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J. G. Kim
USDA-ARS

J. L. Vallet
USDA-ARS

R. K. Christenson
USDA-ARS, christenson@email.marc.usda.gov

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Kim, J. G.; Vallet, J. L.; and Christenson, R. K., "Characterization of Uterine Epidermal Growth Factor During Early Pregnancy in Pigs" (2001). *Roman L. Hruska U.S. Meat Animal Research Center.* Paper 239.
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ELSEVIER

Domestic Animal Endocrinology 20 (2001) 253–265

DOMESTIC
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ENDOCRINOLOGY

Characterization of uterine epidermal growth factor during early pregnancy in pigs☆

J.G. Kim, J.L. Vallet, R.K. Christenson*

USDA-ARS, Roman L. Hruska U.S. Meat Animal Research Center, P. O. Box 166, Clay Center, NE 68933-0166, USA

Received 30 November 2000; accepted 6 February 2001

Abstract

Genomic research has identified a quantitative trait locus for uterine capacity, a component trait contributing to litter size, on porcine chromosome 8. The epidermal growth factor (EGF) gene, on porcine chromosome 8, may influence uterine capacity because of its growth-promoting activities. Using reverse transcription-polymerase chain reaction (PCR) and iterative screening of a porcine reproductive tissue cDNA library, 4932 bp cDNA sequence coding for porcine EGF precursor was obtained. The predicted protein sequence of the EGF precursor contained 1214 amino acids, similar to human EGF precursor (1207 amino acids, 81% identity). Curiously, the sequence of the mature peptide was less homologous between species than other regions of EGF precursor. The presence of conserved regions outside the mature peptide may suggest that these regions are functionally important. Expression of EGF mRNA in the endometrium of White crossbred gilts ($n = 3$ to 5 each) was determined by Northern blotting using 20 μg of total RNA from endometrium of D 10, 13, and 15 cyclic, and D 10, 13, 15, 20, 30, and 40 of pregnant gilts. A 3342 bp probe from EGF precursor was used. The bands corresponding to EGF mRNA were quantified by densitometry and results were analyzed by ANOVA. EGF mRNA expression decreased significantly from D 13 to 15 of the cycle and pregnancy ($P = 0.04$), and from D 30 to 40 of pregnancy ($P = 0.01$). These findings show that EGF mRNA expression is temporally regulated during the cycle and early pregnancy, and this pattern of gene expression may be important during early conceptus development. © 2001 Elsevier Science Inc. All rights reserved.

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* Corresponding author. Tel.: +1-402-762-4192; fax: +1-402-762-4382.
E-mail address: christenson@email.marc.usda.gov (R.K. Christenson).

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PII: S0739-7240(01)00097-2

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1. Introduction

Epidermal growth factor (EGF) is a 53-amino acid polypeptide that has been reported to stimulate epithelial cell growth. EGF and EGF receptor mRNA expression have been shown in the porcine conceptus by reverse transcription-PCR (RT-PCR) [1]. In the endometrium, EGF mRNA expression has been shown by RT-PCR to be higher on D 6 than D 1 of cyclic and pregnant pigs [2]. The presence of EGF protein in uterine fluid [3] and of EGF receptors in endometrium [4] has also been reported. These data suggest a role for EGF in the development of porcine conceptus and endometrium.

Uterine capacity is a component trait contributing to litter size in swine [5], and direct selection for uterine capacity has significantly increased uterine capacity and associated farrowing traits in swine [6,7]. A quantitative trait locus (QTL) for uterine capacity was identified on the long arm of chromosome 8 near 71 cM [8]. This region is near the known location of the gene for EGF precursor [9]. Thus, because of its location and its potential effect on conceptus or endometrial growth, EGF gene is a candidate for the previously reported uterine capacity QTL on chromosome 8.

Partial genomic and cDNA sequences of porcine EGF precursor [10,11], including the coding region of the mature protein, have been reported. However, the entire coding region of the porcine EGF precursor has not been cloned. Furthermore, relative expression of EGF mRNA has not been well characterized during trophoblast elongation, implantation, and early development of the placenta (D 10 to D 40). As a first step in determining whether EGF is the gene responsible for the uterine capacity QTL, the objectives of this study were to clone the full length cDNA for EGF precursor and determine changes in expression in EGF mRNA during early pregnancy.

2. Materials and methods

2.1. Cloning of the porcine EGF precursor cDNA

The cloning strategy and clones of pig EGF precursor obtained in this experiment are illustrated schematically in Fig. 1. From uterine endometrium of D 13 and D 30 pregnant gilts, total RNA was isolated using the RNeasy kit (Qiagen, Santa Clarita, CA). The list of forward and reverse primers at different cloning stages is indicated in Table 1. To obtain the initial clone, 2 μg of total RNA was used for reverse-transcription (RT) with reverse primer 2, and then 1 μl of the resultant product was amplified by PCR with all combinations of the forward and reverse primers for the initial clone. Primer design was based on the known porcine cDNA sequence [11]. A 327 bp PCR product amplified with forward primer 2 and reverse primer 2 was cloned into pCRII vector (Invitrogen, Carlsbad, CA) as the initial clone and was subsequently sequenced. Forward primer 2 and reverse primer 2, based on the initial clone sequence, were used to screen the “Meat Animal Research Center 2 PIG” porcine expressed sequence tag (EST) library (Fahrenkrug et al., manuscript in preparation). Iterative screening of the EST library revealed a partial 3342 bp EGF precursor clone containing the 3′ end of the EGF cDNA. A part of the missing 5′ region of the EGF cDNA was obtained

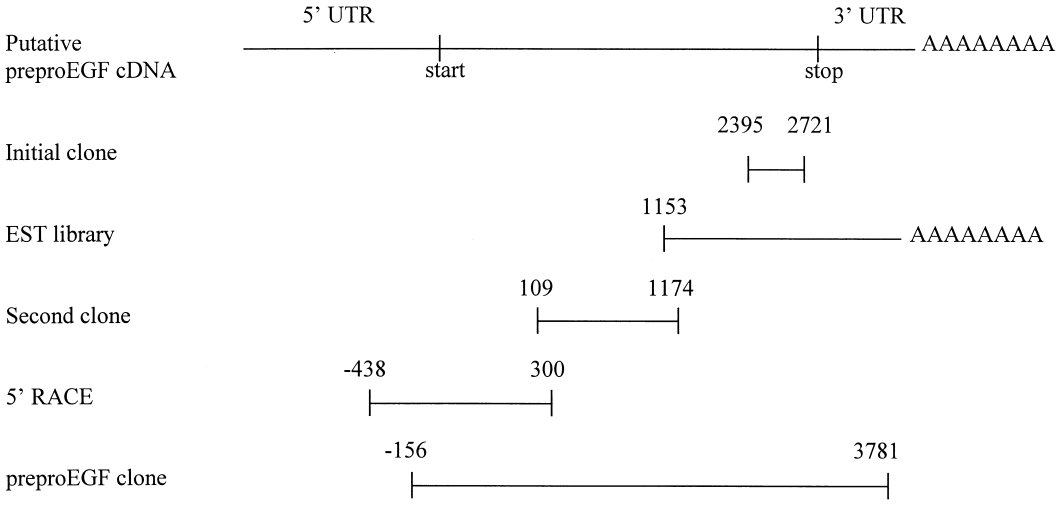

Fig. 1. The cloning strategy and clones of pig epidermal growth factor (EGF) precursor obtained in this experiment are illustrated schematically. Initially, a 329-bp clone was obtained and used to screen the expressed sequence tag (EST) library. Then, 1066-bp and 739-bp clones of further 5′ sequences were obtained by reverse transcription-PCR(RT-PCR) and 5′ rapid amplification of cDNA ends (RACE), respectively. Finally, the entire coding sequence of pig preproEGF including portions of 5′ and 3′ untranslated region (UTR) was confirmed.

