Developmental regulation of alternatively spliced acetyl-CoA carboxylase-α mRNAs encoding isozymes with or without an eight amino acid domain upstream of the Ser-1200 phosphorylation motif in the mammary gland

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ABSTRACT

Expression of a variant acetyl-CoA carboxylase-α (ACC-α) mRNA encoding an isozyme either comprising (+24nt) or lacking (Δ24nt) an eight amino acid domain proximal to the Ser-1200 phosphorylation motif has been investigated in ovine and rat mammary tissue throughout pregnancy and lactation. The ratio of the Δ24nt mRNA: +24nt mRNA in ovine tissues varied from 0·1–0·25 (spleen, lung, muscle, heart, adipose tissue, brain) to 0·6–0·8 (pancreas, liver, kidney) to approximately 5·0 (lactating mammary gland). The sixfold increase in total ACC-α mRNA expression in mammary gland during lactation was due entirely to a tenfold increase in the level of the Δ24nt species as the level of expression of the +24nt species remained unaltered between pregnancy and lactation. This mode of expression of the +24nt and Δ24nt mRNAs was similar in rat mammary gland. Between day 20 of pregnancy and day 4 of lactation the ratio of the Δ24nt : +24nt mRNA increased from 2:1 to 10–20:1. Forced involution reduced the ratio of the two mRNAs to levels observed throughout pregnancy. Treatment of lactating rats with bromocryptine reduced the ratio of the Δ24nt : +24nt mRNA to relative levels observed after forced involution, suggesting that the exonic splicing responsible for the generation of the two mRNA isoforms is prolactin responsive.

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INTRODUCTION

Mammary gland differentiation during pregnancy and lactation results in a marked increase in the capacity for fatty acid synthesis (Mackall & Lane 1977, Lopez-Casillas et al. 1991). Acetyl-CoA carboxylase (ACC), the flux-determining enzyme in the biosynthesis of long chain fatty acids, exists in several isozymic forms, due in part to the presence of two related genes, ACC-α and ACC-β, and gene-specific alternative splicing resulting in coding region variation. The ACC-α gene gives rise to a 265 kDa enzyme which is expressed in all cell types but demonstrates elevated expression in the lipogenic tissues of liver and adipose tissue, and also the mammary gland during lactation (Lopez-Casillas et al. 1991, Abu-Elheiga et al. 1995). The ACC-β gene gives rise to a 275–280 kDa protein which is the major ACC isozyme in heart and skeletal muscle (Ha et al. 1996, Abu-Elheiga et al. 1997, 2001).

Expression of ACC-α is regulated in a complex fashion involving both acute mechanisms, through allosteric effectors and reversible phosphorylation to regulate enzyme activity (Kim et al. 1989), and chronic regulation through gene transcription (Kim & Tae 1994). Transcription of the ACC-α gene is complex, with transcripts resulting from at least two promoters, PI and PII, in mammals and demonstrating both ubiquitous and tissue-specific expression depending on the promoter used (Luo et al. 1989, Ha et al. 1994a, Travers & Barber 1999).

