A novel first exon directs hormone-sensitive transcription of the pig prolactin receptor

Anke Schennink, Josephine F Trott, Bradley A Freking¹ and Russell C Hovey

Department of Animal Science, University of California Davis, 2335 Meyer Hall, One Shields Avenue, Davis, California 95616, USA

¹USDA, ARS, US Meat Animal Research Center, PO Box 166, Clay Center, Nebraska 68933, USA

Correspondence
should be addressed
to A Schennink
Email
aschennink@ucdavis.edu

Abstract

Endocrine, paracrine, and autocrine prolactin (PRL) acts through its receptor (PRLR) to confer a wide range of biological functions, including its established role during lactation. We have identified a novel first exon of the porcine PRLR that gives rise to three different mRNA transcripts. Transcription of this first exon is tissue specific, where it increases during gestation in the adrenal glands and uterus. Within the mammary glands, its transcription is induced by estrogen and PRL, while in the uterus, its expression is downregulated by progesterone. The promoter region has an enhancer element located between –453 and –424 bp and a putative repressor element between –648 and –596 bp. Estrogen, acting through the estrogen receptor, activates transcription from this promoter through both E-box and transcription factor AP-2 α binding sites. These findings support the concept that the multilevel hormonal regulation of PRLR transcription contributes to the various biological functions of PRL.

Key Words

- ▶ promoter
- ▶ prolactin receptor
- ▶ alternative splicing
- ▶ pig

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Introduction

Prolactin (PRL) is a protein hormone that has diverse biological functions including pronounced roles in adaptive stress responses, osmoregulation, uterine function, proliferation and differentiation of the mammary glands, and lactation (Bole-Feysot *et al.* 1998). PRL functions through multiple forms of its membrane-associated receptor (PRLR) that differ primarily within the intracellular domain as described for human, mouse, rat, cow, pig, sheep, goat, and carp. Upon ligand activation, these various isoforms can differentially activate downstream signal transduction pathways (reviewed in Trott *et al.* (2012)). The PRLR is expressed in various tissues and cell types, where its levels vary during states including the estrus cycle, pregnancy, and lactation (Jahn *et al.* 1991, Hovey *et al.* 2001, Trott *et al.* 2009). The PRLR is also

expressed in a majority of human breast cancers (Gill *et al.* 2001, Peirce & Chen 2001), where antagonism of the PRLR potentiates the cytotoxic action of anti-cancer drugs and reduces PRL-induced cell survival (Howell *et al.* 2008).

Expression of the PRLR across various tissues and physiological states is under complex transcriptional regulation. Six alternative non-coding first exons have been reported in humans, including a generic exon 1 (hE1₃) that is orthologous to E1₃ in rat and mouse (Hu *et al.* 1999, 2002). The E1₃ is expressed in the mammary glands, ovaries, Leydig cells, and liver of both humans and rats (Moldrup *et al.* 1996, Hu *et al.* 1999). In addition to E1₃, four other gonad-, liver-, and brain-specific first exons have been identified in rats (Hu *et al.* 1996, Moldrup *et al.* 1996, Hu *et al.* 1997, Tanaka *et al.* 2002, 2005) and mice

(Ormandy *et al.* 1998, Tabata *et al.* 2012). Transcription from these alternative first exons is controlled by upstream promoters that recruit specific *cis*-acting factors. In turn, activation of these promoters could lead to differential expression of the PRLR across tissues, thereby contributing to the diverse biological functions of PRL.

Apart from data for humans, rats, and mice, no literature is available on the 5' UTR of the PRLR or its regulation in livestock where reproduction and lactation underlie a multi-billion dollar industry. At the same time, production animals are increasingly important as biomedical models for cancer, obesity, and infertility (Kuzmuk & Schook 2011). Furthermore, expression of the first exons of the PRLR across a broad array of tissues has not been analyzed in any species, nor has its hormonal control been investigated for established PRL target tissues such as the mammary glands or uterus *in vivo*.

In this study, we identified multiple splice variants of a porcine PRLR E1 (pE1). We investigated the expression of three pE1 splice variants across various tissues in virgin and pregnant females and found that expression levels increased during pregnancy in the adrenal glands and endometrium. Expression analysis in a 'hormone-replacement' model *in vivo* established that pE1 was positively regulated by β -estradiol (E) and PRL in the mammary glands and negatively regulated by progesterin in the uterus. Furthermore, E activated the pE1 promoter in E-responsive breast cancer cells through E-box and transcription factor AP-2 α (TFAP2A) binding sites.

