Journal of Cellular Biochemistry 95:313–327 (2005)

Regulation by the Extracellular Matrix (ECM) of Prolactin-Induced αs1-Casein Gene Expression in Rabbit Primary Mammary Cells: Role of STAT5, C/EBP, and Chromatin Structure

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Abstract The aim of the present study was to understand how the extracellular matrix (ECM) regulates at the gene level the prolactin (Prl)-induced signal transducer and activator of transcription 5 (STAT5)-dependent expression of the αs1-casein gene in mammary epithelial cells. CCAAT enhancer binding proteins (C/EBPs) are assumed regulators of β-casein gene expression. Rabbit primary mammary cells express αs1-casein gene when cultured on collagen and not on plastic. Similar C/EBPβ, C/EBPδ, STAT5, and Prl-activated STAT5 were found under all culture conditions. Thus the ECM does not act through C/EBPs or STAT5. This was confirmed by transfections of rabbit primary mammary cells by a construct sensitive to ovine prolactin (oPrl) and ECM (6i TK luc) encompassing STAT5 and C/EBP binding sites. The mutation of C/EBPs binding sites showed that these sites were not mandatory for Prl-induced expression of the construct. Interestingly, chromatin immunoprecipitation by the anti-acetylhistone H4 antibody (ChIP) showed that the ECM (and not Prl) maintained a high amount of histone H4 acetylation upstream of the αs1-casein gene especially at the level of a distal Prl- and ECM- sensitive enhancer. Alpha6 integrin (a membrane receptor of laminin, the principal active component of the mammary ECM) was found at the surface of cells cultured on collagen but not on plastic. In cells cultured on collagen in the presence of anti-α6 integrin antibody, Prl-induced transcription of the endogenous αs1-casein gene was significantly reduced, without modifying C/EBPs and STAT5. Besides, histone H4 acetylation was reduced. Thus, we propose that the ECM regulates rabbit αs1-casein protein expression by local modification of chromatin structure, independently of STAT5 and C/EBPs. J. Cell. Biochem. 95: 313–327, 2005.

Key words: extracellular matrix; αs1-casein gene expression; chromatin immunoprecipitation; histone acetylation; STAT5; C/EBP; rabbit mammary cell

Milk protein gene expression is regulated by lactogenic hormones (mainly prolactin (Prl) and glucocorticoids) and by the extracellular matrix (ECM). The ECM delivers signals that allow lactogenic hormones to induce milk protein gene expression. Numerous experiments have been performed to elucidate the interactions between lactogenic hormones and ECM transduction pathways. However, controversial or confusing points remain. In mouse primary mammary cells, it was shown that the signal transducer and activator of transcription 5 (STAT5), one of the transcription factors activated by Prl [see for review Hennighausen, 1997; Rosen et al., 1999], was activated by Prl in the presence of EHS (Engelbroth, Holm, Swarm) ECM and not in its absence [Edwards et al., 1998]. However, in rabbit primary mammary cells, others reported that STAT5 was activated in cells cultured both on plastic and on thick floating collagen gel [Tourkine et al., 1995; Jolivet et al., 2001]. Thus STAT5

Abbreviations used: C/EBP, CCAAT enhancer binding protein; ChIP, chromatin immunoprecipitation; ECM, extracellular matrix; EMSA, electrophoretic mobility shift assay; STAT5, signal transducer and activator of transcription 5; Prl, prolactin; oPrl, ovine prolactin.

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Received 25 May 2004; Accepted 9 November 2004
DOI 10.1002/jcb.20397

was identified as a target for the ECM in mouse primary mammary cells only. In rabbit primary mammary cells, the ECM modified transcription factors of the family of CCAAT enhancer binding proteins [Jolivet et al., 2001]. C/EBPβ and especially its binding to DNA binding sites differed in cells cultured on thick collagen gels or on plastic. This led the authors to assume that C/EBPβ was a putative target for ECM. Independently, it was observed that STAT5 isoforms with high molecular weights were detected in cells cultured on collagen, whereas STAT5 with both high and low molecular weights were detected in cells cultured on plastic. Low molecular weight STAT5 isoforms were never detected in cells cultured on collagen. The origin and role of the different STAT5 isoforms with low molecular weights were not elucidated [Jolivet et al., 2001].

In order to clarify the role of ECM in Prl-induced STAT5-dependent casein gene expression at the level of the gene, we re-assessed the effect of the ECM on STAT5 and C/EBP by studying nuclear (NE) and cytosolic extracts (CE) prepared from rabbit primary mammary cells cultured on plastic or on collagen with or without ovine prolactin (oPrl) and by transfection in primary mammary cells of a plasmid 6i TK luc encompassing a Luciferase gene driven by an artificial promoter-enhancer [Pantano et al., 2002] including mutations of STAT5 or C/EBP binding sites. In the present study, to limit cellular protease activity which has been suspected to generate multiple forms of C/EBPs during extraction procedures [Welm et al., 1999; Baer and Johnson, 2000; Dearth et al., 2001], we modified our protocols by using additional protease inhibitors.

Accumulated evidences indicate that the activation of a gene is a multistep process including chromatin opening and formation of an active transcription complex. A few studies suggest that the ECM might contribute to modify chromatin structure. It had previously been reported that the total cellular level of acetylated histones differed in mouse mammary cells cultured on EHS or plastic. In the mouse mammary epithelial cell line Eph4 [Reichmann et al., 1989], the presence of a basement membrane induced widespread chromatin deacetylation [Pujuguet et al., 2001]. In addition, tricostatin A, a histone deacetylase inhibitor, prevented prolactin-induced transcription of the β-casein gene, and increased total histone acetylation. This suggested that the ECM regulates histone acetylation, which in turn modulates β-casein gene transcription. In the present paper, we studied whether local modifications of histone acetylation occur by comparing the level of acetylated histone H4 specifically in the promoter region of the αs1-casein gene in rabbit primary mammary cells cultured on plastic or floating collagen, with or without lactogenic hormones.

Finally, to confirm our findings and simultaneously to investigate further the ECM pathway, we tried to block the ECM activity. In the mammary gland of rodents, laminin is the component of the ECM, which binds to specific membrane receptors (beta 1 and alpha 6 integrins) [Streuli et al., 1995a]. This binding is necessary for Prl to induce casein gene expression [Streuli et al., 1991; Roskelley et al., 1995; Klinowska et al., 1999; Muschler et al., 1999]. In the rabbit primary mammary cells, we attempted to interrupt this pathway by blocking the activity of α6 integrin using a specific function-blocking antibody previously studied by others [Muschler et al., 1999].

MATERIALS AND METHODS

Plasmids

The pGL3 6i TK plasmid (6i TK luc) contains six copies in tandem of the distal enhancer "i" (−3,442, −3,285) of the rabbit αs1-casein gene, added upstream of the TK gene promoter linked to the firefly luciferase (luc) reporter gene in the pGL3 plasmid (Promega, Charbonnieres-les-bains, France) [Pantano et al., 2002]. Mutations were introduced in the STAT5 or C/EBP binding sites present in the "i" enhancer [Jolivet et al., 2001] by PCR using mutated oligonucleotides (the sequence of the sense strand is indicated, with the mutated nucleotides in italic letters: mSTAT5 5′-GATACTTT***G***TTA***C***AAAATTCATGGCG-3′, mC/EBP 5′-TATTCATTAT***C***T***GT***TAGAAAG-3′). Six copies in tandem of the mutated "i" enhancer were then cloned upstream of the TK luciferase construct in pGL3 TK [Pantano et al., 2002], generating the 6i mSTAT5 TK luc and 6i mC/EBP TK luc plasmids.

Cell Cultures, Transfection, and Hormonal Induction

Rabbit primary mammary cell culture. Organoids were isolated from the mammary gland of 14- to 16-day pregnant rabbits, and isolated mammary cells were prepared as

previously described [Devinoy et al., 1991; Jolivet et al., 2001]. Organoids are clusters of mammary cells including ductal and alveoli cells. Isolated mammary cells were prepared from 4-day-cultured organoids treated by trypsin. The isolated cells were seeded on plastic or on thick rat-tail collagen gel at a high density in order to be confluent the following day (mammary epithelial cells undergo no cell division under these culture conditions and the number of cells remains essentially constant over the next 2 days). To maintain the full differentiation of cells, the collagen was gently detached from the bottom of the plate to allow the gel to float.

Transfection and hormonal induction. Transfections were performed as previously described with cells seeded in 35-mm-diameter dishes [Pantano et al., 2002]. Rabbit primary mammary cell transfections were performed on confluent cells seeded the previous day on plastic or on thick collagen gels using 4 μg of luciferase gene expressing plasmid, 1 μg of pCMV-βgal (Pharmacia), and 10 μg of Lipofectin (Invitrogen, Cergy Pontoise, France). Cells were incubated overnight in the presence of a plasmid-Lipofectin mixture in OPTIMEM medium (Invitrogen). The next morning, the transfection medium was discarded and fresh medium containing DMEM/F12, 2% Ultroser, 1 μg/ml insulin, with or without cortisol (100 ng/ml) and with or without oPrl (1 μg/ml) was added. The collagen was detached from the dishes and allowed to float. Luciferase and β-galactosidase assays were performed 48 h later using cell extracts.

