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Molecular Cloning and Endometrial Expression of Porcine Amphiregulin

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ABSTRACT The porcine amphiregulin gene was previously reported to be within the quantitative trait locus (QTL) for uterine capacity on chromosome 8. Because amphiregulin stimulates cell proliferation, the amphiregulin gene might be responsible for this QTL. The objectives of this study were to clone amphiregulin cDNA and compare endometrial expression of its mRNA in pregnant Meishan (M) and White composite (WC) pigs. We obtained two amphiregulin cDNAs, one with 1,221 bp and another with 1,109 bp. The 112 bp difference corresponded to exon 5 of the human amphiregulin gene, which codes for the cytoplasmic domain. Endometrial mRNA expression of amphiregulin was significantly lower in M pigs than in WC pigs during early pregnancy (day 15–40 of gestation). Amphiregulin mRNA expression in the endometrium of both M and WC pigs increased ($P < 0.01$) from days 15 to 20, decreased ($P = 0.01$) from days 20 to 30, and did not change between days 30 and 40. This may result in reduced amphiregulin protein production leading to the slower development of M conceptuses, contributing to greater uterine capacity and litter size in prolific Chinese M pigs. Porcine genomic sequences isolated from a bacterial artificial chromosome genomic library contained exon 5, suggesting that the deletion of exon 5 in the mRNA may be due to differential splicing. The amphiregulin gene consisted of six exons and five introns spanning 10.3 kb. *Mol. Reprod. Dev. 65: 366–372, 2003.*

Key Words: uterine capacity; gene expression; differential splicing

INTRODUCTION

Uterine capacity contributes to litter size in swine (Christenson et al., 1987). A quantitative trait locus (QTL) for uterine capacity has been identified near 71 cM (95% confidence interval 53–107 cM) on chromosome 8 in a population of 1/2 Meishan (M), 1/2 White composite (WC) gilts (Rohrer et al., 1999). The beneficial allele originated from M gilts and substitution of a M allele for a WC allele was estimated to increase uterine capacity by approximately two pigs per litter (Rohrer et al., 1999).

Studies in M pigs have suggested that the greater fertility of this breed is associated with reduced conceptus growth rate. Embryos, placentas, and fetuses have been reported to be smaller in M pigs, and these differences have been suggested to be beneficial to uterine capacity by decreasing both the space taken up in the uterus and the nutrients required from the uterus to maintain each fetus (Christenson, 1993; Wilson et al., 1998; Vallet et al., 2002). Thus, genes influencing growth rate of the conceptus that lie within the QTL region of chromosome 8 may be candidate genes responsible for the QTL.

One such gene is amphiregulin. Amphiregulin is a glycoprotein that is a member of the epidermal growth factor (EGF) family. It binds to EGF receptor with lower affinity than EGF (Shoyab et al., 1989). The amphiregulin gene maps to within the uterine capacity QTL (Kim et al., 2002). It is also expressed by the porcine conceptus (Kennedy et al., 1993). Thus, chromosomal location, its expression by the conceptus, and its potential growth modulating activity make it a good candidate gene for the uterine capacity QTL. To further explore this possibility, the objectives of this study were to clone the complete coding region for porcine amphiregulin and compare endometrial amphiregulin mRNA expression in M and WC pigs.

MATERIALS AND METHODS

Cloning of a Partial Amphiregulin cDNA and Screening of an Expressed Sequence Tag Library

Both M and WC gilts were slaughtered on day 30 of gestation and the uterus was recovered. Endometrium was collected and snap frozen in liquid nitrogen. Total RNA was then isolated from the endometrial samples of

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WC gilts using the RNeasy kit (Qiagen, Santa Clarita, CA). To obtain a partial clone, 2 μg of total RNA was used for reverse-transcription (RT) with reverse primer 2 in a 20-μl reaction, and then 1 μl of the resultant product was amplified with all possible pairs of forward (F1-4) and reverse (R1-4) primers (Table 1). Polymerase chain reaction (PCR) was performed in a 10-μl reaction volume using 30 cycles of 95°C for 1 min, 58°C for 1 min, and 68°C for 1 min. Primer design was based on the known human cDNA sequence (Plowman et al., 1990). Amplification with F3 and R4 resulted in a 479-bp PCR product. This 479 bp product was cloned into pCRII vector (Invitrogen, Carlsbad, CA) and sequenced. The "Meat Animal Research Center 2 PIG" (MARC 2PIG) porcine expressed sequence tag (EST) library, which had been constructed using tissues from both M and WC pigs (Fahrenkrug et al., 2002), was then screened by PCR with primers based on the sequence of this clone. Because iterative screening of the EST library revealed a clone for amphiregulin without a region corresponding to exon 5 of the human amphiregulin gene, experiments were undertaken to investigate this further.

