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Multiple isoforms of porcine aromatase are encoded by three distinct genes ☆

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Abstract

Cytochrome P450 aromatase, a product of the CYP 19 gene and the terminal enzyme in the estrogen biosynthetic pathway, is synthesized by the ovary, endometrium, placenta, and peri-implantation embryos in the pig and other mammals, albeit to varying levels, implying its functional role(s) in pregnancy events. The aromatase produced by the pig tissues exists as three distinct isoforms (type I — ovary, type II — placenta, and type III — embryo), with presumed differences in substrate specificities, expression levels, activity, and mode of regulation. In order to delineate the molecular mechanisms whereby estrogen synthesis is regulated in these diverse tissues, the present study examined if these aromatase isoforms represent products of multiple genes or of a single gene via complex splicing mechanisms. Porcine genomic DNA from a single animal was used as a template in the polymerase chain reaction (PCR) to amplify isoform-specific sequences corresponding to exons 4 and 7, respectively. Nucleotide sequence analysis of the generated fragments revealed the presence of only clones corresponding to the three known aromatase types. Screening a porcine Bacterial Artificial Chromosome (BAC) library for aromatase gene by PCR yielded a single clone ~80 kb in length. Southern blot analysis, using probes specific for exons 1A–1B, 2–3, 4–9, and 10 sequences indicated that the BAC genomic clone contains the entirety of the coding exons as well as the proximal promoter region. Sequence analysis of the fragment generated with exon 4 primers determined that this BAC clone contains only the type II gene. The presence and relative orientation of the untranslated 5′- exons 1A and 1B, previously demonstrated for the type III isoform were evaluated in the BAC clone and genomic DNA by PCR. The 265 bp fragment generated from both PCR reactions was confirmed by sequence analysis to contain exons 1A and 1B that are located contiguous to each other and separated by only three bp. A diagnostic procedure for typing aromatase isoforms was developed, based on the presence of specific restriction sites within isoform-specific exons. The use of this protocol confirmed the existence of only three aromatase isoforms in the porcine genome and indicated changes in aromatase types expressed by the uterine endometrium as a function of pregnancy stage. The presence of distinct genes encoding each of the aromatase isoform predicts important differences in the mechanisms underlying the molecular evolution and regulation of porcine aromatase, unique from those of other mammals, and suggests a critical role for P450 aromatase steroidal products in uterine functions related to pregnancy events.

1. Introduction

Endocrine and paracrine signals mediated by estrogen are critical to the biological functions and/or proper development of multiple tissues ranging from

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the embryonic gonad to adult brain in diverse vertebrate species [1,2]. Androgens are catalyzed to estrogens by cytochrome P450 aromatase, a product of the CYP19 gene and the terminal enzyme in the estrogen biosynthetic pathway [3,4]. Human P450 aromatase, the best characterized aromatase to date and the paradigm for much of the current understanding of aromatase regulation and function, exists as a single gene [1]. Aromatase expression in humans has been reported in a myriad of tissues, with control of tissue, hormonal, and developmental specificity granted through the use of alternatively spliced 5′-untranslated exons [5]. Each of these 5′ untranslated exons possesses unique promoter sequences and is mutually exclusive. Most other species examined (rabbit, mouse, rat, horse, cow) also appear to use a single gene with alternatively spliced untranslated 5′ exons [6–11], albeit these lacked the more detailed characterization of tissue expression observed in the human. In several species (e.g., goldfish, pig), the nucleotide sequences of the cloned cDNAs encoding the protein from multiple tissues do not fit into the paradigm of a single gene with tissue-specific regulation. For these species, distinct differences in the coding regions are apparent, suggestive of the existence of multiple aromatase isoforms and corresponding genes [12–15].

The expression of aromatase in the ovary, placenta, blastocyst and pregnancy endometrium of the pig implies the diverse functional role of this enzyme in reproduction, similar to that demonstrated for other mammals [1,2,10,11]. However, unlike that in other species, the aromatase isoform may vary for each tissue, with type I predominantly expressed in the ovary and type II in the mid- and late-pregnancy endometrium/placenta. Additionally, peri-implantation porcine conceptuses synthesize high levels of estrogens [16], which is a consequence of the tissue's high level of expression of yet another type of aromatase, the type III isoform [9,13,17]. Interestingly, this isoform while exhibiting the typical catalytic activity of its type I and type II counterparts, also has been recently noted to catalyze the formation of 19-norandrogens [18]. The postulated functional significance of embryo-derived estrogenic molecules in the initiation and establishment of maternal-embryo communication [19,20], which occurs to a larger extent in the pig than in any other mammals, suggests that this novel activity may be related to unique aspects of peri-implantation embryo development in this species.