Total RNA Extraction and Northern Blot Analysis

Total RNA extraction was carried out using the SV Total RNA isolation system (Promega). Northern blot analyses were performed as previously described [Bayat-Sarmadi et al., 1995]. Rabbit αs1-casein mRNA was detected after transfer onto nylon membranes and hybridization with a ^{32}P-labeled cDNA probe (DNA fragment extending from nucleotide (nt) 48 to 1,130, corresponding to almost the full-length cDNA [Devinoy et al., 1988]).

Antibodies

Anti-STAT5a and STAT5b mouse monoclonal antibodies (Zymed, San Francisco, CA) were used to detect STAT5a and STAT5b, respectively. The rabbit polyclonal antibody (C-17) (Santa Cruz Biotechnology, Santa Cruz, CA) reacted with both STAT5a and STAT5b ("total STAT5" antibody). The rabbit polyclonal antibody directed against phosphorylated Tyr 694 of STAT5 (Upstate, Lake Placid, NY) reacted with both phosphorylated STAT5a and STAT5b (antibody to PY STAT5). Rabbit polyclonal antibodies directed against C/EBPβ (C-19) and C/EBPδ (C-22) were obtained from Santa Cruz Biotechnology. As explained previously [Jolivet et al., 2001], no antibody directed against C/EBPα is available to study this protein in the rabbit. Monoclonal anti-heterochromatin protein-1α (HP1α) was purchased from Euromedex (Mundolsheim, France). Anti-acetylated histone H4 directed against a tetra-acetylated N-terminal peptide was obtained from Upstate (chromatin immunoprecipitation (ChIP) grade antibody). Rat monoclonal antibody against integrin α6 chain (clone GoH3) and hamster monoclonal antibody against integrin β1 chain (clone Ha2/5) were purchased from PharMingen (Becton Dickinson). Secondary peroxidase-conjugated antibodies (sheep anti-mouse IgG and goat anti-rabbit IgG) were obtained from Sigma (Saint Quentin Fallavier, France). The secondary antibody Texas red dye-conjugated anti-rat IgG was obtained from Jackson ImmunoResearch (Intuchin Montluqon, France).

Preparation of Nuclear Extracts

Nuclear (NEs) and cytosolic extracts (CEs) were prepared from primary mammary cells, as previously described [Jolivet et al., 1996, 2001]. A cocktail of protease inhibitors was added to the previously used protease inhibitors (such as PMSF and benzamidine) in all buffers (Complete protease inhibition cocktail, Roche Diagnostics; four tablets per 100 ml).

Electrophoretic Mobility Shift Assay

Electrophoretic mobility shift assays (EMSAs) were performed as previously described [Jolivet et al., 2001] with modifications concerning F3-C/EBP binding analysis, i.e., NEs from all samples were diluted extemporaneously twofold before incubation in a 2% bovine serum albumin/PBS (phosphate buffer saline) solution. The sequences of the probes were: αs1cas-STAT5 5′-GAGAATTCTTAGAATTTAAA-3′ corresponding to the (−104, −85) fragment of

the rabbit αs1-casein gene, previously reported as a STAT5 binding site in the proximal promoter of the rabbit αs1-casein gene [Jolivet et al., 1996]); F3-C/EBP 5′-TATTCATTATGTAATAGAAAG-3′ corresponding to the (−3,352, −3,333) fragment of the rabbit αs1-casein gene, previously described as a C/EBP binding site [Jolivet et al., 2001]. In supershift experiments, 1 μg of antibody was added to the incubation mixture with the NE or CE. Labeled complexes and free oligonucleotides were separated by electrophoresis on non-denaturing 5% acrylamide/bis-acrylamide gels. Gels were pre-run overnight at a low voltage (50 V) before samples were loaded. After electrophoresis, gels were transferred onto DE81 paper, dried and exposed for autoradiography.

Western Blot Analysis

Western blot analysis of nuclear or cytosolic extracts was performed as previously described [Jahn et al., 1997]. Antibodies were used at a final concentration of 1 μg/ml and incubation was performed overnight at 4°C. After washing the blots several times as previously indicated, the corresponding secondary antibody (anti-mouse or anti-rabbit peroxidase conjugate) was incubated for 1 h at room temperature (anti-rabbit IgG (1:15,000) or anti-mouse IgG (1:2,000)). The blots were developed with ECL reagent (Amersham). In order to avoid possible cross-reactions with the secondary antibodies, and to limit the presence of residual bands between successive hybridizations, blots were treated with the primary antibodies in the following order: PY STAT5, STAT5a, STAT5b, and finally total STAT5.

Immunohistological Procedures

Primary mammary cells seeded on coverslips at the usual density, or fragments of collagen gel containing the seeded primary mammary cells, were incubated for 20 min at room temperature in paraformaldehyde (2.5% in PBS). After several washes in PBS, cells were treated with NH4Cl (50 mM in PBS), washed several times, permeabilized with saponine (0.05% saponine, 2% BSA in PBS), and incubated overnight with the primary antibody α6 integrin antibody GoH3 or β1 integrin Ha2/5 (1 μg/ml, in saponine, 2% BSA in PBS buffer). After several washes in PBS containing saponine (0.05%) and BSA (0.2%), cells were incubated for 1 h with the secondary antibody Texas red dye-conjugated anti-rat IgG at room temperature. Washed samples were treated with DAPI to stain the nuclear DNA.

Chromatin Immunoprecipitation Assay

The ChIP assay was performed according to the method described in the ChIP assay kit for acetylhistone H4 (Upstate). Prior to harvest, primary mammary cells were treated (or not) with oPrl (1 μg/ml in the culture medium) for 15–30 min. Cross-linking and subsequent steps of chromatin extraction were performed as indicated. The samples were sonicated to reduce DNA length to 0.2–1 kb. The DNA concentration of sample was assayed using a Hoechst DNA Assay in a Labsystems fluorimeter (Fluoroscan II). Preparation of cross-linked chromatin from tissue samples (mammary gland and leg muscle) was performed as for cultured cells after rapid dissociation of tissues into small pieces immediately after sampling.

Aliquots containing approximately 10 μg of DNA were used for each immunoprecipitation. Diluted cross-linked samples were pre-cleared with Salmon Sperm DNA/Protein A Agarose-50% slurry (Upstate) for 1 h at 4°C under agitation as described in Upstate protocol. Agarose was pelted by centrifugation and the supernatant collected. An aliquot was retained as input starting (IS) material to quantify the amount of target DNA present in each sample. The antibody directed against acetylated histone H4 (anti-AcH4, 5 μg) was added to a second aliquot and incubation was performed overnight at 4°C under rotation. For a negative control (NC), a no-antibody incubation was performed simultaneously on a third aliquot. The antibody/histone complex was collected in anti-AcH4 treated and NC aliquots by salmon sperm DNA/protein A agarose under rotation at 4°C for 1 h and centrifugation. Beads were washed with low salt, high salt and LiCl washing buffers as described in the ChIP protocol from Upstate. Elution was carried out in elution buffer (1% SDS, 0.1 M $NaHCO_3$) under agitation, and the cross-linking was reversed by heating for 4–20 h at 65°C after the addition of NaCl (0.3 M final concentration) in the presence of DNase-free RNase A (1 μg). DNA was recovered after proteinase K treatment for 1 h at 45°C, followed by phenol/chloroform extraction, and ethanol precipitation. DNA pellets were solubilized in 50 μl of water.

Real-Time PCR and Data Analysis

Real-time PCR was performed on 2–4 μl DNA samples using a ABIPrism 7700 sequence detector and qPCR Mastermix for SybrGreenI (Eurogentec, Angers, France). Real-time PCR was carried out in duplicate at 50°C for 2 min and 95°C for 10 min, followed by 45 cycles of 95°C for 15 s and 60–65°C for 1 min. The temperature of annealing and the $MgCl_2$ concentration in the PCR incubation mixture were optimized for each set of primers. Data were collected at 60 or 65°C. For each sample, five target sequences were assayed in the anti-AcH4 immunoprecipitated (IP) fraction, in the input sample (IS), and in the negative control (NC). Data analysis was performed as described [Litt et al., 2001]. The efficiency of immunoprecipitation was determined by dividing the amount of target sequence in the IP fraction by the amount of target sequence in the input sample (IS). This ratio was calculated by $IP/IS = 2^{(CtIS - CtIP)}$, where CtIS and CtIP represent the cycle threshold number (Ct) given by the sequence detector for the IS and IP fractions, respectively (for details concerning this formula see [Litt et al., 2001]). With the NC fraction, a ratio for nonspecific immunoprecipitation was calculated: $NC/IS = 2^{(CtIS - CtNC)}$. The ratio for specific immunoprecipitation was finally obtained by subtracting the NC/IS ratio from the IP/IS ratio.

