Expression of calcium binding protein D-9k messenger RNA in the mouse uterine endometrium during implantation

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To investigate the molecular mechanisms of implantation, we constructed a cDNA library of mouse uteri enriched with pregnancy-induced genes by subtractive hybridization and polymerase chain reaction (PCR). One of the isolated clones was the cDNA for the calcium binding protein D-9k (CaBP9k), which is considered to regulate intracytoplasmic concentration and transport of free calcium ions. Northern blot and in-situ hybridization analyses demonstrated that the CaBP9k mRNA was expressed in the endometrial epithelia, both luminal and glandular, in the uterus at the time of implantation. On pregnancy day 5 it was detected in the luminal, but not in the glandular, epithelia. In the oophorectomized adult mice, progesterone enhanced CaBP9k mRNA expression in the uterus, whereas oestrogen did not. Consistent with this, a nucleotide change was identified in the first intron of mouse CaBP9k gene corresponding to the oestrogen responsive element in the rat CaBP9k gene. Transfer of embryos into the uterine cavity of pseudopregnant mice reduced the expression of CaBP9k mRNA in the glandular epithelium, suggesting that CaBP9k mRNA expression is also regulated by embryonal signal(s). These findings demonstrated that CaBP9k mRNA is expressed in the endometrial epithelia during the implantation period under the control of progesterone and the presence of embryo, and suggest that CaBP9k plays a role in implantation by regulating the local calcium concentrations.

Key words: calcium binding protein/cDNA subtraction/embryonal signal/endometrium/oestrogen responsive element

Introduction

Successful implantation and development of the embryo requires complex interactions between the embryo and the uterus. In the mouse, when a blastocyst reaches the uterus 4 days after fertilization, the uterine endometrium undergoes certain changes that prepare it to be receptive for implantation such as the closure of the uterine lumen, the increase of vascular permeability and decidualization. Implantation proceeds to the adhesion stage, and the trophoderm cells of the embryo displace the uterine epithelium at the site of implantation, attach and penetrate the underlying basement membrane and invade the uterine stroma in a controlled fashion (Dey, 1996). These processes are regulated by a timely interplay of the maternal hormones, oestrogen and progesterone (Psychoyos, 1973, 1993; Dey, 1996). In addition, some endometrial changes, such as increased permeability of the subepithelial capillaries surrounding the blastocyst, seem to occur in response to the blastocyst. Many signals and molecular pathways have been recognized that induce or regulate the complex series of interactions required for implantation, including cell surface adhesion molecules, cytokines and growth factors and their receptors (Cross et al., 1994; Tabibzadeh and Babaknia, 1995). Leukemia inhibitory factor is revealed to be essential for blastocyst implantation (Stewart et al., 1992). The role of the epidermal growth factor (EGF) family of growth factors in uterine biology and implantation has been studied extensively (Huet-Hudson et al., 1990; Tamada et al., 1991; Das et al., 1994, 1995). The expression of heparin-binding EGF is induced by blastocyst in the uterine epithelium just prior to the attachment reaction and only at the site of blastocyst implantation (Das et al., 1994).

To identify the genes induced in human endometrial stromal cells during decidualization, we previously used the cDNA subtractive hybridization technique and demonstrated that expression of tissue inhibitor of metalloproteinase-3 (Higuchi et al., 1995) and tissue transglutaminase (Fujimoto et al., 1996) is induced by progesterone. In the present study, we sought to identify the genes involved in the implantation process by subtracting cDNA constructed from the uteri of non-pregnant mice from cDNA from those of pregnancy day 5 mice. One of the identified cDNA encoded the calcium binding protein D-9k (Calbindin-D9k, CaBP9k). We examined the expression profile of CaBP9k mRNA in the uteri of pregnant and pseudopregnant mice during the implantation period. Furthermore, we investigated the factors regulating CaBP9k mRNA expression in the uterus.

Materials and methods

Animals

Male and female CD-1 mice were obtained from Charles River Japan Inc. (Kanagawa, Japan). Six week old females were mated with adult...
males, and the presence of vaginal plug after mating was designated day 1 of pregnancy. Pseudopregnant mice were prepared by mating with vasectomized males. Oestrous stage was ascertained by examining vaginal smears as described (Watanabe et al., 1997). Uteri from three animals on each day of pregnancy or pseudopregnancy were used for Northern blotting and in-situ hybridization.

