BIOLOGY OF REPRODUCTION **67,** 351–358 (2002)

Differential Expression of Microsomal Prostaglandin E Synthase at Implantation Sites and in Decidual Cells of Mouse Uterus[1]

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ABSTRACT

Prostaglandin E_2 (PGE_2) is considered important for blastocyst spacing, implantation, and decidualization in the rodent uterus. PGE synthase (PGES) catalyzes the isomerization of PGH_2 to PGE_2. There are two isoforms of PGES, microsomal PGES (mPGES) and cytosolic PGES (cPGES). However, the expression and regulation of mPGES in the mammalian uterus during early pregnancy are still unknown. The aim of this study was to investigate the differential expression of mPGES in mouse uterus during early pregnancy and its regulation under different conditions by in situ hybridization and immunohistochemistry. Microsomal PGES expression in the preimplantation mouse embryos was also performed by reverse transcription polymerase chain reaction (RT-PCR). Expression of mPGES mRNA and protein was at a basal level in the luminal epithelium from Day 1 to Day 4 of pregnancy. However, mPGES mRNA and protein were highly expressed in the stroma immediately surrounding the blastocyst but not in the luminal epithelium on Day 5 of pregnancy. Microsomal PGES mRNA and protein were not detected in the pseudopregnant uterus from Day 1 to Day 5. During delayed implantation, mPGES mRNA and protein were also not detected in the uterus. Once delayed implantation was terminated by estrogen treatment and embryo implantation initiated, both mPGES mRNA and protein were induced to express in the stroma immediately surrounding the blastocyst, which was similar to the expression pattern on Day 5 of pregnancy. From Day 6 to Day 8 of pregnancy, the signals for mPGES mRNA and protein were strongly detected in the decidualized cells. Microsomal PGES mRNA and protein were also highly expressed in the artificially decidualized cells but not in the control horn. Microsomal PGES mRNA was detected in the oocytes and all the stages of preimplantation embryos. The strong mPGES expression in the implantation site and decidual cells suggests that mPGES might play an important role during implantation and more importantly in decidualization.

decidua, early development, female reproductive tract, implantation, uterus

INTRODUCTION

Implantation is a process in which the embryo makes a close physical and physiological contact with the maternal endometrium for the establishment of pregnancy. Successful implantation is the result of cell-cell communication between the blastocyst and uterus [1]. However, in most mammals, there is only a restricted time during the uterine cycle when implantation can occur [2].

Prostaglandins (PGs) comprise a diverse family of autocoids derived from cyclooxygenase (COX) metabolism of arachidonic acid to PGG/H_2, leading to the generation of five principal bioactive PG metabolites: prostaglandin E_2 (PGE_2), $PGF_{2\alpha}$, PGD_2, PGI_2, and TXA_2 [3]. PGs are important modulators of events at the implantation site and during the decidual cell reaction in laboratory rodents [4, 5]. After endogenous PG synthesis is inhibited by indomethacin, PG infused into the lumen of sensitized rat uteri is able to restore the endometrial vascular permeability response and decidualization [6–8]. PGE_2 has a major role in the hatching of mouse blastocysts [9] and is able to induce implantation of mouse blastocysts [10]. Among various PGs, PGE_2 and prostacyclin (PGI_2) have been considered as a primary candidate involved in implantation and decidualization in the rodents [11].

There are two isoforms of PGE synthase (PGES), microsomal PGES (mPGES) and cytosolic PGES (cPGES). cPGES, a constitutive enzyme expressed in a wide variety of cells and tissues, is predominantly linked with COX-1 to promote the immediate response, during which relatively high concentration of arachidonic acid are released in a short period [12]. mPGES, a 16-kDa membrane-associated and inducible perinuclear enzyme with glutathione-dependent activity, is expressed in a variety of tissues including prostate, testes, and small intestine [13]. Microsomal PGES is preferentially coupled with the inducible COX-2 to promote delayed PGE_2 generation and, if COX-2 already exists in cells, also regulates immediate PGE_2 generation [14]. So far the roles of COX-2 in reproduction have been extensively investigated. COX-2 deficiency affects all stages of early pregnancy leading to failure in ovulation, fertilization, implantation, and decidualization [15]. However, the expression and regulation of mPGES during early pregnancy are still unknown. The aim of this study was to investigate the expression and regulation of mPGES gene in mouse uterus during early pregnancy using multiple approaches.

MATERIALS AND METHODS

Animals and Treatments

Mature mice (Kongmin White outbred strain) were caged in a controlled environment with a 14L:10D cycle. All animal procedures were approved by the Institutional Animal Care and Use Committee of Northeast Agricultural University. Adult females were mated with fertile or vasectomized males of the same strain to induce pregnancy or pseudopregnancy, respectively (Day 1 = day of vaginal plug). Pregnancy on Days 1–4 was confirmed by recovering embryos from the reproductive tracts. The implantation sites on Days 5 and 6 were identified by intravenous injection of trypan blue solution.

To induce delayed implantation, the pregnant mice were ovariectomized under ether anesthesia at 0830–0900 h on Day 4 of pregnancy. Progesterone (1 mg/mouse) was injected to maintain delayed implantation from Day 5 through Day 7. Estradiol-17β (100 ng/mouse) was given to progesterone-primed delayed-implantation mice to terminate delayed implantation. The mice were killed to collect uteri 24 h after estrogen treatment. The implantation sites were identified by intravenous injection of trypan blue solution. Delayed implantation was confirmed by flushing the blastocysts from the uterus.

[1]Supported by Chinese National Natural Science Foundation grants 39825120, 39730250, and 30170110, and US CICCR/CONRAD CIG-01-64.

