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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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-isof orm was found throughout the cytoplasm whereas the β-isof orm appeared to be associated with the mitochondria [6], lead to the suggestion that these isoenzymes had different functions; ACC-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.
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 PIIII 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 HSSs in a 1.6 kbp region upstream of the start-site of PIPII, that are associated with an increase in abundance of PIIII-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 PIPII 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.). Transfase 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 × 10⁶) 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 μg 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 PIPII blots, they were probed with a 413 bp BgII–ApaI DNA probe, located within E5A [+63 to +475 relative to the tss (transcription start site)]. A 1.1 kbp Apal–EcoRI 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 HindIII–BglII 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′-CTTGGACCTGCTTCCCCCTGGAGTCTGAAATTC-3′; dC2, 5′-CAGTCTTGTGGTGTCGATCAAAATC-3′; dE1, 5′-GATTTGGTCTCAGAAATGCAT-3′. The NotI 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) fetal 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 μg of plasmid and a 2:1 charge ratio of Transfect 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 μg of plasmid and a 2:1 ratio of Transfect. Overexpression of ADD1 (adipocyte determination and differentiation-dependent factor 1)/SREBP-1 was performed with 0.05 μg 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
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 µg) was added to a 20 µl 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 µg of BSA, 0.1 µg of poly(dI/dC) and 1 ng of [³²P]-labelled oligonucleotide (3×10⁵ c.p.m.), with or without antibodies 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-ended 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×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 µl of 2 µg/µl anti-USF-1 (C-20), anti-USF-2 (C-20) and anti-SREBP-1 (H-160) or NF-YA [CBF-B (CCAAAT-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×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_E1AE 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 Crombrugghe (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 µg) 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 6×10 s pulses, in the presence of 0.1 g of glass beads (212–300 µm), 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 µg 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 PII, 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-fourth 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 PII proximal region: forward primer, 5’-TCTTTGCCTTGCTGCCCTCCT-3’; reverse primer, 5’-CAGGGAATAGCAATAACCTCA-3’; reverse primer, 5’-ATTACGTCAGGAGCTATCT-3’ (annealing temperature 59°C); ACC-α PII distal region flanking the STAT5 motif: forward primer, 5’-CAGCAAGAGCTGCAGCTAGTT-3’; reverse primer, 5’-TTCTCAGGAGCTGAGCTCT-3’ (annealing temperature 58°C); ACC-α PII proximal region: forward primer,

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**Table 1 Oligonucleotides used in EMSA**

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<th>Name</th>
<th>Sequence</th>
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<td>C2_E1</td>
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</tr>
<tr>
<td>C2_E1mutC</td>
<td>G⁻¹⁷⁸CTGTGATTGGCCTCACAGATACCGTGCTTCCC⁻¹³⁸</td>
</tr>
<tr>
<td>C2_E1AE</td>
<td>G⁻¹⁷⁸CTGTGATTGGCCTCACAGATACCGTGCTTCCC⁻¹³⁸</td>
</tr>
<tr>
<td>C1_CCAAT</td>
<td>G⁻¹⁷⁸CTGTGATTGGCCTCACAGATACCGTGCTTCCC⁻¹³⁸</td>
</tr>
<tr>
<td>C2_ICE</td>
<td>G⁻¹⁷⁸CTGTGATTGGCCTACAGATACCGTGCTTCCC⁻¹³⁸</td>
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<tr>
<td>E2</td>
<td>G⁻¹⁷⁸CTGTGATTGGCCTACAGATACCGTGCTTCCC⁻¹³⁸</td>
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<tr>
<td>E2m</td>
<td>G⁻¹⁷⁸CTGTGATTGGCCTACAGATACCGTGCTTCCC⁻¹³⁸</td>
</tr>
<tr>
<td>FIRE1_FAS</td>
<td>G⁻¹⁷⁸CTGTGATTGGCCTACAGATACCGTGCTTCCC⁻¹³⁸</td>
</tr>
</tbody>
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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 × 10⁷) 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 32P-labelled BglII–ApaI probe are diagrammatically represented in (B). These data are representative of three experiments using different preparations of nuclei.

5′-CCCGCCCCCGCTCCCACCTC-3′; reverse primer, 5′-CGGA-CGGCCCGCCCTCTAG-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 μg) 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.
Table 2 Deletion analysis of ACC-α PIII-luciferase constructs in HepG2 hepatoma cells

<table>
<thead>
<tr>
<th>Expression construct</th>
<th>HepG2</th>
<th>HC11</th>
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<tbody>
<tr>
<td>−1404/+68</td>
<td>435 ± 130</td>
<td>14.4 ± 1.0</td>
</tr>
<tr>
<td>−1172/+68</td>
<td>640 ± 190</td>
<td>11.8 ± 5.4</td>
</tr>
<tr>
<td>−879/+68</td>
<td>630 ± 145</td>
<td>21.4 ± 3.9</td>
</tr>
<tr>
<td>−562/+68</td>
<td>850 ± 190</td>
<td>19.4 ± 5.0</td>
</tr>
<tr>
<td>−220/+68</td>
<td>330 ± 240</td>
<td>22.2 ± 2.2</td>
</tr>
<tr>
<td>−159/+68</td>
<td>140 ± 40</td>
<td>3.5 ± 0.4</td>
</tr>
<tr>
<td>−38/+68</td>
<td>30 ± 5</td>
<td>1.3 ± 0.2</td>
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</table>

HepG2 cells were transiently transfected with 1.0 μg 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 32P-labelled pGL3basic vector. Transfections were performed in the presence of insulin (170 nM, HepG2) or insulin (670 nM), dexamethasone (0.5 μM) and prolactin (210 nM) (HC11 cells). Results are means ± S.E.M. for three to five individual experiments using duplicate preparations of plasmid DNA.

