Identification of transcription factors interacting with the Oxytocin gene promoter

by

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Abstract

Oxytocin (OT) is a hormone that plays important functions in the physiology of reproduction. OT is highly expressed in specific neurons of the hypothalamus. OT is also expressed in other peripheral sites in moderate to low levels. The OT gene has been cloned and described in many species. The structural organization of the cloned OT genes is quite similar, irrespective of the species. Hypothalamic expression of OT represents a nice model of tissue specific expression. Indeed, OT and the related hormone vasopressin (AVP) are both expressed in the hypothalamus but are never co-expressed in the same neurons. More intriguing, the OT and AVP genes are believed to derive from one common gene by gene duplication and both genes are adjacent to each other on the chromosome in all species studied. Because of the lack of suitable cell lines expressing the OT gene, scientists have taken advantage of the availability of promoter sequences of numerous species to overcome this deficit. Alignment of the OT promoters available revealed stretches of high homology and further analysis of these stretches revealed that they are binding sites for the nuclear receptor superfamily of transcription factors such as the estrogen receptor (ER), retinoic acid receptor (RAR) and the thyroid hormone receptor (TR). Here, I identify that the orphan receptors, chicken ovalbumin up-stream promoter transcription factor II (COUP-TFII), ErbA-related factor 2 (Ear-2) and retinoid orphan receptor α1 (RORα1) as potential regulators of the OT gene promoter.

In contrast to the positive effect of the ER, RAR and TR on the OT promoter, COUP-TFII and Ear-2 have a negative effect on the human OT promoter. The effects of COUP-TFs on the OT promoter are two-fold; they are able to silence or actively repress basal promoter

activity and secondly, inhibit the activation by other nuclear receptors via a mechanism of competitive binding. Two additive DNA elements were identified to mediate the effect of COUP-TFII and Ear-2. The first one is a direct repeat of the AGGTCA motif spaced by zero nucleotide (DR0) that is embedded in the OT/estrogen response element (OT/ERE). The second element, located downstream of the OT/ERE, consists of three AGGTCA motifs each spaced by four nucleotides. Inhibition of the estrogenic induction only requires the DR0 whereas both elements are required for full silencing of basal activity. DNaseI footprinting and gel shift assays show that recombinant COUP-TFII and Ear-2 are able to bind to the DR0 and the downstream repeats. Mutations that affect the ability of COUP-TFII and Ear-2 to bind to these elements also affect their activity in transient transfection studies. The importance of COUP-TFII and Ear-2 in the regulation of OT gene expression is supported by their expression in the epithelial layer of the rat parturient uterus. The uterus is a known site of OT expression where a dynamic regulation of OT is observed.

The ROR/RZR is a novel subfamily of orphan receptors. They are characterized by their ability to bind DNA as a monomer. Three isoforms (α, β and γ) have been cloned. Although the expression pattern of this family is quite ubiquitous, tissue-specific expression of some members is also observed. Of interest, ROR/RZRβ is expressed mainly in the brain including the hypothalamus, the pineal gland and the thalamus. Since the hypothalamus and pineal gland are known sites of expression of OT, experiments were set up to investigate the possible role of ROR/RZRs in the regulation of OT gene promoter.

Transient transfection studies show the ability of RORα1 to activate the mouse OT promoter in Neuro2a cells. Deletion analysis of the OT promoter indicates that the active element is located between -225 to -118 of the promoter. DNaseI footprinting analysis using

this -225 to -118 region as a probe revealed that recombinant RORα1 protected two regions from the DNaseI digestion. The OT/ERE and a region 5' of the OT/ERE were identified as the protected regions. Mutations of either of the elements affected the transactivation by half and when both elements were mutated, activation was abolished. These results indicate that the two DNA elements are required for full transcriptional activation by RORα1.

Overall, this thesis describes the possible role of orphan receptors in the regulation of the OT gene promoter. The silencing effect of COUP-TFII and Ear-2 on basal promoter activity represents the first description of active repression of the OT promoter. Moreover, this is the first report of the activation of the OT promoter by RORα1.

Résumé

L'hormone ocytocine (OT) joue un rôle important dans la physiologie de la reproduction. L'expression d'OT est très élèvée au niveau de neurones spécifiques de l'hypothalamus. OT est également exprimée à un taux faible à modéré dans d'autres sites périphériques. Le gène de l'OT a été cloné et décrit chez plusieurs espèces. L'expression hypothalamique d'OT représente un bon modèle d'étude de l'expression tissulaire spécifique. En effet, OT et l'hormone apparentée vasopressine (AVP) sont exprimées dans des neurones distincts dans l'hypothalamus. Le plus surprenant est que les gènes OT et AVP dériveraient d'un gène commun par duplication gènique et ils sont adjacents sur le même chromosome (20 chez l'homme et 2 chez la souris) chez toutes les espèces étudiées. L'absence de lignée cellulaire exprimant le gène OT rend difficile l'étude du contrôle de son expression. L'alignement des séquences promotrices d'OT de différentes espèces a révélé l'existence de régions très conservées contenant des sites de liaison pour les récepteurs nucléaires tels que le récepteur des œstrogènes (ER), le récepteur de l'acide rétinoïque (RAR) et le récepteur de l'hormone thyroïdienne (TR). Dans notre présente étude, nous avons identifié les récepteurs nucléaires orphelins, chicken ovalbumin upstream promoter-transcription factor II (COUP-TFII), ErbA-related factor 2 (Ear-2) et retinoid orphan receptor α1 (RORα1) comme étant des régulateurs potentiels de l'expression du gène OT.

ER, RAR et TR ont un effet positif sur la régulation du gène OT tandis que COUP-TFII et Ear-2 ont un effet négatif. COUP-TFII et Ear-2 peuvent réprimer l'activité œstrogénique observée sur le promoteur OT. De même, ces facteurs sont capables de réduire l'activité basale du promoteur du gène OT humain dans la lignée cellulaire Neuro2a. Au

niveau du promoteur du gène OT, nous avons identifié deux éléments responsables de l'activité médiée par COUP-TFII et Ear-2. Le premier élément est une répétition directe du motif AGGTCA espacé de zéro nucléotide (DR0), incluse dans l'élément de réponse aux œstrogènes (ERE). Le second élément est localisé en aval de l'ERE et comprend trois motifs AGGTCA séparés l'un de l'autre par quatre nucléotides. L'inhibition de l'activité œstrogénique nécessite seulement l'élément DR0 alors que la réduction totale de l'activité promotrice basale est obtenue avec les deux éléments. Des expériences d'empreinte à la DNase I et de gel retard ont montré que les facteurs COUP-TFII et Ear-2 peuvent se fixer sur l'élément DR0 et les répétitions situées en aval. Des mutations, affectant la liaison de COUP-TFII et Ear-2 à ces éléments, affectent également leur activité observée en transfection transitoire. L'importance de COUP-TFII et Ear-2 dans la régulation de l'expression du gène OT est renforcée par leur présence au niveau des cellules épithéliales de l'utérus lors de la parturition. L'utérus est connu pour être le site d'une régulation dynamique de l'OT.

ROR/RZR est une nouvelle sous-famille de récepteurs orphelins caractérisés par leur capacité à lier l'ADN sous forme monomérique. Trois isoformes α, β, and γ ont été clonées. Bien que ces récepteurs soient ubiquistes, certains membres présentent une expression tissu-spécifique. Par exemple, ROR/RZRβ est principalement exprimé dans le cerveau incluant l'hypothalamus, la glande pinéale et le thalamus. L'hypothalamus et la glande pinéale étant des sites connus d'expression de l'OT, nous avons entrepris d'étudier le rôle possible des ROR/RZRs dans la régulation du promoteur du gène OT. Des expériences de transfection transitoire en cellules Neuro2a ont montré la capacité de RORα1 à activer le promoteur du gène OT murin. Une analyse par délétion du promoteur a révélé la présence d'un élément actif localisé entre les positions –225 et –118. Une étude d'empreinte à la DNase I sur la

région promotrice –225 à –118 a montré que RORα1 protège deux zones correspondant à l'OT/ERE et à une région en amont de cet ERE. Des mutations de l'un ou l'autre de ces éléments réduisent la transactivation par RORα1 de moitié et, lorsque les deux éléments sont mutés en même temps, l'activation du promoteur OT est abolie. Ces résultats indiquent que ces deux éléments ont un effet additif.

En conclusion, cette thèse décrit le rôle possible des récepteurs orphelins dans la régulation du promoteur du gène OT. L'effet répresseur de COUP-TFII et Ear-2 sur l'activité basale représente la première description d'une répression active du promoteur OT. De même, il s'agit de la première étude décrivant l'activation du promoteur OT par RORα1.

List of abbreviations

ACTH	Adrenocorticotropic hormone
AF-1	Activation function 1
AF-2	Activation function 2
AR	Androgen receptor
ARP-I	Apolipoprotein regulatory protein I
AVP	Arginine vasopressin
cAMP	3',5'-Cyclic adenosine monophosphate
CBP	CREB binding protein
COUP-TFI	Chicken ovalbumin upstream promoter transcription factor I
COUP-TFII	Chicken ovalbumin upstream promoter transcription factor II
COX	Cyclooxygenase
CREB	cAMP response element binding factor
DBD	DNA binding domain
DNA	deoxyribonucleic acid
DR	Direct repeat
Ear-2	Erb-A related receptor 2
ER	Estrogen receptor
ERE	Estrogen response element
ERR2	Estrogen related receptor 2
ES	Embryonic stem
GR	Glucocorticoid receptor

H3	Histone H3
H4	Histone H4
HNF-4	hepatocyte nuclear factor 4
IGF-I	Insulin-like growth factor I
IGF-II	Insulin-like growth factor II
IT	Isotocin
LBD	Ligand binding domain
LH	Luteneizing hormone
MR	Mineralocorticoid receptor
mRNA	messenger ribonucleic acid
N-CoR	Nuclear receptor co-repressor
NEO	Neomycin
NGFI-B	Nerve growth factor inducible factor B
Nurr1	Nur-related factor 1
OT	Oxytocin
OTR	Oxytocin receptor
PGF2α	Prostagandin F 2α
POU	Pit-1, Oct-1, Unc-86
PPAR	Peroxysome proliferator activated receptor
PVN	Paraventricular nucleus
RAR	Retinoic acid receptor
RXR	Retinoic X receptor
SF-I	Steroidogenic factor I

SMRT	Silencing mediator for retinoid and thyroid hormone receptors
SON	Supraoptic nucleus
SRC	Steroid receptor coactivator
STAT	Signal transducer and activator of transcription
SVP	Seven-up
TR	Thyroid hormone receptor
USP	Ultraspiracle
VDR	Vitamin D receptor
VT	vasotocin

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Preface

Guidelines from the thesis office

Candidates have the option of including, as part of the thesis, the text of one or more papers submitted or to be submitted for publication, or the clearly duplicated text of one or more published papers. These texts must be bound as an integral part of the thesis. If this option is chosen, connecting texts that provide logical bridges between the different papers are mandatory. The thesis must be written in such a way that it is more than a mere collection of manuscripts; in other words, results of a series of papers must be integrated.

The thesis must still conform to all other requirements of the "Guidelines for Thesis Preparation". The thesis must include: A table of Contents, an abstract in English and French, an introduction which clearly states the rationale and objectives of the study, a comprehensive review of the literature, a final conclusion and summary, and a thorough bibliography or reference list.

Additional material must be provided where appropriate (e.g. in appendices) and in sufficient detail to allow a clear and precise judgement to be made of the importance and originality of the research reported in the thesis.

In the case of manuscripts co-authored by the candidate and others, the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent. Supervisors must attest to the accuracy of such statements at the doctoral defense. Since the task of the examiners is made more difficult in these cases, it is in the

candidate's interest to make perfectly clear the responsibilities of all the authors of the co-authored papers. Under no circumstances can a co-author of any component of such a thesis serve as an examiner for that thesis.

The above described option was chosen in preparing the present thesis. The first chapter is the introduction containing a review of literature and presenting the objectives and rationale of the present study. Chapters II and III consist of reprints of published papers from Molecular and Cellular Endocrinology and Journal of Molecular Endocrinology. Chapter IV has been accepted for publication in the Journal of Molecular Endocrinology. CHAPTERS II to IV are preceded by short introductory texts that provide a link between them. The references found at the end are valid for Chapters I and V. The references for Chapters II to IV are found at the end of the respective chapter.

Apart from the following exceptions, all of the experimental work, data collection, and analysis described in the present work were performed by Khoi Chu under the supervision of Dr. H.H. Zingg

- In Chapter II, Dr. Jean-Marie Boutin kindly provided the plasmids and antibodies for rat COUP-TFII and Ear-2 before publication.

- In Chapter II, the northern blot membrane used was obtained from J. Neculcea, (Laboratory of Molecular Endocrinology).

- In Chapter II, the immunohistochemical localization of COUP-TFII and Ear-2 in the uterus was performed by Dr. C. Breton.

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Chapter I

Introduction and Literature Review

Chapter I Introduction and Literature Review

1.1 Anatomy of the hypothalamus

The hypothalamo-neurohypophysial neurosecretory system is composed of large neurons of the supraoptic and paraventricular nuclei (SON and PVN), which project to the posterior pituitary. These large neurons are referred to as magnocellular neurons and synthesize mainly the peptidic hormones oxytocin (OT) and arginine vasopressin (AVP). In general, OT and AVP are expressed in distinct and mutually exclusive neurons (Mohr *et al.*, 1988). OT expressing magnocellular neurons project to the posterior pituitary where OT is released into the circulation to serve peripheral functions (see below). Within the PVN, there exists another type of neuron called parvocellular neuron that also expresses OT and projects into the portal system of the median eminence. Here, OT is believed to play a role in anterior pituitary hormone secretion. In addition OT producing parvocellular neurons also project to discrete regions of the brain such as the amygdada, septum, hippocampus, ventral tegmental area, frontal cortex, brainstem, pons, medulla and spinal cord where OT acts as a neurotransmitter (reviewed in Sawchenko and Swanson, 1982; Silverman and Zimmerman, 1983; Swanson and Sawchenko, 1983). Central functions of OT include modulation of social and reproductive behaviors (see below).

1.2 Functions of Oxytocin

1.2.1 Oxytocin and Parturition

Parturition is a well-orchestrated event that leads to the expulsion of the fetus and requires the action of many actors including OT, gonadal steroids, prostaglandins and other

uncharacterized players. OT is the most potent endogenous uterotonic agent known. The effect of OT on uterine smooth muscle contractions has been known for quite a long time. As early as 1906, Dale reported the uterotonic activity of a posterior pituitary extract (Dale, 1906). Since then, OT has been used for the induction of labor. Nevertheless, the exact role of OT in parturition is unclear (Fuchs and Fuchs, 1984). Since it is the most potent uterotonic agent, it has been proposed that OT could be involved in the initiation of labor. Plasma measurements of OT at parturition show moderate increase of OT concentration in most species studied. But as labor progresses there is a significant increase in plasma OT concentration probably attributed to afferent nerve activity, originating from the cervix, uterine and vagina, which leads to stimulation of OT secretion (Ferguson reflex). Thus, it seems from the measurement of plasma OT that it is more important for progression than initiation of labor. Recently, OT mRNA has been detected in epithelial cells of the endometrium of the rat uterus (Lefebvre *et al.*, 1992) and in human decidua (Chibbar *et al.*, 1993; Chibbar *et al.*, 1995) but surprisingly no expression is detected in the mouse uterus (Ho and Murphy, 1997). This local expression of OT has revived the idea that OT might be important in initiating labor. However, mice lacking the OT gene can give birth with no apparent timing problem.

Nevertheless, there is a dramatic increase in the number of OT receptors in the myometrium that occurs at the end of gestation. An increase in the ratio of serum estrogen/progesterone that occurs at the end of gestation is known to increase the sensitivity of the myometrium to OT in rats. The human OT receptor mRNA is detected in uterine endometrium, myometrium, myoepithelial cell of the mammary gland, chorion and decidua, brain and ovary (Kimura *et al.*, 1992; Takemura *et al.*, 1994; Yoshimura *et al.*, 1996). In

human, there is a 300-fold increase in OT receptors mRNA at parturition compared with the nonpregnant myometrium (Kimura *et al.*, 1996). Western blot analysis reveals a significant but less dramatic increase of OT receptors in the myometrium and, surprisingly, significant amounts of OT receptors are detected in the nonpregnant myometrium (Kimura *et al.*, 1996). The rise in OT receptor numbers increases the sensitivity of the myometrium to OT such that even low levels of OT can stimulate uterine contractions, thus bypassing the requirement of an increase in OT peptide. It seems that the increase in OT receptors and consequently sensitivity of the myometrium to OT is an important event for the initiation of labor. The generation of mice devoid of OT receptors will greatly help in clarifying its importance and role in initiation of parturition.

1.2.2 Oxytocin and Lactation

The other important function of OT is its role in milk ejection during lactation. OT is released following stimulation of the nipple and acts on OT receptors localized in myoepithelial cells of the mammary gland. The increased responsiveness of the lactating mammary gland to OT is due to an increase of OT receptors (Soloff *et al.*, 1979).

There is no doubt as to the role of OT in lactation since mice lacking the OT gene cannot nourish their pups, although milk is present in the ducts and alveoli of the dams. Furthermore, injection of exogenous OT to knockout dams restores milk ejection (Nishimori *et al.*, 1996; Young *et al.*, 1996).

1.2.3 Oxytocin and Behavior

A role for OT in short-term maternal behavior was first reported by Pederson and Caldwell (Pedersen and Prange, 1979) and was enhanced by prior treatment with estrogen

(Fahrbach *et al.*, 1985). Although the role of OT in maternal behavior is well accepted, the OT-inducted maternal behavior requires specific experimental conditions. Female rats are responsive to OT only when they are mildly stressed, for example the transfer from the home cage to an unfamiliar cage but are unresponsive when the experiment is performed in their home cage (Fahrbach *et al.*, 1986).

Other described effects of OT include facilitation of lordosis behavior (Arletti and Bertolini, 1985), parental behavior (Shapiro and Insel, 1992), yawning/penile erection (Melis *et al.*, 1986), grooming (Caldwell *et al.*, 1986), feeding (Verbalis *et al.*, 1986), learning and memory (Bohus *et al.*, 1978) and anxiety (McCarthy, 1995; McCarthy *et al.*, 1996).

1.2.4 Other functions

Other peripheral functions of OT include osmoregulation in the kidney (Verbalis *et al.*, 1991), luteolysis in ruminants (Ivell and Richter, 1984), immune functions (Geenen *et al.*, 1986) and stimulation of anterior pituitary hormones secretion like ACTH (Antoni *et al.*, 1983), prolactin (Samson *et al.*, 1986), and LH (reviewed in Evans, 1997). The importance of these non-classical functions remains to be clarified. The OT null mutant mouse should be a very useful model for these studies.

1.3 Peripheral Oxytocin

OT synthesis has also been detected in many peripheral organs such as the uterus (Lefebvre *et al.*, 1992), decidua (Chibbar *et al.*, 1993), adrenal gland (Nussey *et al.*, 1987), thymus (Geenen *et al.*, 1986), ovary (Ivell and Richter, 1984), testis (Foo *et al.*, 1991; Rehbein *et al.*, 1986), placenta (Lefebvre *et al.*, 1992), and pineal gland (Liu *et al.*, 1991). The level of expression is generally marginal compared to the hypothalamus with the exception of

the rat uterus where appreciable amounts are detected during late pregnancy and the ruminant corpus luteum where high expression is detected during luteolysis. It is thought that the role of peripheral OT is to act in a paracrine and/or autocrine regulator of local action.

1.4 The structural organization on the Oxytocin gene

The OT and AVP genes have a very similar structural organization probably reflecting their common ancestral origin. It is generally accepted that the OT/AVP family emerged from a common vertebrate ancestral molecule by gene duplication. The OT gene consists of three exons and two introns and encompasses less that 1 kb of DNA (Fig. 1). The OT gene is linked to the AVP gene in a tail-to-tail orientation and thus the two genes are transcribed in opposite direction. The intergenic region varies between 3 to 12 kb depending on the species (human 12 kb, rat 11 kb, mouse 3.5 kb). High sequence identity between the human OT and AVP genes is observed in the second exon which encodes most of the neurophysin I moiety (96%; see below) and approximately 70% and 50% identity is observed in exons 1 and 2 respectively (Sausville *et al.*, 1985). The first exon of the OT gene codes for the signal peptide, the nonapeptide OT and the N-terminal part of neurophysin I. Exon 2 encodes for most of the neurophysin I part and finally exon 3 encodes the C-terminal part of neurophysin I. Only one copy of the OT gene is detected in the human genome (Lopes da Silva *et al.*, 1993; Sausville *et al.*, 1985). Finally, the genes for OT from rat, mouse, bovine and sheep all show the same genomic structure as the human gene.

1.5 Targeted deletion of the Oxytocin gene

To study the role of the mouse OT during reproduction, two groups have generated null mutants for the mouse OT gene. The first group replaced the OT gene by the PGK-hprt

Human OXYTOCIN-VASOPRESSIN Locus

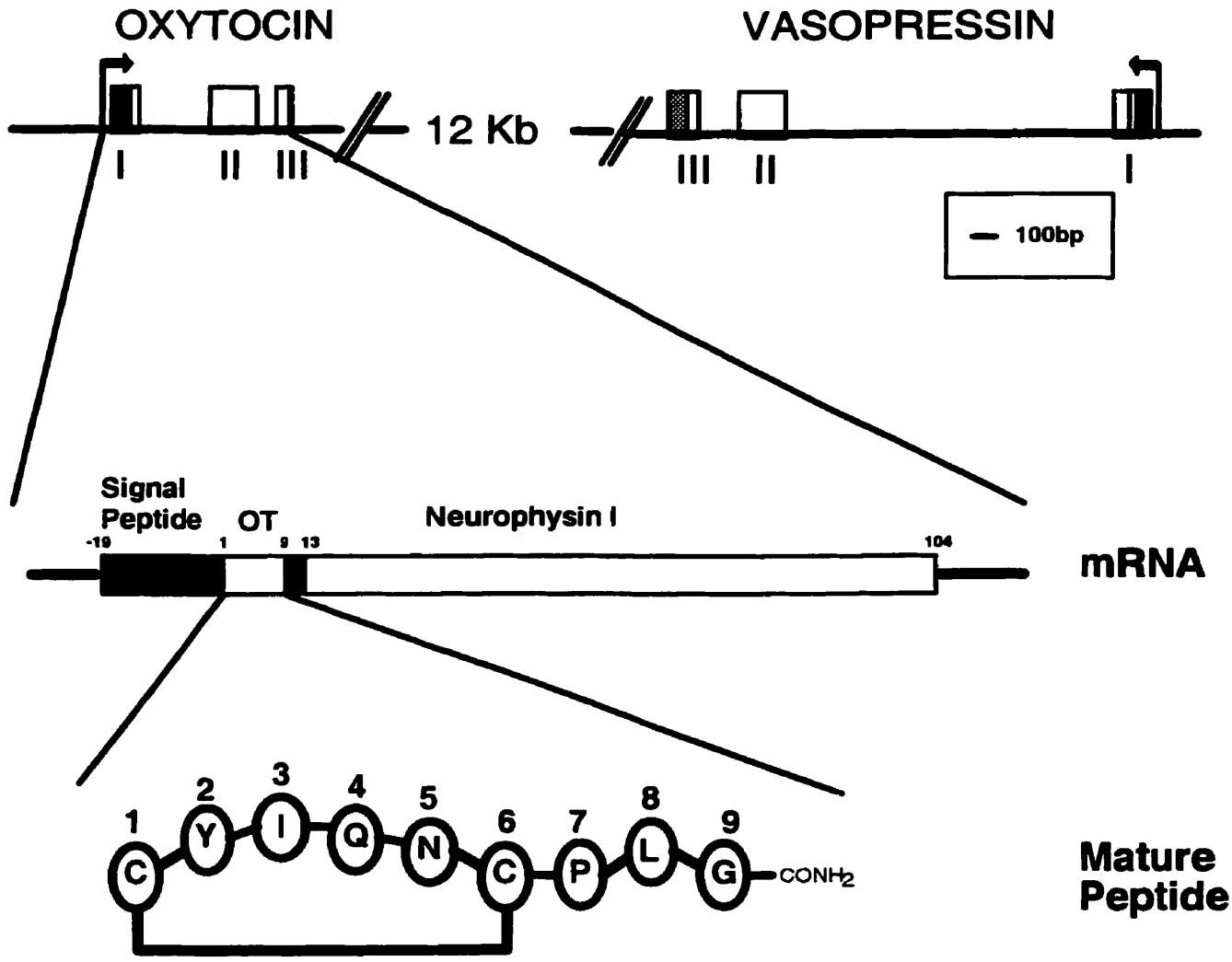

Figure 1. Top: Schematic organization of the Oxytocin and Vasopressin genes. The structural organization of the OT and AVP genes is shown. Both genes consist of three exons and two introns. The two genes are transcribed in opposite direction. Middle: The OT mRNA with the corresponding precursor polypeptide is shown. Bottom: The mature OT polypeptide with the cystine bond between cysteines 1 and 6 and the amidated C-terminal is shown.

expression cassette using homologous recombination in ES cells (Nishimori *et al.*, 1996). Using this strategy, exon 1 was deleted. The second group replaced the first intron and the last two exons of the OT gene by the neomycin resistance cassette, NEO, (Young *et al.*, 1996). Both groups observed similar phenotype. The OT-deficient mice are viable and are fertile. *In situ* hybridization was performed to confirm the absence of OT mRNA expression in the brain of homozygous mice. Using an OT-specific oligonucleotide probe, no signal was detected in the PVN and SON of homozygote mice whereas a strong signal is detected in heterozygote and wild-type animals. The level of AVP in homozygous mice appeared normal.

Parturition in the null mutant female was surprisingly unaffected by the absence of OT. Furthermore, homozygote female displayed normal maternal behavior such as nest building, pup retrieval, pup grooming. The only defect observed in null mutant mice was the complete failure of postpartum homozygote dams to transfer milk to the suckling pups. Offspings from homozygote mothers died within 24 hours of delivery unless the mothers are injected with exogenous OT.

These results clearly show the lack of role of OT in the initiation of parturition and maternal behavior. With respect to parturition, the OT and OT receptor genes are strongly up-regulated in the uterus late in gestation in most species (Lefebvre *et al.*, 1992) with the exception of the mouse uterus, which does not seem to express the OT gene (Ho and Murphy, 1997). Thus, the lack of parturition defect might be specific to the laboratory mouse. It is important to point out that most laboratory mice were artificially selected at some point for their reproductive performance. Consequently, the laboratory mouse might have been selected for efficient parturition. The other explanation for the lack of parturition defect in OT null mice is redundancy. Other systems can take over the role of OT in parturition such as

prostaglandins. Indeed, mice deficient in PGF2α receptor fail to produce labor and also fail to express OT receptor mRNA during late pregnancy and the decline in serum progesterone is not observed (Sugimoto *et al.*, 1997). Furthermore, mice deficient in the enzyme phospholipase A2 also fail to produce labor (Uozumi *et al.*, 1997). And not surprisingly, mice deficient for the COX-1 enzyme, which catalyzes the first committed step in the formation of prostaglandins from arachidonic acid, are delayed in their onset of labor (Gross *et al.*, 1998). These results clearly demonstrate the importance of prostaglandins in the initiation of labor in mice and maybe relegate OT as a supporting role in the maintenance of parturition. Nishimori and colleagues have suggested that OT of brain or uterine origin can help in the induction of parturition but is not necessary since other molecules (e.g., PGF2α) may be more important or potent. Alternatively, a novel OT-like peptide may be up-regulated in the OT-deficient animal and bind to the OTR and induces smooth muscle contraction. Moreover, disruption of the type 1 steroid 5α-reductase enzyme, an enzyme involved in the synthesis of 5α-androstan-3α,17-diol α 5α-reduced androgen in mice leads to prolonged labor (Mahendroo *et al.*, 1996). The parturition defect can be reversed by administration of 5α-androstan-3α,17-diol. Interestingly, the serum progesterone levels drop and the increase in OTR mRNA in late gestation are observed in the knockout mice. This is another example of redundancy. Since a well-executed parturition is a crucial event in the survival of any species, it is not surprising that more than one system will be required to participate in this event.

The lack of maternal behavior defect displayed by the OT-deficient mice is quite a dilemma and suggests that OT is not required for that behavior. Numerous reports over the last 10-15 years have implicated OT in behavioral functions such as maternal behavior and

lordosis. Although, maternal behaviors defect have been observed in FosB (Brown *et al.*, 1996) and dopamine β-hydroxylase (Thomas and Palmiter, 1997) knockout mice, thus the absence of a defect cannot be explained by the fact that most strains of laboratory mice, including the strain used to create the OT knockout, are spontaneously maternal (Gandelman, 1973; Young *et al.*, 1997). Species difference could be one reason for this discrepancy, since most of the behavioral studies of OT have been performed on rats. In addition, the distribution of OTR in the brain is known to be species-specific. In rats, high concentration of OTR is detected in the bed nucleus of the stria terminalis and low concentration in the lateral septum whereas in mice the reverse is observed (Insel *et al.*, 1993).

The OT-deficient mice do show some social behavioral defects (Young *et al.*, 1997) such as decreased olfactory investigation, increased aggressive behavior, and a decrease in infant distress calls. A decrease in the duration of aggressive behaviors was also observed especially in agonistic encounters within neutral arenas (DeVries *et al.*, 1997).

1.6 Molecular biology of Oxytocin

1.6.1 Oxytocin promoters

Alignment of the promoters from human, rat, mouse, bovine, and sheep OT genes (Fig. 2) show regions of high homology within the first 200 nucleotides of the promoters. Beyond the first 200 nucleotides, regions of high homology are low. The most conserved region in the OT promoters is the presence of AGGTCA motifs located around -160 to -180, which includes an estrogen response element (ERE). Also imbedded in the ERE motif is a direct repeat of AGGTCA (referred as DR0) that is present in human and rodent OT promoters but not in the ruminant promoter. Downstream of this DR0, three AGGTCA half-

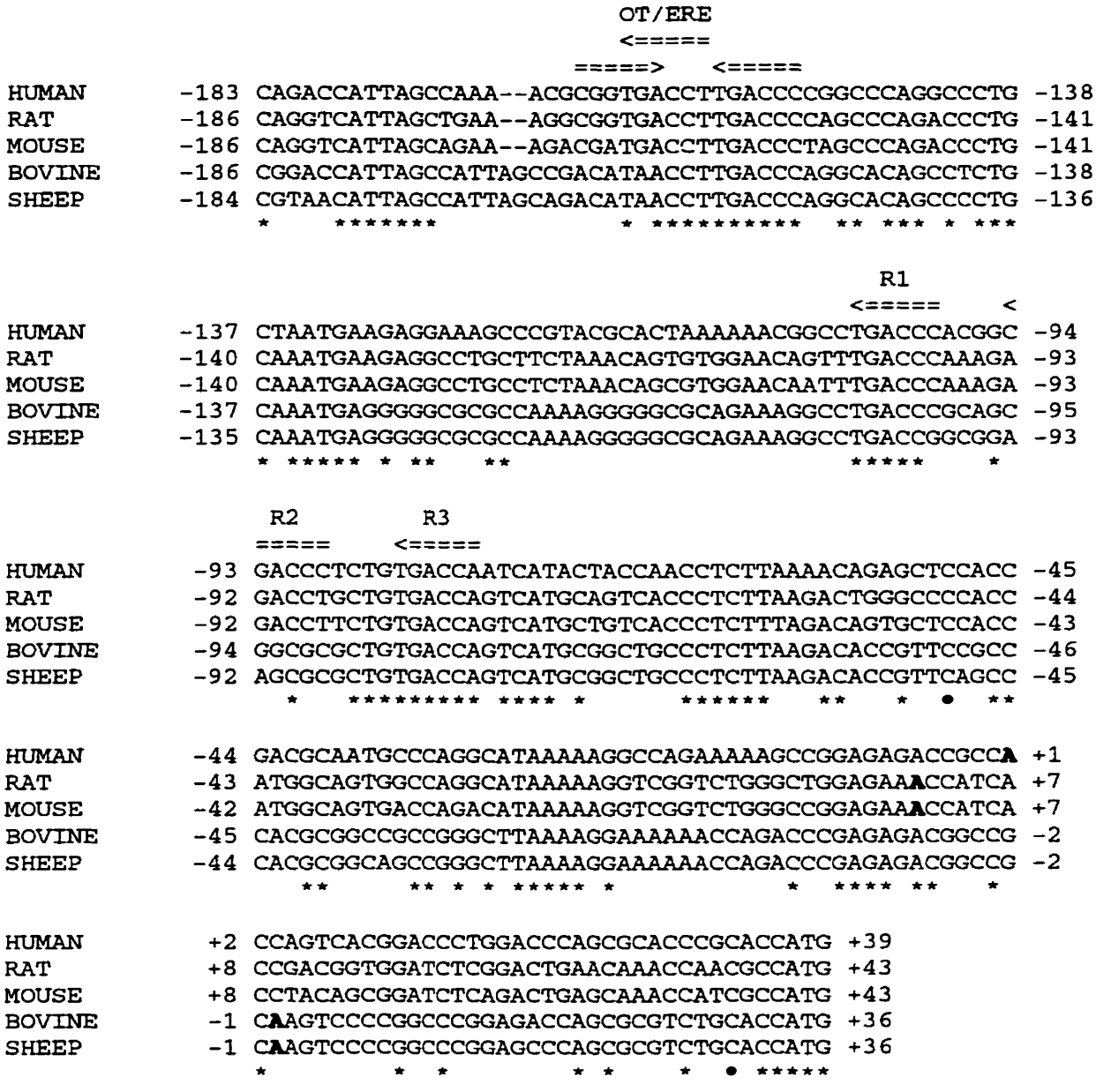

Figure 2. Sequence comparison of OT promoters from different species. The numbering is with respect to the transcriptional start site and the bolded A represent the transcriptional start site. The OT/ERE and the downstream repeats are indicated with the arrows represent the AGGTCA half-site. Sequencing errors were observed in the mouse sequence at position -101 a G instead of a C is observed and in the human sequence at position -13 where a G was missing from the published sequence. The rat sequence used also incorporated the sequencing errors observed by Burchach *et al.* (Burbach JPH *et al.*, 1994). *, indicates sequence identities.

sites are observed that are well conserved and are recognized by nuclear receptors. Other conserved regions include two A/T rich regions flanking the conserved OT/ERE. Close examination of these A/T rich regions indicates their resemblance to binding sites for POU or homeobox transcription factors. Of interest, the 5' putative POU/homeobox binding site is well conserved in the Fugu isotocin (homologue of OT) promoter.

