Regulation of the human oxytocin receptor in the uterus: a molecular approach

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The oxytocin–oxytocin receptor (OTR) system plays important roles in the human uterus, and the effectiveness of oxytocin is greatly influenced by the pattern of receptor expression in vivo. To investigate OTR expression at the molecular level, we have established a bioassay system for the specific transcripts using *Xenopus laevis* oocytes, cloned the OTR cDNA, raised anti-OTR antibodies, and characterized OTR genomic clones and systems to study the transcriptional regulation of the gene. Using these molecular tools, we have examined OTR expression in the endometrium and myometrium of non-pregnant and pregnant women. OTR expression appears mainly to be regulated at the transcription level. Analysis of the 5′ flanking region of this gene indicates constitutionally active promoter activity when transfected into cultured HeLa and SKN cells. We are currently developing these techniques to analyse OTR regulation in the uterus.

Key words: oxytocin receptor/promoter activity/transcription/uterus

Introduction

The oxytocin–oxytocin receptor (OTR) system mediates a variety of physiological phenomena not only in reproductive organs such as the uterus, ovary, testis and mammary gland, but also in the central nervous system, the immune system, and in the control of water and electrolyte balance (reviewed in Ivell and Russell, 1995). The uterus occupies a special place among these different functions: at term of pregnancy the uterus becomes very sensitive to oxytocin, reflecting a massive upregulation of OTR (Soloff et al., 1979; Fuchs et al., 1984). As an oxytocin antagonist is also effective in the treatment of dysmenorrhea (Akerlund et al., 1987), an oxytocin–OTR system would also appear to play an important role in relation to the functioning of the menstrual cycle. These oxytocinergic effects in the uterus seem to be determined mainly by the expression of the receptor, in turn determining uterine activity. We therefore speculated that the mechanism of OTR regulation in the uterus represents the biochemical situation of uterine ‘quiescence’ or ‘activeness’. It was necessary to develop the appropriate molecular techniques in order to study in detail the molecular mechanisms involved from transcriptional control of the gene through to expression of the OTR on the cell surface. We began by cloning the OTR cDNA, then established antibodies to enable detection of the protein, and finally cloned the OTR gene in order to analyse the structural basis of transcriptional regulation. Using these materials as molecular probes, we then investigated the temporal and spatial expression of the OTR in the uterus of non-pregnant and pregnant women, as well as in gynaecological pathological situations. We also performed experiments to elaborate some of the complexity which was evidently involved in OTR transcriptional regulation. The present review describes in detail the techniques and methods used to investigate the human OTR, and summarizes our data on the expression of the OTR in the human uterus in vivo.

Molecular analyses

Several molecular methods were applied to investigate the human OTR system. These included RNA extraction and its use in *Xenopus* oocytes, and cloning the receptor and

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quantifying it by Northern blotting. Final sections cover the detection of low amounts of receptor mRNAs using a variety of approaches.

