UNIVERSITY OF ALBERTA

REGULATION OF THE HUMAN OXYTOCIN RECEPTOR GENE BY INTERLEUKIN-1β AND INTERLEUKIN-6 *IN VITRO*.

by

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ABSTRACT

Uterine contractions during labor are mediated by myometrial oxytocin (OT) receptors, which are significantly upregulated in late pregnancy. The 5'-flanking region of the human OT receptor gene contains various cis-acting elements, including several putative binding sites for nuclear factor IL-6 (NF-IL6). This trans-acting factor is known to modulate the expression of genes involved in acute, immune and inflammatory responses. Pro-inflammatory cytokines, such as IL-1β or IL-6, have been implicated as mediators in both preterm and term labor, particularly in association with intrauterine infection. We hypothesized that IL-1β and IL-6 induce OT receptor gene expression in human myometrial cells and this is mediated by NF-IL6 and cognate response elements in the 5'-flanking region of the OT receptor gene. Contrary to our hypothesis, we have demonstrated that either IL-1ß or IL-6 treatment of immortalized human myometrial cells causes a reduction of OT receptor mRNA levels. In addition, we have shown that NF-IL6 is present in immortalized human myometrial cells and appears to be increased by either IL-1ß or IL-6. Finally, we have characterized 1.3 kb of the 5'-flanking region of the human OT receptor gene in HeLa cells. We have demonstrated that the promoter displays basal activity and we have localized the negative regulation of the OT receptor promoter reporter construct by either IL-1ß or IL-6 to the –1203/-722 region. Our findings suggest a role for both inflammatory cytokines, IL-1ß and IL-6, in the transcriptional regulation of the human oxytocin receptor gene.

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LIST OF ABBREVIATIONS

ANOVA	analysis of variance
AP-1	activator protein-1
APRE	acute-phase response element
APRF	acute-phase response factor
β-gal	beta-galactosidase
CAT	chloramphenicol acetyltransferase
cDNA	complementary deoxyribonucleic acid
cRNA	complementary ribonucleic acid
DAG	diacylglyceride
DEPC	diethylpyrocarbonate
DNA	deoxyribonucleic acid
DTT	DL-dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EMSA	electrophoretic mobility shift assay
G-protein	guanosine-binding protein
HEPES	4-(2-hydroyethyl)-piperazine-1-ethanesulfonic acid
H_2O dist.	distilled water
IL-1β	interleukin-1beta
IL-6	interleukin-6
IP_3	inositol 1,4,5-triphosphate
kb	kilobase
kDa	kilodalton
LPS	lipopolysaccharide

LSC	liquid scintillation counting
MLCK	myosin light chain kinase
mRNA	messenger ribonucleic acid
NF-IL6	nuclear factor interleukin-6
NFκB	nuclear factor kappa B
OT	oxytocin
OTR	oxytocin receptor
PMSF	phenylmethylsulfonyl fluoride
PBS	phosphate buffered saline
PG	prostaglandin
PGHS	prostaglandin-endoperoxide H synthase
pH	negative log of hydrogen ion concentration
PIP_2	phoshatidylinositol 4,5-bisphosphate
PKC	protein kinase C
PLC	phospholipase C
RPA	ribonuclease protection assay
TE	tris/EDTA buffer
TBE	tris/borate/EDTA
TGE	tris/glycine/EDTA
Tris	tris (hydroxymethylaminomethane)
tRNA	transfer RNA

INTRODUCTION

In preparation for birth, coordinated molecular events lead to changes in the cervix, fetal membranes and uterine contractility. The underlying mechanisms are still unclear but may reflect a balance between inhibitory and stimulatory factors (*Mc Coshen JA et al., 1996*). An inappropriate regulation of this equilibrium can cause preterm or delayed labor, respectively. Preterm labor is a major obstetrical problem in western countries where six to ten percent of all babies are born at less than 37 weeks gestation. The consequences of premature delivery are not only one of the leading causes of human perinatal mortality and morbidity but are also enormous in terms of adverse economic and social impact (*Creasy R, 1994).*

CHANGES IN THE UTERUS AT PARTURITION

At the end of gestation, the cervix has transformed from a closed, rigid structure to one that is soft and able to dilate. In the fetal membranes, which are formed by the apposition of amnion and chorion, changes have taken place that lead to their rupture. In both processes, locally produced collagenases are thought to play an important role by degrading connective tissue components (*Winkler M, Rath W, 1999*; *Bryant-Greenwood GD, 1998*).

Uterine contractility at term occurs in two phases: activation and stimulation (Figure 1). During activation uterotrophins, such as estrogens, increase myometrial expression of contraction associated proteins. These include receptors for oxytocin and prostaglandins, connexin 43 and ion channels. During the stimulation phase,

uterotonins, such as oxytocin and prostaglandins, induce contraction of the activated myometrium (*Lye SJ, Challis JRG, 1989*).

The mechanism of uterine contractions is based on electrical and chemical changes within myometrial cells (*Wray S, 1993; Somlyo AP, Somlyo AV, 1994*) (Figure 2). In late gestation, spontaneous electrical activity of myometrial cells becomes more regular. Once their depolarization reaches a threshold, action potentials arise, mainly caused by an influx of calcium ions (Ca^{2+}) via voltage-gated Ca^{2+} channels. In addition, levels of intracellular Ca^{2+} are increased through the activation of oxytocin (OT) receptors, which couple through G-proteins to the phospholipase C/Ca^{2+} signaling pathway (*Sanborn BM, 1995*).

Intracellular calcium ions bind to calmodulin. The Ca^{2+}/calmodulin complex activates myosin-light chain kinase to phosphorylate myosin on its light chains. Actin binds to myosin and activates myosin Mg-ATPase, which causes hydrolysis of ATP with liberation of the energy required for contraction. Dephosphorylation of myosin by phosphatases leads again to muscle relaxation (*Word RA, 1995*).

The uterus contracts as a whole since action potentials propagate from myocyte to myocyte via a large number of "gap junctions". These continuous channels consist of a hexameric arrangement of connexins. In the myometrium, connexin 43 is thought to be the major gap junction protein (*Garfield RE et al., 1977*).

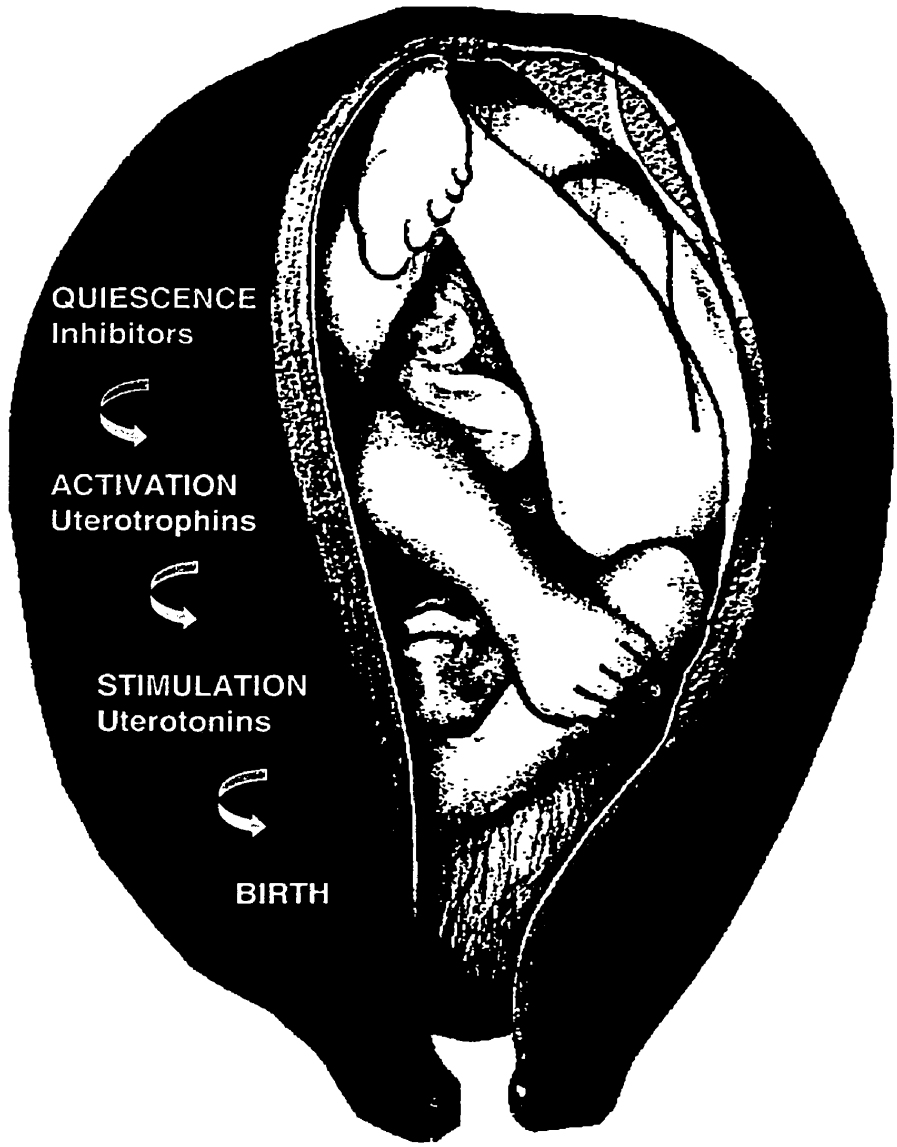

FIG. 1. **The uterus in preparation for birth.** During most part of pregnancy, inhibitors keep the myometrium quiescent. At the end of gestation, the uterus has been activated by uterotrophins. Uterotonins can then stimulate the activated myometrium and the baby is expelled by forceful contractions (*modified after Smith R, 1999*).

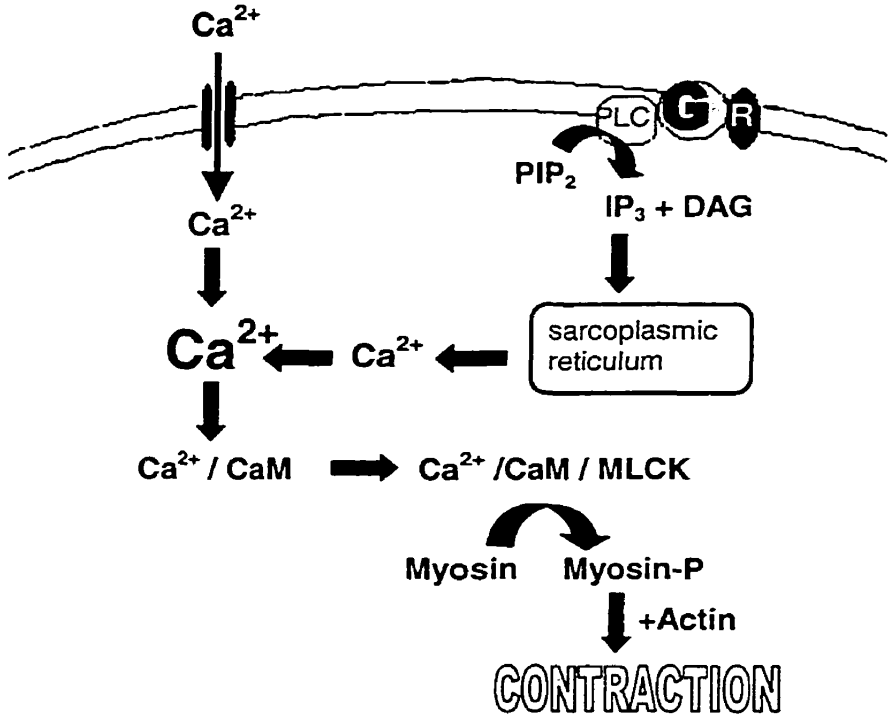

FIG. 2. **Mechanism of myometrial contractions.** Intracellular calcium increases by an influx of Ca^{2+} via voltage operated channels (left) or via IP_3 mediated Ca^{2+} release from the sarcoplasmic reticulum (right). Ca^{2+} /Calmodulin (CaM)-dependent Myosin Light Chain Kinase (MLCK) is activated leading to the phosphorylation of myosin light chain. Actin binds to myosin and activates myosin Mg-ATPase which causes hydrolysis of ATP with liberation of the energy required for contraction (*modified after Sanborn BM, 1995*).

HORMONAL CONTROL OF UTERINE CONTRACTILITY

Pregnancy is regulated in a complex way involving not only endocrine but also auto- and paracrine mechanisms. Recent studies have shown that placenta, decidua and fetal membranes release a variety of hormones, which contribute to gestational development (*Petraglia F et al., 1996*). First, a description will be given on how uterine activation is thought to occur in animals, followed by a recent theory on human parturition. Second, the role of prostaglandins (PG) and oxytocin in uterine stimulation will be discussed.

Activation of the pregnant uterus: Generally, progesterone and estradiol are seen as regulators of pregnancy. While progesterone is thought to keep the uterus quiescent during most of pregnancy, estradiol is believed to increase uterine contractility through its uterotrophic action (*Pepe GJ, Albrecht ED, 1995*).

In animal models of parturition, the myometrium becomes activated when maternal plasma concentrations of progesterone decrease while those of estrogen rise (*Challis JRG, Olson DM, 1988*). In sheep, these changes in maternal steroid levels are triggered by the fetus. In the days preceding birth, the production of fetal adrenal cortisol gets stimulated due to the maturation of the fetal hypothalamic-pituitary-adrenal axis. Cortisol induces placental steroid 17α-hydroxylase, which converts progesterone to estrogen. As a result, the estrogen-to-progesterone ratio in maternal plasma increases and promotes uterine contractility (*Flint APF et al., 1975*; *Nathanielsz PW, 1998*). In rats, it is the progesterone produced by the corpus luteum that is necessary to maintain pregnancy. With luteolysis shortly before term, progesterone

secretion significantly decreases leading to an increased estrogen to progesterone ratio in maternal plasma and thus to myometrial activation.

However, towards the end of human pregnancy no such significant changes in maternal plasma steroid concentrations occur (*Block BSB et al., 1984*). In pregnant women, myometrial activation may be regulated in a paracrine rather than endocrine fashion.

During human pregnancy, estrogen synthesis occurs as a cooperative process between the placenta and the fetal adrenal cortex, referred to as the feto-placental unit (Figure 3). Due to a deficiency in 17α-hydroxylase, the placenta carries out only the initial and final steps of estrogen synthesis, while androgen precursors are coming from the fetus and the mother. On the other hand, the fetal adrenal cortex lacks 3β-hydroxysteroid dehydrogenase and only synthesizes Δ5-3β-hydroxysteroids, principally dehydroepiandrosterone sulfate (DHEAS). In the fetal liver, DHEAS gets metabolized to 16-OH-DHEAS, which gets converted to estriol by the placental aromatase enzyme complex (*Kuss E, 1994*).

In a recent theory, corticotropin-releasing hormone (CRH) is proposed to be important for uterine activation (*Schwartz LB, 1999*). During the last weeks of gestation, placental CRH is proposed to stimulate the fetal pituitary to secrete adrenocorticotropin (ACTH) thus triggering the fetal adrenal to produce cortisol and the estradiol precursor dehydroepiandrosterone sulfate (DHEAS) (*Karalis K et al., 1996*). Cortisol may then bind to placental glucocorticoid receptors further stimulating CRH production. DHEAS may increase placental estradiol production which is thought to activate the myometrium through stimulation of contraction associated proteins. This theory is

supported by the parallel increases in placental CRH and fetal cortisol levels during the last weeks of gestation (*Fencl MD et al., 1980*).

Additionally, the endocrine and immune system might communicate within the placenta by interactions between CRH and cytokines (*Dudley DJ et al., 1999*).

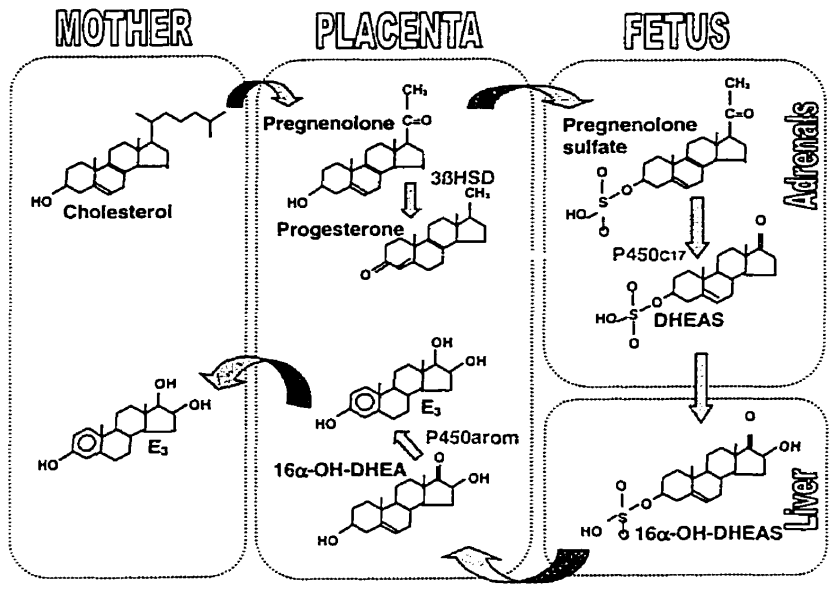

FIG. 3. **The human feto-placental unit.** While the placenta is deficient in 17α-hydroxylase (P450C17) and carries out only the initial and final steps of estrogen synthesis, the fetal zone of the fetal adrenal cortex lacks 3β-hydroxysteroid dehydrogenase (3βHSD) and therefore only synthesizes Δ5-3β-hydroxysteroids, principally dehydroepiandrosterone sulfate (DHEAS). In the fetal liver, DHEAS gets first metabolized to 16α-hydroxy-DHEAS, then converted to 16α-hydroxyandrostenedione and then further aromatized into estriol (E_3) by the placental aromatase enzyme complex (P450arom) (*modified after Kuss E, 1994*).