Finally, the most conserved DNA element in eukaryotic promoters, the TATA box (TATAAAAA), located ≈30 nucleotides upstream from the transcriptional start site, is present with a slight variation (CATAAAAA) in the human and rodent OT promoters. Interestingly, the TATA box is poorly conserved in ruminant species, which probably points to a secondary role for the TATA binding protein or TFIID complex in directing the pre-initiation complex formation. Indeed, point mutations of the TATA box in the human OT promoter only marginally affect the human OT promoter activity in transfection studies (personal observation).

1.6.2 Oxytocin Transgenic studies

Because of the lack of a good *in vitro* model for the study of OT gene expression, transgenic technology offers an interesting avenue for the study of tissue specific promoter, but the drawbacks are the time and the cost to generate the transgenic lines. The first attempt by Young and colleagues to create an OT transgenic mouse was unsuccessful (Young *et al.*, 1990). The first construct, ROT1.63, consisted of 0.36 kb of promoter region, the complete rat OT coding region and 0.4 kb of downstream sequences. When the construct ROT1.63 was injected into fertilized one-cell mouse eggs, no transgenic pups were recovered, suggesting an incompatibility of the transgene. The rat AVP gene (1.4 kb of upstream sequences, the

structural AVP gene and 0.4 kb of downstream sequences) was then fused to the ROT1.63 construct in a tail to head or tail to tail and injected into one-cell mouse eggs (V1 in Fig. 3; Young *et al.*, 1990). This construct produced a specific expression of the rat transgene in OT-expressing hypothalamic magnocellular neurons in addition to ectopic expression sites (testis and lung). The level of the rat OT transgene expression was less than that of the endogenous mouse gene and there was no apparent relationship between copy number and expression in four lines. However, the rat transgene expression was increased in response to lactation, which indicates that the regulatory elements required for increased transcription are present in the transgene. Surprisingly, the AVP moiety of the rat OT/AVP transgene was not expressed in the hypothalamus. These results point to the importance of the linkage of the OT and AVP genes in determining tissue-specific expression of the OT gene. Very similar results were obtained with the Fugu isotocin (IT) and vasotocin (VT) locus (Venkatesh *et al.*, 1997). VT is thought to be the ancestral member of AVP and OT. The IT and VT genes are organized in a tail to head orientation which is unique, since in all other species the OT and AVP are in a tail to tail orientation. The IT and VT are separated by 24.4 kb, and within that intergenic region lie five other genes. A 43 kb cosmid that encompasses the IT-VT locus was microinjected into fertilized one-cell Sprague-Dawley rat eggs. Remarkably, in one line of transgenic rat, the IT transgene was expressed in magnocellular neurons of the hypothalamus and up-regulated upon dehydration analogous to rat OT gene. Furthermore, the IT transgene is expressed in magnocellular neurons distinct from the AVP expressing neurons. Again, the VT gene is not expressed in the hypothalamus similar to the rat transgene. In addition, ectopic expression of IT and VT in the testis was also observed. It is quite remarkable that two species that are

TRANSGENES

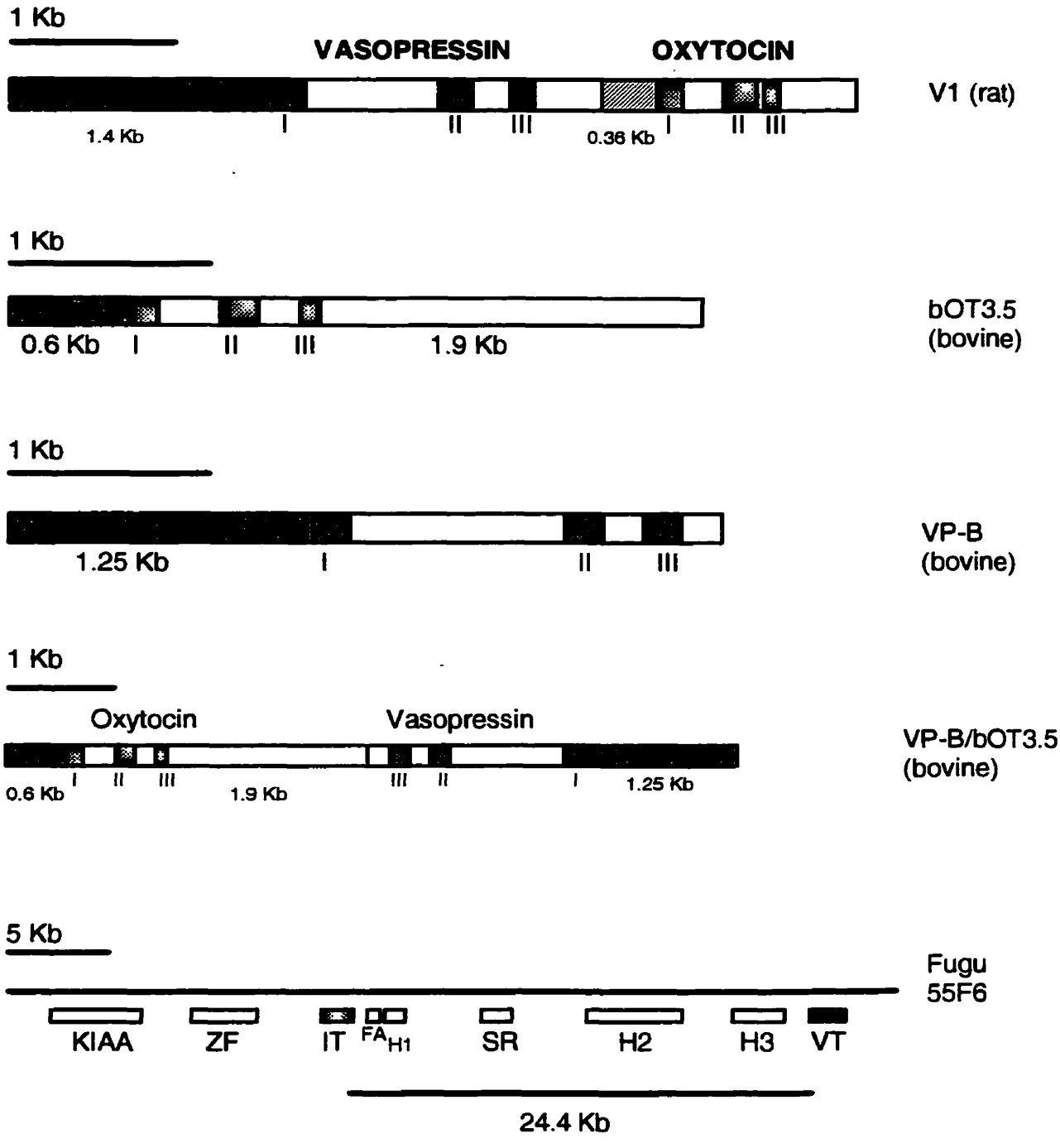

Figure 3. Schematic representation of OT and AVP transgenes used for the production of different transgenic animals. The species of origin is indicated in brackets. Exons encoding the OT and AVP gene are represented by the black and checkered boxes, respectively. Promoter regions for OT and AVP are represented by stippled and gray boxes, respectively. See text for more details.

separated by 400 million years have conserved DNA elements that drive neuron specific expression.

David Murphy's group has made significant contributions to the field of OT transgenic studies. Using the bovine OT gene in transgenic studies, they identified one construct, bOT3.5, which consisted of the OT structural gene flanked by 0.6 kb of upstream and 1.9 kb of downstream sequences and that is specifically expressed in magnocellular neurons of the hypothalamus (Ho *et al.*, 1995), distinct from AVP expressing neurons. This bOT3.5 construct seems to be the exception to the OT/AVP locus hypothesis since specific expression does not require the AVP gene. Furthermore, linkage of the AVP gene with bOT3.5 represses hypothalamic expression (construct VP-B/bOT3). Again, ectopic expression of the bovine OT is frequently observed in the testis and lung.

Conversely, attempts to make an AVP transgene were successful only when the AVP gene was used alone (Ang *et al.*, 1993; Grant *et al.*, 1993) and linkage to the OT gene leads to repression of expression of the AVP transgene (Ho *et al.*, 1995; Venkatesh *et al*, 1997; Young *et al.*, 1990).

To conclude, it seems that the mechanism of regulation of OT transgene in mice is quite complex and involves both activator and repressor sequences that are present throughout the OT/AVP locus.

1.6.3 Estrogen and Oxytocin expression

The relation between the OT system and the gonadal steroids has been described for quite a long time. Both are implicated in important reproductive events such as sexual behavior, mating and parturition. In the brain, the relation between OT and gonadal steroids is

not completely understood. Although an ERE has been identified in the OT promoter of most species cloned, the importance of this ERE in hypothalamic OT expression is unclear, but it probably plays an important role in uterine OT expression (Burbach *et al.*, 1994; Chu *et al.*, 1998). However, there are several observations that point at the possible involvement of estrogens in the up-regulation of hypothalamic OT gene expression. Hypothalamic OT mRNA is up-regulated under physiological conditions where high levels of estrogens are detected in the circulation, i.e., at estrus during the cycle or during late gestation in rats or during puberty (Zingg and Lefebvre, 1988; Van Tol *et al.*, 1988; Miller *et al.*, 1989). On the other hand, direct evidence of an effect of administration of estradiol to ovariectomized rats on OT mRNA levels in the SON or PVN has been lacking (Caldwell *et al.*, 1989; Chung *et al.*, 1991). Instead, an increase in OT mRNA is observed in accessory OT neurons such as in the anterior commisure nucleus (Chung *et al.*, 1991) and the preoptic area (Caldwell *et al.*, 1989). The lack of regulation of OT by estrogens in rat PVN and SON is also supported by the lack of estrogen receptor (ERα; see below) in OT-containing magnocellular neurons (Burbach *et al.*, 1990) and lack of estrogen-concentrating neurons in these nuclei (Rhodes *et al.*, 1981). However, there is some species difference in the localization of ERα in the hypothalamus. In guinea pig, a substantial amount of ERα-immunoreactivity is detected by immunohistochemistry in OT expressing magnocellular neurons of the PVN and SON (Warembourg and Poulain, 1991). Furthermore, estradiol-concentrating OT neurons in the SON have also been reported in the mouse (Sar and Stumpf, 1980). A direct effect by estrogen has recently been reported but requires a special long-term treatment of estrogen and progesterone followed by progesterone withdrawal that mimics the variation in gonadal

steroids observed during the cycle or late gestation (Crowley *et al.*, 1995). The discrepancy between the up-regulation of the OT gene by estrogens and the apparent lack of ERα immunoreactivity in the SON and PVN might be explained by the recent cloning of a novel estrogen receptor referred as ERβ (Kuiper *et al.*, 1996). Indeed, *in situ* hybridization has demonstrated high levels of ERβ mRNA in the hypothalamus and especially the PVN and SON (Shughrue *et al.*, 1996; Li *et al.*, 1997; Shughrue *et al.*, 1997; Shughrue *et al.*, 1997). Furthermore, using an antibody specific to the C-terminus of the rat ERβ, ERβ-immunoreactivity was detected in 35% of OT expressing parvocellular neurons in the PVN (Simonian and Herbison, 1997) whereas very few PVN magnocellular OT neurons exhibited ERβ staining with the antibody. It is possible that these PVN magnocellular neurons have a lower concentration of ERβ proteins compared with the parvocellular neurons. In the SON, it seems that ERβ immunoreactivity is co-localized primarily with AVP expressing magnocellular neurons (Alves *et al.*, 1998).

While there is evidence for an effect of estrogen on OT gene expression, it is fair to say that the effect seen is generally modest. One potential explanation for the low effect observed is the high abundance of OT mRNA in magnocellular neurons that can mask an increase in OT transcription. Finally, although the effect of estrogen of OT is relatively marginal, its effect on brain OT receptors is quite convincing (Tribollet *et al.*, 1990; Young *et al.*, 1997). In addition, most of the behavioral effect of OT is greatly enhanced by prior treatment with estrogens. This effect is likely the result of increased OT receptor population.

More convincing evidence for a role of estrogens in the regulation of OT has been obtained by our group in relation to the uterus (Lefebvre *et al.*, 1994; Lefebvre *et al.*, 1994).

We have previously shown that, during late gestation in the rat, OT mRNA is highly up-regulated in the epithelial cells of the endometrium (Lefebvre *et al.*, 1992). During the rat estrous cycle, OT mRNA in the uterus was shown to be induced at proestrous and estrous similar to what is observed in the hypothalamus (Van Tol *et al.*, 1988). Furthermore, in virgin ovariectomized rats, the expression of OT was up-regulated by estrogen treatment for 2 days but not by a similar progesterone treatment. Unexpectedly, combined treatment with estrogen and progesterone for 2 days leads to a stronger up-regulation of OT mRNA than with estrogen alone (Lefebvre *et al.*, 1994). Removal of either estrogen or progesterone at mid-treatment decreased the level of OT mRNA induction. These results show that uterine OT expression is up-regulated by estrogen and not by progesterone but, when combined, lead to a stronger response than estrogen alone. The mechanism of this synergism is not fully understood but it probably involves a direct effect by estrogen via the ERE present in the OT promoter and an indirect effect by progesterone.

In human, OT is expressed in the amnion, chorion and decidua of the pregnant uterus with highest expression in the decidua (Chibbar *et al.*, 1993). OT mRNA expression is up-regulated 3-4 fold following labor and delivery and can be up-regulated by estrogens *in vitro*. In addition, mRNAs for the ERα and the progesterone receptor were highest in the decidua similar to OT mRNA. A 3-fold increase in ERα mRNA is observed in chorio-decidua tissues obtained after spontaneous labor versus tissues obtained from cesarean section whereas no change in progesterone mRNA is observed (Chibbar *et al.*, 1995).

1.6.4 Orphan receptors and Oxytocin gene expression

The bovine OT gene is strongly up-regulated in large granulosa cells of the early corpus luteum (Ivell *et al.*, 1985; Ivell and Richter, 1984). On a per organ basis, the bovine corpus luteum produces 250 times more of OT mRNA than the hypothalamus (Ivell and Richter, 1984). During the estrus cycle of non-pregnant cows, the level of OT mRNA is maximum following ovulation and decreases rapidly thereafter (Ivell *et al.*, 1985). In addition, *in vitro* cultures of follicles express the OT gene when stimulated by activators of cAMP such as IGF-I, insulin but not IGF-II (Furuya *et al.*, 1990; Holtorf *et al.*, 1989). For these reasons, this system is an attractive one to study OT gene expression. However, one major drawback is the low expression of OT in the ovaries of others species such as human, mouse and rat. Thus the conclusions drawn from these studies might be specific for the bovine system and may not be applicable to other species.

Using nuclear extract prepared from bovine granulosa cells either from pre-ovulatory follicles or from middle to late cycle corpus lutea, gel retardation assays were performed with a DNA probe corresponding to the conserved -160 region of the promoter region (Wehrenberg *et al.*, 1994). Two complexes were found to bind to the probe and specific antibodies to the nuclear receptors SF-1 and COUP-TF revealed their identities. The SF-1 complex seems to bind constitutively to the -160 region during the cycle. In contrast, the repressor COUP-TF complex is only present after ovulation and seems to correlate with down regulation of the OT gene. A simple model can be proposed where two differentially expressed antagonistic factors, SF-1 and COUP-TF, can stimulate or repress the OT gene in granulosa cells depending on the level of expression of one factor versus the other. Consistent with this hypothesis, transcripts for COUP-TFI and COUP-TFII are down-regulated in granulosa cells under conditions where OT is expressed (Wehrenberg *et al.*, 1992). The

presence of SF-1 is not sufficient for high activation of the OT promoter since co-transfection of SF-1 and a reporter containing the putative SF-1 binding site in the OT promoter is only able to stimulate reporter activity by 2 fold which is far from the strong increase in mRNA observed during the estrus cycle. Possible explanations for this discrepancy include increased stability of OT mRNA or that SF-1 is required but not sufficient for high level of transcription and other factors are involved in the massive up-regulation. It is also possible that activation by SF-1 requires a ligand that is absent in the cell culture medium. In addition, methylation and DNaseI hypersensitivity studies of the chromatin structure of the promoter region of the bovine OT gene have identified a previously uncharacterized region located around -1797 (Kascheike *et al.*, 1997). Two complexes were observed in gel retardation assays. Complex II is probably SF-1 whereas the identity of complex I is unknown but is likely a member of the monomeric type of nuclear receptor that binds to extended half-site (see below).

COUP-TFI, COUP-TFII and Ear-2 are three highly related orphan receptors of the COUP-TF subfamily, which were shown to be potent negative regulators of the OT gene. Like in the bovine OT gene, the COUP-TF subfamily can also interfere with the activity of other receptors such as the ER, TR, and RAR in the rat and human OT genes (Burbach *et al.*, 1994; Chu *et al.*, 1998). Again, the mechanism of COUP-TF's action is believed to occur by a competitive binding mechanism. In addition, this thesis shows that COUP-TFII and Ear-2 can also repress the human OT promoter by a mechanism of active repression or silencing (see below (Chu and Zingg, 1997).

To identify potential regulators of OT expression (Lopes da Silva *et al.*, 1995), a RT-PCR approach was used to detect the presence of nuclear receptors in total RNA extracted from microdissected SON. In the SON, the following nuclear receptors were identified TRα>

COUP-TFII> TR4 ≈ COUP-TFI. However, *in situ* hybridization showed low level of expression of COUP-TFI and COUP-TFII in magnocellular neurons of the SON and only TRα was detected in appreciable amounts. The orphan receptor TR4, however, seems to be distributed throughout the hypothalamus with no preference for the SON where it was initially cloned (Chang *et al.*, 1994). The physiological importance of TR4 in the regulation of OT remains to be determined. The localization of Ear-2 has not been performed, and thus it remains possible that another orphan regulator will be involved in the regulation of OT expression in the hypothalamus.

1.6.5 Regulation of Oxytocin by retinoic acid and thyroid hormones

In transient transfection studies using a heterologous system, our group and others have identified a possible role for retinoic acid and thyroid hormone in the regulation of the human and rat OT gene (Adan *et al.*, 1993; Adan *et al.*, 1992; Richard and Zingg, 1991). The regions responsible for the activation by the hormones were localized to nucleotides -180 to -70, a region that contains the OT/ERE and the three downstream repeats. Full activation by RA requires both DNA elements, and mutation of the any half-sites in the OT/ERE or the downstream repeats greatly diminishes activation (Adan *et al.*, 1993; Richard and Zingg, 1991). In contrast, activation by thyroid hormone only requires the OT/ERE and an extra AGGTCA half-site located 5' of the OT/ERE in the rat OT gene (Adan *et al.*, 1992). This extra half-site is not conserved in the human promoter.

The physiological significance of the activation by retinoic acid in the hypothalamus is questionable. Indeed, pups derived from vitamin A deficient rats do not show variation in

hypothalamic OT mRNA as well as pituitary OT content. Furthermore, injection of retinoic acid in vitamin A deficient pups does not affect the levels of OT peptide and mRNA (Adan *et al.*, 1993). In contrast, we were able to show that injection of retinoic acid into day 16 pregnant rats stimulates uterine OT mRNA significantly by 2-fold (Larcher *et al.*, 1995).

Thyroid hormone, when given orally to rats, can stimulate significantly OT mRNA in the hypothalamus, OT peptide content in the pituitary and plasma OT concentration (Adan *et al.*, 1992). These results suggest a (patho-)physiological role for thyroid hormone in the regulation of OT gene expression that is consistent with the presence of the thyroid hormone receptor in OT magnocellular neurons (Bradley *et al.*, 1989).

2. The Nuclear Receptor Superfamily of Transcription Factors

Nuclear receptors were initially identified as receptors for small lipophilic hormones such as steroids. Classically, the small lipophilic steroid hormones are secreted into the circulation to act on target tissues that contain the appropriate receptor (Fig. 4). The small lipophilic steroids diffuse into the intracellular space and bind to its receptor. This interaction will cause a conformational change of the nuclear receptors such that repressive molecules like heat shock proteins are released. The inactive nuclear receptor then translocates to the nucleus, binds to, and activates the promoters of specific genes. This classical view is true but for only a small number of nuclear receptors, such as the steroid hormone receptors. In fact, the majority of nuclear receptors are localized in the nucleus and probably already bound to the DNA in an inactive conformation (or repressive state) and, upon binding of the appropriate ligand, the nuclear receptor is activated (see below). Two other mechanisms of action by hormones were recently described. The first one is very similar to the classical

mechanism except that the hormone is secreted in its apohormone form and is processed by the cell to acquire the active conformation. Finally, the third type of mechanism is one in which the active hormone is generated within the cell and acts upon nuclear receptors in the same cell. The nuclear receptor superfamily can be divided into two subgroups. The first one comprises the nuclear receptors activated by steroids such as estrogen receptor (ER), progesterone receptor (PR), androgen receptor (AR), mineralocorticoid receptor (MR) and glucocorticoid receptor (GR). The second subgroup comprises the thyroid/retinoid/vitamin D (or nonsteroid) members and is characterized by the retinoic acid receptors (RARs), the 9-cis retinoic acid receptors (RXRs), the thyroid hormone receptors (TRs), the vitamin D3 receptor (VDR) and many orphan receptors for which no ligand has been identified. This thesis will mostly deal with orphan receptor especially the chicken ovalbumin upstream promoter transcription factor (COUP-TF) sub-family (see below).

2.1 Functional domains

2.1.1 The A/B domain

Nuclear receptors are organized in a modular fashion (Fig. 5). Numerous groups have identified the different domains A/B, C, D, E and F and each of those domains was shown to play a defined role in the action of nuclear receptors. The N-terminal A/B domain is highly variable in sequence and in length. The A/B domain contains the activation function 1 (AF-1). This activation domain is generally thought to be constitutive, and together with the AF-2 (see below) is required for full activation by ER in response to 17β-estradiol (Kraus *et al.*, 1995; McInerney *et al.*, 1996; Tzukerman *et al.* 1994).

2.1.2 The C or DNA binding domain

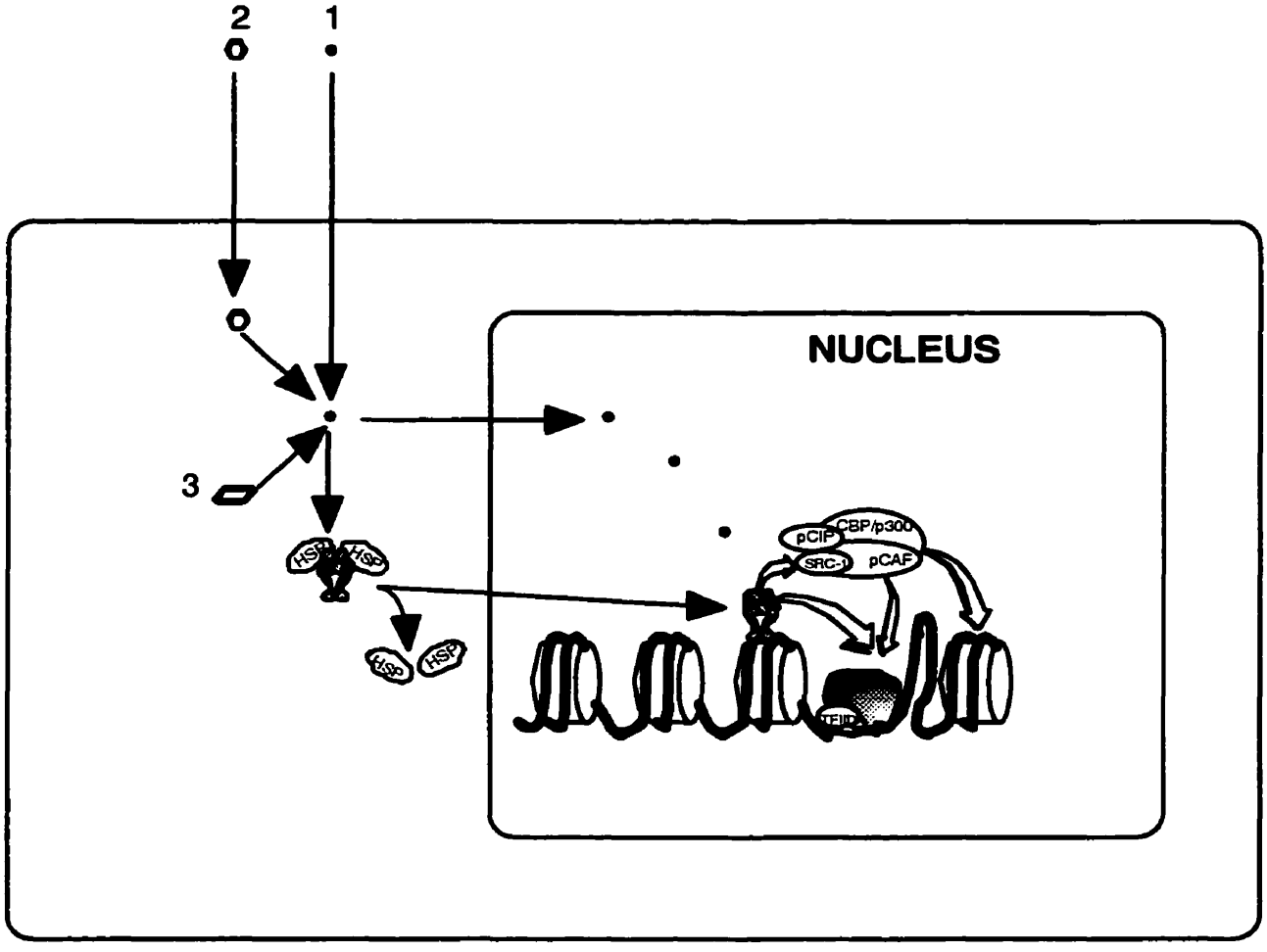

Figure 4. Mechanism of hormone action. The active hormone can be generated in three ways. 1) The classical hormones such as steroids are generated at a location distant from the target site and are secreted into the circulation. At the target cell, due to its lipophilic nature, the hormone can readily cross the membrane and bind to its receptor and activate transcription. The receptor can be localized either in the cytoplasm (e.g. steroid receptors) or in the nucleus (e.g. RAR). Upon ligand binding, the steroid receptors undergo a conformational change such that heat shock proteins bound to the receptor are released. The steroid receptor can then translocate to the nucleus, where it can bind to its target site and activate transcription by recruiting co-activators. 2) This second mechanism is similar to the classical one except that the hormone is in its apohormone form and has to be converted to its active form while in the circulation or in the cytoplasm of the target cell. 3) The active ligand is generated intracellularly and is not secreted.

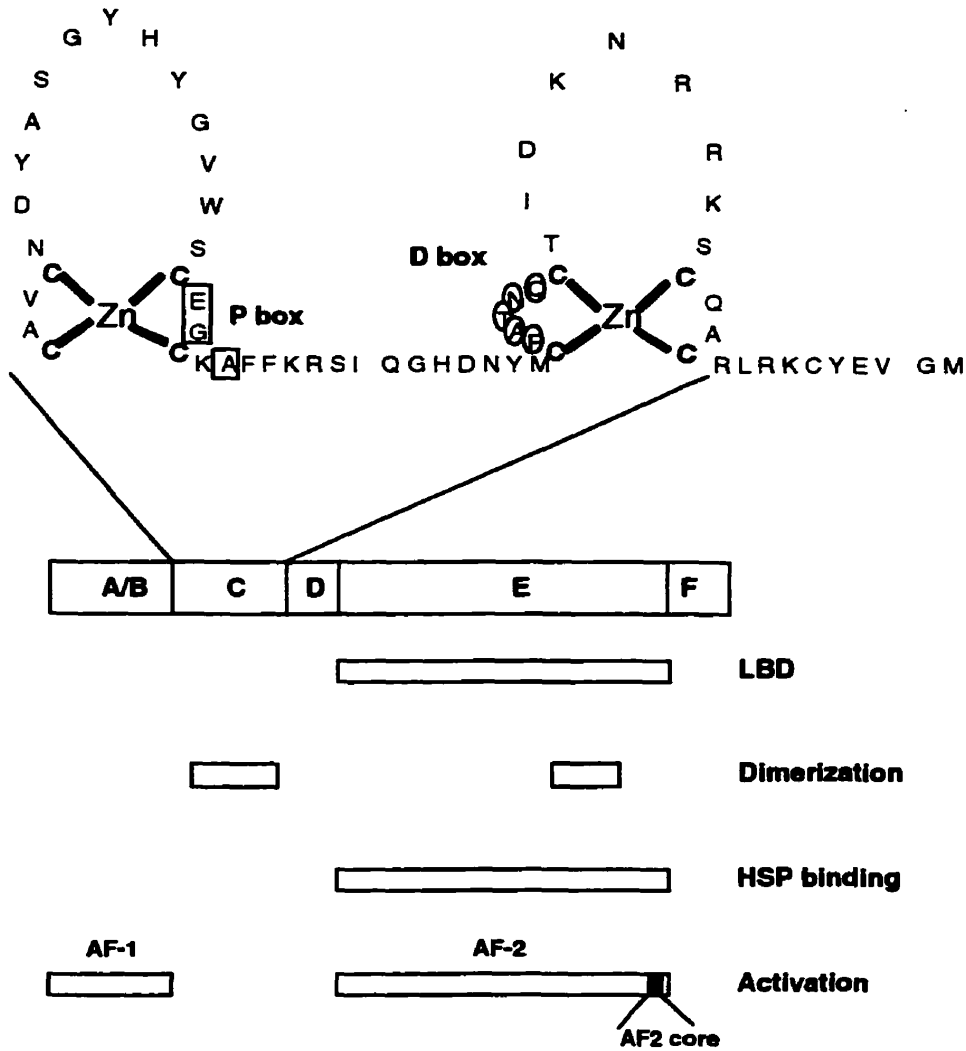

Figure 5. General structure of the nuclear receptor. Top: Nuclear receptors are characterized by six domains (A to F). The most conserved one is the C domain or DNA binding domain. The DNA binding domain of the human estrogen receptor is shown. Amino acids defined as the P and D boxes are indicated. Bottom: Functional domains of nuclear receptors. The A to F domains are shown with some of the functions associated with them. Region C and E represent respectively the DNA binding domain and ligand binding domain. Two activation domains, Activation Function 1 and 2 (AF-1 and AF-2) are present in the A/B and E domains respectively. The AF-2 core, generally associated with helix 12 of the crystal structure, is also shown.

