



HAL
open science

Differential roles for signal transducers and activators of transcription 5a and 5b in PRL stimulation of ERalpha and ERbeta transcription

J. Frasor, K. Park, M. Byers, C. Telleria, T. Kitamura, L.Y. Yu-Lee, Jean Djiane, O.K. Park-Sarge, G. Gibori

► To cite this version:

J. Frasor, K. Park, M. Byers, C. Telleria, T. Kitamura, et al.. Differential roles for signal transducers and activators of transcription 5a and 5b in PRL stimulation of ERalpha and ERbeta transcription. *Molecular Endocrinology -Baltimore-*, 2001, 15 (12), pp.2172-2181. hal-02673573

HAL Id: hal-02673573

<https://hal.inrae.fr/hal-02673573v1>

Submitted on 31 May 2020

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Differential Roles for Signal Transducers and Activators of Transcription 5a and 5b in PRL Stimulation of ER α and ER β Transcription

JONNA FRASOR, KYUNGSOO PARK, MICHAEL BYERS, CARLOS TELLERIA, TOSHIO KITAMURA, LI-YUAN YU-LEE, JEAN DJIANE, OK-KYONG PARK-SARGE, AND GEULA GIBORI

Department of Physiology and Biophysics (J.F., C.T., G.G.), University of Illinois at Chicago, Chicago, Illinois 60612; Department of Physiology (K.P., M.B., O.-K.P.-S.), University of Kentucky, Lexington, Kentucky 40536; Department of Hemopoietic Factors (T.K.), University of Tokyo, Tokyo 108-8639, Japan; Departments of Medicine, Molecular and Cell Biology, and Immunology (L.-Y.Y.-L.), Baylor College of Medicine, Houston, Texas 77030-3411; and Department of Biologie Cellulaire (J.D.), Institut National de Recherche Agronomique, Jouy-en-Josas, F-78350 France

PRL has been shown to stimulate mRNA expression of both ER α and ER β in the rat corpus luteum and decidua of pregnancy. To investigate whether PRL may stimulate ER expression at the level of transcription and which transcription factors may mediate this stimulation, we have cloned the 5'-flanking regions of both rat ER genes. A constitutively active PRL receptor (PRL-R_{CA}) stimulated both ER α and ER β promoter activity, indicating that PRL is acting to stimulate ER transcription. Putative signal transducer and activator of transcription (Stat)5 response elements were identified at -189 in the ER α promoter and at -330 in the ER β promoter. Mutation of these response elements or overexpression of dominant negative Stat5 prevented stimulation of ER α and ER β pro-

motor activity, indicating that PRL regulation of ER expression requires both intact Stat5 binding sites as well as functional Stat5. Interestingly, either Stat5a or Stat5b could stimulate ER α transcription while stimulation of ER β occurred only in the presence of Stat5b. Through mutational analysis, a single nucleotide difference between the ER α and ER β Stat5 response elements was shown to be responsible for the lack of Stat5a-mediated stimulation of ER β . These findings indicate that PRL stimulation of ER expression occurs at the level of transcription and that PRL regulation of ER α can be mediated by either Stat5a or Stat5b, while regulation of ER β appears to be mediated only by Stat5b. (Molecular Endocrinology 15: 2172-2181, 2001)

IN THE PREGNANT rat, E2 is a potent tropic hormone, which stimulates both progesterone biosynthesis and luteal cell hypertrophy (1). However, the stimulatory effect of E2 depends upon previous exposure of the corpus luteum to PRL or PRL-related hormones from placental origin (2). This prerequisite was shown to be due to PRL stimulation of E2 binding activity and mRNA levels for both ER α and ER β (2, 3). In addition to the corpus luteum, PRL has been shown to stimulate E2 binding activity or mRNA levels in the rat decidua (4), mammary gland (5), and liver (6). The mechanism of PRL action on ER expression, however, is not known.

Although PRL has been shown to activate multiple signaling pathways, including MAPK (7-11), PKC δ (12), c-src (13-16), and PI3K (17-20), the major and most comprehensively studied pathway activated by PRL is the janus kinase 2/signal transducer and acti-

vator of transcription 5 (Jak2/Stat5) pathway. PKC δ may be involved in PRL regulation of relaxin expression in the rat corpus luteum (12) whereas PI3K and/or MAPK may regulate PIM-1 expression in Nb2 cells (11). However, the mechanisms of by which PRL regulates gene expression through these pathways are not fully understood. In contrast, PRL has clearly been shown to regulate gene transcription through the Jak2/Stat5 pathway. This pathway has been implicated in the regulation of numerous genes by PRL, including milk proteins in the mammary gland (21), α 2-macroglobulin in the corpus luteum (22), sodium-dependent bile acid cotransporter in rat liver (23), the CIS gene promoter in COS cells (24), the 3 β -hydroxysteroid dehydrogenase gene promoter in HeLa cells (25), the PRL receptor (PRL-R) gene in insulin-producing INS-1 cells (26), and the aP2 promoter in NIH-3T3 cells (27).

In the general Jak/Stat signaling paradigm, the PRL-R dimerizes upon ligand binding (28). This causes activation of the tyrosine kinase, Jak2, which undergoes autophosphorylation and subsequently phosphorylates the receptor on tyrosine residues (28, 29). The phosphorylated tyrosines on the receptors and Jak2 become docking sites for the SH2 domains of

Abbreviations: CA-5a, CA-5b, Constitutively activated Stat5a and Stat5b; DN-5a, dominant negative Stat5a; β -gal, β -galactosidase; GAS, γ -interferon-activating sequence; Jak2, janus kinase 2; Mut-5b, mutant Stat5b; PRL-R, PRL receptor; PRL-R_{CA}, constitutively active PRL-R; Stat5, signal transducer and activator of transcription 5; WCE, whole-cell extracts.

Stat proteins (30, 31). Jak2 can thus phosphorylate and activate the recruited Stat proteins. The phosphotyrosine residues on the Stat proteins can serve as docking sites for the SH2 domain of another Stat protein so that Stats can either homo- or heterodimerize and translocate to the nucleus (32). By binding to cognate response elements located upstream of their responsive genes, Stat proteins can interact with basal transcriptional machinery and thereby regulate transcription (33).

Two forms of Stat5, Stat5a and Stat5b, were shown to transduce PRL signaling (33, 34). Although encoded by different genes, they are approximately 95% homologous at the protein level. These proteins contain a single conserved tyrosine residue in the C terminus (Y694 in Stat5a and Y699 in Stat5b), which becomes phosphorylated by Jak2 in response to PRL and is necessary for regulation of gene transcription (33, 34). Both Stat5a and Stat5b recognize the same DNA binding site, or GAS site (γ -interferon-activating sequence; TTCNNNGAA), and can mediate PRL-induced transcription (34). Stat5a and Stat5b have been knocked out, either independently or together, and several key differences between these two transcription factors were observed (35, 36). Without Stat5a, mammary gland maturation and function is impaired, while male patterns of liver function appear to be disrupted when Stat5b is absent (35–37). The reproductive phenotype in these mice is not clear. In the single Stat5a or Stat5b knockouts, no reproductive defects were observed, while the double knockout was infertile (36). However, in another Stat5b knockout, the ability to maintain pregnancy was reduced (35).

One of the major functions of PRL in luteal function is to stimulate ER expression and thereby maintain luteal responsiveness to E2 (2). To examine whether this stimulation occurs at the level of transcription, a 2-kb genomic fragment of the rat ER β promoter region was isolated and sequenced. The rat ER α promoter was also cloned. Sequence analysis has revealed that both contain putative Stat5 response elements. Both promoters were found to be stimulated by PRL and to require intact Stat5 binding sites and functional Stat5. However, PRL stimulation of ER β could be mediated by Stat5b only. The lack of ER β responsiveness to Stat5a was found to be due to a single nucleotide difference in the ER β Stat5 response element.

