Molecular Interaction of BMP-4, TGF-β, and Estrogens in Lactotrophs: Impact on the PRL Promoter

Damiana Giacomini, Marcelo Páez-Pereda, Johanna Stalla, Günter K. Stalla, and Eduardo Arzt

Laboratorio de Fisiología y Biología Molecular (D.G., E.A.), Departamento de Fisiología, Biología Molecular y Celular, Facultad Ciencias Exactas y Naturales, Universidad de Buenos Aires, 1428 Buenos Aires, Argentina; Max-Planck Institute of Psychiatry (M.P.-P., J.S., G.K.S.), 80804 Munich, Germany; and Affectis Pharmaceuticals (M.P.-P.), 82152 Martinsried, Germany

The regulatory role of estrogen, bone morphogenetic protein-4 (BMP-4), and TGF-β has a strong impact on hormone secretion, gene transcription, and cellular growth of prolactin (PRL)-producing cells. In contrast to TGF-β, BMP-4 induces the secretion of PRL in GH3 cells. Therefore, we studied the mechanism of their transcriptional regulation. Both BMP-4 and TGF-β inhibited the transcriptional activity of the estrogen receptor (ER). Estrogens had no effect on TGF-β-specific Smad protein transcriptional activity but presented a stimulatory action on the transcriptional activity of the BMP-4-specific Smads. BMP-4/estrogen cross talk was observed both on PRL hormone secretion and on the PRL promoter. This cross talk was abolished by the expression of a dominant-negative form for Smad-1 and treatment with ICI 182780 but not by point mutagenesis of the estrogen response element site within the promoter, suggesting that Smad/ER interaction might be dependent on the ER and a Smad binding element. By serial deletions of the PRL promoter, we observed that indeed a region responsive to BMP-4 is located between -2000 and -1500 bp upstream of the transcriptional start site. Chromatin immunoprecipitation confirmed Smad-4 binding to this region, and by specific mutation and gel shift assay, a Smad binding element responsible site was characterized. These results demonstrate that the different transcriptional factors involved in the Smad/ER complexes regulate their transcriptional activity in differential ways and may account for the different regulatory roles of BMP-4, TGF-β, and estrogens in PRL-producing cells. **(*Molecular Endocrinology* 23: 1102–1114, 2009)**

Many actions of the anterior pituitary hormone prolactin (PRL) have been reported, including effects on water and salt balance, growth and development, endocrinology and metabolism, brain and behavior, reproduction, immune regulation and protection, and growth of different forms of cancer (1, 2). Although it is well known that dopamine of hypothalamic origin provides inhibitory control on the secretion of PRL, other factors within the brain, pituitary gland, and peripheral organs have been shown to inhibit or stimulate PRL secretion as well (3).

Removal of the ovaries is followed by a decrease in lactotroph size and number as well as the intracellular abundance of PRL-secretory granules (4, 5). Estradiol (E2) is the strongest ovarian hormone that reverses these effects on lactotroph cell number and subsequently stimulates PRL secretion (6). Estrogen regulates PRL at the transcriptional level acting, as a regulatory steroid hormone (7), on the specific sequence on its promoter (8). Estrogens are also antidopaminergic at the lactotroph level and exert this effect by decreasing the number of dopamine receptors (9). Estrogens have also been shown to stimulate lactotroph proliferation and are involved in prolactinoma development. The participation of the estrogen receptor (ER) in the growth response of pituitary tumor cells to IL-2 has been demonstrated (10). Prolactinomas occur more frequently in women and increase in size during pregnancy or estrogen treatment (11, 12). The last promotes the development of experimental prolactinomas in female rats (13, 14), whereas in dopamine D2-receptor (D2R)-deficient mice ($D2R^{-/-}$), these grow spontaneously or much earlier in females (15–17) but also in aged male animals (17).

Members of the TGF-β superfamily have been implicated in the control of lactotroph physiology. It is well documented that

ISSN Print 0888-8809 ISSN Online 1944-9917
Printed in U.S.A.

doi: 10.1210/me.2008-0425 Received November 10, 2008. Accepted March 27, 2009.
First Published Online April 2, 2009

Abbreviations: BMP-4, Bone morphogenetic protein-4; ChIP, chromatin immunoprecipitation; D2R, dopamine D2-receptor; DTT, dithiothreitol; E2, estradiol; ER, estrogen receptor; ERE, estrogen response element; hPRL, human PRL; MH1, MAD-homology 1; PRL, prolactin; SBE, Smad binding elements; SDS, sodium dodecyl sulfate; Smad-4dn, dominant-negative form of Smad-4.

TGF-β1 inhibits PRL secretion of normal lactotroph in an auto/paracrine manner (18). In addition to its effects on normal lactotrophs, TGF-β1 also inhibits the expression of PRL gene in rat pituitary tumor cell lines (19). TGF-β and other members of the TGF-β superfamily, such as activin, also have inhibitory effects on cell proliferation of normal and tumor lactotrophs (18, 20–22), and a reduction in the expression and action of TGF-β1 on lactotrophs during estrogen-induced anterior pituitary tumorigenesis has been documented (23, 24). TGF-β3, which is increased by E2 administration, has a stimulatory effect on lactotroph cell proliferation (25).

Bone morphogenetic protein-4 (BMP-4) is one of the members of the TGF-β superfamily. Initially, BMP-4 had been studied in the pituitary for its role in the development and patterning of the pituitary from the initial induction of Rathke's pouch to cell specification in the anterior lobe and differentiation of the lactotroph linage (26, 27). More recently, our laboratory has described its role in the lactotroph tumorigenesis process. BMP-4 is overexpressed in different prolactinoma models, including the D2R$^{-/-}$ mice, E2-induced rat prolactinomas, and human prolactinomas, as compared with normal tissue (28). Experiments in nude mice have shown that tumor growth is inhibited when a dominant-negative form of Smad-4 (Smad-4dn) expression was expressed in GH3 lactosomatotroph cells, providing direct evidence that BMP-4 and Smad-4 are involved in prolactinoma development *in vivo* (28). BMP-4 action on lactotroph cells involves a cross talk between intracellular signaling mechanisms of BMP-4 and estrogens (28). By co-immunoprecipitation studies, we have previously demonstrated that BMP-4 or estrogen treatment induces a physical interaction between the ER, Smad4, and the specific BMP signal transducer Smad1. In contrast, TGF-β or estrogens induced a physical interaction between ER, Smad4, and the specific TGF-β signal transducer Smad2 but not Smad1. Thus, these different signaling protein complexes specifically induced by BMP-4 or TGF-β might provide a basis to explain their different effects on lactotroph physiology (28). Although the effects of BMP-4 on proliferation have been described, no studies have shown BMP-4 action on lactotroph hormone secretion.

The expression of the rat PRL gene is under the control of a number of hormones, and its transcriptional activity modulation is regulated through protein interactions within at least two distinct regions of the 5′-flanking sequence, a proximal promoter region and a distal enhancer region located approximately 1.5 kb upstream from the transcription initiation site (29–32). It has been demonstrated that the ER selectively binds to a region located between 1.2 and 2.0 kb upstream from the transcription initiation site rather than to a more proximal position as has been shown for the transcriptional regulatory action of other steroid hormone receptors (33). Furthermore, the ER binding site has been determined; mutagenesis of a response element located at positions −1582 to −1569 of the promoter inhibits the ability of estrogens to stimulate transcription (30), showing the specificity of this site for the ER. Moreover, several studies have pointed out that the distal enhancer activity contributes mainly to the basal level of PRL gene expression and may be involved in pituitary cell-type specificity (32, 34). The requirement of both the specific pituitary transcription factor Pit-1 and the ER for estrogen responsiveness of the rat PRL gene has also been shown (35). Furthermore, Pit-1 interaction with other proteins mediates transcriptional responses of multiple signal transduction pathways on the PRL gene (36–40). Recently, the regulation of the human PRL (hPRL) promoter by estrogens has been studied (41). This study shows that in pituitary cells, hPRL gene expression is regulated by a 5′-flanking region 5000 bp upstream the transcriptional start site and that the ER activates the hPRL promoter through binding to a degenerate estrogen response element (ERE) sequence found at −1189 bp (41). Both rat and human ERE of the PRL promoter differ from the consensus ERE sequence at the same two bases, and both are located in the distal enhancer (41–43). In an attempt to characterize and define the TGF-β1 inhibitory transcriptional mechanism a series of 5′ rat PRL promoter deletions were used, the region between −116 and 54 was shown to be responsible of the TGF-β1 inhibitory response (44).

The aim of this work was to study the regulation of the transcriptional activity of the Smads proteins and the ER to understand the complex regulatory action of BMP-4, TGF-β1, and estrogens in PRL production.

Results

BMP-4 and estrogens cross talk on PRL secretion

We first studied whether BMP-4 has an effect on hormone secretion on GH3 cells. BMP-4 treatment induced PRL secretion (Fig. 1A). We studied whether the cross talk described between BMP-4 and E2 for proliferation (28) was also observed on hormone secretion. A BMP-4 and E2 combination increased PRL hormone secretion (Fig. 1A). When studying GH secretion, we observed that BMP-4 treatment induced GH secretion and also that E2 induced GH secretion as previously described in pituitary primary culture (45). On the other hand, no BMP-4 cross talk with estrogens was observed on GH secretion (Fig. 1B). These results pointed out that BMP-4 and E2 cross talk is specific for cell proliferation and PRL secretion rather that a general effect. When we studied the combination between BMP-4 and TGF-β, we observed a lower PRL secretion induced by BMP-4 (Fig. 1C), probably involving a TGF-β inhibitory effect on the basal transcriptional level that was previously described (44).

BMP-4, TGF, and estrogen cross talk on PRL promoter

We next studied whether the effects on hormone secretion were also observed at the transcriptional level. BMP-4 induced PRL promoter activity, and in the presence of E2, this activation was increased (Fig. 2A). Coexpression of Smad-1dn not only resulted in the inhibition of BMP-4 transcriptional stimulatory effect but also inhibited the cross talk observed between BMP-4 and E2 (Fig. 2A). These results indicate that the cross talk observed on PRL transcriptional activity is dependent on BMP-4 signaling activation, specifically dependent on Smad-1. We also studied the combination effect between both TGF-β and E2 and TGF-β and BMP-4 on the regulation of the PRL promoter transcriptional activity. As shown in Fig. 2B, TGF-β treatment in the

