Expression of the genes encoding CCAAT-enhancer binding protein isoforms in the mouse mammary gland during lactation and involution

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Transcription factors belonging to the CCAAT-enhancer binding protein (C/EBP) family have been implicated in the activation of gene expression in the mammary gland during lactation. We have therefore investigated the detailed expression profile of the C/EBP family during lactation and involution of the mouse mammary gland. The expression of C/EBPβ and C/EBPδ mRNA was low during lactation, increased dramatically at the beginning of involution and remained constant thereafter. In contrast, C/EBPα mRNA expression was relatively high during the early stages of lactation, declined to low levels during the late stages of lactation and at the start of involution, and increased again during involution. Electrophoretic mobility-shift assays showed a close correlation between the expression of the C/EBP genes and the functional C/EBP DNA-binding activity and, additionally, demonstrated the participation of heterodimers, formed from among the three proteins, in DNA–protein interactions. The DNA-binding activity of the activator protein 1 (AP1) family of transcription factors was also induced during involution. These results therefore point to potentially important regulatory roles for both the C/EBP and the AP1 family during lactation and involution of the mammary gland.

INTRODUCTION

Transcription factors belonging to the CCAAT-enhancer binding protein (C/EBP) family all contain a highly conserved C-terminal basic region-leucine zipper domain that consists of a basic region, involved in DNA recognition, and an adjacent helical structure, the leucine zipper, that mediates subunit dimerization [1]. In contrast, the N-termini of these proteins, which carry the regulatory and the *trans*-activation domains, are quite divergent. Six distinct C/EBP isoforms have been identified to date (C/EBPα–ζ), with the majority of the family members able to recognize similar DNA sequences, at least *in vitro*, activate gene transcription *in vivo* and form heterodimers in all intrafamilial combinations [1]. Additionally, in the case of C/EBPα and C/EBPβ, polypeptides of different sizes and *trans*-activating capabilities are known to be produced from the same mRNA, by alternative use of in-frame AUG codons because of the existence of a leaky ribosome scanning mechanism [2,3]. Thus, the range of C/EBP proteins that may be present in any given tissue/cell may be wider than the number of C/EBP-encoding genes might suggest.

Several roles have been ascribed to the C/EBP family, including the regulation of tissue-specific gene expression, cellular growth and differentiation and cytokine-induced gene transcription during inflammation [1,4–6]. More recently, binding sites for the C/EBPs have been identified in the promoter region of the milk protein gene, β-casein [7,8]. Furthermore, in nuclear extracts from the mammary epithelial HC11 cell line, C/EBPβ was identified as the prevalent C/EBP isoform interacting with these sites [7]. In addition, C/EBPδ, but not C/EBPα, was shown to be present in these cells [7]. In contrast, using cell extracts from the whole rat mammary gland, taken at day 10 of lactation, Raught et al. [8] showed that C/EBPα was the major C/EBP isoform that binds to the β-casein promoter. These workers also demonstrated that the presence of C/EBP proteins appeared to be developmentally regulated in the mammary gland during pregnancy and lactation. However, the studies did not examine the expression of C/EBPs at the mRNA level and, most importantly, did not examine the complete range of phases seen during the involution of the mammary gland. In the present study therefore we have examined in detail the expression of C/EBPα, β and δ mRNA and functional protein activity in the mouse mammary gland during lactation and throughout involution.

EXPERIMENTAL PROCEDURES

Preparation of mammary gland

Balb/c mice (Charles River) were maintained in a controlled environment (12 h light:12 h darkness) and allowed a standard rat/mouse maintenance diet (Harlan Teklad) and water *ad libitum*. Mammary gland tissue was collected post mortem from mice, during their first pregnancy, at various stages of lactation (days 2, 6, 12 and 18) and each day after the removal of the pups from the mother, either at the end of the normal lactation cycle (day 18) or, after they had reached a state of full lactation (day 7; referred to as induced involution). After parturition, the number of pups was adjusted to seven for each female. The tissue samples were either used immediately [preparation of nuclear extracts for electrophoretic mobility-shift assay (EMSA)], or frozen in liquid nitrogen and stored at -70 °C until use (isolation of RNA or proteins).

The morphology of the mammary gland in the virgin animal, and during lactation and involution, was examined by histological analysis and found to be similar to previous published reports [9–12] (results not shown).

Abbreviations used: AP1, activator protein 1; C/EBP, CCAAT-enhancer binding protein; DTT, dithiothreitol; EMSA, electrophoretic mobility-shift assay; LPL, lipoprotein lipase; RT-PCR, reverse transcription PCR; STAT, signal transducer and activator of transcription; WAP, whey acidic protein; LAP, liver activator protein; LIP, liver inhibitor protein.