Stimulation of the activated uterus: Once the myometrium is activated, it can be stimulated by uterotonins, such as prostaglandins (PG) and oxytocin (OT).

PG are polyunsaturated fatty acids and found in many tissues where they act as paracrine hormones. They are derived from arachidonic acid, which is mobilized from membrane lipids by phospholipase A_2 (PLA_2) or phospholipase C (PLC) (*Bleasdale JE, Johnson JM, 1984*). Prostaglandin endoperoxide H synthase (PGHS) then mediates the formation of eicosanoids (PGE_2, $PGF_{2\alpha}$, PGI_2, Thromboxane A_2, PGD_2). This key enzyme exists in two forms, a constitutively expressed PGHS-1 and an inducible PGHS-2 form, that have been reviewed (*Smith WL, DeWitt DL, 1996*).

PG exert many different actions via functionally different PG receptors, which have been pharmacologically classified as DP, EP1-EP4, FP and IP receptors for PGD_2, PGE_2, $PGF_{2\alpha}$ and PGI_2, respectively. All mediate their action by G-proteins but are coupled to different signal transducers. IP, EP2, EP4 and DP receptors stimulate adenylate cyclase, while EP3 receptors inhibit it. EP1 and FP receptors couple to the phospholipase C/Ca^{2+} signaling pathway (*Molnar M, Hertelendy F, 1990; Negishi M et al., 1995*). In human myometrium, binding sites for PGE_2 and $PGF_{2\alpha}$ can be found (*Bauknecht T et al., 1981*).

Several lines of evidence suggest that PG play an important regulatory role in the process of parturition. As reviewed by Gibb, there is a major increase in PG concentrations in amniotic fluid and serum of a number of experimental animals (*Gibb W, 1998*). Also in human, PG concentrations in amniotic fluid rise before labor (*Romero R et al., 1996*).

Findings from studies measuring PG synthesis *in vitro* can be artifactual since experimental manipulations are known to cause PG release. Nonetheless, the

upregulation of synthetic key enzymes towards term points to an actual increase in PG production. The expression of PGHS-2 in human fetal membranes and its activity increases at parturition (*Teixeira FJ et al.,1994; Hirst JJ et al., 1995; Mijovic JE et al., 1997*). In mice, the rapid induction of PGHS-2 in uterine tissues by endotoxin is correlated with preterm delivery (*Swaisgood CM et al., 1997*). It is also known that PGHS-2 inhibitors extend gestation and PGE_2 and $PGF_{2\alpha}$ can induce labor at any stage of gestation when administered either systemically or locally (*Challis JRG, Lye SJ, 1994; review*).

There is controversy as to whether PG cause or are the consequence of parturition (*MacDonald PC et al., 1993; Romero R et al., 1994*). In mice deficient in the FP receptor, parturition fails to occur (*Sugimoto Y et al., 1997*).

Since its discovery in 1906 the nonapeptide OT has been clinically used to augment or induce labor (*Dale HH, 1906; Owen J, 1991*). In the hypothalamus, OT is synthesized as part of a longer precursor including neurophysin I (NPI) (Figure 4). Via axonal transport, this complex reaches the neurohypophysis, also known as posterior pituitary, where it is stored. Cleaved from NPI, OT is released by exocytosis into the maternal blood. The active nonapeptide can then bind to its cognate receptor in the myometrium and thus induce uterine contractions (*Pickering BT, 1994*).

Several findings suggest that systemic OT produced in the pituitary may be physiologically irrelevant as an uterotonic agent. OT is mainly released from maternal pituitary in a pulsatile fashion. Although the frequency of this pulsatile release increases near term (*Chard T, 1989; Fuchs AR et al., 1991*), maternal circulating OT levels do not rise prior to labor (*Dawood MY et al., 1979*). This may be partly due to the action of circulating oxytocinase, a primarily placental enzyme that catabolizes OT

(Giraldi A et al., 1990; Burd JM et al., 1987). Additionally, labor occurs normally in women without a functional pituitary, in rats after the administration of OT antiserum and in mice with a disrupted oxytocin gene (*Phelan JP et al., 1978; Kumaresan P et al., 1971; Nishimori K et al., 1996*).

Conversely, there is strong evidence to suggest that OT has an important role as a paracrine regulator of uterine contractility at term. OT may be secreted locally within the uterus. It has been shown that its mRNA is synthesized in maternal decidua and the fetal membranes and that decidual levels of OT mRNA are significantly elevated near labor (*Chibbar R et al., 1993*). This uterine increase in OT mRNA is also seen in pregnant rats (*Fang X et al., 1996*). In addition, human uterine contractility may be controlled by the modulation of myometrial OT receptors, which are highly up-regulated at term (*Fuchs AR et al., 1984*). This is supported by the fact that OT receptor antagonists are effective in women with threatened pre-term labor (*Goodwin TM et al., 1998*). Such increase of uterine OT receptors at term is also seen in rats and does not depend on maternal OT (*Soloff MS et al., 1979; Fang X et al., 1996; Nishimori K et al., 1996*).

Finally, OT may have a bipartite role and regulate uterine contractility not only directly but also indirectly by stimulating PG release. OT has been shown to increase decidual PG production, specifically $PGF_{2\alpha}$ (*Fuchs AR et al., 1982; Soloff MS et al., 1982; Chan WY et al., 1980)*. And PG will lead to increased plasma OT levels and increased uterine sensitivity to OT in pregnant women (*Gillespie A et al., 1972; Saldana IR et al., 1974*).

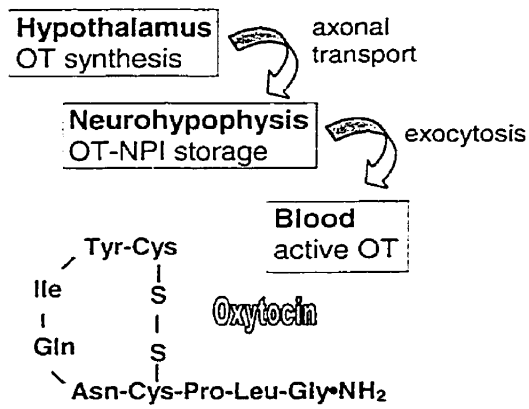

FIG. 4. **The pathway to oxytocin.** In the hypothalamus, oxytocin (OT) is synthesized and bound to neurophysin I (NPI). Via axonal transport, it reaches the neurohypophysis where it is stored. After cleavage from NPI, it is released by exocytosis from the neurohypophysis as the active nonapeptide (*modified after Pickering BT, 1994*).

THE OXYTOCIN RECEPTOR

Uterine contractions during labor are mediated by oxytocin (OT) receptors, which couple through G-proteins to the phospholipase C/Ca^{2+} signaling pathway (*Phaneuf S et al., 1995*). In late human pregnancy, uterine OT responsiveness increases with a concomitant upregulation of OT receptors (*Fuchs AR et al., 1984*). New insights into OT receptor regulation at a molecular level have been gained since its cDNA was cloned in 1992 (*Kimura T et al., 1992*).

OT receptor structure and pharmacology

Xenopus laevis oocytes are known to functionally express membrane receptors after microinjecting appropriate mRNA *(Meyerhof W et al., 1988)*. This system was used as a screening method to clone the 4.1 kb long OT receptor cDNA, which encodes a 389 amino acid protein with an estimated molecular weight of 43 kDa *(Kimura T et al., 1992)*.

The OT receptor is closely related to the vasopressin V_{1a}, V_{1b} and V_2 receptors. Naturally occuring mutations of the human V_2 receptor, mutagenesis and the use of synthetic peptide analogues have helped to shed light on the structural bases of these receptors *(Barberis C et al., 1998)*. They all belong to the superfamily of G-protein-coupled receptors characterized by seven transmembrane domains (TM) joined by alternating extracellular and intracellular loops, an extracellular N-terminal domain and an intracellular C-terminal domain. The extracellular N-terminal domain of the OT receptor was found to be glycosylated on Asn-X-Ser/Thr residues in a species-specific manner. This post-translational modification does not influence receptor function, but

might explain the larger (70 kDa) molecular weight of uterine OT receptors determined by immunoblotting than the estimated one (43 kDa) *(Kimura T et al., 1997)*. Disulfide bridges are found between conserved Cys residues in the second and third extracellular loops and within the C-terminal domain. They are necessary for correct protein folding *(Barberis C et al., 1998)*. In the V_{1a} receptor, ligand binding occurs in a pocket formed by the transmembrane domains via hydrogen bond interactions *(Mouillac B et al., 1995)*. Single amino acids are crucial for proper function of these receptors. In OT receptors, Tyr^{209} in TM V and Phe^{284} in TM VI play a key role in regulating agonist-OT receptor interaction *(Chini B et al., 1996)*. In the V_{1a} receptor, Tyr^{115} in the first extracellular loop was shown to define the agonist selectivity *(Chini B et al., 1995)*.

The binding activity of OT receptors has been characterized using radiolabeled ligands, most commonly [^{3}H]OT. More selective receptor ligands, in particular (d$(CH_2)_5$[Tyr-(Me)^2, Tyr^4, Orn^8, Tyr-NH_2^9]vasotocin ([^{125}I]OTA), have been used for detailed pharmacological characterization of OT receptors in several species *(Ivell R et al., 1997)*. A variety of synthetic peptides acting as OT agonists or antagonists have been designed over the last thirty years *(Manning M et al., 1993)*; with the latter as an approach to inhibit uterine contractions in women with preterm labor Their design has been mainly based on data coming from structure-activity studies. Few antagonists have been tested in animal models and only 1-deamino[D-Tyr(Et)^2,Tyr^4,Orn^8]OT (Atosiban®) has been administered to pregnant women and was shown to have potential clinical use (*Goodwin TM et al., 1998*). Non-peptide OT antagonists are being developed to improve oral availability and duration of action *(Bell IM et al., 1998)*.

Although, structural knowledge of the receptors has grown it is not known how the dynamic changes during ligand binding and G-protein coupling take place *(Barberis C et al., 1998)*.

OT receptor function

Upon ligand binding, the OT receptor activates a G-protein (guanosine binding protein) (Figure 5). This heterotrimeric protein complex consists of an α, β and γ subunit and is classified according to its α species *(Simon MI et al., 1991)*. The OT receptor couples to G-proteins of the $G\alpha_q$ subfamily *(Ku CY et al., 1995)*, which stimulate phospholipase C_β *(Lee CH et al., 1992)*, one of several phospholipase C isoforms *(Rhee SG, Choi KD, 1992)*. These enzymes hydrolize phosphatidylinositol-4,5-bisphosphate (PIP_2) to inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 then releases Ca^{2+} from the sarcoplasmic reticulum into the cytoplasm *(Berridge MJ, 1995)*.

Recently, it was reported that oxytocin induces the activation of mitogen–activated protein (MAP) kinase through an islet-activating protein-sensitive G-protein in cultured human myometrial cells *(Ohmichi M et al., 1995)*. The activation of G-protein-linked receptors is believed to stimulate members of the MAP kinase family, known to phosphorylate proteins on Ser/Thr/Tyr residues *(Zachary I et al., 1991; Simonson MS, Herman WH, 1993)*.

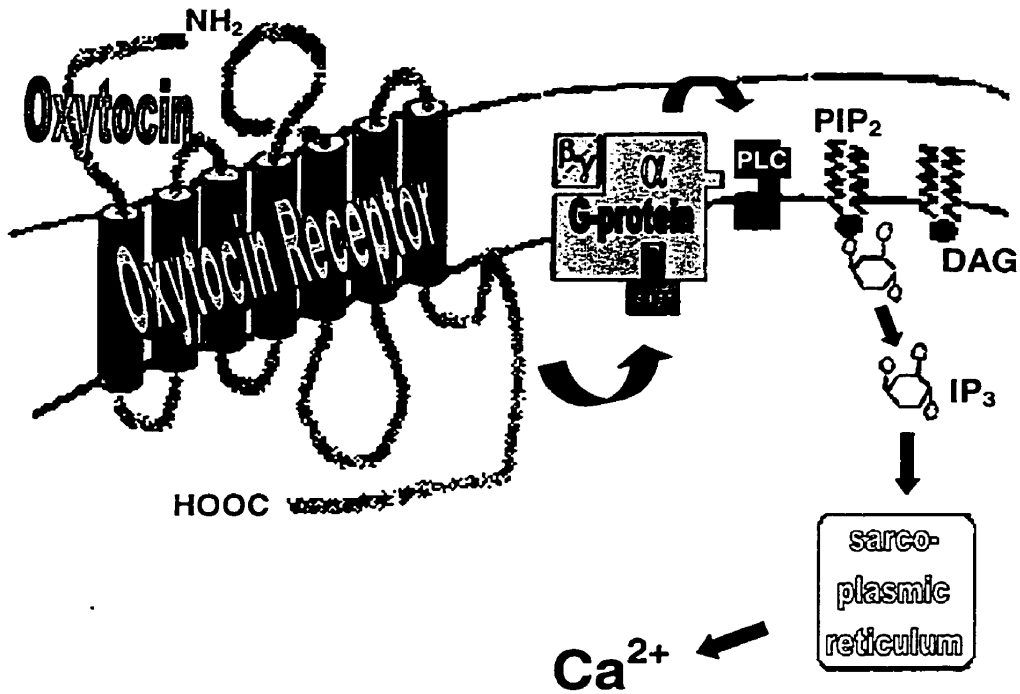

FIG. 5. **The function of the oxytocin receptor.** Upon ligand binding, the OT receptor activates a heterotrimeric G-protein. Its α subunit stimulates phospholipase C to yield diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3) from phosphatidylinositol 4,5-bisphosphate (PIP_2). IP_3 releases Ca^{2+} from the sarcoplasmatic reticulum into the cytoplasm. (*modified after Phaneuf S et al., 1995*).

OT receptor gene

The complete genomic structure of the OT receptor gene is known for humans (*Inoue T et al., 1994*) (Figure 6), bovine (*Bathgate R et al., 1995*), rats (*Rozen F et al., 1995*), mice (*Kubota Y et al., 1996*) and voles (*Young LJ et al., 1996*).

Differences in ligand binding selectivity in uterine tissues have been demonstrated and point to the existence of OT receptor isoforms *(Maggi M et al., 1990, 1992; Chen DL et al., 1994)*. However, so far their existence has not been confirmed by molecular cloning. Post-translational modifications of the receptor or the plasma membrane composition are proposed to account for the differences in ligand binding properties *(Gimpl G et al., 1995; Klein U et al., 1995)*.

The human OT receptor gene is located on chromosome 3p25 and exists as a single copy in the haploid genome *(Simmons CF et al., 1995)*. It spans about 17 kb and consists of four exons and three introns (*Inoue T et al., 1994*). Exons 1 and 2 contain the 5' UTR. Exon 3 starts 142 bp upstream from the ATG start codon spanning 922 bp downstream encoding most of the protein. Exon 4 contains the sequence encoding downstream from the seventh transmembrane domain, the C terminus, and the entire 3' UTR, including the polyadenylation signals.

In its 5'-flanking region a variety of potential nucleotide consensus sequences exist (Figure 7). These include one Sp-1 site, four invert GATA-1 sites, one c-Myb site, one activator protein-2 (AP-2) and two AP-1 sites, three nuclear factor interleukin-6 (NF-IL6) sites, two acute phase reactant-responsive elements (APRE), one nuclear factor kappa B (NFκB) site and several half-palindromic estrogen responsive elements (ERE/2). The proximal promoter region contains a TATAA-like motif, an initiator (Inr)

element, an ets gene family binding site (EBS) and a half cAMP responsive element (CRE/2) (*Inoue T et al., 1994; Hoare S et al., 1999*).