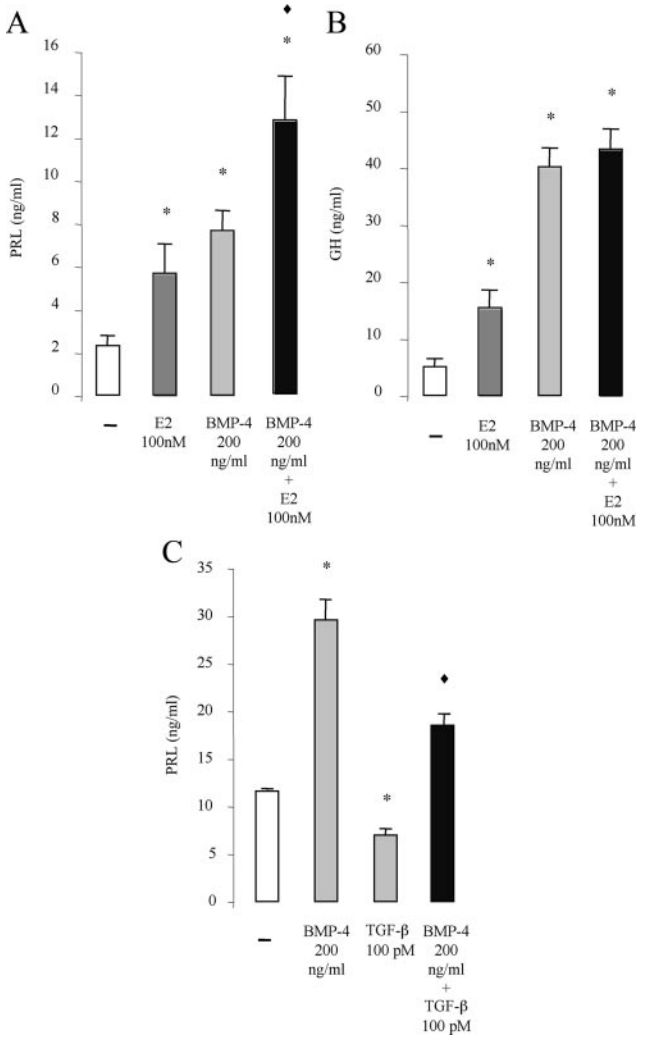

FIG. 1. Cross talk between BMP-4 and estrogens on PRL secretion in GH3 cells. A and B, GH3 cells were treated with BMP-4 or E2 as indicated. GH3 cells were seeded at 45×10^3 cells in 24-well plates. After FCS depletion for 24 h, treatments were added with fresh FCS-depleted medium. After 24 h, the supernatants were collected, and PRL (A) and GH (B) were measured by RIA. Values represent the mean ± SE of quadruplicate determinations of a single representative experiment (total of three with similar results). *, $P < 0.05$ with respect to basal conditions; ♦, $P < 0.05$ with respect to BMP-4 200 ng/ml, ANOVA with Scheffé's test. C, GH3 cells were treated with BMP-4 or TGF-β as indicated. GH3 cells were seeded at 45×10^3 cells in 24-well plates. After FCS depletion for 24 h, treatments were added with fresh FCS-depleted medium. After 24 h, the supernatants were collected, and PRL was measured by RIA. Values represent the mean ± SE of quadruplicate determinations of a single representative experiment from three independent experiments with similar results. *, $P < 0.05$ with respect to basal conditions; ♦, $P < 0.05$ with respect to BMP-4, ANOVA with Scheffé's test.

presence of E2 inhibits PRL transcriptional activity, and this effect was also observed with different concentrations of TGF-β (data not shown).

BMP-4 and TGF action on the transcriptional activity of the ER

We studied whether BMP-4 or TGF-β could alter the transcriptional activity of the ER. As shown in Fig. 3A, BMP-4 did not change the basal transcriptional activity of the ER on a reporter construct that has the EREs. On the other hand, at low doses of E2, BMP-4 inhibited the transcriptional activity of the ER. On the GAL4-ER construct, BMP-4 treatment also inhibited the transcriptional activity of ER (Fig. 3B). TGF-β treatment inhibited the ER transcriptional activity produced by E2 through the ER (Fig. 3, C and D), and in the presence of TGF-β, there is a smaller transcriptional activity of the ER. Thus, the ER/Smads interaction inhibits the transcriptional activity of the ER independently of its composition, although BMP-4 inhibition is weaker.

BMP-4, TGF, and estrogens cross talk on Smad-mediated transcription

Based on these results, we hypothesized that the transcriptional activity of the Smad proteins involved in the complexes might also be affected and studied this in reporter constructions containing Smad binding elements (SBE) responding to BMP-4 or TGF-β. As shown in Fig. 4A, the basal transcriptional activity of the BMPRE-LUC was not affected by the addition of E2 in any of the different doses tested. As expected, BMP-4 induced BMPRE-LUC activity in a dose-dependent manner, and this activation was augmented in the presence of E2. The stimulation of E2 on BMP-4 activity is similar to that observed on the PRL promoter. We then assessed the same situation but for the possible effect of ER signaling on TGF-β activity using the reporter TGF-βRE-LUC. In contrast to that observed for BMP-4 signaling, E2 had no effect on the transcriptional activity of the TGF-β-specific Smad proteins (Fig. 4B).

Characterization of the BMP binding site on the PRL promoter

We next examined the importance of the integrity of the ERE of the PRL promoter in the observed cross talk between BMP-4 and E2. Oligonucleotide-directed mutagenesis was used to alter the sequence of the estrogen receptor binding site located at positions −1582 to −1569. As shown previously (30), mutation of this region completely abolished the stimulatory effect of E2 on the transcriptional activity of the PRL promoter (Fig. 5). On the contrary, this mutation had no effect on BMP-4 action on PRL transcriptional activity, and interestingly, the mutation did not inhibit the cross talk between BMP-4 and E2 (Fig. 5A). These data suggest that the BMP-4/E2 cross talk is independent of the ERE site of the PRL promoter. ICI 182780 treatment inhibited the cross talk between BMP-4 and E2 observed on the PRL promoter (Fig. 5B), confirming that the BMP-4 and E2 cross talk is indeed mediated by ER. We further evaluated TGF-β action on the mutated PRL promoter; as shown in Fig. 5C, the inhibitory role of TGF-β in basal and E2-stimulated conditions was not affected by this mutation of the ERE in the PRL promoter. We next analyzed whether the cross talk between BMP-4 and E2 depends on a putative BMP-4 response element located in the PRL promoter. For this purpose, we first characterized in which region of the PRL promoter was the BMP-4 site of action. We generated a series of 5′ deletions (sequentially truncated fragments of 500 bp) of the PRL promoter from 2500 bp upstream of the transcriptional start site, and these reporter constructs were analyzed. As shown in Fig. 6, BMP-4 treatment induced the transcriptional activity of the −2000 bp PRL-LUC deletion, suggesting that the putative re-

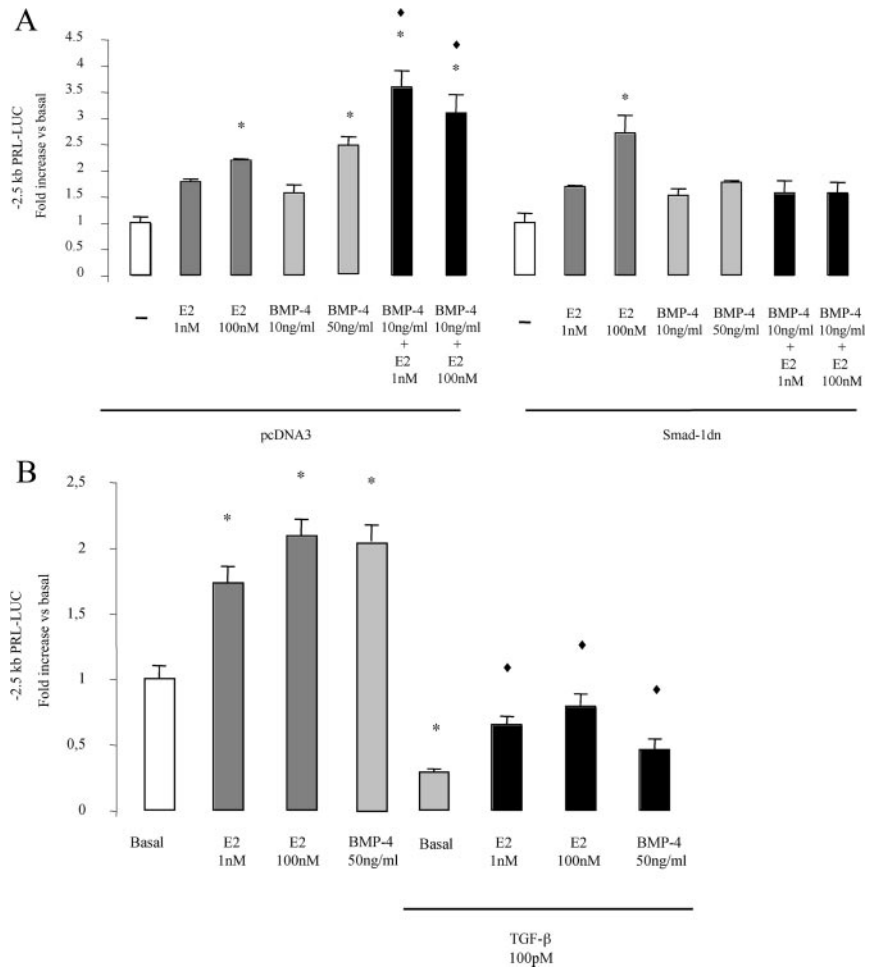

FIG. 2. Cross talk between BMP-4 and estrogens on PRL promoter transcriptional activity. A, GH3 cells were transiently transfected with a plasmid DNA mixture containing 1.5 μg luciferase reporter construct of 2.5 kb PRL-LUC/pcDNA3 or 2.5 kb PRL-LUC/Smad-1dn and 0.5 μg pRSV-βgal. Cells were treated with E2, BMP-4, or the combination of them, and after 24 h treatment, luciferase activity was measured. The values corresponding to luciferase were normalized with respect to β-galactosidase activity. Values over the basal value are expressed as fold increase *vs.* basal (basal = 1). Mean ± SE of triplicate determinations per treatment, of one representative experiment from three independent experiments with similar results, are shown. *, $P < 0.05$ with respect to basal conditions; ♦, $P < 0.05$ with respect to corresponding E2 or BMP-4 alone, ANOVA with Scheffé's test. B, GH3 cells were transiently transfected with a plasmid DNA mixture containing 1.5 μg luciferase reporter construct of 2.5 kb PRL-LUC and 0.5 μg pRSV-βgal. Cells were treated with E2, BMP-4, TGF-β, or the combination of them, and after 24 h treatment, luciferase activity was measured. The values corresponding to luciferase were normalized with respect to β-galactosidase activity. Values over the basal value are expressed as fold increase *vs.* basal (basal = 1). Mean ± SE of triplicate determinations per treatment, of one representative experiment from three independent experiments with similar results, are shown. *, $P < 0.05$ with respect to basal conditions; ♦, $P < 0.05$ with respect to corresponding E2 or BMP-4 alone, ANOVA with Scheffé's test.

sponse element must be located in this region. Also, the BMP-4/E2 cross talk was not affect in this deletion construct, supporting the notion that the response element required was within this region (Fig. 6A). On the other hand, further 5′ deletions of the region between −2000 and −1500 bp (−1500 bp PRL-LUC) completely eliminated the BMP-4 response (Fig. 6B). Similar results were obtained with a −1000 bp PRL-LUC construct (data not shown). This analysis led to the identification of at least one BMP-4 response element upstream of the PRL coding sequence.

Having identified a region for the existence of a potential SBE for BMP-4 with the promoter deletion constructs, we aimed to verify that Smad-4 could bind to it. In a chromatin immunoprecipitation (ChIP) assay, endogenous Smad4-DNA complexes were immunoprecipitated with an anti-Smad4 antibody, and the DNA purified from this coprecipitation was analyzed by PCR with a pair of PCR primers that amplified the −2000/−1500 region where the putative SBE might be located. The experiments were performed with the endogenous Smad-4 to analyze a scenario more similar to the physiological one. In untreated cell lysates, we observed low levels of amplification in Smad-4 immunoprecipitates. However, in cells stimulated with 100 μg/ml BMP-4, we observed amplification of DNA binding sites (Fig. 7). These results demonstrate that BMP-4 treatment induced binding of endogenous Smad-4 to a specific site in the PRL promoter located in the region between nucleotides −2000/−1500.

