Stat5-Mediated Regulation of the Human Type II 3β-Hydroxysteroid Dehydrogenase/Δ^5-Δ^4 Isomerase Gene: Activation by Prolactin

F. Alex Feltus, Bernd Groner, and Michael H. Melner

Vanderbilt University School of Medicine (F.A.F., M.H.M.)
Departments of Obstetrics/Gynecology and Cell Biology
Nashville, Tennessee 37232

Institute for Biomedical Research (B.G.)
Georg-Speyer-Haus
Frankfurt, Germany

Altered PRL levels are associated with infertility in women. Molecular targets at which PRL elicits these effects have yet to be determined. These studies demonstrate transcriptional regulation by PRL of the gene encoding the final enzymatic step in progesterone biosynthesis: 3β-hydroxysteroid dehydrogenase/Δ^5-Δ^4 isomerase (3β-HSD). A 9/9 match with the consensus Stat5 response element was identified at −110 to −118 in the human Type II 3β-HSD promoter. 3β-HSD chloramphenicol acetyltransferase (CAT) reporter constructs containing either an intact or mutated Stat5 element were tested for PRL activation. Expression vectors for Stat5 and the PRL receptor were cotransfected with a −300 → +45 3β-HSD CAT reporter construct into HeLa cells, which resulted in a 21-fold increase in reporter activity in the presence of PRL. Promoter activity showed an increased response with a stepwise elevation of transfected Stat5 expression or by treatment with increasing concentrations of PRL (max, 250 ng/ml). This effect was dramatically reduced when the putative Stat5 response element was removed by 5′-deletion of the promoter or by the introduction of a 3-bp mutation into critical nucleotides in the element. Furthermore, ^{32}P-labeled promoter fragments containing the Stat5 element were shifted in electrophoretic mobility shift assay experiments using nuclear extracts from cells treated with PRL, and this complex was supershifted with antibodies to Stat5. These results demonstrate that PRL has the ability to regulate expression of a key human enzyme gene (type II 3β-HSD) in the progesterone biosynthetic pathway, which is essential for maintaining pregnancy. (Molecular Endocrinology 13: 1084–1093, 1999)

0888-8809/99/$3.00/0
Molecular Endocrinology

INTRODUCTION

PRL is a multifunctional hormone involved in diverse processes such as lactation, electrolyte balance, metabolic processes, behavior, and immunoregulation (1). PRL is a 23-kDa peptide produced by lactotrophic cells of the anterior pituitary and by peripheral sites such as decidual cells of the endometrium (2), lymphocytes (3), and breast cancer cells (4). The importance of PRL in the regulation of reproductive function has been demonstrated in gene knockout experiments of either PRL (5) or the PRL receptor (6). In the case of the PRL receptor knockout experiments, female mice exhibited irregular cycles, reduced fertility, and a lack of pseudopregnancy. Further evaluation of the molecular effects of PRL on gene expression in reproductive target tissues is essential for a full understanding of PRL function.

The role of PRL in human reproduction is evident when circulating PRL is dramatically altered from normal physiological levels. Hypoprolactinemia induced by bromocriptine treatment of normal women has been shown to affect the length of the luteal cycle and circulating levels of progesterone (7). Conversely, hyperprolactinemia is associated with infertility in women (8), and elevated serum PRL levels in these patients may result in galactorrhea and amenorrhea. Functional targets of PRL in the human reproductive tissues are unclear, but a putative site of action is the ovary with effects on folliculogenesis and corpus luteum (CL) function.

PRL effects are mediated by members of the PRL/placental lactogen (PL) family in the rat and PRL/GH/PL family in humans (9). Tissue-specific effects of PRL and PRL-like molecules are regulated by cell surface expression of PRL receptors. The PRL receptor is a single-pass transmembrane receptor of the cytokine receptor superfamily (10), and it is alternatively transcribed from a single gene resulting in expression of at

least two isoforms: long and short (11). The long form of the PRL receptor has been shown to transduce PRL signals primarily by activation of Stat5 through the Jak/Stat pathway (12). PRL activation of Stat5 is thought to occur by ligand-dependent activation of the tyrosine kinase, Jak2, resulting in recruitment of latent Stat5 molecules via SH2 domains from the cytoplasm to the receptor complex. Jak2 subsequently phosphorylates Stat5 on tyrosine 694 (13), and the Stat5 molecules then dimerize via association with SH2 domains, translocate to the nucleus, and bind to Stat5 response elements in the regulatory regions of target genes, thereby activating transcription.

Some PRL-regulated genes in the rat ovary have been identified including several genes involved in ovulation such as α_2-macroglobulin (14), LH receptor (15), tissue plasminogen activator (16), and plasminogen activator inhibitor type-1 (PAI-1) (16). PRL also appears to regulate genes encoding enzymes involved with progesterone synthesis and metabolism in the rat CL including 20α-HSD (17), $P450_{scc}$ (18), and 3β-HSD (19). These examples demonstrate the broad scope of PRL action in the rat CL.

While PRL/placental lactogens play a primary luteotrophic role in rodents, the function of PRL in conjunction with hCG in primates is less defined. Although PRL receptors have been demonstrated in the human ovary (20, 21), functional consequences of this binding have not been fully explored. Owing to the luteotrophic nature of PRL in the rat CL, it is possible that PRL might play a contributing role in the primate CL. PRL has been shown to increase basal progesterone production in antral follicles (22), dispersed corpora luteal cells (23), and in granulosa-lutein cell cultures obtained from women undergoing egg retrieval in *in vitro* fertilization procedures (24, 25). Increases in progesterone production occurred at physiological doses of PRL, but these effects were reversed when doses approached levels seen in hyperprolactinemic patients (22). Therefore, it is postulated that PRL might be capable of regulating genes involved in the progesterone biosynthetic pathway.

The final catalytic step in the production of progesterone is the conversion of pregnenolone into progesterone by the enzyme, 3β-hydroxysteroid dehydrogenase/Δ^5-Δ^4 isomerase (3β-HSD). 3β-HSD exists as two isoforms encoded by two genes in humans. Type I 3β-HSD (26) is primarily expressed in the placenta and with lower expression in peripheral tissues such as the prostate, breast, and skin. Type II 3β-HSD (27, 28) is expressed in the adrenal, ovaries, and testis. Regulation of Type II 3β-HSD by gonadotropins (18, 19) and PRL (29) in the rat has been demonstrated. Human type II 3β-HSD regulation by cAMP and phorbol esters (mimetics of gonadotropin signaling pathways) has been reported to be dependent upon the orphan nuclear receptor, SF-1 (30), thus providing a mechanism for gonadotropin regulation in humans. However, direct regulation of human type II 3β-HSD by PRL has yet to be demonstrated. The results herein provide a molecular mechanism by which type II 3β-HSD expression is regulated by PRL.

