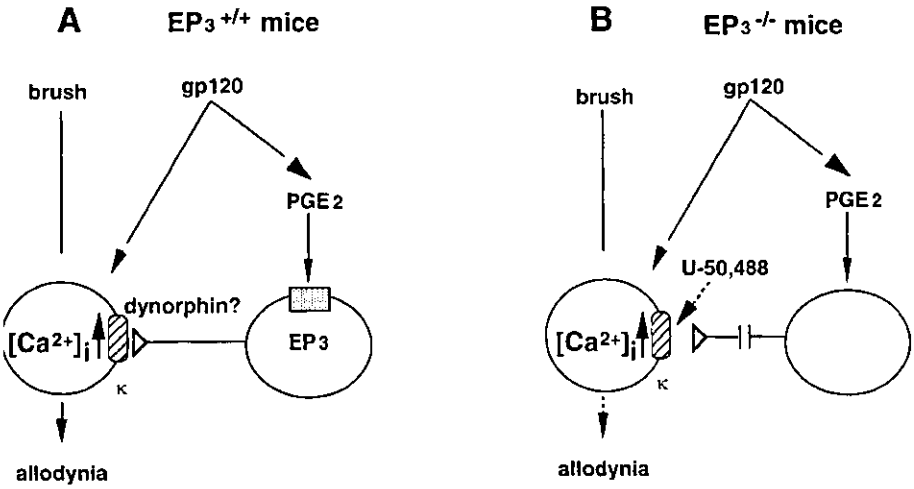

Fig. 8. Postulated mechanism of gp120-induced allodynia in EP3$^{+/+}$ (A) and EP3$^{-/-}$ (B) mice.

and whether COX-1 or COX-2 is responsible for PG production remain unknown. Pain associated with AIDS patients is a prominent and debilitating feature in about 60% of patients but, alarmingly, is undertreated (O'Neill and Sherrard, 1993; Breitbart, 1997; Larue et al., 1997). Evidence showing a link between HIV-1 gp120 and the EP3 receptor clearly warrants further investigations, which may lead to pre-emptive therapy for pain associated with AIDS patients.

Acknowledgements

This work was supported in part by grants from the programs Grants-in-Aid for Scientific Research on Priority Areas and Creative Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan, Grants-in-Aid for Scientific Research (B) and (C) from the Japan Society for the Promotion of Science, and by grants from the Science Research Promotion Fund of the Japan Private School Promotion Foundation, the Ono Foundation, and the Fund for AIDS Prevention of Osaka Association of Public Health.

References

Bagetta, G., Corasaniti, M.T., Paoletti, A.M., Berliocchi, L., Nisticò, R., Giammarioli, A.M., Malorni, W., Finazzi-Agrò, A., 1998. HIV-1 gp120-induced apoptosis in the rat neocortex involves enhanced expression of cyclo-oxygenase type 2 (COX-2). Biochemical Biophysical Research Communications 244, 819–824.

Bezzi, P., Domercq, M., Brambilla, L., Galli, R., Schols, D., De Clercq, E., Vescovi, A., Bagetta, G., Kollias, G., Meldolesi, J., Volterra, A., 2001. CXCR4-activated astrocyte glutamate release via TNFa: amplification by microglia triggers neurotoxicity. Nature Neuroscience 4, 702–710.

Bouhassira, D., Attal, N., Willer, J.C., Brasseur, L., 1999. Painful and painless peripheral sensory neuropathies due to HIV infection: a comparison using quantitative sensory evaluation. Pain 80, 265–272.

Breder, C.D., Dewitt, D., Kraig, R.P., 1995. Characterization of inducible cyclooxygenase in rat brain. Journal of Comparative Neurology 355, 296–315.

Breder, C.D., Smith, W.L., Raz, A., Masferrer, J., Seibert, K., Needleman, P., Saper, C.B., 1992. Distribution and characterization of cyclooxygenase immunoreactivity in the ovine brain. Journal of Comparative Neurology 322, 409–438.

Breitbart, W., 1997. Pain in AIDS. In: Jensen, T.S., Turner, J.A., Wiesenfeld-Hallin, Z., (Eds.), Progress in Pain Research and Management. Proceedings of the 8th World Congress on Pain, Vol. 8. IASP Press, Seattle, pp. 63–100.

Corasaniti, M.T., Strongoli, M.C., Piccirilli, S., Nisticò, R., Costa, A., Bilotta, A., Turano, P., Finazzi-Agrò, A., Bagetta, G., 2000. Apoptosis induced by gp120 in the neocortex of rat involves enhanced expression of cyclooxygenase type 2 and is prevented by NMDA receptor antagonists and by the 21-aminosteroid U-74389G. Biochemical Biophysical Research Communications 274, 664–669.

Cornblath, D.R., McArthur, J.C., 1988. Predominantly sensory neuropathy in patients with AIDS and AIDS-related complex. Neurology 38, 794–796.

Delézay, O., Yahi, N., Tamalet, C., Baghdiguian, S., Boudier, J.A., Fantini, J., 1997. Direct effect of type 1 human immunodeficiency virus (HIV-1) on intestinal epithelial cell differentiation: relationship to HIV-1 enteropathy. Virology 238, 231–242.

Dreyer, E.B., Kaiser, P.K., Offermann, J.T., Lipton, S.A., 1990. HIV-1 coat protein neurotoxicity prevented by calcium channel antagonists. Science 248, 364–367.

Epstein, L.G., Gendelman, H.E., 1993. Human immunodeficiency virus type 1 infection of the nervous system: pathogenetic mechanisms. Annals of Neurology 33, 429–436.

Fields, H.L., 1994. Peripheral neuropathic pain: an approach to management. In: Wall, P.D., Melzack, R. (Eds.), Textbook of Pain, third ed. Churchill Livingstone, London, pp. 991–996.

Hao, J.-X., Yu, W., Wiesenfeld-Hallin, Z., Xu, X.J., 1998. Treatment of chronic allodynia in spinally injured rats: effects of intrathecal selective opioid receptor agonists. Pain 75, 209–217.

Harouse, J.M., Bhat, S., Spitalnik, S.L., Laughlin, M., Stefano, K., Silberberg, D.H., Gonzalez-Scarano, F., 1991. Inhibition of entry of HIV-1 in neural cell lines by antibodies against galactosyl ceramide. Science 253, 320–323.

Lannuzel, A., Liedo, P.-M., Lamghitnia, H.O., Vincent, J.-D., Tardieu, M., 1995. HIV-1 envelope proteins gp120 and gp160 potentiate NMDA-induced [Ca^{2+}]i increase, alter [Ca^{2+}]i homeostasis and induce neurotoxicity in human embryonic neurons. European Journal of Neuroscience 7, 2285–2293.

Larue, F., Fontaine, A., Colleau, S.M., 1997. Underestimation and undertreatment of pain in HIV disease: multicentre study. British Medical Journal 314, 23–28.

Maccarrone, M., Bari, M., Corasaniti, M.T., Nistico, R., Bagetta, G., Finazzi-Agrò, A., 2000. HIV-1 coat glycoprotein gp120 induces apoptosis in rat brain neocortex by deranging the arachidonate cascade in favor of prostanoids. Journal of Neurochemistry 75, 196–203.

Maccarrone, M., Navarra, M., Corasaniti, M.T., Nistico, G., Agro, A.R., 1998. Cytotoxic effect of HIV-1 coat glycoprotein gp120 on human neuroblastoma CHP100 cells involves activation of the arachidonate cascade. Biochemical Journal 333, 45–49.

Medina, I., Ghose, S., Ben-Ari, Y., 1999. Mobilization of intracellular calcium stores participates in the rise of [Ca^{2+}]i and the toxic actions of the HIV coat protein GP120. European Journal of Neuroscience 11, 1167–1178.

Milligan, E.D., Mehmert, K.K., Hinde, J.L., Harvey, L.O. Jr., Martin, D., Tracey, K.J., Maier, S.F., Watkins, L.R., 2000. Thermal hyperalgesia and mechanical allodynia produced by intrathecal administration of the human immunodeficiency virus-1 (HIV-1) envelop glycoprotein, gp120. Brain Research 861, 105–116.

Milligan, E.D., O'Connor, K.A., Nguyen, K.T., Armstrong, C.B., Twining, C., Gaykema, R.P., Holguin, A., Martin, D., Maier, S.F., Watkins, L.R., 2001. Intrathecal HIV-1 envelope glycoprotein gp120 induces enhanced pain states mediated by spinal cord proinflammatory cytokines. Journal of Neuroscience 21, 2808–2819.

Minami, T., Nakano, H., Kobayashi, T., Sugimoto, Y., Ushikubi, F., Ichikawa, A., Narumiya, S., Ito, S., 2001. Characterization of EP receptor subtypes responsible for prostaglandin E_2-induced pain responses by use of EP_1 and EP_3 receptor knockout mice. British Journal of Pharmacology 133, 438–444.

Minami, T., Nishihara, I., Uda, R., Ito, S., Hyodo, M., Hayaishia, O., 1994. Characterization of EP-receptor subtypes involved in allodynia and hyperalgesia induced by intrathecal administration of prostaglandin E_2 to mice. British Journal of Pharmacology 112, 735–740.

Minami, T., Okuda-Ashitaka, E., Hori, Y., Sakuma, S., Sugimoto, T., Sakimura, K., Mishina, M., Ito, S., 1999. Involvement of primary afferent C-fibers in touch-evoked pain (allodynia) induced by prostaglandin E_2. European Journal of Neuroscience 11, 1849–1856.

Narumiya, S., Sugimoto, Y., Ushikubi, F., 1999. Prostanoid receptors: structures, properties, and functions. Physiological Review 79, 1193–1226.

O'Neill, W.M., Sherrard, J.S., 1993. Pain in human immunodeficiency virus disease: a review. Pain 54, 3–14.

Plummer, J.L., Cmielewski, P.L., Gourlay, G.K., Owen, H., Cousins, M.J., 1992. Antinociceptive and motor effects of intrathecal morphine combined with intrathecal clonidine, noradrenaline, carbachol or midazolam in rats. Pain 49, 145–152.

Thomas, F.P., Chalk, C., Lalonde, R., Robitaille, Y., Jolicoeur, P., 1994. Expression of human immunodeficiency virus type 1 in the nervous system of transgenic mice leads to neurological disease. Journal of Virology 68, 7099–7107.

Toggas, S.M., Masliah, E., Rockenstein, E.M., Rall, G.F., Abraham, C.R., Mucke, L., 1994. Central nervous system damage produced by expression of the HIV-1 coat protein gp120 in transgenic mice. Nature 367, 188–193.

Totani, T., Taketani, Y., Ueda, N., Nishisho, T., Ohmoto, Y., Sakata, Y., Muraguchi, M., Wada, K., Yamamoto, S., 1999. Possible involvement of interleukin-1 in cyclooxygenase-2 induction after spinal cord injury in rats. Journal of Neurochemistry 72, 302–309.

Ueno, A., Matsumoto, H., Naraba, H., Ikeda, Y., Ushikubi, F., Matsuoka, T., Narumiya, S., Sugimoto, Y., Ichikawa, A., Oh-ishi, S., 2001. Major roles of prostanoid receptors IP and EP_3 in endotoxin-induced enhancement of pain perception. Biochemical Pharmacology 62, 157–160.

Ushijima, H., Nishio, O., Klőcking, R., Perovic, S., Müller, W.E.G., 1995. Exposure to gp120 of HIV-1 induces an increased release of arachidonic acid in rat primary neuronal cell culture followed by NMDA receptor-mediated neurotoxicity. European Journal of Neuroscience 7, 1353–1359.

Ushikubi, F., Segi, E., Sugimoto, Y., Murata, T., Matsuoka, T., Kobayashi, T., Hizaki, H., Tuboi, K., Katsuyama, M., Ichikawa, A., Tanaka, T., Yoshida, N., Narumiya, S., 1998. Impaired febrile response in mice lacking the prostaglandin E receptor subtype EP_3. Nature 395, 281–284.

Vane, J.R., Bakhle, Y.S., Botting, R.M., 1998. Cyclooxygenases 1 and 2. Annual Review of Pharmacology and Toxicology 38, 97–120.

Watkins, L.R., Maier, S.F., 1999. Implications of immune-to-brain communication for sickness and pain. Proceedings of the National Academy of Sciences of the United States of America 96, 7710–7713.

Woolf, C.J., 1994. The dorsal horn: state-dependent sensory processing and the generation of pain. In: Wall, P.D., Melzack, R. (Eds.), Textbook of Pain, third ed. Churchill Livingstone, London, pp. 101–112.

Yaksh, T.L., Hua, X.Y., Kalcheva, I., Nozaki-Taguchi, N., Marsala, M., 1999. The spinal biology in humans and animals of pain states generated by persistent small afferent input. Proceedings of the National Academy of Sciences of the United States of America 96, 7680–7686.

Zacharowski, K., Olbrich, A., Piper, J., Hafner, G., Kondo, K., Thiemermann, C., 1999. Selective activation of the prostanoid EP_3 receptor reduces myocardial infarct size in rodents. Arteriosclerosis. Thrombosis and Vascular Biolology 19, 2141–2147.

Zimmermann, M., 1983. Ethical guidelines for investigations of experimental pain in conscious animals. Pain 16, 109–110.

BIOLOGY OF REPRODUCTION 69, 195–201 (2003)
Published online before print 5 March 2003.
DOI 10.1095/biolreprod.102.013870

Role of Prostaglandin H_2 Synthase 2 in Murine Parturition: Study on Ovariectomy-Induced Parturition in Prostaglandin F Receptor-Deficient Mice[1]

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ABSTRACT

To determine the prostaglandin (PG) H_2 synthase (generally referred to as cyclooxygenase [COX]) isozyme responsible for producing uterotonic PGs during parturition, we used $PGF_{2\alpha}$ receptor-deficient mice, which exhibit parturition failure due to impaired withdrawal of serum progesterone at term. On ovariectomy-induced parturition in these mice, uterine COX-2 mRNA expression was drastically induced in the myometrium, whereas COX-1 mRNA expression in the endometrial epithelium decreased. The concomitant administration of progesterone with ovariectomy resulted in a delay in parturition and the disappearance of both the increase in COX-2 mRNA and the decrease in COX-1 mRNA. Thus, the expression of myometrial COX-2 and the occurrence of parturition are closely associated in this model. Furthermore, administration of the COX-nonselective inhibitor, indomethacin, or the COX-2-selective inhibitor, Dup-697 or JTE-522, effectively delayed ovariectomy-induced parturition in these mice. These findings suggest that COX-2-derived PGs contribute to the onset of parturition after the decrease in serum progesterone level.

female reproductive tract, parturition, pregnancy, progesterone, uterus

INTRODUCTION

The molecular mechanism that determines the timing of parturition remains elusive despite intensive studies. Preterm parturition, resulting from the disruption of normal parturition signaling, is considered to be the major cause of neonatal mortality. Therefore, coming to an understanding of the molecular events that trigger parturition is essential. Prostaglandins (PGs) are involved in various mammalian reproductive processes, including the induction of parturition [1–4]. PGH_2 synthase, generally referred to as cyclooxygenase (COX), is the rate-limiting enzyme in the biosynthetic pathway of various PGs from arachidonic acid [5]. Aspirin-like drugs, which inhibit the enzymatic activities of the COX isozymes, have been known to cause a delay in parturition in many species, suggesting that PGs are important mediators of the onset of parturition [6]. Of the two isozymes of COX, COX-1 is mostly expressed constitutively, playing housekeeping roles in many tissues, whereas COX-2 is induced on various pathophysiological stimuli such as by cytokines [7].

