A MGF/STAT5 binding site is necessary in the distal enhancer for high prolactin induction of transfected rabbit αs1-casein–CAT gene transcription

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Abstract The rabbit *αs1-casein* gene contains a distal prolactin-dependent enhancer 3442–3285 bp 5′ to the site of initiation of transcription. We have reported previously that four DNA/protein-binding sites (F1–F4) are located within this distal enhancer. We now show that one of this binding site (the F4 site) binds in vitro a MGF/STAT5-like factor. The functional importance of the F4 site was estimated by cotransfection of CHO cells with a chimeric gene containing or not the F4 sequence linked to the (−391/+1774)*CAT* gene and a plasmid encoding the rabbit mammary prolactin receptor. The F4 site is necessary for maximal response of the enhancer to prolactin. However, this site has to be associated to the F1–F3 fragment. It can be replaced by a genuine MGF/STAT5-binding site. A mutational analysis indicates that F4 and F1 sites are simultaneously involved to confer a high prolactin sensitivity.

Key words: Mammary gland; Primary cell; MGF/STAT5; αs1-casein; Milk protein gene expression; Electrophoretic mobility shift assay

1. Introduction

Milk protein gene expression is regulated in the mammary gland by various factors, involving hormones (mainly prolactin and glucocorticoids) and the extracellular matrix. Several gene-regulatory elements involved in the regulation of milk gene transcription have already been identified and a characterization of the nuclear factors bound to these elements has been attempted in some cases. Among these factors, the mammary gland factor (MGF) has been characterized [1], purified [2] and cloned in sheep [3], mice [4,5], rats [6] and humans [7]. This factor belongs to the multigene family of signal transducers and activators of transcription (STAT family), and was renamed STAT5. The MGF/STAT5 factor is clearly involved in the prolactin activation of several milk protein gene transcription in various species. A functional MGF/STAT5-binding site has been identified in the proximal promoters in the rat β-*casein* gene [1], in the rabbit *αs1-casein* gene [8], in the β-*lactoglobulin* gene [9]. In these studies, it was demonstrated that the binding of MGF/STAT5 or of a MGF/STAT5-like nuclear factor to the corresponding proximal binding site is necessary for prolactin sensitivity of the milk protein gene promoter.

Despite the fundamental importance of the proximal promoter in the regulation of milk protein gene transcription, it has been reported that the presence of more upstream regulatory gene elements is essential for high transcription levels in several genes. This was described in the bovine β-*casein* gene [10], in the rabbit *αs1-casein* gene [11] and in the rabbit [12] or rat whey acidic protein (*WAP*) gene [13]. We have previously identified four rabbit mammary gland nuclear factor-binding regions within the distal regulatory −3442/−3285 fragment in the rabbit *αs1-casein* gene [8]. In the present study, we provide evidence that one of these binding sites (F4 (−3333/−3307) site) is a genuine MGF/STAT5-binding site and that binding of a MGF/STAT5-like nuclear factor to the F4 site is necessary for the maximal enhancer activity of the distal regulatory element.

2. Materials and methods

2.1. Cell culture and transient expression procedure

CHO K1 cells were grown and transfected as previously described [14] with modifications [11]. Transfection of plasmid DNA was performed using the calcium phosphate procedure, with 40 μg DNA in each precipitate. Each precipitate was obtained with 6 μg of the CAT plasmid to be tested, 12 μg of the pCH110 plasmid coding for β-galactosidase (Pharmacia), 12 μg of the plasmid pER2-3 carrying the rabbit mammary prolactin receptor cDNA [15], and 10 μg of carrier DNA (genomic sonicated DNA from salmon testes). After 2–5-h incubation in the presence of the precipitate, the cells were washed and cultured in serum-free medium [14] containing hormones. Two dishes were incubated with cortisol (60 nM). Cortisol and ovine prolactin (16 nM; NIH, Bethesda, MD) were added to two other dishes. Cells were harvested 72 h later. CAT assays were performed as previously described [11]. CAT values were normalized to β-galactosidase activity [16]. The prolactin fold inductions were calculated as the ratio of normalized CAT/β-galactosidase activity in prolactin-plus cortisol-treated cells versus cortisol-treated cells.