RESULTS

Regulation of the Human Type II 3β-HSD Promoter by PRL

PRL signal transduction involves the nuclear translocation of tyrosine- phosphorylated Stat5 dimers (12). Therefore, a search of the 5′-flanking sequence of the human type II 3β-HSD gene was performed for Stat5 response elements. A putative Stat5 response element (5′-TTCTGAGAA-3′) was identified from −110 to −118 upstream of the transcription initiation site (Fig. 1). This is a 9/9 match with the consensus Stat5 response element: 5′-TTCNNNGAA-3′ (31).

To test PRL responsiveness of the putative Stat5 response element, a −301 → +46 fragment of the human type II 3β-HSD gene linked to the chloramphenicol acetyltransferase (CAT) reporter gene (−301CAT) was transiently cotransfected into HeLa cells with expression vectors for Stat5 and the PRL receptor (PRL-R_c). As shown in Fig. 2, Stat5 overexpression had little effect on reporter activity in both untreated and PRL-treated (100 ng/ml) cells when compared with reporter alone. PRL treatment with PRL-R_c overexpression resulted in a 13-fold increase in reporter activity relative to cells overexpressing PRL-R_c without treatment. PRL treatment of cells cotransfected with Stat5 and PRL-R_c resulted in approximately 21-fold increased reporter activity relative to untreated cells. These results demonstrate the ability of PRL activation of Stat5 to increase the transcriptional activity of human type II 3β-HSD promoter.

5′-Deletion Mutagenesis of the Type II 3β-HSD Promoter

To determine the region of the promoter conferring PRL responsiveness, 5′-deletion mutants were transiently cotransfected into HeLa cells with expression vectors for Stat5 and PRL-R_c. As shown in Fig. 3,

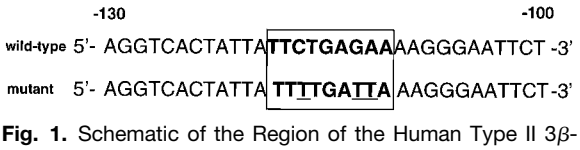

Fig. 1. Schematic of the Region of the Human Type II 3β-HSD Promoter Showing Both the Wild-type (*top*) and Mutated (*bottom*) Stat5 Responsive Elements

The putative Stat5 responsive element is shown in the *box*. Three-point mutations introduced into critical base pairs of the Stat5 consensus sequenced are *underlined*. These mutations were introduced by linking synthesized fragments of the human type II 3β-HSD promoter from −301 → +45 as described in *Materials and Methods*.

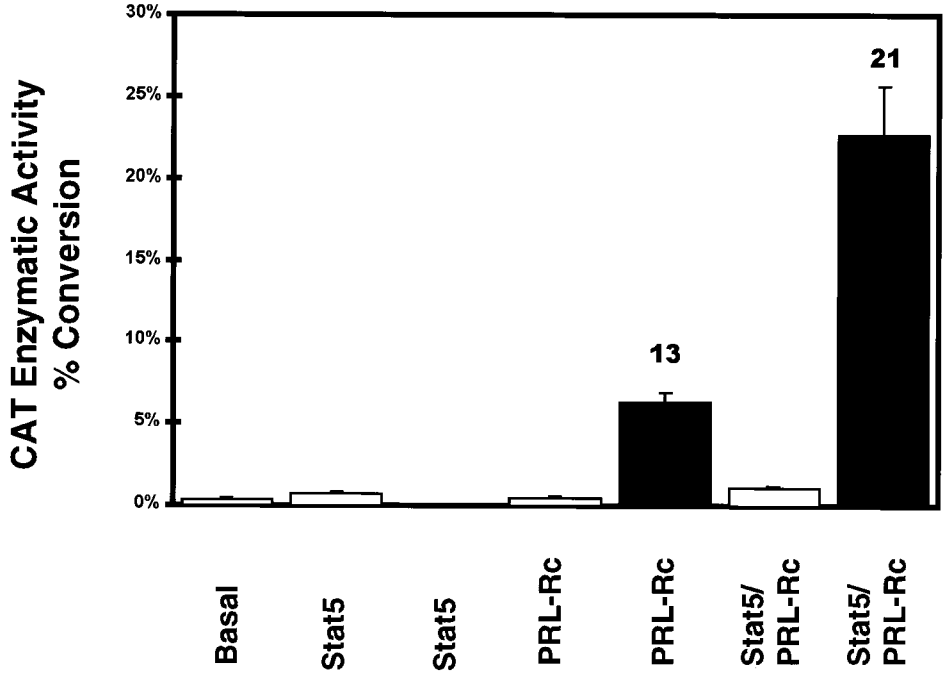

Fig. 2. Requirement of Stat5 and PRL-R_c for Maximal Induction of Human Type II 3β-HSD Promoter Activity by PRL

HeLa cells were cotransfected with −301 → +45 fragment of the type II 3β-HSD promoter fused to a CAT reporter gene (−301 CAT; 5 μg) and cytomegalovirus-driven expression vectors for Stat5 (5 μg) and PRLR-R_c (5 μg) using the calcium phosphate precipitation method followed by treatment for 24 h with PRL (100 ng/ml) as described in *Materials and Methods*. *Black bars* indicate PRL treatment. Number *above* bar is fold-activation as compared with identically transfected group minus PRL. Data represent the mean ± SE of triplicate cultures after correction for transfection efficiency from a representative experiment of three experiments.

basal reporter activity increased for −1051, −701, −301, and −101 CAT promoter constructs but was reduced in the −52 CAT construct. PRL activation of the −1051 CAT construct yielded promoter activity that was 94-fold higher than basal while increases in reporter activity were 23-fold and 21-fold higher in −701 CAT and −301 CAT, respectively. PRL treatment of the −101 CAT ($P > 0.08$) and −52 CAT ($P > 0.79$) constructs resulted in promoter activities that were not statistically different. The putative Stat5 response element was deleted in the −101 and −52 CAT constructs. A similar pattern of activation of the 5′-deletion CAT promoter constructs was seen in experiments in which Stat5 was not overexpressed albeit at decreased basal and PRL-stimulated levels (data not shown). These data suggest that a major region of PRL responsiveness is eliminated with deletion of the region containing the Stat5 response element. In addition, there is one other region of PRL responsiveness located from −1051 → −701 that does not correspond with the presence of a consensus Stat5 response element.

Point Mutation of the Putative Stat5 Response Element

Since 5′-deletion mutagenesis mapped a major region of PRL responsiveness to the −301 → −101 region containing a putative Stat5 response element, the requirement of the Stat5 element for transactivation of type II 3β-HSD promoter by PRL was tested. As shown in Fig. 1, 3-bp point mutations were introduced into critical base pairs in the −301 → +46 CAT reporter construct converting the element from 5′-TTCTGAGAA-3′ to 5′-TT**T**TGA**TT**A-3′. Comparison of the −301(mutant) CAT promoter construct as compared with the −301(wild-type) CAT promoter construct is shown in Fig. 4. Both reporter constructs were transiently cotransfected into HeLa cells overexpressing Stat5 and PRL-R_c. Basal and PRL-activated reporter activities were dramatically reduced by mutation of the Stat5 element.