In the induction of parturition of rodents, a decrease in serum progesterone levels precedes uterine contraction. We previously reported that $PGF_{2\alpha}$ receptor-deficient ($FP^{-/-}$) mice exhibit a phenotype of loss of parturition due to impaired luteolysis and persistently high serum progesterone levels at term [8]. Gross et al. [9] reported that COX-1-deficient mice exhibit similar parturition defects with impaired luteolysis. These studies together indicate that COX-1-derived $PGF_{2\alpha}$ is essential for luteolysis, which is a trigger for the induction of murine parturition. On the other hand, along with luteolytic action of $PGF_{2\alpha}$, PGE_2 and $PGF_{2\alpha}$ have been reported to have potent uterotonic activities in the periparturient uterus [10], suggesting that these PGs may contribute to the parturition process by directly contracting myometrial smooth muscle after the decrease in serum progesterone levels. However, it remains unclear as to which COX isozyme is responsible for producing these uterotonic PGs. We have previously reported that the uterine expression of COX-2 mRNA is induced and the expression of COX-1 mRNA is decreased during parturition in wild-type mice, suggesting the involvement of COX-2 in producing uterotonic PGs during parturition [11]. A significant amount of COX-1 is expressed in intrauterine tissues at term, and the role of COX-2 has been obscured by the observation that a COX-2-selective inhibitor delayed murine parturition only at high doses [12]. COX-2-deficient mice have not been used for the analysis of parturition due to their infertility [13].

To determine the isozyme responsible for producing uterotonic PGs during parturition, we used an ovariectomy-induced parturition model in $FP^{-/-}$ mice. As stated herein, $FP^{-/-}$ mice do not undergo parturition due to persistently high serum progesterone levels. Ovariectomy on the day before the expected term date is able to induce both successful parturition and uterine COX-2 mRNA expression at 20 h after the treatment [11]. In the present study, we show that the expression of COX-2 mRNA in the myometrium is closely correlated with the occurrence of parturition, since myometrial COX-2 mRNA expression at 20 h after ovariectomy greatly decreased when parturition was delayed by treatment with progesterone. Furthermore, both COX-nonselective and COX-2-selective inhibitors effectively delayed ovariectomy-induced parturition in $FP^{-/-}$ mice. Taken together, we propose that COX-2 is the major isozyme responsible for the production of PGs, contributing to the onset of parturition downstream of the decrease in serum progesterone level, presumably via their uterotonic activities.

[1]This work was supported in part by the grants from the Mochida Memorial Foundation for Medical and Pharmaceutical Research; the Sankyo Foundation of Life Science, the Takeda Science Foundation, and Grants-in-aid for Scientific Research from the Ministry of Education, Culture, Sports Science and Technology of Japan.
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Received: 27 November 2002.
First decision: 28 December 2002.
Accepted: 26 February 2003.

ISSN: 0006-3363. http://www.biolreprod.org

MATERIALS AND METHODS

Animal and Tissue Preparations

Female FP$^{-/-}$ mice, with the chimeric background (129/Ola × C57BL/6), were maintained at 23°C under a 12L:12D cycle [8]. To obtain timed-pregnant mice, FP$^{-/-}$ virgin female mice (8 to 12 wk of age) were housed overnight with C57BL/6 males and checked the following morning for vaginal plugs. The day when a vaginal plug was observed was counted as Day 1 of pregnancy. FP$^{-/-}$ mice were anesthetized by ether and bilaterally ovariectomized at 2130–2230 h on Day 19 of pregnancy as previously described [8, 11]. Because not only progesterone withdrawal but also an increase in estradiol level appear necessary for induction of parturition, we investigated the effect of progesterone or estradiol on the ovariectomy-induced parturition and COX gene expression, and we also added both steroid-administrated groups to examine possible interactions of each effect. FP$^{-/-}$ mice were treated subcutaneously with vehicle (0.1 ml of sesame oil), progesterone (1 mg; Research Biochemicals International, Natick, MA), estradiol (17β-estradiol, 250 ng; Research Biochemicals International), or both estradiol and progesterone immediately after the ovariectomy. First, the time of onset of parturition after ovariectomy was examined by infrared videorecording. The onset of parturition was defined as the time of complete delivery of the first pup in each mother. Second, uterine horns were isolated 20 h after ovariectomy and subjected to Northern blot analysis or in situ hybridization analysis. Control animals received a sham operation followed by vehicle treatment. For the groups administered with COX inhibitors, animals were treated subcutaneously with vehicle (0.1 ml of 3.5% dimethyl sulfoxide in sesame oil), the COX-nonselective inhibitor, indomethacin (5 mg/kg; Sigma, St. Louis, MO), or COX-2-specific inhibitors, Dup-697 (5 mg/kg; Cayman Chemical, Ann Arbor, MI) [14] or JTE-522 (15 mg/kg; 4-[4-cyclohexyl-2-methyloxazol-5-yl]2-fluorobenzenesulphonamide; a gift from Japan Tobacco Inc., Osaka, Japan) [15] at 12 h after ovariectomy. Thereafter, the onset of parturition was monitored by infrared videorecording. All mouse breeding and experiments were performed according to the guideline for animal experiments of Kyoto University.

Northern Blot Analysis

Uterine horns were dissected, freed from the conspectuses and placentas, immediately frozen in liquid N_2, and stored at −80°C until use. Total RNA was extracted from both uterine horns derived from one animal by the acid guanidinium thiocyanate-phenol-chloroform method [16]. Total RNA (15 μg) was separated by electrophoresis on a 1.5% agarose gel and transferred onto a nylon membrane (Biodyne-A, Pall, Port Washington, NY). Hybridizations were performed with ^{32}P-labeled cDNA fragments specific for COX-1 [17], COX-2 [17], and connexin-43 (Cx-43) (1.2-kilobase fragment corresponding to the entire coding region) [18] at 65°C in 6 × SSC (1 × SSC is composed of 0.15 M NaCl and 0.015 M sodium citrate), 0.5% SDS, and 5 × Denhardt solution. After hybridization, filters were washed at 65°C in 0.5 × SSC and 0.25% SDS, and the hybrids were detected by autoradiography. The filters were then rehybridized with a ^{32}P-labeled cDNA fragment specific for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Clontech, Palo Alto, CA). Autoradiograms were subjected to densitometric analyses for quantification of COX-1, COX-2, or Cx-43 mRNA levels relative to GAPDH mRNA levels using the National Institutes of Health Image software. For each group of tissues, three animals were analyzed and data were expressed as mean ± SEM.

In Situ Hybridization

In situ hybridization was performed as described previously [11, 19]. Uterine horns were dissected, freed from the fetuses and placentas, and immediately frozen. Sections 10 μm in thickness were cut on a Jung Frigocut 3000E cryostat (Leica Instruments, Nussloch, Germany) and thaw mounted onto poly-L-lysine-coated glass slides. Mouse cDNAs for COX-1 and COX-2 [17] were prepared in the pBluescript II vector (Stratagene, La Jolla, CA), and antisense or sense riboprobes specific for COX-1 and COX-2 were synthesized by transcription with T3 or T7 RNA polymerase (Stratagene) in the presence of [α-^{35}S]CTP. The sections were fixed with 4% formalin and acetylated with 0.25% acetic anhydride. Hybridization was performed in a buffer containing 50% formamide, 2 × SSC, 10 mM tris(hydroxymethyl)aminomethane-Cl (pH 7.5), 1 × Denhardt solution, 10% dextran sulfate, 0.2% SDS, 100 mM dithiothreitol, 500 μg/ml of sheared single-stranded salmon sperm DNA, and 250 μg/ml of yeast tRNA. The antisense riboprobes were added to the hybridization buffer at 1.5 × 10^5 cpm/μl. After incubation at 60°C for 5 h, the slides were washed for 1 h in 2 × SSC. The sections were treated with 20 μg/ml of ribonuclease A, followed by an additional wash in 0.1 × SSC at 60°C for 1 h. The slides were then dipped in nuclear track emulsion (NTB3, Eastman Kodak, Rochester, NY). After exposure for 1.5 (for COX-1) or 4 (for COX-2) wk at 4°C, the dipped slides were developed, fixed, and counterstained with hematoxylin-eosin. These experiments were repeated two or three times with different animals and similar results were obtained.

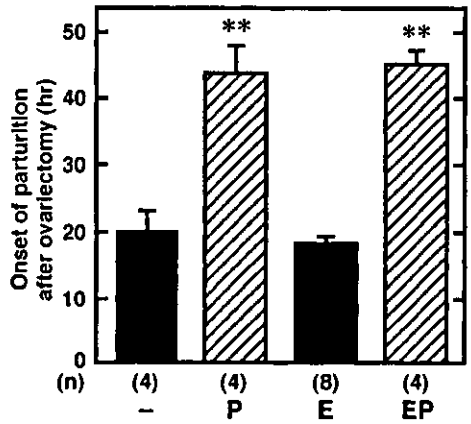

FIG. 1. Effects of the treatment with ovarian steroids on the onset of parturition after ovariectomy in FP$^{-/-}$ mice. FP$^{-/-}$ mice on Day 19 of pregnancy were ovariectomized and immediately treated with vehicle (–), progesterone (P), estradiol (E), or both estradiol and progesterone (EP). The time of onset of parturition after ovariectomy was examined. Data are expressed as mean ± SEM. The number of mice used in each group is indicated in parentheses. **$P < 0.01$ vs. vehicle.

Statistical Analysis

For the statistical analyses of the time of onset of parturition after ovariectomy and the quantified data of Northern blot analyses, one-way ANOVA followed by the Student or Welch t-test were used to evaluate differences among individual groups. Some statistical analyses were performed after logarithmic transformations of the data when they were appropriate. Values were considered statistically significant at $P < 0.05$.

RESULTS

Effect of Steroid Treatment on the Onset of Parturition after Ovariectomy at Term in FP$^{-/-}$ Mice

We examined the effects of progesterone and/or estradiol treatments of FP$^{-/-}$ mice on the timing of ovariectomy-induced parturition by an infrared videorecording system. FP$^{-/-}$ mice treated with vehicle exhibited parturition at 19.9 ± 2.3 h (n = 4) after ovariectomy on the evening of Day 20 (1800 h on the average). On the other hand, the onset of parturition was delayed to 43.6 ± 4.2 h (n = 4) after ovariectomy in the progesterone-treated mice on the evening of Day 21 (1800 h on the average) (Fig. 1). However, treatment of FP$^{-/-}$ mice with estradiol had no effect on the onset of parturition either in the presence or absence of progesterone treatment. These results demonstrated that progesterone but not estradiol has a retardant effect on the onset timing of parturition in this model.

Expression of COX-1 and COX-2 mRNAs after Ovariectomy at Term in FP$^{-/-}$ Mice

To assess possible contribution of COX isozymes to the onset of ovariectomy-induced parturition, we examined uterine expression levels of mRNAs for COX-1, COX-2, and Cx-43, a myometrial major gap junction protein, at 20 h after each treatment by Northern blot analyses (Fig. 2). At this point, vehicle- or estradiol-treated mice underwent parturition but other groups did not (Fig. 1). In FP$^{-/-}$ mice,

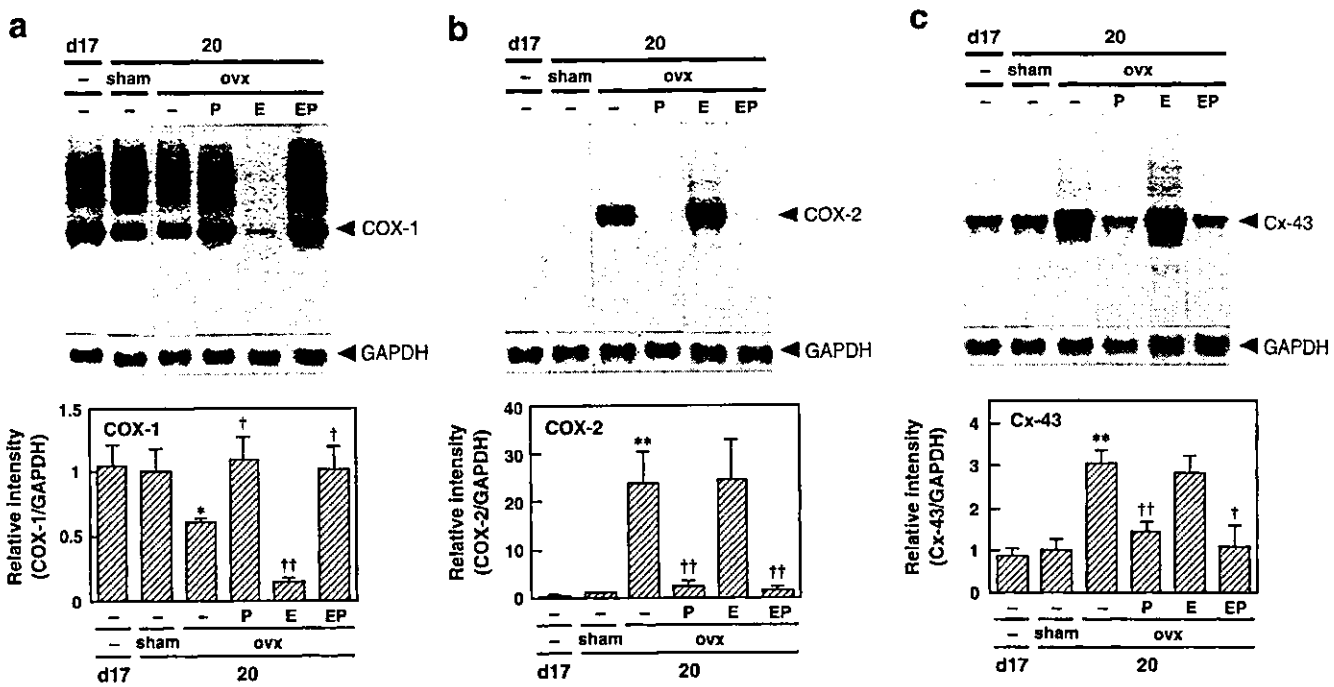

FIG. 2. Effect of ovariectomy and ovarian steroid treatment on uterine expression of COX-1 (a), COX-2 (b), and Cx-43 (c) mRNAs in late pregnant $FP^{-/-}$ mice. $FP^{-/-}$ mice were ovariectomized bilaterally (ovx) or sham operated (sham) on Day 19 of pregnancy and were treated with vehicle (–), progesterone (P), estradiol (E), or both estradiol and progesterone (EP). Uterine horns were collected 20 h after the treatment and subjected to Northern blot analysis (20). Uterine horns of $FP^{-/-}$ mice on Day 17 of pregnancy without treatment were also subjected to Northern blot analysis (d 17). The positions of the major bands are indicated by arrowheads. The same blots were rehybridized with a ^{32}P-labeled cDNA probe for GAPDH. The lower panels show quantified and normalized COX-1, COX-2, and Cx-43 mRNA levels relative to GAPDH mRNA levels (mean ± SEM; n = 3). Values were expressed as the fold of the level of sham-operated mice treated with vehicle. $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. sham-operated mice treated with vehicle. $^{\dagger}P < 0.05$ and $^{\dagger\dagger}P < 0.01$ vs. ovariectomized mice treated with vehicle.

