Induction of transcripts derived from promoter III of the acetyl-CoA carboxylase- α gene in mammary gland is associated with recruitment of SREBP-1 to a region of the proximal promoter defined by a DNase I hypersensitive site

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ACC- α (acetyl-CoA carboxylase- α), a key regulator of fatty-acid metabolism, is encoded by mRNAs transcribed from three promoters, PI, PII and PIII, in the ovine genome. Enhanced expression of transcripts encoded by PIII in mammary gland during lactation is associated with alterations in chromatin structure that result in the detection of two DNase I hypersensitive sites, upstream of the start site. The most proximal site, located between –190 and –10, is characterized by the presence of an inverted-CCAAT box, C2 at –167, and E-boxes, E1 and E2, at –151 and –46. Deletion of these motifs, which bind nuclear factor-Y and upstream stimulatory factors respectively in gel-shift assays, attenuates the activity of luciferase reporter constructs in transfected cells. Chromatin immunoprecipitation demonstrated that these transcription factors were associated with PIII *in vivo* in both lactating and non-lactating mammary tissues. The basic

helix–loop–helix-leucine zipper transcription factor, SREBP-1 (sterol-regulated-element-binding protein-1), transactivated PIII reporter constructs in transfected HC11 mammary cells, and this was dependent on the presence of E1, but not on C2 or E2. SREBP-1 was only associated with PIII in chromatin from lactating animals, which was coincident with a 4-fold increase in the precursor (125 kDa) form of SREBP-1 in microsomes and the appearance of the mature form (68 kDa) in the nucleus. SREBP-1 motifs are also present in the proximal region of PII, which is also induced in lactation. This indicates that SREBP-1 is a major developmental regulator of the programme of lipid synthesis *de novo* in the lactating mammary gland.

Key words: basic helix–loop–helix, chromatin immunoprecipitation, lactation, lipogenesis, STAT5.

INTRODUCTION

ACC (acetyl-CoA carboxylase; EC 6.4.1.2) plays a pivotal role in fatty-acid metabolism. Two principal isoenzymes of ACC have been described and these are transcribed from separate genes termed ACC- α and - β , also known as ACC-1 and -2 respectively [1–3]. The ACC- α gene gives rise to a 265 kDa enzyme which is ubiquitously expressed but found at highest levels in the major lipogenic tissues, adipose, liver and lactating mammary gland [4,5]. The ACC- β gene encodes a 280 kDa enzyme which is the main isoenzyme expressed in the heart and the skeletal muscles [3]. The tissue distribution of these isoenzymes, together with the demonstration that the α -isoform was found throughout the cytoplasm whereas the β -isoform appeared to be associated with the mitochondria [6], lead to the suggestion that these isoenzymes had different functions; ACC- α providing malonyl-CoA for the synthesis of fatty acids, whereas that from ACC- β was used to regulate carnitine palmitoyl-CoA transferase I and therefore the rate of fatty-acyl β -oxidation. Both isoenzymes are regulated acutely by phosphorylation on several important serine residues, leading to inactivation [7–10], and more chronically by regulation of gene transcription [11–13].

Previous studies have shown that the ACC- α isoform was transcribed from two promoters, PI and PII, in a tissue-specific

fashion, PII expression being ubiquitous whereas PI expression was limited to adipose tissue and liver under lipogenic conditions [5,14,15]. Use of these promoters results in the generation of a heterogeneous population of mRNAs containing either exon 1 or exon 2 as the primary exon, spliced on to combinations of exons 3 and 4, before exon 5, the primary coding exon [14,16]. The physiological significance of both the tissue-specific promoter usage and transcript diversity is unclear, although the use of PI may reflect an increased capacity for lipid synthesis for uses other than membrane replenishment in liver and adipose tissue. As transcripts from PI and PII differ only in their 5'-untranslated regions they all essentially encode the same protein product.

During the characterization of ACC- α cDNAs in ovine tissues, we isolated a novel transcript from lactating mammary gland in which exon 5 and the sequence upstream of this were replaced by a 460 nt sequence represented by a single exon, exon 5A (E5A), located between exons 5 and 6 of the ovine gene [17]. Transcription of this variant ACC- α mRNA was shown to be under the control of a unique third promoter, PIII, immediately upstream of E5A, that resulted in a tissue-specific mode of expression; PIII transcripts were found in lung, liver, kidney, brain and mammary gland, but not in a number of other tissues, e.g. spleen, muscle and adipose tissues. In addition, the expression in mammary gland was significantly induced during lactation. PIII transcripts are

Abbreviations used: ACC, acetyl-CoA carboxylase; ADD1, adipocyte determination and differentiation-dependent factor 1; bHLH-ZIP, basic helix–loop–helix-leucine zipper; ChIP, chromatin immunoprecipitation; EMSA, electrophoretic mobility-shift assay; E5A, exon 5A; FAS, fatty-acid synthase; HSS, hypersensitive site; NF-Y/CBF, nuclear factor-Y/CCAAT-binding factor; SRE, sterol-regulated element; SREBP-1, SRE-binding protein-1; STAT5, signal transducer and activator of transcription 5; tss, transcription start site; USF, upstream stimulatory factor.

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distinct in that they encode an ACC- α with an alternate N-terminal sequence; the 76 amino acids encoded by exon 5 being replaced by just 17 residues encoded by E5A [17]. The biochemical functionality conferred by the E5A isoform of ACC- α is not clear at present, as is the physiological relevance to the tissues in which it is expressed. The residue at the -5 position in the AMP-activated protein kinase consensus sequence for phosphorylation of Ser⁷⁹ (Ser⁸⁰ in the ovine sequence) is altered by the use of E5A, and could possibly result in altered kinetics of this form of the enzyme [18]. It is notable that the three principal ACC isoenzymes differ mainly at the N-terminus, suggesting that a major, and possibly unsuspected, functionality is conferred by this domain. Another key question relates to what forms the molecular basis for the tissue-restricted pattern of the PIII mRNA expression. Identification of the co-regulated genes would aid understanding into the physiological basis of PIII expression in these tissues.

Important regulatory regions of genes have been located within chromatin domains that are hypersensitive to DNase I treatment. The presence of DNase I HSSs (hypersensitive sites) in genes can vary with development or hormonal status and can often define the differential accessibility of transcription factors to regulatory elements, or interaction between spatially distinct domains that associate owing to looping [19]. In the present study, we identify regions of altered chromatin structure, defined by the presence of two DNase I HSS in a 1.6 kbp region upstream of the start-site of PIII, that are associated with an increase in abundance of PIII-derived transcripts in mammary gland during lactation. Through a combination of mutagenesis of PIII-reporter constructs in transfected cells, gel-shift analysis (EMSA, electrophoretic mobility-shift assay) and ChIP (chromatin immunoprecipitation), we demonstrate that transcription factors NF-Y (nuclear factor-Y) and USF-1 and -2 (where USF stands for upstream stimulatory factor), associate with the proximal promoter, and functionally interact with the bHLH-ZIP (basic helix-loop-helix-leucine zipper) transcription factor SREBP-1 [sterol-regulated-element (SRE)-binding protein-1], which is recruited to HSS1 in lactating tissue. Furthermore, the identification of SREBP-1-binding motifs in an open-chromatin conformation in the PII promoter, which is also up-regulated in lactation, albeit to a lesser extent [17], represents a mechanism for the co-ordinate regulation of PII and PIII in mammary tissue, and indicates that SREBP-1 is a major regulator of lipid synthesis *de novo* in the lactating mammary gland.

EXPERIMENTAL

Materials

All culture media and supplements were from Gibco BRL (Paisley, Renfrewshire, Scotland, U.K.). Transfast transfection agent, luciferase assay reagents, luciferase reporter vectors, Exonuclease III, the T7 Ribomax reagent, rabbit reticulocyte lysate, Protein Precipitation Solution and *Pfu* DNA polymerase were from Promega (Chilworth, Southampton, U.K.). Oligonucleotides used for the construction of mutant plasmids and for EMSA were synthesized by MWG-Biotech (Ebersberg, Germany). Antibodies to transcription factors were from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). QuickHyb hybridization solution was from Stratagene (Cambridge, U.K.). Biotrans nylon membrane was from ICN-Flow (Paisley, U.K.).

DNase I hypersensitivity

Nuclei were isolated and treated with DNase I as given by the procedures of Whitelaw et al. [20] with minor modifications. Briefly,

nuclei (3.4×10^7) were used immediately after isolation and were digested with 50 units of DNase I at 37 °C for the various times indicated. After overnight digestion with proteinase K, the DNA was purified using Protein Precipitation Solution (Promega) followed by ethanol precipitation. DNA was then resuspended in water and 15 μ g of aliquots from each time point were digested with restriction endonucleases before being resolved on a 1% agarose gel and blotted on to Biotrans membrane. To detect HSSs within PIII blots, they were probed with a 413 bp *Bgl*III-*Apa*I DNA probe, located within E5A [+63 to +475 relative to the tss (transcription start site)]. A 1.1 kbp *Apa*I-*Eco*RI fragment located at +160 to +1273 relative to the tss was used to detect HSSs in PII. DNA was labelled with [α -³²P]dCTP using random primers [21] and hybridized using QuickHyb hybridization solution according to the manufacturer's instructions. Blots were washed, exposed to phosphor screens and scanned using a 441 SI PhosphorImager (Molecular Dynamics, Little Chalfont, Bucks., U.K.).

