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Characterization of Temporal and Cell-Specific Changes in Transcripts for Prostaglandin E_2 Receptors in Pseudopregnant Rat Endometrium[1]

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ABSTRACT

In the rodent uterus, prostaglandin E_2 (PGE_2) is believed to have a major role in implantation and decidualization. The present study investigated the temporal and hormonal control of mRNA expression for the four E-prostanoid ($EP_{1–4}$) receptors in the rat endometrium. For Northern blot analysis and in situ hybridization, samples were obtained from rats on Days 1–10 of pseudopregnancy or from rats differentially sensitized for the decidual cell reaction with estradiol. No EP_1 mRNA signal was detected. Endometrial EP_2 and EP_3 mRNA levels increased to a maximum on Day 5, and the mRNAs were localized to the luminal epithelium at the antimesometrial pole, and in the endometrial stroma and glandular epithelium, respectively. Endometrial EP_4 mRNA levels were unchanged on Days 1–5, but the mRNA was concentrated in the antimesometrial endometrial stroma on Day 5. Cell-specific expression of EP_2, EP_3, and EP_4 on Day 5 was dependent upon a dose of estradiol given on Day 4 that induced differential uterine sensitization on Day 5. After the application of a deciduogenic stimulus on Day 5, mRNA levels for these receptors decreased significantly, while in nonstimulated horns they remained elevated. Overall, these results support a role for PGE_2 in the onset of receptivity and initiation of decidualization in the rat.

INTRODUCTION

Successful implantation of the embryo requires precise synchronization of embryonic development to the expanded blastocyst stage along with the acquisition of a receptive endometrium for blastocyst attachment [1]. One of the first detectable signs of implantation is an increase in endometrial vascular permeability, which is localized to sites of blastocyst apposition [2]; this is followed by the initiation of the decidual cell reaction [2]. Decidualization involves the proliferation and differentiation of endometrial stromal cells into decidual cells, which ultimately form the maternal component of the placenta. The initiation of this process requires provocation by a deciduogenic stimulus that can be of embryonic or artificial origin [3, 4]. In rats, initiation of blastocyst implantation [5] and the induction of decidualization by nontraumatic stimuli [6, 7] can occur within a narrow time period on Day 5 of pregnancy or pseudopregnancy, or after exposure of the uterus to the appropriate combination, and concentrations, of progesterone (P_4) and estrogen [1–3]. Therefore, uterine receptivity and sensitization for decidualization are both time- and hormone-dependent.

Presently, the exact mechanisms acting at the cellular level to control uterine receptivity and sensitization for decidualization remain to be determined. However, considerable evidence exists indicating that prostaglandins (PGs) are important modulators of events at the site of implantation and during the decidual cell reaction in laboratory rodents [8, 9]. The specific PG involved in these processes is currently controversial. While there is considerable evidence from studies in rats that PGE_2 has a role [9], recent studies in cyclooxygenase-2 (COX-2)-deficient mice have suggested that PGI_2 is involved [10, 11]. However, it is clear that when infused into the lumen of sensitized rat uteri in which endogenous PG synthesis is inhibited, PGE_2 is able to restore the endometrial vascular permeability response and decidualization [12–14], thereby demonstrating that the rat endometrium is responsive to PGE_2.

Presently, the exact cellular sites of action of PGE_2 within the rat uterus are unknown. High-affinity PGE-binding sites have been detected in endometrial membrane preparations obtained from rats on Day 5 of pseudopregnancy [15]. However, it is now known that PGE_2 acts on four distinct G protein-coupled receptors, referred to as E-prostanoid (EP) receptors [16, 17]. EP_1 is coupled to diacylglycerol/inositol trisphosphate turnover and an increase in intracellular Ca^{2+} levels, while EP_2 and EP_4 are coupled to the stimulation of adenylyl cyclase. EP_3 has the opposite effect, as it is coupled to the inhibition of adenylyl cyclase. The existence of multiple receptor subtypes for PGE_2 suggests that PGE_2-induced actions within the uterus, at the time of implantation, may depend upon the relative contribution of the various EP receptor subtypes. Therefore, the present study investigated the temporal and cell-specific expression of the mRNA for the four EP receptors, $EP_{1–4}$, within the pseudopregnant rat endometrium (Days 1–10) by Northern blot analyses and in situ hybridization. As the onset of receptivity and uterine sensitization for decidualization both require the coordinated action of ovarian steroid hormones, we also examined the regulation of the PGE_2 receptor genes by estradiol (E_2) and P_4.

MATERIALS AND METHODS

Preparation of Animals

Female Sprague-Dawley rats, obtained from Harlan Sprague-Dawley (Indianapolis, IN) at 200–225 g body mass, were housed under temperature- and light-controlled conditions (lights-on from 0500 to 1900 h) with free access to food and water. All procedures involving animals were performed in accordance with the guidelines of the Canadian Council on Animal Care and the University of Western Ontario Council on Animal Care. Animals were ovariectomized under ether anesthesia and allowed at least 4 days to recover from surgery. To obtain a state equivalent to

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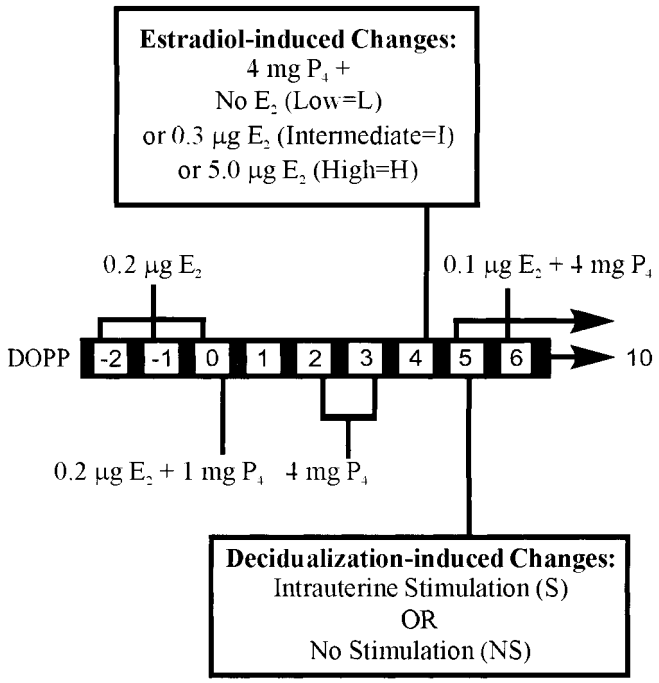

FIG. 1. Schematic representation showing the sequence of hormone administration to ovariectomized rats to obtain uteri differentially sensitized for the decidual cell reaction. Black areas represent periods of darkness; numbers within light areas indicate equivalent day of pseudopregnancy. Temporal changes in uterine state were investigated by collecting tissue from Days 1–4, 5 (intermediate), and 6–10 of pseudopregnancy. E_2-induced changes were investigated by administering different doses of E_2 on Day 4 and collecting tissue on Day 5. (DOPP, equivalent day of pseudopregnancy.)

pseudopregnancy, ovariectomized rats were given injections (s.c.) of E_2 and P_4 in sesame oil according to the protocol illustrated (Fig. 1), as described previously [18]. To investigate the temporal changes in EP receptor transcript expression, animals were killed on the equivalent of Days 1–10 of pseudopregnancy. To investigate deciduali-zation-induced changes in EP receptor transcript expression, some animals given an intermediate dose of E_2 on Day 4 received an artificial deciduogenic stimulus (S), the bilateral intrauterine injection of 0.1 ml sesame oil [19] on Day 5, and were killed on Days 6–10. Other rats were treated identically except that they did not receive the deciduogenic stimulus (NS). To investigate the effects of E_2-induced differential uterine sensitization on the expression of EP receptor transcripts on Day 5 of pseudopregnancy, different amounts of E_2 (0 μg, 0.3 μg, or 5 μg) with a constant amount of P_4 (4 mg) were given to rats on the afternoon of Day 4 of pseudopregnancy, as described previously [20], and animals were killed the next day. These treatments will be referred to as Day 5 low (L), intermediate (I), and high (H) E_2 doses, respectively.

