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Extracellular Matrix Regulates Alpha S1-Casein Gene Expression in Rabbit Primary Mammary Cells and CCAAT Enhancer Binding Protein (C/EBP) Binding Activity

Geneviève Jolivet,[1]* Chantal Meusnier,[1] Gilles Chaumaz,[2] and Louis-Marie Houdebine[1]

[1]Biologie du Développement et Biotechnologies, Institut National de la Recherche Agronomique, 78352 Jouy en Josas, France
[2]Nutrition et Sécurité Alimentaire, Institut National de la Recherche Agronomique, 78352 Jouy en Josas, France

Abstract Previous studies have shown that both the signal transducer and activator of transcription 5 (STAT5) and the CCAAT enhancer binding proteins (C/EBPs) are involved in the regulation of casein gene expression by mammary epithelial cells. Prolactin (Prl) activation of STAT5 is necessary for casein gene expression. The extracellular matrix (ECM) regulates also casein gene expression. Here, we have investigated whether ECM regulates C/EBPs activity in primary rabbit mammary epithelial cells. Isolated primary mammary cells were cultured on plastic or on floating collagen I gel. Prolactin induced αs 1-casein gene expression when cells were cultured on collagen but not on plastic. It is noteworthy that activated STAT5 was detected in both culture conditions. Several STAT5 isoforms (STAT5a, STAT5b, and other STAT5 related isoforms, some with lower molecular weight than the full-length STAT5a and STAT5b) were detected under the different culture conditions. However, their presence was not related to the expression of αs 1-casein gene. The binding of nuclear factors to a C/EBP specific binding site and the protein level of C/EBPβ differed in cells cultured on plastic or on collagen but these parameters were not modified by Prl. This suggests that C/EBP binding activity was regulated by ECM and not by Prl. Interestingly, these modifications were correlated to the expression of the αs 1-casein gene. Hence, the activation of the αs 1-casein gene expression depends on two independent signals, one delivered by Prl via the activation of STAT5, the other delivered by ECM via C/EBP. J. Cell. Biochem. 82: 371–386, 2001. © 2001 Wiley-Liss, Inc.

Key words: extracellular matrix; milk protein gene expression; STAT5; C/EBP; primary mammary cell cultures; rabbit; αs 1-casein

Milk protein gene expression is regulated by lactogenic hormones, essentially prolactin (prl), glucocorticoids and insulin, and by the various components of the extracellular matrix (ECM) (see reviews in Roskelley et al., 1995; Hennighausen, 1997; Rosen et al., 1999). It is now well established that lactogenic hormones activate transcription factors, which in turn bind to specific sites located essentially upstream of the transcription start point (tsp) of milk protein genes. Under Prl stimulation, the transcription factor STAT5 is tyrosine phosphorylated. It then binds specifically to a high affinity STAT5 binding site located around 100 base pairs (bp) upstream of the tsp in rat β-casein, sheep β lactoglobulin, and rabbit αs 1-casein genes [Schmitt-Ney et al., 1991; Burdon et al., 1994; Pierre et al., 1994]. This activation is a necessary step in the cascade of events induced by the binding of Prl to its specific membrane receptor (see Hennighausen, 1997; Teglund et al., 1998; Rosen et al., 1999 for reviews). In response to glucocorticoids, the activated glucocorticoid receptor binds to glucocorticoid responsive elements in the proximal promoter of the rat β-casein gene [Lechner et al., 1997] and enhances STAT5 activation by prolonging STAT5 DNA binding and tyrosine phosphorylation

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

*Correspondence to: Geneviève Jolivet, Biologie du Développement et Biotechnologies Institut National de la Recherche Agronomique, 78352 Jouy en Josas, France.
E-mail: gjolivet@biotec.jouy.inra.fr

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[Stoecklin et al., 1997; Wyszomierski et al., 1999]. The presence of ECM is known to be necessary for the complete differentiation of the mouse mammary gland, and especially for the expression of milk protein genes [Roskelley et al., 1995; Hennighausen, 1997; Rosen et al., 1999]. Beta-casein gene transcription is induced by Prl only when cells are cultured on floating collagen gel [Li et al., 1987], or in the presence of a reconstituted basement membrane matrix derived from the Englebreth-Holm-Swarm mouse sarcoma tumor (EHS gel) [Streuli et al., 1995]. So far, it has not been shown whether ECM activates a specific transcription factor. A transcriptional enhancer, involved in Prl and ECM regulation of bovine β-casein gene expression, was identified within the 5′ flanking region of this gene [Schmidhauser et al., 1992]. Despite the characterization of binding sites for nuclear transcription factors within this enhancer, the regulation of these transcription factors by ECM was not clearly observed [Myers et al., 1998]. In mouse primary mammary cells, ECM inhibits the activity of cellular phosphatases, which enhance STAT5 phosphorylation [Edwards et al., 1998]. Therefore, it is likely that ECM regulates milk protein gene expression by at least modulating the activity of STAT5.

The present study was undertaken in order to investigate whether in the mammary gland ECM regulates the activity of other transcription factors, such as CCAAT enhancer binding proteins (C/EBPs). C/EBPs are involved in the differentiation of several cell types including adipocytes [Rosen et al., 2000], hepatocytes [Runge et al., 2000], myeloid cells [Khanna-Gupta et al., 2000], keratinocytes [Corbi et al., 2000], and mammary epithelial cells [Robinson et al., 1998; Seagroves et al., 1998]. Three isoforms of C/EBPs (C/EBP α, β, and δ) have been identified in rat mammary gland extracts or in cellular extracts prepared from the mouse mammary epithelial cell line HC11 [Doppler et al., 1995; Raught et al., 1995]. In C/EBPβ knock-out mice, the mammary epithelial cells are unable to differentiate [Robinson et al., 1998; Seagroves et al., 1998]. In contrast, mammary gland differentiated normally in C/EBPα knock-out mice which proves that C/EBPα is not necessary for mammary gland differentiation. C/EBPδ regulates mammary cell growth and differentiation, since growth arrest and apoptosis are induced by C/EBPδ overexpression in the mouse mammary epithelial cell line COMMA1D [O'Rourke et al., 1997, 1999; Hutt et al., 2000]. However, it cannot be asserted that C/EBPδ is essential to the in vivo differentiation of mouse mammary gland since no phenotype concerning the morphology and the function of the mammary gland has been identified in C/EBPδ knock-out mice [Tanaka et al., 1997].

C/EBPs are likely involved in the regulation of casein gene expression. The mammary tissue obtained from C/EBPβ knock-out mice and transplanted in wild type mouse mammary fat pad differentiates but produces no significant amount of β-casein [Seagroves et al., 2000]. In vitro, C/EBPβ and δ bind to multiple sites in the rat β-casein gene promoter [Doppler et al., 1995; Raught et al., 1995], which suggests a direct interaction of C/EBPs on casein gene promoter.

It has been already shown that ECM can regulate C/EBPs activity. In hepatoma cells, C/EBPα and β are differentially expressed whether cells are cultured on collagen I-coated dishes or on EHS and the binding activities of these transcription factors are greatly modified according to culture conditions [Rana et al., 1994; Runge et al., 1997]. In the mouse mammary cell line HC11, tenascin-C, a component of ECM, modifies the nuclear level of C/EBPα [Cella et al., 2000]. It had been previously shown that rabbit primary mammary cells are sensitive to ECM, since transferrin and αs 1-casein gene expression is enhanced in cells cultured on floating collagen I gel [Bayat-Sarmadi et al., 1995]. Hence, we analyzed the effect of ECM on C/EBP binding activity and protein level in isolated rabbit mammary cells cultured on floating collagen I gel or on plastic. Simultaneously, cells were treated or not by Prl, and STAT5 activation was measured. We found that αs 1-casein gene was expressed in isolated cells cultured on collagen but not on plastic. STAT5 and C/EBP were modified when cells were cultured either on plastic or collagen. Interestingly, modification of C/EBP but not of STAT5 was related to αs 1-casein gene expression.