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Northern blot analysis

Total RNA was isolated from tissue samples using Tri-Reagent according to the manufacturer's instructions (Molecular Research Center). Each isolated RNA sample (10–20 μg) was size-fractionated on denaturing 1% (w/v) agarose gels containing 2.2 M formaldehyde, and transferred to Hybond Nfp membranes (Amersham). Membranes were then pre-hybridized for 6 h at 65 °C in a solution containing a mixture of 5 × SSC (SSC = 0.15 M NaCl/0.015 M sodium citrate)/2 × Denhardt's (Denhardt's = 0.02% Ficoll 400/0.02% polyvinylpyrrolidone/0.02% BSA)/0.1% (v/v) SDS/100 μg/ml yeast tRNA in a ratio of 1:4 (v/v) with a 50% (w/v) solution of dextran sulphate. cDNA probes were radiolabelled with [α-^{32}P]dCTP by the random priming technique using the Megaprime DNA labelling kit (Amersham). Hybridization with denatured radiolabelled probes was carried out overnight at 65 °C in the pre-hybridization buffer. Blots were then washed at 65 °C in a solution containing 0.1% (v/v) SDS and decreasing concentrations of SSC, ranging from 2 × to 0.1 × SSC, followed by exposure to X-ray film (Kodak).

Reverse transcription (RT)-PCR

Total RNA samples (1 μg) were treated with RNase-free DNase-I (Promega) and then used as templates to generate random-primed cDNA according to the manufacturer's instructions (Clonetech). PCR reactions (50 μl) were carried out using Taq DNA polymerase (2.5 units/reaction) and PCR buffer (Promega), with 0.2 mM dNTPs, 100 pmol of each primer, 1 mM $MgCl_2$, 5% (v/v) DMSO and 0.5–10 μl of each original cDNA template. In the PCR reactions, the initial denaturation was carried out at 94 °C for 2 min and was followed by 20 cycles (β-actin) or 30 cycles (C/EBPs) of denaturation at 94 °C for 2 min, annealing for 2 min at 58 °C and extension at 72 °C for 2 min. The final round of extension was left to proceed for 5 min. These conditions were found to be within the exponential phase of amplification, as judged by the use of three different amounts of cDNA template. The sequences of the primers were: 5′-AAGGCCAAGAAGTCGGTGGA-3′ and 5′-CAGTTCACGGCTCAGCTGTT-3′ for C/EBPα, 5′-GCGCGAGCGCAACAACATCT-3′ and 5′-TGCTTGAACAAGTTCCGCAG-3′ for C/EBPβ, 5′-TGCTTGAACAAGTTCCGCAG-3′ and 5′-CCAGGTCCCGGGTGAGCT-3′ for C/EBPδ, and 5′-TGGAGAAGAGCTATGAGCTGCCTG-3′ and 5′-GTGCCACCAGACAGCACTGTGTTG-3′ for β-actin.

The PCR products were size-fractionated on 2% (w/v) agarose gels, transferred onto Nylon membranes and hybridized to the appropriate radiolabelled cDNA inserts at 42 °C in 50% (v/v) formamide/5 × SSC/0.1% (v/v) SDS/100 μg/ml yeast tRNA. The membranes were then washed, at 65 °C, in 0.1 × SSC/0.1% (v/v) SDS, and exposed to X-ray film (Kodak).

Nuclear extract preparations and EMSA

Nuclear extracts were prepared using a modification of the method described by de Groot and Sassone-Corsi [13]. Briefly, 300 mg of tissue was homogenized in 400 μl of buffer A [10 mM Hepes, pH 7.9/1.5 mM magnesium chloride/10 mM potassium chloride/0.1 mM EDTA/0.5 mM dithiothreitol (DTT)] in the presence of protease inhibitors (5 μg/ml aprotinin/5 mM benzamidine/0.5 mM PMSF). The homogenate was incubated on ice for 10 min, vortexed for 10 s and then centrifuged for 10 min in a microcentrifuge at 12000 ***g*** and 4 °C. The nuclear pellet was then resuspended in 100 μl of buffer C [20 mM Hepes, pH 7.9/10% (v/v) glycerol/420 mM sodium chloride/1.5 mM magnesium chloride/0.1 mM EDTA/0.5 mM DTT] in the presence of the protease inhibitors detailed above, and incubated for 10 min on ice. Cellular debris was removed by ultracentrifugation under the conditions described above, and the supernatant, containing the nuclear extracts, was stored in 10 μl aliquots at −70 °C. The protein concentration of the nuclear extract was determined using the Bio-Rad protein assay kit as described by the manufacturer.