A

Inverted Repeats (IR)	**AGGTCA Nx TGACCT**
Everted Repeats (ER)	**TGACCT Nx AGGTCA**
Direct Repeats (DR)	**AGGTCA Nx AGGTCA**

B

Receptor	P-box	Half-site	Consensus
1 GR, MR, PR, AR	c GS ck V	AGAACA	IR3
2 RAR/RXR	c EG ck G	AGGTCA	DR2, DR5
3 TR/RXR	c EG ck G	AGGTCA	DR4
4 TR/TR	c EG ck G	AGGTCA	IR0
5 VDR/RXR	c EG ck G	AGGTCA	DR3
6 PPAR/RXR	c EG ck G	AGGTCA	DR1
7 RXR/RXR	c EG ck G	AGGTCA	DR1
8 RXR/NGFI-B	c EG ck G	AGGTCA	DR5
9 COUP-TF	c EG ck G	AGGTCA	DR0, DR1, DR4, DR6
10 ROR (RZR)	c EG ck G	AGGTCA	WAWWT AGGTCA
11 ER	c EG ck A	AGGTCA	IR3
12 ERR	c EA ck A	AGGTCA	M:TCA AGGTCA
13 SF-1	c ES ck G	AGGTCA	M: TCA AGGTCA
14 HNF-4	c DG ck G	AGGTCA	DR1

Figure 6. Nuclear response elements and P-boxes. (A) DNA response element of dimeric nuclear receptors. The AGGTCA half-site can be arranged into inverted, everted and directed repeats spaced by N nucleotides. (B) The P box and the corresponding response element of some members of the nuclear receptor family. (W=A or T; M=monomer).

The C region is the most conserved region of the nuclear receptor superfamily. It is characterized by two type II C2C2 zinc (Zn) fingers, and is responsible for DNA binding, dimerization and site-specific recognition (reviewed in Mangelsdorf and Evans, 1995; Tsai and O'Malley, 1994). One Zn atom that is tetrahedrically coordinated to four cysteines characterizes a zinc finger. Within the first Zn finger, lies a small conserved region called the P box (Fig. 5,6). This P box is important for DNA binding specificity and is defined by three amino acid residues surrounding the fourth cysteine of the Zn finger. This P box will discriminate between the two types of DNA hexameric motifs or half-sites recognized by nuclear receptors (Mader *et al.*, 1989). The P box GCckV is specific to steroid receptors, with the exception of the ER, and will bind an AGAACA type of half-site. Most other nuclear receptors including the orphan receptors have a P box consisting of EGckG/A and will recognize an AGGTCA type of half-site. The D box is located in the second Zn finger and is important for phosphate contacts and for dimerization of steroid hormone receptors (Kumar and Chambon, 1988). The importance of the P and D boxes were shown by X-ray crystallographic analysis of the GR, ER and RXR/TR heterodimer bound to their respective DNA (Luisi *et al.*, 1991; Rastinejad *et al.*, 1995; Schwabe *et al.*, 1993). These studies showed a highly conserved globular structure of the DBD, which consists of a pair of amphipathic α helices packed at right angles and crossing near their midpoint followed by a C-terminal extension. The N-terminal α helix, which contains the P box, makes specific contacts with the response element in the major groove. The three conserved amino acids of the P box were also shown to be located on the same face of the α-helix.

Contrarily to the symmetrical DNA binding of steroid hormone receptors, most other receptors will bind to asymmetric sites consisting mainly of direct repeats. The crystal structure of the DBD of RXR/TR heterodimers bound to a DR4 response element was achieved by Rastinejad *et al.* (Rastinejad *et al.*, 1995). It shows that RXR and TR bind to adjacent major grooves on the same face of the DNA. The crystallographic results confirmed the polarity of DNA binding, such that RXR occupies the 5' half-site and TR the 3' half-site, and revealed the presence of two dimerization interfaces in the DBD. The RXR surface is generated by the second Zn finger including one residue from the D box, and the TR contributes a surface encompassing residues in the tip of the first finger and a region N-terminal to the second finger (T box). Furthermore the residues in RXR required for dimerization with TR will slightly differ from the one required for VDR or RAR, as observed in molecular modeling studies (Rastinejad *et al.*,1995).

2.1.3 The D or hinge domain

The D or hinge region is less well conserved and is important for a unique set of nuclear receptors that bind DNA as a monomer, such as the rat nerve growth factor inducible protein B (NGFI-B), the retinoid orphan receptors (RORs) and the steroidogenic factor 1 (SF-1). These small regions referred to as the A box (for NGFI-B; Wilson *et al.*, 1992), or C-terminal extension (CTE for RORs; Giguère *et al.*, 1994; McBroom *et al.*, 1995), or T/A box (for RXRα, TRα and SF-1; Lee *et al.*, 1993; Rastinejad *et al.*, 1995; Wilson *et al.*, 1993) are thought to confer extra contacts adjacent to the consensus half-site between the protein and the DNA. These extra contacts are believed to provide an increase stability and specificity in DNA binding. Indeed the T/A box from TRα does form an α-helical in crystal structures and

makes direct and indirect DNA and phosphate backbone contacts (Rastinejad *et al.*, 1995). There is no homology between the mentioned boxes. In TR and RAR, this region has also been shown to be important for interaction with the corepressors N-CoR and SMRT (see below). Finally, the D region contains the nuclear localization signal for steroid receptors.

2.1.4 The E or ligand binding domain

The E domain or ligand binding domain (LBD), is the most complex region. This domain contains the ligand binding pocket for some members and the strong Activation Function 2 (AF-2), the heat-shock protein interaction, the dimerization, the nuclear localization, and the intermolecular silencing domains. The crystal structure of the LBD of the RAR bound to all trans RA, the TR bound to the thyroid hormone, ligand free hRXRα and β-estradiol bound ERα were recently published (Bourguet *et al.*, 1995; Brzozowski *et al.*, 1997; Renaud *et al.*, 1995; Wagner *et al.*, 1995). The crystal structures revealed 12 α-helixes arranged in an antiparallel α helical sandwich and highlight the hydrophobic nature of the ligand binding pocket. From the crystal structure of RXRα, it is apparent that its homodimerization interface is formed mainly by helix 10 (H10), containing the previously described, ninth heptad (Fawell *et al.*, 1990), and, to a lesser extent by the loop between H7 and H8 (Bourguet *et al.*, 1995; Fawell *et al.*, 1990). The role of this dimerization interface is believed to play a stabilizing role, since X-ray crystallographic studies have clearly shown that the dimerization domain in the DBD is the main determinant of DNA selectivity (see earlier). In addition, this dimerization interface also contains the identity box (I-box) shown to be important for the specificity in heterodimerization or homodimerization (Perlmann *et al.*, 1996). In addition, from these crystallographic studies, it has been proposed that ligand

binding to its receptor induces an allosteric change in the LBD that resulted in the generation of a novel surface required for AF-2 function. Helix 12, which contains a region referred as the AF-2 core, is reoriented across the LBD and is stabilized by the ligand itself and by interaction between H12 and helices 4 and/or 3. The helix 12/AF-2 core is amphipatic in nature and well conserved in most members of the nuclear receptor superfamily and is essential for transactivation (Durand *et al.*, 1994; Wurtz *et al.*, 1996). Optimal transactivation by steroid receptors seems to require both the AF-1 and AF-2 (McInerney *et al.*, 1996; Tzukerman *et al.*, 1994). It is postulated that coactivators such as SRC-1 can mediate such synergism (McInerney *et al.*, 1996).

Finally, the function of the F domain located at the C-terminal end of nuclear receptors, remains largely unknown. In general, this domain is not well conserved among nuclear receptors.

2.2 DNA Binding properties

The DNA sequences that are recognized by nuclear receptors are referred to as response element (RE). Most nuclear receptors will bind to their respective RE as a dimer. Each partner of the dimer recognizes one hexameric DNA sequence via the zinc finger motif. Two types of hexameric or half-site motifs have been identified: AGAACA and AGGTCA (Fig. 6). The former is recognized by the steroid hormone nuclear receptors such as the PR, GR, MR and AR. It is important to note that the ER is not part of the first group but rather recognizes the AGGTCA motif recognized all other members of the nuclear receptor superfamily.

2.2.1 Steroid receptors

The steroid hormone nuclear receptors bind their DNA response element as homodimers. A steroid hormone RE is composed of two half-sites arranged in a palindromic or inverted repeat fashion spaced by three nucleotides: 1) AGAACAnnnTGTTCT for PR, GR, MR and AR 2) AGGTCAnnnTGACCT for ER.

2.2.2 Non-steroid nuclear receptors

The nonsteroid nuclear receptors such as RAR, TR, VDR will recognize directed repeats (DRs) of the AGGTCA half-site with various spacing as heterodimers with RXR. Their binding as homodimer is weak but addition of RXRs greatly enhances their binding and transcriptional activity (Leid *et al.*, 1992; Yu *et al.*, 1991; Zhang *et al.*, 1992). A simple rule, the so-called "3,4,5 rule" was formulated by Umesono *et al.* (Umesono *et al.*, 1991) where a spacing of 3 (DR3), 4 (DR4), 5 (DR5) nucleotides between two AGGTCA half-sites dictates the receptor binding specificity of VDR/RXR, TR/RXR and RAR/RXR, respectively. But recently, other types of spacing were identified as the binding site for other nuclear receptors such as a DR0 for COUP-TF homodimer, DR1 for RXR homodimer, COUP-TF homodimer, RXR/COUP-TF heterodimer, PPAR/RXR heterodimer and DR2 for RAR/RXR heterodimer. Although the "3,4,5 rule" and its extension to other spacing does work in general, there is nevertheless a certain promiscuity in DNA binding by certain nuclear receptors such as the TR which has been known to bind to various type of arrangements of half-site (Umesono *et al.*, 1988). In addition, there is a polarity in heterodimeric binding such that RXR occupies the 5' half-site and the other partner (RAR, or TR, or VDR) the 3' half-site (Kurokawa *et al.*, 1994). One of the most promiscuous nuclear receptors is the COUP-TF sub-family (see below). COUP-TF can bind as a homodimer to AGGTCA repeats with spacing varying from

0 to 11 nucleotides with a relative binding affinity as follow: DR1>DR6>DR4>DR8>DR0>DR11. In addition, COUP-TF can also bind, although with lower affinity, to inverted and everted repeats. The direct consequence of such versatility in binding is COUP-TF's ability to interfere with the binding of other nuclear receptors.

2.3 Mechanism of Activation

The steps leading from the binding to DNA of the ligand-bound nuclear receptor to the initiation of transcription by RNA polymerase II are unknown. Some nuclear receptors were shown to interact with the general transcription factors TFIIB and the TATA binding protein (Baniahmad *et al.*, 1993; Fondell *et al.*, 1996; Ing *et al.*, 1992) and suggested a simple mechanism to recruit and assemble the basal transcriptional machinery over the transcriptional start site (Fig. 7). However, the identification of nuclear receptor-associated proteins has complicated the overall picture of transcription activation by nuclear receptors (Chen and Evans, 1995; Halachmi *et al.*, 1994; Horlein *et al.*, 1995; Oñate *et al.*, 1995; Seol *et al.*, 1995). Most of the cloned coactivators interacted with the AF-2 of the nuclear receptors.

Following the initial cloning of SRC-1, numerous related proteins were subsequently cloned. An impressive amount of coactivators and corepressors was identified by Far-Western and yeast two-hybrid techniques whose binding is ligand-dependent and ligand-independent in the case of coactivators and corepressors, respectively. The mechanism of action of coactivators is not clearly known, but some are though to recruit members of the basal

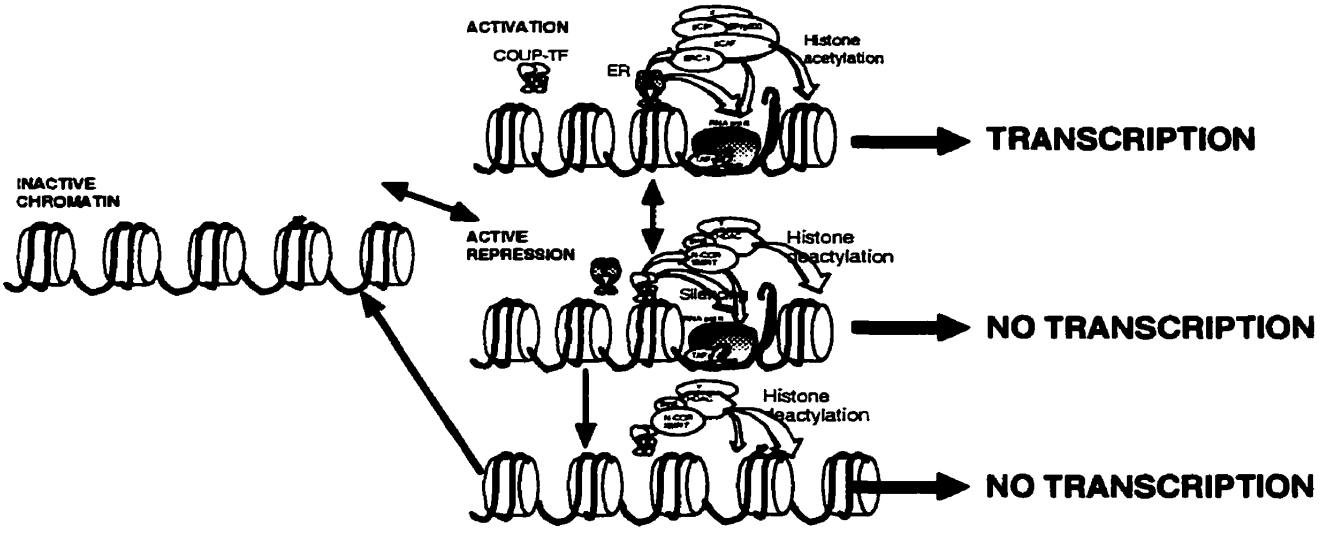

Figure 7. Model of regulation of transcription. A gene can be in two states, transcribed or non-transcribed. The first state is activated transcription and could correspond to what is generally referred as tissue-specific gene expression or basal expression. In this state, a transcription factor binds to its DNA element and recruits either a coactivator complex or general transcription factors (e.g. TATA binding protein) such that there is modification (e.g. acetylation) or/and remodeling of the chromatin structure to allow the RNA polymerase to be positioned near the transcriptional start site. Histone acetylation is believed to destabilize the histone core-DNA interaction. The first state is inactive chromatin where the gene is packaged in nucleosomes. With respect to OT gene expression, this could be the state of the OT gene in non-expressing cells. A second level to this state is active repression. Active repression, requires the participation of a transcription factor that is recruited to a promoter. This repressor can affect the ability of the RNA polymerase II to initiate transcription by either recruiting a corepressor complex with histone deacetylase activity or by directly acting on the RNA polymerase complex. Histone deacetylase is believed to stabilize the association of the octamer to DNA. The relationship between the different states is not well understood and the double arrows between the states were put there to represent the possible dynamic relation between the different states.

machinery, thus facilitating the formation of the pre-initiation complex. Other coactivators seem to act at the level of the chromatin; several coactivators (CBP/p300, SRC-1 family members, and p/CAF) have intrinsic histone acetylase activity (Chen *et al.*, 1997; Spencer *et al.*, 1997; Yang *et al.*, 1996) or are involved in chromatin remodeling like SNF2α (hBRM) and SNF2β (BRG1) (reviewed in Kingston *et al.*, 1996). The chromatin core is composed of two subunits of histone 2A, 2B, 3 and 4. Histone 3 (H3) and histone 4 (H4) are known to be acetylated at the ε-amino group of lysine in the N-terminal tail that extends outwardly from the globular core of the histone octamer (reviewed in Kadanoga, 1998). It is believed that acetylation of H3 and H4 neutralizes the positive charge of lysine, thus causing a reduction in the affinity of histone-DNA interaction and allowing greater accessibility for transcription factors to bind DNA. There are an impressive number of coactivators with the ability to regulate nuclear receptor's activity and it is unlikely that they are all involved in their regulation. Some of these coactivators might only be expressed in specific tissues and periods during development and differentiation and consequently only be available for specific nuclear receptors and other transcription factors present. It is important to emphasize that most of the coactivators will likely be important for the activation of other families of transcription factors. The best example is CBP/p300 which has been implicated in mediating the action of many transcription factors including nuclear receptors, CREB, AP1 (Jun/Fos), STAT, Myo-D, Myb, E1A, TFIIB (reviewed in Goodman *et al.*, 1997). Because of CBP/p300's role in multiple transcriptional routes, they have been referred to as cointegrators. In fact, it seems that coactivators can form different multiprotein complexes in the nucleus that are able to respond to a variety of transcription factors including the RXR/RAR, CREB

and STAT-1 (Korzus *et al.*, 1998; Kurokawa *et al.*, 1998). This increases the level of complexity of transcription, such that one must consider not only the transcription factors present but also the coactivators or corepressors available in the fine tuning of transcriptional regulation. A direct consequence of this is the competition for coactivators by different types of transcription factor in a given cell, like in the case of the mutual antagonistic action of nuclear receptors and the transcription factor AP1 (Kamei *et al.*, 1996). Finally the complexity does not stop here, multiple isoforms of the SRC-1 exist with sometimes antagonistic effects (Kalkhoven *et al.*, 1998). Indeed the isoforms SRC-1a and SRC-1e have completely opposite effects on the estrogenic responsiveness of a rat OT reporter; co-transfection of increasing amounts of SRC-1a inhibited activation by estrogen whereas SRC-1e potentiated the estrogenic induction (Kalkhoven *et al.*, 1998).

2.4 The Orphan Receptors

The term orphan receptor was introduced in the late 1980s to describe a surprising number of novel nuclear receptors cloned by low stringency hybridization and PCR (reviewed in Enmark and Gustafsson, 1996). These novel nuclear receptors had all of the characteristics associated with classical steroid nuclear receptors but no physiological ligands were identified for these novel receptors, thus the term "orphan receptor". To date at least 60 different orphan receptors have been identified, representing more than 75 % of the whole nuclear receptor superfamily (Enmark and Gustafsson, 1996). From an evolutionary point of view, it seems that orphan receptors are the ancestral nuclear receptors and those with known ligands are more recent molecules (Escriva *et al.*, 1997).

Table 1.

	Receptor	Species	Comments	DNA Binding Site	Possible Ligand
	COUP subfamily				
1	COUP-TFI	H, M, B, X		DR1, DR0, DR4	
2	Ear-3	H			
3	Arp-1	H			
4	COUP-TFII	H, M, R	subtype of 1		
5	Seven-up	D			
6	Seven-up-2	D			
7	Ear-2	H, R	subtype of 1		
	NGFI-B subfamily				
8	NGFI-B	R		AAAAGGTCA, DR5* with RXR	
9	N10/nur77	M			
10	NAK1/TR3	H			
11	NOT/TINUR/ MINOR	H	subtype of 8		
12	RNR-1/ HZF-3	R			
13	Nurr1	M		AAAAGGTCA, DR5* with RXR	
14	NOR-1	R	subtype of 8	does not heterodimerize with RXR	
15	XNGFI-B	X			
16	DHR38	D			
	SF-1 subfamily				
17	SF-1/mPtz	M		TCAAGGTCA	25(S)- and 26(S)-Oxysterol
18	ELP	M			
19	A44Bp	C, M			
20	LRH-1	M	subtype of 17		
21	XFFlt	X			
22	XFFlt-B	X	variant of 17		
23	Ptz-F1alpha	D			
24	Ptz-F1beta	D	variant of 23		
25	DHR39	D			
	HNF-4 subfamily				
26	HNF-4	H, R, M		DR1	
27	DHNF-4	D			
	ERR subfamily				
28	ERR-1	H, M		TCAAGGTCA	
29	ERR-2	H	subtype of 28		
	ROR/RZR subfamily				
30	RORalpha1	H		WWAWWTAGGTCA	
31	RORalpha2	H	variant of 30		
32	RORalpha3	H	variant of 30		
33	RORalpha4	H	variant of 30		
34	RZRalpha	R			
35	RZRbeta	R	subtype of 30		
36	RORgamma	H	subtype of 30		
37	DHR3	D			
	TR2 subfamily				
38	TR2	H			
39	TR2-11	H	variant of 38		
40	TR2-9	H	variant of 38		
41	TR2R1	H	variant of 38		
42	DHR78E/F	D			
43	TR4	R		DR4	
44	TR4/TAK1	H			
	Rev/ErbA subfamily				
45	Rev-ErbAalpha	R		WWAWWTAGGTCA	
46	Ear-1	H			
47	Rev-ErbAbeta/RVR	M	subtype of 45		
48	Rev-ErbAlpha/HZF-2	H, R, ch			
49	Rev-erbA-2	R	variant of 47		
50	E75A	D			
51	E75B	D	variant of 50 (lack DBD)		
52	E75C	D	variant of 50		
	LXR/FXR subfamily				
53	OR-1/UR,	R			
54	hUR/NER	H			
55	RIP15	M			
56	RLD-1	R	subtype of 53		
57	LXR	H		DR4* + RXR	24(S),25-epoxycholesterol 22(R)OH- and 24(R)OHCholesterol
58	RIP14	M	subtype of 53	IR-1 + RXR	Retinoids ?
59	FXR	R		IR-1 + RXR	Farnesol, Juvenile Hormone III Bile acid (Chenodroxycholic acid)
	Others				
60	Tailless subfamily	D			
61	Knirp subfamily	D			
62	GCNF/RTR	M		DR0, TCAAGGTCA	
63	MB67	H		DR5 + RXR	
64	mCAR	M			Androstanol
65	SHP	H, M	no A/B and DBD		
66	DAX-1	H	no A/B and DBD	DR5, DNA hairpin structure	
67	BXR	X			4-amino-butyl Benzoate
68	PXR	M			Pregnenolone-16 carbonitrile
69	SXR	M			Rifampicin

Table 1: A compilation of orphan receptors. The orphan receptors are regrouped in subfamily when possible. The species of origin and the DNA binding specificity are shown. Putative ligands for some receptors are listed. B=bovine, D=drosophila H=human, R=rat , M=mouse, X=xenopus,.

Over the past few years, many groups and companies have shown great interest in discovering putative ligands for orphan receptors especially since they might represent novel therapeutic avenues. A general theme seems to appear: orphan receptors that can heterodimerize with the RXRs family of receptors are likely to have a ligand. These novel ligands include fatty acids, oxysterols, benzoates and xenobiotics (reviewed in Blumberg and Evans, 1998; Kliewer, 1999). The identification of these ligands has given support to the concept of intracellular hormones described by O'Malley and Conneely (O'Malley and Conneely, 1992). It emphasized the role of these novel receptors as intracellular sensors of synthesis and metabolism. A new mechanism of action by nuclear receptors was also uncovered, while searching for potential ligands for mCAR. Unlike any of the ligand activated nuclear receptor, mCAR is repressed by its ligands, androstanol and androstenol (Forman *et al.*, 1998). Table 1 summarizes the known orphan receptors and homologues when available. The functions of most orphan receptors are largely unknown but genetic and molecular biology techniques have provided some insight into their possible role. Gene knockout has become an attractive avenue for the identification of a potential physiological role for orphan receptors. These include the HNF-4 (Chen *et al.*, 1994), ERR2 (Luo *et al.*, 1997), NGFI-B (Crawford *et al.*, 1995), Nurr1 (Zetterstrom *et al.*, 1997), PPARα (Lee *et al.*, 1995), SF1 (Luo *et al.*, 1995), RORα (Dussault *et al.*, 1998) and others are expected in the near future. The drawback of such an approach is that one gets unexpected results such as in the case of the liver-enriched HNF-4, where the null mutant mice die in utero at 6.5 days post coitus (Chen *et al.*, 1994). Such a phenotype was unexpected but not unpredictable since, in Drosophila, HNF-4 mRNA of maternal origin is expressed in the early embryo suggesting a

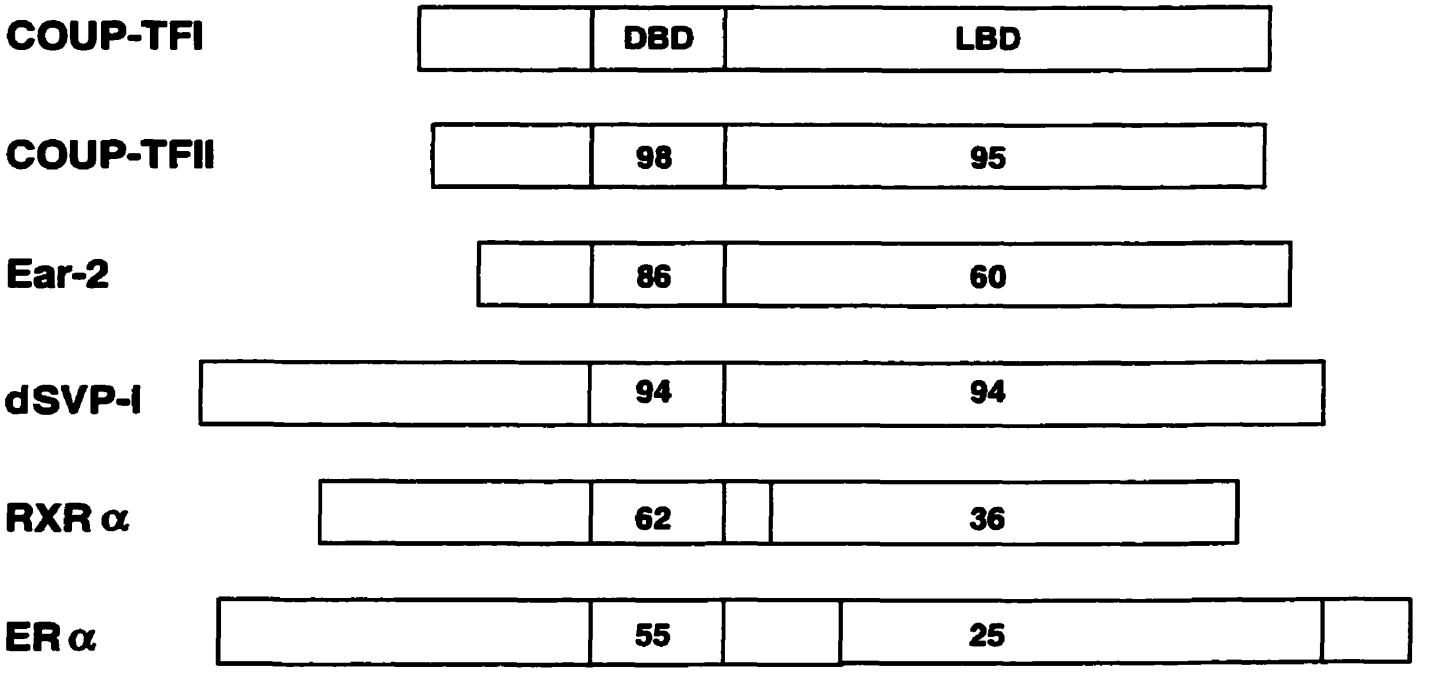

Figure 8. Schematic alignment of COUP-TFs and some nuclear receptors. The percentage homology is indicated for the DBD and LBD .

role in development for HNF-4 (Zhong *et al.*, 1993). The role of HNF-4 in the control of liver specific genes in the adult awaits the generation of a conditional knockout mouse.

Others open new fields, for example the knockout of the NURR1 gene (Zetterstrom *et al.*, 1997). Previous the knockout, NURR1 was known as an immediate early gene related to NGFI-B. The functions associated with NURR1 were not clearly understood. Mice deleted of the NURR1 gene lack midbrain dopaminergic neurons and die soon after birth. The possible relation between NURR1 and neurodegenerative disorders like Parkinson's disease is very intriguing and suggests that modulation of activity or expression of NURR1 could represent an attractive therapeutic avenue.

2.5 The Chicken Ovalbumin Upstream Promoter Transcription Factor

The Chicken ovalbumin upstream promoter transcription factor (COUP-TF) is the most studied orphan nuclear receptors, probably due to its ubiquitous distribution and its promiscuous binding ability. COUP-TF was the first orphan receptor identified (Pastorcic *et al.*, 1986; Sagami *et al.*, 1986; Wang *et al.*, 1989) and was characterized as a positive regulator of the ovalbumin gene (Pastorcic *et al.*, 1986; Sagami *et al.*, 1986; Wang *et al.*, 1987), where COUP-TF was shown to interact with an imperfect DR1. Interestingly, COUP-TF was first identified as a positive regulator, whereas now most of the activities associated with COUP-TF are negative for transcription. COUP-TF was then purified from Hela cells (Wang *et al.*, 1989; Wang *et al.*, 1987) and antibodies were raised against COUP-TF, which helped in the cloning of human COUP-TFI (Wang *et al.*, 1989). hCOUP-TFI is also referred to as ErbA-Related protein 3 (EAR-3) (Miyajima *et al.*, 1988). The human COUP-TFII was then cloned by homology screening (Wang *et al.*, 1991) and Southwestern approach (Ladias

and Karathanasis, 1991). hCOUP-TFII is also known under the name ARP-I (Ladias and Karathanasis, 1991). A third related member is ErbA-Related protein 2 (EAR-2; Miyajima *et al.*, 1988). In general, COUP-TFI, COUP-TFII and Ear-2 have similar actions, so throughout this thesis they will refer to all three members as "COUP-TFs".

The COUP-TF sub-group belongs to the ER/TR sub-family of nuclear receptors and binds to AGGTCA repeats. There is a high conservation in the DBD and LBD of hCOUP-TFI, hCOUP-TFII, dSVP and hEAR-2 (fig. 8). In general, there is >94% and >90% identity in the DBD and LBD, respectively. With the exception of EAR-2, which is generally considered as the closest relative, the homology in the LBD is ~70%. Whereas there are at best <67% and <50% similarities with all other nuclear receptor in the DBD and LBD, respectively.

In Drosophila, the COUP-TF ortholog Seven-up (svp) has been shown to control photoreceptor cell fate and thus provides a role for COUP-TFs in neuronal differentiation. This latter function is supported by the observation that mice, whose gene for COUP-TFI is deleted, display an altered morphogenesis of the ninth cranial ganglion and nerve (Qiu *et al.*, 1997). Furthermore, gene knockout of COUP-TFII revealed an embryonic lethal phenotype with a severe vascularization and cardiac defects (Pereira *et al*, 1999). The physiological role in the adult of COUP-TFI and COUP-TFII remains to be established because of their early lethality, and the generation of a conditional knockout using the CRE-LOXP approach will be very useful in assessing their role in the adult. The two null mutants also indicate that COUP-TFI and COUP-TFII have clearly different functions.

2.6 Actions of COUP-TFs

2.6.1 Competitive binding mechanism

Because of COUP-TFs promiscuous binding ability, COUP-TFs can interfere, by a competitive binding mechanism, with the action of many nuclear receptors including the RARs, TRs, VDR, RXRs, and PPARs. The response elements of these nuclear receptors are all direct repeats (DR2/DR5, DR3, DR4, DR1, DR1 respectively Umesono *et al.*, 1991). Thus it is not surprising that COUP-TFs can bind to such sequences with various affinity and interfere with the hormonal signaling for these nuclear receptors (Cooney *et al.*, 1993; Cooney *et al.*, 1992; Tran *et al.*, 1992). In most cases, the inhibition by COUP-TFs is dose-dependent, implying a competitive binding mechanism. Recently, the importance of this mechanism of action by COUP-TFs has been demonstrated in lung cancer cell lines (Wu *et al.*, 1997). Retinoic acid can inhibit growth in lung cancer cell lines but only in those cell lines that are responsive to retinoic acid as assessed by induction of the RAR gene. In extracts from retinoic acid-sensitive cell lines, COUP-TF binds to the RARE whereas, in non-responsive lung cancer cell lines, no binding was observed. Thus, the postulated function of COUP-TF is to repress basal expression of the RAR gene in the absence of retinoic acid. Finally COUP-TFs have also been shown to inhibit the estrogen mediated activation of the mouse lactoferrin gene (Liu and Teng, 1992; Liu *et al.*, 1993) and the rat and human oxytocin genes (this thesis and Burbach *et al.*, 1994) via competitive binding to overlapping binding sites.