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Received: 17 December 2001.
First decision: 14 January 2002.
Accepted: 6 February 2002.
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ISSN: 0006-3363. http://www.biolreprod.org

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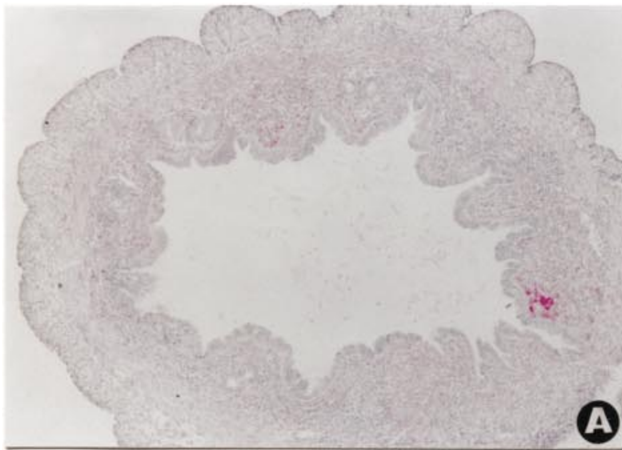

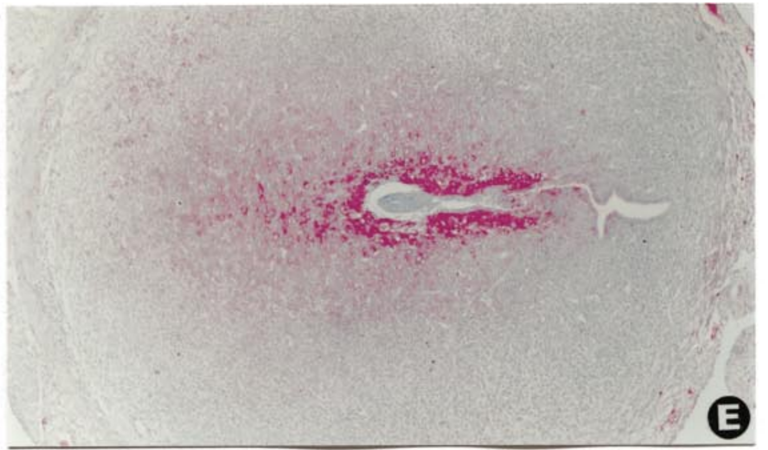