The presence of at least three types of aromatase isoforms with potentially distinct functions and mode of regulation of synthesis has raised the question of whether porcine aromatase is encoded from multiple genes or a single gene with a complex splicing mechanism [13–15]. In this study, we examined the hypothesis that multiple isoforms of porcine aromatase are encoded by multiple genes. Experiments were carried out to: (1) evaluate the existence of different isoform-specific exons in the porcine genome; (2) isolate a genomic clone for a specific aromatase isoform from a Bacterial Artificial Chromosome (BAC) library constructed from porcine genomic DNA; (3) determine the orientation of the known untranslated exons 1A and 1B identified for type III aromatase isoform in the porcine genome; and (4) develop a diagnostic system for rapid identification of P450 aromatase isoform type(s) expressed in porcine tissues. Results presented herein are consistent with the notion that multiple aromatase transcripts are the products of three distinct genes (paralogs) rather than a consequence of complex splicing mechanisms within the porcine genome, and that within the uterus, the endometrial expression of distinct aromatase genes is associated with the stage of pregnancy.

2. Materials and methods

2.1. Materials

All molecular-biology grade reagents were purchased from Fisher Scientific (Pittsburgh, PA). Restriction endonucleases were from Boehringer Mannheim (Indianapolis, IN), porcine genomic DNA from Clontech (Palo Alto, CA), and the nick-translation kit was from Amersham Corp (Arlington Heights, IL). [^{32}P] Deoxycytidine triphosphate (SA 3000 Ci/mmole) and BioTrans nylon membranes were purchased from ICN Radiochemicals (Irvine, CA). Trizol reagent was purchased from GIBCO-BRL (Gaithersburg, MD). Kits for Reverse-transcription (RT) and polymerase chain reaction (PCR) were obtained from Invitrogen (San Diego, CA). All primers for PCR were synthesized by Gemini Biotech (The Woodlands, TX). Porcine endometrial tissues were isolated from timed pregnant pigs, as described previously [21].

2.2. Genomic DNA PCR and analysis

The optimal conditions and primer pairs for detecting exon-specific aromatase isoforms in the porcine genome are presented in Table 1. The expected sizes of all generated PCR products were confirmed by electrophoresis on a 2.0% agarose gel. PCR fragments were purified (Wizard kit, Promega), and subcloned into TOPO™ PCR II vector (Invitrogen). Sequence analyses of both strands of subcloned fragments were carried out by the DNA Sequencing Core facility of the Interdisciplinary Center for Biotechnology Research, using Sp6 and T7 primers, respectively. DNA sequence data were compared to known aromatase sequences by using the BLAST feature on the

National Center for Biotechnology Information website (www.ncbi.nlm.nih.gov/BLAST/).

2.3. Restriction enzyme analysis

PCR products were digested with specific restriction enzymes under conditions determined to be optimal for each enzyme. Restriction digests were analyzed by gel electrophoresis on an agarose gel in 1X Tris–Borate–EDTA (TBE) buffer, using a 100 bp ladder as standards (BRL–GIBCO). Restriction digests of exon 4 PCR products were analyzed by gel electrophoresis in 4.0% Metaphor agarose (FMC Bioproducts, Rockland, Maine) in 1X TBE buffer, with a 20 bp ladder (FMC Bioproducts) as a molecular weight marker.

2.4. Isolation of porcine aromatase gene from a BAC library

The 2X genome coverage BAC library was constructed as previously described [22]. The library was screened for aromatase genes by PCR of pooled genes using a three-tiered strategy [23], with primers (forward: 5′-TATGAGAGCATG-AGGTACCA-3′; reverse: 5′-CTCGAGTCTATG-CATTCTTC-3′) designed from GenBank sequence No. L15471, representing porcine embryo aromatase [9]. PCR conditions were 1 min at 95°C, 2 min at 57°C, and 3 min at 72°C for 30 cycles. A single aromatase clone was isolated from the library and confirmed by PCR under the same conditions as described above.

2.5. Preparation of RNA and reverse transcriptase-polymerase chain reaction (RT-PCR)

Total cellular RNA was isolated from pig tissues by the use of TriZol (GIBCO–BRL), following the manufacturer instructions. The integrity of the RNA was assessed by inspection of 28 and 18 S band intensities after agarose gel electrophoresis. The RT reaction used 5 μg of total cellular RNA and followed protocols described by the manufacturer. Primers used for PCR amplification were as described in Table 1, and the generated PCR fragments were analyzed by restriction enzyme digestion as described above. To confirm the restriction enzyme patterns indicative of each aromatase isoform, representative PCR fragments from each isoform type were subcloned into TOPO™ vector, and resultant clones were subjected to nucleotide sequence analysis, as described above.