Construction of a subtracted cDNA library
The cDNA subtraction was performed as described previously (Higuchi et al., 1995). Total RNA was extracted from the uteri of mice at oestrous or pregnancy day 5 by centrifugation through 4 M guanidinium isothionate and 5.7 M CsCl gradient. Polyadenylated [poly(A)+] RNA was prepared using a Messagemaker Reagent Assembly (Life Technologies, Rockville, MD, USA). After the synthesis of a double-stranded cDNA from each poly(A)+ RNA using a Time Saver cDNA Synthesis kit (Pharmacia, Piscataway, NJ, USA), the cDNA was enzymatically digested into lengths of ~500 bp. The cDNA was then ligated with oligonucleotide adapters specific to each cDNA library and amplified by PCR using primers specific to each adapter. A biotinylated primer was used for cDNA made from the oestrous uteri (competitor cDNA) to label the cDNA with biotin. The cDNA from the pregnant uteri (target cDNA) were then hybridized with an excess of competitor cDNA. Biotinylated hybrids were subtracted by streptavidin incubation and phenol–chloroform extraction. The subtracted cDNA was recovered in the aqueous phase, amplified by PCR using primers for the target cDNA and subjected to another three rounds of subtractive hybridization. The subtracted cDNA were finally cloned into pBluescript SK(−) plasmid (Stratagene, La Jolla, CA, USA), and the DNA sequence of each clone was determined using an autosequencer (DSQ-1000; Shimadzu, Kyoto, Japan).

Northern blotting
For Northern blot analysis, total RNA was extracted from tissues by using Trizol Reagent (Life Technologies) according to the manufacturer’s protocol. Total RNA (10 µg/lane) was electrophoresed on a 1.0% agarose–formaldehyde gel and transferred to a nylon membrane (Hybond-N+, Amersham, Arlington, IL, USA). The membrane was incubated with prehybridization solution (Rapid Hybridization Buffer; Amersham) for 30 min at 65°C, and then hybridized with the 32P-labelled probes for 2 h at 65°C in the same solution. After hybridization, the filter was washed in 2× standard saline citrate (SSC) and 0.1% sodium dodecyl sulphate at 65°C for 30 min, and then subjected to autoradiography. The membrane was washed and rehybridized with mouse beta-actin probe to correct for the amount of loaded RNA (Takabatake et al., 1997b).

In-situ hybridization
Tissues were fixed with freshly prepared 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS) for 24 h, and then embedded in polyester wax after dehydration. In-situ hybridization was performed as described previously (Kaneko et al., 1993). Briefly, a plasmid containing the murine Cabp9k cDNA (clone F7-49) was linearized with the appropriate enzymes to provide sense and antisense templates. Digoxigenin-labelled single strand RNA probes were transcribed with T3 or T7 RNA polymerase, using a DIG RNA Labeling kit (Boehringer Mannheim, Mannheim, Germany). Before hybridization, the sections were dewaxed and hydrated with a graded ethanol series, fixed with 4% paraformaldehyde for 15 min and pretreated successively with proteinase K, 0.2 N HCl, and 0.25% acetic anhydride in 0.1 M triethanolamine. The sections were dehydrated with a graded ethanol series and air-dried. Hybridization was then carried out overnight at 50°C with ~0.5 mg/ml digoxigenin-labelled RNA probes in 50% formamide, 10% dextran sulphate, 10×Denhardt’s solution (10 mg Ficoll, 10 mg polyvinylpyrrolidone and 10 mg bovine serum albumin/ml), 600 mM NaCl, and 250 µg/ml Escherichia coli transfer RNA. After hybridization, the sections were treated with ribonuclease A (1 µg/ml) at 37°C for 30 min and washed twice in 2×SSC and 0.2×SSC for 20 min each at 50°C. The hybridized digoxigenin-labelled probe was detected with an alkaline phosphatase-conjugated anti-digoxigenin antibody and visualized with NBT/BCIP (DIG Nucleic Acid Detection Kit; Boehringer Mannheim). Hybridization with the sense probe was carried out at the same time under identical conditions and served as a negative control.