MATERIALS AND METHODS

Antibodies

Anti-STAT5a and STAT5b mouse monoclonal antibodies were obtained from Zymed (San Francisco, CA). These antibodies were raised

against a peptide corresponding to the specific carboxy-terminus of STAT5a and STAT5b, respectively. A rabbit polyclonal antibody (C-17) raised against a more internal peptide (amino acids 706–722) which reacts with both STAT5a and STAT5b (antibody to total STAT5) was obtained from Santa Cruz Biotechnology (CA). A rabbit polyclonal antibody directed against phosphorylated STAT5 (Tyr 694) (antibody to PY STAT5) which reacts with both phosphorylated STAT5a and STAT5b was purchased from Upstate Biotechnology (NY). Rabbit polyclonal antibodies directed against C/EBPα (14AA), C/EBPβ (C-19), and C/EBPδ (C-22) were obtained from Santa Cruz Biotechnology. Secondary antibodies (sheep anti-mouse IgG and goat anti-rabbit IgG, conjugated to peroxidase) were obtained from Sigma.

Rabbit Primary Mammary Cell Cultures

Organoids were isolated from the mammary gland of 14–21 day pregnant rabbits as previously described [Devinoy et al., 1991]. Organoids prepared from a mammary gland were seeded on 50–100 dishes (8 cm in diameter) in DMEM/Ham's F12 medium (1:1) supplemented with 10% fetal calf serum (FCS, Sigma) and bovine insulin (5 μg/ml). Four to seven days later, some dishes were treated with trypsin to dissociate mammary cells. After elimination of non-dissociated material by rapid decantation or filtration using 100 μm sterile filters (Falcon), isolated cells were plated on plastic or on thick rat-tail collagen gel. From this step until the end of the culture, the medium used was a DMEM/Ham's F12 medium (1:1) supplemented with 2% steroid free (SF) UltroSer (Biosepra, France), insulin (5 μg/ml), and cortisol (10^{-7} M). SF Ultroser is a steroid and Prl free substitute of fetal calf serum. It was used instead of fetal calf serum in order to eliminate any possible stimulation by serum prolactin and steroids. Cells (seeded on plastic or collagen) were rinsed the next morning to remove non-attached material, and fresh medium was added. Collagen was then gently detached from the bottom of the plate to allow the gel to float.

Total RNA Extraction and Northern Blot Analysis

Total RNA extraction and Northern blot analysis were performed as previously described [Bayat-Sarmadi et al., 1995]. Rabbit αs 1-casein mRNA was detected after transfer on nylon membranes and hybridization with a cDNA probe (DNA fragment extending from nucleotide (nt) 48–1130, corresponding to almost the full-length cDNA [Devinoy et al., 1988].

Assessment of DNA Fragmentation

Mammary cell apoptosis was assessed by visualization of DNA fragmentation on agarose gel. Organoids and isolated cells cultured on plastic were rinsed with PBS then scraped. Cells cultured on floating collagen gels were collected after treatment of the gel for 5 min at 37°C with collagenase IV (Sigma) (1 mg/ml). Cells detached from the gel were pelleted by centrifugation and rinsed with PBS. Two hundred μl of lysis buffer (Tris 5 mM pH 8, EDTA 5 mM, 0.3 M sodium acetate, 1% sodium dodecyl sulfate) containing proteinase K (500 μg/ml) were added, and subsequent incubation was performed at 50°C for one and a half hour. After treatment of samples with an equal volume of phenol/chloroform, then of chloroform, DNA was precipitated by ethanol. Ten μg of DNA were treated with 500 ng of DNase free RNase (Roche Diagnostics) at 37°C for 30 min and size fractionated by electrophoresis in a 1.8% agarose gel for 4 h at 40 V using TBE buffer (90 mM Tris, 90 mM borate, 2 mM EDTA pH 8) in the presence of ethidium bromide for visualizing the DNA ladder.

Preparation of Nuclear Extracts

Mammary gland nuclear extracts (NEs) and cytosolic extracts (CEs) were prepared from thoroughly dissected tissue to remove most of the connective and muscle tissues as previously described [Jolivet et al., 1996].

Nuclei were prepared from plated mammary cells as described by Jolivet et al. [1996], with some modifications. All steps were performed at 4°C. After rinsing with phosphate-buffered saline (PBS), cells were scraped and Dounce-homogenized in buffer 1 (10 mM HEPES pH 7.7, 25 mM KC1, 2 mM EDTA, 0.5 mM EGTA, 0.6 M sucrose, 0.2% NP40, 0.5 mM DTT, 0.15 mM spermine, 0.5 mM spermidine, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM sodium fluoride, 1 mM sodium vanadate, 10 μM ammonium molibdate). Cells cultured on collagen were Dounce-homogenized with the collagen gel, and the homogenate was filtered using a Scrynex nylon gauze (pore size: 30 μM) to remove the gel.

Nuclei were pelleted by centrifugation of the homogenates for 15 min at 3,500 rpm.

Cytosolic extracts (CEs) were obtained by centrifugation of the supernatants at 15,000 rpm for 30 min. Nuclear extracts (NEs) were obtained by resuspending crude nuclei pellets in buffer C (20 mM HEPES pH 7.7, 0.4 M Nacl, 1.5 mM MgCl2, 0.2 mM EDTA, 25% glycerol, 0.5mM DTT, 2 mM benzamidine, 0.5 mM PMSF) followed by incubation for 60 min at 4°C under gentle stirring, and a centrifugation at 15,000 rpm for 30 min to remove insoluble material. Protein concentration was determined in extracts by the method of Bradford [1976] using the Bio-Rad reagent (Bio-Rad Laboratories, Richmond, CA) and BSA as standard.

Electrophoretic Mobility Shift Assay

Electrophoretic mobility shift assays (EMSA) were performed as previously described [Pierre et al., 1994; Jolivet et al., 1996]. The sequences of the probes were: αs l cas-STAT5 5′-GAGAATTCTTAGAATTTAAA-3′ corresponding to the (−104,−85) fragment of the rabbit αs l-casein gene; F3-C/EBP 5′-TATTCATTATG-TAATAGAAAG-3′ corresponding to the (−3352, −3333) fragment of the rabbit αs 1-casein gene. The sequence 5′-TGCAGATTGCGCAATCTG-CA-3′, which contained a C/EBP high affinity binding site [Cao et al., 1991] was used as competitor in EMSAs. In supershift experiments, 1 μg of antibody was added to the incubation mixture with the NE or the CE. Labeled complexes and free oligonucleotides were separated by electrophoresis on non-denaturing acrylamide/bis-acrylamide gels (5% when EMSAs were performed with the αs 1cas-STAT5 probe, 8% with the F3-C/EBP probe). Gels were pre-run overnight at low voltage (50 V) before loading samples. After electrophoresis, gels were transferred onto a 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: antibody to PY STAT5, to STAT5a, to STAT5b, and finally to total STAT5.

RESULTS

αs 1-Casein Gene Expression is Regulated by ECM

Isolated primary mammary cells were prepared from rabbit mammary gland organoids cultured on plastic for 4–7 days then dissociated by trypsin treatment. Organoids were composed of different types of cells: epithelial cells, fibroblasts, and myoepithelial cells. Epithelial cells are known to be the only source of casein. After organoids treatment by trypsin, the three types of cells were mixed. Immunocytochemical characterization revealed that in all culture conditions, the epithelial cells were highly predominant, the number of fibroblasts and myoepithelial cells remaining low (data not shown).