3. Results

3.1. Analysis of porcine genomic DNA for aromatase isoform genes

To establish the existence of different isoform-specific exons in the porcine genome, DNA prepared from an individual pig was used as template in PCR, using primer sets designed from sequences located within the previously characterized exon 4 of the porcine aromatase type III gene [9,13]. This region was chosen since the mRNA sequences bordering the 5′ and 3′ junctions of this exon are fairly conserved in all three known aromatase isoforms, whereas the internal sequences exhibit differ-

Table 1
Conditions and primers used in experiments

Location		Primer sequence 5′-3′	Size (bp)	Conditions
				°C Min
Exon 4	Sense	GTCCTCAAGTGTGTTCCATGTAATGAAGCA	144	95 2
	Antisense	TAAGTTCTAACAGCTTTCCAGAGGACTGGA		55 2
				72 3
				30 cycles
Exon 7	Sense	AGGATTTGAAAGAGAACATGG	110	95 2
	Antisense	AGCGGTGACTCAACTAAAACC		55 2
				72 3
				30 cycles
Exon 5	Sense	GCGCATGGTGACCGTCTGTG	649	94 45 s
Exon 9	Antisense	CATGACGAGGTCCACGACAG		62 1
				72 3
				35 cycles
Exon 1A	Sense	AGCAACTACTCGTGCGAAAGATC	2 min 20 s extention time + 20 s/cycle	
	Antisense	GATCTTTCGCACGAGTAGTTGCT		
Exon 1B	Sense	AGAGGGGCAGAGACTTGGTA	Program according to manufacturer′s intructions	
	Antisense	AAGCCAAATGTCCTGGGAGT		

ences which are unique to each aromatase type (Table 2). The PCR products generated from pig DNA were subcloned into TOPO™ vector, and the resultant plasmid DNA prepared from individual clones was analyzed for the presence of the expected insert size (144 bp) by digestion with *Eco*RI. Eight clones exhibiting the correct size insert were arbitrarily chosen for nucleotide sequence analysis. Comparison of the complete nucleotide sequences of the eight clones with those established for type I, II, or III isoforms indicated that four of the clones were of the type I, and four were of the type III, gene.

The lack of clones representative of the type II isoform led to the analysis of additional exon 4 clones generated by PCR using porcine genomic DNA as template. Since confirmation of isolated clones for aromatase type by nucleotide sequence analysis was not cost-effective, a diagnostic procedure was designed, which took advantage of the presence of unique restriction endonuclease sites within exon 4 that are predicted to be characteristic for type I, II, or III isoform genes. Using the Webcutter program (www.first-market.com/cutter/cut2.html), it was determined that the endonuclease *Bsm*I (GAATGC) would specifically digest exon 4 sequence from type I isoform gene once but not type III isoform, while *Nsi*I (ATGCA/T) would digest exon 4 sequence from type I twice and type III isoform once. Exon 4 from type II isoform would have no restriction sites for either enzyme (Fig.1, Table 2). The subsequent analysis of a total of 23 clones classified 8, 5, and 10 of these to be types I, II, and III aromatase isoforms, respectively. No restriction digestion pattern indicative of a novel sequence, distinct from those of the three known types, was observed. Nucleotide sequence determination of representative clones for types I, II, and III confirmed their classification (data not shown).

To further confirm the presence of distinct, albeit limited number of aromatase isoform sequences within the porcine genome, primer pairs were designed from within the 5′ and 3′ junctions of exon 7. Similar to exon 4, the boundaries of exon 7 exhibit a region of high homology for all isoforms, although the internal sequences exhibit differences that are unique to each isoform (data not shown). Nucleotide sequence analysis of 10 clones from the generated PCR fragments, upon sub-cloning into the plasmid vector TOPO™ classified six and four clones to be of the types II and III isoforms, respectively.