RESULTS

Our laboratory has previously shown, using semiquantitative RT-PCR, that PRL stimulates both ER α and ER β mRNA levels in corpora lutea of pregnant rats and in primary cultures of luteinized granulosa cells (3). Since this method does not provide information as to the differential levels of expression between two genes, we have used quantitative real-time RT-PCR. Known amounts of rat ER α and ER β cDNA were used

to generate standard curves for analysis of mRNA levels in experimental samples amplified in parallel reactions (Fig. 1A). In corpora lutea on d 7 of preg-

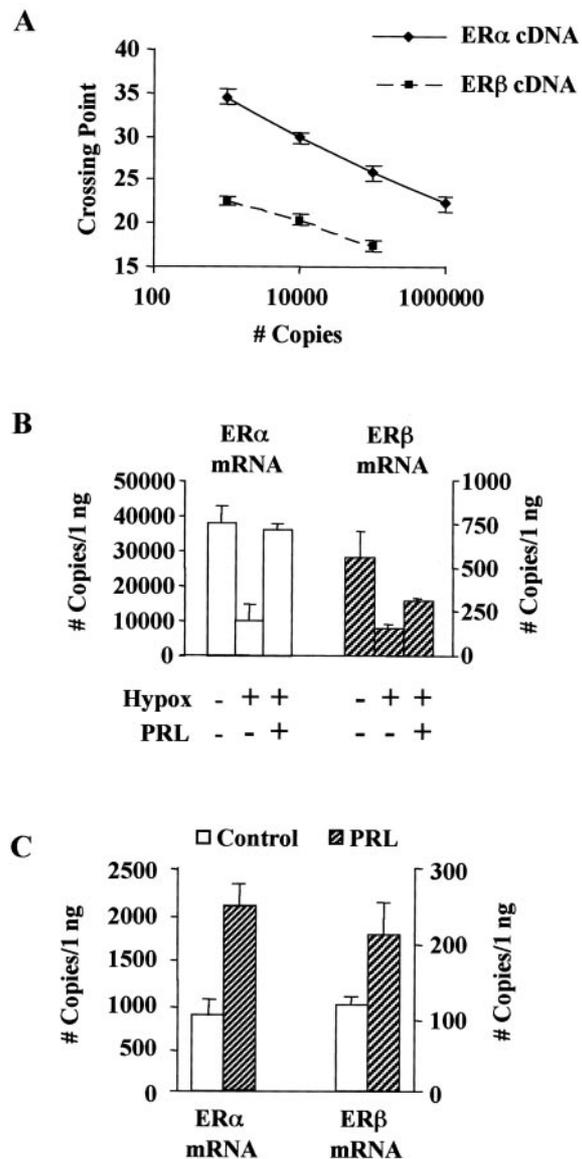


Fig. 1. Standard Curves for Real-Time Quantitative PCR

A, Known amounts of ER α and ER β cDNA, ranging from 10^3 to 10^7 copies/ μ l and 10^3 to 10^6 copies/ μ l, respectively, were amplified as described in *Materials and Methods*. The crossing point represents the number of cycles required to reach a chosen level of fluorescence, at which all standards and samples were in the linear range of amplification. The crossing point was then plotted against the number of copies of cDNA/ μ l, and the linear regression equation through the data points was used to determine the number of copies of ER α or ER β in reverse transcribed RNA samples. B, Real-time quantitative PCR was carried out for ER α and ER β using mRNA from corpora lutea of pregnant rats, hypophysectomized rats, and hypophysectomized rats treated with PRL. C, Real-time quantitative PCR was carried out for ER α and ER β using mRNA from primary luteinized granulosa cells that had been treated with 1 μ g/ml PRL for 12 h.

nancy, approximately 45,000 copies of ER α and 550 copies of ER β were detected in samples corresponding to 1 ng of RNA (Fig. 1B). In rats, hypophysectomized on d 3 of pregnancy, ER α and ER β levels were reduced to approximately 30% of the control levels. Sustained treatment with PRL induced a 3.5-fold induction of ER α expression and a 2-fold induction of ER β expression. In contrast to corpora lutea, in which there was approximately 70 times more ER α than ER β , luteinized granulosa cells cultured for 72 h expressed only 8 times more ER α than ER β , with approximately 850 copies of ER α and 120 copies of ER β per ng of starting RNA (Fig. 1C). After a 12-h treatment with PRL, a 2.4-fold increase in ER α and a 1.8-fold increase in ER β expression was observed. These findings confirm that PRL can stimulate both ER α and ER β mRNA expression and further demonstrate a much higher level of ER α than ER β in corpora lutea of pregnancy and in primary luteinized granulosa cells.

To investigate whether PRL can regulate ER expression at the level of transcription, the ER α and ER β 5'-flanking regions were cloned, and promoter-reporter constructs were prepared as described in *Materials and Methods*. A putative Stat5 response element (5'-TTCTAGGAA-3'), which represents a perfect consensus Stat5 binding site, was located at -180 bp in the ER α promoter region. In addition, a putative Stat5 response element (3'-TTCTGGTAA-5') with one nucleotide difference (*underlined*) from the

consensus sequence was located at -330 bp in the ER β promoter region (Fig. 2). CHO cells were transfected with either the ER α or ER β promoter-luciferase reporter constructs (ER α -luc, ER β -luc) together with an expression vector for a constitutively active PRL-R (PRL-R_{CA}) (38). Control cells were transfected with an expression vector for PRL-R_L, which, in the absence of any exogenous PRL treatment, served as a control. The active PRL-R has previously been shown to signal to the β -casein promoter in much the same way as PRL acting through the long form of its receptor but in the absence of exogenous PRL treatment (38). The presence of PRL-R_{CA} caused a marked phosphorylation of both Stat5a and Stat5b (Fig. 3A) and induced a 10-fold stimulation of ER α -luc activity (Fig. 3B, *left*) and a 5-fold stimulation of ER β -luc activity (Fig. 3B, *right*), indicating that PRL can induce phosphorylation of Stat5a and Stat5b as well as regulate both ER α and ER β expression at the level of transcription.