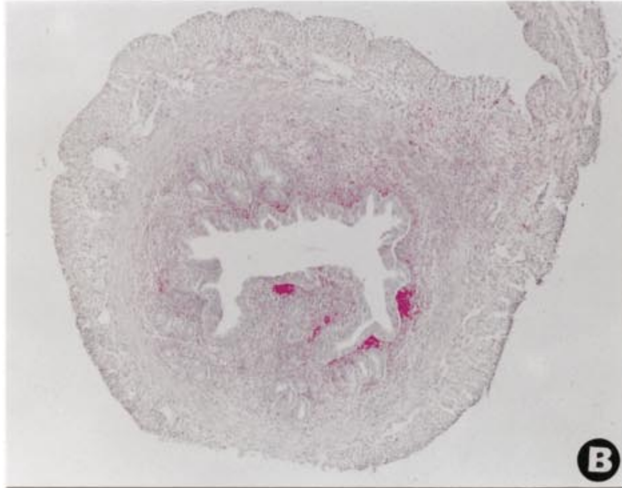

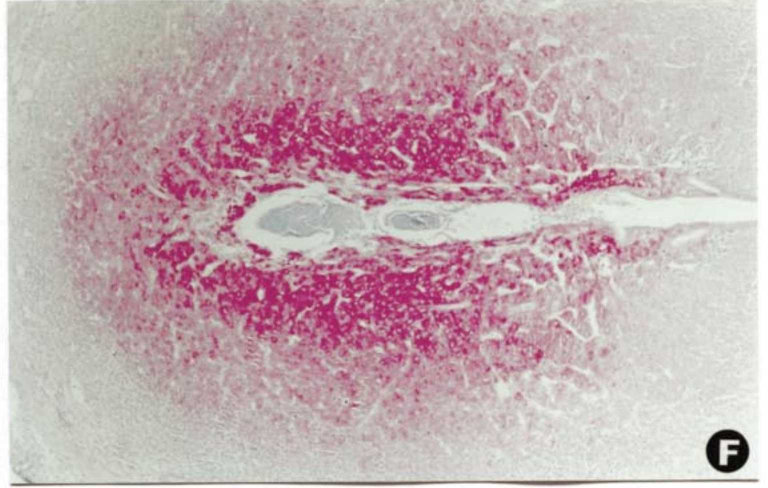

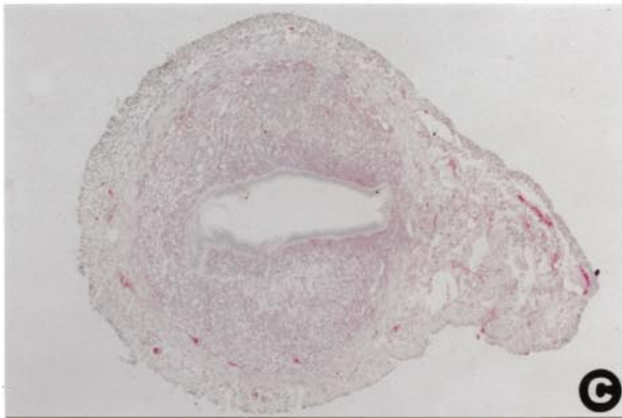

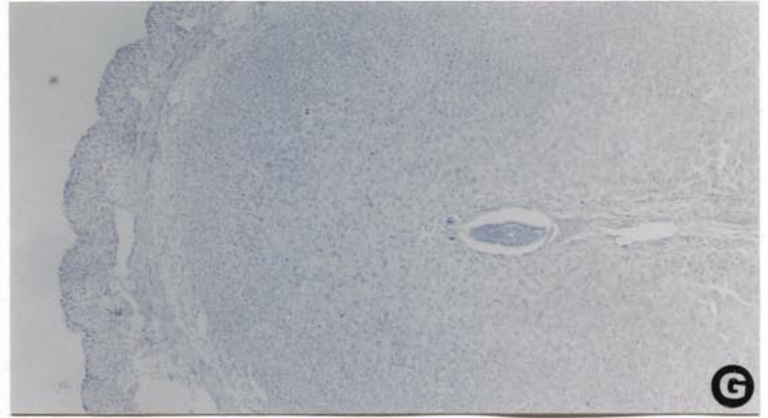

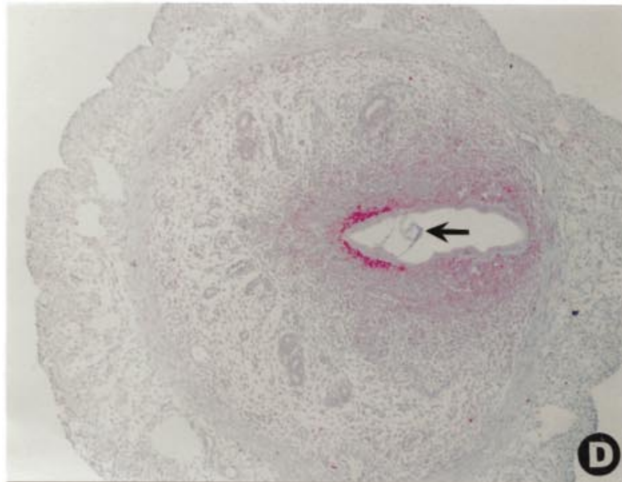

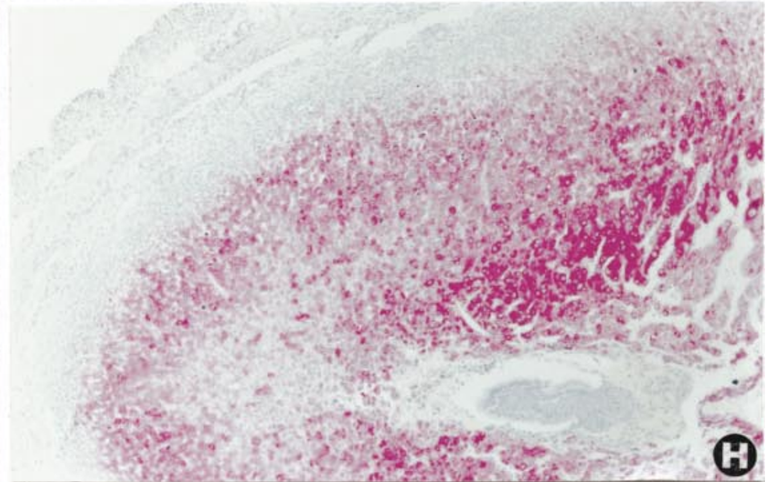

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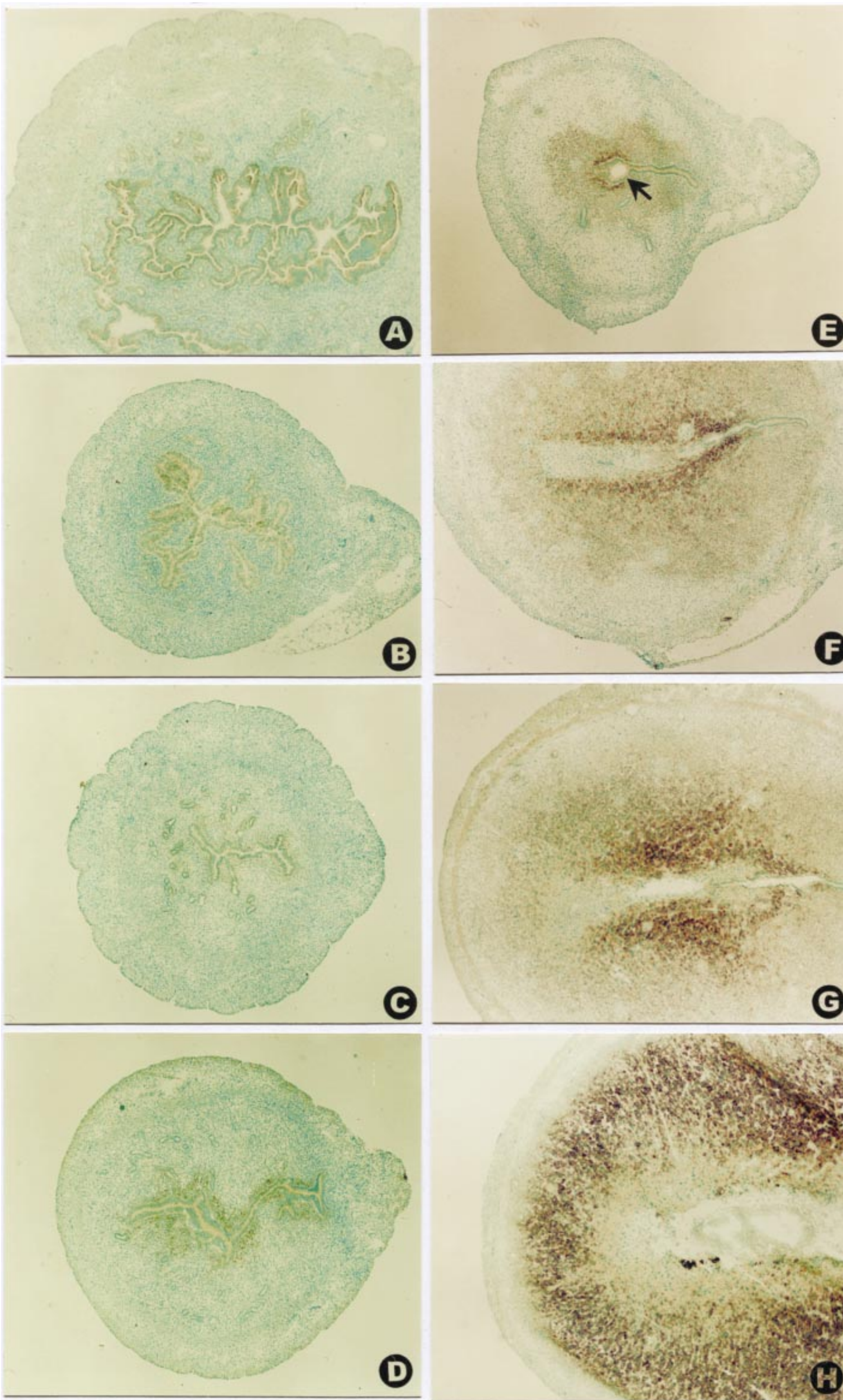