3.2. Analysis of BAC clone

The porcine genomic clone of ~80 kb in length, which was isolated from a BAC library, was analyzed by a series of Southern blot hybridizations to confirm its identity as a bona-fide aromatase gene. DNA was digested with a number of enzymes, singly and in combination, and hybridized on Bio-Trans filters to a ^{32}P-labeled probe (317 bp) corresponding to exons 2 and 3 of type III aromatase cDNA. Specific single and multiple hybridizing bands were detected (Fig. 2A). Two other probes, one corresponding to exons 4 to 9 of Type III aromatase cDNA sequence (1310 bp) (Fig. 2B) and the other, to that of exon 10 sequences (data not shown), also hybridized to genomic fragments distinct from those that hybridized with exons 2 to 3 sequences. Thus, the clone

Table 2
Exon 4 sequence comparison of porcine aromatase isoforms[a]

```
Type I   1  GTCTTCAAGTATTTTCCACATAATGAAGCACAATCATTAC
     II     GTCCTCAAGTGTGTTCCATGTAATGAAGCACAGTCACTAC
     III    GTCCTCAAGTGTGTTCCATGTAATGAAGCATAGTCACTAC

     I  41 ACGTGCCGATTTGGCAGCAAACTTGGGTTGGAATGGATTG
     II     ACATCCCGATTCGGCAGCAAACCTGGGTTGGAGTGCATCG
     III    ACATCCCGATTTGGCAGCAAACCTGGGTTGCAGTTCATTG

     I  81 GCATGCATGAAAAAGGCATCATGTTTAACAATAATCCAGC
     II     GCATGTATGAGAAGGGCATCATATTTAATAATGATCCAGC
     III    GCATGCATGAGAAAGGCATTATATTCAACAATAATCCAGT

     I 121 CCTCTGGAAAGCTGTTCGACCTTA
     II     CCTCTGGAAAGCCGTTAGAACTTA
     III    CCTCTGGAAAGCTGTTAGAACTTA
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[a] The first base of the sense primer is numbered as base one. Primers share 100% sequence identity with type III isoform and are represented by underlined sequences. Shaded bases are isoform-unique. The darkly shaded region represents the recognition site for endonuclease BsmI (GAATGC). Boxed regions represent the recognition site for endonuclease NsiI (ATGCA/T). Porcine aromatase isoform sequences were obtained from Genebank (U92246, U92245 and U37312).

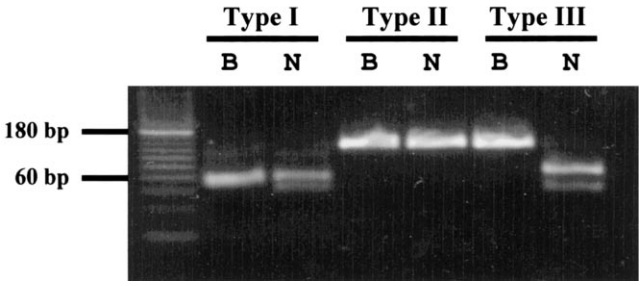

Fig. 1. Representative restriction digests of exon 4 isoforms. PCR fragments generated with exon 4 specific primers using porcine genomic DNA as template (Table 1) were digested with the restriction endonucleases *Bsm* I (B) or *Nsi* (N), and the digestion products were separated on a 4% Metaphor agarose gel. The ethidium bromide-stained gel shows that *Bsm* I (B) will digest type I isoform once, but not types II and III isoforms, and *Nsi* (N) will digest type I isoform twice, type III isoform once but not type II isoform. The migration positions of the 20 bp ladder used as standards are shown.

appears to contain all the coding exons (exons 2 to 10), including exon 4.

To evaluate whether single or multiple exon types are present within this genomic clone, the BAC clone was used as template for PCR using the primer sets designed for exon 4 (Table 1). The PCR fragment was subcloned, and the complete nucleotide sequence of four arbitrarily selected subclones was determined. All analyzed clones (four of four) were of the type II isoform, indicating that this BAC clone contains only the chromosomal gene for type II aromatase. Additionally, the restriction enzymes *Bsm*I and *Nsi*I did not digest the exon 4 PCR product from the BAC clone (data not shown), confirming its isoform type.