Primers were selected from the sequence of the 5′ upstream region of the rabbit αs1-casein gene (GenBank admission number AY284844) using PE Applied Biosystems Primer Express software. As controls, sets of primers were devised around the transcription start point (tsp) of the rabbit alpha myosin heavy chain gene (GenBank accession number AF192305) and the tsp of the rabbit beta blobin gene (GenBank accession number VOO882) (Table I).

RESULTS

STAT5 and C/EBP Transcription Factors Are not Modified by the ECM

To assess the effect of the ECM on STAT5 and C/EBPs in mammary epithelial cells, nuclear and cytosolic extracts were prepared from primary rabbit mammary cells plated as organoids on plastic then isolated by trypsin treatment and seeded on plastic or thick floating collagen gel. As shown previously using mouse mammary cells [Streuli and Bissel, 1990], it is probable that rabbit mammary cells cultured on thick floating rat tail collagen gel synthesized their own ECM, that is a mixture of numerous factors, including laminin. The floating collagen gel system which itself contains no laminin or other factors proved appropriate to study the effects of this endogenously synthesized basement membrane on Prl-induced expression of the αs1-casein gene. Indeed, αs1-casein gene expression was induced by oPrl in cells cultured on floating collagen but not in cells cultured on plastic [Jolivet et al., 2001].

When using the F3-C/EBP probe and NEs in EMSAs, the same retarded complexes were observed in all culture conditions (Fig. 1A). C/EBPβ and δ were detected by supershift with C/EBPβ or δ antibodies. In Western blots experiments, the 48- and 42-kDa C/EBPβ proteins, and the 35-kDa C/EBPδ protein were similar in all NEs prepared from cells cultured either on plastic or on collagen (Fig. 1B). The 20-kDa protein (identified as LIP, one previously

TABLE I. Sequences of Primers Used in Real-time PCR

Rabbit αs1-casein	Position from the tsp	
Sense	−3,434; −3,422	5′-GACTCTGTTGTGAACTGTCTCAGGTTC-3′
Reverse	−3,300; −3,282	5′-GCTAGGACATTCGATAAAACGTTCATG-3′
Sense	−3,258; −3,234	5′ATGAACCTCTGAATTCTTGACTGATAC-3′
Reverse	−3,159; −3,132	5′-AAGCTATCATGTGAAGAGATAAAATTTTAC-3′
Sense	−2,373; −2,351	5′-CTCTCATCGCATATTGGAGTGCC-3′
Reverse	−2,297; −2,274	5′-GGAACCTGGACTACCATCTGTTGC-3′
Sense	−177; −151	5′-CACTCCCTTGTTGAAAACTCTCCTCAG-3′
Reverse	−80; −53	5′-ATTTTGTGGTTTCAGATCAACCAATAGG-3′
Rabbit β-globin		
Sense	−13; +8	5′-GCAGCTGCTGCTTACACTTGC_3′
Reverse	+68; +88	5′-ACCGCAGACTTCTCCTCACTG-3′
Rabbit α-myosin		
Sense	−59; −39	5-GGCTCCAAATTTAGGCAAGGG-3′
Reverse	+11; +32	5′-GCACAAACCACACTTACCTGGG-3′

Numbers refer to the position of primers versus the transcription start point of the rabbit αs1-casein gene (GenBank admission number AY284844), the rabbit β-globin gene (GenBank admission number V00882), and the rabbit α-myosin gene (GenBank admission number AF192305).

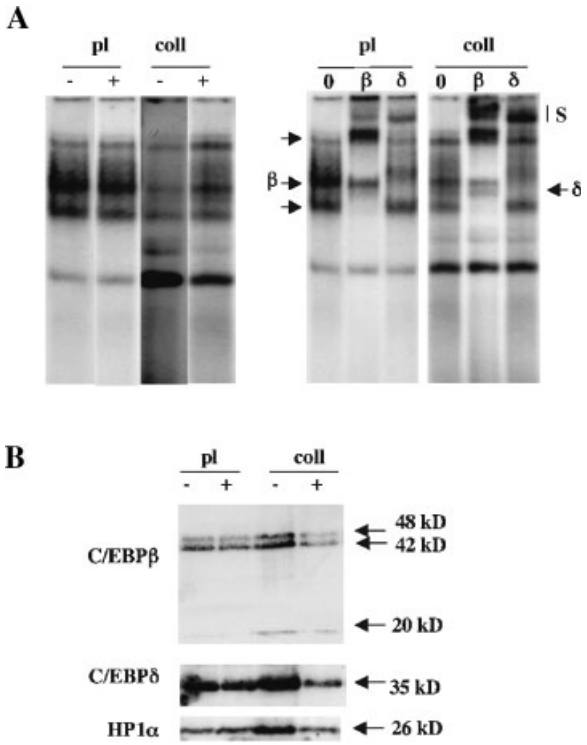

Fig. 1. Characterization of C/EBPβ and δ in NEs from isolated rabbit primary mammary cells cultured on plastic or collagen. Electrophoretic mobility shift assays (EMSAs) were performed with F3-C/EBP as probe and NEs prepared from cells cultured on plastic (pl) or collagen (coll), without cortisol from the beginning of culture. The same amount of nuclear proteins was used in each assay (from 3 to 10 μg, depending on each culture). Autoradiographies were representative of seven independent cultures. **A**, **left panel**: Seven days after mammary gland dissection, isolated cells were treated (+) or not (−) with ovine prolactin (oPrl, 1 μg/ml) for 15 min before NEs preparation. **Right panel**: Supershifts carried out by adding C/EBPβ (β) or C/EBPδ (δ) antibody to the incubation mixture. (0) No-antibody reaction. NEs were prepared from cells cultured without oPrl and without cortisol. Supershifted complexes (S) were visible at the top of the gel. The position of C/EBPβ and δ including complexes (black arrows) was deduced from supershit experiments. **B**: Identification of C/EBPβ and δ by Western blots in NEs from mammary cells cultured on plastic or collagen. Twenty micrograms of nuclear proteins were fractionated by SDS–12% PAGE, transferred to a nitrocellulose membrane and probed with C/EBPβ or δ antibodies. The molecular weight of proteins deduced from migration rates of standards is indicated. As control for the loading of samples, the hybridization obtained with the HP1α antibody is presented.

characterized isoforms of C/EBPβ [Jolivet et al., 2001]) was hardly visible. Similar data were obtained in EMSAs and in Western blots using cells cultured with and without cortisol (not shown). When CEs were used instead of NEs, no retarded complexes were detected in EMSAs and no significant proteins were labeled in Western blots (data not shown), indicating that the nuclear-cytoplasmic distribution of C/EBPs was not modified by the culture conditions. Thus, these data clearly indicate that the ECM did not modify C/EBPs in rabbit primary mammary cells.

When using the αs1cas-STAT5 probe and NEs in EMSA, one retarded complex was characterized in all samples after oPrl stimulation (Fig. 2, lane 0). It was not observed in samples from non-oPrl stimulated cells (data not shown). STAT5b and STAT5a were always detected (Fig. 2, lanes 5a, b). As observed previously [Jolivet et al., 2001], the antibody directed against both STAT5a and STAT5b (lane 5t) totally shifted the αs1cas-STAT5 complex, and induced multiple supershifted complexes. Similar data were obtained using NEs prepared from cells stimulated or not by F, or from CEs (not shown). In Western blot experiments, similar proteins were revealed by the total STAT5 antibody in NEs prepared from cells cultured on plastic or collagen (Fig. 2, total STAT5). The correspondence of the 100 kDa signal with STAT5P and 97 kDa with STAT5b was deduced from successive hybridizations with antibodies directed against STAT5P (Fig. 2, STAT5P) and STAT5b (not shown). The phosphorylated form of STAT5 (100-kDa) was clearly detected in oPrl-stimulated cells, cultured on plastic or collagen. STAT5a was never detected, probably because of the low affinity of the antibody or the small quantity of STAT5a in the samples. The non-phosphorylated form of STAT5b (97 kDa) was detected in all NEs from cells treated or not by oPrl. Similar data were obtained with CEs, or with extracts from cells treated or not by F (data not shown).