The expression of the αs 1-casein gene was studied by Northern blot analysis (Fig. 1). Alpha s1-casein mRNA was detected in the mammary gland of pregnant rabbits, in organoids and in isolated cells cultured on collagen treated by ovine Prl (oPrl) for 48 h. Alpha s 1-casein mRNA was found neither in organoids deprived of oPrl for 4 days, nor in isolated cells cultured on plastic and treated by oPrl. Thus, isolated cells cultured on plastic lost the ability to express the αs 1-casein gene. In contrast, isolated cells cultured on floating collagen still expressed this gene under oPrl stimulation.

Assessment of Mammary Cell Apoptosis

Previous studies have shown that mouse mammary cell apoptosis depends on culture conditions, especially on the nature of the ECM components [Pullan et al., 1996]. Apoptosis might be responsible for the absence of αs 1-casein gene expression in rabbit mammary cells cultured on plastic. The extent of apoptosis was assessed by DNA fragmentation which was visualized by electrophoresis of the genomic DNA (Fig. 2). A faint typical DNA ladder pattern was observed but it was not significantly

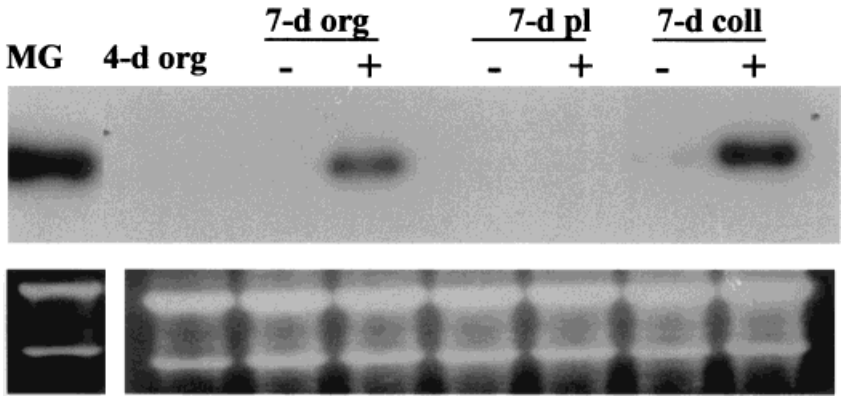

Fig. 1. Expression of αs 1-casein gene in rabbit primary mammary cells. Four day-cultured organoids were treated by trypsin. Isolated cells were seeded on plastic or on thick collagen gels in growing medium containing insulin (5 μg/ml) and cortisol (100 ng/ml). The following morning (Day 5), ovine Prl (1 μg/ml) was added to the culture medium as indicated (+ or −) and collagen gels were allowed to float in the medium. The medium was not replaced for the next 48 h. Cells were collected on Day 7 for total RNA extraction. The amount of αs 1-casein mRNA was evaluated by Northern blot hybridization of total RNA with a rabbit αs 1-casein cDNA probe. MG; 20-day pregnant rabbit mammary gland, org; organoids cultured for 4 days (4-d org) or 7 days (7-d org); pl; isolated cells cultured on plastic, coll; isolated cells cultured on collagen. Ten μg (MG) or 20 μg (other samples) of total RNA were loaded. Equal loading of lanes 2–8 is shown by ethidium bromide staining of 28S and 18S ribosomal RNAs. Exposure lasted 6 h for MG, and 24 h for all other samples. This figure is representative of at least six distinct experiments performed with 14–21-day pregnant rabbits.

different when isolated cells were cultured either on plastic or on collagen. In all culture conditions, only a few cells detached from their support and a small proportion exhibited fragmented and condensed nuclei. This confirms that apoptosis was not related to culture conditions. Hence, the lack of αs 1-casein gene expression in isolated cells cultured on plastic cannot be attributed to apoptosis.

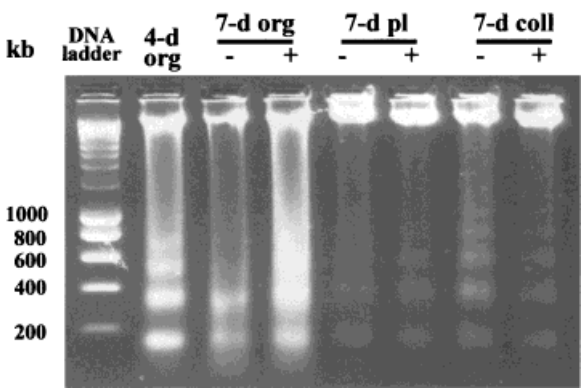

Fig. 2. Effect of culture conditions on DNA fragmentation of rabbit primary mammary cells. Primary mammary organoids and cells were prepared and cultured as previously described in Figure 1. All cells were collected on Day 7 for preparation of the genomic DNA. Ten μg of genomic DNA was loaded in each lane. DNA ladder: numbers on the left indicate the size of the bands in kilobases (kb). Org; organoids, pl; isolated cells cultured on plastic; coll; isolated cells cultured on collagen.

STAT5 was Activated by oPrl in All Culture Conditions

It has been repeatedly observed that after the binding of Prl to its membrane receptor, STAT5 is tyrosine-phosphorylated in the cytoplasm and translocated to the nucleus. The binding of the phosphorylated form of STAT5 to its specific binding site in the proximal promoter of β -or αs 1-casein genes is a necessary step to activate transcription in cultured cells. The lack of expression of the αs 1-casein gene in cells cultured on plastic could result from an impaired STAT5 phosphorylation under Prl stimulation. To evaluate this hypothesis, activation of STAT5 by oPrl was assayed in vitro by EMSA using a specific DNA probe, which binding is characteristic of tyrosine-phosphorylated STAT5 (also called activated STAT5). The probe was the rabbit αs 1-casein proximal binding site (−104, −85; called αs 1 cas-STAT5) previously identified as a specific STAT5 binding site [Pierre et al., 1994]. When EMSAs were carried out with extracts from cells treated for 48 h by oPrl (similar to cells that were used in parallel for Northern blot analysis), the αs 1 cas-STAT5 complex was hardly detected in all culture conditions (data not shown). Previous kinetic studies carried out using oPrl treated mammary cell HC11 [Wartmann et al., 1996] or