The oligonucleotides for EMSA analysis were labelled using 'fill-in' reactions carried out in the presence of [α-^{32}P]dCTP and Klenow DNA polymerase. The sequences of the oligonucleotides used were 5′-TGCAGATTGCGCAAT-3′ and 5′-TGCAATTGCGCAATC-3′ for the C/EBP binding site from the promoter of the albumin gene [5], 5′-CATAGTGGCGCAAACTCCCTTACTG-3′ and 5′-CAGTAAGGGAGTTTGCGCCACTATG-3′ for the C/EBP binding site from the promoter of the C-reactive protein gene [5,6], 5′-GCAGAATTTCTTGGGAAAGAAAA-3′ and 5′-GCTATTTTCTTTCCCAAGAAATT-3′ for the C/EBP binding site from the promoter of the β-casein gene [7,8], 5′-GCAGAATTTAAGCTTAAAGAAAA-3′ and 5′-GCTATTTTCTTTAAGCTTAAATT-3′ for a mutated site that abolishes C/EBP binding [11,12], and 5′-GATCCTTCGTGACTCAGCGGGATCCTTCGTGAG-3′ and 5′-CCGCTGAGTCACGAAGGATCCCGCTGAGTCACG-3′ for the activator protein 1 (AP1) binding site [14].

For EMSA analysis, 5 μl of nuclear extract, containing 8 μg of protein, was incubated in a 20 μl total reaction volume containing 34 mM potassium chloride, 5 mM magnesium chloride, 0.1 mM DTT and 3 μg of poly(dI-dC). After 10 min on ice, ^{32}P-labelled probes (40000 c.p.m.) were added and the incubation continued for 30 min at room temperature. Following addition of 5 μl of a 20% (w/v) Ficoll solution to each sample, the free probe and DNA–protein complexes were resolved on 6% (w/v) polyacrylamide gels in 0.25 × TBE (22.5 mM Tris base/22.5 mM boric acid/0.5 mM EDTA). The gel was dried under vacuum and exposed to X-ray film. For antibody supershift assays, samples of the nuclear extracts and 1:20 dilution of rabbit antiserum against either C/EBPα [5,15], C/EBPβ [5,6] or C/EBPδ [6,15] were incubated for 30 min on ice before the addition of the radiolabelled probe.

RESULTS

Expression of C/EBP mRNA in the mouse mammary gland during lactation and at the commencement of involution

Initially, the levels of the mRNA coding for the three C/EBP members (α, β and δ) were determined in mouse mammary gland during lactation and at the commencement of involution using semi-quantitative RT-PCR. The conditions used were shown to be within the exponential phase of amplification and therefore provided a direct correlation between the amount of amplification products and RNA template abundance in the samples. The PCR products generated were subjected to Southern blot analysis using probes specific for individual C/EBP members. Figure 1 shows the outcome of the analysis in which a similar profile of C/EBP expression was obtained in four independent experimental series (i.e. four sets of animals for each sampling point). The expression of C/EBPα was elevated during the initial phase of lactation (days 2 and 6) and decreased thereafter, remaining at low, but detectable, levels at involution. In contrast, C/EBPβ mRNA was present at low levels in the initial and mid-stages of lactation, but increased dramatically thereafter, particularly at involution. C/EBPδ showed a similar expression profile to C/EBPβ, except that higher levels of mRNA were present at day 2 of lactation. In all three cases, no signals were obtained when

reverse transcriptase was omitted from the RT-PCR reactions (Figure 1), and a product of the expected size was obtained when primers designed against two different exons in the β-actin gene were used (results not shown). In addition, the specificity of the PCR amplification and the profile of C/EBP expression (Figure 1) was confirmed further by sequencing representative amplification products after subcloning into the pGEM-T vector [16] and the use of Northern blot analysis respectively (results not shown).

In order to compare the expression patterns of the C/EBPs with the RNA expression patterns of established, well-documented marker genes of mammary gland function [β-casein, whey acidic protein (WAP) and lipoprotein lipase (LPL)], the expression of such genes was also analysed. For this, 10 μg of total RNA from the same lactating mammary gland samples as used for RT-PCR (Figure 1) was subjected to Northern blot analysis with the appropriate radiolabelled probes. For each of the three genes, the results obtained were consistent with previous reports on the expected RNA and/or protein expression profiles [9,10,14,17,18], thereby indicating a normal lactation cycle (Figure 2). Specifically, two major LPL transcripts (3.4 kb and 1.7 kb) were detected and, similar to previous studies on changes in LPL enzymic activity [17,18], the amount of both transcripts increased during the initial stages of lactation, reaching a peak at day 12, and subsequently returning to low levels at day 18. Similarly, the expression of both β-casein and WAP increased during lactation, reaching a peak during mid-lactation (day 12) and decreasing thereafter.

