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**A Study on the Structure and Function
of the Prostaglandin E Receptor Subtype EP₂**

1998

Masato Katsuyama

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INTRODUCTION

Prostaglandin (PG) E₂ is a major metabolite of arachidonic acid liberated from phospholipids in plasma membranes. PGE₂ is synthesized from arachidonic acid through oxygenation and reduction by prostaglandin H synthase (cyclooxygenase) and isomerization by PGE synthase (Fig. 0-1). PGE₂ produces a broad range of biological actions in diverse tissues through binding to specific receptors on plasma membranes (Samuelsson et al., 1978; Moncada et al., 1985). Pharmacological studies have suggested that there are four subtypes of PGE receptors, EP₁, EP₂, EP₃ and EP₄, which are thought to differ in their signal transduction mechanisms; these receptors are coupled to Ca²⁺ mobilization (EP₁), or the stimulation (EP₂ and EP₄) or inhibition (EP₃) of adenylate cyclase (Coleman et al., 1990; Negishi et al., 1993; Coleman et al., 1994). Various pharmacological actions of PGE₂ via these receptor subtypes have been characterized (Table 1).

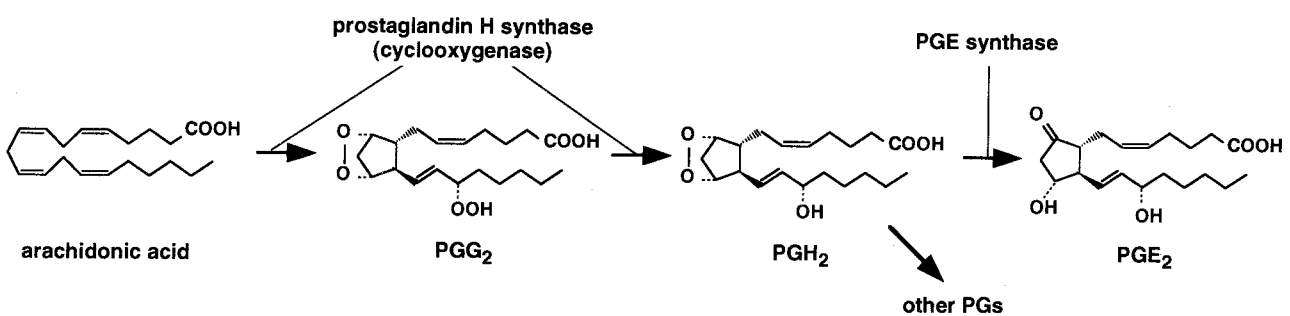


Fig. 0-1. Biosynthesis of PGE₂. PGE₂ is synthesized from arachidonic acid via the actions of prostaglandin H synthase (cyclooxygenase) and PGE synthase.

Table 1. Prostaglandin E receptor subtypes and their functions.

Subtype	Signaling Pathway	Expression Sites	Functions
EP ₁	[Ca ²⁺] _i ↑	Kidney	Inhibition of Water Reabsorption
		Smooth Muscle	Contraction
EP ₃	[cAMP] ↓	Kidney	Inhibition of Na ⁺ Transport
		Uterus	Contraction
		Stomach	Inhibition of Gastric Acid Secretion
		Neurons	Inhibition of Neurotransmitter Release
EP ₂ / EP ₄	[cAMP] ↑	Trachea	Relaxation
		Ileum	Relaxation, Secretion
		Thymus	Negative Regulation of the Immune System
		Blood Vessels	Vasodilation

The receptor subtype EP₂, which is coupled to the stimulation of adenylate cyclase, is known to be involved in PGE₂-induced relaxation of the trachea and ileal smooth muscle (Gardiner, 1986; Botella et al., 1993). In addition, PGE₂ has been shown to increase the cAMP level in many tissues and cells (Samuelsson et al., 1978; Coleman et al., 1990), suggesting that EP₂ is widely distributed and mediates various actions of PGE₂. This idea however, is not conclusive because most pharmacological studies have been performed using PGE₂ as an agonist, but not with ligands selective to EP₂. PGE₂ has been reported to increase the cAMP level through its interaction with EP₂ and/or EP₄ (Honda et al., 1993; Coleman et al., 1994; Nishigaki et al., 1995). Therefore, it is necessary to clarify the functions of EP₂ at the molecular level, and to elucidate which subtype is involved in each cAMP-mediated action of PGE₂.

The author at first isolated a cDNA encoding the mouse EP₂, and then using this cDNA, characterized its structural, binding and signaling properties and its tissue distribution (Katsuyama et al., 1995). The author also found that the mRNAs for the EP₂ as well as other EP subtypes show distinct cellular localizations in pseudopregnant mouse uteri, and that the EP₂ mRNA is specifically expressed on day 5 and its expression is confined to the luminal epithelium (Katsuyama et al., 1997). Furthermore, the author found tissue specific initiation of transcription of the EP₂ gene in the macrophage and the uterus (Katsuyama et al., 1998).

The results obtained are described in the following, divided into three chapters.

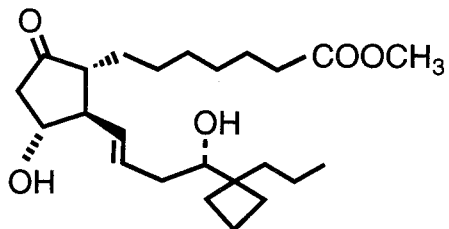
The abbreviations used in the text are as follows;

bp	base pair(s)
C-	carboxy-
cAMP	adenosine 3',5'-cyclic monophosphate
cDNA	complementary DNA
CHO	Chinese hamster ovary
cpm	counts per minute
cRNA	complementary RNA
CTP	cytidine triphosphate
dCTP	deoxy cytidine triphosphate
EDTA	ethylenediamine-N,N,N',N'-tetraacetic acid
Gi	inhibitory guanine nucleotide-binding protein
hCG	human chorionic gonadotropin
HEPES	2-[4-(2-hydroxyethyl)-1-piperazinyl] ethanesulfonic acid
kb	kilobase(s)

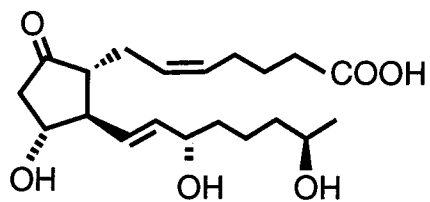
LPS	lipopolysaccharide
mRNA	messenger RNA
N-	amino-
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PG	prostaglandin
PIPES	piperazine-N,N'-bis(2-ethanesulfonic acid)
PMSG	pregnant mare serum gonadotropin
5'-RACE	5'- rapid amplification of cDNA ends
RNase	ribonuclease
RT-PCR	reverse transcriptase-polymerase chain reaction
SDS	sodium dodecyl sulfate
SSC	standard saline citrate
TM	transmembrane
TX	thromboxane
UTP	uridine triphosphate
[Ca ²⁺] _i	intracellular Ca ²⁺ concentration

Subtype-specific PGE analogues used in this study are shown in Fig. 0-2.

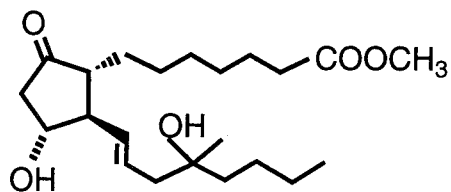
Agonists



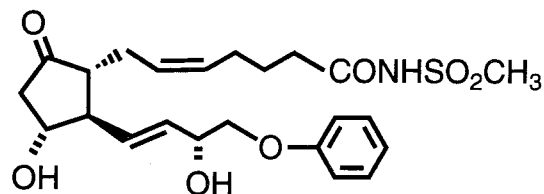
Butaprost



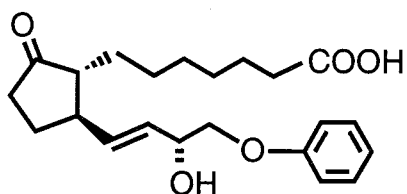
19(R)OH PGE₂



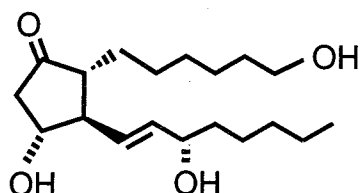
Misoprostol



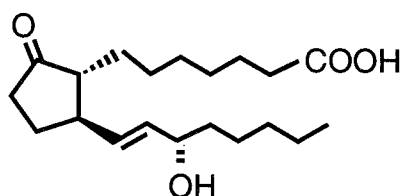
Sulprostone



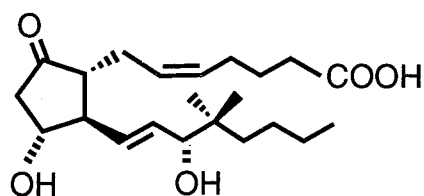
M&B 28767



1-OH PGE₁

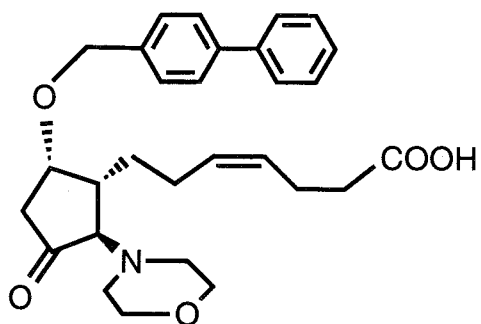


11-deoxy PGE₁

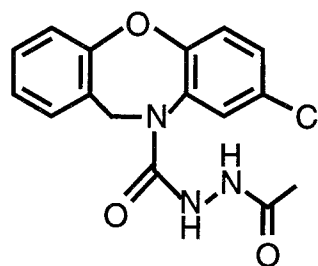


16,16-dimethyl PGE₂

Antagonists



AH 23848B



SC-19220

Fig. 0-2. Structures of PGE ligands used in this study.

1. The Mouse Prostaglandin E Receptor EP₂ Subtype: Cloning, Expression, and Northern Blot Analysis

ABSTRACT

A functional cDNA clone for the mouse PGE receptor subtype EP₂ was isolated from a mouse cDNA library. The mouse EP₂ consists of 362 amino acid residues with seven putative transmembrane domains. [³H]PGE₂ bound specifically to the membrane of Chinese hamster ovary (CHO) cells stably expressing the cloned receptor. This binding was displaced by unlabeled prostanoids, in the order of efficiency of PGE₂ = PGE₁ >> iloprost, a stable PGI₂ agonist > PGF_{2α} > PGD₂. Binding was also inhibited by butaprost (an EP₂ agonist) and to a lesser extent by M&B 28767 (an EP₃ agonist), but not by sulprostone (an EP₁ and EP₃ agonist) or SC-19220 (an EP₁ antagonist). PGE₂ and butaprost increased the cAMP level in the CHO cells in a concentration-dependent manner. Northern blot analysis revealed that EP₂ mRNA is expressed most abundantly in the uterus, followed by the spleen, lung, thymus, ileum, liver, and stomach.