Collection of Tissues

Animals were killed by decapitation between 0900 and 1100 h. To collect endometrial tissue, uterine horns were cut longitudinally, and the endometrium was separated from the myometrium with a spatula as described by Martel and Psychoyos [21]. Endometrium was pooled from 3–6 rats for each day of pseudopregnancy and used for RNA isolation. Alternatively, for in situ hybridization, whole uteri were obtained from 2–3 animals for each of Day 4, Day 5 (L, I, and H E_2 groups), and Day 6 (S and NS) of pseudopregnancy and fixed overnight by immersion in 4% paraformaldehyde at 4°C. After fixation, tissues were dehydrated and embedded in paraffin, and 6-μm sections were mounted onto positively charged Superfrost*/Plus slides (Fisher Scientific, Nepean, ON, Canada).

TABLE 1. Primer pair sequences used for the generation of RT-PCR and the lengths of the predicted PCR fragments.

Primers for:	GenBank/ EMBL Data Bank accession number	Primer sequence (5′ → 3′)	Expected product length (bp)	Start position on cDNA (bp)
EP_1	D88751	ACGGTGGTGTGAGCCTTTA	402	133
		TGTCCCAACTTTCTGTGCC*		534
EP_2	U94708	AGACGGACCACCTCATTCTC	481	818
		CTGACACTTACCACAAAGGGC*		1298
EP_3	D14869	CTTGCTGGCTCTGGTGGT	387	698
		GCATAGTTGGTGTGGTCCCT*		1084
EP_4	U94709	AGACGGTTCAGCACAGCA	370	1077
		TTTCAGCGTTTCACTGGG*		1446

* Denotes anti-sense strand.

Hybridization Probes

Based on published nucleotide sequences listed in the GenBank/EMBL Data Bank, primer pairs (Table 1) were designed to specifically amplify, by reverse transcription (RT) and polymerase chain reaction (PCR), nonhomologous cDNAs for each EP receptor. Total RNA from rat kidneys was used as the template [16]. The resulting PCR fragments were cloned into pBluescript II KS (+/−) phagemids (Stratagene, La Jolla, CA) for sequence analysis to confirm their identity. A cDNA for mouse 18S rRNA was a generous gift from Dr. G. Hammond, University of Western Ontario, London, Ontario. For Northern blot hybridization, probes for the EP receptors were prepared from *Eco*RI and *Hin*dIII digests (Pharmacia Biotech, Baie D'Urfé, PQ, Canada) of the plasmid vectors. The cDNA fragments (25 ng) for EP_1 (402 base pairs [bp]), EP_2 (481 bp), EP_3 (387 bp), and EP_4 (370 bp) were labeled using a random primer DNA labeling system (Gibco BRL, Burlington, ON, Canada) in the presence of [α-^{32}P]dCTP (Amersham, Oakville, ON, Canada) and DNA polymerase I, large (Klenow) fragment (Gibco BRL), and purified using nick columns according to the manufacturer's instructions (Pharmacia Biotech). For in situ hybridization, sense and antisense riboprobes were synthesized from a linearized plasmid template by the appropriate DNA-dependent RNA polymerases (T3 or T7; Boehringer-Mannheim, Laval, PQ, Canada) and incubation at 37°C for 1 h in the presence of [α-^{35}S]UTP (ICN Biomedicals, Toronto, ON, Canada).

RNA Isolation and Northern Blot Hybridization

Total RNA was isolated from endometrial tissue by phenol-chloroform extraction using a single-step guanidine thiocyanate procedure as previously described [22]. Samples of total RNA and an RNA ladder (Gibco BRL) were denatured in 50% formamide and 2.2 M formaldehyde in a single-strength MEA buffer (20 mM 3-[*N*-morpholino]propanesulfonic acid, 50 mM sodium acetate, 10 mM EDTA, pH 7.2) at 65°C for 15 min; they were then separated by denaturing formaldehyde-agarose gel (1.1%) electrophoresis. Ethidium bromide was added to each sample before loading so that RNA integrity could subsequently be

determined. RNAs were transferred overnight to Hybond-N membranes (Amersham) by capillary transfer as previously described [23], and cross-linked to the membranes by UV irradiation (0.12 J/cm^2) with the UVC 500-115 V Mini UV Crosslinker (Hoefer Scientific Instruments, San Francisco, CA). Membranes were prehybridized for 2 h at 42°C in hybridization buffer containing 50% formamide, 5-strength SSPE (20-strength SSPE is 3 M NaCl, 200 mM NaH_2PO_4, 20 mM EDTA, pH 7.4), 5-strength Denhardt's reagent, 0.5% SDS, and 100 μg/ml salmon sperm DNA. ^{32}P-Labeled cDNA probes (specific activity 2–4 × 10^9 cpm/μg), denatured in 0.5 M NaOH for 30 min, were added to the hybridization buffer at a concentration of 1.5–2 × 10^6 cpm/ml; and hybridization was carried out overnight at 42°C. The membranes were then washed two times in each of wash 1 (double-strength SSPE, 0.1% SDS) at 25°C for 5 min, wash 2 (0.2-strength SSPE, 0.1% SDS) at 42°C for 10 min, and wash 3 (0.1-strength SSPE, 0.1% SDS) at 65°C for 15 min. Hybridization blots were subjected to autoradiography for 5–6 days at −70°C with BioMax MS film (Eastman Kodak, Rochester, NY) and intensifying screens (Eastman Kodak). Blots were stripped in stripping buffer (1 mM Tris, 1 mM EDTA, 0.1-strength Denhardt's reagent, pH 7.2) for 2 h at 75°C and then rehybridized with the remaining EP receptor probes and the 18S rRNA probe, which served as a control for equal loading and transfer of RNA.

Densitometry and Data Analysis

All Northern blot experiments were repeated three times with sets of RNA obtained from separate experiments, and similar results were obtained. EP receptor mRNAs along with 18S rRNA signals were quantified by densitometry with Image Master VDS densitometry software (Pharmacia Biotech). The relative levels of the signals for the mRNAs on the autoradiograms were expressed as the mean of the ratios of target mRNA to 18S rRNA signal after these values had been arbitrarily normalized to Day 1 or Day 4 values, depending on the experiment. These means were subjected to a one-way ANOVA with each experiment considered a block. When significant differences were found, a Duncan's new multiple range test was used for group comparisons. A P value of less than 0.05 was considered significant.