To further characterize the putative SBE sites in this region, the BMP-4 regulation of different fragments of the PRL promoter with mutations or deletions of the most similar SBE was analyzed. As shown in Fig. 8A, BMP-4 treatment induced the transcriptional activity of a −2000 bp PRL-LUC deletion that lacks the first putative SBE, suggesting that this first putative response element is not the one responsible for BMP-4 stimulation. Also, BMP-4 induced the transcriptional activity of a PRL-LUC construct that lacks the second putative SBE (Fig. 8B). On the contrary, when analyzing a PRL-LUC construct with the third putative SBE deleted, the stimulatory effect of BMP-4 on the transcriptional activity of this PRL-LUC construct was abolished (Fig. 8C). These data point out that this SBE is responsible of BMP-4 action on the PRL promoter.

Finally, we examine the binding of Smad proteins present in nuclear extracts prepared from GH3 cells under BMP-4 treatment to the SBE3 putative sequence. When we tested the SBE3 sequence of the PRL promoter, we observed the formation of a complex (Fig. 9A), and preincubation with an anti Smad-4 antibody disrupted this complex (Fig. 9B).

Discussion

In this work, we studied the interaction of two members of the TGF-β family that have physiologically opposite actions on lactotrophs (the stimulatory BMP-4 and the inhibitory TGF-β) with estrogens, on their specific DNA response elements, and on PRL, both at the secretion and promoter transcriptional activity

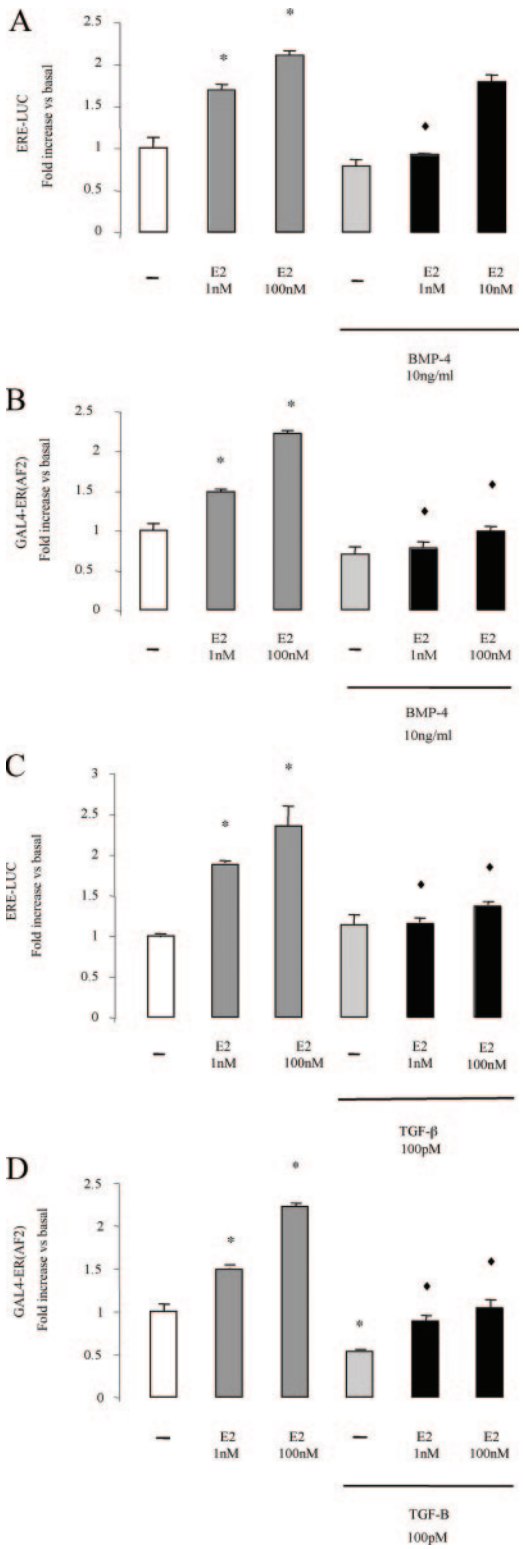

FIG. 3. Transcriptional activity of ERE-LUC and Gal4-ER (AF-2). A–D, GH3 cells were transiently transfected with a plasmid DNA mixture containing 1.5 μg luciferase reporter construct ERE-LUC or Gal4-ER (AF-2)/pG5-LUC and 0.5 μg pRSV-βgal. Cells were treated with E2, BMP-4 (A and B), or TGF-β (C and D) alone or the combination of them, and after 24 h treatment, luciferase activity was measured. The values corresponding to luciferase were normalized with respect to β-galactosidase activity. Values over the basal value are expressed as fold increase *vs.* basal (basal = 1). Mean ± SE of triplicate determinations per treatment, of one representative experiment from three independent experiments with similar results, are shown. *, $P < 0.05$ with respect to basal conditions; ♦, $P < 0.05$ with respect to corresponding E2 alone, ANOVA with Scheffé's test.

level. A cross talk between BMP-4 and E2 occurs at both PRL hormonal secretion and PRL promoter transcriptional activity levels, as demonstrated by a stronger induction in the presence of both ligands. A TGF-β inhibitory effect on the PRL promoter basal transcriptional level, which was previously described (44), might be involved in the lower PRL secretion observed after TGF-β and BMP-4 treatment. Both BMP-4 and TGF-β inhibited the transcriptional activity of the ER on ERE constructs (Fig. 10). On the contrary, estrogen had no effect on TGF-β-specific Smad proteins transcriptional activity but presented a stimulatory action on the transcriptional activity of the BMP-4-specific Smads, indicating a specific interaction with BMP-4. We also analyzed the combination between TGF-β and either estrogen or BMP-4 on the regulation of the PRL promoter transcriptional activity and established that TGF-β inhibitory effect overrides both BMP-4 and estrogen stimulatory action (Fig. 10). The BMP-4/E2 cross talk on the PRL promoter was independent of the ERE but was inhibited when the response to BMP-4 was abolished. We characterized the region within the PRL promoter that contains a potential BMP-4 response site responding to BMP-4 (Fig. 10).

When studying hormone secretion on GH3 cells, we determined that BMP-4 induced GH secretion in a dose-dependent manner, but although estrogen induced GH secretion as previously described in primary cultures (45), no interaction between BMP-4 and estrogen was observed. Recently, it has been shown that BMP-4 increased GH secretion by forskolin (46); nevertheless, the stimulatory basal effect of BMP-4 on GH secretion was not observed, which might be due to the immediate stimulation after 10% fetal calf serum (FCS) medium change (in contrast to 24 h depletion of FCS), in which BMP-4 action might have been masked by factors present in the FCS.

Furthermore, we demonstrated that BMP-4 and E2 cotreatment resulted in a stimulatory cross talk on PRL secretion, which correlates with previous results showing a BMP-4 and estrogen-positive cross talk on GH3 cell proliferation (28). We have recently demonstrated that the stimulatory effect of BMP-4 overrides the inhibitory effect of TGF-β on lactotroph proliferation *in vivo* (28). In this work we demonstrate that the TGF-β inhibitory effect on basal transcription overrides the BMP-4 stimulatory action on PRL secretion. Because these two members of the TGF-β superfamily have opposite roles in lactotroph physiology, we studied in more detail the transcriptional activity of the molecular complexes involved in each signaling pathway.

We studied whether the different molecular mechanisms induced by BMP-4, TGF-β, or estrogen could alter their transcriptional activity to understand in more detail the opposite actions of BMP-4 and TGF-β described on lactotrophs. Using two different transfection reporter assays, we observed that in GH3 cells, the transcriptional activity of the ER was inhibited by both BMP-4 and TGF-β. In contrast, although estrogens have no effect on the transcriptional activity of the specific TGF-β Smad proteins, estrogens enhanced BMP-4-specific Smad-induced transcriptional activity in the presence of BMP-4 but not under basal conditions. Previous findings have shown that Smad proteins and ER physically interact and that their interaction depends on the stimuli. In the presence of BMP-4 and/or estrogens,

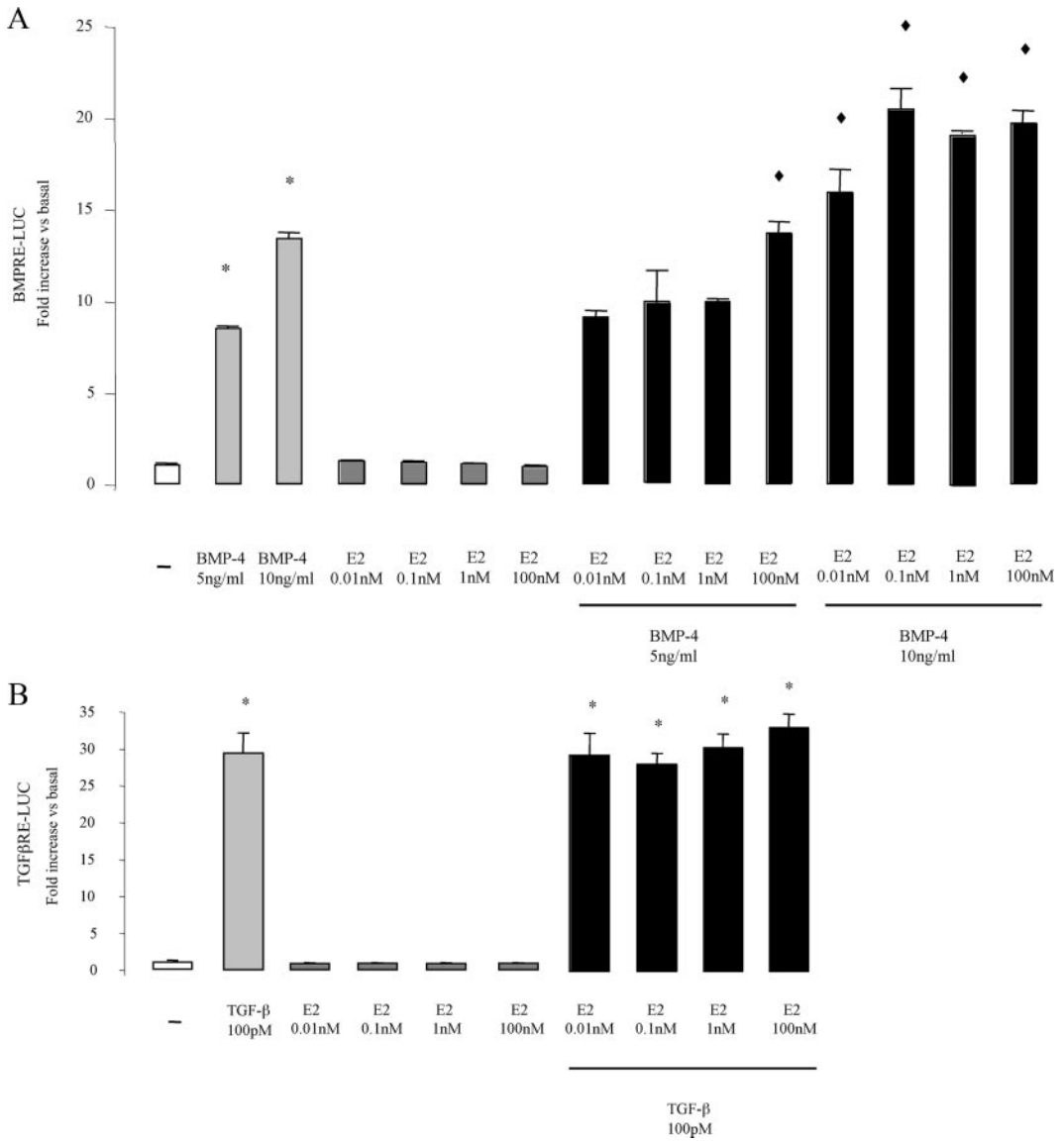

FIG. 4. Transcriptional activity of BMPRE-LUC and TGF-βRE-LUC. A and B, GH3 cells were transiently transfected with a plasmid DNA mixture containing 1.5 μg luciferase reporter construct of BMPRE-LUC or TGF-βRE-LUC and 0.5 μg pRSV-βgal. Cells were treated with E2, BMP-4 (A), or TGF-β (B) alone or the combination of them, and after 24 h treatment, luciferase activity was measured. The values corresponding to luciferase were normalized with respect to β-galactosidase activity. Values over the basal value are expressed as fold increase *vs.* basal (basal = 1). Mean ± SE of triplicate determinations per treatment, of one representative experiment from three independent experiments with similar results, are shown. *, $P < 0.05$ with respect to basal conditions; ♦, $P < 0.05$ with respect to corresponding BMP-4 alone, ANOVA with Scheffé's test.