The role for ACC isozymes and mRNA isoforms in cell function is largely unknown, with the exception that expression of the ACC-β gene is related to the need to regulate fatty acyl β-oxidation (Abu-Elheiga et al. 1997, 2001). Recently, we demonstrated the presence in ovine tissues of a third ACC-α promoter, PIII, which gives rise to a transcript which encodes an enzyme with a variant N terminus such that the primary coding exon, exon
5 (E5), in PI and PII transcripts, is replaced by a downstream exon, exon 5A (ES5A). Expression of the PIII transcript is induced 15-fold throughout pregnancy and lactation in the ovine mammary gland, suggesting that the isozyme produced is important for mammary-specific control of lipogenesis and lipid secretion during lactation (Barber & Travers 1998). Furthermore, Kong et al. (1990) demonstrated the existence of two forms of rat ACC-α mRNA generated through alternative exon splicing that differed in the presence (+24nt) or absence (Δ24nt) of 24 nucleotides coding for eight amino acids upstream of the Ser-1200 phosphorylation motif. Reversible phosphorylation of Ser-1200 (Ser-1201 in the ovine sequence) by cyclic AMP-dependent protein kinase has been implicated by some workers (Ha et al. 1994b), but not by others (Davies et al. 1990), as being a critical regulatory motif in the regulation of the activity of ACC. Kong et al. (1990) showed that the relative abundance of the mRNAs varied in a tissue-specific fashion, such that in liver and adipose tissue the +24nt species predominated whereas in lactating mammary gland the Δ24nt mRNA was in abundance. Here we have shown that the ratio of the abundances of the +24nt and Δ24nt mRNAs is developmentally regulated in both ovine and rat mammary gland. In both species, cellular expression of the Δ24nt mRNA was markedly enhanced during lactation relative to the +24nt species, levels of which remained relatively constant throughout pregnancy and lactation. Furthermore, expression of the Δ24nt mRNA in rat mammary gland could be repressed by reduction of serum prolactin levels in lactation. The significance of the Δ24nt transcript in the regulation of mammary gland milk fat synthesis is discussed.

MATERIALS AND METHODS

Animals

Sheep were Finn–Dorset Horn cross-bred animals, fed on hay and cereals for at least 4 weeks before slaughter. Animals were used either as non-pregnant non-lactating, pregnant or lactating ewes. Pregnant animals were used between days 100 and 105 of gestation and lactating ewes, suckling at least two lambs, were used at about day 18 of lactation. Animals were anaesthetised and exsanguinated, and samples of tissue were obtained, snap frozen and stored in liquid nitrogen. Wistar rats (A Tuck and Son, Rayleigh, Essex, UK) were fed on Labsure irradiated CRM diet (Labsure, Poole, Dorset, UK) and water available ad libitum. They were mated at 2–3 months of age, and the number of pups was adjusted to ten within 24 h of birth. Litters were removed and/or treatments were begun on days 12–14 of lactation. All injections were subcutaneous and were administered twice daily for 2 days as described previously (Barber et al. 1992). On the third day rats were anaesthetised with a combination of Hypnorm and Hypnoid (Jansen Pharmaceuticals, Oxford, Oxon, UK). Rats received the following, either alone or in combinations as stated in the text and Figure legends: bromocryptine (a gift from Sandoz (Basle, Switzerland); 500 μg/injection); γ-globulin fraction of an antiserum to rat growth hormone (220 mg/injection); sheep prolactin (a gift from NIADDK, Bethesda, MD, USA; 1·5 mg/injection); or carrier solutions (Madon et al. 1986). Once rats were anaesthetised, samples of mammary tissue were removed and frozen immediately in liquid nitrogen.

RNAse protection assays

A 438 nucleotide EcoRI–BamHI fragment of ovine ACC-α (nucleotides 3854–4292 of the ovine sequence (Barber & Travers 1995)) was subcloned into pGEM-7zf+ and used to generate a 32P-labelled 483 nucleotide antisense transcript (Kreig & Melton 1987). A rat ACC-α cDNA corresponding to the same region of the mRNA was generated by PCR of adipose tissue cDNA with primers (5′-GTAGAATTCCAGTTCATGCTGCCCAC and 5′-GCCGGATCCCATGCTCACCA GAGTAGCT-3′), and cloned into the pGEM-7zf+ vector as above. Total RNA was isolated from tissue, with the exception of adipose tissue, using guanidinium thiocyanate and centrifugation through caesium chloride (Chirgwin et al. 1979). RNA was isolated from adipose tissue by a modification of Chomczynski and Sacchi’s procedure (Chomczynski & Sacchi 1987, Louveau et al. 1991). Aliquots of total RNA (30 μg) were co-precipitated with 1·5 ng labelled antisense probe. All samples were resuspended in 30 μl hybridisation buffer (80% formamide, 40 mM PIPES, pH 6·7, 0·4 M NaCl, 1 mM EDTA) overlaid with mineral oil and incubated at 85 °C for 5 min and then overnight at 45 °C (Kreig & Melton 1987). After RNase treatment to remove unhybridised RNA, samples were phenol/chloroform extracted and ethanol precipitated. After centrifugation and drying, samples were resuspended and resolved on 8% (w/v) polyacrylamide/7 M urea gels with TBE buffer (0·089 M Tris-borate, 0·089 M boric acid, 2 mM EDTA, pH 7·5). Gels were then dried and exposed to Kodak phosphor screens. The resulting images were then scanned using a Molecular Dynamics phosphorimager 445 SI (Sunnyvale, CA, USA) and the volume of the individual bands was
Samples of total cellular RNA were separated by Northern blot analysis of RNA obtained using ImageQuant software (Molecular Dynamics). Tissue DNA was assayed by the method of Labarca & Paigen (1980). The yield of RNA/g tissue, together with the amount of DNA/g for each sample was used to calculate the levels of ACC-α transcripts in arbitrary units/mg DNA.