by RT-PCR. Total RNA (2 μg) was used for RT with reverse primer 1. Then, 1 μl of the resultant product was amplified by PCR with the combination of four forward primers (forward primer 3–6) based on the human EGF cDNA sequence and two reverse primers (reverse primer 3–4) based on the porcine EGF cDNA sequence. The 1066 bp product obtained after amplification with forward primer 5 and reverse primer 3 was cloned into pCRII vector and sequenced. This clone encoded for amino acids 37–391. The remaining 5′ region was obtained by 5′ rapid amplification of cDNA ends (RACE) following a previously reported procedure [12]. All combinations of the forward (XBAcom and forward primer 7) and reverse primers (reverse primer 5–8) were used for PCR. Forward primer 7 and reverse primers 5 and 6 were designed based on the human sequence, and reverse primer 7 and 8 were designed based on the porcine sequence. The resulting products were then further amplified by PCR with forward primer 7 and reverse primer 8. The resultant 739 bp product was cloned into pCRII vector. At least three separate clones for each cDNA were sequenced in both directions. Finally, the full-length clone was obtained by RT-PCR using primers for preproEGF, which were designed to amplify the entire coding region. Again, total RNA (2 μg) was used for RT with reverse primer 9. Then 1 μl of the resultant product was amplified by PCR with the combination of forward primers 8 and 9, and reverse primers 9 and 10, designed based on the porcine EGF cDNA sequences. The product amplified with forward primer 8 and reverse primer 9 was further amplified with the nested forward and reverse primers. Then the resultant 3937 bp product was cloned into pCRII vector and sequenced.

Table 1
Primers used in the characterization of the preproEGF cDNAs

Stage	Primer	Sequence
Initial	Forward	
	1	GGTCGTGGGAAAGATCTGAG
	2	TGGAGTAATGCCAGTGGACAC
	Reverse	
	1	CATGTGCAGGTGTAGTTTCCC
	2	CCCATCTCCCTGGTAGCCTTCC
Second	Forward	
	3	GTCTCTCAGCACCGCAGCAC
	4	GGAGCTGTCCTGAAGGTACTCTCG
	5	GGGAATTCTACTTGTGTGGGTCC
	6	GGTGGTGGATGCTGGTGTCTC
	Reverse	
	3	CATCAGGGAGCAGAACAAATC
	4	TCAGACACATTGCTTGGGC
5′ RACE	Forward	
	XBAcom	AACGACATTGCCTGACTTGAC
	7	TCTCCATATTTCTTCTTTCAGCCCC
	Reverse	
	5	CCAGCATCCACCACCAATTG
	6	CTTTGCAAAAGTTGTCTTTCTAAATCCAC
	7	GCCTTGTCCCATTCAGAAAAAC
	8	TGCCTTGTCCCATTCAGAAAAAC
preproEGF	Forward	
	8	TCTCCATATTTCTTCTTTCAGCCCC
	9	TGAAATTTGTCATAAGGGTGTCAGG
	Reverse	
	9	GATTTCTTCTGGGGCTGTAGG
	10	ATGAGTTACCGAGTGATTCTCCC
	Nested forward	AGAAGGAGCTGTAGAGGGCAAG
	Nested reverse	CTTTGATGCTGAGTTAGTGATTAGTC

2.2. Northern blotting

Northern blot analysis was performed using 20 μg of total RNA from endometrium of D 10, 13, and 15 cyclic and D 10, 13, 15, 20, 30, and 40 pregnant White crossbred gilts (n = 3 to 5 each). Total RNA was electrophoresed in 1.5% agarose gels prepared in MOPS (3-[N-morpholino] propane-sulfonic acid)/formaldehyde buffer; the gels were then blotted onto Gene Screen membrane (NEN, Life Science Products, Boston, MA). Intactness of RNA and approximately equal gel loading were assessed [13] visually using ethidium bromide stained ribosomal RNA bands (Fig. 4). Probe was generated by T7 RNA polymerase using the MAXIscript kit (Ambion, Austin, TX) in the presence of [^{32}P]UTP using a 3342-bp partial cDNA clone of EGF precursor obtained from the EST library as the template. Membranes were hybridized with ULTRAhyb (Ambion, Austin, TX) containing 1×10^6 cpm radiolabeled probe per milliliter at 65°C overnight. Then the membranes were washed twice with 2× SSC, 0.1% SDS at 65°C, and then with 0.1× SSC, 0.1% SDS at 65°C, and subjected to autoradiography.

2.3. Statistical analysis

Relative expression of EGF precursor mRNA was determined by densitometry and results were analyzed by ANOVA using the General Linear Models procedure of the statistical analysis system (SAS Institute, Inc., Cary, NC). The model included effects of status, day of the cycle or pregnancy, and the status-by-day interaction. However, no effect of status or status-by-day interaction was obtained, so these effects were dropped from the model. Day effects were then more fully evaluated using a set of orthogonal contrasts. Contrasts were 1) D 10 versus D 13; 2) D 10 and 13 combined versus D 15; 3) D 10, 13, and 15 combined versus D 20; 4) D 10, 13, 15, and 20 combined versus D 30; 5) D 10, 13, 15, 20, and 30 combined versus D 40.

3. Results

3.1. Cloning of porcine EGF precursor

Nucleotide sequences and predicted amino acid sequences for putative porcine EGF precursor (Genebank Acc. No AF336151) are shown in Fig. 2. Alignment of the EGF precursor cDNA sequence with the human EGF precursor cDNA sequence indicates that the 5′ end of the porcine sequence starts at 16 bp of the reported human preproEGF cDNA sequence [14]. It has a long open reading frame of 3642 bp that encodes 1214 amino acids compared to 3621 bp and 1207 amino acids for human EGF precursor and 3651 bp and 1217 amino acids for mouse EGF precursor. The 5′ and 3′ untranslated regions (UTR) of cDNA were 438 and 549 bp, respectively. The sequence identity of the 5′ UTR of porcine EGF precursor with the 5′ UTR from mouse, rat, and human ranged from 58 to 77%. The sequence identity of 3′ UTR of EGF precursor cDNA with the 3′ UTR of the other three species ranged from 62 to 79%. Though conserved among species, multiple sequence alignment showed no consensus region in either the 5′ UTR or 3′ UTR except a consensus polyadenylation signal of AATAAA located in the 3′ UTR 16 bp upstream from the polyA tail.