Deletion and site-directed mutagenesis

Deletion and site-directed mutants were constructed from a 1.5 kbp *Hind*III-*Bgl*III DNA fragment encompassing the upstream region of ovine ACC- α PIII, corresponding to nucleotides -1404 to +68. The plasmid has been renumbered to reflect assessment of the tss [17]. 5'-Deletion mutants were created with Exonuclease III [22]. Site-directed mutagenesis was performed with *Pfu* DNA polymerase using a method based on the QuickChange mutagenesis kit (Stratagene). Double-stranded oligonucleotides (top-strand only shown) used to produce targeted deletions of the C1, C2 and E1 motifs are as follows: dC1, 5'-CTTGACCTG-CTCTCCCCTTGGAGTCTGAAATTC-3'; dC2, 5'-CAGTCTT-GTTGTGGTCTCAGAAATCAC-3'; dE1, 5'-GATTGGGTCTC-AGAAATGCATCTTTCCTCCTCCCTC-3'; an *Nsi*I restriction site was introduced into dE1 (underlined). All mutant plasmids were sequenced to confirm the mutation.

Cell culture and transfection

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 on to 24-well plates at a density approximating 50% confluence 24 h before transfection. For the transfection of adherent cells, all cells were transfected in 24-well plates with 1 μ g of plasmid and a 2:1 charge ratio of Transfast reagent per well according to the manufacturer's instructions. Transfected cells were maintained in culture for a further 48 h in the presence or absence of insulin (170 nM). HC11 cells were maintained and differentiated as described previously [23] in 24-well plates. Cells were transfected after 5 days of differentiation, again using 1 μ g of plasmid and a 2:1 ratio of Transfast. Overexpression of ADD1 (adipocyte determination and differentiation-dependent factor 1)/SREBP-1 was performed with 0.05 μ g expression vector encoding the nuclear form of the transcription factor or empty vector (pSG5). Cells were then maintained in culture for a further 48 h in a differentiation medium. Cell extracts of HepG2 and HC11 cells 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 and hybridizing this to the pGL3 basic vector [17,24]. Luciferase activity was then

Table 1 Oligonucleotides used in EMSA

The sequence of the upper strand for each double-stranded molecule is shown. The core transcription factor consensus sequences are shown in bold.

Name	Sequence
C2_E1	G ⁻¹⁷⁸ TCTTGTGTG ATTGGG TCTCAGAAAT CACCTG TCTTTCC ⁻¹³⁸
C2_E1mutC	G ⁻¹⁷⁸ TCTTGTGTGATTAGTCTCAGAAAT CACCTG TCTTTCC ⁻¹³⁸
C2_E1 Δ E	G ⁻¹⁷⁸ TCTTGTGTG ATTGGG TCTCAGAAATGCAT----CTTTCC ⁻¹³⁸
C1_CCAAT	G ⁻²¹⁷ ACCTGCTCTCC CCAAT CTTGGAGTCT ⁻¹⁸⁷
C2_ICE	G ⁻¹⁷⁸ TCTTGTGTG ATTGGG TCTCAGAAATC ⁻¹⁵¹
E2	G ⁻⁵⁹ AGCCTTCCCTGT CACGTG TCCCGGGCAGCC ⁻²⁹
E2m	G ⁻⁵⁹ AGCCTTCCCTGTGAATTC ⁻⁴⁹⁶ TCCCGGGCAGCC ⁻²⁹
FIRE1_FAS [26]	G ⁻⁵¹⁷ CCTCGGTG TCCAAT TGGTCTC ⁻⁴⁹⁶

expressed as the luciferase activity per well divided by the relative amount of plasmid DNA (in arbitrary units) per well.

EMSAs and supershifts

Nuclear extract from HepG2 cells and mammary tissue were prepared as described previously [25]. EMSA was performed with nuclear extract as follows. Nuclear extract protein (3 μ g) was added to a 20 μ l of binding reaction mixture containing 15 mM Hepes (pH 7.9), 3 mM MgCl₂, 60 mM KCl, 1 mM EDTA, 5 mM dithiothreitol, 8 % (v/v) glycerol, 2.5 μ g of BSA, 0.1 μ g of poly(dI/dC) and 1 ng of ³²P-labelled oligonucleotide (3 $\times$ 10⁴ c.p.m.), with and without competitors at 50-fold molar excess. Competitors were added before the addition of nuclear protein, except when dissociation rates were determined (see next section). The total concentration of univalent cation was 105 mM. Probes were made by labelling with [α -³²P]dCTP or non-radioactive dNTPs, and Klenow fragment of *Escherichia coli* DNA polymerase to create blunt-end probes as required. After 20 min of incubation at room temperature (22 °C), the binding reaction mixtures were applied to a 4 % (w/v) non-denaturing polyacrylamide gel run in 1 $\times$ TBE running buffer (90 mM Tris/borate/2.5 mM EDTA, pH 8.3) at 20 mA constant current for 1.5 h at 4 °C. For antibody 'supershift' assays, 2 μ l of 2 μ g/ μ l anti-USF-1 (C-20), anti-USF-2 (C-20) and anti-SREBP-1 (H-160) or NF-YA [CBF-B (CCAAT-binding factor), H-209] antibodies were added to the assay after the reaction was assembled and incubation continued as described above. The antibodies cross-reacted with the corresponding proteins in ovine tissues in Western blot. Gels were dried and exposed to Kodak phosphor screens. The resulting images were scanned with a 445 SI PhosphorImager (Molecular Dynamics).

Half-life determination of DNA-protein complexes

A 6-fold scale-up of the standard EMSA using oligonucleotide C2_E1 (Table 1) and lactating mammary gland nuclear extract was allowed to attain equilibrium at 22 °C. A 1000-fold molar excess was added to the binding reaction and portions were loaded on to a continuously electrophoresing 4 % (w/v) polyacrylamide gel set at 20 mA at 4 °C with 1 $\times$ TBE buffer, at 0, 2, 4, 8, 16 and 32 min after the addition of the competitor. To determine the half-lives of the NF-Y-DNA, and USF-DNA complexes, oligonucleotides C2_E1 Δ E and C2_E1mutC (Table 1) respectively were used as competitors. Half-lives of the complexes were determined from a plot of the fraction of total probe in the specific complex at times during the competition divided by that present in the specific complex at zero time, against time.

Transcription and translation *in vitro*

Plasmids pCiteCBF-A, pCiteCBF-B and pCiteCBF-C [27] (corresponding to rat NF-YA, NF-YB and NF-YC respectively) were gifts from Dr B. de Crombrughe (The University of Texas, Houston, TX, U.S.A.). Plasmids were linearized 3' to the inserted cDNA and transcribed using the T7 Ribomax reagent according to the manufacturer's instructions. Samples of RNA (10 μ g) were translated in rabbit reticulocyte lysate [28]. NF-YA, NF-YB and NF-YC RNA were translated separately, and programmed lysates were combined in equal proportions to reconstitute NF-Y-binding activity [28]. Transcribed RNA and programmed lysates were stored at -80 °C before use.

ChIP

Chromatin was prepared from 0.3 g of samples of lactating and non-lactating ovine mammary tissue as described by Wells and Farnham [29]. Cross-linked chromatin was sheared by sonication to an average length of approx. 400–600 bp using 6 $\times$ 10 s pulses, in the presence of 0.1 g of glass beads (212–300 μ m), using a sonicator (Kontes, Vineland, NJ, U.S.A.). Samples were then centrifuged at 22 300 g for 10 min at 4 °C, and the chromatin transferred to a fresh tube. Chromatin was then pre-cleared using blocked Staph A cells and used for immunoprecipitation [29]. Chromatin from 0.03 g of tissue was used for each immunoprecipitation with 1 μ g of antibody specific for NF-Y, USF-1, USF-2, or SREBP-1 or STAT5 (C-17) (where STAT5 stands for signal transducer and activator of transcription 5). Samples containing non-immune serum or no antibody or dialysis buffer instead of chromatin, were also included to check for non-specific interactions and contamination of immunoprecipitations and wash solutions. After the immunoprecipitation step the supernatant from the sample without the antibody was collected, and a fraction of this was included in the treatments to reverse the cross-linking and clean up the DNA. This DNA represents the total 'input' DNA. To ensure that PCR was performed on the same amount of input DNA from each sample, quantitative PCR was used. The relative level of a region of the promoter of ACC- α PIII, representative of the genome copy number, was determined using a Roche Light cycler and duplicate samples of a 1:500 dilution of input DNAs. PCR product was detected using SYBR green at a temperature just below the melting temperature. The sample volume, representing the same amount of input genomic DNA, was then determined relative to one of the samples from the non-lactating tissue and this normalized volume was used in all the subsequent PCRs, both quantitative and non-quantitative. Non-quantitative PCR was performed on input, specific antibody-precipitated and non-immune serum-precipitated DNAs using PCR master mix (Abgene, Epsom, Surrey, U.K.) and 0.2 nM primers. PCR conditions were as follows: an initial denaturing step of 94 °C for 5 min, followed by 30 cycles of 92 °C for 30 s, the appropriate annealing temperature for 30 s, and 72 °C for 30 s, and a final extension step of 72 °C for 5 min. One-fifth of the PCR assays was then resolved on a 1 % agarose/TBE gel and visualized under UV. Quantitative Light cycler PCR was also performed on the same samples in duplicate. The following primers were used; ACC- α PIII proximal region: forward primer, 5'-TCCTCAGTGGTGGCGTGTCTCATCCTTC-3'; reverse primer, 5'-CAGGGCAAA-AAGAGAAGGCCACGCTTAC-3' (annealing temperature 59 °C); ACC- α PIII distal region flanking the STAT5 motif: forward primer, 5'-TCACCCGAATGTCCTAACATCAAG-3'; reverse primer, 5'-ATTACAGGCACCACAGAAAATTCCT-3' (annealing temperature 58 °C); ACC- α PII proximal region: forward primer,

Figure 1 Determination of lactationally induced DNase I HSSs in the 5'-flanking region of ACC- α PIII promoter

(A) Nuclei were isolated from the mammary glands of non-lactating and lactating sheep and digested with 50 units of DNase I/(3.4×10^7) nuclei. Nuclei were digested for up to 20 min at 37 °C. DNA from DNase-treated nuclei was purified and then digested with *NspI*. The resulting DNA fragments were separated by size on a 1% agarose gel and transferred to BioTrans membrane. The parent band (2.0 kb) and the bands produced by DNase I treatment were detected by indirect restriction fragment end-labelling using a 413 bp *BglII*–*ApaI* probe localized within E5A. DNase I HSSs are labelled 1 and 2 and their location relative to the 32 P-labelled *BglII*–*ApaI* probe are diagrammatically represented in (B). These data are representative of three experiments using different preparations of nuclei.