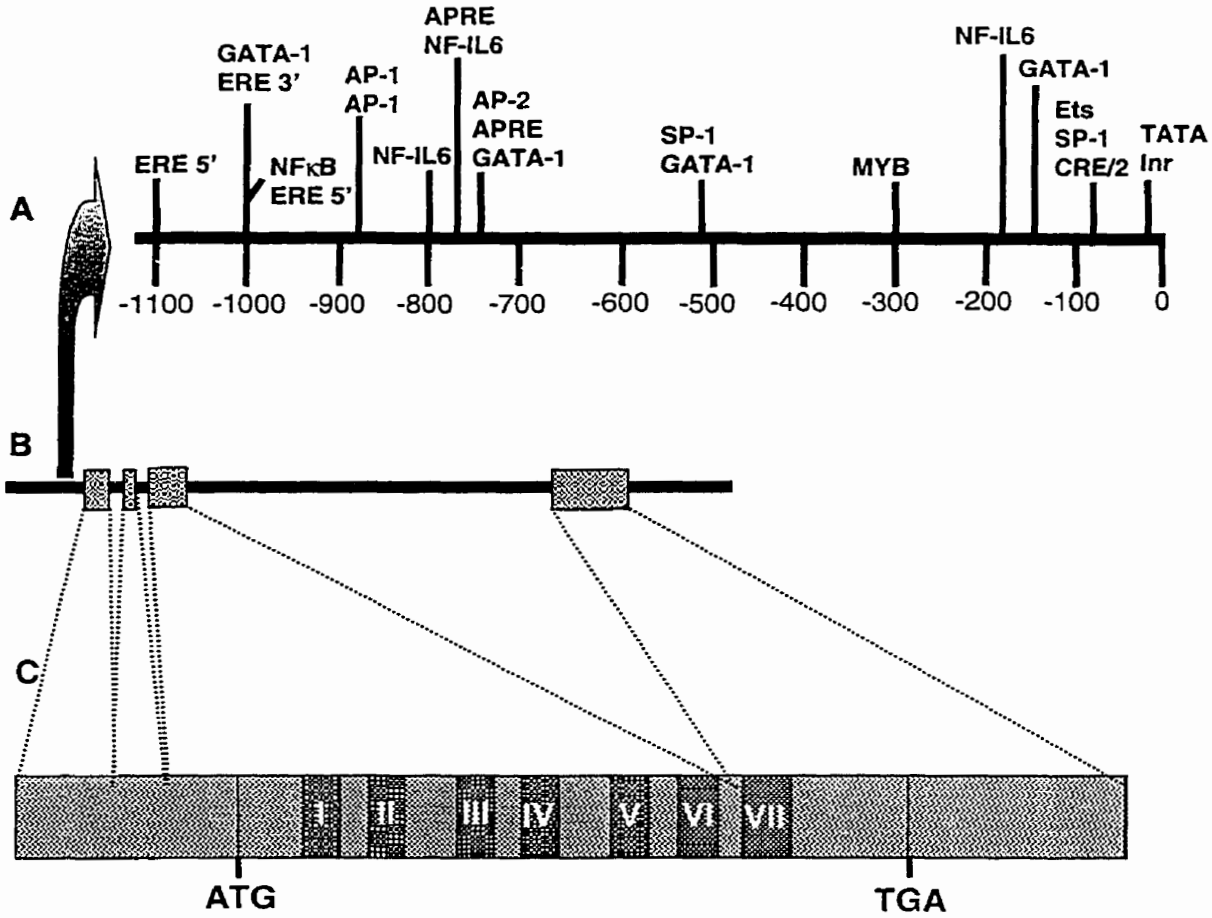

FIG. 6. **Structure of the oxytocin receptor gene.** A, The 5'-flanking region of the oxytocin receptor gene is shown with putative binding sites for transcription factors. B, The oxytocin receptor gene consists of four exons (boxes) and three introns. C, Oxytocin receptor cDNA is shown. Membrane spanning domains are represented by enclosed, numbered boxes. Translation start (ATG) and termination sites (TGA) are indicated (*modified after Inoue T et al., 1994; Hoare S, 1999*).

```
-1227                    cagct gtcttccagg agaaagaagg gcaggcttgg ttctacaggc

-1182 cagtgttttc cttctcacat tcaccggtca ggggctcata gggaaaaatc tgccttcatc
                                ERE5'
-1122 cagccgtggg gtgaggcagg ggtgttttac ctgctaggat tgaaccctca gattgcacag

-1062 attatacagt ttcagaaaac cagagggaca ggacctcaga cattagctat cttgaccagt
                                                      GATA1  ERE3"
-1002 gctctcattt tacatatgag gagactgagg cccaaagaga aggggcttgc ccaaggtcac
                                                 NFκB        ERE5'
-0942 ttcattctca cccgagtcgt ctgtaacttt aatctgtaat tttcgaggcc gataggtact

-0882 attgactaat attgattaat actgcctgcc acccccttggc aatgctgtca agattcccag
       AP-1            AP-1
-0822 cccccattct ggaatgatta ctcagctaga accctgggat ccaggtgctg taaggttggc
                NF-IL6                   APRE                NF-IL6
-0762 ccctgggata tctcggcatg gggctgtaat tgtggattaa ggaaacccag tccttggcta
       AP-2  APRE  GATA1
-0702 actcaagtct ctccacataa taaaaagacg gagaagtgaa atgtcaggag gaaatacaca

-0642 tttaatgcat tttaaagagc cctgtttatt tttgaatcct ggcctttttt tctgacttaa

-0582 ttcttggcca ctgtaaatta cttcaaaaaa tgatttttag aatagagaag gggcagggag

-0522 gctgagaagc tgtctttaac attttatctt cctttggcat catttagaat tttaattccg
       SP-1                   GATA1
-0462 aagcgcgaca aggaggcaga aacggctctt gggcgcagac aagcagaatc actttaaatg

-0402 aagacagtgt tgtgcttcag aatttcctct aaaactaccg aaaaaataac gcctcttcca

-0342 gcactgctta gaatagaggc catttctaat tcctcattaa cgggaatagg aacaaaagta
                                                MYB
-0282 ttccaaagca aagacttatt tgagttcact gctaaagccg ctacatcaag ctggaggtgt

-0222 ggggggagag aaaagcctga aaattaacat catttttggg aaataatcag tttaaatgct
                                                NF-IL6
-0162 tttgtaactt catcactatc tacccgggga agaacattat tattcaagcc tcctatgtgt
                GATA-1
-0102 ctcggagtca agagcttcta aaccaagaaa ggaagaaacg ggcgggttat tgacgagttc
                                        Ets        SP-1 *  •      CRE/2
-0042 cctccctctc gcagttttaa accactgcaa aataaaccca ttTgtTaagg ctctgggacc
                         TATA                        Inr
+0019 aacgctgggc gaaccagctc cgctccggag gggtctgcgc ggctggcctc gcccgccccc

+0079 tagcggaccc gtgcgatagt gcagcctcag ccccaggcac agcgccgcat ccagacgccg

+0139 tccgcgcgcg cagcctggga ggcgctcctc gctcgcctcc tgta
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FIG. 7. **Nucleotide sequence of the 5'-flanking region of the human OT receptor gene.** The transcription start sites are indicated by asterisk. A TATA-like motif and putative binding sites for transcription factors are enclosed by gray boxes (*modified after Inoue T et al., 1994; Hoare S et al., 1999*).

OT receptor regulation

Radiolabeled ligands and antibodies have been used to quantify uterine OT receptors whose expression is highly regulated. In human, their concentration increases over 180-fold during gestation (*Fuchs AR et al., 1984*). OT receptor mRNA expression has been examined by northern blotting and *in situ* hybridization and shown to be increased in a similar magnitude as is the protein (*Kimura T et al., 1996*) (Figure 8).

Most *in vivo* data on the OT receptor regulation come from rat experiments. In these animals, the OT receptor synthesis is mainly modulated by sex steroids. Uterine OT receptors were shown to be upregulated by estrogens and down regulated by progesterone *(Soloff MS et al., 1979; Fuchs AR et al., 1983; Soloff MS et al., 1983*). An estrogen dependent upregulation of OT receptor mRNA was seen in the hypothalamus, the kidney and the uterus (*Bale TL et al., 1995a; Ostrowski NL, Lolait SJ, 1995; Larcher A et al., 1995)*, while progesterone was shown to negatively regulate OT receptor mRNA in the uterus (*Fang X et al., 1997; Ou CW et al., 1998*).

Additionally, mechanical stretch of the rat uterus was demonstrated to cause an upregulation of OT receptor mRNA (*Ou CW et al., 1998*).

In vitro, OT receptors were decreased in bovine endometrium by interferon τ *(Horn S et al., 1998).* In human myometrial cells, OT receptor expression was inhibited by interferon α. The reduction of OT receptor mRNA levels was maximal after 2-3 days and was followed by a parallel decrease in OT binding sites with lowest levels after 3-4 days *(Maggi M et al., 1996).*

OT receptor expression was upregulated in rabbit amnion cells by forskolin and cortisol. Both agents activated OT receptor gene transcription thus leading to increased OT receptor mRNA levels and OT receptor-binding capacity *(Jeng YJ et al.,*

1995). The human breast carcinoma cell line Hs578T was shown to express functional OT receptors, which were up-regulated by FBS and dexamethasone. Up-regulation by FBS was mediated by PKC. In addition, FBS and dexamethasone increased steady-state levels of OT receptor mRNA (*Copland JA et al., 1999*).

In term human myometrium, representing the up-regulated state of OT receptors, a nuclear heteromeric protein complex was shown to exist that is able to bind a sequence in the OT receptor promoter. This sequence was named uterine stimulator motif-1 (US-1) and showed a low and orientation-dependent enhancer activity in transfected SKN cells (cell line derived from human leiomyosarcoma) (*Kimura T et al., 1999*). Recently, Ivell et al. have analyzed approximately 3 kbp of the 5'-flanking region of the human OT receptor gene for its ability to bind nuclear proteins from term myometrium. They divided this sequence into overlapping radiolabeled fragments of approximately 200 bp. Using electrophoretic mobility shift assay (EMSA), these fragments were used to screen for the presence of DNA-protein complexes associated in nuclear extracts of term myometrium. Only two fragments formed a specific DNA-protein complex and the involved cis-elements unknown (*Ivell R et al., 1998*). Surprisingly, no other specific protein-DNA complexes were formed although many potential binding sites for trans-acting factors have been identified in this promoter region (*Inoue T et al., 1994; Hoare S et al., 1999*)

Using promoter-reporter studies, it has been shown that basal and serum induced expression of the OT receptor gene requires a GABP binding site. In addition, the heterodimer GABPα/β seems to upregulate OT receptor gene expression in a synergistic way with AP-1 (*Hoare S et al., 1999*). The rat OT receptor promoter displays a basal transcriptional activity in MCF7 and SK-N-SH cells but can be

markedly induced by forskolin and phorbol 12-myristate 13-acetate (PMA). This induction involves a CRE and a half-SRE element and possibly PKA and/or PKC *(Bale T, Dorsa DM, 1997).* A high degree of constitutive basal activity was also shown for the human OT receptor promoter when HeLa or COS7 cells were transfected. Estradiol treatment of these transfected cells did not up-regulate the OT receptor gene although several ERE half sites are found in the OT receptor promoter within a few kb of the transcription start site *(Ivell R et al., 1998).*

In addition to the regulation of the OT receptor gene via trans-acting factors at the promoter, DNA methylation may play a role. A region near the middle of the third intron of the human OT receptor was found hypomethylated in term myometrium, which highly expresses the OT receptor gene. In tissues not expressing OT receptors, this region is hypermethylated and binds nuclear proteins associated with a suppression of the OT receptor promoter activity (*Mizumoto Y et al., 1997*).

In addition to the regulation of OT receptors at the mRNA level other mechanisms have been shown to exist. Ligand binding does not only desensitize the receptor with a concomitant down-regulation of OT receptor mRNA but also decreases the number of expressed OT receptors by dislocating them from the cell surface to the inside of the cell *(Phaneuf S et al., 1997; Adachi S, Oku M, 1995).*

Finally, it has been recently shown that the function of the rat OT receptor can be affected by progesterone or its metabolites in a non-genomic way. Progesterone was shown to inhibit OT binding to OT receptors *in vitro*, to bind rat OT receptors expressed in CHO cells and to suppress OT-induced inositol phosphate production and calcium mobilization *(Grazzini E et al., 1998).*

Response elements to mediators of inflammation are present in the 5'-flanking region of the OT receptor gene. Consensus sequences present in the OT receptor promoter include (Figure 7): three NF-IL6 (nuclear factor interleukin-6) binding sites at –179/-187, -769/-777 and –808/-816 and two APREs (acute phase reactant-responsive elements) at –755/-760 and –784/-789 *(Inoue T et al., 1994)*. This suggests that the corresponding trans-acting factors regulate the expression of the OT receptor gene. Consensus sequences typically present in genes for acute phase proteins exist. These genes are rapidly upregulated by pro-inflammatory cytokines, including IL-1β and IL-6. However, the role of these cytokines in the induction of the OT receptor gene at the molecular level has not been studied to date and will therefore be investigated.

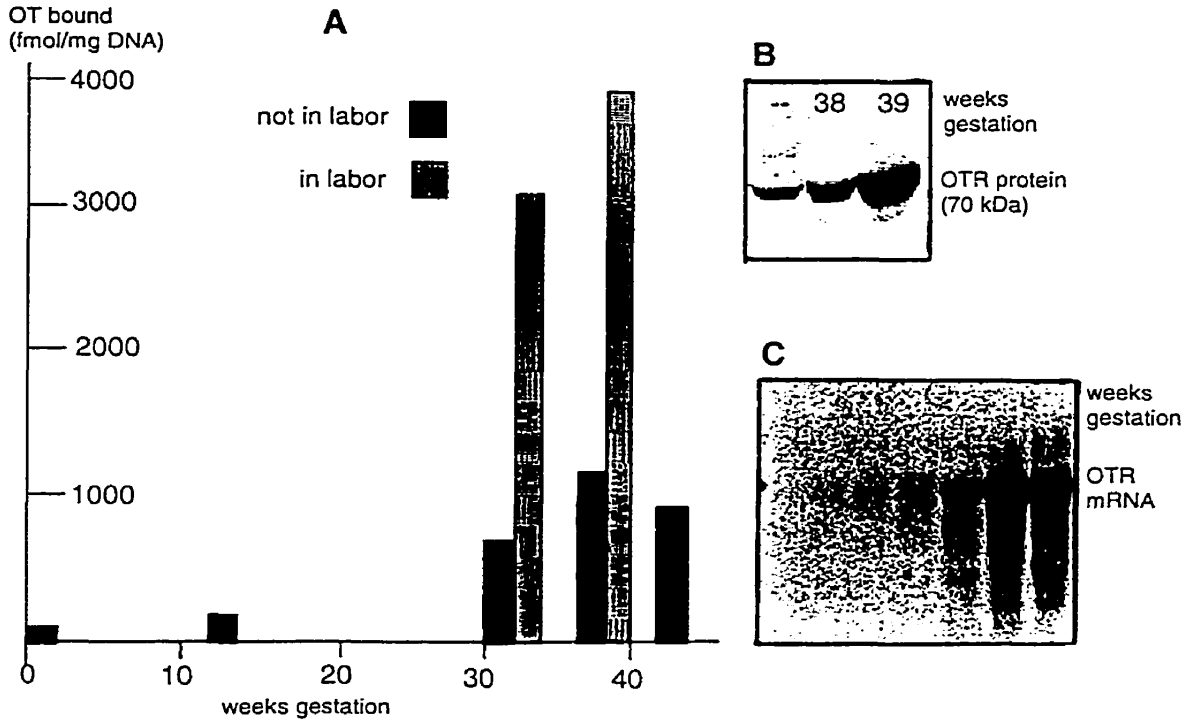

FIG. 8. **Changes in myometrial OT receptor and its mRNA during pregnancy.** A, Significant increase of myometrial OT binding sites at the end of pregnancy (*modified after Fuchs AR, Fuchs F, 1993*). B, Western blot of the OT receptor in nonpregnant (-) and term myometrium using the primary antibody, 2F8. OT receptor was detected at 70 kDa and its amount was increased in term myometrium. C, Northern blot of OTR mRNA in myometrium during pregnancy using a ^{32}P-dCTP labeled 0.8 kb fragment of human OTR cDNA as probe (*modified after Kimura T et al., 1996*)

THE IMMUNE SYSTEM AND PARTURITION

The maternal-fetal interface is known as an immunologically active site. During pregnancy, antibody mediated immunity is enhanced while cell mediated immunity is diminished. This creates a cytokine environment that is favorable for the semi-allogenic fetus (*Robertson SA, Seamark RF and Guilbert LJ, Wegmann TG, 1994*).

Cytokines: regulators of myometrial contractions at term ?

Cytokines may be involved in the activation of the pregnant myometrium. The pro-inflammatory cytokines IL-1β, IL-6 and TNF-α have been demonstrated to be critical in the pathophysiology of infection-associated preterm labor *(Romero R et al., 1991; Dudley DJ et al., 1994)*. But even without infection, their concentrations in amniotic fluid and maternal serum increase with labor at term (*Opsjon SL et al., 1993; Steinborn A et al., 1996*).

