Investigation of the Oxytocin Receptor Gene in Sheep

Thesis submitted for the degree of Doctor of Philosophy

by

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No matter how far and how hard it is,

I would go until I found you……

DECLARATION

In accordance with the requirements of Victoria University of Technology for the degree of Doctor of Philosophy, I hereby declare that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where reference is made in the text of the thesis. This is my original work with the various forms of assistance described in acknowledgments. No part of this thesis has been submitted for any other degree or diploma at any other University.

Helen Chunying Feng

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SUMMARY

The increase in uterine oxytocin receptor (OTR) concentrations over the late luteal phase of the estrous cycle in sheep is thought to play an important role in the regulation of the cycle length by facilitating the effect of oxytocin on uterine prostaglandin release. At the end of pregnancy, a dramatic increase in the number of OTR also indicates the importance for OTR in the process of labor. In order to study the mechanisms underlying the resolution of OTR expression, the aim of this study was to obtain and characterise OTR cDNA and OTR gene transcripts, to determine the OTR gene expression level in different tissue types, to investigate the role of exogenous steroids in the regulation of OTR gene expression, and to develop a gene expression system for OTR protein.

The sequence of OTR cDNA was obtained from ovine uterine endometrium collected on day 15 of the estrous cycle, using RT-PCR techniques. It contained 1176 bp in coding region, from start code to stop code. The putative amino acid sequence derived from this cDNA revealed the occurrence of seven hydrophobic transmembrane regions, characteristic of G-protein coupled receptor. The sequence of this cDNA had a high homology to OTRs of human, rat, pig and cattle, and had differences in seven nucleotide alterations from the one previously reported by Riley *et al.*, 1995. It was demonstrated that the heterogeneity of the different sized OTR transcripts in sheep was due, in part to the alternative use of polyadenylation sites.

Post-translational processing and storage of oxytocin has been reported in secretory granules of corpus luteum in sheep (Sheldrick and Flint, 1989). However, whether OTRs are present in the ovine corpus luteum is still uncertain. In this study Northern hybridization, by detection of OTR mRNA expression, was able to confirm that the OTR gene is expressed in the ovine corpus luteum.

Riley *et al.* (1995) have reported the sequence of the endometrial oxytocin receptor cDNA in sheep but information on the sequences of mRNA transcripts is still lacking.

This study has provided additional sequence information of the 3'untranslated region of OTR cDNA in sheep, which has been the first that has been reported and published.

Previous studies have indicated that there are a number of transcripts of the OTR gene in the endometrium of several species (Kimura *et al.,* 1992; Rozen *et al.*, 1995; Bathgate *et al.,* 1995), but little information is available on the role of these transcripts. Investigation of OTR gene expression in this study showed the presence of multiple transcripts in the endometrium and myometrium, whereas there was a single transcript in the pituitary gland and in the corpus luteum. Based on the different function of oxytocin in these tissues, the results from this study indicate that the pattern of OTR gene expression in sheep is not only tissue-specific, but also highly function-related.

This study provides evidence of mRNA editing in both the coding regions and the 3'untranslated regions of OTR gene transcripts in ovine endometrium, the first demonstration of this phenomenon for OTR mRNA. Many studies have shown that the concentration of OTRs measured in endometrial tissue do not always correlate well with oxytocin-induced prostaglandin F2α (PGF2α) release. Research has shown that the development of OTRs in the luminal epithelium is necessary in order to achieve a maximal PGF2α response to an oxytocin challenge. However, the OTRs located within the caruncular stroma and deep glands do not form until luteolysis is complete. The formation and activity of these receptors are probably estrogen dependent. The explanation for these differences in OTR in the different endometrial sites remains unclear. The results in this study found that the differences in the sequence of OTR mRNA populations in endometrium are due to mRNA editing. It also indicated that mRNA editing of OTR transcripts resulted in amino acid changes in the carboxyl terminus, which may explain the differences in response of the OTR to those physiological stimuli.

Analysis of the secondary structure of the putative amino acid sequence indicated that editing of OTR mRNA resulted in amino acid changes in the carboxyl terminus. it is hypothesized that this may affect G-protein coupling on the pathway of OT signal transduction. The finding of mRNA editing has expanded our understanding of OT signal transduction. Oxytocin-stimulated PGF2α release is associated with an increase

in phospholipase C (PLC) and phospholipase A_2 (PLA_2). However, the relationship between PGF2α release and an increase in PLC has shown considerable discrepancies. On the basis of the results obtained in this study, it is suggested that changes in the C-terminus sequence composition, that results from mRNA editing, may influence the initial coupling of oxytocin to its receptor and/or the oxytocin signal may operate via a different pathway.

Previous studies have shown that OTRs are involved in luteal regression during the estrous cycle, through their roles in stimulating uterine prostaglandin F2α release over the latter stage of the estrous cycle. OTR expression can be regulated by steroid hormones, estrogen and progesterone. In an investigation into the roles of estrogen and progesterone in the regulation of OTR and PGHS gene expression in this study indicated that estrogen has an inhibitory effect on both OTR and PGHS gene expression, although the mechanism involved in estrogen down-regulation of OTR gene expression remains to be investigated. This study also indicated that progesterone withdrawal may lead to an increase in OTR mRNA expression.

PUBLICATIONS

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ABBREVIATIONS

AA	arachidonic acid
APRE	acute phase reactant responsive element
ATP	adenosine triphosphate
bp	base pairs
BSA	bovine serum albumin
°C	degree celcius
cDNA	complementary deoxyribonucleic acid
C-terminus	carboxy-terminus
CHO	Chinese hamster ovary
Ci	curie
cpm	counts per min
CRE	Cyclic AMP response element
DAG	diacylglycerol
dATP	adenosine deoxyribonucleoside triphosphate
dCTP	cytidine deoxyribonucleoside triphosphate
ddATP	adenosine dideoxyribonucleoside triphosphate
ddCTP	cytidine dideoxyribonucleoside triphosphate
ddGTP	guanosine dideoxyribonucleoside triphosphate
ddTTP	thymine dideoxyribonucleoside triphosphate
DDT	dithiothreitol
DEPC	diethyl-pyrocarbonate
dGTP	guanosine deoxyribonucleoside triphosphate
DNase	deoxyribonuclease
dNTPs	equimolar mixture of dATP, dCTP, dGTP, dTTP
ds-DNA	double-stranded DNA
dTTP	thymine deoxyribonucleoside triphosphate
EB	ethidium bromide
EDTA	ethylenediaminotetra-acetic acid
EMSA	electrophoretic mobility shift assays
ERE	estrogen response element
ERK2	extracellular signal-regulated protein kinase 2

G-protein	guanine nucleotide-binding protein
IgG	immunoglobulin G
InsP3	inositol-1, 4, 5-trisphosphate
IRE	interferon response element
kb	kilobase pairs
kDa	kilodaltons
LacZ	*E.coli* gene for β-galactosidase
MAPK	mitogen-activated protein kinase
M	Molar
mM	Millimolar
mg	Milligram
μg	microgram
mL	millilitre
μL	microlitre
min	minute
MOPS	3-(N-morpholino) propanesulphonic acid
mRNA	messenger RNA
ng	nanogram
nm	nanometer
N-terminus	amino-terminus
OD	optical density
OT	oxytocin
OTA	oxytocin antagonist
OTR	oxytocin receptor
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PGF2α	prostaglandin F2α
PGS	prostaglandin synthase
PGHS	prostaglandin H synthase
PGE2	prostaglandin E2 synthesis
PKA	protein kinase A
PKC	protein kinase C
PLA2	phospholipase A2

PLC	phospholipase C
PT	pertussis toxin
RACE	rapid amplify cDNA end
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per min
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate-poly acrylamide gel electrophoresis
SS-DNA	single-stranded DNA
SRE	serum response element
TE	Tris-EDTA buffer
UV	ultraviolet
V	volt
v/v	volume by volume
w/v	weight by volume
EMBL	European Molecular Biology Laboratory
DDBJ	DNA Database Bank of Janpan
PDB	Protein Database Bank

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Chapter 1 Literature review

1.1 Introduction

It is well-known that oxytocin (OT) appears to be involved in the regulation of most biological processes associated with reproductive function. These include uterine contractions at labor, milk release during lactation, control of the length of the estrous cycle and sexual behavior in male and female (Fuchs, 1985; Carter, 1992; Ivell and Russell, 1995). All these effects of OT are thought to be mediated through its specific receptors: the oxytocin receptors (OTR). In sheep, OT stimulates uterine contractions during labor and OT-induced release of prostaglandins promotes luteolysis during the estrous cycle. A large amount of data is now available which has demonstrated the importance of oxytocin receptors. During the estrous cycle, oxytocin binding to the oxytocin receptor in the endometrium is one of the important factors that is involved in the initiation of luteolysis. In pregnancy, the presence of trophoblast-derived proteins in the conceptus has been shown to block endometrial oxytocin receptor gene expression leading to the prolongation of luteal function. During labor, a dramatic increase in OTR concentration in the endometrium and myometrium is considered important in the regulation of myometrial contractility.

This chapter will address the role of oxytocin and its receptor with particular reference to luteolysis, early pregnancy and initiation of parturition. The major factors involved in these events will also be reviewed. An investigation of the OTR at the molecular level is reviewed with particular emphasis on the mechanisms involved in control of OTR gene expression.

1.2 Oxytocin

1.2.1 Oxytocin gene expression

The OT gene, containing three exons separated by two introns, has been reported for the cow (Ruppert *et al.*, 1984), rat (Ivell and Richter, 1984a), human (Sausville *et al.*, 1985) and sheep (Ivell *et al.*, 1990). OT is biosynthetically derived from a 16-kDa precursor that gives rise to the nonapeptide OT as well as the OT-associated neurophysin. As illustrated in Fig 1.2, the gene is encoded, as in all reported mammalian species, in three exons. The first exon (exon A) contains the nucleotide bases that encode the signal peptide, immediately followed by the nonapeptide, then a three-amino acid spacer (Gly-Lys-Arg, the endoprotease cleavage site of the precursors), and the first nine amino acids of the N-terminus of the neurophysin $I_{1\text{-}9}$. The second exon (exon B) contains the highly conserved region of the neurophysin corresponding to neurophysin $I_{10\text{-}76}$. The third exon (exon C) contains the remaining C- terminus of the neurophysin $I_{77\text{-}93}$ (Ruppert *et al.*, 1984; Ivell and Richter, 1984b; Sausville *et al.*, 1985; Ivell *et al.*, 1990; Gainer and Wray, 1994).

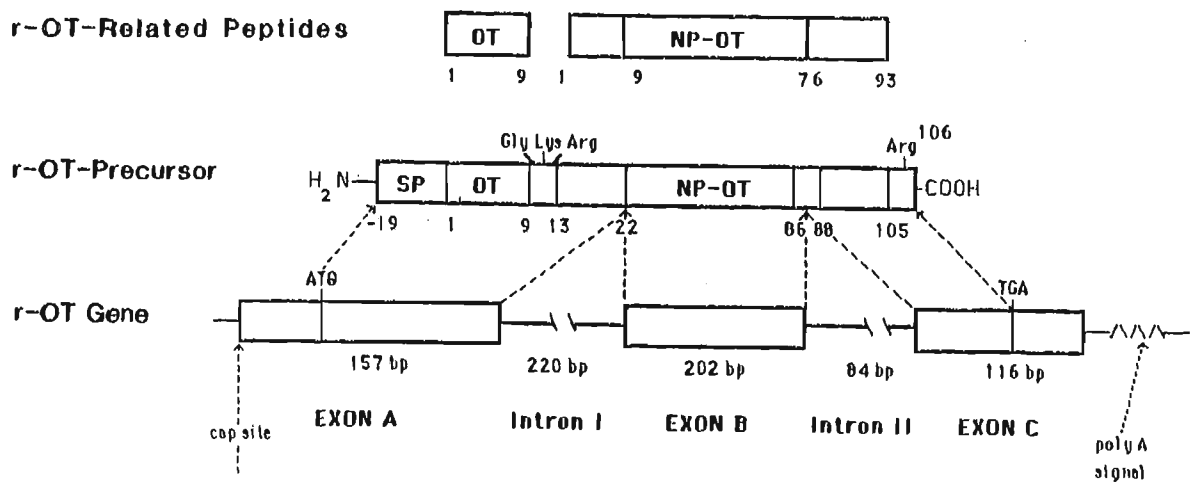


Fig 1.1 Organization of the intron-exon structure of the OT gene in the rat, and the relationships of OT gene to its precursor (preprohormome) and final peptide product (adopted and modified from Gainer and Wray, 1994). Three exons, A, B and C, are

shown as open rectangles. Two introns are shown as disconnected lines between exons. The "cap site" represents the site of transcription initiation, and the ATG signal represents the site of translation initiation. The TGA signal on the gene represents the site of translational termination. The number of nucleotides in each gene component is illustrated as base pairs (bp). The broken arrows illustrate the amino acid sequences in the precursors that are post-translationally modified to produce the peptide products. Abbreviations: SP, signal peptide; NP-OT, oxytocin-associated neurophysin.

1.2.1.1 Hypothalamic oxytocin gene expression

The main sites of oxytocin (OT) synthesis are in mangnocellular neurons located in the supraoptic and paraventricular nuclei of the hypothalamus (Gainer and Wray, 1994). After axonal transport to the posterior pituitary, OT is released into the general circulation. OT is also produced by a subgroup of parvicellular neurons located in the caudal paraventricular nucleus. These neurons project to the median eminence, where OT is released into the pituitary-portal circulation along with the other classical hypothalamic releasing factors (Silvermann, 1983).

1.2.1.2 Ovarian oxytocin gene expression

OT has been found in the corpora lutea of many species. In the ewe and the cow, OT synthesis first appears in the granulosa cells of preovulatory follicles and later resides in the large cells of the corpus luteum (Rodgers *et al.*, 1983; Schams *et al.*, 1985; Khan-Dawood, 1986; Fehr *et al.*, 1987). The synthesis involves the oxytocin-neurophysin prohormone as in the neurohypophysis. Luteal secretion of OT is accompanied by secretion of neurophysin, and as expected on the basis of the structure of the prohormone, the two compounds are released on a ratio of 1:1 (Watkins *et al.*, 1984). In the corpus luteum, as in the neurohypophysis, post-translational processing and storage of the secreted product occur in secretory granules of large luteal cells (Sheldrick and Flint, 1989). The concentrations of OT in the corpus luteum is approximately one-quarter of those in the posterior pituitary. However, a large proportion of stored OT is available for secretion as a single pulse in the corpus luteum compared with the

neurohypophysis. The corpus luteum may therefore contain larger quantities of OT than the posterior pituitary (Flint *et al.*, 1994).

1.2.1.3 Uterine oxytocin gene expression

In situ hybridization and immunocytochemistry have indicated that the uterine epithelium in the pregnant rat is the main site of OT gene expression and OT immunoreactivity accumulation. The uterine and hypothalamic OT transcripts are identical, with the exception that uterine OT mRNA has a shorter poly(A) tail than its hypothalamic counterpart (Lefebvre *et al.*, 1992a). Characterization of uterine OT-like immunoreactivity by HPLC analysis revealed two peaks, one corresponding to the amidated nonapeptide OT and the other to OT-associated neurophysin (Lefebvre *et al.*, 1992a). Zingg *et al.* (1995) found that the highest levels of OT expression were restricted to the last 3-4 days of pregnancy. During this time period, there was >100-fold increase in OT mRNA level. In parallel with the rise in OT mRNA, there was also a 35-fold increase in immunoreactive OT during pregnancy. The highest levels of OT-like immunoreactivity were observed at the time of parturition, whereas the OT mRNA levels reached a peak 1 day prior to parturition (Zingg *et al.*, 1995).

Experiments undertaken by comparing the hybridization signal obtained from uterine mRNA with the signal obtained from hypothalamic mRNA indicated that the rat uterus was the major site of OT gene expression during the later stages of pregnancy (Zingg *et al.*, 1995). It was suggested by Zingg *et al.* (1995) that OT released from endometrial epithelial cells might act in an autocrine fashion on OTR located on the endometrial epithelial cells themselves. Moreover, OT might also pass the thin layer of stroma existing between the tip of the uterine glands and the myometrium and interact directly with myometrial OTRs.

1.2.1.4 Oxytocin gene expression in fetoplacental tissues

Studies have shown that OT is synthesized in the amnion, chorion, placenta and decidua in rats and humans (Lefebvre *et al.,* 1992b; Chibbar *et al.,* 1993; Lefebvre *et al.,* 1993). It was reported that OT was secreted by the fetus and then transferred to the maternal side. A marked increase in fetal OT secretion was found during the first stage of labor prior to the increase of OT level in the maternal plasma (Dawood *et al.,* 1978; Dawood *et al.,* 1979). Because the amnion, chorion and decidua are located close to the myometrium, these tissues act as a paracrine mediator in OT production and therefore, have a unique and important influence on the myometrium contraction (Miller *et al.*, 1993).

1.2.2 The role of oxytocin

It is well known that OT plays an important role in reproductive physiology in all mammalian animals. The roles include uterine contraction at birth, contraction of mammary gland myoepithelial cells during milk ejection and initiation of luteolysis during the estrous cycle in ruminants. In general, OT acts as a hormone released in the form of pulses from its site of synthesis by a regulated pathway (Gainer and Wray, 1994). However, OT may be produced in some local sites by a constitutive, nonpulsatile pathway and can act via autocrine and paracrine mechanisms.

1.2.2.1 The role of oxytocin in the brain

One of major functions of OT is thought to be the stimulation of the sexual behavior (Carter, 1992). The direct microinjection of antisense oligonucluetide to the OTR cDNA sequence attenuated the sexual activity of the mature female rat (McCarth *et al.,* 1994). OT can improve maternal behavior in sheep (Insel and Kendrick, 1992), and modulates social behavior in voles (Insel and Shapiro, 1992). Adult prairie voles form pair bonds after mating with prolonged, repeated bouts of copulation. In female voles, central administration of an OT antagonist prevents pair bonding without interfering with the mating behavior whereas central administration of OT facilitates pair bonding

in the absence of mating (Insel and Shapiro, 1992). In the rat, OT is also implicated in the control of natriuresis, thirst and hunger (Verbalis *et al.,* 1993).

OT can exert several important hypophysiotrophic functions. OT has been shown to act as a releasing factor for gonadotropin, corticotrophin and PRL. These results suggested that the OT effect on lactotrophs was direct, whereas the OT effect on gonadotrophs and corticotrophs were either indirect or mediated via a different receptor, and OT acts as a PRL-releasing factor only towards the end of gestation (Breton *et al.,* 1995). OT also appears to act as a neurotransmitter within the central nervous system (Kovacs, 1986).

1.2.2.2 The role of oxytocin during luteolysis

As mentioned in section 1.2.1.2, OT is synthesized and secreted by the corpus luteum. In ruminants, ovarian OT acts mainly as a hormone. A major function of ovarian OT is to stimulate the release of uterine PGF2α which in turn acts on the corpus luteum to initiate luteolysis (Flint and Sheldrick, 1986a; Wathes *et al.,* 1986).

In primates, it has been shown that the influence of the uterus on luteal life-span is not significant in these species. OT has a paracrine role in the regulation of ovarian function, acting on ovarian tissue to release PGF2α which then diffuses into the adjacent luteal cells to cause luteolysis (Fuchs, 1988).

There is also an oxytocinergic autocrine and /or paracrine system in the ovary and around the ovum that might regulate steroidogenesis and the early stage of fertilization (Sernia *et al.,* 1991).

1.2.2.3 The role of oxytocin during parturition

OT is the strongest natural uteronic substance known. Although it is generally accepted that there is a rise in pituitary OT secretion during parturition, the role of OT in triggering the onset of parturition is still unclear. Specifically, there does not appear to

be a consensus in the literature over whether a rise in circulating OT levels precedes the onset of parturition (Zeeman *et al.*, 1997).

Kumaresan *et al.* (1971) found that circulating OT played an important role in the mediation of the milk ejection reflex but was not crucial for normal parturition. It has been demonstrated in women that the pulse frequency and duration of circulating OT increase during spontaneous labor (Fuchs *et al.*, 1991), indicating that pulses of circulating OT play a role in uterine activation. However, studies have also shown that OT can be produced in both uterine endometrium and fetoplacental tissues in humans and rats (section 1.2.1.3 and 1.2.1.4), but not in sheep (Wathes *et al.*, 1996). The uterine OT mRNA concentration was about 70 times higher than in the hypothalamus at term in rats (Lefebvre *et al.*, 1992b). In addition the observed rise in fetal OT concentrations occurs earlier than in maternal plasma in rats (Dawood *et al.*, 1978). It appears that the local OT production also plays an important role in the initiation of parturition in humans and rats.

Zingg *et al.* (1995) has proposed a model for OT action during parturition mainly based on the studies in rats. The diagram is shown in Fig 1.2.

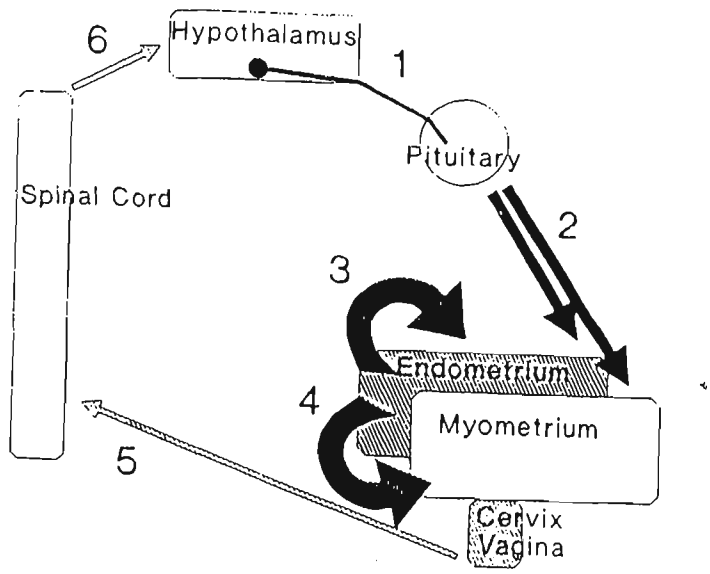


Fig 1.2 Scheme of proposed concepts of oxytocin action during parturition (adopted from Zingg *et al.*, 1995).

Step 1: OT is synthesized in magnocellular hypothalamic neurosecretory neurons, and is carried to the neural lobe of the pituitary via axonal transport.
Step 2: OT is released from the nerve endings into the general circulation. However, the factors responsible for the increased pulsatile secretion of OT in the early stages of labor remain uncertain.
Step 3: OT produced in the endometrium may interact with endometrial OTRs, thereby stimulating prostaglandin production;
or Step 4: OT produced in the endometrium may reach the myometrium and interact with myometrial OTRs to cause myometrial contraction. As found in rats at term, the uterus itself represents a major site of OT production, the activation of uterine contractions by circulating OT may be only one aspect of OT action.
Steps 5 and 6: As initially proposed by Ferguson (1941), OT may be released in response to the distention of the cervix by the descending fetus and this may enhance the release of OT via activation of hypothalamic oxytocinergic neurons.

Current research by Kobayashi *et al.* (1999) has showed that a rapid increase in circulating oxytocin concentrations did occur at the onset of delivery in rats. Plasma oxytocin concentrations and the receptor densities were found to be the same in rats with preterm labor (induced by the combined use of bilateral ovariectomy and estradiol injection) as in rats with term labor. This study provides good biochemical evidence in rats that plasma oxytocin plays an important role in the initiation of parturition in both term and preterm labor.

In sheep, OT gene expression and immunoreactivity in the hypothalamus increase over the late stages of gestation with a further increase at term (Broad *et al.*, 1993). The increase in plasma OT concentrations is associated with the onset of uterine contraction, whereas vaginocervical stimulation caused by passage of the lamb at birth does enhance the rise of OT concentrations (Mitchell *et al.*, 1982; Kendrick *et al.*, 1991). However, there is no evidence for the local uterine production of OT during gestation and at term in sheep (Wathes *et al.*, 1996). The question as to whether or not the increased OT release from the neurohypophysis triggers the onset of labor remains unclear. It has been demonstrated previously that there is an increase in uterine OTR concentrations,

and that OT-induced release of maternal PGF2α is enhanced in late gestation (Wathes *et al.*, 1996; Jenkin *et al.*, 1992). In this respect, it does appear that OT acts through its own receptor to initiate uterine activity at labor. These studies emphasize the importance of OTR in regulating the action of OT at parturition in sheep.

1.3 OT signaling pathway

OT acts as a stimulant for the secretion of PGF2α release from the ovine uterus during luteolysis and for myometrial contractions during parturition (section 1.2.2). In general, OT appears to interact with its receptor which activates a specific signal-transducing G protein (Gilman, 1987). The activated G proteins interact with several enzymes that contribute to PGF2α synthesis and to the increase of intracellular calcium concentration, including phospholipase C, phospholipase A2, prostaglandin synthase (PGS) and extracellular signal-regulated protein kinase 2 (ERK2). Other factors are also involved as reviewed in the text.

The acute OT signaling pathway is still under investigation. Strakova *et al.* (1998) used transfected OTR-CHO (Chinese hamster ovary) cells as a model to illustrate the pathways mediating OT-stimulated increases in intracellular calcium concentration and prostaglandin E2 (PGE2) synthesis. Briefly, there are two pathways: OTR-mediated Gq/PLC pathway and OTR-associated Gi/protein tyrosine kinase pathway. OTR-Gq coupling activates OT/PLC-mediated events, which include InsP3 production, PKC activation, ERK2 phosphorylation and PGE2 synthesis. OTR-Gi coupling initiates tyrosine phosphorylation and Ras activation, resulting in the increase of p38 kinase activity. OTR-Gq coupling also stimulates p38 activity through PKC dependent pathway. The details are as described in Fig 1.3.

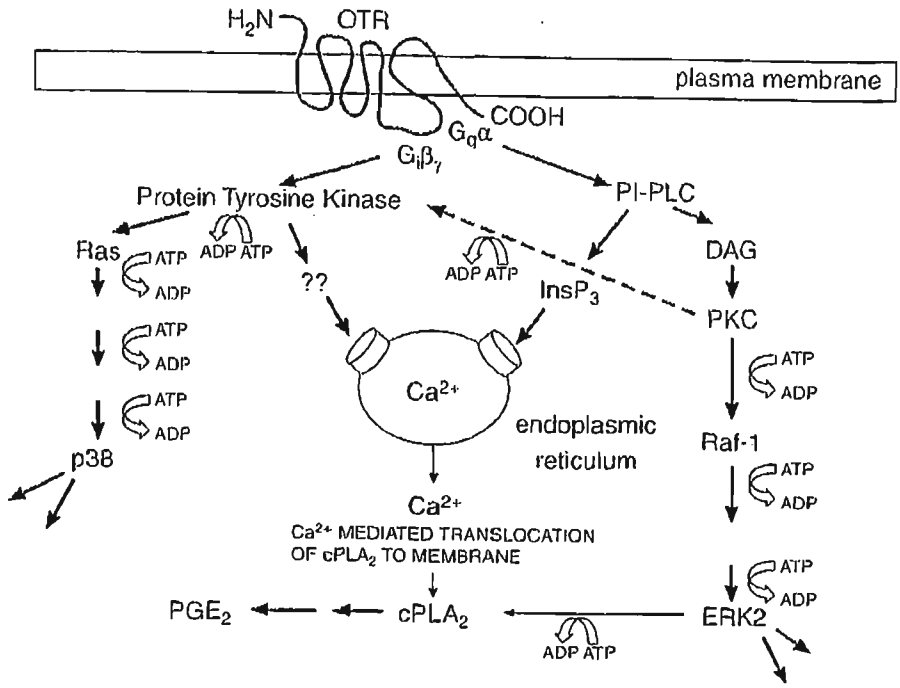


Fig 1.3 Postulated diagram of OT signaling pathways in OTR-CHO cells as adopted and modified from Strakova *et al.* (1998). Two pathways are involved: OTR-mediated Gq/PLC pathway and OTR-associated Gi/protein tyrosine kinase pathway. When OTR is coupled to Gq, it stimulates Phospholipase C (PLC) activity for inositol-1, 4, 5-trisphosphate (InsP3) and diacylglycerol (DAG) production. InsP3 stimulates the release of intracellular calcium and DAG activates protein kinase C (PKC). PKC activation stimulates the activity of Raf-protein-serine/threonine kinase (Raf-1), leading to phosphorylation of its extracellular signal-regulated protein kinase 2 (ERK2). ERK2 phosphorylation activates cytosolic phospholipase A2 (PLA2) and leads to increase prostaglandin E2 synthesis. When OTR is coupled to Gi, it initiates the activity of protein tyrosine kinase. Protein tyrosine kinase catalyzes tyrosine phosphorylation that activates Ras. Ras are plasma membrane-associated guanine nucleotide-binding proteins that take part in intracellular signal transduction. Ras activation initiates p38 mitogen-activated protein kinase (p38) phosphorylation. OTR-Gq coupling also stimulates p38 activity through PKC dependent pathway (as indicated in broken line). Endoplasmic reticulum receptors mediating intracellular calcium release from Gi/protein tyrosine kinase pathways are unknown (as indicated as ??).

1.3.1 AlF_4^-

AlF_4^- is a non-specific stimulator of GTP-binding regulatory proteins (Gilman, 1987). These proteins link some membrane-bound receptors to some second messenger-generating enzymes, such as adenylate cyclase and phospholipase C (PLC) (Gilman, 1987). AlF_4^- is a potent stimulator of PGF2α release from ovine endometrial tissue (Silvia *et al.,* 1994) and is believed to induce cellular responses by increasing the activity of membrane-bound regulatory proteins that bind GTP (G-proteins, see Gilman 1987). The ability of AlF_4^- to stimulate the release of PGF2α suggests that GTP-binding proteins play an important intermediary role in OT signal transduction (Gilman, 1987).

1.3.2 G-protein coupling

G-proteins have been distinguished by bacterial toxins in various systems, since the toxins from *Vibrio cholerae* and *Bordetella pertussis* can ADT-ribosylate G proteins from the adenyl cyclase system (Ui and Katada, 1990). Simon *et al.* (1991) have used pertussis toxin (PT) to show that at least two different classes of G protein are involved in the regulation of the formation of InsP3 (see section 3.3). These include PT-sensitive and PT-insensitive G proteins.

PT-sensitive G proteins (Gi/Go) have the amino acid sequences which are necessary for PT-stimulated ADP-ribosylation. In contrast, a class of G proteins termed Gq do not contain the amino acid sequence which are necessary for PT-induced ADP-ribosylation (Strathmann and Simon, 1990). Two members of this family, Gq and G11 are candidates for the PT-insensitive G protein responsible for stimulation of PLC (Smrcka *et al.* 1991; Taylor *et al.* 1991). The toxin-insensitive G proteins have been classified as: Gq, a toxin-insensitive G protein that activates PLC-β1 and Gh, a toxin-insensitive G protein with transglutaminase activity (Park *et al.* 1998). It has been shown that an 80 kDa Ghα family protein is stimulated by OT-bound receptor in human myometrium, suggesting that the OTR couples to the Ghα family proteins in signal mediation (Baek *et al.* 1996). A potential PT-sensitive G protein that was thought to be involved in the regulation of InsP3 is Go, and was originally identified in the brain. Moriarty *et al.*

(1990) showed that this G protein can serve as the signal transducer of the receptor-regulated PLC in *Xenopus oocytes*.

PT has been reported to inhibit OT-induced formation of inositol phosphates (IPs) and intracellular calcium [Ca^{2+}] mobilization in rat myometrium but not in guinea-pig myometrium (Marc *et al.* 1988; Anwer *et al.* 1989, 1990; Anwer and Sanborn 1989). In the human myometrial cell, Phaneuf *et al.* (1993) reported that the OT-induced production of IPs and [Ca^{2+}] were mediated by a PT-sensitive G protein. It was also shown that part of the PGF2α stimulation of the production of IPs was independent of [Ca^{2+}] and it was suggested that a PT-insensitive G protein, possibly a member of the Gq family was involved in its coupling to PLC. Similarily it has been reported that OTR is coupled to both Gq and Gi in transfected CHO cell line (Strakova *et al.*, 1998) and in the myometrium of the pregnant rat (Strakova and Soloff, 1997).

1.3.3 Phospholipase C (PLC)

Phospholipase C hydrolyses phosphatidylinositol 4,5-bisphosphate (a minor phospholipid in cellular membranes) to diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (InsP3) (Berridge, 1987). Both of these products are active second messengers. InsP3 initiates contractions in the myometrium by releasing calcium from calcium-sequestering compartments within the cell and DAG, along with free calcium, activates protein kinase C (PKC) (Nishizuka, 1984; Berridge, 1987). A number of PKC phosphorylated enzymes are known to be involved in liberating free arachidonic acid (AA) from intracellular storage pools. These pools serve as a substrate for prostaglandin synthesis. Studies have also found that phorbol ester analogues of DAG activate PKC (Kikkawa *et al.* 1983) and stimulate the release of PGF2α from ovine and bovine endometrial tissue *in vitro* (Silvia and Homanics, 1988; Lafrance and Goff, 1990). Alternatively, AA may be cleaved directly from DAG by DAG lipase and monoacylglycerol lipase (Flint *et al.*, 1986b).

Phosphoinositide-specific PLC is a family of isoenzymes consisting of three major groups (named PLC-β, PLC-γ and PLC-δ) and classified by molecular mass, deduced amino acid sequence and immunological cross-reactivity (Rhee *et al.* 1989). PLC-β is

stimulated by the Gq family, which mediates the actions of a variety of transmembrane receptors (Watson and Arkinstall, 1994; Exton, 1994). PLC-γ is thought to be mainly a cytosolic isoenzyme that is phosphorylated by activated tyrosine kinase-containing receptors (Rhee and Choi, 1992; Koch *et al.* 1991). PLC-δ1 has been shown to be modulated not only by Ghα (transglutaminase II)-mediated signaling (Nakaoka *et al.* 1994; Feng *et al.* 1996) but also by thrombin-mediated signaling via a PT-sensitive G protein (Banno *et al.* 1994). It has been demonstrated that PLC-δ1 is an effector of OTR signaling via activation of Ghα in human myometrium and the three key components OTR-Ghα-PLC-δ1 are involved in OT signaling system (Park *et al.* 1998).

1.3.4 Phospholipase A_2 (PLA_2)

Synthesis of prostaglandins is associated with an increase in the activity of PLA_2 in many cell types (Irvine 1982). Arachidonic acid (AA, see section 1.3.3) can be released from phospholipids by increased PLA_2 activity. This enzyme is calcium dependent and can be activated by an increase in intracellular $[Ca^{2+}]$. The PLA_2 activity can also be induced through an increase in PLC and associated second messenger activity or can be induced by the G protein activation directly (Fain *et al.* 1988). The increase in PLA_2 activity without any accompanying rise in InsP3 showed that the two pathways can be separated (Fuse and Tai, 1987; Fain *et al.* 1988). PLA_2 activity has been observed in uterine tissue collected from sheep, guinea-pig, rat and human (Grieves and Liggins, 1976; Dey *et al.* 1982; Downing and Poyser, 1983; Bonney 1985). It has been demonstrated that the stimulatory effect of OT on PGF2α release from ovine endometrial tissue is mediated through PLA_2 (Lee and Silvia, 1994).

1.3.5 Prostaglandin synthase (PGS)

Binding of OT to the OTRs involves the activation of the enzymes that mobilize release of arachidonic acid (AA) from its storage pool. Regulation of this response is possible at two other points in the pathway, i availability of AA reserves and ii the enzyme prostaglandin synthase (PGS) which converts AA to PGF2α (Silvia *et al.*, 1991). AA is released from either phospholipid or triglyceride stores. Two isoforms of PGS have been found, PGHS-1 and PGSH-2 (O'Nill *et al.*, 1993). PGSH-1 has been identified in many

tissues in basal amounts in a constitutively expressed isoform. PGSH-2 can be regulated by exogenous stimulation, such as cytokines, growth factors and tumor promoters (Hersehman *et al.*, 1994).

The endometrial concentration of PGS change during the estrous cycle, with an overall increase in activity on days 12-13 of the estrous cycle in ewes, which occurs at least two days before the increase in responsiveness to oxytocin (Silvia and Raw, 1993). PGS is localized principally in the stromal cells, and is found in the epithelial cells of the lumen and superficial glands (Salamonsen *et al.* 1992). However, it has been suggested by Wathes and Lamming (1995) that PGS availability is not the rate-limiting step in the development of oxytocin induced PGF responsiveness in cyclic ewes.

1.3.6 Extracellular Signal-Regulated Protein Kinase 2 (ERK2)

Extracellular signal-regulated protein kinases (ERKs) are one of the subgroups of mitogen-activated protein kinase (MAPK) family (Cobb *et al.*, 1995). MAPKs have been shown to act as integrators of mitogenic and other signals originating from distinct classes of cell surface receptors, such as tyrosine kinase and G protein-coupled receptors (Van Biesen *et al.*, 1996). There are two activated forms for ERKs, ERK1 and ERK2. Both of them transmit extracellular stimuli by phosphorylating a variety of substrates, including transcriptional factors and other kinases (Anderson *et al.*, 1990). Several studies have indicated that ERKs phosphorylate and activate cytoplasmic phospholipase A_2 ($cPLA_2$) to produce arachidonic acid for PG synthesis (Lin *et al.*, 1993; Sa *et al.*, 1995). The finding that OT-induced phosphorylation of ERK2 can be inhibited by both pertussis toxin and inhibitors of phospholipase C and protein kinase C, indicated that OTRs are coupled to both Gi and Gq (Strakova *et al.*, 1998). OT-stimulated c-fos expression was also mediated by ERK2 phosphorylation. Results are available showing that inhibition of OT-induced ERK2 phosphorylation with an ERK kinase inhibitor (PD-98059) markedly reduced OT-stimulated PGE2 synthesis. These results demonstrate the importance of ERK2 activation in OT-induced PGE2 synthesis (Strakova *et al.*, 1998).

1.4 Oxytocin Receptor

1.4.1 Localization of OTR

Localization of OTR has been studied for many years using different biochemical techniques. In sheep, OTRs have been found in the endometrium, myometrium and oviduct using both a [^{3}H] oxytocin binding assay and an ^{125}I-labelled [1-(β-mercapto-β, β-cyclopentamethylene propionic acid), 2-(ortho-methyl)-Tyr^2, Thr^4, Orn^8, Tyr^9-NH_2]-vasotocin (OTA) binding assay (Roberts *et al.,* 1976; Sheldrick and Flint, 1985; Ayad and Wathes, 1989; Ayad *et al.,* 1990; Ayad *et al.,* 1991). In the ampullary and isthmic regions of the oviduct and the myometrium, ^{125}I-labelled OTA binding sites were confined to smooth muscle. Endometrial binding sites for ^{125}I-labelled OTA were consistently found in the luminal epithelium and epithelial cells lining the secretory glands (Ayad *et al.,* 1991). The location of OTR in the endometrium has been identified by *in situ* hybridization. Using this technique, it was found that OTRs first appeared in the luminal epithelium at onset of luteolysis, then spread to the superficial glands during luteolysis and developed on the caruncular stroma and deep glands at estrus (Stevenson *et al.* 1994; Wathes and Lamming, 1995). OTR is also found in the cervix of nonpregnant ewes (Matthews and Ayad, 1994).

At parturition, OTR expression was demonstrated in both the endometrium and myometrium (Wu *et al.*, 1996). Using the antibodies raised against nonoverlapping sequences of the third intracellular loop of the rat OTR, OTR was demonstrated by immunocytochemistry in the smooth muscle of myometrial blood vessels in sheep (Wu *et al.*, 1996). Localization of OTR mRNA in the ovine uterus, cervix and placentome throughout gestation has been investigated by Wathes *et al.* (1996). It was found that in the intercotyledonary region, OTR was localized to the luminal epithelium and myometrium. The OTR mRNA concentration remained low from day 22 of pregnancy onwards and then increased sharply during labor. In the cervix, OTR mRNA was detected at low concentration from day 22 throughout gestation. In the placentomes, OTR mRNA was found in a stromal capsule surrounding the placental villi. Expression in this region was maximal in mid-gestation, declining in the second half of pregnancy and remaining low during labor.

Although OTRs have been found in human, bovine and porcine ovaries (Fuchs *et al.*, 1990; Pitzel *et al.*, 1993), the question as to whether OTRs exist in the ovine corpus luteum has not been firmly defined. Early reports indicated that specific oxytocin binding could not be demonstrated in luteal tissue during the estrous cycle, although it was found in the corpora lutea of pregnant ewes (Sernia *et al.*, 1989). Northern analysis did not detect OTR gene expression in ovine corpus lutea (Flint *et al.*, 1995).

In the rat, OTRs have been reported previously in the brain, pituitary, mammary gland and uterus by immunocytochemical analysis (Adan *et al.*, 1995). Ultrastructural *in situ* hybridization combined with immunogold labeling indicated that pituitary OTR gene expression is highly cell specific and restricted to lactotrophs in the rat (Breton *et al.*, 1995). OTR gene expression in the rat uterus has also been identified by ^{125}I-labelled OTA binding assay, Northern blot, RT-PCR and *in situ* hybridization (Larcher *et al.*, 1995; Rozen *et al.*, 1995). Specific high-affinity OTRs were found in decidua (Soloff *et al.*, 1979) and OTRs have also been found in the kidney in ovariectomized female rats (Ostrowki & Lolait 1995). When treated with estradiol benzoate, the increase in the expression of the OTR mRNA and ^{125}I-labelled OTA binding to OTRs was found in the renal cortex and medullary collecting ducts.

In human, the expression of the OTR mRNA has been found in the endometrium, ovary and mammary gland, as well as in the myometrium during pregnancy (Kimura *et al.*, 1992). In the non-pregnant human uterus, the OTR is mainly expressed in the endometrium. In the myometrium, little mRNA encoding for OTR could be detected. The OTR mRNA was expressed in glandular epithelial cells, but was not observed in stromal cells (Takemura *et al.*, 1993). OTRs were found in choriodecidua and amnion with significant increases being reported at term (Fuchs *et al.*, 1982; Benedetto *et al.*, 1990). The OTR mRNA was not detected in cerebral cortex, lung, liver, kidney and spleen using Northern analysis (Kimura *et al.*, 1992).

1.4.2 Hormonal changes during the estrous cycle

1.4.2.1 Prostaglandin F2α (PGF2α)

The principal source of PGF2α in the uterus at luteolysis is the endometrium (Caldwell *et al.*, 1968). It is well known that luteolysis results from the episodic secretion of PGF2α by the uterine endometrium. PGF2α is transferred from the uterine vein to the ovarian artery by a process generally attributed to counter current distribution of the lipoidal PGF2α molecule between the two vessels. In domestic ruminants the ovarian artery follows a tortuous course in close apposition to the uterine vein. This anatomical relationship facilitates the transfer of lipoidal solutes including PGF2α between the uterine vein and the ovarian artery (Ginther, 1981), leading to the degeneration of the luteal cells.

Thorburn *et al.*, (1973) was the first to observe a pulsatile release pattern of uterine PGF2α around the time of luteolysis. A detailed study in ewes showed that the first two or three recognisable pulses of PGF2α occurred at a low frequency of around 16h and preceded the first sustained fall in progesterone which began on days 14-15. The period when progesterone concentrations declined was associated with a further six pulses of PGF2α at a higher frequency of about 6-8 hrs (Fairclough *et al.*, 1983; Hooper *et al.*, 1986; Moore *et al.*, 1986). It was suggested on the basis of this evidence that the time of luteal regression is determined by the pulse frequency of PGF2α release (Zarco *et al.*, 1988).

Wiltbank *et al.* (1991) proposed a working hypothesis for the luteolytic action of PGF2α. They suggested that PGF2α, by increasing the hydrolysis of phosphoinositides to yield inositol triphosphate (InsP3) and diacylglycerol, caused a sustained elevation in the concentrations of free intracellular Ca^{2+} in the large luteal cells. Diacylglycerol and free intracellular Ca^{2+} can activate protein kinase C leading to an inhibition of steroidgenesis and progesterone synthesis. The sustained elevation of free intracellular Ca^{2+} concentrations is cytotoxic and causes the degeneration of the large luteal cells (Wiltbank *et al.*, 1991). These authors also suggested that the PGF2α-induced

degeneration of small luteal cells may be caused by the decrease in the luteal blood flow, or the effects of the cytotoxic agents released by the degenerating large luteal cells. The results of a number of other studies are consistent with this hypothesis (Jacobs *et al.,* 1987; Davis *et al.,* 1987 and Hoyer & Marion, 1989).

Endometrial PGF2α secretion is stimulated by oxytocin in most domestic ruminants, as a result of the binding of OT to its receptor followed by activation of PLC and possibly PLA_2 (Flint *et al.,* 1986b). The time course of the response of the endometrium to oxytocin in terms of increased phosphatidyl inositol metabolism is consistent with that of the activation of pulsatile release of PGF2α *in vivo* (Flint *et al.,* 1986b).

1.4.2.2 Oxytocin (OT)

In ruminants, circulating oxytocin concentrations are basal around oestrus, rise from about day 2 of the cycle as luteal oxytocin synthesis becomes established and reach a peak about day 9 (Wathes and Denning Kendall, 1992). During this period, pulses of oxytocin are superimposed on a continuously increased baseline. In ewes the baseline falls on days 12-13 before the onset of luteolysis, although major episodes of secretion continue (Flint *et al.*, 1990; Wathes *et al.,* 1993).

Secretion of oxytocin by the corpus luteum is stimulated by PGF2α of uterine origin, while PGF2α is transported to the ovary via the uterine vein (Flint & Sheldrick 1982; Heap *et al.,* 1989). This response to PGF2α is relatively rapid with maximum rates of secretion occurring within 10 min of treatment. Large luteal cells express the PGF2α receptor and respond to PGF2α with increased production of inositol phosphates and diacylglycerol, and activation of protein kinase C (Fitz *et al.,* 1982; McCann & Flint 1990; Wiltbank *et al.,* 1991).

It has been demonstrated that the pulsatile release of oxytocin and PGF2α or the oxytocin associated neurophysin occurs simultaneously at luteolysis (Fairclough *et al.*, 1980; Flint & Sheldrick 1983). Flint & Sheldrick (1983) suggested that a positive feedback mechanism operated between these two hormones at luteolysis. This model

proposed that a small rise in the secretion of either hormone was amplified and was followed by the secretion of the other hormone. The rapid rise in the rate of secretion of both oxytocin and PGF2α was characteristic of the episodic secretion of these hormones during luteolysis. Cessation of secretion at the termination of each episode may, in principle, be due to one or more of the following: depletion of available oxytocin from the corpus luteum; depletion of a precursor of PGF2α from the endometrium; the development of luteal refractoriness to PGF2α; or the development of endometrial refractoriness to oxytocin. There is evidence for the involvement of all of these events (Flint *et al.,* 1990). The role of oxytocin in the production of luteolytic episodes of PGF2α at luteolysis was investigated by Mann (1999). The results demonstrated that a normal frequency of PGF2α release episodes occurred independently of luteal oxytocin secretion. However, luteal oxytocin was involved in regulating the pattern of release, perhaps causing the release of episodes of the magnitude required for the successful completion of luteolysis (Mann, 1999). The research from Burns *et al.* (1998) also indicated that both extracellular and intracellular calcium concentrations might be required for oxytocin to stimulate PGF2α release in bovine endometrial tissue.

1.4.2.3 Changes in OTR levels during the estrous cycle

The estrous cycle in domestic ruminants consists of two phases, a luteal phase followed by a follicular phase preceding ovulation. The length of the cycle is determined principally by the length of the luteal phase, and the length of luteal phase is determined by the time of luteal regression. The lengths of the estrous cycle is 17±0.9 days in sheep, and 21±0.8 days in cattle (Flint *et al.*, 1994).

Studies using [^{3}H] oxytocin and ^{125}I-labelled OTA binding assays have indicated that OTRs are present in the endometrium, myometrium and oviduct of the non-pregnant ewe (Roberts *et al.,* 1976; Sheldrick and Flint, 1985; Ayad and Wathes, 1989; Ayad *et al.,* 1990; Ayad *et al.,* 1991). All three receptor populations are present at low concentrations during the luteal phase, increasing at the time of luteolysis reaching a maximum on the day of estrus (Wathes & Hamon 1993). Measurements of oxytocin binding in tissue extracts show that concentrations are at the limit of detection (about

10-20 fmol [^{3}H]oxytocin bound mg^{-1} protein) during the luteal phase (days 5-13) of the estrous cycle. Small increases to about 100 fmol mg^{-1} protein can first be detected on day 14, and thereafter rise to a peak of >500 fmol mg^{-1} protein at estrus, and then decline during days 1-4 following estrus (Wathes & Hamon 1993).

Stevenson *et al.* (1994) used a synthetic 45-mer oligonucleotide corresponding to part of the ovine endometrial OTR cDNA to hybridize sections of ovine uterus collected at different stages during the estrous cycle. The quantity of OTR mRNA expression was measured as the optical density (OD) value on autoradiographs using image analysis. OTR mRNA first appeared in the luminal epithelium on days 14-15 of the cycle, increasing to a peak at estrus and then declined between days 2-5. OTR mRNA expression in the superficial glands, deep glands and caruncular stroma increased to a peak between day 15 and estrus, declined by day 2 and then returned to basal level by day 5. This study indicated that in the endometrium receptor mRNA and ^{125}I-labelled OTA binding patterns changed in parallel, and both sets of measurements were significantly correlated. In the myometrium a significant increase in ^{125}I-labelled OTA binding occurred at estrus. However, this was not accompanied by a similar increase in OTR mRNA hybridization.

1.4.2.4 The role of OTR during the estrous cycle

Since maximal concentrations of OT occur in the mid-luteal phase of the cycle and episodic uterine PGF2α secretion is restricted to the period of luteal regression, a mechanism must exist to limit uterine responsiveness to OT during the early and mid-luteal phases of the cycle. The inhibition of endometrial OTR expression (and probably uncoupling of receptor from second messenger systems) appears to be responsible for this process (Flint *et al.*, 1994). Concentrations of OTR and OTR mRNA expression rise on approximately days 13 or 14 of the cycle in sheep, at the time of the first episodes of PGF2α secretion. However, before this time endometrial OTR concentrations are low and the uterus is not responsive to OT-induced PGF2α secretion (Sheldrick and Flint, 1985; Stewart *et al.*, 1993). It is suggested by Flint *et al.* (1994) that availability of the OTR in the endometrium defines the period of responsiveness of

the uterus to OT in terms of PGF2α secretion and determines the ability of the uterus to cause luteal regression.

1.4.3 Early pregnancy

In all spontaneously ovulating mammals with repetitive ovarian cycles luteolysis is blocked following conception. The function of the corpus luteum must be maintained in early pregnancy in order to provide the high circulating progesterone concentrations necessary for stimulation of a secretory endometrium which is required for normal conceptus growth, particularly before implantation (Flint *et al.*, 1994). Thus an early endocrine adaptation to pregnancy is the inhibition of luteolysis, and this process has been termed the maternal recognition of pregnancy (Short, 1969).

In ruminants, the embryo remains unattached to the uterine endometrium until after the second week of pregnancy. The time of the maternal recognition of pregnancy can be determined by flushing embryos from, or transferring them to the uterus (Flint *et al.*, 1994).