5'-CCCGCCCCGCTCCACCTC-3'; reverse primer, 5'-CGGA-CCCGCCCGCCCTCTAG-3' (annealing temperature 67 °C).

Immunoblotting

Protein concentration was determined using the bicinchoninic acid protein assay reagent (Pierce, Rockford, IL, U.S.A.) using BSA as a standard. Samples of nuclear or microsomal extracts (10 μ g) were separated by SDS/PAGE [10% (w/v) gel] and transferred to Hybond C super membranes. Blots were probed with commercially available antisera (1:5000) as described above and revealed with horseradish peroxidase-conjugated anti-rabbit IgG (1:2000) using an enhanced chemiluminescence detection system (Roche).

RESULTS

Two lactation-induced HSS are detected within a 1.6 kbp region upstream of the start-site

As a lactating mammary gland expresses the highest levels of PIII transcripts, both in absolute terms and also as a proportion of total

ACC- α mRNA, with levels increasing 15-fold between pregnancy and lactation [17], nuclei were isolated from lactating and non-lactating mammary gland, and treated with DNase I at different times (Figure 1A). The sites of DNase digestion were mapped by digesting the isolated DNA with *NspI* to generate a 2.0 kb parent band; a 413 bp *BglII*–*ApaI* fragment corresponding to E5A and the 3'-end of the *NspI* restriction fragment was used as a probe to detect the parental and DNase-cleaved digestion fragments (Figures 1A and 1B). The 2.0 kb parent band and two sub-bands, corresponding to DNase I HSS1 and HSS2, were detected in DNA samples from mammary nuclei of lactating animals. HSS1 is located in the proximal promoter between –10 and –190 bp. HSS2 is located between –680 and –870 bp relative to the tss (Figure 1A). Conversely, nuclei from non-lactating mammary gland, even after considerably extended digestion, do not exhibit strong DNase I HSS (Figure 1A). This suggests that the chromatin structure around the PIII promoter is more compact in the mammary nuclei of non-lactating animals when compared with the lactating ones. This result therefore identifies key regulatory regions in the proximal promoter, between –10 and –190 bp, and also at a more distal location, between –680 and –870 bp, that may contribute to the regulation of the PIII promoter in lactation.

-270 CCAAGTTCCTCAGTGGTGGCGTGCTCATCCTCCGACCGACTTGCTTTC
 -220 CCTCTTGACCTGCTCTCCCAATCTTGAGTCTGAAATTCAGTCTTGT
 -170 GTGATTGGGCTCTCAGAAATCACCTGTTCTTCTCCTCCTCCTCGGACTTC
 -120 CTGACCCCATTTATTTCTGGGCATAGCTGTCTCGTAAGCGTGGCCTTCT
 -70 CTTTTTGCCCTGAGCCCTTCCTGTCCCGGGCAGCCTGGAGAGG
 -20 CTGCGGAGCCTCTCTCTAGTGACCGTCAGAAGAGTACCGTTGTTAAA
 +31 GCACTTTTGCTGCAGTAAGGCAGAGCTGCTGAGATC

Figure 2 DNA sequence and location of putative transcription factor binding sites within the ACC- α PIII proximal promoter

The tss [17] is indicated by an arrow. Numbering is relative to the major tss [17]. C1 and C2 denote CCAAT boxes and potential NF-Y binding sites. E1 and E2 denote E-boxes. The underlined portion of C1 shows a palindromic sequence flanking the CCAAT box. The position of potential Inr and Inr-like elements flanking the tss are indicated. Downward arrows show the 5'-extents of the -220, -159 and -38/+68 deletion clones (Figure 1). The broken lines correspond to the position of oligonucleotides used in ChIP analysis (see Figure 5).

Identification of regulatory elements within the 5'-flanking region

Examination of the sequence context around each of the two HSSs for consensus motifs for transcription factors known to modulate gene expression in the mammary gland revealed a potential site for STAT5 (5'-TTCNNNGAA-3'; see also Figure 8) at -833. STAT5 is a key prolactin-mediated transcriptional activator of milk protein genes expressed in lactation [30], and therefore the occupancy of this motif in the distal PIII promoter could account for the formation of HSS2 in the mammary chromatin of lactating animals; STAT5 motifs associate with HSS in milk protein genes [20,31]. In contrast, the sequence corresponding to HSS1 (Figure 2), located immediately upstream of the tss, is characterized by the presence of a CCAAT box at -200 (C1) and an inverted CCAAT box at -167 (C2), and E-box motifs, E1 and E2, binding sites for members of the bHLH family of transcription factors [20,27], located at -151 and -46 respectively. With the exception of C1, these motifs are conserved in the bovine ACC- α PIII promoter [32].

As the motifs present within HSS1, although present in a large number of promoters, had not been previously associated with the specific expression of genes in the mammary gland, attention was focused further on identifying the factors binding to these motifs and their role in the expression of ACC- α PIII in this tissue. Preliminary studies to identify a cell system that expresses the PIII transcript endogenously were unsuccessful; cultured ovine mammary cells [17] lose the ability to synthesize the transcript despite transcribing milk protein mRNAs (results not shown). Human tissues, though not those from rats or mouse, express the PIII transcript at variable levels. However, screening of a number of human cell lines (HepG2, MCF-7, MCF-10a and MDA-MB-231) again failed to identify an endogenously expressing cell (M. C. Barber and M. T. Travers, unpublished work). As we had demonstrated previously that HepG2 and HC11 mouse mammary cells supported high levels of PIII-luciferase reporter gene activity under transient transfection conditions [17], initial experiments were conducted with these cells. A series of 5'-deletion constructs in the pGL3 basic luciferase reporter vector, and based on 1.4 kb (-1404/+68Luc) flanking sequence upstream of the tss, were transiently transfected into the two cell lines with similar results (Table 2). Table 2 demonstrates that progressive deletion of the region from -1404 to -220 bp upstream of the major tss results in a gradual increase in basal activity, suggesting a loss of repressive elements. Deletion from -220 to -159 bp resulted in a 90% reduction in promoter activity. Deletion to -38 bp

Table 2 Deletion analysis of ACC- α PIII-luciferase constructs in HepG2 hepatoma cells

HepG2 cells were transiently transfected with 1.0 μ g of luciferase reporter gene constructs in pGL3basic as indicated. Cells were harvested 48 h after transfection and assayed for luciferase activity. 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 presence of insulin (170 nM, HepG2) or insulin (870 nM), dexamethasone (0.5 μ M) and prolactin (210 nM) (HC11 cells). Results are means $\pm$ S.E.M. for three to five individual experiments using duplicate preparations of plasmid DNA.

Expression construct	Normalized luciferase activity	
	HepG2	HC11
-1404/+68	435 $\pm$ 130	14.4 $\pm$ 1.0
-1172/+68	640 $\pm$ 190	11.8 $\pm$ 5.4
-879/+68	630 $\pm$ 145	21.4 $\pm$ 3.9
-562/+68	850 $\pm$ 190	19.4 $\pm$ 5.0
-220/+68	930 $\pm$ 240	22.2 $\pm$ 2.2
-159/+68	140 $\pm$ 40	3.5 $\pm$ 0.4
-38/+68	30 $\pm$ 5	1.3 $\pm$ 0.2

resulted in further loss of activity. These results demonstrate that the -220/+68Luc construct defines a minimal promoter when transfected into the two cell lines, and this activity notably corresponds to the region associated with HSS1 described above (Figure 1).