Cloning of Amphiregulin cDNA From M and WC Endometrium

To determine whether mRNAs from the endometrium of WC and M gilts contained the region corresponding to exon 5 of the human amphiregulin gene, RT-PCR was performed using total RNA from endometrium collected from day-30 pregnant WC and M gilts. In WC gilts, total RNA (2 μg) was used for RT with primer R2. The resultant product (1 μl) was amplified by PCR with primers F6 and R7, followed by the nested primers, F4 and R5. A minor 314 bp product and a major 202 bp product were obtained. Each was cloned into pCRII vector (Invitrogen), and three clones for each form were sequenced in both directions. In M gilts, primers for RT-PCR were chosen to generate smaller products than the primers used for RT-PCR in WC gilts while still encompassing the deleted region. This was done so that electrophoresis would separate the two products more definitively. In M gilts, total RNA (2 μg) was used for RT with primer R6. The resultant product (1 μl) was amplified by PCR using primers F6 and R6, followed by the nested primers, F7 and R5. A minor 291 bp product and a major 179 bp product were cloned into pCRII vector. Three clones each were sequenced in both directions. The same sets of primers used for RT-PCR in M gilts were then used to demonstrate whether both forms of amphiregulin mRNA are expressed in the endometrium from M and WC gilts (n = 4 each).

Confirmation of the Full Coding Region of Both Forms of Amphiregulin cDNA From M and WC Endometrium

To obtain the full coding region of the amphiregulin cDNA within each breed, RT-PCR was performed using total RNA from endometrium of M and WC gilts (n = 4 each). Total RNA (2 μg) was used for RT with primer R6 and the resultant product (1 μl) was amplified by four rounds of nested primers. The nested primers used were F5 and R6 (1st round), F8 and R8 (2nd round), F8 and R7 (3rd round), and F1 and R7 (4th round). Isolated bands were purified and cloned into pCRII vector. Both long-form (containing the full coding region) and short-form (lacking a region corresponding to exon 5) clones from M

TABLE 1. Primers Used in the Characterization of the Porcine Amphiregulin

Stage	Primer	Sequence
Initial	Forward	
	F1	CAGAGACCGAGACGCCGC
	F2	GCTGCTACCGCCGGC
	F3	GCTCAGGCCATTATGCTGCTG
	F4	GAGCACCTGGAAGCAGTAACATG
	Reverse	
	R1	GAAGTTTCTTTCGTTCCTCAGCTTCTC
	R2	ACCATCATAACAAAAAGGGTCCATTG
	R3	CATGTTACTGCTTCCAGGTGCTC
	R4	GACTTTTCCCCACACCGTTCAC
Both forms (WC)	Forward	
	F6	GCCCAAGAGAAACAGAACAGAAAG
	F7	CTGTAGAGGGCAAGAGCAAAGC
	Reverse	
	R5	AGTGACCCCGATCTGCTACCTAG
	R7	GGTTCTGTCTTCTTATGATCCACTGC
Both forms (M)	Reverse	
	R6	CTTTTTATCTCCGTGCATCTTTATGTACAG
Confirmation	Forward	
	F5	CCGGAGCCCAGGGAATC
	F8	CCTTCGAAAACGATACTTCAGGG
	Reverse	
	R8	AGCATTAAAAAAAGTGACAGTTGG
Real-time RT-PCR	Forward	GATCCTCTGCTCAGCCCATTATG
	Reverse	CCTCACTTCCCGAGGACATC
	Probe	TGCTGGACTGGACGTCAACGGCA

(four long- and two short-form clones from two gilts) and WC (seven long- and one short-form clones from three gilts) were sequenced in both directions.