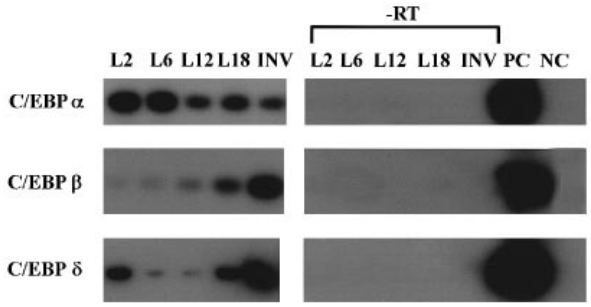

Figure 1 C/EBP mRNA expression in the mammary gland during lactation and at involution

cDNA template, prepared against total RNA isolated from the mouse mammary gland during the stages of lactation indicated, lactational day 2 (L2) to lactational day 18 (L18) and at day 1 of involution (INV), was used in PCR reactions in which primers designed against individual C/EBP members, as described in the Experimental procedures section, were present. The amplification products were then size-fractionated by electrophoresis on 2% (w/v) agarose gels, transferred onto Nylon membrane and probed with radiolabelled cDNA inserts for C/EBPα, β and δ. $-$RT represents samples that were processed in an identical manner except for the omission of the reverse transcriptase enzyme. Positive controls (PC) using recombinant plasmid DNA for each C/EBP isoform and negative controls (NC) lacking any template DNA were also included. The description of changes in band intensity included in the text is based on the replicated outcome of laser densitometric quantification.

Expression of C/EBP mRNA during involution of the mouse mammary gland

Because the evidence presented in Figure 1 indicated that the expression of both C/EBPβ and C/EBPδ was induced at the start of involution, a more detailed investigation of the expression of C/EBPα, β and δ mRNA during the different phases of involution (days 1–5 of weaning) was undertaken. Consistent with the results shown in Figure 1, the mRNA levels of both C/EBPβ and C/EBPδ were present at high levels on day 18 of lactation and at day 1 of involution, and remained relatively constant at subsequent days of weaning (Figure 3A). In addition, the analysis also showed that the expression of C/EBPα mRNA was induced during involution to reach peak levels on days 3–5 of weaning. A similar trend of C/EBP expression during involution was observed in two independent experimental series.

In the experiments detailed above, involution was initiated by the removal of pups after a complete lactation cycle of 18 days had elapsed. In order to examine the link between the observed events and the removal of the suckling stimulus, in a subsequent experiment pups were removed from dams after 7 days of lactation, i.e. when lactation was approaching its peak. The analysis of C/EBP expression was also extended, with samples taken daily until 10 days after the removal of the pups. Additionally, mammary gland from virgin mice was included for comparison. As shown in Figure 3(B), the data obtained confirmed the previously noted changes (Figure 3A). Thus, C/EBPα mRNA was present at relatively low levels at days 1–2 of weaning, increased drastically at day 3 and remained relatively constant thereafter. C/EBPβ and C/EBPδ mRNA were present at high, and relatively constant, levels from day 1 to day 10 of weaning. In addition, the analysis showed the expression of all three C/EBP genes in the virgin animal.

Overall, the RT-PCR analysis shown in Figures 1 and 3 indicates that the mRNA levels of the three C/EBP genes (α, β and δ) are induced during weaning of the mammary gland and expressed throughout involution. Although small variations in the expression of these genes were observed during involution (e.g. slight reductions on days 7–8 for C/EBPβ and day 8 for C/EBPδ) (Figure 3B), further experiments using a larger set of animals will clearly be required to verify whether they represent significant changes.

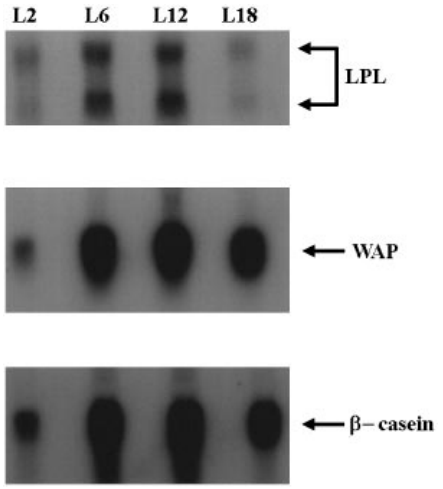

Figure 2 Analysis of marker-gene expression in the mammary gland during lactation

Samples of total RNA (10 μg) were size-fractionated on 1% (w/v) agarose/formaldehyde gels, blotted onto Nylon and probed with ^{32}P-labelled cDNA inserts for LPL, β-casein or WAP (see text). Arrows indicate the positions of the hybridization signals. Equal amounts of total RNA was present in the different lanes, as judged by ethidium bromide staining of 28 S and 18 S rRNA (results not shown).