Electrophoretic Mobility Shift Assays of the Putative Stat5 Response Element

Since point mutations indicated the importance of the Stat5 element for PRL responsiveness, a ^{32}P-end-labeled, double-stranded fragment of the type II 3β-HSD promoter (5′-GTCACTATTA**TTCTGAGAA**AAGGGATTCTG-3′) was incubated with nuclear extracts obtained from HeLa cells overexpressing Stat5 and PRL-R_c under basal or stimulated (100 ng/ml PRL) conditions. As seen in Fig. 5, both basal and PRL-treated nuclear extracts formed complex D (lane 1), which was not further shifted by Stat5 antiserum (lane

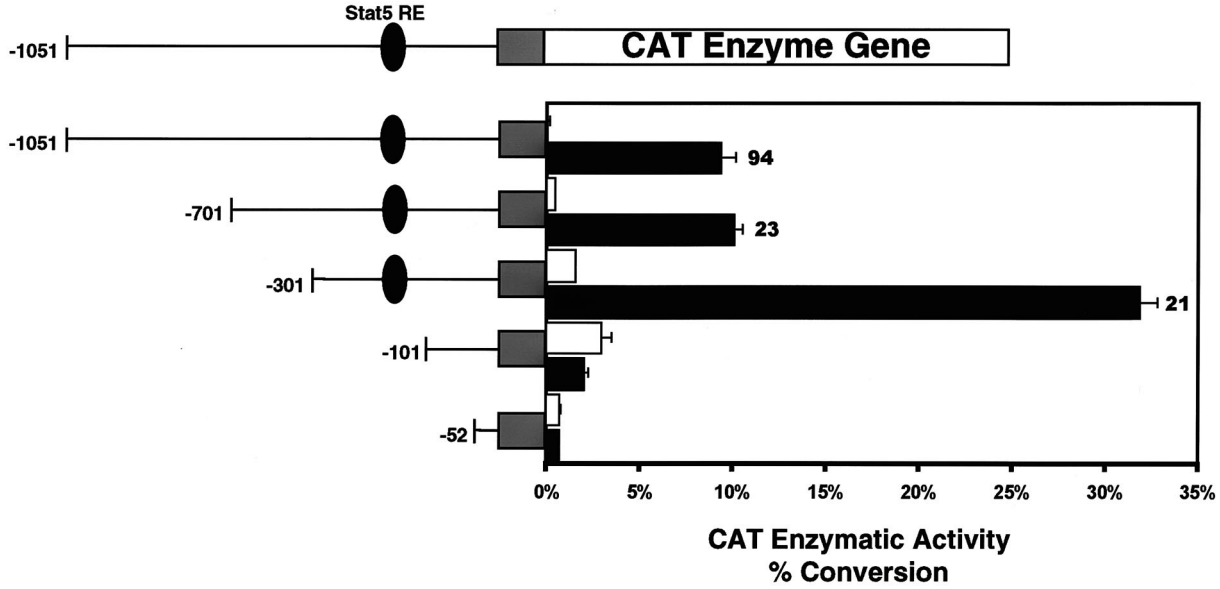

Fig. 3. Deletion Mutagenesis of Human Type II 3β-HSD Promoter CAT Constructs and Promoter Activities in Transiently Transfected HeLa Cells

HeLa cells were transfected with a series of 5′-deletion mutants of the type II 3β-HSD promoter fused to a CAT reporter gene (5 μg) and expression vectors for Stat5 (5 μg) and PRL-R_c (5 μg) followed by treatment for 24 h with PRL (100 ng/ml) as described in *Materials and Methods*. *Black bars* indicate PRL treatment. *Number above bar* is fold-activation as compared with identically transfected group minus PRL. Data represent the mean ± SE of triplicate cultures after correction for transfection efficiency from a representative experiment of three experiments.

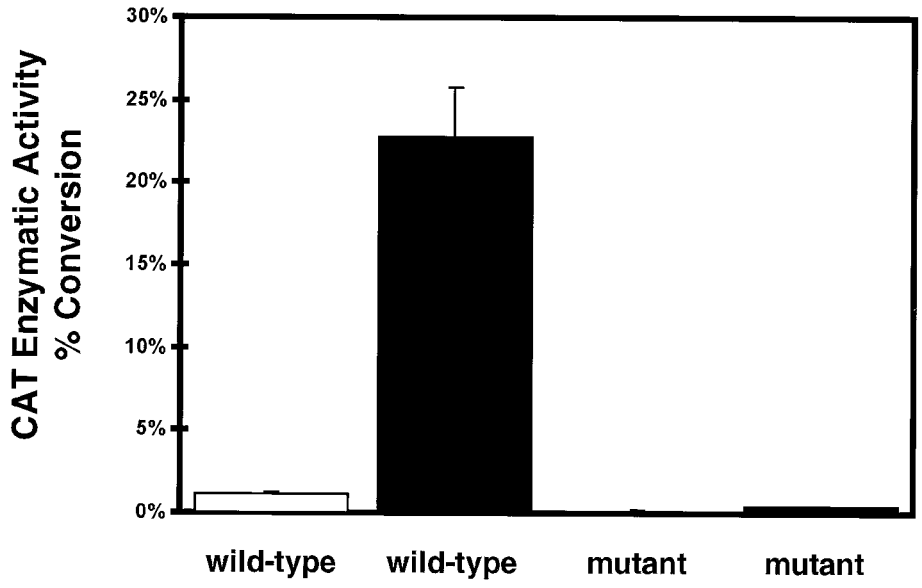

Fig. 4. A Putative Stat5 Regulatory Element Is Required for PRL-Mediated Transactivation of the Type II 3β-HSD Promoter in Transiently Transfected HeLa Cells

HeLa cells were transfected with −301(wild-type) or −301(mutant) CAT reporter constructs (5 μg) and expression vectors for Stat5 (5 μg) and PRL-R_c (5 μg) followed by treatment for 24 h with PRL (100 ng/ml) as described in *Materials and Methods*. *Black bars* indicate PRL treatment. Data represent the mean ± SE of triplicate cultures after correction for transfection efficiency from a representative experiment of three experiments.