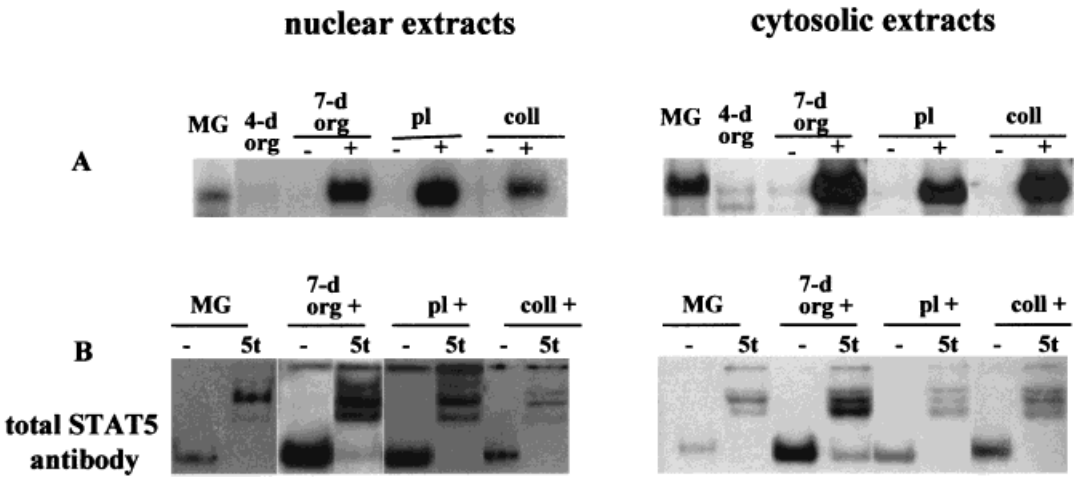

Fig. 3. αs 1 cas-STAT5 binding of nuclear and cytosolic proteins from organoids and isolated cells cultured on plastic or collagen. EMSAs were performed with nuclear (NE) or cytosolic extracts (CE) using αs 1 cas-STAT5 as probe. Each panel represents the data obtained within one culture. Cells were prepared as described in legend of Figure 1. MG: pregnant mammary gland, 4-d org: organoids cultured for 4 days, 7-d org: organoids cultured for 7 days, pl: isolated cells cultured on plastic, coll: isolated cells cultured on collagen. Organoids and cells were treated or not by oPrl for 15 min on Day 7 of culture (+ or −) then collected for nuclei and cytosol preparation. **A**: EMSAs performed with 9 μg of NEs and 19 μg of CEs. The length of exposure was similar for all samples. **B**: supershift characterization of αs 1 cas-STAT5 complexes in oPrl stimulated NEs or CEs. EMSAs were performed with 9–19 μg of nuclear or cytosolic proteins. One μl (1μg) of antibody directed against both STAT5a and STAT5b (total STAT5 or 5t) was added in each reaction mixture. The length of exposure was adapted in each sample to get optimum intensity of the signal on the autoradiography.

in vivo oPrl stimulated rat mammary gland [Jahn et al., 1997] showed that maximum Prl activation of STAT5 was reached after 15–30 min and then rapidly disappeared. Consequently, to obtain optimum STAT5 activation, the rabbit mammary cells were cultured without oPrl for 48 h and then treated for 15 min by oPrl before NEs and CEs were prepared.

An αs 1 cas-STAT5 complex was observed in the NEs and CEs from pregnant rabbit mammary glands, from organoids and from isolated cells cultured on plastic or collagen and stimulated by oPrl for 15 min (Fig. 3A). In all NEs or CEs, the αs 1 cas-STAT5 complex was supershifted by the antibody directed against STAT5 (Fig. 3B) which proves that STAT5 was actually present in the complex. Alpha s1 cas-STAT5 complex was not detected in cells cultured for 4 days without oPrl. Hence, STAT5 was activated by oPrl in cells which expressed the αs 1-casein gene (organoids and isolated cells cultured on collagen) or not (isolated cells cultured on plastic). This indicates that the Prl receptor was always present and retained its ability to induce STAT5 activation. In isolated epithelial cells cultured on plastic, this activation was not sufficient to induce αs 1-casein gene expression.

Prl Activated STAT5 Isoforms Differed With the Culture Conditions

One possible explanation of the data reported above is that, according to culture conditions, different STAT5 isoforms were activated under oPrl stimulation. In the various species previously studied (mouse, rat, human, pig, cow), two isoforms of STAT5 have been identified: STAT5a and STAT5b (see review in Rosen et al., 1999). These two isoforms may not play identical roles in the expression of milk protein genes in vivo [Teglund et al., 1998]. In the rabbit, the STAT5 gene has not yet been cloned. The STAT5a and STAT5b isoforms may exist in the rabbit, as in other species, but it is not known whether they are simultaneously activated in the different experimental conditions. Two antibodies directed specifically against the C-terminal end of STAT5a or STAT5b were used (Fig. 4). Unexpectedly, results varied from one culture to another. Figure 5 reports the data obtained from two cultures (Day 14 and 21 culture) chosen among the various analyzed

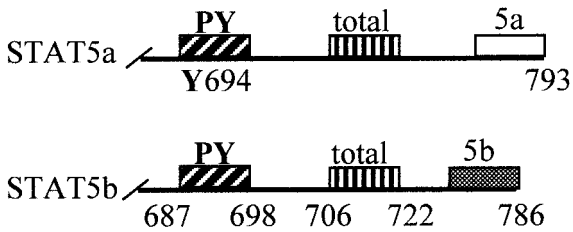

Fig. 4. Representation of the position of STAT5 peptides recognized by the antibodies to the various isoforms of STAT5. The carboxy terminus of STAT5a and STAT5b are represented by boxes indicating the position of the immunopeptides recognized by the antibody to STAT5a (5a), the antibody to STAT5b (5b), the antibody which recognizes both STAT5a and STAT5b (total) and the antibody raised against the peptide including the phosphorylated tyrosine 694 (PY). Numbers represent the position of amino acid residues.

cultures as being representative of the most different patterns. The origin of the variability remains unclear. Each culture was carried out from the mammary gland of one rabbit at 14–21-day of pregnancy. However, the variability was probably not directly related to the stage of pregnancy.

Despite this variability, important features have to be pointed out. First, STAT5b was always detected in NEs and CEs from mammary gland and isolated cells cultured on collagen and treated by oPrl. Second, STAT5a was never found in NEs from isolated cells cultured on plastic and treated by oPrl. Interestingly, in some experiments, STAT5a was detected in CEs of isolated cells on plastic, and not in NEs (see for example pl cells in Day 21 culture, Fig. 5). A defect in the nuclear translocation of activated cytoplasmic STAT5a could explain this, as it has been previously shown that in COS cells, the nuclear translocation of activated STAT5a depended upon the presence of other cofactors [Kazansky et al., 1999]. Third, in several extracts, no supershift was detected in the presence of antibodies to STAT5a or STAT5b, although a clear and total supershift occurred always after addition of the antibody to total STAT5 (an example is given in Fig. 5, Day 14 culture org NE). It is assumed that in these samples, a STAT5-related factor was activated by oPrl, but it was recognized neither by antibody to STAT5a nor to STAT5b. This was observed independently in CEs or in NEs from 7-day organoids (as shown in Fig. 5) or from isolated cells cultured on plastic (not shown) but never from mammary gland or from isolated cells cultured on collagen. In conclusion, the nature of activated STAT5 isoform is clearly modified with the culture conditions. It is

