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

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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

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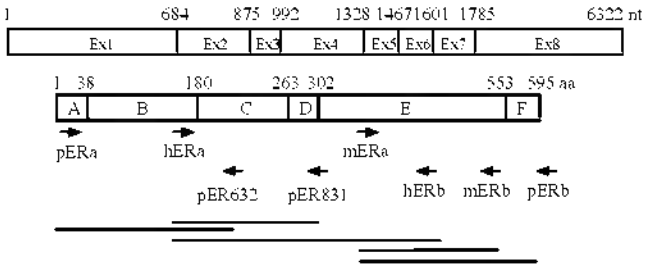

mERa:GGAGATTCTGATGATTGGTCT (Graham et al., 1990)
mERb:CATCTCCAGGAGCAGGCTAT (Graham et al., 1990)
pERa:TGCAGAGAATTCATGACCATGACC
pERb:AGTCCGAATTCTCAGATTGTGGTG
hERa:GTGTCTGTGATCTTGTCC
hERb:TCTGCCAAGGAGACTCGC
pER632:TCTCTTGAAGAAGGCCTTACAG
pER831:CAGATCTCATGTCTCCAG

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

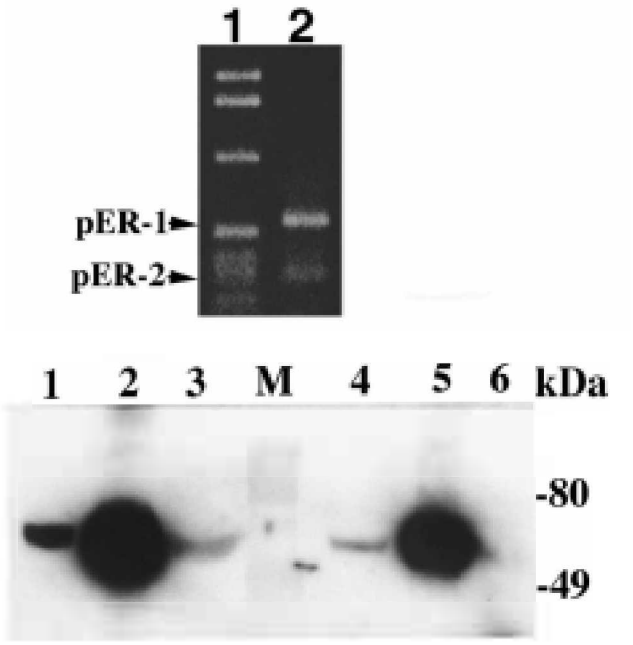

Figure 2. RT-PCR amplification of the 5' end of the ER-alpha cDNA from the uteri of Lan-Yu pig. Total RNA was prepared from pig uteri and amplified with primers pERa and pER632. 1 kb DNA marker (BRL GIBCO) was used as molecular weight markers in lane 1.

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 PCRII 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