An inverted-CCAAT box and an E-box, separated by 11 bp, determine PIII promoter activity

To determine if the two CCAAT boxes, C1 and C2, play a role in the transcriptional activity of the -220 to -159 region, the two motifs were deleted by site-directed mutagenesis in both the context of the -1404/+68Luc and -220/+68Luc plasmids. The E-boxes E1 and E2 were also deleted in further experiments. Mutant plasmids were then transfected into HepG2 and HC11 cells. Table 3 shows that deletion of C2 and E1 individually had a similar effect, resulting in a 75–90% reduction in luciferase activity, in the context of both -1404/+68Luc and -220/+68Luc when transfected into HepG2 cells. Deletion of C1 resulted in a slight increase in the activity of the two constructs in HepG2 cells. The effect of deleting the inverted-CCAAT box C2 was similar in magnitude to the effect of deleting the region between -220 and -159 confirming that C2, rather than C1, is responsible for the transcriptional activity of this region. The effect observed after deleting E1 was not expected, as the E-box is located just downstream of -159. Deletion of C2 appeared to have a less dominant effect compared with E1 deletion on the reporter activity when the constructs were transfected into HC11 cells, in contrast with that predicted by the deletion between -220 and -159 (Table 2) which had a similar effect in both cell lines. This could reflect the relative roles of CCAAT box binding in determining the reporter activity in the two cell lines, and the degree to which the deletion of -159 site affects the binding of factors to E1. Nevertheless, it suggests that factors binding to both C2 and E1, located 11 bp downstream of C2, are necessary for transcription of -1404/+68Luc and -220/+68Luc in HepG2 and HC11 cells.

Identification of factors binding to C2 and E1 was addressed using EMSA. CCAAT box motifs have been shown to bind predominantly to the ubiquitously expressed heterotrimeric (subunits A–C) transcription factor, NF-Y/CBF (hereafter referred to as NF-Y), which is postulated to play a role in the remodelling

Figure 3 CCAAT box, C2, binds NF-Y and E-box, E1, binds USFs from nuclear extracts isolated from lactating sheep mammary gland and HepG2 cells as determined by EMSA and supershift assays

EMSA was performed with either 3 μ g (LSMG) (**A**) or 18 μ g (HepG2) (**B**) of nuclear extract and the C2_E1 oligonucleotide. Competition with oligonucleotides listed in Table 1 was performed as indicated at 50-fold molar excess. Supershift was performed with antisera against USF-1 and -2, NF-YA and SREBP-1 as indicated. In (**B**) supershift was performed after competition with oligonucleotides C2_E1mutC (lanes 3–6) or C2_E1ΔE (lanes 7–10). Bands D–F are non-specific complexes. A denotes the position in the gel of the NF-Y.USF ternary complex formed with LSMG nuclear extract resolved on the same gel (results not shown).

of chromatin through interaction with core-histones and transcriptional co-activators [33]. NF-Y has been implicated in the transcriptional regulation of the FAS (fatty-acid synthase) gene in rat liver, binding at multiple motifs [26,28]. Nuclear protein binding to the region between –178 and –138 using oligonucleotide C2_E1 (Table 1) was investigated using extracts from HepG2 cells and lactating mammary gland. Three major complexes (A–C) (lane 1) were formed with mammary gland nuclear extract (Figure 3A). Use of anti-USF-1 and -2 antisera, to investigate the nature of proteins binding to the E-box motif, demonstrates that band C is supershifted by the specific antisera (lanes 6 and 7), but not by antisera to a related bHLH-ZIP family member, SREBP-1 (lane 9). Band C is not competed by a competitor harbouring a deleted E1 motif (C2_E1ΔE) (lane 4). Together, this indicates that E1 is bound by a heterodimer composed of USF-1 and -2 in this assay *in vitro*. Antisera to USF-1 and -2 also supershift band A (lanes 6 and 7), demonstrating that USFs are also present in this complex. The importance of the E-box to the formation of complexes A and C is demonstrated by the observation that oligonucleotides with an intact E1 but mutated C2 motif (C2_E1mutC) compete with both bands (lane 3). Band B in the mammary extract is a complex containing NF-Y as it is supershifted by an anti-NF-YA antisera (lane 8) and is not competed by an oligonucleotide harbouring a mutation in C2 (C2_E1mutC; 5'-ATTGGG-3' to 5'-ATTAAG-3') (lane 3). Band A is disrupted by both anti-NF-Y (lane 8) and also by oligonucleotides C2_E1ΔE and FIRE1 that compete for NF-Y binding (lanes 4 and 5). Conversely, oligonucleotides comprising C2 (C2_E1 and C2_ICE) but not C1 (C1_CCAAT) (Table 1) compete for NF-Y binding to the FAS FIRE1 motif in rat liver nuclear extract [26], and bind translated NF-Y *in vitro* (results not shown). This indicates that the conserved C2 motif, as

opposed to C1, is a probable functional NF-Y binding site. The context around C1 displays a palindromic sequence (underlined in oligonucleotide C1_CCAAT; Table 1) with a symmetry axis; consensus NF-Y binding sites demonstrate no symmetry axis [33].

Specific complexes comprising NF-Y (band B) and USF-1 and -2 (band C) are also observed when nuclear extracts from HepG2 cells are used with the C2_E1 oligonucleotide (Figure 3B, lane 1). The abundances of these complexes are markedly lower than those observed with mammary gland nuclear extract, necessitating the use of larger amounts of protein (18 versus 3 μ g) in the binding reactions, and also the use of competing oligonucleotides, C2_E1mutC and C2_E1ΔE, to reduce the higher background, exhibited by non-specific complexes D, E and F. Competition with C2_E1mutC (lanes 3, 4 and 6) results in the competition of all complexes with the exception of band B. Band B is supershifted by antisera to NF-Y (lane 5) but not to USF-1 and -2 (lane 4) or SREBP-1 (lane 6). Conversely, competition with C2_E1ΔE results in the competition of all complexes with the exception of band C (lanes 7, 9 and 10); band C is disrupted and supershifted by antisera to USF-1 and -2 (lane 8). A NF-Y.USF ternary complex is not readily visible with the HepG2 nuclear extract, probably due to the higher background and the lower abundance of NF-Y and USF in this extract when compared with the mammary nuclear extract.

To determine if there was any co-operation in binding between NF-Y and USFs to account for the formation of the NF-Y.USF.DNA ternary complex, the relative stabilities of each of the complexes, A–C, formed in lactating mammary nuclear extract with ³²P-labelled oligonucleotide C2_E1 was determined (Figure 4). Figure 4 shows that when using oligonucleotide C2_E1ΔE, which has the E-box deleted and is thus only capable of binding NF-Y as an unlabelled competitor, the NF-Y.DNA

Figure 4 Dissociation rates of USF, NF-Y and USF.NF-Y complexes binding to the C2_E1 oligonucleotide from lactating sheep mammary gland nuclear extract as determined by competitive EMSA and continuous gel electrophoresis

Upper panel: pre-complexed binding reactions were supplemented with a 1000-fold molar excess of C2_E1 Δ E before loading on to the gel at the indicated times (lanes 1–6). Lanes 7–9 correspond to samples in which complexes were allowed to form (lane 7) or prevented from forming by the addition of 50-fold (lane 8) or 1000-fold (lane 9) molar excess of specific competitor and were resolved on the gel with a 32 min time point (lane 6). Lower panel: the time at which the horizontal broken line intersects the dissociation curves is the half-life for that complex. Data are the means $\pm$ S.E.M. from at least three independent experiments.

complex (band B) is very unstable, whereas NF-Y complexed with USFs (band A) is much more stable with a half-life of 2.5 min. The USF.DNA complex (band C) appears to have a half-life similar to that of band A. A similar result (results not shown) was also obtained when the experiment was repeated using oligonucleotide C2_E1mutC, which has a mutated C2 motif and is thus only capable of binding USFs, as a competitor. Thus even in the absence of a specific competitor, the NF-Y.DNA complex is still unstable relative to the NF-Y.USF.DNA complex. Thus binding of USFs to E1 acts to stabilize binding of NF-Y to C2 *in vitro*. This could indicate that occupancy of E1 by USFs, or related factors, may act in a similar fashion *in vivo* to increase the probability of formation of a co-dependent transcription complex, as exhibited by the dependency of both C2 and E1 for the activity of PIII luciferase reporter constructs in both transfected HepG2 and HC11 cells (Table 3).

NF-Y and USF-1 and -2 are associated with the region defined by HSS1 in chromatin from lactating and non-lactating mammary tissue

As EMSA experiments merely determine the potential for interaction of NF-Y and USFs with their cognate-binding motifs as assessed *in vitro*, ChIP was used to confirm the interaction of

Table 3 Effect of deletion of CCAAT boxes, C1 and C2, and E-boxes, E1 and E2, on the activity of ACC- α PIII-luciferase reporter constructs in HepG2 hepatoma and HC11 mammary cells

Cells were transfected with plasmids comprising combinations of deletions of C1, C2 and E1 in the context of both the –1409 and –220 PIII 5'-flanking sequence. Transfections were performed as summarized in the legend to Table 2. Results are means $\pm$ S.E.M. from at least four individual experiments using duplicate preparations of plasmid DNA. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ when compared with wt (wild-type) expression construct.