2.2. Rabbit primary mammary cell culture

Organoids were isolated from the mammary gland of one 18-day pregnant rabbit by limited collagenase (200 U/ml) and hyaluronidase (200 U/ml) digestion for 90 min as previously described [12]. They were seeded on plastic dishes in DMEM–Ham's F12 medium (1 : 1) supplemented with 2% UltroSer SF (Biosepra, France) as serum substitute devoided of steroids and prolactin. Organoids prepared from one mammary gland were seeded on 40 dishes (10 cm diameter) then treated 4 days later for 10 min by insulin (5 μg/ml) or by insulin (5 μg/

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ml) plus ovine prolactin (1 μg/ml). The dishes were then rapidly frozen on dry ice and kept at −80°C until preparation of nuclear extracts.

2.3. Preparation of nuclear extracts

Nuclear extracts were prepared from lactating mammary glands or from cultured mammary cells. The mammary tissue was rapidly dissected to eliminate most of the connective and muscular tissue, then frozen in liquid nitrogen and stored at −80°C.

Nuclei were prepared from frozen mammary tissue as described [17] with modifications. Two buffers (A: 10 mM HEPES (pH 7.7), 50 mM KCl, 2 mM EDTA, 10% glycerol, 0.25 M sucrose; B: similar to A with 0.9 M instead of 0.25 M sucrose) were prepared and kept at 4°C. Extemporaneously, dithiothreitol (0.5 mM DTT), spermine (0.15 mM), spermidine (0.5 mM), protease inhibitor (0.5 mM phenylmethylsulfonyl fluoride (PMSF)) and phosphatase inhibitors (1 mM sodium fluoride, 1 mM sodium vanadate and 10 μM ammonium molibdate) were added to these buffers. All steps were carried out at 4°C. Frozen tissue was pulverized in liquid nitrogen. Buffer A (10 vol/g of frozen tissue) was added onto the frozen powder. The mixture was then homogenized by 4–8 strokes in a motor-driven glass/teflon Potter. The homogenate was filtered on 5 layers of gauze. The resulting homogenate was layered on a 0.9 M sucrose cushion (buffer B). Nuclei were pelleted by centrifugation for 10 min at 3500 rpm.

Nuclei were prepared from frozen cells as follows. Frozen scraped primary cells from 10 dishes were pooled and thawed at 4°C in buffer 1 (10 mM HEPES, pH 7.7, 25 mM KCl, 2 mM EDTA, 0.5 mM EGTA, 0.6 M sucrose, 0.8% NP40, with the same protease and phosphatase inhibitors as previously indicated for mammary gland nuclei preparation) in order to attain a final 10–15 ml volume. After Dounce homogenization (10–15 strokes), the mixture was layered on 10 ml of buffer 2 (same as buffer 1 but with 1 M sucrose and without NP40). The nuclei were pelleted by centrifugation for 10 min at 3500 rpm.

Nuclear extracts were prepared essentially as described [18] with modifications as previously reported [8].

2.4. Partial purification of rabbit mammary gland MGF/STAT5-like factor by oligonucleotide affinity

Three millilitres of nuclear extract from lactating mammary gland were incubated overnight at 4°C with 500 μl of streptavidine agarose coupled with a biotinylated double-stranded W oligonucleotide (Genset, France) the sequence of which matches the −104/−85 position of the rabbit *αs1-casein* gene. As presented in Fig. 1, the sequence of the W oligonucleotide is homologous to the sequence of the consensus for the MGF/STAT5-binding site [8]. The agarose was then washed by 20 ml of buffer D (20 mM Hepes, pH 7.7, 100 mM KCl, 0.2 mM EDTA, 2% glycerol) containing protease inhibitors and sonicated salmon sperm DNA (200 μg/ml) as non-specific competitor. The specific bound factors were eluted twice with 500 μl of buffer D containing 500 mM KCl.

2.5. Electrophoretic mobility shift assay

Electrophoretic mobility shift assay (EMSA) was performed as previously described [8]. Briefly, nuclear extracts were incubated at room temperature for 30 min with 0.5 μg of poly dI–dC/poly (dI–dC, Pharmacia) as non-specific competitor in the reaction mixture with the following final salt concentrations: 20 mM Tris-HCl, pH 7.6, 80 mM KCl, 8% Ficoll, 6% glycerol, 0.2 mM EDTA, 0.5 mM DTT. In competition experiments, a 100-fold molar excess of probe competitor was added. The sequences of the competitor oligonucleotides are given in Fig. 1. In supershift experiments, 1 μl of rabbit immune serum directed against rabbit MGF/STAT5-like factor [19] or anti-phosphotyrosine antibody (Upstate Biotechnology, NY) was added with the nuclear extract to the binding buffer. After addition of 30 000 cpm (30 pg) of a 5′-labelled double-stranded probe, incubation was performed at room temperature. After electrophoresis on a 6% acrylamide/bis-acrylamide non-denaturing gel in 0.25× TBE buffer, the gel was transferred on DE81 paper, dried and exposed for autoradiography.