2). This complex was specific since it was competed away by 10× (lane 3) and 50× (lane 4) identical, unlabeled oligonucleotide. PRL-treated extracts caused shifting of two additional complexes B and C (lane 5). Only complex C was supershifted (forming complex A and possibly increasing complex B) by antiserum to

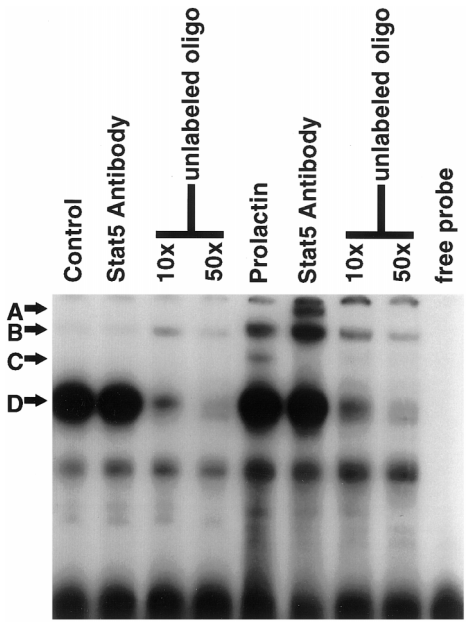

Fig. 5. HeLa Cell Nuclear Proteins Form a Complex with the Stat5 Response Element Present in the Type II 3β-HSD Promoter That is Supershifted by Antibodies to Stat5

Gel shifts were performed using nuclear extracts from control or PRL-treated HeLa cells (20 μg) and labeled oligonucleotide containing the Stat5 regulatory element present in the type II 3β-HSD promoter in the presence or absence of Stat5 antiserum (1 μl) or increasing molar concentrations (10- or 50-fold) of unlabeled oligonucleotide as described in *Materials and Methods*. Band A represents a supershifted Stat5 containing complex. Band C represents a PRL-activated complex. Bands B and D are unidentified complexes.

Stat5 (lane 6). Both B and C were competed out by 10× (lane 7) and 50× (lane 8) of identical, unlabeled oligonucleotide, yet only complex C was completely competed out at 10× concentrations. Lane 9 contains an end-labeled probe that was not incubated with nuclear extract. These experiments indicate that the Stat5 response element forms at least one molecular complex with nuclear extracts expressing Stat5 from cells exposed to PRL, but this complex does not form with extracts expressing Stat5 under basal conditions.

Effects of Increasing Stat5 Expression Levels on the Transcriptional Activity of the Type II 3β-HSD Promoter

Since Stat5 protein levels increase during luteinization in the ovary of the pseudopregnant rat (32), the effect of increasing the amount of Stat5 expression upon promoter activity in this system was examined. As shown in Fig. 6, all groups were transiently transfected with 5 μg of the −301 CAT reporter construct, 5 μg of PRL-R_c, and either increasing amounts of Stat5 or empty expression vector yielding a total transfected plasmid amount of 25 μg for each dish. The first group represents PRL-induced transcriptional activity by endogenous factors. Subsequent groups show PRL-activated promoter activity with increasing levels of Stat5 cotransfected into the cells. Significant activation ($P < 0.001$) over basal occurs with transfection at 100 ng of Stat5 and above. The overexpression of Stat5 at levels used in other experiments (5 μg) resulted in a 5-fold increase in PRL-activated promoter activity. These data demonstrate that increasing the amount of Stat5 overexpressed in this system will increase type II 3β-HSD promoter activity with a maximal response occurring above 100 ng of cotransfected Stat5 expression vector.

PRL Dose Response on Type II 3β-HSD Promoter Activity

Since down-regulation of PRL receptors is a potential mechanism in the regulation of PRL responsiveness (33), it was of interest to examine a dose-response curve of PRL activation of the type II 3β-HSD promoter in HeLa cells overexpressing PRL receptors where down-regulation cannot occur. As shown in Fig. 7, HeLa cells were cotransfected with the −301 CAT reporter construct, and expression vectors for Stat5 and PRL-R_c. Increasing concentrations of PRL resulted in a dose-dependent increase in promoter activity with maximum stimulation seen at 250 ng/ml. The response at higher doses plateau and remain constant. These results suggest that high doses of PRL saturate the receptors overexpressed on the cell surface, and that most likely intracellular mechanisms do not account for inhibitory effects seen at high doses of PRL.

DISCUSSION

These studies clearly show that the human Type II 3β-HSD promoter is activated by PRL through the Stat5 response element. 5′-Deletion of the region containing the element resulted in a dramatic loss of responsiveness that was also seen in point mutants of the Stat5 element. In addition, a PRL-activated complex containing Stat5 bound to an oligonucleotide containing the Stat5 response element. The PRL response is dose dependent and elevated with increasing levels of Stat5 expression. One other region of PRL responsiveness was identified from −1051 → −701 as seen in Fig. 3, and this increased response is interesting in light of the absence of consensus Stat elements. Future studies will address the potential for functional interaction between Stat5 and heterologous transcription factors in this region and for other mechanisms of PRL activation.

Previous studies in the laboratory have identified a potential molecular mechanism by which gonadotropin regulation of 3β-HSD occurs. Gonadotropins act through G protein-coupled receptors and activate

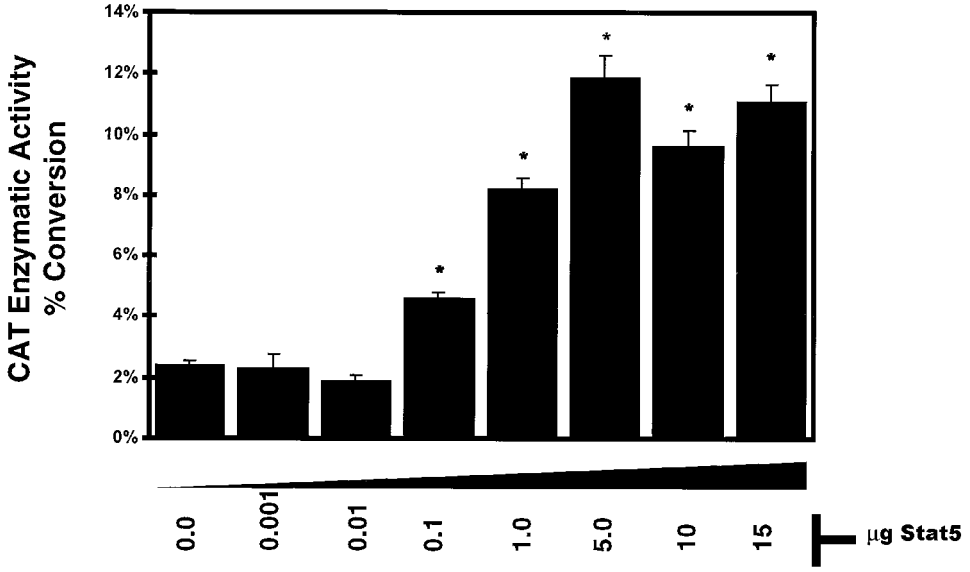

Fig. 6. Increasing Levels of Stat5 Result in a Rise in the Transactivation of Human Type II 3β-HSD Promoter in Transiently Transfected HeLa Cells

HeLa cells were transfected with −301 CAT reporter construct (5 μg), expression vectors for PRLR-R_c (5 μg), and increasing levels of Stat5 (0 → 15 μg) followed by treatment for 24 h with PRL (100 ng/ml) as described in *Materials and Methods*. Data represent the mean ± SE of triplicate cultures after correction for transfection efficiency from a representative experiment of two experiments. An *asterisk* represents statistical significance where $P < 0.001$ relative to the unstimulated control.