the ER can physically interact with Smad4 and the specific BMP signal transducer Smad1. On the contrary, under TGF-β treatment, Smad2 and Smad4, but not Smad1, coimmunoprecipitated with ER (28). These different complexes may explain the differential activity in the presence of E2. Other studies have identified interactions and transcriptional activity regulation between Smad proteins and steroid receptors in many different models, which resulted in the inhibition or enhancement of the transcriptional activity of the proteins involved. For example, a physical interaction has been reported between Smad3 and vitamin D receptor that resulted in enhanced vitamin D receptor transcriptional activity (47). In prostate cancer cells, opposite actions have been described; androgen receptor stimulated TGF-β signaling via direct binding to Smad3 (48), whereas Smad3 repressed androgen receptor-mediated transcription (49). In hepatoma cells, the interaction between glucocorticoid receptor and Smad3 suppressed TGF-β signaling (50). Within the pituitary gland, we have described a cross talk between BMP-4 and retinoic acid that resulted in the inhibition of tumor corticotroph proliferation (51). For ER, several interactions have been described as well. In human kidney carcinoma cells, different from lactotrophs, ER-mediated transcriptional activation was enhanced by TGF-β signaling, whereas ER suppressed Smad3 (52). In lactotrophs, we observed that TGF-β overrides E2 stimulation on the PRL promoter, whereas there is no interaction on the specific TGF-ß reporter. The inhibitory effect of TGF-β on estrogen stimulation on the ERE reporter may probably be involved in the observed inhibitory role of TGF-β over estrogen on the PRL promoter. In line with our results about the positive interaction of BMP-4 and estrogen and the existence of

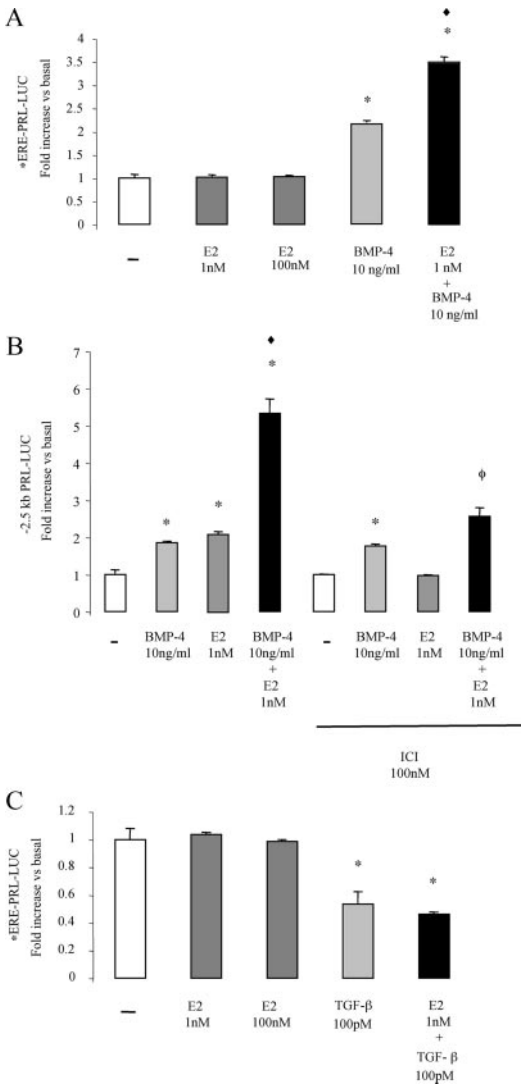

FIG. 5. Cross talk between BMP-4 and estrogens on the transcriptional activity of a PRL promoter that has a point mutation on its estrogen response element. A, GH3 cells were transiently transfected with a plasmid DNA mixture containing 1.5 μg luciferase reporter construct of 2.5 kb *ERE-PRL-LUC and 0.5 μg pRSV-βgal. Cells were treated with estrogens, BMP-4, or the combination of them, and after 24 h treatment, luciferase activity was measured. The values corresponding to luciferase were normalized with respect to β-galactosidase activity. Values over the basal value are expressed as fold increase *vs.* basal (basal = 1). Mean ± SE of triplicate determinations per treatment, of one representative experiment from three independent experiments with similar results, are shown. *, $P < 0.05$ with respect to basal conditions; ♦, $P < 0.05$ with respect to BMP-4 alone, ANOVA with Scheffé's test. B, GH3 cells were transiently transfected with a plasmid DNA mixture containing 1.5 μg luciferase reporter construct of 2.5 PRL-LUC and 0.5 μg pRSV-βgal. Cells were treated with E2, BMP-4, ICI 182780 (ICI), or the combination of them, and after 24 h treatment, luciferase activity was measured. The values corresponding to luciferase were normalized with respect to β-galactosidase activity. Values over the basal value are expressed as fold increase *vs.* basal (basal = 1). Mean ± SE of triplicate determinations per treatment, of one representative experiment from three independent experiments with similar results, are shown. *, $P < 0.05$ with respect to basal conditions; ♦, $P < 0.05$ with respect to corresponding E2 or BMP-4 alone; ϕ, $P < 0.05$ with respect to corresponding BMP-4 plus E2 without ICI, ANOVA with Scheffé's test. C, GH3 cells were transiently transfected with a plasmid DNA mixture containing 1.5 μg luciferase reporter construct of 2.5 kb *ERE-PRL-LUC and 0.5 μg pRSV-βgal. Cells were treated with estrogens, TGF-β, or the combination of them, and after 24 h treatment, luciferase activity was measured. The values corresponding to luciferase were normalized with respect to β-galactosidase activity. Values over the basal value are expressed as fold increase *vs.* basal (basal = 1). Mean ± SE of triplicate determinations per treatment, of one representative experiment from three independent experiments with similar results, are shown. *, $P < 0.05$ with respect to basal conditions, ANOVA with Scheffé's test.

the ER-Smads complexes (28), recent findings have demonstrated that the inhibitory actions of estrogens on BMP-2 function were due to a direct physical and functional interaction between Smad-1 and ER (53). Smads and steroid receptors, particularly the ER, present in the different protein complexes regulate their transcriptional activity in a cell- and promoter-dependent manner. In contrast to our previous result observed on cell proliferation (28), the basal inhibitory effect of TGF-β overrides the stimulatory effect of BMP-4 on PRL transcription as shown in Fig. 2B. In lactotrophs, both BMP-4 and TGF-β-specific Smad proteins inhibited the transcriptional activity of the ER, but the ER enhanced only the transcriptional activity of the BMP-4-specific Smads. This reciprocal regulation, particularly the BMP-4 and E2 potentiation of the specific BMP-4-activated Smad activity, promotes specific PRL regulation in lactotroph cells.

Furthermore, opposite actions have been described between TGF-β1 and TGF-β3 on lactotroph physiology (25) that are independent of Smad proteins. These opposite effects between TGF-β1 and TGF-β3 are a consequence of a different action of both isoforms at the same receptor site; for example, TGF-β1 response on cell proliferation is mediated via the TGF-β type II receptor, but TGF-β transcriptional responses are mediated via TGF-β type I receptor (54).

The BMP-4 and estrogen cross talk effect is relevant at the transcriptional level of the PRL promoter. We found that the BMP-4/estrogen cross talk was not inhibited by a point mutation of the ERE in the PRL promoter but was rather abolished when a dominant-negative form of Smad-1 was coexpressed in the cells. Also, we demonstrate that treatment with ICI 182780 inhibited the BMP-4/estrogen cross talk at the transcriptional level. These results suggest that the cross talk might depend on a potential BMP-4 response element within the promoter that is mediated by ER. As mentioned in the introductory section, Farrow and Gutierrez-Hartmann (44) have studied TGF-β modulation of the PRL promoter using a series of 5′-deletion promoter mutants. They found that the TGF-β inhibitory response was abrogated by deletion of the −116/−54 region of the PRL promoter, but no single response element was sufficient to mediate the TGF-β effect, suggesting that TGF-β may act through a complex signaling pathway that involved multiple DNA elements within the PRL promoter (44). When deleting the region between −2000 and −1500, both the BMP-4 response and the cross talk with estrogens was abolished, suggesting that the BMP-4 action site was located within that region. Furthermore, we performed ChIP experiments to test the ability of Smad-4 to directly bind to the −2000 to −1500 DNA region. Cells treated with BMP-4 showed an enhanced Smad-4 binding, proving that this region might contain a putative SBE. This region also contains the ERE and considering the cross talk and interaction between BMP-4-specific Smad proteins and ER, it might be possible that both response elements sites are located close together to facilitate the observed interaction. Smad MAD-homology 1 (MH1) domains can bind DNA directly, and Smad-4 MH1 domains bind to 5′-GnCT, 5′-CAGACA (CAGA motif, in association especially with Smad-3), and other GC-rich sequences, whereas the Smad1 MH1 binds a 5′-GCCGNCGC