2.6. Plasmid constructions

The plasmid pac(−391/+1774)CAT [8] consisted of 391 bp of the 5′-flanking region of the rabbit *αs1-casein* gene, the first non-coding exon (49 nt), the first intron (1719 nt), 5 nt of the second exon (not including the initiation site of the translation), the coding region of the *CAT* gene (including the initiation site of translation), and the polyadenylation signal of Simian virus-40 early genes cloned in the pPolyIII vector [20].

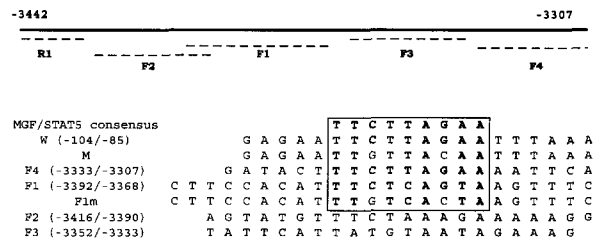

Fig. 1. Sequence of the oligonucleotides used in EMSA experiments. Sequences of the sense strands are given with the consensus sequence previously established for binding of the MGF/STAT5 factor [1]. Box and bold letters indicate the highly homologous region. The positions of the F1, F2, F3 and F4 protected areas in the distal regulatory element (−3442/−3307) are indicated [8]. In M and F1m oligonucleotides, mutated nucleotides are indicated in light letters.

A −3442/−3333 fragment of rabbit *αs1-casein* gene was synthesized by polymerase chain reaction (PCR) using R1 (5′-TGTGAACT-GTCTCAGGTTCC-3′) and C (5′-CTTTCTATTACATAATGAA-TA-3′) (complementary to the F3 oligonucleotide, see Fig. 1) as primers and pac(−3772/+1774)CAT [8] as template. This fragment was subcloned in the *Eco*RV site in pBluescript KSII+ (Stratagene) to generate the S plasmid. Oligonucleotides F4, W or M presented in Fig. 1 were introduced in the *Eco*RI site of the S plasmid after blunting by Klenow polymerase. The new generated plasmids were named S–F4, S–W or S–M. The S and S–F4 plasmids were digested by *Eco*RI and *Hin*dIII, the S–M and S–W plasmids were digested by *Hin*dIII and *Pst*I. The inserts were purified by gel electrophoresis, blunt-ended by Klenow polymerase or T4 DNA polymerase, then subcloned in the *Bgl*II site of pac(−391/+1774)CAT located at the 5′-border of the *αs1-casein* gene fragment.

A double mutation was created in the F1 region by site-directed mutagenesis by PCR using two complementary oligonucleotides F1m (5′-CTTCCACATTTGTCACTAAGTTTCCT-3′) and Am (5′-AG-GAAACTTAGTGACAAATGTGGAAG-3′) whose sequences are homologous to the sequence −3393/−3368 of the rabbit *αs1-casein* gene with one G instead of one C at position −3382 and one C instead of one G at position −3378. The mutated −3442/−3118 fragment was subcloned in the *Bam*H1 site of the polylinker in pBlCAT2 [21]. The mutated −3442/−3333 fragment was obtained by PCR using R1 and C as primers and, as template, the pBLCAT2 containing the mutated −3442/−4118 fragment. The −3442/−3333 fragment was then subcloned in the *Eco*RV site of pBluescript KSII+ to generate the M plasmid. The F4 double-strand oligonucleotide was inserted in the *Eco*RI site of the M plasmid after blunting by Klenow polymerase. The generated plasmid F4–M was digested by *Eco*RI and *Hin*dIII. The insert was purified by gel electrophoresis, blunt-ended by Klenow polymerase then subcloned in the *Bgl*II site of pac(−391/+1774)CAT.