Expression of Amphiregulin mRNA in the Endometrium of M and WC Pigs

Endometrium was collected on days 15, 20, 30, and 40 of pregnancy from M and WC pigs (gilts and sows) and total RNA was isolated from endometrium using cesium chloride gradient purification. To measure specific mRNA, real-time RT-PCR was performed using the one step RT-PCR kit (Applied Biosystems, Foster City, CA) and 100 ng of total RNA (n = 4 to 5 pigs per day) with an ABI Prism 7700 (Applied Biosystems) according to the manufacturer's protocol. To standardize the real-time RT-PCR procedure, 25, 50, 100, 200, and 400 ng of total RNA from endometrium collected on day 20 of pregnancy was assayed for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and amphiregulin mRNA within each assay. TaqMan fluorescent probes for GAPDH and amphiregulin labeled with 6-carboxy-4,7,2′,7′-tetrachloro-fluorescein (TET) and 6-carboxy-fluorescein (FAM), respectively, were used to detect the respective mRNAs. Standard curves were calculated for each mRNA arbitrarily setting the 25, 50, 100, 200, and 400 ng of total RNA as 1, 2, 4, 8, and 16 U, respectively, and then plotting the threshold cycle versus log of the arbitrary units as suggested by the manufacturer's protocol. Amphiregulin (Table 1) and GAPDH primers were designed based on the porcine amphiregulin clone cDNA sequence and porcine GAPDH (GenBank accession no. AF017079), respectively. GAPDH results were included as a covariate in subsequent analyses of amphiregulin mRNA to correct for differences in RNA sample dilutions. Thermal cycling conditions were 48°C for 30 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min.

Screening of Bacterial Artificial Chromosome Library and Sequencing

The RPCI-44 male porcine bacterial artificial chromosome (BAC) genomic library (BACPAC Resources, Buffalo, NY) was screened using a 1,000-bp cDNA probe. The probe was generated by PCR with primers F7 and R7 using the short form amphiregulin cDNA obtained from the EST library as template. Two positive BAC clones (197A1 and 199P1) were digested with EcoRI or PstI, blotted onto nylon membrane, and hybridized using the same cDNA probe for amphiregulin. After autoradiography, both BAC clones displayed the same positive bands. A 1.7 kb EcoRI fragment was subcloned into EcoRI digested pBluescript II SK vector and sequenced. PstI fragments of 5 and 4 kb were subcloned into PstI digested pBluescript II SK vector and sequenced. A positive 3.4 kb EcoRI/XbaI fragment was subcloned into pBluescript II SK vector digested with EcoRI/XbaI and sequenced. Primers were designed to amplify the remaining regions: (1) from a part of intron 1 to a part of exon 3, including exon 2 and intron 2, and (2) a part of intron 4. Amplified products were cloned into pCRII vector and sequenced.

Statistical Analysis

Expression of amphiregulin mRNA was analyzed by the General Linear Models procedure of the statistical analysis system (SAS Institute, Inc., Cary, NC) after the data were log transformed to decrease heterogeneity of variance between breed-day combinations. The log-transformed amphiregulin mRNA data were analyzed with and without correction using the units of GAPDH mRNA as a covariate. The model included effects of day, breed, assay, and the day-by-breed interaction.

RESULTS

Identification of a Porcine Amphiregulin Clone

A clone containing the full coding region for porcine amphiregulin was obtained from the MARC 2PIG EST library, but it lacked a region corresponding to exon 5 of the human amphiregulin gene. Further cloning by RT-PCR and sequencing of the clones revealed that two forms of porcine amphiregulin mRNAs, one with and one without the 112-bp region corresponding to exon 5 of the human amphiregulin gene, were present in the endometrium of M and WC gilts on day 30 of pregnancy. Consistent with this, a 1,221 bp long form and an 1,109 bp short form (GenBank accession no. AY028310 and AY028311, respectively) of porcine amphiregulin cDNA were obtained. Nucleotide sequences and predicted amino acid sequences for both forms of the putative porcine amphiregulin are shown in Figure 1. The short form of amphiregulin mRNA lacking the region corresponding to exon 5 of the human amphiregulin gene appeared to be the predominant form (Fig. 2). There were no consistent differences in the amphiregulin cDNA sequences derived from M (n = 2 gilts) and WC (n = 3 gilts) in either the long or short forms. The long form of porcine amphiregulin cDNA has an open reading frame of 741 bp that encodes 247 amino acids compared to 753 bp and 251 amino acids for the human amphiregulin cDNA, and 746 bp and 249 amino acids for the mouse amphiregulin cDNA. The 5′ and 3′ untranslated regions (UTR) of the porcine cDNAs were 200 and 291 bp, respectively. The sequence identity of the 5′ UTR of porcine amphiregulin cDNA with those of mouse, rat, and human ranged from 52 to 65%. The sequence identity of the 3′ UTR of porcine amphiregulin cDNA with the 3′ UTR of the other three species ranged from 69 to 89%. Multiple sequence alignment showed no consensus regions in the 5′ UTR. However, there were consensus sequences in the 3′ UTR surrounding ATTTA sequences, a motif previously reported to destabilize mRNA (Shaw and Kamen, 1986). Four ATTTA repeat sequences are located in the 3′ UTR of pig, human, mouse, and rat sequences. There was a polyadenylation signal of ATTAAA 12 bp upstream from the polyA tail, which was also conserved. There were three potential N-glycosylation sites (Fig. 1), and these are conserved in