In Situ Hybridization

In situ hybridization was performed as previously described [24], with some modifications. Briefly, deparaffinized and rehydrated tissue sections were digested for 10 min at 25°C with 20 μg/ml proteinase K (Gibco BRL), postfixed in 4% paraformaldehyde (20 min), acetylated, and then dehydrated with ethanol. Sections were prehybridized for 2–4 h at 53–55°C in Nalgene utility boxes (Fisher Scientific), humidified with buffer containing 50% formamide and 4-strength SSC (20-strength SSC is 3 M NaCl, 0.3 M Na_3-citrate, pH 7.0). Labeled and denatured sense and antisense ^{35}S-labeled probes (specific activity 1–3 × 10^5 cpm/ng) were added to tissue sections at a concentration of 0.05 or 0.1 ng/μl. Overnight hybridization was carried out at 53–55°C. After hybridization and washing, nonspecifically bound riboprobe was removed from tissue sections after a 30-min digestion in double-strength SSC with 20 μg/ml RNase A (Boehringer-Mannheim). After final washes and dehydration in ethanol (containing 0.3 M ammonium acetate), hybridized sections were dipped in NTB-2 liquid emulsion (Eastman Kodak), diluted 1:1 with water, and stored at 4°C for 2–3 wk in light-tight boxes containing desiccant. Slides were developed (Kodak D-19), fixed, and poststained with Harris' hematoxylin.

RESULTS

EP Receptor mRNAs in the Rat Endometrium—Northern Blot Analyses

The mRNAs for EP_2, EP_3, and EP_4 were detected by Northern blot analyses in total RNA from endometrium obtained during simulated pseudopregnancy (Fig. 2). The mRNA for the EP_1 receptor was not detected (data not shown). The estimated sizes of the major transcripts were 2.8, 2.3, and 3.8 kilobases (kb) for EP_2, EP_3, and EP_4, respectively, which corresponded to previously reported sizes [25–29]. Minor bands for EP_2 and EP_3 were observed at 2.2 and 7.0 kb, also as previously reported. The level of expression of EP_2, EP_3, and EP_4 transcripts fluctuated with respect to the equivalent day of pseudopregnancy and whether or not decidualization had been induced (Fig. 2). During the preimplantation period (Days 1–5), EP_4 mRNA steady state levels remained relatively unchanged ($P >$ 0.05) and were readily detected, while mRNA for EP_2 was

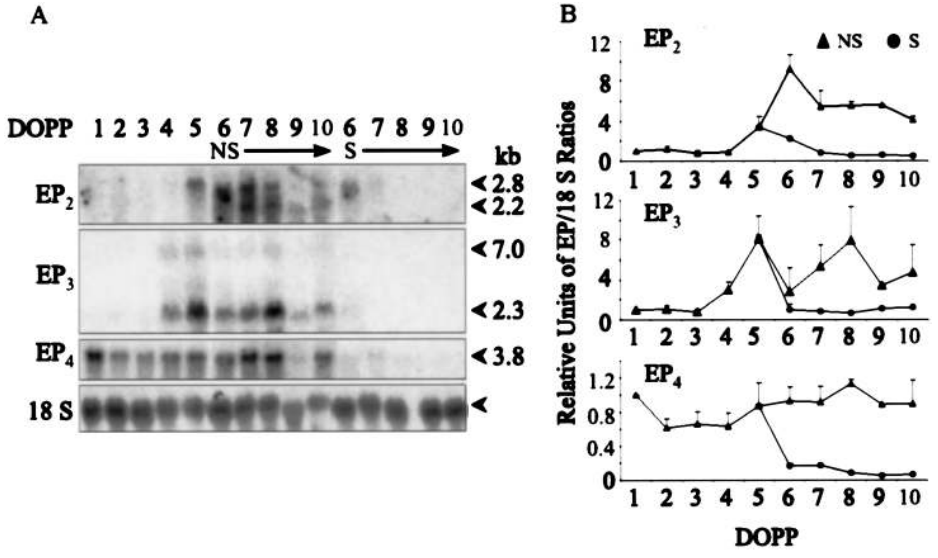

FIG. 2. **A**) Northern blot analyses of EP_{2-4} steady state levels in rat endometrium obtained on the equivalent of Days 1–10 of pseudopregnancy. On the equivalent of Day 5, some rats received a deciduogenic stimulus (S) while others remained nonstimulated (NS). Representative autoradiographs of results are shown; 20 μg total RNA was loaded into each lane. The estimated size of each major transcript is shown in kilobases. **B**) Densitometric analyses of EP receptor signals as detected on the equivalent of Days 1–10 of pseudopregnancy. Results are for three separate experiments. Ratios of EP receptor mRNA to 18S rRNA are displayed as mean ± SEM relative units normalized arbitrarily to Day 1 of pseudopregnancy. (DOPP, equivalent day of pseudopregnancy.)