3. Results

3.1. The F4 (−3333/−3307) fragment and MGF/STAT5-binding sequence W (−104/−85) in the proximal promoter of the rabbit αs1-casein gene bind in vitro similar nuclear factors

The sequence of the F4-binding site in the distal enhancer presents several homologies with the MGF/STAT5-binding consensus and with the W sequence (Fig. 1). We have previously supposed that the W sequence could bind a rabbit mammary gland MGF/STAT5-like nuclear factor [8]. In a first attempt to identify the nuclear factors which bind to the F4 site, we compared the properties of F4- and W-bound complexes in EMSA. Nuclear extracts prepared from lactating rabbit mammary glands or from primary mammary cells were used. Competitions were carried out with various oligonucleo-

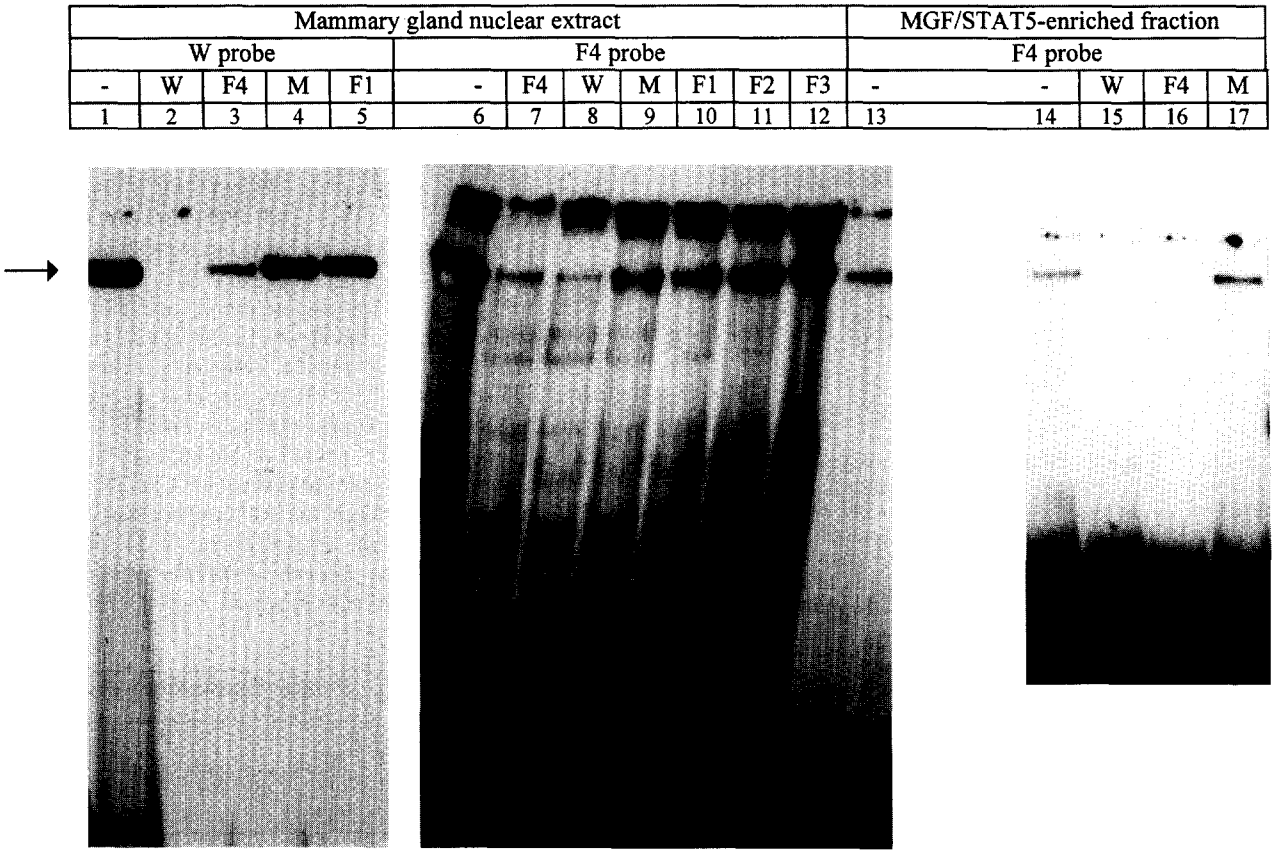

Fig. 2. Binding of nuclear factor to F4 (−3333/−3307) and W (−104/−85) probes. EMSA using mammary gland nuclear extracts was performed with 2.4 μg of proteins in each point with W (lanes 1–5) or F4 (lanes 6–12) as probe. Three microlitres of the affinity column eluted fraction containing partially purified MGF/STAT5-like nuclear factor (mixed with 8 μg of serum bovine albumin in each point) were used in EMSA with F4 as probe (lanes 13–17). The name of each competitor oligonucleotide (100× molar excess of non-labelled double-stranded oligonucleotide) is indicated on top of each lane. The arrow indicates the position of the specific complex. The length of exposure was adapted in each experiment to obtain approximatively similar signals for the specific bands on the autoradiographs.

