 5A cloned into pGL3basic; construct –1441/+35R corresponds to the 1.5 kbp *Hind*III–*Bgl*II fragment cloned into pGL3basic in the reverse orientation. 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 (see the Experimental section). Transfections were performed in the absence or presence of 5 μ g/ml insulin. Results are means $\pm$ S.E.M. for three to seven independent experiments. *** P < 0.001, ** P < 0.01 compared with cultures in the absence of insulin. Abbreviation: n.d., not determined.

Plasmid	Normalized luciferase activity					
	HepG2 cells		HC11 mouse mammary cells		Sheep primary mammary cells	
	– Insulin	+ Insulin	– Insulin	+ Insulin	– Insulin	+ Insulin
pGL3control	3720 $\pm$ 470	6670 $\pm$ 410***	314 $\pm$ 96	318 $\pm$ 122	29.0 $\pm$ 3.1	31.3 $\pm$ 5.9
pGL3promoter	510 $\pm$ 41	693 $\pm$ 120	n.d.	n.d.	0.2 $\pm$ 0.03	0.1 $\pm$ 0.04
pGL3basic	11 $\pm$ 4	14 $\pm$ 3	3 $\pm$ 2	1 $\pm$ 1	n.d.	0.1 $\pm$ 0.01
–1441/+35	391 $\pm$ 50	1215 $\pm$ 87**	22 $\pm$ 5	41 $\pm$ 3**	1.0 $\pm$ 0.2	1.2 $\pm$ 0.3
–1441/+35R	10 $\pm$ 1	48 $\pm$ 23	n.d.	4 $\pm$ 1	n.d.	0.03 $\pm$ 0.02

downstream of the major site (Figure 6B). Fragments in this size range were not detected in the liver RNA. However, in both the lactating sheep mammary gland and liver RNA a band of 327 nt was protected corresponding to the full-length cloned fragment of p3A7z. This probably represents unspliced ACC- α pre-mRNA. This is supported by the fact that in lactating sheep mammary gland total RNA the ratio of the cluster of protected fragments around 141 nt to the 327 nt protected fragment was approximately 10:1 on a molar basis, whereas in poly(A)⁺ RNA the ratio increased to 300:1 (results not shown). In addition, the relative abundances of the 327 nt band in the mammary gland and liver RNA samples, and the fact that the 141 nt cluster was largely absent from the liver RNA, are consistent with the

relative levels of expression of exon 5-containing and E5A-containing ACC- α mRNA in the two tissues (Figure 3). The 141 nt protected fragment indicates that the 5' end of exon 5A lies 36 nt upstream of the sequence corresponding to the 5' terminus of clone 2RP (Figure 6C). The sequence in this region does not conform to that expected of a 3' splice site. However, a TATA box is not present proximal to the start site cluster. Instead, between –45 and –37 a pyrimidine-rich sequence CTCTCTCT shows a seven out of eight match to the consensus *inr* sequence CTCANTCT found in a large number of TATA-less promoters [40]. Interestingly, the cluster of start sites is flanked by this *inr*-like element and a downstream purine-rich sequence element. In addition, consensus sequences for a number

Figure 6 Mapping the 5' end of exon 5A

(A) An RNase protection assay was established to determine the position of the 5' end of exon 5A by using an anti-sense riboprobe (p3A7z) corresponding to nt -219 to +108 relative to the 5' end of clone 2RP (Figure 1). The 424 nt riboprobe is shown relative to the position of exon 5A in the 7 kb *EcoRI* fragment of λ 3A (Figure 5). Various amounts of sense transcript were included in the assay as hybridization controls, resulting in a 398 nt protected fragment. (B) Total RNA (100 μ g) was isolated from lactating mammary gland (LMG) and liver (L) and used in the RNase protection assay; 50 and 100 pg of sense transcript were used as hybridization controls. The position of the anti-sense probe (424 nt), sense-anti-sense hybrid (398 nt) and the principal protected fragments in the RNA samples (327 and 141 nt) are indicated. The asterisks denote minor protected fragments in the LMG sample. M, 32 P-labelled pGEM 7zf(+) *HpaII* markers; the slowest and fastest markers correspond to 765 and 110 nt respectively. (C) The nucleotide sequence of the 5' portion of p3A7z from nt -219 to +35, illustrating the position of the start sites (*) mapped by RNase protection assay. The nucleotide marked by a double asterisk denotes the 141 nt protected fragment in (B). Consensus binding sequences for various transcription factors are underlined. Abbreviations: USF/SREBP, upstream stimulatory factor/serum response element-binding protein; AP1, activator protein 1. ICE denotes an inverted CCAAT box element. The boxed areas flanking the start sites illustrate pyrimidine-rich (Py-box) and purine-rich (Pu-box) sequence elements. The sequence in lower case denotes the 5' extent of clone 2RP (Figure 1).

