Studies of the regulation of the mouse carboxyl ester lipase gene in mammary gland

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The lactating mammary gland and pancreas of mouse constitute the main tissues for synthesis and secretion of a bile-salt-stimulated lipase called carboxyl ester lipase (CEL). In this paper we have analysed the endogenous *CEL* gene expression in mammary gland. It is shown that the gene is expressed at day 14 of pregnancy, which is synchronous with that of the whey acidic protein (*WAP*) gene. Even though the *CEL* and *WAP* genes are induced at the same time during mammary gland differentiation, their regulation is different with respect to dependence on lactogenic hormones. The high induction of the *WAP* gene expression due to the activation of signal transducer and activator of transcription (STAT)5 by prolactin has not been observed for the *CEL* gene, even though it has been demonstrated that both STAT5 isoforms interact with one of the γ-interferon activation sequence sites in the promoter of the *CEL* gene. Hence we have demonstrated that the prolactin/STAT5 signal is not involved in a general and significant activation of 'milk genes'. Instead of a direct effect of the lactogenic hormones, the up-regulation of the *CEL* gene is correlated with an increase in the number of differentiated epithelial cells. Furthermore, promoter studies using the mammary-gland-derived cell line, HC11, show that a major positive element in the *CEL* gene promoter interacts with a member(s) of the CCAAT-binding transcription factor/nuclear factor 1 family, binding to a palindromic site. Binding of this factor(s) is important for the tissue-specific activation of the *CEL* gene in the mammary gland, because no activation by this factor(s) was seen in cells of pancreatic origin.

INTRODUCTION

Unlike most other organs, the mammary gland is one of the few that undergoes repeated cycles of development and regression in the adult animal. Pregnancy induces a massive proliferation of the epithelial component of the gland, which leads to a duct-branching morphogenesis followed by alveolar differentiation. The proliferation and differentiation is governed directly by a variety of steroid hormones, e.g. oestrogen and progesterone, polypeptide hormones, e.g. prolactin [1], and growth factors, e.g. epidermal growth factor (EGF) and transforming growth factor-α [2,3], and by interactions of the cells with the microenvironment, such as the extracellular matrix. Morphogenesis is accompanied by the production and secretion of milk proteins by epithelial cells. After the lactation period, this massively developed epithelium is dismantled during involution by a process that involves programmed cell death (apoptosis) [4–6].

Regulation of milk protein genes has been extensively studied in both transfected mammary epithelial cells and transgenic mice, and has led to the identification of important *cis*-acting elements that are required for the regulation of genes, such as *β-casein*, whey acid protein (*WAP*) and *β-lactoglobulin* genes respectively. Further analysis of these elements has resulted in the identification of interacting transcription factors; both positively acting factors, such as signal transducer and activator of transcription (STAT)5, Ets-1, CCAAT-binding transcription factor (CTF)/nuclear factor 1 (NF1) and CCAAT-enhancer-binding protein (C/EBP)-β [7–10], and a negatively acting factor (Ying and Yang 1, or YY1) have been identified [7]. To date, no tissue-specific factor has been identified.

In addition to the highly expressed *β-casein*, *WAP* and *β-lactoglobulin* genes, several other genes are expressed in the mammary gland during lactation. One protein not so abundantly present is the bile-salt-stimulated lipase, carboxyl ester lipase (CEL) [11]. By studying the regulation of this gene, further insights into both the tissue-specific and developmental regulation of the milk protein genes may be obtained. The *CEL* gene provides excellent possibilities for identifying important regulatory elements, because the gene is expressed mainly in two tissues, the pancreas (where expression is constitutive) and the mammary gland (where expression is induced during pregnancy). By performing comparative analyses on the two tissues, it should be possible to identify important *cis*-elements and *trans*-acting factors that confer the tissue specificity of expression.

EXPERIMENTAL

RNA preparation

At different stages during pregnancy (days 10, 14 and 16) and lactation (day 1 and 10), starting from the discovery of a vaginal plug, the inguinal mammary glands were dissected from C57 Black/6JCBA mice and immediately frozen in liquid N_2. For each developmental stage, two animals were used. Total RNA

Abbreviations used: CAT, chloramphenicol acetyltransferase; C/EBP, CCAAT-enhancer-binding protein; CEL, carboxyl ester lipase; CTF, CCAAT-binding transcription factor; EGF, epidermal growth factor; EMSA, electrophoretic mobility-shift assay; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GAS, γ-interferon activation sequence; GR, glucocorticoid receptor; Luc, luciferase; NF1, nuclear factor 1; RT, reverse transcriptase; STAT, signal transducer and activator of transcription; WAP, whey acid protein.

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Table 1 Hormone treatment of organ cultures

No.	Treatment
1	Insulin (5 μg/ml)
2	EGF (10 ng/ml)
3	Dexamethasone (1 μM)
4	Prolactin (5 μg/ml)
5	Insulin (5 μg/ml) + EGF (10 ng/ml)
6	Dexamethasone (1 μM) + EGF (10 ng/ml)
7	Insulin (5 μg/ml) + dexamethasone (1 μM) + EGF (10 ng/ml)
8	Insulin (5 μg/ml) + dexamethasone (1 μM) + prolactin (5 μg/ml)

was isolated by acid guanidinium thiocyanate/phenol/chlorophorm extraction, essentially as described by Chomczynski and Sacchi [12].

Northern blotting

Aliquots of 10 μg of total RNA were analysed using a Northern blot technique and the filter was hybridized with probes detecting the CEL [13], WAP [14] or β-casein transcripts [15] respectively. Hybridization and washing procedures followed were as recommended by Amersham Pharmacia Biotech (Uppsala, Sweden).