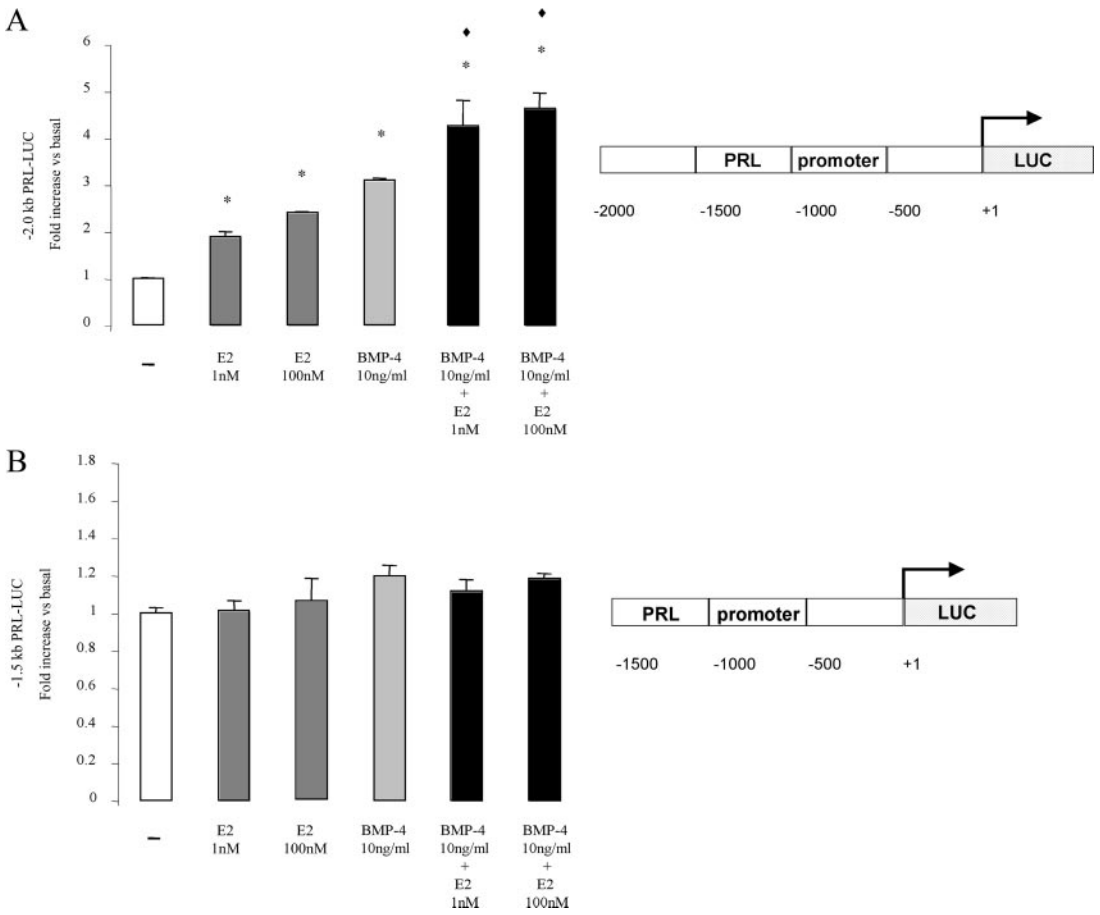

FIG. 6. BMP-4 stimulatory effect on PRL transcriptional activity is dependent of the region −2000/−1500 bp upstream the initiation site. A and B, GH3 cells were transiently transfected with a plasmid DNA mixture containing 1.5 μg luciferase reporter construct of each one of the different deletions of the PRL-LUC, −2000 PRL-LUC (A), −1500 PRL-LUC (B), and 0.5 μg pRSV-βgal. Cells were treated with E2, BMP-4, or the combination of them, and after 24 h treatment, luciferase activity was measured. The values corresponding to luciferase were normalized with respect to β-galactosidase activity. Values over the basal value are expressed as fold increase *vs.* basal (basal = 1). Mean ± SE of triplicate determinations per treatment, of one representative experiment from three independent experiments with similar results, are shown. *, $P < 0.05$ with respect to basal conditions; ♦, $P < 0.05$ with respect to corresponding E2 or BMP-4 alone, ANOVA with Scheffé's test.

motif (55). When analyzing the sequence of the PRL promoter, a CAGACA sequence was found approximately 290 bp upstream of the ERE element, within the region that we found crucial for the BMP-4 response. This might be one of the putative sequences responsible for BMP-4 and BMP-4/estrogen action on the PRL promoter. Other CAGA sequences are also detected in this region of the promoter, and although they differ from the specific CAGACA, they might also be putative SBE candidates. When we studied the transcriptional activity of a −2000-bp PRL-LUC deletion that lacks the first putative SBE sequence (two inverted CAGA separated by 2 bp), BMP-4 stimulation was still observed, suggesting that this first putative response element is not the one responsible for the BMP-4 effect. In addition, BMP-4 induced the transcriptional activity of a PRL-LUC mutated construct that lacks the second putative SBE sequence (one CAGACA and one inverted CAGA separated by 7 bp). However, the BMP-4 stimulatory effect was abolished in a PRL-LUC construct truncated in the third putative SBE sequence (two CAGA separated by 9 bp). Moreover, nuclear extracts prepared from GH3 cells treated with BMP-4 formed a complex with the oligonucleotide corresponding to the SBE3 sequence of the PRL promoter that was disrupted in the presence of an anti-Smad-4 antibody. These results suggest that the BMP-4 stimulatory effect is dependent on the third SBE sequence, although it does not resemble a classical consensus response element. Very likely, as determined for

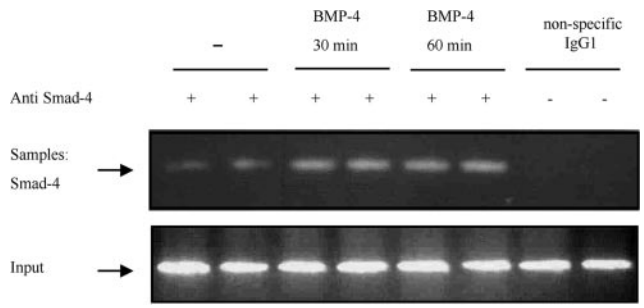

FIG. 7. BMP-4 treatment induces Smad-4 binding to a PRL gene promoter region. ChIP assays were performed from untreated or BMP-4-treated (200 ng/ml, 30 and 60 min) GH3 cells using an antibody specific for Smad-4 or using rabbit control IgG. The precipitated chromatin was analyzed using primers specific for detection of the putative SBE within the PRL promoter. These primers amplified a region from approximately −2000 to −1500 bp of the rat PRL gene promoter encompassing an SBE-like motif (*upper panel*). Input chromatin isolated before the immunoprecipitation was used to control for equal amounts of input DNA (input DNA). The experiment shown is representative of three independent experiments with similar results.

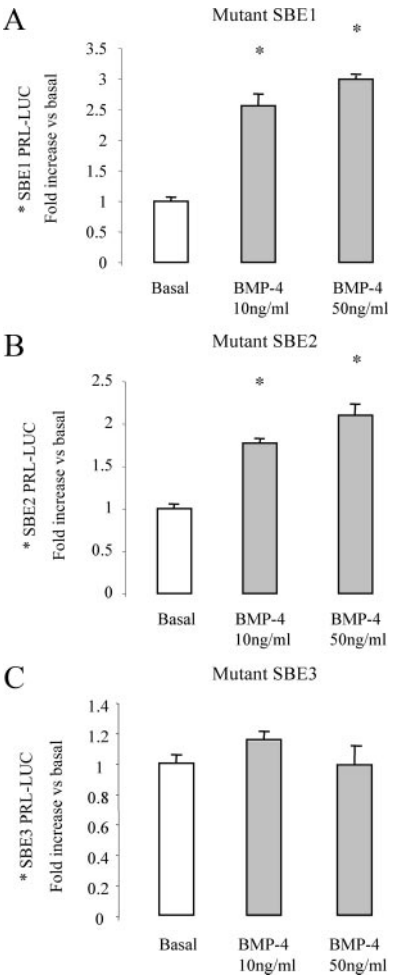

FIG. 8. Characterization of a putative SBE sequence on the PRL promoter. A–C, GH3 cells were transiently transfected with a plasmid DNA mixture containing 1.5 μg luciferase reporter construct of each one of the different mutations of the three putative SBE of the PRL-LUC, *SBE1 PRL-LUC (A), *SBE2 PRL-LUC (B), *SBE3 PRL-LUC (C), and 0.5 μg pRSV-βgal. Cells were treated with BMP-4, and after 24 h treatment, luciferase activity was measured. The values corresponding to luciferase were normalized with respect to β-galactosidase activity. Values over the basal value are expressed as fold increase *vs.* basal (basal = 1). Mean ± SE of triplicate determinations per treatment, of one representative experiment from three independent experiments with similar results, are shown. *, $P < 0.05$ with respect to basal conditions, ANOVA with Scheffé's test.

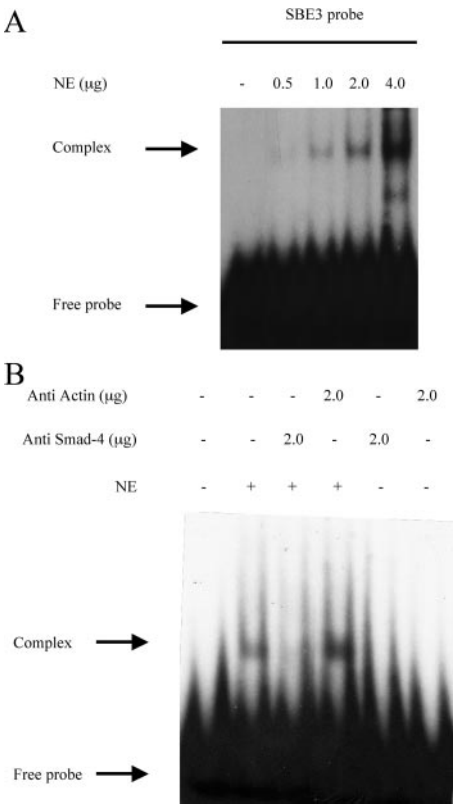

FIG. 9. Complex formed with the SBE3 oligonucleotide contains Smad-4 protein. A, Gel shift analysis of binding reactions using SBE3 sequence of the PRL promoter (wild type *vs.* mutant) as probe and BMP-4-stimulated GH3 cell nuclear extracts (NE) (0.5–4 μg). The experiment shown is representative for three independent experiments with similar results. B, Gel shift analysis of binding reactions using SBE3 sequence of the PRL promoter as probe and BMP-4-stimulated GH3 cells NE (4 μg), in the presence of an anti Smad-4 antibody (2 μg) or an anti-actin antibody (2 μg). The experiment shown is representative of three independent experiments with similar results.

TGF-β action on PRL promoter (44), it involves a complex stimulatory response that might require the interaction with others *cis*-acting factors and/or nonconsensus response elements.

We demonstrate for the first time that a BMP-4-Smad-ER molecular regulatory mechanism exists on PRL promoter transcriptional regulation in a physiologically relevant model where both BMP-4 and estrogens play an important role. We also describe for the first time the region where the binding site for BMP-4-specific Smad proteins is located in the PRL promoter. Given the role of both estrogens and BMP-4 in prolactinomas, their interaction may play a role in prolactinomas in women. It may be acting not only in prolactinoma cells but also in others such as breast and bone, in which both estrogens and the TGF-β family play important roles or in which Smad proteins may mediate transcriptional activation or repression depending on the protein partners associated (56–58).

Materials and Methods

Cell culture and stimulation

Materials and reagents, except where stated, were obtained from Invitrogen Life Technologies (Frederick, MD) and Sigma Chemical Co. (St. Louis, MO). Recombinant rat BMP-4, TGF-β (R&D Systems Inc., Minneapolis, MN), and E2 (Sigma) were used.

GH3, a rat lactosomatotrophic pituitary tumor cell line, obtained from the American Type Culture Collection (Rockville, MD), was used (59). Cells were cultured in DMEM (pH 7.3) supplemented with 10% FCS, 2.2 g/liter $NaHCO_3$, 10 mM HEPES, and 2 mM glutamine until they were confluent under 5% CO_2 atmosphere at 37 C. For proliferation and hormone secretion experiments, the cells were washed twice with PBS, and then medium was replaced by an experimental medium consisting of the same supplemented DMEM without FCS. Before and after the stimulation period, cell viability was routinely controlled microscopically after ethidium bromide/acridine orange staining to ensure that this parameter did not change during the experimental time.

For experiments using E2, cells were cultured in DMEM without phenol red (60) supplemented with 10% stripped serum for a week before the experiment was performed.

Cells were treated, as indicated, with BMP-4, TGF-β, or E2 that was dissolved in 100% ethanol as a 25 mM stock solution and handled in the dark. The diluting mixture was used in all the experiments as control.