Percent identities between the amino acid sequences of pig, human [14], mouse [15], and rat [16] EGF precursors are shown in Table 2. Pig EGF precursor sequence is more homologous to human than to either mouse or rat. The encoded amino acid sequence of porcine EGF precursor cDNA is aligned with those of human, mouse, and rat EGF precursors in Fig. 3. Underlined porcine sequences are the regions first reported in this study, including the 5′-UTR, EGF-like modules 1 and 2 (8 nucleotides of EGF-like module 2 were reported previously, [11]), cytoplasmic domain (4 nucleotides of the cytoplasmic domain were reported previously, [11]), and 3′-UTR with poly A tail. Among the four species, the amino acid identity for the EGF-like module I and II are 66 and 93%, respectively. The mature EGF peptide, as reported previously [10], is indicated in bold letters.

```
   1  TCTCCATATTTCTTCTTTCAGCCCCATCAAGGGTTCTGGTTGGAACTTTCCACCAACTTTTTCCTTGTTTTCCTCTCTCTAGCCTTGGCTCTGCCACTCT   100

 101  CTGCCTGTCACAGTGAAGTCAGCAGCAGGCCTCCAGGGCAAGGCTGTGAAACTGTGAAATTTGCCATAAGGGTGTCAGGTATTTCTTACTGGCTTCCCAG   200

 201  AATCTTAGATCAAGAAACCTTTCCCTGGCATGTCCCCTTCAGGTTGCATTTGGACGGTGTCTTGAGTTGAAGAAGAAGGAGCTGTAGAGGGCAAGAGCAA   300

 301  AGCAGCAGAGGAAAAATGCCCCAGAGCTGCAGGCCAACCCGGGTGGACACGCTGCGCGTCAGACATACCCACCTTGCCCGGGCTATGCTCAAGCAGAGTC   400

 401  AACTTATTTTCTACTGAGAGTTCAAGCCCGTCAAGATAATGCTGCTCTTCCTTATCCTTCTGTTGCCGGTAGTTTTGAAATTTAGTTTTGTCAGCCTCTC   500
   1                                        M  L  L  F  L  I  L  L  L  P  V  V  L  K  F  S  F  V  S  L  S     21

 501  AGCACCAGCGCGCTGGAACTGTCCTGAAGGCTCTCCCTCAGGAAACGGGAATGCCACTTGTGTGGGTCCTGCACCCTTCTTAATTTTCTCCCATGGAAAC   600
  22   A  P  A  R  W  N  C  P  E  G  S  P  S  G  N  G  N  A  T  C  V  G  P  A  P  F  L  I  F  S  H  G  N     54
                                                                  *

 601  AGTATCTTTAGGATTGACTTGGAAGGAACTAATCATGAGCAATTGGTGGCAGATGCTGGAATATCAGTGCTCATGGACTTCCATTACAATGAGGAAAGAA   700
  55  S  I  F  R  I  D  L  E  G  T  N  H  E  Q  L  V  A  D  A  G  I  S  V  L  M  D  F  H  Y  N  E  E  R  I     88

 701  TCTATTGGGTCGATCTAGAAAGACAACTTTTGCAAAGAGTTTTTCTGAATGGGACAAGGCAAGAGAAAGTATGCAATCTAGAGAAAAATGTTTCAGGAAT   800
  89   Y  W  V  D  L  E  R  Q  L  L  Q  R  V  F  L  N  G  T  R  Q  E  K  V  C  N  L  E  K  N  V  S  G  M    121
                                                                  *                                  *

 801  GGCAATAAATTGGATCAATGAAGAACTTATTTGGTCAAATCAACAGGAAGGCACCATTACAGTAACAGATATGAAAGGAAACAATTCCCGTGTTCTCTTA   900
 122   A  I  N  W  I  N  E  E  L  I  W  S  N  Q  Q  E  G  T  I  T  V  T  D  M  K  G  N  N  S  R  V  L  L    154
                                                                                                  *

 901  AGCGCCTTAAAATATCCTGCAAATGTAGCAGTTGATCCAGTAGAAAGGTTGATGTTTTGGTCTTCAGTGGTGGCCGGCAGCCTTCACAGGGCAGATGTTA  1000
 155  S  A  L  K  Y  P  A  N  V  A  V  D  P  V  E  R  L  M  F  W  S  S  V  V  A  G  S  L  H  R  A  D  V  T    188

1001  CAGGTGTGGAAGTGAGACTTCTGTTGGAGACATCAGAGGAAATTGCAGCTGTGTCATTGGATGTGCTTGATAAACGGCTCTTTTGGATTCAGTATAACAG  1100
 189   G  V  E  V  R  L  L  L  E  T  S  E  E  I  A  A  V  S  L  D  V  L  D  K  R  L  F  W  I  Q  Y  N  R    221

1101  AGAAGGAGGCAGTTCTCGTATTTGTTCCTGTGATTATGATGGAGGTTCTGTCCATTTTAGCAAACATCTCACACAGCACAATGTGTTTGCAATGTCCCTT  1200
 222   E  G  G  S  S  R  I  C  S  C  D  Y  D  G  G  S  V  H  F  S  K  H  L  T  Q  H  N  V  F  A  M  S  L    254

1201  TTTGGTGATCACATCTTCTATTCAACATGGAAAAAGAAGACAATTTGGGTAGCTAACAAGCACACTGGGAAGGATATGGTTAAAATGAACCTCAATCCAG  1300
 255  F  G  D  H  I  F  Y  S  T  W  K  K  K  T  I  W  V  A  N  K  H  T  G  K  D  M  V  K  M  N  L  N  P  A    288

1301  CATTTGTACCACCTGGTGGAATTAAAGTAGTGCATCCACTTGTTCAGCCCAAGGCAGAGGGTGATGCTTGGGCCTCTGATCAGAAACTCTGCAAACTGAG  1400
 289   F  V  P  P  G  G  I  K  V  V  H  P  L  V  Q  P  K  A  E  G  D  A  W  A  S  D  Q  K  L  C  K  L  R    321