3.3. Organization of 5′-untranslated exons in the porcine genome and BAC clone

The untranslated 5′ exons 1A and 1B identified for type III aromatase cDNA are presumed to represent alternatively utilized exons within the porcine genome [9,13]; however, the arrangements of these exons and their distance relative to each other within gene(s) encoding aromatase are not known. To examine this, different combinations of sense and anti-sense primers specific for both exons (Table 1) were used in PCR reactions using Long-Run PCR kit (Boehringer Mannheim) and genomic or BAC clone DNA as templates. With both templates, the use of sense 1A with anti-sense 1B resulted in a PCR fragment of 256 bp, while the pair, sense 1B and anti-sense 1A, had no observable PCR product (Fig. 3A, shown only for genomic DNA). The presence of this region in the BAC clone was also confirmed by Southern blot analysis using 1A–1B fragment as probe (Fig. 3B). Specific genomic fragments obtained by digestion of the BAC clone DNA with specific restriction enzymes hybridized to the 1A–1B probe. To confirm the orientation of exon 1A located upstream of exon 1B in the aromatase gene, PCR reactions were carried out using sense 1A and anti-sense corresponding to a region of exon 4 on one hand, and sense 1B and the same anti-sense primer for exon 4 on the other (Table 1). A genomic fragment of approximately 15 kb was obtained with the former, while a slightly smaller fragment was observed for the latter (Fig. 3A). The 1A–1B PCR product was subcloned, and two distinct clones obtained from each template were subjected to nucleotide sequence analysis. The complete sequences of these clones were identical to each other and to the original corresponding cDNA sequence [9] and revealed that exons 1A and 1B are separated by only 3 bp (Fig. 3C).

3.4. Expression of aromatase isoforms in the porcine uterine endometrium of pregnancy

Our previous observations demonstrating a shift in uterine isoform type with pregnancy status [13] indicated that specific transitions in aromatase types may be correlated with uterine development essential to pregnancy events. To more clearly delineate the specific changes in aromatase isoform type with pregnancy, cDNAs prepared from RNAs isolated from uterine endometrium of early pregnancy (days 12–19), and from days 30 to 60, pregnancy endometrium were used as templates in PCR. A primer set corresponding

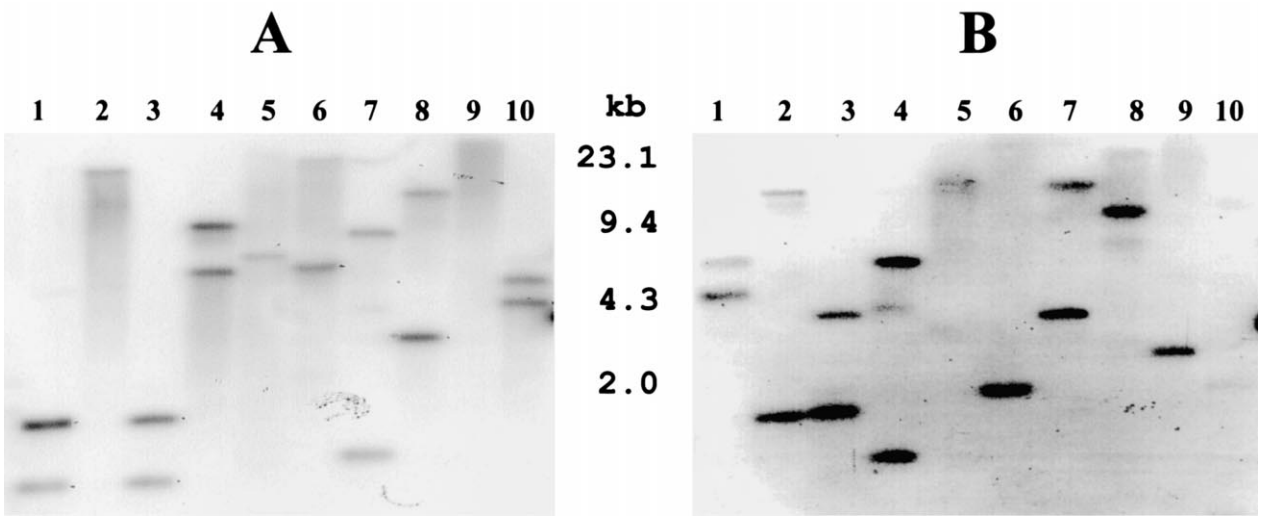

Fig. 2. Southern blot hybridization analysis of the porcine BAC clone. The BAC clone was digested with a number of restriction endonucleases, and the resulting digests were electrophoresed on an agarose gel and analysed by Southern blot using as probes, porcine cDNA sequences corresponding either to exons 2–3 (317 bp) (Panel A) or those corresponding to exons 4–9 (1310 bp) (Panel B). The molecular weight markers are shown in the middle of the two panels. Lanes represent digestions with the restriction enzymes: 1) *Eco*RI, 2) *Eco*RI + *Hin*dIII; 3) *Hin*dIII, 4) *Xba*I, 5) *Xho*I, 6) *Nco*I, 7) *Bgl*II, 8) *Sac*I, 9) *Sma*I, and 10) *Bam*HI.