tides, including F1, F2 and F3, the sequences of which correspond to mammary gland nuclear factor-binding regions within the distal element.

After incubating nuclear extracts from lactating mammary gland with a F4 probe, a retarded complex with a low migrating rate was detected in EMSA (Fig. 2, lane 6). F4 and W oligonucleotides were efficient competitors (lanes 7 and 8). M oligonucleotide (which differs from W by two point mutations) and F1 oligonucleotide were poor competitors (lanes 9 and 10). The heterologous oligonucleotides F2 and F3 had no effect (lanes 11 and 12). Several other complexes with higher migrating rates were visible at the bottom of the gel; they were greatly reduced after addition of various oligonucleotides, except F3. The nature of these complexes remains unclear and was not studied.

When a partially purified MGF/STAT5-like nuclear factor was incubated with F4 as probe, one retarded complex was observed (lane 13). This complex comigrated with that observed with mammary gland nuclear extracts (compare lanes 6 and 13). F4 or W oligonucleotides were efficient competitors. The addition of M oligonucleotide did not modify the binding (lanes 15–17).

As expected, one retarded complex was observed after incubation of W as probe with nuclear extracts from lactating mammary gland (lane 1). A complete competition was observed in the presence of W and F4 oligonucleotides. F1 was a poor competitor. These observations led us to conclude that the F4- and W-bound factors have identical electrophoretic migration rates and DNA-binding specificity.

To further characterize the nature of F4-bound factors, we used the MGF/STAT5-specific antiserum [19]. The F4-bound complex was shifted after addition of the immune serum (Fig. 3, lane 2), indicating that the F4-bound complex and the MGF/STAT5 factor are immunologically related. Moreover, the complex diminished after addition of an antiphosphotyrosine antibody (lane 3), indicating that the bound component is probably tyrosine phosphorylated, as previously described for the rat [22] or rabbit [19] MGF/STAT5 factor. In conclusion, the similarities between the DNA-binding specificity of F4- and W-bound factors as well as the result of the immunological characterization indicate therefore that the F4 fragment binds in vitro a MGF/STAT5-like factor.

3.2. The binding of a MGF/STAT5-like factor to F4 is inducible by prolactin in cultured mammary gland cells

Nuclear extracts from cultured rabbit mammary cells were incubated with F4 as probe and analysed in EMSA. A low migration rate retarded complex was observed only after prolactin treatment of the cells (Fig. 4, lanes 2–3). F4 or W oligonucleotides were efficient competitors (lanes 5 and 6), as it was observed for a lactating mammary gland nuclear extract or the partially purified MGF/STAT5-like nuclear fac-

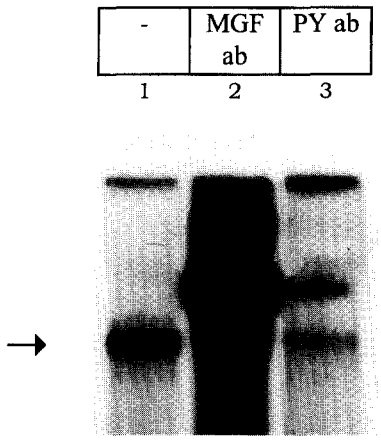

Fig. 3. Immunological identification of the complex formed with the F4 sequence and mammary gland nuclear extract. EMSA was performed with F4 as probe and 2.4 μg of nuclear proteins extracted from a lactating mammary gland. The incubation was performed after addition of 1 μl of the rabbit immune serum directed against rabbit MGF/STAT5-like factor (lane 2) or 1 μm of antiphosphotyrosine antibody (lane 3). The arrow indicates the position of the specific complex.

tor (Fig. 2). Unexpectedly, the F1 oligonucleotide was a more efficient competitor for the F4-binding in mammary cell (Fig. 4, lane 7) than in lactating mammary gland extract (Fig. 2, lane 10). Other complexes showing higher migration rates were observed. They were not affected by the addition of any of the oligonucleotides we employed and, for this reason, they were considered as non-specific. The specific complex was shifted by addition of the rabbit MGF/STAT5-specific antiserum (lane 4). This observation argues in favour of the idea that (1) the F4-bound factor is similar to MGF/STAT5, and (2) the binding of this factor to the F4 sequence is induced by prolactin in cultured mammary gland cells.