2.6.2 Heterodimerization with RXR

RXR is the heterodimeric partner of RARs, TRs, VDRs, PPAR and NGFI-B, [1991 #150; Zhang, 1992 #151; Leid, 1992 #172; Perlman, 1995 #152]. RXR greatly enhances the binding of these nuclear receptors to their cognate DNA response element. Interestingly, COUP-TFI can also heterodimerize with RXR (Kliewer *et al.*, 1992). By heterodimerizing

with RXR, COUP-TFs can regulate the availability of RXR and thus regulate the activity of RARs, TRs, VDRs, and PPARs. In addition, COUP-TFs can also interact with RARs and TRs and disrupt their functions (Berrodin *et al.*, 1992; Leng *et al.*, 1996).

In Drosophila, a similar situation is observed. Svp has been shown to regulate the function of Usp (Drosophila RXR homolog). Usp is the heterodimeric partner of the ecdysone receptor. In this case, the mechanism of inhibition by Svp can be explained as follows: 1) it interferes with the ecdysone signaling by competing with the ecdysone receptor/Usp heterodimer for the same DNA binding site. 2) Svp titers the availability of Usp to form the functional heterodimeric complex ecdysone receptor/Usp, by heterodimerizing with Usp (Zelhof *et al.*, 1995).

2.6.3 Transrepression by COUP-TFs

Transrepression is another mechanism of action that was recently described (Achatz *et al.*, 1997; Leng *et al.*, 1996). This type of repression does not require binding to DNA by COUP-TFs but takes advantage of the ability of COUP-TFs to heterodimerize with RXR, RAR, and TR via their respective LBD. In transient transfection studies, a Gal4-COUP-TFI(LBD) can interact with DNA-bound full length TR, or RAR, or RXR and the consequence of such heterodimeric interaction is the inhibition of basal and activated transcription. Deletion of 15 amino acids in the extreme C-terminal completely abolishes transrepression. The physiological significance of this type of repression is unclear as it is observed in transfection studies with artificially high amount of transfected plasmids.

2.6.4 Active repression or silencing

COUP-TFs possess a repressing activity when bound to DNA (Cooney *et al.*, 1993; Cooney *et al.*, 1992), similar to the effect observed with unliganded TR and RAR (Baniahmad *et al.*, 1992a; Baniahmad *et al.*, 1992b). The silencing of basal activity is promoter-specific and not due to general repression of transcription, such as titrating the availability of TFIIB with whom COUP-TFI has been shown to interact *in vitro* (Ing *et al.*, 1992). In addition, the repression by gal-COUP-TFI chimera of a gal-reporter can be relieved by overexpression of TR, indicating that the repressor protein (co-repressor) mediating this effect is the same for both proteins and that the co-repressor is present in limiting amounts in cells (Shibata *et al.*, 1997)

The repressing domain has been localized to the extreme 35aa C-terminal of the LBD of COUP-TFI (Achatz *et al.*, 1997; Leng *et al.*, 1996; Shibata *et al.*, 1997). This domain contains the putative AF-2 activation domain core associated with most nuclear receptors (Durand *et al.*, 1994; Wurtz *et al.*, 1996). The 35 amino acids C-terminal repressing domain (also referred as corepressor-interacting domain, CID) acts by interacting with the corepressors Nuclear Receptor Corepressor (N-CoR) and Silencing Mediator for Retinoic Acid Receptor and Thyroid Hormone Receptor (SMRT) (Chen and Evans, 1995; Horlein *et al.*, 1995; Shibata *et al.*, 1997). The two corepressors are highly related proteins and were initially identified as unliganded RAR- and TR-interacting proteins. The mechanism of repression by COUP-TFI is thought to involve recruitment, via its extreme C-terminal domain, of a repressor complex that contains the Sin3 protein, a histone deacetylase (HDAC1 or 2) in addition to the corepressors N-CoR or SMRT, (Alland *et al.*, 1997; Heinzel *et al.*, 1997; Nagy *et al.*, 1997). This recruited repressor complex is believed to deacetylate the N-terminals of the acetylated histone H3 and H4. The consequence of this is increased stability

of the nucleosome core, which generates a chromatin structure with limited promoter accessibility (see Fig. 7). However, the mechanism that links hypoacetylation of H3 and H4 to transcriptional repression is poorly understood. Hyperacetylation and hypoacetylation were historically associated with euchromatin and heterochromatin, respectively. In addition, genetic studies in yeast have linked the level of acetylation of specific lysines in the N-terminal of H3 and H4 with gene expression. Thus it is not surprising to discover that coactivators such as CBP, p300, P/CAF have histone acetylase activity and that corepressors such N-CoR and SMRT are associated with histone deacetylase activity (Alland *et al.*, 1997; Heinzel *et al.*, 1997; Nagy *et al.*, 1997).

2.6.5 Activation by COUP-TFs

COUP-TFI was initially characterized as a positive activator of the chicken ovalbumin promoter in *in vitro* transcription assays (Pastorcic *et al.*, 1986). Since the cloning of hCOUP-TFI, most of the activities found to be associated with COUP-TFs were generally negative, i.e. repressor of transcription. However, recent reports by many groups have described a transactivation function for COUP-TFs in numerous genes (reviewed in Tsai and Tsai, 1997). The mechanism of activation is unclear and seems to depend on the system and the cell type used. In some systems, it appears that activation does not require direct binding of COUP-TFs to DNA. Indeed, interactions of COUP-TFs with HNF-4, Oct1/2, and Sp1/Sp3 have been reported (Ktistaki and Talianidis, 1997; Pipaon *et al.*, 1999; Power and Cereghini, 1996; Rohr *et al.*, 1997). Most of these reports do not address the possible squelching, of the corepressors N-CoR or SMRT, a mechanism that has already been reported for COUP-TFI in relieving repression of unliganded gal-TR chimera (Shibata *et al.*, 1997). One can envision a

mechanism, where the bioavailability of corepressor will dictate the level of gene activity. Indeed, some steroid receptor antagonists, such as the antiestrogen tamoxifen or the antiprogestin RU486, have inappropriate agonistic activities in various tissues and cell lines (Horwitz, 1995; Meyer *et al.*, 1990). It has been proposed that the antagonistic activity of some steroids is dependent on the presence of corepressors and inappropriate level of corepressors can result in inappropriate agonistic activity. There is also evidence supporting the importance of the ratio of coactivators to corepressors as a crucial parameter in determining the activity of a ligand (Jackson *et al.*, 1997; Smith *et al.*, 1997). Thus, the fine-tuning of a given promoter may not only depend on the presence of positive and negative transcription factors but also on the level of coactivator to corepressor complexes.

2.6.6 COUP-TFs and Signaling Pathway

Like many nuclear receptors, COUP-TFs are phosphoproteins and their activities can be modulated by phosphorylation. Dopamine D1 agonists such as, dopamine and α-ergocryptine, have been shown to activate a chimeric protein consisting of the A/B, D/E domains of COUP-TFI and the C domain (DBD) of PR when cotransfected with a progesterone responsive reporter (Power *et al.*, 1991). It was also shown that the cAMP-dependent protein kinase activator (8-Br-cAMP) or the inhibitor of protein phosphatase 1 and 2A (okadeic acid) can stimulate the same reporter construct. Such results are consistent the possible phosphorylation of COUP-TFI. Furthermore, a deletion (amino acid 243 to 345) in the LBD of COUP-TFI abolished the dopamine-mediated activation reporter (Power *et al.*, 1991).

Recently, a putative coactivator was cloned by a yeast two-hybrid assay using the rat COUP-TFII as a bait (Marcus *et al.*, 1996). A previously cloned protein, p62, a ligand for the tyrosine kinase signaling molecule p56lck (Joung *et al.*, 1996), was shown to interact with COUP-TFII in yeast. The significance of this interaction remains to be demonstrated.

A phosphorylation mechanism may be used by orphan receptors to regulate gene expression in the absence of ligands and may provide a link to signal transduction pathways and nuclear events mediating cellular growth and differentiation.

2.7 Rationale and outline of the present study

The OT hormone plays important functions in the physiology of reproduction. OT and the related AVP are highly expressed in magnocellular neurons of the hypothalamus but are never co-localized. This exquisite expression pattern represents a superb example of nature's fine-tuning. Nevertheless, little is known about the determinants of this neuron-specific distribution. Our lack of knowledge is due to the absence of a suitable cell line model. The uses of heterologous cell lines have been employed by many groups including ours to bypass the lack of suitable neuroendocrine cell lines. Although the significance of such an approach is uncertain, transfection studies have been useful in identifying potential conserved DNA elements present in the OT promoter. Conserved DNA elements responsive to estrogen, retinoic acid and thyroid hormone were identified in the OT promoter using heterologous cell lines. In addition, our group has identified a cell line, Neuro2a, in which the human OT promoter is highly active. A minimal 100 bp OT promoter was also characterized using the Neuro2a cell line. The rational underlying the present study was to identify potential novel transcription factors that might regulate the OT promoter using the Neuro2a line as a model.

These studies were hindered by the lack of reproducibility of published results and sequencing errors in the published human OT sequence. Thus, a second approach was exploited which consisted in identifying other nuclear receptors that could play a role in the regulation of the OT promoter. In the first two parts of the present study, we provide evidence that orphan receptors COUP-TFII and Ear-2 are potent negative regulator of activated and basal activity of the human OT promoter. The importance of the regulation by COUP-TFII and Ear-2 proteins is supported by their presence in the epithelium layer of the pregnant rat uterus where OT is expressed.

In the third part of this study, we identify the ROR/RZR subfamily of orphan receptors as potential regulators of OT. In transfection studies, RORα1 can transactivate the OT promoter by 6- to 14-fold. The elements responsible for this activation were localized to the OT/ERE and a region 5' of the OT/ERE.

Overall, the present study points to a role of orphan receptors in the regulation of OT. These findings are supported by the high conservation between species of the DNA elements mediating these effects.

Chapter II

Nuclear orphan receptors COUP-TFII and Ear-2: presence in oxytocin-producing uterine cells and functional interaction with the oxytocin gene promoter

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Abstract

We have previously demonstrated that the oxytocin (OT) gene is expressed in the rat uterine epithelium and that its expression is upregulated *in vivo* and *in vitro* by estrogen. This hormonal regulation is mediated by a hormone response element (HRE) located in the OT gene promoter. Here we show that the same OT-HRE is also capable of interacting with two novel members of the orphan nuclear receptor family, rat COUP-TFII and Ear-2, and that this interaction antagonizes the estrogenic induction of the OT promoter. By Northern blot analysis and immunocytochemistry, using specific cDNA probes and antibodies, respectively, we demonstrate furthermore that both orphan receptors are expressed in uterine epithelial cells. Therefore, the present findings indicate that uterine OT gene expression is under stimulatory as well as inhibitory influences which are both mediated by the same HRE. More detailed analysis of the sequences necessary for estrogen receptor action and for orphan receptor action, using site-directed mutagenesis, revealed that the specific recognition sequences are overlapping but distinct: whereas the (imperfect) palindromic structure of the HRE constitutes the estrogen response element (ERE), orphan receptor action relies on an underlying direct TGACC repeat which forms part of the OT-HRE structure and overlaps with the estrogen response element.

1. Introduction

The nonapeptide oxytocin (OT) mediates several important reproductive functions, including milk ejection during lactation and uterine contractions during parturition (Gainer and Wray, 1994; Zingg, 1996). In addition, OT acts as a neurotransmitter and mediates specific social and reproductive behaviours (Insel, 1992). Within the central nervous system, OT is mainly produced in specific subsets of hypothalamic magnocellular and parvocellular neurons and is released at the level of the neural lobe, the median eminence as well as at specific synaptic sites within the brain. In addition to its central site of biosynthesis, OT is also produced in several peripheral organs, including the uterus (Lefebvre *et al.*, 1992; Zingg *et al.*, 1993). In the rat, uterine OT production is highest in the endometrial epithelium (Lefebvre *et al.*, 1992), and, in the human, in the decidua (Chibbar *et al.*, 1993). Uterine OT gene expression is induced by estrogen (Lefebvre *et al.*, 1994) as well as by retinoic acid (Larcher *et al.*, 1995). This effect is mediated via a composite hormone response element (HRE) in the OT gene promoter which encompasses an estrogen response element (ERE) (Richard and Zingg, 1990; Adan *et al.*, 1993) and a retinoic acid response element (RARE) (Richard and Zingg, 1991a). As illustrated in Fig. 1., the HRE in the human and rat OT gene represents an imperfect palindrome. It also encompasses, embedded within its structure a direct TGACC repeat. According to the classification of Umesono *et al.* (1991), this repeat structure represents a natural variant (i.e. with one mismatch and in reverse orientation) of a so called 'DR0 element', which is defined as a direct AGGTCA repeat with a spacing of zero nucleotides.

Several RAREs and EREs have been shown to interact with additional members of the large superfamily of nuclear receptors, specifically with members of the so-called orphan nuclear receptor family (Liu and Teng, 1992; Islam and Toftgard, 1994; Jonk *et al.*, 1994;

Ladias, 1994; Nakshatri and Chambon, 1994; Ben-Shushan *et al.*, 1995; Lee *et al.*, 1995). Furthermore, Liu *et al.* reported the presence in mouse uterine extracts of a DNA-binding activity that reacted with an anti-COUP-TF antibody (Liu and Teng, 1992). These reports prompted us to investigate (1) to what extent members of the orphan receptor family were co-expressed in uterine cells expressing the OT gene, and (2) whether specific members of the orphan receptor family were able to bind to, and functionally interact with, the HRE of the OT gene that had previously been characterized by Richard and Zingg (1990; 1991a; 1991b). In the present report, we focused on two members of the orphan receptor family, COUP-TFII (also referred to as ARP-1) and Ear-2, of which the rat homologue has recently been cloned and sequenced. Our data demonstrate that both members are co-expressed in OT expressing uterine endometrial cells, that they are capable of functionally interacting with the HRE present in the OT gene promoter, but that differences exist with respect to the precise sequence elements recognized by the ER and the orphan receptors studied here.

2. Materials and methods

2.1. *Animals* Control female (200 g) or timed-pregnant Sprague-Dawley rats were obtained from Charles River, St-Constant, Canada. Timed-pregnant rats were at the same weight as control rats at the onset of pregnancy. Animals were kept in a temperature and humidity controlled environment. They were killed by decapitation under ether anesthesia. The procedures were approved by the Royal Victoria Hospital Research Institute Animal Ethics Committee.

2.2. *Northern blot analysis* Total RNA was extracted from uterus by homogenization in guanidium thiocyanate and centrifugation through a cesium chloride cushion. RNA was denatured in 2.2 M formaldehyde and resolved electrophoretically on a 1.2% agarose-formaldehyde gel in phosphate buffer (pH 7.2). After electrophoresis, equal loading of the wells was verified by ethidium bromide staining and RNA was transferred to a Hybond-N filter (Amersham Life Sciences, Arlington, IL) and UV cross-linked. The RNA blots were incubated at 42°C for 4 h with prehybridization buffer containing 50% (vol:vol) formamide, 5X SSPE (1X SSPE, 0.01 M phosphate buffer, pH 7, and 0.15 M NaCl), 5X Denhardt'solution (0.02% (vol:vol) each of bovine serum albumin(BSA), Ficoll 400, and polyvinylpyrrolidone), 0.5% SDS, and 250 mg/ml salmon sperm DNA. Hybridization was carried out overnight in the same buffer with radioactively labeled cDNA probes. Washing was carried out consecutively in 5X SSPE:0.1% SDS, 2X SSPE:0.1% SDS, and 1X SSPE:0.01% SDS at 65°C.Membranes were then exposed with intensifying screens to Kodak XAR-5 films (Eastman Kodak, Rochester, NY). The cDNA probes used were specific for COUP-TFII and Ear-2, respectively. The COUP-TFII probe consisted of a 290 bp cDNA fragment corresponding to the 5' end of the rat COUP-TFII cDNA and the Ear-2 probe

consisted of a 381 bp fragment of the 5' end of the rat Ear-2 cDNA (Boutin *et al.*, 1996; unpublished data). Probes were labeled using the random primer labeling method.

2.3. *Immunohistochemistry* Antibodies were produced in rabbits against GST-fusion proteins containing the N-terminal part of rat COUP-TFII (residues 1–32) or of Ear-2 (residues 1–46), respectively (Boutin *et al.*, 1996; unpublished data). The specificity of the antibodies was established by Western blotting using full-length recombinant COUP-TFII and Ear-2 proteins (Boutin *et al.*, 1996; unpublished data). Antibodies (anti-COUP-TFII, anti-Ear-2 or non-immune serum) were applied in a dilution of 1:50 to 18mm cryostat sections of rat uteri taken either from non-pregnant animals or animals at term (day 22 of pregnancy). The first antibody was visualized using a FITC-conjugated goat anti-rabbit IgG antibody (Bio-Source, Camarillo, CA) applied at a 1:500 dilution. Sections were viewed and photographed using an Olympus immunofluorescence microscope.

2.4. *Plasmids* Plasmid pOT-164CAT contained 164 nucleotides of the 5'-flanking regions of the human OT gene linked to the reporter gene chloramphenicol acetyltransferase (CAT) as described earlier (Fig. 1) (Richard and Zingg, 1990). In plasmid pOT-49+eCAT, the ERE of the OT gene (sequences -164 to -146) was added at its original distance to a plasmid containing the 49 bp minimal OT promoter, as described (Richard and Zingg, 1990). Plasmid pOT-ERE/tkCAT (Fig. 1(B)) contained the ERE element of the OT gene in front of the herpes simplex virus thymidine kinase (tk) promoter (Richard and Zingg, 1990). pOT-mERE/tkCAT was identical to the former plasmid with the exception that the OT-ERE was mutated at three positions (mERE, Fig. 1(A)). These mutations changed the imperfect ERE palindrome into a perfect palindrome but destroyed the first of the two direct TGACC repeat elements, thus destroying the inherent DR0 structure. The last two plasmids were constructed by inserting

double-stranded DNA oligonucleotides upstream of the tk promoter into the *Sal*I and *Hin*dIII sites of a plasmid described earlier (ptkCAT) (Richard and Zingg, 1990), in which the herpes simplex virus thymidine kinase promoter was linked to a CAT cDNA. The sense-strands of the oligonucleotides used had the following sequences:

ERE: TCGAGCCAACGCGGTGACCTTGACC-CGGCCCA;

mERE: TCGAGCCAACGCGGTcACagTGACC-CGGCCCA (differences to the first oligonucleotide in lower case letters).

Plasmid vERE/admlCAT contained a 22 bp fragment containing the vitellogenin ERE placed upstream of the adenovirus major late promoter linked to the CAT gene (Tora *et al.*, 1989). Plasmid HE0 was an expression vector containing the human estrogen receptor (ER), placed under the control of the SV40 virus enhancer and promoter (Kumar *et al.*, 1987). Both plasmids were obtained from Dr P. Chambon, Strasbourg.

pRSV-COUP-TFII and pRSV-Ear-2 were expression vectors encoding rat COUP-TFII and Ear-2, respectively, as described by Boutin *et al.* (1996; unpublished data). They were constructed by using the expression vector (R-EXP) (Glass *et al.*, 1989), in which the inserted cDNA is placed under the control of the Rous sarcoma virus long terminal repeat. For COUP-TFII, a *Bam*HI site at position 177 and an *Xba*I site at position 1432 were created in the rat COUP-TFII cDNA by site-directed *in vitro* mutagenesis, and the *Bam*HI/*Xba*I fragment was inserted between the *Bam*HI and *Xba*I sites of the R-EXP vector. For Ear-2, a *Bam*HI site at position 203 and an *Xba*I site at position 1397 were created similarly and the *Bam*HI/*Xba*I fragment was inserted in the R-EXP vector.

2.5. *Transient transfection assays* Neuro-2a cells (obtained from the American Type Culture Collection, Rockville, MD) were maintained in Earle's minimal essential medium

(phenol red-free) supplemented with 10% dextran-coated charcoal-stripped fetal bovine serum (FBS). Ishikawa cells were obtained from Dr De Grandis, Toronto. Cells were plated on 10 cm plastic petri dishes (10^6 cells/plate) on the day before transfection. All transfections were carried out with plasmid DNA purified 2X by CsCl centrifugation. Unless stated otherwise, a total of 20 μg of DNA was added per plate. This included 10 μg CAT/promoter constructs, 2 μg of plasmid HE0 encoding the human ER, up to 2 μg of expression vectors for COUP-TFII or Ear-2 and 6–8 μg of salmon sperm DNA. The calcium phosphate procedure was used for cell transfection (Ausubel *et al.*, 1996). Estradiol benzoate (10^{-7} M) or vehicle (ethanol 0.01%, final concentration) was added 24 h after transfection. Cells were harvested 40 h following transfection and CAT activity was determined in cell extracts using the phase extraction method (Seed and Sheen, 1988) and normalized to protein extract content (mean/10 μg protein). CAT activity was determined by measuring the radioactivity recovered following phase extraction and was expressed in cpmX10^{-3}.

3. Results

3.1. *Northern blot analysis*

We determined whether two recent members of the COUP family of transcription factors, rat COUP-TFII and rat Ear-2, which have recently been cloned and sequenced by Boutin *et al.* (1996), were expressed in uterine tissues. RNA was extracted from rat uteri and analyzed by Northern blotting, using 5'-directed cDNA probes specific for COUP-TFII and Ear-2. With the 290 bp rat COUP-TFII cDNA probe, a single RNA band of 4.5 kb was detected in uterine extracts (Fig.2(A)). The 381 bp rat Ear-2 cDNA probe hybridized to a single band of 2.5 kb present in uterine extracts (Fig. 2(B)). The sizes observed for both transcripts corresponded to the sizes reported for the human counterparts present in human tissues (Miyajima *et al.*,1988; Ladias and Karathanasis, 1991). Comparison of the signal intensity of extracts obtained from uteri of non-pregnant rats and pregnant rats indicated that COUP-TFII as well as Ear-2 mRNA levels were increased at the end of gestation.

3.2. *Localization of COUP-TFII and Ear- 2 in the uterus*

To determine whether the corresponding protein products were expressed in the uterus and to delineate the tissues in which expression occurred, immunocytochemistry was applied using specific COUP-TFII and Ear-2 antibodies, recently developed by Boutin *et al.*(1996). With both antibodies, the strongest staining was observed in the uterine epithelium (Fig. 3). Some staining was also observed in the stroma and the myometrium, although to a much weaker extent.

3.3. *COUP-TFII and Ear- 2 repress estrogen receptor-mediated activation*

As studies from this laboratory had shown earlier, the uterine epithelium is also a major site of OT gene expression (Lefebvre *et al.*, 1992) and uterine OT gene expression is

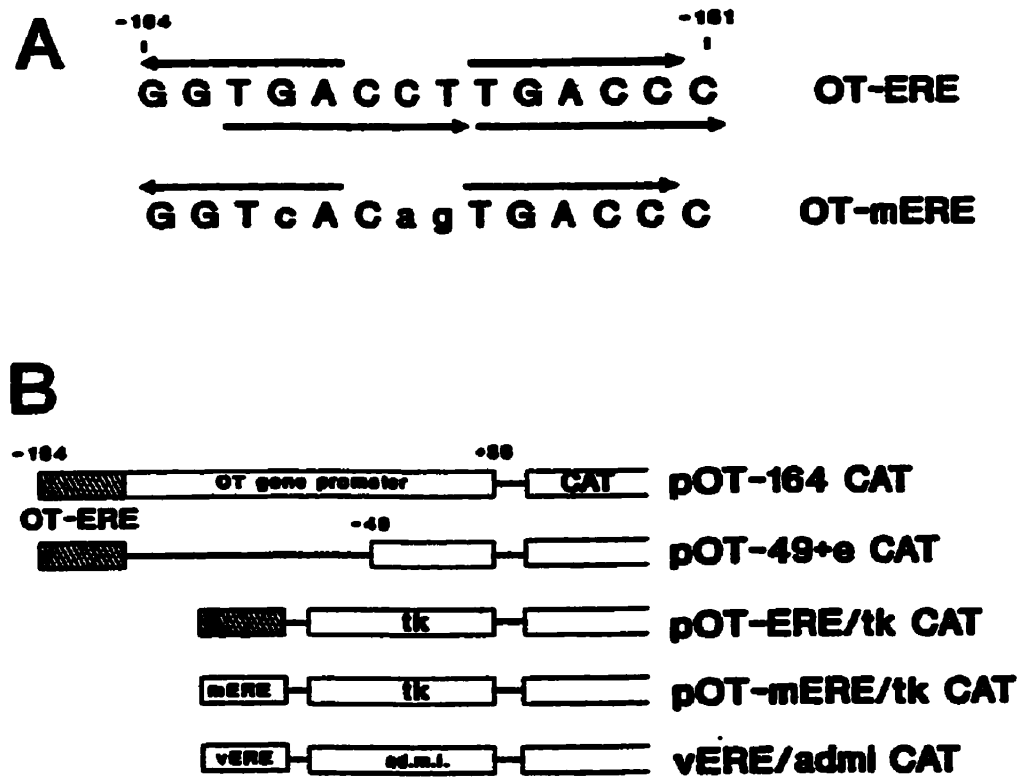


Fig. 1. (A) OT-ERE: sequence of the ERE present in the human and rat OT genes. Numbering according to the human OT gene sequence (Sausville *et al.*, 1985). Arrows above letters indicate the (imperfect) palindrome constituting the ERE. Arrows below letters indicate the direct repeat structure present in the same sequence element. The repeat corresponds (with one mismatch) to a 'DR0' structure according to Umesono *et al.* (1991). OT-mERE: mutation of the OT-ERE. This mutation converts the ERE to a perfect palindrome but destroys the DR0 structure. (B) Constructs used in the present paper are as described in Materials and methods. OT-ERE: sequence element shown in (A); CAT: chloramphenicol-transferase; tk: Herpes simplex virus thymidine kinase promoter; mERE: OT-mERE as shown in (A); vERE: 22 base pair element containing the vitellogenin ERE (Kumar *et al.*, 1987); adml: adenovirus major-late promoter (Kumar *et al.*, 1987).

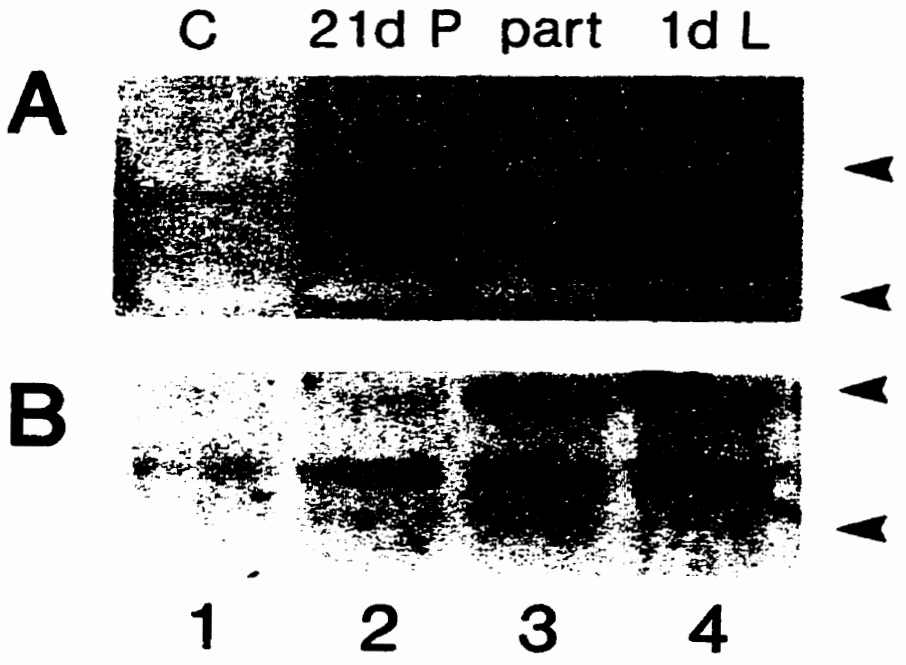


Fig. 2. Northern blot analysis of COUP-TFII (A) and Ear-2 (B) RNA present in extracts of rat uterus (lanes 1–4). Total RNA (50 μg) purified from the uterus of a non-pregnant (lane 1), 21 day pregnant (lane 2), parturient (lane 3) and 1 day lactating rat (lane 4) was hybridized to a 32 P-labeled COUP-TFII-specific DNA probe (A) or an Ear-2 specific cDNA probe (B). Exposure time: 3 day (A) or 14 day (B). The blot shown is representative of three independent experiments.

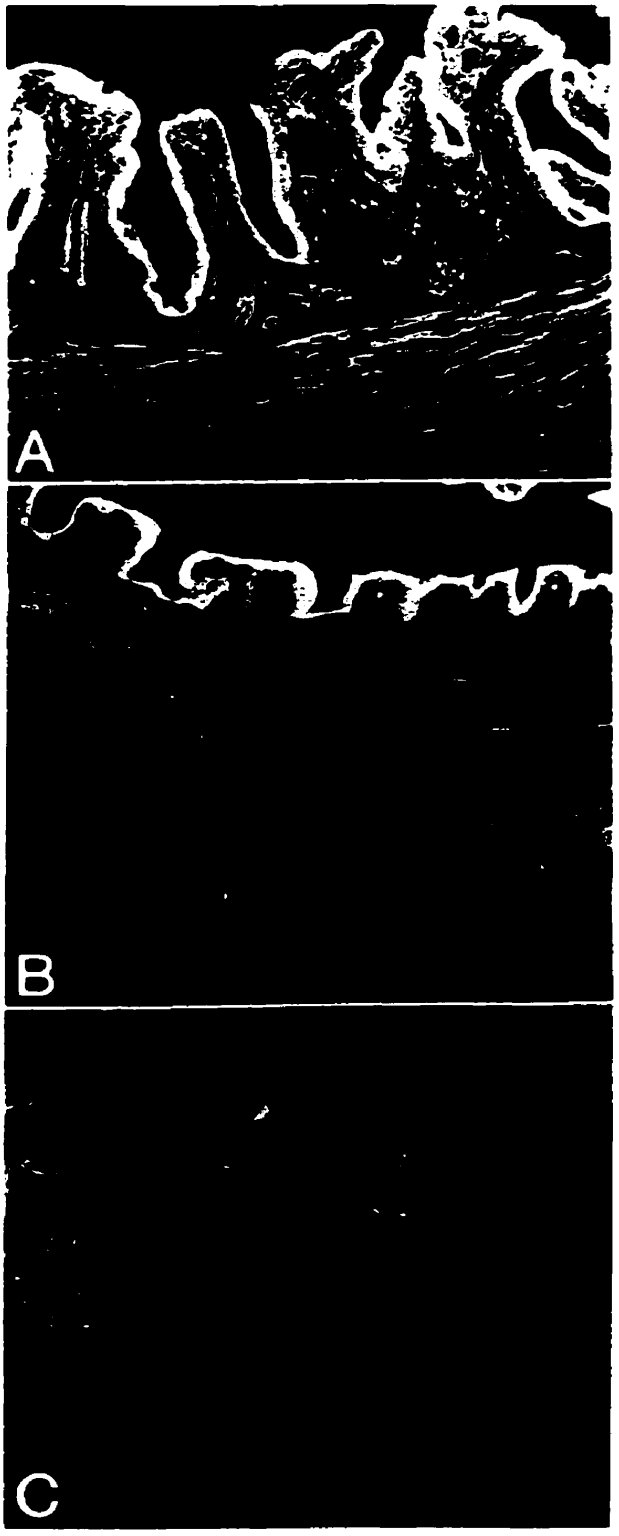
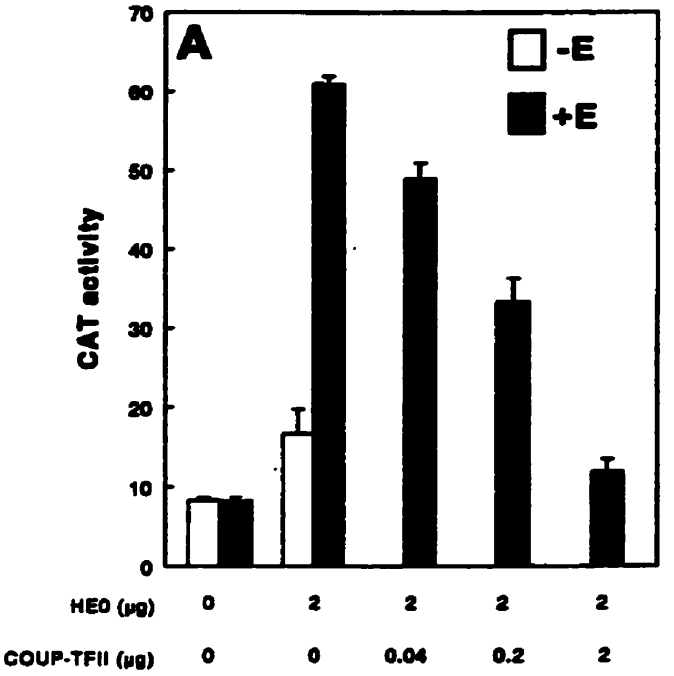


Fig. 3. Immunohistochemical localization of COUP-TFII-like immunoreactivity (A) and Ear-2 like immunoreactivity (B) in sections of a rat uterus at the day of parturition. Sections were incubated with a 1:50 dilution of a specific anti-rat COUP-TFII antibody (A), a specific anti-rat Ear-2 antibody (B) or with non-immune rabbit serum (C) followed by incubation with a FITC-coupled second antibody and visualization under a fluorescence microscope.

strongly inducible by estrogen (Lefebvre *et al.*, 1994). We therefore tested whether COUP-TFII and/or Ear-2 were able to interact with the OT gene promoter, specifically with the previously characterized HRE present in the OT promoter. The host cell lines used included Neuro-2a cells as well as Ishikawa cells. Neuro-2a is a mouse neuroblastoma-derived cell line which expresses both the OT as well as the related vasopressin genes (Richard and Zingg, 1991a; Bamberger *et al.*, 1995). Whereas Neuro-2a cells are presently the only cell line known to express the OT gene and have previously been extensively used for studies on OT gene promoter function (Richard and Zingg, 1990, 1991a,b; Chu and Zingg, 1997), Ishikawa cells represent a well-differentiated human endometrial adenocarcinoma cell line and provide a well-characterized *in vitro* system for the study of human endometrial epithelial cell function (Nishida *et al.*, 1985).