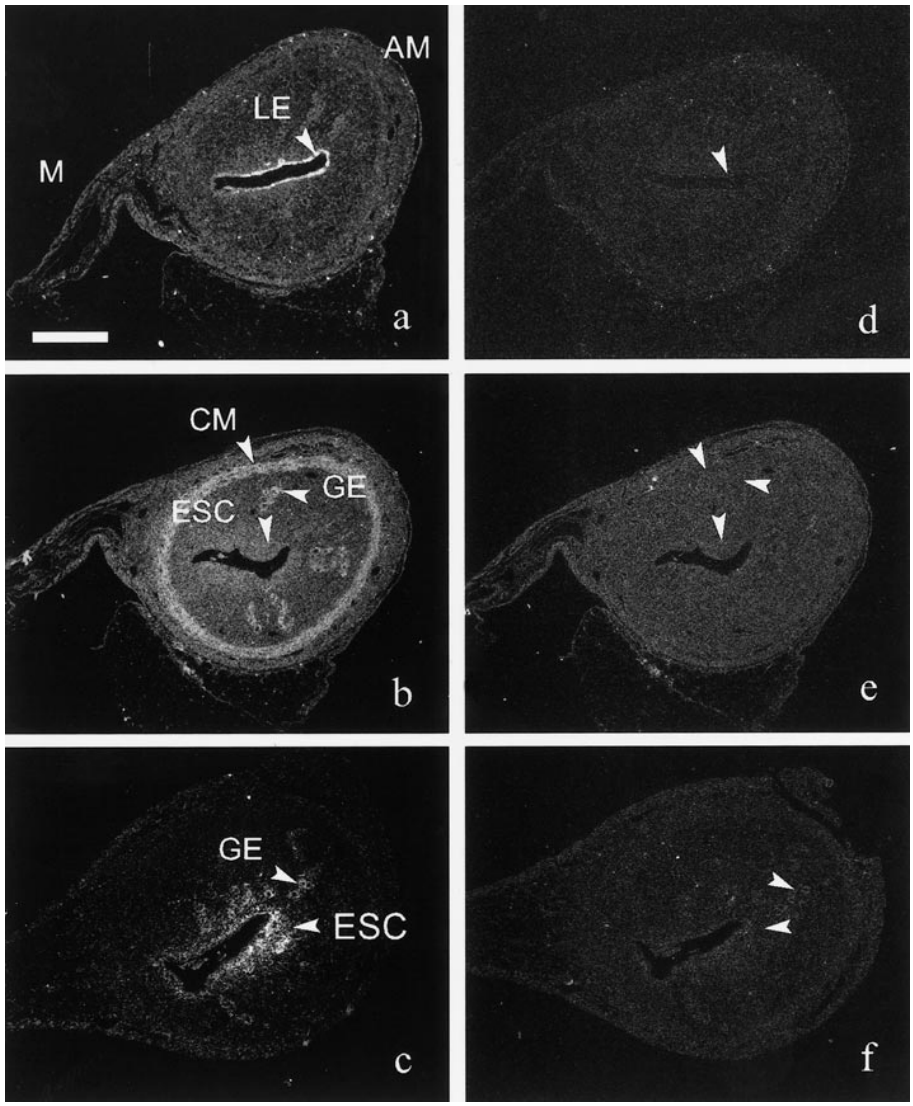

FIG. 3. Darkfield photomicrographs demonstrating the specificity of the in situ hybridization. Photomicrographs (**a–f**) are shown of transverse sections of Day 5 rat uteri hybridized with ^{35}S-labeled antisense (**a–c**) or sense (**d–f**) riboprobes for the EP_2 (**a, d**), EP_3 (**b, e**), and EP_4 (**c, f**) receptor transcripts. Mesometrial pole of the uterus is directed toward the left of each picture. (AM, antimesometrium; CM, circular smooth muscle; ESC, endometrial stromal cells; GE, glandular epithelium; LE, luminal epithelium; M, mesometrium.) Bar = 500 μm.

detected only at a low level until Day 5 of pseudopregnancy, when relative mRNA levels increased by almost 4-fold from previous steady state levels ($P < 0.01$). Similarly, mRNA for EP_3 was barely detectable on Days 1–3, then increased from Day 3 to Day 4, and reached a maximum on Day 5 of pseudopregnancy. On this day, levels of mRNA for EP_3 were almost 10-fold and 3-fold higher than the steady state levels on Day 3 ($P < 0.01$) and Day 4 ($P < 0.01$), respectively.

After application of a deciduogenic stimulus on Day 5 of pseudopregnancy, EP_3 and EP_4 receptor transcripts decreased to almost nondetectable levels ($P < 0.05$) (Fig. 2; S samples). By contrast, EP_2 mRNA levels remained at levels on Day 6 not different ($P > 0.05$) from those on Day 5, and subsequently decreased. In the absence of a deciduogenic stimulus, the levels of EP_2, EP_3, and EP_4 receptor transcripts remained elevated (Fig. 2; NS samples). Furthermore, transcript levels in the nonstimulated endometrium were greater than in stimulated endometrium from the corresponding day of pseudopregnancy ($P < 0.05$). One exception was for the EP_3 receptor, whose mRNA levels on Day 6 in both the stimulated and nonstimulated endometrium were not different from each other ($P > 0.05$) and were lower than Day 5 values ($P < 0.01$).

EP Receptor mRNAs in the Pseudopregnant Rat Uterus—In Situ Hybridization

In situ hybridization of uterine cross sections revealed that each EP receptor gene was expressed in a temporally specific and cell-specific manner except for the EP_1 receptor, as no cell-specific signal for this transcript was detected on any of the days studied (data not shown). The specificity of each probe was confirmed by no specific hybridization with the sense probe (Fig. 3). For the EP_2 receptor (Fig. 4, a–d), no specific signals were detected in any cell type on Day 4 of pseudopregnancy (Fig. 4a). However, by Day 5 of pseudopregnancy (Fig. 4b), strong signals were detected specifically in the luminal epithelium. These signals were concentrated in lateral segments (Fig. 4b) and at the antimesometrial pole (Fig. 5, a and e), but not the mesometrial pole (Fig. 5, b and f), of the epithelium. This clear upregulation of EP_2 mRNA in the luminal epithelium was maintained on Day 6 in both nonstimulated (Fig. 4c) and stimulated (Fig. 4d) uterine horns, but was detected in all luminal epithelial cells irrespective of their position within the endometrium. A weak signal in the glandular epithelium was also detected on Day 6 of pseudopregnancy in both nonstimulated and stimulated uterine horns.

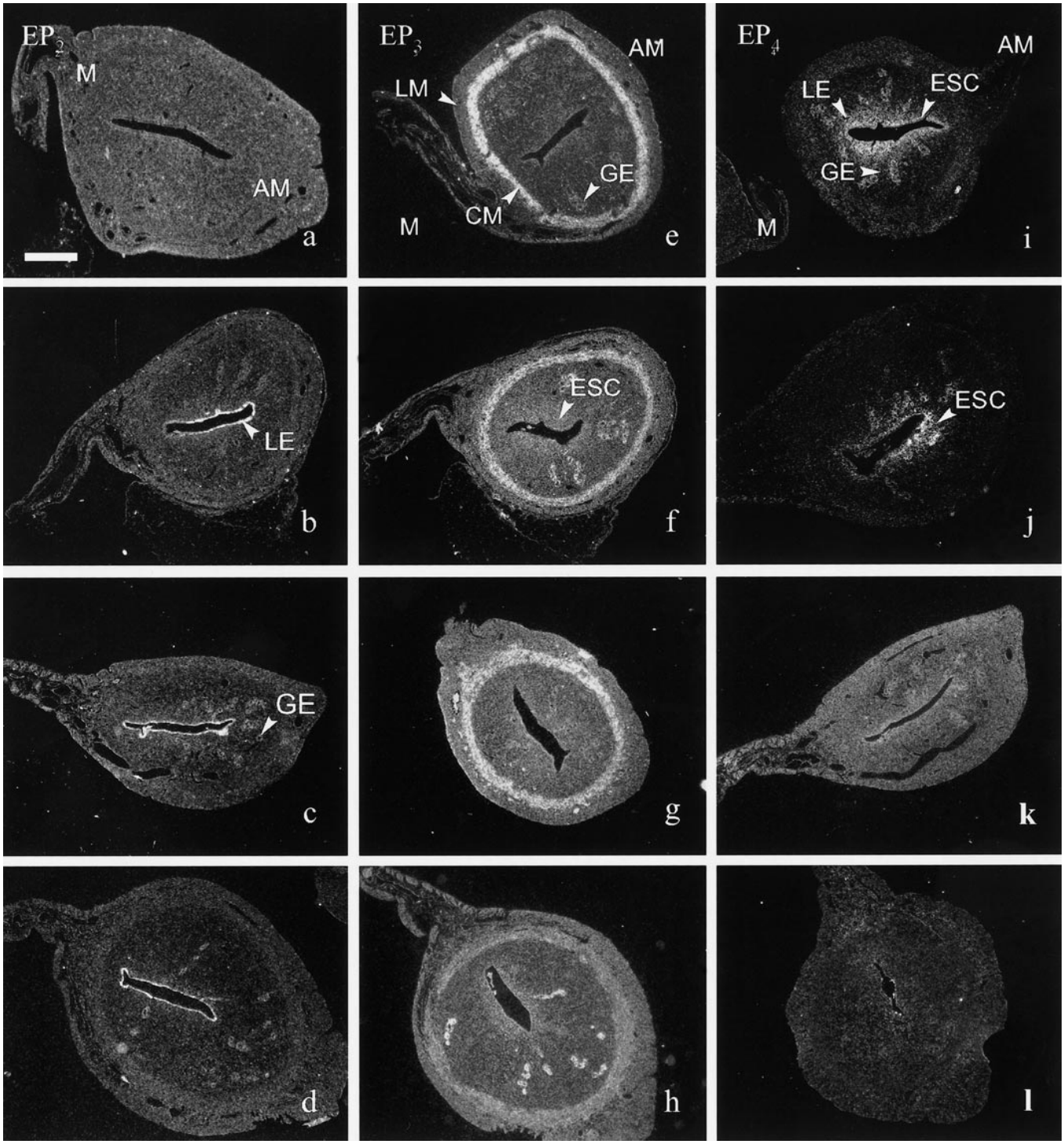

FIG. 4. Regional distribution of uterine mRNA for the EP_2 (**a–d**), EP_3 (**e–h**), and EP_4 (**i–l**) receptors in the pseudopregnant rat uterus as detected by in situ hybridization. Darkfield photomicrographs (**a–l**) are shown of transverse sections hybridized with ^{35}S-labeled riboprobes for each EP receptor. Panels represent sections of uteri obtained on the equivalent of Day 4 (**a, e, i**), 5 (**b, f, j**), and 6 (**c, g, k**) of pseudopregnancy, and from animals on Day 6 that had received a deciduogenic stimulus on Day 5 (**d, h, l**). Mesometrial pole of the uterus is directed toward the left of each picture. Abbreviations are the same as for Figure 3. LM, longitudinal smooth muscle. Bar = 500 μm.

