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Porcine Calbindin-D_{9k} Gene: Expression in Endometrium, Myometrium, and Placenta in the Absence of a Functional Estrogen Response Element in Intron A[1]

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ABSTRACT

The expression of Calbindin-D_{9k} (CaBP-9k) in the pig uterus and placenta was measured by Northern blot analysis and reverse transcription polymerase chain reaction (PCR), respectively. Progesterone (P_4) administration to ovariectomized pigs decreased CaBP-9k mRNA levels. Expression of endometrial CaBP-9k mRNA was high on pregnancy Days 10–12 and below the detection limit on Days 15 and 18. On Day 60, expression could be detected at low levels. In myometrium and placenta, CaBP-9k mRNA expression was not detectable by Northern analysis using total RNA. Reverse-transcribed RNA from both tissues demonstrated the presence of CaBP-9k transcripts by means of PCR. The partial CaBP-9k gene was amplified by PCR and cloned to determine the sequence of intron A. In contrast to the rat CaBP-9k gene, the pig gene does not contain a functional estrogen response element (ERE) within this region. A similar ERE-like sequence located at the identical location was examined by gel retardation analysis and failed to bind the estradiol receptor. A similar disruption of this ERE-like sequence has been described in the human CaBP-9k gene, which is not expressed at any level in placenta, myometrium, or endometrium. It is concluded that the pig CaBP-9k gene is regulated in these reproductive tissues in a manner distinct from that in rat and human tissues. The regulation is probably due to a regulatory region outside of intron A, which in the rat gene contains the key cis element for uterine expression of the CaBP-9k gene.

INTRODUCTION

Calbindin-D_{9k} (CaBP-9k), a member of a family of calcium-binding proteins, is a small (M_r 9000) cytosolic protein with two high-affinity calcium binding sites [1]. In the rat, CaBP-9k is localized mainly in the intestine, uterus, and placenta. CaBP-9k biosynthesis provides an important model for studying the molecular mechanisms of vitamin D (intestine) and estrogen (uterus) action. CaBP-9k is believed to be involved in the vitamin D-stimulated calcium transport across epithelia in the intestine [2–5]. Expression of CaBP-9k in the uterus is not affected by vitamin D [6]. In the rat uterus, 17β-estradiol (E_2) has been shown to stimulate CaBP-9k gene expression via an estrogen response element (ERE) near the promoter region [6]. By virtue of its calcium affinity, high expression in myometrium, and E_2 regulation, it has been suggested that CaBP-9k may be involved in the control of uterine activity during pregnancy [7]. Intestinal expression of CaBP-9k has been reported in rats [8], mice [9], cows [10], pigs [11], and humans [12]. Uterine expression, on the other hand, has been reported only in rats [13] and mice [14]. Recently, we have reported the molecular cloning of the porcine CaBP-9k and demonstrated expression in the uterus [15]. In unpublished experiments from our laboratory, we have also detected CaBP-9k transcripts in the bovine and ovine uterus. Surprisingly, however, the human uterus does not express CaBP-9k [16, 17]. Sequence of the genomic DNA encoding the human CaBP-9k locus [18] has revealed a difference of two nucleotides within the region corresponding to the rat CaBP-9k ERE. Binding studies with the estrogen receptor (ER) have shown that the human element no longer functions as ERE and could be the cause of the absence of CaBP-9k transcripts in human uterus [16, 17].

Data on uterine expression of CaBP-9k have mainly been generated in the rat, where an intricate hormonal system controls expression during reproduction, with E_2 and its receptor as a key stimulus and progesterone (P_4) as inhibitor [19–23]. The present study was aimed at determining whether pig uterine CaBP-9k is subject to steroid hormone regulation similar to that reported in the rat. More specifically, the porcine CaBP-9k gene was analyzed to determine whether a consensus ERE identical to that in the rat gene is involved in control of porcine gene promoter activity.

MATERIALS AND METHODS

Animals and Treatments

A 6-wk-old female Yorkshire piglet was obtained from a local farm. The animal was killed by an injection of pentobarbital sodium in accordance with the guidelines of the Animal Care Committee of the University of British Columbia. The duodenum and a segment of skeletal muscle were removed for isolation of total RNA. A segment of the liver was removed for preparation of genomic DNA.

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For experiments on the regulation of CaBP-9k, gilts that had experienced at least two estrous cycles were used. Ovariectomy was performed two days after the onset of estrus. The animals (n = 3) received 8 consecutive daily injections (s.c.) of either E_2 (0.1 μg/kg), P_4 (0.2 mg/kg), E_2 +P_4, or vehicle (corn oil) as described previously [24]. Timed pregnant gilts (n = 3) were obtained as reported earlier [25, 26], and after hysterectomy tissues were separated into myometrium and endometrium. Placentae were also collected, and total RNA was prepared from all tissues.

RNA Analysis

Total RNA was isolated by the guanidinium isothiocyanate/CsCL procedure [27, 28].

Quantification of CaBP-9k and β-Actin mRNA

Twenty micrograms of total RNA was analyzed by Northern blot analysis using a porcine CaBP-9k [16] and subsequently a bovine β-actin [29] cDNA probe. The probes were labeled by random primer synthesis and incorporation of [α-^{32}P]dCTP (specific activity 1 × 10^9 dpm/μg DNA). The resulting autoradiographs were scanned by laser densitometry. Equal loading and integrity of the RNA was confirmed by addition of 0.5 μg of ethidium bromide to each sample.