3.3. *The F4 sequence is necessary for maximal activity of the distal prolactin-dependent enhancer in the rabbit αs1-casein gene*

To assess the functional importance of the F4-binding site, CHO cells were cotransfected with gene constructs containing (or not) the F4 sequence linked to the (−391/+1774)CAT gene and with the plasmid pER2-3 which encodes the rabbit mammary prolactin receptor. Whatever the transfected *CAT* gene construct, the basal level of CAT activity determined in non-prolactin-treated cells was similar (data not shown). After prolactin treatment of the transfected cells, the CAT activity was elevated only twice when the (−391/+1774)*CAT* gene was transfected (Fig. 5, lane 1). When the −3442/−3333 fragment was linked upstream of the (−391/+1774)*CAT* gene, the prolactin sensitivity remained unchanged (lane 2). The prolactin sensitivity of the (−391/+1774)*CAT* gene was quite significantly enhanced when the −3442/−3307 gene fragment, which contains the F4 sequence, was linked upstream of the reporter construct (5.7-fold induction, lane 3). When the F4 sequence was replaced by the W sequence (the sequence of the proximal binding site of a MGF/STAT5-like factor in the rabbit αs1-casein promoter), the prolactin sensitivity of the construct was high and not significantly different to that of the wild-type construct (7.5-fold induction, lane 4). After substitution of the F4 sequence by a mutated MGF/STAT5-binding sequence (M, previously described as unable to bind a MGF/STAT-like factor by Pierre et al. [8]), the sensitivity of the construct towards prolactin dropped (2.1-fold induction, lane 5). It was concluded that the presence of a F4-binding site is necessary to confer a high prolactin sensitivity to the (−391/+1774)*CAT* gene.

The F4 sequence was by itself not sufficient to induce a high prolactin sensitivity to the (−391/+1774)*CAT* gene (2.3-fold induction, lane 6). Other gene elements contained in the −3442/−3333 fragment are also necessary. We have previously identified three binding sites in this area named F1, F2 and F3 [8]. Until now, the nature of the factors which bind to these sites remains unknown. In this study, we have investigated the possible interactions between F4- and F1-binding sites. The sequence of the F1 site presents several homologies with the consensus sequence for the binding of the MGF/STAT5 factor (Fig. 1). Therefore, we have introduced a double mutation in this site, interverting G and C at positions −3382 and −3378. When the F4 sequence was linked to the −3442/−3333 fragment encompassing a F1 mutated fragment, the sensitivity towards prolactin was not different from that of the minimal construct (−391/+1774)*CAT* (1.9-