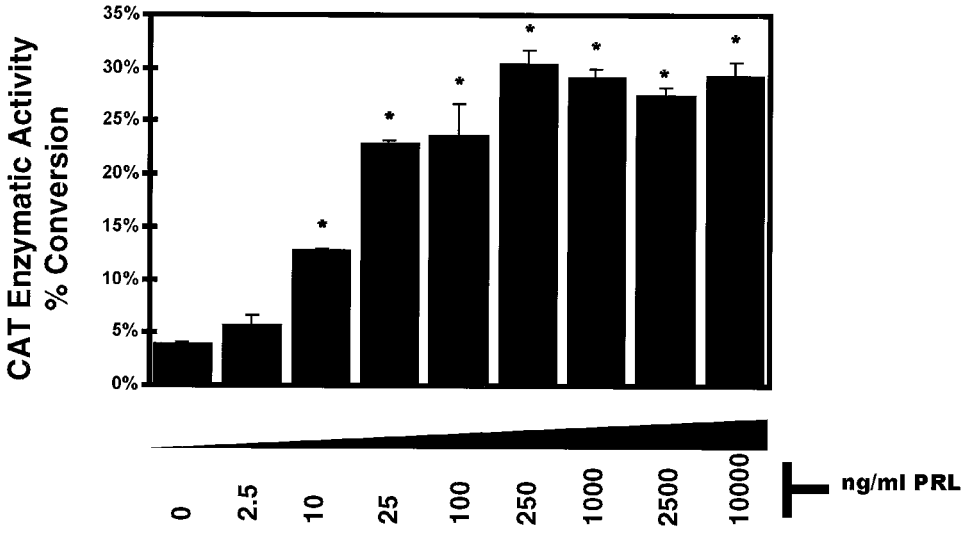

Fig. 7. Increasing Concentrations of PRL Result in Increased Transactivation of Human Type II 3β-HSD Promoter in Transiently Transfected HeLa Cells

HeLa cells were transiently transfected with −301 CAT reporter construct (5 μg) and expression vectors for Stat5 (5 μg) and PRLR-R_c (5 μg) followed by treatment for 24 h with increasing doses of PRL (0 → 10,000 ng/ml) as described in *Materials and Methods*. Data represent the mean ± SE of triplicate cultures after correction for transfection efficiency from a representative experiment of three experiments. An *asterisk* represents statistical significance where $P < 0.001$ relative to the unstimulated control.

cAMP/protein kinase A and Ca^{2+} flux/protein kinase C signaling pathways (34). Treatment of H295R (adrenocortical cell line) cells with phorbol esters (30) or cAMP analogs (35) will stimulate reporter gene expression when linked to the type II 3β-HSD 5′ promoter region. This activity localizes to a response element (5′-TCAAGGTAA-3′) that binds the orphan nuclear receptor, SF-1, located from −64 to −56 in the 5′-flanking sequence of the transcription initiation site. Disruption of this element by inserting point mutations into critical base pairs abrogates the cAMP/phorbol ester responsiveness. It therefore appears that a major portion of

gonadotropin control of 3β-HSD occurs through the SF-1 nuclear receptor.

The relative importance of regulation of type II 3β-HSD through the SF-1 and Stat5 response elements is unclear at this time. These sites may be working in parallel, but it is possible that formation of the transcriptional complex on the Stat5 element might interfere with formation of an SF-1 complex, thus forming the hypothesis that PRL might inhibit progesterone production via inhibition of gonadotropin signaling through the SF-1 element. Another intriguing possibility is that the Stat5 response element might be a target for other signals that activate Stat5. These include growth factors (*i.e.* GH or EGF) or cytokines. These factors might up-regulate type II 3β-HSD enzyme levels under physiological or pathological conditions. Stat5 has also been shown to interact with nuclear receptors. For instance, the glucocorticoid receptor has been shown to functionally interact with Stat5 in up-regulation of β-casein expression in the presence of the lactogenic hormones: insulin, hydrocortisone, and PRL (36). Identification of the Stat5 response element in the type II 3β-HSD promoter opens the possibility that transduction of other signals might occur through this element either by directly activating Stat5 or interacting with Stat5 via protein-protein interactions.

Regulation of the type II 3β-HSD gene by PRL provides a mechanism by which PRL can elicit some of its ovarian effects. Factors that regulate luteal function are either luteotropins or luteolysins that increase or decrease progesterone output by the CL, respectively. The gonadotropins LH (rat) and LH/hCG (human) have been known to play a luteotrophic role by acting through G protein-coupled transmembrane receptors and elevating protein kinase A and protein kinase C activities (34). PRL has been shown to have a dual role in luteal function in the rat. Depending upon experimental conditions, PRL treatment of hypophysectomized rats has been shown to induce either luteolysis (19, 29) or be luteotrophic (37). Further evidence for a luteotrophic role of PRL in the rat ovary comes from studies in which 20α-hydroxysteroid dehydrogenase (20α-HSD), an enzyme that metabolizes progesterone into an inactive metabolite, was shown to be down-regulated by PRL (17). These studies support the idea that PRL plays a role in both the maintenance and degradation of the rodent CL depending upon experimental conditions used.

The role PRL plays in the primate ovary is equally complex. Studies of the effect of PRL exposure to granulosa-lutein cell cultures demonstrated that PRL is required at low doses ($<$20 ng/ml) for progesterone production by these cells, yet progesterone production is inhibited at higher PRL concentrations ($>$20 ng/ml) (22, 24, 25). This reduction in progesterone production occurs with PRL levels that correlate with concentrations seen in women with hyperprolactinemia (8). Other studies have shown that reduction of PRL by bromocriptine treatment resulted in shorter luteal cycles and reduced serum progesterone levels in women (7). These studies suggest an analogous dual-function role of PRL in either maintenance or disruption of progesterone output by the human CL, and that PRL effects upon the CL differ depending on circulating concentrations of the hormone. Regulation of human type II 3β-HSD by PRL could account for the reduction in progesterone production by luteal cells and reduced serum progesterone levels in hypoprolactinemic women.

The importance of the PRL-R_c/Stat5 signaling system in mediating the effects of PRL in the female reproductive system has been demonstrated in gene knockout experiments. Stat5 proteins are expressed in two isoforms that arise from two separate genes: Stat5a and Stat5b (38, 39). Mice deficient in Stat5a do not lactate and are fertile (40). Mice deficient in Stat5b show impaired mammary gland development and spontaneous abortions that can be rescued by the administration of progesterone (41). Apparent compensation of some Stat5 function occurs in either knockout because Stat5a/Stat5b double knockout mice have a more severe reproductive phenotype. These mice are infertile, do not form corpora lutea, and show an up-regulation of 20α-HSD (42). Stat5 levels have also been shown to increase in the ovaries of pseudopregnant rats (32). Mice lacking PRL-R_c are sterile, show irregular cycles, and do not exhibit pseudopregnancy (6), and mice lacking the PRL gene are infertile (5). These experiments clearly demonstrate the importance of Stat5, PRL, and PRL-R_c in regulating luteal function.