to exon 5 (sense) and exon 9 (anti-sense) sequences and homologous to the three known isoform sequences (Table 1) was used, since intronic regions are located between these exons in the porcine aromatase gene [14]. This strategy eliminated the possibility that the PCR product may be due to genomic DNA contamination from the RNA extraction and generated a fragment of 649 bp. Endometrial expression of aromatase, determined by the presence of this PCR product, was very low (one of three animals) or undetectable (two of three animals) at day 12 of pregnancy, but was readily observed on pregnancy days 18–19, 30 and 60 (Fig. 4). Evaluation of the relative aromatase transcript levels across pregnancy, using the transcript for the constitutively expressed GAPDH gene for normalization (data not shown), indicated the specificity of endometrial aromatase expression at day 18 of early pregnancy. To determine which aromatase type(s) was synthesized, *Eco*RI was used for diagnostic analysis since type III isoform is uncut, while type II is digested into two fragments (427 and 222 bp) by this enzyme. RT-PCR products from day 18 to 19 pregnancy endometrium were uncut by *Eco*RI, indicative of type III aromatase. By contrast, RT-PCR products from days 30 and 60 pregnancy endometria, upon digestion with *Eco*RI, yielded the 649, 427, and 222 bp fragments, respectively, indicative of a mix of types II and III isoforms (Fig. 4B).

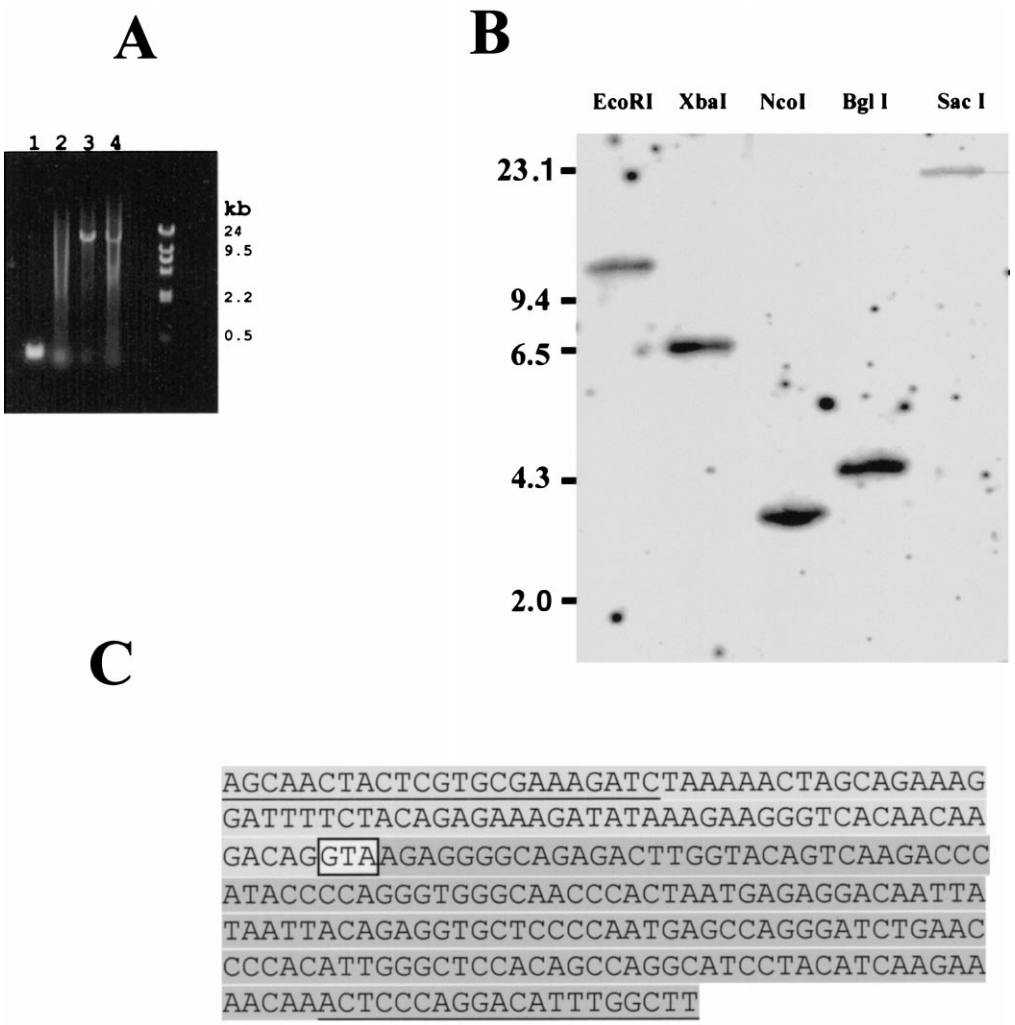

Fig. 3. PCR and sequence analyses of porcine genomic DNA and aromatase BAC clone DNA for 5′ untranslated exons 1A and 1B. Panel A. A representative ethidium bromide-stained agarose gel containing products generated from Long-Run PCR using different primer combinations (Table 1) is shown. Lanes represent PCR products generated using: 1) sense exon 1A and antisense exon 1B primers; 2) sense 1B and antisense 1A primers; 3) sense 1A and antisense exon 4 primers, and 4) sense 1B and antisense exon 4. Panel B. Southern blot hybridization analysis of the BAC clone encoding the porcine aromatase type II gene. The BAC clone DNA was digested with a number of restriction endonucleases, and the resulting digests were electrophoresed on an agarose gel and the gel processed for hybridization with [^{32}P-labeled] exon 1A–1B probe from porcine type III aromatase cDNA. Panel C. Nucleotide sequence of the 256 bp PCR product obtained from genomic DNA using sense 1A and antisense 1B primers. Primer sequences are underlined. The shaded areas have 100% sequence identity with untranslated exons 1A and 1B found in the type III gene [9].

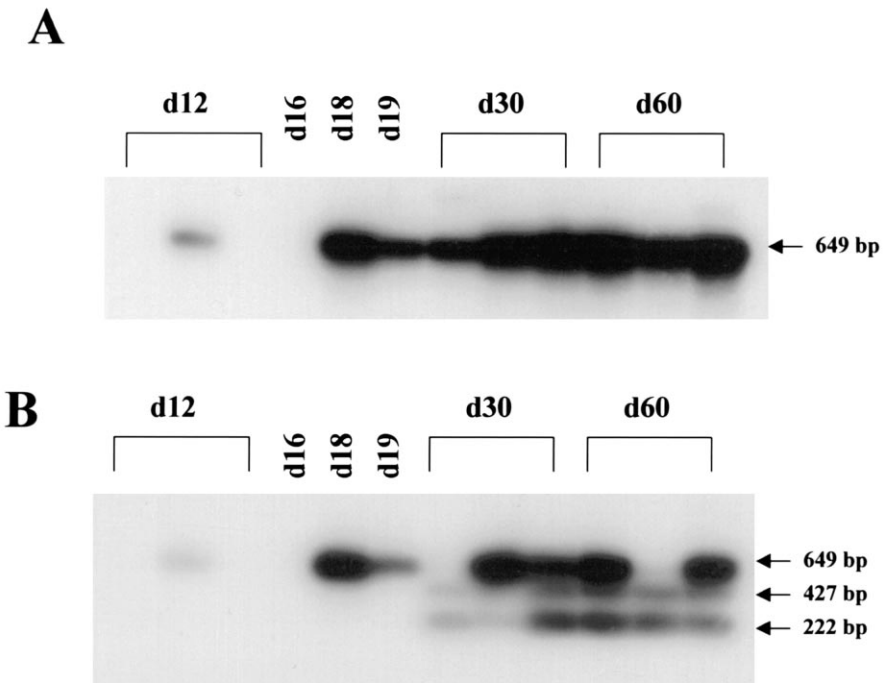

Fig. 4. RT-PCR analysis of porcine endometrial tissues for aromatase gene isoforms. Panel A. Equal aliquots (20 μl) of the RT-PCR reactions generated using primers specific for exons 5–9 of porcine aromatase were electrophoresed in a 1% agarose gel and subjected to Southern hybridization, using a [^{32}P-labeled] exon 5–9 (649 bp) fragment. Panel B. Equal aliquots (20 μl) of the RT-PCR fragments (from Panel A, above) were digested with *Eco*RI. Digested DNA were separated on an agarose gel, transferred onto a nylon membrane, and the membrane was hybridized with a nick-translated 649 bp aromatase probe, following the Southern blot protocol. The sizes of the hybridizing bands (in bp) are shown.

4. Discussion