RESULTS

Structure of the Mouse EP₂ Receptor

A mouse cDNA clone, ML202, was isolated by PCR and subsequent screening of a mouse lung cDNA library. Figure 1-1 shows the nucleotide sequence and deduced amino acid sequence of ML202. ML202 contains a 1.7 kb insert which has an open reading frame of 1,086 bp. The deduced amino acid sequence showed that it encodes a protein of 362 amino acid residues with an estimated molecular weight of 40,478. The ATG assigned as the initiation codon matched well to the Kozak consensus sequence for translation initiation (Kozak, 1989). Analysis of the predicted amino acid sequence indicated that it possesses seven hydrophobic segments typical of guanine nucleotide-binding protein-coupled rhodopsin-type receptors, and is 86% identical to that of the human EP₂ (Regan et al., 1994). A potential *N*-glycosylation site (Bause, 1983) was found in both the N-terminal and the first extracellular loop regions. Five potential sites of phosphorylation by protein kinase C (Woodgett et al., 1986) were found in the second and third intracellular loops and the C-terminal regions.

```

-145 GTGCGAGTTGGGGCCGGAAGGAG -121
CTCTGGATTTCGGTCCCTCCCCTTTCCCTGCTCTGTCTTGGAGCCCTGGGGCCATCAGACCCCTCCGACTGTCTGGTACTTGCCTGGAAGAGATATCATCTCTCCACACCCCTCCACC -1
ATGACAATTTTCTTAATGACTCCAAGCTAATGGAGGACTGCAAGAGTCTGTCAGTGGCTCTTTTCGGGGGAAAGCCAGCCATCAGCTCGGTGATGTTCTCGGCCGGGTTCGGGGAAT 120
M D N F L N D S K L M E D C K S R Q W L L S G E S P A I S S V M F S A G V L G N 40
      Δ
CTCATCGCACTGGCACTGTTGGCGCGCCGCTGGCGTGGGACACCGGGTGTAGCGCCGGCAGCAGGACCTCTATCTCCTTGTTCACGTGCTGGTAACGGAATTTGGTGTCACTGACCTG 240
L I A L A L L L A R R W R G D T G C S A G S R T S I S L F H V L V T E L V L T D L 80
      I
CTGGAACTGCTCATCAGCCCGTGGTGTGGCTTCATATTCAGAAACCAGACCCCTGGTGGCCCTGGCTCCCGAAAGTCACCGGTGTACCTAATTTGCTTTTCACTATGACCTTCTTT 360
L G T C L I S P V V L A S Y S R N Q T L V A L A P E S H A C T Y F A F T M T F F 120
      Δ
AGTCTGGCCACGATGCTCATGCTCTTCGCTATGGCCCTGGAACGCTACCTCTCCATCGGGTACCCTTACTTCTACAGGCGCCACTTATCGCGCCGCGGGGTCTGGCGGTGCTGCCTGTC 480
S L A T M L M L F A M A L E R Y L S I G Y P Y F Y R R H L S R R G G L A V L P V 160
      III
ATCTATGGGGCCTCCTTGTCTTCTGTTCCTGCGCGTGTCTCAACTACGGGAGTACGTCACAGTACTGTCTGGGACATGGTGTCTTATCCGGCACGGGAGGACTGCATACCTTCAGCTG 600
I Y G A S L L L F C S L P L L N Y G E Y V Q Y C P G T W C F I R H G R T A Y L Q L 200
      # IV
TACGCCACGATGCTCTGCTCTTATCGTGGCTGTGCTCGCCTGCAACATCAGCGTATCTCAACTCATTCGCGATGACCGTGGAGCAGAGAAGCCGCTGCGGATTTGCTGCGT 720
Y A T M L L L L L I V A V L A C N I S V I L L N L I R M H R R S R R S R C G L S G S 240
      #
AGCCTAAGAGGCCCTGGATCTCGCAGGAGAGAGAGAGGACTTCGATGGCAGAGGAGAGCGGACCACTCATTCTCTGGCCATTATGACCATCACCTTCGCCATATGCTCCTTGCCTTTC 840
S L R G P G S R R R G E R T S M A E E T D H L I L L A I M T I T F A I C S L P F 280
      #
ACAATCTTTGCCTACATGGATGAAACCTCTTCCCTAAAGGAAAAGTGGGACCTCCGAGCTCTTCGGTTTTATCAGTTAACTCTATAATTGACCCTTGGGTCTTTGCCATACTTAGGCCA 960
T I F A Y M D E T S S L K E K W D L R A L R F L S V N S I I D P W V F A I L R P 320
      VI
CCGGTCTGAGGTTAATGCGCTCAGTCTCTGTGTGTCGGACTTCACTGAGAACAACAAGAAGCTCAGCAAACATCCTGTTCTACCCAGTCCAGTCCAGTAAACAGACTGACCTTTGTGGA 1080
P V L R L M R S V L C C R T S L R T Q E A Q Q T S C S T Q S S A S K Q T D L C G 360
      #
CAGTTGTGAGGATGCGCTTCACGAGGGAACCTCTGAAGAAGTCTTTAAATGGCCCTATTGGAGAAGTGTAAAGGGCTGGAATATAAACAGAATAACCTTGCCTGAGAAGCAGATGAAAC 1200
Q L 362
AGACTTATGAGGTTTGGGCTGCAGATGTGATGACAGAGCCCTTTGTGAAAAGTGTGAGGATATAAAGTTCACATTATGTGACCTTTGAAGGACAACTGCTGCGTCGAAGACCCAGT 1320
TGATTTAAGCTTTCCTGTTGAGTGACAAAGCATGTGGTTTTGTAAATTTGTTTTAAATGCTACATGGTTACTGTTTTAATTTGATGTCAGTCTGTTATAAATGCAGTCAGACCAAATG 1440
AAATTCATCTCTAGGAAGTCAACTCATGCAAGCGATGAAGCAACAGAGCAGACAAGTTGCTGTCTCGTGATTGCTTAATGGACTCTTTATTTGGACCATGAAGTTGAATGCCATTGGC 1560
CCCTTTTAAAAA 1576

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Fig. 1-1. Nucleotide sequence and deduced amino acid sequence of ML202. The deduced amino acid sequence is shown beneath the nucleotide sequence using the single-letter code. The positions of the putative transmembrane segments, I-VII, are underlined. *Triangles*, potential *N*-glycosylation sites. *Parallel crosses*, potential sites of phosphorylation by protein kinase C.

Ligand Binding Specificity of the Mouse EP₂ Receptor

Membranes of CHO cells expressing the cloned receptor showed specific binding of [³H]PGE₂. Scatchard analysis of this binding yielded a dissociation constant of 26.7 nM and maximal binding of 822 fmol/mg protein. Figure 1-2 shows the specificity of this binding. As shown in Fig. 1-2a, specific [³H]PGE₂ binding was displaced by unlabeled prostanoids, in the order of efficiency of PGE₂ = PGE₁ >> iloprost, a stable PGI₂ agonist > PGF_{2α} > PGD₂. This result indicates that ML202 encodes a specific PGE receptor. As shown in Fig. 1-2b, further analyses using several ligands selective to PGE receptor subtypes demonstrated that the binding was also inhibited by butaprost (an EP₂ agonist) and to a lesser extent by M&B 28767 (an EP₃ agonist), but not by sulprostone (an EP₁ and EP₃ agonist) or SC-19220 (an EP₁ antagonist). These results indicate that ML202 encodes the mouse EP₂. The binding specificity of the mouse EP₂ was further characterized using various additional ligands, including additional EP₂ agonists. As shown in Fig. 1-2c, specific [³H]PGE₂ binding was also inhibited by several additional ligands, in the order of efficiency of 16,16-dimethyl PGE₂ (a nonselective EP agonist) (Nishigaki et al., 1995; Regan et al., 1994) > 11-deoxy PGE₁ (an EP₂, EP₃, and EP₄ agonist) (Nishigaki et al., 1995; Regan et al., 1994) > misoprostol (an EP₂, EP₃, and EP₄ agonist) (Honda et al., 1993; Coleman et al., 1988; Reeves et al., 1988) > 1-OH-PGE₁ (an EP₄ partial agonist) (Nishigaki et al., 1995) > 19(R)OH-PGE₂ (an EP₂ agonist) (Regan et al., 1994; Woodward et al., 1993). These binding characteristics were in good agreement with those of the human EP₂ (Regan et al., 1994), except for poor reactivity of 19(R)OH-PGE₂ to the mouse receptor. This may at least in part be due to species differences between the two receptors.

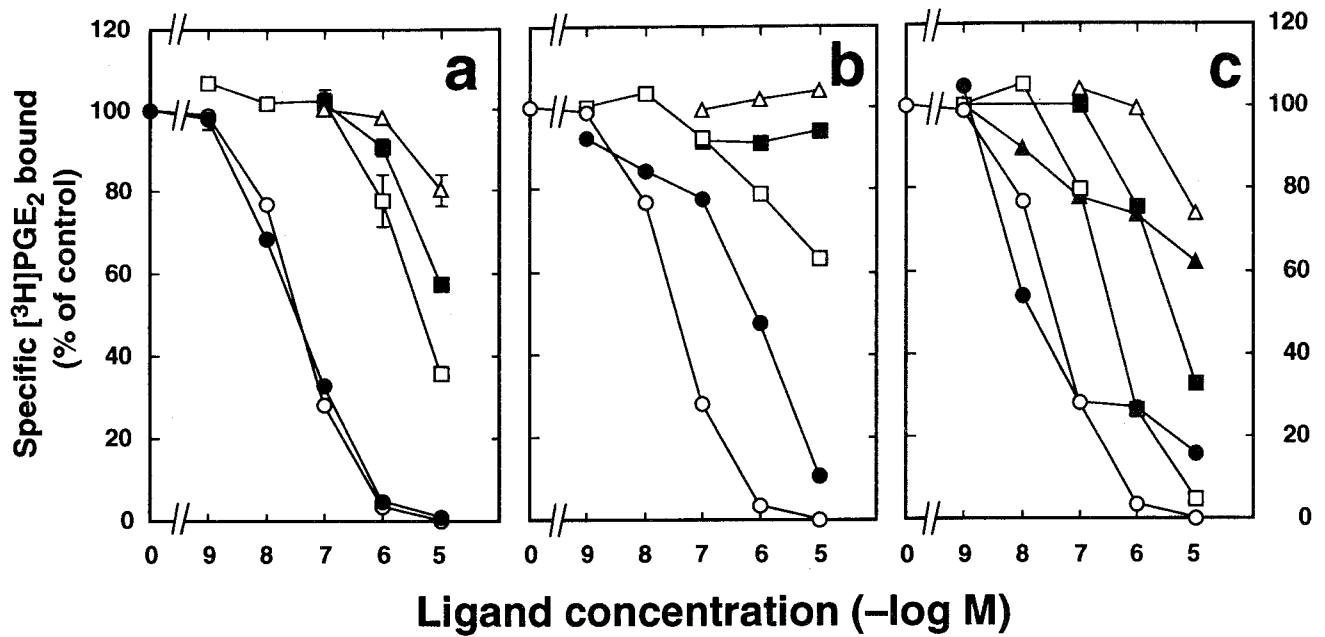


Fig. 1-2. Binding of [³H]PGE₂ to ML202-expressing CHO cell membranes. a, Displacement of [³H]PGE₂ binding by various prostanooids. Membranes of CHO cells expressing ML202 were incubated with 4 nM [³H]PGE₂ and various unlabeled prostanooids at the concentrations indicated. ○, PGE₂; ●, PGE₁; □, iloprost; ■, PGF_{2α}; △, PGD₂. b, Displacement of [³H]PGE₂ binding by ligands for PGE receptor subtypes. ●, butaprost; □, M&B 28767; ■, sulprostone; △, SC-19220. c, Displacement of [³H]PGE₂ binding by other EP agonists. ●, 16,16-dimethyl PGE₂; □, 11-deoxy PGE₁; ■, misoprostol; △, 19(R)OH-PGE₂; ▲, 1-OH-PGE₁. The same data for PGE₂ as shown in "a" are displayed in "b" and "c".

Signal Transduction Pathway of the Mouse EP₂ Receptor

Next, the signal transduction pathway of the mouse EP₂ was examined. As shown in Fig. 1-3, PGE₂ generated cAMP in a concentration-dependent manner in CHO cells expressing EP₂. In addition, butaprost and 11-deoxy PGE₁ were also effective in stimulating cAMP formation in the cells, although their half-maximal concentrations for this effect were about 10-fold higher than that of PGE₂. 19(R)OH-PGE₂ and 1-OH-PGE₁ were less effective than the ligands described above. It was also examined whether PGE₂ induces accumulation of inositol phosphates, but PGE₂ did not evoke phosphatidylinositol hydrolysis in the cells (data not shown), as is the case in EP₄ (Honda et al., 1993).

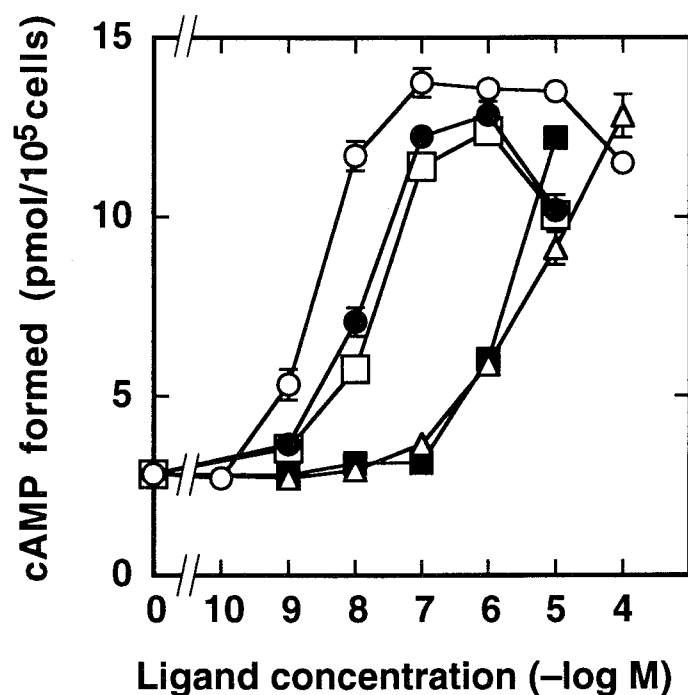


Fig. 1-3. Effects of PGE₂ and several ligands on the cAMP level in ML202-expressing CHO cells. CHO cells were incubated with the indicated concentrations of each ligand, and the cAMP level was determined as described in "EXPERIMENTAL PROCEDURES." ○, PGE₂; ●, butaprost; □, 11-deoxy PGE₁; ■, 19(R)OH-PGE₂; △, 1-OH-PGE₁.

Tissue Distribution of the mRNA for the Mouse EP₂ Receptor

PGE₂ has been shown to increase the cAMP level in many tissues and cells (Samuelsson et al., 1978) through its interaction with EP₂ and/or EP₄. To elucidate which PGE receptor subtype is involved in cAMP-mediating actions of PGE₂ in each tissue, the expression patterns of EP₂ and EP₄ were compared by Northern hybridization. As shown in Fig. 1-4a, the level of EP₂ expression was much lower than that of EP₄ in most of the tissues examined. In a previous report, using standard hybridization analysis, Regan et al. detected EP₂ mRNA only in the mRNA from the human placenta and lung, and not in the other tissues (Regan et al., 1994). Therefore, highly purified poly(A)⁺ RNAs were utilized for more sensitive detection (Fig. 1-4b). As a result, a positive band at 2.2 kb was detected in a number of tissues in which EP₂ had been identified in pharmacological studies (Coleman et al., 1990); the tissues include the uterus, lung, ileum, liver, stomach, thymus and spleen. Another hybridizing band was also seen at 2.8 kb in the uterus. These data demonstrate that EP₂ is widely distributed throughout the mouse tissues.

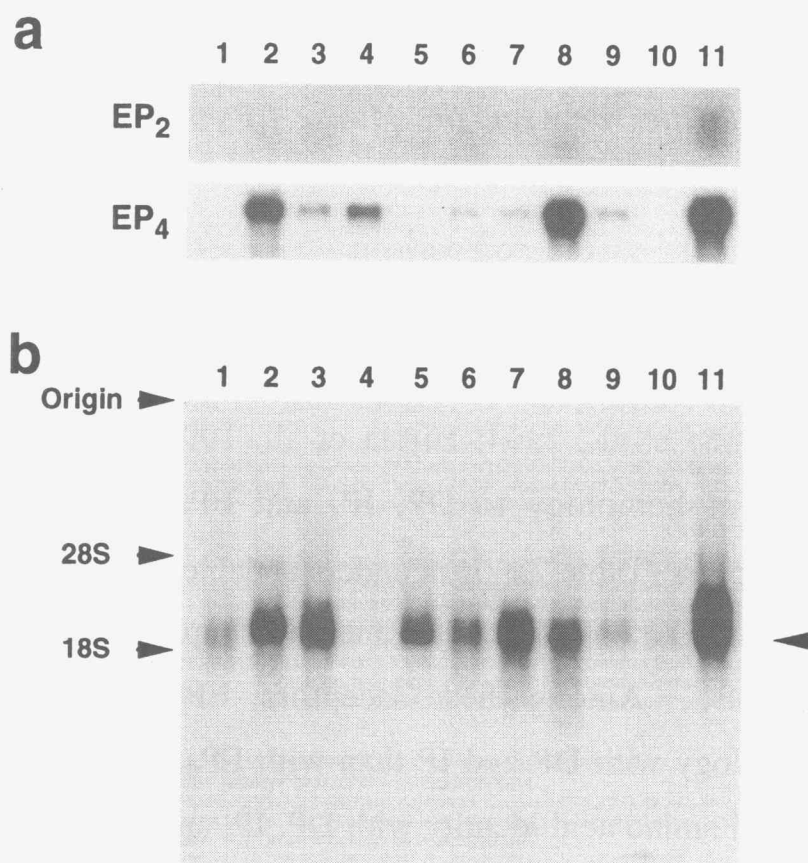


Fig. 1-4. Northern blot analysis of RNAs isolated from various mouse tissues. a, Comparison of the levels of expression of the mouse EP₂ receptor (top) and EP₄ receptor (bottom). Poly(A)⁺-rich RNA (5 μg) was applied to each lane. b, Tissue distribution of the mouse EP₂ receptor. Highly purified poly(A)⁺ RNA (5 μg) was applied to each lane. Hybridization analysis was carried out as described in "EXPERIMENTAL PROCEDURES." Lane 1, brain; lane 2, thymus; lane 3, lung; lane 4, heart; lane 5, liver; lane 6, stomach; lane 7, spleen; lane 8, ileum; lane 9, kidney; lane 10, testis; lane 11, uterus.