1.4.3.1 Trophoblast Interferon-τ (IFN-τ) and its role

In ruminants, the inhibition of luteal regression involves the blocking of release of luteolytic episodes of PGF2α from the uterus (Thorburn *et al.*, 1973; Peterson *et al.*, 1976). This is achieved by the secretion of a group of proteins by the trophoblast of the developing conceptus. There is a large body of evidence that the conceptus secretes a number of proteins (conceptus secretory proteins, CSP), some of which are involved in the maintaining the function of the corpus luteum (Godkin *et al.*, 1984; Bazer *et al.*, 1986; Lacroix & Kann 1986). One of these this inhibitory substances was later referred to as ovine trophoblastic protein-1 (oTP-1) or (more recently) trophoblast Interferon-τ (IFN-τ). IFN-τ is the antiluteolytic protein produced exclusively by trophoblast cells. Northern analysis of RNA extracted from the blastocyst or *in situ* hybridization with trophoblast tissue, shows that IFN-τ mRNA expression is first detectable by days 8-10 of pregnancy. The concentration of IFN-τ increased on day 13 and reached a peak

between day 14-16 in sheep and day 17-19 in cattle but then decreases rapidly (Flint *et al.*, 1991; Farin *et al.*, 1990; Wathes and Lamming, 1995).

Experiments demonstrating the antiluteolytic properties of IFN-τ have been performed using either purified conceptus secretory proteins containing IFN-τ or recombinant ovine IFN-τ. Administration of synthetic IFNs or blastocyst conditioned medium, extended the lifespan of the corpus luteum and inhibited the pulsatile release of PGF2α (Martal *et al.*, 1990; Garverick *et al.*, 1992; Parkinson *et al.*, 1992). Systemically administered IFNs acted in a similar fashion and was more effective when administration started before day 12 of the estrous cycle (Roberts *et al.*, 1992; Mirando *et al.*, 1993). These treatments prevented the normal development of uterine OTRs by day 15 in comparison with control animals.

Evidence for an inhibitory role for IFN-τ on OTR levels also comes from ewes with transected uteri. In unilaterally transected animals the pregnant horn contains high IFN concentrations and low OTR concentrations, whereas the nonpregnant horn has low IFN concentrations and high OTR concentrations, with the observed increase in OTRs limited to the luminal epithelium (Stewart *et al.*, 1992; Flint *et al.*, 1994). Data from unilaterally pregnant ewes indicated that IFN, produced by the blastocyst, can inhibit OTR expression. Furthermore, since in this surgical preparation both uterine horns were exposed to the same concentrations of the steroid hormones via the systemic circulation, any inhibitory effect of IFN-τ on OTR expression must be exerted locally, rather than systemically (Flint *et al.*, 1994). The direct effects of IFN-τ on OTR have been confirmed in endometrial organ culture (Abayasekara *et al.*, 1992).

Trophoblast IFNs are produced for a limited time in early pregnancy as mentioned above. In sheep, synthesis of IFNs is initiated between day 8 and 10 and ceases between day 20 and 23 of the estrous cycle. Before day 8 to 10, OTR concentrations are inhibited by progesterone and the uterus is refractory to oxytocin. After day 20 the corpus luteum is unable to release oxytocin, and is incapable of stimulating episodic secretion of PGF2α from the uterus (Sheldrick and Flint, 1983; Ivell *et al.*, 1985; Jones and Flint, 1988). Interferon production would be redundant beyond this point. The production of IFNs in this particular time period is quite important. The inhibitory

effect of IFN-τ on OTR gene expression has been shown to lead to the maintainance of corpus luteum function during early pregnancy.

Additional studies have now provided more information on the mechanism of action of IFN-τ. Intrauterine injection of recombinant ovine IFN-τ has been shown to prevent luteolysis by inhibiting estrogen-induced increases in endometrial estrogen receptor (ER) and OTR gene expression (Spencer *et al.,* 1995). The antiluteolytic action of IFN-τ does result in the suppression of transcription of the ER gene by a negative-acting transcriptional mechanism (Spencer and Bazer, 1996). This action of IFN-τ appeared to block uterine luteolysis and, therefore, release of luteolytic pulses of PGF2α, but it had no effect on expression of the progesterone receptor. The maintenance of progesterone secretion by the corpus luteum ensures the establishment and maintenance of pregnancy (Bazer *et al.,* 1997). It is proposed that IFN-τ affects endometrial gene expression by activating the Jak/Stat pathway, which results in formation of the ISGF3alpha transcription factor complex. ISGF3alpha binds to interferon-stimulated response elements and activates transcription of interferon-responsive genes such as interferon regulatory factor-1 (IRF-1) which, in turn, activates expression of the negative-acting transcription factor IRF-2. Pregnancy (or intrauterine injection of roIFN-τ) results in a transient increase in endometrial IRF-1 expression followed 36-48 hr later by a sustained increase in IRF-2. IRF-2, or an IFN-τ-induced negative regulatory factor like IRF-2, suppresses the expression of the estrogen receptor gene and directly or indirectly blocks expression of the gene for the oxytocin receptor to abrogate the uterine luteolytic mechanism and ensure the establishment of pregnancy (Bazer *et al.,* 1997). The IFN-induced down-regulation of the OTR occurs at the transcriptional level and appears to be mediated by protein kinase C (Abayasekara *et al.*, 1995). A similar protein kinase C-mediated down-regulation of OTR gene transcription appears to be part of the homologous desensitization response of human myometrial cells to oxytocin (Phaneuf *et al.,* 1997).

1.4.4 Parturition

A common feature in all mammals is that the uterine sensitivity to OT increases with advancing pregnancy and the myometrium becomes most sensitive to OT at the time of

labor (Fuchs, 1985). It is well accepted that an increase in uterine sensitivity to OT may be involved in the stimulus for parturition. In some species, such as rat, rabbit, goat and sheep, there is a very rapid rise in uterine sensitivity to OT preceding parturition (Fuchs, 1985). In humans, the rise in uterine OT responsiveness occurs more slowly and extends over the entire second half of pregnancy (Takashi *et al.*, 1980).

1.4.4.1 Local uterine factors

The uterus is a rich source of growth factors, cytokines, and other bioactive peptides. Several of these products are regulators of normal growth and development of the uterine endometrium. It was suggested by Zingg *et al.* (1995) that these components may have a role in controlling the uterine OT-prostaglandin system. Interleukin-1β (IL-1β) is one of the important factors for the initiation of parturition. Previous studies have shown that IL-1β can stimulate the production of prostaglandins, arachidonic acid and platelet-activating factor in the uterus and fetal tissues (Semer *et al.*, 1991). IL-1β also stimulates the production of other cytokines (Dinarello, 1991). Evidence is available showing that the administration of IL-1β causes preterm delivery in mice (Romero *et al.*, 1991). The nucleofactor interleukin 6 binding consensus sequences (NF IL-6 CS), found in the promoter region of the OTR gene, suggested that interleukins may act as mediators to induce OTR gene expression at parturition (section 1.5.4)

It has also been shown that prostaglandins can elicit uterine contractions at any stage of gestation in woman and inhibitors of prostaglandin synthesis can extend gestation in many species (Wiqvist *et al.*, 1985). Stimulation of decidual OTRs results in prostaglandin release. Oxytocin has been shown to stimulate decidual prostaglandin synthetase activity and to increase PGF2α release (Fuchs *et al.*, 1982). Prostaglandin stimulation can lead to an increase in plasma OT levels in ewes (Flint and Sheldrick, 1982). Prostaglandins have been shown to increase in uterine sensitivity to OT in woman (Saldana *et al.*, 1974) and myometrial OTR levels (Alexandrova and Soloff, 1980).

It is known that prostaglandins are synthesized within the human fetal membranes (amnion and chorion) and decidua and act to ripen the cervix, change membrane structure and contract the myometrium (Gibb, 1998). Romero *et al.* (1996) provided convincing evidence that prostaglandin concentrations increase in amniotic fluid prior to myometrial contractions, and the activity of prostaglandin H synthase (PGHS) increases in the chorion laeve and amnion at labor (Hirst *et al.*, 1995; Mijovic *et al.*, 1997). This increase is due to increased expression of the PGHS-2 isoenzyme rather than the PGHS-1 isoenzyme (Hirst *et al.*, 1995; Mijovic *et al.*, 1997). In both human and animal pregnancies this increase appears to occur in the fetal tissues rather than in the maternal tissues (Rice *et al.*, 1996; M, Macchia *et al.*, 1997). Economopoulos *et al.* (1996) have indicated that glucocorticoids may be important in regulating prostaglandin formation within the human fetal membranes by increasing expression of PGHS-2 in the amnion and decreasing PGDH activity in the chorion. Prostaglandin dehydrogenase (PGDH) activity decreases in certain cases of preterm labor, and at term it decreases in the area of the chorion laeve (Patel *et al.*, 1999a; Patel *et al.*, 1999b).

Oxytocin induced prostaglandin production is thought to involve G-protein-coupled activation of mitogen-activated protein kinase and cyclooxygenase-2 gene expression, leading to increased prostaglandin production in human myometrial cells. This signaling pathway complements the rapid activation of the phosphoinositide cycle and may be responsible for sustained release of prostaglandins in uterine tissues, promoting labor and parturition (Molnar *et al.*, 1999).

1.4.4.2 Oxytocin receptor and its role

There is abundant evidence in all mammalian species studied to date that there is a rise in uterine OTR preceding the onset of parturition. In women at labor, the level of OTR is over 150 times higher than in nonpregnant women and at the onset of labor, there is a doubling of OTR concentration (Fuchs *et al.*, 1984). In cows, there is an abrupt increase in the response to standard doses of OT about 25 to 48 hours before parturition and this increase coincides with a sharp rise in endometrial OTR density (Fuchs *et al.*, 1992). In the rat, concentrations of uterine OTRs rise sharply about one day preceding parturition (Soloff *et al.*, 1979). The level of OTR mRNA measured by Northern blot

analysis increases dramatically during labor in the pregnant sheep myometrium and endometrium (Wu *et al.*, 1995). Northern blot and Western blot analyses of the levels of OTR mRNA and OTR protein in pregnant sheep myometrium and endometrium in controls and glucocorticoid (betamethasone and dexamethasone)-induced labor showed a significant increases in the tissues associated with labor (Wu *et al.*, 1996).

The mechanisms underlying the pre-parturient rise in OTRs remain to be clarified. One hypothesis is that the rise in OTR alone could be sufficient to trigger the onset of parturition by allowing myometrial and endometrial cells to respond to basal levels of circulating OT. Alternatively it has been proposed that the increase in OTR is the result of an increase in OTR gene expression and post-translational modification. Several studies have demonstrated that estrogens (Alexandrova and Soloff, 1980; Fushs *et al.*, 1983; Zhang *et al.*, 1992) and glucocorticoids (Hinko and Soloff, 1993) have positive effects in up-regulating myometrial OTR, whereas progesterone (Zhang *et al.*, 1992; Jenner *et al.*, 1991; Lau *et al.*, 1992a) and OT (Ayad *et al.*, 1993; Sheldrick, 1992) can down-regulate OTR. Studies in the rat indicate that estrogens induce a strong rise in uterine OTR mRNA expression (Zingg *et al.*, 1995). This is in keeping with the reported effects of estrogens on uterine OT binding (Fuchs *et al.*, 1983). Progesterone alone is without effect. Preliminary data indicate that progesterone does not block the estrogen-induced rise in OTR mRNA (Zingg *et al.*, 1995), despite the fact that progesterone completely blocks the estrogen-induced increase in OT binding (Fuchs *et al.*, 1983). It is likely that the sharply increased maternal estrogen concentration (Challis, 1971) and the fall in progesterone that occurs immediately before parturition are together responsible for the induction of OTR mRNA expression during labor.

Experiments have shown that specific immunostaining for OTR was mainly associated with myometrial cells in pregnant sheep myometrium, whereas in the endometrium, the glands comprised the main site for OTR immunostaining (Wu *et al.*, 1996). These results provide histological evidence indicating that OT may stimulate myometrial contraction by two parallel mechanisms. The first mechanism involves direct activation of OTR on myometrial cells, with a resultant change in intracellular calcium concentrations and increased myometrial contractility (Challis and Lye, 1994). The second mechanism involves indirect stimulation of contraction through the release of

stimulatory PGs from the endometrium (Riemer *et al.*, 1986; Fuchs *et al.*, 1982; Chan, 1979).

Sugimoto *et al.* (1998) found that female mice lacking the receptor for prostaglandin F2α did not deliver fetuses at term, although the fetal mice could be successfully rescued by cesarean section. No induction of the oxytocin receptor mRNA was found in the uterus of these mice, and they showed no evidence of uterine contraction following intravenous administration of oxytocin. Furthermore, a decline in serum progesterone levels during the perparturition period was not observed in these animals. Ovariectomy at day 19 of pregnancy restored induction of the oxytocin receptor and caused successful delivery in these animals. These results emphasis the essential role of luteolytic PGF2α action in natural parturitient and confirm the importance of oxytocin receptor induction in this process.

1.4.5 Regulation of OTR by steroid hormones

The role of estradiol in the regulation of OTR appears to be contentious, since both stimulatory and inhibitory effects have been reported. It has been reported that estradiol-17β treatment can stimulate uterine synthesis and release of PGF2α in ovariectomised estrous ewes (McCracken *et al.*, 1984). Hixon and Flint (1987) showed that a pharmacological dose of estradiol-17β given on Days 9 and 10 of the estrous cycle increased uterine OTR concentrations. Beard and Lamming (1994) reported that the dose of exogenous estradiol affected the timing, the magnitude and the pattern of the PGF2α response to oxytocin in progesterone-treated ovariectomised ewes, and that the estradiol concentration was positively correlated with the rise in the concentrations of oxytocin receptors. In contrast, it was reported that estradiol treatment alone decreased the initially high OTR concentration to intermediate values in ovariectomised ewes (Vallet *et al.*, 1990; Fairclough and Lau, 1992). The basis for this difference in action of estradiol in regulating OTR levels is unclear.

When treated with the prostaglandin synthetase inhibitor, Finadyne, it was found that there was an increase in the concentrations of oxytocin receptors at luteolysis in naturally cycling ewes. It was suggested that this effect of Finadyne may be due to the

loss of the inhibitory effects of progesterone on uterine oxytocin receptors (Lau *et al.*, 1992a). In chronically ovariectomized ewe, it was shown that progesterone can exert a suppressive effect on the concentrations of endometrial oxytocin receptors. However, prolongation of progesterone treatment does lead to the oxytocin receptor eventually becoming refractory to the suppressive effect of progesterone and lead to an increase in the concentrations of oxytocin receptor (Lau *et al.*, 1992b).

As discussed above, the effect of progesterone appears to be biphasic. Substantial evidence is available indicating that progesterone may control the expression of the oxytocin receptor in the uterus. Treatment of ovariectomized ewes with estrogen and progesterone, in doses designed to mimic those circulating during the ovarian cycle, showed that progesterone initially reduces endometrial oxytocin receptor concentrations during the mid-luteal phase of the ovarian cycle. After 10 days this inhibitory action of progesterone declines and endometrial oxytocin concentrations rise to the high levels characteristic of luteal regression (McCracken *et al.*, 1981; Vallet *et al.*, 1990; Vallet & Lamming, 1991). The receptors that develop in these circumstance are located exclusively on the luminal epithelium (Wathes and Lamming, 1995).

The biphasic nature of the response of the endometrium to progesterone can be explained in terms of steroid receptor function. Progesterone and estrogen interact, in their actions on target tissues, through effects on the availability both of their own, and each other's receptors. Thus, estrogens can promote a tissue response to progesterone by increasing tissue concentrations of progesterone receptor. Conversely progesterone is known to reduce estrogen receptor availability (Ott *et al.*, 1993). Progesterone, by down regulating its receptor, causes the tissue to become refractory to progesterone itself. The inhibitory action of progesterone on estrogen receptor synthesis, is therefore withdrawn, and the estrogen receptor is then able to activate oxytocin receptor gene expression. These interactions may explain the dependence of oxytocin receptor gene expression on the ratio of progesterone to estrogen in the circulation (Beard & Lamming, 1994).

It has been suggested that the regulation of the oxytocin receptor occurs principally at the level of gene transcription or OTR mRNA stabilization (Mann and Lamming, 1994;

Lamming and Mann, 1995; Zingg *et al.*, 1998). Zingg *et al.* (1995) have shown that estrogens induce a strong rise in uterine OTR mRNA expression. This is in keeping with the reported effects of estrogens on uterine OT-binding (Fuchs *et al.*, 1983). The effects of steroids on uterine OTR mRNA accumulation *in vivo* have been investigated in ovariectomized rat (Umscheid *et al.*, 1998). After 24h of estradiol treatment, OTR mRNA and OT binding sites increased significantly. Progesterone alone had no effect on OTR mRNA expression. However, progesterone combined with estradiol significantly inhibited the up-regulation of OTR mRNA by estradiol alone. It is suggested that estradiol-dependent activation of OTR gene expression and active OTR synthesis in smooth muscle cells account for the increased OTR levels in rat cervix *in vivo*. In this tissue progesterone acted as an antagonist of estradiol action on OTR gene expression. In contrast, studies by Hazzard *et al.* (1998) on the effects of exogenous estradiol and progesterone on uterine concentrations of OTR and OTR mRNA in cycling ewes showed that endometrial concentrations of OTR and OTR mRNA were significantly greater in control than in estradiol or progesterone-treated ewes. These data suggest that the exposure of the uterus to increased concentrations of estradiol or progesterone causes down-regulation of OTR as a consequence of suppression of the OTR gene (Hazzard *et al.*, 1998). The observed discrepancy between the effect of estradiol and progesterone on OTR gene expression remains to be elucidated.

When a semiquantitative reverse transcription-polymerase chain reaction was used to determine OTR mRNA expression, there was a marked increase in OTR at the onset of spontaneous labor at term in the rat. Exogenous progesterone (4 mg/ day) given on day 20 of gestation blocked this increase. Both preterm labor and the rise in myometrial OTR expression were blocked by progesterone (Ou *et al.*, 1998). On the basis of this and other findings, it seems that progesterone is essential for establishing and maintaining pregnancy in mammals (Pepe and Albrecht, 1995; Garfield *et al.*, 1982). One of its functions includes the maintenance of uterine quiescence by decreasing uterine sensitivity to the uterotonic peptide hormone oxytocin (Garfield *et al.*, 1982; Fuchs *et al.*, 1983; Soloff *et al.*, 1983). Although it is generally held that steroid hormones such as progesterone act at a genomic level by binding to nuclear receptors and modulating the expression of specific target genes (Tsai and O'Malley, 1994), the effect of progesterone on uterine sensitivity to oxytocin involves direct, nongenomic

action of progesterone on the uterine OTR (Grazzini *et al.*, 1998). It has been reported that progesterone binds with high affinity to recombinant Chinese hamster ovary cell (CHO) cells that have been shown to express rat OTR in the membrane. It was reported that the binding of progesterone to the recombinant CHO cells suppressed oxytocin-induced inositol phosphate production and calcium mobilization (Grazzini *et al.*, 1998).

1.5 Investigation of the OTR at the molecular level

1.5.1 Cloning of OTR gene

Kimura *et al.* (1992) first reported the structure and expression of the human oxytocin receptor complementary DNA. The encoded receptor is a 389-amino-acid polypeptide with 7 transmembrane domains typical of G protein-coupled receptors. Messenger RNAs for the receptor are of two sizes, 3.6 kb in breast, and 4.4 kb in ovary, uterine endometrium and moymetrium. The OTR has especially high homology (approximately 40-45%) with the receptors for nonapeptide hormones such as vasopressin V1a, V1b,V2 receptors and the teleost fish Arg^8-vasotocin receptor. These receptors have been classified as the nonapeptide hormone receptor family.

Stewart *et al.* (1993) used the polymerase chain reaction to generate a 131 bp cDNA encoding part of the sheep endometrial OTR based on the human OTR cDNA gene to develop oligonucleotide primers. The nucleotide sequence of this cDNA was 93.8% identical to the human OTR sequence in this region. When used to probe Northern blots of sheep endometrial RNA, the PCR product was identified as a 6.7 kb mRNA which appeared and disappeared during the estrous cycle in parallel with the OTR molecule as measured by ligand binding.

A sheep oxytocin receptor cDNA, including the complete coding region, was isolated using mRNA extracted from endometrium collected from a non-pregnant ewe on day 16 after oestrus and was sequenced by a variety of strategies (Riley *et al.*, 1995). The sheep endometrial OTR cDNA encodes a 391 amino acids protein and its mRNA is heterogeneous, usually giving 4 bands on Northern blot of approximately 1.65, 2.5, 3.9 and 6.1 Kb (Riley *et al.*, 1995). The sheep OTR cDNA sequence unexpectedly

revealed that the third intracytoplasmic region of the sheep receptor has 3 and 2 additional amino acids relative to the rat and human receptors, respectively. It was confirmed that additional amino acids are a general phenomenon in ruminants (Kaluz *et al.*, 1996). However, little information has been published to explain the presence of multiple transcripts.

Rozen *et al.*, (1995) have isolated and characterised the rat genomic OTR gene. The rat OTR gene spans >20 kb and contains three exons. A 97 bp intron occurs in the 5' untranslated region and a >12 kb intron interrupts the coding region between transmembrane domains 6 and 7. The predicted amino acid sequence was 93% identical to the human OTR sequence but only 48% and 38% identical to the rat V1 and V2 vasopressin receptor sequences, respectively. At parturition, the OTR gene is highly expressed in the rat uterus and gives rise to at least three transcripts (2.9, 4.8, and 6.7 kb) which differ in the length of their 3' untranslated regions.

The gene for the bovine OTR has been sequenced using a combination of clones derived from a bovine endometrial cDNA library from estrus and a bovine genomic DNA library. The gene structure of OTR was confirmed using RT-PCR programmed by term myometrial RNA. There are two introns, one in the open reading frame between the regions encoding the transmembrane domains VI and VII. Northern blot analysis indicated three major transcripts in myometrium and endometrium *in vivo* at 6.5 kb, 3.5 kb, and 2.0 kb (Bathgate *et al.*, 1995).

1.5.2 Expression of OTR

OTR expression has been extensively studied using Northern blotting and *in situ* hybridization. In section 1.4, the localization of OTR mRNA was addressed and the level of OTR mRNA expression during the estrous cycle, in early pregnant and at parturition was reviewed. This section focuses on the difference between OTR concentrations and the development of OTR responsiveness to OT challenge in the endometrium.

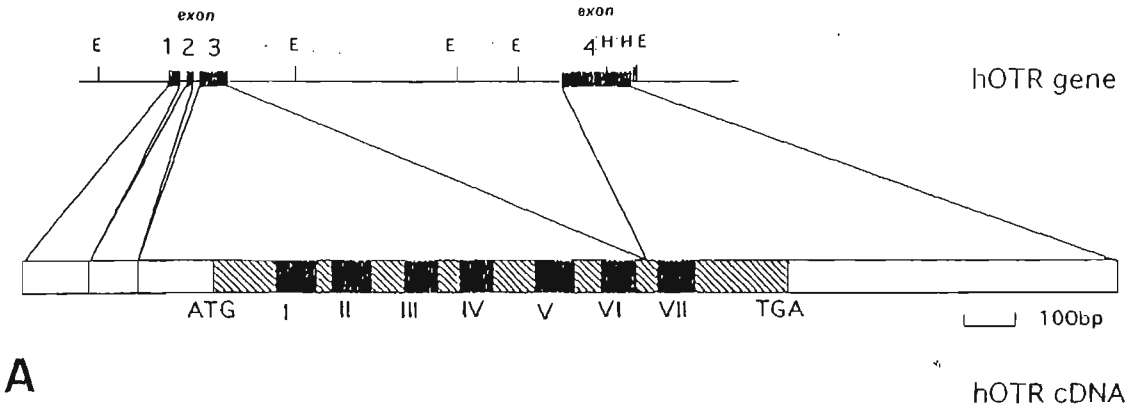
Several investigators have indicated the possibility of OTR subtypes by functionally analyzing the receptor (El *et al.,* 1990; Chan *et al.,* 1993) and by determining mRNA OTR expression (Larcher *et al.*, 1995; Stevenson *et al.*, 1994). Research by Wathes and Lamming (1995), using *in situ* hybridization, has shown that the development of OTRs in a specific cell type, the luminal epithelium, is necessary in order to achieve a maximal PGF2α response to an oxytocin challenge. However, the OTRs in the luminal epithelium constitute a small percentage of the total potential endometrial OTR population. A large proportion of the endometrial OTR population is located within the caruncular stroma and deep glands but the OTRs in these locations do not increase until luteolysis is complete. Moreover, the formation and activity of these receptors are probably estrogen dependent. The explanation for these differences in the different endometrial sites remains unclear. It has been demonstrated that a single copy gene for OTR, as determined by Southern blot analysis of restricted genomic DNA, exists in both the human and the cow (Kimura and Saji, 1995; Bathgate *et al.*, 1995). Whether the existence of the different populations of OTR in the endometrium results from transcriptional modification remains to be investigated.

1.5.3 Structure of OTR

1.5.3.1 Genomic DNA structure

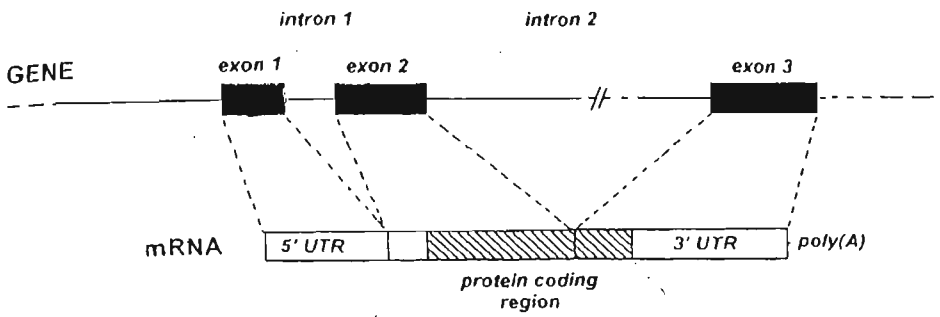
Genomic DNA for OTR have been reported in the human, rat and cattle (Inoue *et al.,* 1994; Rozen *et al.,* 1995; Bathgate *et al.,* 1995). The genomic DNA for OTR can be mainly divided into three segments. Segment 1 contains the 5'non-coding region. Segment 2 starts upstream from the ATG initiation codon and spans downstream encoding most of the receptor protein to just beyond the sixth transmembrane domain of the receptor. Segment 3 contains the sequence encoding downstream from the seventh transmembrane domain of the receptor, the C-terminal region and the entire 3'non-coding region, including multiple polyadenylation signals. There are two introns between the three segments. The largest intron, being about 12 kb in human and rat, interrupts the coding region between transmembrane domain 6 and 7. The human OTR gene resembles those in the rat and cattle except that there are two exons upstream of that containing the ATG initiation codon. This gene is located close to 3p26.2 of human

chromosome 3, a locus where until now, no other protein-encoding genes have been reported to date (Kimura 1995). The genomic DNA structure in sheep has not been reported. The following diagrams (Fig 1.4) indicate the structure and organization of OTR gene in human (A), rat (B) and cattle (C).

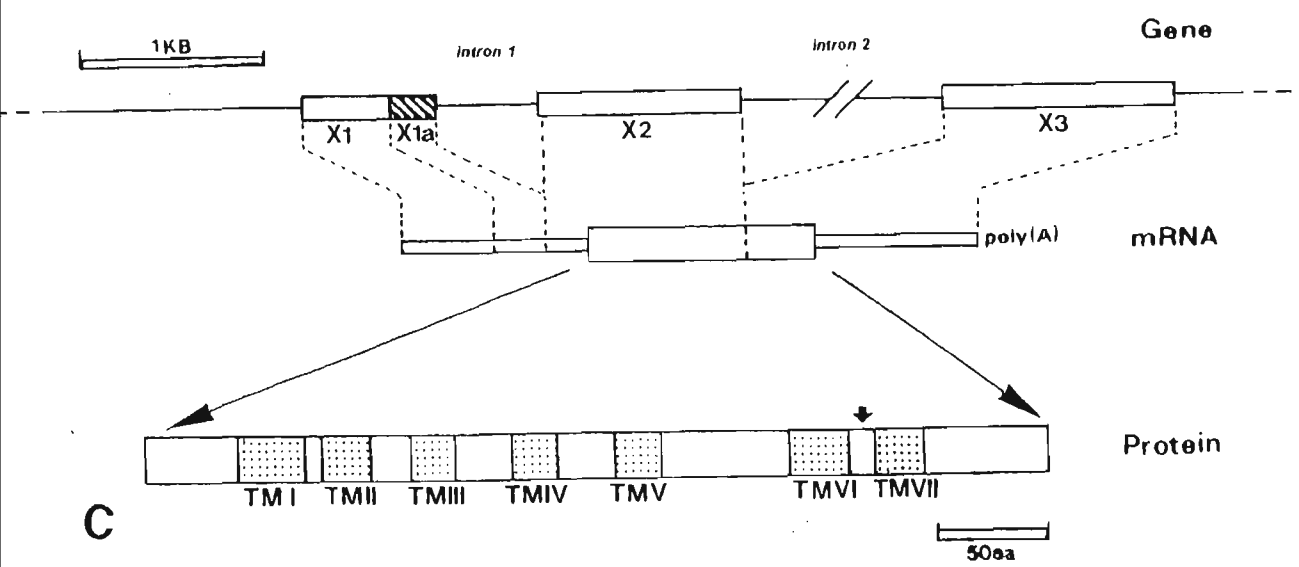


A

hOTR cDNA



B



C

Fig 1.4 Scheme illustrating the structure and organization of OTR gene in human (A, adopted from Kimura and Saji, 1995), rat (B, modified from Rozen *et al.*, 1995) and cattle (C, adopted from Ivell *et al.*, 1995), indicating the probable splicing pattern and structure of the resulting transcript. X=exon, TM I-VII or I-VII= transmembrane domains I to VII. The arrowhead indicates the position of the intron 2 splicing site.

1.5.3.2 Protein structure

It is well known that the OTR is a G-protein coupled receptor with seven transmembrane domains in several species. Translation of the protein-coding region of the OTR gene gives rise to an amino acid sequence which differs mainly in N-terminus, C-terminus and the third intracellular loop in comparison with the putative amino acid sequence in sheep (Riley *et al.,* 1995), cattle (Bathgate *et al.,* 1995), pig (Gorbulev *et al.*, 1993), human (Kimura *et al.,* 1992) and rat (Rozen *et al.*, 1995). It has been demonstrated that the third intracellular loop of OTR is involved in the interaction of OTR with Gαq/11 using interference assays (Qian *et al.*, 1998). Moreover, the data indicated that interactions with more than one intracellular loop of OTR probably mediate its coupling to proteins of the Gαq/11 subclass (Qian *et al.*, 1998). A more recent study has provided evidence that the proximal portion of the carboxyl terminus of the OTR is required for coupling to Gq-protein using CHO-OTR cell lines (Hoare *et al.*, 1999). When 51 amino acid residues from the carboxyl terminus of OTR were deleted, the Gq-mediated events, which include InsP3 formation, PKC translocation, ERK-2 phosphorylation and PGE2 synthesis were reduced. However, these cells still responded to OT through the Gi-mediated pathway. The truncated receptor of 39 amino acid residues from the carboxyl terminus had all the activities of the wild type.

The identification of the OTR domain has been investigated in detail (Postina *et al.*, 1998). A model system of hybrid vasopressin V2/oxytocin receptors with mainly extracellular receptor domains for identification of OTR domains has been established for selective OT and OT antagonist binding. In conclusion: it is suggested that; the first three extracellular domains of the OTR provide one half of the high affinity OT binding site and the transmembrane helices have a role in specific binding of OT. It is also suggested that the extracellular receptor domains have an influence on the signal transduction pathway and that part of the peptide antagonist binding site of OTR is formed by the transmembrane helices 1, 2 and 7 (Postina *et al.*, 1998). The protein secondary structure of the bovine OTR is given in Fig 1.5.

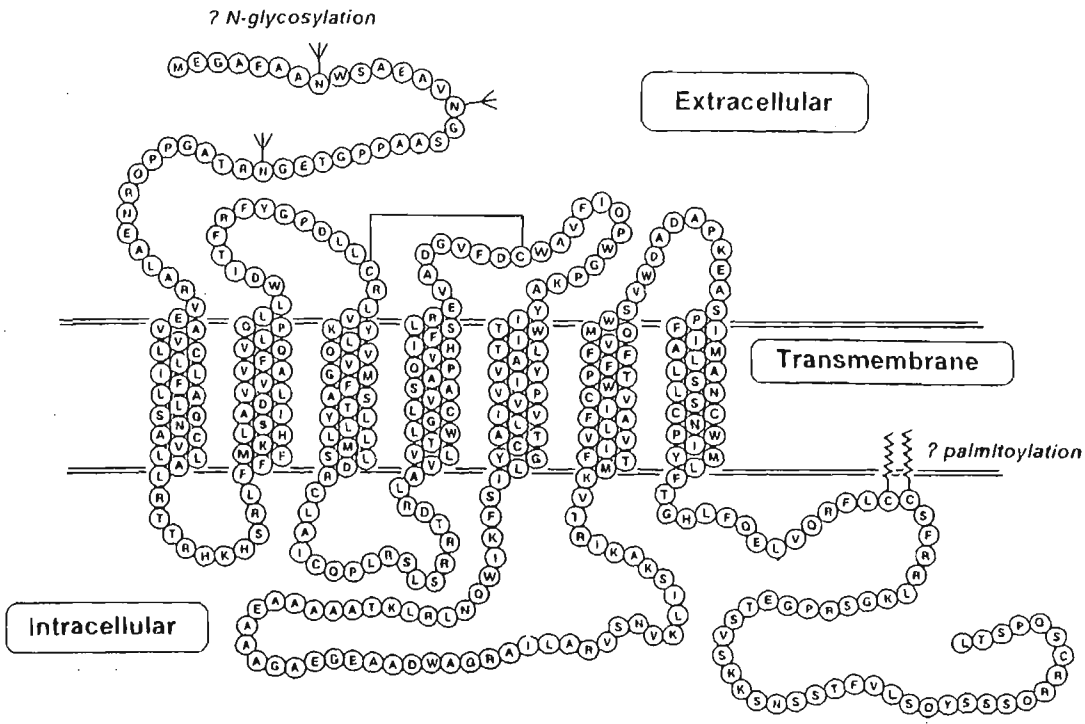


Fig 1.5 The protein secondary structure of the bovine OTR (adopted from Horn *et al.*, 1998). The sites of N-glycosylation and palmitoylation remain putatively.

1.5.4 Regulation of OTR gene

The regulation of OTR expression has been reviewed in section 1.4, particularly with regard to the possible effects of steroids (section 1.4.5) and interferon (section 1.4.3). This section focuses on the OTR gene promoter region.

In the 5' upstream region of the human and rat OTR genes, there are several common consensus sequences for binding sites of transcriptional regulatory proteins. Three nucleofactor interleukin 6 binding consensus sequences (NF IL-6 CS) and two binding sequences for the acute phase reactant responsive element (APRE) were found in the human gene (Inoue *et al.*, 1994). In the rat gene, six NF IL-6 CS motifs and four APRE sites have been identified (Rozen *et al.*, 1995). The presence of APRE and NF IL-6 CS in the OTR gene promoter suggests that the acute induction of OTR expression at the onset of parturition may be a phenomenon mechanistically similar to the fast induction of acute phase response genes, such as α2- macroglobulin and T-kininogen (Hattori *et al.*, 1990; Mann *et al.*, 1991), which are induced as a result of infection and inflammation (Kimura, 1995; Rozen *et al.*, 1995). Interleukin-1β released from macrophages stimulates the production and release of interleukin-6 by uterine stromal cells (Tabibzadeh *et al.*, 1991; Dudley *et al.*, 1992). Interleukin-1 is a central pathophysiological mediator of infection-induced premature delivery (Romero *et al.*, 1992), and premature delivery can be prevented by an interleukin-1 antagonist in mice (Romero and Tartakovsky, 1992). It was speculated by Rozen *et al.* (1995) that under physiological, as well as pathophysiological conditions, inflammatory cytokines might be important inducers of labor and that this mechanism involved the cytokine-induced transcriptional activation of the OTR gene.

A 4 kb fragment of 5'-flanking DNA of the rat OTR gene has been cloned and sequenced, and identified as an internal segment which was not mentioned in the initial publication on the promoter sequence (Bale and Dorsa, 1998). Sequence analysis of this segment, as well as the novel upstream region, revealed the presence of a Cyclic AMP response element (CRE) as well as several other potential regulatory elements, including AP-1, AP-2, AP-3, AP-4 sites, an estrogen response element (ERE), and a half-SRE (serum response element) (SRE/2). Mutational analyses of the CRE and half-

SRE sites in this promoter indicated that these elements function as enhancers and support forskolin and NGF effects on transcription respectively. These studies have identified a novel region of the rat OTR promoter containing elements that impart cAMP and/or phorbol ester inducibility of OTR gene transcription. A potential role of the protein kinase A (PKA) and/or PKC pathways in OTR gene regulation is suggested.

Despite the fact that estrogens are strongly implicated in the up-regulation of uterine and hypothalamic OT binding sites and uterine and hypothalamic OTR mRNA (see section 1.4.1 and 1.4.3), and other data indicate that the role of estrogen on the OTR regulation occurs principally at the level of gene transcription (Fuchs *et al.*, 1995), there is no classical palindromic ERE in the 5'flanking region found in humans and rats. However, there are several half palindromic ERE motifs in this region. In the case of the chicken ovalbumin gene, activation by estrogens is mediated by several widely spaced half palindromes (TGACC or GGTCA), with one such element located in the promoter proximal region (Kato *et al.*, 1992). As has been noted for the rat gene, the human and bovine OTR gene 5'flanking region also contain a total of six widely spaced half palindromes, with one centered 25 bp 5' to the cap site. It is possible that widely spaced half palindromic ERE motifs may act synergistically (Bale and Dorsa, 1997). However, using the heterologous system, Ivell *et al.* (1998) did not observe a hormone-dependent upregulation of the OTR gene in cattle. Similar observation have also been made by others for the human and rat genes (Hoare *et al.*, 1997, Grazzini *et al.*, 1997). The functional activity of these elements should be further examined. The schematic organization of the human and bovine OTR gene promoter regions is shown in Fig 1.6.

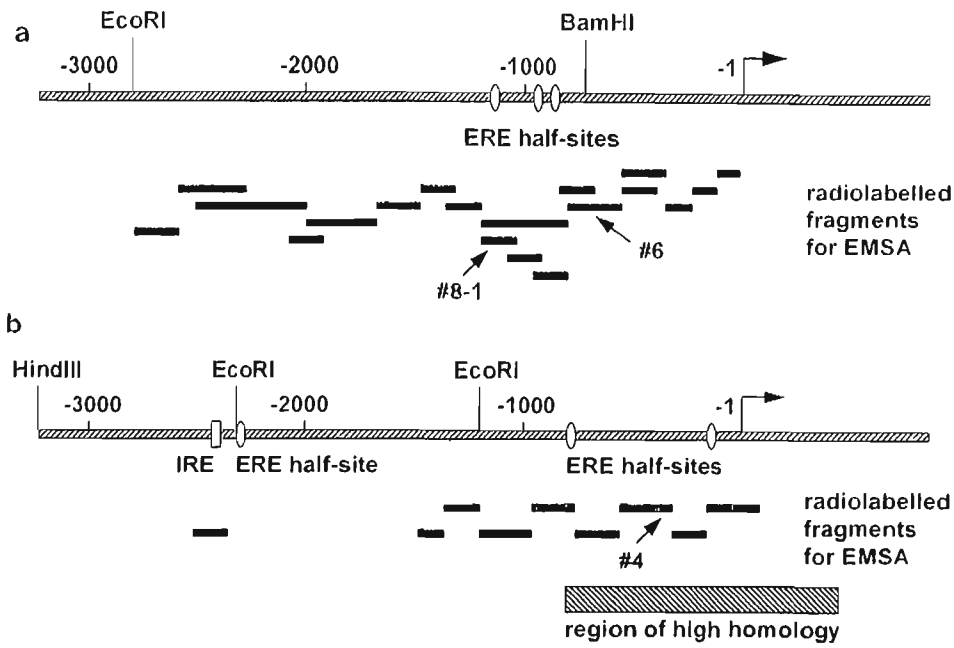


Fig 1.6 Schematic organization of the human (a) and bovine (b) OTR gene promoter regions (adopted from Ivell *et al.*, 1998). The putative estrogen response element half-sites (ERE) and a putative interferon response element (IRE) were identified in a computer search. The radiolabelled fragments used for electrophoretic mobility shift assays (EMSA) are indicated as black bars. The region of high homology between all OTR genes is shown as a horizontal hatched bar.

1.6 Application of OTR

1.6.1 Clinical use

In routine clinical examination of pregnant women, the estimation of the uterine preparedness for parturition is one of the most important tasks in the prediction of preterm labor and the timing of delivery. One of major changes in the uterus during the preparation of labor is an increase in OTR levels. To detect the uterine OTR in a non-invasive manner, Kubota *et al.* (1994) have examined OTR expression in scraped endocervical cells using RT-PCR. This method would lead to biochemical estimation of the status of the pregnant uterus, although technical improvements are required. Kobayashi *et al.* (1999) found that the oxytocin antagonist L-366,509 delayed the initiation of labor in rats with term and preterm in a dose-dependent manner. This oxytocin antagonist was able to delay term and preterm labor, so it might prove useful in clinical practice for the treatment of preterm labor.

1.6.2 Breast cancer research

The presence of OTR in human breast cancer tissue has been demonstrated (Taylor *et al.*, 1990; Cassoni *et al.*, 1994). Monoclonal antibodies raised from the human OTR gene were used in studies on cancer. Among breast cancer patients, high positive results (91.2%) for the detection of OTR in breast cancer was obtained by immunohistochemistry (Ito *et al.*, 1996). In the mammary gland, OT can also act on cell growth and differentiation. In the developing mouse mammary gland, OT increases the proliferation of myoepithelial cells and reduces the number of undifferentiated stem cells (Sapino *et al.*, 1993). On the other hand, OT inhibits cell proliferation of human breast cancer cells both *in vitro* (Cassoni *et al.*, 1994) and *in vivo* (Cassoni *et al.*, 1996). The inhibitory effect of OT on breast carcinoma cells is due to the activation of OTR (Bussolati *et al.*, 1996; Cassoni *et al.*, 1998). Receptor antagonist therapy using anti-estrogen in breast cancer has been quite successful if the cancer tissue expresses the estrogen receptor (Pearson *et al.*, 1982). The regulation of OTR in breast cancer cells could be another area for breast cancer research.

1.6.3 Studies of OTR regulation

Because of the role that OT appears to play in many acute physiology associated with reproduction and reproduction-associated behavioral functions, it is difficult to select the most suitable tissue for studies on the regulation of OTR gene expression. A bovine endometrial epithelial cell system that was collected from bovine uterine tissue during the estrous cycle has been established to study OTR regulation (Horn *et al.*, 1998). These cells were shown to express relative high levels of OTR after about one week in culture. Experiments to investigate the regulation of the OTR and its gene using this cell model, have shown that both estradiol and progsterone, or a combination of these has no effect on the expression of the endogenous OTR gene. However, interferon-τ has downregulation effect on both OTR and its gene transcripts in cultured endometrial epithelial cells. This bovine endometrial system offers a flexible and useful model to investigate the regulation of the OTR gene, although further research should provide more information about the regultory factors involved in the molecular control of OTR gene expression.

1.7 Aim of the thesis

In order to obtain a better understanding of the mechanism underlying uterine OTR regulation, the following studies were undertaken:

To determine the cDNA sequence of the OTR gene in sheep using RT-PCR techniques.

To identify the transcripts for OTR in ovine uterine endometrium, and to characterize the sequence differences among these transcripts.

To determine OTR mRNA expression in the endometrium, corpus luteum and pituitary gland during the estrous cycle, and to detect OTR expression in endometrium, myometrium, cervix and cotyledons at labor.

To investigate the role of estradiol in regulating the OTR mRNA and PGHS mRNA expression in ovariectomised ewes that have been previously primed with exogenous progesterone and estradiol.

To establish an expression system for OTR expression, to obtain the OTR antibody for further research work.

Chapter 2 Materials and methods

2.1 Animals

Merino ewes were used for the experiments documented in this thesis. All procedures were approved by the Animal Experimentation Ethics Committees of Victoria University of Technology and Monash University, Department of Physiology and all genetic manipulation work was approved by the Institutional Biosafety Committee of Victoria University of technology.

2.1.1 Determination of estrous

Estrus was determined using a vasectomized ram fitted with a marking harness. The day of first marking was designated as day 0 of estrous cycle. The following days were determined analogically.

2.1.2 Determination of labor

Ewes were obtained from the Monash University farm at Tooradin. On days 112-116 of gestation these animals were brought into the Department of Physiology animal housing facility, where they were placed into metabolic cages and were settled into an environment of 12 hours light and 12 hours dark. On days 117-121 of gestation these ewes underwent surgery to implant electromyograph (EMG) recording electrodes to the uterine wall (Sigger *et al.*, 1984). Electrodes were made from multi-stranded stainless steel teflon-coated wire (1.2 m long, Cooner Wire Co., Chadsworth, USA), covered with a vinyl catheter (Dural Plastics, Dural, Australia) and were sutured to the body of the uterus in a triangular pattern 5 mm apart. Signals derived from these electrodes were amplified on a Grass 7P3 amplifier (Grass Instruments, Quincy, USA) with high pass filtering at 0.3 Hz. These signals were recorded on a 2 or 4 channel Grass Polygraph (Model 7D). The surgical procedures were carried out by Dr Ross Young and Wendy McLaren. EMG activity was monitored throughout the latter stages of gestation and the animals were characterized as being in labor when contractile activity showed high frequency, high amplitude contractions of low duration (Harding *et al.*, 1982; Jenkin & Nathanielsz, 1994). At this time, the ewes were euthanased using a

lethal dose of Lethabarb (pentobarbitone sodium 325 mg/ml; Virbac Tty, Ltd, NSW, Australia).

2.1.3 Induction of labor

Ewes were mated at the Monash University farm, Tooradin. Mated ewes underwent an ultra-sound to confirm pregnancy between days 60 to 90 of pregnancy. This procedure was carried out between May to August 1995. Ewes were transferred to the Department of Physiology, animal house facilities between days 122-128 of pregnancy. Ewes were randomly divided into two groups. Labor was induced in the ewes on day 133 of pregnancy by an i.m. injection of 6 mg betamethasone. Control ewes were given an i.m. injection of saline on day 132 of pregnancy.

2.1.4 Preparation of steroid injections

Progesterone and oestradiol-17β (Sigma) injections were prepared in vegetable oil. The required quantity of steroid was first dissolved in absolute ethanol at 5% (v/v) of the final volume. Vegetable oil was then added to make a stock solution and the ethanol was evaporated by heating at 60-70°C and constant stirring with a magnetic stirrer. The required doses of the steroid solutions were then prepared in glass flasks and were drawn up into plastic syringes just prior to injection to prevent non-specific binding of the steroids to the plastic.

2.1.5 Euthanasia

At the time of sample collection, the ewes were euthanased using a lethal dose of Lethabarb (pentobarbitone sodium 325 mg/ml; Virbac Tty, Ltd, NSW, Australia).

2.1.6 Tissue collection

Method 1. All tissue samples from the ewes during the estrous cycle were placed immediately on ice for processing. Uterine endometrium, pituitary gland and corpus luteum were dissected respectively, washed with cold PBS (0.01M $NaPO_4$, pH7.4;

0.15M NaCl), blotted on sterile surgical gauze, snap frozen in liquid nitrogen and stored on dry ice before storing in a freezer at -70°C.

Method 2. The uterus collected from the ewe at parturition was removed immediately after slaughter. Tissues were wrapped in aluminium foil and frozen in liquid nitrogen, cooled in isopentane and stored at -70°C. These tissues were collected from pieces of uterus, endometrium, myometrium, cotyledon, section of uterine wall (including intercotyledonary endometrium and myometruim) and the cervix.

2.2 RNA preparation

2.2.1 Total RNA isolation

2.2.1.1 The guanidium thiocyanate extraction method

The protocol used to extract total RNA was taken from the handbook (Current protocols in molecular biology, 1996). The brief protocol was as follows:

Tissue samples (0.2g) were added to a McCartney tube containing 2 ml denaturing solution (100mg tissue/ml). The sample was homogenised immediately using a polytron homogenizer at high speed for less than 10 seconds and the process was repeated 2-3 times. The sample was then quickly put on ice to allow separation of the homogenate. The solution was transferred to a 6ml-polypropylene tube. Sodium acetate (2M, pH4) was added (0.2ml) and the solution was mixed thoroughly by inversion. Water-saturated phenol (2ml) was added and the solution was mixed thoroughly by inversion. Chloroform/isoamyl alcohol (49:1) was added (0.4ml) and the solution was mixed thoroughly by inversion. The tube was left on ice for 15 min, and was centrifuged for 20 min in a JA-20 rotor (Beckman) at 12000 rpm (10000g), 4°C, Break=0. The upper aqueous layer was carefully transferred to a fresh tube. 1 volume of 100% ispropanol was added, and the tube was left at -20°C for 30min (or overnight). After centrifuging for 10 min at 12000rpm, the supernatant was discarded. The pellet was dissolved in 0.6ml denaturing solution, and 1 volume of 100% isopropanol was added, the tube was left at -20°C for 30min. After centrifuging for a further 10 min at

12000rpm, the supernatant was discarded. The pellet was washed in 0.5 ml 75% ethanol for 10-15 min, and was then dried. The pellet was re-dissolved in 200ul DEPC-water. The solution was transferred into a 1.5ml microcentrifuge tube, 10% vol. of 2M sodium acetate pH4, and 3 vol. of 100% ethanol were added. The sample was stored in -80°C pending further experimentation.

2.2.1.2 Total RNA extraction by TRIZOL reagent

Tissue samples were homogenized in 3 ml of TRIZOL reagent (Life Technologies) (50mg/ml of tissue) using Polytron homogenizer. The sample volume did not exceed 10% of the volume of the TRIZOL reagent used for homogenization. The homogenised samples were incubated for 5 min at room temperature to permit the complete dissociation of nucleoprotein complexes. 0.6 ml of chloroform (0.2ml/1ml TRIZOL) was added, and the tube was shaken vigorously for 15 sec and incubated at room temperature for 2-3 min. After centrifuging at 12000rpm for 15 min at 4°C, the upper aqueous phase was transferred to a fresh tube. 1.5ml of isopropanol (0.5ml/1ml TRIZOL) was added. The solution was incubated at room temperature for 10 min, and then centrifuged at 12000rpm for 10 min at 4°C. After removing the supernatant, the RNA pellet was washed with 3ml 75% ethanol (1ml/1ml TRIZOL). The sample was mixed by votexing and was centrifuged at 7500rpm for 5min at 4°C. The RNA pellet was air-dried briefly. The RNA sample was re-dissolved in DEPC-H_2O by passing the solution a few times through a pipette tip, the sample was incubated for 10 min at 55 to 60°C. 10% vol. of 2M sodium acetate pH4 and 3 vol. of 100% ethanol were added. The sample was stored in -80°C for further use.

2.2.2 mRNA isolation (small scale)

mRNA was purified from samples of total RNA using PolyATtract mRNA Isolation System (Promega). This system used a biotinylated oligo(dT) primer to hybridize at high efficiency in solution to the 3' poly(A) region present in most mature eukaryotic mRNA species. The hybrids were captured and washed at high stringency using streptavidin coupled to paramagnetic particles (SA-PMP) and a magnetic separation

stand. The mRNA was eluted from the solid phase by the simple addition of ribonuclease-free deionized water. The protocol used was as follows:

Annealing of the probe 0.1-1.0 mg of total RNA was placed in a sterile, RNase-free tube to a final volume of 500 µl in DEPC-H_2O. The tube was incubated in a 65°C heating block for 10 minutes. 3µl of the biotinylated-oligo(dT) probe and 13µl of 20XSSC were added to the RNA sample. The solution was mixed gently and incubated at room temperature until completely cooled (about 10 minutes).

Washing of streptavidin-paramagnetic particles (SA-PMP) The SA-PMPs were resuspended by gently flicking the bottom of the tube until they were completely dispersed and then captured by placing the tube in the magnetic stand until the SA-PMPs have been collected at the side the tube. The supernatant was carefully removed. The SA-PMPs were washed three times with 0.5XSSC (0.3ml per wash), each time the SA-PMPs were captured using the magnetic stand and then the supernatant was carefully removed. The washed SA-PMPs were resuspend in 0.1ml of 0.5XSSC.