Treatment of mice with sex steroid hormones
Progesterone and 17β-oestradiol were purchased from Sigma (St Louis, MO, USA) and were dissolved initially in 100% ethanol and then diluted in sesame oil. Oophorectomy was performed under anaesthesia with nembutal (Dainabot, Osaka, Japan) as described (Takabatake et al., 1997a). Two weeks after the oophorectomy, the mice were given daily injections s.c. with progesterone (2 mg/mouse) and/or oestradiol (25 mg/mouse) for 4 days. The injected volume was 100 µl per mouse. Oophorectomized mice injected with vehicle (sesame oil) alone served as a control. Each injection was performed at 1700 h. Sixteen hours after the last injection, the animals were killed by cervical dislocation, and their uteri were collected for RNA isolation.

To examine the kinetics of Cabp9k mRNA induction by progesterone, total RNA was extracted from the uteri of oophorectomized mice 1, 3, 6, 12, 24 or 48 h after a single injection with progesterone (2 mg/mouse) or vehicle alone. RNA was also extracted from the uteri of oophorectomized mice 16 h after the last daily injection with progesterone (2 mg/mouse) or vehicle alone for 1, 2, 3, 4 or 5 days. Three animals were used for each treatment group in these experiments.

Embryo transfer
Embryo transfer was performed as described previously (Takabatake et al., 1997a). Donor mice (5 week old) were injected i.p. with pregnant mare serum gonadotrophin (PMSG; Teikoku Zoki, Tokyo, Japan) (5 IU/mouse) followed by human chorionic gonadotrophin (HCG; Teikoku Zoki) (5 IU/mouse) 48 h later to induce ovulation, and mated with male mice. Blastocysts were collected by flushing the uteri of donor mice on pregnancy day 4. The recipient pseudopregnant mice (6 week old) were prepared by natural mating. At 1600 h on pseudopregnancy day 4, 10 blastocysts were transferred to the left uterine horn of the recipient mice. The medium without blastocysts was injected to the right horn of the same animals as a control. Uteri were collected 36 h later, fixed in 4% paraformaldehyde and used for in-situ hybridization. Four and three animals were used as the donor and the recipient, respectively.

Sequence determination of 5′ end of cDNA and intron of the Cabp9k gene
To determine the 5′ end of the murine Cabp9k cDNA sequence, 5′ rapid amplification of cDNA ends technique (5′ RACE) was performed using a Rapid Amplification of cDNA Ends Kit (Boehringer Mannheim) according to the manufacturer’s protocol. First strand cDNA was synthesized from the total RNA derived from the pregnancy day 5 uterus, and the antisense gene-specific primer 1 (GSP 1) [5′-AGGTAGTGCAAGAAGATGTTG-3′] and GSP 2 [5′-ATTTTCTCTCA-CACGATCTCTAT-3′] were used. PCR products were cloned into pBluescript SK(−) plasmid, and sequenced. To determine the nucleotide sequence of full-length cDNA, several clones were analysed after PCR amplification using the 5′-end and 3′-end primers of Cabp9k.
Mouse genomic DNA was prepared from the liver of a 6 week old CD1 female mouse using standard protocols. By using a sense primer [5'-TCACCTGCTGTTCTGTTCTG-3'], an antisense primer [5'-CGTTGCTCCGAACTTGCCTTT-3'] and an Expand High Fidelity kit (Life Technologies), Cabp9k genomic fragment was amplified by PCR. PCR was performed for 30 cycles under conditions recommended by the manufacturer.

Results

Isolation of Cabp9k partial cDNA clone (F7-49)

After analysing the subtracted cDNA library of mouse uterus enriched with pregnancy-induced genes, we isolated one clone (clone F7-49). Northern blot analysis using clone F7-49 as a probe revealed that a 0.5 kb band was clearly observed in the pregnancy day 5 uterus, but hardly in the oestrus uterus (Figure 1). The nucleotide sequence of the 369 bp insert of this clone was determined, which showed 87% homology with that of the rat Cabp9k cDNA (Darwish et al., 1987). The deduced amino acid sequence was highly homologous with that of rat CaBP9k (94% homology) (MacManus et al., 1986), and identical to that of reported mouse intestinal CaBP9k (Hunt et al., 1989). Thus, we concluded that the clone F7-49 was a part of the mouse uterine Cabp9k cDNA. To determine the 5' end of clone F7-49 cDNA sequence, we carried out the 5' RACE technique. After analysing five independent cDNA clones, the 423 bp Cabp9k cDNA sequence was determined (Figure 2A) (deposited in GenBank, accession number: AF028071).