As shown in Fig. 4(A) (bar 1 vs bar 2), the basal promoter activity of the construct pOT-164CAT was increased >6-fold by co-transfection with the ER expression vector HE0 in the presence of 10^{-7} M estrogen. This effect confirmed our results obtained earlier with the same construct (Richard and Zingg, 1990). However, the ER-mediated stimulation was inhibited by co-transfection with an expression vector for COUP-TFII (pRSV-COUP-TFII) (Fig. 4(A)). This effect was dose-dependent. Co-transfection with equal amounts of COUP-TFII and ER expression vectors led to a complete abolition of the ER-induced transcriptional response (Fig. 4(A), bar 5). A significant suppression was also observed when a 50X lower amount of COUP-TFII expression vector was used for transfection (Fig.4(A), bar 3).

To test the effect of Ear-2 on the estrogenic induction of the OT gene promoter, similar experiments were carried out, using the Ear-2 expression vector pRSV-Ear-2. As shown in Fig. 4(B), these experiments demonstrated that Ear-2 was an equally potent



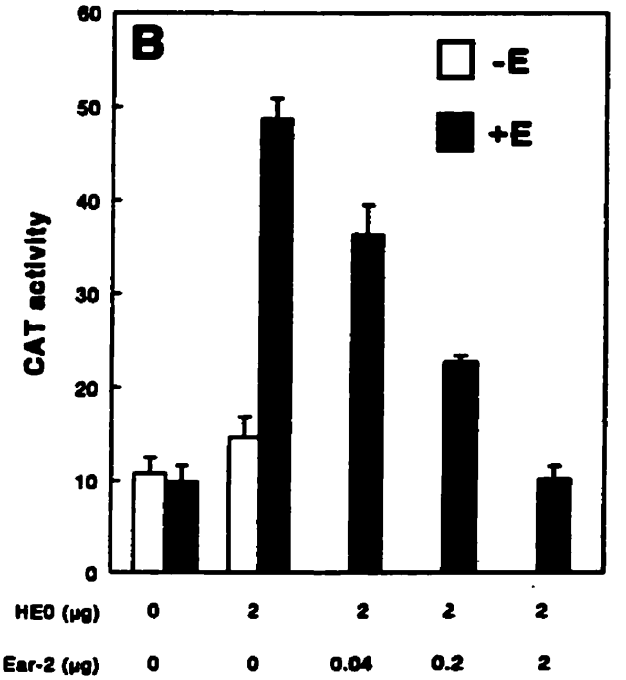
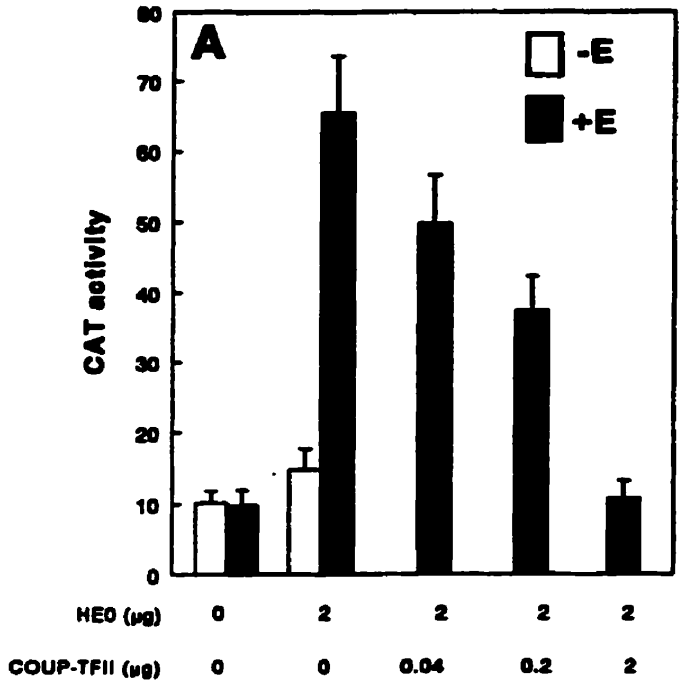


Fig. 4. Repression by COUP-TFII or Ear-2 of the estrogen-induced transcriptional activation of the human OT gene promoter. Neuro-2a cells were transfected with 10 µg of pOT-164CAT and different amounts of the estrogen receptor expression vector HE0 and expression vectors for either COUP-TFII (A) or Ear-2 (B) as indicated. Estradiol was added at a final concentration of 10^{-7} M. CAT activity was determined in cell extracts by the phase extraction method (Seed and Sheen, 1988) and was expressed as cpmX10^{-3}. Each bar represents the mean ± SE of four independent experiments.



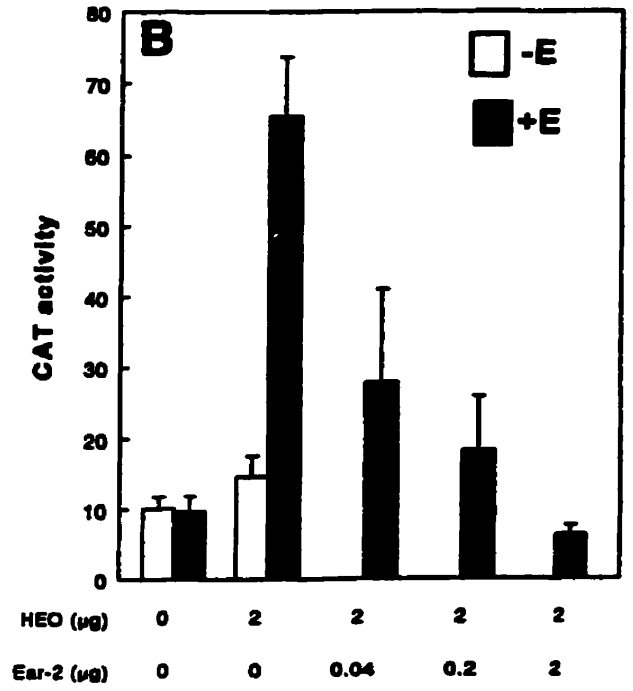


Fig. 5. Effect of COUP-TFII (A) and Ear-2 (B) on the estrogenic induction of the mutant construct pOT-49+eCAT. Transfections and CAT assays were carried out as described in Fig. 4. Each bar represents the mean ± SE of three independent experiments.

suppressor of estrogen-induced OT promoter activity. Co-transfection of the Ear-2 expression vector at amounts 50X lower than the ER expression vector led to a significant suppression of ER-induced transcriptional stimulation. Total suppression was observed when equal amounts of pRSV-COUP-TFII and HEO vectors were used for transfection.

As demonstrated earlier (Richard and Zingg, 1990), only the HRE region (-164 to -146) in conjunction with a minimal region of the OT promoter (-49 to +36) is necessary for full estrogen inducibility of the OT promoter. To test whether these sequences are also sufficient for mediating the COUP-TFII and Ear-2 induced suppressive effects, the preceding experiments were repeated using pOT-49+eCAT as a reporter construct. As shown in Fig. 5(A) and (B), the same dose-dependent repression of estrogen inducibility was observed as with the full-length wild-type construct, suggesting that the HRE region necessary and sufficient for mediating the estrogen response was also sufficient for mediating the COUP-TFII as well as the Ear-2-induced suppression of this response.

To determine to what extent this effect was dependent on the promoter context, the extended HRE region (-172 to -145) was inserted upstream of the viral tk promoter (plasmid pOT-ERE/tkCAT). As shown in Fig. 6(A) (bars 1 and 2), the estrogen response of this plasmid was fully suppressed by co-transfection of an equal amount of COUP-TFII expression plasmid. However, the introduction of three specific point mutations within the HRE region (mERE) prevented COUP-TFII from exerting its full suppressive effect (Fig. 6(A)). In this mutation, the OT ERE was transformed into a perfect ERE palindrome (Fig. 1(A)) and thus, the estrogen response remained unaffected. However, the additional two point mutations in the spaced region between the wings of the palindrome destroyed the DR0 structure by changing the first TGACCT repeat to TCACAG. The fact that this mutation

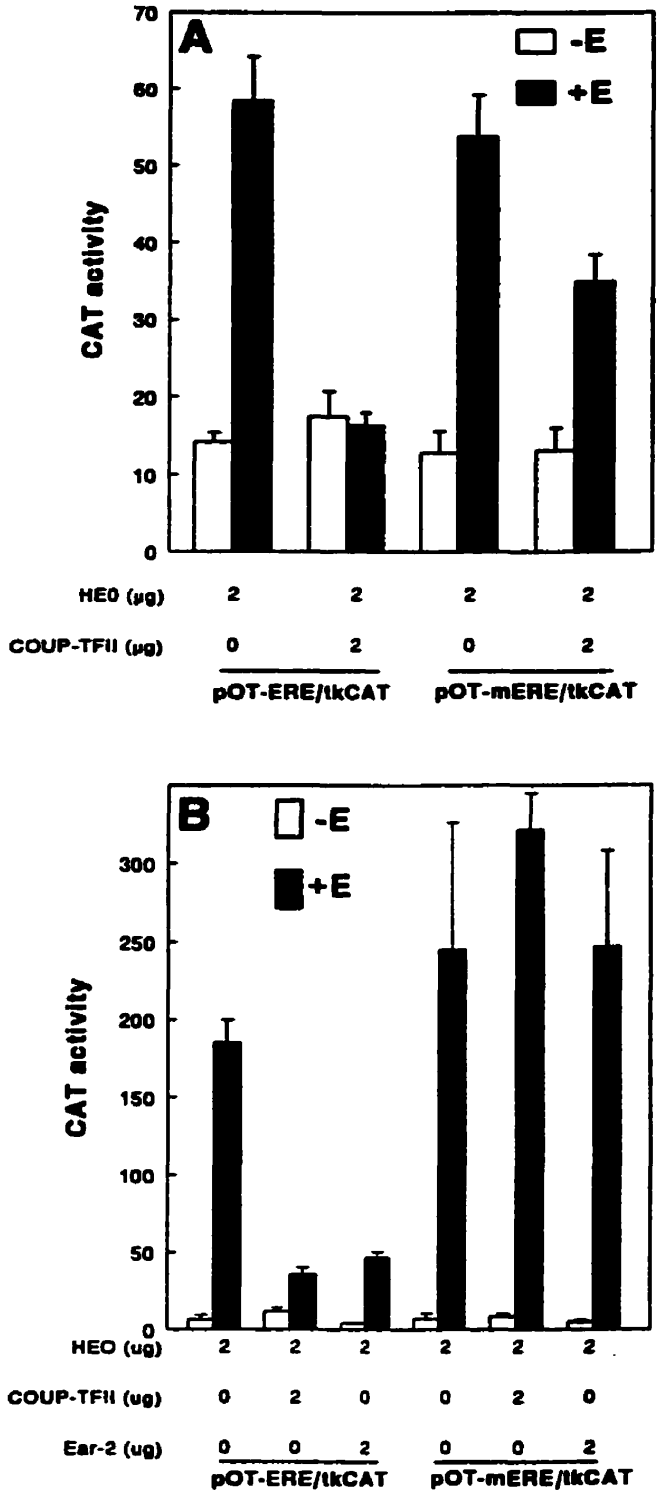


Fig. 6. Effects of COUP-TFII or Ear-2 co-transfection on the induction by estrogen of the constructs pOT-ERE/tkCAT and pOT-mERE/tkCAT in Neuro-2a cells (A) or Ishikawa cells (B). The constructs are as described in Fig. 1. They represent the ERE of the OT gene promoter (pOT-ERE) or a mutated version thereof (pOT-mERE), respectively, linked to the herpes simplex virus tk promoter. The amounts of estrogen receptor expression vector HE0 and of the COUP-TFII expression vector are indicated. Estradiol 10^{-7} M, (closed bars) or vehicle 0.01% ethanol, (open bars) was added following transfection. Each bar represents the mean ± SE of three independent experiments.

maintained estrogen inducibility but markedly affected the suppressive effect of COUP-TFII suggests that the binding determinant for the orphan receptors is not the ERE *per se* but the inherent DR0 structure present in the OT-HRE.

We further wished to determine whether the orphan receptor effects observed above in Neuro-2a cells could also be reproduced in the human uterine epithelium-derived Ishikawa cells. As shown in Fig. 6(B), essentially the same effects of COUP-TFII and Ear-2 could be observed on the estrogenic induction of the OT-ERE. Both orphan receptors were able to suppress E2-induction of the wild-type OT-ERE, but not of the mutated OT-ERE (OT-mERE). This result suggests that the observed effects of COUP-TFII and Ear-2 are not dependent on cell-specific factors and are observable in neuronally-derived as well as epithelial cells. To test whether the interaction of COUP-TFII and Ear-2 with the OT-HRE was specific for this ERE or whether similar interactions occurred with other EREs, we examined the effects of COUP-TFII and Ear-2 on the estrogen-inducibility of the vitellogenin ERE linked to the adenovirus major later promoter (vEREadml-CAT). The estrogen induction of this construct remained completely unaffected by co-transfection with equal amounts of either the expression vector for COUP-TFII or Ear-2 (Fig. 7).

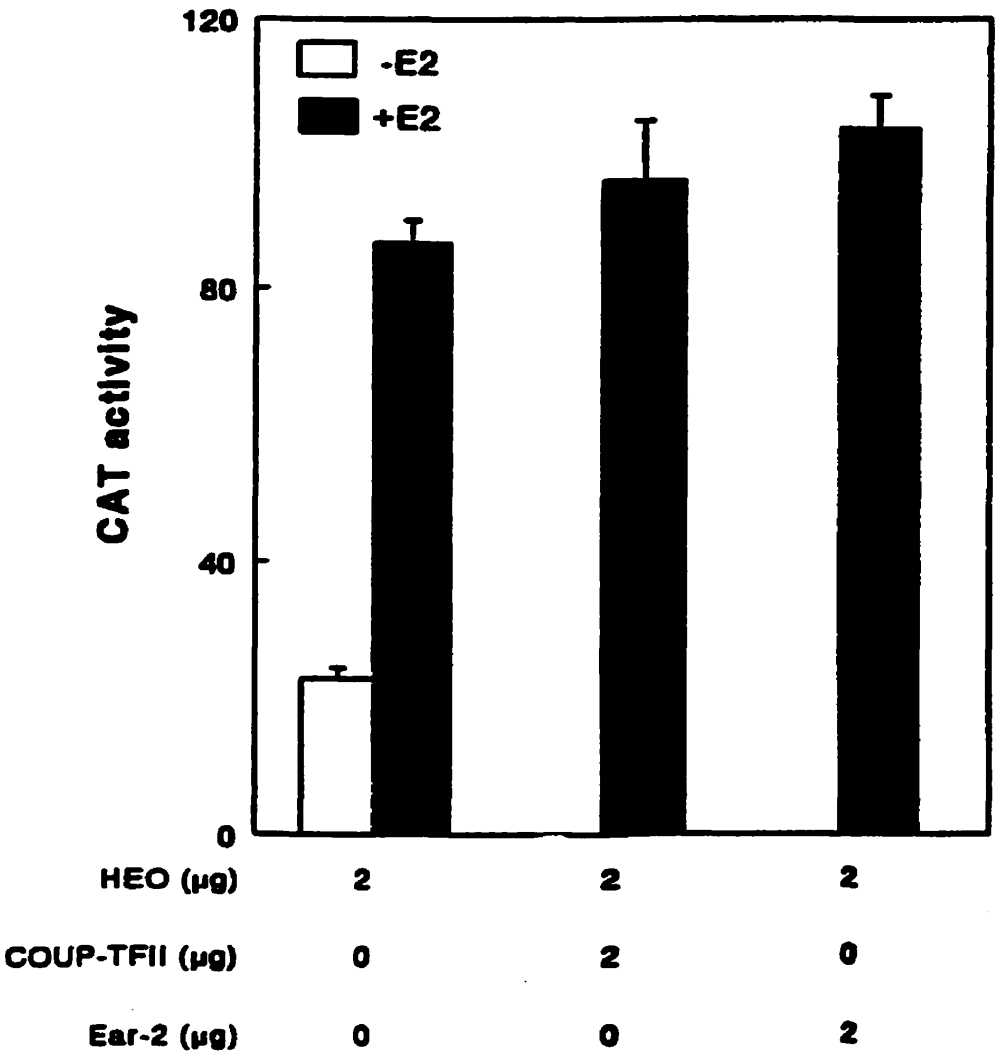


Fig. 7. Absence of an effect of co-transfection with an expression vector for COUP-TFII or Ear-2 on the estrogenic induction of a vitellogenin ERE linked to the adenovirus major-late promoter. Cells were transfected with an expression vector for the ER (HE0) and an expression vector for COUP-TFII or Ear-2 as indicated. Cells were treated with estradiol (10^{-7} M, closed bars) or vehicle (0.01% ethanol, open bar). Each bar represents the mean ± SE of three independent experiments.

4. Discussion

Previous studies from this laboratory showed that the human OT gene promoter is activated by estrogen and retinoic acid, and that this activation involved a HRE located 160 bp 5' to the transcriptional initiation site (Richard and Zingg, 1990, 1991a). We have also shown that this hormonal activation mechanism is functional *in vivo* in as much as both estrogen and retinoic acid are capable of inducing OT gene expression in the uterine epithelium (Lefebvre *et al.*, 1994; Larcher *et al.*,1995).

In the present report we now demonstrate that the rat uterine epithelium is also the site of expression of additional members of the nuclear receptor superfamily, namely of at least two members of the orphan receptor family, COUP-TFII and Ear-2, and that these nuclear factors are able to interact with the OT gene promoter. Whereas interactions of the ER and the RAR with the OT-HRE lead to a stimulation of transcription in the presence of the cognate ligands, the present study indicates that COUP-TFII and Ear-2 exert an opposing, inhibitory effect in the experimental system used. Experiments with the deletion mutant pOT-49+eCAT showed that both COUP-TFII and Ear-2 appear to interact with the same HRE as the ER. This idea was further supported by the finding that inhibition of estrogenic stimulation by COUP-TFII was still observed when the OT-HRE was linked to a herpes simplex virus tk promoter. However, the use of an additional mutant, in which the ERE was converted to a perfect palindrome but in which the inherent direct TGACC(Py) repeat was destroyed, indicated that the precise sequence requirements for the stimulatory effect of the ER differed from these for COUP-TFII. Whereas these and earlier studies (Richard and Zingg, 1990) showed that the ERE palindrome is necessary and sufficient for the estrogenic induction of the OT promoter,

the present results indicate that orphan receptor-mediated suppression involves the underlying TGACC(Py) repeat structure.

Umesono *et al.* (1991) classified the response elements for different nuclear receptors in terms of repeats of the element AGGTCA with different spacings between the repeat element. According to this classification, the present repeat structure in the OT gene corresponds to a variant of the direct repeat with a spacing of zero base pairs (DR0). The present element in the human OT gene differs from a classical DR0 element with respect to one mismatch and with respect to its 5'–3' orientation. Studies with synthetic response elements with different spacings showed that the DR1 element (AGGTCA repeat with a spacing of one nucleotide) is the preferred binding sites for COUP-TF homodimers (Kliewer *et al.*, 1992), but that DR0 sites can also act as sub-optimal COUP-TFII binding sites (Cooney *et al.*, 1992; Kadowaki *et al.*, 1992). These data are compatible with the idea that the inherent DR0 motif in the human OT-HRE is the determinant for the interaction of COUP-TFII and Ear-2 with the OT gene promoter. Moreover, our data obtained with the vitellogenin ERE linked to the adenovirus major-late promoter indicated that the COUP-TFII and Ear-2 transcription factors do not interact indiscriminately with any ERE, since the estrogen response of the vitellogenin ERE remained unaffected by either COUP-TFII or Ear-2 co-transfection. This implies that the OT-ERE is relatively unique in its capacity to interact on a competitive basis with the ER as well as with members of the orphan receptor family. The only other ERE for which a similar competitive interaction has been described is the ERE present in the lactoferrin gene which, interestingly, is also expressed in the uterine epithelium (Liu and Teng, 1992).

The fact that COUP-TFII and Ear-2 are both expressed in the uterine epithelium and that interaction with the OT-ERE occur in uterine epithelium-derived cell lines, as shown in the present study, supports the idea that these transcription factors may play a role *in vivo* as modulators of uterine epithelial OT gene expression. We have previously shown that the uterine expression of the stimulatory nuclear receptors, ER and RAR, is similarly upregulated during pregnancy in the rat uterus (Larcher *et al.*, 1995). Thus, as term approaches, uterine OT gene expression may be under both positive and negative regulatory influences and the balance between these influences may result in a precise tuning of the levels of OT gene expression. Alternatively, it cannot be excluded that the *in vivo* context in the intact animal, the function of the orphan receptors studied here may undergo so far poorly understood regulatory influences which could reveal hidden stimulatory potentials of these receptors. The following possibilities deserve consideration:

An unidentified ligand, released in a paracrine or endocrine fashion, could function as an activator of the orphan receptors studied here. Alternatively, post-translational modifications of the orphan receptors, such as specific phosphorylation, may be necessary to convert them into transcriptional activators. Support for this possibility stems from the studies by Power *et al.* (1991), which demonstrated that a chimera containing the COUP-TFI ligand binding domain linked to the progesterone receptor DNA binding domain was activated by dopamine. Moreover, the *Drosophila* COUP-TFII homologue, *seven-up*, functions as a transcriptional activator in response to activation of the *ras* pathway (Begemann *et al.*, 1995; Kramer *et al.*,1995). Most recently, a positively acting co-activator of COUP-TFII has been identified which allows this orphan receptor to act as a transcriptional activator (Marcus *et al.*, 1966). This co-factor, termed ORCA, is also a ligand for the tyrosine kinase signaling

molecule $p56^{lck}$ and possesses intrinsic or tightly-associated serine/threonine kinase activity (Park *et al.*, 1995). Thus it cannot be excluded that COUP-TFII and Ear-2 may be involved in activating uterine OT gene expression *in vivo* via so far unidentified signaling pathways.

A physiological role for COUP-TFI in the regulation of the OT gene has previously been proposed by Wehrenberg *et al.* (1992) in the bovine corpus luteum and by Lopes da Silva *et al.* (1995) in the hypothalamus. In bovine granulosa cells, unlike the situation in the rat uterus, expression of COUP-TFI and II is inversely correlated to OT gene expression, suggesting that in this cell type these transcription factors act as repressors (Wehrenberg *et al.*, 1994). Based on their findings of interactions of the orphan receptor COUP-TFI with the rat OT promoter, the group of Burbach *et al.* (1994) investigated the expression of COUP-TFI and II in hypothalamic neurons (Lopes da Silva *et al.*, 1995). Their studies showed that, contrary to the uterine epithelium, neither COUP-TFI nor II were co-expressed with the OT gene in hypothalamic neurons of the supraoptic nucleus (Lopes da Silva *et al.*, 1995).

In conclusion, our previous and present studies indicate that, in the rat uterus, OT gene expression is regulated by multiple signaling pathways which include both stimulatory and possibly inhibitory effectors, interacting with overlapping DNA recognition elements. In view of the high expression of COUP-TFII, Ear-2, and possibly other members of the orphan receptor family, the uterus, and specifically the uterine epithelium, may represent a useful model system for further studies on the physiological functions of orphan receptors.

Acknowledgements

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Chapter III

The nuclear orphan receptors COUP-TFII and Ear-2 act as silencers of the human oxytocin gene promoter

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Preface

In Chapter II, we observed the inhibitory effect of COUP-TFII and Ear-2 on the estrogenic induction of the human OT promoter. The mechanism of inhibition by COUP-TFII and Ear-2 is one of competitive binding to overlapping sites. Another known mechanism of action of COUP-TFs is its ability to actively silence or repress a promoter. Therefore, in Chapter III, we investigate the ability of COUP-TFII and Ear-2 to actively repress the human OT promoter. The following studies were performed in the mouse neuroblastoma cell line, Neuro2A, in which the human OT promoter is very active.

ABSTRACT

We have previously shown that COUP-TFII and Ear-2, two members of the nuclear orphan receptor family, are able to repress oestrogen-stimulated transcriptional activity of the human oxytocin (OT) gene promoter by binding to a site that overlaps with the oestrogen response element (ERE) present in the 5' flanking region of the gene. Although most nuclear receptor-mediated transcriptional repression conforms with the paradigm of passive repression and involves competitive binding to an activator site, active repression, i.e. silencing of basal promoter activity, has been observed in a limited number of cases. Here we show by co-transfection experiments using COUP-TFII and Ear-2 expression vectors and reporter constructs containing OT gene promoter fragments linked to the chloramphenicol acetyltransferase gene that both COUP-TFII and Ear-2 are capable of silencing basal OT gene promoter activity by 54 and 75% respectively. 5' Deletion and footprint analyses revealed two areas of functionally important interaction sites: (1) a direct TGACC(T/C) repeat overlapping the ERE and (2) a more promoter-proximal area centered at -90 containing three imperfect direct repeats (R1–R3) spaced by four nucleotides each. Mutagenesis of reporter constructs as well as electrophoretic mobility shift assays demonstrated that each of the three proximal repeats R1–R3 contributed to orphan receptor binding and the silencing effect. Inasmuch as the orphan receptor-binding sites are not involved in mediating basal transcriptional activity of the OT gene promoter, the observed effects are best interpreted as active repression or promoter silencing. Moreover, since COUP-TFII and Ear-2 are both co-expressed in OT-expressing uterine epithelial cells, the novel transcriptional effects described here are likely to be of functional importance in the fine-tuning of uterine OT gene expression *in vivo*.

INTRODUCTION

Oxytocin (OT) is a multifunctional hormone that mediates uterine contractions, milk ejection, specific behavioural patterns as well as natriuresis (Zingg, 1996). The OT gene is expressed centrally in specific neurons, including neuroendocrine cells in the supraoptic and paraventricular nuclei of the hypothalamus as well as peripherally in uterine epithelium (Lefebvre *et al.* 1992), fetal membranes (Chibbar *et al.* 1993, Lefebvre *et al.* 1993) and the corpus luteum (Ivell *et al.* 1985). We and others have demonstrated previously that OT gene expression is hormonally induced by oestrogen and retinoic acid via direct promoter activation (Richard & Zingg 1990, 1991a, Adan *et al.* 1993, Zingg *et al.*1995) and that this induction can be suppressed by the action of members of the COUP family of orphan nuclear receptors (Burbach *et al.* 1994, Chu *et al.* 1998). In the human and rat genes, the repression of oestrogenic induction by COUP-TFs involves direct competition of binding to an oestrogen response element (ERE) centered around 160 bp upstream of the transcriptional initiation site (Burbach *et al.* 1994, Chu *et al.* 1997). In the bovine corpus luteum, the OT gene is induced by steroidogenic factor 1, and this induction is suppressed by COUP-TFI by competition for a common binding site (Wehrenberg *et al.* 1994).

The above-described mechanisms of transcriptional repression are commonly referred to as passive repression (Johnson 1995, Leng *et al.* 1996) and involve competition between an activator and a repressor for a common or overlapping DNA-binding site. In contrast, active repression or silencing occurs by actively interfering with the formation of a functional pre-initiation complex either by directly suppressing the function of general transcription factors or by inhibiting the transactivation function of an activator (Johnson 1995, Leng *et al.* 1996).

Several members of the nuclear receptor family have been found to use one or more of these mechanisms to achieve transcriptional repression (Renkawitz 1990, Johnson 1995). Therefore we investigated in the present study the capacity of two orphan nuclear receptors that are co-expressed with OT in uterine epithelial cells, namely COUP-TFII (also referred to as ARP-1 or COUP-β) and Ear-2 (also referred to as COUP-γ), to exert an active silencing effect on the basal activity of the OT promoter. Our studies show that both orphan receptors are capable of suppressing the basal activity of the OT promoter and that this effect involves specific binding sites distributed over an area spanning 86 nucleotides. Our studies further indicate that the observed effects correspond to active silencing, since the sequences involved in silencing do not contribute positively to basal promoter activity.

MATERIALS AND METHODS

Plasmids The plasmids p164(wt)CAT, p115(wt)CAT and p49(wt)CAT contained portions of the 5' flanking region of the human OT gene (positions –164 to +36, -115 to +36 and -49 to +36 respectively) linked to the bacterial reporter gene chloramphenicol acetyltransferase (CAT). The parent plasmid used for these constructs was pBluescript KS (+). The polylinker region was modified by digestion with SalI and S1 nuclease followed by religation, in order to remove a potential orphan nuclear receptor-binding site in the polylinker region. Point mutations were generated by site-directed mutagenesis using the methods described by Kunkel (Ausubel *et al.* 1996) using appropriate oligonucleotides consisting of 27 bases each and carrying the desired mutations at their centre. All constructs were verified by dideoxy-nucleotide sequencing. Expression constructs for COUP-TF and Ear-2 were generated by ligating full-length cDNAs encoding rat COUP-TF or rat Ear-2 (obtained from Dr J M Boutin, Hôpital Hôtel Dieu de Montréal, Montréal, Québec, Canada) between the XbaI and BamHI sites of the expression vector pcDNA3 (Invitrogen, San Diego, CA, USA). In this vector, the inserted cDNA is placed under the control of a cytomegalovirus promoter.

Transfections Mouse Neuro-2a cells (ATCC CCL131; American Type Culture Collection, Rockville, MD, USA) were maintained in Eagle's minimum essential medium (EMEM; Sigma, St Louis, MO, USA) supplemented with 10% fetal bovine serum (Gibco, New York, NY, USA). Cells were plated on to 60 mm dishes and allowed to reach 70–80% confluency before transfection. Each plate was transfected with a total of 10 μg DNA, including 1 μg CAT reporter plasmid, 0.25 μg expression vectors and 8.75 μg pBluescript plasmid KS(+). Transfection was performed by the calcium phosphate precipitation technique. After 4 h of incubation with the precipitate, a 'glycerol shock' was applied by incubating cells in a

buffered saline solution containing 15% glycerol for 2.5 min. Cells were then washed with PBS and cultured in EMEM/10% fetal bovine serum. At 48 h after the transfection, cells were collected and CAT activity was determined using the phase extraction method (Seed & Sheen 1988). Protein concentration was measured using the Bio-Rad Protein Assay Kit (Bio-Rad, Hercules, CA, USA).

Bacterial expression of proteins Recombinant rat COUP-TFII and Ear-2 were produced in BL21 cells as glutathione S-transferase (GST) fusion proteins. cDNAs encoding rat COUP-TFII or rat Ear-2 were inserted into the bacterial expression vectors pGEXKG and the resulting plasmids were transfected into BL21 cells as described (Novagen, Madison, WI, USA). Cells were grown until they reached an OD 550 of 0.6. At this point, isopropyl β-D-thiogalactoside (IPTG) was added to a final concentration of 0.1 mM. At 3h after IPTG induction, cells were collected and purified using a GST resin (Pharmacia, Piscataway, NJ, USA), according to the manufacturer's instructions. Finally, the GST moiety was removed by thrombin cleavage.