Materials and methods

Animals

Virgin and pregnant (day 105 of gestation) crossbred (Hampshire $\times$ Yorkshire) gilts and boars were housed, treated, killed, and their tissues sampled as described (Trott *et al.* 2011). Tissues from pregnant (day 105 of gestation) gilts, and testis from a boar, were used for 5' RACE. Tissues from virgin gilts were used to determine tissue-specific expression in non-pregnant animals. Pregnant, unilaterally hysterectomized–ovariectomized gilts were killed at day 25, 45, 65, 85, or 105 of gestation and were housed, treated, and sampled as described (Freking *et al.* 2007).

Hormone-treated peripubertal Yucatan miniature pigs were housed, treated, killed, and their tissues harvested as described (Horigan *et al.* 2009). A total of 36 gilts were used, of which four were sham-operated, while 32 were

ovariectomized. One day after surgery, ovariectomized gilts began receiving bromocriptine (Br; 0.1 mg/kg per day i.m.) for 8 days to suppress PRL secretion. Beginning on day 9, gilts were injected i.m. with various combinations of E (0.1 mg/kg per day), medroxyprogesterone 17-acetate (a synthetic progestin (P), 0.25 mg/kg per day), and/or Br or haloperidol (Hal; 1.5 mg/kg per day; to induce endogenous PRL release) for 5 days. The ovariectomized, Br-treated gilts were assigned to one of the eight treatments ($n=4$ per group): Br, Br + E, Br + P, Br + E + P, Hal, Hal + E, Hal + P, and Hal + E + P. Sham-ovariectomized gilts received daily injections of saline for 14 days. All gilts were killed 14 days after surgery.

5' RACE

Poly A+RNA (Oligotex mRNA Mini Kit, Qiagen) was isolated from mammary gland, placenta, liver, kidney, adrenal, ovary, and testis, and 5' RACE was performed using the SMARTer RACE cDNA amplification kit (Clontech) with two gene-specific primers: GSP1a, 5'-CGGAGGTGACTGTCCATTCAGAAGGCTG-3' and NGSP1a, 5'-TTGGCTCCCTTCTCTTTCCACAGGCAG-3' (designed from the *pPRLR* cDNA, GenBank ID: NM_001001868) or the 5'/3' RACE Kit (Roche) with primers reported in Trott *et al.* (2007) using two rounds of nested PCR. 5' RACE products from the nested PCR were identified by electrophoresis on 1.2% agarose gels, gel-purified, and cloned into pCR2.1-TOPO (Invitrogen). Clones were analyzed by restriction enzyme digestion and agarose gel electrophoresis before sequencing. The sequence of the *pPRLR* first exon was confirmed by sequencing a bacterial artificial chromosome (BAC) containing the *pPRLR* (CHORI-242 255C20, a gift from Dan Nonneman, USDA-ARS) using a sequencing primer in the newly discovered pE1.

RNA extraction and RT

Tissues were homogenized in TriReagent (Molecular Research Center, Inc., Cincinnati, OH, USA) and total RNA was extracted according to the manufacturer's instructions. Total RNA (5 μ g) was treated with DNaseI (Roche) and purified using the DNA-Free RNA Kit (Zymo Research Corporation, Orange, CA, USA) before confirmation of its integrity by formaldehyde–agarose gel electrophoresis. Total RNA (500 ng) was denatured at 70 °C for 5 min along with oligo-dT (50 ng, Amersham Biosciences) and random hexamers (2 ng, Amersham Biosciences) and then reverse transcribed using 1 $\times$ RT

Table 1 Primers used for PCR amplification of *pPRLR* E1 promoter fragments cloned into pCR2.1

Oligo	Primer sequence (5'–3')
pPRLRE1_F170	CCAGCCAGGAACAGAAATGT
pPRLRE1_F424	CTTGGCTGCCAGGAGAAATA
pPRLRE1_F651	CCTGGAAGTTTGGGTTTGA
pPRLRE1_F859	TGGTGGTTGTGTGTGGTTT
pPRLRE1_F1002	CCCTCTGGGTTATGAAACGA
pPRLRE1_F1176	AGTGTCTGGGATACGCAGT
pPRLRE1_R	CTGCAAAAGGACGTGGAGAT

Buffer (Promega), dNTP mixture (0.016 mM, Promega), Moloney's murine leukemia virus reverse transcriptase (2 U/μl, Promega), and RNase inhibitor (0.85 U/μl, Promega) at 37 °C for 90 min, followed by denaturation at 95 °C for 5 min.