Corbin et al. [24] first reported the presence of aromatase transcripts containing tissue-specific sequence differences in porcine ovary and placenta, and subsequently, Choi et al. [9] characterized an aromatase transcript distinct from those found in the ovary and placenta and unique to the porcine blastocyst. Several hypothesis were raised to account for the differences in the aromatase nucleotide sequences that give rise to the three distinct aromatase isoforms; these include distinct genes encoding these isoforms, post-transcriptional splicing, and/or post-transcriptional editing of a single gene [15]. In this report, we provide evidence to support the presence of three aromatase genes, each of which encode the aromatase isoform specific to the ovary, late-gestation placenta, and embryo, respectively. Additionally, we report the orientation within the pig genome of the 5′ untranslated exons IA and IB of the embryonic isoform (type III), document its presence and similar orientation within the BAC clone encoding the type II gene, and demonstrate that these exons are separated by only three nucleotides. Finally, using a diagnostic tool for the rapid identification of the three aromatase isoforms using specific primer sets and restriction enzymes, we confirmed previous data that suggested the presence of distinct aromatase isoforms in the porcine uterus as a function of pregnancy stage.

Several lines of evidence are presented in this study to indicate presence of distinct, albeit limited number of aromatase genes: (1) sequence analysis of different exon regions (exons 4 and 7) from porcine genomic DNA generated only those specific for types I, II, and III; (2) the BAC clone of ~80 kb encoded only the gene for type II aromatase isoform; and (3) the characterization of the sequence and orientations of 5′ 1A and 1B untranslated exons for the types II and III genes are consistent with large duplications not just of aromatase exons, but also of 5′ control regions for these genes. The presence of a unique gene for each aromatase isoform in the pig is unique and points to an interesting molecular evolutionary history that has not been reported in any other artiodactyls [5].

A series of primer sets for the study of porcine aromatase gene expression were evaluated in this report. The primer set for exon 4, when used on genomic DNA, appeared to favor the synthesis of types I (ovary) and III (embryo) isoforms. The primer set for exon 7, while designed primarily to the placental aromatase isoform (type II), also amplified type III-specific sequences. A third primer set spanning exons 5–9 was designed to sequences conserved for all aromatase types and spanned multiple exons. This strategy eliminated the possibility of genomic DNA

contamination yielding products in RT-PCR, and with the appropriate restriction enzymes, enabled the rapid typing for aromatase isoforms in uterine endometrium of different pregnancy days. The type(s) of aromatase expressed in the pig uterus was found to vary, depending on the pregnancy stage. Whereas day 12 pregnancy endometrium had very low or undetectable aromatase expression, those at days 18–19, 30, and 60 expressed sufficiently high levels of aromatase, albeit of different types. The relatively high levels of endometrial aromatase expression observed for days 18–19 (in contrast to day 12) of early pregnancy may account, in part, for the reported increase in uterine luminal concentrations of estrogen observed between days 16–25 of pregnancy [25], when very low or undetectable levels