

Co-expression and Sequence Determination of Estrogen Receptor Variant Messenger RNAs in Swine Uterus

C. Ying*, M.-A. Chan, W. T. K. Cheng¹ and W.-F. Hong

Department of Microbiology, Soochow University, Taipei, Taiwan, ROC

ABSTRACT : Steroid hormones and their receptors play an important role in reproductive process. Estrogen is intimately involved with pregnancy and its function is mediated through the estrogen receptor which has been chosen as a candidate gene to study litter size in pigs. In this study, we report that two estrogen receptor variants, designated pER-1 and pER-2 were co-expressed in the uteri of normal cycling Lan-Yu pig (*Sus vittatus*, a small-ear miniature in Taiwan) with the pER-1 expression level appeared to be several times higher than that of pER-2. These receptor variants were isolated using reverse transcription-PCR from the pig uteri and their sequences were determined. The pER-1 and pER-2 sequences, which are homologous to those found in other mammalian estrogen receptors, encode putative proteins consisting of 574 and 486 amino acids, respectively. A deletion in exon I was identified in both sequences, with deletion lengths of 63 bp in pER-1 and 327 bp in pER-2. The deletion in pER-1 is internal to that in pER-2 and both deletions resulted in a truncation of the B domain, which confers the transactivating activity of estrogen receptor protein. This result describes the existence of estrogen receptor variants with a deletion in exon I and implies the possibility that physiological functioning of an estrogen receptor may not require the presence of an intact B domain. (*Asian-Aust. J. Anim. Sci.* 2003, Vol 16, No. 12 : 1716-1721)

Key Words : Gene Expression, Estrogen Receptor, Pig, Reproduction

INTRODUCTION

Estrogen receptors (ER) belong to the superfamily of retinoic acid, vitamin D and steroid and thyroid hormone receptors (Green et al., 1986). The members of this family are ligand-induced transcription factors composed of functional modules that mediate DNA and hormone binding, dimerization and transcriptional activation (Kumar et al., 1987; Evans, 1988; Gronemeyer, 1991). ER and its hormone ligand, the female sex steroid 17-estradiol, play critical roles in the development of secondary female sexual characteristics, the establishment of female reproductive cycle, fertility, and pregnancy maintenance (King, 1991). Estradiol is also thought to be essential to embryonic and fetal development (Rothschild et al., 1996).

Estrogen regulates female reproductive functions and embryonic development primarily through the nuclear estrogen receptor-alpha (ER-alpha). Upon binding to the estrogen ligand, ER-alpha protein is activated and becomes a transcription factor that modulates the expression of target genes (Das et al., 1997; Rissman et al., 1997). In an ER-alpha knockout mouse model, severe reproductive and behavior deficits have been observed. Both male and female mice became completely infertile and the induction of female sexual behaviors by estradiol and progesterone was diminished (Moffatt et al., 1998). Mice lacking the ER-

alpha also exhibited unsuccessful ovulation and the estrogen-induced epithelial mitogenesis in female reproductive organs was impaired (Cooke et al., 1998; Schomberg et al., 1999). The recently cloned estrogen receptor-beta (ER-beta), like ER-alpha, is a member of the steroid receptor superfamily (Kuiper et al., 1996), and low but detectable levels of ER-beta mRNA and protein have been detected in the rodent uterus and vagina (Couse et al., 1997; Kuiper et al., 1997; Saunders et al., 1997). However, the molecular mechanisms that regulate the transcriptional activity of ER-beta may be distinct from those of ER-alpha (Tremblay et al., 1997). Observation with mice lacking ER-beta indicated that ER-beta is essential for normal ovulation efficiency but is not required for female or male sexual differentiation, fertility or lactation (Krege et al., 1998).

The ER present in swine uteri is widely used for studying hormone binding, receptor dimerization, DNA binding, and phosphorylation (Lahooti et al., 1994; Le Goff et al., 1994). However, in contrast to numerous data collected and published on human, rat and mouse ER-alpha and ER-beta sequences and their variants (Green et al., 1986; Koike et al., 1987; Bokenkamp et al., 1994; Kuiper et al., 1996), porcine ER-alpha sequences have only recently been determined in a single strain (Bokenkamp et al., 1994) *Sus scrofa* or Large white, and information on the presence of ER-beta has not been reported. In this study, we report cloning and sequence determination of two co-expressed ER messenger RNA variants of ER-alpha from the uteri of *Sus vittatus*, or Lan-Yu pig, a species found in Taiwan. The Lan-Yu pigs are miniature wild pigs with small ear and black body. A comparison of the cDNA sequences of these two ER variant with those published for other species

* Corresponding Author: Chingwen Ying, Tel: +886-2-2881-9471 (ext) 6858, Fax: +886-2-2883-1193, E-mail: cying@mail1.scu.edu.tw

¹ Department of Animal Sciences, National Taiwan University, Taipei, Taiwan, ROC.