Reverse Transcription Polymerase Chain Reaction (RT-PCR) Analysis

Low-abundance expression was determined by use of an RT-PCR technique, and total RNA from myometrial and placental samples. Both sense and antisense primers were derived from the porcine CaBP-9k cDNA sequence position #5–24 and #161–182. Assuming similar genomic structures among the porcine, rat [30, 31], and human [18] CaBP-9k genes, the two primers encompassed exons I and II, respectively. After 30 cycles of standard PCR, the product was separated on 6% polyacrylamide gels. A negative control was included consisting of all reagents, RNA, and *Taq* polymerase with the exception of reverse transcriptase.

Primer Extension Analysis

The transcription initiation site of the porcine CaBP-9k gene was determined by primer extension analysis. Twenty micrograms of total RNA was used for annealing an end-labeled primer ([γ-^{32}P]ATP) designed after the cDNA sequence #103–123 [15]. Approximately 1–3 × 10^5 cpm were extended according to the method described by Calzone et al. [32]. Extension products were analyzed on a 6% denaturing acrylamide gel alongside a sequencing reaction ([α-^{35}S]dATP) of pUC19.

Genomic DNA Analysis

Amplification and cloning of the intron A region of the porcine CaBP-9k gene were carried out as follows: the primers (100 pmol) described in the RT-PCR analysis section above were used to amplify 500 ng of genomic DNA. PCR conditions were as follows: 96°C for 45 sec, 50°C for 30 sec, and 72°C for 90 sec. The products were analyzed on a 1% agarose gel, filled in with Klenow fragment of DNA polymerase I, and cloned into the *Sma* I site of pUC19. The DNA sequence was determined by the dideoxy chain termination method from three independent clones. Computer analysis of the nucleotide sequence was carried out with the Wisconsin Package program from the Genetics Computer Group, Inc. (Madison, WI).

Gel Retardation Analysis

A DNA element similar to the ERE at the 5′ end of intron A of the rat CaBP-9k gene was tested for potential binding affinity to the ER. The gel retardation assay was as described previously, with rat uterus cytosol used as the source for ER [6]. The DNA elements used in the assay are depicted in Figure 7a. ERE from the rat CaBP-9k and Xenopus vitellogenin A_2 genes and a nonfunctional, palindromic mutant ERE were supplied by H.F. DeLuca, University of Wisconsin-Madison, Madison, WI. All elements were synthesized as oligonucleotides with *Bam*HI overhangs and cloned into pUTKAT1. Plasmids were restricted with *Xba* I and *Eco*RI to release a 45-nucleotide fragment carrying the elements near the center. The DNA was labeled by filling in the ends with Klenow fragments of DNA polymerase I, [α-^{32}P]dATP, and [α-^{32}P]dCTP. Fragments were purified over a 6% polyacrylamide gel and eluted, and radioactivity was determined by Cerenkow counting. ER antiserum was supplied by J. Gorski, University of Wisconsin-Madison, Madison, WI.

Statistical Analysis

Results were analyzed by analysis of variance. Individual groups were compared by means of the Student-Fisher *t*-test. Results are expressed as mean ± SEM.

RESULTS

CaBP-9k Regulation in Uterus and Placenta

Experiment I. In the first experiment, endometrial RNA was analyzed by Northern blot hybridization, with the porcine CaBP-9k and a bovine β-actin cDNA used as probes. The effects of E_2 and P_4 treatment were measured in ovariectomized (OVX) gilts. Animals were treated with either corn oil or the steroid hormones indicated for 8 consecutive days after ovariectomy. Each group consisted of 3 animals. The Northern blot in Figure 1 demonstrates that there was a single transcript detectable in all groups. Three samples of pregnant endometrium were co-electrophoresed to compare levels of expression in treated and pregnant animals. Figure 2 indicates the result of densitometric analysis of CaBP-9k and β-actin mRNA expression in the experimental

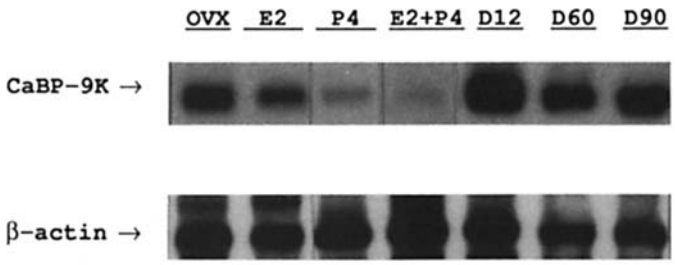


FIG. 1. CaBP-9k mRNA expression in porcine endometrium. Total RNA was analyzed by Northern blot analysis with pig CaBP-9k and bovine β-actin cDNA used as probes. Animals were OVX and treated with vehicle, E_2 (0.1 μg/kg), P_4 (0.2 mg/kg), or E_2 + P_4 for 8 consecutive days. For comparison, RNA from animals at pregnancy Days 12, 16, and 90 was co-electrophoresed (D12, D60, D90).

groups. Ovariectomy did not prevent CaBP-9k expression, and mRNA levels were even slightly higher in OVX animals than in E_2-treated OVX gilts. The difference amounted to about 17%, which was not significant. Both P_4 and E_2 + P_4 treatment significantly decreased the amount of CaBP-9k mRNA, with a stronger repression in the group treated with both steroids. There was no significant difference between the P_4- and E_2 + P_4-treated groups. CaBP-9k expression in the three pregnant samples co-analyzed with these groups indicated a substantially higher expression at Days 12, 60, and 90 of pregnancy. Comparison of expression in endometrium (Day 12) with that found in intestine at weaning (15) showed that the endometrial expression was approximately 20-fold lower (data not shown). The amount of total RNA and its integrity was confirmed by ethidium bromide staining of the ribosomal RNA. When the blots were probed with β-actin, no significant difference was detected in the treated groups, and levels in the nonpregnant samples were slightly higher than in the three pregnant preparations.