To examine whether PRL-R_{CA} activation of Stat5 was required for its stimulatory effect on ER α and ER β promoter activity, CHO cells were transfected with expression vectors for a dominant negative Stat5a (DN-5a) or a mutant Stat5b (Mut-5b). The DN-5a contains a C-terminal deletion in the transactivation domain and is transcriptionally inactive (39). The Mut-5b contains a four-amino acid substitution in the DNA binding domain, which prevents it from entering the nucleus, binding DNA, and therefore regulating tran-

```

-2023  AAAAAAATTT  ATTATTTATT  TTTATGTGTA  TGGTGTTTTG  CTGGCATGTG  TGTAAGTAGA  TGTACCATAT
-1953  ATCGCCGTGC  ATGGAGGCTG  AGAAGACAGT  ATTGGTTCCC  CTGGGCATGG  AGTCAACGAG  GACTGGAGTT
-1883  ACAGGCAAIT  GTGAGCCACC  ATGTGGGTGC  TGGAAGTGGG  CCCAGACCTT  ATGGAAACCC  TTTGCTTTTC
-1813  CTTGGGTTCT  GTGACTAAA  CTCAGGTCGT  TCAGCTAGCT  TGGCTGGTCC  ATTTTATGGT  TGAGCCATCC
-1743  TGGCCTCCCT  CCAGATGAAT  CTTGTTAACA  AGTACCAATA  AATGAACAGA  TTGTTTAAGA  CCTCTACTTC
-1673  AACTTTGCTA  CCCAAGGCAG  AAGGAGGAAG  GACTGAGTGA  TACAGGACAC  ACAGTCTCTC  GGGAAAGGGT
-1603  ACAGGATGT  GCAGATTTGG  GAGTACCAAT  CTCACCCTTT  CCTTTCCTCA  CCGGAATGGT  CTCAGTAGGT
-1533  AGCCCTCTC  CTGTCCACTC  TACCCTTCCT  AACCTCAAG  GATGACATTT  TACCCTATAA  ATGGGGAAGA
-1463  GTACAGGACT  CTTAGCTTAA  CTGAAACGTG  CACATAAGAC  GATGGGGGCA  GTGAGGTTTG  GACGCTTTGT
-1393  ATAGTTTTAA  AGTAGGTTCT  TTTACATATG  GTAGTCTGTG  GCGCTACCTG  TGGGACTCAT  AAACCCAGGC
-1323  CTCAGGAAGG  TCCCAAAAGG  CATTTTGGTT  ACACGTAAAT  GTTGAGGTTG  GCGTTGTACA  AGTTTCCCTC
-1253  CAAATACCCC  TTGGTTTTCT  TCTATTACAT  TTCCATCCAT  CAAGAAACAC  ATTCCGGGCC  TGAAGAGACA
-1183  GCTCCGCTGT  TAAGATAGCA  AGCTGCTCTT  GCATAGAAGC  TGAGTTCTGC  CCCAGCACCC  ATGCCCAGGA
-1113  GGAATCCCA  CACCTATAAT  CTCGGTGGGC  ATCTGCACGC  GTGCCCTCAA  GCCCACGTGT  GCATAATTTA
-1043  AAAGATAAAA  AGTAGATCTT  TGGGGCTGGA  GAGATGGCTC  GAGAGGTTAA  GAGCACTGGC  TGCTCTTCCA
-973  GAGATCTTGA  GTTCAAATCC  CAGCAACCAC  ATGGTGGCTT  CGAACCATCT  GTAATGGGAT  CCGGTGCCCT
-903  CTTCTGGTGT  GCTCGAAGAC  AGCTATAGTG  TACTCATTTA  ATAAATCTTT  AAAAAAAAAA  AGTAGATCTT
-833  TGA AACCCCT  CATAAACGCC  CCATGATGTG  TGCGGATGGC  CTGACCACTA  ATTAGGTACA  CGAGTGACAC
-763  CAGGTCCGCC  TACAGGTCAT  TTGTTTTCTT  TTTAACTTTT  GCCACCACAG  ACTGCCTTAA  AGCCAAATGT
-693  TTCTTAAAAA  AAAACA AAAA  AACAAAAAAA  CAAAAAACA  AAAAACAAG  GCGTGATCCC  TGCCCTTTGA
-623  GAGTAGGGTC  ACTTCAGGTT  AACCGTGTGG  AGCTGGGAGG  ATCCGAGCTG  CCATCAGGGG  ATCCGGTTCG
-553  CCCTTTGGGA  CAAGGAAGAG  AGGAGCCCTG  TAAAAGTGTA  GGCAGATTTT  TTCAGGTGAA  TTCCCTCGTT
-483  TTTTTTTTTT  TCTCTCCACT  TCCATCGCCA  GCCGCTGGTA  GACACGCAT  CAAACCTCCG  CCTCCAGCCC
-413  TTGCCCTTCT  GGGCTCAAG  CTAACACTCC  CTGAAAAGGG  ATCTGATTTT  ATTACTCCGT  TATTACTGCT

          STAT5 RE
-343  TATTTCCGTG  CTATTACCAG  AACCCGGGGC  CTGGCCCATG  CTTCTTAAGG  AGAGTTGTCA  GACCTAAGCA
-273  TTTAATGACA  CGCCCGTGGG  TCAAAGTATG  GAAACAAGAT  GAAGTGTGGT  CCCTGGAGCG  ACGCAGTCAA
-203  GTCCAGAAAT  CCTGGGGATG  GTCAGAAAT  CCCATTCCCG  GGCTCCATCT  GGTTCCTCAT  CGTCAGTCTC
-133  TTGGAAGGGG  GGTGTCCCTA  GTGGATGACT  GTGAAGAGGC  TGGAGGTGCA  CATCCAGTGG  ATCCGGTTGC
-63  GCGGCGGGG  AGTGCCTGAA  ATCTCCGGCT  CCGGGTTTGT  CACGTGGACT  TAGCTACCCT  CCCACTCTCT
+8  TTTC TAGGTC  TTTAAAAGAC  GCAC TAAACAT  CCGTTAGTC  +1

```

Fig. 2. Rat ER β Gene Promoter Region

The rat ER β 5'-flanking region was cloned as described in *Materials and Methods*. This regulatory region of the ER β gene spans from -2,023 bp to +46 bp and contains a putative Stat5 response element at -330 bp (*underlined and labeled*). The second gene-specific primer used for cloning the ER β promoter is *underlined*.

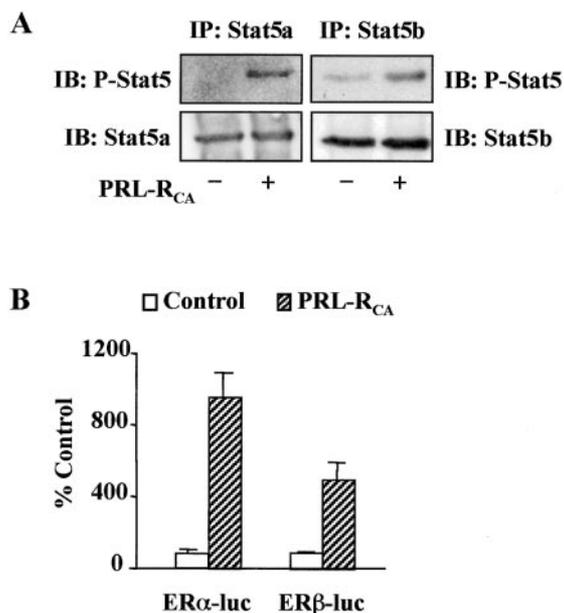


Fig. 3. Effect of PRL-R_{CA} on Stat5 Phosphorylation and ER α and ER β Promoter Activity in CHO Cells

A, CHO cells were cultured in six-well plates and transfected with 2 μ g/well expression vectors for PRL-R_{CA} or PRL-R_L (control). Forty-eight hours after the start of transfection, WCE were prepared and Stat5a and Stat5b were immunoprecipitated using specific antibodies to each form of Stat5. Western blotting was performed first using an antibody, which recognizes the tyrosine-phosphorylated form of both Stat5a and Stat5b (Y694 and Y699, respectively). The blots were stripped and reprobbed using the same antibodies for immunoprecipitation. B, Cells were transfected as described in panel A with the addition of 0.5 μ g/well ER α -luc or ER β -luc. Luciferase activity was measured in each well and normalized to the total protein level within that well. The experiment was repeated three times with triplicate wells for each group. The data represent the combined mean \pm SEM for all three experiments.

scription (40, 41). Both of these Stat5 expression vectors prevented PRL-R_{CA} stimulation of ER α and ER β promoter-driven luciferase activity (Fig. 4), indicating that PRL-R_{CA} regulation of both ER α and ER β transcription requires a functional Stat5.

The ability of PRL-R_{CA} to regulate ER α -luc and ER β -luc was also studied in COS cells. In contrast to CHO cells, COS cells express very low levels of endogenous Stat5a and Stat5b, which could not be detected by Western analysis (Fig. 5A, lanes 1 and 2). When COS cells were transfected with expression vectors for either Stat5a or Stat5b, high levels of Stat5 expression were detected and both became phosphorylated in response to PRL-R_{CA} (Fig. 5A, lanes 3 and 4). We next examined the capacity of each Stat5 to transactivate the ER α and ER β promoters. As shown in Fig. 5B, ER α promoter activity was stimulated 2-fold by PRL-R_{CA} in the presence of either Stat5a or Stat5b. In contrast, ER β promoter activity was stimulated by PRL-R_{CA} only in the presence of Stat5b (Fig. 5B). In the presence of Stat5a, PRL-R_{CA} had little or no effect

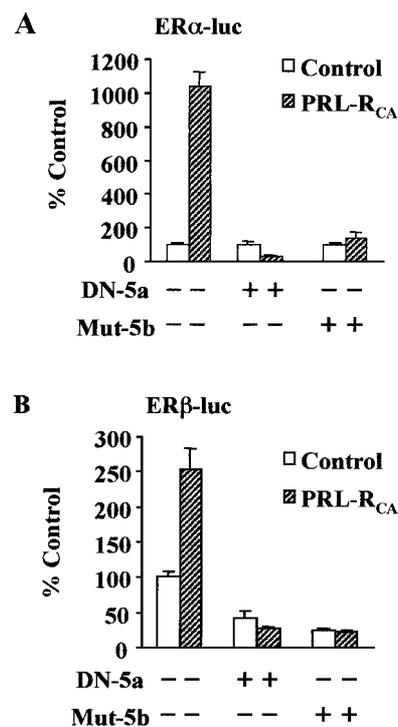


Fig. 4. Effect of Dominant Negative Stat5 on PRL-R_{CA} Stimulation of ER α and ER β Promoter Activity in CHO Cells

CHO cells were cultured and transfected as described for Fig. 3. In addition, each well was transfected with 1 μ g/well DN-Stat5a or Mut-Stat5b. Luciferase activity was measured in each well and normalized to the total protein level within that well. The experiment was repeated three times with triplicate wells for each group. The data represent the combined mean \pm SEM for all three experiments.

on ER β -driven reporter activity. These findings provide additional evidence that PRL can regulate ER expression at the level of transcription. In addition, it appears that ER α is responsive to either Stat5a or Stat5b while ER β may be responsive only to Stat5b.