DISCUSSION

Structural Similarities between the Mouse EP₂ Receptor and Other Prostanoid Receptors

Figure 1-5 shows the comparison of the amino acid sequences among the cloned mouse prostanoid receptors (Sugimoto et al., 1992; Namba et al., 1992; Honda et al., 1993; Watabe et al., 1993; Sugimoto et al., 1994a; Namba et al., 1994; Hirata et al., 1994). EP₂ showed a higher degree of homology to DP, IP, and EP₄ than to the other prostanoid receptors (Table 2). It is understandable from the viewpoint of phylogeny that EP₂, DP, IP, and EP₄ are all coupled to stimulation of adenylate cyclase. Among these receptors, EP₂ possesses higher sequence homology with DP and IP than with EP₄; EP₂ shares 44, 39, and 31% overall amino acid identity with DP, IP, and EP₄, respectively. This indicates that the divergence between EP₂ and EP₄ occurred before the derivation of DP and IP from EP₂ (Toh et al., 1995).

Table 2. Homology between the mouse EP₂ and other prostanoid receptors.

	amino acid sequence homology with EP ₂	signal transduction pathway
DP	44%	[cAMP] ↑
IP	39%	[cAMP] ↑, PI turnover
EP ₄	31%	[cAMP] ↑
.....		
EP ₃	30%	[cAMP] ↓, [cAMP] ↑ (splice variant)
.....		
EP ₁	29%	[Ca ²⁺] _i ↑
TP	25%	PI turnover
FP	24%	PI turnover

	I	II	
mEP2	MDNFLNDSKLMEDCKSRQWLLSGESP-AISSVMFSA-GVLGNLIALALLARWRGDTGCSAGSRTSISLFLHVLVT		73
mDP	MNESYRCQTSITWVERGSSA-TMGAVLFGA-GLLGNLLALVLLARSGLGSCRPGPLHPPP-SVFFVYLV		65
mIP	MKMMASDGHGPPSVTPGSPLSAGGREWQMGAGCWNITVVDQSVGP-ATSTLMFVA-GVVGNGLALGIL-----GARRRSH-PS--A-FAVLVT		84
mEP4	MAEVGGTIPRSNRELQRCVLLTTTMSIPGVNASFSSTPERLN-SPVTIPAVMFIF-GVVGNLVAIVVL-----CKSRKEQKET--T-FYTLVC		84
mEP3 α	MASMWAPHSAAEHSNLS-STTDDCGSVSV-AFPITMMVT-GFVGNLALM-LLVSRSY--RRRESKRKK---FLLCIG		70
mEP1	MSPCGLNLSLADEAATCATPRLPNTSVLPTGDNSTP-ALPI-FSMTLGAVSNVLAALLAQVAGRMRRRSAA---T-FLLFVA		80
mFP	MSMNSKQPVSPAAGLIANTTCQENRLSV-FPSI-IFMTVGIILSNLSLAIAILMKAYQRF-RQKSKA----S-FLLLAS		71
mTP	MWPNGTSLGACFRPVNIT--LQERRAIASP-WFAA-SFCALGLGSLNLLALSVLAGA----RPGAGPRS--S-FLALLC		67
III			
mEP2	ELVLTDLGLTCLISPV-VLASYSRNQTVALAPES--HACTYFAFTMTFFSLATMLMLFAMALERYLSIGYPYFYRRHLRSR-RGGLAVLPVIYGAS	IV	165
mDP	GLTVDLLGKCLISPM-VLAAAYAQNQSLKELLPASGNQLCETFAFLMSFFGLASTLQLLAMAVECWLGLGHPFFYQRHVTL-RRGVLVAPVVAFC		159
mIP	GLAVTDLGLTCLFSPA-VFVAY--G-LAHGGT-M----LCDTFDFAMTFDLASTLILFAMAVECRLALSHPYLYAQLDGP-PCARFALPSIYAF		177
mEP4	GLAVTDLGLTCLVSPV-TIATYMKG-QWPGDQ-A----LCDYSTFILLFFGLSGLSIIICAMSIERYLAINHAYFYSHYVDK-RLAGLTLFAIYASN		172
mEP3 α	WLALTDLVGQLTSPV-VILVYLSQRRWEQLDP-SGR-LCTFFGLTMTVFGLSLLVASAMAVERALAIRAPHWYASHMKT-R-ATPVLLGVWLSV		161
mEP1	SLLAIDLAGHVIPGAL-VLRLYTAGRA-PAGG-A----CHFLLGCGMVFGLCPLLLGCGMAVERCVGVTQPLIHAARVSVAR-ARLALAVLVAFA		167
mFP	GLVITDFFGHLINGGIQVY-YASDKDWIRFD-QSNI-LCSIFGISMVFGLCPLFLGSGAMAIERICIGVTNPIFHSTKITSKH-VKMLISGLVCMFA		163
mTP	GLVLTDFLGLLVTGAI-VASQHAALLDWRATDP-SCR-LCYFMGVAMVFGLCPLLLGAMASERFVGTITRPFSPRTATSR-R-AWATVGLVWVAA		158
V			
mEP2	LLFCSLPLLNIGEY-VQYCPGTWCFIRHGR-----TAYLQLYATMLLLLIVAVLACNIVIL-NLIRMRHRRSRRCGLSGSSLR---		243
mDP	LAFCALPFAGFGKF-VQYCPGTWCFIQMIHKERS-----FSVIGFVLYSSLMALLVLAIVVCLGAMY-NLYDMHRRHRHYPHRCSDRAQ---		244
mIP	CLFCSLPLLGLGEHQ-QYCPGSGWCFIRM-R--SAP-----GGCAFSLAYASLMALLVTSIFFCNGSVTL-SLYHMYRQRRHSGSEVP-----		255
mEP4	VLFICALPNMGLGRSERQY-PGTWCFIDWTT--NVT-----AYAAFVSYMAGFSSFLILATVLCN-VLVCGALLRMRHMQFRMRTSLGTEQHAAAA		258
mEP3 α	LAFALLPVLGVGRYSVQW-PGTWCFISTGPAGNETDPAREPGSVAFASAFACGLLALVVTFACN-LATIKALVSR-----		235
mEP1	LAVALLPLVHVGRYELQY-PGTWCFISLGRG-----GWR-QALLAGLFAGLGLAALLALVLCNTLSGL-ALLRA-RWRRRRRSRFRKTAGPDDR		253
mFP	VFVAVLPILGHRDYQIQV-SRTWCFYNTEHIE-----DWE-DRFYLLFFSFLGLLALGVSFSCNAVTVGV-TLLRV-KF--RSQ-----		235
mTP	GALGLLPLLGLGRYSVQY-PGSGWCFITLGT-----QRGDVVFGLIFALLGASVGLSLLLNTVSVV-TL-----		220
VI			
mEP2	-----GPGSRRRGERTSMAEETDHLI-LL-AIMTITFAICSLPFTIFAYMDETSSL-----KEKW-DL	VII	298
mDP	-----SGSDYRHGSLHPLEELDHFV-LL-ALMTVLTMCSEPLIYRAYYGAFLKLENKAEG--D---SE---DL		302
mIP	-----TSRAREDEVYHLI-LL-ALMTVIMAVCSLPLMIRGFTQAIAPDS-----REMG-DL		303
mEP4	AAVASVACRGHAGASPALQRLSDF-----RRRSFRRIAGAEIQMVI-LLIATS-LVVLICSIPLVVRFVFNQLYQPNVV---KDIS---RNPDL		340
mEP3 α	-----CRAKAAVSQS-----SAQWGRIT-TTETALQLM-GIM-CVLSVCWSPLLIMMLKMIFNQMSVEQCKTQMGKEKCNSTL		305
mEP1	RRWGSRGPRLASASSASSITSATATLRSSRGGGSARRVHAHDVEMVQQLV-GIM-VVSCICWSPLLVLVV-LAIGGWNSNSL-Q---RPL--FL		338
mFP	-----QHRQGRSHHLEMIQQL-AIM-CVSCVCWSPFLVIMANIAINGNNSPVTC-----E---TTL		287
mTP	-----CRVYHT-----R--EATQRPRDCEVEMVQLV-GIM-VVATVCMPLLVFIMQTLTTPPVMSFSGQLLRATEHQ-LL		288
VIII			
mEP2	RALRFLVNSIIDPWVFAILRPPVLRRL-MRSVLCRTSLRTEQAQQTSCSTQSSASKQTDLCGQL		362
mDP	QALRFLVVISVDPWFIFLIRTSVFRM-LFHKVFTTRPLIYRNWSSHSQQSNVESTL		357
mIP	LAFRFNAPNPILDPWVIFLFRKAVFQR-LKFWLCCLCARSVHGDLOAPLSRPAASGRDPPAPTSLQAKEGWSVPLSSWGTGQVA (30)		416
mEP4	QAIRIASVNPILDPWVYILLRKTIVLSKALEKIKCLFCRIGGSGRDSQAHCSESRRTSSAMGHSRSPFLARELKEISSTSQTL (90)		513
mEP3 α	IAVRLASLNQILDPWVYLLRRIILRKL-FCQIRDHNTYASSSTSLPCPCGSSALMWSQQLER		365
mEP1	-AVRLASWNQILDPWVYILLRQAMLRQ-LLRLLPLRVSAGKGPTELGLTKSAWEASSLRSSRHSGFSLH		405
mFP	FALRMTWVNLDPWVYILLRKAVALRN-LYKLASRCCGVNITSLHIEWELSSIKNSLVAAIASEPAAEKESQQASSEAGL		366
mTP	IYLRVATWVNLDPWVYILFRRSVLRRL-LHPRFSSQLQAVSLRPPAQAMLSGP		341

Fig. 1-5. Comparison of the amino acid sequences of mouse prostanoid receptors. The deduced amino acid sequences of the mouse EP₂ (*mEP2*), mouse PGD receptor (*mDP*), mouse PGI receptor (*mIP*), mouse EP₄ (*mEP4*), mouse EP_{3 α} (*mEP3 α*), mouse EP₁ (*mEP1*), mouse PGF receptor (*mFP*), and mouse TXA₂ receptor (*mTP*) are aligned to optimize homology. Identical amino acid residues in four or more sequences are indicated by *bold characters*. The approximate positions of the putative transmembrane regions are indicated *above* the amino acid sequences.

Physiological Function of the EP₂ Receptor

Tissue distribution of the mRNA for the mouse EP₂, together with those for other PGE receptor subtypes, are shown in Fig. 1-6. The highest level of EP₂ mRNA expression is seen in the uterus, in which EP₃ and EP₄ are also expressed. Cellular distributions of these PGE receptor subtypes in the uterus were investigated and their physiological functions are discussed in Chapter 2. Both EP₂ and EP₄ are expressed in the ileum and thymus. PGE₂ and butaprost have been reported to cause relaxation of the ileal smooth muscle (Botella et al., 1993), while PGE₂ also stimulates intestinal secretion via the elevation of mucosal cAMP levels (Musch et al., 1987; Bunce et al., 1987; Homaidan et al., 1995). It is likely that EP₂ and EP₄ are involved in relaxation of the intestinal smooth muscle and stimulation of intestinal secretion, respectively. The EP₂ transcript was detected in the liver, in which no signals for the EP₄ transcript were detected. PGE₂ has been reported to be involved in liver regeneration (Tsuji et al., 1993), and PGE₂ and other cAMP-elevating agents can induce the expression of hepatocyte growth factor (Matsunaga et al., 1994). EP₂ might be involved in these actions of PGE₂. EP₂ was also expressed in the spleen, lung, and stomach. Although other PGE receptor subtypes are also expressed in the lung (Sugimoto et al., 1992; Watabe et al., 1993), EP₂ may be partly involved in the PGE₂-induced relaxation of the trachea (Gardiner, 1986).

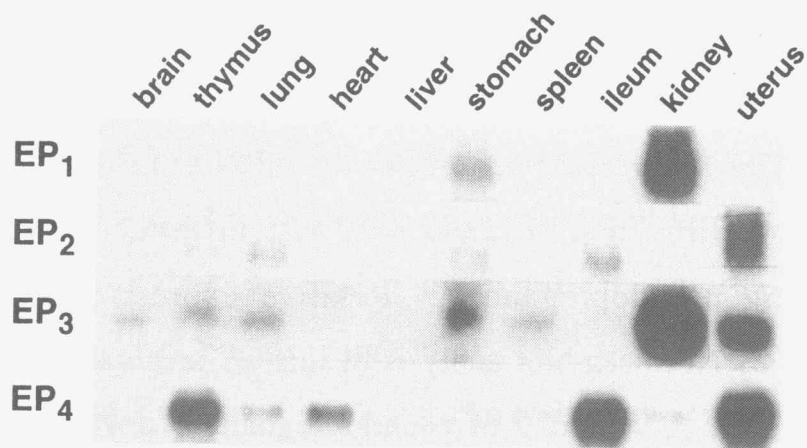


Fig. 1-6. Northern blot analysis of the mRNAs for four subtypes of the mouse PGE receptor.

2. Distinct Cellular Localization of the Messenger Ribonucleic Acid for Prostaglandin E Receptor Subtypes in the Mouse Uterus during Pseudopregnancy

ABSTRACT

As an initial step to clarify the mechanisms of various uterine actions of PGE₂, expression patterns of the mRNAs for four subtypes of PGE receptors, EP₁, EP₂, EP₃, and EP₄, were investigated in the mouse uterus during pseudopregnancy. Relative expression levels were investigated by Northern blot analysis of mRNA levels in uteri obtained on days 0, 1, 3, 5, 7, and 9 of pseudopregnancy (day 0 = 48 h after PMSG injection), and cellular localization was determined by *in situ* hybridization in uteri obtained on days 0 and 5. EP₂ mRNA was specifically expressed on day 5, and its expression was confined to the luminal epithelium. On the other hand, the level of the EP₃ mRNA expression progressively increased until day 5. Cell populations expressing the EP₃ mRNA were confined to the longitudinal smooth muscle on day 0, but they changed to the circular smooth muscle on day 5. The expression level of EP₄ mRNA was low on days 0 and 1, but became high on days 3 and 5. On day 0, EP₄ mRNA was localized to the luminal epithelium. On day 5, EP₄ expression was observed over the endometrial stroma and epithelium. This expression was diffuse, but still significant. No EP₁ mRNA signals were observed. Transient expression of EP₂ on day 5 of pseudopregnancy in the luminal epithelium suggests its involvement in blastocyst implantation signaling. EP₄ in the endometrial stroma is suggested to be involved in decidual transformation of stromal cells, whereas EP₃ in the myometrium is believed to be involved in regulation of myometrial activity.

RESULTS

Changes in the expression levels of the PGE receptor mRNAs in the uterus during pseudopregnancy