Throughout normal gestation, bone marrow-derived immunocompetent cells are present in uterine tissues. They include macrophages, large granular lymphocytes and T cells. In term decidua, they become especially abundant and account for approximately half of the decidual cells (*Hunt JS, 1989*; *Vince GS et al., 1990*).

Placental tissues after spontaneous labor produce more cytokines in culture than tissues after term cesarean section with no labor, suggesting a role for cytokines as regulators of labor (*Steinborn A et al., 1995*). Decidual explants and monolayer cultures not only produce IL-1β and IL-6 (*Romero R et al., 1989; Dudley DJ et al., 1992*) but IL-1β was also shown to stimulate decidua and chorion cells to produce IL-6 (*Dudley DJ et al., 1992*). Interleukins can also increase PG production in cultures of

fetal membranes and decidua (*Mitchell MD et al., 1991*). IL-1β stimulated PGE_2 production in cultures of amnion-derived WISH and decidua cells (*Albert TJ et al., 1994*; *Kennard EA et al., 1995*). As reviewed by Mitchell et al., the underlying mechanism of this increased PG synthesis appears to be an up-regulation of the key enzymes for PG synthesis, PLA_2 and PGHS-2. This prostaglandin production by cytokine stimulated gestational tissues may represent one way, in which cytokines are linked with parturition (*Mitchell MD et al., 1995*).

Cytokine signaling

Cytokines are regulatory peptides that act via specific membrane receptors. In most cases, ligand binding leads to homo- or heterodimerization of receptor molecules. This complex formation triggers signal transduction pathways eventually leading to altered gene expression (*Taga T et al., 1993*).

The mechanism of action of IL-6 provides an example of cytokine signaling (Figure 9). Members of the IL-6 family of cytokines act via receptor complexes that share the gp130 molecule. This component is required to transduce the extracellular cytokine signal to the inside of the cell. Receptors for IL-6 cytokines and gp130 belong to the class I receptor family and share in their extracellular region a conserved domain of approximately 200 aa residues comprising two fibronectin type III modules. This domain appears to be crucial for ternary structure formation. Since gp130 is expressed in many cells, the cellular response to a specific cytokine is likely determined by the regulated expression of cytokine specific receptors and/or the spatial and temporal expression of the specific cytokine (*Taga T, Kishimoto T, 1997*).

The JAK/STAT signaling pathway: The Janus kinase (JAK)/signal transducers and activators of transcription (STAT) pathway is used by the IL-6 family of cytokines. Upon binding of an IL-6 cytokine to its cognate receptor this cytokine/cytokine receptor complex induces the homodimerization of gp130 (Figure 10).

Intracellular JAKs that are already associated with gp130 become activated. Four mammalian members are known to exist: JAK1-JAK3 and Tyk2 (*Briscoe J et al., 1996*). They possess several tandem kinase and pseudokinase domains. Activated JAKs phosphorylate cytoplasmic receptor domains on tyrosine residues thus creating docking sites for SH2 (Src homology 2) proteins, which in turn are also JAK substrates. Examples of JAK substrates include the adaptor molecules Shc and Grb2 and the STAT family of transcription factors.

The MAPK signaling pathway: Cytokine induced gp130 dimerization leads to phosphorylation of docking sites on gp130 for the SH2 domain-containing adaptor proteins Shc and Grb2. Grb2 recruits SOS which activates a G-protein, such as Ras. This G-protein activates a MAPKK kinase initiating the MAPK (mitogen activated protein kinase) pathway consisting of a three-kinase cascade (Figure 11): a MAPKK kinase, such as Raf, activates a MAPK kinase, such as MEK (MAPK/ERK kinase), which in turn activates a MAPK, such as ERK (extracellular regulated protein kinase). Most MAPKs are activated by phosphorylation on one tyrosine and one threonine residue. In turn, they phosphorylate target proteins, such as transcription factors, on Ser/Thr/Tyr residues (*Robinson MJ et al., 1997*).

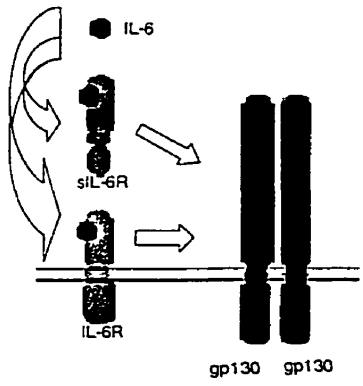

FIG. 9. **The IL-6 receptor (IL-6R) and gp130.** Both molecules, IL-6 and gp130, belong to the class I cytokine receptor family. IL-6 can bind to soluble and membrane-anchored IL-6R. Both complexes IL-6/sIL-6R and IL-6/IL-6R can induce gp130 homodimer formation (*modified after Taga T, Kishimoto T, 1997*).

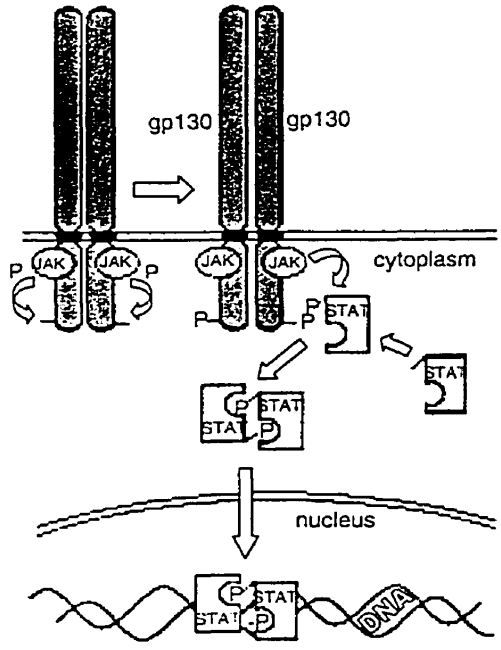

FIG. 10. **The JAK/STAT signaling pathway.** Cytokine induced homodimerization of gp130 activates intracellular JAKs already associated with the receptor. JAKs phosphorylate receptor-tyrosines providing docking sites for STATs, which bind via their SH2 domains. STATs are then phosphorylated by a JAK, form homo- or heterodimers and transclocate to the nucleus (*modified after Yasukawa H et al., 2000*)

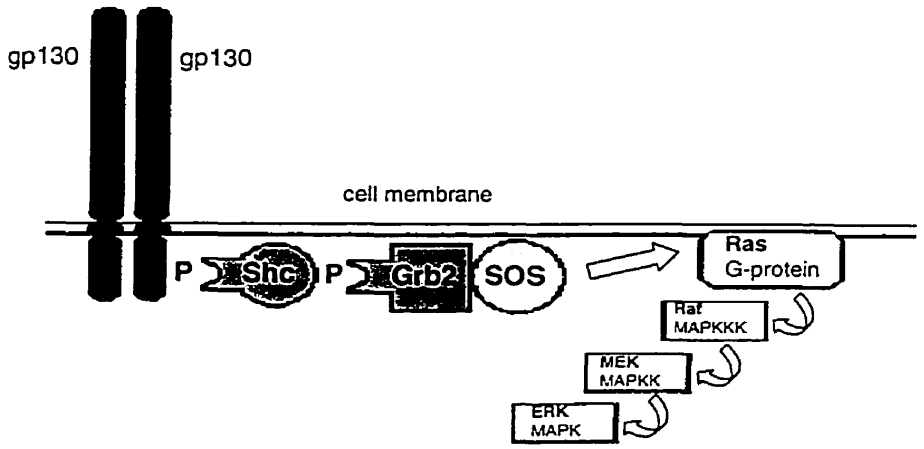

FIG. 11. **The MAPkinase pathway.** Ligand binding leads to gp130 dimerization and autophosphorylation at Tyr. Resulting phosphotyrosine residues on intracellular receptor domains serve as sites for SH2 domain-containing proteins such as Shc and Grb2 adapter proteins. Grb2 recruits SOS which activates Ras, a G-protein. Ras activates Raf kinase initiating a kinase cascade resulting in stimulation of ERK activity (*modified after van Biesen T et al., 1996*)

Transcription factors: The study of IFN-inducible genes led to the discovery of **STATs.** Seven members have been identified so far: STAT1-STAT4, STAT5A, STAT5B and STAT6. Upon phosphorylation by cytokine receptor associated JAKs, STATs dissociate from the receptor and form homo- and hetero-dimers, which translocate to the nucleus (*Darnell JE Jr., 1997; Horvath CM, Darnell JE Jr., 1997*). Most STAT dimers bind semipalindromic motifs, TTCNNNGAA or TTCNNNNGAA, which are known as GAS motifs (INFγ-activated sequences). STATs are inactivated by dephosphorylation, degradation or by inhibition through proteins including the cytokine inducible SH2 protein (CIS). Genes regulated by STAT5 include CIS, oncostatin M, an immediate early gene induced by multiple cytokines, and IL-2Rα. STAT 6 is known to regulate the genes of MHC class II, CD23 and IL-4Rα proteins (*Leonard WJ, O'Shea JJ, 1998*).

The transcription factor **nuclear factor IL-6 (NF-IL6)** was discovered as an IL-1 inducible factor that interacts with an IL-1 responsive element in the IL-6 gene (*Isshiki H et al., 1990*). It is known that NF-IL6 exists in all tissues at very low levels, but is induced by LPS, IL-1, IL-6 or TNFα (*Akira S, Kishimoto T, 1997*). NF-IL6, also known as C/EBPβ, belongs to the CCAAT box/enhancer binding protein (C/EBP) family with the consensus binding sequence T(T/G)NNGNNAA(T/G) (*Akira S et al., 1990*). All family members share a common approximately 80 amino acid carboxy-terminal basic domain. This so-called leucine zipper motif is known to mediate dimerization, DNA binding and has transregulating activity. Different signaling pathways activate NF-IL6: In addition to serine and threonine sites that get phosphorylated by MAP kinase and protein kinase A (PKA), a calcium/calmodulin-dependent kinase site can be found in NF-IL6 *(Akira S, Kishimoto T, 1997)*. NF-IL6 was shown to bind *in vitro* the regulatory sites in a variety of genes, including those for acute phase p roteins (*Akira S, Kishimoto*

T, 1992), c-fos (*Metz R, Ziff E, 1991*), pregnancy-specific glycoprotein (*Chen H et al., 1995*), iNOS (*Nunokawa Y et al., 1994*), PGHS-2 (*Inoue H et al., 1995*) and placental lactogen (*Stephanou A, Handwerger S, 1995*).

The transcription factor **nuclear factor kappa B (NF-κB)** was discovered as an enhancer-binding protein involved in the regulation of Igκ-light chain gene expression in B cells (*Sen R, Baltimore D, 1986*). It is now known to be ubiquitously expressed in many cell types. NF-κB belongs to the Rel family of transcriptional activators and functions as a homo- or heterodimer. Family members share an approximately 300 amino acid Rel homology domain. This region mediates DNA binding, dimerization and nuclear localization (*McKay LI, Cidlowski JA, 1999*). NF-κB exists in the cytosol as an inactive Rel dimer, bound to an inhibitor of the I-κB family of proteins (Figure 12). They share multiple ankyrin repeat motifs involved in the interaction between NF-κB and I-κB, an acidic region and a PEST sequence (pro-glu/asp-ser-thr), both located in the C terminus (*Verma IM et al., 1995*). NF-κB is activated by phosphorylation of I-κB at two serine residues. Phosphorylated I-κB is a target for ubiquitination, which leads to its degradation by proteasomes. Nuclear localization signals are now unmasked and the NF-κB dimer enters the nucleus. The active form of NF-κB is most often formed of p50/p65, which has a high affinity for the consensus NF-κB DNA sequence 5'-GGGRNNYYCC-3' (*Baldwin AS Jr, 1996*). NF-κB can be activated by various stimuli including IL-1β. The mechanism of this effect may involve the activation of an acid sphingomyelinase through DAG production by PLC. Sphingomyelinase then releases ceramide, which activates NF-κB (*Saklatvala J, 1995)*.

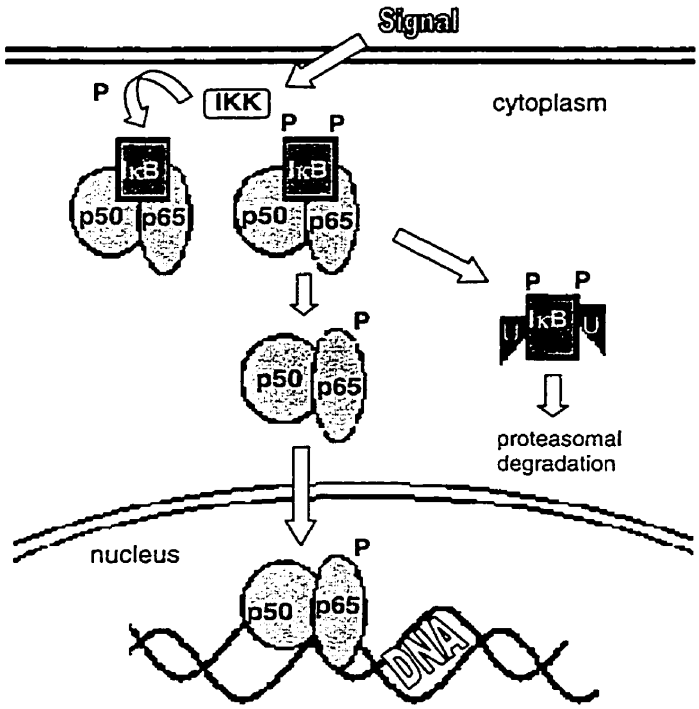

FIG. 12. **Activation of NFκB.** Inactive NFκB heterodimers , often composed of p50/p65, are retained in cytoplasm by the inhibitor IκB. Upon an activating signal, IκB kinase (IKK) phosphorylates IκB, which thus becomes a target for ubiquitination and therefore proteasomal degradation. The activated NFκB heterodimer is now able to enter the nucleus and to modulate gene transcription (*modified after McKay LI, Cidlowski JA, 1999*)

Interaction among transcription factors: Gene transcription may be regulated by protein-protein interactions between different transcription factors. Many factors that interact with NF-κB have been identified, including proteins of the basal transcription machinery, members of the C/EBP and STAT family of transcription factors. McKay and Cidlowski propose that, in the case of the interaction between members of the NF-κB and C/EBP families, the transcriptional effect depends on which members interact and the arrangement of their DNA binding sites in the gene they regulate. Synergism is displayed in the presence of C/EBP binding sites. However, transcriptional activity decreases when NF-κB and C/EBP interact and only NF-κB binding sites exist (*McKay L, Cidlowski JA, 1999*). Such antagonistic effect was observed in the rat angiotensinogen gene. NF-κB and NF-IL6 were shown to compete for overlapping binding sites in the DNA (*Brasier AR et al., 1990*). However, on the promoter for IL-6 and IL-8, these two transcription factors showed a synergistic transactivation, which may have happened through cooperative DNA binding (*Matsusaka T et al., 1993; Stein B, Baldwin AS Jr, 1993*).