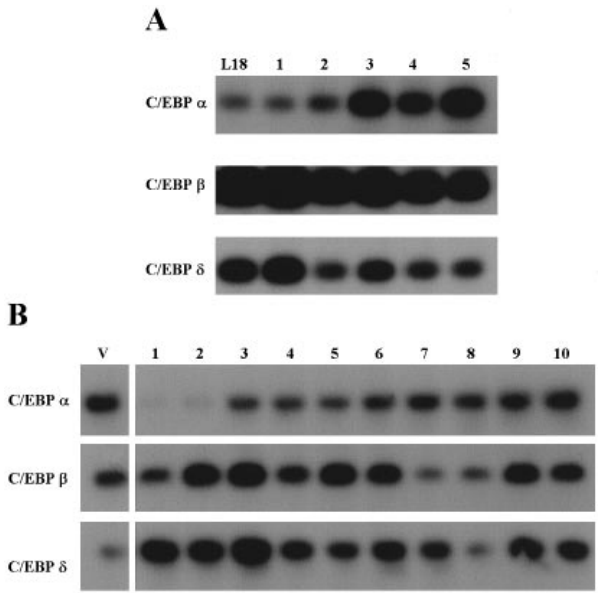

Figure 3 Expression of C/EBPα, β and δ mRNA in the mammary gland during involution

cDNA template was prepared against total RNA isolated from mouse mammary gland at day 18 of lactation (L18) and days 1, 2, 3, 4 and 5 after weaning (lanes 1–5) (**A**), or from virgin mice (V) and days 1–10 after removal of pups after 7 days of lactation (lanes 1–10) (**B**). PCR was carried out using conditions which were in the exponential phase of amplification, and the amplification products were subjected to Southern blot analysis using radiolabelled cDNA inserts for C/EBPα, β and δ as probes. No amplification products were obtained from parallel samples in which reverse transcriptase was omitted. The description of changes in band intensity included in the text is based on the replicated outcome of laser densitometric quantification.

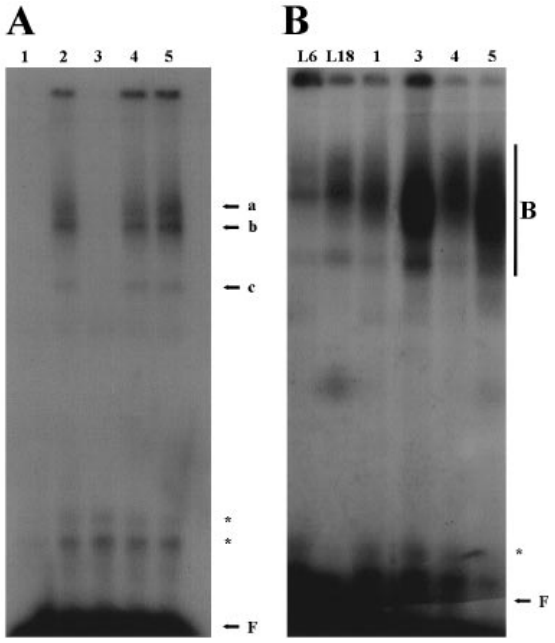

Figure 4 EMSAs with nuclear extracts from the mouse mammary gland

(**A**) Competition EMSAs were carried out using nuclear extracts from day 1 of weaning in the absence (lane 2) or presence of a 100-fold excess of unlabelled oligonucleotides corresponding to either a homologous C/EBP-binding site (lane 3), a heterologous low-affinity C/EBP binding site from the promoter of the C-reactive protein gene (lane 4), or an unrelated AP1 consensus sequence (lane 5). Lane 1 represents a reaction mixture containing the radiolabelled oligonucleotide without any nuclear extracts. The position of the three DNA–protein complexes (a–c) and the free probe (F) are indicated by arrows. (**B**) EMSA was carried out using nuclear extracts from the mammary gland at days 6 and 18 of lactation (L6 and L18 respectively) and days 1, 3, 4 and 5 after weaning. The position of the DNA–protein complexes is indicated by a vertical line, labelled B, and the free probe (F) is shown by an arrow. In both (**A**) and (**B**), asterisks indicate extra bands that are probably derived from a partial denaturation of the probe [19].