**RNA extraction and estimation of OTR mRNA expression using Xenopus laevis oocytes**

Tissues from non-pregnant uterus, and from uteri during the course of pregnancy and at term, were obtained from surgical specimens. Informed consent was obtained from patients prior to tissue sampling. Tissues were washed in ice-cold normal saline or phosphate-buffered saline (PBS), and the myometrium was separated from the serosa and endometrium, dissected, frozen in liquid nitrogen and stored at –70°C. Total RNA was prepared using the guanidinium–CsCl or guanidinium–CsTFA procedures (the latter for construction of a cDNA library). Tissue was pulverized in liquid nitrogen using a mortar and pestle, and then lysed in 5.5 M guanidinium isothiocyanate, 100 mM 2-mercaptoethanol, 0.5% Sarcosyl, 25 mM sodium citrate pH 7.0 by 10 strokes of a glass–Teflon homogenizer. The tissue lysate was then centrifuged at 2000 g for 15 min at room temperature to remove tissue debris. The resulting supernatant was then layered onto 20 ml of 5.5 M CsCl, 0.1 M EDTA pH 7.0 or 17 ml of CsTFA (Pharmacia, Uppsala, Sweden), 0.1 M EDTA pH 7.0 by 10 strokes of a glass–Teflon homogenizer. The tissue lysate was then centrifuged at 2000 g for 15 min at room temperature. The resulting supernatant was then layered onto 20 ml of 5.5 M CsCl, 0.1 M EDTA pH 7.0 or 17 ml of CsTFA (Pharmacia, Uppsala, Sweden), 0.1 M EDTA pH 7.0, adjusted to a density of 1.51 g/ml at room temperature in a polyallomer ultra-centrifuge tube (Beckman). Approximately 5–10 g of tissue was lysed in 100 ml of the guanidinium solution. Total RNA was sedimented by centrifugation at 100 000 g at 20°C in a Beckman SW28 rotor for 24 h. The pelletted RNA was redissolved in 4 M guanidinium isothiocyanate, 100 mM 2-mercaptoethanol, 0.5% Sarcosyl, 25 mM sodium citrate solution and precipitated by adding 0.75 vol. of ethanol, and 0.025 vol. of 1 M sodium acetate at –20°C. RNA was recovered by centrifuging at 14 000 g for 10 min at 4°C, then redissolved in Tris–EDTA (10 mM Tris–HCl, 1 mM EDTA, pH 7.5) buffer, reprecipitated by ethanol, and rinsed in 80% ethanol. For isolation of the poly(A)-enriched mRNA, total RNA was subjected to affinity chromatography on a 1.5 ml oligo(dT)–cellulose column (Type 3; Collaborative Research Inc., Bedford, MA, USA). The eluent was preserved as an ethanol precipitate at –20°C. A portion (150 µg) of the mRNA from term uterus was further size-fractionated by discontinuous sucrose density gradient ultracentrifugation (nine steps of sucrose from 5% to 25% (w/v), 100 mM NaCl, 10 mM Tris–HCl, 1 mM EDTA, 0.1% Sarcosyl pH 7.5). Each fraction was reprecipitated by ethanol and dissolved in water prior to microinjection.

Adult female *Xenopus laevis* were maintained at 18–20°C. Ovarian fragments were excised from frogs anaesthetized by placing them in iced water for 30 min. Oocytes were isolated from the ovarian fragments under a microscope, and washed in modified Barth’s saline [MBS: 88 mM NaCl, 1 mM KCl, 0.33 mM Ca(NO3)2, 0.41 mM CaCl2, 0.82 mM MgSO4, 2.4 mM NaHCO3, 7.5 mM Tris–HCl pH 7.6, 10 µg/ml gentamicin, 5 U/ml mycostatin]. The oocytes were then defolliculated by a 3 h treatment with 2 mg/ml collagenase (Wako Pure Chemical, Osaka, Japan) in MBS from which Ca2+ was omitted. Oocytes were then cultured in MBS overnight and the healthy Dumont stage V and VI oocytes (Dumont, 1972) were selected under the microscope just prior to the microinjection.

Messenger RNA was recovered by microfuge, rinsed with 80% ethanol and dissolved in filtered Milli-Q water at a concentration of 1 mg/ml. 50 nl (50ng) of RNA solution was injected into each oocyte under the microscope. In some experiments, mRNA was diluted with water and the injection volume maintained at 50 nl. Injected oocytes were cultured in MBS overnight at 19°C, after which dying or ruptured oocytes were removed and culture continued in fresh MBS for a further 1–2 days. Whole-cell current measurement was performed with a conventional two-microelectrode voltage–clamp method (Sumikawa et al., 1990). Voltage-clamping and current-injecting glass micropipettes were filled with 3.0 M KCl and had an electrical resistance of 1–4 MΩ. The oocyte was placed in a small bath and perfused continuously with amphibian saline (115 mM NaCl, 1 mM KCl, 5 mM Tris–HCl pH 7.2, 1.8 mM CaCl2). After insertion of the micropipettes, oocytes having a resting membrane potential between –30 and –70 mV were considered as ‘living’ oocytes and the potential controlled to –60 mV using a voltage–clamp amplifier (CEZ-1100; Nihon Koden, Tokyo, Japan). The membrane current signal was filtered through a low path filter (RC = 30 Hz) and recorded on a pen recorder (WT-625G; Nihon Koden). The assayed ligands were perfused at the indicated concentration in amphibian saline for 1 min. The complete assay procedure was performed at room temperature.