1401  GAAGGGTAACTGCAGAGGCAGCATGTGTGGGCAAGAGCCAAAGTCCCACGTGTGCACGTGTGCCGAGGGATACACTTTAAGCCAAGATGGAAGGAAGTGT  1500
 322   K  G  N  C  R  G  S  M  C  G  Q  E  P  K  S  H  V  C  T  C  A  E  G  Y  T  L  S  Q  D  G  R  K  C    354

1501  GAAGATGTCAACGAGTGTGCCTTCTGGAATCATGGCTGTACTCTCGGGTGTGAAAACACTCCTGGATCCTATTATTGCACGTGTCCTGCAGGATTTGTTC  1600
 355  E  D  V  N  E  C  A  F  W  N  H  G  C  T  L  G  C  E  N  T  P  G  S  Y  Y  C  T  C  P  A  G  F  V  L    388

1601  TGCTCCCTGATGGGAAACGGTGTCATCAATTAATTTCCTGCCCAAGCAATGTGTCTGAGTGTAGCCATGACTGTGTTCTGACATCAGATGGTCCCATATG  1700
 389   L  P  D  G  K  R  C  H  Q  L  I  S  C  P  S  N  V  S  E  C  S  H  D  C  V  L  T  S  D  G  P  I  C    421
                                                                  *

1701  TTTCTGTCCTGAAGACTCAGTACTTGAGGCAGATGGAAAAACATGTAGCGGCTGTTCATCACCTGATAATGGTGGGTGCAGCCAGCTCTGCCTTCCTCTC  1800
 422   F  C  P  E  D  S  V  L  E  A  D  G  K  T  C  S  G  C  S  S  P  D  N  G  G  C  S  Q  L  C  L  P  L    454

1801  AGTCCAGTAACCTGGGAATGTGGCTGCTTTCCTGGGTATGACCTACAGTTGGACAAAAAGAGCTGTAGAGCTTCAGGACCACCACCGTTTTTGCTGTTTG  1900
 455  S  P  V  T  W  E  C  G  C  F  P  G  Y  D  L  Q  L  D  K  K  S  C  R  A  S  G  P  P  P  F  L  L  F  A    488

1901  CCAATTCTCAAGATATTCGACACATGCATTTTGACGGAACAGATTATGAAACTCTGCTCAACCAGCAAATAGGAATGGTTTTGGCCCTAGATCATGACCC  2000
 489   N  S  Q  D  I  R  H  M  H  F  D  G  T  D  Y  E  T  L  L  N  Q  Q  I  G  M  V  L  A  L  D  H  D  P    521

2001  TGTGGAAAATAAGGTATACTTTGCCCATACAGCCCTGAAATGGATAGAGAGAGCTAATATGGATGGTTCCCAGCGAGAAAGGCTTTTTGAGGAAGCAGTC  2100
 522   V  E  N  K  V  Y  F  A  H  T  A  L  K  W  I  E  R  A  N  M  D  G  S  Q  R  E  R  L  F  E  E  A  V    554

2101  GATGTGCCAGAAGGTCTTGCCATAGACTGGATTGGCCGTAAATTCTATTGGACAGACAGAGGGAGATCTCTCATTGAAGGGAGTGATTTAAATGGAAAAT  2200
 555  D  V  P  E  G  L  A  I  D  W  I  G  R  K  F  Y  W  T  D  R  G  R  S  L  I  E  G  S  D  L  N  G  K  Y    588

2201  ACCGCGAAATAATCATTAAGGAAGACATCTCTCAGCCACGAGGAATTGCTGTTCATCCAGTGGCCAAGAGATTATTCTGGACTGATATGGGGACTAATCC  2300
 589   R  E  I  I  I  K  E  D  I  S  Q  P  R  G  I  A  V  H  P  V  A  K  R  L  F  W  T  D  M  G  T  N  P    621

2301  ACGAATTGAAAGTTCATCCCTTCAAGGCATTGGCCGACAGGTTATAGCCAGCTCGGATCTGGTCTGGCCCAGCGGAATAACAATTGATTACTTAACTGAC  2400
 622   R  I  E  S  S  S  L  Q  G  I  G  R  Q  V  I  A  S  S  D  L  V  W  P  S  G  I  T  I  D  Y  L  T  D    654

2401  AAGTTGTATTGGTGCGATGCCAAGCAGTCTGTGATTGAAATGTCCAATCTGGATGGTTCGAGACGCCAGAGACTTGCCCAGAACGATGTAGGTCACCCAT  2500
 655  K  L  Y  W  C  D  A  K  Q  S  V  I  E  M  S  N  L  D  G  S  R  R  Q  R  L  A  Q  N  D  V  G  H  P  F    688

2501  TTGCGGTAGCTGTGTTTGAGGATCACGTGTGGTTCTCTGACTGGACTATGCCATCGGTAATAAGGGTGAACAAGAGGACTGGCAAAAATAGGGTACGTCT  2600
 689   A  V  A  V  F  E  D  H  V  W  F  S  D  W  T  M  P  S  V  I  R  V  N  K  R  T  G  K  N  R  V  R  L    721
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Fig. 2. Nucleotide sequence and predicted amino acid sequence for putative porcine epidermal growth factor precursor is shown. Seven ATTTA repeat sequences are underlined. A consensus polyadenylation signal of AATAAA is shown in bold letters. Possible sites for Asn-linked glycosylation are indicated with *.

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2601  CCGAGGCAGCATGCTGAAGCCCTCATCACTGGTTGTAGTTCATCCATTGGCAAAACCAGGAACAAATCCCTGCTTACACCAAAATGGAGGCTGTGAACAT   2700
 722   R  G  S  M  L  K  P  S  S  L  V  V  V  H  P  L  A  K  P  G  T  N  P  C  L  H  Q  N  G  G  C  E  H     754

2701  ATCTGCAAAGAGAGTTTTGGAACTGCTCAGTGTTTGTGTCATGAAGGTTTTCTGAAAGCCCCAGATGGAAAAATGTGTCTGGCTCTGAATGGCCAAGAGA   2800
 755  I  C  K  E  S  F  G  T  A  Q  C  L  C  H  E  G  F  L  K  A  P  D  G  K  M  C  L  A  L  N  G  Q  E  I    788

2801  TATTGGCAGGTCGTGGGAAAGATCTGAGTGATGGAGTAATGCCAGTGGACACCTTACCCAGAAGCAGAGAGCTTGAAGATAACCTTACAGAATCTCAACA   2900
 789   L  A  G  R  G  K  D  L  S  D  G  V  M  P  V  D  T  L  P  R  S  R  E  L  E  D  N  L  T  E  S  Q  H     821
                                                                                       *