In summary, these data demonstrate the stimulatory effect of PRL on human type II 3β-HSD promoter activity. PRL induces promoter activity by the formation of a transcriptionally active complex on the Stat5 response element in the promoter that contains Stat5. This activity is disrupted by the introduction of specific point mutations into the Stat5 element or by its deletion. These data provide a potential mechanism by which human type II 3β-HSD can be up-regulated in response to PRL treatment, accounting for increases in progesterone production by human luteal cells by PRL in culture and reduced progesterone output by women with decreased circulating PRL due to bromocriptine treatment.

MATERIALS AND METHODS

Cell Culture

HeLa (human cervical carcinoma) cells were maintained in DMEM/F-12 (Life Technologies, Gaithersburg, MD) with 10% FCS (HyClone Laboratories, Inc., Logan, UT). Media contained 50 μg/ml gentamicin (Sigma Chemical Co., St. Louis, MO). PRL treatment was with 100 ng/ml ovine PRL (Sigma Chemical Co.) in 1$\times$ PBS solution. Treatment was for 24 h for CAT assays and 20 min for preparation of nuclear extracts.

Transient Transfection

HeLa cells were transiently transfected using a modification of the calcium phosphate coprecipitation method (43). Plasmid constructs employed were ovine Stat5 and the murine PRL receptor (long form) subcloned into pcDNA3 (Invitrogen, San Diego, CA) expression vectors. Human type II 3β-HSD promoter fragments were inserted into pCAT-Basic (Promega Corp., Madison, WI) reporter plasmids as described previously (30). Adherent HeLa cells were cultured to 55–65% confluency in 100-mm tissue culture dishes (Corning, Inc., Corning, NY) in 10 ml of the appropriate medium. Calcium phosphate-DNA coprecipitates were formed by dropwise addition of equal volumes (0.5 ml) of solution A (0.24 M $CaCl_2$ containing 15 μg of appropriate plasmid constructs) to solution B [2× HEPES-buffered saline; 50 mM HEPES, 1.4 mM Na_2HPO_4, 0.28 M NaCl (pH 7.1)]. Calcium phosphate-DNA precipitates were incubated at 23 C for at least 20 min and added to single 100-mm dishes of cells containing 9 ml of fresh medium. HeLa cells were then incubated with precipitate for 4 h at 37 C (5% CO_2 and 95% air), shocked for 1 min with 15% (vol/vol) glycerol in Dulbecco's PBS (D-PBS; 0.137 M NaCl, 0.5 mM $MgCl_2$, 6.45 mM Na_2HPO_4, 1.5 mM K_2HPO_4), washed three times with D-PBS, and incubated at 37 C for 24 h. During the final 24 h of incubation, cells were cultured in the presence or absence of appropriate treatment. Cells were then harvested using trypsin/EDTA (Life Technologies), pelleted, resuspended in 0.25 M Tris-HCl (pH 7.4), and stored at −70 C until assayed for CAT activity. Transfections were performed in triplicate with mock negative controls. Internal transfection efficiency was monitored by cotransfection of 1 μg of either pSEAP2 (secreted alkaline phosphatase) or pCMV-β-galactosidase constructs and measurement of respective enzymatic activities. One hundred percent transfection efficiency was considered to be the group with the highest control enzymatic activity (alkaline phosphatase or β-galactosidase), and all groups were normalized to this value.

CAT Assays

Frozen cell pellets were thawed on ice and lysed by sonication. Extracts were heated to 60 C for 5 min to denature any endogenous acetylase/deacetylase enzymes. Soluble extracts were then separated from cell debris by centrifugation, divided into aliquots for CAT assays, and stored at −70 C before use. Fluorescent CAT assays were performed as described previously (44) with some modifications using the FLASH CAT assay kit (Stratagene, La Jolla, CA). Acetyl coenzyme A (CoA) was synthesized by reaction of CoA (Pharmacia Biotech, Piscataway, NJ) with acetic anhydride (Sigma Chemical Co.) as described elsewhere (45) and stored at −70 C until use. Cell extracts (10–20 μl) were incubated in 0.25 M Tris-HCl (pH 7.4) in a total reaction volume of 50 μl with acetyl-CoA (8.2 μM) and fluorescent borondipyrromethene difluoride (BODIPY) chloramphenicol (CAM) substrate (1:12.5 dilution) at 37 C for 4–8 h. Reactions were terminated by addition of cold ethyl acetate (850 μl) followed by vigorous vortexing. An aliquot (800 μl) of extracted substrate and acetylated products was removed (organic phase), dried under vacuum, and resuspended in ethyl acetate (20 μl) before separation on TLC plates (LK6, Whatman, Clifton, NJ) with chloroform-methanol (9:1) for 30 min. Substrate and products were visualized under long-wave UV light (366 nm) and photographed (type 55 positive/negative film, Polaroid). Substrate and combined product bands were scraped from the plates, extracted, and diluted 1:10 in methanol before quantification by fluorescence spectrophotometry at excitation and emission wavelengths of 490 nm and 512 nm, respectively, using a fluorometer. Percent conversion of BODIPY CAM substrate to 1-, 3-, and 1,3-acetylated BODIPY CAM products was computed.

Preparation of Nuclear Extracts

Crude nuclear extracts were prepared as described previously (46) with modifications (47). Three identical 100-mm tissue culture dishes of HeLa cells were transfected and cultured as described. At 55–65% confluency, cells were cultured in the presence or absence of 100 ng/ml ovine PRL for 20 min and harvested by scraping into D-PBS containing tyrosine phosphatase inhibitors (1 mM Na_3VO_4, 50 μM Na_3MoO_4). Cells were then pelleted and resuspended in Buffer A [10 mM HEPES-KOH (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, 1 mM Na_3VO_4, 50 μM Na_3MoO_4] for 10 min at 4 C followed by vortexing and centrifugation. The supernatant was then discarded and the pellet resuspended in Buffer C (20 mM HEPES-KOH (pH 7.9), 25% glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, 1 mM Na_3VO_4, 50 μM Na_3MoO_4) for 20 min to extract nuclear proteins. The suspension was centrifuged, and the supernatant containing nuclear proteins was aliquoted and stored in liquid nitrogen. Protein concentrations were determined using the BCA method (Pierce Chemical Co., Rockford, IL).