Capture and washing of annealed oligo(dT)-mRNA hybrids The entire content of the annealing reaction was added to the tube containing the washed SA-PMPs. The mixture was incubated at room temperature for 10 minutes. SA-PMPs were captured using the magnetic stand and then the supernatant was carefully removed without disturbing the SA-PMP pellet. The supernatant was saved until it was certain that satisfactory binding and elution of mRNA had occurred. The particles were washed four times with 0.1XSSC (0.3ml per wash) by gently flicking the bottom of the tube until all of the particles were resuspended. After the final wash, the aqueous phase was removed as much as possible without disturbing the SA-PMP particles.

Elution of mRNA The final washed SA-PMP particles were resuspended in 0.1ml of DEPC-H2O by gently flicking the bottom of the tube. The SA-PMPs were magnetically captured and the eluted mRNA aqueous phase was transferred to a sterile, RNase-free tube. The elution step was repeated by resuspending the SA-PMP particles in 0.15ml of DEPC-H_2O. The capture step was repeated, and the eluates were pooled

together (0.25ml total volume). The eluted mRNA samples were stored at -80°C for further use.

2.2.3 Estimation of RNA sample concentration

An aliquot of the RNA sample was diluted to measure the absorbance at 260 nm (A260) using a spectrophotometer. The concentration of the RNA sample was determined using the following formula (Sambrook *et al.*, 1989):

A_{260} x dilution factor x 40* = μg RNA/mL
(*A_{260} = 1 for 40 μg/mL RNA)

2.2.4 Estimation of RNA sample quality

In general, the OD ratio measured at A260 to A280 should be equal or over 1.8 for total RNA sample (A260 /A280 ≥1.8), and for mRNA sample, the OD ratio should be equal or over 2.0 (A260 /A280 ≥2.0).

2.2.5 Gel electrophoresis of RNA

2.2.5.1 Preparation of solutions

1). 10XMOPS buffer (running buffer): 0.2M MOPS
80mM sodium acetate
10 mM EDTA (pH 8.0)

41.2g 4-morpholinopropanesulfonic acid was dissolved in 80ml 1M sodium acetate, sterile-ddH_2O was added up to 800ml, pH was adjusted to 7.0 with NaOH (~15 pellets), then 20 ml 0.5M EDTA was added, the final volume was adjusted to 1000ml with ddH_2O.

2). RNA sample buffer:	250µl	formamide (deionised)
	83µl	formaldehyde 37% (v/v)
	50µl	10X MOPS buffer
	17µl	DEPC-H_2O

3). RNA loading buffer:	50%	glycerol
	1mM	EDTA
	0.4%	bromophenol
	1ug/sample	ethidium bromide

2.2.5.2 Formaldehyde gel preparation

The following formula was used for the mini-gel preparation. For the medium or large gel preparation, the volume of the contents was increased two or three times.

Formaldehyde gel:	0.5g	agarose
	40.5ml	sterile-DEPC H_2O
	10ml	formaldehyde
	5.5ml	10X MOPS buffer

The agarose was melted in DEPC-H_2O by heating. When the agarose had cooled to about 60°C, MOPS buffer and formaldehyde were added, the contents were mixed well and poured immediately into the gel tray.

2.2.5.3 Sample preparation

A volume of RNA sample (~10ug) that was stored in an ethanol solution were taken and centrifuged at 4°C for 15 min in high speed. The pellet was air-dried and dissolved in 4ul DEPC-H_2O. 16ul of sample buffer (1:4) was added. The sample was denatured by heating at 65°C for 10min, and then was placed on ice immediately for 5 minutes. 5ul of RNA loading buffer were added.

2.2.5.4 Electrophoresis

The sample was loaded onto a 1% formaldehyde agarose gel as described above. To avoid spillage of samples the gel was not submerged in the buffer during loading. The gel was run in 1XMOPS running buffer at a high voltage (100V) for 5 minutes until the sample entered the gel. The gel was submerged in 2-3mm running buffer and the electrophoresis was continued at 65V about 2-3 hours until the samples had migrated to the middle of the gel.

2.3 DNA preparation

2.3.1 Isolation of double-stranded plasmid DNA

The double-stranded plasmid DNA preparations from the recombination *E.coli* colonies were carried out by the procedures of Sambrook et al. (1989).

2.3.1.1 Large-scale preparation

A single *E.coli* colony was selected and transferred into a tube containing 3 ml of 2YT medium with ampicillin (50mg/ml). The tube was incubated with shaking at 37° until the A260 reached 0.8-1.0. 1 ml of this culture was transferred to a flask containing 100 ml of LB medium with ampicillin (50mg/ml), the mixture was incubated with shaking (200 cycle/minute) at 37°C overnight. 100 ml of an overnight culture was transferred to a centrifuge tube and was centrifuged in a Beckman JA-20 rotor at 6000 rpm for 5 min, 4°C to collect the cells. As much of the supernatant as possible was removed by aspiration. The pellet was resuspended in 6 ml of ice-cold STE buffer (Solution I) by vigorously vortexing. Lysozyme was added to a final concentration of 2 mg/ml, the mixture was left on ice for 10 min. 12 ml of 0.2 M NaCl/1% SDS (Solution II) was added. The solution was mixed by gently inverting the tube 10 times. The tube was left on ice for 5 min. 7.5 ml of 3M potassium acetate (Solution III) was added and the mixture was mixed by gently inverting the tube 10 times. The tube was left on ice for 30 min. After centrifuging at 15000 rpm for 10 min, 4°C, the supernatant was carefully decanted into a clean tube. 0.7 volume of isopropanol was added, and the mixture was

incubated at room temperature for 10 min. The sample was centrifuged at 15000 rpm for 10 min at 4°C to collect the pellet. The pellet was re-suspended in 1ml of TE buffer. 5ul of RNase A (10ug/ul) was added, and the solution was incubated at 37°C for 30-60 min. The solution was transferred into two fresh Eppendorf tubes (0.5 ml each). The same volume of phenol:chloroform/isoamyl alcohol (1:1) was added. The sample was mixed by vortexing for 1 min and centrifuged at 15000 rpm for 5 min. The phenol/chloroform extraction step was repeated either once or twice as necessary. The upper aqueous phase was transferred to a fresh tube and same volume of chloroform/isoamyl alcohol (24:1) was added. The mixed sample was centrifuged at 15000 rpm for 5 min. The upper aqueous phase was transferted to a fresh tube, and 1/10 volume of 3M sodium acetate and 2 volume of 100% ethanol were added to the tube. The mixture was left at -20°C for 30 min and was centrifuged at 15000 rpm for 10 min. The pellet was washed with 70% ethanol, and was then dissolved in 100µl TE buffer.

2.3.1.2 Small-scale preparation (alkaline method)

Each of the single recombination *E.coli* colonies were incubated in 3 ml LB medium with ampicillin (50 mg/ml) and grown at 37°C with shaking (200 cycle/minute) overnight. 1.5ml x 2 of an overnight culture was transferred to an Eppendorf tube and centrifuged at 6000rpm for 1.5-2 min to pellet the cells. The supernatant was removed as much as possible by aspiration. The pellet was re-suspended in 100 µl of Solution I by vigorously vortexing. A 200 µl of Solution II was added and the content was mixed by inverting the tube 10 times. The tube was left at room temperature for 5 min. 150 µl of Solution III was added and the content was mixed by inverting the tube 10 times. The tube was left at room temperature for a further 10 min. After centrifuging at 14000 rpm for 5 min, the supernatant was carefully decanted into a clean tube. 2 volume 100% ethanol was added, and the tube was incubated at -20°C for 15 min. The mixture was centrifuged at 14000 rpm for 10 min. The pellet was collected and re-suspended in 300 µl of TE buffer. 3µl of RNase A (10ug/ul) was added, and the solution was incubated at 37°C for 30-60 min. 300 µl (same volume) of phenol:chloroform/isoamyl alcohol (1:1) was added and the sample was mixed by vortexing for 1 min. After

centrifuging at 14000 rpm for 5 min, the upper aqueous phase was transferred to a fresh tube and same volume of chloroform/isoamyl alcohol was added. The mixture was centrifuged at 14000 rpm for 5 min. The upper aqueous phase was transferred to a fresh tube. 1/10 volume of 3M sodium acetate and 2 volumes of 100% ethanol were added, the sample was incubated at -20°C for 30 min. After centrifuging at 14000 rpm for 10 min, the pellet was washed with 70% ethanol. The DNA pellet was dissolved in 20μl TE buffer or ddH_2O and stored at -20°C for further use.

2.3.1.3 Small-scale preparation (boiling method)

Each of the single recombination *E.coli* colonies were incubated in 3 ml LB medium with ampicillin (50 mg/ml) and grown at 37°C with shaking (200 cycle/minute) overnight. The cells were harvested by centrifuging at 6000rpm for 1.5-2 min and resuspended in 350 μl of the STET solution. 25μl of a freshly prepared lysozyme solution (10 mg/ml in 10 mM Tris-Cl pH 8.0) was added, and the sample was mixed by vortexing for a few seconds. The tube was placed in a boiling-water bath for 40 seconds and then immediately cooled on the ice for 5 minutes. The tube was centrifuged at 12000 rpm for 10 minutes. The pellet of bacterial debris was removed with a sterile toothpick. 40μl of 2.5 M sodium acetate (pH5.2) and 420 μl of isopropanol were added to the supernatant. The sample was mixed gently and left at room temperature for 5 min. The tube was centrifuged at 12000 rpm for 10 minutes at 4°C. The supernatant was removed by gentle aspiration and the pellet was washed with 70% ethanol. The DNA pellet was dissolved in 20 μl TE buffer or ddH_2O and was stored at -20°C for further use.

2.3.2 Oligonucleotide primer preparation

Most of the oligonucleotide primers used in this thesis were synthesized in our laboratory using a DNA synthesizer 391 (Applied Biosystems). After synthesis, the cleavage of oligonucleotide from the column and deprotection, dehydration and precipitation of the oligonucleotide were carried out following the instructions provided by the supplier (Applied Biosystems).

2.3.2.1 Cleavage of oligonucleotide from the synthesis column

1. 5-7 ml concentrated ammonia solution (30-35%) was dispensed into a clean glass bottle and tightly capped.
2. The tip of a sterile 1ml syringe was inserted into one end of the synthesis column. The syringe had to fit snugly and plunger should be at the needle end.
3. 0.75ml of concentrated ammonia solution was taken into another 1ml syringe.
4. The syringe containing 0.75ml concentrated ammonia was inserted into the other end of the column. The syringe-column-syringe unit was lifted up horizontally with care by holding the syringe in both hands.
5. The plunger of the syringe, containing the ammonia, was pushed gently and the ammonia was allowed to pass through the column into the opposite syringe. Pushing the ammonia back and forth between the syringes was repeated 4-5 times.
6. The syringe-column-syringe unit was incubated for 15 minutes at room temperature on a flat surface, making sure that there was ammonia solution inside the column with no leakage.
7. Step 5 was repeated.
8. The ammonia solution was drawn into one of the syringes, which was removed very carefully from the column and the ammonia solution was expelled into a pre-cleaned 4ml vial. The vial was tightly capped with a teflon lined screw cap.
9. Step 2-8 was repeated three times. Approximately 3ml of the ammonia solution was collected. The cleaved oligonucleotide was either stored at -20°C in ammonia solution or deprotected.

2.3.2.2 Deprotection

The tightly closed collection vial was placed in a 55°C water bath for either 8-12 hours or overnight. The deprotected solution was stored at -20°C or dehydrated directly.

2.3.2.3 Dehydration

The deprotected oligonucleotide in the vial was incubated in water bath at 35-40°C overnight, with a loose cap to allow the ammonia to evaporate. The oligonucleotide solution was frozen at -70°C before dehydration. The deprotected oligonucleotide was dehydrated in a vacuum freeze drier.

2.3.2.4 Precipitation of oligonucleotides

The freeze-dried oligonucleotides were dissolved in water. The volume of water was determined by the synthesis scale (30µl of water, 5µl of 3M sodium acetate and 100µl of ethanol/optical density unit (ODU) of oligonucleotide product). A small scale synthesis at expected ODU 20-30 was chosen for this work. The freeze-dried oligonucleotides were then dissolved in 600µl of water, 100µl of 3M sodium acetate and 2000µl of 100% ethanol were added. The mixture was vortexed briefly and incubated at -20°C for 30 minutes. The sample was centrifuged at 14000 rpm for 5 minutes. The supernatant was carefully removed and the pellet was wash with 70% ethanol. The pellet was dissolved in 200µl water and stored at -20°C.

2.3.3 Estimation of DNA sample concentration

An aliquot of the DNA sample was diluted to measure the absorbance at 260 nm (A260) by a spectrophotometer. The concentration of the DNA sample was determined using the following formula (Sambrook *et al.*, 1989):

A_{260} x dilution factor x * = µg DNA/mL

(*A_{260} = 1 for 50 µg/mL of double stranded DNA

*A_{260} = 1 for 33 µg/mL of single stranded DNA)

2.3.4 Gel electrophoresis of DNA

2.3.4.1 Gel preparation

1. TBE-gels: DNA fragments were separated on a 0.8-1.5% agarose gel containing 0.5X TBE (90mM Tris base, 90mM boric acid, 2mM EDTA). The electrophoretic buffer was 0.5X TBE buffer.

2. TAE-gels: DNA fragments were separated on a 0.8-1% agarose gel containing 1X TAE buffer (0.4M Tris, 0.2M acetate acid, 20mM EDTA). The electrophoretic buffer was 1X TAE buffer.

3. Low-melt temperature agarose gel: 0.7% low-melt temperature agarose was prepared in ddH_2O. This gel was used for the DNA band purification and prepared using a TAE-gel.

2.3.4.2 Sample loading

6X Blue/Orange Loading buffer (Promega) was used. This buffer contained 10% Ficoll 400, 0.25% Bromophenol Blue, 0.25% Xylene Cyanol FF, 0.4% Orange G, 10mM Tris HCl pH7.5 and 50mM EDTA. One part loading buffer for every 5 parts DNA sample was used for loading.

2.3.4.3 Electrophoresis

DNA samples (plasmids, restriction fragments and PCR products) were separated by electrophoresis at 55-85 voltage on either a TAE-agarose gel or a TBE-agarose gel containing 0.1mg/ml ethidium bromide (EB). The gel was viewed using a U.V. transilluminator and photographed using MP4 Land Camera (Polaroid) and 667 positive film or 665 positive/negative film (Polaroid).

2.3.5 DNA purification

2.3.5.1 Plasmid DNA purification

Wizard DNA Clean-up System (Promega) was used to obtain high quality plasmid DNA for cloning or sequencing purposes. This system provides a simple and effective way to purify linear and circular DNA (200-50,000 bp), especially for the plasmid DNA (2-5kb, 50ng-5μg). The procedure was carried out as follows:

1. One Wizard Minicolumn was used for each sample. The plunger was removed from a 3ml disposable syringe. The syringe barrel was attached to the Luer-Lok extension of each Minicolumn.
2. 1ml of well-mixed Wizard DNA Clean-up Resin was added to a 1.5ml microcentrifuge tube, which contained 200μl (2μg) of the plasmid DNA sample (50-500μl) and the sample was mixed by gently inverting several times.
3. The Wizard DNA Clean-up Resin containing the bound DNA was transferred into a syringe barrel. The syringe plunger was inserted slowly and the slurry was gently pushed into the Minicolumn.
4. The syringe from the Minicolumn was detached and the plunger was removed from the syringe. The syringe barrel was reattached to the Minicolumn. 2ml of 80% isopropanol was added into the syringe to wash the column. The syringe plunger was inserted slowly and the slurry was gently pushed into the Minicolumn.
5. The syringe was removed. The Minicolumn was placed into a 1.5ml mircrocentrifuge tube and centrifuged for 20 seconds at 12000rpm to dry the resin. The column was left at room temperature for 5-15 minutes to evaporate the isopropanol.
6. The Minicolumn was placed into a new 1.5ml microcentrifuge tube. 40μl of prewarmed (68°C) ddH_2O or TE buffer was added to the column. The column was left for 10 minutes and centrifuged for 20 seconds at 12000rpm to elute the bound DNA sample. This step can be repeated once if necessary. The sample was stored at -20°C for further use.

2.3.5.2 PCR product purification

Wizard PCR Preps DNA purification System (Promega) was used to purify the PCR products (50ng-16μg) between sizes 200bp to 1500bp. This system enabled purification either from the direct PCR reaction or from the agarose gel. The protocol was as follows:

2.3.5.2.1. Direct purification from PCR reaction

1. For each completed PCR reaction, 50-100μl of the aqueous phase was transferred to a clean microcentrifuge tube without mineral oil. 100μl of direct purification buffer was added and the sample was mixed by briefly vortexing. 1ml of resin was added and vortexed briefly three times for a one minute period.
2. One Wizard Minicolumn was used for each sample. The plunger was removed from a 3ml disposable syringe. The syringe barrel was attached to the Luer-Lok extension of each Minicolumn.
3. The Resin/DNA mixture from step 1) was transferred into the syringe barrel. The syringe plunger was inserted slowly and the slurry was gently pushed into the Minicolumn.
4. The following step was the same as indicated in section 2.3.5.1, steps 4 to 6.

2.3.5.2.2. Purification of DNA from agarose gel

The PCR products were separated by electrophoresis in a TAE agarose gel containing ethidium bromide. The desired DNA band was cut with a clean, sterile blade under the UV light. The band should be in approximately 300μl (300μg) of agarose.

1. Low-melting temperature agarose gel: About 300μg of agarose gel slice was transferred to a 1.5ml mircrocentrifuge tube and incubated at 70°C until the low-melting agarose was completely melted. 1ml of resin was added to the melted agarose slice. The sample was mixed thoroughly for 20 seconds (no vortexing).

2. High-melting temperature agarose gel: 300μg of agarose slice was transferred to a 1.5ml mircrocentrifuge tube and 1ml of resin was added. The tube was incubated at 65°C in a water bath for 5 minutes or until the agarose was completely melted.
3. Steps 3 and 4 as described in section 2.3.5.2, were followed to complete the purification.

2.3.5.3 DNA fragment purification

Two DNA purification systems described above also can be used for DNA fragment purification. It depends on the condition and DNA sample size. For the DNA fragments (≥1500 bp), a Wizard DNA Clean-up System (Promega) was recommended. For the DNA fragments (≤1500 bp), a Wizard PCR Preps DNA purification System (Promega) was recommended. The other two systems used were as described as follows:

1. The GENECLEAN Kit (Bio101 Inc.) This kit was used to purify the small DNA fragments from the gel (<200bp). The following protocol was used:

The band of interest was cut from the TAE gel. The band was weighed and transferred to a 1.5ml mircrocentrifuge tube. 3 volumes of NaI stock solution was added. The sample was incubate at 55°C for 5 minutes and mixed by inverting during the time until the gel slice was completely dissolved. 5μl of GLASSMILK (for<5μg DNA samples) was added. The sample was left on ice for 5 minutes and mixed every 1-2 minutes to ensure that GLASSMILK stayed suspended. The tube was spun in a microcentrifigue for 5 seconds. The supernatant was poured off carefully. The pellet was washed 3 times with NEW WASH, 500μl each time. The DNA fragment was eluted with 20-50μl of TE buffer at 55°C for 5 minutes.

2. Phenol extraction method One other method used to purify the plasmid DNA and DNA fragments from low-melting agarose gel in this thesis was phenol extraction method. The protocol was as follows: 0.7% low-melting agarose gel was prepared with ddH_2O. The DNA sample was separated by electrophoresis in a TAE agarose gel

containing ethidium bromide. The entire TAE agarose gel was transferred to a clean plate. A well was made in front of the desired DNA band and the buffer in the well was carefully removed. The 0.7% low-melting agarose gel was pre-warmed at 65°C and was placed into the well. The gel was left for a while until settled. Electrophoresis was continued until the desired DNA band had migrated to the low-melting agarose gel. The desired DNA band was cut using a clean, sterile blade under the UV light. The band should be approximately 300 μg in weight. The 300 μg agarose slice was transferred to a 1.5 ml mircrocentrifuge tube and the final volume adjusted to 500 μl with TE buffer. The tube was incubated at 65°C until the gel was completely melted. The same volume of phenol solution was added immediately and the sample was mixed thoroughly by vortexing. After centrifuging at 14000 rpm for 10 minutes, the upper aqueous phase was transferred to a clean 1.5 ml mircrocentrifuge tube. The same volume of chloroform/isoamyl alcohol (24:1) solution was added immediately and the sample was mixed thoroughly by vortexing. The sample was centrifuged at 14000 rpm for 5 min and the upper aqueous phase was taken to a fresh tube. 1/10 volume of 3M sodium acetate and 2.5 volume of 100% ethanol was added. The sample was incubated at -20°C for 30 min. After centrifuging at 14000 rpm for 10 min, the pellet was washed with 70% ethanol. The DNA pellet was dissolved in 20μl TE buffer or ddH_2O and stored at -20°C for further use.

2.3.5.4 Estimation of purified DNA concentration on agarose gel

The approximate DNA concentration was estimated on the agarose gel, using the intensity of one band of the marker for comparison, according to the instructions of the manufacturer. PCR Markers (Promega) and 100bp DNA Step Ladder (Life Technologies) were used in this thesis. When 5μl of PCR Markers was loaded and separated on the agarose gel, each band contained around 50ng of DNA. When 4μl of 100bp DNA Step Ladder was loaded and separated on the agarose gel, the 600bp band contained around 60ng of DNA and the rest of the bands contained around 20ng of DNA.

2.4 Polymerase Chain Reaction (PCR)

PCR was used as a method to identify the positive colonies, which contained a part of the known and unknown sequences. The primers were prepared from the known sequence. This methodology has been described as nested PCR. This section only describes the nested PCR used in this thesis. PCR was also used as a method to create the cloning site on the end of the gene interest using specific designed primers (for details, see chapter 7).

2.4.1 Primer design

The following criteria were applied for designing primers (Sambrook et al., 1989):

1. The single-strand primers should typically be 15 to30 bases in length.
2. The proportions of G and C in each primers should be near 50%, to maximize specificity.
3. The primer sequence should not complement within themselves or each other, particularly at the 3' end.

2.4.2 Temperature cycling and cycle optimization

A typical cycle consists of:

1. A melting step (94-96°C), to separate the complementary strands of DNA.
2. An annealing step (37-65°C), to allow hybridization of the primers to the ssDNA and initiation of polymerization.
3. An extension step (68-72°C), to complete the copy initiated during annealing.

Beginning with the template melting step (94-96°C for 2-5 minutes), the PCR was performed for 25-35 cycles. The final extension step was hold for about 7 minutes. Primer concentrations in the range of 0.2-0.5 μM were used for most PCR amplifications. High G and C content DNA needs very high annealing (>65°C) and melting temperatures. Higher annealing temperature (>45°C) generally result in much more specific product. The length of the target sequence will affect the required extension time.

2.4.3 Nested PCR

Nested PCR was performed to screen the positive clones for cloned RACE products using gene specific primers to detect the sequence that contains known and unknown parts. The DNA Polymerase used for PCR in this thesis was obtained from three companies: AmpliTaq DNA Polymerase (Perkin Elmer) and Taq Polymerase (Life Technologies and Promega). Based on the criteria and instruction provided by the suppliers, the PCR amplification was performed with an initial 3min denaturation at 95°C, then 95°C for 40 seconds, 56°C for 40 seconds, 72°C for 90 seconds for 30 cycles. The final extension step was 72°C for 7 minutes. The final concentrations of the components in the reaction were: 10mM Tris-HCl, pH8.3, 50mM KCl, 2mM MgCl2, 200μM dNTP, DNA Polymerase 2.5 units/50μl and primers 30 pmol/50μl.

2.5 Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

RT-PCR reaction was performed using Access RT-PCR System (Promega). The procedure used was provided by the supplier as follows:

2.5.1 First strand cDNA synthesis

10μl of mRNA sample (500ng) was added to a 0.5ml mircrocentrifuge tube. The tube was incubated at 65°C for 10 minutes and then chilled on ice for 2 minutes. The following solutions were added: 10μl of 5X AMV/TF1 Reaction Buffer, 2μl of 25 mM MgSO4, 1μl of 10 mM dNTP mix, 50 pmol downstream primer, 50 pmol upstream primer, 1μl of avian myeloblastoma virus (AMV) reverse transcriptase. Nuclease-free water was then added to a final volume of 50μl. The contents were mixed gently and incubated at 48°C for 45 minutes.

2.5.2 Second strand cDNA synthesis and PCR amplification

The reaction was heated at 95°C for 2 minutes to inactivate AMV reverse transcriptase and to denature the RNA/cDNA/primer. 1μl of TF1 DNA polymerase was added. PCR amplification was then proceeded directly for 35 cycles. The cycling parameters were 95°C 30s, 56-58°C 45s, 68°C 90s. The annealing temperature and time was variable

according to the primer Tm value. The final extension was incubated at 68°C for 7 minutes. After amplification, the PCR products were analyzed by agarose gel electrophoresis with the appropriate molecular size standards and were purified from the gel using a Wizard PCR Preps DNA purification system (section 2.3.5.2). The purified samples were used for sequencing.

2.6 Rapid Amplification of cDNA Ends (RACE)

Two different methods were used to amplify the 3'end of the OTR cDNA. In principle, Rapid Amplification of cDNA 3'Ends (3'RACE) is a procedure for amplification of nucleic acid sequences from a mRNA template between a defined internal site and the 3' end of mRNA. 3'RACE takes advantage of the natural poly(A) tail found in mRNA as a genetic priming site for PCR. In this procedure, mRNAs are converted into cDNA using reverse transcriptase and an oligo-dT tailed primer. Specific cDNA was then amplified by PCR using a gene-specific primer and an anchored or adapter primer. This permits the capture of unknown 3'mRNA sequences that lie between the exon and the polyA tail. Design of the gene-specific primer is described in chapter 3 (section 3.2.6.1). The procedure used was provided by the supplier

2.6.1 3'RACE system I (Life Technologies)

1. First strand cDNA synthesis

11μl of mRNA sample (50ng, in DEPC-H2O) was transferred to a 0.5ml mircrocentrifuge tube. 1μl of AP solution was added, and the mixture was mixed gently and collected by brief centrifugation. The mixture was heated at 70°C for 10 minutes and chilled on ice for 2 minutes. The following solutions were added: 2μl of 10XPCR buffer, 2μl of 25 mM MgCl2, 1μl of 10 mM dNTP mix, 2μl of 0.1 M DTT. The reaction was mixed gently and collected by brief centrifugation. The mixture was equilibrated at 42°C for 5 minutes. 1μl (200unit) of Superscript II reverse transcriptase was added and the reaction was incubated at 42°C for 50 minutes. The reaction was terminated by heating at 70°C for 15 minutes. The reaction was chilled on ice and

collected by brief centrifugation. 1μl of RNase H was added to the tube, the reaction was mixed and incubated at 37°C for 20 minutes before proceeding to step 2.

2. Amplification of the target cDNA

To a fresh 0.5ml mircrocentrifuge tube, the following solutions was added: 5μl of 10XPCR buffer, 3μl of 25 mM MgCl2, 1μl of 10 mM dNTP mix, 1μl (10μM) of abridged universal amplification primer (AUAP), 10pmol gene specific primer, and the sterile distilled water to final volume of 47.5μl. 2μl of the first strand cDNA synthesis reaction was added to the tube. The reaction was incubated at 94°C for 3 minutes, then 0.5μl of Taq polymerase (5 unit) was added. PCR was performed using Peltcer Thermal Cycler (PTC-200) (Bresatec) with the heating lid on. The cycling parameters were 95°C for 1 min, 56°C for 1 min and 72°C for 3 min for 35 cycles.

2.6.2 3'RACE system II (Clontech)

1. First strand cDNA synthesis

4μl of mRNA (0.12μg/μl) and 1μl of CDS3' anchored primer (10μM) were added to a fresh 0.5ml mircrocentrifuge tube, the contents were mixed by brief centrifugation. The mixture was heated at 72°C for 2 minutes and chilled on ice for 2 minutes. The following solutions were added: 2μl of 5X First-strand buffer, 1μl of 10 mM dNTP mix, 1μl of 20mM DTT, 1μl of MMLV reverse transcriptase (100 unit). The reaction was mixed gently and incubated at 42°C for 60 minutes. The next step was performed directly.

2. Amplification of the target cDNA

1μl of the first strand cDNA synthesis reaction was taken to a fresh tube. The following solutions were added: 5μl of 10XPCR buffer, 1μl of 10 mM dNTP mix, 1μl (10μM) of CDS3' anchored primer, 10 pmol gene specific primer, and the sterile distilled water to final volume of 49μl. 1 μl of Advantage polymerase mix was added. PCR amplification was performed with an initial 1min denaturation at 95°C, then 95°C for 15 second, 68°C for 5 min for 35 cycles. 5-10μl of the reaction was analyzed by agarose gel electrophoresis with the appropriate DNA molecular size standards.

2.7 Northern hybridization

Northern hybridization was carried out to determine the OTR gene expression at different stages of the reproductive cycle and in different tissues as described in this thesis.

2.7.1 Gel electrophoresis of RNA samples

For the details of RNA sample preparation and gel electrophoresis, please see section 2.2 RNA preparation. Samples of total RNA (10-15 μg/lane) from each tissue were loaded with RNA markers (G319a, Promega). The samples were separated by electrophoresis on 1% agarose-formaldehyde gels in MOPS buffer at voltage of 65 for about 3 hours or at voltage of 15 overnight. A photo of the RNA gel was taken with a ruler to determine the size of interested bands. The gel was then transferred for blotting.

2.7.2 Setting up a capillary blot

1. A glass tray (it should be pre-baked at 148°C at least 2 hours) was filled with blotting buffer (10XSSC). A platform was made and covered with a wick made from Whatman 3mm filter paper, which saturate with blotting buffer.
2. A membrane (positively charged, Boehringer Mannheim) and three sheets of 3mm paper were cut to the exact size of the gel and saturated with blotting buffer.
3. A ready-cut 3mm sheet was placed on the wick and the bubbles were removed with a glass pipette. The gel was placed on the sheet (well-side up) and the bubbles were carefully removed.
4. The cut membrane was placed on the top of the gel. The membrane was avoided to move once it touched the gel, as the RNA transfer started immediately. Bubbles between the membrane and the gel were removed gently.
5. Another two 3mm sheets were placed on the top of the membrane and the bubbles were removed each time. The gel was surrounded with cling film to prevent the blotting buffer being absorbed directly onto the paper towels.
6. A stack of absorbent paper towels was placed on the top of the 3mm paper. A plate was placed on the top of paper towels and a 0.5-1kg weight bottle was placed on the top. Blotting was undertaken for about 12-16 hours.

7. After blotting, all the papers were removed carefully. A corner of the membrane was labled for correct orientation.
8. The membrane was washed briefly in 2XSSC and air-dried.

2.7.3 Fixation for blot

UV crosslinking: the membrane (sample-side down) was placed on a UV light box for 5 minutes to allow the samples to bind to the membrane. 312nm wavelength was recommended. After fixation, the RNA marker lane was cut off from membrane and stained in 0.04% Methyline Blue in 0.5M sodium acetate, pH5.2 for 10-20 minutes until bands became clear. The slip of stained RNA markers was rinsed in water and was kept with the membrane in clean 3mm paper.

2.7.4 Probes

Probe 1. This probe was a 132 base pair (bp) RT-PCR product encoding part of the sheep endometrial OTR (residues 859-990) and shared 54% identity with the rat vasopressin V1a receptor gene (Stewart *et al.*, 1993). It was kindly provided by Prof. Flint from Department of Physiology and Environmental Science, University of Nottingham. This probe was cloned into PBluescript II SK+ phagemid cloning vector (section 2.9.1).

Probe 2. This probe was a 759bp DNA fragment that was generated by reverse transcriptase-polymerase chain reaction (RT-PCR) corresponding to the region encoding residues 220-978 of sheep endometrial OTR. This probe was cloned into pCR-Script Amp SK(+) Cloning vector (section 2.9.2)

Probe 3. This probe was generated from RT-PCR with primers PC and PD. It is a cDNA fragment encoding residues 411-848 of sheep endometrial OTR.

Probe 4. A control probe that was used for the detection of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. GAPDH gene is a kind of housekeeping gene and is used to normalize the sample loading. GAPDH cDNA was isolated from rat and cloned into pUC vector (Piechaczyk *et al.*, 1984). The reconbinant plasmid DNA

was named as pRGAPDH-1. This probe was digested by *EcorI* and ApaI from the plasmid DNA. It consists of a 950 bp DNA fragment of rat GAPDH cDNA.

2.7.5 Labeling

Two labeling systems were used depending on the size of the probes. For fragments less than 500bp, the probe was labeled by random priming. For fragments greater than 500bp, The probe was labeled by a nick translation system.

2.7.5.1 Random priming (GIGAPRIME DNA Labeling Kit, Bresatec)

A GENECLEAN Kit (section 2.3.5.3) was used to purify the probe 1 (132bp). The labeling procedure was as follows:

50-150 ng of purified DNA fragment was added to a total volume of 6μl with ddH_2O in a 1.5ml tube. The sample was denatured at 95°C for 5 minutes and chilled on ice. The following reagents were added: 6μl of Decanucleotide solution (Tube1), 6μl of Nucleotide/Buffer Cocktail (Tube 2A if using α-[P^{32}]dATP; Tube 2C if using α-[P^{32}]dCTP; Tube 2B if using both), 5μl (50 μCi) of α-[P^{32}]dATP and or α-[P^{32}]dCTP and 1μl of enzyme solution (Tube 4). The reaction was mixed gently and incubated at 37°C for 20 minutes. The reaction was stopped by adding EDTA (pH8.0) to a final concentration of 20 mM.

2.7.5.2 Nick translation system (Promega)

Probe 2 and 3 were labeled by nick translation system. For the sample purification, see section 2.3.5. The protocol was used as follows:

The following reagents were added: 10μl of unlabeled dNTP (1:1:1 in mixture), 5μl of Nick Translation 10Xbuffer, 10μl of sample DNA (100-1000ng) in water, 7μl of α-[P^{32}]dNTP, 5μl of Nick Translation Enzyme mix, and nuclease-free water to final

volume of 50µl. The reaction was incubated at 15°C for 60 minutes. 5µl of the stop solution was added.

2.7.6 Purification of labeling reaction

Unincorporated radioactive dNTPs can be removed either by chromatography or centrifugation using a small column of Sephadex G-50. A MicroSpin S-300 HR Column (Pharmacia) was used for purification of labeling reaction. The protocol was used as follows:

1. The resin in the column was re-suspended by vortexing.
2. The cap was loosened one-fourth turn and the bottom closure was removed.
3. The column was placed in a no-cap microcentrifuge tube and spun at 3000rpm for 1 minute.
4. The column was transferred in a new tube and the labeling reaction (10-100µl) was slowly added to the top-center of the resin. The column was spun at 3000rpm for 2 minutes.
5. The purified sample was collected in the tube and used for hybridization.

2.7.7 Hybridization

1. Pre-hybridization The ready to use blot was placed in a plastic bag. 15ml of hybridization buffer (2× Denhart's solution, 5 mM EDTA, 10 mM Tris-HCl, 5% SDS, 0.5 M sodium phosphate), and denatured salmon sperm DNA (100 ng/ml) (95°C for 10 min, chilled in ice) were added. After the air-bubbles were removed, the bag was sealed and incubated in a rolling oven at 65°C for 2-4 hours.

2. Hybridization The purified labeling sample was denatured at 95°C for 10 min, chilled on ice and transferred into the bag prehybridized at 65°C for 2-4 hours. The bag was carefully heat-sealed and incubated in a rolling oven at 65°C overnight.

3. Washing The hybridization bag was carefully opened and the hybridization buffer was gently poured to the radioactive waste bottle. The blot was transferred to a clean tray and washed sequentially in 1×SSC, 1% SDS at room temperature for 10 min, at

65°C for 10 min, and then in 0.2×SSC, 1% SDS at 65°C for 15 min. The radioactive count was monitored until the background was low. The blot was air-dried and wrapped with cling film.

2.7.8 Autoradiography of hybridization signal

This procedure was carried out under red light in the dark room. The top edge of the hybridized blot and the edge of the X-ray Film (Hyperfilm-MP, Amersham) were aligned. The blot was fixed on the film carefully. The hybridization signal was exposed to X-ray film with intensifying screens at -80°C for 2-3 days. The X-ray film was developed in Developing solution for 2-5 min, rinsed in water and then put in fixing solution for 3-5 min. The developed X-ray film was rinsed with water, then air-dried.

2.7.9 Stripping the blot for reprobing

The blots were incubated 2 x 30 minutes at 68°C in stripping solution which contained 50% dimethyl formamide, 1% SDS, 50 mM Tris-HCl (pH 8). They were then rinsed first in water and then in 2 x SSC and stored wet in 2 x SSC in a sealed plastic bag at 4°C.

2.8 Southern hybridization

Southern hybridization was used to optimize the RT-PCR condition for the primers pair 1 in this thesis (chapter 3). In brief, two types of primers pair 1 was designed to used, F_A and F_B (18 bases), P_A and P_B (30 bases). The reactions using each pair of primers or both with different cycling parameters were performed. Each reaction was loaded to 1% TBE agarose gel with PCR markers. After electrophoresis, a photo of the gel was taken with a ruler on the UV box. The gel was blotted using dry-blotting method. The protocol was:

1. The gel was soaked in 100ml denaturing solution (0.5M NaOH, 1.5M NaCl) for 30 min with gentle shaking.
2. The gel was rinsed in water and soaked in 150ml neutralizing solution (1M ammonium acetate, 0.02M NaOH) for 30 min with gentle shaking.

3. The gel was transferred to a clean plate (well-side down). Bubbles were removed gently with pipette. A membrane (Nybond-N, positively charged, Amersham Life Science) and three sheets of 3mm paper were cut to the exact size of the gel.
4. The membrane was aligned onto the gel. Bubbles were removed gently with pipette.
5. Three sheets of 3mm paper were placed on the membrane and a stack of absorbent paper towels was placed on the top of 3mm papers. A plate was placed on the top of paper towels and a 0.5-1 kg bottle was added on top. It was left overnight.
6. After blotting, all the papers were removed carefully. A corner of the membrane was marked for orientation purpose. The membrane was rinsed briefly in 2XSSC and then partly air-dried (keep moistened). The blot was wrapped with cling film and stored at 4°C.
7. UV cross-linking was made as described in section 2.7.3. The blot was hybridized with Probe1. Labeling and hybridization procedures were the same as described in section 2.7.5.1 and 2.7.7.

2.9 Cloning of partial OTR cDNA

2.9.1 Cloning of 132 bp fragment encoding part of the sheep endometrial OTR

1. **DNA fragment** The products generated by RT-PCR (Stewart *et al.*, 1993) were kindly provided by Prof. Flint. It has *EcorI* restriction enzyme site on 5'end and *BamHI* restriction enzyme site on 3'end. It is about 150 bp DNA fragment.

2. **Cloning vector** PBluescript II SK+ phagemid was used as cloning vector.

3. **Preparation of DNA fragment and Cloning vector** The DNA fragment and Cloning vector were digested with restriction enzymes *EcorI* and *BamHI*. The digestion reaction was incubated at 37°C for 2 hours as follows:

	PCR product 6µl (200ng/µl)	PBluescript II SK+ 5µl (100ng/µl)
10x *EcorI* buffer	2 µl	2 µl
H2O	10.5 µl	11.5 µl
EcorI (20U/µl)	0.5 µl	0.5 µl
BamHI (10U/µl)	1 µl	1 µl

4. **Purification of digested PBluescript II SK+ plasmid and DNA fragment** The total digestion reaction of PBluescript II SK+ and DNA fragment (as described above) were loaded onto a 1% TAE agarose gel. After electrophoresis, the interest bands from low melt agarose gel were collected and put together for band purification using phenol extraction method (for details see section 2.3.5.3). After washing, the DNA pellet was dried briefly under vacuum.

5. **Ligation** 7 µl of sterile distilled water was added to the dried pellet (as described in step 4), then 1 µl of 10 mM ATP, 1 µl of 10X ligation buffer and 1µl of ligase (3 Weiss units/µl) were added. The reaction was mixed well and was incubated at 4°C overnight.
6. **Preparation of competent *E.coli* cells** *E.coli* XL-1 blue strain was used for preparation of competent cells. Competent *E.coli* cells were prepared by $CaCl_2$ treatment as described in Sambrook *et al.* (1989) as follows:

   A single colony of XL-1 blue strain was inoculated into 3 ml of LB medium and incubated at 37°C overnight under shaking (200 cycles/min). 1 ml of this culture was transferred into 100 ml fresh LB medium in a 250 ml flask and incubated at 37°C under vigorous shaking (250 cycles/min). When the A600 reached to 0.6-0.8, the culture was placed on ice for 10 min and the cells were harvested by centrifuging at 5000 rpm, 4°C for 5min. The supernatant was discarded and the cells were suspended in 40ml ice-cold $CaCl_2$ solution (15% glycerol, 0.1 M $CaCl_2$). After incubation on ice for 20 min., the cells were pelleted and resuspended in 10ml ice-cold $CaCl_2$ solution. The suspension was left on ice for 1.5 hours. The cell suspension was aliquoted into 200µl aliquots and stored at -80°C until required.

7. **Transformation of ligation reaction into competent cells** Transformation was as described in Sambrook *et al.* (1989). Frozen competent cells were thawed on ice and incubated with the appropriate DNA ligation reaction (as discribed in step 5, 5μl /200μl cells) for 1 hour on ice. The cells were then heat shocked at 42°C for 2 min, placed on ice to recover for 5 min before the addition of 1ml LB medium. After incubation at 37°C under vigorous shaking (250 cycles/min) for 1 hour, the cells were collected by spinning briefly. With about 300μl LB medium left, the cells were resuspended and the cell suspension (100μl) were plated onto LB agar plates, incubated overnight at 37°C. Each plate contained 50μg/ml ampicillin, 10μl of 10% X-gal and 10μl of 0.1M IPTG.

8. **Screening of recombinant plasmid DNA** Single white colonies of transformed cells were inoculated to 3ml LB plus ampicillin, which were grown at 37°C overnight. Cells were then harvested for small-scale plasmid DNA preparation (section 2.3.1.2). The plasmid DNA samples were digested with restriction enzymes (*EcorI* and *BamHI*). The digestion reactions were electrophoresed on agarose gel to identify the positive clone. The cells of positive clone were then stored in 15% glycerol at -80°C. The positive clones were confirmed by sequencing (section 2.10).

2.9.2 Cloning of RT-PCR product

pCR-Script Amp SK(+) Cloning Kit (Stratagene) was used for cloning of RT-PCR products (759 bp) generated by primer pair 1 (PA and PB). The procedure was used as described by the supplier.

1. **Purifying the PCR products** The PCR products were purified using Wizard PCR Preps DNA Purification System (section 2.3.5.2, direct purification).

2. **Polishing the purified PCR products** The PCR products generated with either Taq DNA polymerase or other low-fidelity DNA polymerase should be polished the end by *Pfu* DNA polymerase. The reaction was: 10μl of the purified PCR products (20ng/μl), 1μl of 10mM dNTP mix, 1.3μl of 10Xpolishing buffer, 1μl of cloned *Pfu*

DNA polymerase (0.5unit). The reaction was incubated in PTC-200 DNA Thermal Cycler at 72°C for 30 minutes.

3. **Inserting the PCR products into the pCR-Script Amp SK(+) Cloning vector** To clone the polished PCR products, the ligation reaction was: 1μl of the pCR-Script Amp SK(+) Cloning vector (10ng/μl), 1μl of pCR-Script 10X reaction buffer, 0.5μl of 10 mM rATP, 5.5μl of the polished PCR products reaction, 1μl of *Srf* I restriction enzyme (5 units/μl), 1μl of T4 DNA ligase to a final volume of 10μl. The ligation reaction was incubated at room temperature for 1 hour before heat treating at 65°C for 10 min. The ideal ratio of insert-to-vector DNA at the range 40:1 to 100:1 was recommended by the supplier. However, the ratio in this reaction was 10:1 which worked well.

4. **Transformation** 40 μl of *E.coli* XL1-Blue MRF'Kan super-competent cells were thawed and transferred into a pre-chilled 15ml Falcon 2059 Polypropylene tube. 0.7μl of the β-mercaptoethanol was added to a final concentration of 25mM. The transformation reaction was incubated on ice with gently swirling the tube every 2 minutes. A 3μl of the ligation reaction was then transformed to the tube. The transformation reaction was incubated on ice for 30 minutes. After heat shocking at 42°C for 45 seconds, the transformation reaction was put on ice for 2 minutes and 0.45ml of the prepared SOC medium was added. The reaction was incubated at 37°C for 1 hour with shaking. The transformed cells were separated on LB-ampicline (20μg/ml)-methicillin (80μg/ml) agar plates containing 10μl of 10% X-gal and 10μl of 0.1M IPTG. The plates were incubated at 37°C overnight.

5. **Screening of recombinant plasmid DNA** Single white colonies of transformed cells were selected for screening. The procedure was the same as the one described in section 2.9.1, step 8, except restriction enzymes BstXI and *EcorI* were used for digestion.

2.9.3 Cloning of 3'RACR products

pGEM-T cloning vector system (Promega) was used for cloning of RACE-PCR products. The procedure used was provided by the supplier.

1. **Purification** The PCR products (600bp and 1200bp, see chapter 3) generated from 3'RACE system were purified using Wizard PCR Preps DNA Purification System (section 2.3.5.2, gel purification).

2. **A-tailing reaction** The purified products were performed with A-tailing reaction as follows: 1μl of 10X Taq DNA polymerase reaction buffer, 1μl of 25mM $MgCl_2$, 1μl of 2mM dATP, 0.5μl of Taq DNA polymerase, 40ng of purified 600bp products or 35ng of purified 1200bp products in water to a final volume of 10μl. The reaction was incubated at 70°C for 20 min.

3. **Ligations using the pGEM-T Vector** The vectors were prepared by cutting Promega's pGEM-5Zf(+) with EcoRV and adding a 3'terminal thymidine to both ends by the supplier. The ligation reaction was: 1μl of 10X T4 DNA Ligase buffer, 1μl of prepared pGEM-T Vector (50ng), 7μl of A- tailing reaction for 600bp products or 1200bp products from step 2, 1μl of T4 DNA Ligase to final volume of 10μl. The reactions were incubated at 4°C overnight.

4. **Transformation** 50μl of JM 109 High Efficiency Competent Cells (from supplier) were thawed in ice. 5μl of the ligation reaction from step 3 was added and incubated on ice for 20 minutes. After heat shocking at 42°C for 45 seconds, the transformation reaction was incubated on ice for 2 minutes and 0.95ml of the prepared SOC medium was added. The reaction was incubated at 37°C for 1.5 hour with shaking. The transformed cells were separated on LB-ampiciline (100μg/ml) agar plates containing 10μl of 10% X-gal and 10μl of 0.1M IPTG. The plates were incubated at 37°C overnight.

5. **Screening of recombinant plasmid DNA** Single white colonies of transformed cells were selected and cultured in 3ml LB plus ampicillin at 37°C overnight. Cells

were then harvested for small-scale plasmid DNA preparation (section 2.3.1.3). The plasmid DNA from each clone was identified by nested PCR (section 2.4). The positive clones were confirmed by sequencing.

2.10 DNA sequencing

A T7 Sequencing kit (Pharmacia) was used to determine the sequence of cloned probe 1 (section 2.9.1 and 2.7.4). A ABI Prism Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer) was used for direct sequencing of RT-TCR products and RT-TCR products cloned into the vector. ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer) was used for sequencing of RACE products cloned into the vector.

2.10.1 Sequencing reaction with T7 DNA polymerase

1. **Annealing of a primer to the double-stranded plasmid DNA** 32 μl of template (1.5-2.0 μg plasmid DNA) and 8 μl of 2 M NaOH were added to a microcentrifuge tube. The tube was vortexed gently and incubated at 37°C for 10 min. 7 μl of 3M sodium acetate (pH 4.8), 4 μl of distilled water and 120 μl of 100% ethanol were added. The solution was mixed well and placed on dry ice for 15 min. The precipitated DNA was collected by centrifuging for 15 min. The pellet was washed with ice-cold 70% ethanol. Following re-centrifugation for 10 min, the pellet was air-dried and dissolved in 10 μl of distilled water. To anneal primer/template, 2 μl of the Universal primer and 2 μl of annealing buffer were added. The reaction was mixed well and then incubated at 65°C for 5 min. The tube was quickly transferred to a 37°C water bath and incubated for 10 min. The tube was left at room temperature for at least 10 min to make sure that annealing was completed, then centrifuged briefly. The sequencing reaction was proceeded immediately.

2. **Labeling reaction** The following reagents were added to the tube containing 14μl of annealed primer/template: 3μl of Labeling mix-dATP, 1μl of α-P^{32}-dATP, 2μl of diluted T7 DNA Polymerase (1:4). The reaction was mixed gently and centrifuged briefly, and then incubated at room temperature for 5 min.

3. **Termination reactions** While the labeling incubation was in progress, each of 2.5μl of the 'A' Mix-Short, 'G' Mix-Short, 'C' Mix-Short, 'T' Mix-Short were added into the corresponding tube respectively, and the tubes were placed at 37°C for at least 1 min. After the 5 min incubation of the labeling reaction, 4.5μl of incubated labeling reaction was transferred into each of the four pre-warmed sequencing mixes. The components were mixed gently and incubated at 37°C for 5 min. 5μl of stop solution was added to each tube and mixed gently.

4. **Gel preparation** 20ml of 20% acrylamide solution, 5ml of 10X TBE buffer and 25ml of 46.7% urea solution were mixed to total volume of 50ml, 8% acrylamide. The mixture was filtered and degased under vacuum for 5 min. 250μl of 10% ammonium persulfate and 50μl of TEMED were added to the mixture, and the container was swirled to mix. The gel solution was immediately poured between the two plates (Silane-treated side facing inward) with syringe, taking care to avoid air bubbles in the gel. The plates were laid in a horizontal position until the gel had been polymerized. The gel was pre-run at 40W constant power for 45-60 min.

5. **Electrophoresis** 3 μl of each stopped reaction was transferred to a fresh microcentrifuge tube respectively. The tubes were heated at 78°C for 2 min. 3 μl of each heated sample was immediately loaded into the shark's-tooth comb (the comb points just touch the surface of the gel) well of a pre-run sequencing gel. The gel was re-run at 40W constant power until the bromophenol blue dye reached the bottom of the gel.

6. **Autoradiography** After gel electrophoresis, the plates were carefully separated so that the gel remained attached to one of them. A Whatman 3mm paper (appropriately sized) was tightly covered to the gel, and the gel was carefully peeled from the plate. The gel-paper was covered with cling wrap. In a darkroom, the covered gel-paper was placed in a film cassette and a X-ray film (Kodak) was put on the top of the gel with intensifying screen. The signal was exposed at -20°C for 1hour. The procedure for developing the film was as described as in section 2.7.8.

2.10.2 Cycle sequencing method using dye-labelled terminators with AmpliTaq DNA polymerase

The ABI Prism Dye Terminator Cycle Sequencing Ready Reaction and ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction were undertaken using the same procedure as described except for the changes in the composition of the reaction mixes (given below). The procedure was:

1. **Sequencing reaction**

Composition	Standard Dye kit	BigDye kit
DNA template	30-90ng PCR products or 300-500ng plasmid DNA	30-90ng PCR products or 300-500ng plasmid DNA
Primer	1μl (3-5 pmol)	1μl (3-5 pmol)
Terminator Ready Reaction Mix (provided with kit)	8μl	6μl
Total volume	20μl	16μl

2. **The cycling parameters** The cycle sequencing reaction was performed on Peltcer Thermal Cycler (PTC-200, Bresatec). The cycling parameters was: 96°C, 30 seconds; 50°C, 15 seconds and 60°C, 4 minutes for 25 cycles.