**Molecular cloning of OTR cDNA**

A full-length cDNA library of 107 independent clones was constructed using Okayama–Berg methodology (Okayama et al., 1987). The cloning vector plasmid, pcDSP6/T7, was derived from original the pcD1 expression cloning vector and the pGL1 linker vector. It differs from the original construction in having an SP6 RNA polymerase promotor sequence in the linker and a T7 RNA polymerase promotor downstream. In addition, there are three unique rare
restriction sites downstream of the insert cDNA; the sites for NotI, BstXI and XhoI. The procedure to construct the cDNA library strictly followed the protocol of Okayama et al. (1987). After circularization of the plasmid and transfection into competent Escherichia coli DH5 cells (prepared by the chemical method; Inoue et al., 1990), the library was amplified in 2 l of LB–ampicillin medium until the OD600 reached 1.0. The plasmid DNA produced was purified, and 50 µg completely digested by the restriction enzyme NotI. After size-fractionation on a 1% low-melting agarose gel, the DNA fraction >7 kb (cDNA >4 kb) was excised, purified by phenol extraction, re-circularized and re-transfected. Among 10^6 clones of this sub-library, 16 pools of 6000 clones each were amplified to OD600 = 1.0. Each pool was titrated, divided and electrophysiological assay in vitro—transcribed RNA was then subjected to the xenopus oocytes as described earlier and the positive pool was isolated. The library was produced progressively smaller sub-libraries, we finally isolated a single clone encoding the putative OTR.

Northern blotting to quantitate OTR mRNA

Total RNA was extracted by the guanidinium–CsCl procedure as described above. In order to load the same amount of mRNA from several tissues, we followed the ‘saturation’ procedure using oligo(dT)-conjugated latex beads (Oligotex DT30, Takara). As 50 µl of Oligotex suspension can adsorb 1 µg of poly(A)+ RNA, an excess of total RNA (200 µg) was applied to 100 µl of Oligotex suspension in 100 mM NaCl, 10 mM Tris–HCl, 1 mM EDTA pH 8.0, centrifuged, and eluted with 10 mM Tris–HCl, 1 mM EDTA pH 8.0. In this process, Oligotex becomes saturated and similar amounts of poly(A)+ RNA are recovered by ethanol precipitation. Equivalent RNA loading was confirmed by ethidium bromide staining after gel electrophoresis in denaturing 2.2 M formaldehyde/1% agarose gels with MOPS buffer (20 mM MOPS, 5 mM sodium acetate, 0.5 mM EDTA pH 7.0). RNA was transferred from the gel onto a nylon membrane by capillary blotting in 20× SSC. Hybridization was performed at 43°C for 16 h in 50% formamide, 5× SSC, 10× Denhardt’s solution, 0.5% sodium dodecyl sulphate (SDS), 100 µg/ml denatured fragmented salmon sperm DNA and 10 mM sodium phosphate buffer pH 7.0, with a 0.8 kb BamHI–PstI fragment of the OTR cDNA, which was labelled using [α-32P]dCTP to high specific activity (>10^8 c.p.m./mg). The membrane was washed briefly in 5× SSC, 2× SSC/0.1% SDS at 60°C for 30 min and 0.2× SSC/0.1% SDS at 60°C for 30 min, and then exposed to autoradiographic film. The membrane was also exposed to an imaging plate (Fuji Photo Film Co., Tokyo, Japan) for 1 h and quantified using an imaging plate scanner (BAS 2000, Fuji Photo Film Co.).

Reverse transcriptase–polymerase chain reaction (RT–PCR) for the detection of small amounts of OTR mRNA

For small amounts of tissue, we prefer to use the acid guanidinium–phenol–chloroform method (Chomczynski and Sacchi, 1987) to extract total RNA. Oligonucleotide primers were designed to amplify a 391 bp (1215–1602) fragment of the human OTR mRNA. As this fragment is interrupted by the 12 kb intron 3 at nucleotide 1289 of the OTR cDNA (Inoue et al., 1994), these primer pairs cannot amplify genomic DNA under the PCR conditions used. As an internal control, we also designed primers specific for a 741 bp (384–1124) internal region of the β-actin mRNA. The primer sequences used were, OTR-F, 5′- CCTTCATCGTG-TGCTGAGCG-3′; OTR-R, 5′- CTAGGACAGGAGCCACCTTAG-3′; BETA-F, 5′- CACGCCGAAGATG-ACCAG-3′; and BETA-R, 5′- CTTCTGTTGCTGTACC-CAT-3′. 1 µg of total RNA was heat-denatured at 65°C for 5 min, reverse-transcribed in 20 µl of a reaction mixture containing 200 U Moloney murine leukaemia virus reverse transcriptase (BRL, Gaithersburg, MD, USA) and 50 pmol of OTR-R and BETA-R primers at 37°C for 30 min. Half of the reaction product was amplified by PCR in 80 µl × 1 PCR buffer (Boehringer) containing 1 U Taq DNA polymerase (Boehringer), 12.5 pmol of OTR-F and OTR-R. The reaction was carried out for 30 cycles (94°C × 1 min, 55°C × 1 min, 72°C × 2 min) using a programmable thermal cycler. The other half of the reverse-transcribed product was also amplified for β-actin using 12.5 pmol of BETA-F and BETA-R under the same buffer, enzyme and temperature conditions for 15 cycles. 10 µl of the reaction products were electrophoresed, stained with ethidium bromide and blotted onto a charged nylon membrane in 0.4 M NaOH for 2 h. The membrane was then hybridized with a 48 bp internal oligonucleotide probe (OX-2, GGACGACGACAG-GAAGCGCTGCACGAGTTCGTGGAAGTGCC CGT) which was end-labelled with [γ-32P]ATP in 5× SSC, 5× Denhardt’s solution, 0.1% SDS and 100 µg/ml salmon sperm DNA at 65°C for 1 h and exposed to autoradiography film.
In-situ hybridization