Because these studies in both CHO and COS cells consist of a reconstructed PRL signaling pathway, we questioned whether PRL utilizes the same pathway to regulate expression of the endogenous ER α and ER β genes. To address this question, primary luteinized granulosa cells were used. PRL is known to induce phosphorylation of Stat5 in these cells; however, it is not known whether this is due to Stat5a or Stat5b activation (22, 42). Primary cells were cultured for 72 h and then treated with PRL for 5 min. After immunoprecipitation and Western blotting, it was found that primary luteinized granulosa cells express both Stat5a and Stat5b and both become highly phosphorylated in response to PRL (Fig. 6A). To examine the possibility that Stat5a and Stat5b can differentially affect stimulation of endogenous ER α and ER β mRNA, primary luteinized granulosa cells were transfected with expression vectors for constitutively active Stat5a or Stat5b (CA-5a or CA-5b). These constitutively active Stat5s were generated by random mutagenesis and

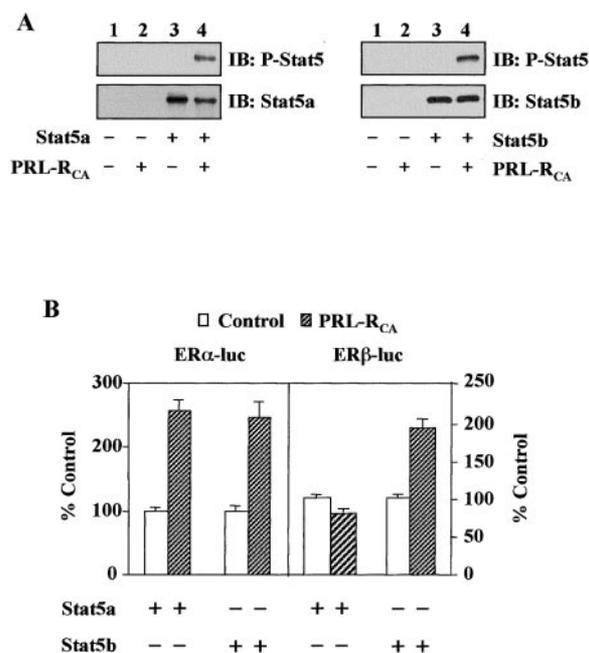


Fig. 5. Effect of PRL-R_{CA} on Stat5 Phosphorylation and ER α and ER β Promoter Activity in COS Cells

A, WCE were prepared from COS cells transfected with 1 μ g/well Stat5a (left panels) or 1 μ g/well Stat5b (right panels) and 2 μ g/well PRL-R_{CA} or PRL-R_L (control). Western blotting was performed as described for Fig. 3. B, Luciferase activity was measured in COS cells that had been transfected with either 0.5 μ g/well ER α -luc (left) or ER β -luc (right), 0.5 μ g/well β -gal, 1 μ g/well Stat5a or Stat5b, and 2 μ g/well PRL-R_{CA} or PRL-R_L (control). Luciferase activity was normalized to the β -gal activity within that well. The experiment was repeated seven times with triplicate wells for each group. The data represent the combined mean \pm SEM for all seven experiments.

found to contain two mutations, one in the DNA binding domain (H299R) and one in the transactivation domain (S711F). They have been shown to be constitutively phosphorylated, located in the nucleus, and capable of binding DNA and regulating gene transcription in the absence of any cytokine stimulation (43). CA-5a induced a 4-fold stimulation of endogenous ER α mRNA while CA-5b stimulated ER α mRNA expression 2-fold (Fig. 6B). In contrast, ER β expression was stimulated 2-fold only in the presence of CA-5b. These results confirm our findings that Stat5b, and not Stat5a, can mediate regulation of ER β while stimulation of ER α can be mediated by either Stat5a or Stat5b.

Because both ER promoters contain putative Stat5 response elements, we next examined whether these were essential for regulation by PRL. Mutations were made to each promoter so that the Stat5 response elements were no longer capable of binding Stat5. Stimulation of both ER α and ER β promoter-driven luciferase activity by PRL-R_{CA} was completely prevented by mutation to the Stat5 response elements (Fig. 7), indicating that PRL stimulation of ER expres-

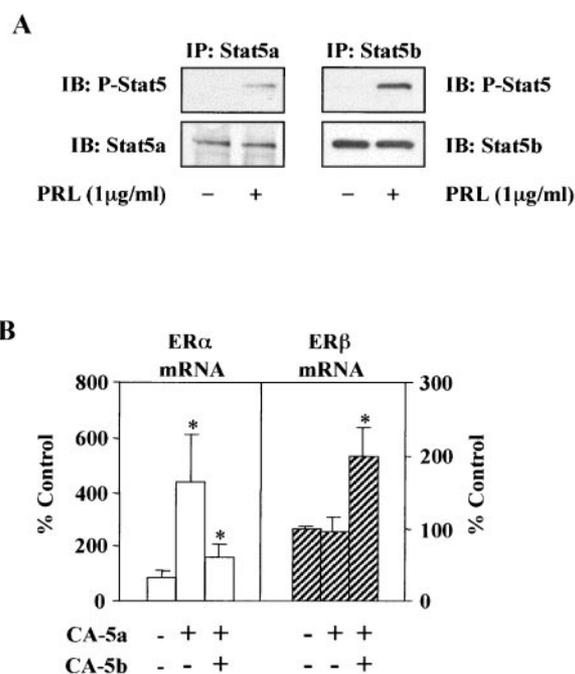


Fig. 6. Stat5 Phosphorylation and Regulation of ER α and ER β mRNA in Primary Luteinized Granulosa Cells

A, Primary luteinized granulosa cells were cultured for 72 h and then treated with PRL (1 μ g/ml) for 5 min. Immunoprecipitation and Western blotting were performed as described for CHO cells. B, Primary luteinized granulosa cells were transfected with expression vectors for CA-5a or CA-5b (1 μ g/well). Thirty-six hours after the start of transfection, total RNA was isolated and quantitative real-time RT-PCR was carried out for ER α and ER β as described in *Materials and Methods* and Fig. 1. The data for the CA-5a and CA-5b groups were normalized to the level of ER α or ER β mRNA expression in the control group and presented here as the mean \pm SEM for six different transfected wells for each group. *, $P < 0.05$ compared with empty vector control as determined by ANOVA followed by Dunnett's multiple range test.

sion requires intact Stat5 DNA binding sites. Because the ER α and ER β promoter Stat5 response elements contain a 1-bp difference (GAA for ER α and TAA for ER β), we next investigated whether this single nucleotide could explain the differential responsiveness of ER α and ER β to Stat5a. A single nucleotide mutation was made to the ER α promoter (ER α -M₁) so that it resembled the ER β response element (GAA to TAA), and the corresponding mutation was made to ER β (ER β -M₁) so that it resembled the ER α response element (TAA to GAA). The mutated ER α promoter (ER α -M₁) containing the ER β Stat5 response element was still stimulated by PRL-R_{CA} in the presence of Stat5a or Stat5b, although the degree of stimulation was markedly reduced (Fig. 8A). Of great interest was our finding that a single nucleotide mutation to the Stat5 response element of the ER β promoter (ER β -M₁) rendered ER β now highly responsive to Stat5a (Fig. 8B). In addition, stimulation of the mutated ER β promoter was significantly increased in the presence of Stat5b

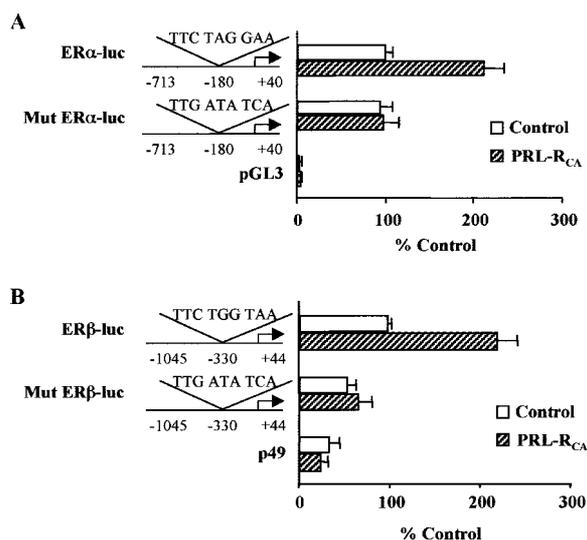


Fig. 7. Role of Putative Stat5 Response Elements in PRL-R_{CA} Stimulation of ER α and ER β Promoter Activity

COS cells were transfected with 0.5 μ g/well wild-type, mutated, or promoter-less reporter constructs for ER α -luc (A) or ER β -luc (B). In addition, each well was transfected with 0.5 μ g β -gal, 1 μ g Stat5b, and 2 μ g PRL-R_{CA} or PRL-R_L (control). Forty-eight hours after the start of transfection, luciferase activity was measured in each well and normalized β -gal activity within that well. The experiment was repeated four times with triplicate wells for each group. The data represent the combined mean \pm SEM for all four experiments.

as well (Fig. 8B). Based on these results, it appears that the nonconsensus Stat5 binding site in the ER β promoter both prevents its responsiveness to Stat5a and limits its responsiveness to Stat5b.