3. **Purification** After cycle sequencing, the products of each reaction were transferred to a 1.5ml microcentrifuge tube. 2μl of 3M Sodium acetate, pH4.8 and 50μl of chilled ethanol were added. The mixture was incubated on ice for 10 minutes. After centrifuging at 15000 rpm for 30 minutes, the precipitated sample was washed with 250μl of 70% ethanol and air-dried.

4. **Electrophoresis and analysis** All the sequence reactions were separated and analyzed using an ABI 373A DNA Sequencer at DNA Sequencing Service, Department of Microbiology, Monash University, Clayton 3168, Australia.

2.11 Western blotting

2.11.1 Protein gel preparation

1. 12.5% acrylamide separating gel: 1.54ml of 30% acrylamide, 1.5ml of 1M Tris HCl (pH 8.8), 0.9ml of ddH_2O, 50µl of 10% SDS, 20µl of 10% APS and 3.5µl of TEMED.

2. Stacking gel: 0.26ml of 30% acrylamide, 0.5ml of 0.5M Tris HCl (pH 6.8), 1.2ml of ddH_2O, 40µl of 10% SDS, 20µl of 10% APS and 3.5µl of TEMED.

2.11.2 Protein gel electrophoresis

Two mini-gels were prepared in parallel. To determine the size and position of gel band, the same samples were loaded in both gels, one gel was used for Commassie Blue stain and the other gel was used for Western blot. The sample preparation is as described in chapter 7, section 7.2.1.6. After incubating for 3 minutes in boiling water and followed by cooling on ice, the samples were loaded along with the low range protein markers (Bio-Rad). Electrophoresis was conducted at a constant voltage of 110 V until the dye had reached bottom of the gel. The gel running buffer (pH 8.3) were: 0.025M Tris, 0.192M glycine and 0.1% SDS.

2.11.3 Gel staining

After electrophoresis, one gel was carefully removed to a tray that contained 100ml of fixing solution (40% ethanol, 10% acetic acid). The gel was soaked in fixing solution at room temperature with gentle shaking for 30 minutes. And then the gel was stained in staining solution (35% ethanol, 10% acetic acid, 5% (w/v) commassie blue) for another 30 minutes at room temperature with gentle shaking. After removing the staining solution, the gel was destained in destaining solution (35% ethanol, 10% acetic acid) overnight. After rinsing in water, the gel was kept between two membranes and dried in gel drier.

2.11.4 Transfer of separated protein samples onto a membrane

When gel electrophoresis was finished, the gel set was removed. One plate was detached from the gel. Three sheets of 3mm Whatman paper that pre-cut to the gel size and pre-soaked in transfer buffer were placed on the top of the gel. The air bubbles were removed carefully. The paper-gel unit was lifted and detached from another plate. A pre-cut nitrocellulose membrane (Bio-Rad) was placed on the top of the gel (do not move the membrane once it touches the gel). Another three sheets of 3mm whatman paper that pre-cut to the gel size and pre-soaked in transfer buffer were placed on the top of the membrane. The paper-gel-membrane unit was place in a cassette. The cassette was put to the apparatus unit with cooling system for electrotransfer. To make sure that the gel side is close to the negative pole and the membrane side is close to the positive pole. The conditions for electroelution were: 15V, 100mA at 10-15°C overnight.

2.11.5 Western blotting

2.11.5.1 Solution preparation

1. TBS (Tris Buffered Saline): 6.057g of Tris was dissolved in 800ml ddH_2O and pH was adjusted to 7.5 with HCl. 8.76 g NaCl was added and mixed with the solution. After the salt had dissolved, ddH_2O was added up to 1000ml to make the final concentrations of Tris-HCl (pH 7.5) at 0.05M and NaCl at 0.15M.

2. TBST (Tris Buffered Saline with Tween 20): 200µl of Tween 20 was added to 1000ml of TBS.

3. Developing solution: one pill (30mg) of 4-chloro-1-naphthol was dissolved in 10ml methanol. After mixing, 50ml of TBS and 35µl of H_2O_2 were added.

2.11.5.2 Procedure

All the procedures were performed at room temperature. The nitrocellulose membrane was blocked in 100ml of TBST and 1% w/v gelatin for 30 minutes with shaking. The membrane was washed in TBST twice (10 minutes each time) and then incubated with the primary antibody (Anti-GST Antibody from Pharmacia Biotech, raised in goat, 1:1000 diluted) for 1 hour using a shaker. The membrane was washed in TBST twice (10 minutes each time) again and incubated with the secondary antibody (Peroxidase-Conjugated Rabbit Anti Goat IgG, 1:500 diluted) for 1 hour using a shaker. The membrane was washed in TBS twice with shaking (10 minutes each time). The membrane was then covered with Developing solution until the colour of band appeared and was immensed immediately in distilled water for 10 minutes.

2.12 Analysis of sequences for OTR cDNA

Sequence analysis was carried out by using the Australia National Genomic Information Service (ANGIS) on WebANGIS interface (Gaeta 1997).

2.12.1 Sequence search from database

QueryDB program was used to search the OTR gene or cDNA sequence in any species available from database.

2.12.2 Comparison of two sequences

The Bestfit program was used to determine the identity between two DNA sequences or amino acid sequences. The conventional conditions for this comparison were used for gap weight 5.00, length weight 0.30, average match 1.00 and average mismatch –0.90. Any two sequences were compared by the Prettybox program to exhibit the locations of the altered nucleotides or amino acids.

2.12.3 Alignment of multiple sequences

Pileup program was performed to compare the multiple (more than two) sequences of DNA or protein. The conventional conditions for this comparison were used for gap weight 3.00, length weight 0.10. The multiple sequence alignments were also analyzed further with the Readseq program and the Prettybox program to exhibit the locations of varied sequences.

2.12.4 Translation of DNA into protein sequences

Etranslation program was used to translate OTR cDNA sequence into putative amino acid sequence.

2.12.5 Analysis of the structure of the deduced protein

A Peptide-structure program was used to analyze the physical properties and structures of the deduced OTR protein with variation sites. This program has been constructed based on the original method by Chou and Fasman in 1978 (Greta, 1997).

2.12.6 Identification of OTR

Motifs program was used to classify the putative amino acid sequence for OTR based on consensus pattern of the amino acid sequence from the majority of the receptors.

2.13 Statistical analysis

Statistical analysis was carried out using Sigma Stat statistical software (SPSS Inc.).

2.13.1 One way analysis of variance (One way ANOVA)

Data are presented as means±the standard error of the mean (SEM) and were analyzed by One Way ANOVA program with time of sample collection taken as the treatment. When the ANOVA indicated a significant treatment effect ($P<0.05$), the individual time

points were subsequently compared by Post-hoc test using Tukey's Multiple Comparison procedure. Statistical significance was tested at the 5% level ($P<0.05$).

2.13.2 Two way analysis of variance (Two way ANOVA)

Two way ANOVA was used to analyze the effects between two factors and time, and the interaction between these factors on each of the measured parameters. Analysis of variance for different factors was taken, using adjusted SS for tests. When the ANOVA indicated as $P<0.05$, this was interpreted as indicating a significant effect between two factors and time.

2.13.3 T-test

A T-test was used to compare the significant difference between the two individual samples.

2.14 Quantitation of mRNA expression

Autoradiographed signals were quantitated by densitometric scanning and the ratios of signals obtained by hybridization with an OTR probe versus the signals obtained with GAPDH probe were calculated.

2.15 Determination of the size of OTR transcripts

RNA markers (G319a, Promega) were loaded with each blot to use as a reference for determining the size of the hybridization bands. The standard curve for the markers was constructed using logarithmic paper. The distance of each band of RNA markers migrated in the gel was measured and used as data X. The molecular weight of each band was taken as the logarithmic value and was used to plot the Y axis. The size of targeted hybridization band was determined from the distance of the migration band from the origin and from the standard curve constructed using the markers.

2.16 Chemicals and reagents

All the chemicals used in this work are listed in the Appendix part 1. All reagents were analytical grade and mainly supplied by BDH (UK) and Sigma (USA), unless otherwise stated. Preparation of buffers and solutions are shown in the Appendix part 2 or in chapter 2.

2.17 Equipment and instrumentation

Beckmann J2-HS centrifuge (Beckmann instruments Inc., CA, USA)

Beckmann Microcentrifuge

UV-Visible Spectrophotometer (LKB)

Dynavac FD 300 freeze-dryer (Dynavac Engineering Pty. Ltd. Inc., Melbourne, Austrilia)

H1 8418 pH Meter (Hanna instruments, NSW, Austrilia)

Vortex mixer MT19 (Chiltern)

Chapter 3 Characterisation of the oxytocin receptor cDNA in sheep

3.1 Introduction

Since Kimura *et al.* (1992) first reported the structure and expression of the human oxytocin receptor (OTR) complementary DNA, characterization of the OTR gene has been widely studied. Investigations of the OTR at the molecular level have been reviewed in detail in chapter 1, section 1.5. The genomic DNA and cDNA for OTR have been isolated and characterized in human, rat, pig, cattle, mouse, sheep and monkey. Among these species, the predicted amino acid sequence of OTR has high homology to one another, but low homology to the V1 and V2 vasopressin receptor sequences. The encoded receptor belongs to G protein-coupled receptor families with 7 transmembrane domains.

The sheep OTR gene has been investigated by a research group in the UK and the research details has been reviewed in chapter 1, section 1.5.1. In brief, a 132 bp cDNA encoding part of the sheep endometrial OTR was generated by RT-PCR (Stewart *et al.,* 1993), and used as a probe to determine the OTR mRNA expression during the oestrous cycle. A 6.7kb transcript of OTR was identified which showed an increase over the latter stage of the oestrous cycle in parallel with the OTR protein molecule, as determined by ligand binding assay (Stewart *et al.,* 1993).

In 1995, a sheep oxytocin receptor cDNA including the complete coding region was isolated from an ovine endometrium cDNA library and was sequenced by a variety of strategies (Riley *et al.*, 1995). However, the presence of multiple OTR transcripts in ovine endometrium (Flint *et al.,* 1995) was different from the single transcript that had been reported by Stewart *et al.* (1993) and little information has been offered to explain these differences.

In order to obtain a better understanding of the mechanism underlying uterine OTR regulation, this study was undertaken to determine the cDNA sequence of OTR gene in sheep using RT-PCR techniques.

3.2 Materials and Methods

3.2.1 Animals

Merino ewes used for this experiment were housed at the animal house, Department of Physiology, Monash University. The ewes were euthanized by intravenous administration of a lethal dose of sodium pentobarbitone for collecting the samples.

3.2.2 Tissue collection

Uterine tissues were obtained from two ewes at oestrus and immediately placed on ice for processing. Uterine endometrium tissues were dissected and collected as described in chapter 2, section 2.1.6. Uterine endometrium and myometrium tissues were also collected respectively from ewes treated at day 133 of gestation with an injection of betamathasone to induce labor and from ewes at natural parturition.

3.2.3 Total RNA isolation

Two methods for RNA isolation were used in this experiment: the guanidium thiocyanate extraction method and the TRIZOL Reagent from GIBCOBRL for total RNA extraction (for details see chapter 2, section 2.2.1). The second system that utilized a mono-phasic solution of phenol and guanidine isothiocyanate to extract total RNA was found to be superior and more convenient than the guanidium thiocyanate extraction method. The brief descriptions of these methods are as follows:

3.2.3.1 The guanidium thiocyanate total RNA extraction method

Tissues were homogenized in a denaturing solution containing 4M guanidium thiocyanate according to the procedure outlined in Current Protocols in Molecular Biology (supplement 14, Ausubel *et al.*, 1994). The homogenate was mixed sequentially with 2M sodium acetate, pH 4, phenol, and chloroform/isoamyl

alcohol. The resulting mixture was centrifuged, yielding an upper aqueous phase containing total RNA. Following isopropanol precipitation, the RNA pellet was dissolved in denaturing solution, reprecipitated with isopropanol, washed with 75% ethanol and dissolved in DEPC-water.

3.2.3.2 Total RNA extraction by TRIZOL Reagent (GIBCOBRL)

Tissues were homogenized in TRIZOL reagent (50mg tissue/ml) using a Polytron homogenizer. The homogenate was incubated for 5 min at room temperature to ensure the complete dissociation of nucleoprotein complexes, and then mixed with chloroform at 0.2 ml/ml TRIZOL reagent. The resulting mixture was centrifuged at 13000 x g for 15 min at 4°C. The upper aqueous phase containing total RNA was removed. Following isopropanol precipitation, the RNA pellet was washed with 75% ethanol and dissolved in DEPC water.

3.2.4 mRNA purification

Two kinds of total RNA samples were used for mRNA purification. One of these samples was endometrial total RNA prepared from two ewes on day 15 of the oestrous cycle respectively. The other samples were mixed total RNA samples from the ewes at parturition. These mixed total RNA samples were: uterine endometrium and myometrium samples from the ewe on day 133 of gestation injected with betamathasone; myometrium samples from the ewes on days 149 and 150 of gestation. PolyATtract mRNA Isolation System (Promega) was used for mRNA purification followed the protocol as descried in chapter 2, section 2.2.2.

3.2.5 Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

3.2.5.1 Primer design

Three different pairs of primers were designed (Fig. 3.1). The sequences of all the primers used for RT-PCR are shown in table 3.1.

Table 3.1 List of primer nomenclature, primer sequence and relevant references

Primer name	Sequence	Reference
F_A	5' TT(CT)[a] TT(CT)[b]TT(TC)[c]ATGAA(GA)[d]CAC 3'	Kimura *et.al.*, 1992; Rozen *et.al.*, 1995
F_B	5' GCAGCAGCTGTTGAGGCT 3'	Steward *et.al.*,1993
P_A	5'CTCTTCTT(CT) [a]TTCATGAAGCACCT(GA) [b]AGCATA 3'	Kimura *et.al.*, 1992; Rozen *et.al.*, 1995
P_B	5'GCAGCAGCTGTTGAGGCTGGCCAGAAGCAT 3'	Steward *et.al.*,1993
P_C	5'CTGCCTGGCCATCTGCCAGCCGCT 3'	Based on sequence generated by pair 1
P_F	5'TCACACCGTGGATGGCTGCGAGCA 3'	Riley *et.al.*,1995
P_E	5'ATGGAGGGCGCGTTTGCGGCAAAC 3'	Riley *et.al.*,1995
P_D	5'ACGACGATGAAGGTCATCTTGACC 3'	Based on sequence generated by pair 1
P_H	5'ATGTGGAGTGTCTGGGATGCCGAT 3'	Based on sequence generated by pair 1
AP (adapter)	5'GGCCACGCGTCGACTAGTACT$(dT)_{16}$ 3'	3'RACE system (Life Technology)
AUAP	5'GGCCACGCGTCGACTAGT 3'	3'RACE system (Life Technology)
	AUAP=abridged universal amplification primer	
AP(anchored)	5'(dT)30N-1N 3' , N-1=A,G,C; N=A,G,C,T	3'RACE system (Clontech)

[a, b. c, d] indicates the mixture on the site

For pair 1, two sets of primers were designed to optimize the RT-PCR working condition. These were labeled as F_A, F_B and P_A, P_B. The forward primers (P_A, 30 nucleotides; F_A, 18 nucleotides) were designed based on the human and rat OTR DNA sequence corresponding to the region encoding residues 220-249 or 223-240 respectively with mixed bases on certain sites. In choosing these sequences the objective was to select regions of maximum similarity between the human and rat OTR (Kimura *et al.*, 1992; Rozen *et al.*, 1995), on the assumption that regions conserved in these receptor mRNAs would be likely to be represented in the sheep OTR mRNAs. There was no sheep OTR gene sequence data available when this work started. The reverse primer (P_B, 30 nucleotides; F_B, 18 nucleotides) was designed based on the partial sheep OTR cDNA sequence corresponding to the region encoding residues 949-978 or 949-966 respectively (Steward *et al.*, 1993).

For pair 2, the forward primer P_E was located at the beginning of coding region (residues 1-24) (Riley *et al.*, 1995) and the reverse primer P_D was complementary to the region encoding residues 825-848. This data was based on the sequence of RT-PCR product generated from pair 1.

For pair 3, the forward primer P_C was located at the region encoding residues 411-434. The data was based on the sequence of RT-PCR product generated from pair 1. The reverse primer P_F was complementary to the end of the stop codon (Riley *et al.*, 1995).

3.2.5.2 Optimization of RT-PCR conditions

GeneAmp RNA PCR Kit (Perkin Elmer) was used to optimize the RT-PCR condition. The different reactions for RT-PCR using different set of primer pair 1 and amplification reactions were listed in Table 3.2. The products were identified by Southern analysis as described in chapter 2, section 2.8. In brief, each reaction was loaded to 1% TBE agarose gel with PCR markers. After electrophoresis, a photo of the gel was taken with a ruler on the UV box. The gel was blotted using the dry-blotting method (chapter 2, section 2.8). The blot was hybridized with Probe 1 (chapter 2, section 2.7.4). Labeling and hybridization procedures were the same as described in chapter 2, section 2.7.5 and 2.7.7.

Table 3.2 Experimental protocols used for the optimization of RT-PCR conditions

reaction	1	2	3	4	5	6	7	8	9	10	11
mRNA	0.7µg	0.7µg	0.7µg	0.7µg	0.95µg	0.95µg	0.7µg	0.7µg	0.7µg	0.7µg	0.7µg
primers for RT	P_B,F_B	dT	dT	dT	P_B	F_B	dT,P_B,F_B	dT,P_B,F_B	F_B	P_B,F_B	P_B
incubation time for RT	37°C, 10 minutes, then 42°C, 15 minutes						37°C, 10 minutes, then 42°C, 15 minutes				
primers for PCR	F_A,F_B	P_A,P_B	F_A,F_B	P_A,P_B	P_A	F_A	F_A	P_A	F_A	F_A	P_A
cycling parameters	95°C, 40 seconds 55°C, 40 seconds 72°C, 60 seconds		95°C, 40 seconds; 55°C, 40 seconds; 72°C, 70 seconds							95°C, 40 seconds 60°C, 40 seconds 72°C, 70 seconds	
cycles	40	40	45	50	45	45	45	45	45	45	45
result	-	-	-	++	-	-	-	-	-	-	+

RT: reverse transcription

3.2.5.3 Templates

mRNA was used as templates in this experiment (as described previously in section 3.2.4). For the RT-PCR reaction generated by primer pair 1 (P_A, P_B), mixed endometrium and myometrium mRNA samples at parturition were used as the template. For the RT-PCR reactions generated by primer pair 2 (P_C, P_F) and primer pair 3 (P_E, P_D), a mRNA sample prepared from uterine endometrium of a sheep at oestrus was used as a template.

3.2.5.4 RT-PCR amplifications

The RT-PCR reaction was performed using a RT-PCR kit (Promega). For details see chapter 2, section 2.5. First-strand cDNA was synthesized using 0.5 μg mRNA, 5 units avian myeloblastoma virus reverse transcriptase and 50 pmol primers. The reaction was incubated at 48°C for 45 min. PCR amplification was then performed directly for 35 cycles using 5 units Taq polymerase. For primer pair 1 (P_A, P_B), the cycling parameters were 95°C 30s, 58°C 45s, 68°C 90s. For primers pair 2 (P_C, P_F) and primers pair 3 (P_E, P_D), the cycling parameters were 95°C 30s, 56°C 45s, 68°C 90s.

After amplification, the PCR products were separated on a 1% agarose gel and purified from the gel using a Wizard PCR Preps DNA purification system (Promega). The purified samples were used for DNA sequencing. For details see chapter 2.

3.2.6 Rapid Amplification of cDNA Ends (RACE)

Two different 3'RACE systems (Life Technologies and Clontech) were used to amplify the 3'end of the OTR cDNA. The details of the procedure for amplification is described in chapter 2, section 2.6 and in chapter 4, section 4.2.5 in brief. Primers used for RACE were indicated in Fig.3.1 and listed in Table 3.1.

3.2.7 Optimization of RACE conditions

In order to obtain the large size of OTR cDNA based on the result of Northern hybridization (chapter 5), the reaction for amplification of cDNA end (Life Technologies) was optimized as listed in Table 3.3. 3'RACE system from Clontech was not used for optimization since the limited reactions were available.

Table 3.3 Experimental protocol used to determine the optimal condition for the 3'RACE (Life Technology)

reaction	1	2	3
mRNA template	486 ng	486ng	486ng
Reverse transcription	Followed instruction provided by the manufacturer	Followed instruction provided by the manufacturer	Followed instruction provided by the manufacturer
Primer for PCR Cycling parameters	PH 30pmol AUAP 30pmol 95°C 1 min 58°C 1 min 72°C 3 min	PH 10pmol AUAP 10pmol 95°C 1 min 56°C 1 min 72°C 3 min	PH 30pmol AUAP 30pmol 95°C 40 second 56°C 40 second 72°C 5 min
Result (product size) 600bp 1300bp >1300bp	 ++ ± -	 ++ + -	 ++ + -

3.2.8 Cloning of PCR products

pCR-Script Amp SK(+) Cloning Kit (Stratagene) was used for the cloning of PCR products generated by primer pair 1. For details, see chapter 2, section 2.9. The PCR products were purified using Wizard PCR Preps DNA Purification System (Promega). The purified products (200 ng) were polished by Pfu DNA polymerase at 72°C for 30 min to blunt the end of PCR products. 4 µl of polishing reaction was added to 10 µl of a ligation containing pCR-Script 1x reaction buffer, 0.5 mM rATP and 10ng of Srf1-digested pCR-Script Amp SK(+) cloning vector. The enzymes Srf1 and T4 DNA ligase were added. The reaction was incubated at room temperature for 1 hour before heat treating at 65°C for 10 min. A 3µl of the reaction was then used to

transform to 40µl of E.coli XL1-Blue MRF'Kan super-comptent cells. White colonies were selected and identified.

3.2.9 Cloning of RACE products

The pGEM-T cloning vector system (Promega), the pCR-Script Amp SK(+) Cloning vector (Stratagene) and the Original TA Cloning Kit (Invitrogen) were used to clone the RACE products. The detailed protocols are described in chapter 2, section 2.9.3. However, only the pGEM-T cloning vector system (Promega) worked successfully. For the reasons, given in chapter 4, section 4.3 and 4.4. The pGEM-T cloning method is described in brief as follows:

The pGEM-T cloning vector system (Promega) was used for the cloning of RACE-PCR products. The PCR products generated from the RACE system were purified using the Wizard PCR Preps DNA Purification System (Promega). The purified products were performed with A-tailing reaction by Taq DNA polymerase at 70°C for 20 min. A-tailed products were therefore, ligated with pGEM-T vector by T_4 DNA ligase at 4°C overnight. JM109 high efficiency competent cells were used for transformations. Recombinant plasmid DNAs were identified by DNA sequencing.

3.2.10 DNA sequencing

A ABI Prism Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer) was used for direct sequencing of RT-TCR products and RACE products. A ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer) was used for sequencing of cloned RACE products. The protocol supplied by the manufacturer was followed for cycle sequencing (chapter 2, section 2.10). After cycle sequencing on Peltcer Thermal Cycler (PTC-200, Bresatec), the products were purified by ethanol precipitation as per the instructions.

3.2.11 Analysis of sequences for OTR cDNA

DNA Sequence editing, comparison, alignment, translation and analysis of the physical properties and structures of the deduced protein were carried out using the

Australia National Genomic Information Service (ANGIS) on WebANGIS interface (Gaeta 1997).

3.3 Results

3.3.1 RT-PCR

The results of the optimization of the RT-PCR showed a strong hybridization signal appeared in reaction 4 and showed an expected size of 750bp. In addition a signal was also present in reaction 11 (Table 3.2, Fig 3.2). These reactions were performed with primers P_A and P_B. This primer pair 1 (P_A and P_B) was chosen for RT-PCR and the cycling parameters designed were partly based on these results.

The three main bands of 750bp, 760bp and 840bp were generated with primer pairs 1, 2 and 3, respectively (Fig 3.1 and Fig 3.3). These three fragments were named as fragment 1, fragment 2, fragment 3, respectively (Fig. 3.4). The sequence of fragment 1 covered the major coding region of OTR gene (from transmembrane domain II to XI) (Fig 3.1 and 3.4). No sequence variation was observed in both direct sequencing of fragment 1 or the sequencing of the cloned products. Because this fragment was generated from mixed endometrial and myometrial mRNA samples at labor, it was considered important to ensure that no sequence variation occurred between endometrial and myometrial OTR mRNAs before a sequence comparison was made. These three fragments have accurate sizes of 759bp, 765bp, and 848bp based on the sequencing results, and encode the regions at position 220-978, 411-1176 and 1-848, respectively.

3.3.2 3'RACE

Two bands were generated by both 3'RACE systems, a major band size of 600 bp and a minor band size of 1300 bp (chapter 4, Fig 4.5). Direct sequencing of the 600 bp band showed some divergence at certain sites and this result was repeatable. The RACE products were then cloned for further analysis. The reason of the sequence variations in the sequence of the 600 bp of RACE products is explained in chapter 4.

The larger size of OTR cDNA transcripts was not observed by optimizing the amplification conditions using the 3'RACE system from Life Technologies (Table 3.3).

3.3.3 Analysis of the coding region for OTR cDNA

The sequence data of all three fragments was combined to form a 1176 bp of the OTR cDNA, from the beginning codon of ATG to the stop codon of TGA. The overlapping of these fragments was used to compose the OTR cDNA segment shown in Fig3.4. Each sequence data was confirmed from two fragments that were generated by a different pair of primers, except the region at position 1-220. The sequence of this region (1-220) was obtained from fragment 3 only and was identical to the published sheep endometrial OTR cDNA (Riley *et al.*, 1995). The sequence of this combined OTR cDNA was 89.48%, 86.12%, 90.13% and 96.34% identical to OTR cDNA of human, rat, pig and cattle respectively (Fig 3.5) (Bestfit, ANGIS) (Kimura *et al.*, 1992; Rozen *et al.*, 1995; Gorbulev *et al.* 1993; Bathgate *et al.*, 1995). The nucleotide and amino acid sequence of this sheep endometrial OTR cDNA is shown in Fig 3.6.

Comparison of the published sheep endometrial OTR cDNA sequence (Riley *et al.*, 1995) with the sequence found in this study showed seven different nucleotide alterations in the coding region in positions 727, 886, 887, 1076, 1077, 1094, 1095 (putative amino acid positions 243, 296, 359, 365) (Fig 3.5 and Fig 3.7).

3.3.4 Analysis of the 3'Untranslated Region (3'UTR)

As described above, the products generated by the 3'RACE systems showed two sizes at 600 bp and 1300 bp respectively. These products contained part of the OTR cDNA coding region (positions 892-1176) and about 300 bp and 1000 bp of the 3'untranslated region. The sequences of these two products are identical, except for variations at certain sites, which are addressed at greater length in Chapter 4. The 1300 bp product had a longer sequence in the 3'untranslated region (Fig. 3.2). When the sequence in coding region with the 3'untranslated region were taken together, the

cDNA for OTR had fragment sizes of 1475bp and 2196bp, which were similar to the two lower Northern hybridization bands with sizes of 1.5 kb and 2.1 kb (see Fig.5.1 and Fig 5.2 in chapter 5).

The sequences of four cDNA species generated by the 3'RACE system, when compared with the published sheep OTR cDNA sequence in the 3'untranslated region, showed that several variations occurred randomly (Fig 3.8). The changes not only involved in base replacement, but also involved in base addition / deletion.

3.3.5 Analysis of the putative amino acid sequence

The putative amino acid sequence derived from this cDNA revealed the occurrence of seven hydrophobic transmembrane regions, which is characteristic of a G-protein coupled receptor (analysed by Motifs, ANGIS). Sequence analysis indicated the presence of three N-glycosylation sites at Asn-8, Asn-15 and Asn-26 located on the extracellular side of the membrane at the N-terminus (analysed by Plotstructure, ANGIS). There are five possible serine and threonine phosphorylation sites as indicated in Fig. 3.7. One is in the carboxyl terminus and the other four are in the third intracellular loop (Kennelly and Krebs, 1991). Comparison of the OTR putative amino acid sequence between sheep (present work), sheep1 (Riley *et al.,* 1995), cattle (Bathgate *et al.,* 1995), pig (Gorbulev *et al.* 1993), human (Kimura *et al.,* 1992) and rat (Rozen *et al.*, 1995) is shown in Fig. 3.7. The areas that are absolutely conserved among the five species are in the first and second extracellular loops (between transmembrane domain II and III, IV and V), and little change was observed in the third extracellular loop (between transmembrane domain VI and VII). There are also a few changes in the first and second intercellular loop (between transmembrane domains I and II, III and IV). The big differences in sequence variation among the five species are in N-terminal, C-terminal and, especially in the third intercellular loop (between transmembrane domains V and VI). There are few amino acid changes in the seven transmembrane domains among the five species. The various amino acids are almost within the same group expect in transmembrane domain I (Phe to Leu change) and transmembrane domain V (Ala to Thr change).

3.3.6 Cloning of RT-PCR and RACE products

The RT-PCR products generated by primer pair 1 (P_A and P_B) were cloned into pCR-Script Amp SK(+) Cloning vector (Stratagene). Six positive colonies were identified by restriction enzymes BstXI and EcoRI. Two of them were confirmed by sequencing. Positive colonies No.8 and 10 have the same sequence, but different orientation. The cloning map is shown in Fig 3.9

Because of the sequence variation at particular sites from the direct sequencing results (as mentioned in section 3.3.2), the RACE products were then cloned into a pGEM-T cloning vector system (Promega). For the details, see chapter 4, section 4.3.

3.4 Discussion

Three N-glycosylation sites at Asn-8, Asn-15 and Asn-26 in the N-terminus are present in all species with the exception of the site at Asn-15 (Fig. 3.7) in rats (analysed by Plotstructure, ANGIS). These sites are thought to contribute to the biosynthesis and internalization of the membrane protein (Jans *et al.* 1992). In the three extracellular loops (between transmembrane domains II and III, IV and V, and VI and VII; Fig 3.7), little change was found among the five species, thus implying that their amino acid sequence structure is essential for their function and specific ligand-receptor binding properties throughout mammalian evolution. All of the intracellular domains are proposed to have a role in efficient G-protein coupling. The reported variations among the five species are in the three intracellular loops but these changes are thought to be tolerated through evolution without affecting biological function (Fig 3.7). Short stretches of the membrane proximal region of the third intracellular loop (between transmembrane domains V and VI), and possibly the carboxyl terminus, appear to be particularly critical in determining the specificity of G-protein coupling (Probst *et al.* 1992). The differences in the third intracellular loop and the carboxyl terminus among the five species may imply that each species has their own specific structure for receptor-protein binding.

Comparison of a published sheep endometrial OTR cDNA sequence (Riley *et al.*, 1995) to the present one shows seven different nucleotides alterations in coding region in positions 727, 886, 887, 1076, 1077, 1094, 1095 (putative amino acid positions 243, 296, 359, 365) (see Fig 3.5 and Fig. 3.7). The possibility of these being PCR-generated errors has been excluded since the same data was obtained in at least two PCR products generated independently using different primers. No sequence variation is present in the designed primer regions. When the putative amino acid changes are compared with other published OTR sequences at these particular sites, the present data indicates that the sheep OTR sequence obtained in this study has more similarity with the sequence found in other species. In the 3'untranslated region, the DNA sequences showed a high similarity with the published literatures, except for certain sites (Fig 3.9). Whether these sequence differences are due to the variations between different sheep breeds remains to be investigated.

A sequence search on the 3'untranslated region of clones 600A, 600B and 600C did not find any sequence element which determined the site of cleavage and polyadenylation in pre-mRNA (Edwalds-Gilbert *et al.* 1997). Only one highly conserved poly A signal, the hexanucleotide AATAAA was found in 1300D near the 3'end (Fig.4, chapter 4). Another consensus sequence ATTTA, known to be associated with transcript destabilization (Chen and Shu, 1995), was not found in any of the PCR-RACE products in the 3'untranslated region. However, two secondary structures, i e., stem loops were present in 600A, 600B and 600C prior to the poly A site, indicating that these structures may be necessary for cleavage and polyadenylation.

The DNA sequence obtained from the RT-PCR products generated from the mixed endometrial and myometrial mRNA samples at labor and endometrial mRNA samples on day 15 of the oestrous cycle were the same. No sequence variation was found. These data indicate that the OTR gene expressed in either the endometrium or the myometrium is the same, which is compatible with the conclusion found after Northern hybridization (see Fig.5a Northern hybridization analysis of uterine OTR gene expression at parturition).

The results from the Northern hybridization showed four transcripts of OTR gene expressed in both the endometrium and the myometrium. These transcripts were determined to be 5.6 kb, 3.3 kb, 2.1 kb and 1.5 kb in size, respectively (for details, see chapter 5, section 5.2). When the sequence in the coding region was combined with the 3'untranslated region, they resulted in a cDNA fragment of size 1475 bp and 2196 bp, which corresponded with the two lower Northern hybridisation bands of around 1500 bp and 2100 bp (Fig 5b, chapter 5). These data indicate that these two transcripts may contain a short 5'untranslated region. Sequence analysis indicated that the size differences of OTR mRNA were possibly due to the alternative use of a different polyadenylation site (Fig 4, chapter 4). This result is consistent with previous findings in rat (Rozen *et al.*, 1995).

Both RACE systems did not capture the two large cDNA bands that would correspond with the two higher Northern hybridization bands of size 5.6kb and 3.3kb. The possible reasons are: first, in comparison with the small size of mRNA template, the large size of mRNA template is harder to capture using the reverse transcription reaction. Second, the amplification conditions provided by both RACE systems may not be suitable to amplify the larger size of cDNAs. Theoretically, it is possible to amplify the large size of cDNAs, however, the reagents for the reverse transcription and amplification need to be chosen carefully, and amplification conditions need to be optimized. In this work, although the reaction conditions were optimized, no larger size cDNA band was captured. However, because of time and financial constraints, further work was not undertaken.

In next chapter (chapter 4), it is proposed that the differences in the sequence of the OTR mRNA in the endometrium are due to mRNA editing.

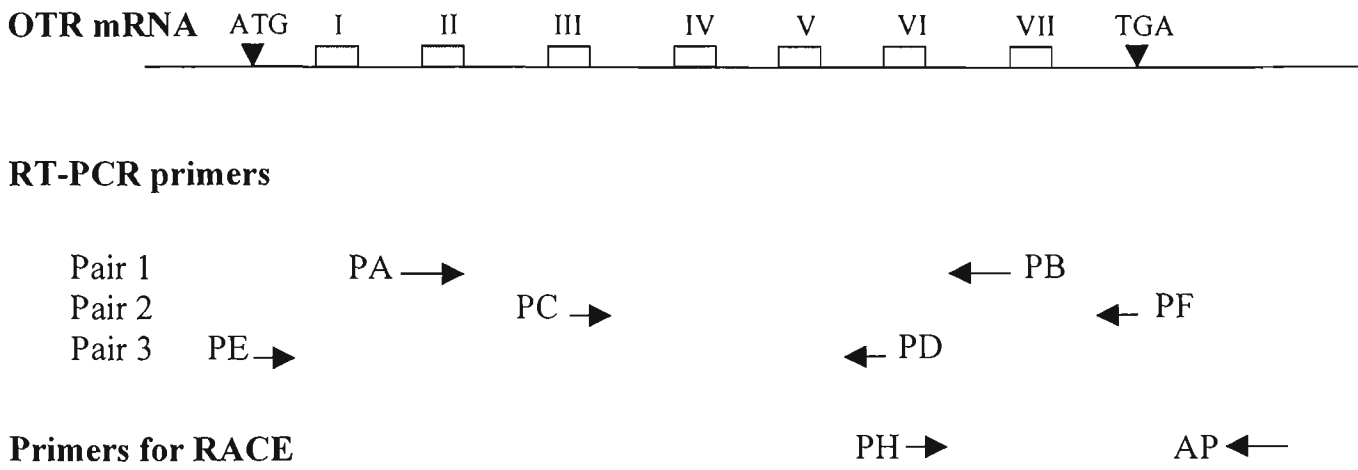


Fig 3.1 Schematic representation of the PCR primers used for the amplification of OTR cDNA. Each box indicates the transmembrane domain labeled with a Roman numeral. AP, adaptor primer or anchored primer.

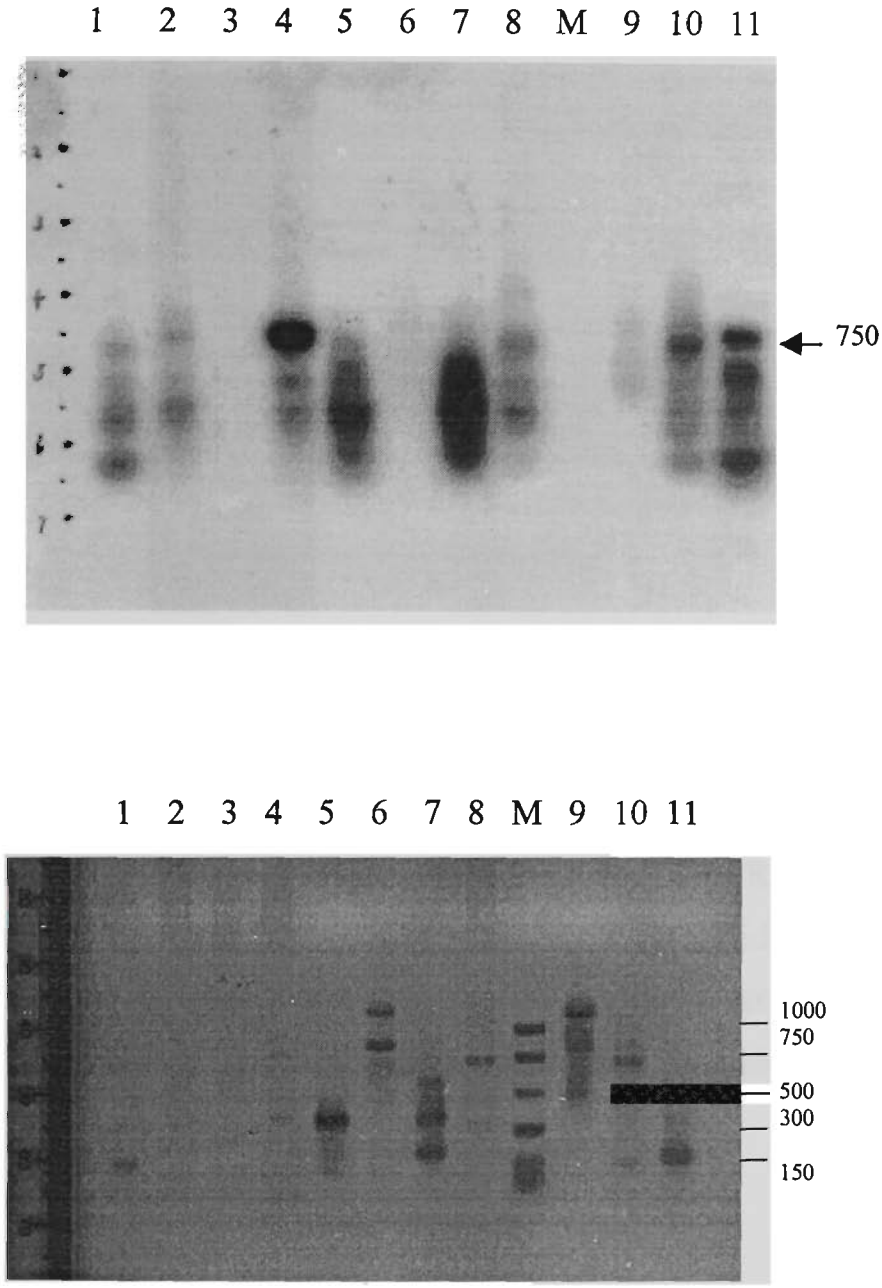


Fig 3.2 The result of Southern hybidization with Probe 1 used to detect the positive RT-PCR conditions. Upper panel: hybidization signals in the different reactions. Bottom panel: gel electrophoresis of the RT-PCR products from the different reactions listed in Table 3.3. 1-11: the number of the reaction. M: PCR markers (Promega) indicated the size at 1000 bp, 750 bp, 500 bp, 300 bp and 150 bp (from top to bottom). ➔indicates the expected RT-PCR product band.

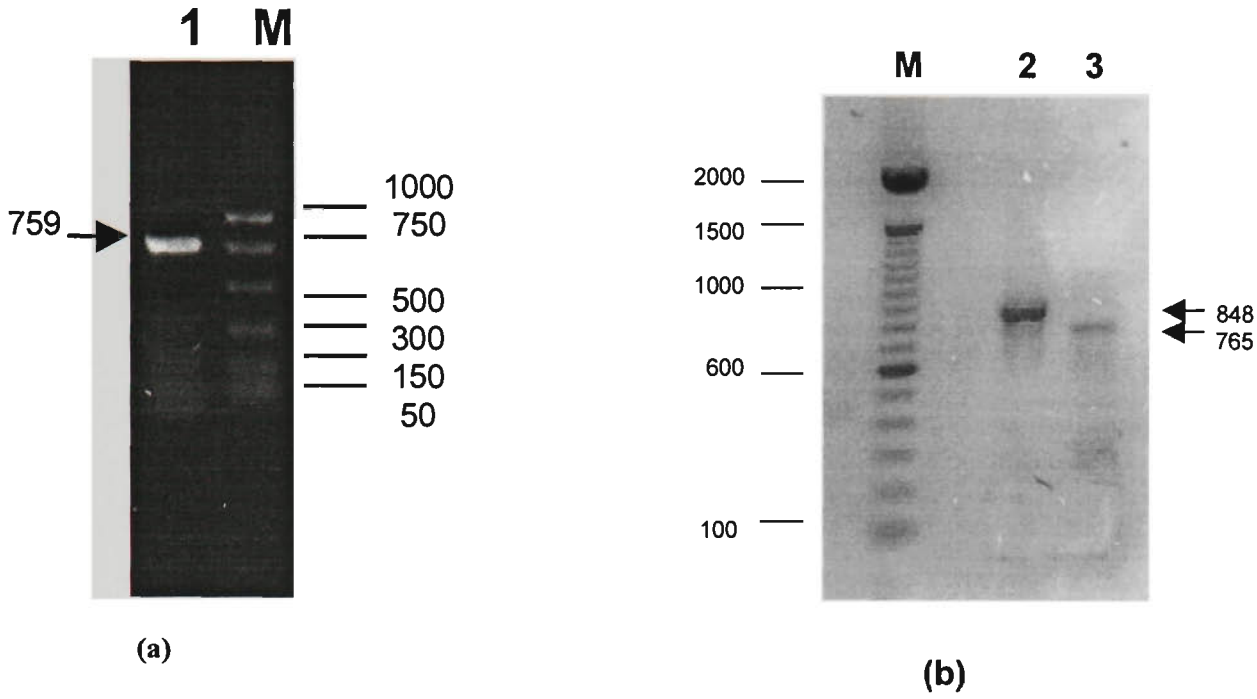


Fig.3.3 PCR products generated from the RT-PCR system. (a), M: PCR Marker (Promega); Lane 1 showed PCR fragments generated by primer pairs 1 (5 μl/Lane). → indicates size (bp) of each fragment. (b), M: 100 bp DNA ladder Marker (Life Technologies). Lanes 2 and 3 show the PCR fragments generated by primer pairs 2, 3 respectively (5 μl/Lane).

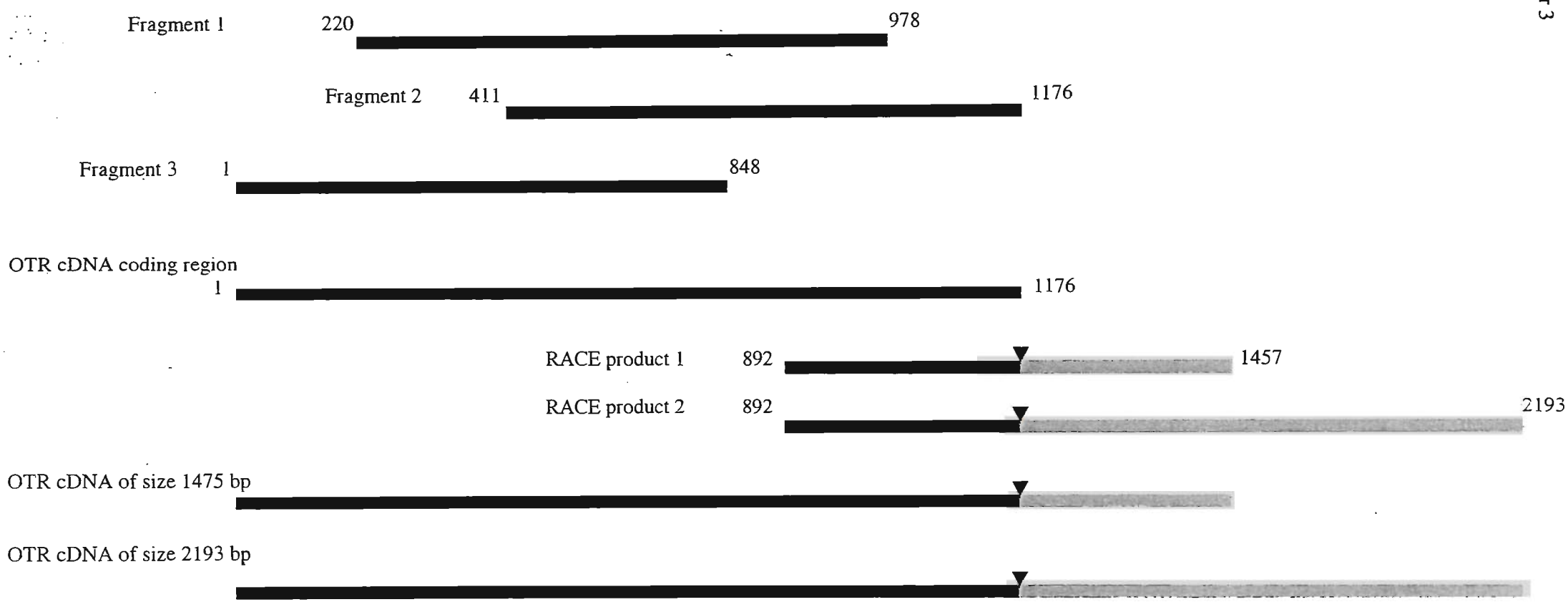


Fig 3.4 Schematic representation of the PCR fragments and RACE products used to compose the OTR cDNA (coding region in black and 3' untranslated region in grey). ▼: stop code. Numbers indicate the position on each fragment.

```
sheep  ATGGAGGGCG CGTTTGCGGC AAACTGGAGC GCGGAGGCGG TCAACGGGAG CGCGGCGCCT    60
sheep1 ---------- ---------- ---------- ---------- ---------- ----------    60
cattle --------T- ---------- T--------- --T------- ---------- ---------G    60
  pig  --------G- T-C----A-- C--------- --C------- -----TC--- ---------G    60
human  ---------- --C-C--A-- C--------- --C-----A- C-----CC-- ---C-----G    60
  rat  ---------A --CCA--A-- C--------T -TC---TT-- A-CT------ T-GA-T---A    60

sheep  CCGGGAACCG AGGGCAATCG CACTGCCGGG CCGCCACAGC GCAACGAGGC CCTGGCGCGG   120
sheep1 ---------- ---------- ---------- ---------- ---------- ----------   120
cattle ---------- ---------- ---------- ---------- ---------- ----------   120
  pig  --A-AGG--- -------C-- ---C------ -----G---- ---------- ---------T   120
human  -----GG--- -------C-- ---C-----A --C--G-G-- ---------- ---------C   120
  rat  --A--GGAG- -------C-- ---G------ --A-----A- ---------- ---------C   120

sheep  GTGGAGGTGG CCGTGCTGTC CCTCATCCTG TTCCTGGCGC TGAGCGGCAA CGCGTGCGTG   180
sheep1 ---------- ---------- ---------- ---------- ---------- ----------   180
cattle ---------- ---------G ---------- ---------- ---------- ----------   180
  pig  ---------- -G---T---G ------T--C -----A--T- ---------- T---------   180
human  ---------- -G-------G T--------- C--------- -------G-- ------T---   180
  rat  ---------- -G--C----G ------T--- --------T- ----T----- ------T---   180

sheep  CTGCTGGCGC TGCGCACCAC GCGCCACAAG CACTCGCGCC TCTTCTTCTT CATGAAGCAC   240
sheep1 ---------- ---------- ---------- ---------- ---------- ----------   240
cattle -----A---- ---------- ---------- ---------- ---------- ----------   240
  pig  ---------- ---------- ---------- --T------- ---------- ------A---   240
human  ---------- ---------- A-----G--- ---------- ---------- ----------   240
  rat  ---------- ---------- ---------- ---------- -------T-- ----------   240

sheep  CTGAGCATAG CCGACCTGGC GGTGGCGGTG TTCCAGGTGC TGCCGCAGCT TCTGTGGGAC   300
sheep1 ---------- ---------- ---------- ---------- ---------- ----------   300
cattle ---------- ---------T A--------- ---------- ---------- ----------   300
  pig  ---------- ---------T ---------- ---------- ----T----- A--------T   300
human  --A-----C- ---------T ------A--- --T------- --------T- G---------   300
  rat  --------CC GT-------T ------T--- --T------- -T-------- G---------   300

sheep  ATCACGTTCC GTTTCTACGG GCCCGACCTG CTCTGCCGCC TCGTCAAGTA CCTGCAGGTG   360
sheep1 ---------- ---------- ---------- ---------- ---------- ----------   360
cattle ---------- -C-------- ---------- --G------- ---------- ---------T   360
  pig  -----C---- -C-------- A------T-- --G------- ---------- ---C------   360
human  -----C---- -C-------- ---------- --G------- -G-------- -T--------   360
  rat  -----C---- -C-----T-- ---------- --G--T--T- -G-----A-- -T--------   360

sheep  GTGGGCATGT TCGCGTCCAC CTACCTGCTG CTGCTCATGT CGCTCGACCG CTGCCTGGCC   420
sheep1 ---------- ---------- ---------- ---------- ---------- ----------   420
cattle ---------- ---------- ---------- ---------- ---------- ----------   420
  pig  ---------- ----T----- T--------- ---------- ---------- ---T------   420
human  ---------- ----C----- ---------- ---------- -C--G----- ----------   420
  rat  ---------- ----C----- ---------- -----G---- ---------- ---T------   420
```

continue to the next page

```
sheep  ATCTGCCAGC CGCTGCGCTC GCTGCGCCGC CGCACCGACC GCCTGGCGGT ACTCGCCACC   480
sheep1 ---------- ---------- ---------- ---------- ---------- ----------   480
cattle ---------- ---------- ----A----- ---------- ---------- -----T---A   480
  pig  ---------- --T-----G- ---------- -C-G-G--T- -T-----A-- G--A-----A   480
human  ---------- ---------- ---------- ---------- -------A-- G--------G   480
  rat  ---------- ---------- C--------- --A------- ---------- G--G-GG--G   480

sheep  TGGCTCGGCT GCCTGGTGGC CAGCGCGCCG CAGGTGCACA TCTTCTCGCT GCGCGAGGTG   540
sheep1 ---------- ---------- ---------- ---------- ---------- ----------   540
cattle ---------- ---------- ---------- ---------- ---------- ----------   540
  pig  -----G---- ---------- ---------- ---------- ---------- ----------   540
human  ---------- ---------- ---------- ---------- -------T-- ----------   540
  rat  -----T---- ---------- ---T--T--- ---------- -T-------- ------A---   540

sheep  GCCGACGGCG TCTTCGACTG CTGGGCCGTT TTCATTCAGC CCTGGGGGCC CAAGGCCTAC   600
sheep1 ---------- ---------- ---------- ---------- ---------- ----------   600
cattle --------T- ---------- ---------- --------A- ---------- ----------   600
  pig  ---------- -------T-- ---------C -----C---- ---------- ----------   600
human  --T------- ---------- ---------C -----C---- -------A-- ----------   600
  rat  --G-----A- -------T-- ------G--C -----C--A- -------A-- ----------   600

sheep  ATCACGTGGA TCACGCTCGC CGTCTACATT GTGCCGGTCA TCGTGCTTGC CGCCTGCTAC   660
sheep1 ---------- ---------- ---------- ---------- ---------- ----------   660
cattle ---------- ---------- ---------- -----C---- ----C----- -A-------T   660
  pig  ---------- ----A--T-- G--------C ---------- -------C-- ----------   660
human  -----A---- -------A-- T--------C ---------- -------C-- TA--------   660
  rat  G----A---- ---------- ---------- --A------- -------G-- ----------   660

sheep  GGCCTTATCA GCTTCAAGAT CTGGCAGAAC TTACGGCTCA AGACGGAGGC GGCGGCGGCA   720
sheep1 ---------- ---------- ---------- ---------- ---------- ----------   720
cattle ---------- ---------- ---------T ---------- ------C--- ----------   720
  pig  ---------- ---------- ---------- --G------- ----T-C--- --AA------   720
human  ---------- ---------- ---------- --G------- ----C-CT-- A--------G   720
  rat  -----C---- ---------- ---------T C-GA-A---- ------CA-- ----------.   719

sheep  GAGGCTGCCG CGGGGGCTGA GGGCGCGGCG GCAGACTGCG CGGGGCGCGC GGCTCTGGCC   780
sheep1 ------T--- ---------- ---------- ---------- ---------- ----------   780
cattle ---------- ---------- -----A---- --------G- ---------- -AT-------   780
  pig  -----GAT-- ----AA-C-- ---------- --G-G-A--C G---C--A-- ----------   780
human  -.......--- A--C-C-A-- ---------- --T-G-GAT- G--------T ---C-----G   774
  rat  ..-------- A----AA--- ......C--- --G-GTG-A- -T-----T-- ---GT----A   771

sheep  CGCGTCAGCA ACGTCAAGCT CATCTCTAAG GCTAAGATCC GCACGGTCAA GATGACCTTC   840
sheep1 ---------- ---------- ---------- ---------- ---------- ----------   840
cattle ---------- ---------- ---------- --C------- ---------- ----------   840
  pig  ---------- G--------- ------C--- --C------- ----C----- ----------   840
human  --T------- G--------- ------C--- --C------- ---------- ------T---   834
  rat  --G-----T- GT-------- T-----C--- --C--A---- -------G-- ----------   831
```

continue to the next page

```
sheep  ATCGTCGTGC TGGCCTTCAT CGTGTGCTGG ACGCCATTCT TTTTCGTGCA GATGTGGAGT   900
sheep1 ---------- ---------- ---------- ---------- -----AA--- ----------   900
cattle ---------- ---------- ---------- ---------- ---------- ----------   900
  pig  ---A-T---- ---------- ---A------ ---------- ---------- ---------C   900
human  ---A------ ---------- ---------- -----T---- -C-------- ---------C   894
  rat  ---A----A- ---------- ---------- --A--T---- -C-------- ---------C   891

sheep  GTCTGGGATG CCGATGCGCC CAAGGAAGCC TCGGCTTTCA TCATCGCCAT GCTTCTGGCC   960
sheep1 ---------- ---------- ---------- ---------- ---------- ----------   960
cattle ---------- ---------- ---------- --AC------ ---------- ---C------   960
  pig  --------C- ---------- ---------- --A------- ----T--A-- ---C------   960
human  ---------- --A-C----- ---------- -----C---- ------T--- ---C------   954
  rat  --T-----C- T-A------- ---------T --T--C---- ----T----- ---CT-----   951

sheep  AGCCTCAACA GCTGCTGCAA CCCCTGGATC TACATGCTCT TCACGGGCCA CCTCTTCCAA  1020
sheep1 ---------- ---------- ---------- ---------- ---------- ----------  1020
cattle ---------- ---------- ---------- ---------- ---------- ----------  1020
  pig  ---------- ---------- ---------- ---------- ----A----- ---------T  1020
human  ---------- ---------- ---------- --------G- ---------- ---------C  1014
  rat  ---------- ---------- ---G------ ---------- ----A--T-- ---------C  1011

sheep  GACCTTGTGC AGCGCTTCCT CTGCTGCTCA TTCCGCCGCC TGAAAGGCAG CCAGCCTGGG  1080
sheep1 ---------- ---------- ---------- ---------- ---------- -----TG---  1080
cattle --A------- ---------- ---------- ---------- ---------- --G-------  1080
  pig  --A------- ---------- T--------T -C-A---A-- ----GAC--- --G---C---  1080
human  --A--C---- ---------- G--------C GC-A--TA-- ----G----- A-GC-TG--A  1074
  rat  --A--C---- --------T- ---------T GCT--TTA-- ----G----- --G---C--C  1071

sheep  GAGACGAGCG TCAGCAAAAA GATCCATTCG TACACCTTTG TCCTGAGTCG GCACAGCTCC  1140
sheep1 ---------- ---CG----- ---------- ---------- ---------- ----------  1140
cattle -----A---- ---------- --G-A-C--- -CT------- -------C-A -T--------  1140
  pig  --------T- ---------- --G-A-C--- -C-------- --T----C-A ----------  1140
human  --------T- C--------- --G-A-C--- -C-T------ -------C-A T-G-------  1134
  rat  --------T- -------G-- --G-A-C--A -C-------- -------C-- T-G------A  1131

sheep  AGCCAGAGAA GCTGCTCGCA GCCATCCACG GTGTGA                            1176
sheep1 ---------- ---------- ---------- ------                            1176
cattle ---------- -A-------- ---------- C-----                            1176
  pig  -------AG- --------T- -......... ......                            1161
human  --------G- -------C-- ---------- -C----                            1170
  rat  --------G- -------T-- ------TT-A -CA---                            1167
```