Organ culture

Inguinal mammary glands from mid-pregnant C57Black/6JCBA mice (10–13 days of gestation) were dissected out and placed in ice-cold Hanks balanced salt solution. After gently cutting the glands into small pieces, the explants were transferred to Millipore (Bedford, MA, U.S.A.) filters (0.45-mm pore sizes). The filters were placed on glass supports, which were, in turn, placed in Costar Petri dishes containing pre-equilibrated culture medium. The explants were maintained *in vitro* for 5 days under maximal humidity conditions at 37 °C in an atmosphere containing 5 % CO_2/95 % air. The BGJb medium (Life Technologies AB, Täby, Sweden) was supplemented with 0.1 mg/ml ascorbic acid, 2 mM L-glutamine, 0.1 % (w/v) BSA, 50 units/ml penicillin and 0.05 mg/ml streptomycin. The explants were divided into eight groups, which were treated with hormones (Table 1). The medium was changed the day after the start of the culture, and then every second day. The explants were then either processed for histological examination or prepared for RNA extraction, as described above. For morphological examination, explants were fixed in Bouin's fixative and prepared for paraffin embedding. Sections were stained with Ladewig's Haematoxylin.

RNase protection analysis

In order to detect CEL mRNA by RNase protection analysis, a 211-bp fragment that corresponds to the mouse CEL cDNA from nt 851 to 1062 [13] was subcloned into pBluescript SK$^-$ (Stratagene, Heidelberg, Germany) to create pSK211. *Sst*I-linearized pSK211 was transcribed *in vitro* with T7 RNA polymerase in the presence of [α-^{32}P]CTP. The resulting radiolabelled antisense RNA probe was hybridized overnight at 42 °C to 10 μg of total RNA from the different tissue cultures. Transcription *in vitro* was performed using the protocol of Boehringer Mannheim (Indianapolis, IN, U.S.A) and the RNase protection analysis was performed as described in the RPA II TM Ribonuclease Protection Assay Kit (Ambion, Austin, TX, U.S.A). As an internal control, the pTRI RNA 18 S antisense control template was used, purchased from Ambion. The radioactivity associated with each band was quantified using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA, U.S.A.).

Reverse transcriptase (RT)–PCR

RT–PCR experiments were carried out using the Titan™ One Tube RT–PCR System (Boehringer Mannheim). The sequences of the primers used were: 5′-AGATGGCTGCTGGTCTGA-AGATCA-3′ and 5′-CAGTGTGTCCACTGCTGTAGCCTG-TA-3′, yielding a 309-bp fragment of the mouse CEL cDNA [13]; 5′-TGTATCATCTGCCAAACCAACGAG-3′ and 5′-GAAG-GGTCTTGCTGTATAGACTTGGGCTGG-3′, producing a 349-bp fragment from the mouse WAP cDNA [16]; 5′-GTGT-CGACCTAGCCAAGATCC-3′ and 5′-CTCGCTGGTAGCT-CAGATGGC-3′, yielding a 490-bp fragment of the mouse keratin 19 cDNA [17]; and 5′-CACCACCATGGAGAAGG-CCGGGGCC-3′ and 5′-TTGAAGTCGCAGGAGACAACC-TGGT-3′, producing a 554-bp fragment from the mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA [18]. The RT–PCR reactions were first incubated at 50 °C for 30 min, followed by ten cycles of 1 min at 96 °C, 1 min at 62 °C and 4 min at 68 °C, and 20 cycles of 30 s at 96 °C, 30 s at 62 °C and 1 min at 68 °C.

Cloning and reporter gene constructs

To obtain the mouse CEL gene promoter, the genomic clone mouse CEL [13] was digested with *Xba*I/*Kpn*I and the resulting 2775-bp fragment, extending from nt −2175 to +600, was inserted into the *Xba*I/*Kpn*I sites of the cloning vector pTZ18R (Amersham Pharmacia Biotech). This fragment was further subcloned and sequenced by the dideoxy chain-termination method using an automated laser fluorescent sequencer (Amersham Pharmacia Biotech). The nucleotide sequence was generated from double-stranded templates using the Auto Read T7 sequencing kit with either the universal/reverse primers or specific primers derived from the mouse *CEL* gene promoter.

In order to generate a construction that included the promoter region only, the recombinant (−2175/+600)/pTZ18R was digested with *Nco*I, treated with mung-bean nuclease to eliminate the ATG start codon and then cut with *Bgl*II to yield compatible ends for the insertion of the resulting 1184-bp fragment into a *Hin*dIII-blunted/*Bcl*I-digested luciferase (Luc) vector. Sequencing verified that the start codon was eliminated in the −1184/Luc construction. More upstream promoter sequence was introduced by *Sac*I/*Kpn*I digestions of both the −1184/Luc construction and the (−2175/+600)/pTZ18 construct respectively.

The 5′-deletion constructs of the promoter were mainly prepared by using internal restriction-enzyme sites, but for some of the constructs PCR amplification was necessary. Site-directed mutagenesis was performed using the Quik change™ site-directed mutagenesis kit (Stratagene). The primers used in the different mutagenesis reactions were the double-stranded oligonucleotides, NF1:1 mut, NF1:2 mut and Glu mut, used in the electrophoretic mobility-shift assay (EMSA) reactions (see Figure 5B) and the (−1480/−1831)/pTZ18R construct was used as template. The sequence of the mutated regions were verified by sequencing of double-stranded templates. The mutated sequence −1480/−1831 NF1:1 mut was then introduced into the −1480/Luc construct.

Cell culture

The mouse mammary epithelial cell line HC11 was grown on collagen-coated plastic at 37 °C in a 5 % CO_2/95 % air at-

mosphere and in RPMI-1640 medium supplemented with 10 % (v/v) fetal calf serum, 1 % (v/v) penicillin–streptomycin, 5 μg/ml insulin and 10 ng/ml EGF. The cultures were kept for 2 days at confluency before the medium was switched to RPMI-1640 medium containing 10 % (v/v) heat-inactivated fetal-calf serum, 1 % (w/v) penicillin–streptomycin and either insulin (5 μg/ml) and EGF (10 ng/ml) or insulin, dexamethasone (1 μM) and prolactin (5 μg/ml). After addition of hormones the cells were cultured for an additional 4-day period.