Tissues subjected to in-situ hybridization were fixed with freshly prepared 4% paraformaldehyde in 0.1 M sodium phosphate buffer (PB; pH 7.4) for 24 h at 4°C and embedded in paraffin wax after dehydration through descending ethanol. In order to generate an OTR-specific probe, we used a mixture of in-vitro-transcribed digoxigenin-labelled single-strand cRNAs from three different subclones in pBluescript plasmids (Stratagene, La Jolla, CA, USA) containing respectively a 388 bp PstI–ApaI fragment (686–1074 of the cDNA), a 484 bp PstI–PstI fragment (1079–1562), and a 255 bp BglII–DraI fragment (3035–3289). Recently, we found that equivalent results could be obtained using only the cRNA from the subclone PstI–ApaI. The plasmids were linearized with the appropriate enzymes and transcribed with a DIG-RNA labelling kit (Boehringer Mannheim). Before hybridization, sections were rehydrated with xylene, followed by 99, 95, 90, 80 and 70% ethanols, after which they were postfixed with freshly prepared 4% paraformaldehyde in 0.1 M PB pH 7.4 for 20 min. Sections were then treated with 0.2 M HCl to inactivate endogenous alkaline phosphatase, acetylated with 0.25% acetic anhydride in 0.1 M PB twice for 5 min, dehydrated with 70, 80, 90, 95 and 99.5% ethanol, and air-dried. 50 µl of the hybridization solution (50% formamide, 10% dextran sulphate, 10× Denhardt’s solution, 0.6 M NaCl, 10 mM DTT, 0.25% SDS, 250 µg/ml E.coli tRNA, 0.5 µg/ml mixture of three cRNA probes) were placed on each section, covered by Parafilm and the sections incubated at 50°C for 16 h in a moist chamber. After hybridization, the sections were washed briefly with 5× SSC, then 2× SSC, 50% formamide for 30 min at 50°C. The signal was detected with a Nucleic Acid Detection kit (Boehringer Mannheim) according to the manufacturer’s instructions. When sufficient staining intensity was attained, the slides were rinsed with 10 mM Tris–HCl, 1 mM EDTA pH 8.0 and coverslips mounted.

Monoclonal and polyclonal antibodies

Three synthetic polypeptides (peptide A, C-MEGA-LAANWSAEAOANASAA; amino acids 1–19 from the N-terminus of the OTR primary sequence: peptide 2F8, C-PGAEGRATGGPRNREALAR; amino acids 20–40 from the N-terminal domain: peptide 1-2, C-TFRFYGPDDLCLRLVKKYLQ; amino acids 102–119 from the first extracellular loop) were custom synthesized. The cysteine residue added to the N-terminus was used to conjugate the peptide antigens to chicken egg albumin, grade V (ovalbumin; Sigma, St Louis, MO, USA) or to keyhole lipid haemocyanin (KLH; Calbiochem, Cambridge, MA, USA) by the method of Hashida et al. (1984).

To prepare the monoclonal antibodies, 50 µg of KLH conjugate (2F8 or 1-2) with 0.1 ml of Freund’s complete adjuvant (Difco Laboratories Inc., Detroit, MI, USA) was injected s.c. into a Balb/C mouse for the first immunization; for the second, carried out 10 days after the first, 5 µg/mouse of the conjugate was injected intraperitoneally. At 3 days after the second immunization, spleen cells were collected and fused with the myeloma cell line P3X63Ag8U1 (American Type Culture Collection, Rockville, MD, USA). The hybridomas obtained were cloned by limiting dilution. The antibody titre was evaluated by determining binding to the antigen–peptide OVA conjugate coated on the enzyme-linked immunosorbent assay (ELISA) plate. The monoclonal antibodies were purified from the culture supernatant by salting out with ammonium sulphate, and were designated 2F8 and 1-2, respectively.