In the uteri from pseudopregnant mice, the mRNAs encoding the three PGE receptor subtypes, EP₂, EP₃, and EP₄, were detected by Northern blot analyses (Fig. 2-1a); the major transcripts were 2.8, 2.3, and 3.9 kb for EP₂, EP₃, and EP₄, respectively. Minor bands for the EP₂ and EP₃ mRNAs were also observed, as reported previously (Sugimoto et al., 1992; Katsuyama et al., 1995). No expression of the EP₁ transcript was detected (data not shown). The level of expression of the EP₂ transcript showed marked variations during the period examined. The transcript was barely detectable on days 0 and 1 of pseudopregnancy. On day 3, EP₂ mRNA was detected at a very low level, and the increase was evident on day 5: the mRNA level was 11-fold the level on day 3. The EP₂ mRNA thereafter sharply decreased to that seen on day 3. EP₃ mRNA was hardly detected on day 0. Beginning on day 1, the transcript progressively increased in abundance to reach the maximum on day 5, which was about 13-fold the level observed on day 1 (Fig. 2-1b). On days 7 and 9, expression decreased to lower levels than those on day 3. The expression of EP₄ mRNA, which was low on days 0 and 1, progressively increased and was maintained at a high level after day 5, which was approximately 10- to 15-fold the level on day 0 (Fig. 2-1b).

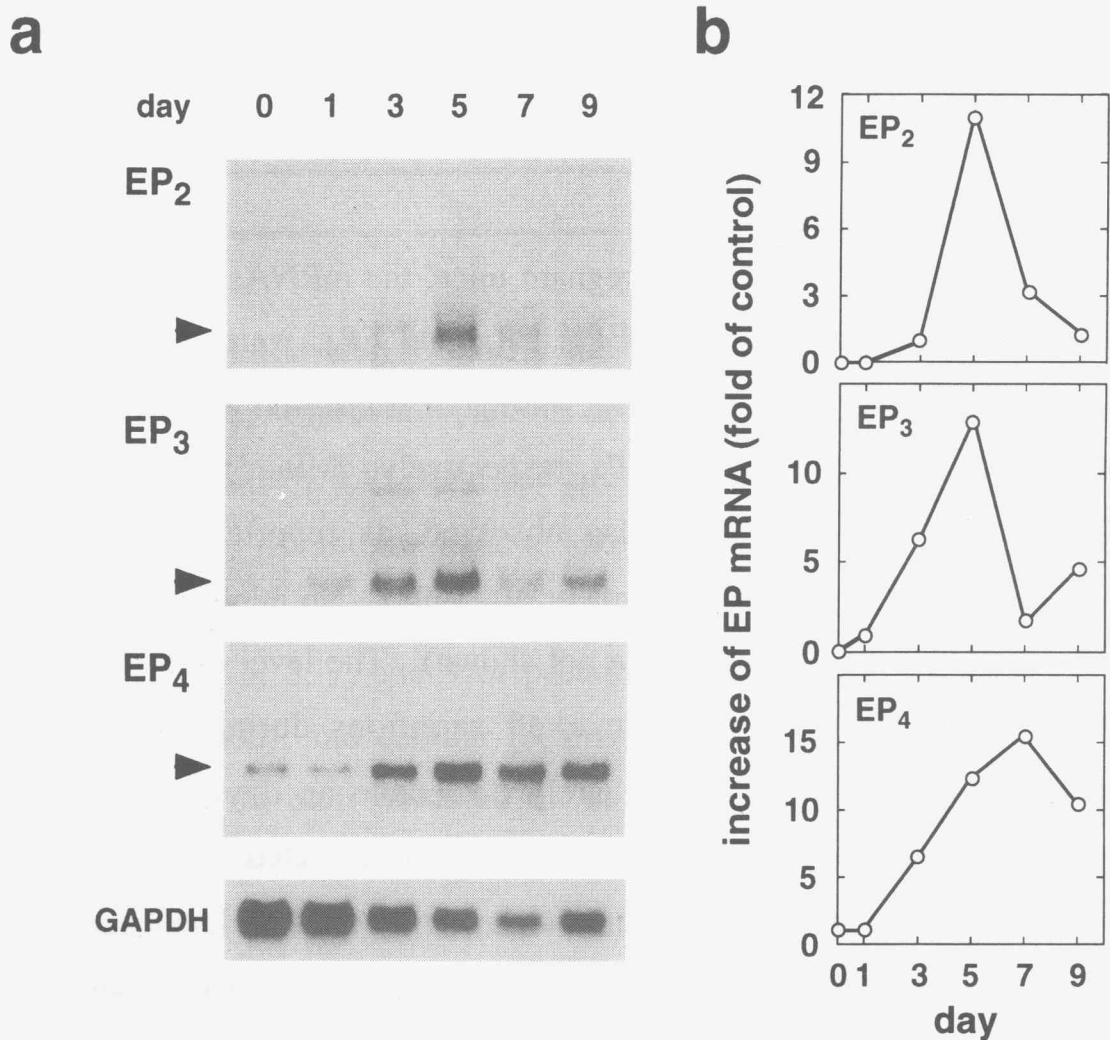


Fig. 2-1. a, Time-dependent expression of the mRNAs for PGE receptor subtypes in the mouse uterus during pseudopregnancy. Uteri were collected from three mice for each day of pseudopregnancy studied, and total RNAs were isolated. mRNA values were determined by Northern blot analysis using ³²P-labeled antisense riboprobes as described in "EXPERIMENTAL PROCEDURES." The same blots were rehybridized with ³²P-labeled DNA probe for GAPDH, and the representative results are shown. The day of pseudopregnancy is shown at the *top*. Day 0 represents 48 h after PMSG injection. The positions of the major bands are indicated by *arrowheads*. b, Quantitation of the signals for the PGE receptor (EP) mRNAs in a. The autoradiograms were subjected to densitometric scanning, and EP mRNA levels were normalized to GAPDH mRNA levels. The increase in EP mRNA levels is expressed as the fold increase over the level on the control day (days 3, 1, and, 0 for EP₂, EP₃, and EP₄, respectively). Northern blot analyses were independently repeated three times, and similar results were obtained. Representative results are shown.

Cellular localization of PGE receptor mRNAs in the pseudopregnant uterus

To define the cell populations expressing the PGE receptor subtype mRNAs, *in situ* hybridization analysis was performed on uteri on days 0 and 5 of pseudopregnancy. *In situ* hybridization using ³⁵S-labeled riboprobes revealed distinct regional distributions of EP₂, EP₃, and EP₄ mRNAs in the uterus. No signals for the EP₁ mRNA were detected in uteri on days 0 and 5 (Fig. 2-4b for day 5).

On day 0 of pseudopregnancy, EP₃ and EP₄ mRNAs were detected in the uteri (Fig. 2-2, c and d), but no significant signals for EP₂ were seen (Fig. 2-2b). Hybridization signals for the EP₃ mRNA were found in the myometrium (Fig. 2-2c). Microscopic examination of the myometrium exhibited weak, but significant, labeling of the longitudinal smooth muscle cells, but the circular smooth muscle cells were hardly labeled with the EP₃ probe (Fig. 2-3, a and b). As shown in Fig. 2-3, c and d, signals for the EP₄ mRNA were localized in the luminal epithelium.

On day 5 of pseudopregnancy, strong hybridization signals for EP₂ mRNA were observed in the luminal epithelial cells (Figs. 2-4c and 2-5, a and b). As shown in Fig. 2-4d, strong hybridization signals for EP₃ mRNA were seen in the myometrium. Microscopic examination of the myometrium exhibited intense labeling of the circular smooth muscle cells, but the longitudinal smooth muscle cells were not labeled (Fig. 2-5, c and d). Diffuse, but significant, EP₄ expression was observed in the endometrium (Fig. 2-4e). Microscopic examination of the endometrium revealed significant labeling of the luminal epithelial cells like that on day 0 of pseudopregnancy. In addition, endometrial stromal cells and the glandular epithelium were also labeled (Fig. 2-5, e and f).

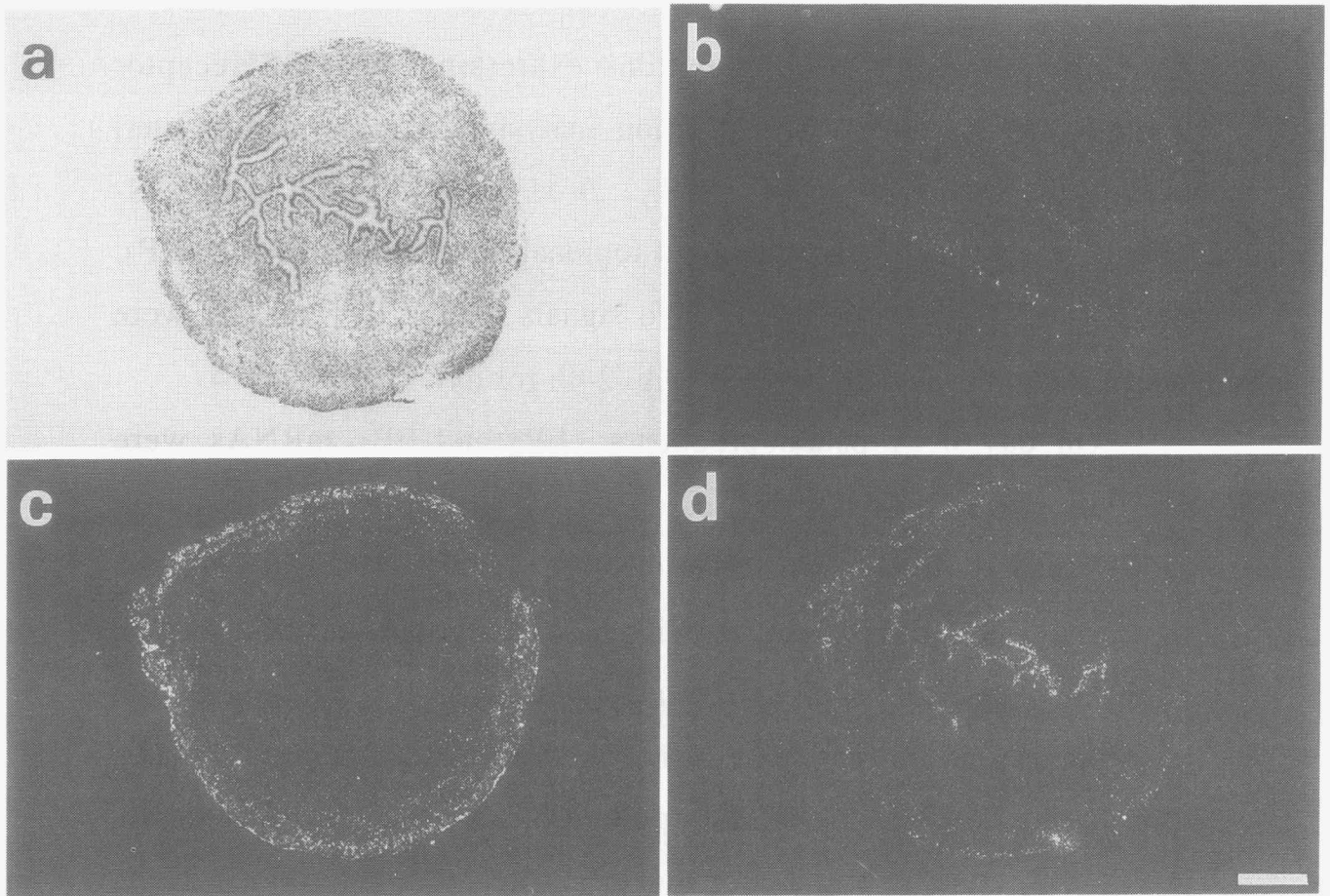


Fig. 2-2. Regional distribution of the mRNAs for PGE receptor subtypes in the mouse uterus on day 0 of pseudopregnancy (48 h after PMSG injection). a, Brightfield transverse section through the uterus. b-d, Darkfield photomicrographs of transverse sections hybridized with ^{35}S -labeled antisense riboprobes for EP₂ (b), EP₃ (c), and EP₄ mRNA (d). *Scale bar* = 300 μm .

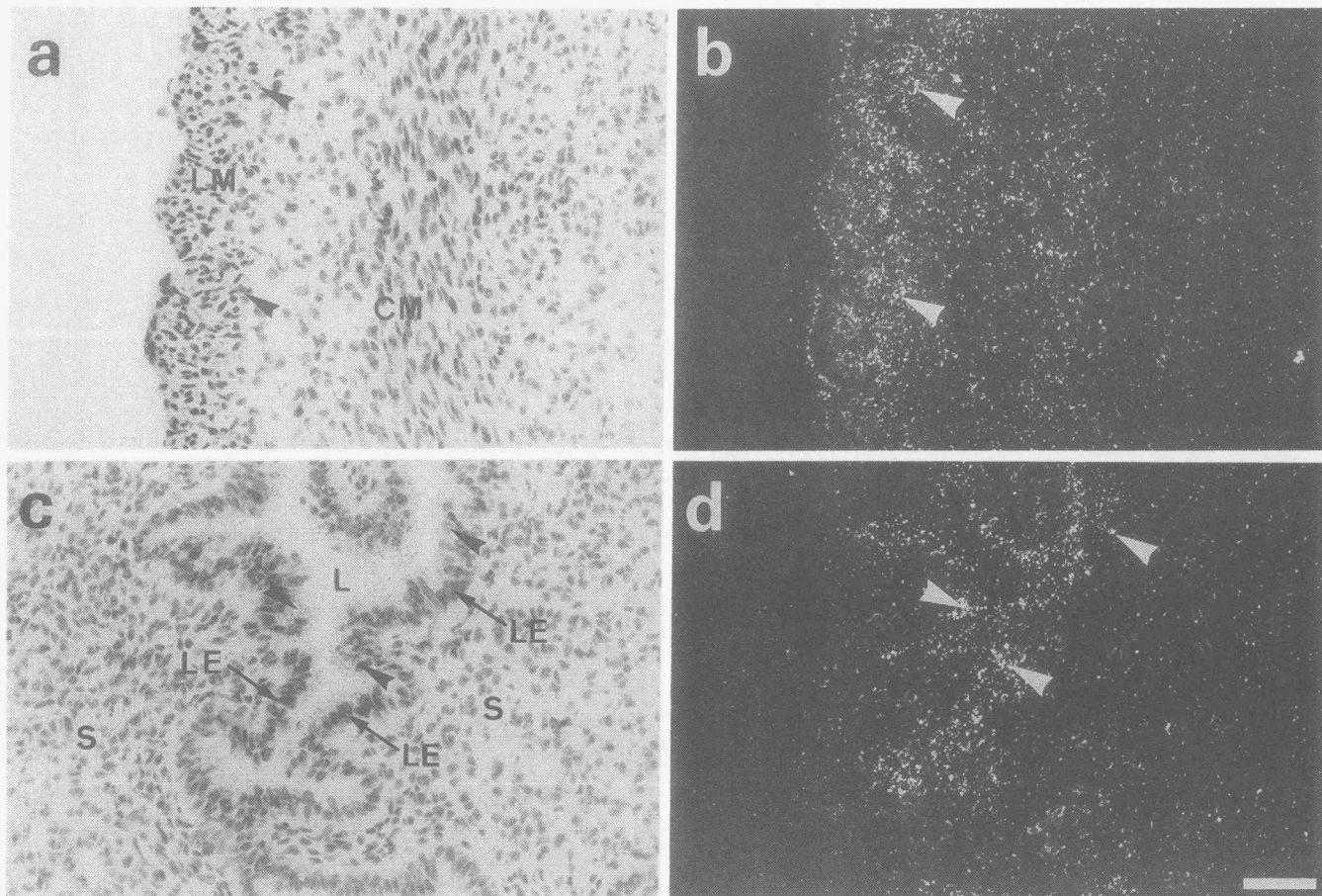


Fig. 2-3. Cellular localization of the mRNAs for PGE receptor subtypes in the mouse uterus on day 0 of pseudopregnancy. a and b, Bright- and darkfield photomicrographs of the myometrium, respectively, showing hybridization signals for EP₃ mRNA. c and d, Bright- and darkfield photomicrographs of the luminal epithelium, respectively, showing hybridization signals for EP₄ mRNA. *Arrowheads*, Cells labeled with each probe. CM, circular smooth muscle; LM, longitudinal smooth muscle; L, uterine lumen; LE, luminal epithelium; S, stroma. *Scale bar* = 50 μ m.

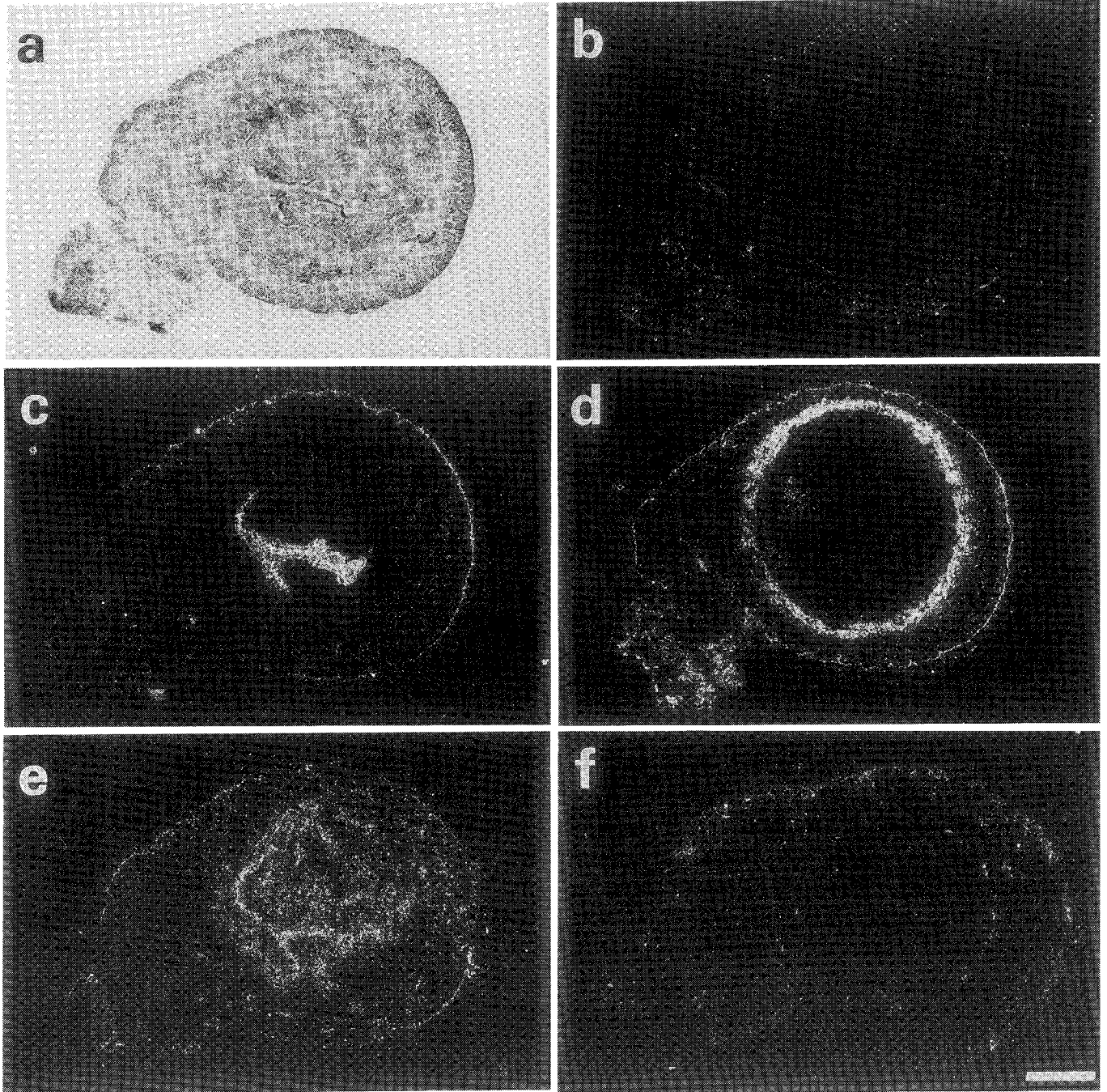


Fig. 2-4. Regional distribution of the mRNAs for PGE receptor subtypes in the mouse uterus on day 5 of pseudopregnancy. a, Brightfield transverse section through the uterus. b-e, Darkfield photomicrographs of transverse sections hybridized with ^{35}S -labeled antisense riboprobes for EP₁ (b), EP₂ (c), EP₃ (d), and EP₄ mRNA (e). Hybridization signals for EP₃ shown in d were abolished in the presence of excess unlabeled probe (f). *Scale bar* = 300 μm .

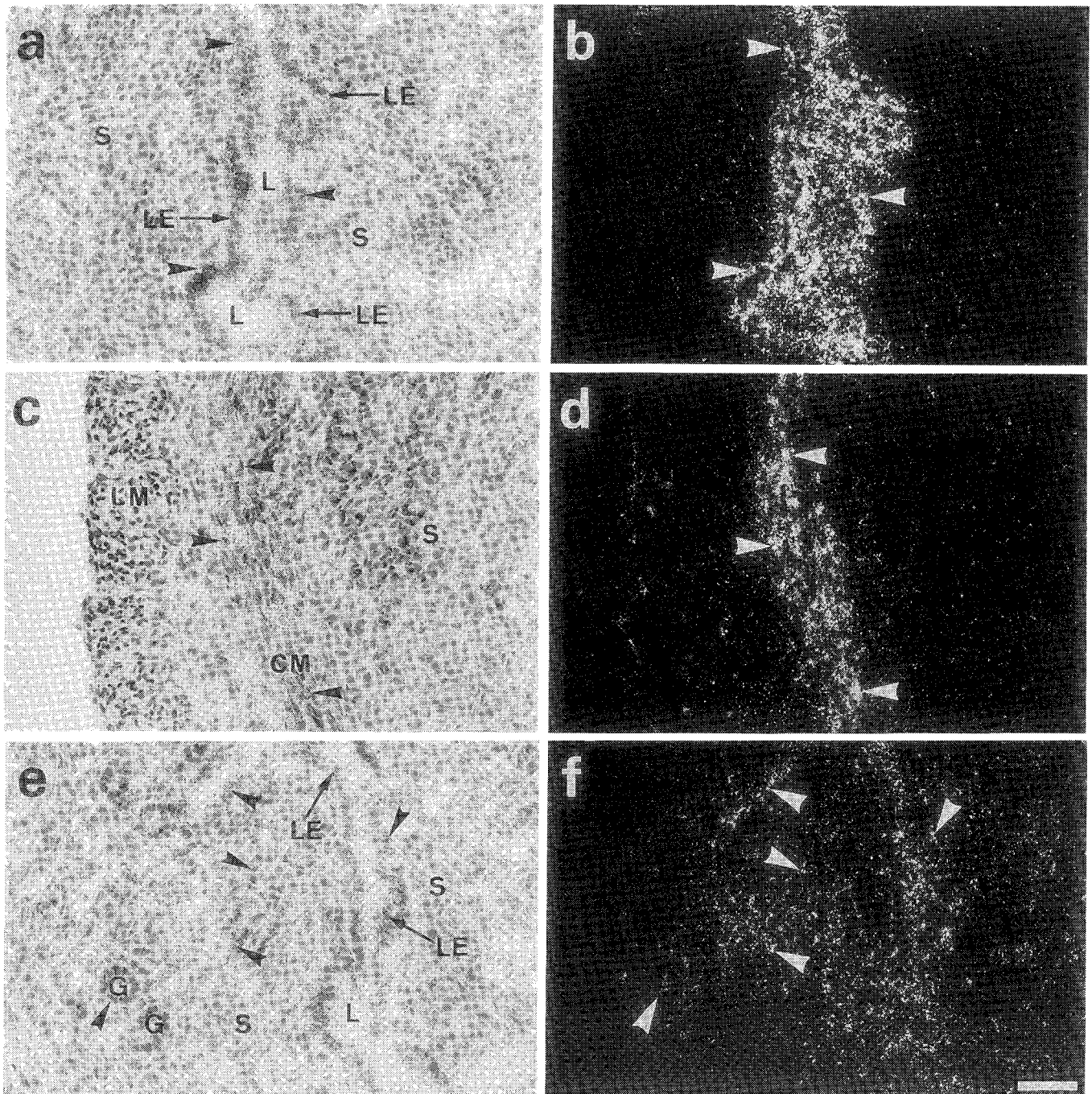


Fig. 2-5. Cellular localization of the mRNAs for PGE receptor subtypes in the mouse uterus on day 5 of pseudopregnancy. a and b, Bright- and darkfield photomicrographs of the luminal epithelium, respectively, showing hybridization signals for EP₂ mRNA. c and d, Bright- and darkfield photomicrographs of the myometrium, respectively, showing hybridization signals for EP₃ mRNA. e and f, Bright- and darkfield photomicrographs of the endometrium, respectively, showing hybridization signals for EP₄ mRNA. *Arrowheads*, Cells labeled with each probe. L, uterine lumen; LE, luminal epithelium; S, stroma; G, glandular epithelium; CM, circular smooth muscle; LM, longitudinal smooth muscle. *Scale bar* = 50 μ m.

DISCUSSION

PGE₂ is a major metabolite of arachidonic acid synthesized by cyclooxygenase pathways in the uterus, and has been demonstrated to be produced at implantation sites and the amnion and decidua at the onset of labor (Parr and Parr, 1989; Soloff, 1989). PGE₂ regulates various uterine functions, such as contraction and relaxation of the uterine smooth muscles, cervical ripening and labor induction, elevation of endometrial vascular permeability, and induction of decidualization (Weitlauf, 1988; Parr and Parr, 1989; Soloff, 1989; Huszar and Walsh, 1989; Coleman et al., 1990). PGE₂-binding activity has been determined in the myometrium in various species (Coleman et al., 1990; Senior et al., 1991; Goureau et al., 1992; Senior et al., 1993), and changes in the number of binding sites, depending on the state of pseudopregnancy or the estrous cycle, have been reported regarding the endometrium (Kennedy et al., 1983a; Hofmann et al., 1985; Kennedy et al., 1986). However, the contributions of the four receptor subtypes to PGE₂-induced uterine actions have not yet been well established, except for the contractile action on the myometrium, which is probably mediated by EP₃ (Senior et al., 1991; Goureau et al., 1992; Senior et al., 1993). Using isolated cDNAs encoding four PGE receptor subtypes (Sugimoto et al., 1992; Honda et al., 1993; Watabe et al., 1993; Nishigaki et al., 1995; Katsuyama et al., 1995), their cellular distribution in pseudopregnant mouse uteri was investigated by Northern blot and *in situ* hybridization analyses.

The present study demonstrated time-dependent changes in expression levels and distinct cellular localizations of the mRNAs encoding the three subtypes of PGE receptors, EP₂, EP₃, and EP₄, in the mouse uterus during pseudopregnancy. Such time-dependent

fluctuation of PGE receptor mRNAs has not been identified in other tissues or systems other than the uterus. In this study, mice were administered PMSG; on day 0, defined as 48 h after injection, mice were regarded as being in the proestrous stage by analysis of vaginal smears or by confirmation of ovulation on the following morning. On the other hand, day 5 was regarded as the time of maximal uterine sensitivity to the presence of blastocysts (Moulton and Koenig, 1986; Markoff et al., 1995). In the present study, superovulation of immature mice and induction of pseudopregnancy were used as a model system to examine the changes in PGE receptor expression. Although this system is known to include nonphysiological hormonal stimuli, preliminary *in situ* hybridization was performed on pseudopregnant day 5 mice that were generated from mature and cycling females, and consequently almost the same results as achieved using superovulated day 5 mice were obtained (data not shown).

The mRNA encoding EP₂, which is coupled to the stimulation of adenylate cyclase, was expressed almost exclusively on day 5 of pseudopregnancy, and its expression was confined to the luminal epithelium. This observation is inconsistent with the previous report that no [³H]PGE₂-binding sites were detected in the luminal epithelium (Kennedy et al., 1983b). In this previous study, however, binding experiments were performed using the uterine epithelium of progesterone-treated ovariectomized rats, not that of rats on day 5 of pseudopregnancy. The high level selective expression of EP₂ on day 5 suggests the involvement of PGE₂ in blastocyst implantation, which occurs during days 4 and 5 of pregnancy in the mouse (Markoff et al., 1995; Das et al., 1992; Schiff et al., 1993). It has been noted that cyclooxygenase-2 (COX-2) is transiently expressed on day 4.5 of pregnancy in the luminal epithelium (Jacobs et al., 1994). Considering