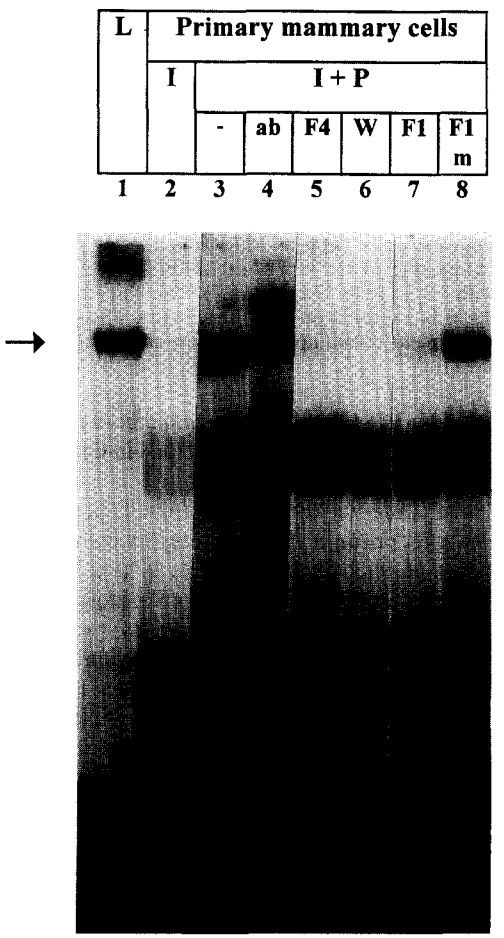

Fig. 4. Prolactin induction of the F4-bound complex in nuclear extracts from cultured mammary gland cells. Rabbit primary mammary cells were maintained in culture medium for 4 days then treated for 10 min with insuline (I, 5 μg/ml) or with insuline plus ovine prolactin (I+P, 1 μg/ml). Nuclear extracts were prepared and EMSA was performed with F4 as probe and 4–5 μg of nuclear protein in each point. The arrow indicates the location of the specific complexes. L, nuclear extracts from lactating mammary gland; ab, EMSA with MGF/STAT5-specific antiserum.

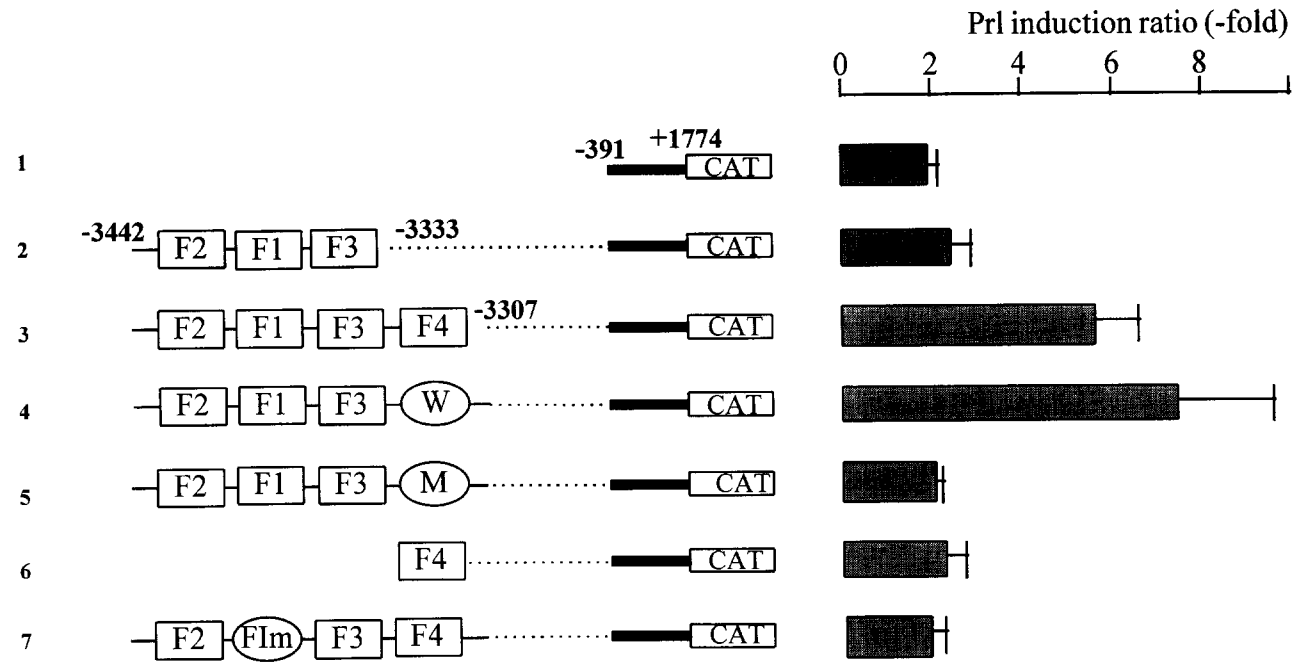

Fig. 5. The F4 sequence is necessary for maximal activity of the distal prolactin-dependent enhancer in the rabbit *αs1-casein* gene. Plasmids containing (or not) the F4 sequence were transfected into CHO cells with the plasmid pER2-3 expressing the rabbit mammary prolactin receptor. Transfected cells were treated for 72 h by cortisol or cortisol + prolactin (Prl) as described in Section 2. Prl induction was calculated as the ratio of CAT activity in Prl + cortisol-treated cells versus cortisol-treated cells. For each construct, at least two different DNA preparations were used in three independent experiments. Values are means+SEM.

fold induction, lane 7), indicating that the mutated distal fragment has no prolactin-dependent enhancer activity. Thus, the F4- and F1-binding sites interact simultaneously to confer a high prolactin sensitivity to the transfected gene construct.