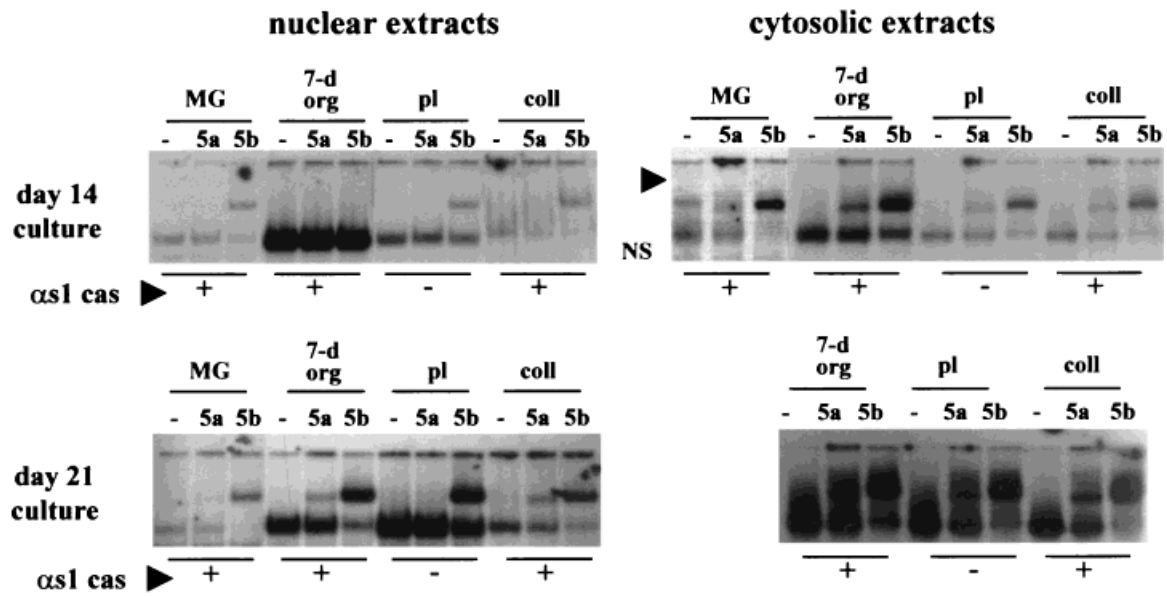

Fig. 5. Supershift characterization of STAT5a and STAT5b in αs 1 cas-STAT5 complex in organoids and isolated cells cultured on plastic or thick collagen gels. Six different cultures were analyzed. Two distinct patterns respectively obtained in Day 14 culture (14-day pregnant mammary gland) and Day 21 culture (21-day pregnant mammary gland) are shown. Nine–nineteen μg proteins were assayed as described in Figure 3. Antibodies to STAT5a (5a) or STAT5b (5b) were added in each reaction mixture (1 μl, 1 μg/sample). The length of exposure was adapted for each sample to ensure an optimum intensity of the signal on the autoradiography. NS; non-specific complex. The expression or the absence of expression of αs 1-casein gene in samples is indicated below by + or −.

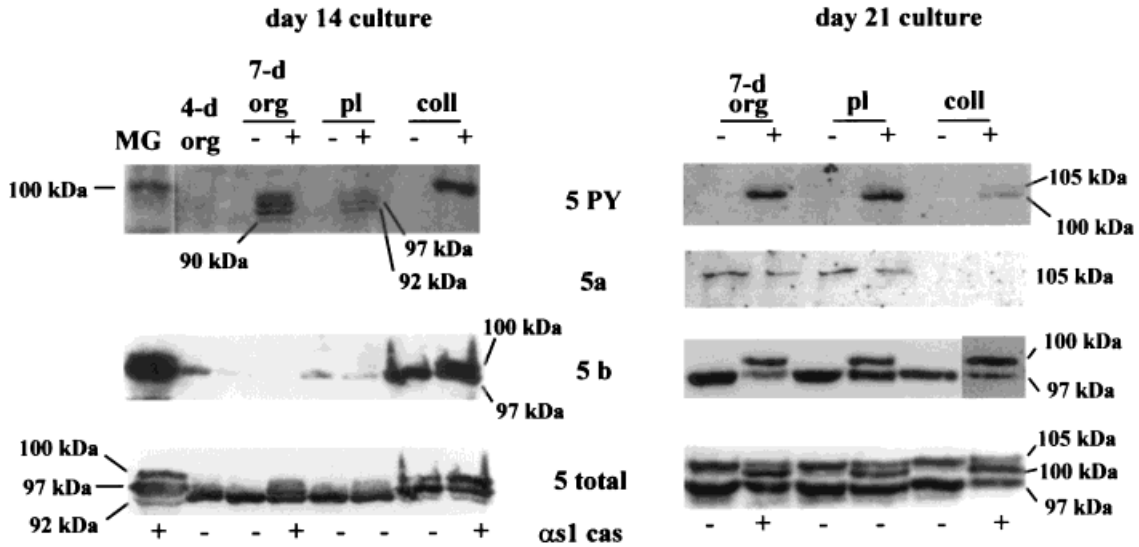

Fig. 6. Western blot analysis of STAT5 proteins in CEs prepared from Day 14 and 21 cultures. Cytosolic extracts were those analyzed in EMSAs. CEs (80 μg) were fractionated by 5% SDS PAGE, transferred to a nitrocellulose membrane and probed with antibodies raised against the phosphotyrosine form of STAT5 (5PY), STAT5a (5a), STAT5b (5b), and both STAT5a and b isoforms (5 total). Molecular weight of proteins (deduced from migration rates of standards) is indicated. MG; dissected pregnant mammary gland. Pl; isolated primary mammary cells cultured on plastic. Coll; isolated mammary cells cultured on collagen. Cells were treated or not by oPrl for 15 min (− or +). Below each sample is indicated the expression (+) or the absence of expression (−) of αs 1-casein gene.

noteworthy that in cells cultured on plastic, neither activated STAT5a nor αs 1-casein mRNA were detected. In contrast, in organoids from several cultures, no activated STAT5a was detected but αs 1-casein mRNA was detected. Thus, the presence of activated STAT5a is not strictly related to the expression of αs 1-casein gene.

To further characterize the activated STAT5 detected using EMSA, we performed a Western blot analysis on CEs. Antibodies to STAT5a, to STAT5b, to total STAT5 and an antibody to PY STAT5, which recognized both phosphotyrosine STAT5a and STAT5b were used (Fig. 4). The latter antibody was expected to reveal the molecular weight of the PY STAT5 characterized in EMSA. In Figure 6 the Western blots obtained respectively from Day 14−21 cultures are given.

A 105 kDa protein was revealed by the antibodies to STAT5a and to PY STAT5 in CES from mammary gland, from organoids and from isolated cells cultured on plastic (Fig. 6, Day 21 culture). It was not detected by the antibody to STAT5a in CEs from cells cultured on collagen, even in CEs when the antibody to STAT5a induced the formation of a supershifted complex in EMSA. More, the 105 kDa protein was not detected in all cultures, as for example in Day 14 culture. It is likely that STAT5a was present in all the samples but at a too low concentration to be detectable by Western blot assay. It is noteworthy that the 105 kDa protein was not always detected in samples prepared from cells which expressed αs 1-casein gene (as for example in Day 21 culture, mammary gland, organoids, and cells cultured on collagen). Thus, the presence of large amounts of STAT5a is not required for αs 1-casein gene expression.

In mammary gland or isolated cells cultured on collagen, STAT5b (97 kDa protein) and PY STAT5b (100 kDa) were always found (Figs. 6, 5b and 5PY panels). In organoids and in isolated cells cultured on plastic, STAT5b (tyrosine phosphorylated or not) was sometimes detected clearly (Fig. 6, Day 21 culture), and sometimes detected in low amounts or not at all (Fig. 6, Day 14 culture). It is important to note that in all the cultures, organoids expressed αs 1-casein gene at a similar level under oPrl stimulation. This strongly suggests that the presence of large amounts of STAT5b is not required for αs 1-casein gene expression.

In organoids and in isolated cells cultured on plastic, multiple STAT5 related isoforms were sometimes detected (Fig. 6, Day 14 culture). A

90-kDa protein was recognized by the antibody to PY-STAT5 (Fig. 6, PY STAT5 panel). A 92 and a 97-kDa proteins were labeled both by the antibodies to PY-STAT5 and to total STAT5. It might correspond to the activated STAT5 observed in EMSA, which was not supershifted after addition of the antibodies to STAT5a and to STAT5b. These STAT5 related isoforms were never detected in isolated cells cultured on collagen or in mammary gland. Hence, the presence of STAT5 related isoforms depended on the culture condition. However, it was not related to the ability of cells to express αs 1-casein gene. Indeed, these forms were detected both in organoids which expressed αs 1-casein gene and in isolated cells cultured on plastic which did not express this gene.

The Binding of Nuclear Factors to the F3-C/EBP Binding Site is Modified With the Culture Conditions

In a previous study [Pierre et al., 1992; Jolivet et al., 1996], we had identified the sequence of one binding site (named F3) homologous to the sequence of the consensus site for the binding of C/EBP [Akira et al., 1990]. This sequence was found in a distal Prl-dependent enhancer located 3442-3307 bp 5′ to the tsp of the rabbit αs 1-casein gene. Here, using EMSA, we investigated whether the F3-C/EBP complexes obtained by incubation of rabbit mammary NEs with the F3-C/EBP probe differed with the culture conditions.