**Northern blot analysis of RNA**

Samples of total cellular RNA were separated by electrophoresis in 1·2% agarose gels containing 2·2 M formaldehyde and then transferred to Biotrans nylon membranes (ICN Biochemicals, Irvine, CA, USA). Details of the hybridisation have been described previously (Barber & Travers 1995). The rat α-casein cDNA was a gift from Professor J Rosen, Baylor College of Medicine, Houston, TX, USA.

**Statistical analysis**

Results are means ± s.e.m. and were compared by ANOVA.

**RESULTS**

Reverse transcription-polymerase chain reaction (RT-PCR) of RNA from the lactating sheep mammary gland using primers flanking the region encoding the Ser-1201 phosphorylation site and corresponding to nucleotides 3853–3873 and nucleotides 4025–4024 of ovine ACC-α mRNA (Barber & Travers 1995) resulted in two DNA fragments of 192 and 168 bp. RT-PCR of RNA from adipose tissue resulted in the amplification of the 192 bp fragment only (results not shown). Cloning and sequencing of the two bands produced by RT-PCR of lactating mammary gland RNA confirmed that the 168 bp fragment lacked a 24 bp sequence upstream of the Ser-1201 codon. The nucleotide sequence around the Ser-1201 codon in the context of the two mRNA species is shown in Fig. 1. These two species are now referred to as +24nt and Δ24nt ACC-α mRNA respectively. To determine the tissue distribution of these two ACC-α mRNAs (Fig. 2), an RNAse protection assay was established using a 438 bp EcoRI–BamHI fragment corresponding to nucleotides 3854–4291 relative to the 5′ terminus of the transcript (Barber & Travers 1995). Transcription of the cloned fragment using SP6 RNA polymerase resulted in the production of a 483 nucleotide cRNA probe that allows discrimination from protected RNA:DNA hybrids. Hybridisation of the probe to +24nt mRNA species resulted in a 438 nucleotide band, whereas hybridisation to the Δ24nt mRNA species resulted in protected bands of 382 nucleotides and 33 nucleotides because of the 24 nucleotide sequence contained within the cRNA forming a single-stranded loop structure which is removed by the action of RNAseA and RNAseT1. The 33 nucleotide band runs off the end of the poliacrylamide gel and is not visualised. Figure 2 shows that the +24nt species is the major transcript in all ovine tissues with the exception of lactating mammary gland where the Δ24nt mRNA is the dominant transcript. The ratio of the Δ24nt mRNA : +24nt mRNA in ovine tissues varies from 0·1–0·25 (spleen, lung, muscle, heart, adipose tissue, brain) to 0·6–0·8 (pancreas, liver, kidney) to approximately 5·0 (lactating mammary gland). In mammary gland prior to pregnancy and at 100 days of pregnancy, the ratio of the Δ24nt mRNA : +24nt mRNA is approximately 1:1 (Fig. 3A and B). The sixfold increase in ACC-α mRNA expression during lactation is due entirely to a tenfold increase in the level of the Δ24nt species (P<0·001) as the level of expression of the +24nt species remains unaltered between pregnancy and lactation (Fig. 3B). Conversely, although total expression of ACC-α mRNA declines in adipose tissue (Fig. 3C) and liver (Fig. 3D) during pregnancy and into lactation there is no apparent change in the ratio of Δ24nt mRNA : +24nt mRNA in these tissues.