The present data show clearly that, in rabbit primary mammary cells, the ECM modifies neither the amount nor the in vitro ability of STAT5 and C/EBPs to bind DNA. The ECM does not modulate Prl activation of STAT5. These findings differed from those presented in our previous paper [Jolivet et al., 2001]. Most likely, the addition of supplementary protease inhibitors in the present experiments limited the extent of protease activity during the preparation of NEs and CEs. Indeed, in the previous experiments, PMSF and benzamidine were the only protease inhibitors added in extraction buffers, when the present inhibitor cocktail contains pepstatin, aprotinin, and leupeptin. The most extensive inhibition of protease activity

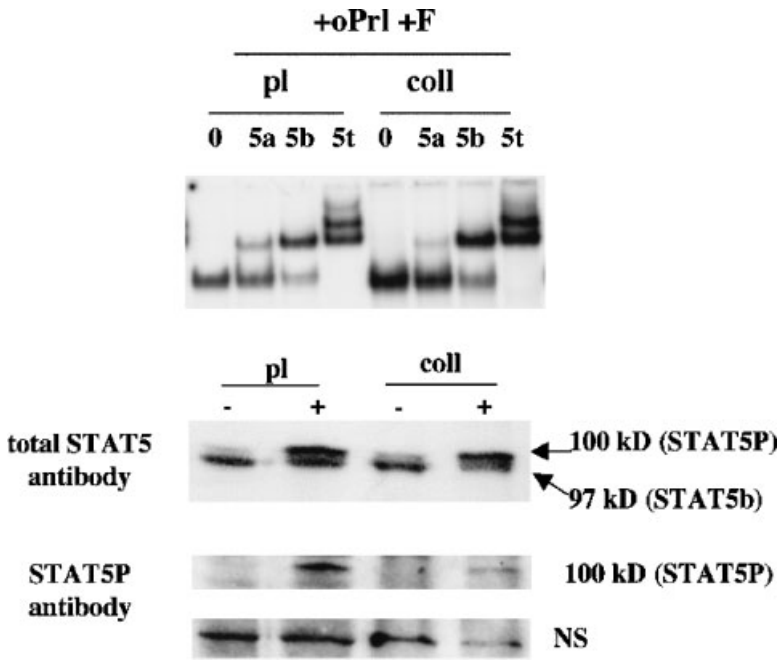

Fig. 2. Characterization of STAT5 in NEs from isolated rabbit primary mammary cells cultured on plastic or collagen. **Upper panel**: EMSAs were performed with αs1-cas STAT5 as probe and NEs prepared from cells cultured on plastic (pl) or on collagen (coll) treated with cortisol (+F) from the beginning of culture and with oPrl (+oPrl) for 15 min prior to NE preparation. A similar amount of NE proteins (3–10 μg, depending on the experiment) was incubated without (0) or with antibodies directed against STAT5a (5a), STAT5b (5b), or both STAT5a and b (5t). The autoradiographies were representative of seven independent cultures. **Lower panel**: Identification of STAT5 by Western blots in NEs from mammary cells cultured on plastic and collagen. One hundred micrograms of nuclear proteins were fractionated by SDS–7% PAGE, transferred to a nitrocellulose membrane and probed with antibodies directed against STAT5a and STAT5b (total STAT5, upper panel) and STAT5P (lower panel). As control for the loading of samples, a non-specific hybridization signal (NS) obtained with the STAT5P antibody is presented. The molecular weight of proteins deduced from migration rates of standards is indicated. The 100 kDa signal corresponds to STAT5P, the 97 kDa to STAT5b. The NEs were the same than those analyzed in Figure 1.

prevented the formation of isoforms previously observed.

STAT5 but not C/EBP Binding Sites Are Mandatory for Induction by oPrl and ECM of a Reporter Gene

In order to determine if the binding of STAT5 or C/EBP to their specific sites is involved in vivo in the ECM regulation of Prl-induced transcription, we mutated the STAT5 or C/EBP binding sites of a construct sensitive to Prl and ECM (6i TK luc). This construct contained a six-copy repeat of a distal enhancer located upstream of the rabbit αs1-casein gene [Jolivet et al., 1996] linked to the promoter of the Herpes simplex virus thymidine kinase (TK) and to a firefly luciferase gene. The enhancer encompasses one C/EBP binding site flanked by at least one STAT5 binding site [Jolivet et al., 1996]. This composite promoter was shown to be sensitive to Prl and ECM in rabbit primary mammary cells [Pantano et al., 2002]. In the present study, we transfected the 6i mC/EBP TK luc plasmid with three point mutations inside each F3-C/EBP binding site (Fig. 3). The mutations were sufficient to prevent the binding of nuclear proteins to the F3-C/EBP site, since in EMSA, no retarded complexes were visible when mF3-C/EBP was used as the probe (data not shown). The expression of the reporter luciferase gene was measured 48 h after transfection during the transient phase of expression. In rabbit primary mammary cells (Fig. 3), 6i TK luc and 6i mC/EBP TK luc exhibited similar sensitivity towards oPrl and ECM. With both constructs, oPrl strongly enhanced luciferase expression in cells cultured on collagen, and cortisol enhanced oPrl activity. In primary mammary cells cultured on plastic, the luciferase gene expression with 6i TK luc and 6i mC/EBP TK luc was not induced.

A mutation in each STAT5 binding site (which flanks the C/EBP binding site in "i") totally abolished the sensitivity of the construct towards oPrl (Fig. 3, 6i mSTAT5 TK luc panel).

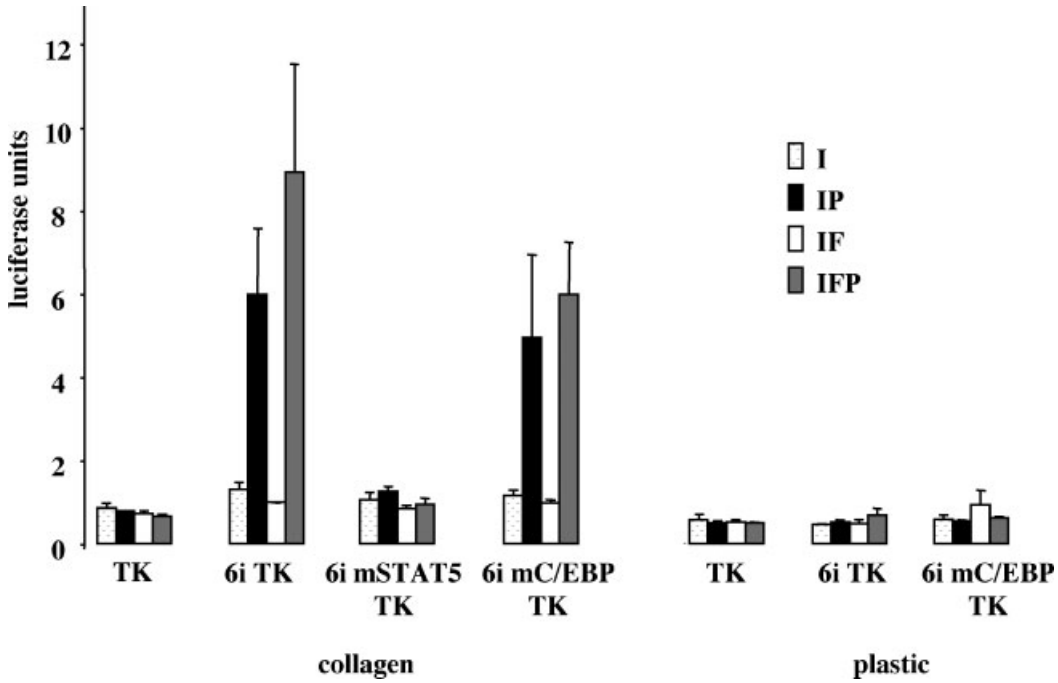

Fig. 3. Luciferase gene expression in primary mammary cells transfected by 6i TK luc constructs with mutated STAT5 or C/EBP binding sites. Isolated rabbit primary mammary cells cultured on plastic or collagen were transfected as described in Experimental Procedures. Cells were treated the following day with insulin (I, 400 ng/ml), cortisol (F) (100 ng/ml), or oPrl (P) (1 μg/ml) for 48 h. Luciferase and β-galactosidase gene expressions were measured 2 days after transfection. Luciferase values were normalized to β-galactosidase activities, then normalized to the value measured in 6i TK luc transfected cells cultured on collagen and treated with I plus F within the same culture. The values are means and standard errors of the mean calculated from three to nine independent experiments.

This result is in agreement with our previous data obtained by transfecting CHO cells with a construct containing the "i" enhancer linked upstream of the proximal rabbit αs1-casein gene promoter and upstream of the chloramphenicol acetyl transferase reporter gene [Pierre et al., 1994]. Clearly, the STAT5 binding site is mandatory for oPrl and ECM-dependent enhancer activity of "i" but not the C/EBP binding site.