DNase I footprinting assays Probes used for Dnase I footprinting were obtained by PCR. The primer pair used for the amplification reaction consisted of oligonucleotide R48 (complementary to a part of the pBluescript polylinker region; sense strand direction) and a reverse primer complementary to the 5'-end of the CAT cDNA (antisense direction). The oligonucleotide R48 was labelled with ^{32}P using polynucleotide kinase. For the PCR, p164(wt)CAT was used as a template in conjunction with 10 pmol of each primer. After 35 cycles of amplification (94 °C for 1 min; 58 °C for 1 min; 72 °C for 1 min), the PCR product was gel purified after PAGE and diluted to 10 000 c.p.m./μl. For DNase I footprinting

analysis, 0.06–5 µl recombinant protein were incubated with 10 000 c.p.m. DNA probe in binding buffer (12 mM Hepes, pH 7.9, 12% glycerol, 70 mM KCl, 3 mM MgCl2, 250 ng poly(dI-dC)). After incubation of the binding reaction mixture for 30 min at room temperature, 0.25 unit DNase I (RQ1-DNase I; Promega, Madison, WI, USA) was added for 90 s. The endonuclease reaction was stopped by the addition of EDTA to a final concentration of 5 mM. The binding reaction mixture was phenol extracted, precipitated with ethanol and resuspended in 90% formamide loading buffer. Equal amounts of recovered probe were then loaded on a 10% denaturing polyacrylamide gel.

Electrophoretic mobility-shift assay (EMSA) For EMSAs, *in vitro* translated COUP-TFII and Ear-2 were used. Translation products were obtained *in vitro* by using plasmids pcDNA3-COUP-TFII and pcDNA3-Ear-2 as templates in an *in vitro* transcription/translation reaction mix (TNT lysate; Promega) according to the manufacturer's instructions. All proteins were [^{35}S]-methionine-labelled and their sizes verified by SDS–PAGE (10% gels).

Double-stranded oligonucleotides (10 pmol) were labelled using T4 kinase and [γ-32 P]ATP. Labelled probes were purified by PAGE. Then 1 µl *in vitro* translated COUP-TFII or Ear-2 was incubated in binding buffer with 10 to 25 fmol probe for 30 min at room temperature and electrophoresed on a non-denaturing 4% polyacrylamide gel in 0.25X Tris/borate/EDTA at 150 V. Antibodies against rat COUP-TFII and Ear-2 were obtained from Dr J Boutin.

RESULTS

Previous studies in this laboratory have shown that the neuroblastoma-derived Neuro-2a cells express the endogenous OT gene and support OT gene promoter activity in transient transfection assays (Richard & Zingg 1990, 1991a). By 5' and 3' deletion analyses, we

previously found that basal promoter activity is mediated by a defined region extending from -49 to +36 of the OT promoter (Richard & Zingg 1991b). In the present study, we used co-transfection of expression vectors encoding the nuclear orphan receptors COUP-TFII or Ear-2 with different OT/CAT reporter constructs in order to determine whether these orphan receptors were capable of influencing basal OT promoter activity and to delineate the areas in the promoter region that were mediating this interaction.

As shown in Fig. 1, co-transfection of expression vectors for COUP-TFII or Ear-2 with a reporter construct containing 164 bp of 5' flanking region of the human OT gene (p164(wt)CAT) repressed basal promoter activity by 54 and 75% respectively. The shorter deletion mutant p115(wt)CAT was also suppressed by both orphan receptors, but to a lesser extent: Ear-2 suppressed by 54% and COUP-TFII by 15% only. Finally, a construct containing only the minimal promoter region extending from –49 to +36 was not suppressible by co-transfection with either of the orphan receptor expression constructs. These results showed that both orphan receptors tested here were indeed capable of significantly silencing the OT promoter activity and that sites mediating this effect were present in an area between -164 and -115 as well as in an area extending from -115 to -49.

In order to determine more precisely the sites at which COUP-TFII and Ear-2 interacted with the OT promoter sequence, we performed a footprinting analysis using recombinant COUP-TFII and Ear-2 proteins produced by bacterial expression. As shown in Fig. 2, both proteins produced footprints in two distinct areas. The first footprint extended from nucleotides -164 to -145. The second footprint was observed at slightly higher protein concentrations and extended from -110 to -79. The first footprint overlapped with the area containing the palindromic ERE characterized earlier (Richard & Zingg 1990). This ERE

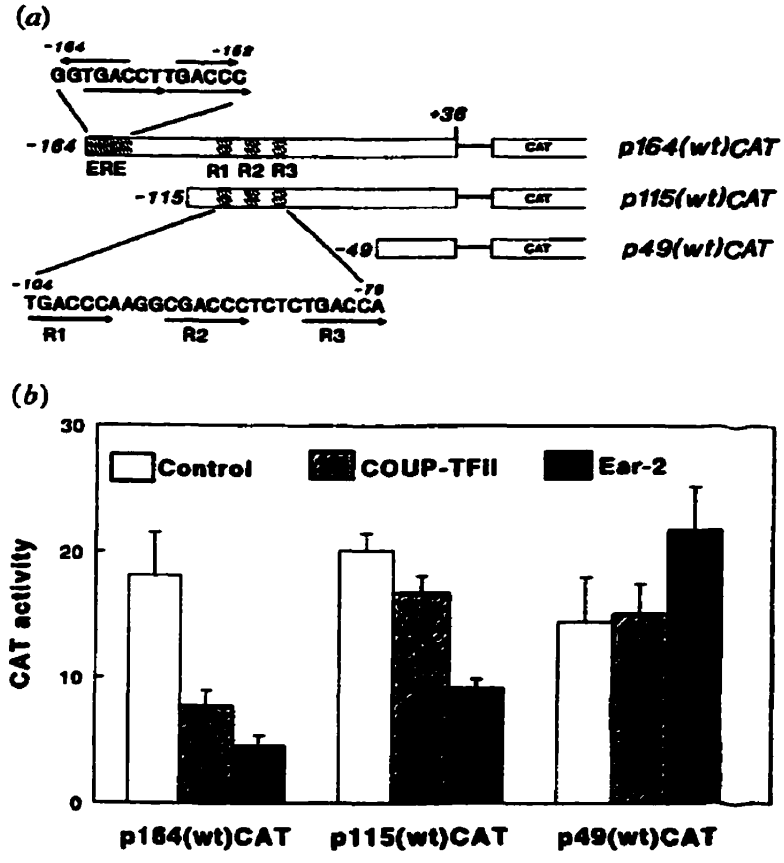

Figure 1. Silencing effects of COUP-TFII and Ear-2 on basal OT gene promoter activity. (a) Schematic diagram of constructs used. Different length segments of the 5' flanking region of the human OT gene were linked to the structural gene for CAT. Sequence positions are indicated with respect to the transcriptional initiation site. Arrows above letters indicate the imperfect palindrome, and arrows below letters indicate the overlapping TGACC(T/C) repeat. R1, R2 and R3, (T/C)GACC repeats present in the -103 to –78 region. (b) The constructs shown in (a) were co-transfected with expression vectors for COUP-TFII (hatched bars), Ear-2 (closed bars) or the parent expression vector pcDNA3 (open bars) in Neuro-2a cells, and CAT activity was assayed in cell extracts. CAT activity was normalized to protein concentration. Each bar represents the mean & S.E. of three or more independent experiments, each performed in duplicate.

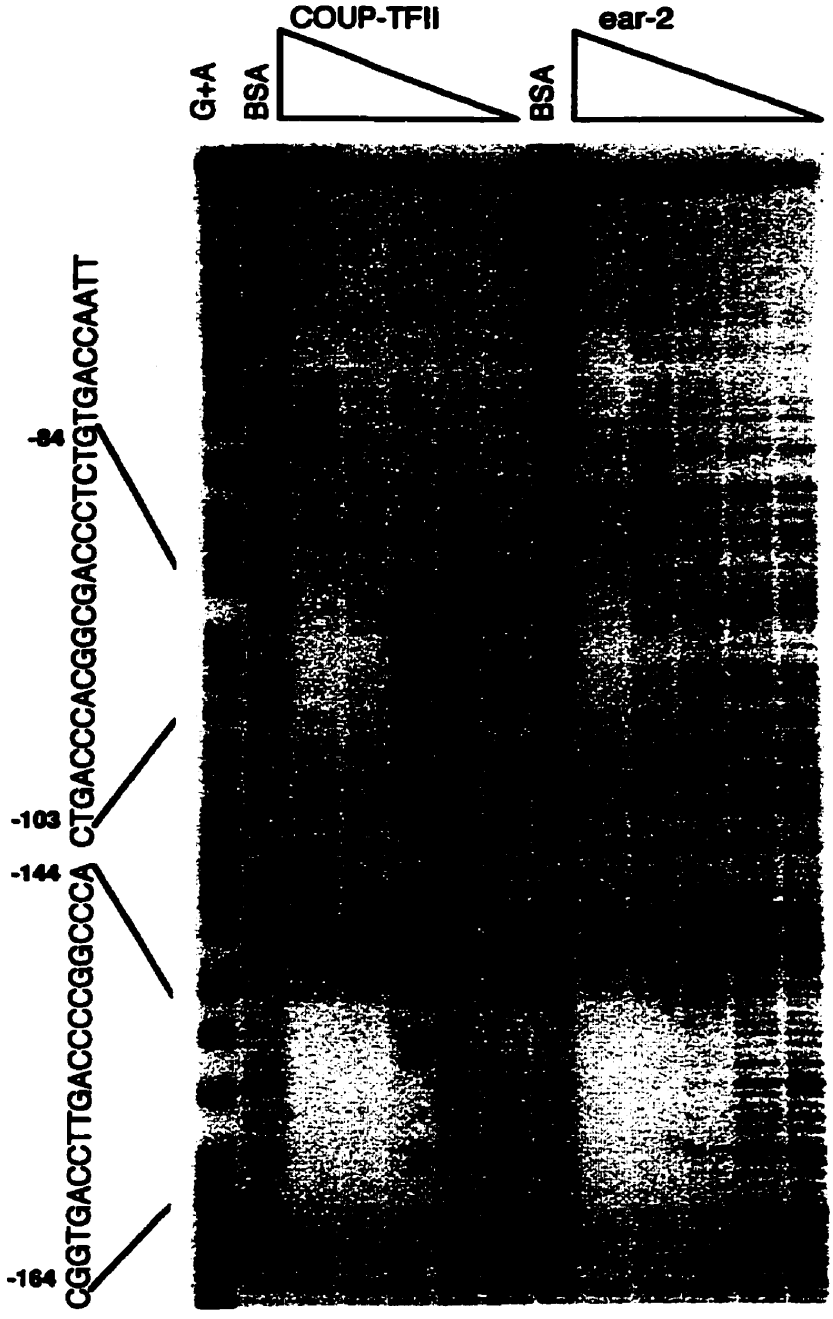

Figure 2. DNase I footprinting analysis of COUP-TFII- or Ear-2-binding sites on the human OT gene promoter using 1000, 333, 111, 37 and 12 ng recombinant rat COUP-TFII (lanes 3–7) or Ear-2 (lanes 9–13) in conjunction with a 5'-end (sense strand) labelled 200 bp OT promoter fragment. BSA was used instead of recombinant protein. G+A, Maxam–Gilbert sequencing ladder (G+A reaction). Relevant sequence portions are indicated on the left (sense strand).

overlaps with a direct TGACCPy (Py=pyrimidine) repeat which, according to the classification by Umesono *et al.* (1991), corresponds to a natural variant of a so-called DR0 repeat (see Discussion). The second footprint corresponded to a region containing three repeats of the same general structure TGACCPy. These results are compatible with the functional results shown in Fig. 1 and imply that there are two distinct areas in the OT promoter that serve as COUP-TFII and Ear-2 DNA-binding sites and which both contribute to the observed silencing effect.

Based on the results obtained by the footprinting experiments, we designed further point mutants. In Fig. 3, the effects of mutations in repeats 1, 2 and as well as in repeats 1 and 3 combined were tested. The results showed that a combined mutation of repeats 1 and 3 (p115(Δr1,Δr3)) completely abrogated the capacity of either Ear-2 or COUP-TFII to silence the promoter. By contrast, the suppressive action of Ear-2 was not abrogated by any mutation that affected only one of the three repeats. The silencing effects of COUP-TFII were weaker in general, and mutations in either R1 or R2 alone were sufficient to abrogate the effect of COUP-TFII. Taken together, these data indicate that within the -115 to +36 region, the triple repeat structure R1–R3 is essential for mediating the silencing effects of Ear-2 and COUP-TFII.

EMSAs with *in vitro* transcribed/translated COUP-TFII and Ear-2 were used to further characterize COUP-TFII and Ear-2 interactions with the OT gene promoter. First we focused on the ERE region at-160 and investigated which sequences were relevant for mediating the silencing effect. Figure 4 shows that a double-stranded oligonucleotide containing the ERE plus eight flanking nucleotides on each side acted as a strong and specific binding site for COUP-TFII as well as for Ear-2. Whereas competition with the homologous unlabelled

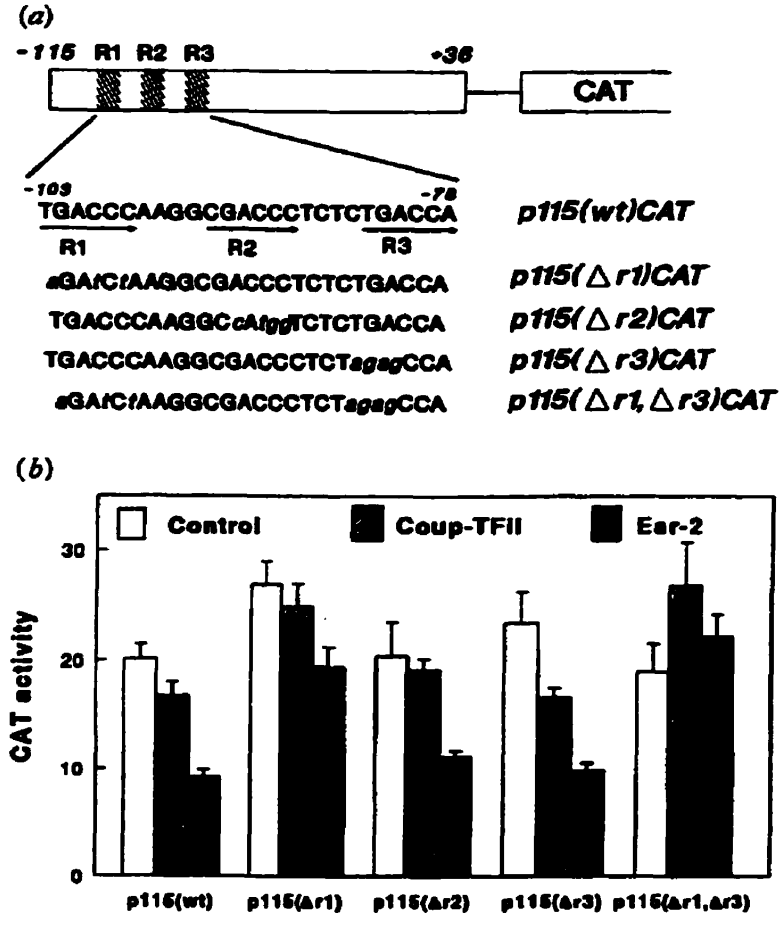

Figure 3. Effect of mutations in repeats R1, R2 and R3 on silencing by COUP-TFII or Ear-2. (a) Schematic representation of constructs used. Mutations are indicated by lower-case italic letters. (b) The constructs shown in (a) were co-transfected with expression vectors for COUP-TFII (hatched bars), Ear-2 (closed bars) or the parent expression vector pcDNA3 (open bars) in Neuro-2a cells, and CAT activity was determined. Each bar represents the mean & S.E. of three or more independent experiments, each performed in duplicate.

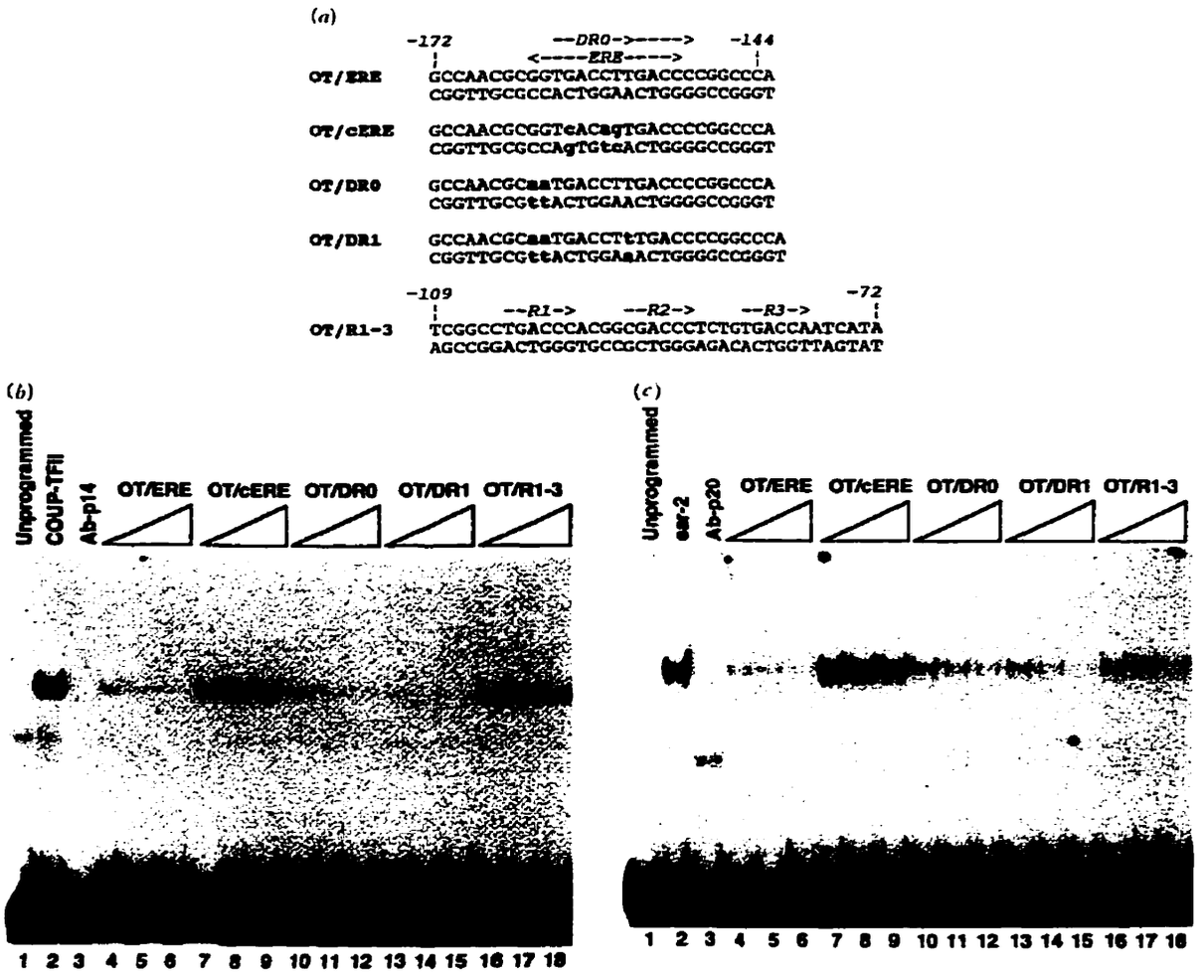

Figure 4. EMSAs of COUP-TFII and Ear-2 binding to the OT/ERE and mutants thereof. (a) Double-stranded oligonucleotides used. The ERE palindrome (ERE) as well as the overlapping TGACCPy repeat are indicated. Mutations are indicated in lower-case bold letters. Oligonucleotide OT/ERE was used as 32 P-labelled probe throughout. (b) *In vitro* transcribed/translated COUP-TFII (lanes 2–18) or unprogrammed reticulocyte lysate (lane 1) was incubated with 32 P-labelled OT/ERE double-stranded oligonucleotide. Lane 3, co-incubation with anti-COUP-TFII antibody. The binding was competed for with increasing concentrations (6X, 12·5X and 25X excess) of unlabelled homologous oligonucleotide OT/ERE (lanes 4–6), OT/cERE (lanes 7–9), OT/DR0 (lanes 10–12),OT/DR1 (lanes 13–15) or OT/R1-3 (lanes 16–18). (c) As in (b) but *in vitro* transcribed/translated Ear-2 was used instead of COUP-TFII and an Ear-2 antibody in lane 3.

oligonucleotide readily displaced bound COUP-TFII or Ear-2 (Fig 4*b* and c, lanes 4–6), an oligonucleotide containing a triple-point mutation that converted the naturally occurring imperfect palindrome into the 'classical' perfectly palindromic vitellogenin ERE (cERE) was unable to compete (lanes 7–9). Since the ERE to cERE mutation destroyed the inherent DR0 repeat structure (Fig. 4a), this result suggests that the structural determinant mediating orphan receptor binding to the OT/ERE is not the ERE structure *per se* but the underlying DR0 repeat. To test this hypothesis further, we examined the capacity of the oligonucleotide OT/DR0 to act as a competitor. In this double-point mutant, the ERE palindrome was destroyed, whereas the DR0 structure was maintained (Fig. 4a). As shown in Fig. 4*b* and *c* (lanes 10–12), this oligonucleotide mutant successfully competed for OT/ERE binding. Since for COUP-TFI, the DR1 configuration appears to represent the preferred DNA-binding site (Kadowaki *et al.* 1992), we tested the effect of increasing the repeat spacing in OT/DR0 by 1, creating OT/DR1 (Fig. 4a). This oligonucleotide represented even as lightly stronger competitor, compatible with the notion that DR1 represents also for COUP-TFII and Ear-2 a strong DNA-binding motif (Fig. 4*b* and c, lanes 13–15).

To compare the relative efficiency of COUP-TFII and Ear-2 to bind to the downstream repeat structure of -103 to -78 (R1–R3), we also used an oligonucleotide corresponding to this region as a competitor. As shown in lanes 16–18 (Fig. 4*b* and c), this oligonucleotide was indeed able to compete, albeit with lesser affinity. This finding is in accordance with the footprint data shown above, where the region -103 to -78 required a higher protein concentration than the ERE area for DNaseI protection.

To analyse further COUP-TFII and Ear-2 binding to the R1–R3 repeat structure, we used oligonucleotides OT/R1-3 as a labelled probe and tested the capacity of mutants in

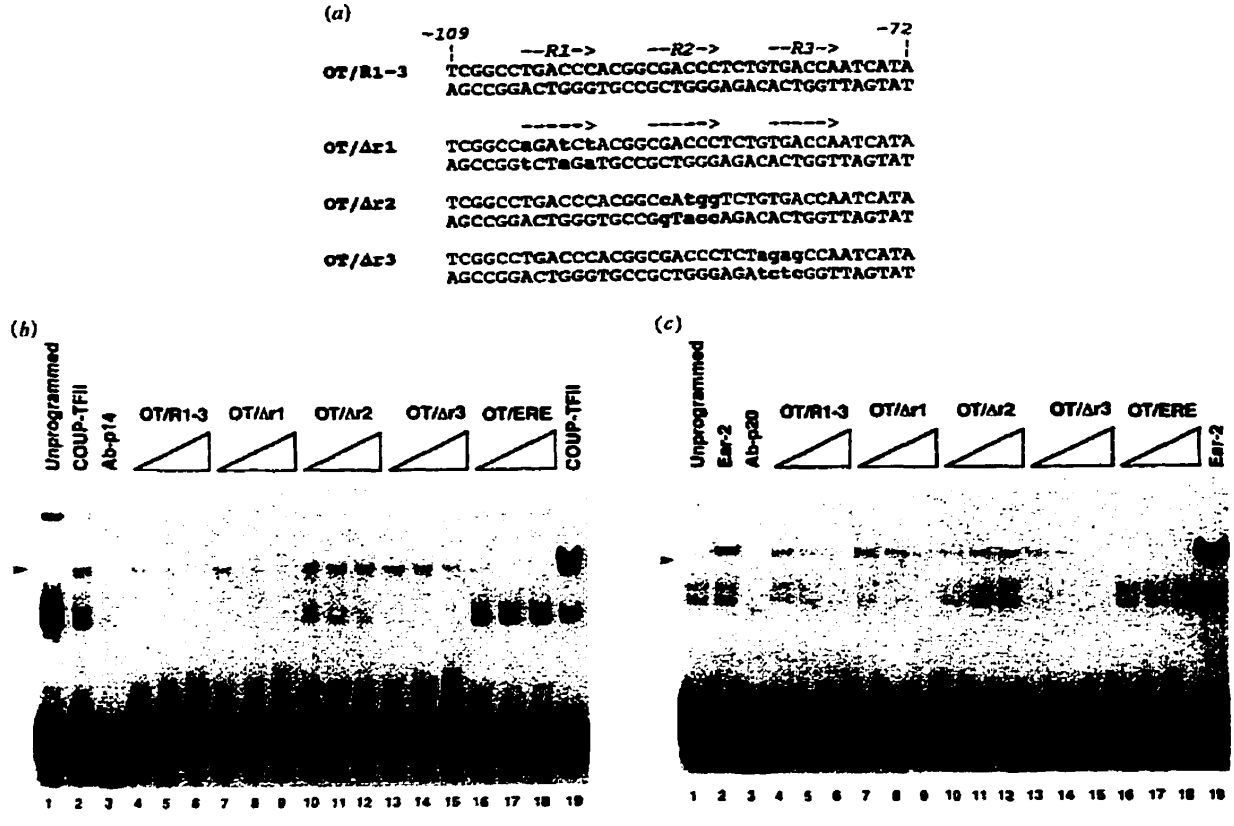

Figure 5. EMSA of COUP-TFII and Ear-2 binding to OT/R1-3 and mutations thereof. *(*a) Oligonucleotides used. Oligonucleotides OT/Δr1, OT/Δr2 and OT/Δr3 contain mutations in repeats R1, R2 and R3 respectively. Base pairs differing from the wild-type sequence are indicated in lower-case bold letters. *(*b) Unprogrammed reticulocyte cell lysate (lane 1) or *in vitro* transcribed/translated COUP-TFII (lanes 2–19) was incubated with labelled oligonucleotide OT/R1-3 (lanes 1–18) or labelled OT/ERE (lane 19). In lane 3, a COUP-TFII antibody was co-incubated. Binding was competed for with increasing concentrations of unlabelled homologous oligonucleotide OT/R1-3 (lanes 4–6) or OT/Δr1 (lanes 7–9), OT/Δr2 (lanes 10–12), OT/Δr3 (lanes 13–15) or OT/ERE (lanes 16–18). The band resulting from specific binding of COUP-TFII is indicated by an arrowhead. *(*c) As in *(*b), but *in vitro* transcribed/translated Ear-2 was used instead of COUP-TFII. In lane 3, an Ear-2 antibody was co-incubated.

repeats 1, 2 or 3 to compete for binding to the wild-type sequence (Fig. 5a). Fig. 5*b* and *c* show that both COUP-TFII and Ear-2 bound specifically to oligonucleotide OT/R1-3 (lanes 2 and 4–6). Mutations in repeats 1,2 or 3 either decreased (OT/Δr1 and OT/Δr3) or eliminated (OT/Δr2) the capacity to act as a competitor, indicating that each of the three repeats contributed to some extent to binding of COUP-TFII and Ear-2 to this segment. Although repeat R3 was only poorly protected in the footprint analysis, the EMSA with COUP-TFII in Fig. 5*b* showed that a mutation in this repeat (OT/Δr3) significantly decreased the capacity of the corresponding oligonucleotide to act as a competitor (Fig. 5b, lanes 13–15), implying a limited functional role also for repeat R3. As expected, oligonucleotide OT/ERE represented a very strong competitor. Comparing lanes 16–18 with 4–6 in Fig. 5*b* and *c* shows that OT/ERE competed even more strongly for protein binding than the homologous OT/R1-3 oligonucleotide. Similarly, binding to labelled OT/ERE (lane 19) is stronger than to labelled OT/R1-3 (lane 2). This is compatible with the EMSA results in Fig. 4*b* and *c* which showed that, conversely, OT/R1-3 was a relatively weak competitor for COUP-TFII and Ear-2 binding to OT/ERE.

DISCUSSION

The present series of experiments demonstrated a novel mechanism of OT gene regulation by two members of the nuclear orphan receptor family. Whereas our previous observations characterized a passive mechanism of repression by displacing an activator, namely the oestrogen receptor, from its binding site, the present study indicates that the orphan receptors COUP-TFII and Ear-2 are, in addition, capable of actively silencing the OT promoter. This silencing activity is mediated by binding sites extending over an 86 bp area and includes a previously identified binding site overlapping the ERE as well as additional proximal binding sites. If the observed silencing effect exerted by the nuclear orphan receptors is by competitive displacement of an activator, one would expect that removal of the presumptive activator-binding site(s) would result in a decrease in basal promoter activity. The results from the present and previous (Richard & Zingg 1991b) 5'deletion analyses exclude this hypothesis, since removal of the sites of orphan receptor/DNA interactions leads to an inability of the orphan receptors to silence the promoter, but not to any loss of basal promoter activity. Therefore the orphan receptor-binding sites do not appear to correspond to binding sites for activators of basal transcription. Our earlier deletion and mutational analyses indicate that these latter interactions take place mainly in more proximal sites, within the boundaries of -49 to +36 (Richard & Zingg 1991b). Thus binding of the orphan receptors appears to repress basal transcription actively, most probably by negatively affecting the formation of a functional pre-initiation complex or by recruiting a limiting cofactor (Leng *et al.* 1996). The precise mechanisms of COUP-TFII-and Ear-2-mediated active transcriptional repression of the OT gene remain to be elucidated. For COUP-TFII, a direct interaction with the general transcription factor TFIIB has been demonstrated (Tsai *et al.* 1987, Ing *et al.* 1992,

Malik & Karathanasis 1995). According to this scenario, the interaction of COUP-TFII with TFIIB may induce a non-productive TFIIB conformation, leading to transcriptional repression. Recently, another mechanism of active repression was proposed with the identification of the corepressors Silencing Mediator for Retinoic acid receptor and Thyroid hormone receptor (SMRT) and Nuclear receptor-Corepressor (N-CoR) (Chen & Evans, 1995, Horlein *et al.* 1995, Shibata *et al.* 1997). It is postulated that SMRT and N-CoR, recruited by COUP-TFs, have an effect on the chromatin structure and more specifically on the level of acetylation of histones. SMRT and N-CoR have been shown to be part of a protein complex that has a histone deacetylase activity. Hypo-acetylated histones are generally associated with gene silencing. Thus, it is not unexpected that COUP-TFs are able to associate with such protein complex. In the case of the human OT gene, our earlier studies identified in the -49 to +36 region of the gene several sites that were important for basal promoter activity (Richard & Zingg 1991b). Based on EMSA, these sites bind nuclear proteins that presumably function as transcriptional activators (Richard & Zingg 1991b). It is therefore conceivable that COUP-TFII and Ear-2 may in addition to their possible interactions with TFIIB and histone deacetylases also contact one or several of these transactivators and modulate their transcription function via allosteric interactions. To what extent additional post-translation modifications such as phosphorylation are necessary for the silencing function remains to be elucidated. The fact that recombinant COUP-TFII and Ear-2 are capable of binding to their corresponding response elements indicates that for DNA binding *per se* such modifications are not required.

Active silencing by members of the COUP family of nuclear receptors has only been described in a limited number of genes. These include the cellular retinol-binding protein II

(Nakshatri & Chambon 1994), members of the apolipoprotein gene family (Ladias & Karathanasis 1991, Ladias *et al.* 1992, Mietus-Snyder *et al.* 1992, Malik & Karathanasis 1995), the cholesteryl ester transferase gene (Gaudet & Ginsburg 1995) and the Oct3/4 and Oct4 genes (Sylvester & Scholer 1994, Ben-Shushan *et al.*1995). Among hormonal genes, active silencing by COUP-TFII has been described so far in the case of the human placental lactogen gene (Stephanou *et al.* 1996). As we have shown previously, COUP-TFII as well as Ear-2 are co-expressed with OT in uterine epithelial cells and are therefore likely to play a physiological role in the regulation of endogenous OT gene expression.