Cloning pE1 transcripts

Mammary gland cDNA was amplified with GoTaq Green Master Mix (Promega) using 0.2 μM each of the primers pE1_F, 5'-GACTGATACTGCAGACTGACTTTGCT-3' and pE3/4_R, 5'-GGAGGTGACTGTCCATTCAGA-3' (spanning exons 3 and 4) to confirm the existence of splice variants involving the first three exons of the *pPRLR*. Different-sized PCR products were agarose gel-purified, cloned into pCR2.1-TOPO, and sequenced.

Quantitative PCR

The cDNA was diluted 1:1, and 4 μl was used as a template for real-time quantitative PCR (qPCR) containing Fast SYBR Green Master Mix (Applied Biosystems) and 0.2 μM of each primer on a 7500 Fast Real-Time PCR System (Applied Biosystems), using primers named for the exons in which they are located: pE1/3_F, 5'-CCGGCAAAATGAACCTCTGA-3' and pE3_R, 5'-TGTGGATGGCACA-TTTCCTT-3'; pE1_F, 5'-GACTGATACTGCAGACTGACTTTGCT-3' and pE2/3_R, 5'-GGTCAGAGGTTCACTTCAGGGGTCAT-3'; pE1_F, 5'-GACTGATACTGCAGACTGACTTTGCT-3' and pE2/2.1_R, 5'-GGATGGGAGCCTTCAGGG-3', for transcripts pE1/3, pE1/2/3, and pE1/2/2.1/3 respectively. Samples were analyzed as duplicates. A standard curve with a range from 10 to 10⁷ copies was prepared for each *pPRLR* transcript using mouse liver cDNA spiked with the respective number of copies of *pPRLR* cDNA cloned into pCR2.1-TOPO. The standard curve was spiked into mouse cDNA to ensure that the standard curve and samples had a similar composition.

The same standard curve was used in duplicate or triplicate across multiple plates for each transcript. Standard curves were generated by linear regression of Ct vs log₁₀ (dilution factor). Melting curve analysis was used to confirm the specificity of PCR products, and a minus-RT control was used to ensure that products were from cDNA rather than genomic DNA. Gene expression levels were normalized for 18S rRNA expression (Trott *et al.* 2011).

Construction of promoter plasmids

A 1171-nucleotide promoter sequence was obtained by sequencing BAC CHORI-242 255C20 using a reverse primer in pE1 and primer walking upstream. Promoter fragments of various lengths were generated by PCR from this BAC by one of the two methods. First, fragments were generated using primers listed in Table 1 and Extensor PCR mix (ABgene, Epsom, UK) followed by subcloning into pCR2.1-TOPO. Promoter fragments were then excised and ligated into the promoterless pGL3-Basic vector (Promega) using the Quick Ligation Kit (New England BioLabs, Ipswich, MA, USA). Further deletions of the pE1 promoter were generated by PCR using primers listed in Table 2 and GoTaq Green Mastermix (Promega). These fragments were ligated into pGL3-basic using the In-Fusion PCR Cloning system (Clontech). Mutations in the TFAP2A and E-box binding sites of the 201 and 242 bp pE1 promoter fragments were generated from pGL3-pPRLR E1_1176 by PCR using primers listed in Table 3. PCR was performed using GoTaq Green Mastermix (Promega) and products were ligated into pGL3-basic using the Gibson Assembly

Table 2 Primers used for PCR amplification of *pPRLR* E1 promoter fragments cloned using the In-Fusion PCR cloning system

Oligo	Primer sequence (5'–3')
pPRLRE1_201F	TATCGATAGGTACCGTTCAGGCTGAAG-AAAATCA
pPRLRE1_242F	TATCGATAGGTACCGCTCCCTCCCTCACCT-GGTA
pPRLRE1_293F	TATCGATAGGTACCGGGGAATCCGTGCTC-TCTCT
pPRLRE1_353F	TATCGATAGGTACCGCTTTTCTGGTGCAG-TCTCAGC
pPRLRE1_453F	TATCGATAGGTACCGAGGAAATTTGACA-CGGAGACA
pPRLRE1_500F	TATCGATAGGTACCGCCCCAAGGCTGTCA-AGATAC
pPRLRE1_567F	TATCGATAGGTACCGCTGTGCCCCCTTC-ACTCT
pPRLRE1_596F	TATCGATAGGTACCGCTCAGGTACAGG-GGTCAT
pPRLRE1_infR	TCGAGCCCCGGCTAGCTGCAAAAGGAC-GTGGAGAT