Histone H4 Hyperacetylation of the Upstream Sequence of Rabbit αs1-Casein Gene Is Regulated by the ECM

It is now broadly accepted that the acetylation of histones H3 and H4 is coincident with a partial opening of chromosomal domains and transcriptional competence [see for review Eberharter and Becker, 2002; Orlando and Jones, 2002]. Moreover, transcriptional activation within an open domain frequently correlates with an additional acetylation of histones in the promoter region. We, therefore, wondered if the ECM acts on αs1-casein gene transcription by regulating the level of acetylated histones in its upstream sequences. Consequently, we measured the level of histone acetylation in the upstream sequences of the αs1-casein gene under various culture conditions. After ChIP by an antibody directed against the acetylated form of histone H4, real-time PCR was performed to quantify the amount of immunoprecipitated DNA. Extracts were prepared from rabbit primary mammary cells, from mammary gland and from a control tissue (muscle) where the αs1-casein gene is not expressed. One set of primers (localized around −177 nt upstream of the tsp) amplified a sequence encompassing the proximal binding site for STAT5 [Pierre et al., 1994]. This site, present in the proximal promoter of αs1- and β-casein genes in rabbit, mouse and rat is essential to transcriptional activation by Prl [see for review Hennighausen, 1997; Rosen et al., 1999]. The −2,373 amplified sequence is localized within a region which contains no known binding site for transcription factor, as judged by TFSearch software analysis. Moreover, deletion of a large region encompassing this sequence does not modify the transcriptional activity of the rabbit αs1-casein gene promoter in transfected CHO cells [Pierre

et al., 1994]. The amplified sequences −3,258 and −3,434 upstream of the tsp of rabbit αs1-casein gene are localized inside the "i" distal enhancer (−3,434) or close to the 3′ end of this enhancer (−3,258). A set of primers was chosen at the level of the tsp of the rabbit β-globin gene. The expression of this gene is erythroid cell-specific, not regulated by the ECM, and consequently suitable to correct for ChIP efficiency in each sample. Thus, IP/IS values obtained using the four αs1-casein sets of primers were normalized to the IP/IS values obtained using the β-globin set of primers. Finally, a set of primers was devised at the level of the tsp of the rabbit α-myosin heavy chain gene to compare results obtained with a non-mammary related region and αs1-casein regions.

In the control myosin region as in −2,373 and −177 αs1-casein regions, the level of acetylated H4 was not significantly different in cells cultured on plastic or on collagen (Fig. 4). Besides, in the far upstream αs1-casein regions −3,434 and −3,258, the level of acetylated H4 was lower in cells cultured on plastic. This effect was more pronounced in the −3,434 region, with a significant difference between plastic and collagen cultured cells. The 15-min oPrl-treatment did not modify clearly the level of acetylated histone H4 (compare the −Prl and +Prl points). Similar results were obtained with or without F in the culture medium (not shown). Thus, in cells cultured on plastic in the absence of a basement membrane, the far upstream αs1-casein −3,434 region undergoes a local histone de-acetylation.

The ECM Regulates the Presence of Integrins at the Surface of Primary Mammary Cells

To further confirm the role of the ECM, we tried to block the ECM transduction pathway using integrin-blocking antibodies. It is well known that in mouse mammary cells, α6 and β1 integrins are required for ECM action on Prl-induced casein gene transcription [Streuli et al., 1991; Muschler et al., 1999]. In Scp2 mouse mammary cells cultured with a laminin-enriched medium, the amount of cellular

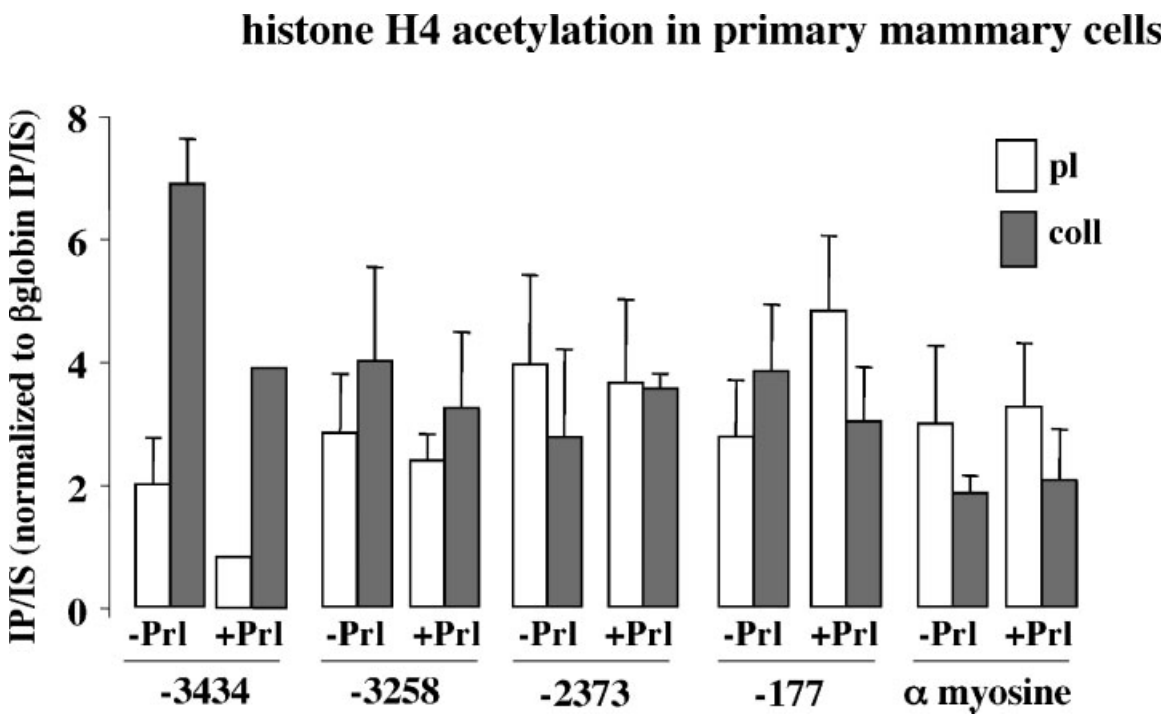

Fig. 4. Level of acetylated histone H4 upstream of the αs1-casein gene in mammary gland and cells cultured on collagen or plastic. Chromatin immunoprecipitation of acetylated histone H4 was performed using chromatin extracts from isolated cells cultured on plastic (pl) or on collagen (coll) treated (+Prl) or not (−Prl) with oPrl for 15 min before extraction. Quantitative PCR was performed to amplify four regions upstream of the tsp of the rabbit αs1-casein gene and the α-myosin gene. Each αs1-casein sequence is noted by the position of the 5′ end of the amplified fragment versus the tsp. A region of the rabbit β-globin gene was amplified as control to normalize data. For each sample, αs1-casein and α-myosin IP/IS values were normalized to the β-globin IP/IS value. Four distinct cultures were analyzed. Each represented value is the mean of three to four data except for the −3,434 +Prl samples where two data were available. Vertical bars represent standard errors of the mean.

αs1-casein was significantly reduced by treatment with the α6 or β1 integrin-blocking antibody [Muschler et al., 1999]. Alpha6 integrin has been found at the surface of mouse mammary epithelial cells cultured on EHS but not cultured on plastic [Streuli et al., 1991]. We, therefore, searched for α6 and β1 integrins in rabbit primary mammary cells cultured on plastic or collagen.

Alpha6 integrin was easily detected at the surface of primary mammary cells cultured on collagen but not in cells cultured on glass coverslips (Fig. 5). The addition of hormones (oPrl or cortisol) did not modify α6 integrin distribution (not shown). Beta1 integrin was not detected in either type of cells (data not shown), probably because the antibody did not recognize correctly rabbit β1 integrin. For this reason, we did not studied further β1 integrin.

The Function-Blocking Integrin Antibody GoH3 Inhibits the oPrl-Induced Transcription of αs1-Casein Gene Without Modifying STAT5, C/EBPs but Decreases Histone Acetylation at the αs1-Casein Gene Level

We first evaluated the effect of the α6 integrin function-blocking antibody on the accumulation of endogenous αs1-casein mRNA. The expression of the αs1-casein gene was induced by oPrl in cells cultured on floating collagen but not on plastic (Fig. 6), a finding in agreement with our previous work [Jolivet et al., 2001]. This result was obtained in both the presence and absence of cortisol, which is known to amplify Prl action. After the addition of anti-α6 integrin antibody GoH3 to the culture medium of primary cells cultured on collagen and treated with oPrl, the amount of αs1-casein mRNA was markedly reduced.

It was, therefore, worthwhile assessing whether the function-blocking integrin antibody GoH3 was acting through modifications of STAT5 or C/EBP transcription factors. The addition of function-blocking antibody GoH3 for 48 h to collagen and culture medium before the 15 min-stimulation by oPrl modified neither STAT5 nor C/EBPs in NEs (Fig. 7) or CEs (unshown data). STAT5 activation by oPrl was not affected by the presence of the antibody (Fig. 7, αs1cas-STAT5 probe). The nuclear (Fig. 7, total STAT5 and STAT5P antibody panels) or cytosolic (not shown) amounts of STAT5 were not modified. The binding of C/EBPs (Fig. 7, F3-C/EBP probe), the nature of C/EBPs included in retarded F3-C/EBP complexes (not shown) and the nuclear amount of C/EBPs (Fig. 7, C/EBPβ and δ antibody panels) remained unchanged. Thus, the inhibitory activity of the function-blocking antibody GoH3 did not involved STAT5 or C/EBPs.