FIG. 2. In situ hybridization of mPGES mRNA in mouse uterus during early pregnancy. A basal level of mPGES mRNA was seen in the luminal epithelium on Days 1 (**A**), 2 (**B**), 3 (**C**), and 4 (**D**). Microsomal PGES was strongly detected in the stromal cells immediately surrounding the implanting blastocyst (arrow) on Day 5 (**E**) and in the decidualized cells on Days 6 (**F**), 7 (**G**), and 8 (**H**). ×400.

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FIG. 1. Immunostaining of mPGES protein in mouse uterus on Days 1 (**A**), 2 (**B**), 4 (**C**), 5 (**D**), 6 (**E**), 7 (**F**), and 8 (**H**) of pregnancy. On Day 5, mPGES immunostaining was mainly detected in the stromal cells immediately surrounding the implanting blastocyst (arrow) at the implantation site (**D**). Microsomal PGES immunostaining was strongly shown in the decidualized cells from Day 6 to Day 8. However, no mPGES immunostaining was observed in the Day 7 uterus when rabbit anti-human mPGES was replaced by normal rabbit IgG (**G**). ×400.

Artificial decidualization was induced by intraluminally infusing 25 μl sesame oil into one uterine horn on Day 4 of pseudopregnancy, while the contralateral uninjected horn served as a control. The uteri were collected on Day 8 of pseudopregnancy. Decidualization was confirmed by weighing uterine horns and by histological examination of uterine sections [15].

Immunohistochemistry

Mouse uteri were immediately cut into small pieces, fixed in Bouin solution, dehydrated, and embedded in paraffin. Sections (8 μm) were cut, de-

paraffinized, and rehydrated. Nonspecific binding was blocked in 10% normal horse serum in PBS for 1 h. The sections were incubated with rabbit anti-human mPGES (Cayman Chemical, Ann Arbor, MI) in 10% horse serum overnight at 4°C. After washing in PBS 3 times for 5 min each time, the sections were incubated with biotinylated secondary antibody followed by an avidin-alkaline phosphatase complex and Vector Red according to the manufacturer's protocol (Vectastain ABC-AP kit; Vector Laboratories, Burlingame, CA). Vector Red was visualized as a red color. Endogenous alkaline phosphatase activity was inhibited with levamisole (Sigma, St. Louis, MO). In some sections, rabbit anti-human mPGES was replaced with normal rabbit IgG as a negative control. The sections were counterstained with hematoxylin and mounted. The degree of staining was assessed subjectively by blinded examination of the slides by two investigators.

In Situ Hybridization

A 427-basepair (bp; 108–518 bp) mPGES cDNA fragment was reverse transcribed and amplified with forward primer 5′-CGGAATTCACACTG-CTGGTCATCAAG and reverse primer 5′-CGGGATCCTTCAGCTGCT-GGTCACAG (Genebank accession number AB041997) with the total RNA from mouse uterus. In these primers, protection bases (CG) and *Eco*RI sites were added at the 5′ end of the forward primer and protection bases (CG) and *Bam*HI sites at the 5′ end of the reverse primer. The PCR fragment for mPGES was recovered from the agarose gel and cloned into pGEM-3Zf (+) plasmid through *Eco*RI and *Bam*HI sites, respectively. The cloned mPGES fragment was further verified by sequencing. These plasmids were linearized with appropriate enzymes for labeling. Digoxigenin (DIG)-labeled antisense or sense cRNA probes were transcribed in vitro using a DIG RNA labeling kit (T7 for sense, SP6 for antisense; Boehringer Mannheim, Mannheim, Germany).