The rat pancreatoma cell line, AR4-2J, and the African green monkey kidney cell line, COS-7, respectively were obtained from A.T.C.C. Both of these cell lines were cultured at 37 °C in a 5 % CO_2/95 % air atmosphere in Dulbecco's modified Eagle's medium containing 2 mM glutamine and 4.5 g/l glucose, and supplemented with 10 % (v/v) heat-inactivated fetal-calf serum and 1 % (w/v) penicillin–streptomycin.

Nuclear protein preparation

Preparation of nuclear extracts for EMSA and DNase I footprinting experiments were carried out as described previously [19]. Protein concentrations of the extracts were determined by the method of Bradford [20], and the extracts were stored in aliquots at −70 °C before use.

EMSA

The oligonucleotides used in the EMSA reactions (see Table 2 and Figure 5B) were synthesized using a Beckman Oligo 1000. For each reaction, 25000 c.p.m (by the method of Čerenkov) of labelled double-stranded oligonucleotide were incubated in a 20 μl reaction volume containing 20 mM Hepes (pH 7.9)/50 mM KCl/10 % (v/v) glycerol/2 mM $MgCl_2$/0.5 mM EDTA/0.1 mg/ml BSA/0.5 mM dithiothreitol, with 1.5 μg of poly(dI–dC) and 8 μg of nuclear extracts. In some cases, unlabelled double-stranded oligonucleotides were included in the incubation mixture as competitors. Super-shifts were performed by adding antibodies raised against STAT3, STAT5a, STAT5b and STAT6 (from Santa Cruz Biotechnology Inc., Santa Cruz, CA, U.S.A.) to the binding reactions.

Stable and transient transfections

The cell line HC11 was stably transfected using lipofectin with 10 μg of the different 5′-deletion constructs and 1 μg of pTX-1 neo^R vector per 4-cm culture dish. Two days after transfection, the antibiotic G418 (350μg/ml) was added to the cells. G418-resistant colonies (20–100) were pooled and expanded in G418-containing medium, and finally frozen or used immediately for different hormone treatments, as indicated above.

The AR4-2J cells were transiently transfected by the lipofectin method, using 9 μg of mouse CEL Luc constructs and 3 μg of pCMV-chloramphenicol acetyltransferase (CAT) reporter plasmid as internal control. In the case of COS-7 cells, 9 μg of mouse CEL Luc constructs and 0.5 μg of pCMV-β-galactosidase reporter plasmid were used for the transfection.

Cell extract and reporter gene assays

Luc assays were performed using the Promega kit with 50 μl of extract, and assayed in a luminometer (Berthold, Pforzheim, Germany). Protein concentration of the extracts was determined by the method of Bradford [20]. The Luc activity was normalized to the protein concentration of each extract.

The cell extracts from the transiently transfected AR4-2J and COS-7 cells were also prepared using the Promega kit, and Luc activity was also measured as described above. For the CAT assay, endogenous deacetylation activities were removed by heat inactivation before the reaction was measured using 75 μl of extract, [^{14}C]butyryl-CoA and chloramphenicol in an automatic scintillation counter (Beckman). The assay for β-galactosidase activity was performed using the Clontech protocol for luminescent β-galactosidase assay. The Luc activity was normalized to either the CAT activity (for AR4-2J cells) or the luminescent β-galactosidase activity (for COS-7 cells).

DNase I footprinting

DNA templates were isolated from a pTZ18R plasmid containing the region from nt −1831 to −1480 of the mouse CEL promoter or pTZ18R (−1831/−1480) in which the NF1:1 mut, NF1:2 mut or Glu mut mutants have been introduced as described above. Probes (10000 c.p.m., by the method of Čerenkov) were incubated with 20 μg of nuclear extracts from HC11 cells, treated with either insulin/EGF or insulin/dexamethasone/prolactin, AR4-2J cells and COS-7 cells. DNase I digestion and work-up procedures were used as described by Jones et al. [21].

RESULTS

Expression of the *CEL* gene during mammary gland development

To investigate the developmentally regulated expression of the mouse *CEL* gene in mammary gland, we measured steady-state levels of corresponding mRNA on days 10, 14 and 16 of pregnancy, and on days 1 and 10 of lactation. Northern analysis revealed that the *CEL* gene is expressed on day 14 of pregnancy (Figure 1A), which is similar to that of the *WAP* gene, and that the β-casein gene is expressed on day 10 of pregnancy (Figures 1B and 1C).

A common feature in the regulation of expression of milk protein genes is that during pregnancy, milk protein genes are positively controlled by the lactogenic hormones, insulin, dexamethasone and prolactin. In order to see if this also includes the *CEL* gene, we examined its hormonal regulation *in vitro* by use of organ cultures derived from mammary glands. The explants that were obtained from animals on gestational days 10–13 were cultured under serum-free conditions in the presence of various combinations of the hormones insulin, EGF, dexamethasone and prolactin (Table 1) for 5 days. Treatment of the explants with EGF, prolactin, dexamethasone or EGF/dexamethasone

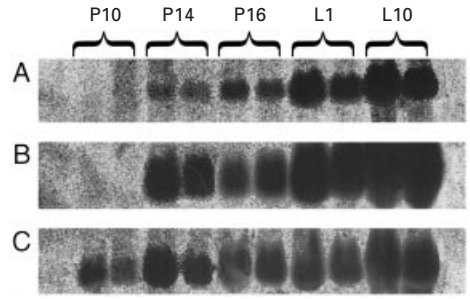


Figure 1 Accumulation of CEL, WAP and β-casein mRNAs during mammary gland development

Aliquots of 10 μg of total RNA from different stages of the mammary gland development [days 10 (P10), 14 (P14) and 16 (P16) of pregnancy, and days 1 (L1) and 10 (L10) of lactation] were analysed using the Northern blot technique. The filter was hybridized with probes detecting CEL (**A**) or WAP (**B**) or β-casein (**C**) transcripts respectively.