The level of acetylated histone H4 in the upstream sequence of the αs1-casein gene was determined in isolated cells cultured on collagen, treated with GoH3 for 48 h and then

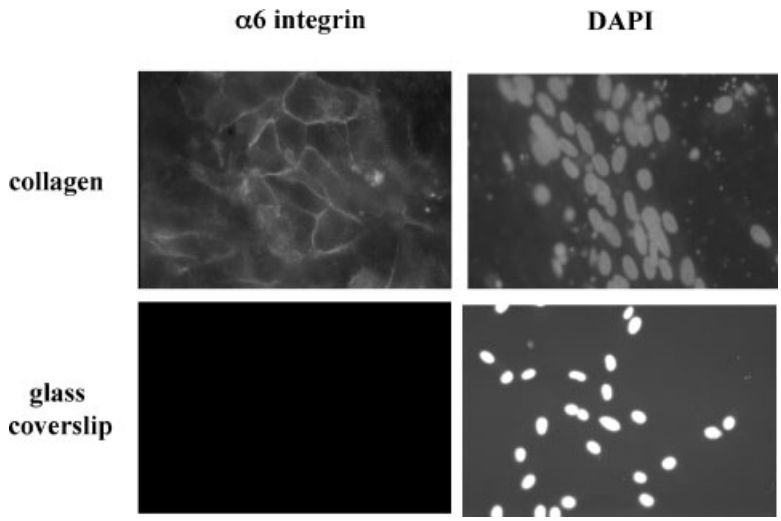

Fig. 5. Immunohistological localization of the α6 integrin. Cells isolated by trypsin treatment and seeded on thick collagen gel or on glass coverslips were incubated with anti-α6 integrin antibody and stained as described in Material and Methods. Cells were treated with cortisol from the beginning of culture and with oPrl 15 min before fixation in paraformaldehyde. Nuclei were counterstained by DAPI.

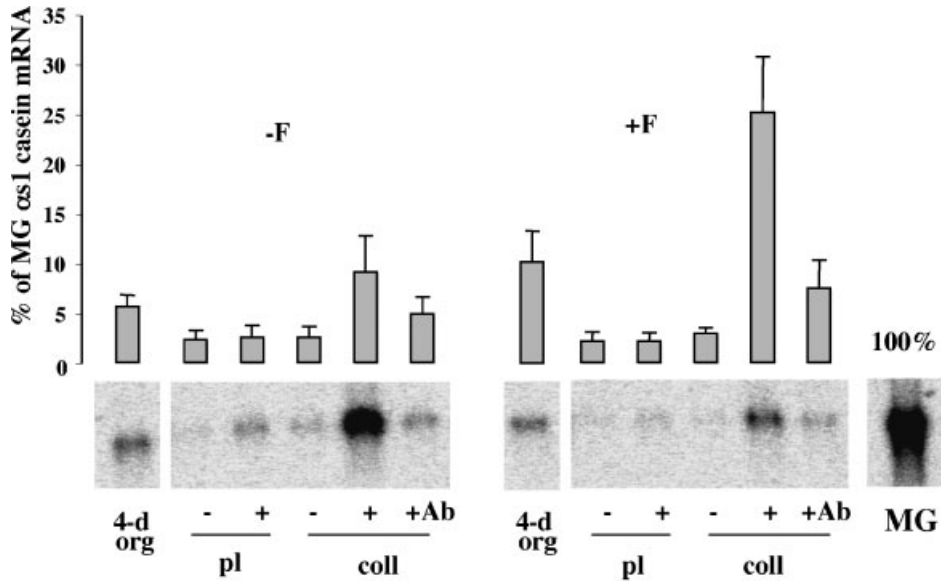

Fig. 6. Inhibition of prolactin-induced αs1-casein gene transcription by anti-α6 integrin antibody. The amount of αs1-casein mRNA was evaluated by Northern blot hybridization of total RNA (10 μg) with a rabbit cDNA probe. After mammary gland dissection, organoids were cultured for 4 days on plastic (4-day org) with (+F) or without (−F) cortisol. After trypsin treatment of organoids, isolated cells were cultured on plastic (pl) or collagen (coll) and treated (+) or not (−) with oPrl for 48 h, as described in Experimental Procedures. Cortisol treatment was maintained (+F or −F) until the end of culture. MG: 14-day pregnant rabbit mammary gland. Loading of lanes was checked by ethidium bromide staining of 28S and 18S ribosomal RNA (not shown). The autoradiography was obtained using samples extracted within one representative experiment over seven independent experiments. The radioactivity of each signal was measured using a StormImager then normalized to the value of the MG (100%) for each culture. Vertical bars represent the mean of seven independent cultures with the standard error of the mean. +Ab; isolated cells cultured on collagen with oPrl and anti-α6 integrin antibody (GoH3) in collagen (3 μg/ml) and culture medium (7 μg/ml) for 48 h.

activated by a 15-min treatment with oPrl (Fig. 8). Interestingly, the levels were lower in cells treated by GoH3 and prolactin than in those treated by prolactin alone especially far upstream the as1-casein gene (regions −3,434, −3,258, and −2,373). Noticeably, the −177 and the control α myosin region were not modified. Thus the function-blocking antibody GoH3 acts on αs1-casein gene transcription by a pathway, which involves histone H4 acetylation. This supports the idea that the ECM regulates histone acetylation at the level of αs1-casein gene upstream sequence through α6 integrin independently of prolactin transduction pathway.

DISCUSSION

One essential result of this work is that STAT5 is not a direct target of the ECM in cultured rabbit primary mammary cells. STAT5 activation by oPrl occurred in isolated cells cultured on plastic or on collagen. This fact was reported in our previous studies [Tourkine et al., 1995; Jolivet et al., 2001]. Despite the presence of activated STAT5 in cells cultured on plastic, no αs1-casein gene transcription occurred. Thus essential signals, possibly delivered by the ECM, were missing. These data differ from those previously reported [Streuli et al., 1995b; Edwards et al., 1998]. In primary mouse mammary cells cultured on plastic and on collagen I-coated dishes, no STAT5 activation occurred after oPrl stimulation. One possible explanation for this discrepancy is that different species were used for these two studies. It is conceivable that mouse and rabbit mammary cells do not display the same sensitivity towards ECM and Prl.

The second important result of this work is that C/EBPs are not direct targets of the ECM. Thus, the function of C/EBPs remains to be determined in the rabbit mammary gland. Numerous studies have ascribed a role to C/EBPs in the rodent mammary gland. C/EBPβ is crucial to mammary development and to the differentiation of mammary epithelial cells [Robinson et al., 1998; Seagroves et al., 1998, 2000; Zahnow et al., 2001]. Invalidation of the *C/EBPβ* gene leads to an abnormal development of the mouse mammary gland and an uneven