Uteri were cut into 4- to 6-mm pieces and flash frozen in liquid nitrogen. Frozen sections (10 μm) were mounted on 3-aminopropyltriethoxysilane-coated slides and fixed in 4% paraformaldehyde solution in PBS. The sections were treated in 0.2 N HCl for 10 min followed by 2 washings in PBS, digestion with 0.1 μg/ml proteinase K at room temperature for 10–15 min, and incubation in 0.2% glycine in PBS twice for 5 min each. Postfixation was performed in 4% paraformaldehyde for 2 min. After acetylation in freshly prepared 0.25% acetic anhydride in 0.1 M triethanolamine (pH 8.0) for 10 min, the sections were washed in 2× SSC (1× SSC is 0.15 M sodium chloride, 0.015 M sodium citrate) twice for 5 min each time, dehydrated in serial dilutions of ethanol, and air dried. Following the prehybridization in hybridization buffer (4× SSC, 50% formamide, 5% dextran sulfate, 1× Denhardt solution, 0.5 mg/ml denatured salmon sperm DNA, 0.25 mg/ml yeast tRNA) at 25°C for 2–3 h, the sections were hybridized in hybridization buffer with 1–5 μg/ml DIG-labeled antisense or sense RNA probe for mouse mPGES at 49°C for 16 h. After hybridization, the sections were washed in 4× SSC at room temperature for 10 min and digested in 10 μg/ml RNase A (Boehringer Mannheim) in 0.01 M Tris-HCl and 0.5 M NaCl (pH 8.0) at 37°C for 30 min. The sections were washed in 4× SSC, 1× SSC, and 0.5× SSC twice for 10 min each at 42°C. After nonspecific binding was blocked in 0.5% block mix (Boehringer Mannheim), the sections were incubated in sheep anti-DIG antibody conjugated with alkaline phosphatase (1:200; Boehringer Mannheim). The signal was visualized with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium. Endogenous alkaline phosphatase activity was inhibited with levamisole (Sigma). All of the sections were counterstained with methyl green. All of our in situ hybridizations performed with DIG-labeled sense probe were negative.

Reverse Transcription PCR

Female mice were superovulated with eCG (10 IU/mouse; Sigma) followed by an injection of hCG (10 IU/mouse, Sigma) 48 h later. The mice were caged with fertile males after hCG injection. The embryos at the stages of zygote, 2-cell, 4-cell, 8-cell, morula, and blastocyst were collected by flushing oviducts or uteri at 23, 42, 54, 68, 80, and 92 h post-hCG injection, respectively. Mature oocytes were collected from the unmated mice following eCG and hCG injection 14 h later. The granulosa and cumulus cells were removed by digestion with hyaluronidase (Sigma). A total of 100 oocytes or embryos at each stage of development were treated with TRIZOL (Gibco BRL, Gaithersburg, MD) with an addition of 100 μg yeast tRNA (Boehringer Mannheim) into each sample as a carrier RNA. The total RNA was extracted from oocytes or embryos according to the manufacturer's instruction. Final RNA was dissolved in DEPC-dH$_2$O and digested with RQ1 DNase I (Promega Corp., Madison, WI). Compared with the supplemented yeast tRNA, the RNA concentration from oocytes or embryos was much less. The RNA concentration of each sample was measured to calculate the recovery rate of sample RNA only based on the concentration of supplemented yeast tRNA. Extracted RNA was then diluted to have 2 oocytes or embryos in each sample prior to use.

The primers for mPGES were used to amplify mPGES mRNA in the preimplantation embryos. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a control for the quality of cDNA preparations. Mouse GAPDH primers were 5′-ACCACAGTCCATGCCATCAC-3′ and 5′-TCCACCACCCTGTTGCTGTA-3′ for a 452-bp fragment [16]. The RNA samples were reverse transcribed and PCR amplified using Takara BcaBEST RNA PCR kit (Takara Biotechniques, Dalian, China) according to the manufacturer's protocol. The amplification of mPGES cDNA was done for 45 cycles at 94°C for 30 sec, 60°C for 30 sec, and 72°C for 45 sec. The conditions for GAPDH amplification were the same as for m-PGES except that there were 36 cycles. PCR products were run on 1.5% (w/v) agarose gels containing 0.5 μg/ml ethidium bromide. To check for the specificity of RT-PCR, 3 controls were set for testing the contamination of genomic DNA: 1) RNA samples were directly amplified without reverse transcription, 2) reverse transcription was done without adding reverse transcriptase followed by PCR amplification, 3) RNA samples were replaced by DEPC-dH2O in RT-PCR. All the RT-PCR reactions were carried out only after no DNA contamination was detected in all 3 controls. All the RT-PCR reactions were repeated at least 3 times with 3 batches of oocytes and embryos.

RESULTS

Immunohistochemistry of mPGES Protein During Early Pregnancy

No mPGES immunostaining was seen in Day 1 uteri (Fig. 1A). On Days 2 and 3, mPGES was localized to a very limited area of subluminal stroma (Fig. 1B). A low level of signal was uniformly distributed in the uterus on Day 4 of pregnancy (Fig. 1C). On Day 5, mPGES protein was strongly observed in the subluminal stroma surrounding the implanting blastocyst, while no corresponding signal was seen at the mesometrial side in the implantation site (Fig. 1D). Moreover, there was no mPGES immunostaining at the interimplantation areas on Day 5 of pregnancy. On Day 6, mPGES protein was strongly localized in the secondary decidua (Fig. 1E). On Days 7 and 8, a high level of mPGES immunostaining was observed throughout the whole decidua (Fig. 1, F and H). However, no mPGES immunostaining was observed in the Day 7 uteri when rabbit anti-human mPGES was replaced by normal rabbit IgG (Fig. 1G).