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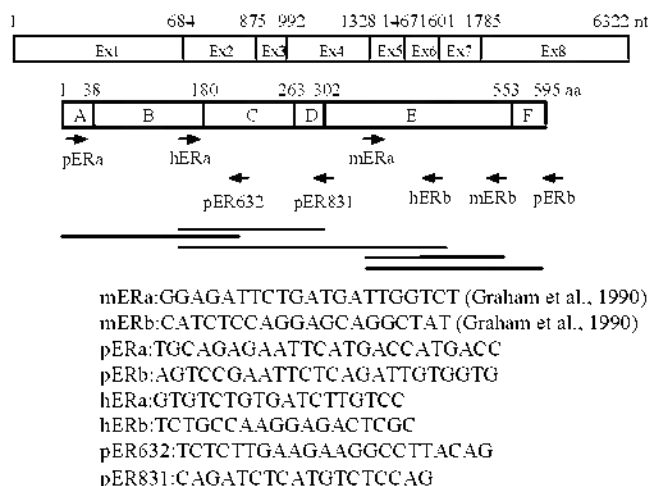


Figure 1. Positions of various primers used to amplify ER-alpha mRNA from the uteri of Lan-Yu pig. The schematic representation of ER-alpha mRNA (top) and protein modified from Gotteland et al. (1995) shows the map position of the eight exons and six domains, respectively. The heavy lines represent the pig ER cDNA fragments amplified by individual primer sets.

revealed unexpected and novel exon I deletion.

MATERIALS AND METHODS

Materials

TRIzol reagent and superscript II reverse transcriptase were obtained from GibcoBRL Life Tech (Gaithersburg, MD, USA). The pCR-Script SK (+) and TA cloning kit used in this study were purchased from Stratagene (La Jolla, CA, USA) and Invitrogen (Leek, Netherlands), respectively.

Porcine uterine RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR) assays

Lan-Yu pig (*Sus vittatus*; a small-ear miniature gilt (Mason, 1996)) uteri from four-month-old animals were obtained from the Department of Animal Sciences at the National Taiwan University, the location of conservation of Lan-Yu pigs, and stored at -80°C until use. The Lan-Yu pigs, also known as Yan-Yu small-ear miniature pigs or Taiwan small-ear miniature pigs become fertile earlier than Large White at 97 ± 42 days of age. The length of pregnancy is 114 days in average for Lan-Yu pigs. The visible characteristics of Lan-Yu pigs included small and erect ear; straight tail; long and slightly concave face; very short legs with thick strong pasterns; stiff bristles; hanging belly and curving back and boars showing attack behavior under stress. Uterus RNA was prepared according to procedures described by Chomczynski and Sacchi (1987). For cDNA synthesis, 5 g of porcine uterus RNA was mixed with 4 l of 5X first strand buffer, 2 l of 0.1 M DTT, 1 l of 10 mM

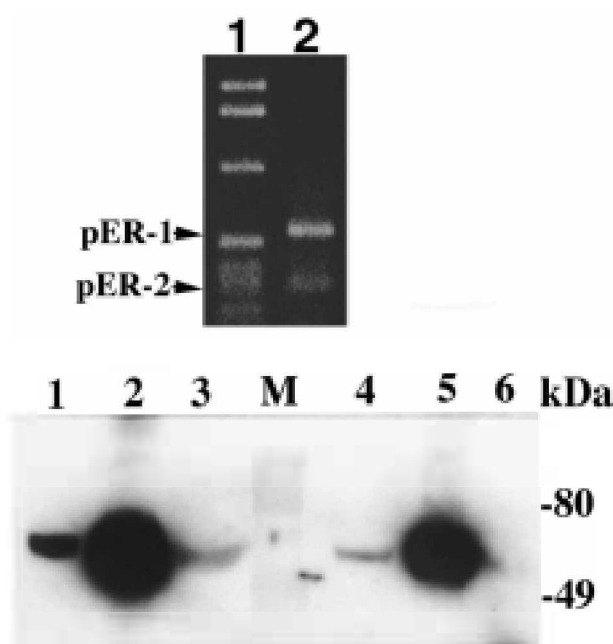


Figure 2. RT-PCR amplification of the 5' end of the ER-alpha cDNA from the uteri of Lan-Yu pig. Total RNA was prepared from pig uteri and amplified with primers pERa and pER632. 1 kb DNA marker (BRL GIBCO) was used as molecular weight markers in lane 1.

dNTPs and 1 l (200 units) of reverse transcriptase in a total reaction volume of 20 l. Reaction mixtures were first incubated at 45°C for 1 h and then at 70°C for 15 min. Various pig ER cDNA fragments were amplified from a 100 l reaction mixture containing 1 X PCR buffer, 0.1 mM dNTPs, 0.5 g primers, 2.5 U Taq DNA polymerase, 1.5 l of RT product and a pre-determined concentration of MgCl_2 (4 mM for the primer sets of mERa/mERb and mERa/pERb; 3 mM for the primer set of hERa/hERb; 1 mM for the primer sets of hERa/pER831 and pERa/pER632). Primers used in this study and the DNA fragments amplified are listed (Figure 1). Amplified DNA fragments were analyzed by agarose gel electrophoresis and their identification was confirmed by Southern blot analysis using a digoxigenin (DIG) labeled human ER cDNA probe.

Cloning and Sequencing of cDNA fragments

Amplified cDNA fragments were cloned either into the PCR II using a TA cloning kit or into the pCR-Script SK(+) after flushing the cDNA ends with cloned *pfu* DNA polymerase according to manufacture's suggestion (Stratagene, La Jolla, CA, USA). Both strands of the cloned cDNA fragments were sequenced using an ALF express DNA Sequencer (Pharmacia Biotech, Uppsala, Sweden).