Table 3 Primers used for PCR amplification of mutant transcription factor binding sites in the *pPRLR* E1 promoter, cloned using the Gibson Assembly cloning kit. Altered nucleotides are lower case. The E-box (242 bp promoter) and TFAP2A (201 bp promoter) sites are underlined

Oligo	Primer sequence (5'–3')
pPRLRE1_201mut	CGATAGGTACCGAGCTTTGAGGCTGAAGA-AAATCACTGTTT <u>TaatTCC</u>
pPRLRE1_242mut	CGATAGGTACCGAGCTCTCCCTCCT <u>gtCTG</u> -GTAAAC
pPRLRE1_gibR	AGATCTCGAGCCCGGGCTGCAAAAGGACGT-GGAGAT

cloning kit (New England BioLabs). All promoter fragments were sequence-verified.

Transient transfection of MCF-7 cells and reporter gene assays

Human MCF-7 breast cancer cells were cultured in DMEM supplemented with 10% FBS, 100 U penicillin and 100 µg streptomycin/ml, and 2.42 g/l HEPES. Cells were plated into 12-well plates at 2×10^5 cells/well, then 1 day later were transiently transfected with 0.8 µg of either pE1 promoter-pGL3 plasmid or pGL3-basic DNA and 0.2 µg of either pEF-lacZ or pCMV-eGFP plasmid using 3 µl FuGene6 (Roche). In some studies, cells were transfected with 0.4 µg of either pE1 promoter-pGL3 plasmid or pGL3-basic plasmid and 0.1 µg of pCMV-eGFP, using 1.5 µl Lipofectamine LTX and 0.5 µl PLUS (Invitrogen). In the promoter deletion experiment, cells were cultured in complete growth medium and harvested after 24 h. In experiments examining induction of the pE1 promoter by E, the culture medium was changed to hormone-deficient medium (containing 10% charcoal-stripped FBS; Omega Scientific, Tarzana, CA, USA) the day after transfection. Cells were then exposed to E (1 nM) or ethanol vehicle for 48 h. In the experiment using the estrogen receptor (ESR) antagonist ICI 182 780 (ICI; Ascent Scientific, Cambridge, MA, USA; 100 nM in ethanol), the culture medium was changed to hormone-deficient medium the day after transfection. Cells were then exposed to either ICI (100 nM) or vehicle for 24 h before treatment with either E, E+ICI, or vehicle for 48 h in hormone-deficient medium. Cells were lysed with 200 µl Glo Lysis buffer (Promega) and lysates were assayed for luciferase and either β-galactosidase or eGFP activity. Luciferase activities were corrected for transfection efficiency as determined by the relative level of β-galactosidase or fluorescence in each lysate.

Transcription factor binding sites

Putative binding sites for transcription factors in the pE1 promoter sequence were identified using TESS (<http://www.cbil.upenn.edu/cgi-bin/tess/tess>) and AliBaba 2.1 (<http://www.gene-regulation.com/pub/programs/alibaba2/index.html>). For TESS, only sites with a Log-likelihood score (La) ≥ 10 were considered.

Statistical analysis

Data were analyzed using the General Linear Models procedure in SAS (SAS version 9.3, SAS Institute, Inc., Cary, NC, USA). Expression data were $\log_{10}$ or power transformed for normalization where appropriate. Expression data from virgin, hormone-treated gilts were analyzed within each tissue by ANOVA followed by Tukey's *post hoc* test. Main effects of hormones and their interaction were tested by factorial ANOVA. Expression data from pregnant gilts were analyzed within tissue by linear regression. Luciferase activity data from the promoter deletion and mutation studies were analyzed by ANOVA. Significance was declared at $P < 0.05$.

Results

Identification of a novel pPRLR first exon

Using 5' RACE, we obtained 35 clones of 200–250 bp from reverse-transcribed RNA from mammary gland, liver, placenta, adrenal gland, kidney, ovary, and testis. All clones were identified as spanning from a novel exon 1 (pE1) to exon 3 of the *pPRLR*, where 54% of the clones also contained a 62 bp non-coding exon 2. One clone contained 162 bp of pE1, as confirmed by its alignment with genomic sequence. The 162 bp of pE1 is located more than 91 kb upstream of exon 2 on *Sus scrofa* chromosome 16 (SSC16; Fig. 1A). Among the clones, 23% had a transcription start site 23 bp downstream that conferred a length of 139 bp for pE1, while 15% of clones had a transcription start 59 bp downstream that conferred a pE1 length of 80 bp. Using RT-PCR, we further identified three distinct splice variants involving exon pE1. Two were identical to our 5' RACE products, namely pE1/3 and pE1/2/3. The largest PCR product contained a novel exon of 113 bp that is located between exons 2 and 3, which was designated as non-coding exon 2.1 (Fig. 1A), giving rise to the transcript pE1/2/2.1/3. A multiple sequence alignment revealed that pE1 is homologous to the generic E1₃ in humans, rats, and mice (Fig. 1B).