In Situ Hybridization of mPGES mRNA During Early Pregnancy

Microsomal PGES mRNA localization in mouse uteri from Day 1 to Day 8 of pregnancy was shown by in situ hybridization (Fig. 2). A low level of mPGES mRNA was detected in the luminal and glandular epithelium in the uteri from Day 1 to Day 4 of pregnancy (Fig. 2, A–D). On Day 5, a strong signal was seen in the subluminal stroma surrounding the implanting blastocyst, although there was a low level of signal in the primary decidua (Fig. 2E). From Day 6 to Day 7, mPGES mRNA was strongly shown in the primary decidua (Fig. 2, F and G), while a strong signal

FIG. 3. Microsomal PGES immunostaining and mRNA in mouse uterus during pseudopregnancy and under artificial decidualization. No mPGES immunostaining was seen on Days 1 (**A**) and 5 (**C**) of pseudopregnancy. The signals for mPGES mRNA were also not detected on Days 1 (**B**) and 5 (**D**) of pseudopregnancy. Both mPGES protein (**E**) and mRNA (**F**) were not detected in the control uterine horn. After decidualization was artificially induced by injecting sesame oil into the pseudopregnant uterine horn on Day 4, a high level of mPGES protein (**G**) and mRNA (**H**) expression was seen in the decidualized cells. *Uterine lumen. ×400.

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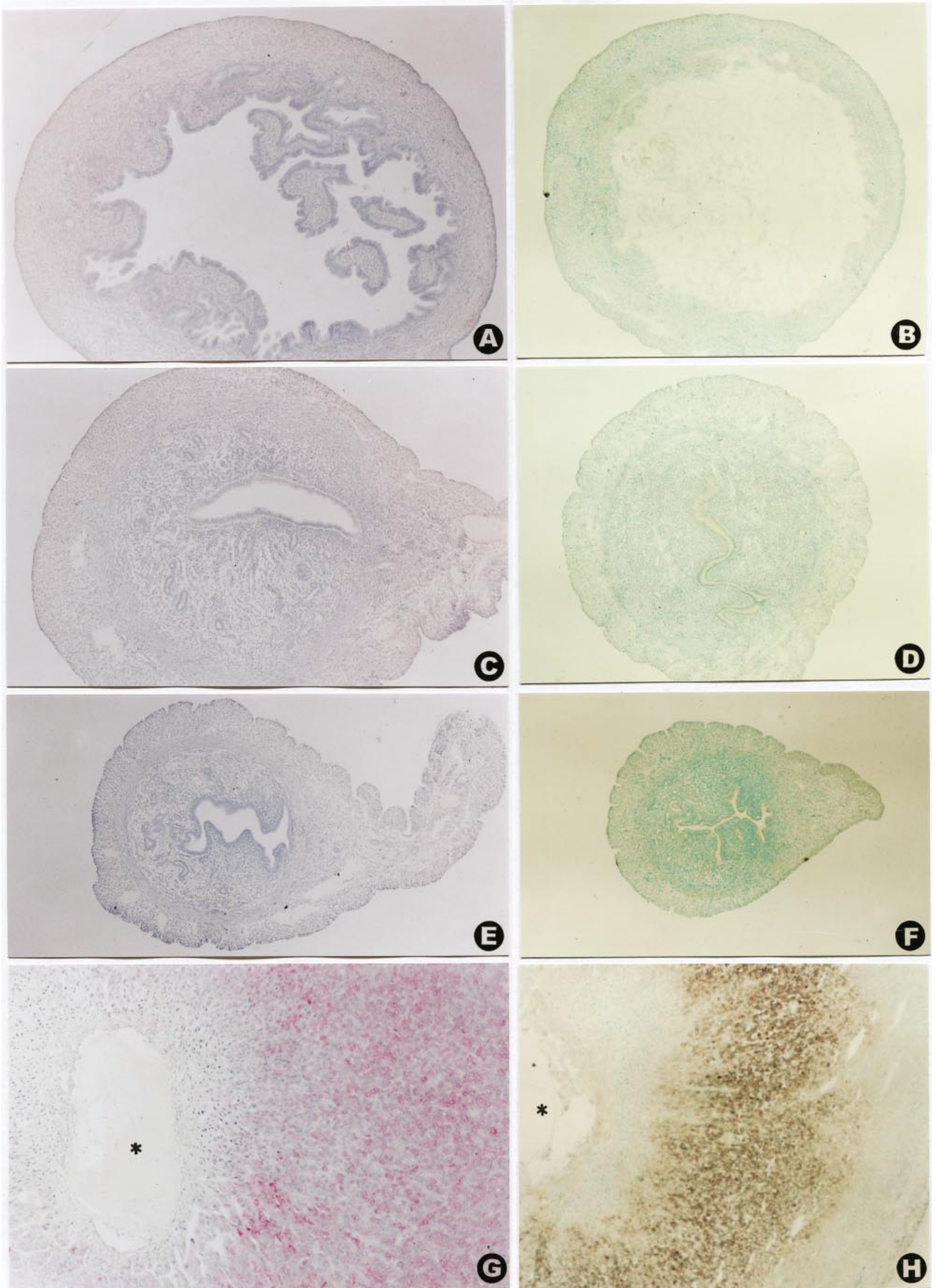