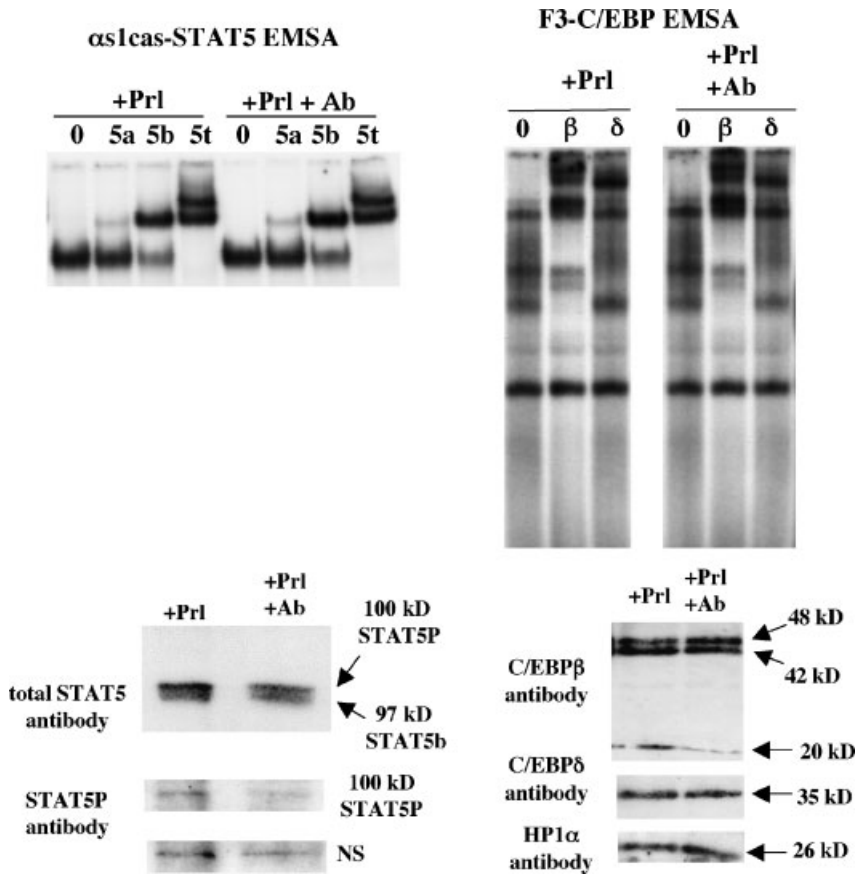

Fig. 7. Effect of function-blocking α6 integrin antibody GoH3 on C/EBPs and STAT5. Primary mammary cells cultured on collagen were treated with cortisol from the beginning of the culture; oPrl was added (+Prl) to the culture medium for 15 min before NE preparation. +Prl + Ab: integrin antibody GoH3 (3–4 μg/ml in collagen, 5–7 μg/ml in culture medium) was added for 48 h, then oPrl for 15 minutes before NE preparation. EMSAs were performed using αs1cas-STAT5 (αs1cas-STAT5 EMSA) or F3-C/EBP (F3-C/EBP EMSA) as probe. Western blots (**lower panels**) showing STAT5 (total STAT5 and STAT5P antibody) and C/EBPs (C/EBPβ and δ antibody) were performed as described in the legends to Figures 2 and 4. Data are representative of seven independent experiments. Non-specific hybridized material (NS) and HP1α hybridization are shown as controls for sample loading respectively in STAT5 and C/EBP Western blots.

differentiation of epithelial cells. A direct effect of C/EBPβ on rodent milk protein gene expression has been also extensively described. Mammary fat pads from C/EBPβ deficient mouse transplanted in wild-type mammary fat pads undergo partial differentiation but never correctly express β-casein gene [Robinson et al., 1998]. Primary cultures of mammary cells from C/EBPβ−/− mice were unable to produce significant amounts of β-casein [Seagroves et al., 1998], which led the authors to conclude that C/EBPβ was directly involved in β-casein gene transcription. Further experiments have been performed to support this hypothesis. After the over-expression of C/EBPβ (LAP or LIP) isoforms in COS cells, oPrl was able to stimulate transcription of the β-casein gene, but exclusively when plasmids expressing STAT5 and glucocorticoid receptor (GR) were transfected at the same time [Wyszomierski and Rosen, 2001]. Mutations in C/EBP binding sites within the distal or proximal promoters of rat or bovine β-casein genes induced a dramatic decrease in the sensitivity of the promoter towards oPrl induction [Doppler et al., 1995; Myers et al., 1998].

It is an apparent contradiction between these previous works showing the role of C/EBPs in the regulation of the transcriptional activity of milk protein genes and ours. Possibly, C/EBPs may have different functions in rodents and in the rabbit. However, our data do not exclude that C/EBPs are potential regulators of the transcriptional activity of the rabbit αs1-casein gene acting through other C/EBP binding sites, which are numerous in the proximal promoter

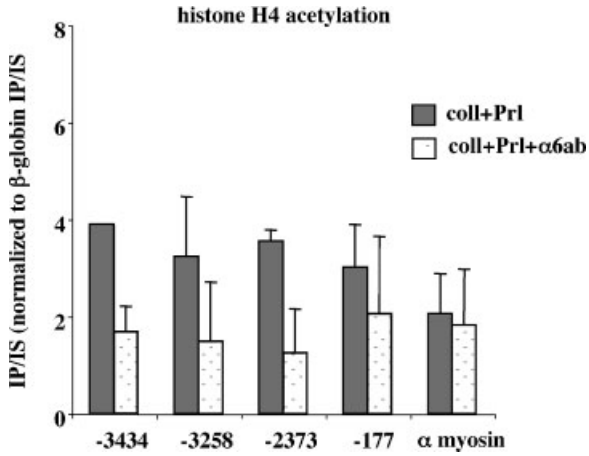

Fig. 8. Effect of function-blocking α6 integrin antibody GoH3 on histone H4 acetylation upstream of the αs1-casein gene. Primary mammary cells were cultured on collagen with (+α6ab) or without integrin antibody GoH3 (3–4 μg/ml in collagen, 5–7 μg/ml in culture medium) for 48 h. Cells were treated (+Prl) with oPrl for 15 min. Chromatin immunoprecipitation (ChIP) and the presentation of results were similar to those shown in the legend to Figure 4. Means and standard errors of the mean are calculated from three independent experiments.

of the rabbit αs1-casein gene but this regulation is independent of ECM transduction pathway.

Another explanation is worth being considered. Myers et al. noticed that stably integrated and non-integrated transfected constructs gave different results. Prolactin strongly stimulated reporter gene activity in CID9 cells cultured on a laminin-rich basement, but only after stable integration of the transfected constructs. The authors concluded that only the integrated gene was subjected to ECM-dependent chromatin regulation [Myers et al., 1998]. During our transient transfection experiments, the "i" enhancer in the 6i TK luc construct was kept out of a chromatin context. For this reason, it was perhaps impossible to observe the C/EBPs effects that were observed by others.

The regulation of gene expression at the chromatin level is tightly linked to the covalent modification of histones, such as acetylation, methylation, and ubiquitination. Histone acetylation leads to modifications of chromatin structure, which generally becomes permissive to transcription [see for review Eberharter and Becker, 2002 for review]. Many authors have shown that a high level of acetylated histones is correlated with the accessibility of DNA to transcription factors and recruitment of the basal transcription machinery. To our knowledge, the present study shows for the first time that the ECM is necessary to maintain a high level of acetylated histone H4 in the 5′ upstream region of a gene, the αs1-casein gene. Notably, the ECM does not act simultaneously on the four regions that we have analyzed in the αs1-casein gene upstream sequences. Indeed, a more pronounced effect was observed at the level of the distal STAT5 binding sites (the distal enhancer located at −3,434) than at the level of the proximal STAT5 site (the proximal promoter located at −177) or at the level of the intermediary region (−2,373), which is not necessary for αs1-casein gene transcription.

These data output the idea that in rabbit mammary epithelial cells, the ECM maintains the chromatin in a structure that enables the transcription factors to bind in vivo to the αs1casein gene promoter and to recruit the transcription machinery. In cells cultured on plastic, the low level of acetylated histones around the 5′ upstream region of the αs1-casein gene may lead to a low accessibility of transcription factors and RNA polymerase. This could explain why in rabbit primary mammary cells cultured on plastic, oPrl did not induce transcription of the αs1-casein gene despite the presence of activated STAT5.

Recently, the molecular mechanism of cytokine induced transcriptional activation by STAT5 has been investigated [Rascle et al., 2003; Rascle and Lees, 2003; Xu et al., 2003]. These studies have shown that cytokine treatment induces STAT5 binding, gene promoter histone acetylation, and chromatin remodeling. Moreover, a non-histone protein deacetylase activity is required, which could result in deacetylation of C/EBPβ and activation of this transcription factor [Xu et al., 2003]. In rabbit primary mammary cells, it is conceivable that oPrl acts through similar mechanisms, but only in the presence of the ECM which is necessary to maintain the chromatin in a permissive status.

On the basis of our findings, we propose that ECM regulates Prl-induced αs1-casein gene transcription by modifying chromatin structure at the level of the upstream gene sequences. The first step of this pathway involves α6 integrin. The subsequent mechanisms, which lead to maintain a high histone acetylation level, remain to be clarified. The possibility to measure chromatin opening at the αs1-casein gene level offers new opportunity to study ECM transduction pathway on milk protein gene expression. Besides, it is interesting to know if the ECM

maintains high histone acetylation specifically on small gene portions, such as several kilobases, upstream of the αs1-casein gene or on larger genomic domains (several hundreds of kb) encompassing several genes. Milk protein gene loci encompass milk protein genes but also unrelated genes. This is the case for the casein gene locus [Rijnkels et al., 2003] and the whey acidic protein gene locus [Rival-Gervier et al., 2002, 2003]. It should be of great interest to determine if the ECM regulates also the transcriptional activity of the nearby genes and whether the hyperacetylation of histone H4 induced by the ECM spreads on a large domain including simultaneously milk protein and other genes. These experiments are in progress in our laboratory.

REFERENCES

Baer M, Johnson PF. 2000. Generation of truncated C/EBP beta isoforms by in vitro proteolysis. J Biol Chem 275: 26582–26590.

Bayat-Sarmadi M, Puissant C, Houdebine LM. 1995. The effects of various kinase and phosphatase inhibitors on the transmission of the prolactin and extracellular matrix signals to rabbit alpha S1-casein and transferrin genes. Int J Biochem Cell Biol 27:707–718.

Dearth LR, Hutt J, Sattler A, Gigliotti A, DeWille J. 2001. Expression and function of CCAAT/enhancer binding proteinbeta (C/EBPbeta) LAP and LIP isoforms in mouse mammary gland, tumors, and cultured mammary epithelial cells. J Cell Biochem 82:357–370.