The repeats involved in mediating COUP-TFII and Ear-2 binding to the OT gene promoter represent copies and natural variants of the core element TGACCPy, which represents the reverse complement of the PuGGTCA (Pu=purine) element described by Umesono *et al.* (1991) and constitutes an essential building block of several nuclear receptor response elements (NRREs). According to the classification by Umesono *et al.* (1991), the repeat inherent in the ERE of the OT gene would correspond to a 'DR0 element' since the repeated core elements are separated by zero nucleotides. In contrast, the repeat structure R1–R3 present in the more proximal-103 to-78 area would correspond to a double-DR4 structure since, at this site, the repeat elements are separated by four nucleotides. Although *in vitro* binding studies indicate that the DR1 structure appears to be the preferred binding site for members of the COUP-TF family, COUP-TFs are also known to bind *in vitro* to artificial DR0 elements (Kadowaki *et al.* 1992). The DR0 element described here, however, represents to our knowledge a unique example of a functional silencing element corresponding to a DR0 structure in a gene that is expressed in differentiated cells. Another DR0 element acting as a silencer has been described in the Oct-4 gene, a gene that is expressed in undifferentiated

embryonic cells (Sylvester *et al.* 1994). Most repeat elements in the OT gene represent variations of the TGACCPy structure. The 'A' present in position 6 in R3 is also found in other NRREs described in the mouse transthyretin gene and the ApoCIII promoter (Kimura *et al.* 1993), whereas the 'C' found in position 1 of R2 is a novel variant.

It is interesting to note that R2 mutants were still able to act as binding sites for Ear-2 (Fig. 5b) and induce Ear-2-mediated suppression (Fig. 3b). This implies that Ear-2 is able to interact with the remaining two repeats which are spaced 14 nucleotides apart (DR14). As reported by Carter *et al.* (1994), an everted repeat with the same spacing of 14 nucleotides (ER14) forms part of the NRRE of the medium-chain ethyl coenzyme A dehydrogenase gene promoter and functions as a COUP-TFI homodimer-binding site. Obviously, COUP family homodimers are able to interact with a variety of NRREs, some of which consist of widely spaced repeat sequences. In the case of the present DR14 or ER14 motifs, the centres of the repeats are spaced 20 nucleotides apart, which places them at the same site of a B-DNA helix, a fact that may play an important role in facilitating DNA contact formation. Data from transfection studies (Fig. 1b) and binding studies (Figs 2, 5*b* and c) indicate that the DR0 element embedded in the ERE acts as a stronger repressor element than the R1–R3 repeat structure and that this is probably a consequence of the higher affinity of the orphan receptors to the DR0 site than the DR4 sites present in the R1–R3 segment.

In conclusion, our studies demonstrate an additional mechanism of OT gene regulation. Since both COUP-TFII and Ear-2-like immunoreactivities are present in OT-producing uterine epithelial cells (Chu *et al.* 1998), the mechanism analysed here is likely to be of physiological importance in the fine-tuning of uterine OT gene regulation. Moreover, the OT gene promoter represents a naturally occurring model system for the study of orphan

nuclear receptor function and should provide a useful tool for further studies on the mechanism of transcriptional repression by members of the COUP-TF orphan receptor family.

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Chapter IV

Activation of the mouse oxytocin promoter by the orphan receptor RORα1

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Preface

The two previous chapters demonstrate a role for the COUP-TF subfamily of nuclear orphan receptors in the regulation of the OT gene. We were interested in identifying other orphan receptors that could be involved in the regulation of the OT gene. Here we identify in the orphan receptor subfamily, ROR/RZR, as potential regulators of OT. One member of this subfamily, ROR/RZR, was shown to be expressed in the hypothalamus and the pineal gland, two sites of OT expression. These observations prompted us to investigate the possible role of this subfamily in OT gene regulation. In this chapter, we show that RORα1 can activate the mouse OT promoter. We also identify the mouse OT/ERE as the DNA element responsible for RORα1 activation.

ABSTRACT

The nuclear orphan receptor superfamily has undergone recent rapid expansion, but the number of known naturally occurring genes that are directly regulated by orphan receptors is still low. We have shown previously that the gene encoding the neuropeptide oxytocin (OT) is negatively regulated by the orphan receptors COUP TF I and II. Here we show that the mouse OT gene promoter is activated by RORα1, a representative of a novel orphan receptor subfamily. Using promoter/chloamphenicol acetyl transferase constructs in heterologous transfection assays, we determined that RORα1 action induces a >6-fold increase in promoter activity. By 5' and 3' deletion analysis, DNaseI footprint analysis and mobility shift assays, we found that RORα1 action is mediated by two 14 bp regions centered at 160 and 180 nucleotides upstream of the transcriptional initiation site. Both sites contain significant sequence identities with an established RORα1 recognition sequence. Mutations in either or both these sites reduce significantly RORα1–induced activation of the OT promoter. In view of the strong transcriptional activation exerted by RORα1 on the OT gene promoter and the widespread distribution of different members of the ROR/RZR family, interactions between ROR/RZR isoforms and the OT gene may form part of the multifactorial regulatory mechanisms that control OT gene expression in different tissues.

INTRODUCTION

The nonapeptide hormone oxytocin (OT) acts as both a neurotransmitter as well as a peripheral hormone. Whereas central effects of OT involve induction of specific reproductive behaviors, peripheral effects include uterine contractions, milk ejection, natriuresis and vasodilation (Zingg 1996, Thibonnier *et al.* 1999). Centrally, the OT gene is expressed in specific hypothalamic neuroendocrine cells as well as in the pineal gland (Fisher & Fernstrom 1981, Liu & Burbach 1987, Liu *et al.* 1991, Liu & Burbach 1987, Liu *et al.* 1988, Milcu *et al.* 1963). Peripherally, the gene is expressed in uterine epithelium (Lefebvre *et al.* 1992), fetal membranes (Mitchell *et al.* 1998) and the corpus luteum (Ivell *et al.* 1998). OT gene expression is hormonally induced by estrogen and retinoic acid (Larcher *et al.* 1995). In addition, we and others have shown that orphan nuclear receptors, specifically members of the COUP-TF family, antagonize hormone-mediated transcriptional activation of the OT gene and actively silence the OT gene promoter (Burbach *et al.* 1994, Chu *et al.* 1997, Chu & Zingg 1997). These nuclear receptor/promoter interactions are mediated by a well conserved region centered at -160 relative to the transcriptional initiation site (Adan 1993, Richard & Zingg 1990). This region was initially identified as an estrogen response element (ERE) (Richard & Zingg 1991) and is here referred to as OT/ERE.

In the present study, we have examined the role of the recently identified orphan receptor RORα1 in the regulation of the OT promoter. The ROR/RZR subfamily of orphan nuclear receptors is a recent addition to the nuclear receptor family and comprises the following members: RORα1, RORα2, RORα3, RORß/RZRß and RORγ (Carlberg *et al.* 1994, Giguère *et al.* 1994, Hirose *et al.* 1994). The members of this subfamily bind as

monomers preferentially to an AGGTCA core motif preceded by an A/T-rich region (Giguère *et al.* 1994). Although the expression pattern of this receptor family is ubiquitous, high expression of specific members is observed in certain tissues. Thus, RORα1 is highly expressed in Purkinje cells of the mouse cerebellum (Matsui *et al.* 1995), and a mutation in the RORα1 gene gives rise to the *staggerer* mouse phenotype (Dussault *et al.* 1998, Hamilton *et al.* 1996). RORß/RZR is abundantly expressed in the hypothalamus, the pineal gland and the thalamus (Becker-André *et al.* 1994). RORγ is highly expressed in skeletal muscle and may play a role in muscle development (Hirose *et al.* 1994).

Although structural information on the orphan receptor superfamily is rapidly expanding, relatively few genes have been identified that are directly regulated by orphan receptors. Identified target genes for members of the ROR/RZR family include the gene for γF-crystallin, apoliprotein A-I, 5-lipoxygenase, cellular retinol binding protein, retinoic acid receptor ß, $p21^{WAF/CIP1}$, and Purkinje cell protein-2 (Schrader *et al.* 1996, Steinhilber *et al.* 1995, Tini *et al.* 1995, Vu-Dac *et al.* 1997). Here we report that the mouse OT gene represents a novel target for ROR receptors by demonstrating that its promoter is strongly stimulated by RORα1 via a DNA element that has been conserved throughout species evolution.

MATERIALS AND METHODS

Plasmids

Plasmid p1064CAT contained nucleotides -1064 to +38 of the mouse OT promoter (Hara *et al.* 1990) linked to the gene encoding chloramphenicol acetyltransferase (CAT). The deletion mutants p428CAT, p225CAT, p118CAT and p42CAT were derived from p1064CAT. p225ΔOT/ERE, p225ΔHS1 and p225ΔΔ mutants were obtained by site-directed mutagenesis (Kunkel 1985). The mutagenic oligonucleotides were also used in the gel shift assays (see below). Plasmids p225/118-TATA-CAT and p134/34-TATA-CAT contained fragments -225 to -118 and -134 to -34, respectively, of the mouse OT 5' flanking region linked to a TATA-box derived from the adenovirus 1b followed by the gene encoding CAT. The parent plasmid ß-CAT containing the TATA-box linked to the CAT gene was obtained from Dr. V. Giguère, Montréal. All constructs were verified by dideoxynucleotide sequencing. Expression vectors CMX and CMX-RORα1 were also obtained from Dr. Giguère (Giguère *et al.* 1994). These contained a cytomegalovirus-derived promoter which, in CMX-RORα1, was linked to a cDNA encoding mouse RORα1. The vector HSP-ß-gal contained a heat shock protein gene-derived promoter linked to the ß-galactosidase gene (obtained from Dr. Giguère).

Transfections

Mouse Neuro-2a cells (American Type Culture Collection, Rockville, MD; line CCL 131) were maintained in Eagle's minimum essential medium (EMEM; Sigma) supplemented

with 10% fetal bovine serum (GIBCO). Cells were plated on 60 mm dishes and allowed to reach 70-80% confluency before transfection. Plasmids used in transfection studies were purified twice by ultracentrifugation through 5.2M cesium chloride. Each plate was transfected with a total of 10 μg of DNA, including 5 μg CAT reporter plasmid, 3 μg expression vectors, 100 ng HSP-ß galactosidase plasmid, and 1.9 μg pBluescript plasmid KS(+). Transfection was performed by the calcium phosphate precipitation technique. After 4 h of incubation with the precipitate, a glycerol shock was applied by incubating cells in a buffered saline solution containing 15% glycerol for 2.5 min. Cells were then washed with phosphate buffered saline and cultured in EMEM/10% fetal bovine serum. Forty-eight hours after the transfection, cells were collected and CAT activity was determined, using a phase extraction method (Seed & Sheen 1988). Unless stated otherwise, transfections were performed in duplicate at least 3 times. CAT activity was normalized with respect to ß-gal activity recovered from cell extracts. Co-transfection with the RORα1 expression vector remained without effect on the transcriptional activity of the HSP-ß-galactosidase expression vector.

Bacterial expression of proteins

Recombinant human RORα1 and rat COUP-TFII were produced in BL21 cells as GST-fusion proteins. The cDNAs encoding human RORα1 or rat COUP-TFII were amplified by PCR and inserted in-frame with the glutathione-S-transferase (GST) coding region in the bacterial expression plasmid pGEXKG (Novagen, Madison, WI). The resulting plasmid was used to transform BL21 cells as described (Novagen, Madison, WI). Cells were grown until

they reached an OD_{550} of 0.6 and IPTG was added to a final concentration of 0.1 mM. Three hours after IPTG induction, cells were collected and purified using a glutathione resin (Pharmacia), according to the manufacturer's instructions. Finally, the GST moiety was removed by thrombin cleavage

DNAse I footprinting assays

Probes for DNase I footprinting experiments were generated by PCR. The primer pair used for the amplification reaction consisted of oligonucleotide R48 (complementary to a part of the pBluescript polylinker region, sense strand direction) and a reverse primer complementary to the 5'-end of the CAT cDNA (anti-sense direction). The oligonucleotide R48 was labeled with ^{32}P using [^{32}P]γATP and T4 polynucleotide kinase. For the PCR reaction, p225CAT was used as a template in conjunction with 10 pmoles of each primer. Following 35 cycles of amplification (94C, 1 min; 58C, 1 min; 72C, 1 min), the PCR product was gel purified and diluted to 10,000 cpm/ml. For DNase I footprinting analysis, 0.06-5 μl of recombinant protein was incubated with 10,000 cpm of DNA probe in binding buffer (12 mM HEPES [pH 7.9] 12% glycerol, 70 mM KCl, 3 mM $MgCl_2$, 250 ng polyIdC). Following incubation of the binding reaction for 30 min at room temperature, 0.25 units of DNAse I (RQ1-DNase I, Promega) was added for 90 s. The endonuclease reaction was stopped by the addition of EDTA to a final concentration of 5 mM. The binding reaction was phenol extracted, precipitated with ethanol and resuspended in 90% formamide loading buffer. Equal amounts of recovered probe were then loaded on a 10% denaturing polyacrylamide gel.

Electrophoretic mobility shift assay (EMSA)

For EMSAs, *in vitro* translated CMX or CMX-RORα1 were used. Translation products were obtained *in vitro* by using plasmids CMX or CMX-RORα1 as templates in an *in vitro* transcription/translation reaction mix (TNT lysate, Promega) according to the manufacturer's instruction. All proteins were [^{35}S]-methionine labeled and their sizes verified by electrophoresis in 10% SDS-polyacrylamide gels.

Ten pmoles of double-stranded oligonucleotides were labeled using T4 kinase and [^{32}P]γATP. Two ml of *in vitro* translated RORα1 was incubated in binding buffer with 10 fmoles of probe for 30 min at room temperature and electrophoresed on a non-denaturing 4% polyacrylamide gel in 0.25 x TBE buffer at 150 volts. A polyclonal antibody against rat RORα1 was obtained from Dr. V. Giguère (Dussault *et al.* 1998).

RESULTS

We tested the effect of RORα1, a member of the family of orphan receptors, on OT promoter activity by transfecting 5' deletion mutants of mouse OT-CAT reporter plasmids in mouse Neuro2a cells in conjunction with an expression plasmid for RORα1 (CMX-RORα1). With the longest reporter construct used (p1064CAT), co-transfection of the RORα1 expression vector CMX-RORα1 lead to a 6-7 fold increase in CAT activity compared to cells transfected with the parental plasmid CMX (Fig. 1). Similar or greater activation was also observed with shorter constructs (p428CAT and p225CAT). However, with the construct p118CAT, RORα1 elicited only a 2.2.-fold increase in activity. No significant activation was observed with the construct p42CAT. These studies indicate that the sequence interval -225 and –42 harbors a region involved in conferring inducibility by RORα1.

To delineate further the 3' end of the area mediating this response, regions -225 to -118 (p225/118TATA-CAT) and -134 to -34 (p134/34TATA-CAT) were cloned upstream of a minimal adenovirus 1b TATA-box/CAT reporter and transfected in Neuro2a cells (Fig. 2). Cotransfection of RORα1 elicited a strong 14-fold increase in CAT activity mediated by the construct p225/118TATA-CAT. Only a very weak effect was observed with p134/42TATA-CAT or the parental reporter construct (ß-CAT). These results narrow the area mediating activation by RORα1 further down to the region -225 to -118.

DNAse I footprinting with bacterially expressed RORα1 was used to identify more precisely the regions involved in RORα1 binding. Two adjacent regions were found to be resistant to DNAse I digestion following incubation with RORα1 (Fig. 3). One region was

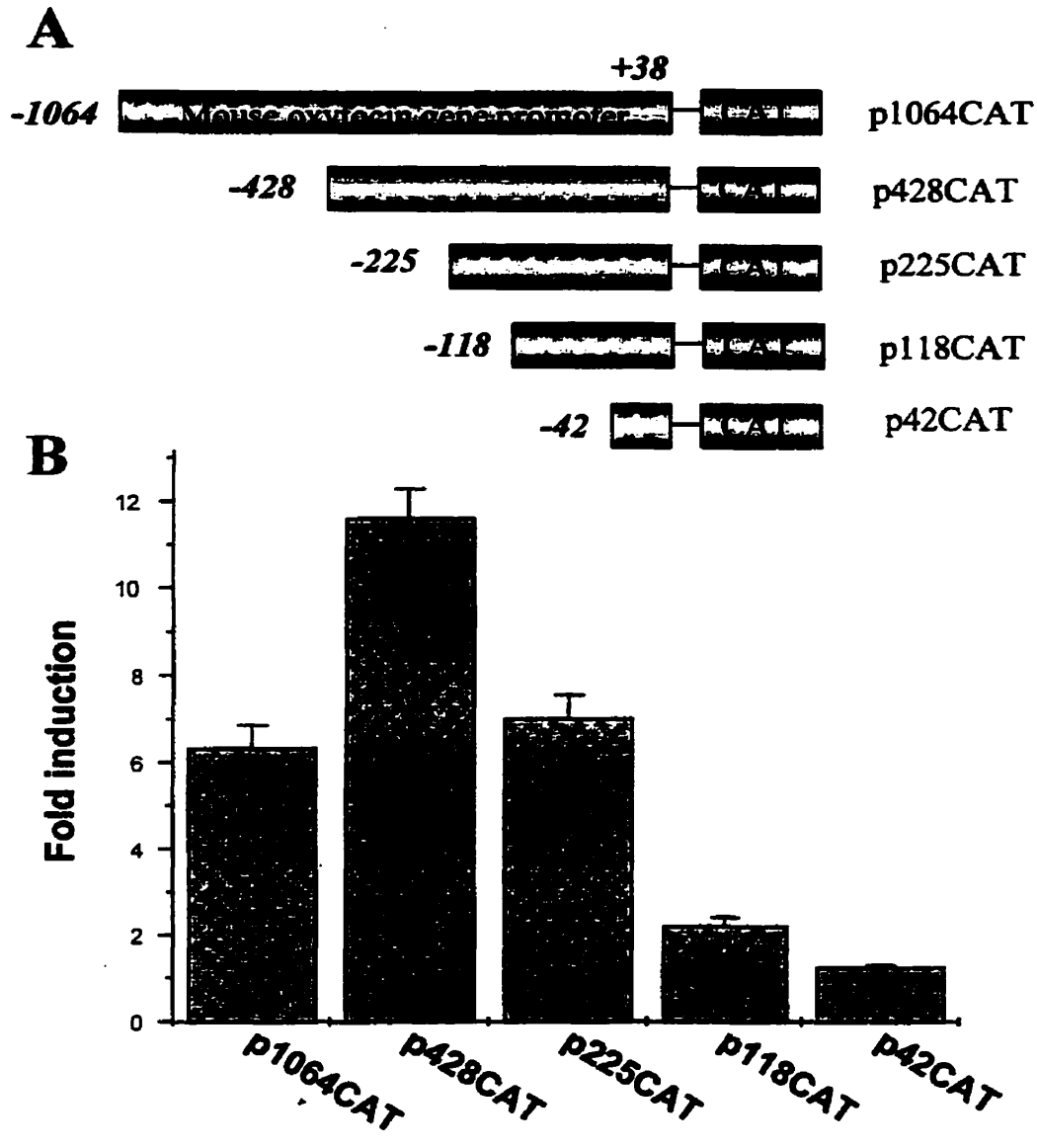

Fig. 1 Activation of the mouse OT promoter by RORα1. A: Schematic diagram of constructs used. Different length segments of the 5'-flanking region of the mouse OT gene were linked to the structural gene for CAT. Sequence positions are indicated with respect to the transcriptional initiation site. B: The constructs shown in A were transfected in mouse Neuro-2a cells together with a lacZ expression vector and the parent expression vector CMX or the RORα1 expression vector CMX RORα1. CAT activity was determined in cell extracts and was normalized with respect to ß-gal activity. RORα1-mediated transcriptional activation was expressed as the ratio of CAT activity in cells transfected with vector CMX- RORα1 *versus* cells transfected with the parent expression vector CMX. Each bar represents the mean ± S.E. of three or more independent experiments, each performed in duplicate.

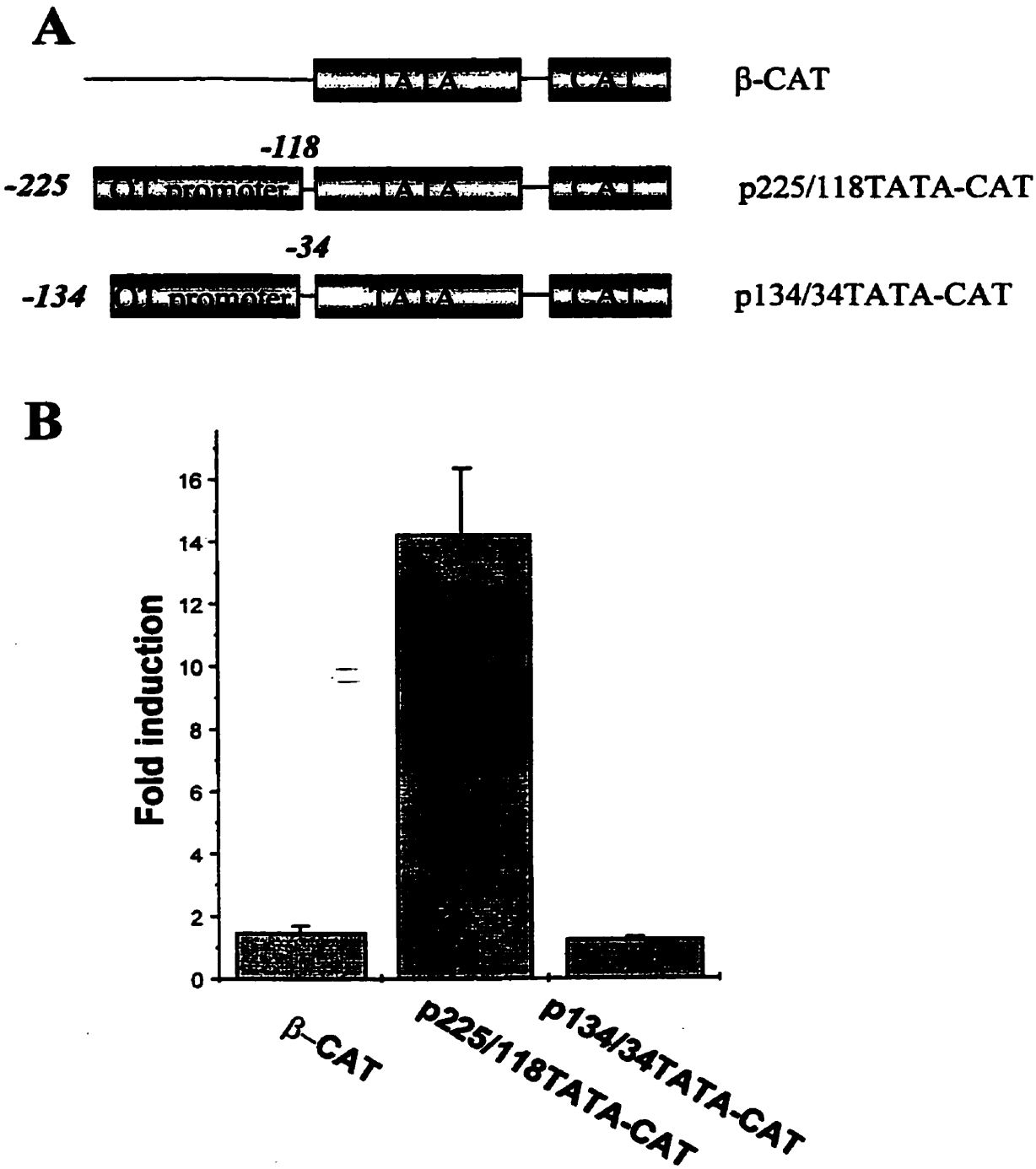

Fig. 2 The region -225 to -118 of the OT promoter is sufficient for RORα1-mediated activation. A: Schematic diagram of constructs used. Regions -225 to -118 (p225/118TATA-CAT) and -134 to -34 (p134/34TATA-CAT) were cloned upstream of the adenovirus 1b TATA box and transfected into Neuro2a cell together with the vector CMX or CMX- RORα1. B: CAT activity was normalized with respect to ß-gal activity and was expressed as -fold induction observed in CMX- RORα1-transfected cells over CMX-transfected cells, as in Fig.1. Each bar represents the mean ± S.E. of three or more independent experiments, each performed in duplicate.

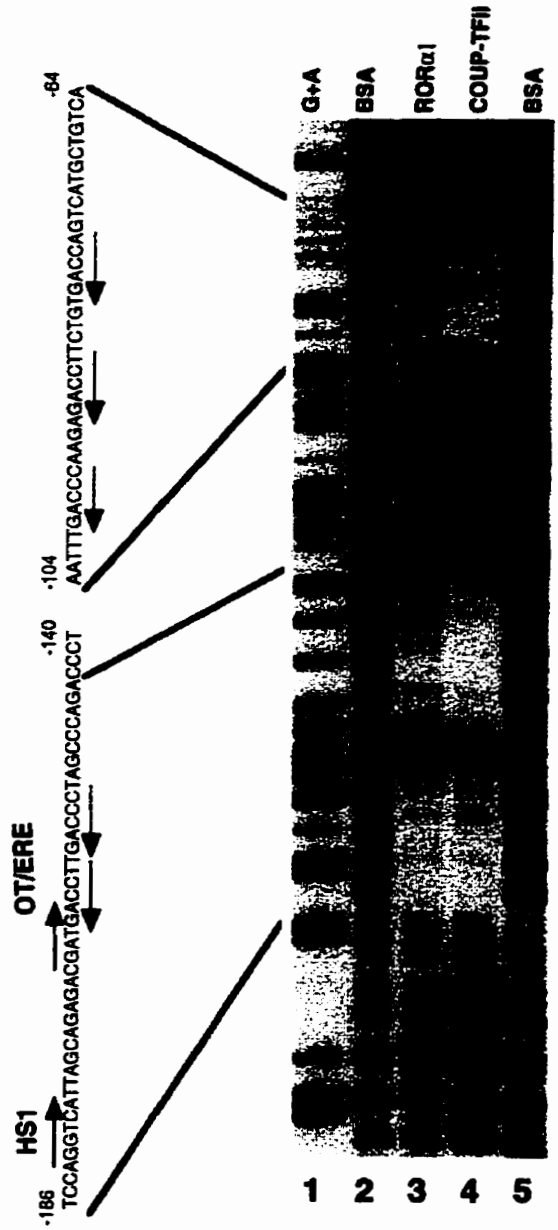

Fig. 3 DNaseI footprinting analysis of RORα1 binding sites on the mouse OT gene promoter. Recombinant human RORα1 (lane 3) and rat COUP-TFII (lane 4) were used in conjunction with a 5'-end (sense strand) labeled 320 bp OT promoter fragment. BSA: Bovine serum albumin was used instead of recombinant protein. G + A: Maxam-Gilbert sequencing ladder (G + A reaction). Relevant sequence portions are indicated on the left (sense strand).

centered at position -180 and the other at position -160. The first region (-188 to –174) includes the sequence element AGGTCA. As shown by Umesono *et al.* (1991), this element forms the building block of several types of nuclear receptor response elements (see Discussion). The second region encompasses the OT/ERE and contains three AGGTCA-related motifs which can be viewed either as an inverted repeat (HS2 and HS3) or as a direct repeat (HS3 and HS4) (Fig. 4a). The center of the footprint appeared to be located over HS4 (Fig 3).

For comparison, the binding of the orphan receptor COUP-TFII to this same promoter area was also examined. COUP-TFII is a prototype for dimer binding. We have previously shown that COUP-TFII binds to the human OT/ERE (Chu *et al.* 1997). Lane 4 in Fig. 3 shows that bacterially expressed COUP-TFII also binds to the mouse OT/ERE and HS1, but the regions protected by COUP-TFII are larger than the ones protected by RORα1. This is compatible with the accepted concept that COUP-TFII binds as a homodimer, whereas RORα1 binds as a monomer. In addition, COUP-TFII is also able to bind to a more downstream area (-75 to –100) containing three (inverted) AGGTCA repeats (Fig. 3) as demonstrated earlier for the human gene (Chu *et al.* 1997). A noticeable but weak footprint is also formed by RORα1 in this region.

EMSA was performed to further characterize RORα1 binding to the OT/ERE and the HS1. *In vitro* translated RORα1 protein was incubated with oligonucleotides encompassing either the OT/ERE (Fig. 4B) or the HS1 (Fig. 4C). RORα1 readily bound specifically to the OT/ERE (Fig. 4B, lanes 3-6). In contrast to the wild-type OT/ERE oligonucleotide, an oligonucleotide carrying a mutated OT/ERE (ΔOT/ERE) was unable to compete for binding

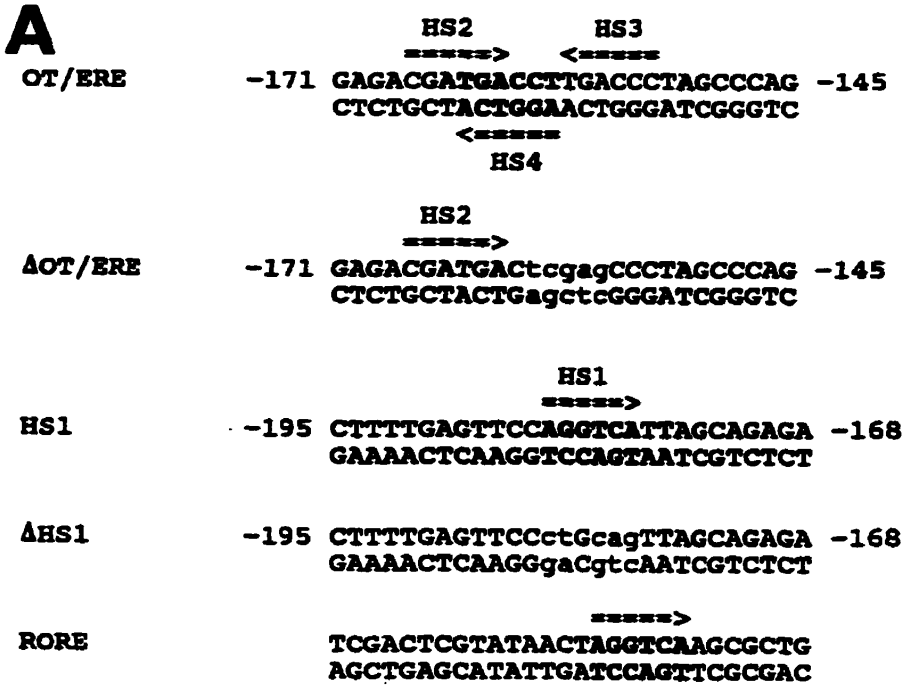

Fig. 4 Electrophoretic mobility shift assays of RORα1 binding to the OT/ERE and mutants thereof. A: Double-stranded oligonucleotides used in EMSA. The OT/ERE with its intrinsic half-sites (HS2, HS3 and HS4) as well as the upstream half-site HS1 are shown. B: *In vitro* transcribed/translated RORα1 (lanes 2-15) or unprogrammed reticulocyte lysate (lane 1) was incubated with ^{32}P-labelled OT/ERE double-stranded oligonucleotide. Lane 3: Co-incubation with an anti- RORα1 antibody. Binding was competed with increasing concentrations (5x, 25x and 100x-fold excess) of unlabelled homologous oligonucleotide OT/ERE (lanes 4-6), OT/ERE-M1 (lanes 7-9), HS1 (lanes 10-12), or RORE (lanes 13-15). C: As in B but the probe HS1 was used instead of OT/ERE.