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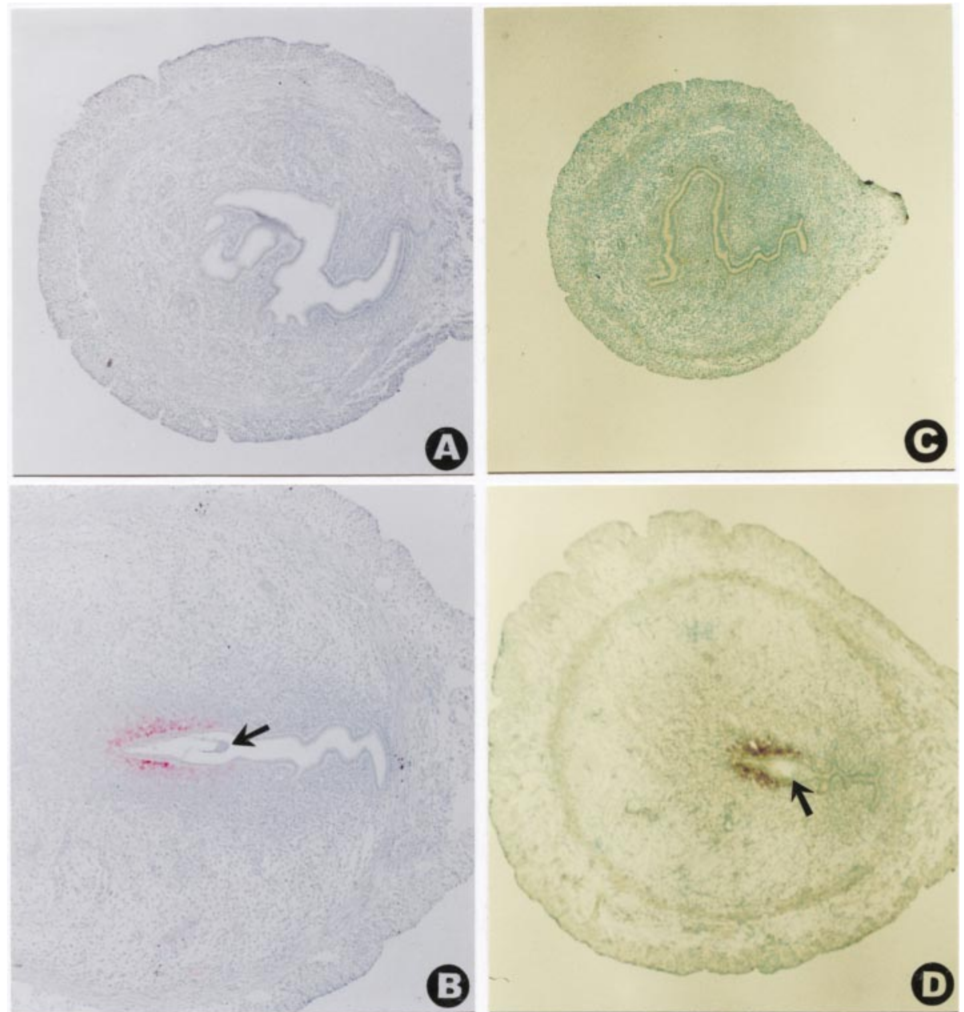

FIG. 4. Both mPGES protein (**A**) and mRNA (**C**) were not detected in the uterus during delayed implantation. After delayed implantation was terminated by estrogen treatment and the embryo was implanted, both mPGES protein (**B**) and mRNA (**D**) were strongly shown in the stromal cells immediately surrounded the implanting blastocyst (arrows). ×400.

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was detected in the secondary decidua on Day 8 of pregnancy (Fig. 2H). No mPGES signals were seen in the embryos from Day 5 to Day 8 of pregnancy by in situ hybridization (Fig. 2, E–H).

Expression of mPGES mRNA and Protein During Pseudopregnancy

From Day 1 to Day 8 of pseudopregnancy, mPGES immunostaining was not observed (Fig. 3, A and C). No mPGES mRNA signal was detected during the pseudopregnancy (Fig. 3, B and D).

Expression of mPGES mRNA and Protein under Artificial Decidualization

In the uninjected control horn, neither mPGES mRNA nor protein was detected in the whole uterus (Fig. 3, E and F). However, a very strong signal for both mPGES mRNA and protein was observed in the decidualized cells adjacent to myometrium 4 days after the pseudopregnant uterine horn was induced to be artificially decidualized by injecting sesame oil into the uterine lumen (Fig. 3, G and H). In the decidualized cells near the lumen, the signals for mPGES mRNA and protein were not observed.

Expression of mPGES mRNA and Protein in the Delayed Implanting Uterus Before and after the Initiation of Implantation

In the uterus under the progesterone-primed, delayed implantation, neither mPGES mRNA nor protein was detected (Fig. 4, A and C). Microsomal PGES immunostaining was strongly localized in the primary decidual zone immediately surrounding the implanting blastocyst 24 h after delayed implantation was terminated by estrogen treatment and embryo implantation initiated (Fig. 4B). The pattern of mPGES mRNA localization was the same as for the mPGES protein (Fig. 4D). There were no detectable mPGES mRNA and protein in the mesometrial side of the same implantation site, which was opposite to the implanting blastocyst (Fig. 4, B and D).

Microsomal PGES mRNA Expression in the Oocytes and Preimplantation Embryos by RT-PCR

The RT-PCR was performed to examine the expression of mPGES and GAPDH in the mouse oocytes and embryos at the stages of zygote, 2-cell, 4-cell, 8-cell, morula, and blastocyst. The predicted 452-bp fragment was seen with GAPDH primers (Fig. 5). GAPDH was used to verify the integrity of cDNA preparations. The predicted 427-bp fragment was obtained with mPGES primers. Microsomal PGES mRNA was expressed in all the stages examined

(Fig. 5). From our results, it seems clear that the levels of mPGES mRNA at the stages of oocyte, morula, and blastocyst were higher than those at other stages, but no quantification was performed.

DISCUSSION

PGE_2 is one of the principal prostaglandins produced and is unique among the prostanoids in that it interacts with multiple G-protein-coupled receptors. Currently, there are four PGE_2 receptors (EP1–EP4), which were initially classified by their ability to relax or contract smooth muscle. These receptors have been characterized functionally and cDNAs encoding each of these receptor subtypes have been cloned [17–20].