Expression of functional C/EBP protein during lactation and involution of the mammary gland

Western blot analysis of samples from representative stages of lactation and involution, using antisera specific for C/EBPα, β and δ, showed the presence of proteins for all three genes (results not shown). In addition, this analysis indicated the expression of the different C/EBPα and C/EBPβ polypeptides that are produced by alternate use of translation-initiation codons [i.e. p42 and p30, and LAP*, LAP (liver activator proteins) and LIP (liver inhibitor protein) respectively] [2,3]. In order to evaluate the potential functional consequences of this C/EBP protein expression on the DNA binding activity, EMSAs were carried out using nuclear extracts prepared from samples of the lactating and involuting mammary gland and a ^{32}P-labelled C/EBP-binding site oligonucleotide. This analysis was concentrated on samples obtained from mice that underwent a normal lactation cycle of 18 days, followed by involution. However, the mammary gland samples were obtained from a different set of mice to those used for the analysis of mRNA expression detailed above (Figures 1–3). Figure 4(A) shows the EMSA profile using extracts from mammary tissue taken 1 day after weaning. Consistent with other studies on the C/EBP family [e.g. 5–8], multiple DNA–protein complexes were present, of which at least three (a, b and c) were particularly prominent in this and the other experiments detailed below. Competition EMSA showed that all three complexes could be competed out using an excess of the C/EBP binding site oligonucleotide (Figure 4A, lane 3), but not by an unrelated AP1 binding site oligonucleotide (Figure 4A, lane 5). Because the nature of the C/EBP-regulated genes in the mammary gland during involution is not known, we have used here a high-affinity C/EBP binding site from the albumin promoter, the choice being based on previous studies [5,6]. Nevertheless, the three complexes can also be competed out, at least partially, using an excess of oligonucleotide containing the low-affinity C/EBP binding sequence from the promoter of the C-reactive protein and the β-casein genes, but not with a mutant site from the β-casein promoter that has previously been demonstrated to abolish C/EBP binding (Figure 4A, lane 4; other results not shown).

EMSA analysis using nuclear extracts from the mammary gland samples taken at representative stages of lactation and involution showed that there were drastic changes in both the number of DNA–protein complexes and the total C/EBP binding activity (Figure 4B). Thus, consistent with the mRNA expression profile shown in Figures 1 and 3, the total C/EBP DNA binding activity was low at day 6 of lactation and increased dramatically at day 18 of lactation and, particularly, during involution.

In order to identify the nature of the C/EBP isoforms that were participating in the formation of a particular DNA–protein complex, antibody interference EMSA experiments were carried out using nuclear extracts from tissue samples collected at days 1, 3 and 5 after weaning and specific antisera against C/EBPα, β and δ. Both previous studies [5–8,15] and current Western blot analysis (results not shown) have demonstrated that each of the antisera was specific for a particular C/EBP isoform and did not

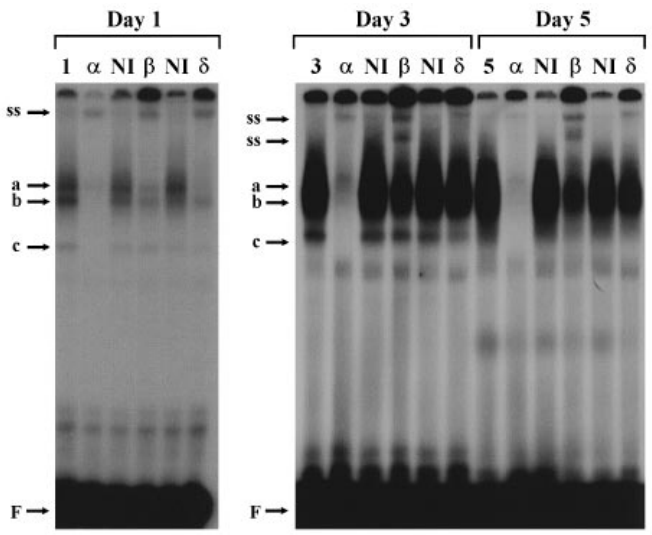

Figure 5 Identification of the C/EBP isoforms involved in DNA–protein interactions

EMSAs were carried out using extracts from mammary gland samples taken at days 1, 3 and 5 after weaning, either in the absence of any added antisera (1, 3 and 5) or in the presence of antiserum against C/EBPα (α), C/EBPβ (β) or C/EBPδ (δ), or preimmune serum (NI). a–c indicate the position of the three DNA–protein complexes, and arrows labelled ss and F show the 'super-shifted' complexes and the free probe respectively.

cross-react with the other family members. As shown in Figure 5, three complexes (a–c) were apparent with extracts from tissue taken at days 1 and 3 after weaning, and two complexes (a and b) were observed with similar extracts obtained at day 5 after weaning. Similar to the results presented in Figure 4(A), the specificity of the DNA–protein interactions obtained using extracts from days 3 and 5 of weaning was confirmed by competition EMSAs (results not shown).