Interactions between NF-κB and the STAT family of transcription factors was shown for the E selectin gene expression. STAT6 repressed the action of NF-κB, supposedly by competing for overlapping binding sites (*Bennett BL et al., 1997*). In mouse fibroblasts, STAT1 and NF-κB synergistically induce gene transcription of proinflammatory cytokines (*Ohmori Y et al., 1997*).

When NF-κB interacts with AP-1, a heterodimeric transcription factor consisting of the proteins Jun and Fos, the transactivation function of both was enhanced (*Stein B et al., 1993*).

The review of McKay and Cidlowski describes NF-κB interactions with steroid hormone receptors. In particular, they shed light on the interaction between NF-κB and the glucocorticoid receptor-signaling pathway. They even suggest that the NF-κB-GR antagonism found at a cellular level may represent an important mechanism regulating immune homeostasis (*McKay LI, Cidlowski JA, 1999*).

Interactions between NF-IL6 and numerous transcription factors have been reviewed by Akira and Kishimoto (*Akira S, Kishimoto T, 1997*). NF-IL6 was shown to cooperate with AP-1 through a promoter region containing NF-IL6 and AP-1 binding sites. Interestingly, NF-IL6 can bind to both NF-IL6 and AP-1 sites. On the contrary, AP-1 cannot bind to most NF-IL6 sites. *In vitro*, the bZIP region of NF-IL6 interacts with the bZIP regions of Fos and Jun (*Hsu W et al., 1994*). Furthermore, interactions occur between NF-IL6 and the general transcription factor SP-1, GATA and Myb (*Akira S, Kishimoto T, 1997*).

In summary, although OT does not seem to be essential for parturition, the modulation of its receptor may be an important mechanism involved in the activation of the uterus. In late human pregnancy, uterine responsiveness to OT is markedly increased and coincides with a rise in both OT receptor protein and its mRNA. The 5'-flanking region of the OT receptor gene contains response elements for nuclear factor IL-6 (NF-IL6), which are usually found in cytokine induced genes. Pro-inflammatory cytokines have been associated with the pathophysiology of infection-associated preterm labor. Although the cDNA of the human OT receptor gene was cloned in 1992 to date no studies on the transcriptional regulation of the OT receptor gene by pro-inflammatory cytokines have been carried out.

OBJECTIVE AND HYPOTHESIS

The **objective** of our research was to assess the potential role of IL-1β and IL-6 in the transcriptional regulation of the human OT receptor gene, *in vitro*.

We **hypothesized** that IL-1β and IL-6 induce OT receptor gene expression in human myometrial cells and this is mediated by the transcription factor nuclear factor IL-6 (NF-IL6) and cognate response elements in the 5'-flanking region of the OT receptor gene.

MATERIAL AND METHODS

CELL CULTURE

The human myometrial cell line ULTR (*Perez-Reyes N et al., 1992*) was kindly provided by Dr. J. K. Mc Dougall's laboratory (Fred Hutchinson Cancer Research Center, Seattle, WA). This cell line originates from myometrium of a non–pregnant woman and was immortalized with a retroviral vector containing the E6/E7 open reading frames of human papillomavirus type 16. HeLa cells were used for transient transfection experiments.

Both cell lines were grown in phenol red-free Dulbecco's modified Eagle's medium (D-MEM, Gibco, Rockville, WI), supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin sulfate and 250 ng/ml amphotericin B (Gibco, Rockville, WI) at 37°C in humidified 5% CO_2/95% air.

CYTOKINE TREATMENT OF ULTR CELLS

For cytokine stimulation, ULTR cells were grown to 70-90% confluence and kept over night in phenol red-free D-MEM without serum. In RPA experiments, cells (~5-7.5 x 10^5) were incubated for 1.5, 3 and 6 h in fresh phenol red-free D-MEM in the presence or absence of IL-1β and IL-6 at 10 ng/ml each. In EMSA experiments, cells (~4 x 10^6) were incubated for 0.25 and 1 h in fresh phenol red-free D-MEM in the presence or absence of 10 ng/ml IL-1β and 100 ng/ml IL-6. Cytokine concentrations were chosen based on results from preliminary studies.

RIBONUCLEASE PROTECTION ASSAY (RPA)

RNA isolation: Total RNA was extracted from cells using Trizol reagent according to the supplier's instructions (Gibco, Rockville, WI). RNA concentrations and purities were assessed by measuring their optical densities at 260 nm and 280 nm, respectively.

Preparation of template DNA: The DNA template for the synthesis of the human OT receptor riboprobe had been prepared in our laboratory. Briefly, using an RT-PCR kit (Invitrogen, Carlsbad, CA) and human decidual RNA, a 237 bp fragment corresponding to a region extending from the third to the fifth transmembrane domain coding sequence was amplified. The primers used were 5'-CTA CCT GCT GCT GCT CAT GTC-3' (sense) and 5'-AGC GTG ATC CAT GTG ATG TAG G-3' (antisense) (*Helmer H et al, 1995*). The fragment was subcloned into pCR-Script using a cloning kit (Stratagene, La Jolla, CA) and linearized with BamHI. A linearized plasmid template of human cyclophilin inserted into the pTRIPLEscript vector (Ambion, Austin, TX) was used to generate a probe that served as an internal standard.

***In vitro* transcription:** Antisense riboprobes for human OT receptor (333 bp) and cyclophilin (138 bp) were generated using T3 RNA polymerase (Promega, Madison, WI) and [α-^{32}P] CTP (Amersham, UK). The reaction mixture of 10 µl contained 1x transcription buffer (40 mM Tris-Cl; 8 mM $MgCl_2$; 2 mM spermidine; 50 mM NaCl), 1.2 U/µl placental RNase inhibitor (RNasin, Promega, Madison, WI), 6 mM dithiothreitol (Promega, Madison, WI), 0.5 mM of each ATP, GTP, and UTP, 100 µCi [α-^{32}P] CTP, 1 µg of the linearized DNA template and 17 U/µl T3 RNA polymerase and was incubated at 37°C for 1 h. The DNA template was digested by adding 6 µl of RNase-free DNase I (10-50x 10^3 U/ml) (Boehringer Mannheim) and incubated 20 min at 37°C. To extract the labeled cRNA probe, 100 µl 1x TE (10 mM Tris, pH 7,4; 1 mM EDTA), 4 µl carrier

tRNA (50 μg/ml) and 150 μl Tris-saturated phenol and chloroform containing isoamyl alcohol were added. The reaction was vortexed and centrifuged for 5 min. For precipitation, 50 μl 1xTE, 50 μl ammonium acetate (10 mM) and 800 μl 95% (v/v) ethanol were added. The reaction was vortexed and incubated at -20°C overnight. The precipitated cRNA was pelleted by centrifugation for 15 min, dried at room temperature for 5-10 min and resuspended in 3 μl 1xTE and 16 μl loading buffer (80% v/v formamide; 1 mM EDTA, pH 8; 0.1% (w/v) bromphenol blue; 0.1% (w/v) xylene cyanol). Labeled cRNA probe was then separated from unincorporated nucleotides by electrophoresis at 350 V in 1x TBE (89 mM Tris, 89 mM boric acid, 2 mM EDTA; pH 8.0) on a pre-warmed 6% (w/v) denaturing polyacrylamide gel. After electrophoresis, the band of interest was excised from the gel with a scalpel, collected in a 1.5 ml microfuge tube and eluted from the gel in 400μl prewarmed buffer (2 M ammonium acetate; 1% (w/v) SDS; 25 μg/ml tRNA) for 3 h at 37°C. After ethanol precipitation, the probe was dried at room temperature, redissolved in 3 μl TE and taken up in 100 μl hybridization buffer (40 mM PIPES, pH 6.4; 0.4 M NaCl; 1 mM EDTA; 80% (v/v) formamide).

Hybridization: Total RNA (3-5 μg) was ethanol precipitated in the presence of 40 μg tRNA. In each experiment a sample containing tRNA alone served as negative control. The vacuum dried precipitates were redissolved in 2 μl 1x TE. Hybridization was then carried out in a 30 μl reaction containing RNA samples in 2 μl 1x TE, $3x10^5$ cpm OTR and cyclophilin riboprobes in hybridization buffer. Microfuge tubes were sealed with parafilm and incubated for at least 14 h at 55°C.

RNAse treatment: To remove free probe, 0.75 μg RNase A and 300 U RNase T1 were added and reactions were incubated for 30 min at 30°C in buffer containing 10

mM Tris-Cl (pH 7.5), 300 mM NaCl and 5 mM EDTA. RNases were inactivated by adding 100 μg proteinase K in the presence of 0.6% (w/v) sodium dodecyl sulfate for 20 min at 37°C. After a phenol-chloroform extraction, hybridized [^{32}P] RNA fragments were dried, dissolved in loading buffer and electrophoresed on a pre-warmed 6% denaturing polyacrylamide gel. The gel was dried and exposed to Kodak XAR-5 film. Exposure times were chosen to give RNA band intensities in a linear range. The intensity of the RNA bands was then quantified using the SCION IMAGE program (Scion Corporation, NIH, 1998) and normalized to cyclophilin mRNA.

Data analysis: OTR densitometric values were divided by those of cyclophilin measured in the same RNA preparations. Data were analyzed using ANOVA and Tukey-Kramer Multiple Comparisons Test.

NUCLEAR EXTRACTS

After cytokine treatment, nuclear proteins were isolated as described by Schreiber et al. (*Schreiber E et al., 1989*). Briefly, myocyte monolayers were rinsed three times with ice-cold PBS and scraped in PBS in 1.5 ml microfuge tubes. Cells were pelleted by centrifugation at 1500 g for 3 min. Pellets were resuspended in 400 μl of buffer A (10 mM HEPES, pH 7.9; 10 mM KCl; 0.1 mM EGTA; 1 mM dithiothreitol; 1 mM phenylmethylsulfonylfluoride) and swelled for 15 min at 4°C. Twenty-five μl of 10% (v/v) NP-40 were added and tubes were vortexed. Homogenates were centrifuged 30 sec at 13,800g in a microfuge. The nuclear pellets were resuspended in 50 μl ice-cold buffer C (20 mM HEPES, pH 7.9; 0.4 M NaCl; 1 mM EDTA; 1 mM EGTA; 1 mM dithiothreitol; 1mM phenylmethylsulfonyl-fluoride) and the extracts were vigorously

rocked at 4°C for 15min. The nuclear extracts were then centrifuged for 5 min at 13,800g in a microfuge at 4°C and the supernatant was stored at -70°C.

Concentrations of nuclear proteins in extracts were determined with the Micro BCA™ protein assay reagent as recommended by the supplier (Pierce Chemical Company, Rockford, IL).

ELECTROPHORETIC MOBILITY SHIFT ASSAY (EMSA)

The NUSHIFT kit (Geneka, Montreal, Quebec) was used to screen for the presence of nucleofactor-IL6 (NF-IL6) in nuclear extracts of cytokine stimulated myometrial cells. The double-stranded DNA probe provided had the following sequence with the consensus motif shown in italics: CTA GGG C*TT GCG CAA T*CT ATA TTC G. This nucleotide motif is recognized by different members of the C/EBP family of leucine zipper (bZIP) transcription factors including C/EBPα, C/EBPβ (NF-IL6), C/EBPδ and C/EBPε (bandshift). To specifically show the presence of NF-IL6, nuclear extracts were incubated with rabbit polyclonal antibody against NF-IL6 (supershift).