2901  CATTCTAGTGGCTGAAATCATGGTTTCAGATGACGAGGACTGCGGCGCTGCGGGATGCAGTGCGCAGGCGCGGTGTGTTACAGAGGGAGAGGACGCCACG   3000
 822   I  L  V  A  E  I  M  V  S  D  D  E  D  C  G  A  A  G  C  S  A  Q  A  R  C  V  T  E  G  E  D  A  T     854

3001  TGTCAGTGTTTGAAAGGATTTGCTGGAGATGGAAACCTGTGTTCTGATATAGATGAGTGTGAGCTGGGCACCTCGGTGTGCCCTCCTACCTCCTCCGAGT   3100
 855  C  Q  C  L  K  G  F  A  G  D  G  N  L  C  S  D  I  D  E  C  E  L  G  T  S  V  C  P  P  T  S  S  E  C    888

3101  GCATCAATACCGAAGGTGGTCACGTCTGCCGCTGCTCGGAAGGCTACCAGGGAGATGGGATCCACTGTCTCGATATTGATGAGTGCCAACTAGGTGTGCA   3200
 889   I  N  T  E  G  G  H  V  C  R  C  S  E  G  Y  Q  G  D  G  I  H  C  L  D  I  D  E  C  Q  L  G  V  H     921

3201  CACCTGTGGGGAAAATGCCACCTGTACAAATACGGAGGGAAACTACACCTGCACATGTGCTGGCCGCCCCTCTGAACCCGGACGGATTTGCCCTGACCCT   3300
 922   T  C  G  E  N  A  T  C  T  N  T  E  G  N  Y  T  C  T  C  A  G  R  P  S  E  P  G  R  I  C  P  D  P     954
                  *                                 *

3301  ACTCCACCCTCTCACCTCGGGGAGGATGGCCGCTATTCTGTGAGAAATAGTTACTCTGAATGCCCGCCGTCCCACGACGGGTACTGCCTCCACGGTGGTG   3400
 955  T  P  P  S  H  L  G  E  D  G  R  Y  S  V  R  N  S  Y  S  E  C  P  P  S  H  D  G  Y  C  L  H  G  G  V    988

3401  TGTGTATGTATATTGAAGCCGTCGACAGCTATGCCTGCAACTGTGTTTTTGGCTACGTTGGCGAGCGATGTCAGCACAGAGACTTGAAATGGTGGGAGCT   3500
 989   C  M  Y  I  E  A  V  D  S  Y  A  C  N  C  V  F  G  Y  V  G  E  R  C  Q  H  R  D  L  K  W  W  E  L    1021

3501  GCGCCACGCTGGCCTCGGGCGACAGTGGAACGTCACGGTGGTGGCCGTCTGCGTGGTGGTGCTGGTCCTGCTGCTGCTCCTGGGGCTGTGGGGGGCTCAC   3600
1022   R  H  A  G  L  G  R  Q  W  N  V  T  V  V  A  V  C  V  V  V  L  V  L  L  L  L  L  G  L  W  G  A  H    1054
                                  *

3601  TACTACAGGACTCAGAAGCTGCTATCAAAAAATCCAAAGAATCCTTATGAAGAGTCAGGCAGAGATGTGAGTGGTATCAGGCCTGCAGACGGCGAGGCTG   3700
1055  Y  Y  R  T  Q  K  L  L  S  K  N  P  K  N  P  Y  E  E  S  G  R  D  V  S  G  I  R  P  A  D  G  E  A  G   1088

3701  GGATGTCCTCTTGCCCCCAACCTTGGTTTGTGGTGATAAAGGAACATCAAAATCTCAGGAATGGGAGTCAACCTGGAGCCCCCAAGGACGGCCTGGGAGC   3800
1089   M  S  S  C  P  Q  P  W  F  V  V  I  K  E  H  Q  N  L  R  N  G  S  Q  P  G  A  P  K  D  G  L  G  A    1121
                                                                  *

3801  AGATGTTGGCCAGTTTTCTTCCCTGGAGCCTGGGTCATTGCAGCCGACATCATGGAGGAAGGAACCCCAGATGTATATGGACACAGAACAAGGATGCTGT   3900
1122   D  V  G  Q  F  S  S  L  E  P  G  S  L  Q  P  T  S  W  R  K  E  P  Q  M  Y  M  D  T  E  Q  G  C  C    1154

3901  ATTCCATCCTCCAGTGATAAAGGCTCTGGTCCCCAGGGAATAGGGTATAGCTTTCATCTCCCCTCCTATGGGGCACGGTCCATTGCTGTCGGGGTTGAAA   4000
1155  I  P  S  S  S  D  K  G  S  G  P  Q  G  I  G  Y  S  F  H  L  P  S  Y  G  A  R  S  I  A  V  G  V  E  K   1188

4001  AATCCCATTCTCTCCTGTCAGCTAACCCATTACGGCAACAAAGGGCTCCAGATCCACCACACCAAATGGAGCTGACTCAGTGAAAACTGGAATTAAAAGT   4100
1189   S  H  S  L  L  S  A  N  P  L  R  Q  Q  R  A  P  D  P  P  H  Q  M  E  L  T  Q                          1214

4101  AAGATCAAGGAGAACAAACTGTACCGATGGACAGTAATACTTCCTTTCAAAAAGTAGAAGAAAAATACAGATTTTTGTTCTACAATCTCTATGACTAATC   4200

4201  ACTAACTCAGCATCAAAGACAAATATTTACTGTGCTTTTATTTCTTCCTTCAAGCAGTCTCACTGCAGGTTGTCAAGTAAAAGTAATTGGGAGAATCACT   4300

4301  CGGTAACTCATTAGAAACCAAAATGGGGACAGTAGTGTCTGTAAAAAGTGTTGTCTTCAATAATCAATACAGAGAGGTTTTTGTTTTTGTTTTTACCTAC   4400

4401  AGCCCCAGAAGAAATCTGGAGTTACGGCAATCATTCATACTGGTTTGGTCAGTTACAAAGTAATTTCTTTGATTTGGATAGAACATTTAGCTCAATCTTA   4500

4501  TGAAATGGTTGGCATATTAAAATTATTGTTACATAGGCATTTAACTTCTCCTTGCTATGGTCCATGCTGGTAATTTTTCAGACTGAATTATGATAAATTA   4600

4601  ATGAGCAATGTATTAACCTAGAACCTTGATTTCTTCAGAATTAGACGGCTTAATTTTTTCTATTTTAAAATCTTTGAACGAAGACATTTAATCTTTAAAA   4700

4701  CATTACCCAAGAGACTTTTGTGTTTCTCATTCATTACTGTCTTTTTCCCCACAGAATTTATTTCTAATGAAAATGGTAAATTTCCCCTTTGTGTGTTTGC   4800

4801  ACAGAATTTTTACTTATTTTCAAAATATGAGATTGTAAGCAGATTGCCTGATTTATTTTCATTTTGAACAACAATGAAGTTCTTCTAATTATTTAAATAA   4900

4901  AATCAGCATAAAGCATTAAAAAAAAAAAAAAA  4932
```