Northern blot analysis of Cabp9k expression in the uteri during early pregnancy

To examine uterine Cabp9k mRNA expression during early pregnancy and pseudopregnancy, Northern blot analysis was carried out (Figure 3). In the uteri of oestrus phase and pregnancy day 1 or 2 mice, the Cabp9k mRNA expression was very weak. On pregnancy day 3, the expression of Cabp9k mRNA was increased, reached a peak on day 4, keeping the high level until day 6 and then decreased on day 7. In pseudopregnant mice, the expression of Cabp9k was almost identical to that in pregnant mice.

Effects of progesterone and oestradiol on Cabp9k mRNA expression in the uteri of oophorectomized mice

To determine whether the Cabp9k mRNA expression is regulated by sex steroid hormones in the uterus, we administered progesterone and/or oestradiol to oophorectomized mice. By Northern blot analysis, an increase in the level of Cabp9k mRNA was observed in the groups treated with progesterone for 4 days with or without oestradiol (Figure 4A). Although oestradiol is known to induce expression of Cabp9k mRNA in the rat uterus (L’Horset et al., 1990), oestradiol treatment alone did not affect the Cabp9k mRNA expression in the mouse uterus.

We next examined the time course of Cabp9k mRNA expression induced by progesterone in the oophorectomized mice. After a single administration of progesterone, an increase in the level of Cabp9k mRNA was observed within 12 h, and the expression level reached a peak at 24 h, and then it decreased (Figure 4B). Daily injection of progesterone resulted in progressive increase in the level of Cabp9k mRNA until day 3, then the level decreased in spite of the continued administration of progesterone (Figure 4C).

In-situ hybridization analysis of Cabp9k mRNA expression in the uterus of pregnant mice

To identify the cells expressing Cabp9k mRNA in the uteri of pregnant mice, in-situ hybridization analysis was carried out. In pregnant mice, Cabp9k mRNA expression was observed in the luminal and/or glandular epithelia of uterus, but not in the stroma or myometrium (Figure 5A–C). In the luminal epithelium, the expression level was low on day 3 (data not shown), increased during the following 2 days and remained high on day 6. However, no signal was detected around the implantation site on day 6. In the glandular epithelium, the expression of Cabp9k mRNA was observed within 12 h, and the expression level reached a peak on day 4, but it completely disappeared on day 5.

In pseudopregnant mice, Cabp9k mRNA was also specifically localized in the luminal and glandular epithelia, and no significant expression was detected in the stroma or myometrium (Figure 5D–F). The expression level in the luminal epithelium was low on day 3 (data not shown), and increased during the following 2 days and remained high on day 6. In contrast to the findings in pregnant mice, the expression of Cabp9k mRNA in the glandular epithelium was observed from day 3 to day 5, and in some glandular epithelium it was still detected on day 6. Sections from two other animals for each group showed the same expression patterns as described above (data not shown).

Figure 1. Northern blot analysis of clone F7-49 gene expression in the mouse uterus. Lane 1, 10 µg RNA from uterus of mice at oestrus; lane 2, pregnancy day 5; F7-49 cDNA probe (upper panel). The arrow indicates the hybridized band. β-Actin probe to correct for the amount of RNA loaded (lower panel).
Figure 2. Sequence of mouse Cabp9k. (A) The nucleotide sequence of the identified full-length mouse Cabp9k cDNA and the deduced amino acids. (B) Comparison of the amino acid sequence deduced from mouse uterine Cabp9k cDNA with mouse intestine CaBP9k (Hunt et al., 1989) and rat CaBP9k (MacManus et al., 1986). The single letter amino acid code is used, and residues common to all sequences are indicated by asterisks.

Effects of the presence of embryo on the expression of Cabp9k mRNA in the uterus

To determine whether Cabp9k mRNA expression in the glandular epithelium is affected by the presence of the embryo, we transferred blastocysts into the uteri of mice on pseudopregnancy day 4. In-situ hybridization analysis showed that the glandular epithelium of the embryo-transferred uterine horn did not express Cabp9k mRNA 36 h after the embryo transfer either at the implantation site or interimplantation site although the luminal epithelia continued to express Cabp9k (Figure 6A, B). In contrast, the glandular epithelium near the lumen continued to express Cabp9k mRNA 36 h after transfer of medium alone in the control horn of the same animal (Figure 6C).