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1     CCTTGTGCGGGAGCCGGAGCCCAGGGAATCCCGAGGCGAGCTCCAGGCGCTGCCTCCAGCGCCCGAGCCCGGCCGGGCCTCTGAGCACAGCTGCTCGGCC     100

101   CCCCTGGTCCCCGGTCACCTCCTCCCGCCCGCCCGCAGCTCCCGGCCGAGTTGCCCCAGAGACCGCGACGCCACAGCTCCGCGGGACCAATGAGAGCTCC     200
1                                                                                                 M  R  A  P       4

201   GCTGCTGCCGCCGGCGCCCGTGGTGCTGTCACTCTTGATCCTCTGCTCAGCCCATTATGCTGCTGGACTGGACGTCAACGGCACCTCCTCTGGGAAAGGA     300
5      L  L  P  P  A  P  V  V  L  S  L  L  I  L  C  S  A  H  Y  A  A  G  L  D  V  N  G  T  S  S  G  K  G       37
                                                                                          *

301   GAACCATTTTCTGGGGACCATGGTGCTGAGGCATTTGAGGTGACCTCCAGAAGTGAGATGTCCTCGGGAAGTGAGGCTCCCCCTGCTAGCGAAATGCCTT     400
38    E  P  F  S  G  D  H  G  A  E  A  F  E  V  T  S  R  S  E  M  S  S  G  S  E  A  P  P  A  S  E  M  P  S      71

401   CTGGTAGCGACTATGACTATGCTGAAGAGTACGATAACGAACCGCACATATCTGGCTATATTGTAGATGATTCAGTCAGAGTTGAACAGGTAGTTAAGCC     500
72      G  S  D  Y  D  Y  A  E  E  Y  D  N  E  P  H  I  S  G  Y  I  V  D  D  S  V  R  V  E  Q  V  V  K  P     104

501   CAAGAGAAACAGAACAGAAAGTGAAAATACTTCAGATAAACCCAAAAGAAAGAAAAAGGGAGGCAAAAGTGGAAAAAATAGAAGAAACAGAAAGAAGAAA     600
105    K  R  N  R  T  E  S  E  N  T  S  D  K  P  K  R  K  K  K  G  G  K  S  G  K  N  R  R  N  R  K  K  K     137
          *              *

601   AATCCATGTGATGCAGAATTCCAAAACTTCTGCATTCACGGAGATTGCAAATATATAGAGCACCTGGAAGCAGTAACCTGCAAATGTTACCAGGATTACT     700
138   N  P  C  D  A  E  F  Q  N  F  C  I  H  G  D  C  K  Y  I  E  H  L  E  A  V  T  C  K  C  Y  Q  D  Y  F     171

701   TTGGTGAACGATGTGGGGAAAAGTCCATGAAGACTCACACCATGGTCCACAGCGATTTATCAAAAATTGCTTTAGCAGCCATTGCTGCTTTTGTCTCTGC     800
172     G  E  R  C  G  E  K  S  M  K  T  H  T  M  V  H  S  D  L  S  K  I  A  L  A  A  I  A  A  F  V  S  A     204

L801  CATGAGCTTCACAGCTATTGCTGTTGTTATTACAATCTACCTTCGAAAACGATACTTCAGGGAATATGAAGGTGCAGCTGAAGAACGAAAGAAaCTTCGA     900
205    M  S  F  T  A  I  A  V  V  I  T  I  Y  L  R  K  R  Y  F  R  E  Y  E  G  A  A  E  E  R  K  K  L  R     237
S801  CATGAGCTTCACAGCTATTGCTGTTGTTATTACAATCTAG   840----------------------------------------------------------
205    M  S  F  T  A  I  A  V  V  I  T  I  Stop  216

L901  CAAGAAAATGCAAATGCACATGCCATAGCATGACTGAAGATAATATTACAGGGTAGCAGATCGGGGTCACTGCCAAGTCATAACCATGACTGATGAGTTG    1000
238   Q  E  N  A  N  A  H  A  I  A  Stop  247
      ------------------------------------------------841  GTAGCAGATCGGGGTCACTGCCAAGTCATAACCATGACTGATGAGTTG     888