DISCUSSION

In the rat corpus luteum of pregnancy, PRL is known to stimulate expression of both ER α and ER β mRNA, leading to a functionally significant increase in the number of E2 binding sites (3, 44). Results from our current studies indicate that PRL causes this increase in ER expression at the level of transcription, which is mediated by the transcription factor Stat5. Using real-time quantitative RT-PCR, we provide evidence that ER α mRNA expression is far more abundant in both corpora lutea and luteinized granulosa cells than is ER β . This is expected since previous work from Dr. Park-Sarge's group (45) has shown that the LH surge specifically down-regulates ER β expression but has no effect on ER α levels. Also, Dr. Joanne Richards' laboratory (46) found low expression of ER β mRNA and no ER β DNA binding activity in luteinized granulosa cells. In addition, although the ER β knockout mouse is subfertile, it is capable of supporting a full-term pregnancy, suggesting that luteal function is not compromised in the absence of ER β and that ER α is

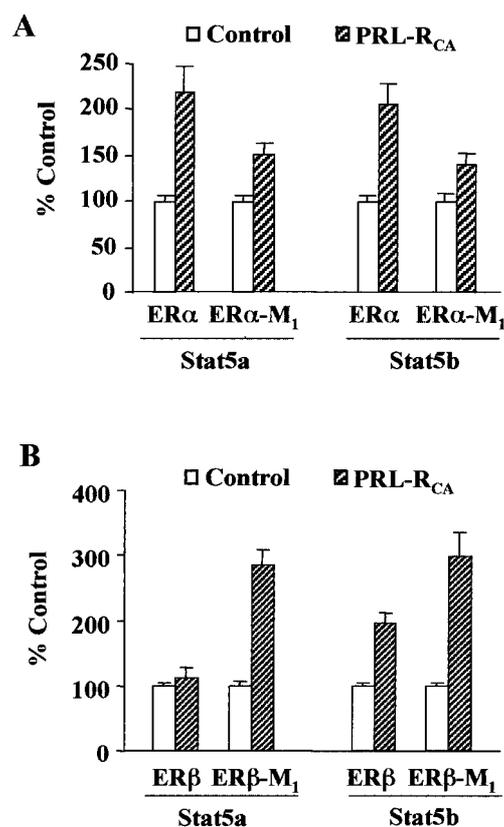


Fig. 8. Comparison of ER α and ER β Consensus and Non-consensus Stat5 Response Elements in Stat5-Mediated Regulation by PRL-R_{CA}

COS cells were transfected with 0.5 μ g/well ER α -luc, ER α M1-luc, ER β -luc, or ER β M1-luc, 0.5 μ g β -gal, 1 μ g Stat5a or Stat5b, and 2 μ g PRL-R_{CA} or PRL-R_L (control). ER α constructs are shown in panel A and ER β constructs are shown in panel B. Forty-eight hours after the start of transfection, luciferase activity was measured in each well and normalized β -gal activity within that well. The experiment was repeated six times with triplicate wells for each group. The data represent the combined mean \pm SEM for all six experiments.

sufficient to sustain pregnancy (47). Nevertheless, PRL is capable of stimulating expression of both ER α and ER β at the level of transcription. Although ER β may not be crucial for luteal function, the ability of PRL to stimulate ER β may be important in other PRL target tissues. In addition, multiple hormones and cytokines are capable of activating Stat5 and may therefore be capable of regulating ER expression through a similar pathway.

Perhaps the most intriguing finding from these studies was that ER α and ER β appear to be regulated differently by Stat5a and Stat5b. In COS and primary luteinized granulosa cells, the ER α promoter and endogenous gene were responsive to both Stat5a and Stat5b. In contrast, in each of the models used, the findings support the conclusion that ER β is far more responsive to Stat5b than to Stat5a. In CHO cells, dominant negative expression vectors for both Stat5a and Stat5b were capable of preventing stimulation of

both ER α and ER β . This finding suggests then that the DN-5a may prevent stimulation of ER β by forming heterodimers with endogenous Stat5b. Alternatively, overexpression of these Stats may be capable of preventing the endogenous Stat5 from being activated through competition for Jak2 substrate binding sites. This lack of stimulation by Stat5a on ER β expression, however, may not be an issue in the corpus luteum since the major Stat5 expressed is Stat5b (data not shown). Whether PRL can or cannot regulate ER β transcription in other tissues, such as the mammary gland, where Stat5a plays a major role in PRL signaling, remains to be investigated. The differential regulation of ER α and ER β by Stat5a and Stat5b may be one mechanism that contributes to the tissue-specific pattern of ER expression.

The lack of ER β stimulation by Stat5a was attributed to a single nucleotide in the Stat5 response element, which both prevented ER β responsiveness to Stat5a and limited its responsiveness to Stat5b. When this single nucleotide was introduced into the ER α response element, responsiveness to both Stat5a and Stat5b was significantly reduced. These results are somewhat expected since a nonconsensus binding site should be less effective at driving gene expression than would be a consensus site. Also, when this single nucleotide was mutated in ER β , so that it resembled the consensus ER α Stat5 response element, ER β transcription in the presence of either Stat5a and Stat5b was markedly increased. These findings could explain, in part, why ER β expression is so much lower in the corpus luteum than is ER α . However, these results do not explain the complete lack of ER β responsiveness to Stat5a. If this nucleotide were the sole explanation, then it would be expected that ER α would be unresponsive to Stat5a once it too had the same nucleotide as ER β . This suggests that within the ER α promoter some additional regulatory region may be capable of enhancing Stat5a action. It is possible that the differential action of Stat5a and Stat5b on these two promoters may be explained by the ability of Stat5 to form stable tetramers through protein-protein interactions involving a tryptophan residue, which is conserved in all Stats and a lysine residue in the Stat5 N-terminal domain (48). Interestingly, only Stat5a tetramers and not Stat5b tetramers were shown to bind the multiple GAS sites in the CIS gene promoter (24). In this same promoter, Stat5b preferentially bound as a dimer. The study of Stat5a dimer and tetramer DNA binding sites revealed that Stat5a tetramers could bind to a wider range of nonconsensus sites, which a Stat5a dimer could not bind. A spacing of 6 bp between tandem GAS sites was the preferred distance for Stat5a tetramer binding (49). Also, it appears that two full GAS sites are not completely necessary for Stat5a tetramer binding since one of the sites could be replaced with a GAS half-site (24). Although we have not found additional GAS sites or half-sites in either promoter, it is possible that some nonconsensus site in the ER α promoter could be essential for its respon-

siveness to Stat5a while the ER β promoter may not be capable of supporting Stat5a tetramer binding.

In conclusion, it is clear that PRL regulation of ER expression is at the level of transcription and that Stat5 mediates this regulation. Furthermore, the Stat5 response elements within the ER α and ER β are necessary for this stimulation. Our data also demonstrate that ER α and ER β are differentially responsive to Stat5a and Stat5b and that a single nucleotide in the ER β promoter can explain its lack of responsiveness to Stat5a. And finally, this increase in ER transcription represents a functional stimulation of ER expression since only a modest increase of E2 binding sites by PRL in the corpus luteum is sufficient to render the corpus luteum responsive to E2.