the similarity in fluctuation between EP₂ and COX-2 expression, PGE₂ synthesized in the luminal epithelium may exert its effect through EP₂ in an autocrine manner. EP₂ might mediate signals for morphological changes in the luminal epithelium essential for blastocyst implantation (Parr and Parr, 1989). In fact, treatment with indomethacin, a COX-inhibitor, has been shown to elicit ultrastructural changes in the luminal epithelium and to inhibit blastocyst implantation (Lundkvist and Nilsson, 1980). Thus, EP₂ expressed in the luminal epithelium might be involved in transmission of the signal from the blastocyst to the underlying stromal cells.

The expression of EP₄, which is also positively coupled to adenylate cyclase, sharply increased on day 3 of pseudopregnancy. This increase may be reflecting the change in stromal cells; EP₄ mRNA was expressed in the stroma on day 5, but not on day 0. As the serum progesterone level was reported to rise and reach its maximum on day 5 of pseudopregnancy in the rat (Pepe and Rothchild, 1974), it is possible that EP₄ expression in stromal cells is induced by progesterone, as reported previously (Kennedy et al., 1983b). PGE₂ was reported to cause an increase in alkaline phosphatase activity in the stromal cells during decidualization via elevation of the cAMP level (Yee and Kennedy, 1991). This effect may be mediated by EP₄. Thus, EP₄ may mediate signals for decidualization of stromal cells.

The mRNA encoding EP₃, which is coupled to the inhibition of adenylate cyclase, was specifically localized to the myometrium. Previous pharmacological studies demonstrated that PGE₂ causes uterine contraction through elevation of the intracellular Ca²⁺ concentration (Goureau et al., 1992). Mouse EP₃ expressed in CHO cells is coupled not only to the inhibition of adenylate cyclase, but also to Ca²⁺ mobilization via Gi (Irie et al., 1994). Therefore, PGE₂-induced

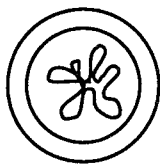
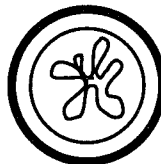



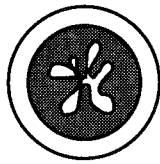
uterine contraction may be mediated by an EP₃-mediated Ca²⁺ signal in the myometrium.

As in the cases of EP₂ and EP₄, the level of EP₃ expression also changed during pseudopregnancy; EP₃ expression was low on day 0, but increased to reach a maximal level on day 5. The temporal changes in EP₃ expression coincided well with the change in myometrial activity in pregnant and pseudopregnant rats; the myometrial activity was reported to increase after mating and reached a maximal level on day 5 (Crane and Martin, 1991a). Therefore, the level of EP₃ expression might affect myometrial activity. The cell populations expressing EP₃ also changed in a temporally specific manner; EP₃ transcripts were localized to the longitudinal smooth muscle on day 0, but expression was confined to the circular smooth muscle on day 5. To date, there have been no reports concerning changes in PGE₂-induced myometrial contractility in these two distinct types of smooth muscle during pregnancy or pseudopregnancy. However, EP₃ expressed in the longitudinal smooth muscle on day 0 might be involved in longitudinal contraction observed during the periovulatory period (Crane and Martin, 1991b). EP₃-mediated longitudinal contraction might contribute to sperm transport (Chalubinski et al., 1993). On the other hand, EP₃ highly expressed in the circular smooth muscle on day 5 might be involved in circular contraction necessary for retention and spacing of embryos (Crane et al., 1991a). At present, there is no information regarding the mechanism underlying such time-dependent changes in the expression of EP₃ mRNA in two distinct types of smooth muscle cells. More detailed analyses are therefore required.

In summary, the results presented here indicate distinct cellular localizations and time-dependent changes in the expression levels of PGE receptor subtypes in mouse uteri during pseudopregnancy. It is

also suggested that PGE₂ modulates uterine functions through at least three receptors; EP₂ is involved in blastocyst implantation signaling, EP₄ is involved in decidual transformation of stromal cells, and EP₃ is involved in regulation of myometrial contractility (Table 3).

Table 3. Summary of the distribution of three PGE receptor subtypes in pseudopregnant mouse uteri.

	EP ₂	EP ₃	EP ₄
Day 0			
		longitudinal smooth muscle	luminal epithelium
Day 5			
	luminal epithelium	circular smooth muscle	stroma luminal epithelium glandular epithelium
Function	blastocyst implantation signaling	regulation of myometrial contractility	decidual transformation of stromal cells

3. Characterization of the Gene for the Mouse Prostaglandin E Receptor Subtype EP₂: Tissue Specific Initiation of Transcription in the Macrophage and the Uterus

ABSTRACT

Genomic DNA clones for the mouse PGE receptor subtype EP₂ were isolated and characterized. The mouse EP₂ gene is composed of 2 exons and 1 intron, and spans 16 kb. The intron which is approximately 12 kb in length is located at the end of the sixth transmembrane domain, as with other prostanoid receptor genes. Based on this structure, transcripts were analyzed in endotoxin-treated macrophages and pseudopregnant mouse uteri, in which abundant expression of EP₂ mRNA was observed. Sequence analysis of cDNA clones from these origins and Northern hybridization of these RNAs revealed that the uterine EP₂ mRNA (U-type) has a longer 5'-untranslated region than the macrophage EP₂ transcript (M-type). The major transcription initiation sites for M-type and U-type EP₂ are located 124 bp and 769 bp upstream of the translation start site, respectively. The M-type was expressed in various tissues, whereas the U-type was found only in the uterus. The 2 kb segment containing the immediate 5'-flanking and 5'-noncoding regions contain three consensus sequences for the NF-IL6 binding site, one consensus sequence for the NF-κB binding site, four AP-2 consensus sequences, one AP-4 consensus sequence, one potential cAMP response element, and one potential progesterone response element. These results suggest that EP₂ gene expression in the macrophage and uterus is under the control of distinct mechanisms involving alternative promoters.

RESULTS

Structure of the mouse EP₂ gene

Structural analysis of isolated clones revealed that the mouse EP₂ gene spans 16 kb in length and contains 2 exons (Fig. 3-1). Exon 1 is composed of the 5'-untranslated region and the coding region from ATG to the putative sixth transmembrane domain of the receptor. Exon 2 encodes the residual part of the coding region and the 3'-untranslated region. The sequences of the exons were in complete agreement with that of the cDNA. The exon/intron boundaries conform perfectly with the GT/AG rule (Table 4) (Breathnach and Chambon, 1981; Mount, 1982). In Southern blot analysis of mouse genomic DNA, digestion by BamHI, NcoI, NdeI, or ScaI yielded a single hybridization band, suggesting that the EP₂ is specified by a single gene (data not shown). Using the genomic DNA structure as a basis, the mRNA species from the macrophage and uterus were further characterized.

Table 4. Exon-intron organization of the mouse EP₂ gene.

	Exon size (bp)	Exon	Intron	Exon
1	macrophage; 966 uterus; 1611	TTCACA	<u>g</u> taagtcacc..(12 kb ^a).. <u>cttctttcag</u>	ATCTTT IlePhe
2	916 (3'-short form) 1976 (3'-long form)			

Exon sequences are in upper case letters; intron sequences are in lower case letters.
The deduced amino acids are shown below the nucleotide sequence.

^aThe length of the intron was determined by PCR.

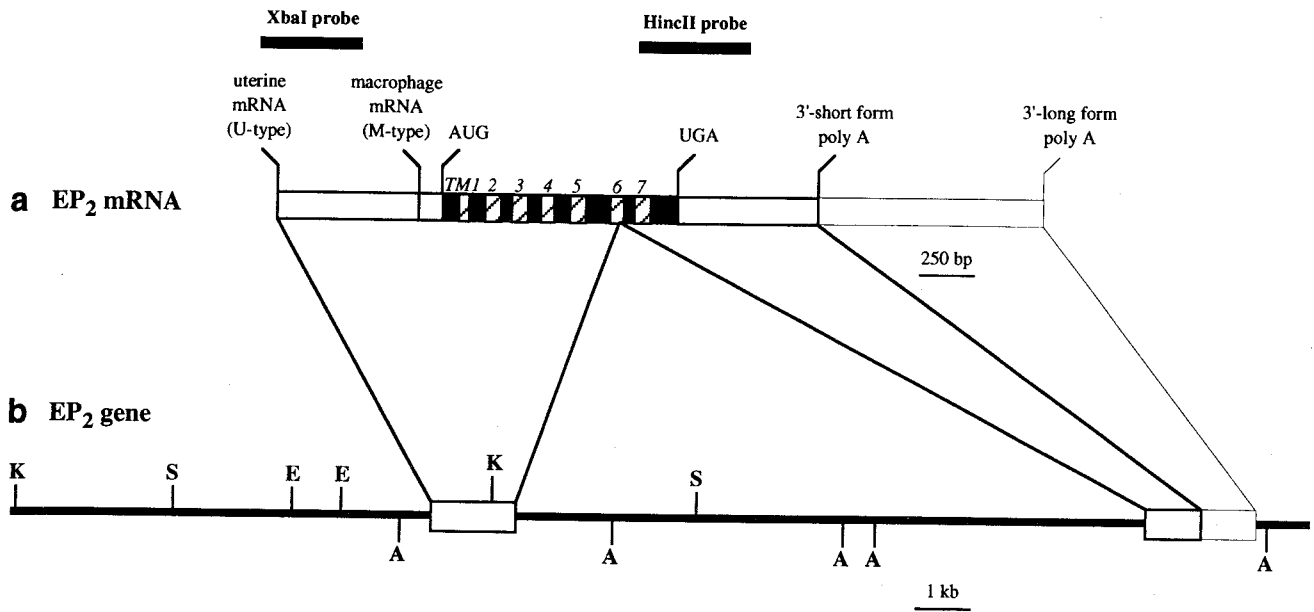


Fig. 3-1. Schematic diagram of the mouse EP₂ mRNA and gene. a, Structure of the mouse EP₂ mRNA. The noncoding regions are represented by *open boxes*, the putative transmembrane regions are indicated by *hatched boxes* and *numbered*, and the other coding regions are shown by *closed boxes*. The translation initiation site, AUG, the translation termination site, UGA, and two polyadenylation sites are indicated. Positions of probes used in the Northern blot analysis are depicted by *bold bars* and *labelled*. b, A diagram of the mouse EP₂ gene. The restriction sites are indicated as follows: AatI (A), EcoRI (E), KpnI (K), SmaI (S). Exons are represented by *open boxes*.

Characterization of cDNAs from a mouse uterus library

As described in Chapter 1, the 2.2 kb species of EP₂ mRNA was distributed in various tissues, whereas the 2.8 kb transcript was abundantly expressed only in the uterus, and hence appeared to be uterus-specific (Katsuyama et al., 1995). To obtain structural information on the uterus-derived EP₂ transcript, EP₂ cDNA clones were isolated from a mouse uterus cDNA library. Thirteen positive clones were isolated, and 9 clones contained the full-length of the coding region. Restriction analysis of these clones revealed that the sequence of the coding region was the same as that of ML202, a representative EP₂ cDNA clone derived from the lung (Katsuyama et al., 1995). However, 5 out of 9 uterine clones contained an additional upstream genomic sequence (640 - 545 bp in length) at their 5'-ends compared with the lung cDNAs (Figs. 3-1 and 2). Due to these findings, it was further examined whether the additional sequence observed only in the uterine EP₂ cDNA corresponds to the extra sequence in the uterine-specific 2.8 kb transcript.