![Image of Figure 1](https://www.endocrinology.org/)

**Figure 1.** Partial sequence of two ovine ACC-α mRNAs in the region translated as the Ser-1201 codon in which a 24nt region encoding an eight amino acid domain is either included (+24nt) or excluded (Δ24nt) in the mature transcript. The nucleotide positions relate to the 5′ terminus of ovine ACC-α mRNA (Barber & Travers 1995). The nucleotide sequence encoding the eight amino acid domain and the protein sequence deleted in the isozyme encoded by the Δ24nt mRNA are shown in bold typeface.
To determine the exact timing of the induction of the Δ24nt ACC-α mRNA in mammary gland throughout pregnancy and lactation, and to investigate the role of the endocrine environment in the induction of this transcript, experiments using rats were conducted. A rat ACC-α cRNA probe, corresponding to the same region of the mRNA as used for detection of the ovine transcripts, was used in RNAse protection assays to detect the +24nt and Δ24nt transcripts in rat mammary gland. In rat mammary gland at day 4 of pregnancy the ratio of Δ24nt mRNA : +24nt mRNA was approximately 2·0 and remained at this level throughout pregnancy. The additional band ascribed to the Δ24nt mRNA species was probably due to inefficient removal of the 24nt loop structure by RNAseA and RNAseT1 in the sequence context of the rat ACC-α cRNA probe. At the onset of lactation the ratio of the two transcripts markedly increased to approximately 8–10. At the onset of forced involution due to pup removal the ratio of Δ24nt mRNA : +24nt mRNA declined to levels observed in pregnancy (Fig. 4A and B). Expression of total ACC-α mRNA (Fig. 4C) increased sevenfold from day 4 of pregnancy to early lactation (P<0·001) in a manner similar to that of the expression of the α-casein gene (Fig. 4D). Similar to ovine mammary gland, the increase in total ACC-α mRNA in the rat mammary gland at the onset of lactation was due principally to an increase in the level of Δ24nt transcripts as levels of +24nt transcripts were similar to those observed throughout most of pregnancy (results not shown).

Figure 5 shows the effect of depleting serum prolactin (by administration of bromocryptine) and growth hormone (by a neutralising antibody) either singly or in combination, on the ratio of Δ24nt mRNA : +24nt mRNA in the lactating rat mammary gland. Bromocryptine injection reduced the ratio from approximately 22:1 in lactating rats to approximately 14:1 (P<0·001) in treated rats, whereas anti-rat growth hormone treatment had no effect. Treatment of rats with anti-rat growth hormone serum plus bromocryptine reduced the ratio from 14:1 in bromocryptine-treated rats to approximately 8:1 (P<0·05) in rats on the combined treatment, and resulting in a similar ratio of Δ24nt mRNA : +24nt mRNA as that observed in rats after 2 days of involution. Simultaneous injection of bromocryptine plus prolactin prevented the alteration in the ratio of Δ24nt mRNA : +24nt mRNA seen with bromocryptine alone. It is notable that the ratio of Δ24nt mRNA : +24nt mRNA in both lactating and involuting rats in this experiment was approximately twice that observed in animals in these physiological states in the first experiment with rats above. These two experiments used animals of the same strain and age, and therefore an explanation for the difference in the observed ratio of Δ24nt mRNA : +24nt mRNA in these experiments is not obvious.

**DISCUSSION**

We have demonstrated that the ratio of the abundances of the +24nt and Δ24nt mRNAs is developmentally regulated in both ovine and rat mammary gland, such that the increased rate of
fatty acid synthesis in lactation is associated with a marked increase in expression of the Δ24nt ACC-α mRNA. Our results confirm and extend

**Figure 3.** Expression of the Δ24nt ACC-α mRNA is increased in ovine mammary gland during lactation. (A) Total RNA was isolated from non-pregnant non-lactating (C), 100 days pregnant (P) and 18 days lactating (L) mammary gland and included in the RNase protection assay. Bands corresponding to the +24nt and Δ24nt ACC-α mRNAs are shown. (B) Results derived from (A) are normalised/mg tissue DNA and correspond to the mean ± s.e.m. from five animals for each developmental stage. The open and solid bars correspond to the expression, as arbitrary units, of Δ24nt and +24nt ACC-α mRNAs/mg tissue DNA respectively. Expression of the +24nt and Δ24nt ACC-α mRNAs in (C) adipose tissue and (D) liver throughout pregnancy and lactation. The RNase protection assay was performed as described above and is representative of three to five animals for each developmental stage.