MATERIALS AND METHODS

Materials

PMSG, human CG, DMEM/F12 (1:1), DMEM, horseradish peroxidase conjugated secondary antibodies, and all other reagent grade chemicals were obtained from Sigma (St. Louis, MO). OptiMem, LipofectAMINE, α -MEM medium, and Trizol were purchased from Life Technologies, Inc. (Gaithersburg, MD). The Advantage RT-for-PCR kit and the chemiluminescence β -gal substrate were from CLONTECH Laboratories, Inc. (Palo Alto, CA). FBS was from HyClone Laboratories, Inc. (Logan, UT). DNA Master SYBR Green I was purchased from Roche Molecular Biochemicals (Indianapolis, IN). Trypsin-EDTA, antibiotics, and antimycotics were from Mediatech (Herndon, VA). Antibodies to Stat5a, Stat5b, and phosphorylated Stat5a/5b were from Upstate Biotechnology, Inc. (Lake Placid, NY). Protein A/G agarose beads and the enhanced chemiluminescence detection reagents were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The luciferase assay substrate and reporter lysis buffer were purchased from Promega Corp. (Madison, WI).

Animal Models

Pregnant and immature female Sprague Dawley rats were obtained from Sasco Animal Labs (Madison, WI) and housed under controlled conditions of light and temperature with free access to standard rat chow and water. All experiments were conducted in accordance with the principles and procedures of the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee.

To determine the effect of PRL on ER expression, mRNA was obtained from corpora lutea from pregnant rats that had either been left intact, hypophysectomized, or hypophysectomized and treated with PRL as previously published (3). To obtain primary luteinized granulosa cells, follicular development was induced in immature rats (24–26 d of age) by injection of 15 IU PMSG ip. An ovulatory dose of human CG (10 IU, ip) was given 48 h later. Luteinized granulosa cells were harvested and cultured as previously described (42). Transfection of primary cells was carried out in OptiMem using LipofectAMINE according to the manufacturer's directions.

Real-Time, Quantitative RT-PCR

RNA from tissue and cell cultures was isolated using Trizol according to the manufacturer's instructions. Reverse tran-

scription was carried out using reagents from the Advantage RT-for-PCR kit according to the manufacturer's instructions. One microgram of total RNA was used for the reverse transcription reaction, and the product was diluted to a final volume of 100 μ l by adding diethyl pyrocarbonate-treated H₂O. To generate standard curves for quantitative PCR, rat ER α and ER β cDNA, which was kindly provided by Dr. Maruyama (50), were diluted to concentrations ranging from 10³ to 10⁷ copies/ μ l. Five-microliter aliquots of standards or diluted reverse transcription products were combined with 2 μ l 10 \times DNA Master SYBR Green I, 1.6 μ l MgCl₂ (3 mM final concentration), and specific primers for rat ER α or ER β (0.5 μ M final concentration). The primers used have been previously published (3). Reactions were carried out in glass capillary tubes in a total volume of 20 μ l. The DNA Master SYBR Green I mix contains Taq DNA polymerase, reaction buffer, deoxynucleotide triphosphate, 10 mM MgCl₂, and SYBR Green I dye, which is a specific fluorescence dye for double-stranded DNA. PCR reactions were performed in the Roche Lightcycler instrument and the accompanying software was used for data analysis (Roche Molecular Biochemicals, Mannheim, Germany). After a 2-min denaturation, PCR cycles were carried out as follows: 0 sec at 95 C, 10 sec at the annealing temperature, and 15 sec at 72 C. For ER α , 40 cycles at an annealing temperature of 63 C were used; for ER β , 35 cycles at an annealing temperature of 69 C were used. At the end of each cycle, the amount of double-stranded DNA was monitored by measuring the level of SYBR Green I fluorescence. After the completion of all cycles, a level of fluorescence was selected at which all of the standards and samples were within the linear range of amplification. The crossing point, or the number of cycles necessary for each sample or standard to obtain the selected level of fluorescence, was calculated using the Roche Lightcycler software. Based on these crossing points, a standard curve was generated, and the number of ER α or ER β copies was calculated for each sample. The data presented represent the number of copies of ER α or ER β in 1 ng of total RNA.

Cloning of the ER α and ER β Promoters

The 5'-flanking region of the rat ER α gene was cloned using the rat PromoterFinder DNA Walking kit (CLONTECH Laboratories, Inc.). Five different genomic libraries were generated by digesting genomic DNA with 5 different restriction enzymes, namely EcoRV, Scal, Dral, PvuII, and SspI followed by ligation with a specifically designed PromoterFinder adapter. The ER α promoter region was amplified by nested PCR using the five different genomic libraries as templates and two sets of primers designed according to the published sequence of the ER α promoter (51). The first PCR reaction was carried out using the following primers: 5'-CCACTCATAAATCTCTT-GGTAACGGC-3' and 5'-GAAGGAAGGAATGTGCTCGAAT-GATC-3'. A second PCR reaction was carried out using product from the first reaction and the following primers: 5'-CTGGGGTTGCAATTAGTC-ATTAGGC-3' and 5'-TCGC-GAATTCGAGTGGCGCGGTGTGTGATCAAG-3'. The second primer also included an attached EcoRI site for subsequent cloning. All five sources of genomic DNA yielded an amplified product of the expected size (880 bp). The PCR products were pooled, and the internal KpnI site at -769 in the ER α promoter region and the added EcoRI site were used to subclone the PCR product into the pBluescript DNA vector (Stratagene, La Jolla, CA). Subcloning of the ER α promoter region into the pGL3-basic luciferase reporter vector (Promega Corp.) was carried out utilizing the KpnI and BglII sites in the pGL3 vector and the KpnI and BamHI sites in the pBluescript vector. Both strands of the ER α promoter generated from different colonies were sequenced. Sequence analysis revealed two differences from the originally published sequence (G to C at -494, A to G at -346) (51).

To isolate the regulatory region of the rat ER β gene, we used the touchdown PCR amplification approach using the

GenomeWalker kit (CLONTECH Laboratories, Inc.) according to the manufacturer's procedure. Two gene-specific primers were designed against the most 5'-end of sequences of the rat ER β mRNA (gene-specific primer 1: 5'-AAGCTGCAAA-GATTACCCACGACTA-3' and gene-specific primer 2: 5'-GACTAACGGATGTTAGTGCCTT-3') (52). Thus, the expected gene-regulatory DNA would contain 46 bp of the 5'-end of the ER β mRNA. The primary PCR amplification was carried out using the combined GenomeWalker libraries (1 μ l) and the primer set of gene-specific primer 1 and adapter primer 1, under PCR conditions of 72 C for 4 min (7 cycles) and 67 C for 4 min (33 cycles). The secondary PCR amplification was carried out using 1 μ l of the diluted primary PCR products (1:100) and the primer set of gene-specific primer 2 and adapter primer 2, under PCR conditions of 72 C for 4 min (5 cycles) and 67 C (22 cycles). This procedure yielded two prominent PCR fragments (~1 and ~2 kb) that were subsequently isolated, subcloned into PCR2.1 T/A overhang vector (CLONTECH Laboratories, Inc.), and sequenced using M13 forward and backward primers. Both contained the adapter 2 sequences at their 5'-end and the gene-specific primer 2 at their 3'-end. The inserts of these clones were isolated by restriction digestion using EcoRV/SpeI and subsequent fill-in reactions with Klenow, and inserted into the SmaI arms of the pUBT-luc vector (53). For these studies, the ~1 kb promoter region of the rat ER β gene was used.

Mutations to ER α and ER β Promoters

The first set of mutations made to the ER α and ER β promoters consisted of six and five nucleotides, respectively, being changed to abolish the Stat5 binding sites. Oligonucleotide primers for these mutations were made as follows (mutated nucleotides *underlined*): ER α 5'-GCCAAGGGGGCTG-GAGTTTCTTGATATCATGCTGA-TTCTAGTGGTACTACT-GCCG-3' and ER β 5'-ATTACTGCTTATTTCCGGTGCATGATATCAACCCGGGGCCTGGCCCATGC-3'. The second set of mutations made to the ER α and ER β promoters consisted of a single nucleotide being changed. The consensus Stat5 site of the ER α promoter (TTCnnnGAA) was changed so that it resembled the Stat5 response element of the ER β promoter (TTCnnnTAA). The nonconsensus ER β Stat5 response element (TTCnnnTAA) was mutated so that it would resemble the ER α Stat5 response element (TTCnnnGAA). Oligonucleotide primers for these mutations were made as follows (mutated nucleotides *underlined*): ER α 5'-GGCTGGAGTT-TCTTCTAGTAAT-GCTGATTCTAGTGG-3' and ER β 5'-CGGTGCTATTCAGGACCCGGG-GGCCTGG-3'. All mutations were made using the QuikChange Site-directed Mutagenesis kit according to the manufacturer's directions (Stratagene). The presence of the correct mutations was confirmed by DNA sequencing.