For the EP_3 receptor (Fig. 4, e–h), there was clear localization of signal in the circular smooth muscle on the equivalent of Days 4 (Fig. 4e), 5 (Fig. 4f), and 6 of pseudopregnancy in nonstimulated uteri (Fig. 4g), with lower levels on Day 6 following a deciduogenic stimulus (Fig. 4h). Within the endometrium, high levels of signal were apparent in the glandular epithelium on Days 4 and 5, and also in Day 6 stimulated endometrium, with a slight decrease in these cells on Day 6 in nonstimulated endometrium. There was also accumulation of signal on Day 5 of pseudopregnancy that was restricted to the endometrial stromal cells directly surrounding the luminal epithelium (Fig. 4f), i.e., the subepithelial stromal cells. This signal was low and diffuse, but by comparison to the sense control (Fig. 3, b and e) it appears to reflect a signal for EP_3.

For the EP_4 receptor, in situ hybridization revealed differ-

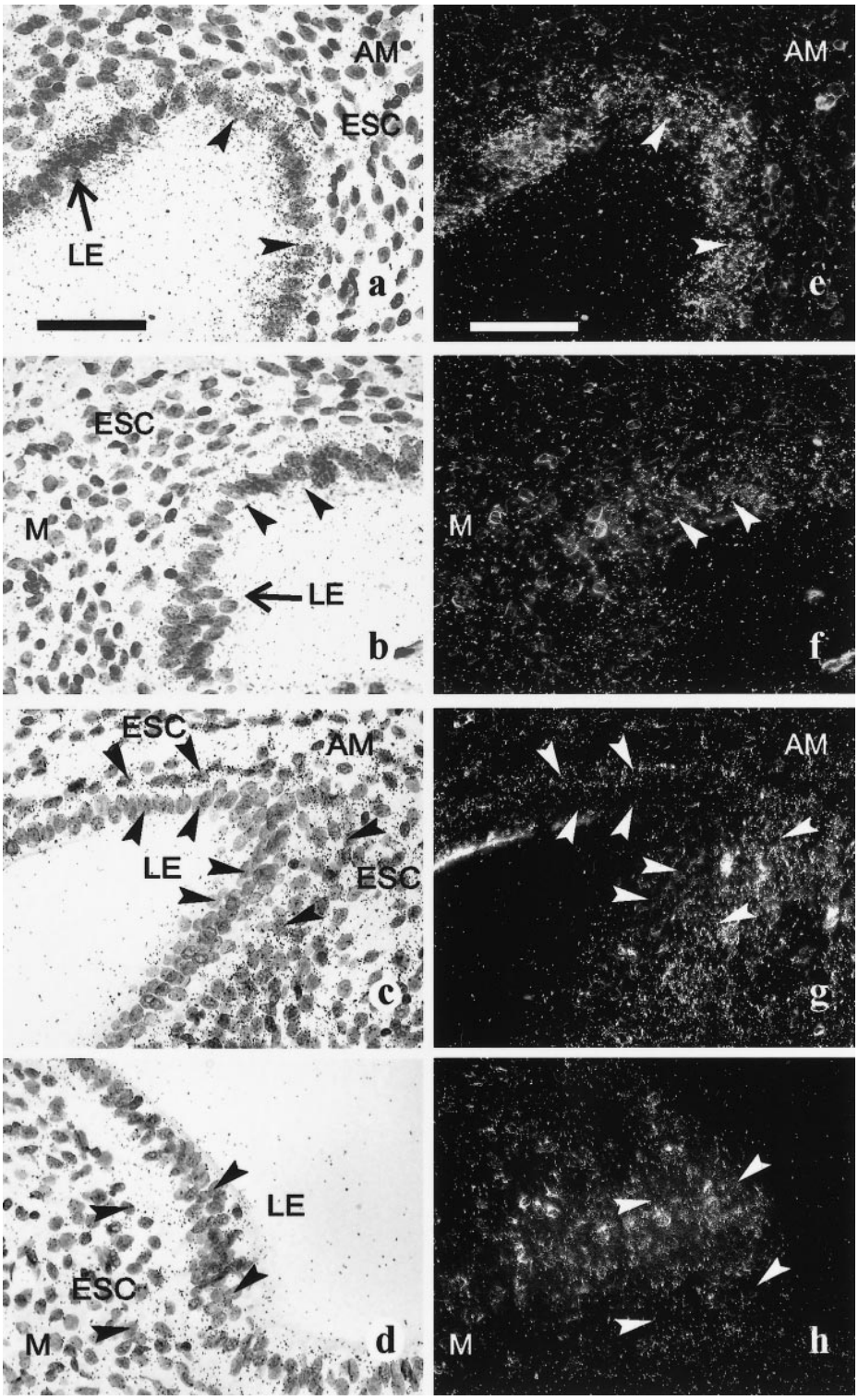

FIG. 5. Cellular localization of mRNA for the EP_2 (**a, b, e, f**) and EP_4 (**c, d, g, h**) receptors differed between the antimesometrial and mesometrial poles of the rat uterus on the equivalent of Day 5 of pseudopregnancy, as detected by in situ hybridization. Brightfield (**a–d**) and darkfield (**e–h**) photomicrographs are shown of transverse sections hybridized with ^{35}S-labeled antisense riboprobes for the EP receptors. (Arrowheads, cells labeled with the EP probes; abbreviations are the same as for Figure 3.) Bar = 50 μm.

ences between days of pseudopregnancy in localization of this transcript (Fig. 4, i–l). High levels of EP_4 signal accumulated in the subepithelial endometrial stromal cells, and lower levels were detected in the glandular epithelium on the equivalent of Days 4 (Fig. 4i), 5 (Fig. 4j), and 6 (Fig. 4k; NS samples) of pseudopregnancy. The mRNA for EP_4 was also localized to the luminal epithelium on Days 4 and 6, but on Day 5 it was down-regulated compared to what occurred in the subepithelial stroma (Fig. 5, g and h). Another feature of EP_4 mRNA localization on Day 5 was that the signal in the subepithelial endometrial stromal cells was stronger and more concentrated at the antimesometrial (Fig. 5, c and g) than the mesometrial (Fig. 5, d and h) pole. No signal was detectable in uterine sections obtained from animals on Day 6 following a deciduogenic stimulus (Fig. 4l).