Electrophoretic Mobility Shift Analysis (EMSA)

EMSA experiments were performed as described (48) with some modification. Single-stranded, complementary 30-bp oligonucleotides (5′-GTCACTATTA**TTCTGAGAA**AAGGGATTCTG-3′) containing the putative Stat5 response elements were synthesized (Life Technologies, Inc.). Double-stranded probes were prepared by annealing 50 ng of each oligonucleotide strand for 2 min at 95 C, followed by slow cooling to room temperature. The probe was then end-labeled using [γ-^{32}P]ATP (3000 Ci/μmol; Amersham, Arlington Heights, IL) and T4 polynucleotide kinase (New England BioLabs, Inc., Beverly, MA) and purified using Nuc-Trap columns (Stratagene). Nuclear extracts (20 μg) from cells were preincubated in the presence (1 μl) or absence of anti-Stat5 (C-17; Santa-Cruz Biotechnology, Inc.,) for 30 min on ice before the addition of poly(dIdC)·poly(dIdC) (2 μg, Pharmacia Biotech) in 15.0 mM HEPES (pH 7.9), 50 mM KCl, 42 mM NaCl, 0.15 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, 2.5% glycerol, 4% Ficoll, ^{32}P-labeled oligonucleotide (~4 × 10^4 cpm) to a final reaction volume of 20 μl and incubated for an additional 30 min on ice. In additional competition experiments, reactions contained unlabeled, double-stranded oligonucleotide (10× or 50× molar excess). DNA-protein complexes were resolved using native PAGE (5% acrylamide-bisacrylamide, 37.5:1) with 0.5× Tris borate-EDTA (44.5 mM Tris, 44.5 mM boric acid, 1 mM EDTA) for 2 h at 150 V. Gels were then dried under vacuum at 70 C for 1 h and exposed to Kodak BioMax MR film (Eastman Kodak Co., Rochester, NY) for 24 h at −70 C.

Generation of Mutated Stat5 Response Element

The −301 (mutant) CAT promoter construct was generated by synthesizing (Life Technologies) overlapping top and complementary DNA strands with directional restriction endonuclease sites at either end. The oligonucleotides, ranging in size from 19 to 45 bases, modified the Stat5 element from 5′-TTCTGAGAA-3′ to 5′-TTTTGATTA-3′. The oligonucleotides were phosphorylated by T_4 polynucleotide kinase, ligated with T_4 DNA ligase, and then filled with the Klenow fragment of DNA polymerase. The final blunt fragment was cleaved with restriction endonucleases, agarose gel-purified, and ligated into pCAT-basic. The final −301 (mutant) CAT promoter construct was sequenced on both strands to verify the point mutations and the fidelity of the remaining sequence.

Statistical Analysis

Statistical significance was determined by single-factor ANOVA followed by Bonferroni correction for multiple comparisons. Sample differences were not considered to be statistically significant unless $P < 0.05$ (divided by the number of treatment groups) as per the Bonferroni correction for multiple comparisons.

Acknowledgments

The authors wish to thank Dr. Susan Ruff for invaluable advice on EMSAs.

Received February 1, 1999. Revision received March 19, 1999. Accepted March 31, 1999.

Address requests for reprints to: Dr. Michael H. Melner, Department of Obstetrics/Gynecology, B-1100 Medical Center North, Vanderbilt University, Nashville, Tennessee 37232. E-mail: mike.melner@mcmail.vanderbilt.edu.

Frank A. Feltus was supported in part by NIH Training Grant 5T3-HD-07043.

REFERENCES

1. 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
2. Lee DW, Markoff E 1986 Synthesis and release of glycosylated prolactin by human decidua in vitro. J Clin Endocrinol Metab 62:990–994
3. Montgomery DW, LeFevre JA, Ulrich ED, Adamson CR, Zukoski CF 1990 Identification of prolactin-like proteins synthesized by normal murine lymphocytes. Endocrinology 127:2601–2603
4. Ginsburg E, Vonderhaar BK 1995 Prolactin synthesis and secretion by human breast cancer cells. Cancer Res 55:2591–2595
5. Horseman ND, Zhao W, Montecino-Rodriguez E, Tanaka M, Nakashima K, Engle SJ, Smith F, Markoff E, Dorshkind K 1997 Defective mammopoiesis, but normal hematopoiesis, in mice with a targeted disruption of the prolactin gene. EMBO J 16:6926–6935
6. Ormandy CJ, Camus A, Barra J, Damotte D, Lucas B, Buteau H, Edery M, Brousse N, Babinet C, Binart N, Kelly PA 1997 Null mutation of the prolactin receptor gene produces multiple reproductive defects in the mouse. Genes Dev 11:167–178
7. Kauppila A, Martikainen H, Puistola U, Reinila M, Ronnberg L 1988 Hypoprolactinemia and ovarian function. Fertil Steril 49:437–441
8. Blackwell RE 1992 Hyperprolactinemia: evaluation and management. Endocrinol Metab Clin North Am 21:105–124
9. Soares MJ, Muller H, Orwig KE, Peters TJ, Dai G 1998 The uteroplacental prolactin family and pregnancy. Biol Reprod 58:273–284
10. Bazan JF 1989 A novel family of growth factor receptors: a common binding domain in the growth hormone, prolactin, erythropoietin and IL-6 receptors, and the p75 IL-2 receptor β-chain. Biochem Biophys Res Commun 164:788–795
11. Lesueur L, Edery M, Ali S, Paly J, Kelly PA, Djiane J 1991 Comparison of long and short forms of the prolactin receptor on prolactin-induced milk protein gene transcription. Proc Natl Acad Sci USA 88:824–828
12. Darnell Jr JE 1997 STATs and gene regulation. Science 277:1630–1635
13. Gouilleux F, Wakao H, Mundt M, Groner B 1994 Prolactin induces phosphorylation of Tyr694 of Stat5 (MGF), a prerequisite for DNA binding and induction of transcription. EMBO J 13:4361–4369
14. Dajee M, Kazansky AV, Raught B, Hocke GM, Fey GH, Richards JS 1996 Prolactin induction of the alpha 2-Macroglobulin gene in rat ovarian granulosa cells: stat5 activation and binding to the interleukin-6 response element. Mol Endocrinol 10:171–184
15. Casper RF, Erickson GF 1981 *In vitro* heteroregulation of LH receptors by prolactin and FSH in rat granulosa cells. Mol Cell Endocrinol 23:161–171
16. Liu YX, Peng XR, Liu HZ, Chen YJ, Ny T 1998 Prolactin regulation of tissue type plasminogen activator and plasminogen activator inhibitor type-I gene expression in eCG-primed rat granulosa cells in culture. Biol Reprod 59:409–416
17. Zhong L, Parmer TG, Robertson MC, Gibori G 1997 Prolactin-mediated inhibition of 20α-hydroxysteroid dehydrogenase gene expression and the tyrosine kinase system. Biochem Biophys Res Commun 235:587–592
18. Doody KJ, Lephart ED, Stirling D, Lorence MC, Magness RR, McPhaul MJ, Simpson ER 1991 Expression of mRNA species encoding steroidogenic enzymes in the rat ovary. J Mol Endocrinol 6:153–162
19. Martel C, Gagne D, Couet J, Labrie Y, Simard J, Labrie F 1994 Rapid modulation of ovarian 3β-hydroxysteroid dehydrogenase/Δ^5-Δ^4 isomerase gene expression by prolactin and human chorionic gonadotropin in the hypophysectomized rat. Mol Cell Endocrinol 99:63–71
20. Schwarzler P, Untergasser G, Hermann M, Dirnhofer S, Abendstein B, Berger P 1997 Prolactin gene expression and prolactin protein in premenopausal and postmenopausal human ovaries. Fertil Steril 68:696–701
21. Saito T, Saxena BB 1975 Specific receptors for prolactin in the ovary. Acta Endocrinol (Copenh) 80:126–137
22. McNatty KP, Sawers RS, McNeilly AS 1974 A possible role for prolactin in control of steroid secretion by the human Graafian follicle. Nature 250:653–655
23. Alila HW, Rogo KO, Gombe S 1987 Effects of prolactin on steroidogenesis by human luteal cells in culture. Fertil Steril 47:947–955
24. Lee MS, Ben-Rafael Z, Meloni F, Mastroianni Jr L, Flickinger GL 1986 Effects of prolactin on steroidogenesis by human luteinized granulosa cells. Fertil Steril 46:32–36
25. Soto EA, Tureck RW, Strauss III JF 1985 Effects of prolactin on progestin secretion by human granulosa cells in culture. Biol Reprod 32:541–545
26. Simard J, Melner MH, Breton N, Low KG, Zhao HF, Periman LM, Labrie F 1991 Characterization of macaque 3β-hydroxy-5-ene steroid dehydrogenase/Δ^5-Δ^4 isomerase: structure and expression in steroidogenic and peripheral tissues in primate. Mol Cell Endocrinol 75:101–110
27. Lachance Y, Luu-The V, Verreault H, Dumont M, Rheaume E, Leblanc G, Labrie F 1991 Structure of the human type II 3β-hydroxysteroid dehydrogenase/Δ^5-Δ^4 isomerase (3β-HSD) gene: adrenal and gonadal specificity. DNA Cell Biol 10:701–711
28. Rheaume E, Lachance Y, Zhao HF, Breton N, Dumont M, de Launoit Y, Trudel C, Luu-The V, Simard J, Labrie F 1991 Structure and expression of a new complementary DNA encoding the almost exclusive 3β-hydroxysteroid dehydrogenase/Δ^5-Δ^4-isomerase in human adrenals and gonads. Mol Endocrinol 5:1147–1157
29. Martel C, Labrie C, Dupont E, Couet J, Trudel C, Rheaume E, Simard J, Luu-The V, Pelletier G, Labrie F 1990 Regulation of 3β-hydroxysteroid dehydrogenase/Δ^5-Δ^4 isomerase expression and activity in the hypophysectomized rat ovary: interactions between the