Experiment II. In the second experiment, RNA was analyzed from endometrium and myometrium during early pregnancy. Placental RNA from Days 45, 75, 90, and 112 of gestation was examined. Figure 3 shows a Northern blot of total RNA probed sequentially with CaBP-9k and β-actin. Figure 4 depicts the results of quantitative analysis using densitometric scanning of the autoradiographs. Expression of CaBP-9k was high on Days 10–12 of pregnancy, and there was a slight, nonsignificant increase of expression from Day 10 to Day 12. Strikingly, on Days 15 and 18 the transcripts decreased below the detection limit of the assay. On Day 60 CaBP-9k mRNA was again detectable. Beta-actin mRNA expression indicated a different pattern with no significant changes between pregnancy Days 10 and 18. On Day 60, β-actin mRNA was significantly decreased compared to that on Day 10 ($p < 0.02$).

When myometrial RNA from the same animals was analyzed, CaBP-9k transcript was not detectable in any of the samples (data not shown). When samples were probed with β-actin, very high expression was apparent. There was no change of expression between pregnancy Days 10 and 18.

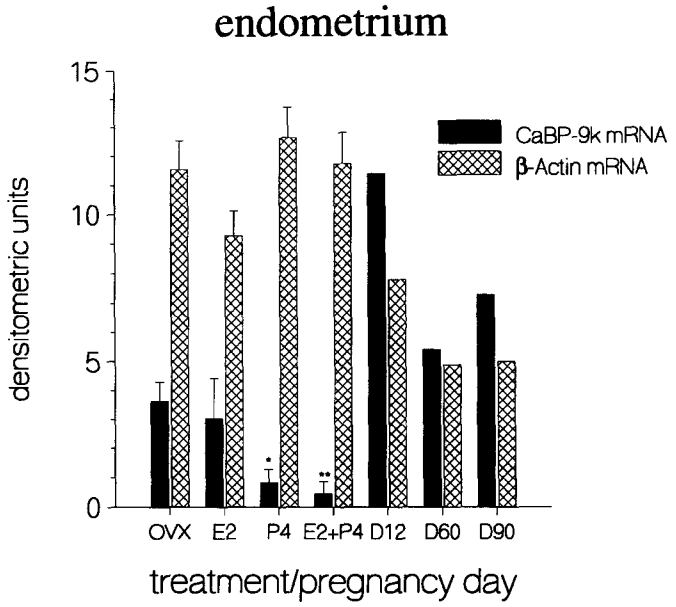


FIG. 2. Quantification of CaBP-9k mRNA in endometrium after hormone treatment. Northern blots were performed with RNA from the groups as in Figure 1 (n = 3). The autoradiographs were scanned by laser densitometry. Results are shown as mean ± SE. For comparison, pregnant endometrium from one individual at Days 12, 60, and 90 was analyzed. * $p < 0.05$; ** $p < 0.02$ compared to OVX).

On Day 60, β-actin mRNA was significantly lower than on Day 10 ($p < 0.02$). Overall, β-actin mRNA levels in myometrium were 2–3-fold higher than in endometrium.

As in the myometrial samples, there was no CaBP-9k expression detectable in placenta when total RNA was subjected to Northern blot analysis using up to 20 μg of RNA and after 1 wk of exposure (data not shown). In order to determine whether there was any CaBP-9k expression in myometrium and placenta, RT-PCR experiments were carried out. When myometrial RNA was amplified in 25 cycles of PCR, a strong amplification product was obtained from samples taken at Days 10–12 of pregnancy (Fig. 5). Interestingly, on Day 15 and in particular on Day 18 the amount of product decreased substantially. This decrease was similar to the pattern seen in endometrial CaBP-9k regulation

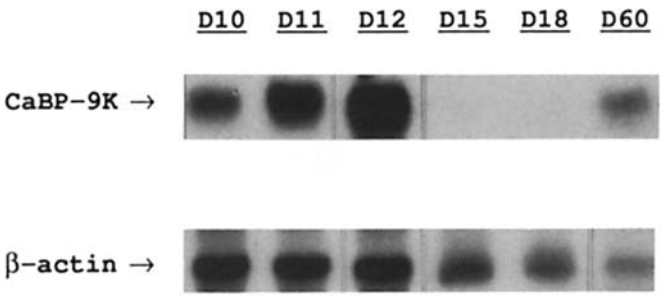


FIG. 3. CaBP-9k mRNA expression during early pregnancy in porcine endometrium. Total RNA was analyzed as described in Figure 1. Tissues were removed at pregnancy Days 10, 11, 12, 15, 18, and 60.