Expression construct	Normalized luciferase activity Cells	
	HepG2	HC11
–1404/+68 wt	435 $\pm$ 70	14.4 $\pm$ 1.0
–1404/+68 Δ C1	695 $\pm$ 70	n.d.
–1404/+68 Δ C2	40 $\pm$ 5***	9.8 $\pm$ 2.3*
–1404/+68 Δ E1	120 $\pm$ 35***	4.9 $\pm$ 0.9**
–220/+68 wt	805 $\pm$ 110	17.3 $\pm$ 2.1
–220/+68 Δ C1	1030 $\pm$ 60	n.d.
–220/+68 Δ C2	195 $\pm$ 25***	10.8 $\pm$ 2.5*
–220/+68 Δ E1	210 $\pm$ 30***	8.4 $\pm$ 1.5**
–220/+68 Δ E2	85 $\pm$ 5***	4.3 $\pm$ 0.6***

Abbreviation: n.d., not determined.

these factors with the proximal promoter *in vivo*. ChIP allows DNA–protein interactions *in vivo*, which are fixed by formaldehyde cross-linking, to be interrogated by immunoprecipitation with specific antisera, and the presence of the PIII promoter in the immunoprecipitates to be analysed by PCR using DNA primers specific to the proximal region of PIII. This is particularly important for USF-1 and -2, abundant members of the large family of bHLH proteins that bind the core E-box motif, where a possibility exists that interactions observed *in vitro* are not favoured *in vivo*. Figure 5 shows that, after 30 cycles of PCR, the predicted 206 bp DNA fragment is amplified from equal amounts of chromatin, from both lactating and non-lactating tissues, immunoprecipitated with antisera against NF-Y or USF-1 or USF-2, but not when rabbit IgG or no DNA was included in the reaction. The lower panel shows a 1:500 dilution of input chromatin, before immunoprecipitation. DNA corresponding to the proximal promoter appeared to be present at slightly higher levels in immunoprecipitates, generated by each of the antisera, from the chromatin of the lactating animals when compared with non-lactating animals. These differences were confirmed by quantitative real-time PCR of the immunoprecipitates. The association of NF-Y with the proximal promoter increased ($P = 0.035$) from 50 ± 15 arbitrary units in the chromatin from non-lactating animals to 110 ± 20 arbitrary units in the chromatin of lactating animals. Similarly, the association of USF-1 and -2 with the proximal promoter increased 2-fold ($P = 0.01$) and 4-fold ($P = 0.014$) respectively in lactation; USF-1 increased from 80 ± 8 to 160 ± 25 arbitrary units and USF-2 increased from 70 ± 15 to 270 ± 100 arbitrary units. It is important to note that these are expressed as arbitrary units due to the probable differences in antisera affinities, and as such no judgements can be made about the stoichiometry of the association of these factors. The signal generated by the pre-immune IgG was greater than 20-fold but lower than that achieved with the specific antisera, and no significant differences were observed between the chromatin obtained from lactating and non-lactating animals. Thus these results demonstrate that USF-1 and -2, and NF-Y are associated with the PIII promoter in non-lactating tissue, and their association is slightly modulated with lactation.

Figure 5 Transcription factor association with the proximal region of the ACC- α PIII promoter

Chromatin was prepared from mammary tissue from non-lactating (lanes 1–3) and lactating (lanes 4–6) animals ($n=3$ for each developmental stage). Equal amounts of cross-linked chromatin, determined by real-time PCR, were incubated with antisera against NF-YA, USF-1, USF-2 and SREBP-1. Rabbit IgG was used as a control. The input chromatin corresponds to a 1:500 dilution of DNA extracted before immunoprecipitation. DNA extracted from each immunoprecipitate was analysed by 30 cycles of PCR using primers corresponding to the proximal promoter and an equal portion of each sample was resolved on a 1% (w/v) agarose gel. The PCR product is 206 bp. *Primer-dimers formed in the absence of chromatin (lane 7).

Figure 2 shows that two E-box motifs, E1 and E2, are present within the proximal promoter region. Deleting the proximal E-box, E2 at -46 , in the context of $-220/+68$ Luc also resulted in a significant attenuation in luciferase activity when HepG2 and HC11 cells were transfected (Table 3), suggesting that the E2 motif is also functionally relevant. However, as E1 is within the sequence flanked by the primers used in the ChIP method, and E2 lies only a few bp downstream of the 3'-primer, but within the resolution of the technique, it is not possible to determine with certainty by this method whether both E-box motifs bind USF-1 and -2 *in vivo*. To determine the relative affinities of E1 and E2 for USFs, a competitive EMSA was performed using an oligonucleotide comprising E2 as a probe. E2 corresponds closely to an optimal canonical USF binding E-box [34]. This experiment was performed in the presence of magnesium chloride, which restricts the ability of USF to bind to suboptimal motifs [34]. Figure 6 shows a specific complex is formed (lane 1) between mammary gland nuclear extract and the E2 oligonucleotide, which can be disrupted and supershifted by antisera against USF-1 (lane 7) and USF-2 (lane 8). The USF complex is competed effectively by E2 at increasing molar excess (lanes 2 and 3, and the lower panel), and also, less effectively by the C2_E1 oligonucleotide at equivalent concentrations (lanes 5 and 6, and the lower panel), but not by an oligonucleotide, E2m (lane 4), harbouring a disrupted E-box motif identical with that in the $-220/+68\Delta E2$ luciferase reporter construct (Table 3). These results indicate that E1 has a lower affinity for USF binding than the E2 motif when assessed by EMSA *in vitro*.

SREBP-1 is recruited to the proximal promoter during lactation

Lack of major differences in the association of NF-Y, USF-1 and -2 with the proximal promoter *in vivo* between lactating and

Figure 6 E2 displays higher affinity for USF binding *in vitro* than E1

EMSA was performed with 3 μ g of nuclear extract from lactating mammary gland and the E2 oligonucleotide. Competition with oligonucleotides E2 and C2_E1 (Table 1) was performed at 5- (results not shown), 10- and 50-fold molar excess. Competition with the E2m oligonucleotide was performed at 50-fold molar excess. Supershift was performed with antisera against USF-1, -2 and SREBP1. In the lower panel, results are expressed as the mean $\pm$ S.E.M. of the initial level of USF complex formation in the absence of competitor from three independent experiments. * $P < 0.05$ when values for the competition with C2_E1 are compared with the E2 oligonucleotide.

non-lactating mammary tissue prompted us to investigate other factors. The lipogenic transcription factor SREBP-1 is known to transactivate motifs related to E-boxes in a restricted number of lipogenic enzyme genes in liver. However, the presence of E-boxes in promoters does not necessarily predict transactivation by SREBP-1, indicating that the configuration of these motifs with other sequence elements is probably very important in this respect [35]. As SREBP-1 activation of some E-box-containing promoters is related to the proximity of the E-box with NF-Y-binding sites, we investigated whether SREBP-1 was associated with chromatin in the vicinity of the PIII proximal promoter in lactating and non-lactating mammary tissues. Figure 5 shows that antisera to SREBP-1 immunoprecipitate DNA corresponding to the proximal promoter in lactating tissue, but these sequences are not detectable in immunoprecipitates of chromatin from non-lactating mammary tissue. Real-time quantitative PCR of the immunoprecipitates demonstrates that sequences corresponding to the proximal promoter are detected at >20 -fold higher levels in the chromatin of lactating animals compared with non-lactating animals. Therefore these results indicate that the increase in PIII

A Microsomes

B Nuclei

Figure 7 Induction of the precursor (125 kDa) and the mature (68 kDa) form of SREBP-1 in mammary gland during lactation

Microsomes (A) and nuclear extracts (B) were prepared from non-lactating (NL; lanes 1–3) and lactating (L; lanes 4–6) mammary tissue. Three animals from each developmental stage were used. Extracts (10 μ g) were resolved on 10% (w/v) polyacrylamide gels, blotted, and incubated with antisera against SREBP-1. Membranes containing nuclear proteins were sequentially stripped and incubated with antisera against USF-1, USF-2 and NF-YA. Duplicate membranes were incubated with antisera for STAT5A and STAT5B.

transcripts during lactation is associated with the recruitment of SREBP-1 to the proximal promoter.

Figure 7 shows that the recruitment of the transcription factor to the proximal promoter of PIII in lactation is associated with increases in both the precursor (125 kDa) microsomal and mature nuclear (68 kDa) forms of SREBP-1. The 125 kDa form is increased ($P < 0.001$) 4-fold from 535 ± 80 arbitrary units in the microsomal fraction of non-lactating tissue to 2350 ± 165 arbitrary units in lactating tissue. The mature transcriptional activator is increased ($P < 0.02$) 9-fold from 90 ± 20 arbitrary units in the nuclear fraction from non-lactating tissue to 725 ± 200 arbitrary units in nuclei from lactating tissue. The 125 kDa precursor is also detected in the crude nuclear fraction, probably as a result of microsomal association with the nuclear envelope. In comparison, the abundance of the transcription factors, NF-Y, USF-1, USF-2, STAT5A and STAT5B in nuclei was not altered in lactation. USF-2 is detected as 44 and 38 kDa proteins, which is consistent with the molecular masses of the USF-2a and -2b isoforms [36].

To determine if SREBP-1 can stimulate PIII-luciferase gene expression in HC11 cells, a plasmid construct comprising promoter sequences from -220 to $+68$ ($-220/+68$ Luc) was co-transfected with either control plasmid or the same vector encoding ADD1/SREBP-1c. Owing to alternative promoter usage, two isoforms of SREBP-1, 1a and 1c, are potentially

Table 4 E1, a target of SREBP-1 transactivation, after co-transfection of HC11 mammary cells by ACC- α PIII luciferase reporter constructs and an expression vector encoding ADD1/SREBP-1c

Cells were transfected with plasmids comprising deletions of C2, E1 and E2 in the context of the $-220/+68$ PIII 5'-flanking sequence. Transfections were performed in the presence of insulin (870 nM), dexamethasone (0.5 μ M) and prolactin (210 nM). Results are means $\pm$ S.E.M. from six individual experiments using duplicate preparations of plasmid DNA. *** $P < 0.001$ when compared with wt (wild-type) expression construct, ** $P < 0.05$ when compared with wt and $-220/+68\Delta C2$ expression constructs.