To prepare polyclonal antibodies, 0.5 mg of OVA-conjugated peptide A was administered to Japanese White rabbits, together with 1 ml of Freund’s complete adjuvant (Difco) for the initial immunization. Six subsequent immunizations were performed once weekly thereafter with a similar amount of antigen and 1 ml of Freund’s incomplete adjuvant (Difco). At one week after the last immunization, whole-blood samples were taken. After salting out by ammonium sulphate, the serum was purified further by affinity chromatography on protein A–Sepharose CL-4B (Pharmacia). This antibody was designated poly-A.

Immunohistochemistry

For immunohistochemical staining, we used three fixation procedures. (i) Tissues were embedded immediately in OCT compound (Miles Inc., Elkhart, IN, USA), then frozen on dry ice or in liquid nitrogen. The frozen tissues were sectioned using a cryotome (4 µm), fixed with acetone for 10 min onto the glass slides at 4°C, and treated with methanol containing 0.3% hydrogen peroxide for 30 min to eliminate intrinsic peroxidase activity. (ii) Tissues were soaked in diluted aldehyde solution (2% v/v formaldehyde, 0.05% v/v glutaraldehyde, 0.025% w/v CaCl2, 0.05 M cacodylate buffer pH 7.35) and then irradiated with 2450 MHz microwave energy at 500 W, twice for 10 s with a 5 s interval between each. After dehydration with ethanol and xylene, tissues were embedded in paraffin. The tissues were sectioned (3–4 µm), dewaxed in xylene three times for 5 min each and rehydrated through graded ethanols (100% to 60%). The slides were then rinsed in PBS, and incubated in 0.3% v/v H2O2, 0.1% w/v NaN3 (sodium azide) for 30 min at room temperature. (iii) Dissected tissues were snap-frozen in liquid nitrogen, thawed and fixed.
in buffered formalin (4 g NaH₂PO₄, 7.5 g Na₂HPO₄, 100 ml 37% formaldehyde/pH 7.0) overnight at room temperature, rinsed in 70% ethanol twice for 12 h at 4°C, and embedded in paraffin. Dewaxing, rehydration and quenching of endogenous peroxidase were performed as above. When paraffin-embedded tissue was used with monoclonal antibodies, non-specific binding was blocked with 10% normal rabbit serum or 10% normal human serum. Immunohistochemical staining was performed using the Vectorstain ABC kit (Vector Laboratories Inc., Burlingame, CA, USA) or Histofine SAB-PO kit (Nichirei Co., Tokyo, Japan) according to the manufacturer’s instructions. The primary antibody (2F8 or 1-2, 10 µg/ml; poly-A, 20 µg/ml) was applied to the slides and incubated at 4°C overnight in a moist chamber. In the buffer for monoclonal antibodies (2F8 and 1-2), the concentration of NaCl was increased to 0.45 M to reduce the background staining. After rinsing with PBS, the second antibody was applied to the slides and incubated for 30 min at room temperature. The slides were then washed with PBS, and incubated with peroxidase-conjugated streptavidin for 30 min at room temperature. The signal was visualized by incubating slides in 50 mM Tris–HCl pH 7.6 containing 0.02% (w/v) 3,3′-diaminobenzidine tetrahydrochloride and 0.03% (v/v) H₂O₂. For the determination of the signal specificity, antigen peptide (5 µg/ml in PBS) was added to the primary antibody, and co-incubated with the neighbouring section overnight at 4°C.

**Western blotting**

Frozen samples were pulverized and lysed in 10-fold (w/v) of 1× SDS buffer (50 mM Tris–HCl pH 6.8, 2% SDS, 100 mM DTT, 0.1% bromphenol blue, 10% glycerol). The concentration of protein lysate was measured using the BioRad protein assay kit (Bio-Rad, Hercules, CA, USA). 5 to 200 µg of lysates were denatured by boiling at least for 10 min, resolved by electrophoresis on 8% SDS–polyacrylamide gels and transferred onto a nitrocellulose membrane (0.45 µm; Schleicher & Schuell, Dassel, Germany) using a mini trans-blot electrophoretic transfer cell (BioRad). The OTR signal was detected using the ECL Western blotting analysis system (Amersham, Buckinghamshire, UK) according to the manufacturer’s instructions. For blocking, we used 5% (w/v) of the Blocking Reagent from Boehringer Mannheim in TTBS [0.1M Tris–HCl pH 7.5, 0.9% (w/v) NaCl, 0.1% (v/v) Tween 20]. The primary antibody, 2F8, was used at a final concentration of 1.4 µg/ml in TTBS.