Devinoy E, Schaerer E, Jolivet G, Fontaine LM, Kraenhenbuhl JP, Houdebine LM. 1988. Sequence of the rabbit aS1-casein cDNA. Nucl Acids Res 16:11813.

Devinoy E, Malienou-N'Gassa R, Thèpot D, Puissant C, Houdebine LM. 1991. Hormone responsive elements within the upstream sequences of the rabbit whey acidic protein (*WAP*) gene direct chloramphenicol acetyl transferase (CAT) reporter gene expression in transfected rabbit mammary cells. Mol Cell Endocrinol 81:185–193.

Doppler W, Welte T, Philipp S. 1995. CCAAT/enhancer-binding protein isoforms beta and delta are expressed in mammary epithelial cells and bind to multiple sites in the beta-casein gene promoter. J Biol Chem 270:17962–17969.

Eberharter A, Becker PB. 2002. Histone acetylation: A switch between repressive and permissive chromatin. Second in review series on chromatin dynamics. EMBO Rep 3:224–229.

Edwards GM, Wilford FH, Liu XW, Hennighausen L, Djiane J, Streuli CH. 1998. Regulation of mammary differentiation by extracellular matrix involves protein-tyrosine phosphatases. J Biol Chem 273:9495–9500.

Hennighausen L. 1997. Molecular mechanisms of hormone controlled gene expression in the breast. Mol Biol Rep 24:169–174.

Jahn GA, Daniel N, Jolivet G, Belair L, BoleFeysot C, Kelly PA, Djiane J. 1997. In vivo study of prolactin (PRL) intracellular signalling during lactogenesis in the rat: JAK/STAT pathway is activated by PRL in the mammary gland but not in the liver. Biol Reprod 57:894–900.

Jolivet G, Lhotte C, Pierre S, Tourkine N, Houdebine LM. 1996. A MGF/STAT5 binding site is necessary in the distal enhancer for high prolactin induction of transfected rabbit alpha s1-casein-CAT gene transcription. FEBS Lett 389:257–262.

Jolivet G, Meusnier C, Chaumaz G, Houdebine LM. 2001. Extracellular matrix regulates alpha s1-casein gene expression in rabbit primary mammary cells and CCAAT enhancer binding protein (C/EBP) binding activity. J Cell Biochem 82:371–386.

Klinowska TCM, Soriano JV, Edwards GM, Oliver JM, Valentijn AJ, Montesano R, Streuli CH. 1999. Laminin and beta 1 integrins are crucial for normal mammary gland development in the mouse. Dev Biol 215:13–32.

Litt MD, Simpson M, Recillas-Targa F, Prioleau MN, Felsenfeld G. 2001. Transitions in histone acetylation reveal boundaries of three separately regulated neighboring loci. EMBO J 20:2224–2235.

Muschler J, Lochter A, Roskelley CD, Yurchenco P, Bissell MJ. 1999. Division of labor among the alpha 6 beta 4 integrin, beta 1 integrins, and an E3 laminin receptor to signal morphogenesis and beta-casein expression in mammary epithelial cells. Mol Biol Cell 10:2817–2828.

Myers CA, Schmidhauser C, Mellentin Michelotti J, Fragoso G, Roskelley CD, Casperson G, Mossi R, Pujuguet P, Hager G, Bissell MJ. 1998. Characterization of BCE-1, a transcriptional enhancer regulated by prolactin and extracellular matrix and modulated by the state of histone acetylation. Mol Cell Biol 18:2184–2195.

Orlando V, Jones KA. 2002. Wild chromatin: Regulation of eukaryotic genes in their natural chromatin context. Gene Dev 16:2039–2044.

Pantano T, Jolivet G, Prince S, Menck-Le Bourhis C, Maeder C, Viglietta C, Rival S, Houdebine LM. 2002. Effect of the rabbit alphas1-casein gene distal enhancer on the expression of a reporter gene in vitro and in vivo. Biochem Biophys Res Co 290:53–61.

Pierre S, Jolivet G, Devinoy E, Houdebine LM. 1994. A combination of distal and proximal regions is required for efficient prolactin regulation of transfected rabbit alpha s1-casein chloramphenicol acetyltransferase constructs. Mol Endocrinol 8:1720–1730.

Pujuguet P, Radisky D, Levy D, Lacza C, Bissell MJ. 2001. Trichostatin A inhibits beta-casein expression in mammary epithelial cells. J Cell Biochem 83:660–670.

Rascle A, Lees E. 2003. Chromatin acetylation and remodelling at the Cis promoter during STAT5-induced transcription. Nucl Acids Res 31:6882–6890.

Rascle A, Johnston JA, Amati B. 2003. Deacetylase activity is required for recruitment of the basal transcription machinery and transactivation by STAT5. Mol Cell Biol 23:4162–4173.

Reichmann E, Ball R, Groner B, Friis RR. 1989. New mammary epithelium and fibroblastic cell clones in coculture form structures competent to differentiate functionally. J Cell Biol 108:1127–1138.

Rijnkels M, Elnitski L, Miller W, Rosen JM. 2003. Multispecies comparative analysis of a mammalian-specific genomic domain encoding secretory proteins. Genomics 82:417–432.

Rival-Gervier S, Viglietta C, Maeder C, Attal J, Houdebine LM. 2002. Position-independent and tissue-specific expression of porcine whey acidic protein gene from a bacterial artificial chromosome in transgenic mice. Mol Reprod Dev 63:161–167.

Rival-Gervier S, Thepot D, Jolivet G, Houdebine LM. 2003. Pig whey acidic protein gene is surrounded by two ubiquitously expressed genes. Biochim Biophys Acta 1627: 7–14.

Robinson GW, Johnson PF, Hennighausen L, Sterneck E. 1998. The C/EBP beta transcription factor regulates epithelial cell proliferation and differentiation in the mammary gland. Gen Dev 12:1907–1916.

Rosen JM, Wyszomierski SL, Hadsell D. 1999. Regulation of milk protein gene expression. Annu Rev Nutr 19:407–436.

Roskelley CD, Srebrow A, Bissell MJ. 1995. A hierarchy of ECM-mediated signalling regulates tissue-specific gene expression. Curr Opin Cell Biol 7:736–747.

Seagroves TN, Krnacik S, Raught B, Gay J, BurgessBeusse B, Darlington GJ, Rosen JM. 1998. C/EBP beta, but not C/EBP alpha, is essential for ductal morphogenesis, lobuloalveolar proliferation, and functional differentiation in the mouse mammary gland. Gene Dev 12:1917–1928.

Seagroves TN, Lydon JP, Hovey RC, Vonderhaar BK, Rosen JM. 2000. C/EBP beta (CCAAT/enhancer binding protein) controls cell fate determination during mammary gland development. Mol Endocrinol 14:359–368.

Streuli CH, Bissel M. 1990. Expression of extracellular matrix components is regulated by substratum. J Cell Biol 110:1405–1415.

Streuli CH, Bailey N, Bissell MJ. 1991. Control of mammary epithelial differentiation: Basement membrane induces tissue-specific gene expression in the absence of cell–cell interaction and morphological polarity. J Cell Biol 115:1383–1395.

Streuli CH, Schmidhauser C, Bailey N, Yurchenco P, Skubitz APN, Roskelley C, Bissell MJ. 1995a. Laminin mediates tissue-specific gene expression in mammary epithelia. J Cell Biol 129:591–603.

Streuli CH, Edwards GM, Delcommenne M, Whitelaw CBA, Burdon TG, Schindler C, Watson CJ. 1995b. Stat5 as a target for regulation by extracellular matrix. J Biol Chem 270:21639–21644.

Tourkine N, Schindler C, Larose M, Houdebine LM. 1995. Activation of STAT factors by prolactin, interferon-gamma, growth hormones, and a tyrosine phosphatase inhibitor in rabbit primary mammary epithelial cells. J Biol Chem 270:20952–20961.

Welm AL, Timchenko NA, Darlington GJ. 1999. C/EBP alpha regulates generation of C/EBP beta isoforms through activation of specific proteolytic cleavage. Mol Cell Biol 3:1695–1704.

Wyszomierski SL, Rosen JM. 2001. Cooperative effects of STAT5 (signal transducer and activator of transcription 5) and C/EBPbeta (CCAAT/enhancer-binding protein-beta) on beta-casein gene transcription are mediated by the glucocorticoid receptor. Mol Endocrinol 15:228–240.

Xu M, Nie L, Kim SH, Sun XH. 2003. STAT5-induced Id-1 transcription involves recruitment of HDAC1 and deacetylation of C/EBPbeta. EMBO J 22:893–904.

Zahnow CA, Cardiff RD, Laucirica R, Medina D, Rosen JM. 2001. A role for CCAAT/enhancer binding protein beta-liver-enriched inhibitory protein in mammary epithelial cell proliferation. Cancer Res 61:261–269.