Fig 3.5 Multiple alignments to show similarities and differences in the OTR cDNA sequence (coding region only) between sheep (present work), sheep1 (Riley *et al.*, 1995), cattle (Bathgate *et al.*, 1995), pig (Gorbulev *et al.* 1993), human (Kimura *et al.*, 1992) and rat (Rozen *et al.*, 1995). – indicates identical nucleotide.

```
ATGGAGGGCGCGTTTGCGGCAAACTGGAGCGCGGAGGCGGTCAACGGGAGCGCGGCGCCT        60
M  E  G  A  F  A  A  N  W  S  A  E  A  V  N  G  S  A  A  P        20

CCGGGAACCGAGGGCAATCGCACTGCCGGGCCGCCACAGCGCAACGAGGCCCTGGCGCGG       120
P  G  T  E  G  N  R  T  A  G  P  P  Q  R  N  E  A  L  A  R        40

GTGGAGGTGGCCGTGCTGTCCCTCATCCTGTTCCTGGCGCTGAGCGGCAACGCGTGCGTG       180
V  E  V  A  V  L  S  L  I  L  F  L  A  L  S  G  N  A  C  V        60

CTGCTGGCGCTGCGCACCACGCGCCACAAGCACTCGCGCCTCTTCTTCTTCATGAAGCAC       240
L  L  A  L  R  T  T  R  H  K  H  S  R  L  F  F  F  M  K  H        80

CTGAGCATAGCCGACCTGGCGGTGGCGGTGTTCCAGGTGCTGCCGCAGCTTCTGTGGGAC       300
L  S  I  A  D  L  A  V  A  V  F  Q  V  L  P  Q  L  L  W  D       100

ATCACGTTCCGTTTCTACGGGCCCGACCTGCTCTGCCGCCTCGTCAAGTACCTGCAGGTG       360
I  T  F  R  F  Y  G  P  D  L  L  C  R  L  V  K  Y  L  Q  V       120

GTGGGCATGTTCGCGTCCACCTACCTGCTGCTGCTCATGTCGCTCGACCGCTGCCTGGCC       420
V  G  M  F  A  S  T  Y  L  L  L  L  M  S  L  D  R  C  L  A       140

ATCTGCCAGCCGCTGCGCTCGCTGCGCCGCCGCACCGACCGCCTGGCGGTACTCGCCACC       480
I  C  Q  P  L  R  S  L  R  R  R  T  D  R  L  A  V  L  A  T       160

TGGCTCGGCTGCCTGGTGGCCAGCGCGCCGCAGGTGCACATCTTCTCGCTGCGCGAGGTG       540
W  L  G  C  L  V  A  S  A  P  Q  V  H  I  F  S  L  R  E  V       180

GCCGACGGCGTCTTCGACTGCTGGGCCGTTTTCATTCAGCCCTGGGGGCCCAAGGCCTAC       600
A  D  G  V  F  D  C  W  A  V  F  I  Q  P  W  G  P  K  A  Y       200

ATCACGTGGATCACGCTCGCCGTCTACATTGTGCCGGTCATCGTGCTTGCCGCCTGCTAC       660
I  T  W  I  T  L  A  V  Y  I  V  P  V  I  V  L  A  A  C  Y       220

GGCCTTATCAGCTTCAAGATCTGGCAGAACTTACGGCTCAAGACGGAGGCGGCGGCGGCA       720
G  L  I  S  F  K  I  W  Q  N  L  R  L  K  T  E  A  A  A  A       240

GAGGCTGCCGCGGGGGCTGAGGGCGCGGCGGCAGACTGCGCGGGGCGCGCGGCTCTGGCC       780
E  A  A  A  G  A  E  G  A  A  A  D  C  A  G  R  A  A  L  A       260

CGCGTCAGCAACGTCAAGCTCATCTCTAAGGCTAAGATCCGCACGGTCAAGATGACCTTC       840
R  V  S  N  V  K  L  I  S  K  A  K  I  R  T  V  K  M  T  F       280

ATCGTCGTGCTGGCCTTCATCGTGTGCTGGACGCCATTCTTTTTCGTGCAGATGTGGAGT       900
I  V  V  L  A  F  I  V  C  W  T  P  F  F  F  V  Q  M  W  S       300

GTCTGGGATGCCGATGCGCCCAAGGAAGCCTCGGCTTTCATCATCGCCATGCTTCTGGCC       960
V  W  D  A  D  A  P  K  E  A  S  A  F  I  I  A  M  L  L  A       320

AGCCTCAACAGCTGCTGCAACCCCTGGATCTACATGCTCTTCACGGGCCACCTCTTCCAA      1020
S  L  N  S  C  C  N  P  W  I  Y  M  L  F  T  G  H  L  F  Q       340

GACCTTGTGCAGCGCTTCCTCTGCTGCTCATTCCGCCGCCTGAAAGGCAGCCAGCCTGGG      1080
D  L  V  Q  R  F  L  C  C  S  F  R  R  L  K  G  S  Q  P  G       360

GAGACGAGCGTCAGCAAAAAGATCCATTCGTACACCTTTGTCCTGAGTCGGCACAGCTCC      1140
E  T  S  V  S  K  K  I  H  S  Y  T  F  V  L  S  R  H  S  S       380

AGCCAGAGAAGCTGCTCGCAGCCATCCACGGTGTGA                              1176
S  Q  R  S  C  S  Q  P  S  T  V  *                                391
```

Fig 3.6 Nucleotide and deduced amino acid sequence of sheep endometrial OTR cDNA

```
                ♦       ♦           ♦          __________ I __________
  sheep  MEGAFAANWS AEAVNGSAAP PGTEGNRTAG PPQRNEALAR VEVAVLSLIL FLALSGNACV   60
 sheep1  ---------- ---------- ---------- ---------- ---------- ----------   60
 cattle  ---------- ---------- ---------- ---------- ------C--- ----------   60
    pig  ---VL----- -----S---- -EA------- ---------- ------C--- ----------   60
  human  ----L----- ---A-A---- --A------- --R------- ------C--- L---------   60
    rat  ---TP----- V-LDL--GV- --E------- ---------- ------C--- ----------   60

         _____         _______ II _______                                _
  sheep  LLALRTTRHK HSRLFFFMKH LSIADLAVAV FQVLPQLLWD ITFRFYGPDL LCRLVKYLQV  120
 sheep1  ---------- ---------- ---------- ---------- ---------- ----------  120
 cattle  ---------- ---------- ------V--- ---------- ---------- ----------  120
    pig  ---------- ---------- ------V--- ---------- ---------- ----------  120
  human  --------Q- ---------- ------V--- ---------- ---------- ----------  120
    rat  ---------- ---------- ---R--V--- ---------- ---------- ----------  120

         _______ III _______                      ______ VI ______
  sheep  VGMFASTYLL LLMSLDRCLA ICQPLRSLRR RTDRLAVLAT WLGCLVASAP QVHIFSLREV  180
 sheep1  ---------- ---------- ---------- ---------- ---------- ----------  180
 cattle  ---------- ---------- --------S- --------V- ---------- ----------  180
    pig  ---------- ---------- ------A--- PA-------- ---------- ----------  180
  human  ---------- ---------- ---------- ---------- ---------- ----------  180
    rat  ---------- ---------- ---------- --------G- ---------- ----------  180

                               _______ V _______             •
  sheep  ADGVFDCWAV FIQPWGPKAY ITWITLAVYI VPVIVLAACY GLISFKIWQN LRLKTEAAAA  240
 sheep1  ---------- ---------- ---------- ---------- ---------- ----------  240
 cattle  ---------- ---------- ---------- -------T-- ---------- -----A----  240
    pig  ---------- ---------- ---------- ---------- ---------- -----A-E--  240
  human  ---------- ---------- ---------- -------T-- ---------- -----A---.  239
    rat  ---------- ---------- V--------- ---------- ---------- -----A---.  239

                                 •     •      •  ________ VI ________
  sheep  EAAAGAEGAA ADCAGRAALA RVSNVKLISK AKIRTVKMTF IVVLAFIVCW TPFFFVQMWS  300
 sheep1  --S------- ---------- ---------- ---------- ---------- -----K----  300
 cattle  --------E- --W----I-- ---------- ---------- ---------- ----------  300
    pig  --I--T---- -GSR------ ---S------ ---------- -I-------- ----------  300
  human  .--EAP---- -GDG--V--- ---S------ ---------- -I-------- ----------  298
    rat  .--.AEGND- -GG------- ---S------ ---------- -I-------- ----------  297

                    ________ VII ________
  sheep  VWDADAPKEA SAFIIAMLLA SLNSCCNPWI YMLFTGHLFQ DLVQRFLCCS FRRLKGSQPG  360
 sheep1  ---------- ---------- ---------- ---------- ---------- --------L-  360
 cattle  ---------- -P-------- ---------- ---------- E--------- -------R--  360
    pig  ---------- ---------- ---------- ---------H E--------- SSH--T-R--  360
  human  ----N----- -----V---- ---------- ---------H E--------- ASY---RRL-  358
    rat  ---VN----- ---------- ---------- ---------H E-----F--- A-Y----R--  357

                            •
  sheep  ETSVSKKIHS YTFVLSRHSS SQRSCSQPST V                                  391
 sheep1  ----T----- ---------- ---------- -                                  391
 cattle  -------SN- S-----QY-- ---R------ L                                  391
    pig  -------SN- S-----Q--- --K---.... .                                  386
  human  ---A---SN- SS----HR-- ---------- A                                  389
    rat  -------SN- S------R-- ---------S A                                  388
```

Fig. 3.7 Alignment showing similarities and differences of the putative OTR amino acid sequence between sheep (present work), sheep1 (Riley *et al.*, 1995), cattle (Bathgate *et al.*, 1995), pig (Gorbulev *et al.* 1993), human (Kimura *et al.*, 1992) and rat (Rozen *et al.*, 1995). Putatived N-linked glycosylation sites (♦) and phosphorylation sites (•) are shown above the sequences. Transmembrane domains are indicated above the sequences with Roman numerals (I-VII). – indicates identical amino acids.

```
3utra  .......... .......... .......... ...TGACTGA TCGGACCAGG ACTGCCCTCC  1200
3utrb  ---------- ---------- ---------- ---------- ---------- --C-------  1200
3utrd  ---------- ---------- ---------- ---------- ---------- --C-------  1200
3utrc  ---------- ---------- ---------- ---------- --A-G----- --C-------  1200
sheep1                                    ------- ----C-AG-- ...-------  1197

3utra  TTGGCCTCGA CTGTGCGGCA GC.GACAGTC TGGCTCTTGG TGGCTGTGTA CATGTGTGT.  1260
3utrb  -------T-- ---------- ---------- ---------- ---------- --A-------  1260
3utrd  ---------- ---------- ---------- ---------- ---------- ----------  1260
3utrc  -----T---- -------A-- ---------- ---------- ---------- ---------A  1260
sheep1 -----T---- -------A-- --A------- ---------- ---------- ---------A  1257

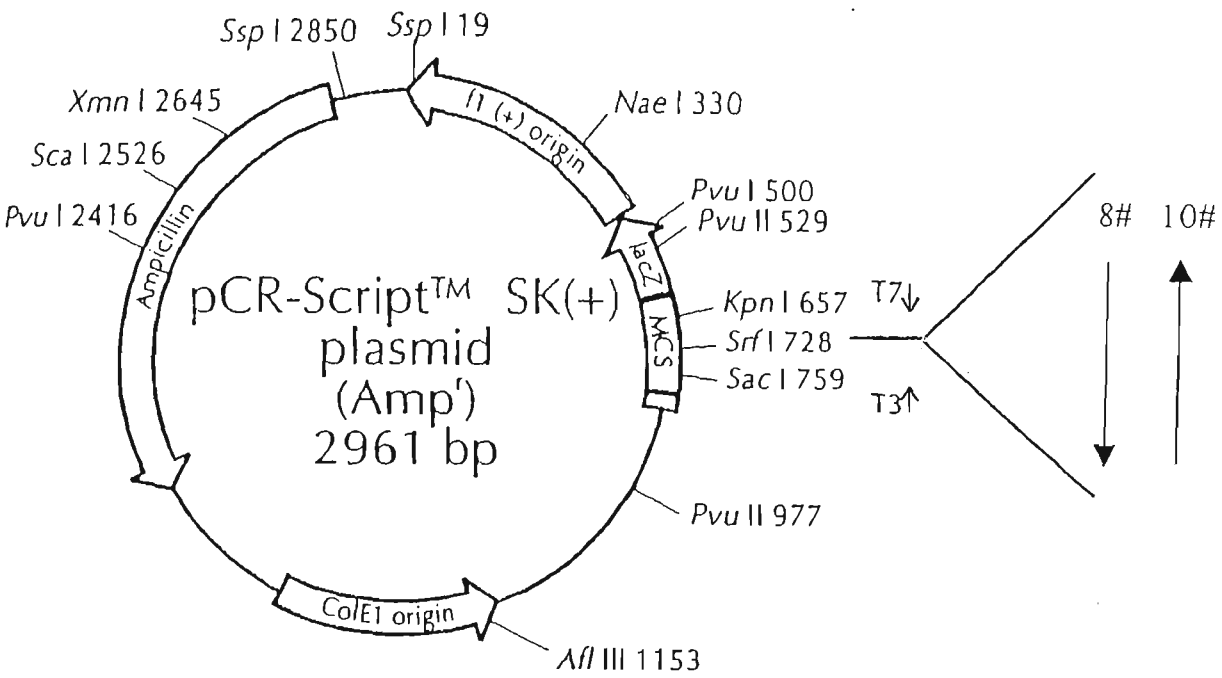
3utra  .AAGGTACCT TCCAGTTTGT ACCCCCCTGC ACCTTGGGCA GCTGGAGTTG GGTGGGGAAG  1320
3utrb  ---------- ---------- ---------- ---------- ---------- ----------  1320
3utrd  ---------- ---------- ---------- ---------- ---------- ----------  1320
3utrc  C--------- ---------- ---------- ---------- ---------- ----------  1320
sheep1 C--------- ---------- -.-------- ---------- ----..AG-T --GT------  1314

3utra  CAACCCCCAC GGGGAAGATG GTAGGGTGAC TCAACCATCA AGCCAAGCCC CAGATCTTCC  1380
3utrb  ---------- ---------- ---------- ---------- ---------- ----------  1380
3utrd  ---------- ---------- ---------- ---------- ---------- ----------  1380
3utrc  ---------- ---------- ---------- ---------- ---------- ----------  1380
sheep1 ------A-.. .--------- ---------- ---------- ---------- ---------T  1371

3utra  GTGAGCCCCC ATGGGTACTC CTGCCCTGAC CCCACTGCTG CCTTCCTTTA ACAGTAAGTC  1440
3utrb  ---------- ---------- ---------- ---------- ---------- ----------  1440
3utrd  ---------- ---------- ---------- ---------- ---------- ----------  1440
3utrc  ---------- ---------- ---------- ---------- ---------- ----------  1440
sheep1 ---.-G---- ---------- ---------- ---------- ---------- ----------  1430

3utra  CCCTACATAA GAATGAGTTC TGTTCTGAGA AAAAAAAA.. .......... ..........  1500
3utrb  ---------- ---------- ---------- GC------AA ---------- ----------  1500
3utrd  ---------- ---------- ---------- GC-CGTTTGT AAGTCTCATT TGTTAGTAAA  1500
3utrc  ---------- ---------- -----C--A- --------A- ---------- ----------  1500
sheep1 ---------- ---------- ---------- --------AA AAAAAAAAA- ----------  1490
```

Fig 3.8 Sequence comparison of 3' untranslated region between present work and published work. 3utra,b,c and d, sequence of 3' untranslated region for cloned RACE products 600A, 600B, 600C and 1300D, respectively. sheep1, sequence of 3' untranslated region for published one (Riley *et al.*, 1995).



Restriction enzyme map of RT-PCR product (fragment 1)

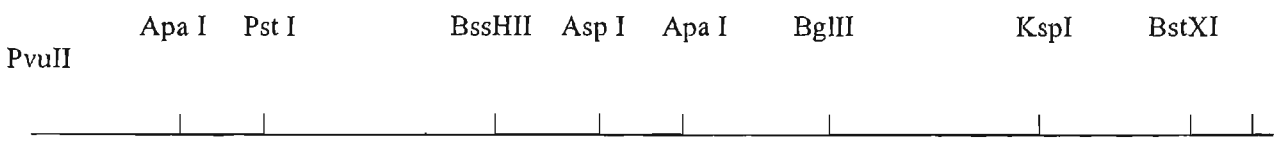


Fig 3.9 Circular map of the pCR-Script ™ SK(+) cloning vector. The pCR-Script ™ SK(+) cloning vector was designed by incorporating a *Srf* I site into the polylinker region of the pBluescript II SK(+) phagemid. → indicates the orientation of cloned RT-PCR products, from 5'end to 3'end. Restriction enzyme sites of RT-PCR products that were cloned into the vector are indicated.

Chapter 4 mRNA editing of OTR: A new mechanism for gene regulation

4.1 Introduction

The increase of uterine oxytocin receptor (OTR) concentrations over the late luteal phase of the estrous cycle is thought to play an important role in regulating the time of luteolysis (Flint *et al.*,1994). In non-pregnant ewes, OTRs first develop in the luminal epithelium on day 14 of the estrous cycle, extending to the superficial and deep glands and caruncular stroma at estrus, declining again by day 2 (Ayad *et al.*, 1991; Wallace *et al.*, 1991; Stevenson *et al.*, 1994). As mentioned in section 1.5.1, the OTRs are involved in luteal regression during the estrous cycle through their roles in stimulating uterine prostaglandin F2α (PGF2α) release. However, many studies have shown that the concentration of OTRs measured in endometrial tissue do not correlate well with oxytocin-induced PGF2α release (Sheldrick and Flint, 1985; Silvia *et al.*, 1991; Lau *et al.*, 1992; Mirando *et al.*, 1993). Research has shown that OTRs were required only on the luminal epithelium to evoke a full luteolytic response and these constitute only a small percentage of the total potential endometrial OTR population (Wathes and Lamming, 1995). A large proportion of the endometrial OTR population in caruncular stroma and deep glands does not form until luteolysis is complete and the formation and activity of these receptors is probably oestrogen dependent. The development of OTRs in the luminal epithelium is an important biological event necessary to achieve a maximal PGF2α response to an oxytocin challenge (Wathes and Lamming, 1995). The regulation of this OTR population is different from that in the rest of the endometrium such as caruncular stroma and deep glands. However, the basis for the differences in regulatory mechanisms remains unclear.

Oxytocin-stimulated PGF2α release is associated with an increase in phospholipase C (PLC, section 1.3.3) and phospholipase A_2 (PLA_2, section 1.3.4) (Mirando *et al.*, 1993; Silvia and Raw, 1993; Lee and Silvia, 1994; Silvia *et al.*, 1994). However, the relationship between PGF2α release and an increase in PLC and PLA_2 has again shown discrepancies (Silvia and Raw, 1993; Lee and Silvia, 1994). Graf *et al.* (1999)

have shown that cytosolic phospholipase A_2 ($cPLA_2$) enzymic activity is not a limiting factor for PGF2α synthesis during the time of the development of uterine secretory responsiveness to oxytocin. It is still not clear what factors influence the regulation of the OT signaling pathway to the initial coupling of oxytocin to its receptor.

Several investigators have indicated the possibility of OTR subtypes by functionally analyzing the receptor (El *et al.*, 1990; Chan *et al.*, 1993). Using two OT antagonists, it was found that myometrial uterotonic OTRs and endometrial/decidual PG-releasing OTRs were two distinct subtypes and could be differentiated (Chan *et al.*, 1993). The possibility of OTR subtypes was also suggested by the researchers who used either *in situ* hybridization or Northern blots to determine OTR mRNA expression in endometrium and myometrium (Stevenson *et al.*, 1994; Larcher *et al.*, 1995). However, strong evidence of OTR subtypes at the molecular level still appears to be lacking.

Northern hybridization has shown four transcripts for OTR in ovine uterine endometrium and myometrium (see Chapter 5). However, the sequence information of those transcripts is still lacking. There is also a question over whether OTR subtypes exist in the uterus?

The aim of this work was to identify the transcripts for OTR in the ovine uterine endometrium, and to characterise the sequence differences among these transcripts.

4.2 Materials and Methods

4.2.1 Sample preparation

Merino ewes used for this experiment were housed in the animal house, Department of Physiology, Monash University. The ewes were killed on day 15 of the estrous cycle by intravenous administration of a lethal dose of sodium pentobarbitone (for details, see Chapter 2, section 2.1.1 and 2.1.5). Uterine tissues from two ewes on day 15 of the estrous cycle were collected and uterine endometrium was dissected and cut

into small pieces (about 1 cm x 0.5 cm). The procedure was as described in Chapter 2 (section 2.6.1, method 1). Endometrial tissues were homogenized in TRIZOL reagent (50 mg tissue/ml) to prepare total RNA samples. The procedure is as described in Chapter 2, section 2.2.1. Endometrial RNA from two ewes on day 15 of estrous cycle was used for mRNA purification (PolyATtract mRNA Isolation System, Promega) following the protocol described in Chapter 2, section 2.2.2.

4.2.2 Rapid Amplification of cDNA Ends (RACE)

Two different 3'RACE system (Life Technologies and Clontech) were used to amplify the 3'end of the OTR cDNA. The details of the procedure for amplification is described in Chapter 2, section 2.6.

4.2.2.1 Primer designation

For both 3'RACE systems, an oligo-dT adapter primer and a gene-specific primer are required. A gene-specific primer (PH) (residues 892-915 for OTR cDNA) was chosen from a region of known exon sequences of OTR cDNA. The sequences of oligo-dT adapter primers and a gene-specific primer (PH) were listed in Chapter 3, Table 3.1. PH was required to have balanced AT/GC ratio and the length of 22-24 bases for the reaction. BLAST search (Non-redundant GenBank+EMBL +DDBJ+PDB sequences) showed that this part of OTR cDNA sequence (positions 892-1176) has low homology with the other genes, such as vasopressin receptor genes, especially in forward primer region.

4.2.2.2 3'RACE

In the first 3'RACE system (Life Technologies), first strand cDNA was synthesized by using 0.2μg of mRNA from sheep uterine endometrium collected at day 15 of the estrous cycle and PCR amplification was performed as described in Chapter 2, section 2.6.1.

In the second 3'RACE system, 100 units of MMLV reverse transcriptase (CLONTECH) was used with 10 pmol of anchored primer for the first strand cDNA synthesis and PCR amplification was performed as described in Chapter 2, section 2.6.2.

4.2.3 Cloning of RACE products

pGEM-T cloning vector system (Promega), pCR-Script Amp SK(+) Cloning vector (Stratagene) and the Original TA Cloning Kit (Invitrogen) were used in this experiment. For details, see Chapter 2, section 2.9.3.

4.2.4 DNA sequencing

A ABI Prism Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer) was used for direct sequencing of RACE products and a ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer) was used for sequencing of cloned RACE products following the protocol supplied by the manufacturer to run cycle sequencing For details, see Chapter 2, section 2.10.3.

4.2.5 Analysis of sequences for OTR cDNA

Analysis of the structures of the deduced protein for OTR, especially in comparison with edited and unedited amino acid sequence in the C-terminus, were carried out using the Australia National Genomic Information Service (ANGIS) on WebANGIS interface (Gaeta 1997) (Chapter 2, section 2.11.5).

4.3 Results

4.3.1 Amplification of 3' untranslated region by the 3'RACE systems

Two bands were generated by both 3'RACE systems, a major band size of 600 bp and a minor band size of 1300 bp (Fig. 4.1). However, the size of these two bands that were present in the TAE-agarose gel was slightly higher than expected. A

possible reason for this result could be that the loading marker and samples behaved slightly differently in the presence of different salt concentrations, which may have affected the rate of migration.

4.3.2 Direct sequencing of RACE products

Direct sequencing of the 600 bp band resulted in the expected sequence in the coding region, up to the stop codon. However, the sequence appeared to be divergent and consisted of mixed or various bases at certain sites after the stop codon (Fig 4.2). Similar results were obtained inspite of several independent experiments. In order to obtain a more definitive result for the sequence variations in these reactions, the RACE products were then cloned.

4.3.3 Cloning of RACE products

To clone the RACE products, three cloning systems were used. They were: pCR-Script Amp SK(+) Cloning vector (Stratagene), the Original TA Cloning Kit (Invitrogen) and pGEM-T cloning vector system (Promega). All the protocols were followed according to the supplier's instructions. However, only pGEM-T cloning vector system (Promega) worked successfully. When pCR-Script Amp SK(+) Cloning vector (Stratagene) was used for cloning of RACE products, very low yield and no positive colonies were obtained. When the Original TA Cloning Kit (Invitrogen) was used, the recombinant plasmid DNA only contained the coding regions of the RACE products. The part of the 3'untranslated region that starts immediately after the stop codon was missing. The reason for these failures remains a mystery. The map of cloned RACE products is shown in Fig 4.3a and the restriction enzyme map of cloned 3'RACE products 600A, 600B, 600C and 1300D is shown in Fig 4.3b.

4.3.4 Sequencing of cloned RACE products

Three types of clones (arbitrarily named 600A, 600B and 600C) were derived from the 600 bp of RACE products. The 1300 bp of RACE products was also cloned. Because of technical difficulties, only one positive clone was obtained (arbitrarily

named 1300D). Sequence analysis indicated fourteen potential polymorphic sites with respect to the sequence given in Fig 4.4, four in coding region and the others in 3'untranslated region. These consisted of T/C conversions, A/G conversions, T/A conversion and an AC insertion/deletion. The nucleotide differences in four types of the clones are listed in Table 4.1.

The T/C conversion in positions 995 and 1103 replaced the codon AT*G-995 (Met-331) to AC*G-995 (Thr-331), and the codon AT*C-1103 (Ile-368) to AC*C-1103 (Thr-368). The A/G conversion in position 1032 encoding CAG* (Gln-343) appeared in place of CAA* (Gln) codon and T/C conversion in position 1128 encoding AGC* (Ser-376) appeared in place of AGT* (Ser) codon, respectively, which did not cause a change in its amino acid.

Table 4.1. Listing of polymorphic sites in cloned RACE products 600A, 600B, 600C and 1300D

Name of clone	Nucleotide base in positions:													
	995	1032	1103	1128	1183	1185	1193	1206	1208	1218	1252	1259	1465	1468
600A	T	G	T	T	G	A	T	C	C	G	T	--	T	G
600B	T	G	T	T	G	A	C	C	T	G	A	--	T	G
600C	T	G	T	C	A	G	C	T	C	A	T	AC	C	A
1300D	C	A	C	T	G	A	C	C	C	G	T	--	T	G

4.4 Discussion

In comparison with the sequence obtained from clones 600A, 600B, 600C and 1300D, the results did show the presence of sequence variations in both coding regions and untranslated regions in the 3'RACE products. Since the OTR gene is a single-copy gene per haploid genome and is well conserved among vertebrate species (Simmons *et al.*,1995; Bathgate *et al.*, 1995), the occurrence of polymorphic sequences in the RACE generated products from OTR mRNA suggest the presence of post-transcriptional modifications, probably through mRNA editing. The possibility of PCR generated error cannot be excluded although this possibility is unlikely due to the reproducibility of the results obtained from several independent reactions. Several pieces of evidence support the view that the sequence differences found are not artifactual. Firstly, the forward gene specific primer region (PH) has almost no similarity to any other gene sequences available in the database (Non-redundant GenBank+EMBL+DDBJ+PDB sequences), thus minimizing the possibility of other unrelated cDNA being generated in the RACE reactions. Secondly, the chances that PCR-introduced errors would occur randomly over the length of the sequence are not high. However, our observations strongly indicate a high degree of heterogeneity at specific positions in the 3'untanslated region, suggesting that the mRNA transcripts are divergent at these bases.

RNA editing has been widely studied. It can be divided into two major classifications, conversion/substitution editing and insertion/deletion editing (Benne, 1996). Four classes of mRNA editing have been reported in mammalian cells (Ashkenas, 1997). They are: cytosine (C) to uracil (U); adenine (A) to inosine/guanine (I)/(G); U to C and U to A. It is known that C to U conversion is due to the deamination of C performed by enzyme apo B-editing catalytic subunit (APOBEC-1), and A to I (G) conversion is due to the deamination of A performed by enzyme double-stranded RNA adenosine deaminases (DRADAs). The mechanisms for the other classes of editing are not known, but may involve base substitution rather than covalent modification (Ashkenas, 1997).

mRNA editing was first reported for serotonin-2C receptor, a G-protein-coupled receptor (Burns *et al.*, 1997). Sequence analysis of cDNA that was isolated from dissected brain regions, has indicated the tissue-specific expression of seven major serotonin-2C receptor isoforms encoded by eleven distinct RNA species. Multiple transcripts encoding serotonin-2C receptor undergo RNA editing by the transition of genomically encoded adenosine to inosine, which performed by two separable double-stranded RNA adenosine deaminases: dsRNA-specific adenosine deaminase (dsRAD) and dsRNA-specific editase1 (RED1). Discrepancies between the site selectivity resulted from differences in enzyme activity or from requirements for additional regulatory factors (Burns *et al.* (1997). These investigators found that region-specific editing of serotonin-2C receptor transcripts generated multiple receptor isoforms, the fully edited one had a lower efficiency to intracellular signal transduction. This reduced receptor-G protein coupling may alter receptor structure or receptor phosphorylation at the introduced serine moiety.

As discussed in Chapter 3, no sequence element which determined the site of cleavage and polyadenylation in pre-mRNA (Edwalds-Gilbert *et al.* 1997) was found in the 600A, 600B and 600C clones. However, there were two mRNA editing sites, which occurred just prior to the poly A site in a stem loop in 600A, 600B and 600C (Fig 4.4), thus indicating that these sites may cooperate with alternative polyadenylation site selection.

Based on the polymorphic sequence results, it seems that mRNA editing not only involved conversion/substitution editing, but also insertion/deletion editing. This is the first report that mRNA editing exists in the 3'untranslated region. The similar finding was reported in *C18orf1* mRNA in 5'untranslated region (Yoshikawa *et al.*, 1998). The significance of mRNA editing in untranslated region remains unclear. As gene expression can be regulated through alternative splicing of pre-mRNAs and alternative polyadenylation site choice (Curtis *et al.*, 1995; Ross, 1995), mRNA editing in untranslated region might be another post transcriptional modification to coordinate with polyadenylation site selection.

In comparison with the determined sequences for the clones 600A, 600B and 600C, the clone 1300D shown the presence of AC*G-995 (Thr-331) and AC*C-1103 (Thr-

368) codons. In addition, AT*G-995 (Met-331) and AT*C-1103 (Ile-368) codons were found in the corresponding positions in the clones 600A, 600B and 600C (Fig 4.4). Analysis of the secondary structure of putative amino acid sequence (Plotstructure, ANGIS) indicated that the sequence with two edited sites (clone 1300D) would lack an α-helices at the C-terminal (position 358 to 371) in comparison with the unedited one (Fig.4.5). The vicinity of this α-helix at the G-protein coupling site indicates possible alterations to protein structure of the edited protein. The presence of serine and threonine in the carboxyl terminus are well known to be potential phosphorylation sites which have been identified as being important mechanism for the regulation of G-protein coupling (Hargrave *et al.* 1982, Probst *et al.* 1992). The present results thus suggest that editing of OTR mRNAs may affect G-protein coupling. A current study has provided evidence that the proximal portion of the carboxyl terminus of the OTR is required for coupling to G-protein Gq (Hoare *et al.*, 1999). It was reported that deletion of 51 amino acid residues from the carboxyl terminus of OTR resulted in reduced affinity for OT. It has been shown that the truncated receptor was uncoupled from Gq-mediated pathways when OT failed to increase phospholipase A activity or activate protein kinase C. However, the truncated receptor was coupled to Gi, which mediated intracellular Ca^{2+} by protein tyrosine kinase pathways (Hoare *et al.*, 1999). This result indicated different G-protein coupling pathway occurred due to the structure change in the C-terminal. In view of the finding of mRNA editing in this study, it is suggested that different G-protein coupling pathways could be regulated through mRNA editing.

In previous studies both Stevenson *et al.* (1994) and Wathes and Lamming, (1995) conducted autoradiography experiments using an oxytocin antagonist (^{125}I-labelled OTA) to localize OTR binding sites and *in situ* hybridization to detect the location of mRNA encoding OTR in the ovine uterus. The results they obtained indicated that OTRs first appeared in the luminal epithelium at the onset of luteolysis, spread to the superficial glands during luteolysis and then to the caruncular stroma and deep glands at estrous (Wathes and Lamming, 1995). The authors also indicated that only epithelial receptors were needed to achieve a maximal $PGF_{2\alpha}$ response to an oxytocin challenge, but those receptors on the caruncular stroma and deep glands needed positive regulation and appeared dependent on an oestrogenic environment. These

results may be able to be explained by mRNA editing. In this study two different type of mRNAs were found: edited and unedited. Edited mRNAs encoding OTR induced two Thr sites, which might be the potential phosphorylation site in carboxyl terminus and these composed a small percentage of the entire mRNA population. When this information is combined with the present results, it is hypothesized that edited OTR mRNAs might correspond to the OTRs in the luminal epithelium. The edited sites co-relating with the potential phosphorylation site might activate G-protein coupling, and those OTRs might respond to an oxytocin challenge. Unedited OTR mRNAs might correspond to the OTRs in the caruncular stroma and deep gland and respond to oestrogen stimulation. Further work needs to be undertaken by determining whether mRNA editing occurred in the rest of the coding region at different stages of the estrous cycle and from different types of cells. The work conducted during this study has provided some evidence to suggest that the difference in OTR population in the endometrium may be due to mRNA editing and the existence of oxytocin receptor subtypes, may result from mRNA editing. It is also suggested that the regulation could occur at the site of G-protein coupling.

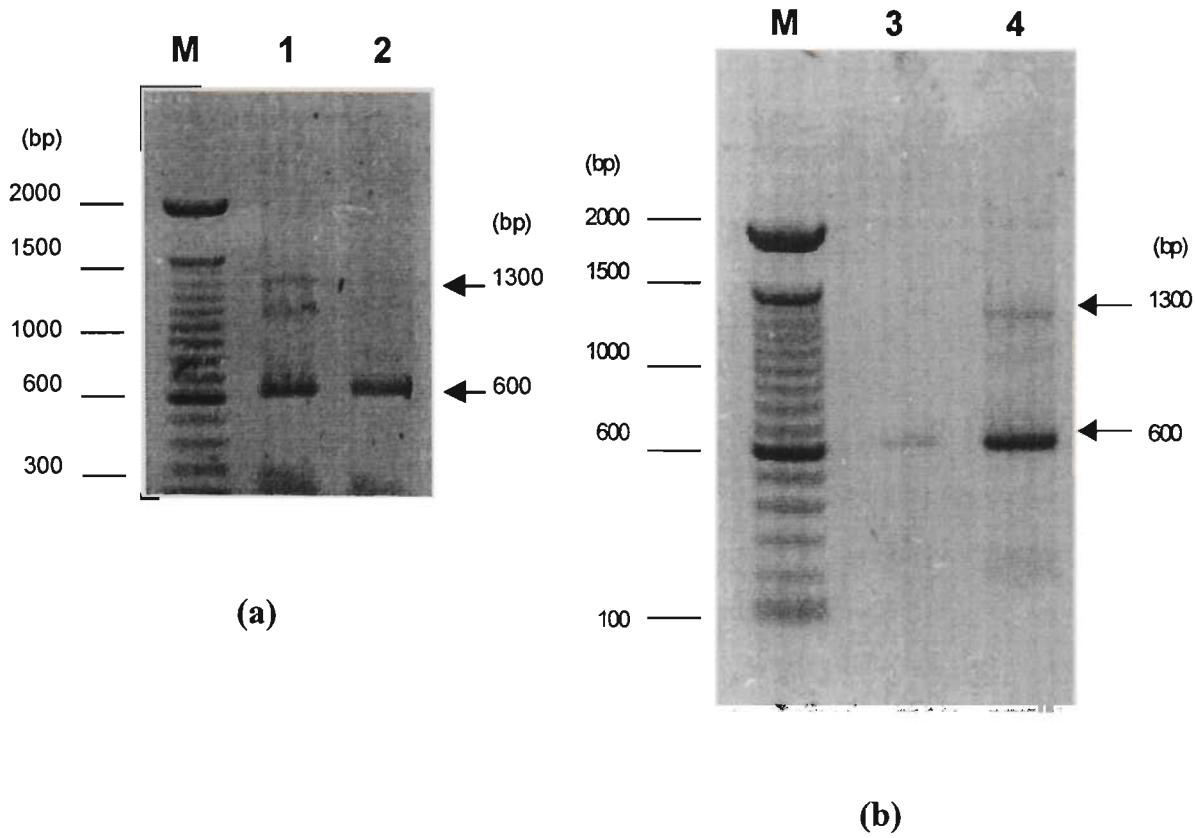


Fig. 4.1 The products generated from 3'RACE systems. (a), reactions from the first 3'RACE system (Life Technologies). (b), reactions from the second 3'RACE system (Clontech). M, 100bp DNA ladder Markers (Life Technologies). Lane 1, 2, 3 and 4 are represented independent reactions (5 μl/Lane). → indicates the size of products.

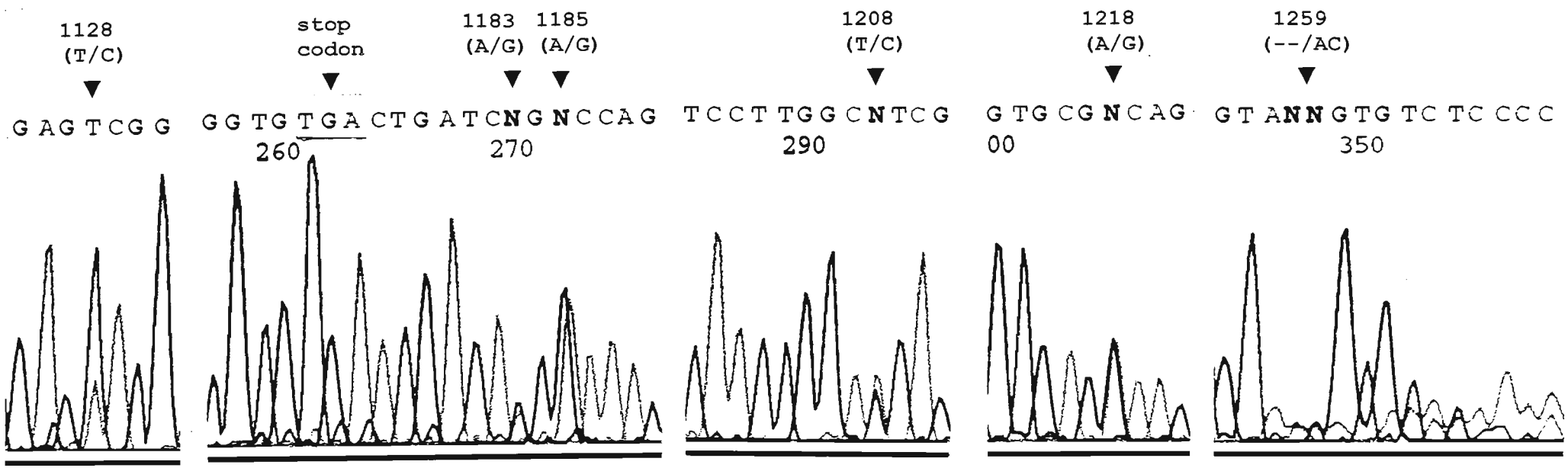


Fig 4.2 Direct sequencing data for the 600bp band. ▼: indicates the mixed bases at certain sites. In position 1128, there is a C peak appeared in the peak of T. In positions 1183,1185,1208 and 1218, N displays the mixed site as indicated. In position 1259, after two N sites, the background of the sequencing figure became higher, indicating that the different products existed in the sequence reaction.

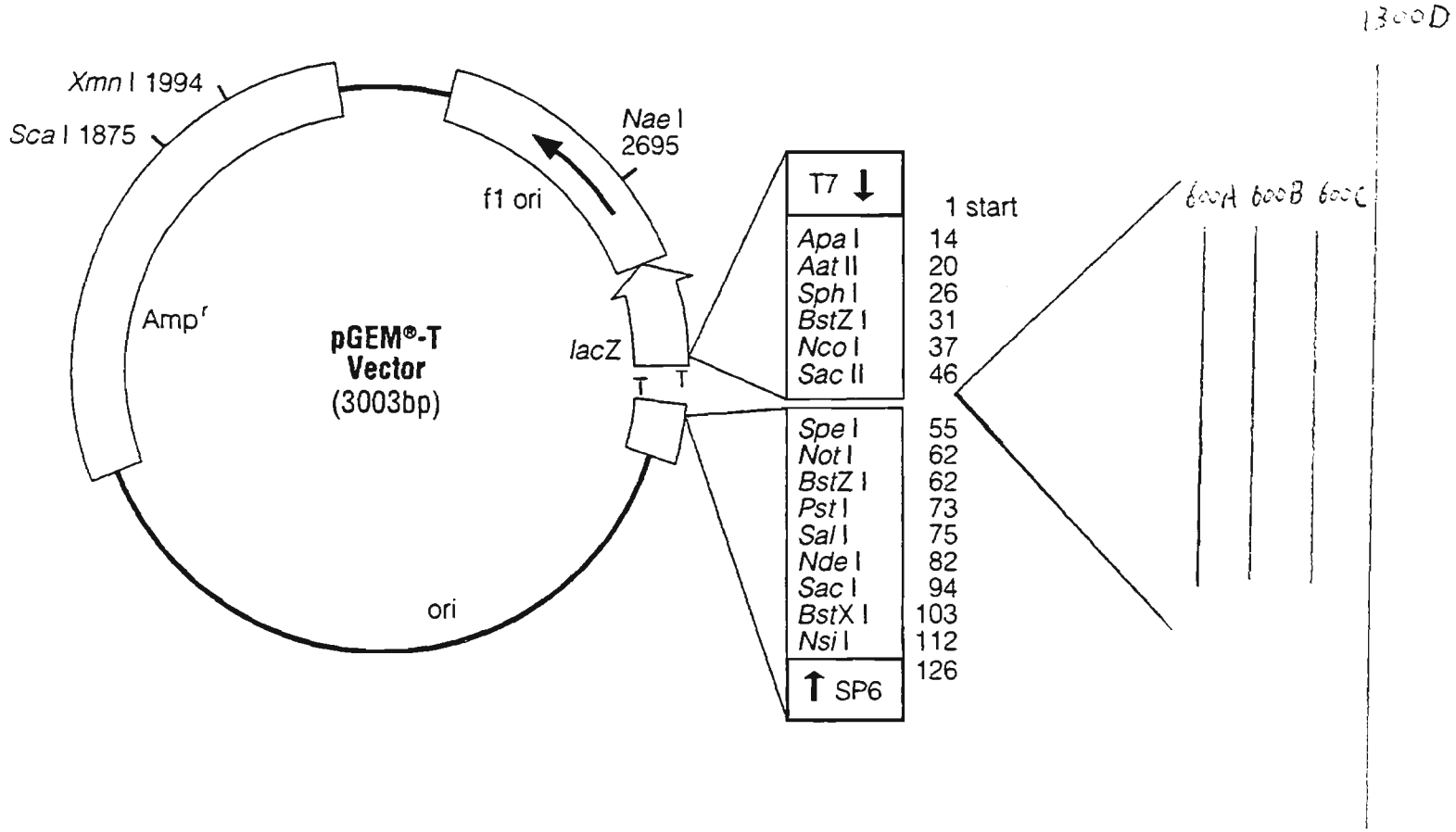


Fig 4.3a The cloning map of cloned 3'RACE products.