Culture and Transfection of CHO and COS Cells

CHO and COS cells were routinely cultured in α -MEM and DMEM/F12 (1:1), respectively. All media were supplemented with 10% FBS, 100 IU/ml penicillin G, 100 μ g/ml streptomycin, and 0.25 μ g/ml Amphotericin. Cultures were carried out at 37 C in a 5% CO₂, humidified atmosphere. For transient transfections, 100,000 cells were seeded per well in six-well plates and cultured for 24 h. Both CHO and COS cells were transfected using calcium phosphate DNA precipitation and were approximately 50% confluent at the start of transfection (54). In general, a total of 4–5 μ g DNA were transfected per well, and the total amount of DNA was equalized with empty vector when necessary. Twenty-four hours after the start of transfection, media were changed to standard culture media supplemented with 1% FBS, and cells were cultured for an additional 24 h at 5% CO₂. The entire length of the experiments was standardized to 48 h from the start of transfection.

Reporter Assays

Luciferase and β -galactosidase (β -gal) activities were measured by first preparing cell lysates in 1 \times reporter lysis buffer. Luciferase activity driven by the ER α or ER β promoter was measured by combining lysate with Firefly luciferase assay substrate and measuring luminescence for 10 sec on a Lumat LB 9507 Luminometer (EG&G Berthold, Oak Ridge, TN). As a control, cells transfected with the ER α or ER β promoter were cotransfected with an expression vector for β -gal. β -gal Activity was measured in a separate aliquot of lysate by incubating with a luminescent β -gal substrate for 1 h at room temperature and then measuring luminescence for 5 sec. The luciferase activity was normalized to β -gal activity within the same well. In experiments done in CHO cells, luciferase activity was normalized to total protein levels in each well because of inconsistent β -gal expression.

Immunoprecipitation and Western Blotting

Whole cell extracts (WCE) from primary luteinized granulosa cells and cell lines were prepared by lysing cells in RIPA buffer (1 \times PBS, 1% Nonidet, 0.5% sodium deoxycholate, 0.1% SDS) containing 1 μ M sodium orthovanadate, 10 μ g/ml phenylmethylsulfonyl fluoride, and 30 μ l/ml aprotinin. For immunoprecipitation, 500 μ g of WCE were incubated with 4 μ l anti-Stat5a or anti-Stat5b antibodies for 1 h at 4 C. Protein A/G agarose beads were added, and the mixture was incubated overnight at 4 C on a rocking platform. The beads were washed four times in PBS, resuspended in 2 \times electrophoresis buffer, and boiled for 5 min. For Western blots performed on WCE, protein was diluted in an equal volume of 2 \times electrophoresis buffer and boiled for 5 min. Twenty microliters of immunoprecipitated protein or 20 μ g of WCE were separated on a 10% SDS-PAGE gel and transferred to a nitrocellulose membrane. Western blotting was performed using protocols provided with the Stat5 antibodies.

Acknowledgments

The authors thank Drs. George Kuiper and Jan-Ake Gustafsson for sharing the information on the mouse promoter sequences before their publication and Dr. Alice Mui for the dominant negative Stat5a.

Received May 23, 2001. Accepted August 28, 2001.

Address all correspondence and requests for reprints to: Geula Gibori, 835 South Wolcott, M/C 901, Department of Physiology and Biophysics, University of Illinois at Chicago, Chicago, Illinois 60612. E-mail: ggibori@uic.edu.

This work was supported by NIH Grants HD-11119 (to G.G.), HD-12356 (to G.G.), HD-36879 (to O.-K.P.-S.), and HD-01135 (to O.-K.P.-S.).

REFERENCES

- Gibori G 1993 The corpus luteum of pregnancy. In: Adashi EY, Leung PCK, eds. The ovary. New York: Raven Press, Ltd.; 261–317
- Gibori G, Richards JS, Keyes PL 1979 Synergistic effects of prolactin and estradiol in the luteotropic process in the pregnant rat: regulation of estradiol receptor by prolactin. *Biol Reprod* 21:419–423
- Telleria CM, Zhong L, Deb S, Srivastava RK, Park KS, Sugino N, Park-Sarge OK, Gibori G 1998 Differential expression of the estrogen receptors α and β in the rat corpus luteum of pregnancy: regulation by prolactin and placental lactogens. *Endocrinology* 139:2432–2442
- Tessier C, Deb S, Prigent-Tessier A, Ferguson-Gottschall S, Gibori GB, Shiu RP, Gibori G 2000 Estrogen receptors α and β in rat decidua cells: cell-specific expression and differential regulation by steroid hormones and prolactin. *Endocrinology* 141:3842–3851
- Edey M, Imagawa W, Larson L, Nandi S 1985 Regulation of estrogen and progesterone receptor levels in mouse mammary epithelial cells grown in serum-free collagen gel cultures. *Endocrinology* 116:105–112
- Norstedt G, Wrangé O, Gustafsson JA 1981 Multihormonal regulation of the estrogen receptor in rat liver. *Endocrinology* 108:1190–1196
- Carey GB, Liberti JP 1995 Stimulation of receptor-associated kinase, tyrosine kinase, and MAP kinase is required for prolactin-mediated macromolecular biosynthesis and mitogenesis in Nb2 lymphoma. *Arch Biochem Biophys* 316:179–189
- Das R, Vonderhaar BK 1996 Involvement of SHC, GRB2, SOS and RAS in prolactin signal transduction in mammary epithelial cells. *Oncogene* 13:1139–1145
- Piccoletti R, Bendinelli P, Maroni P 1997 Signal transduction pathway of prolactin in rat liver. *Mol Cell Endocrinol* 135:169–177
- Goupille O, Barnier JV, Guibert B, Paly J, Djiane J 2000 Effect of PRL on MAPK activation: negative regulatory role of the C-terminal part of the PRL receptor. *Mol Cell Endocrinol* 159:133–146
- Buckley AR 2000 Transcriptional regulation of pim-1 by prolactin: independence of a requirement for Jak2/Stat signaling. *J Neuroimmunol* 109:40–46
- Peters CA, Maizels ET, Robertson MC, Shiu RP, Soloff MS, Hunzicker-Dunn M 2000 Induction of relaxin messenger RNA expression in response to prolactin receptor activation requires protein kinase C δ signaling. *Mol Endocrinol* 14:576–590
- Clevenger CV, Medaglia MV 1994 The protein tyrosine kinase P59fyn is associated with prolactin (PRL) receptor and is activated by PRL stimulation of T-lymphocytes. *Mol Endocrinol* 8:674–681
- Fresno Vara JA, Carretero MV, Geronimo H, Ballmer-Hofer K, Martin-Perez J 2000 Stimulation of c-Src by prolactin is independent of Jak2. *Biochem J* 345:17–24
- Mangoura D, Pelletiere C, Leung S, Sakellariadis N, Wang DX 2000 Prolactin concurrently activates src-PLD and JAK/Stat signaling pathways to induce proliferation while promoting differentiation in embryonic astrocytes. *Int J Dev Neurosci* 18:693–704
- Berlanga JJ, Fresno Vara JA, Martin-Perez J, Garcia-Ruiz JP 1995 Prolactin receptor is associated with c-src kinase in rat liver. *Mol Endocrinol* 9:1461–1467
- Al-Sakkaf KA, Mooney LM, Dobson PR, Brown BL 2000 Possible role for protein kinase B in the anti-apoptotic effect of prolactin in rat Nb2 lymphoma cells. *J Endocrinol* 167:85–92
- al-Sakkaf KA, Dobson PR, Brown BL 1996 Activation of phosphatidylinositol 3-kinase by prolactin in Nb2 cells. *Biochem Biophys Res Commun* 221:779–784
- al-Sakkaf KA, Dobson PR, Brown BL 1997 Prolactin induced tyrosine phosphorylation of p59fyn may mediate phosphatidylinositol 3-kinase activation in Nb2 cells. *J Mol Endocrinol* 19:347–350
- Yamauchi T, Kaburagi Y, Ueki K, Tsuji Y, Stark GR, Kerr IM, Tsushima T, Akanuma Y, Komuro I, Tobe K, Yazaki Y, Kadowaki T 1998 Growth hormone and prolactin stimulate tyrosine phosphorylation of insulin receptor substrate-1, -2, and -3, their association with p85 phosphatidylinositol 3-kinase (PI3-kinase), and concomitantly PI3-kinase activation via JAK2 kinase. *J Biol Chem* 273:15719–15726
- Schmitt-Ney M, Doppler W, Ball RK, Groner B 1991 β -Casein gene promoter activity is regulated by the