Figure 1

Identification of the 5' UTR and promoter region of *pPRLR*. (A) Top panel, genomic structure of exons 1, 2, 2.1, and 3 of the *pPRLR* on *Sus scrofa* chromosome 16 (SSC16; according to *Sus scrofa* build 10.2, Ensembl release 67). The translation initiation site is located in exon 3. GPG, Golden Path Gap. Bottom panel, the three newly identified *pPRLR* splice variants. All transcripts starting with exon 1 are spliced onto exon 3, with or without the non-coding exon 2. Transcripts that splice onto exon 2.1 always included exon 2 (GenBank ID: JX429229). (B) Cladogram generated from all six human E1

(hE1), five rat E1 (rE1), and four murine E1 (mE1), and pE1, showing that the pE1 is homologous to hE1₃, rE1₃, and mE1₃. (C) Nucleotide sequence of the pE1 and its 5' flanking region (GenBank ID: JX429230). Numbering is relative to the main transcription start site depicted by an arrow. Predicted transcription factor binding sites are boxed. ETS, E26 transformation specific; Pit-1, pituitary 1; ERE1/2, estrogen response element half-site; C/EBP, CCAAT/enhancer binding protein; AP-2, transcription factor AP-2 α ; Sp1, specificity protein 1; DSE, downstream sequence element.

Tissue-specific expression of *pPRLR* E1 in virgin and pregnant gilts

Expression of these three pE1 transcripts in a wide range of tissues from virgin gilts was determined by qPCR. The pE1/3 and pE1/2/3 transcripts ($> 10^5$ copies/ μ g RNA) were most abundant in the adrenal glands, endometrium, mammary glands, pituitary, and placenta (Fig. 2A and B), while the pE1/2/2.1/3 transcript was most abundant in the

adrenal glands, heart, mammary glands, pituitary, and placenta (Fig. 2C). Very low or no expression of pE1 was detected in liver, lymph node, pancreas, skeletal muscle, and thymus. Notably, expression of the pE1/2/2.1/3 transcript was ~ 100 -fold lower than that for the two other pE1 transcripts in all tissues.

Given the crucial role for PRL during gestation, we further examined expression of the pE1 transcripts in

Figure 2

Expression of different *pPRLR* exon 1 transcripts across various tissues in virgin gilts. Number of *pPRLR* mRNA copies was measured by qPCR with normalization for the corresponding level of 18S rRNA. (A) Transcript pE1/3, (B) transcript pE1/2/3, (C) transcript pE1/2/2.1/3. Endom, endometrium; Hypoth, hypothalamus; MG, mammary gland; Lymph, lymph node; Sk musc, skeletal muscle; Sm int, small intestine. Data are means $\pm$ S.E.M. ($n=3$).

various tissues from gilts at days 25, 45, 65, 85, and 105 of gestation (Fig. 3). The highest expression of pE1 was recorded in the adrenal glands, endometrium, and mammary glands, while it was barely detectable in the liver. Expression of all three pE1 transcripts in adrenal glands and endometrium increased linearly during

gestation (Fig. 3). Expression of the pE1/3 and pE1/2/3 transcripts also increased linearly across gestation in the hypothalamus, as did expression of the pE1/3 transcript in the kidney, liver, mammary glands, and pituitary. These data reveal that pE1 expression is upregulated during gestation, particularly in known targets for PRL, leading us to hypothesize that pE1 is hormonally regulated.

Hormonal regulation of *pPRLR* E1

Given our previous finding that expression of the long form of the *pPRLR* (*pPRLR*-LF) is regulated by PRL in the mammary glands, and by E and P in the uterus (Trott *et al.* 2009), alongside findings by others that E regulates E1 expression in rats and human breast cancer cells, we further examined the regulation of pE1 transcription by E, P, and PRL in the uterus, liver, and mammary glands *in vivo* (Fig. 4). The female pigs were hormone deficient before treatment with either Br (hypoprolactinemic) or Hal (hyperprolactinemic) in the presence or absence of E and/or P. There was a negative main effect of P on the expression of all pE1 transcripts in the uterus ($P<0.0001$). For the pE1/3 transcript, there was also a positive main effect of E ($P<0.04$) and a negative interaction between the effects of E and P ($P=0.02$), and PRL and P ($P=0.05$). For the liver, there was a small but significant negative main effect of P on the expression of all three transcripts ($P=0.01$, 0.01, and 0.04 for pE1/3, pE1/2/3, and pE1/2/2.1/3 respectively), as well as a positive main effect of PRL on the expression of pE1/3 ($P=0.01$) and pE1/2/3 ($P=0.002$). Both E and Hal exerted positive main effects ($P<0.01$) on expression of all three pE1 transcripts in the mammary glands, and there was an interaction between the effects of PRL and P ($P=0.01$) for expression of the pE1/3 transcript.