L1001 GTTCCTCTTTGCAGTGGATCATAAGAAGACAGAACCTTTTTGTTCTGGTGGTTTTAAACTTCCAACTGTCACTTTTTTTAATGCTAAGTCTTATTTCTGT    1100
S889  GTTCCTCTTTGCAGTGGATCATAAGAAGACAGAACCTTTTTGTTCTGGTGGTTTTAAACTTCCAACTGTCACTTTTTTTAATGCTAAGTCTTATTTCTGT     988
                        ↓                                                   ↓
L1101 ACATAAAGATGCACGGAGATAAAAAGTATTTTTTCAAGTTGTAAATAATTTATTTAATATTTTAATGGAAGTGTATTTATTTTACAGTTCATTAAACTTTT    1200
S989  ACATAAAGATGCACGGAGATAAAAAGTATTTTTTCAAGTTGTAAATAATTTATTTAATATTTTAATGGAAGTGTATTTATTTTACAGTTCATTAAACTTTT    1088

L1201 TTAATCAAAAAAAAAAAAAAA   1221
S1089 TTAATCAAAAAAAAAAAAAAA   1109
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Fig. 1. The nucleotide sequences and predicted amino acid sequences for the putative porcine amphiregulin precursors are shown. The cDNA sequences of the short (S) and long (L) forms of the porcine amphiregulin differ in a 112 bp region (indicated by -----). A 47 bp AT-rich region (44 AT and 3 GC) is indicated by two arrows (↓). Four ATTTA repeat sequences in the 3′ UTR are underlined. A consensus polyadenylation signal of ATTAAA is shown in bold, italicized letters. Three possible Asn-linked glycosylation sites are indicated with *.

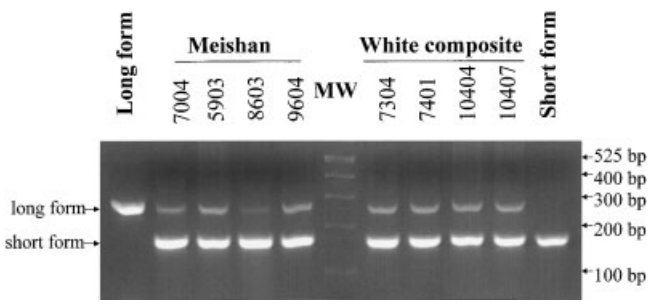

Fig. 2. Two different forms of amphiregulin mRNAs were present in both Meishan (M) and White composite (WC) pig endometrium on day 30 of pregnancy (n = 4 each) as shown by reverse transcription-polymerase chain reaction (PCR). The short form appeared to be the predominant form in both breeds. Arrows (→) on the left indicate the bands resulting from amplification of the long and short forms of amphiregulin cDNAs. Molecular weight (MW) markers are shown in the middle. Arrows (←) on the right indicate MW markers of 525 (appearing close together with 500-bp), 400, 300, 200, and 100-bp.

humans (Plowman et al., 1990); however, only the first two N-glycosylation sites are conserved in rodents (Kimura et al., 1990; Das et al., 1995) and in sheep (Forsyth et al., 1997). A 47-bp AT-rich region (44 AT and 3 GC) is present in the 3′ UTR of the porcine amphiregulin cDNA.

Percent identities between the amino acid sequences of pig and human (Plowman et al., 1990), mouse (Das et al., 1995), or rat (Kimura et al., 1990) amphiregulin precursors are 83, 71, and 67%, respectively. The encoded amino acid sequence of porcine amphiregulin cDNA is aligned with those of human, mouse, and rat amphiregulin precursors in Figure 3. Five structural domains are predicted in the porcine amphiregulin precursor based on the sequence of the human amphiregulin precursor (Plowman et al., 1990): a signal peptide (aa 1–19); an amino terminal pro-region (aa 20–94); mature amphiregulin (aa 95–178, EGF-like domain 138-178); a transmembrane domain (aa 193–216); and a cytoplasmic domain (aa 217–247). Cysteine residues are conserved in the EGF-like domains of porcine

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        1                                                  51                                                 100
        |→ signal peptide ←|→                                amino terminal domain                              ←|
pshort  MRAPLLPPAP VVLSLLILCS AHYAAGLDVN GTSSGKGEPF SGDHGAEAFE VTSRSEMSSG SEAPPASEMP SGS......D YDYAEEYDNE PHISGYIVDD
plong   MRAPLLPPAP VVLSLLILCS AHYAAGLDVN GTSSGKGEPF SGDHGAEAFE VTSRSEMSSG SEAPPASEMP SGS......D YDYAEEYDNE PHISGYIVDD
human   -------------------G- G-------L- D-Y---R--- ----S-DG-- ---------- --IS-V---- -S-EPSSGA- ---S------ -Q-P------
mouse   --T----L-R S--L--V-G- G----A-EL- DP------SL ----S-GGL- LSVGR-V-TI --M-SG--LS T-.......- ---S------ -Q--------
rat     --T-S-SL-L S-----V-G- G------EL- ---------S ----S-GGLV -S...-V-TI --M-SG--LS T-.......- ---S------ -Q--------