**Isolation of the oxytocin receptor gene**

Using standard plaque hybridization procedures, we screened a human genomic library (constructed in EMBL3-Sp6/T7 from placental DNA; Clontech, Palo Alto, CA, USA). To isolate the 3’ end of this gene, we further screened a library in Charon 4A from human mononuclear cells in peripheral blood (a kind gift from Dr T.Maniatis, Harvard University). Four positive clones were further characterized by restriction mapping, and sequencing after subcloning. The transcriptional start site was determined by primer extension analysis, employing two different synthetic oligonucleotides (PE-1, 5′-TGCACTATCGACGGGTCCG-3′; PE-2, 5′-TGCCCTGGGGCTGAGGCTGAC-3′) end-labelled with [γ³²P]ATP. With 300 ng of mRNA from term human myometrium as template, 0.5 pmol of labelled primer were hybridized in 10 µl of 0.4 M NaCl, 10 mM PIPES pH 6.4, overlaid with mineral oil, at 65°C for 6 h. The annealed primers were then extended with murine leukaemia virus reverse transcriptase (BRL) in the presence of dNTPs at 42°C for 1 h. The length of the products was determined by electrophoresis in 7 M urea–8% polyacrylamide gel comparing the migration relative to a sequencing reaction, which was obtained from the corresponding genomic fragment using the same labelled primer.

**Transfection experiments to determine promoter activity**

To construct an appropriate luciferase reporter vector, a BamHI–BssHII fragment (786 bp upstream of the first transcriptional start site to 144 bp downstream of this site, –786 to +144) was excised from a subclone (EcoRI–PstI) of the human OTR gene and further subcloned into the pSE280 vector (Invitrogen) from which subsequently a BamHI–NheI fragment was excised. A 446 bp fragment from –1213 bp to –766 bp was amplified by PCR from the same EcoRI–PstI subclone using as the forward primer, 5′-agctctcgagAAAGAAGGGCAGGCTTG-3′, and as the reverse primer, 5′-tcgaagatctAACCTTACAGCACCTGGGGCTGAGGCTGCAC-3′ (lower-case letters are the tags for directional subcloning of the insert). The amplified product was digested with XhoI and BglII, then subcloned into a XhoI–BamHI-digested pBluescript II vector. The KpnI–BamHI fragment (–1213 bp to –767 bp) was excised by PCR from the same EcoRI–PstI vector simultaneously (–1213/+144GL3). The vector was digested with KpnI and NdeI, and a part of the DNA was blunt-ended by T4DNA polymerase and religated (–989/+144GL3). Another portion of this DNA was
Molecular cloning of the OTR cDNA

Since we had found that the term human myometrium had very high activity corresponding to OTR mRNA, we constructed a cDNA library from this tissue. Because we already knew the approximate size of OTR mRNA, we made a plasmid library and prepared from this a sublibrary enriched in clones containing longer cDNA inserts. For this purpose, a plasmid library proved more advantageous than a bacteriophage cDNA library, since we could then use normal agarose gel electrophoresis for the size fractionation. This process, as well as the sub-selection procedure, involved serial amplification of the cDNA library. Uneven amplification can often cause severe problems for the screening process; therefore, to overcome this problem we always stopped the liquid culture at OD$_{600}$ = 1.0. Recently, a multifunctional plasmid cloning vector has become available which has T3 and T7 RNA polymerase promoter sequences, as well as the rare cutting sites NcoI and XbaI (pSPORT 1) in conjunction with a directional cDNA synthesis kit (SUPERSCRIPT Plasmid system; Gibco BRL). This type of kit would be very helpful in avoiding the complicated procedures inherent in the cloning process of the original Okayama–Berg method.