the levels of uterine COX-1 mRNA expression were persistent during late pregnancy, as reported previously [11]. Ovariectomy on Day 19 resulted in a significant decrease in COX-1 mRNA levels compared with the levels on sham treatment and on Day 17. Estradiol treatment enhanced the decrease in COX-1 mRNA expression. However, the inhibition of ovariectomy-elicited parturition by progesterone or both steroid treatments reversed the COX-1 gene expression to the levels on sham treatment. In contrast to COX-1, ovariectomy led to highly induced uterine COX-2 mRNA levels (on Day 20, 23.8- and 43.3-fold of the levels on sham treatment, and on Day 17, respectively; $P < 0.01$ for both), accompanied with the induction of parturition. The expression of uterine COX-2 mRNA was down-regulated to faint levels, when the parturition was inhibited by progesterone treatment. Estradiol treatment had no effect on uterine COX-2 mRNA levels in either the presence or absence of progesterone treatment. The change in expression levels for Cx-43 mRNA was similar to but showed more modest changes than that for COX-2 mRNA on treatment with ovarian steroids. Thus, the expression of uterine COX-2 mRNA and the occurrence of parturition were closely associated.

Cellular Localization of COX-1 and COX-2 mRNAs after Ovariectomy at Term in $FP^{-/-}$ Mice

We next examined the distribution of COX-1 and COX-2 mRNAs in uterine tissues at 20 h after ovariectomy and administration of ovarian steroids in $FP^{-/-}$ mice (Figs. 3 and 4). In situ hybridization analyses revealed a marked spatial separation between COX-1 and COX-2 mRNAs in these tissues. Signals for COX-1 mRNA were found in the endometrial epithelium in vehicle-, progesterone-, and both estradiol- and progesterone-treated mice (Fig. 3b, c, and e). Consistent with the results of the Northern blot analysis, COX-1 signals were weak in estradiol-treated mice (Fig. 3d). In contrast to COX-1 signals, hybridization signals for COX-2 mRNA were observed in circular myometrium in vehicle-treated mice (Fig. 4b). However, consistent with the results of the Northern blot analysis, these signals disappeared when parturition was inhibited by progesterone treatment (Fig. 4c). Estradiol treatment had no effect on the distribution and signal intensity of COX-2 mRNA in the absence of progesterone treatment (Fig. 4d) and did not induce COX-2 mRNA signals in the presence of progesterone treatment (Fig. 4e). Hybridization signals were abolished when an excess amount of unlabeled probe was added (Figs. 3f and 4f, for controls of Figs. 3b and 4b, respectively; not shown for others) or the sense probe was hybridized (data not shown).

Delaying Effects of COX-Nonselective or COX-2-Selective Inhibitors on the Onset of Parturition after Ovariectomy in $FP^{-/-}$ Mice

To determine whether COX-2-derived PGs have a role in the onset of parturition after ovariectomy in $FP^{-/-}$ mice, animals were treated with COX-nonselective or COX-2-selective inhibitors at term after ovariectomy, and the onset of parturition was monitored. The time to delivery after ovariectomy was significantly prolonged by treatment with the COX-nonselective inhibitor, indomethacin, or the COX-2-selective inhibitor, Dup-697 or JTE-522 (Fig. 5), suggesting a role of COX-2-derived PGs in the onset of parturition in this system.

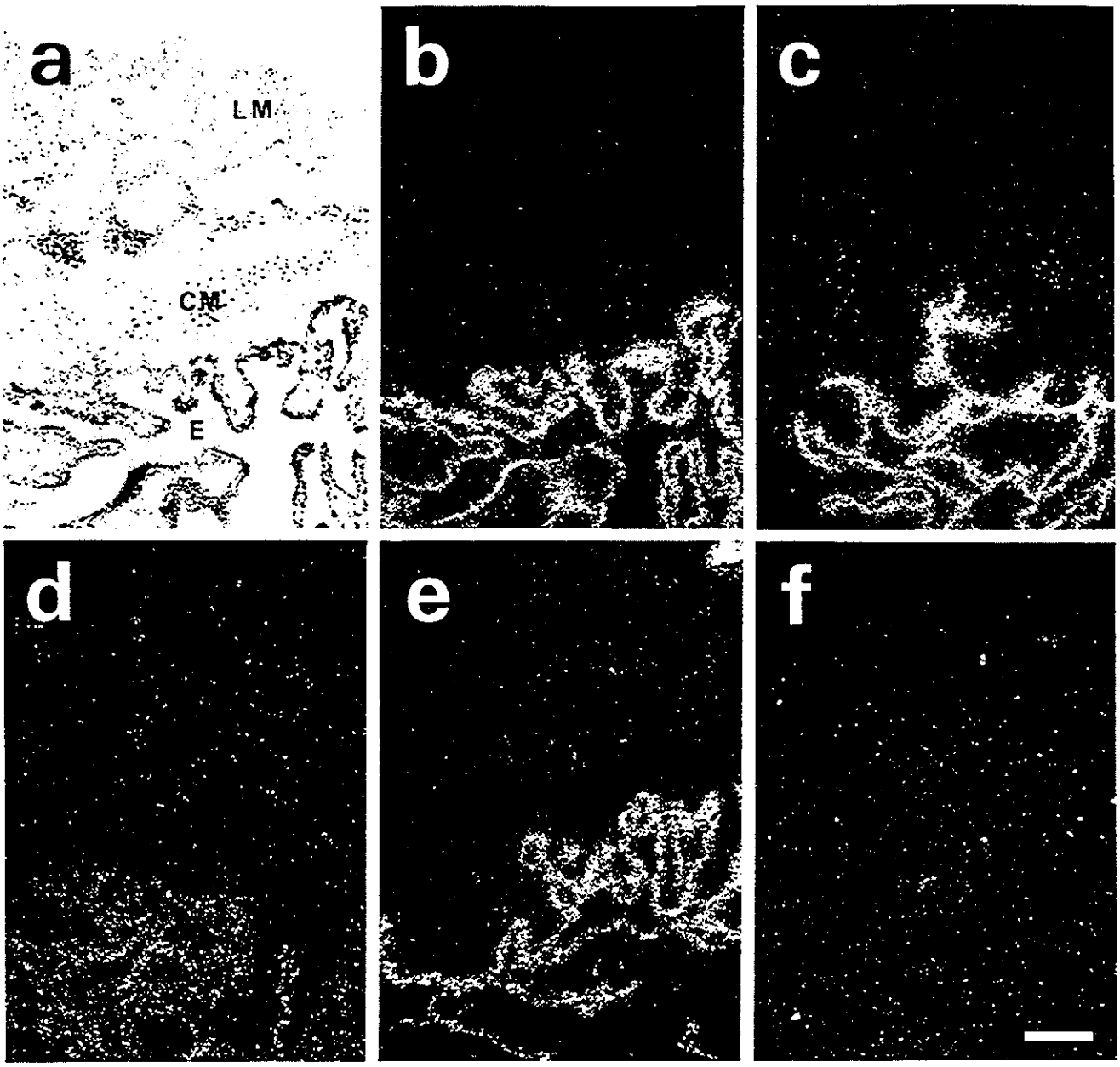

FIG. 3. Bright-field (a) and dark-field (b–f) photomicrographs showing hybridization signals for COX-1 mRNA in uterine tissues after ovariectomy and administration of ovarian steroids in late pregnant $FP^{-/-}$ mice. $FP^{-/-}$ mice on Day 19 of pregnancy were ovariectomized and treated with vehicle (a, b, f), progesterone (c), estradiol (d), or both estradiol and progesterone (e). Uterine horns were collected at 20 h after the treatment, and sections were subjected to in situ hybridization analyses. The specificity of hybridization signals was verified by disappearance of the signals in (b) in the presence of an excess amount of unlabeled probe (f) or by absence of the signals with the sense probe (data not shown). LM, Longitudinal smooth muscle layer; CM, circular smooth muscle layer; E, endometrial epithelium. Bar = 150 μm.

DISCUSSION

The principal finding in the present study is that induction of the COX-2 gene in the myometrium is closely associated with the onset of ovariectomy-induced parturition in $FP^{-/-}$ mice, whereas expression of the COX-1 gene in the uterus is down-regulated at that time. This observation supports the important role of COX-2, which is induced after the decline of serum progesterone levels in the signaling pathway of murine parturition. Indeed, COX-2-selective inhibitors were able to delay ovariectomy-induced parturition in $FP^{-/-}$ mice. Reese et al. [12] recently proposed that COX-2 may not be involved in mouse parturition because its mRNA is undetectable in the myometrium on the morning of Day 19 of pregnancy. Although we have a similar result that shows the absence of the COX-2 mRNA in the myometrium of wild-type mice on the morning of Day 19, strong signals for COX-2 mRNA were detected in the myometrium during both natural parturition on Day 20 in wild-type mice and ovariectomy-induced parturition in $FP^{-/-}$ mice, occurring an average of 20 h after operation. Indeed, the expression of COX-2 mRNA could be induced 16 h but not 12 h after ovariectomy in $FP^{-/-}$ mice [11]. Thus, induction of COX-2 gene in the myometrium presumably starts only a few hours before the onset

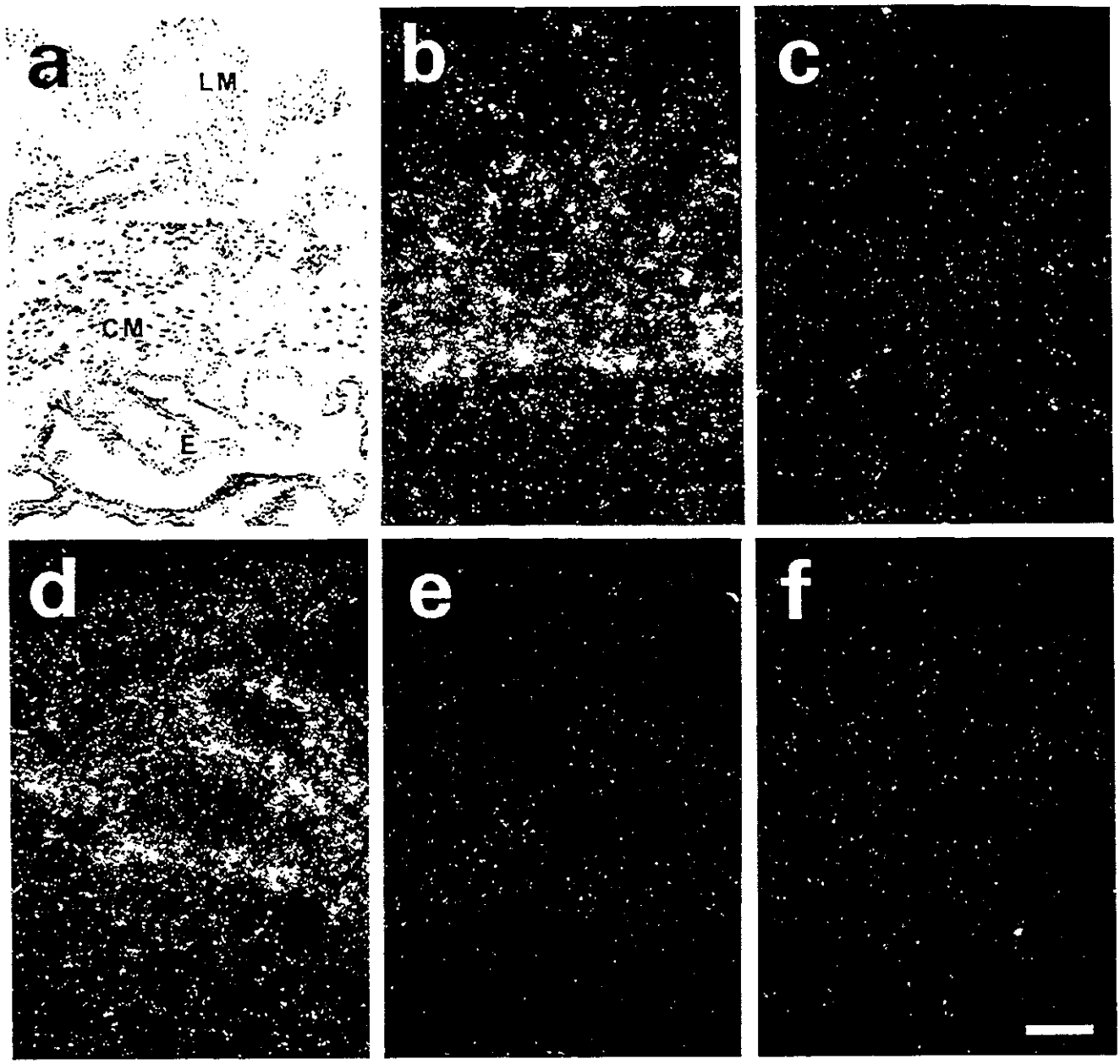

FIG. 4. Bright-field (a) and dark-field (b–f) photomicrographs showing hybridization signals for COX-2 mRNA in uterine tissues after ovariectomy and administration of ovarian steroids in late pregnant $FP^{-/-}$ mice. $FP^{-/-}$ mice on Day 19 of pregnancy were ovariectomized and treated with vehicle (a, b, f), progesterone (c), estradiol (d), or both estradiol and progesterone (e). Uterine horns were collected at 20 h after the treatment, and sections were subjected to in situ hybridization analyses. The specificity of hybridization signals was verified by disappearance of the signals in (b) in the presence of an excess amount of unlabeled probe (f) or by absence of the signals with the sense probe (data not shown). LM, Longitudinal smooth muscle layer; CM, circular smooth muscle layer; E, endometrial epithelium. Bar = 150 μm.

of parturition. Based on these results, we concluded that COX-2-derived PGs other than $PGF_{2\alpha}$ contribute to the onset of parturition after the decrease in circulating progesterone levels.