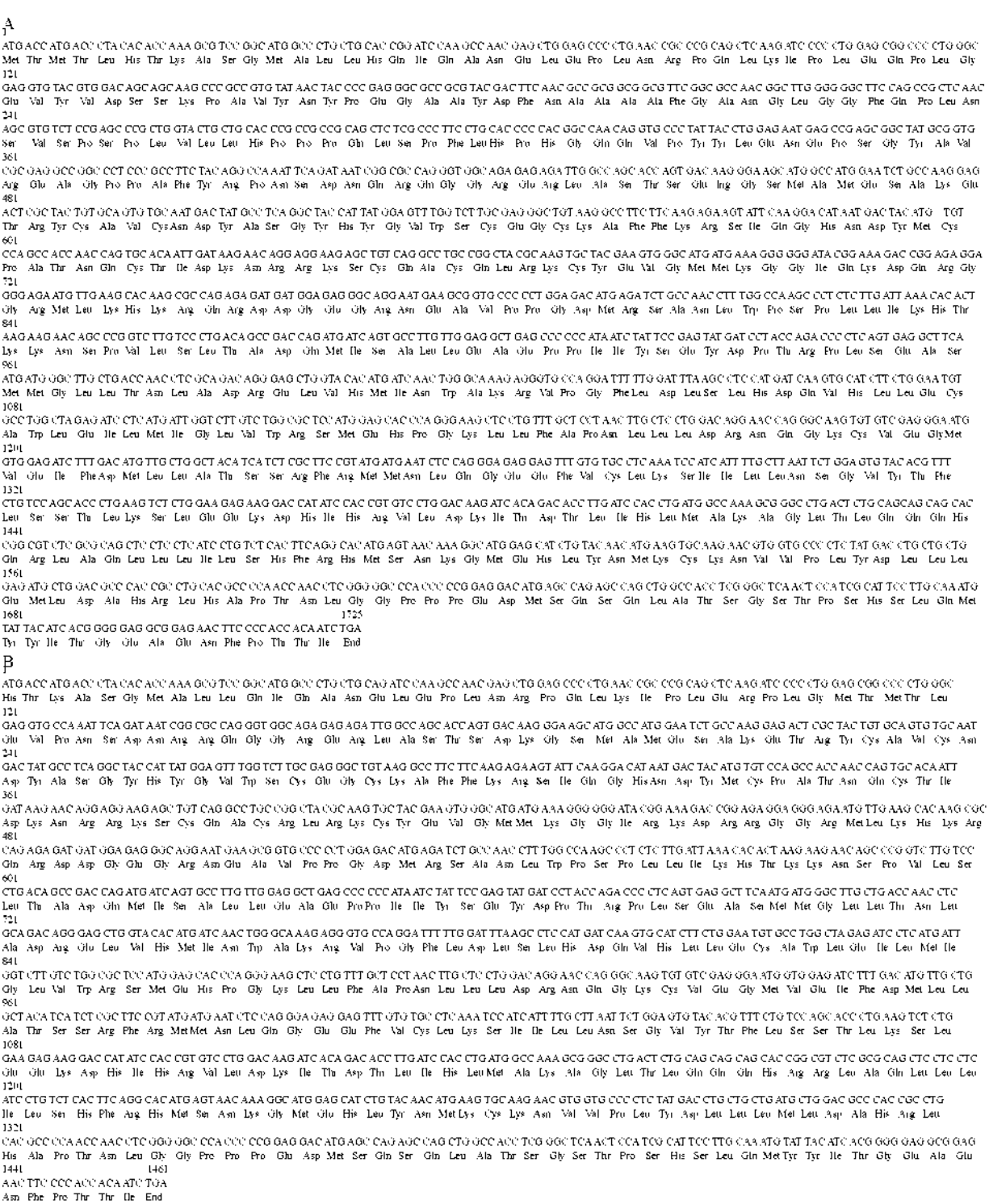

Figure 3. DNA sequences and deduced amino acid sequences of the ER-alpha variants pER-1 (A) and pER-2 (B) in the uteri of Lan-Yu pig.

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

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ftnbjolsx0    1                                   50
Human         MTMTLHTKASGMALLHQIQGNELEPLNRPQLKIPLERPLGEVYLDSSKPA
pER-1         ::::::::::::::::::::A::::::::::::::::::::::V::::::
pER-2         ::::::::::::::::::::A::::::::::::::::::::::--------
S.scrifa      ::::::::::::::::::::A::::::::::::::::::::::V::::::
Rat           ::::::::::::::::::::::::::::::::::M:M::A:::::::N::::
Mouse         ::::::::::::::::::::::::::::::::::M:M::A:::::::N:::T

              51                                              100
Human         VYNYPEGAAYEFNAAAAA-----NAQVYGQTGLPYPPGSEAAAFGSNGLG
pER-1         ::::::::::D::------------------------------------::::::::::
pER-2         ----------------------------------------------------
S.scrifa      ::::::::::D::::::::::-----S:P::::S::A::::::::::A::::
Rat           :F::::::::::::::::AAAGA::::::::SIT::::::::::::S::
Mouse         :F::::::::::::::::AAAA-::::::::::I::::::::::S::S::

              101                                             150
Human         GFPPLNSVSPSPLMLLHPPPQLSPFLQPHGQQVPYYLENEPSGYTVREAG
pER-1         ::Q::::::::::V::::::::::::H::::::::::::::::::A:::::
pER-2         ----------------------------------------------------
S.scrifa      ::Q::::::::::V::::::::::::H::::::::::::::::::A:::::
Rat           A::Q::::::::::::::::HV::::::::H::::::::::A::::DT:
Mouse         A::Q::::::::::::::::::::::::::::::::::::::::::::::::

              151                                             200
Human         PPAFYRPNSDNRRQGGRERLASTNDKGSMAMESAKETRYCAVCNDYASGY
pER-1         ::::::::::::::::::::::S::::::::::::::::::::::::::::
pER-2         ------::::::::::::::::::S::::::::::::::::::::::::::
S.scrifa      ::::::::::::::::::::::S::::::::::::::::::::::::::::
Rat           ::::::S:::::::N:::::S:SSE::N:I::::::::::::::::::::
Mouse         ::::::S:::::::N:::::S:S:E::N:I::::::::::::::::::::
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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. Detained 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 varients 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 occuring 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.

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