Correlation of Uterine Sensitization and Endometrial EP Receptor Transcripts

To correlate uterine sensitization for the decidual cell reaction with expression of EP receptors, rats were killed

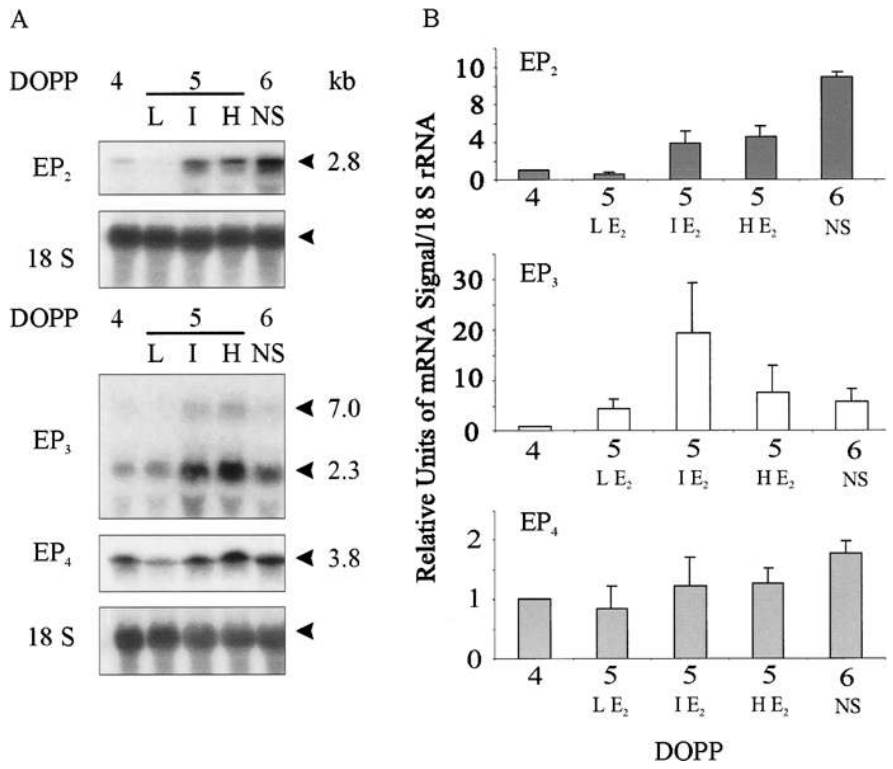

FIG. 6. **A**) Northern blot analyses of EP_{2-4} steady state levels in rat endometrium obtained from animals that were differentially sensitized for the decidual cell reaction. Endometrial tissue was collected on the equivalent of Day 4, 5, and 6 of pseudopregnancy. For rats on Day 5, some had been treated on the afternoon of Day 4 with either a low (L, 0 μg), intermediate (I, 0.3 μg), or high (H, 5 μg) dose of E_2. Rats on Day 6 received the intermediate dose of E_2 on Day 4 but did not receive a stimulus (NS). Representative autoradiographs of results are shown; 20 μg total RNA was loaded into each lane. The estimated size of each major transcript is shown in kilobases. **B**) Densitometric analyses of EP receptor signals; results are for three separate experiments. Ratios of EP receptor mRNA to 18S rRNA are displayed as mean ± SEM relative units normalized arbitrarily to Day 4 of pseudopregnancy. (DOPP, equivalent day of pseudopregnancy.)

on Day 4 or 6 of pseudopregnancy, or on Day 5 after having received a low, intermediate, or high dose of E_2 on the afternoon of Day 4 of pseudopregnancy. Day 5 I E_2 animals are maximally sensitized for the decidual cell reaction. For EP_2, Northern blot analysis indicated that steady state mRNA levels on Day 5 L E_2 endometrium were low and were similar ($P > 0.05$) to those on Day 4 of pseudopregnancy (Fig. 6), and by in situ hybridization no specific signals were detected (Fig. 7a). For the Day 5 I E_2 and H E_2 endometrium, transcripts for EP_2 were increased by almost 7-fold and 8-fold, respectively, over Day 5 L E_2 levels, although the increase was significant ($P < 0.05$) only for the H E_2 group (Fig. 6). In situ hybridization revealed that strong signals for EP_2 mRNA accumulated in the luminal epithelium of both these groups (Fig. 7, b and c). However, while the signal in the luminal epithelium of the Day 5 I E_2 uterus was concentrated in the lateral and antimesometrial regions of the lumen (Fig. 7b), in the Day 5 H E_2 uterus it was seen throughout the luminal epithelium, irrespective of cell position within the uterus (Fig. 7c). The relative mRNA levels for EP_2 on Day 6 of pseudopregnancy were significantly greater ($P < 0.05$) than on Day 4 of pseudopregnancy and in all three Day 5 E_2 groups, but its localization (Fig. 4c) was similar to that observed in the Day 5 H E_2 uterus.

For EP_3, compared to Day 4 and Day 6 (NS samples), there was a dramatic up-regulation in transcript levels on Day 5 of pseudopregnancy in response to the I E_2 dose given on Day 4 ($P < 0.01$) (Fig. 6). These levels were also approximately 5-fold ($P < 0.01$) and 3-fold ($P > 0.05$) greater than in Day 5 L E_2 and Day 5 H E_2 endometrium, respectively. By in situ hybridization, signal was detected in the glandular epithelium of all three Day 5 E_2 groups (Fig. 7, d–f). However, sections obtained from animals given the I E_2 dose on Day 4 had a greater accumulation of signal in the glandular epithelium, as well as up-regulation of expression throughout the subepithelial stroma (Fig. 7e), compared to the Day 5 L E_2 (Fig. 7d) and H E_2 (Fig. 7f) sections.

In contrast to Northern blot analysis of total endometrial RNA from differentially sensitized rat uteri, which indicated that relative mRNA levels for EP_4 did not differ ($P > 0.05$) between groups (Fig. 6), in situ hybridization indicated changes in the distribution of these transcripts (Fig. 7, g–i). The localization of the signals for EP_4 in Day 5 L E_2 (Fig. 7g) and H E_2 (Fig. 7i) uterine sections was similar to that on Day 4 and Day 6 of pseudopregnancy (Fig. 4, i and k)—that is, in the glandular epithelium and all cells of the luminal epithelium and subepithelial endometrial stromal cells. However, in Day 5 I E_2 sections (Fig. 7h), luminal epithelial expression was less than expression in the endometrial stroma. Additionally, mRNA expression for EP_4 in the endometrial stromal cells was stronger and more abundant at the antimesometrial pole on Day 5 in response to the intermediate dose of E_2 given on Day 4 (Fig. 7h), compared to the Day 5 L E_2 (Fig. 7g) and H E_2 (Fig. 7i), and also compared to uteri obtained on Days 4 and 6 (NS samples) of pseudopregnancy (Fig. 4, i and k).