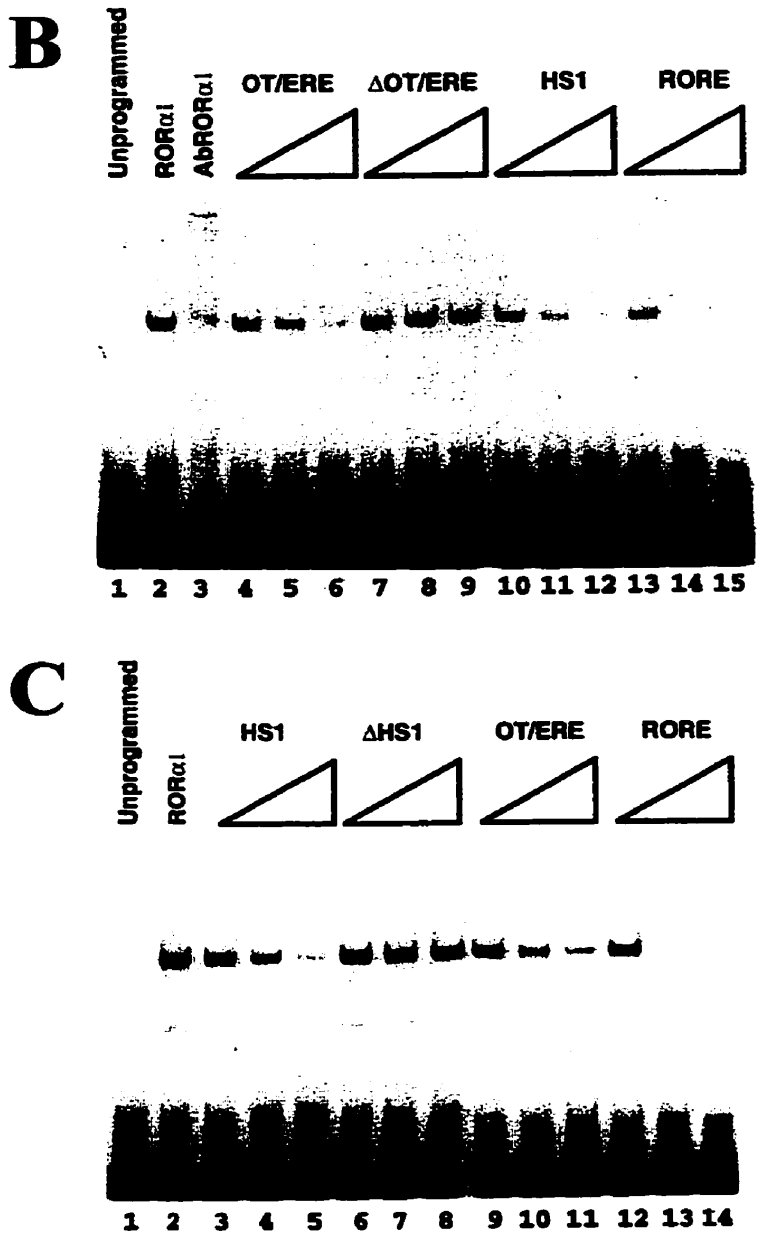
B
Unprogrammed
RORα1
AbRORα1
OT/ERE
ΔOT/ERE
HS1
RORE
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
C
Unprogrammed
RORα1
HS1
ΔHS1
OT/ERE
RORE
1 2 3 4 5 6 7 8 9 10 11 12 13 14

Fig.4B and C

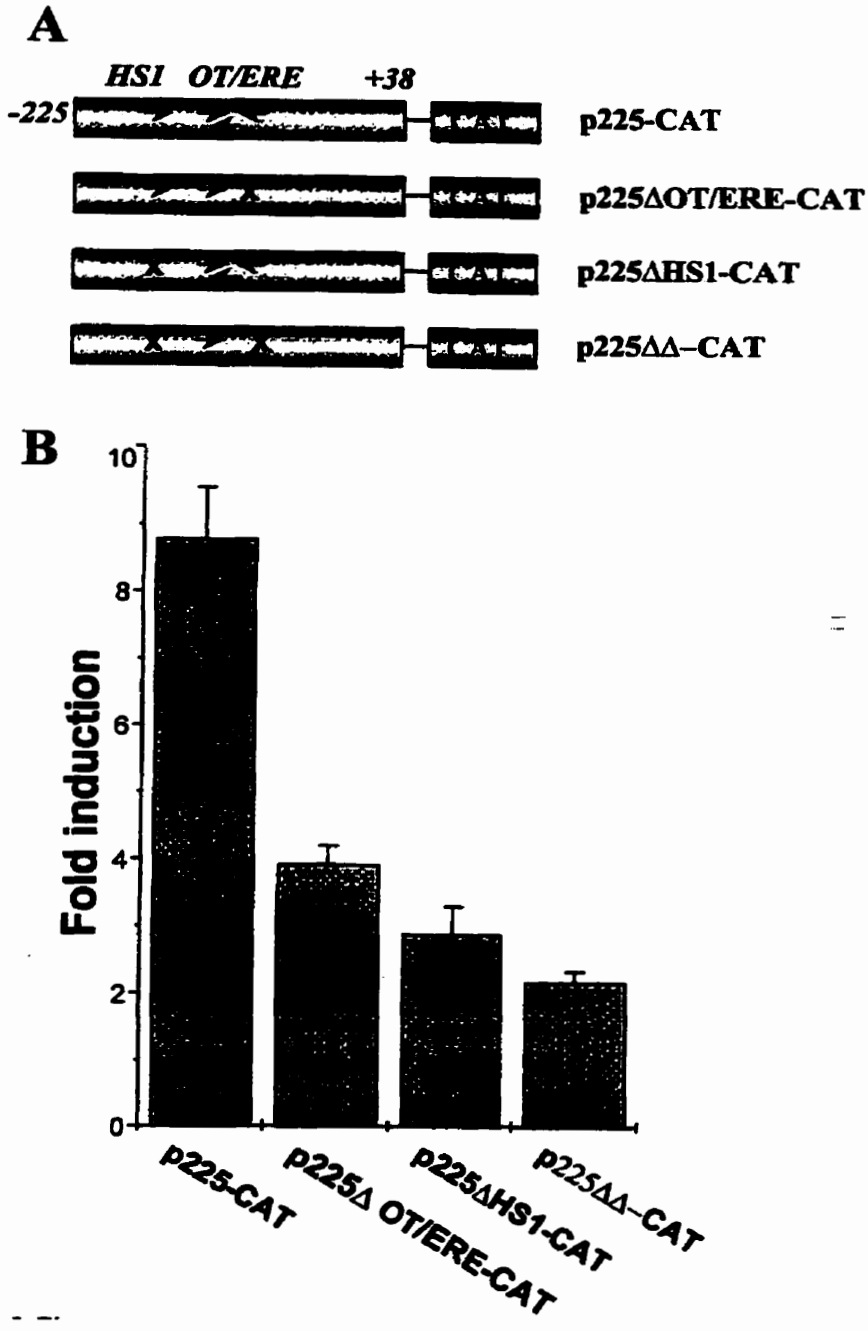

Fig. 5 Effect of mutations in OT/ERE and HS1 on activation by RORα1. A: Schematic representation of constructs used. Mutations were the same as in Fig. 4 and are denoted by "x". Half sites are indicated by arrows. B: Constructs shown in A were cotransfected with the expression vector CMX or CMX- RORα1 in Neuro-2a cells and CAT activity was determined. RORα1-mediated transcriptional activation was expressed as the ratio of CAT activity in cells transfected with vector CMX- RORα1 *versus* cells transfected with the parent expression vector CMX. Each bar represents the mean ± S.E. of three or more independent experiments, each performed in duplicate.

(lanes 4-6 vs. 7-9). RORα1 binding to the OT/ERE probe is also competed by an oligonucleotide containing HS1 (lanes 10-12). The consensus RORE represented an even stronger competitor. Whereas a 25-fold excess of cold RORE oligonucleotide readily competed away binding of the OT/ERE or the HS1 probes, a 100-fold excess of homologous cold probe was required to observe the same effect (Fig. 4B and C).

We next tested mutations that abrogate RORα1 binding with respect to their effect on promoter function. The construct p255ΔOT/ERE-CAT carried the mutations present in the oligonucleotide ΔOT/ERE used in the EMSA studies above. Similarly, the mutations in construct p225ΔHS1-CAT corresponded to the mutations in the oligonucleotide ΔHS1. Finally, the construct p255ΔΔ–CAT contained the combined mutations of ΔHS1 and ΔOT/ERE. Whereas wild-type p225CAT activity was stimulated 9-fold by RORα1, the mutant p225ΔOT/ERE-CAT was only stimulated 4-fold (Fig. 5). Similarly, construct p225ΔHS1-CAT was only induced 3-fold (Fig. 5). Introduction of a double mutation (p225ΔΔ–CAT) led to a further decrease in inducibility to about 2-fold. These results suggest that the OT/ERE and the HS1 sites both contribute additively, but not synergistically, to RORα1–mediated induction.

DISCUSSION

We and others have shown that selected members of the nuclear receptor are potent regulators of OT expression. This includes the nuclear receptors for estrogens, retinoids and thyroid hormone, as well as the orphan receptors COUP-TFI, COUP-TFII, Ear-2 and SF-1 (Adan *et al.* 1992, Adan *et al.* 1993, Burbach *et al.* 1994, Richard *et al.* 1990, Richard *et al.* 1991, Wehrenberg *et al.* 1994). Here we demonstrate that a member of the orphan receptor family ROR/RZR acts as a regulator of OT expression. In contrast to all other orphan receptors that have so far been shown to act as inhibitors or, in the case of SF-1, as only weak activators of the OT gene promoter (Wehrenberg *et al.* 1994), RORα1 acts as a strong activator of the OT gene promoter.

We have demonstrated that the OT promoter is stimulated by the orphan receptor RORα1 via a small region of the promoter which can be transferred to a heterologous adenovirus 1b TATA promoter. This small region contains the conserved OT/ERE and an AGGTCA half-site (HS1) located 5' of the OT/ERE. Mutation of any of these elements leads to reduced activation by RORα1. In addition, using DNase I footprinting and EMSA experiments, RORα1 was shown to bind specifically to these two elements.

By PCR-mediated site selection, Giguère *et al.* have determined a consensus sequence for RORα1 binding as follows: $(^{A}/G/_{T})^{T}/_{A}{}^{A}/_{T}{}^{T}/_{A}C^{T}/_{A}$AGGTCA (Fig. 6) (Giguère *et al.* 1994). The OT/ERE contains three half-sites, HS2, HS3 and HS4 (Fig. 6). According to the results from our footprinting studies, HS4 seems to be preferentially recognized by RORα1. Comparison of HS4 with the consensus RORα1 binding site indicates that, in addition to the conserved AGGTCA half-site, nucleotides -1, -2, and -3 preceding the AGGTCA also

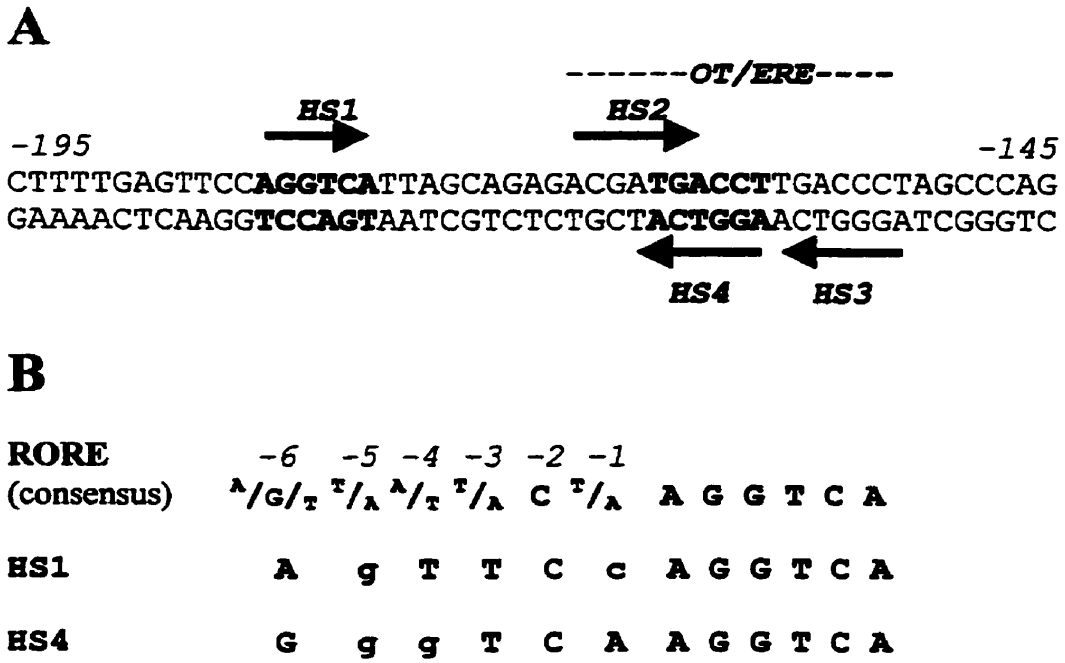

Fig. 6 A: Region –145 to –195 of the mouse OT gene promoter. HS1 and the OT/ERE, containing HS2, HS3, and HS4, are indicated. Arrows indicate the AGGTCA-related half-sites. B: Comparison of the HS4 of the OT/ERE and HS1 with the consensus RORE. Upper and lower case letters indicate matches and mismatches, respectively.

conform to consensus binding site (Fig 6). Nucleotide -4 however differs from the consensus sequence. Comparison of the HS1 with the consensus RORα1 binding site reveals that positions -2, -3, -4 and -6 relative to the AGGTCA motif all conform to the consensus sequence. Of note is the presence of a C at position -2, which is conserved throughout in the OT/ERE, the HS1 and the RORα1 consensus binding sequence.

Mutation of the OT/ERE or HS1 that abolished binding to RORα1 reduced RORα1-mediated induction by 56% and 68% respectively and a combined mutation of both the OT/EE and the HS1 lowered the induction by 76% (Fig. 5B). The residual activity of the double mutant is probably due to the downstream AGGTCA repeats, a region is also weakly protected in the DNAse I footprinting assay.

What is the potential physiological significance of the RORα1-induced activation of the OT gene promoter described here? Recently, it has been proposed that melatonin can activate RORα under special experimental conditions (Becker-André *et al.*, 1994; Wiesenberg *et al.* 1995). Thus, it is possible that melatonin or a related molecule will act as a ligand for RORα. Moreover, a synthetic RZR/ROR-ligand, the thiazolidine dione CGP 52608 is also able to activate RORα (Wiesenberg *et al.* 1995). It is also possible that melatonin acts via its membrane bound receptor (a seven transmembrane-type of receptor) to activate the orphan receptor RORα by second messenger signaling such as phosphorylation. Furthermore, melatonin has also been shown to act as a stimulator of OT release in the intact animal (Ross *et al.* 1985) as well as *in vitro* from human granulosa cells (Schaeffer & Sirotkin 1997). In the rat, extirpation of the pineal gland, the main site of melatonin production, leads to a decrease in osmotically stimulated OT release (Windle *et al.* 1996). Interestingly, the pineal itself is

also a site of OT production (Liu *et al.* 1987, Liu *et al.* 1987). Recently, OT binding sites have been detected and characterized in the ovine pineal gland (Rahmani *et al.* 1997), and OT has been shown to modulate melatonin secretion in the rat (Simonneaux *et al.* 1990). Thus, reciprocal interactions may exists between the melatonin and OT systems, two hormonal systems that are both involved in controlling reproductive functions and undergo diurnal rhythms.

It remains to be determined to what extent other members of the ROR/RZR family have the same or similar effects on OT gene promoter activity. Studies by Carlberg *et al.* (1994) indicate that RORα1 and RORß/RZRß have indistinguishable binding characteristics and suggest considerable functional overlap between these ROR/RZR isoforms. In view of the expanding list of isoforms present in the ROR/RZR family and the widespread distribution of these members, it is conceivable that interactions similar to the one characterized here for the case of RORα1 may take place in other OT-expressing tissues, involving different ROR/RZR isoforms. Considering the strong, ligand-independent activation function exerted by RORα1, these interactions may form an important part of the multifactorial regulatory mechanisms that control the neuronal and non-neuronal expression of the oxytocin gene.

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Chapter V

General Discussion

The work presented in this thesis focuses on the role of nuclear receptors in the regulation of the OT gene promoter. Because of the lack of cell lines expressing the OT gene, heterologous systems have been used to study the OT promoter. Although the scope of this approach is limited, it allows one to dissect basic regulatory DNA element of the promoter. We and others have identified, using such an approach, that nuclear receptors are potent regulators of the OT promoter. In this thesis, the possible role for the orphan receptors COUP-TFII, Ear-2 and RORα1 is studied.

5.1 Estrogens and COUP-TFs

The role of estrogens in the regulation of OT gene expression is rather ambiguous with respect to hypothalamic OT gene expression, whereas there is little doubt for its role in uterine OT expression. In the endometrium of the uterus, the ERα is expressed in both the epithelial and stromal layers of cells, where it regulates uterine growth and differentiation. Northern blot analysis using whole uterus RNA of timed pregnant rats showed a steady rise of ERα mRNA from day 14 to parturition (day 22-23) with a 3.7 fold increase in ERα mRNA (Larcher *et al.*, 1995). In human, a 3-fold increased in ERα mRNA is observed in chorio-decidua tissues obtained after spontaneous labor versus tissues obtained from cesarean section (Chibbar *et al.*, 1995). This increase parallels the increase of OT mRNA in the rat and human uterus during parturition (Lefebvre *et al.*, 1992; Chibbar *et al.*, 1993; Chibbar *et al.*, 1995). Since an ERE has been identified in the human and rodent OT promoters, it is likely that the up-regulation of OT mRNA observed during late gestation is due to estrogen via its receptor.

In the hypothalamus, the regulation of OT by estrogens is possibly mediated by the ERβ. As discussed previously, the rat ERβ does co-localize with OT in discrete neurons of the hypothalamus thus it is reasonable to assume that within those neurons, the ERβ is able to activate the OT promoter.

The presence of COUP-TFII and Ear-2 in the epithelial layer of the rat uterus strongly suggests a role for these orphan receptors in the regulation of the OT gene. Transcripts for COUP-TFII and Ear-2 are detected in the uterus and the level of expression is increased as gestation progresses. COUP-TFII and Ear-2 proteins are also detected primarily in the epithelial layer of the uterus of the parturient rat uterus. Since OT is a strong uterotonic agent, it is important to tightly regulate the level of OT during gestation. Inhibitory factors such as COUP-TFs and activating factors like ERα might play an important role in the fine-tuning of OT levels during gestation. Further studies focussing on the levels of expression of ERα, COUP-TFII and Ear-2 proteins during pregnancy might yield clues to the intricacies of OT regulation during pregnancy.

In chapter 2, we describe the negative role of COUP-TFII and Ear-2 on the estrogenic induction of the human OT promoter. The inhibition of the estrogenic induction is readily observed when COUP-TFII or Ear-2 and ERα plasmids are transfected at a ratio of 1:50. In these studies, different viral promoters were used to drive the expression of COUP-TFII or Ear-2 (cytomegalovirus) and ERα (SV40), but similar results were obtained when both components were subcloned downstream of the same viral cytomegalovirus promoter (data not shown). The OT/ERE is sufficient to mediate the repression since the pOT-49+eCAt and pOT-ERE/tkCAT constructs are repressed by COUP-TFII and Ear-2. The inhibition by COUP-TFs is abolished when the OT/ERE is mutated from CGGTGA CCT TGACCC to

CGGTcA Cag TGACCC. These mutations convert the imperfect OT/ERE to a perfect palindromic ERE and abolish the DR0 present within the OT/ERE. This result clearly indicates the importance of the DR0 for COUP-TFII and Ear-2 repression. This effect is also specific for the OT/ERE since the ERE of the xenopus vitellogenin gene is not affected by COUP-TFII and Ear-2.

5.2 Basal repression

The high activity of the human OT reporter constructs in Neuro2a cells allowed our group to discover the silencing effect of COUP-TFII and Ear-2 on the human OT promoter (chapter 3). This silencing effect of COUP-TFII and Ear-2 is mediated by two distinct DNA elements: the OT/ERE and the downstream repeats that consist of three imperfect AGGTCA half-sites (R1, R2 and R3) each spaced by four nucleotides. Recombinant COUP-TFII and Ear-2 can bind to the DR0 motif embedded within the OT/ERE and the three downstream repeats *in vitro* as shown by DNaseI footprinting and in EMSA studies. Mutations that affect the DR0 within the OT/ERE greatly affect the ability of COUP-TFII to repress OT promoter activity whereas Ear-2 is less sensitive to those same mutations and is still able to repress the promoter activity by 50%. It is not possible to conclude whether Ear-2 is a stronger repressor than COUP-TFII or whether the difference in activity is due to difference in expression levels in transfection. The three downstream repeats seem to contribute marginally to the repression by COUP-TFII but appreciable repression is still observed with Ear-2. Mutagenesis of reporter constructs as well as EMSAs demonstrated that each of the three proximal repeats contributed to COUP-TFII and Ear-2 binding and silencing effect. These results also demonstrate the promiscuous DNA ability of COUP-TFII and Ear-2.

The physiological importance of the silencing of COUP-TFII and Ear-2 on the OT gene remains to be established. Are COUP-TFs involved in the silencing of OT expression in non-OT expressing cells or are they involved in physiological situations where a more dynamic regulation of OT is required? The expression pattern of COUP-TFI and COUP-TFII suggests that they are important in the second situation. COUP-TFs seem to be co-localized with OT in peripheral tissues such as the ovary and uterus. In those tissues, a more dynamic regulation of OT gene expression is required. Thus, it is very likely that COUP-TFs may be involved in the tight regulation of OT expression in those tissues.

5.3 Model of dynamic regulation of the OT promoter

The effects of COUP-TFs on the OT promoter are two-fold; they are able to silence or actively repress basal promoter activity and secondly, inhibit the activation by other nuclear receptors via a mechanism of competitive binding. These two mechanisms are not completely independent, since both require binding of COUP-TFs to the OT/ERE and downstream repeats. In the case of silencing of basal promoter activity, binding is sufficient to allow the intrinsic silencing activity of COUP-TFs to act on the basal machinery and/or chromatin structure. In contrast, the competitive binding mechanism requires one to consider two important parameters: the level of expression of the two antagonistic players and their relative affinity and stability for the DNA site. The transfection studies in chapter 2 suggest that COUP-TFs have a stronger affinity and/or stability for the OT/ERE than the ERα. In addition, there are two binding sites for COUP-TFs compared to one for ER on the human OT promoter. Another possible mechanism to explain the high inhibitory activity of COUP-TFs is the formation of non-productive heterodimers. Such heterodimers were not observed on DNA in gel retardation assays (Burbach *et al.*, 1994) but were observed in solution in GST-pull

down assays (Klinge *et al.*, 1997). Non-productive heterodimers between COUP-TFs and ER might prevent ER to bind to the OT/ERE and may explain the potent inhibition of COUP-TFs in transfection assays. Interestingly, COUP-TFs seem to co-purify with ERα on numerous chromatographic columns from bovine uterus and MCF-7 cells extracts (Klinge *et al.*, 1997).

5.4 Regulation of the OT promoter by the orphan receptor RORα1

The results shown in chapter 4 also implicate the orphan receptor subfamily ROR/RZR in the regulation of the mouse OT promoter. Deletion analysis of the mouse promoter revealed that a region between -225 to -118 of the promoter is responsible for the activation. This region, when linked to a minimal TATA box containing promoter-reporter, is also sufficient for activation. This activation is specific since a region of the mouse promoter (-134 to -34), which contains three TGACCpy (or the reverse complement puGGTCA) motifs, is not responsive to RORα1. Within the -225 to -118 region lies the OT/ERE and DNaseI footprinting analysis shows that recombinant RORα1 can bind to the OT/ERE. In addition, a half-site 5' of the OT/ERE (referred to as HS1) was also protected by RORα1 and *in vitro* gel shift studies showed binding of RORα1 to an OT/ERE or HS1 probe

RORα1 binds to DNA as a monomer to an extended half-site motif (A/G/T, T/A, A/T, T/A, C, T/A, AGGTCA). The HS4 and OT/ERE were shown to interact with RORα1 *in vitro*. Three half-sites HS2, HS3 and HS4 are present within the OT/ERE (see Fig. 6, chapter 4). We believe that the HS4 and not HS2 or HS3 is the binding site for RORα1 in the mouse OT/ERE. Sequence alignment of the three half-site with the consensus RORα1 binding site revealed that the important C residue at position -2 is not conserved in either HS2 or HS3. This C at position -2 was shown to be critical for RORα1 binding (Giguère *et al.*, 1994) and thus makes the HS2 and HS3 elements poor binding sites RORα1.

In gel shift studies, cold consensus RORE binding site oligonucleotides are better competitors of binding than the OT/ERE and HS1 oligonucleotides. This is indicative that the HS1 and HS4 are weak binding sites for RORα1 but appreciable activation (between 6- to 14-fold) is observed in transfection studies. Thus, the presence of two binding sites can compensate for the weak binding of RORα1 to HS1 and HS4. Full activation in transfection studies required both the OT/ERE and HS1 elements since mutation of either the HS1 or the HS4 element decreases the activation by half. These results are indicative of an additive effect between the two sites.

The absence of COUP-TFI and COUP-TFII in OT-expressing neurons in the hypothalamus rules out their involvement in hypothalamic OT expression (Lopes da Silva *et al.*, 1995). However, this does not exclude that other orphan receptors might be involved. The ROR/RZR subfamily is an emerging family of nuclear receptor comprising three subtypes (α, β, and γ). Although the expression pattern of this family is ubiquitous, tissue-specific expression of specific members is also observed. Of particular interest for the present study is the fact that ROR/RZRβ is expressed in the hypothalamus, the pineal gland and the thalamus (Becker-Andre *et al.*, 1994). Both the hypothalamus and the pineal gland are known sites of OT gene expression. Thus, the pineal OT gene expression might be regulated by members of the ROR/RZR family of nuclear receptor. Mice with a targeted disruption of ROR/RZRβ display a duck-like gait, transient male inability to reproduce and a severe disorganization of the retina (André *et al.*, 1998). It would be interesting to look at the level of pineal OT mRNA expression in these null mutant mice.

5.5 Hypothalamic expression of transcription factors

The expression pattern of COUP-TFI and COUP-TFII in the hypothalamus does not support a role of these factors in OT expression (Lopes da Silva *et al.*, 1995). On the other hand, it remains to be determined, whether Ear-2 is expressed in OT expressing neurons of the hypothalamus. To date, only few nuclear receptors have been localized in the SON and PVN. These include TRα (Bradley *et al.*, 1989), ERβ (Shughrue *et al.*, 1996; Li *et al.*, 1997; Shughrue *et al.*, 1997a; Shughrue *et al.*, 1997b; Alves *et al.*, 1998), VDR (Prüfer and Jirikowski, 1997). Of those, only ERβ and VDR were specifically co-localized with OT.

Another class of transcription factors, the POU (for Pit-1, Oct-1, 2, and Unc96) domain transcription factors, Brain-1, Brain-2 and Brain-4 (Brn1, Brn2, Brn4) are localized to the hypothalamus (He *et al.*, 1989; Li *et al.*, 1993; Malik *et al.*, 1996). The role of numerous POU proteins in determining cell-specific phenotypes is well established (reviewed in Ryan and Rosenfeld, 1997). Pit-1 is required for proliferation and survival of three anterior pituitary cell types. Unc86 is important for neural cell fate in C. elegans and finally Oct-2 is also a major determinant of B-cell development and survival. Since Brn1, Brn2 and Brn4 are expressed in overlapping and defined regions of the hypothalamus; it is tempting to speculate a possible involvement of these proteins in determining specific neuronal phenotypes. Moreover, Brn2 knockout in mice completely lack OT and AVP expressing neurons in the PVN and SON (Nakai *et al.*, 1995; Schonemann *et al.*, 1995) but AVP expressing neurons are still present in the suprachiasmatic nucleus. In addition Brn-2 co-localizes with OT in the PVN and SON (Malik *et al.*, 1996; Schonemann *et al.*, 1995) and two highly conserved A/T rich regions are conserved within the proximal promoter of the human OT promoter. However, co-transfection of a Brn-2 expression vector with either the human or the mouse OT promoter CAT reporters does not elicit any activation (personal observation).

Recently, the bHLH-PAS transcription factor Single-minded 1 (SIM1) has been implicated in the development of magnocellular neurons of the PVN and SON (Michaud *et al.*, 1998). Indeed knockout of the SIM1 locus in mice led to a complete absence of magnocellular neurons of the PVN and SON and of parvocellular neurons of the anterior periventricular nucleus. The phenotypes of SIM1 null mutants are very similar to that of the Brn-2 knockout mice. Interestingly, SIM1 seems to act upstream of Brn-2 possibly by regulation the expression of the latter. It will be interesting to test the effect of SIM1 on the OT promoter.

5.6 Perspectives

The physiological significance of the results presented in this thesis remains to be established. The importance of the role of the COUP-TF and/or RORα1 subfamilies in regulating OT expression is not known. Because of the lack of OT expressing cell lines, heterologous cell lines (the neuroblastoma Neuro2a and the uterine epithelial Ishikawa cell lines) were used to study the human and mouse OT promoters. With the advances of molecular biology, new techniques have become available, such as genomic dimethyl sulphate footprinting coupled to ligation-mediated polymerase chain reaction that permits one to study protein-DNA interaction *in vivo*. Such an approach was used by Beato and colleagues (Scholz *et al.*, 1998) to study the expression of the rabbit uteroglobin gene in endometrial epithelium. It is easily conceivable that such an approach could be extended to the study of OT in the uterus of the human or rodent species, since epithelial cells from the endometrium can be readily isolated in sufficient quantities and purity. The results of such studies would likely support a role for the OT/ERE in mediating the massive up-regulation of OT mRNA observed in the rat uterus during the late stage of pregnancy. This type of study might not be

applicable to the hypothalamus because of the difficulty in isolating a homogenous population of OT magnocellular neurons.

An alternative approach for further studies would be to generate an OT-expressing cell line using targeted immortalization with the SV40 large T antigen oncogene. Such an approach has previously shown to be efficient in immortalizing GnRH hypothalamic neurons (Mellon *et al.*, 1990). Targeted expression of OT transgenics in magnocellular neurons was achieved by various groups using the rat (Young *et al.*, 1990), the bovine (Ho *et al.*, 1995) OT gene and the Fugu (Venkatesh *et al.*, 1997) Isotocin gene. In most cases, correct expression in magnocellular neurons is complex and requires multiple DNA elements scattered within the OT promoter, the OT structural gene as well as the OT/AVP intergenic region and finally linkage to the AVP gene (see chapter 1). To circumvent the complexity of this cell-specific regulation, one can envision a polycistronic construct in which the cDNA of the SV40 large T antigen would be placed 3' of the last exon of the OT gene. Translational initiation of the large T antigen will require an internal ribosome binding site that is located 5' of the large T antigen cDNA. This construct would contain all the regulatory elements present in the OT/AVP locus and, in addition, the large T antigen cDNA. If tissue specific expression of OT is achieved, the large T antigen would also be expressed in those cells and it might induce immortalization of OT expressing neurons. Finally, a similar polycistronic approach could be used for the AVP gene. The creation of such OT or AVP cell line would represent a valuable tool for the study of the molecular biology of OT and AVP. Alternatively, the Brn-2 or SIM1 promoter could be used to drive the expression of the large T antigen protein since both proteins were shown to be important in the ontogeny of the SON and PVN during development (see earlier)

The role of nuclear receptor in the regulation of hypothalamic OT expression remains somewhat elusive. It seems that nuclear and orphan receptors play an important role in the regulation of the OT gene especially at peripheral sites where a more dynamic type of regulation is observed. This raises the question of what regulates OT hypothalamic expression? The answer likely lies in other nuclear receptors, since there are at least 100 members in this superfamily. It is possible that some other member(s), known or unknown, is (are) involved. The notion that nuclear receptors are important in regulating OT gene expression is further supported by the high conservation of the DNA elements mediating their action in the promoter region of the human, rat, mouse, bovine and sheep OT genes.

There is no doubt that other classes of transcription factors are also involved in the regulation of OT gene expression. Unfortunately, the lack of a cell line expressing OT has delayed the discovery of tissue-specific transcription factors implicated in the regulation of OT in the hypothalamus. It is thus imperative to generate such cell lines. To find support for this idea, one only has to look at the field of pancreatic gene expression and especially at the hormone, insulin. There exist many insulin-producing cell lines both naturally-occurring and experimentally-generated. Such cell lines have been central in the identification of many important transcription factors involved not only in insulin gene expression but also in pancreas genesis and islet of Langerhans development. Although, cell lines will not replace the multitude of information obtained from transgene and knockout studies, they will permit in the end enormous monetary and time savings.

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Claim of original contribution

The following items constitute genuine novel contribution:

1) Immunolocalization of the orphan receptors COUP-TFII and Ear-2 in the parturient rat uterus.

2) The active repression of COUP-TFII and Ear-2 observed on the human OT gene promoter

3) The activation of the mouse OT promoter by the orphan receptor RORα1