RESULTS

Our strategy for cloning the ER from porcine uteri is

A
 ATG ACC ATG ACC CTA CAC ACC AAA GGG TCC GGC ATG GGC CTG CTG CAC CGG ATC CAA GCC AAC GAG CTG GAG CCC CTG AAC CGC CCG CAG CTC AAG ATC CCC CTG GAG CGG CCC CTG GGC
 Met Thr Met Thr Leu His Thr Lys Ala Ser Gly Met Ala Leu Leu His Gln Ile Gln Ala Asn Glu Leu Glu Pro Leu Asn Arg Pro Gln Leu Lys Ile Pro Leu Glu Gln Pro Leu Gly
 121
 GAG GTG TAC GTG GAC AGC AGC AAG CCC GGC GTG TAT AAC TAC CCC GAG GGC GGC GCG TAC GAC TTC AAC GGC GCG GCG GCG TTC GGC GCC AAC GGC TTG GGG GGC TTC CAG CCC CTC AAC
 Glu Val Tyr Val Asp Ser Ser Lys Pro Ala Val Tyr Asn Tyr Pro Glu Gly Ala Ala Tyr Asp Phe Asn Ala Ala Ala Ala Phe Gly Ala Asn Gly Leu Gly Gly Phe Gln Pro Leu Asn
 241
 AGC GTG TCT CCG AGC CCG CTG GTA CTG CTG CAC CCG CCG CCG CAG CTC TCG CCC TTC CTG CAC CCC CAC GGC CAA CAG GTG CCC TAT TAC CTG GAG AAT GAG CCG AGC GGC TAT GCG GTG
 Ser Val Ser Pro Ser Pro Leu Val Leu Leu His Pro Pro Pro Gln Leu Ser Pro Phe Leu His Pro His Gly Gln Gln Val Pro Tyr Tyr Leu Glu Asn Glu Pro Ser Gly Tyr Ala Val
 361
 CCG GAG CCC GGC CCT CCC GCC TTC TAC AGG CAA AAT TCA GAT AAT CCG CCG CAG GGT GGC AGA GAG AGA TTG GCC AGC ACC AGT GAC AAG GGA AGC ATG GCC ATG GAA TCT GCC AAG GAG
 Arg Glu Ala Gly Pro Pro Ala Phe Tyr Arg Pro Asn Ser Asp Asn Gln Arg Gln Gly Gly Arg Glu Arg Leu Ala Ser Thr Ser Glu Ing Gly Ser Met Ala Met Glu Ser Ala Lys Glu
 481
 ACT CCG TAC TGT GCA GTG TGC AAT GAC TAT GGC TCA GGC TAC CAT TAT GGA GTT TGG TCT TGC GAG GGC TGT AAG GCC TTC TTC AAG AGA AGT ATT CAA GGA CAT AAT GAC TAC ATG TGT
 Thr Arg Tyr Cys Ala Val Cys Asn Asp Tyr Ala Ser Gly Tyr His Tyr Gly Val Trp Ser Cys Glu Gly Cys Lys Ala Phe Phe Lys Arg Ser Ile Gln Gly His Asn Asp Tyr Met Cys
 601
 CCA GCC ACC AAC CAG TGC ACA ATT GAT AAG AAC AGS AGS AAG AGC TGT CAG GCC TGC CCG CTA CCG AAG TGC TAC GAA GTG GGC ATG ATG AAA GGG GGG ATA CCG AAA GAC CCG AGA GGA
 Pro Ala Thr Asn Gln Cys Thr Ile Asp Lys Asn Arg Arg Lys Ser Cys Gln Ala Cys Gln Leu Arg Lys Cys Tyr Glu Val Gly Met Met Lys Gly Gly Ile Gln Lys Asp Gln Arg Gly
 721
 GGG AGA ATG TTG AAG CAC AAG CCG CAG AGA GAT GAT GGA GAG GGC AGG AAT GAA GCG GTG CCC CCT GGA GAC ATG AGA TCT GCC AAC CTT TGG CCA AGC CTT CTC TTG ATT AAA CAC ACT
 Gly Arg Met Leu Lys His Lys Arg Gln Arg Asp Asp Gly Glu Gly Arg Asn Glu Ala Val Pro Pro Gly Asp Met Arg Ser Ala Asn Leu Trp Pro Ser Pro Leu Leu Ile Lys His Thr
 841