        101                                                151                                                200
        |→                      amphiregulin  mature  peptide                                        ←|          |→
pshort  SVRVEQVVKP KRNRTESENT SDKPKRKKKG GKSGKNRRN. RKKKNPCDAE FQNFCIHGDC KYIEHLEAVT CKCYQDYFGE RCGEKSMKTH TMVHSDLSKI
plong   SVRVEQVVKP KRNRTESENT SDKPKRKKKG GKSGKNRRN. RKKKNPCDAE FQNFCIHGDC KYIEHLEAVT CKCYQDYFGE RCGEKSMKTH TMVHSDLSKI
human   ---------- PQ-K------ ---------- --N------. -------N-- --------E- ---------- ---Q-E---- ---------- S-ID-S----
mouse   -------I-- -K-K--G-KS TE-------- --N--G---. K------T-K --------E- R---N--V-- -N-H------ ---------- SEDDK-----
rat     -------I-- -E-K--G-KS -E-------- --G--G---R K------A-K --------E- R---N--V-- -H-H------ -----T---Q KKDD------

        201                                                256
           transmembrane domain  ←|→      cytoplasmic domain      ←|
pshort  ALAAIAAFVS AMSFTA..IA VVITI
plong   ALAAIAAFVS AMSFTA..IA VVITIYLRKR YFREYEGAAE ERKKLRQENA NAHAIA
human   --------M- -VIL--..V- .---VQ--RQ -V-K---E-- ---------G -V----
mouse   -VV-VTI--- -IILA-IG-G I---VH-W-- -------ET- --RR-----G TV----
rat     -----IV--- -V-VA-..-G IITAVL---- F------.E-- --RR-----G T-----
```

Fig. 3. A comparison of the predicted amino acid sequences for putative porcine amphiregulin precursor with human, mouse, and rat amphiregulin sequences is shown. Bold letters represent the coding region corresponding to mature amphiregulin. The beginning (| →) and the end (← |) of each domain are indicated.

amphiregulin, EGF (Kim et al., 2001), and TGF-α (Vaughan et al., 1993) (Table 2). The region comprised of predominantly basic residues, thought to be involved in heparin-binding ability (Thorne and Plowman, 1994), is also well conserved. The porcine amino terminal domain contained 75 residues compared to 81 residues in the human and 74 residues in the mouse. Both porcine and rat transmembrane domains contained 24 residues compared to 23 residues in the human and 26 residues in the mouse.

Expression of Amphiregulin mRNA in the Endometrium of M and WC Pigs

Analysis of real-time RT-PCR data indicated that there were significant day ($P < 0.01$) and breed ($P < 0.01$) effects with no day-by-breed interaction. Amphiregulin mRNA expression in the endometrium of both M and WC pigs increased ($P < 0.01$) from days 15 to 20, decreased ($P = 0.01$) from days 20 to 30, and did not change between days 30 and 40 (Fig. 4). Amphiregulin mRNA expression was lower in M pigs than WC pigs throughout the days measured.

There was no difference between the M (mean 2.40 ± 0.25 arbitrary units) and WC (2.39 ± 0.25) pigs in overall GAPDH mRNA expression as determined by real-time RT-PCR. However, GAPDH mRNA expression did change across the days of gestation studied. It increased ($P = 0.02$) from day 15 (1.98 ± 0.33) to day 20 (3.19 ± 0.33), did not change on day 30 (2.68 ± 0.37), and tended to decrease ($P = 0.08$) on day 40 (1.73 ± 0.33). There was no day-by-breed interaction. Therefore, it is statistically valid to adjust amphiregulin mRNA expression across day of gestation using GAPDH as a covariate.

When amphiregulin mRNA expression was statistically adjusted using GAPDH mRNA expression as a covariate, the changes in amphiregulin mRNA expression became smaller, but were still significant. The adjusted values of amphiregulin mRNA expression on days 15, 20, 30, and 40 were 0.31, 2.61, 1.36, and 0.95 for M pigs and 1.34, 6.93, 1.49, and 2.31 for WC pigs, respectively. These adjusted amphiregulin mRNA expression values still depicted significant day (of gestation) and breed effects (Fig. 4).