Fig. 2. *(continued)*

Table 2
Percent identities between the amino acid sequences of the pig, human, mouse and rat preproepidermal growth factor (EGF)

	Human	Rat	Mouse
Pig	81	67	66
Mouse	67	79	
Rat	68		

```
         1     |→ signal peptide     ←|                50
pigEGF   ......MLLF LILLLPVVLK FSFVSLSAPA RWNCPEGSP. .SGNGNSTCV
humegf   .......--T --I-----S- ---------Q H-S----TL. .A--------
rategf   .......M-- SLTF-S-F-- ITVL-VT-QQ TR--QS-PLE R--TTTYAAA
mouse    MPWGRRPTWL -LAF-L-F-- I-IL-VT-WQ TG--QP-PLE R-ERSG.--A

         51                                               100
pigEGF   GPAPFLIFSH GNSIFRIDLE GTNHEQLVAD AGISVLMDFH YNEERIYWVD
humegf   ---------- ----------T- ---Y----V- --V--I---- ---K------
rategf   --PR----LQ -------N?D ----Q---V- --V--V---- -K--K--L---
mouse    ------V--Q -K--S---PD ----Q---V- ----AD--I- -KK--L----

         101                                              150
pigEGF   LERQLLQRVF LNGTRQEKVC NLEKNVSGMA INWINEELIW SNQQEGTTTV
humegf   ---------- ---S--R-- -I-------- -------V-- ------I---
rategf   ---------- F--SG--T-- KVD-----L- ----DG-ILR TDRWK-V---
mouse    V---V-L--- ----GL---- -V-RK---L- -D--DD-VL- VD--N-V---

         151                                              200
pigEGF   TDMKGNNSRV LLSALKYPAN VAVDPVERLM FWSSVVAGSL HRADVTGVEV
humegf   --------HI ---------- --------FI ----E----- Y---LD--G-
rategf   ---N------ ---S--R--- IL---T---I ------T-N- ----LG-MD-
mouse    ---T-K---- ---S--H-S- I----I---- ----E-T--- ---HLK--D-

         201                                              250
pigEGF   RLLLETSEEI AAVSLDVLDK RLFWIQYNRE GGSSRICSCD YDGGSVHFSK
humegf   KA------K- T--------- ---------- -SN-L---- -------I--
rategf   KT---AP-R- SVLI--I--- ----A-DG-- -SHGY-H--- -N---I-HIR
mouse    KT----GG.- SVLT------ ----V-DSG- -SHAY-H--- -E----RLIR

         251                                              300
pigEGF   HLTQHNVFAM SLFGDHIFYS TWKKKTIWVA NKHTGKDMVK MNLNPAFVPP
humegf   -P---L---- -----R---- ---M---I-- ---------R I--HSS---L
rategf   -QAR-DLLT- AI---K-L-- AL-E-A--I- D-----NV-R V--D--S---
mouse    -QAR-SLSS- AF---R---- VL-S-A--I- -------T-R I--H-SF-T-

         301                  |→      EGF-like 1          350
pigEGF   GGIKVVHPLV QPKAEGDAWA SDQKLCKLRK GNCRGSMCGQ EPKSHVCTCA
humegf   -EL------A -----D-T-E PE-------- ---SSTV--- DLQ--L-M--
rategf   RELR---LHA --GT-NR-Q- --SER--Q-R -Q-LY-LSER D-N-DSSA--
mouse    -KLM----RA --RT-DA-KD P-PE-L-Q-G RP--FGL-ER D----SSA--

         351          ←|→Ca2+ binding  EGF-like 2          400
pigEGF   EGYTLSQDGR KCEDVNECAF WNHGCTLGCE NTPGSYYCTC PAGFVLLPDG
humegf   ---A--R-RK Y--------- ---------K ---------- -V--------
rategf   ------R-RK Y--------L Q--------- -I-------- -T--------
mouse    ------R-RK Y--------T Q--------- ------H--- -T--------

         401                                      |→  450
           ←|→            EGF-like 3                ←|
pigEGF   KRCHQLISCP SNVSECSHDC VLTSDGPICF CPEDSVLEAD GKTCSGCSSP
humegf   ------V--- R--------- ----E--L-- ---G----R- ----------
rategf   ----E-VA-- G-R------- I------L-I --AG---GK- ----T---FS
mouse    -Q--E-V--- G---K---G- -------R-I --AG---GR- ----T-----

         451   EGF-like 4                 ←|                500
pigEGF   DNGGCSQLCL PLSPVTWECG CFPGYDLQLD KKSCRAS.GP PPFLLFANSQ
humegf   ---------V ---------D ---------- E---A--.-- Q--TL----N-
rategf   -------I-- ---LA----D ---------- R---A--M-- Q----------
mouse    -------I-- --R-G----D --------S- R---A--.-- Q-L-------

         501                                              550
pigEGF   DIRHMHFDGT DYETLLNQQI GMVLALDHDP VENKVYFAHT ALKWIERANM
humegf   ---------- --G---S--M ---Y------ ----I----- ----------
rategf   ---------- --K---SR-M ---F---Y-- --S-I---Q- ---------L
mouse    ---------- --KV--SR-M ---F---Y-- --S-I---Q- ----------

         551                                              600
pigEGF   DGSQRERLFE EAVDVPEGLA IDWIGRKFYW TDRGRSLIEG SDLNGKYREI
humegf   --------I- -G-------- V-----R--- ----R---GR ------RSK-
rategf   -------RIT -G--T----- V------I-- --S-K-V--- ---S--HHQ-
mouse    --------IT -G--TL---- L------I-- --S-K-VVG- ---S--HHR-

         601                                              650
pigEGF   IIKEDISQPR GIAVHPVAKR LFWTDMGTNP RIESSSLQGI GRQVIASSDL
humegf   -T--N----- ------M--- -----T-I-- ---------L --L-------
rategf   ---S--R-R- ------K-R- -----T-MS- ---------S D-TL----N-
mouse    --Q-R--R-- ------R-R- -----V-MS- ----A----S D-VL----N-

         651                                              700
VWPSGITIDY LTDKLYWCDA KQSVIEMSNL DGSRRQRLAQ NDVGHPFAVA
I--------F ---------- -------A-- ---K-R--T- ----------
LE----A--- ---T-----T -L-----AD- ---K-R--T- -------SL-
LE-------- ---T-----T -R-----A-- ---K-R--I- -------SL-

                                                         |→
         701                                              750
VFEDHVWFSD WTMPSVIRVN KRTGKNRVRL RGSMLKPSSL VVVHPLAKPG
----Y----- -A-------- -----D---- Q--------- ----------
---------- -AI------- ----Q----- ---------- ----------
-----L-V-- -AI------- ----Q----- Q--------- ----------

         751      EGF-like 5                  ←|          800
TNPCLHQNGG CEHICKESFG TAQCLCHEGF LKAPDGKMCL ALNGQEILAG
AD---Y---- ------KRL- --W-S-R--- M--S---T-- --D-HQL---
AD----R--- -----Q--L- ------R--- V--------- TRKDDQ----
AD---YR--- -----Q--L- --R---R--- V--W------ .PQDYP--S-

         801                                      |→      850
RGKDLSDGVM PVDTLPRSRE LEDNLTESQH ILVAEIMVSD ...DEDCGAA
GEV--KNQ-T -L-I-SKT-V S---I----- M--------- ...QD--APV
DNA---KE-A SL-NS-KAYV PD-DR---S. T--------G LNYED---PG
ENA---KE-T SLSNSTQAEV PD-DG---S. T--------G MNYED---PG

         851    EGF-like 6                ←|→Ca2+ binding    900
GCSAQARCVT EGEDATCQCL KGFAGDGNLC SDIDECELGT SVCPPTSSEC
---MY---IS ---------- -------K-- -------M-V P----A--K-
--GSH-H-IS ---A-V---- --------- --------S -D------R-
--GSH----S D--T-E---- ----R----- ------V-AR -D--S---R-

         901     EGF-like 7     ←|→Ca2+ binding  EGF-like 8 950
INTEGGHVCR CSEGYQGDGI HCLDIDECQL GVHTCGENAT CTNTEGNYTC
------Y--- ---------- ---------- ---------S -----G---
------Y--Q -----E---- Y---V----Q -S-G-S---- ------G-N-
------Y--- -----E---- S-F------R -A-N-A---A ------G-N-

         951          ←|                  |→     EGF      1000
TCAGRPSEPG RICPDPTPPS HLGEDG.RYS VRNSYSECPP SHDGYCLHGG
M----L---- L----S---P --R--DHH-- ----D---L --------D-
----C--A-- LP---S-S-- L--K--CHW. ----NTG--- -Y-----N--
-------S-- -S---S-A-- L-----HHL. D----PG--S -Y-----N--

         1001                                  ←|          |→ 1050
VCMYIEAVDS YACNCVPGYV GERCQHRDLK WWELRHAGLG RQWNVTVVAV
-------L-K ------V--I -----Y---- --------H- Q-QK-I----
----V-S--R -V----I--I ---------R --K----DY- QRHDI---S-
---H--SL-- -T----I--S -D---T---R --------Y- QKHDIM----

 Transmembrane
         1051 domain   ←|→       Cytoplasmic domain        1100
CVVVLVLLLL LGLWGAHYYR TQKLLSKNPK NPYEESGRDV SGIRPA.DGE
------M--- -S-------- --------- -------S--- RSR----.-T-
---A-A---- --M--TY--- -R-Q--ESS- K-S---SSS- -SNG-D.SSG
-M-A------ --M--TY--- -R-Q--NP-- --CD-PSGS- -SSG-DSSSG

         1101                                             1150
AGMSSCPQPW FVVYIKEHQNL RNGSQPGAPK DGLGADVGQF SSLEPGSLQP
D--------- --------D- K--G--V-GE --QA-+.... ......--M--
--V--G---- ---LE---QP K--RL-A-GT N-AVVEA-LS ----------
-AVA------ ---LEK--DP K---L-ADGT N-AVV-A-LS P--QL--VHL

         1151                                             1200
TSWRKEPQMY .MDTEQGCCI PSSSDKGSGP QGIGYSFHLP SYGARSIAVG
----Q---LC G-G-----W- -V------C- -VMER---M- ---TQTLEG-
.......... .......... .......... .......... ----------
----QK-HID G-G-G-S-W- -P---R-..- -E-EGNS--- --RP.....-

                                        ←|
         1201                          1229
VEKSHSLLSA NPLRQQRAPD PPHQMELTQ
---P------ ---W----L- ---------
.......... .......... .........
P--L---Q-- -GSCHE---- L-R-T-PVK
```