```

-2000  ACAAAC TTCCTGCACCAGGGAATTCCTTGCTTAAACCCATGTCTGGGGCTTCATAGTGGCTTCTTCCCCCAGGATCCCCAAGACTGTCTCACATGCAGG
          NF-kB
-1900  GAGTGCATACCCCTAGGGTCTGAGGGGTTGTTGAGGAAGGGGTATGGACAGAGCTTAGTAATCAGAACTAGATTGTCACACACCTCTGTGAGAATCTTC
          NF-IL6
-1800  TCACAGTGTGCTACTAAGTTGGGGTAGAGACTAGCAGAAAAAGGCAGAGGGTATAGGGATGAGATCTCGAATTTTCTCTGGTATCCATTAAAGTCAGCAG
-1700  AGTCATGTCACCTCATGTAGTGAAAAGGTAGAGACAATTATATAGAAATAGAGCCACAAGGGGCTTTTGTATAAGGTGACCATGGAACAGAAAGGGTAT
-1600  AAAAGCCTTTTGGAAAGTAAACACAGTGGAAACAGCATCAGAGTCTTGCCTGTTCTGCTCAT'TCCCCAACCATGTGGGAGGAGATGGCACAAGATGGACAT
          NF-IL6
-1500  GGGAGAGTCCACTTTGGGGTGGCTGTGTGGAGGACGCTACTGCCTGCAGTTTACAGCCTGAAAGGAAACTAGAGGAGTTTGACACCCACTGCATCCATGC
-1400  CCTACAAACTTAGCAATGGAGTGGATAGCTTCAGCTGTGGGAAATGAGCCTGTAGGCCCTCCCAAATGGATTCTGCTTCCCGTATTTTGCTAGAAAACCCC
          AP-4
-1300  TCCAGTCCCCAGGCCAGAAGCATGGTGAGCTAAGTCATGAGAAGCAAGGTACTCCATAAATAATCTTTCCATTGCTCTTCTTTTTACTCCTGGGCAA
          AP-2
-1200  ACCTTTCTTTTCTCAGCACACTCTCTTCTCTGCATGAAATACACTTTTTCTAGTAGTATACCCTTGCCCTACGTTGTCTGTGTCAGGCTGGACTGAGAAAACC
-1100  AGAAACACTGCTGCATTTTAACTACTCTGTAACACTATGGCAACAATGATAAATAACTATTTATGCCAGCTTCATTTGTGAAACTGGCCAAATTACCAAAAC
          CCAAT-box
-1000  ACTCCGGGAATAAATCCTTCGAATGGTTAACTGACTGTGGAGAGACGAATGTGAACAATGTATTAACAGATTCTTCAAGATTGAGCCTCAATAGAGAACA
-900   CTTAAGTGCCATTTGTTTTCCATCTGGGGCCACAAACATGACTCTAGAGGATTACAAGAACAGTTCTCTGTTTGTGTTTGTAGGATTGCTTAAAGCAAGTC
          XbaI                PRE                NF-IL6
-800   GTTGTATAGGAAGGCAAAGGCCACTCTGTG
          U-type
          -769 GTGTGCACGGTTCATTTTCTGGATGGATAGGTTTGCTTTTTCAGTTCCCTCCAGTTTGTGCTCCACCTT
          EP2UR primer
-700   TGTGGGTATCTTTCTGGATGTTTGTGTTTGTGTTTAAAGGAAAACAACAACCTCTCTCATATTCTTACTTCAAGTTGTCTTGATGTTTGTG
-600   CCTCTCCACACCTCACAGGGCTGGACTCTCTTAACCTCAGTTTGGTGAGTTCTCTGTACCCCAAGTCTTATTAACACTGTGCTGGTTCCAGCCTTCTGCT
-500   AGGGCTAAAGGCTTCAGAGCTGAAAGTCAGAGGCAC'TGCAACTTCCAACCATAGGAAGGACCTTATGGGCCAGCTCAGGGTGGAGCCTGGGAACGCC
          CRE
          ▼
-400   CCCAGTCCCTTCTAGAGCCCTGTCAGTGTGTGCTGCCCCGCTGGCCACAGCAGCTCGCAAGCAATCTAAATFGCTTTCCTGGATTCCTACTAC
          XbaI                AP-2
          ▼
-300   TGGACTTGCCCCCTGAAGGCGCTGGAGGGAGCAGCTGCTCTGGCAAGCACCCCTGCTAGGGCAGGTGAGGCACAGAAGCACCGAGAGCGACCGGATATT
-200   GTAGTGAAGAGGCCACTGTACGTACAGGCAGGAGACCCAAACAAGTCTGTCTTGGTGGCAGTTGGGGGCGGGAAG
          M-type
          -124 GGAGCTCTGGATTTCCGGTCCCTCC
-100   CCTTTTCCCCTGCTCTGTCTTGGAGCCCTGGGGCCATCAGACCCTCCGACTGTCTGGTACTTGCCTGGAAGAGATATCATCTCTCCTCCACACCTCCACC
          EP2NR primer
1  ATGGACAATTTTCTTAATGACTCCAAGCTAATGGAGACTGCAAGAGTGTGCTAGTGGCTCCTTTCCGGGGAAAGCCCAGCCATCAGCTCGGTGATG...
M D N F L N D S K L M E D C K S R Q W L L S G E S P A I S S V M

```

Fig. 3-2. Nucleotide sequence of the 5'-flanking and 5'-noncoding regions of the mouse EP₂ gene. Numbers on the left are relative to the adenosine in the ATG start codon. Representative cDNA clones ML202 and MU14 start at positions -145 and -743, respectively. The major transcription initiation sites for macrophage (M-type) and uterine (U-type) transcripts are indicated. Two other potential transcription start sites for macrophage transcripts are shown by *open triangles*. Potential binding sites for the indicated transcription factors are *underlined*. The two XbaI restriction sites are *italicized* and *underlined*. Locations of the primers used in primer extension analysis are shown by *lines above the sequence*.

Northern hybridization with region-specific probes

The preliminary investigation showed that the expression of the 2.2 kb EP₂ transcript was higher in LPS-treated J774.1 cells, a macrophage-like cell line, than in the lung. To examine whether the uterus-specific 2.8 kb transcript is derived due to a difference in the length of the 5'-noncoding sequence, Northern blot analysis was performed on the J774.1 cells and uteri using a probe specific for the additional sequence. First a HincII fragment covering the seventh transmembrane and C-terminal regions of EP₂ was used as a probe (Fig. 3-1). As shown in Fig. 3-3a, a major band was observed at 2.2 kb and 2.8 kb in LPS-treated J774.1 cells and pseudopregnant day 5 uteri, respectively. In J774.1 cells, a minor band was also observed at 4.0 kb. On the other hand, when a XbaI fragment specific for uterine EP₂ cDNA was used as a probe (Fig. 3-1), the 2.2 kb transcript from the J774.1 cells was not detected (Fig. 3-3b). These results showed that only the uterine EP₂ transcript has an additional 5'-untranslated region, suggesting that the transcription of the uterine EP₂ mRNA starts approximately 600 bp upstream of that in the lung.

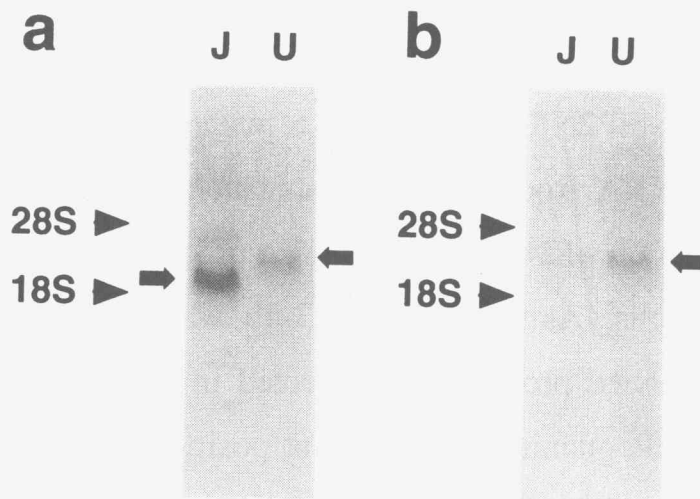


Fig. 3-3. Northern blot analysis of RNAs isolated from LPS-treated J774.1 cells and pseudopregnant day 5 mouse uteri. LPS-treatment of J774.1 cells and achievement of pseudopregnancy in virgin mice were performed as described in "*EXPERIMENTAL PROCEDURES*." Total RNA (10 μ g) was applied to each lane. a, EP₂ mRNAs were detected by a 543 bp-HincII fragment covering the seventh transmembrane and C-terminal regions. A major band was detected at 2.2 kb and 2.8 kb in J774.1 cells and uteri, respectively. b, A 469 bp-XbaI fragment from the 5'-noncoding region of uterine EP₂ cDNA was used as a probe. Only the 2.8 kb transcript was detected. J, LPS-treated J774.1 cells; U, pseudopregnant day 5 uteri.

Determination of the transcription start site

In order to confirm uterus-specific transcription initiation, the transcription initiation sites for the macrophage (M-type) and uterus (U-type) EP₂ transcripts were investigated. Primer extension analysis was first performed with two antisense oligonucleotides, EP2NR (corresponding to position +27 - -3) and EP2UR (position -610 - -644) (Fig. 3-2). When EP2NR was used as a primer, an extended product was detected in RNA from J774.1 cells, which started at the guanine nucleotide at position -124 (Fig. 3-4a). The product was not detected in RNA from the uterus. As shown in Fig. 3-4b, when EP2UR was used as a primer, an extended product was detected in RNA from the uterus, which started at the guanine nucleotide at position -769. This product was barely detected in RNA from J774.1 cells. Thus, the major transcription initiation sites for M-type and U-type EP₂ were identified as positions -124 and -769, respectively. Two additional minor transcription initiation sites for the M-type were further identified by the 5'-RACE experiment (Fig. 3-2). From these results it is concluded that the transcription start site of uterine EP₂ mRNA is approximately 600 bp upstream of that in the other tissues.

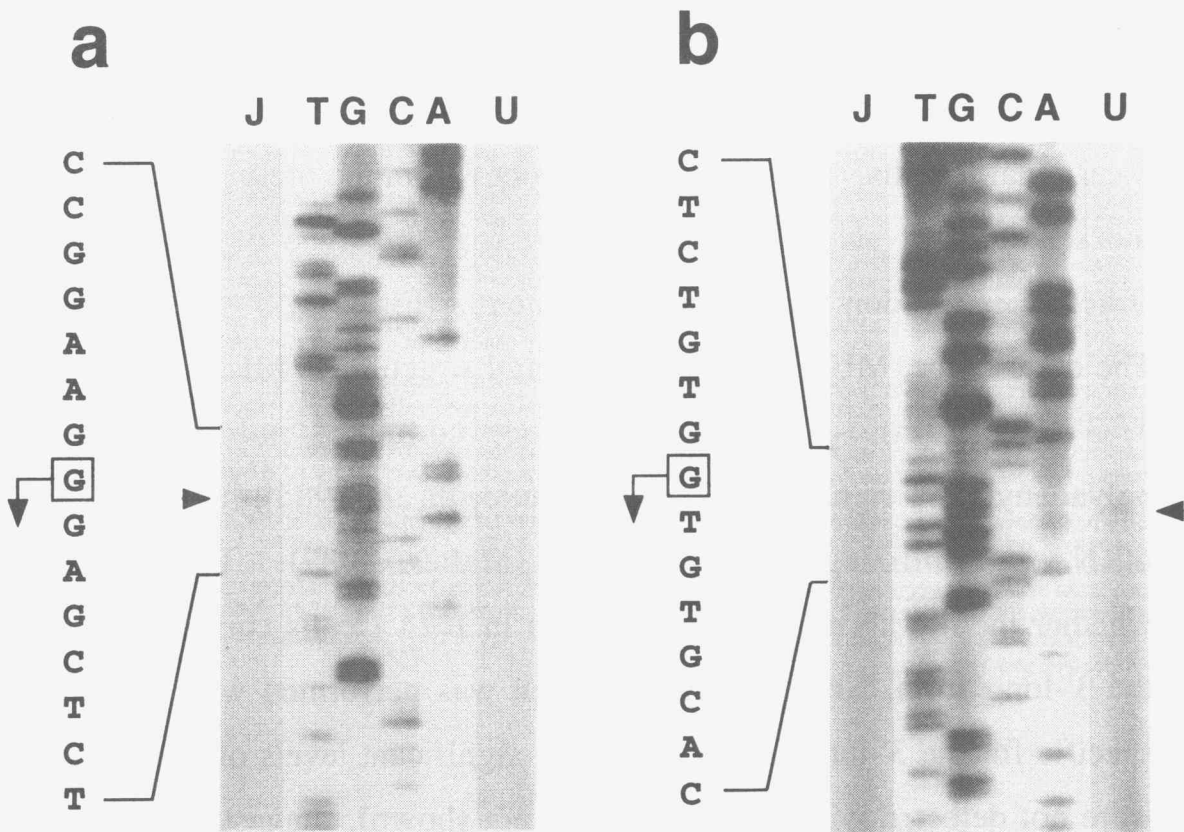


Fig. 3-4. Determination of the transcription start sites of the mouse EP₂ gene by primer extension analysis. ³²P-labeled antisense oligonucleotides were annealed to poly (A)⁺ RNAs as described in "EXPERIMENTAL PROCEDURES." a and b, Extension by EP2NR and EP2UR primers, respectively. Arrowheads indicate the extended products. J, LPS-treated J774.1 cells; U, pseudopregnant day 5 uteri.

Diversity of the EP₂ transcripts in their 3'-noncoding region

Heterogeneity was also found in the length of the 3'-noncoding sequence in EP₂ cDNA (Figs. 3-1 and 5). When cloning the EP₂ cDNA from a mouse lung library, isolated clones were subdivided into three groups, represented by ML202, ML203, and ML204, according to their 3'-terminal lengths. Eleven out of 12 clones from the uterus library had the same 3'-tail as ML203, suggesting that this type of 3'-terminal structure is the dominant form in the uterus (Fig. 3-5, 3'-short form). The other clone, MU6, had the same 3'-tail structure as ML204 (Fig. 3-5, 3'-long form). This mRNA species contained not only three polyadenylation signals, AATAAA (Manley et al., 1985), but also five mRNA destabilizing motifs, ATTTA (Malter, 1989). To examine whether the 4.0 kb minor band detected in J774.1 cells corresponds to the 3'-long form, Northern hybridization was performed with a probe specific for the 3'-long form. However, significant levels of transcripts were not detected with this probe (data not shown), suggesting that the 3'-long form was expressed at a very low level, or was susceptible to degradation. The 4.0 kb minor band detected in J774.1 cells might be an immature form of the 2.2 kb mRNA.

1 ATCTTTGCCCTACATGGATGAAACCTCTCCCTAAAGGAAAAGTGGGACCTCCGAGCTCTTCGGTTTTTATCAGTTAACTCTATAATTGAC
282 I F A Y M D E T S S L K E K W D L R A L R F L S V N S I I D
VI VII

91 CCTTGGGTCTTTGCCATACTTAGGCCACCGGTCTGAGGTTAATGCGCTCAGTCCCTCTGTTGTCGGACTTCACTGAGAACAACAAGAAGCT
312 P W V F A I L R P P V L R L M R S V L C C R T S L R T Q E A

181 CAGCAAACATCCTGTTCTACCCAGTCCAGTGCCAGTAAACAGACTGACCTTTGTGGACAGTTGTGAGGATGCGCTTACAGAGGAAACCTC
342 Q Q T S C S T Q S S A S K Q T D L C G Q L

271 TGAAGAAGTCTTTAAATGGCCTCATTTGGAGAAGTGAAGGGGCTGGAATATAAACAGAATAACCTTGGCCCTGAGAAGCAGATGAAACAGA
361 CTTTATGAGGTTTGGGCTGCAGATGTGATGACAGAGCCCTTTGTGGAAAGTGTGAGGATATAAAGTTCACATTATGTGACCTTTGAAG
451 GACAATCTGCTGCGTGAAGACCCAGTTGATTTAAGCTTTCCCTGTTGAGTGACAAAGCATGTGGTTTTGTAATTTGTTTTAAATGCTAC
541 ATGGTTACTGTTTTAATTTTGATGTCACCTGCTGTTATAAATGAGTCCAGACCAATGAAATTCATTCTCTAGGAAGTCAACTCATGCAAG
631 CGATGAAGCAACCAGAGCAGACAAGTTGCTGCTCGTGATTGCTTAATGGACTCTTATTTGGACCATGAAGTTGAATGCCATTGGCCCC
721 TTTTACTGTGCGTGTTCATGTGGGAGTCTCTCTGGTAACGAAAGGATGCACTGATTTGGTTAATGCTAGTTAATCCTTTAGGAGCTCTG
811 TCATGTTGTCAGATTTATTTCTGTCTTGTCTATTGTGATAAGACTACTGAAGAAAACCTTCAGAAAATTTGTGTCTCACAAAGGAATAAT
901 TAAAAAATCAATTTT (3'-short form poly A site)

AATTAGTGTCTTTCAGTTTTTTATAGAGGAAGAGTTTTTCACAGTTGTATCTGAGATCAGATTTTAGGATCAAAC
991 TCTGAAAATTCAGTCTATTTTCAGGTTCTCTAGAATCCTTCTAGTGTGGTACTAGACATAACTAACATACACATACACTACAAGATG
1081 TTTGATTGTGCCACGGCAAACGCTTTTGCTGTATTTTATTTAGGGAACATATTTTGATTCATACAACAAAAGAAATCATGTAACCTGTG
1171 AGTCATATCTTATATTTCCAAACGTTTGGTACGGTATAAACTCAGCATTAAGATATTCCAGTGAGCGTGTACATTTAGTCACTATAGTG
1261 TAAACACATCTGAAATGTTACAAGCTAAATATAAAAACAAAAAGTAGAAGTCTTCAATTAATTCACATCCCAAGGACAGTTTGCAGTTA
1351 ATGTAGACATGCCATCTTTTAGGTAATAAAGTTTTTAAAAAATTCATAATATTTTGGCAAGTTTTTAAATGTACAGTTGTTATAA
1441 ATTAATAATTTAAGATATCCAGTGGAGTCTTGTATATGAAAGAGAGTTTTGCTATCAGTTTTCACCTCTCTGACATGGTCAATTAGAATA
1531 ATATAACCATCGTATTTGAGGGCAGATAGAATTGTAGGATGGCTGTTAATCTTAAATTTAGTTGTTTCTCATGCTTCATGTTTACCCA
1621 GAGCTGTTTTTTATAGTTAGTTATCAATTTAGAAAGACTCTGTGTAACATAATATCTCTGATACAAAATATGGGAGTCAAAGCAGCTCTTA
1711 CTTTTACAAGAAATATATAAATAAGTGGTTCGTGTTGTATAAAATTTACAGAGCAGACAAAGTTTAAATTTAACTAAATCTAGGCAGGTA
1801 TAAATAAATGCCACCTCTTCAACCGGTGTTGCATGCCAAGAAATTCATTCAGGGGAGAAATGAGGCCCGTGGTATGACTGAAGCAGA
1891 AATTTCTGGTTACTTTGCACCTGGAATTTTGATTCCTTTTATCAGATGTCGTTGTAATAATCTCAGAAATAAAAAAATAACTAC
(3'-long form poly A site)

Fig. 3-5. Nucleotide sequence of exon 2 of the mouse EP₂ gene. The deduced amino acid sequence is shown beneath the nucleotide sequence using the single-letter code. The positions of the putative transmembrane segments, VI and VII, are *underlined*. AATAAA polyadenylation signals are *double underlined*. ATTTA mRNA destabilizing motifs are *italicized and underlined*. The previously reported cDNA clone ML202 stopped at nucleotide 724 and was polyadenylated.

Tissue distribution analysis of EP₂ mRNA variants

Tissue specificity of the expression of EP₂ mRNA with a long 5'- or 3'-untranslated region was examined by RT-PCR (Fig. 3-6). As for the 5'-end variants, when the MG2721 primer, which amplifies both the M-type and U-type EP₂ was used, a 977 bp fragment was detected in all tissues examined. In contrast, when amplified with the MG2723 primer specific for the U-type, a 1334 bp fragment was detected only in pseudopregnant day 5 uteri (Fig. 3-6b). These results suggest that the M-type was expressed in various tissues, whereas the U-type was expressed exclusively in the uterus. As for the 3'-end variants, the long form was expressed in all tissues examined (Fig. 3-6c), suggesting that tissue specificity does not exist in the expression of the EP₂ mRNA with the long 3'-untranslated region.

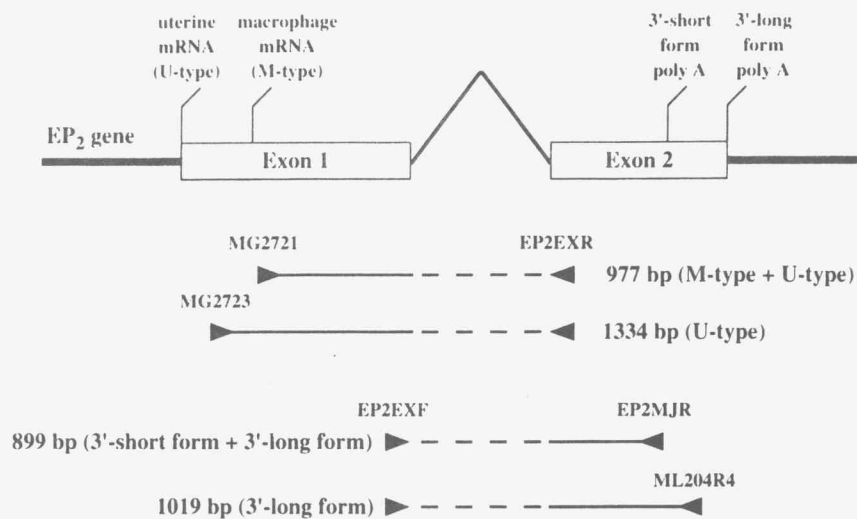
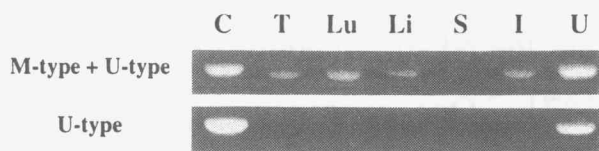
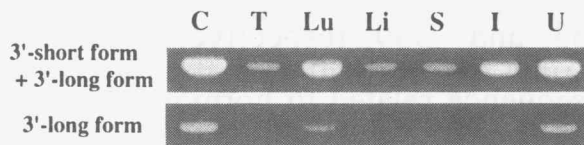
a**b****c**

Fig. 3-6. Tissue specificity of EP₂ mRNA variants. RT-PCR was performed as described in "EXPERIMENTAL PROCEDURES." a, Diagram of the locations of the PCR primers and amplified products. The MG2721 primer in combination with the EP2EXR primer amplifies both the M-type and U-type EP₂ transcripts, and a 977 bp fragment is detected. On the other hand, the MG2723 primer amplifies only the U-type mRNA and a 1334 bp fragment is detected. As for the 3'-end variants, the EP2MJR primer in combination with the EP2EXF primer amplifies both the 3'-short form and the 3'-long form, and a 899 bp fragment is detected. On the other hand, the ML204R4 primer amplifies only the 3'-long form, and a 1019 bp fragment is detected. b, Distribution of the two types of 5'-end variants. A uterine EP₂ cDNA clone was used as a control of the amplification. The M-type transcript is expressed in various tissues, whereas the expression of U-type mRNA is restricted to the uterus. c, Distribution of two forms of 3'-end variants. ML204 was used as a control of the amplification. The long form is expressed in all tissues examined. C, control DNA; T, thymus; Lu, lung; Li, liver; S, spleen; I, ileum; U, pseudopregnant-day-5 uterus.

Analysis of the promoter sequence

The 2 kb nucleotide sequence containing the immediate 5'-flanking and 5'-noncoding regions of the mouse EP₂ gene was examined (Fig. 3-2). No consensus TATA-box sequence was found in the presumed promoter region of either the M-type or U-type EP₂. Although one consensus TATA-box sequence is present at position -1603, it seems too distant to be involved in transcription. A CCAAT-box is present 247 bp upstream of the major transcription start site for the U-type. Consensus motifs relevant to LPS stimulation, such as NF-IL6 and NF-κB binding sites, are found in the 5'-flanking region. Consensus sequences for the NF-IL6 binding site are present at positions -811, -1593, and -1871. One consensus sequence for the NF-κB binding site is found at position -1982. In addition, in the promoter region for the M-type, one putative cAMP response element (CRE) and a cluster of three AP-2 consensus sequences were found at positions -480 and -352, -353, and -359, respectively. On the contrary, no apparent consensus sequence related to hormone-dependent expression in the uterus, such as an estrogen response element (ERE) or a progesterone response element (PRE), was found in the 5'-flanking region. However, one potential PRE was found at position -844, 75 bp upstream of the major transcription start site for the U-type. In addition, one AP-2 consensus sequence and one AP-4 consensus sequence are present at positions -1295 and -1370, respectively.

DISCUSSION

Although both EP₂ and EP₄ are coupled to the stimulation of adenylate cyclase, expression of EP₂ mRNA is much lower than that of EP₄ in almost all tissues. However, abundant EP₂ mRNA expression was observed in the macrophage and uterus. As described in Chapter 2, EP₂ mRNA is induced in the luminal epithelium of the mouse uterus on day 5 of pseudopregnancy, suggesting that the EP₂ gene is hormonally regulated in the uterus (Katsuyama et al., 1997). The dominant EP₂ transcript detected in the uterus was 2.8 kb in length. On the other hand, EP₂ mRNA in J774.1 cells, a macrophage-like cell line, is highly induced by stimulation with LPS (unpublished data). In this cell line, the major EP₂ transcript detected was 2.2 kb in length, as was observed in tissues other than the uterus. These observations lead to the hypothesis that expression of EP₂ is induced by hormonal or pathophysiological stimuli, and that distinct activation systems of EP₂ gene expression exist in these two types of stimuli. As an initial step to clarify the mechanisms of EP₂ induction, genomic DNA clones encoding EP₂ were isolated and characterized. As a result, differences were found in transcription initiation of the EP₂ gene between the macrophage and uterus.

The mouse EP₂ gene is composed of 2 exons. Exon 1 contains the 5'-untranslated region, the translation initiation codon, and most of the EP₂ receptor coding region. Intron 1, which is approximately 12 kb in length, divides the coding region at the putative sixth transmembrane domain. The location of this intron in the sixth transmembrane domain is completely conserved in the genes of eight types and subtypes of prostanoid receptors (Nüsing et al., 1993; Hirata et al., 1994; Båtshake et al., 1995; Ogawa et al., 1995; Arakawa et al., 1996; Kotani et al.,

1997; Hasumoto et al., 1997). On the other hand, the EP₂ gene does not have an intron in the 5'-noncoding region, which was found to be present in the genes of five types of prostanoid receptors. Among the prostanoid receptors, the human TP receptor gene and the bovine FP receptor gene have been reported to generate two mRNA species by alternative promoter usage (Nüsing et al., 1993; Ezashi et al., 1997). However, these two mRNA species appeared to be co-expressed in the same tissue. In the present study, diversity of EP₂ mRNA was identified in both the 5'- and 3'-ends. RT-PCR and Northern analyses revealed that the 3'-end variants, the short form and the long form (Fig. 3-5), are co-expressed in various tissues and that the short form is dominant. On the other hand, expression of the 5'-end variants (Fig. 3-2) is tissue-specific; 5'-long (U-type) mRNA is expressed only in the uterus and 5'-short (M-type) mRNA is expressed in other tissues and the macrophage. Thus, the EP₂ gene performs tissue-specific transcriptional initiation. The significance of the long 5'-noncoding sequence of the U-type is yet unknown, but it might be involved in the alteration of the stability or translation efficiency of the transcript. Indeed, the 5'-noncoding region of the U-type contains four AUG codons. Presence of these start codons upstream of the main open reading frame is known to inhibit cap-dependent translation (Kozak, 1991). Therefore, the translation efficiency of the U-type might be lower than that of the M-type. Strict control of translation, in addition to transcriptional regulation, might be necessary for precise expression of EP₂ in the uterus.