The role of COX-2 in preterm parturition has been proposed by earlier studies. Specifically, COX-2 mRNA has been induced in the uterus on lipopolysaccharide- or ethanol-induced preterm parturition [20, 21]. Furthermore, the pharmacological inhibition of COX-2 but not the genetic ablation of COX-1 delayed lipopolysaccharide-induced preterm parturition [20, 22]. Thus, COX-2 may have an important role in preterm parturition and in our ovariectomy-induced term parturition in $FP^{-/-}$ mice. On the other hand, Reese et al. [12] reported that a COX-2-selective inhibitor did not significantly delay murine spontaneous term parturition, except at high doses that may also inhibit COX-1. They thus suggested that COX-2 does not play a primary role in the signaling pathway of parturition, which is inconsistent with our results. This discrepancy may have arisen from the higher sensitivity of our system for detecting the tocolytic effects of the COX-2-selective inhibitor than by the analysis of spontaneous parturition. The lower variability in the time of ovariectomy-induced parturition on vehicle treatment in our system than that seen with spontaneous parturition potentiates the sensitivity for detecting the tocolytic effects and thus

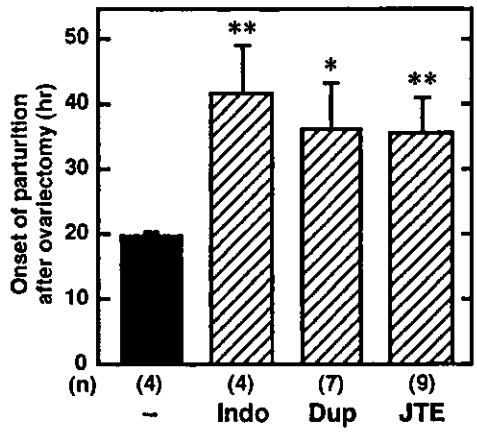

FIG. 5. Delaying effects of COX-nonselective and COX-2-selective inhibitors on the onset of parturition after ovariectomy in $FP^{-/-}$ mice. $FP^{-/-}$ mice were ovariectomized on Day 19 of pregnancy. At 12 h after ovariectomy, animals were treated with vehicle (-), the COX-nonselective inhibitor, indomethacin (Indo), or the COX-2-selective inhibitors, Dup-697 (Dup) or JTE-522 (JTE). The time of onset of parturition after ovariectomy was examined. Data are expressed as mean ± SEM. The number of mice used in each group is indicated in the parentheses. $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. vehicle, respectively.

should be useful for studies such as this that investigate potential tocolytic treatments.

Although COX inhibitors are an effective treatment for preterm parturition in humans, their use is limited by the adverse effects of inducing fetal ductus arteriosus (DA) closure in utero [23]. It has been reported that 100% of mice lacking both COX-1 and COX-2 died within 12 h of birth due to an abnormality in the DA, whereas the mortality rate was only 35% in the COX-2-deficient mice [24]. This result, together with our studies, suggests that the use of COX-2-selective inhibitors should provide a therapeutic advance toward the treatment of preterm parturition with fewer adverse effects than COX-nonselective inhibitors if the extent of the roles of each COX isozyme on DA are conserved across animal species. Indeed, the COX-2 inhibitor has been reported to possibly prevent human preterm delivery [25]. However, caution is needed on using COX-2 inhibitors for the treatment of preterm labor due to the remaining risk on the DA and the reported adverse effects on the fetal kidney [26].

One of the intriguing findings in this study is that administration of progesterone was sufficient to completely suppress uterine COX-2 mRNA induced by ovariectomy in $FP^{-/-}$ mice. Therefore, periparturient withdrawal of circulatory progesterone may be crucial for the induction of uterine COX-2 mRNA in wild-type and ovariectomized $FP^{-/-}$ mice. In contrast, administration of estradiol failed to show any effects on ovariectomy-induced and progesterone-suppressed COX-2 expression, as observed in the timing of parturition. These results may reflect that the controls of gene expression by progesterone predominate those by estradiol during late pregnancy. Indeed, in the present study, COX-2 showed a similar expression pattern to that of Cx-43, a proposed important gap junctional protein during parturition, which was demonstrated to be induced by progesterone withdrawal in the periparturient uterus [27]. The negative regulation of COX-2 mRNA by progesterone in vivo has also been suggested by Critchley et al. [28], who reported that the cessation of progesterone administration elevates COX-2 mRNA expression in the nonpregnant human uterus. However, progesterone did not decrease COX-2 mRNA levels in bovine myometrial or epithelial cells in vitro [29, 30]. These differences suggest that the negative regulation of COX-2 mRNA by progesterone is by an indirect fashion and may require some paracrine factors.

The result that COX-2 localizes in the myometrium during parturition, which is mostly consistent with previous findings [31], may be of help in considering the possible contribution of immune cells and paracrine factors in the negative regulation of COX-2 gene expression by progesterone. Mackler et al. [32, 33] reported that macrophages are accumulated in murine myometrium, especially stroma surrounding muscle bundles in late pregnancy. Intrauterine macrophages are known to produce interleukin-1β (IL-1β), which is locally acting on neighboring cells, but this cytokine production is inhibited by progesterone [34–36]. Furthermore, like IL-1β, it was suggested that macrophage-derived chemokines, such as monocyte chemotactic protein 1 and RANTES (regulated on activation, normal T-cell expressed and secreted), are present within uterine tissues, and their expression was also suppressed by progesterone [37, 38]. Such suppression in paracrine factor production in macrophages may mediate the negative regulation of COX-2 gene expression by progesterone. Indeed, IL-1β has been shown to induce COX-2 expression at the transcriptional level in rat myometrial cells [39].

In our study, ovariectomy treatment decreased uterine COX-1 mRNA expression, which was restored by the administration of progesterone, suggesting that progesterone is necessary for elevated COX-1 mRNA expression during late pregnancy. This stimulatory effect of progesterone is consistent with other reports using nonpregnant animals. Specifically, antiprogestin treatment was found to abolish COX-1 mRNA expression in the baboon endometrial epithelium during the luteal phase of the menstrual cycle [40], and the administration of progesterone elicited a modest induction of uterine COX-1 mRNA expression in ovariectomized nonpregnant mice [41]. However, this stimulatory action of progesterone may have species-specific differences because the treatment of progesterone had no effect on uterine COX-1 in ovariectomized nonpregnant sheep [42]. In contrast to progesterone, the administration of estradiol greatly decreased uterine COX-1 mRNA in ovariectomized $FP^{-/-}$ mice. We previously reported that uterine COX-1 mRNA decreases sharply during spontaneous parturition in wild-type mice, in which serum estradiol levels rise at term [11]. Thus, a rise in serum estradiol concentrations, in addition to the withdrawal of progesterone, is thought to be required for the rapid down-regulation of uterine COX-1 mRNA during spontaneous parturition. The inhibitory effect of estradiol on COX-1 mRNA is surprising because estradiol has been reported to increase COX-1 mRNA in the uterus of ovariectomized nonpregnant mice [41, 43]. It is unknown why this difference arises, but it may suggest that the regulation of COX-1 mRNA by estradiol is indirect and is mediated by other factors specific for each condition.

In summary, our results suggest that COX-2 is induced in the myometrium and COX-2-derived PGs other than $PGF_{2\alpha}$ play a pivotal role in the onset of parturition under the control of progesterone withdrawal. However, the exact mechanism by which COX-2-derived PGs contribute to the parturition process requires further investigation.

ACKNOWLEDGMENTS

We thank Drs. N. Eguchi and Y. Urade for their generous assistance with the infrared videorecording technique. We also thank Ms. H. A. Po-

piel for her careful reading of the manuscript and Ms. S. Terai for secretarial assistance.

REFERENCES

1. Goldberg VJ, Ramwell PW. Role of prostaglandins in reproduction. Physiol Rev 1975; 55:325–351.
2. Challis JRG, Lye SJ. Parturition. In: Knobil E, Neill JD (eds.), The Physiology of Reproduction, vol. 2, 2nd ed. New York: Raven Press; 1994: 985–1031.
3. Soloff MS. Endocrine control of parturition. In: Wynn RM, Jollie WP (eds.), Biology of the Uterus, 2nd ed. New York: Plenum Medical; 1989: 559–607.
4. Thorburn GD, Challis JR. Endocrine control of parturition. Physiol Rev 1979; 59:863–918.
5. DeWitt DL. Prostaglandin endoperoxide synthase: regulation of enzyme expression. Biochim Biophys Acta 1991; 1083:121–134.
6. Chester R, Dukes M, Slater SR, Walpole AL. Delay of parturition in the rat by anti-inflammatory agents which inhibit the biosynthesis of prostaglandins. Nature 1972; 240:37–38.
7. Dubois RN, Abramson SB, Crofford L, Gupta RA, Simon LS, Van De Putte LBA, Lipsky PE. Cyclooxygenase in biology and disease. FASEB J 1998; 12:1063–1073.
8. Sugimoto Y, Yamasaki A, Segi E, Tsuboi K, Aze Y, Nishimura T, Oida H, Yoshida N, Tanaka T, Katsuyama M, Hasumoto K, Murata T, Hirata M, Ushikubi F, Negishi M, Ichikawa A, Narumiya S. Failure of parturition in mice lacking the prostaglandin F receptor. Science 1997; 277:681–683.
9. Gross GA, Imamura T, Luedke C, Vogt SK, Olson LM, Nelson DM, Sadovsky Y, Muglia LJ. Opposing actions of prostaglandins and oxytocin determine the onset of murine labor. Proc Natl Acad Sci U S A 1998; 95:11875–11879.
10. Vane JR, Williams KI. The contribution of prostaglandin production to contractions of the isolated uterus of the rat. Br J Pharmacol 1973; 48:629–639.
11. Tsuboi K, Sugimoto Y, Iwane A, Yamamoto K, Yamamoto S, Ichikawa A. Uterine expression of prostaglandin H_2 synthase in late pregnancy and during parturition in prostaglandin F receptor-deficient mice. Endocrinology 2000; 141:315–324.
12. Reese J, Paria BC, Brown N, Zhao X, Morrow JD, Dey SK. Coordinated regulation of fetal and maternal prostaglandins directs successful birth and postnatal adaptation in the mouse. Proc Natl Acad Sci U S A 2000; 97:9759–9764.
13. Lim H, Paria BC, Das SK, Dinchuk JE, Langenbach R, Trzaskos JM, Dey SK. Multiple female reproductive failures in cyclooxygenase 2-deficient mice. Cell 1997; 91:197–208.
14. Gans KR, Galbraith W, Roman RJ, Haber SB, Kerr JS, Schmidt WK, Smith C, Hewes WE, Ackerman NR. Anti-inflammatory and safety profile of DuP 697, a novel orally effective prostaglandin synthesis inhibitor. J Pharmacol Exp Ther 1990; 254:180–187.
15. Matsushita M, Masaki M, Yagi Y, Tanaka T, Wakitani K. Pharmacological profile of JTE-522, a novel prostaglandin H synthase-2 inhibitor, in rats. Inflamm Res 1997; 46:461–466.
16. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 1987; 162:156–159.
17. Takahashi Y, Taketani Y, Endo T, Yamamoto S, Kumegawa M. Studies on the induction of cyclooxygenase isozymes by various prostaglandins in mouse osteoblastic cell line with reference to signal transduction pathways. Biochim Biophys Acta 1994; 1212:217–224.
18. Beyer EC, Steinberg TH. Evidence that the gap junction protein connexin-43 is the ATP-induced pore of mouse macrophages. J Biol Chem 1991; 266:7971–7974.
19. Sugimoto Y, Namba T, Shigemoto R, Negishi M, Ichikawa A, Narumiya S. Distinct cellular localization of mRNAs for three subtypes of prostaglandin E receptor in kidney. Am J Physiol 1994; 266:F823–F828.
20. Gross G, Imamura T, Vogt SK, Wozniak DF, Nelson DM, Sadovsky Y, Muglia LJ. Inhibition of cyclooxygenase-2 prevents inflammation-mediated preterm labor in the mouse. Am J Physiol Regul Integr Comp Physiol 2000; 278:R1415–R1423.
21. Cook JL, Zaragoza DB, Sung DH, Olson DM. Expression of myometrial activation and stimulation genes in a mouse model of preterm labor: myometrial activation, stimulation, and preterm labor. Endocrinology 2000; 141:1718–1728.
22. Sakai M, Tanebe K, Sasaki Y, Momma K, Yoneda S, Saito S. Evaluation of the tocolytic effect of a selective cyclooxygenase-2 inhibitor in a mouse model of lipopolysaccharide-induced preterm delivery. Mol Hum Reprod 2001; 7:595–602.
23. Moise KJ Jr. Effect of advancing gestational age on the frequency of fetal ductal constriction in association with maternal indomethacin use. Am J Obstet Gynecol 1993; 168:1350–1353.
24. Loftin CD, Trivedi DB, Tiano HF, Clark JA, Lee CA, Epstein JA, Morham SG, Breyer MD, Nguyen M, Hawkins BM, Goulet JL, Smithies O, Koller BH, Langenbach R. Failure of ductus arteriosus closure and remodeling in neonatal mice deficient in cyclooxygenase-1 and cyclooxygenase-2. Proc Natl Acad Sci U S A 2001; 98:1059–1064.
25. Sawdy R, Slater D, Fisk N, Edmonds DK, Bennett P. Use of a cyclooxygenase type-2-selective non-steroidal anti-inflammatory agent to prevent preterm delivery. Lancet 1997; 350:265–266.
26. Peruzzi L, Gianoglio B, Porcellini MG, Coppo R. Neonatal end-stage renal failure associated with maternal ingestion of cyclo-oxygenase-type-1 selective inhibitor nimesulide as tocolytic. Lancet 1999; 354: 1615.
27. Petrocelli T, Lye SJ. Regulation of transcripts encoding the myometrial gap junction protein, connexin-43, by estrogen and progesterone. Endocrinology 1993; 133:284–290.
28. Critchley HOD, Jones RL, Lea RG, Drudy TA, Kelly RW, Williams ARW, Baird DT. Role of inflammatory mediators in human endometrium during progesterone withdrawal and early pregnancy. J Clin Endocrinol Metab 1999; 84:240–248.
29. Doualla-Bell F, Guay JM, Bourgoin S, Fortier MA. Prostaglandin G/H synthase (PGHS)-2 expression in bovine myometrium: influence of steroid hormones and PGHS inhibitors. Biol Reprod 1998; 59:1433–1438.
30. Xiao CW, Liu JM, Sirois J, Goff AK. Regulation of cyclooxygenase-2 and prostaglandin F synthase gene expression by steroid hormones and interferon-τ in bovine endometrial cells. Endocrinology 1998; 139:2293–2299.
31. Takanami-Ohnishi Y, Asada S, Tsunoda H, Fukamizu A, Goto K, Yoshikawa H, Kubo T, Sudo T, Kimura S, Kasuya Y. Possible involvement of p38 mitogen-activated protein kinase in decidual function in parturition. Biochem Biophys Res Commun 2001; 288:1155–1161.
32. Mackler AM, Iezza G, Akin MR, McMillan P, Yellon SM. Macrophage trafficking in the uterus and cervix precedes parturition in the mouse. Biol Reprod 1999; 61:879–883.
33. Mackler AM, Green LM, McMillan PJ, Yellon SM. Distribution and activation of uterine mononuclear phagocytes in peripartum endometrium and myometrium of the mouse. Biol Reprod. 2000; 62:1193–1200.
34. Polan ML, Daniele A, Kuo A. Gonadal steroids modulate human monocyte interleukin-1 (IL-1) activity. Fertil Steril 1988; 49:964–968.
35. Polan ML, Loukides J, Nelson P, Carding S, Diamond M, Walsh A, Bottomly K. Progesterone and estradiol modulate interleukin-1β messenger ribonucleic acid levels in cultured human peripheral monocytes. J Clin Endocrinol Metab 1989; 69:1200–1206.
36. De M, Sanford TH, Wood GW. Detection of interleukin-1, interleukin-6, and tumor necrosis factor-alpha in the uterus during the second half of pregnancy in the mouse. Endocrinology 1992; 131:14–20.
37. Wood GW, Hausmann EHS, Kanakaraj K. Expression and regulation of chemokine genes in the mouse uterus during pregnancy. Cytokine 1999; 11:1038–1045.
38. Zhao D, Lebovic DI, Taylor RN. Long-term progestin treatment inhibits RANTES (regulated on activation, normal T cell expressed and secreted) gene expression in human endometrial stromal cells. J Clin Endocrinol Metab 2002; 87:2514–2519.
39. Dong YL, Gangula PR, Fang L, Yallampalli C. Differential expression of cyclooxygenase-1 and -2 proteins in rat uterus and cervix during the estrous cycle, pregnancy, labor and in myometrial cells. Prostaglandins 1996; 52:13–34.
40. Kim JJ, Wang J, Bambra C, Das SK, Dey SK, Fazleabas AT. Expression of cyclooxygenase-1 and -2 in the baboon endometrium during the menstrual cycle and pregnancy. Endocrinology 1999; 140:2672–2678.
41. Chakraborty I, Das SK, Wang J, Dey SK. Developmental expression of the cyclo-oxygenase-1 and cyclo-oxygenase-2 genes in the peri-implantation mouse uterus and their differential regulation by the blastocyst and ovarian steroids. J Mol Endocrinol 1996; 16:107–122.
42. Wu WX, Ma XH, Zhang Q, Buchwalder L, Nathanielsz PW. Regulation of prostaglandin endoperoxide H synthase 1 and 2 by estradiol and progesterone in nonpregnant ovine myometrium and endometrium *in vivo*. Endocrinology 1997; 138:4005–4012.
43. Jun SS, Chen Z, Pace MC, Shaul PW. Estrogen upregulates cyclooxygenase-1 gene expression in ovine fetal pulmonary artery endothelium. J Clin Invest 1998; 102:176–183.