**Figure 4.** Expression of the +24nt and Δ24nt ACC-α mRNAs in rat mammary gland throughout pregnancy, lactation and involution. (A) Total RNA was isolated from rat mammary gland from several stages of pregnancy (P; days 4, 8, 12, 16 and 20), lactation (L; days 4, 8, 12, 16 and 20), and for 1 and 2 days after the commencement of involution (I) following pup removal. RNase protection assay was performed with a rat ACC-α cDNA (nt 3532–3969 relative to AUG translation initiation codon) (Lopez-Casillas et al. 1988). Bands corresponding to the +24nt and Δ24nt ACC-α mRNAs are shown. (B) Results derived from (A) are plotted as the ratio of the Δ24nt transcript to the +24nt transcript, and (C) the total levels of ACC-α transcripts are expressed as arbitrary units/mg tissue DNA. (D) The expression of α-casein mRNA/mg DNA throughout pregnancy, lactation and involution in rat mammary gland as determined by Northern analysis. Data are the means ± s.e.m. from four animals for each developmental stage.
increased transcription of the gene during pregnancy and the enhancement of the exonic splicing process during lactation, which results in the marked increase in the levels of Δ24nt mRNA compared with +24nt mRNA, are independent modalities subject to differing temporal controls. ACC-α mRNA expression during pregnancy follows the expression of α-casein mRNA closely, suggesting a degree of co-regulation of these loci during the development of the mammary gland. By reference to DNA sequences deposited in databases as part of the human genome project, the 24 nucleotide sequence has been localised to a single exon, at least in human DNA (accession number: AC016482), thereby confirming that changes in the ratio of the two mRNA variants is due to a regulated exon-splicing process which is subject to tissue-specific and endocrine regulation. Our results in rats would suggest that prolactin responsiveness is a major regulator of this process in the mammary gland, in addition to regulating the abundance of total ACC-α mRNA during lactation (Barber et al. 1992, Travers et al. 1996), and thereby implicates the presence of prolactin response domains in the splicing machinery, in addition to the transcriptional machinery. This change in the ratio of the two ACC-α transcripts parallels temporally the increases in serum prolactin (Cowie et al. 1980) and prolactin receptor number and mRNA expression (Jahn et al. 1991) in the rat mammary gland observed at the onset of parturition. Also STAT5 phosphorylation in the mammary gland is markedly increased at late pregnancy (Liu et al. 1996), prior to the onset of the marked change in the ratio of the two ACC-α mRNAs, suggesting that serum prolactin levels are reflected in the potential for mammary intracellular signalling by prolactin. Additionally, lack of response, or delayed response relative to the onset of STAT5 phosphorylation and casein mRNA expression (Liu et al. 1996), of the ACC-α splicing machinery to prolactin signalling may be a reflection of the state of the chromatin structure around the splice site, which may need to be modified to result in an appropriate response. Further work will be necessary to determine the signalling pathway and factors involved in the regulation of this splicing event.