As described in Chapter 2, it was found that in the synchronized mouse uterus, EP₂ mRNA expression is induced in the luminal epithelial (LE) cells only during the periimplantation period (Katsuyama et al., 1997). This observation suggests the involvement of PGE₂ and the EP₂ receptor in blastocyst implantation. Furthermore, preliminary analyses

using ovariectomized mice revealed that the expression of EP₂ mRNA was induced in uterine LE cells by exogenously-added progesterone (unpublished data). These results suggest that uterine expression of the EP₂ gene is under the control of ovarian progesterone. In fact, there is one potential PRE in the 5'-flanking sequence, 75 bp upstream of the uterine transcription start site. However, it is yet unknown whether this PRE works as an uterus-specific promoter in regulation of EP₂ gene expression. All that is known is that uterine transcription of the EP₂ gene starts 645 bp upstream of the transcription initiation site in the macrophage cell line. Therefore, one possible explanation is that the PRE in the EP₂ gene may be involved in activation of the closer transcription start site in uterine LE cells in response to progesterone.

On the other hand, expression of the 2.2 kb species of the EP₂ transcript was widely observed in various tissues. This EP₂ transcript was induced by LPS-stimulation in J774.1 cells. PGE₂ is known to inhibit LPS-induced cytokine production in the macrophage via cAMP accumulation (Zhong et al., 1995). LPS-induced EP₂ might be involved in such inhibitory actions of PGE₂ in the activated macrophage. Indeed, in the 2 kb immediate 5'-flanking region of the EP₂ gene, three consensus sequences for the NF-IL6 binding site and one consensus sequence for the NF- κ B binding site were found (Fig. 3-2). It is possible that the 2.2 kb transcript is induced by these inflammatory transcription factors (Akira and Kishimoto, 1992; Matsuoka et al., 1993). If this is the case, it seems reasonable that the expression levels of the 2.2 kb transcript were extremely low in healthy mouse tissues other than the uterus.

The most remarkable finding in this study is that uterine transcription initiation of the EP₂ gene is different from that in other tissues. This suggests that distinct promoters are involved in EP₂ gene

expression in the uterus and other tissues. Such tissue specific expression respondent to various physiological stimuli has been reported in the mouse FP receptor gene; the 7.3 kb fragment of the 5'-flanking region of the FP gene contains enough promoter sequence for its expression in the stomach and kidney, but not in the corpora lutea (Hasumoto et al., 1997). In the case of the EP₂ gene, however, it remains unknown at present whether the uterine promoter and the promoter for other tissues work independently from each other. It is also unclear whether some of the putative promoter regions in both systems overlap and share some basic transcription factors. Thus, further systematic studies on EP₂ gene expression in the uterus and other tissues are essential.

CONCLUSION

The author performed a study on the structure and function of the prostaglandin E receptor subtype EP₂. A summary of the results obtained here are as follows:

1. Expression of EP₂ mRNA was much lower than that of EP₄ in almost all tissues. EP₂ mRNA was expressed most abundantly in the uterus.
2. The mRNAs for EP₂, EP₃, and EP₄ showed distinct cellular localizations and time-dependent changes in the level of expression in individual cells in pseudopregnant mouse uteri. Confined expression of EP₂ mRNA in the luminal epithelium on day 5 of pseudopregnancy suggests the involvement of PGE₂ and the EP₂ receptor in blastocyst implantation signaling (Fig. 4-1).
3. Transcriptional initiation of the EP₂ gene in the uterus was different from that in other tissues and that in LPS-stimulated macrophages (Fig. 4-1).

Information obtained from the present study is bound to facilitate the understanding of the physiological functions of PGE₂, as well as the development of more subtype-specific PGE₂ analogues for therapeutic purposes.

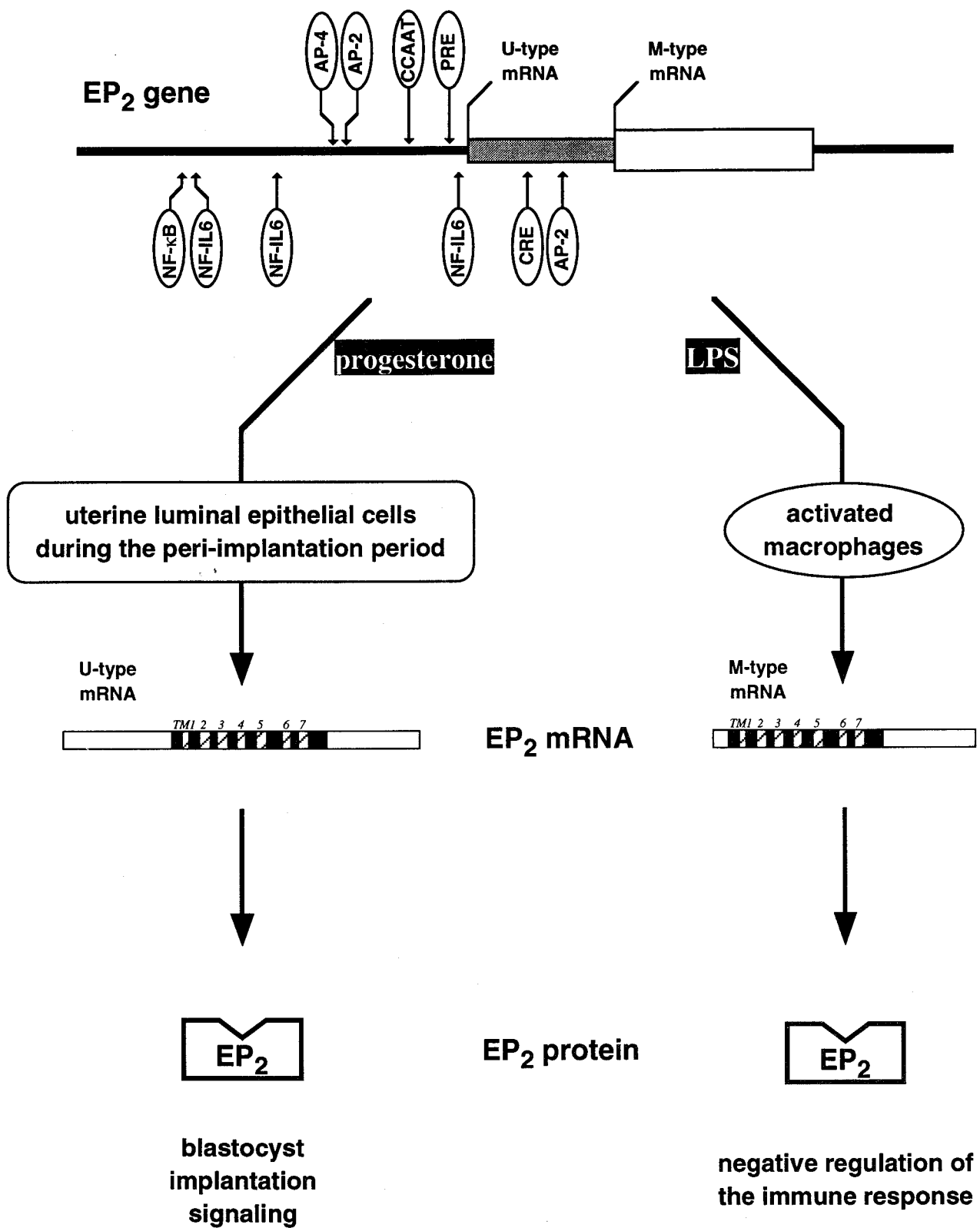


Fig. 4-1. Regulation of gene expression and potential physiological functions of the prostaglandin E receptor subtype EP₂.

EXPERIMENTAL PROCEDURES

Chapter 1

1. *Materials*

Butaprost, SC-19220 and misoprostol, M&B 28767, and sulprostone were generous gifts from Dr. P. J. Gardiner of Bayer U. K. Ltd., Dr. P. W. Collins of Searle, Dr. M. P. L. Caton of Rhone-Poulenc Ltd., and Schering, respectively. [5,6,8,9,11,12,14,15-³H]PGE₂ (181 Ci/mmol), iloprost, and the ¹²⁵I-labeled cyclic AMP assay system were obtained from Amersham Corp. [α-³²P]dCTP (3,000 Ci/mmol), [α-³²P]UTP (3,000 Ci/mmol), [α-³⁵S]CTP (1,000 Ci/mmol), and [γ-³²P]ATP (3,000 Ci/mmol) were obtained from Du Pont-New England Nuclear. PGE₂, 11-deoxy PGE₁, 19(R)OH-PGE₂, 1-OH-PGE₁, and 16,16-dimethyl PGE₂ were purchased from Cayman Chemical (Ann Arbor, MI). PGE₁, PGF_{2α}, and PGD₂ were obtained from Funakoshi Pharmaceuticals (Tokyo, Japan). Sources of other materials are shown in the text.

2. *cDNA cloning of the mouse prostanoid EP₂ receptor*

2-1. *Amplification of a cDNA Fragment of the Mouse EP₂*

First-strand cDNA was synthesized from mouse lung total RNA using random hexanucleotides as primers. Degenerate PCR primers, 5'-CTGGGATCCATGCTCATGCT(CG)TT(CT)GC(ACGT)ATGGC-3' and 5'-GCAGAATTCAGACGGCGAA(CG)GT(AG)AT(GT)GTCAT-3', corresponding to the putative third and sixth transmembrane domains of the human EP₂ (Regan et al., 1994) were synthesized. The thermal cycle program was 2 cycles of 94°C for 0.8 min, 48°C for 1 min, and 72°C for 3 min, 3 cycles of 94°C for 0.8 min, 50°C for 1 min, and 72°C for 2 min, followed by 27 cycles of 94°C for 0.8 min, 55°C for 1 min, and 72°C

for 3 min. Amplified cDNAs were subcloned into pBluescript II SK(+) (Stratagene). A 466-base pair clone showed a sequence highly homologous to the human EP₂ receptor cDNA.

2-2. Isolation of a Functional cDNA Clone

A mouse lung cDNA library was prepared by the oligo(dT) priming method. Approximately 3.0×10^5 recombinant clones from this library were screened by hybridization with the cDNA fragment obtained by PCR. Hybridization was carried out at 68°C for 15 h in $6 \times$ SSC (900 mM NaCl and 90 mM sodium citrate) containing $5 \times$ Denhardt's solution (0.1% Ficoll, 0.1% polyvinylpyrrolidone, and 0.1% bovine serum albumin) and 0.5% SDS, and filters were washed twice at 68°C for 30 min in $2 \times$ SSC containing 1% SDS. Four positive clones were isolated, and a representative clone, ML202, was subjected to sequence and expression analyses. Nucleotide sequencing was carried out on both strands by the dideoxy chain termination method.

3. Stable Expression in CHO Cells

The EcoRI insert of ML202 was subcloned into pdKCR-dhfr (Oikawa et al., 1989), and the resultant plasmid DNA was transfected into CHO cells deficient in dihydrofolate reductase activity (CHO-dhfr⁻) by the lipofection method (Felgner et al., 1987). Cell populations expressing the cDNA together with dihydrofolate reductase were selected in α -modification of Eagle's medium lacking ribonucleosides and deoxyribonucleosides and containing penicillin (100 units/ml), streptomycin (100 μ g/ml), and 10% dialyzed fetal bovine serum (Cell Culture Laboratory). From these cell populations, clonal cell lines were isolated by single-cell cloning. Expression of the mouse cDNA was assessed by Northern blot analysis.

4. Ligand Binding Assay

Using the crude membrane fraction (300,000 × g pellet fraction) prepared from a CHO cell clone expressing the receptor, [³H]PGE₂ binding was determined as described by Nishigaki et al. (1995). The membrane (40 μg) was incubated with 4 nM [³H]PGE₂ (397,000 dpm) at 30°C for 1 h in 100 μl of buffer A (20 mM HEPES-NaOH, pH 7.4, 1 mM EDTA, 10 mM MgCl₂). The reaction was terminated by the addition of ice-cold buffer A, and the resultant mixture was immediately filtrated through Whatman GF/C filter. The filter was then washed twice with 3 ml of ice-cold buffer A, and the radioactivity associated with the filter was measured by a liquid scintillation counter. Non-specific binding was determined using a 1,000-fold excess of unlabeled PGE₂ in the reaction mixture. The specific binding was calculated by subtracting the non-specific binding from the total binding.

5. Measurement of cAMP Formation

The cAMP levels in the CHO cell clone were measured as described by Nishigaki et al. (1995). The cells cultured in 24-well plates (5 × 10⁵ cells/well) were incubated at 37°C for 10 min in HEPES-buffered saline (140 mM NaCl, 4.7 mM KCl, 2.2 mM CaCl₂, 1.2 mM MgCl₂, 1.2 mM KH₂PO₄, 11 mM glucose, and 15 mM HEPES, pH 7.4). Reactions were started by the addition of the test agents along with 100 μM Ro-20-1724. After incubation at 37°C for 10 min, reactions were terminated by the addition of 10% trichloroacetic acid. The cAMP formed was measured by the radioimmunoassay kit.

6. Northern Blot Analysis

Total RNAs from various mouse tissues were isolated by the acid guanidinium thiocyanate-phenol-chloroform method (Chomczynski and

Sacchi, 1987), and poly(A)⁺ RNAs were purified using Oligotex dT30 <Super> (Takara Shuzo, Kyoto, Japan). For the detection of EP₂ transcripts, 5 µg of poly(A)⁺ RNAs purified twice with the Oligotex from various tissues were separated by electrophoresis on a 1.5% agarose gel, and transferred onto a nylon membrane (Hybond-N, Amersham). An antisense RNA probe was synthesized with T7 RNA polymerase using linearized ML202 as the template. For the detection of the EP₄ mRNA, 5 µg of poly(A)⁺-rich RNA (purified once with the Oligotex) was used. An antisense RNA probe was synthesized with T7 RNA polymerase using linearized MP412 with the XhoI fragment deleted as the template. Hybridization was carried out at 70°C for 4 hr in 5 × SSC, 50% formamide, 5 × Denhardt's solution, 0.2% SDS, 0.05 M sodium phosphate (pH 6.5), 250 µg/ml heat-denatured salmon sperm DNA, 200 µg/ml yeast transfer RNA, and the ³²P-labeled probe. Filters were washed at 70°C in 2 × SSC and 1% SDS for 10 min, in 0.2 × SSC and 0.5% SDS for the next 10 min, and then in 0.1 × SSC and 0.1% SDS for 20 min. The filter for detecting the EP₄ mRNA was treated with 1 µg/ml RNaseA at 20°C for 10 min, and washed again at 70°C in 0.1 × SSC and 0.1% SDS for 10 min.

Chapter 2

1. Treatment of Animals

ddY mice were purchased from Japan SLC Inc. (Hamamatsu, Japan). Pseudopregnancy was achieved in virgin mice as follows. Immature (21-day-old) female mice were obtained and housed under a 12-h light, 12-h dark photoperiod, with lights on at 0800 h. The mice received a single ip injection of 5 IU PMSG. After 48 h, mice were injected ip with 5 IU hCG and paired overnight with a vasectomized male of the same inbred line. The morning on which a vaginal plug was

detected was taken as day 0.5 of pseudopregnancy. Mice were killed at different intervals after mating, and the uteri were isolated, frozen immediately in liquid nitrogen (for RNA isolation) or in 2-methylbutane at -50°C (for preparation of uterine sections), and stored at -80°C until use. For RNA isolation, uteri were collected from three mice for each day of pseudopregnancy studied, and Northern blot experiments were independently repeated three times. *In situ* hybridizations were repeated twice or three times for each day of pseudopregnancy studied.

2. Hybridization Probes

Mouse cDNAs for EP₁, EP₂, EP₃, and EP₄ were subcloned into pBluescript II (Stratagene, La Jolla, CA) for synthesis of both sense and antisense cRNA probes (Sugimoto et al., 1992; Honda et al., 1993; Watabe et al., 1993; Katsuyama et al., 1995). For Northern hybridization, antisense ³²P-labeled cRNA probes were generated, whereas for *in situ* hybridization, sense and antisense ³⁵S-labeled cRNA probes were generated using the appropriate polymerases. Cold antisense riboprobes were also synthesized by the same procedure with unlabeled nucleotides.

3. Northern Blot Analysis

Uteri were collected from three mice for each day of pseudopregnancy studied. Uterine total RNAs were isolated as described above. Total RNA (15 μg) was separated by electrophoresis on a 1.5% agarose gel and transferred onto a nylon membrane (Hybond-N, Amersham). Four different blots were prepared and separately hybridized with ³²P-labeled probes for EP₁, EP₂, EP₃, and EP₄. Hybridization was carried out essentially as described above. After hybridization, the blots were washed under stringent conditions, and the

hybrids were detected by autoradiography. Then the blots were stripped and rehybridized with ^{32}P -labeled DNA probe for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Hybridization was carried out at 68°C in $6 \times \text{SSC}$, and filters were washed at 68°C in $2 \times \text{SSC}$ as described above. Autoradiograms were subjected to densitometric scanning (AE-6900M, ATTO, Tokyo, Japan) for quantitation of PGE receptor mRNA levels relative to GAPDH mRNA levels. Northern blot experiments were independently repeated three times, and similar results were obtained. Representative results are shown in Fig. 2-1.

4. Preparation of Uterine Sections

Uterine sections $10 \mu\text{m}$ thick were cut on a Jung Frigocut 2800E cryostat and mounted onto poly-L-lysine-coated glass slides. The sections were fixed in 4% formalin in PBS for 10 min, rinsed in PBS, and acetylated with 0.25% acetic anhydride in 0.1 M triethanolamine-0.9% NaCl for 10 min at room temperature. After dehydration sequentially in 70%, 95%, and 100% ethanol, the sections were air-dried and stored at -80°C until use.

5. In Situ Hybridization

Riboprobes were prepared essentially as described above by transcription in the presence of $[\alpha\text{-}^{35}\text{S}]\text{CTP}$ to specific activities of 1.0×10^9 cpm/ μg . After removing unincorporated nucleotides, riboprobes were degraded to about 150 bases by alkaline hydrolysis. Hybridization was carried out essentially as described by Sugimoto et al. (1994b). The specificity of the signal with each antisense probe was verified either by its disappearance with the addition of an excess of unlabeled probe (shown for EP₃ in Fig. 2-4f, not shown for others), or by absence of

specific hybridization with sense probes (data not shown). Hybridized sections were dipped in nuclear track emulsion (NTB3, Eastman Kodak, Rochester, NY) diluted 1:1 with water. After exposure for about 4 weeks at 4°C, the dipped slides were developed and fixed, then counterstained with hematoxylin and eosin. These experiments were repeated twice or three times on each day of pseudopregnancy studied.

Chapter 3

1. Isolation of genomic clones

A mouse genomic library (approximately 5.0×10^5 recombinants from the 129SVJ λ FIXII library, Stratagene) was screened by hybridization with a 466 bp fragment of the mouse EP₂ cDNA spanning the putative third to sixth transmembrane domains. Hybridization was carried out under the conditions described above. Ten positive clones were isolated and subjected to sequence and restriction analyses. These clones contained the nucleotide sequence upstream of the sixth transmembrane domain of the mouse EP₂ but not the seventh transmembrane and 3'-untranslated regions. Therefore, the library was then screened with a 543 bp-HincII fragment covering the putative seventh transmembrane and C-terminal regions. Five other genomic clones were isolated and subjected to sequence and restriction analyses. Nucleotide sequencing was carried out on both strands by the dideoxy chain termination method. Intron size was determined by PCR using mouse liver genomic DNA as the template and the LA PCR Kit Ver. 2 (Takara Shuzo, Kyoto, Japan). Oligonucleotides (35-mer) corresponding to the exon-intron junctions of the EP₂ gene were used as PCR primers, and amplification was performed under these conditions in the following order; (1) 94°C for 1 min, (2) 98°C for 10 sec and 68°C for 15 min for 14 cycles, (3) 98°C for 10 sec and 68°C for 15

min, with an addition of 15 sec/cycle at 68°C for 16 cycles, (4) 72°C for 10 min. The amplified DNA fragment was subjected to restriction analysis.

2. Isolation of cDNA clones from a mouse uterus cDNA library

A mouse uterus cDNA library was prepared by the oligo(dT)-priming method. Approximately 4.0×10^5 recombinant clones from this library were screened by hybridization with the insert DNA of ML202 (Katsuyama et al., 1995). Thirteen positive clones were isolated and subjected to sequence and restriction analyses.

3. Northern Blot Analysis

J774.1 macrophage-like cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 µg/ml). The cells were treated with 100 ng/ml LPS (*B. E. coli* 055:B5, Difco Laboratories, Detroit, MI) for 3 hr. Pseudopregnancy was achieved in virgin mice essentially as described above. Total RNA was isolated from LPS-treated J774.1 cells and pseudopregnant day 5 uteri as described above. The RNAs (10 µg) were separated by electrophoresis on a 1.5% agarose gel, and transferred onto a nylon membrane (BIODYNE, Pall Biosupport Division, East Hills, NY). Hybridization probes used were as follows; a HincII fragment (543 bp) covering the seventh transmembrane and C-terminal regions, a XbaI fragment (469 bp) corresponding to the 5'-noncoding region of uterine EP₂ cDNA clones, and an EcoRI/SphI fragment (377 bp) of the 3'-terminus of an EP₂ cDNA clone with a long 3'-untranslated region (ML204). Hybridization was carried out under the conditions described above.

4. Determination of the transcription start sites