Identification and characterization of a novel progesterone receptor-binding element in the mouse prostaglandin E receptor subtype EP2 gene

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Abstract

***Background*: Gene expression of prostaglandin E receptor EP2 is induced in the luminal epithelium of the mouse uterus during peri-implantation period (day-5 of pseudopregnancy), suggesting the involvement of progesterone and its receptor (PR) in this expression. However it remains unclear whether PR affects EP2 gene expression through its binding.**

***Results*: We investigated transcriptional regulation of EP2 gene expression with reporter gene analysis using HeLa cells with or without expression of the PR. The 5′-flanking region (−3260 to −27, upstream of the translation initiation site) exhibited progesterone-induced promoter activation and basal promoter activity in the presence of PR. Using successive deletion analysis, we determined the six regulatory regions in the EP2 gene. Three regions were found to be involved in progesterone-induced promoter activation, whereas the other three regions were involved in basal promoter activity in the presence of PR. We identified a novel PR-binding sequence, 5′-G(G/A)CCGGA-3′, in the two basal promoter regions and Sp1- and Sp3-binding in the other basal promoter region.**

***Conclusions*: We identified a novel PR-binding sequence, which may be involved in the regulation of basal promoter activity in the EP2 gene.**

Introduction

Prostaglandin E_2 (PGE2) receptor subtype EP2 is coupled to Gs, and activation of the EP2 leads to an elevation of cAMP concentration, consistent with its ability to relax smooth muscle *in vivo* (Gardiner 1986; Regan *et al.* 1994). In addition, EP2 has been shown to be involved in various PGE2 actions, such as ovulation, implantation, and inflammation (Coleman *et al.* 1990; Sugimoto *et al.* 2000). Accumulating evidence suggests that EP2 mRNA is induced in a variety of tissues in response to various physiological and pathological stimuli (Katsuyama *et al.* 1998a; Hizaki *et al.* 1999; Ikegami *et al.* 2001; Sonoshita *et al.* 2001; Segi *et al.* 2003). Indeed, it has been demonstrated that EP2 mRNA is transiently expressed in the luminal epithelium of the mouse uterus only during the implantation period (day-5 of pseudopregnancy) (Katsuyama *et al.* 1997). This induction was accompanied by an increase in serum progesterone levels, suggesting the probable involvement of progesterone in the expression of the EP2 gene in the uterus (Lim & Dey 1997). Kennedy *et al.* (1983) have in fact reported that [^{3}H]PGE_2 binding to endometrium membranes is increased by progesterone treatment in ovariectomized rats. However, there has been no direct evidence showing that expression of the EP2 gene is transcriptionally regulated by progesterone.

The actions of progesterone are mediated through its binding to progesterone receptors (PRs) in the target cells. The PRs belong to the nuclear hormone receptor family, which serve as ligand-activated transcription factors. PRs consist of two isoforms, PR-A and PR-B, being generated by alternative promoter usage (Kastner *et al.* 1990). Both PR isoforms have the ability to recognize and bind to a common sequence, known as the progesterone responsive element (PRE) located in the gene promoters. To date, the sequence, AGAACANNNTGTTCT, is thought to be a typical PRE (Forman & Samuels 1990), but PR has also been shown to elicit enhancer activity through its binding to quite different sequences on promoter regions of several genes in

Communicated by: Masayuki M. Yamamoto
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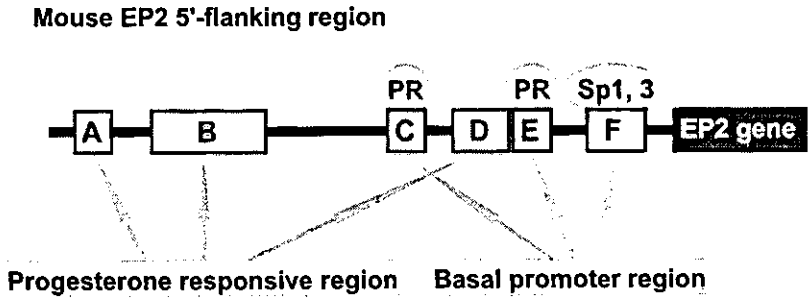


Summary Figure A schematic presentation of the regulatory regions in the mouse EP2 5′-flanking region.

progesterone-responsive cells (Lamian *et al.* 1993; Kepa *et al.* 1996). Therefore, to understand progesterone activity it is essential to analyse the sequences bearing basal or PR-inducible promoter activity.

We recently demonstrated that the sequence in the 5′-flanking region of the mouse EP2 gene contains no canonical consensus sequence for basal promoters such as the TATA-box and GC-box (Katsuyama *et al.* 1998b). Although one PRE-like region is present at −844 to −830, the involvement of this sequence in EP2 gene expression has not been addressed.

In the current study, we designed experiments for two main purposes as follows; (i) to identify which regions are responsible for progesterone-induced and basal promoter activities (ii) to identify which regions show direct PR-binding activity in the 5′-flanking region of the mouse EP2 gene. We identified a novel PR-binding sequence, which may be involved in the regulation of basal promoter activity.

Results

Uterine expression of EP2 mRNA in ovariectomized mice treated with progesterone or 17-β-oestradiol

Female mice were ovariectomized and maintained for a further two weeks to eliminate the influence of endogenous progesterone. A subcutaneous injection of 1 mg progesterone resulted in an increase of EP2 mRNA expression in the uterus, which started 6 h after injection and linearly increased for 24 h. On the other hand, the injection of 250 ng 17-β-oestradiol did not increase but rather slightly decreased EP2 mRNA expression (Fig. 1).

Progesterone-induced transcriptional activity of the 5′-flanking region of the EP2 gene

To determine whether the effect of progesterone is mediated by direct transactivation of the EP2 gene, we evaluated promoter activity of the 5′-flanking region of the EP2 gene (3.2 kbp, −3260 to −27) by reporter gene analysis in the presence of mouse progesterone receptors, PR-A and PR-B. Treatment with progesterone for 24 h markedly increased relative luciferase activity in the cells co-transfected with PR-B cDNA, and slightly in cells with PR-A cDNA (Fig. 2). In the absence of progesterone, the increase of basal luciferase activity in both PR-A- and PR-B-expressing cells was significant.

Identification of the progesterone responsive region in the 5′-flanking region of the EP2 gene

We previously demonstrated that the 5′-flanking region of the EP2 gene including the 3260 base pairs upstream of the translational initiation site, contains one putative PRE but no typical TATA-box, GC-box or initiator sequence (Katsuyama *et al.* 1998b). To determine the *cis*-regulatory elements for progesterone-induced expression of the EP2 gene, a series of 5′-deletion mutants fused to the luciferase gene as shown in Fig. 3 were constructed, and they were subjected to reporter gene analysis using HeLa cells co-transfected with mouse PR-B cDNA. As a result, we identified six regulatory elements, which were classified into regions A to F in the 5′-flanking region of the EP2 gene (Fig. 3). Successive deletion of three regions, −226 to −206 (region C), −146 to −126 (region E), and −117 to −76 (region F), leads to the reduction of promoter activity in the absence of progesterone (Fig. 3, inset), whereas deletion of the other three regions, −528 to −504 (region A), −472 to −409 (region B), and −186 to −176 (region D), leads to the reduction of promoter activity in the presence of progesterone. In HeLa cells co-transfected with empty vector or PR-A cDNA, the profile of basal promoter activity exhibited a similar pattern to that of the cells co-transfected with PR-B cDNA (data not shown). On the other hand, deletion of the region containing the putative PRE-like sequence did not induce any significant changes in either progesterone-induced or basal promoter activity. These results suggested that the regions C, E and F may be involved in basal promoter activity and the regions A, B

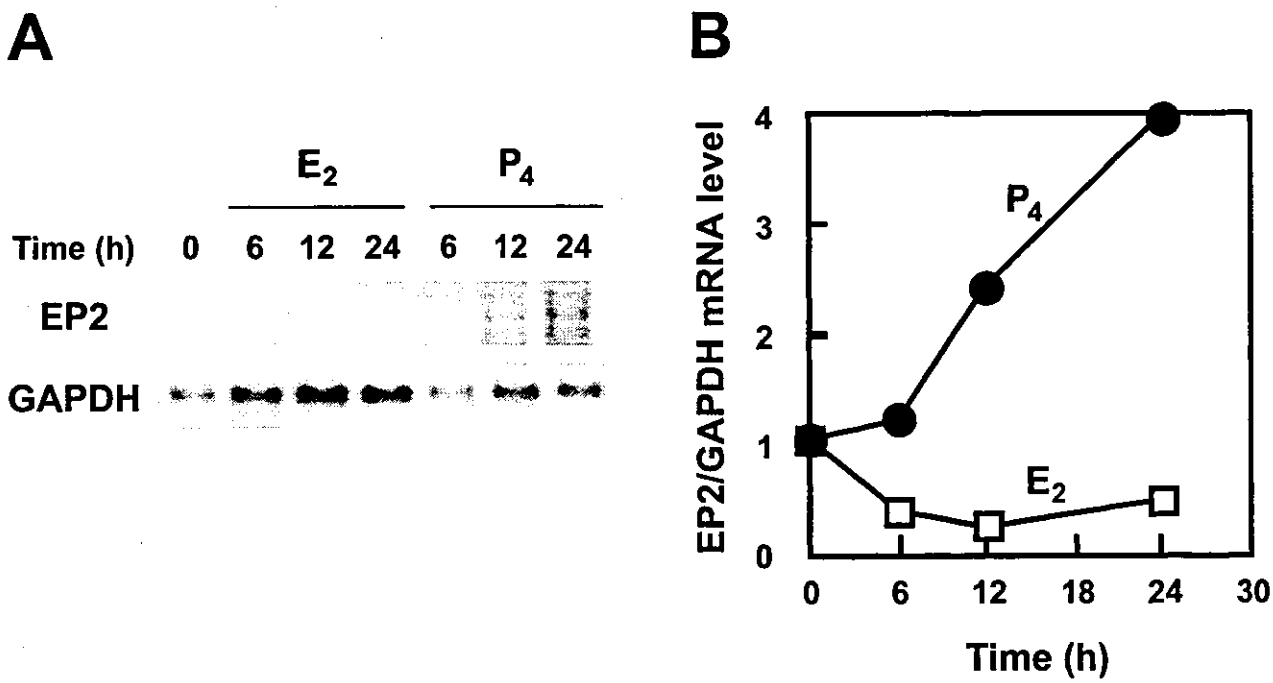


Figure 1 Expression of EP2 mRNA in the uteri of ovariectomized mice treated with progesterone and 17-β-oestradiol. (A) Female ddY mice (9 weeks of age) were ovariectomized and maintained for a further 2 weeks. The mice were subcutaneously treated with 17-β-oestradiol (E2, 250 ng/mouse) or progesterone (P4, 1 mg/mouse) for the indicated times. Control mice were treated with vehicle alone. Total RNA was extracted from each uteri of three mice and Northern blot analysis was performed using the ^{32}P-labelled specific probe for the mouse EP2 receptor, as described under Experimental procedures. The probe for GAPDH was used as a loading control. The data are a representative of three similar experiments. (B) Relative expression levels of EP2 mRNA obtained in (A) are shown (● P4, □ E2). The blots were subjected to densitometric analysis, and EP2 mRNA levels were normalized to GAPDH mRNA levels. The EP2/GAPDH values are represented as fold of the value at 0 h.