of transcription factors are present in the region proximal to the start-site cluster. These include E-box-binding proteins, upstream stimulatory factor 1 and serum response element-binding protein, and activator protein 1. Therefore it is likely that the 141 nt protected cluster delineates the transcription initiation sites for E5A ACC- α transcripts.

DISCUSSION

Previous studies by Kim et al. [1,2,17] in rodents and humans have demonstrated that ACC- α , the principal ACC isoenzyme in lipogenic tissues, is translated from a heterogeneous population of mRNA species by transcription from two promoters in a tissue-specific fashion and differential splicing of exons contributing to the 5' UTR of the mRNA species. These ACC- α mRNA isoforms essentially encode the same protein product because exon 5, the primary coding exon, is present in all ACC- α transcripts characterized before this present study. Such a pattern of promoter usage is also evident in sheep tissues, with transcripts derived from promoter I being principally restricted to adipose tissue [3] and transcripts derived from promoter II having a more ubiquitous distribution that includes elevated

expression in mammary gland during lactation. In the present study we have characterized an additional sheep ACC- α mRNA isoform in which exon 5 is replaced by a 424 nt sequence (460 nt after taking into account the start-site mapping) (Figure 6) that corresponds to exon 5A at the 5' terminus of the variant mRNA. Transcription of the variant ACC- α mRNA isoform is under the control of a unique promoter immediately upstream of exon 5A, PIII, that results in a tissue-restricted mode of expression. This is in contrast with the only other ACC- α isoenzyme variant characterized that corresponds to an enzyme with an eight-residue deletion upstream of the Ser-1200 codon of lactating rat mammary gland ACC- α and in which regulation of RNA splicing seems to be the primary mechanism for the expression of the variant isoenzyme [20,21]. This isoenzyme is implicated in the alteration of the acute control of enzyme activity through reversible phosphorylation by cAMP-dependent protein kinase at Ser-1200 [20].

The 460 nt sequence corresponding to exon 5A encodes a 17-residue N-terminal domain that contrasts with the 76-residue N-terminus of ACC- α derived from transcripts comprising exon 5. If we assume that there are no further differences in the coding regions of ACC- α isoenzymes encoded by transcripts comprising

E5A and exon 5 as the primary coding exons, the E5A-type isoenzyme would be approx. 6 kDa smaller than the exon 5-type isoenzyme (259 kDa compared with 265 kDa). This difference would be difficult to detect by gel electrophoresis of purified ACC. Transcripts comprising truncated E5A-type RNA (the 309 bp *PstI*–*NsiI* fragment of 2RP) are translated *in vitro* in rabbit reticulocyte lysates to give a predominant protein product that is indicative of translation initiation at the predicted Met codon; this product is immunoprecipitated by anti-(rat ACC) antisera (results not shown). However, rabbit reticulocyte lysate is a low-stringency translation system and thus gives little indication of the extent to which E5A-type mRNA might be translated *in vivo*. It might be important to note that the sequence context around the predicted initiator Met codon in E5A-type mRNA demonstrates a sub-optimal Kozak initiator sequence (GATATGG) compared with the sequence around the predicted initiator Met codon in exon 5 containing transcripts (ACAA-TGG) that correspond to the Kozak consensus sequence (ACCA-TGG) [41]. This suggests that there might be differences in translatability between the two ACC transcripts. We are currently addressing these questions.