With extracts from all three stages of involution, both the appearance of slower migrating 'super-shifted' complexes (arrows marked ss in Figure 5; in the autoradiographic exposures shown, this is particularly apparent with extracts from day 1 of involution) and the inhibition of specific DNA–protein complexes were observed in the presence of each of the three antibodies. This indicated that all three C/EBP family members were participating in DNA–protein interactions. Thus, functional C/EBPα protein was also expressed at day 1 of weaning, despite relatively low levels of mRNA being present at this stage (Figures 1 and 3A). In addition, the analysis demonstated the involvement of heterodimers among these three proteins and the various polypeptides produced by alternate use of translation-initiation codons [2,3], in DNA binding. For example, with extracts from tissue taken at day 1 after weaning, the presence of complex (a) was inhibited completely by antiserum against either C/EBPα or C/EBPδ and inhibited partially by antiserum against C/EBPβ. This observation indicated that the α–δ heterodimer could be considered to be the predominant species in this complex, with the β homo-/hetero-dimer representing only a minor component. Complexes (b) and (c) were extensively inhibited in the presence of C/EBPα antiserum, and partially so by the presence of antisera against either C/EBPβ or C/EBPδ, thereby implicating the α homo-/hetero-dimer as the predominant species in these complexes. The difference in the mobility of complex (a)/(b) from that of complex (c) suggests that the different-sized polypeptides characteristic of both the C/EBPα and C/EBPβ family members, and which are produced by alternative use of initiation codons [2,3], are all likely to participate in the observed DNA–protein interactions. With extracts taken at day 3 of weaning, the formation of complex (a) appeared to be prevented in the presence of C/EBPα antiserum and partially affected by the presence of antiserum against C/EBPβ and C/EBPδ, thus indicating that the α homo-/hetero-dimer was likely to be the predominant species present in the complex. Complex (b) was 'super-shifted' completely by both the C/EBPα and the C/EBPβ antiserum, and partially by the C/EBPδ antiserum, thereby suggesting the α–β heterodimer as the likely predominant species in this complex. These observations are particularly apparent in lower autoradiographic exposures. The formation of complex (c) was inhibited in the presence of antiserum against both C/EBPα and C/EBPδ, and partially by the C/EBPβ antiserum, thus suggesting the α–δ heterodimer as the predominant species in this complex. For extracts from tissue taken at day 5 after weaning, only two complexes, (a) and (b), were apparent (Figure 5). Both of these were inhibited completely in the presence of the C/EBPα antiserum and partially so by the C/EBPβ and C/EBPδ antiserum, thus suggesting that the homo-/hetero-dimer of C/EBPα was the most prominent participating protein in these complexes.

As expected, preimmune serum from two different sources failed to affect the mobility of any of the complexes formed (lanes labelled NI in Figure 5), thereby confirming the specificity of the assays.

Previous studies have indicated that involution of the mammary gland is characterized by the induced expression of several genes [9–12,14], including members of the AP1 transcription factor family [14]. Although involution in the mice used for the current studies was judged to be normal on the basis of histological analysis (results not shown), the comparative expression of the AP1 family was also undertaken by EMSA. The AP1 DNA-binding activity was also found to be induced at day 18 of lactation and during the early stages of involution (i.e. days 1 and 3) (results not shown). In addition, preliminary RT-PCR analysis also showed expression of the c-*fos* and c-*jun* mRNA during involution (results not shown).

DISCUSSION

The postnatal development of the mouse mammary gland is complex and can be divided into at least three phases: ductal growth from 6 to 8 weeks of age, development of the lobular-alveolar structures during pregnancy and lactation, and involution during weaning. Transcription factors belonging to the C/EBP family have been shown recently to be expressed in the mammary gland and to interact with a highly conserved 'milk box region' present in the promoter region of the β-casein gene [7]. Additionally, using Western blot analysis, Raught et al. [8] showed that the expression of the C/EBPα, β and δ proteins changed in the rat mammary gland during pregnancy and lactation. We have extended these studies considerably by examining the changes in the mRNA level and DNA-binding activity of C/EBPs during lactation and throughout the involution of gland following weaning in mice. Where our data on the changes in C/EBP mRNA expression overlap with the changes in C/EBP protein expression determined by Raught et al. [8] (i.e. during lactation and the commencement of involution) (Figure 1), the results from the two studies are in agreement. In addition, we demonstrate the induction of C/EBPα expression during weaning (Figures 3 and 5). Thus, in common with the transcription factors belonging to the STAT family [20], the C/EBP family is expressed during both lactation and involution of the mammary gland.