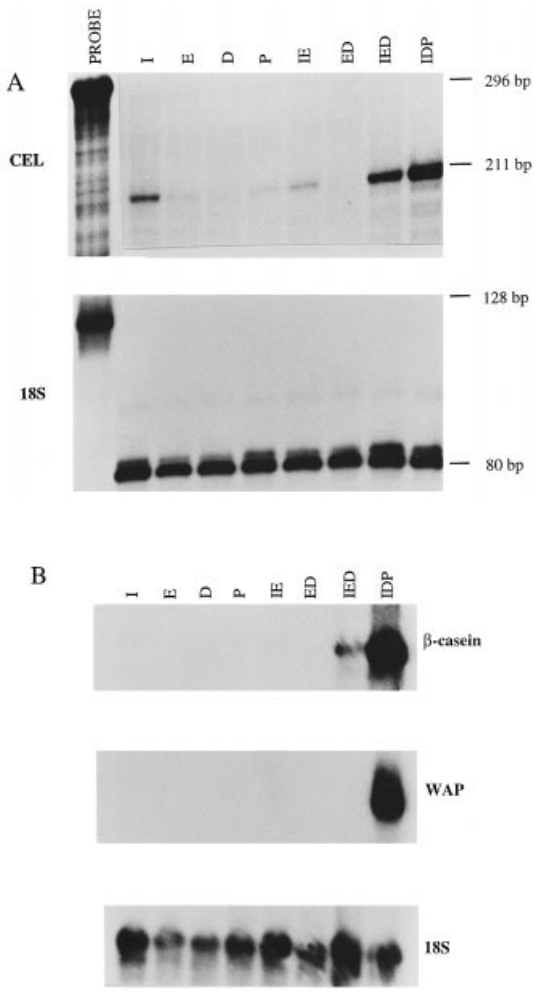


Figure 2 Effect of hormone treatment on CEL, β-casein and WAP mRNAs in cultured mammary tissue

The concentrations of the different hormones: insulin (I), EGF (E), dexamethasone (D) and prolactin (P), are shown in Table 1. After 5 days of cultivation, total RNA was extracted and the CEL mRNA was analysed using the RNase protection assay (**A**). Antisense RNA probes transcribed *in vitro*, which protect 211 bp of the CEL mRNA or 80 bp of the 18 S RNA, were used. The β-casein mRNA, the WAP mRNA and the 18 S RNA were analysed using the Northern blot technique (**B**).

Table 2 STAT oligonucleotides

Sequence of the double-stranded oligonucleotides	
5'-CTACCCT**TTC**TCT**GAA**GAGGCCA-3' 3'-GATGGGA**AAG**AGA**CTT**CTCCGGT-5'	Stat CEL1[a]
5'-GCTCTGG**TTC**TCA**GAA**TCACACC-3' 3'-CGAGACC**AAG**AGT**CTT**AGTGTGG-5'	Stat CEL2[a]
5'-GCTCTGG**cc**CTCAGAATCACACC-3' 3'-CGAGACC**gg**GAGTCTTAGTGTGG-5'	Stat CEL2 mut[b]
5'-AGAT**TTC**TAG**GAA**TTCAATCC-3' 3'-TCTA**AAG**ATC**CTT**AAGTTAGG-5'	Stat β-casein[a]
5'-AGCACTTTGTGGCACTTGCTAG-3' 3'-TCGTGAAACACCGTGAACGATC-5'	Unspecific

[a] The Stat binding sequence is shown in bold.
[b] Mutated sequences are shown in bold lower-case letters.

resulted in tissue involution, while treatment of the explants with insulin or insulin/EGF did not affect the differentiation at all, i.e. the cells were maintained at approximately the same developmental stage as when they were explanted. However, when insulin/EGF/dexamethasone were added together, the cells developed, resulting in ductal branching and some secretion. Addition of the lactogenic hormones insulin/dexamethasone/prolactin led to terminally differentiated cells and lobulo-alveolar development (results not shown).

Total RNA from these extracts were prepared and the level of expression of the *CEL* gene was estimated using the RNase protection assay. As can be seen in Figure 2(A), no *CEL* gene expression was detected in explants treated with EGF, dexamethasone, prolactin or EGF/dexamethasone, but treatment of the cells with insulin or insulin/EGF resulted in a low level of CEL mRNA transcript production. The expression was increased by 3-fold by treating the explants with insulin/EGF/dexamethasone and, ultimately, the level of CEL mRNA was found to be a further 3-fold higher in cells treated with insulin/dexamethasone/prolactin. To ensure that the organ culture assay reflected the condition reported by others, we also investigated the amounts of β-casein and WAP transcripts in the different explants. A Northern blot with the same amount of RNA as in the RNase protection assay was performed and the results revealed that the expression pattern was as expected for both genes, with their highest expression level occurring in cells treated with the lactogenic hormones (Figure 2B) [22,23]. Taken together, these results show that the *CEL* gene is expressed by day 14 of pregnancy, which is synchronous with the expression of the *WAP* gene. However, the lactogenic hormone response of *CEL* gene expression is only about 10-fold compared with the 1000-fold induction of *WAP* gene expression.

Evidence for an interaction between STAT5a/STAT5b and the *CEL* gene promoter

As it is known that activation of STAT5 by prolactin is important for the induction of *WAP* gene expression [24], we wanted to investigate whether this factor is also involved in the 10-fold up-regulation of the *CEL* gene. Therefore a fragment of the mouse *CEL* gene promoter, including 2.2 kb upstream of the translational initiation site, was isolated from the genomic clone mouse CEL1 [13]. The DNA sequence was determined, and computer analysis revealed that there are two putative γ-interferon activation sequence (GAS) sites, as well as binding sites for other transcription factors, such as CTF/NF-1, Oct-1, Ets-1, YY-1, C/EBP-β and the glucocorticoid receptor (GR) respectively, in this promoter region.