Radiolabeling of the oligonucleotide probe: To generate radioactive probes, 2 µl (50 ng) oligonucleotide probe, 4 µl (40 µCi) of γ-[^{32}P] ATP (3000 Ci/mM) (New England Nuclear-DuPont, Boston, MA), 1 µl (10 U) T_4 polynucleotide kinase (Promega, Madison, WI) and sterile water to a final volume of 20 µl were incubated for 30 min at 37°C. T_4 polynucleotide kinase was inactivated by adding 5 µl 1% (w/v) SDS/100 mM EDTA. The ^{32}P-labeled probe was purified on a Micro Spin™ G_{25} column (Geneka, Montreal, Quebec, CA).

Formation of antibody-NF-IL6 complex: For the supershift assay, nuclear extracts were incubated for 20 min on ice in the presence or absence of rabbit NF-IL6 polyclonal antibody in buffer provided by the kit (Geneka, Montreal, Quebec, CA).

Binding of C/EBP nuclear proteins to the labeled oligonucleotide probe: A probe premix containing approximately 10^5 cpm of the labeled oligonucleotide probe in a buffer provided by the kit was added to the pre-incubated extract/antibody premix. Nuclear extract from rat liver tissue was used as positive control (Geneka, Montreal, Quebec, CA). Everything was mixed and incubated for 20 min on ice.

Gel electrophoresis and band detection: After the binding reaction, the entire mix was electrophoresed on a 5% (w/v) nondenaturing polyacrylamide gel, pre-cooled to 4° in 1x TGE buffer (6.06 g Tris-base, 28.54 g glycine, 0.78 g EDTA in 1l deionized water; pH 8.5). After electrophoresis, the gel was dried and exposed to Kodak XAR-5 film for suitable exposure times.

PROMOTER CONSTRUCTS

The OT receptor promoter construct and its 5'-deletions were prepared by PCR using selected sense/antisense primers (Figure 13). The cDNA template (Dr. T. Kimura, Osaka, University, Medical School, Osaka, Japan) ranged from approximately 3 kb upstream of the transcription start site to the middle of the third intron. The first transcriptional start site (*Inoue T et al, 1994*) was assigned to be +1. The 5' end primers consisted of 20-22 nucleotides complementary to the bottom strand of the template. Upstream of the matched sequence a HindIII restriction site was attached, flanked by 4 nucleotides. The sense primer sequences were: ATC GAA GCT TGG CAG GCT TGG TTC TAC AGG, ATC GAA GCT TCT GTA ATT TTC GAG CCG ATA

G, ATC GAA GCT TGG AAA CCC AGT CCT TGGCTA and ATC GAA GCT TGC GCA GAC AAG CAG AAT CAC. The 3' end primer consisted of 20 nucleotides complementary to the top strand, upstream of which was a XbaI restriction site with 4 flanking nucleotides. Its sequence was: ATC GTC TAG ACT GAG GCT GCA CTA TCG CAC. All constructs had the same 3' end since the sam 3' end primer was used in each of the PCR reactions. DNA was amplified in a 100 µl reaction consisting of 10 µl 10x polymerase buffer, 200 µM each of dNTP, 1.5 mM $MgCl_2$, 0.2 µM each of the primers, 2 ng of cDNA template and 5 U of Platinum Taq DNA Polymerase (Gibco, Rockville, WI). After denaturing the template for 1 min at 94°C DNA was amplified through 30 cycles for 1 min at 94°C, 1 min at 58°C and 2min at 72°C. A final extension step was done at 72°C for 6 min. PCR reactions were electrophoresed on a 1% (w/v) agarose gel in TAE buffer (2 M Tris-HCl, 250 mM sodium acetate, 1 M EDTA) at 120 V and yielded the following products (Figure 13): a 1.31 kbp fragment (-1203 bp/+108 bp), a 1.01 kbp fragment (-909 bp/+108 bp), an 830 bp fragment (-722 bp/+108 bp) and a 511 bp fragment (-403 bp/+108 bp). Bands of interest were excised with a scalpel and DNA was eluted from the gel with a kit (Promega, Madison, WI). The purified products were resuspended in H_2O and digested with HindIII and XbaI for 1 h at 37°C. Enzymes were inactivated by heating at 65°C for 15min and each product was inserted in the promoterless pCAT®-Basic Vector (Promega, Madison, WI) upstream of the CAT cDNA.

The reporter vector was opened at the polylinker with HindIII and XbaI (Gibco, Rockville, WI) and dephosphorylated with Calf Intestinal Alkaline Phosphatase according to the supplier's instructions (Gibco, Rockville, WI). Then, the vector was purified on a 1% (w/v) low-melting agarose gel followed by elution with a kit as

recommended by the manufacturer (Promega, Madison, WI). The ligation reaction was done with T_4 Ligase (Gibco, Rockville, WI) overnight at 12°C according to the supplier's protocol. Competent HB101 were transformed with 10 µl of a five fold dilution of the ligation reaction. Bacteria were then grown in LB medium for 1 h at 37°C and streaked on LB plates containing 100 µg/ml ampicillin. Bacterial colonies were transferred to sterile 15 ml tubes with 2 ml LB medium containing 100 µg/ml ampicillin and grown overnight at 37°C. Plasmid DNA was extracted with the QIAGEN minipreparation technique (QIAGEN, Germany). To confirm cloning of the PCR products into the pCAT®-Basic Vector, the plasmid DNA was digested with HindIII and XbaI and electrophoresed on a 1% agarose gel at 120V (Figure 13). From clones containing the PCR products endotoxin free DNA was prepared on a large scale with a Plasmid Maxi Kit (QIAGEN, Germany) and was used for transient transfections.

TRANSIENT TRANSFECTIONS

Initially, ULTR cells were transiently transfected with the FUGENE6 transfection reagent. However, the promoter activity of the full length human OT receptor promoter assessed with the CAT system was low. HeLa cells were evaluated in following transfections using FUGENE6. As compared to ULTR cells, CAT activity of full length human OT receptor promoter was higher in HeLa cells. Therefore, HeLa cells were used in the following transfection studies.

Transient transfections of HeLa cells were performed in 6-well plates using FuGENE6 transfection reagent (Boehringer Mannheim, Germany). Cells were plated into culture dishes one day before transfection and transfected at approximately 70% confluency. Three µl of FuGENE6 reagent were incubated with 97 µl of serum free medium for 5

min at room temperature. Diluted FuGENE6 was added to 1 µg plasmid construct and 1 µg pSV-ß-galactosidase vector (Promega, Madison, WI) and incubated for 15 min at room temperature. The FuGENE6-DNA complex was added dropwise to the cells covered with 2 ml serum-containing medium. At 5 and 24 h post-transfection, medium was exchanged for medium alone or with IL-1ß (10 ng/ml) or IL-6 (10 ng/ml), respectively. Forty-eight hours post-transfection, cells were harvested with 300µl reporter lysis buffer (Promega, Madison, WI). Cell lysates were vortexed for 10-15sec and split in two equal portions; one for CAT assay, the other for ß-galactosidase assay. The former was heated at 60°C for 10 min to inactivate endogenous deacetylase. Then, all lysates were centrifuged for 2 min in a microcentrifuge and the supernatant was assayed.

For the CAT assay, 100 µl of cell lysate were incubated with 3 µl [^{14}C] chloramphenicol (50 µCi/ml) (Amersham, UK), 5 µl n-butyryl Coenzyme A (5 mg/ml) and 17 µl H_2O for up to 20 h at 37°C. After a quick centrifugation, reactions were split in two portions; one for analysis by thin layer chromatography (TLC), the other for analysis by liquid scintillation counting (LSC). Five hundred µl ethyl acetate were added to the former and 300 µl mixed xylenes to the latter. For the TLC assay, samples were vortexed for 1 min and centrifuged for 3 min in a microcentrifuge. The organic phase was transferred to a fresh tube and evaporated to dryness. The residue was resuspended in 20 µl ethyl acetate and spotted on a TLC plate (Silica Gel 60A, Whatman, USA) and dried. The plate was run in a closed chamber pre-equilibrated for 1hour with chloroform/methanol (95:5) until the solvent was at least half-way up the plate. The dried plate was covered with plastic wrap and exposed with intensifying screen to an x-ray film (Kodak XAR 5, Rochester, NY) for 7 days at room temperature. For the LSC assay, samples were

vortexed for 30 sec and centrifuged for 3 min. One hundred µl of fresh 0.25 M Tris-HCl , pH 8.0 were added to the xylene phase. Again, samples were vortexed for 30 sec and centrifuged for 3min at top speed in a microcentrifuge (Eppendorf, Germany). Two hundred µl of the xylene phase were counted in 2 ml scintillation cocktail (Scinti Safe Plus, Fisher Scientific, USA) in a scintillation counter (LS5000TD, Beckman Instruments, USA).

For the ß-galactosidase assay, 30 µl lysate were mixed with 20 µl reporter lysis buffer (Promega, USA) or 50 µl undiluted lysate and pipetted into the wells of a 96 well plate. 50 µl of 2x Assay Buffer were added to each well and the plate was incubated at 37°C for 30 min or until a faint yellow color had developed. One hundred and fifty µl 1M sodium carbonate were added to each well to stop the reaction and the absorbance of the samples was read at 405 nm in a UV_{max} Kinetic Microplate Reader (Molecular Devices Corp., USA).

Data analysis: CAT values were normalized to β-gal activity from a co-transfected β-gal plasmid and divided by protein the protein concentration. Data were analyzed using ANOVA and Tukey-Kramer Multiple Comparisons Test.

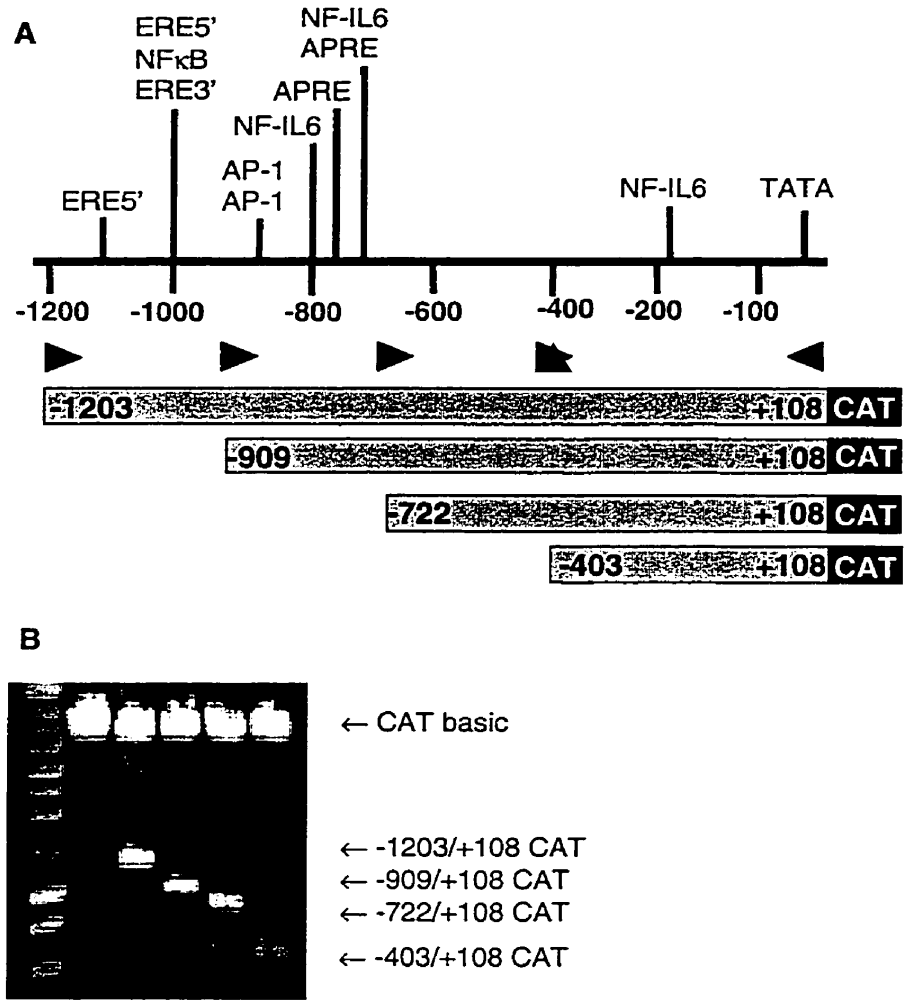

FIG. 13. **5'-deletion constructs by polymerase chain reaction (PCR).** A, 1.3 kb of the 5'-flanking region of the OT receptor gene with putative binding sites for transcription factors is shown. Desired 5'-deletions were obtained using different forward primers (▶) and the same reverse primer (◀). The fragments were approximately 1.3 kb, 1 kb, 0.83 kb and 0.54 kb in length and were inserted in the pCAT Basic vector upstream of the CAT reporter. B, Densitometric scan of ethidium bromide-stained 1% agarose gel. pCATBasic vector deletion constructs were digested with HindIII/XbaI. The vector runs at approximately 4.3 kb. The cloned products run at 1.3 kb, 1 kb, 0.83 kb and 0.54 kb.

RESULTS

RIBONUCLEASE PROTECTION ANALYSIS

The effects of IL-1β and IL-6 treatment on hOTR mRNA levels are shown in Figure 14 and Figure 15, respectively. Using the RPA, we detected a protected band for hOTR at 237 bp as expected. In response to both, IL-1β and IL-6, we found a decrease in hOTR mRNA. IL-1β treatment significantly ($p<0.05$) decreased hOTR mRNA levels after 3 h (Figure 14). IL-6 treatment significantly ($p<0.05$) decreased hOTR mRNA levels at 1.5 and 3 h (Figure 15). At 6 h, hOTR mRNA levels were significantly ($p<0.05$) decreased to approximately 25-30% of levels at time 0 in response to both, IL-1β and IL-6.

DETECTION OF NF-IL6 BY EMSA

The detection of NF-IL6 (C/EBPβ) using a ^{32}P-end labeled C/EBP consensus motif (Geneka, Dallas, TX) as probe and nuclear extracts from ULTR cells treated with IL-1β (10 ng/ml) or IL-6 (100 ng/ml) for 0.25 and 1 h is shown in Figure 16 and Figure 17, respectively. With or without IL-1β or IL-6 stimulation, a DNA-protein complex was detected. The addition of anti-NF-IL6 antibody created a supershift in extracts of both IL-1β or IL-6 stimulated and unstimulated ULTR cells. Preliminary experiments, showed no changes over time in unstimulated controls (not shown). This supershift suggests the presence of NF-IL6 within this DNA-protein complex and seems stronger in nuclear extracts of IL-1β or IL-6 treated ULTR cells as compared to 0h control cells.