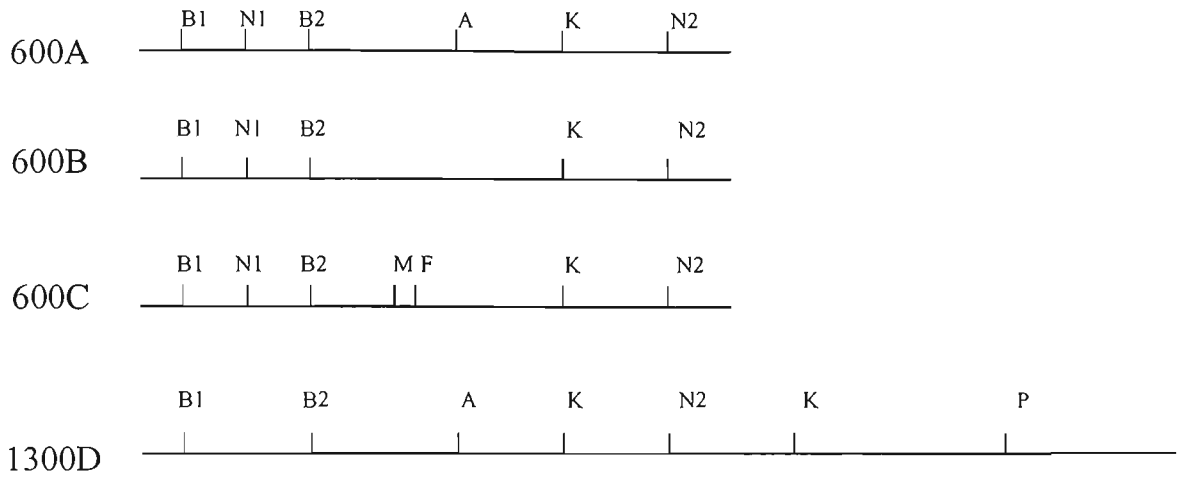


Fig 4.3b Restriction enzyme maps for the cloned 3'RACE products 600A, 600B, 600C and 1300D. Six bases or more restriction enzyme sites are chosen. B1: BstXI, N1: NspI, B2: BglI, M: MroNI, F: FbaI, A: AflIII, K: KpnI, N2: NcoI, P:PaeI.

```
600A  ATGTGGAGTG TCTGGGATGC CGATGCGCCC AAGGAAGCCT CGGCTTTCAT CATCGCCATG CTTCTGGCCA   961
600B  ---------- ---------- ---------- ---------- ---------- ---------- ----------   961
1200D ---------- ---------- ---------- ---------- ---------- ---------- ----------   961
600C  ---------- ---------- ---------- ---------- ---------- ---------- ----------   961
                                        (Met)                                (Gln)
600A  GCCTCAACAG CTGCTGCAAC CCCTGGATCT ACATGCTCTT CACGGGCCAC CTCTTCCAAG ACCTTGTGCA  1031
600B  ---------- ---------- ---------- ---------- ---------- ---------- ----------  1031
1200D ---------- ---------- ---------- --ACG----- ---------- ---------- ----------  1031
600C  ---------- ---------- ---------- ---------- ---------- ---------- ----------  1031
                                        (Thr)
600A  GCGCTTCCTC TGCTGCTCAT TCCGCCGCCT GAAAGGCAGC CAGCCTGGGG AGACGAGCGT CAGCAAAAAG  1101
600B  ---------- ---------- ---------- ---------- ---------- ---------- ----------  1101
1200D A--------- ---------- ---------- ---------- ---------- ---------- ----------  1101
600C  ---------- ---------- ---------- ---------- ---------- ---------- ----------  1101
     (Ile)                    (Ser)
600A  ATCCATTCGT ACACCTTTGT CCTGAGTCGG CACAGCTCCA GCCAGAGAAG CTGCTCGCAG CCATCCACGG  1171
600B  ---------- ---------- ---------- ---------- ---------- ---------- ----------  1171
1200D ACC------- ---------- ---------- ---------- ---------- ---------- ----------  1171
600C  ---------- ---------- ------C--- ---------- ---------- ---------- ----------  1171
     (Thr)
        *
600A  TGTGA CTGAT CGGACCAGGA CTGCCCTCCT TGGCCTCGAC TGTGCGGCAG CGACAGTCTG GCTCTTGGTG  1241
600B  ----- ----- ---------- -C-------- ------T--- ---------- ---------- ----------  1241
1200D ----- ----- ---------- -C-------- ---------- ---------- ---------- ----------  1241
600C  ----- ----- -A-G------ -C-------- ----T----- ------A--- ---------- ----------  1241

600A  GCTGTGTACA TGTGTGT..A AGGTACCTTC CAGTTTGTAC CCCCCTGCAC CTTGGGCAGC TGGAGTTGGG  1309
600B  ---------- A--------- ---------- ---------- ---------- ---------- ----------  1309
1200D ---------- ---------- ---------- ---------- ---------- ---------- ----------  1309
600C  ---------- -------AC- ---------- ---------- ---------- ---------- ----------  1311

600A  TGGGGAAGCA ACCCCCACGG GGAAGATGGT AGGGTGACTC AACCATCAAG CCAAGCCCCA GATCTTCCGT  1379
600B  ---------- ---------- ---------- ---------- ---------- ---------- ----------  1379
1200D ---------- ---------- ---------- ---------- ---------- ---------- ----------  1379
600C  ---------- ---------- ---------- ---------- ---------- ---------- ----------  1381

600A  GAGCCCCCAT GGGTACTCCT GCCCTGACCC CACTGCTGCC TTCCTTTAAC AGTAAGTCCC CTACATAAGA  1449
600B  ---------- ---------- ---------- ---------- ---------- ---------- ----------  1449
1200D ---------- ---------- ---------- ---------- ---------- ---------- ----------  1449
600C  ---------- ---------- ---------- ---------- ---------- ---------- ----------  1451

600A  ATGAGTTCTG TTCTGAGAAA AAAAAA.... .......... .......... .......... ..........  1475
600B  ---------- --------GC ------AA-- ---------- ---------- ---------- ----------  1477
1200D ---------- --------GC -CGTTTGTAA GTCTCATTTG TTAGTAAATC CAACAAAGTT AGCCTGGGTA  1519
600C  ---------- ---C--A--- ------A--- ---------- ---------- ---------- ----------  1478

1200D CCCAGCTCAC ACAGTCAGCT ATATAGTGCT GTACTAGTAT ACCATACAGT ATAGCGCTGT ATGCAGCCCT  1589
1200D GCACTGTGCT AGTATGTTAC TGTGCTGAGT AGGCTTGTAA GACTTTTCAC GCAAGTAATA CATACAAGGC  1659
1200D AGAAAGTAAA ACACTTCAGT CTTAGAGTAC CTTGAAAAGT ACACCTGGAA TACAGGGGCA GAAAGTCAAA  1729
1200D CATTTCAGTC TTATAGTAGA GTACCTTGAA AAGTACACCT GAAATACAAG GGCTGGCATG CCTCTTTGAA  1899
1200D AGTTTGCAAC TAGAAGGTTT GTGTGTCGAG GACTTACTGT ATTCTGGGAT CTTGAGAAAG GTAGTAAGTA  1869
1200D TAAGATTGGT GACCAGGTGG GTACGGAAGC CTAGTGTTCT GAGCTTGGGG TGAGGAATAG GGCCTTGCTG  1939
1200D GTGGTGGTGC TAATATCCTC CCGGCCTCAA AGAGCTTTTG CCTTTGAGTG GACTGGTGCT AGCATCCTTG  2009
1200D AGCACCAACT TATCTGGTGG TGGCCCTAGA GCAGGGGATT CTCTTAGGAG ACTGTCTGGC ACAGGCAGCC  2079
1200D AAGTAAAACT GAGGTGAAAA ATGTTATGCT AATACTTCAG AAGCATTTGG GAAAGAAAAA GGAAATAAAA  2149
1200D GACATCTAAA TTTGTAAAAA AAAAAAAAAA AAAAAAAAAA TATT                              2193
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Fig 4.4 A comparison of nucleotide sequences of 3'RACE-PCR products. OTR cDNA sequences in 3' untranslated region are shown. Editing site of nucleotide sequence is coloured and encoded amino acid is indicated in ( ). Stop codon is underline and indicated by *. The poly A signal AATAAA is highlighted and the secondary structures are underline. - indicates identical base. The stem loop areas are underline.

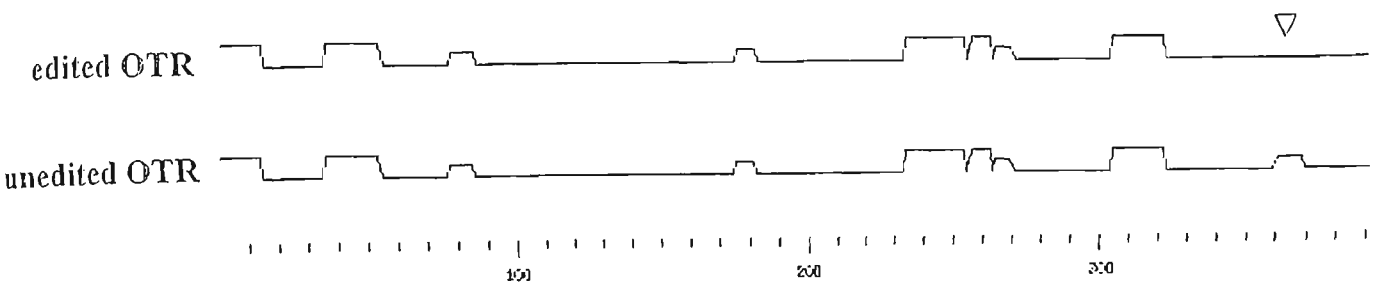


Fig 4.5 A comparison of α-helices structure for OTR putative amino acid sequence. One α-helix structure is abolished in the edited OTR indicated by ∇. Numbers indicate the amino acid positions.

Chapter 5 Determination of OTR mRNA expression in the uterus, corpus luteum and pituitary gland during the estrous cycle and at parturition in sheep

5.1 Introduction

Uterine endometrial OTRs play an important role in the control of PGF2α secretion during the estrous cycle (Chapter 1, section 1.4.2.3). The uterine OTRs bind to OT from the peripheral circulation to stimulate PGF2α release in the endometrium over days 15-17 of the estrous cycle (Roberts *et al.*, 1976; Flint *et al.*, 1994). Previous studies in sheep have shown that the uterus is not responsive to oxytocin in terms of PGF2α secretion until OTR concentrations rise on day 14-15 of post-oestrus (Sheldrick and Flint, 1985; Stewart *et al.*, 1993). Quantitation of OTR mRNA expression in ovine endometrium by *in situ* hybridization during estrous cycle have shown that OTR mRNAs are present at low concentrations during the luteal phase, increasing on day 14 of the estrous cycle before reaching a maximum on the day of estrus (Stewart *et al.*, 1994).

It is well established that oxytocin is synthesized and released from corpus luteum and neurohypophysis by a similar mechanism. Post-translational processing and storage of oxytocin in secretory granules of corpus luteum in sheep has been reported (Sheldrick and Flint, 1989). However, whether OTRs are present in the ovine corpus luteum is still uncertain. Early reports have indicated that specific oxytocin binding could not be demonstrated in luteal tissue during the estrous cycle, although specific oxytocin binding has been found in the corpora lutea of pregnant ewes (Sernia *et al.*, 1989). OTR gene expression has not been detected in the ovine corpus luteum during the estrous cycle using Northern blot analysis (Flint *et al.*, 1995).

The corpus luteum is thought to be the major source of OT that is involved in stimulating uterine PGF2α secretion. Whether OT from the pituitary gland is involved in this event remains to be elucidated. OTRs have been reported previously in

pituitary gland in rat (Breton *et al.*, 1995). However, the detection of OTR expression in corpus luteum and in pituitary gland during the estrous cycle in sheep has not been reported.

As it indicated in Chapter 2, OT has a dual action in the uterus during labor: a uterotonic action on myometrial cells to stimulate uterine contractions (Challis and Lye, 1994) and is also involved in releasing of PGF2α from endometrial cells to promote myometrial gap junction formation in preparation for labor (Fuchs *et al.*, 1982). OT acts on both endometrial and myometrial cells through specific OTRs. These studies indicate that OT has different functions depending on the target tissue.

Previous studies have indicated that there are a number of transcripts of the OTR gene in human, rat and cattle (Kimura *et al.*, 1992; Rozen *et al.*, 1995; Bathgate *et al.*, 1995). However, little information is available on the nature and the role of these transcripts.

The purpose of this study was to determine OTR mRNA expression in the endometrium, corpus luteum and pituitary gland during the estrous cycle, and to detect the OTR expression in endometrium, myometrium, cervix and cotyledons at labor.

5.2 Materials and methods

5.2.1 Sample collection

Twenty Merino ewes were allocated at random to 5 groups of 4 ewes on day 0, 2, 7, 12 and 15 of the estrous cycle. Estrus was determined as described in Chapter 2, section 2.1.1. At each of the times indicated above, the ewes were killed by intravenous administration of a lethal dose of sodium pentobarbitone prior to the sample collection. Samples of endometrium, pituitary gland and corpus luteum were then collected as described in Chapter 2, section 2.6.1, Method 1.

Parturition was monitored by EMG activity as described in Chapter 2, section 2.1.2. Samples were then collected at different stage of labor as described in Chapter 2, section 2.6.1, Method 2. Samples at labor were collected from sheep induced by betamethasone or injected with saline as described in Chapter 2, section 2.1.3.

5.2.2 RNA isolation

The guanidium thiocyanate extraction method was used to prepare the total RNA samples at parturition, and TRIZOL Reagent (GIBCOBRL, Life Technologies) was used to prepared the total RNA samples from endometrium, pituitary gland and corpus luteum during the estrous cycle. For details, see Chapter 2, section 2.2.1.

5.2.3 Northern blot analysis

5.2.3.1 Samples

Three series of samples were prepared for Northern blot. These included:

1. Endometrium and myometrium samples obtained from sheep induced to deliver by an i.m. injection of 6 mg betamethasone given on day 133 of gestation. Endometrium and myometrium samples obtained from additional sheep injected with saline on day 132 of gestation. Myometrium and cotyledon samples collected from a sheep that delivered on day 150 of gestation during the early stages of labor. Myometrium, cervix, cotyledons and full uterus samples collected from a sheep on day 149 of gestation during the late stages of labor.

2. Endometrium samples collected on day 0 (n=4), 2 (n=4), 7 (n=4), 12 (n=4) and 15 (n=4) of the estrous cycle.

3. Pituitary gland samples collected on day 0 (n=4), 2 (n=4), 7 (n=4), 12 (n=4) and 15 (n=4) of the estrous cycle and corpus luteum on day 12 of the estrous cycle.

5.2.3.2 Probes

For the detection of OTR mRNA levels at labor by Northern blot analysis, Probe 1 was used (Chapter 2, section 2.7.4). The DNA fragments were digested by *EcoRI* and *BamHI* from plasmid DNA (Chapter 2, section 2.9.1) and were purified using GENECLEAN Kit (Bio101 Inc) (Chapter 2, section 2.3.5.3). This probe was labeled by random priming (GIGAPRIME DNA Labeling Kit, Bresatec) with α-[P^{32}]dATP (Chapter 2, section 2.7.5).

Probe 3 was used for the detection of OTR mRNA expression levels in uterine endometrium, pituitary gland and corpus luteum during the estrous cycle. This probe was generated by RT-PCR with primers PC and PD (Chapter 2 section 2.7.4) and was purified directly by RT-PCR using Wizard PCR Preps DNA purification System (Promega) (Chapter 2, section 2.3.5.2). The probe was also labeled by random priming (GIGAPRIME DNA Labeling Kit, Bresatec) with α-[P^{32}]dCTP (Chapter 2, section 2.7.5).

Probe 4 was used as a control for the detection of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA (Chapter 2, section 2.7.4). This probe was digested by EcoRI and ApaI from plasmid DNA and was purified from the agarose gel using Wizard PCR Preps DNA purification System (Promega) (Chapter 2, section 2.3.5.2). It was labeled using a nick translation system (Promega) with α-[P^{32}]dATP (Chapter 2, section 2.7.5).

5.2.3.3 Northern hybridization

Northern hybridization was adapted from Sambrook *et al.* (1989) and was described in Chapter 2, section 2.7 in details. In brief, total RNA samples (10 μg/lane for the samples collected during the estrous cycle or 15 μg/lane for the samples collected at parturition) were denatured in sample buffer and separated by electrophoresis on 1% agarose-formaldehyde gels (Chapter 2, section 2.7.1). After electrophoresis, RNA was transferred to a nylon membrane by capillary blotting overnight (Chapter 2, section 2.7.2 and 2.7.3). Pre-hybridization (4h) and hybridization (overnight) was

carried out at 65°C in the same buffer with the probe labeled by the isotope (Chapter 2, section 2.7.7). Hybridization signals were detected by exposing membranes to X-ray film, with intensifying screens, at -80°C for 2-3 days (Chapter 2, section 2.7.8).

5.2.4 Quantitation of mRNA expression

Autoradiograph signals were quantitated as described in Chapter 2, section 2.13. The size of OTR transcripts were determined as described in Chapter 2, section 2.14. One Way ANOVA (Chapter 2, section 2.12.1) was used to analyze the significant difference between the endometrium samples or between the pituitary gland samples collected on days 0, 2, 7, 12 and 15 of the estrous cycle.

5.3 Results

5.3.1 Analysis of transcripts for OTR gene expression in uterus, pituitary gland and corpus luteum

Northern hybridization analysis of OTR gene expression from uterine samples at parturition and at estrus revealed four different hybridizing transcripts: two major bands were present at approximately 1.5kb and 5.6kb, and the other two minor bands were present at approximately 2.1kb and 3.3kb (Fig.5.1, 5.2). In contrast, a single faint band was present at approximately 4.3kb in the pituitary gland (Fig.5.3). A transcript of a size similar to that in pituitary gland was also expressed in corpus luteum (Fig.5.3).

5.3.2 Analysis of OTR gene expression in uterine tissues at parturition

As showed in Fig.5.1, the OTR gene is highly expressed at day 133 of gestation in endometrium and myometrium samples following an injection of betamethasone, but was not detectable in the same tissues collected from sheep given an injection of saline. Betamethasone is known to induce the labor, while saline was used as the negative control. The pattern of mRNA expressed in endometrium and myometrium samples

showed the same four transcripts. To estimate sample loading, a photograph of the ethidium bromide stained agarose gel was provided and the density of ribosomal RNA bands from each sample was shown (Fig 5.1). The highly expressed OTR mRNA signal was also present in the myometrium on day 149 of gestation during the late stages of labor, and on day 150 of gestation during the early stages of labor, and in full uterus samples on day 149 of gestation during the late stages of labor. However, it was not detectable in the cervix and in the cotyledons.

5.3.3 Analysis of OTR mRNA expression level in the endometrium and pituitary gland during the estrous cycle

Northern hybridization results showed differences in the hybridization signal in endometrial samples taken during the estrous cycle, and appeared as a faint band in the pituitary gland samples throughout the cycle (Fig5.2 and 5.3). The OTR mRNA expression levels on days 0, 2, 7, 12 and 15 of the estrous cycle in uterine endometrium and pituitary gland were calculated using densitometric scanning. The GAPDH hybridization signal was used as a control to normalize total RNA loading. Data calculated from each sample are listed in Tables 5.1 and 5.2.

Table 5.1 Relative values of OTR mRNA expression from each endometrial sample collected from sheep during the estrous cycle

Day	Day 0	Day 2	Day 7	Day 12	Day 15
Sample 1	4.60	0.49	0.15	0.25	1.31
Sample 2	6.20	0.62	0.18	0.03	2.50
Sample 3	2.60	0.81	0.32	0.88	2.20
Sample 4	1.60	0.38	0.24	0.12	IC*
means±SEM	2.85±1.25	0.57±0.18	0.22±0.07	0.31±0.38	2.00±0.61

* Insufficient capacity to run sample on gel.

Table 5.2 Relative values of OTR mRNA expression from each pituitary gland sample during the estrous cycle

Day	Day 0	Day 2	Day 7	Day 12	Day 15
Sample	0.14	0.19	0.17	0.20	0.18
	0.30	0.25	0.19	0.15	0.28
	0.13	0.10	0.16	0.18	0.13
	IC*	0.19	0.22	IC*	IC*
means±SEM	0.18±0.09	0.18±0.06	0.18±0.02	0.17±0.02	0.19±0.07

Data are present as the ratios of signals obtained by hybridization with an OTR probe versus the signals obtained with GAPDH probe.

The main 5.6kb band in each of the endometrial samples was scanned as the hybridization signal to determine the OTR mRNA expression levels. The level of OTR mRNA expression in uterine endometrium was low on days 2, 7 and 12 (day 2 vs. day 7, P=0.939), showed a significant increase on day 15 (day 15 vs. day 7, P=0.023; day 15 vs. day 12, P=0.033), and reached a peak at oestrus (day 0 vs. day 2, P=0.002; day 0 vs. day 7, P<0.001; day 0 vs. day 12, P<0.001) and declined again on day 2 (Fig.5.4). The level of OTR mRNA expression remained relatively constant in the pituitary gland throughout the estrous cycle and there was no significant difference between the samples (Fig.5.4) (P=0.997).

5.3 Discussion

The observed changes in OTR mRNA expression in ovine endometrium during the estrous cycle in this study were consistent with the result reported by Stewart *et al.* (1993) showing an increase in endometrial OTR mRNA level over days13-15 of the oestrous cycle. The level of OTR mRNA expression in the pituitary gland during the estrous cycle did not show any significant changes and remained stable at a relatively low level. The findings of the presence of OTR in the corpus lutuem is consistent with observations of other investigators in the cow, human and pig (Fuchs *et al.*, 1990; Kimura *et al.*, 1992; Pitzel and Wuttke, 1993). Although the function of OTR in the corpus lutuem is still uncertain, it has been suggested that the presence of OTR in corpus lutuem may play a role in luteal regression in the ewe (Sernia *et al.*, 1989).

Alternatively, it has been suggested by Sernia *et al.* (1989) that ovarian oxytocin may have a regulatory role in progesterone secretion from the corpus luteum.

The OTR transcripts in both the corpus luteum and the pituitary gland were similar in size at around 4.3 kb. The corpus luteum and the pituitary gland are known to be involved in the storage and release of oxytocin. The uterine endometrium and myometrium, which are the target sites of OT action, share the same pattern of OTR transcripts, but these differ from those found in the corpus luteum and pituitary gland. Similar tissue-specific OTR transcripts have been found in human (Kimura *et al.,* 1992) and rat (Rozen *et al.*, 1995; Breton *et al.*, 1995). In humans, a single OTR transcript of size 3.6 kb was found in mammary gland, and a single 4.4 kb OTR transcript was found in endometrium and myometrium, but multiple OTR transcripts of size 6.1 kb, 4.4 kb, 3.6 kb and 2.9 kb were reported in the myometrium at parturition (Kimura *et al.,* 1992). In the rat, three OTR transcripts at sizes of 6.7 kb, 4.8 kb and 2.9 kb were found in uterus at term, and two OTR transcripts at sizes of 6.7 kb and 4.8 kb were found in the pituitary gland (Rozen *et al.*, 1995; Breton *et al.*, 1995). In the cytoplasm, it is thought the poly A tail on the mRNA plays a role in stability and translatability (Curtis *et al.,* 1995; Ross, 1995; Sachs and Wahle, 1993). If the different forms of mRNA have different stability or translatability, then the use of alternative poly A sites can positively or negatively impact on the final amount of protein per unit precursor RNA transcribed. A particular splicing or polyadenylation site can serve as an important control point for gene expression in a tissue or developmental stage-specific manner (Edwalds-Gilbert *et al.,* 1997). On the basis of these findings it is suggested from our results, that the pattern of OTR gene expression is not only tissue-specific, but also highly function-related.

Previous studies have reported OTR mRNA expression in the uterus at term using Northern hybridization. A strong hybridization signal was observed in the myometrium, but not in the endometrium at term in the rat (Larcher *et al.*, 1995). It was suggested that the differences in results between endometrium and myometrium might be due to two different subtypes (Larcher *et al.*, 1995). A similar suggestion was made by Stevenson *et al.* (1994), when they failed to detect a significant increase of OTR mRNA expression in myometrium, but a remarked rise in endometrial OTR mRNA expression at estrus in sheep. The tissue localization of OTR and OTR

mRNA expression have been observed in both endometrium and myometrium of pregnant sheep using *in situ* hybridisation and immunolocalisation (Wu *et al.*, 1996). The results in this Chapter support the view that the patten of transcripts of the OTR gene expressed in endometrium and myometrium are similar. This result also provides evidence supporting the functions of OT in sheep uterus since OT stimulates myometrial contraction in two different ways: a direct activation of OTR on myometrial cells, causing a resultant change in intercellular calcium concentration and an indirect stimulation of contraction by release of PGs from endometrium. The findings that OTR mRNA expression occurs in both endometrium and myometrium suggest the importance of OTR in the transduction of OT's effects on its target cells. In accordance with the findings of mRNA editing (Chapter 4), OTR subtypes may exist within endometrium, but small differences in nucleotide size cannot be distinguished by Northern hybridization.

Previous studies which determined the location and expression of OTR at parturition, using in situ hybridization, have shown that the concentration of OTR mRNA in the cross section of cotyledons in ewes was high in luminal epithelium (Wathes *et al.*, 1996). However, in this study the OTR mRNA signal was not detected in the cotyledon by Northern hybridization. It is possible that the population of OTR mRNA in the entire cotyledon tissue was low in comparison with those in endometrium and myometrium, which could offer an explanation as to why OTR mRNA expression was not detectable in cotyledons.

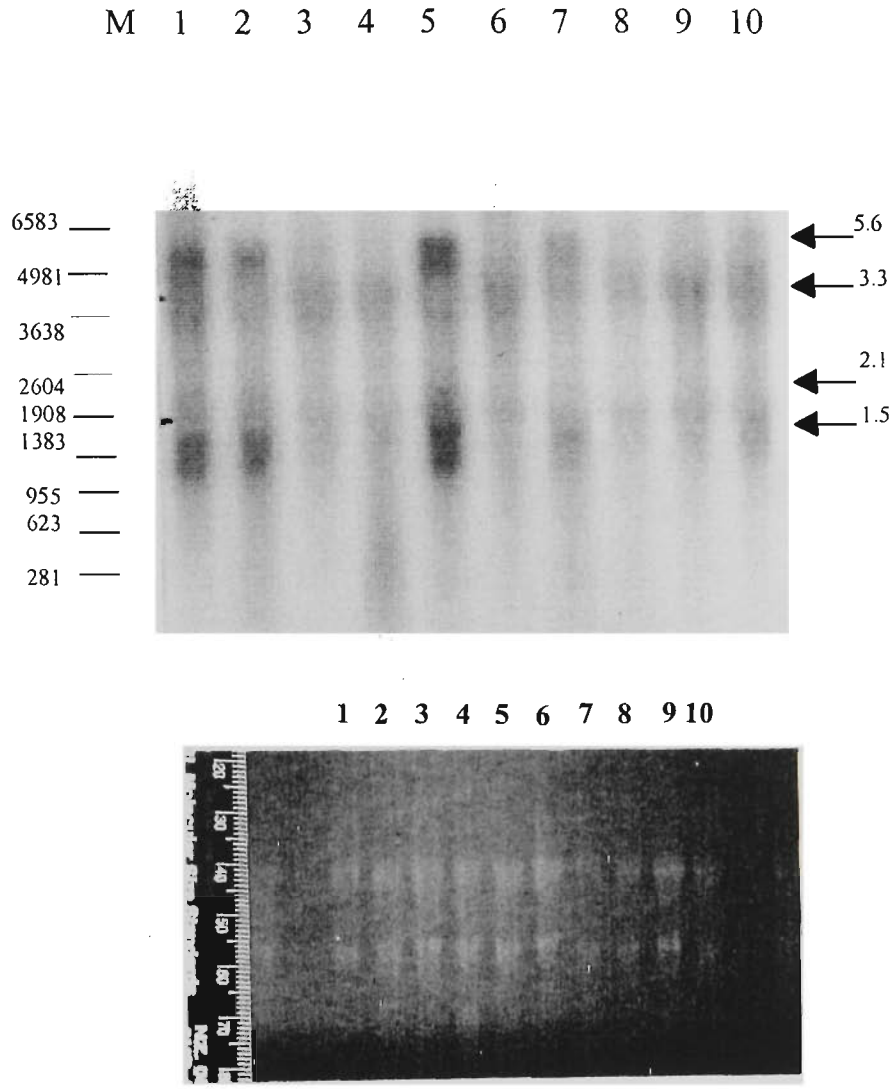


Fig. 5.1 Northern hybridization analysis of uterine OTR mRNA expression from uterine tissue at parturition in sheep. Lane 1 and 2: endometrium and myometrium, collected from sheep induced to delivery with 6 mg betamethasone on day 133 of gestation; Lane 3 and 4: endometrium and myometrium samples collected from a control sheep on day 132 of gestation injected with saline; Lane 5 and 6: myometrium and cotyledons samples from a sheep on day 150 of gestation; Lane 7, 8, 9 and 10: myometrium, cervix, cotyledons and full uterus samples from sheep on day 149 of gestation, respectively. 15 μg of total RNA was loaded per lane. Size of RNA molecular weight Markers (bp) (G319a, Promega) is indicated on the left. → indicates the size of OTR transcripts. Bottom panel: photograph of the ethidium bromide stained agarose gel, showing ribosomal RNA bands. Two dots on the left in top panel indicate the location of ribosomal RNA bands. The migration of ribosomal RNA bands are different to any of those OTR mRNA bands.

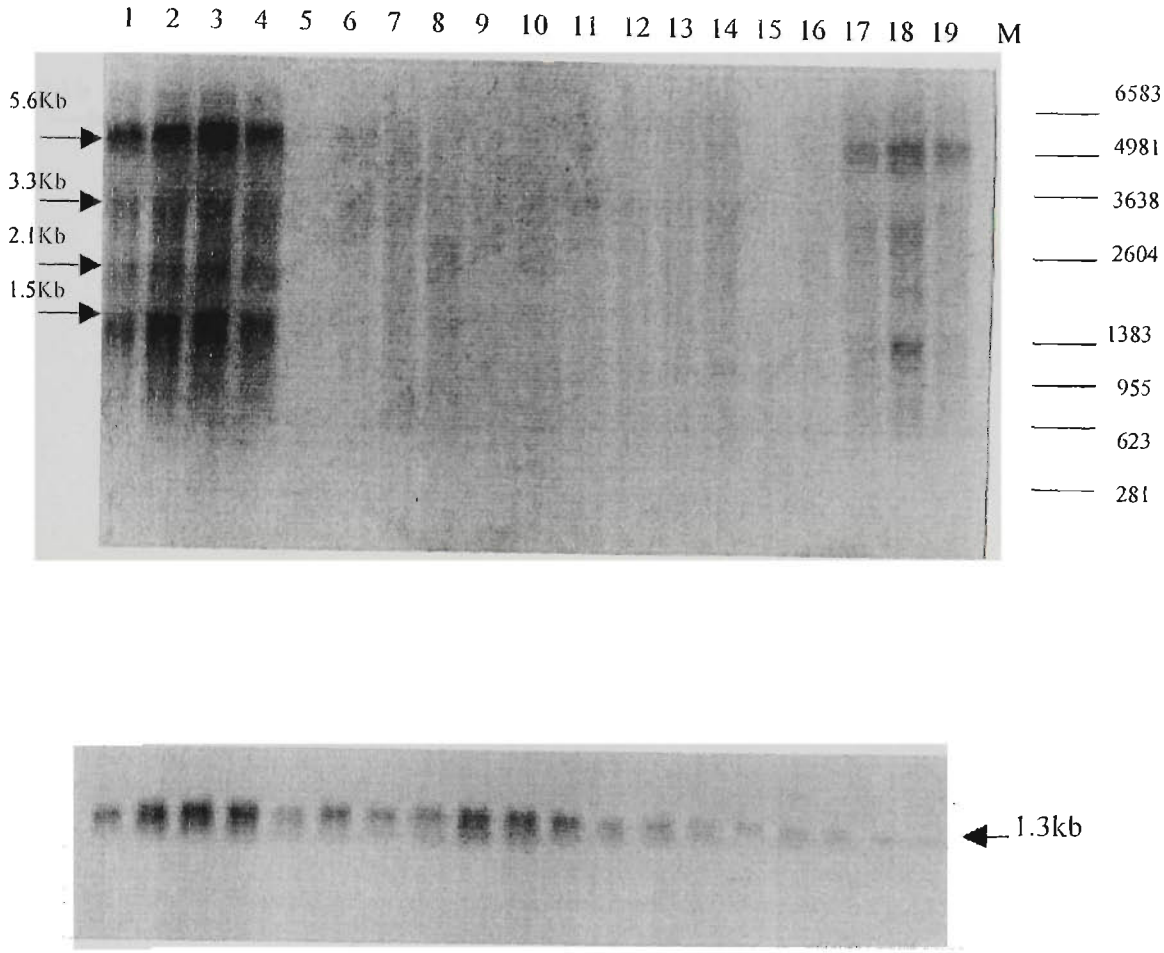


Fig. 5.2 Northern hybridization analysis of ovine endometrial OTR mRNA expression during the estrous cycle. Lane 1-4, day 0; Lane 5-8, day 2; Lane 9-12, day 7; Lane 13-16, day 12; Lane 17-19, day 15. 10 μg of total RNA was loaded in each lane. Size of RNA molecular weight Markers (bp) (G319a, Promega) is indicated on the right. Upper panel: Hybridisation with OTR cDNA. → indicates the size of OTR transcripts. Bottom panel: hybridisation with GAPDH cDNA. → indicates the size of GAPDH transcripts.

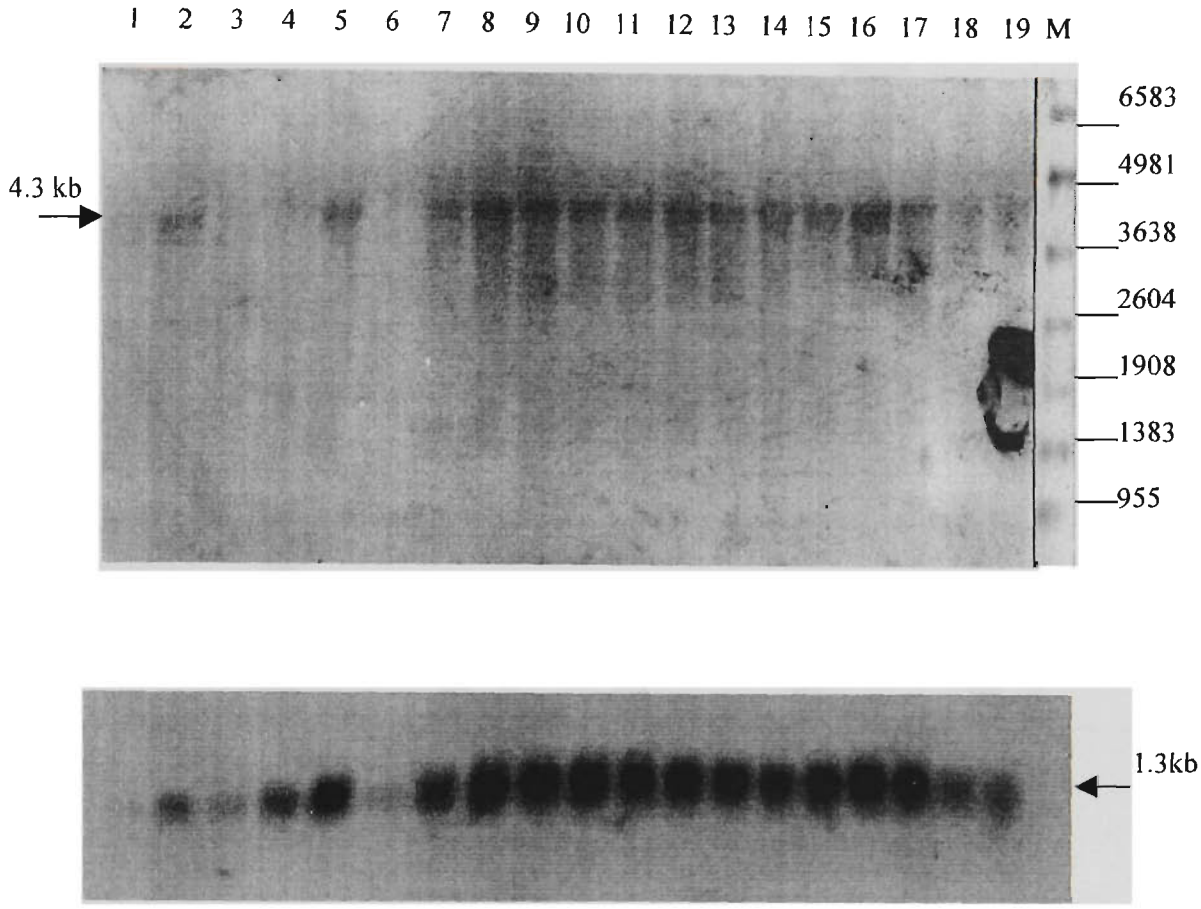


Fig. 5.3 Northern hybridization analysis of ovine pituitary gland OTR mRNA expression during the estrous cycle and detection of OTR gene expression in corpus luteum. Lane 1-17 pituitary gland samples. Lane 1-3, day 0; Lane 4-7, day 2; Lane 8-11, day 7; Lane 12-14, day 12; Lane 15-17, day 15. Lane 18-19 corpus luteum samples from two sheep on day 12 of the estrous cycle. 10 μg total RNA was loaded per lane. Size of RNA molecular weight markers (bp) (G319a, Promega) is indicated on the right. Upper panel: Hybridisation with OTR cDNA. → indicates the size of OTR transcript. Bottom panel: hybridisation with GAPDH cDNA. → indicates the size of GAPDH transcript.

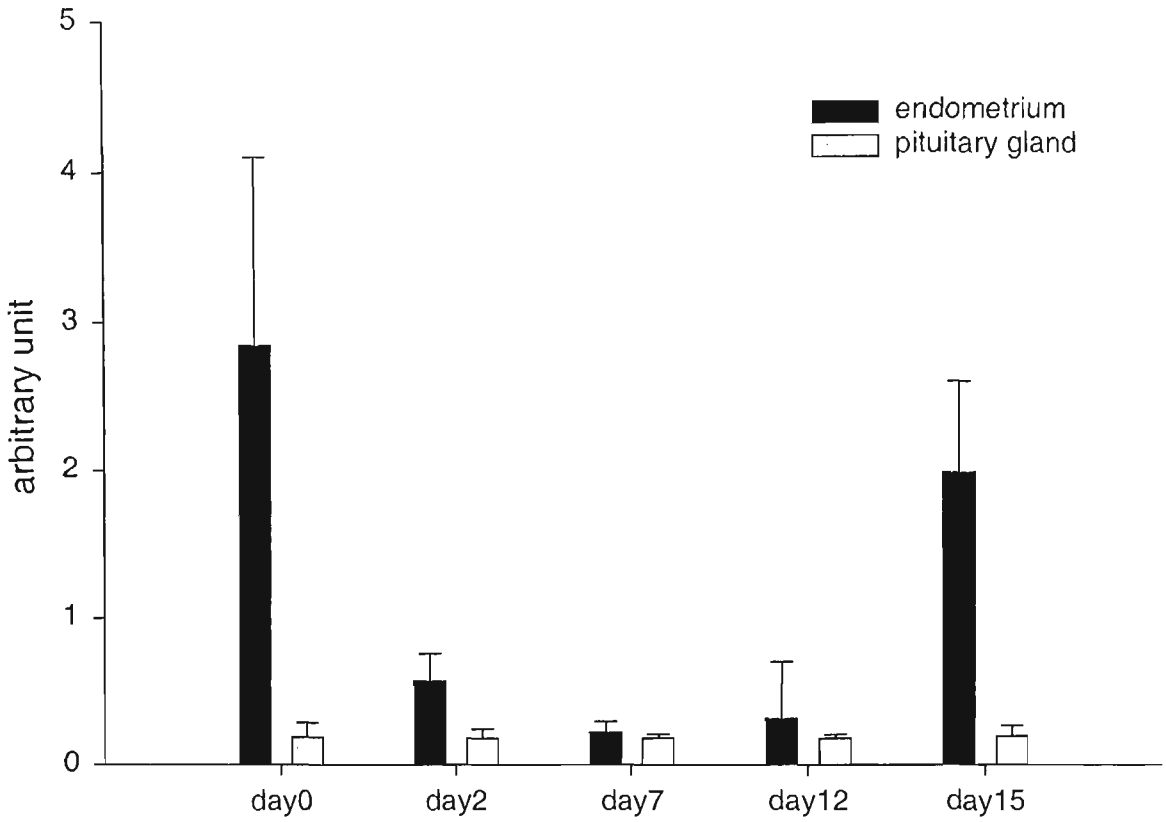


Fig. 5.4 The level of OTR mRNA expression in the endometrium (black bar) and pituitary gland (grey bar) during the estrous cycle in sheep. The signals for OTR mRNA and GAPDH mRNA on the autoradiographs were quantified by densitometry and expressed as arbitrary units for the ratio of OTR mRNA to GAPDH mRNA. Significant differences ($P<0.05$) between groups in the endometrium are represented by the different lettering. An increase in the level of OTR mRNA expression was shown on day 15 and day 0 compared to days 2, 7 and 12. There is no statistically significant difference in OTR mRNA expression between groups of pituitary gland samples ($P=0.997$). ^{a,b,c}Different scripts differ at $P< 0.05$.

Chapter 6 The role of oestrogen in the regulation of oxytocin receptor and prostaglandin synthetase gene expression

6.1 INTRODUCTION

The possible role of uterine oxytocin receptors (OTR) in regulating the timing of luteolysis is now well established. The pulsatile release of PGF2α from the uterus causing luteolysis (Goding, 1974) is triggered by oxytocin acting on OTR in the endometrium (Flint and Sheldrick, 1983; Bazer *et al.*, 1991) and mediated by steroid hormones and other factors (Flint *et al.*, 1994).

During the normal estrous cycle in sheep, the concentrations of OTR remain low during the luteal phase, increasing to a peak at oestrus and then declining again after estrus (Sheldrick and Flint, 1985). The increase of uterine OTR concentrations during the late luteal phase coincides with the maximum pulsatile release of PGF2α as measured by changes in the levels of the PGF metabolite, PGFM (Fairclough *et al.*, 1980). At this time, the circulating plasma concentrations of progesterone fall and estradiol concentrations rise (Sheldrick and Flint, 1985).

The uterine endometrium is the main site for PGF2α production. There are three key steps in the production of PGF2α. Firstly, there is an increase in the release of free arachidonic acid from phospholipid precursors. Secondly, there is a conversion of arachidonic acid into PGH2, and finally the conversion of PGH2 into PGF2α. The second step in the cascade is catalyzed by prostaglandin G/H synthase (PGHS). Two isoforms of PGHS have been identified: PGHS-1 and PGHS-2. PGHS-1 has been found in many tissues at basal amounts with generally little response to exogenous stimulation (O'Neill and Ford, 1993). PGHS-2 is present in low or undetectable amounts under normal physiological conditions and can be stimulated by hormones and growth factors (Herschman, 1994).

The steroid-treated ovariectomized ewe has been widely used as a model to determine the roles of oestrogen and progesterone in regulating the concentrations of OTR.

However, the role of steroids in the regulation of uterine OTR response is unclear, and both stimulatory and inhibitory effects have been reported as reviewed in chapter 1. In some instances, it seems that estradiol exerts a stimulatory effect on OTR regulation, and this occurs principally at the level of gene transcription (Mann and Lamming, 1994; Lamming and Mann, 1995). Progesterone has an inhibitory effect on OTR regulation after around day 10 of the estrous cycle, and the progesterone-induced oxytocin receptor down-regulation occurs via a different, perhaps non-genomic mechanism (Zingg *et al.*, 1995).

In previous studies reported by Sheldrick and Flint (1985) and Leavitt *et al.* (1985), the uterine OTR concentrations in the ovariectomised ewe increased after pretreatment with progesterone followed by an injection of estradiol or after continuously infusing with estradiol. In contrast, Vallet *et al.* (1990) showed that when ewes were given estradiol after a 12-day progesterone treatment, no further increase in OTR concentration occurred in comparison with ewes given the progesterone treatment alone. It was also reported that short-term treatment with estradiol resulted in a depression of OTR concentrations (Vallet *et al.*, 1990).

Previous studies have shown that the increase in the concentration of uterine OTRs was closely correlated with the uterine prostaglandin F response to an oxytocin challenge during the estrous cycle (Roberts *et al.*, 1976). However, many studies indicate that this correlation does not always occur. In ovariectomized ewes, the maximum OT-induced uterine prostaglandin F response occurred on day 14 of progesterone treatment, whereas the highest OTR concentration did not occur until day 16-17 of the estrous cycle (Lau *et al.*, 1992b). McCracken *et al.*, (1984) reported that estradiol-17β treatment can stimulate uterine synthesis and release of $PGF_{2\alpha}$ in ovariectomised ewes. In contrast, Vallet *et al.* (1990) found that estradiol-17β treatment of ovariectomised anestrous ewes had no effect on the OT-induced prostaglandin F response, but decreased uterine OTR concentrations.

Little work has been undertaken to study the effects of steroids on the regulation of uterine mRNA expression of OTR and PGHS. This study aimed to investigate the role of estradiol in regulating OTR mRNA and PGHS mRNA expression in

ovariectomized ewes that have been previously primed with exogenous progesterone and estradiol.

6.2 Materials and Methods

6.2.1 Treatment of animals

Forty Merino ewes (weight 40-58kg) were ovariectomized at least two months prior to this study. Ewes were allocated at random to 8 groups (n=5). One group (named PT) received no steroid treatment and remained housed throughout the treatment period. This group of ewes was slaughtered as control samples before steroid priming. The remaining 35 ewes (named AT) were treated with steroids over 23 days to mimic a natural estrous cycle (as described in Table 6.1a). Preparation of steroid injections is described in Chapter 2, section 2.1.4. On day 24, five of these ewes (samples designated as OC) were slaughtered as controls after steroid priming. Of the remaining 30 ewes, 15 were given estradiol in an oil solution and 15 were given oil solution only. At each of the times +3, +12, +48 hours after the last injection of progesterone, 5 ewes given estradiol injections (samples were named as 3T, 12T, 48T respectively based on the time of the injection) and 5 ewes given oil injections (samples were named as 3C, 12C, 48C respectively based on the time of the injection) were slaughtered for sample collection (as described in Table 6.1b).

Table 6.1a Dose and timing of steroid treatment in ovariectomized ewes

Group	n	Days	Injection time	Estradiol (μg)	Progesterone (mg)	Slaughter
PT	5	1-23		0	0	n=5
AT	35	1-4	0800h	0	12	
		5	1600h	5	0	
			2400h	10	0	
		6	0800h	20	0	
			1600h	10	0	
			2400h	5	0	
		7		0	0	
		8	0800h	0	0.5	
			2000h	0	0.63	
		9	0800h	0	0.75	
			2000h	0	1	
		10	0800h	0	1.25	
			2000h	0	1.5	
		11	0800h	0	2	
			2000h	0	2.5	
		12	0800h	0	3	
			2000h	0	4	
		13	0800h	0	5	
			2000h	0	6	
		14-22	0800h	0	12	
			2000h	0	12	
		23		0	0	

Table 6.1b Dose and timing of steroid treatment in ovariectomized ewes

Group	Injection time on day 24	Estradiol (μg)	oil	Progesterone (mg)	Slaughter number and time after injection
OC	0800h	0	0	0	n=5, T=0h
3T	0800h	50	0	0	n=5, T=3h
3C	0800h	0	+	0	n=5, T=3h
12T	0800h	50	0	0	n=5, T=12h
12C	0800h	0	+	0	n=5, T=12h
48T	0800h	50	0	0	n=5, T=48h
48C	0800h	0	+	0	n=5, T=48h

After euthanasia (chapter 2, section 2.1.5), the uteri were placed immediately on ice for processing. Endometrium was dissected from the uterine tissue, washed with cold PBS and stored (as described in chapter 2, section 2.1.6, method 1).

6.2.2 RNA isolation

Total RNA samples were isolated by the guanidium thiocyanate extraction method as described in chapter 2, section 2.2.1.1. Each total RNA sample was diluted (1:100) in DEPC-treated water to measure the absorbance at 260nm by spectrophotometer and the concentration of each sample was determined by the formula described in chapter 2, section 2.2.3. These samples were used for Northern blotting.

6.2.3 Probes

Probe 1 (chapter 2, section 2.7.4) was used for specific detection of OTR mRNA by Northern blot analysis. This probe was labeled by random priming (GIGAPRIME DNA Labeling Kit, Bresatec) with α-[P^{32}]dATP (chapter 2, section 2.7.5.1). The probe 3 (chapter 2, section 2.7.4) was used as a control for detection of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. It was labeled using nick translation (Promega) with α-[P^{32}]dATP (chapter 2, section 2.7.5.2).

6.2.4 Northen blot analysis

Samples of total RNA (15 μg/lane) from each endometrial tissue were denatured in RNA sample buffer for 10 min at 65°C and separated by electrophoresis on 1% agarose-formaldehyde gels in MOPS buffer (chapter 2, section 2.2.5). After electrophoresis, RNA samples were transferred to a membrane (positively charged, Boehringer Mannheim) by capillary blotting overnight in 10×SSC and cross-linked by UV irradiation (chapter 2, section 2.7.2 and 2.7.3). Hybridization with probe 1 was carried out as described in chapter 2, section 2.7.7. Hybridization signals were detected by exposing membranes to X-ray film (Hyperfilm-MP, Amersham), with intensifying screens at -80°C for 3 days. After hybridization with probe 1, probe 1 was stripped off as described in chapter 2, section 2.7.9. The membrane was re-

hybridized with a GAPDH cDNA probe (probe 3) for normalization of OTR mRNA levels.

6.2.5 Semiquantitative RT-PCR analysis

The semiquantitative RT-PCR methodology was adapted from Tsai *et al.* (1996). In choosing this method the objective was to select regions of mRNA used for amplification, on the basis that the standard curves of mRNA were linear across more than two orders of magnitude and the final PCR products would lie within the linear region of the standard curve.

6.2.5.1 Primer design

Three different pairs of primer were used for semiquantitative RT-PCR analysis of PGHS expression (as listed in Table 6.2). The primers pair AB used to detect ovine PGHS-1 mRNA were designed by Tsai and Wiltbank (1997). The primer pairs CD and EF, which used to detect ovine PGHS-2 and GAPDH mRNA respectively, were designed by Tsai *et al.* (1996) based on the sequences from the cattle.

Table 6.2 List of primer sequences used for semiquantitative RT-PCR analysis of PGHS expression

Primer name	Sequence	Reference
A (forward)	5' GGAGATATGGACCTGGCTC 3'	Tsai and Wiltbank, 1997
B (reverse)	5' GGAACTCCGCATCTGGCAA 3'	Tsai and Wiltbank, 1997
C (forward)	5'AGGTGTATGTATGAGTGTAGGA 3'	Tsai *et al.*, 1996
D (reverse)	5'GTGCTGGGCAAAGAATGCAA 3'	Tsai *et al.*, 1996
E (forward)	5' TGTTCCAGTATGATTCCACCC 3'	Tsai *et al.*, 1996
F (reverse)	5' TCCACCACCCTGTTGCTGTA 3'	Tsai *et al.*, 1996

6.2.5.2 mRNA preparation

Total RNA samples were pooled from each group and the mRNAs were then extracted for purification because only small amounts of tissue was available. PolyATtract mRNA Isolation Systems (Promega) was used for mRNA purification as described in chapter 2, section 2.2.2. After final elution, 80μl of each pooled mRNA sample was collected and the concentration was determined as described in chapter 2, section 2.2.3. However, the absorbance measured at 260nm from each sample was too low. The samples were then freeze-dried and eluted in 25μl of DEPC water. The concentration of each sample is shown in Table 6.3.

Table 6.3 List of sample concentration and sample volume of each group used for semiquantitative RT-PCR.

Group name	OD (1:100)	Concentration (μg/μl)	Sample volume for RT reaction (μl)	Final mRNA amount for PCR (ng)
PT	0.011	0.044	2.73	30
OC	0.006	0.024	5	30
3C	0.014	0.056	2.14	30
3T	0.008	0.032	3.75	30
12C	0.010	0.04	3	30
12T	0.003	0.012	10	30
48C	0.011	0.044	2.73	30
48T	0.005	0.02	6	30

6.2.5.3 RT-PCR

The mRNA samples were prepared as described above and the volume of each sample used for the reactions are indicated in Table 6.3. 120ng of mRNA/sample and 50ng

of Oligo $(dT)_{12-18}$ in 11.5μl DEPC-water were denatured at 65°C for 10 minutes. The tubes containing denatured mRNA samples were put on ice immediately and the following contents were added: 4μl of 5X First Strand Buffer, 2μl of 0.1 M DTT, 1μl of 10mM dNTP mix and 0.5μl of RNasin to the final volume of 19μl. The contents in the tube were mixed gently and were incubated at 42°C for 2 minutes. First-strand cDNA was synthesized using 200 units (1μl) of SuperScript II RNase H^- Reverse Transcriptase (Life Technologies). The reaction was incubated at 42°C for 50 min, and then was inactivated by heating at 70°C for 15 minutes. PCR amplification was proceeded by adding the following contents: 2.5μl of 10X PCR buffer, 1.5μl of 25mM $MgCl_2$, 0.5μl of 10mM dNTP mix, 1μl of specific forward primer, 1μl of specific reverse primer, 5μl of first strand cDNA synthesis reaction and 13μl of DEPC-water. The contents were mixed gently and heated at 95°C for 3 minutes, then were processed directly for 40 cycles using 2.5 units of Taq polymerase (Life Technologies). The cycling parameters were 95°C 30s, 57°C 30s, 72°C 60s. After amplification, 4μl of each sample was loaded and separated on a 1.2% agarose gel. The gel was photographed and the density of bands was quantified by scanning.

6.2.6 Quantification of mRNA expression

Autoradiographed signals from Northern hybridization for detection of OTR mRNA expression were quantitated by densitometric scanning. The signal of the mRNA expression for OTR was compared with the signal for GAPDH to standardize the value obtained.

The band intensity of the RT-PCR products generated by specific PGHS-1 and PGHS-2 cDNA primers was quantitated by densitometric scanning and compared with the band intensity of GAPDH RT-PCR products. The data was present as the ratio of either PGHS-1 or PGHS-2 band intensity to GAPDH band intensity.

In each group, the average data were calculated with the standard deviation. Data was presented as means ± the standard error of the mean (SEM).

6.2.7 Statistical analysis

A one-way ANOVA (chapter 2, section 2.12.1) was used to analyze the overall effect of steroid priming and withdrawal of priming factors (for example PT, OC, 3C, 12C and 48C group of ewes) on each of the measured parameters. It was also used to analyze the overall effect of steroid priming and oestrogen treatment factors (for example PT, OC, 3T, 12T and 48T group of ewes) on each of the measured parameters. A T-test was used to determine the statistical differences between the two control group samples PT and OC.