We first investigated whether the F3-C/EBP probe actually bound C/EBPs in mammary nuclear extracts. Data shown in Figure 7 represent the F3-C/EBP complexes obtained with NEs prepared from the mammary gland of 4 pregnant rabbits at Day 14–18 of pregnancy. In all EMSAs carried out with the F3-C/EBP probe and NEs prepared from pregnant mammary gland, two complexes 1 and 2 were detected. The F3-C/EBP complexes were not detected after addition of an excess of a competitor DNA sequence specific for the high affinity binding of C/EBPs [Cao et al., 1991] (lane +cont). Antibodies to C/EBPα, β, and δ were used to define which C/EBP isoform was present in each complex. The antibody to C/EBPα recognized no protein in the F3-C/EBP complexes obtained with the NE from pregnant rabbit mammary gland (lane α), although it induced a supershift in EMSA performed with F3-C/EBP as probe and NEs from lactating rat

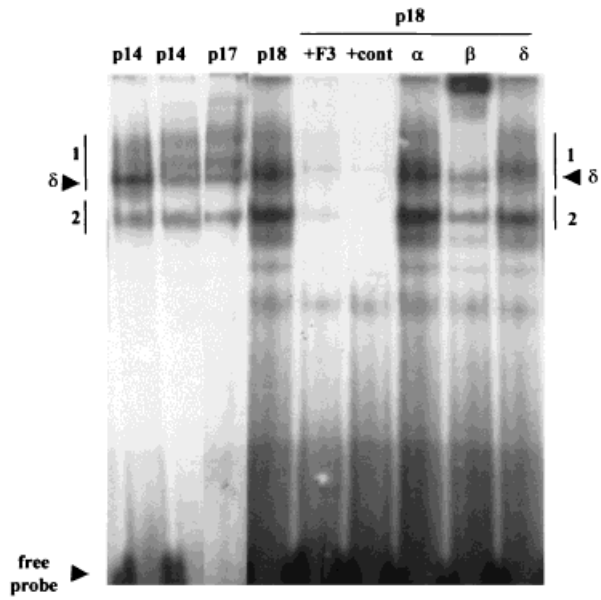

Fig. 7. Binding of nuclear proteins from 4 pregnant rabbit mammary glands to the F3-C/EBP probe. EMSAs were performed with 3–9 μg of NE from 14-, 17-,and 18-day pregnant rabbit mammary glands (p14, p17, p18) using F3-C/EBP as a probe. Non labeled double strand oligonucleotides were added in excess (× 100) as competitors. F3; non-labeled F3-C/EBP oligonucleotide. Cont; non-labeled high affinity C/EBP binding site (Cao et al., 1991]. One μl (1 μg) of antibody was added for supershift reactions. α; antibody to C/EBPα. β; antibody to C/EBPβ. δ; antibody to C/EBPδ.

mammary gland (data not shown). The same antibody to C/EBPα recognized a F3-C/EBP complex neither in NEs of lactating rabbit mammary gland nor in NEs of rabbit primary mammary cells regardless of the culture condition (data not shown). This antibody raised against mouse C/EBPα probably did not recognize rabbit C/EBPα. For this reason, we did not further analyze this C/EBP isoform in EMSA.

After addition of the antibody to C/EBPβ, the two complexes 1 and 2 were strongly modified (laneβ). A higher molecular weight complex (supershifted complex) was simultaneously formed, which hardly entered the gel. After addition of the antibody to C/EBPδ (lane δ), the complex 2 was unchanged, but the lower band of complex 1 was significantly reduced. This suggests that C/EBPβ was involved in the complexes 1 and 2, and C/EBPδ was mainly involved in complex 1.

The two F3-C/EBP complexes 1 and 2 were observed when EMSAs were carried out with the NEs of isolated cells cultured on collagen (Fig. 8A). As in mammary gland NE, complexes

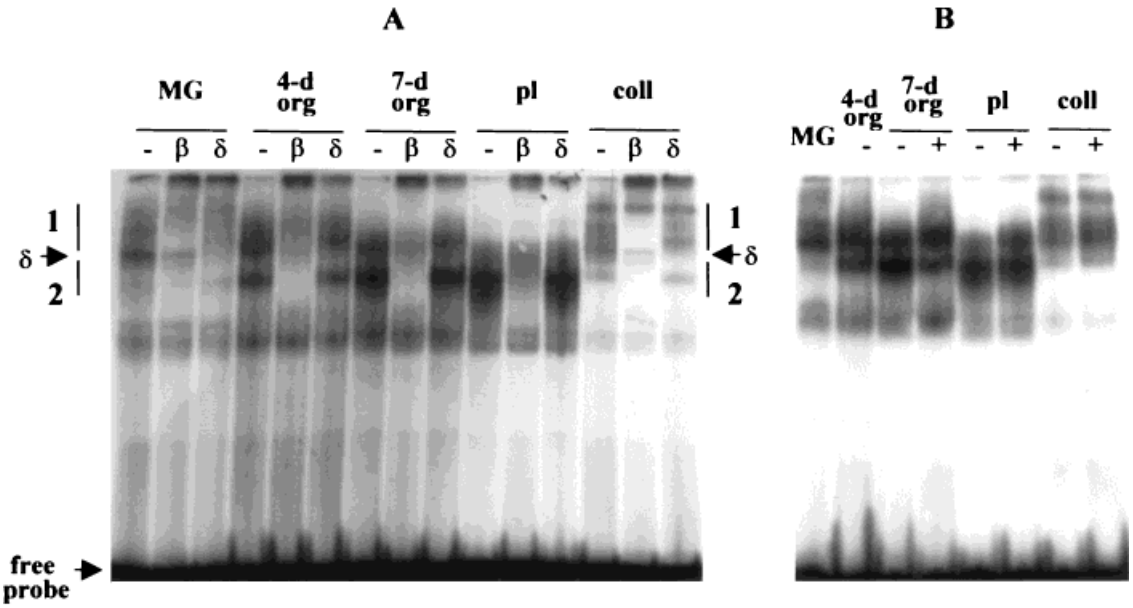

Fig. 8. Binding of nuclear proteins from organoids and isolated cells cultured on plastic or collagen to the F3-C/EBP probe. EMSAs were performed with 6 μg of NEs prepared from rabbit mammary tissue or cultured cells and F3-C/EBP as a probe. Cells and nuclear extracts were prepared as indicated in Figure 1. Each panel represents the data obtained within one culture. These data are representative of all the culture tested from 14–21-day pregnant rabbits. **A:** supershift characterization of F3-C/EBP complexes. One μg of antibody (directed against C/EBPβ or δ) was added in each reaction mixture. Nuclear extracts used in these EMSAs were prepared from organoids or cells not stimulated by oPrl. Similar results were obtained when the NEs were prepared from cultured organoids and cells treated by oPrl for 15 min or 48 h. **B:** F3-C/EBP bound complexes in NEs from MG, organoids and isolated cells cultured on plastic or collagen, treated or not by oPrl for 15 min. NEs (6 μg in each point) were prepared from samples obtained within the same culture.