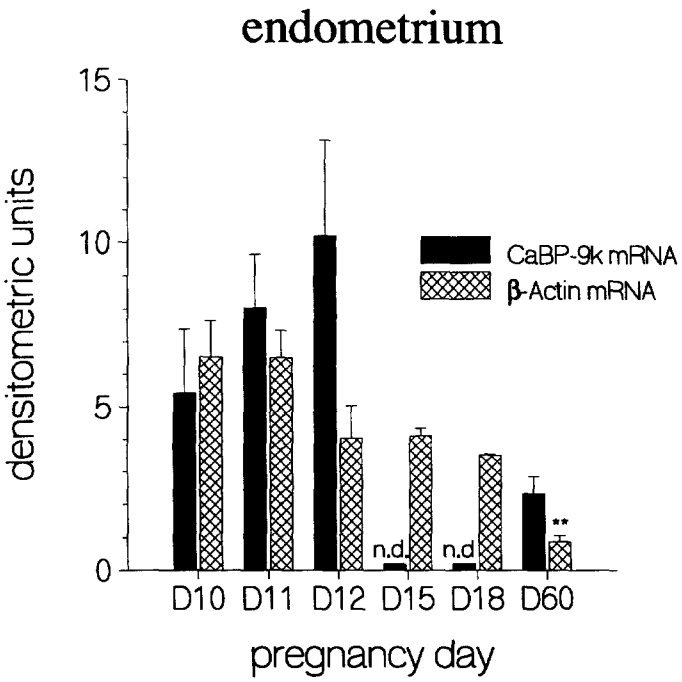


FIG. 4. Quantification of CaBP-9k mRNA in endometrium during early pregnancy. Groups (n = 3) of animals were as in Figure 3. CaBP-9k and β-actin mRNA was quantified as in Figure 2 n.d., non detectable; ** $p < 0.02$ compared to D10.

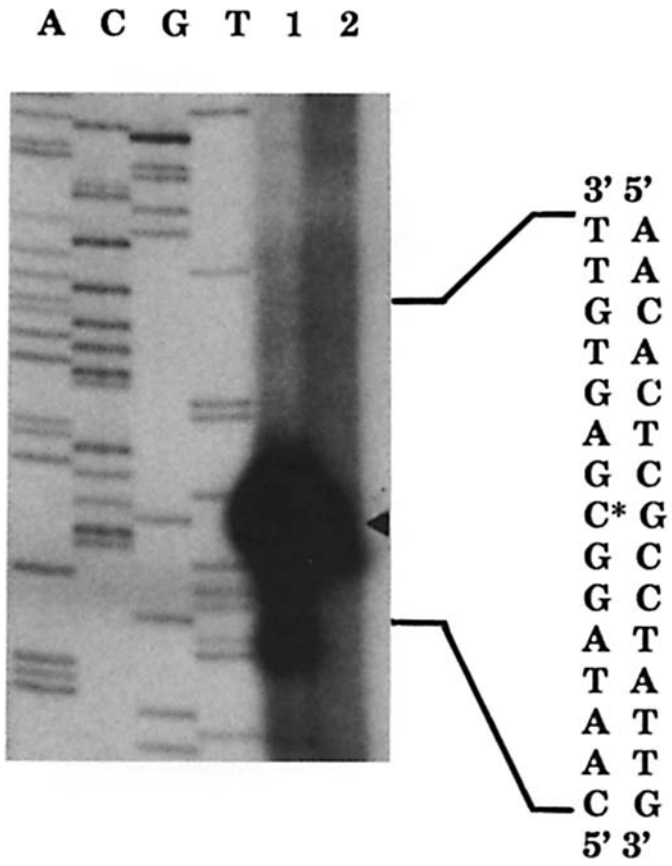


FIG. 6. Transcription initiation site of porcine CaBP-9k gene. Primer extension analysis was performed using an [α-^{32}P] end-labeled primer and intestinal (1) or skeletal muscle (2) total RNA. Extension product was electrophoresed on 6% denaturing acrylamide gel alongside an [α-^{35}S]-dATP labeled pUC19 sequencing reaction as size marker. Sequence of plasmid is indicated on right. Gel was overexposed to show weaker [^{35}S] isotope. * Band equivalent to size of extension product.

(Fig. 3). On Day 60, the product formation increased again in comparison to that on Days 15 and 18.

All placental samples analyzed (two separate samples from gestation Days 45, 75, 90, and 112) revealed expression of CaBP-9k mRNA. No major changes were apparent under the same PCR conditions described for analysis of myometrial RNA as a function of pregnancy state.

Characterization of the CaBP-9k Gene

Primer extension analysis. As a first step in characterizing the gene encoding the porcine CaBP-9k, the transcription initiation site and position and sequence of the first intron were determined. Figure 6 shows the result of a primer extension experiment. When duodenal RNA was used with the primer described in *Materials and Methods*, a prominent extension product of 123 nucleotides was detected, which agrees with the predicted size based on the cDNA sequence [15]. No extension product was detected when porcine skeletal muscle RNA was used as a negative control on short exposures of the gel (Fig. 6), which

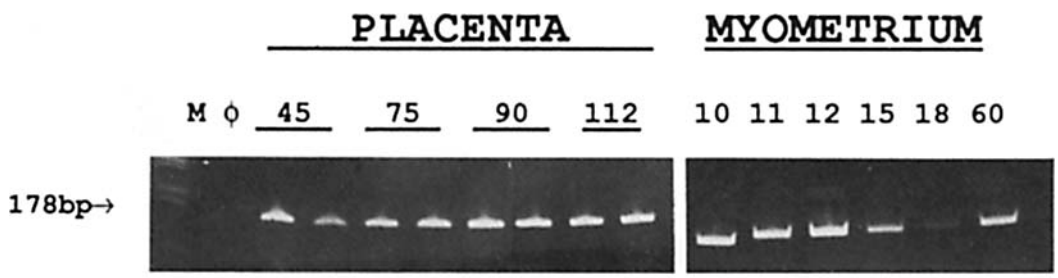


FIG. 5. Expression of CaBP-9k mRNA in porcine placenta and myometrium. Total RNA from placenta and myometrium at various times of pregnancy (Days 10–112) were subjected to RT-PCR. Amplification products were electrophoresed on 6% polyacrylamide gel. Each sample was reverse-transcribed and amplified at the same time under the same conditions. M, molecular weight standards, 0, negative control without reverse transcriptase.

did not allow detection of the ^{35}S sequencing signals. The experiment confirmed that the previously published cDNA contains the complete 5′ end of the transcript.