Plasmids constructs and cloning

The plasmid constructs that we used were kindly provided and/or previously described, as indicated. The luciferase (LUC) reporter gene

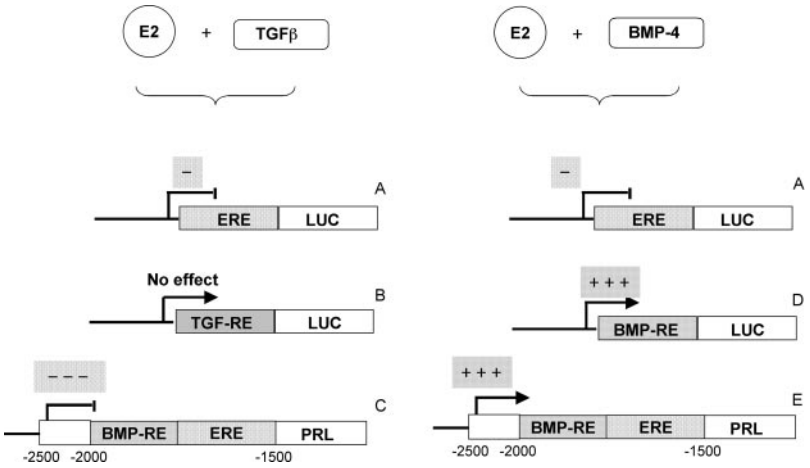

FIG. 10. Transcriptional regulation by BMP-4 or TGF-β stimulation. A, Under BMP-4 or TGF-β stimulation, both proteins inhibit the transcriptional activity of the ER on ERE sites. B, On the contrary, estrogen had no effect on TGF-β-specific Smad proteins transcriptional activity. C, On the other hand, TGF-β inhibitory effect overrides both BMP-4 and estrogens stimulatory action on PRL promoter transcriptional activity (C). D, Furthermore, a cross talk system between BMP-4 and E2 occurs on the transcriptional activity of the BMP-4-specific Smads in the presence of both ligands. E, Finally, the BMP-4/E2 cross talk system is also active on the regulation of the PRL promoter transcriptional activity, and BMP-4 acts through a specific response element.

under the control of −2.5 kb of the PRL promoter (PRL-LUC) construct was generously provided by Dr. A. Gutierrez-Hartmann (61). To measure ER activity, the ERE-tk-LUC was used, provided generously by Dr. Christian Behl (62). To test the transcriptional activity of the Smad proteins, we used two different reporter constructs. The BRE-LUC construct, used to evaluate BMP-specific Smad protein activity was kindly provided by Dr. Peter ten Dijke (63) and consists of two repeats of the −1052/−1032 region of the Id1 gene promoter that contains several specific SBE coupled to another region of the Id1 gene promoter (−1105/−1080), which contains just the GGCGCC palindrome sequence flanked by CAGC-3 and CAGC-4, and all this response region is cloned upstream of a minimal promoter reporter coupled to the luciferase gene. These fused Id1 fragments provided a strong BMP-specific enhancer function. To study the transcriptional activity of TGF-β-specific Smad proteins, the TGF-βRE-LUC construct kindly provided by Dr. Joan Massagué was used. This construct consists of a CAGA consensus sequence (*underlined*) repeated three times under a special insert (5′-TCGAGAGCCAGACAAAAAGCCAGACATTTAGCCAGACAC-3′), and this insert is repeated four times.

To assess the specificity of the BMP-4 effects on PRL transcriptional activation, we used a dominant-negative form of Smad-1 (Smad-1dn). The Smad-1dn expression vector generously provided by Dr. Sergio Roman-Roman consists of a truncated Rmad-1 cDNA, the mouse homolog of human Smad1, expressed under the control of the CMV promoter in the pcDNA3.1 vector. This truncated fragment was achieved by truncating the C-terminal phosphorylation site, introducing a stop codon at alanine 422, thus inhibiting its further heterodimerization and translocation to the nucleus (64).

Another approach to study the transcriptional activity of the ER was using a chimeric protein consisting of the DNA-binding domain of GAL4 and the ER fragment representing the AF-2 domain. The Gal4-ER (AF-2) expression vector was kindly provided by Dr. Donald McDonnell and consists of the DNA-binding domain of GAL4 and the ER fragment representing the AF-2 domain (amino acids 312–595) (65) containing the ligand-binding domain. This chimeric protein was assayed for transcriptional activity in a transient transfection system using a reporter plasmid containing five GAL4 DNA-binding sites placed upstream of a TATA sequence element initiating transcription of the luciferase gene (pG5-LUC).

Point mutagenesis, promoter deletions, and cloning of the PRL promoter were performed with standard PCR-based techniques as previously described (66). For point mutation of the ERE site in the PRL promoter, the region previously shown to bind the ER (67) was modified using oligonucleotides with the mutant sequence 5′-AATGCATTTTGGGCACGAGGGCCGAGAGTGCTTT-3′ (primer 2) and 5′-AAAGCACTCTCGGCCCTCGTGCCCAAAATGCATT-3′ (primer 3) (Fig. 11). Two additional primers were used, 5′-TGCTTAAGTGGTTTGAATCTTGACTTTGATTACA-3′ (primer 1) and 5′-ACCCATGGCTCCAACTGATGTAGCAGAAGATGAC-3′ (primer 4). The mutation was introduced via primers 2 and 3, whereas primers 1 and 4 were used to attach the overlap regions. The full-length fragment with the mutation was then subcloned by *Afl*III/*Nco*I in the original −2.5-kb PRL-LUC plasmid. For deletions of the −2.5-kb PRL-LUC, the primers used were 5′-AAAGGTACCCTGCTCTGGAGTCTTAC-3′ (primer 5, −2000 bp forward), 5′-AAAGGTACCCGTGTAACTCTAGCTG-3′ (primer 6, −2000 bp forward that lacks the first putative SBE, two inverted CAGA separated by two base pairs), 5′-AAAGGTACCGGTTAGAATTGTGATTGATTG-3′ (primer 7, −1500 bp forward), 5′-AAAGGTACCGTTACATACAAAACCAAAAG-3′ (primer 8, −1000 bp forward), and 5′-AAAAGATCTTAAGAGAACCACTGCTTTC-3′ (primer 9, reverse). The specific fragments were then subcloned by *Bgl*II/*Kpn*I in the pGL3 reporter plasmid (Promega, Madison, WI). For point mutation of the second putative SBE in the PRL promoter (CAGACA and CAGA sequences separated by 7 bp), these sites were modified as previously described (68, 69) using oligonucleotides with the mutate sequence 5′-TGTGTTAGGATGTGTGGAATTCTGTGTATGTAGG-3′ (primer 10) and 5′-CACCTACATACACAGAATTCCACACATCCTAACACA-3′ (primer 11). Two additional primers were used, primer 1 and primer 4. The mutation was introduced via primers 10 and 11, whereas primers 1 and 4 were used to attach the overlap regions. The full-length fragment with the mutation was then subcloned by *Afl*III/*Nco*I in the original −2.5-kb PRL-LUC plasmid. For deletion of the third SBE (two CAGA sequences separated by 9 bp) together with the ERE site, the primers used were 5′-AAAGGTACCTAATGGCAGATTTCCTTAG-3′ (primer 12), 5′-GTTATGACCCACTCATGTTTTAGAGATTTC-3′ (primer 13), 5′-GAGTGGGTCATAACGATTTATTTATCTATATCTT-3′ (primer 14), and primer 9. The deletion was introduced via primers 13 and 14, whereas primers 12 and 9 were used to attach the overlap regions. The specific fragments were then subcloned by *Bgl*II/*Kpn*I in the pGL3 reporter plasmid (Promega) (Fig. 11).

The nucleotide sequences of all constructs obtained by PCR were confirmed by DNA sequencing.

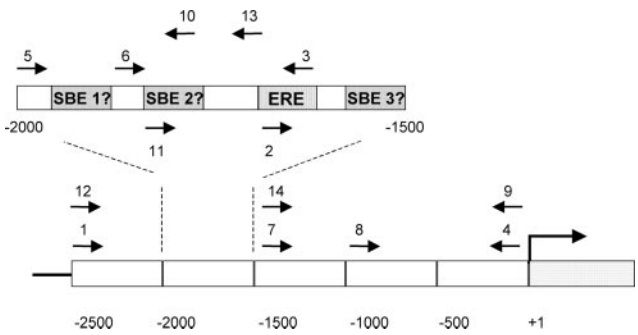

FIG. 11. Schematic representation of the 2.5-kb PRL promoter. A schematic representation of the 2.5-kb PRL promoter and the different primers used to mutate the ERE and the putative SBEs and to generate the different PRL deletions is shown. The region between −2000/−1500 bp is enlarged to make it more comprehensive.

Transfection of GH3 cells and reporter assays

Cell transfection was performed with Lipofectamine using standard procedures, as previously described (70). After plating the cells in six-well plates, the cells were transfected in DMEM without FCS for 6 h using 5 μl Lipofectamine (Invitrogen, Frederick, MD) per well with 1.5 μg for each plasmid combination, in conjunction with a control RSV-βgal construction (0.5 μg). Cells were then washed and left overnight in DMEM supplemented with 10% FCS. Then the following day, cells were washed and stimulated for 6 or 24 h in DMEM supplemented with 2% FCS with the indicated compounds. After treatment, LUC activity in cell lysates was measured as previously described (70), using the LUC measure kit (Promega) with a Junior luminometer (Berthold, Bad Wildbad, Germany). β-Gal activity was measured as previously described (70) as control for transfection efficiency. Results were standardized for β-gal activity.

ChIP assays

For ChIP analysis performed as previously described (71) with modifications, GH3 cells were stimulated under activating conditions for Smad-4 protein (BMP-4 100 ng/ml) for 30 min or 1 h. GH3 cells were fixed with 1% formaldehyde for 10 min at room temperature, washed with ice-cold PBS containing protease inhibitors, and lysed first with 1 ml cytoplasmic lysis buffer [5 mM PIPES (pH 8.0), 85 mM KCl, 0.5% Nonidet P-40, and protease inhibitors] for 30 min on ice. Nuclei were centrifuged (5000 rpm) for 10 min at 4 C and resuspended in 0.4 ml nuclear lysis buffer [50 mM Tris-HCl (pH 8.1), 10 mM EDTA, 1% sodium dodecyl sulfate (SDS), and protease inhibitors] to lyse the nucleus. The chromatin was sheared to 250-1000 bp length by sonication with three pulses of 10 sec at 30% amplitude (Sonic Dismembrator; Fisher Scientific, Troy, NY). After centrifugation, 15 μl of the supernatants were used as inputs. The remainder sonicated cell supernatant was diluted 5-fold in ChIP dilution buffer [15 mM Tris-HCl (pH 8.1), 1% Triton X-100, 0.01% SDS, 1 mM EDTA, 150 mM NaCl, and protease inhibitors]. The cell sonicated samples were precleared by adding 20 μl protein A-Sepharose (Sigma) bead slurry (50%) per 1 ml cell lysate and incubating at 4 C for 2 h on a rocker shaker. These beads were prepared by three washings in PBS and resuspension in 1 mM EDTA, 10 mM Tris-HCl (pH 8.1). Mouse monoclonal anti-Smad4 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was added (1 μg per immunoprecipitation) and incubated overnight at 4 C. Immunoprecipitation was carried out using protein A-Sepharose beads for 2 h at 4 C. Proteins were collected by gentle centrifugation at 700 rpm for 1 min at 4 C, and the A-Sepharose/antibody/protein complex was sequentially washed (4 min on a rocker shaker) with each of the following buffers (1 ml): low-salt wash buffer [0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl (pH 8.1), and 150 mM NaCl], high-salt wash buffer [0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl (pH 8.1), and 500 mM NaCl], and LiCl wash buffer [0.25 M LiCl, 1% Nonidet P-40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris-HCl (pH 8.1)]. This was followed by two washes with Tris-EDTA. After each wash, the proteins were collected by centrifugation (700 rpm, 1 min at 4 C). The antibody complexes were eluted twice in 250 μl elution buffer (1% SDS, 0.1 M NaHCO3), and the cross-links were reversed by adding ChIP digestion buffer [50 mM Tris-HCl (pH 8), 1 mM EDTA, 100 mM NaCl, 0.5% SDS, and 100 μg/ml proteinase K] to each sample and heating 3 h at 60 C and then 6 h at 65 C. The DNA was phenol/chloroform extracted and precipitated with 20 μg glycogen as a carrier. The DNA was resuspended in 20 μl ribonuclease-free water and was used for PCR analysis. For each experiment, PCRs were performed with different numbers of cycles or with dilution series of input DNA to determine the linear range of the amplification. All the results shown fall within this range and were normalized to the DNA input used for each individual immunoprecipitation. The ChIP experiment shown is one representative result of three independent experiments with similar results. For the putative Smad-4 binding site within the −2000-/−1500-bp PRL promoter, the following PCR primers were used: forward, 5′-ACCTGCTCTGGAGTCTTAC-3′, and reverse, 5′-TAGATGCAACACATAGTGC-3′. Mock control conditions were performed without antibodies, and a nonspecific IgG_1 antibody was added as control.