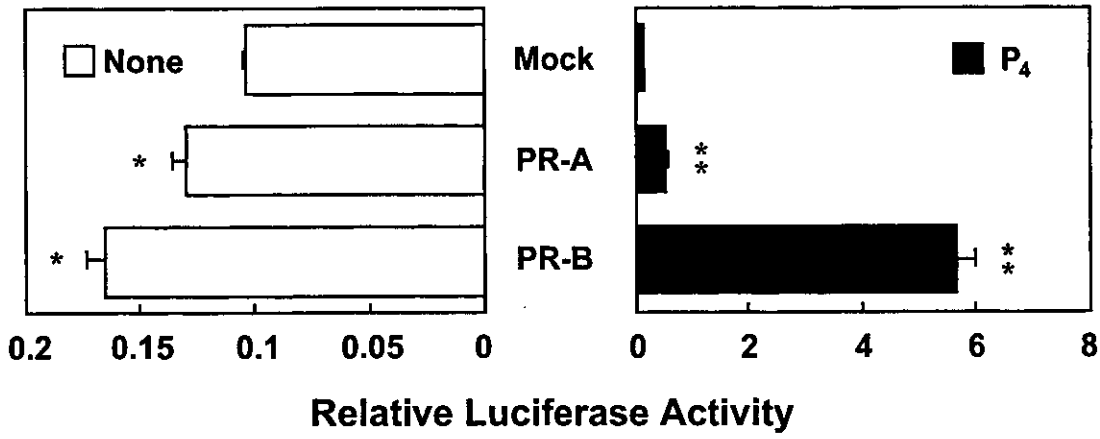


Figure 2 Progesterone-induced transcriptional activities of the 5′-flanking region of the mouse EP2 gene in the presence of mouse PR subtypes. HeLa cells were transiently co-transfected with pGL3/EP2 −3260/−27, pcDNA3/mPR-A or B, and pRL-TK. The transfected cells were treated with (P4, ■) or without 1 μM progesterone (None, □) for 24 h and then harvested. The cell extracts were assayed for *Photinus pyralis* luciferase activity to monitor the transcriptional activity as described in Experimental procedures. The efficiency of transfection was normalized by the assay for *Renilla reniformis* luciferase activity. Each value represents the mean ± SD (n = 3). *P < 0.001 compared with Mock/None, **P < 0.0001 compared with Mock/P_4. The data are a representative of three similar experiments.

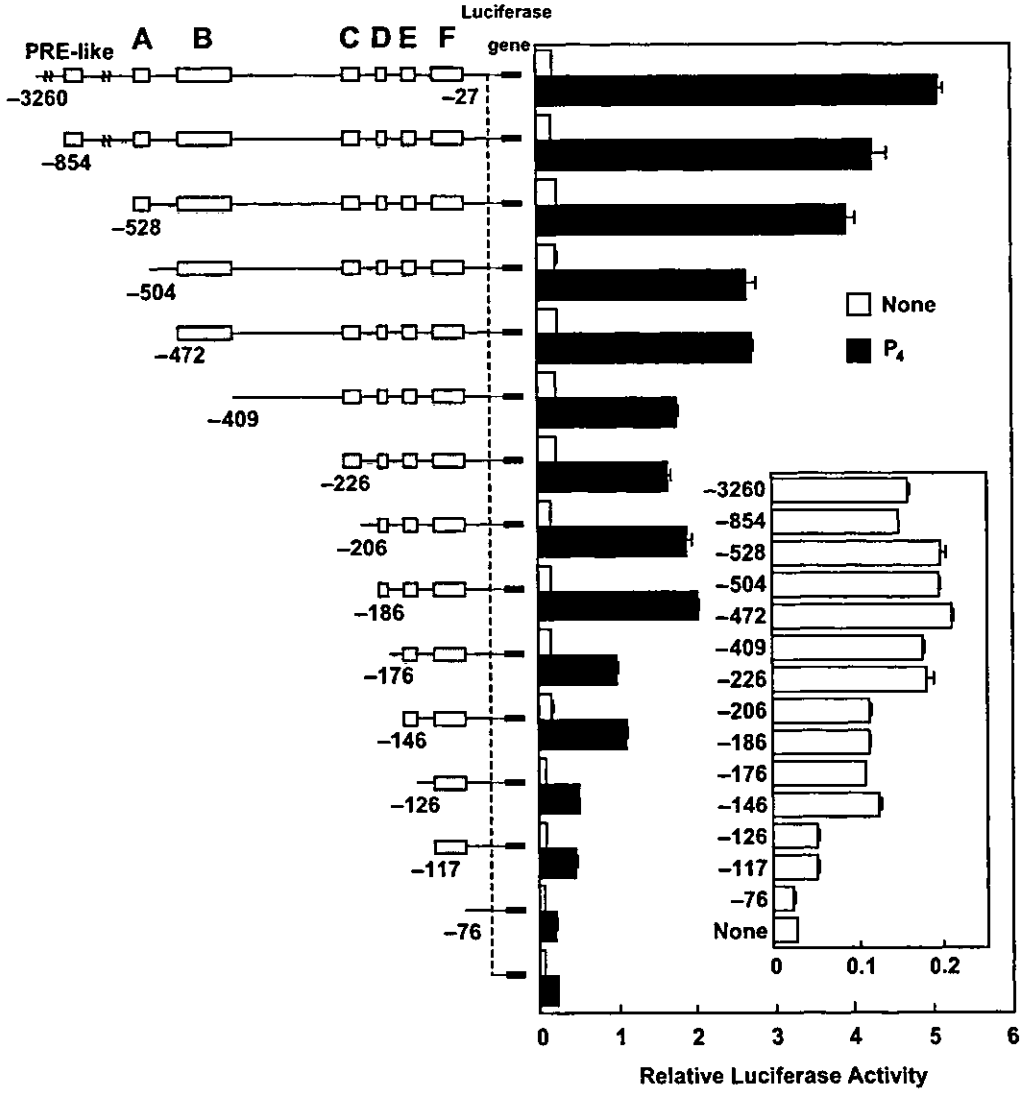


Figure 3 Progesterone-induced transcriptional activities of a series of 5′-deletion mutants of the mouse EP2 promoter region in the presence of mouse PR-B. HeLa cells were transiently co-transfected with pcDNA3/PR-B and a series of 5′-deletion mutants of pGL3/EP2 as indicated on the left. The cells were treated for 24 h with (P4, ■) or without 1 μM progesterone (None, □). Luciferase activity was measured to monitor promoter activity of each construct as described under the Experimental procedures. Each value represents the mean ± SD (n = 3). The data are a representative of three similar experiments. The inset shows the values in the absence of progesterone illustrated with a different scale.

and D may contribute to progesterone-induced promoter activity of the EP2 gene.

Determination of the regions showing PR-binding activity in the 5′-flanking region of the EP2 gene

Since none of the regions A-F contained a consensus PRE, PR-binding sequences were explored by electrophoretic mobility shift assay (EMSA) using each region as a probe to detect PR-binding. The ^{32}P-labelled probe C (−225 to −195) corresponding to the region C resulted in two specific retarded bands detected in the nuclear extracts of T-47D cells, which are known to abundantly express human PR proteins (Horwitz *et al.* 1982) (Fig. 4A). Treatment with an antibody against PR resulted in disappearance of the upper band and appearance of two bands with larger mobility shifts, the so called super-shifted bands. This result indicates that the upper band may contain human PR. On the other hand, the nuclear extracts from HeLa cells transfected with

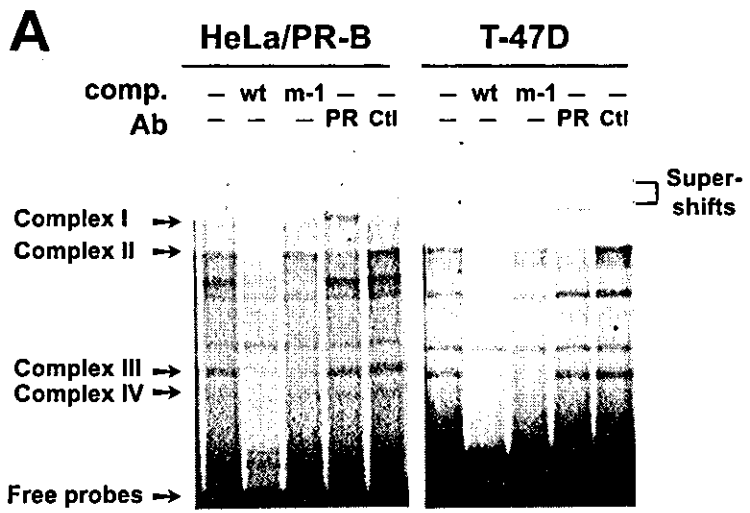
PR-B cDNA produced four specific bands (Complexes I to IV). Among them, the complex II was super-shifted and the mobility of the complex II seemed to be similar to that in the T-47D cells. These complexes and the supershifts were also detected in the nuclear extracts of mock-transfected cells (data not shown). Indeed, we confirmed on immunoblot analyses using the anti-PR antibody that PR-A was detectable in the nuclear extracts of HeLa cells and expression of PR-B was obtained only in the cells transfected with PR-B cDNA (data not shown). Next, we investigated PR-binding by EMSA using the ^{32}P-labelled probe E (−152 to −118) containing the region E, and found that the results were roughly the same as that using ^{32}P-labelled probe C shown in Fig. 4A. The formation of one specific band and two super-shifted bands with the anti-PR antibody were detected in the nuclear extracts of T-47D cells, respectively (Fig. 4B). Furthermore, in the nuclear extracts of HeLa cells transfected with PR-B cDNA, four specific bands (Complexes I′ to IV′) were detected. Addition of the anti-PR antibody resulted in the decrease of the complex II′ level, and conversely in the formation of super-shifted bands. EMSA performed with ^{32}P-labelled probes containing the region A, B, D or F resulted in several specific bands, which were not super-shifted by addition of the anti-PR antibody (data not shown).

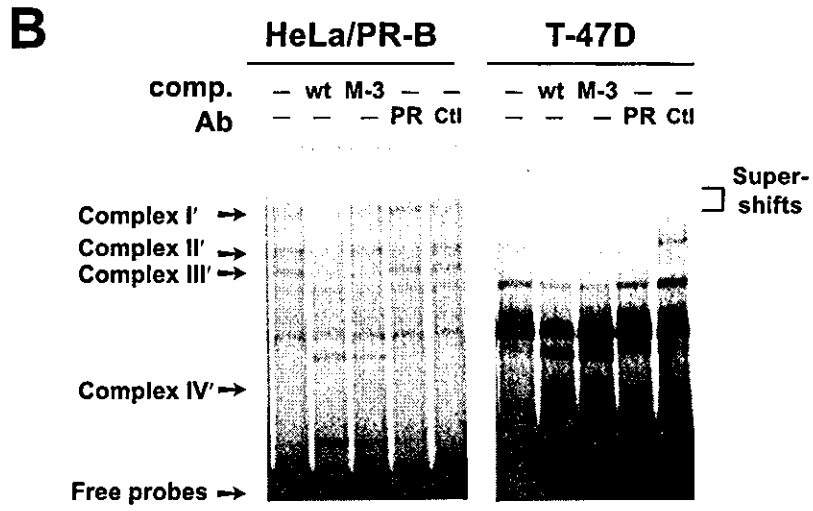
Identification of the PR-binding sequence in the EP2 promoter region

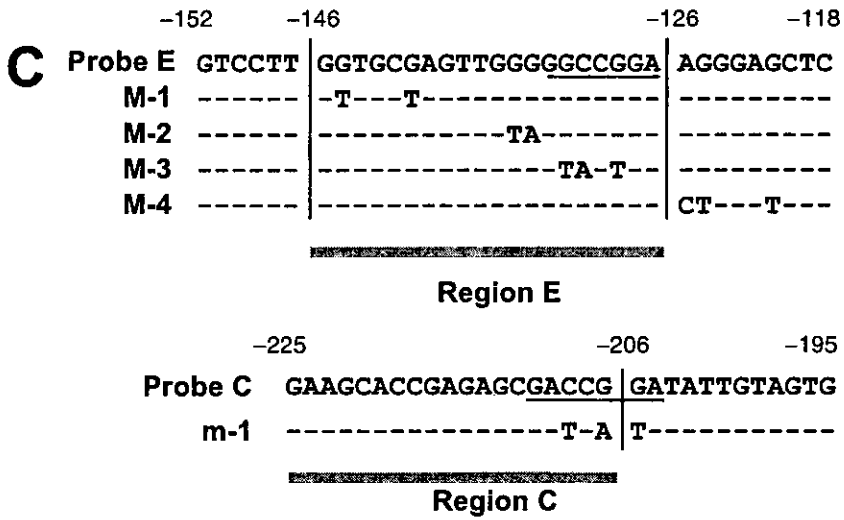
To determine the PR-binding sequence in probe E, four kinds of mutated probe E, named M-1 to M-4 (Fig. 4C) were prepared. Since this region is GC-rich, point mutations targeting the GC bases were introduced into each sample. The formation of PR-probe E complexes in the extracts of T-47D cells, corresponding to the complex II′ (Fig. 4D), was evaluated in the presence of these mutant competitors. PR-binding to the probe E was completely inhibited in the presence of M-1, -2 and -4, and not at all in the presence of M-3 (Fig. 4D), indicating that the sequence, 5′-GGCCGGA-3′ (−133 to −127), around the mutated bases in M-3, might be a candidate for a novel PRE. A similar sequence was also found in the probe C with only a single nucleotide substitution (Fig. 4C). In the presence of excess amounts of the mutated cold probe m-1 and M-3, the intensities of the complex II and the complex II′ remained unchanged, respectively (Fig. 4A,B), indicating that PR may bind to the sequences, 5′-G(G/A)CCGGA-3′. Indeed, we then performed EMSAs using the ^{32}P-labelled m-1 and M-3 probes and found no PR-binding was detected in HeLa and T-47D cells with either probe (data not shown). Furthermore, we investigated enhancer activity of the regions covering the region C or E by placing it upstream of thymidine kinase promoter. The thymidine kinase promoter itself was found to be responsive to progesterone in HeLa cells in the presence of PR-B (control; 0.492 ± 0.0611, +progesterone; 1.80 ± 0.172, 3.66-fold increase, relative luciferase activity, $n = 3$), which is consistent with the previous study (Thomson *et al.* 1990). In case of fusion constructs with the region C or E, the basal promoter activity was increased, but no drastic enhancer activity was observed (Region C, control; 1.08 ± 0.115, +progesterone; 2.84 ± 0.425, 2.63-fold increase, Region E, control; 1.31 ± 0.163, +progesterone; 4.33 ± 0.598, 3.31-fold increase).