The initial attachment reaction between the uterine luminal epithelium and the blastocyst trophectoderm in mouse occurs at 2200–2300 h on Day 4 of pregnancy and is marked by a localized increase in endometrial vascular permeability around the blastocyst [21]. The attachment reaction is rapidly followed by proliferation and differentiation of stromal cells into decidual cells (decidualization). The decidual reaction can also be induced experimentally by an intraluminal injection of a small amount of oil into the uteri of pseudopregnant mice. In our study, both mPGES mRNA and protein were highly expressed in the decidual cells on Days 6–8 of pregnancy and in the decidual cells artificially induced, suggesting that mPGES may have an important role during the decidualization. PGE_2 produced in the decidual cells may act through the vasodilating receptor subtype EP2 and/or EP4, resulting in the local increase of endometrial vascular permeability and preparing for angiogenesis and placentation. EP2 mRNA expression was exclusively localized in the luminal epithelium primarily on Day 4 (the day of implantation) and Day 5 (early decidualization) of pregnancy [22]. We previously demonstrated that EP4 expression was detected in the luminal epithelium and stromal cells on Days 3 and 4 of pregnancy and also in the luminal epithelium and decidualizing stroma around the implanting blastocyst [23]. Since PGs are poor inducers of edema, they may induce this reaction through other factors. PGs are known as inducers of vascular endothelial growth factor (VEGF), which is angiogenic and stimulates vascular permeability [24, 25]. It is possible that increased endometrial vascular permeability at the implantation sites is mediated by VEGF, which is induced by PGE_2 via EP2 and/or EP4. Additionally, the localization of mPGES protein and mRNA within the endometrium was not always identical. Although a low level of mPGES was detected in the luminal and glandular epithelia on Days 1–4 of pregnancy, mPGES protein was localized to a limited area of the subluminal stroma on Days 2 and 3. The difference between mPGES mRNA and protein localization may result from a posttranscriptional regulation on mPGES expression since mPGES mRNA localization was wider than mPGES protein.

In this study, both mPGES mRNA and protein were specifically localized in the subluminal stroma surrounding the implanting blastocyst. Because this pattern of mPGES expression was not present in the corresponding pseudopregnant uterus or in the interimplantation areas of the pregnant uterus, the implantation site-specific expression must have been regulated by the blastocyst. Additionally, mPGES mRNA and protein were not detected in the delayed uterus and were only induced to express at the implantation site once the delayed implantation was terminated by estrogen treatment and delayed blastocysts implanted. PGE_2, a major product of COX, is elevated in the uterus following the application of a deciduogenic stimulus [5]. In the rat, prostacyclin and PGE_2 are higher in implantation sites than in the surrounding uterus [26, 27]. COX-2 was localized in the luminal epithelium of mice as early as 2 h following an intraluminal oil injection on Day 4 of pseudopregnancy [15]. Although COX-1 was expressed in uterine epithelial cells on the morning of Day 4 of pregnancy, the expression became undetectable by the time of the attachment reaction. COX-2 was expressed in the luminal epithelium and underlying stromal cells solely at the sites of blastocyst attachment reaction [28]. At the implantation site, mPGES expression was very similar to COX-2 in the subluminal stroma surrounding the implanting blastocyst, but mPGES was not expressed in the luminal epithelium. Previous studies have shown that a de-epitheliated stroma fails to decidualize after application of a deciduogenic stimulus, suggesting that the stimulus must transduce a decidual signal via the luminal epithelium [29]. It has been shown that mPGES is preferentially coupled with the inducible COX-2 to promote delayed PGE_2 generation and, if COX-2 already exists in cells, also regulates immediate PGE_2 generation (likely to be a reflection of the priming response) [14]. COX-2-derived PGE_2 could be involved in implantation by acting via EPs. EP receptor subtypes were expressed in the preimplantation mouse uterus in a spatiotemporal manner [22, 23], suggesting roles of PGE_2 in periimplantation events. However, COX-2-derived PGI_2 was also shown to be the primary PG that is essential for implantation and decidualization [11]. It is possible that implantation and decidualization are mediated by both PGE_2 and PGI_2.

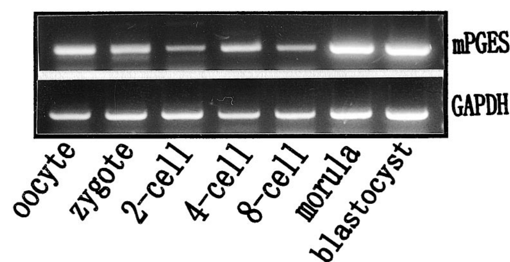

FIG. 5. A representative RT-PCR of mPGES and GAPDH mRNA expression in the oocytes and embryos at the stages of zygote, 2-cell, 4-cell, 8-cell, morula, and blastocyst, respectively.

Blastocysts from mouse [30], rat [31], rabbit [32], sheep [33], and cow [34] appear to have the capacity for transforming arachidonic acid into its biologically active derivatives via a COX, resulting in the production of PGE_2 and/or $PGF_{2\alpha}$. Holmes et al. [35] found that all the stages of human embryos studied (4-cell embryos and blastocysts) produced PGE_2 when preimplantation human embryos were cultured for 48 h. The largest amount of PGE_2 was found in the medium from late blastocysts. In our study, mPGES mRNA was detected in oocytes and all of the embryonic stages from zygote to blastocyst by RT-PCR. PGs produced by the blastocyst have been considered to serve as embryonic signals to the uterus. However, it still remains to be determined whether blastocyst-derived PGs mediate the endometrial responses [5].

In summary, the strong mPGES expression in the implantation site and decidual cells suggests that mPGES might play an important role during implantation and decidualization.

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ACKNOWLEDGMENT

The authors are grateful to Dr. Michael J.K. Harper for his very helpful suggestions and critical review.

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