To investigate whether either of the putative GAS sites, located at nt -753 to -745 and at -1340 to -1332 respectively, are capable of interacting with STAT5 or any other member of the STAT family, a gel-retardation experiment was performed. Nuclear extracts from mouse mammary gland on day 1 of lactation were incubated with either the STAT CEL 1 or the STAT CEL 2 oligonucleotides (Table 2). The result of this study indicated that there is specific binding of STAT proteins to the GAS site located between nt -1340 to -1332 (STAT CEL 2) (results not shown). The specificity of the interaction was demonstrated by the ability to efficiently compete for the interaction using a 200-fold molar excess of the specific com-

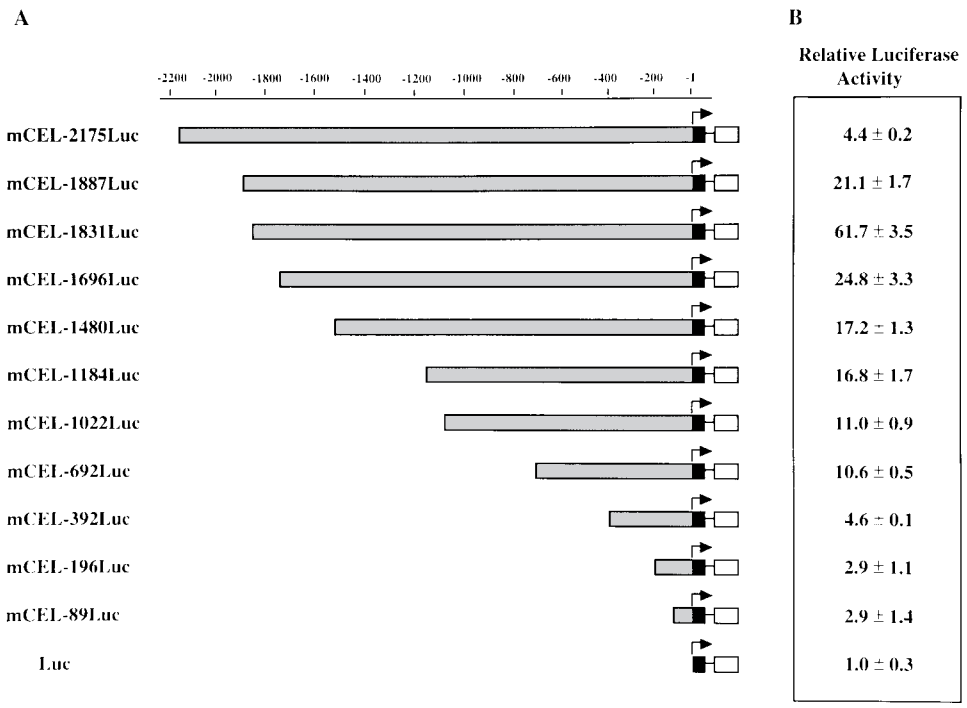


Figure 3 5′-deletion analysis of the mouse *CEL* gene promoter

HC11 cells were stably transfected with a series of mouse *CEL* gene promoter Luc constructs with different 5′-borders (**A**) and then subjected to treatment with either insulin/EGF or insulin/dexamethasone/prolactin. Since the Luc activity did not differ significantly between both hormone treatments, only the values representing the insulin/EGF treatment are shown (**B**). The values of Luc activities of the various constructs have been normalized to the activity of the Luc construct (taken as 1). Each value is the mean for two or three independent transfections.

petitor (oligonucleotide STAT CEL 2), while the same amount of an oligonucleotide containing two base substitutions within the GAS site (oligonucleotide STAT CEL 2 mut) was unable to compete (results not shown). Furthermore, a super-shift experiment using antibodies against STAT3, STAT5a, STAT5b and STAT6 indicated that the GAS-binding activity involves STAT5a and STAT5b (results not shown). To confirm further that it is STAT 5a/STAT 5b that interacts with STAT CEL 2, a gel-shift experiment was performed in which an oligonucleotide, including the GAS site derived from the bovine *β-casein* gene promoter, was used as the probe; a 200-fold molar excess of STAT CEL 2 was found to efficiently compete with the interaction of STAT5a/STAT5b (results not shown). These results indicate that STAT5a, as well as STAT5b, binds to one of the GAS sites in the *CEL* gene promoter.

Transfection studies of the *CEL* gene promoter in the mouse mammary-gland-derived cell line HC11

For further analysis of the STAT5 binding and identification of other regulatory elements important for the expression of the *CEL* gene in the mammary gland, a series of mouse *CEL* gene promoter–Luc reporter constructs with different 5′-borders (Figure 3) were analysed in the mouse mammary-gland-derived cell line, HC11 [25]. This cell line has been used for identification of important elements for the regulation of both the *β-casein* and the *WAP* genes [16,26].

The promoter constructs (Figure 3) were stably transfected into HC11 cells and then subjected to either EGF/insulin or the lactogenic hormones for 4 days. The Luc activity was measured, and a stepwise increase in Luc activity was obtained for constructs extending as far as −1831 nt, but when the promoter was further extended, a reduction in the Luc activity was observed (Figure 3). The same Luc activity was obtained with both hormone treatments for every construct, even the constructs including the STAT CEL 2 site. This suggests that binding of STAT5 is not important for the expression of the *CEL* gene. To confirm this further we analysed the expression of the *CEL* gene in STAT5a-deficient mice, which clearly demonstrated that the expression level of the gene is not influenced by the absence of STAT5a (results not shown).

The explanation for the marginal up-regulation by the lactogenic hormones could be that the induction is not a result of a direct hormonal effect, but rather reflects an indirect lactogenic hormone response resulting in an increase in the number of differentiated epithelial cells. To confirm this, we analysed the expression of keratin 19, which is considered to be a marker for differentiated epithelial cells [27]. To meet this aim, RNA from the explants was analysed with RT–PCR and results show that the increase in keratin expression correlates with that of the *CEL* gene. This is in contrast with the dramatic increase in *WAP* gene expression seen in explants treated with the lactogenic hormones, suggesting that the up-regulation of the *CEL* gene is a result of an increasing number of differentiated epithelial cells (Figure 4).