A two-way ANOVA (chapter 2, section 2.12.2) was used to investigate the overall effects of oestrogen treatment and the time after oestrogen treatment and interaction between these factors (for example 3C, 3T, 12C, 12T, 48C and 48T group of ewes) on each of the measured parameters.

6.3 RESULTS

6.3.1 Northern hybridization analysis of OTR mRNA expression in the samples treated with steroids

Northern hybridization showed that four transcripts of OTR were present in all the endometrium samples (shown in Fig 6.1). This result is in keeping with the results found in endometrium during the natural estrous cycle, and in endometrium and myometriun at labor (see chapter 5 for details). A single transcript of 1.3kb was detected for GAPDH. The band of the first transcript (5.6kb) was quantified by scanning and the ratio to GAPDH was expressed for each sample. The hybridization signals were scanned in duplicate and data was shown in Table 6.4.

Table 6.4 List of the parameters quantified from hybridization signals for OTR mRNA expression

sample	PT	OC	3C	3T	12C	12T	48C	48T
1	0.22*	1.17	0.39	1.51	0.84	0.72	1.62	0.3
2	0.39*	0.68	1.0	0.70	1.02	0.4	2.83	0.64
3	2.24	0.75	0.55	1.14		0.51	1.6	0.2
4	1.91	1.6	0.42					
means±	2.1±	1.05±	0.59±	1.12±	0.93±	0.54±	2.01±	0.38±
SEM	0.23	0.42	0.28	0.4	0.12	0.16	0.7	0.23

The data is present as ratio of OTR hybridization band intensity to GAPDH hybridization band intensity. * data was ignored for calculation

In chapter 5, it has been found that four transcripts of the OTR gene were present in the uterus: two major bands at size of 5.6kb and 1.5kb, and two minor bands of size 3.3kb and 2.1kb. However, in this experiment, the density of the first transcript (5.6kb) in two samples (marked as *) in PT group were much lower than another main band (1.5kb). When a comparison was made with the other two samples in the PT group, both hybridization signals (5.6kb and 1.5kb) in those two samples were high and even as expected. The reason might be that the hybridization signal in marked samples was interrupted during the processing of Northern blot. Because those two marked samples were located close to the edge of the blot, RNA samples might not have been transferred completely from the gel to the membrane, or they may be contaminated by fingerprints. Because of this result, the two samples were considered to be abnormal and removed from the calculations.

The result of the t-test indicated a significant difference (P=0.032) between the two control samples before steroid priming and after steroid priming. One-way ANOVA for analysis of the samples in control groups indicated that after injection with oil, the OTR mRNA expression increased significantly (3C vs. 48C, P=0.01, Fig 6.2a). In the estradiol treatment group, there was no statistical significant difference (3T vs. 48T, P=0.117) after injection with estradiol (Fig 6.2b).

Two-way ANOVA indicated a significant effect (P=0.001) of estrogen treatment with time (Fig 6.3). As shown in Fig 6.3, the OTR mRNA level was reduced in the samples that were treated with estradiol in ovariectomised ewes, whereas in the control groups that were injected with oil only, the OTR mRNA expression level increased after treatment with steroids over 23 days to simulate a natural estrous cycle.

6.3.2 Semiquantitative RT-PCR for detection of PGSH-1 and PGSH-2 mRNA expression in the samples treated with steroids

The RT-PCR products generated by primer pairs AB, CD and EF were presented at the approximate sizes of 310 bp, 480 bp and 850 bp; the expected sizes are 314 bp, 484 bp and 850 bp respectively (Tsai *et al.*, 1996; Tsai and Wiltbank 1997) (Fig 6.4). The reaction was repeated twice and the same result was obtained. The parameters of the scanned bands are shown in Table 6.5. The results from semiquantitative RT-PCR for detection of PGSH mRNA level showed that both PGSH-1 and PGSH-2 mRNA levels did decline in the group treated with estradiol, but these results were not statistically significant because samples were pooled (Fig 6.5 and Fig 6.6). In the control group, the PGSH-1 and PGSH-2 mRNA levels increased after 3 hour estradiol treatment, then declined (Fig 6.5 and Fig 6.6).

Table 6.5 Quantification of the RT-PCR products for PGHS-1 and PGHS-2 mRNA expression.

sample	PT	OC	3C	3T	12C	12T	48C	48T
PGSH-1	0.63	0.38	0.93	0.56	0.45	0.048	0.29	0.036
PGSH-2	1.91	1.33	4.67	2.46	1.40	1.07	0.87	0.015

The data are presented as a ratio of either PGHS-1 or PGHS-2 band intensity to GAPDH band intensity.

6.4 DISCUSSION

When Northern blotting was carried out for OTR mRNA expression, only a 14-well gel tank was available so that not all the samples were used for measurement. Samples in group OC were loaded in two gels (two samples/gel) respectively and the two gels were exposed to X-ray film at the same time. Similar hybridization signals were obtained from both gels (see Table 6.4, sample 1, 2 and sample 3, 4 in group OC), which indicated that the procedures used for Northern hybridization in this experiment were stable and the data obtained from the hybridization result was reliable.

In the semiquantitative RT-PCR experiment, the amount of mRNA used for reverse transcription and PCR amplification was from 30 ng of each sample, which is less than 0.3 picomoles. This is much lower than the amount of native PGHS-2 mRNA (25.6-0.2 attomoles) used by Tsai and Wiltbank (1997). Different concentrations of RT-PCR bands were found when the same amount of mRNA was used for reverse transcription and PCR amplification in each sample. These data demonstrated that the data from each sample in this study is comparable.

Results showed that the OTR mRNA concentrations in the ovariectomized ewes before steroid priming were high. As suggested previously by Lau *et al.* (1992b), high concentrations of uterine OTR found in ovariectomized ewes were due to the ceasation of progesterone secretion from ovary. One-way ANOVA indicated that the OTR mRNA expression level did not change after estradiol treatment, but did increase after injection with oil. Two-way ANOVA indicated that there was a significant difference ($P=0.001$) between oestrogen and oil (control) treatments and with time after treatment. When the results of these two analyses are taken together, it indicates that estrogen has an inhibitory effect on oxytocin receptor expression. Similar results have been observed by Vallet *et al.* (1990). They showed that estradiol treatment alone in ovariectomized ewes had a long-term inhibitory effect on oxytocin receptor in endometrium. It is suggested that the estrogen-induced oxytocin receptor down-regulation may occur.

Previous studies have shown that the concentration of OTR increased when progesterone administration was stopped (Leavitt *et al.*, 1985; Vallet *et al.*, 1990). It was suggested by Lau *et al.* (1992a) that the increase in OTR concentrations was due to the withdrawal of the inhibitory influence of progesterone. The data obtained in this study showed that progesterone withdrawal did result in a significant increase in OTR mRNA expression. This finding indicates that the observed increase in OTR mRNA levels may be due to the loss of the inhibitory effects of progesterone. Previous studies have shown that the progesterone-induced inhibitory effect on oxytocin receptor involved a direct interaction with the OTR at the level of the cell membrane (Grazzini *et al.*, 1998). It has been shown that progesterone binds to the OTR and thus inhibits its ligand binding and signaling function (Grazzini *et al.*, 1998). Zingg *et al.* (1998) showed that progesterone alone had no significant effect on unstimulated OTR mRNA levels. However, progesterone had a strong inhibitory effect on oestrogen-induced OT binding, but not on oestrogen-induced OTR mRNA accumulation. Previous studies have shown that progesterone can also act at a genomic level by binding to nuclear receptors and modulating the expression of specific target gene (Tsai *et al.*, 1994). The rise in OTR mRNA expression level in the controls (oil only) indicates that progesterone withdrawal may lead to an increase in OTR mRNA expression. Whether it is possible that progesterone acts via either genomic or nongenomic mechanisms to regulate OTR gene expression indirectly, however, remains to be investigated.

The results of the semiquantitative RT-PCR for the detection of PGSH mRNA level showed a tendency for estradiol to inhibit both PGSH-1 and PGSH-2 mRNA expression, although these results were not statistically significant, since pooled samples were used. Other investigators have shown that prostaglandin synthetase mRNA levels in the endometrium of ovarectomized ewes were inhibited by estradiol treatment (Salamonsen *et al.*, 1991). In this study, the reduction of PGSH mRNA concentrations correlated with the potential decrease in the concentrations of OTR mRNA after estradiol treatment. In the control groups that were injected with oil, both PGSH-1 and PGSH-2 mRNA levels showed a decline after a short-term increase, although the changes were not as great as found in the groups that injected with estradiol. It seems that progesterone withdrawal has a short-term effect on

prostaglandin synthetase mRNA expression and the increase of OTR mRNA expression is uncoupled to prostaglandin synthetase mRNA expression.

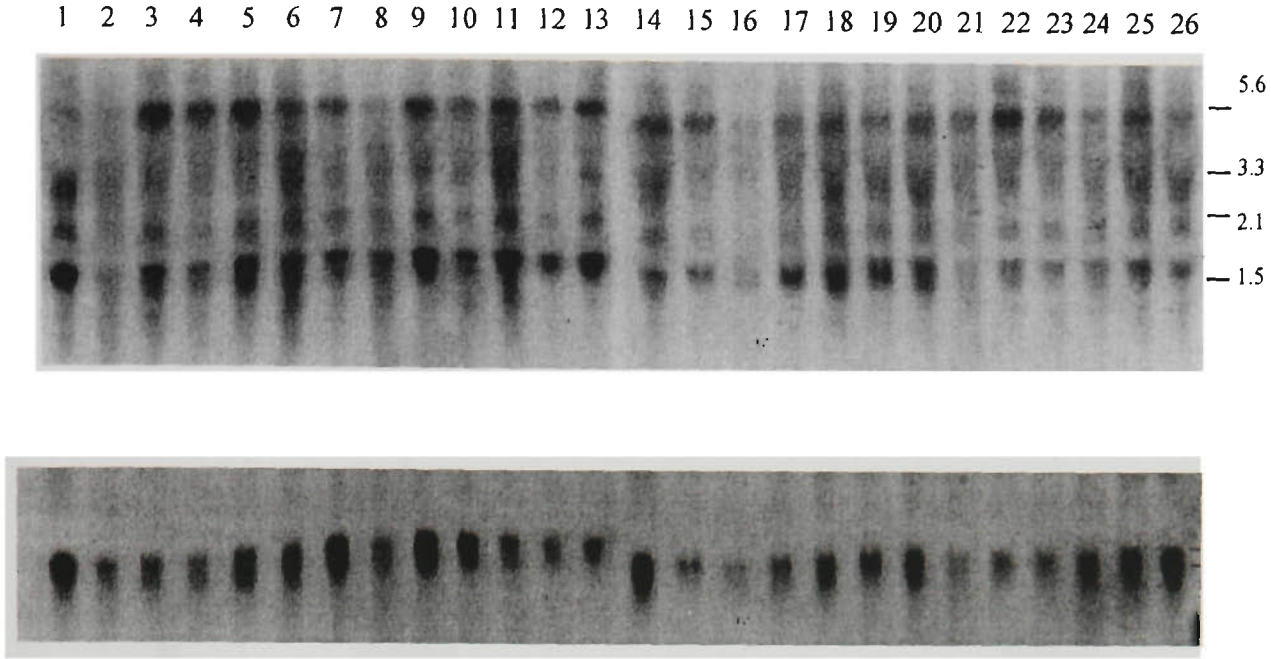


Fig 6.1 Northern hybridization analysis of ovine endometrium OTR mRNA expression in ovariectomized ewes treated with steroids. Lane 1-4, PT samples collected after ovariectomy, but before steroid priming. Lane 5-6 and 14-15, OC samples collected after steroid priming. Lane 7-10, 3C samples collected after oil treatment for 3 hours. Lane 11-13, 3T samples collected after estradiol treatment for 3 hours. Lane 16-17, 12C samples collected after oil treatment for 12 hours. Lane 18-20, 12T samples collected after estradiol treatment for 12 hours. Lane 21-23, 48C samples collected after oil treatment for 48 hours. Lane 24-26, 48T samples collected after estradiol treatment for 48 hours. Upper panel, hybridization with OTR cDNA probe. Bottom panel, hybridization with GAPDH cDNA probe. - indicates the size of OTR transcripts (Kb).

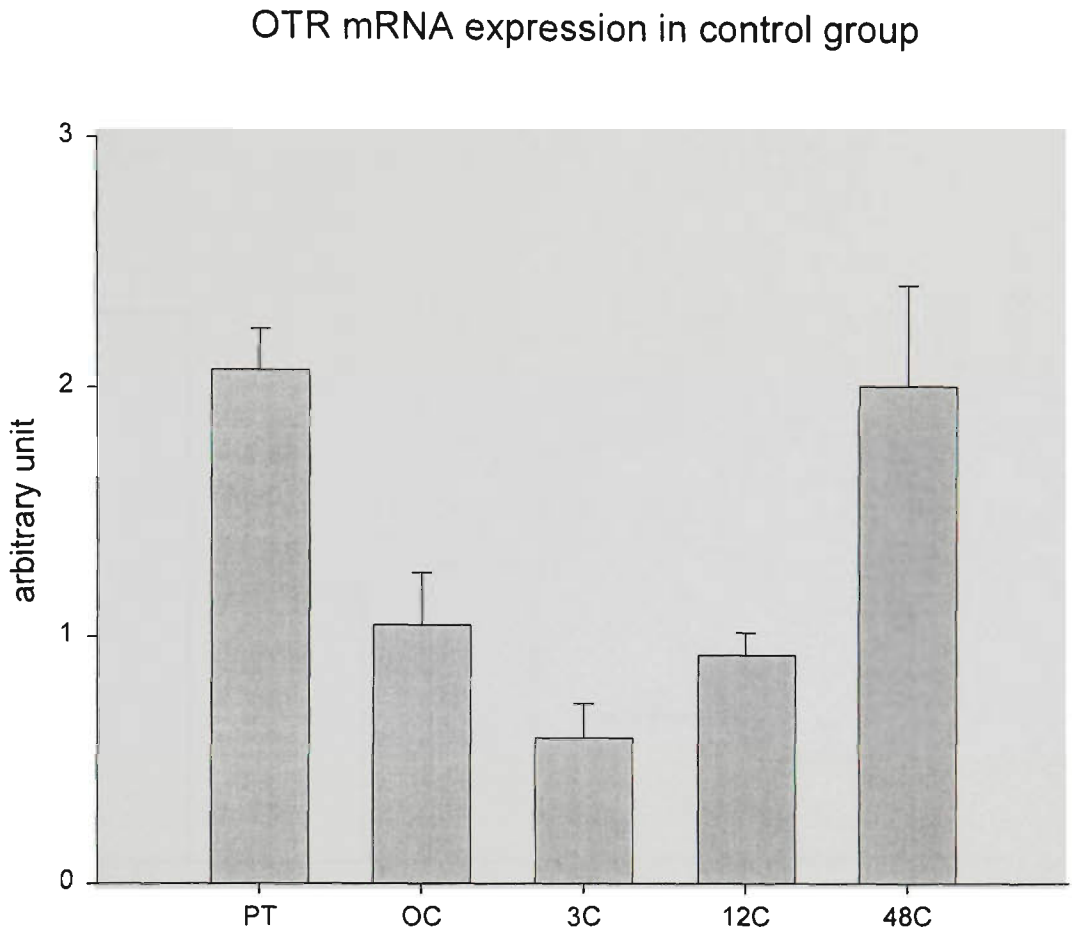


Fig 6.2a OTR mRNA expression in control sheep treated with oil. The signals for OTR mRNA and GAPDH mRNA on the autoradiographs were quantified by densitometry and expressed as arbitrary units for the ratio of OTR mRNA to GAPDH mRNA. PT, samples collected before steroid priming. OC, samples collected after steroid priming. 3C, 12C and 48C, samples collected at the time 3, 12, 48 hours after the last oil injection. Data were analyzed by one-way ANOVA analysis. The significant differences ($P<0.05$) between groups in the endometrium are represented by the different lettering. There were statistical differences between groups PT and 3C ($P=0.017$), and between groups 3C and 48C ($P=0.001$). There was no statistically significant difference between groups 3C and 12C ($P=0.577$). ^{a,b} Different scripts differ at $P< 0.05$.

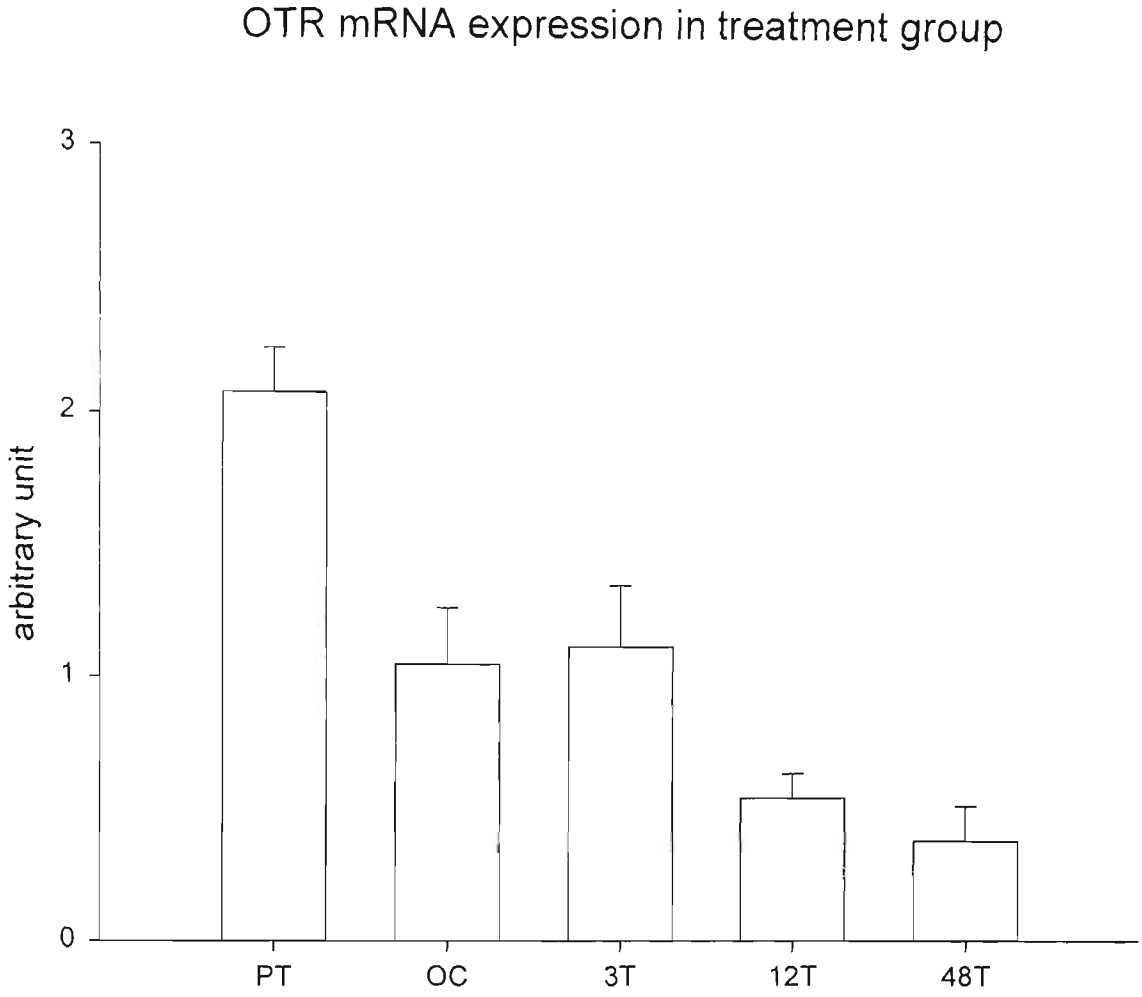


Fig 6.2b OTR mRNA expression in steroid-primed ovariectomized ewes treated with estradiol-17β. The signals for both the OTR mRNA and GAPDH mRNA were quantified by densitometry and expressed as arbitrary units, being the ratio of OTR mRNA to GAPDH mRNA. PT, samples collected before steroid priming. OC, samples collected after steroid priming. 3TC, 12T and 48T, samples collected at the time 3, 12, 48 hours after the last estradiol injection. Data were analyzed by one-way ANOVA analysis. Significant differences ($P<0.05$) between groups are represented by the different lettering. There was a statistical significant difference between groups PT and OC ($P=0.031$), groups PT and 12T ($P=0.003$) and between groups PT and 48T ($P=0.002$). There was no statistical significant difference between groups PT and 3T ($P=0.59$) and between groups 3T and 48T ($P=0.117$). ^{a,b} Different scripts differ at $P< 0.05$.

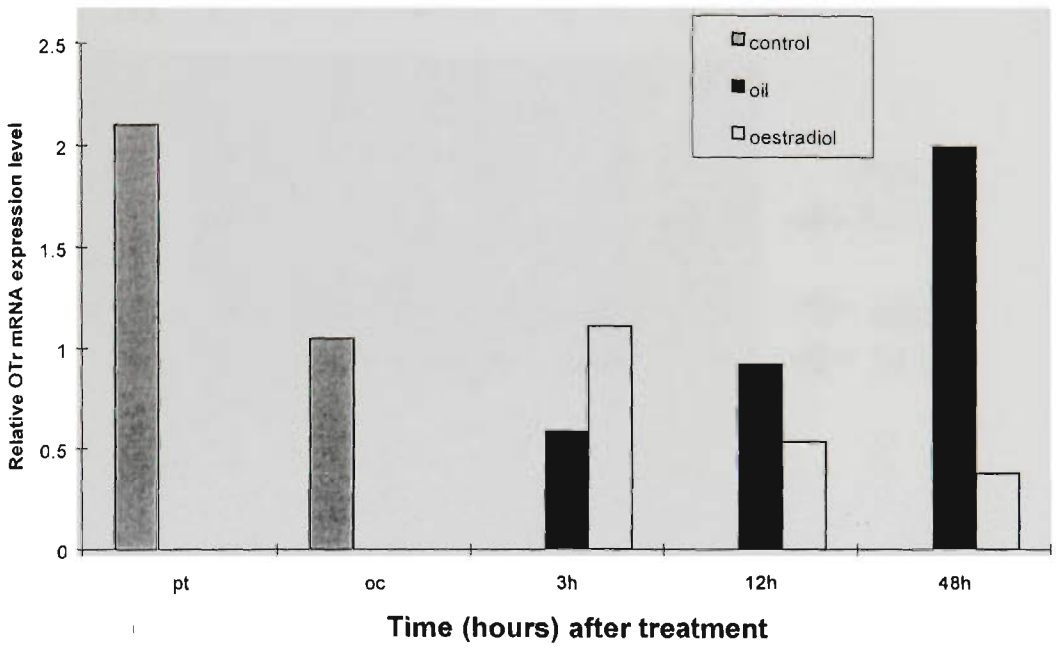


Fig 6.3 OTR mRNA expression in steroid-primed ovariectomized ewes given either oil only or estradiol treatment. Data were analyzed by two-way ANOVA analysis. Grey bar indicate the samples used as control. PT, samples collected before steroid priming. OC, samples collected after steroid priming. Black bars indicate the samples given oil only, and white bars indicate the samples given estradiol treatment at the same time. There was a significant effect (P=0.001) between estradiol and oil treatment and the time after treatment. The OTR mRNA level appeared to decrease in the samples that treated with estradiol, whereas in the oil only treatment groups, the OTR mRNA level appeared to increase after treated with steroids over 23 days to mimic a natural estrous cycle.

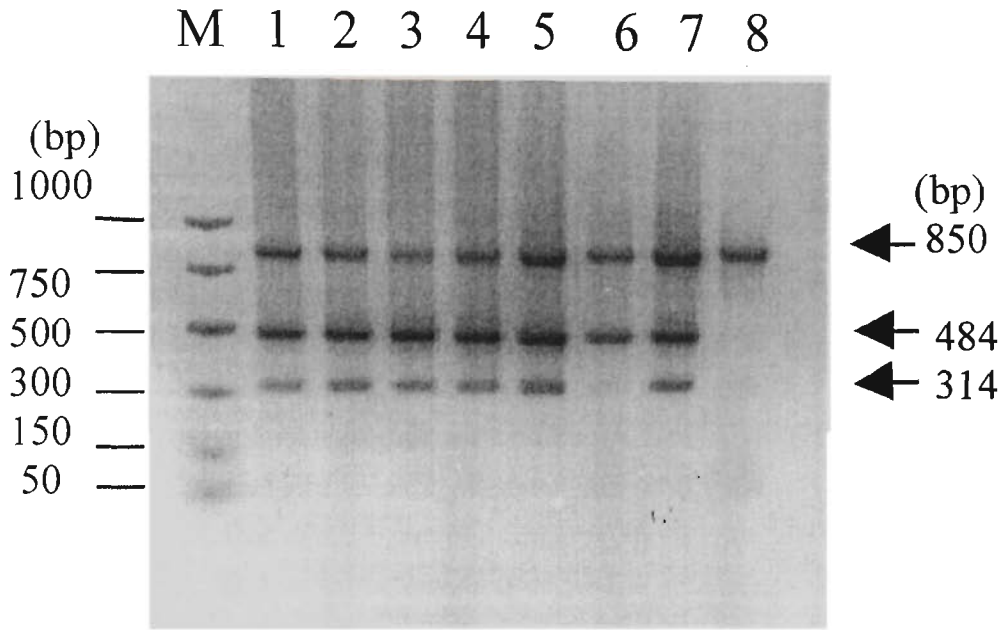


Fig 6.4 An agarose gel showing a semiquantitative RT-PCR for the detection of PGSH-1 and PGSH-2 mRNA expression in steroid-primed ovariectomized ewes given either oil only or estradiol treatment. M, PCR markers (Promega). Lane 1-8, are the pooled samples collected from PT, OC, 3C, 3T, 12C, 12T, 48C and 48T groups respectively (see text for details). – indicates the size of PCR markers. → indicates the size of RT-PCR products. The 850bp band is the GAPDH cDNA generated by primers E and F. The 484bp band is the PGHS-2 cDNA generated by primers C and D. The 314bp band is the PGHS-1 cDNA generated by primers A and B.

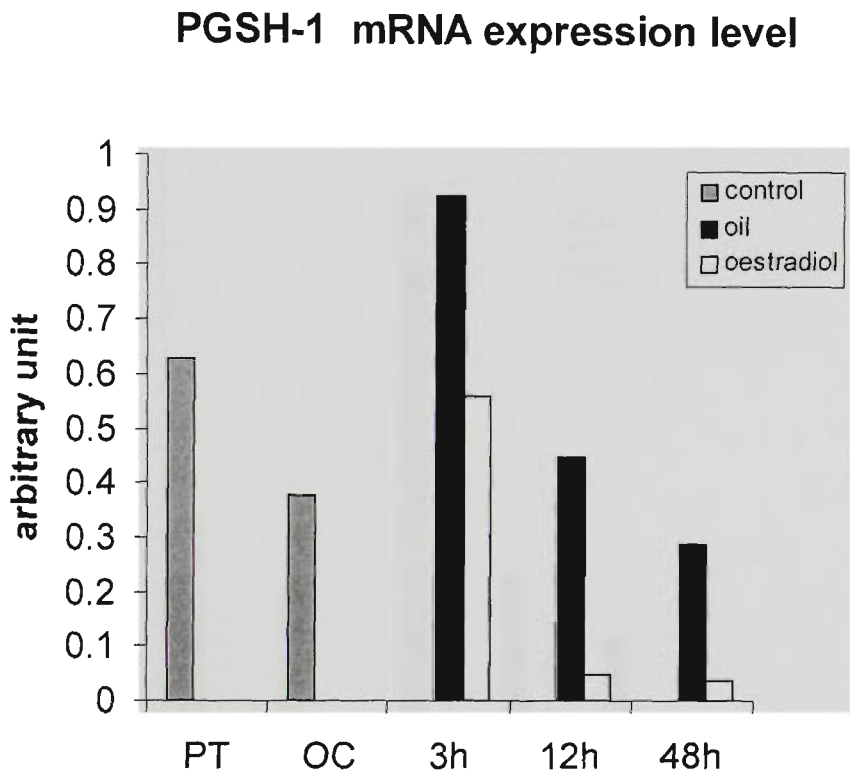


Fig 6.5 PGSH-1 mRNA expression in steroid-primed ovariectomized ewes given either oil only or estradiol treatment (pooled samples). PT, OC were the samples collected before and after steroid priming respectively. Result showed that PGSH-1 mRNA levels tended to decrease in the groups treated with estradiol. In the groups treated with oil only, the PGSH-1 mRNA levels tended to show a small increase 3 hours after oil injection and then declined.

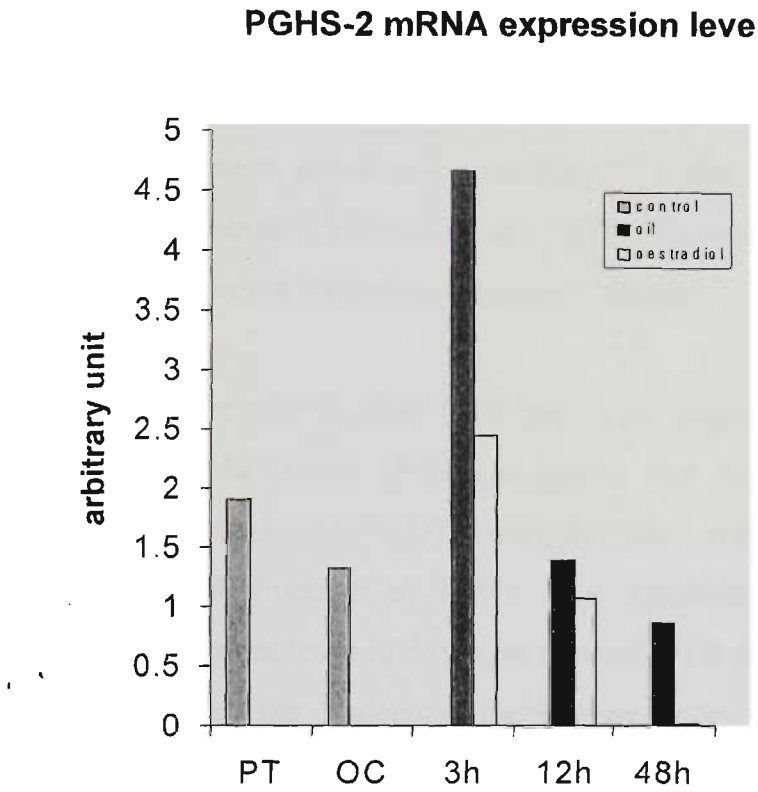


Fig 6.6 PGSH-2 mRNA expression in steroid-primed ovariectomized ewes given either oil only or estradiol treatment (pooled samples). PT,OC were the samples collected before and after steroid priming respectively. Result showed that PGSH-2 mRNA levels tended to decrease in the groups treated with estradiol. In the groups treated with oil only, the PGSH-1 mRNA levels tended to show a small increase 3 hours after oil injection, then declined.

Chapter 7 Expression of oxytocin receptor protein using prokaryotic and eukaryotic expression systems

7.1 Introduction

Several studies have shown that oxytocin (OT) is involved in the regulation of biological processes associated with a number of reproductive functions, including uterine contraction, milk ejection and sexual behavior (Fuchs, 1985; Carter, 1992; Ivell and Russell, 1995). It has been found in the rat that OT is also implicated in the control of natriuresis, thirst and hunger (Verbalis *et al.*, 1993). These effects of OT are thought to be mediated through specific oxytocin receptors (OTRs).

Numerous studies have shown that OTR plays an important role in a number of biochemical events in the uterus. It is also known that rising OTR levels results in a marked increase in the sensitivity to OT during the labor, reflecting a marked increase in the upregulation of OTR (Fuchs *et al.*, 1984). The oxytocinergic effects in the uterus are illustrated by the marked increase in the expression of OTR during the oestrous cycle. In order to study in detail the molecular mechanisms involved in the expression and regulation of the OTR protein on the cell surface, it is necessary to develop the appropriate techniques to obtain the OTR abtibodies.

Vasopressin V1a, V1b, V2 receptor and OTR share a high degree of homology in the transmembrane regions, but differ significantly in the intracellular loops (Morel *et al.*, 1992, Kimura *et al.*, 1992; Lolait *et al.*, 1992; Sugimoto *et al.*, 1994). Previous studies on the characterization of OT binding sites by autoradiography and in vitro binding studies have suggested that there may be more than one OTR subtype (Elands *et al.*, 1991; Chan *et al.*, 1993). Other studies have shown that there is a single copy gene for OTR (Kimura and Saji, 1995; Bathgate *et al.*, 1995). Whether the pharmacological differences in OTR are due to changes at the molecular level needs to be investigated.

Two peptides with non-overlapping sequences of the third intracellular loop of the rat OTR were chosen to raise polyoclonal antibodies for OTR (Adan *et al.*, 1995). Using these antisera, OTR expression was demonstrated in the brain, pituitary, mammary gland, and uterus in rat by immunocytochemistry (Adan *et al.*, 1995). Using the same antisera, the location of OTR was determined by immunocytochemistry in the smooth muscle of myometrial blood vessels in sheep (Wu *et al.*, 1996).

The polyclonal antibody generated by a synthetic peptide corresponding to the N-terminus (amino acids 1-19) of the putative human OTR protein sequence was prepared (Kimura, 1997). Two peptides, amino acids 20-40 from the N-terminal domain and amino acids 102-119 from the first extracellular loop of the putative human OTR protein sequence, were used to raise the monoclonal antibodies for OTR (Kimura, 1995; Kimura, 1998). Both antibodies were used for immunocytochemistry and Western blot analysis. Using these antibodies, the expression and distribution of the OTR in human female reproductive organs and in rat brain was investigated (Kimura, 1995).

Immunocytochemistry with specific antibodies, has been used to detect the location of OTR in the specific tissue sections as mentioned above. However to date, all the anti-OTR antisera have been generated by synthetic peptides. No antibodies that raised against a large partial protein of OTR or the intact OTR have been reported.

The aim of this study was to establish an expression system for OTR, then to use the purified OTR to obtain the antibodies for further biological studies.

7.2 Materials and Methods

7.2.1 Expression of oxytocin receptor cDNA using a prokaryotic gene fusion system

7.2.1.1 Expression Vector

pGEX-5X-2 plasmid (AMRAD Co.Ltd) was chosen as an expression vector (kindly provided by Dr Yaping Chen). pGEX-5X-2 is from the glutathione S-transferase (GST) gene fusion system. This system is an integrated system for the expression, purification and detection of fusion proteins produced in *E.coli*. The system consists of three major components: pGEX plasmid vectors, two GST purification modules and the GST detection module. The pGEX plasmids are designed for inducible, high-level intracellular expression of genes or gene fragments as fusions with *Schistosoma japonicum* GST. Fusion proteins are easily purified from bacterial lysates by affinity chromatography using the GST purification modules. Fusion proteins can be detected using a colorimetric assay or immunoassay provided in the GST detection module.

pGEX-5X-2 has a tac promoter for IPTG inducible, high-level expression; an internal lac I^q gene for use in any *E.coli* host; factor Xa protease recognition site for cleaving the desired protein from the fusion product, and very mild elution conditions for release of fusion proteins from the affinity matrix.

7.2.1.2 Insert (OTR cDNA fragment)

The RT-PCR product generated by primers PA and PB was used as an insert (Chapter 3, section 3.2.5). This product has a predicted size of 759bp and the putative amino acid sequence derived from this DNA sequence covers the region of six transmembrane domains (from II-VII transmembrane domain). The 759bp DNA fragment was cloned into pCR-Script SK(+) plasmid (Chapter 2, section 2.9.2). The positive colony named C8 was used.

7.2.1.3 PCR for creating cloning site

To achieve successful cloning, it is necessary to choose the appropriate cloning site, which is unique, enable to expression the protein in frame. By comparison of the sequence of the multiple cloning site in the plasmid pCR-Script SK(+) that contains OTR cDNA fragment and in vector plasmid pGEX-5X-2 (Fig 7.1), restriction enzyme sites *SmaI* and *XhoI* were chosen as the appropriate cloning sites. The downstream primer Ped (5'CG CTC GAG CCC TCA GCT GTT GAG 3') was designed to create a stop codon (TGA) and the *XhoI* site at the C-terminal. The upstream primer was T7 (5'GTA ATA CGA CTC ACT ATA GGG C 3') and M13-20 (5'GTA AAA CGA CGG CCA GT 3'), respectively. Using a cloned 759bp OTR cDNA insert in the plasmid pCR-Script SK(+) as a template (plasmid named C8), the PCR was carried out with initial denaturation at 95°C for 2 minutes, and then at 95°C for 45 seconds, 50°C for 30 seconds, 68°C for 90 seconds with Taq polymerase (promega) for 30 cycles.

7.2.1.4 Cloning of the PCR product into pGEX-5X-2 vector

The PCR products E5 (generated by primers Ped and M13-20) and E6 (generated by primers Ped and T7), and the pGEX-5X-2 plasmid DNA were digested with restriction enzymes *SmaI* and *XhoI*, respectively. The digestion reactions were listed in Table 7.1. After digestion, the DNA fragment E5 and E6 were purified using a Wizard PCR Preps DNA purification System (Promega), and the digested plasmid DNA PGEX-5X-2 was purified using Wizard DNA Clean-up System (Promega) (Chapter 2, section 2.3.5.2 and 2.3.5.3). The purified DNA fragments and plasmid DNA were then ligated with ligase at 16°C overnight. The ligation reaction was: 3μl of digested plasmid DNA pGEX-5X-2 (150ng), 15μl of digested E5 and E6 mixture (150ng), 2μl of 10X ligase buffer and 0.5μl of ligase (5 weiss unit/μl, NEB).

Table 7.1 The digestion reactions with restriction enzymes *SmaI* and *XhoI*.

	PCR product E5 10μl (10ng/μl)	PCR product E6 10μl (10ng/μl)	PGEX-5X-2 3μl (300ng/μl)
10x Y buffer+PBS	1.5μl	1.5μl	1.5μl
H_2O	2.5μl	2.5μl	9.0μl
SmaI (10U/μl)	1μl	1μl	1.5μl
	Incubate at 30°C for 2 hours		
10x R buffer+PBS	3μl	3μl	3μl
H_2O	11μl	11μl	10.5μl
XhoI (10U/μl)	1μl	1μl	1.5μl
	Incubate at 37°C for 3 hours		

7.2.1.5 Transformation of ligated plasmid DNA

E. coli cell lines XL1-Blue and TG2 were chosen as the host cells in this experiment. Competent cells were prepared as described in Chapter 2, section 2.9.1.6. 10μl of ligation reaction was transformed into the cells, according to the procedure described in Chapter 2, section 2.9.1.7. The recombinant cells were screened by blue/white selection. The plasmid DNA samples from the white colonies were prepared and digested with restriction enzymes (*EcoRI* and *XhoI*) to identify the size of the insert. Ten out of thirteen colonies were positive. The plasmid DNA from the positive clones (named E15 and E16) were prepared a using large-scale plasmid DNA preparation method (Chapter 2, section 2.3.1.1) and purified by a Wizard DNA clean-up system (Promega, Chapter 2, section 2.3.5.1). The purified plasmid DNAs were then sequenced to confirm that the insert DNA was cloned in right site without causing a shift in the reading frame (Chapter 2, section 2.10).

7.2.1.6 Expression of fusion proteins

IPTG was used to induce the expression of fusion proteins. The procedure was as follows: A single colony was taken into 5ml 2X YTA medium and was cultured with shaking at 37°C overnight. About 100-150μl of overnight culture was then transferred into another fresh tube containing 5ml 2X YTA medium to the OD_{600}=0.1. The culture was incubated with shaking at 30-37°C until the OD_{600} reached an absorbance of 0.6-1.0. 1 ml of culture was taken and was spun at high speed for 1 minute to collect the cells. The pellet was stored at -20°C till use. Another 1 ml of culture was transfered to an empty sterile tube and 10μl of 0.1M IPTG was added. The tube was incubated with shaking at 37°C. After 90-120 minutes, 1-5ml of culture was taken for cell collection.

The collected samples described above could be either treated with sonication or Protein Sample Buffer for SDS-PAGE analysis. The procedure of small-scale sonication was as follows: 5ml of collected culture described above was centrifuged at 5000 rpm for 1 minute at 4°C. The cell pellet was completely suspended in 250μl ice-cold 1X PBS using a pipet. The suspended cells were sonicated in ice in less than 10 second each time until the suspension became partial clear. The suspension was centrifuged at 12000 rpm for 10 minute at 4°C. The supernatant was saved for SDS-PAGE analysis as follow:

The collected cells or bacterial sonicates were suspended in 20μl of H_2O and 20μl of 2X protein sample buffer and were boiled at 95°C for 3 minutes. 10μl of boiled cell supernatant from each sample was loaded into a well of 12.5% SDS-protein acrylamide gel together with a low range protein marker. After electrophoreses, the protein gel was either stained with coomassie blue (Chapter 2, section) for further analysis, or transferred to a Nitrocellulose membrane by Western blot using Anti-GST Antibody raised in goat as the primary antibody and Peroxidase-Conjugated Rabbit Anti Goat IgG as the secondary antibody for protein detection (Chapter 2, section 2.11).

7.2.2 Expression of OTR cDNA fragments using BAC-TO-BAC Baculovirus Expression System

BAC-TO-BAC Baculovirus Expression System (Life Technologies) is based on site-specific transposition of an expression cassette into a baculovirus shuttle vector (bacmid) propagated in *E. coli* (Luckow *et al.*, 1993). This system contains: the pFASTBAC donor plasmid, the competent DH10 BAC *E. coli* cells that contain the bacmid and the helper plasmid. The pFASTBAC donor plasmid has a mini-Tn7 element that contains an expression cassette consisting of a baculovirus-specific promoter, a multiple cloning site, and an SV40 poly(A) signal inserted between the left and right arms of Tn7. The gene to be expressed is cloned into the multiple cloning site of a pFASTBAC donor plasmid downstream from the baculovirus-specific promoter. When recombinant pFASTBAC donor plasmid is transformed into the competent DH10 BAC *E. coli* cells, the mini-Tn7 element on the pFASTBAC donor plasmid can transpose to the mini-attTn7 target site on the bacmid in the presence of transposition proteins provided by the helper plasmid. Insertion of the mini-Tn7 into the mini-attTn7 attachment site on the bacmid disrupts expression of the LacZα peptide, so colonies containing the recombinant bacmid are white in the background of blue colonies. Recombinant bacmid DNA can be rapidly isolated from small scale cultures and then used to transfect insect cells. Viral stocks harvested from the transfected cells can then be used to infect fresh insect cells for further protein expression, purification and analysis.

7.2.2.1 pFASTBAC vector

pFASTBAC1 and pFASTBAC HTb were chosen as the vector to clone the OTR cDNA fragment. The multiple cloning site sequences of pFASTBAC1 and pFASTBAC HTb vector are shown in Fig 7.2.

7.2.2.2 Insert DNA

The DNA fragment between the restriction enzymes *SalI* and *NotI* in the recombinant plasmid C8 (as described in section 7.2.1.2), and the DNA fragment between the restriction enzymes *EcoRI* and *XhoI* in the recombinant plasmids E15 and E16 (as described in section 7.2.1.5), were used as the gene of interest to be expressed.

7.2.2.3 Cloning of the gene of interest into the vector

The restriction enzymes *SalI* and *NotI* in the expression vector pFastBac1 were chosen as the appropriate cloning site. The expression vector pFastBac1 and the recombinant plasmid C8 were digested with restriction enzymes *SalI* and *NotI*, respectively. The digestion reactions are listed in Table 7.2. After digestion, the DNA fragment excised from plasmid C8 (about 840bp) was purified using Wizard PCR Preps DNA purification System (Promega), and the digested plasmid pFastBac1 was purified using Wizard DNA Clean-up System (Promega) (Chapter 2, section 2.3.5.2 and 2.3.5.3). The purified DNA fragment and plasmid DNA were then ligated together with ligase at 14°C overnight. The ligation reaction was: 2μl of digested pFastBac1 plasmid DNA (40ng), 4μl of digested DNA fragment from plasmid C8 (40ng), 1μl of 10X ligase buffer, 2μl of H_2O and 1μl of ligase (5 weiss unit/μl, NEB).

Table 7.2 The digestion reactions with restriction enzymes *SalI* and *NotI*.

	Plasmid C8 5μl (100ng/μl)	pFastBac1 3μl (100ng/μl)
10x O buffer	4μl	4μl
H_2O	28μl	30μl
SalI (10U/μl)	1.5μl	1.5μl
NotI (10U/μl)	1.5μl	1.5μl
	Incubate at 37°C for 3 hours	

The sites for restriction enzymes *EcoRI* and *XhoI* in the expression vector pFastBac HTb were chosen as the appropriate cloning sites. The expression vector pFastBac HTb and the recombinant plasmid E15 and E16 were digested with restriction enzymes *EcoRI* and *XhoI*, respectively. The digestion reactions are listed in Table 7.3. After digestion, the DNA fragment digested from plasmid E15 and E16 (size about 770bp) were purified using Wizard PCR Preps DNA purification System (Promega), and the digested plasmid pFastBac HTb was purified using Wizard DNA Clean-up System (Promega) (Chapter 2, section 2.3.5.2 and 2.3.5.3). The purified DNA fragments and plasmid DNA were then ligated with ligase at 14°C overnight. The ligation reactions were:

1) 8μl of digested pFastBac HTb plasmid DNA (40ng), 9μl of excised DNA fragment from plasmid E15 (30ng), 2μl of 10X ligase buffer and 1μl of ligase (5 weiss unit/μl, NEB).
2) 8μl of digested pFastBac HTb plasmid DNA (40ng), 4.5μl of excised DNA fragment from plasmid E16 (22.5ng), 1.5μl of 10X ligase buffer and 1μl of ligase (5 weiss unit/μl, NEB).

Table 7.3 The digestion reactions with restriction enzymes *EcoRI* and *XhoI*.

	Plasmid E15 5μl (100ng/μl)	Plasmid E16 4μl (150ng/μl)	pFastBacHTb 3μl (100ng/μl)
10x R buffer	2μl	2μl	2μl
H_2O	11μl	12μl	13μl
EcoRI (10U/μl)	1μl	1μl	1μl
XhoI (10U/μl)	1μl	1μl	1μl
Incubate at 37°C for 3 hours			

7.2.2.4 Transformation of ligated plasmid DNA

Competent *E. coli* cells (TG2) were prepared as described in Chapter 2, section 2.9.1.6. 100μl of pre-prepared competent cells was taken from the -80°C freezer for the transformation. Half of the ligation reaction from each independent reaction described in section 7.2.2.3 was transformed following the procedure as described in Chapter 2, section 2.9.1.7. The transformant DNA samples were prepared and digested with restriction enzymes (*SalI* and *NotI*, or *EcoRI* and *XhoI*). The digestion reactions were electrophoresed on agarose gel to identify the positive clones.

7.2.2.5 Transposition

The pre-prepared DH 10BAC competent cells were taken from -80°C and thawed on ice. 100μl of the cells was dispensed into 1.5 ml eppendorf tubes. 100ng of recombinant donor plasmid DNA (the expression vectors pFASTBAC1 and pFASTBACHTb that were cloned with OTR cDNA fragment respectively) were added with gently mixing. The mixture was incubated on ice for 30 minutes. The mixutre was heated at 42°C for 2 minutes, and then was chilled on ice. 450μl of LB medium was added and the tube was placed in a shaking incubator at 37°C with medium agitation for 4 hours. The cells were diluted in 1:2, 1:4 and 1:10 with LB. 100μl of each dilution was placed on the Luria Agar plates and spread evenly over the surface. The plates were incubated at 37°C for at lease 24 hours according to the instruction.

7.2.2.6 Isolation of recombinant bacmid DNA

Each of white recombinant clones was inoculated into 3 ml LB medium containing kanamycin (50 μg/ml), gentamicin (7μg/ml) and tetracycline (10μg/ml) and the culture grew at 37°C with shaking (200 cycle/minute) overnight. 1.5ml of the culture was transferred to an Eppendorf tube and was centrifuged at 6000rpm for 1.5-2 min to pellet the cells. The supernatant was removed by aspiration, leaving the pellet as dry as

possible. The pellet was resuspended in 300 µl of Solution I (15 mM Tris-HCl pH 8.0, 10 mM EDTA, 100µg/ml RNase A) by repeated pipetting. 300 µl of Solution II (0.2N NaOH, 1% SDS) was added and the solution was mixed by inverting, the tube was then incubated at room temperature for 5 min. 300 µl of Solution III (3 M potassium acetate pH 5.5) was added slowly, and the tube was mixed gently and put on ice for 10 min. After centrifuging at 14000 rpm for 10 min, the supernatant was carefully decanted into a clean tube. 0.8ml of absolute isopropanol was added and the solution was mixed gently, then the tube was incubated on ice for 10 min. The sample was centrifuged at 14000 rpm for 15 min. The supernatant was discarded and the pellet was washed several times with 70% ethanol. After centrifuging at 14000 rpm for 5 min, the supernatant was removed. The pellet was air-dried briefly and was dissolved in 20 µl of TE buffer. The sample was stored at -20°C for further use.

7.2.2.7 Analysis of recombinant bacmid DNA by PCR

The PCR was carried out with the pUC/M13 amplification primers. The reaction was: 5µl of 10X PCR buffer, 1.5µl of 50mM $MgCl_2$, 2.5µl of primer mix (1.25µl each, 10pmol/µl), 1µl of template DNA and 0.5µl of Taq polymerase (2.5 units). After incubation at 93°C for 3 minutes, the amplification reaction was performed over 35 cycles as follows: 94°C for 45 seconds, 55°C for 45 seconds and 72°C for 5 minutes.

7.3 Results and Discussion

7.3.1 Expression of oxytocin receptor cDNA fragment in the pGEX vector

The PCR reactions used for creating the cloning site produced a PCR product E5 generated by primer M13-20 and Ped, and a PCR product E6 generated by primer T7 and Ped. Both of these products were of the expected size (about 950bp) with E5 about 30bp larger than E6 (Fig 7.2). After cloning into the PGEX-5X-2 vector, ten of the thirteen colonies were found to be positive. The positive clones Number 6, 7, 13, 14, 15 and 16

were induced with IPTG for fusion protein expression. The host cells of these positive clones were *XL-1 blue*. As shown in Fig 7.4, there was no induced band present after IPTG induction in SDS-PAGE analysis. Western blot also did not detect any positive band at the appropriate size. Although the different induction conditions were chosen by changing the induction temperature from 37°C to 30°C and by starting induction at OD_{600}=0.3 instead of OD_{600}=0.6, the same negative results were obtained.

The recombinant plasmid DNA prepared from positive clones Number 6 and 16 were transformed into *TG2* cells for the detection of fusion protein expression. The plasmid DNA prepared from positive clone Number 6 were transferred into GT2 cells. Positive clones named TG2-6-21, TG2-6-23, TG2-6-31, TG2-6-32 and TG2-6-33 were selected for fusion protein expression analysis. A band of size around 56 kDa was found after IPTG induction using SDS-PAGE analysis (Fig 7.5 A, B). A faint band at a similar position was also detected by Western blotting. However, this band lost its colour very quickly after development that did not allow time for photography (as shown in Fig 7.5 C). A similar result was obtained when the recombinant cells where transferred from positive clone Number 16 were induced with IPTG for fusion protein expression analysis (data not shown).

According to the instruction from the manufactures, any *E. coli* cells can be used as a host cell. In this study, *XL-1Blue* and *TG2* cells were selected. When recombinant plasmid DNA was transferred into *XL-1Blue* cells, the colony appeared to be transparent and the cells grew slowly in comparison with the control cell that contained PGEX-5X-2 plasmid DNA without the DNA insert. When recombinant plasmid DNA was transferred into *TG2* cells, an induced band at the appropriate size of 56 kDa was found after IPTG induction in SDS-PAGE analysis (Fig 7.5), although this band lost its colour very quickly following Western blotting and the level of fusion protein expression was very low. Results from this study indicated that not all *E. coli* cells are suitable host cells for fusion protein expression.

DNA sequencing result (Fig 7.6) has shown that the OTR cDNA fragment was cloned into pGEX-5X-2 vector in a reading frame with a stop codon at C-terminal. The reason for the low expression of the fusion protein remains unclear. Possible reasons could include: firstly, the cloned insert may be toxic to the host cells that cause catabolite repression. Secondly, the fusion protein may be insoluble. Although the cells were disrupted by sonication, only the samples collected from sonicated supernatants were used for SDS-PAGE analysis in this study. The insoluble part of the cell pellet could have been used for SDS-PAGE analysis. Thirdly, for future studies the sequence of recombinant plasmid DNA should be analyzed carefully, in order to determine if there was a base addition/deletion which could cause a shift in the reading frame. Fourthly, further induction conditions would be needed and optimised to increase possible expression.

7.3.2 Expression of OTR cDNA fragments in Bac-to-Bac baculovirus expression system

When the insert of the OTR cDNA fragment from plasmid DNA C8, was cloned into the expression vector pFASTBAC1, one positive colony was identified by screening of the recombinants by restriction enzyme digestion (as shown in Fig 7.7). Five and three positive colonies were identified by restriction enzyme digestion when the insert from plasmid DNA E15 and E16 was cloned into expression vector pFASTBAC HTb respectively (as shown in Fig 7.7). After transposition, both white and blue colonies appeared and the white colonies were selected for recombinant bacmid DNA preparation. The recombinant bacmid DNAs were analyzed by agarose gel electrophoresis and PCR. The agarose gel electrophoresis analysis did not determine categorically if the recombined mini-Tn7 element on the pFastBac donor plasmid had transposed to the mini-attTn7 target site on the bacmid successfully. The results from PCR did not show any positive colonies from transposition (as shown in Fig 7.8). The possible reasons for failure to detect a number of positive clones could be:

1) The quality of recombinant donor DNA used for transposition was poor, since resulting in an unsuccessful transposition.
2) The *DH 10Bac* competent cells prepared in this study were not very efficient in comparison with commercially competent cells that were not available for the experiment due to financial constraints.
3) The PCR conditions for identification were not fully optimised leading to negative results.

After a number of attempts at obtaining an efficient expression system, no further experiments were undertaken because of time constraints.

Cloned sequence of OTR cDNA fragment (759bp) in PCR-Script SK(+) plasmid