Sequence analysis of intron A in the mouse Cabp9k gene

The intron located between exon I and exon II (intron A) of the rat Cabp9k gene contains an oestrogen responsive element (ERE), and Cabp9k transcription has been shown to be
increased by oestradiol in the rat (Darwish et al., 1991). Since we did not observe such an increase in Cabp9k expression by oestradiol in the mouse, we analysed the mouse genomic DNA sequence corresponding to the rat intron A. Sequence analysis of the mouse intron A obtained by PCR revealed that the candidate ERE was positioned in the same region as in the rat Cabp9k gene, near the boundary of exon I and intron A (Figure 7A). However, in this imperfect palindromic sequence, one nucleotide was different from that of rat (Figure 7B).

**Discussion**

In the present study, we have constructed a subtracted cDNA library of mouse uteri enriched with pregnancy-induced genes, and isolated one clone whose nucleotide sequence was highly homologous to that of the rat Cabp9k cDNA. Northern blot analysis showed that the size of the transcript was the same as rat Cabp9k mRNA, and that it was expressed in the uterus of pregnancy day 5 mice. The deduced amino acid sequence of this clone was identical to that of the reported mouse Cabp9k. We determined the full-length nucleotide sequence of Cabp9k cDNA obtained from the mouse uterus and found that the deduced complete amino acid sequence consisted of 79 amino acids. In the mouse intestine, two isoforms (major and minor proteins) of CaBP9k have been detected and their partial amino acid sequences have been determined by tandem mass spectrometry (Hunt et al., 1989). The reported 77-amino acid sequence from the carboxy-terminus of the major isoform was identical to the deduced carboxy-terminal 77 amino acid sequence of the uterine CaBP9k, although the latter had two additional amino acids at the amino-terminus. The minor isoform of intestinal CaBP9k is reported to have one amino acid inserted into the major isoform (Hunt et al., 1989). Since no cDNA clone corresponding to the minor isoform was isolated in the present study, the mouse uterus is considered to express mainly the major isoform of the intestinal CaBP9k.

CaBP9k is a small intracellular calcium binding protein (M, 9000) with two high-affinity calcium binding domains (Wasserman et al., 1968). It was originally isolated from the rat intestine as vitamin D-dependent gene, and found to be expressed in mammalian intestine, duodenum, placenta and uterus (Bruns et al., 1978; Delorme et al., 1983; Mathieu et al., 1989). In the uterus of non-pregnant rats, Cabp9k is expressed in the myometrium and endometrial stroma (Delorme et al., 1983). In the uterus of pregnant rat, Cabp9k gene expression decreases in the preimplantation period and is absent on pregnancy day 5 (Krisinger et al., 1994). It increases after mid-pregnancy, being localized in late gestation in the myometrium, luminal epithelium and the juxtaposed yolk sac epithelium as well as the intraplacental yolk sac (Warendorn et al., 1987; Mathieu et al., 1989). In the mouse uterus after mid-pregnancy, the expression pattern of Cabp9k has been shown immunohistochemically to be similar to that of rat, being localized in the luminal epithelium and the yolk sac (Bruns et al., 1985). In the present study, we found that the Cabp9k mRNA was strongly induced in the endometrial epithelial cells during the peri-implantation period in pregnant mice. Since mRNA level does not necessarily parallel with the level of protein expression, the CaBP9k protein level remains to be determined.

The uterine receptive phase for embryos, the so-called implantation window, is considered to be regulated by sex steroid hormones (Psychoyos, 1973, 1993; Dey, 1996). We found that the expression of Cabp9k mRNA increased on day 3 of pregnancy (Figure 3) when the serum progesterone concentration is high (Psychoyos, 1973, 1993). This suggests that Cabp9k mRNA expression is regulated by sex steroid hormones in the uterus. In the rat uterus, oestradiol has been shown to stimulate Cabp9k gene expression at the transcriptional and post-transcriptional levels (L’Horset et al., 1990), and the imperfect ERE present in the intron A, which is slightly different from the consensus ERE and has lower activity for oestradiol response, has been implicated in transcriptional regulation (L’Horset et al., 1994). The effect of oestradiol has been blocked by the simultaneous administration of progesterone or testosterone (Bruns et al., 1988; L’Horset et al., 1993). To investigate the regulatory mechanism of Cabp9k mRNA expression in the mouse uterus, we examined the effect of progesterone and oestradiol using the oophorectomized mouse model. In contrast to the findings in the rat uterus, progesterone induced a marked increase in the expression of Cabp9k mRNA, and oestradiol alone exhibited no significant effect on Cabp9k mRNA expression at a dose comparable to that used in the rat, suggesting that the expression of Cabp9k in the uterus is regulated in a different manner between mouse and rat.