Evidence for an interaction between CTF/NF1 and a palindromic binding site

We next wanted to see if we could identify transcription factors that are important for the expression of the *CEL* gene. Results from the transfection assay suggest that the region between nt −1696 and −1831 includes *cis*-acting element(s) that is/are important for high expression of the *CEL* gene. Computer analysis of this region revealed that there are potential binding

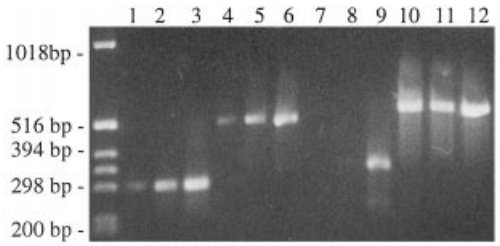
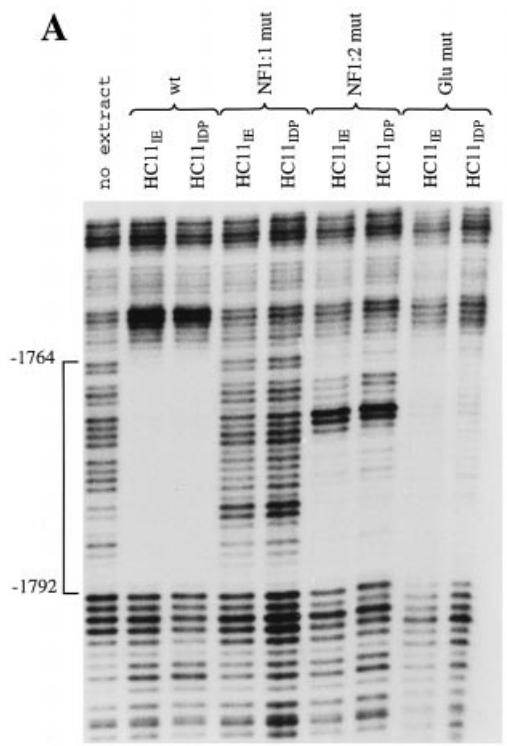


Figure 4 Comparison of the expression of the *CEL*, *keratin 19*, *WAP* and *GAPDH* genes in organ cultures of mammary glands

The endogenous expression of the *CEL*, *keratin 19*, *WAP* and *GAPDH* genes in organ cultures of mammary glands treated with either insulin/EGF (lanes 1, 4, 7 and 10) or insulin/EGF/dexamethasone (lanes 2, 5, 8 and 11) or insulin/dexamethasone/prolactin (lanes 3, 6, 9 and 12) was measured. The expression level of the four genes was estimated using RT–PCR, and the sizes of the amplified fragments were 309 bp of CEL cDNA (lanes 1–3), 490 bp of keratin 19 cDNA (lanes 4–6), 349 bp of WAP cDNA (lanes 7–9) and 554 bp of GAPDH cDNA (lane 10–12) respectively.

sites for several different transcription factors, such as CTF/NF-1, C/EBP-β, Oct-1 and GR respectively. To investigate if any of these are interacting within this region, a DNase I footprinting experiment was carried out. Nuclear extracts from HC11 cells treated with either insulin/EGF or insulin/dexamethasone/prolactin were included in the different footprint reactions. One distinct footprint (F1; Figure 5A)was observed in both reactions, and it extended from nt −1792 to −1764, which includes specifically the potential CTF/NF-1 binding site and one of the GR half-sites described previously. To demonstrate whether the protein(s) bind(s) to the potential CTF/NF-1 site or to the GR half-site, nt −1480/−1831 fragments containing the three different mutations NF1:1 mut, NF1:2 mut and Glu mut (Figure 5B) were included in the footprint reactions. When the fragment containing the mutation in the first part of the CTF/NF-1 site (−1480/−1831 NF1:1 mut) was used, the protein–DNA interaction was totally prevented, as shown by the loss of protection (Figure 5A). In the reactions containing the fragment with mutations in the second part of the CTF/NF-1 site (NF1:2 mut) there was, however, still an interaction, but the protected region was slightly extended from nt −1792 to −1776, which includes the first part of the partially palindromic sequence (Figure 5A). Also, it can be seen that the protein interaction with this sequence is not as strong as it is to the wild-type sequence. In the reaction containing the oligonucleotide with the mutation in the GR-binding site, the original footprint F1 is formed, but it seems as if the interaction is weaker with this DNA sequence than with the wild-type sequence (Figure 5A). Hence it is possible that the bases between the two partially palindromic sequences are important for strong interaction. These different footprint reactions show that it is a CTF/NF-1 member(s) that is responsible for the interaction.

The F1 region was further analysed with a gel-retardation experiment with nuclear extracts from the HC11 cells, together with a labelled oligonucleotide, corresponding to the F1 region (Figure 5B). Multiple protein–DNA interactions were observed in the extracts from insulin/EGF-treated HC11 cells and in extracts from HC11 cells treated with insulin/dexamethasone/prolactin (Figure 6). These multiple complexes observed using EMSA are characteristic of the gel-retardation pattern exhibited by CTF/NF1 in mammary gland, in view of its known heterogeneity [28]. The specificity of these interactions was demonstrated by the ability to compete efficiently for the interactions



B

5'-GTTCTGCT**TGGC**GTGTT**ATCAA**GGTTGCT-3'	F1 (wt)
5'-GTTCTGCT**Tata**GTGTT**ATCAA**GGTTGCT-3'	NF1:1 mut
5'-GTTCTGCT**TGGC**GTGTT**ATgcA**GGTTGCT-3'	NF1:2 mut
5'-GTTCTGCTTGGCGT**acg**ATCAAGGTTGCT-3'	Glu mut
5'-CTGGGAAAAAATGGACCATCCCACAC-3'	Unspecific
5'-TGTTCTTT**TGGA**ATTTA**TCCA**AATCTTAT-3'	NF-1 MMTV
5'-CACGTCG**TGGCA**CATCGTAGCCT-3'	NF-1 half site