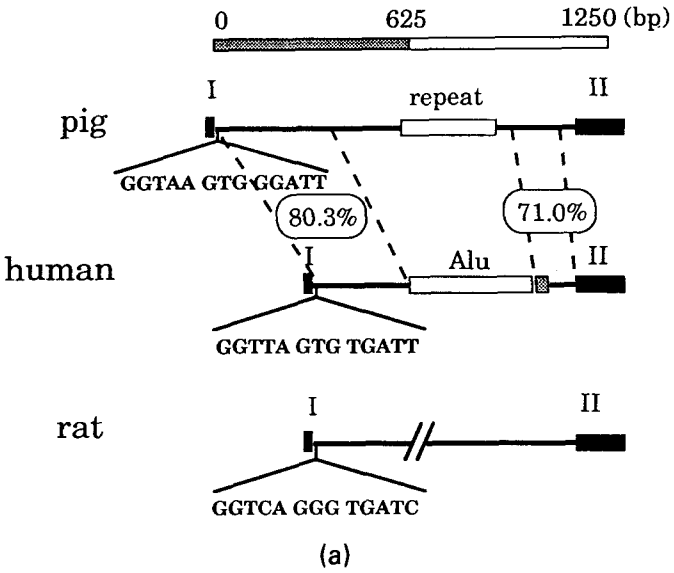
Amplification of intron A. For amplification of the porcine CaBP-9k, PCR (30 cycles) was carried out with P5 and P3 primers and with use of genomic DNA from pig liver. A Southern blot of the PCR product was hybridized with an oligonucleotide within the region synthesized with the amplification primers. The prominent product had a size of approximately 1250 nucleotides, which included 178 nucleotides of exonic DNA (data not shown). Therefore, this fragment appeared to contain about 1.1 kb of genomic DNA representing intron A of the pig CaBP-9k gene. The product was cloned into the *Sma* I site of pUC19, and the sequence was determined. Results of this experiment are presented in Figure 7. The sequence was subjected to computer analysis, and both the 5′ and 3′ ends matched the cDNA sequence, indicating that exons I and II are separated by intron A (1061 nucleotides). The sequence was compared to the GenBank/EMBL data base. A number of reported porcine gene sequences scored high identity to one region within the pig CaBP-9k gene, which was identified as a "short interspersed repeat." Interestingly, when the sequences of intron A of the human and porcine CaBP-9k genes were compared, two segments of relative high identity were detected. The first segment covers the 5′ end of intron A and stretches over 356 bp with 80.3% identity. The other region is located at the 3′ end and scored 71% identity over 152 bp. The sequence was also examined for a motif resembling the ERE located at +51 on the intron A of the rat CaBP-9k gene. A motif similar to the rat ERE was identified at the boundary of exon I and intron A of the pig gene:

rat CaBP-9k ERE: GGTCA GGG TGATC

porcine CaBP-9k: GGTAA *GTG GGATT*

The ability of this sequence to bind the ER was tested in a gel retardation experiment.

Gel retardation analysis. Figure 8 presents the results of gel retardation analysis using the ERE-like sequence from the pig CaBP-9k gene. As shown in Figure 8B, both the ER-Evit and EREr CaBP sequences formed a retardation complex that increased in intensity upon heat transformation of the cytosol. The EREpCaBP failed to form such a complex, as did the negative control element EREm. To provide further evidence that the complex observed in this assay was due to the ER, an ER antiserum was added to the incuba-



(a)