Sequencing of BAC Subclones

The porcine amphiregulin gene sequence was derived from the positive subclones after restriction enzyme digestion of a clone containing the amphiregulin gene from the porcine BAC library, and the sequence obtained was compared with the human amphiregulin gene sequence. The exons of the amphiregulin gene in both species were homologous. An EcoRI digested 1.7 kb fragment contained a part of exon 3, intron 3, exon 4, and a part of intron 4 (Fig. 5). The 5 kb PstI fragment contained the region 5′ of the amphiregulin gene, exon 1, and a part of intron 1. The PstI digested 4 kb fragment

TABLE 2. Cysteine Residues Conserved in the EGF-Like Domains of Porcine EGF Family Growth Factors Are Shown in Bold Letters; Other Conserved Amino Acids Are Underlined

Amphiregulin	**C**DAEFQNF**C**I<u>HG</u>D-**C**KYIEHLEAVT**C**K**C**YQD<u>Y</u>F<u>G</u>E<u>R</u>**C**GEK
EGF	**C**PPSHDGY**C**L<u>HG</u>G V**C**MYIEAVDSYA**C** N**C**VFG<u>Y</u>V<u>G</u>E<u>R</u>**C**QHR
TGF-α	**C**PDSHSQF**C**F<u>HG</u>T-**C**R F LVQEDKPA**C**V**C**HSG<u>Y</u>V<u>G</u>A<u>R</u>**C**EHA

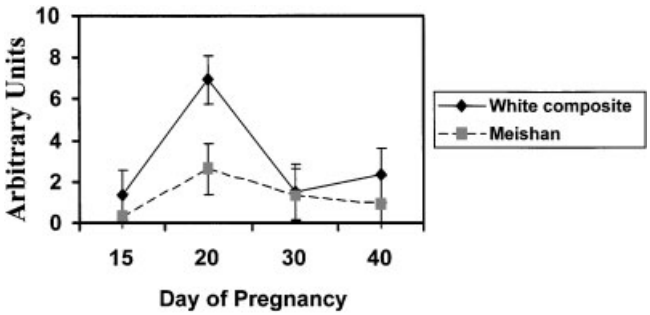

Fig. 4. Least-square means (± standard error means) of arbitrary units, adjusted using GAPDH, from real-time RT-PCR for amphiregulin mRNA in endometrium of M and WC pigs during early pregnancy are illustrated. WC pigs had higher ($P < 0.01$) amphiregulin mRNA expression than M pigs. Amphiregulin mRNA expression increased significantly ($P < 0.01$) from day 15 to 20 of pregnancy, decreased from days 20 to 30 ($P = 0.01$), but did not change between days 30 and 40.

contained a part of intron 4, exon 5, intron 5, exon 6, and the 3′ flanking region (Fig. 5). A positive 3.4 kb EcoRI/XbaI fragment contained a part of intron 1, exon 2, intron 2, and a part of exon 3. Alignment of these sequences, along with the sequences obtained from PCR amplification and cloning, revealed the gene structure consisted of six exons and five introns and exon–intron organizations (Fig. 5). In the long form, exon 4 is spliced to exon 5. However, in the short form, exon 4 is spliced to exon 6, causing a frame shift. The resulting stop codon TAG predicts that the resulting protein lacks the cytoplasmic domain (Table 3).

Amplification of the variant region (a part of intron 4, exon 5, intron 5, and a part of exon 6) by PCR using genomic DNA of M × WC crossbred pigs (n = 12) as template followed by Southern blotting resulted in a single band matching the size of the full genomic sequence. This result confirms that no gene duplication or deletion of exons at the genome level is present in the pigs examined (data not shown).

DISCUSSION

This is the first report of the full coding region for porcine amphiregulin cDNA. In addition, two forms of mRNA were observed in pig endometrium. The short form of the amphiregulin mRNA would result in an amphiregulin precursor protein that lacks the cytoplasmic domain due to differential splicing of exon 5. Finally, the expression of amphiregulin mRNA in the endometrium was elevated ($P < 0.01$) on day 20 of pregnancy compared to day 15 and was lower ($P < 0.01$) in M pigs compared to WC pigs on all days examined.