 AAG AAG AAC AGC CCG GTC TTG TCC CTG ACA GCC GAC CAG ATG ATC AGT GCC TTG TTG GAG GCT GAG CCC CCC ATA ATC TAT TCC GAG TAT GAT CTT ACC AGA CCC CTC AGT GAG GCT TCA
 Lys Lys Asn Ser Pro Val Leu Ser Leu Thr Ala Asp Gln Met Ile Ser Ala Leu Leu Glu Ala Glu Pro Pro Ile Ile Tyr Ser Glu Tyr Asp Pro Thr Arg Pro Leu Ser Glu Ala Ser
 961
 ATG ATG GGC TTG CTG ACC AAC CTC CCA GAC AGG GAG CTG GTA CAC ATG ATC AAC TGG CCA AAG AAG GGT CCA GGA TTT TTG GAT TTA AGC CTC CAT GAT CAA GTG CAT CTT CTG GAA TGT
 Met Met Gly Leu Leu Thr Asn Leu Ala Asp Arg Glu Leu Val His Met Ile Asn Trp Ala Lys Arg Val Pro Gly Phe Leu Asp Leu Ser Leu His Asp Gln Val His Leu Leu Glu Cys
 1081
 GCC TGG CTG GAG ATC CTC ATG ATT GGT CTT GTC TGG GGC TCC ATG GAG CAC CCA GGG AAG CTC CTG TTT GCT CCT AAC TTG CTC CTG GAC AGG AAC CAG GGC AAG TGT GTC GAG GGA ATG
 Ala Trp Leu Ala Gln Leu Met Ile Gly Leu Val Trp Arg Ser Met Glu His Pro Gly Lys Leu Leu Phe Ala Pro Asn Leu Leu Leu Asp Arg Asn Gln Gly Lys Cys Val Glu Gly Met
 1201
 GTG GAG ATC TTT GAC ATG TTG CTG GCT ACA TCA TCT CCG TTC CBT ATG ATG AAT CTC CAG GGA GAG GAG TTT GTG TGC CTC AAA TCC ATC ATT TTG CTT AAT TCT GGA GTG TAC AGC TTT
 Val Glu Ile Phe Asp Met Leu Leu Ala Thr Ser Ser Arg Phe Arg Met Met Asn Leu Gln Gly Glu Glu Phe Val Cys Leu Lys Ser Ile Ile Leu Leu Asn Ser Gly Val Tyr Thr Phe
 1321
 CTG TCC AGC ACC CTG AAG TCT CTG GAA GAG AAG GAC CAT ATC CAC CGT GTC CTG GAC AAG ATC ACA GAC ACC TTG ATC CAC CTG ATG GCC AAA GCG GGC CTG ACT CTG CAG CAG CAG CAC
 Leu Ser Ser Thr Leu Lys Ser Leu Glu Glu Lys Asp His Ile His Arg Val Leu Asp Lys Ile Thr Asp Thr Leu Ile His Leu Met Ala Lys Ala Gly Leu Thr Leu Gln Gln Gln His
 1441
 CCG CGT CTC GCG CAG CTC CTC CTC ATC CTG TCT CAC TTC AGG CAC ATG AGT AAC AAA GGC ATG GAG CAT CTG TAC AAC ATG AAG TCC AAG AAC GTG GTG CCC CTC TAT GAC CTG CTG CTG
 Gln Arg Leu Ala Gln Leu Leu Leu Leu Ser His Phe Arg His Met Ser Asn Lys Gly Met Lys Ser Asn Lys Gly Met Lys His Leu Tyr Asn Met Lys Lys Lys Asn Val Pro Leu Tyr Asp Leu Leu Leu
 1561
 GAG ATG CTG GAC GGC CAC CCG CTG CAC GCC CCA ACC AAC CTC GGG GGC CCA CCC CCG GAG GAC ATG AGC CAG AGC CAG CTG GCC ACC TGG GGC TCA ACT CCA TCG CAT TCC TTG CAA ATG
 Glu Met Leu Asp Ala His Arg Leu His Ala Pro Thr Asn Leu Gly Gly Pro Pro Pro Glu Asp Met Ser Gln Ser Gln Leu Ala Thr Ser Gly Ser Thr Pro Ser His Ser Leu Gln Met
 1681
 TAT TAC ATC ACG GGG GAG GCG GAG AAC TTC CCC ACC ACA ATC TGA
 Tyr Tyr Ile Thr Gly Glu Ala Glu Asn Phe Pro Thr Thr Ile End
 1725