Analysis of the putative *pPRLR* E1 promoter

The 1.2 kb region upstream of pE1 was sequenced from BAC clone CHORI-242 255C20 (Fig. 1C). The promoter region is numbered from the transcription start site defined by the longest 5' RACE transcript of pE1 (162 bp). We did not identify a TATA box within 40 bp upstream of this, or the other two, potential transcription start sites. We used MCF-7 epithelial breast cancer cells, a widely used model for *trans*-species E-responsiveness *in vitro*, to study pE1 promoter activity. Deletion of the region -1171 to -648 bp of the pE1 promoter did not affect its transcriptional activity in transiently transfected MCF-7

Figure 3

Expression of the different *pPRLR* exon 1 transcripts across tissues from gilts at different stages of gestation. Copy number of total *pPRLR* mRNA was measured by qPCR with normalization for the corresponding level of 18S rRNA. (A) Transcript pE1/3. (B) Transcript pE1/2/3. (C) Transcript pE1/2/2.1/3.

Endom, endometrium; Hypoth, hypothalamus; MG, mammary gland. Data are means $\pm$ s.e.m. ($n=4-5$). Where a significant linear relation existed between expression and time of gestation, the R^2 for the correlation is shown.

Figure 4

Expression of the different *pPRLR* exon 1 transcripts in uterus, liver, and mammary glands of ovariectomized, bromocriptine (Br)-treated pigs administered all combinations of E, medroxyprogesterone 17-acetate (P), and/or haloperidol (Hal). Copy number of total *pPRLR* mRNA was measured

by qPCR with normalization for the corresponding level of *18S* rRNA.

(A) Transcript pE1/3. (B) Transcript pE1/2/3. (C) Transcript pE1/2/2.1/3.

Sham, sham-operated; Sal, saline treated. Data are means $\pm$ s.e.m. ($n=4$).

a,b,c Means without a common superscript differ.

cells. Deleting the region -648 to -424 bp increased promoter activity by 130%, suggesting the likely presence of a repressor site (Fig. 5A). Further deleting the region -424 to -170 bp reduced transcriptional activity to that

of the promoterless construct. We further analyzed the region -651 to -170 bp to identify putative *cis* elements that might regulate expression from the pE1 promoter (Fig. 5B). The aforementioned putative repressor site was

Figure 5

Promoter analysis for pE1 in MCF-7 cells transiently transfected with plasmids containing varying lengths of the pE1 promoter upstream of a luciferase reporter. Luciferase activity in cells transfected with pGL3-Basic that contained 170 bp of the promoter was set to 1. (A) Cells were transfected in complete growth medium and harvested 24 h later. Data are means $\pm$ s.e.m. ($n=3$ independent experiments). (B) Deletion constructs were transfected into cells and then treated for 48 h with 1 nM β -estradiol (E) or vehicle in hormone-deficient media. Data are means $\pm$ s.e.m. ($n=3-4$; four independent experiments). A significant difference between the response to vehicle and E is indicated by *. ^{a,b,c,d,e,f}Means without a common superscript differ ($P<0.05$).

localized between -648 and -596 bp while a putative activation site was identified between -453 and -424 bp. Using TESS and Alibaba, we also predicted transcription factor binding sites in this region. Candidate binding sites for pituitary-1 (Pit-1) and E26 transformation-specific (ETS) family members were identified at -629 to -624 and -645 to -640 respectively (Fig. 1C). Furthermore, CCAAT/enhancer binding protein (C/EBP) and specificity protein 1 (Sp1) binding sites that were previously shown to regulate PRLR expression in humans, rats, and mice are

partially conserved in pE1 at -151 to -141 and at -139 to -131 . The smallest pE1 promoter fragment (-170), which contains both the C/EBP and Sp1 binding sites, failed to promote transcription in our analyses. The downstream sequence element (-77 to -68) that is required for E1₃ basal activity in rats (Hu *et al.* 1998) is partially conserved in the pE1 promoter but is also located in the region of the promoter that was inactive in MCF-7 cells.