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1     ATTCCTATTT GATTCTTCCA GCTGCCGCAC GACTGGACGA CCAGGTAAGT
51    GGGATTTGAG TGCTACCCGC ACCTCCTCCA GCTGCCTTTC AGAACGGCGG
101   GAAAGAACAA AGTGGGTTTG TGCCCATCTA AAAACGTCAC CGGTAACTTC
151   AGAGGCTAGA AGCTGTGGTC GAGTTTCTTT AAGACCACAG CCTACAGAGT
201   GCAGAGCAGT TACATGAAAA GTGAAGAATG TCCAAGTTAC TAGTAAAAGT
251   GTTTGGCGCC AGAGACTCTC TTCTAACAGA TGTACTCGAA GAACAGACAC
301   TGAATGTACA ATCTAAGATG ATTCTTCTAT ATTAAATATA TGTATATTTT
351   TTTGTCTTTT TACAGCTGTA CCTGGGACAT ATGAAGTTCC CAGGCTAGGG
401   GTCGAATCTG AGTTGCCAGC TGCCAGCTTA CACCACAGGC CACGTTGGAT
451   CCGAGCCGAG TCTGCCATCT ACACCCCAGC TCAAGGCCAC GCGACCCTTA
501   ACCCACTGAG CGAGGCCCAG GGATCGAACC GAATCCTCAT GGATACTAGT
551   CAGGTTATTA ACCTGCTTGA GCCACAAAAG GAAACTCTAA GATTTTTTTT
601   TTTTTTTTTA AAAGGTTGTT AGCAGGAGTT CCCACTGTGG CTCGGTGGTA
651   ATCAACCCGA CCAGTATCAG CAAGAGGGGG GCTTCATTCC CTGGCCTCAC
701   TCAGTAGGTT AAGGAATCTG CATGGCCTTG AGCTGTGGTT GTAGATGGCA
751   GACTCAGCTC GGGATCCAGC GTGGATGTGG TGTAGCGCGG CGGCTACAGC
801   TCTGATTCAA CCCCTAGCCC GGGAACTTTC CATATGCCAT GGATGCAGCC
851   TTAAAAAGGA CTGGAAAAAA AAAAAAAAAA AAAAAAGGTG TGAGCAGTCA
901   CTGGTGCATT GTCGTTCCCA CAGTTCCCCG CCCCGTCTCT CCTTCTCCTC
951   TTTATCCTCA CCCTCCCCTT TTCTTTATTC TCTCTCACTG TGCCAGGGAA
1001  ACGGCCCCCC CCCCCCACGC CAACGCACTC ACCTCCCGCT GGCAATGATA
1051  AGTATAAAAC CCCACTTCGG CTTTTTGGTA AGTTCATGTC TTTCTCCATT
1101  TACAGGACAC CAAAATGAGT GCCCAAAAGT CTCCTGCAGA ACTGAAGAGC
1151  ATTTTTGAAA AATATGCAGC CAAAGAAGGG GATCCAAACC AGCTGTCGAA
1201  GGAGGACGTG AAGCAACTGA TTCAGTCTGA ATTCCCCAG
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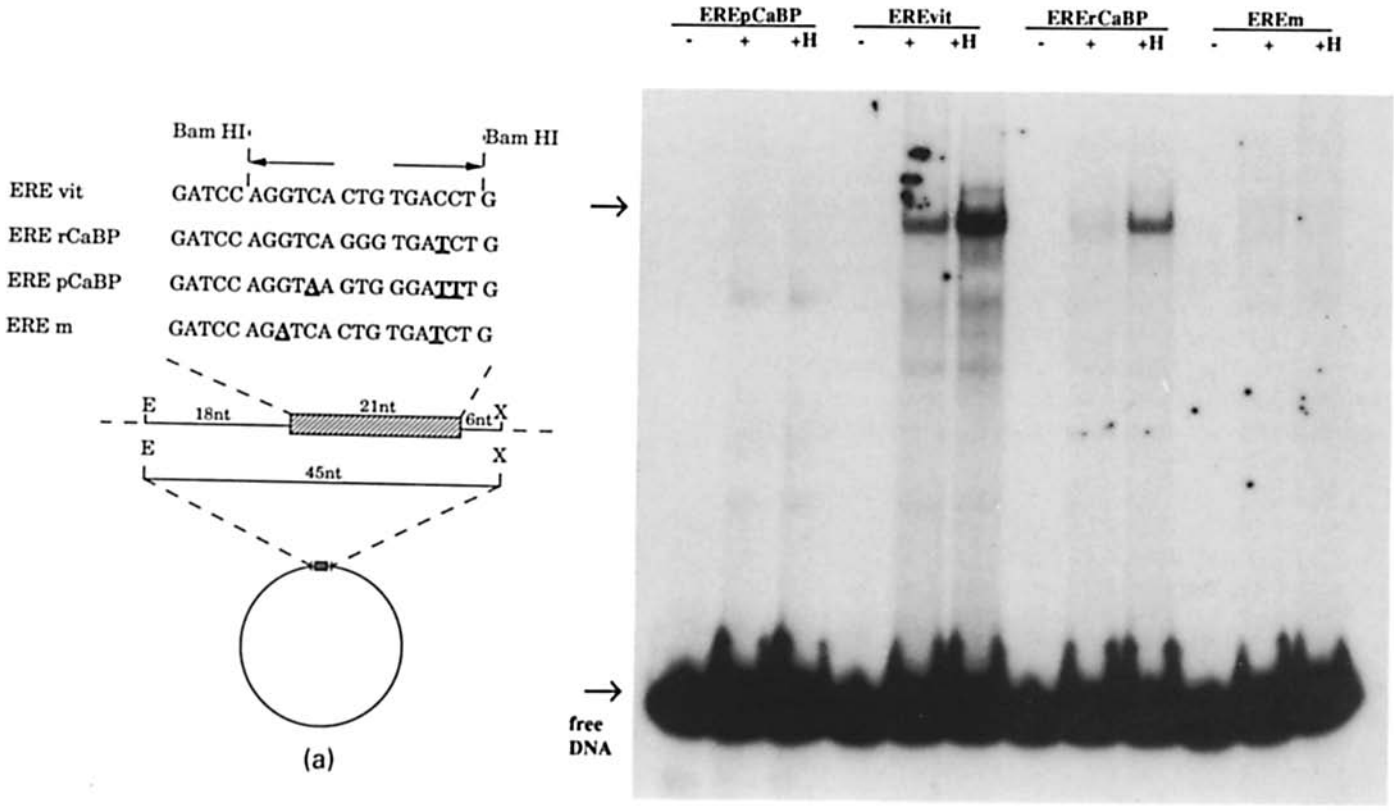
(b)

FIG. 7. A) Schematic structure of pig CaBP-9k intron A region. Schematic structures of CaBP-9k gene in pig, human, and rat covering part of exons I and II and intron A are indicated. Percent identity over two regions between pig and human genes are indicated. A sequence at boundary of exon I and intron A representing an ERE (rat) or ERE-like sequence is given on top of each gene. Black boxes, exons; open boxes, repeats; lines, remaining intronic sequences. B) Nucleotide sequence of amplification product containing intron A of pig CaBP-9k gene. Exonic sequence is printed in bold and italic; PCR primers are underlined; ERE-like sequence is boxed; repeat sequence is double-underlined.