5'-DELETION ANALYSIS

The results of the 5'-deletion constructs and transfection experiments are shown in Figure 18. The level of CAT expression decreased significantly ($p<0.05$) when the sequence between –1203 and –909 was deleted. When the sequence between –909 and -722 was deleted another significant decrease ($p<0.05$) in the level of CAT expression occured as compared to both the –1203/+108 and the –909/+108 construct. The deletion of –722 to –403 led to a decrease in the level of CAT activity, which was significant from both the –1203/+108 and the –909/+108 construct but not from the –722/+108 construct.

To determine whether cytokine responsive regions are present in the hOTR promoter, the effects of IL-1ß and IL-6 on CAT activity of 5'-deletion constructs of the hOTR promoter transfected in HeLa cells were investigated (Figure 19). For both, the-1203/+108 and the –909/+108 construct, CAT activity was significantly ($p<0.05$) decreased by IL-1ß and IL-6 treatment of transfected HeLa cells, as compared to untreated cells. With both constructs, -1203/+108 and –909/+108, no significant difference was seen between CAT activity of cells treated with IL-1ß as compared to cells treated with IL-6. Neither IL-1ß nor IL-6 treatment showed a significant effect on CAT activity of cells transfected with either the –722/+108 or the –403/+108 construct.

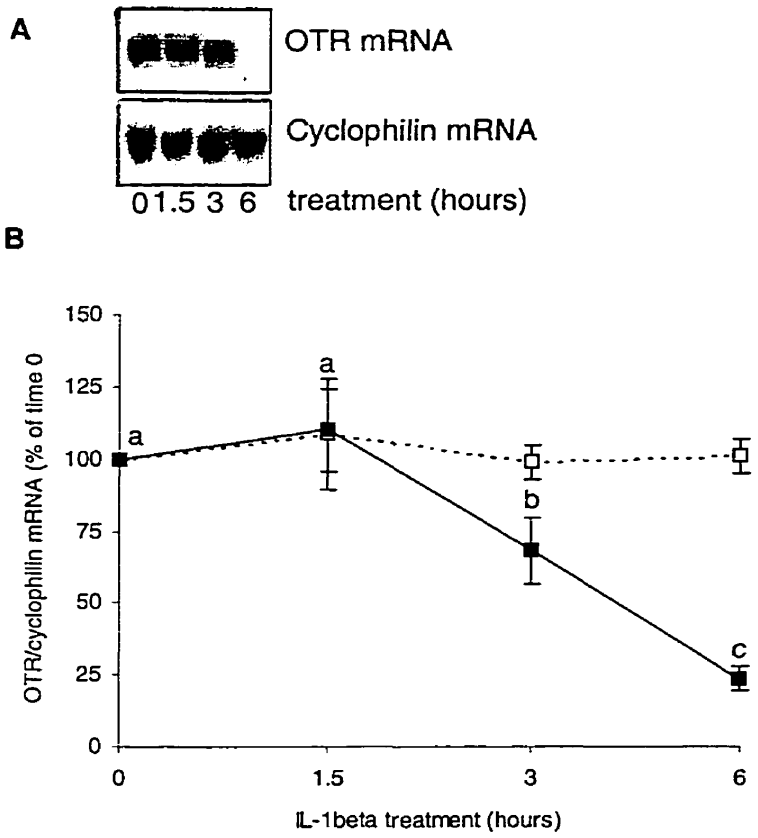

FIG. 14. **Effect of IL-1ß (10 ng/ml) treatment for varying time intervals (1.5, 3 or 6 h) on hOTR mRNA levels in ULTR cells.** RNase protection analysis: Total RNA was extracted from ULTR cells, hybridized with hOTR antisense riboprobe and analyzed as described in MATERIAL AND METHODS. A, representative autoradiography of a 6% (w/v) polyacrylamide gel. B, graphical representation of RNase protection analysis of IL-1β treated (■) and control (□) cells. RNA band intensities were quantified as described in MATERIAL AND METHODS. Results are shown as mean ± SE (n=6) and expressed as percent of time 0. Points with different superscripts are significantly different from each other ($p<0.05$).

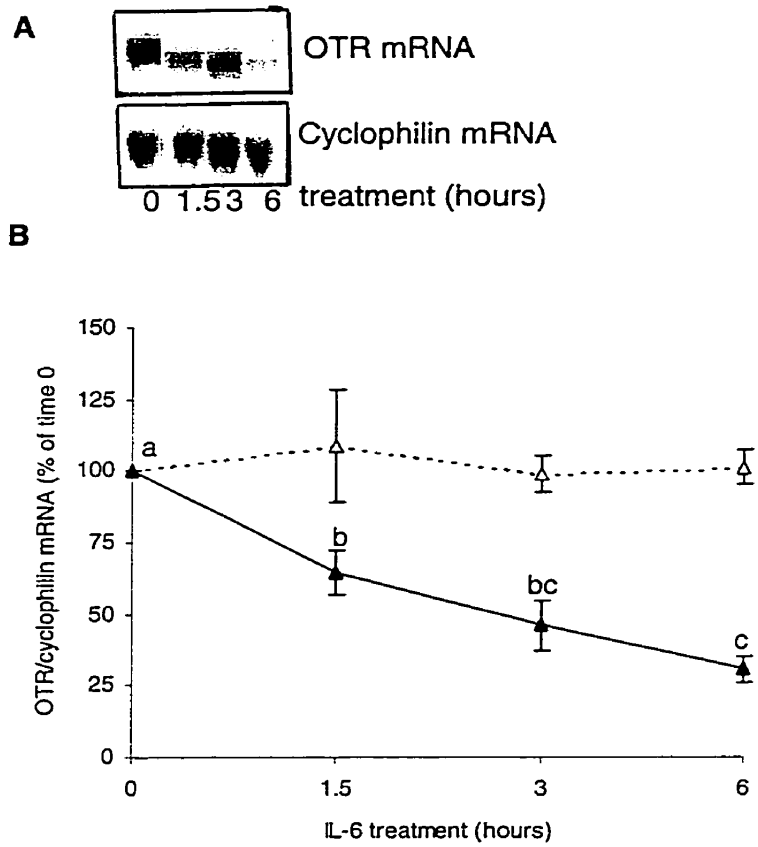

FIG. 15. **Effect of IL-6 (10 ng/ml) treatment for varying time intervals (1.5, 3 or 6 h) on hOTR mRNA levels in ULTR cells.** A, RNase protection analysis: Total RNA was extracted from ULTR cells, hybridized with hOTR antisense riboprobe and analyzed as described in MATERIAL and METHODS. A representative autoradiography of a 6% (w/v) polyacrylamide gel is shown. B, graphical representation of RNase protection analysis of IL-6 treated (▲) and control (Δ) cells. RNA band intensities were quantified as described in MATERIAL AND METHODS. Results are shown as mean ± SE (n=6) and expressed as percent of time 0. Points with different superscripts are significantly different from each other ($p<0.05$).

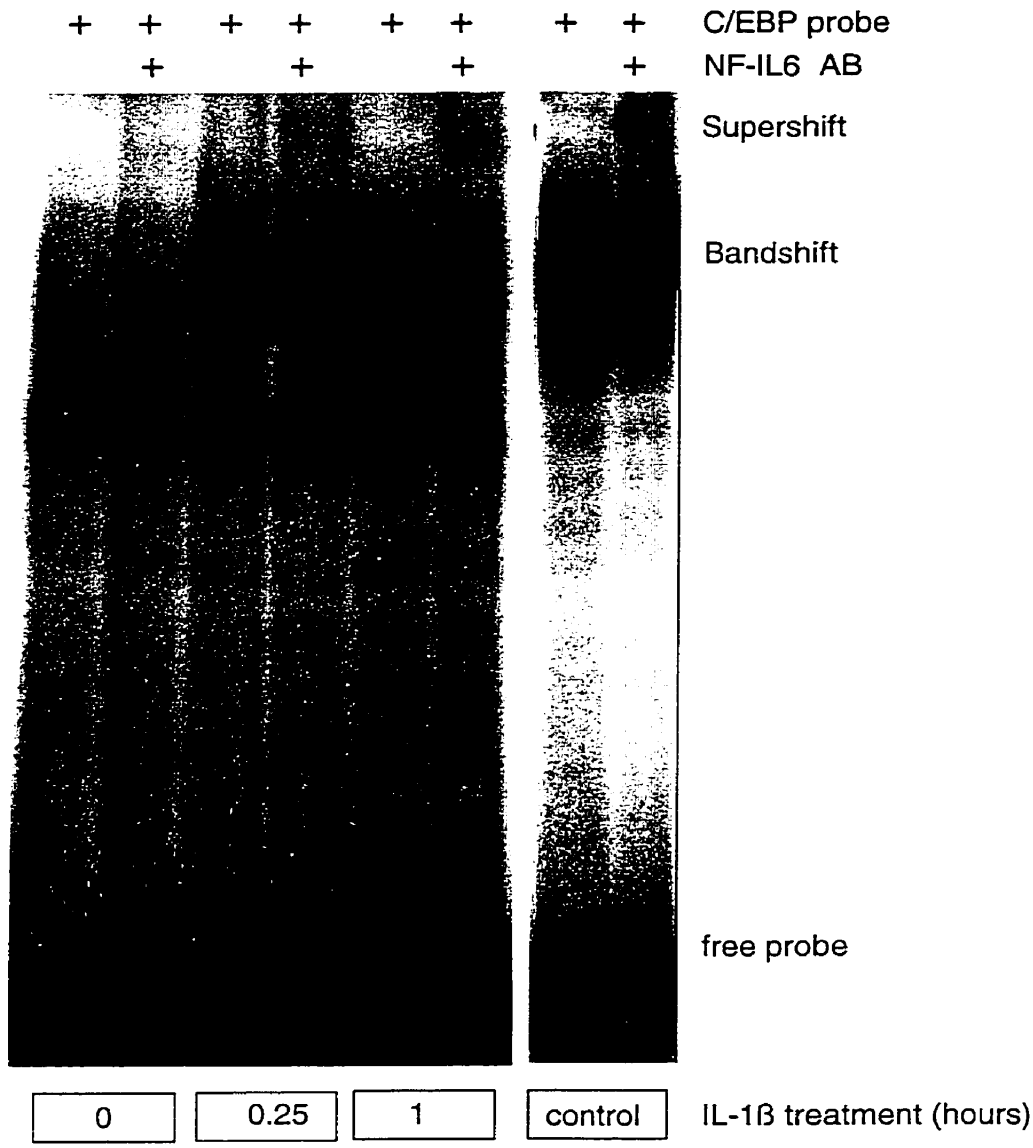

FIG. 16. **Detection of NF-IL6 (C/EBPß) binding to a C/EBP probe by EMSA in nuclear extracts of ULTR cells treated with IL-1ß.** Using nuclear extracts of ULTR cells treated for 0, 0.25 and 1 h with IL-1ß (10 ng/ml) and a consensus C/EBP motif as probe, a DNA-protein complex was identified. The addition of anti-NF-IL6 antibody created a supershift suggesting the presence of NF-IL6 within this DNA-protein complex. Nuclear extract from rat liver tissue served as control.

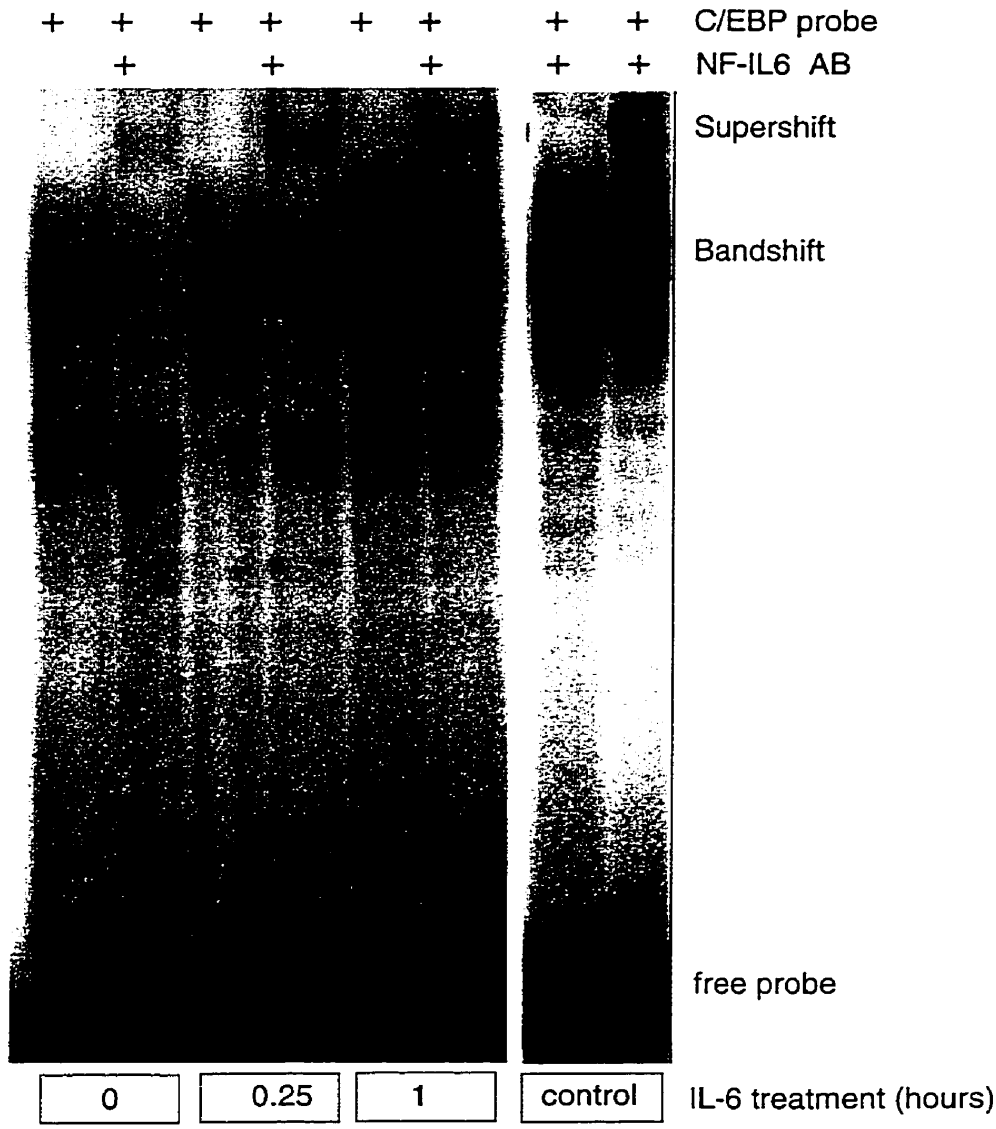

FIG. 17. **Detection of NF-IL6 (C/EBPß) binding to a C/EBP probe by EMSA in nuclear extracts of ULTR cells treated with IL-6.** Using nuclear extracts of ULTR cells treated for 0, 0.25 and 1 h with IL-6 (100 ng/ml) and a consensus C/EBP motif as probe, a DNA-protein complex was identified. The addition of anti-NF-IL6 antibody created a supershift suggesting the presence of NF-IL6 within this DNA-protein complex. Nuclear extract from rat liver tissue served as control.

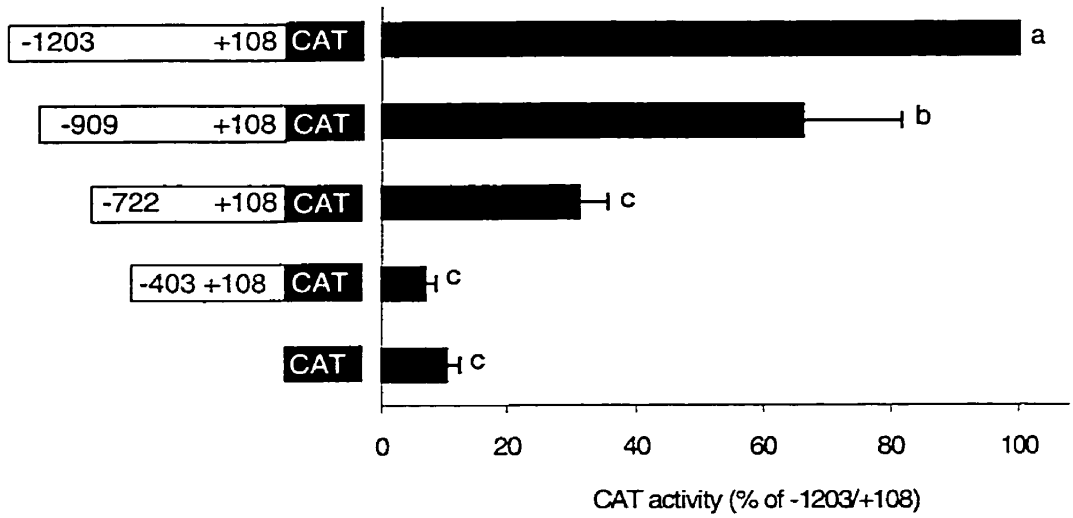

FIG. 18. **Effect of 5'-deletions of the hOTR gene promoter on CAT activity in HeLa cells.** Deletion constructs were linked to a CAT reporter and transfected into HeLa cells. CAT values were normalized to ß-gal activity from a cotransfected ß-gal plasmid and expressed as percent change ±SE (n=5), relative to the -1203/+108 construct. Data were analyzed using ANOVA and Tukey Kramer Multiple Comparisons Test. Histograms with different letters were significantly different from each other ($p<0.05$).

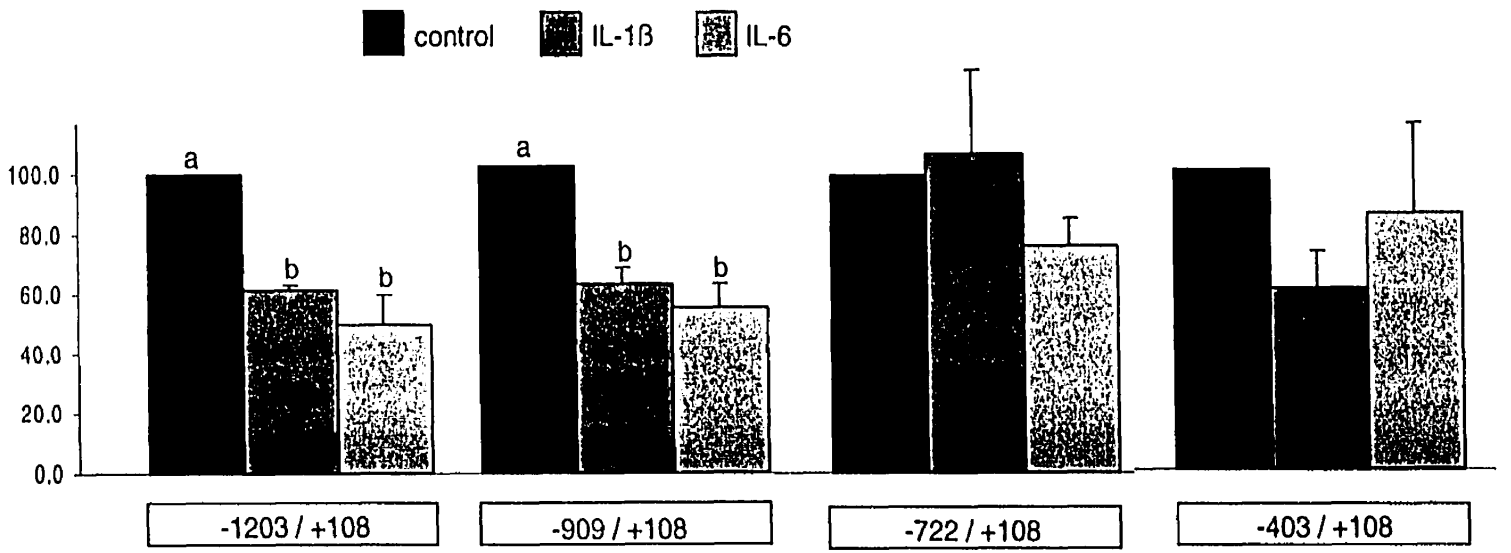

FIG. 19. **Effects of IL-1ß and IL-6 on CAT activity of 5'-deletion constructs of the hOTR promoter transfected in HeLa cells.** Deletion constructs (-1203/+108, -909/+108, -722/+108, -403/+108) were linked to a CAT reporter and transfected into HeLa cells. After transfection, cells were treated for 48 h with IL-1ß (10 ng/ml) or IL-6 (10 ng/ml), respectively. CAT values were normalized to ß-gal activity from a cotransfected ß-gal plasmid and expressed as percent change ±SE (n=4), relative to the -1203/+108 construct. Where applicable, data were analyzed using ANOVA and Tukey Kramer Multiple Comparisons Test. Points with different superscripts were significantly different from each other ($p<0.05$).

DISCUSSION

Uterine contractions are mediated by oxytocin receptors, which are highly expressed in human myometrium at term. The significant upregulation occurs principally at the transcriptional level by mechanisms that are still poorly understood. However, the structure of the OT receptor gene promoter region implicates a role for pro-inflammatory cytokines as modulators of OT receptor gene transcription.

It is very likely that different external signals are transduced into a myometrial smooth muscle cell and that various signaling cascades become activated. The transducers of these cascades may interact with one another at more proximal or more distal levels. Generally, the promoter regions of eukaryotic genes consist of multiple binding sites for transcriptional activators and repressors that act in combination to regulate the expression of a specific gene. In the 5'-flanking region of the OT receptor gene several putative binding sites for the nuclear factor IL-6 can be found among various other cis-acting elements. There are several mechanisms through which NF-IL6 could be involved in the regulation of the OT receptor gene. NF-IL6 could be activated by post translational modifications and directly act on the OT receptor promoter or it could recruit co-regulators to the promoter.

We have used ULTR cells as a model to study the regulation of OT receptor mRNA synthesis by IL-1β and IL-6. This cell line originates from non-pregnant human myometrium and was immortalized with a retroviral vector containing the E6/E7 open reading frames of human papillomavirus type 16 (*Perez-Reyes N et al., 1992*). The cells produce OT receptor mRNA but have not been demonstrated to express functional OT receptors. Contrary to our hypothesis, we have shown that treatment of ULTR cells with either IL-1β or IL-6 significantly decreases OT receptor mRNA within a few hours. These studies were performed using a cell line as model. These cells lack

tissue integrity and therefore may behave differently from myometrial cells found *in vivo*. In this regard, IL-6 was recently shown to significantly increase OT receptor mRNA in uterine explants from pregnant rats (*Fang X et al., in press*). Interestingly, in these experiments, no effect of IL-6 was seen when explants from non-pregnant animals were incubated with IL-6. These results suggest that the response to cytokines may depend on the presence of the intact tissue matrix and to the endocrine environment.

When compared to findings by an other group (*Helmer H et al., 1998*) we have obtained the same effect for IL-1β. However, while they observed an up-regulation of OT receptor mRNA by IL-6 we found a significant down-regulation. The discrepancies between the results may be related to the use of different cells and cell culture conditions. We used ULTR cells while they used primary uterine smooth muscle cells and PHM1-41 cells (*Monga M et al., 1996*).

A down-regulation of OT receptor mRNA in myometrial cells has been reported by two other groups. Maggi et al. have shown that INF-α decreases OT receptor mRNA in cultured human myometrial cells in a time- and dose- dependent way. They found that OT mRNA levels are maximally inhibited 2-3 days post-treatment (*Maggi M et al., 1996*). Although, all three cytokines, IL-1β, IL-6 and INF-α, decrease mRNA for OT receptors in myometrial cells the time frame is different. This may suggest that the mechanisms leading to this suppression are different or again the cell context is crucial.

Phaneuf et al. have reported a down-regulation of OT receptor mRNA during receptor desensitation in human primary myometrial cells. Such agonist-induced decrease is

common for many receptor mRNAs but occurs within different time intervals depending on the cell type (*Phaneuf S et al., 1997*).

IL-1β or IL-6 treatment of ULTR cells could decrease OT receptor mRNA levels through transcriptional suppression of the OT receptor gene or through activation of factors responsible for mRNA destabilization. Mizimoto et al. have reported that the third intron of the OT receptor gene contains an element that may mediate transcriptional suppression of the human OT receptor gene. This region is hypomethylated in term myometrium, which highly expresses the OT receptor gene. In tissues not expressing OT receptors, it is hypermethylated and binds nuclear proteins associated with a suppression of the OT receptor promoter activity (*Mizimoto Y et al., 1997*). IL-1β or IL-6 treatment of ULTR cells may alter the methylation pattern of the OT receptor gene and thus lead to its transcriptional suppression.

Alternatively, IL-1β or IL-6 could activate cell mechanisms leading to a destabilization of OT receptor mRNA. It is known that AU-rich sequences in the 3' UTR mediate mRNA degradation by RNases (*Zubiaga AM et al., 1995; Wennborg A et al., 1995*). Such AU-rich elements can be found in the 3'-UTR of the human OT receptor gene (*Kimura T et al., 1992*). Therefore, IL-1β or IL-6 treatment of ULTR cells may induce RNases or increase their synthesis resulting in OT receptor mRNA destabilization.

Using anti NF-IL6 antibodies in supershift experiments, we have shown that NF-IL6, is present in nuclear extracts from ULTR cells, with or without cytokine treatment. However, a short stimulation (15 min, 1 h) of ULTR cells with either IL-1β or IL-6 appears to increase NF-IL6 levels. Normally, this transcription factor is found in all tissues at very low levels but can rapidly be induced by LPS and inflammatory cytokines, including IL-1β and IL-6 (*Akira S, Kishimoto T, 1997*).