Expression construct	Fold induction by co-transfected ADD1/SREBP-1c
$-220/+68$ wt	2.5 ± 0.3
$-220/+68\Delta C2$	2.3 ± 0.3
$-220/+68\Delta E1$	$1.1 \pm 0.1^{***}$
$-220/+68\Delta E2$	$3.3 \pm 0.2^{**}$

present in tissues. These differ in the length of the N-terminal transactivation domain. SREBP-1c mRNA, the transcript that results in the form with the shorter transactivation domain, is the major SREBP-1 mRNA in lactating mammary gland, at least in the mouse (results not shown). Overexpression of SREBP-1c, when compared with co-transfection with the same vector minus the SREBP-1 coding sequence, resulted in a 2.5-fold increase in luciferase activity ($P < 0.001$) (Table 4). To determine whether C2, E1 and E2 are involved in the response to SREBP-1c, reporter constructs comprising deletions of these motifs were also transfected into HC11 cells. Although the deletion of C2 and E2 reduced the activity of reporter constructs in HC11 cells (Table 3), the fold induction (2.5–3-fold) in response to ADD1/SREBP-1c was not impaired. In contrast, the stimulation by SREBP-1 is inhibited in constructs bearing a deletion in E1. These results indicate that the lower affinity USF-binding motif, E1, mediates the stimulatory effect of SREBP-1 on the $-220/+68$ PIII reporter construct in transfected HC11 cells, and provides evidence, in conjunction with ChIP, that the E1 motif may be functionally relevant *in vivo* in this respect.

DISCUSSION

The present study has demonstrated that the marked increase in abundance of transcripts derived from the PIII promoter of the ACC- α gene that occurs in the mammary gland of ruminants [17,32] during lactation is associated with the recruitment of the bHLH-ZIP transcription factor, SREBP-1, to a region of the proximal promoter. The proximal region (HSS1), together with an upstream region at approx. -800 (HSS2), is defined as lactation-induced DNase I HSS, indicating that increased transcription from this promoter is accompanied by substantial chromatin remodelling. SREBP-1 is a major regulator of nutrient-modulated lipogenic gene expression in rodent liver [37]. It is widely expressed in mammalian tissues [38], and recent studies have identified orthologues as key regulators of lipid metabolism in *Clostridium elegans* [39] and *Drosophila* [40]. SREBP-1 is synthesized as a membrane precursor that resides in the endoplasmic reticulum, and is subject to regulated proteolysis by two proteases under appropriate physiological conditions, that result in the release and translocation of an N-terminal portion of the molecule to the nucleus, and its recruitment to the promoters of target lipogenic genes [35]. Expression of the endoplasmic reticulum and nuclear forms are increased 4- and 10-fold in the

microsome and nuclear fraction respectively in lactating relative to non-lactating mammary tissue, indicating that the state of lactation influences the size of the SREBP-1 precursor pool, and also the proportion of precursor that is subject to cleavage. Lactation is not characterized by the relative hyperinsulinaemia or increased serum glucose which define the action of SREBP-1 in liver, suggesting that other factors, probably related to the particular endocrinology of this physiological state, are involved in the increased expression of this transcription factor in the lactating mammary gland.

The proximal promoter, corresponding to HSS1, and the target of SREBP-1 recruitment, is characterized by two E-box motifs, E1 and E2, and an inverted CCAAT-box motif, C2, a helical turn upstream of E1. These motifs appear to be functionally relevant in the context of maintaining the activity of PIII-luciferase constructs when transfected into HepG2 hepatoma and HC11 mammary cells, in a qualitatively similar fashion. ChIP has been used to confirm the prediction from EMSA experiments that NF-Y and USF-1 and -2 associate with the proximal promoter *in vivo*. This is particularly important for USF-1 and -2, abundant members of the large family of bHLH proteins that bind the core E-box motif, where a possibility exists that interactions observed *in vitro* are not necessarily favoured *in vivo*. It is important to note that the ChIP procedure confirms the association of factors with chromatin with an effective resolution of approx. 200 bp flanking the sequence defined by the PCR primers [29], and thus sites other than C2 and E1 and E2 could bind NF-Y and USFs *in vivo* respectively. However, inspection of the DNA sequence of PIII in this region indicates no other sites of high probability of binding for these factors. Consequently, it is not possible to determine with certainty by ChIP whether both E-box motifs bind USF-1 and -2 *in vivo*, especially in the non-lactating mammary gland before the recruitment of SREBP-1. E2 corresponds most closely to the optimal USF-binding motif, defined by Bendall et al. [34], and has a slightly higher affinity for the transcription factors *in vitro* and, therefore, a higher probability of binding the USFs *in vivo*. E1 corresponds to a non-canonical E-box. Non-canonical E-boxes have been demonstrated to recruit USFs *in vivo* in a number of promoters [41], suggesting E1 may also be a physiological target for USFs. Interestingly, it may be inferred that the association of NF-Y and USFs with the proximal promoter *in vivo* in non-lactating mammary tissue is contributory, as predicted by the transfection of mutant reporter constructs, to the lower level of expression of ACC- α transcripts observed in this state. The role of these factors in driving basal promoter activity is also supported by the observation that the association of NF-Y and USFs with the proximal promoter was only slightly modulated by lactation, which could be explained by an increase in the proportion of cells in lactating tissue expressing the promoter.

Co-transfection of the -220/+68 reporter construct with an expression vector for SREBP-1 into HC11 mammary cells suggested that E1, as opposed to E2, is a target for this transcription factor. SREBP-1 is a member of a small group of bHLH transcription factors in which a conserved arginine residue in the DNA-binding domain is replaced by a tyrosine residue (Tyr³²⁰ of SREBP-1c). This has the effect of modifying the DNA-binding specificity of this class of bHLH factors, such that SREBP family members are capable of binding SRE motifs corresponding to direct repeats of CAC in the configuration 5'-atCA-CccCAC-3', in addition to canonical E-boxes of CAC dyad symmetry [38]. Indeed, well-established physiological targets for SREBP-1, and also its paralogue SREBP-2, generally correspond to the SRE motif present in a number of genes involved in the synthesis of cholesterol, and also a more limited number of genes regarded as primarily lipogenic. However, there is some

debate as to whether E-boxes or E-box-like motifs are physiological targets of SREBP-1. Recombinant SREBP-1, as opposed to nuclear extracts comprising physiological levels of the transcription factor [35], will bind canonical E-boxes *in vitro* in the absence of cofactors, though it is generally apparent that it will not predictably transactivate promoters containing E-boxes of this type. Significantly, SREBP-2 will also bind E-boxes *in vitro*, but fail to transactivate a comprehensive number of E-box-containing promoters [35]. This is consistent with a restricted role of this paralogue in regulating SRE-containing promoters. Indeed, this suggests that the transactivation potential of SREBP family members is conferred by the mode of interaction with its increasingly diverse binding motifs, and that the consequent ability to interact with cofactors and co-activators, in a promoter-specific fashion, is obligatory for a physiological response. Cofactor requirements and SREBP-binding motifs are especially diverse for SREBP-1-responsive lipogenic promoters in terms of their identity and relative spacing [35]. Generally, interaction distances between the SREBP-binding motif, which can vary from an E-box-like motif, as in the rat S14 gene [42], to SRE-like motifs, as in FAS [43], to SRE half-sites [5'-CCAT(n)₁₀TCAC-3'], as in ACC- α PII [44,45], and the cofactor binding motif, generally NF-Y or stimulating protein-1, are in the order of 10–30 bp apart. Shortening this spacing to 7 bp, at least in the context of SREs, can impair the response to SREBP-1 [35]. Also, there are some SREBP-1-responsive promoters, e.g. glucose 6-phosphate dehydrogenase, in which it is apparent that a cofactor binding motif cannot be easily identified [35]. The SREBP-1-responsive region of PIII has been identified as the non-canonical E-box or SRE half-site (5'-ATCACCTg-3') E1, 11 bp downstream of the C2 motif that binds NF-Y. In contrast with a large number of SREBP-1-responsive promoters that show a large degree of co-operativity between SREBP-1 and NF-Y in both the regulation of basal-promoter activity and SREBP-1 responsiveness [42,46], the C2 and E1 motifs within the proximal region of PIII appear to act independently, as deletion of C2 or E1 in the context of reporter vectors transfected into HC11 cells suppress luciferase activity in a similar fashion, whereas only loss of E1 has any effect on SREBP-1 inducibility. However, the rat ATP-citrate lyase promoter appears to demonstrate a more complex motif in which the SREBP-1-responsive region comprises two SRE-motifs and a CCAAT box [47]. Mutation of individual motifs reduces promoter activity, although affecting SREBP-1 inducibility only to a limited extent. Attenuation of SREBP-1 responsiveness in the ATP-citrate lyase promoter is observed only when more than one of the motifs is disrupted, indicating the composite nature of the SREBP-1-responsive region and the potential for functional redundancy. A similar situation, involving functional redundancy, may occur in the PIII promoter to account for the apparent lack of co-operativity between the inverted-CCAAT box motif, C2, and the SREBP-binding motif, E1, in supporting a response to SREBP-1, at least in the context of the HC11 cell line. However, how unique the SREBP-1-responsive region of the ACC- α PIII promoter is in this respect will require the identification of an expanded number of SREBP-1-responsive promoters to determine the evolution of the motif, and its interacting motifs, in lipogenic genes.