The two antisense oligonucleotides used in primer extension analysis were the EP2NR primer corresponding to nucleotides +27 to -3 of ML202 (Katsuyama et al., 1995) and the EP2UR primer corresponding to 610-644 bp upstream of the ATG start codon. The primers were labeled with [γ - ^{32}P]ATP (Du Pont-New England Nuclear) by T4 polynucleotide kinase. Poly(A)⁺ RNAs were purified from LPS-treated J774.1 cells and pseudopregnant day 5 uteri using Oligotex dT30 <Super> (Takara Shuzo, Kyoto, Japan). The ^{32}P -labeled EP2NR primer was annealed overnight to 5 μg of poly(A)⁺ RNAs in hybridization buffer (40 mM PIPES, pH 6.4, 1 mM EDTA, 0.4 M NaCl, 80% formamide) at 30°C. The ^{32}P -labeled EP2UR primer was annealed to 10 μg of poly(A)⁺ RNAs. The annealed RNA/primer mixture was extended by Superscript II reverse transcriptase (Life Technologies) at 42°C for 1 h. The extension products were electrophoresed on a 6% polyacrylamide gel containing 7 M urea. Resultant fragment sizes were determined by sequencing ladders generated by the same primers.

The 5'-RACE experiment was performed essentially as described by Hasumoto et al. (1997). Poly(A)⁺ RNA (1 μg) from LPS-treated J774.1 cells was reverse transcribed with the EP2NR primer, and the resultant first strand cDNA was polyadenylated by terminal deoxynucleotidyl transferase. The cDNA was amplified with an oligo(dT) primer (25-mer) and the EP2NR3 primer complementary to nucleotides 96 - 125 of ML202. The resultant products were then amplified using another primer, EP2NR4, complementary to nucleotides 71 - 95 of ML202. The amplified products were separated on a 2% agarose gel, and two major products, approximately 350 bp and 270 bp in length, were subcloned into a plasmid vector for sequencing.

5. Detection of EP₂ mRNA variants by RT-PCR

Total RNAs from various tissues were transcribed with M-MLV reverse transcriptase (Life Technologies) using hexanucleotides as primers, and the resultant cDNAs were used for PCR. For the detection of 5'-end variants, amplification was performed with the EP2EXR primer complementary to the 35 nucleotides at the 5'-terminus of exon 2, in combination with either the MG2721 primer corresponding to nucleotides -99 - -72 or the MG2723 primer corresponding to nucleotides -456 - -429. A uterine EP₂ cDNA clone (MU14) was used as a positive control of the amplification. For the amplification of 3'-end variants, PCR was performed with the EP2EXF primer corresponding to the 35 nucleotides at the 3'-terminus of exon 1, in combination with either the EP2MJR primer complementary to nucleotides 840 - 864 of exon 2 or the ML204R4 primer complementary to nucleotides 955 - 984 of exon 2. ML204 was used as a positive control of the amplification. Amplified products were separated by electrophoresis on 1% agarose gels.

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REFERENCES

- Akira, S. and Kishimoto, T. (1992) IL-6 and NF-IL6 in acute-phase response and viral infection. *Immunol. Rev.* 127, 25-50.
- Arakawa, T., Laneuville, O., Miller, C. A., Lakkides, K. M., Wingerd, B. A., DeWitt, D. L., and Smith, W. L. (1996) Prostanoid receptors of murine NIH3T3 and RAW264.7 cells: structure and expression of the murine prostaglandin EP4 receptor gene. *J. Biol. Chem.* 271, 29569-29575.
- Bause, E. (1983) Structural requirements of *N*-glycosylation of proteins: studies with proline peptides as conformational probes. *Biochem. J.* 209, 331-336.
- Botella, A., Delvaux, M., Fioramonti, J., Frexinos, J., and Bueno, L. (1993) Stimulatory (EP₁ and EP₃) and inhibitory (EP₂) prostaglandin E₂ receptors in isolated ileal smooth muscle cells. *Eur. J. Pharmacol.* 237, 131-137.
- Breathnach, R. and Chambon, P. (1981) Organization and expression of eukaryotic split genes coding for proteins. *Annu. Rev. Biochem.* 50, 349-383.
- Bunce, K. T., Clayton, N. M., Knight, M., Sheppard, D. N. and Spraggs, C. F. (1987) Differential effects of prostaglandins on unidirectional absorption and secretion in rat ileum. *Gastroenterology* 92, 1332.
- Båtshake, B., Nilsson, C., and Sundelin, J. (1995) Molecular characterization of the mouse prostanoid EP₁ receptor gene. *Eur. J. Biochem.* 231, 809-814.
- Chalubinski, K., Deutinger, J., and Bernaschek, G. (1993) Vaginosonography for recording of cycle-related myometrial contractions. *Fertil. Steril.* 59, 225-228.
- Chomczynski, P. and Sacchi, N. (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* 162, 156-159.
- Coleman, R. A., Humphray, J. M., Sheldrick, R. L. G., and White, B. P. (1988) Gastric antisecretory prostanoids: actions at different prostanoid receptors. *Br. J. Pharmacol.* 95, suppl. 724P.
- Coleman, R. A., Kennedy, I., Humphrey, P. P. A., Bunce, K., and Lumley, P. (1990) Prostanoids and their receptors. in: *Comprehensive Medicinal Chemistry* (Hansch, C., Sammes, P. G., Taylor, J. B. and Emmett, J. C. eds) Vol. 3, pp. 643-714 Pergamon, Oxford.
- Coleman, R. A., Grix, S. P., Head, S.A., Louttit, J. B., Mallett, A., and Sheldrick, R. L. G. (1994) A novel inhibitory prostanoid receptor in piglet saphenous vein. *Prostaglandins* 47, 151-168.
- Crane, L. H. and Martin, L. (1991a) *In vivo* myometrial activity during early pregnancy and pseudopregnancy in the rat. *Reprod. Fertil. Dev.* 3, 233-244.
- Crane, L. H. and Martin, L. (1991b) *In vivo* myometrial activity in the rat during the oestrous cycle: studies with a novel technique of video laparoscopy. *Reprod. Fertil. Dev.* 3, 185-199.

- Das, S. K., Flanders, K. C., Andrews, G. K., and Dey, S. K. (1992) Expression of transforming growth factor- β isoforms (β 2 and β 3) in the mouse uterus: analysis of the periimplantation period and effects of ovarian steroids. *Endocrinology* 130, 3459-3466.
- Ezashi, T., Sakamoto, K., Miwa, K., Okuda-Ashitaka, E., Ito, S., and Hayaishi, O. (1997) Genomic organization and characterization of the gene encoding bovine prostaglandin F_{2 α} receptor. *Gene* 190, 271-278.
- Felgner, P. L., Gadek, T. R., Holm, M., Roman, R., Chan, H. W., Wenz, M., Northrop, J. P., Ringold, G. M., and Danielsen, M. (1987) Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure. *Proc. Natl. Acad. Sci. USA* 84, 7413-7417.
- Gardiner, P. J. (1986) Characterization of prostanoid relaxant/inhibitory receptors (ψ) using a highly selective agonist, TR4979. *Br. J. Pharmacol.* 87, 45-56.
- Goureau, O., Tanfin, Z., Marc, S., and Harbon, S. (1992) Diverse prostaglandin receptors activate distinct signal transduction pathways in rat myometrium. *Am. J. Physiol.* 32, C257-C265.
- Hasumoto, K., Sugimoto, Y., Gotoh, M., Segi, E., Yamasaki, A., Yamaguchi, M., Honda, H., Hirai, H., Negishi, M., Kakizuka, A., and Ichikawa, A. (1997) Characterization of the mouse prostaglandin F receptor gene: a transgenic mouse study of a regulatory region that controls its expression in the stomach and kidney but not in the ovary. *Genes to Cells* 2, 571-580.
- Hirata, M., Kakizuka, A., Aizawa, M., Ushikubi, F., and Narumiya, S. (1994) Molecular characterization of a mouse prostaglandin D receptor and functional expression of the cloned gene. *Proc. Natl. Acad. Sci. USA* 91, 11192-11196.
- Hofmann, G. E., Rao, Ch. V., De Leon, F. D., Toledo, A. A., and Sanfilippo, J. S. (1985) Human endometrial prostaglandin E₂ binding sites and their profiles during the menstrual cycle and in pathologic states. *Am. J. Obstet. Gynecol.* 151, 369-375.
- Homaidan, F. R., Zhao, L., and Burakoff, R. (1995) Characterization of PGE₂ receptors in isolated rabbit colonic crypt cells. *Am. J. Physiol.* 268, G270-G275.
- Honda, A., Sugimoto, Y., Namba, T., Watabe, A., Irie, A., Negishi, M., Narumiya, S., and Ichikawa, A. (1993) Cloning and expression of a cDNA for mouse prostaglandin E receptor EP₂ subtype. *J. Biol. Chem.* 268, 7759-7762.
- Huszar, G. and Walsh, M. P. (1989) Biochemistry of the myometrium and cervix. in: *Biology of the Uterus* (Wynn, R. M. and Jollie, W. P. eds.) 2nd ed., pp. 355-402, Plenum Medical, New York.
- Irie, A., Segi, E., Sugimoto, Y., Ichikawa, A., and Negishi, M. (1994) Mouse prostaglandin E receptor EP₃ subtype mediates calcium signals via Gi in cDNA-transfected Chinese hamster ovary cells. *Biochem. Biophys. Res. Commun.* 204, 303-309.
- Jacobs, A. L., Hwang, D., Julian, J., and Carson, D. D. (1994) Regulated expression of prostaglandin endoperoxide synthase-2 by uterine stroma. *Endocrinology* 135, 1807-1815.
- Katsuyama, M., Nishigaki, N., Sugimoto, Y., Morimoto, K., Negishi, M., Narumiya, S., and Ichikawa, A. (1995) The mouse prostaglandin E receptor EP₂ subtype: cloning, expression, and Northern blot analysis. *FEBS Lett.* 372, 151-156.

Katsuyama, M., Sugimoto, Y., Morimoto, K., Hasumoto, K., Fukumoto, M., Negishi, M., and Ichikawa, A. (1997) Distinct cellular localization of the messenger ribonucleic acid for prostaglandin E receptor subtypes in the mouse uterus during pseudopregnancy. *Endocrinology* 138, 344-350.

Katsuyama, M., Sugimoto, Y., Okano, K., Segi, E., Ikegami, R., Negishi, M., and Ichikawa, A. (1998) Characterization of the gene for the mouse prostaglandin E receptor subtype EP₂: tissue specific initiation of transcription in the macrophage and the uterus. *Biochem. J.*, in press.

Kennedy, T. G., Martel, D., and Phychoyos, A. (1983a) Endometrial prostaglandin E₂ binding: characterization in rats sensitized for the decidual cell reaction and changes during pseudopregnancy. *Biol. Reprod.* 29, 556-564.

Kennedy, T. G., Martel, D., and Phychoyos, A. (1983b) Endometrial prostaglandin E₂ binding during the estrous cycle and its hormonal control in ovariectomized rats. *Biol. Reprod.* 29, 565-571.

Kennedy, T. G., Keys, J. L., and King, G. J. (1986) Endometrial prostaglandin E₂-binding sites in the pig: characterization and changes during the estrous cycle and early pregnancy. *Biol. Reprod.* 35, 624-632.

Kotani, M., Tanaka, I., Ogawa, Y., Usui, T., Tamura, N., Mori, K., Narumiya, S., Yoshimi, T., and Nakao, K. (1997) Structural organization of the human prostaglandin EP(3) receptor subtype gene (PTGER3). *Genomics* 40, 425-434.

Kozak, M. (1989) The scanning model for translation: an update. *J. Cell Biol.* 108, 229-241.

Kozak, M. (1991) An analysis of vertebrate mRNA sequences: intimations of translational control. *J. Cell Biol.* 115, 887-903.

Lundkvist, Ö. and Nilsson, B. O. (1980) Ultrastructural changes of the trophoblast-epithelial complex in mice subjected to implantation blocking treatment with indomethacin. *Biol. Reprod.* 22, 719-726.

Malter, J. S. (1989) Identification of an AUUUA-specific messenger RNA binding protein. *Science* 246, 664-666.

Manley, J. L., Yu, H., Ryner, L. (1985) RNA sequence containing hexanucleotide AAUAAA directs efficient mRNA polyadenylation in vitro. *Mol. Cell. Biol.* 5, 373-379.

Markoff, E., Henemyre, C., Fellows, J., Pennington, E., Zeitler, P. S., and Cedars, M. I. (1995) Localization of insulin-like growth factor binding protein-4 expression in the mouse uterus during the peri-implantation period. *Biol. Reprod.* 53, 1103-1109.

Matsunaga, T., Gohda, E., Takebe, T., Wu, Y. L., Iwao, M., Kataoka, H., and Yamamoto, I. (1994) Expression of hepatocyte growth factor is up-regulated through activation of a cAMP-mediated pathway. *Exp. Cell Res.* 210, 326-335.

Matsusaka, T., Fujikawa, K., Nishio, Y., Mukaida, N., Matsushima, K., Kishimoto, T. and Akira, S. (1993) Transcription factors NF-IL6 and NF- κ B synergistically activate transcription of the inflammatory cytokines, interleukin 6 and interleukin 8. *Proc. Natl. Acad. Sci. USA* 90, 10193-10197.

Moncada, S., Flower, R. J., and Vane, J. R. (1985) Prostaglandins, prostacyclin, thromboxane A₂, and leukotrienes. in: *The Pharmacological Basis of Therapeutics*

(Gilman, A. G., Goodman, L. S., Rall, T. W., and Murad, F., eds) 7th Ed., pp. 660-673, Macmillan Publishing Co., New York.

Moulton, B. C. and Koenig, B. B. (1986) Biochemical responses of the luminal epithelium and uterine sensitization. *Ann. N. Y. Acad. Sci.* 476, 95-109.

Mount, S. M. (1982) A catalogue of splice junction sequences. *Nucleic Acids Res.* 10, 459-475.

Musch, M. W., Field, M., Miller, R. J., and Stoff, J. S. (1987) Homologous desensitization to prostaglandins in rabbit ileum. *Am. J. Physiol.* 252, G120-G127.

Namba, T., Sugimoto, Y., Hirata, M., Hayashi, Y., Honda, A., Watabe, A., Negishi, M., Ichikawa, A., and Narumiya, S. (1992) Mouse thromboxane A₂ receptor: cDNA cloning, expression and Northern blot analysis. *Biochem. Biophys. Res. Commun.* 184, 1197-1203.

Namba, T., Oida, H., Sugimoto, Y., Kakizuka, A., Negishi, M., Ichikawa, A., and Narumiya, S. (1994) cDNA cloning of a mouse prostacyclin receptor: multiple signaling pathways and expression in thymic medulla. *J. Biol. Chem.* 269, 9986-9992.

Negishi, M., Sugimoto, Y., and Ichikawa, A. (1993) Prostanoid receptors and their biological actions. *Prog. Lipid Res.* 32, 417-434.

Nishigaki, N., Negishi, M., Honda, A., Sugimoto, Y., Namba, T., Narumiya, S., and Ichikawa, A. (1995) Identification of prostaglandin E receptor "EP2" cloned from mastocytoma cells as EP4 subtype. *FEBS Lett.* 364, 339-341.

Nüsing, R. M., Hirata, M., Kakizuka, A., Eki, T., Ozawa, K., and Narumiya, S. (1993) Characterization and chromosomal mapping of the human thromboxane A₂ receptor gene. *J. Biol. Chem.* 268, 25253-25259.

Ogawa, Y., Tanaka, I., Inoue, M., Yoshitake, Y., Isse, N., Nakagawa, O., Usui, T., Itoh, H., Yoshimasa, T., Narumiya, S., and Nakao, K. (1995) Structural organization and chromosomal assignment of the human prostacyclin receptor gene. *Genomics* 27, 142-148.

Oikawa, S., Inuzuka, C., Kuroki, M., Matsuoka, Y., Kosaki, G., and Nakazato, H. (1989) Cell adhesion activity of non-specific cross-reacting antigen (NCA) and carcinoembryonic antigen (CEA) expressed on CHO cell surface: homophilic and heterophilic adhesion. *Biochem. Biophys. Res. Commun.* 164, 39-45.

Parr, M. B. and Parr, E. L. (1989) The implantation reaction. in: *Biology of the Uterus* (Wynn, R. M. and Jollie, W. P. eds.) 2nd ed., pp. 233-277, Plenum Medical, New York.

Pepe, G. J. and Rothchild, I. (1974) A comparative study of serum progesterone levels in pregnancy and in various types of pseudopregnancy in the rat. *Endocrinology* 95, 275-279.

Reeves, J. J., Bunce, K. T., Sheldrick, R. L. G., and Stables, R. (1988) Evidence for the PGE receptor subtype mediating inhibition of acid secretion in the rat. *Br. J. Pharmacol.* 95, suppl. 805P.

Regan, J. W., Bailey, T. J., Pepperl, D. J., Pierce, K. L., Bogardus, A. M., Donello, J. E., Fairbairn, C. E., Kedzie, K. M., Woodward, D. F., and Gil, D. W. (1994) Cloning of a novel human prostaglandin receptor with characteristics of the pharmacologically defined EP₂ subtype. *Mol. Pharmacol.* 46, 213-220.

- Samuelsson, B., Goldyne, M., Granström, E., Hamberg, M., Hammarström, S., and Malmsten, C. (1978) Prostaglandins and thromboxanes. *Annu. Rev. Biochem.* 47, 997-1029.
- Schiff, R., Arensburg, J., Itin, A., Keshet, E., and Orly, J. (1993) Expression and cellular localization of uterine side-chain cleavage cytochrome P450 messenger ribonucleic acid during early pregnancy in mice. *Endocrinology* 133, 529-537.
- Senior, J., Marshall, K., Sangha, R., Baxter, G. S., and Clayton, J. K. (1991) *In vitro* characterization of prostanoid EP-receptors in the non-pregnant human myometrium. *Br. J. Pharmacol.* 102, 747-753.
- Senior, J., Marshall, K., Sangha, R. and Clayton, J. K. (1993) *In vitro* characterization of prostanoid receptors on human myometrium at term pregnancy. *Br. J. Pharmacol.* 108, 501-506.
- Soloff, M. S. (1989) Endocrine control of parturition. in: *Biology of the Uterus* (Wynn, R. M. and Jollie, W. P. eds.) 2nd ed., pp. 559-607, Plenum Medical, New York.
- Sugimoto, Y., Namba, T., Honda, A., Hayashi, Y., Negishi, M., Ichikawa, A., and Narumiya, S. (1992) Cloning and expression of a cDNA for mouse prostaglandin E receptor EP₃ subtype. *J. Biol. Chem.* 267, 6463-6466.
- Sugimoto, Y., Hasumoto, K., Namba, T., Irie, A., Katsuyama, M., Negishi, M., Kakizuka, A., Narumiya, S., and Ichikawa, A. (1994a) Cloning and expression of a cDNA for mouse prostaglandin F receptor. *J. Biol. Chem.* 269, 1356-1360.
- Sugimoto, Y., Namba, T., Shigemoto, R., Negishi, M., Ichikawa, A., and Narumiya, S. (1994b) Distinct cellular localization of mRNAs for three subtypes of prostaglandin E receptor in kidney. *Am. J. Physiol.* 266, F823-F828.
- Toh, H., Ichikawa, A., and Narumiya, S. (1995) Molecular evolution of receptors for eicosanoids. *FEBS Lett.* 361, 17-21.
- Tsujii, H., Okamoto, Y., Kikuchi, E., Matsumoto, M., and Nakano, H. (1993) Prostaglandin E₂ and rat liver regeneration. *Gastroenterology* 105, 495-499.
- Watabe, A., Sugimoto, Y., Honda, A., Irie, A., Namba, T., Negishi, M., Ito, S., Narumiya, S., and Ichikawa, A. (1993) Cloning and expression of cDNA for a mouse EP₁ subtype of prostaglandin E receptor. *J. Biol. Chem.* 268, 20175-20178.
- Weitlauf, H. M. (1988) Biology of implantation. in: *Biology of the Uterus* (Wynn, R. M. and Jollie, W. P. eds.) 2nd ed., pp. 231-262, Plenum Medical, New York.
- Woodgett, J. R., Gould, K. L., and Hunter, T. (1986) Substrate specificity of protein kinase C: use of synthetic peptides corresponding to physiological sites as probes for substrate recognition requirements. *Eur. J. Biochem.* 161, 177-184.
- Woodward, D. F., Protzman, C. E., Krauss, A. H.-P., and Williams, L. S. (1993) Identification of 19 (R)-OH prostaglandin E₂ as a selective prostanoid EP₂-receptor agonist. *Prostaglandins* 46, 371-383.
- Yee, G. M. and Kennedy, T. G. (1991) Role of cyclic adenosine 3',5'-monophosphate in mediating the effect of prostaglandin E₂ on decidualization in vitro. *Biol. Reprod.* 45, 163-171.
- Zhong, W. W., Burke, P. A., Drotar, M. E., Chavali, S. R., and Forse, R. A. (1995) Effects of prostaglandin E₂, cholera toxin and 8-bromo-cyclic AMP on

lipopolysaccharide-induced gene expression of cytokines in human macrophages.
Immunology 84, 446-452.