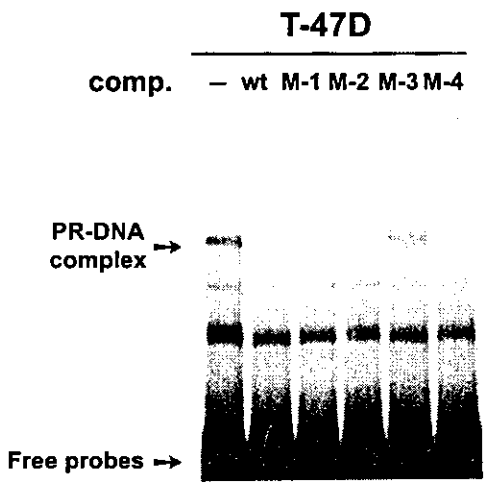
Sp1-binding in the EP2 promoter region

It has previously been reported that PR can directly interact with the Sp1 transcription factor (Owen *et al.* 1998), and that PR regulates transcription of some genes through the GC-box (Gao *et al.* 2001; Tang *et al.* 2002). These results indicate that PR may indirectly bind to this sequence via Sp1 family members. We then performed EMSA using the consensus GC-box, 5′-GATTCG ATC GGG GCG GGG CGA GC-3′, as a probe to investigate the involvement of the Sp1 family in the regulation of the EP2 gene (Fig. 5). Four specific bands (Complexes I″ to IV″) were detected in the HeLa cell nuclear extracts. The complex I″ was super-shifted in the presence of an anti-Sp1 antibody, whereas the complexes II″ and III″ were super-shifted in the presence of an anti-Sp3 antibody, which is consistent with previous observations (LeVan *et al.* 2001). Addition of the antibody against Sp2 or Sp4 was found not to alter the mobility shift patterns. Even in the presence of an excess of the probe C or E which possess PR-binding activity (Fig. 4), complexes I″, II″, and III″ still appeared. In addition to the regions C and E, the region F was found to exhibit basal promoter regulation of the EP2 gene (Fig. 3). In the presence of an excess of the probe F (−124 to −96), formation of the complexes I″, II″, and III″ was completely abolished. These results indicate that Sp1 and Sp3 can bind to the probe F, but not to the probes C and E. Using the ^{32}P-labelled probe F, EMSA was performed to confirm binding of Sp1 and Sp3 (Fig. 6). Four specific bands (Complexes I# to IV#) appeared with the probe F. Among them, complex I# was super-shifted in the presence of the anti-Sp1 antibody, whereas complexes II# and III# were super-shifted in the presence of the anti-Sp3 antibody. To determine the Sp1/Sp3-binding sequence, competition analysis using an excess of









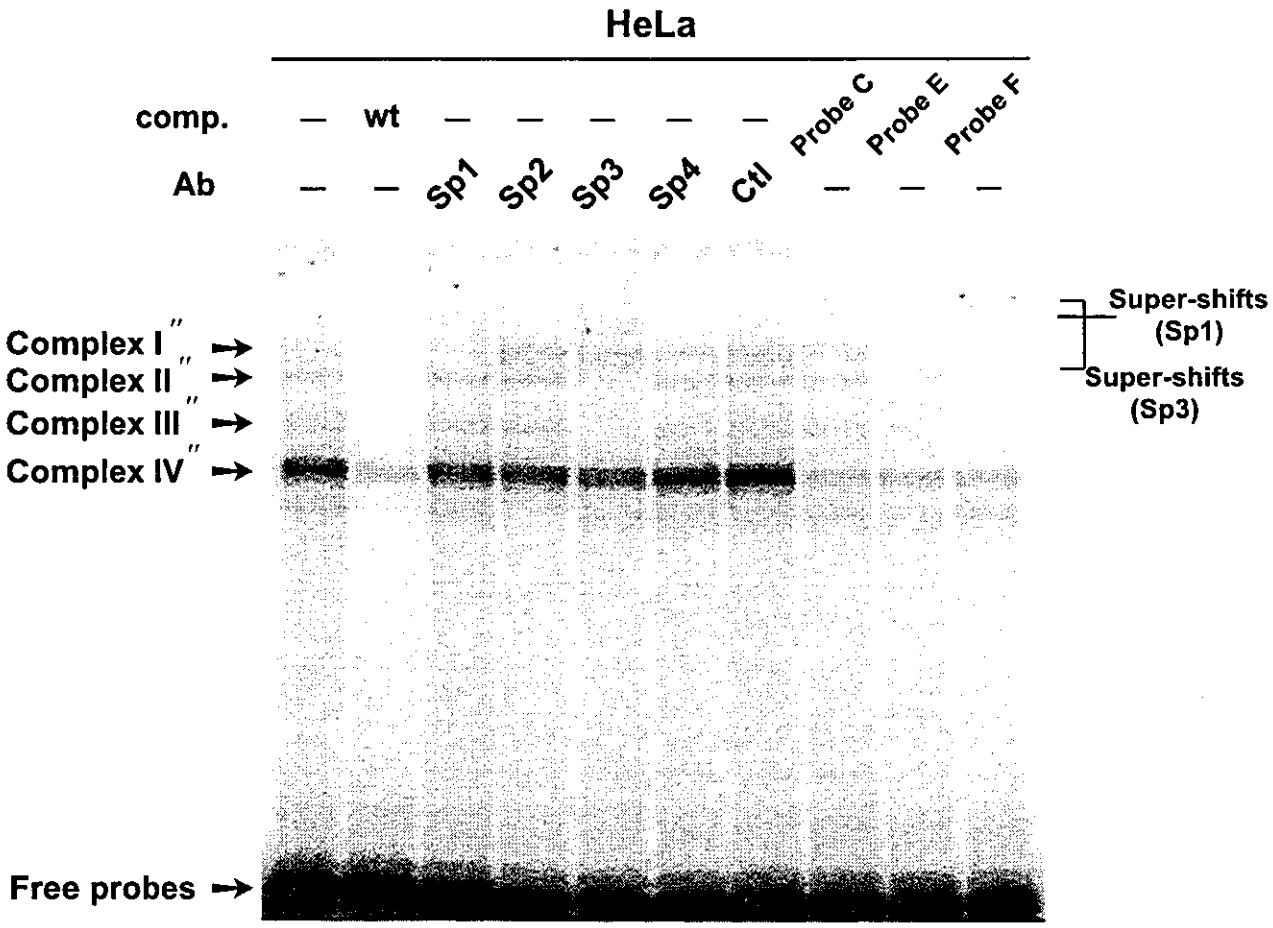


Figure 5 Binding of Sp1 and Sp3 to the EP2 basal promoter region. EMSA was performed using the ^{32}P-labelled consensus GC-box probe with the nuclear extracts of HeLa cells (5 μg) as described under Experimental procedures. Competition analysis was performed to determine specific binding using an excess of unlabelled probe C, E or F (−124 to −96). Supershift analysis was also performed using the antibodies raised against Sp1, Sp2, Sp3 and Sp4 (Sp1-Sp4) and the control non-immune antibody (Ctl). The bands for the specific bindings are indicated as the Complexes I″ to IV″ with the arrows on the left and super-shifted bands are shown on the right. This panel shows representative data of three independent experiments.

Figure 4 PR-binding activity of the basal promoter region in the 5′-flanking region of the mouse EP2 gene. HeLa cells were transfected with pcDNA3/PR-B 48 h before preparation of the nuclear extracts. EMSA was performed using ^{32}P-labelled oligonucleotides corresponding to the regions −225 to −195 (probe C) (A), or −152 to −118 (probe E) (B). Nuclear extracts of HeLa cells transfected with PR-B cDNA (HeLa/PR-B, 30 μg) and T-47D cells (T-47D, 7.5 μg) were prepared 2 h after the addition of 1 μM of progesterone. Preincubation with a 200-fold molar excess of the same, unlabelled probes (wt, Probe C in (A) and Probe E in (B)) or the mutated probes (m-1 in (A) and M-3 in (B), the sequences are presented in (C)) was performed to determine the specificity of binding. Supershift analyses were performed using an anti-PR antibody, which can bind to both human and mouse PR (PR) or control non-immune antibody (Ctl). The bands for specific bindings are indicated as the Complexes I to IV in (A), and the Complexes I′ to IV′ in (B) by the arrows on the left. The super-shifted bands are also indicated on the right. The data are a representative of three similar experiments. (C) Sequences of probe E and C, and the mutated oligonucleotides (M-1 to M-4, and m-1) are presented. The bar indicates the regions E and C. (D) EMSA was performed using the ^{32}P-labelled probe E with the nuclear extracts of T-47D cells (7.5 μg) pretreated with 1 μM of progesterone for 2 h. Preincubation with an excess of each unlabelled probe designed as M-1 to M-4 in (C) was performed to determine the PR-binding sequence. The PR-probe E complex is indicated as PR-DNA complex by the arrow on the left. The data are a representative of three similar experiments.

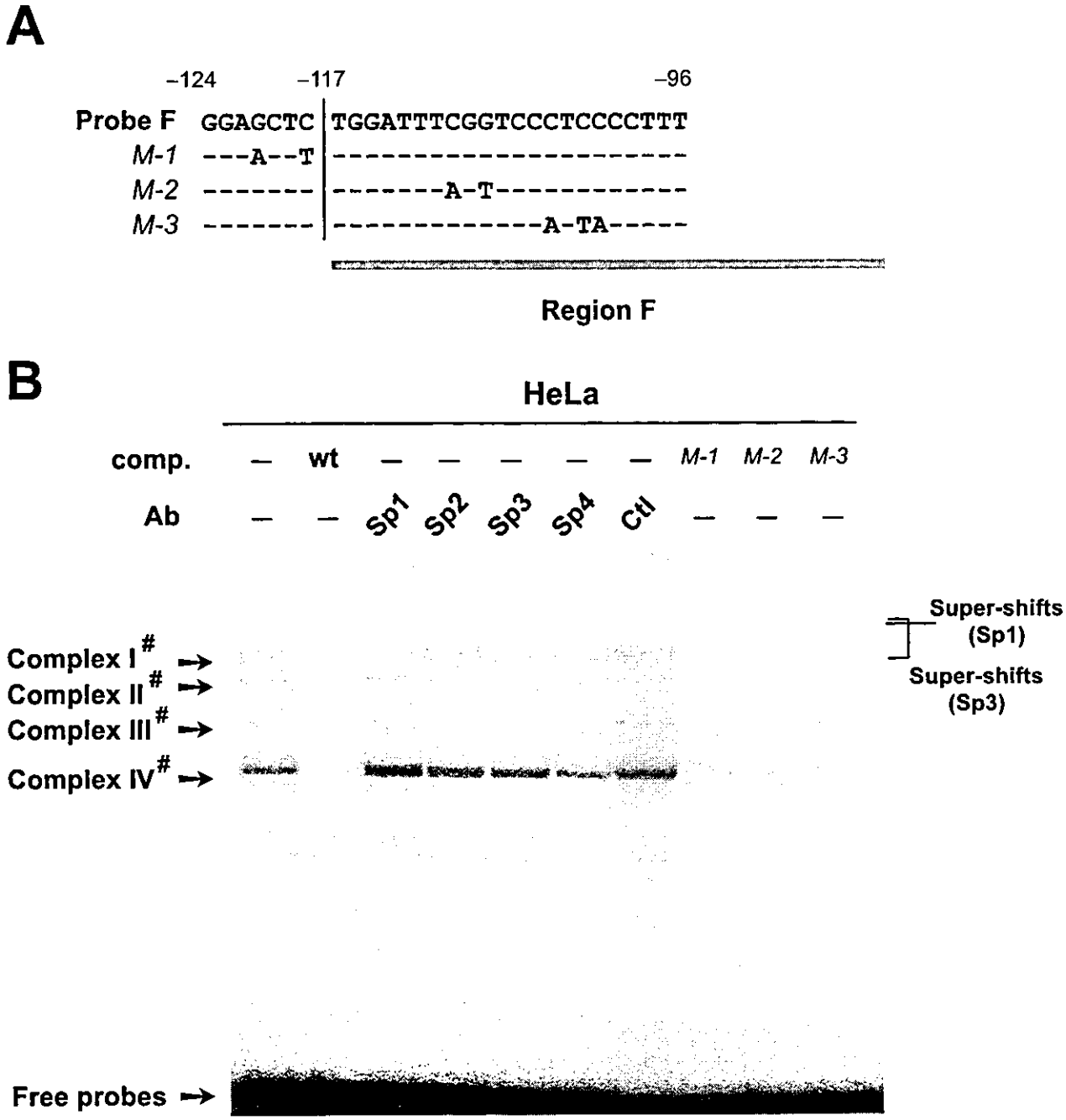


Figure 6 Sp1- and Sp3-binding sequence in region F of the EP2 promoter region. (A) Sequences of the probe F and the mutated oligonucleotides (*M*-1 ~ *M*-3) are presented. (B) EMSA was performed using the ^{32}P-labelled probe F with the nuclear extracts of HeLa cells (5 μg) as described under Experimental procedures. Competition analysis was performed to determine specific binding using an excess of unlabelled probes corresponding to the consensus GC-box, the mutated probes, *M*-1, -2, or -3. Supershift analysis was performed using either an anti-Sp1, an anti-Sp2, an anti-Sp3, an anti-Sp4 (Sp1-Sp4), or the control non-immune antibody (Ctl). The bands for specific binding are indicated as the Complexes I# to IV# with the arrows on the left and the super-shifted bands are indicated on the right. The data are a representative of three similar experiments.

unlabelled probe was performed (Fig. 6A). Formation of complexes I#, II#, and III# were all abolished in the presence of an excess of *M*-1 or *M*-2, whereas these complexes were still detected in the presence of *M*-3.

Discussion

Progesterone has been suggested to affect the EP2 gene expression in the uterine epithelial cells (Katsuyama *et al.* 1997; Lim & Dey 1997), also there has been no direct evidence in terms of transcriptional regulation of the EP2 gene by progesterone. The current study demonstrated that the 5′-flanking region of mouse EP2 gene (−3260 to −27) is activated by progesterone in the presence of PR, especially of the B form (Fig. 2). Preference for the B form has been reported in many progesterone responsive genes (Wen *et al.* 1994), which is consistent with our results. Increased promoter activity was also found in the absence of progesterone in cells co-transfected with PR. This observation indicates that PR may be involved in both progesterone-induced and basal transcription of the EP2 gene.