Given our finding that pE1 expression in the mammary glands is E-regulated, we examined whether, and then how, E activated the pE1 promoter in MCF-7 cells. Transcription from the pE1 promoter was E-inducible for all promoter fragments that contained more than the first 170 nucleotides. ERs bind with highest affinity to palindromic estrogen response elements (EREs), although many EREs contain weaker binding sequences and some only consist of half-palindromic sites (Bourdeau *et al.* 2004). We identified putative ERE half-sites in the pE1 promoter at -549 to -545 , -583 to -579 , and -592 to -588 , but none between -170 and -549 . Given that the magnitude of E-activation from the -242 fragment was higher than from the -201 fragment, we examined the -170 to -242 region for putative transcription factor binding sites. We identified a putative TFAP2A binding site between -166 and -174 (consensus G/C C C N N A/C/G G/A G G/C/T; McPherson & Weigel 1999) and an E-box sequence (5'-CANNTG-3') from -232 to -227 . We next generated two constructs that contained either a mutated TFAP2A site in the -201 promoter or a mutated E-box in the -242 promoter. Estrogen-induced activation of the -201 promoter was ablated when the TFAP2A site was mutated (Fig. 6A), while E-activation of the -242 promoter was ablated by mutation of the E-box (Fig. 6B). The basal transcription levels were also reduced by mutating both the TFAP2A site (Fig. 6A) and the E-box (Fig. 6B). Given that the E-box and TFAP2A sites are not classical EREs, we used the ESR antagonist ICI 182 780 and confirmed that the ESR is involved in the E-induced activation of these -201 and -242 bp pE1 promoter fragments (Fig. 7).

Discussion

We find that the pE1 is structurally and functionally similar to the hE1₃, rE1₃, and mE1₃ with regard to sequence homology, conserved *cis*-regulatory elements, and level of expression across tissues. These first exons in other species are regarded as the principal first exon for the PRLR gene. Furthermore, we identified that exon 2 is a variably spliced

Figure 6

Analysis of transcription factor mutants for the pE1 promoter in MCF-7 cells transiently transfected with plasmids containing the promoter upstream of a luciferase reporter. Luciferase activity in cells transfected with pGL3-Basic and not stimulated by β -estradiol (E) was set to 1. Cells in hormone-deficient media were treated for 48 h with either 1 nM E or vehicle. (A) The 201 bp promoter was mutated at the TFAP2A binding site (-201mut). Data are means $\pm$ S.E.M. ($n=3$) from three independent experiments. (B) The 242 bp promoter was mutated at the E-box sequence (-242mut). Data are means $\pm$ S.E.M. ($n=3$) from three independent experiments. A significant difference between the response to vehicle and E is indicated by *. ^{a,b,c}Means without a common superscript differ ($P<0.05$).

constituent of transcripts arising from pE1. Splicing of exons 1–3, while omitting exon 2, has also been reported for both the rPRLR (Hu *et al.* 1996) and the hPRLR (Hu *et al.* 1999). To our knowledge, the existence of an exon 2.1 has not been reported in any other species, although a 70% homologous sequence is present within the intron between E2 and E3 in the hPRLR gene. Alternative splicing of exon 2 and/or 2.1 would further diversify the PRLR 5' UTR by deleting regulatory *cis* elements. This could affect PRLR mRNA stability or the rate of protein translation.

Among the various tissues we examined, expression of pE1 was most abundant in PRL-sensitive tissues and increased during gestation, similar to the expression profile for the pPRLR-LF (Trott *et al.* 2009). Previous analysis of the hormonal regulation of E1 transcription *in vivo* have been limited to select tissues such as the liver, pituitary gland and in a subset of brain regions from rats and mice or in murine and human mammary epithelial cell lines *in vitro* (Pi *et al.* 2003, Tanaka *et al.* 2005, Dong *et al.* 2006, Kobayashi *et al.* 2007, Nogami *et al.* 2007, Goldhar *et al.* 2011, Tabata *et al.* 2012). The induction of pE1 expression by E and its repression by P in the uterus of pigs aligns with the effect of these hormones on pPRLR-LF mRNA expression. Alternating concentrations of serum hormones during gestation may account for the changes in pE1 expression during this period (Eldridge-White *et al.* 1989). Here, we show that E increases pE1 mRNA expression in the mammary glands of pigs, consistent with our previous finding that E specifically induces pPRLR-LF expression in the mammary epithelium. It is noteworthy that the E-induction of pE1 expression here was much higher than that for pPRLR-LF (Trott *et al.* 2009). These results also align with our findings in breast cancer cells herein and similar reports by others (Dong *et al.* 2006). Investigations into the effect of progestins on E1 expression have been limited to mice and transiently transfected T47D