Fig. 3. The similarity between the predicted amino acid sequences for putative porcine epidermal growth factor (EGF) precursor compared with human, rat, and mouse sequences is shown. Underlined porcine sequences are the regions first reported in this study. Bold letters are the coding region corresponding to the mature EGF. The beginning (|→) and the ending (←|) of each domain are shown. Calcium binding consensus sequences are shown in bold and italicized characters.

3.2. Northern blotting

A representative autoradiograph of a Northern blot of endometrial total RNA probed with [^{32}P]labeled EGF cRNA is shown in Fig. 4 along with an ethidium bromide stained agarose gel. Least-square means of densitometry units (± standard error means) for EGF precursor mRNA in endometrium during the estrous cycle and pregnancy are illustrated in Fig. 4C. EGF mRNA expression decreased significantly from D 13 to 15 ($P = 0.04$) of the cycle and pregnancy and from D 30 to 40 ($P = 0.01$) of pregnancy. These findings show that EGF mRNA expression is temporally regulated.

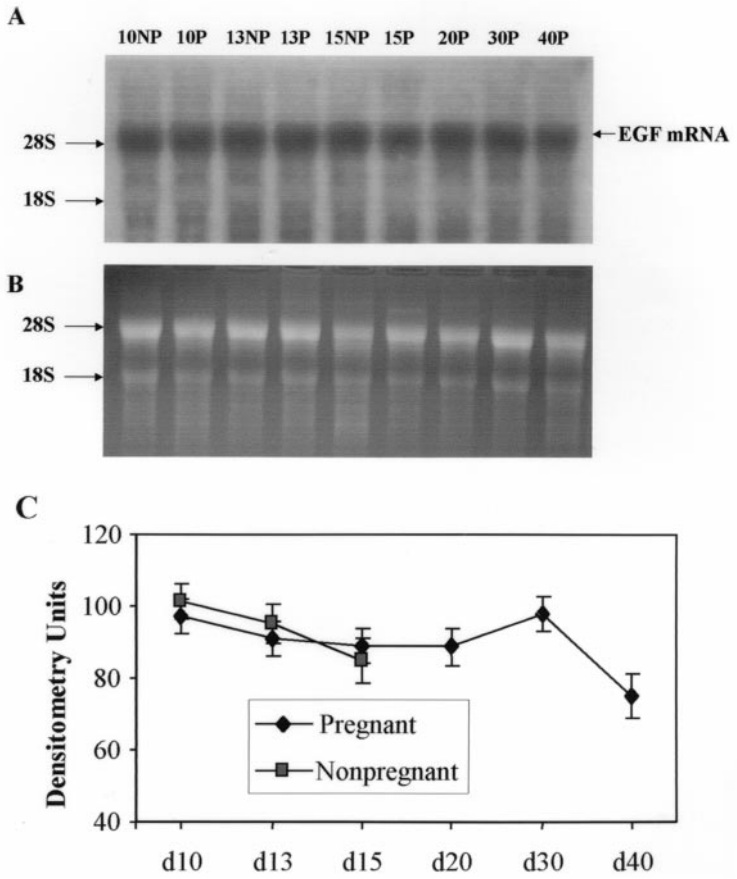

Fig. 4. Representative autoradiograph of Northern blot (A) and photograph of agarose gel loaded with total cellular RNA (20 μg) from endometrium of nonpregnant (NP) gilts on D 10, 13, and 15, and pregnant (P) gilts on D 10, 13, 15, 20, 30, and 40 (B). Arrows indicate 28S and 18S ribosomal bands. Epidermal growth factor (EGF) mRNA band (approximately 5-kb) is also indicated. Least-square means ($\pm$ standard error means) of densitometry units for EGF precursor mRNA in endometrium during estrous cycle and pregnancy are illustrated (C). EGF mRNA expression tends to decrease gradually during the estrous cycle and pregnancy with significant drops from D 13 to 15 ($P = 0.04$) and from D 30 to 40 ($P = 0.01$). These findings show that EGF mRNA expression is temporally regulated.