Co-operativity of interaction between SREs and cofactors can also be confirmed by ChIP. In the LDLR gene, recruitment of SREBP-2 to the promoter by sterol deprivation also results in increased association of NF-Y [48]. Conversely, a lack of co-operativity, at least in the association of these factors with the proximal promoter of ACC- α PIII, is suggested by the observation that recruitment of SREBP-1 in lactation is not associated with a significant increase in NF-Y binding. However, ChIP also yields a potential problem of interpretation in that an attractive model of

Figure 8 Co-ordinate induction of PIII and PII in lactation: putative interacting proximal and distal regions of each promoter

Alignment of the 5'-flanking regions of ACC- α PIII and PII showing the localization of common transcription factor binding motifs within regions of open chromatin structure, as defined by lactationally induced DNase I HSSs (downward pointing arrows).

SREBP-1 replacing USFs at E1 in lactation is not supported by the results, as a reduction, relative to non-lactating tissue, of USF association with the promoter is not observed. Three possibilities could account for this. First, USFs do not occupy E1 in non-lactating tissue, indicating that E1 is occupied by another factor to stabilize NF-Y binding, or alternatively is unoccupied in this state, suggesting that the EMSA results (Figure 4) is an *in vitro* phenomenon. Secondly, USFs could form higher-order structures, tetramers as opposed to dimers, in the chromatin of lactating tissue, as observed with recombinant USF-1 at physiological concentrations [49], which could perhaps facilitate chromatin remodelling. Thirdly, SREBP-1 could heterodimerize with USFs, though we are not aware of any results to support this. Clearly, future experiments are required to address these possibilities.

The association of increased expression of ACC- α PIII transcripts in mammary gland during lactation with reorganization of the chromatin structure in the region flanking the promoter is consistent with a large number of studies showing that rearrangement of chromatin is essential for the correct positioning and functioning of transcription factors in modulating promoter activity [19]. Interestingly, a significant alteration in chromatin structure exhibited by the presence of three lactation-induced

DNase I HSS upstream of exon 2, is also associated with the increased expression of transcripts derived from the PII promoter. HSS1 is located within the proximal promoter and HSS2 and HSS3 are located distally at -700 and -1300 respectively and are shown schematically in Figure 8. The chromatin around PII, as it is with PIII, is markedly inaccessible to DNase I in non-lactating mammary tissue and, intriguingly, the marked increase in the accessibility of chromatin in the region of PII with lactation is only associated with a 2- to 3-fold increase in PII transcripts relative to non-lactating tissue, in comparison with the 15-fold increase in PIII transcripts [17]. Together, this indicates that the co-ordinate up-regulation of these promoters is probably achieved through communication, possibly via productive looping through the action of co-activators, between the proximal and more distal HSS for each promoter. This could be achieved in part by the recruitment of common transcriptional activators to both PII and PIII. Inspection of the DNA sequence (Figure 8) within HSS1 of PII also reveals the presence of an SREBP-1-binding motif characterized as two SRE half-sites, separated by 10 bp, in conjunction with stimulating protein-1 and NF-Y motifs. Indeed the proximal promoter region of PII defined by HSS1 and including the SRE-like motifs is highly conserved in a number

of species [50]. The SRE-like motifs have been demonstrated to mediate the upregulation of the PII promoter in response to ectopically expressed SREBP-1 in CV-1 [44] and MIN6 β -cells [45]. Significantly, a number of genes for lipogenic enzymes (FAS, ATP-citrate lyase and 'malic' enzyme) that demonstrate increased activity in the mammary gland during lactation [51] are also defined by the presence of SRE motifs in their proximal promoter regions and have been demonstrated to be responsive to ectopic expression of SREBP-1 in the context of transfected cells and in the livers of transgenic mice [35]. The marked increase in SREBP-1 expression in the lactating mammary gland may thus provide a mechanism for the co-ordinate upregulation of these genes to meet the increased metabolic requirement to synthesize milk. Attempts to confirm that SREBP-1 was recruited to the PII promoter in lactation by ChIP were not successful, probably due to the technical difficulty of performing PCR across a region of high-percentage GC content (77% in a 400 bp region upstream of the tss). Interestingly, the region around HSS2, in both PII and PIII, is characterized by common motifs that bind the transcription factor STAT5 in EMSA with nuclear extracts of lactating mammary gland (results not shown). STAT5 is a downstream target of prolactin signalling complexes in lactating mammary gland, and a co-ordinator of mammary development and milk protein gene expression. [30]. The association of STAT5 with the region defined by HSS2 in PIII with lactation could not be confirmed by ChIP (results not shown), even though the antiserum detects STAT5 in nuclear extracts by Western-blot analysis (Figure 7). However, Mao et al. [32] have shown recently that mutation of the STAT5 motif in the bovine ACC- α PIII promoter attenuates lactogenic hormone induction of reporter constructs when transfected into HC11 cells, thereby lending some support to the functional relevance of the STAT5 motif in the regulation of PIII transcription in the mammary gland.

In conclusion, the present study has identified SREBP-1 as a major regulator of lipid synthesis *de novo* in the lactating mammary gland, through its regulation of ACC- α PIII, achieved in concert with NF-Y and USFs. SRE-like motifs are also present in the proximal region of PII indicating that SREBP-1 could play an important role in the co-ordinate regulation of both promoters. Inductions of both of the promoters are defined by interaction between largely unknown proximal and distal elements in each promoter, and therefore more studies are required to define these interactions.

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REFERENCES

- Lopez-Casillas, F., Bai, D. H., Luo, X. C., Kong, I. S., Hermanson, M. A. and Kim, K. H. (1988) Structure of the coding sequence and primary amino acid sequence of acetyl-coenzyme A carboxylase. *Proc. Natl. Acad. Sci. U.S.A.* **85**, 5784–5788
- Ha, J., Lee, J. K., Kim, K. S., Witters, L. A. and Kim, K. H. (1996) Cloning of human acetyl-CoA carboxylase- β and its unique features. *Proc. Natl. Acad. Sci. U.S.A.* **93**, 11466–11470
- Abu-Elheiga, L., Almaraz-Ortega, D. B., Baldini, A. and Wakil, S. J. (1997) Human acetyl-CoA carboxylase 2. Molecular cloning, characterization, chromosomal mapping, and evidence for two isoforms. *J. Biol. Chem.* **272**, 10669–10677
- Winz, R., Hess, D., Aebersold, R. and Brownsey, R. W. (1994) Unique structural features and differential phosphorylation of the 280-kDa component (isozyme) of rat liver acetyl-CoA carboxylase. *J. Biol. Chem.* **269**, 14438–14445
- Lopez-Casillas, F., Ponce-Castaneda, M. V. and Kim, K. H. (1991) *In vivo* regulation of the activity of the two promoters of the rat acetyl coenzyme-A carboxylase gene. *Endocrinology* **129**, 1049–1058
- Abu-Elheiga, L., Brinkley, W. R., Zhong, L., Chirala, S. S., Woldegiorgis, G. and Wakil, S. J. (2000) The subcellular localization of acetyl-CoA carboxylase 2. *Proc. Natl. Acad. Sci. U.S.A.* **97**, 1444–1449
- Davies, S. P., Sim, A. T. R. and Hardie, D. G. (1990) Location and function of three sites phosphorylated on rat acetyl-CoA carboxylase by the AMP-activated protein kinase. *Eur. J. Biochem.* **187**, 183–190
- Ha, J., Daniel, S., Broyles, S. S. and Kim, K. H. (1994) Critical phosphorylation sites for acetyl-CoA carboxylase activity. *J. Biol. Chem.* **269**, 22162–22168
- Boone, A. N., Rodrigues, B. and Brownsey, R. W. (1999) Multiple-site phosphorylation of the 280 kDa isoform of acetyl-CoA carboxylase in rat cardiac myocytes: evidence that cAMP-dependent protein kinase mediates effects of β -adrenergic stimulation. *Biochem. J.* **341**, 347–354
- Dyck, J. R. B., Kudo, N., Barr, A. J., Davies, S. P., Hardie, D. G. and Lopaschuk, G. D. (1999) Phosphorylation control of cardiac acetyl-CoA carboxylase by cAMP-dependent protein kinase and 5'-AMP activated protein kinase. *Eur. J. Biochem.* **262**, 184–190
- Travers, M. T. and Barber, M. C. (1999) Insulin-glucocorticoid interactions in the regulation of acetyl-CoA carboxylase- α transcript diversity in ovine adipose tissue. *J. Mol. Endocrinol.* **22**, 71–79
- Zhang, S. and Kim, K. H. (1996) Acetyl-CoA carboxylase is essential for nutrient-induced insulin secretion. *Biochem. Biophys. Res. Commun.* **229**, 701–705
- Kim, K. H. and Tae, H. J. (1994) Pattern and regulation of acetyl-CoA carboxylase gene expression. *J. Nutr.* **124**, 1273S–1283S
- Luo, X. C., Park, K., Lopez-Casillas, F. and Kim, K. H. (1989) Structural features of the acetyl-CoA carboxylase gene: mechanisms for the generation of mRNAs with 5'-end heterogeneity. *Proc. Natl. Acad. Sci. U.S.A.* **86**, 4042–4046
- Kim, T. S., Leahy, P. and Freake, H. C. (1996) Promoter usage determines tissue specific responsiveness of the rat acetyl-CoA carboxylase gene. *Biochem. Biophys. Res. Commun.* **225**, 647–653
- Barber, M. C. and Travers, M. T. (1995) Cloning and characterisation of multiple acetyl-CoA carboxylase transcripts in ovine adipose tissue. *Gene* **154**, 271–275
- Barber, M. C. and Travers, M. T. (1998) Elucidation of a promoter activity that directs the expression of acetyl-CoA carboxylase α with an alternative N-terminus in a tissue-restricted fashion. *Biochem. J.* **333**, 17–25
- Michell, B. J., Stapleton, D., Mitchell, K. I., House, C. M., Katsis, F., Witters, L. A. and Kemp, B. E. (1996) Isoform-specific purification and substrate specificity of the 5'-AMP-activated protein kinase. *J. Biol. Chem.* **271**, 28445–28450
- Krebs, J. E. and Peterson, C. L. (2000) Understanding 'active' chromatin: a historical perspective of chromatin modelling. *Crit. Rev. Eukaryot. Gene Expr.* **10**, 1–12
- Whitelaw, C. B. A., Harris, S., McClenaghan, M., Simons, J. P. and Clark, A. J. (1992) Position-independent expression of the ovine β -lactoglobulin gene in transgenic mice. *Biochem. J.* **286**, 31–39
- Feinberg, A. P. and Vogelstein, B. (1983) A technique for radiolabelling DNA restriction fragments to high specific activity. *Anal. Biochem.* **132**, 6–13
- Henikoff, S. (1984) Unidirectional digestion with exonuclease III creates targeted breakpoints for DNA sequencing. *Gene* **28**, 351–359
- Bail, R. K., Friis, R. R., Schoenenberger, C. A., Doppler, W. and Groner, B. (1988) Prolactin regulation of β -casein gene expression and of a cytosolic 120-kD protein in a cloned mouse mammary epithelial cell line. *EMBO J.* **7**, 2089–2095
- Abken, H. and Reifensath, B. (1992) A procedure to standardize CAT reporter gene assay. *Nucleic Acids Res.* **20**, 3528
- Travers, M. T., Vallance, A. J., Gourlay, H. T., Gill, C. A., Klein, I., Bottema, C. B. K. and Barber, M. C. (2001) 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. *Biochem. J.* **359**, 273–284
- Roder, K., Wolf, S. S., Beck, K.-F. and Schweizer, M. (1997) Cooperative binding of NF-Y and Sp1 at the DNase I-hypersensitive site, fatty acid synthase insulin-responsive element 1, located at –500 in the rat fatty acid synthase promoter. *J. Biol. Chem.* **272**, 21616–21624
- Kim, I. S., Sinha, S., de Crombrughe, B. and Maity, S. N. (1996) Determination of functional domains in the C subunit of the CCAAT (CBF)-binding factor necessary for formation of a CBF-DNA complex: CBF-B interacts simultaneously with both the CBF-A and CBF-C subunits to form a heterotrimeric CBF molecule. *Mol. Cell. Biol.* **16**, 4003–4013
- Roder, K., Wolf, S. S., Beck, K.-F., Sickinger, S. and Schweizer, M. (1997) NF-Y binds to the inverted CCAAT box, an essential element for cAMP-dependent regulation of the rat fatty acid synthase (FAS) gene. *Gene* **184**, 21–26
- Wells, J. and Farnham, P. J. (2002) Characterizing transcription factor binding sites using formaldehyde crosslinking and immunoprecipitation. *Methods* **26**, 48–56