DISCUSSION

In the present study, we show that mRNAs for the EP_2 and EP_4 receptors, which are coupled to adenylyl cyclase activation [26, 28], as well as the EP_3 receptor, which is coupled to inhibition of adenylyl cyclase [25], are expressed in the pseudopregnant rat endometrium at the time of uterine sensitization for decidualization. However, by the methods used in the present study, mRNA for the EP_1 receptor was not detected; but we cannot conclude that the EP_1 receptor is absent from the endometrium. Nevertheless, the results for the EP_2, EP_3, and EP_4 receptors do provide a basis for the potentially diverse effects of PGE_2 within the endometrium as a consequence of expression of different EP receptors, in different populations of cells, and at

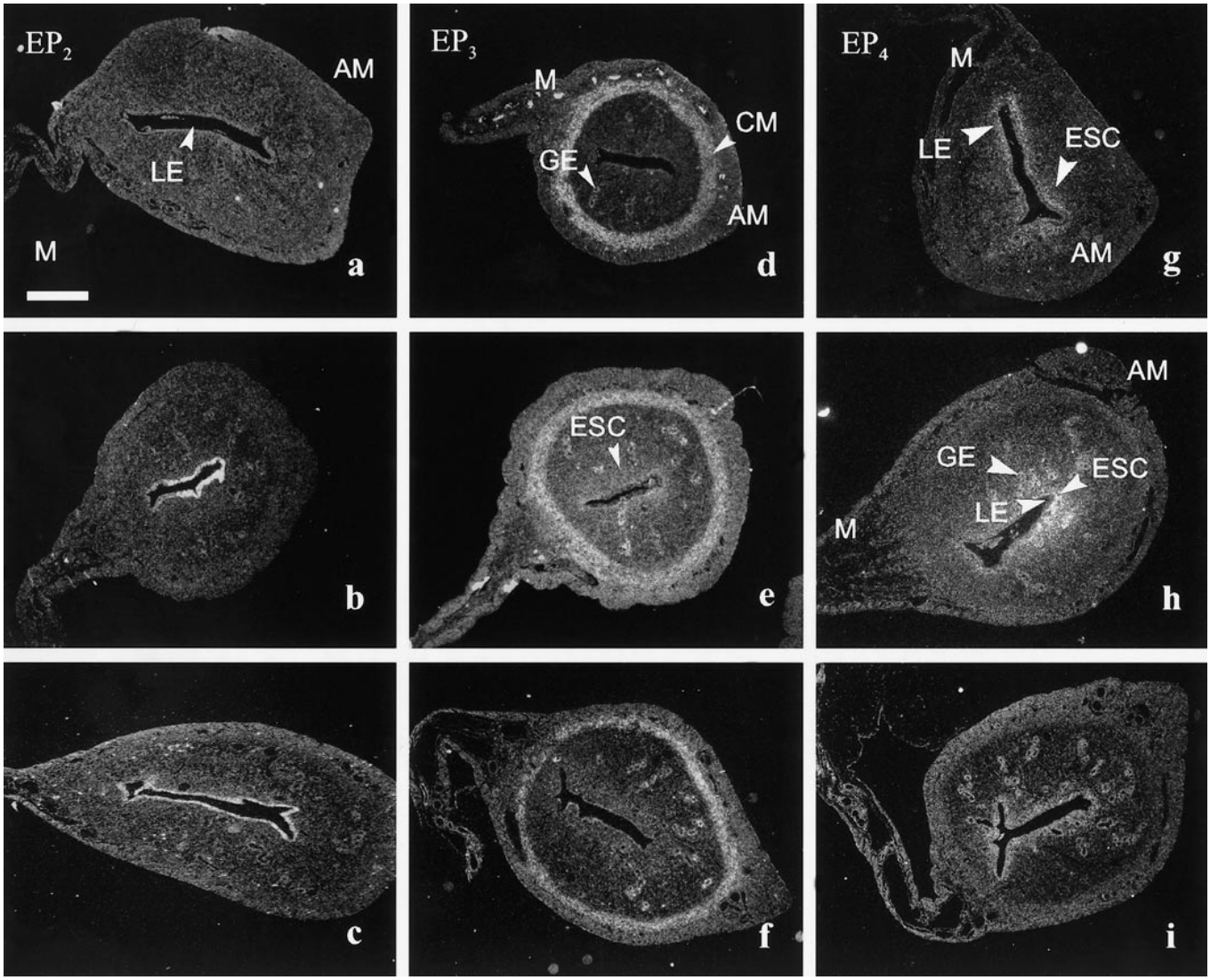

FIG. 7. Change in regional distribution of mRNA for the EP_2 (**a–c**), EP_3 (**d–f**), and EP_4 (**g–i**) receptors in the uteri of pseudopregnant rats differentially sensitized for the decidual cell reaction, as detected by in situ hybridization. Darkfield photomicrographs (**a–i**) are shown of transverse sections hybridized with ^{35}S-labeled antisense riboprobes for the EP receptor transcripts. Panels represent sections of uteri obtained from rats on the equivalent of Day 5 of pseudopregnancy that had been treated on the afternoon of Day 4 with E_2 at a low (0 μg; **a, d, g**), intermediate (0.3 μg; **b, e, h**), or high (5 μg; **c, f, i**) dose. Mesometrial pole of the uterus is directed toward the left of each picture. Abbreviations are the same as for Figure 3. Bar = 500 μm.

specific stages of early pregnancy/pseudopregnancy. A highlight of the study was the correlation of the time- and E_2-dependent changes in mRNA levels and cellular localization of EP transcripts with uterine sensitization for the decidual cell reaction.

In the rat endometrium, on the equivalent of Day 5 of pseudopregnancy the uterus is maximally sensitized for decidualization [1, 2]. This is also the time when implantation is initiated in the rat [1, 2]. With regard to the temporal and cell-specific expression of mRNA for EP_2, our results are similar to findings from previous studies in the mouse in which mRNA for EP_2 was up-regulated on Days 4 (day of implantation in mice) and 5 of pregnancy [30] and Day 5 of pseudopregnancy in the absence of a stimulus [31]. In these studies, expression was also localized to the luminal epithelium; however, at the time of implantation and maximal uterine sensitization in mice, there was no distinct difference between expression of EP_2 transcripts between the antimesometrial and mesometrial poles, as was observed in the rat. Therefore, the results of the present study are consistent with previous evidence that PGE_2 is involved in blastocyst implantation and the initiation of decidualization, which occur at the antimesometrial pole of the uterus in both mice and rats [3]. Furthermore, this process may be regulated by the action of PGE_2 on the EP_2 receptor. Previous studies have shown that a de-epitheliated stroma fails to decidualize after application of a deciduogenic stimulus [32], suggesting that the stimulus must transduce a ''decidual'' signal via the luminal epithelium. Additionally, COX-2 has been localized in the luminal epithelium of mice as early as 2 h following intraluminal oil injection on Day 4 of pseudopregnancy [10]; and PGE_2, one of the products of the COX enzyme, is elevated at implantation sites and following the application of a deciduogenic stimulus [9]. Therefore, activation of luminal epithelial EP_2 by PGE_2, following a deciduogenic stimulus, may be a requirement for signaling to the underlying stroma and initiation of the process of decidualization.

The cell-specific pattern of expression of mRNA encoding the EP_4 receptor observed in the present study was sim-

ilar to findings by Yang et al. [33] showing that the level of EP_4 mRNA expression was high in pregnant mice during the preimplantation period and localized to the subepithelial endometrial stromal cells and the luminal epithelium at the time of implantation. However, as for EP_2 expression, in the rat a greater level of EP_4 receptor mRNA is located at the antimesometrial pole of the uterus at the time of uterine sensitization, while mRNA levels for EP_4 were negligible following a deciduogenic stimulus. These results are in contrast to previous studies in the mouse on Day 4 of pseudopregnancy in which stromal EP_4 was not concentrated at the antimesometrial pole [31] until after implantation in the pregnant mouse [33, 34]. Nevertheless, results of the present study suggest that PGE_2 activation of EP_4 receptors may be involved in triggering decidualization of the subepithelial stroma at the antimesometrial pole.