```
    M13-20      T7
5'.....----.....-----  .....GAA TTC CTG CAG CCC GGG GGA TCC GCC → continue to
                                                                  next line
                                          SmaI

                                        (A)      (C)
          [CTC TTC..... AGC CTC AAC AGC TGC*]GGGCTA*GAGCGGCCG.....3'
Ped primer sequence  3'TCG GAG TTG TCG ACT  CCCGAG CTCGC.....5'
                                             XhoI
```

Cloning site in expression vector pGEX-5X-2

```
5'..... GGG ATC CCC GGA ATT CCC GGG TCG ACT CGA GCG GCC GCA TCG TGA ... 3'
                            SmaI         XhoI
```

Fig 7.1 Sequences show the cloned OTR cDNA fragment (759bp) in PCR-Script SK(+) plasmid and the cloning site in expression vector pGEX-5X-2. Primer Ped was designed to create the stop codon TGA and *XhoI* site as shown above. *SmaI* and *XhoI* were chosen to clone the OTR cDNA fragment from recombinant plasmid DNA C8 into the expression vector pGEX-5X-2. --- indicates the position of universal primers M13 and T7. * indicates the mutant site. The sequence labelled by [ ] is the OTR cDNA sequence.

Restriction enzyme sites in recombinant plasmid DNA E15 and E16 (Cloned sequence of PCR products E5 and E6 in pGEX-5X-2 Vector)

```
5'..... GGG ATC CCC GGA ATT CCC GGG GGA TCC GCC [CTC TTC TTT TTC ATG.....CTT
        BamHI        EcoRI
CTG GCC AGC CTC AAC AGC TGA] GGG CTC GAG CGG CCG CAT CGTGA.....3'
                          stop       XhoI
```

Cloning site in expression vector pFastBac HTb

```
5'.....ATG TCG TAC TAC CAT CAC CAC CAT CAT CAC GAT TAC GAT ATC CCA ACG ACC
    met ser tyr tyr his his his his his his asp tyr asp lie pro thr thr
                              6xHis                      spacer region
GAA AAC CTG TAT TTT CAG * GGC GCC ATG GGA TCC GGA ATT CAA AGG CCT ACG.....
glu asn leu tyr phe gln   gly          BamHI    EcoRI
 rTEV protease cleavage site
GCC TGC AGT CTC GAG GCA TGC GGT ACC AAG CTT GTC GAG AAG TAC TAG.....3'
    PstI      XhoI   SphI    KpnI   HindIII                  stop
```

Restriction enzyme sites in recombinant plasmid DNA C8

```
5'.....GTC GAC GGT ATC GAT AAG CTT GAT ATC GAA TTC CTG CAG CCC GGG GGA TCC
     SalI               HindIII          EcoRI   PstI    SmaI
GCC[CTC TTC TTT TTC ATG.....CTC AAC AGC TGC]GGG CTA GAG CGG CCG CGG TGG 3'
                                                    NotI
```

Cloning site in expression vector pFastBac1

```
5'.....AGTATT.....CGG AAT TCA AAG GCC TAC GTC GAC GAG CTC ACT AGT CGC GGC CGC
                EcoRI      StuI       SalI    SstI    SpeI      NotI
TTT CGA .....GAT CATAA .....3'
                stop
```

Fig 7.2 Sequences showing the cloning sites in expression vectors pFastBac HTb and pFastBac1. *EcoRI* and *XhoI* were chosen to clone the OTR cDNA fragment from recombinant plasmid DNA E15 and E16 into pFastBac HTb. The cloning sites are highlight in red. *SalI* and *NotI* were chosen to clone the OTR cDNA fragment from the recombinant plasmid DNA C8 into pFastBac1. The cloning sites are highlighted in green. The original translational start site is highlight in yellow. * indicates the rTEV protease cleavage site between the amino acid site gln and gly. The sequence labelled by [ ] is the OTR cDNA sequence.

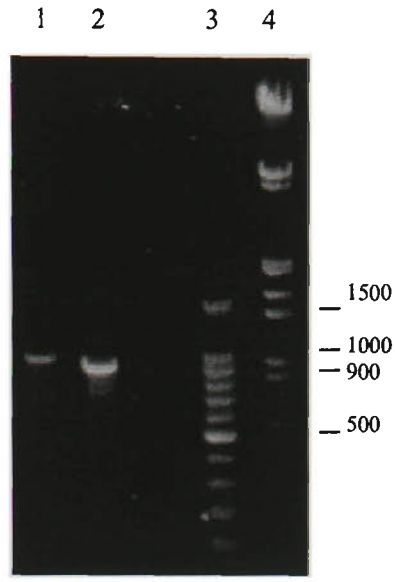


Fig 7.3 Agarose gel electrophoresis showing the PCR products generated by primers Ped, T7 and primers Ped, M13-20 using plasmid C8 as a template. Lane 1, the PCR product generated by primers Ped and M13-20. Lane 2, the PCR product generated by primers Ped and T7. Lane 3, 100bp DNA markers (Life Technologies). Lane 4, λ DNA/*EcoRI*+ HindIII markers. → indicates the size of markers (bp).

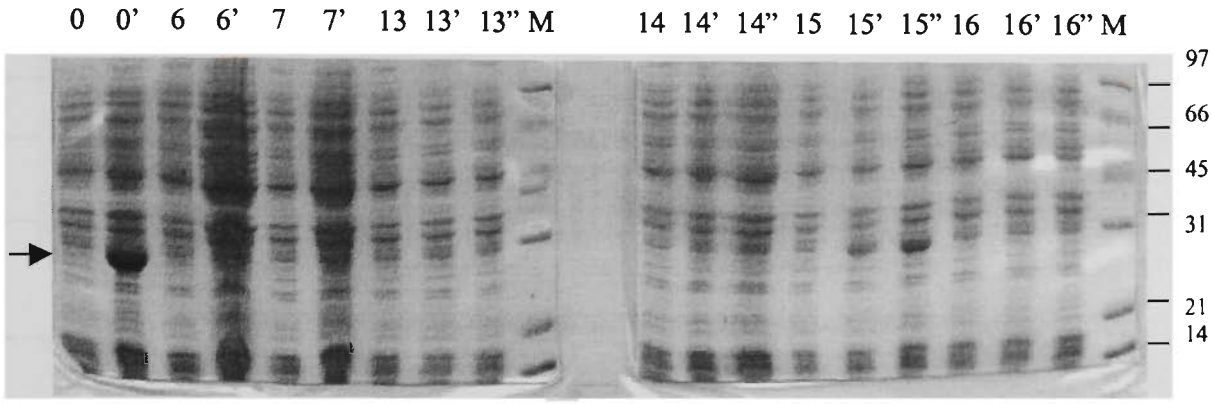
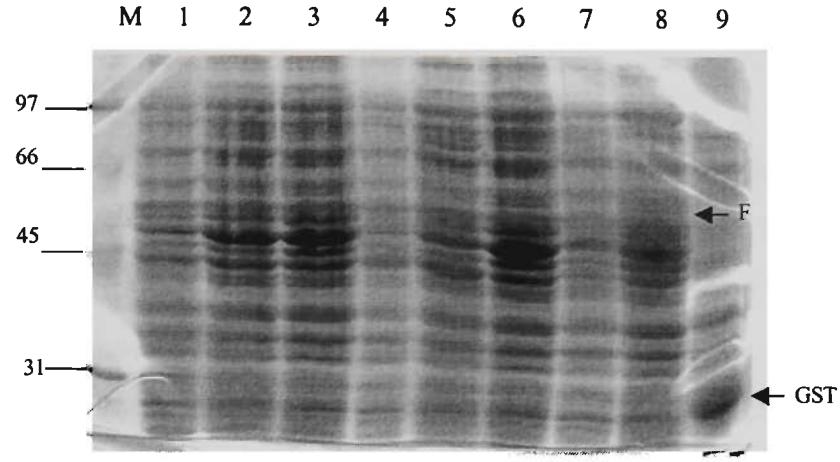
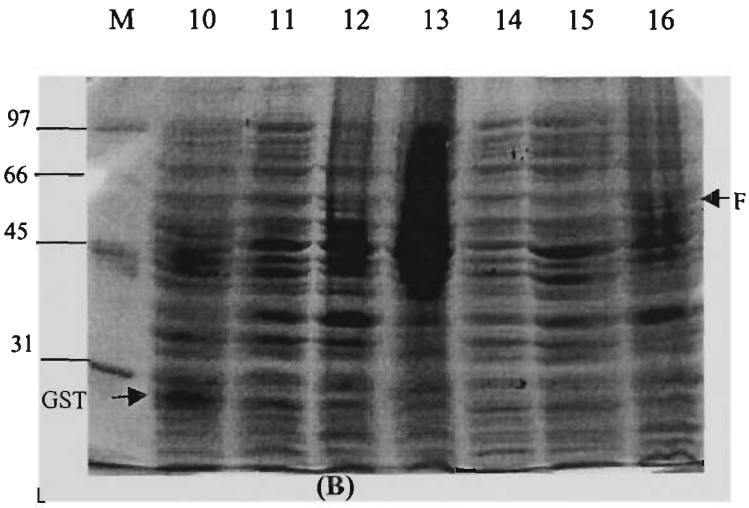


Fig 7.4 SDS-PAGE analysis of fusion protein expression in XL-1 blue host cells. The IPTG induction started at OD600 = 0.3. Number 0 represents the cells with PGEX-5X-2 vector used as control. The other numbers represent the positive colonies. The numbers without any labels indicate the induction time as 0 hour. The numbers with label ' and " indicate the induction time as 1.5 hours and 2.5 hours, respectively. Cells of clones number 0, 6, 7, 13 were induced at 37°C and cells of clones number 14, 15, 16 were induced at 30°C. M, low range protein markers (Bio-Rad). – indicates the molecular weight of the markers (kDa). → indicates the band of GST fusion protein.

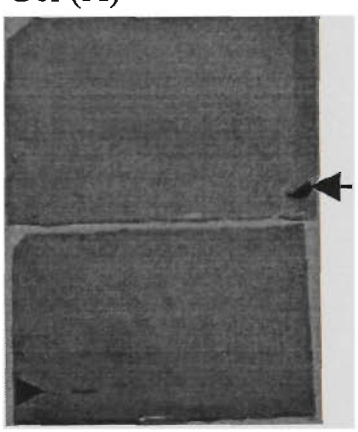


(A)



(B)

Gel (A)



Gel (B) (C)

Fig 7.5 SDS-PAGE and Western blotting analysis of fusion protein expression in TG2 host cells. All the IPTG induction started at OD600 = 0.6. Lane 9 and 10, the colonies with PGEX-5X-2 vector used as control that the cells were induced by IPTG for 2 hours and 1.5 hours, respectively. Lane 1-3, clone TG2-6-21; Lane 4-6, clone TG2-6-23; Lane 7-8, clone TG2-6-33; Lane 11-13, clone TG2-6-31; Lane 14-16, clone TG2-6-32. In each positive clone the cells were induced by IPTG for 0, 1.5 and 3 hours respectively, except clone TG2-6-33 in Lane 7-8 where the cells were induced by IPTG for 0 and 3.5 hours. F, possible fusion protein band. GST, GST fusion protein band. ➔ in picture C indicates the positive GST fusion protein band from Western blotting.

```
5'... GGG ATC CCC GGA ATT CCC GGG GGA TCC GCC[CTCTTCTTCTTCATGAAGCAC
                        SmaI                 L  F  F  F  M  K  H

CTGAGCATAGCCGACCTGGCGGTGGCGGTGTTCCAGGTGCTGCCGCAGCTTCTGTGGGAC
L  S  I  A  D  L  A  V  A  V  F  Q  V  L  P  Q  L  L  W  D

ATCACGTTCCGTTTCTACGGGCCCGACCTGCTCTGCCGCCTCGTCAAGTACCTGCAGGTG
I  T  F  R  F  Y  G  P  D  L  L  C  R  L  V  K  Y  L  Q  V

GTGGGCATGTTCGCGTCCACCTACCTGCTGCTGCTCATGTCGCTCGACCGCTGCCTGGCC
V  G  M  F  A  S  T  Y  L  L  L  L  M  S  L  D  R  C  L  A

ATCTGCCAGCCGCTGCGCTCGCTGCGCCGCCGCACCGACCGCCTGGCGGTACTCGCCACC
I  C  Q  P  L  R  S  L  R  R  R  T  D  R  L  A  V  L  A  T

TGGCTCGGCTGCCTGGTGGCCAGCGCGCCGCAGGTGCACATCTTCTCGCTGCGCGAGGTG
W  L  G  C  L  V  A  S  A  P  Q  V  H  I  F  S  L  R  E  V

GCCGACGGCGTCTTCGACTGCTGGGCCGTTTTCATTCAGCCCTGGGGGCCCAAGGCCTAC
A  D  G  V  F  D  C  W  A  V  F  I  Q  P  W  G  P  K  A  Y

ATCACGTGGATCACGCTCGCCGTCTACATTGTGCCGGTCATCGTGCTTGCCGCCTGCTAC
I  T  W  I  T  L  A  V  Y  I  V  P  V  I  V  L  A  A  C  Y

GGCCTTATCAGCTTCAAGATCTGGCAGAACTTACGGCTCAAGACGGAGGCGGCGGCGGCA
G  L  I  S  F  K  I  W  Q  N  L  R  L  K  T  E  A  A  A  A

GAGGCTGCCGCGGGGGCTGAGGGCGCGGCGGCAGACTGCGCGGGGCGCGCGGCTCTGGCC
E  A  A  A  G  A  E  G  A  A  A  D  C  A  G  R  A  A  L  A

CGCGTCAGCAACGTCAAGCTCATCTCTAAGGCTAAGATCCGCACGGTCAAGATGACCTTC
R  V  S  N  V  K  L  I  S  K  A  K  I  R  T  V  K  M  T  F

ATCGTCGTGCTGGCCTTCATCGTGTGCTGGACGCCATTCTTTTTCGTGCAGATGTGGAGT
I  V  V  L  A  F  I  V  C  W  T  P  F  F  F  V  Q  M  W  S

GTCTGGGATGCCGATGCGCCCAAGGAAGCCTCGGCTTTCATCATCGCCATGCTTCTGGCC
V  W  D  A  D  A  P  K  E  A  S  A  F  I  I  A  M  L  L  A

AGCCTCAACAGCTGCTGA]GGG CTCGAG CGGCCGCATCGTGA...3'
S  L  N  S  C  C       XhoI
```

Fig 7.6 DNA sequence showing the sequence of the recombinant plasmid DNA pGEX-5X-2 in the region of the cloning sites. The sequence labelled by [ ] is the OTR cDNA sequence.

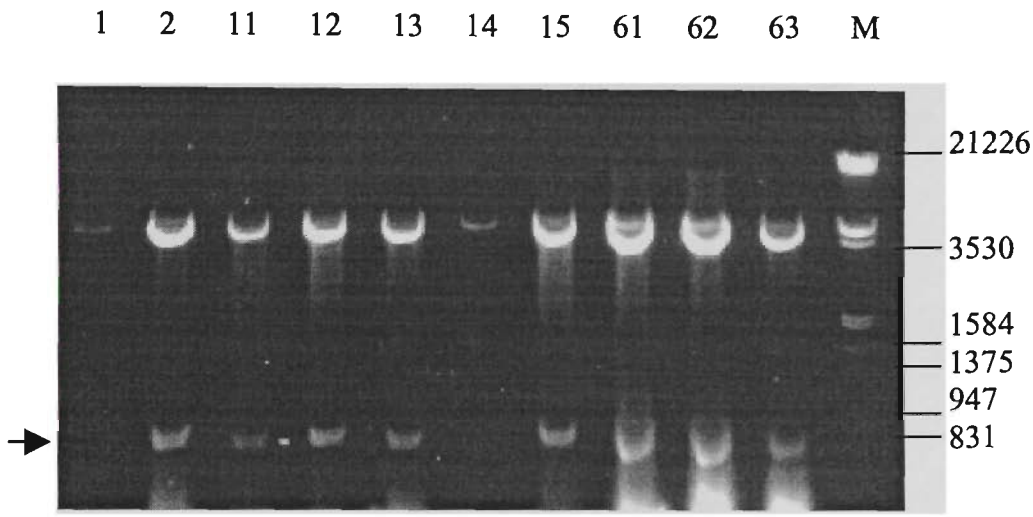


Fig 7.7 Agarose gel to show screening of the recombinants with restriction enzyme digestion. Numbers indicate the individual clones. M, λ DNA/*EcoRI*+ *HindIII* Molecular Weight. — indicates the size of markers (bp). Clones 1, 2, pFastBac1+DNA fragment from recombinant plasmid DNA C8 digested by *SalI* and *NotI*. Clones 11-15, pFastBac HTb +DNA fragment from recombinant plasmid DNA E15 digested by *EcoRI* and *XhoI*. Clones 61, 62, 64, pFastBac HTb+DNA fragment from recombinant plasmid DNA E16 digested by *EcoRI* and *XhoI*. ➔ indicates the expected DNA digestion bands.

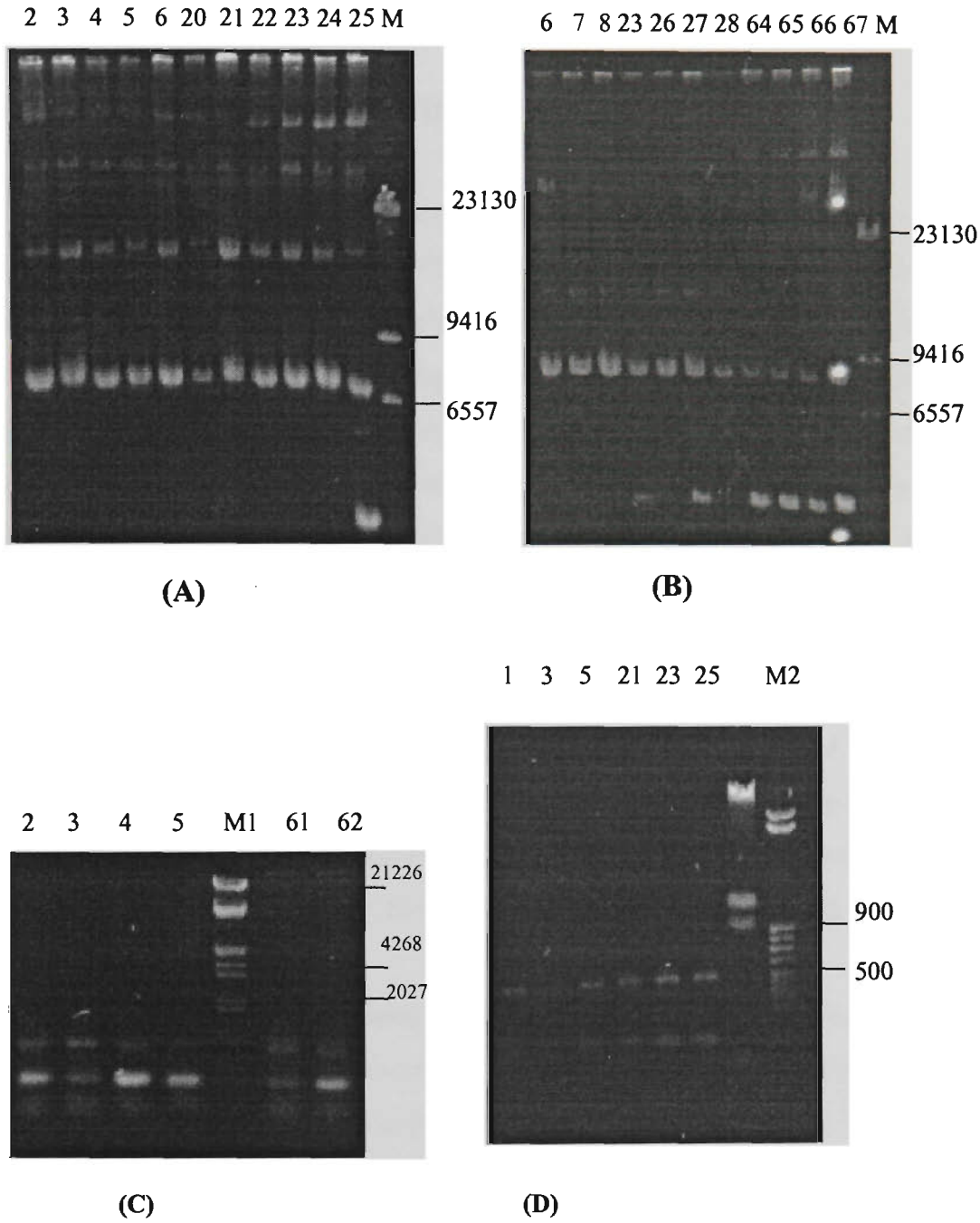


Fig 7.8 Analysis of recombinant bacmid DNA using agarose gel eletrohporesis and PCR. A and B: agarose gel eletrohporesis showing the migration of recombinant bacmid DNA. C and D: PCR results for identification of each recombinant bacmid DNA. The expected size of PCR products should be around 3.0 Kb. Numbers indicate the individual clone of recombinant bacmid DNA. M, λ DNA/*HindIII* markers. M1, λ DNA/*EcoRI*+*HindIII* markers. M2, 50bp DNA markers. - indicates the DNA size.

Chapter 8 Conclusions and Recommendation

8.1 Characterization of OTR cDNA

In this study, OTR mRNA was extracted from ovine uterine endometrium collected on day 15 of the estrous cycle, and OTR cDNA was obtained from mRNA using RT-PCR techniques. Data based on the determined sequence of OTR cDNA gave a size of 1176 bp for the OTR gene in the coding region. The putative amino acid sequence derived from this cDNA revealed the occurrence of seven hydrophobic transmembrane regions, which is characteristic of a G-protein coupled receptor. The sequence of this cDNA has high homology with OTRs of human, rat, pig and cattle respectively. A comparison of the cDNA sequence of OTR in this study with the published sheep endometrial OTR cDNA sequence of Riley *et al.* (1995) showed seven nucleotides alterations in the coding region. The possibility of these being PCR-generated errors was considered to be unlikely as indicated in chapter 3. This study has provided additional sequence information of OTR cDNA in sheep, particularly in the 3'untranslated region, to that reported previously. The additional OTR cDNA sequence information (917 bp) in the 3'untranslated region in sheep has been first reported and published.

Northern hybridization indicated multiple transcripts of sizes 5.6kb, 3.3kb, 2.1kb and 1.5kb in the uterine tissues in sheep. In this study it was shown that the heterogeneity of the different sized OTR transcripts in sheep was due in part, to the alternative use of polyadenylation sites. The sequences of two smaller transcripts were confirmed by combining the OTR cDNA sequence in the coding region with that in the 3'untranslated region. However, the sequences of the other two transcripts in uterus and the single transcript in both the pituitary gland and in the corpus luteum remain to be identified. The genomic DNA sequence in sheep has to our knowledge not been previously reported. In order to have a better understanding of the mechanisms involved in the regulation of OTR gene expression, further work should be conducted to determine the DNA sequence of the OTR in sheep, especially in the promoter region. Technically, it is

possible to achieve this using a combination of screening the cDNA and genomic DNA libraries with different PCR strategies.

8.2 Determination of OTR gene expression

In situ hybridization and OT binding assay were used in previous studies to determine the level of both OTR mRNA expression and OTR protein expression during the estrous cycle. Northern hybridization was used in this study to detect OTR gene expression and to determine the level of OTR mRNA expression in different tissues at different stages of the estrous cycle and at parturition. Experiments undertaken during the estrous cycle indicated that OTR mRNA expression in the endometrium was high at estrus compared with days 2, 7 and 12 of the cycle. No work in detecting the levels of OTR mRNA expression in the pituitary gland during the estrous cycle have been reported previously. This work indicated there was no significant difference in OTR mRNA expression in the pituitary gland during the estrous cycle. The results of this study showed that OTR mRNAs were highly expressed in the both endometrium and myometrium at the time of labour. It was found that OTR mRNA was expressed in the corpus luteum on day 12 of the estrous cycle. This is the first demonstration of OTR mRNA being expressed in the corpus luteum in sheep by Northern hybridization. The results also showed the presence of multiple transcripts in the endometrium and myometrium, whereas there was a single transcript in the pituitary gland and in the corpus luteum. It is suggested from the results of this study, that the pattern of OTR gene expression is not only tissue-specific, but also highly function-related.

Northern hybridization was unable to localize gene expression in different type of cells. To obtain a clear picture of gene expression in different type of cells, *in situ* hybridization would be the ideal technique. However the use of *in situ* hybridization to detect OTR gene expression was not possible, because the equipment for this work had not been installed at Victoria University of Technology when this work was undertaken.

8.3 Analysis of sequence variation in OTR transcripts

The results obtained in this study showed evidence of mRNA editing in both the coding region and 3'untranslated region (3'UTR) of OTR gene transcripts in ovine endometrium when 3'RACE systems were used to amplify the cDNA of OTR transcripts. This is the first demonstration of this phenomenon for OTR mRNA. Analysis of the secondary structure of the putative amino acid sequence (Plotstructure, ANGIS) indicated that editing of OTR mRNA resulted in amino acid changes in the carboxyl terminus which may affect G-protein coupling on the pathway of OT signal transduction. The finding of mRNA editing in OTR transcripts in this study has provided some evidence to suggest that the differences in the sequence of OTR mRNA populations in endometrium might be due to mRNA editing and that the existence of OTR subtypes, may result from mRNA editing. Further work needs to be carried out to determine whether mRNA editing occurred in the rest of the coding region at different stages of the estrous cycle or from different cell types.

The results also indicated that mRNA editing of OTR transcripts resulted in amino acid changes in the carboxyl terminus which might explain the differences in response of the OTR to physiological stimuli. To investigate the role of the edited OTR molecule on G-protein coupling, the following experiments might be considered for future work.

1. Combine the DNA fragments generated by RT-PCR from OTR mRNA into a entire OTR cDNA reconstruct.
2. Clone both edited and unedited OTR cDNA into an expression vector for transfection.
3. Choose a natural non-OTR secretory cell line as a suitable host cell for OTR expression, so that only the cloned OTR protein could be identified to reach the purpose without intracellular interruption.
4. Investigate the role of edited and unedited OTR on G-protein coupling and signal transduction. This could be conducted by:
   1) Determining the affinity of the expressed OTR for OT.

2) Detecting OT-stimulated PGE synthesis and an OT-stimulated increase of intracellular calcium concentrations to investigate the signal pathway.
3) Estimating the sensitivity of G-protein-OTR coupling to a pertussis toxin, a known inhibitor of Gi-OTR coupling, to identify the type of G-protein coupling for edited and unedited OTR proteins.

It has been demonstrated in this study that the sequence variation in OTR transcripts was due to mRNA editing. However, it is still unclear what bases have been altered in the polymorphic site and which enzymes are involved in the editing events. To identify the base changes, DNA sequences can be amplified from genomic DNA of OTR in specific regions using PCR techniques, given that the OTR cDNA sequence is now available to assist with the primer design.

8.4 Investigation of the role of oestradiol in the regulation of OTR and PGHS gene expression

In this study, the results showed that the OTR mRNA expression level did not change after estradiol treatment, but did increase in the controls (given oil only). Statistical analysis of the results indicated that there was a significant difference ($P<0.001$) between the estrogen and the oil (control) treatments and also an effect of time after treatment. It was shown that estrogen has an inhibitory effect on oxytocin receptor expression. However, the mechanism by which estrogen down-regulates OTR gene expression remains to be investigated.

The results obtained in this study showed that the OTR mRNA level in the endometrium did increase when treatment with progesterone was stopped. The rise in OTR mRNA expression levels in the oil-injected samples indicates that progesterone withdrawal may lead to an increase in OTR mRNA expression. Although previous studies have shown that the inhibitory effect of progesterone on the oxytocin receptor involves a direct interaction with the OTR at level of the cell membrane (Grazzini *et al.*, 1998), the possibility exists that progesterone may act via other genomic or nongenomic

mechanisms to regulate OTR gene expression indirectly. This hypothesis remains to be investigated.

8.5 Expression of OTR cDNA *in vitro*

No OTR protein expression work has been reported previously. All antibodies represented previously against OTR were generated from synthetic peptides (Adan *et al.*, 1995; Kimura 1995, 1997). The original aim of this study was to obtain OTR protein using commercially available expression systems, and then to prepare polyclonal antibodies. For this purpose, the cDNA fragment encoding II to VII transmembrane domains of OTR was cloned into both a glutathione S-transferase (GST) gene fusion system and Bac-to-Bac baculovirus expression system. However, very low level of fusion protein was expressed in the GST fusion protein expression system, and transposition experiment was not successful using Bac-to-Bac baculovirus expression system. The reason for these unsuccessful results is not clear and has been discussed in chapter 7. Although unsuccessful in providing large quantity of the expressed OTR protein, this study provided a clear indication that expression of OTR cDNA *in vitro* was achievable, and also provided valuable experience for OTR protein expression using the GST gene fusion system and Bac-to-Bac baculovirus expression systems for future studies.

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APPENDIX

1. Solutions and Buffers

Acrylamide/bis acrylamide solution (30% T, 2.67% C)

Acrylamide (29.2 g) and N'N'-bis-methylene-acrylamide (0.8 g) were made up to 100 mL with dH_2O. The solution was stored at 4°C in an amber bottle.

Acrylamide/bis solution (30% T, 0.45% C)

Acrylamide (29.865 g) and N'N'-bis-methylene-acrylamide (0.135 g) were made up to 100 mL with dH_2O. The solution was stored at 4°C in an amber bottle.

Denhardt's reagent X 50

An amount of 5 g of Ficoll (Type 400, Pharmacia), 5 g of polyvinylpyrrolidone and 5 g of bovine serum albumin was dissolved and made up to 500 mL with DEPC water, filter sterilised (0.2 μm filter) and stored at -20°C.

DEPC water

Deionised water containing 0.1% of DEPC was shaken vigorously, incubated overnight at 37°C and autoclaved for 30 minutes.

DNA extraction buffer

The buffer constituted of Tris (pH 8.0) 100 mM, EDTA (pH 8.0) 50 mM, NaCl 100 mM, SDS 1% and 10 mM –mercaptoethanol added fresh. The solution was autoclaved.

EDTA, 0.5 M (pH 8.0)

Disodium ethylenediaminetetra-acetate.$2H_20$ (186.1 g) was dissolved in 800 mL dH_2O by continuous stirring and the pH was adjusted to 8.0 with NaOH pellets. The volume was made up to 1 litre with dH_2O and autoclaved.

Electrode buffer (Running buffer) X 5, (pH 8.3)

Tris base (9 g), glycine (43.2 g) and SDS (3 g) to 600 mL with dH_2O. The solution was stored at 4°C. The working solution is 1 X buffer.

Ethidium Bromide (10 mg/mL)

Ethidium bromide (10 mg/mL) in autoclaved dH_2O was stirred for several hours to ensure that the dye has dissolved. The solution was stored in an amber bottle at 4°C.

Formaldehyde gel-loading buffer

The buffer constituted of 50% autoclaved glycerol, 1 mM EDTA (pH 8.0), 0.25% bromophenol blue and 0.25% xylene cyanol FF.

Gel loading buffer (for starch electrophorsis)

Bromophenol blue (0.25 %) was prepared in 40% (w/v) sucrose in water and stored at 4°C.

MOPS X 10

The buffer constituted of 200 mM of 3-[N-Mopholino] propane sulfonic acid, 80 mM sodium acetate and 10 mM EDTA (The pH was adjusted to 7 with 2N NaOH). Following 0.2 μm filter sterilisation, the stock buffer was stored in an amber bottle at room temperature.

Phenol-chloroform-isoamyl alcohol (25:24:1; v / v)

Liquified phenol (melted at 65°C), to which 8-hydroxyquinoline was added at a concentration of 0.1% (w/v), was equilibrated by extracting several times with 50 mM Tris base and 50 mM Tris-Cl (pH 8) until the pH was 8. One part of this phenol was mixed with one part of chloroform-isoamyl alcohol (24:1) and stored under 50 mM Tris-Cl (pH 8) buffer in an amber glass bottle at 4°C.

Prehybridisation solution

The solution constituted of 2 parts of Denhardt's reagent, 5 mM EDTA, 10 mM Tris-Cl, 0.5 M Na_3PO_4 (pH = 7), 5% SDS and treated salmon sperm DNA solution (100 ng/mL) added fresh.

RNAase (DNAase-free)

Ribonuclease A (from bovine pancreas) was dissolved at a concentration of 10 mg/mL in 0.01 M sodium acetate (pH 5.2) and heated at 100°C for 15 minutes. It was then cooled slowly to room temperature, dispensed into aliquots and stored at -20°C.

RNA marker stain

Methylene blue (0.04% w/v) dissolved in 0.5 M sodium acetate (pH 5.2).

Salmon sperm DNA

Salmon sperm DNA (ssDNA) solution (from Gibco-BRL) was prepared at a concentration of 10 mg/mL by dissolving in autoclaved dH_2O. The required amount for prehybridisation was denatured in a boiling water bath for 10 minutes and chilled for 2-3 minutes.

Sample buffer (for RNA)

The buffer was made up by mixing 250 µL formamide, 83 µL of 37% formaldehyde (pH > 3.5), 50 µL of 10 X MOPS buffer and the volume was adjusted to 400 µL with DEPC water and stored -20°C.

SDS 10%

Sodium dodecyl sulphate (100 g) was dissolved in 900 mL dH_2O by heating it to 68°C and the pH was adjusted to 7.2 with concentrated HCL. The volume was made up to 1 litre.

SDS gel-loading buffer (for protein)

The buffer constituted of 50 mM Tris.Cl (pH 6.8), 100 mM dithiothreitol, 2% SDS, 0.1% bromophenol blue and 10% glycerol. The buffer was stored at –20°C.

Sodium acetate 3 M (pH 5.2)

Sodium acetate.$3H_2O$ (408.1 g) was dissolved in 800 mL dH_2O, the pH was adjusted to 5.2 with glacial acetic acid and the volume made to 1 litre with dH_2O. The solution was autoclaved.

Sodium phosphate 1 M (pH 7)

To prepare 100 mL of the solution, 68.4 mL of 1 M Na_2HPO_4 was added to 31.6 mL of 1 M NaH_2PO_4.

SSC X 20

Sodium chloride (175.3 g) and sodium citrate (88.2 g) were dissolved in 800 mL of dH_2O. The pH was adjusted to 7 with 10 N NaOH and the volume was made up to 1 litre with dH_2O and autoclaved.

Stripping Solution

The solution constituted of dimethylformamide 50%, SDS 1% and Tris-HCL (pH 8.0) 50 mM.

Tris-acetate buffer (TAE) X 10

An amount of 400 mM Tris (pH 7.4), 200 mM sodium acetate and 20 mM EDTA (pH 8.0) were mixed and the pH was adjusted to 7.4 with glacial acetic acid. The volume was made up to 1 litre with dH_2O and the solution was autoclaved. The working solution is 1 X TAE.

Tris-borate buffer 0.1 M (pH 8.3) X 5

Tris base (54 g) and boric acid (27.5 g) were made up to 1 litre. The working solution is 1 X Tris-borate.

Tris-HCl 1 M

Tris base (121.1 g) was dissolved in 800 mL of dH_2O. The pH was adjusted with concentrated HCl as follows when solution was at room temperature:

pH	HCl
7.4	70 mL
7.6	60 mL
8.0	42 mL

The volume was made up to 1 litre and the solution was autoclaved.

Tris-HCl 1.5 M (pH 8.8)

Tris-base (18.15 g) was dissolved in 60 mL dH_2O, the pH was adjusted to 8.8 with 1N HCl and the volume was made to 100 mL with dH_2O. The solution was stored at 4°C.

Tris-HCl 0.5 M (pH 6.8)

Tris base (6 g) was dissolved in ~60 mL dH_2O, the pH was adjusted to 6.8 with 1 N HCl and the volume was made up to 1 litre.

TE Buffer (pH 7.4)

The solution constituted of 10 mM Tris-Cl (pH 7.4) and 1 mM EDTA (pH 8.0). The solution was autoclaved.

X-ray developer

400 mL developer concentrate (AGVA GEVART)

2 litres water

2. Reagents for plasmid DNA isolation

Solution I: 100 mM NaCl, 10 mM Tris-Cl (pH 7.4) and 1 mM EDTA (pH 8.0).

Solution II: 0.2M NaOH, 1% (w/v) SDS

Solution III: 3M potassium acetate (pH4.8-5.2). To prepare 100 mL, mix 60mL of 5M potassium acetate, 11.5 mL of glacial acid and 28.5 mL of ddH2O.

3.Restriction enzyme buffers

Buffer O: 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 100mM NaCl

Buffer R: 10 mM Tris-HCl (pH 8.5), 10 mM $MgCl_2$, 100mM KCl

Buffer Y: 33mM Tris-acetate (pH 7.9), 10 mM Mg-acetate, 66mM K-acetate

4. Bacterial media

LB:	Trypton	10g/L
	Yeast extract	5g/L
	Sodium chloride	10g/L
SOB:	Trypton	20g/L
	Yeast extract	5g/L
	Sodium chloride	0.5g/L

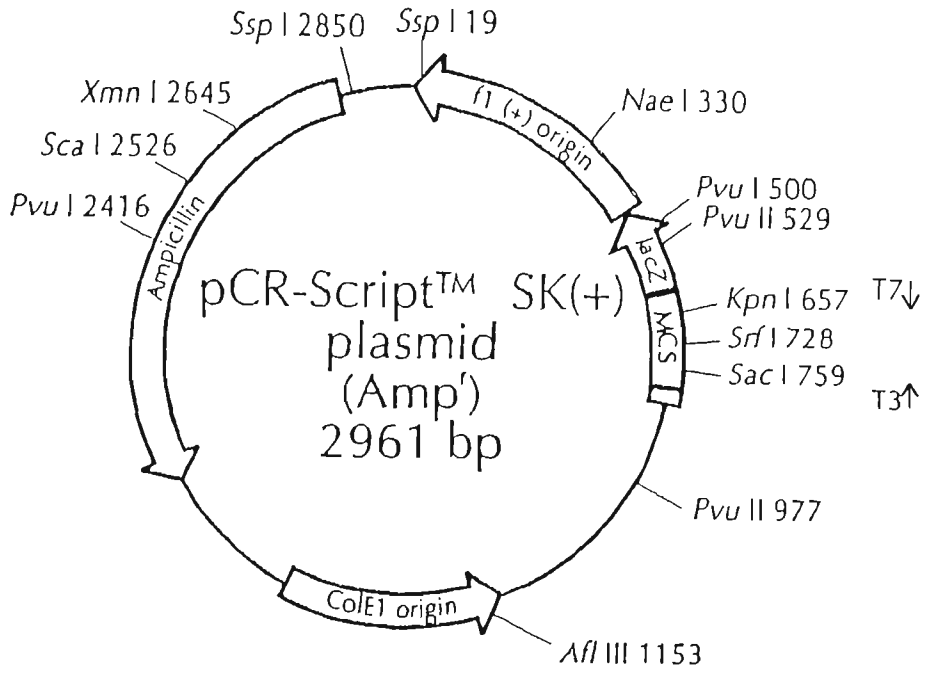
SOC: to 100mL SOB, add 1 mL of sterile 1M $MgCl_2$, 1mL of sterile 250 mM KCl and 2.78 mL of sterile 36% glucose before use.

LBA: LB+ 100μg/mL ampicillin

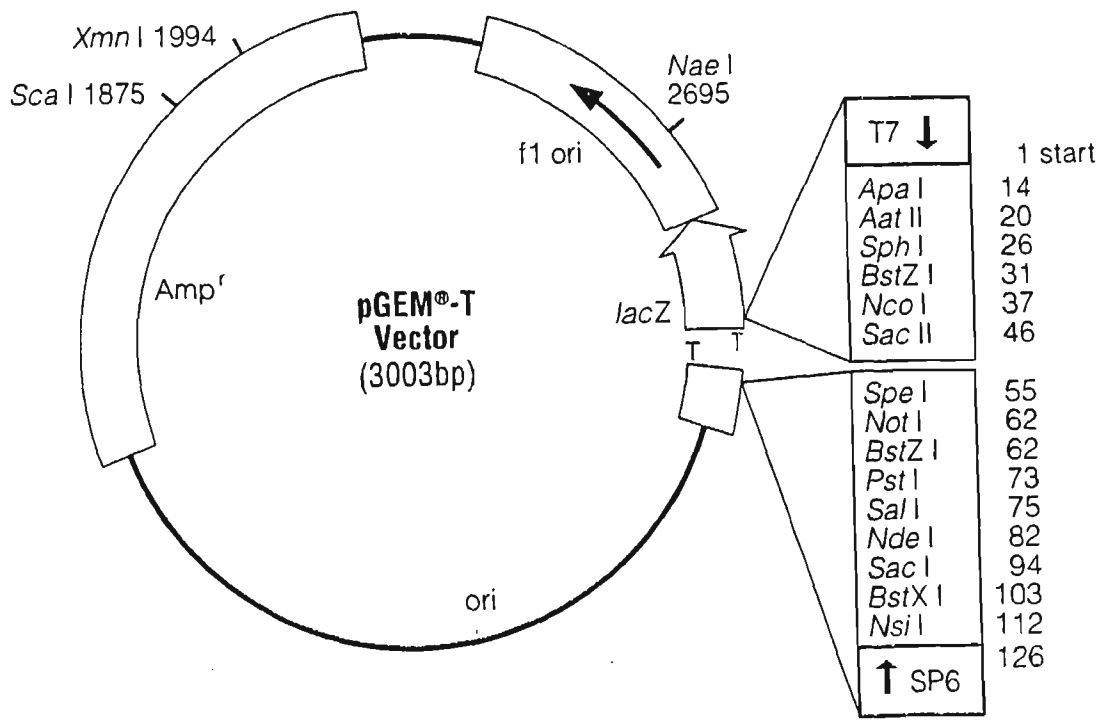
* note: all mediums used for bacterial culture should be autoclaved at 120 °C for 20 minutes.

5. Maps of the Cloning and the expression vectors

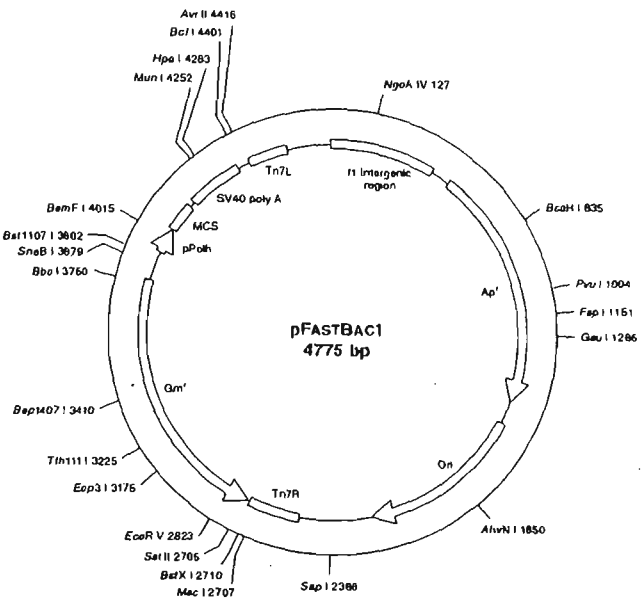
A. Map of the pCR-Script Amp SK(+) cloning vector (Stratagene)



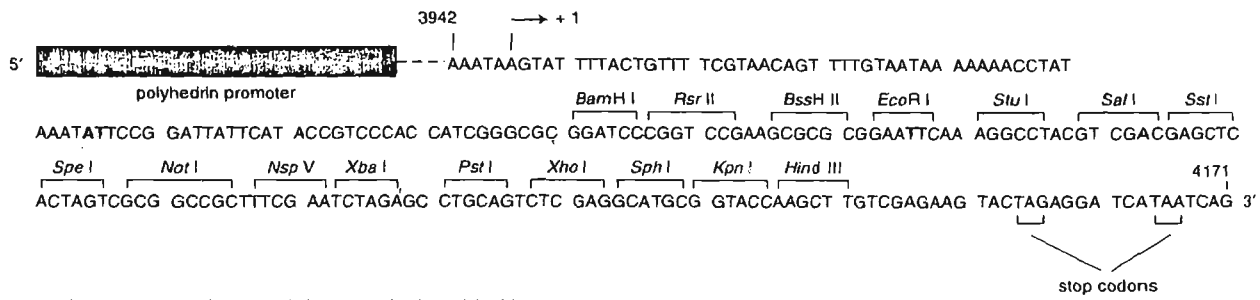
B. Map of the pGEM-T cloning vector (Promega)



C. Map of the pFastBac1 expression vector including multiple cloning site and promoter region (Life Technologies)



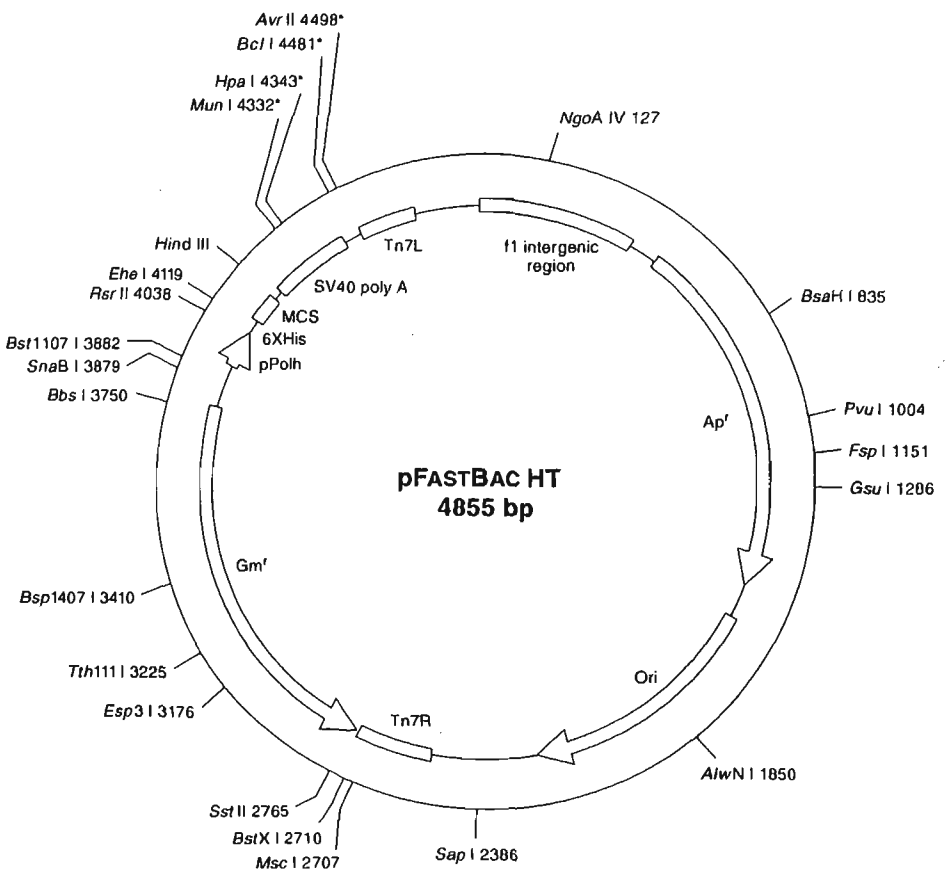
pFastBac1 MCS and promoter region:



+1 corresponds to the transcriptional start for the polyhedrin promoter.

ATT—site of original translational start. The ATG was mutated to an ATT.

D. Map of the pFastBacHTb expression vector including multiple cloning site and promoter region (Life Technologies)



pFastBac HTb

Rsr II

CTCGGTCCGAAACC ATG TCG TAC TAC CAT CAC CAT CAC CAT CAC GAT TAC GAT ATC CCA ACG ACC GAA AAC
met ser tyr tyr his his his his his his asp tyr asp ile pro thr thr glu asn
6xHis spacer region rTEV

Ehe I Nco I BamH I EcoR I Stu I Sal I Sst I Spe I

CTG TAT TTT CAG** GGC GCC ATG GGA TCC GGA ATT CAA AGG CCT ACG TCG ACG AGC TCA CTA GTC GCG
leu tyr phe gln gly ala met gly ser gly ile glu arg pro thr ser thr ser ser leu val ala
protease cleavage site

Not I Nsp V Xba I Pst I Xho I Sph I Kpn I Hind III

GCC GCT TTC GAA TCT AGA GCC TGC AGT CTC GAG GCA TGC GGT ACC AAG CTT GTC GAG AAG TAC TAG
ala ala phe glu ser arg ala cys ser leu glu ala cys gly thr lys leu val glu lys tyr stop