B
 ATG ACC ATG ACC CTA CAC ACC AAA GGG TCC GGC ATG GGC CTG CTG CAG ATC CAA GCC AAC GAG CTG GAG CCC CTG AAC CGC CCG CAG CTC AAG ATC CCC CTG GAG CGG CCC CTG GGC
 His Thr Lys Ala Ser Gly Met Ala Leu Leu Gln Ile Gln Ala Asn Glu Leu Glu Pro Leu Asn Arg Pro Gln Leu Lys Ile Pro Leu Glu Arg Pro Leu Gly Met Thr Met Thr Leu
 121
 GAG GTG CCA AAT TCA GAT AAT CCG CCG CAG GGT GGC AGA GAG AGA TTG GCC AGC ACC AGT GAC AAG GGA AGC ATG GCC ATG GAA TCT GCC AAG GAG ACT CCG TAC TGT GCA GTG TGC AAT
 Glu Val Pro Asn Ser Asp Asn Arg Arg Glu Arg Leu Ala Ser Thr Tyr Ser Asp Lys Gly Ser Met Ala Met Glu Ser Ala Lys Gln Thr Arg Tyr Cys Ala Val Cys Asn
 241
 GAC TAT GCC TCA GGC TAC CAT TAT GGA GTT TGG TCT TGC GAG GGC TGT AAG GCC TTC TTC AAG AGA AGT ATT CAA GGA CAT AAT GAC TAC ATG TGT CCA GCC ACC AAC CAG TGC ACA ATT
 Asp Tyr Ala Ser Gly Tyr His Tyr Gly Val Trp Ser Cys Glu Gly Cys Lys Ala Phe Phe Lys Arg Ser Ile Gln Gly His Asn Asp Tyr Met Cys Pro Ala Thr Asn Gln Cys Thr Ile
 361
 GAT AAG AAC AGG AGG AAG AGC TGT CAG GCC TGC CCG CTA CCG AAG TGC TAC GAA GTG GGC ATG ATG AAA GGG GGG ATA CCG AAA GAC CCG AGA GGA GGG AGA ATG TTG AAG CAC AAG CCG
 Asp Lys Asn Arg Arg Lys Ser Cys Gln Ala Cys Arg Leu Arg Lys Cys Tyr Glu Val Gly Met Met Lys Gly Gly Ile Arg Lys Asp Arg Gly Gly Arg Met Leu Lys His Lys Arg
 481
 CAG AGA GAT GAT GGA GAG GGC AAG AAT GAA GCG GTG CCC CTT GGA GAC ATG AGA TCT GGC AAC CTT TGG CCA AGC CTT CTC TTG ATT AAA CAC ACT AAG AAG AAC AGC CCG GTC TTG TCC
 Gln Arg Asp Asp Gln Glu Gly Arg Asn Glu Ala Val Pro Pro Gly Asp Met Arg Ser Ala Asn Leu Trp Pro Ser Pro Leu Leu Ile Lys His Thr Lys Lys Asn Ser Pro Val Leu Ser
 601
 CTG ACA GCC GAC CAG ATG ATC AGT GCC TTG TTG GAG GCT GAG CCC CCC ATA ATC TAT TCC GAG TAT GAT CTT ACC AGA CCC CTC AGT GAG GCT TCA ATG ATG GGC TTG CTG ACC AAC CTC
 Leu Thr Ala Asp Gln Met Ile Ser Ala Leu Leu Glu Ala Glu Pro Pro Ile Ile Tyr Ser Glu Tyr Asp Pro Thr Arg Pro Leu Ser Glu Ala Ser Met Met Gly Leu Leu Thr Asn Leu
 721
 GCA GAC AGG GAG CTG GTA CAC ATG ATC AAC TGG CCA AAG AGG GTG CCA GGA TTT TTG GAT TTA AGC CTC CAT GAT CAA GTG CAT CTT CTG GAA TGT GCC TGG CTA GAG ATC CTC ATG ATT
 Ala Asp Arg Glu Leu Val His Met Ile Asn Trp Ala Lys Arg Val Pro Gly Phe Leu Asp Leu Ser Leu His Asp Gln Val His Leu Leu Glu Cys Ala Trp Leu Glu Ile Leu Met Ile
 841
 GGT CTT GTC TGG GGC TCC ATG GAG CAC CCA GGG AAG CTC CTG TTT GCT CCT AAC TTG CTC CTG GAC AGG AAC CAG GGC AAG TGT GTC GAG GGA ATG GTG GAG ATC TTT GAC ATG TTG CTG
 Gly Leu Val Trp Arg Ser Met Glu His Pro Gly Lys Leu Leu Phe Ala Pro Asn Leu Leu Leu Asp Arg Asn Gln Gly Lys Cys Val Glu Gly Met Val Glu Ile Phe Asp Met Leu Leu
 961
 GCT ACA TCA TCT GGC TTC CBT ATG ATG AAT CTC CAG GGA GAG GAG TTT GTG TCC CTC AAA TCC ATC ATT TTG CTT AAT TCT GGA GTG TAC AGC TTT CTG TCC AGC ACC CCG AAG TCT CTC
 Ala Thr Ser Ser Arg Phe Arg Met Met Asn Leu Gln Gly Glu Glu Phe Val Cys Leu Lys Ser Ile Ile Leu Leu Asn Ser Gly Val Tyr Thr Phe Leu Ser Ser Thr Leu Lys Ser Leu
 1081
 GAA GAG AAG GAC CAT ATC CAC CBT GTC CTG CAG AAG ATC ACA GAC ACC TTG ATC CAC CTG ATG GCC AAA GCG GGC CTG ACT CTG CAG CAG CAG CAC CCG GGT CTC GCG CAG CTC CTC CTC
 Glu Glu Lys Asp His Ile His Arg Val Leu Asp Lys Ile Thr Asp Thr Leu Ile His Leu Met Ala Lys Ala Gly Leu Thr Leu Gln Gln Gln His Arg Arg Leu Ala Gln Leu Leu Leu
 1201
 ATC CTG TCT CAC TTC AGG CAC ATG ATG AAC AAA GGC ATG GAG CAT CTG TAC AAC ATG AAG TGC AAG AAC GTG GTG CCC CTC TAT GAC CTG CTG CTG ATG CTG GAC GCC CAC CCG CTG
 Ile Leu Ser His Phe Arg His Met Ser Asn Lys Gly Met Glu His Leu Tyr Asn Met Lys Cys Lys Asn Val Val Pro Leu Tyr Asp Leu Leu Leu Met Leu Asp Ala His Arg Leu
 1321
 CAC GCC CCA ACC AAC CTC GGG GGC CCA CCC CCG GAG GAC ATG AGC CAG AGC CAG CTG GCC ACC TGG GGC TCA ACT CCA TCG CAT TCC TTG CAA ATG TAT TAC ATC ACG GGG GAG GCG GAG
 His Ala Pro Thr Asn Leu Gly Gly Pro Pro Pro Glu Asp Met Ser Gln Ser Gln Leu Ala Thr Ser Gly Ser Thr Pro Ser His Ser Leu Gln Met Tyr Tyr Ile Thr Gly Glu Ala Glu
 1441
 AAC TTC CCC ACC ACA ATC TGA
 Asn Phe Pro Thr Thr Ile End