EREpCaBP
EREvit
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Bam HI
ERE vit GATCC AGGTCA CTG TGACCT G
ERE rCaBP GATCC AGGTCA GGG TGATCT G
ERE pCaBP GATCC AGGTΔA GTG GGATTT G
ERE m GATCC AGΔTCA CTG TGATCT G
E
18nt
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6nt
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(a)
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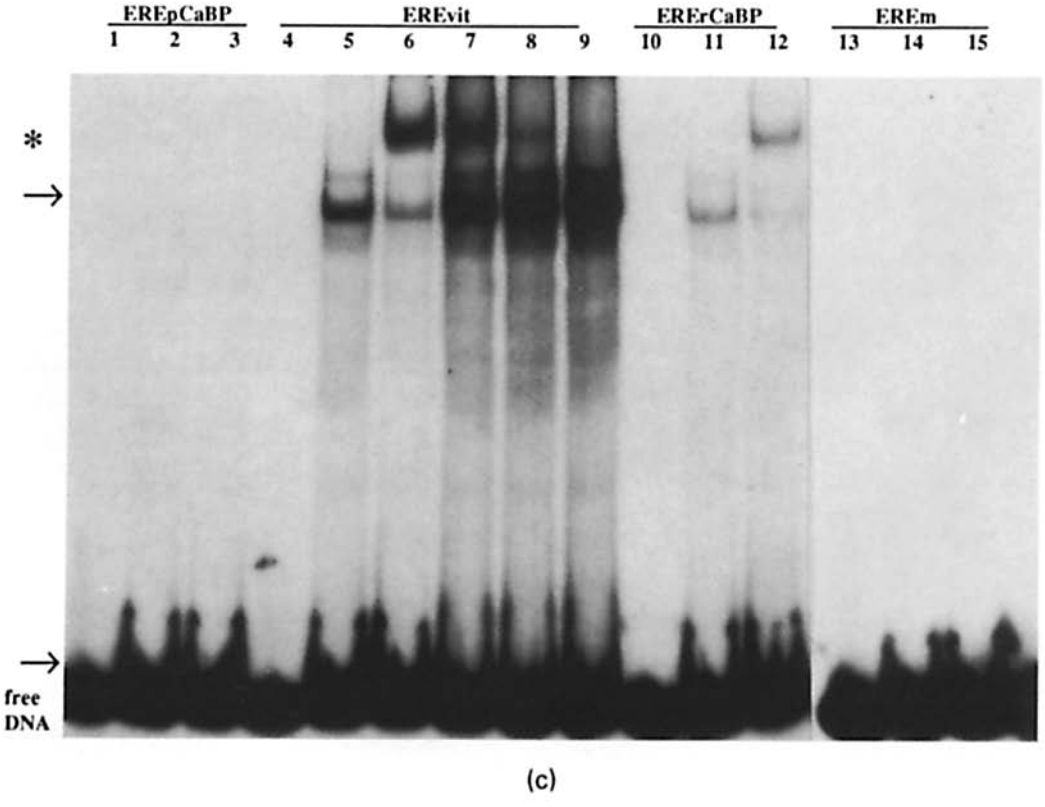
EREpCaBP
EREvit
EREr CaBP
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1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
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free DNA
(c)

tion. Lanes 6–9 in Figure 8C demonstrate the effect of decreasing amounts of antiserum. A 1:5 dilution was able to immunoshift most of the complexes with EREvit and ERErCABP to higher molecular weight. Addition of immune serum to the EREpCaBP and EREm had no effect.

DISCUSSION

The aim of the present study was to determine whether the porcine uterus does express CaBP-9k in a steroid hormone/gestational-dependant manner as has been shown in the rat [19–23]. In the rat CaBP-9k gene, a strong ERE has been characterized [6] that provides the key element within the promoter region in mediating of CaBP-9k expression in the uterus (and possibly placenta). Interestingly, this is one of the few genes carrying an enhancer element in an intronic region downstream of the promoter.

The present data indicate that the pattern of the porcine CaBP-9k gene expression in the uterus is distinct from that of rats and humans. There is a striking difference in expression levels between endometrium and myometrium. Expression in endometrium is high, although much less than in intestine, with sufficient concentrations to allow detection in 10 μg total RNA upon short-exposure autoradiography. In contrast, myometrial levels of CaBP-9k are very low and required a more sensitive technique for detection. In this case we used RT-PCR to detect CaBP-9k transcripts. From these semi-quantitative experiments it appeared that although the levels of expression are different, the overall pattern of expression as a function of pregnancy is similar in endometrium and myometrium. This contrasts with the rat uterus, where expression of CaBP-9k in endometrium and myometrium has been shown by in situ hybridization [33] and immunohistochemistry [34] to occur at similar levels. In unpublished experiments we also observed identical concentrations of CaBP-9k mRNA in 21-day pregnant rat myometrium and endometrium by Northern blot analysis.