A second ACC gene has been characterized that encodes the 280 kDa ACC- β isoenzyme [5,6–8] expressed in heart and skeletal muscle, where it is thought to control fatty acid β -oxidation by means of the ability of malonyl-CoA to inhibit carnitine palmitoyltransferase I, the flux-determining step in fatty acid uptake and oxidation by mitochondria [18,42,43]. The β isoenzyme is highly homologous to the α -isoenzyme except for a 218-residue N-terminal domain. This N-terminal domain is located immediately upstream of sequences encoded by the β -isoenzyme homologue of ACC- α exon 6 [7,8]. The N-terminal domain of ACC- β is hydrophilic, with a hydrophobic terminus that has been suggested to target the β isoenzyme to the outer mitochondrial membrane [7]. Similarly the E5A ACC- α isoenzyme diverges upstream of the exon 6-encoded sequence but, in comparison with the β isoenzyme, has a short hydrophilic helix at the N-terminus that suggests that this isoenzyme is not specifically targeted to membranes. ACC activity is regulated by covalent phosphorylation and dephosphorylation of the enzyme [15]. It has been demonstrated that phosphorylation at serine residues at positions 77, 79, 1200 and 1215 in rat ACC affect enzyme activity [12–16]. Positions 77 and 79 reside within exon 6 and are targets *in vitro* for cAMP-dependent protein kinase and AMP-activated protein kinase respectively [12–16]. These kinases have loose consensus sequences that span the boundary of exon 6 and the upstream exon, i.e. exon 5 in ACC- α : for example, in the AMP-activated protein kinase the loose consensus sequence corresponds to a hydrophobic residue at positions –5 and +4 relative to the serine residue [44]. In Ser-79 in ACC- α (Ser-80 in sheep ACC) the hydrophobic residue at –5 is encoded by exon 5; this residue is replaced by a glutamine residue in the E5A variant. Such an altered primary amino acid sequence in the E5A variant might result in altered substrate specificities for the AMP-activated kinase on Ser-80 or indeed cAMP-dependent protein kinase on Ser-78, with the result that the E5A variant would exhibit altered enzyme kinetics. Michell et al. [45] have demonstrated that peptides containing glutamine at the +4 position are good substrates for the AMP-activated protein kinase, implying that glutamine in this position is able to fit into the hydrophobic pocket of the kinase; whether this applies to the –5 position remains to be established. Interestingly, in ACC- β the Met codon at position –5 is conserved, implying that this isoenzyme could be a good substrate for AMP-activated protein kinase [7,8].

ACC- α transcripts of the E5A type are principally restricted to

kidney, lung, liver and mammary gland. During lactation, expression of E5A transcripts in mammary gland are increased 15-fold relative to mammary tissue of non-pregnant non-lactating animals. This contrasts with ACC- α transcripts derived from PII that comprise exon 5 as the primary coding exon, which are increased only 3-fold in lactation. These observations imply that the PIII promoter is tightly regulated in a tissue-specific and temporally related fashion. The PIII promoter lacks TATA and CCAAT boxes proximal to the transcription start site, which could account for E5A mRNA's being a minority ACC- α transcript, contributing less than 10% of the total ACC- α mRNA in most tissues that it is expressed in; only in lactating mammary gland does it exceed 30% of the total.

The physiological role of the E5A transcript is difficult to deduce at present, as is the diversity in ACC transcripts and isoenzymic forms in general [1–8,17]. So far three isoenzymic forms of ACC- α , including the E5A variant, have been characterized [20,46]; given the estimated size (400 kbp) of the ACC- α gene and the probably large number of exons [1,4] the possibility of more ACC- α isoenzymes cannot be excluded. Such arguments would also apply to ACC- β . Such transcript and isozymic diversity might be related to the increasingly diverse functions of fatty acids ascribed to physiological processes [18,47,48] that could necessitate the physical and metabolic compartmentalization of ACC molecules within cells.

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