Involution of the mammary gland is characterized by several events, including the loss of epithelial cells via apoptosis, proteolytic degradation of the extracellular matrix and infiltration by adipocytes [9–12,14]. The activation of C/EBP isoforms during involution is not surprising, given the crucial involvement of this family in the regulation of the cell cycle and cell proliferation [4,21–23]. C/EBP isoforms have also been implicated in apoptosis [24] and, additionally, shown to play a pivotal role in adipocyte differentiation [4,15,25]. It is interesting to note that the differential induction profile of the different C/EBP members during involution (i.e. the expression of C/EBPβ and C/EBPδ preceding that of C/EBPα) (Figure 3) is similar to what is seen in cell-culture model systems of adipocyte differentiation [4,25]. The presence of high numbers of adipocytes in the mammary gland of virgin mice and the expression of all three C/EBP genes at this stage is also consistent with the crucial role of this family of transcription factors in adipogenesis. In addition, consistent with previous studies on the expression of the AP1 family in Moro mice [14], we have also observed increased DNA-binding activity in this family of transcription factors during involution of the Balb/c mice (results not shown). In common with the C/EBP family, the AP1 family of factors has also been implicated in the control of cell proliferation and apoptosis [26] and, additionally, binding sites for AP1 are present in the promoter regions of several genes that are expressed characteristically in adipose tissue [4].

Because the total C/EBP DNA-binding activity (Figure 4) followed the mRNA levels (Figures 1 and 3) closely, it is likely that changes in mRNA metabolism (i.e. transcription or mRNA stability) are responsible primarily for the regulation of C/EBP isoform expression during lactation and involution. However, other mechanisms may also play an important role. For example, EMSA analysis (Figure 5) suggests possible modulation of the DNA binding and/or *trans*-activation potential via changes in the composition of C/EBP heterodimers. Additionally, other proteins that are known to interact with and regulate C/EBP function (e.g. AP1, p53) are also induced during involution [9,14]. Furthermore, post-translational modification of C/EBPα by several protein kinases that are activated during involution [e.g. 14] may be responsible for the avid binding of C/EBPα to its consensus recognition sequence at the start of involution (day 1; Figure 5), despite the relatively low levels of mRNA being present (Figures 1 and 3).

While this manuscript was in preparation, Gigliotti and DeWille [27] described the induction of C/EBPβ and C/EBPδ mRNA expression during involution of FVB/N mice. Their results on mRNA expression are therefore in agreement with those shown in Figure 1. In addition, we have extended the analysis to include changes in the functional DNA-binding activity of C/EBP isoforms (Figures 4 and 5). In contrast to the present study, Gigliotti and DeWille [27] observed that involution, however, had no effect on the low, but detectable, levels of C/EBPα mRNA in the mammary gland. The precise reason(s) for this discrepency is unclear currently, but may reflect the use of either different strains of mice (FVB/N), the less sensitive technique of Northern blot analysis for measurement of mRNA expression and/or analysis of samples from restricted stages during involution (i.e. 0 h, 6 h, 12 h, 18 h, 24 h, 48 h and 72 h) [27]. It is interesting to note that these authors [27] also detected low levels of C/EBPα mRNA expression during lactation. In contrast, our studies in mice (Figure 1) and those of Raught et al. [8] in rats clearly demonstrated high expression levels of this gene during lactation.

In conclusion, we have shown here that the three C/EBP isoforms are expressed differentially during lactation and involution of the mammary gland. Milk protein genes, the expression of which are induced during lactation (Figure 2), and which contain C/EBP-recognition sequences in their promoter regions [7,8], represent known targets for C/EBP-action during lactation. The potential downstream effector genes, the expression of which is induced by the family during involution, remain to be determined, but may include genes involved in cell proliferation and differentiation (e.g. c-*myc*, p53, transforming growth factor-β1), tissue remodelling (gelatinase A, stromelysin-1, tissue inhibitor of metalloproteinase), stress/cell death (e.g. interleukin-1β converting enzyme, sulphated glycoprotein-1), and, other transcription factors (e.g. AP1) [9–12,14], Indeed, C/EBPβ has already been shown to interact with and activate the promoter of the c-*fos* gene [28].

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