Figure 7

The β -estradiol (E) receptor antagonist ICI 182 780 (ICI) inhibits E-induced activation of the pE1 promoter in MCF-7 cells transiently transfected with plasmids containing the promoter upstream of a luciferase reporter. Luciferase activity in cells transfected with pGL3-Basic and not stimulated by E was set to 1. Cells were pre-treated for 24 h with 100 nM ICI or vehicle in hormone-deficient media followed by 48 h treatment with vehicle, 1 nM E, or E+ICI. Data are means $\pm$ S.E.M. ($n=3$) from three independent experiments. A significant difference between the response to vehicle and E is indicated by *. ^{a,b,c}Means without a common superscript differ ($P<0.05$).

breast cancer cells where progesterone induced mE1₃ transcription (Goldhar *et al.* 2011). Expression of pE1 in the liver is far less sensitive to hormonal regulation compared with the uterus or the mammary glands. This finding is similar to the complete lack of hormonal regulation for pPRLR-LF mRNA expression in the liver (Trott *et al.* 2009) and the finding that neither gonadectomy nor sex steroid hormone treatment affected E1₃ expression in the liver of rats (Tanaka *et al.* 2005). Others have suggested that E regulates rE1₃ expression in some regions of the brain such as the hypothalamus and the choroid plexus, although discrepancies exist regarding the existence and the direction of this effect (Pi *et al.* 2003, Kobayashi *et al.* 2007, Nogami *et al.* 2007, Tabata *et al.* 2012). This differential hormone responsiveness may reflect a varied presence of tissue-specific transcription factors across different stages of development.

While C/EBP and SP1 sites are critical for E-induced hPRLR expression (Hu *et al.* 2002, Dong *et al.* 2006), our results indicate that the proximal C/EBP and Sp1 binding sites located between –151 and –131 are not involved in either the basal promoter activity for pE1 or its responsiveness to E. We find that the sequence between –648 and –170 of the pE1 promoter is the primary regulator of transcription and that two proximal non-ERE but E-sensitive sites, an E-box and an TFAP2A site, contribute to ER-induced transcription directed by the pE1 promoter. Both also contribute to basal transcription. Dong *et al.* (2006) also failed to identify functional EREs in the homologous hE1₃ and proposed that transcription resulted from ligand-bound ER recruited to C/EBP β and Sp1 response elements in the proximal part of the promoter while forming complexes with additional coactivators. We suggest two ways in which E-activation of the ER might activate the E-box to induce transcription from the pE1 promoter. First, E may directly activate upstream stimulator factors (USFs) to bind the E-box or it may activate the ESR to complex with USFs at the E-box. USF-1 binds an imperfect E-box through a complex involving USF1, ESR1, and SP1 proteins that is absolutely required for transcription from the ESR1 promoter (deGraffenried *et al.* 2004). Secondly, the ESR can directly activate USFs to bind an E-box in the cathepsin D promoter (Xing & Archer 1998). Meanwhile, it is less clear how E-activation of the ER acts on the putative TFAP2A site to induce pE1 promoter activity. Others showed that TFAP2A is a major regulator of E-signaling in MCF-7 cells (Woodfield *et al.* 2007) and in ESR-positive breast cancer cells where it regulates transcription of the ER promoter upstream of the P1 cap site (deConinck *et al.* 1995). However, our

discovery that a putative TFAP2A site in the porcine PRLR promoter is required for ESR-induced transcription is the first report of E-activation of a putative TFAP2A site.

The region between –648 and –596 bp of the pE1 promoter includes a candidate repressor region that contains putative binding sites for Pit-1 and ETS. Pit-1 is primarily expressed in the pituitary but has also been detected in the extrapituitary tissues such as placenta, lymphoid, and hematopoietic tissues, as well as in the normal and tumorous breast, and in MCF-7 cells. This factor is responsible for the transcriptional regulation of GH, PRL, and TSH expression (Holloway *et al.* 1995, Gil-Puig *et al.* 2005). ETS factors act as transcriptional repressors and/or activators and can be either ubiquitous or tissue specific (Sharrocks 2001, Wei *et al.* 2010).

In summary, we have identified species-specific differences in the PRLR E1 promoter that may underlie the functional diversity of PRL across species. The complex multilevel transcriptional control of the PRLR could be crucial to the many biological functions of PRL.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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