- 30 Watson, C. J. (2001) Stat transcription factors in mammary gland development and tumorigenesis. *J. Mammary Gland Biol. Neoplasia* **11**, 503–509
- 31 Millot, B., Fontaine, M. L., Thepot, D. and Devinoy, E. (2001) A distal region, hypersensitive to DNase I, plays a key role in regulating rabbit whey acidic protein gene expression. *Biochem. J.* **359**, 557–565
- 32 Mao, J., Molenaar, A. J., Wheeler, T. T. and Seyfert, H. M. (2002) STAT5 binding contributes to lactational stimulation of promoter III expressing the bovine acetyl-CoA carboxylase α -encoding gene in the mammary gland. *J. Mol. Endocrinol.* **29**, 73–88
- 33 Mantovani, R. (1999) The molecular biology of the CCAAT-binding factor NF-Y. *Gene* **239**, 15–27
- 34 Bendall, A. J. and Molloy, A. J. (1994) Base preferences for DNA binding by the bHLH-Zip protein USF: effects of MgCl₂ on specificity and comparison with binding of Myc family members. *Nucleic Acids Res.* **22**, 2801–2810
- 35 Amemiya-Kudo, M., Shimano, H., Hasty, A. H., Yahagi, N., Yoshikawa, T., Matsuzaka, T., Okazaki, H., Tamura, Y., Iizuka, Y., Ohashi, K. et al. (2002) Transcriptional activities of nuclear SREBP-1a, -1c, and -2 to different target promoters of lipogenic and cholesterologenic genes. *J. Lipid Res.* **43**, 1220–1235
- 36 Viollet, B., Lefrançois-Martinez, A.-M., Henrion, A., Kahn, A., Raymondjean, M. and Martinez, A. (1996) Immunochemical characterization and transacting properties of upstream stimulatory factor isoforms. *J. Biol. Chem.* **271**, 1405–1415
- 37 Foufelle, F. and Ferre, P. (2002) New perspectives in the regulation of hepatic glycolytic and lipogenic genes by insulin and glucose: a role for the transcription factor sterol regulatory element binding protein-1c. *Biochem. J.* **366**, 377–391
- 38 Yokoyama, C., Wang, X., Briggs, M. R., Admon, A., Wu, J., Hua, X., Goldstein, J. L. and Brown, M. S. (1993) SREBP-1, a basic-helix-loop-helix-zipper protein that controls transcription of the low density lipoprotein receptor gene. *Cell (Cambridge, Mass.)* **75**, 185–197
- 39 McKay, R. M., McKay, J. P., Avery, L. and Graff, J. M. (2003) *C. elegans*: a model for exploring the genetics of fat storage. *Dev. Cell* **4**, 131–142
- 40 Seegmiller, A. C., Dobrosotskaya, I., Goldstein, J. L., Ho, Y. K., Brown, M. S. and Rawson, R. B. (2002) The SREBP pathway in *Drosophila*: regulation by palmitate, not sterols. *Dev. Cell* **2**, 229–238
- 41 Salero, E., Gimenez, C. and Zafra, F. (2003) Identification of a non-canonical E-box motif as a regulatory element in the proximal promoter of the apolipoprotein E gene. *Biochem. J.* **370**, 979–986
- 42 Jump, D. B., Thelen, A. P. and Mater, M. K. (2001) Functional interaction between sterol regulatory element-binding protein-1c, nuclear factor Y, and 3,5,3'-triiodothyronine nuclear receptors. *J. Biol. Chem.* **276**, 34419–34427
- 43 Magana, M. M. and Osborne, T. F. (1996) Two tandem binding sites for sterol regulatory element binding proteins are required for sterol regulation of fatty acid synthase promoter. *J. Biol. Chem.* **271**, 32689–32694
- 44 Magana, M. M., Lin, S. S., Dooley, K. A. and Osborne, T. F. (1997) Sterol regulation of acetyl coenzyme A carboxylase promoter requires two interdependent binding sites for sterol regulatory element binding proteins. *J. Lipid Res.* **38**, 1630–1638
- 45 Andreolas, C., da Silva Xavier, G., Diraison, F., Zhao, C., Varadi, A., Lopez-Casillas, Ferre, P., Foufelle, F. and Rutter, G. A. (2002) Stimulation of acetyl-CoA carboxylase gene expression by glucose requires insulin release and sterol regulatory element binding protein 1c in pancreatic MIN6 β -cells. *Diabetes* **51**, 2536–2545
- 46 Jackson, S. M., Ericsson, J., Mantovani, R. and Edwards, P. A. (1998) Synergistic activation of transcription by nuclear factor Y and sterol regulatory element binding protein. *J. Lipid Res.* **39**, 767–776
- 47 Moon, Y. H., Lee, J. J., Park, S. W., Ahn, Y. H. and Kim, K. S. (2000) The roles of sterol regulatory-element binding proteins in the transactivation of the rat ATP citrate-lyase promoter. *J. Biol. Chem.* **275**, 30280–30286
- 48 Bennett, M. K. and Osborne, T. F. (2000) Nutrient-regulation of gene expression by the sterol regulatory element binding proteins: increased recruitment of gene-specific coregulatory factors and selective hyperacetylation of histone H3 *in vivo*. *Proc. Natl. Acad. Sci. U.S.A.* **97**, 6340–6344
- 49 Ferre-D'Amare, A. R., Pognonec, P., Roeder, R. G. and Burley, S. K. (1994) Structure and function of the b/HLH/Z domain of USF. *EMBO J.* **13**, 180–189
- 50 Mao, J. and Seyfert, H. M. (2002) Promoter II of the bovine acetyl-coenzyme A carboxylase- α -encoding gene is widely expressed and strongly active in different cells. *Biochim. Biophys. Acta* **1576**, 324–329
- 51 Grigor, M. R., Geursen, A., Sneyd, M. J. and Warren, S. M. (1982) Regulation of lipogenic capacity in lactating rats. *Biochem. J.* **208**, 611–618

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