Figure 5 DNase I footprinting of the mouse *CEL* gene promoter

(**A**) The footprinting reactions were carried out with a *CEL* gene promoter fragment comprising either the region from nt −1831 to −1480 (wt) or nt −1480/−1831 fragments containing one of the three mutations, NF1:1 mut, NF1:2 mut or Glu mut respectively. Included in the different reactions were nuclear extracts from HC11 cells treated with either insulin/EGF ($HC11_{IE}$) or insulin/dexamethasone/prolactin ($HC11_{IDP}$). The footprints obtained in the reactions containing the wt and the Glu mut fragments are indicated by an extended square bracket. The footprint in the NF1:2-containing reaction is not marked, but extends from nt −1792 to −1776. (**B**) The upper-strand sequence of the footprint region (F1), the mutated sequences NF1:1 mut, NF1:2 mut and Glu mut, as well as the sequences of the oligonucleotides included in the gel-retardation experiment (Figure 6), are shown. The partially palindromic CTF/NF1-binding sequence is shown in bold and the half-GR binding sequence is underlined. Mutated sequences are shown in bold lower-case letters. The CTF/NF 1 half-site is also shown in bold.

using a 200-fold molar excess of the specific competitor [oligo F1 (wt)], while the same amount of a non-specific oligonucleotide was unable to compete (Figure 6). Furthermore, we have shown that these CTF/NF-1 complexes have a greater affinity for a palindromic binding site than a half-palindromic site, since an oligonucleotide containing a palindromic CTF/NF-1-binding site derived from the murine-mammary-tumour virus long terminal repeat [29] completely out-competed the protein–DNA interactions, whereas a half-palindromic binding site was unable to compete (Figure 6).

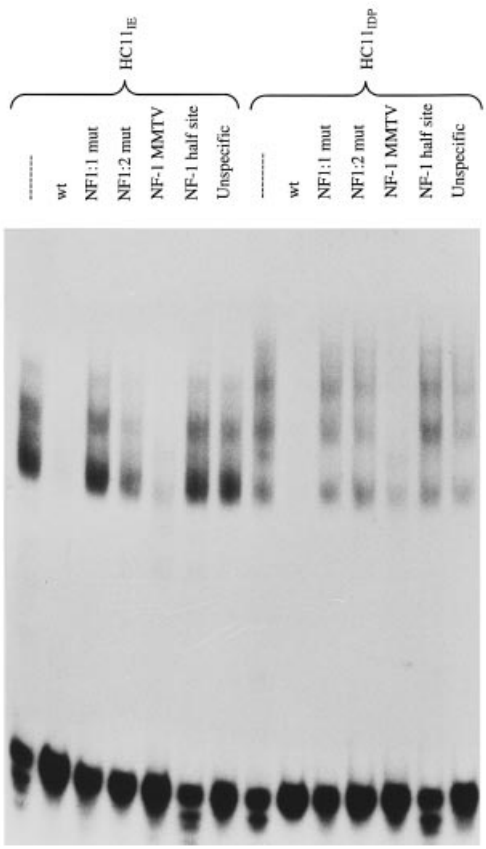


Figure 6 The interacting CTF/NF1 complexes have higher affinity for a palindromic-binding site than for a half-palindromic site

Gel-retardation analysis using the F1 (wt) oligonucleotide as probe; nuclear extracts were obtained from HC11 cells treated with either insulin/EGF or insulin/dexamethasone/prolactin. A 200-fold molar excess of various competitor oligonucleotides were added to the reaction mixtures, as indicated above the different lanes.

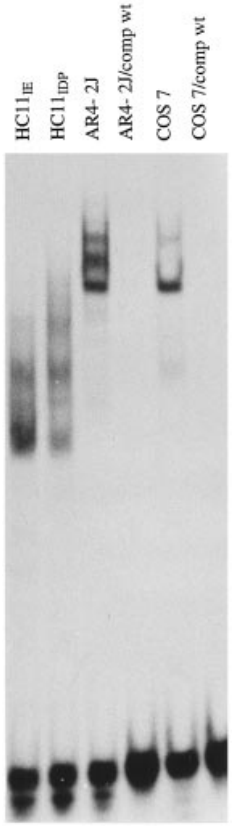


Figure 7 Interacting CTF/NF1 complexes in AR4-2J and COS-7 cells compared with those in HC11 cells

Gel-retardation analysis using the F1 (wt) oligonucleotide as probe; nuclear extracts from HC11 cells treated with either insulin/EGF or insulin/dexamethasone/prolactin, AR4-2J cells or COS-7 cells were assayed. A 200-fold molar excess of unlabelled F1 (wt) was included as indicated.

Table 3 Effect of binding of CTF/NF1 and *CEL* gene expression in different cells

	Relative Luc activity		
Different constructs	HC11 cells	AR4-2J cells	COS-7 cells
Mouse CEL −1831 Luc	61.7 ± 3.5	41.1 ± 3.3	3.5 ± 0.3
Mouse CEL −1831 NF1:1 mut Luc	21.0 ± 4.0	39.6 ± 6.5	3.3 ± 0.2
Luc	1.0 ± 0.3	1.0 ± 0.2	1.0 ± 0.4

CTF/NF-1 is required for mammary gland expression, but not for that in pancreas

To determine if binding of CTF/NF-1 complexes is required for *CEL* gene promoter activity in mammary gland, the mutation corresponding to the first part of the CTF/NF-1 site was introduced into the nt −1831 Luc construct. The functional activity of this mutant was examined in the HC11 cells. The activity was reduced to the same level as that of the construct nt −1696, showing that the CTF/NF-1 complexes are responsible for the increase in activity (Table 3).