The cDNA for OTR proved to be 4.1 kb, and encoded a 389 amino acid protein of theoretical molecular weight 43 kDa. It shows the seven-transmembrane domain structure typical of the G protein-coupled receptors (Kimura et al., 1992b). This molecular size was very close to that determined by photoaffinity labelling/SDS–PAGE gel electrophoresis after deglycosylation (Kojro et al., 1991). The predicted polypeptide also includes three putative N-glycosylation sites in the N-terminal domain, in agreement with the findings of Kojro et al. (1991) that in vivo the OTR molecule appears to be highly glycosylated. It is of great interest that alternative glycosylation could be a cause of receptor subtype, the existence of which has been indicated by several authors (e.g. Chan et al., 1993). Disruption of the N-glycosylation sites by specifically mutating the Asn-
X-Ser/Thr sites to Asp-X-Ser/Thr did not appear to alter the pharmacological properties of the receptor relative to OT and related ligands (Kimura et al., 1997). As only a single OTR gene seems to be present in the genome of most mammals by Southern blotting and genomic library screening, one explanation for this phenomenon could be alternative receptors resulting from differential post-translational modification of the primary polypeptide. Our results show, however, that this finding cannot be explained by an alteration of the receptor N-glycosylation.

When the OTR-encoding DNA is expressed in Cos7 cells, there appeared to be relatively low binding selectivity between OT and Arg8-vasopressin (the IC₅₀ differs by <10-fold). However, the biological activity of OT determined by the amplitude of elicited membrane current in the Xenopus oocytes is 100 times higher than that of Arg8-vasopressin (Kimura et al., 1994). This is comparable with the physiological experiment using rat uterine muscle strip, indicating that the EC₅₀ value of Arg8-vasopressin was about 25-fold higher than that of OT (Chan et al., 1996). Thus, at physiological concentrations, Arg8-vasopressin could act as a partial antagonist rather than as a partial agonist to the OTR. Permanently transfected cell lines are also available (Jasper et al., 1995; Kimura et al., 1997) and these should be useful in the development by the pharmaceutical industry of improved agonists and antagonists.

Temporal and spatial expression of OTR in the human uterus

In the endometrium of normal-cycling women, OTR expression is localized to the glandular epithelium. The expression level of OTR mRNA was highest at the ovulatory phase on day 14 of the menstrual cycle. In the late secretory phase, the OTR mRNA was faint. We have been unable to detect an OTR mRNA signal in the stromal cells of the endometrium (Takemura et al., 1993). When we stained late proliferative endometrium using the poly-A antibody raised against the N-terminal 19 amino acids, the glandular epithelial layer was stained, as also were the transiently transfected Cos7 cells. The protein localization was consistent with the mRNA localization and, since the N-terminal antibody could recognize the OTR protein, this peptide moiety appears not to be processed from the receptor in the form of a signal peptide. On the other hand, in the endometrium of term uterus, where the glandular epithelial cells are compressed and only the stroma-derived decidual cells can be seen in most sections, OTR mRNA and protein were localized in the decidual cells as well as in the chorion laeve originating from the fetal trophoectoderm. The expression level of OTR mRNA is upregulated during the course of parturition (Takemura et al., 1994). These two observations suggest that there should be some transition point when the cellular OTR localization shifts from the glandular epithelium to the stromal cells. The physiological role of OTR in the decidua is considered to subserve the production of prostaglandin-F₂α upon oxytocin stimulation (Fuchs et al., 1981). Extraneurohypophysial oxytocin synthesis has been observed in the decidual cells and chorion laeve at term, and the expression of the peptide gene appears also to be upregulated during parturition (Chibbar et al., 1993). This indicates that there would appear to be an oxytocin–OTR autocrine/paracrine circuit within the choriodecidual layer, possibly to modulate the progression of parturition. On the other hand, the role of OTR in the endometrium of the human cycle is largely unknown. In the ruminant, the endometrial OTR at the end of the oestrous cycle appears to control luteolysis by inducing prostaglandin-F₂α secretion in response to oxytocin stimulation. The oestrous cycle is prolonged by hysterectomy and shortened by oxytocin administration into the uterine cavity (Anderson, 1973). This role is unlikely in humans. OTR mRNA and protein have also been observed in the ectopic endometrium within endometrial cysts of the ovary or adenomyosis of the uterus (Mizumoto et al., 1995). The level of the message was not altered by gonadotrophin releasing hormone (GnRH) agonist treatment which depletes endogenous oestrogen (Y. Mizumoto et al., unpublished results). These data suggest that the endometrial oxytocin–OTR system may be concerned with the proliferation of the glandular endometrial cells.