**Mouse CaBP9k expression during implantation**

Figure 4. Northern blot analysis of the effects of oestradiol and progesterone on the Cabp9k mRNA expression in the uterus of oophorectomized mice. (A) Effects of oestradiol and progesterone. Uterine RNA from oophorectomized mice injected with vehicle alone (lane 1), progesterone (lane 2), oestradiol (lane 3), or progesterone and oestradiol (lane 4) for 4 days. (B) Kinetics of induction by progesterone. Uterine RNA from the oophorectomized mice 12 h after injection with vehicle alone (lane 1), or 1, 3, 6, 12, 24 or 48 h after single injection with progesterone (lanes 2, 3, 4, 5, 6 and 7, respectively). (C) Effect of daily injection with progesterone. Uterine RNA from the oophorectomized mice injected with vehicle alone for 1, 2, 3, 4 or 5 days (lanes 1, 2, 3, 4 and 5, respectively) or with progesterone for 1, 2, 3, 4, or 5 days (lanes 6, 7, 8, 9 and 10, respectively).
Figure 5. In-situ hybridization analysis of Cabp9k mRNA expression in mouse uterus. Sections of the uterus made from mice on pregnancy day 4, 5 or 6 (A, B and C, respectively) or mice on pseudopregnancy day 4, 5 or 6 (D, E and F, respectively) and hybridized with antisense probe for mouse Cabp9k. Expression was observed in the endometrial epithelium and no significant signal was observed in the endometrial stroma or in the myometrium. Note the absence of signals on the glandular epithelium of pregnant mice on days 5 and 6 (B and C, respectively), and its presence on that of pseudopregnant mice (E and F). Hybridization with sense probe did not give significant signal (data not shown). le = luminal epithelium; ge (arrow heads) = glandular epithelium; imp (an arrow) = implantation site. Bars = 100 μm.

et al., 1992). Studies using transgenic mice have revealed that different tissues utilize distinct cis-acting elements to direct the expression of the rat Cabp9k gene (Blin et al., 1996; Romagnolo et al., 1996).

Species differences in the regulation of Cabp9k gene expression have also been described. The mouse kidney expresses Cabp9k, but the rat kidney does not (Schreiner et al., 1983; Li and Chiristakos, 1991). In the uterus of non-pregnant cows, Cabp9k mRNA expression is 3-fold higher during the progesterone-dominated luteal phase than oestrogen-dominated follicular phase (Inpanbutr et al., 1994). A similar hormonal regulation has been observed in human myometrium (Miller et al., 1994). In accord with this, the ERE-like sequence in the human Cabp9k gene is suggested not to bind to the oestrogen receptor (Jeung et al., 1994). We isolated the mouse Cabp9k gene and compared the intron A sequence with that of rat. The ERE of rat Cabp9k present in the intron A is an imperfect palindromic sequence of 15 bp recognized by the oestrogen receptor (Darwish et al., 1991). The mouse intron A contained a slightly different sequence of 15 bp at the corresponding position. For the response to oestrogen, the third nucleotide ‘A’ in the latter half of the palindrome is essential and conserved in the rat Cabp9k gene (Klock et al., 1987; Beato et al., 1989, 1991). However, it is changed to ‘G’ in the mouse Cabp9k gene, which possibly explains the absence of response to oestradiol in the mouse uterus, although the
Mouse CaBP9k expression during implantation

Figure 6. Effects of the presence of embryo on expression of Cabp9k mRNA. Transverse sections of the uterus were made from a pseudopregnant mouse 36 h after the embryo transfer, and analysed by in-situ hybridization. Implantation site (A) and interimplantation site (B) of the embryo-transferred horn and control horn (C) hybridized with antisense probe. Insets: higher power views of glandular epithelia indicated by white squares. Note the absence of Cabp9k mRNA expression in the glandular epithelium of the embryo-transferred horn, and its presence in the control horn. The localized partial staining of luminal epithelium in (B) was not reproducible and complete staining was observed in repeated experiments. le = luminal epithelium; ge (arrow heads) = glandular epithelium. Bars in lower power views = 100 µm; bars in insets = 20 µm.

effect of this change for response to oestradiol has not been examined (Truss et al., 1991).