Figure 3. DNA sequences and deduced amino acid sequences of the ER-alpha variants pER-1 (A) and pER-2 (B) in the uteri of Lan-Yu pig.

presented in Figure 1. Fragments of pig ER cDNA were amplified and cloned into appropriate plasmids. Surprisingly, two cDNA fragments encoding the N-terminal region of the ER protein were amplified using the primer set of pERa and pER632 (Figure 2). In addition to a DNA fragment of 600 bp (similar to the expected size of 632 bp),

one minor DNA fragment of 300 bp was also amplified in the PCR products. The amount of the major DNA fragment, designated 5' end of the pER-1 appeared to be about four times that of the minor DNA fragment, designated 5' end of the pER-2 presented in the uterus RNA. All attempts to improve the specificity of the PCR technique failed to

eliminate the pER-2 DNA fragment from the PCR products. Southern analysis using a human ER cDNA fragment as a probe revealed hybridization with both the major and minor DNA fragments (data not shown). Since the pER-1 and pER-2 cDNA fragments are potentially two variants of ER messenger RNA, the decision was made to clone and determine the sequences of both. By combining the sequences of amplified pig ER cDNA fragments, the lengths of pER-1 and pER-2 were determined (Figure 3). Fragment lengths were measured at 1.725 bp for pER-1 and 1.461 bp for pER-2. Both sequences were found to be highly homologous to those reported for other species (Figure 4).

A more detailed comparison of the pER-2 sequences with ER cDNA sequences from other mammals identified a 327 bp deletion from nucleotide 126 to 452. A putative protein of 486 amino acids with a deletion of 109 amino acid residue (from 43 to 151 aa) in the B domain of the ER

protein was deduced from the sequences of pER-2. A deletion corresponding to the 193-255 bp region which was internal to the deletion in pER-2 was also observed in the pER-1 sequences. The pER-1 amino acid sequences was therefore deduced as consisting of 574 amino acids, with the B domain of the ER shortened by 21 amino acids (65-85 aa) compared to that reported by Bokenkamp et al. (1994) for the Large White pig species. A comparison with ER cDNA taken from Large White pig (*Sus scrofa*) revealed alterations in four bases which did not result in changes in amino acid sequences: at 264 (G/A), 606 (T/C), 1,602(T/C) and 1,692 (A/G).

DISCUSSION

Two ER-alpha messenger RNA variants with deletions in the exon I were identified in the uterus of Lan-Yu pigs. Their sequences were determined and compared with the

ftnbjolsx0	1	50
Human	MTMTLHTKASGMALLHOIOGNELEPLNRPQLKIPLERPLGEVYLDSSKPA	
pER-1A:.....V:.....	
pER-2A:.....	
<i>S. scrofa</i>A:.....V:.....	
RatM:M:A:.....N:.....	
MouseM:M:A:.....N:.....T	
	51	100
Human	VYNYPEGAAYEFNAAAAA-----NAQVYGQTGLPYPPGSEAAAFGSNGLG	
pER-1D:-----	
pER-2	-----	
<i>S. scrofa</i>D:-----S:P:.....S:A:.....A:.....	
Rat	:F:.....AAAGA:.....SIT:.....S:.....	
Mouse	:F:.....AAAA-.....I:.....S:.....S:.....	
	101	150
Human	GFPLNSVSPSPLMLLHPPQLSPFLQPHGQQVPYYLENEPSGYTVREAG	
pER-1	:Q:.....V:.....H:.....A:.....	
pER-2	-----	
<i>S. scrofa</i>	:Q:.....V:.....H:.....A:.....	
Rat	A:Q:.....HV:.....H:.....A:.....DT:.....	
Mouse	A:Q:.....	
	151	200
Human	PPAFYRPNSDNRQGRERLASTNDKGSMAESAKETRYCAVCNDYASGY	
pER-1S:.....	
pER-2	-----S:.....	
<i>S. scrofa</i>S:.....	
RatS:.....N:.....S:SSE:.....N:I:.....	
MouseS:.....N:.....S:S:E:.....N:I:.....	

Figure 4. Comparison of the amino acid sequence of Lan-Yu pig ER-alpha variants with those from human, large white pig, mouse and rat. "-" indicated the absence of corresponding amino acid and ":" indicates that the same amino acid is present compared to the sequence of human ER.

ER cDNAs of other species. The major variant pER-1 contained a deletion of 63 nucleotide deletion and the minor variant pER-2 contained a 327 nucleotide deletion. Both variants stay in frame and code for a ER protein missing aa 65-85 and aa 43-151 in the B domain, respectively. The B domain of ER protein confers transactivation activity and is not as conserved as domains C (involved in DNA binding) or E (required for ligand binding).

To our knowledge, ER-alpha messenger RNA variants with deletions in exon I have not been previously reported. However, data has been published on observed deletions in exons 2-7 in various tumor tissues and established cell lines (Graham et al., 1990; McGuire et al., 1992; Koehorst et al., 1993; Pfeffer et al., 1993) and whether these ER variants are physiologically significant in terms of tumor progress remains unknown (Wang and Miksicek, 1991; Pfeffer et al., 1995). That the ER variants reported previously were resulted from a precise deletion of either a single exon (exons 2, 3, 4, 5, or 7), or two exons (exon 3 and 4) suggests an alternative splicing mechanism was involved. In addition to tumor tissues, Gotteland et al. also observed the presence of at least six ER messenger RNA variants in normal breast tissues (Gotteland et al., 1995). In the present study, we observed the co-expression of two ER messenger RNA variants in pig uteri. Unlike the previously described "exon-skipping" mutants, these variants most likely reflect changes in genomic ER sequence, especially since the two messenger RNAs do not correspond to the normal splicing patterns of ER mRNA. Detailed genomic sequence characterization would facilitate the understanding of whether such changes take place in the ER gene. The putative proteins translated from these two pig ER RNAs observed in this study exhibit internal in-phase deletion in B domain and thus generated a in-frame truncation of the ER protein. Very few cases of truncated protein synthesis have been previously reported in the literature. A 46 and a 47 kDa with characteristics of ER protein were identified by Diaz-Chico et al. (1988) and Jozan et al. (1991). They suggested that these truncated proteins were generated by deletion in exons 3 and 4. A 80 k Da ER variant containing an in-frame duplication of exons 6 and 7 due to genomic rearrangement was reported by Pink et al. (1996). Graham et al. (1990) also described ER mutations that include two frame-shift/termination mutants plus an additional mutant with a large in-frame deletion spanning the hinge region and some of the hormone-binding domains (Gronemeyer, 1991). They suggest that these mutations were generated by chromosomal rearrangement. Whether similar mechanism is involved in generating the pER-1 and pER-2 variants requires further investigation.