In the myometrium of the cyclic uterus, the expression level of OTR varies greatly between patients. The relationship between OTR expression and phase of the cycle, or the presence of dysmenorrhea should be analysed further. In the myometrium of pregnancy, OTR is massively upregulated towards the onset of labour. The level of OTR protein and mRNA was further increased after the onset of labour when we examined samples from corpus uteri (Kimura et al., 1996). Interestingly, when we examined the OTR mRNA expression in mouse uteri using mouse OTR cDNA, we could not detect the message in the mid term of pregnancy by Northern blot; the signal became prominent only at day 20, within 24 h of parturition (Kubota et al., 1996). This may reflect a species-specific temporal OTR expression profile. For women, even at the maximum expression of OTR at term in labour, there is a heterogeneity of the OTR expression in the myometrial layer: not all of the cells express the receptor, and the receptor-positive cells are scattered through the myometrium. These observations suggest that there is a complex regulatory mechanism for OTR, even within a single organ.
**Gene structure of the human OTR**

OTR expression is mainly regulated at the level of transcription in the uterus, since the fluctuating level of OTR protein as determined by Western blotting is very comparable with that of OTR mRNA measured by Northern blot analysis (Kimura et al., 1996). In order to investigate regulation at the transcriptional level, a knowledge of the OTR gene structure is essential. The human OTR gene localizes to chromosome 3 (3p26.2), and spans 17 kb and contains four exons and three introns (Inoue et al., 1994). The mouse OTR has a similar structure (Kubota et al., 1996), although it was reported that there were only three exons (the exon 2 of human and mouse being missing) in the rat, bovine and vole OTR genes (Bathgate et al., 1995; Rozen et al., 1995; Young et al., 1996). Computer analysis indicated several consensus transcription factor-binding DNA sequences, such as the sites for SP-1, c-Myb, GATA-1, NF-IL6 and APRE in the 5' flanking promoter sequences of these genes in most species. We could find no consensus sequence for the oestrogen-responsive element (ORE) in the human gene, even though we have now sequenced 7.2 kb upstream from the transcriptional start site (Y.Mizumoto et al., unpublished data).

**Regulatory mechanism of the human OTR transcription in the uterus**

In order to investigate the regulatory mechanism of the human OTR gene in the uterus, we applied two different strategies to this gene. One was to transfect the OTR gene upstream into appropriate (uterine-derived) cell lines. Another approach was to search the difference of the genomic DNA methylation or nuclear protein–DNA binding pattern using actual tissue extracts from upregulated and downregulated tissues.

SKN cells from human uterine leiomyosarcoma express faint amounts of OTR mRNA endogenously, although we have been unable to detect any specific binding of [125I]-d(CH2)5[Tyr(Me)2Thr4,Om8,Tyr9-NH2]-vasotocin to membrane fractions. On the other hand, there is no endogenous OTR message expression in HeLa cells derived from human uterine cervical carcinoma. These cells might thus represent the OTR-positive/-negative situation in the uterus, as we have used both these cell lines for transfection studies to analyse the function of the human OTR gene 5' flanking region. As shown in Figure 1, each clone has 8- to 10-fold higher activity of luciferase as compared with that of the pGL3 vector itself. Interestingly, the luciferase activity was the same or slightly higher in the HeLa cells, which do not naturally express the OTR mRNA. This finding suggests that the OTR gene promoter might be constitutively ‘on’, and that it is normally downregulated by some inhibitory mechanism. For the second approach, we performed Southern blotting and electromobility shift assay (EMSA) for determination of the differential methylation of the genome DNA and DNA-binding protein. In the Southern blotting using high-molecular weight DNA extracted from leukocytes, non-pregnant myometrium and term myometrium digested with HpaII (methylation-sensitive) and MspI (methylation-insensitive), we found that there is a hypomethylated region within the third intron of the human OTR gene in the non-pregnant and term uterus (Figure 2a–c). Close to this region, there is one protein-binding DNA element (GTCTCCCACCCCAAC) where the DNA-binding proteins are differentially expressed in these three tissues. As some of the DNA–protein complexes are upregulated in the OTR downregulated tissues (Figure 2d), these proteins might be involved in transcriptional suppression of the gene (Mizumoto et al., 1997). On the other hand, at the term of pregnancy when upregulation of the gene is >200-fold, the promoter activity must be further enhanced in addition to any release from inhibition.

**Conclusions**

We have developed a series of methods to analyse OTR expression at the molecular level. This gene appears to be regulated mainly at the transcriptional level, although the
5’ flanking sequence also appears to be constitutively active. As there is no cell line available which mimics the massive upregulation of OTR at parturition, we can analyse this phenomenon only by mapping the DNA-binding proteins on the gene, making use of nuclear proteins from tissue samples in which OTR is massively upregulated (activated uterus) or downregulated (quiescent uterus). This research is currently in progress and we hope that with such methods we may be able to analyse the biochemical background of uterine activity and identify a molecular basis for the dramatic physiological changes associated with OTR expression.

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