stimulatory effect of human chorionic gonadotropin and the luteolytic effect of prolactin. Endocrinology 127:2726–2737
30. Leers-Sucheta S, Morohashi K, Mason JI, Melner MH 1997 Synergistic activation of the human type II 3β-hydroxysteroid dehydrogenase/Δ^5-Δ^4 isomerase promoter by the transcription factor steroidogenic factor-1/adrenal 4-binding protein and phorbol ester. J Biol Chem 272:7960–7967
31. Standke GJ, Meier VS, Groner B 1994 Mammary gland factor activated by prolactin on mammary epithelial cells and acute-phase response factor activated by interleukin-6 in liver cells share DNA binding and transactivation potential. Mol Endocrinol 8:469–477
32. Ruff SJ, Leers-Sucheta S, Melner MH, Cohen S 1996 Induction and activation of Stat 5 in the ovaries of pseudopregnant rats. Endocrinology 137:4095–4099
33. Augustine EC, Kensinger RS 1990 Endocytosis and degradation of ovine prolactin by Nb2 lymphoma cells: characterization and effects of agents known to alter prolactin-induced mitogenesis. Endocrinology 127:200–210
34. Segaloff DL, Ascoli M 1993 The lutropin/choriogonadotropin receptor . . . 4 years later. Endocr Rev 14:324–347
35. Melner MH, Morohashi K, Mason JI, Feltus FA, Leers-Sucheta S, Activation of the human 3β-hydroxysteroid dehydrogenase promoter by the orphan nuclear receptor steroidogenic factor-1 (SF-1) is enhanced in a synergistic manner by cAMP as well as phorbol esters. Program of the 16th Joint Meeting of the British Endocrine Societies, Harrogate, England, 1997 (Abstract)
36. Stocklin E, Wissler M, Gouilleux F, Groner B 1996 Functional interactions between Stat5 and the glucocorticoid receptor. Nature 383:726–728
37. Albarracin CT, Gibori G 1991 Prolactin action on luteal protein expression in the corpus luteum. Endocrinology 129:1821–1830
38. Liu X, Robinson GW, Gouilleux F, Groner B, Hennighausen L 1995 Cloning and expression of Stat5 and an additional homologue (Stat5b) involved in prolactin signal transduction in mouse mammary tissue. Proc Natl Acad Sci USA 92:8831–8835
39. Wakao H, Gouilleux F, Groner B 1995 Mammary gland factor (MGF) is a novel member of the cytokine regulated transcription factor gene family and confers the prolactin response. EMBO J 14:854–855
40. Liu X, Robinson GW, Wagner KU, Garrett L, Wynshaw-Boris A, Hennighausen L 1997 Stat5a is mandatory for adult mammary gland development and lactogenesis. Genes Dev 11:179–186
41. Udy GB, Towers RP, Snell RG, Wilkins RJ, Park SH, Ram PA, Waxman DJ, Davey HW 1997 Requirement of STAT5b for sexual dimorphism of body growth rates and liver gene expression. Proc Natl Acad Sci USA 94:7239–7244
42. Teglund S, McKay C, Schuetz E, van Deursen JM, Stravopodis D, Wang D, Brown M, Bodner S, Grosveld G, Ihle JN 1998 Stat5a and Stat5b proteins have essential and nonessential, or redundant, roles in cytokine responses. Cell 93:841–850
43. Low KG, Chu HM, Schwartz PM, Daniels GM, Melner MH, Comb MJ 1994 Novel interactions between human T-cell leukemia virus type I Tax and activating transcription factor 3 at a cyclic AMP-responsive element. Mol Cell Biol 14:4958–4974
44. Young SL, Barbera L, Kaynard AH, Haugland RP, Kang HC, Brinkley M, Melner MH 1991 A nonradioactive assay for transfected chloramphenicol acetyltransferase activity using fluorescent substrates. Anal Biochem 197:401–407
45. Stadtman ER 1957 Preparation and assay of acyl coenzyme A and other thiol esters; use of hydroxyamine. Methods Enzymol 3:931–941
46. Dignam JD, Lebovitz RM, Roeder RG 1983 Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res 11:1475–1489
47. Andrews NC, Faller DV 1991 A rapid micropreparation technique for extraction of DNA-binding proteins from limiting numbers of mammalian cells. Nucleic Acids Res 19:2499
48. Morohashi K, Omura T 1990 Tissue-specific transcription of P-450(11 β) gene *in vitro*. J Biochem (Tokyo) 108:1050–1056