Mammary gland differentiation during pregnancy and lactation results in a marked increase in the capacity for fatty acid synthesis (Mackall & Lane 1977, Lopez-Casillas et al. 1991). The present study, and a recent study in which an mRNA isoform encoding an ACC-α isozyme with a variant N terminus was characterised (Barber & Travers 1998), would suggest that fatty acid synthesis in the mammary gland during lactation is associated with observations made by Kong et al. (1990) who noted that the relative abundance of the +24nt and Δ24nt ACC-α mRNAs varied in a tissue-specific fashion in adipose tissue, liver and the lactating mammary gland of rats. Furthermore, our results demonstrate that, in a variety of ovine tissues, expression of the +24nt mRNA predominates by as much as tenfold compared with the Δ24nt mRNA, suggesting that the Δ24nt : +24 nt ratio >1:0 observed in the lactating mammary gland is uniquely related to that tissue. It is notable that the ratio of the two ACC-α mRNA variants observed in our experiments with lactating rats (10–20:1) differed markedly from the ratio observed in the experiments of Kong et al. (1990) (2.5:1). The reason for this is not entirely clear, although it could possibly be explained by the use of different strains of rats or through the use of slightly different methodology to derive the data; S1 nuclease analysis was used in the experiments of Kong et al. (1990).

Our results in rats have demonstrated, in the rat mammary gland during development, that the

FIGURE 5. Effect of serum depletion of prolactin and growth hormone on the expression of the +24nt and Δ24nt ACC-α mRNAs in the rat mammary gland during lactation. Lactating rats were administered bromocryptine (CB154) to deplete serum prolactin and an antiserum to rat growth hormone (AGS) to deplete serum growth hormone, either alone or in combination. Control animals received saline or simultaneous injection of CB154 plus prolactin. (A) Total RNA was isolated and the RNase protection assay was performed as described in Fig. 4. Bands corresponding to the +24nt and Δ24nt ACC-α mRNAs are shown. (B) The ratios of Δ24nt : +24nt ACC-α mRNAs are shown. Data are the means ± s.e.m. from four animals for each experimental determination.
increased expression of largely mammary-restricted ACC-α mRNAs, at least in sheep. To date, the E5A mRNA variant (Barber & Travers 1998) has not been characterised in any other species, although the present study has demonstrated that similar changes with respect to the Δ24nt-mRNA are occurring in both sheep and rats during mammary gland development. A major question can be asked as to the physiological significance of these changes in the pattern of these ACC-α mRNA isoforms during lactation. As these changes in expression of these mRNA isoforms are related to lactation it would appear they must relate to a set of unique metabolic requirements to synthesise and secrete lipids in milk. Both the ACC-α and ACC-β genes are transcribed from multiple promoters and demonstrate diverse mRNA isoforms that vary in the 5’UTR as well as a limited number of coding region variations. It has been speculated that some of the ACC-α mRNA isoforms encode isozymes with altered kinetics (Kong et al. 1990, Barber & Travers 1998). For example, it has been demonstrated that a recombinant protein expressing a domain of ACC-α flanking the eight amino acid domain (+24nt) is resistant to phosphorylation on Ser-1200 by cAMP-dependent protein kinase in vitro when compared with a protein lacking the eight amino acid domain (Δ24nt) (Kong et al. 1990). Thus, if this effect is maintained in the context of the entire protein then this would result in an ACC-α isozyme which would be more phosphorylation prone and therefore more dependent on allosteric effectors, such as citrate, for activity. In addition, the amino acid glutamate has also recently been shown to act in a bimodal fashion by promoting the activity of an ACC-α protein phosphatase, PP2A, and also by activating the dephosphorylated enzyme in an allosteric fashion (Gaussin et al. 1996, Boone et al. 2000). Glutamate transport into mammary epithelial cells is markedly increased in lactation, and repressed by involution (Millar & Shennan 1999). Such a regulated transport activity is likely to result in a situation where the demand for milk protein synthesis is reflected by the cytosolic concentration of glutamate. Such dependence on allosteric effectors, if operating, could be a mechanism to match fatty acid synthesis to the overall metabolic activity of mammary cells and more specifically to the requirement to produce milk components. Such speculation may be difficult to investigate in the context of ACC enzyme isolated from mammary gland in which the potential for four ACC-α isozymes (E5 ± 24nt, E5A ± 24nt) plus an unknown number of ACC-β isozymes exists. Such a situation is likely to be kinetically complex, and therefore a more fruitful approach to investigate the function of these isozymes may be to express defined recombinant isozymes in cell lines that have low levels of endogenous ACC activity (Ha et al. 1994b). This approach is being considered.

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