In contrast to the rat, ovariectomy of pigs did not lead to a disappearance of CaBP-9k transcripts, as determined 10 days after surgery. When OVX rats are treated with a single injection of E_2, CaBP-9k mRNA is rapidly induced to very high levels [6]. This mode of regulation appears to be different in the pig. It remains unclear whether the ovariectomy in these animals was sufficient to eliminate endogenous E_2 to same extent as in the rat model. The present experiment differs from others reported in the rat also in that E_2 was injected on 8 consecutive days and not as a single dose 24–48 h prior to RNA quantification. Aliquots of the same RNA used for the present study have been analyzed for expression of antileukoproteinase (ALP) expression [35]. In these experiments, E_2 treatment caused a strong induction of ALP expression. The effects of P_4 on CaBP-9k expression, however, are similar in both species, displaying a strong inhibitory effect on CaBP-9k expression. This has been shown by P_4 treatment of cycling [19] or immature [20] rats and is seen under physiological conditions during early pregnancy [36] and lactation [23] in the rat. Progesterone dominance in rat pregnancy from Days 2 to 10 and in lactation suppressed CaBP-9k expression nearly completely. A similar effect was seen in the present data using early-pregnant pigs. After high expression was found on Days 10–12 of pregnancy, a rapid down-regulation was seen on Days 15 and 18. Similar to the situation during rat pregnancy, where on Day 10 CaBP-9k mRNA began to reappear, in pregnant pigs low-level expression was detected at Day 60. In unpublished experiments, we have also detected CaBP-9k mRNA at high levels in late-pregnant endometrium (Days 75, 90, and 105). The cause of suppression of CaBP-9k at Days 15 and 18 is unclear. Despite high P_4 and low E_2 plasma levels at this time in pig pregnancy [37], uterine E_2 levels were high. The mechanism by which P_4 suppresses CaBP-9k mRNA expression in the rat appears to involve down-regulation of the ER in uterus. The rat promoter seems to require relatively high levels of ER for CaBP-9k gene transcription. A reduction below a critical amount of ER levels by P_4 results in inhibition of CaBP-9k gene expression [23, 36].

Genomic porcine DNA was analyzed to determine whether the pig CaBP-9k gene carries an ERE near the promoter similar to that reported in the rat. First, the transcription initiation site was determined to confirm that the cDNA sequence determined previously was full-length and the 5′ part of the gene (exon I) could be targeted. The primer extension experiment clearly produced a prominent product of the predicted size. Under the assumption of similar exon/intron structure, two primers were used to span intron A of the pig CaBP-9k gene. As in the human CaBP-9k gene [18], the pig intron A was substantially larger than the corresponding rat intron [30, 31]. This is partly due to the presence of an Alu-type repeat in the human and a "short interspersed repeat" in the pig gene. This type of repeat is commonly found in genomic DNA and 3′ noncoding mRNA sequences in the pig. Two of those genes are the FSH-β subunit [38] and the sarcoplasmic/endoplasmic-reticulum calcium pump [39]. The inhibin β_A mRNA carries

FIG. 8. Gel retardation analysis of ERE-like element in pig CaBP-9k gene. A) Schematic structure and DNA sequence of DNA elements used for gel retardation. Test sequences were synthesized as oligonucleotides with *Bam*HI compatible ends and cloned into pUTKAT1. DNA was prepared by releasing test sequence through *Eco*RI/*Xba* I (E/X) digest, producing 45-nucleotide (nt) fragment. As positive controls, Xenopus vitellogenin A_2 ERE (EREvit) and rat CaBP-9k ERE (ERErCaBP) were used. As negative control, palindromic double-point mutant of EREvit (EREm) was used. B) Gel retardation with rat uterine cytosol with and without heat treatment. DNA fragments labeled with [^{32}P] were incubated and electrophoresed in absence (−) or presence (+) of cytosol. To heat-transform ER in vitro, cytosol was incubated at 25°C for 1 h prior to assay (+H). → Retardation complex and free DNA. C) Gel retardation in presence of ER-antiserum. To identify involvement of ER in retardation complex, ER antiserum was added in lanes 3, 6–9, 12, and 15. Lanes 1, 4, 10, and 13 were without cytosol; lanes 2, 5, 11, and 14 had cytosol added. Lanes 6–9 indicate decreasing amounts of antiserum included in the assay: 1:5, 1:25, 1:125, 1:625. * Immunoshifted complex.

such a repeat in the 3′ noncoding region [40]. When the sequence was compared to the human CaBP-9k intron A, two regions of significant identities and substantial size were found. At present, an interpretation of this finding is lacking. Most interesting, however, is the similarity of the sequence positioned at the boundary of exon I and intron A. The rat version of this motif represents a strong ERE-type enhancer that is able to cause estrogen inducibility to a heterologous promoter in the absence of other transcription factor binding sites linked to it [16]. Although the porcine gene had 9 of the 13 residues of this region conserved, the element failed to bind the ER when analyzed in gel retardation experiments. It is surprising that the ERE-like sequence in the pig gene was inactive as shown previously for the human CaBP-9k gene, since expression of CaBP-9k in pig uterus and placenta was observed. Similar changes in an ERE that directs expression of the oxytocin gene in several species including human have been shown [41, 42]. Because of the presence of multiple EREs in the oxytocin gene [41], however, the disruption of one ERE in the human oxytocin gene did not eliminate expression. Such a disruption of an enhancer, causing elimination of expression, appears to have occurred in the human CaBP-9k gene. There is no ERE in intron A, and the gene is not expressed in uterus or placenta. Both human and rat CaBP-9k gene sequences have been determined up to 2500 or 1300 nucleotides upstream [31, 43] of the initiation site, and no potential ERE motif in either flanking region was identified. The porcine CaBP-9k gene appears to be different from both these genes. Intron A does not harbor an ERE, but the gene is expressed in uterus and placenta. In addition, there is a striking difference in levels of myometrial and endometrial expression not found in the rat. We speculate that the pig gene does in fact contain an ERE responsible for this expression pattern either in the 5′ flanking region or elsewhere in the locus. It will be of interest to locate this ERE and determine the differential requirements for additional transcription factor and the potency of such an enhancer. We predict that the putative porcine ERE is distinct from the rat CaBP-9k ERE and might represent the key element in understanding the distinct differences in the expression of this gene in the three species.

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