Although this promoter region contains one progesterone responsive element (PRE)-like sequence (−844 to −830), the deletion of this region did not influence progesterone-induced reporter gene activation (Fig. 3), indicating the existence of other progesterone responsive elements. Successive deletion analysis of the 5′-flanking region of the EP2 gene revealed six regulatory elements (Fig. 3), which can be divided into two categories; one is involved in progesterone-dependent transcriptional activation (regions A, B and D), and the other in basal promoter activity (regions C, E and F). We failed to detect any PR-binding to the former three regions, although EMSA using each region as a probe demonstrated specific binding proteins (data not shown). These results indicate that the inducible effects of progesterone may be mediated via other unknown transcription factors binding to these elements, which contain no homologous sequences with the known regulatory elements. On the other hand, PR-binding to the latter two regions (C and E), both of which have no canonical PRE (Forman & Samuels 1990), was observed (Fig. 4). Supershift analyses using the anti-PR antibody and mutational analyses revealed that the sequence, 5′-G(G/A)CCGGA-3′, is a potent candidate for the novel PR-binding sequence (Fig. 4). The porcine uteroferrin gene was also found to be regulated by progesterone through its 5′-flanking region, which contains no canonical PRE (Lamian *et al.* 1993), although the PR-binding sequence has not yet been confirmed in this region. Since we can find the same sequence as the novel PR-binding element of the EP2 gene in this region, this sequence may be functional not only in the EP2 gene but also in the other progesterone responsive genes.

The nuclear extracts of HeLa cells were demonstrated to contain several specific binding proteins to the novel PR-binding element in addition to PR, whereas those of T-47D cells were not (Fig. 4). It is possible that PR-binding to this element may be indirect and be mediated via binding(s) of other transcription factor(s) in HeLa cells. Since Owen *et al.* (1998) reported that PR can interact with Sp1 and CBP/p300 in *in vitro* and since Tang *et al.* (2002) reported that human PR mediates its own promoter activity through the Sp1 site, PR-binding to the novel PR-binding element in the EP2 gene, which is a GC-rich sequence, may be enhanced by binding of the Sp1 family. Supershift analysis using the consensus GC-box revealed that Sp1 (complex I″ in Fig. 5) and Sp3 (complex II″ and III″ in Fig. 5) are included in the nuclear extracts of HeLa cells, and both bindings were still detected in the presence of an excess of the probe C or E. These observations indicate that neither Sp1 nor Sp3 binds to the novel PR-binding element of the EP2 gene. On the other hand, the probe F, which contains a partial sequence of the putative basal promoter, the region F, was found to compete with Sp1- and Sp3-binding to the GC-box. Mutational analysis revealed that the cytidine-rich sequence in the region F is involved in Sp1- and Sp3-binding (Fig. 6). These results suggest that PR-binding to the novel PR-binding element may regulate basal promoter activity of the EP2 gene through interaction with Sp1 and/or Sp3 bound to the cytidine-rich sequence of the region F (5′-CCCTC-CCC-3′), which is completely conserved in the human EP2 gene (Smock *et al.* 1999). We have previously reported that both EP2 and COX(cyclooxygenase)-2 mRNA expression is induced upon treatment with lipopolysaccharide in mouse macrophages, and upon treatment with gonadotropins in ovarian cumulus cells (Hizaki *et al.* 1999; Ikegami *et al.* 2001). Since Sp1 and Sp3 were also found to bind to the Sp1 element (5′-GGGAGG-3′) in the human COX-2 promoter region (Xu *et al.* 2000), Sp1/Sp3-binding may be one of the key regulators in the simultaneous expression of the EP2 and COX genes.

Significant amounts of bands corresponding to the complex I″ and the complex I# were still detected in the presence of the anti-Sp1 antibody, and they appear to be due to the incomplete supershift (Figs 5 and 6). However, the existence of remaining band has also been reported in HeLa cells using the consensus GC-box as a probe with the anti-Sp1 antibodies obtained from the different sources (LeVan *et al.* 2001; Cohen *et al.* 1997).

These results suggest that this complex may be a doublet and the remaining band may not be Sp1, although we could not identify this complex using the antibodies raised against the other Sp1 family (Fig. 5).

Excess amounts of cold probe C, E, or F were found to inhibit formation of the complex IV″ to a similar degree to those of the GC-box, which indicates the presence of another transcription factor that can bind to the probes C, E, and F, in addition to the consensus GC-box. Similar complexes with small mobility shifts have also been detected in addition to the Sp1 family using the consensus GC-box as a probe in the other cell lines (Skak & Michelsen 1999; Stoner *et al.* 2000), although these complexes were not identified in these studies. We could not identify this complex by supershift analyses using available antibodies including those against the Sp1 family proteins (Fig. 5).

In summary, we determined the six regulatory regions in the 5′-flanking region of the mouse EP2 gene; three regions were found to be involved in progesterone-induced promoter activation, whereas the other three regions were involved in basal promoter activity in the presence of PR. Furthermore, we identified the novel PR-binding sequence, 5′-G(G/A)CCGGA-3′, in the two basal promoter regions and Sp1- and Sp3-binding to the other basal promoter region.

Experimental procedures

Materials

The following materials were obtained from the sources indicated: [α-^{32}P]dCTP (3000 Ci/mmol) from Du Pont New England Nuclear (Boston, MA, USA), 17-β-oestradiol and progesterone from Research Biochemical International (Natick, MA, USA), progesterone-water soluble and Poly(deoxyinosinic-deoxycytidylic) acid sodium salt (Poly(dI-dC)·Poly(dI-dC)), from Sigma-Aldrich (Saint Louis, MO, USA), Biodyne A membranes from Pall (East Hills, NY), pGL3-Basic Vector and Dual-luciferase® Reporter Assay System from Promega (Madison, WI, USA), Time Saver from Amersham Pharmacia (Uppsala, Sweden), FuGENE™ 6 transfection reagent from Roche (Mannheim, Germany), specific antibodies against PR, Sp1, Sp2, Sp3 and Sp4 and control non-immune antibody from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). All other chemicals were commercial products of reagent grade.

Northern blot analysis

ddY mice were purchased from Japan SLC (Hamamatsu, Japan). Adult female mice (9 weeks of age), which were not conditioned to the same estrous cycle, were ovariectomized. Two weeks after the ovariectomy, they were subcutaneously injected with 17-β-oestradiol (250 ng/mouse), or progesterone (1 mg/mouse). All steroids were dissolved in sesame oil (0.1 mL/mouse). The control animal received vehicle only. Uteri were collected at the indicated time points from three mice. Total RNAs were isolated according to the acid guanidinium thiocyanate-phenol-chloroform method (Chomczynski & Sacchi 1987) and separated by electrophoresis on a 1.5% agarose gel (40 μg/lane) followed by transfer on to a Biodyne A membrane. Synthesis of the EP2 anti-sense RNA probe and hybridization was performed as previously described (Katsuyama *et al.* 1995). The same filter was rehybridized with the ^{32}P-labelled probe for glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Construction of reporter genes

The 5′-flanking region of the mouse EP2 gene was isolated from a mouse genomic library as reported previously (GenBank accession no. AB007695) (Katsuyama *et al.* 1998b). The 3.2 kbp-fragment (nucleotide residues −3260 to −27, the translation start site = 1) of the 5′-flanking region of the mouse EP2 gene was subcloned into the *Sma*I site of the pGL3 basic vector (pGL3/EP2 −3260/−27). Successive 5′-deletion mutants were generated by polymerase chain reaction (PCR) using forward primers, containing the cassette sequence 5′-CGC GTG CTA GCC C-3′ at the 5′-end, and the reverse primer 5′-TTC TTT ATG TTT TTG GCG TCT TCC A-3′ (+113 to +89 in pGL3 basic vector). The PCR products were digested with *Nhe*I and *Xho*I, and subcloned into the pGL3 basic vector. In enhancer analyses, the thymidine kinase promoter in pBLCAT2 was digested with *Bam*HI and *Bgl*II, and subcloned into the *Bgl*II site of pGL3 basic vector. Oligonucleotides corresponding to the sequence −225 to −195 and −152 to −118 of the 5′-flanking region of the EP2 gene were synthesized and inserted into the *Sma*I site upstream of the thymidine kinase promoter.

Cloning of mouse PR cDNA

A uterus cDNA library was constructed from mice stimulated with PMSG for 48 h and mouse PR cDNAs were cloned using Time Saver. The mouse PR-A and PR-B cDNAs were subcloned into pcDNA3 (pcDNA3/PR-A and pcDNA3/PR-B) according to the procedures previously described (Schott *et al.* 1991).

Cell culture

HeLa, a human cervical carcinoma cell line, and T-47D, a human breast cancer cell line, were cultured in phenol red-free Eagle's Minimum Essential Medium containing 10% foetal bovine serum, 2 mM L-glutamine, 0.06 mg/mL kanamycin, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 °C in a fully humidified 5% CO_2 atmosphere.

Transfection and luciferase assay

HeLa cells were seeded on to six-well plates at a density of 1×10^5 cells per well in phenol red-free Eagle's Minimum Essential

Medium containing 10% charcoal-stripped and heat-inactivated (40 min, 55 °C) foetal bovine serum, 2 mM L-glutamine and 0.06 mg/mL kanamycin. After 24 h, cells were transiently co-transfected with 0.67 μg of a reporter gene construct (pGL3) containing 5′-flanking regions of the EP2 gene, 0.25 μg of a mouse PR-subtype expression vector (pcDNA3/PR) or 0.07 μg of pRL-TK using FuGENE™ 6 according to the manufacturer's instructions. Twenty-four hours after transfection, cells were treated with or without 1 μM of progesterone. Cells were harvested in 1 × luciferase lysis buffer 48 h after transfection, and reporter gene expression was measured and normalized using a dual luciferase assay kit. All experiments were repeated at least three times in triplicate, and the data are expressed as mean ± SD.

Nuclear protein extraction

HeLa cells were seeded on to a plastic dish (Φ = 150 mm) at a density of 2×10^6 cells in phenol red-free Eagle's Minimum Essential Medium containing 10% charcoal-stripped and heat-inactivated foetal bovine serum, 2 mM L-glutamine and 0.06 mg/mL kanamycin. After 24 h, cells were transiently transfected with 20 μg of pcDNA3/PR-B by FuGENE™ 6. Forty-six hours after transfection, cells were treated with 1 μM of progesterone. Forty-eight hours after transfection, cells were washed with cold phosphate-buffered saline, and harvested. The cell pellets were then resuspended in 20 mM HEPES, pH 7.9, containing 5 mM KCl, 0.5 mM $MgCl_2$, 2 mM dithiothreitol (DTT), and 0.6 mM phenylmethanesulphonyl fluoride (PMSF), and incubated on ice for 30 min. The pellets were lysed with a Dounce homogenizer (type B) and centrifuged at 7500 *g* for 30 min at 4 °C to obtain the nucleus fraction. The nucleus fraction was then resuspended in 20 mM HEPES, pH 7.9, containing 500 mM NaCl, 0.2 mM EDTA, 1.5 mM $MgCl_2$, 2 mM DTT, 0.6 mM PMSF, and 25% glycerol and incubated at 4 °C for 1.5 h with gentle shaking. Nuclear extracts were then obtained by centrifugation of the nucleus fraction at 18 000 *g* for 30 min at 4 °C, followed by twice dialysis against 25 mM HEPES, pH 7.9, containing 50 mM KCl, 0.5 mM EDTA, 2 mM DTT, 0.6 mM PMSF, and 10% glycerol for 3 h at 4 °C. The nuclear extracts were then re-centrifuged at 18 000 *g* for 30 min at 4 °C. The resultant nuclear extracts were stored at −80 °C until use. Protein concentrations were determined according to the method of Bradford (1976) using bovine serum albumin as a standard.

Electrophoretic mobility shift assay (EMSA)

Oligonucleotide probes corresponding to the sequence −225 to −195, −152 to −118 and −124 to −96 of the 5′-flanking region of the EP2 gene and the Sp1 consensus sequence (Cohen *et al.* 1997) were synthesized and radiolabelled with [α-^{32}P]dCTP using the Klenow fragment. The nuclear extracts from HeLa cells (30 μg protein) or from T-47D cells (7.5 μg protein) pretreated with 2 μg of Poly(dI-dC)·Poly(dI-dC) for 15 min at 30 °C were incubated with 20 000 c.p.m. of ^{32}P-labelled probes for 45 min at 30 °C. The reaction mixtures were separated on native 4% polyacrylamide gels at 150 V for 2.5 h at 4 °C in 50 mM Tris-HCl, pH 8.5, containing 380 mM glycine and 2 mM EDTA. EMSA for Sp1-binding was performed according to the procedure as previously described by Shou *et al.* (1998). The dried gels were subjected to autoradiography.

Acknowledgements

We thank Drs E. Segi and K. Tsuboi for their invaluable advice. This work was supported in part by grants from the Sankyo Foundation of Life Science, the Takeda Science Foundation, the Mochida Memorial Foundation for Medical and Pharmaceutical Research, and grants-in-aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

References

Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248–254.

Chomczynski, P. & Sacchi, N. (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**, 156–159.

Cohen, H.T., Bossone, S.A., Zhu, G., McDonald, G.A. & Sukhatme, V.P. (1997) Sp1 is a critical regulator of the *Wilms' tumor-1* gene. *J. Biol. Chem.* **272**, 2901–2913.

Coleman, R.A., Kennedy, I., Humphery, P.P.A., *et al.* (1990) Prostanoids and their receptors. In: *Comprehensive Medicinal Chemistry* (eds C. Hansch, P.G. Sammes, J.B. Taylor & J.C. Emmett), Vol. 3, pp. 643–714. Oxford: Pergamon Press.

Forman, B.M. & Samuels, H.H. (1990) Interactions among a subfamily of nuclear hormone receptors: the regulatory zipper model. *Mol. Endocrinol.* **4**, 1293–1301.

Gao, J., Mazella, J., Seppala, M. & Tseng, L. (2001) Ligand activated hPR modulates the glycodelin promoter activity through the Sp1 sites in human endometrial adenocarcinoma cells. *Mol. Cell. Endocrinol.* **176**, 97–102.

Gardiner, P.J. (1986) Characterization of prostanoid relaxant/inhibitory receptors (Ψ) using a highly selective agonist, TR4979. *Br. J. Pharmacol.* **87**, 45–56.

Hizaki, H., Segi, E., Sugimoto, Y., *et al.* (1999) Abortive expansion of the cumulus and impaired fertility in mice lacking the prostaglandin E receptor subtype EP_2. *Proc. Natl. Acad. Sci. USA* **96**, 10501–10506.

Horwitz, K.B., Mockus, M.B. & Lessey, B.A. (1982) Variant T47D human breast cancer cells with high progesterone-receptor levels despite estrogen and antiestrogen resistance. *Cell* **28**, 633–642.

Ikegami, R., Sugimoto, Y., Segi, E., *et al.* (2001) The expression of prostaglandin E receptors EP_2 and EP_4 and their different regulation by lipopolysaccharide in C3H/HeN peritoneal macrophages. *J. Immunol.* **166**, 4689–4696.

Kastner, P., Krust, A., Turcotte, B., *et al.* (1990) Two distinct estrogen-regulated promoters generate transcripts encoding the