The pER-1 and pER-2 fragments were the only two specific DNA fragments that could be detected via RT-PCR technique in this study and attempts at identifying the

presence of non-deleted ER mRNA species were unsuccessful. Therefore, it appeared that either the non-deleted ER RNA is not expressed in pig uterus or its expression level is below detection limit used in the present experiment. In addition, the deletion in pER-1 did not appear to be an artifact that may occur using culture cells as starting materials since it was isolated from the uteri of normal cycling pig. In previous reports, Rothschild et al. studied the role of the ER gene on litter size in pigs and detected genetic difference at the ER among different pig species. They also suggested that such genetic difference is associated with litter size in pigs and ER is the best predictor of litter size differences (Rothschild et al., 1996). At farrowing, the average litter size of Lan-Yu pigs have been reported to be 4.71-5.89 and the survival rates of 8 week piglets at weaning was in the range of 84-91%. Therefore, it is interesting to speculate but remains to be studied the potential association for these ER variants occurring in Lan-Yu pig (a miniature) but not in Large White Pig or sheep (Madigou et al., 1996). In summary, the two ER variants from the uteri of Lan-Yu pig both contained an unusual exon I deletion. Although an affinity for estradiol is retained in the C-terminal half of the molecules (Murdoch et al., 1990) that appears to be intact in the two ER variants, it remains to be shown what effects these deletions, especially in pER-2, have on normal ER functions.

REFERENCES

- Bokenkamp, D., P. W. Jungblut, H. H. Thole. 1994. The C-terminal half of the porcine estradiol receptor contains no post-translational modification: determination of the primary structure. *Mol. Cell Endocrinol.* 104:163-172.
- Chomczynski, P., N. Sacchi. 1987. Single-step method of RNA isolated by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* 162:156-159.
- Cooke, P. S., D. L. Buchanan, D. B. Lubahn, G. R. Cunha. 1998. Mechanism of estrogen action: Lessons from the estrogen receptor-alpha knockout mouse. *Biol. Reprod.* 59:470-475.
- Couse, J. F., J. Lindzey, K. Grandien, J. A. Gustafsson. 1997. Tissue destruction and quantitative analysis of estrogen receptor-(ER) and estrogen receptor-(ER) messenger ribonucleic acid in the wild-type and ER-, knockout mouse. *Endocrinology*, 138:4613-4621.
- Das, S. K., J. A. Taylor, K. S. Korach, B. C. Paria, S. K. Dey, D. B. Lubahn. 1997. Estrogenic responses in estrogen receptor-alpha deficient mice reveal a distinct estrogen signaling pathway. *Proc. Natl. Acad. Sci. USA.* 94:12786-12791.
- Diaz-Chico, B. N., Y. Ogasawara, G. C. Chamenss, M. Salman, W. L. McGuire. 1988. A 46-kDa antigen associated with estrogen receptor in human breast cancer. *J. Steroid. Biochem.* 30:315-320.
- Evans, R. M. 1988. The steroid and thyroid hormone receptor superfamily. *Science*, 240:889-895.
- Green, S., P. Walter, V. Kumar, A. Krust, J-M. Bornert, P. Argos, P. Chambon. 1986. Human oestrogen receptor cDNA: sequence,