We have not demonstrated that the increase in NF-IL6 levels actually causes changes in the transcription of the OT receptor gene. Additionally, post-translational modifications, such as phosphorylation of NF-IL6 could increase its DNA binding activity thus modulating OT receptor gene transcription. Finally, NF-IL6 may not directly bind to the OT receptor promoter but form complexes with other transcription factors. This possibility is supported by a detailed analysis of about 3000 nucleotides of the 5'-flanking region of the OT receptor (*Ivell R et al., 1998*). By using EMSA, nuclear extracts of term myometrium representing the up-regulated state of OT receptors were compared to non-pregnant one. In term myometrium, only two fragments were found to form specific DNA-protein complexes and the involved cis elements did not include binding sites for NF-IL6.

We have functionally characterized 1.3 kb (-1203/+108) of the promoter of the human OT receptor gene by using 5'-deletion mutants and transfection experiments in HeLa cells, which do not express OT receptor mRNA. We have shown that this promoter exhibits basal transcriptional activity in HeLa. Contrary to our hypothesis but in keeping with our experiments using ULTR cells, we have shown that elements within the –1203/-722 region of the OT receptor promoter mediate the suppression of promoter activity in HeLa cells treated either with IL-1β or IL-6.

It is important to note that the specific chromatin structure found in the natural context of the cell nucleus likely does not exist in our promoter constructs. Therefore, it may not be appropriate to extrapolate our *in vitro* findings to the *in vivo* environment.

In future studies, this –1203/-722 region may be further characterized and factors binding to it may be identified. The signaling pathways of IL-1β and IL-6 and the presence of binding sites for NFκB and APRF in this –1203/-722 region (*Kimura T et*

al., 1994; Hoare S et al., 1999) suggest NFκB and APRF as candidates mediating the actions of IL-1β and IL-6 (Figure 20).

Our findings that the OT receptor promoter exhibits basal transcriptional activity is in agreement with results from studies done by other groups using OT receptor promoters from rat or human in different cell lines (*Bale T, Dorsa DM, 1997; Ivell R et al., 1998; Kimura T et al., 1998*). This basal transcriptional activity of the human OT receptor promoter occurs independently of whether cells naturally express OT receptor mRNA (*Kimura T et al., 1998*). This suggests that the OT receptor promoter is constitutively active due to the presence of general transcription factors required for basal transcription and the absence of suppressors. However, in non-pregnant myometrium or during the myometrial quiescent phase of pregnancy, representing the down-regulated state of the OT receptor, it is possible that inhibitory mechanisms suppress OT receptor promoter activity. If so, activation of the myometrium prior to labor may involve de-repression rather than, or in addition to stimulation of transcription of the OT receptor gene promoter.

In conclusion, we have demonstrated, contrary to our hypothesis, that IL-1β and IL-6 treatment of immortalized human myometrial cells results in a significant decrease in OT receptor mRNA within a few hours of treatment. We have also shown that, in human immortalized myometrial cells, the ubiquitous transcription factor NF-IL6 is present at low levels and appears to be increased by IL-1β and IL-6 treatment. Furthermore, we have demonstrated that the 5'-flanking region of the OT receptor gene is functional in HeLa cells and again, contrary to what we hypothesized, treatment with IL-1β and IL-6 of transfected HeLa cells decreased promoter activity. Taking these findings together, we conclude that IL-1β and IL-6 may be involved in the

transcriptional regulation of the OT receptor gene. Better insight into the mechanism of action of how these cytokines in modulation of OT receptor gene expression requires a more detailed characterization of the –1203/-722 region of the OT receptor promoter and an identification of trans-acting factors able to bind OT receptor DNA.

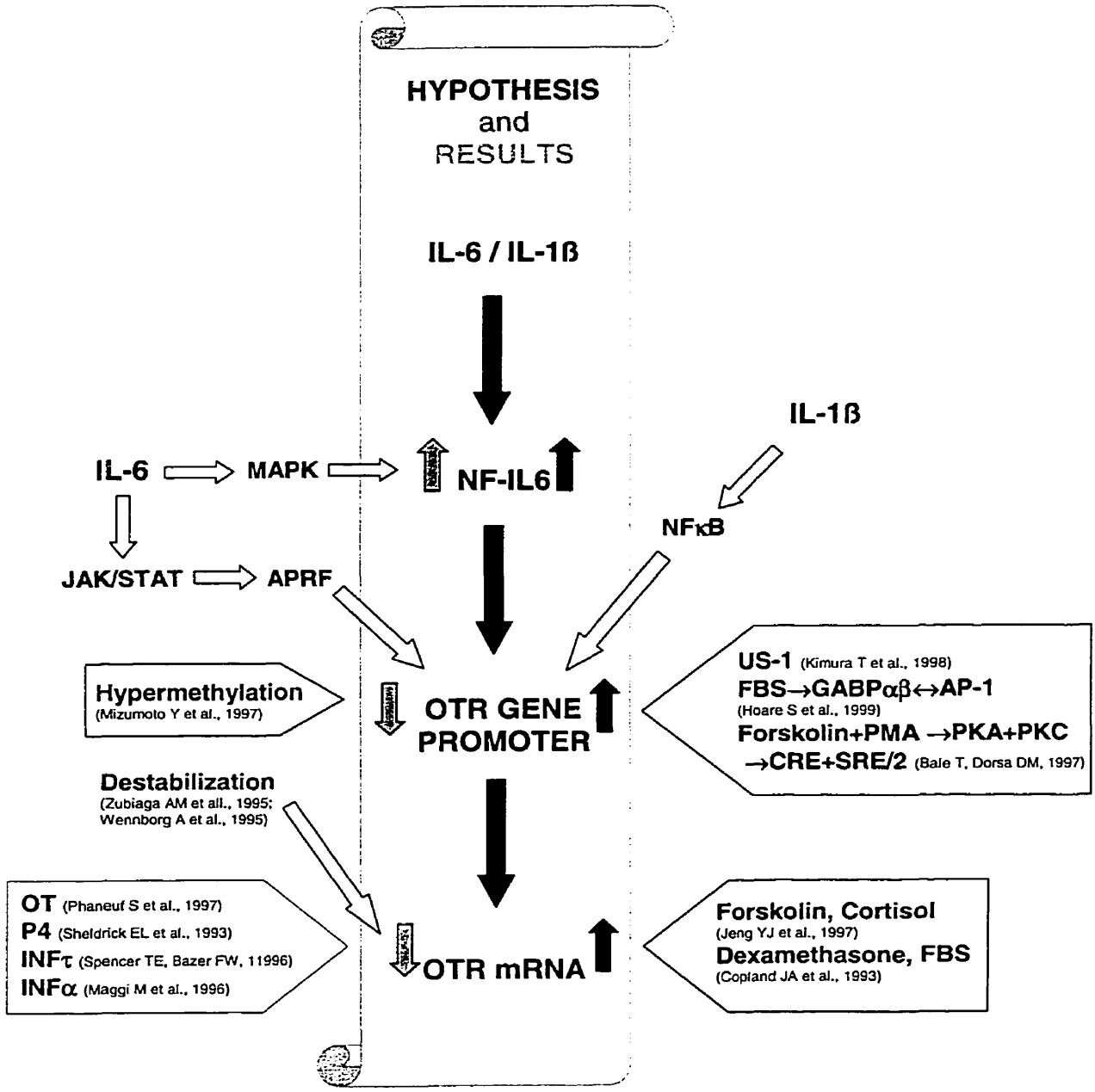

FIG. 20. **Scheme integrating our hypothesis, findings from our experiments as well as from other groups.** Our hypothesis is presented on the gray background in black letters and arrows. Our results are shown with gray arrows. Block arrows indicate findings from other groups and white arrows represent potential pathways involved in the regulation of OT receptor expression.

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APPENDIX

Originally, the aim was to investigate the effect of IL-1β and IL-6 on the regulation of several of the contraction associated proteins in primary cultures of human myometrial smooth muscle cells.

In our laboratory, template cDNA for the generation of riboprobes used in the RPA for the estrogen, progesterone and oxytocin receptor had been previously prepared. In addition, the template for the FP receptor was generated. Riboprobes of all cDNA templates were then used in RPAs first with cells isolated from myometrial samples later with ULTR cells. Preliminary results obtained from RPAs using ULTR cells were more consistent as compared to those obtained with primary cells. Therefore, ULTR cells were used for RPA and EMSA experiments.

ISOLATION OF SMOOTH MUSCLE CELLS FROM HUMAN MYOMETRIUM

Myometrial tissue samples were collected through the Perinatal Research Center from women at the Royal Alexandra Hospital, Edmonton. Informed consent from each woman was obtained prior to myometrial sampling. Pregnant term human myometrium was taken at the time of cesarean section and myometrial tissue from non-pregnant premenopausal women was obtained at hysterectomy. Samples were excised with a scalpel from normal muscle in areas free of macroscopically visible anomalies. After collection, the biopsy specimens were placed in Hank's balanced salt solution (HEPES 20 mM; pH 7.4) and transported immediately to the laboratory. All work following tissue removal was done under sterile conditions.

Cells were isolated from myometrial samples according to a protocol modified after Casey et al. (*Casey ML et al., 1978*). Briefly, the tissue was minced into 1mm^3 cubes and incubated in Hank's balanced salt solution containing collagenase type I (1

mg/ml), collagenase type IA (1 mg/ml), DNase (0.2 mg/ml), penicillin (200 U/ml), streptomycin (200 μg/ml), and fungizone (0.5 μg/ml) in a 37°C water bath with agitation. After 1 h, the supernatant containing fibroblasts was discarded and the remaining tissue was incubated for further 4 h with fresh enzyme solution. Smooth muscle cells from supernatant were collected by centrifugation at 1500g for 5-10 min, washed 2x in PBS, resuspended in 20x pcv Dulbecco's modified Eagle's medium (D-MEM, Gibco, Rockville, WI) and incubated at 37°C in humidified 5% CO_2/95% air.

Smooth muscle cells in culture were differentiated immunologically from contamining fibroblasts. To detect smooth muscle specific actin, immunocytochemistry was performed by the Department of Pathology at the University of Alberta Hospital (Figure 21).

PROSTAGLANDIN FP RECEPTOR TEMPLATE FOR RPA GENERATED BY POLYMERASE CHAIN REACTION

The generation of the RNA probe requires an appropriate cDNA template, which is either cloned into a vector in reverse orientation downstream of an RNA polymerase promoter or synthesized by polymerase chain reaction (PCR) with primers containing an RNA polymerase promoter site. In the latter case, a linear DNA product, which contains a promoter sequence at one end is generated and can then be used to synthesize the RNA probe for the RPA assay.

Primer design: The antisense PCR primer sequence was designed to generate an antisense RNA probe (cRNA) ranging from nucleotide position 294 to nucleotide position 486 of the cDNA sequence for the human prostaglandin FP receptor (*Abramovitz M et al., 1994*). The 5' end of the complementary sequence of the

antisense primer was extended by attaching the T7 bacteriophage RNA polymerase promoter sequence. The primer used were: 5'-CAC AAC CTG CCA GAC GGA AAA C-3' (sense) and 5'-GAA TTT AAT ACG ACT CAC TAT AGG GCG AGA TGA GAT GGC CAA AGA AAT CAG TG-3' (antisense). The T7 promoter sequence is underlined.

Generation of DNA template: Total RNA was extracted from human myometrial cells using Trizol reagent according to the supplier's instructions (Gibco, Rockville, WI) and template DNA was synthesized by reverse transcriptase polymerase chain reaction (RT-PCR). The RT reaction of 60 µl contained 3.3% DMSO, 5 mM $MgCl_2$, 1x PCR buffer (Gibco, Rockville, WI), 1 mM of each dATP, dCTP, dGTP, dTTP, 1 µg human total human RNA, was heated for 5 min at 95°C and chilled for 2 min on ice. 1 U/µl placental RNase inhibitor (Gibco, Rockville, WI), 2.5 U/µl M-MLV reverse transcriptase (Gibco, Rockville, WI) and 2.5 µM random hexamers were added and the mixture was incubated for 10 min at room temperature. The reaction was then incubated for 45 min at 42°C followed by a heat-inactivation of the reverse transcriptase for 5 min at 99°C. The PCR reaction of 100 µl contained 0.2 µM of each primer, 1 mM $MgCl_2$, 1x PCR buffer (Gibco, Rockville, WI) and 2.5 U Platinum Taq Polymerase (Gibco, Rockville, WI) and 20 µl of the RT reaction. DNA was amplified through 35 cycles for 1 min at 94°C, 1 min at 58°C and 2 min at 72°C. The extension step was done for 10 min at 72°C. The 203 bp PCR product was purified on a G_{50} fine sephadex column and ethanol precipitated (Figure 22).

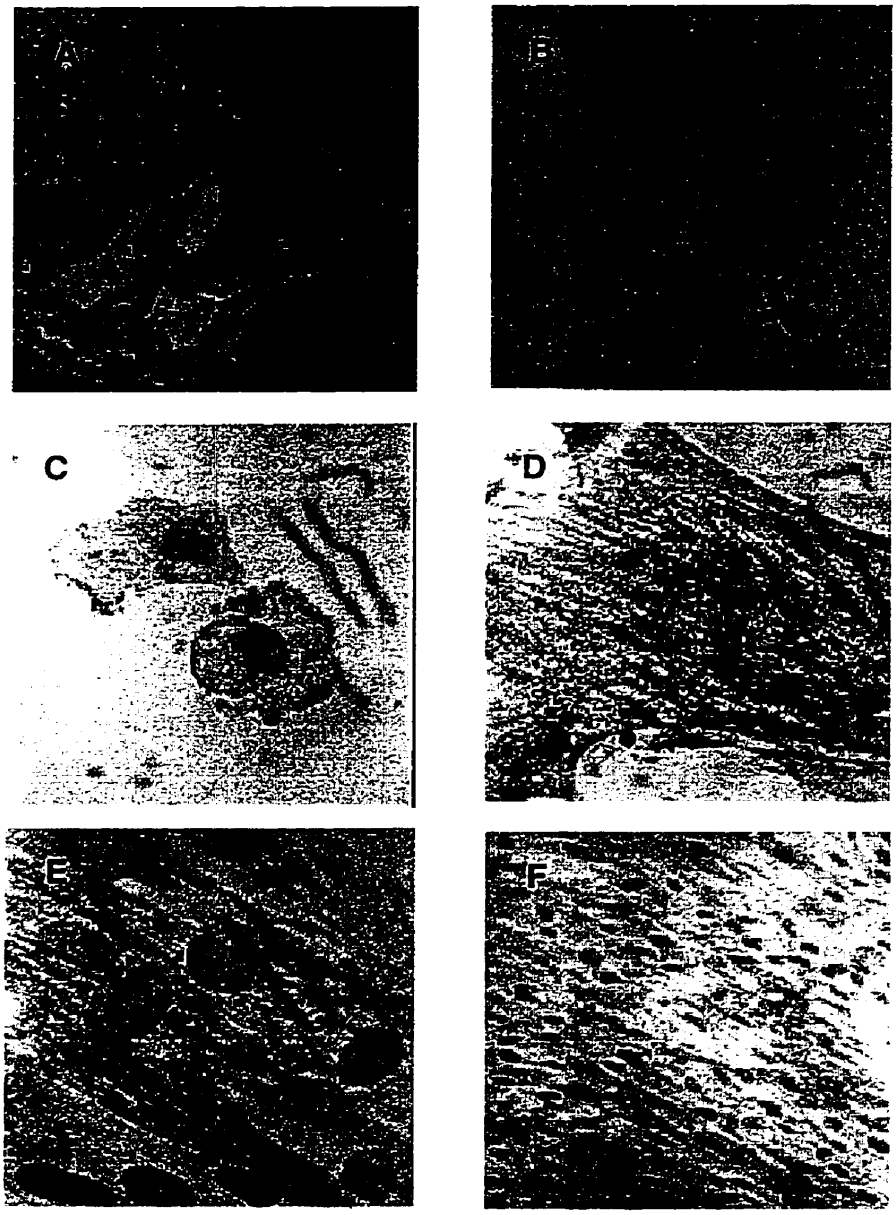

FIG. 21. **Immunocytochemistry to detect smooth muscle specific actin.** Human smooth muscle cells (SMC) were isolated from myometrium and identified using a specific antibody against smooth muscle specific actin. A, human myometrium (400x). B, isolated SMC (1000x). C, isolated SMC after 6 h in culture (1000x). D, isolated SMC after 48 h in culture (1000x). E and F isolated SMC at confluency (1000x) and (250x), respectively.

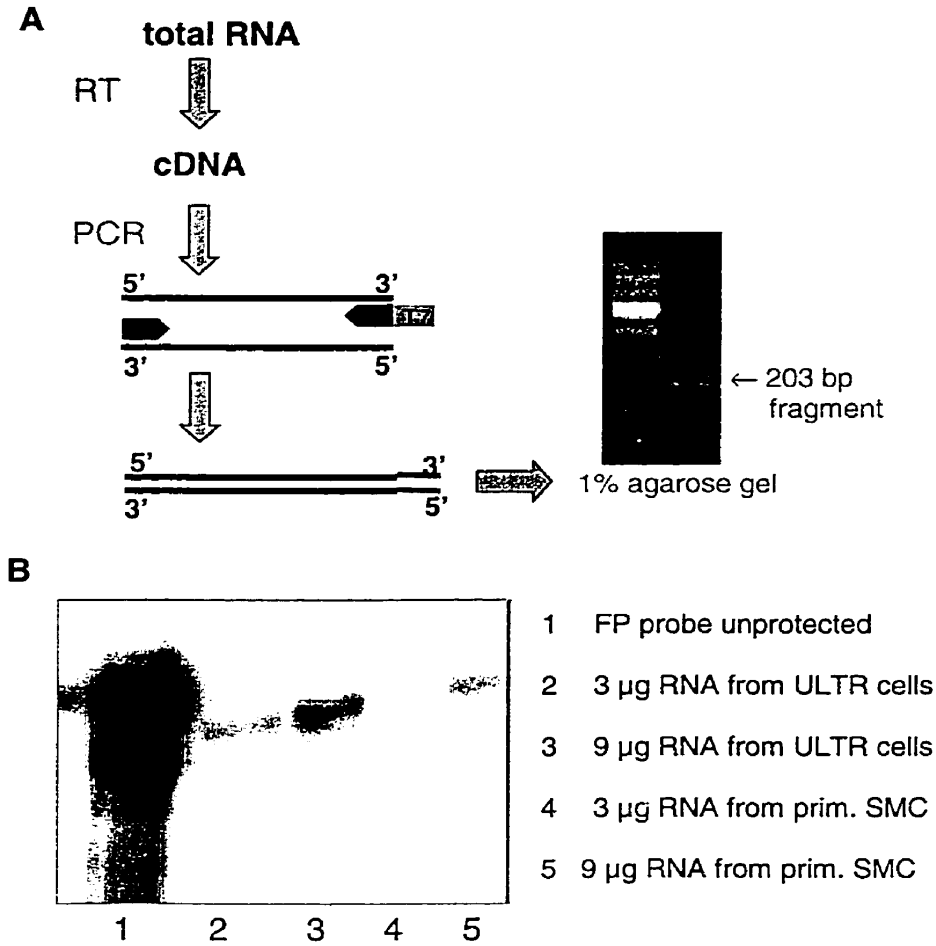

FIG. 22. **Generation of prostaglandin FP receptor template by PCR and its use in RPA.** A, cDNA is generated by reverse transcription (RT) of total RNA. Then, the double stranded 203 bp DNA template that has a T7 RNA polymerase promoter incorporated at one side is synthesized using polymerase chain reaction (PCR) and a specific primer pair. B, RNase protection analysis: 3 or 9 µg of total RNA extracted from ULTR and primary smooth muscle (SMC) cells were hybridized with hFP receptor antisense riboprobe. The autoradiography of the 6% polyacrylamide gel is shown.