of conceptus aromatase mRNA were noted [26]. Since the aromatase isoform type synthesized by the pregnancy endometrium at this time is predominantly type III, the functional consequence(s) of these estrogens (and other steroid products) is likely to be similar to that synthesized by the embryo itself. The shift in expression of aromatase types (from type III to type II) around mid-pregnancy (days 30–60 of pregnancy) may be seen as an indication of the pivotal nature of this period in uterine differentiation correlated with important transitions in fetal–maternal interactions. The elucidation of the molecular mechanisms underlying changes in the expression of aromatase types represents an important direction for future studies.

The presence of multiple aromatase isoforms is not unique to the pig. Indeed, in the past few years several reports have demonstrated the presence of tissue-specific aromatase isoforms in fish and of an untranslated pseudogene in the cow. Callard and Tchoudakova [12] using Southern blotting of genomic DNA in the goldfish, showed that separate genes existed for ovarian and brain aromatase. The goldfish represents an interesting model because the putative duplication of aromatase in this species occurred much earlier than those in mammals [27]. Like in the pig, the expression of these aromatase isoforms are also tissue-specific [12]. Aromatase activity in goldfish brain tissues appears to be regulated differently from ovarian tissues, since brain tissues exhibited aromatase activity 100–1000-fold greater than that in the ovary. To date, no novel functions for the goldfish brain aromatase have been proposed, despite the fact that it exhibits only 64% homology with the ovarian form [12].

The bovine placenta transcribes, but does not translate, an aromatase pseudogene consisting of sequences highly homologous to those of exons 2–3, 5, and 8–9 of the functional bovine aromatase, but interspersed with a conserved bovine repeat element [28]. Brunner et al. [29] have speculated that this pseudogene represents a duplication of the proximal regulatory region and the coding area of the CYP19 gene. Using the technique of DNA fiber FISH, the functional and pseudo-genes were estimated to lie 24 kb apart and to span a locus of at least 130 kb. Although this pseudogene exhibits interesting features with significant implications for the generation of aromatase isoforms in the pig [27], it is highly unlikely that this pseudogene was derived from the same duplications yielding the present porcine aromatase isoforms.

In conclusion, this report describes the presence of multiple isoforms of porcine aromatase existing as three separate genes. Additionally, we have described a rapid diagnostic tool, using specific restriction enzymes, for characterizing the qualitative expression of porcine aromatase isoforms in different tissues. Given the accumulating evidence that these isoforms are functionally different [18,30], the existence and differential expression of multiple aromatase genes suggest that a further understanding of the molecular basis for the temporal and tissue-specific regulation of aromatase gene expression could provide important insights into the physiological processes influenced by their steroidal products.

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