In rodents, the interactions between embryo and endometrium are necessary for complete decidualization of the endometrial stroma (Parr and Parr, 1989; Dey, 1996). In this study, in-situ hybridization histochemistry showed that the expression of Cabp9k mRNA disappeared in the glandular epithelium on day 5 of pregnancy. In the pseudopregnant mice, the expression of Cabp9k was observed in the glandular epithelium on day 5, suggesting that it is regulated not only by the sex steroid hormones but also by the embryonal signal(s). To further examine this possibility, we performed the embryo transfer experiments. While Cabp9k mRNA was detected by in-situ hybridization in the glandular epithelium of the control horn, it was not detected in the embryo-transferred horn 36 h after embryo transfer. These results indicated that the expression of Cabp9k mRNA in the glandular epithelium was suppressed by the presence of blastocysts on day 5 of pregnancy.

CaBP9k has been proposed to regulate the intracytoplasmic concentration and transport of free Ca$^{2+}$ (Wasserman et al., 1968; Mathieu et al., 1989). In the mammalian myometrium, CaBP9k probably maintains uterine quiescence during pregnancy by acting as cytoplasmic Ca$^{2+}$ buffers (Mathieu et al., 1989; Miller et al., 1994). The induction of CaBP9k in the rat placenta and endometrial epithelium at the time of maximal fetal bone growth and accumulation of calcium suggests its involvement in maternal–fetal calcium transport (Mathieu et al., 1989). In this study, we showed that the Cabp9k mRNA expression was increased in the mouse uterus during the period of implantation. Ca$^{2+}$-dependent signal transduction mechanisms have been implicated in the induction of the murine decidualization (Kyd and Murdoch, 1992). Sakoff et al. (1994) reported that the decidualized areas contained significantly greater amounts of Ca$^{2+}$ than non-decidualized areas, and that the content was decreased on day 5 of pregnancy.

Figure 7. Nucleotide difference between mouse and rat in the oestrogen responsive element (ERE) of Cabp9k gene. (A) Nucleotide sequence of intron A (boxed) of the mouse Cabp9k gene. The ERE-like sequence (underlined) is located at the boundary of exon I and intron A. (B) Comparison of the ERE-like sequence of mouse with the ERE of rat Cabp9k gene and the ERE consensus sequences.

| Mouse CaBP9k | AGGTCA GGT TCGTCT |
| Rat CaBP9k | AGGTCA GGG TCGCTC |
| ERE consensus | AGGTCA nnn TCGCTC |

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Since CaBP9k mRNA expression was observed in the luminal epithelium during the period of implantation, it may play a role in implantation by regulating the Ca\(^{2+}\) content and/or decidualization.

The conditions in the uterine cavity are important for successful implantation (Dey, 1996) and are influenced by the secretory activity of the glandular epithelium, which increases at the time of implantation (Given and Enders, 1980, 1981). To protect the implanting embryo from any hypercalcaemic effect and promote a quiescent state of the uterus, the Ca\(^{2+}\) concentration should be strictly regulated in the uterine cavity (Kyd and Murdoch, 1992). Calcitonin has been shown to be produced in the glandular epithelium at the time of implantation and has been proposed to be one of the local regulators of Ca\(^{2+}\) concentration in the uterine cavity (Ding et al., 1994).

In this study, the CaBP9k mRNA level was found to change in the glandular epithelium during the peri-implantation period and this alteration was influenced by the presence of embryos. These findings are consistent with a notion that CaBP9k plays a role as an intracytoplasmic calcium transporter in the glandular epithelium and is involved in regulation of calcium concentration in the uterine cavity during the peri-implantation period. Clarification of the precise roles played by CaBP9k should facilitate our understanding of the molecular mechanisms of successful decidualization and early embryogenesis.

Acknowledgement

This work was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan.

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Received on June 22, 1998; accepted on October 22, 1998.