- expression and homology to v-erb-A. *Nature (Lond.)* 320:134-139.
- Gotteland, M., G. Desauty, J. C. Delarue, L. Liu and E. May. 1995. Human estrogen receptor messenger RNA variants in both normal and tumor breast tissues. *Mol. Cell Endocrinol.* 112:1-13.
- Graham, M. L., N. L. Krett, L. A. Miller, K. K. Leslie, D. F. Gordon, W. M. Wood, L. L. Wei and K. B. Horwitz. 1990. T47Dco cells, genetically unstable and containing estrogen receptor mutations, are a model for the progression of breast cancers to hormone resistance. *Cancer Res.* 50:6208-6217.
- Gronemeyer, H. 1991. Transcription activation by estrogen and progesterone receptors. *Ann. Rev. Gen.* 25:89-123.
- Jozan, S., A. M. Julia, A. Carretie, N. Eche, V. Maisongrosse, B. Fouet, B. Marques and J. F. David. 1991. 65 and 47 kDa forms of estrogen receptor in human breast cancer: relation with estrogen responsiveness. *Breast Cancer Res. Treat.* 19:103-109.
- King, R. J. B. 1991. A discussion of the roles of oestrogen and progestin in human mammary carcinogenesis. *J. Steroid Biochem Mol Biol.* 39:811-818.
- Koehorst, S. G. A., H. M. Jacobs, J. H. H. Thijssen and M. A. Blankenstein. 1993. Wild type and alternatively spliced estrogen receptor messenger RNA in human meningioma tissue and MCF7 breast cancer cells. *J. Steroid. Biochem. Mol. Biol.* 45:227-233.
- Koike, S., M. Sakai and M. Muramatsu. 1987. Molecular cloning and characterization of rat estrogen receptor cDNA. *Nuc Acids Res.* 15:2499-2513.
- Krege, J. H., J. B. Hodgkin, J. F. Couse, E. Enmark, M. Warner, J. F. Mahler, M. Sar, K. S. Korach, J. A. Gustafsson and O. Smithies. 1998. Generation and reproductive phenotypes of mice lacking estrogen receptor beta. *Proc. Natl. Acad. Sci. USA.* 95:15677-15682.
- Kuiper, G. G. M. J., E. Enmark, M. Peltö-Huikko, S. Nilsson and J. A. Gustafsson. 1996. Cloning of a novel estrogen receptor expressed in rat prostate and ovary. *Proc. Natl. Acad. Sci. USA.* 93:5925-5930.
- Kumar, V., S. Green, G. Stack, M. Berry, J.-R. Jin and P. Chambon. 1987. Functional domains of the human estrogen receptor. *Cell.* 51: 941-951.
- Kuiper, G. G. J. M., B. Carlsson, K. Grandien, E. Enmark, J. Haggblad, S. Nilsson and J.-A. Gustafsson. 1997. Comparison of ligand binding specificity and transcript tissue distribution of estrogen receptors and *Endocrinology*, 138:863-870.
- Lahooti, H., R. White, P. Danielian and M. G. Parker. 1994. Characterization of ligand-dependent phosphorylation of the estrogen receptor. *Mol. Endocrinol.* 8:182-188.
- LeGoff, P., M. M. Montano, D. J. Schodin, B. S. Katzenellenbogen. 1994. Phosphorylation of the human estrogen receptor: identification of hormone-regulated sites and examination of their influence on transcriptional activity. *J. Biol. Chem.* 269:4458-4466.
- Madigou, T., C. Tiffoche, G. Lazenec, J. Pelletier and M. L. Thiéulant. 1996. The sheep estrogen receptor: Cloning and regulation of expression in the hypothalamo-pituitary axis. *Mol. Cell Endocrinol.* 121:153-163.
- Mason, I. L. 1996. A world dictionary of livestock breeds, types and varieties, CAB International, Rome, Italy.
- McGuire, W. L., G. C. Chamness and S. A. W. Fuqua. 1992. Abnormal estrogen receptor in clinical breast cancer. *J. Steroid. Biochem. Mol. Biol.* 43:243-247.
- Moffatt, C. A., E. F. Rissman, M. A. Shupnik and J. D. Blaustein. 1998. Induction of progestin receptors by estradiol in the forebrain of estrogen receptor-alpha gene-disrupted mice. *Neuroscience*, 18:9556-9563.
- Murdoch, F., D. Meier, D. Furlow, K. Grunwald and J. Gorski. 1990. Estrogen receptor binding to a DNA response element *in vitro* is not dependent upon estradiol. *Biochemistry* 29:8377-8385.
- Pfeffer, U., E. Fecarotta, L. Castagnetta and G. Vidali. 1993. Estrogen receptor variant messenger RNA lacking exon 4 in estrogen-responsive human breast cancer cell lines. *Cancer Res.* 53:41-743.
- Pfeffer, U., E. Fecarotta and G. Vidali. 1995. Coexpression of multiple estrogen receptor variant messenger RNAs in normal and neoplastic breast tissues and in MCF-7 cells. *Cancer Res.* 55:21582-165.
- Pink, J. J., S. Q. Wu, D. M. Wolf, M. M. Bilimoria and V. C. Ordan. 1996. A novel 80 kDa human estrogen receptor containing a duplication of exons 6 and 7. *Nucl. Acids Res.* 24:962-969.
- Rissman, E. F., S. R. Wersinger, J. A. Taylor and D. B. Lubahn. 1997. Estrogen receptor function as revealed by knockout studies: Neuroendocrine and behavioral aspects. *Hormone Behav.* 31:232-243.
- Rothschild, M., C. Jacobson, D. Vaske, C. Tuggle, L. Wang, T. Short, G. Eckardt, S. Sasaki, A. Vincent, D. McLaren, O. Southwood, H. Van Der Steen, A. Mileham, G. Plastow. 1996. The estrogen receptor locus is associated with a major gene influencing litter size in pigs. *Proc. Natl. Acad. Sci. USA.* 93:201-205.
- Schomberg, D. W., J. F. Course, A. Mukherjee, D. B. Lubahn, M. Sar, K. E. Mayo and K. S. Korach. 1999. Targeted disruption of the estrogen receptor-alpha gene in female mice: characterization of ovarian responses and phenotype in the adult. *Endocrinology* 140:2733-2744.
- Saunders, P. T. K. L., S. M. Maguire, J. Gaughan and M. R. Millar. 1997. Expression of oestrogen receptor beta in multiple rat tissues visualized by immunohistochemistry. *J. Endocrinol.* 154:13-16.
- Tremblay, G. B., A. Tremblay, N. G. Copeland, D. J. Gilbert, N. A. Jenkins, F. Labrie and V. Giguere. 1997. Cloning, chromosomal localization and functional analysis of the murine estrogen receptor beta. *Mol. Endocrinol.* 11:353-365.
- Wang, Y., R. J. Miksick. 1991. Identification of a dominant negative form of the human estrogen receptor. *Mol. Endocrinol.* 5:1707-1715.