- hormone-mediated relief of transcriptional repression and a mammary-gland-specific nuclear factor. *Mol Cell Biol* 11:3745–3755
22. Dajee M, Kazansky AV, Raught B, Hocke GM, Fey GH, Richards JS 1996 Prolactin induction of the α 2-macroglobulin gene in rat ovarian granulosa cells: Stat 5 activation and binding to the interleukin-6 response element. *Mol Endocrinol* 10:171–184
 23. Ganguly TC, O'Brien ML, Karpen SJ, Hyde JF, Suchy FJ, Vore M 1997 Regulation of the rat liver sodium-dependent bile acid cotransporter gene by prolactin. Mediation of transcriptional activation by Stat5. *J Clin Invest* 99:2906–2914
 24. Verdier F, Rabionet R, Gouilleux F, Beisenherz-Huss C, Varlet P, Muller O, Mayeux P, Lacombe C, Gisselbrecht S, Chretien S 1998 A sequence of the CIS gene promoter interacts preferentially with two associated STAT5A dimers: a distinct biochemical difference between STAT5A and STAT5B. *Mol Cell Biol* 18:5852–5860
 25. Feltus FA, Groner B, Melner MH 1999 Stat5-mediated regulation of the human type II 3β -hydroxysteroid dehydrogenase/ δ 5- δ 4 isomerase gene: activation by prolactin. *Mol Endocrinol* 13:1084–1093
 26. Galsgaard ED, Nielsen JH, Moldrup A 1999 Regulation of prolactin receptor (PRLR) gene expression in insulin-producing cells. Prolactin and growth hormone activate one of the rat prlr gene promoters via STAT5a and STAT5b. *J Biol Chem* 274:18686–18692
 27. Nanbu-Wakao R, Fujitani Y, Masuho Y, Muramatsu M, Wakao H 2000 Prolactin enhances CCAAT enhancer-binding protein-beta (C/EBP β) and peroxisome proliferator-activated receptor gamma (PPAR γ) messenger RNA expression and stimulates adipogenic conversion of NIH-3T3 cells. *Mol Endocrinol* 14:307–316
 28. Rui H, Lebrun JJ, Kirken RA, Kelly PA, Farrar WL 1994 JAK2 activation and cell proliferation induced by antibody-mediated prolactin receptor dimerization. *Endocrinology* 135:1299–1306
 29. Lebrun JJ, Ali S, Sofer L, Ullrich A, Kelly PA 1994 Prolactin-induced proliferation of Nb2 cells involves tyrosine phosphorylation of the prolactin receptor and its associated tyrosine kinase JAK2. *J Biol Chem* 269:14021–14026
 30. Goupille O, Daniel N, Bignon C, Jolivet G, Djiane J 1997 Prolactin signal transduction to milk protein genes: carboxy-terminal part of the prolactin receptor and its tyrosine phosphorylation are not obligatory for JAK2 and STAT5 activation. *Mol Cell Endocrinol* 127:155–169
 31. Pezet A, Ferrag F, Kelly PA, Ederly M 1997 Tyrosine docking sites of the rat prolactin receptor required for association and activation of stat5. *J Biol Chem* 272:25043–25050
 32. Ali S 1998 Prolactin receptor regulates Stat5 tyrosine phosphorylation and nuclear translocation by two separate pathways. *J Biol Chem* 273:7709–7716
 33. 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
 34. 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
 35. 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
 36. 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
 37. Grimley PM, Dong F, Rui H 1999 Stat5a and Stat5b: fraternal twins of signal transduction and transcriptional activation. *Cytokine Growth Factor Rev* 10:131–157
 38. Gourdou I, Gabou L, Paly J, Kermabon AY, Belair L, Djiane J 1996 Development of a constitutively active mutant form of the prolactin receptor, a member of the cytokine receptor family. *Mol Endocrinol* 10:45–56
 39. Mui AL, Wakao H, Kinoshita T, Kitamura T, Miyajima A 1996 Suppression of interleukin-3-induced gene expression by a C-terminal truncated Stat5: role of Stat5 in proliferation. *EMBO J* 15:2425–2433
 40. Luo G, Yu-Lee L 1997 Transcriptional inhibition by Stat5. Differential activities at growth-related vs. differentiation-specific promoters. *J Biol Chem* 272:26841–26849
 41. Herrington J, Rui L, Luo G, Yu-Lee LY, Carter-Su C 1999 A functional DNA binding domain is required for growth hormone-induced nuclear accumulation of Stat5B. *J Biol Chem* 274:5138–5145
 42. 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
 43. Onishi M, Nosaka T, Misawa K, Mui AL, Gorman D, McMahon M, Miyajima A, Kitamura T 1998 Identification and characterization of a constitutively active STAT5 mutant that promotes cell proliferation. *Mol Cell Biol* 18:3871–3879
 44. Gibori G, Richards JS, Keyes PL 1979 Prolactin control of receptor for estradiol in corpora lutea of pregnant rats. *Adv Exp Med Biol* 112:53–58
 45. Byers M, Kuiper GG, Gustafsson JA, Park-Sarge OK 1997 Estrogen receptor- β mRNA expression in rat ovary: down-regulation by gonadotropins. *Mol Endocrinol* 11:172–182
 46. Sharma SC, Clemens JW, Pisarska MD, Richards JS 1999 Expression and function of estrogen receptor subtypes in granulosa cells: regulation by estradiol and forskolin. *Endocrinology* 140:4320–4334
 47. Krege JH, Hodgin JB, Couse JF, Enmark E, Warner M, Mahler JF, Sar M, Korach KS, Gustafsson JA, Smithies O 1998 Generation and reproductive phenotypes of mice lacking estrogen receptor β . *Proc Natl Acad Sci USA* 95:15677–15682
 48. John S, Vinkemeier U, Soldaini E, Darnell Jr JE, Leonard WJ 1999 The significance of tetramerization in promoter recruitment by Stat5. *Mol Cell Biol* 19:1910–1918
 49. Soldaini E, John S, Moro S, Bollenbacher J, Schindler U, Leonard WJ 2000 DNA binding site selection of dimeric and tetrameric Stat5 proteins reveals a large repertoire of divergent tetrameric Stat5a binding sites. *Mol Cell Biol* 20:389–401
 50. Maruyama K, Endoh H, Sasaki-Iwaoka H, Kanou H, Shimaya E, Hashimoto S, Kato S, Kawashima H 1998 A novel isoform of rat estrogen receptor β with 18 amino acid insertion in the ligand binding domain as a putative dominant negative regulator of estrogen action. *Biochem Biophys Res Commun* 246:142–147
 51. Freyschuss B, Grandien K 1996 The 5' flank of the rat estrogen receptor gene: structural characterization and evidence for tissue- and species-specific promoter utilization. *J Mol Endocrinol* 17:197–206
 52. O'Brien ML, Park K, In Y, Park-Sarge OK 1999 Characterization of estrogen receptor- β (ER β) messenger ribonucleic acid and protein expression in rat granulosa cells. *Endocrinology* 140:4530–4541
 53. de Martin R, Strasswimmer J, Philipson L 1993 A new luciferase promoter insertion vector for the analysis of weak transcriptional activities. *Gene* 124:137–138
 54. Southern PJ, Berg P 1982 Transformation of mammalian cells to antibiotic resistance with a bacterial gene under control of the SV40 early region promoter. *J Mol Appl Genet* 1:327–341