4. Discussion

In our previous work [8], we observed that the −3442/−3285 fragment of the rabbit *αs1-casein* gene was sufficient to confer a high prolactin sensitivity to the (−391/+1774)CAT construct. A nuclear factor-DNA binding study led us to localize four protein/DNA-binding areas in this fragment, which were named F1–F4 (Fig. 1). The sequence of the binding area F4 is highly homologous to the sequence of the binding site of the already identified MGF/STAT5 factor (Fig. 1). It was therefore postulated that the F4 site could bind a factor similar to the MGF/STAT5 factor. The aim of the present study was to compare the F4-bound factor and a MGF/STAT5-like factor.

Two types of experiments were carried out: first, an in vitro protein-binding DNA study and, secondly, a functional study. The specificity of the binding of nuclear factors to the F4 and W oligonucleotides were similar (W contains the proximal MGF/STAT5-binding site in the rabbit αs1-casein promoter). The binding of the nuclear factor to the F4 sequence was prolactin inducible in cultured rabbit mammary cells, as it has been already described for the binding of rabbit primary mammary cells nuclear factor to a MGF/STAT5 sequence [19]. The migration rate of the F4 complex was modified by the addition of an antiserum directed against MGF/STAT5. All these data are strongly in favour of the idea that the F4 sequence binds in vitro a MGF/STAT5-like factor. Data obtained by the functional study confirmed this hypothesis. The F4 sequence can be replaced by a W sequence (but not by a mutated W sequence) in the transfected constructs without any significant modification of the prolactin sensitivity of the chimeric gene. We thus suggest that the F4 and W fragments bind in vivo similar nuclear factors, i.E., a MGF/STAT5-like factor.

Interestingly, the F4-bound complex appears wider in EMSA with cellular extracts compared to lactating mammary gland extracts (Fig. 4, lanes 1 and 3). This may be explained by the fact that the F4-bound complex is composed of several components in cell extracts. Following the results of the present study, one of this component is a MGF/STAT5-like factor. The nature of the other components remains unknown. It is possible that another form of the MGF/STAT5-like factor binds to the same F4 probe. Several forms of MGF/STAT5 have been identified in the mouse [4,5], in the early myeloid cell line FDC-P1 [23], in rats [6] or in humans [7]. Another explanation could be that the MGF/STAT5-like factor binds to the F4 probe in association with a second transcription factor, the nature of which is not elucidated. All these hypotheses have to be tested.

A distal MGF/STAT5-binding site has already been identified in the rat *WAP* gene [24]. According to the authors, the presence of this distal binding site is necessary to support a high expression of the gene in transgenic animals. The mutation of the distal MGF/STAT5-binding site considerably diminished the level of expression of the transgene in mice without altering the tissue specificity. The presence of two NF1-binding sites was necessary as well. The authors concluded that the distal enhancer was composed of several interacting elements.

Similarly to the rat *WAP* gene, the high level of prolactin-induced expression of the rabbit *αs1-casein* gene depends on

the presence of a distal enhancer encompassing one MGF/STAT5-binding site. The F4-binding site alone is not sufficient to confer a high prolactin sensitivity to the chimeric *αs1-casein* gene–*CAT* gene. The 3′ portion of the distal fragment encompassing the F2, F1 and F3 sites is quite necessary. In a first attempt, we have demonstrated that the F4- and F1-binding sites interacted. The F1 sequence is homologous to the sequence of the MGF/STAT5-binding site (Fig. 1). In a preliminary study, we have observed several complexes in EMSA performed with F1 as probe and rabbit lactating mammary gland nuclear extract (unpublished data). One of these complexes had binding characteristics similar to that of a MFG/STAT5–DNA complex and was inducible by prolactin in cultured mammary gland cells. It is thus possible that this F1 sequence also binds a MGF/STAT5-like factor. This hypothesis is now currently being studied.

In our previous study, we demonstrated that the presence of a proximal MGF/STAT5-binding site was necessary for the enhancer activity of the distal fragment [8]. Our data indicate that the high prolactin sensitivity of the rabbit *αs1-casein* gene depends on the interaction of multiple MGF/STAT5-binding sites, located in the proximal promoter and in the distal enhancer. The F2- and F3-binding sites could also play an important role. Their involvement in the high-level prolactin-dependent enhancer activity of the distal enhancer is under study.

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