Several molecular weight forms of amphiregulin, which differ in glycosylation and in N-terminal peptide core length, have been reported in humans (Johnson et al., 1993). However, the presence of a naturally occurring amphiregulin that is missing the cytoplasmic domain has not been reported. The directed deletion of the cytoplasmic domain in human amphiregulin did not affect the secretion of the bioactive material (Thorne and Plowman, 1994); however, the cytoplasmic domain is required for basolateral sorting in polarized Madin–Darby canine kidney cells (Brown et al., 2001). Whether the lack of a cytoplasmic domain in the short form of porcine amphiregulin affects its cleavage from the membrane or basolateral sorting remains to be determined.

The porcine amphiregulin gene was mapped (Kim et al., 2002) within the previously identified uterine capacity QTL (Rohrer et al., 1999). In the crossbred M × WC population used to identify the QTL, the allele that increases uterine capacity originates from the M breed. However, no differences in the amphiregulin cDNA sequences, which included the protein coding regions, were obtained from M (n = 2) and WC (n = 3)

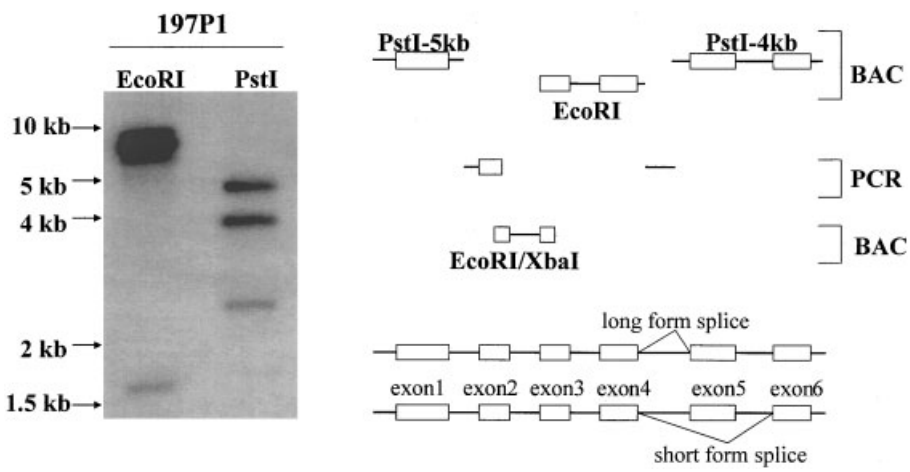

Fig. 5. Identification of the porcine amphiregulin gene organization. A BAC clone containing the amphiregulin gene was digested with EcoRI or PstI, and Southern blotting was performed (**left panel**). The positive fragments were subcloned and sequenced. The remaining areas were PCR amplified, subcloned, and sequenced. Alignment of sequences revealed the organization of the amphiregulin gene (**right panel**): exons are indicated in rectangles, while introns, and 5′ and 3′ flanking sequences are indicated in lines. The 5-kb fragment digested with PstI contained 5′ flanking sequence and exon 1. The 4-kb fragment digested with PstI contained the exons 5 and 6. The 1.7-kb fragment digested with EcoRI contained a part of exon 3 and exon 4. The 3.4-kb EcoRI/XbaI fragment contained a part of intron 1, exon 2, intron 2, and a part of exon 3. Differential splicing of exon 5 for the long and short forms of amphiregulin mRNA is indicated.

TABLE 3. Exon–Intron Splicing in the Long and Short Form of the Porcine Amphiregulin Gene

Exon number	5′ splice donor	3′ splice acceptor	Amino acid interrupted
Long form			
4	ACA ATC TA gtaagtagg..	..ctttgaag C CTT C	Ty r 216
5	CAGG tttgagtttt.....	..atttttcaacag GGTA	none—3′ UTR
Short form			
4	ACA ATC TA gtaagtagg..	..atttttcaacag GGTA	Sto p

gilts. In contrast, amphiregulin mRNA expression was significantly greater ($P < 0.01$) in WC pigs compared to M pigs. One could hypothesize that less amphiregulin mRNA expression in the endometrium of M pigs might result in the production of less amphiregulin protein, which could play a role in the slower development of M conceptuses during early pregnancy (Ford and Youngs, 1993). This could lead to increased uterine capacity and litter size. Thus, differences in endometrial amphiregulin expression between M and WC pigs are consistent with, but do not prove, that amphiregulin may be responsible for the previously reported QTL. Further experiments to compare gene sequences controlling amphiregulin expression in M and WC gilts are needed to fully evaluate this possibility.

CONCLUSIONS

In conclusion, the full coding region for porcine amphiregulin precursor cDNA from endometrium is reported. While we were unable to detect mutations in the cDNA sequences that may be responsible for the uterine capacity QTL in either M or WC pigs, the breed difference in amphiregulin mRNA expression is consistent with the hypothesis that amphiregulin is a candidate for the previously reported QTL.

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