1 and 2 were composed of C/EBPβ and δ since after incubation with the antibodies to C/EBPβ and δ, these complexes were modified (Fig. 8A). In contrast, the F3-C/EBP complexes were different when the NEs from 4-day organoids, 7-day organoids, and isolated cells cultured on plastic were used (Fig. 8A, B). The broadness of the complex 1 was reduced and simultaneously, the complex 2 was enhanced. These modifications were modest in organoids and marked in isolated cells cultured on plastic. In the latter case, the complex 1 was almost not detected. C/EBPβ was clearly characterized in the complex 1 in organoids, and in the complex 2 in organoids, and in cells cultured on plastic. The addition of the antibody to C/EBPδ induced no modification, which suggests that C/EBPδ was absent in NEs from organoids and from isolated cells on plastic. An oPrl treatment for 15 min (Fig. 8B) or for 48 h (data not shown) had no influence on the pattern of the F3-C/EBP complexes. These modifications were reversible since in isolated cells cultured on collagen which had been prepared from 4-day organoids, the complexes 1 and 2 were similar to those characterized in MG. In conclusion, different F3-C/EBP complexes were detected according to the culture conditions. Interestingly, the complex 1 was absent in cells cultured on plastic, which never expressed the αs 1-casein gene.

A characterization of nuclear C/EBPs was carried out by Western blot analysis of NE. Using the antibody directed against C/EBPα, as it could be expected, no specific signal was detected (data not shown). Thus, the characterization of C/EBPα was not further pursued.

A 35 kDa protein, specifically immunoreactive after incubation with the antibody to C/EBPδ, was detected in all NEs prepared from the mammary gland of pregnant rabbits, from organoids, or from isolated cells cultured on plastic or collagen, and treated or not by oPrl (Fig. 9, C/EBPδ panel). The molecular weight of this protein was similar to that of the mouse C/EBPδ previously identified in the mouse mammary cell line HC11 [O'Rourke et al., 1997]. Thus, this protein is most likely the rabbit C/EBPδ protein. Clearly, Figure 9 indicates that the amount of C/EBPδ was

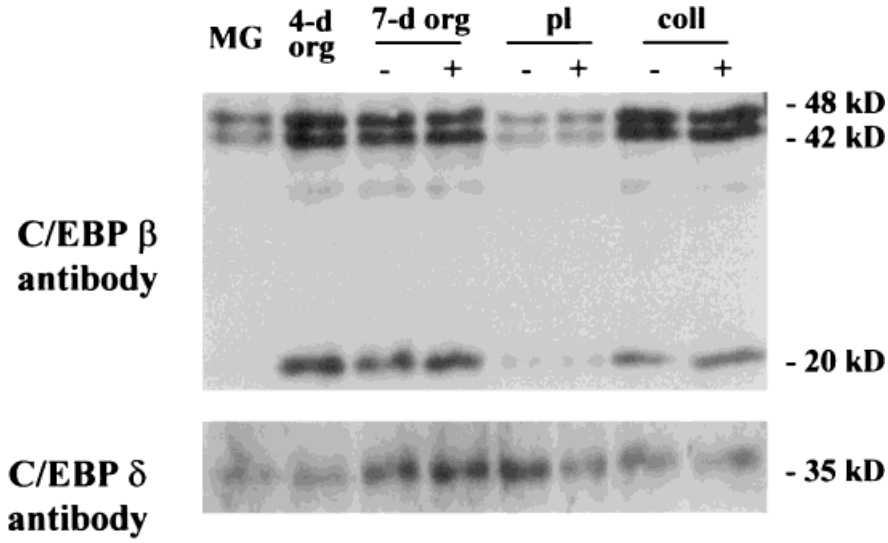

Fig. 9. Western blot of C/EBP proteins in mammary cell NEs. NEs were prepared from cells cultured as previously described in Figure 1. Aliquots of nuclear proteins (25 μg) were fractionated by SDS-12% PAGE, transferred to a nitrocellulose membrane and probed with antibodies raised against C/EBPβ or C/EBPδ. Molecular weight of proteins (deduced from migration rates of standards) is indicated.

unaffected by the culture conditions and the hormonal treatment.

The antibody to C/EBPβ labeled three proteins (48, 42, and 20 kDa) in the NEs from organoids and isolated cells (Fig. 9). The 20-kDa protein was not detected in NE from MG. With a longer exposure of the blot, this 20 kDa protein was hardly visible (not shown). The labeling of these proteins was totally inhibited after addition of an excess of the C/EBPβ peptide used to obtain the antiserum (data not shown). In rats and in mice, two isoforms of C/EBPβ have been characterized [Descombes and Schibler, 1991]: a LAP isoform (liver-enriched transcriptional activator protein), which has a stimulatory activity on the transcription of all its target genes, and a LIP isoform (liver-enriched transcriptional inhibitor protein) which has an inhibitory activity. In whole cell extracts from mouse and rat mammary gland, a 35 kDa protein was referred to as the LAP isoform, a 20 kDa protein as the LIP isoform [Raught et al., 1995], and a 45 kDa as a cross-reactive material which reacts specifically to the antibody but which was not generated by the transcription of the C/EBPβ gene [Robinson et al., 1998]. The antibody to C/EBPβ used in the present study could recognize both mouse LAP and LIP isoforms. In rabbit primary mammary cell NEs, the 20-kDa protein is a candidate to be LIP. No specific labeling was detected around 35 kDa as in the rat or the mouse (the faint labeling detected around 30 kDa was not specific). The 48 and 42 kDa proteins likely represent two other isoforms of the rabbit C/EBPβ.

The amount of the three C/EBPβ isoforms was similar in NEs from organoids and in NEs from isolated cells cultured on collagen (Fig. 9). In contrast, it was strongly reduced in NEs from isolated cells cultured on plastic. Fifteen minutes-oPrl treatment induced no modification. Clearly, this study indicates that the protein level of the three isoforms of C/EBPβ was modified according to the culture conditions. In isolated cells cultured on plastic, which did not express αs 1-casein gene, the amount of C/EBPβ was at the lowest.

DISCUSSION

The present paper shows that isolated rabbit mammary cells expressed the αs 1-casein gene under oPrl stimulation only when cultured on a thick floating collagen gel (Fig. 1). As previously shown in primary mouse mammary cell cultures, Prl induced expression of casein is tightly regulated by the presence of ECM. In all cases, apoptosis of the rabbit cultured epithelial cells was limited and not dependent on culture conditions (Fig. 2). This points out a main difference between rabbit and mouse mammary cell cultures. In mouse, a marked apoptosis occurred as soon as cells were cultured on plastic and EHS prevented this phenomenon

[Pullan et al., 1996]. Isolated rabbit mammary cells cultured on plastic were probably much less susceptible to apoptosis than mouse mammary cells.

The first important observation of this paper is that in cultured rabbit mammary cells, oPrl activated STAT5 in all conditions, even in isolated cells cultured on plastic although they did not express the αs-1casein gene. This fact was already reported in a previous work using rabbit primary mammary cell cultures [Tourkine et al., 1995]. In contrast, in isolated mouse mammary cells cultured on plastic and stimulated by Prl, neither EMSA nor Western blot analysis made it possible to characterize activated STAT5 able to bind to a specific STAT5 binding sequence, or PY STAT5, [Streuli et al., 1995]. Species-specific differences may explain this discrepancy.

In isolated mouse mammary cells cultured on collagen I, Prl did not induce STAT5 activation [Streuli et al., 1995; Edwards et al., 1998]. In contrast, in isolated rabbit mammary cells cultured on collagen, oPrl activated STAT5 was always detected. Both data are apparently inconsistent. However, it should be stressed that collagen I coated dishes were used in mouse mammary cell cultures whereas thick floating collagen I gel was used in rabbit mammary cell cultures. Therefore, the cells were not cultured on the same matrix despite the presence of collagen I in both experiments.