4. Discussion

This is the first reported cloning of the full coding region for the porcine EGF precursor. The coding sequence of porcine EGF precursor is most similar to human EGF precursor. It is much less homologous to the rodent EGF sequence as reported previously using partial EGF precursor sequence [11]. Interestingly, amino acids within the mature peptide are not more conserved than the rest of the coding sequence. Highlighting of the altered amino acids on a three-dimensional view of mature human EGF (not shown) indicated that the differences appear to be distributed uniformly over the surface of the peptide. This may reflect the lack

of specificity of the EGF receptor, which can be activated by many ligands of the EGF family including transforming growth factor-α [17].

The high amino acid sequence identity in the nonEGF portions of the EGF precursor suggests that these regions may play a role in the processing or function of EGF. The role of nonEGF portions has been speculated based on a comparison between mouse EGF precursor and the human low density lipoprotein (LDL) receptor [18–20]. The region of the mouse EGF precursor (amino acids 22–392) between the signal peptide and the region homologous to human LDL receptor might constitute the ligand binding domain [14]. The cytoplasmic domain of mouse EGF precursor (amino acids 1058–1207) might function in receptor mobilization and internalization [14]. However, the lack of conservation of the cytoplasmic domain of EGF precursor argues against this region being functional. The functional significance of these domains in EGF precursor is not clear. EGF precursor has 8 EGF-like modules in addition to EGF [14]. The EGF-like modules may be involved in protein-protein interactions [21]. A subclass of EGF-like modules exhibits the consensus sequence Cys-X-Asp/Asn-X-X-X-X-Tyr/Phe-X-Cys required for the postribosomal hydroxylation of the Asp/Asn residue [22]. Only EGF-like modules 2, 7, and 8 of human EGF precursor contain the consensus sequence for Asp/Asn hydroxylation. All three of these EGF-like modules have a calcium binding site with the consensus sequence of Asp-Ile/Val-Asp-Glu. Studies on human clotting factors have also shown that EGF-like modules bind Ca^{2+} [23–25]. Synthetic EGF-like module 2 binds Ca^{2+} with low affinity similar to other isolated EGF-like modules. EGF-like module 7, when part of the intact protein, binds Ca^{2+} with higher affinity than that of isolated EGF-like modules [22]. Porcine EGF precursor amino acid sequences for EGF-like modules 2, 7, and 8 are conserved in corresponding sites as indicated in Fig. 3; thus, the putative Asp/Asn residue hydroxylation sites and potential Ca^{2+} binding sites are likely to be functional. This is of interest as estradiol secreted by the conceptus stimulates a rapid release of calcium into the uterine lumen [26] at the time of trophoblast elongation on D 12. The interaction of EGF precursor with calcium warrants further study.

Seven ATTTA repeat sequences are located in the 3′-UTR (compared to four repeats in human and five repeats in mouse and rat). These sequences have been reported to influence the turnover of mRNA [27]. The higher number of ATTTA repeats in porcine sequence may increase the rate of degradation of EGF precursor mRNA. Disruption of one or more of the ATTTA sequences using site directed mitogenesis could help determine whether the ATTTA sequences are involved in the turnover of the mRNA.

Human EGF precursor sequence has nine possible sites for Asn-linked glycosylation and all are located in the region that would be predicted to project from the cell surface if it were anchored in the membrane [14]. Porcine EGF precursor sequence has seven of ten potential sites for Asn-linked glycosylation in corresponding positions, while in the mouse only two of the six sites are in corresponding positions [14].

Glutamine (amino acid 634) coded by codon CAG in the porcine EGF precursor sequence was obtained from both the EST library and the 3937-bp preproEGF clone, while arginine coded by CGG was reported previously using RNA from Danish Landrace kidney [11]. This possible polymorphism is outside the EGF-like modules. Therefore, it is not likely to change the function of EGF precursor.

Growth factors, including EGF, in the uterine fluid may regulate conceptus or endometrial development during early pregnancy. At the time of trophoblast elongation on D 12, the conceptus secretes estradiol, which stimulates a rapid release of calcium into the uterine lumen [26]. The expression of EGF in mice [28] and EGF receptor in rats [29] are stimulated by estrogens.

Elevated EGF precursor mRNA expression on D 30 and the drop in expression on D 40 of pregnancy are correlated with estradiol concentrations in uterine vein [30]. However, the EGF precursor mRNA expression pattern in this study, which is elevated from D10 to 13 and decreased from D 13 to D 15, is not correlated with the luminal estradiol profiles of either cyclic or pregnant gilts [26]. The role of EGF mRNA expression in endometrial function or conceptus development is not known. Expression of EGF mRNA is high previous to and during conceptus elongation and decreases as elongation nears completion (D 15). Thus, EGF may play a role in conceptus elongation. Between D 30 and D 40 of pregnancy, EGF mRNA expression is associated with placental areolae development. Areolae develop over the openings of the uterine glands between D 30 and D 40, and are responsible for uptake of uterine products. Thus it is possible that the changes in EGF mRNA expression may play a role in some aspects of areolae development. A complicating factor in any proposed role for EGF during this period is that the EGF precursor requires proteolytic processing to generate the mature peptide. EGF precursor has been reported to be cleaved by kallikrein, a serine protease [31]. Both kallikrein gene expression and kallikrein activity have been observed in endometrium in swine [32]. Thus, kallikrein may be involved in the processing of EGF precursor, and processing likely influences the role of EGF in both conceptus and endometrial development. Further study is required to determine how the EGF precursor is processed, how proteolytic enzymes control EGF in the uterine environment, and the role of EGF in conceptus or endometrial development.

Wollenhaupt et al. [2] have shown by RT-PCR that EGF mRNA expression in the endometrium was higher on D 6 than D 1 of cyclic and pregnant pigs by RT-PCR. Because our results do not cover the same period, it is difficult to compare.

In conclusion, the full coding region for the porcine EGF precursor cDNA is reported. The high amino acid sequence identity in the nonEGF portions of the EGF precursor suggests that these regions may play a role in the processing or function of EGF. Northern blot analysis shows that EGF mRNA expression is temporally regulated during the cycle and early pregnancy; this pattern of gene expression may be important during early conceptus development, thus contributing to the uterine capacity.

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