Since the *CEL* gene is mainly expressed in mammary gland and pancreas, we wanted to determine if CTF/NF1 interactions are also involved in the regulation of the *CEL* gene in pancreas. Hence the footprint experiment was repeated with nuclear extracts from the rat pancreatic cell line, AR4-2J, that expresses the *CEL* gene [30], as well as from the non-*CEL*-gene-expressing African green monkey kidney cell line, COS-7. It was found that an identical footprint was formed in extracts from both cell lines, even though the interaction in the COS-7 cell extract is much weaker in comparison with the interactions obtained in the other two cell lines, HC11 and AR4-2J respectively (results not shown). Since the CTF/NF-1 family consists of many different CTF/NF-1 forms that interact with a similar binding site, it was interesting to investigate whether the same CTF/NF-1 form(s) is/are involved in the interaction in different tissues. This was examined by using EMSA containing the labelled oligonucleotide F1 and nuclear extracts from the three cell lines. As shown in Figure 7, the complexes formed in the reactions containing AR4-2J and COS-7 extracts are totally different as compared with those formed in the HC11 extracts.

The effect of CTF/NF-1 binding was then examined in both AR4-2J and COS-7 cells, by transient transfection of the −1831 mouse CEL Luc and −1831 NF1:1 mut Luc constructs. Because the mutation in the first part of the CTF/NF-1-binding site did not affect the Luc activity at all in either the COS 7 or AR4-2J cells (Table 3), this suggests that binding of CTF/NF-1 is

important for *CEL* gene expression in mammary gland, but not in pancreas.

DISCUSSION

The mammary gland is an useful model system for studying developmental biology. During development, specific genetic programmes are activated by transcription factors that are often both developmental- and tissue-specific in their distribution. To date, very little is known about the genes that determine the lineage, differentiation characteristics and growth potential of mammary epithelial cells.

As nothing was known about *CEL* gene expression in mammary gland, we analysed the steady-state level of CEL mRNA transcript in this organ during pregnancy and lactation, and compared the results with the steady-state levels of mRNA transcripts of two other milk genes, those of WAP and the β-casein. This analysis revealed that *β-casein* gene expression is activated at day 10 of pregnancy, and that the other two genes, *CEL* and *WAP*, were expressed at day 14. Even though the *CEL* and *WAP* genes are apparently induced at about the same time during mammary gland development, the 'lactogenic-hormone-dependent' regulation of these genes is different. The organ-culture assay revealed that the level of *CEL* gene is only increased 10-fold by the lactogenic hormones, in comparison with *WAP* gene expression, which is induced approx. 1000-fold. The fact that this high level of *WAP* gene expression is due to the activation of STAT5a by prolactin has been shown recently in STAT5a-deficient mice, where the *WAP* levels are reduced to 10% [24]. However, transfection studies in HC11 cells and analysis of the expression of *CEL* gene in STAT5a-deficient mice revealed that this gene is not directly affected by the activation of STAT5 isoforms, even though there are interactions between these factors and one of the GAS binding sites in the promoter. This demonstrates that STAT5 activation by prolactin is not involved in a general and direct activation of milk genes. The differences in the regulatory mechanisms of these milk genes might be explained by the sugggestion that STAT5 activation by prolactin is only important for milk protein genes that are highly expressed. Therefore the up-regulation of the *CEL* gene is suggested not to be the result of a direct hormonal effect, but instead is merely reflecting the increased number of differentiated epithelial cells.

In this study we have identified a transcription factor that is important for the expression of the *CEL* gene in mouse mammary gland and this factor is a member of the CTF/NF-1 family. It has been reported previously [31–33] that members of this family are involved in the regulation of other milk genes. Apparently, different forms exist in the mammary gland: one that recognizes a half site and one that recognizes a palindromic site. The half-site-recognizing form has been shown to bind to both sheep *β-lactoglobulin* [31] and rat *WAP* [32] gene promoters, where its binding site is located in close proximity with other important factors, such as STAT5, GR and the mammary activation factor, MAF [33]. The other form, binding to a palindromic site, has also been implicated in the regulation of the *WAP* gene [32]. Li and Rosen [32] reported that interaction between CTF/NF-1 and a palindromic site plays a significant role in determining the overall activity of the *WAP* gene *in vivo*, since mutation of this site totally abolished the expression.

In this paper both gel-retardation and footprint experiments revealed that the CTF/NF-1 complexes involved in the activation of *CEL* gene expression bind to a palindromic CTF/NF-1 site. Furthermore, by performing the same experiment with nuclear extracts from a pancreatic cell line that also expresses the gene, we could show that mammary-gland-specific CTF/NF-1 complexes seem to exist, since protein–DNA interactions observed in the extract from the pancreatic cell line are totally different compared with the interactions observed in extracts from the HC11 cells. It is not known whether these complexes are composed of different CTF/NF-1 family members expressed in the two tissues, or whether the complexes reflect tissue-specific combinations. However, the formation of these complexes is only important for *CEL* gene activity in the mammary gland, since it does not affect the expression of this gene in cells of pancreatic origin. Kumar et al. [34] have shown that a CTF/NF-1 complex, interacting with a palindromic site, might be responsible for cell-specific expression in a paper that describes the importance of a palindromic CTF/NF-1-binding site for glial-cell-specific expression of the human JC virus. Furthermore, a report describing the involvement of CTF/NF-1 in the epithelial specificity of the human papilloma virus type 16 enhancer lends support to the hypothesis that CTF/NF-1 proteins might vary in a cell-specific manner as a function of their differential expression, splicing or heterodimerization [35].

Before these studies were carried out, different factors involved in milk protein gene expression had been identified; however, no tissue-specific factor had been found. The most likely hypothesis is that tissue specificity is not controlled by either a single factor or a family of factors that are expressed exclusively in mammary epithelial cells, but by multiple factors whose co-operative interactions dictate the specific pattern of expression. In this work, we have identified such a factor. We have shown that a CTF/NF-1 complex is important for the tissue-specific activation of the *CEL* gene in mammary gland. Future studies, also involving transgenic animals, will be designed to identify which form(s) of the CTF/NF-1 family are involved in the regulation of the *CEL* gene.

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