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 predominately 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
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_E1mutC) (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′-ATTTGGG-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_ICE) 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_ICE; 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 µg) 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–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.

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 µg (LSMG) (A) or 18 µg (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).
E1mutC, which has a mutated C2 motif and related factors, may act in a similar fashion in vitro.

The binding of USFs to E1 acts to stabilize the binding of NF-Y to C2. However, even in the absence of a specific competitor, the NF-Y-DNA complex is still capable of binding USFs, as a competitor. Thus, even with the addition of a 50-fold excess of specific competitor and resolved on the gel with a 32 min time point (lane 5), the 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 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 the signal generated by the pre-immune IgG.

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

<table>
<thead>
<tr>
<th>Expression construct</th>
<th>Normalized luciferase activity</th>
<th>Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>−1404/+68 wt</td>
<td>435 ± 70</td>
<td>14.4 ± 1.0</td>
</tr>
<tr>
<td>−1404/+68ΔC1</td>
<td>695 ± 70</td>
<td>n.d.</td>
</tr>
<tr>
<td>−1404/+68ΔC2</td>
<td>40 ± 5***</td>
<td>9.8 ± 2.3*</td>
</tr>
<tr>
<td>−1404/+68ΔE1</td>
<td>120 ± 35***</td>
<td>4.9 ± 0.9**</td>
</tr>
<tr>
<td>−220/+68 wt</td>
<td>805 ± 110</td>
<td>17.3 ± 2.1</td>
</tr>
<tr>
<td>−220/+68ΔC1</td>
<td>1030 ± 60</td>
<td>n.d.</td>
</tr>
<tr>
<td>−220/+68ΔC2</td>
<td>195 ± 25***</td>
<td>10.8 ± 2.5*</td>
</tr>
<tr>
<td>−220/+68ΔE1</td>
<td>210 ± 30**</td>
<td>8.4 ± 1.5**</td>
</tr>
<tr>
<td>−220/+68ΔE2</td>
<td>85 ± 5**</td>
<td>4.3 ± 0.6***</td>
</tr>
</tbody>
</table>

Abbreviation: n.d., not determined.
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 5

EMSA was performed with 3 µg 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 % ± 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.

Figure 6

E2 displays higher affinity for USF binding in vitro than E1

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 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...
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/+68Luc) 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 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

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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

<table>
<thead>
<tr>
<th>Expression construct</th>
<th>Fold induction by co-transfected ADD1/SREBP-1c</th>
</tr>
</thead>
<tbody>
<tr>
<td>−220/+68 wt</td>
<td>2.5 ± 0.3</td>
</tr>
<tr>
<td>−220/+68ΔC2</td>
<td>2.3 ± 0.3</td>
</tr>
<tr>
<td>−220/+68ΔE1</td>
<td>1.1 ± 0.1***</td>
</tr>
<tr>
<td>−220/+68ΔE2</td>
<td>3.3 ± 0.2**</td>
</tr>
</tbody>
</table>
micromsome 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 helix-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 USFs-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′-atCAC-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)6TCAC-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 PII has been identified as the non-canonical E-box or SRE half-site (5′-ATCACGc-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 PII 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 affect 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 PII 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 PII 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 PII, 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
SREBP-1 Regulation of Acetyl-CoA Carboxylase-α Promoter III

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 chromation structure, as defined by lactationally induced DNase I HSSs (downward pointing arrows).

Figure 8 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 PII 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 ‘mālic’ 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 PII 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 PII 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 in concert with NF-Y and USFs. SRE-like motifs are also present in the mammary gland, through its regulation of ACC-α de novo synthesis [51].

REFERENCES


30. Watson, C. J. (2001) Stat transcription factors in mammary gland development and

hypersensitive to DNase I, plays a key role in regulating rabbit whey acidic protein gene

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

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. Anemiyi-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
cholesterogenic genes. J. Lipid Res. 43, 1220–1235

Martinez, A. (1996) Immunochemical characterization and transacting properties of
upstream stimulatory factor isoforms. J. Biol. Chem. 271, 1405–1415

and lipogenic genes by insulin and glucose: a role for the transcription factor sterol

Brown, M. S. (1997) SREBP-1, a basic-helix–loop–helix-zipper protein that controls
regulatory element binding protein-1c in pancreatic MIN6 β-cells. Diabetes 56, 2536–2545

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
not sterols. Dev. Cell 2, 229–238

as a regulatory element in the proximal promoter of the apolipoprotein E gene.
Biochem. J. 370, 979–986

regulatory element-binding protein–1c, nuclear factor Y, and 3,5,3′-triiodothyronine
nuclear receptors. J. Biol. Chem. 276, 34419–34427

element binding proteins are required for sterol regulation of fatty acid synthase promoter.
J. Biol. Chem. 271, 32689–32694

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,
gene expression by glucose requires insulin release and sterol regulatory element
binding protein 1c in pancreatic MIN6 β-cells. Diabetes 51, 2530–2545

activation of transcription by nuclear factor Y and sterol regulatory element binding
protein. J. Lipid Res. 39, 767–776

regulatory-element binding proteins in the transactivation of the rat ATP citrate-lyase
promoter. J. Biol. Chem. 275, 30280–30286

sterol regulatory element binding proteins: increased recruitment of gene-specific
Acad. Sci. U.S.A. 97, 6340–6344

function of the b/HLH/Zip domain of USF. EMBO J. 13, 180–189

carboxylase-α-encoding gene is widely expressed and strongly active in different cells.
Biochim. Biophys. Acta 1576, 324–329

capacity in lactating rats. Biochem. J. 208, 611–618

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