A second important observation is that the presence of activated STAT5a and STAT5b was variable with the culture conditions. Moreover, Western blot analysis made it possible to characterize in some organoids and isolated cells cultured on plastic additional STAT5 isoforms, some of them having lower molecular weight than the full-length 105 kDa STAT5a and 97 kDa STAT5b. These isoforms were probably tyrosine phosphorylated under oPrl stimulation as the full-length STAT5. The origin of the low molecular weight forms remain unknown. It was previously shown that carboxy-terminally deleted forms of STAT5 were generated in vitro during the preparation of mammary cell extracts, presumably by the action of proteases which were not fully inhibited by the protease inhibitors added to the extraction buffer [Garimorth et al., 1999]. However, the fact that in some rabbit mammary cell cultures (as in Day 21 culture, Fig. 6) the low molecular weight STAT5 isoforms were never detected strongly suggests that proteolysis did not systematically occur during extraction.

Alternatively, it can be suggested that the STAT5-related isoforms were generated in vivo by the cleavage of the C-terminal end of the full-length STAT5. Likewise, the presence of carboxyl-truncated STAT5 isoforms has already been reported in early hematopoietic or myeloid cell lines [Wang et al., 1996; Azam et al., 1997; Meyer et al., 1998]. These isoforms were generated by the cleavage of the C-terminal end of STAT5 under the activity of specific cytoplasmic or nuclear proteases [Lee et al., 1999]. These natural carboxyl-truncated variants of STAT5a and STAT5b were tyrosine phosphorylated as the full-length STAT5 under hormonal stimulation. Interestingly, they were potent competitive inhibitors of full-length PY STAT5 [Wang et al., 1996]. In contrast, the present study shows that the STAT5 related isoforms had no significant inhibitory activity, since they were detected in similar amounts in organoids which expressed the αs 1-casein gene and in isolated cells on plastic which did not express this gene.

Interestingly, the STAT5 related isoforms were never detected in large amounts in MG or in isolated cells cultured on collagen, as though collagen prevented their generation. Likely, the presence of the STAT5 related isoforms was the consequence of the culture on plastic. In organoids, a transitory situation was encountered. Organoids exhibited both the characteristics of cells cultured on plastic (such as the presence of STAT5 related isoforms) and of cells cultured on collagen (such as the expression of the αs 1-casein gene).

The third important observation of this paper is that large amounts of full length activated STAT5a or STAT5b were not required for the expression of αs 1-casein gene by rabbit mammary cells. In the absence of full length STAT5a and STAT5b, the other STAT5 related isoforms mediated Prl transduction (as for example in organoids presented in Figs. 5 and 6, Day 14 culture, which expressed αs 1-casein gene under oPrl stimulation). However, in cells cultured on plastic, the presence of the STAT5 related isoforms was not sufficient to induce αs 1-casein gene expression. These data strongly suggest a) that the expression of the αs 1-casein gene is not strictly related to the presence of one or the other STAT5 isoform and, b) that an essential

pathway dependent on ECM was not activated in cells cultured on plastic.

The present study shows that C/EBPs are probably involved in this pathway. It is described for the first time that in rabbit primary mammary cell cultures, a modification of ECM coincides with modifications of F3-C/EBP complexes. Despite these modifications, C/EBPβ was still the main component of these complexes, C/EBPδ being not clearly detected. Opposite results have previously been obtained in the mammary cell line CID-9, subline of the spontaneously immortalized mouse mammary epithelial cell line COMMA 1D. In CID-9 cells, the migration pattern of C/EBP complexes was similar whether cells were grown on EHS or on plastic [Myers et al., 1998]. However, CID-9 cells were able to respond to ECM since the expression of transfected reporter genes directed by the bovine β-casein regulatory sequences was enhanced by EHS. One possible explanation for this discrepancy is that the present work was carried out in rabbit primary cells instead of mouse immortalized cells.

The modifications of the pattern of F3-C/EBP complexes between isolated cells cultured on plastic or on collagen deserve comments. The Western blot analysis revealed that the same C/EBPβ isoforms (20, 45, and 48 kDa) were present in both culture conditions. The only difference was the significantly reduced amount of these three isoforms in cells cultured on plastic. Considering that all C/EBP isoforms can bind to the same C/EBP probe as homodimers and heterobimers, and that the number and the migration rate of the C/EBP DNA complexes are tightly related to the relative amount of each C/EBP isoform [Descombes and Schibler, 1991; An et al., 1996; Osada et al., 1996], it is probable that the modifications of the F3-C/EBP complexes result from the reduction of the amount of the three isoforms of C/EBPβ. Alternatively, a modification of the binding affinity of C/EBPs for their specific recognition sequence occurred. Indeed, it has already been reported that the binding of C/EBPs to DNA may change without any concomitant modification of the amount of nuclear C/EBPs. During differentiation of adipocytes, the binding activity of C/EBPs increased progressively whereas the nuclear amount of C/EBPs was not altered [Tang and Lane, 1999]. The binding affinity of C/EBPs for their DNA recognition sequence is probably modulated by serine phosphorylation under protein kinase A or C activation, numerous potential serine phosphorylation sites being present in C/EBPs [Mahoney et al., 1992; Trautwein et al., 1993]. Hence, it should be of interest to study whether post-transcriptional modifications of C/EBPs occurred differently in organoids and in isolated cells cultured on plastic or collagen.

Interestingly, the 20 kDa protein (presumably the LIP isoform) was absent or detected in very low amounts in NE from mammary gland, although it was clearly characterized in NE from cultured cells and organoids. In the mouse and the human, it has been proposed that LIP and LAP are both translated from the same C/EBPβ mRNA, by alternative utilization of internal AUG codons [Descombes and Schibler, 1991]. Alternatively, LIP was suggested to be generated by in vitro proteolysis of C/EBPβ [Baer and Johnson, 2000]. In the present study, the origin of the 20 kDa-LIP protein was not elucidated, but its presence was clearly related to the fact that cells were cultured, whatever the culture conditions. LIP was previously characterized as an inhibitory transcription factor. Its abundance in organoids and in isolated cells cultured on collagen (which both expressed αs 1-casein gene under oprl stimulation) is thus unexpected. However, it has been previously suggested that in HC11 cells, the transcriptional activity of β-casein gene was determined by the relative amount of LAP and LIP [Raught et al., 1995]. Hence, in organoids and isolated cells cultured on collagen, the stimulatory activity of other C/EBPβ forms may have counteracted the inhibitory activity of the 20 kDa LIP. We suggest that these stimulatory forms were the 45 and 48 kDa proteins characterized in Western blot analysis (Fig. 9). In agreement with this hypothesis, the 45 and 48 kDa proteins were abundant in NEs from mammary gland, organoids and isolated cells cultured on collagen, which all expressed the αs 1-casein gene. In contrast, they were hardly detected in isolated cells cultured on plastic, which did not express this gene.

Finally, an important observation of this study is that the modification of the binding activity of C/EBPs and more precisely, of the nuclear amount of 45 and 48 kDa C/EBPβ isoforms, is correlated to αs 1-casein gene expression. It is therefore probable that these C/EBPs isoforms, which amount is modified with ECM are involved in ECM regulation of αs

1-casein gene expression. In contrast, STAT5, which binding activity is also influenced by ECM, is not directly involved in this pathway. This mechanism differs from that previously described in mouse mammary epithelial cells [Edwards et al., 1998]. In mouse mammary cells, ECM regulated STAT5 activation by Prl, and these modifications of STAT5 activity regulated directly casein gene expression. In conclusion, this work shows that in rabbit mammary cells, at least two independent signaling pathways must be simultaneously activated to induce αs 1-casein gene expression: one is induced by Prl through STAT5 activation and the other by ECM, probably in part through C/EBPs. These two independent pathways must combine their effects in promoter and enhancer elements of milk protein genes which include both STAT5 and C/EBP binding sites, such as the proximal promoter of β-casein gene [Raught et al., 1995], or the distal enhancer of bovine [Myers et al., 1998] and rabbit [Jolivet et al., 1996] αs 1-casein gene.

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