In the present study, elevated steady state levels of endometrial EP_3 mRNA on Day 5 of pseudopregnancy, as detected by Northern blot analysis, were localized to the stromal cells and glandular epithelium. These results are similar to EP_3 transcript localization in the endometrium of pregnant mice on Days 3 and 4 that was also specific to the stroma [10, 33]. However, in this case, it is the mouse uterus in which EP_3 was localized to a specific region of the endometrium, a subpopulation of cells at the mesometrial pole, while expression in the rat uterus was independent of the position of the stromal cells within the uterus. The findings of this study are consistent with EP_3 receptor expression in the endometrium and add further support for a role for PGE_2 in triggering decidualization of the subepithelial stroma at the time of implantation by acting upon the EP_3 receptor, in addition to stromal EP_4. The coexpression of EP receptors with opposite effects, such as EP_3 and EP_4, has been deemed important for buffering cellular responses to transient extremes of agonists, i.e., a homeostatic control mechanism for the actions of PGs at the cellular level [35]. Although undetermined by the present study, colocalization of EP_3 in combination with EP_4 in the same cells in the antimesometrium may act to precisely regulate localized high levels of cAMP that are restricted to a subpopulation of antimesometrial cells and may be required for the regionally specific initiation of decidualization. The progression of the decidual cell reaction, from the antimesometrium to the mesometrium, may then be dependent on the generation of a smooth concentration gradient of cAMP, regulated by coactivation of the EP_3 and EP_4 receptors, and maintained by the expression of endometrial gap junctions. This theory is consistent with expression of connexin 26 and 43 in the luminal epithelium and subepithelial endometrial stromal cells of the antimesometrium, respectively, on Day 5 of pregnancy in rats, and in decidual cells on Day 6 of pregnancy and pseudopregnancy following an artificial deciduogenic stimulus [36, 37].

After the onset of receptivity the uterine environment becomes refractory to implantation of blastocysts and detrimental to their survival [5]. In the present study, mRNA levels remained elevated in nonstimulated uteri, suggesting that termination of endometrial responsiveness in the rat cannot be explained simply by a decrease in EP receptor expression, but may be related to the change in localization of these receptors. In rodents, there is also evidence for the involvement of PGs throughout the decidual cell transformation [38, 39]. However, in the present study, endometrial expression of mRNA for EP_2, EP_3, and EP_4 was negligible in stimulated uteri. The only exception was for the EP_2 receptor, whose mRNA levels remained elevated on Day 6 (although localization was in all cells of the luminal epithelium) and declined thereafter. Therefore these findings differ from previous results in the mouse that documented a persistence in EP_3 transcript expression in the mesometrial stroma on Day 5 (following implantation on Day 4 of pregnancy) and in the secondary decidual zone on Day 7 [33]. Furthermore, those studies detected mRNA for EP_4 in the primary decidual zone on Day 5 as well as in the secondary decidual zone on Days 6–8 of pregnancy in the mouse. Therefore in the mouse, EP receptor expression was correlated with the progression of the decidual cell reaction, which occurs similarly in the rat and in the mouse, although with a slight difference in timing [3]. It is possible that in rat endometrium, protein expression remains elevated despite low levels of mRNA for the EP receptors and that PGE_2 acts through these receptors throughout the decidual cell reaction. Alternatively, PGs other than PGE_2 may be involved in propagating later stages of the decidual response. On the other hand, in support of a role for PGE_2 in the process of decidualization, Lim et al. [10] observed that on Day 4 of pregnancy, mice deficient in Hoxa-10 displayed abnormally low levels of mRNA encoding the stromal EP_3 and EP_4 receptors, while mRNA encoding the EP_2 receptor was normally expressed and localized to the luminal epithelium. From this study it was suggested that the reduced stromal cell proliferation in these mutant mice was a consequence of altered PG signaling due to abnormally low levels of stromal EP_3 and EP_4, and that this may have contributed to the defective process of decidualization seen in these mice. These results support a role for PGE_2 throughout the decidual cell reaction, at least in mice, and suggest that for a full decidual response functional stromal EP_3 and EP_4 receptors are required.

In rodents, the onset of uterine receptivity to blastocyst attachment coincides with uterine sensitization for decidualization, and both these conditions are time- and hormone-dependent (reviewed by Psychoyos [2]). It has been previously suggested that the onset of receptivity and capacity of the endometrium to proliferate and differentiate into decidual tissue may depend on the uterine expression of EP receptors, rather than the ability of the uterus to produce PGs [40, 41]. Therefore, we determined whether the specific localization of mRNA for the EP receptors on Day 5 of pseudopregnancy was influenced by the amount of E_2 given on Day 4. Suboptimal, optimal, and suprathreshold doses of E_2 were given on Day 4 of pseudopregnancy, along with a constant dose of P_4, to induce uterine states equivalent to the endometrial environment during delayed implantation, the receptive state, or the refractory state, respectively, on Day 5. We found that the optimal dose of E_2 given along with P_4 is responsible for directing expression of EP_2 and EP_4 toward the antimesometrial pole of the endometrium on Day 5 of pseudopregnancy while it simply up-regulates EP_3 throughout the endometrium. The results of the present study are consistent with studies in the mouse in which a cooperative interaction was seen between E_2 acting with P_4 to augment basal EP_2 levels in the luminal epithelium and EP_3 mRNA levels in the endometrial stroma [10, 30, 33]. However, in contrast to the rat, in the mouse the distribution of EP_4 transcripts was the same for P_4 alone as for P_4 and E_2 in combination [10, 33]. Although the exact mechanisms by which E_2 and P_4 interact to achieve the receptive state (and uterine sensitization) are unknown, the most significant finding of the present study is that there appears to be a correlation between EP_2, EP_3, and EP_4 transcript expression and cellular localization with uterine sen-

sitization and the onset of receptivity in the rat. These findings lend support to previous studies suggesting that estrogen acts on a P_4-dominated endometrium to direct epithelial differentiation [2, 42] and to increase the number of stromal cells capable of undergoing decidualization [1, 43]. The up-regulation of the EP_2 receptor may allow the epithelium to transduce signals to the underlying stroma, and the up-regulation of the EP_4 receptor may allow for the initiation of decidualization and endometrial stromal cell mitogenesis, respectively, by increasing cAMP levels following PGE_2 activation. The involvement of a cAMP-dependent mechanism in mediating the effects of PGE_2 at the time of implantation and decidualization has been supported by previous studies [44–46].

In conclusion, we have shown that mRNAs encoding three subtypes of PGE_2 receptors, EP_2, EP_3, and EP_4, are coexpressed in neighboring or identical regions of the rat endometrium on Day 5 of pseudopregnancy in response to the optimal dose of E_2 and P_4 that induces maximal uterine sensitization for decidualization. Overall, this suggests that these receptors may act in combination to modulate the levels of cAMP in the uterus at the time of implantation by acting as a homeostatic control mechanism for the effects of PGE_2. Furthermore, the change in expression pattern of these receptors among the different cell populations of the uterus may provide a basis for the change in uterine state from a ''pre-receptive'' to a receptive endometrium, and then to a refractory state. However, studies looking at levels and localization of EP receptor protein remain to be completed.

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