Hormone determination

Hormones were measured by RIA as previously described (59). For rat GH and PRL, reagents were kindly provided by Dr. A. F. Parlow from the National Hormone and Pituitary Program (Baltimore, MD). In the hormone secretion experiments, similar results were observed in experiments stimulated for 6 and 12 h, in which proliferative effects could be ruled out.

Preparation of nuclear extracts

GH3 cells (2×10^7) were treated with BMP-4 for 1 h and then harvested in 2 ml cold PBS containing 0.6 mM EDTA and pelleted at 5000 × *g* for 30 sec. Cell pellets were gently resuspended in 350 μl cold buffer A [10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, and 1 μg/ml pepstatin] and allowed to swell on ice for 10 min; then 5 μl of a 10% Nonidet P-40 solution was added to the cell suspensions, and the cells were lysed on ice for 10 min and vortexed for 5 sec every 2 min. After centrifugation at 12,000 × *g* for 20 min, the nuclear pellets were resuspended in 100 μl cold buffer B [20 mM HEPES (pH 7.9), 600 mM KCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, and 1 μg/ml pepstatin, and 25% glycerol) and incubated for 40 min on ice and vortexed every 10 min. After removal of nuclear debris by centrifugation at 16,000 × *g* for 10 min at 4 C, the microextracts were stored at −90 C.

EMSA

Oligonucleotides were synthesized as single-strand, and the annealing of the complementary strands was allowed. Briefly, each oligonucleotide was dissolved to a final concentration of 1 mg/ml before use, the annealing reaction was performed in oligo annealing buffer [100 mM Tris-HCl (pH 7.5), 1 M NaCl, and 10 mM EDTA], and equal volumes of both oligonucleotides were mixed and heated in a standard heat block at 95 C. Then the heat block was allowed to cool to room temperature on the workbench. Oligonucleotides (1 μg) were labeled with [α-^{32}P]ATP and large-fragment DNA polymerase I (Klenow fragment). For binding reactions, embryonic extracts were incubated for 25 min on ice in a total volume of 15 μl containing 25 mM HEPES (pH 7.2), 150 mM KCl, 5 mM DTT, 10% glycerol, and 2 μg poly(deoxyinosinic-deoxycytidylic) used as nonspecific competitor DNA, with 50000 cpm labeled probe. The samples were resolved on 5% polyacrylamide gels (acrylamide/bisacrylamide ratio of 29:1), buffered, and run for about 2 h in 0.5% TBE at 4 C (1× TBE is 45 mM Tris/borate/1 mM EDTA). For antibody supershift experiments, the anti-Smad-4 antibody (Santa Cruz Biotechnology) or anti-actin antibody (Santa Cruz Biotechnology) as control was added to the nuclear extracts and incubated for 30 min at room temperature before addition of the probe. The binding reaction mixture was further incubated for 15 min at room temperature and applied to the gel as described above.

The following double-strand oligonucleotide was used: oligo SBE3 wild type, 5′-TCATGATCTATCTGCCTGCCTCTTCTGACC-3′.

Statistics

Statistics were performed by ANOVA in combination with the Scheffé's test. Data are shown as mean ± SE.

Acknowledgments

We thank Dr. Arthur Gutierrez-Hartmann for the −2.5 kb of the PRL promoter (PRL-LUC), Dr. Christian Behl for the ERE-tk-LUC construct, Dr. Peter ten Dijke for the BRE-LUC construct, Dr. Sergio Roman-Roman for the Smad-1 dominant-negative expression vector, Dr. Joan Massagué for the TGF-βRE-LUC, and Dr. Donald McDonnell for the Gal4-ER(AF-2) expression vector.

Address all correspondence and requests for reprints to: Dr. E. Arzt, Laboratorio Fisiología y Biología Molecular, Departamento de Fisi-

ología, Biología Molecular y Celular, Facultad Ciencias Exactas y Naturales, Universidad de Buenos Aires, Ciudad Universitaria, Pabellon II, 1428 Buenos Aires, Argentina. E-mail: earzt@fbmc.fcen.uba.ar.

This work was supported by grants from the University of Buenos Aires, the Argentine National Research Council (Consejo Nacional de Investigaciones Científicas y Técnicas), and Agencia Nacional de Promoción Científica y Tecnológica-Argentina.

D.G. and E.A. are members of the IFIBYNE-Argentine National Research Council (CONICET).

Disclosure Summary: The authors have nothing to disclose.

References

1. **Goffin V, Bernichtein S, Touraine P, Kelly PA** 2005 Development and potential clinical uses of human prolactin receptor antagonists. Endocr Rev 26: 400–422
2. **Bole-Feysot C, Goffin V, Edery M, Binart N, Kelly PA** 1998 Prolactin (PRL) and its receptor: actions, signal transduction pathways and phenotypes observed in PRL receptor knockout mice. Endocr Rev 19:225–268
3. **Freeman ME, Kanyicska B, Lerant A, Nagy G** 2000 Prolactin: structure, function, and regulation of secretion. Physiol Rev 80:1523–1631
4. **De Paul AL, Pons P, Aoki A, Torres AI** 1997 Heterogeneity of pituitary lactotrophs: immunocytochemical identification of functional subtypes. Acta Histochem 99:277–289
5. **Sasaki F, Sano M** 1986 Roles of the arcuate nucleus and ovary in the maturation of growth hormone, prolactin, and nongranulated cells in the mouse adenohypophysis during postnatal development: a stereological morphometric study by electron microscopy. Endocrinology 119:1682–1689
6. **Chen CL, Meites J** 1970 Effects of estrogen and progesterone on serum and pituitary prolactin levels in ovariectomized rats. Endocrinology 86:503–505
7. **Burnstein KL, Cidlowski JA** 1993 Multiple mechanisms for regulation of steroid hormone action. J Cell Biochem 51:130–134
8. **Waterman ML, Adler S, Nelson C, Greene GL, Evans RM, Rosenfeld MG** 1988 A single domain of the estrogen receptor confers deoxyribonucleic acid binding and transcriptional activation of the rat prolactin gene. Mol Endocrinol 2:14–21
9. **Raymond V, Beaulieu M, Labrie F, Boissier J** 1978 Potent antidopaminergic activity of estradiol at the pituitary level on prolactin release. Science 200: 1173–1175
10. **Newton CJ, Arzt E, Stalla GK** 1994 Involvement of the estrogen receptor in the growth response of pituitary tumor cells to interleukin-2. Biochem Biophys Res Commun 205:1930–1937
11. **Gillam MP, Molitch ME, Lombardi G, Colao A** 2006 Advances in the treatment of prolactinomas. Endocr Rev 27:485–534
12. **Garcia MM, Kapcala LP** 1995 Growth of a microprolactinoma to a macroprolactinoma during estrogen therapy. J Endocrinol Invest 18:450–455
13. **Heaney AP, Horwitz GA, Wang Z, Singson R, Melmed S** 1999 Early involvement of estrogen-induced pituitary tumor transforming gene and fibroblast growth factor expression in prolactinoma pathogenesis. Nat Med 5:1317–1321
14. **Wiklund J, Wertz N, Gorski J** 1981 A comparison of estrogen effects on uterine and pituitary growth and prolactin synthesis in F344 and Holtzman rats. Endocrinology 109:1700–1707
15. **Kelly MA, Rubinstein M, Asa SL, Zhang G, Saez C, Bunzow JR, Allen RG, Hnasko R, Ben-Jonathan N, Grandy DK, Low MJ** 1997 Pituitary lactotroph hyperplasia and chronic hyperprolactinemia in dopamine D2 receptor-deficient mice. Neuron 19:103–113
16. **Asa SL, Kelly MA, Grandy DK, Low MJ** 1999 Pituitary lactotroph adenomas develop after prolonged lactotroph hyperplasia in dopamine D2 receptor-deficient mice. Endocrinology 140:5348–5355
17. **Saiardi A, Bozzi Y, Baik JH, Borrelli E** 1997 Antiproliferative role of dopamine: loss of D2 receptors causes hormonal dysfunction and pituitary hyperplasia. Neuron 19:115–126
18. **Sarkar DK, Kim KH, Minami S** 1992 Transforming growth factor-β1 messenger RNA and protein expression in the pituitary gland: its action on prolactin secretion and lactotropic growth. Mol Endocrinol 6:1825–1833
19. **Delidow BC, Billis WM, Agarwal P, White BA** 1991 Inhibition of prolactin gene transcription by transforming growth factor-β in GH3 cells. Mol Endocrinol 5:1716–1722
20. **Lacerte A, Lee EH, Reynaud R, Canaff L, De Guise C, Devost D, Ali S, Hendy GN, Lebrun JJ** 2004 Activin inhibits pituitary prolactin expression and cell growth through Smads, Pit-1 and menin. Mol Endocrinol 18:1558–1569
21. **Ramsdell JS** 1991 Transforming growth factor-α and -β are potent and effective inhibitors of GH4 pituitary tumor cell proliferation. Endocrinology 128: 1981–1990
22. **Coya R, Alvarez CV, Perez F, Gianzo C, Diéguez C** 1999 Effects of TGF-β1 on prolactin synthesis and secretion: an in-vitro study. J Neuroendocrinol 11: 351–360
23. **Pastorcic M, De A, Boyadjieva N, Vale W, Sarkar DK** 1995 Reduction in the expression and action of transforming growth factor β1 on lactotropes during estrogen-induced tumorigenesis in the anterior pituitary. Cancer Res 55:4892–4898
24. **Burns G, Sarkar DK** 1993 Transforming growth factor β1-like immunoreactivity in the pituitary gland of the rat: effect of estrogen. Endocrinology 133: 1444–1449
25. **Hentges S, Pastorcic M, De A, Boyadjieva N, Sarkar DK** 2000 Opposing actions of two transforming growth factor-β isoforms on pituitary lactotropic cell proliferation. Endocrinology 141:1528–1535
26. **Scully KM, Rosenfeld MG** 2002 Pituitary development: regulatory codes in mammalian organogenesis. Science 295:2231–2235
27. **Davis SW, Camper SA** 2007 Noggin regulates Bmp4 activity during pituitary induction. Dev Biol 305:145–160
28. **Paez-Pereda M, Giacomini D, Refojo D, Nagashima AC, Hopfner U, Grubler Y, Chervin A, Goldberg V, Goya R, Hentges ST, Low MJ, Holsboer F, Stalla GK, Arzt E** 2003 Involvement of bone morphogenetic protein 4 (BMP-4) in pituitary prolactinoma pathogenesis through a Smad/estrogen receptor crosstalk. Proc Natl Acad Sci USA 100:1034–1039
29. **Day RN, Maurer RA** 1989 The distal enhancer region of the rat prolactin gene contains elements conferring response to multiple hormones. Mol Endocrinol 3:3–9
30. **Kim KE, Day RN, Maurer RA** 1988 Functional analysis of the interaction of a tissue-specific factor with an upstream enhancer element of the rat prolactin gene. Mol Endocrinol 2:1374–1381
31. **Gutierrez-Hartmann A, Siddiqui S, Loukin S** 1987 Selective transcription and DNase I protection of the rat prolactin gene by GH3 pituitary cell-free extracts. Proc Natl Acad Sci USA 84:5211–5215
32. **Nelson C, Crenshaw 3rd EB, Franco R, Lira SA, Albert VR, Evans RM, Rosenfeld MG** 1986 Discrete *cis*-active genomic sequences dictate the pituitary cell type-specific expression of rat prolactin and growth hormone genes. Nature 322:557–562
33. **Maurer RA** 1985 Selective binding of the estradiol receptor to a region at least one kilobase upstream from the rat prolactin gene. DNA 4:1–9
34. **Simmons DM, Voss JW, Ingraham HA, Holloway JM, Broide RS, Rosenfeld MG, Swanson LW** 1990 Pituitary cell phenotypes involve cell-specific Pit-1 mRNA translation and synergistic interactions with other classes of transcription factors. Genes Dev 4:695–711
35. **Day RN, Koike S, Sakai M, Muramatsu M, Maurer RA** 1990 Both Pit-1 and the estrogen receptor are required for estrogen responsiveness of the rat prolactin gene. Mol Endocrinol 4:1964–1971
36. **Howard PW, Maurer RA** 1995 A composite Ets/Pit-1 binding site in the prolactin gene can mediate transcriptional responses to multiple signal transduction pathways. J Biol Chem 270:20930–20936
37. **Bradford AP, Conrad KE, Tran PH, Ostrowski MC, Gutierrez-Hartmann A** 1996 GHF-1/Pit-1 functions as a cell-specific integrator of Ras signaling by targeting the Ras pathway to a composite Ets-1/GHF-1 response element. J Biol Chem 271:24639–24648
38. **Bradford AP, Conrad KE, Wasylyk C, Wasylyk B, Gutierrez-Hartmann A** 1995 Functional interaction of c-Ets-1 and GHF-1/Pit-1 mediates Ras activation of pituitary-specific gene expression: mapping of the essential c-Ets-1 domain. Mol Cell Biol 15:2849–2857
39. **Diamond SE, Gutierrez-Hartmann A** 1996 A 26-amino acid insertion domain defines a functional transcription switch motif in Pit-1β. J Biol Chem 271: 28925–28932
40. **Bradford AP, Brodsky KS, Diamond SE, Kuhn LC, Liu Y, Gutierrez-Hartmann A** 2000 The Pit-1 homeodomain and β-domain interact with Ets-1 and modulate synergistic activation of the rat prolactin promoter. J Biol Chem 275:3100–3106
41. **Adamson AD, Friedrichsen S, Semprini S, Harper CV, Mullins JJ, White MR, Davis JR** 2008 Human prolactin gene promoter regulation by estrogen: convergence with tumor necrosis factor-α signaling. Endocrinology 149:687–694
42. **Murdoch FE, Byrne LM, Ariazi EA, Furlow JD, Meier DA, Gorski J** 1995 Estrogen receptor binding to DNA: affinity for nonpalindromic elements from the rat prolactin gene. Biochemistry 34:9144–9150
43. **Berwaer M, Monget P, Peers B, Mathy-Hartert M, Bellefroid E, Davis JR, Belayew A, Martial JA** 1991 Multihormonal regulation of the human prolactin gene expression from 5000 bp of its upstream sequence. Mol Cell Endocrinol 80:53–64

44. **Farrow KN, Gutierrez-Hartmann A** 1999 Transforming growth factor-β1 inhibits rat prolactin promoter activity in GH4 neuroendocrine cells. DNA Cell Biol 18:863–873
45. **Komolov IS, Perez-Arce JA, Fedotov VP** 1980 The effects of estradiol on prolactin and growth hormone secretion in cultured pituitary cells from intact and ovariectomized rats. Endokrinologie 75:278–284
46. **Miyoshi T, Otsuka F, Otani H, Inagaki K, Goto J, Yamashita M, Ogura T, Iwasaki Y, Makino H** 2008 Involvement of bone morphogenetic protein-4 in GH regulation by octreotide and bromocriptine in rat pituitary GH3 cells. J Endocrinol 197:159–169
47. **Yanagisawa J, Yanagi Y, Masuhiro Y, Suzawa M, Watanabe M, Kashiwagi K, Toriyabe T, Kawabata M, Miyazono K, Kato S** 1999 Convergence of transforming growth factor-β and vitamin D signaling pathways on SMAD transcriptional coactivators. Science 283:1317–1321
48. **Kang HY, Lin HK, Hu YC, Yeh S, Huang KE, Chang C** 2001 From transforming growth factor-β signaling to androgen action: identification of Smad3 as an androgen receptor coregulator in prostate cancer cells. Proc Natl Acad Sci USA 98:3018–3023
49. **Hayes SA, Zarnegar M, Sharma M, Yang F, Peehl DM, ten Dijke P, Sun Z** 2001 SMAD3 represses androgen receptor-mediated transcription. Cancer Res 61:2112–2118
50. **Song CZ, Tian X, Gelehrter TD** 1999 Glucocorticoid receptor inhibits transforming growth factor-β signaling by directly targeting the transcriptional activation function of Smad3. Proc Natl Acad Sci USA 96:11776–11781
51. **Giacomini D, Páez-Pereda M, Theodoropoulou M, Labeur M, Refojo D, Gerez J, Chervin A, Berner S, Losa M, Buchfelder M, Renner U, Stalla GK, Arzt E** 2006 Bone morphogenetic protein-4 inhibits corticotroph tumor cells: involvement in the retinoic acid inhibitory action. Endocrinology 147:247–256
52. **Matsuda T, Yamamoto T, Muraguchi A, Saatcioglu F** 2001 Cross-talk between transforming growth factor-β and estrogen receptor signaling through Smad3. J Biol Chem 276:42908–42914
53. **Yamamoto T, Saatcioglu F, Matsuda T** 2002 Cross-talk between bone morphogenic proteins and estrogen receptor signaling. Endocrinology 143:2635–2642
54. **Sarkar DK, Pastorcic M, De A, Engel M, Moses H, Ghasemzadeh MB** 1998 Role of transforming growth factor (TGF)-β type I and TGF-β type II receptors in the TGF-β1-regulated gene expression in pituitary prolactin-secreting lactotropes. Endocrinology 139:3620–3628
55. **Kusanagi K, Inoue H, Ishidou Y, Mishima HK, Kawabata M, Miyazono K** 2000 Characterization of a bone morphogenetic protein-responsive Smad-binding element. Mol Biol Cell 11:555–565
56. **Massagué J** 2000 How cells read TGF-β signals. Nat Rev Mol Cell Biol 1:169–178
57. **Massagué J, Blain SW, Lo RS** 2000 TGFβ signaling in growth control, cancer, and heritable disorders. Cell 103:295–309
58. **Chen CR, Kang Y, Siegel PM, Massagué J** 2002 E2F4/5 and p107 as Smad cofactors linking the TGFβ receptor to c-myc repression. Cell 110:19–32
59. **Arzt E, Buric R, Stelzer G, Stalla J, Sauer J, Renner U, Stalla GK** 1993 Interleukin involvement in anterior pituitary cell growth regulation: effects of interleukin-2 (IL-2) and IL-6. Endocrinology 132:459–467
60. **Berthois Y, Katzenellenbogen JA, Ktzenellenbogen BS** 1986 Phenol red in tissue culture media is a weak estrogen: implications concerning the study of estrogen-responsive cells in culture. Proc Natl Acad Sci USA 83:2496–2500
61. **Farrow KN, Manning N, Schaufele F, Gutierrez-Hartmann A** 1996 The c-Jun δ-domain inhibits neuroendocrine promoter activity in a DNA sequence and pituitary-specific manner. J Biol Chem 271:17139–17146
62. **Zschocke J, Manthey D, Bayatti N, van der Burg B, Goodenough S, Behl C** 2002 Estrogen receptor α-mediated silencing of caveolin gene expression in neuronal cells. J Biol Chem 277:38772–38780
63. **Korchynskyi O, ten Dijke P** 2002 Identification and functional characterization of distinct critically important bone morphogenetic protein-specific response elements in the Id1 promoter. J Biol Chem 277:4883–4891
64. **Spinella-Jaegle S, Roman-Roman S, Faucheu C, Dunn F, Kawai S, Galléa S, Stiot V, Blanchet AM, Courtois B, Baron R, Rawadi G** 2001 Opposite effects of bone morphogenetic protein-2 and transforming growth factor-β1 on osteoblast differentiation. Bone 29:323–330
65. **Norris JD, Fan D, Kerner SA, McDonnell DP** 1997 Identification of a third autonomous activation domain within the human estrogen receptor. Mol Endocrinol 11:747–754
66. **Carbia-Nagashima A, Gerez J, Perez-Castro C, Paez-Pereda M, Silberstein S, Stalla GK, Holsboer F, Arzt E** 2007 RSUME, a small RWD-containing protein, enhances SUMO conjugation and stabilizes HIF-1α during hypoxia. Cell 131:309–323
67. **Maurer RA, Notides AC** 1987 Identification of an estrogen-responsive element from the 5′-flanking region of the rat prolactin gene. Mol Cell Biol 7:4247–4254
68. **Stopa M, Anhuf D, Terstegen L, Gatsios P, Gressner AM, Dooley S** 2000 Participation of Smad2, Smad3, and Smad4 in transforming growth factor β (TGF-β)-induced activation of Smad7. The TGF-β response element of the promoter requires functional Smad binding element and E-box sequences for transcriptional regulation. J Biol Chem 275:29308–29317
69. **Li W, Chen F, Nagarajan RP, Liu X, Chen Y** 2001 Characterization of the DNA-binding property of Smad5. Biochem Biophys Res Commun 286:1163–1169
70. **Páez-Pereda M, Kovalovsky D, Hopfner U, Theodoropoulou M, Pagotto U, Uhl E, Losa M, Stalla J, Grübler Y, Missale C, Arzt E, Stalla G** 2001 Retinoic acid prevents experimental Cushing syndrome. J Clin Invest 108:1123–1131
71. **Liberman AC, Refojo D, Druker J, Toscano M, Rein T, Holsboer F, Arzt E** 2007 The activated glucocorticoid receptor inhibits the transcription factor T-bet by direct protein-protein interaction. FASEB J 21:1177–1188